

## **Polymerase Chain Reaction**

The polymerase chain reaction (PCR) is arguably the most powerful laboratory technique ever invented. The ease with which it can be done, the relatively low cost, and its unique combination of specificity and sensitivity coupled with great flexibility has led to a true revolution in genetics. PCR has opened doors to areas hidden to all but a few for most of the history of genetics. Yet, along with the everyman power of PCR has come a tacit assumption that everyone knows how to do it and understands it. My experience is that this is not the case. There is a great deal about PCR that most practitioners do not know and many other things that they should know better than they do. The advent of kinetic, or Real-Time PCR has served to add yet another dimension to this cognitive dissonance, particularly in the realms of experimental as well as primer and probe design along with optimization of experimental conditions. Moreover, as correctly pointed out by Bustin [1] “The comparative ease and rapidity with which quantitative data can be acquired using real-time RT-PCR assays has generated the impression that those data are reliable and can be subjected to objective analysis.” What has occurred in fact is an even higher level of sophistication that has either been taken for granted or ignored entirely.

In this tutorial the fundamentals of the polymerase chain reaction are discussed.

### **A Brief (Very) History of PCR**

Any attempt to document the development of the polymerase chain reaction will encounter nearly as much myth as science. The strict fact, at least as reiterated in the literature, is that the polymerase chain reaction was conceptualized and operationalized by Kary Mullis and colleagues at Cetus Corporation in the early 1980's [2]. The method was first formally presented at the American Society of Human Genetics Conference in October of 1985 and the first clinical application for PCR, an analysis of sickle cell anemia, was published the same year [3]. In its initial form, PCR was tedious and labor intensive. However, the advent of a method by which a specific DNA sequence could be isolated from its genomic context and amplified virtually without limit would not long remain a tool of graduate student and post-doc abuse. The breakthrough came with the isolation and purification of thermostable DNA polymerases [4]. This allowed for PCR to be automated and soon the first programmable PCR thermal cyclers appeared on the market. Since that time, PCR has spread to literally every corner of the world and to every conceivable aspect of biology and chemistry. So profound was the impact of PCR

that Kary Mullis was awarded the 1993 Nobel Prize in Chemistry, not even ten years after its introduction.

### **The PCR Reaction Components**

Despite the numerous variations on the basic theme of PCR, the reaction itself is composed of only a few components. These are as follows:

Water

PCR Buffer

MgCl<sub>2</sub>

dNTPs

Forward Primer

Reverse Primer

Target DNA

Polymerase

Considering each of these components, we can begin with **Water**. While it may seem trivial, water can be a source of concern and frustration. Water is present to provide the liquid environment for the reaction to take place. It is the matrix in which the other components interact. For most people and in most labs sterile, deionized water is the choice. However, water purification systems can fail, the cartridges might not get changed often enough, or contaminants may still get through. In order to eliminate this as a potential problem, we have switched to HPLC-grade bottled water for every application in the lab. This includes reagents. Thus, even our gel buffers are made with HPLC-grade bottled water (see [www.idtdna.com](http://www.idtdna.com) on-line catalog for purchasing water).

The next component is the **PCR Reaction Buffer**. This reagent is supplied with commercial polymerase and most often as a 10x concentrate. The primary purpose of this component is to provide an optimal pH. Many commercially supplied PCR buffers already contain **magnesium chloride** (MgCl<sub>2</sub>). MgCl<sub>2</sub> supplies the Mg<sup>++</sup> divalent cations required as a cofactor for Type II enzymes, which include restriction endonucleases and the polymerases used in PCR. The standard final concentration of this reagent for polymerases used in PCR is 1.5mM. Sometimes it is necessary to change this concentration in order to optimize the PCR reaction. For this reason we choose to obtain PCR buffer without MgCl<sub>2</sub> and to add it ourselves. 3.0ul of the standard

25mM MgCl<sub>2</sub> provided commercially will yield a 1.5mM final concentration in a 50ul reaction volume.

