CHAPTER 2

DNA Fingerprinting: Multi-locus Analysis

Introduction

The goal of this experiment is to carry out multi-locus DNA fingerprinting. For this procedure you will use your own DNA and a probe that recognizes a family of DNA mini-satellites with the core sequence GAGGGTGGNG GNTCT. The typing of your DNA will require performing five procedures. The essential steps of the typing procedure are as follows.

1. DNA is digested into fragments using restriction endonuclease enzymes. We will use the enzyme *Hae*III, which is widely used in forensic work. Experiment 1 describes this procedure.

2. DNA fragments resulting from digestion are separated based on size by agarose gel electrophoresis. Experiment 2 describes this technique.

3. DNA fragments are transferred from gel onto a nylon membrane by a process termed Southern blotting. The membrane will contain the DNA fragment located in exactly the same position as it was present in the gel. This procedure will be performed in experiment 3.

4. Immobilized DNA fragments are hybridized with labeled DNA probe complementary to core mini-satellite sequences. Labeling of the probe and hybridization will be carried out in experiment 4.

5. Hybridized DNA fragments are detected by chemiluminescence. This is described in the signal detection experiment.

The entire experiment will be done during five laboratory periods. Figure 2.1 presents the overall timetable for these experiments.

Background

The human genome contains approximately 3 billion bp. This genome, similar to all other higher eucaryotes, may be divided into classes based very broadly on their functional properties. Approximately 10 percent of the genome constitutes DNA sequences harboring genetically relevant

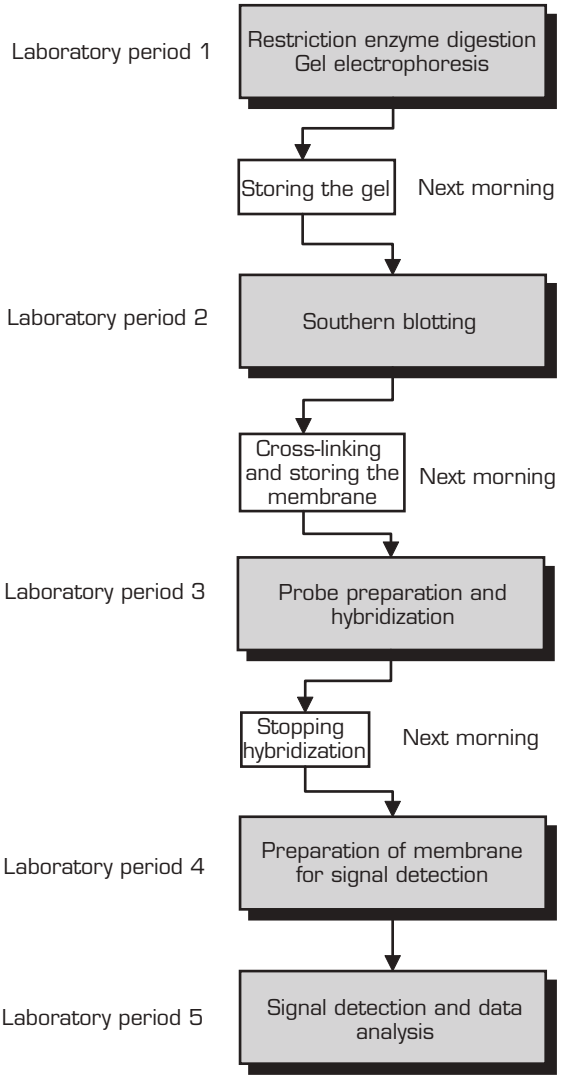

Figure 2.1 Schematic outline of the procedures used in the multi-locus DNA fingerprinting experiment.

information that is essential for each individual, i.e. the gene-coding region of the DNA. The remaining DNA constitutes non-coding regions of the genome. This part of the genome, largely due to its non-coding nature, is the major source of variability or polymorphism-responsible differences between individuals. The non-coding segment of the genome consists of two classes of DNA sequences: unique low copy number DNA and repetitive DNA. Repetitive DNA can in turn be further subdivided into interspersed repetitive DNA and tandemly repeated DNA.

Tandemly repetitive sequences, commonly known as "satellite DNAs" are classified into three major groups.

1. Satellites. These are very highly repetitive DNA sequences with repeat lengths of one to several thousand base pairs. These sequences are typically organized as large clusters (up to 100 million bp) in the heterochromatic regions of chromosomes, centromeres, and telomeres. Human centromeric and pericentromeric DNA consists predominantly of satellites II, III, IV, alphoid, and I that are specific or nearly specific for particular chromosomes. In contrast, telomeric tandem repeats are conserved and identical for all chromosomes. In addition, specific satellite DNA sequences are present on the long arm of the Y chromosome.

2. Micro-satellites. These are moderately repetitive DNA sequences composed of arrays of short repeats (2–10 bp). The human genome contains at least 30,000 micro-satellite loci located in euchromatin. The number of repeats is characteristically variable within a population for each micro-satellite, typically with mean array sizes in the order of ten to 100 repeats.

3. Mini-satellites. These are moderately repetitive, tandemly repeated arrays of 10–100 bp spanning 0.5 kb to several kilobases. They are found in euchromatic regions of the genome and are predominantly clustered towards chromosome ends. They are highly variable in array size.

In general, satellite DNAs can be variable among individuals and, thus, form excellent tools for genetic individualization, particularly with regard to the number of repeats at a given locus. Mini-satellite loci are the most highly polymorphic sequence elements yet discovered in the human genome and delineating the repeat lengths of these loci is the basis of most DNA typing systems used in forensic medicine. These loci are usually referred to as variable number tandem repeat (VNTR) loci. The VNTRs can be grouped into families of independent loci that are related to each other by small variation in their common core sequence. Some VNTR loci are hypervariable and contain between 100 and 1,000 repeats. The variability of these loci is not limited to differences in the number of the repeated unit, but also the sequence of the repeat can vary in different members of an array. Thus, any given hypervariable VNTR allele can be monomorphic for length, but may still be polymorphic in structure. At present approximately 300 human mini-satellite families have been typed and less than ten of them are hypervariable (Nakamura et al., 1987; Armour et al., 1990; Amarger et al., 1998; Vergnaud and Denoeud, 2000).

Hypervariable VNTR loci are used in genetic typing by means of two methodologies: a **multi-locus analysis** or DNA fingerprinting and a **single-locus analysis** or single-locus DNA typing. Jeffreys et al. (1985a, b) first introduced DNA fingerprinting for individual identification in 1985. Soon after this the first case, which involved a UK immigration dispute, was satisfactorily resolved by DNA fingerprinting. Shortly after the method was used in an unusual paternity dispute in a UK court. DNA fingerprinting

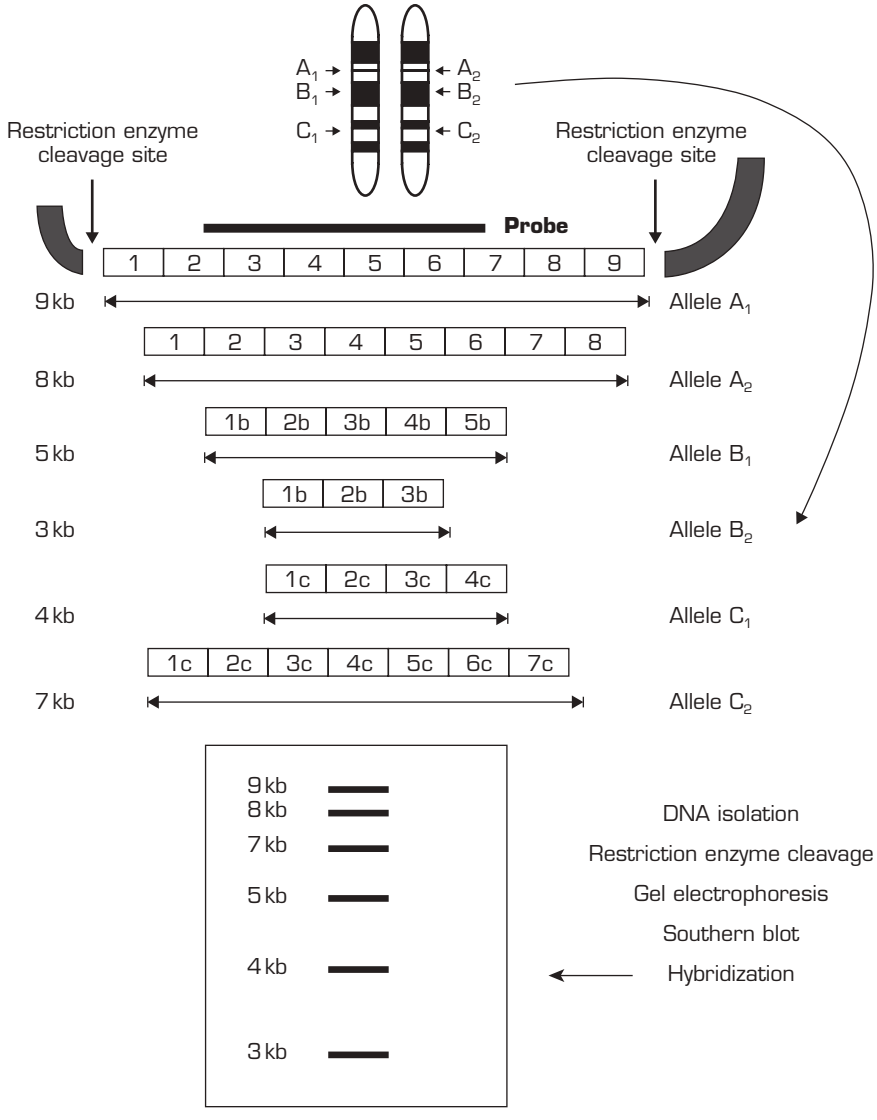

Figure 2.2 Principle of multi-locus DNA fingerprinting. Independent mini-satellite loci A, B, and C are members of one VNTR family related to each other by a small variation in the common core sequence of 1,000 bp. Each locus has a different number of repeats on each homologous chromosome, designated as A_1 and A_2, B_1 and B_2, and C_1 and C_2. In order to generate a DNA fingerprint, DNA is cut with restriction endonuclease that does not have a recognition site on any repeat. This generates a set of DNA fragments of different sizes, which is a consequence of the different number of repeats present at a particular locus. Thus, locus A_1 will be represented by 9 kb fragments, locus A_2 by 8 kb fragments, locus B_1 by 5 kb fragments, etc. These fragments are separated by agarose gel electrophoresis, transferred to a membrane, and hybridized to a probe complementary to the repeated element. The autogram shows a set of hybridization bands that represent hybridization to each member of the VNTR family. This is called a DNA fingerprint.

made its debut in a criminal case in 1986 in the Enderbery murder case (Jeffreys and Pena, 1993). After a period of initial disputes as to the validity of DNA fingerprinting for unequivocal identification of an individual, the method became established worldwide in forensic medicine and criminal investigations (Benecke, 1997). DNA typing has found many uses other than forensic applications (Kirby, 1990). These include animal and plant breeding, conservation biology, patent identification, genototoxicity studies, etc.

