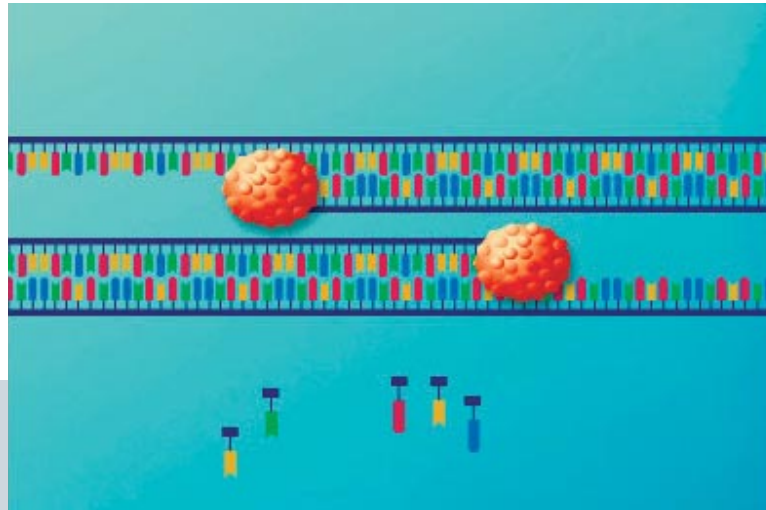


# PCR: an outstanding method



Scarcely any invention has altered biological science so radically in such a short period as the polymerase chain reaction, or PCR. With this technique, minute amounts of DNA can be replicated very rapidly and thereby amplified to such an extent that the DNA becomes easy to detect, study and use for any given purpose. The potential of this technique in medicine has long been known, and ever more applications are being developed. Wherever genes provide clues to the cause or natural history of a disease, PCR is the method of choice.

Long car journeys can sometimes be a godsend. Driving along a monotonous stretch of dark road one April weekend in 1983, American chemist Kary Mullis was struck by an idea that was later to earn him the Nobel Prize: the principle of the polymerase chain reaction. Among the instruments and glassware of his laboratory Mullis might never have had the most momentous and far-reaching idea of his life.

Within a few years PCR – short for ‘polymerase chain reaction’ – took the world’s biological laboratories by storm. By the mid-

1980s the technique was used for the first time to diagnose a disease, when researchers identified the gene for sickle cell anemia. At about the same time the method was introduced into forensic medicine. The polymerase chain reaction reaped the highest scientific honour for its inventor in record time: In 1993, just ten years after his historical car journey, Kary Mullis received the Nobel Prize for Chemistry. The reason for this extraordinary success is that the technique provided a solution to one of the most pressing problems facing biology at the time – the replication of DNA.

### Terms

**DNA** deoxyribonucleic acid; the chemical substance of our genes

**RNA** ribonucleic acid; the chemical substance that makes up the working copies of genes (mRNA), among other things

**Nucleic acids** a chemical term that covers both DNA and RNA; nucleic acids are molecules consisting of long chains of nucleotides linked together

**Nucleotides** the building blocks of DNA; they comprise the four bases adenine, thymine, cytosine and guanine (A, T, C, G; in RNA thymine is replaced by uracil [U]), a sugar and at least one phosphate group; without the phosphate group these building blocks are referred to as nucleosides

**Sequence** the order of the nucleotides in DNA (DNA sequence) or RNA (RNA sequence)

**Primer** a short DNA fragment with a defined sequence that serves as an extension point for polymerases

**Polymerases** enzymes that link individual nucleotides together to form long DNA or RNA chains

**Hybridisation (annealing)** the joining of two complementary DNA (or RNA) strands to form a double strand

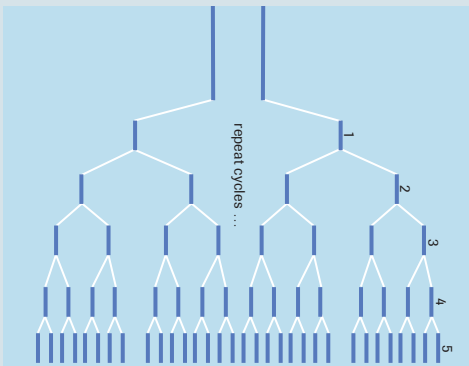
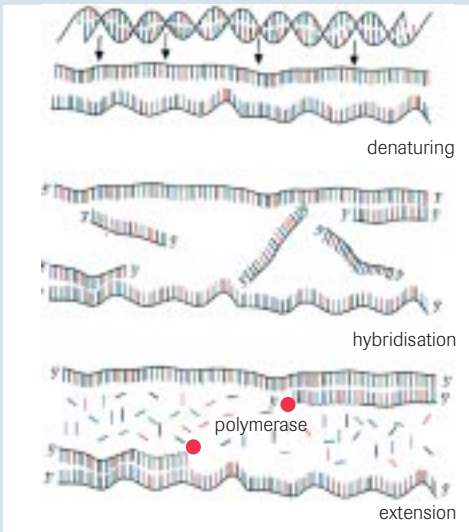
**Complementary DNA** The building blocks of DNA and RNA form specific pairings. Two strands whose building blocks form a sequence of perfect pairings are able to form a stable double strand and are referred to as complementary strands

