### SBC G852: INTRODUCTION TO MOLECULAR BIOLOGY FOR PROGRAMMERS

**Molecular biology techniques**.

PCR, Gel Electrophoresis (agarose gel and PAGE [native and denatured]), Recombinant DNA technology – Kivumbi Mark and Manase Aloo

**THE POLYMERASE CHAIN REACTION (PCR)**

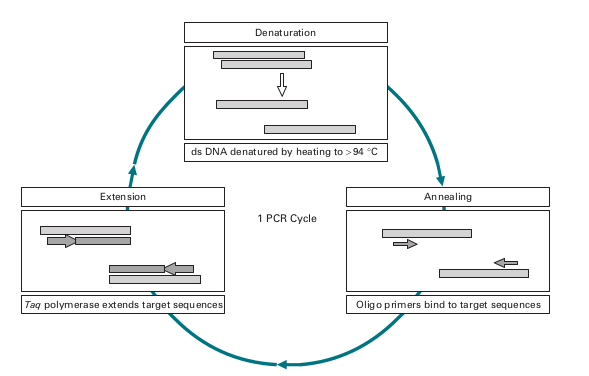
**Basic concept of the PCR**

The polymerase chain reaction or PCR is one of the mainstays of molecular biology. The PCR is used to amplify a precise fragment of DNA from a complex mixture of starting material usually termed the template DNA and in many cases requires little DNA puriﬁcation. It does require the knowledge of some DNA sequence information which ﬂanks the fragment of DNA to be ampliﬁed. From this information two oligonucleotide primers may be chemically synthesised each complementary to a stretch of DNA to the 3 0 side of the target DNA, one oligonucleotide for each of the two DNA strands. It may be thought of as a technique analogous to the DNA replication process that takes place in cells since the outcome is the same: the generation of new complementary DNA stretches based upon the existing ones. It is also a technique that has replaced, in many cases, the traditional DNA cloning methods since it fulﬁls the same function, the production of large amounts of DNA from limited starting material; however, this is achieved in a fraction of the time needed to clone a DNA fragment.

**Stages in the PCR**

The PCR consists of three deﬁned sets of times and temperatures termed steps: (i) denaturation, (ii) annealing and (iii) extension. Each of these steps is repeated 30–40 times, termed cycles. In the ﬁrst cycle the double-stranded template DNA is (i) denatured by heating the reaction to above 90 C. Within the complex DNA the region to be speciﬁcally ampliﬁed (target) is made accessible. The temperature is then cooled to 40–60 C. The precise temperature is critical and each PCR system has to be deﬁned and optimised. One useful technique for optimisation is touchdown PCR where a programmable cycler is used to incrementally decrease the annealing temperature until the optimum is derived. Reactions that are not optimised may give rise to other DNA products in addition to the speciﬁc target or may not produce any ampliﬁed products at all. The annealing step allows the hybridisation of the two oligonucleotide primers, which are present in excess, to bind to their complementary sites that ﬂank the target DNA. The annealed oligonucleotides act as primers for DNA synthesis, since they provide a free 3 0 hydroxyl group for DNA polymerase. The DNA synthesis step is termed extension and is carried out by a thermostable DNA polymerase, most commonly Taq DNA polymerase.

DNA synthesis proceeds from both of the primers until the new strands have been extended along and beyond the target DNA to be ampliﬁed. It is important to note that, since the new strands extend beyond the target DNA, they will contain a region near their 3 0 ends that is complementary to the other primer. Thus, if another round of DNA synthesis is allowed to take place, not only the original strands will be used as templates but also the new strands. Most interestingly, the products obtained from the new strands will have a precise length, delimited exactly by the two regions complementary to the primers. As the system is taken through successive cycles of denaturation, annealing and extension all the new strands will act as templates and so there will be an exponential increase in the amount of DNA produced. The net effect is to selectively amplify the target DNA and the primer regions ﬂanking it. One problem with early PCR reactions was that the temperature needed to denature the DNA also denatured the DNA polymerase. However the availability of a thermostable DNA polymerase enzyme isolated from the thermophilic bacterium Thermus aquaticus found in hot springs provided the means to automate the reaction. Taq DNA polymerase has a temperature optimum of 72 C and survives prolonged exposure to temperatures as high as 96 C and so is still active after each of the denaturation steps. The widespread utility of the technique is also due to the ability to automate the reaction and as such many thermal cyclers have been produced in which it is possible to program in the temperatures and times for a particular PCR reaction.



