Medical Biomethods Handbook

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Edited by

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and

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Preface

There have been numerous advances made in many fields throughout the biosciences in recent years, with perhaps the most dramatic being those in our ability to investigate and define cellular processes at the molecular level. These insights have been largely the result of the development and application of powerful new techniques in molecular biology, in particular nucleic acid and protein methodologies.

The purpose of the *Medical Biomethods Handbook* is to introduce the reader to a wide-ranging selection of those molecular biology techniques that are most frequently used by research workers in the field of medical and clinical research. Clearly, within the constraints of a single volume, we have had to be selective. However, all of the techniques described are core methods and in regular research use. We have aimed to describe both the theory behind, and the application of, the techniques described. A companion volume, the *Molecular Biomethods Handbook*, published in 1998, provides similar details on a range of other basic molecular biology techniques. For those who require detailed laboratory protocols, these can be found in the references cited in each chapter and in the laboratory protocol series *Methods in Molecular Biology*™ and *Methods in Molecular Medicine*™ published by Humana Press.

The *Medical Biomethods Handbook* should prove useful to undergraduate students (especially project students), postgraduate researchers, and all research scientists and technicians who wish to understand and use new techniques, but do no yet have the necessary background to set up specific techniques. In addition, it will be useful for all those wishing to update their knowledge of particular techniques. All chapters have been written by well-established research scientists who run their own research programs and who use the methods on a regular basis. In sum, then, our hope is that this book will prove a useful source of information on all the major molecular biomedical techniques in use today, as well as a valuable text for those already engaged in or just entering the field of molecular biology.

John M. Walker
Ralph Rapley

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1

Basic Techniques in Molecular Biology

Ralph Rapley

1. Introduction

There have been many developments over the past three decades that have led to the efficient manipulation and analysis of nucleic acid and proteins. Many of these have resulted from the isolation and characterization of numerous DNA-manipulating enzymes, such as DNA polymerase, DNA ligase, and reverse transcriptase. However, perhaps the most important was the isolation and application of a number of enzymes that enabled the reproducible digestion of DNA. These enzymes, termed *restriction endonucleases* or *restriction enzymes*, provided a turning point for not only the analysis of DNA but also the development of recombinant DNA technology and provided a means for the detection and identification of disease and disease markers.

1.1. Enzymes Used in Molecular Biology

Restriction endonucleases recognize specific DNA target sequences, mainly 4–6 bp in length, and cut them, reproducibly, in a defined manner. The nucleotide sequences recognized are of an inverted repeat nature (typically termed *palindromic*), reading the same in both directions on each strand ***(1)***. When cut or cleaved, they produce a flush or blunt-ended, or a staggered, cohesive-ended fragment depending on the particular enzyme as indicated in **Fig. 1**. An important property of cohesive ends is that DNA from different sources (e.g., different organisms) digested by the same restriction endonuclease will be complementary (termed "sticky") and so will join or anneal to each other. The annealed strands are held together only by hydrogen-bonding between complementary bases on opposite strands. Covalent joining of nucleotide ends on each of the two strands may be brought about by the introduction of the enzyme DNA ligase. This process, termed *ligation*, is widely exploited in molecular biology to enable the construction of recombinant DNA molecules (i.e., the joining of DNA fragments from different sources). Approximately 500 restriction enzymes have been characterized to date that recognise over 100 different target sequences. A number of these, termed *isoschizomers*, recognize different target sequences but produce the same staggered ends or overhangs. In addition to restriction enzymes a number of other enzymes have proved to be of value in the manipulation of DNA and are indicated at appropriate points within this chapter.

2. Extraction and Separation of Nucleic Acids

2.1. DNA Extraction Techniques

The use of DNA for medical analysis or for research-driven manipulation usually requires that it be isolated and purified to a certain degree. DNA is usually recovered from cells by methods that include cell rupture but that prevent the DNA from fragmenting by mechanical shearing. This is generally undertaken in the presence of EDTA, which chelates the magnesium ions needed

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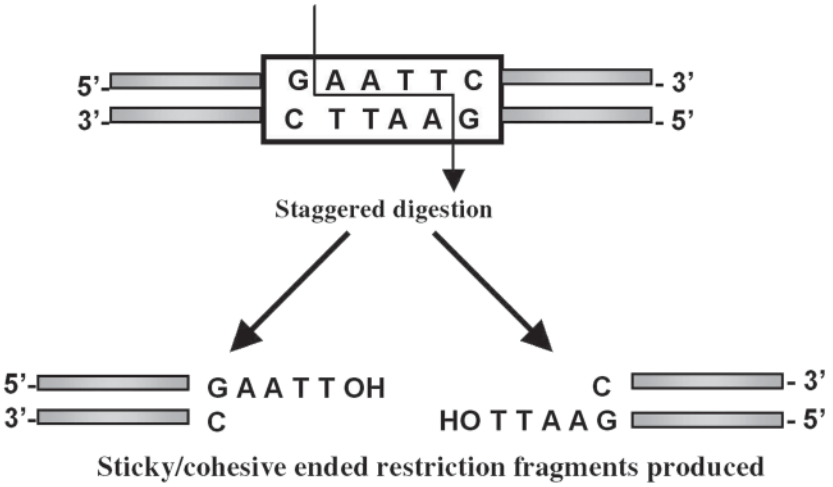

Fig. 1. The cleavage of a DNA strand with a target site for the restriction enzyme *Eco*R1 indicating the ends of the DNA formed following digestion.

as cofactors for enzymes that degrade DNA, termed *DNase*. Ideally, cell walls, if present, should be digested enzymatically (e.g., lysozyme in the bacteria or bacterial). In addition the cell membrane should be solubilized using detergent. Indeed, if physical disruption is necessary, it should be kept to a minimum and should involve cutting or squashing of cells, rather than the use of shear forces. Cell disruption and most of the subsequent steps should be performed at 4°C, using glassware and solutions that have been autoclaved to destroy DNase activity.

After release of nucleic acids from the cells, RNA can be removed by treatment with ribonuclease (RNase) that has been heat treated to inactivate any DNase contaminants; RNase is relatively stable to heat as a result of its disulfide bonds, which ensure rapid renaturation of the molecule on cooling. The other major contaminant, protein, is removed by shaking the solution gently with water-saturated phenol, or with a phenol/chloroform mixture, either of which will denature proteins but not nucleic acids. Centrifugation of the emulsion formed by this mixing produces a lower, organic phase, separated from the upper, aqueous phase by an interface of denatured protein. The aqueous solution is recovered and deproteinized repeatedly, until no more material is seen at the interface. Finally, the deproteinized DNA preparation is mixed with 2 vol of absolute ethanol, and the DNA is allowed to precipitate out of solution in a freezer. After centrifugation, the DNA pellet is redissolved in a buffer containing EDTA to inactivate any DNases present. This solution can be stored at 4°C for at least a month. DNA solutions can be stored frozen, although repeated freezing and thawing tends to damage long DNA molecules by shearing. A flow diagram summarizing the extraction of DNA is given in **Fig. 2**. The above-described procedure is suitable for total cellular DNA. If the DNA from a specific organelle or viral particle is needed, it is best to isolate the organelle or virus before extracting its DNA, because the recovery of a particular type of DNA from a mixture is usually rather difficult. Where a high degree of purity is required, DNA may be subjected to density gradient ultracentrifugation through cesium chloride, which is particularly useful for the preparation plasmid DNA. It is possible to check the integrity of the DNA by agarose gel electrophoresis and determine the concentration of the DNA by using the fact that 1 absorbance unit equates to 50 µg/mL of DNA:

$$50A_{260} = \text{Concentration of DNA sample (µg/mL)}$$

The identification of contaminants may also be undertaken by scanning ultraviolet (UV)-spectrophotometry from 200 nm to 300 nm. A ratio of 260 nm : 280 nm of approx 1.8 indicates that the sample is free of protein contamination, which absorbs strongly at 280 nm.