### Rapid DNA cloning

In the PCR procedure trace amounts of DNA can be quickly and repeatedly copied to produce a quantity sufficient to investigate using conventional laboratory methods. In this way, for example, it is possible to sequence the DNA, i.e. determine the order of its building blocks. Theoretically, a single DNA molecule is sufficient. PCR is therefore one of the most sensitive biological techniques ever devised. Given these capabilities, Mullis’s method ultimately ushered in the age of genomics. From the Human Genome Project to the search for targets to the development of gene tests, there are few areas of

genetic research today that do not depend on PCR. Only with the advent of increasingly sensitive DNA chips in recent years has PCR faced any notable competition (see chapter on DNA chips). But even then it is often necessary to first copy, or amplify, the DNA of interest. For this reason PCR and DNA chips often go hand in hand.

### Simple and effective: the PCR principle



The polymerase chain reaction serves to copy DNA. It uses repeated cycles, each of which consists of three steps:

1. The reaction solution containing DNA molecules (to be copied), polymerases (which copy the DNA), primers (which serve as starting DNA) and nucleotides (which are attached to the primers) is heated to 95°C. This causes the two complementary strands to separate, a process known as denaturing or melting.
2. Lowering the temperature to 55°C causes the primers to bind to the DNA, a process known as hybridisation or annealing. The resulting bonds are stable only if the primer and DNA segment are complementary, i.e. if the base pairs of the primer and DNA segment match. The polymerases then begin to attach additional complementary nucleotides at these sites, thus strengthening the bonding between the primers and the DNA.
3. Extension: The temperature is again increased, this time to 72°C. This is the ideal working temperature for the polymerases used, which add further nucleotides to the developing DNA strand. At the same time, any loose bonds that have formed between the primers and DNA segments that are not fully complementary are broken.

Each time these three steps are repeated the number of copied DNA molecules doubles. After 20 cycles about a million molecules are cloned from a single segment of double-stranded DNA.

The temperatures and duration of the individual steps described above refer to the most commonly used protocol. A number of modifications have been introduced that give better results to meet specific requirements.

## **Copies of copies of copies**

The basic PCR principle is simple. As the name implies, it is a chain reaction: One DNA molecule is used to produce two copies, then four, then eight and so forth. This continuous doubling is accomplished by specific proteins known as polymerases, enzymes that are able to string together individual DNA building blocks to form long molecular strands. To do their job polymerases require a supply of DNA building blocks, i.e. the nucleotides consisting of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G). They also need a small fragment of DNA, known as the primer, to which they attach the building blocks as well as a longer DNA molecule to serve as a template for constructing the new strand. If these three ingredients are supplied, the enzymes will construct exact copies of the templates (see box on page 67). This process is important, for example, when DNA polymerases double the genetic material during cell division. Besides DNA polymerases there are also RNA polymerases that string together RNA building blocks to form molecular strands. They are mainly involved in making mRNA, the working copies of genes.

## **Copying unknown DNA**

These enzymes can be used in the PCR to copy any nucleic acid segment of interest. Usually this is DNA; if RNA needs to be copied, it is usually first transcribed into DNA with the help of the enzyme reverse transcriptase – a method known as reverse transcription PCR (RT-PCR). For the copying procedure only a small fragment of the DNA section of interest needs to be identified. This then serves as a template for producing the primers that initiate the reaction. It is then possible to clone DNA whose sequence is unknown. This is one of the method's major advantages.

Genes are commonly flanked by similar stretches of nucleic acid. Once identified, these patterns can be used to clone unknown genes – a method that has supplanted the technique of molecular cloning in which DNA fragments are tediously copied in bacteria or other host organisms. With the PCR method this goal can be achieved faster, more easily and above all in vitro, i.e. in the test-tube. Moreover, known sections of long DNA molecules, e.g. of chromosomes, can be used in PCR to scout further into unknown areas.