Both, DNA typing (single-locus analysis) and DNA fingerprinting (multi-locus analysis) use the same technique for revealing the polymorphic variation in the number of tandem repeats. DNA is digested with restriction enzyme that cuts externally to the tandem repeat, fragments are separated by gel electrophoresis, and Southern blots are hybridized to a probe either recognizing an internal core sequence (DNA fingerprinting) or a locus-specific sequence (single-locus analysis). The principle of multi-locus DNA fingerprinting is presented in Fig. 2.2.

The quantity of non-degraded DNA needed for these analyses is in the order of 1–10 μg. Because, with the exception of parentage studies, the quantity and integrity of DNA in a typical forensic specimen is limited, a PCR (polymerase chain reaction)-based technique was developed. This technique requires only a few nanograms of DNA and, indeed, the DNA of a single cell is usually sufficient for successful DNA typing. An efficient PCR is possible for fragments of approximately 1 kb in size. Most classical VNTR alleles are much longer than that limit and cannot be reliably amplified. However, PCRs can be used for amplifying tandem repeats of micro-satellites, named short tandem repeats (STRs), for which the repeat arrays are in the range of 50–300 bp. At present forensic DNA analysis uses STR loci exclusively for individual identification. These loci are composed of tri-, tetra-, and pentameric core units and are evenly distributed throughout all chromosomes. Because the primer used is unique for a given STR locus, this method uses a single-locus approach for data analysis.

FIRST LABORATORY PERIOD

In this laboratory period two experiments will be performed. First, you will digest your DNA with restriction enzyme endonuclease. Second, you will carry out agarose gel electrophoresis of restricted DNA.

Experiment 1: Restriction Enzyme Digestion

Introduction

In this experiment you will digest DNA purified in Chapter 1 using type II restriction enzyme endonuclease *Hae*III. You will digest your DNA and your partner's DNA as well as two control DNAs that will be given to you by your instructor. This experiment will take a single laboratory period.

The *Hae*III restriction enzyme endonuclease is the most often used restriction endonuclease in human DNA fingerprinting. The recognition sequence for this enzyme is GGCC assuring extensive digestion of human DNA. This recognition site is not present in the M13 15 bp tandem repeat element (GAGGTGGNGGNTCT) that we will use for the multi-locus fingerprint of your DNA.

Background

Restriction endonucleases catalyze sequence-dependent, double-stranded breaks in DNA yielding a homogeneous population of DNA fragments. These enzymes are used in a number of applications in molecular biology, including establishment of an endonuclease map of DNA, fragmentation of genomic DNA prior to Southern blotting, generation of fragments that can be subcloned in appropriate vectors, and generation of fragments for labeled probes.

The most frequently used restriction endonucleases belong to the type II endonucleases discovered by Smith and co-workers (Smith and Wilcox, 1970). These enzymes are small, monomeric proteins that require only Mg^{2+} for activity. Type II enzymes recognize a short nucleotide sequence with dyad symmetry (the 5′ to 3′ nucleotide sequence of one DNA strand is identical to that of the complementary strand sequences). Most sites consist of 4, 5, or 6 bp (Brooks, 1987), but a few have a recognition site of 8 bp or larger or sites smaller than 4 bp (Roberts and Macelis, 1991). In general, there are three possible cleavage positions within a recognition sequence: at the center of the axis of symmetry, yielding "flush" or "blunt" ends (e.g. CCC3′|5′GGG), to the left of the center giving cohesive termini with a protruding 5′-phosphate (e.g. C3′|5′CGG), or to the right of the center giving cohesive termini with protruding 3′-phosphates (e.g. CTGCA3′|5′G).

An estimate of the number of cleavage sites for a restriction endonuclease within a given piece of DNA, assuming an even distribution of bases, is described by the equation site number = $N/4^n$, where N is a number of base pairs in the DNA and n is the number of bases in the recognition site of the restriction endonuclease. This should be treated only as an approximation of the number of expected sites (Rodriguez and Tait, 1983).

Owing to the complicated nature of the restriction reaction and differences in substrates, it is difficult to define universal units of activity for these enzymes. For this reason, a convention was adopted for defining a unit of enzyme activity as the amount of enzyme required to digest 1 µg of bacteriophage lambda DNA completely in 1 hour.

Despite the diversity of the sources and of the specificity of over 1,000 type II restriction endonucleases identified to date, restriction enzyme reaction conditions are remarkably similar. Each restriction enzyme has its own optimal reaction conditions, which are usually given on the information sheet provided by the manufacturer. The major variables are the temperature of incubation and the composition of the buffer. The temperature requirements of type II restriction enzymes are very strict, whereas the differences between salt and pH requirements are often only slight. Presently the manufacturer of the enzyme supplies the appropriate buffer for each enzyme.

Technical tips

The general rules for working with restriction enzymes and preparing a digestion reaction are as follows.

1. Store restriction endonuclease at –20°C in a freezer that is not frost free at a concentration of 10 u μl^{-1} or higher.

2. The volume of the digestion reaction should be large enough that the restriction enzyme constitutes no more than 10 percent of the total volume. A 30 µl reaction volume should be routinely used.

3. Use a DNA amount no greater than 10 µg added in a volume that is not to exceed one-third of the reaction volume. Addition of a large volume of DNA dissolved in TE buffer will decrease the Mg^{2+} ion concentration in the reaction, thereby inhibiting restriction enzyme activity.

4. Use ten units or more of enzyme per microgram of DNA. Although this is far more enzyme than is theoretically required, this excess assures complete digestion in the case of impurities in the DNA, decreased enzyme activity from storage, pipetting errors during enzyme addition, etc. Some enzymes cleave their defined sites with different efficiency, largely due to differences in the flanking nucleotides and cleavage rates. Different sites recognized by a given enzyme can differ by a factor of ten. Using excess enzyme does compensate for these differences. Use 20-fold excess enzyme when digesting human genomic DNA.

Protocol

Always observe the following rules when preparing the reaction mixture. First, thaw all reagents at room temperature and place them on ice. Second, calculate the amounts of all reagents needed. Do not include water in these calculations. Next calculate the amount of water needed to obtain the desired reaction volume.

Start the assembly of the reaction mixture by addition of water. Remember the rule for reaction mixture assembly: **the amount of water is always calculated last, but water is always added first.** Add the remaining ingredients in the following order: buffer, co-factors, and substrate. Start the reaction by the addition of enzyme.

1. Label four sterile 1.5 ml microfuge tubes as follows: 1H, 2H, 3H, and 4H.

2. Calculate the volume of DNA solution to be drawn in order to have 3 µg of DNA per reaction. Use your DNA concentration as determined in the DNA isolation experiment. Your instructor will give you the concentrations of control DNAs. Record the results of these calculations in Table 2.1.

3. Calculate the amount of enzyme and buffer to be added to each reaction. The concentration of the buffer stock solution is ten times and the enzyme concentration in the stock solution is $10\,u\,\mu l^{-1}$. You will need 30 units of enzyme per reaction. Record these calculations in Table 2.1.

4. Calculate the amount of water needed to obtain a final reaction volume of 30 µl.

5. Add the calculated amount of water to each tube.

6. Add 3 µl of ten times buffer to each tube. Mix by pipetting up and down.

7. Add the appropriate DNA for each tube. Consult Table 2.1 for the amount and type of DNA to be added to each tube. For the addition of each DNA you will need to prepare a wide-bore yellow tip. Prepare each tip by cutting off 5–6 mm from the end of the tip with a razor blade. Set a P20

Table 2.1 Restriction enzyme digestion of DNA

Tube number	1H	2H	3H	4H
Buffer (ten times)	3.0 µl	3.0 µl	3.0 µl	3.0 µl
DNA control 1		–	–	–
DNA control 2	–		–	–
Your DNA	–	–		–
Partner's DNA	–	–	–	
*Hae*III enzyme ($10\,u\,\mu l^{-1}$)	3.0 µl	3.0 µl	3.0 µl	3.0 µl
Water				
Total	30.0 µl	30.0 µl	30.0 µl	30.0 µl

Pipetman to the required volume for each DNA type. Draw that amount of TE buffer into the tip and mark the level of the liquid with a marking pen. Discard TE buffer from the tip. Using the same tip draw the DNA solution to this mark and transfer it to the reaction mixture. Pipette up and down several times in order to remove the viscose DNA solution from the inside of the pipette tip. **Note:** it is important to follow the procedure described above in order to prevent DNA shearing during pipetting.

8. Start reactions with the addition of enzyme. Mix the enzyme with the reaction mixture by pipetting up and down several times.

9. Centrifuge the tube for 5–10 seconds in order to remove air bubbles and collect liquid at the bottom of the tube.

10. Transfer the tubes to a 37°C water bath and incubate for 1–2 hours.

11. Begin preparing the agarose gel as described in the protocol of step 2 in experiment 2.

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Experiment 2: agarose gel electrophoresis

Introduction

The procedure describes the use of a large agarose gel in the separation of human DNA fragments. DNA band separation in such gels is sufficient for detecting a single copy gene in a human genome. These gels are well suited for Southern blotting. In order to attain higher resolution, electrophoresis will be run at a low voltage gradient of 1 V cm^{-1}. Thus, the electrophoresis time will be longer than usual, but the resolution of DNA fragments will be better, in particular for higher molecular weight DNA.

Background

Principle of electrophoresis

When a molecule is placed in an electric field it will migrate to the appropriate electrode with a velocity or free electrophoretic mobility (M_0), which is described by the equation:

$$M_0 = \frac{E}{d}\frac{q}{6\pi R\eta} \tag{2.1}$$

where E is the potential difference between electrodes measured in volts, q is the net charge of the molecule, d is the distance between electrodes (cm), η is the viscosity of the solution, R is Stock's radius of the molecule, and E/d is the field strength.

