Basic Molecular Biology Techniques

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1– ENZYMES USED

The discovery and characterisation of a number of key enzymes have permitted the development of various techniques for the analysis and manipulation of DNA.
In particular, the enzymes termed type II restriction endonucleases have come to play a key role in all aspects of molecular biology. These enzymes **recognise specific DNA sequences, usually 4–6 base pairs (bp) in length**, and cleave them in a defined manner. **The sequences recognised are palindromic or of an inverted repeat nature**, that is, they read the same in both directions on each strand. When cleaved they leave a **flush-ended or staggered** (also termed a **cohesive-ended**) fragment depending on the particular enzyme used (Figure 1.1).
An important property of staggered ends is that those produced from different molecules by the same enzyme are complementary (or 'sticky') and so will anneal to each other (Table 1.1).

The annealed strands are held together only by hydrogen bonding between complementary bases on opposite strands. Covalent joining of ends on each of the two strands may be brought about by the **enzyme DNA ligase**. This is widely exploited in molecular biology to allow the construction of recombinant DNA, *i.e.* the joining of DNA fragments from different sources.

Approximately 500 restriction enzymes have been characterised that recognise over 100 different target sequences. **A number of these, termed isoschizomers, recognise different target sequences but produce the same staggered ends or overhangs.** A number of other enzymes have proved to be of value in the manipulation of DNA, as summarized in Table 1.2, and are indicated at appropriate points within the text.

2– ISOLATION AND SEPARATION OF NUCLEIC ACIDS

2.1 Isolation of DNA

The use of DNA for analysis or manipulation usually requires that it is isolated and purified to a certain extent.
DNA is recovered from cells by the gentlest possible method of cell rupture to prevent the DNA from fragmenting by mechanical shearing.
This is usually in the presence of EDTA which chelate the Mg^{2+} ions needed for enzymes that degrade DNA termed DNase.

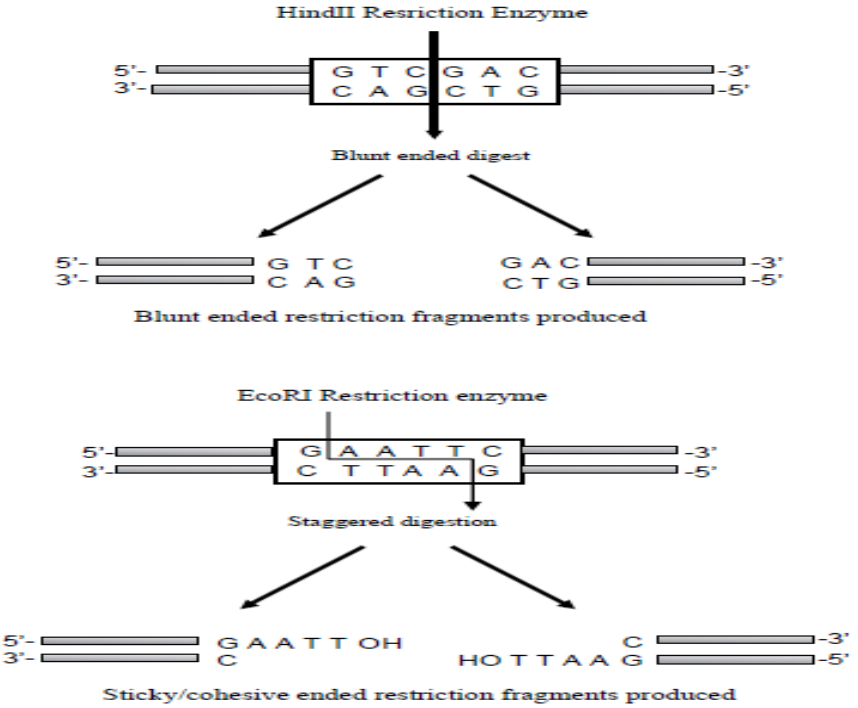

Figure 1.1 Examples of digestion of DNA by restriction endonucleases. The upper panel indicates the result of a restriction digestion forming blunt fragments with the enzyme *Hin*dIII. The bottom panel indicates the cohesive fragments produced by digestion with the enzyme *Eco*R1.

Table 1.1 Examples of restriction endonucleases that recognise different target sequences and the resulting fragments following digestion.

