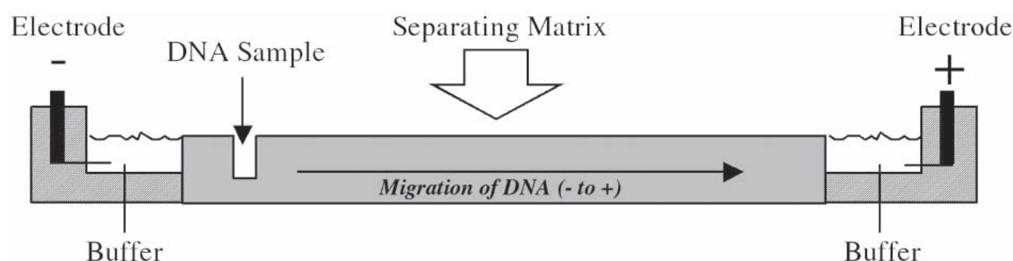

Fig. 1.3. Schematic illustration of a typical horizontal gel electrophoresis setup for the separation of nucleic acids.

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 base pairs (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 that 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 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 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, inclusions 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. 1.4**. 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 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

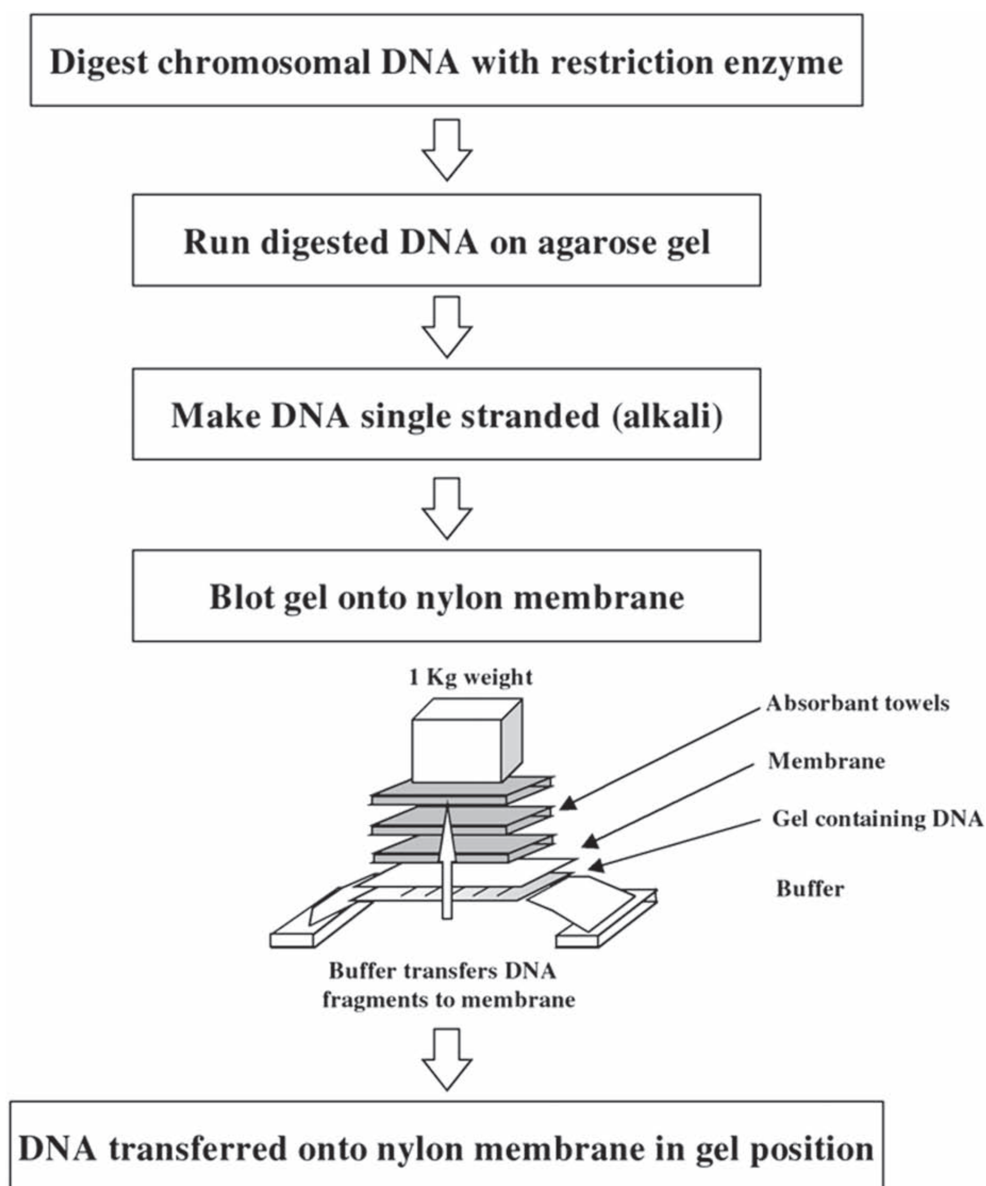

Fig. 1.4. The procedure involved in a typical Southern blot indicating the construction of a simple capillary transfer setup.