## Help from hot springs

Soon after its discovery the PCR method was refined in several ways. One of the first modifications of the original protocol concerned the polymerases used. Like all enzymes, polymerases function best at the body temperature of the organism in which they originate – 37°C in the case of polymerases isolated from humans. Below this temperature the enzyme's activity declines steeply, above this temperature it is quickly destroyed. In PCR, however, the two strands of the DNA molecule must be separated in order to permit the primers to anneal to them. This is done by raising the temperature to around 95°. At such temperatures the polymerases of the vast majority of organisms are permanently destroyed. As a result, new enzyme had to be added in the first reaction step of each cycle – a time-consuming and expensive proposition.

A solution was found in hot springs. Certain microorganisms thrive in such hot pools under the most inhospitable conditions, at temperatures that can reach 100°C and in some cases in the presence of extreme salt or acid concentrations. The polymerases of these organisms are adapted to high temperatures and are therefore ideal for use in PCR. Today the polymerases used in nearly all PCR methods the world over are derived from such microorganisms. This prominent bacterium goes by the name of *Thermus aquaticus*, and its heat-stable polymerase, called Taq poly-

merase, supports an entire industry. The organism was originally discovered in a 70°C spring near Great Fountain Geyser in Yellowstone National Park in the USA. Employees of Cetus, who Kary Mullis was working for at the time of his discovery, isolated the first samples from the hot spring and then cultivated in the laboratory one of the most useful bacterial strains known today. Meanwhile *Thermus aquaticus* has been found in similar hot springs all over the world.



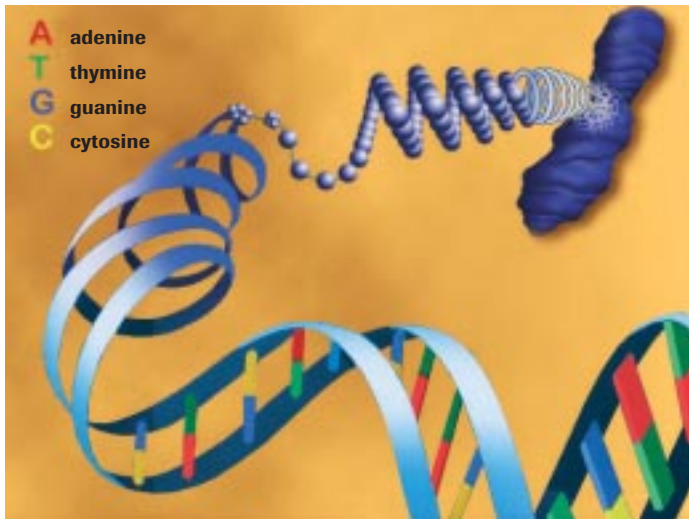
**Hot spring: *Thermus aquaticus*.** Nearly all PCR techniques in the world now use the Taq polymerases isolated from the bacterium *Thermus aquaticus*, a microorganism that dwells in hot springs at about 70°C. The first *T. aquaticus* strain from which polymerases for PCR were obtained was found in Yellowstone National Park in the USA.

### **Further developments around the world**

The introduction of Taq polymerase has certainly not been the only modification to the PCR method. This was helped by the fact that Mullis published his discovery relatively early – though not without some difficulty. Both *Science* and *Nature*, the two most renowned scientific journals, failed to recognise the significance of PCR and rejected the paper describing the method. Moreover, despite global patent protection, the use of the PCR technique is still free and unrestricted for basic researchers thanks to Roche, which owns the rights to the method. In 1991 Roche obtained an exclusive license from Mullis's former employer Cetus for 300 million dollars. Scientists from all over the world have modified the PCR method in many ways and adapted it for routine diagnostic testing and molecular research. At the same time, more and more new applications are emerging.