Name	Recognition Sequence	Digestion Products	
	Four Nucleotide Recognition Sequence		
*Hae*III	5′-GGCC-3′ 3′-CCGG-5′	5′-GG CC-3′ 3′-CC GG-5′	**Blunt End Digestion**
*Hpa*II	5′-CCGG-3′ 3′-GGCC-5′	5′-C CGG-3′ 3′-GGC C-5′	**Cohesive End Digestion**
	Six Nucleotide Recognition Sequence		
*Bam*HI	5′-GGATTC-3′ 3′-GGCC-5′	5′-G GATCC-3′ 3′-CCTAG G-5′	
*Eco*RI	5′-GAATTC-3′ 3′-CTTAAG-5′	5′-G AATCC-3′ 3′-CTTAA G-5′	
*Hin*dIII	5′-AAGCTT-3′ 3′-TTCGAA-5′	5′-A AGCTT-3′ 3′-TTCGA A-5′	
	Eight Nucleotide Recognition Sequence		
*Not*I	5′-GCGGCCGC-3′ 3′-CGCCGGCG-5′	5′-GC GGCCGC-3′ 3′-CGCCGG CG-5′	

Table 1.2 Comparison of the various labelling methods for DNA.

Labelling method	*Enzyme*	*Probe Type*	*Specific Activity*
5′ end labelling	Alkaline Phosphatase Polynucleotide Kinase	DNA	Low
3′ end labelling	Terminal Transferase	DNA	Low
Nick Translation	DNase I DNA Polymerase I	DNA	High
Random Hexamer	DNA Polymerase I	DNA	High
PCR	*Taq* DNA Polymerase	DNA	High
Riboprobes (cRNA)	RNA Polymerase	RNA	High

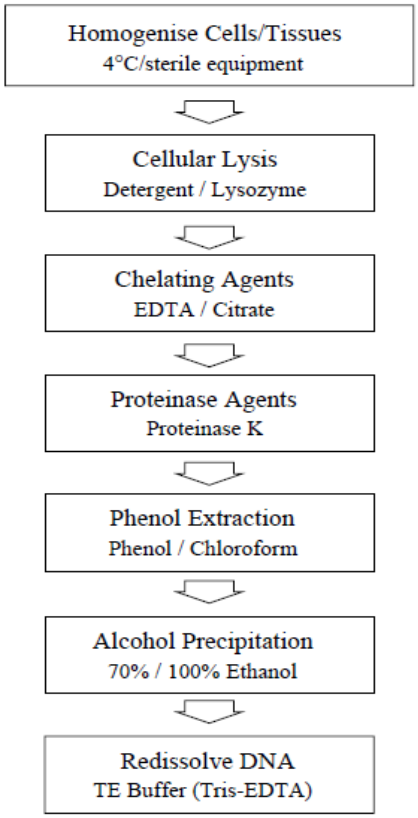

Figure 1.2 Flow diagram of the main steps involved in the extraction of DNA.

Ideally, cell walls, if present, should be digested enzymatically (e.g. lysozyme treatment of bacteria) and the cell membrane should be solubilized using detergent. 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 subsequent steps) should be performed at 4 ^{0}C, using glassware and solutions which have been autoclaved to destroy DNase activity (Figure 1.2).

After release of nucleic acids from the cells, RNA can be removed by treatment with ribonuclease (RNase) which 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 deproteinised repeatedly, until no more material is seen at the interface. Finally, the deproteinised DNA preparation is mixed with two volumes of absolute ethanol and the DNA 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 ^{0}C for at least 1 month. DNA solutions can be stored frozen, although repeated freezing and thawing tend to damage long DNA molecules by shearing.

The procedure described above 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, since 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 caesium chloride, which is particularly useful for the preparation of 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 $^{-1}$ of DNA and so

$$50 \times A_{260} = \text{concentration of DNA sample } (\mu g ml^{-1}) \qquad (1)$$

Contaminants may also be identified by scanning UV spectrophotometry from 200 to 300 nm. A ratio of 260 nm:280nm of approximately 1.8 indicates that the sample is free of protein contamination, which absorbs strongly at 280 nm.

2.2 Isolation of RNA

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 more vigorous. 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 denature immediately any RNases. Subsequent deproteinisation should be particularly rigorous, since RNA is often tightly associated with proteins.[3] DNase treatment can be used to remove DNA and RNA can be precipitated by ethanol. One reagent in which 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 analysing it by agarose gel electrophoresis. The most abundant RNA species are rRNA molecules, 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. At 260 nm, 1 absorbance unit equates to 40 μgml^{-1} of RNA and therefore

$$40 \times A_{260} = \text{concentration of RNA sample } (\mu gml^{-1}) \qquad (2)$$

Contaminants may also be identified in the same way as for DNA by scanning UV spectrophotometry; however, in the case of RNA a 260 nm:280nm ratio of approximately 2 would be expected for a sample containing no contamination. 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 with further high-salt solution. Finally, the bound mRNA can be eluted using a low concentration of salt. 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.