light in the presence or 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. 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 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 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, fluorescein, 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. 1.5**). 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}PadATP or biotin-labeled dNTP), is added to the 3' end of the DNA by the enzyme terminal transferase as indicated in **Fig. 1.6**. Although this is a simpler 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

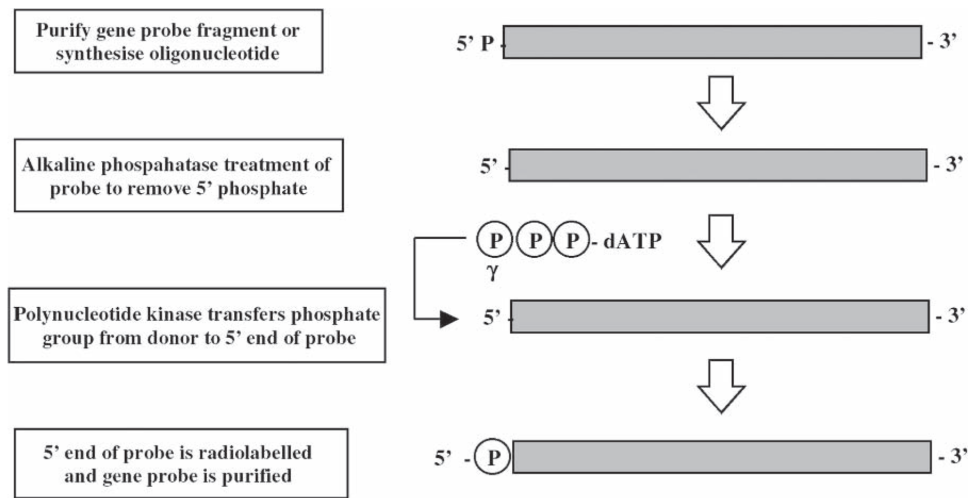

Fig. 1.5. End labeling of a gene probe at the 5' end with alkaline phosphatase and polynucleotide kinase.

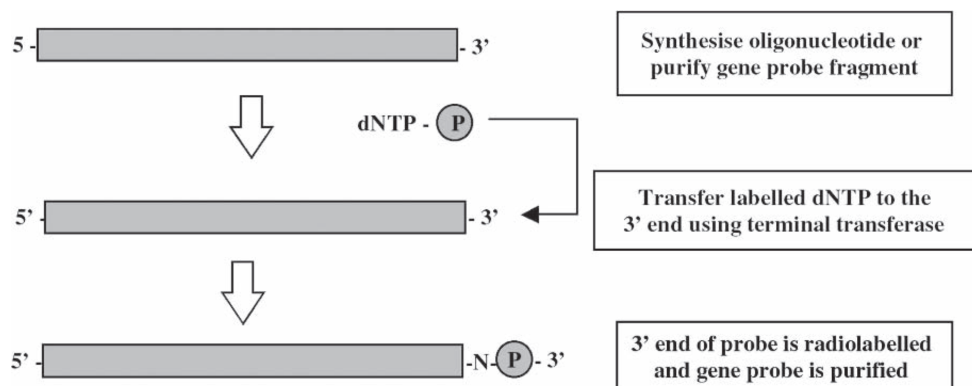

Fig. 1.6. End labeling of a gene probe at the 3' end using terminal transferase. Note that the addition of a labeled dNTP at the 3' end alters the sequence of the gene probe.