Since under physiological conditions phosphate groups in the phosphosugar backbone of DNA (RNA) are ionized, these polyanions will migrate to the positive electrode (anode) when placed in an electric field. Due to the repetitive nature of the phosphosugar backbone, double-stranded DNA molecules have a net charge to mass ratio that is approximately the same. Consequently, DNA molecules have approximately the same free electrophoretic mobility (M_0) irrespective of their size. It is apparent from equation (1) that the effects of friction on the mobility of the molecules can be accentuated by changing the viscosity (η) of the electrophoretic medium. If the viscosity is very large, the mobility of the molecules subjected to electrophoresis will depend largely on their shape and size. Equation (2.1) simplifies to:

$$M_0 = \frac{E}{d}\frac{1}{R} \tag{2.2}$$

Specific support matrixes are used for increasing the viscosity of an electrophoretic medium. These include agarose and polyacrylamide. Varying the pore size using various agarose concentrations or different cross-linking ratios of polyacrylamide alters the viscosity of these materials. The mobility of DNA molecules is profoundly influenced by the size and shape of the molecules, as well as by the size of the matrix pores. Using these gels, DNA molecules are fractionated by their size and conformation in a relatively fast and inexpensive way.

Principle of agarose gel electrophoresis of DNA

Agarose is a polysaccharide consisting of basic agarobiose repeat units of 1, 3-linked β-D-galactopyranose and 1, 4-linked 3, 6-anhydro-α-L-galactopyranose. Units form long chains of approximately 400 repeats, reaching a molecular weight of approximately 120,000 Da. Long polymer chains contain small amounts of charged residues consisting largely of pyruvate and sulfate that are responsible for agarose properties that are important in gel electrophoresis. To these belongs the phenomenon of electroendosmosis (Adamson, 1976; Hiemenz, 1977).

During electrophoresis only hydrated positive ions, which are normally associated with the fixed anionic groups of agarose (pyruvate or sulfate residues), can move towards the cathode. Water is therefore pulled with these positive ions towards the negative electrode and negative molecules, such as DNA migrating towards the positive electrode, are slowed down. Thus, for maximum separation of DNA molecules by agarose gel electrophoresis, agarose with the lowest possible electroendosmosis should be used.

The electrophoretic migration rate of DNA through agarose gel depends on the following parameters: the size of the DNA molecules, the concentration of agarose, the voltage applied, the conformation of the DNA, and the buffer used for electrophoresis.

On first approximation, DNA molecules travel through gel at a rate inversely proportional to the logarithm of their molecular weight or number of base pairs. Therefore, a plot of mobility against the log of the size should give a straight line for all DNA sizes. However, this is true for a narrow size range. A better linear relationship between mobility and DNA size is obtained in plots of DNA base pair number (DNA size) versus 1/mobility (Hiemenz, 1977; Sealey and Southern, 1982).

The useful linear range of mobility depends on the gel concentration used and voltage applied. A DNA fragment of a given size migrates at different rates in gels containing different concentrations of agarose. A model for gel structure predicts that the log of the mobility of different DNA molecules (M) as a function of gel concentration (C) should result in a straight line with different slopes called retardation coefficients (K_r) and intercepts the

so-called free mobility (M_0), i.e. the mobility of DNA molecules at zero concentration of agarose. This can be expressed mathematically by the following equation:

$$\text{Log } M = \log M_0 - CK_r \tag{2.3}$$

It is possible to resolve a wide range of DNA fragment sizes using gels of different agarose concentrations provided that the voltage gradient applied to the gel is chosen correctly. Normally the migration rate of DNA fragments is directly proportional to the voltage applied. However, with increased voltage large DNA molecules migrate at a rate proportionally faster than small molecules. Consequently, the field strength applied to most gels should be between 0.5 and 10 V cm^{-1}. In general, higher resolution is achieved at a low voltage gradient, particularly if higher molecular weight DNA is used.

The amount of DNA in a sample will also affect its apparent mobility. Overloaded bands will appear to move faster than bands with the correct amount of DNA. For that reason, the amount of DNA loaded should be similar when comparing the mobility of DNA fragments. The useful separation ranges of various gel concentrations do overlap and different electrophoretic conditions can shift their useful range (see Surzycki (2000) for details).

Electrophoresis buffers

Several different buffers are used for agarose gel electrophoresis. These are TAE (Tris-acetate EDTA) buffer, TBE (Tris-borate EDTA) buffer, and TPA (Tris-phosphate EDTA) buffer.

TAE buffer is the most frequently used buffer for DNA electrophoresis. This buffer has a rather low buffering capacity, but permits the application of high-voltage gradients resulting in shorter running times. The ratio of voltage applied to current (mA) is approximately 1.0 for a wide variety of gel sizes and buffer volumes when this buffer is used (Perbal, 1988). The tracking dye, bromophenol blue, will travel in this buffer at a rate of approximately 1 cm h^{-1} at 1–10 V cm^{-1} field strength. Thus, this marker dye co-migrates with the smallest DNA molecules at each agarose concentration.

TBE buffer has a very high buffering capacity. It can be used when DNA of less than 12,000 bp is electrophoresed, but gives superior results, as compared to TAE buffer, in electrophoresis of DNA fragments of less than 1,000 bp. The DNA mobility in this buffer is approximately two times slower than in TAE buffer. This is due to the lower porosity of agarose gel when agarose polymerizes in the presence of borate. The ionic strength of TBE buffer is high, resulting in a 4 : 1 ratio of voltage to current (mA) during

electrophoresis for a wide variety of gel sizes and buffer volumes (Perbal, 1988). In general, DNA bands are sharper when TBE buffer is used, but the time of electrophoresis is considerably longer.

TPA buffer has high buffering capacity, comparable to that of TBE buffer. However, the DNA mobility in this buffer is similar to that in TAE buffer due to a similar pore size formed during agarose polymerization. The buffer has a high ionic strength resulting in a voltage to current (mA) ratio similar to that obtained in TBE buffer.

Gel size

Agarose gel electrophoresis is commonly carried out using submerged horizontal slab gels (submarine gels). The best separation between DNA bands in such a system is achieved in gels that are approximately 20 cm long, 15 cm wide, and approximately 4 mm thick. To obtain maximum resolution of many bands electrophoresis should be continued until the tracking dye (for example bromophenol blue) has moved 70–80 percent of the length of the gel.

The size of the sample well can also affect the resolution of DNA bands. The optimal length of the sample well for a large gel is between 0.5 and 1 cm and the optimal width of the well is 1–2.0 mm. The sample well bottom should be 0.5–1.0 mm above the gel bottom. Most of the commercially available submarine electrophoresis gel boxes fulfill the above requirements.

Sample concentration

The amount of DNA loaded into one well can vary considerably without affecting the mobility of the DNA. For standard large gels, the DNA load can vary from 1 to 10 ng of DNA per band. The total amount of DNA loaded per well should not exceed 10 µg. In general, decreasing amounts of DNA should be used with increasing voltage.

Sample loading solutions

DNA samples are prepared for electrophoresis by the addition of loading dye solution. The composition of loading dye solution plays an essential role in obtaining sharp DNA bands. This solution serves three vital functions: it is used to terminate enzymatic reactions before electrophoresis (stop solution), it provides density for loading the sample into the well, and it provides a way of monitoring the progress of electrophoresis.

Most loading dye solutions contain EDTA in order to stop enzymatic reactions. However, this is frequently not sufficient for fully dissociating DNA–protein complexes, the presence of which will affect not only the

mobility of DNA fragments, but can also cause an excessive smearing and widening of the bands. In order to remove these complexes, loading dye solutions should contain a protein-denaturing agent. Urea, at a concentration of 5 M, is the best protein-denaturing agent because it does not interact with agarose or affect DNA mobility.

Glycerol or sucrose, at concentrations of 5–10 percent, is used to provide the sample density for loading. However, using these low molecular weight compounds results in U-shaped DNA bands due to sample streaming up the side of the well before beginning electrophoresis (Sealey and Southern, 1982). This effect is particularly pronounced when electrophoresis is carried out at low field strength. To increase the sharpness of the bands and prevent their U-shape appearance, Ficoll 400 should be added at a concentration of 15–20 percent.

Gel staining

In order to visualize DNA, agarose gels are usually stained with ethidium bromide. This is the most rapid, sensitive, and reproducible method currently available for staining single- and double-stranded DNA (Sharp et al., 1973). Ethidium bromide binds to double-stranded nucleic acid by intercalation between stacked base pairs. The mobility of linear DNA in gel electrophoresis is reduced by approximately 15 percent. Ultraviolet (UV) irradiation of ethidium bromide at 302 and 366 nm is absorbed and re-emitted as fluorescence at 590 nm. Similarly, energy absorbed by DNA irradiated at 260 nm is transmitted to intercalated dye and re-emitted as fluorescence at 590 nm. The intensity of fluorescence of dye bound to DNA is much greater than that of free dye suspended in agarose. This results in very low background and a high intensity of fluorescence from DNA bands. The best staining results are obtained by incorporating ethidium bromide into the gel at a concentration of 0.5 $\mu g\,ml^{-1}$. This permits direct observation of the progress of electrophoresis and limits the amount of ethidium bromide-contaminated liquid waste. The most sensitive photographs of ethidium bromide-stained DNA are obtained when DNA is illuminated with UV light at 254–260 nm rather than by ethidium bromide direct illumination at 300 nm.

Photographing gels

Photographs of the gels provide not only a permanent record of the experiment, but also permit analysis of the data and visualization of DNA bands not visible to the unaided eye. Polaroid cameras, equipped with appropriate filters, are usually used for this purpose. The most commonly used fast Polaroid film type 667 (ASA 3000) can record a DNA band containing 2–4 ng when loaded into a well of 1 cm.

A more sensitive method of recording gel results is the use of a computer imaging system equipped with a charge-couple device digital camera. The sensitivity of the computer imaging system is approximately ten times greater than the sensitivity of photography. This permits visualization and recording of as little as 0.1 ng of DNA per band.

Safety precautions

Ethidium bromide is a mutagen and suspected carcinogen. Contact with skin should be avoided. Wear gloves when handling ethidium bromide solution and gels containing ethidium bromide.

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.