The purpose of the **deoxynucleotide triphosphates (dNTPs)** is to supply the “bricks.” Since the idea behind PCR is to synthesize a virtually unlimited amount of a specific stretch of double-stranded DNA, the individual DNA bases must be supplied to the polymerase enzyme. This much is obvious. What might not be as obvious is the fact that the PCR reaction requires energy. The only source of that energy is the  $\beta$  and  $\gamma$  phosphates of the individual dNTPs. One useful hint here: while the PCR buffer and the MgCl<sub>2</sub> will stand up to repeated freezing and thawing, the dNTPs are a different story. It is best to obtain them commercially as a 10 mM dNTP mix and to immediately aliquot them into smaller working volumes such that only a fraction of the original dNTP supply is ever thawed out at any one time. The remainder remains frozen until needed and should not cause any difficulty.

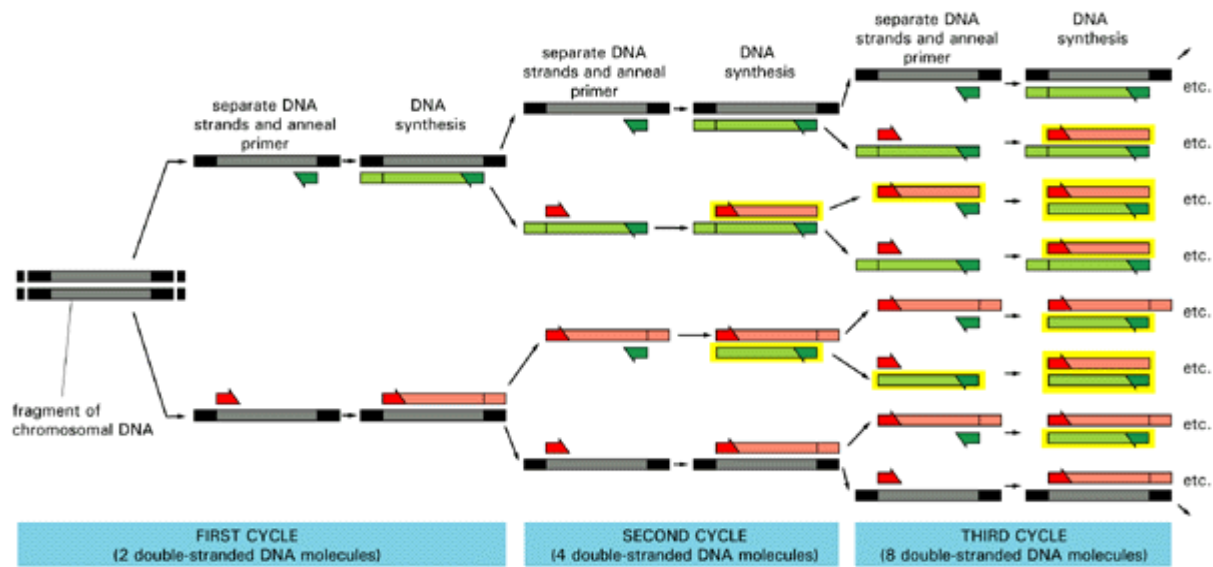
The next component is, of course, the **target DNA**. The quality and quantity of the target DNA is important. The phrase “garbage in-garbage out” is apt. The DNA used as the PCR target should be as pure as possible but also it should be uncontaminated by any other DNA source. The PCR reaction does not discriminate between targets. That is, DNA is DNA is DNA as far as the reaction is concerned. Thus care must be taken to ensure that the target DNA only contains the target of interest. As far as target concentration goes, it depends upon both the source and the method. Plasmid DNA is small and highly enriched for the specific target sequence while genomic DNA will usually contain only one copy of the target sequence per genome equivalent. Thus it is necessary to use more of the latter than the former to present a sufficient number of targets for efficient amplification. We have settled upon a maximum of 100ng of genomic DNA for PCR amplifications from a genomic background. For mammalian genomes, this represents about 10,000 genome equivalents in the reaction. For many reactions substantially smaller amounts can be used. Here, it is really a function of how much DNA you have, how easy is it to replace, and how good it is.

