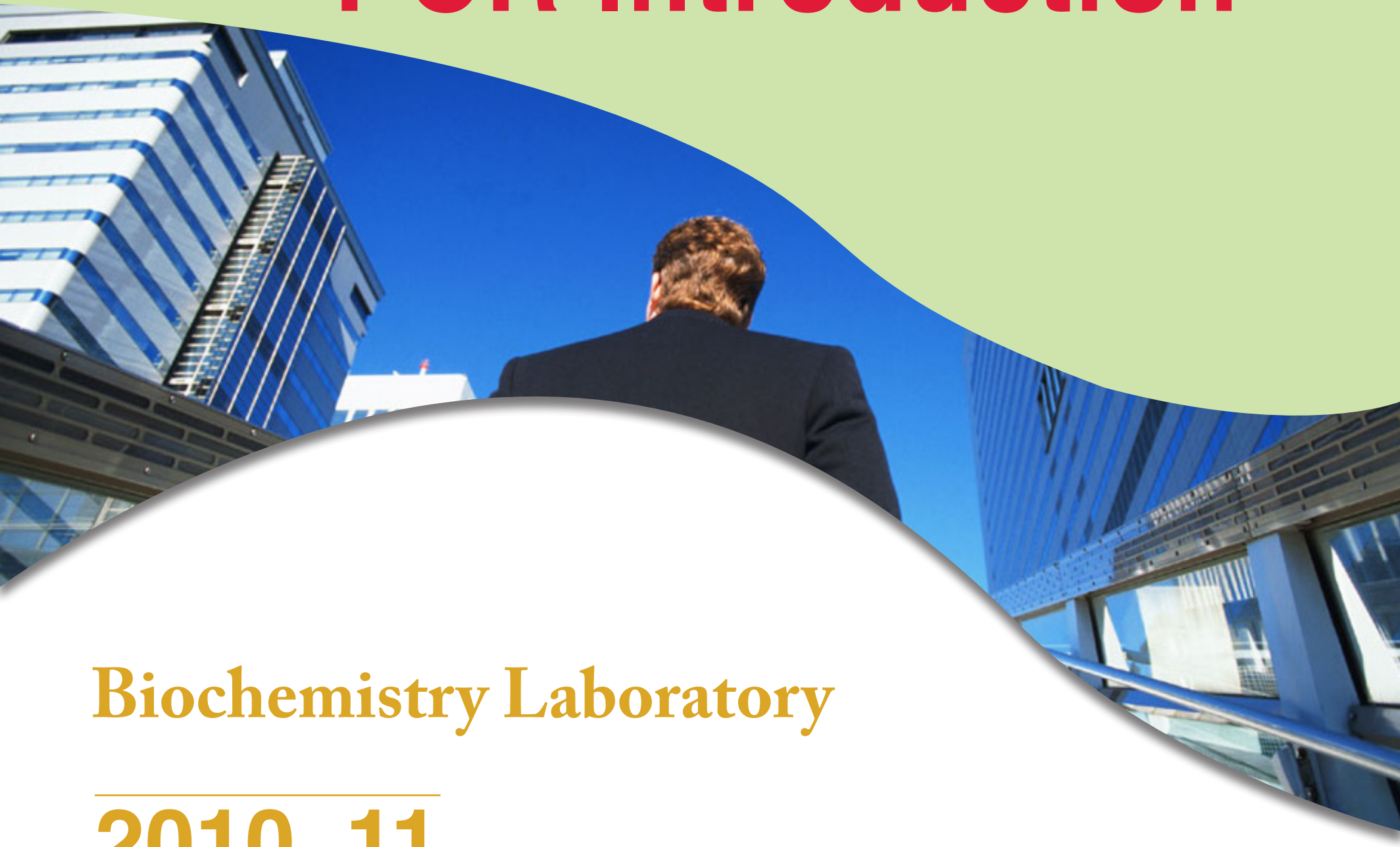
PCR Introduction



Biochemistry Laboratory

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Mechanism of DNA Synthesis

DNA polymerase extends the primer by sequentially adding a single dNTP (dATP, dGTP, dCTP or dTTP) that is complementary to the existing DNA strand

The sequence of the newly synthesized strand is complementary to that of the template strand.

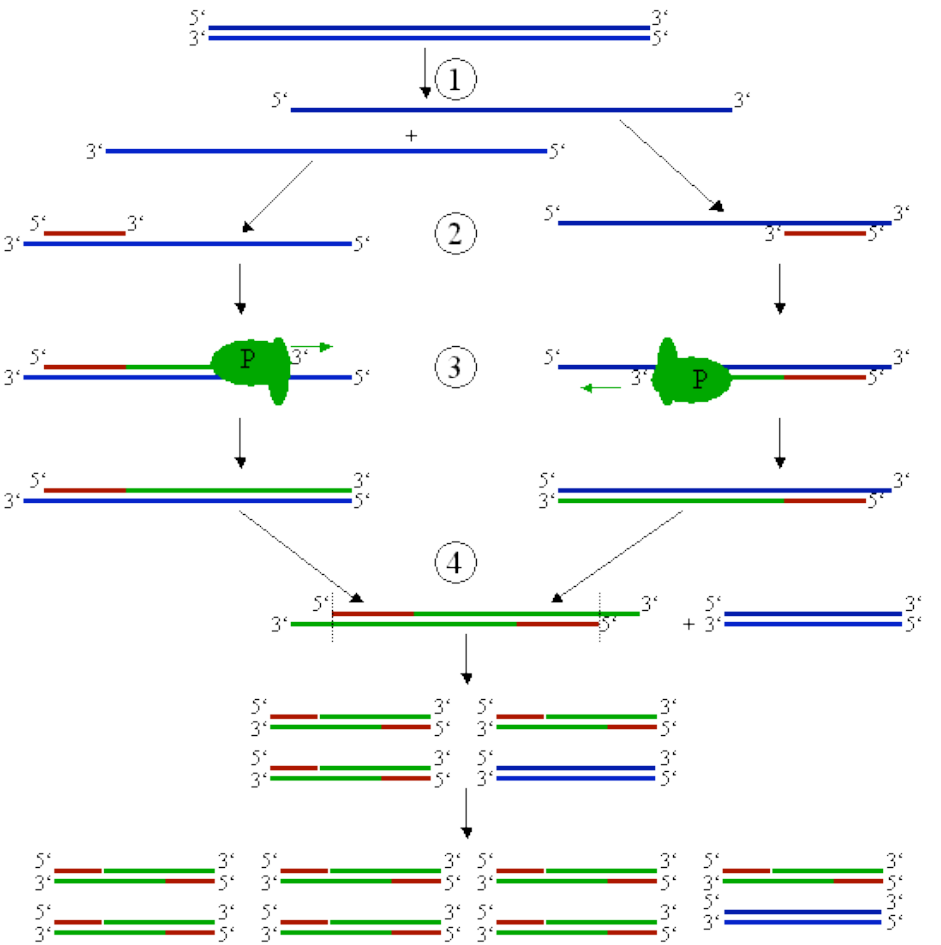
The dNTP is added to the 3´ end of the growing DNA strand, so DNA synthesis occurs in the 5´ to 3´ direction.

Polymerase Chain Reaction

> Rapid, inexpensive and simple way of making millions of copies of a gene starting with very few copies

> Does not require the use of isotopes or toxic chemicals

> It involves preparing the sample DNA and a master mix with primers, followed by detecting reaction products



Advantages of PCR

Sensitive

Versatile – easy to test new genes (Primers are inexpensive)

Reliable (Much more than microarrays for individual transcripts)

Standardized competitor templates or standard curves (allow comparison between expts)

Internal standards (Addresses variation in tissue starting amounts or loading errors)

Disadvantages of PCR

- Optimizations required
  - Annealing temperature
  - Number of cycles
- Small order of magnitiude sensitivity for detection

Components of PCR

DNA: the template used to synthesize new DNA strands.

DNA polymerase: an enzyme that synthesizes new DNA strands.

Two PCR primers: short DNA molecules (oligonucleotides) that define the DNA sequence to be amplified.

Deoxynucleotide triphosphates (dNTPs): the building blocks for the newly synthesized DNA strands.

Reaction buffer: a chemical solution that provides the optimal environmental conditions.

Magnesium: a necessary cofactor for DNA polymerase activity.

PCR Primers

Primers define the DNA sequence to be amplified—they give the PCR specificity.

Primers bind (anneal) to the DNA template and act as starting points for the DNA polymerase, since DNA polymerases can only extend existing DNA molecules and cannot initiate DNA synthesis without a primer.

The distance between the two primers determines the length of the newly synthesized DNA molecules.