### **Forerunner of genomics: DNA sequencing**

In the 1990s biology was faced with one overriding preoccupation: the unravelling of the genome. Thanks to huge technical and organisational efforts, first viruses and bacteria, then yeasts, plants and animals relinquished the secrets of their genetic material. This accomplishment would have been unthinkable without PCR, which made it possible to prepare large amounts of DNA within a short time. The simple cloning of DNA has therefore remained one of the main uses of the method. Thus PCR is used whenever the exact sequence of DNA building blocks needs to be determined: e.g. in other genome sequencing projects, in gene research, in the investigation of genomic changes, in the search for targets, etc. An important topic in the field of genomics today is SNPs (pronounced 'snips'), single nucleotide changes in the genome which appear to account for a large proportion of the genetic differences between individuals (see chapter on SNPs). Among other things, SNPs are responsible for disease susceptibility and for differences in the way patients respond to drugs. In order to detect such hereditary and often widespread variations, scientists have to sequence the genome of many different people in parallel. Genes with SNPs are also potential targets for new drugs. PCR therefore plays a key role in this important area of drug research.



**DNA, our genetic material.**

**Sensitive determination:  
qualitative PCR**

When PCR is used only for detecting a specific DNA segment, the method is referred to as qualitative PCR. Usually the standard protocol is used. Qualitative PCR is an extremely sensitive method which is theoretically able to detect a single DNA molecule in a sample solution. In many cases specific genes are copied in this way, e.g. in order to identify pathological changes. As mentioned earlier, the first gene identified by PCR was the gene responsible for sickle cell anemia. Countless other gene tests have meanwhile been devised. Qualitative PCR is also used around the world in forensic medicine to identify individuals. Usually individual regions of the genome are amplified and examined. However, although these regions differ between people, they reveal nothing about the traits or character of the person in question.

PCR can of course be used to detect not only human genes but also genes of bacteria and viruses. One of the most important medical applications of the classical PCR method is therefore the detection of pathogens. Here PCR is replacing immunological methods, in which antibodies against a pathogen are used to identify the pathogen in a patient's blood. Antibodies are not detectable until several weeks after the onset of an infection, whereas PCR is able to detect the DNA or RNA of the pathogens much more quickly. Moreover, antibodies can remain in the bloodstream long after an infectious disease has resolved. Hence, only qualitative PCR can determine whether an infection





**Human immunodeficiency virus.**

has been eradicated, whether it is chronic (and might therefore progress unnoticed) and whether the individual has been reinfected with a different but related pathogen. Many viruses contain RNA rather than DNA. In such cases the viral genome has to be transcribed before PCR is performed, and RT-PCR is therefore used.

Sometimes it is also necessary to detect pathogens outside the body. Fortunately, the PCR method can detect the DNA of microorganisms in any sample, whether of body fluids, foodstuffs or drinking water. PCR is therefore used in all these areas. One of the most urgent problems PCR is helping to solve is to determine if donated blood is contaminated. Blood banks are one of the major transmission sources of hepatitis C, for example, and sometimes of HIV. Fast, simple and above all inexpensive testing is essential – and PCR ideally meets all these criteria.

**More than just yes or no:  
quantitative PCR**

Quantitative PCR provides additional information beyond mere detection of DNA. It indicates not just whether a specific DNA segment is present in a sample, but also how much of it is there. This information is required in a number of applications ranging from medical diagnostic testing through target searches to basic research. Consequently, although quantitative PCR was not described until the 1990s, the method already exists in a number of variants and protocols to meet a broad range of requirements. Theoretically it is possible to calculate the amount of DNA originally present in a sample directly from the amount found at the end of a PCR run. If, for example, there were not one but two double strands at the start of the reaction, exactly twice as much will be present after each cycle. However, this simple approach founders on the fact that conditions for the polymerases are not optimal at the start or end of PCR. At the start the performance of the enzymes is limited by the small amount of template present, while in the final cycles the enzymes' activity declines as a



result of continuous temperature changes. Moreover, in these later cycles the amount of available nucleotides falls and the newly formed templates increasingly bind to each other rather than to the primers. The effects of all these factors vary greatly depending on small differences in the reaction conditions (temperature, duration of the steps, concentrations of the reagents, etc.). In practical terms it is therefore impossible to draw direct conclusions about the number of molecules in the original sample from the amount of DNA present at the end of PCR.

Instead, researchers have developed various methods that determine the number of new DNA molecules formed in the reaction, i.e. after each cycle (see box on p. 74). Because this approach affords continuous observation of the reaction, it is referred to as real-time PCR. Ultimately such experiments involve conjugating the new DNA copies (but not the primers or free DNA building blocks) to a dye, thus making it possible to determine the quantity of template.