### **The PCR Reaction Itself**

Assume the perfect PCR reaction. The water is HPLC-grade, the buffer, MgCl<sub>2</sub>, dNTPs, and polymerase are brand new, the target DNA is as clean as it can get, and you have designed an optimal primer pair. What's next? The first thing is to specify the reaction conditions. PCR is a three step cycling process. The first step is to denature the target DNA so as to make it single-

stranded and open up the complementary sequences of the primers. This is routinely done at 94°C or 95°C for up to one minute with 30 seconds being the norm. The second step is to choose a primer **annealing temperature**,  $T_a$ . The melting temperature of the primers determines this temperature. The usual place to set the  $T_a$  is about 2 °C lower than the lowest  $T_m$  of the primers. Thus, if the primer melting temperatures are 58.5 °C and 59.2 °C, the  $T_a$  should be set at 56.5 °C as a starting point. This temperature can, and should, be changed up or down depending upon the results. With regard to duration, the norm is around 30 seconds. This leaves the final step in the cycle, the **polymerase extension** step. The convention here is to set this temperature to 72 °C, the optimal temperature for Taq polymerase. However, some protocols, especially those for long PCR, lower this to 68 °C to reduce depurination of longer amplicons. The duration of this final step is determined by the length of the amplicon. The rule of thumb is 30 seconds for every 500bp of product. Since most PCR products are less than 500bp, setting the polymerase extension step to 30 seconds works well. For a product of, say, 2,300bp, the duration should be increased to two and one-half minutes to give the polymerase ample time to make the desired product. A generic PCR cycling profile is presented in Figure 6.

Now, the perfect PCR reaction has been assembled, the cycling parameters have been chosen, entered into the thermal cycler, and you have pushed “RUN.” What is actually happening in that tube? A detailed cartoon of the first four cycles of a PCR reaction is presented in Figure 7. During the initial **denaturation** step there are actually two things happening. First, all of the target DNAs in the reaction are becoming single-stranded. Second, the heat is setting up convection currents in the reaction mix that will start all of the molecules in motion; i.e., **Brownian Motion**. This motion will ebb and flow during all of the subsequent temperature changes but the motion will never cease. The reaction components will exist in a constant state of mixing. When the denaturation step terminates, the temperature in the tube will be lowered toward the **annealing** temperature. During this period the primers will be passing through temperature zones in which random transient duplexes will be tried and discarded until, nearing the  $T_m$  of the primers, more and more of the primer molecules will find their perfect complement and



**Figure 6. A generic three-step PCR cycle profile.**

will begin to anneal. As the temperature in the tube passes through the  $T_m$  range and settles at the  $T_a$  temperature, the maximum possible number of primer molecules relative to the number of available targets will have found those targets and will lay down in stable duplexes. At the same time, the DNA polymerase will have been activated by the requisite  $Mg^{++}$  ions and will zero in their preferred substrates, the primer/target duplexes. As these substrates are acquired the polymerase will bind and immediately begin to extend in the  $5' \rightarrow 3'$  direction from the primer. As the complementary dNTPs are captured and set in place the  $\beta$  and  $\gamma$  phosphates will be released as a pyrophosphate,  $PP_i$ . This reaction provides the energy needed for the polymerase to move and begin to capture and set the next complementary dNTP. This process will continue up to and through the polymerase extension step albeit at a slower pace and will only cease when the temperature in the tube reaches the level needed for denaturation.

During the very first PCR cycle the only templates available for primer annealing are the target DNAs. As each primer finds its complement on these target DNAs the polymerase will bind and begin to extend. However, on these templates there is no extension stopping point! The polymerase will continue to extend until the denaturation temperature is reached to begin the next cycle. These PCR products form a population of template molecules that are bounded on only one end. There are various terms that can be used to describe these molecules; **anchored** and **semi-bounded** are two that we have used. In the second cycle, both the original target DNAs and the anchored DNAs will serve as templates. The former will continue to make anchored

products in every cycle of the reaction while the latter will bind the complementary primer and form the first defined PCR amplicon. In every subsequent cycle, the template DNAs, the anchored DNAs, and the amplicons will serve as targets for the PCR primers. The upshot of this is that it is not the actual target DNAs that produce PCR amplicons but, rather, the anchored DNAs and other amplicons! The implications of this are that the relative mix of forward and reverse anchored DNA molecules is actually a variable that can be manipulated by the relative amounts of forward and reverse primers in the reaction. In the vast majority of PCR reactions the forward and reverse primer concentrations are equal but there are circumstances when it is advantageous to alter the ratios.

