CHAPTER TWENTY-THREE

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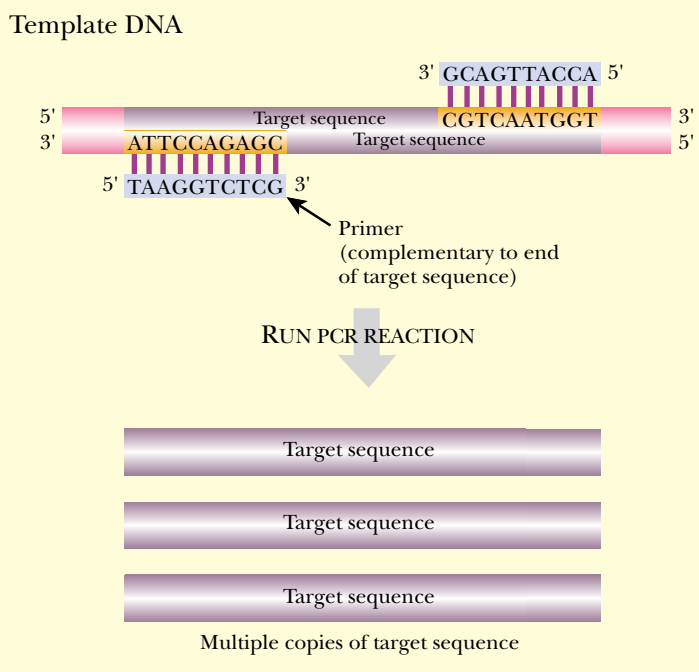

FIGURE 23.01 ***The Polymerase Chain Reaction (PCR)***

During PCR, two primers anneal to complementary sequences at either end of a target sequence on a piece of denatured template DNA. DNA polymerase synthesizes DNA, elongates the primers and makes two new strands of DNA, thus duplicating the original target sequence. In further cycles, the newly made DNA molecules are denatured in turn and duplicated by the same sequence of events, resulting in multiple copies of the original target sequence.

Fundamentals of the Polymerase Chain Reaction

The PCR allows trace amounts of a DNA sequence to be amplified giving enough DNA for cloning, sequencing or other analyses.

Of all the technical advances in modern molecular biology, the **polymerase chain reaction (PCR)** is one of the most useful. The PCR provides a means of amplifying DNA sequences. Starting with incredibly tiny amounts of any particular DNA molecule, the PCR can be used to generate microgram quantities of DNA. PCR is sufficiently sensitive that it can amplify the DNA from a single cell into amounts sufficient for cloning or sequencing. Consequently, PCR is used in clinical diagnosis, genetic analysis, genetic engineering and forensic analysis. In particular, PCR has revolutionized and speeded up the whole area of recombinant DNA technology. Previously, cloned DNA was made by growing up bacterial cultures and extracting and purifying the DNA. PCR allows the rapid generation of large amounts of specific DNA sequences that are easier to purify and less damaged. In this chapter we will examine how the polymerase chain reaction works. As the name indicates, DNA polymerase is used to manufacture DNA using a pre-existing DNA molecule as template. Each new DNA molecule synthesized becomes a template for generating more, thus creating a chain reaction. The PCR actually amplifies only a chosen segment (the **target sequence**) within the original DNA template, not the whole template DNA molecule (Fig. 23.01).

The components involved in the polymerase chain reaction are as follows:

1. The original DNA molecule that is to be copied is called the template and the segment of it that will actually be amplified is known as the target sequence. A trace amount of the DNA template is sufficient.
2. Two **PCR primers** are needed to initiate DNA synthesis. These are short pieces of single-stranded DNA that match the sequences at either end of the target DNA segment. PCR primers are made by chemical synthesis of DNA as described in Ch. 21.

polymerase chain reaction (PCR) Amplification of a DNA sequence by repeated cycles of strand separation and replication
PCR primers Short pieces of single-stranded DNA that match the sequences at either end of the target DNA segment and which are needed to initiate DNA synthesis in PCR
target sequence Sequence within the original DNA template that is amplified in a PCR reaction

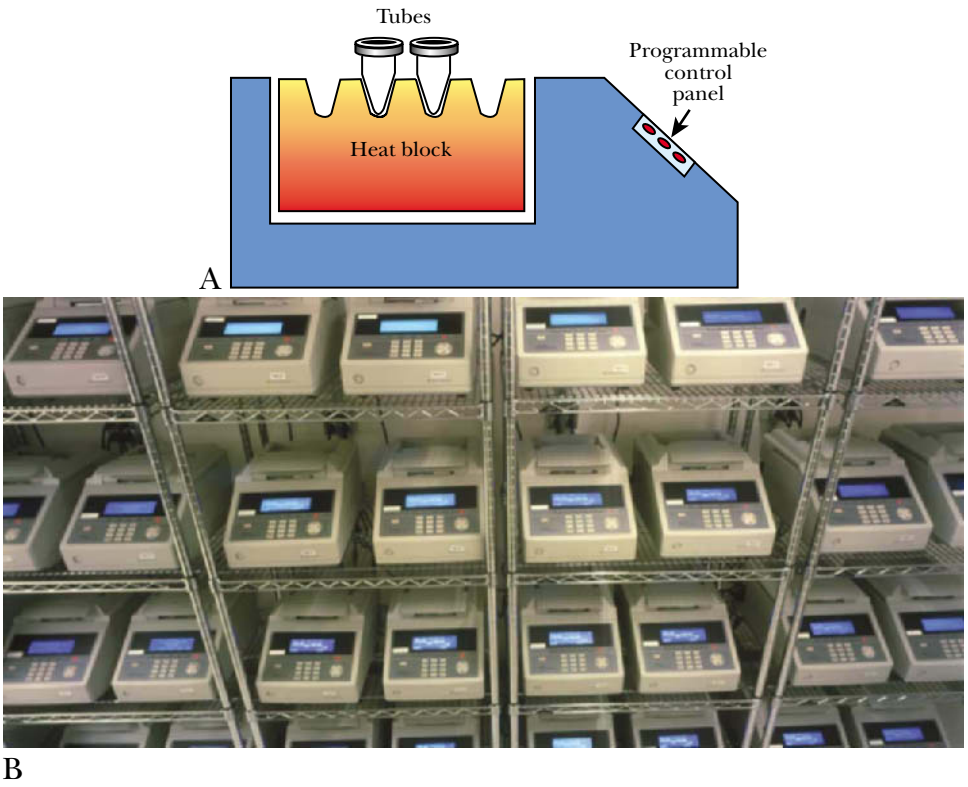

FIGURE 23.02 ***PCR Machine or Thermocycler***

(A) The thermocycler or PCR machine can be programmed to change temperature rapidly. The heat block typically changes from a high temperature such as 90°C (for denaturation) to 50°C (for primer annealing), then back to 70°C (for DNA elongation) in a matter of minutes. This may be repeated for many cycles. (B) Rows of GeneAmp PCR machines copying human DNA at the Joint Genome Institute, in Walnut Creek, California, a collaboration between three of the US Department of Energy's National Laboratories. Credit: David Parker, Science Photo Library.

3. The enzyme DNA polymerase is needed to manufacture the DNA copies. The PCR procedure involves several high temperature steps so a heat resistant DNA polymerase is required. This came originally from heat resistant bacteria living in hot springs at temperatures up to 90°C. **Taq polymerase** from ***Thermus aquaticus*** is most widely used.
4. A supply of nucleotides is needed by the polymerase to make the new DNA. These are supplied as the nuceoside triphosphates.
5. Finally we need a **PCR machine** to keep changing the temperature (Fig. 23.02). The PCR process requires cycling through several different temperatures. Because of this, PCR machines are sometimes called **thermocyclers**.

PCR needs primers to start DNA synthesis which means that we must know some DNA sequence in or close to the region of interest.

The requirement for primers means that some knowledge of the sequence of the DNA template is needed. As described in Chapter 24, ever-increasing quantity of genomic DNA sequences is now available. Unknown sequences are dealt with in a variety of ways (for some specialized approaches see below). However, since binding of a primer need not be perfect, related sequences can often be used successfully, especially if longer primers are used.

When Kary Mullis invented PCR in 1987, he used normal DNA polymerase. Since the temperature needed to separate DNA into single strands destroys this enzyme, he had to add a fresh dose of polymerase to each tube every cycle! Luckily, heat resistant DNA polymerase was purified from *Thermus aquaticus* just a year or two later. Taq polymerase can be added to the reaction mixture at the beginning and survives all of the heating steps. It actually requires a high temperature to manufacture new DNA.

PCR machine See thermocycler
Taq polymerase Heat resistant DNA polymerase from *Thermus aquaticus* that is used for PCR
thermocycler Machine used to rapidly shift samples between several temperatures in a pre-set order (for PCR)
Thermus aquaticus Thermophilic bacterium found in hot springs and used as a source of thermostable DNA polymerase

Kary Mullis Invents PCR after a Vision

"Science, like nothing else among the institutions of mankind, grows like a weed every year. Art is subject to arbitrary fashion, religion is inwardly focused and driven only to sustain itself, law shuttles between freeing us and enslaving us."—Kary Mullis

Kary Mullis won the Nobel Prize in Chemistry in 1993 for developing the polymerase chain reaction (PCR). The PCR is one of modern biology's most useful techniques and has been used in virtually every area of molecular biology and biotechnology. Kary Mullis is one of sciences true eccentrics. In addition to molecular biology he has also contributed to other areas of science. While a doctoral candidate working on bacterial iron transport, he published an article entitled "The Cosmological Significance of Time Reversal" (*Nature 218*:663 (1968)), which deals with his notion that about half of the mass in the universe is going backward in time.

Kary Mullis invented PCR while working as a scientist for the Cetus Corporation. He conceived the idea while cruising in a Honda Civic on Highway 128 from San Francisco to Mendocino in April 1983. Mullis recalls seeing the polymerase chain reaction as clear as if it were up on a blackboard in his head. In lurid pink and blue. He pulled over and started scribbling. One basic ingredient of the PCR is that it amplifies DNA by constant repetition—rather like the computer programs Mullis was then involved in writing. Kary Mullis was given a $10,000 bonus by Cetus, who at first failed to realize the significance of the discovery. Later they sold the technology to Roche for $300,000,000.

In 1999, Kary Mullis mentioned the computer DNA connection again, "It is interesting that biochemistry developed alongside computers. If computers had not come along at about the same time as the structure of DNA was discovered, there would be no biochemistry. You always needed the computer to process the information. Without it we would have rooms and rooms full of monks writing out the sequences."

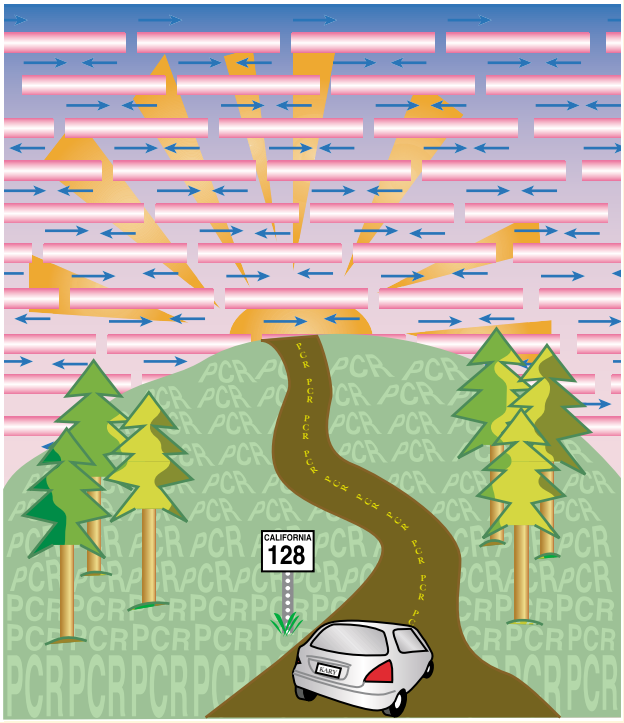

FIGURE 23.03 ***Kary Mullis Sees PCR in a Vision***

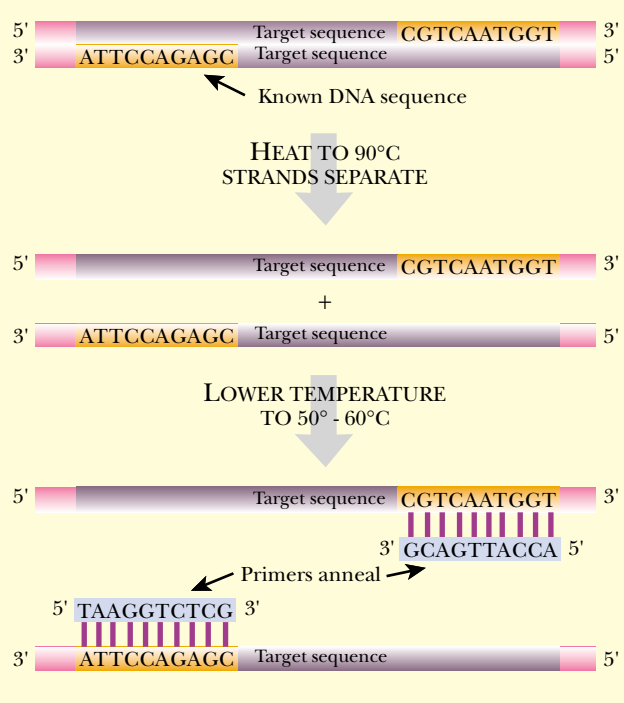

FIGURE 23.04 ***Denaturing the Template and Binding the Primers***

In the steps of PCR, a very small amount of template DNA is heated to 90°C, which separates the two strands of the double helix. When the temperature is lowered to 50–60°C, the primers can anneal to the ends of the target sequence. Since the primer is present in large excess over the template DNA, essentially all template strands will bind to primers rather than re-annealing to each other.

