Chapter 18

DNA PROBES

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Introduction

There is no doubt that radioimmunoassays have contributed a lot to the diagnosis and understanding of many diseases. There are two problems with them:

(a) they use radioisotopic label and

(b) they are difficult to automate because of the separation step, which is indispensable.

To bring the immunoassays in the fold of clinical chemistry, a large amount of effort is now directed towards developing non-isotopic assays.

On the other hand, In vitro nuclear techniques are gradually moving in new directions opened up by modern molecular biology. Two approaches are promising in this respect:

(a) DNA probes and

(b) proteins of biological interest produced by recombinant techniques.

The creation of DNA probes for detection of specific nucleotide segments differs from ligand detection in that it is a chemical rather than an immunological reaction. Complementery DNA or RNA is used in place of the antibody and is labelled with ^{32}P. So far, DNA probes have been successfully employed in the diagnosis of inherited disorders, infectious diseases, and for identification of human oncogenes.

Recombinant techniques have already provided a number of pure human proteins in large quantities including insulin, growth hormone, lymphokines etc. Immunoassays, mostly RIA, have been critical in monitoring therapeutic blood transfusion.

The latest approach to the diagnosis of communicable and parasitic infections is based on the use of deoxyribonucleic acid (DNA) probes. The genetic information of all cells is encoded by DNA and the DNA probe approach to identification of pathogens is unique because the focus of the method is the nucleic acid content of the organism rather than the products that the nucleic acid encodes. Since every properly classified species has some unique nucleotide sequences that distinguish it from every other species, each organism's genetic composition is in essence a finger print that can be used for its identification. In addition to this specificity, DNA probes offer other advantages in that pathogens may be identified directly in clinical specimens; the method does not depend on the expression of an antigen, and organisms that may have undergone spontaneous mutation can still be identified

since single mutations rarely result in a major change in nucleic acid composition. In order to monitor the end result of the DNA probe test, a label is incorporated into the probe. The most common label is the radionuclide ^{32}Phosphorus, that is detected in the end product of the test either by autoradiography or with a scintillation counter.

Clinical problems both diagnostic and therapeutic are being solved using new approaches made possible by DNA techniques. These techniques are over a decade old, yet many clinicians and biomedical personnel are unaware of the potential impact of these techniques on their ability to diagnose diseases rapidly and accurately. During the next decade, these techniques are expected to become the backbone of diagnostic laboratories. Preliminary reports from laboratories already using commercially available DNA probe kits are encouraging. This Chapter tries to introduce these technology to the physicians, describe their potential for medical diagnosis and point out how relevant and practicable they are for the developing countries.

The DNA Molecule

The basis of molecular biology is deoxyribonucleic acid or DNA. DNA is a double-stranded helical molecule composed of pairs of nucleotide bases: adenine (A) guanine (G) thymine (T) and cytosine (C). Within a strand of DNA, the bases are linked by a sugar-phosphate backbone. The DNA molecule resembles a twisted ladder with the A and T and G and C linked together by hydrogen bonds. The two strands can be separated by heat (thermal denaturation) or by raising the pH or lowering the ionic strength of the DNA solution. The DNA molecule is most stable in its native double-stranded state. Thus when single-stranded DNA is placed in solution, under appropriate temperature and salt conditions, the complementary strands will recombine to form a duplex molecule **(Fig. 18.1)**

A region of DNA, which encodes a protein is termed a gene. The genetic information is encoded by a sequence of bases via a non-overlapping code in which three bases (a triplet) determine a particular amino acid. For a gene to be expressed, an enzyme, RNA polymerase II, copies or transcribes one strand of the DNA into mRNA (messanger RNA), which is then decoded or translated by the protein synthesis machinery in the cytoplasm. The mRNA comprises of a single-stranded polynucleotide chain with a sugar-phosphate backbone in which the order of bases is the complement of the transcribed DNA strand of the gene. In RNA, thymine (T) is replaced by a closely related base uracil (U) which will also pair with or hybridize to Adenine (A).

DNA probes, then, are pieces of nucleic acid, labeled in some fashion, that can seek out and bind to stretches of DNA or RNA that have complementary sequences (adenine opposite thymine (or uracil), cytosine opposite guanine. The two strands of nucleic acid must be in contact and have sufficient complementary base sequences so that a stable double-stranded molecule is formed. Complementary sequences of DNA can bind to RNA counterparts. Thus probes may also be labeled RNA strands directed towards DNA targets or labeled DNA sequences directed towards RNA targets.

Nucleic acid molecules will recombine only when the two strands are composed of complementary sequences. It is this property of nucleic acids that is the basis of the DNA or RNA probe reaction. The site of action is shown in **Fig. 18.2.**

The hybridization reaction consists of four components: the probe, the target (which is contained in the sample), the reporter molecule, and the hybridization method. The sample serves as a source of nucleic acid to be analysed and can consist of a suspension of an unknown organism, (for culture confirmation) or a clinical specimen such as sputum, or serum. The nucleic acid in the sample is referred to as the target DNA or RNA, and the radionuclide label on the probe, the reporter molecule.