In a PCR reaction the amount of template DNA does not change while the number of anchored products increases arithmetically each cycle beginning with cycle 1. Beginning with cycle 2, when the first defined amplicons are formed, the number of defined amplicons increases at a geometric rate. This, then, is the explosive chain reaction from which PCR derives its name (Figure 8). At the end of 35 cycles there are more than 34 billion copies of the amplicon for every copy of the original template sequence in the reaction! Thus, if there are 10,000 copies of the target sequence, there are more than 340 trillion copies of the amplicon.

## **Nested polymerase chain reaction**

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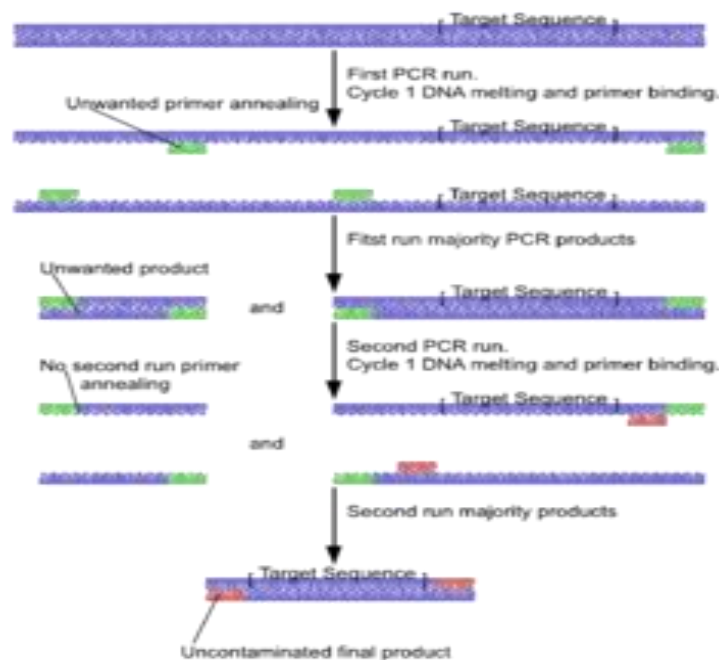
**Nested polymerase chain reaction (Nested PCR)** is a modification of [polymerase chain reaction](#) intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites.

[Polymerase chain reaction](#) itself is the process used to amplify [DNA](#) samples, via a temperature-mediated [DNA polymerase](#). The products can be used for [sequencing](#) or analysis, and this process is a key part of many [genetics](#) research laboratories, along with uses in [DNA fingerprinting](#) for [forensics](#) and other human genetic cases. Conventional PCR requires [primers](#) complementary to the termini of the target DNA. A commonly occurring problem is primers binding to incorrect regions of the DNA, giving unexpected products.

Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product.

### Processes

1. The target DNA undergoes the first run of polymerase chain reaction with the first set of primers, shown in green. The selection of alternative and similar primer binding sites gives a selection of products, only one containing the intended sequence.
2. The product from the first reaction undergoes a second run with the second set of primers, shown in red. It is very unlikely that any of the unwanted PCR products contain binding sites for both the new primers, ensuring the product from the second PCR has little contamination from unwanted products of primer dimers, hairpins, and alternative primer target sequences.



## Inverse polymerase chain reaction

**Inverse polymerase chain reaction (Inverse PCR)** is a variant of the [polymerase chain reaction](#) that is used to amplify [DNA](#) with only one known sequence. One limitation of conventional PCR is that it requires [primers](#) complementary to both [termini](#) of the target DNA,

but this method allows PCR to be carried out even if only one sequence is available from which primers may be designed.