Cycling Through the PCR

PCR is a procedure involving multiple cycles of DNA strand separation, binding of primers, and synthesis of new DNA.

The first step of the PCR is to separate the strands of the template DNA by heating the template DNA to 90°C or so for a minute or two. Although the primers are present from the beginning, they cannot bind to the template DNA at 90°C. So, the temperature is dropped to around 50°C to 60°C, allowing the primers to anneal to the complementary sequences on the template strands (Fig. 23.04). Although the illustration shows 10 base primers, in real life they would be longer, say 15 to 20 bases. A longer primer is more specific for binding to the exact target sequence.

Next, the temperature is maintained at 70°C for a minute or two to allow the thermostable polymerase to elongate new DNA strands starting from the primers (Fig. 23.05). Remember that DNA polymerase cannot initiate the synthesis of a new strand, but can only elongate. In a living cell, RNA primers are used (Ch. 5) but artificial primers of single-stranded DNA are perfectly acceptable *in vitro*. Note that DNA synthesis goes from 5′ to 3′ for both new strands. This gives two partly double stranded pieces of DNA. Notice that the two new strands are not as long as the original templates. They are each missing a piece at the end where synthesis started. However, they are double-stranded over the region that matters, the target sequence. The cycle of events is then repeated many times.

The second cycle is shown in Figure 23.06. There are now four partly double-stranded pieces of DNA. Note again that although they vary in length, they all include double-stranded DNA from the target region.

As the cycles continue, the single strand overhangs are ignored and are rapidly outnumbered by segments of DNA containing only the target sequence. During the third cycle (Fig. 23.07), the first two pieces of double-stranded DNA that correspond exactly to the target sequence are made. These do not have any dangling single-stranded ends. Once past the first two or three cycles, the vast majority of the product is double-stranded target sequence with flush ends. Finally, the DNA generated is run on an agarose gel to assess the size of the PCR fragment.

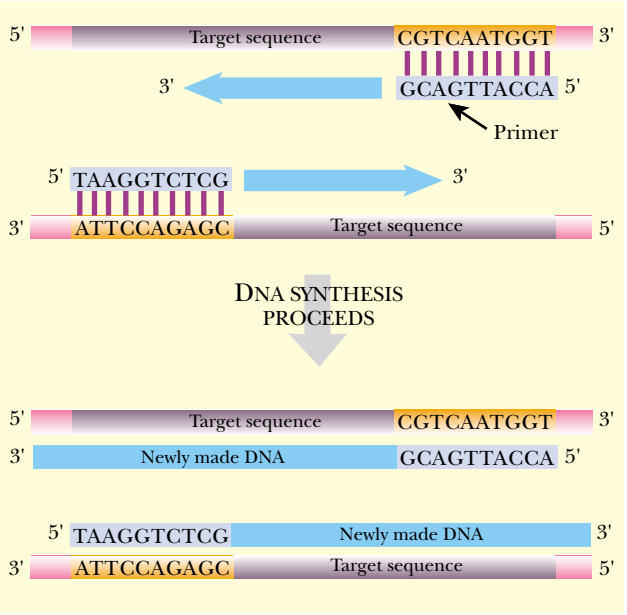

FIGURE 23.05 ***Elongation of New Strand by Taq Polymerase***

Once the primers have annealed to the template, the temperature is increased to 70°C. This is the optimum temperature for the thermostable Taq polymerase to elongate DNA. The polymerase synthesizes new strands of DNA using the 3′ end of the primer as a starting point. A pool of nucleotide precursors is also necessary for this step of the reaction.

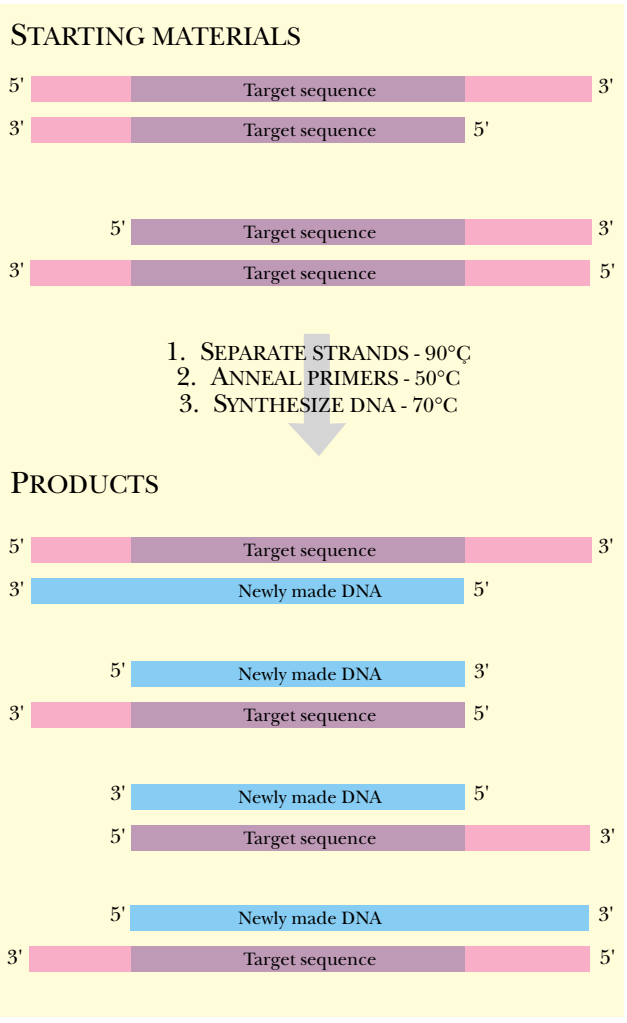

FIGURE 23.06 ***The Second Cycle of the PCR***

The entire cycle is repeated starting with the two DNA pieces produced in the first cycle. The two double-stranded pieces of DNA are denatured into four single-stranded pieces at 90°C. The temperature drops to 50°C in order for the primers to anneal. Finally, the polymerase extends the DNA at 70°C to convert the four single-stranded templates into double-stranded DNA.

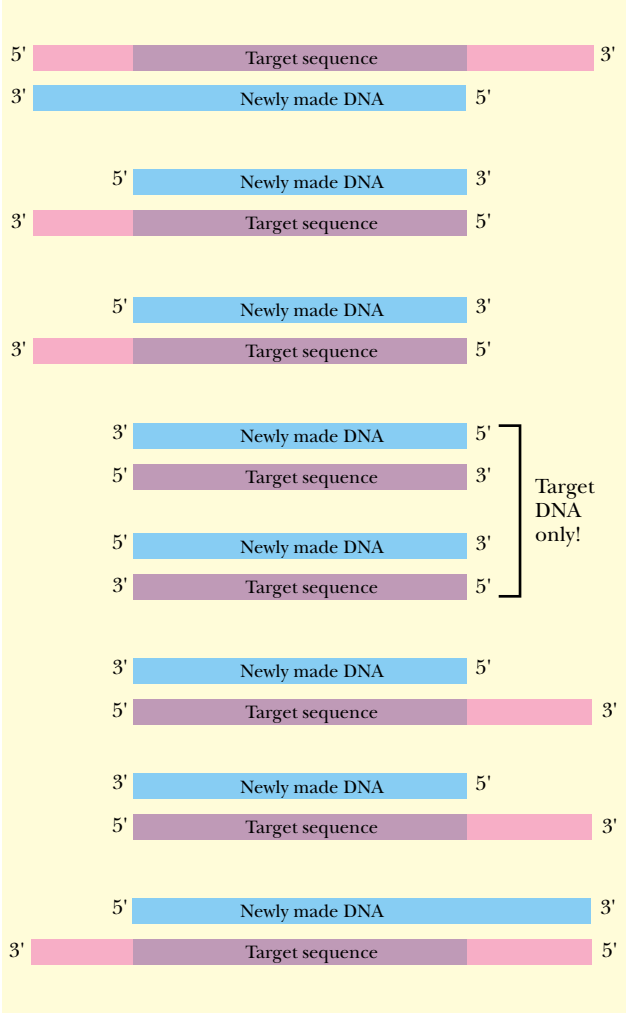

FIGURE 23.07 ***The Third Cycle of the PCR***

The products from the second cycle go through the same process as before. The four double-stranded pieces are denatured into eight single-stranded pieces. The primers anneal and DNA polymerase makes the complementary strands. After this cycle, the number of sequences containing only the target DNA grows exponentially, far exceeding any other product shown in this figure.

Degenerate Primers

The major problem with PCR is obvious. In order to make the PCR primers, some sequence information is required, at least at the ends of the target sequence. **Degenerate primers** are used when partial sequence information is available, but the complete sequence is unknown. For example, we may possess the sequence for a gene from one organism and be interested in obtaining the corresponding gene from another organism. If two organisms are related, their DNA sequences for a particular gene will be close, although rarely identical. Furthermore, the genetic code is degenerate and several codons can encode the same amino acid (see Ch. 8). In particular, many codon families share the first two bases and vary only in the third position. Since the sequence of the protein, rather than the DNA, is most important for function, most of the variation between closely related genes is in the third codon position.

Therefore, degenerate or redundant DNA primers are made that have a mixture of all possible bases in every third position. A degenerate primer is actually a mixture of closely related primers (Fig. 23.08). Presumably one of the primers in this mixture will recognize the DNA of the gene of interest. In addition, a perfect match is not really necessary. If, say, 18 or 19 of 20 bases pair up, a primer will work quite well. Many segments of DNA have been amplified successfully by PCR using sequence data from close relatives.

If only protein sequences are available, degenerate primers are used for PCR.

degenerate primer Primer with several alternative bases at certain positions

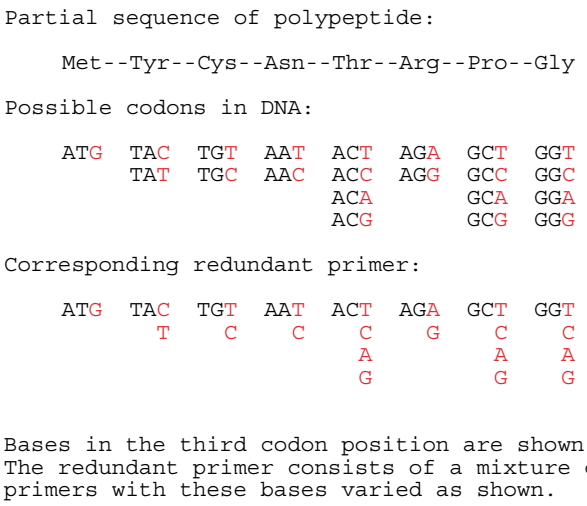

FIGURE 23.08 ***Degenerate DNA Primers***

Degenerate primers are used if only partial DNA sequence information is available. Often, as here, a short amino acid sequence from a protein is known. Because many amino acids are encoded by several alternative codons, the deduced DNA coding sequence is ambiguous. For example, the amino acid tyrosine is encoded by TAC or TAT. Hence the third base is ambiguous and when the primer is synthesized a 50 : 50 mixture of C and T will be inserted at this position. This ambiguity occurs for all the bases shown in red, resulting in a pool of primers with different, but related sequences. Hopefully, one of these primers will have enough complementary bases to anneal to the target sequence that is to be amplified.