**PCR primer design and bioinformatics**

The speciﬁcity of the PCR lies in the design of the two oligonucleotide primers. These have to not only be complementary to sequences ﬂanking the target DNA but also must not be self-complementary or bind each other to form dimers since both prevent DNA ampliﬁcation. They also have to be matched in their GC content and have similar annealing temperatures. The increasing use of bioinformatics resources such as Oligo, Generunner and Geneﬁsher in the design of primers makes the design and the selection of reaction conditions much more straightforward. These resources allow the sequences to be ampliﬁed, primer length, product size, GC content, etc. to be input and, following analysis, provide a choice of matched primer sequences. Indeed the initial selection and design of primers without the aid of bioinformatics would now be unnecessarily time-consuming. It is also possible to design primers with additional sequences at their 5 0 end such as restriction endonuclease target sites or promoter sequences. However modiﬁcations such as these require that the annealing conditions be altered to compensate for the areas of non-homology in the primers. A number of PCR methods have been developed where either one of the primers or both are random. This gives rise to arbitrary priming in genomic templates but interestingly may give rise to discrete banding patterns when analysed by gel electrophoresis. In many cases this technique

may be used reproducibly to identify a particular organism or species. This is sometimes referred to as random ampliﬁed polymorphic DNA (RAPD) and has been used successfully in the detection and differentiation of a number of pathogenic strains of bacteria. In addition primers can now be synthesised with a variety of labels such as ﬂuorophores bound to them allowing easier detection and quantitation using techniques such as qPCR.

**PCR ampliﬁcation templates**

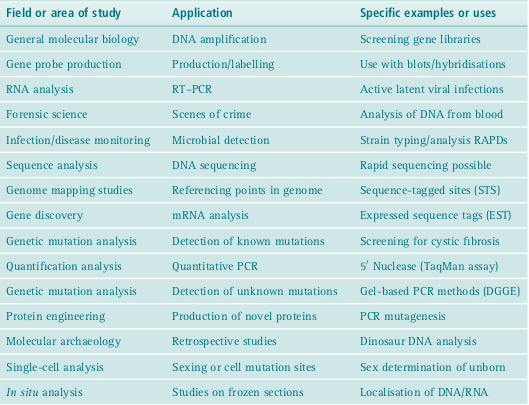
DNA from a variety of sources may be used as the initial source of ampliﬁcation templates. It is also a highly sensitive technique and requires only one or two molecules for successful ampliﬁcation. Unlike many manipulation methods used in current molecular biology the PCR technique is sensitive enough to require very little template preparation. The extraction from many prokaryotic and eukaryotic cells may involve a simple boiling step. Indeed the components of many extraction techniques such as SDS and proteinase K may adversely affect the PCR. The PCR may also be used to amplify RNA, a process termed RT–PCR (reverse transcriptase–PCR). Initially a reverse transcription reaction which converts the RNA to cDNA is carried out. This reaction normally involves the use of the enzyme reverse transcriptase although some thermostable DNA polymerases used in the PCR such as Tth have a reverse transcriptase activity under certain buffer conditions. This allows mRNA transcription products to be effectively analysed. It may also be used to differentiate latent viruses (detected by standard PCR) or active viruses which replicate and thus produce transcription products and are thus detectable by RT–PCR. In addition the PCR may be extended to determine relative amounts of a transcription product.

