Introduction to PCR

ABCF Training Module

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What is PCR?

Polymerase Chain Reaction

- An *in vitro* process that detects, identifies, and copies (amplifies) a specific piece of DNA in a biological sample.
- Dependent on an enzyme for the reaction to proceed…. DNA Polymerase

- Polymerase Chain Reaction (PCR) conceptualized in 1983 by American biochemist Dr Kary Banks Mullis
- Nobel Prize-winner in chemistry in 1993,for the invention of the PCR.

- "***Beginning with a single molecule of genetic material DNA, PCR can generate 100 billion similar molecules in an afternoon. The reaction is easy to execute. It requires no more than a test tube, a few simple reagents, and a source of heat. “***

-Kary Mullis, Scientific American

Uses of PCR

- Medicine: detecting infectious organisms, discovering variations and mutations in genes
- Genome Projects: DNA sequencing
- The law: Genetic fingerprinting
- Evolutionary biology: taxonomic classification
- Zoology: research on animal behaviour
- Ecology: studies on seed dispersal, reducing illegal trade in endangered species, monitoring release of GMOs
- Archaeology and palaeontology: ancient DNA, analyzing genetic variations in animals and plants

Powledge, *Advan. Physiol. Edu.* 28: 44-50, 2004

Theory of PCR

- The Driver of PCR is the **Polymerase** enzyme
- A polymerase will synthesize a complementary sequence of bases to any single strand of DNA **providing it has a double stranded starting point**

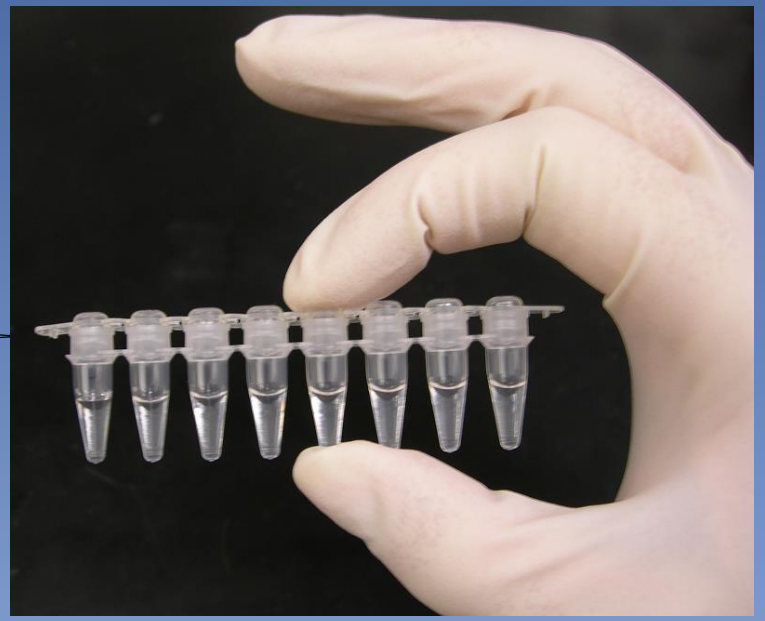
- Any gene can be specifically amplified by the polymerase in a mixed DNA sample by adding small pieces of complementary DNA
- These small pieces of DNA are known as **primers** because they prime the DNA sample ready for the polymerase to bind and begin copying the gene of interest.
- During a PCR, **changes in temperature** are used to control the activity of the polymerase and the binding of primers.

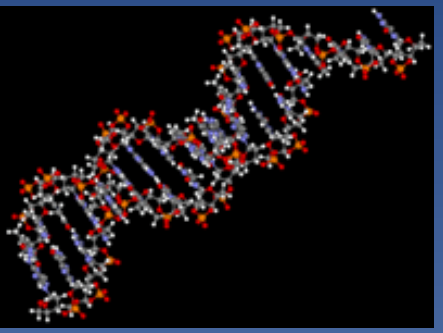
PCR Basics 1

PCR Requires the following:

- Template DNA to be amplified
- Pair of DNA primers
- Thermostable DNA polymerase
- dNTPs
- Buffer to maintain pH and to provide Magnesium Ions

