PCR & RT-PCR

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PCR & RT-PCR

- What is the Polymerase Chain Reaction (PCR)?
- History of PCR
- How PCR works?
- Optimising PCR
- PCR primer design
- What is the Reverse Transcriptase-PCR (RT-PCR)?

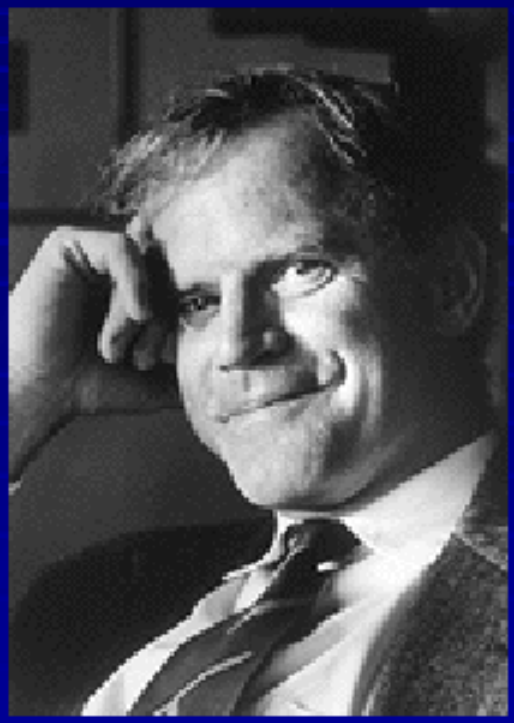
What is the Polymerase Chain Reaction?

- It's a means of selectively amplifying a particular segment of DNA.
- The segment may represent a small part of a large and complex mixture of DNAs: *e.g.* a specific exon of a human gene.
- It can be thought of as a molecular photocopier.
- A photocopier capable of duplicating a part of a sentence

How Powerful is PCR?

- PCR can amplify a usable amount of DNA (visible by gel electrophoresis) in ~2 hours.
- The template DNA need not be highly purified.
- The PCR product can be digested with restriction enzymes, sequenced or cloned.
- PCR can amplify a single DNA molecule.

Kary B Mullis: Inventor of PCR



The Invention of PCR

- Invented by Kary Mullis in 1983.
- First published account appeared in 1985.
- Awarded Nobel Prize for Chemistry in 1993.

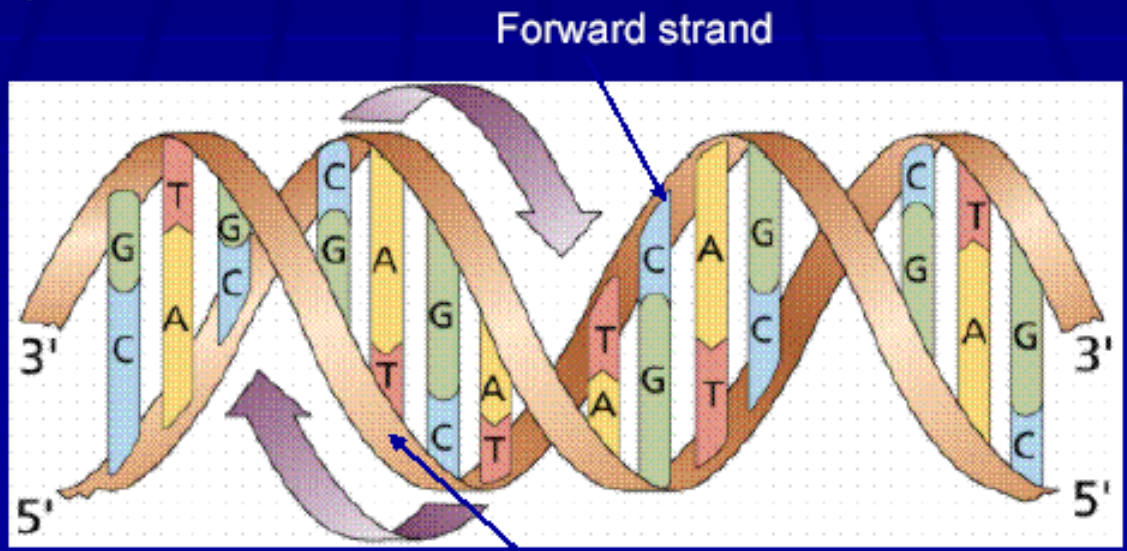
DNA

- Double stranded
- Each strand is made up of polynucleotides
- dATP. dTTP, dGTP, dCTP
- **Purines: A**denine and **G**uanine **Pyrimidines: C**ytosine and **T**hymine (Uracil in RNA)

- A, T, G, C

A = T

C ≡ G



PCR

- Polymerase chain reaction (PCR)
- PCR is an *in vitro* technique for the amplification of a region of DNA which lies between two regions of known sequence.
- PCR amplification is achieved by using oligonucleotide primers.
  - These are typically short, single stranded oligonucleotides (DNA) which are complementary to the outer regions of known sequence.

Steps in PCR analysis of a sample

DNA extraction from a sample

PCR

Run the PCR product on agarose gel

Visualize the PCR product under UV light

DNA (template) extraction methods

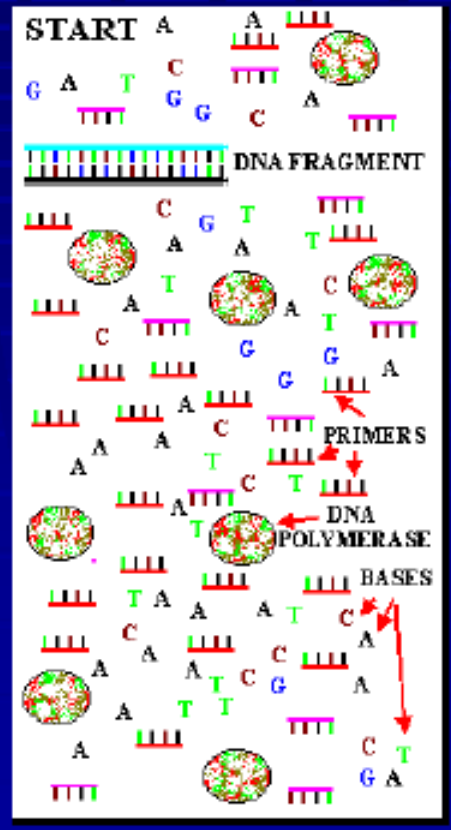
- Phenol chloroform method
- Silica matrix method √
- Boiling method
- Chelex/ Instagene method

What's in the PCR Reaction mix?

- Template DNA
- Reaction buffer (Tris, ammonium ions (and/or potassium ions) & optional (bovine serum albumin, DMSO…)
- Magnesium ions (MgCl)
- Nucleotides (dNTPs: A, T , C, G)
- Primers
- DNA polymerase (usually *Taq polymerase*)

PCR reaction components

- Excess of primer, dNTPS and enzymes
- Primers are actually extended in to PCR products



http://www.wsu.edu:8001/~hurlbert/pages/Chap10.html#PCR

DNA polymerases

DNA Polymerase	Natural or recombinant	Source
Taq	Natural	*Thermus aquaticus*
Amplitaq®	Recombinant	*T. aquaticus*
Amplitaq (Stoffel fragment)®	Recombinant	*T. aquaticus*
Hot *Tub*™	Natural	*Thermus flavis*
Pyrostase™	Natural	*T. flavis*
Vent™	Recombinant	*Thermococcus litoralis*
Deep Vent™	Recombinant	*Pyrococcus GB-D*
Tth	Recombinant	*Thermus thermophilus*
Pfu	Natural	*Pyrococcus furiosus*
ULTma™	Recombinant	*Thermotoga maritima*

Can withstand 95°C and active at 72°C

Primer design

- Generally, primers used are 18 - 30 mer (bases) in length.
- 35 to 65 % GC content
- Primers should avoid stretches of polybase sequences (e.g. poly dG) or repeating motifs - these can hybridize with inappropriate register on the template.
  AAAAAAA, TTTTTT, GGGGG, CCCCC

- Inverted repeat sequences should be avoided so as to prevent formation of secondary structure in the primer, which would prevent hybridization to template , such primer will form a hairpin loop structure

  eg. CCCCTAGGGGG

CCCCC T
I I I I I I A
GGGGG G

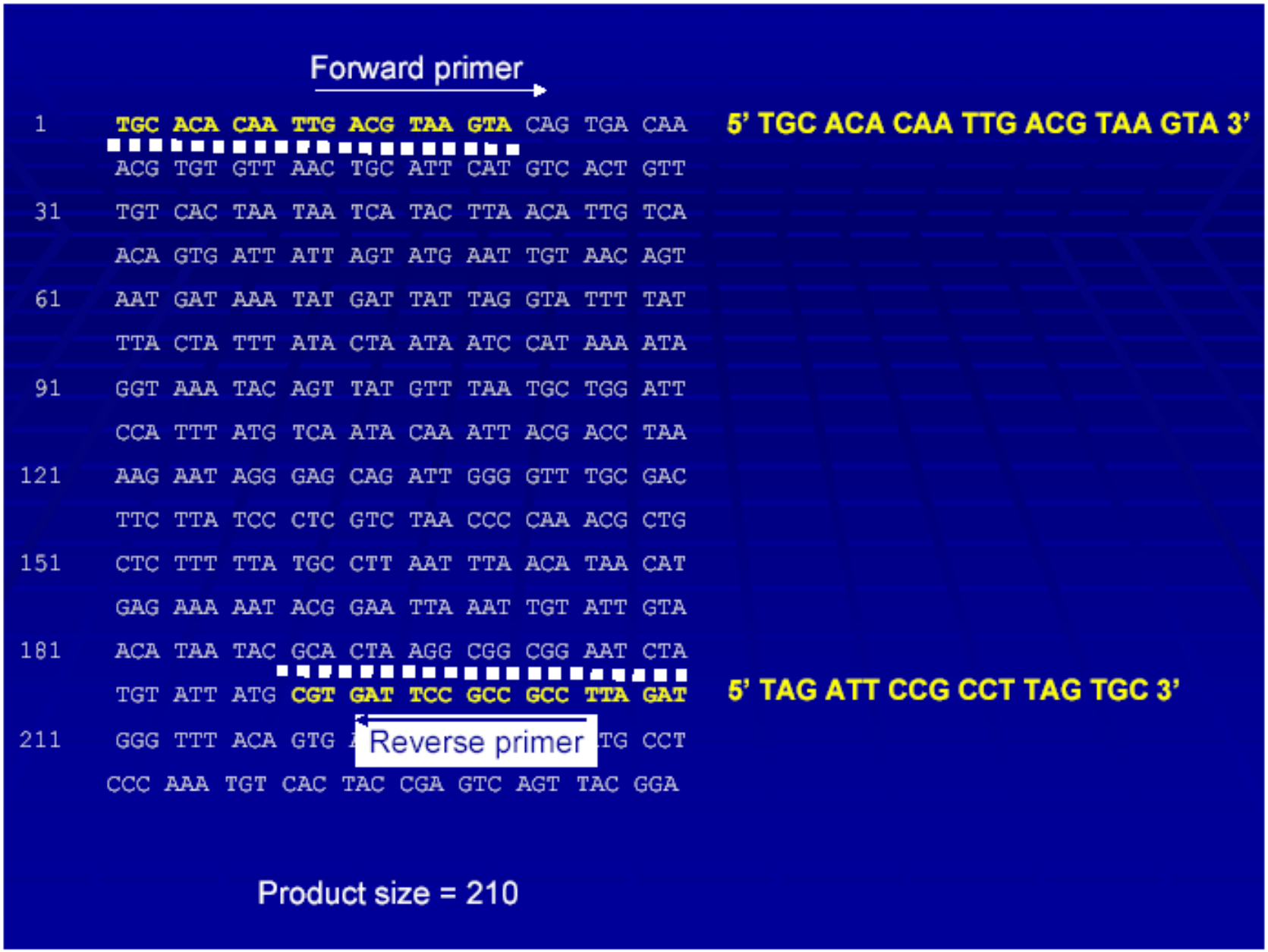
Primer design cont..

