Techniques in Molecular Biology

Prof. Suraksha Agrawal

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Prof. Suraksha Agrawal
Head
Department of Medical Genetics
Sanjay Gandhi Post Graduate Institute of Medical Sciences,
Raebareli Road, Lucknow - 226 014, U.P. [India]

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Preface

Completion of the human genome project is an important mile stone in the understanding of the complex processes involved in modern biology. It is a step towards understanding more precisely the function of each gene which may in turn lead in future to practice molecular and individualized medicine. Practice of molecular medicine is a two fold interaction of intensive basic and applied research. All the techniques which are being used in molecular biology are highly sophisticated and advanced. Moreover these techniques are unique for a set of hypothesis being proposed to answer a specific question.

Various molecular techniques have application not only in the field of biotechnology forensic science, and pharmaceutical research but also have a wide potential in medical sciences. In this manual we have made an attempt to provide the basic back ground of each technique followed by detailed recipe and then how to carryout different experiments.

The topics dealt in this book are preparation of Nucleic Acids that includes Deoxyribose Nucleic Acid (DNA) Extraction, Ribose Nucleic Acid (RNA) Extraction and Plasmid DNA Extraction; Basic Molecular techniques including Polymerase Chain Reaction (PCR) and Electrophoretic Techniques; Gene expression analysis using Reverse transcriptase PCR (RT-PCR); Molecular genotyping of Short Tandem Repeats (STR), Alu insertions; Mutation detection techniques including Restriction Fragment Length Polymorphism (RFLP), Amplification Refractory Mutation Detection System (ARMS) and Single Stranded Conformation Polymorphism (SSCP); Nucleic Acid Blotting: Dot Blotting and Southern Blotting; Latest molecular techniques like DNA Sequencing, Multiplex PCR and Automated DNA Fragment size analysis by Gene scanning; DNA Recombinant Technology and Microarray.

A separate chapter on a brief overview of all the statistical methods which can be used to analyze the biological data has been included.

This book has been written keeping in mind the bench workers and also the researchers who are going to establish their own laboratories. We have tried to give point wise description of various techniques. Although every effort has been made to make this book as up to date as possible, there may still be some deficiencies and mistakes. The comments and suggestions from users are solicited for improvement.

This book would have not taken the present shape without the constant help from all my research students who have worked in the past and also those who are presently working with me. I would like to thank Mr. Sanjay Kumar Johari for typing this manuscript.

I hope that with all its imperfections this book will still be of some use to all the users in this field.

Suraksha Agrawal

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Chapter 1

Nucleic Acid Extraction

- **Preparation of genomic DNA**
- **Ribose Nucleic Acid Extraction**
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Preparation of genomic DNA

*D*eoxyribonucleic acid is commonly known as DNA. DNA is the blueprint of an organism's genetic make-up. F. Griffith in his experiment in 1928 proved that DNA is the genetic material. In late 1953, James Watson and Francis Crick presented a model describing the structure of DNA.

Discovery of DNA

DNA was first identified in 1868 by Friedrich Miescher, a Swiss biologist, in the nuclei of pus cells obtained from discarded surgical bandages. He called the substance nuclein, he noted the presence of phosphorous, and separated the substance into a basic part (which is now known as DNA) and an acidic part (a class of acidic proteins that bind to basic DNA).

DNA as a genetic material

Various experiments in first half of the 20th century lead to the discovery of DNA as a genetic material.

1. In 1943, Oswald Avery, Colin Macleod, and Maclyn McCarty, at the Rockefeller Institute, discovered that different strains of the bacterium *Streptococcus pneumonae* could have different effects on a mouse. One virulent strain when injected can kill the mouse, and another virulent with the name a strain had no effect. When the virulent strain was heat-killed and injected into mice, there was no effect. But when a heat-killed virulent strain was co-injected with the virulent strain, the mice died. They suggested that this was because of transforming principle behind it.

2. F. Griffith carried out one of the classic experiments that proved that virulence (disease causing capacity) of pneumonia causing bacteria could be transferred from one strain of bacteria to another. There are two types of pneumonia bacteria "rough strain" - causes no disease and "smooth strain" - that causes pneumonia. Therefore, dead smooth strain transferred some substance to the non-virulent rough strain, which transformed the rough strain into the more virulent smooth strain. The transforming substance was later discovered to be the DNA.
3. Finally, in 1952, Alfred Hershey and Martha Chase performed the definitive experiment that showed that DNA was, in fact, the genetic material. By radio labeling sulphur in one of the culture, they could tag the path of proteins and not DNA, because there is no sulphur in DNA, however, there is sulphur in the amino acids methionine and cysteine. By radio labeling phosphorous, the opposite effect could be achieved. DNA could be traced and not the protein, because there is phosphorous in the phosphate backbone of DNA and none in any of the amino acids. Cultures could be grown in each of these two ways and the phage purified from the host bacteria, resulting in one culture in which only the phage protein was labeled, and another culture in which only the phage DNA was labeled.

Structure of DNA

The structure of DNA was not completely determined until 1953 when James Watson and Francis Crick took information from several sources and came up with a structure. However, there are three major different experiments which helped in determining the structure of DNA.

1. Chargaff's Rules

Erwin Chargaff took purified DNA and broke it down into nucleotide subunits. (A) Adenine, (T) Thymine, (G) Guanine, (C) Cytosine and carefully measured the amount of each type of nucleotide. Chargaff found that the relative amount of nucleotides varied from species to species and that the amount of adenine always was the same as the amount of thymine and the amount of guanine was the same as the amount of cytosine.

A = T and C = G

2. X-ray Crystallography

Another source of information regarding the structure of DNA was from X-ray crystallography. Work on X-ray diffraction patterns by Maurice Wilkins and Rosalind Franklin revealed that the molecule had a "helical shape" with repeating distances of 0.34 nm.

3. Double Helix structure

Watson and Crick determined that the DNA molecule was a double helix comprised of nucleotides with complementary base pairs. The composition of DNA is a series of nitrogenous bases (known as base pairs), and sugar and phosphate strands. DNA consists of 4 nitrogen bases. Adenine and Guanine (both Purines) Cytosine and Thymine (both Pyrimidine). In **Figure 1.1** their structure is shown.

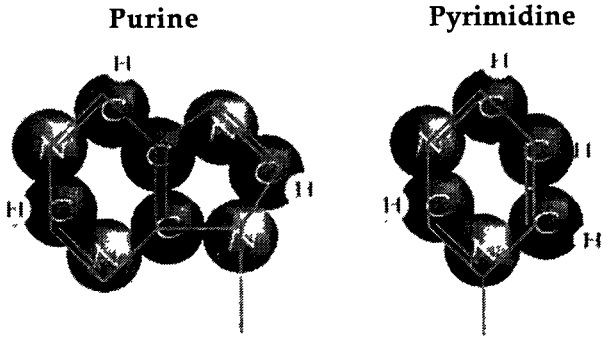


Figure 1.1 Structure of Purine and Pyrimidine bases

In the ladder model of DNA, the sugar and phosphate strands compose the sides of the DNA model, or molecule, while the actual rungs of the ladder are made up of the 4 nitrogen bases. For a molecule of DNA to maintain its shape there are number of rules between pair bonds, and the strands of phosphates, these are given below:

- The four pair bonds, Cytosine, Guanine, Adenine, and Thymine, do not entirely intermix. Only Cytosine can attach with Guanine, and only Adenine can attach to Thymine or only C's can attach to G's, and only T's to A's.
- The Phosphate and Sugar strands (edges of the ladder), are arranged as follows, S - P - S - P - S - P, etc. **(Figure 1.2)**

DNA is a double helical in its structure. The outer edges are formed of alternating ribose sugar molecules and phosphate groups. The two strands run in opposite directions. The nitrogenous bases are "inside"

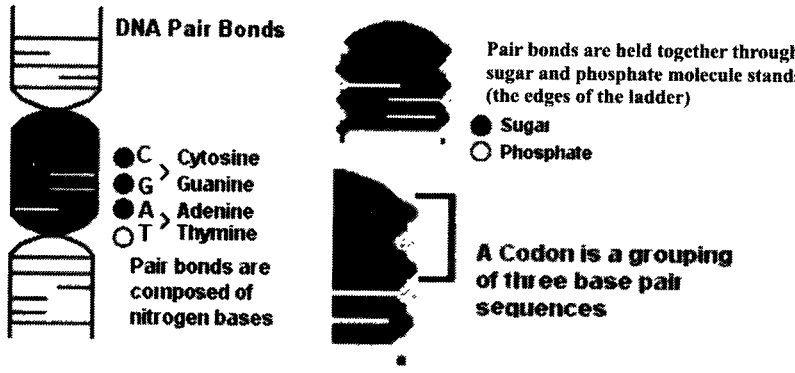


Figure 1.2: Different bonding patterns between both nitrogenous bases and sugar backbone decides the sequence of DNA.

like rungs on a ladder. Adenine on one side pairs with thymine (Uracil in RNA) by hydrogen bonding, and cytosine pairs with guanine. The C-G pair has three hydrogen bonds while the A-T pair has only two. This dictates side-to-side pairing, but nothing about the order along the molecule can be depicted.

For their outstanding work for discovering the double helical structure of DNA, Watson and Crick shared the Nobel Prize for Physiology and Medicine with Maurice Wilkins. Rosalind Franklin in 1962, she had also contributed greatly to this key discovery.

All the characters are inherited from one progeny to the next and these units of inheritance are known as Genes. Study of genetic material i.e. DNA is important to study the function of genes. Various techniques involved in the DNA extraction and availability of good quality DNA has made it possible to decipher the whole human genome. Once the human genome is available, we can study the function of genes more precisely at molecular level. Human genome project has led to the era of proteomics and transcriptomics. In this chapter, we will discuss about various methods of DNA extraction.

DNA extraction methods. After discussing briefly the structure of DNA. DNA extraction procedures are given below. For every genetic analysis, we require a good quality DNA. Now a days readymade kits are available in the market by which the DNA can be extracted but some time it is difficult for the laboratories to use the commercial kits because of the cost.

There are various manual protocols about the DNA extraction. However, in each protocol several steps are involved in the preparation

of DNA. These are – (i) cell breakage (ii) removal of protein (iii) removal of RNA (iv) concentration of DNA (v) determination of the purity and quantity of DNA.

1. Cell breakage

Cell breakage is one of the most important steps in the purification of DNA. The usual means of cell disruption, such as sonication, grinding, blending, or high pressure, cannot be used in DNA isolation. These procedures apply strong force for the disruption of cells which get sheared, as a result the DNA is fragmented. The best procedure for opening cells and obtaining intact DNA is through application of chemical (detergents) and / or enzymatic procedures. Detergents can solubilize protein as well as lipids in cell membranes resulting into gentle cell lysis. In addition, detergents have an inhibitory effect on all cellular DNAses and can denature proteins, thereby aiding in the removal of proteins from the solutions. The lysis of animal cells is usually performed using anionic detergents such as SDS (sodium deodecyl sulfate) or Sarcosyl (sodium deodecyl sarcosinate).

2. Removal of protein

The second step in purification involves removal of major contaminant, namely protein, from the cell lysate. This procedure is called deproteinization. Removal of proteins from the DNA solution depends on differences in the physical properties between nucleic acids and proteins. These differences are in solubility, in partial specific volume, and in sensitivity to digestive enzymes.

(i) Deproteinization using organic solvents

The most frequently used methods for removing proteins explore the solubility differences between proteins and nucleic acids in organic solvents. Nucleic acids are hydrophilic molecules and are easily soluble in water. Proteins, on the other hand, contain many hydrophobic residues making them partially soluble in organic solvents. There are several methods of deproteinization based on this difference and they vary by the choice of the organic solvent.

The organic solvents commonly used are phenol and chloroform containing one percent isoamyl alcohol. The application of phenol is based on the following principle. Phenol is crystalline at room temperature, but in the presence of 20 percent water, it forms an aqueous suspension containing phenol micelles surrounded by water molecules. Protein molecules generally contain many hydrophobic

residues, which are concentrated in the center of the molecule. When an aqueous protein solution is mixed with an equal volume of phenol, some phenol molecules are dissolved in the aqueous phase (approximately 20 percent water and 80 percent phenol). Yet the phenol molecules are extremely hydrophobic. Consequently, they tend to be more soluble in the hydrophobic cores of the protein than in water. Hence, phenol molecules are extremely hydrophobic. As a result, phenol molecules diffuse into the core of the protein causing the protein to swell and eventually to unfold or denature. The denatured protein, with its hydrophobic groups exposed and surrounded by micelles of phenol, is far more soluble in the phenol phase than in the aqueous phase. Proteins are partitioned into the phenol phase leaving the nucleic acids in the aqueous phase. Nucleic acids do not have hydrophobic groups at all and are insoluble in the phenol phase.

Application of the phenol method does require mixing the phenol phase with the water phase. This introduces some shearing of DNA molecules. Since, only relatively small amounts of protein can dissolve in a given volume of phenol, repeated extraction of the aqueous phase with phenol is required in order to remove all the protein. Because the phenol phase at saturation contains 20 percent water, every phenol extraction will remove 20 percent of the DNA into the phenol phase. Even more DNA is lost by entrapment in the interphase layer of precipitated proteins or when the pH of phenol drops below pH 8.0. The oxidation product of phenol can react chemically with DNA (and RNA) molecules. In addition, phenol is highly toxic and requires special disposal procedures.

In order to minimize these effects, several modifications have been introduced:

1. **The use of ionic detergents:** These detergents, by unfolding the protein, help to expose hydrophobic regions of the polypeptide chains to phenol micelles, thereby aiding partitioning of proteins into the phenol phase.
2. **Enzymatic removal of proteins before phenol extraction**: This reduces the number of extractions needed, thus, limiting the loss and shearing of DNA.
3. **Addition of 8HQ (8-Hydroxy-Quinoline) to the phenol**: This increases the solubility of phenol in water. In the presence of this compound phenol remains liquefied at room temperature with only 5 percent water. In addition, 8HQ is easily oxidized and, therefore, it plays the role of an anti-oxidant, protecting

phenol against oxidation. Since the reduced form of 8HQ is yellow and the oxidized form is colorless, the presence or absence of yellow color is an excellent visual indicator of the oxidation state of phenol.

4. **Removal of oxidation products from phenol and prevention of oxidation upon storage or during phenol extraction**: Because water-saturated phenol undergoes oxidation rather easily, particularly in the presence of buffers such as Tris, Phenol used for DNA purification is twice distilled, equilibrated with water, and stored in the presence of 0.1 percent 8HQ.
5. Adjusting the pH of water-saturated phenol solution to above pH8 by equilibration of the liquefied phenol with a strong buffer or sodium borate. DNA obtained by this method is usually of high molecular weight, but contains approximately 0.5 percent protein impurities that can be removed by another method.

The application of chloroform: isoamyl alcohol (CIA) mixture can also be used which is also known as the deproteinization method. This is based on a characteristic of this organic solvent that differs from phenol. The chloroform is not miscible with water and, therefore, even numerous extractions do not result in DNA loss into the organic phase. The deproteinization action of chloroform is based on the ability of denatured polypeptide chains which partially enter or be immobilized at the water-chloroform interphase. The resulting high concentration of protein at the interphase causes protein to precipitate. Since the deproteinization action of chloroform occurs at the chloroform-water interphase, efficient deproteinization depends on the formation of a large interphase area. To achieve this, one has to form an emulsion of water and chloroform. Chloroform does not mix with water. This can only be done by vigorous shaking. An emulsifier, isoamyl alcohol, is added to chloroform to help to form the emulsion and to increase the water- chloroform surface area.

A substantial improvement in the method can be accomplished by limiting the number of extractions. This saves time and limits the DNA shearing. This can be done by enzymatically removing most of the protein before extraction. Another modification frequently used is combining phenol and chloroform extraction into one step.

Proteins can be removed from DNA preparations using a protease that can digest all proteins, i.e. a general-purpose protease. Two such enzymes are in use, proteinase K and pronase. Both enzymes are

very stable, general specificity proteases that are secreted by fungi. Commercial preparations of these enzymes are inexpensive and devoid of DNase contamination, making them available to be used in the purification of nucleic acids. These proteases are active in the presence of low concentrations of anionic detergent, high concentrations of salts, and EDTA and exhibit broad pH (6.0-10.0) and temperature (50-70^{0}C) optima. They can digest intact (globular) and denatured (polypeptide chain) proteins and do not require any co-factors for their activities. Proteinase K and pronase are usually used in DNA purification procedures at final concentrations of 0.1-0.8mgml^{-1}. The difference between these two enzymes lies in their activities towards self; pronase is a self-digesting enzyme, whereas proteinase K is not. The fact that proteinase K is not a self-digesting enzyme makes it a more convenient enzyme to use than pronase, because it is unnecessary to continually add it during the prolonged course of the reaction.

The major drawback in using these enzymes is that enzymatic treatment can only remove 80-90 percent of the proteins present. This is because protein digestion is an enzymatic reaction that is dependent on substrate and enzyme concentrations. In practice, the deproteinization rate depends only on the protein (substrate) concentration, because it is not practical to add a large amount of enzyme to accelerate the reaction at low substrate concentration. Therefore, as the reaction proceed the concentration of substrate decreases progressively, thereby slowing the reaction rate and, indeed, enzymatic reactions will go to completion only given infinite time. At high substrate concentrations and sufficient concentration of enzyme, the reaction proceeds at a maximal rate until 80-90 percent of the substrate has been removed. The reaction rate then becomes too slow to be practical for the removal of remaining protein in a reasonable time.

The characteristics of enzymatic removal of proteins make enzymatic deproteinization an ideal and indispensable first step in nucleic acid purification. This treatment is used when a large amount of protein is present, i.e. right after cell lysis. The remaining proteins can be removed with a single extraction using organic solvent.

(ii) Removal of RNA

The removal of RNA from DNA preparations is usually carried out

using an enzymatic procedure. Consequently, this procedure does not remove all RNA and, therefore, yield DNA preparations with a very small amount of RNA contamination. Two ribonucleases that can be easily and cheaply prepared free of DNase contamination are ribonucleases A and ribonucleases T1.

(a) Ribonuclease A (RNase A) is an endoribonuclease that cleaves RNA after C and U residues. The reaction generates 2′: 3′ –cyclic phosphate which is hydrolyzed to 3′ –nucleoside phosphate producing oligonucleotides ending with 3′-phosphorylated pyrimidine nucleotide.

(b) Ribonuclease T1 (RNase T1) is an endoribonuclease that is very similar to RNase A in a reaction conditions and stability. The enzyme cleaves double-stranded and single-stranded RNA after G residues, generating oligonucleotides ending in a 3′-phosphorylated guanosine nucleotide.

(iii) Concentrating the DNA

Precipitating with alcohol is usually performed for concentration of DNA from the aqueous phase of the deproteinization step. Two types of alcohols can be used for DNA precipitation: **ethanol** and **isopropanol**.

Alcohol precipitation is based on the phenomenon of decreasing the solubility of nucleic acids in water. Polar water molecules surround the DNA molecules in aqueous solutions. The positively charged dipoles of water interact strongly with the negative charges on the phosphodiester groups of DNA. This interaction promotes the solubility of DNA in water. Ethanol is completely miscible with water, yet it is far less polar than water. Ethanol molecules cannot interact with the polar groups of nucleic acids as strongly as water, making ethanol a very poor solvent for nucleic acids.

DNA precipitation is customarily carried out with 70 percent ethanol (final concentration) in the presence of the appropriate concentration of sodium or ammonium salts. The use of each of these salts has its advantages and disadvantages. The major advantage of using sodium chloride, in addition to convenience and low cost, is that SDS remains soluble in ethanol in the presence of 0.2M NaCl. The use of sodium chloride is therefore recommended if a high concentration of SDS has been used for lysing the cells. The disadvantage of sodium chloride

is its limited solubility in 70 percent ethanol making it difficult to completely remove from the DNA samples. This is particularly true when the precipitated DNA is collected by centrifugation.

Sodium acetate is more soluble in ethanol than sodium chloride and therefore, is less likely to precipitate with the DNA sample. Its higher solubility in 70 percent ethanol makes it easier to remove from a DNA preparation by repeated 70 percent ethanol washes. Sodium acetate is the most frequently used salt in DNA precipitation.

Ammonium acetate is highly soluble in ethanol and easy to remove from precipitated DNA due to the volatility of both ammonium and acetate ions. The use of ammonium acetate instead of sodium acetate is also recommended for removing nucleotide triphosphates or small single- or double-stranded oligonucleotides (less then 30 bp), since these molecules are less likely to precipitate at high ethanol concentrations. In addition, precipitation of DNA with ammonium acetate has proven to be more efficient for the removal of heavy metals, detergents, and some unknown impurities that are potent inhibitors of restriction endo nucleases and other enzymes used for DNA manipulation.

Usually, ethanol precipitation is carried out at a temperature of -20 °C or lower, however, even if the precipitation is carried out at room temperature or 4 °C fairly good amount of DNA can be obtained. -70 °C gives the worse DNA amount. These temperatures are important when we require higher concentrations of DNA but for lower concentrations temperature is not very critical.

(iv) Determination of purity and quality of DNA

The last step in DNA isolation is the quality of the DNA being isolated. UV spectrometry is used for determining the DNA concentration. The DNA has maximum absorbance at 260nm and minimum absorbance at 234 nm. This can get affected by the PH of the medium in which the DNA is dissolved.

DNA concentration (N) =A260/ε260; ε260 is the DNA extinction coefficient. This is 0.02 µg -1 cm -1 when measured at neutral or little basic PH for double helical DNA. Thus, an absorbance of 1.0 at 260 nm gives a DNA concentration of 50 µg ml -1 (1/0.02 =50 mg ml-1). However, this may differ because of GC percent. If the DNA is found in small concentrations then dust particle scattering may effect the

measurements. This can be checked by taking one reading at 320 nm, DNA is not absorbed at 320 nm, hence, if there is any recorded reading, it is because of dust contamination. If there is no contamination then at 320 the reading should be 5% less than the absorbance reading at 260. At 280 nm the protein concentration is measured as the protein is absorbed maximally at 280 nm. This is due to tyrosine, phenylalanine and tryptophan. For DNA purity the ratio at A260:A280 is taken. The best purity is 1.8 to 2.

Precautions

All the chemicals should be handled with care specially phenol; if skin comes in contact with phenol flush off with large amount of water and then apply polyethylene glycol, **never apply ethanol.** If using blood or any human tissue precaution should be taken that it is pre-tested and free from HIV. Further, always wear gloves.

Technical tips

Low yield of DNA could be because of two reasons; i) hydrodynamic shearing, ii) DNA degradation due by non specific DNAases.

To avoid hydrodynamic shearing, DNA in solution should be pipetted with wide bore pipettes (approximately 3-4 mm orifice diameter). DNA should never be vigorously shaken or vortexed. To avoid non specific DNAse it is recommended to use DNAses inhibitors in all solutions. These are EDTA and detergents. 50-100mM EDTA is usually sufficient. Detergent used is SDS or sodium Deodedecyl sulfate and Sarcosyl. DNA should be stored properly. Even when the DNA is stored properly, it is expected that one phosphodiaster bond breaks per 200 kb DNA per year. For long term storage, pH should be above 8.5 in order to minimize deamidation and should contain at least 0.15 M NaCl and 10 mM EDTA. Following factors are highly responsible for DNA degradation: DNAse contamination, presence of heavy metals, presence of ethidium bromide (it causes photo-oxidation) and temperature (for long storage, it should be kept at -70°C working DNA can be kept at 4-8°C.

1. **DNA extraction from venous blood sample:** The list and the composition of various buffers and solutions is given below. The DNA solution can be kept at4°C while they are in use. However, for long term storage, they are best kept frozen. The blood should be collected in EDTA as heprin inhibits the PCR reaction.

(1) Lyses buffer buffer

For 500ml
Sucrose = 54.8 gm
Tris HCl = 0.73 gm
MgCl2 = 0.52 gm
Triton X100 = 5 ml
Dissolved in DDW
Do not Autoclave, sterilize it by filtration

(2) Proteinase

For 500ml
NaCl = 11.0 gm
EDTA = 22.4 gm
Dissolved in DDW
pH should be 8.0
Sterilize it by Autoclaving

(3) 5M Nacl

For 100 ml
Nacl = 29 gm
Dissolved in DDW
Sterilize it by Autoclaving

(4) 10% SDS

For 100 ml
SDS = 10 gm in 100 ml of solution
Do not Autoclave

Procedure:- Stepwise isolation procedure is shown below. All steps should be carried out at 4^0C.

(i) 1ml EDTA blood and 1ml Lysis buffer, (ii) mix gently by inverting the tubes, spin at 11,000 rpm for 5 minutes, (iii) decant the supernatant add 400µl of lysis buffer into the pellet, mix with 1ml tips until pellet is broken, spin at 12,000 rpm for 2 minutes, (iv) decant supernatant add 400µl of lysis buffer to the pellet, mix with 1ml tips until pellet gets broken, spin at 12,000 rpm for 2 minutes, (v) Decant supernatant add 400µl of autoclaved double distilled water (DDW), mix with 1ml tip until pellet gets broken, spin at 12,000 rpm for 2 minutes, (vi)Decant supernatant and add 100µl proteinase K buffer, mix 5-6 times with 1 ml tips than add 10 µlof 10% SDS, keep on mixing till whole pellet gets dissolved completely. Add 120µl of 5M Nacl mix with finger tapping then add 300µl double distilled water (DDW). Mix it by inverting the tubes and add 400µl of phenol: chloroform (in a ratio of 4:1), Spin at 12,000 rpm for 10 minutes.

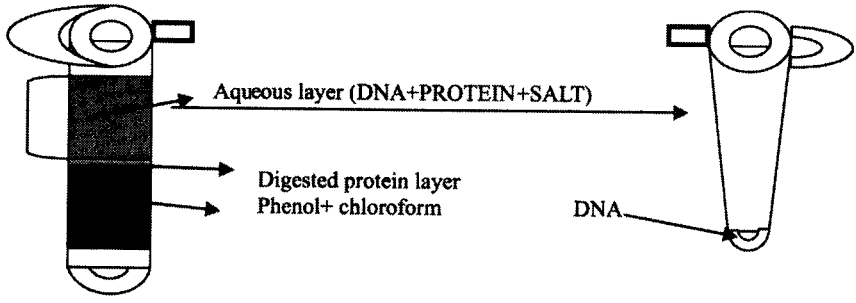


Figure 1.3 Removal of Aqueous layer

(vii) Transfer the aqueous layer into 1.5 ml autoclaved micro centrifuge with the help of 200 µl of tip (Figure 1.3). The edges of the tip should be blunt and the tips should be freshly autoclaved. (viii) Add chilled absolute alcohol double the amount of aqueous phase, (ix) Spin at 12,000 rpm for 4 minutes. Decant supernatant, add 100 µl of 70% alcohol, detach the pellet with finger tapping from the tube wall, Spin at 12,000 rpm for 2 minutes, (x) Decant supernatant with the help of 200ul tip, (xi) Dry the pellet: Drying can be carried out at room temperature: 6-12hrs, at 56^0C: 2 - 3 hrs, at 74^0C: 10-15 minutes.

Reconstitute the pellet in TE buffer. The reconstituted pellet can be left at room temperature for 12-18 hrs, at 56^0C for overnight, at 74^0C for 10-15 minutes.

Preparation of genomic DNA from blood in heparin

Some time the blood received in the laboratories is in heparin. The following procedure should used for heparinised blood:

1. Transfer blood to 50 ml centrifuge tube. (disposable plastic Falcon tubes.) Centrifuge at approximately 3000 RPM for 10 minutes at 4°C.
2. Carefully remove the plasma with a plastic Pasteur pipette, taking care not to disturb the buffy coat. (Leave a small amount of plasma above the buffy coat.)
3. Add 20-30ml distilled; mix thoroughly to ensure good lysis of the red cells.
4. Spin at 3000 RPM for 10 minutes.
5. Carefully remove the supernatant and add 10ml of lysis buffer (100ml NaCl, 25mM EDTA) to the pellet. Mix and add 0.5ml 10% SDS solution.
6. Add 0.5mg of Proteinase K (20µl containing 0.5mg). Mix and incubate at 37°C overnight.
7. Add 1/2 volume of chloroform (5ml) and 1/2 volume of phenol (5ml) previously saturated with 1M Tris-HCl pH8.0.
8. Mix thoroughly and spin.
9. Remove and discard lower phenol layer with plastic Pasteur pipette.
10. In the second step phenol extraction: repeat steps 7, 8, and 9.
11. Add 1/2 volume (5ml) of chloroform mix and centrifuge as in the first step.

12. Remove and discard lower chloroform layer.
13. Second chloroform extraction: Repeat step 11, this time transferring the upper aqueous layer into a clean Falcon tube.
14. Precipitate the DNA by adding 5ml 7.5M ammonium acetate and 2 volumes of absolute ethanol. Mix thoroughly.
15. If DNA precipitates as a fibrous lump, collect by centrifugation. Remove supernatant and wash the precipitate with 1ml of 70% ethanol. Remove ethanol by draining.
16. The DNA pellet is dried by draining and then dissolved in distilled water. The DNA is left at 37°C to redissolve thoroughly (taking up to 2 hours).
17. If no precipitate is observed, the solution is placed at -20°C for at least 1 hour before centrifugation. The precipitate is then redissolved in 1ml of distilled water and the DNA is reprecipitated by adding 0.5ml of 7.5M ammonium acetate and 2 volumes of ethanol.
18. The DNA concentration is measured by observing the optical density of DNA solution (eg. 10µl) made up to 1ml in a 1ml curvette at 260nm (a 1mg/ml solution gives a reading of 20).

2. DNA extraction from wax blocks

When a patient is the only source of material is from histopathology labs from which DNA can be extracted. This DNA is present on the wax blocks which are booked into the lab system in the usual way and are stored in the cupboard labelled wax blocks in the extraction lab. If the whole block is received, the method used to extract DNA usually results in damage to the block and as many pathology departments want the blocks back after we have used them permission to carry out the procedure must be obtained from the pathologist who sends the block or from the clinical geneticist involved.

Wax block material is not the best material for DNA extraction for a number of reasons

(i) The samples are usually old which increases the likelihood of DNA degradation.

(ii) The tissue samples have often been stored in formalin for extended periods prior to being embedded in wax which increases the likelihood of DNA degradation. As the samples are usually old it is difficult to find out exactly how the tissue was treated.

(iii) Degraded DNA is much more difficult to PCR and requires **more amplification cycles.**

(iv) DNA extracted from wax blocks can be contaminated with chemicals e.g. formamide, wax, which may inhibit PCR completely.

(v) The amount of DNA extracted is usually low and combined with degradation problems. Southern blotting with wax block DNA is usually impossible.

Method of extraction

1. Using a scalpel blade, remove some of the tissue from the block, cut and keep in a clean weighing boat and transfer into a 15ml polypropylene tube. If shavings have been received, transfer them straight into the polypropylene tube.
2. Add 2ml of white cell lysis solution and 20µl of proteinase K. Vortex till mixed.
3. Incubate overnight at 55^{O}C.
4. Check the digestion in the morning. If the solution has gone cloudy and thick then proceed with the protocol. If not, then add 20µl more of proteinase K and leave the sample at 37^{O}C until it is digested. If after 2 days the sample is still the same, proceed with the protocol.
5. Cool on ice for 5 minutes.
6. Add 700µl of protein precipitation solution and vortex for 20 seconds.
7. Centrifuge at 3,300 rpm for 10 minutes.
8. Transfer supernatant to a fresh tube. This is difficult to do as the wax sometimes floats on the top.
9. Add 2ml of iso-propanol and 10µl of glycogen (20mg/ml). Mix well.
10. Incubate at -20^{O}C for 2 hours.
11. Centrifuge at 3,300 rpm for 10 minutes to pellet the DNA.
12. Carefully pour off supernatant and add 2ml of 70% ethanol to wash the DNA.
13. Centrifuge at 3,300 rpm for 10 minutes to pellet the DNA.
14. Pour off the supernatant and repeat the wash and centrifugation.
15. Pour off the supernatant and leave the DNA to dry in the fume cupboard.
16. Add 50-100µl of TE and leave the DNA to re-suspend in the

usual way. The amount of TE added depends on the size of the pellet of the DNA. The pellet will probably be contaminated with some wax, so add only 50µl, if uncertain. The sample can be diluted at a later stage, if necessary.

17. Estimate the concentration of DNA using the spectrophotometer.
18. Check the molecular weight of the DNA by carrying out electrophoresis of 5µl of DNA on an agarose gel. The DNA extracted is usually degraded and it is unusual to see high molecular weight DNA.
19. The DNA is now ready to use.

DNA preparation by cryotom tissue dissection

Preparations/ Materials

Cool cryostat down to -20 to -30°C about 3 hours prior to dissection.
Label eppendorf tube (2 ml, e.g. Safe Lock) and microscopic slides with the case number.
Digestion buffer (50 ml Tris, pH 8.5, 1 mM EDTA, 0.5% Tween 20)
Proteinase K (500 µl aliquots of a 20mg/ml stock solution, keep aliquots at -20°C).
Melt the tip of long Pasteur pipettes (one pipette per case).
Dissect only one block per time in the cryostat.
Keep other tissue block frozen (e.g. in styropor box with liquid nitrogen or dry ice) during dissection.
Take care to replace tissue block in the correct vial after dissection.

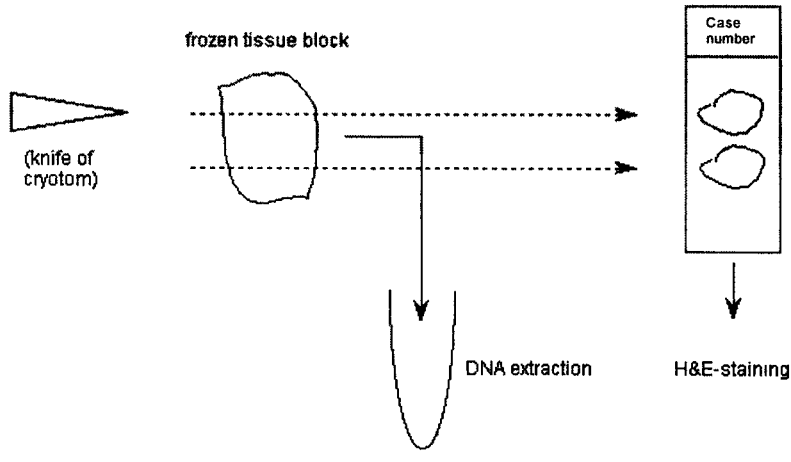


Figure 1.4 DNA extraction from frozen tissue

Steps

1. Freeze tissue block (0.5 - 1 cm^2) on the cryostat plate, e.g. by applying few drops of isotonic NaCl or water and placing the tissue immediately on the cooled cryostat plate.
2. Cut the tissue block and place first section (5-8 µm) on labeled glass slide (close to case No. label).
3. Cut about 20-30 section (20-30µm) and transfer them in the labeled eppendorf tube which contains 900µl digestion buffer. The transfer is facilitated by picking the cool sections by the tip of the pasteur pipette. Change pipette after each case.
4. Transfer last section (5-8 µm) on the glass slide (distal to case No. label).
5. Add 30-50 µl of Proteinase K (stock solution of 20 mg/ml).
6. Incubate for at least 2 hours at 50°C, check the digestion by the disintegration of the tissue, add new Proteinase K if the digestion is bad. The Proteinase K digestion can be extended over night or even for several days.
7. Add 1000 µl of Phenol/Chloroform/Isoamylalcohol, mix e.g. by inverting for about 10 min, centrifuge in a table top centrifuge for 10-20 minute until the two phases have clearly separated. Discard upper phase.
8. Repeat step 7 at least once (better: twice).
9. Add 1/10 vol (about 90 µl) 3 M NaCl, mix. Add 1 vol (about 1000 µl) ice cool isopropanol. Mix by inverting (white DNA pellet visible).
10. Centrifuge DNA pellet for about 5 min, discard supernatant, wash once with 100% EtOH and once with 70% EtOH. Finally, air dry pellet or dry it by a SpeedVac (5-10 min). If you use a Speed Vac, take care not to dry the DNA too much, since high molecular, ultra dry DNA may be difficult to dissolve.
11. Dissolve pellet in about 100-200µl HPLC grade H_2O depending on the amount of DNA.
12. Determine DNA concentration by a photometer (Final concentration for nick translation should by higher than 150 µg/ml).

DNA extraction from fresh tissue

Material required for DNA extraction from tissue are :

Liquid nitrogen
Digestion buffer
PBS, ice-cold
25:24:1 phenol/chloroform/isoamyl alcohol (Unit 2.1A)
7.5 M ammonium acetate
100% and 70% ethanol
TE buffer, pH 8
Sorvall H 1000B rotor or equivalent

Beginning with whole tissue

1a. Excise and immediately mince tissue quickly and freeze in liquid nitrogen.

If working with liver, remove gall bladder, which contains high levels of digestive enzymes.

2a. Grind 200mg to 1g tissue with pre chilled mortar and pestle, or crush with hammer to fine powder. Suspend in 1.2 ml digestion buffer per 100 mg of tissue.

DNA extraction from cultured cells.

1b. Centrifuge cells 5 min at 500 x g and discard supernatant. Trypsinize adherent cells first.

2b. Re-suspend cells in 1 to 10 ml of ice-cold PBS. Centrifuge 5 minute at 500 x g, discard supernatant, and repeat. Re-suspend cells in 1 vol. digestion buffer.

For $<3 \times 10^7$ cells, use 0.3 ml digestion buffer; for larger numbers of cells, use 1 ml digestion buffer / 10^8 cells.

3. Incubate samples, shaking, in tightly capped tubes, 12 to 18 hour at 50^0C.

4. Extract samples with an equal volume of phenol / chloroform / isoamyl alcohol. Centrifuge 10 minute at 1700 x g. If phases do not resolve well; add another volume of digestion buffer, omitting proteinase K, and repeat centrifugation. If thick white material appears at interface, repeat organic extraction. Transfer the layer (aqueous) to a new tube.

5. Add ½ vol of 7.5 M ammonium acetate and 2 vol of 100% ethanol. Centrifuge for 2 min at 1700 x g.

   To prevent shearing of high molecular-weight DNA, remove organic solvents and salt by two dialyses against 100 vol TE buffer for ≥ 24 hr; omit step 6.

6. Wash with 70% ethanol, air dry, and re suspend in TE buffer at ~ 1 mg/ ml.

   Remove residual RNA by adding 0.1% SDS and 1 µg/ml DNAse –free RNAse, incubating 1 hr. at 37^{0}C, and repeating steps 4 and 5.

The expected yield from 1 g cells is ~ 2mg DNA.

DNA quantification assays

Some DNA tests are very sensitive to the initial DNA concentration. For this reason, DNA is stored at a standard concentration of 500µg/ ml where possible. If this is not possible, then it is stored at 250µg/ml and the final concentration is written on the tube and also recorded properly.

Solutions required

1. DNA standard: 10mg/ml DNA standard,
2. Salmon or herring sperm DNA

Equipment required

1. Spectrophotometer and cuvettes
2. Standard curve (see below)
3. Blue pipette tips (200µl -1ml)
4. Yellow pipette tips (50-200µl) (Regional Supplies)
5. Automatic pipettes

Detailed method

(1) Switch on the spectrophotometer and leave to warm up for five minutes.

(2) Set the wavelength at 260nm.

(3) Preparing a Standard Curve

A standard curve needs to be plotted for each spectrophotometer as every machine is different with regard to lamps, mirrors, lenses and optical alignment. A standard solution of salmon or herring sperm

DNA is prepared in 18M water to a concentration of 10mg/ml. This should be stored at -20^{0}C for future use. Prepare from this stock solution 2ml standards of the following concentrations: 500, 1000, 1500, 2000, 2500 and 3000 mg/ml by diluting down using 18M water. Read the absorbance, as detailed below, of each standard and use the readings to plot a standard curve of DNA concentration against absorbance. This standard curve needs to be done only once provided further no change to be made.

(4) Initial Blank and Calibration of the Spectrophotometer:

a) Pipette 990µl of water into cuvette and place into the spectrophotometer with frosted side of glass facing towards you. *Always hold the cuvette on the frosted sides and ensure that the clear glass is clean.*

b) Press *MODE* button until *Abs* appears on the screen.

c) Press *Calibrate* to set Zero absorbance. The spectrophotometer is now ready to use to take the OD of the DNA samples.

(5) Taking Absorbance Measurements:

a) Add 10µl of DNA sample to the 990 µl of water in the cuvette. (*N.B. DNA sample is diluted by a factor of 100.)*

b) Invert cuvette several times with the little lid in place.

c) Place cuvette in the spectrophotometer and read off the absorbance reading.

d) Determine the concentration of the DNA by reading off the value from the standard curve or if no curve.

e) Record the result on the sample worksheet.

f) Empty the cuvette and wash it with distilled water.

(6) For every subsequent DNA sample, the Spectrophotometer must be initially brought to zero (As below).

a) Pipette 990 µl of water into cuvette and place in the spectrophotometer.

b) Calibrate absorbance to Zero.

c) Continue with the taking absorbance measurements section.

(7) Adjusting DNA samples to a Standard Laboratory Concentration.

Read from the appropriate graph, 500 g/ml or 250 g/ml, the extra amount of TE to be added to the re suspended DNA. Record the final concentration on the sample work-sheet. If the DNA can not be

made up to 500 µg/ml, then record the final concentration on the DNA tube. Place on rotator to mix DNA completely.

(8) Calculation of DNA concentrations if no standard curve is available:

1 OD = 50µg/ml of double stranded DNA. The DNA sample is diluted by a factor of 100 in the cuvette. Therefore, DNA Concentration (µg/ml) = OD reading X 50 X 100, or DNA Concentration (µg/ml) = OD Reading X 5000.

Assessing the purity of DNA

Assessing the purity of DNA means checking the presence of protein and RNA in the final solution. The purity is assessed by taking the OD at 260/280 ratio.

Method

a) Measure the OD of the sample, as described in the method above, at a wavelength of 260nm. Do not throw the sample away.

b) Alter the wavelength to 280nm after 260nm.

c) Auto zero, the spectrophotometer at this new wavelength using a separate cuvette containing water (as described above).

d) Measure the OD of the same sample, in your first cuvette, at this new wavelength.

To assess the purity of the DNA, the ratio is of the two absorbencies can be calculated.

$$\text{Ratio} = \frac{\text{OD at wavelength 260nm}}{\text{OD at wavelength 280nm}}$$

1: A ratio of 1.8 denotes pure DNA. A range of 1.6 - 2.0 is taken as sufficiently pure DNA.

2: A ratio above 2 denotes the presence of RNA in the DNA sample.

3: A ratio below 1.8 denotes the presence of protein in the DNA sample.

Analysis of DNA

Analysis of DNA is carried out on Agarose Gel Electrophoresis. Presence of DNA is visualized by size fractionating the DNA which is dissolved in DDW or TBE buffer in 0.8% Agarose gel. Electrophoresis is carried out at 50V for 30 minutes. Samples are finally

visualised using ethidium bromide staining on UV trans-illuminator.

DNA from Plasmids

Plasmids are circular, supercoiled DNA molecules present in most species, but not all strains of bacteria. They are very small, ranging from 0.2 to 4 percent of the bacterial chromosome.

(1) Plasmid carry their own site of origin of replication as shown in **(Figure 1.5).**

(2) Plasmid carry one to many antibiotic resistance site like pUC18 carries Ampicillin resistance site, pBR322 carries both ampicillin and tetracycline resistance site **(Figure 1.5).**

(3) Plasmid have F-factor genes which helps in reproduction of bacteria.

(4) Plasmid DNA carry number of restriction sites which makes them highly suitable for recombinant DNA technology and cloning **(Figure 1.5).**

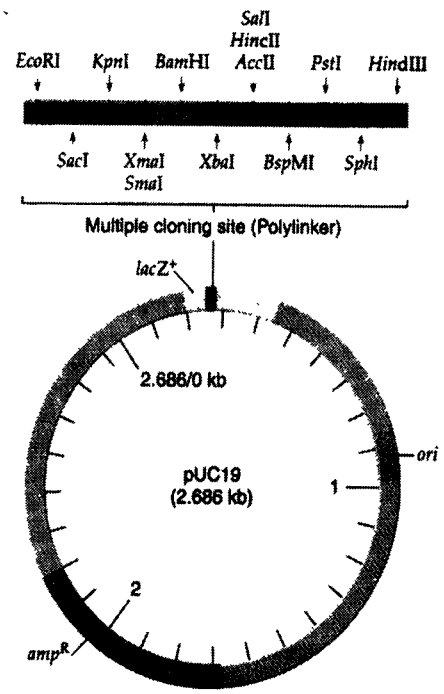


Figure 1.5: Plasmid with its origin of replication, ampicillin resistance and multiple restriction sites

Under most of the conditions required for growth, plasmids are dispensable to their host cells.

However, many plasmids contain genes that have value in particular environment, and often these genes are the main indication that a plasmid is present. For example, R plasmids render their host cells resistant to certain antibiotics, so in nature, a cell containing such a plasmid can better survive in environment in which a fungal antibiotic is present.

In many bacterial species, plasmids are responsible for a particular type of gene transfer between individual cells, a property that is accounted for the initial interest in the 1950s. Furthermore, like phages, plasmids are heavily dependent on the metabolic function of the host cell for their reproduction. They normally use most of the replication machinery of the host and hence, have been useful model for understanding certain features of bacterial DNA replication. Finally, they have been exceedingly valuable to the microbial geneticist in constructing partial diploids and as a gene-cloning vehicle in genetic engineering.

Types of plasmids

Several different types of plasmids can coexist in a single cell, e.g., up to seven in E. coli. Two plasmids can be incompatible, resulting into the destruction of one of them. Therefore, plasmids can be assigned into *incompatibility groups*, depending on their ability to coexist in a single cell. An obvious way of classifying plasmids is by function. There are five main classes:

1. F, the sex, or fertility, plasmids. These plasmids mediate the ability to transfer chromosomal genes (that is genes not carried on the plasmid) from a cell containing an F plasmid to one that does not. Their only function is to initiate conjugation. Bacterial conjugation is the process of bacteria mating, the sexual exchange of the DNA. One of the bacteria must carry the F-plasmid, other one may not. The F-plasmid (also called as F factor is an episome (a plasmid that can integrate into the bacterial chromosome by genetic recombination) of about 100 kb length. It carries its own origin of replication, called as "ori". There can only be one copy of the F-plasmid in a bacterium (which is called as F positive) either free or integrated.
2. R, the drug-resistance plasmids. These plasmids make the host cell resistant to one or more antibiotics, and many R plasmids can transfer the resistance to cells lacking R.
3. Col, the colicinogenic factors, Col plasmids synthesize proteins, collectively called colicins that can kill closely related bacterial strains that lack a Col plasmid of the same type. The mechanism of killing is different for different Col plasmids.
4. Degrative plasmids, which enable the digestion of unusual substances, e.g., toluole or salicylic acid.
5. Virulence plasmids, which turn the bacterium into a pathogen.

With only a single exception, the killer - plasmid of yeast, which is an RNA molecule. The molecular weights of the DNA range from about 10^6 for the smallest plasmid to slightly more than 10^8 for the largest one. **Table 1.1** lists the molecular weights for several plasmids that are actively being studied.

Table 1.1 Different plasmids and their properties

Plasmid	Mass x 10^6	No. copies / chromosome	Self - transmissible	Phenotypic features
Col plasmids				
ColE1	4.2	10 - 15	No	Colicin E1 (membrane changes)
ColE2 (Shigella)	5.0	10 - 15	No	Colicin E2 (DNase)
ColE3	5.0	10 - 15	No	Colicin E3 (ribosomal RNase)
Sex plasmids				
F	62	1 - 2	Yes	F pilus
F'/ac	95	1 - 2	Yes	F pilus; / ac operon
R plasmids				
R100	70	1 - 2	Yes	Cam -r Str-r Sul-r Tet-r
R64	78	(limited)	Yes	Tet-r Str-r
R6K	25	12	Yes	Amp-r Str-r
pSC101	5.8	1 - 2	No	Tet-r
Phage plasmid				
λ dv	4.2	≈ 50	No	λ genes cro, cI, O, P
Recombinant plasmids				
pBR322	2.9	≈ 20	No	High - copy - number
pBR345	0.7	≈ 20	No	ColE1-type replication

Extraction of plasmid DNA

Plasmid DNA can be isolated from bacteria in a simple way.

1. Plasmid -containing bacteria are opened by the lysozyme - detergent treatment, and the resulting translucent solution, called a cell lysate, is centrifuged.
2. The bacterial chromosome complex, which contains protein and RNA, is very large and compact and sediment to the bottom of the centrifuge tube; the smaller plasmid DNA remain in the clear supernatant, which is called a clear lysate.
3. Some chromosomal DNA is usually present in the clear lysate, but since most of the plasmid DNA is covalently circular, this contaminating material can be easily removed by the following procedure.

4. CsCl plus ethidium bromide **(Figure 1.6)** is added to the clear lysate, and the lysate is centrifuged to equilibrium. When ethidium bromide is present, the covalently circular DNA has a higher density then the linear chromosomal fragments, so the plasmid DNA is purified. The only disadvantage of this most commonly used technique is that both nicked circles (which often form accidentally during isolation of plasmid DNA) and non-super coiled replicating molecules are discarded, since they come to equilibrium within the chromosomal - DNA band.

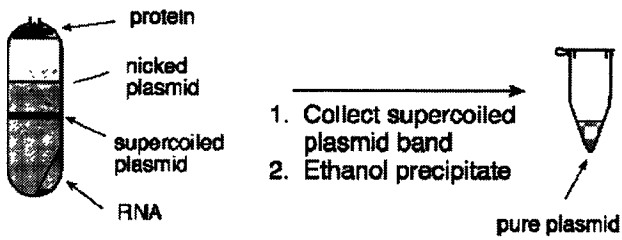


Figure 1.6: CsCl gradient method for extraction of plasmid DNA

Alkaline Lysis method:

The isolation of DNA usually entails a deproteinization step (for example, treatment with phenol). When the DNA molecules of some E. coli plasmids are isolated without such a step, about half of the super coiled DNA molecules contain three tightly bound protein molecules. This DNA-protein complex is called a relaxation complex. If this complex is heated or treated with alkali, proteolytic enzymes, or detergents, one of these proteins, which is a nuclease, nick one DNA strand, thereby "relaxing" the super coil to the nicked circular form **(Figure 1.7)**.

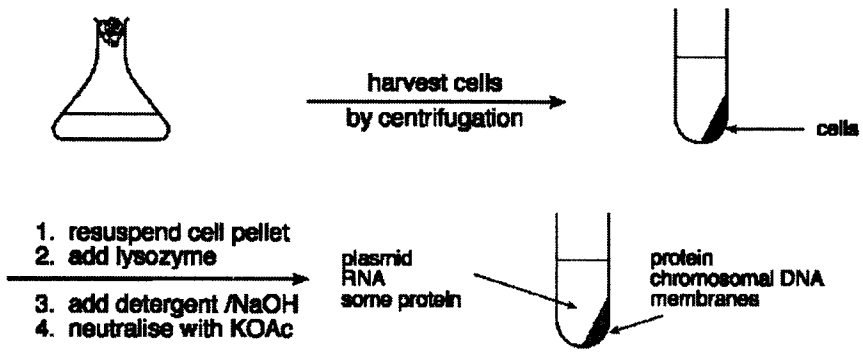


Figure 1.7: Plasmid Extraction by Alkaline lysis method

This nick occurs in only one strand and at a unique site. During relaxation, the two smallest proteins are released, but the largest protein becomes covalently linked to the 5'-P terminus of the nick. If prior to relaxation, the super coiled DNA can be nicked by any of the laboratory techniques, the relaxation nuclease is unable to make its site-specific nick, indicating that the nuclease is active only on super coiled DNA. This nicking, which presumably also occur within cells, is an early step in plasmid transfer. Plasmid DNA can then be precipitated using Ethanol precipitation **(Figure 1.8).**

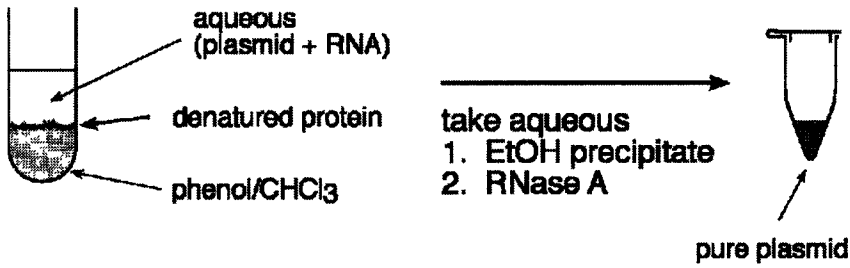


Figure 1.8: Ethanol extraction of plasmid DNA following ethanol precipitation

Protocol for Plasmid isolation
Reagents

(A) STE

NaCl	0.1M
Tris Cl	10mM
EDTA	1mM

(B) Solution - 1

Glucose	50mM
Tris Cl	25mM
EDTA	10mM

(C) Solution - 2

NaOH freshly diluted from 1N NaOH	0.2N
SDS	1%

(D) Solution - 3

K-acetate	5M
Glacial acetic acid	11.5%

Protocol

1. Wash bacterial cell pellet with 2 ml STE by centrifuging at 6,000 rpm for 4 minutes.
2. Add 100µl ice-cold solution -1
   200µl ice-cold solution -2
   150µl ice-cold solution -3 (freshly prepared)
3. Vortex 4-5 times to resuspend the pellet
4. Keep on ice for 5 minutes
5. Centrifuge at 12,000 rpm for 5 minutes
6. Take supernatant (~ 400µl)
7. Add 2 vol ethanol (absolute)
8. Keep for 2 - 3 minutes at room temperature (RT)
9. Centrifuge at 12,000 rpm for 5 minutes
10. Decant the supernatant
11. Rinse with 1ml 70% ethanol by centrifuging at 12,000 rpm for 2 minutes
12. Air dry
13. Dissolve in 30µl HPLC grade water
14. Add 1µl RNAse (10mg/ml)
15. Keep at 37^0C for 1 hour
16. Run on 1% agarose gel
17. Store at - 70^0C till use

Most important points to remember while handling DNA

Precautions for handling DNA	
Handling fresh and stored material before extraction of DNA	For the isolation of genomic and plasmid DNA from cells and tissues, use either fresh samples or samples that have been quickly frozen in liquid nitrogen and stored at -70^0C. This procedure minimizes degradation of the DNA by limiting the activity of endogenous nucleases. For best results, use fresh blood or blood stored for <2 days at room temperature. Blood stored for 7 days at 4^0C or for < 1 month at -20^0C will result into 10 to 15% reduction of yield.

	Collect blood samples in tubes containing EDTA as anticoagulant, not heparin as heparin can cause attenuation or inhibition of amplification during PCR. However, if heparin cannot be avoided, the High Pure PCR Template Preparation Kit can be used to remove the heparin from the sample.
Pipetting DNA	Avoid vigorous pipetting. Pipetting genomic DNA through small tip openings causes shearing or nicking. Use tips with wide openings, specially designed for genomic DNA, or cut the end of tips and make them blunt. Regular pipette tips pose no problem for plasmid DNA and other small DNA molecules.
Storage of DNA	Store genomic DNA at 4^{0}C, storage at -20^{0}C can cause shearing of the DNA. Plasmid DNA and other small DNA molecules can be stored at 4^{0}C for short term storage. In aliquots at -20^{0}C for long term storage. Keep plasmids for transformation purposes at 4^{0}C to avoid nicks. Store modified DNA at 4^{0}C.
Manipulation of DNA	Always keep the DNA sample on ice when preparing an experiment.
Drying DNA	Avoid over drying of genomic DNA after ethanol precipitation. Let the DNA air dry. Plasmid DNA and other small DNA molecules can be air or vacuum dried.
Dissolving DNA	Dissolve DNA in tris buffer (e.g. 10 mM Tris, pH 7.0-pH 8.0). To help to dissolve the DNA, carefully invert the tube several times after adding buffer and / or tap the tube gently on the side. Alternatively, let the DNA stand in buffer overnight at 4^{0}C. Do not vortex genomic DNA. Heat DNA for 10 min at 65^{0}C to dissolve and inactivate DNAses.

Most useful calculations

Calculation of molecular weights in Dalton	Average molecular weight (MW) of a deoxynucleotide : 330 Dalton (Da) Average molecular weight (MW) of a DNA basepair : 660 Dalton (Da) MW of dsDNA = [number of basepairs] x [660 Da]

e.g. MW of pBR322 dsDNA (4,363 basepairs) =
4,363 x 660 Da = 2.9 x 106 Da = 2.9 x 103 Da
MW of ssDNA = [number of bases] x [330 Da]
e.g. MW of M13mp 18 (7,249 bases, ssDNA form) =
7,249x330Da = 2.4 x 106Da = 2.4 x 103kDa
Da (Dalton) is a unit of mass very nearly equal to that of a hydrogen atom (precisely equal to 1,0000 on the atomic mass scale). Named after John Dalton (1766-1844) who developed the atomic theory of matter.

Calculation of pmol of 5′ (or 3′) ends

pmole of ends of a dsDNA molecule

$$= \frac{2 \times 10^6 \times \mu g \text{ (of dsDNA)}}{\text{MW (in Da)}} = \frac{2 \times 10^6 \times \mu g \text{ (of dsDNA)}}{N_{bp} \times 660 \text{ Da}}$$

e.g pmol of 5′ or 3′ ends of 1 µg of a 100 basepairs dsDNA fragment = $2 \times 10^6 \times 1 / 100 \times 660 = 30.3$
pmole of ends of a ssDNA molecule

$$= \frac{1 \times 10^6 \times \mu g \text{ (of ssDNA)}}{\text{MW (in Da)}} = \frac{1 \times 10^6 \times \mu g \text{ (of ssDNA)}}{N_{bp} \times 330 \text{ Da}}$$

e.g pmol of 5′ or 3′ ends of 1 µg of a 250 basepairs ssDNA fragment = $1x\ 10^6 \times 1 / 250 \times 330 = 12.12$
pmol of ends generated by restriction endonucleases cleavage.
* Circular DNA: 2 x (pmol of DNA) x (number of sites)
* Linear DNA: [2x (pmol of DNA) x (number of sites)] + 2 x (pmol of DNA)

Conversion of µg to pmol

pmol of dsDNA = µg (of dsDNA)

$$\times \frac{10^6 \text{ pg}}{1 \mu g} \times \frac{1 \text{ pmol}}{660 \text{ pg}} \times \frac{1}{N_{bp}}$$

= µg (of dsDNA) x 1,515 / N_{bp}
e.g. 1µg of a 100 basepairs dsDNA fragment = 1 x 1,515 / 100 = 15.2 pmol
pmol of ssDNA = µg (of ssDNA)

$$\times \frac{10^6 \text{ pg}}{1 \mu g} \times \frac{1 \text{ pmol}}{330 \text{ pg}} \times \frac{1}{N_b}$$

= µg (of ssDNA) x 3030 / N_b
e.g. 1µg of a 1000 bases ssDNA fragment = 1 x 3030 / 1000 = 3.03 pmol

Conversion of pmol of µg

µg of dsDNA =pmol (of dsDNA)

$$\times \frac{660 \text{ pg}}{1 \text{ pmol}} \times \frac{1 \mu g}{10^6 \text{ pg}} \times N_{bp}$$

= pmol (of dsDNA) x N_{bp} x 6.6 x 10-4
e.g. 1 pmol of a 100 basepairs dsDNA fragment
= 1 x 100 x 6.6x 10-4 = 0.066µg
µg of ssDNA = pmol (of ssDNA)

$$x \frac{330\ pg}{1\ pmol} x \frac{1\ \mu g}{10^6\ pg} x\ N_{bp}$$

= pmol (of ssDNA) x N_b x 3.3 x 10^{-4}
e.g. 1 pmol of a 250 bases ssDNA fragment = 1 x 250 x 3.3 x 10^{-4} = 0.0825 µg
[N_{bp} = number of base pairs (dsDNA) and N_b = number of bases (ssDNA)]

Table 1.2 Type of DNA

Type	Size	From	MW (in kDa)	Pmol /µg	µg/ pmol	pmol of 5′ or 3′ ends/µg
dsDNA fragment	100 bp	Linear	66	15.2	0.066	30.3
dsDNA fragment	500 bp	Linear	330	3.03	0.33	6.06
dsDNA fragment	1000 bp	Linear	660	1.52	0.66	3.03
		Restriction enzyme Digest, 1 site		1.52	0.66	6.06
		Restriction enzyme Digest, 2 sites		1.52	0.66	9.12
pUC18/19 dsDNA	2686 bp	Circular	1.8x 10^3	0.57	1.77	-
		Restriction enzyme Digest, 1 site		0.57	1.77	1.14
		Restriction enzyme Digest, 2 sites		0.57	1.77	2.28
		Restriction enzyme Digest, 3 sites		0.57	1.77	3.42
pBR322 dsDNA	4363 bp	Circular	2.9 x 10^3	0.35	2.88	-
		Restriction enzyme Digest, 1 site		0.35	2.88	0.7
		Restriction enzyme Digest, 2 sites		0.35	2.88	1.4
		Restriction enzyme Digest, 3 sites		0.35	2.88	2.1

If the DNA is from different sources one can use different kits to isolate the DNA or different methodologies can be used. However, the principle remains the same.

The size and molecular weight of DNA from different organism is shown in table 1.3.

Table 1.3 Sizes and weights of DNA from different organism

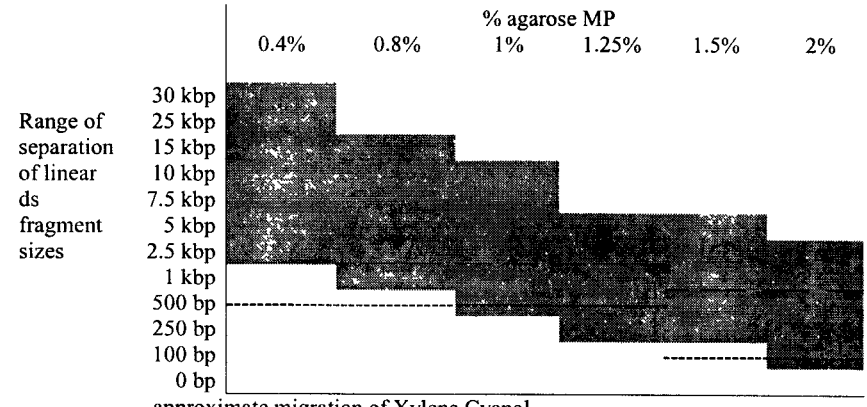
Organism	Size (bp)	Molecular weight (in kDa)	Number of chromosomes
pBR-322, E. coli plasmid	4363	2.9×10^3	
SV 40, simian virus	5243	3.5×10^3	
Φ X 174, E. coli bacteriophage	5386	3.5×10^3	
Adenovirus 2, human virus	35937	23.7×10^3	
Lambda, E. coli bacteriophage	48502	32.0×10^3	
E. coli, bacterium	4.7×10^6	3.1×10^6	
Saccharomyces cerevisiae, yeast	1.5×10^7	9.9×10^6	32 (diploid)
Dictyostelium discoideum, mold	5.4×10^7	3.6×10^7	7 (haploid)
Caenorhabditis elegans, worm	8.0×10^7	5.3×10^7	11/12 (diploid)
Drosophila melanogaster, fruitfly	1.4×10^8	9.2×10^7	8 (diploid)
Mus musculus, mouse	2.7×10^9	1.8×10^9	40 (diploid)
Xenopus leavis, frog	3.1×10^9	2.0×10^9	36 (diploid)
Homo sapiens, human	3.3×10^9	2.2×10^9	46 (diploid)
Zea mays, maize	3.9×10^9	2.6×10^9	20 (diploid)
Nicotiana tabacum, tabacum plant	4.8×10^9	3.2×10^9	48 (diploid)

Most important aspect in DNA prepration is to have good quality DNA with high concentration. This can be checked by OD measurement as shown in Table 1.4.

Table 1.4 Concentration and purity via OD measurement

Concentration of DNA	1 A_{260} unit of dsDNA = 50 µg/ ml H_2O 1 A_{260} Unit of ssDNA = 33 µg/ml H_2O
Notes	OD value should lie between 0.1 and 1.0 to ensure an optimal measurement. The above mentioned values are based on extinction coefficients of nucleic acids in H_2O, note that these coefficients - and hence the above mentioned values - differ in other buffers and/ or solutions. Example of calculation: Volume of dsDNA sample: 100 µl Dilution: 25 µl of this sample +475 µl H_2O (1/20 dilution) A_{260} of this dilution: 0.44 Concentration of dsDNA in sample: 0.44 x 50 µg/ml x 20 (=dilution factor) = 440 µg/ml Amount of dsDNA in sample: 440 µg/ml x 0.1 ml (= sample volume) = 44 µg
Purity of DNA	Pure DNA: A_{260} / $A_{280} \geq 1.8$
Notes	An A_{260} / $A_{280} < 1.8$ indicates that the preparation is contaminated with proteins and aromatic substances (e.g. phenol). An A_{260} / $A_{280} > 2$ indicates a possible contamination with RNA. The OD gives no information about the size of the DNA.

Size of DNA can be estimated by using agrose gel or by polyacrylamide gel electrophoresis as shown in **Figure 1.9** and **Figure 1.10**.



* Agarose Multi purpose – available from different sources.

Figure 1.9 Size estimation of DNA fragments in Agarose MP gels

Further, the size estimation can also be carried out on acrylamide gels.

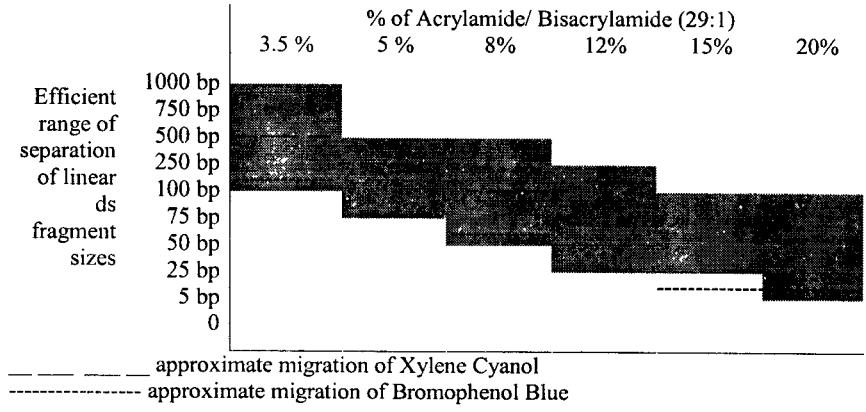


Figure 1.10 Size estimation of DNA fragments in acrylamide gels

Ribose Nucleic Acid Extraction

Ribose nucleic acid is the second type of nucleic acid found in the cell. The role of RNA is three-fold: as a structural molecule, as information transfer molecule and also as an information-decoding molecule. RNA molecules read and interpret the information in DNA. RNA molecules are key players in the reactions that turn information into useful work.

RNA Structure and function:

The tertiary structure of RNA is similar to DNA, but there are several important differences:

- Presence of ribose sugar instead of deoxy ribose and Uracil instead of thymine as a pyrimidine nitrogenous base.
- RNA usually forms intra molecular base pairs.
- The information carried by RNA is not redundant because of these intra molecular base pairs.
- The major and minor grooves are less pronounced.
- The structural, informational adaptor and information transfer roles of RNA are all involved in decoding the information carried by DNA.

Types of RNA

There are four basic types of RNA found in cells, each carry out different and specific functions: (i) tRNA - transfer RNA (ii) mRNA - messenger RNA (iii) rRNA - ribosomal RNA and (iv) snRNA - small nuclear RNA.

Messenger RNA (mRNA)

Messenger or mRNA is a copy of the information carried by a gene on the DNA. The role of mRNA is to move the information contained in DNA to the translation machinery. mRNA is heterogeneous in size and sequence. It always has a 5 ' cap composed of a 5' to 5' triphosphate linkage between two modified nucleotides: a 7-methylguanosine and a 2' O-methyl purine.

This cap serves to identify this RNA molecule as an mRNA to the translational machinery. In addition, most mRNA molecules contain a poly-Adenosine tail at 3' end. Both the 5' cap and the 3' tail are added after the RNA is transcribed and contribute to the stability of the mRNA in the cell. mRNA is not made directly in a eukaryotic cell. It is transcribed as heterogeneous nuclear RNA (hnRNA) in the nucleus. hnRNA contains introns and exons. The introns are removed

by RNA splicing leaving the exons, which contain the information, joined together. In some cases, individual nucleotides can be added in the middle of the mRNA sequence by a process called RNA editing. In figure 1.11 the exons are represented as the region of variable sequence.

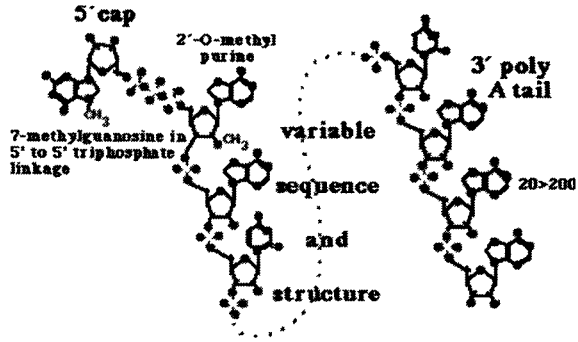


Figure 1.11 Structure of mRNA molecule

hnRNA and mRNA are never found free in the cell. Like DNA, they are bound by cations and proteins. These complexes are termed ribonucleoproteins or RNPs. The variability in sequence and structure means that no structure has been determined for a mRNA **(Figure 1.12)**.

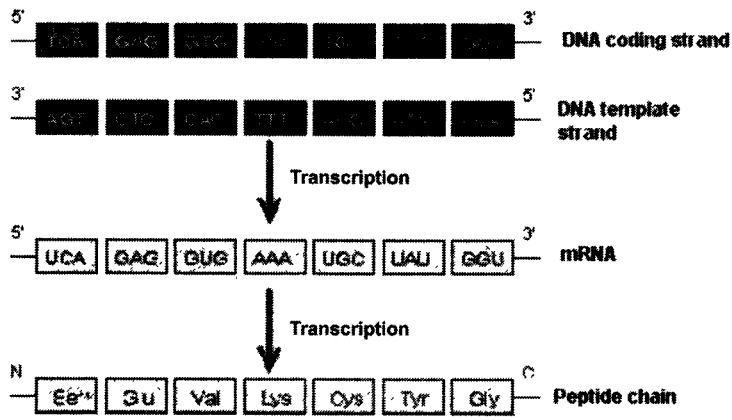


Figure 1.12: The sequence relationship of DNA, mRNA and the encoded peptide. The sequence of mRNA is complementary to DNA's template strand, and thus the same as DNA's coding strand, except that T is replaced by U.

Transfer RNA (tRNA)

tRNA is the information of the adapter molecule. It is the direct

interface between amino-acid sequence of a protein and the information in DNA. Therefore, it decodes the information in DNA. There are > 20 different tRNA molecules. These are between 75-95 nucleotides. All tRNA's form in all organisms has a similar structure, indeed a human tRNA can function in yeast cells.

Transfer RNA has 4 arms and 3 loops. The acceptor, D, T pseudouridine C and anticodon arms, and D, T pseudouridine C and anticodon loops (**Figure 1.13**).

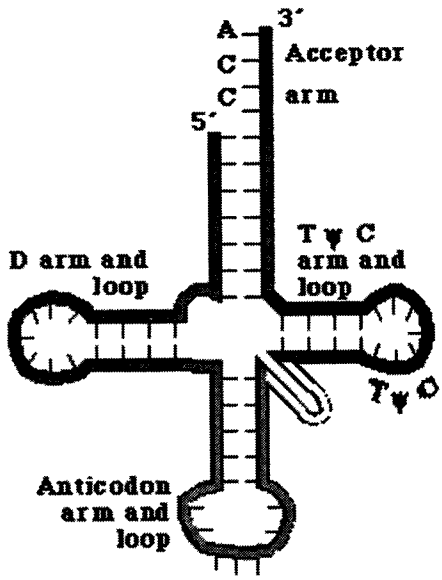


Figure 1.13: Structure of a tRNA molecule

Sometimes tRNA molecules have an extra or variable loop. tRNA is synthesized in two parts. The body of the tRNA is transcribed from a tRNA gene. The acceptor stem is same for all tRNA molecules and is added after the body is synthesized. It is replaced often during lifetime of a tRNA molecule.

The major role of tRNA is to **translate** mRNA sequence into amino acid sequence. A tRNA molecule consists of 70-80 nucleotides. Some nucleotides in tRNA have been modified, such as **dihydrouridine (D), pseudouridine (Y)**, and **inosine (I)**. In dihydrouridine, a hydrogen atom is added to each C5 and C6 of uracil. In pseudouridine, the ribose is attached to C5, instead of the normal N1. Inosine plays an important role in codon recognition. In addition to these modifications, a few nucleosides are methylated.

Ribosomal RNA (rRNA)

Ribosomal RNA (rRNA) is a component of the ribosomes, the protein synthetic factories in the cell. Eukaryotic ribosomes contain four different rRNA molecules: 18 s, 5.8 s, 28 s, and 5 s rRNA. Three of the rRNA molecules are synthesized in the nucleolus, and one is synthesized elsewhere. rRNA molecules are extremely abundant.