- Thermal cycler





www.accelrys.com

Reaction Components

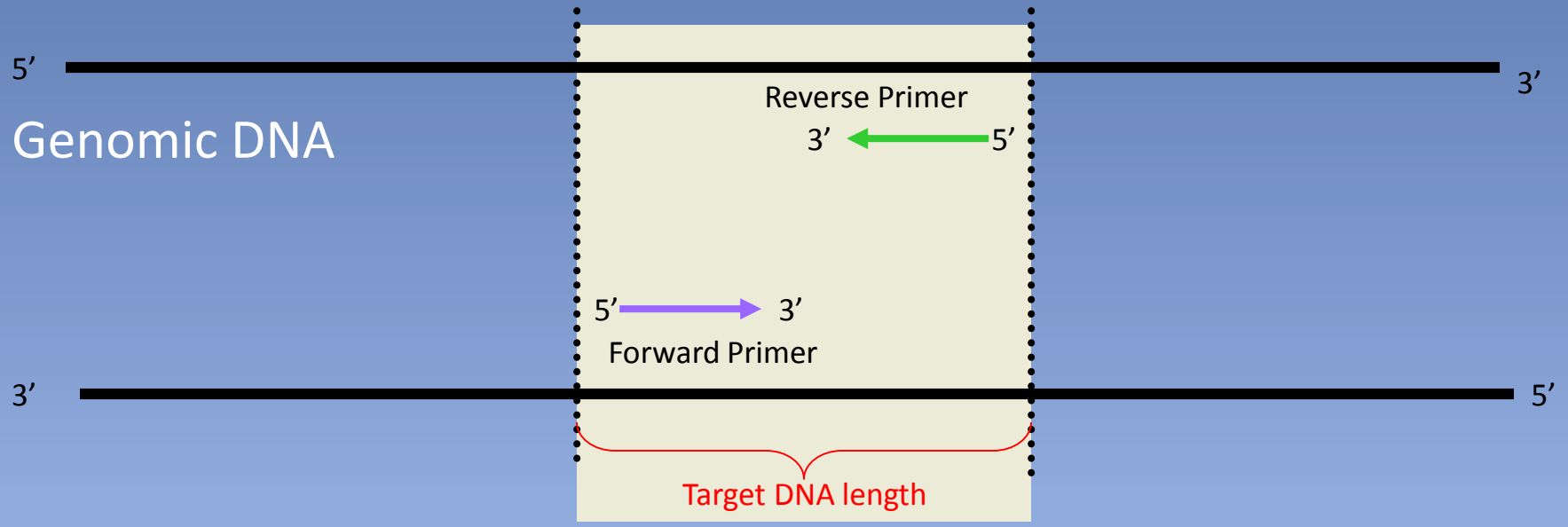
1. Template DNA
   - A sequence of DNA that is to be copied. Also called *target* DNA.
   - Can amplify (copy) a piece of DNA ~50 to ~4000 base pairs long (maybe more, depending on ingredients).
   - DNA must be isolated from an organism before it can be copied (remember Cell lysis, Protein denaturation, DNA precipitation)
   - DNA strand is normally double stranded molecule(double helix), held together by hydrogen bonds between bases attached to two strands
     - A=T
     - C≡ G

Reaction Components

2. A Pair of DNA primers

In the cell (*in vivo*), primers are short RNA strands that serve as a starting point for DNA replication

In a PCR reaction (*in vitro*), Primers are short synthetic strands of single stranded DNA that exactly match the beginning and the end of the DNA fragment to be amplified.



Reaction Components

3. DNA polymerase

Polymerase builds a new DNA strand in the 5’ to 3’ direction.

The newly-polymerized molecule is complementary to the template strand and identical to the template's partner strand.

DNA polymerase

DNA polymerase must be Thermostable (Heat–stable) because of high temperatures used in PCR

Called Taq polymerase because it is isolated from the bacteria *Thermus aquaticus* (they live in hot springs)

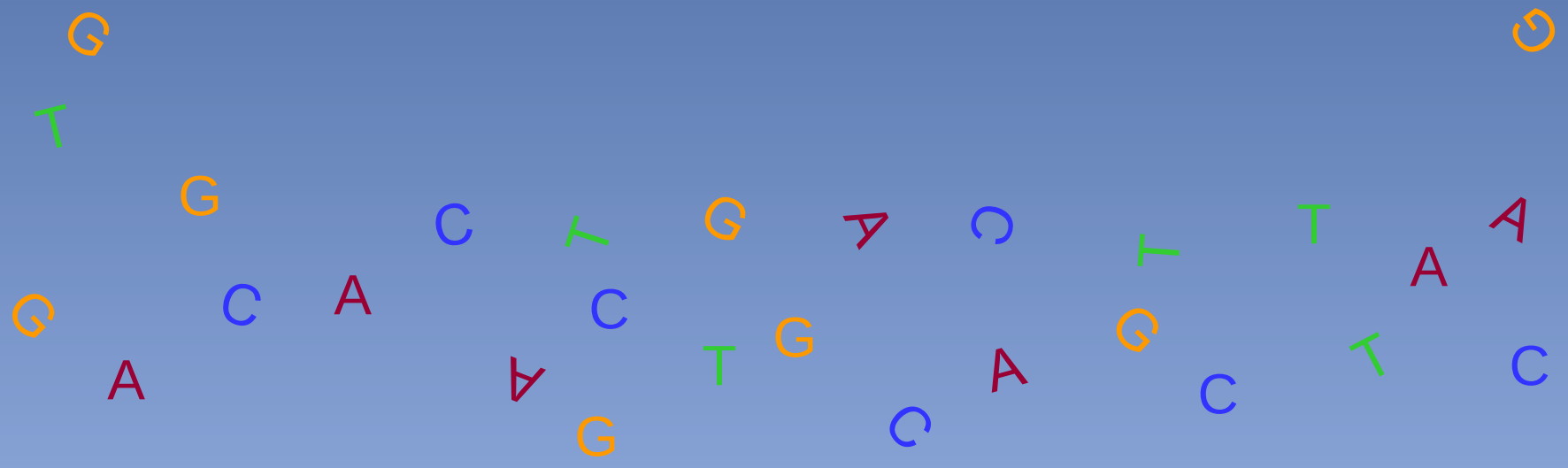
Brock & Freeze, 1969

Reaction Components

4. dNTPs

dNTPs (deoxynucleosides) are the building blocks in the PCR Reaction.