PCR primers are short, single stranded DNA molecules (15-40 bp)

They are manufactured commercially and can be ordered to match any DNA sequence

Primers are sequence specific, they will bind to a particular sequence in a genome

As you design primers with a longer length (15 - 40 bp), the primers become more selective.

DNA polymerase requires primers to initiate replication.

GC content 50-60%, Avoid simple sequences and primer self complementary

A Review of Probability

A COIN THROW

The probability of a heads (H) or a tails (T) is always 0.5 for every throw. What is the probability of getting this combination of tails in a row?

Event	Probability	
Tails	0.5	= 0.5
T,T	0.5 x 0.5	= 0.25
T,T,T	0.5 x0.5 x 0.5	= 0.125
T,T,T,T,T	$(0.5)^5$	= 0.03125
T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{11}$	= 0.0004883
T,T,T,T,T,T,T,T,T,T,T,T,T,T,T,T	$(0.5)^{16}$	=0.00001526

So it become increasing unlikely that one will get 16 tails in a row (1 chance in 65536 throws). In this same way, as the primer increases in size the chances of a match other than the one intended for is highly unlikely.

Probability in Genetics

- There are 4 bases in the DNA molecule A,C,G,T
- The probability of encountering any of these bases in the code is 0.25 (1/4)
- So let us look at the probability of encountering a particular sequence of bases

Event	Probability	
A	0.25	= 0.25
A,T	0.25 x 0.25	= 0.0625
A,T,A	0.25 x0.25 x 0.25	= 0.015625
A,T,A,G,G	$(0.25)^5$	= 0.0009765
A,T,A,G,G,T,T,T,A,A,C	$(0.25)^{11}$	= 0.000002384
A,T,A,G,G,T,T,T,A,A,C,C,T,G,G,T	$(0.25)^{16}$	=0.0000000002384

So it become increasing unlikely that one will get 16 bases in this particular sequence (1 chance in 4.3 billion). In this same way, one can see that as the primer increases in size, the chances of a match other than the one intended for is highly unlikely.

DNA Polymerase

If DNA is the master blueprint of life, DNA polymerase is the molecule most responsible for replication and dissemination of the blueprint. Without DNA polymerase, most living organisms could not generate future progeny or evolve.

The principal function of DNA polymerase is to synthesize new strands of DNA in a 5´-3´ direction from a single-stranded template. Most native DNA polymerases, however, are polyfunctional. In addition to their strand synthesis activity, many DNA polymerases are able to remove nucleotides sequentially from either end of the strand (5´ exonuclease and/or 3´ exonuclease activities).

Molecular biologists have based an incredible number of laboratory applications on the multiple activities of DNA polymerase.

In April 1983, Kary Mullis of Cetus Corporation took a drive on a moonlit California road and came up with the simplest, most elegant application of all. During that drive, he conceived the basic idea for the Polymerase Chain Reaction (PCR), which is a technique for amplifying a specific target DNA sequence in vitro. Ten years later, Dr. Mullis received the 1993 Nobel Prize in Chemistry, underscoring the importance of that one simple idea.

Enzymes for PCR and RT-PCR

• Taq DNA Polymerase and FastStart Taq DNA Polymerase

The primary requirements for a DNA polymerase used in PCR are optimal activity at temperatures around 75°C and the ability to retain that activity after prolonged incubation at even higher temperatures (95°C). The first thermostable DNA polymerase to be widely used for PCR was Taq DNA Polymerasetaq dna polymerase. For many conventional PCRs that do not require extensive optimization, Taq DNA Polymerase is still a good choice. High quality, recombinant Taq DNA Polymerase (such as the preparation available from Roche Applied Science) produces the best results. Nevertheless a major drawback of standard Taq DNA Polymerase is its activity at temperatures below its optimum of 72°C. In non-optimized systems, this will lead to formation of primer-dimers due to elongation of primers annealed to each other before the first DNA denaturation step has occurred.

More recently, modifications of the Taq enzyme were developed which make it more useful for PCR. These so-called "hot start" preparations of Taq DNA Polymerase (e.g., FastStart Taq DNA Polymerase available from Roche Applied Science) are inactive at low temperatures, but readily activated at DNA denaturing temperatures. Thus, hot start polymerases minimize the formation of troublesome primer-dimers during reaction setup.

FastStart Taq DNA Polymerase is a chemically modified form of thermostable recombinant Taq DNA Polymerase that shows no activity up to 75°C. The enzyme is active only at high temperatures, where primers no longer bind non-specifically. The enzyme is complete-

ly activated (by removal of blocking groups) in a single pre-incubation step (95°C, 2-4 minutes) before cycling begins. Activation does not require the extra handling steps typical of other hot-start techniques.

Today, therefore, FastStart Taq DNA Polymerase is the best enzyme for most basic PCRs (i.e., those that amplify normal or GC-rich templates up to 3 kb in length).

• Proofreading DNA Polymerases

Standard Taq DNA Polymerase and its chemically modified form FastStart Taq DNA Polymerase lack the ability to detect the incorporation of a wrong nucleotide during polymerization and cannot remove such misincorporated nucleotides. Thus, these DNA polymerases have a relatively high PCR error rate. Furthermore, misincorporation during replication may lead to stalling of the DNA polymerase and thus to shortened PCR products. If your application requires a better fidelity than provided by (FastStart) Taq DNA Polymerase (e.g., if you want to clone or sequence a PCR product), you need to use a thermostable DNA polymerase with "editing" or "proofreading" activity. Proofreading DNA polymerases possess an additional 3´-5´ exonuclease activity which removes misincorporated nucleotides.

Pwo SuperYield DNA Polymerase (available from Roche Applied Science in most countries, but not available in the U.S.) has a much (up to 18-fold) lower error rate than Taq DNA Polymerase, which makes this enzyme ideal for high fidelity amplification of targets with length up to 3 kb.

Information Note: A drawback of all known proofreading DNA polymerases is, that they cannot be used for carryover prevention using the Uracil-DNA N-glycosylase (UNG) Method. Proofreading DNA polymerases are of archaeal origin and belong to the Family B-type DNA polymerases. All B-type DNA polymerases possess a proofreading (3'->5' exonclease) activity but lack a 5'->3' exonuclease activity. In contrast to Taq DNA Polymerase, archaeal DNA polymerases cannot copy DNA strands containing uracil residues: they possess a "read-ahead" function that detects dU residues in the template strand and stalls DNA synthesis (Martin A. Greagg et al., 1999). Thus, exchanging dTTP by dUTP would lead to inhibtion of PCR when using a B-type DNA polymerase.

• PCR Enzyme Mixtures and Blends

As PCR became more sophisticated, polymerase mixtures and blends began to be used. For specific purposes, these blends actually outperformed the individual DNA polymerases. Several examples are given below.

One approach to overcoming the limited accuracy of Taq DNA Polymerase alone was to combine the Taq DNA Polymerase with a thermostable, proofreading polymerase or another protein that has proofreading activity. Such a blend (e.g., the Expand High Fidelity PCR System from Roche Applied Science) transcribes DNA approximately threefold more accurately than the Taq DNA Polymerase alone and can be used for high fidelity amplification of moderately long (up to 5 kb) targets.

For amplification of GC-rich sequences with high yield, the GC-RICH PCR System* from Roche Applied Science is the best mix to choose.

For amplification of multiple targets in a single reaction (multiplex PCR), FastStart High Fidelity PCR System offers several advantages. This enzyme mixture transcribes more accurately (up to fourfold higher fidelity) than Taq DNA Polymerase alone and is better able to amplify sequences with high (40 – 60%) GC content. Also, the blend is very sensitive, producing good yields of amplicon from small amounts of target DNA.

Certain blends of enzymes, buffers and additives allow accurate amplification of very long templates (e.g., up to 25 kb targets with the Roche Applied Science Expand Long Range dNTPack).

To allow high-fidelity PCR in combination with prevention of carryover contamination using the Uracil DNA Glycosylase method, Roche Applied Science introduced the Expand High FidelityPLUS PCR System and the FastStart High Fidelity PCR System: these enzyme blends consist of Taq DNA Polymerase and a novel proofreading protein, isolated and characterized by Roche Applied Science. This protein mediates proofreading activity but has no polymerase activity itself and thus PCR is not inhibited by dU-containing DNA.

• Reverse Transcriptases

RT-PCR extends the power of PCR to the amplification of RNA by using an RNA-dependent DNA polymerase, commonly called a reverse transcriptase, to convert an RNA into a cDNA, then using a thermostable DNA polymerase to amplify the cDNA to detectable levels. This

combination of reactions can be performed as either a one-step (consecutively, in a single tube) or a two-step (consecutively, in separate tubes) process. There are a variety of reverse transcriptases available for both one-step and two-step RT-PCR.