Degenerate DNA primers must also be used if only a protein sequence is available. In this case, the protein sequence is translated backwards to give the corresponding DNA sequence. Due to the degeneracy of the genetic code, several possibilities will exist for the sequence of DNA that corresponds to any particular polypeptide sequence. Again, most of the ambiguity is in the third codon position. This ambiguous sequence may be used to make degenerate primers, as before (Fig. 23.08). Although proteins are rarely sequenced in their entirety nowadays, short stretches of N-terminal sequence are often obtained. After separating proteins, automated N-terminal sequencing may yield a sequence of the first dozen or more amino acids. This is often sufficient to allow design of a degenerate probe for hybridization screening of a gene library (see Ch. 22) or degenerate primers for PCR.

Inverse PCR

Another approach that uses incomplete sequence information to amplify a target gene is **inverse PCR**. In this case a sequence of part of a long DNA molecule, say a chromosome, is known. The objective is to extend the analysis along the DNA molecule into the unknown regions. To synthesize the primers for PCR, the unknown target sequence must be flanked by two regions of known sequence. The present situation is exactly the opposite of that. To circumvent this problem, the target molecule of DNA is converted into a circle. Going around a circle brings you back to the beginning. In effect, even though, only one small stretch of sequence is known, the circular form allows you to have that one region on both sides of the target sequence.

Performing PCR on a circularized DNA template allows access to neighboring regions of unknown sequence.

A restriction enzyme, usually one that recognizes a six-base sequence, is used to make the circle. This enzyme must not cut into the known sequence, therefore, eventually, this enzyme will cut either upstream or downstream from the known region.

inverse PCR Method for using PCR to amplify unknown sequences by circularizing the template molecule

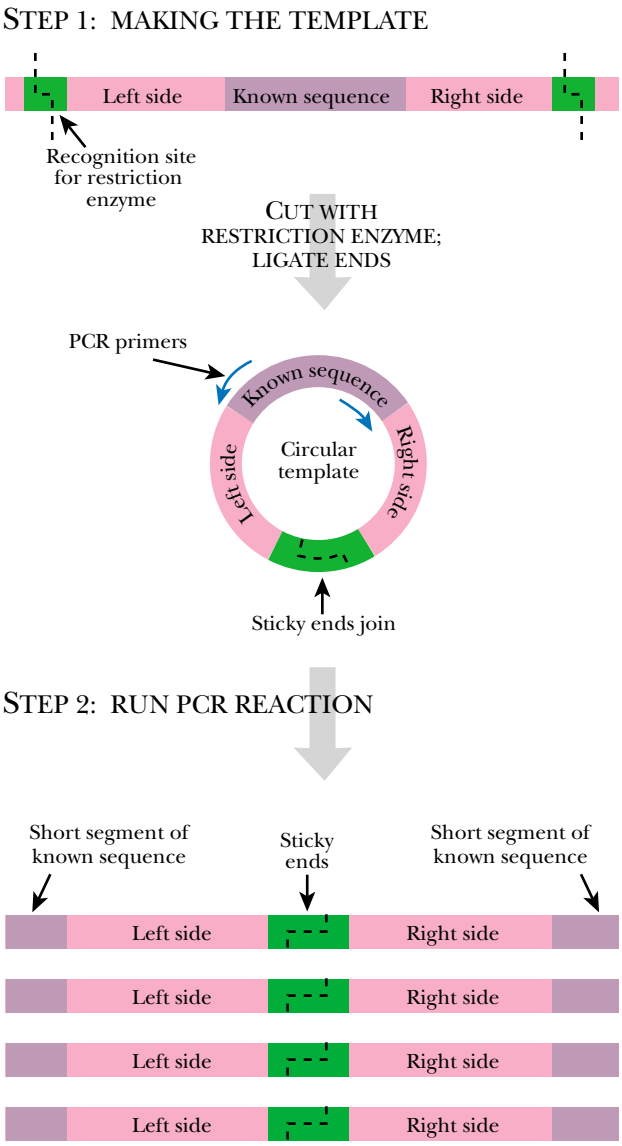

FIGURE 23.09 ***Inverse PCR***

Inverse PCR allows unknown sequences to be amplified by PCR provided that they are located next to DNA whose sequence is already known. The DNA is cut with a restriction enzyme that does not cut within the region of known sequence, as shown in Step I. This generates a fragment of DNA containing the known sequence flanked by two regions of unknown sequence. Since the fragment has two matching sticky ends, it may be easily circularized by DNA ligase. Finally, PCR is performed on the circular fragments of DNA (Step 2). Two primers are used that face outwards from the known DNA sequence. PCR amplification gives a single linear product that includes unknown DNA from both left and right sides. This PCR product can now be cloned and/or sequenced.

The resulting fragment will have unknown sequence first, the known sequence in the middle, followed by more unknown sequence. The two ends of the fragment will have compatible sticky ends that are easily ligated together to make a circle of DNA (Fig. 23.09). Two primers corresponding to the known region and facing outwards around the circle are used for PCR. Synthesis of new DNA will proceed around the circle clockwise from one primer and counter-clockwise from the other. Overall, inverse PCR gives multiple copies of a segment of DNA containing some DNA to the right and some DNA to the left of the original known region.

Adding Artificial Restriction Sites

Artificial restriction sites are often added to the ends of PCR products to aid in cloning.

Once a segment of DNA has been amplified by PCR it may be sequenced (see Ch. 24) or cloned. For cloning it is often convenient to use restriction enzymes to generate sticky ends on both insert and vector (see Ch. 22). However, it is unlikely that such sites will be located just at the ends of any particular target sequence. One way to create convenient restriction cut sites at the end of PCR fragments is to incorporate them into the primers. When designing the primers, artificial

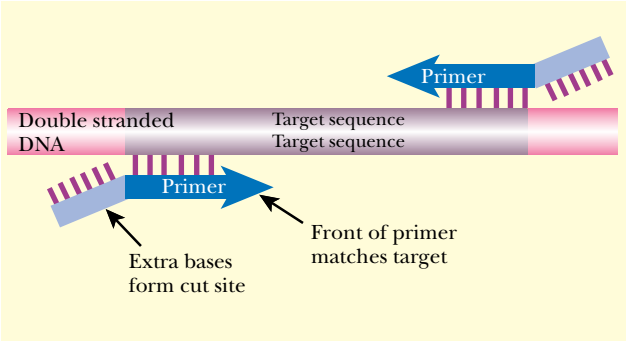

FIGURE 23.10 ***Incorporation of Artificial Restriction Sites***

Primers for PCR can be designed to have non-homologous regions at the 5′ end that contain the recognition sequence for a particular restriction enzyme. After PCR, the amplified product has the restriction enzyme site at both ends. If the PCR product is digested with the restriction enzyme, this generates sticky ends that are compatible with a chosen vector.

restriction enzyme recognition sites are added at the far ends of the primers (Fig. 23.10). As long as the primer has enough bases to match its target site, adding a few extra bases at the end will not affect the PCR reaction. The bases making up the restriction site get copied and appear on the ends of all newly manufactured segments of DNA. After the PCR reaction has been run, the PCR fragment is cut with the chosen restriction enzyme to generate sticky ends. The fragment is then cloned into a convenient plasmid.

TA Cloning by PCR

Single base overhangs may be used to clone PCR products.

Another approach to cloning PCR products takes advantage of the properties of the Taq polymerase itself. This enzyme adds a single adenosine to the 3′-ends of double-stranded DNA. This reaction does not depend on the sequence of the template or primers. The **TA cloning** procedure exploits this terminal transferase activity, which is shared by Taq polymerase and several other thermophilic DNA polymerases. Thus most of the DNA molecules amplified by Taq polymerase possess single 3′-A overhangs (Fig. 23.11). Consequently, they can be directly cloned into a vector that has matching single 3′-T overhangs on both ends (after being linearized). The same **TA cloning vector** can be used to clone any segment of amplified DNA. For that matter, DNA from other sources can have single 3′-A overhangs added to its ends by using Taq polymerase and can then be cloned by the same mechanism. This procedure is especially useful when convenient restriction sites are not available.

Randomly Amplified Polymorphic DNA (RAPD)

Randomly Amplified Polymorphic DNA, or RAPD, is usually found in the plural as RAPDs and is pronounced "rapids," partly because it is a quick way to get a lot of information about the genes of an organism under investigation. The purpose of RAPDs is to test how closely related two organisms are. In practice, DNA samples from unknown organisms are compared with DNA from a previously characterized organism. For example, traces of blood from a crime scene may be compared to

randomly amplified polymorphic DNA (RAPD) Method for testing genetic relatedness using PCR to amplify arbitrarily chosen sequences
TA cloning Procedure that uses Taq polymerase to generate single 3′-A overhangs on the ends of DNA segments that are used to clone DNA into a vector with matching 3′-T overhangs
TA cloning vector Vector with single 3′-T overhangs (in its linearized form) that is used to clone DNA segments with single 3′-A overhangs generated by Taq polymerase

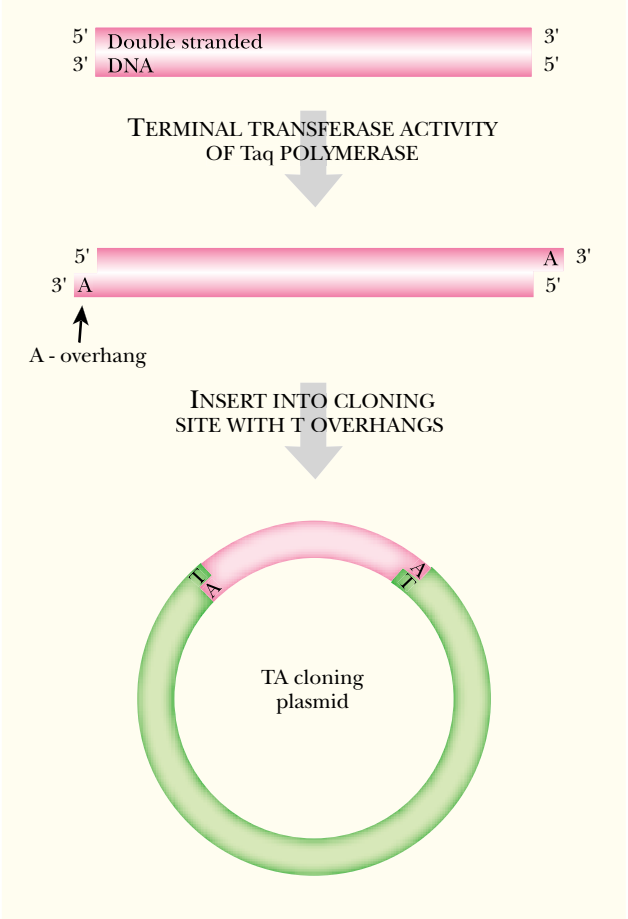

FIGURE 23.11 ***TA Cloning***

When Taq polymerase amplifies a piece of DNA during PCR, the terminal transferase activity of Taq adds an extra adenine at the 3′ end of the PCR product. The TA cloning vector was designed so that when linearized it has single 5′ thymidine overhangs. The PCR product can be ligated into this vector without the need for special restriction enzyme sites.

possible suspects, or disease-causing microorganisms may be related to known pathogens to help trace an epidemic.

PCR may be performed with arbitrary primers. Comparing results from two samples of DNA reveals their relatedness.

The principle of RAPDs is statistically based. Given any particular five-base sequence, such as ACCGA, how often will this exact sequence appear in any random length of DNA? Since there are four different bases to choose from, one in every 4^5 (or $4 \times 4 \times 4 \times 4 \times 4 = 1{,}024$) stretches of five bases will—on average—be the chosen sequence. Any arbitrarily chosen 11-base sequence will be found once in approximately every 4 million bases. This is approximately the amount of DNA in a bacterial cell. In other words, any chosen 11-base sequence is expected to occur by chance once only in the entire bacterial genome. For higher organisms, with much more DNA per cell, a longer sequence would be needed for uniqueness.