Designing a Probe

All organisms contain some unique sequences of DNA or RNA within their genome that distinguish them from all other organisms. The key to developing a nucleic acid probe (i.e. either a DNA or a RNA probe) is to isolate these sequences, reproduce them in large quantities, and attach a reporter molecule to them so they can be incorporated into a hybridization reaction. Hybridization is a process whereby two single strands of nucleic acid come together to form a stable double-stranded molecule. As long as the sequences of bases along each stretch of nucleic acid are complementary, they will bind and stay together.

To produce the unique sequences, cloning vectors are used. The most commonly used cloning vectors are Plasmids. These plasmids are co-valently closed circular pieces of DNA that replicate independently of the bacterial chromosome. They are not required for cell replication but often give the host cell some advantage such as antimicrobial resistance, etc. Plasmids range in size from a few thousand base pairs (bp) to as large as 400 000 bp (400 kilobases or kb). One set of plasmids often used in recombinant DNA technology is called cloning vectors. Cloning vectors are small plasmids often just two to five kb that contain a selectable marker such as ampicillin resistance and a stretch of DNA that can be cleaved by many different restriction endonucleases. These are enzymes, found in bacteria, which cut DNA at specific sequences. For example, the enzyme EcoRI cuts the DNA chain between G and A in the sequence GAATTC. Each time a particular DNA is cleaved by an enzyme, precisely the same set of fragments is generated. Many such enzymes are now available.

Examples: pßr322, a 4.6 kb plasmid with ampicillin and tetracycline resistance; pUC18 and pU19 a pair of plasmids with ampicillin resistance, and multiple restriction sites and an indicator system utilizing a ß galactisidase gene where plasmids with foreign DNA inserts produce colourless colonies, while plasmids with no inserts produce blue colonies **(Figs. 18.3 and 18.4).**

The usual method of isolating and reproducing the unique sequence that will become the probe begins by cleaving that stretch of nucleotide bases away from the remaining nucleic

acid in the cell, using a set of enzymes known as restriction endonucleases. These enzymes provide the molecular scissors to cleave DNA at specific sequences of nucleotide bases. For instance, the enzyme Bam H1, obtained from Bacillus amyloliquefaciens, will cleave the DNA molecule between the Guanine - Guanine bases, Hae III, from Haemophilus aegyptius, between guanine and cytosine, and the ECORI, from Escherichia coli, between Guanine and adenine. Endonucleases are named by the first letter of the genus, the first 2 letters of the species, the strain and the number indicates whether it is the first, second etc. enzyme discovered in the organism. The single-stranded segments produced by restriction endonuclease cleavage are referred to as sticky ends because they can recombine with any other piece of DNA that has been cleaved with the same enzyme, regardless of the source of that DNA. Thus DNA from a virus such as herpes simplex (HSV) type 2 can be cleaved and inserted into a small plasmid from E. coli if the plasmid has been cleaved with the same enzyme. The sticky ends are then sealed with a second enzyme known as DNA ligase, to produce a double-stranded circular molecule and introduced into E. coli by transformation (addition of $CaCl_2$). The plasmid containing the DNA insert of HSV type 2 will replicate in E. coli making hundreds of copies. This process is referred to as cloning. In the simplest terms, cloning is the process of isolating a piece of DNA and placing it in a vector that allows hundreds of copies of that DNA sequence to be produced, when the plasmid replicates **(Figs. 18.5 and 18.6)**.

The plasmid now greatly amplified in copy number can be purified from the bacterial cell by centrifugation or filtration or by column filtration. The plasmid can be labeled directly by random primer method or the foreign DNA sequence isolated by restriction endonuclease digestion followed by centrifugation and the probe labelled by nick translation.

The unique sequence that will constitute a diagnostic probe need not be a whole gene nor need it be from within a sequence that actually encodes a protein.

Hybridization Reactions

After a double-stranded DNA molecule is denatured to single strands, it is capable of reassociating with either a DNA or a RNA strand of complementary sequence. The degree and specificity of binding depends on temperature, pH, use of a denaturant such as formamide, and salt concentration of the reaction buffer. Nucleic acid molecules can tolerate a certain number of mismatched base pairs (such as adenine molecules lining up opposite cytosine or guanine molecules opposing thymines) and still form stable duplexes as long as a significant number of base pairs do match and form bonds. However, the greater the degree of mismatched bases along the strands of nucleic acid, more likely that the two molecules would come apart. The degree of mismatch that can be tolerated in a hybridization reaction and still maintain a double-stranded molecule (and produce a positive hybridization signal) is referred to as the "stringency" of hybridization.

The concept of stringency is very important in understanding the specificity of DNA probe reactions. If the salt concentration or temperature of hybridization is altered, the specificity of the probe will change. The range of conditions that can be tolerated without

affecting the specificity of a probe vary depending on the length of the probe and the percentage of guanine and cytosine residues in the probe. The shorter the probe, the more narrow the range of temperature and salt concentration that can be tolerated.

Under conditions of high stringency, only exact matches of DNA will anneal and stay together. Under conditions of low stringency (i.e. reactions carried out at low temperature, in high salt concentration, or in low concentration of formamide) two DNA strands that are only 80 to 90% homologous may bind together and result in a positive hybridization signal. For example, under conditions of high stringency, a probe developed to Campylobacter jejuni would hybridize only to DNA from that organism while under conditions of low stringency the same probe would bind to DNA from Campylobacter coli and C. fetus but still not bind to DNA from E. coli or Shigella flexneri.