They are the monomers that DNA polymerase uses to form DNA…..the A's, T's, C's and G's that will build the new strand of DNA.



Reaction Components

5. Buffer

To work properly, Taq needs mg^{++}

The concentration of magnesium ions needs to be optimized with each target and primer combination.

Too little magnesium could equal little or no PCR product, too much could mean unwanted product….a fine line.

Buffer also maintains pH

PCR Basics 2

- PCR cycling components
- Equipment
  - Laminar flow cabinet/clean bench area
  - Pipettes, tips, vials/PCR tubes, Vortex mixer, micro-centrifuge, ice buckets/cold blocks etc.
  - Thermocycler
  - Gel electrophoresis units
  - UV trans-illuminator /Gel doc system

How Does PCR Work?
A Three-Step Process

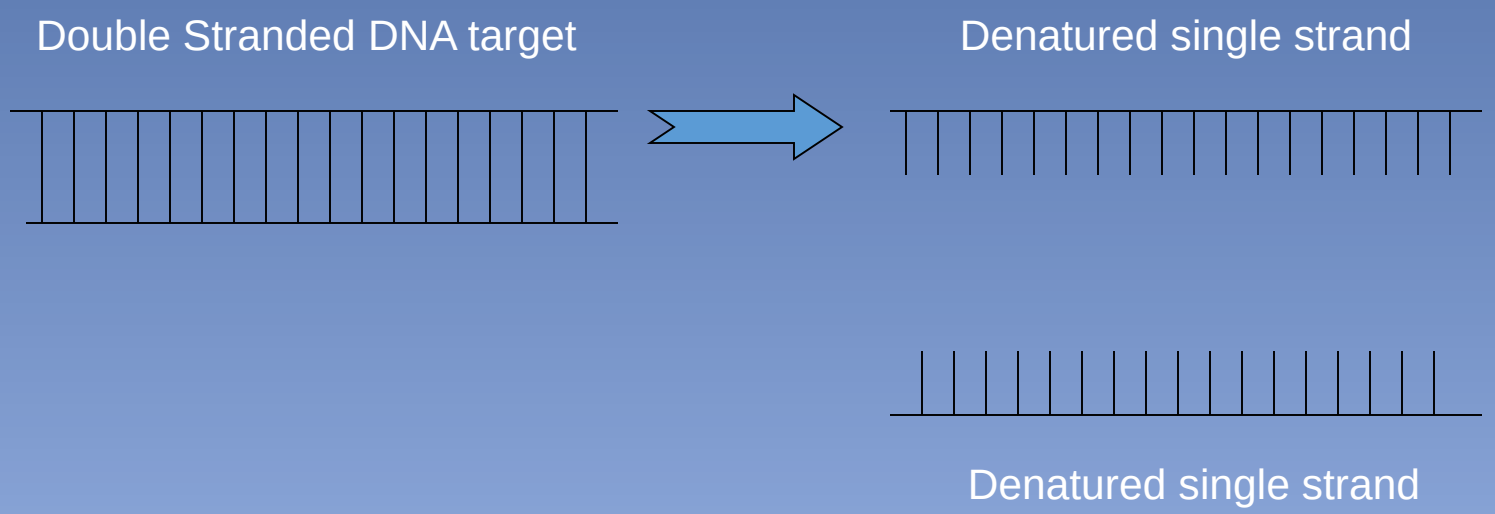
Each step happens at a different temperature

Step 1: Denaturation
Step 2: Annealing
Step 3: Extension

1 cycle

How Does PCR Work?
Step 1: Denaturation

- Heat over 90ºC breaks the hydrogen bonds of DNA and separates double-stranded molecule into two single strands