Protocol

1. Stop restriction reactions by the addition of 5 µl of stop solution. Mix well by pipetting up and down several times. Centrifuge for 5–10 seconds to collect liquid at the bottom of the tube. The reaction is now ready to be loaded onto an agarose gel as described in experiment 2. **Note:** restriction digestion can also be stopped by the addition of 1 µl of 0.5 M EDTA. The reaction can be stored at –20°C for an indefinite time and used for gel electrophoresis when needed.

2. Seal the opened ends of the gel-casting tray with tape. Regular labeling tape or electrical insulation tape can be used. Check that the teeth of the comb are approximately 0.5 mm above the gel bottom. To adjust this height, it is most convenient to place a plastic charge card (e.g. MasterCard) under the comb and adjust the comb height to a position where the card is easily removed from under the comb.

3. Prepare 1,500 ml of one times TAE buffer by adding 30 ml of a 50 times TAE buffer stock solution to a final volume of 1,500 ml of deionized water.

4. Place 150 ml of the buffer into a 500 ml flask and add the appropriate amount of agarose. Weigh 1.5 g of agarose for a 1 percent agarose gel. Melt the agarose by heating the solution in a microwave oven at full power for approximately 3 minutes. Carefully swirl the agarose solution to ensure that the agarose is dissolved, that is no agarose particles are visible. If evaporation occurs during melting, adjust the volume to 150 ml with deionized water.

5. Cool the agarose solution to approximately 60°C and add 5 µl of ethid-

ium bromide stock solution. Slowly pour the agarose into the gel-casting tray. Remove any air bubbles by trapping them in a 10 ml pipette.

6. Position the comb approximately 1.5 cm from the edge of the gel. Let the agarose solidify for approximately 30–60 minutes. After the agarose has solidified, remove the comb with a gentle back and forth motion, taking care not to tear the gel.

7. Remove the tape from the ends of the gel-casting tray and place the tray on the central supporting platform of the gel box. For safety purposes, the electrophoresis apparatus should be always placed on the laboratory bench with the positive electrode (red) facing away from the investigator, that is away from the edge of the bench.

8. Add electrophoresis buffer to the buffer chamber until it reaches a level of 0.5–1 cm above the surface of the gel.

9. Load the samples into the wells using a yellow tip. Place the tip **under** the surface of the electrophoresis buffer just **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 to avoid placing the end of the tip into the sample well or touching the edge of the well with the tip. This can damage the well, resulting in uneven or smeared bands. **Note:** samples must be loaded in sequential sample wells. When loading fewer samples than the number of wells it is preferable to leave the wells nearest the edge of the gel empty.

10. First load 8 µl of the 1 kb ladder standard DNA. Next load the entire sample (35 µl) using a P200 Pipetman. Load the samples in the following order: 1H, 2H, 3H, and 4H.

11. Place the lid on the gel box and connect the electrodes. DNA will travel towards the positive (red) electrode positioned away from the edge of the laboratory bench. Turn on the power supply. Adjust the voltage to approximately 1 V cm^{-1}. For example, if the distance between electrodes (not the gel length) is 40 cm the voltage should be set to 40 V in order to obtain a field strength of 1 V cm^{-1}.

12. Continue electrophoresis until the tracking dye moves at least two-thirds of the gel length. It will take the tracking dye approximately 17 hours to reach this position on a gel 20 cm long.

Next day

1. Turn the power supply off and disconnect the positive (red) lead from the power supply. Remove the gel from the electrophoresis chamber. To avoid electric shock always disconnect the red (positive) lead first.

2. Wrap the gel-casting tray with saran wrap and store in a 4°C refrigerator. Gels can be stored this way for two to four days.

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SECOND LABORATORY PERIOD

Experiment 3: Southern blotting

Introduction

You will continue multi-locus analysis of your DNA by transferring DNA from the gel prepared in experiment 2 to the nylon membrane. This process is called Southern blotting. The DNA fragments are transferred to the membrane at the same positions as they are on the gel. The blotting technique that we will be using relies on a flow by capillary action of a neutral transfer solution from a reservoir through the gel to a membrane overlaid by a stack of dry paper towels. Prior to transfer, DNA fragments will be denatured *in situ* to single-stranded DNA that can be bound to the membrane and hybridized to probe. DNA fragments will be immobilized to the membrane by UV light irradiation.

Background

Southern (1975) introduced immobilizing target DNA to a membrane for hybridization studies. The technique permits hybridization of various probes to immobilized target DNA under controlled conditions. The Southern blot method, originally described by Southern (1975), combines the high resolving power of agarose gel electrophoresis in the separation of DNA fragments with the specificity of DNA–DNA hybridization reactions. The basic principle of the technique is that DNA fragments, which are separated by agarose gel electrophoresis, are transferred and immobilized to a solid support, such as a nylon membrane. Once immobilized, the DNA is available for hybridization with labeled DNA or RNA probes. This technique is applicable to the analysis of small, cloned DNA fragments, as well as to the analysis of genomic DNA. DNA transfer to solid support is generally accomplished by capillary methods, but electroblotting, positive pressure, and vacuum transfer procedures can also be used (Peferoen et al., 1982; Smith et al., 1984). These other methods are in general faster than capillary transfer, but are less efficient and require expensive equipment.

There are two capillary transfer methods: upward capillary transfer and downward capillary transfer. Upward capillary or "standard" transfer results in very efficient transfer of DNA or RNA of all sizes but requires overnight exposure. Downward capillary transfer is just as efficient as upward transfer and requires a much shorter transfer time (3–4 hours). Capillary transfer can be carried out with neutral or alkaline transfer solutions (Chomczynski, 1992).

Alkaline transfer of DNA is only possible with a positively charged nylon

membrane. Capillary alkaline transfer is faster because the neutralization step is omitted from the procedure and the time required for transfer is shorter. An alkaline transfer solution can cause some depurination of the DNA and frequently results in weaker hybridization signals than the neutral transfer. Alkaline blotting is not recommended for genomic DNA transfers or when reprobing of the membrane with another probe is planned.

Safety precautions

The agarose gel contains ethidium bromide, which is a mutagen and suspected carcinogen. Students should wear gloves when handling these gels. Powder free gloves only should be used because the procedure described uses chemiluminescent for the detection of hybridization. The presence of talcum powder will result in the formation of a "spotted" background. Discard the used gel into the designated container.

When viewing and photographing the gel with a UV transilluminator, gloves, UV-protective glasses, and a facemask should be used all times.

Technical tips

Different types of support membranes can be used for DNA fingerprinting experiments. However, the use of chemiluminescent detection requires a positively charged nylon membrane. These membranes have a very low background with chemiluminescent detection. Some producers have developed a chemically optimized, positively charged nylon membrane for chemiluminescent detection. Evaluation of all types of commercially available positively charged nylon membranes showed that the best membrane for chemiluminescent detection is a Magna Graph membrane, which is manufactured by Osmonics/MSI Co. (Surzycki, 2000).

The best signal to noise ratio for a Magna Graph membrane is achieved when DNA is cross-linked to a membrane by UV light irradiation.

Protocol

1. Transfer the gel to a glass Pyrex dish and trim away any unused areas of the gel with a scalpel. Cut off the gel below the bromophenol blue dye. This part of the gel does not contain DNA fragments. Cut the lower corner of the gel at the bottom of the lane with size standards. This will provide a mark with which to orient the hybridized bands on the membrane with the bands in the gel. **Note:** because the gel is thinner in the well area, the transfer solution may pass preferentially through this part of the gel causing uneven DNA transfer. Remove this part of the gel.

2. Transfer the gel to a UV transilluminator. Place an acetate sheet on top of the gel and draw an outline of the gel with a felt marker pen. Mark the

positions of the wells and the position of the cut corner. **It is very important that your drawing be as precise as possible.** Label the contents of each well on the acetate sheet and mark the bottom left corner of the gel (under well number 1). This will help you locate the positions of the hybridization signals in your Southern blots. Turn on the transilluminator and mark the positions of standard DNA bands on the acetate sheet.

3. Photograph the gel. Use a setting of 1 second at F8 when using a Polaroid 667 film pack. One can also use a computer-imaging system for recording the results.

4. Transfer the gel back to the Pyrex dish and add enough 0.25 N HCl to allow the gel to move freely in the solution. This will take approximately 150–200 ml of solution for a standard gel size.

5. Place the dish on an orbital shaker and incubate for 10 minutes rotating at 10–20 r.p.m. **Note:** this procedure breaks large DNA molecules by depurination, thereby facilitating their efficient transfer onto the membrane. It is important not to let the hydrolysis reaction proceed too far, otherwise the DNA is cleaved into fragments that are too short to bind efficiently to the membrane (less then 200 bp).

6. Decant the acid carefully holding the gel with the palm of your gloved hand.

7. Rinse the gel for 10–20 seconds in 200 ml of distilled water. Discard the water and proceed immediately to the next step.

8. Add 200 ml of denaturation solution to the dish and gently agitate it for 20 minutes on a rotary shaker.

9. Decant the denaturation solution as described above and repeat step 8 one more time.

10. Add 200 ml of water and rinse the gel for 10–20 seconds in order to remove most of the denaturation solution trapped on the surface of the gel. Decant the water, holding the gel with the palm of your gloved hand. Be very careful during this procedure because the denaturation solution contains NaOH making the gel very slippery.

11. Add 100–200 ml of neutralization solution to the dish and treat the gel for 20 minutes with gentle agitation.

12. Discard the neutralization solution and repeat step 11 one more time.

13. While the gel is being treated, prepare the nylon membrane for transfer. Cut the nylon membrane to the size of the gel. Use the outline of the gel drawn on the acetate sheet as a guide. Use gloves and only touch the edges of the nylon membrane. Place the membrane in a separate Pyrex dish filled with distilled water. Leave the membrane in water for 1–2 minutes. Decant the water and immerse the membrane in ten times SSC. Cut three sheets of Whatman 3 MM paper to the size of the nylon membrane.

14. Prepare a long strip of Whatman 3 MM paper to use as a wick. The wick should be approximately 30 cm long and 10 cm wide.