Transcriptor Reverse Transcriptase (available from Roche Applied Science) can be used in two-step RT-PCR to amplify RNA targets up to 14 kb long.

You can choose from several one-step RT-PCR Systems, depending on the requirements of your RT-PCR assay (e.g., length of amplicon, GC content, error rate). The Titan One Tube RT-PCR System is best for amplicons up to 6 kb length but moderate GC content. The C. therm. Polymerase (both available from Roche Applied Science) is an efficient enzyme for one-step RT-PCR of RNA targets up to 3 kb long.

Tth DNA Polymerase (available from Roche Applied Science) will reversely transcribe RNA templates (in the presence of Mn^{2+} ions) and thus may be used for one-step, one-tube RT-PCR of short (up to 1 kb) RNA templates.

The PCR Cycle

PCR amplification can turn a few molecules of a specific target nucleic acid (too little to be analyzed directly or used in biochemical reactions) into as much as a microgram of DNA. PCR is closely patterned after the natural DNA replication process (Saiki et al., 1985).

Two oligonucleotide primers flank and define the target sequence to be amplified. These primers hybridize to opposite strands of the DNA to serve as initiation points for the synthesis of new DNA strands. A thermostable DNA polymerase, such as Taq DNA Polymerase, catalyzes this synthesis.

Each round of PCR synthesis involves three steps: denaturation, annealing and extension. This three-step "PCR cycle" is repeated several times (Mullis and Faloona,1987).

The repetitive nature of the PCR process is the key to its amplifying power. Because the primer extension products synthesized in a given cycle can serve as templates in the next cycle, the number of target DNA copies approximately doubles every cycle. Thus, in only 20 cycles, PCR can produce about a million (2^{20}) copies of the target.

The Evolution of PCR

The potential of the PCR technique increased dramatically when scientists identified a thermostable DNA polymerase, Taq DNA Polymerase, that was optimally active and stable at the high elongation temperature (around 72°C) used in PCR. Because the Taq enzyme was stable during the repeated PCR cycles, researchers no longer had to interrupt the PCR process to add fresh enzyme (Saiki et al., 1988).

1989: **The native Taq enzyme was purified in 1986 by David Gelfand and Susanne Stoffel (who were then employed by Cetus Corporation, but later assumed important roles at Roche Molecular Diagnostics). Boehringer Mannheim (now Roche Applied Science) was one of the companies that provided a convenient recombinant version of this important enzyme (in 1989).**

Taq DNA polymerase still had drawbacks however. One such drawback was that it lacked a proofreading activity that could correct the occasional transcriptional errors (and potential mutations) that occurred during amplification. For many applications, these occasional mutations were of little concern. However, in some applications (e.g., the amplification of genomic products for sequencing and the study of allelic polymorphisms), any transcriptional errors can lead to misleading results. The commercial availability of thermostable enzymes that had proofreading activity solved this problem, providing a high fidelity version of PCR for those applications that required very accurate transcription.

1994: A thermostable, proofreading enzyme, Pwo DNA Polymerase, was introduced by Boehringer Mannheim (later Roche Applied Science) in 1994.

Another approach to solving the accuracy problem was to combine Taq DNA polymerase with a thermostable enzyme (such as Tgo DNA polymerase) or other protein that had proofreading activity.

1995: One such enzyme blend, the Expand High Fidelity PCR System, was introduced by Boehringer Mannheim (later Roche Applied Science) in 1995.

Replacing a single enzyme with an enzyme blend also led to another important advance in the evolution of PCR, the ability to amplify much longer targets (Barnes, 1994).

1996: By skillfully purifying and blending enzymes, then pairing the blend with carefully optimized reaction components, Roche Applied Science was able to offer one enzyme blend (Expand Long Template PCR System, introduced in 1994) that could amplify up to 20 kb targets and another (Expand 20 kbPLUS PCR System, introduced in 1996) that could amplify up to 35 kb targets.

By modifying Taq DNA polymerase so it was inactive at room temperature, but readily activated at DNA denaturing temperatures, researchers made possible a “hot start” version of PCR that minimized the formation of troublesome primer dimers (Birch et al, 1996).

2000: Roche Applied Science introduced (in 2000) FastStart Taq DNA Polymerase for “hot start” applications. By including FastStart Taq DNA Polymerase in

an enzyme blend (FastStart High Fidelity PCR System, introduced in 2003), Roche Applied Science created a high fidelity hot start PCR system that can be used in such demanding applications as multiplex PCR.

In 1991, Holland et al. described a technique (5´ nuclease assay) to simultaneously amplify and detect specific DNA sequences with a fluorescent DNA-binding dye. This technique (a 5' nuclease assay) uses so called hydrolysis probes and similar FRET (fluorescence resonance energy transfer)-based techniques made it possible to analyze PCR products as they were being formed (so-called "kinetic" or "real-time" PCR analysis).

PCR Applications

Because of PCR, "insufficient nucleic acid" is no longer a limitation in research and many medical diagnostic procedures. Most importantly, innovative researchers have continually updated and expanded the definition of "PCR applications," increasing the usefulness and scope of the technique. Even a brief review of these innovative applications is beyond the scope of this article. However, here is just a random sampling of PCR applications that are currently important:

> Automated PCR instruments that permit real-time detection and analysis of many products in a single run (e.g., the LightCycler® Carousel-based System, the LightCycler®480 Real-Time PCR System, and the COBAS® TaqMan® 48 Analyzer).

> Clonal amplification of genomic DNA in an emulsion (emPCR) to make ultrarapid sequencing of the genome possible (e.g., in the Genome Sequencer 20 System).

> Introduction of molecular "tags" (such as biotin and digoxigenin) into the PCR product during amplification, allowing these products to be used as sensitive hybridization probes in medical diagnostic tests (e.g., in the COBAS® AMPLICOR® Analyzers).

> Simultaneous amplification of multiple sequences in a single sample (multiplex PCR) (e.g., for identification of human single nucleotide polymorphisms in genomic DNA).

> Studies of genetic variability (e.g., to determine the genetic basis of diseases).

> Amplification of DNA for identity testing (e.g., DNA fingerprinting).

> Study of epigenetic mechanisms (such as DNA methylation, histone acetylation, and RNA interference) involved in activation and inactivation of genes (e.g., addition of T7 promoters to DNA templates to allow generation of double-stranded RNA for gene knockdown studies).

> Creation of novel DNAs by in vitro mutagenesis.

> Exploration of evolutionary relationships via examination of ancient DNA from fossils.

Sources of Contamination

Contamination may be defined as "accidental contact or mixing of the sample with exogenous material (nucleic acids or microbial organisms) that make the sample impure or corrupt." As stated above, any source of contamination, no matter how small, can lead to false positive results.

Contamination usually arises from two sources:

> Cross-contamination, or contamination of the sample with non-amplified material from the environment (e.g., aerosols, cloned DNA molecules that carry the target gene) or other samples. This may be due to parallel storage, handling or processing of intermediate samples.

> End-product (i.e., carryover) contamination, or contamination of the sample with amplicons from earlier PCRs, leading to subsequent amplification of both target and contaminant amplicons. This may be due to storage, handling or processing of samples between PCRs.

Things you can optimize

- Temperature and time to activate Taq polymerase
- Temperature and time to allow primer annealing
- Temperature and time for extension
- Concentration of reagents, especially primers, dNTPs, and $MgCl_2$
- Concentration of template DNA
- Number of replication cycles
- etc…

Quality and Amount of DNA Template

The amount of template in a reaction also strongly influences performance in PCR. For standard PCR, follow these recommendations:

The maximum amount of human genomic DNA should be 200 ng. Use less if possible. Low amounts of genomic template (e.g., <10 ng human genomic DNA) will require specific reaction modifications, such as increases in cycle number, redesign of primers, use of a "Hot Start" reaction, etc. For initial experiments, use:

1–10 ng bacterial DNA

0.1–1 ng plasmid DNA

2 µl cDNA (as template for a 50 µl PCR)

When using cDNA as template, do not let the volume of the cDNA exceed 10% of the volume of the PCR mixture (e.g., for a 50 µl PCR mixture, use no more than 5 µl cDNA from a reverse transcriptase reaction). Greater amounts of cDNA may inhibit the PCR.

High Quality Nucleotides

Nucleotides are vital components in amplification reactions and the purity of these reagents significantly influences PCR results. Be aware that not all preparations of nucleotides are acceptable for PCR.

Specifically, many nucleotide preparations contain trace amounts of contaminants (pyrophosphate, mono-, di- and tetraphosphate nucleotides and organic solvents) that can inhibit amplification reactions. Such nucleotide preparations are suboptimal for PCR.