They make up at least 80% of the RNA molecules found in a typical eukaryotic cell.

Synthesis of the three nucleolar rRNA molecules is unusual because they are made on one primary transcript that is chopped up into three mature rRNA molecules. These rRNA molecules and the 5S rRNA combine with the ribosomal proteins in the nucleolus to form pre 40S and pre 60S ribosomal subunits. These pre-subunits are exported to the nucleus where they mature and assume their role in protein synthesis.

In prokaryotes, the ribosomal RNA (rRNA) has three types: 23S, 5S, and 16S. In mammals, four types of rRNA have been found: 28S, 5.8S, 5S and 18S. The unit "S" stands for Svedberg, which is a measure of the sedimentation rate. After rRNA molecules are produced in the nucleus, they are transported to the cytoplasm, where they combine with tens of specific proteins to form a ribosome. In prokaryotes, the size of a ribosome is 70S, consisting of two subunits: 50S and 30S. The size of a mammalian ribosome is 80S, comprising a 60S and a 40S subunit. Proteins in the larger subunit are designated as L1, L2, L3, etc. (L = large). In the smaller subunit, proteins are denoted by S1, S2, S3, etc.

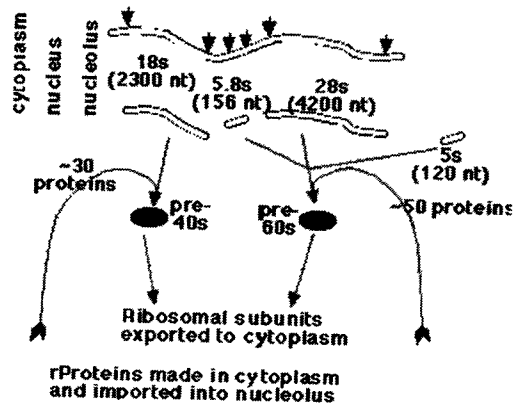


Figure 1.14 Production of r Proteins and

The rRNA molecules have several roles in protein synthesis. First, the 28S rRNA has a catalytic role, it forms part of the peptidyl transferase activity of the 60S subunit. Second, 18S rRNA has a recognition role, involved in correct positioning of the mRNA and the peptidyl tRNA. Finally, the rRNA molecules have a structural role.

They fold into three-dimensional shapes that form the scaffold on which the ribosomal proteins assemble. (**Figure 1.14**)

Small nuclear RNA (snRNA)

Small nuclear RNA (snRNA) is the name which refers to a number of small RNA molecules found in the nucleus. These RNA molecules are important in number of processes including RNA splicing (removal of the introns from hnRNA) and maintenance of the telomers, or chromosomal ends. They are always found associated with specific proteins and the complexes are referred to as small nuclear ribo-nucleo-proteins (SNRNP) or sometimes as snurps. Antibodies against snurps are found in large number of autoimmune diseases.

A simple and quick procedure for obtaining a sufficient quantity of cellular RNA is given below. The analyses of its purity, carrying out RNA gel electrophoresis, and studying gene expression using an RT-PCR are mentioned below.

Background

There are various techniques for purification of total RNA which are now in use and their application depends on the nature of the RNA required. For example, if RNA is going to be used for a quantitative RT-PCR, the intactness of the purified RNA is not critical, whereas intact RNA is required for cDNA library preparation or Northern blot analysis. Complete removal of DNA contamination is critical if RNA is used in an RT-PCR, but is not important in*in vitro* translation.

The physical and chemical properties of RNA and DNA are very similar. Thus, the basic procedures used in RNA purification are similar to those of DNA. All of the RNA purification methods incorporate the following steps:

1. Disruption of cells or tissue.
2. Effective denaturation of nucleoprotein complexes and removal of proteins.
3. Concentration of RNA molecules.
4. Determination of the purity and integrity of isolated RNA.

In addition, methods must include procedures that remove co-purified DNA from the preparation. In contrast to DNA purification, guarding against physical shearing of RNA molecules is not necessary because RNA molecules are much smaller and much more flexible than DNA molecules. In RNA protocols, strong physical forces during cell and tissue breakage are frequently used and the use of wide-mouth pipettes is not required. However, RNA isolation is much more difficult than DNA purification largely due to the sensitivity of RNA to

degradation by internal and external ribonucleases. These enzymes are omnipresent and are very stable molecules that do not require any co-factors for their function. A crucial aspect of any procedure for RNA purification is fast and irreversible inactivation of endogenous RNAses and protection against contamination with exogenous RNAse during the isolation procedure. To these ends, all extraction buffers include powerful RNAse inhibitors and all solutions and equipment used are treated to remove exogenous RNAse.

Elimination of RNAse

The most commonly used inhibitors included in extraction buffers for inhibiting endogenous RNAse are as follows:

Strong protein Denaturation agents. These include guanidinium hydrochloride and guanidinium isothiocyanate used at a concentration of 4M. These chaotropic agents can quickly inactivate endogenous RNAses and contribute to denaturation of nucleoprotein complexes. In order to denature RNAse irreversibly by these compounds, a high concentration of 2-mercapthoethanol is also included. This type of inhibitor will be used in our RNA preparation procedure.

Vandyl-Ribonucleoside complexes. Oxovanadium IV ions form complexes with any ribonucleoside and bind to most RNAses, inhibiting their activity. A 10mM solution is used during cell breakage and is added to other buffers used in RNA isolation. Complexes can be used with deproteinizing agents (phenol or chloroform: isoamyl alcohol or CIA) and with chaotrophic agents. The compound is difficult to be removed from purified RNA. Any residual amount of vanadyl will inhibit many enzymes used in subsequent RNA manipulations.

Aurintricarboxylic acid (ATA). This compound binds selectively to RNAse and inhibits its activity. ATA is usually incorporated into extraction buffer used for bacterial RNA preparations. The inhibitor can affect certain enzymes and is not used if RNA will be used in primer extension or S1 nuclease experiments.

Macaloid. This is naturally occurring clay (sodium magnesium lithofluorosilicate). Being negatively charged, it strongly absorbs all RNAse. The macaloid and bound RNAse are removed from the preparations by centrifugation (Marcus and Halvorson, 1967).

Protein RNAse inhibitors such as RNasin. A protein originally isolated from human placenta, it inhibits RNAse by non competitive binding. It cannot be used in extraction buffer containing a strong

denaturant. It is usually included in solutions used in the later stages of purification or in buffers used in storage or subsequent RNA manipulations.

The most frequent sources of exogenous RNAse contamination are one's hands and bacteria and fungi present on airborne dust particles. The most frequently used inhibitors for removing exogenous RNAse contamination are as follows:

Diethyl pyrocarbonate (DEPC). DEPC causes enzyme inactivation by denaturing proteins. Inactivation of RNase is irreversible. The compound is used for removing RNAse from solutions and glassware used in RNA preparation. DEPC should be used with care because it is highly flammable and a suspected strong carcinogen.

RNAseZap™ or RNAse off solutions. These are commercially available reagents which destroy RNAses on contact and act very effectively. The decontamination solutions are not toxic and can be used for removing RNAse from all surfaces and equipment. The compositions of these reagents are trade secrets.

Methods of RNA isolation

Three methods of RNA isolation or their modification are most frequently used: a guanidinium hot phenol method, a high-salt lithium chloride method, and a TRI-Reagent™ method.

The guanidinium hot-phenol method is a modification of the procedure first described by Chirgwin et al. (1979) and Chomczynski and Sacchi (1987). This single-step extraction procedure takes advantage of the characteristic of RNA to remain in the aqueous phase under acidic conditions containing 4M guanidine thiocyanate, while DNA and proteins are distributed into the phenol-chloroform organic phase. Distribution of DNA into the organic phase is particularly efficient if the DNA molecules are small. The method therefore uses a procedure for fragmenting DNA into molecules not larger than 10kb. This method is used for isolating total RNA from a variety of prokaryotic and eukaryotic cells. The efficiency of this method is very high (80-90 percent), affording purification of a large quantity of high quality RNA.

The high-salt lithium chloride method is frequently used for isolating RNA from plant tissues that are particularly rich in various secondary products such as anthocyanins, phenolic compound, polysaccharides,

and latex. It has been shown that it is very difficult to isolate pure RNA from such plants using chaotropic agents. The procedure involves cell breakage in low pH, high salt buffer in the presence of RNAse inhibitors. Protein and DNA are removed by acidic phenol - CUA extraction and RNA is recovered by lithium chloride precipitation.

The TRI-Reagent™ method is a single step method of RNA isolation using a monophasic solution of phenol and guanidine isothiocyanate combined with precipitation of RNA by isopropanol in the presence of high salt. The method is particularly useful for fast isolation of RNA from numerous small samples and can be used with all types of cells and tissues. We will use a modification of this method (RNAwiz™).

Safety precautions

RNAwiz™ contains phenol and guanidinium isothiocyanate. Both reagents are harmful to the skin. There reagents are rapidly absorbed by and are highly corrosive to the skin. It initially produces a white softened area, followed by severe burns. Because of the local anesthetic properties of phenol, skin burns may not be felt until there has been serious damage. Gloves should be worn when working with this reagent. Because some brands of gloves are soluble or permeable to phenol, they should be tested before use. If TRI-Reagent™ is spilled on the skin, flush off immediately with a large amount of water and treat with a 70 percent solution of PEG (polyethylene glycol) 4000 in water. Used reagent should be collected into a tightly closed glass receptacle and stored in a chemical hood until proper disposal.

Technical tips

The success of the experiment critically depends on rigorous control of RNAse contamination. In order to prevent contamination of equipment and solutions with RNAse, the following precautions should be taken:

1. Students should wear gloves all time. Because gloves can be easily contaminated with RNAse, they should be changed frequently.
2. All tubes should be kept closed at all times.
3. Whenever possible disposable, certified RNAse-free tubes, pipette tips, and plasticware should be used. Regular microfuge tubes and tips usually are not contaminated with RNAse and

they do not require special treatment if they are used from unopened bags.

4. All glassware should be treated with 0.1 percent DEPC water solution and autoclaved to remove DEPC. It is also possible to inactivate RNAse by baking glassware at 180^{0}C for at least 2 hours or overnight. Alternatively, RNAse can easily be and efficiently be eliminated from glassware, countertops, pipettrs, and plastic surfaces using RnaseZapTM solution.
5. All solution should be made with DEPC-treated water. Deionized water from a MilliQ RG apparatus can be used directly in all applications instead of DEPC-treated water because it does not contain RNAse.
6. Since RNAse treatment is frequently used in DNA isolation procedures, gel electrophoresis of DNA can cause electrophoresis tray and gel box contamination. Before their use for RNA gel electrophoresis, the gel tray and box should be decontaminated. To decontaminate the gel apparatus and gel-casting trays, treat them with RnaseZap^{TM} solution. Instead of RnaseZapTM solution, the electrophoresis equipment can be treated with 0.2N NaOH for 15 minutes and rinsed before use with RNAse-free water.

Protocol

Tissues

Blood / or any tissue can be used for preparation of RNA. If we are using tissue for RNA prepration then the tissue must be stored immediately till further use by the following procedure Snap Freezing in Liquid Nitrogen:

i) Following immersion, keep the tissue in the Nitrogen until the procedure is completed.

ii) Upon completion of the harvest procedure, transfer the tissues to empty falcon tubes stored on dry ice.

iii) Keep the tissue frozen until the homogenization procedure is ready to be performed.

In case liquid nitrogen facility is not available then you can store the tissue (until homogenization) according to the following procedure initially - overnight at 2-8 degree C, then indefinitely < -20 degree C, up to four weeks at 2-8 degree C, up to 7 days at 2-8 degree C, up to 1 day at 37 degree C. If you are going to extract the RNA from cultured

cells pellet out of growth media, wash 3 X PBS, and resuspend in RNA extraction solutions. **DO NOT FREEZ THE CELLS.**

RNA purification

1. Collect the cells and add 700µl of RNAwizTM. Close the tube and mix by vortexing. Incubate for 5 minutes at room temperature. Transfer the solution into a microfuge tube.
2. Add 140µl (0.2 volumes) of chloroform (not CIA) and mix by vortexing for 20 seconds. Incubate at room temperature for 10 - 15 minutes.
3. Centrifuge for 10 minutes at room temperature. After centrifugation, the mixture will be separated to two phases: a bottom phase containing chloroform and an upper aqueous phase containing RNA. Note: if an aqueous phase does not appear, add 100µl of chloroform, vortex it for 20 seconds and repeat step 3.
4. Without disturbing the interphase, transfer the top aqueous phase to a fresh RNAse-free water and mix well by inverting the tube several times. Divide the solution into two new tubes. Note: if the combined volume of aqueous phase and water is less then 800 µl, it is not necessary to divide this mixture into two tubes. Instead, add 700µl of isopropanol to the tube, mix well, and let it stand at room temperature for 10 minutes. Next, follow the procedure from step 10.
5. Precipitate RNA by the addition of 350 µl of isopropanol to each tube. Mix by inverting the tubes several times and incubate at room temperature for 10 minutes.
6. Place the tubes into the centrifuge, orienting the attached end of the tube lid away from the center of rotation. Centrifuge for 15 minutes at room temperature to pellet the RNA.
7. Remove the tubes from the centrifuge. Remove the supernatant using a P200 *ul* pipette. Wash the pellet with 700µl of cold 70 percent ethanol. Add ethanol to each tubes and mix by inverting several times.
8. Centrifuge for 5 minutes at room temperature. Remove ethanol with a P200 Pipette man.
9. Place the tubes into the centrifuge, making sure that the side containing the pellet faces away from the center of rotation. Start the centrifuge until it reaches 500 rpm. (1-2 seconds). This will

collect ethanol from the sides of the tube. Remove ethanol using a P200 Pipette man equipped with capillary tip.

10. Prewarm RNAs solution in a 60^0C water bath for 5 minutes. Add 15 µl of this solution to one tube and dissolve the RNA pellet. Transfer the solution to the second tube and dissolve the pellet.
11. As fast as possible heat the sample to 60^0C for 10 minutes to inactivate potential RNAse contamination. Store the RNA sample at -70^0C. Note: since RNA secure only inactivates RNAse at 60^0C, the best results are obtained using pre-warmed solution and transferring the tube as quickly as possible to a 60^0C water bath.
12. Determine the concentration of RNA by measuring the absorbance at 260nm. Initially, use a 1:100 dilution of the sample in PBS. The absorbance reading should be in the range 0.1 - 1.5. Calculate the concentration of RNA using the equation $N= A_{260}/ e_{260}$, where N is the RNA concentration in micrograms per milliliter, e_{260} is the RNA extinction coefficient, and A260 is the absorbance reading (corrected for dilution). The absorption coefficient for total RNA is usually taken to be $0.025\mu g^{-1}$ cm^{-1} giving a solution of 40µgml-1 of RNA an absorbance of 1.0 (e.g. $1/0.025 = 40\mu g$ ml^{-1}).
13. To determine the purity of the RNA, measure the absorbance at 260nm, 280nm, and 234nm, and calculate the 260nm: 280nm and 260nm: 234nm ratios. The concentration of RNA should be between 0.1 and $0.3\mu g$ μl^{-1}.

RNA agarose gel electrophoresis

Introduction

Agarose gel electrophoresis is used to analyse the total RNA. Native gels can be used as an analytical tool for assessing the efficiency of RNA purification.

Background

Gel electrophoresis of RNA molecules requires techniques that are different from those used for DNA. In order to separate RNA molecules according to their size, it is necessary to denaturation before and during electrophoresis. Non-denatured RNA can form secondary structures such as "hairpins" that profoundly influence their electrophoretic mobility. A number of denaturants have been used.

Among these are glyoxal with DMSO, formaldehyde, and methylmercuric hydroxide. Formaldehyde and glyoxal -DMSO are presently used more often than the highly toxic methylmercuric hydroxide.

The buffers used for RNA electrophoresis differ from those used for DNA. These buffers are of very low ionic strength, frequently resulting in the creation of a pH gradient along the length of the gel that causes overheating of the gel and distortion of RNA bands. To prevent this, RNA gels are usually run at low field strength (<5V cm-1) using a large volume of buffer and constant stirring to prevent gradient formation.

It is also possible to run native RNA agarose gels that do not include toxic denaturants in the agarose gel. The native gel system is simple and, in general, does not affect electrophoretic separation of RNA.

The glyoxal -DMSO method does not use toxic chemicals, but it is more difficult to use the native method. This method requires very careful control of pH during electrophoresis to a pH below 8. This is because glyoxal denatures RNA by binding covalently to the guanine residue, forming products that are stable only at a pH below 8. At a pH above 8, glyoxal dissociates from RNA. Submarine gels require continuous recirculation and mixing of electrophoresis buffer in order to maintain the pH within an acceptable limit. In addition, commercially available glyoxal must be purified before use in order to remove gloxylic acid, which is readily formed by oxidation and degrades RNA. The electrophoresis time is longer than for native gels.

Safety precautions

Agarose gel contains ethidium bromide, which is a mutagen and suspected carcinogen. Contact with the skin should be avoided. Students should wear gloves when handling ethidium bromide solution and gels containing ethidium bromide. Discard the used gel into the designated container.

For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, which is away from the edge of the bench. To avoid electric shock always disconnect the red (positive) lead first.

Ultraviolet (UV) light can damage the retina of the eye and cause severe sunburn. Always use safety glasses and a protective face shield to view the gel. Work in gloves and wear a long - sleeved shirt or laboratory coat when operating UV illuminators.

Technical tips

The most common problems with gel electrophoresis of RNA are inadequate denaturation of the samples and overloading the gel with RNA. Inadequate denaturation will appear either as multiple rRNA bands or rRNA bands appear as smear but at a correct ratio. Overloading the gel will result in to very broad rRNA bands that run on the gel with excessive "smearing". Bands could have a U-shape appearance and their mobility might be faster than expected from their base number.

Sample degradation will be indicated by an incorrect ratio between 28S, 18S rRNA or, in more severe cases, the total disappearance of these bands. Students can use even a severely degraded sample for the RT-PCR experiment.

Electrophoresis

Protocol

1. Prepare a mini-gel using a casting tray not larger than 7.5 cm x 7.5 cm and a thin gel (0.2 cm). Seal the ends of the gel -casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Use a mini-gel comb with wells 0.2-0.5cm long and 1mm (or less) wide. Check that the bottom of the comb is approximately 0.5mm above the gel bottom. To adjust this height, it is most convenient to place a plastic charge card (for example, Master Card) at the bottom of the tray and adjust the comb height to a position where it is easy to remove the card from under the comb. Wipe the comb with RNaseZap^{TM} immediately before use.
2. Prepare 500 ml of one times TBE (Tris-borate EDTA) buffer by adding 50 ml of a ten times TBE stock solution to 490 ml of RNAse-free water.
3. Prepare a 1 percent agarose gel. Place 15 ml of the buffer into a 100 ml flask and add 150 mg of agarose powder. Melt the agarose by heating the solution in a microwave oven at full power for 1 -2 minutes until the agarose is fully dissolved. If evaporation or spillage occurs during melting, adjust the volume to 15 ml with

deionized water.

4. Cool the agarose solution to approximately 60^0C and add 1 µl of ethidium bromide stock solution. Slowly pour the agarose into the casting tray. Remove any air bubbles by trapping them in a 10 ml pipette. Place the comb 1 cm away from one end of the gel. Allow the gel to solidify for 20 - 30 minutes. Note: native agarose gels should be as thin as possible (2-3 mm) in order to shorten the electrophoresis time.
5. Remove the tape from the ends of the gel-casting tray and place the tray on the central supporting platform of the gel box. Add electrophoresis buffer until it reaches a level approximately 2- 3 mm above the surface of the gel.
6. Prepare the RNA sample for electrophoresis in a 0.2 ml thin - wall, RNAse -free tube. Prepare the sample as follows: Add 2 µl of five times RNA sample buffer to a sterile micro centrifuge tube and 1 or 2 µl of RNA and fill it up with sterile RNAse-free water to a total volume of 10 µl.
7. Place the tube into a Light Cycler and incubate at 65^0C for 10 minutes. Transfer it immediately to ice. Incubate on ice for at least 2 minutes. Centrifuge the tube for 20 seconds to collect condensation and place the tube back on ice until ready to load onto the gel.
8. Load the samples into the wells using a yellow, RNAse-free tip. Place the tip under the surface of the electrophoresis buffer and above the well. Expel the sample slowly, allowing it to sink to the bottom of the well. Take care not to spill the sample into a neighboring well. During sample loading, it is very important not to place the end of the tip into the sample well or touch the edge of the well with the tip. This can damage the well, resulting in uneven or smeared bands.
9. Place the lid on the gel box and connect the electrodes RNA will travel towards the positive (red) electrode. Turn on the power supply. Adjust the voltage to approximately 5V cm^{-1}. For example, if the distance between electrodes (not the gel length) is 20 cm, in order to obtain field strength of 5V cm-1 the voltage should be set to 100 V. continue electrophoresis until bromophenol blue moves at least two-thirds of the length of the gel. It will take the tracking dye approximately 30 minutes to reach this position.
10. Turn the power supply off and first disconnect the positive (red) and then the negative lead from the power supply. This order of

disconnecting leads prevents the occurrence of accidental electrical shock. Remove the gel from the electrophoresis chamber. You can photograph the gel using a computer imaging system to record the results.

11. Two sharp bands will appear on the gel, 28S RNA (4.7kb) and 18S RNA (1.9 kb). The 5S and 5.8S RNA bands are located on the leading edge of the gel, running together with tRNA. Note: the integrity of the prepared RNA samples and lack of RNA degradation can be easily judged from the appearance of rRNA bands. Degradation of the sample appears as diffused 28S and 18S bands or an incorrect ratio of stain between rRNA bands. This ratio should be approximately 2:1 for 28S and 18S, respectively.

Most important points to remember while handling RNA

Precautions for handling RNA

General information: Working with RNA is more demanding than working with DNA, because of the chemical instability of the RNA and the ubiquitous presence of RNAses.

Unlike DNAses, RNAses do not need metal ion co-factors and can maintain activity even after prolonged boiling or autoclaving.

Therefore, special precautions should be taken when working with RNA.

Gloves and contact: Always wear gloves when working with RNA.

After putting on gloves, do not touch surfaces and equipment to avoid reintroduction of RNAses to decontaminated material.

Workspace and working surfaces: Designate a special area for RNA work only.

Treat surfaces of benches and glassware with commercially available RNAse inactivating agents. Clean benches with 100% ethanol.

Equipment and disposable items: Use sterile, disposable plastic ware.

Electrophoresis tanks for RNA analysis can be cleaned with 1% SDS, rinsed with H_2O, then rinsed with absolute ethanol and finally soaked in 3% H_2O_2 for 10 min. Rinse tanks with DEPC- treated H_2O before use.

Glass and plastic ware: Glassware should be baked at 180^0 - 200^0C for at least 4 hours. Autoclaving glassware is not sufficient to eliminate RNAses.

Use commercially available RNAse free plastic ware.

If plastic ware should be reused, soak (2h, 37^{0}C) in 0.1 M NaOH / 1 mM EDTA (or absolute ethanol with 1% SDS), rinsed with DEPC treated H_2O and heated to 100^{0}C for 15 min.

Corex tubes should be treated with DEPC treated H_2O overnight at room temperature, then autoclave for 30 min. to destroy unreacted DEPC.

Cleaning of pH electrodes: Incubate for 30 s in 70% ethanol, 5 min in 1 M NaOH, and rinse with DEPC - treated H_2O. DEPC = diethylpyrocarbonate

Reagents: Purchase reagents that are free of RNAses.

Reserve separate reagents for RNA work only: Wear gloves, use baked spatulas and untouched weigh boats or weigh paper.

All solutions should be made with DEPC- treated H_2O

Handiling fresh and stored material before extraction of RNA: Extract RNA as quickly as possible after obtaining samples.

For best results, use either fresh samples or samples that have been quickly frozen in liquid nitrogen and stored at - 70^{0}C. This procedure minimizes degradation of crude RNA by limiting the activity of endogenous RNAses.

Blood and bone marrow samples can be stabilized with commericial available stabilization reagents.

All required reagents should be kept on ice.

RNAse inhibitors: RNAse inhibitors can be used to protect RNA from degradation during isolation and purification and in all downstream applications.

The most commonly used inhibitor is a natural protein from human placenta* that inactivates RNAses, via a non-covalent and reversible binding.

To keep the inhibitor active, avoid solutions with strong denaturing agents such as SDS or urea, maintain reducing conditions (1mM DTT) and hold the temperature below 65^{0}C.

Other inhibitors include Macaloid and Vanadyl-ribonucleoside complexes.

Storage of RNA: Store RNA, aliquot in ethanol or isopropanol at - 70^{0}C. Most RNA is relatively stable at this temperature.

Remove ethanol or isopropanol by centrifugation and resuspend in the appropriate RNAse-free buffer.

Manipulation of RNA: Always keep the RNA sample on ice when preparing an experiment.

Drying RNA: Avoid over drying of RNA after ethanol precipitation.

Dry in vacuum or for 15 min at room temperature in a clean space.

Dissolving RNA: Dissolve RNA by adding RNase-free buffer or H_2O and incubate the tube on ice for 15 minutes. Gently tap the tube or vortex with care.

Pipetting RNA: Use RNAse free tips or autoclave regular tips.

Keep pipettes clean.

Temperature sensitivity: RNA is not stable at elevated temperatures, therefore, avoid high temperatures (>65^{0}C), since these affect the integrity of the RNA.

To melt out secondary structures, heat RNA to 65^{0}C for 15 min in the presence of denaturing buffers.

Commonly used formulas

Calculation of molecular weights in Dalton	➢ Average molecular weight (MW) of a ribonucleotide: 340 Dalton (Da) MW of ssRNA = [number of bases] x [340 Da] eg. MW of tRNA from E. coli (75 bases) = 25.5 x 10^3 Da= 25.5 kDa
Conversion of µg to pmol	➢ pmol of ssRNA = µg (of ssRNA) $\times \frac{10^6 \text{ pg}}{1 \text{ µg}} \times \frac{1 \text{ pmol}}{340 \text{pg}} \times \frac{1}{N_b}$ = µg (of ssRNA) x 2,941 / N_b eg. 1 µg of a 100 base ssRNA molecule = 1 x 2941/ 100 = 29.4 pmol ➢ The value calculated with this formula also represents the pmoles of 5′ or 3′ ends. N_b = number of bases
Calculation of pmol of 5′ (or 3′) ends	➢ See above calculation of conversion of µg to pmol
Conversion of pmol to µg	➢ µg of ssRNA = pmol (of ssRNA) x 340 / 1 pmol x 1 µg / 10^6 pg x N_b = pmol (of ssRNA) x N_b x 3.4 x 10^{-4} eg. 1 pmol of a 250 base ssRNA molecule = 0.085 µg N_b = number of bases

Example

Organism	Type	Length (in bases)	MW (in kDa)
E. coli	tRNA	75	26
	5S rRNA	120	41
	16S rRNA	1541	523
	23S rRNA	2904	987
Drosophila	18S rRNA	1976	672
	28S rRNA	3898	1.3×10^3
Mouse	18S rRNA	1869	635
	28S rRNA	4712	1.6×10^3
Human	18S rRNA	1868	635
	28S rRNA	5025	1.7×10^3
Rabbit	18S rRNA	2366	804
	28S rRNA	6333	2.15×10^3

Use this chart to select a product according to the type and origin of RNA

Type	Origin	Recommendation*
mRNA	Cultured cell, tissues, total RNA	mRNA isolation kit mRNA Capture Kit
	Human whole blood/ bone marrow	mRNA isolation kit for blood / bone marrow
	White blood cell fraction of human blood	mRNA isolation kit for white blood cell
	Tissue, cultured cells	mRNA isolation kit for tissue
Total RNA	Cultured cells, bacteria, yeast, blood	High pure RNA isolation kit
	Tissue	High pure RNA tissue kit
	Cultured cells, tissues, bacteria, yeast, blood	TriPure Isolation reagent
Viral RNA	Serum, plasma, other body fluids, cell culture supernatant	High pure viral RNA kit
RNA fragments	Removal of unincorporated nucleotides from labeled RNA molecules	Mini Quick Spin RNA columns

* The amount of RNA that can be isolated with the kits depends on a variety of variables like the amount of sample applied. Concentration of RNA within the sample, buffer systems etc.

Use this chart to select a product according to the main application in which the RNA will be used

Product	RT-PCR	DD*-RT-PCR	cDNA synthesis	Nothern blotting	RNAse protection	In vitro trans-lation
High Pure RNA tissue kit	*	*	*	*	*	*
High pure RNA isolation Kit	*	*	*	*	*	*
High Pure Viral RNA Kit	*		*			
mRNA isolation kit for blood / bone marrow	*			*	*	*
mRNA Isolation kit for White blood cells	*		*	*	*	*
mRNA isolation kit for tissue	*		*	*	*	*
mRNA isolation kit	*		*	*	*	*
mRNA capture kit	*					
TriPure isolation Reagent	*	*	*	*	*	*
Mini Quick Spin RNA columns				*		

* DD = differential display

Use this chart to select a product according to its characteristics

Product	Quantity of starting material	Typical yield
High pure RNA Tissue kit	➢ Tissue: 50 mg - 1g ➢ Cells: 2 x 10^5 - 10^8 ➢ Total RNA: 250 µg- 2.5 mg	➢ 7-14 µg /100 mg tissue[a] ➢ 0.3-25 µg /10^7 cells[b] ➢ 1-5 µg / 100 µg total RNA
mRNA Capture Kit	➢ Tissue: up to 20 mg ➢ Cells: up to 5 x 10^5 ➢ Total RNA: up to 40 µg	➢ Product detectable by RT-PCR
TriPure Isolation reagent	➢ RNA from liver, spleen: 50 mg - 1g ➢ Cultured epithelial cells, fibroblasts: 10^6 - 10^7	➢ 6-10 µg /mg tissue[a] ➢ 8-15 µg/10^6 cells[b]
Mini quick spin RNA columns	➢ 20 - 75 µl labeling mixture	➢ > 80% ➢ Exclusion limit: ➢ G-50: 72b

[a] depends on type of tissue . [b] depends on type of cells

RNA content in various cells and tissues

10^7 cells	3 T3	Hela	COS
Total RNA (µg)	~ 120	~ 150	~ 350
mRNA (µg)	~ 3	~ 3	~ 5

Mouse tissue (100 mg)	Brain	Heart	Intestine	Kidney	Liver	Lung	Spleen
Total RNA (µg)	~ 120	~ 120	~ 150	~ 350	~ 400	~ 130	~ 350
mRNA (µg)	~ 5	~ 6	~ 2	~ 9	~ 14	~ 6	~ 7

Concentration via OD Measurement

Concentration of RNA
- 1 A_{260} Unit of ssRNA = 40 µg/ml H_2O

Notes
- OD value should lie between 0.1 and 1.0 to ensure an optimal measurement.
- The above mentioned value is based on the extinction coefficient of RNA in H_2O.
- Please note that this coefficient - and hence the above mentioned value may differ in other buffers and / or solutions.
- Cuvettes should be RNAse free.
- Example of calculation:
- Volume of ssRNA sample: 100 µl
- Dilution: 25 µl of this sample + 475 µl H_2O (1/20 dilution)
- A260 of this dilution : 0.56
- Concentration of ssRNA in sample: 0.56 x 40 µg/ml x 20 (=dilution factor) = 448 µg/ml
- Amount of ssRNA in sample:
- 448 µg/ml x 0.1 ml (=sample volume) = 44.8 µg

Purity of RNA
- Pure RNA: A_{260} / $A_{280} \geq 2.0$

Notes
- Buffered solutions provide more accurate values than water since the A_{260} / A_{280} ratio is influenced by pH. Therefore, it is recommended to measure the ratio in a low salt buffer.
- Pure RNA has a ratio of 1.9 - 2.1 in a 10mM Tris buffer.
- An A_{260}/A_{280} smaller than 2.0 means that the preparation is contaminated with proteins and aromatic substances (e.g. phenol). In this case, it is recommended to purify the RNA (again) before using it in subsequent applications.
- The OD value gives no indication about the size of the RNA.

Size estimation of RNA fragments

Agarose electrophoresis	➢ In most cases, 1% Agarose MP gels (or variations between 0.8 and 1.2%) are used to separate ssRNA molecules. ➢ The nature of denaturing conditions is a crucial factor for the resolution and visualization of the different fragments in a given gel.
Acrylamide electro-phoresis	➢ Efficient range of separation of ssRNA molecules: ➢ 3.5% of Acrylamide/ Bisacrylamide (29/1): 750-2000 ➢ 5.0% of Acrylamide / Bisacrylamide (29/1): 200-1000 ➢ 8.0% of Acrylamide / Bisacrylamide (29/1): 50-400
Size markers	➢ a variety of different molecular weight markers are available from different suppliers. ➢ Bacterial 23S (~ 2900 b) and / or 16S (~ 1550 b) ribosomal RNA's can also be used as size markers.

Reaction components: Template

Avoiding contamination	➢ "Amplification of DNA: Avoiding contamination" for the different sources of possible contaminations before and during amplification reactions and how to most optimally avoid them.
Type	➢ If possible, use purified mRNA as a template rather than total RNA. ➢ Starting with poly (A+) mRNA will improve the likelihood of successful amplification of rare mRNA's since the proportion of mRNA's in a total RNA preparation is very low (typically 1- 5% of total RNA for a mammalian cell).
Integrity	➢ If a mRNA template is used an enough material is available, the integrity of the mRNA can be checked by gel electrophoresis before using it is a RT-PCR. ➢ The mRNA should appear as a smear between 500 bp and 8 kb. Most of the mRNA should be between 1.5 and 2 kb.
Avoiding RNAse contamination	➢ To minimize the activity of RNAses that are released during cell lysis, include RNAse inhibitors in the lysis mix or use methods that simultaneously disrupt cells and inactivate RNAses. ➢ "Precautions for handling RNA" for a comprehensive overview how to avoid RNAse contamination.

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Chapter 2

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is an in vitro method for amplifying selected nucleic acid sequences. PCR is a test tube system for DNA replication that allows a "target" DNA sequence to be selectively amplified, or enriched, several million-fold in just few hours.

Sometimes referred as "molecular photocopying," PCR can characterize, analyze, and synthesize any specific piece of DNA or RNA. It works even on extremely complicated mixtures, seeking out, identifying, and duplicating a particular bit of genetic material from blood, hair, or tissue specimens, from microbes, animals, or plants, some of them may be thousands-or possibly even millions-of years old.

Principle of PCR

To target the amplification to a specific DNA segment, two primers bearing the complementary sequences that are unique to the target gene are being used. These two primers hybridize to opposite strands of the target DNA, thus, enabling DNA polymerase to extend the sequence between them. Each cycle produces a complementary DNA strand to the target gene. Consequently, the product of each cycle is doubled, generating an exponential increase in the overall number of copies synthesized.

The primers define the boundaries and the specificity of the desired amplicon (the amplified DNA segment). A typical PCR reaction consists of template DNA (genomic DNA), a pair of primers, $MgCl_2$, dNTPs (deoxynucleotide triphosphates) and a thermo stable DNA polymerase are added to the mixture. The PCR reaction is performed in a thermocycler, capable of changing its temperature quickly, precisely, and in reproducible manner. Each PCR cycle consists of denaturation, annealing, and extension steps.

PCR cycle

There are three major steps in a PCR (**Figure 2.1**), which are repeated for ~30 or 40 cycles. This is done on an automated Thermo cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

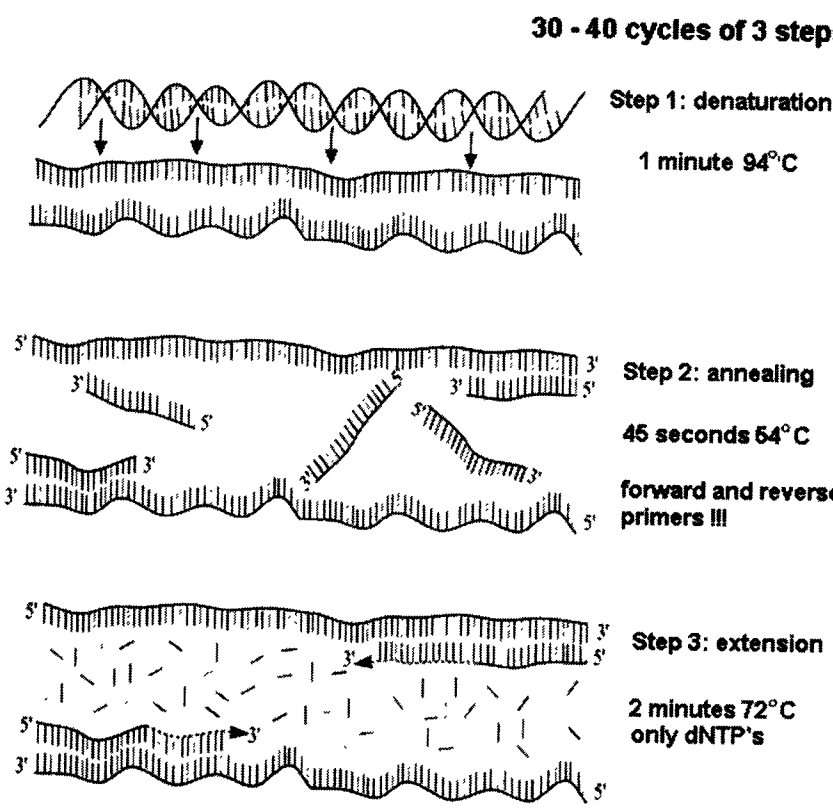

Figure 2.1: Three steps in a normal PCR reaction: denaturation, annealing and extension.

1. **Denaturation** at 90-94°C: During the denaturation, double strand melt open to single stranded DNA and the entire enzymatic reaction stops (for example: the extension from a previous cycle).
2. **Annealing** at 50-65°C: During annealing, primers hybridize or "anneal" (by way of hydrogen bonds) to their complementary sequences on either side of the target sequence. The primers normally jiggle around due to Brownian motion. Hydrogen bonds are constantly formed and broken between the single

stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and start copying the template. Once there are few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break anymore.

3. **Extension** at 72°C: This is the ideal working temperature for the polymerase. The primers, where there are few bases built in, have a stronger attraction to the template, created by hydrogen bonds, than the forces breaking these attractions. Primers that are on positions with no exact match get loose again (because of the higher temperature) and don't give an extension of the fragment.

Exponential growth of DNA segment in PCR

The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side; bases are added complementary to the template).

Because, both strands are copied during PCR, there is an exponential increase of the number of copies of the gene (**Figure 2.2** and **Figure 2.3**). Suppose there is only one copy of the wanted gene before the

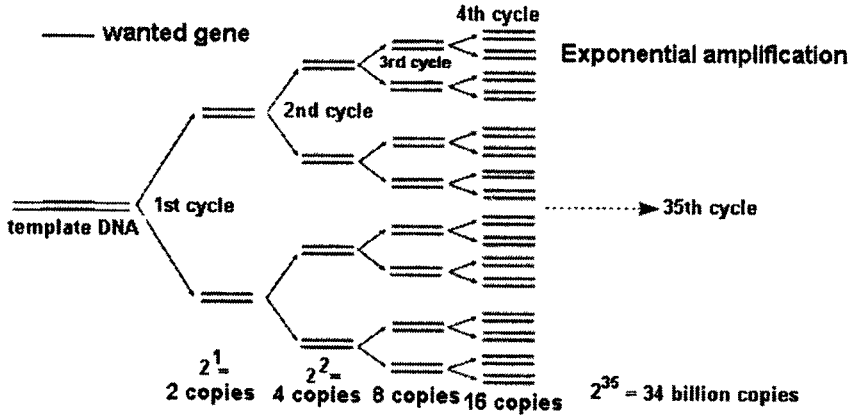

Figure 2.2: Exponential growth of the target gene from template DNA during 30 cycles of a PCR reaction.

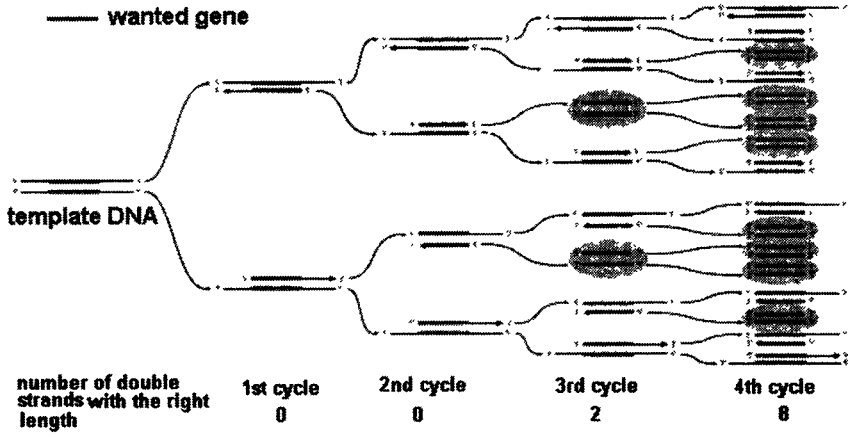

Figure 2.3: The first 4 cycles of a PCR reaction in detail. In the 3rd cycle, two double strands of the right length are copied (the forward and reverse strand are the same in length). In the 4th cycle, 8 double strands of the right length are copied.

cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, and three cycles will result into 8 copies and so on. At the end of the 'n' cycles total no of copies will be 2^n.

Constituents of a standard PCR reaction:

1. Nucleic acid template

Most PCR uses DNA as template molecule for amplifying a target sequence, rather than RNA, because of the stability of the DNA molecule and the ease with which DNA can be isolated. Corresponding cDNA can be taken to investigate a given RNA. The essential criteria for any DNA sample are that it contains at least one intact DNA strand encompassing the region to be amplified and that any impurities are sufficiently diluted so as not to inhibit the polymerization step of the PCR reaction.

2. Oligonucleotide Primers

Other important component in PCR is primers that are segment of nucleotides complementary to a section, flanking the DNA which is to be amplified in the PCR reaction. Primers are annealed to the

denatured DNA template to provide an initiation site for the elongation of the new DNA molecule. Primers can either be specific to a particular DNA nucleotide sequence or they can be "universal." Universal primers are complementary to nucleotide sequences that are very common in a particular set of DNA molecules. Thus, they are able to bind to a wide variety of DNA templates.

3. Deoxynucleotide Triphosphates

Deoxynucleotide Triphosphates (dNTPs) can be used seperately or as a mixture of all the four- dATP (Adenine), dCTP (Cytosine), dTTP (Thymine) and dGTP (Guanine). These dNTPs are required by Taq Polymerase during synthesis of new strand complementary to the template DNA.The four dNTPs should be used at equivalent concentrations to minimize misincorporation errors. Both the specificity and the fidelity of PCR are increased by using accurate dNTP concentrations.

4. Taq Polymerase

A heat-stable DNA polymerase isolated from the bacterium *Thermus aquaticus,* which is found in hot springs is called as Taq polymerase. This remains active despite repeated heating during many cycles of amplification. Taq polymerase amplifies the new strand of DNA by incorporating dNTPs complementary to the template DNA strand. Taq polymerase binds to the 3' end of the oligonulceotide primer and synthesizes the new strand during extension step.

5. Magnesium Chloride

Magnesium Chloride is one of the major constituents responsible for the fidelity of the PCR reaction. It provides Mg++ ions, which acts as co-activator of Taq polymerase. Mg++ ions also help in primer annealing and incorporation of dNTPs at the time of extension.

6. PCR additives

A variety of PCR additives and enhancing agents have been used to increase the yield, specificity and consistency of PCR reactions. These additives may have beneficial effects on some amplification. It is impossible to predict which agents will be useful in a particular context and therefore, they must be empirically tested for each combination of template and primers. Some of the more popular of these additives and their uses are listed in **Table 2.1.**

Table 2.1: Various additives used in PCR and their application

S. No.	Additives	Applications
1	DMSO (dimethyl sulfoxide)	DMSO reduces secondary structure and is particularly useful for GC rich templates. DMSO at 2-10% may be necessary for amplification of some templates, however, 10% DMSO can reduce *Taq* polymerase activity by up to 50%.
2	Betaine	Betaine or Betaine monohydrate ((N, N, N-trimethylglycine = [carboxy methyl] trimethylammonium) enhances Taq polymerase activity especially in GC rich templates.
3	Formamide	It stops the re-annealing of the two DNA strands after denaturation, thus helping primer annealing. 1-5% Formamide is recommended.
4	Non-ionic detergents e.g. Triton X-100, Tween 20 or Nonidet P-40 (NP-40)	Non-ionic detergents stabilize *Taq* polymerase and may also suppress the formation of secondary structure. 0.1-1% Triton X-100, Tween 20 or NP-40 may increase yield but may also increase non-specific amplification. As little as 0.01% SDS contamination of the template DNA (left-over from the extraction procedure) can inhibit PCR by reducing *Taq* polymerase activity to as low as 10%, however, inclusion of 0.5% Tween-20 or NP-40 will effectively neutralize this effect.
5	TMAC (tetramethylam monium chloride)	TMAC is generally used at a final concentration of 15-100mM to eliminate non-specific priming. TMAC is also used to reduce potential DNA-RNA mismatch and improve the stringency of hybridization reactions.
6	7-deaza-2-deoxyguanosine (dC^7GTP)	The base analogue 7-deaza-2'-deoxyguanosine may facilitate amplification of templates with stable secondary structures when used in place of dGTP in a ratio of 3: 1, 7-deaza-2'-deoxyguanosine: dGTP.
7	BSA (bovine serum albumin)	BSA has proven particularly useful when attempting to amplify ancient DNA or templates which contain PCR inhibitors such as melanin.

Standard PCR protocol

Time required

1. PCR reaction: 3-6 hours
2. PCR product analysis:
   (i) Agarose Gel electrophoresis: 45 minutes to 1 hr.
   (ii) Polyacrylamide gel electrophoresis using small gel apparatus: 2.5 hours.
   (iii) Ethidium bromide staining and photography: 45 minutes

Reagents required

1. Synthetic oligonucleotide primer pair flanking the sequence to be amplified.
2. 5X PCR Buffer (250 mM KCl, 50 mM Tris-HCl pH 8.3, 7.5 mM MgCl2).
3. Mixture of four dNTPs (dGTP, dATP, dTTP, dCTP) (Ultrapure dNTP set,). The dNTP mixture is made by adding equal volumes of 10 mM solution of each of the 4 separate dNTPs.
4. Taq DNA Polymerase
5. Light mineral oil
6. Required section of template DNA (genomic DNA).

Equipment required

1. Thermal Cycler
2. Sterile thin-wall 0.2ml micro centrifuge tubes
3. Micropipettes

Recommendations for choosing oligonulceotide primers

The most critical parameter for successful PCR is the designing of primers. The primer sequence and length determines the melting temperature and ultimately the yield. A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. Several variables must be taken into account when designing PCR primers among the most critical ones are:

1. Primer length

Since both specificity and temperature and also the time of annealing

are at least partly dependent on primer length, it is a critical parameter for successful PCR. In general, oligonucleotides between 18 and 24 bases are extremely sequence specific, provided that the annealing temperature is optimal. Primer length is also proportional to annealing efficiency in general, longer the primer, the more inefficient is the annealing. With fewer templates primed at each step, this can result in a significant decrease in amplified product.

2. Melting Temperature (T_m)

It is important to keep in mind that there are two primers added to a PCR reaction. Both of the oligonucleotide primers should be designed in such a manner that they have similar melting temperatures. If primers are mismatched in terms of T_m, amplification will be less efficient or may not work at all since the primer with the higher T_m will mis-prime at lower temperatures and the primer with the lower T_m may not work at higher temperatures. The melting temperatures of oligos are most accurately calculated using nearest neighbor thermodynamic calculations with the formula:

$$T_m^{primer} = \Delta H\ [\Delta S + R \ln (c/4)] - 273.15°C + 16.6 \log_{10} [K^+]$$

Where H is the enthalpy and S is the entropy for helix formation, R is the molar gas constant and C is the concentration of primer. This is most easily accomplished using any number of primer design software packages like DNADIST. Fortunately, a good working approximation of this value (generally valid for oligos in the 18–24 base range) can be calculated using the Wallace formula:

$$T_m = 2(A+T) + 4(G+C).$$

Table 2.2: Primers of different base length and their respective T_m

Primer Length	T_m = 2 (A+T) + 4(G+C)	Primer Length	T_m = 2 (A+T) + 4(G+C)
4	12°C	20	60°C
6	18°C	22	66°C
8	24°C	24	72°C
10	30°C	26	78°C
12	36°C	28	84°C
14	42°C	30	90°C
16	48°C	32	96°C
18	54°C	34	102°C

T_m values for primers of various lengths shown in **Table 2.2** are calculated by the Wallace formula, and assuming a 50% GC content. The temperatures calculated using Wallace's rule are inaccurate at the extremes of this chart. In addition to calculating the melting temperatures of the primers, care must be taken to ensure that the melting temperature of the product is low enough to ensure 100% melting at 92°C. This parameter will help to ensure a more efficient PCR, but is not always necessary for successful PCR. In general, products between 100–600 base pairs are efficiently amplified in many PCR reactions. If there is doubt, the product T_m can be calculated using the formula:

$$'T_m = 81.5 + 16.6\ (\log_{10}[K+]) + 0.41\ (\%G+C) - 675/length.$$

Under standard PCR conditions of 50 mM KCl, this reduces to:

$$T_m = 59.9 + 0.41\ (\%G+C) - 675/length$$

3. Specificity

As mentioned above, primer specificity increases with the length of primer. It is evident that there are many more unique 24 base oligos than there are 15 base pair oligos. That being said, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying the genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.

As Taq Polymerase is active over a broad range of temperatures, primer extension will occur at the lower temperatures of annealing. If the temperature is too low, non-specific priming may occur which can be extended by the polymerase if there is a short homology at the 3' end. In general, a melting temperature of 55°C -72°C gives the best results.

4. Complementary Primer Sequences

Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, "snap back", partially double-stranded structures, can occur which will interfere with annealing to the template.

Another related danger is inter-primer homology. Partial homology in the middle regions of two primers can interfere with hybridization. If there is homology at the 3' end of either of the primer, Primer

dimer formation will occur which will prevent the formation of the desired product via competition.

5. G/C content and Polypyrimidine (T, C) or polypurine (A, G) stretches

The base composition of primers should be between 45% and 55% GC. The primer sequence must be chosen such that there is no Poly G or Poly C stretches that can promote non-specific annealing. Poly A and Poly T stretches are also to be avoided as these will "breath" and open up stretches of the primer-template complex. This can lower the efficiency of amplification. Polypyrimidine (T, C) and polypurine (A, G) stretches should also be avoided. Ideally, the primer will have a near random mix of nucleotides, a 50% GC content and be ~20 bases long. This will put the T_m in the range of 56°C - 62°C.

6. 3'-end Sequence

It is well established that the 3' terminal position in PCR primers is essential for the control of mis-priming. The presence of a G or C residue at the 3' end of primers helps to ensure correct binding at the 3' end due to the stronger hydrogen bonding of G/C residues. It also helps to improve the efficiency of the reaction by minimizing any "breathing" that might occur.

Primer reconstitution

Sometimes, primer units are listed in optical density reading (OD). If this is a problem one needs to convert to molarity using the following equations: Change optical density reading of primer to molarity (µM units)

1. N = no of bases in primer
2. SIGMA 260 =~ 10,000 X N/ m X cm
3. Molecular weight =~ 330 X N
4. OD_{260} / SIGMA 260 X 10^6 = Concentration (µM)

For example- primer is 20 bases long and OD_{260} = 10.

1. N = 20
2. SIGMA 260 =~ 10,000 X 20/m X cm = 20,000/m X cm
3. molecular weight =~ 330 X 20 = 6,600
   10 OD_{260}/20,000 $m^{-1}cm^{-1}$ X 10^6 = 50uM

Procedure for polymerase chain reaction

The PCR reaction can be performed in volumes from 5 µl to 200 µl or more. This volume is recommended when the purpose of the experiment is diagnostic. A scaled up volume can be used if the PCR product is recovered from the gel or used for sequencing. The 5-µl reaction is performed in a 200µl PCR tube and covered by a drop of oil before placing in to the thermal cycler (if heating, LID option is absent in Thermo cycler). The components shown in **Table 2.3** will make up one reaction (5 µl total volume), but a cocktail of everything except the DNA will be made first.

Table 2.3: PCR reaction cocktail for 1 and 10 sample respectively (5 µl each)

Reagents	Requirement for 1 reaction	Cocktail for 10 reactions
5X PCR Buffer	1.0 µl	10 µl 5X PCR Buffer
dNTP mixture (each at 2.5 mM)	0.4 µl	4 µl dNTPs
Primer pair (each primer at 25 µM) (The primer pair solution is 1:1 mixture of the 50 µM primer solutions)	0.2 µl*	2 µl Primer pair
Taq polymerase	0.1 µl	1 µl Taq polymerase
DdH2O	2.3 µl	23 µl ddH20
Total	**4 µl**	**40 µl, divided into 10 PCR with 4 µl each**
DNA (100 ng genomic template DNA or < 50 ng cloned template)	1.0 µl	1.0 µl each sample

*The range of final primer pair concentrations in a normal reaction mix is 0.25 - 2.5 µM and 0.5 µM is sometime ideal.

Steps involved in setting up a PCR

1. Plan your experiment before adding any reagents (primer pairs to be used, number of DNA templates, etc.). After doing so, make the appropriate cocktail/s and ensure complete mixing by tapping the tube and quick spinning. (N.B. Caution should be used to avoid contamination of reactions with even small amounts of DNA. In addition, care should be taken to avoid contamination of pipette with carryover amplification products from previous reactions).

2. Pipette 4.0 µl of the appropriate cocktail directly into the bottom of a sterile microeppendorf tube for each reaction. The tubes should be labeled by placing a round sticker on the cap to prevent smearing by oil in subsequent steps.
3. Add 1.0 µl of the DNA directly into the drop of cocktail in each tube and ensure adequate mixing. Quick spin to collect the reaction mixture in the bottom of the tube.
4. Overlay each reaction with one drop of light mineral oil using a Pasteur pipette. The samples may be quick spun if necessary before placing in the Thermo cycler.
5. Place a drop of mineral oil into each well in the thermal cycler temperature block to be used for the samples (this ensures rapid temperature equilibration during cycling).
6. Place the tightly capped tubes in the temperature block and make sure each is firmly seated by pressing on the tubes individually.
7. The PCR machine must now be programmed for the specific reaction conditions desired (See brief operating instructions for PCR machine). Each cycle in the polymerase chain reaction involves three steps (denaturing, primer annealing, extension), and the products are amplified by performing many cycles one after the other with the help of the automated thermal cycler. The Taq polymerase is heat stable, and remains active despite the high denaturing temperature of each cycle. A representative set of reaction conditions for 25-35 cycles is shown in **Table 2.4.**

Table 2.4: Representative set of PCR conditions for PCR cycle

I.	**Denature**	93-94 degrees C	1.5 minutes
II.	**Anneal**	50-65 degrees C	2 minutes
III.	**Extension**	72 degrees C	2 minutes

8. After completion of the PCR reaction, remove the tubes from the temperature block and wipe from outside, so that, tubes are free of excess oil before placing in an eppendorf rack.
9. The reaction products are conveniently separated according to size by electrophoresis through a 10% polyacrylamide or 2% Agarose gel, and visualized after staining the gel with ethidium bromide (see "Electrophoretic Technique" section).

Optimization of PCR

Enzyme concentration

Recommended concentration range for Taq DNA polymerase is between 1 and 2.5-units/100 µl reaction when other parameters are optimum. However, enzyme requirements may vary with respect to individual target templates or primers. When optimizing a PCR, it is recommended that enzyme concentrations ranging from 0.5 to 5-units/ 100 µl should be tested and results assayed by gel electrophoresis. If the enzyme concentration is too high, nonspecific background products may accumulate, and if too low, an insufficient amount of desired product is made.

Note: Taq DNA polymerase from different suppliers may behave differently because of different formulations or conditions and / or unit definitions.

Deoxynucleotide Triphosphates

Stock dNTP solutions should be neutralized to pH 7.0, and their concentrations should be determined spectrophotometrically. Primary stocks of 100 mM are aliquoted and stored at -20^0 C. A working stock containing 10mM of each dNTP is usually recommended. The stability of the dNTPs during repeated cycles of PCR is such that approximately 50% remains as dNTP after 50 cycles.

Deoxynucleotide concentrations between 20 and 200 mM result in to the optimal balance among yield, specificity, and fidelity. The four dNTPs should be used at equivalent concentrations to minimize misincorporation errors. Both the specificity and the fidelity of PCR are increased by using lower dNTP concentrations than those originally recommended for Klenow mediated PCR (1.5 mM each). Low dNTP concentrations minimize mispriming at non-targeted sites and reduce the likelihood of extending misincorporated nucleotides. One should decide on the lowest dNTP concentration appropriate for the length and composition of the target sequence.

Magnesium concentration

It is beneficial to optimize the magnesium ion concentration. The magnesium concentration may affect all or one of the following; primer annealing, strand dissociation temperatures of both template and PCR product, product specificity, formation of primer dimer, artifacts, enzyme activity and fidelity. Taq DNA Polymerase requires free magnesium apart from that bound by template DNA, primers and

dNTPs. Accordingly, PCRs should contain 0.5 to 2.5 mM magnesium over the total dNTP concentration. An optimum of Mg++ concentration usually exists in the 1-10 mM range. Too low Mg++ concentration may result in no products and excess may result in a variety of unwanted products.

*Note: **The presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent magnesium optimization.***

Other reaction components

A recommended buffer for PCR is 10-50 mM Tris-HCl (between pH 8.3 and 8.8 when measured at 20^0C). However, an extensive survey of other buffers has not been performed. Tris is a dipolar ionic buffer having a pK_3 of 8.3 at 20^0 C, and a delta pK_3 of -0.021/ ^{0}C. Thus, the true pH of 20 mM Tris (pH 8.3) at 20^0 C varies between 7.8 and 6.8 during typical thermal cycling conditions.

Up to 50 mm KCl can be included in the reaction mixture to facilitate primer annealing. NaCl at 50 mM, or KCl above 50 mM, inhibits Taq DNA polymerase activity. Commercially, Taq DNA polymerase compatible 10x PCR buffer is provided along with corresponding Taq DNA polymerase. This 10x buffer should be used as 1x in final concentration.

Primer annealing

Temperature and time required for primer annealing depend upon the base composition, length, and concentration of the amplification primers. An applicable annealing temperature is 5^0 C-10^0 C below the true Tm of the amplification primers. Because, Taq DNA polymerase is active over a broad range of temperatures, including the annealing step. The range of enzyme activity varies by two orders of magnitude between 20^0C and 85^0 C. Annealing temperatures in the range of 55^0C to 72^0C generally yield the best results. At typical primer concentrations (0.2 μM), annealing will require only a few seconds.

Primer extension

Extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extensions are traditionally performed at 72^0C, because, this temperature was near optimal for extending primers on an M13-based model template. An estimate for the rate of nucleotides per second depends upon buffer, pH, salt concentration, and the nature of the DNA template. An

extension time of one minute at 72^0C is considered sufficient for products up to 2 kb in length. However, longer extension time may be helpful in early cycles if the substrate concentration is very low, and in the late cycles when product concentration exceeds enzyme concentration (approximately 1 nM).

Denaturation time and temperature

The most likely cause for failure of a PCR is incomplete denaturation of the target template and/or the PCR product. Typical denaturation conditions are 95^0 C for 30 seconds, or 97^0 C for 15 seconds; however, high temperatures may be appropriate, especially for GC rich targets. It only takes a few seconds to denature DNA at its strand-separation temperature (Ts); however, there may be lag time involved in reaching Ts inside the reaction tube. Incomplete denaturation allows the DNA strands to "snap back", and thus, reduces product yield. In contrast, denaturation steps that are too high and/or too long lead to unnecessary loss of enzyme activity. The half-life of Taq DNA polymerase activity is > 2 hours, at 92.5 ^{0}C and 40 min at 95.5 ^{0} C.

Cycle number

The optimum number of cycles will depend mainly upon the starting concentration of target DNA, when other parameters are optimized. A common mistake is to execute too many cycles. Kary Mullis has stated, "If you have to go more than 40 cycles to amplify a single copy gene, there is something seriously wrong with your PCR." Too many cycles can increase the amount and complexity of nonspecific background products (Plateau effect). Of course, too few cycles give low product yield. Some guidelines for number of cycles versus starting target concentration are provided in **Table 2.5.**

Table 2.5: Number of cycles depending upon the target concentration

Number of initial target molecules	Number of cycles
3×10^5	25 to 30
1.5×10^4	30 to 35
1×10^3	35 to 40
50	40 to 45

Preventing cross-contamination with amplified DNA sequences

As the PCR technique permits amplifications starting from one template molecule, steps need to be taken to prevent unintended

transfer of amplified sequences to items used in the preparation of fresh DNA extracts for PCRs. Cross-contamination becomes evident when amplification occurs in negative controls that did not receive template and should be suspected when more than one ladder is obtained upon sequencing an amplified fragment expected to reveal only one genotype. Precautions should be intensified when performing PCR and sequencing from samples with a small number of initial template molecules.

Cross-contamination may occur with amplified sequences via aerosols normally generated during sample processing, as in mixing by vortex action, opening of micro-centrifuge tubes, pipetting, ejecting pipette tips from micropipettes, and centrifuging under vacuum.

Segregation of laboratory space for all procedures up to and including the setting up of amplification reactions from those following amplification is helpful in preventing undesirable contamination by PCR products. Allocating a laminar flow hood for the handling of PCR products provides enhanced containment of amplified sequences. **Table 2.6** lists some of the specifications for the PCR lab.

The shaft of a conventional air-displacement micropipette is a likely vehicle for carry over of amplified sequences. This part of the pipette is exposed to aerosols produced during suction of samples into and out of the disposable tip. Therefore, a set of pipettes used exclusively for handling amplified DNA is recommended. In this regard, an extra precaution consists of assigning a set of positive-displacement micropipettes. Such pipettes employ a disposable piston that moves within a precisely molded capillary, making direct contact with the sample and thus eliminating the air-to liquid interface between the sample and the shaft of the device.

Table 2.6: Laboratory space allocation for PCR

Area A (Pre-PCR work)	Area B (Post-PCR work)
Weighing chemicals	Electrophoresis of PCR products
Buffer preparation	Processing of PCR products
DNA extraction	Restriction analysis and sequencing of PCR products
Preparation of stocks for PCR	Loading of amplified products for second stage amplification
Preparation of PCR	

Other potential sources of DNA, resulting in the intrusion of sequences that will confound amplification experiments are plasmid preparations with inserts containing a sequence amplified via PCR, purified restriction fragments containing a target sequence centrifuge chambers, and instruments (e.g. forceps, scissors, and microtome blades) used to prepare and analyze tissues.

A simple way of avoiding cross-contamination is to use reagents prepared in small aliquots, so that, stocks can be discarded when evidence of contamination arises. As far as possible, stocks should be prepared and stored in disposable plastic ware. Extractions for DNA destined to be amplified should also be performed in disposable tubes. The budget for disposable gloves should also be generous, as shed skin cells might very well contribute to "background" template activity, especially, when amplifying sequences present in multiple copies per cell, e.g., mitochondrial DNA.

PCR troubleshooting: Sometime nonspecific long products are present. These can be avoided by performing following steps:

1. Decrease annealing time, increase annealing temperature.
2. Decrease extension time, decrease extension temperature to 62-68° C.
3. Increase KCl (buffer) concentration to 1.2x-2x, but keep $MgCl_2$ concentration at 1.5-2mM.
4. Increase $MgCl_2$ concentration up to 3-4.5 mM but keep dNTP concentration constant.
5. Take less primer, take less DNA template, take less Taq polymerase.
6. If none of the above works, check the primer for repetitive sequences (BLAST align the sequence with the databases) and change the primer(s).
7. Combine some/all of the above.

In case shorter nonspecific products are present then these can be avoided by taking into consideration the following points:

1. Increase annealing temperature, increase annealing time, increase extension time, increase extension temperature to 74-78° C.
2. Decrease KCl (buffer) concentration to 0.7-0.8X, but keep $MgCl_2$ concentration at 1.5-2mM.
3. Increase $MgCl_2$ concentration up to 3-4.5 mM, but keep dNTP concentration constant.

4. Take less primer, take less DNA template, take less Taq polymerase.
5. If none of the above works, check the primer for repetitive sequences (BLAST align the sequence with the databases) and change the primer(s).
6. Combine some/all of the above.

Reaction was working before, but now you are not getting any product

1. Make sure all PCR ingredients are taken in the reaction (buffer, template, Taq, etc).
2. Change the dNTP solution (very sensitive to cycles of thawing and freezing, especially, in multiplex PCR).
3. If you just bought new primers, check for their reliability (bad primer synthesis?)
4. Increase primer amount, increase template amount.
5. Decrease annealing temperature by 6-10° C and check if you get any product.
6. Check all your PCR ingredients.
7. Combine some/all of the above.

If PCR product is weak. Is there a way to increase the yield?

1. Gradually, decrease the annealing temperature to the lowest possible.
2. Increase the amount of PCR primer, increase the amount of DNA template, increase the amount of Taq polymerase.
3. Change buffer (KCl) concentration (higher if product is lower than 1000 bp or lower if product is higher than 1000 bp).
4. Add adjuvants. Best, use BSA (0.1 to 0.8 µg/µL final concentration). You can also try 5% (v/v, final concentration) DMSO or glycerol.
5. Check primer sequences for mismatches and/or increase the primer length by 5 nucleotides.
6. Combine some/all of the above.

If two primers have very different melting temperatures (Tm) but the locus cannot be changed. What can be done to improve PCR amplification?

An easy solution is to increase the length of the primer with low Tm. If you need to keep the size of the product constant, add few bases at the 3' end. If size is not a concern, add few bases at either 3' or the 5' end of the primer.

Most important points

Avoiding contamination

Sources: Laboratory benches, equipment and pipetting devices can be contaminated by previous DNA preparations, plasmid DNA or by purified restriction fragments. Cross contamination between samples during isolation of nucleic acids or from the products from the previous PCR amplification.

Sample handling: Use sterile techniques and always wear fresh gloves.

Always use new and/ or sterilized glassware, plastic ware and pipettes to prepare the PCR reagents and template DNA.

Sterilize all reagents and solutions by filtration through a 0.22 μm filter.

Have your own set of PCR reagents and solutions and use them only for PCR reactions. Store these reagents in small aliquots.

Always include a negative (all reaction components without DNA) and a positive control (e.g. a PCR that has been successfully used in previous experiments).

Laboratory facilities: Set up physically separated working places for

Template preparation.

Setting up PCR reactions.

Post-PCR analysis

Use dedicated (PCR use only) pipettes, micro-centrifuges and disposable gloves.

Use aerosol resistant pipette tips.

Set up a PCR reaction under a fume hood equipped with UV light.

Reaction components: Template

Purity: The purity of the template largely influences the outcome of the PCR.

Large amounts of RNA in the sample can chelate Mg^{2+} and reduce the yield of the PCR.

Impure template may contain inhibitors that decrease the efficiency of the reaction.

Always use a purification product and / or procedure specially designed to purify DNA for PCR reactions.

Integrity: Template DNA should be of high molecular weight.

Check the size and integrity of the DNA, run an aliquot on an agarose gel.

Amount: The amount of template in a reaction strongly influences the performance of the PCR.

The recommended amount of template for a standard PCR is

maximum 500 ng for human genomic DNA.

1 -10ng of bacterial DNA

0.1 - 500 ng of plasmid DNA

The optimal amount of template depends on the application and the allowed error rate within the amplified DNA: lower the amount of template, higher the cycle number and the higher the probability of errors within the amplified DNA.

Lower amounts of template will require specific reaction modifications like changes in cycle numbers, redesign of primers, etc.

Tip: When testing a new template, include a positive control with primers that amplify a product of about the same size and produce a good yield.

Avoid dissolving the template in TE buffer since EDTA chelates Mg2+. Use 5-10 mM Tris (pH 7-8) instead.

Reaction components: Primers

General: Length of the primers should be 18 to 24 nucleotides.

GC content: 40-60%, with a balanced distribution of G/C and A/T rich domains.

Contain no internal secondary structure.

Primers are not complementary to each other at the 3' ends to avoid primer-dimer formation.

Melting temperature melting temperature T_m can be calculated as shown below :

T_m = [2^0C x (number of A & T bases)] + [4^0C x(number of G & C bases)]

Design primers with similar T_m values.

Annealing temperature: Optimal annealing temperatures are ~ 5 to 10^0C lower than the T_m values of the primers and have to be determined empirically.

Design primers as such that an annealing temperature of 55- 65^0C is allowed, for maximum specificity, use temperatures of 62 - 72^0C.

Concentrations of primers: Concentrations should be kept between 0.1 and 0.6 µM which are generally optimal.

Higher concentrations may promote mispriming and accumulation of non-specific products.

Lower concentrations may be exhausted before the reaction is completed, resulting in lower yields of the desired product.

Storage: Stock solutions are the lyophilized primers in a small volume of 5 mM Tris, pH 7.5 and store at -20^0C.

Working solution: to avoid repeated freezing and thawing, prepare small aliquots of 10 pmol/µl and store at -20^0C.

Tip: When testing new primers, include a positive control reaction with a template that has been tested in PCR.

Reaction components: $MgCl_2$ concentration

Function of Mg^{2+} ions	➢ Mg^{2+} ions form soluble complexes with dNTP's and template DNA to produce the actual substrate that the polymerase recognizes.
Concentration of Mg^{2+} ions	➢ The concentration of free Mg2+ ions depends on the concentrations of compounds like dNTP's, free pyrophosphates (PPi) and EDTA (eg. From TE buffer). These compounds bind to the ions via their negative charges. ➢ Therefore, the concentration of Mg2+ should always be higher than the concentration of these compounds.

- The most optimal concentration should be determined empirically and may vary from 1mM to 5mM.
- The most commonly used $MgCl_2$ concentration is 1.5 mM, with a dNTP concentration of 200 µM each.
- Excess Mg2+ in the reaction can increase non-specific primer binding and increase the non-specific background of the reaction.
- Too little Mg2+ in the reaction can result in a lower yield of the desired product.

Tip

- A PCR Optimization Kit allows an easy and straightforward optimization of this reaction component.

Reaction components: dNTP concentration

Concentration of dNTP

- Always use balanced solutions of all four dNTP's to minimize the error rate.
- The final concentration of each dNTP should be between 50 and 500 µM, the most commonly used concentration is 200 µM.
- Increase concentration of Mg2+ when increasing the concentration of dNTP's. Increase in dNTP concentrations reduce the concentration of free Mg2+ thereby interfering with the activity of the polymerase enzyme.
- For carry-over prevention, dNTP is substituted by dUTP. A three times higher concentration of dUTP (eg. 600 µM) compared with the other nucleotides normally results in a good amplification.

Storage

- Store stock solutions at -20°C.
- Working solutions (eg. 100 µl mixture of 10mM of each nucleotide) are also stored at -20°C.

Tip

- Special PCR grade nucleotides are specially purified and tested to ensure optimal results and maximum sensitivity in all PCR applications.

Reaction components: other factors

Concentration of polymerase	➢ For most assays, the optimal amount of thermostable DNA polymerase or blend of polymerases is between 0.5 and 2.5 units per 50 µl reaction volume. ➢ Increased enzyme concentrations sometimes lead to decreased specificity.
pH	➢ Generally, the pH of the reaction buffers supplied with the corresponding thermostable DNA polymerase (pH 8.3 – 9.0) gives optimal results. ➢ For some systems, raising the pH may stabilize the template and improve the reaction.
Reaction additives	➢ In some cases, the following compounds can enhance the specificity and /or efficiency of a PCR. ➢ Betaine (0.5 -2 M) ➢ Bovine serum albumin (100 ng/50 µl reaction mix) ➢ Dimethylsulfoxide (2 – 10%, v/v) ➢ Pyrophosphatase (0.001 -0.1 units/ reaction) ➢ Spermidine, Detergents, gelatine, T4 gene 32 protein

Cycling profile for standard PCR

Initial denaturation: It is very important to denature the template DNA completely by initial heating of the PCR mixture. Normally, heating for 2 minutes at 94 -95^{0}C is enough to denature complex genomic DNA. If the template DNA is only partially denatured, it will tend to "snap-back" very quickly, thereby, preventing efficient primer annealing and extension, or leading to "self-priming" which can lead to false positive results.

Denaturation during cycling: Denaturation at 94 -95^{0}C for 20 to 30 seconds is usually sufficient but must be adapted for the thermal cycler and tubes being used. If the denaturation temperature is too low, the incompletely melted DNA "snaps-back", preventing efficient primer

annealing and extension. Use a longer denaturation time or higher denaturation temperature for GC rich template DNA. Never use a longer denaturation time than absolutely required, unnecessary long denaturation times decreases the activity of the polymerase.

Primer annealing: The choice of the primer annealing temperature is a very critical factor in designing a high specificity PCR and - for most purposes-has to be optimized empirically. If the temperature is too high, no annealing occurs. If the temperature is too low, non-specific annealing will increase dramatically.

If the primers have complementary bases, primer-dimer effects will occur.

Primer extension: For fragments up to 3kb, primer extension is carried out at 72^0C. Taq DNA polymerase adds approximately 60 bases per second at 72^0C. A 45 second extension is sufficient for fragments up to 1 kb. For extension of fragments up to 3 kb, allow about 45 seconds per kb. However, this may need to be adjusted for specific templates. To improve yield, use the cycle extension time (e.g. 45 seconds for a 1 kb product).