For RAPDs, the arbitrarily chosen sequence should be rare but not unique. PCR primers are made using the chosen sequence and a PCR reaction is run using the total DNA of the organism as a template. Every now and then a primer will find a correct match, purely by chance, on the template DNA (Fig. 23.12). For PCR amplification to occur there must be two such sites facing each other on opposite strands of the DNA. The sites must be no more than a few thousand bases apart for the reaction to work well. The likelihood of two correct matches in this arrangement is quite low.

In practice, the length of the primers is chosen to give five to 10 PCR products. For higher organisms, primers of around 10 bases are typical. The bands from PCR are separated by gel electrophoresis (see Ch. 21) to measure their sizes. The procedure is repeated several times with primers of different sequence. The result is a diagnostic

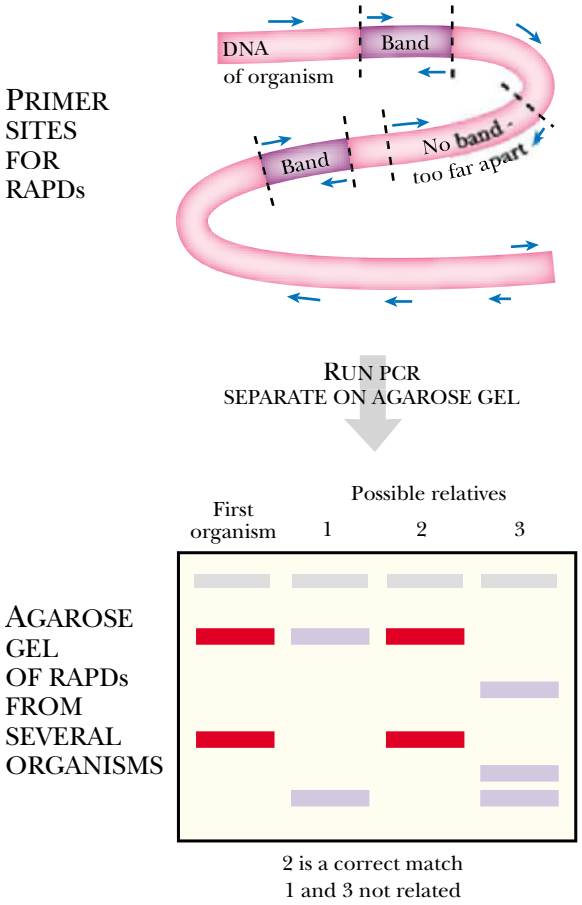

FIGURE 23.12 ***Randomly Amplified Polymorphic DNA***

The first step of RAPD analysis is to design primers that will bind to genomic DNA at random sites that are neither too rare or too common. In this example, the primers were sufficiently long to bind the genomic DNA at a dozen places. For PCR to be successful, two primers must anneal at sites facing each other but on opposite strands. In addition, these paired primer sites must be close enough to allow synthesis of a PCR fragment in a reasonable time. In our example, there are three pairs but only two of these pairs were close enough to actually make the PCR product. Consequently, this primer design will result in two PCR products as seen in the first lane of the gel (marked "First organism"). The same primers are then used to amplify genomic DNA from other organisms that are suspected of being related. In this example, suspect #2 shows the same banding pattern as the first organism and is presumably related. The other two suspects do not match the first organism and are therefore not related.

pattern of bands that will vary in different organisms, depending on how closely they are related. Although we do not know in which particular genes the PCR bands originate, this does not matter in measuring relatedness. Diagnosis therefore relies on having a primer (or set of primers) that reliably give a band of a particular size with the target organism and give different bands with other organisms, even those closely related. RAPD results using such a primer are shown in Figure 23.13. Grey mold, due to *Botrytis cinerea*, is one of the most destructive infections of strawberries and also attacks other plants. Classical diagnosis involves culturing the fungus on nutrient agar. It is slow and difficult due to the presence on the plants of other harmless fungi, which often grow faster in culture. As can be seen, RAPD analysis clearly identifies the pathogens from other related fungi, including other species from the genus *Botrytis*.

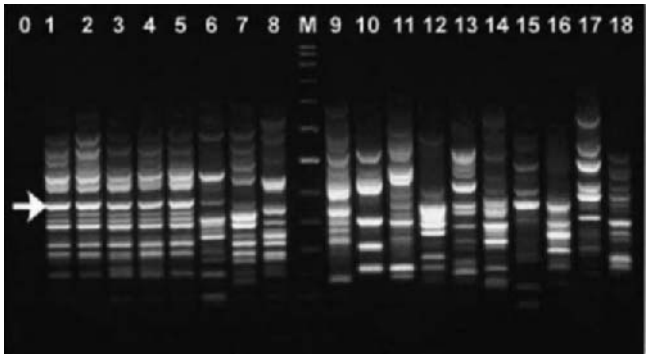

FIGURE 23.13 ***Identification of Fungal Pathogens by RAPD***

RAPD banding patterns generated using the 10 base primer, AACGCGCAAC, on genomic DNA of five closely related strains of the pathogen *Botrytis cinerea* (lanes 1–5), three other strains from the genus *Botrytis* (lanes 6, 7 & 8) and several less related harmless fungi *Alternaria* (9), *Aspergillus* (10), *Cladosporium* (11), *Epicoccum* (12), *Fusarium* (13), *Hainesia* (14), *Penicillium* (15), *Rhizoctonia* (16 & 17), and the host plant, strawberry (18). Lane 0: negative control (no DNA). Lane M: molecular mass marker. From: Rigotti et al., FEMS Microbiology Letters (2002) 209:169–174.

Reverse Transcriptase PCR

Reverse transcription followed by PCR allows cloning of genes starting from the messenger RNA.

The coding sequence of most eukaryotic genes is interrupted by intervening sequences, or introns (see Ch. 12 for introns and RNA processing). Consequently, the original version of a eukaryotic gene is very large, difficult to manipulate and virtually impossible to express in any other type of organism. Since mRNA has had the introns removed naturally, it may be used as the source of an uninterrupted coding sequence that is much more convenient for engineering and expression. This involves converting the RNA back into a DNA copy, known as **complementary DNA (cDNA)** by **reverse transcriptase** (see Ch. 22). Thus, when "cloning" eukaryotic genes the cDNA version is often used (rather than the true chromosomal gene sequence) as this lacks the introns. Once the cDNA has been made, PCR can be used to amplify the cDNA and generate multiple copies (Fig. 23.14). This combined procedure is referred to as **reverse transcriptase PCR (RT-PCR)** and allows genes to be amplified and cloned as intron-free DNA copies starting from mRNA.

RT-PCR has other uses. A specific mRNA molecule is only made when the gene for that protein is turned on and expressed. Therefore extraction and purification of the mRNA gives several mRNA copies of every gene that is being expressed under the particular growth conditions. RT-PCR can then performed on the mixture of mRNA using PCR primers that match some particular gene of interest. If this gene was expressed under the specific growth conditions, a PCR product will be produced, whereas, if the gene was switched off, none of this particular mRNA will be present and no band will be generated (Fig. 23.15). Carrying out RT-PCR on an organism under different growth conditions reveals when the gene under scrutiny was switched on. This allows analysis of which environmental factors bring about expression of any chosen gene.

complementary DNA (cDNA) Version of a gene that lacks the introns and is made from the corresponding mRNA by using reverse transcriptase
reverse transcriptase Enzyme that starts with RNA and makes a DNA copy of the genetic information
reverse transcriptase PCR (RT-PCR) Variant of PCR that allows genes to be amplified and cloned as intron-free DNA copies by starting with mRNA and using reverse transcriptase

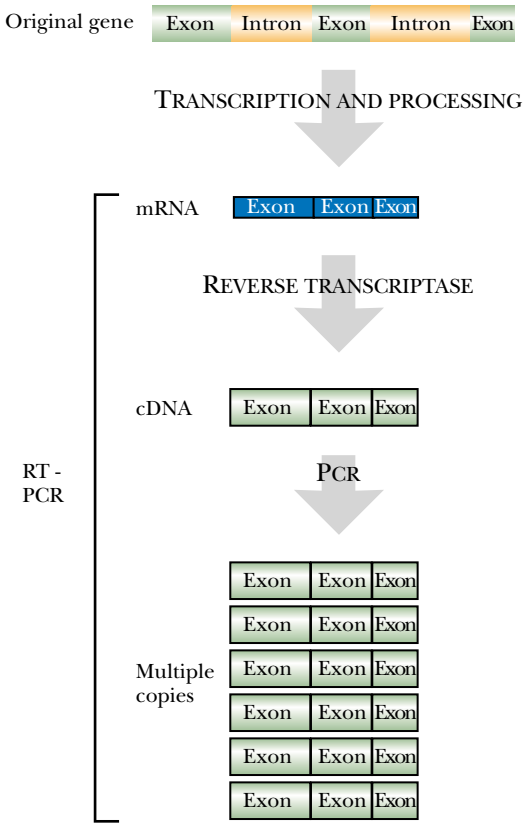

FIGURE 23.14 ***Reverse Transcriptase PCR***

RT-PCR is a two-step procedure that involves making a cDNA copy of the mRNA, then using PCR to amplify the cDNA. First, a sample of mRNA (which lacks introns) is isolated. Reverse transcriptase is used to make a cDNA copy of the mRNA. The cDNA sample then amplified by PCR. This yields multiple copies of cDNA without introns.

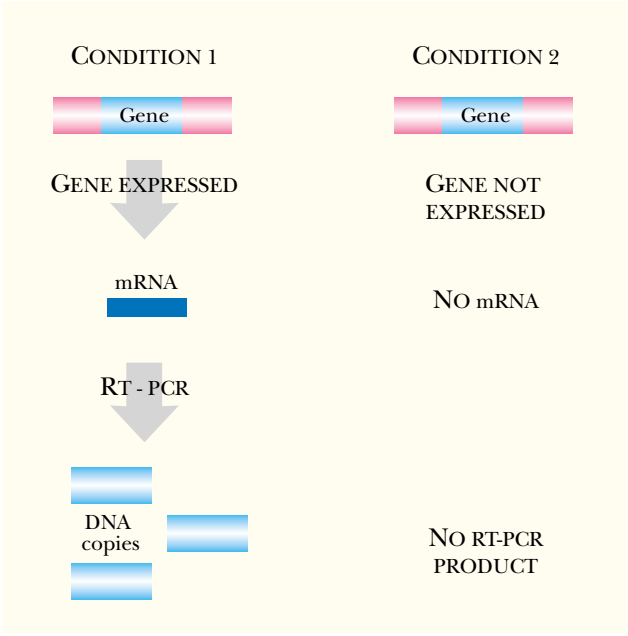

FIGURE 23.15 ***RT-PCR for Gene Expression***

RT-PCR can be used to determine whether or not mRNA corresponding to a particular gene is present. In other words, gene expression may be tested for an organism grown under two different conditions. In this example, the gene of interest is expressed in condition 1 but not in condition 2. Therefore in condition 1 mRNA from the gene of interest is present and reverse transcriptase generates the cDNA. The PCR primers specific for this gene can now bind to the cDNA and PCR will amplify a DNA band corresponding to the original mRNA. In condition 2 the mRNA is absent and so the RT-PCR procedure does not generate the corresponding DNA band.

Differential Display PCR

The mixture of mRNA made by a cell can be surveyed by a PCR based approach.