Conc. of Deoxynucleoside Triphosphate (dNTPs)

During PCR, always use balanced solutions of all four dNTPs to minimize polymerase error rate. Imbalanced (i.e., unequal) concentrations of dNTP will reduce the fidelity of the thermostable DNA polymerase. An exception to this rule is the use of dUTP for carryover prevention. A higher concentration of dUTP is usually used in place of dTTP. This is due to differences in the rates at which dTTP and dUTP are incorporated in DNA.

The usual concentration of dNTP in standard PCR is 200 µM (each nucleotide). For some applications, concentrations ranging from 50 to 500 µM may be acceptable. If you increase the concentration of dNTPs, you must also increase the concentration of Mg^{2+} ion in the reaction. Increases in dNTP concentration reduce free Mg^{2+}, thus interfering with polymerase activity and decreasing primer annealing.

Conc. of Magnesium Ions

Most thermostable DNA polymerases require a source of divalent cations to function. In most cases, the divalent cation required is Mg^{2+}. Mg^{2+} influences enzyme activity and increases the Tm of double-stranded DNA. Mg^{2+}forms soluble complexes with dNTPs to produce the actual substrate that the polymerase recognizes.

In general, lower Mg^{2+} concentrations lead to specific amplification and higher concentrations produce more nonspecific amplification. A few DNA polymerases (such as Tth DNA Polymerase) use Mn^{2+} rather than Mg^{2+}. However, in general, DNA polymerase reactions in the presence of Mn^{2+} make DNA copies with significantly lower fidelity than in reactions in the presence of Mg^{2+}.

The concentration of free Mg^{2+} depends on the concentrations of compounds that bind the ion, including dNTP, template DNA, primers, free pyrophosphate (PPi) and EDTA. Therefore, determining the correct Mg^{2+} concentration in a PCR is not easy.

The most commonly used concentration of Mg^{2+} used in standard PCR with Taq DNA Polymerase is 1.5 mM (with 200 µM dNTPs). However, for best results, always determine the optimal Mg^{2+} concentration for each reaction system empirically. Try a range of Mg^{2+} concentrations, from 1 mM to 10 mM.

Appropriate Buffers

Always use only the purest buffers in PCR. These buffers should be designated “PCR Grade.” In most cases, the reaction buffer supplied with the PCR enzyme(s) will give optimal results with that enzyme. The pH of that buffer will generally be between pH 8.3 and 9.0.

Reaction Additives and Enhancers

In some cases, adding the following compounds can enhance the efficiency, specificity or yield of standard PCR with Taq DNA Polymerase:

Effect of Additives on PCR

Additive/	Concentration in Reaction [a]	Effect of Additive [a]
Ammonium sulfate [$(NH_4)_2SO_4$] [b]	5 - 30 mM	Facilitates DNA strand separation
Bovine serum albumin *	50 - 500 ng per 50 µl reaction	Binds many PCR inhibitors found in tissue samples
Dimethylsulfoxide (DMSO)[b]	2 - 10% v/v	Lowers T_m of target DNA to enhance annealing
Dimethylformamide (DMF)	<10% v/v [c]	Lowers T_m of target DNA to enhance annealing
Betain		Lowers T_m of target DNA to enhance annealing
Formamide	1.25 - 10% v/v [c]	Changes T_m of primer-template hybridization to increase specificity and yield Stabilizes Taq DNA Polymerase
Gelatin [b]	0.01- 0.10% w/v	Stabilizes Taq DNA Polymerase
Glycerol [b]	5 - 15% v/v	Stabilizes Taq DNA Polymerase
PEG 6000	5 - 15% v/v	Stabilizes Taq DNA Polymerase
SDS	less than 0.01% w/v [c]	Prevents aggregation of polymerase
Spermidine		Reduces nonspecific binding of polymerase to template DNA
T4 Gene 32 protein *	0.05 - 0.1 nmol per 50 µl reaction	Changes T_m of primer-template hybridization to increase specificity and yield
Triton X-100	0.01% v/v	Prevents aggregation of polymerase
Urea	1 - 1.5 M [c]	Lowers T_m of target DNA to enhance annealing

a) Part of the information in this table is from Aoyagi (2001). The effects of the additives were determined only for reactions with Taq DNA Polymerase.

b) Component of the Roche Applied Science PCR Optimization Kit. For details on optimizing PCR with the kit, see Chapter 4, section 4.7.

c) Higher concentrations are inhibitory.

* Available from Roche Applied Science.

Effect of Cycling Factors

The denaturation time must be long enough to fully denature the template, but short enough not to inactivate the thermostable DNA polymerase.

The optimal annealing temperature depends on the melting temperature of the primers. The optimal elongation time depends on the length of the target to be amplified. The optimal number of cycles depends on the abundance of the target in the starting sample (e.g., rarer targets require more cycles to amplify).

Because the optimal cycling parameters vary with each experimental system, the thermal cycling program given in any publication (including the protocols of this manual) should be considered guidelines only. For optimal results, these parameters should be optimized empirically for your particular experimental system and equipment.

The table below summarizes the effects of under- or overshooting the optimal time and temperature for each stage of the thermal cycle. You can use that information to help you determine the optimal PCR parameters for your system.

Cycling Parameters Affect PCR

Cycling Parameter	Value Used in Standard PCR [a]	Effect if Parameter Value Is Lower than Optimal [b]	Effect if Parameter Value Is Higher than Optimal [b]
Initial Denaturation	94°C 2 min	Few or no PCR products	Premature denaturation of poly-merase, leading to reduced yield
Denaturationduring Cycling	94°C 15 – 30 s	Reduced yield	Reduced yield
Primer Annealing	50 to 65°C [c] 30 – 60 s	Reduced yield	Formation of nonspecific products
Elongation	72°C 45 s – 2 min [d]	Reduced yield	Reduced yield Increased error rate
Total Number of Cycles	25 – 30	Reduced yield	Formation of nonspecific products
Final Elongation	72°C 7 min	Products are not fully double-stranded	Formation of nonspecific products

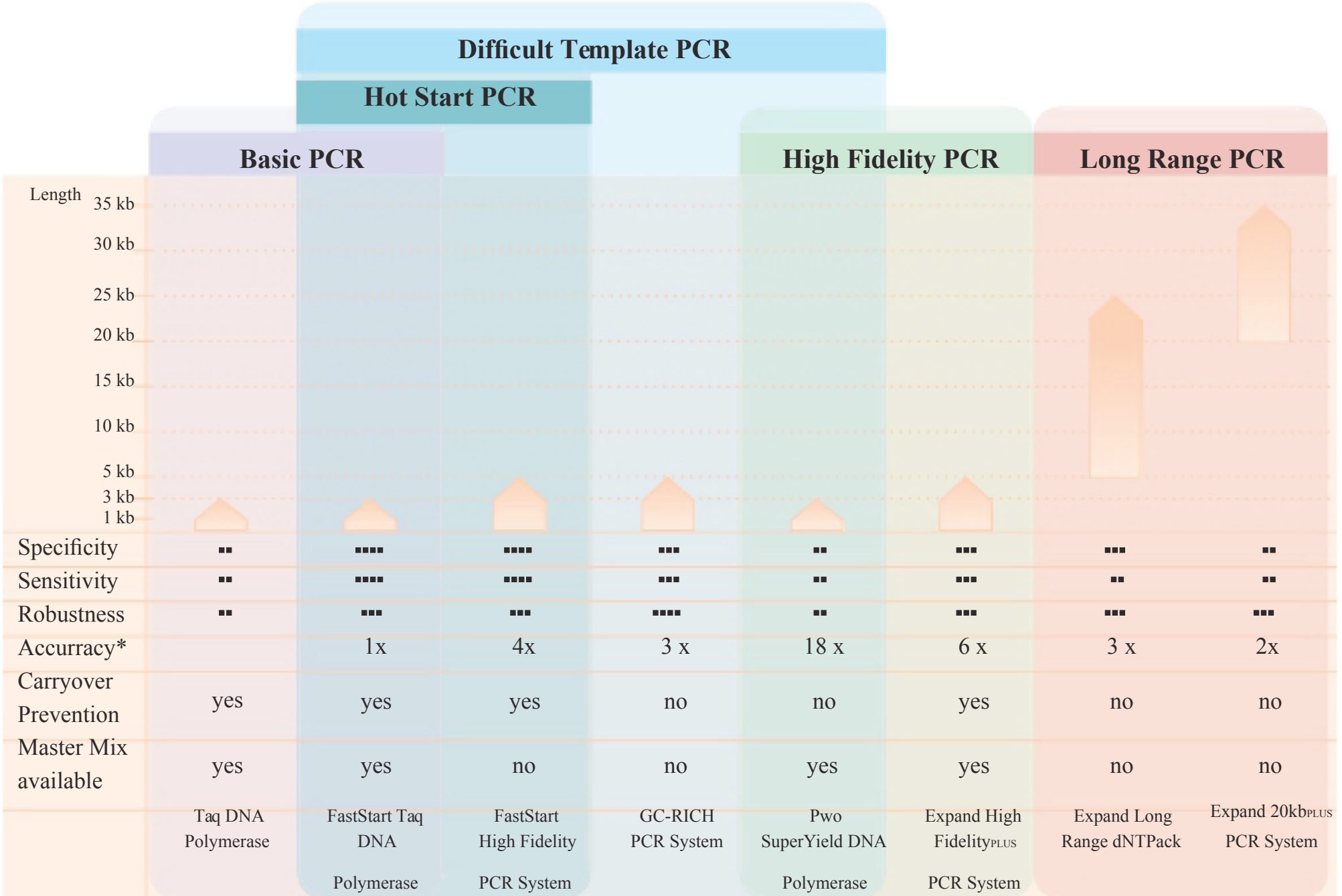
a) With Taq DNA Polymerase; copied from standard PCR protocol (Chapter 4, section 4.1). Other enzyme systems will require different parameter values.