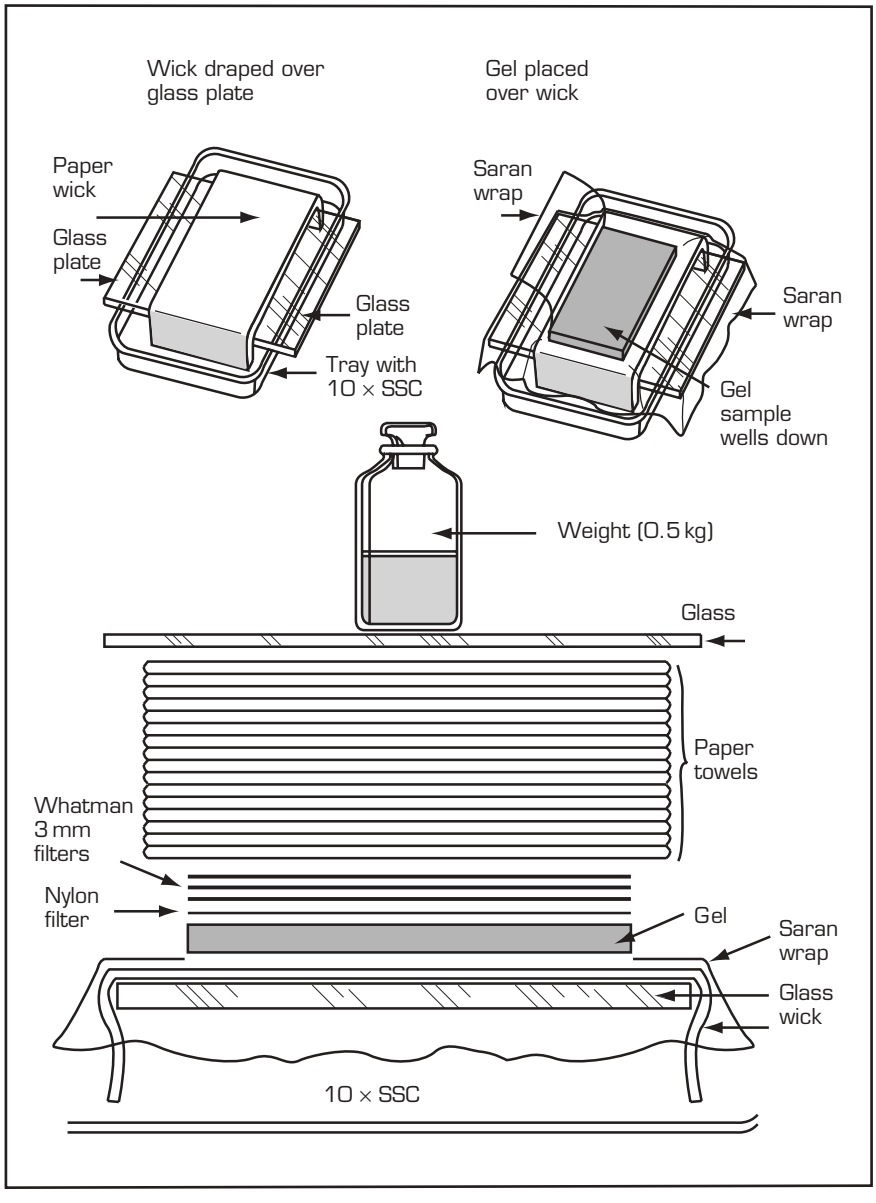

Figure 2.3 Preparation of a Southern blot.

15. Assemble the blot sandwich. Refer to Fig. 2.3 for illustration of the assembly. Add 400–600 ml of ten times SSC to a large Pyrex dish. Place a glass platform across the center of the dish and cover it with the wick. Make sure that both ends of the wick are immersed in SSC solution. Wet the wick with 10–20 ml of ten times SSC and remove trapped air bubbles by rolling a 10 ml glass pipette over it. Carefully lift the gel from the Pyrex dish and place it in

the center of the wick with the **sample wells down**. Smooth the gel and remove trapped air bubbles by gently rolling a glass pipette over the surface. **Note:** the gel is now upside-down with the well openings facing the wick. This is necessary in order to obtain the best results during transfer (sharper resolution due to less diffusion during the transfer) and to maintain the left-to-right sample orientation on the membrane.

16. Cover the entire dish, including the surface of the gel, with saran wrap. With a razor blade "cut away" the saran wrap covering the gel itself and discard it. This will leave an opening over the gel while the remaining area of the wick will be covered by saran wrap.

17. Place the nylon membrane on top of the gel. Add 5 ml of ten times SSC to the top of the membrane and remove air bubbles by rolling a pipette over it. Cut the left bottom corner of the membrane to coincide with the cut made in the gel.

18. Place three sheets of dry Whatman 3 MM paper, prepared in step 13, on the top of the membrane. Place several inches of paper towels on top of the 3 MM paper. Because the wick area is protected by saran wrap, it is not necessary to cut the paper towels to the size of the gel. Place a glass plate on top of the paper towel stack and weigh it down with a 1 l Erlenmeyer flask filled with 500 ml of water. Allow a minimum of 17 hours for the transfer. **Note:** to prevent the gel from collapsing, the weight placed on the top of the stack should never exceed 500 g (i.e. approximately 500 ml of water).

Next day

1. Disassemble the blot. Remove the weight, glass plate, and paper towels. Using forceps remove the membrane and place it "DNA side" up (the side that was in contact with the gel) on a clean sheet of Whatman 3 MM paper. Mark the DNA side with pencil on the corner of the membrane.

2. Place the membrane on a sheet of dry Whatman 3 MM paper. Do not allow the membrane to dry at any time. Place the membrane into a UV oven.

3. UV irradiate the damp membrane to cross-link DNA to the membrane using the automatic setting of the UV oven. Irradiate **both sides** of the membrane. Alternatively, you can wrap the membrane in aluminum foil and bake it in an oven at 80°C for 1 hour. The baking step immobilizes DNA on the membrane.

4. Place the membrane into a plastic bag and store it at room temperature.

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THIRD LABORATORY PERIOD

In this laboratory period you will carry out two procedures. First, you will prepare probe for hybridization using a PCR. Second, you will hybridize the probe to membrane containing *Hae*III-restricted DNA prepared previously.

Experiment 4: preparation of probe and hybridization

Introduction

The hybridization probe will be labeled with digoxigenin (DIG) using dUTP-DIG as labeled substrate. In this substrate dUTP is linked via a spacer arm to the steroid hapten DIG. In this reaction, the ratio of dUTP-DIG to dTTP will be 1 : 2, assuring high density labeling of the probe. You will amplify tandem repeats present in the RF form of bacteriophage M13 genome using specific primers that span the tandem repeat region. An amplified tandem repeat with core sequence GAGGGTGGNGGNTCT is complementary to a large number of hypervariable mini-satellites present in the human genome.

Hybridization will be carried out using a hybridization oven in specially designed hybridization roller bottles. The hybridization procedure described uses low stringency conditions for hybridization and washing. These conditions of hybridization make it possible to hybridize probe to a family of hypervariable mini-satellites that are closely related to the core sequence.

Background

DNA probe preparation

Preparation of probes for hybridization involves *in vitro* incorporation of reporter molecules into nucleic acids. These reporters can be incorporated at one or both ends of nucleic acid molecules, giving specific, low-density labeled probes. High-density labeling is usually achieved by incorporating the reporters uniformly throughout the entire length of nucleic acid molecules. For hybridization work, internally labeled probes are preferred since they provide the strongest hybridization signal.

Two types of reporter molecules are presently in use, radioactive reporters and non-radioactive reporters.

Radioactive reporters are usually tagged with ^{32}P or ^{35}S isotopes and can be directly detected using X-ray film in a process called autoradiography.

Non-radioactive reporters can be fluorescent tags, permitting their direct detection or specific ligands such as biotin, haptens, and hapten-like molecules, the detection of which is indirect.

Two methods are used for detecting non-radioactive reporters: direct and indirect. The direct method uses reporter enzyme conjugation to the probe. Probes obtained by these methods have lower levels of background, thus allowing longer exposure time and, consequently, better sensitivity. However, the presence of enzyme (conjugated to the probe) during hybridization requires modification of the standard hybridization protocol in order to protect the enzyme from inactivation. We will use this method for single-locus analysis.

Indirect detection is carried out using enzymatic reactions catalyzed by either horseradish peroxidase or alkaline phosphatase. These enzymes can be conjugated directly to a secondary molecule that has a very high affinity for a specific ligand (for example biotin–avidin complexes) or they can be conjugated to antibody against a hapten tag. Since enzymes are only used for the detection of labeled hybrids and are not present during hybridization, standard hybridization protocols can be used. This method will be used in multi-locus analysis.

Two types of substrates are used for detecting enzyme activity at the site of hybridization: colorimetric substrate and chemiluminescent substrate.

Colorimetric detection uses a soluble, colorless substrate that is converted into an insoluble, colored product precipitated directly on the membrane. We will use this method for determining the concentration of DIG label in our probe.

Chemiluminescent detection uses a chemiluminescent substrate that is converted by enzyme into a light-emitting substance easily detected by standard photographic film. The sensitivity of the chemiluminescent method is much greater than the colorimetric method and even exceeds the sensitivity of radioactive labeling (Höltke et al., 1990; Kessler et al., 1990). The chemiluminescent substrates now in use allow the detection of 0.03 pg or less DNA on the membrane in approximately 1–2 hours. The same amount of DNA would require 17–20 hours of exposure to detect a ^{32}P-labeled probe. We will use this substrate for detecting hybridization in multi-locus analysis.

Preparation of uniformly labeled probes, independent of the nature of the reporter, is carried out using four basic procedures: nick translation, random priming, RNA probe synthesis, and PCR.

Nick translation

In this method, labeled DNA is synthesized using *Escherichia coli* DNA polymerase. Single-stranded nicks are introduced at random in the double-stranded DNA template with the DNA exonuclease DNase I. DNA

polymerase I initiates DNA synthesis at the 3′-end of the nick, whereas at the 5′-end of the nick the 5′ to 3′ exonuclease activity of this enzyme excises nucleotides. The position of the nick is "translated" downstream, with labeled nucleotides replacing non-labeled nucleotides between the original site of the nick and the new position of the nick. The resulting probe is labeled on both strands with labeled (newly synthesized) DNA interspersed with unlabeled (original template) DNA. The method works best with linear DNA fragments that are larger than 500 bp. The specific activity of these probes is somewhat lower, as compared to other methods, but nick-translated probes generally give very strong hybridization signals (Davis et al., 1994).