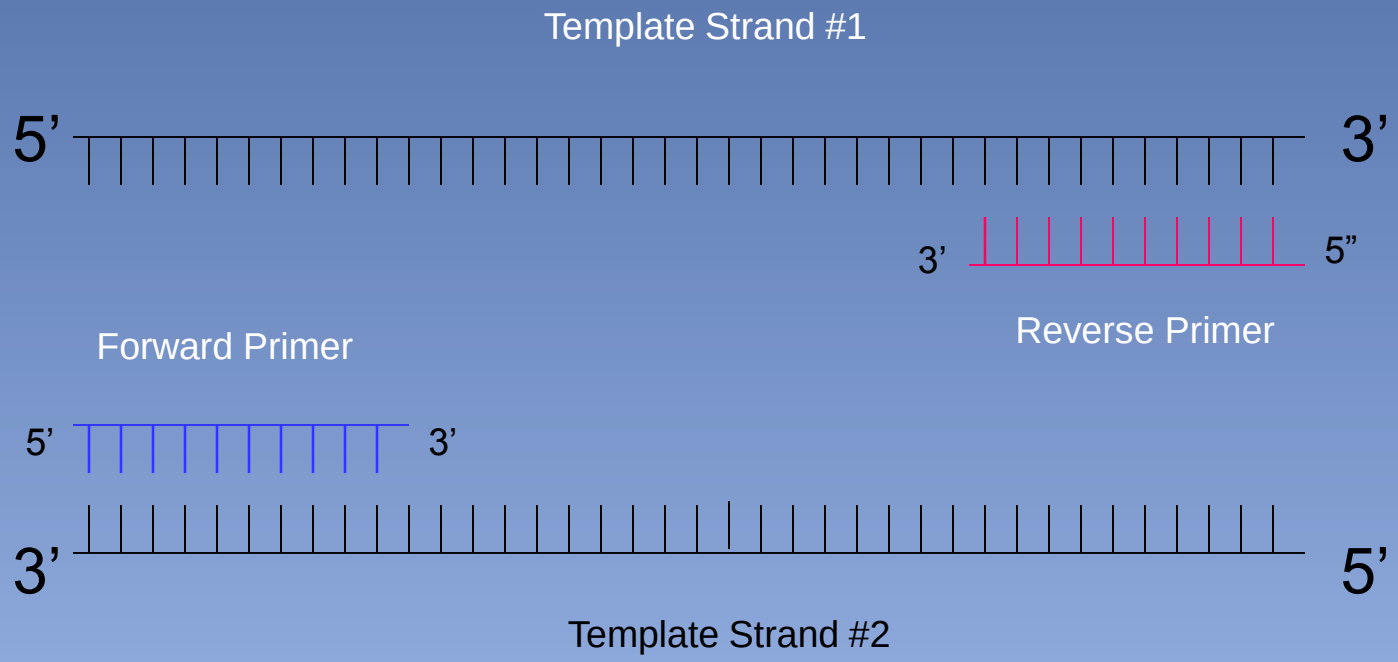


How Does PCR Work?

Step 2: Annealing - Primer Binding to Target also called Hybridization

Temperature is reduced ≈ 50-65ºC

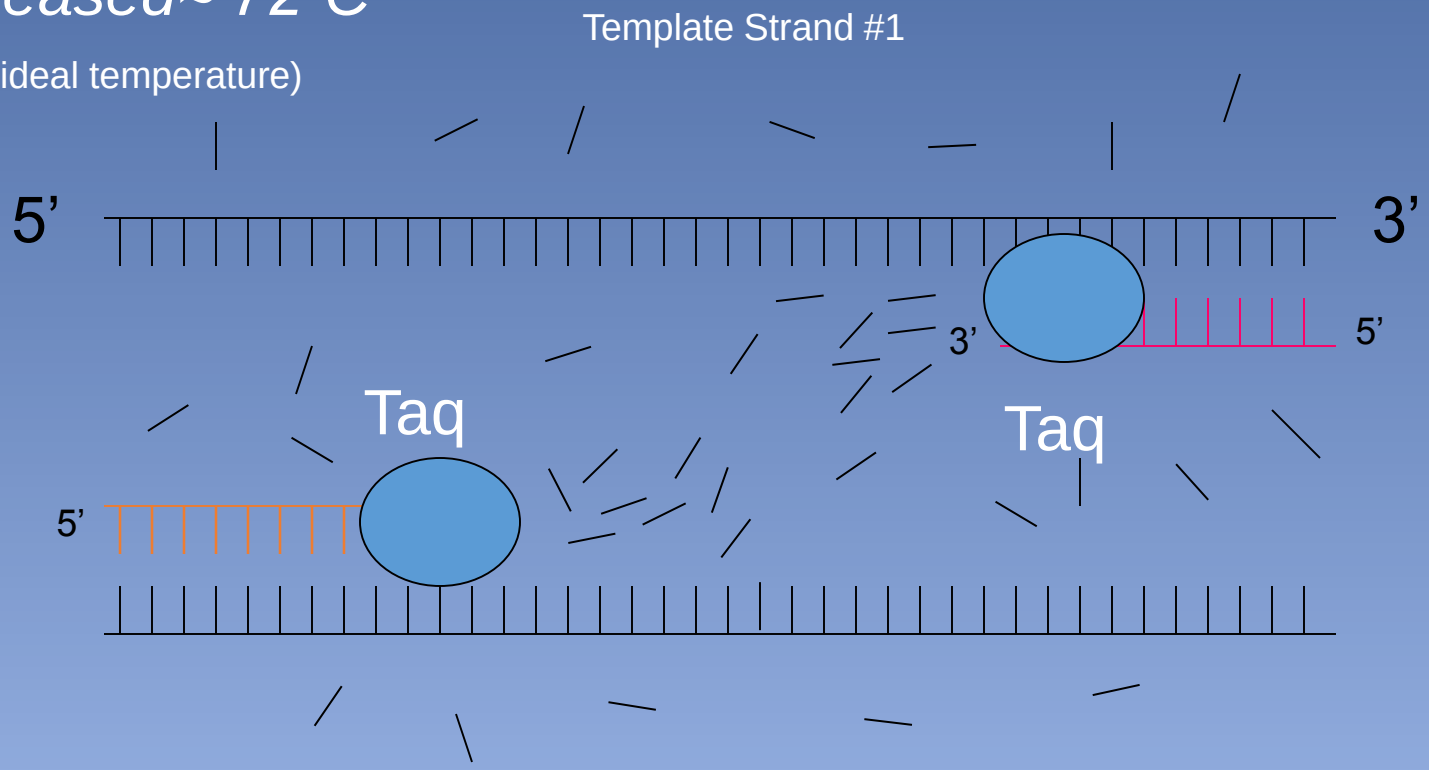
(Annealing temperature depends on primer length and G-C content.)