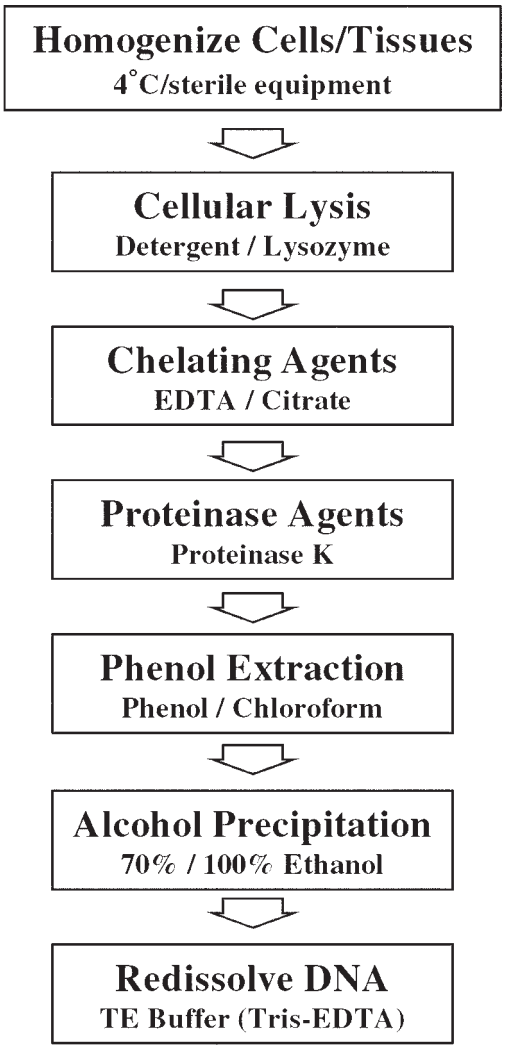

Fig. 2. General steps involved in extracting DNA from cells or tissues.

2.2. RNA Extraction Techniques

The methods used for RNA isolation are very similar to those described above for DNA; however, RNA molecules are relatively short and, therefore, less easily damaged by shearing, so cell disruption can be rather more vigorous *(2)*. RNA is, however, very vulnerable to digestion by Rnases, which are present endogenously in various concentrations in certain cell types and exogenously on fingers. Gloves should therefore be worn, and a strong detergent should be included in the isolation medium to immediately denature any RNases. Subsequent deproteinization should be particularly rigorous, because RNA is often tightly associated with proteins. DNase treatment can be used to remove DNA, and RNA can be precipitated by ethanol. One reagent in particular that is commonly used in RNA extraction is guanadinium thiocyanate, which is both a strong inhibitor of RNase and a protein denaturant. It is possible to check the integrity of an RNA extract by analyzing it by agarose gel electrophoresis. The most abundant RNA species, the rRNA molecules, are 23S and 16S for prokaryotes and 18S and 28S for eukaryotes. These appear as discrete bands on the agarose gel and indicate that the other RNA

components are likely to be intact. This is usually carried out under denaturing conditions to prevent secondary structure formation in the RNA. The concentration of the RNA may be estimated by using UV spectrophotometry in a similar manner to that used for DNA. However in the case of RNA at 260 nm, 1 absorbance unit equates to 40 µg/mL of RNA. Contaminants may also be identified in the same way by scanning UV spectrophotometry; however, in the case of RNA, a 260 nm : 280 nm ratio of approx 2 would be expected for a sample containing little or no contaminating protein *(3)*.

In many cases, it is desirable to isolate eukaryotic mRNA, which constitutes only 2–5% of cellular RNA from a mixture of total RNA molecules. This may be carried out by affinity chromatography on oligo(dT)-cellulose columns. At high salt concentrations, the mRNA containing poly(A) tails binds to the complementary oligo(dT) molecules of the affinity column, and so mRNA will be retained; all other RNA molecules can be washed through the column by further high-salt solution. Finally, the bound mRNA can be eluted using a low concentration of salt *(4)*. Nucleic acid species may also be subfractionated by more physical means such as electrophoretic or chromatographic separations based on differences in nucleic acid fragment sizes or physicochemical characteristics.

2.3. Electrophoresis of Nucleic Acids

In order to analyze nucleic acids by size, the process of electrophoresis in an agarose or polyacrylamide support gel is usually undertaken. Electrophoresis may be used analytically or preparatively and can be qualitative or quantitative. Large fragments of DNA such as chromosomes may also be separated by a modification of electrophoresis termed *pulsed field gel electrophoresis* (PFGE), which uses alternating directions of DNA migration *(5)*. The easiest and most widely applicable method is electrophoresis in horizontal agarose gels as indicated in **Fig. 3**. In order to visualize the DNA, staining has to be undertaken, usually with a dye such as ethidium bromide. This dye binds to DNA by insertion between stacked base-pairs, termed *intercalation*, and exhibits a strong orange/red fluorescence when illuminated with UV light. Alternative stains such as SYBRGreen or Gelstar, which have similar sensitivities, are also available and are less hazardous to use.

In general, electrophoresis is used to check the purity and intactness of a DNA preparation or to assess the extent of an enzymatic reaction during, for example, the steps involved in the cloning of DNA. For such checks "mini-gels" are particularly convenient, because they need little preparation, use small samples, and provide results quickly. Agarose gels can be used to separate molecules larger than about 100 basepairs (bp). For higher resolution or for the effective separation of shorter DNA molecules, polyacrylamide gels are the preferred method. In recent years, a number of acrylic gels have been developed which may be used as an alternative to agarose and polyacrylamide.

When electrophoresis is used preparatively, the fragment of gel containing the desired DNA molecule is physically removed with a scalpel. The DNA is then recovered from the gel fragment in various ways. This may include crushing with a glass rod in a small volume of buffer, using agarase to digest the agrose leaving the DNA, or by the process of electroelution. In this method, the piece of gel is sealed in a length of dialysis tubing containing buffer and is then placed between two electrodes in a tank containing more buffer. Passage of an electrical current between the electrodes causes DNA to migrate out of the gel piece, but it remains trapped within the dialysis tubing and can, therefore, be recovered easily.

3. Nucleic Acid Blotting and Gene Probe Hybridization

3.1. Nucleic Acid Blotting

Electrophoresis of DNA restriction fragments allows separation based on size to be conducted, however, it provides no indication as to the presence of a specific, desired fragment among the complex sample. This can be achieved by transferring the DNA from the intact gel onto a piece of nitrocellulose or Nylon membrane placed in contact with it. This provides a

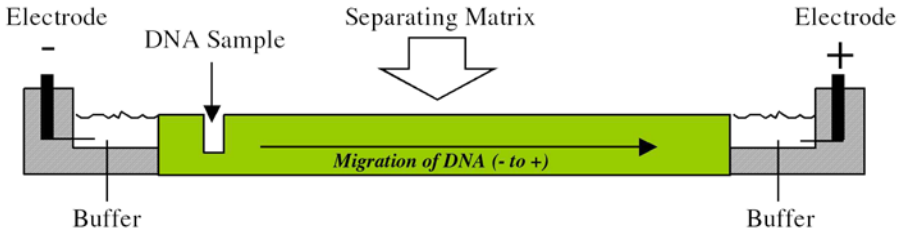

Fig. 3. Schematic illustration of a typical horizontal gel electrophoresis setup for the separation of nucleic acids.

more permanent record of the sample because DNA begins to diffuse out of a gel that is left for a few hours. First the gel is soaked in alkali to render the DNA single stranded. It is then transferred to the membrane so that the DNA becomes bound to it in exactly the same pattern as that originally on the gel *(6)*. This transfer, named a Southern blot (see Chapter 4) after its inventor Ed Southern, is usually performed by drawing large volumes of buffer by capillary action through both gel and membrane, thus transferring DNA from the gel to the membrane. Alternative methods are also available for this operation such as electrotransfer or vacuum-assisted transfer. Both are claimed to give a more even transfer and are much more rapid, although they do require more expensive equipment than the capillary transfer system. Transfer of the DNA from the gel to the membrane allows the membrane to be treated with a labeled DNA gene probe. This single-stranded DNA probe will hybridize under the right conditions to complementary single-stranded DNA fragments immobilized onto the membrane.