### Target research

Quantitative PCR is used, for example, to help search for and evaluate targets, i.e. the sites in the body at which new drugs can act. This primarily relates to the discovery of new genes, a task in which PCR is basically used as a DNA copying tool. The same applies to already known genes that come in a number of variants and that are fairly widespread in the population (polymorphisms; see chapter on SNPs). However, for a gene truly to be a target for new drugs, its products must be involved in the development or progress of a disease. The common occurrence of specific gene variants in affected individuals can only serve as an initial signpost. The question ultimately is not whether a specific form of a gene is present or not, but whether observed variations – i.e. changes in a gene sequence, multiple occurrences of a gene or its absence – really have different effects in healthy and ill people.

To investigate this – a procedure known as target validation – we need to consider the gene's products rather than the gene itself. Gene products are usually proteins. Protein research is therefore devising increasingly sophisticated methods to detect, identify and assay its subjects of enquiry (see chapter on proteomics). But the latter task, quantity determination, cannot be satisfactorily performed with available proteomic methods. Furthermore, proteins often differ markedly in their life cycle and activity. As a result, the quantity of a protein provides only lim-

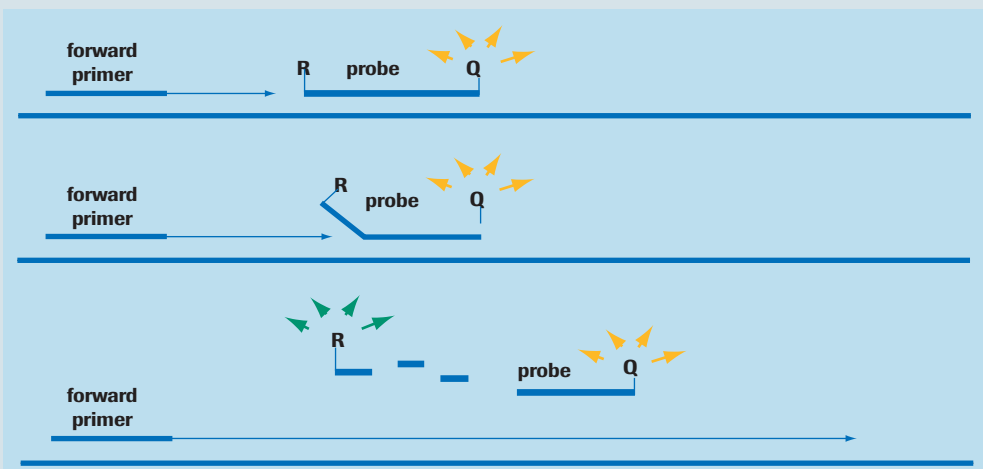
## Shining examples: quantitative real-time PCR

Many applications require the amount of DNA originally present in a PCR sample to be determined. Many techniques are available for calculating the number of DNA copies formed during the individual steps of the PCR procedure and thus for deriving the quantity originally present in the sample. They are usually based on the middle, or exponential, phase of the PCR in which the amount of DNA template is approximately doubled in each cycle.

- Example: competitive PCR – This method is now largely of historical significance only. It was one of the first quantitative PCR methods developed. In addition to the template of interest, another DNA template having a very similar sequence was added to the same reaction vessel. Both DNA strands were then cloned simultaneously under identical conditions. The amount of template and the amount of 'competitive' DNA formed provided at least a rough estimate of the amount of DNA present in the original sample.
- Example: real-time PCR – Most of the quantitative PCR methods in use today are based on a 1922 discovery by the American Russ Higuchi, who used the dye ethidium bromide (EtBr). Embedded in double-stranded DNA, EtBr fluoresces when stimulated by light. The observed fluorescence therefore indicates the amount of DNA formed and does so at any given time during the PCR reaction, hence the name real-time PCR. In this method parallel runs are performed with the same known quantity of DNA and a comparative curve is plotted under identical conditions. This presupposes that the sequence of the DNA to be copied is known. Moreover, it is not possible to distinguish directly between the correctly formed product and primers that have annealed to form

a double strand. Nevertheless, the principle is still used, though usually with other dyes that specifically interact only with the desired DNA product. The experiments have been simplified by the introduction of special equipment such as the Roche LightCycler, which automates the entire procedure, heating and cooling the solutions, stimulating the dye to fluoresce and continuously monitoring the fluorescence. Special computer programs help to analyse the data.