b) From Aoyagi (2001). Effects are generally independent of the enzyme system used.

c) Optimal temperature depends on primer melting temperature.

d) Elongation time depends on fragment length; for Taq DNA Polymerase, the recommended times are 45 seconds for targets that are 1 kb or shorter, 1 minute for 1.0 – 1.5 kb, and 2 minutes for 1.5 – 3.0 kb.

Table 2.1: Comparison of PCR- and RT-PCR Enzymes.



	Taq DNA Polymerase	FastStart Taq DNA Polymerase	FastStart High Fidelity PCR System	GC-RICH PCR System	Pwo SuperYield DNA Polymerase	Expand High Fidelity^{PLUS} PCR System	Expand Long Range dNTPack	Expand 20kb^{PLUS} PCR System
Specificity	▪▪	▪▪▪▪	▪▪▪▪	▪▪▪	▪▪	▪▪▪	▪▪▪	▪▪
Sensitivity	▪▪	▪▪▪▪	▪▪▪▪	▪▪▪	▪▪	▪▪▪	▪▪	▪▪
Robustness	▪▪	▪▪▪	▪▪▪	▪▪▪▪	▪▪	▪▪▪	▪▪▪	▪▪▪
Accurracy*		1x	4x	3 x	18 x	6 x	3 x	2x
Carryover Prevention	yes	yes	yes	no	no	yes	no	no
Master Mix available	yes	yes	no	no	yes	yes	no	no

* compared to Taq DNA Polymerase

	Two-Step RT-PCR		One-Step RT-PCR	
Product Size				
Yield	▪▪▪▪	▪▪▪▪	▪▪▪	▪▪
Sensitivity	▪▪▪	▪▪▪	▪▪▪▪	▪▪▪
Difficult Templates	▪▪▪▪	▪▪▪▪	▪▪	▪▪▪▪
Performance qRT-PCR	▪▪▪▪	▪▪▪▪	—*	—*
Reaction Temperature	42 — 65°C	42 — 65°C	40 — 60°C	60 — 70°C
Full-Length cDNA	▪▪▪▪	▪▪▪▪	▪▪▪	▪▪
	Transcriptor Reverse Transcriptase	Transcriptor First Strand cDNA Synthesis Kit	Titan One Tube RT-PCR System / Kit	*C. therm* One Step RT-PCR System

* for One-Step RT-PC we recommend to use our LightCycler® kits

Abbreviations: kb, kilobase pairs

Processivity: Number of bp that can be added to a copy by one molecule of polymerase before it falls off the template.

Length: Maximum PCR target length that can be amplified from a human genomic DNA template with good yield.

Specificity: Amplifies only the target of interest.

Yield: Produces large amounts of products in a given number of PCR cycles.

Reproducibility: Gives the same results from reaction to reaction.

Sensitivity: Amplifies template present at a low copy number (fewer than 500 copies0

Robustness: Amplifies template even in the presence of contaminating agents or high GC content.

Accuracy: Amplifies template without introducing excessive transcriptional errors.

Some Problems with PCR

- Inhibitors in template DNA
- Amplification bias
- Gene copy number
- Limited by primer design
- Differential denaturation efficiency
- Chimeric PCR products may form
- Contamination w/ non-target DNA
- Potentially low sensitivity and resolution
- General screw-ups

PCR Equation

$$X_n = X_0(1 + E)^n$$

X_n =PCR product after cycle n
X_0 =initial copy number
E =amplification efficiency
n =cycle number

A difference of **0.1** in amplification efficiency create a **five-fold** difference in the final ratio of PCR products after 30 cycles.

Case 1: E = 0.9

$X_n = 100\ (1+0.9)^{30}$

$X_n = 2.3 \times 10^{10}$

Case 2: E = 0.8

$X_n = 100\ (1+0.8)^{30}$

$X_n = 4.6 \times 10^{9}$

RT-PCR

In 1987, Powell et al. described a technique that extended the power of PCR to the amplification of RNA. This technique, RT-PCR, used a reverse transcriptase to convert the rare RNA into a cDNA, then used a thermostable DNA polymerase to amplify the cDNA to detectable levels. This technique made it possibe to use PCR to detect and analyze rare mRNA transcripts and other RNAs present in low abundance.

Step 1: Heat (usually >90°C) separates double-stranded DNA into two single strands. Since the hydrogen bonds linking the bases to one another are weak, they break at high temperatures, whereas the bonds between deoxyribose and phosphates, which are stronger covalent bonds, remain intact. This process takes place in a thermal cycler, an instrument that automatically controls the alternating cycles of heating and cooling required for PCR.

Step 2: The goal is not to replicate the entire strand of DNA but to replicate a target sequence of approximately 100-35,000 base pairs that is unique to the organism. Primers define the ends of that target sequence. Primers are short, synthetic sequences of single-stranded DNA typically consisting of 20-30 bases. [Theoretically, a 16-mer is long enough to represent all unique primer sequences (416) in a random sequence of 3 billion base pairs.] Annealing usually takes place between 40°C and 65°C, depending

on the length and sequence of the primers. This allows the primers to anneal specifically to the target sequence.

Step 3: Once the primers anneal to the complementary DNA sequences, the temperature is raised to approximately 72°C and a thermostable polymerase (e.g., Taq DNA Polymerase) begins to synthesize new double-stranded DNA molecules which are identical to the original target DNA. It does this by facilitating the binding and joining of complementary nucleotides that are free in solution (dNTPs). Synthesis always begins at the 3' end of the primer and proceeds exclusively in the 5' to 3' direction. Thus, the new synthesis effectively extends the primers, creating a complementary, double-stranded molecule from a single-stranded template.

End of cycle: At the end of the first PCR cycle, there are now two new DNA strands identical to the original target. Note, however, that the DNA polymerase does not recognize the end of the target sequence. The newly formed strands have a beginning, which is precisely defined by the 5' end of the primer, but not a precisely defined 3' end. Each subsequent cycle repeats and multiplies this copying process. End of the first PCR cycle: Two copies of target sequence However, as the number of cycles increases, a strand with more defined length frequently serves as the template for the newly synthesized sequence. The DNA strand synthesized from such a template then has a precisely defined length that is limited at either end by the 5' end of each of the two primers. These DNA strands are called an amplicon. After only a few cycles, DNA strands which correspond to the target sequence are present in much larger numbers than the variable

length sequences. In other words the sequence flanked or defined by the two primers is the section that is amplified. After the appropriate number of PCR cycles (usually between 30 and 40 cycles), this repetitive, sequential process exponentially generates up to a billion of copies of the target, all within just a few hours.