How Does PCR Work?
Step 3: Extension

Temperature is increased≈ 72ºC

(taq's ideal temperature)



Thermal Cycler

Also known as:

- Thermocycler
- PCR machine
- DNA amplifier

A very early PCR machine
Samples moved mechanically

Modern Machines
Using the Peltier effect
1834 by Jean-Charles Peltier

Thermal cycler

Is needed for PCR

Thermal cyclers have metal heat blocks with holes where PCR reaction tubes can be inserted. The thermalcycler then raises and lowers the temperature of the block at each step (denaturation ~95℃, annealing ~55℃ and extension 72℃)

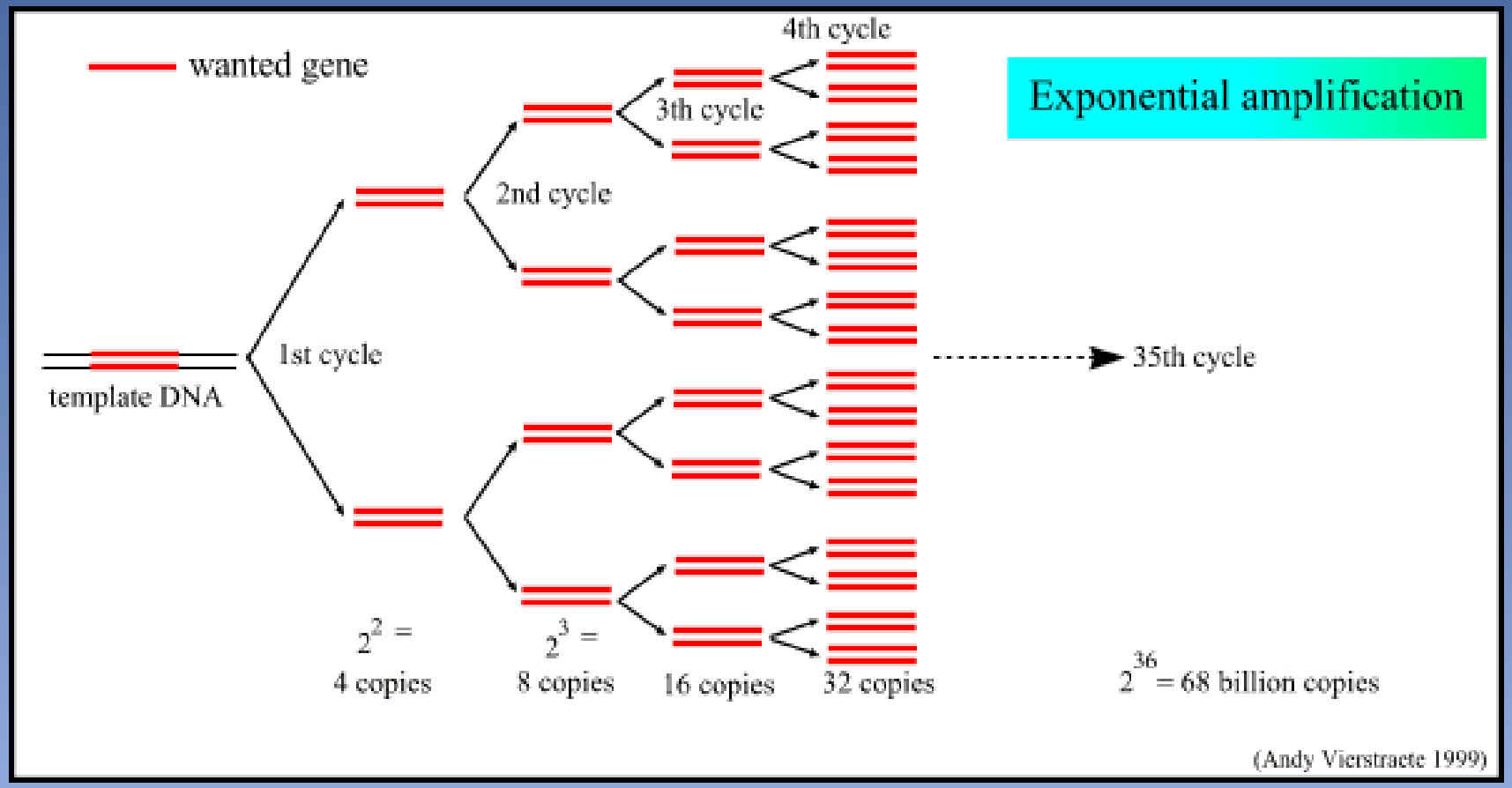
Component	Volume	Final conc.
10X PCR buffer minus Mg	10 µl	1X
10 mM dNTP mixture	2 µl	0.2 mM each
50 mM $MgCl^2$	3 µl	1.5 mM
Primer mix (10 µM each)	5 µl	0.5 µM each
Template DNA	1–20 µl	<1000ng
Taq DNA Polymerase (5 U/µl)	0.2–0.5 µl	1.0–2.5 units
Autoclaved distilled water	to 100 µl	n/a

PCR
a CHAIN reaction

- At the end of the first PCR cycle, there are now two new DNA strands identical to the original target
- Multiple Cycles (30-40)
- Exponential Growth
  - # of Copies $=2^n$

    (Where ^{n} is the number of cycles)

Exponential Amplification of the Target DNA Sequence



BEWARE !