- Example: TaqMan probes: One way to measure only the desired DNA product during PCR is to use TaqMan probes, short DNA fragments that anneal to a middle region of the template DNA (see below). The probes bear a reporter dye (R) at one end and a quencher (Q) at the other. Quenchers are molecules that quench the fluorescence of dyes in their proximity. The polymerases in the PCR solution are able to break down the TaqMan probes during the doubling of the DNA template. In so doing they free the reporter dye, which then migrates away from the influence of the quencher. Hence the fluorescence of the dye is measurable only if the polymerase has in fact copied the desired DNA strand. Each freed molecule of reporter dye represents a DNA strand that has been formed. TaqMan probes can therefore be used to measure the amount of DNA formed at any given time.



ited information about the expression of the corresponding gene. A far more sensitive method is available in the form of quantitative PCR, which measures not the proteins themselves but rather the working copies of the corresponding genes, the mRNA.

In accordance with the principle of RT-PCR, the mRNA is reverse-transcribed into DNA. Its original quantity is then determined by quantitative real-time PCR. This provides an informative picture of how vigorously a gene is being transcribed and in what form, since in many cases one and the same gene can give rise to different products at different sites in the body. The initial working copies of such genes are cut and stitched back together by the cells in various ways. Consequently, the products can have markedly different properties.

**Example: STEP**

One method of determining the quantity and nature of working copies of genes in various samples is 'single target expression profiling', or STEP. In this technique attention is focused on a specific gene (a target), for which an expression profile is prepared, i.e. its expression is measured in various tissues of healthy and/or ill persons (see box on p. 74). When the results are entered in a diagram it can be readily seen in which areas of the body the gene in question is particularly active or inactive. A diagram of this kind is therefore referred to as a body map. A specific tissue from different people can also be examined. Several hundred individual values can be meas-

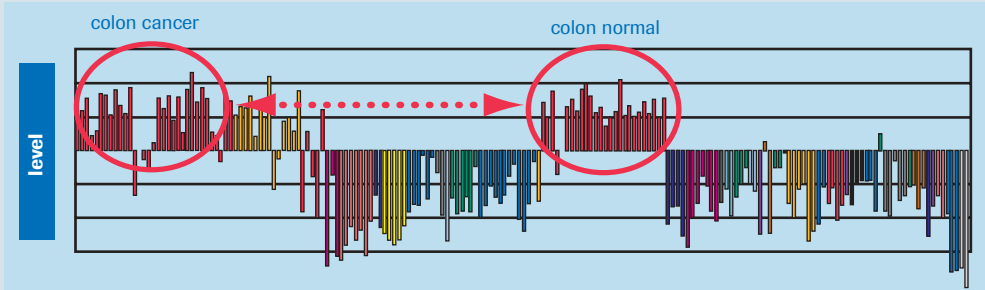


**Quantitative analysis of human DNA using the LightCycler.**

### PCR and target evaluation: STEP

Single Target Expression Profiling, or STEP for short, provides an overview of the expression of a specific gene in various samples. In this method the number of working copies of a specific gene is measured by quantitative PCR. The samples can come from different tissues of an individual or from tissues of many different donors. By comparing the profiles in healthy and ill people it is possible to determine, for example, whether a gene in question really is associated with the development or progression of a disease.

The values obtained are plotted and compared. The example below is a comparative diagram showing the expression profile of various tissues, including samples from the colon of a healthy subject and of a patient with cancer of the colon (circles). The gene in question was suspected of being more active in the presence of colon cancer. However, the STEP procedure showed that the colon-specific gene is equally active in healthy and ill individuals.



ured and plotted. In many cases this reveals whether a target really is associated with a disease.

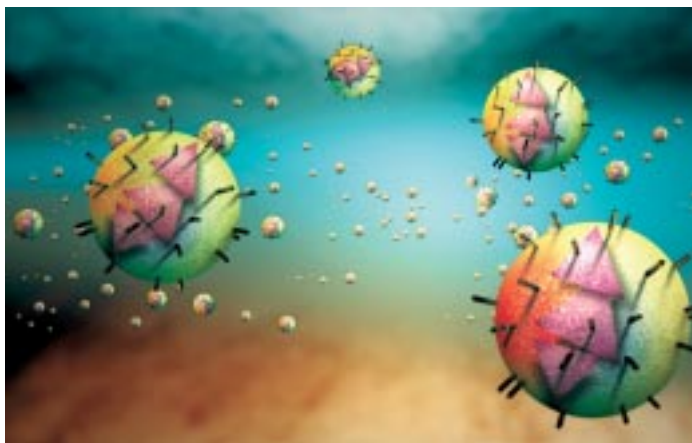
In addition to target evaluation, the STEP method – like other quantitative PCR techniques – is also used in other areas of biological science, e.g. in the development of model systems (for example new cell lines) and in basic research. The establishment of whether, when and how a gene is expressed provides important information on the role of the corresponding gene product in the body's molecular network. This approach has shed light on many biological processes, e.g. the body's response to external factors such as administration of drugs.