Formats for Hybridization Reactions.

Hybridization reactions can be performed in four formats: on a solid support (filter), in solution, in situ, or by using the Southern Blot hybridization procedure after gel electrophoresis. The majority of DNA probes reported in the literature have used the solid support format. However, both solution and solid support formats are utilized by the new commercially available probe kits.

To carry out the hybridization using the solid support format, the sample, which can be purified DNA, a suspension of a microorganism isolated in culture, or a clinical specimen, is either spotted directly on the filter or placed in a vacuum manifold which concentrates the specimen in a small area. The sample is lysed and the DNA denatured by the addition of NaOH or by steaming the filter above a beaker of ammonium acetate. Acid-fast organisms may require pretreatment with anti-microbial agents or other reagents to aid in disrupting their cell wells. Once denatured, the DNA is attached to the filter by baking it in a vacuum oven at 80°C for two hours. The filter is prehybridized with a non-homologous DNA such as salmon sperm DNA or calf thymus DNA, to prevent the non-specific binding of probe to the filter. After hybridization, the filters are washed at various temperatures determined by the stringency of the reaction. Although nitrocellulose filters are traditionally used for such assays, Whatman No. 541 paper and synthetic nylon filters are also frequently used **(Figs. 18.7 and 18.8)**.

The second format is to carry out the hybridization reaction in solution. In this format, both the target and the probe nucleic acid are free to move, maximizing the chance that complementary sequences will align and bind. Solution hybridizations go to completion 5 to ten fold faster than on solid supports. In several of the commercially available solution hybridization reactions samples are incubated at high temperature (72°C) in a sonicating water bath to disrupt the cells and cause them to release their nucleic acids. The addition of glass beads to the sample often aids in the disruption of the cell wall. After the hybridization step, the nascent duplexes are removed from solution by the addition of hydroxyapatite, which selectively binds duplex nucleic acid leaving single-stranded nucleic acids in solution. In some procedures the duplex DNA is removed from the hydroxyapatite by increasing the salt

concentration or by heating, while in other procedures the hydroxyapatite-bound doulbe-stranded nucleic acid is collected by centrifugation and the pellet is washed and quantitated by scintillation counting.

Polymerase chain reaction (PCR)

At times the amount of target nucleic acid present in a sample may be extremely small and thus beyond the limits of detection for conventional hybridization reactions. The recent development of the polymerase chain reaction is one of the most substantial technical advances in molecular genetics in the past decade. The procedure amplifies DNA by chemical proliferation in vitro of a predetermined stretch of DNA. It is possible to amplify specific DNA sequences, from as short as 50 base pairs to over 2000 base pairs in length, more than a million fold in only a few hours.

The PCR method was developed by Saikai and his co-workers at Cetus Corporation. It is based on the repetitive cycling of three simple reactions, the conditions of which vary only in the temperature of incubation. All three reactions occur in the same small tube and with temperature stable reagents. The repetitive cycle is therefore self contained and fully automated.

In addition to the target DNA to be amplified, the important reagents are two single stranded oligonucleotide (Primers), synthesized to be complementary to known sequences of the target DNA; larger amounts of the four deoxyribonucleoside triphosphates, and the heat stable Taq DNA polymerase, isolated from the thermophilic bacterium Thermus aquaticus.

The first step in the procedure is the heat denaturation of native (target) double-stranded DNA, which can be used virtually straight from any clinical, laboratory, or forensic specimen. The target DNA melts at high temperature (90 - 100°C) to liberate single-stranded DNA, which can subsequently reanneal to any other DNA that has complementary sequences. Recent experience suggests that amplification may begin in a sample containing only a single target molecule of DNA, making the PCR the most sensitive detection technique for specific DNA sequences.

In the second step of the cycle, performed at reduced temperatures, two short DNA primers are annealed to complementary sequences on opposite strands of the target DNA. These primers are chosen to encompass the desired genetic material; they define the two ends of the amplified stretch of DNA. The two primers must not anneal to each other and their sites of annealing must be sufficiently distant from each other to allow the subsequent synthesis of new products. The specificity of the PCR method is derived from the precision of this DNA-DNA annealing reaction.

The cycle's third step is the actual synthesis of a complementary second strand of new DNA, which occurs through the extension of each annealed primer by Taq polymerase in the presence of excess deoxyribonucleoside triphosphates. A new single strand of DNA is synthesized for each annealed primer. Each new strand consists of the primer at its 5' end

trailed by a string of linked nucleotides that are complementary to those of the corresponding template. An essential feature of the PCR procedure is that all previously synthesized products act as templates for new primer extension reaction (i.e. DNA synthesis) in each ensuing cycle. The result, aptly named "chain reaction" is the geometric amplification of new DNA products **(Fig. 18.9)**.

Since the primers form the kernals of all new DNA strands, each of the two different primers, as well as the four deoxyribonucleoside triphosphates must initially be present in massive amounts relative to the quantity of target DNA.

After extension of the primers, the cycle is repeated first by raising the temperature to convert double-stranded to single-stranded DNA, and then by lowering the temperature to allow the steps of annealing and extension.