- Forward primer should not be complementary to reverse primer

```
5' ATG TAC TAT TAC TGA  CAT  GAT 3'
                  I I I  I I I I I I I
                3' ACT  GTA  CTA  GTA CAA ATC CA 5'
```

- 3' end of the primer should be complementary to the target DNA sequence, 5' end of the primer can have other sequences like restriction enzyme sites, promoter site etc.,

- The distance between primers should be less than 10 Kb in length. Typically, substantial reduction in yield is observed when the primers extend from each other beyond ~3 Kb.


Forward primer
1 TGC ACA CAA TTG ACG TAA GTA CAG TGA CAA
ACG TGT GTT AAC TGC ATT CAT GTC ACT GTT
31 TGT CAC TAA TAA TCA TAC TTA ACA TTG TCA
ACA GTG ATT ATT AGT ATG AAT TGT AAC AGT
61 AAT GAT AAA TAT GAT TAT TAG GTA TTT TAT
TTA CTA TTT ATA CTA ATA ATC CAT AAA ATA
91 GGT AAA TAC AGT TAT GTT TAA TGC TGG ATT
CCA TTT ATG TCA ATA CAA ATT ACG ACC TAA
121 AAG AAT AGG GAG CAG ATT GGG GTT TGC GAC
TTC TTA TCC CTC GTC TAA CCC CAA ACG CTG
151 CTC TTT TTA TGC CTT AAT TTA ACA TAA CAT
GAG AAA AAT ACG GAA TTA AAT TGT ATT GTA
181 ACA TAA TAC GCA CTA AGG CGG CGG AAT CTA
TGT ATT ATG CGT GAT TCC GCC GCC TTA GAT
211 GGG TTT ACA GTG
TG CCT
CCC AAA TGT CAC TAC CGA GTC AGT TAC GGA
Reverse primer
5' TGC ACA CAA TTG ACG TAA GTA 3'
5' TAG ATT CCG CCT TAG TGC 3'
Product size = 210

Guidelines for the design and use of primers

Sequence:

- Avoid runs of 3 or more G or C at the 3′ end
- Avoid a T at the 3′ end
- Avoid mismatches at the 3′ end
- Avoid complementary sequences within a primer and between primers

Length: 18–30 nucleotides

GC content: 40–60%

T_m: $T_m = 2°C \times (A+T) + 4°C \times (C+G)$

Conc.: 0.1–0.5 µM (0.2 µM)

Mg^{+} ions concentration

- The MgCl2 concentration in the final reaction mixture is usually between 0.5 to 5.0 mM, and the optimum concentration is determined empirically (typically between 1.0 - 1.5 mM). **Mg2+ ions**
- form a soluble complex with dNTP's which is essential for dNTP incorporation
- Mg+ ions are essential for polymerase activity
- increase the Tm (melting temperature) of primer/template interaction (i.e. it serves to stabilize the duplex interaction
- For longer template more Mg+ are required
- **low Mg2+ leads to low yields (or no yield)**
- **high Mg2+ leads to accumulation of nonspecific products (mispriming).**

Steps of PCR

- *Template denaturation*
- *Primer annealing*
- *Primer extension*

- *These three steps comprise a single "cycle" in the PCR amplification*

Template denaturation

- The *initial* denaturation of template is accomplished at 95-100 °C.
  - Denaturation during the PCR experiment (i.e. second cycle onward) is usually accomplished at temperatures of 92-95 °C (usually empirically determined).

Primer annealing temperature

- It is an important parameter in the success of the PCR experiment.
- The annealing temperature is characteristic for each oligonucleotide:
  - it is a function of the length and base composition of the primer as well as the ionic strength of the reaction buffer.
  - These calculated annealing temperatures are a *starting point* for the PCR experiment, but ideal annealing temperatures are determined empirically.
  - Tm = (A+T)2 + (G+C)4 *

* Applicable only primer for about 20bases in length

Primer extension

- Primer extension is usually performed at 72 °C, or the *optimum temperature of the Taq DNA polymerase*.
- The length of time of the primer extension steps can be increased if the region of DNA to be amplified is long. The rule of the thumb is one minute extension time per kb of DNA

Number of cycles

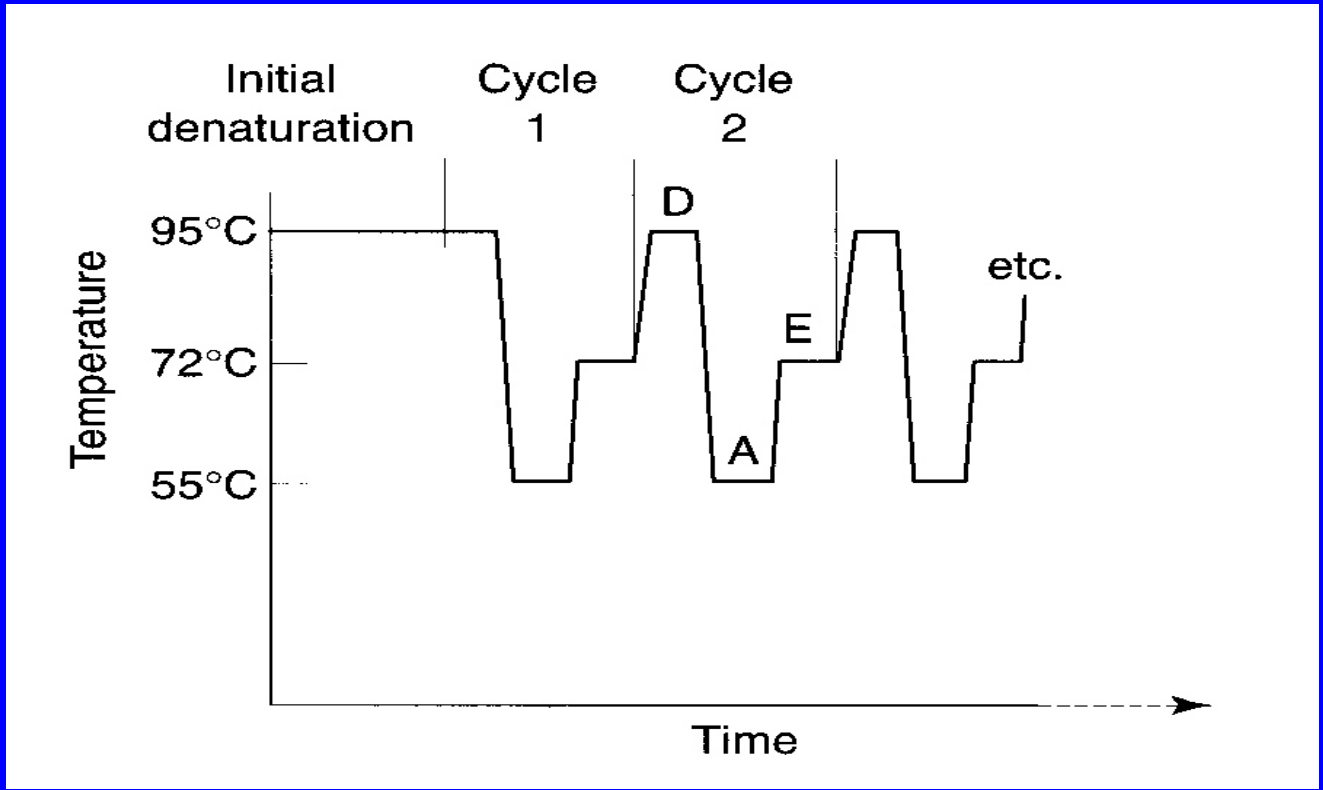
- The number of cycles is usually between 25 and 35.
  - More cycles mean a greater yield of product.
  - However, with increasing number of cycles the greater the probability of generating various artifacts (e.g. mispriming products).
  - It is unusual to find procedures which have more than 40 cycles.

PCR program

Name	Temperature		Time
First denaturing	94 ° C		3-5min
Denaturing	94 ° C		30-60 sec
Annealing	54° C		30-60 sec
Extension	72 ° C	1 min/kb	30-90 sec
Last extension	72 ° C		5 min