3 ELECTROPHORESIS OF NUCLEIC ACIDS

Electrophoresis in agarose or polyacrylamide gels is the most usual way to separate DNA molecules according to size (Figure 1.3). The technique can 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). The easiest and most widely applicable method is electrophoresis in horizontal agarose gels, followed by staining with ethidium bromide. This dye binds to DNA by insertion between stacked base pairs (intercalation) and it exhibits a strong orange/red fluorescence when illuminated with ultraviolet light. Very often electrophoresis is used to check the purity and intactness of a DNA preparation or to assess the extent of a enzymatic reaction during, for example, the steps involved in the cloning of DNA. For such checks ‘mini-gels’ are particularly convenient, since they need little preparation, use small samples and give results quickly. Agarose gels can be used to separate molecules larger than about 100 bp. For higher resolution or for the effective separation of shorter DNA molecules, polyacrylamide gels are the preferred method.[4]

When electrophoresis is used preparatively, the piece of gel containing the desired DNA fragment is physically removed with a scalpel. The DNA may be 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 agarose thus leaving the DNA, or by the process of electroelution. In the latter 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 electric 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.

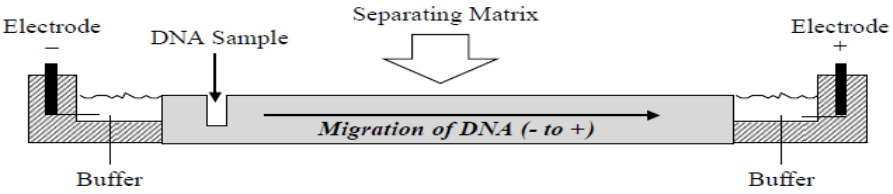

Figure 1.3 A typical setup required for agarose gel electrophoresis of DNA. The upper panel indicates a cross-section of the unit used for gel electrophoresis.

More commonly, commercial spin columns can be used which contain an isolating matrix used in conjunction with a benchtop microcentrifuge. The use of such standardized 'kits' in molecular biology is now commonplace. An alternative to conventional analysis of nucleic acids by electrophoresis is through the use of microfluidic systems. These are carefully manufactured chip-based units where microliter volumes may be used and with the aid of computer analysis provide much of the data necessary for analysis. Their advantage lies in the fact that the sample volume is very small, allowing much of an extract to be used for further analysis.[5]

4– RESTRICTION MAPPING OF DNA FRAGMENTS

Restriction mapping involves the size analysis of restriction fragments produced by several restriction enzymes individually and in combination.[6] The principle of this mapping is illustrated, in which the restriction sites of two enzymes, A and B, are being mapped. Cleavage with A gives fragments 2 and 7 kilobases (kb) from a 9 kb molecule, hence we can position the single A site 2 kb from one end. Similarly, B gives fragments 3 and 6 kb, so it has a single site 3 kb from one end; but it is not possible at this stage to say if it is near to A's site or at the opposite end of the DNA. This can be resolved by a double digestion. If the resultant fragments are 2, 3 and 4 kb, then A and B cut at opposite ends of the molecule; if they are 1, 2 and 6 kb, the sites are near each other. Not surprisingly, the mapping of real molecules is rarely as simple as this and computer analysis of the restriction fragment lengths is usually needed to construct a map (Figure 1.4).

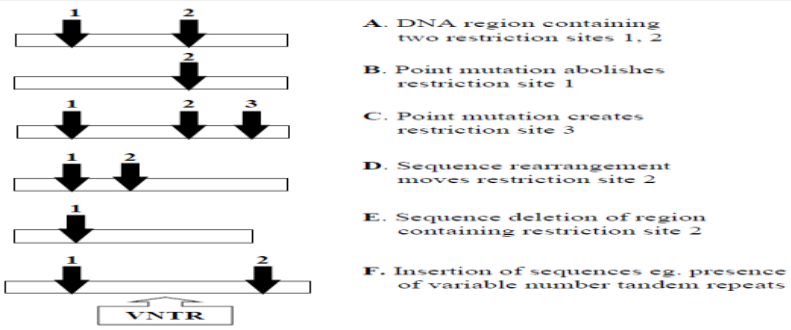

Figure 1.4 Restriction fragment length polymorphisms (RFLP). The schematic panels A–F indicate the various fragments obtained following digestion as a result of differences in the position of restriction endonuclease target sequences.

5– NUCLEIC ACID ANALYSIS METHODS

There are numerous methods for analysing DNA and RNA; however, many of them are solution based or more recently include the use of chip-based array systems. Indeed, the lab-on-a-chip approach is developing rapidly and it is possible to envisage many detection and analysis methods being developed in this format in the future.[7] However, traditional methods are still employed in many laboratories and much is still made of producing a hard copy of digested and separated single stranded DNA fragments attached to a matrix such as nylon for analysis with an appropriate labelled probe.