Next 20 cycles: increase the extension time by 2 -5 seconds per cycle (eg. 50 seconds for cycle 11, 55 seconds for cycle 12/ onward cycles). This allows the enzyme more time to do its job because, as PCR progresses, there is more template to amplify and less active enzyme (due to denaturation during the prolonged high PCR temperatures) to do the extension.

Cycle number: In an optimal reaction, less then 10 template molecules can be amplified in less than 40 cycles to a product detectable by gel electrophoresis. Most PCR's should only include 25-35 cycles. As cycle number increases, non-specific products can accumulate.

Final extension: Usually, after the last cycle, the reaction tubes are held at 72^0C for 5-15 minutes to promote completion of partial extension products and annealing of single –stranded complementary products. After the final extension, immediately put the samples on ice or store at 4^0C.

Product characteristics

	Product size (kb)[a]	Error rate (10^{-6})[b]	Sensitivity[c]	Specificity[d]	Robustness[e]	Yield[f]	Labeling[g]	Cloning[h]	Carry-over prevention
Taq	3	26	++	++	+	++	++	TA	+
Pwo	3	3.2	+	+	+	+	++	Blunt	-
Tth	3	30	++	++	+	++	++	TA	+
Expand High Fidelity PCR system	5	8.5	+++	+++	++	+++	+++	TA	-
Expand long template PCR system	20	13	++	++	+++	+++	+++	TA	-
Expand 20 kb^{PLUS}	35	8.5	++	++	+++	+++	+++	TA	-
GC Rich PCR system	5	8.5	+++	+++	++++	+++	+++	TA	-

[a] Maximum fragment length that can be amplified from a human genomic template with good yield.
[b] Error rate calculated by the Lac I assay .
[c] Amplification of a template present at a low copy number.
[d] Amplifies only the fragment of interest.
[e] Amplification of template independent of contaminating agents, GC content, etc.
[f] Produces large amount of product in a given number of cycles.
[g] Efficient incorporation of modified dNTP's like Biotin dUTP, Dig dUTP, Fluo dUTP.
[h] Taq, Tth and the Expand blends produce fragments with 3'A overhangs and TA cloning is applicable.

Oligonucleotides: Commonly used formulas

Concentration ➢ 1 A_{260} Unit of an Oligonucleotide: 20-30 µg/ml H_2O

Molecular weight (in Dalton) ➢ $MW = (N_A \times 312.2) + (N_G \times 328.2) + (N_C \times 288.2) + (N_T \times 303.2) + P$

N_X = number of residues of respective nucleotide in the Oligonucleotide

P= +17 for phosphorylated oligonucleotide

P= - 61 for dephosphorylated oligonucleotide

Conversion of µg to pmol ➢

$$\text{pmol of oligo} = \mu g(\text{of oligo}) \times \frac{10^6 \text{ pg}}{1 \mu g} \times \frac{1 \text{ pmol}}{330 \text{pg}} \times \frac{1}{N}$$

$$\frac{\mu g \text{ (of oligo)} \times 3030}{N}$$

eg. 1 µg of a 20 bases oligo = 151.5 pmol

Conversion of pmol to µg

➢ µg of oligo = pmol (of oligo)

$$\times \frac{330\ pg}{1\ pmol} \times \frac{1\ \mu g}{10^6 pg} \times N$$

= pmol (of oligo) x N x 3.3 x 10^{-4}

e.g 1 pmol of a 20 bases oligo = 0.0066 µg

N= number of bases

Conversion of µM to pmol

➢ 1 µl of a X µM primer solution = X pmol primers

example:

Question

Given primer solution : 2.5 µM

Amount of primers needed for experiment: 2 pmol

Answer

1 µl of a 2.5 µM primer solution = 2.5 pmol

2 pmol = 0.8 µl

Conversion of pmol to µM

➢ 1 µM of a X pmol / µl primer solution = X µM primers

example:

Question

Given primer solution : 20 pmol / µl

Amount of primers needed for experiment: 5 µM

Answer

1 µl a 20 pmol/ µl primer solution = 20 µM primers

5µM = 0.25 µl

Chapter 3

Electrophoresis Techniques

A day without electrophoresis is very rare in molecular biology labs, because this technique is the standard method used for analyzing, identifying and purifying fragments of DNA. It is also used for separating and analyzing RNAs and oligonucleotides.The term electrophoresis describes the migration of charged particles under the influence of an electric field. Many important biological molecules such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Under the influence of an electric field, these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

General principles

In a gel (Agarose or Polyacrylamide), the negatively charged DNA fragments will move toward the positive electrode at a rate, which is inversely proportional to their length. After the electric field is applied for a certain period, DNA fragments with different lengths will be separated, which can be visualized by autoradiography or by treatment with a fluorescent dye (e.g., ethidium bromide) as shown in **Figure 3.1**. The relationship between the size of a DNA fragment and the distance it migrates in the gel is

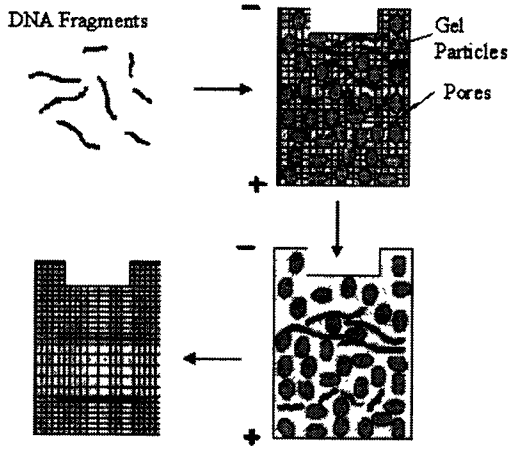

Figure 3.1: General principle of Nucleic Acid Gel Electrophoresis

logarithmic. Therefore, from the band positions, the lengths of DNA fragments can be determined.

Electrophoretic separation

In order to understand fully how charged species separate, it is necessary to look at some simple equations relating to electrophoresis.

1. When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient, E, which is applied voltage, V, divided by the distance, d, between the electrodes.
2. When this potential gradient E is applied, the force on a molecule bearing a charge of q coulombs is Eq newtons. It is this force that drives a charged molecule towards an electrode.
3. However, there is also a frictional resistance that retards the movement of this charged molecule. This frictional force is a measure of the hydrodynamic size of the molecule, the shape of the molecule, the pore size of the medium in which electrophoresis is taking place and the viscosity of the buffer.
4. The velocity, v, of a charged molecule in an electric field is therefore given by the equation:

$$U = Eq/f$$

Where, f is the frictional coefficient.

More commonly the term electrophoretic mobility (μ), of an ion is used, which is the ratio of the velocity of the ion to field strength (v/E). When a potential difference is applied, molecules with different overall charges will begin to separate due to their different electrophoretic mobilities. Even molecules with similar charges will begin to separate, if they have different molecular sizes, since they will experience different frictional forces. Electrophoresis is an incomplete form of electrolysis. The separated samples are then located by staining with an appropriate dye or by autoradiography, if the sample is radio-labeled.

The current in the solution between the electrodes is conducted mainly by the buffer ions with a small proportion being conducted by the sample ions. Ohm's law expresses the relationship between current (I), voltage (V) and resistance (R):

$$V/I = R$$

It therefore, appears that it is possible to accelerate an electrophoretic separation by increasing the applied voltage, which would result into

a corresponding increase in the current flowing. The distance migrated by the ions will be proportional to both current and time. However, this would ignore one of he major problems for most forms of electrophoresis, namely the generation of heat.

During electrophoresis, the power (W, watts) generated in the supporting medium is given by :

$$V/I = R$$

Most of this power generated is dissipated as heat. Heating of the electrophoretic medium can have the following effects:

i. An increased rate of diffusion of sample and buffer ions leading to broadening of the separated samples.

ii. The formation of convection currents, which leads to mixing of separated samples.

iii. Thermal instability of samples, that is rather sensitive to heat. This may include denaturation of proteins or loss of activity of enzymes.

iv. A decrease of buffer viscosity, and hence a reduction in the resistance of the medium.

If a constant voltage is applied, the current increases during electrophoresis due to this decrease in resistance and raised current increases the heat output still further. For this reason, workers often use a stabilized power supply, which provides constant power and thus, eliminates fluctuations in heating.

Electrophoretic equipment

The equipment required for electrophoresis consists of basically two items, a power pack and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel systems.

1. Vertical slab gel units

Vertical gel unit, shown in **Figure 3.2** are commercially available and routinely used to separate microsatellites regions, sequencing and proteins in acrylamide gels. The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. Gel dimensions are typically 12cm x 14cm, with a thickness of 1 - 2 mm. A plastic comb is placed in the gel solution and is removed after polymerization to provide loading wells for samples. When the apparatus is assembled, the lower electrophoresis tank buffer surrounds the gel plates and affords some cooling of the gel plates.

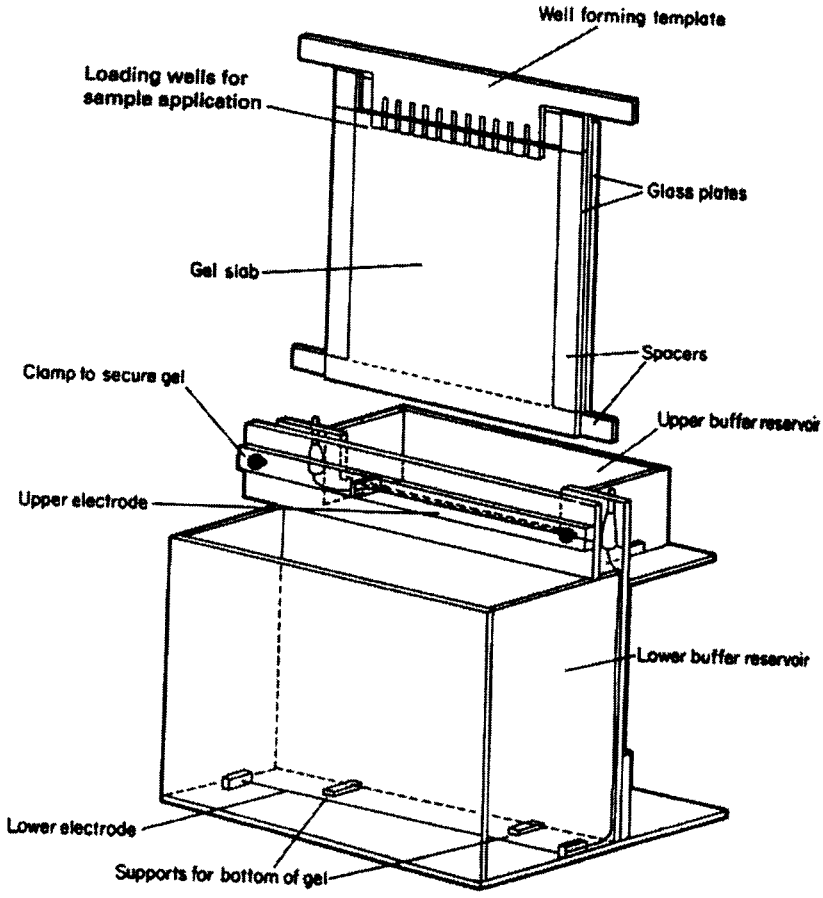

Figure 3.2: Vertical Gel Electrophoresis apparatus

2. Horizontal Gel Unit

A typical horizontal gel system is shown in **Figure 3.3**. The gel is casted on a glass or plastic sheet and placed on a cooling plate (an insulated surface through which cooling water is passed to conduct away generated heat). Both electrodes are present at the opposite end of unit, Agarose gels for DNA electrophoresis are run submerged in the buffer.

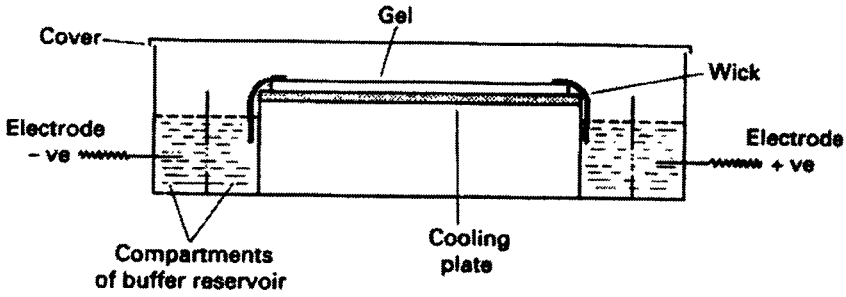

Figure 3.3: Horizontal electrophoresis apparatus

3. Power pack supply

The power pack supplies a direct current between the electrodes in the electrophoresis unit. All electrophoresis is carried out in an appropriate buffer, which is essential to maintain a constant state of ionization of the molecules being separated. Any variation in pH would alter the overall charge and hence, the mobilities (rate of migration in the applied field) of the molecules being separated.

Agarose electrophoresis

Agarose is a linear polysaccharide (average relative molecular mass about 12000) made up of the basic repeat unit Agarobiose, which comprises alternating units of galactose and 3, 6-anhydrogalactose. Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at concentrations of between 1% and 3% and are used for the electrophoresis of both proteins and nucleic acids. For proteins, the pore sizes of a 1% agarose gel are large relative to the sizes of proteins. Agarose gels are, therefore, used in techniques such as immuno electrophoresis or flat-bed isoelectric focusing, where the proteins are required to move unhindered in the gel matrix according to their native charge. Such large pore gels are also used to separate much larger molecules such as DNA or RNA, because the pore size in the gel are still large enough for DNA or RNA molecules to pass through the gel. Now, however, the pore size and molecule size are more comparable and frictional effects begin to play a role in the separation of these molecules. A further advantage of using agarose is the availability of low melting temperature agarose (62-65^{0}C). As the name suggests, these gels can be re-liquefied by heating at 65^{0}C and, thus, for example, DNA samples separated in a gel can be returned to solution and recovered.

Agarose gel electrophoresis of DNA

For the majority of DNA samples, electrophoretic separation is carried out in agarose gel. This is because most DNA molecules and their fragments that are analyzed routinely are considerably larger than proteins, hence, most of the DNA fragments would be unable to enter a polyacrylamide gel, therefore, the larger pore size of an agarose gel is required.

The charge per unit length (due to the phosphate groups) in any given fragment of DNA is the same, all DNA samples should move towards the anode with the same mobility under an applied electrical field. However, separation in agarose gel is achieved due to resistance to their movement caused by the gel matrix. The large molecules will have more difficulty in passing through the gel pores and very large molecules may even be blocked completely, whereas, the smaller molecules will be relatively unhindered. Consequently, the mobility of DNA molecules during gel electrophoresis will depend on size, for e.g. the smaller molecule moves faster.

Agarose gel formation

The gelling properties are attributed to both inter-and intra molecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anticonvectional properties. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentrations and smaller pore size are formed from the higher concentrations. Agarose is sold in different purity grades, based on the sulphate concentration, lower the sulphate content, higher is the purity.

1. Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution is formed.
2. Loading wells are formed by placing a plastic well-forming template or comb in the poured gel solution, and removing this comb once the gel has set.
3. This is poured on to a glass or plastic plate, surrounded by the wall of adhesive tape or a plastic frame to provide a gel about 3mm in depth.
4. It is allowed to cool to room temperature to form a rigid gel.
5. The gel is placed in the electrophoresis tank, covered with buffer, and samples loaded by directly injecting the sample into the wells.

Agarose concentration

When the proper concentration of agarose is selected, there is a linear relationship between the migration rate of a given DNA fragment and the logarithm of its size (in base pairs). Larger is the molecule, the more friction there is on a fragment as it attempts to move through the pores of the agarose gel slab; hence, more slowly it will migrate. **Table 3.1** indicates the appropriate agarose concentrations for several different ranges of fragment sizes. If an agarose gel of inappropriate concentration is prepared, the linear relationship between migration distance and the log of the base pairs will not be observed.

Loading of samples

DNA gels are invariably run as horizontal, submarine or submerged gels; so named because such a gel is totally immersed in buffer. Samples are prepared by dissolving them in a buffer solution that contains:

1. Sucrose, glycerol or ficoll, which makes the solution dense and allows it to sink to the bottom of the well.
2. A dye such as bromophenol blue is also included in the sample solvent; it makes it easier to see the sample that is being loaded and also acts as a marker of the electrophoresis front.

Table 3.1: Different Agarose gel concentration and size of the DNA that can be separated

Percentage of Agarose in gel	Efficient range of separation
0.3%	DNA fragments 5-60 kbp long
0.6%	DNA fragments 1-20 kbp long
0.7%	DNA fragments 0.8-10 kbp long
0.9%	DNA fragments 0.5-7 kbp long
1.2%	DNA fragments 0.4-6 kbp long
1.5%	DNA fragments 0.2-3 kbp long
2.0%	DNA fragments 0.1-2 kbp long

Running of agarose gel

General-purpose gels are approximately 25cm long and 12cm wide, and are run at a voltage gradient of about 1.5 V /cm overnight. A higher voltage would cause excessive heating. For rapid analyses that do not need extensive separation of DNA molecules, it is common to use mini-gels that are less than 10cm long. In this way, information can be obtained in 2-3h.

Visualization of DNA bands on Agarose gels

Once the electrophoresis is complete, the DNA in the gel needs to be stained and visualized. The reagent most widely used is the fluorescent dye ethidium bromide. The gel is rinsed gently in a solution of ethidium bromide (0.5μg cm^{-3}) and then viewed under ultraviolet light (300 nm wavelength). Ethidium bromide is a cyclic planar molecule that binds between the stacked basepairs of DNA (i.e. it intercalates). The ethidium bromide concentration therefore, builds up at the site of the DNA bands and under ultraviolet light the DNA bands fluoresce orange-red.

Protocol for Agarose Gel electrophoresis

Following protocol is for preparing 0.8% Agarose Gel used for checking the presence and quality of DNA extracted (Chapter 1.1), casting of the gel, preparation of electrophoresis buffers, preparation of loading and staining dye, loading of samples and finally visualizing the DNA bands. Same protocol can be used to prepare 2% of Agarose gel that is used to check the amplification product after PCR.

Solutions required

1. Agarose

0.8% Agarose (not low melting point)

8g per litre of 1XTAE (Tris Acetate) Buffer

2. 1kb ladder

From Invitrogen use 1μl per lane (to check the size of the DNA fragment)

3. Loading dye

0.25% bromophenol blue	2.5g per litre
0.25% xylene cyanol	2.5g per litre
25% Ficoll 400	250g per litre

Dissolved in 10XTAE (Tris Acetate) Buffer

4. Running buffer (TAE Buffer)

0.4M Tris	48.5g per litre
0.19M Glacial Acetic Acid	11.4mls per litre
10mM EDTA	3.7g per litre or 20 mls 0.5 M EDTA

1XTAE can be made by using 100ml of TAE (10X) made up to 1 litre with water

5. Staining dye (Ethidium Bromide)

Ethidium bromide solution (10mg/ml) 100 µl per litre

Equipment required

1. Microwave oven
2. Horizontal Agarose gel tank, comb, casting tray and Power Supply Unit
3. 1 ml micro tips
4. 200µl micro tips
5. Various sized automatic micro pipettes
6. 55^0C Water Bath
7. Biohazard tape
8. Transilluminator and photographic equipment

Method

1. Mix the appropriate weight of agarose with TAE buffer.
2. Place the container in the microwave at full power for 4 min 30 sec, heat, protecting gloves should be used when heating the agarose. Agarose should be heated till it gets dissolved, but it should not be boiled.
3. Once fully melted, leave in the microwave for 2 minutes to cool as immediate removal can result into boiling over.
4. Place at 55^0C waterbath to allow it to cool without setting.
5. Seal the edges of a small gel-casting tray with biohazard tape.
6. Add 100µl (per litre) of ethidium bromide (caution, it is carcinogenic) to the cooled agarose solution about 60^0C and mix by gentle swirling.
7. Insert a fine comb into the casting tray.
8. Pour agarose to a depth of about 1cm and allow it to get it solidified.
9. Remove the biohazard tape.
10. Take 10µl of 500µl DNA sample, add 2.5µl loading dye.
11. Fill tank to just above gel bed using TAE buffer (2 litres).
12. Place gel in tank ensuring that the gel is just submerged.
13. Add the samples to the sample wells and run at 80V for about 1hr, or until front dye is near the bottom of the gel.

14. Check electrodes are bubbling so you know the circuit is complete (or check that you have amps as well as volts by pressing the button on the power pack).
15. After the electrophoresis, photograph gel under UV (300nm) Tran illumination.

Polyacrylamide gels (PAGE)

Polyacrylamide is a cross-linked polymer of acrylamide. The length of the polymer chains is dictated by the concentration of acrylamide used, which is typically between 3.5 and 20%. Polyacrylamide gels are significantly more annoying to prepare than agarose gels. Because oxygen inhibits the polymerization process, they must be poured between glass plates (or cylinders). Polyacrylamide gels have a rather small range of separation, but very high resolving power. In the case of DNA, polyacrylamide is used for separating fragments of less than about 500 bp. However, under appropriate conditions, fragments of DNA differing in length by a single base pair are easily resolved. In contrast to agarose, polyacrylamide gels are used extensively for separating and characterizing mixtures of proteins. Polyacrylamide gels are the most sensitive and resolving method of electrophoresis that can be used to separate molecules that differ in size by as little as 2% of their molecular weight.

1. Cross-linked polyacrylamide gels are formed from the polymerization of **Acrylamide monomer** in the presence of smaller amounts of **N, N' –Methylene-bis-acrylamide** that is two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent.
2. Acrylamide monomer is polymerized in a head-to-tail fashion into long chains and occasionally, a Bis-acrylamide molecule is built into the growing chain, thus, introducing a second site for chain extension.
3. In this way, a cross-linked matrix of fairly well defined structure is formed.
4. The polymerization of acrylamide is an example of free-radical catalysis, and is initiated by the addition of **Ammonium Persulphate** and the base **N,N,N',N'-tetramethylenediamine (TEMED)**.
5. TEMED catalyses the decomposition of the persulphate ion to give a free radical (i.e., molecule with an unpaired electron):
   $S_2O_8^{2-} + e^- \rightarrow SO_4^{2} + SO_4^-$

If this free radical is represented as $R^{\bullet}$ (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule, then the polymerization can be represented as follows:

$R^{\bullet} + M \rightarrow RM$

$RM + M \rightarrow RMM$

$RMM + M \rightarrow RMMM$ etc.

In this way, long chains of acrylamide are built up, being cross-linked by the introduction of the occasional Bis-acrylamide molecule into the growing chain. Oxygen removes free radicals and therefore, all gel solutions are normally degassed (the solutions are briefly placed under vacuum to remove loosely dissolved oxygen) prior to use.

Advantages of PAGE

1. Polyacrylamide forms gels with pores of a much more controlled and uniform size than does agarose.
2. Consequently, polyacrylamide gels can be used to separate molecules that differ in size by as little as 2% of their molecular weight.
3. The range of pore sizes possible is also much broader. Acrylamide is particularly superior when very small pore sizes are needed. It can be used effectively to separate small molecules (i.e. DNA oligonucleotides of 100 bases or less) that tend to run right through agarose gels.
4. Gel matrix of PAGE is much stronger than agarose, producing gels that do not tear that easily.
5. It is also possible to load larger quantities of material onto acrylamide gels and (because of the relative purity of the gel ingredients) to recover the material in a very pure (often still biologically active) form.

Disadvantages of PAGE

1. Polyacrylamide gels are difficult to prepare when compared with agarose gels and the risk associated with their preparation is much greater.
2. When un-polymerized, polyacrylamide acts as a cumulative neurotoxin. This means that it can cause serious neurological damage, and that the damage caused by exposure is compounded by each subsequent exposure.

3. It is most dangerous in its powder form, when it is easily inhaled. Masks, gloves and safety glasses must always be worn when working with polyacrylamide powder.

Concentration of PAGE and its resolving power

Acrylamide gels are defined in terms of the total percentage of acrylamide present. Changing the concentrations of both the acrylamide and Bis-acrylamide can vary the pore size in the gel. Acrylamide gels can be made with a content of between 3% and 30% acrylamide. Thus, low percentage gels (e.g. 3%) have large pore size and are used, for example, in the electrophoresis of proteins, where free movement of the proteins by electrophoresis is required without any noticeable frictional effect, e.g. in flat-bed iso electric focusing or the stacking gel system of an SDS polyacrylamide gel. Low percentage acrylamide gels are also used to separate DNA. Gels of between 10% and 20% acrylamide are used in techniques such as SDS gel electrophoresis, where the smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size. As was the case with agarose gels, the gel concentration selected is a function of the size range of the molecules to be separated. The relationship between acrylamide concentration and linear range of separation for DNA is shown in **Table 3.2**

Table 3.2: Different PAGE concentration and size of the DNA that can be separated

PAGE %	Efficient range of separation
5-6%	DNA fragments 15-500 kbp long
8%	DNA fragments 8-350 bp long
9-10%	DNA fragments 4-200 bp long
12%	DNA fragments 2-80bp long
15%	DNA fragments 1-30bp long

Protocol for Polyacrylamide Gel Electrophoresis:

Following protocol is for preparing 8% PAGE with 30% Acrylamide solution (29:1 Acrylamide, Bis-acrylamide ratio) used for separating STR loci having 8bp or more repeat length unit (Chapter 6), casting of the PAGE and loading of samples. Rest of the requirements like Buffers, loading and staining dye are similar to that of agarose gel electrophoresis.

Solutions required

1. 30% acrylamide solution

29gms acrylamide and 1gm Bis-acrylamide dissolved in 100 ml solution of water. The mixture is dissolved on a magnetic stirrer for 4-6 hrs.

2. 10bp ladder

From Invitrogen use 1µl per lane (to carry out the allele sizing), Dissolve 1 µl of 10bp ladder in 9 µl of water and 2.5 µl of loading dye.

3. 10% Ammonium per-sulphate solution

1gm of $(NH4)_2SO_4$ dissolved in 10 ml of solution of water.

4.TEMED

N, N, N', N'-tetramethylenediamin provided by SIGMA Ltd.

Rest of the things like TAE buffer, loading dye (Bromophenol Blue) and staining dye (Ethidium Bromide) are similar to that of agarose electrophoresis. However, the final Buffer concentration used in Agarose gel electrophoresis is 0.5X while that in PAGE is 1X.

Equipment required

1. Vertical Electrophoresis unit, Glass plates, 0.75mm spacers, 0.75mm Comb, Gel caster and Power Supply unit.
2. 1 ml micro tips (Eppendorf) and 200µl micro tips (Regional Supplies).
3. Various sized automatic micro pipettes (Eppendorf).
4. Transilluminator and photographic equipment.

Method

1. Wash the glass plates, vertical gel assembly and gel cast thoroughly and dry with tissue paper so that no dust particle or stain remains there.
2. Put 0.75 mm spacer between the two glass plates (Notched and Plane).
3. Hold the two plates separated by the spacer tightly in the gel caster.
4. Seal the gap between the plates from bottom with the help of warm 2% Agarose solution (described above). This step is required only if gel caster is not available.
5. Another option is putting another 0.75mm spacer at the bottom in between two plates and tight it with the clips.

6. Prepare 8% PAGE solution (10ml)
   (i) 30% acrylamide solution 3ml
   (ii) 5X TAE Buffer 2ml
   (iii) 10% APS 100µl
   (iv) TEMED 10µl
   (v) DDH2O 5ml
7. Pour the PAGE solution immediately between the two glass plates.
8. Pouring should be done carefully, so that, sealing should not disturb, otherwise the liquid solution will flow down.
9. In case, a spacer has been placed at bottom (step 5), then pouring should be done horizontally keeping the plates in slightly bent condition on the palm of your hands. If sealing is done or gel caster is available then, PAGE solution should be poured vertically.
10. Put the comb into the solution from top, between two plates.
11. Step 7-9 should be done as soon as TEMED is added; otherwise the gel will be solidified in the flask itself.
12. Allow the gel to get solidified for 30-45 minutes.
13. Mark the wells, on the glass plate with permanent marker, this will help in recognizing the well's position while sample loading.
14. Remove the comb carefully.
15. Remove the clips, or else take out the plates with gel between them from the gel caster.
16. Assemble the plates on vertical gel apparatus in such a manner that notched plates should be inside (i.e. towards the cathode tank).
17. Fill both the tanks (cathode and anode) with 1X TAE buffer.
18. Give a run without loading the samples at 100V for 10 minutes.
19. Take 10µl of the PCR product and add 3.0µl loading dye.
20. Use 1µl of 10bp ladder and add 9µl water and 3.0µl loading dye as a marker.
21. Run the gel for 8-10 hrs for 100V.
22. Once the blue colour bromophenol dye reaches the anode tank, stop the electrophoresis.
23. Remove the plates from the gel apparatus.

24. Remove the spacer and open up the plane plate with the help of plate separator.
25. Stain the gel placed on the notched plate in a tray carrying the 5ul Ethidium Bromide solution dissolved in 200ml ddH_2O.
26. Shake it for a while till the gel comes out of the notched plate.
27. Take out the gel and take the photograph under UV (300nm) with the help of gel documentation system.

RNA electrophoresis

Native agarose gel electrophoresis may be sufficient to judge the integrity and overall quality of a total RNA preparation by inspection of the 28S and 18S rRNA bands. The secondary structure of RNA alters its migration pattern in native gels, so that, it will not migrate according to its true size. Bands are generally not as sharp as in denaturating gels, and a single RNA species may migrate as multiple bands representing different structures.

We generally load 1 µg and 2.5 µg samples on 1% agarose gels in TBE (89 mM Tris-HCl pH 7.8, 89 mM borate, 2 mM EDTA) with 0.5 µg/ml ethidium bromide added to the gel.

Add 10X native agarose gel loading buffer (15% ficoll, 0.25% xylene cyanol, 0.25% bromophenol blue) to the RNA samples to a final concentration of 1X.

On native gels, the samples can be loaded directly without heating.

An aliquot of intact RNA should always be run as a positive control to rule out unusual results due to gel artifacts.

Run the gel at 5-6 V/cm measured between the electrodes. After running it on to the gel the RNA will look like as depicted in **Figure 3.4**.

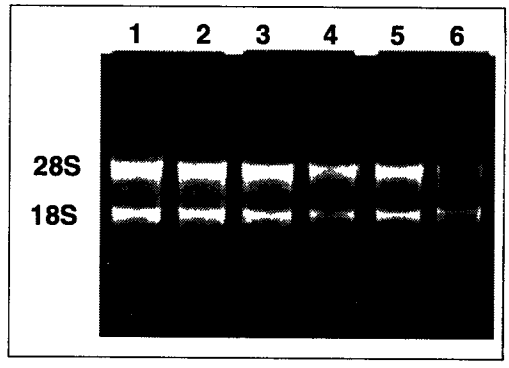

Figure 3.4 Expected results of RNA after running it on Native agrose. Two prominent rRNA Bands. Slight smear of various sized mRNA molecules in background

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Chapter 4

Reverse transcriptase PCR (Gene Expression Analysis)

Sensitive method for the detection and analysis of rare mRNA transcripts or other RNAs present in low abundance are an important aspect of most cell/molecular biology studies. RNA cannot serve as a template for PCR, so it must first be reverse transcribed into DNA known as the "complementary DNA or cDNA." Powell et al. first described a combined technique, now commonly known as RT-PCR in which reverse transcription (RT) is coupled with PCR amplification of the resulting cDNA as shown in **Figure 4.1**

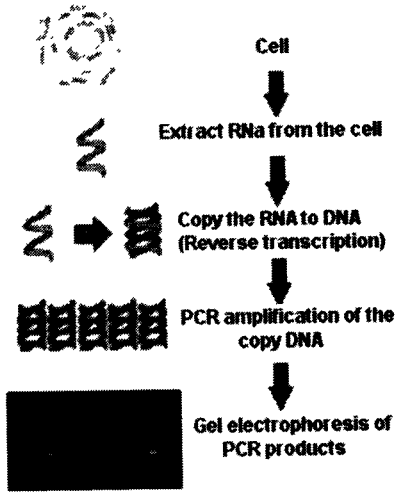


Figure 4.1: Diagrammatical representation of the procedure of RT-PCR

Obtaining pure RNA is an essential step in the analysis of pattern of genes expression and understanding the mechanism of gene expression. Isolation of pure, intact RNA is one of the central techniques in today's molecular biology.

Two strategies of RNA isolation are usually employed: isolation of total RNA and isolation of mRNA. A typical eukaryotic cell contains approximately 10 - 20pg of RNA, most of which is localized in the cytoplasm. Approximately, 80-85 percent of eukaryotic RNA is ribosomal RNA, while 15-20 percent is composed of a variety of stable low molecular weight species such as transfer RNA and small nuclear RNA. Usually, approximately 1-3 percent of the cell RNA is messenger RNA (mRNA) that is

heterogeneous in size and base composition.

Almost all eukaryotic mRNAs are monocistronic and contain a post-transcriptionaly added polyadenylic acid (poly A) tail at their 3′ -terminal. This poly -A 3′ -tail permits separation and isolation of mRNA from all other classes of RNA present in the cell. RT PCR is a two step process.

In the first step of RT-PCR, called the "first strand reaction," complementary DNA is made from a messenger RNA template using dNTPs and an RNA-dependent DNA polymerase (reverse transcriptase) through the process of reverse transcription. RT-PCR exploits a characteristic of mature mRNAs known as the 3' polyadenylated region, commonly called the poly (A) tail, as a common binding site for poly (T) DNA primers. In the case of bacterial mRNA, which lack a poly (A) tail sequence-specific primers can be generated to amplify the target mRNA sequence. These primers will anneal to the 3' end of every mRNA in the solution, allowing 5'-3' synthesis of complementary DNA by the reverse transcriptase enzyme.cDNA can also be prepared from mRNA by using gene specific primer or random hexamer primers.

After the reverse transcriptase reaction is complete, and complementary DNA has been generated from the original single-stranded mRNA, standard polymerase chain reaction, termed the "second strand reaction," is initiated. If the initial mRNA templates were derived from the same tissue, subsequent PCR reactions can be used to probe the cDNA library that was created by reverse transcription. Primers can be designed to amplify target genes being expressed in the source tissue. Quantitative real-time PCR can then be used to compare levels of gene expression.

1. A thermostable DNA polymerase and the upstream and downstream DNA primers are added.
2. The reaction is heated to temperatures above 37°C to facilitate sequence specific binding of DNA primers to the cDNA
3. Further, heating allow the thermostable DNA polymerase to make double-stranded DNA from the primer bound cDNA.
4. The reaction is heated to approximately 95°C to separate the two DNA strands.
5. The reaction is cooled enabling the primers to bind again and the cycle repeats.

After approximately 30 cycles, millions of copies of the sequence of interest are generated. The original RNA template is degraded by RNase H, leaving pure cDNA (plus spare primers).

This process can be simplified into a single step process by the use of wax beads containing the required enzymes for the second stage of the process which are melted, releasing their contents, on heating for primer annealing in the second strand reaction.

Usage of reverse transcription polymerase chain reaction

The exponential amplification via reverse transcription polymerase chain reaction provides for a highly sensitive technique, where a very low copy number of RNA molecules can be detected. Reverse transcription polymerase chain reaction is widely used in the diagnosis of genetic diseases and, quantitatively, in the determination of the abundance of specific different RNA molecules within a cell or tissue as a measure of gene expression. Northern blot is used to study the RNA's gene expression further.

Procedure of RT-PCR

Introduction (Analysis of β-actin genes)

There are six known actin proteins in mammalian cells: two sarcomeric muscle actins (α - skeletal and α -cardiac), two smooth muscle actins (α and γ), and two non-muscle cytoskeletal actins (β and γ). Three genes have been mapped for human actin protein: β-actin gene on chromosome 7, α -skeletal actin gene on chromosome 1 and α-cardiac antigen on chromosome 15. The β-actin gene on chromosome 7 was mapped to the 7p 15 position. In addition to one functioning β -actin gene, there are approximately 20 pseudo-genes widely distributed in the human genome. Only four β -actin pseudo-genes were mapped to other chromosomes (chromosomes 5, 13, and 18).

RT-PCR Kit" is used for carrying out RT-PCR. This kit is designed for sensitive, quick, and reproducible analysis of RNA with high fidelity. Preferably, use one step reaction kit. The one step reaction system uses avian myoblastosis virus (AMV) transcriptase for first -strand synthesis and Expend^{TM} High Fidelity enzyme blend, which consists of *Taq* DNA polymerase and *Pwo* DNA polymerase, for the PCR.

The expected length of the product of our RT-PCR reaction should be 557 bases. Products of different size can also appear. This will depend on the structure of gene one is looking for (a missing processing site

at the intron- exon boundary) or partial expression of pseudo-genes (mRNA synthesis).

Sensitive methods for the detection and analysis of RNA molecules are an important aspect of most cell/molecular biology studies. Commonly used methods include *in situ* hybridization, Northern blots, dot or slot blot analysis, S1 nuclease analysis, and RNAse protection assays. In situ hybridization is very sensitive, but is a rather difficult technique. Other common methods lack sensitivity for detecting small amounts of RNA.

The adaptation of PCR methodology to the investigation of RNA provides a method having speed, efficiency, specificity, and sensitivity. Since RNA cannot serve as a template complementary DNA (cDNA) suitable for PCR. The combination of both techniques is named RT-PCR.

The process of RT-PCR has proven to be invaluable for detecting gene expression, for amplifying RNA sequences prior to subcloning and analysis, and for the diagnosis of infectious agents or genetic disease. This technique is the most sensitive procedure for determining the presence or absence of RNA templates or quantifying the level of gene expression. The use of RT-PCR for analyzing genetic diseases is particularly advantageous, because, it circumvents inefficient amplification of long DNA fragments caused by long introns and provides additional information aboutphenomena such as alternative splicing.

Two different techniques are used for RT-PCR.

1. Two step RT-PCR. The synthesis of cDNA is performed with reverse transcriptase from AMV, *moleney murine leukemia virus* (M-MuLV), or *Thermus thermophilus* (Tth) DNA polymerase in the first step, followed by PCR with an appropriate thermostable DNA polymerase. The two-step reaction requires that the reaction tube is opened after cDNA synthesis and reagents are added for the PCR part of the procedure. This is inconvenient and increases the risk of contamination.
2. One-step RT-PCR. The cDNA synthesis and the PCR are performed together in a single tube. Two techniques are in use for running one-step RT-PCRs. The first technique uses Tth DNA Polymerase for carrying reverse transcription and PCR reactions. The second uses AMV reverse transcriptase and Taq DNA Polymerase.

The first method relies on the ability of the Tth DNA Polymerase to use an RNA template for DNA synthesis (reverse transcription) as well as to use a DNA template (PCR). This enzyme is thermostable allowing both reactions to be carried at elevated temperature. Carrying out the reaction at elevated temperature helps to unravel secondary structures of RNAs, there by allowing the synthesis of longer products and increasing the efficiency synthesis of the first strand of DNA. However, low processivity of the Tth DNA Polymerase limits the RT-PCR products to less than 1.0kb.

The second method uses AMV reverse transcriptase and Taq DNA Polymerase. This technique allows amplification of fragments of up to 2.0 kb with lower error rates as compared to the use of Tth DNA polymerase. However, the reverse transcription step of the reaction has to be performed at 42^0C and therefore, is strongly affected by the secondary structure of mRNA.

Reverse transcriptases

Reverse transcriptases are RNA-dependent DNA polymerases that have predominantly been used for catalyzing first-strand cDNA (complementary DNA) synthesis. However, reverse transcriptases are also capable of synthesizing a DNA strand complementary to a primed single-stranded DNA.

Three different enzymes with reverse transcriptase activity are now commercially available: the viral reverse transcriptases (RTases) from avian myoblastosis virus (AMV), M-MuLV,and the heat -stable DNA Polymerase derived from T. thermophilus. All these enzymes require different pH, salt concentration, and incubation temperatures for optimal activity.

The AMV and m-MuLV viral RTases are highly processive and are able to synthesize cDNAs of up to 10 kb. Tth DNA Polymerase is able to synthesize cDNA in the range of 1.0 - 2.0 kb, which is sufficient since fragments of <1 kb are usually used for PCRs.

The unique advantage of the Tth DNA Polymerase is its ability to perform both reverse transcription and PCR amplification in a one-step reaction.

Priming of a reverse transcriptase reaction

There are three types of primers that may be used for reverse transcription.

1. Oligo (dT) 12-18 primer. This primer binds to the endogenous poly(A) +tail at the 3′ -end of mammalian mRNA. A reaction with this primer frequently produces a full-length cDNA product.
2. Random hexanucleotide primers. These primers can bind to mRNA templates at any complementary site and will give partial length (short) cDNAs. These primers may be better for overcoming the difficulties caused by template secondary structure. The random primers may also transcribe more 5′ -regions of the RNA.
3. Specific oligonucleotide primers. These primers can be used for selectively priming the RNA of interest. This approach has been used very successfully in diagnostic assays, as well as in basic research.

Analysis of the results of the RT-PCR

1. Prepare a mini-gel using a casting tray not larger than 7.5 cm x 7.5 cm. Seal the ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Check that the bottom of the comb is approximately 0.5 mm above the gel bottom. To adjust this height, it is most convenient to place a plastic charge card (For example Master Card) at the bottom of the tray and adjust the comb height to a position where it is easy to remove the card from under the comb.
2. Prepare a 1.6 percent agarose mini-gel in one times TAE (Tris - acetate EDTA) buffer. Use 30 ml of agarose solution. Weigh 480 mg of agarose and add it to 30 ml of one time TAE buffer. Dissolve agarose in a microwave oven, adjust the volume to 30 ml with water, and add 1 µl of ethidium bromide. Pour the agarose into the casting tray. Allow the gel to solidify for 20 -30 minutes.
3. Add 5 µl of stop solution to each tube with PCR reactions. Mix by pipetting up and down. Load prepared samples onto the gel. If only two or three samples are loaded onto the gel, use wells in the center of the gel.
4. Load 6 µl of a size standard (1kb ladder) into the well to the left of your samples. Run gel electrophoresis for 20-30 minutes at 60-80 V. Photograph the gel and analyze the results.

RT-PCR amplification of a particular mRNA sequence requires two PCR primers that are specific for that mRNA sequence. The primer design should also allow differentiation between the amplified product

of cDNA and an amplified product derived from contaminating genomic DNA. There are two different strategies to design the required primers (**Figure 4.2**).

Strategy 1 Design primers that anneal to sequences in exons on both sides of an intron. With these primers, any product amplified from genomic DNA will be much larger than a product amplified from intron less mRNA. (Panel -1)

Strategy 2 Design primers that span exon/exon boundaries on the mRNA. Such primers should not amplify genomic DNA. (Panel -2)

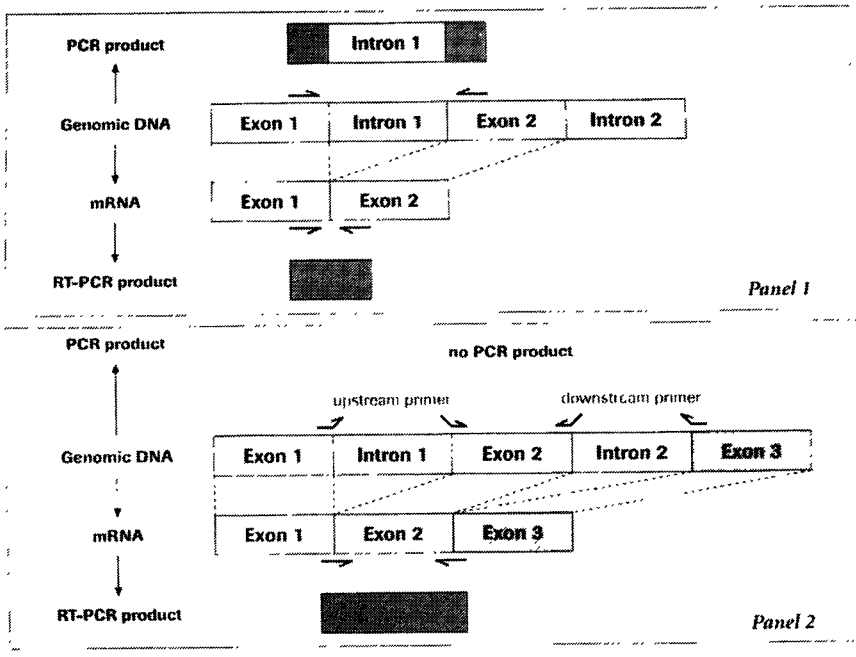


Figure 4.2 Primer designing

Most Important points

Working with RNA

Reaction Components: Choice of enzymes

Temperature optima

- Higher incubation temperatures help to eliminate problems of template secondary structures.
- High temperature improves the specificity of reverse transcription by decreasing false priming.

	➢ Thermoactive reverse transcriptases that can be incubated at higher temperatures (50-70°C) are more likely to produce accurate copies of mRNA, especially, if the template has a high GC content. *Note:* use only specific primers at high temperatures; do not use oligo (dT) or random hexamer primers.
RNAse H Activity	➢ AMV and M-MuLV reverse transcriptases have RNAse H activity. ~ This activity will specifically degrades RNA in a RNA:cDNA hybrid and this can be detrimental if degradation of template RNA competes with DNA synthesis during production of first-strand cDNA. ~ M-MuLV has lower RNAse H activity than AMV Reverse transcripatase. ~ Mutated enzymes without RNAse H activity are available.
Divalent Ion requirement	➢ Most reverse transcriptases require divalent ions for activity. ➢ Enzymes that use Mg2+ are likely to produce more accurate cDNA copies than those that use Mn2+ since Mn2+ adversely affects the fidelity of the DNA synthesis.
Sensitivity	➢ Reverse transcriptases have different ability to copy smaller amount of template.
Specificity	➢ Reverse transcriptases differ in their ability to transcribe RNA secondary structures accurately.
Reverse Trancriptase - DNA polymerase combinations	➢ Using different reverse transcriptase - DNA polymerase combination will allow to optimally amplify certain target length.

Procedures

Two Step procedure	One tube One Step procedure
➢ **Two tube, two step procedure:** ❖ In the first tube, the first strand cDNA synthesis is performed under optimal conditions. ❖ An aliquot of this RT reaction is then transferred to another tube containing all reagents for the subsequent PCR.	➢ Both cDNA synthesis and PCR amplification are performed with the same buffer and site-specific primers, eliminating the need to open the reaction tube between the RT and PCR steps.

Two Step procedure	One tube One Step procedure
➢ **One tube, two step procedure:** ❖ In the first step, the reverse transcriptase produces first stranc cDNA synthesis in the presence o Mg2+ ions, high concentrations of dNTP's and primers. ❖ Following this reaction, PCR buffer (without Mg2+ ions), a thermostable DNA. Polymerase and specific primers are added to the tube and the PCR is performed.	

Advantages of Two Step procedure	Advantages of One tube One Step procedure
➢ **Optimal reaction conditions:** ❖ The two step format allows both reverse transcription and PCR to be performed under optimal conditions ensuring an efficient and accurate amplification.	➢ **Minimal time required:** ❖ The one step reaction has fewer pipetting steps than the two step reaction, thereby significantly reducing the time needed for the experiment and eliminating pipetting errors.
➢ **Flexibility:** ❖ Two step procedures allow the product of a single cDNA synthesis reaction to be used in several PCR reactions for analysis of multiple transcripts. This flexibility is valuable for specialized applications as rapid amplification of cDNA ends and differential display reverse transcription.	➢ **Reduced risks for contamination:** ❖ The entire one step reaction takes place in a single tube with no transfers required and no need to open the reaction tube. (steps where contamination can occur).
➢ **Amplifies long sequences:** ❖ With the right combination of reverse transcriptases and thermostable DNA polymerases, two step RT-PCR can amplify RNA sequences up to 14 kb long.	➢ **Improved sensitivity and specificity:** Two characteristics of the one step reaction provide increased yield and efficiency: ❖ the cDNA reaction is performed at a high temperature, thereby, eliminating problems with secondary RNA structures. ❖ the entire cDNA sample is used as template for the PCR.

Reaction Components: Choice of enzyme for One Step RT-PCR

Use this table to select a product according to the size

			Sensitivity						
Product size	Type of primers	Primer annealing temperature	mRNA	Viral RNA / RNA with secondary structures	Specificity	Fidelity	Influence of nonspecific RNA	Influence of serum components	Recommendation
up to 1 kb	Specific primers	55 - 70°C	+	+	+	-	+++	+++	Tth DNA Polymerase
up to 3 kb	Specific primers	55 - 70°C	++	++	++	+	+	+	C. thermo Polymerase One Step RT-PCR System"
up to 6 kb	Specific primers	45 - 60°C	+++	+	+	++	++	+++	Titan One Tube RT-PCR System

"C. therm Polymerase yields optimal results with GC-rich RNA templates

Choice of enzyme for Two Step RT-PCR

Use this table to select a product according to the size

			Sensitivity[b]					
Product size	**Type of primers**	**Primer annealing temperature**	**Total RNA**	**Viral RNA**	**Specificity[c]**	**Fidelity**	**GC rich structures**	**Recommendation**
up to 1 kb	specific primers	55 - 10°C	nd	nd	nd		nd	>- Tth DNA Polymerase
up to 4 kb	specific primers oligo (dT) primers hexamer primers	55 – 10°C	+	++	++	++	+++	>- C. thermo Polymerase for reverse transcription in two step RT-PCR
up to 10 kb	specific primers oligo (dT) primers hexamer primers	31°C	+	+	nd	+	+	>- M-MuLV Reverse Transcriptase
up to 12 kb	specific primers hexamer primers	42°C (up to 60°C)	++	+	+	+	+	>- AMV Reverse Transcriptase >- First Strand cDNA Synthesis Kit
up to 14 kb	specific primers hexamer primers	42°C (up to 60°C)	++	+	+	+	+	Expand Reverse Transcriptase

b Sensitivity was comparatively tested for fragments in the range of 0.1 - 3 kb. c Reverse transcription was performed with a sequence specific primer that also was used for the subsequent PCR step. d Each of the products in this columns should be used for first strand cDNA only (first step). To choose the appropriate product for the PCR step (second step).

Product characteristics

	Maximum size of template (kb)	Temperature Optimun[a]	Ion requirements	RNAse H activity	Use as combined reverse transcriptase and DNA polymerase	Units needed for standard reaction
Expand Reverse Trancriptase	up to 14 kb	42°C (up to 50°C)	Mg2+	-	-	50
AMV Reverse Transcriptase	up to 12 kb	42°C (up to 60°C)	Mg2+	++	-	40
M-MuLV Reverse Transcriptase	up to 10 kb	37°C	Mg2+	+	-	40
C. therm Polymerase for Reverse Transcription in Two Step RT-PCR	up to 4 kb	60 - 70°C	Mg2+	-	-[b]	6
Tth Polymerase	up to 1 kb	60 - 70°C	Mn2+	-	+	4

[a] Number in parentheses indicate maximum temperature tolerated by enzyme.

[b] C. thermal Polymerase for reverse transcription in two step RT-PCR does not survive incubation at 95°C.

For RT-PCR. use the polymerase together with a second thermostable enzyme such as Taq DNA Polymerase or Expand High Fidelity PCR System.

	Full Length cDNA	High Fidelity cDNA for cloning	Amplification of viral RNA	DD-RT[c]	RACE[c]	PTT[c]	One Step RT-PCR	One Step RT-PCR with carry-over prevention
Expand Reverse Trancriptase[e]	+++	+	++	+++	++	+++	++	nd
AMV Reverse Transcriptase	++	+	++	+	++	+++	++ (+++)[d]	+ (-)[d]
M-MuLV Reverse Transcriptase	+	+	++	++	+	++	+	nd
C. therm Polymerase for Reverse Transcription in Two Step RT-PCR	+	++	+++	+	++	++	+++	+
Tth Polymerase	-	-	+++	+	%	%	++	+

[c] PTT = Protein Truncation Test, DD-RT = differential display reverse transcription, RACE = rapid amplification of cDNA ends.

[d] When used as a component of the Titan One Tube RT-PCR System.

[e] not for sale in the US.

nd = not determined

Pipetting scheme for One Step RT-PCR

Mix 1 (for 1 reaction)

Reagent	Titan One Tube RT-PCR System	C. thermo Polymerase One-Step RT-PCR System	Final concentration
H_2O	variable	variable	200 µM of each dNTP
10 mM Nucleotide mix	4µl		400 µM of each dNTP
25 mM Nucleotide mix		0.8µl	0.4 µM each, 0.3 µM each
Upstream and downstream primer	Variable	Variable	7%
DMSO		2.5µl	5mM
100 mM DTT Solution	2.5µl	2.5µl	5 -10 units/ reaction
RNase Inhibitor (optional) (5 U/Ill)	1µl	1 µl	1 pg- 1 µg RNA/ reaction
Template RNA	Variable	Variable	50 pg - 1µg RNA / reaction
Volume	**25µl**	**25µl**	

* The reaction buffer for the C. thermo Polymerase One-Step RT-PCR System adds 2% DMSO to the final reaction mix.

Add enough additional DMSO (2.5 Ill) to increase the final concentration to 7%. For some applications, the final DMSO concentration may need to be as low as 3% or as high as 10% (determined empirically).

Mix 2 (for 1 reaction)

Reagent	Titan one tube RT-PCR system	C. therm. Polymerase one -step RT-PCR system	Final concentration
H_2O	14µl	13µl	
RT-PCR Buffer, 5X	10µl	10µl	1X
Enzyme mix	1 µl	2 µl	
Volume	25 µl	25 µl	

Combine mix 1 and 2 in a thin-walled PCR tube. Gently vortex the mixture to produce a homogenous reaction, then centrifuge briefly to collect the sample at the bottom of the tube.

For first strand synthesis, place the sample into an equilibrated thermal cycler and incubate as follow:

- For Titan One Tube RT-PCR system: incubate at 50 - 60^{0}C for 30 min.

- For C. therm Polymerase One-Step RT-PCR System: incubate at 60 - 72^{0}C for 30 min.

Immediately, following cDNA synthesis, begin PCR. For temperature profiles, please refer to the next page.

Thermal profile for one step RT-PCR

Titan one tube RT-PCR system

	Temperature	Time	Cycle number
Initial denaturation	94^{0}C	2 min	
Denaturation	94^{0}C	30 s	10
Annealing	45-65^{0}C	30s	
Elongation	68^{0}C	45 s -4 min[b]	
Denaturation	94^{0}C	30 s	25[c]
Annealing	45-65^{0}C	30s	
Elongation	68^{0}C	45 s -4 min[b] +5 s cycle longation for each successive cycle	
Final elongation	68^{0}C	7 min	

[a] The exact annealing temperature depends on the melting temperature of the PCR primers.
[b] Use 45 s for gragments up to 1kb; 1 min for 1.5 kb gragments; 2 min for 3 kb fragments; 3 min for 4.5 kb fragments and 4 min for 6 kb fragments.
[C] The number of cycles depends upon the abundance of the respective mRNA. For rare mRNA messages, 40 cycles or a second (nested) PCR could be necessary.

C. therm. Polymerase one -step RT -PCR system

	Temperature	Time	Cycle number
Initial denaturation	94^{0}C	2 min	
Denaturation	94^{0}C	30 s	10
Annealing	60-70^{0}C[d]	30s	
Elongation	70- 72^{0}C	45 s -4 min[e]	
Denaturation	94^{0}C	30 s	20-25[f]
Annealing	60-70^{0}C[d]	30s	
Elongation	70- 72^{0}C	45 s -4 min[e] +5 s cycle elongation for each successive cycle	
Final elongation	70 - 72^{0}C	7 min	

[d] The exact annealing temperature depends on the melting temperature of the PCR primers.
[e] The elongation time for each cycle depends upon the length of the expected product. To choose the appropriate elongation time for your template, assume an elongation rate of 1 min /kb.
[f] The number of cycles depends upon the abundance of the respective mRNA. For rare mRNA messages. 40 cycles or a second (nested) PCR could be necessary.

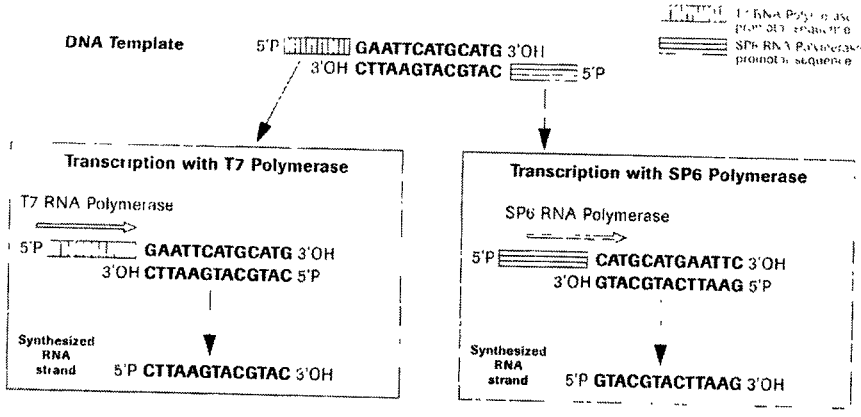
Labeling of RNA

Overview of different techniques

Application	Labeling method	Relative sensitivity
Northern blotting	➢ Labeling RNA by in vitro transcription	+++
Southern blotting	➢ 5′ End labeling*	++
	➢ 3′ End labeling *	++
Dot /Slot Blotting	➢ Labeling RNA by in vitro transcription	+++
	➢ 5′ End labeling*	++
	➢ 3′ End labeling *	++
In situ Hybridization	➢ Labeling RNA by in vitro transcription	+++
	➢ 5′ End labeling*	++
	➢ 3′ End labeling *	++

* for an extensive overview of these techniques, " Labeling of DNA and oligonucleotides".

Principle of *in vitro* transcription with DNA dependent RNA polymerases is given below:



Labeling of RNA

SP6, T7 and T3 polymerases

Properties
- DNA dependent RNA polymerases, specific for their corresponding promotor.

10x Transcription buffer
- 400 mM Tris (pH 8.0), 60 mM $MgCl_2$, 100 mM DTT, 20 mM spermidine
- Buffer without nucleotide is stable at - 20°C, store in aliquots.

Application and typical results
- Generate homogeneously labeled single stranded RNA molecules as probes for hybridization experiments.
- Radioactive nucleotides (^{32}P, ^{35}S) and non radioactive nucleotides (biotin, digoxigenin, fluorochromes) can be incorporates.
- **Standard Assay:**

Components	Radioactive	Non-radioactive	Cold (without label)
Template DNA	0.5µg	1 µg	1 µg
Nucleotides, final concentration	ATP, GTP, UTP, each 0.5 mM final	ATP, GTP, CTP, each 1 mM final UTP 0.65 mM final	ATP, GTP, CTP, UTP, each 1 mM final
Labeled nucleotide, final concentration	[α^{32}P] CTP (400 Ci/ mmol), 50 µCi (1.85 MBq / mmol)	Dig, biotin or fluorochrome UTP, 0.35 mM final	
10x transcription buffer	2 µl	2 µl	2 µl
RNA polymerase	20 units	40 units	40 units
RNase inhibitor	20 units	20 units	20 units
H_2O	Add H_2O to 20 µl	Add H_2O to 20 µl	Add H_2O to 20 µl
Incubation	20 min / 37°C	2 hours / 37°C	2 hours / 37°C

Inactivation of enzyme
- Add 2 µl 0.2 M EDTA and / or heat to 65°C for 10 min.

Safety precautions

The agarose gel contains ethidium bromide, which is a mutagen and suspected carcinogen. Contact with the skin should be avoided. Students should wear gloves when handling ethidium bromide solution and gels containing ethidium bromide. Discard the used gel

into the designated container.

For safety purposes, the electrophoresis apparatus should always be placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench. To avoid electric shock always disconnect the red (positive) lead first.

UV light can damage the retina of the eye and cause severe sunburn. Always use safety glasses and a protective face shield to view the gel. Work in gloves and wear a long-sleeved shirt or laboratory coat when operating UV illuminators.

Chapter 5

Real Time PCR

Introduction to Real Time PCR

If we need to quantify the PCR product then the approach used is to do the quantative PCR. Various dyes can be used which bind to the double stranded DNA to quantify the amount of product being produced. Another approach is to use molecular beacons. These are short reporter oligonucleotides with a flurochrome bound to one end and a quencher molecule on the other end that hybridize in the middle of the target sequence to be amplified. As long as the short oligonucleotide is intact, the fluorochrome and quencher are close enough in proximity, so that, the fluorescence is quenched. When the DNA polymerase synthesizes a new strand of DNA complementary to the template, its exonuclease activity degrades the reporter oligonucleotide. The fluorochrome is then separated from the quencher and fluoresces. The amount of fluorochrome liberated in a given PCR cycle is directly proportional to the number of template molecules being copied by the polymerase. This system of PCR is different from the PCR described before it is called real time PCR.

As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in a given time. A relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. Real Time PCR is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. Real time PCR allows the detection of PCR product during the early phases of the reaction. This ability of measuring the reaction kinetics in the early phases of PCR which provides a distinct advantage over traditional PCR detection. Traditional methods used are gel electrophoresis for the detection of PCR amplification in the final phase or at the end-point of the PCR reaction. While in real time PCR we use optical detection system as shown in **Figure- 5.1**.

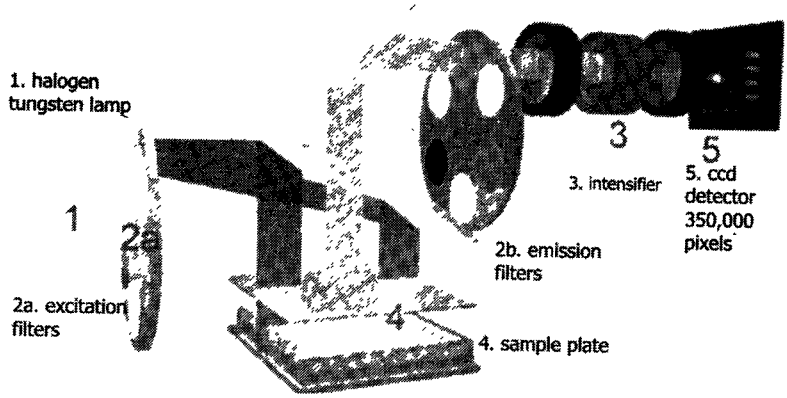


Figure 5.1 Representation of optical detection system layout.

In real time PCR, there occurs an accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). Real time PCR facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. Real time PCR assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation. One of the biggest advantages of real time PCR is that only small amount of mRNA is required to get the desired results. PCR methods are particularly valuable when amounts of RNA are low, since the fact that they involve an amplification step means they are more sensitive. Real time PCR has different applications like i) Quantitative mRNA expression studies. ii) DNA copy number measurements in genomic or viral DNAs. iii) Allelic discrimination assays or SNP genotyping. iv) Verification of microarray results. v) Drug therapy efficacy. vi) DNA damage measurement.

Limitations of real time PCR

In a PCR reaction as the reaction progresses, the reagents are being consumed as a result of amplification. Now, the PCR product is no longer being doubled at each cycle due to this reagent constraint.

This depletion will occur at different rates for each replicate. Thus, the samples begin to diverge in their quantities. This diminished amplification is the linear phase of the reaction. The plateau for each tube will differ due to the different reaction kinetics for each sample. It is in this phase where traditional PCR takes its measurement, also known as the end-point. This End-Point Detection has some problems such as low resolution, poor precision, low sensitivity and the need for post PCR processing. Also, the results of this detection are not expressed in numbers and there is no scope for automation. However, Real time PCR can detect i) Non-specific detection using DNA binding dyes ii) Specific detection target specific probes.

Non-specific detection using DNA binding dyes

In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase. If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during real time PCR, a linear relationship is observed.

SYBR® Green is the most widely used double-strand DNA-specific dye reported for real time PCR. SYBR® Green binds to the minor groove of the DNA double helix. In the solution, the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double strand DNA. SYBR® Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Ethidium bromide can also be used for detection but its carcinogenic nature renders its use restrictive. The advantage of SYBR green upon ethedium bromide is that the ratio of fluorescence in the presence of double-stranded DNA to the fluorescence in the presence of single-stranded DNA is much higher than that for ethidium bromide. Although, these double-stranded DNA-binding dyes provide the simplest and cheapest option for real time PCR, the principal drawback to intercalation based detection of PCR product accumulation is that both specific and nonspecific products generate signal.

Real-Time PCR Chemistries

Currently four different chemistries, TaqMan® Molecular Beacons, Scorpions® and SYBR® Green (Molecular Probes), are available for real-time PCR. All of these chemistries allow detection of PCR products via the generation of a fluorescent signal. TaqMan probes, Molecular Beacons and Scorpions depend on Förster Resonance Energy Transfer (FRET) to generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher moiety to the same or different oligonucleotide substrates. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution, but emits a strong fluorescent signal upon binding to double-stranded DNA.

TaqMan Probes

TaqMan probes depend on the 5'- nuclease activity of the DNA polymerase used for PCR to hydrolyze an oligonucleotide that is hybridized to the target amplicon. TaqMan probes are oligonucleotides that have a fluorescent reporter dye attached to the 5' end and a quencher moiety coupled to the 3' end. These probes are designed to hybridize to an internal region of a PCR product. In the unhybridized state, the proximity of the fluor and the quench molecules prevents the detection of fluorescent signal from the probe. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5'- nuclease activity of the polymerase cleaves the probe. This decouples the fluorescent and quenching dyes and FRET no longer occurs. Thus, fluorescence increases in each cycle, proportional to the amount of probe cleavage. Well-designed TaqMan probes require very little optimization. In addition, they can be used for multiplex assays by designing each probe with a spectrally unique fluor/quench pair. However, TaqMan probes can be expensive to synthesize, with a separate probe needed for each mRNA target being analyzed.

Molecular Beacons

Like TaqMan probes, Molecular Beacons also use FRET to detect and quantitate the synthesized PCR product via a fluor coupled to the 5' end and a quench attached to the 3' end of an oligonucleotide substrate. Unlike TaqMan probes, Molecular Beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement. Molecular Beacons form a stem-loop structure when free in solution. Thus, the close proximity of the fluor and quench molecules prevents the probe from

fluorescing. When a Molecular Beacon hybridizes to a target, the fluorescent dye and quenchers are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation.

Molecular Beacons, like TaqMan probes, can be used for multiplex assays by using spectrally separated fluor/quench moieties on each probe. As with TaqMan probes, Molecular Beacons can be expensive to synthesize, with a separate probe required for every target.

Scorpions

With Scorpion probes, sequence-specific priming and PCR product detection is achieved using a single oligonucleotide. The Scorpion probe maintains a stem-loop configuration in the unhybridized state. The fluorophore is attached to the 5' end and is quenched by a moiety coupled to the 3' end. The 3' portion of the stem also contains sequence that is complementary to the extension product of the primer. This sequence is linked to the 5' end of a specific primer via a non-amplifiable monomer. After extension of the Scorpion primer, the specific probe sequence is able to bind to its complement within the extended amplicon, thus, opening up the hairpin loop. This prevents the fluorescence from being quenched and a signal is observed.

SYBR Green

SYBR Green provides the simplest and most economical format for detecting and quantization of PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The advantages of SYBR Green are that it is inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green is that it binds to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles.

SYBR Green is the most economical choice for real-time PCR product detection. Since, the dye binds to double-stranded DNA, there is no need to design a probe for any particular target being analyzed. However, detection by SYBR Green requires extensive optimization. Since, the dye cannot distinguish between specific and non-specific product accumulated during PCR, follow up assays are needed to validate results.

Taqman vs. SYBR Green: The Data

An example of the precision and accuracy of real-time RT-PCR is shown in **Figure 5.2**. Panels A and B (left) are the amplification profiles of serial 10^7-fold dilutions of a cDNA synthesis reaction (10^7 fold range), amplified with Ambion's QuantumRNA™ 18S Universal primers and detected by either SYBR Green (panel A) or TaqMan (panel B) chemistries. Standard curves for both are shown on the right. The only practical difference between the performances of these two methods is the occasional presence of a false signal late in the SYBR Green amplification. This usually corresponds to a signal lower than that expected from femtograms of RNA, and is of little consequence to accuracy except if a target RNA is extremely rare.

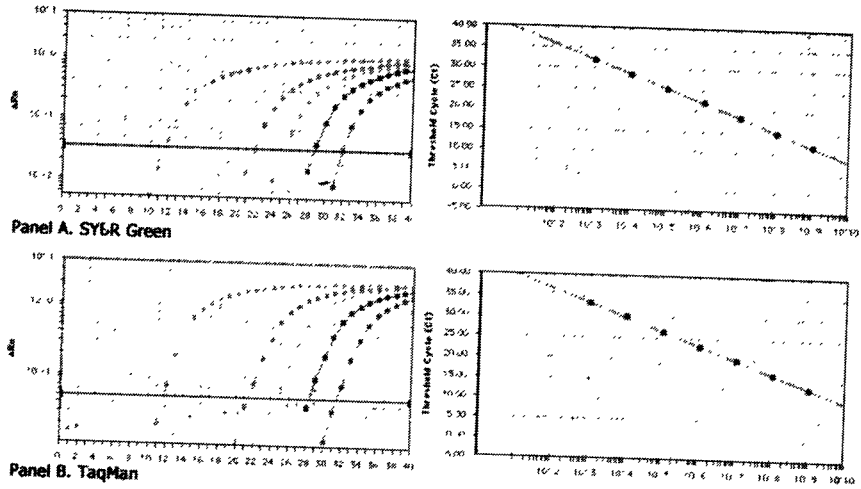


Figure 5.2. Precision and Accuracy of Real-Time RT-PCR. 18S rRNA amplification profiles and standard curves generated using A) SYBR Green, and B) TaqMan chemistries.

Primer and probes selection guidelines are shown in **Table 5.1.**

Real-time Reporters for Multiplex PCR

TaqMan probes, Molecular Beacons and Scorpions allow multiple DNA species to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be co-amplified and permits allele discrimination in single-tube, homogeneous assays. These hybridization probes afford a level of

Table 5.1 Primer and Probes Selection Guidelines for Quantitative Assays.

TaqMan Probe Guidelines	Sequence Detection Primer Guidelines (SYBR Green or TaqMan Assays)
Select the probe first and design the primers as close as possible to the probe without overlapping it (amplicons of 50–150 base pairs are strongly recommended).	Select the probe first and design the primers as close as possible to the probe without overlapping it (amplicons of 50–150 base pairs are strongly recommended).
Keep the G/C content in the 20–80% range.	Keep the G/C content in the 20–80% range.
Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.	Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
When using Primer Express software the Tm should be 68–70 °C.	When using Primer Express software the Tm should be 58–60 °C.
No G on the 5′ end.	The five nucleotides at the 3′ end should have no more than two G and/or C bases.
Select the strand that gives the probe more C than G bases.	

discrimination impossible to obtain with SYBR Green, since, they will only hybridize to true targets in a PCR and not to primer-dimers or other spurious products.

Primer designing

Real-Time PCR primers sets may be designed using standard primer design algorithms without any modification. As with all PCR amplifications, however, the specific reaction conditions for each set must be optimized, particularly primer concentration, annealing temperature and magnesium chloride concentration.

The method of real-time has been described below. The image at the left (**Figure 5.3**) shows a virtual northern blot with two lanes, one with RNA from control cells, and the other with RNA from the experimental sample (e.g. drug treated cells). For the sake of argument, let's say that there is 10 times the amount of signal in the

experimental sample compared to the control sample for the target gene. This could mean that expression of the gene has increased 10-fold in the experimental cells or it could mean that there is simply 10 times as much RNA in the experimental lane; in other words we have a loading artifact. To check for this, we usually do a so-called 'Loading Control' in which the blot is probed for expression of a gene which does not change. In this case, let's say that the loading control shows that there is twice as much RNA in the experimental lane. This means that the real change in the target gene is 10/2 = 5 fold.

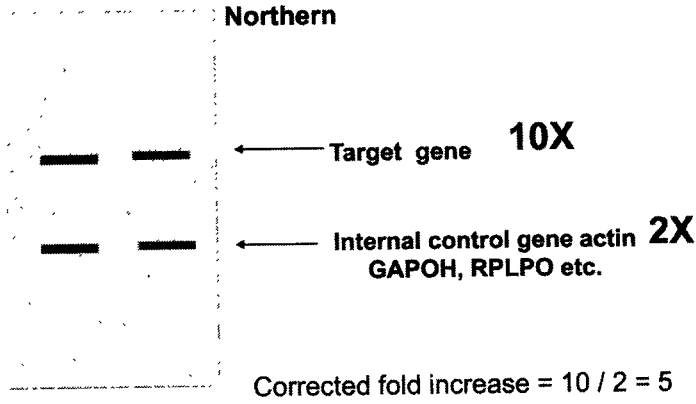


Figure 5.3 Northern blot

We can express this in a more general fashion:

ratio target gene expression (experimental/ control) =

fold change in target gene expression (expt/control)

fold change in reference gene expression (expt/control)

This brings us to the topic of standard or reference genes. A gene that is to be used as a loading control (or internal standard) should have various features.

- The standard gene should have the same copy number in all cells.
- It should be expressed in all the cells.
- A medium copy number is advantageous, since the correction should be more accurate.

However, the perfect standard does not exist; therefore, whatever you decide to use as a standard or standards should be validated for your tissue - If possible, you should be able to show that it does not change significantly in expression when your cells or tissues are

subjected to the experimental variables you plan to use.