25-35 cycles

The Basics of PCR Cycling

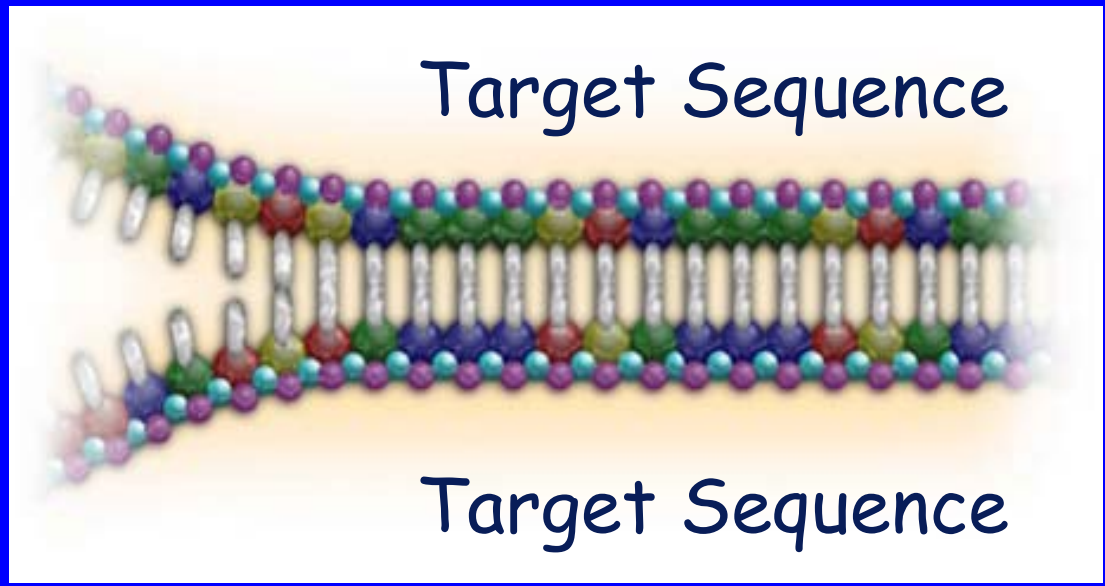


PCR In Detail

- **Denature, anneal, extend and repeat the cycle 30 to 35 times.**

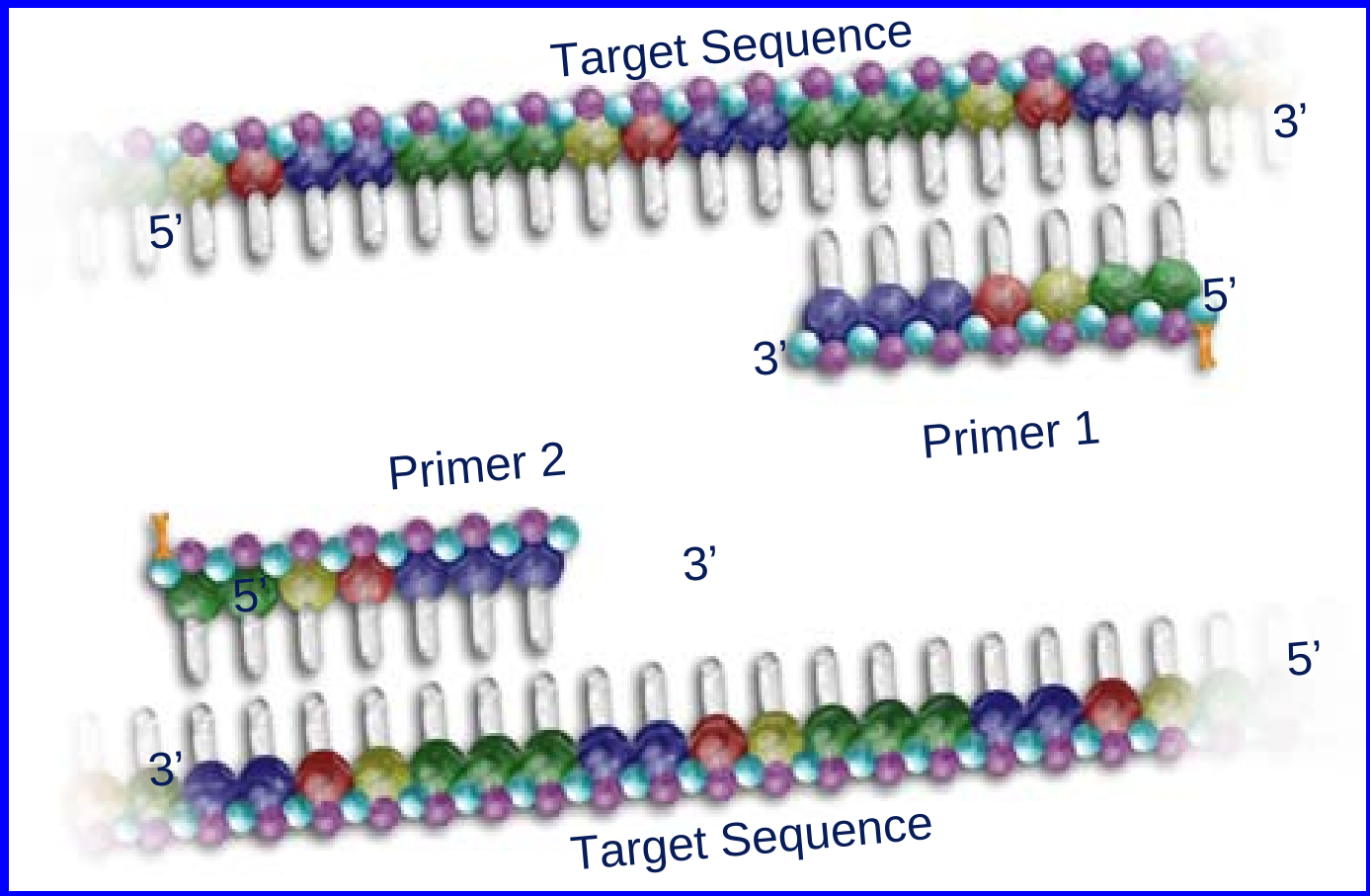
- **PCR animation movie**

PCR Cycle - Step 1
- Denaturation Template DNA by Heat (94°C)

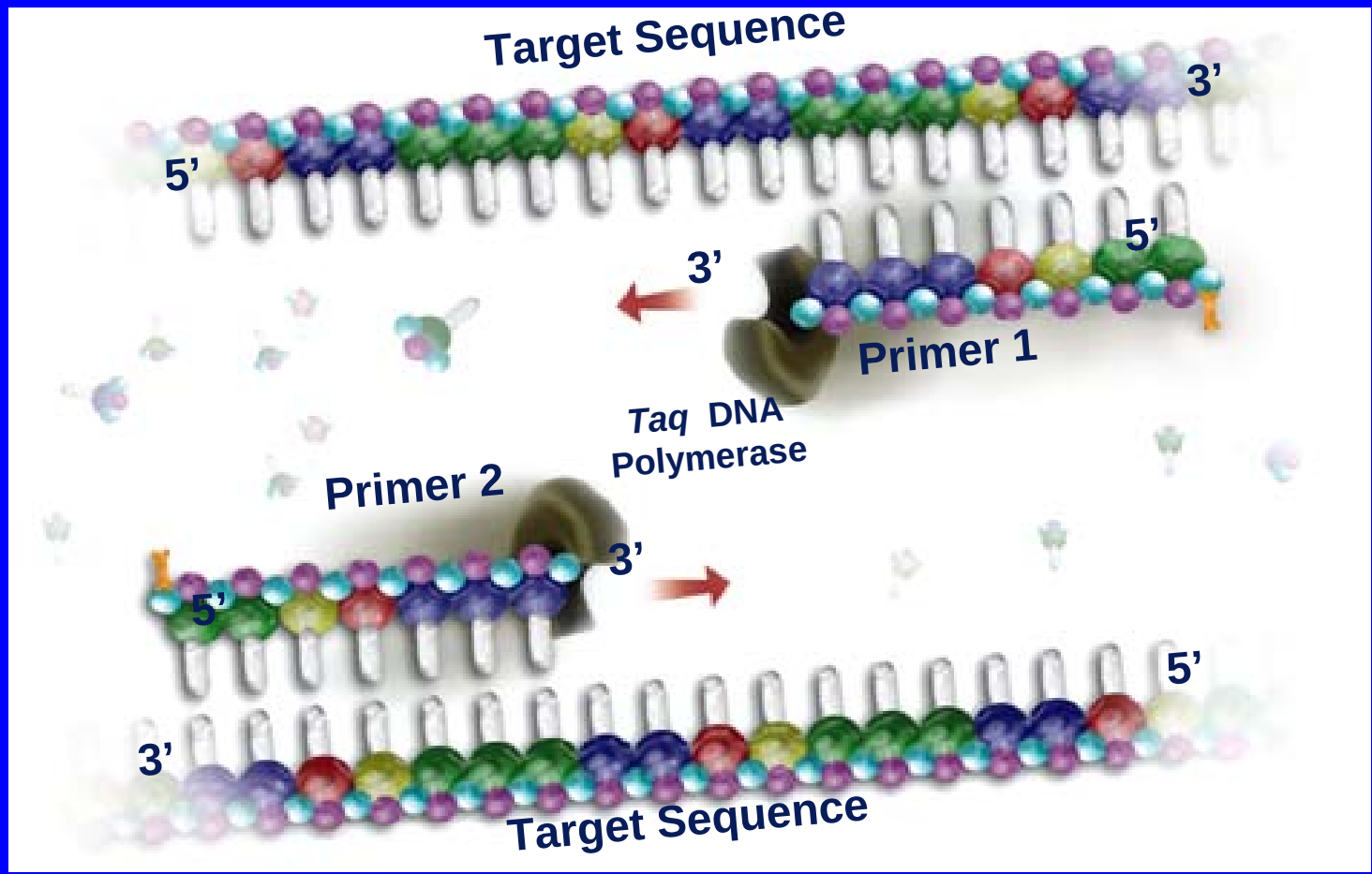


PCR Cycle - Step 2

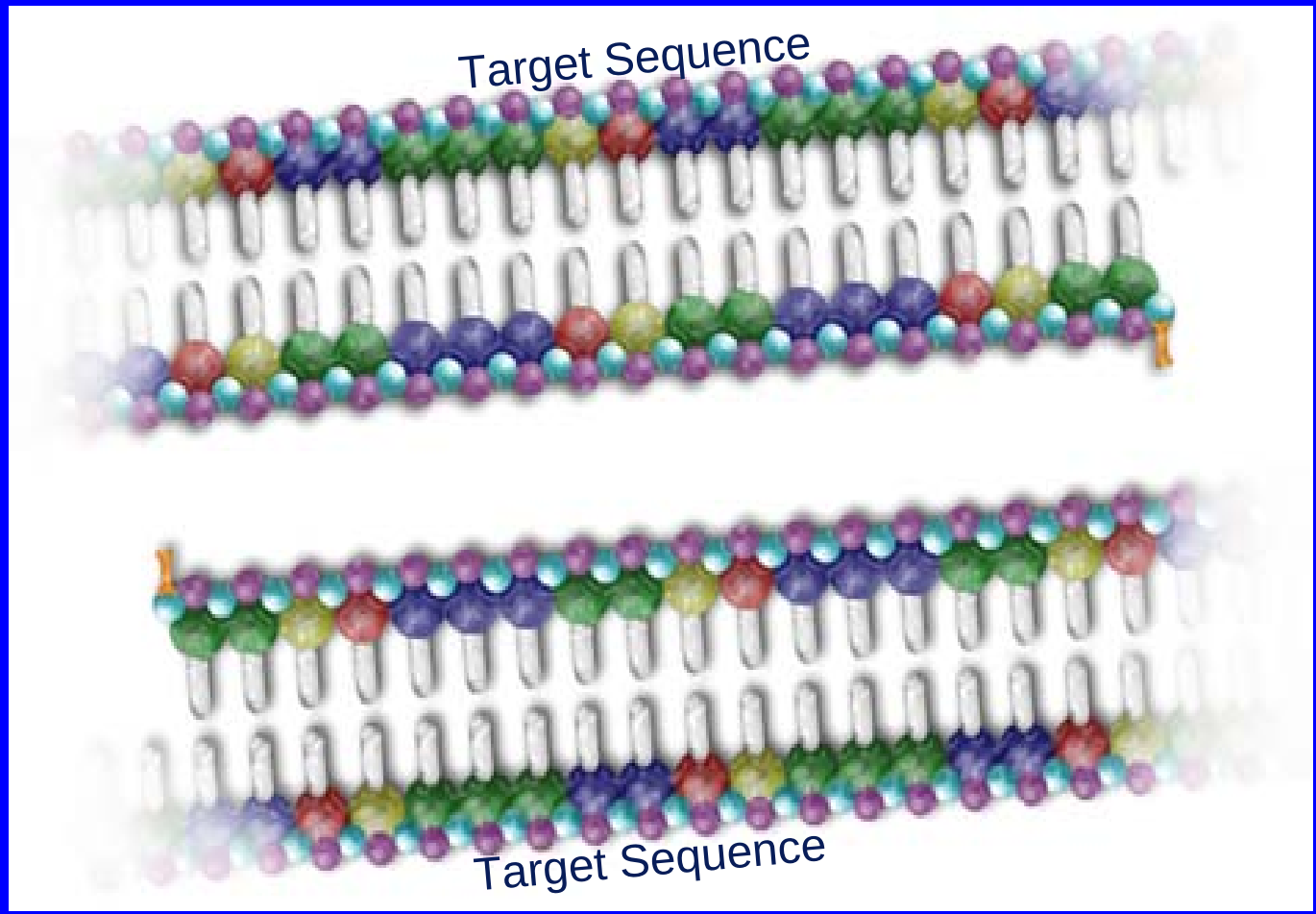
-Temperature is lowered (54ºC) and primers anneal to target sequences



PCR Cycle - Step 3
- At 72 ºC *Taq* DNA polymerase catalyses primer extension as complementary nucleotides are incorporated

