Lecure.2

1. Gene cloning

The term ´´DNA cloning`` refers to the insertion of a DNA fragment (foreign DNA) into the host genome, resulting in the generation of new strains with acquired traits that are not present in the wild type. The foreign DNA is isolated from an external source and inserted into the host genome through transformation.

The first cloning experiments

Hamilton Smith and co-workers demonstrated unequivocally that restriction endoncleases cleave a specific DNA sequence. Later, Daniel Nathans used restriction endonucleases to map the simian virus 40 (SV40) genome and to locate the origin of replication. These major breakthroughs underscored the great potential of restriction endonucleases for DNA work. Building on their discoveries, the cloning experiments of Herbert Boyer, Stanley Cohen, Paul Berg, and their colleagues in the early 1970s ushered in the era of recombinant DNA technology. One of the first recombinant DNA molecules to be engineered was a hybrid of phage λ and the SV40 mammalian DNA virus genome. In 1974 the first eukaryotic gene was cloned. Amplified ribosomal RNA (rRNA) genes or "ribosomal DNA" (rDNA) from the South African clawed frog *Xenopus laevis* were digested with a restriction endonuclease and linked to a bacterial plasmid. Amplified rDNA was used as the source of eukaryotic DNA since it was well characterized at the time and could be isolated in quantity by CsCl-gradient centrifugation. Within oocytes of the frog, rDNA is selectively amplified by a rolling circle mechanism from an extrachromosomal nucleolar circle (see Fig. 6.17). The number of rRNA genes in the oocyte is about 100- to 1000-fold greater than within somatic cells of the same organism. To the great excitement of the scientific community, the cloned frog genes were actively transcribed into rRNA in *E. coli.* This showed that recombinant plasmids containing both eukaryotic and prokaryotic DNA replicate stably in *E. coli*. Thus, genetic engineering could produce new combinations of genes that had never appeared in the natural environment, a feat which led to widespread concern about the safety of recombinant DNA work (Focus box 8.1).

2. The requirements for gene cloning are as follows:

1. Target DNA fragment.
2. Restriction enzymes (traditional method), Ligase.
3. Cloning vector.
4. Host cells.

To become familiar with gene cloning, the following are the principle steps illustrated in the **Figure 5** of gene cloning:

1. Determine the **targeted gene sequences (foreign DNA fragment)** to design primers and amplify the target gene, taking into consideration gene elements, such as promoter, open reading frame and terminator, for successful gene expression.
2. Select an appropriate cloning **vector** that can bind the foreign DNA fragment and ensure the generation of a recombinant DNA molecule.
3. Transfer the **recombinant DNA** molecule into the **host** cell, such as bacterial or fungal cells.
4. The vector replicates inside host cells, allowing multiple copies of the foreign DNA to be produced along with the vectors.
5. The new generation (progeny) of host cells is generated through cell division, passing on the recombinant DNA molecule (vector with foreign DNA) to the new cells.
6. Selection of transformed host cells and identification of the clone containing the gene of interest.

Fate of inserted gene

1. Multiplication and Expression of the introduced Gene inside host.

Consequently:

* Isolation of multiple gene copies/Protein expressed by the gene.