Commonly used standards are:

- Glyceraldehyde-3-phosphate dehydrogenase mRNA
- Beta actin mRNA
- MHC I (major histocompatibility complex I) mRNA
- Cyclophilin mRNA
- mRNAs for certain ribosomal proteins e.g. RPLP0 (ribosomal protein, large, P0). This is also known as 36B4, P0, L10E, RPPO, PRLP0, 60S acidic ribosomal protein P0, ribosomal protein L10, Arbp or acidic ribosomal phosphoprotein P0.
- 28S or 18S rRNAs (ribosomal RNAs)

Now we need to think about the nature of the PCR reaction to understand real time **QUANTITATION**. The amount of DNA theoretically doubles with every cycle of PCR, as shown at the left. After each cycle, the amount of DNA is twice what it was before, so after two cycles, we have 2 X 2 times as much, after 3 cycles - 2 X 2 X 2 times as much or 8 (2^3) times as much, after 4 cycles 2 X 2 X 2 X 2 times as much or 16 times (2^4) as much. Thus, after N cycles we shall have 2^N times as much.

But, of course, the reaction cannot go on forever, and it eventually tails off and reaches a plateau phase, as shown by the figures in red.

If we plot these figures in the standard fashion (**Figure 5.4**). We cannot detect the amplification in the earlier cycles because the changes do not show up on this scale. Eventually, you see the last few cycles of the linear phase (pink) as they rise above the baseline and then the non-linear or plateau phase (red) - Actually this starts somewhat earlier than is shown here.

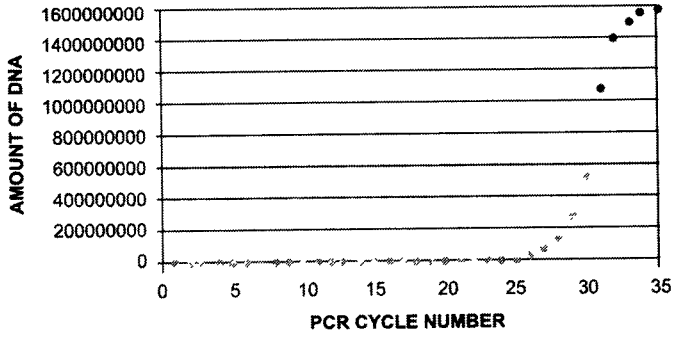


Figure 5.4 Linear and plateau phase in real time PCR

However, if we plot these values on a logarithmic scale, we can see the small differences at earlier cycles. In real time PCR, we use both types of graph to examine the data. Note that there is a straight line relationship between the amount of DNA and cycle number when you look on a logarithmic scale. This is because PCR amplification is an exponential reaction.

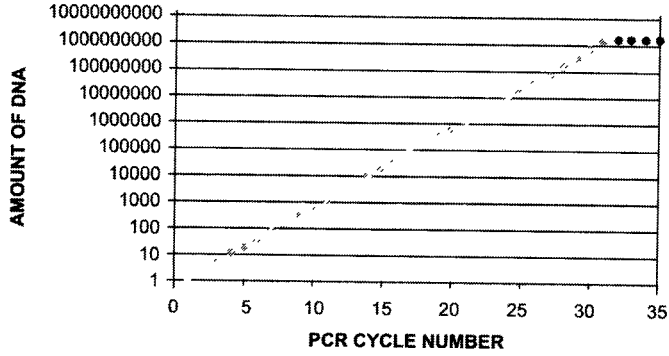


Figure 5.5 PCR amplification in an exponential manner

In real time PCR trace, we can use 96-well plate; cycle number is shown along the X-axis and arbitrary fluorescence units (actually these are fold increase over background fluorescence) are shown on the Y-axis. You can see that this mimics our theoretical graph (inset) - except that the transition to the plateau phase is more gradual. This experiment - and everything we are going to discuss - is with SYBR Green, which has very low fluorescence in the absence of double stranded DNA and very high fluorescence in the presence of double stranded DNA.

Here is the same real time PCR trace shown on a logarithmic scale - again it mimics our theoretical curve (inset).

As we saw with the theoretical curves, you should get a straight line relationship in the linear part of the PCR reaction. In this case, the reaction is linear in the series of 10 fold dilution as shown in **Figure 5.6**.

If we look at the same region on a regular scale, we see the linear part is, in fact, the very early part of the curve. Note that it is **NOT** the region which looks linear in this graphical view. This is a very important point in real-time PCR because we wish to examine the reaction while it is still in the linear phase.

Thus, Real Time PCR is a kinetic approach in which you look at the reaction in the early stages while it is still linear. There are many real

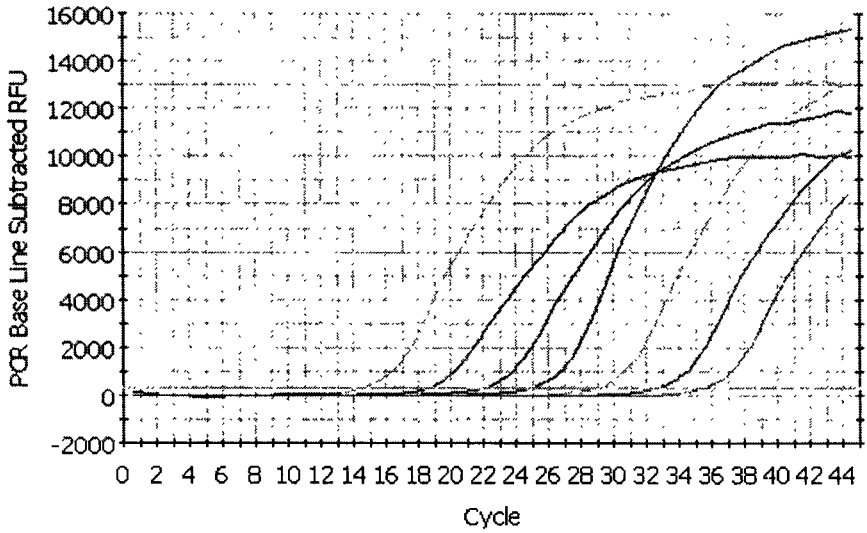


Figure 5.6 Series of 10- Fold dilutions

time machines available. The machines contain a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction. As DNA is synthesized, more SYBR Green will bind and the fluorescence will increase. The basic measure of good results is shown in **Figure 5.7**. We can plot the Ct values for the dilutions against concentration - the result is a linear graph. It should have an excellent correlation coefficient (certainly more than 0.990). The slope of this graph is a measure of efficiency, and can be readily used to calculate efficiency.

Comparative C_t Method

Another quantitation approach is termed the comparative C_t method. This involves comparing the C_t values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The C_t values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene.

The comparative C_t method is also known as the $2^{-[delta][delta]Ct}$ method, where,

$$[delta][delta]C_t = [delta]C_{t,sample} - [delta]C_{t,reference}$$

Here, $[delta]C_{T,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[delta]C_{t,\ reference}$ is the C_t value

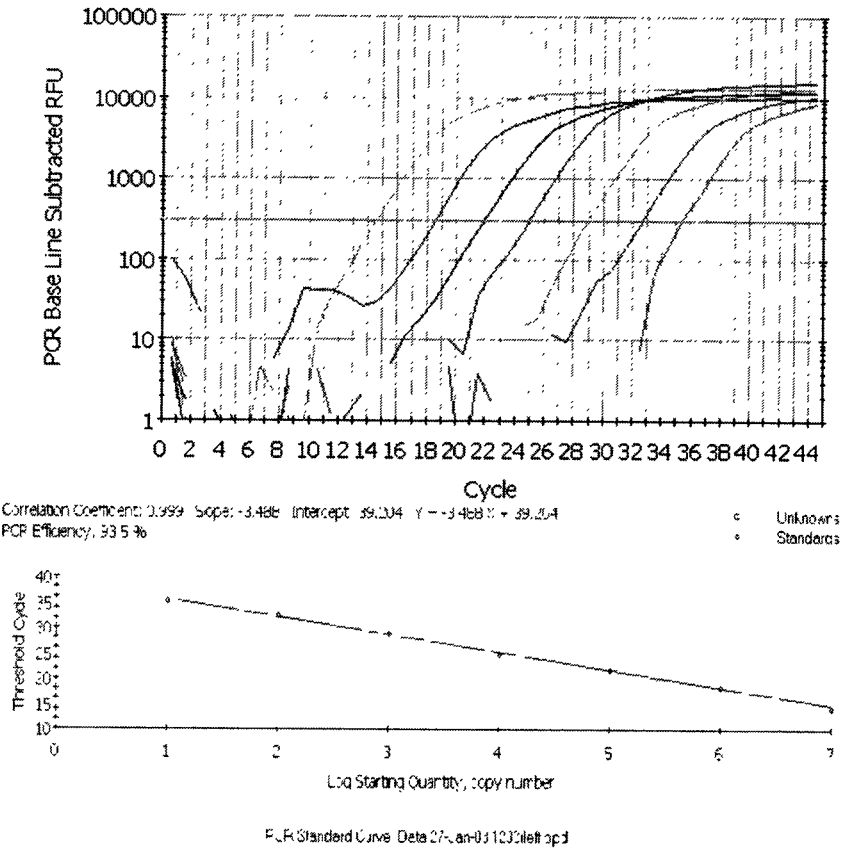


Figure 5.7 Basic measure for good result

for the calibrator also normalized to the endogenous housekeeping gene.

For the [delta][delta]C_t calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how [delta]C_t varies with template dilution. If the plot of cDNA dilution versus delta C_t is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred.

Instrumentation for Real-Time PCR

Real-time PCR requires an instrumentation platform that consists of a thermal cycler, a computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software. These machines, available from several manufacturers, differ in sample capacity (some are 96-well standard format, others process fewer samples or require specialized glass capillary tubes), method of excitation (some use lasers, others broad spectrum light sources with tunable filters), and overall sensitivity. There are also platform-specific differences in how the software processes data. Real-time PCR machines are not inexpensive but are well within purchasing reach of core facilities or labs that have the need for high throughput quantitative analysis.

Competitive RT-PCR

Competitive RT-PCR precisely quantitates a message by comparing RT-PCR product signal intensity to a concentration curve generated by a synthetic competitor RNA sequence. The competitor RNA transcript is designed for amplification by the same primers and with the same efficiency as the endogenous target. The competitor produces a different-sized product, so that, it can be distinguished from the endogenous target product by gel analysis. The competitor is carefully quantitated and titrated into replicate RNA samples. Pilot experiments are used to find the range of competitor concentration, where the experimental signal is most similar. Finally, the mass of product in the experimental samples is compared to the curve to determine the amount of a specific RNA present in the sample.

Some protocols use DNA competitors or random sequences for competitive RT-PCR. These competitors do not effectively control for variations in the RT reaction or for the amplification efficiency of the specific experimental sequence, as do RNA competitors.

Comparative RT-PCR

While exquisitely sensitive, both relative and competitive methods of qRT-PCR have drawbacks. Relative RT-PCR requires extensive optimization to ensure that the PCR is terminated when both the gene of interest and an internal control are in the exponential phase of amplification. Competitive RT-PCR requires that an exogenous "competitor" be synthesized for each target to be analyzed. However, comparative RT-PCR achieves the same level of sensitivity as these

standard methods of qRT-PCR, with significantly less optimization. Target mRNAs from 2 samples are assayed simultaneously, each serving as a competitor for the other, making it possible to compare the relative abundance of target between samples. Comparative RT-PCR is ideal for analyzing target genes discovered by screening methods such as array analysis and differential display.

Data and Analysis

Results will be available in two forms. The first is a hard copy of the Experimental Report as provided by the Sequence Detection System Analysis Software v. 1.7. The output is presented in a tabular form in which each row corresponds to information generated for each well position. For each well, information regarding the sample name, CT value, and quantity or copy number is provided. If a standard curve was run, its slope, fit (R), and y-intercept is provided in the header information. Alternatively, these data can be provided in an electronic format as a Microsoft Excel workbook file. Additional data analysis, such as DDCT calculations, is the responsibility of the user.

Chapter 6

Short Tandem Repeat (STR) Genotyping

Non-coding DNA generally contains DNA sequences for which no function has yet been established. Such sequences may occur in single copy or in multiple copies, thus, being called repetitive DNA. Some ~ 45% of the human genome is comprised of repetitive DNA.

Repetitive DNA can be subdivided into:

1. Tandemly arrayed- Microsatellites, Minisatellites and Telomeres.
2. Interspersed - Mobile elements and processed pseudogenes.

The repetitive DNA, if not existing in order than termed as Dispersed or Interspersed while, if they are arranged one after other then called tandem repeats. The central and top portions of chromosomes normally contain these repeat sequences.

Tandem repetitive DNA

Tandem repeats appear in genomic DNA with a wide variety. A tandem repeat in DNA is two or more similar copies of a DNA sequence. This type of repeats in DNA is also called satellite DNA, because, DNA fragments containing tandemly repeated sequences form 'satellite' bands when genomic DNA is fractionated by density gradient centrifugation. Tandem repeats may not have any functional role. Tandemly repeated DNA sequences are widespread throughout the human genome. Tandem repeats are usually classified as:

1. Satellites- spanning mega bases of DNA, associated with heterochromatin.
2. Minisatellites- repeat units in the range 8-60 bp, spanning hundreds of base pairs. These are also known as Variable Number of Tandem Repeats (VNTR).
3. Microsatellites - Repeat units in the range 1-5 bp, spanning a few tens of nucleotides). The microsatellites are also called "Short Tandem Repeats" or STRs.

Short Tandem Repeats (STR)

Short Tandem Repeats (STR), with a repetitive sequence ranging from 2-6 base pairs is amongst the most polymorphic markers reported till date (**Figure 6.1**). They exhibit substantial allelic variability due to high rate of germline mutations. These STR loci have a uniform and dense distribution throughout the genome. The short sequence length makes PCR detection and analysis feasible and also facilitates multiplexing by fragment analyzers. Moreover, they exhibit high level of relatively stable polymorphism. Total number of STR loci reported till date is approximately 10^6.

The STR loci carry lots of desirable features which makes them an ideal candidate for diverse applications including forensic applications, individual identification, true paternity/maternity detection, and fine scale genetic mapping and inter and intra group phylogenetic reconstruction. Some of these properties include:

1. Easy PCR detection
2. Genome wide distribution
3. Large number of alleles with relatively stable polymorphism

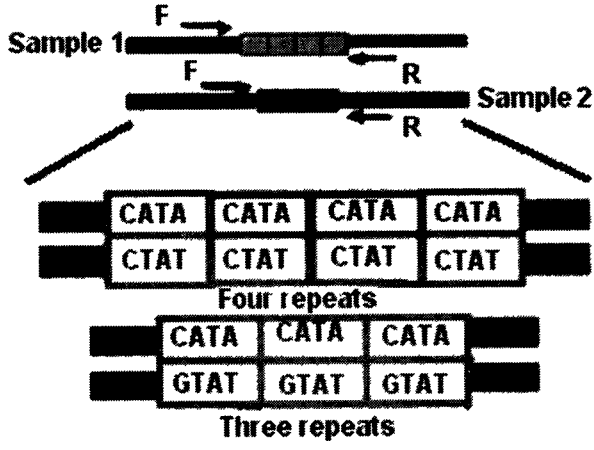


Figure 6.1: An STR locus with CATA repeats in two different samples homozygous for 4 and 3 repeats respectively.

Basis of STR genotyping

STR loci carry certain features, using which, it has become easier to genotype different alleles. These features include:

1. Core sequence of STR is 2-6 bp long (**Figure 6.1**).
2. Core repeating sequence always remains same in a particular STR.
3. This particular sequence gets repeated number of times.
4. Number of times sequence is repeating varies between chromosomes as well as between individuals.
5. Different repeats results into different alleles.
6. The repeat region is variable between samples.
7. The flanking regions where PCR primers bind are constant.
8. Homozygote sample is one with both alleles of the same length.
9. Heterozygote sample is the one in which alleles differ and can be resolved from one another.

Nomenclature for STR alleles and repeat structure

In October 1993, the DNA Commission of the International Society of Forensic Human genetics (ISFH) recommended the nomenclature for STR systems, which is commonly used today.

Alleles are generally named by the number of repeats, which they contain. When an allele does not confirm to the standard repeat motif of the system in question, the number should designate it complete repeat units and the number of base pairs of the partial repeat. These two values should be separated by a decimal point . For example, the STR locus **HUMTH01** contains a non-consensus allele, which is relatively common in Caucasians. This allele is 1 bp shorter than 10 repeat units, due to the loss of an adenine at the 7th repeat unit, and is thus, designated 9.3. The ISFH also recommends using allelic ladders containing the commonly occurring alleles and establishing the size and sequence of those alleles through sequencing. **Table 6.1** includes some of the well-known STR loci, their location and repeats motifs.

Table 6.1: List of various STR loci, their chromosomal location and repeat sequence

Marker	Repeat	Chromosomal Location	Gene	Size
D13S767	GATA	13	Unknown	155-179
D5S818	GATA	5	Unknown	133-165
FGA	TCTT	4q28	Alpha fibrinogen (at intron 3, nucleotide 2912)	250-298
HPRT	TCTA	Xq26	Hypoxanthine phosphoribosyltransferase (at 22 928 bp intron 3)	143-175
D3S1358	AGAT	3p	Unknown	113-145
vWA	ATTA	12q13.3-q13.2	Von Willebrand (at intron 40)	125- 165
Tho-1	TCAT	11p15.5	Tyrosine hydroxylase (at 1170 bp. Intron I)	242-262
ACPP	AAAT	3q21-qter	Prostatic acid phosphatase (at 2342 bp)	260-292
VWF	TCTA	12q13.3-q13.2	Von Willebrand (at intron 40)	126-162
TPO	AATG	2p25–p24	Thyroid peroxidase (at intron 10)	106-134
FES	ATTT	15q25-qter	Cfeslfps proto-oncogene (at 4713 bp. Intron V)	143-167
F13A1	AAAG	6p24.2-p23	Coagulation factor XIII (at 248 bp. Intron A)	180-244
DHFRP2	AAAC	6	Dihydrofolate reductase psi-2 pseudogene (at 103 bp.)	164-184

How STR genotyping is done

PCR amplification of STR loci is a highly sensitive technique which identifies up to 0.1-0.01% of individuals specific DNA. Individual loci are amplified using flanking primers and the amplicon is size fractionated to determine the allele length and eventually to interpret genotype of that individual as shown in **Figure 6.2.**

Protocol for STR genotyping

Autosomal STR- TPO

TPO is an autosomal STR with tetrameric unit of AATG. The STR is

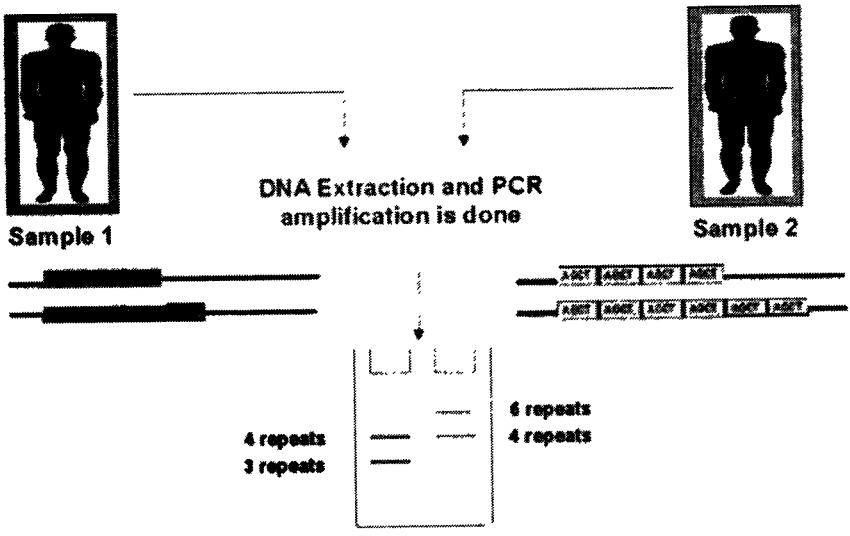


Figure 6.2: Methodology for STR genotyping

located in the intron ten of the thyroid peroxidase gene at chromosome 2. Total 12 alleles have been reported in the forensic literature for this locus, thus, a range of 106-134 bp is observed for different alleles when amplified by PCR and size fractionated on PAGE. The summary of the methodology is summarised in **Figure 6.2**.

1. PCR amplification TPO STR

(a) TPO STR is amplified by PCR, using following primers:

Forward: 5' CACTAGCACCCAGAACCGTC 3'

Reverse: 5' CCTTGTCAGCGTTTATTTGCC 3'

(b) Reaction mix is prepared as shown in **Table 6.2**

Table 6.2: PCR reaction mix for amplification of TPO

Reagents	For 1 reaction (15 µl)	For 5 reactions (15x5=75 µl)
10 X buffer	1.5 µl	7.5 µl
dNTP mix (10µM/ul)	1.6 µl	0.5 µl each or 2.0 µl mix
F-primer (10pm/ul)	0.3 µl	1.5 µl
R-Primer (10pm/ul)	0.3 µl	1.5 µl
Taq DNA polymerase (3U/ul)	0.8 µl	4.0 µl
DdH2O	11 µl	55 µl
DNA (100ng/ul)		1x5 µl

(c) PCR is carried out in a Thermo cycler according to the conditions shown in **Table 6.3.**

Table 6.3: PCR conditions for amplification of Factor HUM TPO gene

Step	Temperature	Time	Cycles
Initial	94°C	5 min	
Denaturation	94°C	1 min	29 Cycles
Annealing	59°C	30 sec	
Extension	72°C	1.30 min	
Final extension	72°C	5 min	1 Cycle
Hold	15°C	Forever	

(d) PCR product are run on 2% Agarose to check the amplification.

2. Analysis of the amplified product

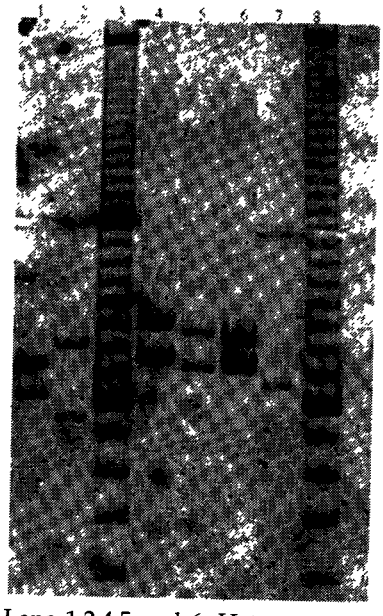
(a) All the samples are size fractionated on 9% PAGE in midi- or large vertical assembly.

(b) 10 bp Ladder and if allelic sizing ladder for Hum TPO is available, is also run for sizing of the bands. Allele size ladder is generally prepared in the laboratory by the investigator.

(c) Band size is analyzed for the corresponding number of repeats as mentioned in the STR database http//: www. STRbase.html. This database shows different allele repeats for each STR marker corresponding to the base pair size for primer pairs.

(d) Band patterns are observed for presence or absence of mutation as shown below:

(i) Heterozygous: showing two-bands, hence, represent different repeat units on different chromosomes **(Figure 6.3).**



Lane 1,2,4,5 and 6: Heterozygous samples
Lane 3 and 8 and 10 bp Ladder
Lane 7: Homozygous sample

Figure 6.3: Genotyping of TPO with the help of 10 bp Ladder fractionated on 9% PAGE

(ii) Homozygous: Having only one band, which means same repeat unit on both the chromosomes **(Figure 6.3).**

STR genotyping can be performed by using automated sequencer. We have described this under the section of gene scanning. Recently, it has been reported that STR genotyping can also be done by MALDI-TOF. Mass spectrometry offers unprecedented analysis times—on the order of seconds per sample—with excellent accuracy in measuring DNA fragment size. This is one of the short coming of the manual STR genotyping. Substantial improvements have been made in recent years with the development of an effective ionization procedure, known as matrix-assisted laser de sorption ionization (MALDI), and the discovery of new matrices, such as 3-hydroxypicolinic acid. In MALDI, DNA samples are mixed with an organic matrix and allowed to co-crystallize in a spatial array on a sample plate with each assay at a separate location. After the sample plate is placed in the mass spectrometer, which is under vacuum, a pulse of laser energy liberates a small portion of the DNA sample. While the generated ions travel to the detector in a matter of microseconds, multiple spectra are averaged for signal processing, which extends the measurement time to a few seconds. The DNA size is calculated by the time-of-flight to the detector in comparison to mass standards. Due to the increased accuracy with mass spectrometry, STR alleles may be reliably typed without comparison to allelic ladders as is done in the manual polyacrylamide gel electrophoresis. Hence, the STR results from time-of-flight mass spectrometry analysis may be obtained more accurately than gel electrophoresis and orders of magnitude is much faster.

In the high-throughput approach, one can amplify 384 samples in parallel using a robotic workstation containing a 96-tip pipetting head. The samples are then transferred to the mass spectrometer where each sample can be analyzed in a few seconds. In this fashion, several thousand samples can be processed daily with a single robotic workstation and automated mass spectrometer. However, before accepting the robustness of the technique, it is important to keep in mind the purpose of the STR genotyping. One should adopt MALDI if one is going to generate a large data set for different purposes.

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Chapter 7

Alu Insertion Genotyping

Alu elements are the most successful and largest class of repetitive mobile sequences that are dispersed ubiquitously throughout the primate genome. They were named after the Alu-I restriction enzyme site within the consensus Alu sequence.

1. Consensus *Alu* sequences are ~280 bp in length.
2. Full length *Alu* elements are ~300 bp long and commonly found in introns, 3'UTR and intergenic genomic regions.
3. They are the most abundant SINES comprising ~10% of the genome reaching a high copy number of 1.1 million during the last 65 Myr.
4. *Alu* elements have amplified by duplicating via an RNA intermediate that is reverse transcribed by target primed reverse transcription and integrated into the genome, hence, *Alus* are postulated to be the retro transposons.
5. Alu elements are unable to retropose autonomously, so they are thought to appropriate the necessary mobilization machinery from the long interspersed elements retro transposons family, which encodes a protein possessing endonuclease and reverse transcriptase activity.

Structure of Alu elements

An Alu repeat contains approximately 300 bp and two homologous halves connected with an A-rich linker. The right (R) half contains an additional segment of 31 bp (**Figure 7. 1**). Both halves have a common ancestral nucleotide sequence, homologous to 7SL nuclear RNA. The monomeric variant of Alu repeat was discovered in rodents and designated as B1. Despite the differences in their organization, both B1 and Alu repeats retained common features. Both repeats contain an internal two-module promoter (A and B boxes) that is recognized

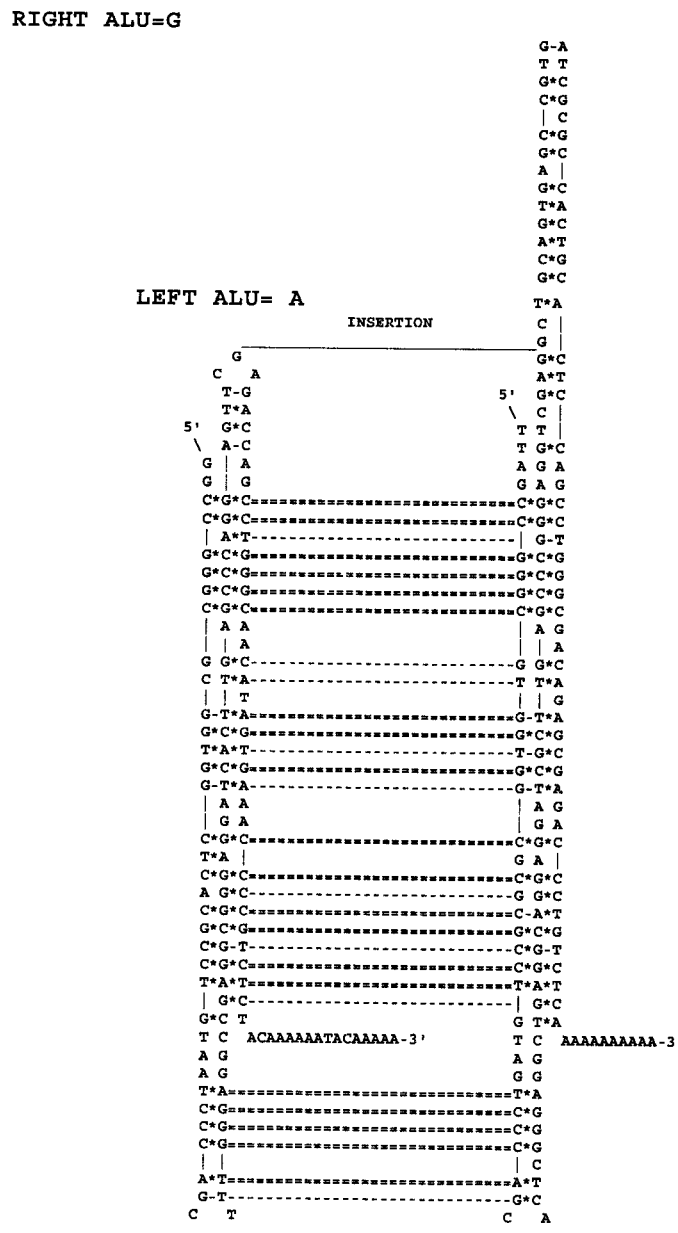

Figure 7.1 Triplex structures of the left (À:Ò:À) and right (G:C:G) monomers of Alu-J repeat (according to the classification of Jurka [2]) with invariant paired structures (=) and compensatory mutations (-). The right monomer contains an insertion of 31 nt. (*), complementary pairs; (-) GT pairs.

by RNA polymerase III and a poly (A) sequence. It is supposed that the common structural features of B1 and Alu repeats determine their capability of transposition within the genome. Dimeric structure, typical of primates, appeared about 65 million years ago.

Alu repeats are divided into several families, differing in their evolutionary age, basing on DNA divergence and certain "diagnostic" substitutions. The families of Alu repeats differ in their CpG dinucleotide content, the ability to bind RNA polymerase III, and the length of poly (A) regions. Despite drastic differences in the primary structure, all the members of Alu repeat superfamily preserve common tRNA-like secondary structure (**Figure 7. 2**). This peculiarity of Alu repeats is of special interest, since the hairpin-forming DNA/RNA structures are functionally important for the 3D structure of chromatin: they are involved in binding HMG proteins, recombination and modification enzymes, in splicing, and regulation of various genetic processes (for example, termination and delays in transcription, selective blocking of mRNA transcription that depends on the secondary structure of the leader sequence, etc.). Hence, the increasing volume of data concerning the role of Alu repeats in regulation of cell differentiation and tissue-specific gene expression are not unexpected.

Alu family classification

The human specific *Alu* are further classified as sub families according to diagnostic nucleotide substitution along their sequence. Due to this characteristic, they can serve as unique evolutionary milestones. Phylogenetic studies of *Alu* elements suggest that only a small number of *Alu* elements deemed "master" or source genes are retropositionally competent, overtime the eventual accumulation of new mutations within the master or source genes created the hierarchy of Alu subfamilies. Diagnostic mutation sites can be used to classify each individual element according to subfamily. Alu classification is based upon age which is:

1. The oldest (J) to
2. Intermediate (S) and
3. Youngest (Y).

Some young Alu subfamilies have amplified so recently that they are virtually absent from the genome of nonhuman primates, as the result of the recent integration of young Alu subfamily members with in the human genome, individual human can be polymorphic for the

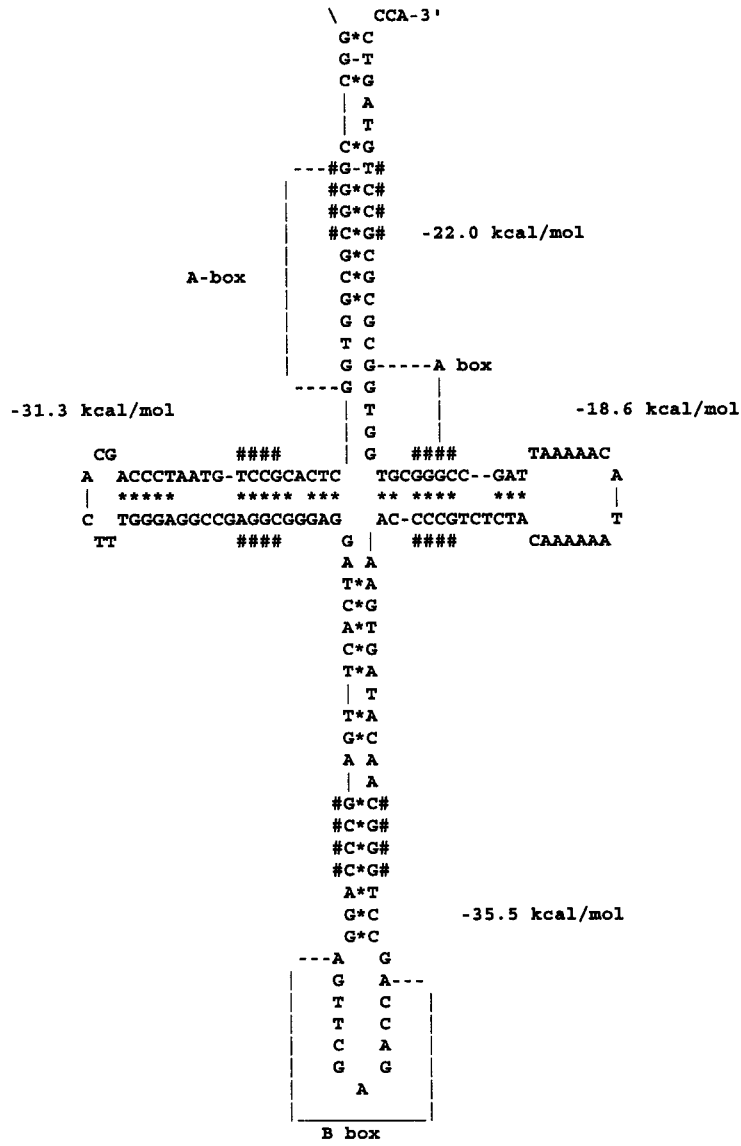

Figure 7. 2. tRNA like structure of the left (L) and a part of the right monomer: core sequences are indicated with ###; two A boxes and one B box, which are the recognition sites of RNA polymerase III, are shown. Energies of interaction for the paired regions are −31.3, −35.5, −18.6, and −22.0 kcal/mol, respectively. B box corresponds to anticodon loop of tRNA; poly(A) linker between L and R monomers, to y loop.

presence of Alu element at particular loci. Almost all of the recently integrated human Alu elements belong to one of the several small and closely related young Alu subfamilies known as Y, Yc1, Yc2, Ya5, Ya5a2, Ya8, Yb8, Yb9 and Yd.

Alu insertion polymorphisms

The largely human specific *Alu* subfamilies represent only ~ 0.5% of all of the *Alu* repeats in the human genome in the past 4 - 6 million years after the divergence of humans and African Apes, but most of them integrated before the African radiation of humans, so these Alu repeats are monomorphic for their insertion sites among diverse human genome. However, approx 25% of the young *Alu* repeats have inserted so recently that they are dimorphic for the presence or absence of the insertion, which makes them useful source of genomic polymorphisms.

Alu elements that are present in the genomes of some individuals and absent from others are referred as **Alu insertion polymorphisms**.

These are the autosomal markers that thus reflects both the maternal and paternal history of a population, moreover, they are the stable markers that unique evolutionary events namely the insertion of an Alu element into a new chromosomal location. These polymorphisms have several characteristics that make them unique markers for the study of human population genetics.

Individuals that share Alu insertion polymorphism have inherited the *Alu* elements from a common ancestor, which makes the Alu insertion alleles identical by descent. Also, because the likelihood of two Alu elements independently inserting into the same location of the genome is extremely small, and as there is no known biological mechanism for the specific excision of Alu elements from the genome, so Alu insertions can be considered homoplasy free characteristic. Furthermore, the ancestral states of this polymorphism are known to be the absence of Alu elements at a particular genomic location. Thus, this facilitates to construct the population phylogeny.

Protocol for detection of an Alu insertion polymorphism by polymerase chain reaction

Step 1: Blood sample (5 ml by venipuncture in EDTA) to be drawn from individuals with prior consent.

Step 2: DNA is isolated by phenol-chloroform method as mentioned in Chapter 1.1.

Step 3: PCR amplification of the human DNA for Alu insertion polymorphism.

Amplification protocol for the PLAT Alu insertion polymorphism is shown below:

Alu Insertion Polymorphism

Here, the protocol for 10 ALU markers is shown.

A set of 10 human specific Alu elements which were located at different autosomal loci. All the Alu markers included here are either of insertion or deletion type. The studied Alu markers are dispersed throughout the genome and are present in different chromosomes i.e. chromosome-1, -3, -8, -11, -12, -16 and -17. The average size of Alu element is ~ 300 bp. Analysis of insertion or deletion of Alu element can be carried out by using polymerase chain reaction (PCR) with Alu specific primers and separating insertion or deletion allele on agarose gel electrophoresis. Depending upon insertion or deletion of Alu element, the amplified product could be ~300 bp more or less, respectively.

(a) Selection criteria of the Alu markers

Alu insertion polymorphisms and other SINE elements are robust markers for evolutionary and phylogenetic studies, because, they have a unique mutational mechanism, an absence of back mutation, and a lack of recurrent forward mutation. A specific Alu insertion and nearby flanking sequences are identical by descent in all individuals in whom they occur. Thus, sets of related chromosome regions marked by an Alu insertion event can be distinguished from a pool of ancestral chromosomes that lack the element. These features give each locus genetic polarity that allows the independent assignment of an ancestral state and a root for phylogenetic analyses. One should take care to include only those markers which show high hetrozygosity.

(b) Alu indel polymorphism genotyping

The Polymerase Chain Reaction (PCR) is based on locus specific amplification. Size fractionation of amplified product is done on agarose gel electrophoresis. Size of insertion and deletion allele depended upon the insertion or deletion of Alu element at the locus. Both the alleles vary by the size of Alu element studied at the locus.

PCR amplification

Presence of various Alu elements at different loci was detected by PCR amplification using flanking primers against unique sequences.

Both forward and reverse primers for each marker are shown in **Table-7.1.**

Table 7.1: Alu repeat Primer sequence

S.No.	Alu Element	Primer	Primer sequence (5′ to 3′)
1	TPA25	F	GTAAGAGTTCCGTAACAGGACAGCT
		R	CCCCACCCTAGGAGAACTTCTCTTT
2	APO A1	F	AAGTGCTGTAGGCCATTTAGATTAG
		R	AGTCTTCGATGACAGCGTATACAGA
3	PV92	F	AACTGGGAAAATTTGAAGAGAAAGT
		R	TGAGTTCTCAACTCCTGTGTGTTAG
4	ACE	F	CTGGAGACCACTCCCATCCTTTCT
		R	GATGTGGCCATCACATTCGTCAGAT
5	FXIII B	F	TCAACTCCATGAGATTTTCAGAAGT
		R	CTGGAAAAAATGTATTCAGGTGAGT
6	DI	F	TGCTGATGCCCAGGGTTAGTAAA
		R	TTTCTGCTATGCTCTTCCCTCTC
7	CD4	F	AGGCCTTGTAGGGTTGGTCTGATA3
		R	TGCAGCTGCTGAGTGAAAGAACTG
8	HS3.23	F	GGTGAAGTTTCCAACGCTGT
		R	CCCTCCTCTCCCTTTAGCAG
9	HS4.32	F	GTTTATTGGGCTAACCTGGG
		R	TGACCTGCTAACTTGTACTTTAACC
10	B65	F	ATATCCTAAAAGGGACACCA
		R	AAAATTTATGGCATGCGTAT

A PCR reaction mixture of 15µl was prepared for each of the loci. The reaction mixture comprise of 10X PCR buffer (Banglore genei), primers (10 pm/µl), dNTP mix (10mM), Taq polymerase (3U/µl) in a fixed proportion for each Alu marker as mentioned in the **Table 7.2.** The PCR cycling conditions varied for different loci and are mentioned in **Table 7.3.**

Table 7.2: PCR reaction mix composition for Alu markers

PCR Components	PCR reaction composition
10X PCR Buffer (Banglore genei)	1.5 µl
Primer 1 (10pm/µl)	0.3 µl
Primer 2 (10pm/µl)	0.3 µl
dNTP mix (10mM)	1.2 µl
Taq polymerase (3U/µl)	0.3 µl
HPLC grade water	9.9 µl
Genomic DNA (~50-100ngm)	1.5 µl
Total	**15 µl**

Table 7.3: PCR conditions for various Alu polymorphic markers

PCR conditions	Alu markers	Temperature (°C)	Time
Initial denaturation		94	2 min
Denaturation		94	40 sec
Annealing	ACE	59	30 sec
	APO	50	30 sec
	B65	52	30 sec
	HS4.32	55.5	30 sec
	PV92	54	30 sec
	TPA25	58	30 sec
	HS3.23	55.5	30 sec
	CD4	55	30 sec
	FXIIIB	57	30 sec
	D1	56	30 sec
Extension		72	60 sec
Additional cycles		34 cycles	
Final extension		72	10 min

Size fractionation of the amplicons and genotyping of the alleles

PCR products vary in length depending on the insertion or the deletion of Alu elemen. Amplicons can be resolved on 2% agarose gels in 0.5x Tris-borate EDTA. Finally, insertion and deletion alleles are visualized by ethidium bromide staining. For the exact assigning of alleles molecular markers are included in each gel. Depending on the size of bands, insertion and deletion alleles at various loci were assigned. Various Alu indel polymorphisms are shown in **Figure 7.3, 7.4 and 7.5.**

Table 7.4: Size of insertion and deletion alleles of various Alu markers

S. No.	Alu element	Size of insertion allele (bp)	Size of deletion allele (bp)
1	TPA25	424	113
2	APO B	409	96
3	PV92	437	122
4	ACE	480	191
5	FXIII B	463	167
6	DI	676	333
7	CD4	1501	1245
8	HS3.23	527	214
9	HS4.32	601	289
10	B65	420	81

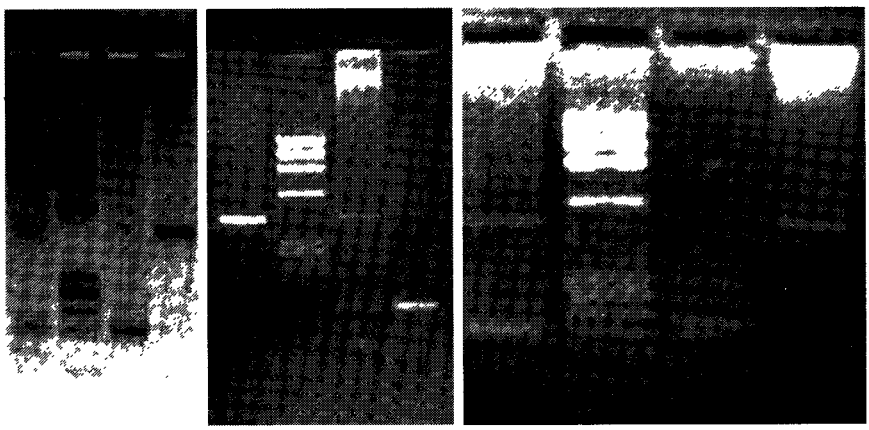

Figure 7.3: Gel picture shows the insertion and deletion alleles of ACE, Apo and HS3.23 Alu markers from left to right, respectively. Ladder used here is ÆX174 Hae III digest. Base pair sizes of various alleles are given in Table-6.1.

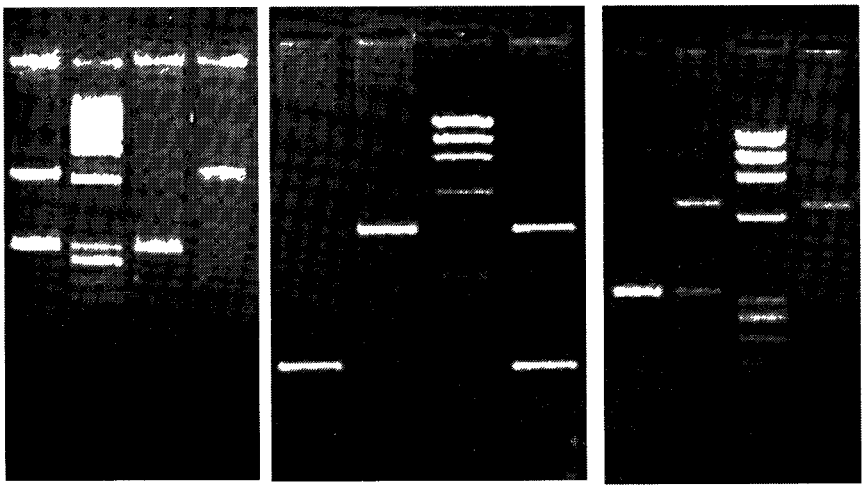

Figure 7.4: Gel picture shows the insertion and deletion alleles of HS4.32, TPA and D1 Alu markers from left to right, respectively. Ladder used here is ÆX174 Hae III digest. Base pair sizes of various alleles are given in Table-1.

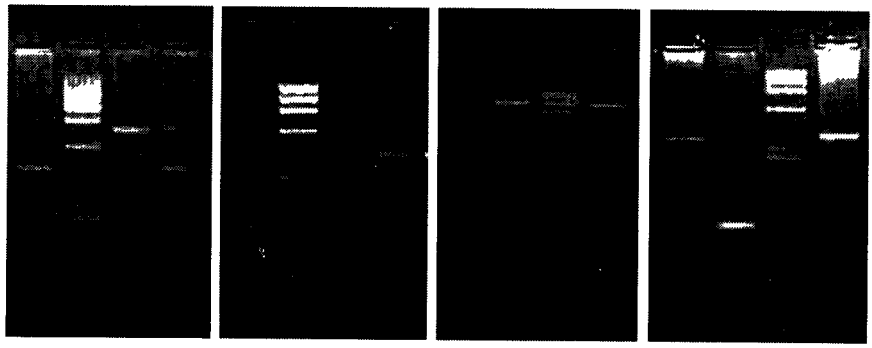

Figure 7.5: Gel picture shows the insertion and deletion alleles of FXIIIB, B65, CD4 and PV92 Alu markers from left to right, respectively. Ladder used here is ÆX174 Hae III digest. Base pair sizes of various alleles are given in Table-1.

Chapter 8

Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular *Restriction Endonuclease,* (RE) the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another. This technique is mainly based on the special class of enzyme i.e. Restriction Endonucleases (RE).

Restriction endonucleases

Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Restriction enzymes are isolated from a wide variety of bacterial genera and are thought to be the part of the cell's defense against invading bacterial viruses. These enzymes are named by using the first letter of the genus, the first two letters of the species, and the order of discovery.

1. More than 500 restriction endonucleases are known.
2. Hundreds of these are commercially available.
3. Sites range from 4 to 8 or more bp.
4. Cut frequently or very rarely (~250 bp to ~ 66 kb).
5. Supplied with buffer and instructions are provided by the manufacturing companies.

Generally, shorter the recognition sequence, greater the number of fragments generated. If molecules differ in nucleotide sequence, fragments of different sizes may be generated. The fragments can be separated by gel electrophoresis. RFLP analysis takes advantage of the cutting enzymes. In southern blotting, the genomic DNA is digested with different enzymes. The pathogenic and non-pathogenic

conditions are examined by analyzing the fragment of the DNA. In **Table 8.1** some of the important restriction endonucleases and the organism from which these are isolated and restriction recognizing sequence are shown.

How Restriction Endonucleases works

RE hydrolyse both strands of a double stranded DNA within a (normally symmetrical) recognition sequence to give a 5′-phosphate and a 3′-hydroxyl at each break point as shown in **Figure 8.1.**

Table 8.1: Different REs and their recognition sequences

Restriction Endonuclease	Bacteria	Recognition Sequence
RE with 4 bp recognition sequence		
HaeIII	Haemophilus *egyptius*	GG ¹ CC CC ₁ GG
HhaI	Haemophilus *homolyticus*	GCG ¹ C C ₁ GCG
HpaII	Haemophilus *paraenfluenzae*	C ¹ CGG GGCC ₁
Sau3A	Staphylococcus *aureus* 3A	G ¹ ATC CTAG ₁
RE with 6 bp recognition sequence		
BamHI	Bacillus *amyloliquefaciens* H	G ¹ GATCC CCTAG ₁ G
BglII	Bacillus *globigi*	A ¹ GATCT TCTAG ₁ A
EcoRI	Escherichea *coli* RY13	G ¹ AATTC CTTAA ₁ G
HaeII	Haemophilus a*egyptius*	RGCGC ¹ Y Y ₁ CGCGR
HindIII	Haemophilus *influenzae* R_d	A ¹ AGCTT TTCGA ₁ A
PstI	Providencia *stuartii*	CTGCA ¹ G G ₁ ACGTC
SalI	Streptomyces *albus*	G ¹ TCGAC CAGCT ₁ G
SmaI	Serratia *marcescens*	CCC ¹ GGG GGG ₁ CCC
RE with 8 bp recognition sequence		
NotI	Nocardia *otitidis*-caviarum	GC ¹ GGCCGC CGCCGG ₁ CG
RE with non symmetrical recognition sequence		
BstXI	Bacillus *stearothermophilus*	CCANNNNN ¹ NTGG GGTN ₁ NNNNNACC

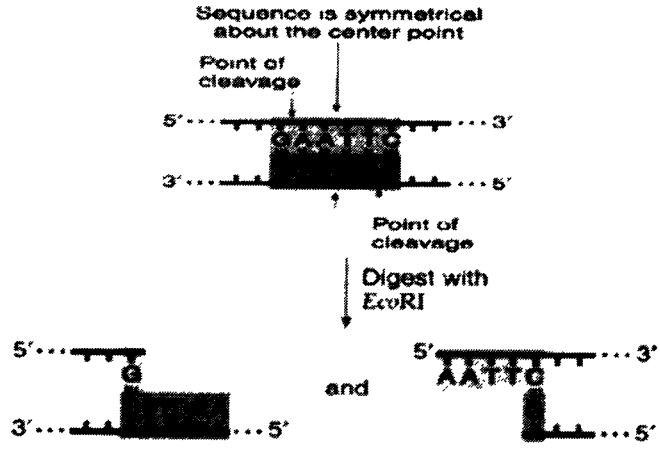

Figure 8.1: DNA digested by EcoRI restriction enzyme at its recognition site

When RE cuts the two strands of DNA at nucleotide on the same position, then it results into two fragments with BLUNT ends **(Figure 8.2A)**, but if RE cut the two strands at nucleotide not on the same position then it results into two fragments with STICKY ends **(Figure 8.2B)**. The later one is highly useful in DNA recombinant technology and is used to insert the DNA of interest into the vector.

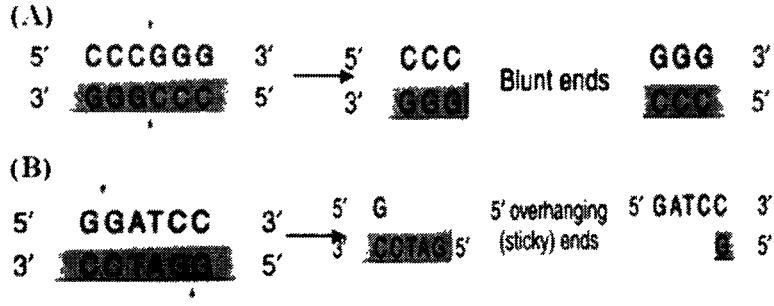

Figure 8.2: (A) DNA digested with SmaI RE resulting into BLUNT end fragments. (B) DNA digested with BamHI RE resulting into STICKY end fragments

Isolation of sufficient DNA for RFLP analysis is time-consuming and labour intensive. However, PCR can be used to amplify very small amount of DNA, usually in 2-3 hours, to the levels required for RFLP analysis. Therefore, more samples can be analyzed in a shorter time.

Restriction Fragment Length Polymorphism of PCR-amplified fragments

PCR has greatly improved the sensitivity of detecting mutations in genomic DNA. The general scheme of PCR-based restriction fragment length polymorphism (PCR-RFLP) includes:

1. Amplification of DNA containing the mutated sequence using flanking primers.
2. This is followed by enzyme restriction of the PCR product.
3. Mutations are detected by simple gel electrophoresis of the PCR product cleaved with the restriction endonuclease.
4. Control sample is always run to have digestion pattern of wild type allele.
5. Undigested products are also run as negative control.
6. There are two ways by which RFLP based mutation detection technique operates:
   (a) If a mutation has destroyed the recognition sequence of an RE, then the wild type samples will show digestion pattern and mutated samples will show intact band as in the case of k-*ras* codon 12 mutations in gastric cancer or codon 6 mutation in b-Globin gene in Sickle cell anemia **(Figure 8.3).**
   (b) If a mutation has been created the recognition sequence of an RE, then the wild type samples will show intact band and mutated samples will show digestion pattern as in the case of Factor V-Leiden Mutation.

This way of transforming any alternation of DNA sequence into an allele-specific enzyme recognition site obviates the use of radio isotopic hybridization, and has been used successfully to detect multiple mutations in the cystic fibrosis gene as well as *ras* oncogenes in gastrointestinal cancers. In addition, primer-mediated

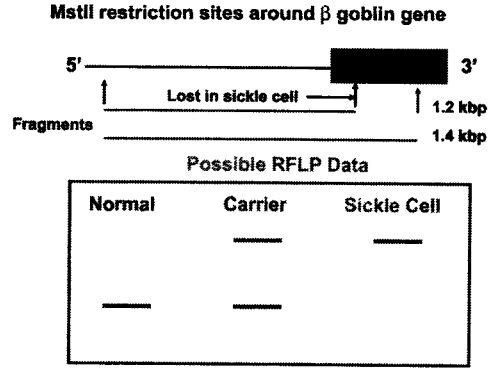

Figure 8.3: RFLP analysis of β- Globin gene mutation in Sickle cell anemia using MstII restriction enzyme.

restriction polymorphism has enhanced by 20% the sensitivity of detection as compared to allele- specific oligonucleotide hybridization technique.

Protocol for PCR-RFLP

The protocol is explained by taking the example of exon 4 of HLA-G.

HLA-G polymorphism has been associated with recurrent spontaneous abortion. Exon 4 of HLA-G has two polymorphic sites at codon 188 and 258, where cytosine is replaced by thymine. This mutation can be detected by PCR-RFLP as mutation at any codon results into the creation of the digestion site of XceI restriction enzyme. This can be done in three steps:

1. PCR amplification

Exon 4 of HLA-G is amplified by PCR, using following primers:

Forward: 5' CCATGAGAGATGCAAAGTGCT-3'

Reverse: 5' TGCTTTCCCTAACAGACATGAT-3'

(a) Reaction mix is prepared as shown in **Table 8.2**

Table 8.2: PCR reaction mix for amplification of HLA-G exon-4

Reagents	For 1 reaction (25ul)	For 5 reactions (25x5=125ul)
10 X buffer	2.5ul	12.5ul
dNTP mix (10uM/ul)	2.0ul	10ul
F-primer (10pm/ul)	1.2ul	6ul
R-Primer (10pm/ul)	1.2ul	6ul
Taq DNA polymerase (3U/ul)	0.8ul	4ul
DdH2O	16.3ul	81.5ul
DNA (100ng/ul)	1ul	1x5ul

(b) PCR is carried out in according to the conditions shown in **Table 8.3.**

Table 8.3: PCR conditions for amplification

Step	Temperature	Time	Cycles
Initial denaturation	94°C	5 min	1 cycle
Denaturation	94°C	30 sec	30 Cycles
Annealing	55°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	1 Cycle
Hold	15°C	Forever	

(c) PCR product are run on 2% Agarose to check the amplification.

2. Restriction digestion of the PCR product

(a) Amplified samples are then digested as per the reaction mix shown in **Table 8.4.**

Table 8.4: Restriction digestion mix for mutation detection

Reagents	For 1 reaction (15ul)	For 5 reactions (15x5=75ul)
10 X buffer	1.5ul	7.5ul
XceI RE (10U/ul)	0.2ul	1ul
DdH2O	9.8ul	49ul
PCR product	3.5ul	5x3.5ul

(b) All the samples are incubated at $37^{O}C$ for 16-20 hrs.

3. Analysis of the digested product

(a) All the samples are size fractionated on 9% PAGE.

(b) 50 bp Ladder is used for sizing of the bands.

(c) Band patterns are observed for the presence or absence of mutation in following manner:

(i) Normal sample: Intact undigested band of 364 bp **(Figure 8.4).**

(ii) Heterozygous (mutation): One band of 364 bp for undigested chromosome.

Bands of 300 bp and 64 bp for mutation at codon 188.

Or Bands of 273 bp and 91 bp for mutation at codon 258 **(Figure 8.4).**

(iii) Homozygous (mutation): Bands of 300 bp and 64 bp (mutation at codon 188) or band of 273 bp and 91 bp (mutation at codon 258).

(iv) homozygous (mild type: no mutation) : Band of 364 bp size only is seen.

How RFLP can be used in different situations can be used is shown below:

Case 1: Screening for the sickle-cell gene

Sickle cell disease is a genetic disorder in which both genes in the patient encode the amino acid **valine** (Val) in the sixth position of the beta chain ($beta^{S}$) of the hemoglobin molecule. "Normal" beta chains

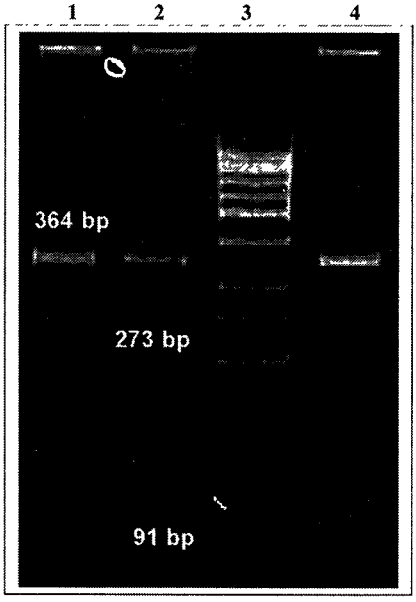

Figure 8.4: PCR-RFLP analysis to detect mutation in exon-4

(beta^A) have **glutamic acid** at this position.

The only difference between the two genes is the substitution of a T for an A in the middle position of codon 6.

	Thr	Pro	Glu	Glu	beta^A chain
...A C T	C C T	G A G	G A G...	beta^A gene	
Codon #	4	5	6	7	
...A C T	C C T	G T G	G A G...	beta^S gene	
	Thr	Pro	Val	Glu	beta^S chain

Figure 8.5 Screening of sickle cell gene by RFLP

This converts a GAG codon (for Glu) to a GTG codon for Val and abolishes a sequence (CTGAGG, which spans codons 5, 6, and 7) recognized and cut by one of the restriction enzymes.

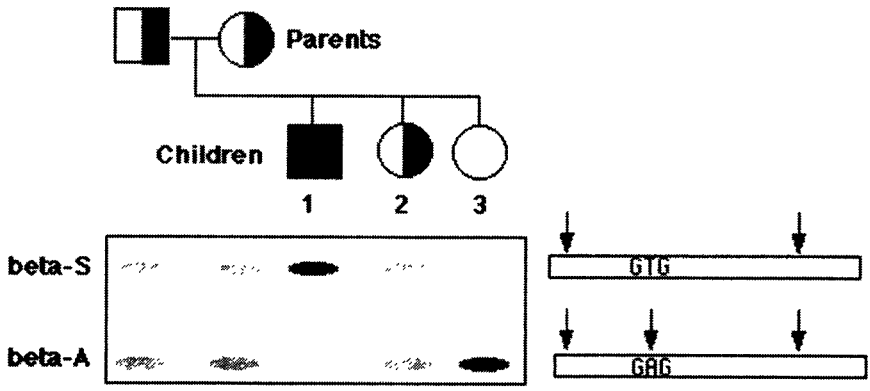

Figure 8.6 Family analysis of beta-S and beta-A by RFLP

When the **normal** gene (beta^A) is digested with the enzyme and the fragments separated by electrophoresis, the probe binds to a **short** fragment (between the red arrows).

However, the enzyme cannot cut the **sickle-cell gene** at this site, so the probe attaches to a much larger fragment (between the blue arrows).

The figure shows the pedigree of a family whose only son has sickle-cell disease. Both his father and mother were heterozygous (semifilled box and circle respectively) as they had to be to produce an afflicted child (solid box). The electrophoresis patterns for each member of the family are placed directly beneath them. Note that the two homozygous children (1 and 3) have only a single band, but these are more intense because there is twice as much DNA in them.

In this example, a change of a single nucleotide produced the RFLP. This is a very common cause of RFLPs and now such polymorphisms are often referred to as **single nucleotide polymorphisms** or **SNPs**. (However, not all RFLPs arise from SNPs.

How can these tools be used?

By testing the DNA of prospective parents, their genotype can be determined and their odds of producing an afflicted child can be determined. In the case of sickle-cell disease, if both parents are heterozygous for the genes, there is a 1 in 4 chance that they will produce a child with the disease. Amniocentesis and chorionic villus sampling make it possible to apply the same techniques to the DNA of a fetus early in pregnancy. The parents can learn whether the

unborn child will be free of the disease or not. They may choose to have an abortion rather than bring an afflicted child into the world.

Three problems:

The mutations that cause most human genetic diseases are more varied than the single mutation associated with sickle-cell disease. Over a thousand different mutations in the cystic fibrosis gene can cause the disease. A probe for one will probably fail to identify a second. A mixture of probes, one for each of the more common mutations, can be used. But there remains the problem of "false negatives": people who are falsely told they do not carry a mutant gene.

There are many diseases which result from several mutant genes working together to produce the disease phenotype.

There are still genetic diseases for which no gene has yet been discovered. Until the gene can be located, cloned, and sequenced, no probe can be made to detect it directly. However, it is sometimes possible to find a genetic "marker" that can serve as a surrogate for the gene itself. Let's see how.

Case 2: Screening for a RFLP "marker"

If a particular RFLP is usually associated with a particular genetic disease, then the presence or absence of that RFLP can be used to counsel people about their risk of developing or transmitting the disease. The assumption is that the gene they are really interested in, is located so close to the RFLP that the presence of the RFLP can serve as a surrogate for the disease gene itself. But people wanting to be tested cannot simply walk in off the street. Because of crossing over, a particular RFLP might be associated with the mutant gene in some people, with its healthy allele in others. Thus, it is essential to examine not only the patient but as many members of the patient's family as possible.

The most useful probes for such analysis bind to a unique sequence of DNA; that is, a sequence occurring at only one place in the genome. Often this DNA is of unknown, if any, function. This can actually be helpful as this DNA has been freer to mutate without harm to the owner. The probe will hybridize (bind to) different lengths of digested DNA in different people depending on where the enzyme cutting sites are that each person has inherited. Thus, a large variety of alleles (polymorphisms) may be present in the population. Some people will be homozygous and reveal a single band; others (e.g., all

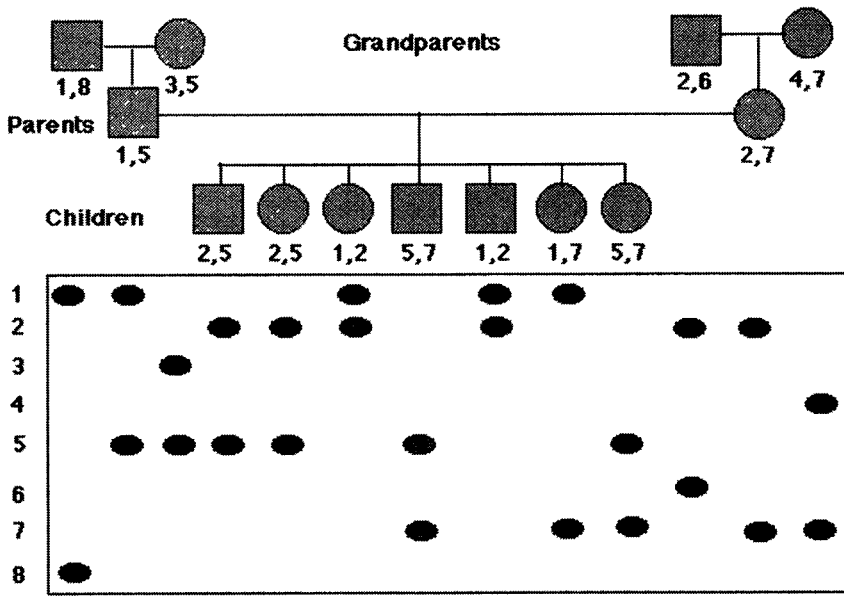

Figure 8.7 Three generation pedigree analysis by RFLP markers

the family members shown below) will be heterozygous with each allele producing its band.

The pedigree shows the inheritance of a RFLP marker through three generations in a single family. A total of 8 alleles (numbered to the left of the blots) are present in the family. The RFLPs of each member of the family are placed directly below his (squares) or her (circles) symbol and RFLP numbers.

If, for example, everyone who inherited RFLP 2 also has a certain inherited disorder, and no one lacking RFLP 2 has the disorder, we deduce that the gene for the disease is closely linked to this RFLP. If the parents decide to have another child, prenatal testing could reveal whether that child was apt to come down with the disease.

But note that crossing over during gamete formation could have moved the RFLP to the healthy allele. So, the greater the distance between the RFLP and the gene locus, the lower the probability of an accurate diagnosis.

Case 3: DNA "typing"

Each human cell contains 6×10^9 base pairs of DNA. Some of this represents structural genes (e.g., for the beta chain of hemoglobin)

that are identical in a large proportion of people. But long stretches of DNA do not encode for anything and are free to mutate extensively. It seems certain that if we could read the entire sequence of DNA in each human, we would never find two that were identical (unless the samples were from identical siblings; i.e., derived from a single zygote).

So each person's DNA is as unique as a fingerprint.

This truth has not escaped the law enforcement and legal professions. Analysis of DNA, called DNA typing, is widely used to find out the criminals e.g. identify rapists and other criminals; determine paternity; that is, who the father of the child really is; determine whether a hopeful immigrant is, as he or she claims, really a close relative of already established residents.

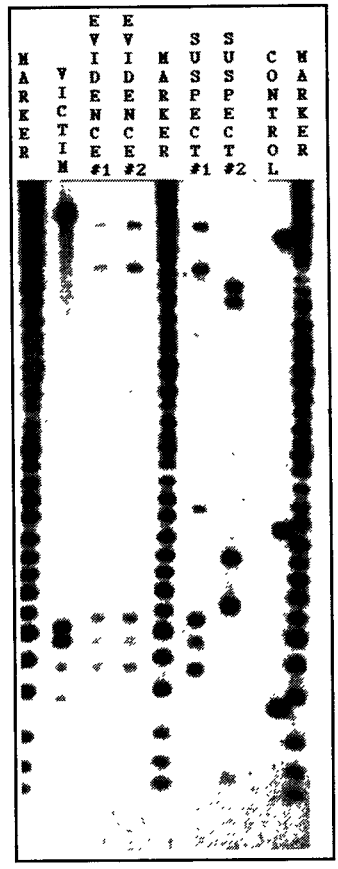

Figure 8.8 Semen analysis

The **Figure 8.8** shows the test results in a rape case. Two probes were used: one revealing the bands at the top, the other those at the bottom.

DNA was tested from semen removed from the vagina of the rape victim (EVIDENCE #2); a semen stain left on the victim's clothing (EVIDENCE #1); the DNA of the victim herself (VICTIM) to be sure that the DNA didn't come from her cells; DNA from two suspects (SUSPECT #1, SUSPECT #2); a set of DNA fragments of known and decreasing length (MARKER). They provide a built-in ruler for measuring the exact distance that each fragment travels. The cells of a previously-tested person to be sure the probes are performing properly (CONTROL).

One the basis of this test, suspect #2 can clearly be ruled out. None of his bands matches the bands found in the semen.

Is suspect #1 guilty?

We can never be certain. The best we can do is to estimate the probability that another person, picked at random, could provide the same DNA fingerprint.

As a conservative estimate, a given allele (band) might be found in 25% of the people tested. The probability of a random match of two alleles is $(0.25)^2$ or 1 in 16. The probability that 6 alleles match, as in this case, is $(0.25)^6$ or 1 in 4096. But the suspect was not picked at random, so you may feel that the evidence of guilt is strong.

The more probes you use, the more confident you can be that you have gotten the right man. If, for example, a set of probes revealed 14 bands in a suspect's DNA identical to those in the semen sample, the probability that you have the wrong man drops to less than 1 in 268 million $(0.25)^{14}$ = 1/268,435,456, which is more than the entire population, males and females, in the United States.

Most important points to carry out Restriction Fragment Length Polymorphism analysis

Restriction enzymes: General Information

Unit definition: One unit of restriction endonucleases is the amount of enzyme required to completely digest 1 μg substrate DNA in 60 min. at the appropriate assay conditions as stated for each restriction enzyme.

The type of the substrate DNA, the correct reaction temperature and the specific activity of each enzyme are stated on the pack-inserts that come with the products.

Stability: All restriction enzymes - supplied by various companies - contain expiry dates on the label. 100% activity of the enzyme is guaranteed until the date of expiry.

Storage: Restriction enzymes are, like all proteins, susceptible to denaturation and can lose their specific activity when exposed to higher temperatures.

Therefore, the enzymes are supplied as a glycerol solution and should be stored at -20^{0}C in a non-defrosting freezer. During usage, it is recommended to keep the enzyme on ice or in specially developed benchcoolers.

Buffers system supplied with various enzymes from different compnies

contain 10x buffer and different components and final concentration is shown in **Table 8.5**.

Table 8.5 Buffer supplied as 10x concentrated solutions:

Buffer components	Final concentration in mM				
	A	B	L	M	N
Tris Acetate	33				
Tris HCl		10	10	10	50
Magnesium Acetate	10				
$MgCl_2$		5	10	10	10
Potassium Acetate	66				
NaCl		100		50	100
1,4 - Dithioerythritol (DTE)			1	1	1
1,4 - Dithiothreitol (DTT)	0.5				
2 - mercaptoethanol		1			
pH at 37°C	7.9	8.0	7.5	7.5	7.5

Table 8.6 Special incubation buffers

Buffer components	Final concentration in mM			
	Mae I	Mae II	Mae III	Nde II
Tris HCl	20	50	20	50
NaCl	250	220	275	75
$MgCl_2$	6	6	6	5
2 - Mercaptoethanol	7	7	7	
Bovine Serum Albumin (BSA)		100 µg/ml		
1,4- Dithiothreitol (DTT)				0,5
pH	8.0 (45°C)	8.8 (50°C)	8.2 (55°C)	7.6 (37°C)

Important tips for restriction enzyme activities are shown in **Table 8.7.**

Table 8.7 Restriction enzymes: Activity

Definition	The ability of restriction enzymes to cleave - under non-optimal conditions - DNA sequences that are similar but not identical to the recognition site of the enzyme.
Enzymes affected	The following enzymes can exhibit star activity under certain non-optimized condition: Bam HI, Bss HII, Dde I, Eco RI, Eco RV, Hind III, Hin fI, Kpn I, Mam I, Pvu II, Sal I, Sau 3AI, Sgr AI, Taq I However, reports suggest that star activity may be a general property of all restriction enzymes.
Factors causing star activity	High glycerol concentration (> 5% v/v). Large excess of enzyme. Non-optimal ionic strength, pH and divalent cations of buffer system. Presence of organic solvents.
Avoiding star activity	Use the optimal buffer for each enzyme as recommended by its suppliers. Use the optimal amount of enzyme as recommended by its suppliers. Make sure that the DNA preparation is free of organic solvents that may have been used during isolation and / or purification of the DNA.

Restriction enzymes: inactivation and removal

Inactivation	Via heat treatment: Certain enzymes can be inactivated by heating. Via EDTA treatment. Alternatively, the enzyme can be inactivated by adding 0.5 M EDTA (pH 8.0) to a final concentration of 10 mM.
Removal	Via phenol / chloroform extraction: Extract the sample with phenol/ chloroform and once with chloroform, precipitate the DNA with ethanol or iso-propanol. Via silica absorption: Alternatively, the tedious and cumbersome phenol/ chloroform extraction procedure can be omitted by using the high pure PCR product purification kit.