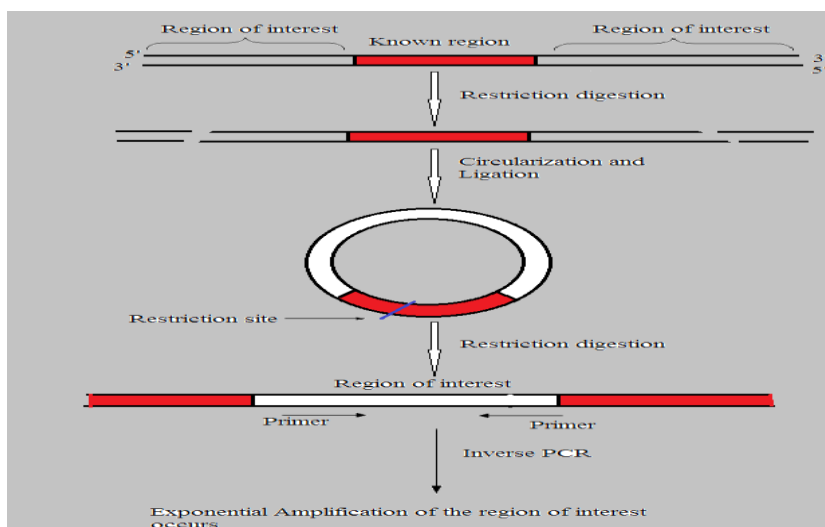
Inverse PCR is especially useful for the determination of [insert](#) locations. For example, various [retroviruses](#) and [transposons](#) randomly integrate into [genomic DNA](#). To identify the sites where they have entered, the known, "internal" viral or transposon sequences can be used to design primers that will amplify a small portion of the flanking, "external" genomic DNA. The amplified product can then be sequenced and [compared](#) with DNA databases to locate the sequence which has been disrupted.

The inverse PCR method involves a series of [restriction digests](#) and [ligation](#), resulting in a looped fragment that can be primed for PCR from a single section of known sequence. Then, like other polymerase chain reaction processes, the DNA is amplified by the temperature-sensitive [DNA polymerase](#):

1. A target region with an internal section of known sequence and unknown flanking regions is identified
2. Genomic DNA is digested into fragments of a few [kilobases](#) by a usually low-moderate frequency (6-8 base) cutting [restriction enzyme](#).
3. Under low DNA concentrations, self-ligation is induced to give a circular DNA product.
4. PCR is carried out as usual, with primers complementary to sections of the known internal sequence.\*

Finally the sequence is compared with the sequence available in the data base.

- Note: although the figure suggests that the circularized ligation product is digested prior to PCR, this is not the case. PCR does not require linear products and the use of another restriction enzyme to cut the known sequence could also cut within the unknown region, resulting in a failed PCR.



## Multiplex PCR

**Multiplex PCR:** Multiplex PCR is the term used when more than one pair of primers is used in a PCR. The goal of multiplex PCR is to amplify several segments of target DNA simultaneously and thereby to conserve template DNA, save time, and minimize expense (1). It is a PCR strategy that enables the amplification of multiple DNA targets in one run. This PCR technique is used for genetic screening, microsatellite analysis, and other applications where it is necessary to amplify several products in a single reaction. This technique often requires extensive optimization because having multiple primer pairs in a single reaction increases the likelihood of primer-dimers and other nonspecific products that may interfere with the amplification of specific products. In addition, the concentrations of individual primer pairs often need to be optimized since different multiplex amplicons are often amplified with differing efficiencies, and multiple primer pairs can compete with each other in the reaction.

## Touchdown polymerase chain reaction

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The touchdown polymerase chain reaction or touchdown style polymerase chain reaction is a method of [polymerase chain reaction](#) by which [primers](#) will avoid amplifying nonspecific sequences. The [annealing temperature](#) during a polymerase chain reaction determines the specificity of primer annealing. The [melting point](#) of the primer sets the upper limit on annealing temperature. At temperatures just below this point, only very specific base pairing between the primer and the template will occur. At lower temperatures, the primers bind less specifically. Nonspecific primer binding obscures polymerase chain reaction results, as the nonspecific sequences to which primers anneal in early steps of amplification will "swamp out" any specific sequences because of the [exponential](#) nature of [polymerase](#) amplification.

The earliest steps of a touchdown polymerase chain reaction cycle have high annealing temperatures. The annealing temperature is decreased in increments for every subsequent set of cycles (the number of individual cycles and increments of temperature decrease is chosen by the experimenter). The primer will anneal at the highest temperature which is least-permissive of nonspecific binding that it is able to tolerate. Thus, the first sequence amplified is the one between the regions of greatest primer specificity; it is most likely that this is the sequence of interest. These fragments will be further amplified during subsequent rounds at lower

temperatures, and will out compete the nonspecific sequences to which the primers may bind at those lower temperatures. If the primer initially (during the higher-temperature phases) binds to the sequence of interest, subsequent rounds of polymerase chain reaction can be performed upon the product to further amplify those fragments. Touchdown increases specificity of the reaction at higher temperatures and increases the efficiency towards the end by lowering the annealing temperature. This greatly improves the final outcome of polymerase chain reaction process.