Other DNA can contaminate the PCR reaction

Sources:

- The person who is setting up the reaction
- The tubes
- The enzymes, buffers or water used in the reaction

VALIDATION

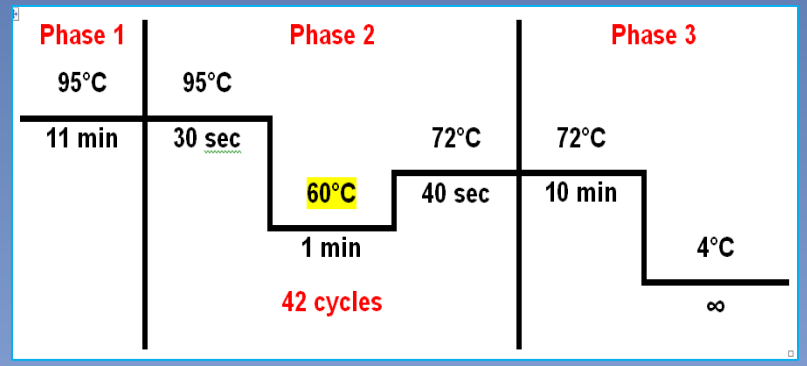
Do a negative control (no DNA) to validate that the PCR product is amplified from the intended DNA, not some other source of DNA.

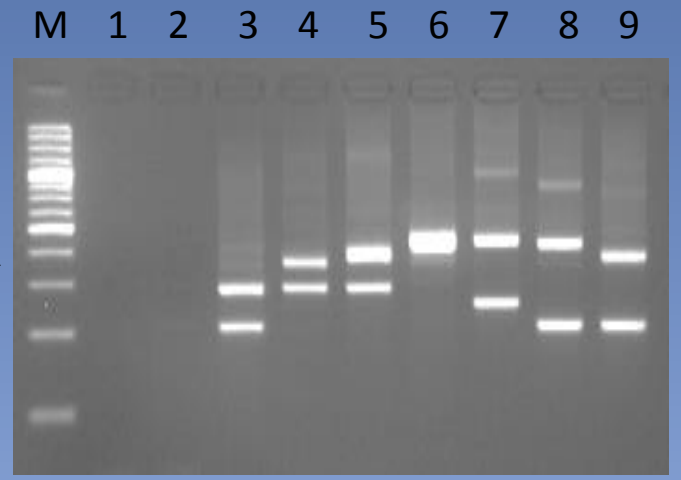
A positive control using DNA with good primers validates that the reaction conditions and thermal cycler work properly.

PCR: Analysis

At the end of a PCR reaction, there is a A LOT more of your target DNA than before the reaction started…billions of copies!