Principles of Real-Time PCR

Based on the detection and quantitation of a fluorescent reporter

The first significant increase in the amount of PCR product (C_T - threshold cycle) correlates to the initial amount of target template

Advantages of Real-Time PCR

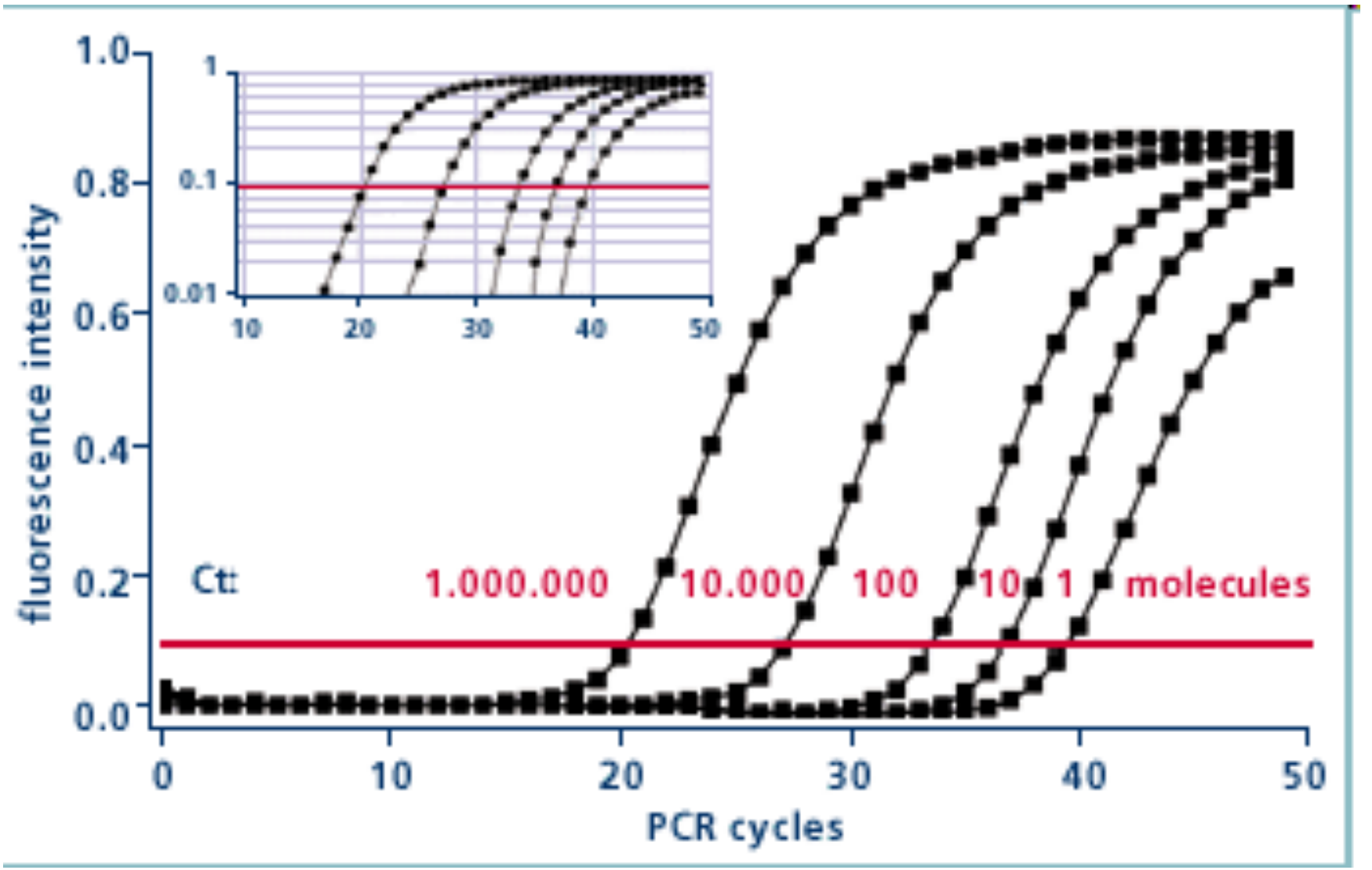
- Not influenced by non-specific amplification
- Amplification can be monitored real-time
- No post-PCR processing of products
- (High throughput, low contamination risk)
- Ultra-rapid cycling (30 minutes to 2 hours)
- Wider dynamic range of up to 1010-fold
- Requirement of 1000-fold less RNA than conventional assays
- (6 picogram = one diploid genome equivalent)
- Detection is capable down to a two-fold change
- Confirmation of specific amplification by melting curve analysis
- Most specific, sensitive and reproducible
- Not much more expensive than conventional PCR (except equipment cost)

Disadvantages of Real-Time PCR

- Not ideal for multiplexing
- Setting up requires high technical skill and support
- High equipment cost
- Intra- and inter-assay variation
- RNA lability
- DNA contamination (in mRNA analysis)

Quantitative (Real Time) PCR

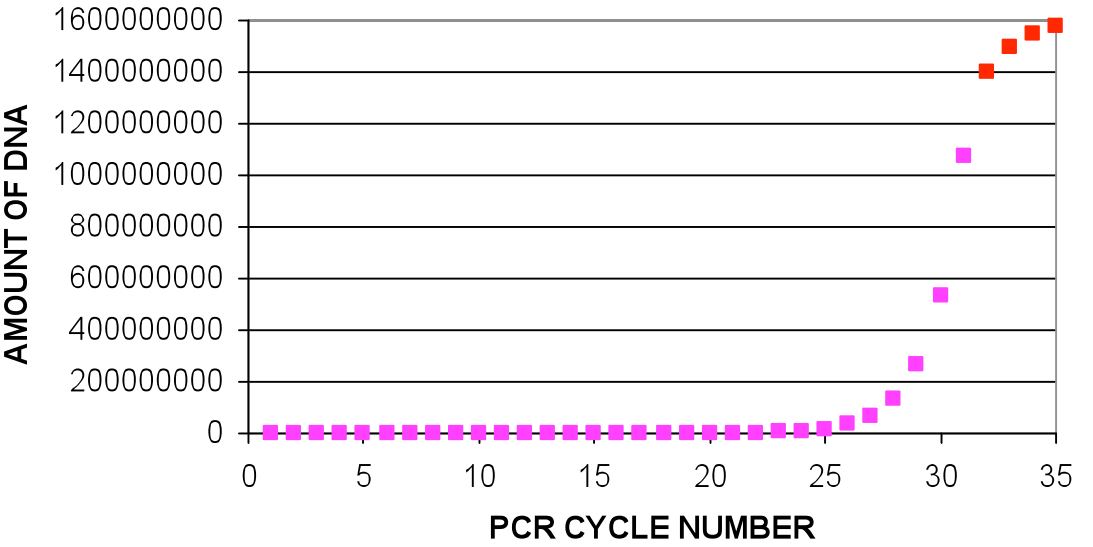
Real time PCR monitors the fluorescence emitted during the reactions as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection.