**Sensitivity of the PCR**

The enormous sensitivity of the PCR system is also one of its main drawbacks since the very large degree of ampliﬁcation makes the system vulnerable to contamination. Even a trace of foreign DNA, such as that even contained in dust particles, may be ampliﬁed to signiﬁcant levels and may give misleading results. Hence cleanliness is paramount when carrying out PCR, and dedicated equipment and in some cases dedicated laboratories are used. It is possible that ampliﬁed products may also contaminate the PCR although this may be overcome by UV irradiation to damage already ampliﬁed products so that they cannot be used as templates. A further interesting solution is to incorporate uracil into the PCR and then treat the products with the enzyme uracil N-glycosylase (UNG) which degrades any PCR amplicons with incorporated uracil rendering them useless as templates. In addition most PCRs are now undertaken using hotstart. Here the reaction mixture is physically separated from the template or the enzyme: when the reaction begins mixing occurs and thus avoids any mispriming that may have arisen.

**Applications of the PCR**

Many traditional methods in molecular biology have now been superseded by the PCR and the applications for the technique appear to be unlimited. The success of the PCR process has given impetus to the development of other ampliﬁcation techniques that are based on either thermal cycling or non-thermal cycling (isothermal) methods. The most popular alternative to the PCR is termed the ligase chain reaction or LCR. This operates in a similar fashion to the PCR but a thermostable DNA ligase joins sets of primers together which are complementary to the target DNA. Following this a similar exponential ampliﬁcation reaction takes place producing amounts of DNA that are similar to the PCR.



**Quantitative PCR (qPCR)**

One of the most useful PCR applications is quantitative PCR or qPCR. This allows the PCR to be used as a means of identifying the initial concentrations of DNA or cDNA template used. Early qPCR methods involved the comparison of a standard or control DNA template ampliﬁed with separate primers at the same time as the speciﬁc target DNA. However these types of quantitation rely on the fact that all the reactions are identical and so any factors affecting this may also affect the result. The introduction of thermal cyclers that incorporate the ability to detect the accumulation of DNA through ﬂuorescent dyes binding to the DNA has rapidly transformed this area. In its simplist form a PCR is set up that includes a DNA-binding cyanine dye such as SYBR green. This dye binds to the major groove of double-stranded DNA but not single-stranded DNA and so as amplicons accumulate during the PCR process SYBR green binds the double-stranded DNA proportionally and ﬂuorescence emission of the dye can be detected following excitation. Thus the accumulation of DNA amplicons can be followed in real time during the reaction run. In order to quantitate unknown DNA templates a standard dilution is prepared using DNA of known concentration. As the DNA accumulates during the early exponential phase of the reaction an arbitrary point is taken where each of the dilluted DNA samples cross. This is termed the crossing threshold on Ct value. From the various Ct values a log graph is prepared from which an unknown concentration can be deduced. Since SYBR green and similar DNA-binding dyes are non-speciﬁc, in order to determine if a correctly sized PCR product is present most qPCR cyclers have a built-in melting curve function. This gradually increases the temperature of each tube until the double-stranded PCR product denatures or melts and allows a precise although not deﬁnitive determination of the product. Conﬁrmation of the product is usually obtained by DNA sequencing.