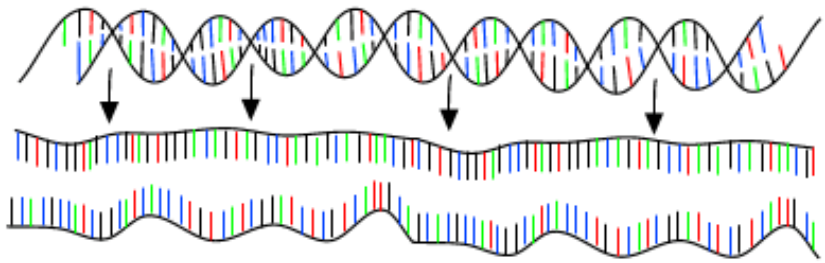


End of the 1st PCR Cycle
- Results in two copies of target sequence



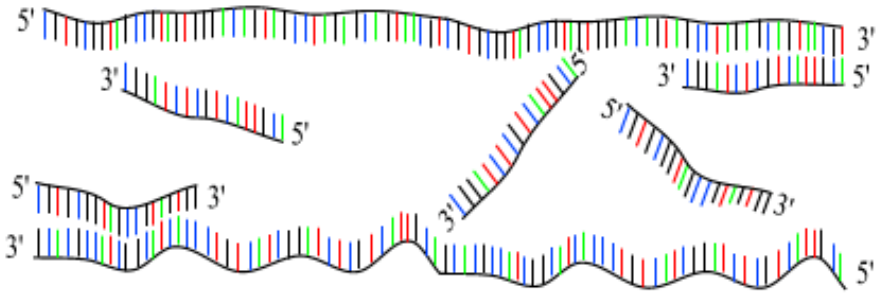
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

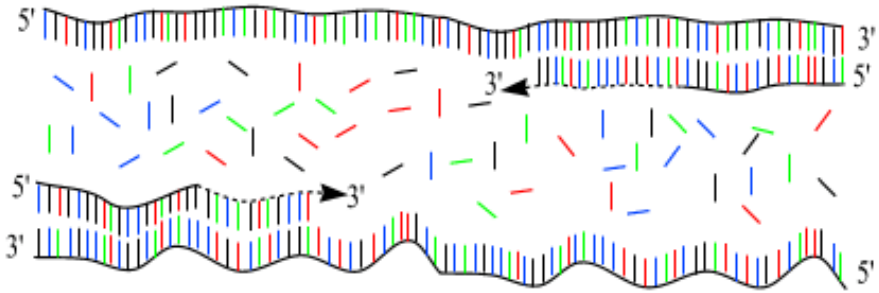
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

2 minutes 72 °C

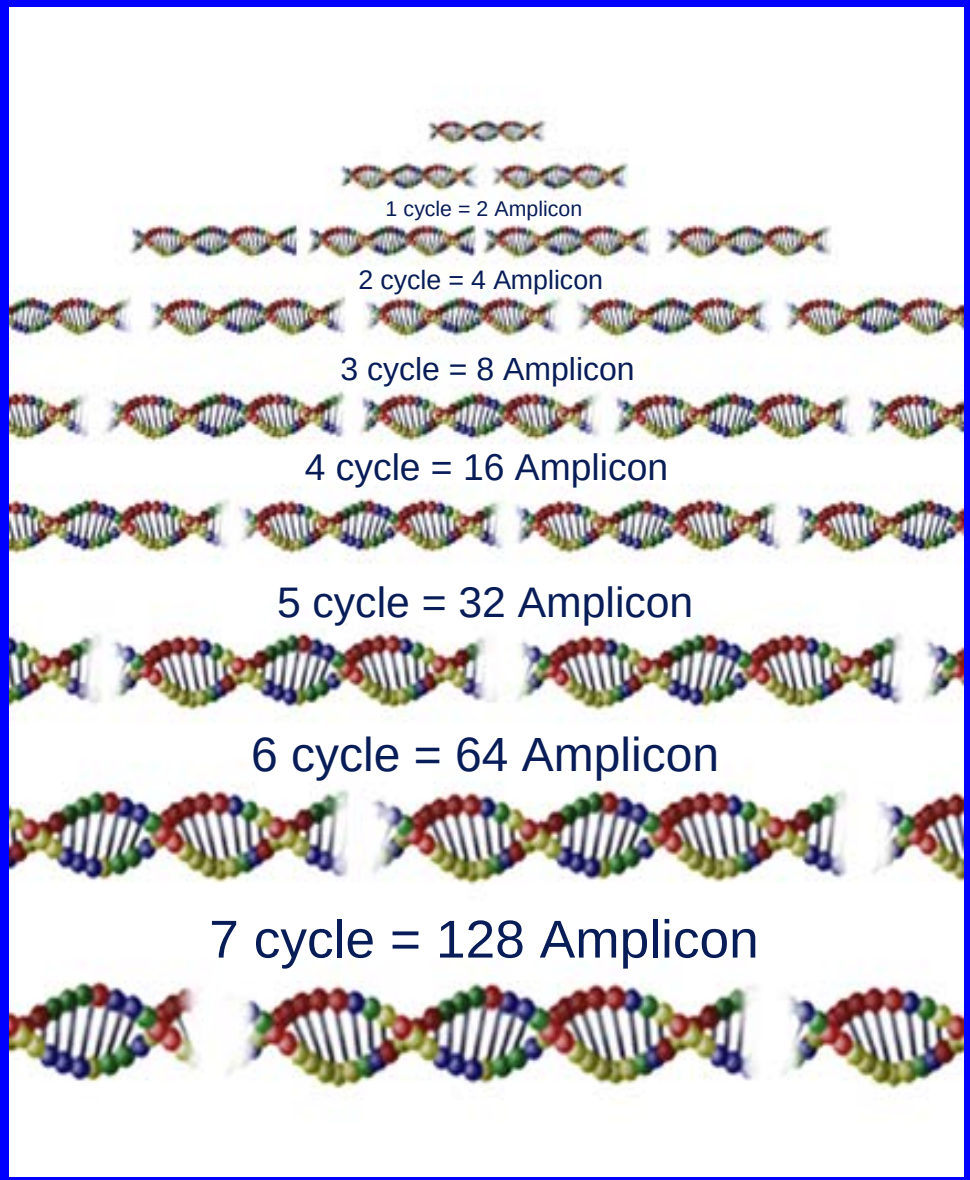
only dNTP's

(Andy Vierstraete 1999)

How many copies?

- No TARGET COPIES are made until the third cycle (see PCR amplification graph).
- At 30 cycles there are 1,073,741,824 DNA COPIES (~1×10^9).

Target Amplification



Cycles #	No. Amplicon Copies of DNA
1	2
2	4
3	8
4	16
5	32
6	64
20	1,048,576
30	1,073,741,824

So Then, it's Easy?

- Cycling performed with three water baths for denaturation, annealing and extension temperatures.
- Thermal cyclers introduced in 1986.
- Early polymerases were not thermostable, so had to be replenished each cycle.
- *Taq* (*Thermus aquaticus*) DNA polymerase first described in 1988.

Thermal Cyclers

- PCR Thermal cyclers available from many suppliers.
- Many block formats and multi-block systems.
- Reactions in tubes or 96-well micro-titre plates.

Thermal Cyclers

PCR machine

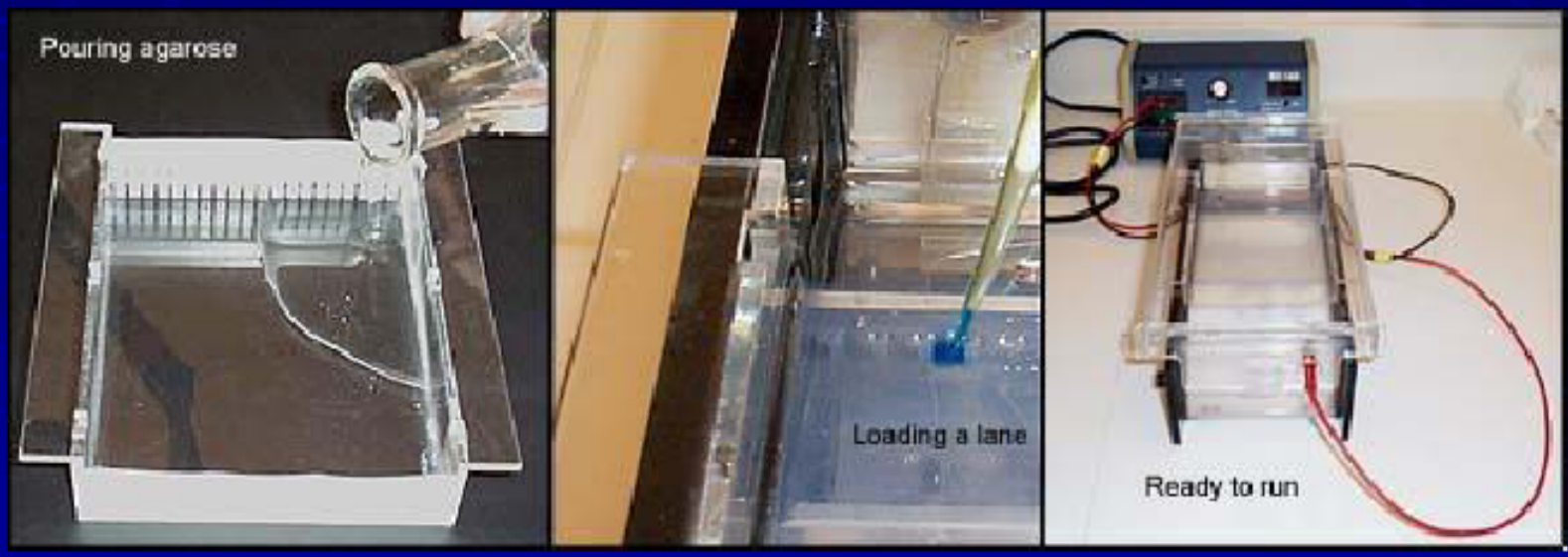
Has It Worked?
Detection of amplified product

- Agarose Gel Detection
- Solid Phase Hybridization and Colour Detection
- Real-time Detection (Real Time PCR)

Agarose gel Detection of amplified product

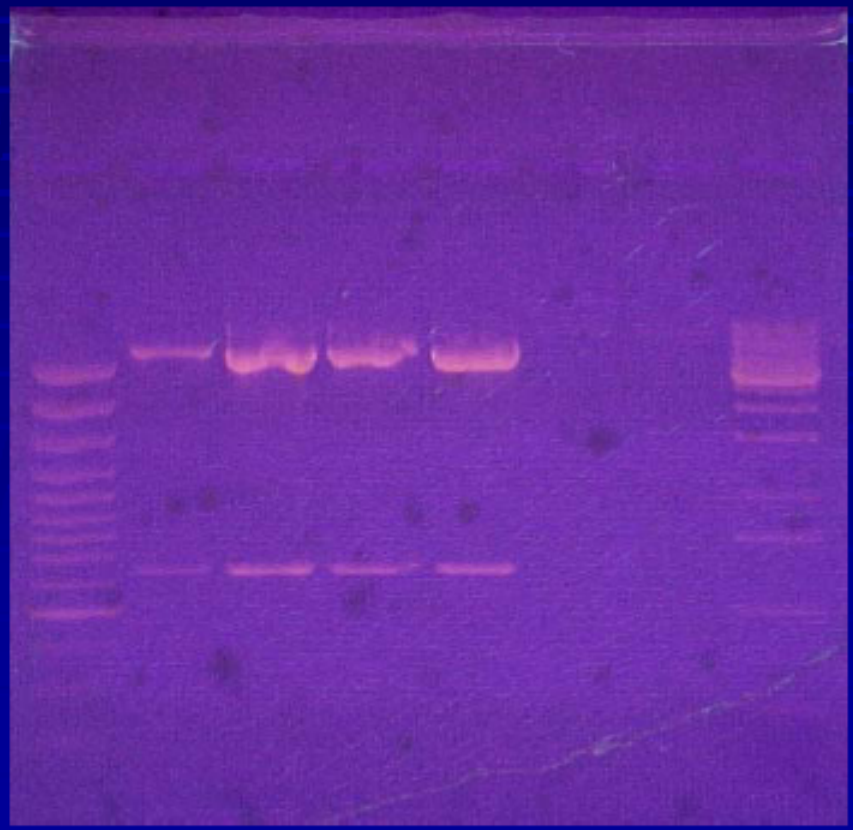
- Check a sample by gel electrophoresis.
- Is the product the size that you expected?
- Is there more than one band?
- Is any band the correct size?
- May need to optimize the reaction conditions.