The success of the procedure depends on knowing the sequence of the target DNA, at least at the site of primer annealing to allow the synthesis of appropriate complementary primers. Synthesizing the olignucleotide primers is itself an automated procedure and relatively inexpensive. If a RNA sequence is to be amplified a cDNA copy of it must be synthesized first using reverse transcriptase, before the PCR is begun.

A PCR reaction cycle typically takes five to seven minutes and is repeated 30 to 40 time to give a million copies.

Oligonucleotide Probes

Some of the currently available probe kits utilize very small stretches of nucleotides as probes. These short probes (oligonucleotide probes) are synthesized de novo in the laboratory on one of the several instruments designed for this purpose. Normally, only 14 to 40 base pair in length, these probes display exquisite specificity. Under stringent conditions they may be capable of detecting a change in a single base pair of a DNA or RNA sequence, which is enough to prevent binding of the probe to the targets.

Olinucleotide probes are very stable over time, and relatively simple to prepare. One batch of oligonucleotide probes is often enough to last in a laboratory for several years since only picogram amounts of probe are required for hybridization. Because of their small size and low complexity (base ratios) these short probes hybridize to target DNA at very rapid rates often with reaction times of less than 30 mins. This is in contrast to long probes that often require 4 to 16 hours.

The disadvantage of short probes is that each oligonucleotide can be labelled with only a single reporter molecule. Thus they are often 10 to 100 fold less sensitive than long probes.

Labelling Probes

Traditionally the most commonly used detection system is the ^{32}P label directly incorporated into the probe by nick translation. Other methods and other labels may also be used (Table I).

TABLE I: COMMON RADIOLABELS FOR DNA PROBES

Isotope	Emission Type	Energy	T½	dpm/mol (100% isotopic enrichment)
^{32}P	ß	1.71 MeV	14.3 d	2.02×10^{19}
^{125}I	γ	0.035 MeV	60 d	3.94×10^{18}
^{3}H	ß	0.0181 MeV	12.3 d	6.39×10^{16}
^{35}S	ß	0.167 MeV	87.4 d	3.33×10^{18}

Nick translation

Incorporation of labelled bases into long probes by nick translation involves nicking one of the two strands of double-stranded DNA with DNASE 1, and then excising stretches of the single strands at the nicks with the 5'-3' exonuclease activity of E. coli DNA polymerase I. In the process the polymerase enzyme also adds nucleotides to the 3' OH. The native bases are replaced with labelled bases in the reaction mixture as the nick moves along the DNA strand. When ^{32}P-labelled bases are incorporated, specific activities of 5 x 108 dpm/μg can be obtained with labelled strands 400 to 800 nucleotides in length. Probes synthesized with ^{3}H labelled bases have specific activities of 0.5 x 108 to 1.5 x 108 dpm/μg. Labelling by nick translation is a fairly simple reaction resulting in uniformally labelled probes with high specific activity (**Fig. 18.10**).

Random Primer Method.

This method is based on the hybridization of the DNA to be labelled with a mixture of all the possible hexanucleotides. The complementary strand is synthesized from the 3' end of the primer hexanucleotide by the Klenow fragment of E. coli DNA polymerase I. The unique sequence need not be separated from the plasmid. The plasmid DNA containing the unique sequence to be used as a probe is heated to separate into single-stranded DNA. The solution is then placed on ice and two to five μl of random primers are added. These primers will anneal to portions of the two single-stranded circular DNAs. Then 5 μl of ^{32}P dATP and unlabelled dCTP, dGTP and dTTP are added with DNA polymerase I. The primers thus extend and a new strand of DNA is produced incorporating the ^{32}P label. The unbound ^{32}P dATP is removed by passing through a spin column (a small sephadex bead column placed on top of a centrifuge tube and spun). The DNA comes through the ^{32}P dATP and other triphosphates remain on the column.

The labelled plasmid DNA is boiled shortly before the hybridization reaction and a drop placed on the spotted target DNA on nitrocellulose paper.

Detection Systems

Autoradiography

The extent of hybridization with radiolabelled probes on filters and in situ is monitored with autoradiographic methods. The procedure is sensitive with good resolution and does not destroy the hybridization support matrix. It lacks the exact quantitative ability of liquid scintiliation methods, but it can reveal artifacts not seen in counting.

The hybridized filter is exposed to X-ray film (Kodak XAR 5 or X-OMAT AR) in a light tight cassette. Exposures range from hours to days, depending on the level of radioactivity present. In general, an area of a dot 3 mm across with about 100 dpm of ^{32}P. The sensitivity of the method can be increased with the use of intensifying screens (Du Point Cronex Quanta III, Cronex Lightning Plus, or Fugi Mach II) by eight to ten fold with two screens.

The film, placed between the hybridized filter and screens, is exposed at -70°C in order to prolong the fluoresence emitted in response to a decay event. As little as 5dpm/7mm^2 area can be visualized overnight.

Scintillation Counting

If the hybridized support is sufficiently radioactive, the area of interest can be cut out and counted in a liquid scintillation counter.

Probes in a Diagnostic Laboratory

Current DNA probes require one to three hours to complete. This is still considered an advantage when compared to culture methods that may take four to six weeks. Probes enable the direct detection of pathogens in clinical specimens although often not the sensitivity of the pathogen to drugs. In certain cases, e.g. P. falciparum and S. mansoni, Neisseria gonorrhoeae sensitivity to pyrimethamine, oxaminiquine and pencillin can be determined by using probes designed for this.