Differential display PCR is used to specifically amplify messenger RNA from eukaryotic cells. The technique is valuable because it allows the researcher to assess the expression of many different mRNA molecules simultaneously. This technique is a

differential display PCR Variant of RT-PCR that specifically amplifies messenger RNA from eukaryotic cells using oligo(dT) primers

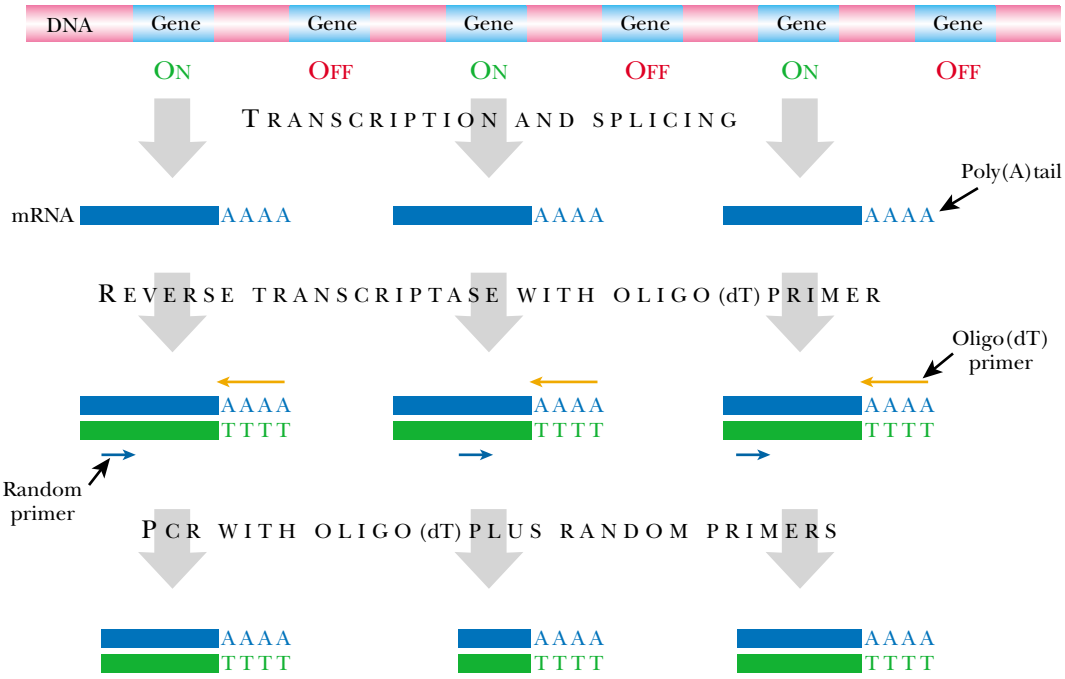

FIGURE 23.16 ***Differential Display PCR***

Differential Display PCR allows simultaneous measurement of the expression of many different mRNA molecules. The example shows that under the conditions used, three of the genes are turned on and three are turned off. Those mRNA molecules that are expressed are converted to cDNA by reverse transcriptase using an oligo(dT) primer, then amplified by PCR. The first PCR primer is oligo(dT) and so binds to the poly(A) sequences. The second PCR primer is a mixture of random sequences, calculated to anneal approximately once per cDNA. These primers ensure that many different cDNA molecules are amplified rather than just one. In this example, three PCR products are produced, corresponding to the original genes that are expressed.

combination of RAPD (see above) with RT-PCR and has one clever modification of its own, the use of oligo(dT) primers. Since almost all eukaryotic mRNA molecules have a 3′-tail of poly(A), an artificial primer made only of dT will base pair to this tail. This method of PCR allows the researcher to compare two different growth conditions on many different genes, rather than just one gene as in RT-PCR.

As in RT-PCR the RNA is extracted from the cells and the corresponding cDNA is made by the use of reverse transcriptase. Then a PCR reaction is run with two primers (Fig. 23.16):

1. An oligo(dT) primer that binds to the 3′ end of all cDNA copies of messenger RNA
2. Since the sequences at the other end of the mRNA molecules are unknown, the second primer is actually a mixture of random primers similar to those used in RAPDs.

These two primers ensure that there are not too many or too few amplified fragments.

The result is the amplification of many different DNA segments corresponding to each of the messenger RNA molecules in the original mixture. As usual, gel electrophoresis is used to separate the different components. This gives a series of DNA bands corresponding to each of the mRNAs being made in the cells that were analyzed. If the growth conditions are then altered, the pattern of DNA bands will change. In many cases multiple bands will appear, and multiple bands will disappear, thus allowing multiple random genes to be analyzed rather than just a single gene of interest as in RT-PCR.

Rapid Amplification of cDNA Ends (RACE)

Rescuing the "lost" ends of cloned genes may be done by a complex PCR based procedure.

Using only reverse transcriptase, full-length cDNA copies may be hard to get, especially from mRNA that is present only in very low amounts or unusually long. Reverse transcriptase often fails to reach the end of a long RNA template due to hindrance by RNA secondary structure. Thus the 5′-end is often incomplete. Consequently some means of recovering the complete cDNA is needed. The **RACE** technique generates the complete cDNA in two halves; hence the name **rapid amplification of cDNA ends**. It is necessary to know part of the internal sequence of the mRNA/cDNA in order to design the internal primers; therefore, the technique is generally used when an incomplete cDNA was isolated by other techniques such as library screening (see Ch. 22). The RACE procedure is essentially a modification of RT-PCR, but unique so-called **anchor sequences** are added to each end of the cDNA to facilitate the PCR portion of the reaction (Fig. 23.17).

The 3′-reaction of RACE-PCR primes reverse transcriptase to synthesize a DNA copy from the poly(A) tail of the mRNA by using an oligo(dT) primer that has a unique anchor sequence at the 5′ end. Since the internal sequence is known, an internal primer is designed so that PCR will amplify from the poly(A) tail to the middle of the gene. In the 5′-reaction, the internal primer is used to initiate DNA synthesis using reverse transcriptase. Next, an artificial poly(A) tail is added to the 3′ end of the DNA by terminal transferase and dATP. The same oligo(dT)/anchor primer as used to initiate the 3′-reaction is then used again during the PCR amplification cycles for the 5′-reaction. The anchor sequence primer and internal primers are generally designed to include convenient restriction sites to allow further cloning and sequencing.

PCR in Genetic Engineering

A whole plethora of modifications has been made to the basic PCR scheme. Some of these are used to alter genes rather than to analyze them. We can divide these modifications into two broad categories: a) rearranging large stretches of DNA and b) changing one or two bases of a DNA sequence. The latter is discussed below as "directed mutagenesis".

As already illustrated, we can amplify any segment of DNA provided we have primers that match its ends. Such segments of DNA may be joined or rearranged in a variety of ways. In order to make a hybrid gene, segments of two different genes must be amplified by PCR and then joined together. There are several protocols that vary in their details. But the crucial point is to use an **overlap primer** that matches part of both gene segments (Fig. 23.18).

Using overlap primers whose sequence matches the ends of two DNA molecules allows them to be fused end to end by PCR.

The PCR reaction is run using a primer for the front end of the first gene, a primer for the rear end of the second gene, and the overlap primer. The result is a hybrid gene. Some variants of this **"molecular sewing"** make the two halves separately and mix and join them later; other versions of this technique mix all three primers plus both templates in a single large reaction. By making hybrid genes using components from various sources it is sometimes possible to work out in detail which regions of a gene or protein are responsible for precisely which properties. The approach can also be used in biotechnology to construct artificial genes made up of modules from different sources.

anchor sequence Sequence added to primers or probes that may be used for binding to a support or may incorporate convenient restriction sites, primer binding sites for future manipulations, or primer bindings sites for subsequent PCR reactions
molecular sewing Creation of a hybrid gene by joining segments from multiple sources using PCR
overlap primer PCR primer that matches small regions of two different gene segments and is used in joining segments of DNA from different sources
RACE See rapid amplification of cDNA ends
rapid amplification of cDNA ends (RACE) RT-PCR-based technique that generates the complete 5′ or 3′ end of a cDNA sequence starting from a partial sequence

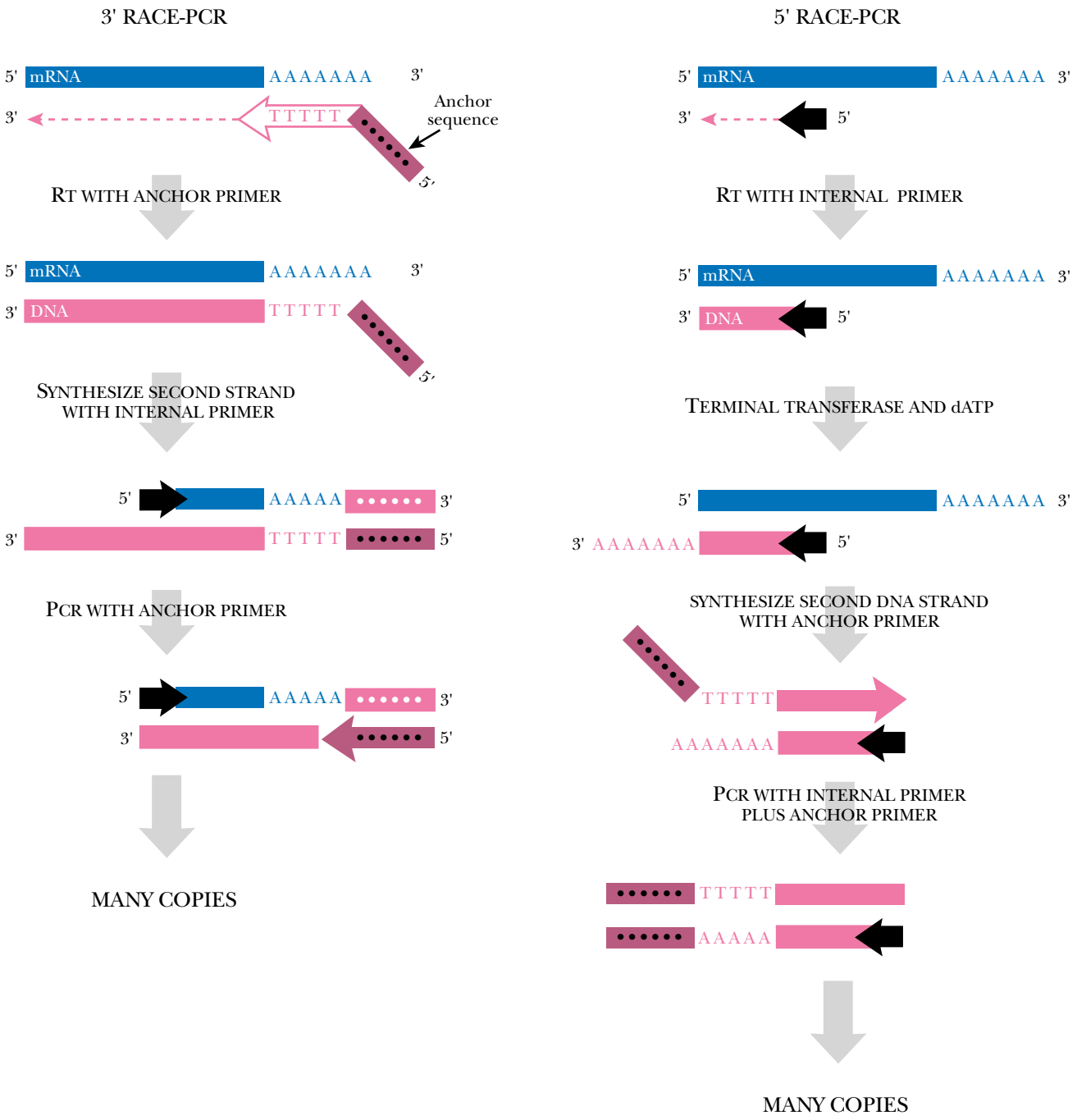

FIGURE 23.17 ***Rapid Amplification of cDNA Ends (RACE)***

RACE can be used to isolate the 5′ and/or 3′ ends of a cDNA that is incomplete. The method to amplify the 3′ end of the cDNA is shown on the left side of the figure. This requires an oligo(dT) primer that has an anchor sequence at the 5′ end. This primer is used with reverse transcriptase to make the mRNA : DNA hybrid molecule. The mRNA portion of this is removed and a second strand of DNA is synthesized. Instead of priming the second strand from the beginning of the DNA, an internal primer closer to the 3′ end of the gene is used. The same internal primer and a primer corresponding to the anchor sequence are then used in a standard PCR reaction to amplify just the 3′ end of the cDNA.

The right side of the figure shows how the 5′ end of a cDNA is isolated. An internal primer is designed to prime reverse transcriptase and make a hybrid molecule of mRNA : DNA. In order to add a primer binding site upstream of the end of the hybrid molecule, the enzyme, terminal transferase, is added together with dATP. This enzyme adds a run of adenines to the 3′ end of the DNA half of the hybrid. The mRNA half of the hybrid is then removed and replaced with DNA by using an oligo(dT) primer carrying the anchor sequence. The oligo(dT) binds the newly synthesized poly(A) stretch on the DNA, and primes the polymerase to make a cDNA. Subsequent PCR using the internal primer and the anchor primer amplify only the 5′ end of the cDNA.