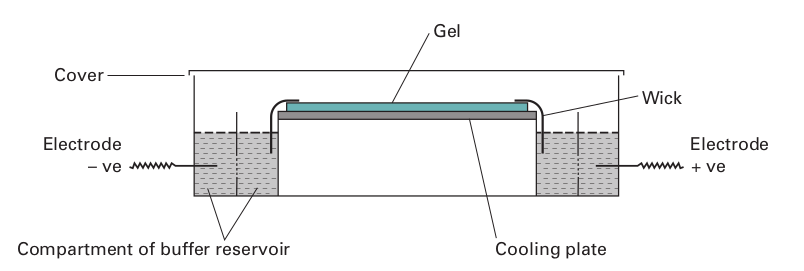
**The TaqMan system**

In order to make qPCR speciﬁc a number of strategies may be employed that rely on speciﬁc hybridisation probes. One ingenious method is called the TaqMan assay or 5 0 nuclease assay. Here the probe consists of an oligonucleotide labelled with a ﬂuorescent reporter at one end of the molecule and quencher at the other end. The PCR proceeds as normal and the oligonucleotide probe binds to the target sequence in the annealing step. As the Taq polymerase extends from the primer its 5 0 exonuclease activity degrades the hybridisation probe and releases the reporter from the quencher. A signal is thus generated which increases in direct proportion to the number of starting molecules and ﬂuorescence can be detected in real time as the PCR proceeds. Although relatively expensive in comparison to other methods for determining expression levels it is simple, rapid and reliable and now in use in many research and clinical areas. Further developments in probe-based PCR systems have also been used and include scorpion probe systems, ampliﬂuor and real-time LUX probes

**Gel Electrophoresis**

The term electrophoresis describes the migration of a charged particle under the inﬂuence of an electric ﬁeld. Many important biological molecules, such as amino acids, peptides, proteins, nucleotides and nucleic acids, possess ionisable groups and, therefore, at any given pH, exist in solution as electrically charged species either as cations (+) or anions (-). Under the inﬂuence of an electric ﬁeld these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The equipment required for electrophoresis consists basically of two items, a power pack and an electrophoresis unit. Electrophoresis units are available for running either vertical or horizontal gel systems. Vertical slab gel units are commercially available and routinely used to separate proteins in acrylamide gels. The gel is formed between two glass plates that are clamped together but held apart by plastic spacers. The most commonly used units are the so-called minigel apparatus. Gel dimensions are typically 8.5 cm wide # 5 cm high, with a thickness of 0.5#1 mm. A plastic comb is placed in the gel solution and is removed after polymerisation to provide loading wells for up to 10 samples. When the apparatus is assembled, the lower electrophoresis tank buffer surrounds the gel plates and affords some cooling of the gel plates. A typical horizontal gel system is shown in. The gel is cast on a glass or plastic sheet and placed on a cooling plate which is an insulated surface through which cooling water is passed to conduct away generated heat. Connection between the gel and electrode buffer is made using a thick wad of wetted ﬁlter paper note, however, that agarose gels for DNA electrophoresis are run submerged in the buffer. The power pack supplies a direct current between the electrodes in the electrophoresis unit. All electrophoresis is carried out in an appropriate buffer, which is essential to maintain a constant state of ionisation of the molecules being separated. Any variation in pH would alter the overall charge and hence the mobilities of the molecules being separated.



**Agarose gels**

Agarose is a linear polysaccharide made up of the basic repeat unit agarobiose, which comprises alternating units of galactose and 3,6-anhydrogalactose. Agarose is one of the components of agar that is a mixture of polysaccharides isolated from certain seaweeds. Agarose is usually used at concentrations of between 1% and 3%. Agarose gels are formed by suspending dry agarose in aqueous buffer, then boiling the mixture until a clear solution forms. This is poured and allowed to cool to room temperature to form a rigid gel. The gelling properties are attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains. This cross-linked structure gives the gel good anticonvectional properties. The pore size in the gel is controlled by the initial concentration of agarose; large pore sizes are formed from low concentrations and smaller pore sizes are formed from the higher concentrations. Although essentially free from charge, substitution of the alternating sugar residues with carboxyl, methyoxyl, pyruvate and especially sulphate groups occurs to varying degrees. This substitution can result in electro-endosmosis during electrophoresis and ionic interactions between the gel and sample in all uses, both unwanted effects.

Agarose is therefore sold in different purity grades, based on the sulphate concen- tration – the lower the sulphate content, the higher the purity. Agarose gels are used for the electrophoresis of both proteins and nucleic acids. For proteins, the pore sizes of a 1% agarose gel are large relative to the sizes of proteins. Agarose gels are therefore used in techniques such as ﬂat-bed isoelectric focussing, where the proteins are required to move unhindered in the gel matrix according to their native charge. Such large pore gels are also used to separate much larger molecules such as DNA or RNA, because the pore sizes in the gel are still large enough for DNA or RNA molecules to pass through the gel. Now, however, the pore size and molecule size are more comparable and fractional effects begin to play a role in the separation of these molecules. A further advantage of using agarose is the availability of low melting temperature agarose (62#65 # C). As the name suggests, these gels can be reliquiﬁed by heating to 65 # C and thus, for example, DNA samples separated in a gel can be cut out of the gel, returned to solution and recovered.