Tests using probes when used in a batch format are relatively inexpensive, although use of probes for individual specimens can be expensive (**Table II**).

TABLE II. COMPARISON OF COSTS FOR 5 AND 50 SAMPLES IN THE FILTER HYBRIDIZATION ASSAY FOR CAMPYLOBACTER JEJUNI

Cost Factor		Cost per	
		5 samples	50 samples
DNA probe kit	($ 16)	3.20	0.32
Reagents	($ 5)	1.00	0.10
Labour	($ 19.50)	3.90	0.39
Total		8.10	0.81

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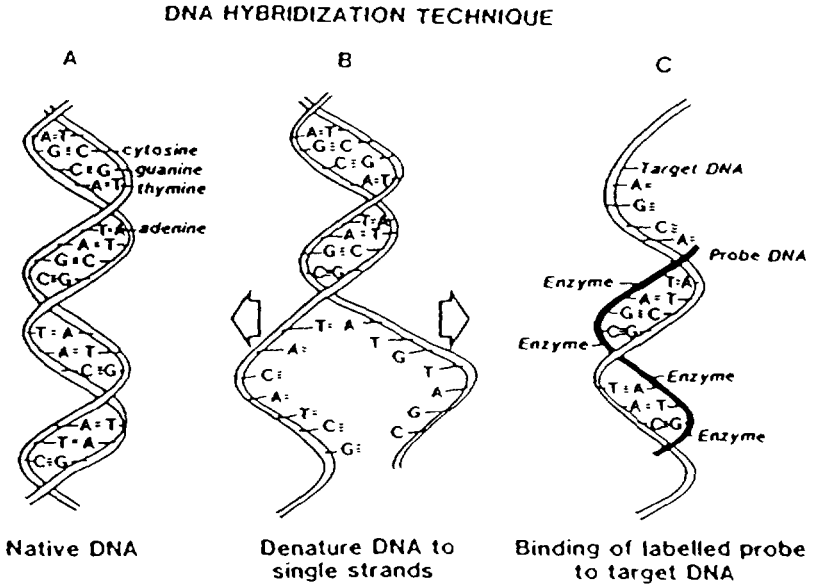

Fig. 18.1 Structure of deoxyribonucleic acid, effects of denaturation and binding of probe to target DNA [from Tenover, F.C., Clin. Microbiol. Rev. 1, 83 (1988)].

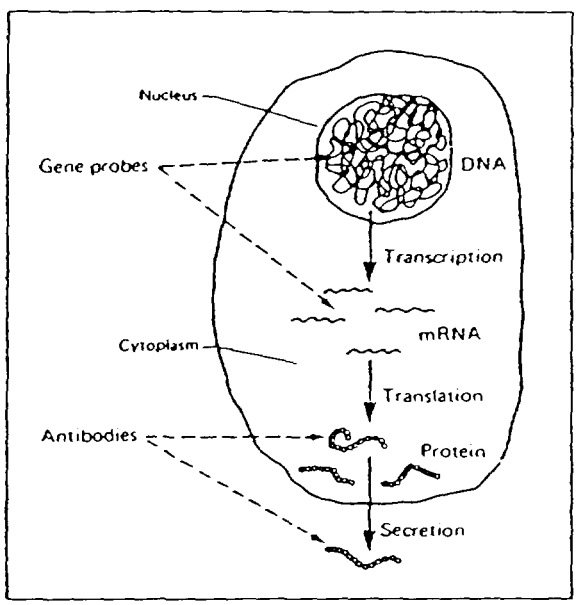

Fig. 18.2 Site of action of gene probes.

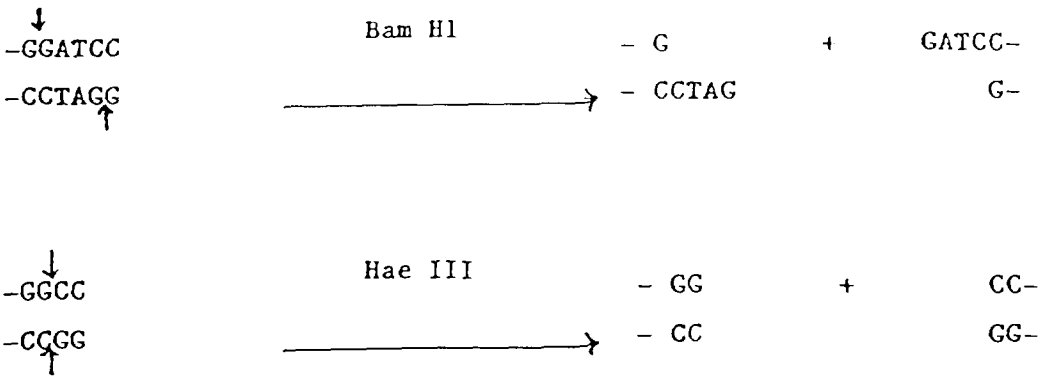

Fig. 18.3 Sequences cleaved by the restriction enzymes Bam H1 and Hae III.