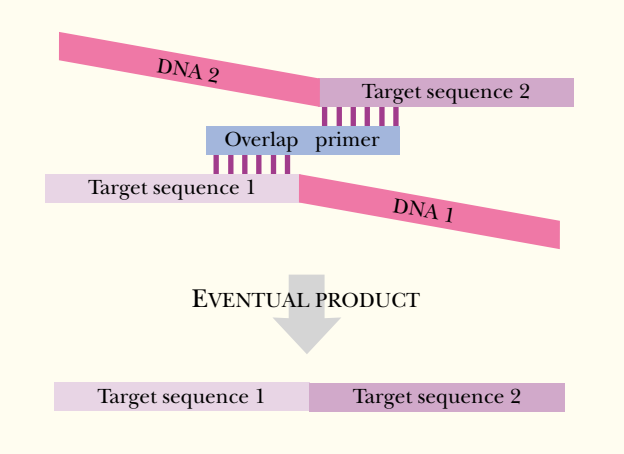

FIGURE 23.18 ***Synthesis of Hybrid Gene by Using Overlap Primers***

Overlapping primers can be used to link two different gene segments. In this scheme, the overlapping primer has one end with sequences complementary to target sequence 1, and the other half similar to target sequence 2. The PCR reaction will create a product with these two regions linked together.

Directed Mutagenesis

Mutations may be inserted artificially into DNA by altering a few bases in a PCR primer.

The term **directed mutagenesis** refers to a wide variety of *in vitro* techniques that are used to deliberately change the sequence of a gene. Several of these techniques use a PCR approach. The most obvious way to change one or two bases in a segment of DNA is to synthesize a PCR primer that carries the required alterations. Consider the sequence AAG CCG G**A**G GCG CCA. Suppose we wish to alter the A in the middle of this sequence to an T. Then we make a PCR primer with the required base alteration; that is, AAG CCG G**T**G GCG CCA. This mutant primer is used as one of a pair of PCR primers to amplify the appropriate segment of DNA using wild type DNA as template. The PCR product will contain the desired mutation, close to one end. As long as this primer is long enough to bind to the correct location on either side of the mutation, the DNA product will incorporate the change made in the primer. The mutant PCR product must then be reinserted into the original gene at the correct place.

Another less controlled way to introduce mutations into PCR products is to use manganese. Taq polymerase requires magnesium ions for proper function. Replacement of magnesium by manganese allows the enzyme to continue to synthesize DNA but the accuracy is greatly reduced. This approach introduces random base changes and yields a mixture of different mutations from a single PCR reaction. The error rate depends on the manganese concentration, so it is possible to get single or multiple mutations as desired.

Engineering Deletions and Insertions by PCR

Overlap primers may be used to precisely insert segments of foreign DNA into a chromosome or other DNA molecule.

PCR is widely used to generate DNA cassettes that can be introduced into chromosomes by homologous recombination. In this procedure, a convenient marker gene, usually an antibiotic resistance gene, is inserted into the chromosome of the host organism where it replaces any chosen gene. In order to target the incoming cassette to the correct location it must first be flanked with DNA sequences homologous to DNA both upstream and downstream of the chosen gene. This is done by using PCR primers that overlap the resistance cassette and also contain about 40–50 bp of DNA homologous in sequence to the target location (Fig. 23.19). The cassette is transformed into the host organism and is inserted into the chromosome by homologous recombination. Antibiotic resistance is then used to select those organisms that have gained the cassette. This approach may be used to generate deletions of any desired chromosomal gene and works especially well in yeast and bacteria.

directed mutagenesis Deliberate alteration of the DNA sequence of a gene by any of a variety of artificial techniques

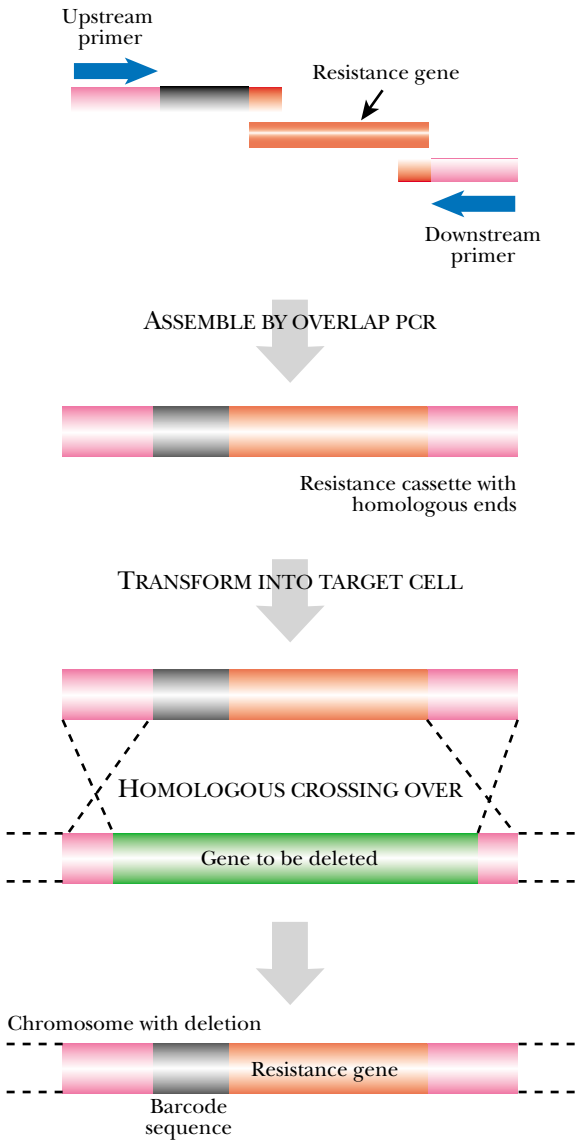

FIGURE 23.19 ***Generation of Insertion or Deletion by PCR***

In the first step, a specifically targeted cassette is constructed by PCR. This contains both a suitable marker gene and upstream and downstream sequences homologous to the chromosomal gene to be replaced. The engineered cassette is transformed into the host cell and homologous crossing over occurs. Recombinants are selected by the antibiotic resistance carried on the cassette.

A collection of yeast strains deleted for all approximately 6,000 known genes has been generated by this procedure. Each strain has had a single coding sequence replaced by a cassette comprising the *npt* gene plus a barcode sequence. The *npt* gene encodes neomycin phosphotransferase which confers resistance to neomycin and kanamycin on bacteria and resistance to the related antibiotic geneticin on eukaryotic cells, such as yeast. A barcode sequence is a unique sequence of around 20 bp that is included as a molecular identity tag. Each insertion has a unique barcode sequence allowing it to be tracked and identified. Such barcode or zipcode sequences are increasingly being used in high volume DNA screening projects where it is necessary to keep track of many similar constructs.

Clearly, the above procedure also generates an insertion of whatever gene is carried on the cassette. Thus any foreign gene may be inserted by this approach. There is no need to delete a resident gene if the objective is the insertion of an extra gene. All that is necessary is that the incoming gene must be flanked with appropriate lengths of DNA homologous to some location on the host chromosome.

Use of PCR in Medical Diagnosis

Traces of DNA can be amplified by PCR and used for diagnosis or forensic analysis.

PCR can be used to identify an unknown sample of blood, tissue, or hair. First, a specific sequence of DNA must be determined from the organism. For example, if we want

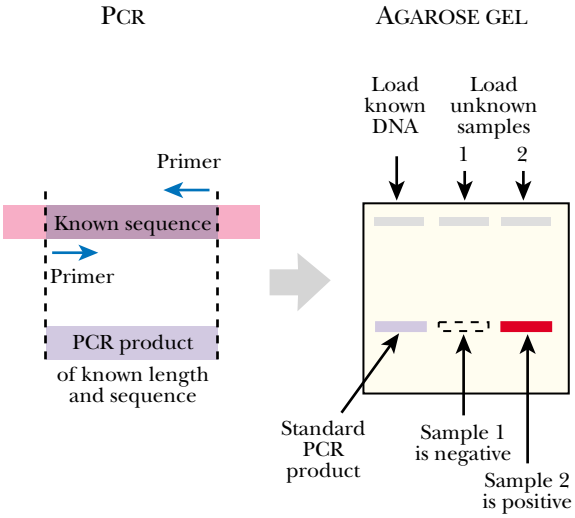

FIGURE 23.20 ***PCR is Used to Diagnose Genetic Relatedness***

PCR can determine the identity of an unknown DNA sample. In this figure, two unknown DNA samples are isolated and amplified using PCR. The primers used to test these DNA samples are specific for a known sequence (pink). In sample 1, the primers did not anneal and no PCR product was made. Therefore, the sample must not contain any DNA with the known sequence (pink). Sample 2 showed a PCR product of the predicted size for the known sequence, therefore, the primers must have annealed and amplified this sequence.

to determine whether the unknown sample of blood is from a human, than a unique sequence from humans must be determined. Since many different genomes are currently being sequenced, this data is easily obtained. Next, two primers must be designed and synthesized using the sequence information. Small samples of DNA of unknown origin can then be tested by PCR using these primers. After the PCR reaction the DNA generated is run on an agarose gel to separate it according to size. If the DNA sample tested contains the target or known sequence, the PCR product will be of the predicted length (e.g., Fig. 23.20; unknown sample No. 2). If the test DNA is not from the same organism, no band will be generated (e.g., Fig. 23.20; unknown sample No. 1). The key to this experiment is the primers and how well they anneal to the target sequence. The primers may bind to closely related sequences, but the DNA made by PCR can be sequenced to determine whether or not this occurred. Increasing the temperature in which the primers anneal to the template can increase the stringency for this reaction also.

Clearly, PCR can be used in a variety of diagnostic tests. For example, visible symptoms of AIDS only appear a long time after infection, often several years. However, using PCR primers specific for sequences found only in the HIV genome, scientists can test for HIV DNA in blood samples, even when no symptoms are apparent. Another example is tuberculosis. Unlike many bacteria, *Mycobacterium*, which causes this disease, grows very slowly. Originally, to test for tuberculosis, the bacteria were cultured on nutrient plates, but this test took nearly a month. In contrast, PCR identification of mycobacterial DNA can be done in a day. Faster medical diagnoses are critical to help prevent the spread and progression of these diseases.

PCR is a powerful tool for amplifying small amounts of DNA. The DNA from 1/100th of a milliliter of human blood contains about 100,000 copies of each chromosome. If the target sequence for PCR is 500 base pairs, then there is about one-tenth of a picogram (10^{-12} gram) by weight of a target sequence. A good PCR run will amplify the target sequence and yield a microgram (10^{-6} gram) or more of DNA. A microgram may not seem much but is plenty for complete sequencing or cloning. Obviously, it is possible to identify an organism from an extremely small trace of DNA-containing material. In fact, the DNA from a single cell can be used to amplify a specific target sequence. This technique has revolutionized the criminal justice system by allowing highly accurate identification of individuals from very small samples.

Environmental Analysis by PCR

It is possible to extract DNA directly from environmental samples, such as soil or water, without bothering to isolate and culture the living organisms that contain it first.

DNA sequences may be amplified by PCR directly from environmental samples.

Such environmental samples of DNA may be amplified by PCR. Because PCR is so sensitive, DNA can be amplified and sequenced from microorganisms that are present in such very low numbers that they cannot be detected by other means. Furthermore, it is not necessary for the microbial cells to be culturable or even viable. If specific PCR primers are used, it is possible to amplify genes from a single bacterium out of the billions that might be present in an environmental sample. As explained in Ch. 20, molecular based classification of organisms is based primarily on the sequence of the ribosomal RNA. Consequently, for identification of microorganisms from environmental samples by PCR, primers to the gene for 16 S ribosomal RNA are usually used. One fascinating result of environmental PCR analysis has been the discovery of many novel microorganisms that have never been cultured or identified by any other means. Such "microorganisms" are known only as novel ribosomal RNA sequences and it is presumed that the corresponding organisms do actually live and grow in the environment even though they do not grow in culture in the laboratory.

RT-PCR allows detection of RNA from environmental samples and so reveals whether the target gene is being transcribed.

By using primer sets specific for any chosen gene, PCR also allows us to check whether or not that particular gene is present in the environment being sampled. For example, suppose that we wish to know whether or not there are microorganisms capable of photosynthesis in a lake. A sample of lake water would be analyzed by PCR using primers specific for a gene that encodes an essential component of the light-harvesting mechanism. [Primer sets that are based on genes involved in a specific metabolic pathway are called "metabolic primers".] If a positive result is obtained we may conclude that there are organisms capable of photosynthesis in the lake. However, this approach does not tell us whether these organisms are actually growing by this mechanism or even still alive. A further step in analysis is to extract RNA from the environment and subject it to RT-PCR. This converts any messenger RNA present in the sample into the corresponding cDNA, which is then amplified. This reveals whether the corresponding genes are being actively transcribed, although, strictly speaking we still do not know if the corresponding enzyme or protein is present.