5.1– DNA Blotting

Electrophoresis of DNA restriction fragments allows separation based on size to be carried out; however, it provides no indication as to the presence of a specific, desired fragment among the complex sample (Figure 1.5). This can be achieved by transferring the DNA from the intact gel on to a piece of nitrocellulose or nylon membrane placed in contact with it.[8] This provides a more permanent record of the sample since 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. This transfer, named a Southern blot after its inventor Ed Southern, can be performed electrophoretically or by drawing large volumes of buffer through both gel and membrane, thus transferring DNA from one to the other by capillary action. The point of this operation is that the membrane can now be treated with a labelled DNA molecule, for example a gene probe. This single-stranded DNA probe will hybridise under the right conditions to complementary fragments immobilized on the membrane. The conditions of hybridisation, including the temperature and salt concentration, are critical for this process to take place effectively. This is usually referred to as the stringency of the hybridisation and it is particular for each individual gene probe and for each sample of DNA. A series of washing steps with buffer are then carried out to remove any unbound probe and the membrane is developed, after which the precise location of the probe and its target may be visualised. It is also possible to analyse 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 **zoo blot** or **garden blot** is a type of Southern blot that demonstrates the similarity between specific, usually protein-coding, DNA sequences of different species. A zoo blot compares animal species while a garden blot compares plant species. The purpose of the zoo blot is to detect the conservation of the gene(s) of interest throughout the evolution of different species).

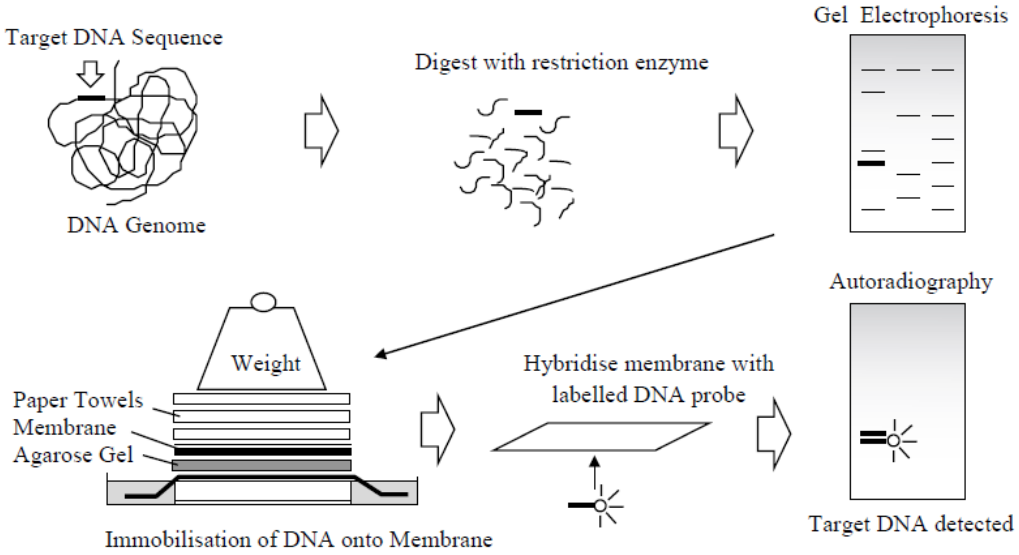

Figure 1.5 The steps involved in the production of a Southern blot and the subsequent detection of a specific DNA sequence following hybridisation with a complementary labelled gene probe.

5.2– RNA Blotting

The same basic process of nucleic acid blotting can be used to transfer RNA from gels on to similar membranes. This allows the identification of specific mRNA sequences of a defined length by hybridization to a labelled gene probe and is known as Northern blotting.[9] With this technique it is not only possible to detect specific mRNA molecules but it may also be used to quantify the relative amounts of the specific mRNA. It is usual to separate the mRNA transcripts by gel electrophoresis under denaturing conditions since 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.

A further method of RNA analysis that overcomes the problems of RNA blotting is termed the ribonuclease protection assay. Here the RNA from a sample is extracted and then mixed with a probe representing the sequence of interest in solution. The probe and the appropriate RNA fragment hybridize to form a double-stranded sequence. RNase is then added, which cleaves any single-stranded RNA present but leaves the double-stranded RNA intact. The intact RNA can then be separated by electrophoresis and an indication of the size of the fragment generated. The efficient removal of the background of RNA and the improved sensitivity make the ribonuclease protection assay a popular choice for the analysis of specific RNA molecules.

An important step in the field of RNA analysis was the development of RNAi (RNA interference), which inhibits gene expression. Here double-stranded DNA promotes the degradation of mRNA. Double-stranded RNA in the cell is cleaved by

a dicer enzyme, resulting in the formation of small 21–25 bp interfering RNAs (siRNA). The siRNA are complementary to a target RNA strand. Small RNAi proteins are guided by the siRNA to the appropriate mRNA, where the target is then cleaved and is unable to be translated. Many areas are now benefiting from the adoption of this technique in the molecular biology and biotechnology fields.