Agarose gel electrophoresis



Visualize the PCR product on a UV transilluminater

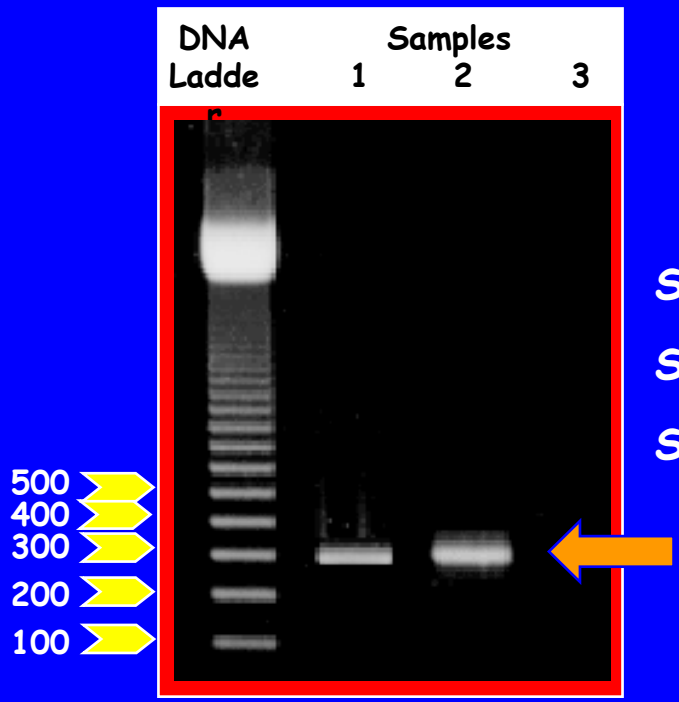
PCR product stained with ethidium bromide



The presence of DNA amplified PCR product in the sample is confirmed by two criteria:

1. A band of DNA must be present

2. This DNA must be of a size which is known to be specific for the target under investigation (300 bases in this example)

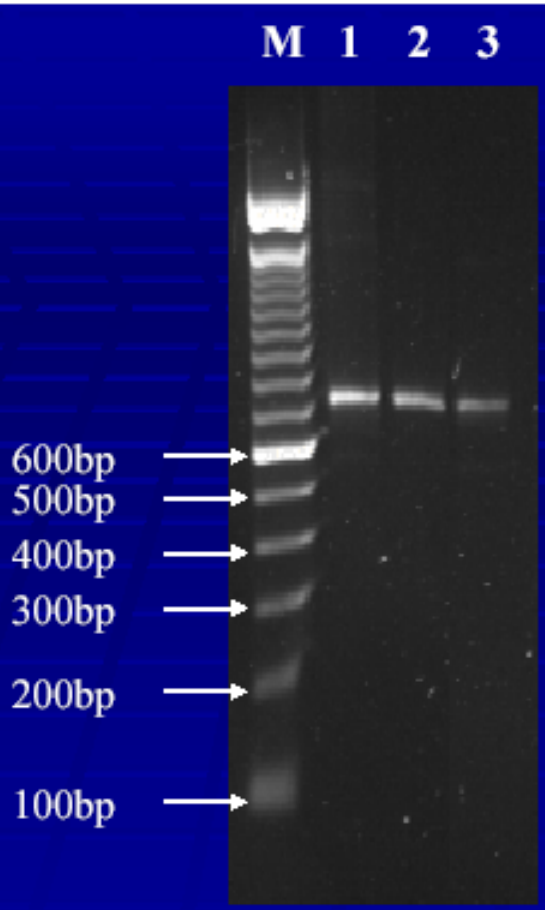


INTERPRETATION OF RESULTS

Sample 1 - TEST (POSITIVE)

Sample 2 - POSITIVE CONTROL

Sample 3 - NEGATIVE CONTROL



- Positive control has known template DNA
- Negative control has no template DNA
- Test will have unknown sample DNA template
- If you see product in positive control, it indicates, the PCR reaction has worked.
- If you see a product in the negative control, it indicates DNA contamination and it is false positive, The result is not valid

Lane M: 100 bp molecular weight standard
Lane 1 : Test
Lane 2 : Positive control
Lane 3 : Negative control

Precautions

- PCR is very sensitive to contamination
- Template DNA has to be prepared in a separate area
- PCR reaction should be set up in a different clean environment
- Use different sets of pipets for template DNA preparation and PCR setting up.
- Nonspecific amplification is often a problem
  - Polymerase works at lower temperatures (*e.g.*, while setting up reactions)
- Always use aerosol barrier tips

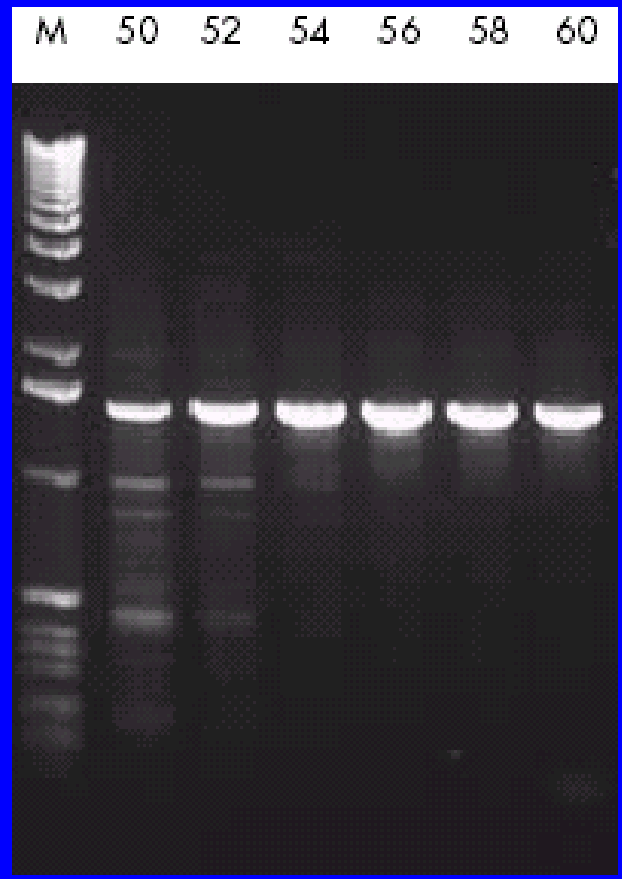
- Maximum product size is about 5000 bases for standard PCR

Optimising the PCR Reaction

- Annealing temperature of the primers.
- The concentration of Mg^{2+} in the reaction.
- The denaturing, annealing and extension times.
- The extension temperature.
- The amount of template and polymerase.

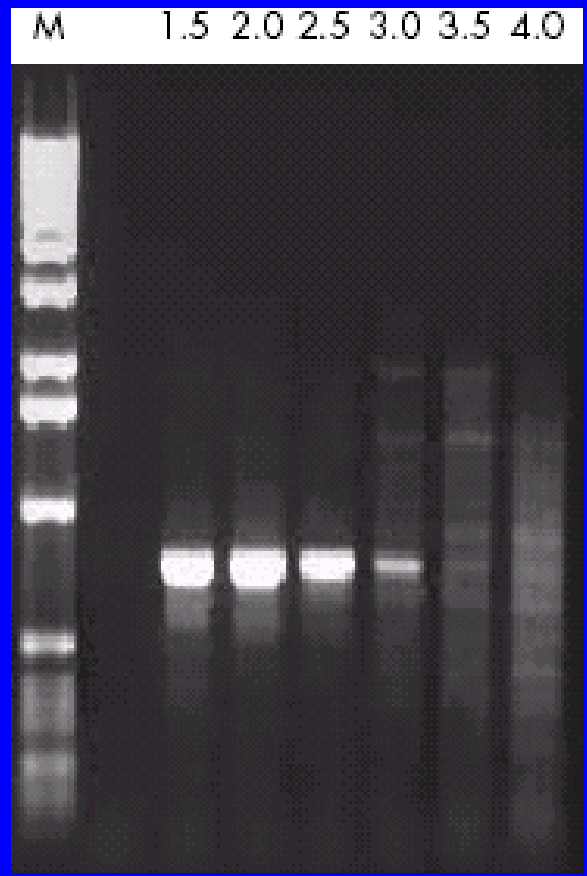
Optimising the Annealing Temperature

- Primers have a calculated annealing temperature (*e.g.* 54°C).
- Temperature must be confirmed practically.
- Temperature steps of 2°C above and below.



Optimising the Mg^{2+} Concentration

- The fidelity of the PCR depends on $[Mg^{2+}]$.
- Vary $[Mg^{2+}]$ in steps of 0.5 mM.



Fidelity of the Reaction

- *Taq* DNA polymerase lacks the 3´→5´ proof-reading activity commonly present in other polymerases.
- *Taq* mis-incorporates 1 base in 10^4.
- A 400 bp target will contain an error in 33% of molecules after 20 cycles.
- Error distribution will be random.

Do Errors Matter?

- Yes, if you want to clone the amplified DNA — an individual molecule may harbour several mutations.

- No, if you want to sequence the amplified DNA or cut it with restriction enzymes.

How Big A Target?

- Amplification products are typically in the size range 100-1500 bp.
- Longer targets are amplifiable → 25 kb.
- Requires modified reaction buffer, cocktails of polymerases, and longer extension times.

Confirmation and Identification of PCR Products

- Gel electrophoresis

- Confirmatory methods
  - ✓Sequencing of the amplification product
  - ✓Southern blotting
  - ✓Restriction fragment length polymorphism (RFLP) analysis

Hot-start PCR - to reduce non-specific amplification. Can also be done by separating the DNA mixtures from enzyme by a layer of wax which melts when heated in cycling reaction. A number of companies also produce hot start PCR products.

"Touch-down" PCR - start at high annealing temperature, then decrease annealing temperature in steps to reduce non-specific PCR product. Can also be used to determine DNA sequence of known protein sequence.

Applications of PCR

- **Mutation testing, *e.g.* cystic fibrosis.**
- **Diagnosis or screening of acquired diseases, *e.g.* AIDS.**
- **Genetic profiling in forensic and legal applications.**
- **Quantitation of mRNA in cells or tissues.**

Variations of PCR in the Diagnostic Lab

The most common variations of standard PCR used in the diagnostic laboratory are:

- ✓ **Reverse Transcriptase PCR (RT-PCR)**
- ✓ **Nested PCR**
- ✓ **Semi-nested PCR**
- ✓ **Multiplex PCR (m-PCR)**
- ✓ **Quantitative Real-time PCR**

Can PCR Amplify RNA?
Reverse Transcriptase PCR (RT-PCR)

- Not directly — the DNA polymerase requires a DNA template and will not copy RNA.
- mRNA can first be copied into cDNA using reverse transcriptase.
- cDNA is a template for PCR — it need not be double-stranded.