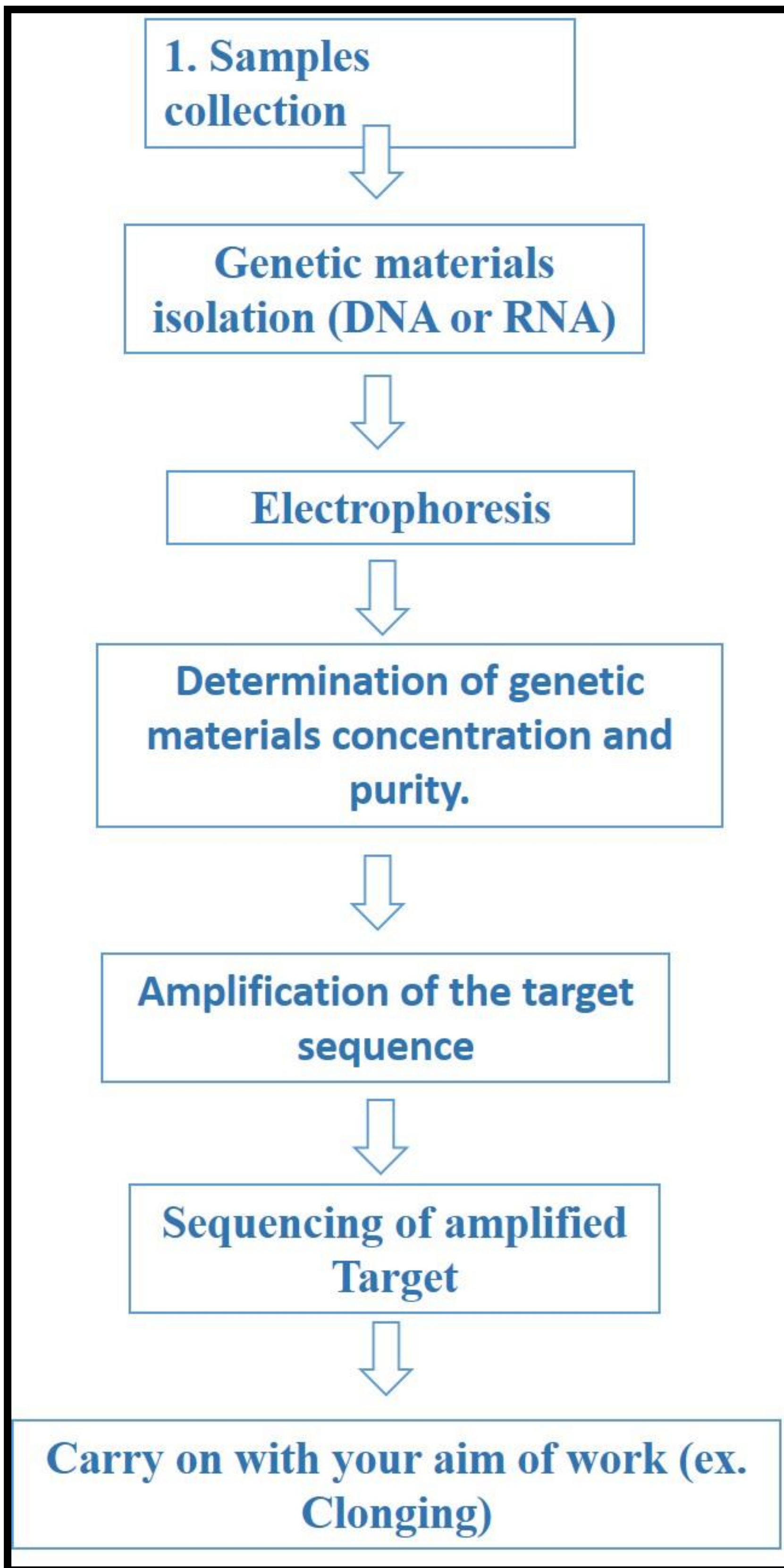

Figure.3 highlights the principle steps necessary to initiate genetic engineering work.

What is PCR?

PCR is carried out in a single test tube simply by mixing DNA with a set of reagents and placing the tube in a thermal cycler, a piece of equipment that enables the mixture to be incubated at a series of temperatures that are varied in a preprogrammed manner. The basic steps in a PCR experiment are as follows