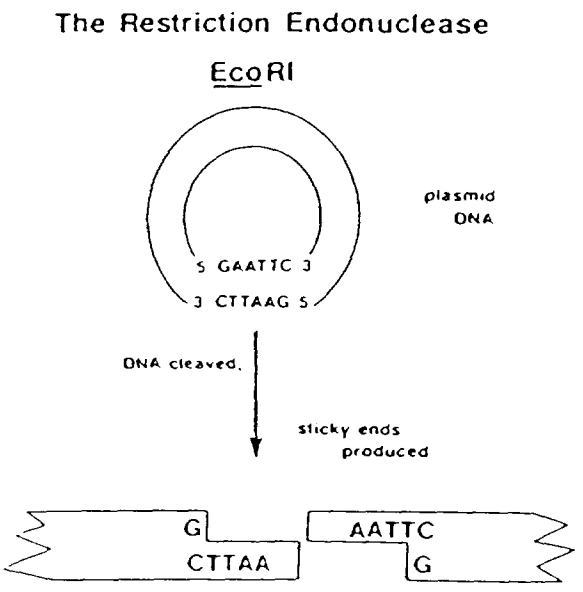

Fig. 18.4 The restriction endonuclease Eco RI. [From Tenover, F.C., Clin. Microbiol. Rev. 1, 83 (1988)].

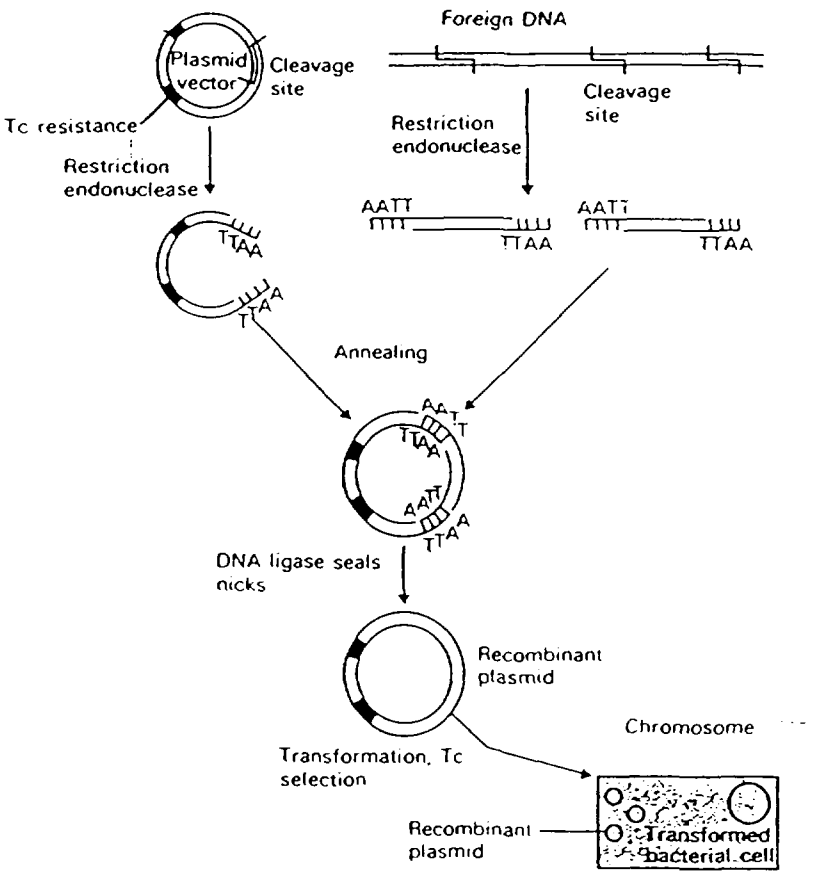

Fig. 18.5 Use of DNA ligase to create a covalent DNA recombinant joined through association of termini generated by Eco RI.