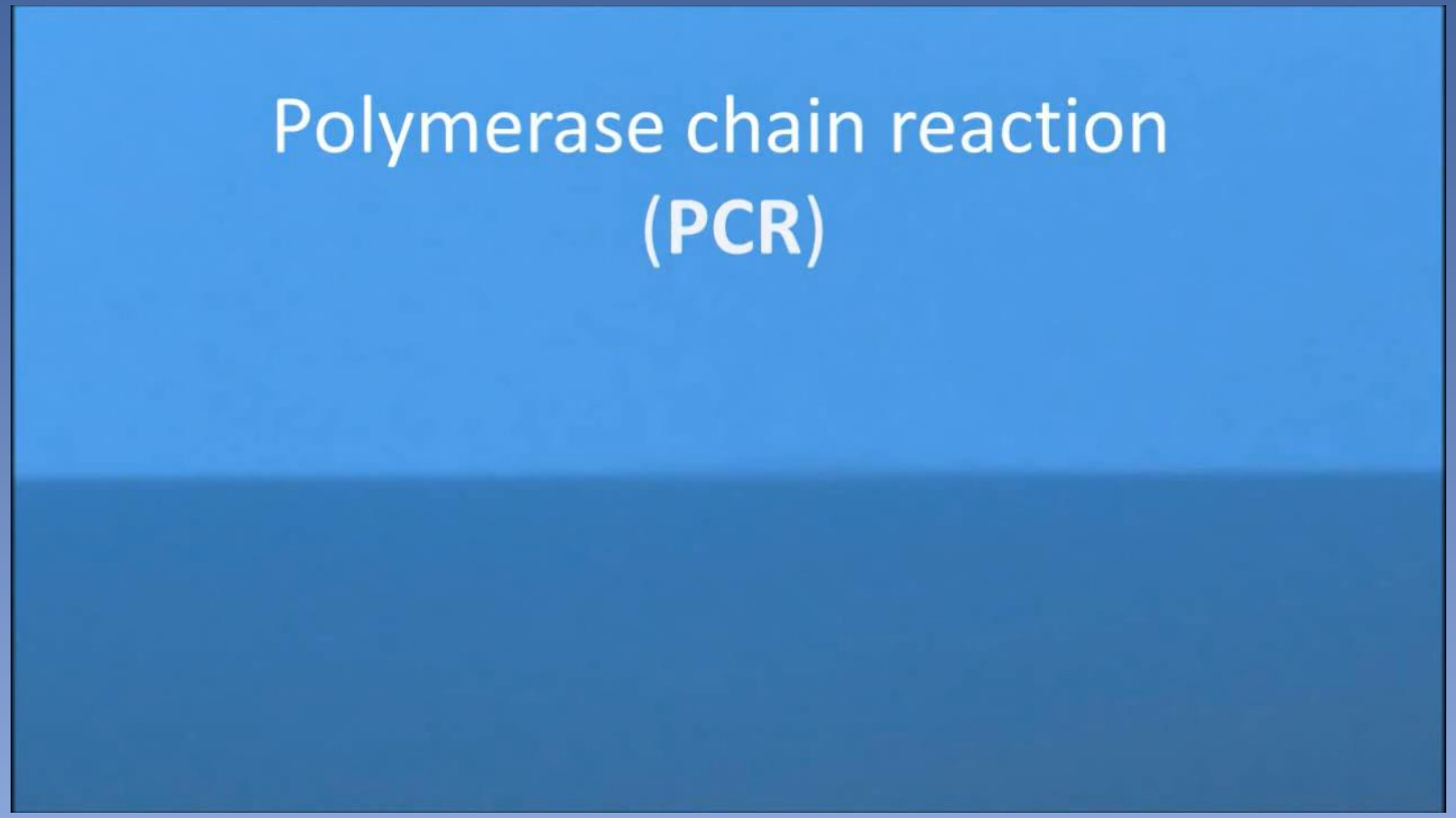
Now the sample is large enough to be seen on a gel and analyzed.





Gel electrophoresis image

PCR Animation



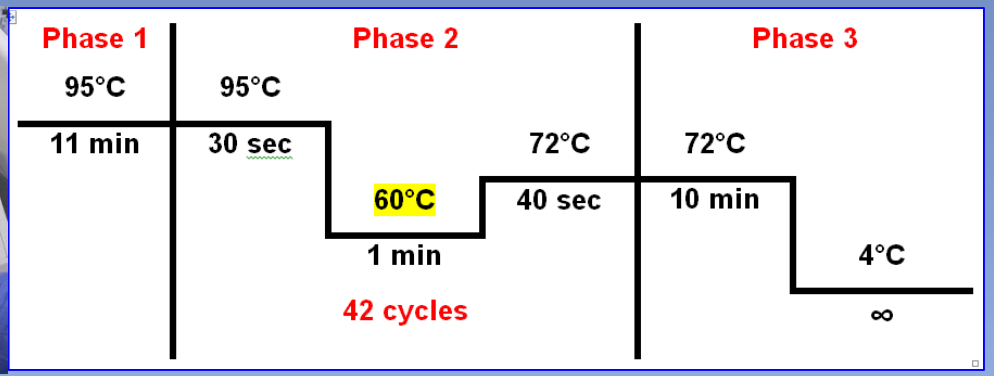
PCR Optimization

Main Areas for PCR Optimization

1- PCR reaction mix

gDNA ---------- 3ul (100-300ng)
10xBuffer ---------- 10ul
25mM $MgCl_2$ ---------- 10ul
25mM dNTP ---------- 1ul
Fwd (100ng/ul) ---------- 1ul
Rev (100ng/ul) ---------- 1ul
AmpliTaq Gold ---------- 0.5ul
H_2O ---------- 75.7 ul
Total ---------- 100ul

2- PCR cycling conditions



Parameters that affect PCR

The specificity, yield, and fidelity of PCR are influenced by the nature of target sequence, as well as by each component of PCR.

Essential components of polymerase chain reactions:

1. Enzyme
2. Primer
3. Deoxynucleoside triphosphates (dNTPs)
4. Divalent cations
5. Buffer to maintain pH
6. Monovalent cations
7. Template DNA
8. PCR additives

gDNA	**3ul (100-300ng)**
10xBuffer	**10ul**
25mM $MgCl_2$	**10ul**
25mM dNTP	**1ul**
Fwd (100ng/ul)	**1ul**
Rev (100ng/ul)	**1ul**
AmpliTaq Gold	**0.5ul**
H_2O	**75.7 ul**
Total	**100ul**

Enzyme: Taq DNA Polymerase

- A thermostable DNA polymerase to catalyze template-dependent synthesis of DNA
- Depending on the ability, fidelity, efficiency to synthesize large DNA products, a wide choice of enzymes is now available
- For routine PCRs, Taq (0.5-2.5 units per standard 25-50 µl reaction) remains the enzyme of choice
- Do not exceed 2.5 U/50 µl, especially for amplicons that are > 5kb
- The efficiency of Taq becomes limiting when too much molecules of amplified product have accumulated in the reaction

Primers

- A pair of synthetic oligonucleotides to prime DNA synthesis
- Influence the efficiency and specificity of the amplification reaction
- The ratio of primer/template is also important regarding the specificity of PCR. If the ratio is too high, PCR is more prone to generate unspecific amplification products
- Typically 0.1-0.5µM of each primer ($6x10^{12}$ to $3x10^{13}$ molecules) recommended
- Higher concentrations of primers favour mispriming which may lead to nonspecific amplification.