The following procedures can be applied to inactivate restriction enzymes

Table 8.8 Restriction enzymes: characteristics (for abbreviations)

	Sequence	A	B	L	M	H	Temp	HI	MS	PFGE	PCR
Aat	GACGT↓C	100	0-10	0-10	10-25	0-10	37	Y	CG^+	♿	25%
AccI	GT↓(A,C)(T,G) AC	100	0-10	10-25	0-10	0-10	37	N	CG^+		<5%
Acs I	(A,G)↓AATT(T,C)	50-75	100	0-10	75-100	50-75	**50**	Y			
Acy I	G(A,G)↓CG(C,T)C	10-25	100	10-25	50-75	25-50	**50**	N			
Afl III	A↓C(A,G) (T,C)GT	50-75	75-100	50-75	75-100	100	37	N			20%
Alu I	AG↓CT	100	50-75	25-50	25-50	0-10	37	Y			100%
Alw 44 I	G↓TGCAC	100	25-50	75-100	100	10-25	37	N			
Apa I	GGGCC↓C	100	10-25	50-75	50-75	0-10	**30**	Y	dcm^+, CG^+	♿	100%
Asn I	AT↓TAAT	100	100	25-50	50-75	75-100	37	N		♿	
Asp I	GACN↓NNGTC	50-75	100	25-50	75-100	75-100	37	N			
Asp 700	GAANN↓NNTTC	50-75	100	10-25	50-75	0-10	37	N			10%
Asp 718	G↓GTACC	75-100	100	0-10	25-50	50-75	37	N	Dcm^+		100%
Asp EI	GACNNN↓NNGTC	10-25	10-25	100	25-50	0-10	37	Y			
Asp HI	G(A,T) GC (T,A)↓C	25-50	100	25-50	50-75	50-75	37	N			
Ava I	C↓(T,C) CG (A,G)G	100	100	10-25	50-75	10-25	37	Y	CG^+	♿	20%
Ava II	G↓G(A,T)CC	100	50-75	75-100	100	10-25	37	Y	Dcm^+, CG^+		<5%
Avi II	TGC↓GCA	50-75	75-100	10-25	50-75	100	37	N		♿	30%
Bam HI	G↓GATCC	100	100	75-100	100	25-50	37	N			100%
Ban II	G(A,G)GC(T,C)↓C	75-100	100	50-75	50-75	25-50	37	Y			
Bbr PI	CAC↓GTG	75-100	100	75-100	75-100	25-50	37			♿	100%
Bcl I	T↓GATCA	100	100	25-50	100	100	**50**		dam^+		
Bfr I	C↓TTAAG	25-50	25-50	75-100	100	25-50	37	Y			100%
Bgi I	GCC(N)$_4$↓NGGC	25-50	50-75	10-25	25-50	100	37	Y			30%
Bgl II	A↓GATCT	100	100	25-50	100	100	37				
Bln I	C↓CTAGG	25-50	50-75	0-10	25-50	100	37			♿	
Bmy I	G(G,A,T)GC(C,T,A)↓C	100	0-10	100	25-50	0-10	37				
Bpu AI	GAAGAC(N)$_{2/6}$	10-25	100	25-50	25-50	50-75	37				
Bse AI	T↓CCGGA	75-100	100	0-10	50-75	25-50	**55**			♿	

	Sequence	A	B	L	M	H	Temp	HI	MS	PFGE	PCR
Bsi WI	C↓GRACG	25-50	100	10-25	75-100	100	**55**	Y[a]	CG[+]	✓	100%
Bsi YI	CCNNNNN↓NNGG	100	100	50-75	100	25-50	**55**				
Bsm I	GAATGCN↓N	0-10	50-75	0-10	25-50	100	**65**				
Bsp LU11I	A↓CATGT	100	100	25-50	50-75	100	**48**				
Bss HII	G↓CGCGC	100	100	75-100	100	75-100	**50**		CG[+]	✓	
Bst 1107I	GTA↓TAC	25-50	50-75	0-10	25-50	100	37		CG[+]		
Bst EII	G↓GTNACC	75-100	100	25-50	50-75	50-75	**60**				100%
Bst XI	CCA(N)$_5$↓NTGG	10-25	100	0-10	10-25	100	**45**				
Cel II	GC↓TNAGC	25-50	50-75	25-50	25-50	100	37			✓	
Cfo I	GCG↓C	75-100	50-75	100	50-75	25-50	37	N			100%
Cfr 10I	(A,G) ↓CCGG(T,C)	25-50	100	0-10	25-50	25-50	37	N	CG[+]		
Cla I	AT↓CGAT	100	100	75-100	100	100	37	N	CG[+],dam+	✓	100%
Dde O	C↓TNAG	50-75	75-100	25-50	25-50	100	37	N			40%
Dpn I	G[m]A↓TC	100	75-100	50-75	75-100	75-100	37	N			100%
Dra I	TTT↓AAA	100	75-100	100	100	50-75	37	Y		✓	100%
Dra II	(A,G)G↓GNCC(T,C)	100	50-75	100	50-75	0-10	37	Y	dcm+		
Dra III	CACNNN↓GTG	50-75	75-100	50-75	75-100	100	37	N			50%
Dsa I	C↓C(A,G)(C,T)GG	0-10	10-25	0-10	10-25	100	**55**	N			
Eae I	(T,C)↓GGCC(A,G)	100	25-50	75-100	50-75	10-25	37	Y	CG[+],dcm+		
Ecl XI	C↓GGCCG	25-50	100	25-50	25-50	50-75	37	N		✓	
Eco 47 III	AGC↓GCT	25-50	50-75	0-10	25-50	100	37	Y	CG[+]	✓	
Eco RI	G↓AATTC	100	100	25-50	50-75	100	37	N		✓	5%
Eco R II	↓GG(A,T)GG	50-75	75-100	0-25	50-75	100	37	Y	dcm+		
Eco RV	GAT↓ATC	25-50	100	0-10	25-50	50-75	37	N			10%
Fok I	GGATG(N)$_{9/13}$	100	50-75	75-100	100	25-50	37	Y			
Hae II	(A,G)GCGC↓(T,C)	100	50-75	25-50	50-75	10-25	37	N	CG[+]		
Hind III	GG↓CC	50-75	50-75	75-100	100	25-50	37	N			100%
Hind II	GT(T,C)↓(A,G)AC	100	100	25-50	100	50-75	37	Y			100%
Hind III	A↓AGCTT	50-75	100	25-50	100	50-75	37	Y			10%
Hinf I	G↓ANTC	100	100	50-75	75-100	100	37	N			50%

	Sequence	A	B	L	M	H	Temp	HI	MS	PFGE	PCR
Hpa I	GTT↓AAC	100	25-50	25-50	50-75	25-50	37	N	CG+		100%
Hpa II	C↓CGG	50-75	25-50	100	50-75	10-25	37	Y	CG+		40%
Ita I	GC↓NGC	0-10	25-50	0-10	0-10	100	37	Y			
Kpn I*	GGTAC↓C	75-100	10-25	100	25-50	0-10	37	N			50%
Ksp I	CCGC↓GG	0-10	0-10	100	0-10	0-10	37	N			
Ksp 6321	CTCTTC(N)¼	100	0-10	25-50	25-50	0-10	37	N			
Mae I**	C↓TAG	25-50	25-50	0-10	0-10	10-25	**45**	N			
Mae II**	A↓CGT	0-10	25-50	0-10	25-50	75-100	**50**	N	CG+		
Mac III**	↓GTNAC	0-10	10-25	0-10	0-10	10-25	**55**	N			
Mam I	GATNN↓NNATC	75-100	75-100	75-100	75-100	100	37	Y	dam+		20%
Mlu I	A↓CGCGT	10-25	25-50	0-10	10-25	100	37	N	CG+		<5%
MLu NI	TGG↓CCA	100	0-10	10-25	10-25	0-10	37	Y			
Mro I	T↓CCGGA	100	0-10	50-75	50-75	0-10	37	N			
Msp I	C↓CGG	100	100	100	100	50-75	37	Y			40%
Mun I	C↓AATTG	50-75	0-10	100	100	10-25	37	N			
Mva I	CC↓(A,T)GG	100	50-75	25-50	25-50	100	37	N			
Mvn I	CG↓CG	50-75	0-10	50-75	100	10-25	37	N			30%
Nae I	GCC↓GGC	100	0-10	100	0-10	0-10	37	Y	CG+		
Nar I	GG↓CGCC	100	75-100	75-100	50-75	0-10	37	Y	CG+		
Nco I	C↓CATGG	50-75	50-75	50-75	50-75	100	37	Y			50%
Nde I	CA↓TATG	25-50	75-100	10-25	50-75	100	37	Y			
Nde II**	↓GATC	10-25	10-25	0-10	0-10	10-25	37	N	dam+		
Nhe I	G↓CTAGC	100	25-50	100	100	10-25	37	Y	CG+		100%
Not I	GC↓GGCCGC	10-25	50-75	0-10	25-50	100	37	Y			
Nru I	TCG↓CGA	10-25	100	0-10	10-25	75-100	37	Y	dam+, CG+		75%
Nsi I	ATGCA↓T	50-75	100	10-25	50-75	100	37	Y			100%
Nsp I	(A.G)CATG↓(T,C)	25-50	50-75	75-100	100	0-10	37	N			
Pin AI	A↓CCGGT	100	100	10-25	50-75	50-75	37	Y			
Psp 1406I	AA↓CGTT	100	100	100	10-25	0-10	37	Y	CG+		
Pst I	CTGCA↓G	25-50	25-50	10-25	25-50	100	37	N			90%

	Sequence	A	B	L	M	H	Temp	HI	MS	PFGE	PCR
Pvu I	CGAT↓CG	50-75	75-100	25-50	50-75	100	37	N	CG+	[b]	<5%
Pvu II	CAG↓CTG	25-50	25-50	25-50	100	25-50	37	N			100%
Rca I	T↓CATGA	75-100	100	25-50	50-75	25-50	37	Y			
Rsa I	GT↓AC	100	50-75	100	50-75	0-10	37	Y	CG+		100%
Rsr II	CG↓G(A,T)CCG	75-100	10-25	100	75-100	0-10	37	N	CG+	[b]	
Sac I	GAGCT↓C	100	0-10	100	50-75	0-10	37	Y			100%
Sal I	G↓TCGAC	0-10	25-50	0-10	10-25	100	37	Y	CG+	[b]	
Sau 3A I	↓GATC	100	25-50	25-50	75-100	0-10	37	N	CG+		100%
Sau 96 I	G↓GNCC	100	50-75	25-50	25-50	25-50	37	N	dcm+, CG+		
Sca I	AGT↓ACT	0-10	100	0-10	75-100	100	37	N			<5%
Scr FI	CC↓NGG	10-25	100	10-25	10-25	50-75	37	Y	dcm+		
Sex AI	A↓CC(A,T)GGT	100	100	50-75	50-75	25-50	37	Y	dcm+	[b]	
Sfi I	GGCC(N)$_4$↓NGGCC	25-50	25-50	75-100	100	25-50	**50**	N		[b]	10%
Sfu I	TT↓CGAA	25-50	50-75	10-25	25-50	100	37	N		[b]	
Sgr AI	C(A,G)↓CCGG(T,C)G	100	0-10	100	10-25	0-10	37	N		[b]	
Sma I	CCC↓GGG	100	0-10	0-10	0-10	0-10	**25**	Y	CG+	[b]	100%
Sna Bi	TAC↓GTA	75-100	25-50	100	100	10-25	37	N	CG+	[b]	50%
Spe I	A↓CTAGT	75-100	75-100	75-100	100	100	37	Y		[b]	
Sph I	GCTAG↓C	50-75	75-100	25-50	100	75-100	37	Y			<5%
Ssp I	AAT↓ATT	75-100	75-100	10-25	75-100	100	37	Y		[b]	
Ssp BI	T↓GTACA	100	100	10-25	50-75	10-25	37	Y[b]			
Stu I	AGG↓CCT	100	100	100	75-100	50-75	37	Y	dcm+		30%
Sty I	C↓C(A,T)(A,T)GG	50-75	100	10-25	75-100	100	37	Y			<5%
Swa I	ATTT↓AAAT	0-10	10-25	0-10	0-10	100	25	N		[b]	
Taq I	T↓CGA	50-75	100	25-50	50-75	50-75	65	N	dam+		100%
Tru 9I	T↓TAA	100	25-50	100	100	25-50	65	N			
Van 91I	CCA(N)4 ↓NTGG	25-50	100	0-10	25-50	0-10	37	Y			
Xba I	T↓GTAGA	100	75-100	75-100	75-100	100	37	N	dam+	[b]	60%
Xho I	C↓TCGAG	25-50	75-100	10-25	25-50	100	37	N	CG+	[b]	<5%
Xho II	(A,G)↓GATC(T,C)	50-75	25-50	100	75-100	0-10	37	N			
Xma CI	C↓CCGGG	50-75	0-10	100	75-100	0-10	37	N			

Abbreviations and icons used in the tables:

A Percentage activity of enzyme in SuRE / Cut buffer A

B Percentage activity of enzyme in SuRE / Cut buffer B

L Percentage activity of enzyme in SuRE / Cut buffer L

M Percentage activity of enzyme in SuRE / Cut buffer M

H Percentage activity of enzyme in SuRE / Cut buffer H

Temp Incubation temperature of enzyme, temperatures in bold differ from the "classical" 37°C.

HI Hear inactivation of enzyme: Y: Indicates that the enzyme can be inactivated by heat (15 min at 65°C unless otherwise stated).

N: Indicates that the enzyme can not be inactivated by heat (see previous page for alternative procedures).

MS Indicates sensitivity of enzyme for methylation; dcm+: indicates that the enzyme is blocked by dcm methylation, dam+: indicates that the enzyme is blocked by dcm methylation, CG+ : indicates that the enzyme is blocked by eukaryotic methylation, No indication: the enzyme is not sensitive to any form of methylation.

PFGE Function tested for Pulse Field Gradient Electrophoreses. The enzyme is function tested for PFGE experiments .

No indication : the enzyme is not function tested.

PCR Percentage of enzyme activity in a standard PCR Mix (=10 mM Tris HCl , pH 8.3 at 20°C, 50 mM KCl, 1.5 mM $MgCl_2$.

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Chapter 9

Amplification Mutation Detection System (ARMS)

The amplification refractory mutation system (ARMS) is an amplification strategy in which a polymerase chain reaction (PCR) primer is designed in such way that it is able to discriminate among templates that differ by a single nucleotide residue. ARMS has also been termed as, allele specific amplification (ASA), PCR amplification of specific allele (PASA), and allele specific PCR (ASP).

Strategy of ARMS

The basis of this strategy is the hypothesis that a mismatch at the 3′ end of one or both of the used oligonucleotides, with high probability, prevents the 3′ elongation of the primer by Taq polymerase. Thus, an ARMS primer can be designed to amplify a specific member of a multi allelic system while remaining refractory to amplification to another allele that may differ by as little as a single base from the former.

In simplest form, two complementary reactions can be carried out:

1. One containing a primer specific for the normal allele and
2. Other containing primer for the mutant allele (both have a common second primer).

One of the PCR primers perfectly matches one allelic variant of the target but is mismatched to the other variant. Mismatch in the primer is located at the 3' end of primer; this makes preferential amplification of the perfectly matched allele. Genotyping is based on whether there is amplification in one or both reactions. Band in reaction with normal primer indicates normal allele, band in reaction with mutant primer indicates mutant allele, and band in both reactions indicate a heterozygote. **Figure 9.1** shows that how allele specific primer results into amplification in one of the tube and not in the other.

The main advantage of ARMS is that the amplification step and diagnostic steps are combined, in that the presence of an amplified

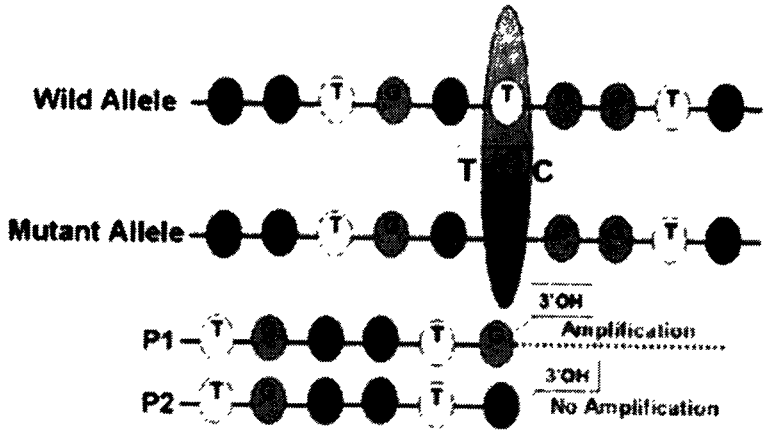

Figure 9.1: Allele specific amplification in ARMS

product which indicates the presence of a particular allele and *vice versa*. This characteristic feature of this technique is that it has made this technique quite efficient method for detection of mutations.

Principle of ARMS

The basic principle of this technique is that the *Thermus aqaticus* (Taq) poymerase, the DNA polymerase commonly used in PCR, lacks a 3′ to 5′ exonuclease activity. Thus, DNA polymerase cannot carryout the proof reading with 3′ mismatched primer and this results in to greatly reduced amplification efficiency.

Thus, an ARMS typing system can be designed by synthesizing primers with their 3′ nucleotide overlying the polymorphic residue, where one of the ARMS primer can specifically amplify one of the allele of a multi allelic system. Hence, for typing system with *n* alleles, *n* ARMS primers will be required with the typing achieved in *n* reactions. For many diagnostic applications a second set of primers from another region of genome are included as an internal control to exclude false negatives.

Important consideration in ARMS

Primers

1. "Wallace temperature" i.e. [(A +T) x 2°C + (G + C) x 4 ° C] should be 48-50°C.

2. GC content of ~50%, if possible.
3. Should not have self-complementary sequences of 4 bp or more.
4. Should not have > 4 nucleotide complementary between their 3' ends.

Double ARMS

Double ARMS is very useful system to reveal the relationship of two polymorphic sites or clarifying the haplotypes. Here in single reaction both primers used are allele specific ARMS primers; hence, amplification will only occur when the allele specific for each primer is present on the same chromosome (**Figure 9.2**).

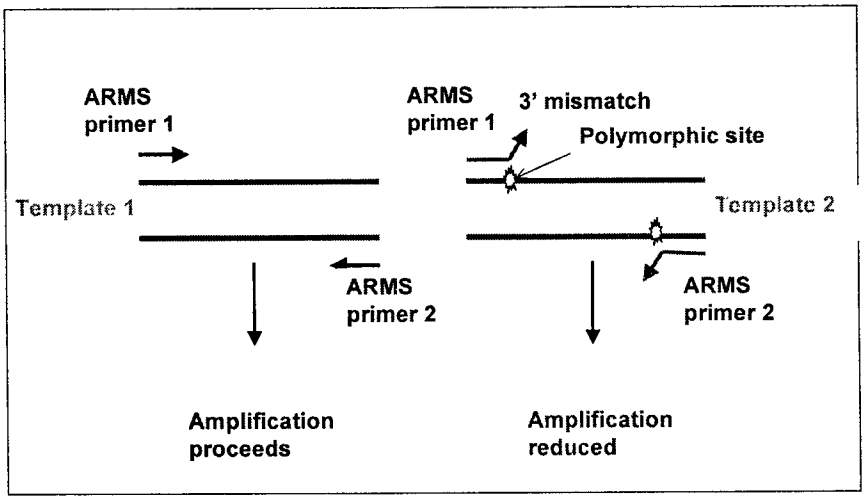

Figure 9.2: Double ARMS

Modification of the ARMS technique

There are two main modifications that have been carried out in the technique of ARMS, based on the application.

1. Multiplex ARMS

Multiplex ARMS is designed to reduce the number of PCR necessary to resolve multi allelic system using ARMS. In multiplexing the number of ARMS primers, each specific for a particular allele, is included in a single reaction in a single tube (Figure 8.2). Here different allele produces PCR product of different length, which can be distinguished by running on the gel. A number of formats for multiplex ARMS PCR

are potentially possible, one example is of tetra primer ARMS, which is a system for typing a biallelic system. Apart from two-allele specific internal ARMS primer, this also consists of a pair of external primers that produces an internal control (**Figure 9.3**).

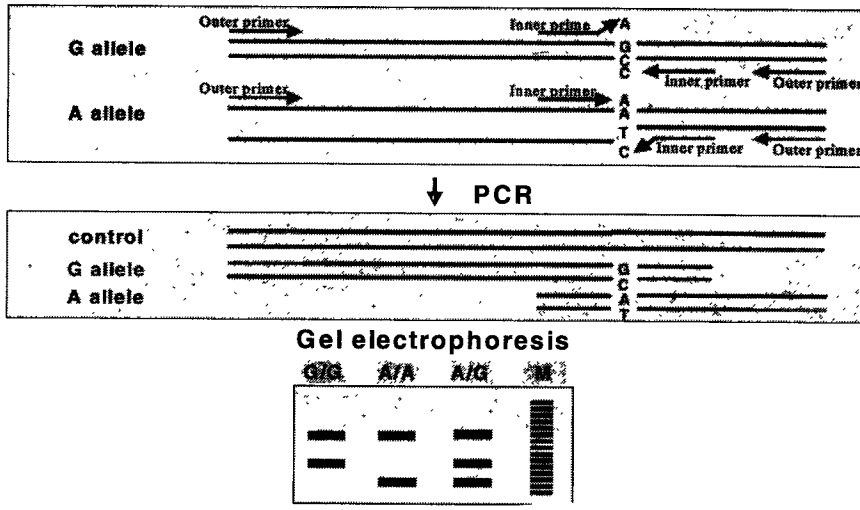

Figure 9.3 Multiplex of ARMS PCR. The single nucleotide polymorphism used here as an example is a G®A substitution, but the method can be used to type other types of single base substitutions. Two allele-specific amplicons are generated using two pairs of primers, one pair (indicated by pink and red arrows, respectively) producing an amplicon representing the G allele and the other pair (indicated by indigo and blue arrows, respectively) producing an amplicon representing the A allele. Allele specificity is conferred by a mismatch between the 3'-terminal base of an inner primer and the template. To enhance allelic specificity, a second deliberate mismatch (indicated by an asterisk) at position -2 from the 3'-terminus is also incorporated in the inner primers. The primers are 26 nt or longer, so as to minimize the difference in stability of primers annealed to the target and non-target alleles, ensuring that allele specificity results from differences in extension rate, rather than hybridization rate. By positioning the two outer primers at different distances from the polymorphic nucleotide, the two allele-specific amplicons differ in length, allowing them to be discriminated by gel electrophoresis.

Computer software to design primers for tetra-primer ARMS-PCR

As primer designing is a critical part of multiplex ARMSPCR and it is time-consuming. There are computer programs for the primer

designing. The program, outlined below is accessible through the Internet at http://cedar.genetics.soton.ac.uk/public_html/primer1.html. Users need to input the target DNA sequence, specify the polymorphic site and define criteria for the primers (T_m, %GC, length and complementarity) and product sizes.

The following flow chart can be utilized for getting the primers designed:

Users input target DNA sequence, specify polymorphic site, and define criteria for primers (including primer Tm, GC%, length and complementarity, as well as product sizes).

↓

Computing by software

1. Compute all possible inner forward and inner reverse primers that meet criteria specified by users.

↓

2. Pick an "optimal" inner primer pair with their Tm closest to the optimal Tm specified by users and with a minimal Tm difference between the two primers.

↓

3. Pick outer reverse primers that satisfy user specified criteria and that have a Tm equal to the mean Tm value of the two inner primers selected in step 2.

↓

4. Pick outer forward primers that satisfy user specified criteria and that have a Tm equal to the mean Tm value of the two inner primers selected in step 2.

↓

Out put:

Either primer details or error messages

ARMS PCR is used in many situations one of these is "Sequence Specific Primer (SSP)" based molecular typing of HLA class I and II loci.

Precautions of ARMS PCR in general

1. A mismatch directs at the 3′ end or within the first 3-5 bases of the 3′ end yields the highest specificity.

2. Primer lengths between 14 and 17 bp yield better results, due to increased relative influence of the mismatch on the annealing behaviour.
3. The DNA concentration should be chosen as low as possible, since concentrations that are too high reduce the specificity.
4. The primer concentrations should be reduced to 0.05μM.
5. Lowering of the magnesium concentration to approximately 1.5μM, and of the dNTP concentration to 25-50μM.
6. If we reduce the Taq polymerase amount to 0.2-0.3 U/25μl PCR mixture the desirable results may be obtained.
7. The addition of 2-5% formamide may improve the specificity of the assay, especially if G-C rich regions are present.

ARMS PCR is important for SNP genotyping. Recent approaches for improved throughput SNP typing include, particularly, microarray-based approaches and mass spectrometric-based techniques [such as matrix-assisted laser de sorption/ ionisation time-of-flight (MALDI-TOF) mass spectrometry]. Relative to tetra-primer ARMS-PCR, both incur much higher capital costs; however, the former allows distribution in many smaller centres. All methods incur an assay set-up phase, but microarrays lend themselves better to re-sequencing or multiplex SNP typing of single samples. Tetra-primer ARMS-PCR with MADGE permits single SNP studies in many samples in parallel, whereas, MALDI-TOF permits very rapid serial genotyping analyses, but requires significant sample 'clean-up' prior to analysis. All methods should be capable of 10^4–10^5 calls per week. The method described here may best fit the 'middle ground' of ease of availability to any laboratory both for scale up of smaller projects and secondary investigations of leads from extremely high throughput centers requiring major capital expenditure.

An allele-specific amplification method with a TaqMan fluorogenic probe (TaqMan-ASA) for the detection of point mutations is also available now. Pair wise PCR amplification using two sets of allele-specific primers in the presence of a TaqMan probe can be monitored in real time with a fluorescence detector. Difference in amplification efficiency between the two PCR reactions can be determined by "threshold" cycles to differentiate mutant and normal alleles without post-PCR processing. The method measures the efficiency of amplification rather than the presence or absence of end-point PCR products, therefore, allowing greater flexibility in designing allele-

specific primers and an ample technical margin for allelic discrimination. The method can be automated and may be applicable to the DNA diagnosis of various genetic diseases. Thus, it may be concluded that the ARMS is a rapid, simple, inexpensive and accurate method for detecting the most common mutations, if required, all these can be confirmed by sequencing.

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Chapter 10

Single Stranded Conformation Polymorphism (SSCP)

SSCP is the electrophoretic separation of single-stranded nucleic acids based on subtle differences in sequence (often a single base pair) which results in a different secondary structure and a measurable difference in mobility through a gel.

The mobility of double-stranded DNA in gel electrophoresis is dependent on strand size and length but is relatively independent of the particular nucleotide sequence. The mobility of single strands, however, is noticeably affected by very small changes in sequence, possibly one changed nucleotide out of several hundred. Small changes are noticeable because of the relatively unstable nature of single-stranded DNA; in the absence of a complementary strand, the single strand may experience intrastrand base pairing, resulting in loops and folds that give the single strand a unique 3D structure, regardless of its length. A single nucleotide change could dramatically affect the strand's mobility through a gel by altering the intrastrand base pairing and its resulting 3D conformation.

Single-strand conformation polymorphism analysis takes advantage of this quality of single-stranded DNA. First announced in 1989 as a new means of detecting DNA polymorphisms, or sequence variations, SSCP analysis offers an inexpensive, convenient, and sensitive method for determining genetic variation.

Like restriction fragment length polymorphisms (RFLPs), SSCPs are allelic variants of inherited, genetic traits that can be used as genetic markers. Unlike RFLP analysis, however, SSCP analysis can detect DNA polymorphisms and mutations at multiple places in DNA fragments. As a mutation scanning technique, though, SSCP is more often used to analyze the polymorphisms at single loci, especially, when used for medical diagnoses.

Principle of SSCP

The SSCP method is based on the principle that single-stranded DNA in a non denaturing condition has a folded structure that is determined by intra molecular interactions. This sequence based secondary structures (conformation) affect the mobility of the DNA during electrophoresis on a nondenaturing polyacrylamide gel.

A DNA molecule containing a mutation, even at a single base substitution, will have a different secondary structure than the wild type, resulting in to a different mobility shift during electrophoresis than that of the wild type.

In this method, the DNA sequence of interest can be amplified and simultaneously radio labeled by PCR. The PCR sample is further denatured prior to being separated on a sequencing size polyacrylamide gel and visualizing the bands by autoradiography. Subsequent improvements in method have eliminated the radio labeling for visualizing the bands with silver staining.

This method is widely acclaimed to be useful because it detects the unknown mutations in a piece of DNA, provided that temperature and power supply for electrophoresis is carried out in a highly controlled fashion. In **Figure 10.1** various steps involved in a SSCP analysis are illustrated.

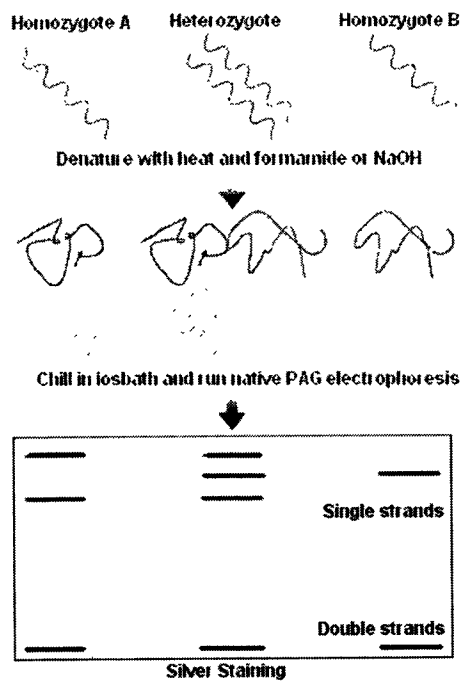

Figure 10.1: Principle and steps

Sensitivity of SSCP

The sensitivity of SSCP analysis range from 35% to nearly 99%. The most important factor that has the greatest effect on SSCP sensitivity is the size of the DNA fragment. As the DNA fragment increases in

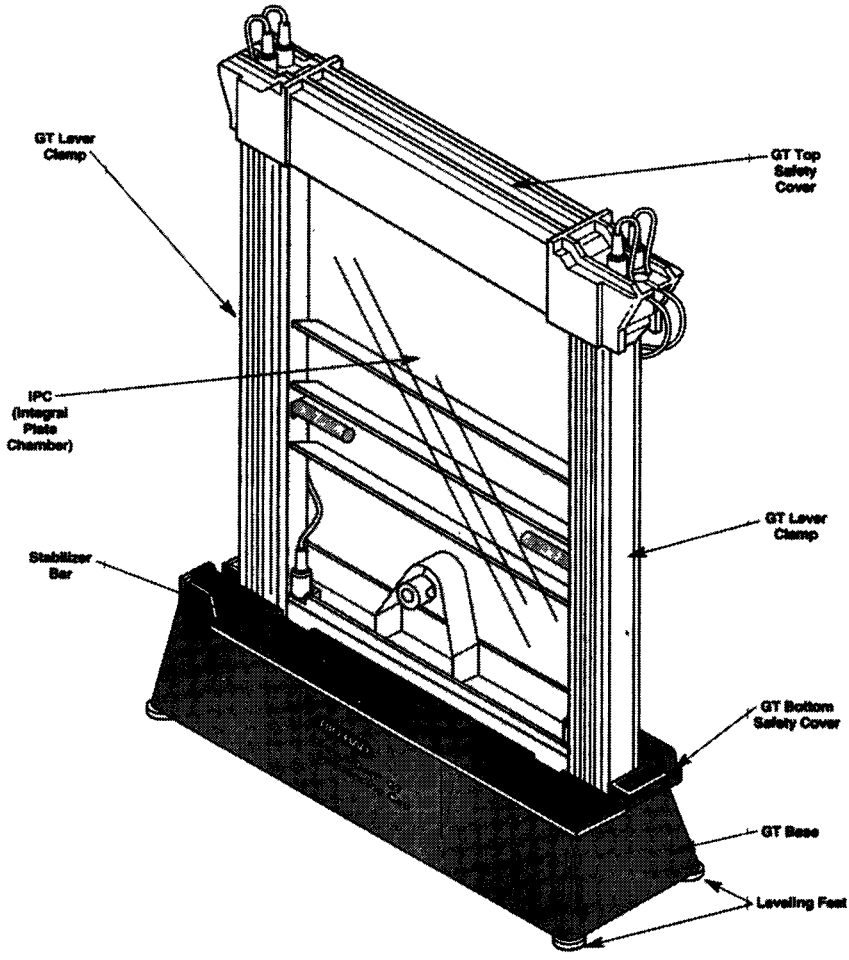

Figure 10.2 Sequi-Gen GT nucleic acid electrophoresis cell

length, the sensitivity of SSCP analysis decreases. An optimal size of 200bp or less is the most sensitive for single-base substitutions.

The electrophoretic mobility of the single-strand conformation may also be affected by temperature, ionic strength, gel additives (glycerol), acrylamide concentration and type of mutation. The description of major parts of electrophoresis apparatus are shown in **Figure 10.2.**

Description of major parts: (Figure 10.2)

Gel reagents and electrophoresis buffers

For most DNA sequencing or nucleic acid separations, a 49:1 acrylamide: bis-acrylamide solution is required. A 1x TBE (Tris, boric acid and EDT A) solution is the preferred electrophoresis buffer. Reproducibility is affected by the quality of the gel and buffer reagents. A full line of high quality polyacrylamide gel reagents and nucleic acid electrophoresis buffers is available from Bio-Rad. Premixed reagents and buffers are also available and offer convenience, time savings, and reproducible results. Each reagent and buffer is purified to meet rigorous quality control standards.

Electrical path

Both electrode wires are positioned near the bottom of the gel. The upper buffer carries the current from the cathode up to the top of the plates near the fill spout, where the gel is exposed. The lower buffer contacts the gel at the bottom edge of the plates in the standard fashion (**Figure 10.3**).

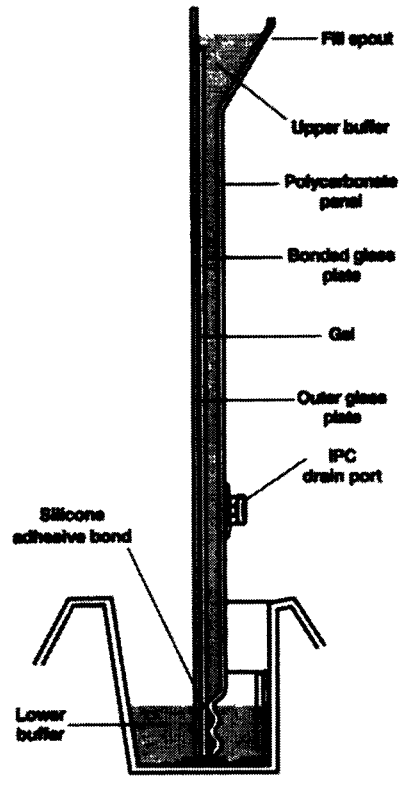

Figure 10.3 Electrical paths through IPC to lower buffer reservoir

Cleaning and maintenance

1. Cleaning and Siliconizing Plates

Important

To insure "bubble-free" gels using the Sequi-Gen GT precision caster, the glass plates must be thoroughly cleaned and the outer (long) glass plate siliconized or coated before each use.

1. Clean both Sequi-Gen GT glass plates (IPC and

outer plates) thoroughly before each use.

- Carefully place the plate into the sink and rinse with warm water.
- Pour powdered lab detergent (Alconox] or Micro [International Products]) into a gloved hand and add sufficient water to make a paste.
- Apply the paste and scrub the entire glass surface with a gloved hand, using circular motions.
- Rinse all of the detergent with warm water.
- Rinse with deionized water.

2. Inspect the plates carefully for pieces of detergent, dried polyacrylamide, or other particles. Rewash if necessary.
3. Perform siliconization under a fume hood, to reduce the hazard from breathing reagents. Alternatively, several non-toxic, non-corrosive glass plate coating solutions are commercially available. Siliconizing or coating only the outer (long) plate is generally recommended, so that, when the plates are separated, the gel sticks to the IPC-bound glass plate.

- Use a glass Pasteur pipette to dispense 2 ml of the silconizing reagent onto the front plate. Coat the plate completely and evenly by spreading the silconizing reagent on the plate surface with a large lint free tissue, using a motion that travels from the top to the bottom of the plate.

Caution: Do not siliconize the IPC plate unless hexane, heptane, or water is used as a solvent in the silanizing reagent. Other organic solvents will crack or damage the IPC plastic and weaken the adhesive bond.

- Never heat an IPC in an oven. Severe damage will result to the adhesive bond. Use siliconizing compounds that react, or cure, at room temperature.

Note: If the gels will be fixed or stained, the IPC (short) plate should be siliconized or coated, since its immersion into fixing or staining solutions is not recommended.

4. Prior to assembling the plates, apply a small amount of ethanol to each plate and rub to dryness with a tissue. Using the same tissue, clean the spacers.

Cleaning Sequi-Gen GT components

1. Rinse the universal base buffer chamber, stabilizer bar, combs, spacers and precision caster base, gasket, syringe and tubing assembly with a mild detergent solution in warm water. Use a soft-bristled brush or sponge to remove polyacrylamide gel pieces.

Note: Do not snag or break the electrode wire in the universal base while cleaning.

2. Rinse thoroughly with warm water and air dry.

Compatible cleaning agents for polycarbonate parts

Chemically compatible cleaners must be used to ensure long life of parts. These include.

- Aqueous solutions of soaps and mild detergents
- Organic solvents
- Hexane
- Aliphatic hydrocarbons
- Alcohols
- Methanol
- Ethanol
- Isopropyl alcohol
- Dilute acids

Caution: Do not touch plastic molded parts with solvents that contain chlorinated hydrocarbons or aromatic hydrocarbons *(e.g.,* carbon tetrachloride, toluene, methyl ethyl ketone, and acetone).

Do not use abrasive or highly alkaline cleaners on the polycarbonate plastic IPC panel. (The glass may be cleaned with abrasive or strong alkaline detergents, if adequate care is taken to avoid contact with the plastic panel.)

Do not soak plastic parts in detergents more than 30 minutes.

Cleaning the clamps

Rinse the clamps with warm water, and wipe any polymerized acrylamide off the clamping surfaces. Drain the banana plug mounts at the top of the clamps, and wipes the clamping surfaces dry before each use. Do not use organic solvents to clean the clamps.

Operating instructions

1. Before assembly
    1. Thoroughly clean all parts.

    Caution: Certain solvents and cleaning agents should be avoided.

    2. Depending on the size of the Sequi-Gen GT IPC, make up the appropriate amount of electrode buffer (typically I x TBE) from **Table 10.1**.

Table 10.1 Electrode buffer volumes

IPC Size Total Buffer Required Upper Lower

IPS size	Total buffer required	Upper	Lower
21 x 40 cm	850 ml	500 ml	350 ml
21 x 50 cm	925 ml	575 ml	350 ml
38 x 30 cm	1000 ml	650 ml	350 ml
38 x 50 cm	1750 ml	1400 ml	350 ml

Assembling the glass plate sandwich

After the Sequi-Gen GT components have been washed and the glass plates siliconized or coated, assemble the Sequi-Gen GT apparatus. Always wear gloves while handling the glass plates during assembly to avoid fingerprints on the glass plates. Fingerprints will cause bubbles during gel casting.

Important: Before assembling the Sequi-Gen GT cell, inspect all plastic parts, glass plates, electrical cables, jacks, and receptors for loose connections, cracks, chips, charring, or corrosion. Do not use any part that is damaged. These parts may cause buffer leaks or arcing.

1. Clean and siliconize the glass plates.
2. Place the IPC flat on the bench with glass plate facing upward.
    - Position one spacer along each long edge of the IPC glass plate. The bottom edges of the spacer and the glass plate should be flush and the long edge of the spacer should be next to the plastic lip of the IPC panel.
3. Place the front (outer, long) glass plate onto the IPC and spacers with the siliconized or coated surface facing down.
    - With both hands, stand the IPC/glass plate sandwich on the benchtop with the outer glass plate facing away from you.

- Allow the glass plates and spacers to touch the bench top, to temporarily align the assembly for gel casting.

4. Slide the clamps over the IPC assembly.
   - The levers of the clamps should be on the IPC panel side of the assembly and need to be facing away from the unit (perpendicular to the IPC panel) for the clamps to slide easily onto the assembly. Secure the clamps to the IPC/glass plate sandwich by moving the levers toward the IPC panel.
5. Lay the IPC assembly on the bench top with the IPC panel (drain port side) facing up.
   - Check the alignment of the glass plates, spacers and clamps. The bottom of the glass plates, spacers and clamps should be flushed. If either glass plate, spacer, or clamp is not properly aligned or flushed, adjust the alignment by loosening the clamps and move clamps, glass plates and spacers into alignment.
   - Tighten the clamps by moving the levers back down towards the IPC after the assembly is flushed.
6. To avoid incompatibility problems between combs and spacers after the gel is cast; check the fit of the combs in the assembled Sequi-Gen GT cell by trying to place them between the plates.
   - If the combs clearly will not fit between the plates without damaging the comb, try a different comb. Optimally, combs should demonstrate slight resistance to being placed between the glass plates.

Casting the Gel

1. Prepare the gel solution.
   - Degas the gel solution for 5-15 minutes under a strong vacuum ($\geq$ 26 in/Hg) to insure reproducible gel porosity **Table 10.2.**

Table 10.2 Required gel volumes using the precision caster assembly

IPC Size	0.25 mm spacers	0.4 mm spacers	0.75 mm spacers	0.25 - 0.75 mm wedge spacers	0.40 - 1.2 mm wedge spacers
21x40 cm	25 ml	35 ml	70 ml	50 ml	60 ml
21x50 cm	30 ml	45 ml	90 ml	65 ml	85 ml
38x30 cm	40 ml	50 ml	90 ml	-	-
38x50 cm	55 ml	85 ml	170 ml	120 ml	140 ml

2. Place the precision caster base on the bench with its open cavity facing up. Place the gray precision caster gasket into the base. The cam pegs in the precision caster must be pulled out to accommodate the apparatus.

Note: If the gasket is wet, remove any remaining water from the gasket by squeezing it with a paper towel.

3. Place the bottom edge of the IPC assembly into the precision caster base with the bottom edge of the assembly resting against the gray gasket of the precision caster base as shown in **Figure 10.4**.
4. When the IPC assembly is seated in the caster base, use the cam pegs to connect the base to the clamps.

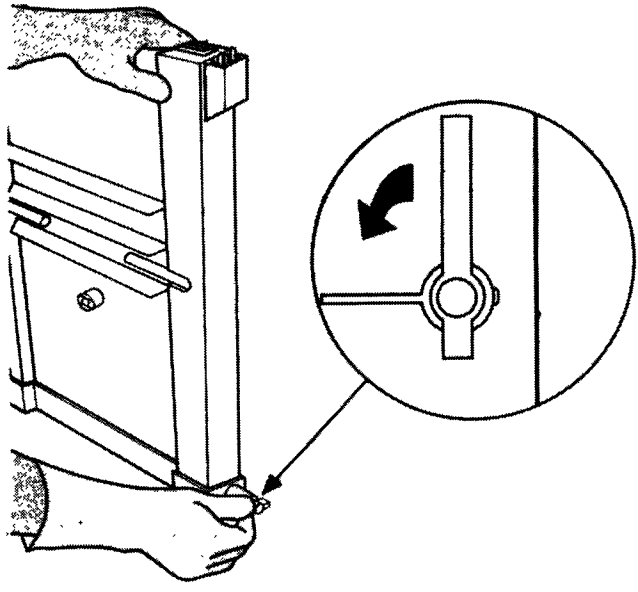

Figure 10.4 Attaching precision caster base to IPC assembly.

- Push each cam peg into the corresponding hole on the clamp with the lever in the up position. Slight downward pressure applied to the top of the IPC assembly may be required to engage each cam peg.

5. When both pegs are engaged, turn them evenly until moderate resistance is felt or the handles of the Cam pegs are perpendicular to the benchtop. This action causes the precision caster base to fit tightly against the plate assembly.

- Lay the IPC assembly flat on the benchtop with the precision caster base facing toward you.
- Look through the injection port of the base. If the precision caster has been attached properly, a space should be seen between the two green glass plates.
- If the space cannot be seen, loosen the caster base by rotating the Cam pegs upward. Adjust the caster base either up or down until the space between the green glass plates can be seen and is in the middle of the injection port hole.
- While securing the precision caster base in place with one hand, turn the cam pegs back to their original position to secure the base to the bottom of the IPC assembly.

6. Lay the IPC assembly and attached precision caster base flat on a bench with the IPC panel (drain port) facing up and the long edges of the clamps running parallel with the edge of the benchtop.
   - The most even pouring can be obtained by insuring that the assembly is level on the benchtop. Failure to level the assembly may result in gel leakage. A leveling bubble is provided to facilitate leveling the IPC assembly. Props (approximately 2 cm) will be required at the top of the IPC to level the unit for casting. The unit is now ready for gel casting. An alternative to the use of props is to cast the gel with the precision caster positioned off the edge of the lab bench.

Note: If casting a 38 x 50 cm IPC, place the 38 x 50 cm IPC assembly at an incline, with the top of the apparatus approximately 4-5 cm higher than the bottom. (The bottom of the apparatus contains the attached precision caster base). After the gel is cast, level the assembly for gel polymerization.

7. While the gel solution is degassing, prepare a fresh 25% ammonium persulfate solution.
   - Choose the appropriate syringe and tubing assembly (tubing and luer taper) provided with the precision caster. Insert the luer taper into the one end of the tubing. Secure the other end of the tubing onto the luer end of the syringe.
8. When the gel solution has degassed, add 25% APS and TEMED in the recommended amounts.

- Swirl the solution gently to mix.
- **Slowly** pull the required gel volume into the syringe.
- Tap air bubbles to the top of the syringe and gently force them out. If bubbles are inadvertently introduced into the tubing, pinch the portion of the tubing where the bubbles exist while forcing some of the gel solution out. This should allow the bubble to exit the tubing with the gel solution.

9. When all air bubbles are removed from the tubing, place the luer taper into the injection port of the precision caster base. Tighten the luer taper fitting in place on the injection port of the precision caster base and begin to slowly inject the gel solution. Slow and even pressure on the syringe plunger will insure uniform gel casting with no bubbles.

Note on gel bubble formation

- The following injection times (from the bottom of IPC to the top) were found to result in bubble-free gels: for 50 cm gels with 0.4 mm spacers, between 40-45 seconds; for 50 cm gels with 0.25 spacers, between 50-65 seconds. Injection times of 10 seconds or less can result in bubble formation in the gel.
- Bubbles can form at the gel front because of soiled areas or uneven siliconization or coating of the glass plates.
- To achieve bubble free gels, thoroughly clean both plates and siliconize the outer glass plate before each use.
- If bubbles begin to form at the gel front, hard tapping on top of the IPC assembly (above the bubble formation) while slowly injecting the gel solution should eliminate the bubble. Alternatively, the comb end of the IPC assembly can be momentarily lifted at an angle to facilitate elimination.

10. Continue to slowly inject the gel solution until the gel solution emerges a few centimeters from the top of the notched (shorter) glass plate (across the entire width of the gel).

Important: If pouring a 38 x 50 cm IPC, remove the support that created an incline and lay the unit level on the bench top (use the Leveling Bubble provided). An additional 2 cm support will be needed to level the PC assembly. Some users find it convenient to use two 1.5 ml tube racks as props.

When the gel is past the short plate, lay the syringe on top of IPC assembly until gel polymerization is complete. **Do not** remove the

luer taper from the precision caster base injection port, or the gel solution will drain out of the plates. Do not adjust the syringe plunger after the gel has been cast (**Figure 10.5**).

11. Insert the comb(s) between the plates to the desired depth.
    - If a sharkstooth comb is used, insert the flat edge of the comb no more than 5 mm past the short glass plate.
    - Clamp the comb(s) in place with three large metal binder clamps.
    - Alternatively, prior to injecting the gel solution, insert the corner of the comb to facilitate comb placement and insertion after gel casting.
12. Let the gel polymerize for 30-60 minutes.
    - After gel polymerization, remove the luer taper from the precision caster base. The syringe, tubing, and luer taper can be cleaned of any remaining polymerized gel.
    - Solution by rinsing with hot tap water, followed by a distilled water rinse.
13. Remove the precision caster base from the IPC assembly and clean the caster base and gasket of polymerized gel solution with tap water, followed by a distilled water rinse.

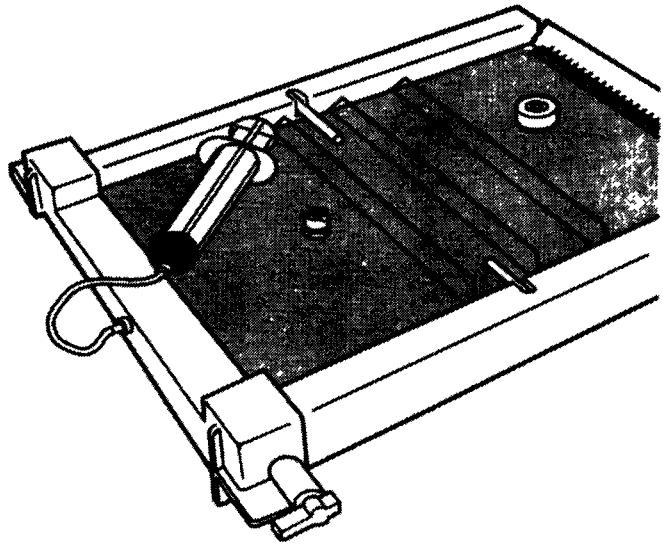

Figure 10.5 Syringe position for gel polymerization

Preparing for operation

1. Adhere a gel temperature indicator onto the outside of the outer plate, somewhere near the center, to monitor the gel temperature during electrophoresis.
   - Place the IPC assembly into the universal base, against the back wall, between the alignment tabs.
2. Insert the stabilizer bar.
   - The stabilizer bar should slide into place with a snug fit, locking the IPC to the base in a vertical position.
   - The heads of the screws on the stabilizer bar should push against the front wall of the base to press the IPC clamps against the back wall of the universal base.

Note: When first setting up your Sequi-Gen GT cell, adjust the screws on the stabilizer bar if the fit seems too loose or too tight (turning the screws counterclockwise makes the stabilizer bar fit more tightly). Too much pressure will make it difficult to insert and remove the stabilizer bar. Too little pressure will result in the stabilizer bar sliding in and out of position without pressing the IPC against the back wall of the base.

3. To avoid buffer spills and cell tipping accidents, adjust the leveling screws on the universal base, as necessary.
   - To insure that the unit will not tip over during electrophoresis, make sure the leveling feet threaded rods are at least 1 cm deep into the threaded boss of the base.
   - At this time, test whether the IPC assembly is properly aligned in the universal base by attaching the top and bottom safety covers. The IPC assembly may have to be shifted to the right or the left to properly attach the safety covers. After this final alignment is complete, remove the safety covers.
4. Fill the upper buffer chamber (the IPC) with running buffer (Ix TBE) using the flared portion of the panel as a fill spout.
   - The level of the buffer should be about 1 cm from the top of the fill spout at all times during the run.
   - Remove the comb(s) from between the glass plates.
   - Thoroughly rinse the resulting well(s) or gel front using a

syringe with a needle, or disposable plastic transfer pipette.

- If using a sharkstooth comb, insert the comb with the teeth facing the gel front. Lower the comb toward the gel surface until the teeth of the comb just touch the gel surface.

5. Fill the lower buffer chamber with 350-500 ml of the running buffer.

Caution: Do not fill the lower chamber with more than 500 ml of buffer. The lower buffer chamber holds the entire volume of the upper buffer chamber should a leak develop in the IPC. Buffer levels over 500 ml will not allow the entire volume of the upper buffer chamber to be contained in the universal base.

6. Attach the top and bottom safety covers and pre-electrophorese the gel at normal operating voltage or power, if desired, to increase the gel temperature.

    - Pre-electrophoresis prior to sample loading will create a uniform gel temperature and bring the gel temperature to the recommended run temperature. This will help eliminate any smile patterns from developing early in the run.

Note: Gel electrophoresis buffers can be heated to 50°C in a microwave before adding buffer into the upper buffer chamber. This will reduce the time needed to bring the gel to the appropriate run temperature before sample loading, and will greatly reduce pre-electrophoresis time.

- Warning: The upper buffer level may drop slightly due to evaporation as the system becomes warmer. Make sure that the upper chamber is always filled with buffer during electrophoresis. Do not allow the buffer level to drop below the level of the notched (shorter) IPC glass plate at any time during electrophoresis, as this may cause arcing and cell damage. Additionally, never allow the gel to exceed 60°C under any circumstance. This excessive heat may crack the plates or cause the IPC/glass bond to deteriorate.

Loading the Gel

1. Turn off the power supply, and remove the top safety cover.

    - Rinse the well(s) with a syringe with needle, or disposable plastic transfer pipette, (to remove urea) before applying the samples to the gel.

2. Load samples on the gel.
   - Samples may be applied with a 5 ~l Hamilton syringe, or a pipette fitted with gel loading tips.
   - Syringe loading requires rinsing the needle between samples.
   - Be sure to reconnect the upper safety cover before turning on the power supply.

Note: Sample loading is the key to high resolution gels.

- Rinse wells thoroughly before sample loading begins.
- Deposit samples directly on the gel surface.
- Electrophoreses the samples into the gel soon after loading (every 4 lane sets) to reduce sample diffusion and enhance band resolution.

Gel electrophoresis

1. . Make sure both safety covers are in place.
   - Apply the voltage by pressing the Start or Run button on the power supply.
   - Verify that current is flowing (note bubbles forming at the cathode wire in the IPC), and that all electrical connections are solid.

Running the gel with constant power (watts) will result in a constant gel temperature during the run and reproducible gel electrophoresis.

Power conditions for DNA sequencing gels are usually dictated by gel running temperature. Run sequencing gelsat 50°C for best results. Refer to the **Table 10.3** for typical power (watts) settings that result in 50°C runs. These settings are only guidelines-optimal settings for gels should be determined empirically. Use a temperature indicator (one is included with this unit) to monitor running temperatures. If the temperature goes above 55°C, reduce the power output of the supply. Alternatively, use a power supply with temperature control functions (Power Pac 3000 with temperature probe) to monitor and control gel temperature.

Table 10. 3 Approximate Power (Watts) Setting for Operation Sequi-Gen Cells

Sequi-Gen GT Cell Size	Gel Thickness	Recommended Power Setting
21 x 40 cm	0.25 mm	35 - 45 W
21 x 40 cm	0.40 mm	40 - 50 W
21 x 40 cm	0.75 mm	45 - 55 W
21 x 40 cm	0.25 - 0.75 mm wedge	45 - 55 W
21 x 40 cm	0.4 - 1.2 mm wedge	45 - 55 W
21 x 50 cm	0.25 mm	45 - 55 W
21 x 50 cm	0.40 mm	50 - 60 W
21 x 50 cm	0.75 mm	55 - 65 W
21 x 50 cm	0.25 - 0.75 mm wedge	55 - 65 W
21 x 50 cm	0.4 - 1.2 mm wedge	55 - 65 W
38 x 30 cm	0.25 mm	70 - 75 W
38 x 30 cm	0.40 mm	70 - 75 W
38 x 30 cm	0.75 mm	70 - 75 W
38 x 30 cm	0.25 - 0.75 mm wedge	70 - 75 W
38 x 30 cm	0.4 - 1.2 mm wedge	70 - 75 W
38 x 50 cm	0.25 mm	70 - 80 W
38 x 50 cm	0.40 mm	75 - 85 W
38 x 50 cm	0.75 mm	75 - 85 W
38 x 50 cm	0.25 - 0.75 mm wedge	75 - 85 W
38 x 50 cm	0.4 - 1.2 mm wedge	75 - 85 W

Important: Never allow the gel temperature to exceed 60^0C. Severe damage to the glass or adhesive bond may result.

Caution: Periodically check the level of the upper buffer to make sure that it is at least 1 cm above the short glass plate.

2. Continue gel electrophoresis until the desired fragment size separation is achieved. Typically, gel electrophoresis times are monitored by observing the dye front mobility of either the bromophenol blue ("fast blue") or Xylene-cyanol ("slow blue") during the course of electrophoresis. Fragment and dye front mobility as a function of polyacrylamide percentage are shown in **Table 10.4** below, and should be used as a guide for gel electrophoresis monitoring.

Table 10.4 Migration of single-stranded DNA in denaturing polyacrylamide gels in relation to dye marker gel migration*

Polyacrylamide gel percentage	Bromophenol blue	Xylene cyanol
5 %	35 bases	130 bases
6 %	26 bases	106bases
8 %	19 bases	75 bases
10 %	12 bases	55 bases

Disassembly

The disassembling of the equipment is shown in **Figure 10.6**

1. When the desired dye front mobility has been achieved, turn off the power supply, and remove both safety covers.
   - The upper buffer chamber can be partially emptied by inserting the drain port connector(and any attached tubing) into the drain port on the IPC. A "click" will be heard when the drain port/tubing connector has been properly inserted.
   - Buffer will begin to drain from the IPC immediately after the connector is inserted into the drain port.

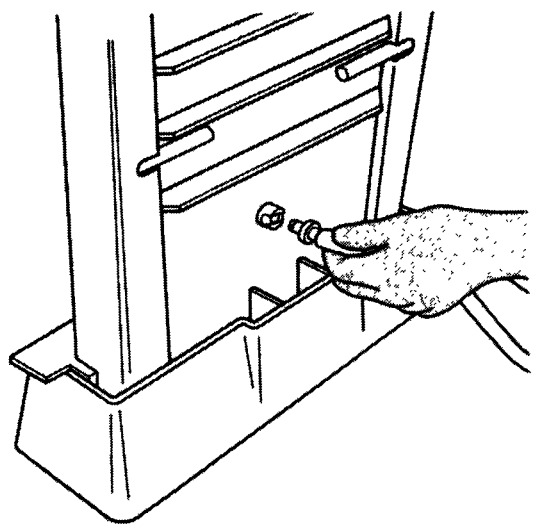

Figure 10.6 Inserting the drain port connector for upper buffer chamber drainage.

2. Carefully pour the remaining upper buffer out of the IPC assembly into a sink. Slowly and carefully pour the lower buffer contained in the universal base into the appropriate sink.
3. Remove the clamp from the IPC assembly by first pulling the levers away from the IPC then sliding the clamps off the IPC assembly.
4. When the gel is secured onto one glass plate, carefully place a piece of filter paper onto the gel surface.
5. The gel is now ready for drying, autoradiography, and interpretation of the results.

Protocols

Samples, preparation and electrophoresis

Samples for combined SSCP are usually PCR products amplified under standard laboratory conditions. No special preparatory steps are necessary, the only requirement being that the amplification should be strong and specific. A minimum of 10 µl of product is required for combined SSCP electrophoresis. The PCR products should ideally be less than 300 bp, although, good results can be obtained with fragments up to 500 bp. Large fragments can be analysed by cutting at internal restriction sites with appropriate restriction endonuleases. It can be advantageous to run restricted and unrestricted DNA simultaneously, provided the complete fragment is below 500 bp, since the sensitivity of mutation detection can be compromised close to the restriction site.

Endonuclease digestion

1. When restriction digestion is required, place 60% of the reaction mixture in a separate micro centrifugation tube and digest according to the suppliers instructions.
2. Add an equal volume of formamide loading buffer with sample and mix well.
3. Pre-load the gel with 1-2 µl of the sample. Electrophoresis MUST NOT be commenced at this stage.
4. Pace the remainder of the samples on a heated block or thermal cycler set at 95^0C for 3 minutes and snap cool in a bath of crushed ice to prevent reannealing of the single stranded DNA.
5. Re-load the wells with 6-10 µl of each sample using thin gel loading tips (the optimum volume will depend on the size of the

wells and the amplification efficiency).

6. Carry out the electrophoresis at 180-500V (depending on fragment size and run time). Double stranded DNA and xylene cyanol co-migrate but there can be considerable sequence dependent variability. Optimum electrophoresis times are best determined empirically.

DNA amplification

If a radioisotope is to be incorporated during the PCR reaction, at either end -labeled primers or deoxynucleotides may be used. For radiolabeled PCR-SSCP amplification is performed in total volume of 10 - 20µl as shown in **Table 10.5**.

Table 10.5 PCR reaction mix for mutation detection in exon 4 of ApoB gene

Reagents	1 sample (10µl)	5 samples (10x5µl)
10 x PCR buffer	1.0µl	5.0µl
$MgCl_2$ (2.0mm)	0.8µl	4.0µl
dNTPs mix*	0.8µl	4.0µl
F-Primer (10pm/µl)	0.1µl	0.5µl
R-Primer (10pm/µl)	0.1µl	0.5µl
Taq (3U/ul)	0.5µl	2.5µl
^{32}PdCTP (300Ci/mmol)	0.1µl	0.5µl
ddH_2O	4.5µl	22.5µl
DMSO (100%)	0.5µl	2.5µl
Template DNA (50 -100ng)	2µl	2x5µl

Note:

1. Synthesize primers 21 - 30 nt in length for products of 200 - 350 bp.
2. Use standard PCR conditions; however, the Tm has to be calculated from the specific primer pair.

* dNTP mix (final concentration): dATP 2.5mM; dTTP 2.5mM; dGTP 2.5mM; dCTP 1.25mM.

DNA denaturation

Complete denaturation of the amplified sample prior to loading it onto the gel for electrophoresis is essential for achieving optimal sensitivity. To ensure complete denaturation, the amplified sample can be diluted (1:1) in a stop solution (95% formamide, 10mM NaOH, 0.1% bromophenol blue, 0.1% Xylene cyanal).

Denaturation is accomplished by heating the diluted samples at 90 - 96^0C for 2-6 min. Samples are immediately placed on ice for 2-5 min. Then 1-3µl of samples loaded onto the electrophoresis gel. Include non-denatured controls. Run gel in 0.5X TBE buffer.

Electrophoresis

Other parameters which affect the sensitivity of SSCP analysis are related to the gel composition and electrophoresis conditions.

Recent reports have shown that a reduced concentration of cross-linker to acrylamide (49:1 to 99:1 acrylamide; N, N' -methylene bisacrylamide) improves separation of single stranded DNA conformers other electrophoretic conditions are temperature, glycerol, acrylamide concentration, and ionic strength.

1. **Equipment required**

   Cooling Water bath (e.g. Grant)
   Biorad Protean Gel System
   Photographic trays for silver staining
   Gel dryer

2. **Preparation of poly acrylamide gels**

   Prepare stock solutions
   50% acrylamide (49:1)

Acrylamide	49g
N- N'- methylene bisacrylamide	1g

Add H_2O to 100ml

Precaution: Acrylamide is highly toxic, use gloves and a mask.

3. Prepare 5x TBE buffer

Buffer	working Solution	concentrated stock solution (/liter)
Tris -borate (TBE)	0.089M Tris-borate 0.089M Boric acid 0.002M EDTA	54g Tris base 5X 27.5 g boric acid 20ml 0.5M EDTA (pH 8.0)

4. **5% Glycerol**

Glycerol	5ml
H_2O	95ml

5. **10% Ammonium persulfate**

Ammonium Persulfate	-	10g
H_2O	-	100ml

The solution may be stored at 4^0C for periods of up to one week.

6. **Formamide loading buffer**

Formamide	10ml
0.5M EDTA	200 µl
Xylene cyanol	1.5 mg
Bromophenol blue	3 mg

7. **TEMED (N,N,N^1,N^1-tetramethylethylenediamine)**

8. **Gel preparation**

8% SSCP GEL

To make 50 ml of gel

1. Switch on the cooling water bath set to 8°C.
2. Place 8 ml of deionised 50% 49:1 stock acrylamide solution into a small beaker.
3. Add 25 ml of 1x TBE buffer followed by 14.5 ml distilled water and 3 ml of 5% glycerol.
4. Mix well then add 25 µl of TEMED followed by 200 µl of amonium-persulphate mix well.
5. Draw the mixture carefully into a 100 ml disposable syringe without introducing air bubbles.
6. Place the assembled glass plates according to the Biorad instruction manual and casting tray vertically on the bench and carefully fill with the acrylamide solution from a corner.
7. Insert an appropriate comb, taking care not to trap any air bubbles.
8. Leave the gel for 40 minutes and monitor for leakages. Care should be taken when inserting the comb into the gel to avoid gel solution spurting out into your eyes.
9. When the gel has set, allow to precool by placing in the gel tank and adding TBE stored in the refrigerator.

The composition for other gels is shown below in **Table 10.6.**

9. Polyacrylamide gels

Table 10.6 Polyacrylamide gel preparation

Reagents	Polyacrylamide gel (%)		
	8% (100 -500 bp)	10% (60 - 400 bp)	12% (40-200bp)
50% (49:1) acrylamide	8.0 ml	10.0ml	12.0ml
1X TBE	25.0 ml	25.0ml	25.0ml
5% glycerol	2.5 ml	2.5ml	2.5ml
Mill Q water	14.5 ml	12.5ml	10.5ml
APS	200 µl	200µl	200µl
TEMED	25 µl	25µl	25µl
Total	**50 ml**	**50ml**	**50ml**

10. Electrophoretic condition

1. Fragment size; 150 - 200bp
- 6 watts
- 10 - 12 hours
- Room temperature

2. Fragment size: >200bp
- 8 watts
- 10 - 16 hrs
- Room temperature

Exposure

(a) Dry gel can be exposed at -70^{0}C for 2 hours or at room temperature for 16 - 18 hrs.

(b) The best conditions for gels to run at room temperature are:

1. The range of acrylamide concentration (5-10%) depends upon the size of PCR product at an acrylamide to bis-acrylamide ratio of 49:1. The gel should be prepared in 0.5 X TBE (Tris Base, Boric Acid and EDTA).

   For same DNA sequences, the addition of 5 - 10% glycerol to the gel can increase the sensitivity of the SSCP analysis.
2. For the best heat dissipation, thin gels of 0.4mm or less should be used. A constant temperature of 20 - 25^{0}C is essential for obtaining reproducible SSCP analysis and sequencing apparatus with aluminium plates, water Jacketed cooling is the best for heat dissipation.
3. Run the SSCP gel in 0.5 XTBE (pH 8.3).
4. Prerun the gel for 10 min at 8 to 20W.
5. Load 1- 3µl of the denatured samples onto the gel for sharp bands, use a shark tooth comb with 5mm teeth.
6. The power supply should be set at a constant to maintain

even heat dissipation. For good heat dissipation, 8-20 W sustain a constant temperature of 20-25^{0}C. The run time for a 200bp fragment at (8W) should be 14-16 hrs. Marker dyes in the stop solution can be used to visualize and monitor movement of the DNA fragments.

7. Once electrophoresis is complete, transfer the gels to 3MM whatman paper, dry and expose to X-ray film for 2 - 3 hr, if 32p is used and longer for lower specific activity labels.

Silver staining of polyacrylamide gels

Slide the spacers from between the glass plates and gently separate the plates apart, avoid the use of metal implements which may scratch or chip the glass. Ensure that the gel is attached to the lower plate before removing the upper plate. Mark the orientation of the gel by cutting the corner adjacent to lane number 1 and place the glass plate with gel attached into a staining tray. Very carefully lift the bottom edge of the gel and fold over a 10 cm length, repeat this action until the gel is completely rolled up. The gel can now be easily dislodged from the plate into the staining tray and stained.

Guide to interpretation

1. Single stranded DNA (ssDNA) has a much lower mobility than double stranded DNA (dsDNA) in the type of native acrylamide gels used for SSCP/HA. Further, the band migrates the greater the potential for resolving small mobility shifts, but the limiting factor in any combined SSCP/HA gel is the need to retain dsDNA on the gel. As a rough guide, on an 8% (49:1) gel, xylene cyanol migrates at approximately 180 bp.
2. The silver staining technique tends to stain ssDNA, which forms the SSCPs, a brown/orange shade. The dsDNA, which can form heteroduplexes, is stained dark grey/black.
3. SSCPs from a homozygote typically give rise to two distinct migrating bands corresponding to the two complementary DNA strands. Often more than two SSCP bands occur, some of which may stain more weakly than others. Providing the PCR amplification has been clean and these specific bands are most likely to be due to alternative stable configurations.
4. Rarely, there is a discrete SSCP band formed by one or both ssDNA strands. If this occurs a faint, in track, smear is usually observed. This corresponds to the fragment adopting a whole

series of stable configurations each of slightly different mobility. The presence of a mutation often destroys this delicate balance, creating a SSCP band of typical appearance.

5. Occasionally, a fragment produces a single SSCP band. This occurrence is due to the strand and its complement having coincidentally similar mobilities and should not compromise sensitivity.
6. When a genuine sequence difference gives rise to a SSCP the intensity of staining of each individual band will be reduced relative to other samples of the same concentration, as the same quantity of DNA is distributed over a greater gel volume. This reduction in intensity can be used to justify the exclusion of artefacts which may have originated from spurious amplification.
7. Excess primers reannealing to the ssDNA after gel loading can cause a third set of bands intermediate in mobility to the ssDNA and dsDNA. Their pattern of migration usually shadows that of the corresponding SSCPs although they tend to stain, like dsDNA, grey/black. They can serve as a useful adjunct to interpretation.

Troubleshooting

1. Formaldehyde, which is used in silver staining solution C, is usually supplied as a 37% solution (formalin) because pure formaldehyde is a gas at room temperature. Remember to take this into account when calculating volumes, since the formaldehyde concentration is critical.
2. The presence of only small quantities of contaminating salt in dH_2O can cause $AgNO_3$ solutions to change to a milky white appearance. If this occurs, the standard of staining will be poor. The most common reason for this is the exhaustion of the ion exchange cartridge used for desalting the mains water supply, replacing the cartridge almost always cures the problem.
3. Holding gels in place whilst pouring silver staining solution away may cause the gels to become marked. This appears to be due to the talc used in some brands of latex gloves. Microtouch gloves seem to be satisfactory. The problem can be avoided by holding the gel in place with an old piece of X-ray film.
4. If a gel in silver staining solution B (0.1% $AgNO_3$) comes into contact with a strong salt or alkaline solution, it may give a milky white precipitate. Attempting to continue under these

circumstances is futile. The gel can often be saved by washing twice with dH_2O then immersing in a 2.5% ammonia solution followed by two further rinses in dH_2O. Silver staining can then be restarted with solution A.

5. Silver stained gels can be heat sealed between layers of plastic for temporary storage. Care must be taken while handling gels stored in this way since they are delicate and are especially susceptible to pressure damage.
6. Leakage from the upper buffer chamber can be a problem with the Protean system. Dampening the seal on the upper chamber can help.
7. If solution D is made up incorrectly then any bands present on the gel may fade or disappear completely duringthe drying step.
8. Gels containing more than 8.5% acrylamide are prone to cracking as they are dried down. To minimise this problem they should be dried at room temperature.
9. Occasionally, a white powdery deposit may build up on the surface of the dried gels. This can be removed by spraying with any household furniture polish and rubbing with a soft cloth.
10. Replicates of gels may be markedly different in appearance. This can usually be traced to gel and environmental variability. Take great care in measuring reagents before pouring gels. Since buffer and gel temperatures are such important parameters, it is best to use a strict routine when setting up SSCP/HA experiments. Pour the gels at a fixed time of the day, similarly, place the gel and electrophoresis buffer in the cold room at the same time and load the gels at the same time of day for every experiment. Your results will then become much more reproducible, although, a certain degree of variability should be expected.

SSCP procedure is summarised below

The procedure used during the development of SSCP is digestion of genomic DNA with restriction endonucleases denaturation in an alkaline (basic) solution electrophoresis on a neutral polyacrylamide gel transfer to a nylon membrane hybridization with either DNA fragments or more clearly with RNA copies synthesized on each strand as probes. Since then, more convenient procedures have been developed, taking into account other molecular techniques, although sometimes, it is simpler to amplify the double strand and then

denature it into single strands instead of trying to find suitable primers for the below PCR method, if the targeted sequence is unknown.

Most experiments involving SSCP are designed to evaluate polymorphisms at single loci and compare the results from different individuals.

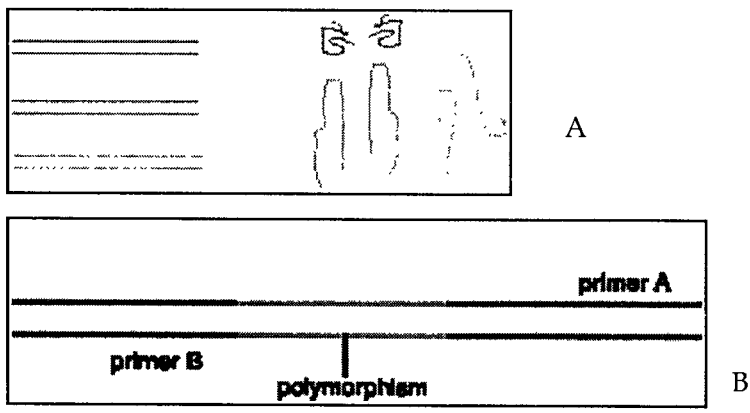

Figure 10.7 The three equal-length double-stranded DNA fragments are shown with the corresponding single-stranded structures, the red fragment folding into the smallest molecule and the green the largest (Panel A). The desired polymorphism is selected with PCR primers; primer A is in excess to amplify only a single strand (Panel B). Both the double-stranded and single-stranded fragments are run through gel electrophoresis (Panel C). If not for the color labels, there would be no distinction between the double-stranded fragments. The single-stranded fragments, however, show considerable variation in mobility; the small red molecule migrates more quickly through the gel than either the blue or the large green molecule. Using this SSCP result, it becomes clear that the different lanes (red, blue, or green) contain strands with different sequences; the more far apart the bands, the less similar to the nucleotide sequences.

Procedure as illustrated in Figure 10.7

1. A specific pair of PCR primers (forward and reverse) is used to amplify the desired DNA fragments from individuals.
2. Single-stranded DNA is produced by asymmetric PCR: The primer on one side of the fragment is greatly in excess over the other primer. After the low-concentration, primer supply is exhausted, continued PCR produces only the target single strand.
3. The mobilities of the single stranded fragments are compared

by electrophoresis on a neutral polyacrylamide gel.