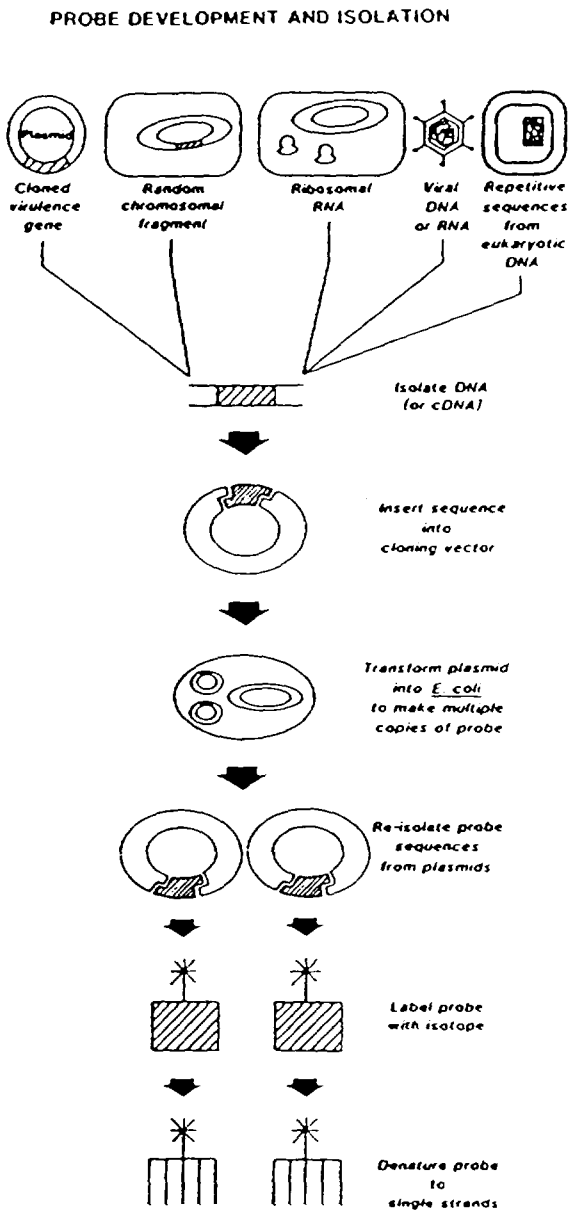

Fig. 18.6 The development of probes from diverse infectious agents. [From Tenover, F.C., Clin. Microbiol. Rev. 1, 83 (1988)].

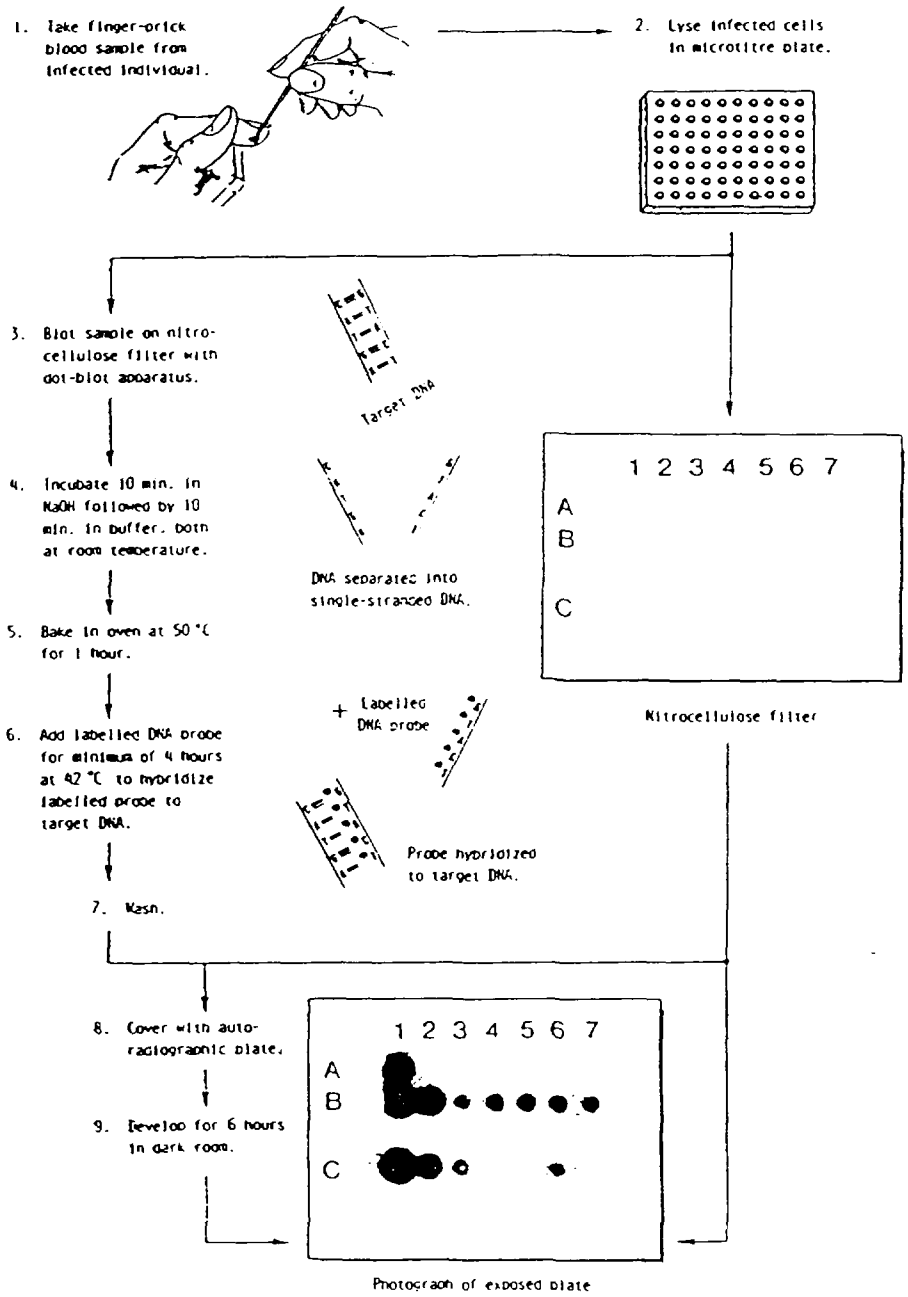

Fig. 18.7 Method used for filter hybridization of DNA probes to P. falciparum DNA.