Deoxynucleoside triphosphates (dNTPs)

- Standard PCRs contain equimolar amounts of all four dNTPs
- Standard conc. of 20-250µM of each dNTP in reactions
- Lower concentrations of dNTPs may increase both the specificity and fidelity of the reaction while excessive dNTP concentrations can actually inhibit PCR(>4mM)
- For longer PCR-fragments, a higher dNTP concentration may be required
- Freeze-thawing affect stability of dNTPs (stored at -20^{o}C in small aliquots)
- To minimize changes in concentration (due to condensation), spin vials dNTP solutions, after thawing, for a few seconds in a micro centrifuge

Divalent cations: Mg^{2+}

- All Taq require free divalent cations- usually Mg^{2+} for activity(co-factor)
- Because dNTPs and primers bind Mg^{2+}, the molar conc. of the cation must exceed the molar conc. of phosphate groups contributed by dNTPs and primers
- Standard conc. of 1.5 mM of Mg^{2+} is routinely used . The optimal conc. of Mg^{2+} must therefore be determined empirically for each combination of primers, template, dNTP conc. and buffer composition
- In general, the optimal Mg^{2+} conc. is 0.5 to 1 mM over the total dNTP conc. for standard PCR
- Increasing conc. of Mg^{2+} to 4.5 mM or 6mM has been reported to decrease nonspecific priming in some cases and to increase it in others
- Excessive Mg^{2+} stabilizes the DNA double strand and prevents complete denaturation of DNA
- The preparations of template DNA should not contain significant amount of chelating agents (like EDTA, EGTA or negatively charged ions like PO_4^{3-}), which can sequester Mg^{2+}

Buffer to maintain pH

- Tris -Cl, adjusted to a pH between 8.3 and 8.8 at room temperature is included in standard PCRs at a conc. of 10mM
- When incubated at 72^{o}C (extension phase of PCR), the pH of the reaction mixture will drop by more than a unit

Monovalent cations

- Standard PCR buffer contains 50mM KCl and works well for amplification of segments of DNA >500bp in length
- Raising the KCl concentration to ~70-100mM often improves the yield of shorter DNA segments
- If the desired amplicon is below 1000 bp and long non-specific products are forming, specificity may be improved by titrating KCl
- Increasing the salt concentration permits shorter DNA molecules to denature preferentially to longer DNA molecules.

Template DNA

- DNA quality and purity affect a PCR experiment. Presence of DNA contaminants interfere with PCR reaction(protein, RNA, organic solvents, and detergents)
- Template DNA can be added to PCR in single or double stranded form
- Closed circular DNA templates are amplified slightly less efficiently than linear DNA>>be improved by digesting the template with a restriction enzyme that doesn't cleave within the target sequence
- At best, PCR requires only a single copy of target sequence as template. Thus:
- 1 pg – 10 ng / 50 µl reaction with low complexity DNA (e.g. plasmid, lambda)
- 50-250 ng / 50 µl reaction with high complexity genomic DNA
- For mammalian genomic DNA, up to 1µg of DNA is utilized per reaction

PCR Additives

- DMSO can be added at 3% in reaction for GC-rich templates, which aids in the denaturing of templates with high GC contents
- For further optimization, DMSO should be varied in e.g. 2% increments
- DMSO can also be used for supercoiled plasmids to relax for denaturation
- If high DMSO concentration is used, the annealing temperature must be lowered, as DMSO decreases the melting point of the primers
- Other PCR additives are formamide (1-10%), glycerol (5-20%), betaine (1-2%), BSA (10-100ug/ml), PEG (5-15%), etc.

Modifying PCR Cycling Conditions

1. **Denaturation** of the template by heat
2. **Annealing** of the oligonucleotide primers to the single stranded target sequence(s), and
3. **Extension** of the annealed primers by a thermostable DNA polymerase

Initial Denaturation: some templates may require longer initial denaturation and the length of the initial denaturation time can be extended up to 3 minutes

Final Extension: completing partial synthesis

Annealing Temperature

- Optimization of annealing temperature with calculation of the $T_m = 4(G + C) + 2(A + T)$
- A single-base mismatch lowers the T_m by ~5°C
- Set the annealing Temperature Ta 5°C below the calculated T_m as a first approximation of the optimum Ta

PCR artifacts and Plateau Effects

- The concentration of the target DNA should be balanced with the number of cycles in the reaction
- Using an elevated concentration of the target combined with the normal or higher number of cycles can cause nonspecific products
- The accumulation of nonspecific products is often observed in a reamplification PCR, when the high initial concentration of the PCR fragment is accompanied by a high number of cycles. Reducing the number of cycles may help