Reverse Transcriptase

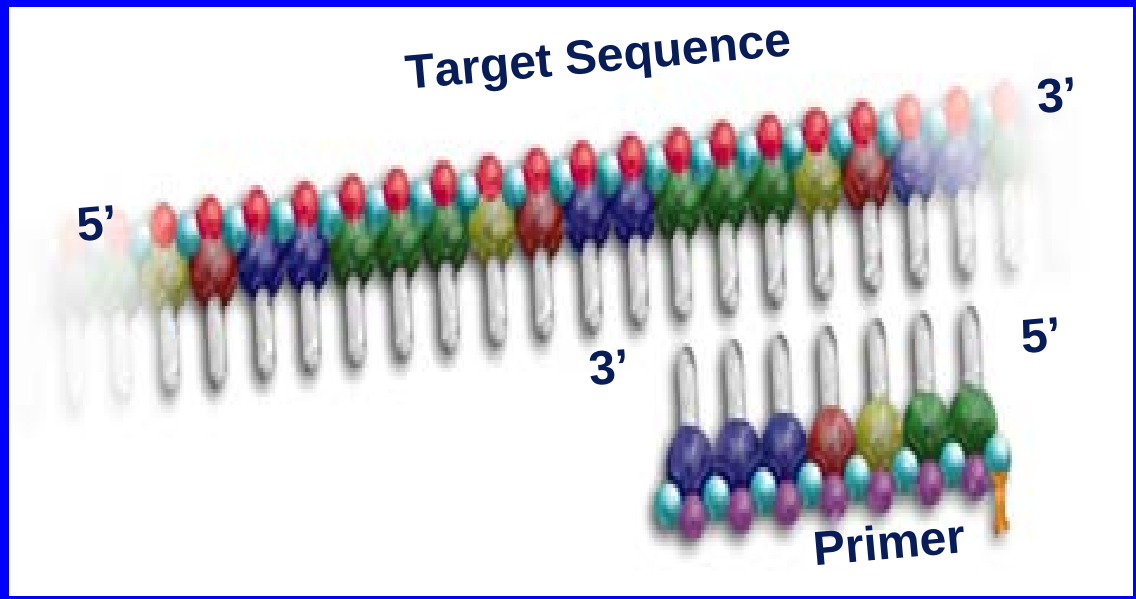
- An enzyme involved in the replication of several kinds of virus.
- RT unique in that it uses as a template not DNA but RNA.
- This enzyme synthesize a DNA strand complementary to an RNA template.

-RNA cannot serve as a template for PCR, so it must first be reverse transcribed into cDNA [e.g. with reverse transcriptase from Moloney murine leukaemia virus (M-MuLV) or avian myeloblastosis virus (AMV)].

-Powell *et al.* first described a combined technique (now commonly known as RT-PCR) in which reverse transcription (RT) is coupled with PCR amplification of the resulting cDNA.

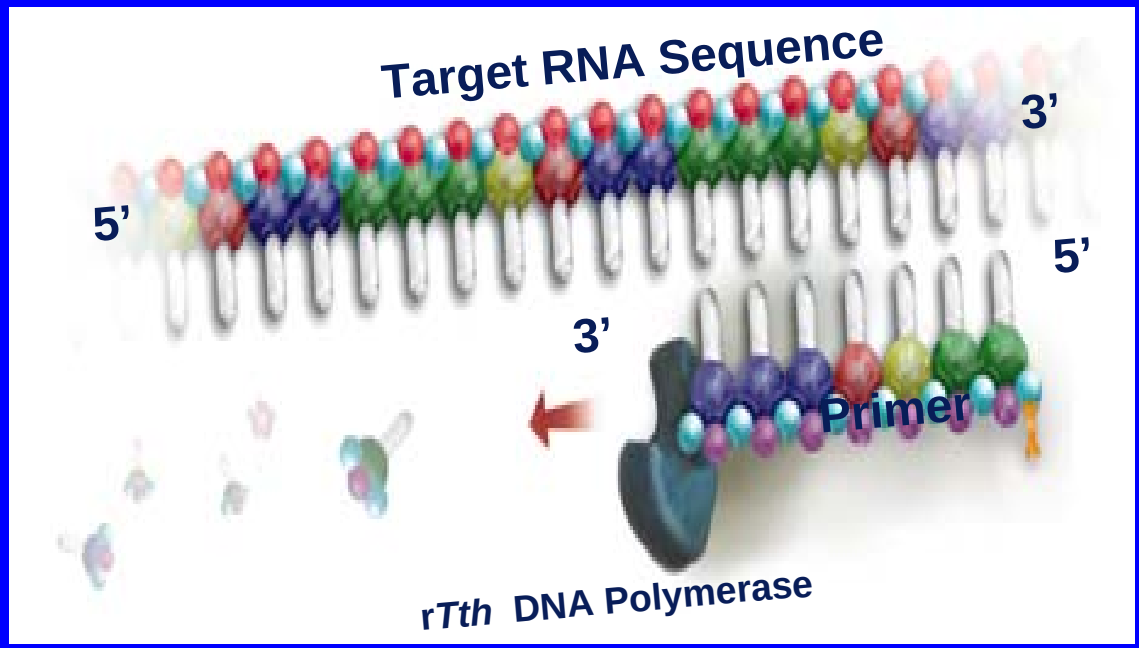
Reverse Transcription - Step 1

-Primer Anneals to Target RNA Sequence



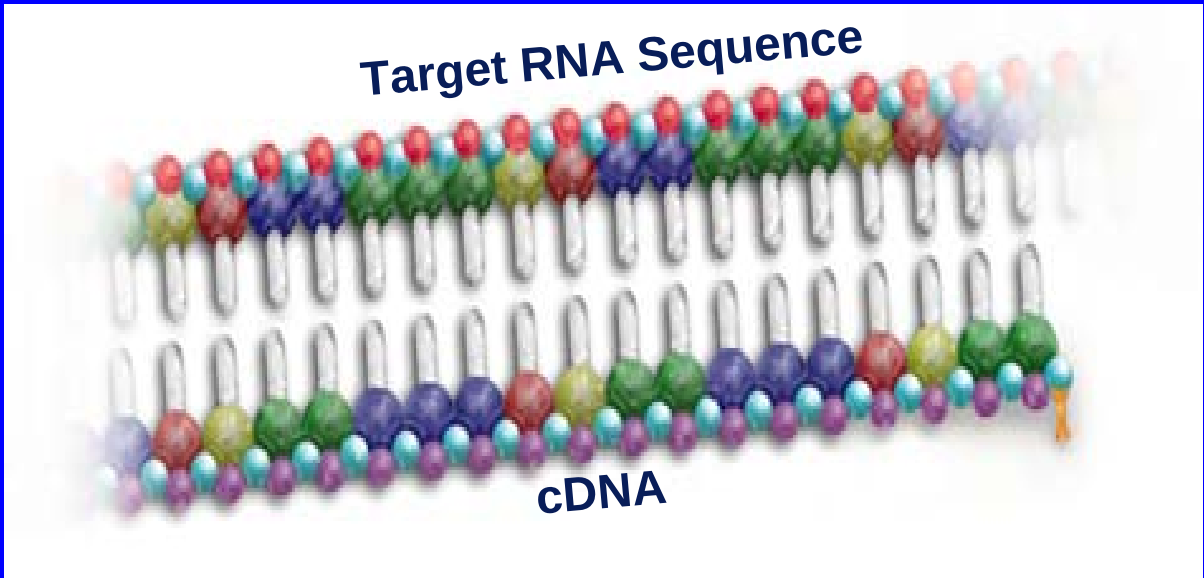
Reverse Transcription - Step 2

-r*Tth* DNA Polymerase also has RT activity Catalyses Primer Extension by Incorporating Complementary Nucleotides



End of Reverse Transcription - Step 3

Results in Synthesis of Complementary DNA (cDNA) to the RNA Target Sequence



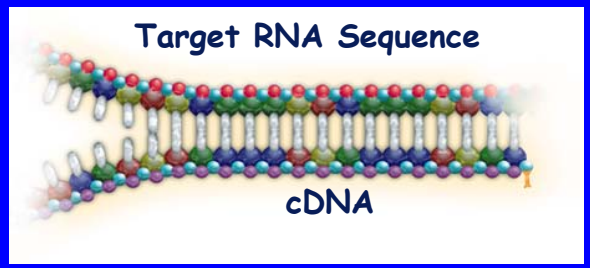
PCR Step 1 - Denaturation by Heat

PCR Step 2 - Annealing of Primer to cDNA

PCR Step 3 - rT*th* DNA Polymerase Catalyses Primer Extension

End of 1st PCR Cycle - Yields a Double-Stranded DNA Copy (Amplicon) of the Target Sequence

1



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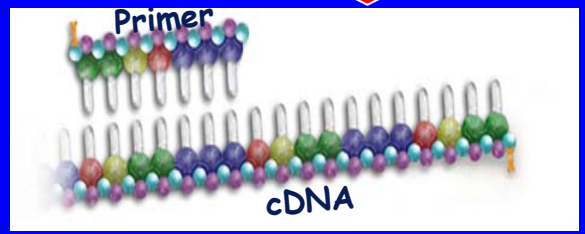

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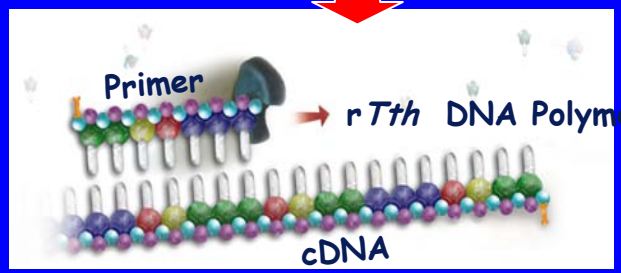

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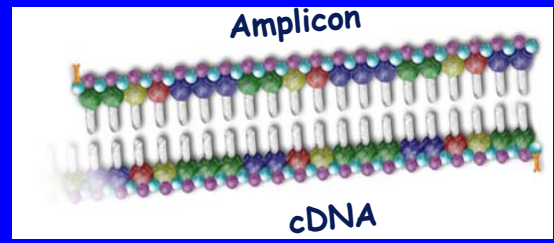
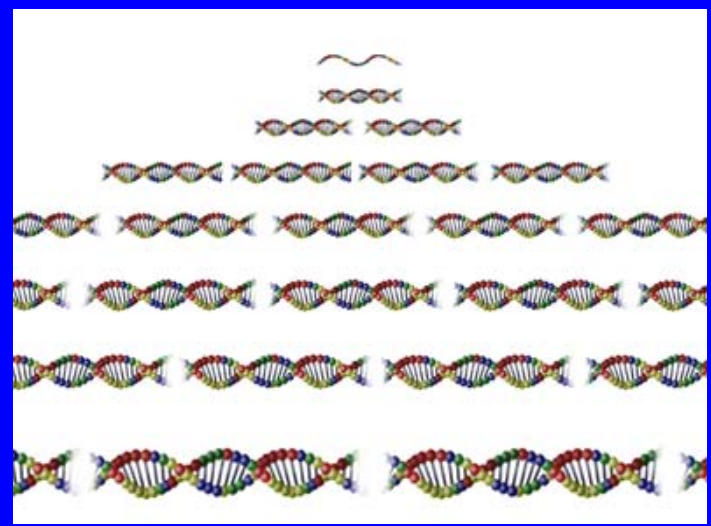




Polymerase Chain Reaction: Introduction

The polymerase chain reaction (PCR) is a powerful new technique that allows scientisits to amplify a specific DNA sequence millions of times in just a few hours. The technique was invented by Dr. Kary Mullis in 1983, for which he received the Nobel Prize in Chemistry ten years later. PCR has already been cited in well over 5,000 scientific papers (as of 1992) and is revolutionizing many areas of genetic research including; genetic disease diagnosis, forensic medicine, and molecular evolution.