3.2. Hybridization and Stringency

The conditions of hybridization are critical for this process to take place effectively. This is usually referred to as the stringency of the hybridization and it is particular for each individual gene probe and for each sample of DNA. Two of the most important components are the temperature and the salt concentration. Higher temperatures and low salt concentrations, termed *high stringency*, provide a favorable environment for perfectly matched probe and template sequences, whereas reduced temperatures and high salt concentrations, termed *low stringency*, allow the stabilization of mismatches in the duplex. In addition, inclusion of denaturants such as formamide allow the hybridization temperatures to be reduced without affecting the stringency.

A series of posthybridization washing steps with a salt solution such as SSC, containing sodium citrate and sodium chloride, is then carried out to remove any unbound probe and control the binding of the duplex. The membrane is developed using either autoradiography if the probe is radiolabeled or by a number of nonradioactive methods. The precise location of the probe and its target may be then visualized. The steps involved in Southern blotting are indicated in **Fig. 4**. It is also possible to analyze DNA from different species or organisms by blotting the DNA and then using a gene probe representing a protein or enzyme from one of the organisms. In this way, it is possible to search for related genes in different species. This technique is generally termed Zoo blotting.

A similar process of nucleic acid blotting can be used to transfer RNA separated by gel electrophoresis onto membranes similar to that used in Southern blotting. This process, termed *Northern blotting*, allows the identification of specific mRNA sequences of a defined length by hybridization to a labeled gene probe *(7)*. It is possible with this technique to not only detect specific mRNA molecules, but it may also be used to quantify the relative amounts of the specific mRNA present in a tissue or sample. It is usual to separate the mRNA transcripts by gel electrophoresis under denaturing conditions because this improves resolution and allows a more accurate estimation of the sizes of the transcripts. The format of the blotting may be altered from transfer from a gel to direct application to slots on a specific blotting apparatus containing

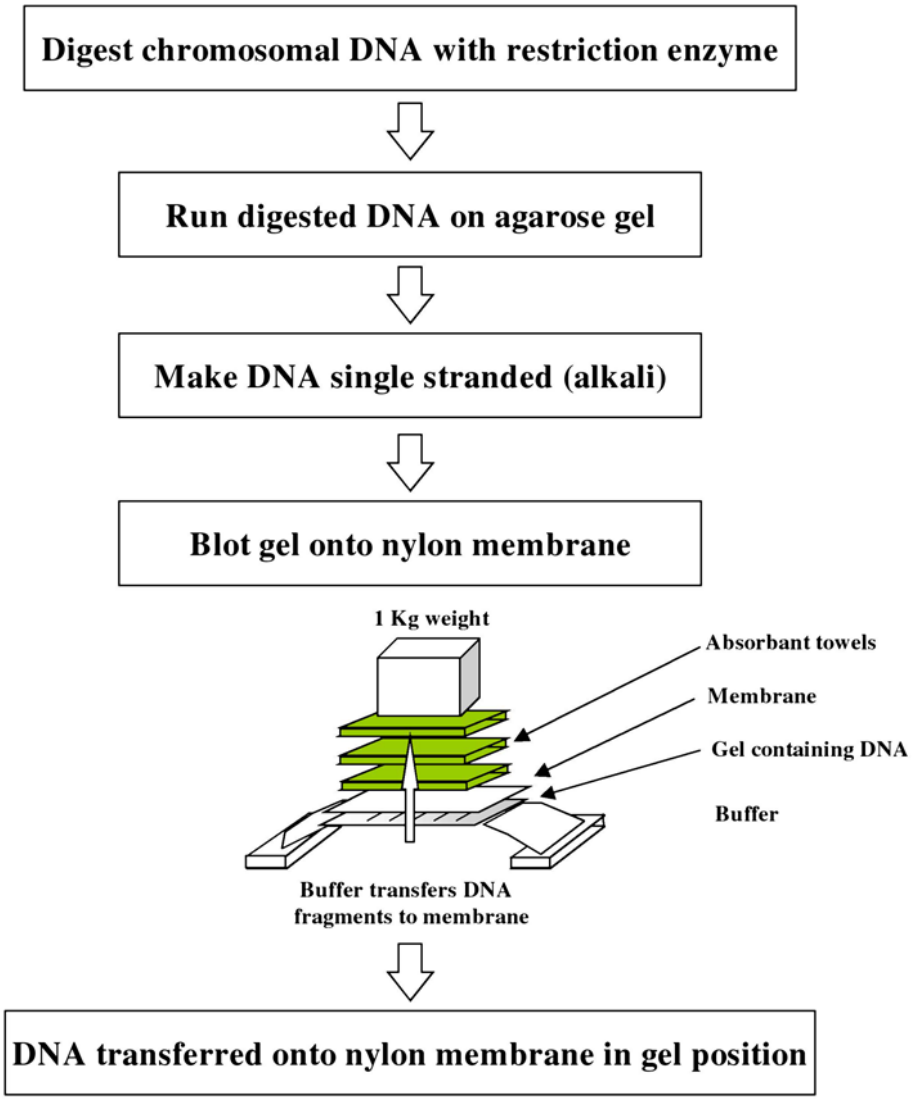

Fig. 4. The procedure involved in a typical Southern blot indicating the construction of a simple capillary transfer setup.

the Nylon membrane. This is termed *slot* or *dot blotting* and provides a convenient means of measuring the abundance of specific mRNA transcripts without the need for gel electrophoresis, it does not, however, provide information regarding the size of the fragments.

Hybridization techniques are essential to many molecular biology experiments; however, the format of the hybridization may be altered to improve speed sensitivity and throughput. One interesting alternative is termed *surface plasmon resonance* (SPR). This is an optical system based on difference between incident and reflected light in the presence of absence of hybridization. Its main advantage is that the kinetics of hybridization can be undertaken in real time and without a DNA label. A further exciting method for hybridization is also in use which uses arrays of single-stranded DNA molecules tethered to small hybridization chips. Hybridization to a DNA sample is detected by computer, allowing DNA mutations to be quickly and easily identified.

3.3. Production of Gene Probes

The availability of a gene probe is essential in many molecular biology techniques; yet, in many cases, it is one of the most difficult steps (see Chapter 2). The information needed to produce a gene probe may come from many sources, but with the development and sophistication of genetic databases, this is usually one of the first stages (*8*). There are a number of genetic databases such as those at Genbank and EMBL and it is possible to search these over the Internet and identify particular sequences relating to a specific gene or protein. In some cases, it is possible to use related proteins from the same gene family to gain information on the most useful DNA sequence. Similar proteins or DNA sequences but from different species may also provide a starting point with which to produce a so-called heterologous gene probe. Although, in some cases, probes are already produced and cloned, it is possible, armed with a DNA sequence from a DNA database, to chemically synthesize a single-stranded oligonucleotide probe. This is usually undertaken by computer-controlled gene synthesizers, which link dNTPs together based on a desired sequence. It is essential to carry out certain checks before probe production to determine that the probe is unique, is not able to self-anneal, or is self-complementary, all of which may compromise its use.