Random priming

In this method DNA polymerase synthesizes a newly labeled DNA by template-dependent extensions of random hexamer primers (Feinberg and Vogelstein, 1983). Polymerases lacking 5′ to 3′ exonuclease activity, such as the Klenow fragment of *E. coli* DNA polymerase I, are used in this reaction. The template strand remains unlabeled whereas the newly synthesized strand is completely labeled. The enzyme can initiate and synthesize several new strands from every template, resulting in net synthesis of large amounts of labeled product, many times exceeding the amount of template input. The specific activity of probe produced by random priming is very high. The method is relatively insensitive to the purity of the DNA template or its size and creates probes of 100–600 bp long. Random priming is particularly well suited for the preparation of non-radioactive probes of very high specific activity.

PCR probes

Two alternative techniques are used for preparing probes using PCR. In the first procedure, uniformly labeled probes are generated by incorporation of a tagged nucleotide during PCR (Kessler, 1992; McCreery and Helentijaris, 1994; Yamaguchi at al., 1994). In the second approach, a large number of specific DNA target molecules are synthesized by PCR, which are subsequently used as a template for preparing random-primer or nick-translated probes (Rost, 1995). PCR labeling can be done using either a genomic template without cloning the DNA fragment in question or from a DNA fragment cloned into plasmid. Specific primers are required for the former but not for the latter PCR procedure. The advantages of generating DNA probes by PCR are numerous. First, large amounts of probe with high label density can be synthesized from very little DNA. Second, probes can be prepared using either purified or partially purified DNA as the source of the template. Third, preparation of the probe is highly flexible and does not depend on restriction enzyme site location. Fourth, it is possible to prepare

specific, single-stranded probes using a single primer. The main disadvantage is the difficulty in amplifying most sequences larger than 3,000 bp in length. We will prepare multi-locus probe using the PCR method.

DNA hybridization

The hybridization reaction is the formation of partial or complete double-stranded nucleic acid molecules by sequence-specific interaction of two complementary single-stranded nucleic acids. The hybridization reaction, using labeled probes, is the only practical way of detecting the presence of specific nucleic acid sequences in a complex nucleic acid mixture. The most frequently used hybridization technique is the membrane hybridization technique. Hybrid formation between complementary strands is commonly called a re-association, renaturation, or re-annealing reaction. The reverse reaction is called strand separation, dissociation, or melting of the DNA.

Kinetics of the hybridization reaction

The hybridization reaction proceeds in two steps: the nucleation reaction and "zippering" reaction. Nucleation is the formation of short hybrids of a few bases long between reacting strands. The nucleation reaction, with some approximation, is a diffusion-limited reaction defined by the Smoluchowski and Deby equation. Therefore, the reaction rate depends on the solvent viscosity, temperature, and ionic strength of the medium (Wetmur and Davidson, 1968; Chang et al., 1974).

Many nucleation events will take place until by chance the correct base pair is formed. A rapid zippering process follows this. The zippering reaction is an extension of the hybrid from the nucleation site throughout the entire molecule. This reaction is very fast and largely independent of the factors mentioned above. Thus, the limiting step in a hybridization reaction is the nucleation reaction and not the zippering process. The overall hybridization rate is dependent on a nucleation rate constant (k_n), the probe length, and target complexity as described by the equation

$$k_2 = \frac{k_n}{\sqrt{L}} \tag{2.4}$$

where k_n is the nucleation rate constant and L is the probe length in bases. The nucleation rate k_n for monovalent cation concentrations commonly used in filter hybridization (0.2–4.0 M) and solvent viscosity comparable to 1.0 M NaCl can be evaluated using the equation (Orosz and Wetmur, 1977)

$$k_n = (4.35 \log[M^+] + 3.5)10^5 \tag{2.5}$$

Temperature of the hybridization

In order to determine the temperature of hybridization, the melting temperature (T_m), which is defined as the temperature at which the DNA is 50 percent denatured, must be known. Melting of DNA is an intramolecular, first-order reaction and, therefore, is independent of substrate concentration. It depends only on the base composition of the duplex and the composition of the solvent. Equation (2.6), which was first empirically established by Marmur and Doty (1962) for molecules shorter than 500 bp, describes the T_m for DNA:DNA duplexes:

$$T_m = 81.5 + 0.41(\%GC) + 16.6\log[M^+] \quad (2.6)$$

where $[M^+]$ is the molar concentration of the monovalent cations Na^+ or K^+ and %GC is the percent of GC bases in hybrid DNA. This equation was modified to incorporate DNA:RNA and RNA:RNA hybrids and extends the monovalent cation concentration range from 0.01 to 4.0 M, the concentrations at which most hybridization reactions are carried out (for a detailed discussion of modified equations see Wetmur (1991) and Surzycki (2000)).

It is obvious that hybridization should be carried out at temperatures below the T_m temperature calculated from equation (2.6). The difference between the T_m temperature and hybridization temperature is defined as the "criterion of hybridization" (Britten et al., 1974). The overall rate of the hybridization reaction k_2 is strongly affected by temperature. This dependence has a bell-shaped curve and increases as the criterion is increased, reaching a broad maximum between 20 and 25°C below the T_m for the DNA:DNA hybrid. As the temperature of hybridization falls further below the T_m, the hybridization rate decreases very fast due to intramolecular base pair formation that decreases the availability of nucleation sites. Therefore, the optimal temperature for hybrid formation (T_h) for a DNA:DNA hybrid is

$$T_h = T_m - (20–25°C) \quad (2.7)$$

At T_h temperature, not only does hybrid formation occur at maximum speed, but it is most perfect. This is because at T_m the formation of perfectly matched hybrids by the zippering reaction is faster than the formation of mismatched hybrids (10 percent or more mismatches). Moreover, the T_m of imperfectly matched hybrids is lower than perfectly matched hybrids (approximately 1°C for each percent of mismatch). Consequently, the maximum hybridization rate of mismatched hybrids occurs much below the hybridization temperature of a perfect hybrid. The formation of poorly matched hybrids shows a similar bell-shaped dependence on temperature,

but the maximum rate (k_2) is several orders slower than for a well-matched hybrid and the entire curve is displaced towards lower temperatures.

Hybridization time

Membrane blot hybridization is usually performed with a large excess of probe DNA. For example, approximately 5–10 µg of eucaryotic genome is used in Southern blot analysis. Assuming that a single gene size is 2,000 bp, the genome size is 3.15×10^9 bp (e.g. human genome), and only 2 percent of the DNA bound to the nylon membrane is open for hybridization (Vernier et al., 1996), the amount of a single gene present on a Southern blot is approximately 0.06×10^{-6} µg. The concentration of probe is usually 20–25 ng ml^{-1}. If the probe is the same size as the genomic target and 10 ml of hybridization solution is used, the amount of probe present is 0.2–0.25 µg or a nearly 3×10^6-fold excess of probe DNA over target DNA. Using these hybridization conditions, the reaction is pseudo first order and its half time is

$$t_{1/2} = 2/k_2 C_0 \tag{2.8}$$

where k_2 is rate of hybridization as calculated from equation (2.4) and C_0 is the initial concentration of probe in moles of nucleotides per liter (Wetmur, 1991, 1995). Since most hybridization reactions are carried out at 1 *M* Na^+ the k_n for this reaction is equal to $3.5\ 10^5\ M^{-1}\ s^{-1}$ (from equation 2.5). The concentration of the probe used in Southern hybridization is usually equal to $C_0 = 6.1\ 10^{-8}$ M (20 ng ml^{-1}) and k_2 is $7.8\ 10^3\ M^{-1}\ s^{-1}$ for a single gene probe calculated from equation (2.4). Using equation (2.8), the $t_{1/2}$ of the reaction can be calculated to be approximately 1 hour.

If an excess of double-stranded probe is hybridized to an immobilized target, self-annealing of the probe limits the time of effective hybridization to approximately two to three times the $t_{1/2}$ calculated for a single-stranded probe. Thus, the above reaction will reach completion in approximately 2–3 hours due to self-annealing of the probe. The half-time for pseudo first-order hybridization can be approximated (in hours) when double-stranded probe is used with standard conditions of hybridization (i.e. 1 M salt at T_h equal to 20–25°C below T_m) from the equation (Sambrook et al., 1989)

$$t_{1/2} = \frac{1}{X} \times \frac{Y}{5} \times \frac{Z}{10} \times 2 \tag{2.9}$$

where X is micrograms of probe added, Y is the probe complexity in kilobases (length of probe), and Z is the volume of hybridization in milliliters. For example, for the DNA hybridization reaction described above, that is hybridization with a 2,000 bp probe at a concentration of 0.02 µg ml^{-1} in

10 ml, the $t_{1/2}$ calculated from equation (2.9) is 2 hours, a value close to that calculated from equation (2.8).

However, hybridization reactions are usually carried out for 13–17 hours in order to increase the signal strength. This is because probes can form extended networks in which the single-stranded tails from one duplex hybridize to a complementary single-stranded tail of another duplex. This network formation occurs five to six times slower than the annealing of a single-stranded probe and its formation will increase the hybridization signal intensity (Geoffrey et al., 1987).

The hybridization kinetics described above only apply strictly to liquid hybridization. When nucleic acid is immobilized on a membrane, the hybridization rate is decreased because the rate of access of probe to target is decreased significantly. Consequently, the nucleation rate (k_n) is two to four times lower than the nucleation rate in liquid hybridization, but the effect of T_m and ionic strength are not changed.

Hybridization reaction solution

Because the rate of the nucleation reaction strongly depends on salt concentration, hybridization should not be carried out at low salt concentration. In order to achieve fast hybridization rates and, consequently, to form the most perfect hybrid, a monovalent cation concentration between 0.75 and 1.0 M is used. Because DNA used in most hybridization reactions has approximately 50 percent GC, the calculated T_h is usually between 72 and 78°C, as calculated from equation (2.7). Single-stranded DNA (probe) is particularly prone to depurination at temperatures higher than 50°C. Prolonged hybridization at high temperature will result in degradation of probe and target DNAs (Blake, 1995). In order to lower the hybridization temperature, the hybridization reactions are usually carried out in the presence of denaturing solvents while maintaining high ionic strength (Hutton, 1977). The most commonly used solvent in membrane hybridization is formamide. This solvent lowers, on average, the T_m of DNA by 0.7°C per 1 percent formamide. The effect of formamide is greater on AT nucleotide pairs than on GC pairs (Anderson and Young, 1985). Formamide has no apparent effect on the rate of hybridization at concentrations between 30 and 50 percent for membrane hybridization, making it an ideal solvent for lowering the incubation temperature. The concentration of formamide most frequently used is 50 percent. This lowers the temperature required for hybridization by approximately 32°C, thereby allowing hybridization of most DNA at temperatures below 50°C without substantially lowering the rate of hybridization.