## Real-time polymerase chain reaction

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A real-time polymerase chain reaction is a [laboratory technique](#) of [molecular biology](#) based on the [polymerase chain reaction](#) (PCR), which is used to amplify and simultaneously detect or quantify a targeted [DNA](#) molecule.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in "[real time](#)". This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for the detection of products in quantitative PCR are: (1) non-specific [fluorescent dyes](#) that [intercalate](#) with any double-stranded DNA, and (2) sequence-specific [DNA probes](#) consisting of [oligonucleotides](#) that are labelled with a [fluorescent](#) reporter which permits detection only after [hybridization](#) of the probe with its complementary sequence to quantify [messenger RNA](#) (mRNA) and [non-coding RNA](#) in cells or tissues.

The MIQE guidelines propose that the abbreviation qPCR be used for quantitative real-time PCR and that RT-qPCR be used for reverse transcription–qPCR [1]. The acronym "RT-PCR" commonly denotes [reverse transcription polymerase chain reaction](#) and not real-time PCR, but not all authors adhere to this convention.

## Background

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Cells in all organisms regulate [gene expression](#) by turnover of gene transcripts ([messenger RNA](#), abbreviated to [mRNA](#)): The amount of an expressed gene in a cell can be measured by the number of copies of an mRNA transcript of that gene present in a sample. In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene

transcript is necessary. The **polymerase chain reaction** (PCR) is a common method for amplifying DNA; for mRNA-based PCR the RNA sample is first reverse-transcribed to **cDNA** with **reverse transcriptase**.

In order to amplify small amounts of DNA, the same methodology is used as in conventional PCR using a DNA template, at least one pair of specific **primers**, **deoxyribonucleotides**, a suitable **buffer solution** and a thermo-stable **DNA polymerase**. A substance marked with a **fluorophore** is added to this mixture in a **thermal cycler** that contains **sensors** for measuring the **fluorescence** of the fluorophore after it has been excited at the required **wavelength** allowing the generation rate to be measured for one or more specific products. This allows the rate of generation of the amplified product to be measured at each PCR cycle. The data thus generated can be analysed by computer software to calculate *relative gene expression* (or *mRNA copy number*) in several samples. Quantitative PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples.<sup>[2]</sup> This measurement is made after each amplification cycle, and this is the reason why this method is called real time PCR (that is, immediate or simultaneous PCR). In the case of RNA quantitation, the template is **complementary DNA** (cDNA), which is obtained by **reverse transcription** of **ribonucleic acid** (RNA). In this instance the technique used is quantitative RT-PCR or Q-RT-PCR.

Quantitative PCR and **DNA microarray** are modern methodologies for studying **gene expression**. Older methods were used to measure mRNA abundance: **Differential display**, **RNase protection assay** and **Northern blot**. **Northern blotting** is often used to estimate the expression level of a gene by visualizing the abundance of its mRNA transcript in a sample.

## Basic principles

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**Quantitative PCR** is carried out in a **thermal cycler** with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the fluorescence emitted by the excited **fluorophore**. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the **nucleic acids** and **DNA polymerase**.

The PCR process generally consists of a series of temperature changes that are repeated 25 – 40 times. These cycles normally consist of three stages: the first, at around 95 °C, allows the separation of the nucleic acid's double chain; the second, at a temperature of around 50-60 °C, allows the binding of the primers with the DNA template;<sup>[6]</sup> the third, at between 68 - 72 °C, facilitates the [polymerization](#) carried out by the DNA polymerase.

## Classification

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The type of quantitative PCR technique used depends on the DNA sequence in the samples, the technique can either use non-specific fluorochromes or [hybridization probes](#).