Where little DNA information is available to prepare a gene probe, it is possible in some cases to use the knowledge gained from analysis of the corresponding protein. Thus, it is possible to isolate and purify proteins and sequence part of the N-terminal end of the protein. From our knowledge of the genetic code, it is possible to predict the various DNA sequences that could code for the protein and then synthesize appropriate oligonucleotide sequences chemically. Because of the degeneracy of the genetic code, most amino acids are coded for by more than one codon, therefore, there will be more than one possible nucleotide sequence that could code for a given polypeptide. The longer the polypeptide, the larger is the number of possible oligonucleotides that must be synthesized. Fortunately, there is no need to synthesize a sequence longer than about 20 bases, as this should hybridize efficiently with any complementary sequences and should be specific for one gene. Ideally, a section of the protein should be chosen that contains as many tryptophan and methionine residues as possible, because these have unique codons and there will therefore be fewer possible base sequences that could code for that part of the protein. The synthetic oligonucleotides can then be used as probes in a number of molecular biology methods.

3.4. DNA Gene Probe Labeling

An essential feature of a gene probe is that it can be visualized by some means. In this way, a gene probe that hybridizes to a complementary sequence may be detected and identify that desired sequence from a complex mixture. There are two main ways of labeling gene probes, traditionally this has been carried out using radioactive labels, but gaining in popularity are nonradioactive labels. Perhaps the most used radioactive label is phosphorous-32 (^{32}P), although for certain techniques sulfur-35 (^{35}S) and tritium (^{3}H) are used. These may be detected by the process of autoradiography where the labeled probe molecule, bound to sample DNA, located, for example, on a Nylon membrane, is placed in contact with an X-ray-sensitive film. Following exposure, the film is developed and fixed just as a black-and-white negative and reveals the precise location of the labeled probe and, therefore, the DNA to which it has hybridized.

3.5. Nonradioactive DNA Labeling

Nonradioactive labels are increasingly being used to label DNA gene probes. Until recently, radioactive labels were more sensitive than their nonradioactive counterparts. However, recent developments have led to similar sensitivities, which, when combined with their improved safety, have led to their greater acceptance.

The labeling systems are either termed direct or indirect. Direct labeling allows an enzyme reporter such as alkaline phosphatase to be coupled directly to the DNA. Although this may alter the characteristics of the DNA gene probe, it offers the advantage of rapid analysis because

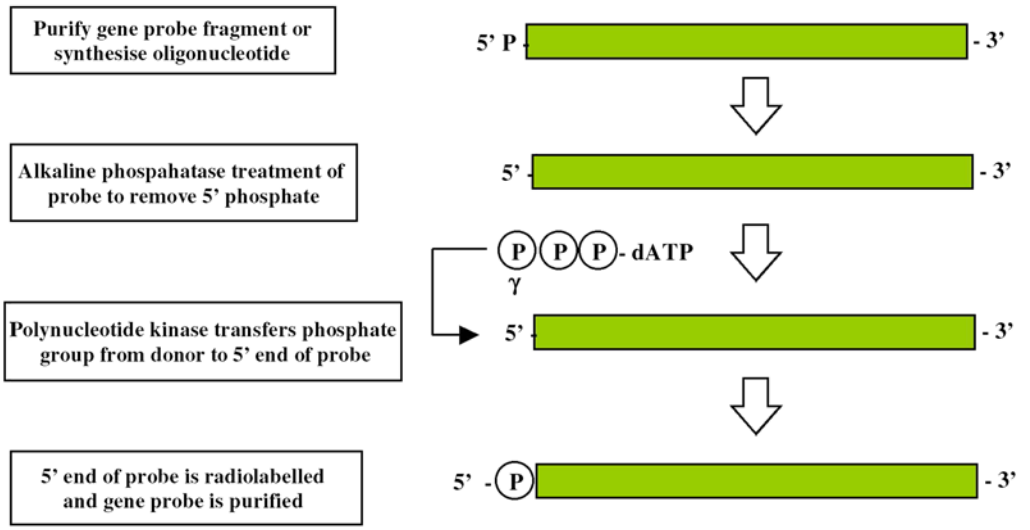

Fig. 5. End labeling of a gene probe at the 5' end with alkaline phosphatase and polynucleotide kinase.

no intermediate steps are needed. However indirect labeling is, at present, more popular. This relies on the incorporation of a nucleotide that has a label attached. At present, three of the main labels in use are biotin, flourescein, and digoxigenin. These molecules are covalently linked to nucleotides using a carbon spacer arm of 7, 14, or 21 atoms. Specific binding proteins may then be used as a bridge between the nucleotide and a reporter protein such as an enzyme. For example, biotin incorporated into a DNA fragment is recognized with a very high affinity by the protein streptavidin. This may either be coupled or conjugated to a reporter enzyme molecule such as alkaline phosphatase or horseradish peroxidase (HRP). This is usually used to convert a colorless substrate into a colored insoluble compound and also offers a means of signal amplification. Alternatively, labels such as digoxigenin incorporated into DNA sequences may be detected by monoclonal antibodies, again conjugated to reporter molecules, including alkaline phosphatase. Thus, rather than the detection system relying on autoradiography, which is necessary for radiolabels, a series of reactions resulting in either a color or a light or a chemiluminescence reaction takes place. This has important practical implications because autoradiography may take 1–3 d, whereas color and chemiluminescent reactions take minutes. In addition, no radiolabeling and detection minimize the potential health and safety hazards encountered when using radiolabels.

3.6. End Labeling of DNA

The simplest form of labeling DNA is by 5'- or 3'-end labeling. 5'-End labeling involves a phosphate transfer or exchange reaction, where the 5' phosphate of the DNA to be used as the probe is removed and in its place a labeled phosphate, usually ^{32}P, is added. This is usually carried out by using two enzymes, the first, alkaline phosphatase, is used to remove the existing phosphate group from the DNA. Following removal of the released phosphate from the DNA, a second enzyme polynucleotide kinase is added that catalyzes the transfer of a phosphate group (^{32}P labeled) to the 5' end of the DNA (*see* **Fig. 5**). The newly labeled probe is then purified, usually by chromatography through a Sephadex column and may be used directly.

Using the other end of the DNA molecule, the 3' end, is slightly less complex. Here, a new dNTP, which is labeled (e.g., ^{32}PαdATP or biotin-labeled dNTP), is added to the 3' end of the DNA by the enzyme terminal transferase as indicated in **Fig. 6**. Although this is a simpler

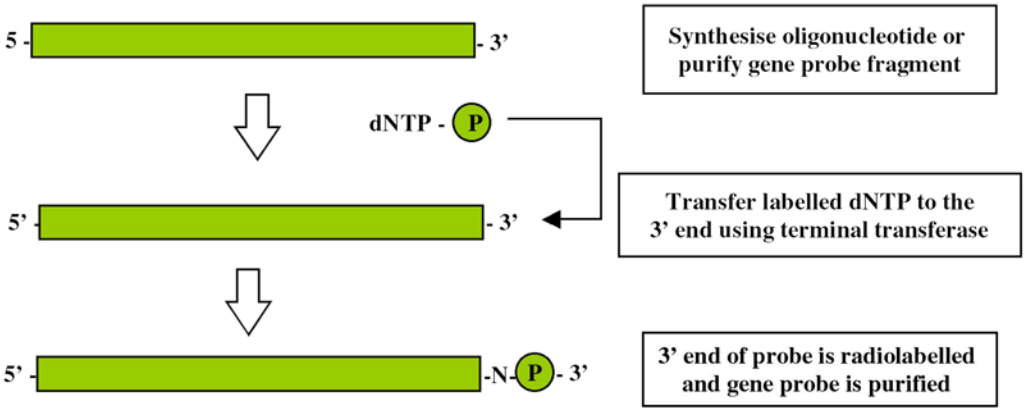

Fig. 6. End labeling of a gene probe at the 3' end using terminal transferase. Note that the addition of a labeled dNTP at the 3' end alters the sequence of the gene probe.

reaction, a potential problem exists because a new nucleotide is added to the existing sequence and so the complete sequence of the DNA is altered, which may affect its hybridization to its target sequence. End labeling methods also suffer from the fact that only one label is added to the DNA, so these methods are of a lower activity in comparison to methods that incorporate labels along the length of the DNA.