Because of the high toxicity of formamide, a new non-toxic compound was recently introduced. This is Dig Easy Hyb solution, which is manufactured by Roche Molecular Biochemicals. The temperature of hybridization

in this solution should be calculated with the same equations that are used for hybridization in 50 percent formamide and 1 M Na^+. Thus, the equation for T_m simplifies to

$$T_{m(\text{Dig Easy})} = 49.82 + 0.41(\%\text{GC}) \tag{2.10}$$

We will use Dig Easy Hyb solution in our hybridization experiment.

Washing reaction

The hybridization reaction is followed by a washing reaction that removes any unhybridized probe and melts mismatched hybrids. This reaction, unlike the hybridization reaction, is a first-order reaction and depends on the thermal stability of the hybrid (T_m). The T_m of the hybrid is lowered by approximately 1°C for each percent of mismatch (Bonner et al., 1973). In order to obtain 95 percent faithful hybrids the washing reaction is carried out at a washing temperature expressed by the equation

$$T_w = T_m - 5°\text{C} \tag{2.11}$$

where T_m is melting temperature calculated from equation (2.6) or (2.10). Reactions carried out at this temperature are called high stringency washes. Reactions performed at temperatures lower than this are usually referred to as "low stringency" washing reactions.

Technical tips

Hybridization can be performed in roller bottles, glass or plastic dishes, or in sealed plastic bags. The hybridization and subsequent procedure for signal detection described here uses a hybridization oven and large roller bottles. This is the most convenient method to use in a large-class situation. No more than one membrane should be placed into the roller bottle. Some membrane overlapping will not affect the hybridization results.

The volume of the reagents used in each step will be substantially increased by using other containers for hybridization and subsequent membrane treatment. The minimum volume of the hybridization probe solution should be 0.2 ml solution per 1 cm^2 of membrane surface area. The volume of all other solutions should be approximately four to five times larger than for hybridization. Care should be taken that the membrane is sufficiently covered with solutions at all times and that it can float freely in the container.

The concentration of DIG-labeled probe should be 10–15 ng DIG label per milliliter. Increasing the probe concentration will not increase the hybridization signal, but will substantially increase the background. Careful

determination of the DIG concentration in the probe is crucial for the success of this experiment.

One of the great advantages of the DIG system is the stability of the labeled probe. The probe can be stored practically for an indefinite time at –20°C. Moreover, the hybridization solution contains unannealed DIG-labeled probe and can be reused several times. This solution can be stored for at least one to two years at –20°C.

The hybridization membrane prepared in this experiment will be used in experiment 3. The membrane should be stored after exposure to X-ray film in the bag with chemiluminescent substrate at 4°C. If longer storage is required (e.g. several months) the chemiluminescent substrate should be removed from the bag and replaced with a solution containing two times SSC and 0.1 percent SDS (sodium deodecyl sulfate).

Protocol

Start this laboratory with pre-hybridization as described in steps 1–3 of the pre-hybridization and hybridization procedure. Pre-hybridization should be carried out for 2–3 hours. During this time, prepare and determine the concentration of probe as described in the following protocol.

Probe preparation and determination of digoxigenin label concentration

1. Place a sterilized 1.5 ml microfuge tube on ice and add the ingredients following Table 2.2. First, calculate the amount of water necessary for the desired volume and add it to the tube. Then add buffer and the remaining components. Add enzyme last and mix by pipetting up and down several times. Never mix by vortexing. Taq polymerase is **very sensitive to vortexing**.

2. Load the reaction into 20 µl capillary tubes. Insert the open end of the capillary tube into the white silicon tip of the micro-dispenser approx-

Table 2.2 Preparation of DIG-labeled probe

Ingredient	Add	Final concentration
Buffer high Mg^{2+} (ten times)	2.0 µl	one times
4dNTP labeled (ten times)	2.0 µl	200.0 mM
Taq polymerase (1–5 u μl^{-1})	1.0 µl	1–5.0 u
Primer M13 V F (5 µM)	2.0 µl	5.0 pM
Primer M13 V R (5 µM)	2.0 µl	5.0 pM
DNA RF M13 (2 ng μl^{-1})	2.0 µl	0.4 ng
Water	9.0 µl	
Total	20.0 µl	

imately 5 mm deep and draw the reaction mixture into it by slowly turning the micro-dispenser knob counterclockwise. Position liquid in the middle of the capillary and seal it by flaming the ends. Only a few seconds of heating the extreme tip of the capillary is necessary. Check the seal by gently turning the micro-dispenser knob back and forth. If the liquid does not move in the tube, the tube is sealed. Flame seal the other end of a capillary tube. Place the sealed tube into a microfuge tube **with the end sealed last on top**.

3. Set the cycling conditions as follows: D (denaturation) = 94°C for 15 seconds, A (annealing) = 55°C for 10 seconds, and E (elongation) = 72°C for 60 seconds. Start with 94°C for 2 minutes. End with 72°C for 5 minutes. The total time of the PCR will be approximately 50 minutes.

4. Place the capillary tubes into the DNA thermal cycler inserting the end sealed first into the holder. Start the machine and observe the end of the capillary tube extruding from the holder carefully. If the second end is improperly sealed, liquid will rise to the top of the capillary tube when the temperature reaches approximately 90°C. If this happens, stop the cycler and reseal the open end. Restart the cycling again.

5. After cycling is complete, remove the liquid from the capillary tube into an appropriately labeled 1.5 ml centrifuge tube. Holding the capillary tube horizontally, gently snap off one end. Insert the open end of the capillary tube into the white silicon tip of the micro-dispenser, approximately 5 mm deep. Snap off the other end of the capillary tube. Remove the amplified sample from the capillary tube by slowly turning the micro-dispenser knob clockwise.

6. Add 9 µl of water to ten microfuge tubes. Prepare a tenfold serial dilution of the newly labeled DNA. Serially dilute as follows: 1 : 10, 1 : 100, 1 : 1,000, 1 : 10,000, and 1 : 100,000. Take 1 µl of the labeled probe and add it to the first dilution tube (S1). Mix well by pipetting up and down. Withdraw 1 µl from this tube and add it to the second dilution tube (S2). Mix well by pipetting up and down. Using the same procedure, prepare 1 : 1,000 (S3), 1 : 10,000 (S4), and 1 : 100,000 (S5) dilutions. Use the same yellow tip for all serial dilutions. Using the remaining five tubes, prepare a similar serial dilution for the DIG-labeled standard (5 ng μl^{-1} DIG-labeled plasmid).

7. Cut a piece of nylon membrane 7 cm × 2 cm and place it at the bottom of a Petri dish. Spot 1 µl of each DIG-labeled dilution onto the membrane. Start from the largest dilution (1 : 100,000) and continue spotting towards the less dilute samples. Use the same yellow tip for spotting the entire series. Repeat this procedure using standard DNA. Place each standard directly under the appropriate dilution of your labeled DNA. Arrange the spots on the membrane in the following way:

Sample DNA	1 : 100,000	1 : 10,000	1 : 1,000	1 : 100	1 : 10
Standard DNA	1 : 100,000	1 : 10,000	1 : 1,000	1 : 100	1 : 10

8. Let the spotted samples dry completely under a heat lamp for 2–4 minutes. Wet the membrane with ten times SSC by placing it onto wet

Whatman 3 MM filter paper. Transfer the membrane to dry Whatman 3 MM filter paper and immobilize the DNA on the damp membrane by UV cross-linking in a UV cross-linking oven. The membrane must be damp in order to cross-link the DNA. Best results are obtained by application of a calibrated UV light source such as a Stratalinker UV oven. Use the "autocross-linking" function of the UV oven.

9. Transfer the membrane to a Petri dish and add 15 ml of buffer A. Incubate on a rotary shaker at 10–20 r.p.m. for 5 minutes. Discard buffer A.

10. Add 15 ml of buffer B and incubate on a rotary shaker as above for 5 minutes. Discard buffer B.

11. During incubation in buffer B, prepare anti-DIG-AP solution. Add 10 ml of buffer B and 2 µl of anti-DIG-AP conjugate antibody to a sterile 15 ml plastic conical centrifuge tube. Mix well by inverting the tube several times.

12. Add anti-DIG-AP solution to the membrane and incubate with gentle rotation for 5 minutes at room temperature. Discard the antibody solution.

13. Add 15 ml of buffer A to the membrane and wash with gentle rotation for 5 minutes. Discard buffer A and repeat the wash once more.

14. Add 15 ml of buffer C and incubate on the rotary shaker for 2 minutes. Discard buffer C.

15. Prepare color development solution as follows. Add 10 ml of buffer C to a 15 ml plastic conical centrifuge tube. Add 45 µl of NBT solution and 35 µl of X-phosphate solution to the tube. Close the tube and mix by inverting several times. Protect the solution from direct light.

16. Add color development solution to the membrane. Make sure the solution covers the membrane. Cover the dish with aluminum foil to protect it from light. Incubate **without shaking** for 30–60 minutes checking occasionally for color development.

17. Compare the spot intensities of probe with control DNA and estimate the concentration of DIG-labeled probe.

Pre-hybridization and hybridization procedure

1. Place the dry membrane into a roller bottle. Make sure that the side of the membrane with DNA is facing away from the glass.

2. Pour 10 ml of Dig Easy Hyb solution into the roller bottle. Close the bottle tightly and label it with your group number.

3. Place the roller bottle into the hybridization oven and allow it to rotate at a **slow speed** (2–4 r.p.m.) for 1–3 hours at 34°C.

4. Ten minutes before the end of pre-hybridization begin to prepare the probe for hybridization. Add 10 ml of Dig Easy Hyb solution to a 15 ml conical centrifuge tube. Add DIG-labeled probe to the Dig Easy Hyb to a final concentration of 15–20 ng ml^{-1}. Close the tube tightly. Place the tube into an 80°C water bath and incubate for 10 minutes to denature the double-stranded DNA probe.

5. Retrieve the roller bottle from the hybridization incubator. Open the bottle and pour the pre-hybridization solution into a storage bottle. Pre-hybridization solution can be stored and used again.
6. Remove the tube with the probe from the 80°C water bath and add the labeled probe to the hybridization tube. Return the roller bottle to the oven and allow it to **rotate slowly** at 34°C overnight.