Owing to the poor elasticity of agarose gels and the consequent problems of removing them from small tubes, the gel rod system sometimes used for acrylamide gels is not used. Horizontal slab gels are invariably used for isoelectric focussing or immunoelectrophoresis in agarose. Horizontal gels are also used routinely for DNA and RNA gels, although vertical systems have been used by some workers.

**Polyacrylamide gels**

Electrophoresis in acrylamide gels is frequently referred to as PAGE, being an abbreviation for polyacrylamide gel electrophoresis. Cross-linked polyacrylamide gels are formed from the polymerisation of acrylamide monomer in the presence of smaller amounts of N,N 0 -methylene-bisacrylamide (normally referred to as ‘bis’-acrylamide). Note that bisacrylamide is essentially two acrylamide molecules linked by a methylene group, and is used as a cross-linking agent. Acrylamide monomer is polymerised in a head-to-tail fashion into long chains and occasionally a bis-acrylamide molecule is built into the growing chain, thus introducing a second site for chain extension. Proceeding in this way a cross-linked matrix of fairly well-deﬁned structure is formed. The polymerisation of acrylamide is an example of free-radical catalysis, and is initiated by the addition of ammonium persulphate and the base N,N,N 0 ,N 0 -tetramethylenediamine (TEMED).

Free radicals are highly reactive species due to the presence of an unpaired electron that needs to be paired with another electron to stabilise the molecule. R therefore reacts with M, forming a single bond by sharing its unpaired electron with one from the outer shell of the monomer molecule. This therefore produces a new free radical molecule R # M , which is equally reactive and will attack a further monomer molecule. In this way long chains of acrylamide are built up, being cross-linked by the introduction of the occasional bis-acrylamide molecule into the growing chain. Oxygen mops up free radicals and therefore all gel solutions are normally degassed (the solutions are brieﬂy placed under vacuum to remove loosely dissolved air) prior to use. The degassing of the gel solution also serves a second purpose. The polymerisation of acrylamide is an exothermic reaction (i.e. heat is liberated) and the warming up of the gel solution as it sets can liberate air bubbles that become trapped in the polymerised gel. The degassing step prevents this possibility. Photopolymerisation is an alternative method that can be used to polymerise acrylamide gels. The ammonium persulphate and TEMED are replaced by riboﬂavin and when the gel is poured it is placed in front of a bright light for 2#3 h. Photodecomposition of riboﬂavin generates a free radical that initiates polymerisation. Acrylamide gels are deﬁned in terms of the total percentage of acrylamide present, and the pore size in the gel can be varied by changing the concentrations of both the acrylamide and bis-acrylamide. Acrylamide gels can be made with a content of between 3% and 30% acrylamide. Thus low percentage gels (e.g. 4%) have large pore sizes and are used, for example, in the electrophoresis of proteins, where free movement of the proteins by electrophoresis is required without any noticeable frictional effect, for example in ﬂat-bed isoelectric focusing or the stacking gel system of an SDS–polyacrylamide gel. Low percentage acrylamide gels are also used to separate DNA. Gels of between 10% and 20% acrylamide are used in techniques such as SDS–gel electrophoresis, where the smaller pore size now introduces a sieving effect that contributes to the separation of proteins according to their size.

Proteins were originally separated on polyacrylamide gels that were polymerised in glass tubes, approximately 7 mm in diameter and about 10 cm in length. The tubes were easy to load and run, with minimum apparatus requirements. However, only one sample could be run per tube and, because conditions of separation could vary from tube to tube, comparison between different samples was not always accurate. The later introduction of vertical gel slabs allowed running of up to 20 samples under identical conditions in a single run. Vertical slabs are now used routinely both for the analysis of proteins and for the separation of DNA fragments during DNA sequence analysis. Although some workers prepare their own acrylamide gels, others purchase commercially available ready-made gels for techniques such as SDS–PAGE, native gels and isoelectric focusing (IEF).