Within a dividing cell, DNA replication involves a series of enzyme-mediated reactions, whose end result is a faithful copy of the entire genome. Enzymes first unwind (denature) the DNA double helix into single strands. Then, an RNA polymerase synthesizes a short stretch of RNA complementary to one of the DNA strands at the start site of replication. This DNA/RNA heteroduplex acts as a "priming site" for the attachment of the DNA polymerase, which then produces the complementary DNA strand.

During PCR, high temperature is used to separate the DNA molecules into single strands, and synthetic sequences of single-stranded DNA (20-30 nucleotides) serve as primers. Two different primer sequences are used to bracket the target region to be amplified. One primer is complementary to one DNA strand at the beginning of the target region; a second primer is complementary to the other strand at the end of the target region.

MENU

Continue

Polymerase Chain Reaction: Introduction

To perform a PCR reaction, a small quantity of the target DNA is added to a test tube with a buffered solution containing DNA polymerase, short oligonucleotide primers, the four deoxynucleotide building blocks of DNA, and the cofactor $MgCl_2$. The PCR mixture is taken through replication cycles consisting of:

- one to several minutes at 94-96°C, during which the DNA is denatured into single strands,
- one to several minutes at 50-65°C, during which the primers hybridize or "anneal" (by way of hydrogen bonds) to their complementary sequences on either side of the target sequence, and,
- one to several minutes at 72°C, during which the DNA polymerase binds and extends a complementary strand from each primer.

As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty such cycles, a theoretical amplification factor of one billion is attained.

Two important innovations were responsible for automating PCR. First, a heat-stable DNA polymerase was isolated from the bacterium *Thermus aquaticus*, which inhabits hot springs. This enzyme, called the "*Taq*" DNA polymerase, remains active despite repeated heating during many cycles of amplification. Second, DNA thermal cyclers have been invented in which a computer controls the repetitive temperature changes required for PCR.

MENU Go Back Next Section

Polymerase Chain Reaction: Amplification

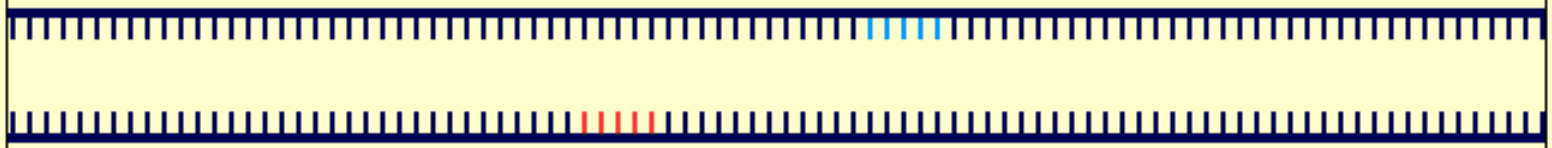
DENATURE DNA

94-96°C

Samples are heated to 94-96°C for one to several minutes to denature (separate into single strands) the target DNA.

MENU

Polymerase Chain Reaction: Cycle One



ANNEAL PRIMERS

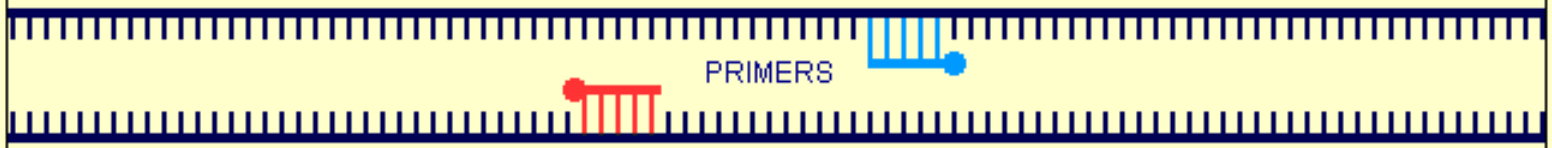
REPEAT PREVIOUS STEP

50-65°C

The temperature is lowered to 50-65°C for one to several minutes allowing the left and right primers to anneal (basepair) to their complementary sequences.

MENU

Polymerase Chain Reaction: Cycle One



EXTEND PRIMERS

72°C

REPEAT PREVIOUS STEP

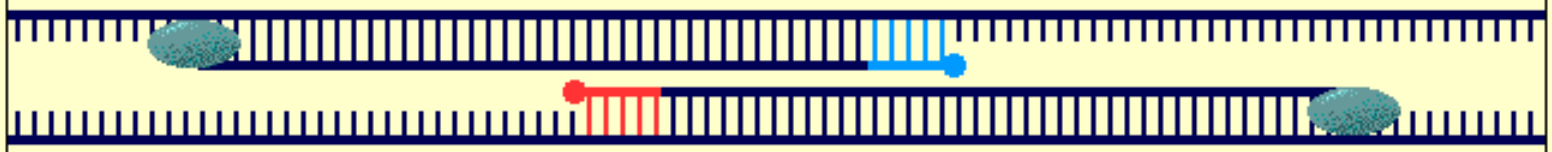
The temperature is raised to 72°C for one to several minutes, allowing *Taq* polymerase to attach at each priming site (where primers have annealed) and extend (synthesize) a new DNA strand.

MENU

Polymerase Chain Reaction: Cycle One

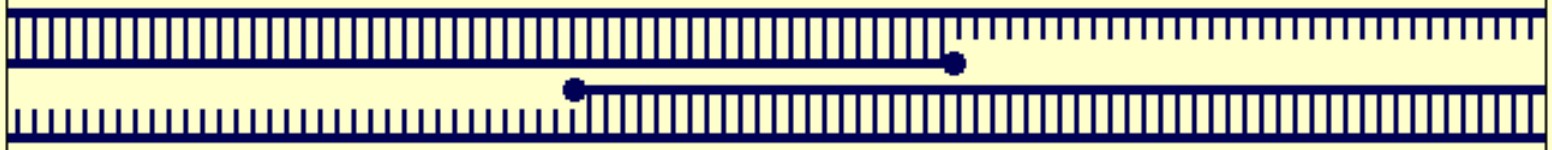
MENU

Polymerase Chain Reaction: Cycle One



MENU

Polymerase Chain Reaction: Cycle One

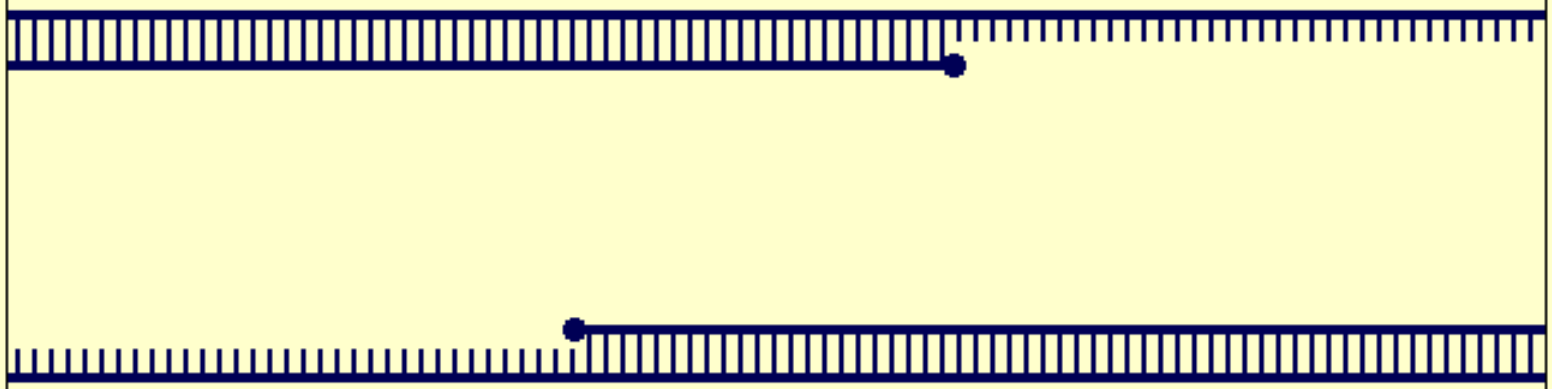


GO TO SECOND CYCLE

REPEAT PREVIOUS STEP

MENU

Polymerase Chain Reaction: Cycle Two

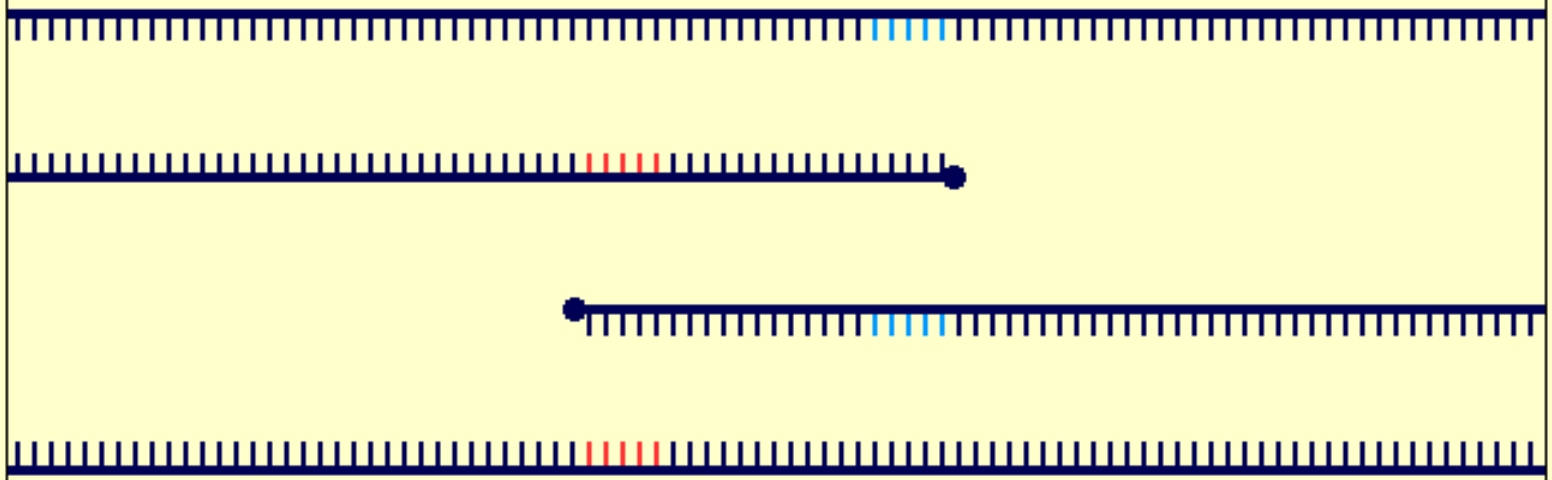


DENATURE DNA

The temperature is raised to 94-96°C, denaturing the target DNA as in cycle one.