3.7. Random Primer Labeling of DNA

The DNA to be labeled is first denatured and then placed under renaturing conditions in the presence of a mixture of many different random sequences of hexamers or hexanucleotides. These hexamers will, by chance, bind to the DNA sample wherever they encounter a complementary sequence and, thus, the DNA will rapidly acquire an approximately random sprinkling of hexanucleotides annealed to it. Each of the hexamers can act as a primer for the synthesis of a fresh strand of DNA catalyzed by DNA polymerase because it has an exposed 3' hydroxyl group, as seen in **Fig. 7**. The Klenow fragment of DNA polymerase is used for random primer labeling because it lacks a 5'–3' exonuclease activity. This is prepared by cleavage of DNA polymerase with subtilisin, giving a large enzyme fragment that has no 5' to 3' exonuclease activity, but which still acts as a 5' to 3' polymerase. Thus, when the Klenow enzyme is mixed with the annealed DNA sample in the presence of dNTPs, including at least one that is labeled, many short stretches of labeled DNA will be generated. In a similar way to random primer labelling, polymerase chain reaction (PCR) may also be used to incorporate radioactive or nonradioactive labels.

3.8 Nick Translation Labeling of DNA

A traditional method of labeling DNA is by the process of nick translation. Low concentrations of DNase I are used to make occasional single-strand nicks in the double-stranded DNA that is to be used as the gene probe. DNA polymerase then fills in the nicks, using an appropriate deoxyribonucleoside triphosphate (dNTP), at the same time making a new nick to the 3' side of the previous one. In this way, the nick is translated along the DNA. If labeled dNTPs are added to the reaction mixture, they will be used to fill in the nicks, as indicated in **Fig. 8**. In this way, the DNA can be labeled to a very high specific activity.

4. Medical Applications of Basic Molecular Techniques: Restriction Mapping of DNA Fragments

Restriction mapping involves the size analysis of restriction fragments produced by several restriction enzymes individually and in combination. Comparison of the lengths of fragments

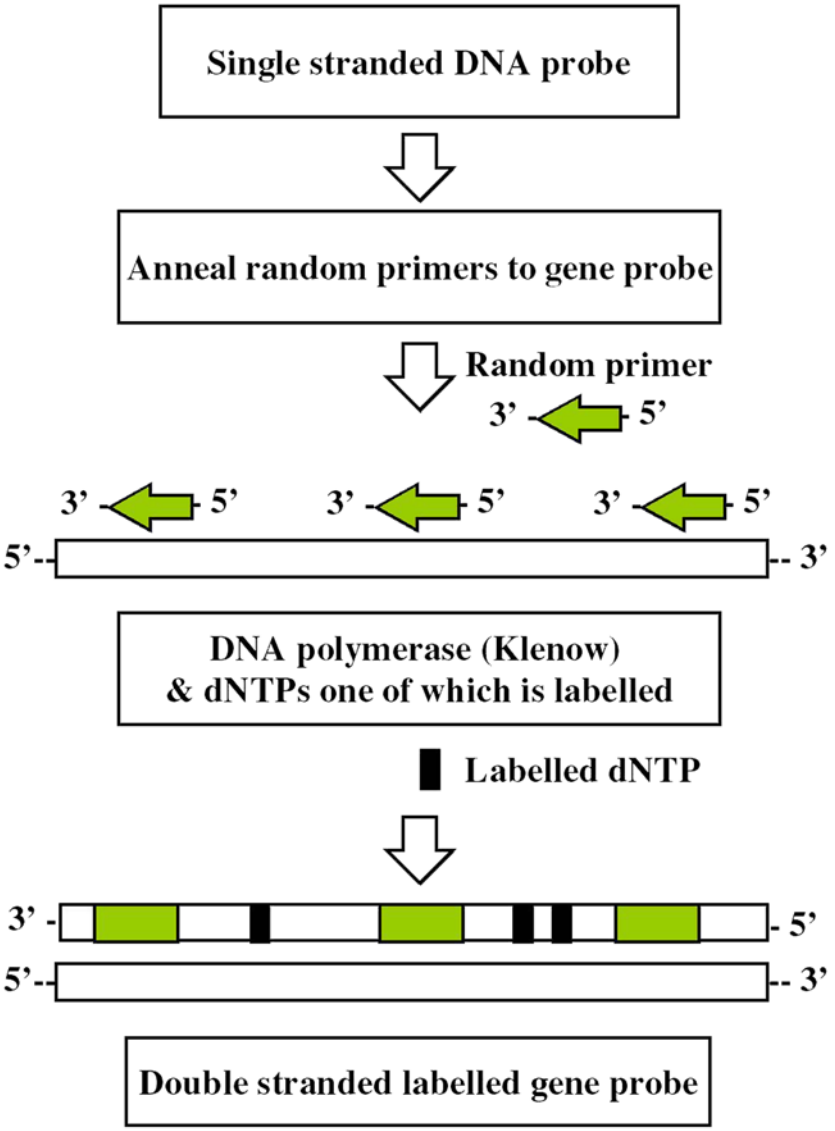

Fig. 7. Random primer gene probe labeling. Random primers are incorporated and used as a start point for Klenow DNA polymerase to synthesize a complementary strand of DNA while incorporating a labeled dNTP at complementary sites.

obtained allows their relative positions within the DNA fragment to be deduced. Any mutation that creates, destroys, or moves the recognition sequence for a restriction enzyme leads to a restriction fragment length polymorphism (RFLP) (*9*). An RFLP can be detected by examining the profile of restriction fragments generated during digestion. Conventionally, this required the purification of the original starting DNA sample before digestion with single or multiple restriction enzymes. The resultant fragments were then size-separated by gel electrophoresis and visualized by staining with ethidium bromide. Routine RFLP analysis of genomic DNA samples generally also involves hybridization with labeled gene probes to detect a specific gene fragment. The first useful RFLP was described for the detection of sickle cell anemia. In this case, a difference in the pattern of digestion with the restriction endonuclease *Hha*I could