**Agarose gel electrophoresis of DNA**

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because most DNA molecules and their fragments that are analysed routinely are considerably larger than proteins and therefore, because most DNA fragments would be unable to enter a polyacrylamide gel, the larger pore size of an agarose gel is required. For example, the commonly used plasmid pBR322 has an M r of 2.4 # 10 6 . However, rather than use such large numbers it is more convenient to refer to DNA size in terms of the number of base-pairs. Although, originally, DNA size was referred to in terms of base-pairs (bp) or kilobase-pairs (kbp), it has now become the

accepted nomenclature to abbreviate kbp to simply kb when referring to doublestranded DNA. pBR322 is therefore 4.36 kb. Even a small restriction fragment of 1 kb has an M r of 620 000. When talking about single-stranded DNA it is common to refer to size in terms of nucleotides (nt). Since the charge per unit length (owing to the phosphate groups) in any given fragment of DNA is the same, all DNA samples should move towards the anode with the same mobility under an applied electrical ﬁeld. However, separation in agarose gels is achieved because of resistance to their movement caused by the gel matrix. The largest molecules will have the most difﬁculty passing through the gel pores (very large molecules may even be blocked completely), whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size, the smallest molecules moving fastest. This is analogous to the separation of proteins in SDS polyacrylamide gels, although the analogy is not perfect, as double stranded DNA molecules form relatively stiff rods and while it is not completely understood how they pass through the gel, it is probable that long DNA molecules pass through the gel pores end-on. While passing through the pores, a DNA molecule will experience drag; so the longer the molecule, the more it will be retarded by each pore. Sideways movement may become more important for very small double-stranded DNA and for the more ﬂexible single-stranded DNA. It will be obvious from the above that gel concentrations must be chosen to suit the size range of the molecules to be separated. Gels containing 0.3% agarose will separate double-stranded DNA molecules of between 5 and 60 kb size, whereas 2% gels are used for samples of between 0.1 and 3 kb. Many laboratories routinely use 0.8% gels, which are suitable for separating DNA molecules in the range 0.5#10 kb. Since agarose gels separate DNA according to size, the M r of a DNA fragment may be determined from its electrophoretic mobility by running a number of standard DNA markers of known M r on the same gel. This is most conveniently achieved by running a sample of bacteriophage l DNA (49 kb) that has been cleaved with a restriction enzyme such as EcoRI. Since the base sequence of l DNA is known, and the cleavage sites for EcoRI are known, this generates fragments of accurately known size. DNA gels are invariably run as horizontal, submarine or submerged gels; so named because such a gel is totally immersed in buffer. Agarose, dissolved in gel buffer by boiling, is poured onto a glass or plastic plate, surrounded by a wall of adhesive tape or a plastic frame to provide a gel about 3 mm in depth. Loading wells are formed by placing a plastic well-forming template or comb in the poured gel solution, and removing this comb once the gel has set. The gel is placed in the electrophoresis tank, covered with buffer, and samples loaded by directly injecting the sample into the wells. Samples are prepared by dissolving them in a buffer solution that contains sucrose, glycerol or Ficoll, which makes the solution dense and allows it to sink to the bottom of the well. A dye such as bromophenol blue is also included in the sample solvent; it makes it easier to see the sample that is being loaded and also acts as a marker of the electrophoresis front. No stacking gel is needed for the electrophoresis of DNA because the mobilities of DNA molecules are much greater in the well than in the gel, and therefore all the molecules in the well pile up against the gel within a few minutes of the current being turned on, forming a tight band at the start of the run. General purpose gels are approximately 25 cm long and 12 cm wide, and are run at a voltage gradient of about 1.5 V cm #1 overnight. A higher voltage would cause excessive heating. For rapid analyses that do not need extensive separation of DNA molecules, it is common to use mini-gels that are less than 10 cm long. In this way information can be obtained in 2#3 h. Once the system has been run, the DNA in the gel needs to be stained and visualised. The reagent most widely used is the ﬂuorescent dye ethidium bromide. The gel is rinsed gently in a solution of ethidium bromide (0.5 mg cm #3 ) and then viewed under ultraviolet light (300 nm wavelength). Ethidium bromide is a cyclic planar molecule that binds between the stacked base-pairs of DNA (i.e. it intercalates). The ethidium bromide concentration therefore builds up at the site of the DNA bands and under ultraviolet light the DNA bands ﬂuoresce orange-red. As little as 10 ng of DNA can be visualised as a 1 cm wide band. It should be noted that extensive viewing of the DNA with ultraviolet light can result in damage of the DNA by nicking and base-pair dimerisation. This is of no consequence if a gel is only to be viewed, but obviously viewing of the gel should be kept to a minimum if the DNA is to be recovered. It is essential to protect one’s eyes by wearing goggles when ultraviolet light is used. If viewing of gels under ultraviolet is carried out for long periods, a plastic mask that covers the whole face should be used to avoid ‘sunburn’.

**Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis**

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) is the most widely used method for analysing protein mixtures qualitatively. It is particularly useful for monitoring protein puriﬁcation and, because the method is based on the separation of proteins according to size, it can also be used to determine the relative molecular mass of proteins. SDSðCH 3 # ðCH 2 Þ 10 # CH 2 OSO # 3 Na Þ is an anionic detergent. Samples to be run on SDS–PAGE are ﬁrstly boiled for 5 min in sample buffer containing b-mercaptoethanol and SDS. The mercaptoethanol reduces any disulphide bridges

present that are holding together the protein tertiary structure, and the SDS binds strongly to, and denatures, the protein. Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. On average, one SDS molecule binds for every two amino acid residues. The original native charge on the molecule is therefore completely swamped by the negatively charged SDS molecules. The rod-like structure remains, as any rotation that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to the rod shape. The sample buffer also contains an ionisable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored, and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well. Once the samples are all loaded, a current is passed through the gel. The samples to be separated are not in fact loaded directly into the main separating gel. When the main separating gel (normally about 5 cm long) has been poured between the glass plates and allowed to set, a shorter (approximately 0.8 cm) stacking gel is poured on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded. The purpose of this stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilising differences in ionic strength and pH between the electrophoresis buffer and the stacking gel buffer and involves a phenomenon known as isotachophoresis. The stacking gel has a very large pore size (4% acrylamide), which allows the proteins to move freely and concentrate, or stack, under the effect of the electric ﬁeld. The band-sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have a lower electrophoretic mobility than do the protein–SDS complexes, which, in turn, have lower mobility than the chloride ions (Cl # ) of the loading buffer and the stacking gel buffer. When the current is switched on, all the ionic species have to migrate at the same speed otherwise there would be a break in the electrical circuit. The glycinate ions can move at the same speed as Cl # only if they are in a region of higher ﬁeld strength. Field strength is inversely proportional to conductivity, which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that [Cl # ] > [protein–SDS] > [glycinate]. There is only a small quantity of protein–SDS complexes, so they concentrate in a very tight band between glycinate and Cl # boundaries. Once the glycinate reaches the separating gel it becomes more fully ionised in the higher pH environment and its mobility increases. (The pH of the stacking gel is 6.8, that of the separating gel is 8.8.) Thus, the interface between glycinate and Cl # leaves behind the protein–SDS complexes, which are left to electrophorese at their own rates. The negatively charged protein–SDS complexes now continue to move towards the anode, and, because they have the same charge per unit length, they travel into the separating gel under the applied electric ﬁeld with the same mobility. However, as they pass through the separating gel the proteins separate, owing to the molecular sieving properties of the gel. Quite simply, the smaller the protein the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of the gels. Being a small molecule, the bromophenol blue dye is totally unretarded and therefore indicates the electrophoresis front. When the dye reaches the bottom of the gel, the current is turned off, and the gel is removed from between the glass plates and shaken in an appropriate stain solution (usually Coomassie Brilliant Blue) and then washed in destain solution. The destain solution removes unbound background dye from the gel, leaving stained proteins visible as blue bands on a clear background. A typical minigel would take about 1 h to prepare and set, 40 min to run at 200 V and have a 1 h staining time with Coomassie Brilliant Blue. Upon destaining, strong protein bands would be seen in the gel within 10#20 min, but overnight destaining is needed to completely remove all background stain. Vertical slab gels are invariably run, since this allows up to 10 different samples to be loaded onto a single gel. Typically, the separating gel used is a 15% polyacrylamide gel. This gives a gel of a certain pore size in which proteins of relative molecular mass (M r ) 10 000 move through the gel relatively unhindered, whereas proteins of M r 100 000 can only just enter the pores of this gel. Gels of 15% polyacrylamide are therefore useful for separating proteins in the range M r 100 000 to 10 000. However, a protein of M r 150 000, for example, would be unable to enter a 15% gel. In this case a larger-pored gel (e.g. a 10% or even 7.5% gel) would be used so that the protein could now enter the gel and be stained and identiﬁed. It is obvious, therefore, that the choice of gel to be used depends on the size of the protein being studied. The fractionation range of different percentage acrylamide gels is shown in Table 10.1. This shows, for example, that in a 10% polyacrylamide gel proteins greater than 200 kDa in mass cannot enter the gel, whereas proteins with relative molecular mass (M r ) in the range 200 000 to 15 000 will separate. Proteins of M r 15 000 or less are too small to experience the sieving effect of the gel matrix, and all run together as a single band at the electrophoresis front.