### Applications in the field of infectious diseases

Another important application of quantitative PCR is in molecular diagnosis, i.e. the diagnosis of diseases based on molecular findings rather than on physiological symptoms. In this connection the diagnosis of viral diseases is an area that is gaining increasing importance. For simple diagnostic testing, i.e. to determine if a pathogen is present in the patient's body, qualitative PCR is sufficient. However, to follow the progress of a disease and to help

choose the right treatment doctors often need to know the actual concentration of pathogens present. PCR is one of the few techniques available today that is able to measure the pathogen load. This is an important parameter, for example, in viral infections, which often follow a chronic course and produce no clinical effects for some time, despite infection and in some cases ongoing physical damage. In this case the viral load in the bloodstream can provide an indication of how the disease is progressing.

In addition, quantitative determinations serve to monitor the success of treatment. If a drug works in a patient, his/her pathogen load will decline sharply. However, some viruses change so rapidly that they cannot be completely eradicated by the drugs used: they become resistant to them. This often occurs, for example, in viral hepatitis C infection. The hepatitis C virus (HCV) often causes chronic inflammation of the liver, leading to liver cirrhosis or even cancer in a substantial proportion of those affected. Damage to the liver often accumulates over decades without being directly detectable. As mentioned earlier, qualitative PCR has been used for some years now for diagnosing HCV infection. But the quantitative form of the technique opens up whole new perspectives for the treatment of the disease. With the help of this method it is possible to monitor the success of treatment as well as determine how rapidly the disease is progressing, if at all. Unlike with conventional methods, it is also possible to ascertain whether the disease has been eradicated or has become chronic.



**Hepatitis C viruses.**

### **Example: HIV**

Another important application in which quantitative PCR is used in the field of infectious diseases is AIDS. Those infected with the causative agent of the disease, the human immunodeficiency virus (HIV), have to take a cocktail of usually three drugs indefinitely, this being the only way to keep the virus in check, if only for a while. HIV mutates extremely rapidly, quickly becoming resistant to drugs. Viruses that are resistant to all three drugs at the same time sometimes even occur, requiring a new cocktail to be used.

In order that this moment, known as a viral breakthrough, should not pass unnoticed – rapid proliferation of the viruses could cause the disease to flare up again – the quantity of viral particles in the blood must be measured periodically. A rise in the viral load indicates that the drugs being used are losing their efficacy. Quantitative PCR permits such monitoring and helps doctors adjust the treatment optimally.

### **Applications in cancer therapy**

Genetic factors are always involved in the development of cancer. Their contribution varies greatly depending on the type of cancer. Genes not only help to determine progression of the disease but can also have a substantial influence on the effectiveness of the available treatments. Identifying the genes that play a role in the development of cancer is therefore an important step towards improving treatment. Both qualitative and quantitative PCR play a crucial role in the fight against cancer. PCR can identify genes that have been implicated in the development of cancer. Often the genes exist in a number of variants with significantly different effects. One example is the gene known as p53, whose product is a central monitor of cellular division. If the function of this monitor is disrupted in a cell, the cell can become cancerous relatively easily. Variants of p53 and similar genes can be detected by qualitative PCR, giving doctors and patients an indication of their personal risk of developing cancer or – if the patient already has cancer – how aggressively it can be expected to progress. Because multiple changes have usually accumulated before cancer actually develops, a reliable test must examine a large number of gene variants. For this reason DNA chips are being increasingly used to screen people for genetic changes (see chapter on DNA chips).

**Example:**  
**promoter methylation**

Meanwhile, quantitative PCR also is gaining importance in the fight against cancer. This is the case where a cancer has a genetic basis but is not due to an altered gene. In some cancers the genes that control cellular division are intact but have been switched off. This can occur through a process known as promoter methylation. In the DNA region containing the start information for reading the downstream gene (the promoter) the cell attaches small molecules (methyl groups) to specific building blocks of the DNA (the cytosine bases). As a result, polymerases that normally read the genes and produce working copies of them are no longer able to dock to the start region. The gene therefore remains silent and no gene product is formed.