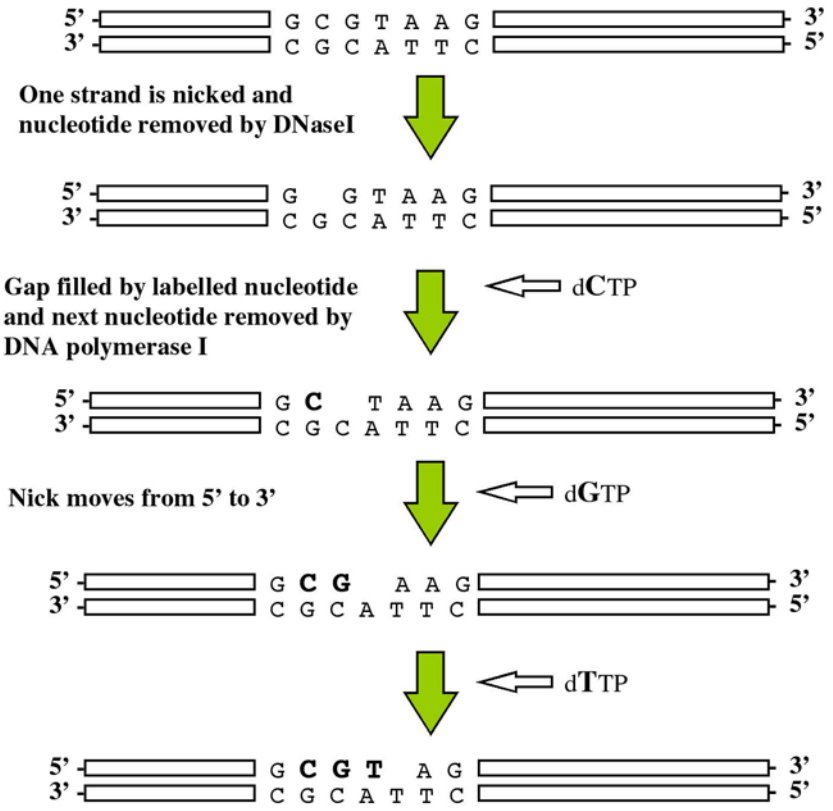

Fig. 8. Nick translation. The removal of nucleotides and their subsequent replacement with labeled nucleotides by DNA polymerase I makes the gene probe more labeled as nick translation proceeds.

be identified between DNA samples from normal individuals and patients with the disease. This polymorphism was later shown to be the result of a single base substitution in the gene for β-globin that changed a codon, GAG specific for the amino acid glutamine to GUG, which encoded valine. A further example is the use of the restriction enzyme *Mnl*I to detect factor V Leiden. This arises from a point mutation in exon 13 of the factor V gene and, in particular, constitutes the most frequent genetic risk factor for venous thrombosis. Although this is a good example of how a basic molecular biology technique could be used for the identification of a genetic mutation, the occurrence is infrequent and thus of limited use.

RFLPs may arise by a number of different means that alter the relative position of restriction endonuclease recognition sequences. In general, most polymorphisms are randomly distributed throughout a genome; however, there are certain regions where many polymophisms exist. These are termed *hypervariable regions* and have been found in regions flanking structural genes from several sources. These were first identified as differences in the numbers of short repeated sequences, termed *minisatellites*. These occurred within the genomes from different individuals, as evidenced by RFLP analysis using specific gene probes. Several types of minisatellite sequence such as variable numbers of tandem repeats (VNTR) have now been described.

An additional source of polymorphic diversity present in the human genome is termed *single-nucleotide polymorphism* (SNP) (pronounced snip). SNPs are substitutions of one base at a precise location within the genome. Those that occur in coding regions are termed cSNPs.

Estimates indicate that an SNP occurs once in every 300 bases and there are thought to be approx 10 million in the human genome. Interest in SNPs lies in the fact that these differences can account for the differences in disease suseptability, drug metabolism, and response to environmental factors between individuals. There are now a number of initiatives to identify SNPs and produce a genome SNP map. A number of maps have been partially completed and a number of bioinformatics resources have been developed, such as the SNP consortium ***(10,11)***.

There are numerous disorders that arise as a result of point mutations or deletions/insertions, and it is possible given an appropriate gene probe complementary to the genetic lesion to identify and detect it successfully following DNA blotting. However, the development and refinement of the PCR has led to many Southern-blot-based methods now being superseded by amplification techniques and will no doubt be ultimately overtaken by the continued development of microarray technology ***(12)***.

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2

Probe Design, Production, and Applications

Marilena Aquino de Muro

1. Introduction

A probe is a nucleic acid molecule (single-stranded DNA or RNA) with a strong affinity with a specific target (DNA or RNA sequence). Probe and target base sequences must be complementary to each other, but depending on conditions, they do not necessarily have to be exactly complementary. The hybrid (probe–target combination) can be revealed when appropriate labeling and detection systems are used. Gene probes are used in various blotting and *in situ* techniques for the detection of nucleic acid sequences. In medicine, they can help in the identification of microorganisms and the diagnosis of infectious, inherited, and other diseases.

2. Probe Design

The probe design depends on whether a gene probe or an oligonucleotide probe is desired.

2.1. Gene Probes

Gene probes are generally longer than 500 bases and comprise all or most of a target gene. They can be generated in two ways. Cloned probes are normally used when a specific clone is available or when the DNA sequence is unknown and must be cloned first in order to be mapped and sequenced. It is usual to cut the gene with restriction enzymes and excise it from an agarose gel, although if the vector has no homology, this might not be necessary.

Polymerase chain reaction (PCR) is a powerful procedure for making gene probes because it is possible to amplify and label, at the same time, long stretches of DNA using chromosomal or plasmid DNA as template and labeled nucleotides included in the extension step (*see* **Subheadings 2.2.** and **3.2.3.**). Having the whole sequence of a gene, which can easily be obtained from databases (GenBank, EMBL, DDBJ), primers can be designed to amplify the whole gene or gene fragments (see Chapter 28). A considerable amount of time can be saved when the gene of interest is PCR amplified, for there is no need for restriction enzyme digestion, electrophoresis, and elution of DNA fragments from vectors. However, if the PCR amplification gives nonspecific bands, it is recommended to gel purify the specific band that will be used as a probe.

Gene probes generally provide greater specificity than oligonucleotides because of their longer sequence and because more detectable groups per probe molecule can be incorporated into them than into oligonucleotide probes ***(1)***.

2.2. Oligonucleotide Probes

Oligonucleotide probes are generally targeted to specific sequences within genes. The most common oligonucleotide probes contain 18–30 bases, but current synthesizers allow efficient synthesis of probes containing at least 100 bases. An oligonucleotide probe can match perfectly

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its target sequence and is sufficiently long to allow the use of hybridization conditions that will prevent the hybridization to other closely related sequences, making it possible to identify and detect DNA with slight differences in sequence within a highly conserved gene, for example.

The selection of oligonucleotide probe sequences can be done manually from a known gene sequence using the following guidelines *(1)*:

- The probe length should be between 18 and 50 bases. Longer probes will result in longer hybridization times and low synthesis yields, shorter probes will lack specificity.
- The base composition should be 40–60% G-C. Nonspecific hybridization may increase for G-C ratios outside of this range.
- Be certain that no complementary regions within the probe are present. These may result in the formation of "hairpin" structures that will inhibit hybridisation to target.
- Avoid sequences containing long stretches (more than four) of a single base.
- Once a sequence meeting the above criteria has been identified, computerized sequence analysis is highly recommended. The probe sequence should be compared with the sequence region or genome from which it was derived, as well as to the reverse complement of the region. If homologies to nontarget regions greater than 70% or eight or more bases in a row are found, that probe sequence should not be used.

However, to determine the optimal hybridization conditions, the synthesized probe should be hybridized to specific and nonspecific target nucleic acids over a range of hybridization conditions.

These same guidelines are applicable to design forward and reverse primers for amplification of a particular gene of interest to make a gene probe. It is important to bear in mind that, in this case, it is essential that the 3' end of both forward and reverse primers have no homology with other stretches of the template DNA other than the region you want to amplify. There are numerous software packages available (LaserDNA™, GeneJockey II™, etc.) that can be used to design a primer for a particular sequence or even just to check if the pair of primers designed manually will perform as expected.