The M r of a protein can be determined by comparing its mobility with those of a number of standard proteins of known M r that are run on the same gel. By plotting a graph of distance moved against log M r for each of the standard proteins, a calibration curve can be constructed. The distance moved by the protein of unknown M r is then measured, and then its log M r and hence M r can be determined from the calibration curve.

SDS–gel electrophoresis is often used after each step of a puriﬁcation protocol to assess the purity or otherwise of the sample. A pure protein should give a single band on an SDS–polyacrylamide gel, unless the molecule is made up of two unequal subunits. In the latter case two bands, corresponding to the two subunits, will be seen. Since only submicrogram amounts of protein are needed for the gel, very little material is used in this form of purity assessment and at the same time a value for the elative molecular mass of the protein can be determined on the same gel run, with no more material being used.

**Native (buffer) gels**

While SDS–PAGE is the most frequently used gel system for studying proteins, the method is of no use if one is aiming to detect a particular protein (often an enzyme) on the basis of its biological activity, because the protein (enzyme) is denatured by the SDS–PAGE procedure. In this case it is necessary to use non-denaturing conditions. In native or buffer gels, polyacrylamide gels are again used (normally a 7.5% gel) but the SDS is absent and the proteins are not denatured prior to loading. Since all the proteins in the sample being analysed carry their native charge at the pH of the gel (normally pH 8.7), proteins separate according to their different electrophoretic mobilities and the sieving effects of the gel. It is therefore not possible to predict the behaviour of a given protein in a buffer gel but, because of the range of different charges and sizes of proteins in a given protein mixture, good resolution is achieved. The enzyme of interest can be identiﬁed by incubating the gel in an appropriate substrate solution such that a coloured product is produced at the site of the enzyme. An alternative method for enzyme detection is to include the substrate in an agarose gel that is poured over the acrylamide gel and allowed to set. Diffusion and interaction of enzyme and substrate between the two gels results in colour formation at the site of the enzyme. Often, duplicate samples will be run on a gel, the gel cut in half and one half stained for activity, the other for total protein. In this way the total protein content of the sample can be analysed and the particular band corresponding to the enzyme identiﬁed by reference to the activity stain gel.