4. Bands are detected by radioactive labeling or (more often) silver staining, and the pattern is interpreted.

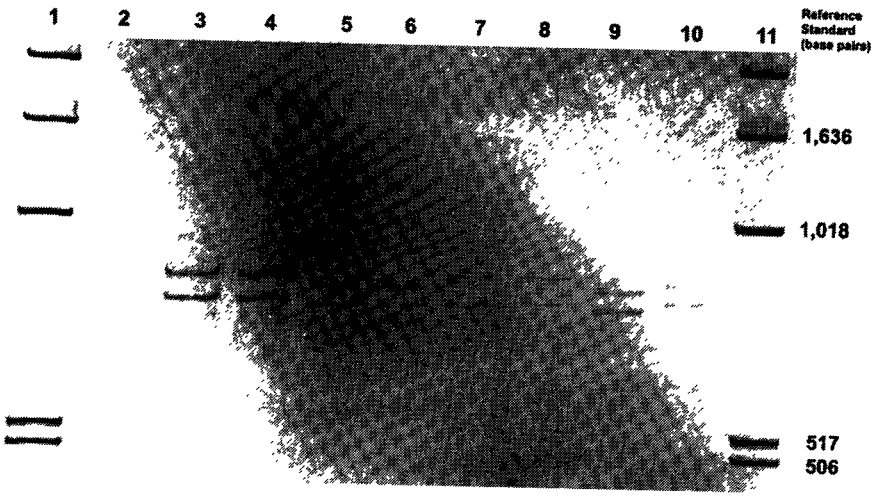

Figure 10.8: Sample SSCP Gel result and interpretation. DNA was isolated and amplified from blood sample of CAD patient. SCCP analysis of the DNA shows multiple haplotypes, or sets of alleles usually inherited as a unit. Lanes 3 and 4 were identical haplotypes from two individuals. The difference in band migration in adjacent lanes is associated with the number of nucleotide differences.

Limitations of SSCP and considerations

Single-stranded DNA mobility is dependent on temperature. For best results, gel electrophoresis must be run in a constant temperature.

Sensitivity of SSCP is affected by pH. Double-stranded DNA fragments are usually denatured by exposure to basic conditions: a high pH. It was found that adding glycerol to the polyacrylamide gel lowers the pH of the electrophoresis buffer—more specifically, the Tris-borate buffer—and the result is increased SSCP sensitivity and clearer data.

Fragment length also affects SSCP analysis. For optimal results, DNA fragment size should fall within the range of 150 to 300 bp, although, SSCP analysis of RNA allows for a larger fragment size. The presence of glycerol in the gel may also allow a larger DNA fragment size at acceptable sensitivity.

Under optimal conditions, approximately, 80 to 90% of the potential base exchanges are detectable by SSCP (Wagner, 2002).

If the specific nucleotide responsible for the mobility difference is known, a similar technique called Single Nucleotide Polymorphism (SNP) may be applied.

Chapter 11

Nucleic Acid Blotting Techniques

This chapter deals with basic concepts and techniques in nucleic acid blotting. Many of the techniques involved with Southern blotting and Northern blotting are similar. Negatively charged, purified nucleic acids from prokaryotic or eukaryotic cells are separated according to size by electrophoresis through an agarose gel matrix. The RNA or denatured DNA is subsequently transferred and immobilized onto a membrane composed of nitrocellulose or nylon. The nucleic acids on the membrane are then hybridized to a specific labeled "probe," which consists of homologous single-stranded nucleic acids that carry molecules, allowing detection and visualization of the hybridized probe. Hybridization between the immobilized nucleic acids and labeled probe allows detection of specific DNA or RNA sequences within a complex mixture of DNA or RNA. The specific method of detection and visualization is dependent on the nature of the labeled probe; radioactive probes enable auto radiographic detection, and probes labeled with enzymes facilitate chemiluminescent or colorimetric detection. Nucleic acid blotting yields valuable information pertaining to gene integrity and copy number (Southern blot) and provides a means of analyzing gene.

Southern

Southern blots are used in gene discovery and mapping, gene incorporation, evolution studies, and diagnostics. Dr. E.M. Southern introduced the process in 1975 as a method of size separation of restriction digested genomic DNA in gels. After denaturing the DNA, the DNA is transferred to a support membrane where the DNA fragments can then be identified by hybridization with labeled gene-specific probes. This same technique is used for analysis of plasmid digests and PCR fragments with labeled gene-specific probes.

Northern

Northern blots, developed for RNA methodologies, are typically used for characterizing one or more specific mRNA transcripts within multiple RNA samples. RNA, usually total RNA, is denatured, size separated by electrophoresis, and transferred to nylon membrane. The blot is then hybridized with target-specific labeled DNA or RNA probes. Northerns may be used to quantitative analysis of a target gene to determine mRNA transcript size, to determine the presence of alternative splice variants of the gene, and to identify the presence of closely related species.

Gene Arrays

Gene arrays are used to rapidly compare the gene expression patterns of two or more samples. They are used for DNA sequencing, genetic analysis and drug discovery. The arrays consist of hundreds to thousands of gene-specific nucleic acids, most often cDNA clones, spotted on nylon membrane. As an example, after activators or inhibitors are added to cells, the levels of expressed mRNA from the samples are converted to cDNA by reverse transcription. The cDNA in turn is used as a probe for the gene array. The signal from any spot in the array is representative of the abundance of mRNA present in the probe population. Therefore, comparisons between different samples can be accomplished.

Dot Blots & Slot Blots

DNA or RNA populations are transferred to a membrane using a vacuum manifold system. This method is used for a rapid and sensitive screen for quantitating and comparing multiple samples.

Nucleic Acid Blotting

General principles

1. DNA can be denatured into single stranded molecules.
2. Transferred and immobilized to a solid membrane.
3. Duplexes (i.e., double-stranded molecules) can be reformed *in vitro* from complementary nucleic acids.
4. Hybridization with a homologous 'probe' can be used to detect specific nucleic acid molecules or fragments.
5. Commonly carried out in the 'blotting' format. (After electrophoresis, nucleic acids are transferred to a positively

charged nylon membrane.)

(i) Southern Blotting (digested DNA is electrophorased and transferred.)

(ii) Northern Blotting (RNA is electrophorased and transferred.)

(iii) Dot/Slot Blots (no electrophoresis, amplified product is directly blotting on the positively charged nylon membrane.)

All these steps are illustrated in **Figure 11.1.**

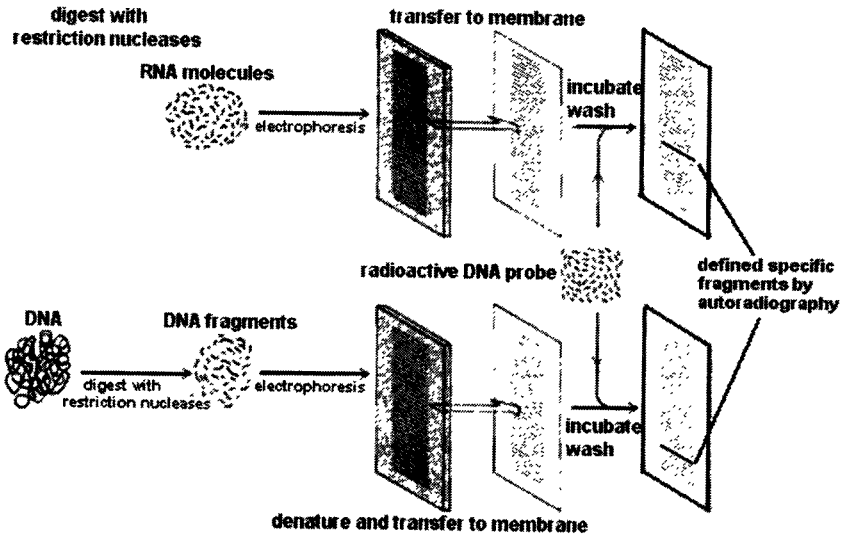

Figure 11.1: General scheme of nucleic acid blotting

Generic blotting protocol

1. Digest DNA or isolate RNA.
2. Electrophoresis denaturing gels for RNA.
3. Depurinate (optional) treatment with dilute HCl will cause random breaks and promote a more efficient transfer of very large DNA molecules.
4. Denature dsDNA treat with alkali (NaOH) to separate strands.
5. Transfer to membrane capillary action (original method, slow) vacuum apparatus (rapid).
6. Fix nucleic acid to membrane heat (80°C)
   UV crosslink
7. Prehybridize incubate with non-specific DNA to 'block' free sites on the membrane.

8. Incubate with probe generally carried out at lower stringency.
9. Wash generally carried out at higher stringency.
10. Detect (autoradiography or develop with substrates).

Factors affecting hybridization

Temperature, ionic strength (Na+ concentration) chaotropic agents (% formamide) probe length probe mismatch % GC content.

Stringency and melting temperature

1. Melting temperature (Tm) is the temperature at which a particular DNA will separate into single strands.
2. Stringency refers to the relative conditions of the hybridization as compared to the Tm.
3. It is related to the homology between probe and target.
4. Effective Tm is estimated from the formula:
   (i) For cloned DNA fragment
   $Tm = 81.5^0 + 16.6\log [Na+] + 0.41(\%GC) - 0.65(\%formamide) - 1.4(\%mismatch)$
   (ii) For synthetic oligonucleotides
   $Tm = 2(A + T) + 4(C + G)$

Labeling DNA probes

Radioactive probes (Figure 11.2 A and B)

1. Random priming (cloned DNA fragments).
2. T4 polynucleotide kinase (synthetic oligonucleotides).

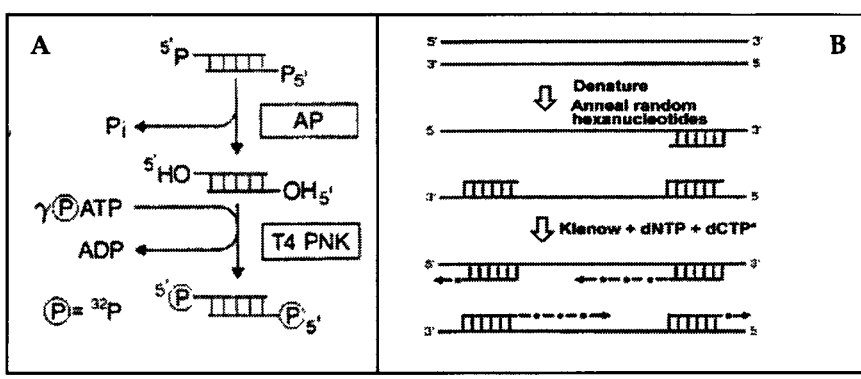

Figure 11.2 (A and B): Radioactive Probe labeling method for nucleic acid blotting.

Non-radioactive probes (enzyme-linked detection)

1. Biotin-Avidin (conjugated with enzyme)- **Figure 11.3** (A and B)
2. Digoxigenin-11-dUTP + enzyme conjugated antibody
3. Direct cross-linking of enzyme (HRP)
4. Often detected with substrates that emitts light.

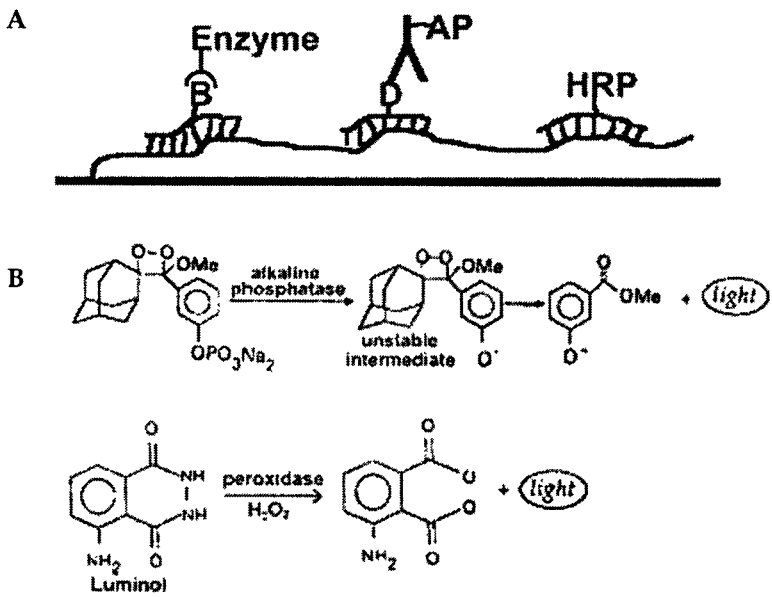

Figure 11.3 (A and B): Enzyme-linked detection method for nucleic acid blotting

Dot blotting of isolated DNA and PCR products

Dot Blot technique is an important blotting technique to detect the known mutations or sequences based on ASOP or SSOP. This technique requires amplified or some time genomic DNA directly on the membrane without digestion and capillary transfer procedure as required in Southern Blotting. DNA is blotted onto nitrocellulose or Nylon filters either by hand orby using a manifold blotting apparatus (**Figure 11.4**).

When the sample is human genomic DNA and the probe recognizes a single-copy gene, a minimum of 10 μg DNA should be loaded per sample. If the target sequence is present in more copies or in case of PCR products, the amount can vary.

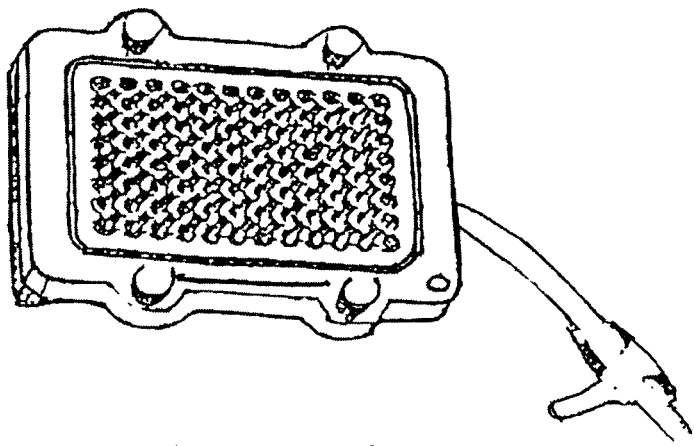

Figure 11.4: Dot Blot apparatus

Dot Blot procedure:

1. Prior to blotting, it is necessary to denature the DNA by boiling. This treatment partially depurinates the DNA, so that, subsequent alkali treatment causes the phosphodiester bond to break at the site of depurination. This is particularly important for samples containing supercoiled DNA, which denatures very rapidly.
2. When the samples are dotted onto the filter with an automatic pipette, it is important not to press on the filter as this may cause background signals.
3. Nitrocellulose membrane must be wetted in water and equilibrated in 20X SSC. Samples are denatured by boiling and an equal volume of 20X SSC is added to the samples before application.
4. The use of high salt solution is not recommended with positively charged nylon membranes; instead 0.4 N-NaOH may be used.

Dot spoting of amplified DNA (Protocol)

Reagents required

10X SSPE

1. NaCl 1.5 M
2. Sodium Phosphate 0.1 M
3. EDTA 10mM
4. pH should be 7.4.
5. Prepared from stock solution of 30 X SSPE (autoclaved).

2X SSC

1. NaCl 0.3 M
2. Sodium Citrate 30 mM
3. Prepared from stock solution of 20X SSC
4. Place the filter on a filter paper (Whatman 3 MM) to remove excess fluid.

Method

1. Amplify the loci for which alleles has to be determined by PCR.
2. The concentration of amplified DNA should be monitored by gel electrophoresis.
3. Denature the PCR samples.
4. Equal amount of amplified DNA should be spotted on to the nylon membrane filter (e.g. Hybond N+ from Amersham).
5. Records of each dot blot should be properly maintained.
6. Use 10 to 50 ng (ordinary 30 ng) of amplified DNA per spot. (i.e. approximately 2-5 µl of PCR product).
7. Make enough number of the replicate filters (depending on the number of sequence-specific Oligonucleotide probes).
8. Prewet the nylon filter in autoclaved dH_2O for 10 minutes.
9. Soak the nylon filter in 10X SSPE (or 2X SSC) for 15 min.
10. Arrange wetted filter in blotting apparatus.
11. Spot 2 to 5 µl of amplified denatured DNA onto the filter according to the order of spotting (Record this step).
12. Dry the filter at room temperature.
13. Rewet the filter with 0.4N NaOH for 10 minutes on alkali wet towel (blotting paper sheets).
14. Keep the side of the filter carrying the blotted DNA on upper side.
15. Soak the filter in 10X SSPE for 10 minutes wet towel (blotting paper sheets).
16. Remove and rinse the membrane with 2x SSC for 2 minutes.
17. Dry the filter to completion at 80^0C for 1 hr (Baking).
18. Alternatively, dry the filter at room temperature and expose it to 254 nm-UV lamp for 5 min.

Alternative method for dot-spot

1. To make 5 replicate filters, about 15 µl of the amplified sample is mixed with 250 µl of 0.4 N NaOH, 25 mM EDTA and incubate for 10 min at room temperature.
2. Transfer the sample on ice.
3. Load 50 µl of the sample into the wells of "Dot-Blotter" manifolds containing a nylon filter that is prewetted in distilled water.

NOTE: Avoid any bubbles between the sample and the filter.

4. Vaccuum-pull the samples through the filter.
5. Apply 100 µl of TE (10 mM Tris-HCl. 1 mM EDTA, pH 8.0) to each well to wash the sample.
6. During the continuous vaccuum, open the manifold and remove the filter
7. Dry the filter to completion at 80^0C for 1 h. (Backing). Alternatively, expose the filter to 254 nm UV lamp for 5 min.

Probe Labelling

Labelling of SSO with ^{32} P

Reagents required:

1. 10X Kinase buffer

Tris-HCl (pH7.6)	0.5 M
$MgCl_2$	0.1 M
DTT	50mM

To make 10p mole oligonulceotide (SSO)

1. Take 2.5 µl of 10X Kinase buffer.
2. Add 60 to 100 µ Ci (sp. act. > 4,000 Ci/mmol) of [g^{32} P] ATP Add ddH_2O up to 25 µl.
3. Incubate at 37^0 C for 30 minutes.
4. Add 1 µl of 0.5 M EDTA (pH 8.0) to stop the reaction.
5. The labeled oligonucleotides can be used directly or after elimination of unincorporated radioactive nucleotides by passage through a disposable column (e.g. Qiagen tip).

Labelling of SSO with dig-ddUTP (kit from Boehringer)

Reagents required

10X tailing buffer

Na-Cacodylate	1.4 mM
Tris-HCl (pH7.2)	300 mM
$CoCl_2$	10 mM

To make 50 pmoles of probe (SSO)

1. Take 2.5 µl of 10 x tailing buffer
2. Add 1 µl of 1 mM dig-11-ddUTP
3. Add dH_2O up to 25 µl
4. Add 50 units of terminal deoxynucleotidyl transferase (1 µl).
5. Incubate at 37^0 C for 30 min.
6. Use labelled SSO directly as probe.

Note: *dig- ddUTP labelled probe is stable at -20^0 C.*

Labelling of SSO with biotin

When synthesized on the DNA-synthesizer, SSOs could be labeled with a biotin molecule at their 5' prime site.

Hybridization

Method with Tetra Methyl Ammonium Chloride (TMAC)

Solutions required

1. 30X SSPE

NaCl	4.5 M
NaH_2PO_4	0.3 M
EDTA	30 mM

Adjust pH with NaOH to 7.4.

2. 20X SSC

NaCl	3 M
Sodium citrate	0.3 M

3. 50X Denhardt's solution

1. Prepare 100 ml of 1% PVP 40 (Polyvinylpirolidone) and1% Ficoll 400.
2. Autoclave it for 10 min. at 120^0 C and cool it to room temperature.
3. Add 2gm of BSA (Fraction V. Boehringer) and sterile ddH2O to make up the volume to 200 ml of the solution.
4. Store in aliquots at -20^0 C until use.

4. TMAC solution

Tris HCl (pH 8.0)	50 mM
Tetramethylammonium chloride (TMAC)	30 mM
EDTA	2 mM
SDS	0.1 %

The use of TMAC facilitates hybridization and washing independently of the GC content of the probe.

5. Stock solutions:

Tris-HCl (pH 8.0)	1 M
Tetramethylammonium chloride	5 M
EDTA (pH 8.0)	0.5 M
SDS	10%

6. Herring sperm DNA (Boehringer)

1. Dissolved in 10 mM Tris-HCl (pH 8.0), 1mM EDTA, 0.1 M NaCl to a concentration of 10 mg/ml.
2. Sonicate the solution
3. Heat at 95^0 C for 5 minutes and cool rapidly on ice, just prior to use.

7. Hybridization Buffer

Tris-HCl (pH 8.0)	50 mM
Tetramethylammonium chloride	3.0 M
EDTA (pH 8.0)	2 mM
Denhardt's solution	5 X
SDS	0.1%
Heat-denatured herring sperm DNA	100 ug/ml

Hybridization procedure

1. Place the nylon membrane in a 50 ml Falcon tube with 10 ml of hybridization buffer. Incubate at 54^0C for at least 30 minutes.
2. Add 30 to 60 ng (5 to 10 pmole) of ^{32}P or dig-ddUTP labelled oligonulceotide probe.
3. Incubate for at least 1h to 16h at 54^0 C with constant agitation. Wash with constant gentle agitation.
4. Rinse the filter in 100 ml (per filter) of 2X SSPE or 2X SSC, 0.1% SDS at room temperature for 10 minutes, twice to remove excess probe.
5. Wash the filter in 100 ml of pre-heated TMAC solution for 30 minutes at appropriate temperature.
6. Several filters may be washed at one time. For ten filters, about 500 ml of the washing solutions may be required.

7. The temperature used for hybridization and washing depends on the length of oligonucleotides as shown in **Table 11.1.**

Table 11.1: Effect of size of probe on temperatures of hybridization and washing

Size of the probe (SSO)	Temperature for hybridization	Temperature for washing
18 nucleotide	54^{0}C	59^{0}C
19 nucleotide	55^{0}C	59-60^{0}C
20 nucleotide	56^{0}C	60^{0}C

Note: *The hybridization solution may be stored at -20^0 C and can be reused at least two times.*

Alternative Method without TMAC

Solution required:

Hybridization Buffer

1. 6X SSPE
2. 5X Denhardt's solution
3. 0.5 % SDS
4. 100 µg/ml denatured herring sperm DNA

Hybridization Procedure

1. Prehybridize with 10 ml of hybridization buffer at 42^0 C for at least 1hr.
2. Add end labeled sequence specific oligonucleotide (SSO) (0.5 pmoles per ml) and incubate at 42^0 C for 2hr to 16 hr with constant agitation.

Washing

1. Rinse the membrane in 100 ml of 2X SSPE, 0.1% SDS at room temperature for 10 min., two times.
2. Rinse the membrane in 100 ml 6X SSPE, 0.1% SDS below (0.5^{O}C) the melting temperature of the probe.
3. Subject the filter to autoradiography to test the amount of SSOs on the filter (all the spots should give enough signals).

Detection of hybridized probes

1. Autoradiography for ^{32}P labelled probes

1. Place the membrane on a Whatman 3 MM filter paper to remove

excess fluid.

2. Place the membrane in a plastic bag or cover it with Saran wrap.
3. Expose to an X-ray film (Kodak, XAR-5) with an intensifying screen at room temperature for 1 to 5 h.
4. Clear signals should be obtained in 30 min to 2 h.

2. Detection of dig- ddUTP labelled probes

This can be done either by (a) Color development or (b) Chemiluminescent detection.

Solutions required:

1. Buffer 1 — 100 mM Tris-HCl (pH 7.2); 150 mM NaCl
2. Buffer 2 — Buffer 1 containing 1% Blocking reagent (Boehringer); Dissolve Blocking reagent 70^{0}C
3. Buffer 3 — 100 mm Tris-HCl (pH 9.5); 100 mM NaCl; 50 mM $MgCl_2$
4. Buffer 4 — 10 mm Tris-HCl (pH 8.0); 1 mm EDTA
5. Alkaline phosphatase conjugated anti-dig Antibody (750 µg/ml, Boehringer)
6. Color solution:
   (i) NBT stock (100 mg/ml Nitroblue Tetrazolium salt in 70% Dimethyl Formamide (DMF)
   (ii) X-Phosphate stock (50 mg/ml 5-bromo-4-chloro - 3/indoyl phosphate in DMF)
   (iii) Lumigen PPD (Boehringer): 10 mg/ml

Add 30 µl of NBT stock and 30 µl of X-phosphate stock to each 10 ml of Buffer 3.

(a) Color development for alkaline phosphatase

1. All procedures should be done at room temperature. After hybridization and washing, rinse the filter in Buffer 1 for 5 min.

2. Incubate the filter in 100 ml of Buffer 2 for 30 min.
3. Rinse the filter briefly in Buffer 1.
4. Dilute AP conjugated anti-Dig antibody to 150mμ/ml by adding 1.5μl of antibody per 15 ml of Buffer 1 [1:10000 dilution].
5. Incubate the filter with the antibody for 30 min.
6. Wash the filter in 100 ml of Buffer 1 for 15 min two times.
7. Put the filter in a heat-sealed plastic bag.
8. Add 10 ml of Buffer 3 and incubate for 2 min.
9. Add 10 ml of colour solution and seal the plastic bag.
10. Do not shake and keep away from strong light during the color development (e.g. put the plastic bag in a pan covered with aluminum foil).
11. Colour precipitates will be formed in 10 min. to 2h.
12. Continue the colour development until enough signals are obtained (usually it takes 1h. to 16h.).
13. Wash the filter with 50 ml of Buffer 4 to stop the colour development.
14. Take a photograph for record.

(b) Chemiluminescent detection

Steps 1-8 are performed at room temperature with shaking or mixing. The volumes of the solutions are calculated for a membrane size of 100 cm^2 and should be adjusted to other membrane sizes.

1. Wash membrane briefly (1-5 min) in washing buffer.
2. Incubate for 30 min in 100 ml buffer 2.
3. Dilute anti-digoxigenin-AP, Fab fragments to 75 mμ/ml (1: 10000) in buffer 2. Diluted antibody conjugate solutions are stable only for 12 h at 4^0 C.
4. Incubate membrane for 30 min in 20 ml of diluted antibody conjugate solution.
5. Remove unbound conjugate by washing 2X 15 min with 100 ml washing buffer.
6. Equilibrate 2-5 min in 20 ml buffer 3.
7. Dilute stock solution of Lumigen PPD (10 mg/ml) 1: 100 in buffer 3.
8. Incubate membrane for 5 min (1-5 min) in approx. 10 ml substrate solution (diluted substrate solution should be stored at 4^0 C in

the dark and can be re-used for at least 5 times).

9. Let excess liquid drip off the membrane; blot for few seconds on a sheet of dry Whatman 3 MM paper but not to complete dryness.
10. Seal the damp membrane in a hybridization bag.
11. Incubate the sealed membrane for 5-15 min at 37^0C.
12. Expose for 15-25 min at room temperature to X-ray or Polaroid b/w film.
13. For reprobing, the membranes should be kept wet.

3. Detection of biotin-labelled probes

Solutions required

(a) Washing buffer — 1X SSPE 0.1 % SDS

(b) Buffer B (pH: 7.3) — 1 M Urea
0.1 M NaCl
5% Triton X-100
1% Dextransulphate (Store buffer B at 4^0 C)

(c) Horse radish peroxidase streptavidine conjugate (Pierce Catrn).

(d) Enhanced chemiluminescence's Kit – (Amersham Pharmacia).

Important: *Don't use powdered latex gloves when manipulating the membranes with reagents for chemiluminescence's, use vinyl gloves or forceps. The powder of the latex gloves can quench the signal completely.*

Method:

1. After finishing the critical wash, the membranes are washed twice separately with 5 ml washing buffer for 5 min at room temperature (washings are performed on a shaking platform).
2. Combine the filters and soak the 4 filters together in the HRP SA working solution (add 2 µl of a HRP-streptavidine solution (2 mg/ml) to 20 ml of washing buffer) for 15 min at room temperature on a shaking platform.
3. Wash the filter paper twice in washing buffer for 5 min.
4. Wash the 4 membranes for 5 min in 20 ml buffer-B at RT.
5. Wash the membranes for 5 min in washing buffer.
6. Use the Amersham ECL kit (catnr RPM 2105): mix 10 ml of

solution 1 of the ECL-kit with 10 ml of solution 2; incubate 4 filters together in this mix for 1 min (Post mixing work should be quick since the mix is unstable).

7. Wrap the filter in foil and tape them into a cassette (dotted side up).
8. In the dark room, when the results are not satisfactory, expose the film for longer or shorter duration or as the case may be.

Dehybridization of probe

This method is available for ^{32}P labelled probe and for dig ddUTP labelled probe (chemiluminescent detection).

1. Immerse the membrane in 10 mM Tris-HCl (pH 8.0), 0.5% SDS at 70^0 C for 45 min., once or twice.
2. Remove excess fluid on a filter paper.
3. Subject it to autoradiography to confirm that the probe is removed sufficiently for rehybridization.
4. Put in a heat sealed plastic bag and store at 4^0C until next use.

Interpretation of signals

For every SSO known positive and negative controls should be included. In case, a given SSO has one nucleotide mismatch with certain sequence a control cell should be included possessing this particular sequence to serve as a negative control for the SSO. So, any spot stronger than the negative control should be considered positive. Weak positive spots should be evaluated by taking into account the amount of amplified DNA in the spot. This is achieved by comparison to the signal obtained with "all" SSO, i.e. an SSO which is positive with all allelic forms, thus, representing a "constant" sequence **(Figure 11.5)**. The signal obtained is the maximal signal for that spot and should be related/ compared to the signal obtained with the typing SSO.

When the differences between positive and negative signal are very small, an additional critical wash with a slightly increased temperature should be carried out as to make the differences between the signals of the positive and negative controls more meaningful. In general, we use for each SSO, the exposure time whereby the negative control on the film shows a very weak spot or no spot at all. The intensity of the spots should be represented by grades as shown in **Table 11.2.**

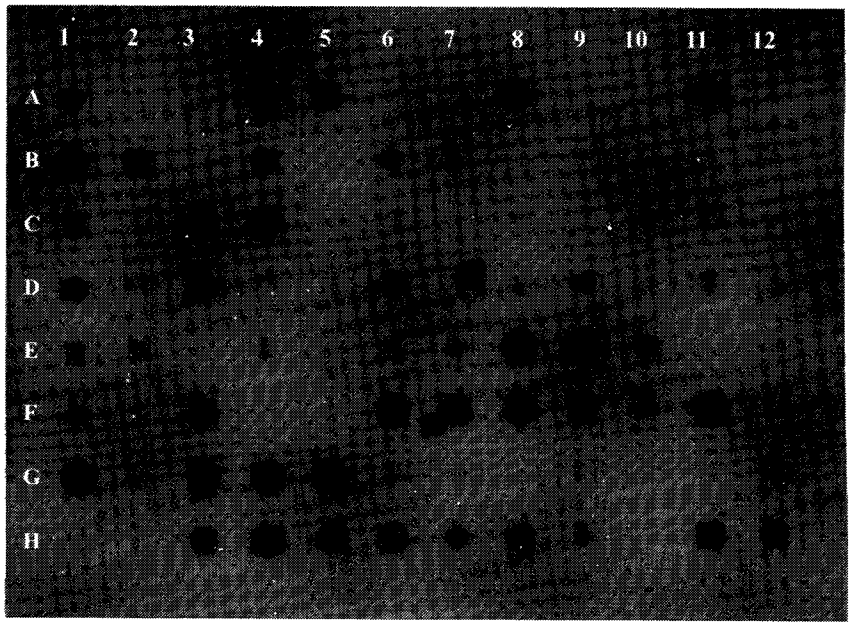

Figure 11.5: An Autoradiogram showing Dot Blot analysis

Table 11.2: Different grades and their interpretations in allele assigning based on the intensity of the spot.

Grade	Interpretation
0	Not spoted
1	Negative (definitely)
2	Negative (probably)
4	Positive/Negative
6	Positive (probably)
8	Positive (definite)
9	Positive (definite)

Southern Blotting

Southern blotting is a technique for detecting specific **DNA** fragments in a complex mixture (**Figure 11.6**). Edward Southern invented the technique in mid-1970s. It has been applied to detect Restriction Fragment Length Polymorphism (**RFLP**) and Variable Number of Tandem Repeat Polymorphism (**VNTR**). The latter is the basis of DNA fingerprinting.

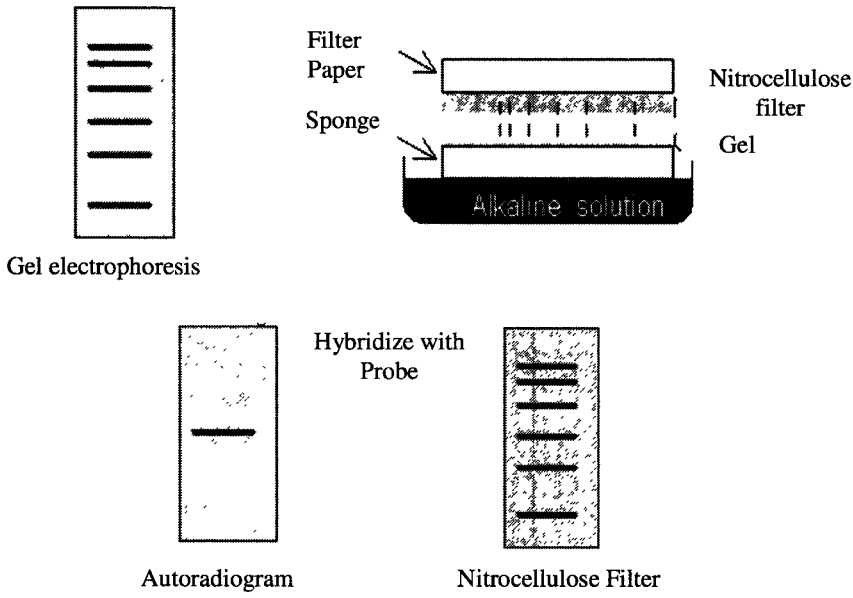

Figure 11.6: Diagrammatical representation of Southern Blotting procedure

Procedure of southern blotting

1) DNA (genomic or other source) is digested with a restriction enzyme and separated by gel electrophoresis, usually an agarose gel. Because, there are so many different restriction fragments on the gel, it usually appears as a smear rather than discrete bands. The DNA is denatured into single strands by incubation with NaOH.

2) The DNA is transferred to a membrane, which is a sheet of special blotting paper. The DNA fragments retain the same pattern of separation they had on the gel.

3) The blot is incubated with many copies of a probe which is single-stranded DNA. This probe will form base pairs with its complementary DNA sequence and bind to form a double-stranded DNA molecule. The probe cannot be seen but it is either radioactive or has an enzyme bound to it (e.g. alkaline phosphatase or horseradish peroxidase).

4) The location of the probe is revealed by incubating it with a coloruless substrate that the attached enzyme converts to a colored product that can be seen or gives off light that will expose X-ray film. If the probe was labeled with radioactivity, it can be exposed to X-ray film directly **(Figure 11.7).**

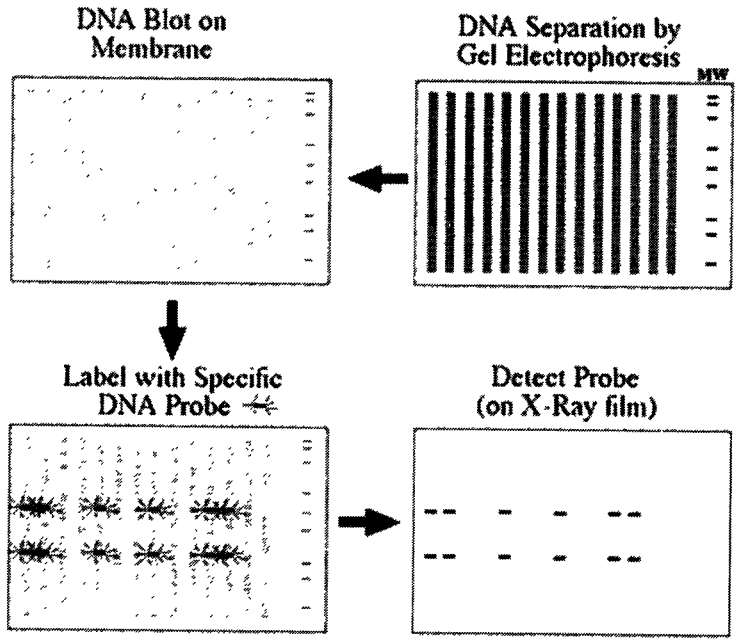

Figure 11.7: Southern blotting technique

Southern Hybridization protocol:

Steps:

1. Restriction digestion of DNA
2. Gel electrophoresis of digested DNA
3. Southern blotting
4. Radio labeling of probe
5. Hybridization

(1) Restriction enzyme digestion of DNA

Reaction mix: (50µl)

10 X restriction buffer	5µl
DNA (should be 10-15 µg)	15µl
Restriction enzyme	5µl (40 – 60 U)
HPLC water or ddH_2O (autoclaved)	make vol. up to 50µl

Incubate at 37^0C for overnight.

(2) Agarose gel electrophoresis

1. Prepare 1% agarose gel for electrophoresis of digested genomic DNA.
2. Before running the final gel for southern transfer, a aliquot of digested DNA (4-5µl) must be electrophoresed to confirm the proper digestion of genomic DNA.
3. Load the whole digested DNA in wells of the gel.
4. A suitable molecular weight marker must run along with digested product for sizing of the fragments.
5. Run the gel at around 2V/cm for O/N, the current must be in the range for 14-15 mA.
6. Next day stain the gel with ethidium bromide; check the proper electrophoresis in UV light.

(3) Southern Blotting

Solutions required

Neutralizing solution

NaCl	1.5 M
Tris	0.5 M
EDTA	1 mM

Denaturation solution

NaOH	0.5 M
NaCl	1.5M

Depurination solution

HCL	0.25N

Method

1. Gel treatment

Wash the gel with ddH_2O (autoclaved) in a tray by continuous shaking with slow speed for 5 min.

i. Depurination

- Treat the gel with depurination solution for 15min by continuous shaking.
- Wash with ddH_2O for 5 min.

ii. Denaturation

- Treat the gel with denaturation solution for 25 min. Change the solution and repeat it again.
- Wash with ddH_2O for 5 min.

iii. Neutralization

- Treat the gel with neutralization solution for 15 min. Change the solution and repeat it again.

2. Southern transfer (Figure 11.8)

- Cut the nylon membrane according to the size of the gel. Dip the membrane in ddH2O and then in 6 x SSC. Arrange the materials as shown in **Figure 11.8**, transfer the gel in 10 x SSC for overnight.

3. Fixing

- Next day take out the blot; dip in 6xSSC and air dry. Fix the membrane by UV in UV cross linker at 12,00 energy.

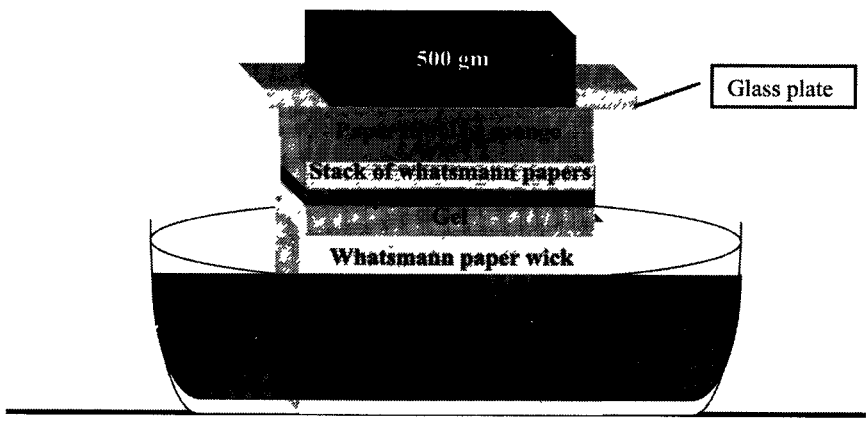

Figure 11.8: Southern transfer procedure

4. Radio labeling of probe

Reaction mix: (50µl)

10x labeling buffer	5µl
dATP	4µl
dGTP	4µl

dTTP	4µl
Klenow enzyme	2µl
d*CTP (p32 labeled)	5µl
Probe DNA (denatured)	21µl (Incubate at 37^{0}C for 2- 3 hrs.)

Note: *Denature the DNA probe by keeping it in boiling water for 10 min. Chill immediately on ice water bath.*

Precipitation of radiolabelled probe

1. Add
   (i) Carrier DNA (0.75 mg/ml) 1µl
   (ii) 0.2 M EDTA 2.5 µl
   (iii) 3M Na-acetate 6µl
2. Mix and add 2.5-vol ethanol.
3. Mix and keep at -70^{0}C for 2 - 3 hrs or -20^{0}C for overnight.
4. Spin at 13000 rpm for 30min.
5. Rinse with 70% ethanol by centrifuging at 13000 for 5 min and with absolute ethanol for 3 min.
6. Air dry and dissolve in 200 µl TE.
7. Take 1 or 2 µl aliquot of labeled probe; count the radioactivity to check the percent incorporation of p32 labeled dCTP.

(5) Hybridization Procedure

(a) Pre-hybridization

Incubate the blot in pre-hybridization solution in shaking condition at 65^{0}C for at least 3 hrs.

Pre-hybridization solution

Na_2HPO_4	0.4 M
NaH_2PO_4	0.1 M
SDS	20%

(b) Hybridization

1. Decant the prehybridization solution.
2. Denature the probe in boiling water for 10 min. Add the denatured probe in fresh pre-hybridization solution.
3. Incubate the membrane in hybridization solution (pre-hybridization solution + Probe) for overnight at 65^{0}C in continues shaking condition.

Post hybridization washing

1. Remove the hybridization solution and store at -20^{0}C for further use.
2. Wash the blot two times with 2xSSC + 0.1% SDS at 65^{0}C for 10 min.
3. Wash with 1X SSC +0.1% SDS at 65^{0}C for 5 -10 min.
4. Wraps the blot by Saran wrap, expose the X-ray film and keep at -70^{0}C for 1 or 2 days.
5. Develop the blot.

Northern Blotting

Suppose you have cloned a cDNA (a DNA copy of an RNA) and want to know how actively the corresponding gene (gene X) is transcribed in a number of different tissues of organism Y. This can be done through Northern Blotting.

You would begin by collecting RNA from several tissues of the organism in question. Then you electrophorese these RNAs in an agarose gel and blot them on to a suitable support. Because a similar blot of DNA is called a Southern blot, it was natural to name a blot of RNA a Northern blot.

Next, you hybridize the Northern blot to a labelled cDNA probe. Wherever an mRNA complementary to the probe exists on the blot, hybridization will occur, resulting in a labelled band that you can detect with X-ray film. If you run marker RNAs of known size next to the unknown RNAs, you can tell the sizes of the RNA bands that "light up" when hybridized to the probe.

Furthermore, the Northern blot tells you how abundant the gene X transcript is. The more RNA the band contains, the more probe it will bind and the darker the band will be on the film. You can quantify this darkness by measuring the amount of light it absorbs in a densitometer.

Northern Hybridization Protocol

Electrophoresis of RNA

Solutions required

1. 5x Formaldehyde gel - running buffer

0.1 M 3-(N-morphotino) propanesulfonic acid (MOPS) pH 7.0

40 mM Na-acetate

5 mM EDTA (pH- 8.0)

2. Formaldehyde gel -loading buffer

50 %	glycerol
1mM	EDTA (pH 8.0)
0.25%	Bromophenol blue
0.25%	Xylene cyanol

Method

(a) Preparation of formaldehyde gel

Prepare the gel by melting the appropriate amount of agarose in water, cooling it to 60^0C and adding 5x formaldehyde gel-running buffer and formaldehyde to give final concentration of 1x and 2.2 M respectively.

(b) Preparation of sample

1. Prepare samples by mixing the following in a sterile tube

RNA (upto 30µg)	4.5µl
5x formaldehyde gel-running buffer	2.0µl
Formaldehyde	3.5µl
Formamide	10.0µl

2. Incubate 65^0C for 15 min, and then chill them on ice.
3. Add 2µl formaldehyde gel-running buffer.
4. Before loading the samples, pre run the gel for 5 min at 5V/Cm. Load the samples into the lanes of the gel along with suitable RNA molecular weight marker.
5. Run the gel submerged in 1x formaldehyde gel -running buffer at 3-4 V /cm. (run till bromophenol blue dye has migrated approx 8 cm)

Transfer of denatured RNA to the membrane (Figure 11.9)

1. Rinse the gel in several changes of DEPC treated water to remove formaldehyde.
2. Soak the gel for 20 min in 0.05 N NaOH.
3. Rinse with DEPC water and then soak it for 45 min in 20% SSC.
4. Cut the membrane according to size of gel and wet in deionized water and then immerse in 20x SSC for 5 min.
5. Like Southern transfer arrange the procedure accordingly for 16-18 hours.
6. After transfer, soak the membrane in 6 x SSC for 5 min.
7. Dry the membrane and fix it under UV light or by baking.

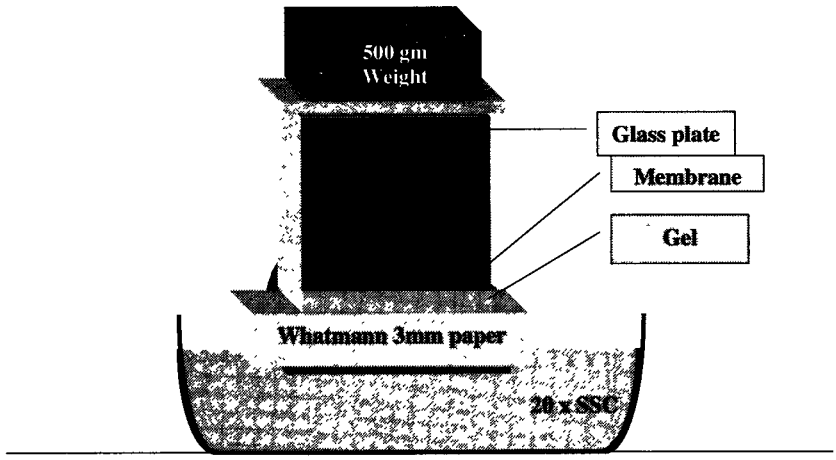

Figure 11.9: Northern Transfer procedure

Hybridization

Solution required

Prehybridization solution (at 42^0C)

50 x formamide

5 x SSPE

2 x Denhardt's solution

0.1 % SDS

Prehybridization solution (at 68^0C)

6 x SSC

2 x Denhardt's solution

0.1% SDS

Method

The conditions for prehybridization and hybridization are essentially the same as those used for DNA.

(a) Prehybridize the membrane in Prehybridization solution either at 42^0C or 68^0C for 2-3 hrs.

(b) For hybridization add denatured radiolabelled probe to prehybridization fluid.

(c) To detect low-abundance mRNA, use at least 0.1 μg of probe

whose specific activity exceeds 2 x 10^8 cpm/µg continues incubation for 16 - 24 hrs. at the appropriate temperature.

(d) Wash the filter for 20 min at room temperature with 1 x SSC + 0.1% SDS followed by 2-3 wash of 20 min each at 68^0C in 0.2 x SSC +0.1%SDS.

(e) Expose the membrane for 24-48 hrs to X-ray film at -70^0C with an intensifying screen and take autoradiograph.

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Chapter 12

Role of Microarray Techniques in Present Day Molecular Biology

After the whole genome being totally explored, the interest is now towards how particular gene is expressed in the pathological and normal condition. The conventional methods used are RFLP, southern hybridization, dot blot analysis, immunohistochemistry, quantitative measurements, or electron microscopy etc. Owing to the rapid developments in molecular biology in recent years, many clinicians and basic researchers are focusing on the analysis of the genetic alterations and gene expression of normal and diseased tissue which underlie their biological properties and probably

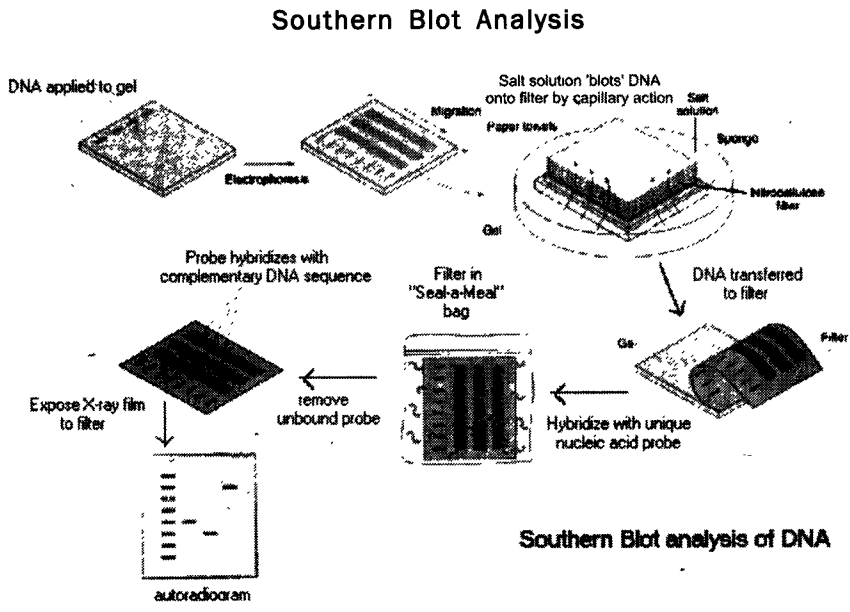

Figure 12.1 Southern blot analysis

their clinical behaviour. This type of molecular analysis can be useful for diagnosis making and prognosis assessment in differential gene expression profiles or genetic alterations correlatation with the patients' prognoses and use of this knowledge in the clinical follow up strategies. A simple comparison of southern blot analysis and microarray is shown in **Figure 12.1 and 12.2.**

After the development of microarray techniques a huge interest in the monitoring of (global) gene expression and the genetic alterations of various multifactorial diseases and tumours is tremendously increasing. Until now, laboratory techniques were not suitable for the measurement of the expression of large numbers of genes at the

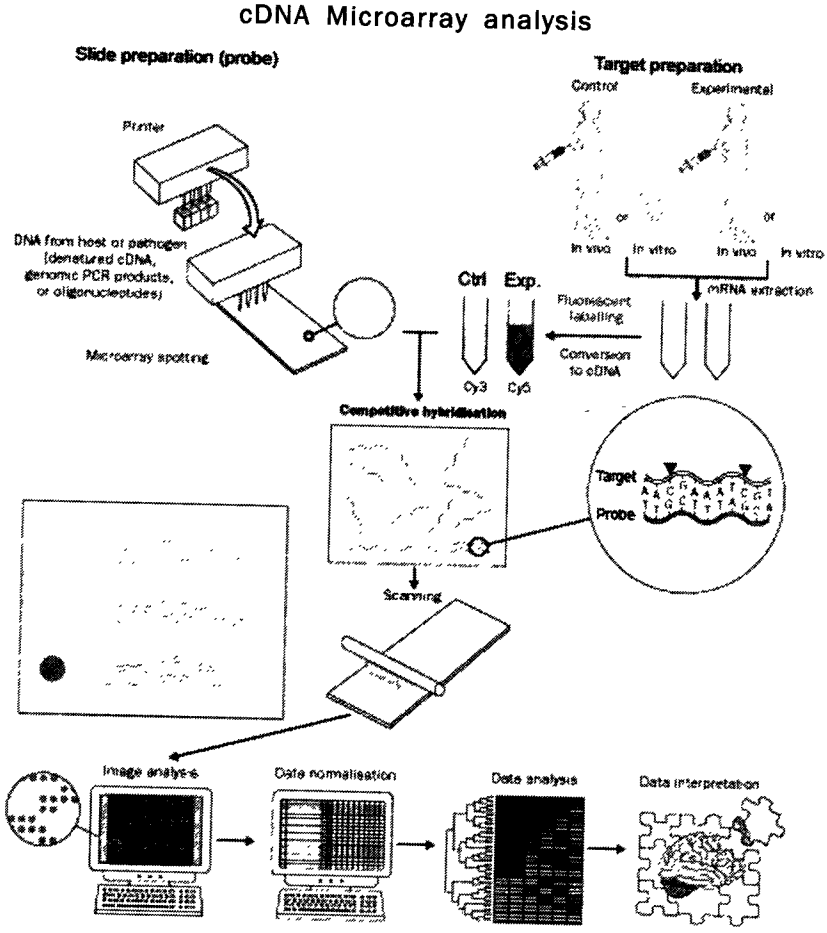

Figure 12.2 Southern blot analysis cDNA microanalysis

mRNA level (for example, northern blotting, the reverse transcriptase polymerase chain reaction (PCR), and in situ hybridisation) or at the protein level (western blotting and immunohistochemistry). One has to realize that, on an average, 10% of the estimated 20,000 genes present in the human genome are expressed in a given cell. Similarly, for the measurement of genetic alterations, the choice was between high resolution but with a limited overview (for example, changes in single bases evaluated by mutation analysis, Southern blot hybridisation, direct sequencing, and in situ hybridisation), or a global overview with a limited resolution (for example, analysis of changes at the chromosomal level by comparative genomic hybridisation[5] or karyotyping of cultured cells). Furthermore, all these techniques are relatively complex and require special equipment and expertise that are not available in many laboratories. Moreover, these techniques are labour intensive, time consuming and therefore, expensive. These drawbacks have hampered the introduction of these useful techniques in diagnosis at molecular level for various diseases.

Recently, a microarray technology has been developed that might overcome many of these drawbacks. It increases the possibilities both for the analysis of gene expression and the monitoring of genetic changes. It provides the best of both worlds: it is not labourious, has high resolution, and a global overview. A microarray is a series of DNA target sequences spotted on to a carrier (a glass slide, a silica "chip", or a membrane) in a logical and orderly fashion, on which nucleic acid probes derived from (for example) the tumour are hybridised. Four types of microarrays can be discerned and each has different applications (**Table 12.1**): (1) arrays of human genomic DNA, (2) arrays of human cDNA, (3) arrays of human oligonucleotides, and (4) arrays of bacterial or viral DNA or cDNA.

Table 12.1 Overview of the four types of microarray

Target	Application
Human genomic DNA	Microarray CGH: genomic gains or losses
Human cDNA	Expression arrays: gene expression patterns
Human oligonucleotides	Mutation analysis
	Single nucleotide polymorphisms (SNPs)
Bacterial or viral DNA/ RNA	Detection and classification of microorganisms

Application of microarray technique is quite common in cancer genetics, hence, here all techniques would be discussed by taking tumour as an example.

Principle of the Microarray Technique

The theory for microarrays is the same as for southern blot analysis/ northern blot analysis. The differences are as follows: the gene fragments (probes) are fixed on a glass slide instead of a membrane support; the size and density of the arrays are smaller and higher. The analysis of genetic expression of the hybridized RNA (target) is not done with isotopes but with florescent dye markers. The number of probes and number of genes can be increased, and it is possible to analyze the gene expression of the comprised genes. Conversely, the probes used in the macroarrays give no information on the expression of unfixed genes. Therefore, from the point of view of analyzing the expression of the genes in the genome, the number of probes required should be maximum. Currently, there are two methods that are used for the microarrays.

The GeneChip™

This is totally different from a cDNA microarray spotting DNA clones on glass slide. For constructing the GeneChip™, approximately 25-mer oligoDNAs, are directly synthesized on a glass slide using photolithography. Nucleotide sequence data are used to create a set of probes for each gene, which contain two types of probes: 25-mer oligoDNAs perfectly matching with 16 (~ 20) locations in each gene and the oligo DNAs of the same stretches except containing a single base substitution at the 13^{th} position of the 25^{th} nucleotides. Once the chip is designed using data from published databases, it is possible to use the chip without maintaining and spotting DNA clones. The GeneChip™ is theoretically an ideal method for quantitative analysis of gene expression, because the length and GC content of probes can be uniformed to make Tm value, the determinant for stringency of hybridization, even between probes. There are three drawbacks in the GeneChip™ method: it is impossible to construct the GeneChip™ in one's own laboratory; if there is no information about nucleotide sequence of a gene, it is impossible to make probes for the GeneChip™; if one embark on further analysis about interensting genes based on the results of GeneChip™ experiment, it is necessary to isolate the DNA clones of genes because the probes are the short oligomers manufactured from information in nucleotide databases, not DNA clones.

cDNA Microarray

cDNA microarray, the method to use cDNA clones fixed on glass

slide as probes, is originally developed by the laboratory of Dr. Patrick O. Brown, Stanford University School of Medicine. There are various methods for spotting cDNA to glass slides, such as mechanical microspotting with capillary shaping pen(s) or noncapillary pen(s) (there are a variety of pen chapes) and ink jetting with miniature nozzle(s). The advantages of cDNA microarray system are that it is possible to prepare microarray at any time by spotting cDNA clones on glass slides and that all of the cDNA clones on microarray are already available to perform further analysis about interesting genes based on the results of microarray experiment. The disadvantages of these systems are that the equipments used for spotting and scannning and chemicals for labeling are expensive and that a large number of cDNA clones need to be prepared for spotting.

Microarray is a powerful tool for gene function analysis. For nearly all of the organisms whose genome are now being or have been sequenced, the cDNA sequencing analyses have also simultaneously been in progress or done. The functions for most of the genes are still unknown though some of the genes show the similarities to the genes/cDNAs with known functions by the results of homology search in the databases based on cDNA sequences. The microarray technique allows one to study how the transcription levels of all genes on microarray change between different biological conditions, with a small number of repeated experiments. (Use of microarray for gene expression profiling). The technique also provide ways to look for the DNA clones that show the transcriptional changes under different conditions regarding a phenomenon that researchers are interested in (for example, response to environmental/biological stress, gene expression specificity in tissue/developmental stage, etc.), from all gene/cDNA clones on microarray. (Use of microarray as method to identify interesting/useful genes). In short, the microarray technique is a method to obtain very large amounts of gene expression data with a minimum number of experiments.

Generally speaking, the experimental techniques in molecular biology have been used to the biological phenomena, whose genetic analyses are easy to perform and/or the cDNAs/genes involved in are easy to isolate. If the microarray technique apply to the interesting biological phenomena that are difficult to elucidate, such as heterosis (= hibrid vigor) and the totipotency of cultured plant cell, the breakthrough in molecular biology on these phenomena will occur.

Microarray as primary differential screening method

As mentioned above, the microarray technique is a method to obtain a large quantity of gene expression data with an experiment and to identify interesting/useful genes. However, it should be stressed that "the data from microarray experiments do not provide all the answers. The microarray is a simple and quick method for determining which of the large number of genes on microarray should be selected for further analysis." By microarray experimental data, it is possible to detect the significant differences of transcription levels, which is generally more than twofold (or threefold), of many genes between different samples. But it is difficult to conclude that all of the differences are authentic, based on only microarray data. To resolve this difficulty, there are following issues: how to standardize the data, how to normalize the data obtained from differrent glass slides, how to set up positive/negative controls and use their values for data analysis, how to find the housekeeping genes showing constant expression levels. Although standardizing the data is possible in a few cases that would be extremely difficult in the cases which show low expression of most of the genes in an organism, such as the states showing cell death in most of the cells. Because the perfect solving of these issues pointed out above is practically impossible, the microarray should be regarded as primary screening method. And it would be advantageous to use the Northern hybridization method or the RT-PCR method for re-examining the levels of transcriptionfor each gene selected by microarray data.

The data analysis of the results obtained by microarray is highly complex and various algorithms are being followed by different workers.

Draw backs of microarray

It is based on the availability of gene sequences arrayed on a solid surface and it allows parallel expression analysis of thousands of genes. DNA microarray technology is high throughput method for gaining information on gene function. To define transcriptional signatures bound to a pathological condition, to find out molecular mechanisms tightly bound to transcription. Since our actual knowledge on genes function in high eukaryotes is quite limited, hence, Microarray analysis frequently does not imply a final answer to a biological problem but allows the discovery of new research paths which enable us to explore these by a different perspective.

In this technique, the expression of the gene at protein level is not possible. We can test the expression at DNA/ RNA level only. The data generated during microarray analysis can be influenced by number of factors like chip type, sample preparation, and data analysis. Data analysis is the most difficult part as it requires sound knowledge of bioinformatics.

Data Analysis

Statistical validation usually implies the selection from the user of statistical significance parameters. For example: SAM (Significance Analysis of Microarrays) always requires the input of a "delta" value which defines the threshold of false positive in the validated dataset. If the stringency of the statistical validation is too high, biologically meaningful genes can be lost making more difficult to rule out functional correlations between the differentially expressed genes. If the stringency of the statistical validation is too loose, the increase of false positives creates background noise from which it is difficult to extract trustful functional correlations between the differentially expressed genes. It is important to note that simple bioinformatics also some time is not able to answer the desired question. Molecular function of the gene can be at DNA/ RNA, chromatin binding, it can be at enzyme level, hence, correlation of the data at all these levels is required before reaching at the final conclusion.

To conclude, microarray is a useful technique in the modern day molecular biology but with limitations of interpretation of data in a right manner of direction.

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Chapter 13

DNA Sequencing

The process of determining the order of the nucleotide bases along a DNA strand is called **Sequencing**. DNA sequencing enables us to perform a thorough analysis of DNA, because it provide us with the most basic information of all i.e. the exact order of the bases A, T, C and G in a segment of DNA. With this knowledge, for example, we can locate regulatory gene sequences and can make comparisons between homologous genes across species and identify mutations.

In 1974, two methods were independently developed by an American team and an English team. The Americans team was lead by **Maxam and Gilbert**, who used **"chemical cleavage protocol"**, while the English team was lead by **Sanger**, designed a procedure similar to the natural process of DNA replication. These methods are known as the **chemical degradation** the **chain termination method** and were equally popular to begin with and even both teams shared the 1980 Nobel Prize, but Sanger's method became the standard because of its practicality.

Sanger's chain termination method

This method is based on the principle that single-stranded DNA molecule that differ in length by just a single nucleotide can be separated from one another using polyacrylamide gel electrophoresis.

The key to the method is the use of modified bases called Dideoxy nucleotide, due to which, this method is also known as **"Sanger's Dideoxy sequencing method"**. The dideoxy method gets its name from the critical role played by these synthetic nucleotides that lack the -OH at the 3' carbon atom of De-oxy ribose sugar. A dideoxynucleotide-for ex-dideoxythymidine triphosphate or ddTTP **(Figure 13.1)** can be **added** to the growing DNA strand but when, chain elongation stops as there is no 3' -OH for the next nucleotide to be attached. Hence, the dideoxy method is also called the **chain termination method**.

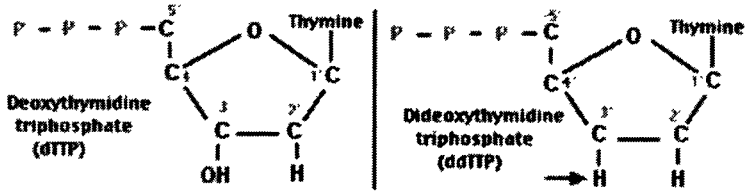

Figure 13.1: Structure of Deoxy and Dideoxy dTTP

Principle of chain termination method

For sequencing, we don't start from genomic DNA (like in PCR) but mostly from PCR fragments or cloned genes. Thus, sequencing gives us the sequence of nucleotide in a piece of DNA.

The DNA to be sequenced is known as the **template DNA**, which is first prepared as a single-stranded DNA. Next, a short oligonucleotide is **annealed**, or joined, to the same position on each template strand. The oligonucleotide acts as a primer for the synthesis of a new DNA strand that should be complimentary to the template DNA. This technique requires that four nucleotide-specific reactions—one each for G, A, C, and T be performed on four identical samples of DNA. The four sequencing reactions require the addition of all the components necessary to synthesize and label new DNA, including:

a) A **DNA template**.

b) A **primer** tagged with a mildly radioactive molecule or a light-emitting chemical.

c) **DNA polymerase**—an enzyme that drives the synthesis of DNA.

d) Four **deoxynucleotides** (G, A, C, T) in all the tubes.

e) One **dideoxynucleotide**, ddGTP, ddATP, ddCTP, or ddTTP, each in specific tube.

How does it work?

1. The reaction is initiated by heating until the two strands of DNA separate, then the primer sticks to its intended location and DNA polymerase starts elongating the primer. This continues till the completion of a new strand of DNA would be formed. If we start with a billion identical pieces of template DNA, we will get a billion new copies. It is similar to a normal PCR. The only difference is that we start from a **PCR product as a template** and **only one primer is used**.

2. After the first deoxynucleotide is added to the growing complementary sequence, DNA polymerase moves along the template and continues to add base after base. The strand synthesis reaction continues until a dideoxynucleotide is added, blocking further elongation.
3. PCR reaction consists of normal deoxynucleotide and chain terminating dideoxynucleotide as well. MOST of the time the enzyme gets normal deoxynucleotide and extension continues. However, 5% of the time, the enzyme will get a dideoxy-dNTP, making growing strand break away from the enzyme, and that strand can never again be elongated.
4. Only a small amount of a dideoxynucleotide is added to each reaction, allowing different reactions to proceed for various lengths of time, until, by chance, a dideoxy NTP is incorporated.
5. Sooner or later all the copies will get terminated by a ddNTP, but each time the enzyme makes a new strand, the place it gets stopped will be random.
6. To read the newly generated sequence, the four reactions are run side-by-side on a polyacrylamide sequencing gel. The families of molecules generated in the presence of ddATP are loaded into one lane of the gel and the other three families, generated with ddCTP, ddGTP, and ddTTP, are loaded into three adjacent lanes. After electrophoresis, the DNA sequence can be read directly from the positions of the bands in the gel. Complete chain termination method is illustrated in **Figure 13.2**.

Automated DNA sequencing

Advancement in technology that we have achieved since 1974, it is not surprising that the Sanger method has become outdated. However, the new technology that has emerged to replace this method is based on the same principles of Sanger's method. Automated sequencing has been developed so that more DNA can be sequenced in a shorter period of time. With the automated procedures the reactions are performed in a single tube containing all four ddNTP's, each labeled with a different color dye.

As in Sanger's method of manual sequencing, the DNA is separated on a gel. In automated sequencing using ABI 377 Automated Genetic Analyzer which uses Gel slab system of electrophoresis. The more recent one, ABI 310 Fragment Size Genetic Analyzer analyzes the sequencing product by capillary electrophoresis based system.

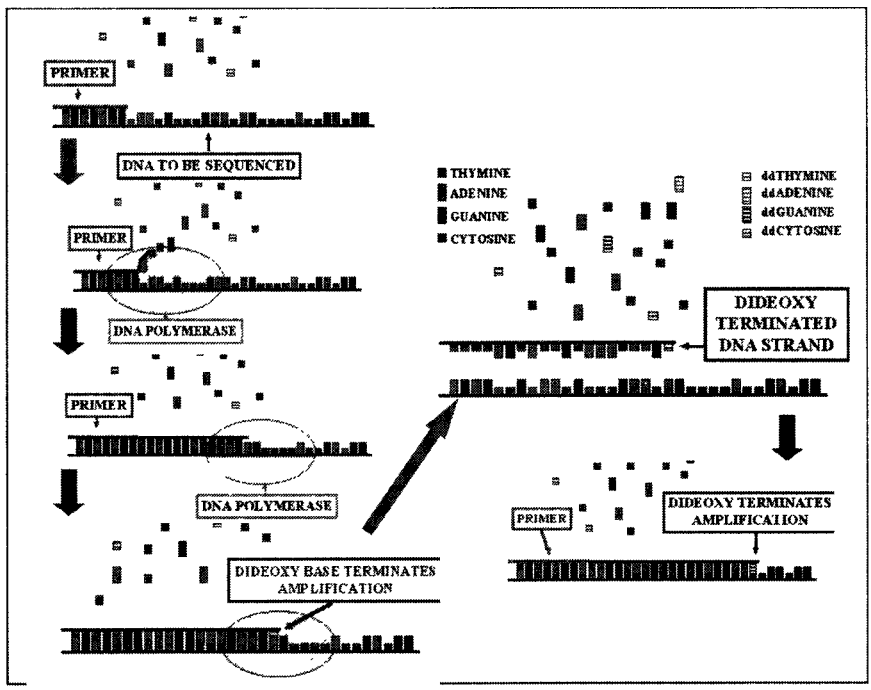

Figure 13.2: Chain termination sequencing involves the synthesis of new strands of DNA complementary to a single-stranded template (step I). The template DNA is supplied with a mixture of all four deoxynucleotides, four dideoxynucleotides – each labeled with a different colour fluorescent tag, and DNA polymerase (step II). As all four deoxynucleotides are present, chain elongation proceeds until, by chance, DNA polymerase inserts a dideoxynucleotide. The result is a new set of DNA chains all of different lengths (step III). The fragments are then separated by size using gel electrophoresis (step IV). As each labeled NA fragment passes through a detector at the bottom of the gel, the coluor is recorded. The DNA sequence is then reconstructed from the pattern of colours representing each nucleotide sequence (step V).

Since the four dyes fluoresce at different wavelengths, then a laser reads the gel to determine the identity of each band according to the wavelengths at which it fluoresces. The results are then depicted in the form of a chromatogram, where coloured peaks correspond to the nucleotides at the position of the sequences **(Figure 13.3).**

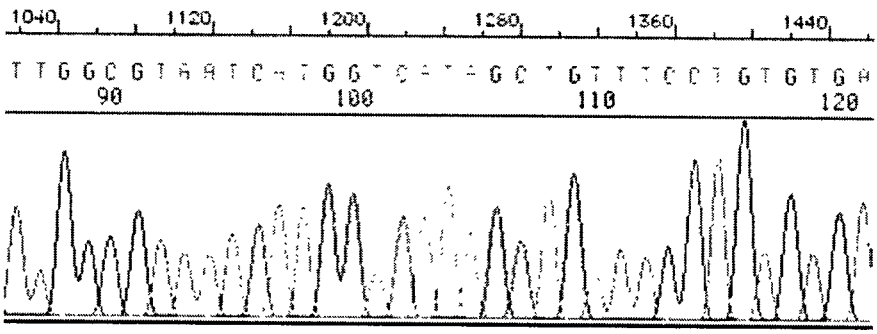

Figure 13.3: A DNA sequence chromatogram obtained on ABI 310 genetic analyzer

Methodology and essentials of automated DNA sequencing

1. Fluorescent Dyes

Each ddNTP is labeled with a different coloured dye. The most recent dyes, which we use in ABI 310 sequencer is, are **BIG DYE v 3.1.1** supplied by Applied Biosystems. The dyes include:

1. **dR110:** Blue colour dye labels **"ddCTP"** and gives blue colour peaks.
2. **dR6G:** Green colour dye labels **"ddATP"** and gives green colour peaks.
3. **dTamara:** Yellow colour dye labels **"ddGTP"** and gives black colour peaks.
4. **Drox:** Red colour dye labels **"ddTTP"** and gives RED colour peaks.

2. Ampli *Taq* Gold -FS

It is a special type of Taq polymerase, which is used specifically for DNA sequencing because it lacks:

(a) **5'-3'exonuclease** activity and consists of increased **synthetase activity**.

(b) It gives low noise, clean data and uniform peaks.

3. Cycle sequencing

The cycle sequencing is a simple method in which successive rounds of denaturation, annealing and extension in a thermal cycler results into a linear amplification of extension product **(Figure 13.4). The important points are:**

(a) It uses only one primer either forward to have a sequence 5'to3' of the template DNA or reverse primer to have sequence from 3' to 5' of the template DNA.

(b) While extension when ddNTP I incorporate, chain termination occur.

(c) While denaturant terminate chain because of ddNTP incorporation.

(d) In successive step of annealing template is again ready for next cycle of extension.

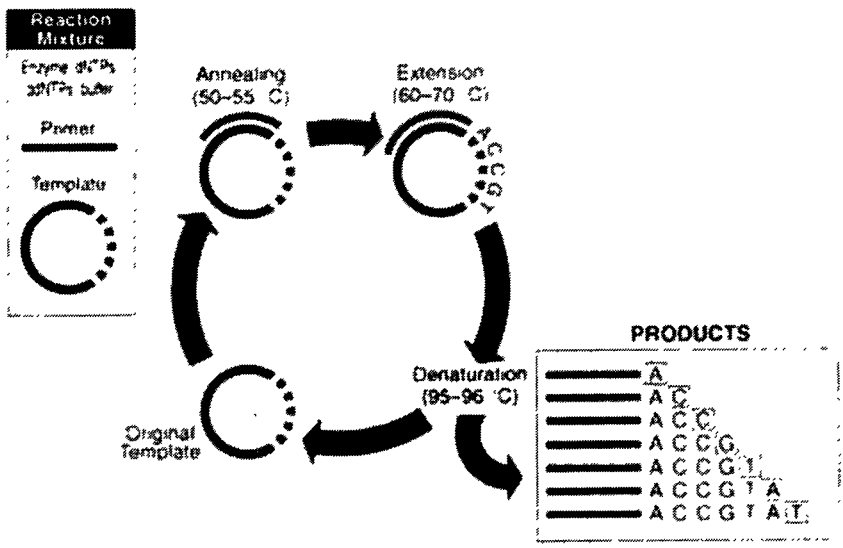

Figure 13.4: Cycle sequencing protocol

3. ABI 310 Genetic fragment size analyzer

The ABI 310 Genetic Fragment Size analyzer is an automated instrument for analyzing fluorescent-labeled DNA fragment by capillary electrophoresis **(Figure 13.5)**. It carries following essential parts:

a) An auto sampler tray to carry the sample tubes.

b) Cathode and Anode electrode through which current is passed and electrophoresis is carried out.

c) Capillaries which carry a separation medium required for electrophoresis.