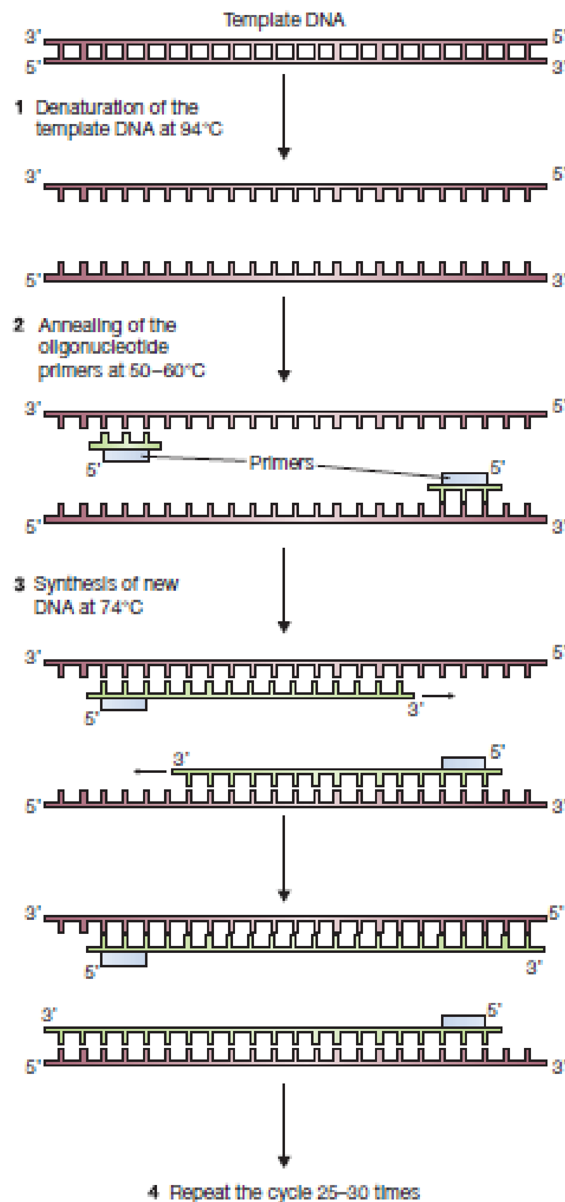

Figure.4 The basic steps in the polymerase chain reaction

The mixture is heated to 94°C, at which temperature the hydrogen bonds that

hold together the two strands of the double-stranded DNA molecule are broken, causing the molecule to **denature**.

2 The mixture is cooled down to 50–60°C. The two strands of each molecule could join back together at this temperature, but most do not because the mixture contains a large excess of short DNA molecules, called **oligonucleotides** or **primers**, which **anneal** to the DNA molecules at specific positions.

3 The temperature is raised to 74°C. This is a good working temperature for the ***Taq* DNA polymerase** that is present in the mixture. We will learn more about **DNA polymerases** on p. 48. All we need to understand at this stage is that the *Taq* DNA polymerase attaches to one end of each primer and synthesizes new strands of DNA, complementary to the **template** DNA molecules, during this step of the PCR. Now we have four stands of DNA instead of the two that there were to start with.

4 The temperature is increased back to 94°C. The double-stranded DNA molecules, each of which consists of one strand of the original molecule and one new strand of DNA, denature into single strands. This begins a second cycle of denaturation–annealing–synthesis, at the end of which there are eight DNA strands. By repeating the cycle 30 times the double-stranded molecule that we began with is converted into over 130 million new double-stranded molecules, each one a copy of the region of the starting molecule delineated by the annealing sites of the two primers.

There are two methods utilized in the gene cloning:

1. Traditional gene cloning

In this methods, a recombinant DNA molecule is generated before transformation. The DNA fragments and circular vectors are subjected to restriction enzymes to generate specific complementary ends sequences. The aim of creating complementary ends is to bind the fragments and generate a recombinant DNA molecule (a vector with foreign DNA) using ligase. Following, this step, transformation is carried out.