3. Labeling and Detection

3.1. Types of Label

3.1.1. Radioactive Labels

Nucleic acid probes can be labeled using radioactive isotopes (e.g., ^{32}P, ^{35}S, ^{125}I, ^{3}H). Detection is by autoradiography or Geiger–Muller counters. Radiolabeled probes used to be the most common type but are less popular today because of safety considerations as well as cost and disposal of radioactive waste products. However, radiolabeled probes are the most sensitive, as they provide the highest degree of resolution currently available in hybridization assays *(1,2)*. High sensitivity means that low concentrations of a probe–target hybrid can be detected; for example, ^{32}P-labeled probes can detect single-copy genes in only 0.5 µg of DNA and Keller and Manak *(1)* list a few reasons:

- ^{32}P has the highest specific activity.
- ^{32}P emits β-particles of high energy.
- ^{32}P-Labeled nucleotides do not inhibit the activity of DNA-modifying enzymes, because the structure is essentially identical to that of the nonradioactive counterpart.

Although ^{32}P-labeled probes can detect minute quantities of immobilized target DNA (<1 pg), their disadvantages is the inability to be used for high-resolution imaging and their relatively short half-life (14.3 d); ^{32}P-labeled probes should be used within a week after preparation.

The lower energy of ^{35}S plus its longer half-life (87.4 d) make this radioisotope more useful than ^{32}P for the preparation of more stable, less specific probes. These ^{35}S-labeled probes, although less sensitive, provide higher resolution in autoradiography and are especially suitable for *in situ* hybridization procedures. Another advantage of ^{35}S over ^{32}P is that the ^{35}S-

labeled nucleotides present little external hazard to the user. The low-energy β-particles barely penetrate the upper dead layer of skin and are easily contained by laboratory tubes and vials.

Similarly, ^{3}H-labeled probes have traditionally been used for *in situ* hybridization because the low-energy β-particle emissions result in maximum resolution with low background. It has the longest half-life (12.3 yr).

The use of ^{125}I and ^{131}I has declined since the 1970's with the availability of ^{125}I-labeled nucleoside triphosphates of high specific activity. ^{125}I has lower energies of emission and a longer half-life (60 d) than ^{131}I, and are frequently used for *in situ* hybridization.

3.1.2. Nonradioactive Labels

Compared to radioactive labels, the use of nonradioactive labels have several advantages:

- Safety.
- Higher stability of probe.
- Efficiency of the labeling reaction.
- Detection *in situ.*
- Less time taken to detect the signal.

Concern over laboratory safety and the economic and environmental aspects of radioactive waste disposal have been key factors in their development and use. Some examples are as follows:

- Biotin: This label can be detected using avidin or streptavidin which have high affinities for biotin. Because the reporter enzyme is not conjugated directly to the probe but is linked to it through a bridge (e.g., streptavidin–biotin), this type of nonradioactive detection is known as an indirect system. Usually, biotinylated probes work very well, but because biotin (vitamin H) is a ubiquitous constituent of mammalian tissues and because biotinylated probes tend to stick to certain types of Nylon membrane, high levels of background can occur during hybridizations. These difficulties can be avoided by using nucleotide derivatives, including digoxigenen-11-UTP, -11-dUTP, and -11-ddUTP, and biotin-11-dUTP or biotin-14-dATP. After hybridization, these are detected by an antibody or avidin, respectively, followed by a color or chemiluminescent reaction catalysed by alkaline phosphatase or peroxidase linked to the antibody or avidin ***(1,2)***.
- Enzymes. The enzyme is attached to the probe and its presence usually detected by reaction with a substrate that changes color. Used in this way, the enzyme is sometimes referred to as a "reporter group," Examples of enzymes used include alkaline phosphatase and horseradish peroxidase (HRP). In the presence of peroxide and peroxidase, chloronaphtol, a chromogenic substrate for HRP, forms a purple insoluble product. HRP also catalyzes the oxidation of luminol, a chemiluminogenic substrate for HRP ***(2,3)***.
- Chemiluminescence. In this method, chemiluminescent chemicals attached to the probe are detected by their light emission using a luminometer. Chemiluminescent probes (including the above enzyme labels) can be easily stripped from membranes, allowing the membranes to be reprobed many times without significant loss of resolution.
- Fluorescence chemicals attached to probe fluoresce under ultraviolet (UV) light. This type of label is especially useful for the direct examination of microbiological or cytological specimens under the microscope—a technique known as fluorescent *in situ* hybridization (FISH). Hugenholts et al. have some useful considerations on probe design for FISH ***(4)***.
- Antibodies. An antigenic group is coupled to the probe and its presence detected using specific antibodies. Also, monoclonal antibodies have been developed that will recognize DNA–RNA hybrids. The antibodies themselves have to be labeled, using an enzyme, for example.
- DIG system. It is the most comprehensive, convenient, and effective system for labeling and detection of DNA, RNA, and oligonucleotides. Digoxigenin (DIG), like biotin, can be chemically coupled to linkers, and nucleotides and DIG-substituted nucleotides can be incorporated into nucleic acid probes by any of the standard enzymatic methods. These probes generally yield significantly lower backgrounds than those labeled with biotin. An anti-digoxigenin an-

Table 1
Types of Label

Radioactive labels
^{32}P
^{35}S
^{125}I
^{131}I
^{3}H
Nonradioactive labels
Biotin
Chemiluminescent enzyme labels (acridinium ester, alkaline phosphatase, β-D-galactosidase, horseradish peroxidase [HRP], isoluminol, xanthine oxidase)
Fluorescence chemicals (fluorochromes)
Antibodies
Digoxigenin system

tibody–alkaline phosphatase conjugate is allowed to bind to the hybridized DIG-labeled probe. The signal is then detected with colorimetric or chemiluminescent alkaline phosphatase substrates. If a colorimetric substrate is used, the signal develops directly on the membrane. The signal is detected on an X-ray film (as with ^{32}P- or ^{35}S-labeled probes) when a chemiluminescent substrate is used. Roche Biochemicals has a series of kits for DIG labeling and detection, as well as comprehensive detailed guides ***(5,6)*** with protocols for single-copy gene detection of human genome on Southern blots, detection of unique mRNA species on Northern blots, colony and plaque screening, slot/dot blots, and *in situ* hybridization.

The one area in which nonradioactive probes have a clear advantage is *in situ* hybridization. When the probe is detected by fluorescence or color reaction, the signal is at the exact location of the annealed probe, whereas radioactive probes can only be visualized as silver grains in a photographic emulsion some distance away from the actual annealed probe ***(7)***.

3.2. Labeling Methods

The majority of radioactive labeling procedures rely upon enzymatic incorporation of a nucleotide labeled into the DNA, RNA, or oligonucleotide.

Table 1 summarizes the various types of label ***(2)***.

3.2.1. Nick Translation

Nick translation is one method of labeling DNA, which uses the enzymes pancreatic Dnase I and *Escherichia coli* DNA polymerase I. The nick translation reaction results from the process by which *E. coli* DNA polymerase I adds nucleotides to the 3'-OH created by the nicking activity of Dnase I, while the 5' to 3' exonuclease activity simultaneously removes nucleotides from the 5' side of the nick. If labeled precursor nucleotides are present in the reaction, the preexisting nucleotides are replaced with labeled nucleotides. For radioactive labeling of DNA, the precursor nucleotide is an [α-^{32}P]dNTP. For nonradioactive labeling procedures, a digoxigenin or a biotin moiety attached to a dNTP analog is used ***(2)***.

3.2.2. Random-Primed Labeling (or Primer Extension)

Gene probes, cloned or PCR-amplified, and oligonucleotide probes can be random-primed labeled with radioactive isotopes and nonradioactive labels (e.g., DIG). Random-primed labeling of DNA fragments (double- or single-stranded DNA) was developed by Feinberg and Volgestein ***(8,9)*** as an alternative to nick translation to produce uniformly labeled probes.