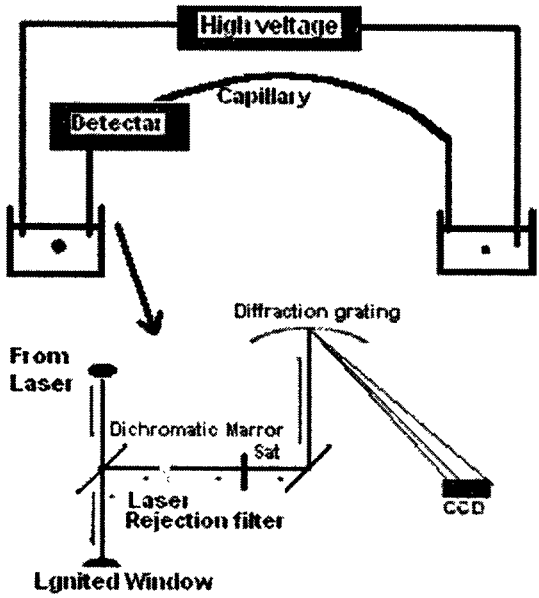

Figure 13.5: Electrophoresis and detection system in ABI 310 genetic analyzer

d) A laser torch which hits the fluorescent labeled fragments.

e) Charged Coupled Device (CCD) camera which records the emission of light when laser hits the labeled fragments.

f) A Power Macintosh computer system to record the digital signals.

g) An array of software files to carry out the sequencing operation and to analyze the obtained sequences.

4. Capillary

Capillary is very narrow in size. It is made up of fused silica. Outer coating of the capillary is made up of Polymide, which is burnt away to form Detection window at which laser hits. It carries the separation medium - High performance optimized polymer **(POP-6)**.

5. Software files

Different software file plays a very important role in successful sequencing operation as well as in analyzing the obtained data. These files and their roles are mentioned in **Table 13.1**.

Table 13.1: Different software files and their functions

Software File	Function
Sequence Sample sheet	It keeps the sample information and sample tube position in the auto sampler tray.
Sequence Injection List	Defines the order of the sample to be run and according to which module.
Sequence Module Files	Defines the run conditions like voltage, temperature and current of the electrophoresis.
Mobility Files	Defines that which dye chemistry is used like Big Dye 3.4.1 and which virtual filter set should be used like Filter set "E".
Big Dye 3.4.1 Matrix File	Avoids overlaps of the peak of different colour, specific for particular dye chemistry.

Protocol for automated DNA sequencing

M-173 SNP on Y-chromosome

Let us take the example of explain sequencing. It is an A®C transversion, which is very common among the Caucasian populations. In the following section, we have explained the whole technique by taking an example of different alleles for M-173 loci.

The total protocol for automated DNA sequencing is divided into five major portions:

1. Amplification of the desired segment of gene Ist PCR.
2. Cycle sequencing using the PCR product of Ist PCR as template, labeled ddNTPs and using only one primer.
3. Purification of the sequencing PCR product.
4. Capillary electrophoresis of the product in ABI 310 genetic analyzer.
5. Analysis of the sequencing data.

1. PCR amplification M-173 loci

(a) M-173 to be amplified by PCR, using following primers:

Forward: 5' CAGTAGGTCAGCAGAACCGTC 3'

Reverse: 5' CCTTTGCTGGAACCTTTATCC 3'

(b) Reaction mix is prepared as shown in **Table 13.2.**

Table 13.2: PCR reaction mix for amplification for M-173

Reagents	For 1 reaction (10µl)	For 5 reactions (10x5=50µl)
10 X buffer	1µl	5µl
dNTP mix (10uM/ul)	1.0µl	5µl
F-primer (10pm/ul)	0.5µl	2.5µl
R-Primer (10pm/ul)	0.5µl	2.5µl
Taq DNA polymerase (3U/ul)	0.5µl	2.5µl
DdH_2O	5.5µl	30µl
DNA (100ng/ul)	1µl	1x5µl

(c) PCR is to be carried out in MJ-Research Thermo cycler according to the conditions shown in **Table 13.3.**

Table 13.3: PCR conditions for amplification of M-173

Step	Temperature	Time	Cycles
Denaturation	94°C	30 sec	30 Cycles
Annealing	58°C	30 sec	
Extension	72°C	1 min	
Final extension	94°C	5 min	1 Cycle
Hold	15°C	Forever	

(d) PCR products are run on 2% Agarose to check the amplification.

2. Cycle Sequencing PCR

(a) M-173 to be amplified by PCR, using only forward primer:
Forward: 5' CAGTAGGTCAGCAGAACCGTC 3'

(b) Reaction mix to be prepared as shown in **Table 13.4.**

Table 13.4: Cycle sequencing PCR reaction mix for sequencing of M-173

Reagents	For 1 reaction (20µl)	For 5 reactions (20x5=100µl)
Ready Reaction mix*	8µl	40µl
F-primer (10pm/ul)	0.5µl	2.5µl
DdH2O	5.5µl	30µl
Ist PCR product	1µl	1x5µl

*** Ready Reaction mix is supplied by Applied Biosystems and carries all the four dNTPs, fluorescent labeled ddNTPs, Ampli Taq Gold, Buffer and required KCl, $MgCl_2$ in a definite proportion.**

(c) PCR is carried out in MJ-Research Thermo cycler according to the conditions shown in **Table 13.5.**

Table 13.5: Cycle sequencing PCR conditions

Step	Temperature	Time	Cycles
Hot Start	96^{0}C	2 min	1 Cycle
Denaturation	96^{0}C	10 sec	30 Cycle
Annealing	50^{0}C	15 sec	
Extension	60^{0}C	4 min	
Hold	4^{0}C	Forever	

3. Purification of the sequencing PCR product

Solution required

1. Solution A:
   100% Ethanol 1ml
   3MM Sodium Acetate 150µl
2. **Solution B:** 80% Ethanol
3. **Hi-Di- Formamide**

Procedure of purification

1. Take out the PCR tubes from the Thermo cycler after the cycle sequencing is completed.
2. Add 25 µl of solution A in each tube.
3. Leave the tubes for 15 mins at room temperature.
4. Centrifuge the tubes at 4000 rpm for 20 mins.
5. Discard the supernatent.
6. Add 100 µl of Solution B in each tube.
7. Centrifuge the tubes at 4000 rpm for 15 mins.
8. Discard the supernatent.
9. Incubate the tubes at 56^{0}C for 10-20 mins so that all the alcohol gets dried off.
10. Add 15 µl of Hi-di-formamide in each tube and vortex it properly.

4. Capillary electrophoresis in ABI 310 genetic analyzer

The purified PCR product is then transferred into sequencing tubes and closed with the septa (care should be taken to check that septa are dissected from the middle).

1. Switch on the Computer.
2. Put the capillary upwards.
3. Switch on the ABI 310 genetic analyzer.
4. Launch the ABI 310 data collection software in the computer.
5. Allow the syringe to go to home for about 5 minutes.
6. Go to the **"WINDOW"** option and launch **"MANUAL CONTROL"** and **"STATUS"** options.
7. Set the temperature from "Manual control" to 50^0C.
8. Go to the **"INSTRUEMENT"** option and launch **"AUTOSAMPLER CALLIBRATION"** window.
9. Keep the capillary 0.5 cm down the cathode.
10. Calibrate the capillary against the black dot placed on the auto sampler tray.
11. Fill the capillary with **POP-6** from **"SEQ FILL CAPILLARY"** option in **"MANUAL CONTROL"** window.
12. Go to the **"FILES"** option and launch **"SEQUENCE SAMPLE SHEET"** options for 48 tubes tray.
13. Type the sample name and details.
14. Select the **"MOBILITY FILE"** and **"MATRIX FILE"**.
15. Save and close the file by the date name.
16. Go to the **"FILES"** option and launch **"SEQUENCE INJECTION LIST"** options for 46 tubes tray.
17. Open the recently made sample sheet.
18. Select the **"MODULE FILE"** and **"VIRTUAL FILTER"**.
19. Perform the **CCD-TEST** to check the performance of laser.
20. Close the file and save it to Desktop.
21. Restart the computer.
22. Place the samples in auto sampler tray according to their positions mentioned in Sample sheet.
23. Launch the **"SEQUENCE INJECTION LIST"** saved on desktop.
24. Start the run.

5. How the capillary electrophoresis proceeds

It works in following manner:

a) The sequencing reaction tubes are placed in an auto sampler tray carrying either 48 or 96 tubes.

b) The auto sampler successively brings each sample in contact with cathode electrode and one end of glass capillary filled with polymer.

c) The other end of the capillary immersed in the buffer carrying the anode electrode.

d) The sample enters the capillary as current flows from cathode to anode. This short period is termed as **"Electrokinetic Injection"**. The sample forms a tight band in the capillary during this injection.

e) The end of the capillary and electrode is then placed into buffer.

f) Current is applied again to continue the electrophoresis.

g) When DNA fragments reaches ignition window in the capillary, a laser ignites the fluorescent-labeled fragment.

h) Emitted fluorescence from dyes is collected by a cooled **Charged Coupled device (CCD) camera** at a particular wavelength band **(Virtual filters)**.

i) This is recorded as digital signals in a **computer system.**

j) Base calling is done by **"Sequence analysis software 1.4.1".**

6. Sequence analysis

Sequence analysis of the obtained data and its alignment to detect the presence of mutation is discussed in the Chapter "Sequence analysis and alignment softwares." In this chapter sequencing has been explained by taking an example of single capillary sequencer as an example but the same principle applies to other sequencers like 4 capillary, 16 capillary or 96 capillary systems. The laboratory should purchase the equipment as per the workload.

Chapter 14

Multiplex PCR and Automated DNA Fragment Analysis by Gene Scanning

Multiplex PCR is an advanced system of Polymerase Chain reaction where two or more unique DNA sequences of different locus in the same specimen are amplified simultaneously in a single PCR reaction.

Types of multiplex PCR

1. Multiplex PCR is uses most frequently in diagnostic assays that use one set of primers to amplify an internal control to verify the integrity of the PCR, while the second set of primers is targeted to the DNA sequence of interest. A positive result with the control primers ensures conditions for a successful PCR are present and confirms the integrity and availability of nucleic acid for the detection of the target gene.
2. 2. Multiplex PCR used for individual identification to simultaneously probe an individual for several different polymorphisms (like STRs) in a single PCR tube (**Figure 14.1**).

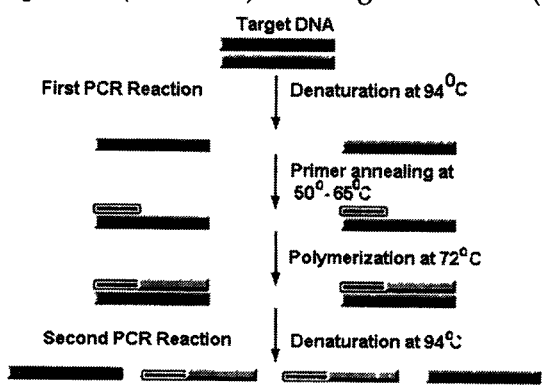

Figure 14.1: An example of multiplex PCR

Requirement for multiplex PCR

1. Primers of each locus should be having nearly same Tm, so that each PCR system can work simultaneously at the same annealing temperature.
2. GC contents of primers of each locus should be same or nearly same.
3. The amplification product of each PCR system should not fall in same range, if analysis has to be done by manual electrophoresis. However, manual electrophoresis is never recommended for large number of loci in a multiplex PCR. The most successful example of the manual multiplex PCR analysis is that of Y-chromosome STR system as only one allele is observed at each locus.
4. In case of automated analysis, one primer of each locus is labeled with fluorescent dye so that the loci of near by size range can be co-amplified and analyzed.
5. Proper denaturation time is required, usually; "Hot Start" is given, so that in initial cycle of PCR, all primers can bind to their respective sequences.
6. All primer pairs (for each locus) should be used in equal amount to avoid the weak amplification for some loci and strong for other.

STR- Multiplex system

Most short tandem repeat loci used to identity testing have repeats that are four base pairs in length, with allele sizes between 100bp and 300bp. The relatively small size of STR alleles reduces the effect of preferential amplification. STRs tend to be less discriminating than VNTRs, so a greater number of loci must be examined to obtain the same level of discrimination.

Multiplex PCR, which involves adding more than one set of PCR primers to the reaction in order to target multiple locations throughout the genome, is an ideal technique for DNA typing because the probability of identical alleles in two individuals' decreases with an increase in the number of polymorphic loci examined. The advent of fluorescent labeling with multiple fluorescent detection permits the multiplexing of STR loci, which may have alleles that fall in the same size range.

Multiplexing can save time and money, but difficulties may arise when co-amplifying several loci. Primers for one locus can complex with

those of other loci and completely inhibit amplification. This effect may be exhibited by dropout of a specific STR locus under certain conditions (e.g., sample mixtures). Finding the optimum PCR conditions, particularly the annealing temperature and the primer concentrations, can be challenging and time-consuming.

Procedure for STR multiplex and manual analysis

Y-chromosome STR analysis

Three Y-chromosome STR can be co-amplified in a single tube reaction and can be analyzed by manual PAGE using commercial ladders to assign proper genotyping. The three loci given here as an example are:

1. **DYS 390:** 190-230
2. **DYS 391:** 270-295
3. **DYS 393:** 108-132

1. **PCR amplification of DYS 390, DYS391 and DYS393**

   (a) All three loci are amplified by using following primer pair:

   DYS 390:
   Forward: 5' CAGTAGGTCAGCAGAACCGTC 3'
   Reverse: 5' CCTTTGCTGGAACCTTTATCC 3'
   DYS 391:
   Forward: 5' GACTGCTGGTTCAGGTCCAATCAG3'
   Reverse: 5' GTTCGGTTACGTCCGTAACCGTT3'
   DYS 393:
   Forward: 5' TAGCAGGTCAACCGTACGTC 3'
   Reverse: 5' TCTGTCGAACCTCTGTTGCCATT3'

   (b) Reaction mix is prepared as shown in **Table 14.1**

Table 14.1: PCR reaction mix for Y-STR mutiplex PCR

Reagents	For 1 reaction (25µl)	For 5 reactions (25x5=125µl)
10 X buffer	2.5µl	12.5µl
dNTP mix (10uM/µl)	2.0µl	10µl
Each F-primer (DYS390, DYS391, DYS393) 10pm/µl	1.0x3µl	1.0x3x5µl
Each R-primer (DYS390, DYS391, DYS393) 10pm/µl	1.0x3µl	1.0x3x5µl
Taq DNA polymerase (3U/µl)	1.2µl	5µl
DdH2O	14µl	70µl
DNA (100ng/µl)	2µl	2x5µl

(c) PCR is to be carried out in a Thermo cycler according to the conditions shown in **Table 14.2**

Table 14.2: PCR conditions for Y-STR multiplex PCR

Step	Temperature	Time	Cycles
Denaturation	94°C	30 sec	30 Cycles
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final extension	94°C	5 min	1 Cycle
Hold	15°C	Forever	

(d) PCR product are run on 2% Agarose to check the amplification.

2. Genotyping of each STR loci:

Manual PAGE electrophoresis

(a) 9% PAGE is prepared in Midi vertical electrophoresis assembly.

(b) All the samples are electrophorased on 9% PAGE for 12-16 hrs at 60-100V.

(c) 10 bp commercial ladder and allelic ladder of each locus is also run for proper sizing of allele.

(d) The bands are visualized on UV transilluminator **(Figure 14.2).**

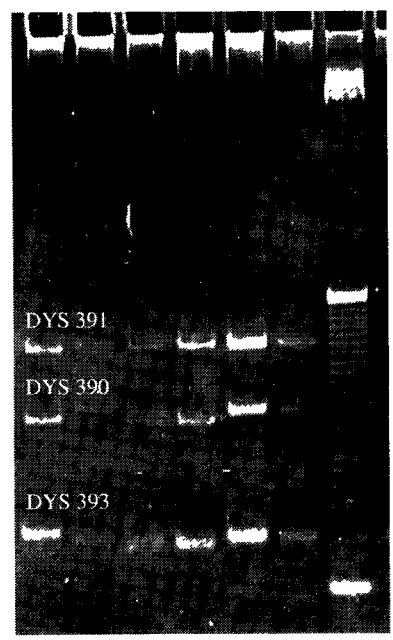

Figure 14.2: Analysis of three Y-STR loci by Multiplex PCR

Automated fragment analysis

Automated fragment analysis involves labeling of one of the primer pairs amplifying a specific segment of DNA, by a fluorescent dye. When this labeled PCR product is subjected to capillary electrophoresis, it emits signal while passing through the ignition window. The Fragment analyzer reads the size of the fragment, which emits the signal every time

it passes through the ignition window.

All automated sequencer - ABI 377, ABI 310, ABI 3700 Automated Genetic Analyzer, can be used. The difference is that ABI 377 is slab gel based while 310 is capillary electrophoresis based system, where ABI 310 is a single capillary while ABI 3700 is 96 capillary system.

STR multiplexes by automated fragment size analyzer

Detection due to fluorescent dye makes STR multiplex analysis by automated fragment analyzer, a great success. 16 loci can be co-amplified and can be analyzed by Gene scanning or fragment size analyzer using 5-dye system and 12 loci by 4-dye system. **Figure 14.3** explains the procedure that how STR multiplexing can be done by gene scanning procedure.

Since the four dyes fluoresce at different wavelengths, a laser then reads the gel to determine the identity of each band according to the wavelengths at which it fluoresces. The results are then depicted in the form of a chromatogram, where each locus is shown by a particular colour peak (depending on the fluorescent dye with which primer is labeled) as shown in **Figure 14.4.**

1. Two peaks of same locus if sample is heterozygous.
2. One peak if sample is homozygous.

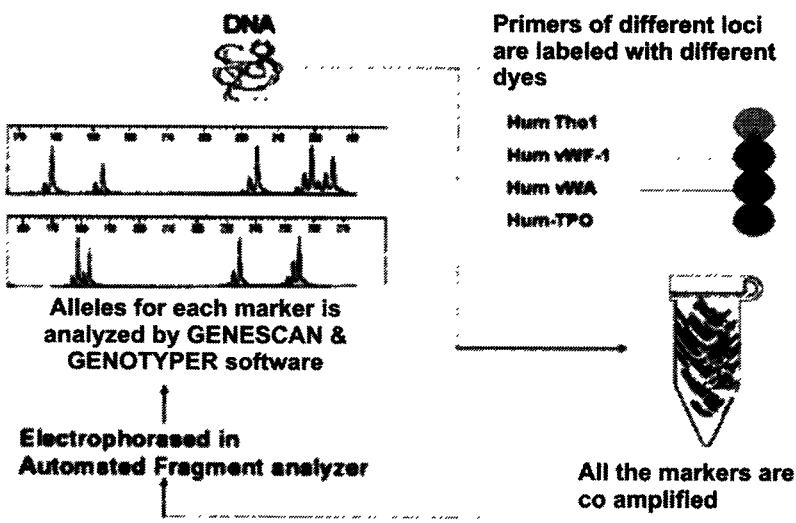

Figure 14.3: Scheme of automated fragment analysis of STR multiplex

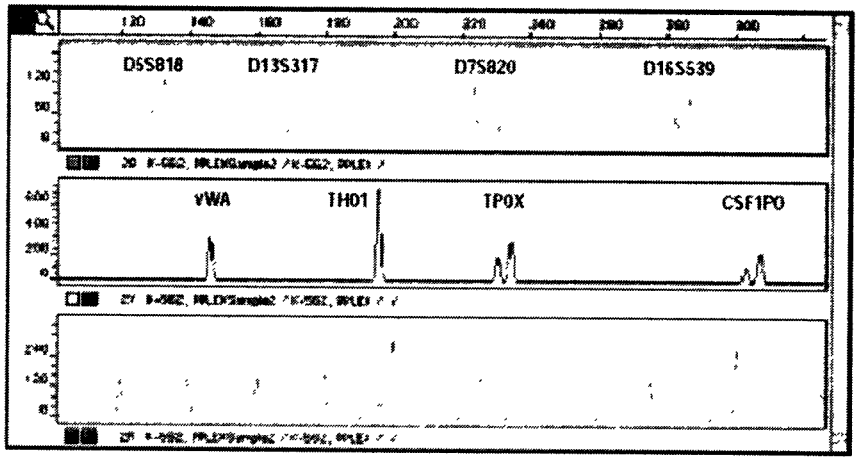

Figure 14.4: A chromatogram of three STR loci amplified by multiplex PCR and analyzed by Gene scanning on ABI 310 genetic analyzer

3. Few relatively smaller peaks of stutter bands.
4. Sizing of each peak can be done by a base pair size ruler, which is labeled with a separate fluorescent dye (mainly ROX which gives RED peaks)

Methodology and essentials of automated DNA analyzer:

1. Fluorescent dyes labeled primers:

One of the primers in each PCR system is labeled with one fluorescent dye:

1. FAM: Blue colour dye, which gives blue colour peaks.
2. VIC: Green colour dye, which gives green colour peaks.
3. NED: Yellow colour dye, which gives black colour peaks.
4. JOE: Orange colour dye, which gives orange colour peaks.
5. ROX: Red colour dye which use to label the base pair sizing ruler.

2. Ampli *Taq* Gold -FS

It is a special type of Taq polymerase, which is used specifically for DNA fragment size analysis because of following reasons:

(a) It carry two point mutations: **first removes 5'-3'Exonuclease** activity while second point mutation increase **Synthetase activity.**

(b) It gives low noise, clean data and uniform peaks.

(c) It works successfully with large size fluorescent dyes in a multiplex system in a single reaction.

3. ABI 310 genetic fragment size analyzer

The ABI 310 Genetic Fragment Size analyzer is an automated instrument for analyzing fluorescent-labeled DNA fragment by capillary electrophoresis **(Figure 14.5)**. It carries following essential parts:

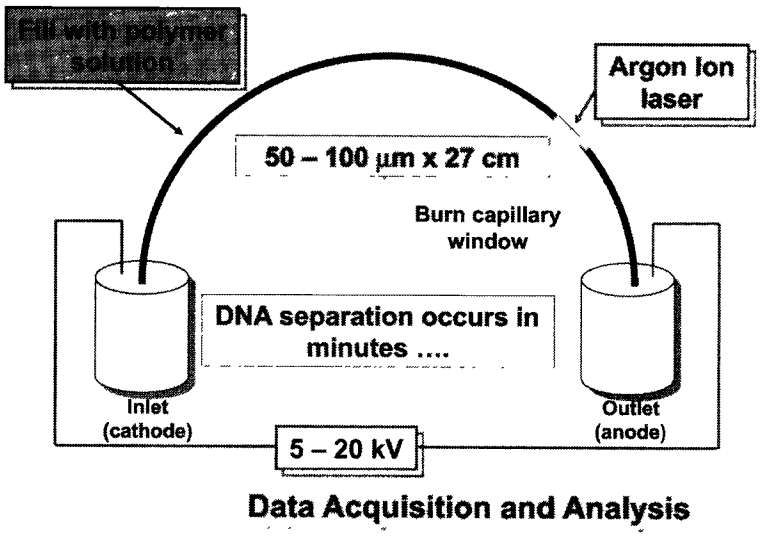

Figure 14.5: Electrophoresis and detection system in ABI 310 genetic analyzer

4. Capillary

Capillary is very narrow in size. It is made up of fused Silica. Outer coating of the capillary is made up of polyimide, which is burnt away to form Detection window at which laser hits. It carries the separation medium - High performance optimized polymer **(POP-4).**

5. Software files

Different software files play a very important role in successful sequencing operation as well as in analyzing the data obtained. These files and their role are mentioned in **Table 14.3.**

Table 14.3: Different software files and their functions

Software File	Function
Gene Scan Sample sheet	It keeps the sample information and sample tube position in the auto sampler tray
Gene Scan Injection List	Defines the order of the sample to be run and on which module
Gene Scan Module Files	Defines the run conditions like voltage, temperature and current of the electrophoresis
Mobility Files	Defines that which dye chemistry is used and which virtual filter set should be used like filter set "D" should be use with the above mentioned dyes
Matrix File	Avoids overlap of the peak of different color, specific for particular dye chemistry

Protocol for Automated DNA fragment sizing

Multiplexing of three autosomal loci

We will multiplex three autosomal STR loci labeled with three different dyes.

1. D5S3156 Labeled with NED
2. D18S848 Labeled with FAM
3. D11S2010 Labeled with VIC

The total protocol for automated DNA fragment analysis (Gene scanning) is divided into five major stages:

1. Multiplex PCR of the three loci labeled with different dyes
2. Processing of the PCR product for capillary electrophoresis
3. Capillary electrophoresis of the product in ABI 310 genetic analyzer.
4. Analysis of the Gene scan data

1. Multiplex PCR of the three loci labeled with different dyes

(a) Three loci will get co-amplified using the following primer pairs:

D5S818:
Forward: 5' GGGTGATTTTCCTCTTTGGT3'
Reverse: 5' TGATTCCCATCATAGCCACA 3'
D18S848:
Forward: 5' TGACAAAGAAACTAAAATGTCCC 3'
Reverse: 5' AAAGCTACATCCAAATTAGGTAGG 3'
D11S2010:
Forward: 5' TTTTCAGGCTTTATCTCATTCA 3'
Reverse: 5' GGGACATATGAGGGCTCTCT 3'

(b) Reaction mix is prepared as shown in **Table 14.4.**

Table 14.4: PCR reaction mix for Y-STR mutiplex PCR

Reagents	For 1 reaction (25µl)	For 5 reactions (25x5=125µl)
10 X buffer	2.5µl	12.5µl
dNTP mix (10uM/ul)	2.0µl	10µl
Each F-primer (D5S3156, D8S12540, D11S316) 10pm/ul	1.0x3µl	1.0x3x5µl
Each R-primer (D5S818, D18S848, D11S2010) 10pm/ul	1.0x3µl	1.0x3x5µl
Ampli Taq Gold (5U/ul)	1.0µl	5µl
DdH2O	14µl	70µl
DNA (100ng/ul)	2µl	2x5µl

(c) PCR is to be carried out in a Thermo cycler according to the conditions shown in **Table 14.5.**

Table 14.5: PCR conditions for Y-STR multiplex PCR

Step	Temperature	Time	Cycles
Denaturation	94°C	30 sec	30 Cycles
Annealing	48°C	30 sec	
Extension	72°C	1 min	
Final extension	94°C	5 min	1 Cycle
Hold	15°C	Forever	

(d) PCR product is run on 2% Agarose to check the amplification.

2. Processing of the PCR product

1. Take out the PCR tubes from the Thermo cycler after the PCR is completed.
2. Take 5 µl of PCR product in 0.5ml tubes.
3. Add 0.5 µl of ROX labeled base pair size ruler in each tube.
4. Add 15 µl of Hi-di-formamide in each tube and vortex it properly.
5. Denature the mix at 96°C for 5 minutes.
6. Snap cool in ice for 10 minutes and load the sample in auto sampler tray for electrophoresis.

3. Capillary electrophoresis in ABI 310 genetic analyzer

The processed PCR product is then transferred into genes scanning tubes and closed with the septa (care should be taken to check that septa are dissected from the middle).

1. Switch on the computer set.
2. Put the capillary upwards.
3. Switch on the ABI 310 genetic analyzer.
4. Launch the ABI 310 data collection software in the Macintosh computer.
5. Allow the syringe to go to home for about 5 minutes.
6. Go to the **"WINDOW"** option and launch **"MANUAL CONTROL"** and **"STATUS"** options.
7. Set the temperature from **"MANUAL CONTROL"** to $60^{O}C$.
8. Go to the **"INSTRUEMENT"** option and launch **"AUTOSAMPLER CALLIBRATION"** window.
9. Keep the capillary 0.5 cm down the cathode.
10. Calibrate the capillary against the black dot placed on the auto sampler tray.
11. Fill the capillary with **POP-4** from **"SEQ FILL CAPILLARY"** option in **"MANUAL CONTROL"** window (this step is followed only when required).
12. Go to the **"FILES"** option and launch **"GENE SCAN SAMPLE SHEET"** options for 48 tubes tray.
13. Type the sample name and details.
14. Select the **"MOBILTY FILE"** and **"MATRIX FILE"**.
15. Save and close the file by the date name.
16. Go to the **"FILES"** option and launch **"GENE SCAN INJECTION LIST"** options for 48 tubes tray.
17. Open the recently made sample sheet.
18. Select the **"MODULE FILE"** and **"VIRTUAL FILTER"**.
19. Perform the **CCD-TEST** to check the performance of laser.
20. Close the file and save it to Desktop.
21. Restart the computer.
22. Place the samples in auto sampler tray according to their positions mentioned in Sample sheet.
23. Launch the **"GENE SCAN INJECTION LIST"** saved on desktop.
24. Start the run.

4. How the capillary electrophoresis proceeds

It works in the following manner:

a) The Gene scanning reaction tubes are placed" in an auto sampler tray carrying either 48 tubes.

b) The auto sampler successively brings each sample in contact and brings each with cathode electrode and one end of glass capillary

filled with polymer.

c) The other end of the capillary gets immersed into the buffer carrying the anode electrode.

d) The sample enters the capillary as current flows from cathode to anode. This short period is termed as **"Electrokinetic Injection"**. The sample forms a tight band in the capillary during this injection.

e) The end of the capillary and electrode is then placed into buffer.

f) Current is applied again to continue the electrophoresis.

g) When DNA fragments reach ignition window in the capillary, a laser ignites the fluorescent-labeled fragment.

h) Emitted fluorescence from dyes is collected by a cooled **Charged Coupled device (CCD) camera** at a particular wavelength band **(Virtual filters)**.

i) This is recorded as digital signals in a **Power Macintosh computer system.**

j) The data is analyzed by **"GeneScan software 3.2"** and Genotyping can be done by **"Genotyper software 4.1.2".**

k) The result obtained on analysis are shown in **Figure 14.6.**

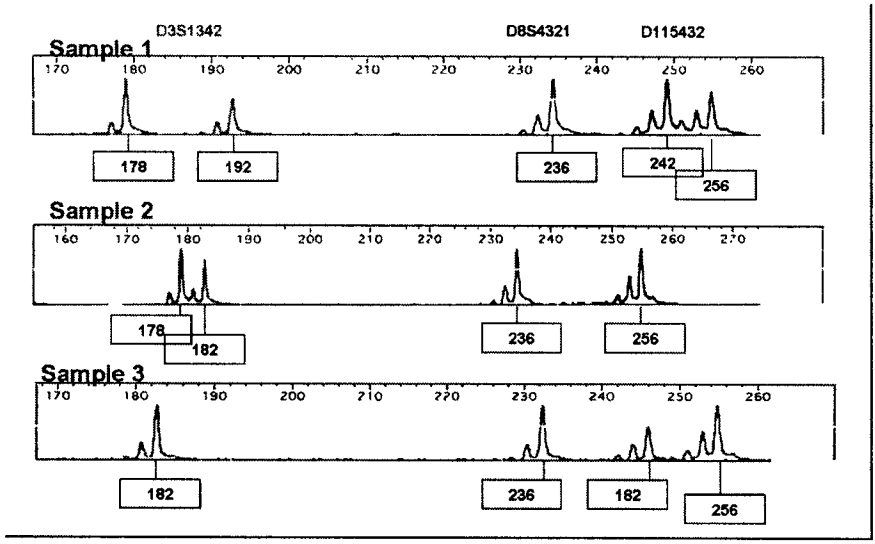

Figure 14.6: A gene scan result of three loci amplified in three different samples

5. Data analysis and genotyping:

Gene Scanning and Genotyping of the obtained data is discussed in the Chapter "GeneScanning and Genotyper software" in latter chapters of the book.

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Chapter 15

DNA Recombinant Technology

In nature, gene transfers are rather imprecise, and their range, in different species is involved, however, it is remarkably limited. The above problems are circumvented by the recombinant DNA technology. A recombinant DNA molecule is produced by joining together two or more DNA segments usually originating from different organisms. More specifically, a recombinant DNA molecule is a vector into which the desired DNA fragment has been inserted to enable its cloning in an appropriate host. This is achieved by using specific enzymes for cutting the DNA (restriction enzymes) into suitable fragments and then for joining together the appropriate fragments (ligation). During last twenty years, studies of cloned DNA sequences have given us a detailed knowledge of gene structure and organization, and have provided clues to the regulatory pathways by which the cell controls gene expression in the multiple cell types comprising the basic vertebrate body plan. Genetic engineering, by which an organism can be modified to include new genes designed with desired characteristics, is now routine practice in basic research laboratories. It has provided the means to produce large amounts of highly purified normal and mutant proteins for detailed analysis of their function in the organism.

Recent advances in this technology have also changed the course of medical research. Exciting new approaches are being developed to exploit the enormous potential of recombinant DNA research in the analysis of genetic disorders. The new ability to manipulate human genetic material has opened radically new avenues for diagnosis and treatment, and has far-reaching consequences.

Recombinant DNA molecules are produced with one of the following three objectives: (1) To obtain a large number of copies of specific DNA fragments, (2) To recover large quantities of the protein produced by the concerned gene, or (3) To integrate the gene in question into the chromosome of a target organism where it expresses itself. Even for the latter two objectives, it is essential to first obtain

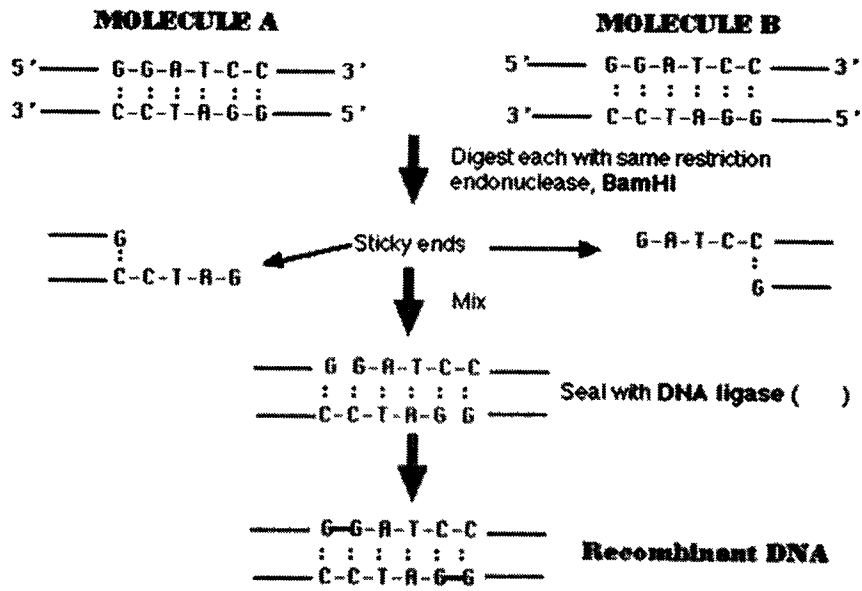


Figure 15.1: Creation of recombinant DNA

a large number of copies of the concerned genes. All these steps are explained in **Figure 15.1.**

To achieve this, the DNA segments are integrated into a self-replicating DNA molecule called vector; most commonly used vectors are either bacterial plasmids or DNA viruses. All these steps concerned with piecing together DNA segments of diverse origin and placing them into a suitable vector together constitute recombinant DNA technology.

The DNA segment to be cloned is called DNA insert. Recombinant DNAs are introduced into a suitable organism, usually a bacterium; this organism is called host, while the process is called transformation. The transformed host cells are selected and cloned.

The recombinant DNA present in such clones would replicate either in synchrony with or independent of the host cell; the gene present in 'the vector may or may not express itself, i.e., direct the synthesis of concerned polypeptide. The step concerned with transformation of a suitable host with recombinant DNA, and cloning of the transformed cells is called DNA cloning or gene cloning.

However, often DNA or gene cloning is taken to include both the development of recombinant DNAs as well as their cloning in a

suitable host. Similarly, often the term recombinant DNA technology is used as a synonym for DNA or gene cloning used in the broader sense. A rather popular term for these activities is genetic engineering.

A clone consists of asexual progeny of a single individual or cell, while the process/technique of producing a clone is called cloning. As a result, all the individuals of a clone have the same genotype, which is also identical with that of the individual from which the clone was derived.

Therefore, the genomes present in members of a single clone are also identical; this applies to the recombinant DNA as well. Therefore, gene or DNA cloning produces large numbers of copies of the gene/ DNA being cloned.

Recombinant DNA technology, often known as Gene cloning or cloning which involves - isolation of a fragment of a genome (an entire gene, or other sequence of interest), and incorporation in a "replicon or vector", that is replicated independently of the original DNA molecule. It includes following steps:

1. **Isolation** of plasmid DNA (or any vector)
2. **Digestion** with restriction enzymes
3. **Purification** of the required fragment
4. **Ligation** of fragment into new vector
5. **Transformation** of *E.coli* strain with new plasmid
6. **Selection** of recombinant plasmids
7. **Analysis** of recombinant plasmids
8. **Replication** of recombinant strains
9. **Production** of identical copies

Requirements for Gene Cloning or DNA recombinant technology:

1. Vector
2. Host Organisms
3. Restriction enzymes
4. Selection of markers for cloned colony screening

1. Vectors in Cloning:

In every case, the recombinant DNA must be taken up by the cell in a form in which it can be replicated and expressed. This is achieved by incorporating the DNA in a **Vector**. Some of the important vectors used for cloning includes:

(a) Plasmids:

Plasmids are molecules of DNA that are found in bacteria but are separate from the bacterial chromosome. They are circular DNA molecule, a few thousand base pairs and usually carry only one or a few genes. DNA fragment of upto 5Kb can be inserted into a plasmid **(Figure 15.2)**.

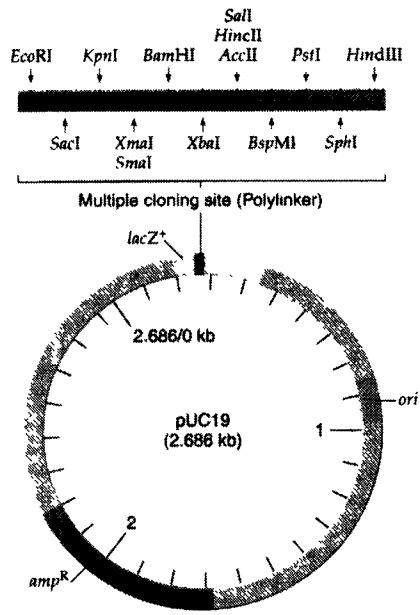


Figure 15.2: Plasmid, its selection site and insert size

The properties of Plasmids which make them ideal for cloning includes:

1. Origin of DNA replication -

That helps in replication of the DNA fragment once inserted into the plasmid.

2. A dominant selectable marker -

Usually resistant to an antibiotic Ampicilin - R or Tetracycline-R) that helps in detecting the presence or absence of recombinant DNA in the host organisms.

3. Unique restriction cleavage sites: -

This helps in the digestion of the plasmid and DNA of interest from the same site, so that they can be ligated together. Moreover, these restriction sites sometimes present in a gene like Lac-Z gene, whose disruption on digestion acts as scorable marker for confirmation of recombination.

(b) Cosmids:

A genetically engineered plasmid that contains the *COS* sites of ë-phage DNA, a drug-resistance gene, and a replication of origin **(Figure 15.3)**.

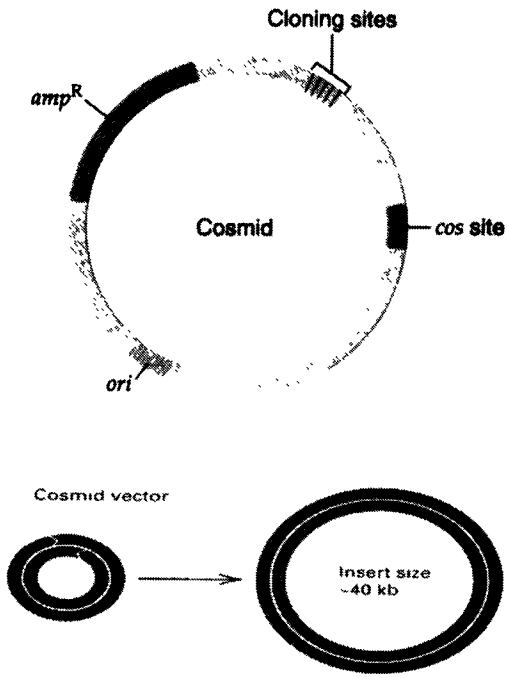


Figure 15.3: Cosmid Vector

1. Cos sites are DNA sequences that allow DNA up to 50 kb in length to be packaged into the λ-phage head.
2. Allows large fragments to be cloned.

(c) Phage λ DNA:

Phage λ DNA is Bacteriophage DNA, which has copy numbers, an Ampicilin-resistance gene and multiple cloning site or poly-linker.

1. Gene can be inserted into λ phage and the recombinant phage is used to infect E. coli.
2. Insert could be upto a gene of 50Kb.
3. The inserted gene will be expressed by the bacteria.

(d) Retroviruses:

Retroviruses can infect virtually any type of mammalian cell and is a common vector to clone DNA in mammalian cells.

1. Contains reverse transcriptase, RNA converted to DNA sequence.
2. The viral DNA integrates into host chromosomal DNA where it

is retained as a provirus, replicating with host DNA at each cell division.

3. Some of the retroviral genes are removed so the vector cannot produce viral particles.
4. Strong promoter region located at the 5′end in a sequence called long terminal repeat (LTR) are left intact.
5. If gene is placed in retroviral vector and the vector is used to infect mammalian cells, the gene will be expressed under control of retroviral promoter region.

(e) Yeast artificial chromosome (YAC):

Yeast Artifical Chromosomes, or YACs, are often the only way to clone extremely large genes including huge introns all in one continuous piece. YACs also provide a way to propagate DNA in a eukaryotic cell, where DNA modification, takes place in an important part of the eukaryotic genetic regulatory machinery, these changes are more likely to be retained **(Figure 15.4)**. YAC carries number of essential features suitable for cloning:

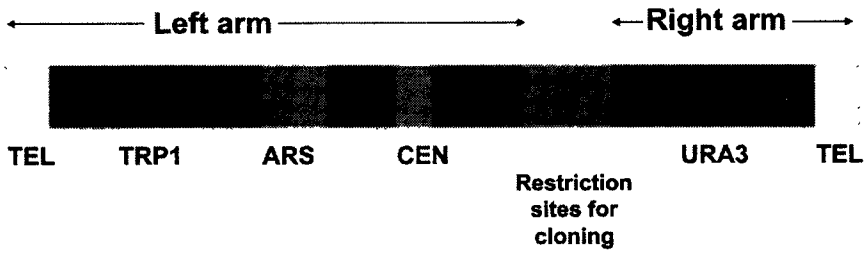


Figure 15.4: Yeast artificial chromosome as a vector

1. A yeast telomere (TEL)
2. A yeast centromere (CEN)
3. A selectable marker (TRP1 and URA3)
   (a) TRP1 (Tryptophan and uracil is dependent)
   (b) URA3 (Mutant strain)
4. Origin of replication sequences (ARS)
5. 10 unique restriction site are present.

II- Host organisms

Number of host organism are used in which the cloned gene or the recombinant DNA is transformed or transfected. The host organism

provides the cellular environment and replication machinery required for the replication of the recombinant DNA. Some of the host organisms are :

1. Standard host- *Escherichia coli*
2. Other bacteria - *Bacillus subtilis, Streptomyces* species
3. Yeast- *Saccharomyces cerevisiae*
4. Insect, animal and plant cells in culture
5. Whole animals and plants

III- Enzymes required:

A battery of enzymes are required to carry out the process. They include:

1. Restriction Endonucleases

These are called DNA scissors, which cut DNA at specific sequences, called restriction sites, within the sequences. They are totally different from DNases, which do not recognize specific sites and randomly cleave DNA into variable series of small fragments (**Figure 15.5**).

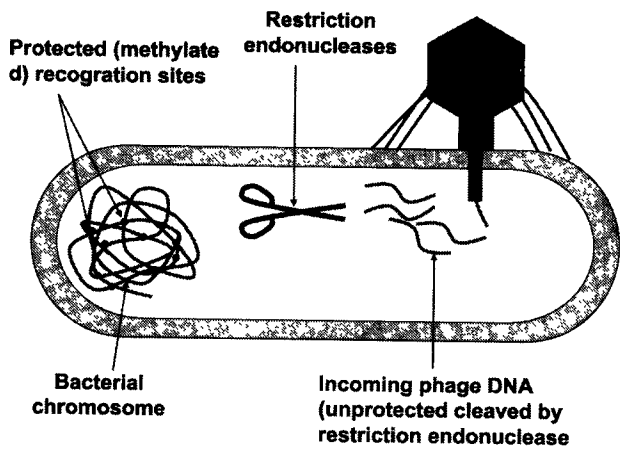


Figure 15.5: Restriction enzymes acts like DNA scissors

2. DNA ligase (from phage T4)

These enzymes are responsible for ligating the vector and DNA fragment already digested with the same restriction enzyme. **Figure 15.6** explains how DNA restriction Endonuclease and DNA ligases works in tandem to form the recombinant DNA.

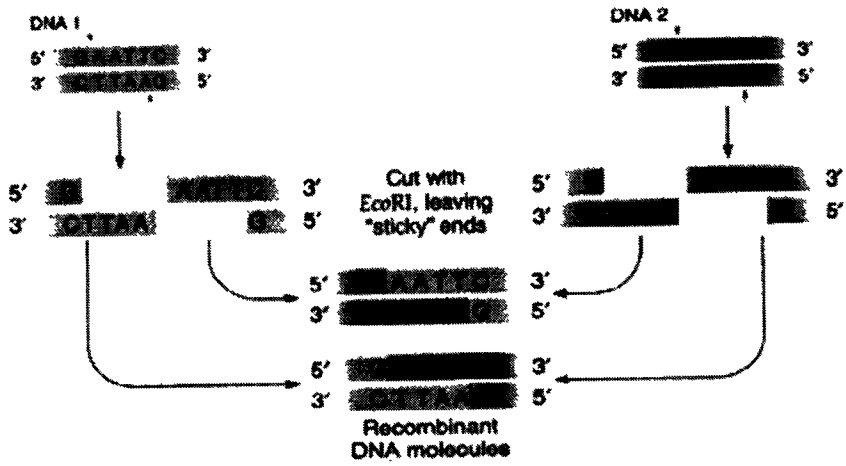


Figure 15.6: Function of restriction Endonuclease and ligase

3. Alkaline phosphatase

Removes Phosphate groups at the 3′ end of the fragments, which helps in ligation of the two fragments by adapters.

4. Polynucleotide kinase

Adds Phosphate groups at the 3′ end of the fragments, which helps in the ligation of the two fragments by Poly linkers.

5. Terminal transferase

Add Poly C at the 3′ end of vector and Poly G at 5′ end of the fragments, so that they can be ligated without the help of linkers or adapters.

6. Reverse transcriptase

This particular enzyme carry the c-DNA synthesis from the m-RNA. This helps in cloning of mature transcript sequence in the form of cDNA, as prokaryotic host i.e. bacteria can't process eukariotic gene II cloned as DNA.

7. *Taq* polymerase

Used for PCR amplification of the DNA fragment which is then eluted from the gel and is used in cloning.

8. RNAse A/RNAse H

It removes RNA from the sample mixture.

9. Nuclease S1

This enzyme digest the genomic fragment into smaller fragment, required for complete genome sequencing that is carried out by inserting each of these fragments in vectors.

Competent cells

They are the host organism cells in which recombinant DNA is transformed and replicated, these cells are highly capable of accepting DNA inserts, soaking in $CaCl_2$, which makes their cell membranes permeable to DNA inserts.

Overview of recombinant DNA formation

1. Creation of recombinant DNA

1. A plasmid vector is digested with a Restriction Endonuclease (RE) at a single site to produce two sticky ends.
2. A sample of human DNA is also digested with same RE to produce pieces with the same sticky ends.
3. Human DNA- or cDNA copied from mRNA using reverse transcriptase from retroviruses.
4. The two samples are mixed and allowed to hybridize, some molecules will get inserted into the plasmid vector at the particular RE site.
5. DNA ligase is used to covalently link the fragments **(Figure 15.7).**

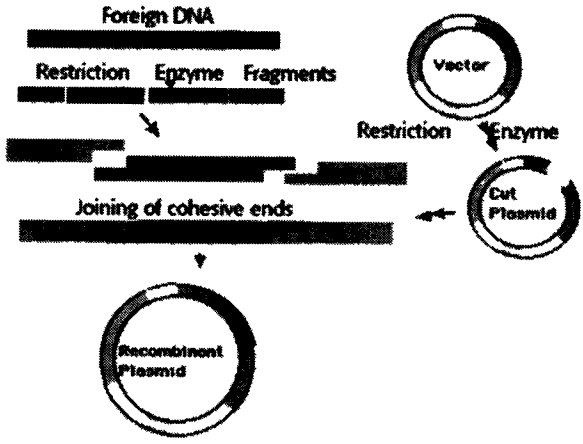


Figure 15.7: Formation of Recombinant DNA

2.Transformation

Recombinant DNA is then transformed into the competent cells of E. coli by giving it a heat shock at 42°C **(Figure 15.8).**

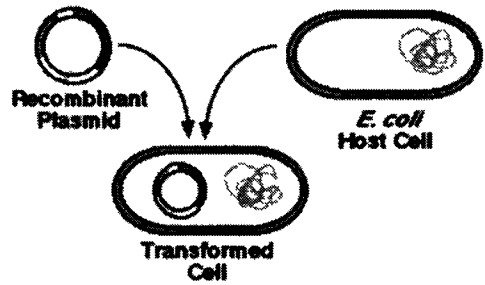


Figure 15.8: Transformation of vector into the E. coli cell

3. Selecting transgenic cells resistant to antibiotic

Plasmid vector contains an ampicillin resistance gene making the cell resistant.

Growth of transformed cells (cells receiving the plasmid) can be identified on agar medium containing (e.g.) ampicillin **(Figure 15.9).**

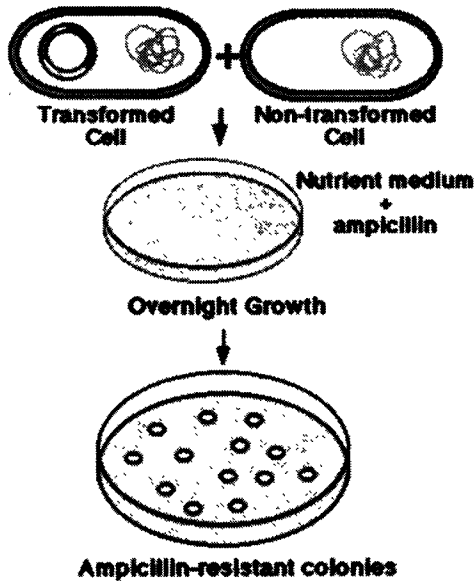


Figure 15.9: Selection of transgenic cells using Ampicillin resistance

4.Identifying clones

1. The plasmid vector contains another identifiable gene (e.g., a second drug resistance or an enzyme activity), with the coding sequence of this gene containing the restriction site for insertion.
2. Insertion of the foreign DNA at this site interrupts the reading frame of the gene and result in the insertional inactivation.
3. In the example shown below, the B-galactosidase gene is inactivated. The substrate "X-gal" turns blue if the gene is intact i.e. makes active enzyme. White colonies in X-gal imply the presence of recombinant DNA in the plasmid (**Figure 15.10**).

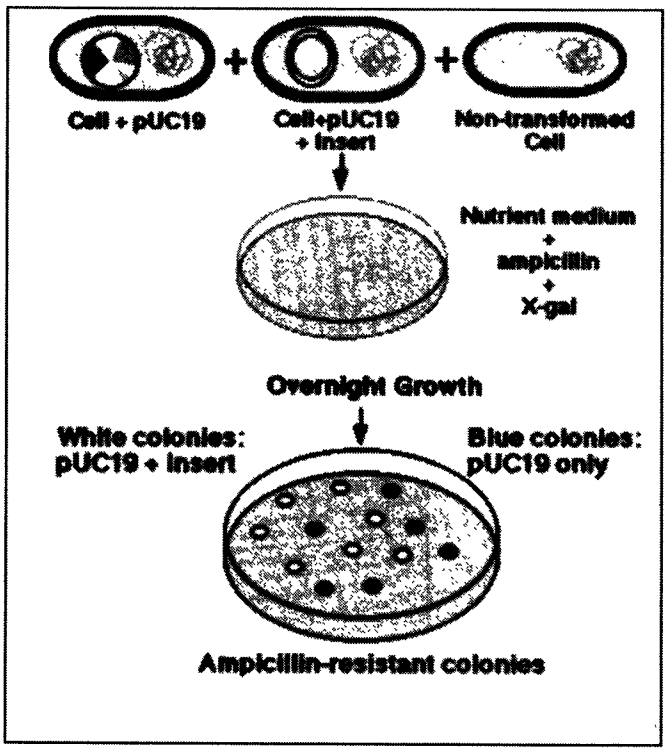


Figure 15.10: Identification of the clones

Protocol

Preparation of recombinant DNA

Cleavage with RE plasmid vector (PIC) and target DNA heating desired sequence is digested with appropriate restriction end nuclease.

This restriction digestion is carried out by incubating the DNA with RE at 37^0C.

Ligation

Example taken here is of HLA-F DNA fragment of 0.8kb (HLA F-probe) will be inserted into pUC vector in the following manner:

1. Ligation reaction mix

10X ligation buffer	2µl
Dephosphorylated pUC vector	2µl (0.5µg/µl)
Insert DNA (HLA-F probe)	2µl (0.5 µg/µl)
T4 DNA Ligase	1 Weiss unit
ddH_2O (make the volume up to)	20 µl

2. Mix and incubate at 15^0C for 2-3 hrs.
3. Inactivate the Ligase by heating it at 65^0C for 5 minutes.

Competent cell preparation

1. A single colony of E. coli DH5α strain in 5ml of LB broth is taken and grown at 37^0C in a shaking water bath for overnight.
2. Next day: transfer 5ml culture in a 100ml LB broth and keep at 37^0C for 3 hrs in shaken water bath.
3. Quickly chilled on ice and keep at 4^0C for 20 min.
4. Transfer into 4 tubes (25ml each), and centrifuge at 6000 rpm for 5 min at 4^0C. Discard the supernatant.
5. Keep on ice, add 1.5ml of 0.1M $CaCl_2$ (6% of the vol), suspend the pallet, keep on ice for 30 min.
6. Centrifuge at 6,000 rpm for 5 min at 4^0C, discard the supernatant.
7. Add 125µl (~ 8% of the vol) of $CaCl_2$ (0.1M), resuspend the pallet.
8. Transfer the total suspension (500µl) into 5, 2ml eppendorf tubes (100µl each).
9. Keep at 4^0C for 1 or 2 days and at - 70^0C for long-term storage.

Transformation

1. Take a vial of competent cell, keep on ice, add plasmid DNA of 20 - 50 ng in a vol. of 4 -10 µl mix by tipping, keep on ice for 20 min.
2. Keep the vial at 42^0C water bath for 2 min, and immediately cool it on ice for 20 min.
3. Add 1ml of LB broth and incubate at 37^0C in a shaking water bath for 1 hr.

Plating

1. Take 100µl of broth, plate on LB Amp. Plate.
2. Take a negative control (with plasmid) and plate on LB Amp plate.
3. Keep at 37^0C for overnight.

Preparation of LB amp plate

1. Weigh 3.5 g LB agar mix in 100 ml of ddH_2O in a 250 ml flask.
2. Autoclave at 121^0C for 30 min.
3. Allow to cool at 50 -55^0C, add 100µl Ampicillin (100mg /ml) and mix.
4. Pour in 4-culture plate (25ml each allow to settle) carrying 350 µl of IPTG (24mg/ml) and 70µl x Gal (50mg/ml).

Isolation of recombinant DNA (Plasmid+insert) by MINI PREP

1. Reagents required

(A) STE

NaCl	0.1M
Tris Cl	10mM
EDTA	1mM

(B) Solution - 1

Glucose	50mM
Tris Cl	25mM
EDTA	10mM

(C) Solution - 2

NaOH freshly diluted from 1N NaOH	0.2N
SDS	1%

(D) Solution - 3

K-acetate	5M
Glacial acetic acid	11.5%

Protocol

1. Wash bacterial cell pallet with 2 ml STE by centrifuging of 6,000rpm for 4 min.

2. Add 100µl ice-cold solution -I
   200µl ice-cold solution -II
   150µl ice-cold solution -III (freshly prepared)
3. Vortex 4 -5 time to re-suspend the pallet.
4. Keep on ice for 5 min.
5. Centrifuge at 12,000 rpm for 5 min.
6. Take supernatant (~ 400µl).
7. Add 2 vol Ethanol (absolute).
8. Keep for 2 - 3 min at room temperature (RT).
9. Centrifuge at 12,000 rpm for 5 min.
10. Decant the supernatant.
11. Rinse with 1ml 70% ethanol by centrifuging 12,000 rpm for 2 min
12. Air dry.
13. Dissolve in 30µl HPLC water.
14. Add 1µl RNase (10mg/ml).
15. Keep at 37^0C for 1 hour.
16. Run on 1% agarose gel.
17. Store at - 70^0C.

Purification of plasmid DNA by Cs-Cl gradient method

1. Plasmid DNA (70 ngms/µl) is dissolved in 250 µl of sterile water.
2. 1 ml of TE buffer (pH 8.0) is added to make final volume of 1.25 ml.
3. 1.8 gms of finely powdered CsCl is added and dissolved properly. Then volume of reaction mixture was set at 2 ml.
4. It is then filled in BECKMAN 2 ml quick sealed tubes and the tubes are sealed properly.
5. 40 µl of Ethidium bromide (10 mg/ml) is then added to these tubes with a syringe and needle.
6. It is then centrifuged at 60,000 rpm for 16 hrs in ultracentrifuge.
7. DNA band formed in the middle is then recovered by piercing a 18 gauge needle in tube and the band is sucked out.
8. This sample is then dialyzed at 4^0C for overnight against TE-buffer (pH 8.0).
9. This plasmid solution is then dialyzed at 4^0C for overnight against TE-buffer (pH 8.0).

10. Plasmid is then precipitated in ethanol and 3 M sodium acetate (3:0.1) and incubated for overnight at -20^{0}C.
11. Centrifuged at 10,000 rpm for 10 min at room temperature.
12. Pellet is dissolved in 200 µl of sterile water and checked by gel electrophoresis using 0.8% agarose gel running at 50 V at 1 hr.

Recovery of cloned DNA / Gene from plasmid

Restriction digestion of plasmid

1. PUC containing 0.8kb of HLA-F probe
2. Take following in 0.5ml eppendorf tube
   (a) 10x buffer : 2µl
   (b) Plasmid DNA: 8µl (~8µg)
   (c) Hind III : 2µl (10U/µl)
   (d) Sac I: 2µl (10U/µl)
   (e) HPLC g water: 6µl
   (f) (ddH_2O) 20 µl
3. Mix and keep at 37^{0}C for 2 - 3 hrs.

Gel electrophoresis

1. Prepare 1% Agarose gel.
2. Add 5µl gel loading dye in digested plasmid load on to the well, also load MW marker in a adjacent well.
3. Run at 4 V/cm for 1.5 - 2hrs.

Purification of DNA from gel

1. Cut the band of interest from the gel, weigh and put in a 2ml of eppendorf tube.
2. Add 1ml Agarose solublization buffer (1ml / 100mg).
3. Add 10µl silica gel and mix it.
4. Keep at 56 - 60^{0}C for 10 min, vortex at every 2 -3 min.
5. Spin for 30 sec, discard supernatant.
6. Add 500 µl Nucleic Acid, binding buffer vortex, spin for 30 sec; discard the supernatant.
7. Add 500µl of washing buffer, resuspend the pallet by vortex.
8. Spin for 30 sec, discard the supernatant.
9. Repeat the washing one more time.

10. Add 25 - 30 µl of TE (pH - 8), vortex and keep at 56-60^{0}C for 10 min with vortex at every 2 -3 min.
11. Spin for 30 sec and take the supernatant in a new 0.5ml eppendorf tube.
12. Again add the TE (30µl), keep at 56 - 60^{0}C for 10min with repeated vortexing.
13. Spin for 30 sec and collect the supernatant in eppendorf.
14. Check the eluted product on 2% Agarose gel.

Important steps in DNA recombinant technology

Ligation with T4 DNA Ligase

Properties	Catalyzes the formation of phosphodiester bonds between adjecent 3′ -OH and 5′ -P ends in dsDNA. Closes single-stranded nicks in dsDNA.Needs ATP as co-factor.
Ligation buffer, 10x	660 mM Tris, 50 mM $MgCl_2$, 50 mM DTT, 10 mM ATP, pH 7.5 at 20^{0}C. Buffer is stable at -20^{0}C, store in aliquots. Note: ATP is not stable and decreased concentrations of ATP largely influence the ligation efficiency: aliquot ATP, store at -20^{0}C and add to ligation mix before use.
Tip	The Rapid DNA Ligation Kit enables the ligation of sticky and blunt ends in just 5 minutes at room temperature. The system contains a specially formulated- ready - to -use -T4 DNA Ligation buffer including all necessary components in a stabilized form. Note: This kit contains PEG and cannot be used - without further purifications - for transformation of bacteria via electroporation.
Molar ratio of vector and fragment DNA	Stick ends: When vector DNA and insert DNA are ~ similar in length: a molar ratio of 1:3 (vector versus insert DNA) is recommended. When vector DNA and insert DNA are not similar in length: a molar ratio of 1:1 or 1: 2 (vector versus insert DNA) is recommended. Blunt ends: a molar ratio of vector DNA to insert DNA of 1: 5 is recommended.

Application and typical results

Standard assay : Ligation of DNA fragments is shown in **Table 15.1.**

Table 15.1

Components	Sticky ends	Blunt ends
Template DNA	Up to 1 µg digested DNA	Up to 1 µg digested DNA
10 x Ligation buffer	3 µl	3 µl
T4 DNA Ligase	1 -5 units	1 - 5 units
H_2O	Add up to 30 µl	Add up to 30 µl
Incubation	4 to 16°C, overnight	16 to 25°C, overnight

Sticky ends: >95% of the DNA is ligated, depending on type and quality of restriction enzyme.

Blunt ends: > 80% of the DNA is ligated, depending on type and quality of restriction enzyme.

Inactivation of enzyme

T4 DNA ligase can be completely inactivated by a 10 min incubation at 65°C. Heat inactivation should only be done if the ligation reaction mixture is used in experiments other than transformation assays. Other - wise, a drastic decrease of (> factor 20) transformats is possible.

Dephosphorylation with shrimp alkaline phosphatase

Properties

- Catalyzes the dephosphorylation of 5′ - protruding, 5′ - recessive and 5′ blunt ends from ssDNA, dsDNA, ssRNA and dsRNA.
- Is completely and irreversible inactivated by heat treatment for 15 min at 65°C.

Dephosphorylation buffer, 10x

- 0.5 M Tris, 50 mM $MgCl_2$, pH 8.5 at 20°C
- Buffer is stable at -20°C.

Inactivation of enzyme

- Unlike calf intestinal phosphatase, SAP is completely and irreversible inactivated by heat treatment for 15 min at 65°C.
- The total procedure including restriction enzyme digestion, dephosphorylation, enzyme inactivation and ligation can be performed in one single tube by using Roche Molecular Biochemical's Shrimp alkaline Phosphatase and Rapid DNA Ligation kit.

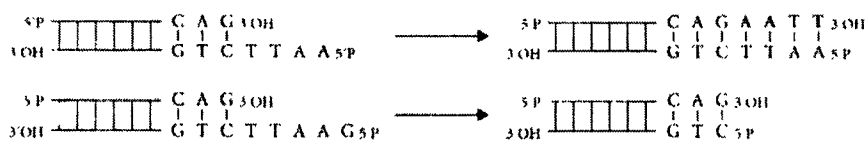
Application and typical results

- Standard Assay: Dephosphorylation of 5'ends of dsDNA fragments to prevent self-annealing of vector DNA prior to the insertion of DNA fragments is shown in **Table 15.2.**

Table 15.2

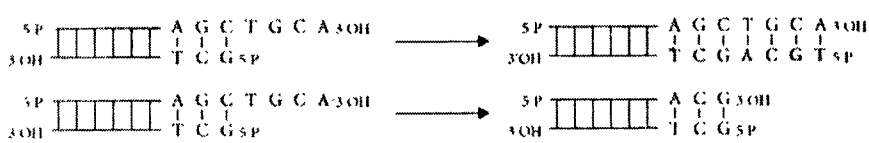
Components	Recessive or protruding ends	Blunt ends
Template DNA	1 pmol	0.2 pmol
10 x Dephosphorylation buffer	2 µl	2 µl
Shrimp Alkaline Phosphatase	1 unit	1 unit
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	10 min at 37°C	60 min at 37°C

Modifying sticky ends to blunt ends: 5′ protruding and 3′ recessed ends:



	Klenow	T4 DNA polymerase	T7 DNA Polymerase	Mung Bean Nuclease
Fill -in 3′ recessed ends	✓	✓	✓	✗
Remove 5′ protruding ends	✗	✗	✗	✓

Modifying sticky ends to blunt ends: 3′ protruding and 5′ recessed ends:

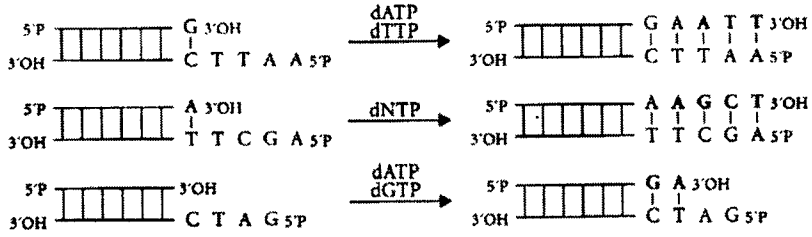


	Klenow	T4 DNA polymerase	T7 DNA Polymerase	Mung Bean Nuclease
Fill -in 3′ recessed ends	✗	✗	✗	✗
Remove 5′ protruding ends	✗	✓	✓	✓

Klenow - for partial or complete filling of 3′ recessed ends

Properties

- DNA dependent 5′ - 3′ polymerase with 3′ - 5′ exonuclease activity catalyzing the addition of mononucleotides from dNTP's to the 3′ OH terminus of a primer / template DNA.



Filling buffer, 10 x

- 500 mM Tris (pH 7.5), 100 mM $MgCl_2$, 10 mM DTT, 500 µg/ml BSA.
- Buffer is stable at - 20°C, store in aliquots.

Application and typical results

- Standard Assay: Partial or complete filling of 3′ recessed ends.

Components	Complete filling	Partial filling
Template DNA	1 µg DNA	1 µg DNA
Nucleorides, final concentration	1mM of desired dNTP's each	1 mM of desired dNTP's each
10x filling buffer	2 µl	2 µl
Klenow	1 unit	1 unit
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	15 min at 37°C	15 min at 37°C

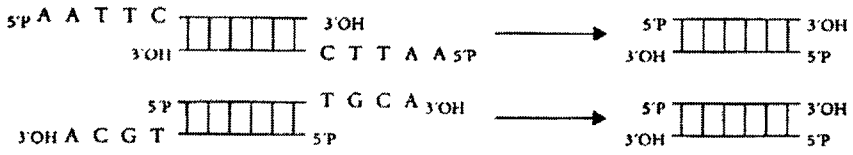
Inactivation of enzyme

- Add 2 µl 0.2 M EDTA and / or heat to 65°C for 10 min.

Mung Bean Nuclease - for removing of 3′ and 5′ protruding ends

Properties

- Degrades ssRNA and ssDNA to produce 5′ - phosphoryl oligo - and mononucleotides.
- dsDNA, dsRNA and DNA : RNA hybrids are relatively resistant to the enzyme.



Nuclease buffer, 10x

- 300 mM sodium acetate, pH 4.6, 500 mM NaCl, 10 mM zinc acetate, 0.01% Trition X-100.
- Buffer is stable at - 20°C, store in aliquots

Application and typical results

- Standard Assay:
- Removing 5′ and 3′ protruding ends of DNA creating blunt ends.

Components	Removing ends
Template DNA	1 µg DNA
10x Nuclease buffer	10 µl
Mung Bean Nuclease	5 units
H_2O	Add up to 100 µl
Incubation	1 hour at 25°C

Inactivation of enzyme

- Add EDTA to a final concentration of 1 mM or SDS to a final concentration of 0.01%.

Miscellaneous

Competent cell and transformation

- For a good overview of the different procedures, please refer to
- Ready -to - use competent cells can also be obtained from various commercial suppliers.

Commonly used E. coli strains

- For a good overview on the genotypes and references of the most commonly used E. coli strains, please refer to "Molecular Biology LABFAX, edited by T.A. Brown, Bios scientific publishers, ISBN, 1 872748 00 7".

Commercially available cloning kits

- A wide variety of different cloning systems and kits are available from different suppliers.
- Roche Molecular Biochemicals offers three systems:
- PCR Cloning Kit: for cloning blunt-end PCR products up to 10kb.
- Expand Cloning Kit: for cloning blunt-end PCR products from 7 to 36 kb.
- Rapid DNA Ligation Kit: enabling ligation of sticky and blunt-end fragments within 5 minutes.

General considerations

Template

- The higher the purity of the DNA template, the better the labeling efficiency.
- For the random primed DNA labeling method, it is critical that the template is linearized and completely heat-denatured prior to the labeling reaction.

Choice of labeling method

- The choice of labeling method depends on:
- The type of application (e.g. Southern, Dot blot...)
- The available template (e.g. Cloned insert. Oligonucleotide,...)
- The requested sensitivity (e.g. single copy gene detection, ...)

Purification of labeled probe

- Unincorporated labeled nucleotides should be removed from the labeling mix:
- Removal from DNA fragments via the High Pure PCR Product Purification Kit or via ethanol precipitation.
- Removal from oligonucleotides: via ethanol precipitation with Glycogen (20 μg/reaction) or G-25 columns.
- This enables a more accurate quantification of the incorporated label and reduces background in hybridization experiments.

Type of label

- Several non-radioactive methods have been developed. The use fo these labels (e.g. biotin, digoxigenin, fluorescein, ..) offers several advantages:
- The technology is safe.
- Probes can be stored for a longer period compared to radioactive probes.
- Hybridization solutions can be reused several times.
- For an overview of the non-radioactive systems available.

Overview of different techniques

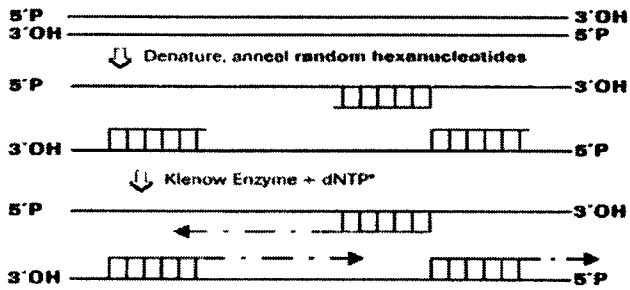
Application	Labeling methods	Relative sensitivity
Southern blotting	Random primed labeling	+++
Northern blotting	PCR labeling	+++
	5′ end labeling	++
	3′ end labeling	++
Dot/Slot Blotting	Random primed labeling	+++
	PCR labeling	+++
	5′ end labeling	+++
	3′ end labeling	+++
Colony / plaque hybridization	Random primed labeling	+++
	PCR labeling	+++
	5′ end labeling	+++
	3′ end labeling	+++
In Situ Hybridisation	Random primed labeling	+++
	PCR labeling	++
	5′ end labeling	++
	3′ end labeling	++

Random primed labeling with Klenow

Principle

- Based on the random hybridization of a mixture of all possible hexanucleotides to the ssDNA form of the DNA that needs to be labeled.
- The complementary strand is synthesized from the 3′ OH termini of the hexanucleotide primers using the 5′ – 3′ polymerase activity of Klenow.

Incorporation of
1. Non-radioactive nucleotides: digoxigenin, biotin, fluorochromes
2. radioactive nucleotides: [^{32}P], [^{35}S], [^{3}H]



Labeling buffer, 10x

- 500mM Tris (pH 7.2), 100 mM $MgCl_2$, 1 mM DTT and 2 mg / ml BSA
- Buffer is stable at -20^{0}C, store in aliquots.

Application and typical results

- DNA is denatured by heating for 10 min at 100°C and immediately put on ice.
- Standard assay - prepare reaction mix on ice:

Components	Non-radioactive	Radioactive
Template DNA	10 ng - 3000 ng DNA	10 ng - 2000 ng DNA
Nucleotides, final concentration	100 µM of dATP, dCTP, dGTP each 65 µM dTTP	25 µM dATP, dGTP, dTTP each
Labeled nucleotide final concentration	35 µM DIG-, biotin -, or Fluorochrome - dUTP	[α^{32} P] dCTP (3000 Ci/mmol), 50 µCi (1.85 Bq)
10 x hecanucleotide mix (62.5 A_{260} Units / ml)	2 µl	2 µl
Klenow enzyme	2 units	2 units
10 x Lableing buffer	2 µl	2 µl
H2O	Add up to 20 µl	Add up to 20 µl
Incubation	At least 60 min at 37°C	30 min at 37°C

- Size of DNA fragment to be labeled: from 200 up to 50,000 bp.
- Size of labeled DNA fragment ranges from 80 - 200 bp.

Inactivation of enzyme

- Add 2 µl 0.2 M EDTA (pH 8.0) and / or heat to 65°C for 10 minutes.