Only in recent years has it emerged that this mechanism of promoter methylation shuts down vital genes in many cancer cells. ‘Methylation status’ is therefore of crucial importance because it provides information on the chance of a tumour becoming malignant and giving rise to metastases. A simple and reliable method used for detecting these crucial DNA changes is methylation-specific PCR (MSP). In this relatively new technique cellular DNA is first treated with sodium bisulphite, which converts normal cytosine to the RNA building block uracil but leaves methylated cytosine intact. This results in different products depending on the methylation status of the DNA (see box). Specific primers for those products are used in the subsequent PCR procedure. In this way it can be determined if the original DNA was methylated or not.

**A host of other applications**

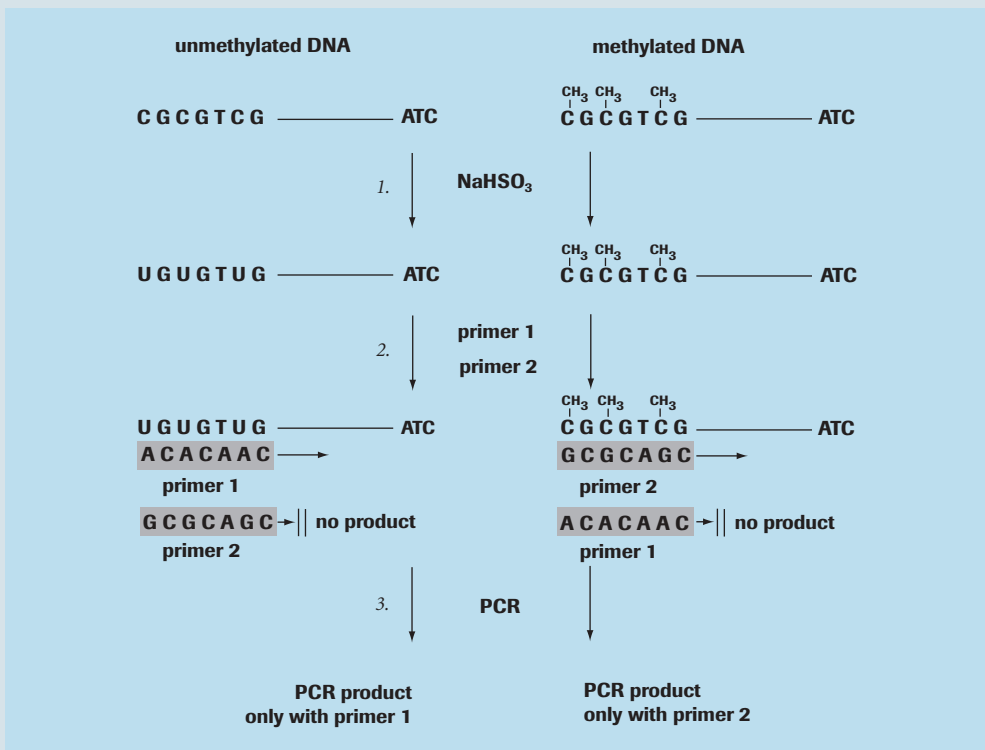
New applications for PCR are still emerging, particularly in the field of medicine. The search for genetic predispositions to diseases is an especially important area of research. In many cases the onset of a disease can be prevented or at least delayed by lifestyle modification or the taking of medications. One example of this is osteoporosis, a loss of bone density that is especially common in postmenopausal women. Because the disease tends to run in families, it is clear that genetic factors are involved in its development and progression. An intensive search for the genes involved is currently under way and has already produced some results. Several promising candidate genes have been identified that appear to be involved in osteoporosis. In this context PCR is not only an aid in the search for the culprit genes, but also of-



fers the possibility of identifying the responsible gene variants in patients.

Similarly, PCR is helping in the investigation and diagnosis of a growing number of diseases. It has also long been a standard method in all laboratories that carry out research on or with nucleic acids. Even competing techniques such as DNA chips often require amplification of DNA by means of PCR as an essential preliminary step.

### Identifying inactivated genes: methylation-specific PCR (MSP)



In many tumours important genes that control cell growth are switched off by methylation of the promoter region. These changes can be detected by means of methylation-specific PCR (MSP). In MSP all the normal cytosines (C) of the original DNA are converted to the RNA building block uracil. The methylated

cytosines, by contrast, remain unchanged (1). The subsequent PCR procedure then uses specific primers for the various products formed (2). Hence, either the original methylated or the unmethylated DNA is copied. The original DNA was therefore methylated or not (3) depending on the primer used to obtain a product.

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