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. 1.7. 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 labeling, 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

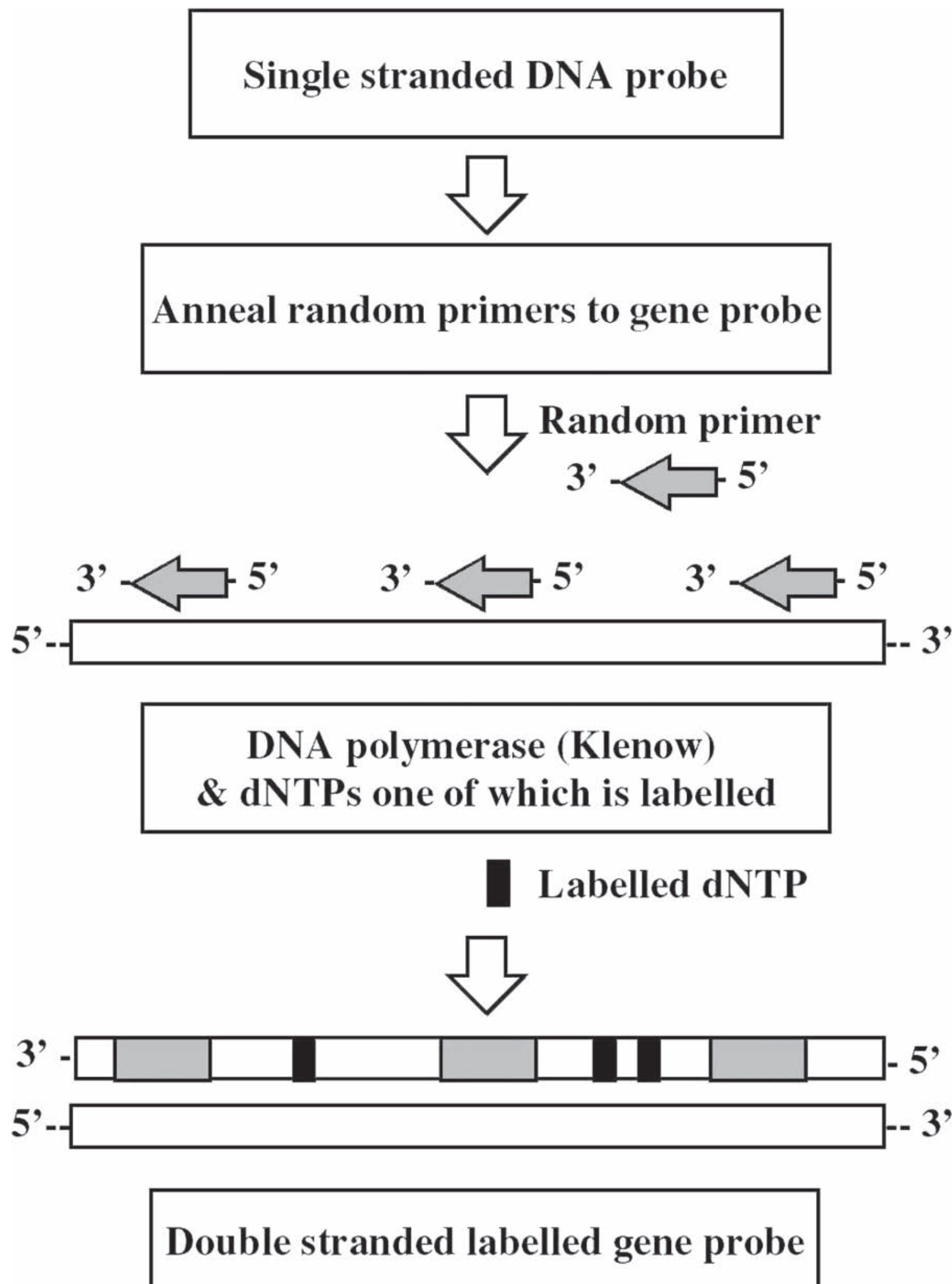

Fig. 1.7. Random primer gene probe labeling. Random primers are incorporated and used as a start point for Klenow DNA polymerase to synthesize a complementary strand of DNA while incorporating a labeled dNTP at complementary sites.

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. 1.8. In this way, the DNA can be labeled to a very high specific activity.

4. RNA Interference

Another of the important developments of recent times was the discovery of RNA interference (RNAi), which has been extensively used as a tool in the identification of the function of specific genes and the effect of gene silencing.