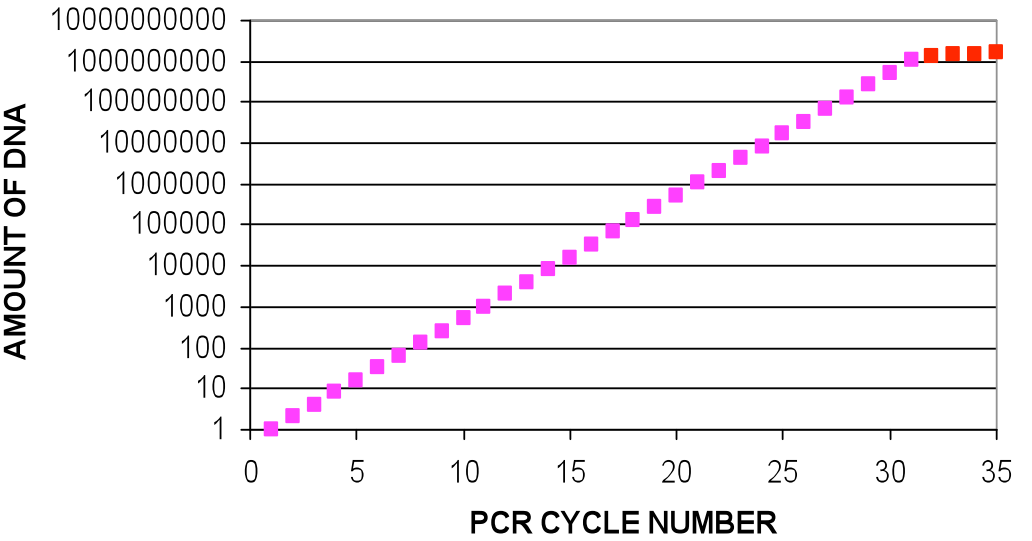


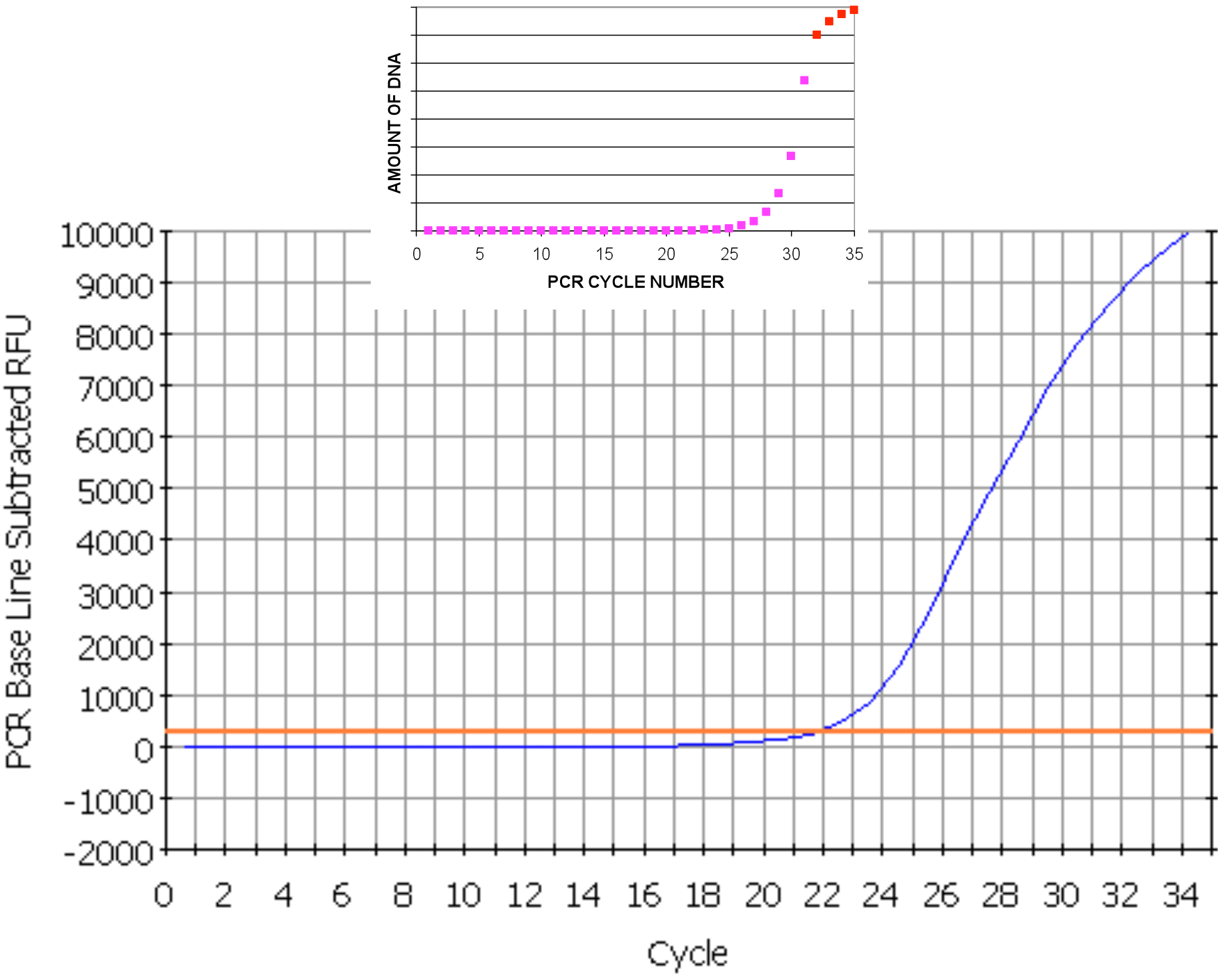
Quantitative (Real Time) PCR

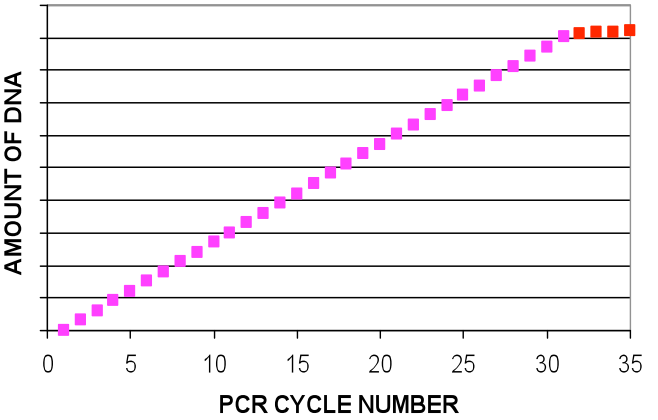
- Detection of “amplification-associated fluorescence” at each cycle during PCR
- No gel-based analysis
- Computer-based analysis
- Compare to internal standards

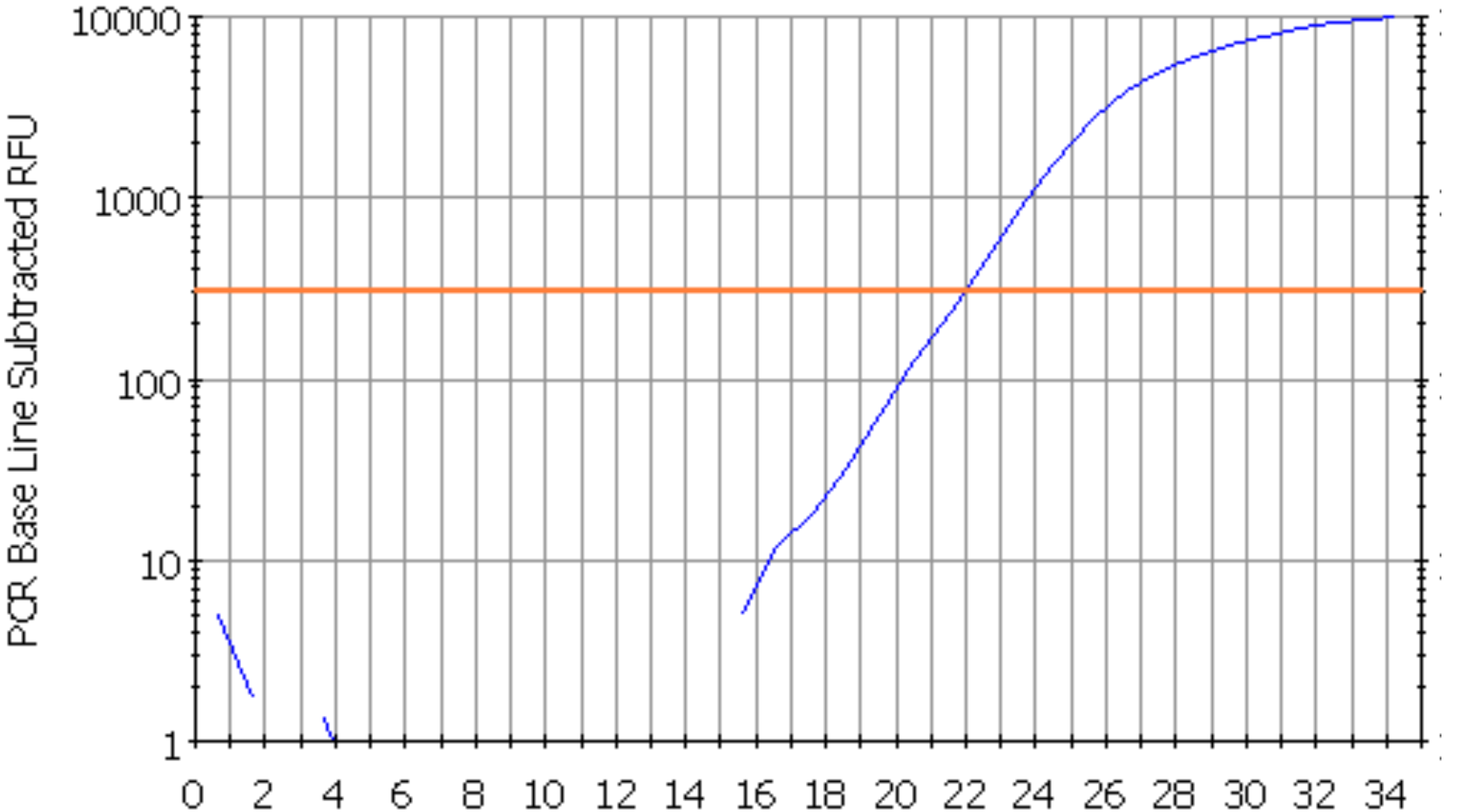
CYCLE NUMBER	AMOUNT OF DNA
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000






AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER
PCR Base Line Subtracted RFU
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
-2000
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Cycle


AMOUNT OF DNA
0
5
10
15
20
25
30
35
PCR CYCLE NUMBER


PCR Base Line Subtracted RFU
10000
1000
100
10
1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34

PCR Equation

$$X_n = X_0(1 + E)^n$$

X_n =PCR product after cycle n
X_0 =initial copy number
E =amplification efficiency
n =cycle number

A difference of **0.1** in amplification efficiency create a **five-fold** difference in the final ratio of PCR products after 30 cycles.