Community profiling by PCR involves assessing the abundance and diversity of bacteria in an environment. In the simplest approach, total genomic DNA is first isolated from an environmental sample. All of the bacterial 16 S rRNA genes in that sample are then amplified by PCR, cloned and sequenced. The sequences are analyzed to identify the bacteria present in that environment. Metabolic profiling using primers specific for genes in particular metabolic pathways may also be performed. The relative abundance of the various organisms in the environment may then be estimated by hybridization.

Genes encoding useful proteins may be cloned from environmental samples without knowing which organism they came from.

It is also possible to isolate useful genes directly by environmental PCR—an approach sometimes referred to as eco-trawling. DNA isolated directly from the environment is amplified by PCR and the PCR fragments are cloned into a suitable plasmid that will allow expression of any successfully captured genes. The plasmids are transformed into a suitable bacterial host cell and the captured genes are expressed. The PCR primers are generally chosen to correspond to some known gene of interest. This results in variant versions of that particular gene, which were present in the environment sampled, being obtained. For example, primers corresponding to the ends of DNA polymerase could be used with DNA extracted from a sample of water from a hot spring. The desired result would be genes encoding novel DNA polymerases able to function at high temperature. This approach is obviously well suited to finding variants of known enzymes that function under novel or extreme conditions.

Rescuing DNA from Extinct Life Forms by PCR

Since any small trace of DNA can be amplified by PCR and then cloned or sequenced, some scientists have looked for DNA in fossils. Stretches of DNA long enough to yield valuable information have been extracted from museum specimens such as Egyptian mummies and fossils of various ages. In addition, DNA has been extracted from mammoth and plant remains frozen in the Siberian permafrost. This data has helped in studying molecular evolution and is discussed more fully in Ch. 20.

FIGURE 23.21 ***Mosquito Preserved in Amber***

Photo by Karen Fiorino.

DNA may be amplified from fossil material and used in identification.

In the sci-fi best seller, "Jurassic Park", the DNA was not obtained directly from fossilized dinosaur bones. Instead, it was extracted from prehistoric insects trapped in amber (Fig. 23.21). The stomachs of bloodsucking insects would contain blood cells complete with DNA from their last victim, and if preserved in amber, this could be extracted and used for PCR. DNA has indeed been extracted from insect fossils preserved in amber. However, the older the fossil, the more decomposed the DNA will be. Normal rates of decay should break the DNA double helix into fragments less than 1,000 bp long in 5,000 years or so. So, though we will no doubt obtain gene fragments from an increasing array of extinct creatures, it is unlikely that any extinct animal will be resurrected intact.

Realtime Fluorescent PCR

PCR has been modified for rapid diagnosis by using fluorescent dyes to follow DNA accumulation.

Recently methods have been developed that allow PCR reactions to be followed in real time by monitoring the increased emission from fluorescent probes (see Ch. 21). Instruments for combined PCR and fluorescence detection carry out the PCR reaction in glass capillary tubes. This allows the penetration of light to activate the fluorophore and the monitoring of the fluorescence emission as the PCR reaction is occurring. Modern instruments can monitor several different fluorescent dyes simultaneously, allowing several reactions to be run in the same tube.

DNA-binding fluorescent probes whose fluorescence increases upon binding to DNA are included in the PCR reaction mixture. As the amount of newly synthesized target DNA increases, the probe binds to the target DNA, and the fluorescence emission increases. The simplest DNA-binding fluorescent probes are not sequence specific. An example is the dye **SYBR® Green I** (Molecular Probes, Eugene, OR), with fluorescence emission at 520 nm. This binds only to double-stranded DNA and becomes fluorescent only when bound (Fig. 23.22).

SYBR® Green monitors the total amount of double-stranded DNA but cannot distinguish between different sequences. To be sure that the correct target sequence is being amplified a sequence specific fluorescent probe is needed. An example is the **TaqMan® probe** (Applied Biosystems, Foster City, CA). The TaqMan® probe consists of two fluorophores linked by a DNA sequence that will hybridize to the middle of the target DNA. **Fluorescence resonance energy transfer (FRET)** transfers the energy from the short-wavelength fluorophore on one end to the long wavelength fluorophore at the other end. This quenches the short wave emission (Fig. 23.23).

fluorescence resonance energy transfer (FRET) Transfer of energy from short-wavelength fluorophore to long-wavelength fluorophore so quenching the short wave emission
SYBR® Green I A DNA-binding fluorescent dye that binds only to double-stranded DNA and becomes fluorescent only when bound
TaqMan® probe Fluorescent probe consisting of two fluorophores linked by a DNA probe sequence. Fluorescence increases only after the fluorophores are separated by degradation of the linking DNA

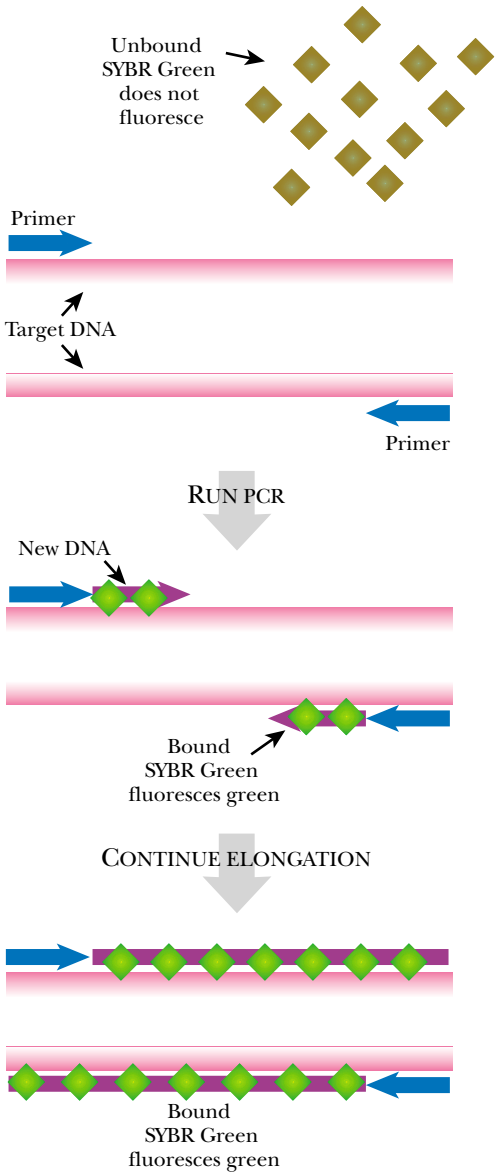

FIGURE 23.22 ***Realtime Fluorescent PCR with SYBR® Green***

When the fluorescent probe SYBR® green is present during a PCR reaction, it binds to the double-stranded PCR product and emits light at 520 nm. The SYBR® Green dye only fluoresces when bound to DNA. Hence, the amount of fluorescence correlates with the amount of PCR product produced. This allows the accumulation of PCR product to be followed through many cycles.

Specific probes can be included in fluorescent PCR procedures to ensure that only specific target DNA sequences give rise to fluorescence.

During PCR the TaqMan® probe binds to the target sequence after the denaturation step that separates the two DNA strands. As the Taq polymerase extends the primer during the next PCR cycle it will eventually bump into the TaqMan® probe. The Taq polymerase is not only capable of displacing strands ahead of it but also has a 5′-nuclease activity that degrades the DNA strand of the probe. This breaks the linkage between the two fluorophores and disrupts the FRET. The short-wavelength fluorophore is now free from quenching and its fluorescence increases. In this case the increase in fluorescence is directly related to the amount of the specific target sequence that has been amplified.

Inclusion of Molecular Beacons in PCR—Scorpion Primers

Molecular beacons may be used in conjunction with PCR primers to give a highly specific amplification plus detection system. **Scorpion primers** consist of a molecular

molecular beacon A fluorescent probe molecule that contains both a fluorophore and a quenching group and that fluoresces only when it binds to a specific DNA target sequence

Scorpion primer DNA primer joined to a molecular beacon by an inert linker. When the probe sequence binds target DNA, the quencher and fluorophore are separated allowing fluorescence

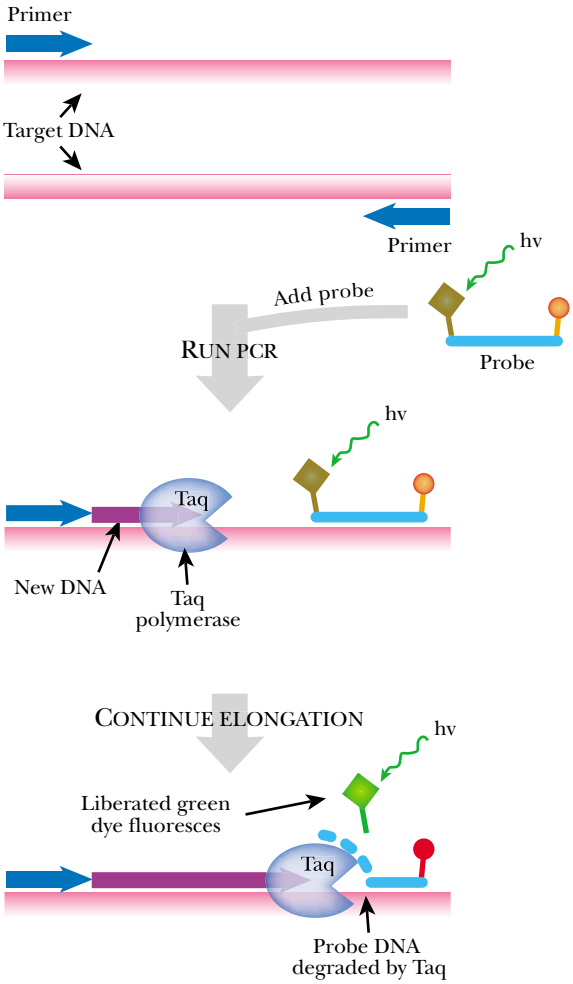

FIGURE 23.23 ***Realtime Fluorescent PCR with TaqMan® Probe***

The TaqMan® probe has three elements: a short-wavelength fluorophore on one end (diamond), a sequence that is specific for the target DNA (blue), and a long-wavelength fluorophore at the other end (circle). The two fluorophores are so close that fluorescence is quenched and no green light is emitted. This probe is designed to anneal to the center of the target DNA. When Taq polymerase elongates the second strand during PCR, its nuclease activity cuts the probe into single nucleotides. This releases the two fluorophores from contact and abolishes quenching. The short-wavelength fluorophore can now fluoresce and a signal will be detected that is proportional to the number of new strands synthesized.

beacon (see Ch. 21) joined to a single-stranded DNA primer by an inert linker molecule (e.g. hexethylene glycol). When the beacon is in its hairpin structure, the **quencher** (e.g. methyl red) binds to the fluorophore (e.g. fluorescein) and prevents fluorescence. The loop portion of the stem and loop structure has sequences complementary to the target DNA, and constitutes the probe segment. When the probe sequence binds to target DNA, the hairpin is disrupted, the quencher and fluorophore are separated and fluorescence occurs.

During PCR, the Scorpion primer binds to the target DNA and is elongated by the Taq polymerase. The two strands are separated in the next denaturation cycle. The Scorpion probe sequence then hybridizes to the single-stranded DNA in the middle of the target sequence. This releases the fluorophore from the quencher and promotes fluorescence (Fig. 23.25).

Rolling Circle Amplification Technology (RCAT)

Several novel methods other than PCR have been proposed for amplifying DNA. These methods all use DNA polymerase to amplify DNA but they avoid the high temperature requirement for DNA denaturation and the consequent temperature cycling. Which of these methods will prove useful in the long run is still undecided. Perhaps

quencher Molecule that prevents fluorescence by binding to the fluorophore and absorbing its activation energy

On Site Diagnosis of Plant Disease by Realtime PCR

The development of realtime PCR has resulted in a great decrease in the time needed for detection of DNA from infectious microorganisms. Classical methods often required 3 or 4 days to isolate the microorganism and another week to confirm its identity—always assuming the pathogen can be cultured. Standard PCR methods not only cut the time needed to 2 to 3 days but also work directly on tissue samples without requiring that the microorganisms should be cultured. Realtime PCR with fluorescent detection has cut the time required for diagnosis even further, although the technique was originally lab-based and relatively expensive. However, portable realtime PCR machines have been developed recently that allow DNA identification in a couple of hours. An example is the Smart Cycler® TD made by Cepheid Corporation of Sunnyvale, California.