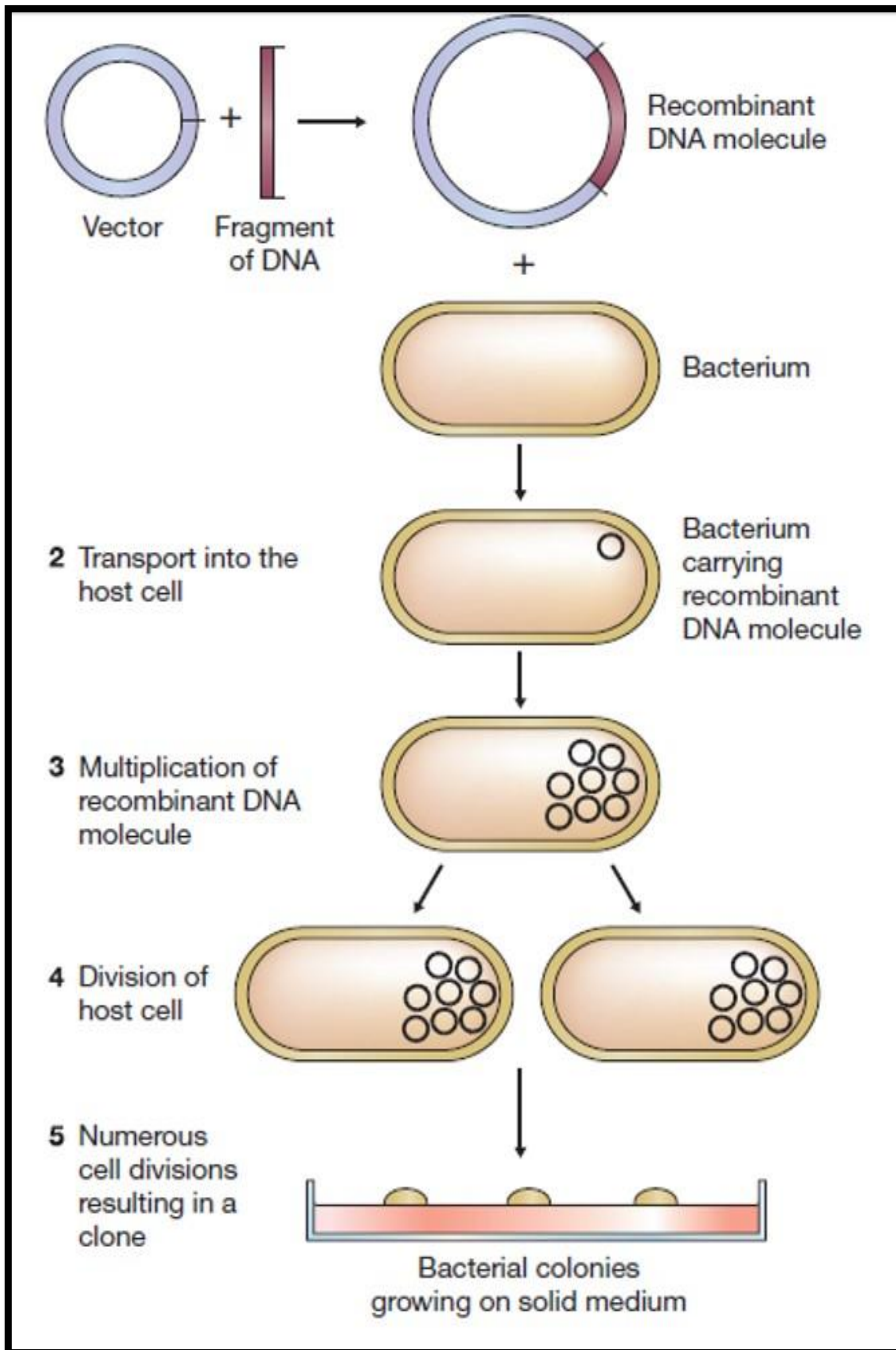

Figure 5: The schematic diagram illustrates the principle steps of gene cloning.

2. PCR Cloning Method

A new method has been developed that saves time, effort and cost. The procedure involves ligating foreign DNA and a cloning vector using he PCR technique, without the use of restriction enzymes (RE). The ligation of fragments can be carried out using blunt or single-base overhang ligation.

During the amplification of the fragments, restriction sites are added to the ends of the fragments by incorporating *EcoR*I and *Not*I sites into the designed primer pairs. As shown in the **Figure 2.2**, the restriction site sequences of the vector are derived from (MRS) and are added to the primer pair sequences, which are used to amplify foreign DNA.