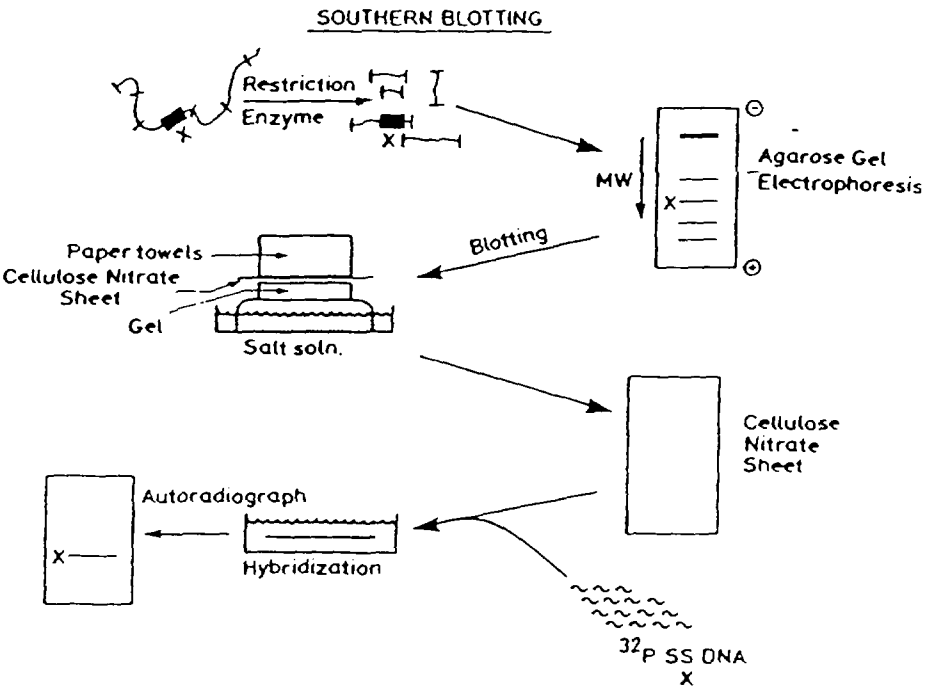

Fig. 18.8 Technique of southern hybridization.

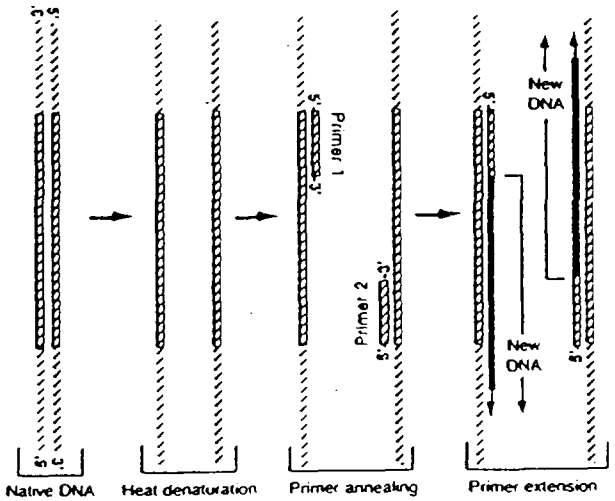

Fig. 18.9 First Round of the Polymerase Chain Reaction. The basic polymerase-chain-reaction cycle consists of three steps performed in the same closed container but at different temperatures. The elevated temperature in the first step melts the double-stranded DNA into single strands. As the temperature is lowered for the second step, the two oppositely directed oligonucleotide primers anneal to complementary sequences on the target DNA, which acts as a template. During the third step, also performed at a lower temperature, the Taq- polymerase enzymatically extends the primers covalently in the presence of excess deoxyribonucleoside triphosphates, the building blocks of new DNA synthesis. The native DNA target sequences, which will be massively amplified as "short products" in the ensuing cycles, are boxed. The vector of action of the DNA polymerase is denoted by the arrows projecting from the newly synthesized DNA, indicated by the dark bars.

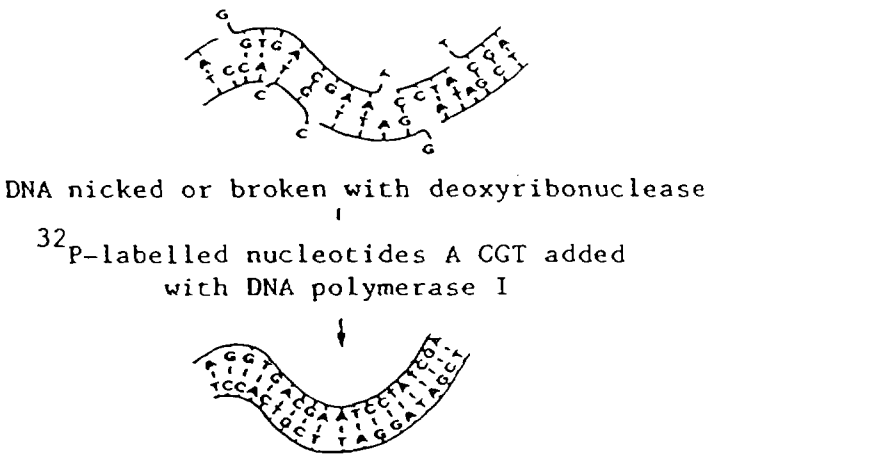

Fig. 18.10 Nick translation to make a DNA fragment sequence highly radioactive.