Next day

1. The next day, remove your roller bottle from the hybridization oven and pour off the hybridization solution into a 15 ml centrifuge tube. The probe can be stored in a –20°C freezer and reused several times.
2. Add 20 ml of washing solution II (two times SSC and 0.1 percent SDS) to the roller bottle. Place the bottle into the hybridization oven and allow it to rotate at slow speed until the next laboratory period.

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FOURTH LABORATORY PERIOD

In this laboratory period you will continue the hybridization experiment. First, you will remove mismatched hybrids from the membrane using a washing procedure. Second, you will prepare the membrane for signal detection by chemiluminescence.

Protocol

Washing reaction

1. Retrieve your roller bottle from the hybridization oven and discard solution II.
2. Add 20 ml of washing solution II to the bottle and place it back into the hybridization oven. Rotate it at **maximum speed** for 10 minutes.
3. Remove the bottle from the hybridization oven and pour off and discard solution II. Drain the liquid well by placing the bottle on end on a paper towel for 1 minute.
4. Add 20 ml of washing solution III prewarmed to 65°C. Place the bottle into the hybridization oven preheated to 65°C. Rotate it at **a slow speed** for 20 minutes.
5. Pour off solution III and discard it. Drain the solution well by placing the bottle on end on a paper towel for 1 minute. Repeat the wash with solution III one more time.
6. Remove solution III and drain the roller bottle well as described above.

Preparation of membrane for detection

1. Add 20 ml of buffer A (washing buffer) to the roller bottle. Cool the oven to room temperature and rotate the bottle at **maximum speed** for 2–5 minutes.
2. Retrieve the roller bottle from the oven and discard buffer A. Add 10 ml of blocking solution (buffer B) to the bottle. Incubate for 1–2 hours **rotating slowly** at room temperature.
3. Pour off and discard buffer B. Invert the roller bottle over a paper towel and let it drain well for 1 minute.
4. Measure 10 ml of buffer B in a plastic conical centrifuge tube and add 1 µl of anti-DIG-alkaline phosphatase solution (antibody solution). Mix well and add the solution to the bottle with the membrane. Incubate for 30 minutes **rotating slowly** at room temperature. **Note:** the working antibody solution is stable for approximately 12 hours at 4°C. Do not prolong the incubation with antibody over 30 minutes. This will result in high background.

5. Pour off and discard the antibody solution. Drain the liquid well by placing the bottle on end on a paper towel for 2 minutes.
6. Add 20 ml of buffer A to the bottle and wash the membrane at room temperature rotating at slow speed for 20 minutes.
7. Add 30 ml of buffer C to the roller bottle and place it in the hybridization oven. Let it rotate at slow speed until the next laboratory period.

FIFTH LABORATORY PERIOD

In this laboratory period you will carry out the signal detection procedure and analyze data obtained in the DNA fingerprinting multi-locus experiment.

Protocol

Signal detection

1. Retrieve the roller bottle from the oven and move the membrane towards the bottle opening by gently shaking the bottle. Discard buffer C. Remove the membrane to a Pyrex dish, place on a rotary shaker, and add 200 ml of buffer C. Wash the membrane by rotating it slowly for 25 minutes. Discard buffer C and repeat the wash one more time.
2. Place a plastic bag on a sheet of Whatman 3 MM paper and add 10 ml of buffer C. Wearing gloves, transfer the membrane from the Pyrex dish into the plastic bag. Open the bag and insert the membrane into it. Place the membrane into the pool of buffer and move it with your fingers to the end of the bag. Leave as little space as possible between the membrane and the end of the bag. This will limit the amount of expensive chemiluminescent substrate necessary for filling the bag.
3. Pour off buffer C from the bag. Remove the remaining liquid gently pressing it out with a Kimwipe tissue. **Note:** do not press strongly on the membrane because this will increase the background. Most of the liquid should be removed from the bag, leaving the membrane slightly wet. At this point, a very small amount of liquid will be visible at the edge of the membrane.
4. Open the end of the bag slightly, leaving the membrane side that does not contain DNA attached to the side of the bag. Add 0.9–1 ml of CDP-Star solution directing the stream towards the side of the bag. Do not add solution directly onto the membrane.
5. Place the bag on a sheet of Whatman 3 MM paper with the DNA side up and distribute the liquid over the surface of the membrane by gently moving the liquid around with a Kimwipe tissue. Make sure that the entire membrane is evenly covered. Do not press on the membrane because this will cause "press marks" on the film. Gently remove excess CDP-Star from the bag by guiding excess solution towards the open end of the bag and onto the Whatman paper with a Kimwipe. Make sure that the membrane remains damp. **Note:** at this point small liquid droplets will be visible on the edge of the membrane, but liquid should not be present on the membrane surface. Seal the bag with a heat sealer.

6. Place the bag in an X-ray film cassette, with the DNA side of the membrane up. In a darkroom, place X-ray film over the membrane. Expose the film for 5–10 minutes at room temperature. Open the cassette and develop the film using standard procedures for film development. **Note:** maximum light emission for CDP-Star is reached in 20–30 minutes, the light emission remains constant for approximately 24 hours, and the blot can be exposed to film a number of times during this period. The best results are usually obtained when the membrane is exposed the next day.
7. After exposure, store the bag with membrane at 4°C. This membrane will be used again in experiment 3. The membrane can be stored this way for several weeks.
8. Place the acetate sheet, with the outline drawing of your gel prepared previously, on top of the X-ray film. Carefully match the top of the drawing with the outline of the membrane on the film. Mark the positions of DNA standard bands on the film with a marker pen. Analyze the date as described in the next section.

Data analysis

The most important parameter of multi-locus DNA fingerprinting is the band-sharing coefficient or similarity indices. This coefficient quantifies differences between individual DNA fragment profiles. Using these coefficients one can compare two individuals with each other. In addition, a single individual can be compared to several different populations each characterized by their unique band-sharing values. For example, average band sharing between unrelated individuals is 0.25 in both the North European and Indian subcontinent populations when *Hae*III endonuclease-digested DNA is probed with 33.15 or 33.6 probes. Thus, if the band-sharing coefficient of your DNA is 0.25 when compared to the average of North European population, you probably belong to this population.

Band-sharing coefficient D is described by the equation

$$D = 2N_{ab}/(N_a + N_b) \tag{2.12}$$

and the similarity coefficient X is described by the equation

$$X = 1/2 \times [(N_{ab}/N_a)] + (N_{ab}/N_b) \tag{2.13}$$

where N_{ab} is the number of scorable bands common to DNA of individuals a and b, N_a is the number of scorable bands in individual a, and N_b is the number of scorable bands in individual b.

The band-sharing values D and similarity coefficient X yield rather similar results though D is biased slightly downward as compared to the value of

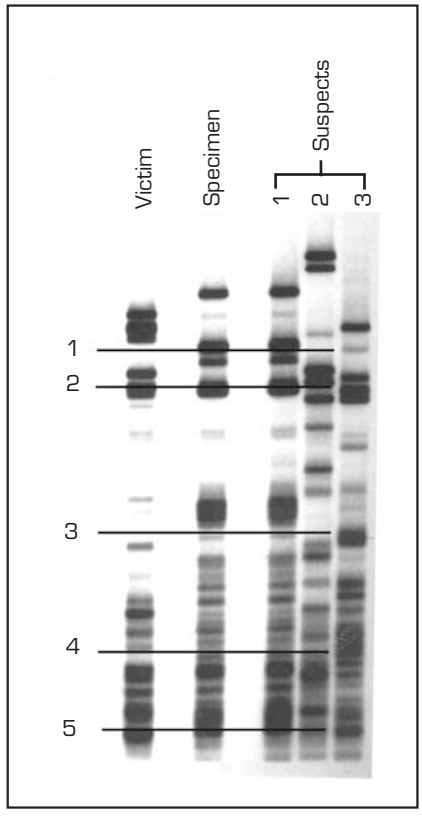

Figure 2.4 Example of multi-locus DNA fingerprinting using a 33.15 probe. DNA fingerprinting of a victim, a specimen, and three suspects is shown. Suspect 1 matches the specimen for all bands. Lines subdivide the autoradiogram into five regions for ease of analysis.

X. Knowing the *D* value (or *X* value), it is possible to estimate the chance of finding the same DNA fingerprint in two individuals. This can be calculated using the equation

$$P_n = D^n \tag{2.14}$$

where *n* equals the average number of scored bands per individual.

From this it is obvious that, when more bands are scored, it is less probable that they will be identical in unrelated individuals. For example, when $D = 0.25$ and nine bands are scored the probability that all of them will be identical in individuals that are not related is one in 4 million ($D^9 = 3.8 \times 10^{-6}$). For 25 bands scored the probability that they are all identical by chance is one in 10^{15} ($D^{25} = 8.8 \times 10^{-16}$).

An example of such analysis is described below. Figure 2.4 presents multi-

locus DNA fingerprinting of a victim, a specimen, and three suspects. This autoradiogram shows that suspect 1 is matching the specimen for all bands. The number of bands in common that can be scored is 21. We can now calculate P_n knowing that, for this probe, the band-sharing coefficient is equal to 0.25. Thus, P_n is equal to $P_n = 0.25^{21} = 2.2 \times 10^{-13}$.

The chance that an identical pattern will be found is 4×10^{12}. Since there are only 5×10^9 human beings living, suspect 1 is the one person on earth who can have an identical pattern to the specimen. What is the probability that suspect 2 is this person? This person has five bands identical with the specimen. Thus, for suspect 2 P_n is equal to $P_n = 0.25^5$ or 9.7×10^{-4}.

This match will be found in one person per 1,024 tested ($1/9.7 \times 10^{-4}$), indicating that suspect 2 cannot be guilty of this crime.

Using the example above, calculate the chance of finding the same DNA fingerprint comparing your DNA and your partner's DNA to two standard DNAs (P_n). Assume that one of the standard DNAs given to you is the DNA of the victim and the second DNA represents a specimen found on the crime scene. The DNA fingerprints of you and your partner represent the DNA of the two suspects. Calculate the probability that you or your partner is "guilty" of this crime. The band-sharing coefficient for the M13 minisatellites family is 0.25. Can you or your partner be fully exonerated of this crime on the basis of these calculations?