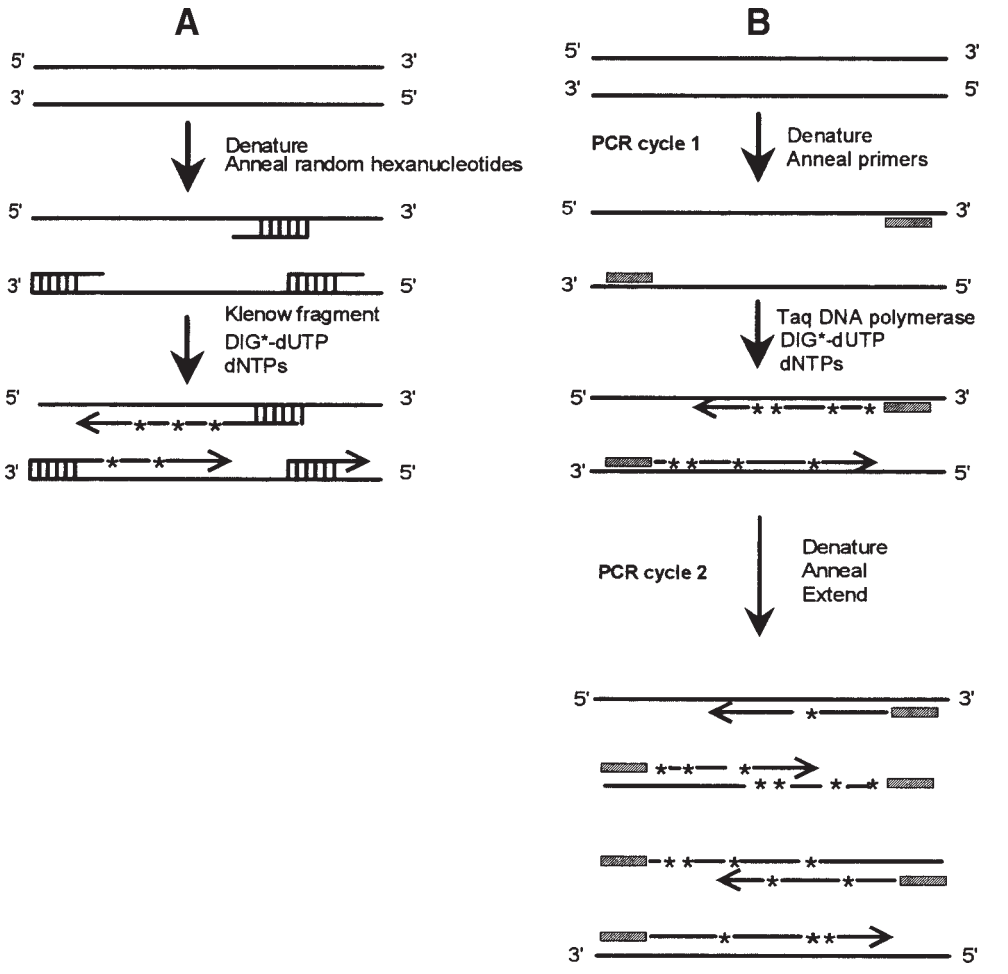

Fig. 1. Steps involved in the following: **(A)** random-primed DIG labeling: Double-stranded DNA is denatured and annealed with random oligonucleotide primers (6-mers); the oligonucleotides serve as primers for the 5' to 3' Klenow fragment of *E. coli* DNA polymerase I, which synthesizes labeled probes in the presence of DIG–dUTP. **(B)** PCR–DIG labelling: DIG–dUTP is incorporated during PCR cycles into the DNA strands amplified from the DNA target. The asterisk represents the digoxigenin molecule incorporated along the DNA strands.

Double-stranded DNA is denatured and annealed with random oligonucleotide primers (6-mers). The oligonucleotides serve as primers for the 5' to 3' polymerase (the Klenow fragment of *E. coli* DNA polymerase I), which synthesizes labeled probes in the presence of a labeled nucleotide precursor. **Figure 1A** shows the steps involved in random-primed DIG labeling as an example.

3.2.3. DIG–PCR Labeling

A very robust method for labeling a gene probe with DIG uses PCR. The probe is PCR-amplified using the appropriate set of primers and thermocycling parameters, however, the dNTP mixture has less dTTP because the labeled DIG–dUTP will also be added to the reaction. (Similarly, when this method is used with [α-^{32}P]dCTP, the dNTP mixture will not have dCTP.) The advantage of PCR–DIG labeling, over random-primed DIG labeling, is the incorporation

of a higher number of DIG moieties along the amplified DNA strands during the PCR cycles. It is worth noting that the random incorporation of large molecules of DIG–dUTP along the DNA strands during the PCR cycles makes the amplified fragment run slower on an agarose gel. A control PCR reaction, without DIG–dUTP, should also be prepared at the same time to verify whether the size of the amplified fragment with incorporated DIG (labeled probe) corresponds to the desired gene fragment. **Figure 1B** shows the steps involved in PCR–DIG labeling, and refs. *(10–12)* describe successful examples of use of PCR–DIG labeling.

3.2.4. Photobiotin Labeling

Photobiotin labeling is a chemical reaction, not an enzymatic one. Biotin and DIG can be linked to a nitrophenyl azido group that is converted by irradiation with UV or strong visible light to a highly reactive nitrene that can form stable covalent linkages to DNA and RNA *(2)*. The materials for photobiotin labeling are more stable than the enzymes needed in nick translation or oligonucleotide labeling and are less expensive, and it is a method of choice when large quantities of probe but not very high sensitivities *(3,13)*.

3.2.5. End Labeling

End labeling of probes for hybridization is mainly used to label oligonucleotide probes *(*for a review, see **ref.** *14)*.

Roche Biochemicals *(6)* has developed three methods for labeling oligonucleotides with digoxigenin:

- The 3'-end labeling of an oligonucleotide 14–100 nucleotides in length with 1 residue of DIG-11-ddUTP per molecule
- The 3' tailing reaction, where terminal transferase adds a mixture of unlabeled nucleotides and DIG-11-dUTP, producing a tail containing multiple digoxigenin residues
- The 5' end labeling in a two-step synthesis with first an aminolinker residue on the 5' end of the oligonucleotide, and then after purification, a digoxigenin-*N*-hydroxy-succinimide ester is covalently linked to the free 5'-amino residue.

Oligonucleotides can also be labeled with radioisotopes by transferring the γ-^{32}P from [γ-^{32}P]ATP to the 5' end using the enzyme bacteriophage T4 polynucleotide kinase. If the reaction is carried out efficiently, the specific activity of such probes can be as high as the specific activity of [γ-^{32}P]ATP itself *(2)*.

Promega has a detailed guide *(15)* with protocols on radioactive and nonradioactive labeling of DNA. The choice of probe labeling method will depend on the following:

- Target format: Southern, Northern, slot/dot, or colony blot (*see* **Subheading 4.**)
- Type of probe: gene or oligonucleotide probe
- Sensitivity required for detection: single-copy gene or detection of PCR-amplified DNA fragments

For example, 3'- and 5'-end labeling of oligonucleotides give good results on slot and colony hybridization in contrast with poor sensitivity when using Southern blotting.

4. Target Format

4.1. Solid Support

A convenient format for the hybridization of DNA to gene probes or oligonucleotide probes is immobilization of the target nucleic acid (DNA or RNA) onto a solid support while the probe is free in solution. The solid support can be a nitrocellulose or Nylon membrane, Latex or magnetic beads, or microtiter plates. Nitrocellulose membranes are very commonly used and produce low background signals; however, they can only be used when colorimetric detection will be performed and no probe stripping and reprobing is planned. For these purposes, positively charged Nylon membranes are recommended, and they also ensure an optimal signal-to-noise ratio when the DIG system is used. Although nitrocellulose membranes are able to bind