The Smart Cycler® has been used to diagnose plant diseases, on-site in the fields where the crops are growing. For example, watermelon fruit blotch is a bacterial disease that causes major economic losses of watermelon crops worldwide. The causative agent, *Acidovorax avenae* subsp. *citrulli* requires 10–14 days for diagnosis by classical procedures. Portable, realtime PCR allows on-site identification within an hour of taking samples from plants with suspected infections. This rapid diagnosis is of great value both in managing crop diseases and also in deciding whether quarantine is required when facing a possible outbreak of a transmissible plant disease that could threaten crops in other locations.

FIGURE 23.24 ***Portable Realtime PCR***

The Cepheid Smart Cycler® system has been used for rapid on-site detection of plant diseases. Courtesy Cepheid Corporation.

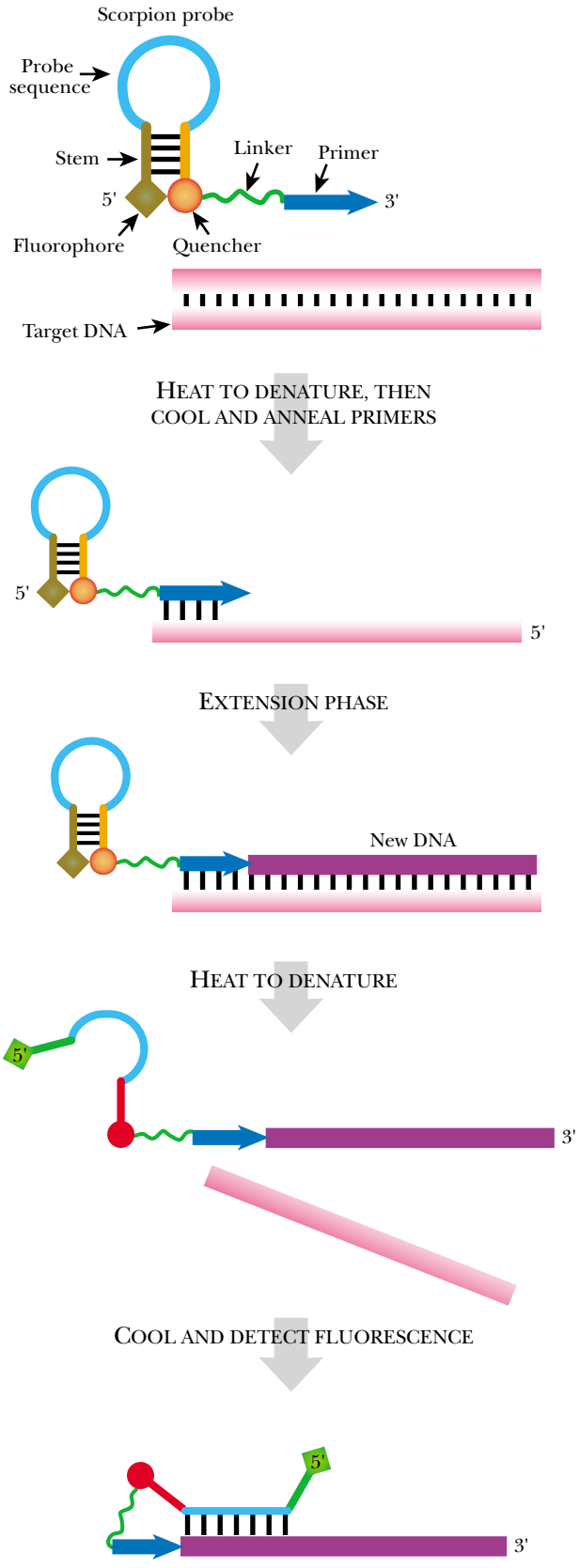

FIGURE 23.25 ***Scorpion Primer with Combined Fluorescent Probe***

Scorpion primers provide another method to detect the PCR product by fluorescence. The Scorpion probe has a stem loop structure that keeps the fluorophore molecule (diamond) in close proximity to the quencher (circle). The loop has a sequence complementary to the target DNA. The stem/loop is linked to a regular PCR primer designed to amplify the target DNA. During the extension step of PCR, the primer portion of the probe anneals to the template and Taq polymerase makes new DNA. During the next denaturation step, the whole probe plus new DNA strand become single-stranded. The loop (blue) can now anneal to the single-stranded target DNA, releasing the fluorophore from the quencher. The fluorescence emitted is a direct measure of the amount of PCR product produced.

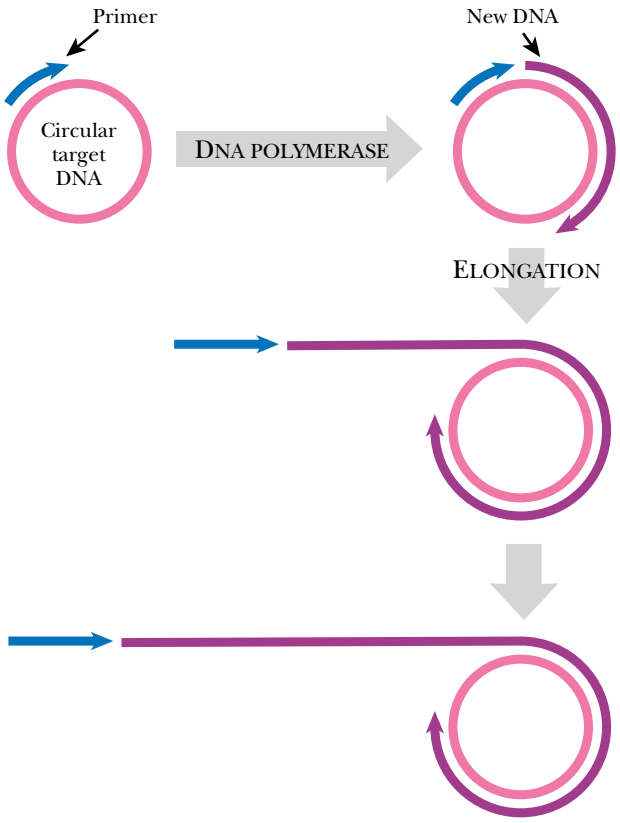

FIGURE 23.26 ***Rolling Circle Amplification of DNA—Linear Version***

Just like rolling circle replication in bacteria, RCAT produces many copies of a circular target DNA. This process only requires one primer for DNA polymerase, and the temperature does not need to be elevated. The result is a long linear piece of DNA.

Amplification of DNA by the rolling circle mechanism avoids the high temperature step of PCR.

the most promising is **Rolling Circle Amplification Technology (RCAT)** (marketed by Molecular Staging, New Haven, CT).

This technique is based on the rolling circle mechanism for DNA replication used by many plasmids and viruses (see Ch. 17); therefore, a circular DNA template is needed. This process occurs at a single temperature and does not require thermostable DNA polymerase. In linear RCAT, a DNA primer binds to the circular DNA, which is then copied many times to give a long single-stranded product that may consist of up to 100,000 tandem repeats of the target sequence (Fig. 23.26). Since the RCAT product remains attached to the original circular template, the method may be used in combination with DNA microarrays (see Ch.24 for DNA arrays).

In exponential RCAT (E-RCAT) a second primer is used that binds to the opposite strand—in other words it binds to the newly made linear strand. When this primer binds to neighboring tandem copies of the target sequence elongation results in strand displacement. This creates single-strand branches that in turn can bind primer number 1 and so be converted to double-stranded DNA. Alternate extension using two primers results in multiple branching of the amplified DNA (Fig. 23.27). The exponential version of RCAT can manufacture 10^{12} copies of each original circle in an hour and can detect a single target molecule. It is thus superior to PCR in both aspects.

rolling circle amplification technology (RCAT) Method based on rolling circle replication that uses DNA polymerase to amplify target DNA at normal temperatures

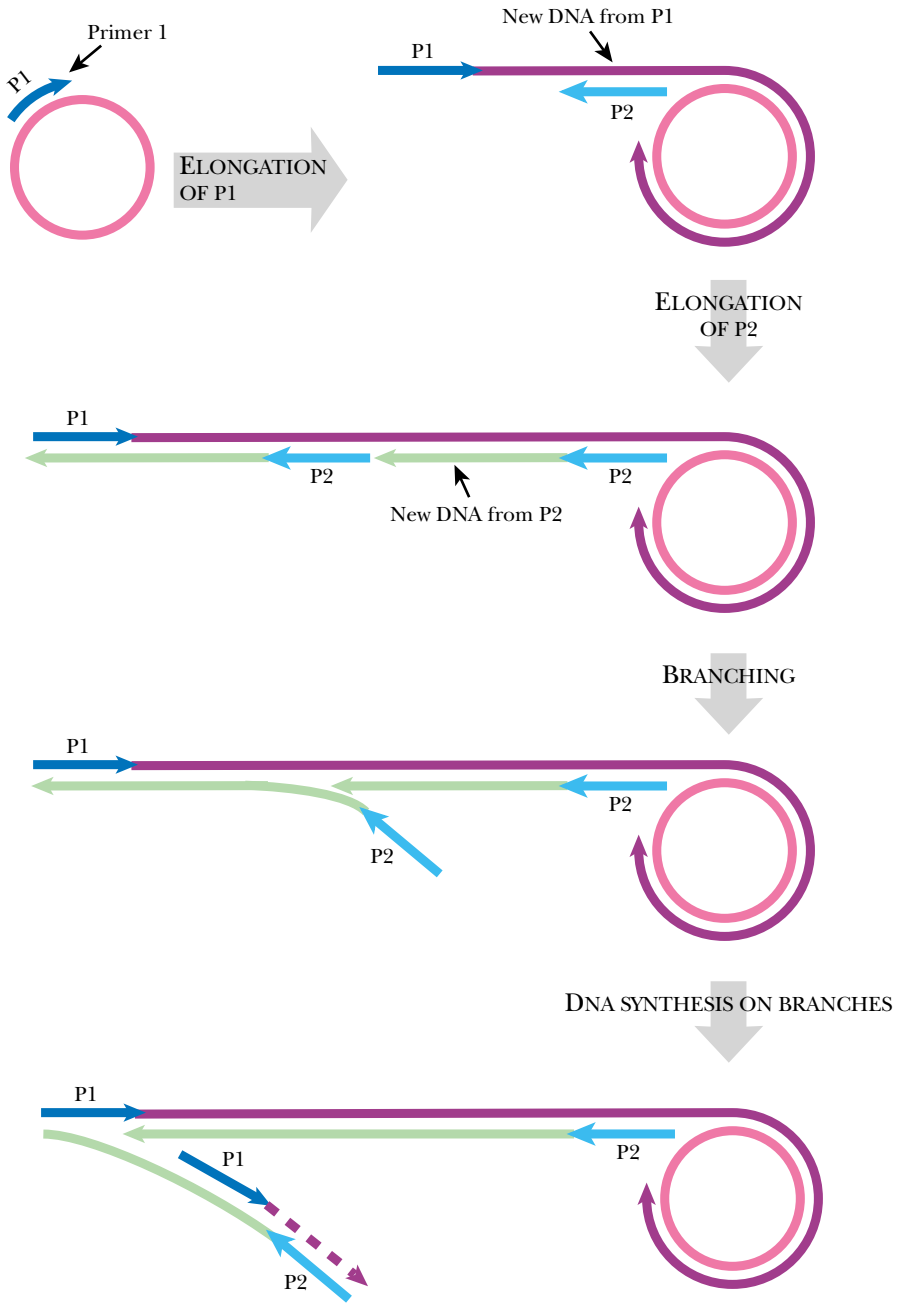

FIGURE 23.27 ***Rolling Circle Amplification of DNA—Exponential Version***

As in the linear version, E-RCAT reactions start with polymerase and the first primer (P1). Rolling circle replication produces tandem single-stranded copies of the circular target DNA. Next, a second primer (P2) anneals at the end of each tandem segment. Complementary strands of DNA are then synthesized starting from primer P2, which makes the entire DNA region double-stranded. As the DNA polymerase passes the end of a target segment, it displaces the DNA strand in front of it, making a single-stranded branch. The first primer, P1, can anneal to the inside end of the branch and polymerase makes the second strand.

The major limitation of RCAT is the need for a circular template. However, variants that involve preliminary circularization of a non-circular target molecule have been developed. As with PCR itself, the RCAT technique can also be combined with fluorescence detection.