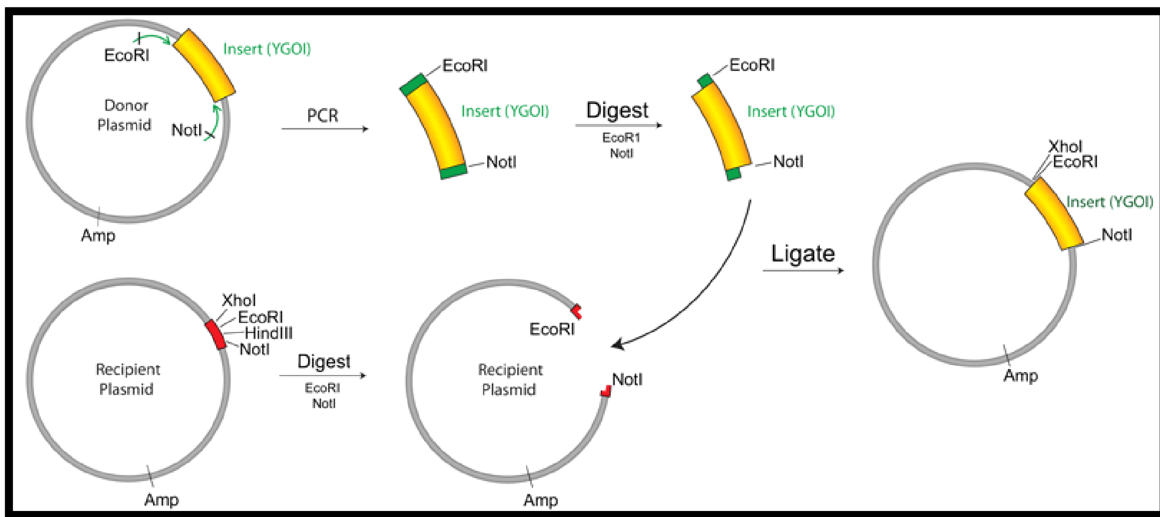

Figure 6: A schematic diagram illustrating two methods of gene cloning. A) the restriction enzyme-based method, B) the PCR-base method.

Why gene cloning and PCR are so important

To understand exactly how cloning can provide a pure sample of a gene, consider the basic experiment from Figure 5, but drawn in a slightly different way (Figure 7). In this example the DNA fragment to be cloned is one member of a mixture of many different fragments, each carrying a different gene or part of a gene. This mixture could indeed be the entire genetic complement of an organism—a human, for instance. Each of these fragments becomes inserted into a different vector molecule to produce a family of recombinant DNA molecules, one of which carries the gene of interest. Usually only one recombinant DNA molecule is transported into any single host cell, so that although the final set of clones may contain many different recombinant DNA molecules, each individual clone contains multiple copies of just one

molecule. The gene is now separated away from all the other genes in the original mixture, and its specific features can be studied in detail.

In practice, the key to the success or failure of a gene cloning experiment is the ability to identify the particular clone of interest from the many different ones that are obtained. If we consider the **genome** of the bacterium *Escherichia coli*, which contains just over 4000 different genes, we might at first despair of being able to find just one gene among all the possible clones (Figure 7). The problem becomes even more overwhelming when we remember that bacteria are relatively simple organisms and that the human genome contains about five times as many genes. However, a variety of different strategies can be used to ensure that the correct gene can be obtained at the end of the cloning experiment. Some of these strategies involve modifications to the basic cloning procedure, so that only cells containing the desired recombinant DNA molecule can divide and the clone of interest is automatically **selected**. Other methods involve techniques that enable the desired clone to be identified from a mixture of lots of different clones.

Once a gene has been cloned there is almost no limit to the information that can
be obtained about its structure and expression. The availability of cloned material has
stimulated the development of analytical methods for studying genes, with new techniques
being introduced all the time. Methods for studying the structure and expression of a cloned
gene.

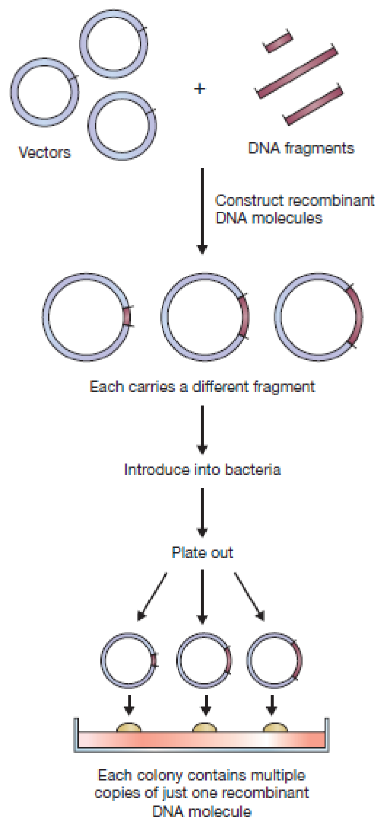

Figure. 7: Cloning allows individual fragments of DNA to be purified.