Case 1: E = 0.9

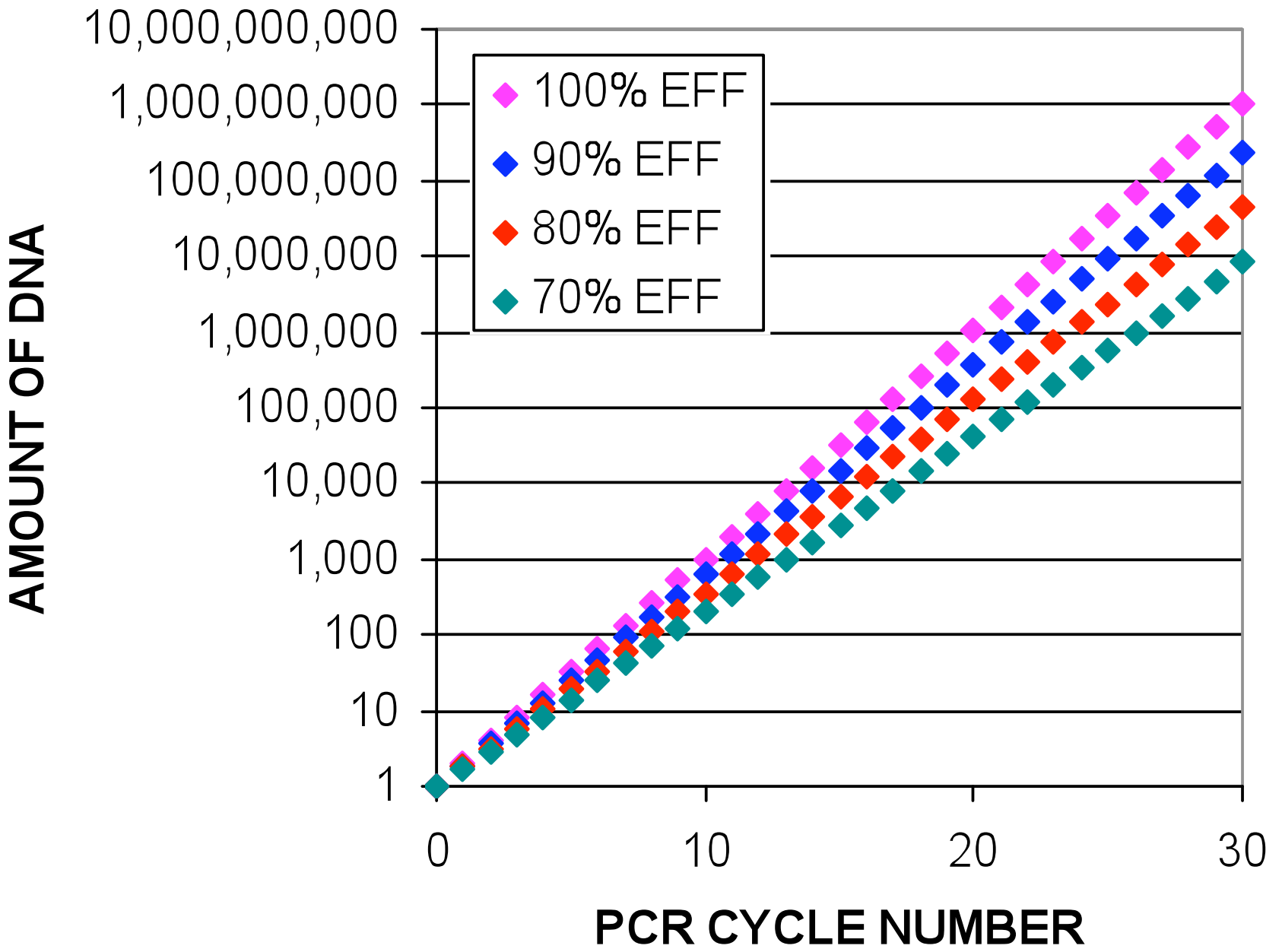
$X_n = 100\ (1+0.9)^{30}$

$X_n = 2.3 \times 10^{10}$

Case 2: E = 0.8

$X_n = 100\ (1+0.8)^{30}$

$X_n = 4.6 \times 10^{9}$


10,000,000,000
1,000,000,000
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
AMOUNT OF DNA
100% EFF
90% EFF
80% EFF
70% EFF
0
10
20
30
PCR CYCLE NUMBER

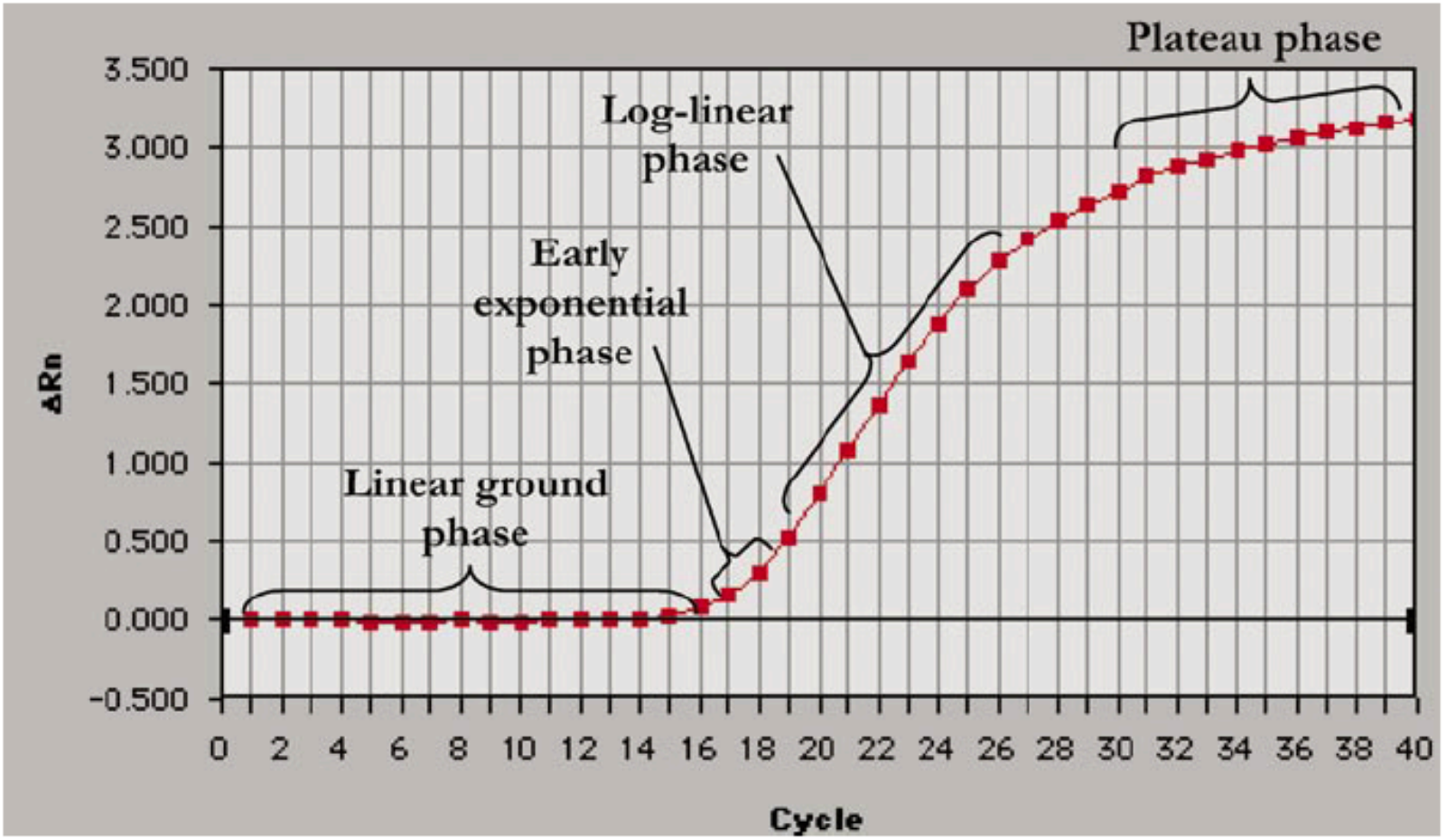


Figure 2. Phases of the PCR amplification curve. The PCR amplification curve charts the accumulation of fluorescent emission at each reaction cycle. The curve can be broken into four different phases: the linear ground, early exponential, log-linear, and plateau phases. Data gathered from these phases are important for calculating background signal, cycle threshold (C_t), and amplification efficiency. Rn is the intensity of fluorescent emission of the reporter dye divided by the intensity of fluorescent emission of the passive dye (a reference dye incorporated into the PCR master mix to control for differences in master mix volume). ΔRn is calculated as the difference in Rn values of a sample and either no template control or background, and thus represents the magnitude of signal generated during PCR. This graph was generated with ABI PRISM SDS version 1.9 software (Applied Biosystems).