### Quantitative PCR with double-stranded DNA-binding dyes as reporters

A DNA-binding dye binds to all double-stranded (ds) [DNA](#) in PCR, causing fluorescence of the dye. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified. However, dsDNA dyes such as [SYBR Green](#) will bind to all dsDNA PCR products, including nonspecific PCR products (such as [Primer dimer](#)). This can potentially interfere with, or prevent, accurate quantification of the intended target sequence. The SYBR Green is excited using blue light ( $\lambda_{\text{max}} = 488 \text{ nm}$ ) and it emits green light ( $\lambda_{\text{max}} = 522 \text{ nm}$ ).

1. The reaction is prepared as usual, with the addition of fluorescent dsDNA dye.
2. The reaction is run in a [quantitative PCR instrument](#), and after each cycle, the levels of fluorescence are measured with a detector; the dye only fluoresces when bound to the dsDNA (i.e., the PCR product). With reference to a standard dilution, the dsDNA concentration in the PCR can be determined.

### Fluorescent reporter probe method

This method has the advantage of only needing a pair of primers to carry out the amplification, which keeps costs down; however, it is only possible to amplify a product using a chain reaction.

[Fluorescent](#) reporter probes detect only the DNA containing the probe sequence; therefore, use of the reporter probe significantly increases specificity, and enables quantification even in the presence of non-specific DNA amplification. Fluorescent probes can be used in multiplex assays—for detection of several genes in the same reaction—based on specific probes with

different-coloured labels, provided that all targeted genes are amplified with similar efficiency. The specificity of fluorescent reporter probes also prevents interference of measurements caused by [primer dimers](#), which are undesirable potential by-products in PCR. However, fluorescent reporter probes do not prevent the inhibitory effect of the primer dimers, which may depress accumulation of the desired products in the reaction.

1. The PCR is prepared as usual (see [PCR](#)), and the reporter probe is added.
2. As the reaction commences, during the [annealing](#) stage of the PCR both probe and primers anneal to the DNA target.
3. Polymerisation of a new DNA strand is initiated from the primers, and once the polymerase reaches the probe, its 5'-3'-exonuclease degrades the probe, physically separating the fluorescent reporter from the quencher, resulting in an increase in fluorescence.
4. Fluorescence is detected and measured in a real-time PCR machine

## Reverse transcription polymerase chain reaction

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Reverse transcription polymerase chain reaction (RT-PCR) is one of many variants of [polymerase chain reaction \(PCR\)](#). This technique is commonly used in molecular biology to detect [RNA](#) expression.<sup>[1]</sup> RT-PCR is often confused with [real-time polymerase chain reaction \(qPCR\)](#) by students and scientists alike.<sup>[2]</sup> However, they are separate and distinct techniques. While RT-PCR is used to qualitatively detect gene expression through creation of [complementary DNA \(cDNA\)](#) transcripts from RNA, qPCR is used to quantitatively measure the amplification of DNA using fluorescent probes. qPCR is also referred to as quantitative PCR,<sup>[2]</sup> quantitative real-time PCR,<sup>[3]</sup> and real-time quantitative PCR.<sup>[4]</sup>

Although RT-PCR and the traditional PCR both produce multiple copies of particular DNA isolates through amplification, the applications of the two techniques are fundamentally different. Traditional PCR is used to exponentially amplify target DNA sequences. RT-PCR is used to clone expressed genes by [reverse transcribing](#) the RNA of interest into its DNA complement through the use of [reverse transcriptase](#). Subsequently, the newly synthesized cDNA is amplified using traditional PCR.

In addition to the qualitative study of gene expression, **quantitative PCR** can be utilized for quantification of RNA, in both relative and absolute terms,<sup>[5]</sup> by incorporating qPCR into the technique. The combined technique, described as quantitative RT-PCR<sup>[6]</sup> or real-time RT-PCR<sup>[7]</sup> (sometimes even quantitative real-time RT-PCR<sup>[8]</sup>), is often abbreviated as qRT-PCR,<sup>[9]</sup> RT-qPCR,<sup>[10]</sup> or RRT-PCR.<sup>[11]</sup> Compared to other RNA quantification methods, such as northern blot, qRT-PCR is considered to be the most powerful, sensitive, and quantitative assay for the detection of RNA levels. It is frequently used in the expression analysis of single or multiple genes, and expression patterns for identifying infections and diseases.