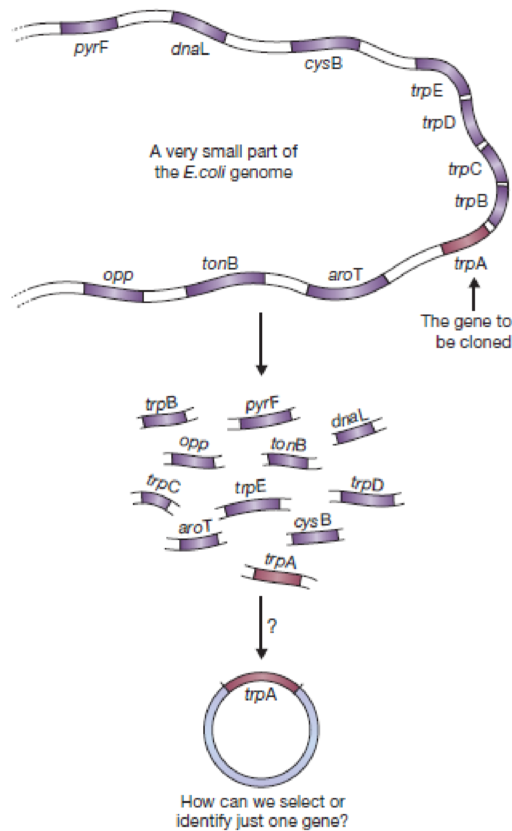

Figure.8 The problem of selection.

2. PCR can also be used to purify a gene

The polymerase chain reaction can also be used to obtain a pure sample of a gene. This is because the region of the starting DNA molecule that is copied during PCR is the segment whose boundaries are marked by the annealing positions of the two oligonucleotide primers. If the primers anneal either side of the gene of interest, many copies of that gene will be synthesized (Figure 9). The outcome is the same as with a gene cloning experiment, although the problem of selection does not arise because the desired gene is automatically "selected" as a result of the positions at which the primers anneal.

A PCR experiment can be completed in a few hours, whereas it takes weeks if not months to obtain a gene by cloning. Why then is gene cloning still used? This is because PCR has two limitations:

l In order for the primers to anneal to the correct positions, either side of the gene of interest, the sequences of these annealing sites must be known. It is easy to synthesize a primer with a predetermined sequence, but if the

sequences of the annealing sites are unknown then the appropriate primers cannot be made. This means that PCR cannot be used to isolate genes that have not been studied before—that has to be done by cloning.

There is a limit to the length of DNA sequence that can be copied by PCR.
Five kilobases (kb) can be copied fairly easily, and segments up to forty kb can be dealt with by using specialized techniques, but this is shorter than the lengths of many genes, especially those of humans and other vertebrates. Cloning must be used if an intact version of a long gene is required.
Gene cloning is therefore the only way of isolating long genes or those that have
never been studied before. But PCR still has many important applications. For example, even if the sequence of a gene is not known, it may still be possible to determine the appropriate sequences for a pair of primers, based on what is known about the sequence of the equivalent gene in a different organism. A gene that has been isolated and sequenced from, say, mouse could therefore be used to design a pair of primers for isolation of the equivalent gene from humans.
In addition, there are many applications where it is necessary to isolate or detect
genes whose sequences are already known. A PCR of human globin genes, for example, is used to test for the presence of mutations that might cause the blood disease called thalassaemia. Design of appropriate primers for this PCR is easy because the sequences of the human globin genes are known. After the PCR, the gene copies are sequenced or studied in some other way to determine if any of the thalassaemia mutations are present. Another clinical application of PCR involves the use of primers specific for the DNA of a disease-causing virus. A positive result indicates that a sample contains the virus and that the person who provided the sample should undergo treatment to prevent onset of the disease. The polymerase chain reaction is tremendously sensitive: a carefully set up
reaction yields detectable amounts of DNA, even if there is just one DNA molecule in the starting mixture. This means that the technique can detect viruses at the earliest stages of an infection, increasing the chances of treatment being successful. This great sensitivity means that PCR can also be used with DNA from forensic material such as hairs and dried bloodstains or even from the bones of long-dead humans.