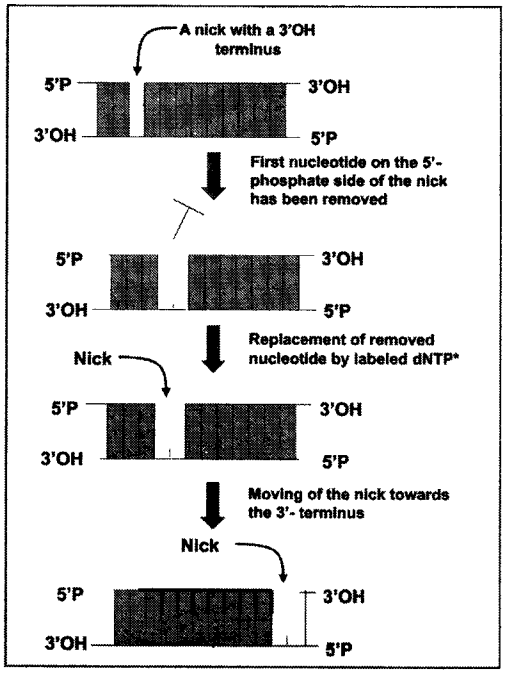
Tip

- Do not solubilize the DNA to be labeled in buffers containing EDTA since EDTA inhibits the random primed reaction.
- Optimized High Prime premixed solutions and kits.

Nick translation with DNA polymerase I and DNAse I

Principle

- Based on the ability of DNAse I to introduce random nicks in dsDNA at low enzyme concentrations and in the presence of Mg^{2+}.
- The E. coli DNA polymerase I synthesize DNA complementary to the intact strand in the 5′ - 3′ direction using the 3′ OH termini of the nicks as primers. The 5′ - 3′ exonuclease activity of the enzyme simultaneously removes nucleotides in the direction of synthesis that are replaced by nucleotides supplemented to the reaction.



NT buffer, 10x
- 500mM Tris (pH 7.2), 100 mM $MgCl_2$, 1 mM DTT and 2 mg /ml BSA
- Buffer is stable at -20°C, store in aliquots.

Application and typical results
- Standard assay – prepare reaction mix on ice:

Components	Non-radioactive	Radioactive
Template DNA	30 ng - 2000 ng DNA	30 ng - 2000 ng DNA
Nucleotides, final concentration	100 µM of dATP, dCTP, dGTP each 60 µM dTTP	20 µM dATP, dGTP, dTTP each
Labeled nucleotide final concentration	40 µM DIG-, biotin -, or Fluorochrome -dUTP	[α^{32} P] dCTP (3000 Ci/mmol), 20 µCi (0.74 MBq)
Mixture of DNA polymerase I and DNAse I[a]	2 µl	2 µl
10 x NT buffer	2 µl	2 µl
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	90 min at 15°C	30 min at 15°C

- Size of DNA fragment to be labeled : from 400 up to 800 bp.

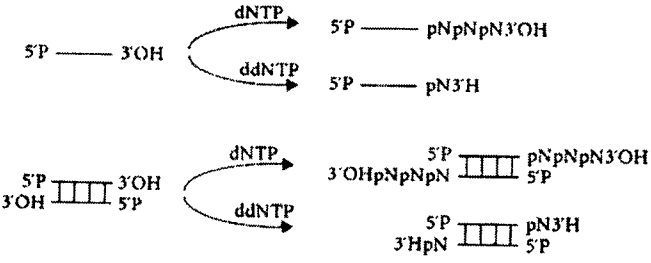
Inactivation of enzyme
- Add 2 µl 0.2 M EDTA (pH 8.0) and / or heat to 65°C for 10 minutes.

Tip
- Premixed Nick Translation mixes and different kit systems.

3′ End labeling with terminal transferase

Principle

- Catalyzes the template independent addition of dNTP's or ddNTP's to the 3′ OH ends of ds and ssDNA and oligonucleotides.



Transferase buffer

- 2 M potassium cacodylate, 250 mM Tris 2.5 mg/ml BSA, pH 6.6 at 25°C.
- Buffer is stable at -20°C, store in aliquots.

Application and typical results

- Standard assay – prepare reaction mix on ice:

Components	Tailing with dNTP's	3′ end labeling with ddNTP's
Template DNA	10 to 100 pmol 3′ ends	10 to 100 pmol 3′ ends
Radioactive nucleotides, final concentration	[α^{32}P] dNTP (3000 Ci/mmol), 50 µCi (1.85 MBq)	[α^{32}P] dNTP (3000 Ci/mmol), 50 µCi (1.85 MBq)
Non-radioactive nucleotides, final concentration	50 µM Dig, biotin or fluorochrome dUTP mixed with 500 µM dATP* 6.25 µM of dATP or dTTP* 5 µM of dGTP or dCTP	50 µM Dig, biotin or fluorochrome dUTP
$CoCl_2$	A or T Tails: 1.5 mM G or C Tails: 0.75 mM	5 mM
Terminal transferase	50 units	50 units
10 x transferase buffer	2 µl	2 µl
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	15 min at 37°C	15 min at 37°C

- For more information on length of tail, type of tail (homo-or heteropolymeric).

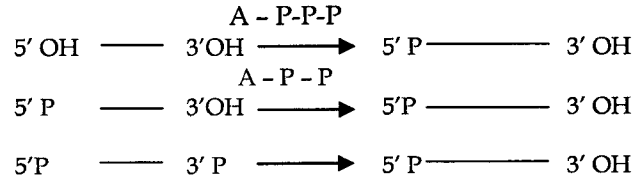
Inactivation of enzyme

- Add 2 µl 0.2 M EDTA (pH 8.0) and / or heat to 75°C for 10 minutes.

5′ end labeling with polynucleotide Kinase

Principle

- Catalyzes the transfer of the terminal phosphate group of ATP to the 5′ OH termini of ds and ssDNA and RNA.
- Catalyzes the exchange of terminal 5′ P groups of ds and ssDNA and RNA.
- Catalyzes the removal of phosphate groups from the 3′ termini of ds and ssDNA and RNA.



Phosphorylation buffer, 10 x

- 500 mM Tris, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM dithiothreitol, imM spermidine, pH 8.2 at 25°C.
- Buffer is stable at - 20°C, store in aliquots

Exchange buffer, 10 x

- 500 mM Imidazole- HCl, 100 mM $MgCl_2$, 1 mM EDTA, 50 mM dithiothreitol, imM spermidine, 3 mM ADP, pH 6.6 at 25°C.
- Buffer is stable at - 20°C, store in aliquots.

Application and typical results

- Standard assay - prepare reaction mix on ice:

Components	Phosporylation of 5′OH groups	Exchange of 5′P groups
Template DNA	20 pmol 5′OH ends	20 pmol of 5′P termini
Nucleotides final concentration	20 pmol [γ^{32}P] ATP	40 pmol [γ^{32}P] ATP
Polynucleotide kinase	10 units	10 units
Buffer	2 µl, phosphorylation buffer, 10x	2 µl, exchange buffer, 10x
H_2O	Add up to 20 µl	Add up to 20 µl
Incubation	30 min at 37°C	30 min at 37°C

- Phosphorylation: More than 30% of [^{32}P] from [γ^{32}P] ATP is incorporated.
- Exchange: More than 10% of [^{32}P] from [γ^{32}P] ATP is incorporated.

Inactivation of enzyme

- Stop phosphorylation and exchange reaction by putting the sample on ice.

Tip

- A special polynucleotide kinase, lacking the 3′ phosphatase activity is available.

Chapter **16**

Most Important Buffers and Media used in Molecular Biology Laboratory

Buffers
Definitions

Term	Symbol	Meaning	Example
Mole	mol	An amount containing avogadro's number of whatever units are being considered. Avogadro's number = 6.023×10^{23}	1 mol H_2O molecules = 6.023×10^{23} H_2O molecules.
Molar	mol/liter or M	Concentration of substance in a liquid. $\text{Molarity} = \frac{\text{Moles of substance}}{\text{Liter of solution}}$	An 1 M (mol/liter) solution of Tris in H_2O is prepared by dissolving 1 mol Tris molecules in H_2O to a final volume of 1 liter.
Molar weight	g/mol	Weight of 1 mol (=6.023×10^{23} parts) of a molecule, as defined by the molecular weight of this molecule.	Molecular weight of Tris 121.1 g/mol. An 1 M (mol / liter) solution of Tris in H_2O is prepared by dissolving 121.1 g Tris in H_2O to a final volume of 1 liter.

Example: How many grams of NaOH are needed to make up a 100 ml solution of 5M NaOH (=5 mol/l)?

Formula: Needed grams = Concentration x Molecular weight x Final volume

= 5 mol/ liter x 40 g/mol x 0.1 liter

= 20 g.

* Named after Amedeo Avogadro (1776 - 1856), a famous Italian physical chemist.

Precautions

Reagents

- Use the highest possible purity grade of reagents.

H_2O

- Prepare all solutions with fresh double - distilled or deionized H_2O.
- Only use H_2O from a distillation or deionization unit with a resistance value of 18 MOhm / cm.
- Regularly clean the units following instructions from the manufactures.

Preparing buffers

- Remove reagents from parent vials by shaking, do not use spatulas.
- Wear gloves.
- Take the water of crystallization into account when calculating the amount of reagent needed to make solutions with a given molarity.
- E.g. Molecular weight of $MgSO_4 - 7H_2O$ = 120.37 (MW of $MgSO_4$) = 126.11 (MW of 7 molecules H_2O)
  = 246.48

Sterilizing

- By autoclaving:
- 0.5 liter solution: ~ 15 minutes at 121^{0}C at 1 bar
- 1 liter solution : ~ 20 minutes at 121^{0}C at 1 bar.
- Caution: certain buffers (e.g. containing glucose, SDS, β -mercaptoethanol...) should not be autoclaved.
- Use filtration through an 0.22 µm filter as an alternative.

pH measurement

- Always fix the pH electrode in a vertical position.
- Gently stir the solution when adjusting the pH.
- Never put the electrode in solutions containing SDS.

- Periodically calibrate the electrode with at least two calibration solutions.
- Store the pH electrode in solution, keep the diaphragm wet and make sure that there is always electrolyte solution inside the electrode.
- Clean the pH electrode with distilled water after usage.
- Caution: the pH of most solutions is temperature -dependent:

  e.g.: 5^0C: pH of a 0.05 M Tris solution = 8.07

  25^0C: pH of a 0.05 M Tris solution = 7.50

  37^0C: pH of a 0.05 M Tris solution = 7.22

Safety

- Carefully consult the product safety information and material safety data sheets of chemicals.
- Take local safety and laboratory regulations into account when working with chemicals.

Buffering ranges of commonly used buffers (at 20^0C).

Recipes for stock solutions

10 M NH_4OAc - 1 liter

- Dissolve 770.8g ammonium acetate (NH_4OAc, MW = 77.08) in 800 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

1 M $CaCl_2$ - 1 liter

- Dissolve 219.08g calcium chloride - $6H_2O$ ($CaCl_2$ - $6H_2O$, MW = 219.08) in 800 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

250 x Denhardt solution - 1 liter

- Dissolve 50g Ficoll 400, 50g polyvinylpyrrolidone and 50 g bovine serum albumin (BSA) in 600 ml H_2O.

- Adjust volume to 1 liter with H_2O.
- Divide in aliquots of 25 ml and store at - 20^0C.

1 M DTT - 20 ml

- Dissolve 3.085 g 1,4- dithio-DL-threitol (DTT, MW = 154.25) in 20 ml 10 mM sodium acetate (pH 5.2).
- Divide in aliquots of 1 ml and sterilize by filtration.
- Store at - 20^0C.

0.5 M Na_2 EDTA (pH 8.0) - 1 liter

- Dissolve 186.12g disodium ethylendediaminetetraacetate- $2H_2O$ (Na_2 EDTA - $1H_2O$, MW = 372.24) in 800 ml H_2O; stir vigorously on a magnetic stirrer.
- Adjust to pH 8.0 with NaOH (~ 20g NaOH pellets) and adjust volume to 1 liter with H_2O.
- Divide into aliquots and sterilize by autoclaving.
- Store at room temperature.

  Note: The disodium salt of EDTA will only solute when the pH of the solution is adjusted to 8.0 by the addition of NaOH.

0.1 M IPTG - 50 ml

- Dissolve 1.19 g isopropyl β -D-thiogalactopyranoside(IPTG, MW = 238.3) in 40 ml H_2O.
- Adjust volume to 50 ml with H_2O.
- Divide in 5 ml aliquots and sterilize by filtration.
- Store at -20^0C, this solution is stable for 2 - 4 months.

1 M $MgCl_2$ - 1 liter

- Dissolve 203.31 g magnesium chloride - $6H_2O$ ($MgCl_2$ - $6H_2O$, MW = 203.31) in 800 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Divide in aliquots of 100 ml and sterilize by atutoclaving.
- Store at room temperature.

10 x MOPS - 1 liter

- Add 41.85 g 4 - morpholinepropane-sulfonic acid (MOPS - free acid, MW = 209.27) and 6.80g sodium acetate - $3H_2O$ (NaOAc - $3H_2O$, MW = 136.08) to 800 ml DEPC treated H2O and stir until completely dissolved.
- Add 20 ml of a DEPC treated 0.5 M Na_2 EDTA solution and adjust pH to 7.0 with 10 M NaOH.
- Adjust volume to 1 liter with DEPC treated H_2O.
- Divide into 200 ml aliquots and store - protected from light - at 4^0C.
- If the solution turns yellow, use a new aliquot.

3 M NaOAc - 1 liter

- Dissolve 408.24g sodium acetate - $3H_2O$ (NaOAc - $3H_2O$, MW= 136.08) in 800 ml H_2O. Adjust to pH 5.2 with glacial acetic acid or to pH 7.0 with diluted acetic acid.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

5 M NaCl - 1 liter

- Dissolve 292.2 g sodium chloride (NaCl, MW = 58.44) in 800 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

10% SDS - 1 liter

- Dissolve 100 g sodium dodecyl sulfate crystals (SDS) in 900 ml H_2O.
- Heat to 68^0C to solute the crystals.
- Adjust pH to 7.2 with HCl (~ 50µl).
- Adjust volume to 1 liter with H_2O.
- Dispense into aliquots and store at room temperature.

  Note: the fine crystals of SDS disperse easily, wear a mask when weighing SDS and clean the weighing area and balance after use.

When SDS crystals precipitate (e.g. due to cold temperature), redissolve by warming the solution at 37^0C.

100% (w/v) TCA

- Add 500g trichloroacetic acid (TCA) to 227 ml H_2O.
- This solution contains 100% (w/v) TCA.

1 M Tris - 1 liter

- Dissolve 121.14g tris (hydroxymethyl) aminomethane (Tris, MW = 121.14) in 800 ml H_2O.
- Adjust pH to the desired value by adding concentrated HCl:

  pH 7.4 : ~70ml

  pH 7.6; ~ 60ml

  pH 8.0: ~ 42ml
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

X - gal (20 mg/ml) 20 ml

- Dissolve 400 mg 5- Bromo-4-Chloro-3-Indolyl-β-D-galactoside (X-gal) in 20 ml N, N' -dimethyl formamide.
- Divide into 500µl aliquots and store in a glass or polypropylene tube protected from light at - 20^0C. This stock solution is stable for 2 - 4 months.

Recipes for buffers

10x PBS - 1 liter

- Dissolve 80g NaCl, 2g KCl, 26.8g Na_2HPO_4 - $7H_2O$ and 2.4g KH_2PO_4 in 800 ml H_2O.
- Adjust to pH 7.4 with HCl.
- Adjust volume to 1 liter with H_2O.
- Divide in aliquots and sterilize by autoclaving.
- Store at room temperature.

20 x SSC - 1 liter

- Dissolve 175.3g NaCl and 88.2 g sodium citrate - $2H_2O$ in 800 ml H_2O.
- Adjust pH to 7.0 wtih HCl.
- Adjust volume to 1 liter with H_2O.

- Divide in aliquots and sterilize by autoclaving.
- Store at room temperature.

20 x SSPE - 1 liter

- Dissolve 175.3 g NaCl, 27.6g NaH_2PO_4 - $1H_2O$ and 7.4g Na_2 EDTA in 800 ml H_2O.
- Adjust pH to 7.4 with NaOH (~6.5 ml of a 10 M solution).
- Adjust volume to 1 liter with H_2O.
- Divide in aliquots and sterilize by autoclaving.
- Store at room temperature.

1 x TE - 1 liter

- Add 10 ml 1 M Tris (pH 8.0, 7.6 or 7.4) and 2 ml 0.5 M Na2 EDTA* (pH 8.0) to 800ml H_2O.
- Mix and adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving.
- Store at room temperature.

* For certain applications (e.g. storage of DNA that will be used in PCR or other enzymatic reactions). Add 200µl 0.5M Na_2 EDTA instead of 2 ml.

Recipes for buffers with desired pH

0.1 M NaOAc - 100 ml

- **0.2 M acetic acid:**
- Mix 11.55 ml glacial acetic acid in 500 ml H_2O and adjust to 1 liter with H_2O.
- **0.2 M Sodium acetate:**
- Dissolve 27.21g sodium acetate - $3H_2O$ (NaOAc - $3H_2O$, MW = 136.08) in 800 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed to obtain a 100 ml solution of 0.1 M NaOAc with a specific desired pH.

0.1 M KOAc - 100 ml

- **0.2 M acetic acid:**
- Mix 11.55 ml glacial acetic acid in 500 ml H_2O and adjust to 1 liter with H_2O.
- **0.2 M potassium acetate:**

- Dissolve 19.62 g postassium acetate (KOAc, MW= 98.14) in 800 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed to obtain a 100 ml solution of 0.1 M KOAc with a specific desired pH.

pH table for acetate buffers

Desired pH[a]	0.2 M acetic acid solution (ml)	0.2 M sodium or potassium acetate solution (ml)	H_2O (ml)
3.6	46.3	3.7	50
3.8	44.0	6.0	50
4.0	41.0	9.0	50
4.2	36.8	13.2	50
4.4	30.5	19.5	50
4.6	25.5	24.5	50
4.8	20.0	30.0	50
5.0	14.8	35.2	50
5.2	10.5	39.5	50
5.4	8.8	41.2	50
5.6	4.8	45.2	50

[a] It is strongly recommended to check the final pH.

0.1 M Na Phosphate 200 ml

- **0.2 M sodium phosphate, mono -sodium salt:**
- Dissolve 27.6g NaH_2PO_4 - $1H_2O$ (MW = 138) in 500 ml H_2O and adjust to 1 liter with H_2O.
- **0.2 M sodium phosphate, di-sodium salt:**
- Dissolve 53.62 g Na_2HPO_4 - $7H_2O$ (MW= 268.1) in 500 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed together to obtain a 200 ml solution of 0.1 M Na phosphate with a specific desired pH.

0.1 M K Phosphate 200 ml

- **0.2 M potassium phosphate, mono - potassium salt:**
- Dissolve 27.2 g KH_2PO_4 (MW = 136.09) in 500 ml H_2O and adjust to 1 liter with H_2O.
- **0.2 M potassium phosphate, di-potassium salt:**
- Dissolve 34.8g K_2HPO_4 (MW= 174.18) in 500 ml H_2O and adjust to 1 liter with H_2O.
- The table below gives the volumes in ml of these solutions and H_2O that should be mixed together to obtain a 200 ml solution of 0.1 M K Phosphate with a specific desired pH.

pH table for phosphate buffers[a]:

Desired pH[a]	Sodium or potassium, phosphate mon salt solution (ml)	Sodium or potassium, phosphate di salt solution (ml)	H_2O (ml)
5.7	93.5	6.5	100
5.8	92.0	8.0	100
5.9	90.0	10.0	100
6.0	87.7	12.3	100
6.1	85.0	15.0	100
6.2	81.5	18.5	100
6.3	77.5	22.5	100
6.4	73.5	26.5	100
6.5	68.5	31.5	100
6.6	62.5	37.5	100
6.7	56.5	43.5	100
6.8	51.0	49.0	100
6.9	45.0	55.0	100
7.0	39.0	61.0	100
7.1	33.0	67.0	100
7.2	28.0	72.0	100
7.3	23.0	77.0	100
7.4	19.0	81.0	100
7.5	16.0	84.0	100
7.6	13.0	87.0	100
7.7	10.5	90.5	100
7.8	8.5	91.5	100
7.9	7.0	93.0	100
8.0	5.3	94.7	100

[a] It is strongly recommended to check the final pH with a pH meter.

0.05 M Tris - 100 ml

- Table below gives the volumes of 0.1 M Tris buffer, 0.1 M HCl and H_2O that should be mixed together to obtain a 100 ml solution of 0.05 M Tris with a specific desired pH[a]:

pH table for Tris buffers

Desired pH[a]	0.1 M HCL (ml)	0.1 M Tris (ml)	H_2O (ml)
7.3	43.4	50	6.6
7.4	42.0	50	8.0
7.5	40.3	50	9.7
7.6	38.5	50	11.5
7.7	36.6	50	13.4
7.8	34.5	50	15.5
7.9	32.0	50	18.0
8.0	29.2	50	20.8
8.1	26.2	50	23.8
8.2	22.9	50	27.1
8.3	19.9	50	30.1
8.4	17.2	50	32.8
8.5	14.7	50	35.3
8.6	12.4	50	37.6

[a] It is strongly recommended to check the final pH with a pH meter.

Electrophoresis of DNA

10x agarose gel sample buffer 100 ml

- Dissolve 250mg bromophenol blue and / or 250 mg xylene cyanol in 33 ml 150 mM Tris pH 7.6.
- Add 60 ml glycerol and 7 ml H_2O.
- Store at room temperature.

10 x TBE (Tris -borate) 1 liter

- Dissolve 108g Tris and 55g Boric acid in 900 ml H_2O.
- Add 40ml 0.5 M Na_2 EDTA (pH 8.0) and adjust volume to 1 liter with H_2O.
- Store at room temperature.

50x TAE (Tris -acetate) 1 liter

- Disssolve 242g Tris in 500 ml H_2O.

- Add 100 ml 0.5 M Na_2 EDTA (pH 8.0) and 57.1 ml glacial acetic acid.
- Adjust volume to 1 liter with H_2O.
- Store at room temperature.

10 x TPE (Tris - phosphate) 1 liter

- Dissolve 108 g Tris in 700 ml H_2O.
- Add 15.5 ml 85% Phosphoric acid (1.679 g /ml) and 40 ml 0.5 m Na_2 EDTA (pH 8.0).
- Adjust volume to 1 liter with H_2O.
- Store at room temperature.

Electrophoresis of RNA

RNA sample buffer

- Mix 10 ml deionized formamide, 3.5 ml 37% formaldehyde and 2 ml 5 x MOPS.
- Divide into 500 µl aliquots and store at - 20^0C.
- The buffer is stable for 6 months.
- Use 2 parts sample buffer for each part of RNA.
- Note: Formamide is a teratogen and formaldehyde is a carcinogen.

Work in a fumehood and follow laboratory safety procedures.

RNA Loading buffer

- Prepare in DEPC -treated H_2O.
- 50% glycerol, 1 mM Na_2EDTA, and 0.4% Bromophenol Blue.
- Use the highest possible grade of glycerol to avoid ribonuclease contamination.
- Divide into 500 µl aliquots and store at - 20^0C.
- Use 2 µl loading buffer per 10 -20 µl RNA sample (RNA pluse sample buffer).

DEPC - treatment

DEPC -treatment per 100 ml solution

- Add 0.1 - 0.2 ml diethylpyrocarbonate (DEPC) to 100 ml of a solution (e.g. H_2O).
- Shake vigorously and incubate overnight in a fume hood.

- Autoclave the solution to inactivate the remaining DEPC.
- Store treated solution at room temperature.
- Note: Wear gloves and use a fume hood when using DEPC (suspected carcinogen).
- All chemical substances containing amino groups (e.g. Tris, MOPS, EDTA, HEPES, etc.) cannot be treated directly with DEPC. Prepare these solutions in DEPC-treated H_2O.

Staining of Nucleic Acids

Ethidium Bromide 100 ml

- Prepare a stock solution of 10 mg/ ml by adding 1 g ethidium bromide to 100 ml H_2O. Stir until the dye has completely dissolved.
- Store in the dark place at 4^0C.
- During electrophoresis: add 0.5 - 1 µg per ml agarose solution.
- After electrophoresis: add 0.5 - 2 µg per ml staining solution.
- Caution: Ethidium bromide is a mutagen and is toxic. Wear gloves when working with the solution and wear a mask when dissolving the powder.

SYBR Green 1 Nucleic acid gel stain[a]

- Supplied[a] as a stock solution in DMSO, stable at - 20^0C for 6- 12 months.
- Working solution:
- Dilute the stock solution 1: 10,000 in TE, TBE or TAE buffer.
- Sensitivity:
- As low as 80 pg per band dsDNA using 312 nm transillumination with the Lumi-Imager F1 system[a].

SYBR green II RNA Gel Stain[a]

- Supplied[a] as a stock solution in DMSO stable at - 20^0C. for 6 -12 months.

- Working solution:
- For nondenaturating gels and denaturing polyacrylamide / urea gels: 1: 10,000 dilution in TBE
- Sensitivity: as low as 5 ng per band of RNA using 312 nm transillumination with the Lumi-Imager F1 system[a].

[a] Available from Roch Molecular Biochemicals

Phenol for the extraction of Nucleic Acids

Phenol - 1 liter

- Dissolve 500 g phenol in 500 ml 1 M Tris (pH 8.0).
- Once dissolved, let phases separate and remove upper aqueous phase.
- Add 500 ml 100mM Tris (pH 8.0); stir to emulsify and let phases separate.
- Repeat procedure with TE until pH of upper, aqueous phase is less than pH 7.2.
- Store at 4^0C protected from light.
- Discard when the solution turns red/ brown.

Phenol (acid) - 1 liter For RNA only

- Dissolve 500 g phenol in 500 ml 50 mM sodium acetate (pH 4.0).
- Once dissolved, let phases separate and remove upper aqueous phase.
- Add 500 ml 50 mM sodium acetate (pH 4.0); stir to emulsify and let phases separate.
- Repeat procedure until the pH of upper, aqueous phase is less than pH 4.1.
- Store at 4^0C protected from light.

Caution

- Phenol is highly corrosive and can cause severe burns.
- Always wear gloves, protective clothing and safety glasses when working with phenol.

- If skin comes in contact with phenol, immediately wash with a large volume of water and soap.

Electrophoresis of Proteins

10% Ammonium persulfate - 10ml

- Dissolve 1 g ammonium persulfate (APS) in 8 ml H_2O.
- Adjust volume to 10ml with H_2O.
- Solution is stable at 4^0C for two weeks.

30% acryl-bisacryl-amide mix - 100ml

- Dissolve 29g acrylamide and 1 g N, N' - methylenebisacrylamide in 60 ml H_2O.
- Heat the solution to 37^0C to dissolve the chemicals.
- Adjust volume to 100 ml with H_2O.
- Store at 4^0C protected from light.
- Caution: Acrylamide is a neurotoxin and is absorbed through the skin. Always wear gloves and a mask when preparing the solutions.

2 x SDS PAGE sample buffer 100 ml

- Mix 10ml 1.5 M Tris (pH 6.8), 6 ml 20% SDS, 30 ml glycerol, 15ml β - mercaptoethanol and 1.8 mg bromophenol blue.
- Adjust volume to 100 ml with H_2O.
- Aliquot in 10 ml stock solution and store at - 20^0C.
- Store working solution at 4^0C.

10 x SDS PAGE Running buffer 1 liter

- Dissolve 10 g SDS, 30.3 g Tris and 144.1 g glycin in 800ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Store at room temperature.

Coomassie Blue staining solution 1 liter

- Dissolve 2.5 g "Coomassie Brilliant Blue R - 250" in a mixture of 450 ml methanol, 100 ml acetic acid and 400 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Store at room temperature.

Coomassie blue destaining stlution 1 liter

- Mix 450 ml methanol, 100ml acetic acid and 400 ml H_2O.
- Adjust volume to 1 liter with H_2O.
- Store at room temperature.

Resolving gels for denaturing SDS - PAGE

Gel	Components	Volume of components (ml) per gel mold volume of							
		5ml	10ml	15ml	20ml	25ml	30ml	40ml	50ml
6%	H_2O	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.50
	30% acryl-bisacrylamide mix	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.00
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8%	H_2O	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.20
	30% acryl-bisacrylamide mix	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.30
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	1.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10%	H_2O	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.80
	30% acryl-bisacrylamide mix	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.70
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12%	H_2O	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.50
	30% acryl-bisacrylamide mix	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.00
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	.015	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15%	H_2O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.50
	30% acryl-bisacrylamide mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.00
	1.5 M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.50
	10% SDS	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	10% ammonium persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.50
	TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

5% stacking gels for denaturing SDS -PAGE

Components	Volume of components (ml) per gel mold volume of							
	1ml	2ml	3ml	4ml	5ml	6ml	8ml	10ml
H_2O	0.6	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acryl-bisacrylamide mix	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.5 M Tris (pH 8.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium persulfate	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

Western Blotting

1x Transfer buffer for wet blots - 1 liter

- Dissolve 2.9 g Glycine, 5.8g Tris and 0.37 g SDS in 200ml methanol.
- Adjust volume to 1 liter with H_2O.
- Store at 4^0C.

1 x Transfer buffer for semi -dry blots

- Scheme:

Layer	Content and buffer
5 (top)	3 x 3 MM papers soaked in 300 mM Tris, pH 10.4
4	Membrane
3	Gel
2	3 x3 MM paper soaked in 25 mM Tris, pH 9.4 and 40mM amino hexane acid
1 (bottom)	3 x3 MM pepers soaked in 25mM Tris, pH10.4

Ponceau S Solution 100 ml

- Mix 2 g Ponceau S, 30g trichloracetic acid and 30g sulfosalicylic acid in 80 ml H_2O.
- Adjust volume to 100 ml with H_2O.
- Store at room temperature.

1 x TBS (Tris buffered saline) 1 liter

- Dissolve 6.05g Tris (50mM) and 8.76g NaCl (150mM) in 800 ml H_2O.
- Adjust pH to 7.5 with 1 M HCl (~ 9.5ml).
- Adjust volume to 1 liter with H_2O.
- TBS is stable at 4^0C for three months.
- Note: Since sodium azide inhibits peroxidase activity, it is not recommended for use as an antimicrobial reagent.

1 x TBST (Tris Buffered saline Tween) - 1 liter

- Dissolve 1 ml Tween 20 in 1 liter TBS buffer.
- TBST is stable at 4^0C for three months.
- Note: Tween 20 suitable for most applications but depending on the type of membrane and antibodies used, different detergents (like SDS, Triton X 100 or Nonidet P40) and concentrations between 0.01 - 1% may lead to better results.

Blocking solution

- A wide variety of different blocking solutions are described in the literature.
- A solution of 2.5 to 5% (w/v) non fat dried milk in TBS or PBS is sufficient for most blots.
- If background is high, use alternative blocking solutions[a] containing 1% of e.g. BSA, gelatine or casein.

Selection of prokaryotic cells

Antibiotic	Mode of action	Mechanism of Resistance	Working Cencentration[a]	Stock Solution[b]
Ampicillin (Amp)	Inhibits cells wall synthesis by inhibiting formation of the peptidoglycan cross-link.	The resistance gene (bla) specifies an enzyme, β -lactamase which cleaves the β - lactam ring of the antibiotic.	20 -100 µg/ ml (50 µg/ml)	100 mg/ml in H_2O store at -20°C
Chlor-amphenicol (Cm)	Prevents peptide bond formation by binding to the 50S subunit of ribosomes.	The resistance gene (cat) specifies an acetyltransferase that acetylates and thereby inactivates the antibiotic.	25 - 170 µg/ml (100 µg/ml)	34mg/ml in Ethanol store at -20°C
Kanamycin (Kan)	Causes misreading of mRNA by binding to 70S ribosomes.	The resistance gene (kan) specifies an aminoglycoside phosphotransferase that inactivates the antibiotic.	10 - 50µg/ml (30µg/ml)	30 mg/ml in H_2O store at -20°C.
Streptomycin (Sm)	Causes misreading of mRNA by binding to 30S subunit of ribosomes.	The resistance gene (str) specifies an enzyme that modifies the antibiotic and inhibits its binding to the ribosomes.	10 - 125 µg/ml (50 µg/ml)	50 mg/ml in H_2O Store at -20°C.

Antibiotic	Mode of action	Mechanism of Resistance	Working Cencentration[a]	Stock Solution[b]
Tetracycline (Tet)	Prevents protein synthesis by preventing binding of the aminoacyl tRNA to the ribosome a site.	The resistance gene (tet) specifies a protein that modifies the bacterial membrane and prevents transport of the antibiotic into the cell.	10 – 50 µg/ml (10 µg/ml in liquid culture - 12.5µg/ml in plates)	12.5 mg/ml in Ethanol store at -20°C
Geneticin (G418)	Interferes with the function of 80S ribosomes and blocks protein synthesis in eukaryotic cells.	The resistance gene (neo) encodes a bacterial aminoglycoside phosphotransferase that inactivates the antibiotic.	50 – 1000 µg/ml; optimal concentration is to be tested experimentally[b]	5 – 50 mg/ml in culture medium or physiological buffers store at -20°C.
Gentamycin	Inhibits protein synthesis by binding to the L6 protein of the 50 S ribosomal subunit.	The resistance gene specifies an aminoglycoside phosphotransferase that inactivates the antibiotic.	100 µg/ml	10 -50 mg/ml solution in H_2O. store at 4°C.
Hygromycin B	Inhibits protein synthesis of bacteria, fungi and eukaryotic cells by interfering with translocation and causing mistranslation.	The resistance gene (hyg or hyh) codes for a kinase that inactivates Hygromycin B through phosphorylation.	50 – 1000 µg/ml; optimal concentration is to be tested experimentally[b]	50 mg/ml in PBS store at 4°C

[a] stock solution in H_2O, culture medium, physiological buffers or PBS: sterilize by filtration, store protected from light.

Stock solutions in ethanol should not be sterilized.

[b] a table with optimal cell-line specific concentration is available for Roche molecular biochemicals.

Media for Bacteria

Recipes for 1 liter

LB

- Mix 10g Bacto-Typtone, 5g Bacto-Yeast extract and 10g NaCl in 900 ml H_2O.
- Adjust the pH to 7.0 with 10 M NaOH (~ 200 µl).
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

M9 minimal

- Mix 12.8g $Na_2 HPO_4 - 7H_2O$, 3g KH_2PO_4, 0.5 g NaCl and 1 g $NH_4 Cl$ in 750 ml H_2O.
- Adjust the pH to 7.4 with 10 M NaOH (~ 100µl).
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and cool to room temperature.
- Add 2 ml 1 M $MgSO_4 - 7H_2O$, 0.1 ml 1M $CaCl_2$ and 10 ml 20% glucose.
- Sterilize by filtration and store at room temperature.

SOB

- Mix 20 g Bactro -Tryptone, 5 g Bacto-Yeast extract, 0.5g NaCl and 2.5 ml 1 M KCl in 900 ml H_2O.
- Adjust pH to 7.0 with 10 M NaOH (~ 100µl) and add H_2O to 990 ml.
- Sterilize by autoclaving and store at room temperature.
- Before use, add 10 ml sterile 1 M $MgCl_2$.

SOC

- Identical to SOB medium, except that it additionally contains 20 ml sterile 1 M glucose.

TB

- Mix 12 g Bacto-Trytone, 24 g Bacto-Yeast extract and 4 ml glycerol in 900 ml H_2O.
- Sterilize by autoclaving and cool to $<60^0C$.
- Add 100 ml of sterile 10x TB phosphate* and store at room temperature.

YT

- Mix 8 g Bacto-Tryptone, 5 g Bacto-Yeast extract and 2.5g NaCl in 900 ml H_2O.
- Adjust pH to 7.0 with 10M NaOH (~200 µl).
- Adjust volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

* Made by dissolving 2.31 g KH_2PO_4 (=0.17 M) and 12.54 K_2HPO_4 (= 0.72M) in 90 ml H_2O. Adjust volume to 100 ml with H_2O and sterilize by autoclaving. Store at room temperature.

Recipes for 1 liter (for ~ 40 plates of 90 mm, ~25 ml per plate)

Agar plates

- Prepare liquid media according to the recipes given on page 109.
- Add 15g Bacto-Agar to 1 liter liquid medium and sterilize by autoclaving.
- Allow the medium to cool to 50^0C and add the appropriate amount of antibiotic.
- Gently mix the medium by swirling and pour into sterile plates.
- Flame the surface of the medium in the plates with a Bunsen burner to remove air bubbles.
- Allow the medium to solidify and store the dishes at 4^0C in the inverted position.
- Store tetracycline plates in the dark.
- Use a colour code to indicate the type of antibiotic in the plate (e.g. black stripe for ampicillin plates, blue stripe for kanamycin plates, etc.)
- Before use, incubate plates at 37^0C in the inverted position for 1 hour to remove condensation within the plate.

X -gal/ IPTG indicator plates

- Before pouring the plates, add:
- 2 ml of IPTG stock solution (0.1M) - final concentration: 0.2mM.
- 2 ml of X -gal stock solution (20 mg/ml) - final concentration: 40 µg/ml.

Top agar overlay

- Prepare liquid media according to the recipes described already.
- Add 7 g Bacto -agar to 1 liter liquid medium and sterilize by autoclaving.

Glossary

Absolute quantification: The absolute quantitation assay is used to quantitate unknown samples by interpolating their quantity from a **standard curve** (as in determination of viral copy number).

Adenine (A) : A nitrogenous base, one member of the base pair AT (adenine-thymine).

Allele : Alternative form of a genetic locus; a single allele for each locus is inherited from each parent (e.g., at a locus for eye colour the allele might result in blue or brown eyes).

Allelic discrimination assay: Assays designed to type for gene variants. Either differentially labeled (TaqMan®) probes (one for each variant) or a single probe and melting curve analysis can be used for this purpose. Alternative methods include dsDNA-binding dyes (in combination with melting curve analysis). TaqMan®-based allelic discrimination assays are analyzed by differences in threshold cycles or by endpoint fluorescence value for each allele. The results are plotted by fluorescence intensity or by C_t values for each allele at X and Y axes.

Alternative splicing : Different ways of combining a gene's exons to make variants of the complete protein.

Amino acid : Any of a class of 20 molecules that are combined to form proteins in living things. The sequence of amino acids in a protein and hence, protein function are determined by the genetic code.

Amplicon: The amplified sequence of DNA in the PCR process.

Amplification : An increase in the number of copies of a specific DNA fragment; can be in vivo or in vitro.

Amplification plot: The plot of cycle number versus fluorescence signal which correlates with the initial amount of target nucleic acid during the exponential phase of PCR.

Anchor & reporter probes: Two partnering Light Cycler (hybridizing) probes that hybridize on the target sequence in close proximity. The anchor probe (donor) emits fluorescence to excite the reporter probe (acceptor) to initiate **FRET**. In allelic discrimination assays,

it is important that the reporter probe spans the mutation and has a lower Tm than the anchor probe.

Annotation : Adding pertinent information such as gene coded for, amino acid sequence, or other commentary to the database entry of raw sequence of DNA bases.

Autoradiography : A technique that uses X-ray film to visualize radioactively labeled molecules or fragments of molecules; used in analyzing length and number of DNA fragments after they are separated by gel electrophoresis.

Autosomal dominant : A gene on one of the non-sex chromosomes that is always expressed, even if only one copy is present. The chance of passing the gene to offspring is 50% for each pregnancy.

Autosome : A chromosome not involved in sex determination. The diploid human genome consists of a total of 46 chromosomes: 22 pairs of autosomes, and 1 pair of sex chromosomes (the X and Y chromosomes).

Bacterial artificial chromosome (BAC) : A vector used to clone DNA fragments (100- to 300-kb insert size; average, 150 kb) in *Escherichia coli* cells. Based on naturally occurring F-factor plasmid found in the bacterium *E. coli*.

Base : One of the molecules that form DNA and RNA molecules.

Base pair (bp) : Two nitrogenous bases (adenine and thymine or guanine and cytosine) held together by weak bonds. Two strands of DNA are held together in the shape of a double helix by the bonds between base pairs.

Base sequence : The order of nucleotide bases in a DNA molecule; determines structure of proteins encoded by that DNA.

Base sequence analysis : A method, sometimes automated, for determining the base sequence.

Baseline: The initial cycles of PCR during which there is little change in fluorescence signal (usually cycles 3 to 15).

Baseline value: During PCR, changing reaction conditions and environment can influence fluorescence. In general, the level of fluorescence in any one well corresponds to the amount of target present. Fluorescence levels may fluctuate due to changes in the reaction medium creating a background signal. The background signal is most evident during the initial cycles of PCR prior to

significant accumulation of the target amplicon. During these early PCR cycles, the background signal in all wells is used to determine the 'baseline fluorescence' across the entire reaction plate. The goal of data analysis is to determine when target amplification is sufficiently above the background signal, facilitating more accurate measurement of fluorescence.

Biotechnology : A set of biological techniques developed through basic research and now applied to research and product development. In particular, biotechnology refers to the use by industry of recombinant DNA, cell fusion, and new bioprocessing techniques.

BLAST : A computer program that identifies homologous (similar) genes in different organisms, such as human, fruit fly, or nematode.

Calibrator: A single reference sample used as the basis for relative-fold increase in expression studies (assuming constant reaction efficiency). This calibrator should be included in each assay.

Capillary array : Gel-filled silica capillaries used to separate fragments for DNA sequencing. The small diameter of the capillaries permit the application of higher electric fields, providing high speed, high throughput separations that are significantly faster than traditional slab gels.

Carrier : An individual who possesses an unexpressed, recessive trait.

cDNA library : A collection of DNA sequences that code for genes. The sequences are generated in the laboratory from mRNA sequences.

Cell : The basic unit of any living organism that carries on the biochemical processes of life.

Centimorgan (cM) : A unit of measure of recombination frequency. One centimorgan is equal to a 1% chance that a marker at one genetic locus will be separated from a marker at a second locus due to crossing over in a single generation. In human beings, one centimorgan is equivalent, on average, to one million base pairs.

Clone : An exact copy made of biological material such as a DNA segment (e.g., a gene or other region), a whole cell, or a complete organism.

Cloning : Using specialized DNA technology to produce multiple, exact copies of a single gene or other segment of DNA to obtain

enough material for further study. This process, used by researchers in the Human Genome Project, is referred to as cloning DNA. The resulting cloned (copied) collections of DNA molecules are called clone libraries. A second type of cloning exploits the natural process of cell division to make many copies of an entire cell. The genetic makeup of these cloned cells, called a cell line, is identical to the original cell. A third type of cloning produces complete, genetically identical animals such as the famous Scottish sheep, Dolly.

Cloning vector : DNA molecule originating from a virus, a plasmid, or the cell of a higher organism into which another DNA fragment of appropriate size can be integrated without loss of the vector's capacity for self-replication; vectors introduce foreign DNA into host cells, where the DNA can be reproduced in large quantities. Examples are plasmids, cosmids, and yeast artificial chromosomes; vectors are often recombinant molecules containing DNA sequences from several sources.

Coefficient of variation (CV): Used as a measure of experimental variation. It is important that a linear value (e.g., copy numbers) is used to calculate the CV (but not C_t values which are logarithmic). Intra-assay CV quantifies the amount of error seen within the same assay (in duplicates) and inter-assay CV quantifies the error between separate assays.

Complementary sequence : Nucleic acid base sequence that can form a double-stranded structure with another DNA fragment by following base-pairing rules (A pairs with T and C with G). The complementary sequence to GTAC for example, is CATG.

Cosmid : Artificially constructed cloning vector containing the cos gene of phage lambda. Cosmids can be packaged in lambda phage particles for infection into *E. coli*; this permits cloning of larger DNA fragments (up to 45kb) than can be introduced into bacterial hosts in plasmid vectors.

C_t (threshold cycle): Threshold cycle reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. It is inversely correlated to the logarithm of the initial copy number. The C_t value assigned to a particular well, thus, reflects the point during the reaction at which a sufficient number of amplicons have accumulated. Also called crossing point (C_p) in LightCycler terminology.

Cytosine (C) : A nitrogenous base, one member of the base pair GC (guanine and cytosine) in DNA.

Derivative curve: This curve is used in Tm analysis. It has the temperature in the x axis and the negative derivative of fluorescence (F) with respect to temperature (T), shown as dF/dT, on the y axis. The reproducibility of a derivative melting curve is high with a standard deviation of only 0.1°C between runs.

Directed sequencing : Successively sequencing DNA from adjacent stretches of chromosome.

Disease-associated genes : Alleles carrying particular DNA sequences associated with the presence of disease.

DNA (deoxyribonucleic acid) : The molecule that encodes genetic information. DNA is a double-stranded molecule held together by weak bonds between base pairs of nucleotides. The four nucleotides in DNA contain the bases adenine (A), guanine (G), cytosine (C), and thymine (T). In nature, base pairs form only between A and T and between G and C; thus, the base sequence of each single strand can be deduced from that of its partner.

DNA bank : A service that stores DNA extracted from blood samples or other human tissue.

DNA probe : *See:* probe

DNA repair genes : Genes encoding proteins that correct errors in DNA sequencing.

DNA replication : The use of existing DNA as a template for the synthesis of new DNA strands. In humans and other eukaryotes, replication occurs in the cell nucleus.

DNA sequence : The relative order of base pairs, whether in a DNA fragment, gene, chromosome, or an entire genome. *See also:* base sequence analysis.

Domain : A discrete portion of a protein with its own function. The combination of domains in a single protein determines its overall function.

Dominant : An allele that is almost always expressed, even if only one copy is present. *See also:* gene, genome

Double helix : The twisted-ladder shape that two linear strands of DNA assume when complementary nucleotides on opposing

strands bond together.

Draft sequence : The sequence generated by the HGP as of June 2000 that, while incomplete, offers a virtual road map to an estimated 95% of all human genes. Draft sequence data are mostly in the form of 10,000 base pair-sized fragments whose approximate chromosomal locations are known.

dsDNA-binding agent: A molecule that emits fluorescence when bound to dsDNA. The prototype is SYBR® Green I. In real-time PCR, the fluorescence intensity increases proportionally to dsDNA (amplicon) concentration. The problem with DNA-binding agents is that they bind to all dsDNA products specific amplicon or non-specific products (misprimed targets and primer-dimers included). For this reason, analysis using DNA-binding agents is usually coupled with melting analysis.

Dynamic range: The range of initial template concentrations over which accurate C_t values are obtained. If endogenous control is used for DDC_t quantitation method, dynamic ranges of target and control should be comparable. In absolute quantitation, interpolation within this range is accurate but extrapolation beyond the dynamic range should be avoided. The larger the dynamic range, the greater the ability to detect samples with high and low copy number in the same run.

Efficiency of the reaction: The efficiency of the reaction can be calculated by the following equation: $E = 10^{(-1/slope)} - 1$. The efficiency of the PCR should be 90-100% meaning doubling of the amplicon at each cycle. This corresponds to a slope of -3.1 to -3.6 in the C_t vs log-template amount standard curve. In order to obtain accurate and reproducible results, reactions should have efficiency as close to 100% as possible (e.g., two-fold increase of amplicon at each cycle), and in any case, efficiency should be similar for both target and reference (normalizer, calibrator, endogenous control, internal control). A number of variables can affect the efficiency of the PCR. These factors can include length of the amplicon, presence of inhibitors, secondary structure and primer design. Although valid data can be obtained that fall outside of the efficiency range, if it is < 0.90, the quantitative real-time PCR should be further optimized or alternative amplicons designed.

Electrophoresis : A method of separating large molecules (such as DNA fragments or proteins) from a mixture of similar molecules.

An electric current is passed through a medium containing the mixture, and each kind of molecule travels through the medium at a different rate, depending on its electrical charge and size. Agarose and acrylamide gels are the media commonly used for electrophoresis of proteins and nucleic acids.

Endogenous control: This is an RNA or DNA that is naturally present in each experimental sample. By using an invariant endogenous control as an active 'reference', quantitation of a messenger RNA (mRNA) target can be normalized for differences in the amount of total RNA added to each reaction and correct for sample-to-sample variations in reverse transcriptase PCR efficiency.

End-point analysis: As opposed to quantitative analysis using the data collected during exponential phase of PCR, real-time applications can also be used to collect end-point data for qualitative assays. These are either allelic discrimination assays (genotyping) or absence/presence assays (pathogen detection).

Exogenous control: This is a characterized RNA or DNA spiked into each sample at a known concentration. An exogenous active reference is usually an in vitro construct that can be used as an internal positive control (IPC) to distinguish true target negatives from PCR inhibition. An exogenous reference can also be used to normalize for differences in efficiency of sample extraction or complementary DNA (cDNA) synthesis by reverse transcriptase. Whether or not an active reference is used, it is important to use a passive reference dye (usually ROX) in order to normalize for non-PCR-related fluctuations in fluorescence signal.

Exonuclease : An enzyme that cleaves nucleotides sequentially from free ends of a linear nucleic acid substrate.

FAM: 6-carboxy fluorescein. Most commonly used reporter dye at the 5' end of a TaqMan® probe.

Fast PCR: A modified PCR protocol that allows shortening of overall reaction time to less than the typical 90 minutes (usually 40 minutes or less) thanks to recent developments in amplicon design, reagent chemistry, thermocycling conditions as well as the PCR machines with fast ramping rates.

Fingerprinting : In genetics, the identification of multiple specific alleles on a person's DNA to produce a unique identifier for that person.

Fluorescence resonance energy transfer (FRET): The interaction between the electronic excited states of two dye molecules. The excitation is transferred from one (the donor) dye molecule to the other (the acceptor) dye molecule. FRET is distance-dependent and occurs when the donor and the acceptor dye are in close proximity.

GC-rich area : Many DNA sequences carry long stretches of repeated G and C which often indicate a gene-rich region.

Gene : The fundamental physical and functional unit of heredity. A gene is an ordered sequence of nucleotides located in a particular position on a particular chromosome that encodes a specific functional product (i.e., a protein or RNA molecule).

Gene amplification : Repeated copying of a piece of DNA; a characteristic of tumor cells.

Gene chip technology : Development of cDNA microarrays from a large number of genes. Used to monitor and measure changes in gene expression for each gene represented on the chip.

Gene pool : All the variations of genes in a species.

Genetic engineering : Altering the genetic material of cells or organisms to enable them to make new substances or perform new functions.

Genetic polymorphism : Difference in DNA sequence among individuals, groups, or populations (e.g., genes for blue eyes versus brown eyes).

Genetics : The study of inheritance patterns of specific traits.

Genotype : The genetic constitution of an organism, as distinguished from its physical appearance (its phenotype).

Guanine (G) : A nitrogenous base, one member of the base pair GC (guanine and cytosine) in DNA.

Haploid : A single set of chromosomes (half the full set of genetic material) present in the egg and sperm cells of animals and in the egg and pollen cells of plants. Human beings have 23 chromosomes in their reproductive cells. *See also:* diploid.

Haplotype : A way of denoting the collective genotype of a number of closely linked loci on a chromosome.

Heterozygosity : The presence of different alleles at one or more loci

on homologous chromosomes.

High-throughput sequencing : A fast method of determining the order of bases in DNA.

Homology : Similarity in DNA or protein sequences between individuals of the same species or among different species.

Homozygote : An organism that has two identical alleles of a gene.

Housekeeping gene: Genes that are widely expressed in abundance and are usually used as reference genes for normalization in real-time PCR with the assumption of 'constant expression'. The current trend is first to check which housekeeping genes are suitable for the target cell or tissue and then to use more than one of them in normalization in qPCR assays.

Human artificial chromosome (HAC) : A vector used to hold large DNA fragments.

Hybrid : The offspring of genetically different parents.

Hybridization : The process of joining two complementary strands of DNA or one each of DNA and RNA to form a double-stranded molecule.

Hybridization probe: One of the main fluorescence-monitoring systems for DNA amplification. LightCycler probes are hybridization probes and are not hydrolyzed by Taq Polymerase. For this reason, melting curve analysis is possible with hybridization probes.

Hydrolysis probe: One of the main fluorescence-monitoring systems for DNA amplification. TaqMan® probes are an example. These kinds of probes are hydrolyzed by the 5' endonuclease activity of Taq Polymerase during PCR.

Internal positive control (IPC): An exogenous IPC can be added to a multiplex assay or run on its own to monitor the presence of inhibitors in the template. Most commonly the IPC is added to the PCR master mix to determine whether inhibitory substances are present in the mix. Alternatively, it can be added at the point of specimen collection or prior to nucleic acid extraction to monitor sample stability and extraction efficiency, respectively.

Intron : DNA sequence that interrupts the protein-coding sequence of a gene; an intron is transcribed into RNA but is cut out of the message before it is translated into protein.

Junk DNA : Stretches of DNA that do not code for genes; most of the genome consists of so-called junk DNA which may have regulatory and other functions. Also called non-coding DNA.

Kilobase (kb) : Unit of length for DNA fragments equal to 1000 nucleotides.

LATE (Linear After The Exponential)-PCR: A new form of asymmetric PCR that uses primer pairs deliberately designed for use at unequal concentrations. Unlike typical asymmetric PCR, LATE-PCR, amplification is efficient due to improved primer design LATE-PCR begins with an exponential phase in which amplification efficiency is similar to that of symmetric PCR. Once the limiting primer is depleted, the reaction abruptly switches to linear amplification, and the single-stranded product is made for many additional thermal cycles. LATE-PCR consistently generates strong signals because the absence of product strand reannealing permits unhindered hybridization of the molecular beacon to its target strand and continued accumulation of that strand beyond the cycle at which symmetric reactions typically plateau. By eliminating the exponential phase, LATE-PCR generates less error scatter among replicates. When used in conjunction with molecular beacons, LATE-PCR results in increased signal intensity and reduced sample variation. These features are particularly useful for real-time PCR initiated with single cells. LATE-PCR has been used to directly amplify ssDNA for pyrosequencing.

Library : An unordered collection of clones (i.e., cloned DNA from a particular organism) whose relationship to each other can be established by physical mapping.
See also: genomic library, arrayed library

Light-up probe: The light-up probe is a peptide nucleic acid (PNA) oligomer to which an asymmetric cyanine dye thiazole orange (a single reporter dye) is tethered. Upon hybridization the thiazole orange moiety interacts with the nucleic acid bases and the probe becomes brightly (up to 50-fold) fluorescent. Being based on an uncharged analog (PNA), the light-up probe hybridizes faster and binds target DNA much stronger than oligonucleotide-based probes. The alternative is the log-view, which expands the initiation of exponential amplification phase (and also the baseline period activity). Either can be used for threshold setting.

Linkage : The proximity of two or more markers (e.g., genes, RFLP

markers) on a chromosome; the closer the markers, the lower the probability that they will be separated during DNA repair or replication processes (binary fission in prokaryotes, mitosis or meiosis in eukaryotes), and hence, the greater the probability that they will be inherited together.

Linkage disequilibrium : Where alleles occur together more often than can be accounted for by chance. Indicates that the two alleles are physically close on the DNA strand.

Linkage map : A map of the relative positions of genetic loci on a chromosome, determined on the basis of how often the loci are inherited together. Distance is measured in centimorgans (cM).

Localize : Determination of the original position (locus) of a gene or other marker on a chromosome.

Locked Nucleic Acid (LNA®) Probes: A new generation of sequence-specific probes designed using LNA (a novel nucleic acid analogue), which has enhanced hybridization performance and biological stability.

Locus (pl. loci) : The position on a chromosome of a gene or other chromosome marker; also, the DNA at that position. The use of locus is sometimes restricted to mean expressed DNA regions.

Log-dilution: Serial dilutions in powers of 10 (10, 100, 1000 etc).

Mass spectrometry : An instrument used to identify chemicals in a substance by their mass and charge.

Megabase (Mb) : Unit of length for DNA fragments equal to 1 million nucleotides and roughly equal to 1 cM.

Melting curve (dissociation) analysis: Every piece of dsDNA has a melting point (Tm) at which temperature 50% of the DNA is single stranded. The temperature depends on the length of the DNA, sequence order, G: C content and Watson-Crick pairing. When DNA-binding dyes are used, as the fragment is heated, a sudden decrease in fluorescence is detected when Tm is reached (due to dissociation of DNA strands and release of the dye). This point is determined from the inflection point of the melting curve or the melting peak of the derivative plot (what is meant by derivative plot is the negative first-derivative of the melting curve). The same analysis can be performed when hybridization probes are used as they are still intact after PCR. As hydrolysis probes (e.g.,

TaqMan®) are cleaved during the PCR reaction, no melting curve analysis possible if they are used (because of their specificity, there is no need either). Mismatch between a hybridization probe and the target results in a lower Tm. Melting curve analysis can be used in known and unknown (new) mutation analysis as a new mutation will create an additional peak or change the peak area.

Mendelian inheritance : One method in which genetic traits are passed from parents to offspring. Named for Gregor Mendel, who first studied and recognized the existence of genes and this method of inheritance.

Microarray : Sets of miniaturized chemical reaction areas that may also be used to test DNA fragments, antibodies, or proteins.

Minor groove binders (MGBs): These dsDNA-binding agents are attached to the 3′ end of TaqMan® probes to increase the Tm value (by stabilization of hybridization) and to design shorter probes. Longer probes reduce design flexibility and are less sensitive to mismatch discrimination. MGBs also reduce background fluorescence and increase dynamic range due to increased efficiency of reporter quenching (these probes use non-fluorescent quenchers at the 3'end). By allowing the use of shorter probes with higher Tm values, MGBs enhances mismatch discrimination in genotyping assays.

Minus reverse transcriptase control (- RTC): A quantitative real-time PCR control sample that contains the starting RNA and all other components for one-step reaction but no reverse transcriptase. Any amplification suggests genomic DNA contamination.

Molecular beacons: These hairpin probes consist of a sequence-specific loop region flanked by two inverted repeats. Reporter and quencher dyes are attached to each end of the molecule and remain in close contact unless sequence-specific binding occurs and reporter emission (FRET) occurs.

Monte Carlo effect: Problems with reproducible quantification of low abundance targets (<1000 copies) by qPCR. It is a limitation of PCR amplification from small amounts of any complex template due to differences in amplification efficiency between individual templates in an amplifying cDNA population. The Monte Carlo effect is dependent upon template concentration; the lower the abundance of any template, the less likely its true abundance will

be reflected in the amplified product.

Multiplexing: Simultaneous analysis of more than one target. Specific quantification of multiple targets that are amplified within a reaction can be performed using a differentially labeled primer or probes. Amplicon or probe melting curve analysis allows multiplexing in allelic discrimination if a dsDNA-binding dye is used as the detection chemistry.

Mutation : Any heritable change in DNA sequence.

Nitrogenous base : A nitrogen-containing molecule having the chemical properties of a base. DNA contains the nitrogenous bases adenine (A), guanine (G), cytosine (C), and thymine (T).*See also:* DNA.

No amplification controls (NAC, a minus enzyme control): In mRNA analysis, NAC is a mock reverse transcription containing all the RT-PCR reagents, except the reverse transcriptase. If cDNA or genomic DNA is used as a template, a reaction mixture lacking Taq polymerase can be included in the assay as NAC. No product should be synthesized in the NTC or NAC. If the absolute fluorescence of the NAC is greater than that of the NTC after PCR, fluorescent contaminants may be present in the sample or in the heating block of the thermal cycler.

No template controls (NTC, a minus sample control): NTC includes all of the RT-PCR reagents except the RNA template. No product should be synthesized in the NTC or NAC; if a product is amplified, this indicates contamination (fluorescent or PCR products) or presence of genomic DNA in the RNA sample. NTC is not equivalent to H_2O controls and H_2O controls are not used in qPCR experiments.

Normalization: A control gene that is expressed at a constant level is used to normalize the gene expression results for variable template amount or template quality. If RNA quantitation can be done accurately, normalization might be done using total RNA amount used in the reaction. The use of multiple housekeeping genes that are most appropriate for the target cell or tissue is the most optimal means for normalization. This normalization is performed by the experimenter and should not be mixed up with the normalization of fluorescence signal using the passive reference dye (usually ROX) performed by the equipment.

Normalized amount of target: A unitless number that can be used to compare the relative amount of target in different samples.

Northern blot : A gel-based laboratory procedure that locates mRNA sequences on a gel that are complementary to a piece of DNA used as a probe.

Nuclear transfer : A laboratory procedure in which a cell's nucleus is removed and placed into an oocyte with its own nucleus removed so the genetic information from the donor nucleus controls the resulting cell. Such cells can be induced to form embryos. This process was used to create the cloned sheep "Dolly".

Nucleic acid : A large molecule composed of nucleotide subunits.

Nucleic acid sequence based amplification (NASBA): NASBA is an isothermal nucleic acid amplification procedure based on target-specific primers and probes, and the coordinated activity of THREE enzymes AMV reverse transcriptase, RNase H and T7 RNA polymerase. NASBA allows direct detection of viral RNA by nucleic acid amplification.

Nucleic acid target: (also called "target template") - DNA or RNA sequence that is going to be amplified.

Nucleolar organizing region : A part of the chromosome containing rRNA genes.

Nucleotide : A subunit of DNA or RNA consisting of a nitrogenous base (adenine, guanine, thymine, or cytosine in DNA; adenine, guanine, uracil, or cytosine in RNA), a phosphate molecule, and a sugar molecule (deoxyribose in DNA and ribose in RNA). Thousands of nucleotides are linked to form a DNA or RNA molecule.

Nucleus : The cellular organelle in eukaryotes that contains most of the genetic material.

Oligonucleotide : A molecule usually composed of 25 or fewer nucleotides; used as a DNA synthesis primer.

Passive reference (reference dye): A fluorescence dye that provides an internal reference to which the reporter dye signal can be normalized during data analysis by the software. This type of normalization is necessary to correct for fluctuations from well to well caused by changes in concentration or volume. **ROX** is the most commonly used passive reference dye.

Peltier element: The element used for heating and cooling in a qPCR machine. Peltier coolers (in ABI machines) use electron flow between semiconductor couples to heat or cool one side of a plate depending on the direction of current. Other systems use liquid or air flow or mechanical transition between blocks of different temperatures to cycle the samples.

Physical map : A map of the locations of identifiable landmarks on DNA (e.g., restriction-enzyme cutting sites, genes), regardless of inheritance. Distance is measured in base pairs. For the human genome, the lowest-resolution physical map is the banding patterns on the 24 different chromosomes; the highest-resolution map is the complete nucleotide sequence of the chromosomes.

Plasmid : Autonomously replicating extra-chromosomal circular DNA molecules, distinct from the normal bacterial genome and nonessential for cell survival under nonselective conditions. Some plasmids are capable of integrating into the host genome. A number of artificially constructed plasmids are used as cloning vectors.

Platform: Refers to hardware that performs real-time PCR. For a current list of available machines **PNA** (peptide nucleic acid oligomer)

Polymerase chain reaction (PCR) : A method for amplifying a DNA base sequence using a heat-stable polymerase and two 20-base primers, one complementary to the (+) strand at one end of the sequence to be amplified and one complementary to the (-) strand at the other end. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce rapid and highly specific amplification of the desired sequence. PCR also can be used to detect the existence of the defined sequence in a DNA sample.

Polymerase, DNA or RNA : Enzyme that catalyzes the synthesis of nucleic acids on preexisting nucleic acid templates, assembling RNA from ribonucleotides or DNA from deoxyribonucleotides.

Polymorphism : Difference in DNA sequence among individuals that may underlie differences in health. Genetic variations occurring in more than 1% of a population would be considered useful polymorphisms for genetic linkage analysis.

Population genetics : The study of variation in genes among a group of individuals.

Positional cloning : A technique used to identify genes, usually those that are associated with diseases, based on their location on a chromosome.

Primer : Short preexisting polynucleotide chain to which new deoxyribonucleotides can be added by DNA polymerase.

Probe : Single-stranded DNA or RNA molecules of specific base sequence, labeled either radioactively or immunologically, that are used to detect the complementary base sequence by hybridization

Protein : A large molecule composed of one or more chains of amino acids in a specific order; the order is determined by the base sequence of nucleotides in the gene that codes for the protein. Proteins are required for the structure, function, and regulation of the body's cells, tissues, and organs; and each protein has unique functions. Examples are hormones, enzymes, and antibodies.

Proteome : Proteins expressed by a cell or organ at a particular time and under specific conditions.

Proteomics : The study of the full set of proteins encoded by a genome.

Purine : A nitrogen-containing, double-ring, basic compound that occurs in nucleic acids. The purines in DNA and RNA are adenine and guanine.

Pyrimidine : A nitrogen-containing, single-ring, basic compound that occurs in nucleic acids. The pyrimidines in DNA are cytosine and thymine; in RNA, cytosine and uracil.

Quencher: The molecule that absorbs the emission of fluorescent reporter when in close vicinity. Most commonly used quenchers include TAMRA, DABCYL and BHQ. The quenchers are usually at the 3′ end of a dual-labeled fluorescent probe. Quencher dye is also called acceptor.

R: In illustrations of real-time PCR principles, 'R' represents fluorescent Reporter (fluorochrome).

r coefficient: Correlation coefficient, which is used to analyze a standard curve (ten-fold dilutions plotted against C_t values) obtained by linear regression analysis. It should be = 0.99 for gene quantitation analysis. It takes values between zero and -1 for

negative correlation and between zero and +1 for positive correlations.

R^2 coefficient: Usually mixed up with 'r' but this is R-squared (also called coefficient of determination). This coefficient only takes values between zero and +1. R^2 is used to assess the fit of the standard curve to the data points plotted. The closer the value to 1, the better the fit.

Rapid-cycle PCR: A powerful fast PCR technique for nucleic acid amplification and analysis that is completed in less than half an hour. Samples amplified by rapid-cycle PCR are immediately analyzed by melting curve analysis in the same instrument. In the presence of fluorescent hybridization probes, melting curves provide 'dynamic dot blots' for fine sequence analysis, including SNPs.

Real-time PCR: The continuous collection of fluorescent signal from polymerase chain reaction throughout cycles.

Recombinant clone : Clone containing recombinant DNA molecules.*See also:* recombinant DNA technology

Recombinant DNA molecules : A combination of DNA molecules of different origin that are joined using recombinant DNA technologies.

Recombinant DNA technology : Procedure used to join together DNA segments in a cell-free system (an environment outside a cell or organism). Under appropriate conditions, a recombinant DNA molecule can enter a cell and replicate there, either autonomously or after it has become integrated into a cellular chromosome.

Recombination: The process by which progeny derive a combination of genes different from that of either parent. In higher organisms, this can occur by crossing over. *See also:* crossing over, mutation

Reference: A passive or active signal used to normalize experimental results. Endogenous and exogenous controls are examples of active references. Active reference means the signal is generated as the result of PCR amplification.

Reference dye: Used in all reactions to obtain normalized reporter signal (Rn) adjusted for well-to-well variations by the analysis software. The most common passive reference dye is **ROX** and is

usually included in the master mix.

Regulatory region or sequence : A DNA base sequence that controls gene expression.

Relative quantitation: A relative quantification assay is used to analyze changes in gene expression in a given sample relative to another reference sample (such as relative increase or decrease - compared to the baseline level- in gene expression in response to a treatment or in time etc). Includes comparative C_t (??C_t) and relative-fold methods.

Repetitive DNA : Sequences of varying lengths that occur in multiple copies in the genome; it represents much of the human genome.

Reporter dye (fluorophore): The fluorescent dye used to monitor amplicon accumulation. This can be attached to a specific probe or can be a dsDNA-binding agent (see for example **SYBR® Green I**).

Resolution : Degree of molecular detail on a physical map of DNA, ranging from low to high.

Restriction enzyme, endonuclease: A protein that recognizes specific, short nucleotide sequences and cuts DNA at those sites. Bacteria contain over 400 such enzymes that recognize and cut more than 100 different DNA sequences.

Restriction fragment length polymorphism (RFLP) : Variation between individuals in DNA fragment sizes cut by specific restriction enzymes; polymorphic sequences that result in RFLPs are used as markers on both physical maps and genetic linkage maps. RFLPs usually are caused by mutation at a cutting site.

Restriction-enzyme cutting site : A specific nucleotide sequence of DNA at which a particular restriction enzyme cuts the DNA. Some sites occur frequently in DNA (e.g., every several hundred base pairs); others much less frequently (rare-cutter; e.g., every 10,000 base pairs).

Reverse transcriptase : An enzyme used by retroviruses to form a complementary DNA sequence (cDNA) form.

Ribosomal RNA (rRNA): Commonly used as a normalizer in quantitative real-time RNA. It is not considered ideal due to its expression levels, transcription by a different RNA polymerase and possible imbalances in relative rRNA-to-mRNA content in different cell types.

Ribosomal RNA (rRNA) : A class of RNA found in the ribosomes of cells.

Ribosomes : Small cellular components composed of specialized ribosomal RNA and protein; site of protein synthesis.

Rn (delta Rn, dRn): The magnitude of the fluorescence signal generated during the PCR at each time point. The Rn value is determined by the following formula (Rn+) - (Rn-).

Rn (normalized reporter signal): The fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye. Rn+ is the Rn value of a reaction containing all components, including the template and Rn- is the Rn value of an unreacted sample. The Rn- value can be obtained from the early cycles of a real-time PCR run (those cycles prior to a significant increase in fluorescence), or a reaction that does not contain any template.

RNA (Ribonucleic acid) : A chemical found in the nucleus and cytoplasm of cells; it plays an important role in protein synthesis and other chemical activities of the cell. The structure of RNA is similar to that of DNA. There are several classes of RNA molecules, including messenger RNA, transfer RNA, ribosomal RNA, and other small RNAs, each serving a different purpose.

ROX: 6-carboxy-X-rhodamine. Most commonly used passive reference dye for normalization of reporter signal. The emission recorded from ROX during the baseline cycles (usually 3 to 15) is used to normalize the emission recorded from the reporter due to amplification in later cycles. The use of ROX improves the results by compensating for small fluorescent fluctuations such as bubbles and well-to-well variations that may occur in the plate. Not using ROX or not designating it as the passive reference dye in the analysis may cause trailing of the clusters in the allelic discrimination plot.

Sanger sequencing : A widely used method of determining the order of bases in DNA.

Scorpion: A fluorescence detection system consists of a detection probe with the upstream primer with a fluorophore at the 5' end, followed by a complementary stem-loop structure also containing the specific probe sequence, quencher dye and a PCR primer on the 3' end. Between the primer and its tail (the probe), a blocking

agent (DNA spacer, hexaethylene glycol) is placed. This structure makes the sequence-specific priming and probing a unimolecular event that creates enough specificity for allelic discrimination assays.

Sequencing : Determination of the order of nucleotides (base sequences) in a DNA or RNA molecule or the order of amino acids in a protein.

Sequencing technology : The instrumentation and procedures used to determine the order of nucleotides in DNA.

Single nucleotide polymorphism (SNP) : DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered.

Slope: Mathematically calculated slope of standard curve, e.g., the plot of C_t values against logarithm of ten-fold dilutions of target nucleic acid. This slope is used for efficiency calculation. Ideally, the slope should be -3.3 (-3.1 to -3.6), which corresponds to 100% efficiency (precisely 1.0092) or two-fold (precisely, 2.0092) amplification at each cycle. Also called gradient.

Southern blotting : Transfer by absorption of DNA fragments separated in electrophoretic gels to membrane filters for detection of specific base sequences by radio-labeled complementary probes.

Standard: A sample of known concentration used to construct a standard curve. By running standards of varying concentrations, a standard curve is created from which the quantity of an unknown sample can be calculated.

Standard curve: Obtained by plotting C_t values against log-transformed concentrations of serial ten-fold dilutions of the target nucleic acid. Standard curve is obtained for quantitative PCR and the range of concentrations included should cover the expected unknown concentrations range. It is used to find out the dynamic range of the target (and/or normalizer), to calculate the slope (therefore, efficiency), r and R^2 coefficients and also to help with quantitation.

Sunrise™ primers: Originally created by Oncor, sunrise™ primers are similar to molecular beacons. They are self-complementary primers that dissociate through the synthesis of the complementary strand and produce fluorescence signals. Currently known as Amplifluor® fluorescent detection system. A fluorogenic minor

groove binding dye that emits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. It is used as a cheaper alternative in real-time PCR applications. It does not bind to ssDNA but because of the lack of sequence specificity it binds to any dsDNA product. Its use usually requires melting curve analysis to assure specificity of the results (and if multiplexing is attempted).

TAMRA: 6-carboxy-terta-methyl-rhodamine. Most commonly used quencher at the 3' end of a TaqMan® probe.

Tandem repeat sequences : Multiple copies of the same base sequence on a chromosome; used as markers in physical mapping.

TaqMan® probe: A dual-labeled specific hydrolysis probe designed to bind to a target sequence with a fluorescent reporter dye at one end (5') and a quencher at the other (3'). Assays using Taqman probes are also called 5' nuclease assays. The threshold should be set in the region associated with an exponential growth of PCR product (which may be easier is the log-view of the amplification plot is used). It is assigned for each run to calculate the C_t value for each amplification.

Unknown: A sample containing an unknown quantity of template. This is the sample of interest (experimental sample as opposed to positive controls or standards) whose quantity is being determined.

Uracil : A nitrogenous base normally found in RNA but not DNA; uracil is capable of forming a base pair with adenine.

Virus : A noncellular biological entity that can reproduce only within a host cell. Viruses consist of nucleic acid covered by protein; some animal viruses are also surrounded by membrane. Inside the infected cell, the virus uses the synthetic capability of the host to produce progeny virus.

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