MENU

Polymerase Chain Reaction: Cycle Two



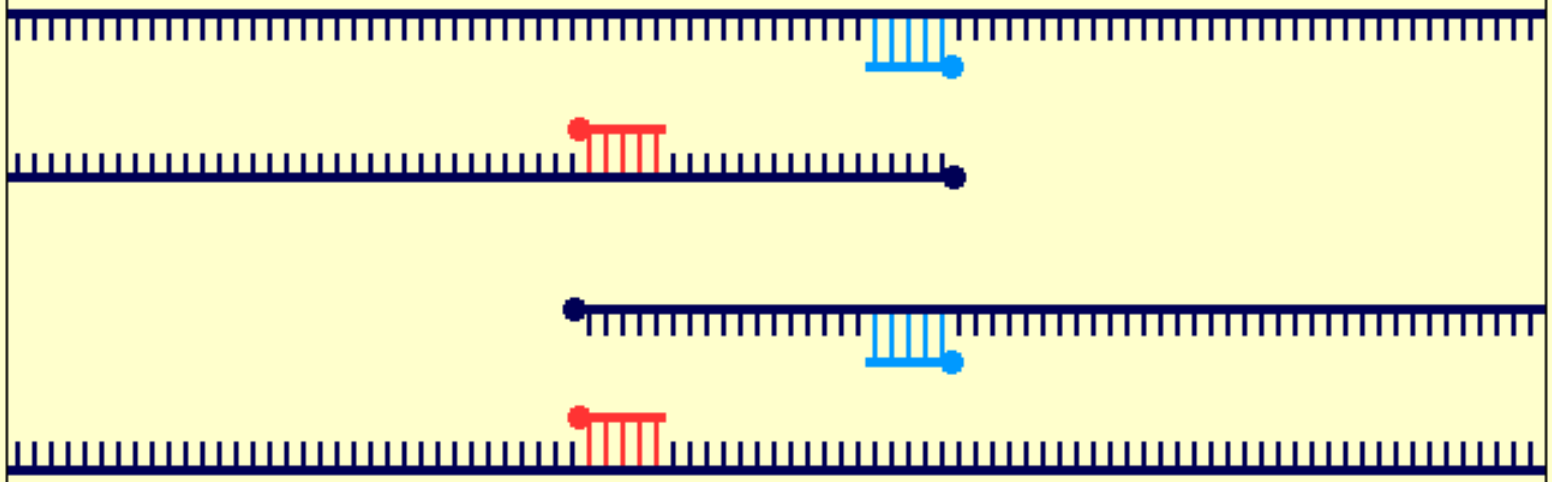
ANNEAL PRIMERS

REPEAT PREVIOUS STEP

As before, the temperature cools to 50-65°C allowing primers to anneal.

MENU

Polymerase Chain Reaction: Cycle Two



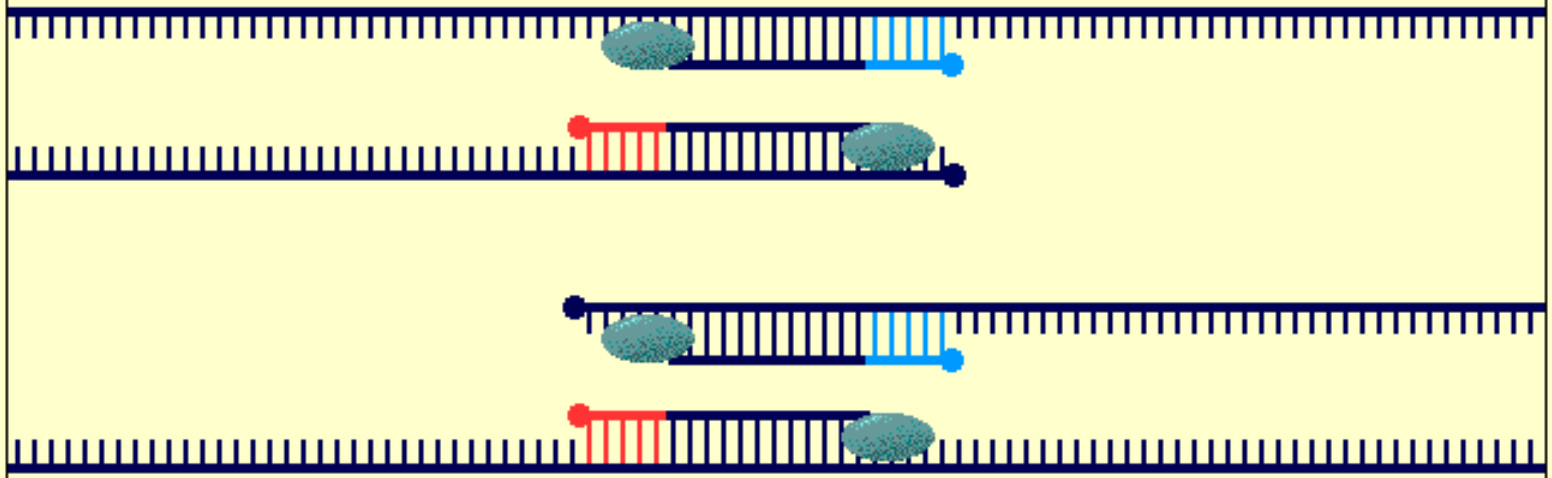
EXTEND PRIMERS

REPEAT PREVIOUS STEP

The temperature is again raised to 72°C. *Taq* polymerase binds to each priming site and synthesizes a new DNA strand.

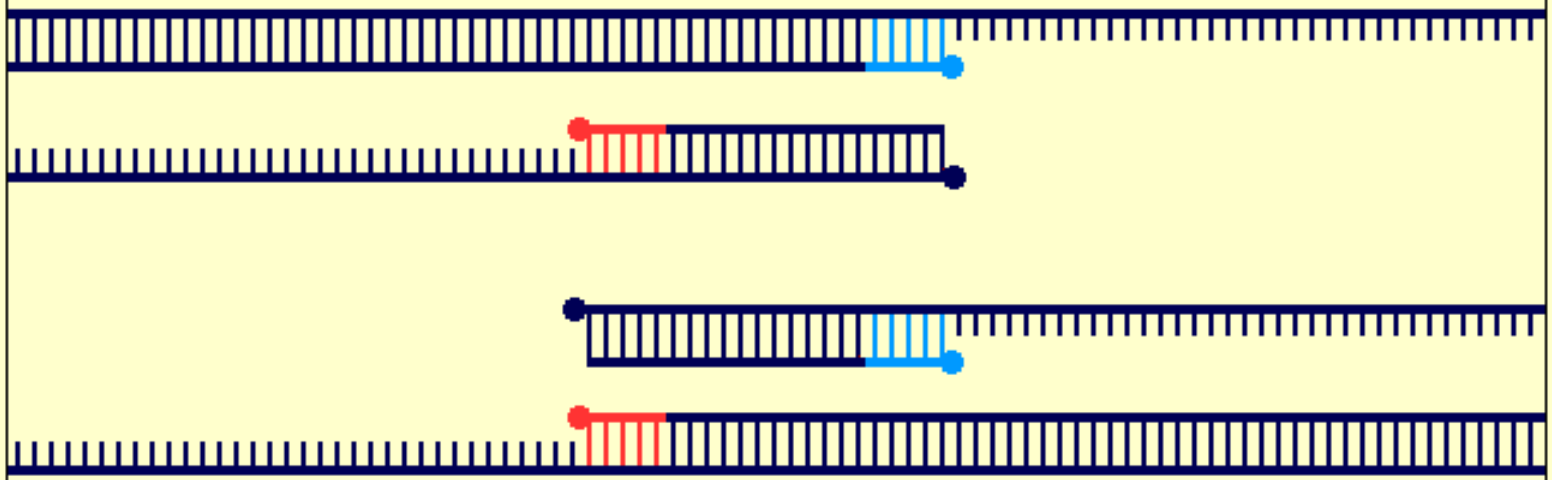
MENU

Polymerase Chain Reaction: Cycle Two



MENU

Polymerase Chain Reaction: Cycle Two

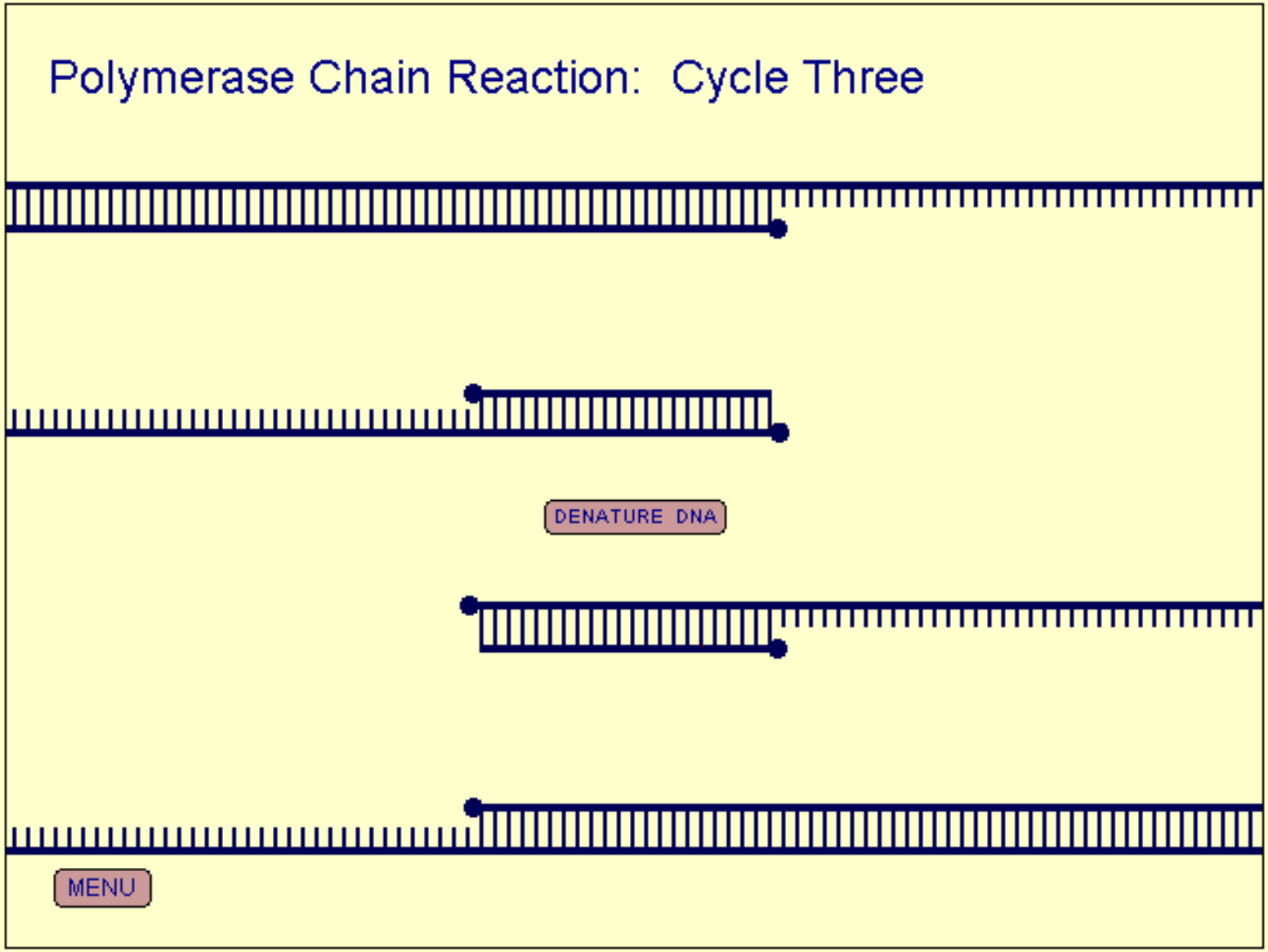


GO TO THIRD CYCLE

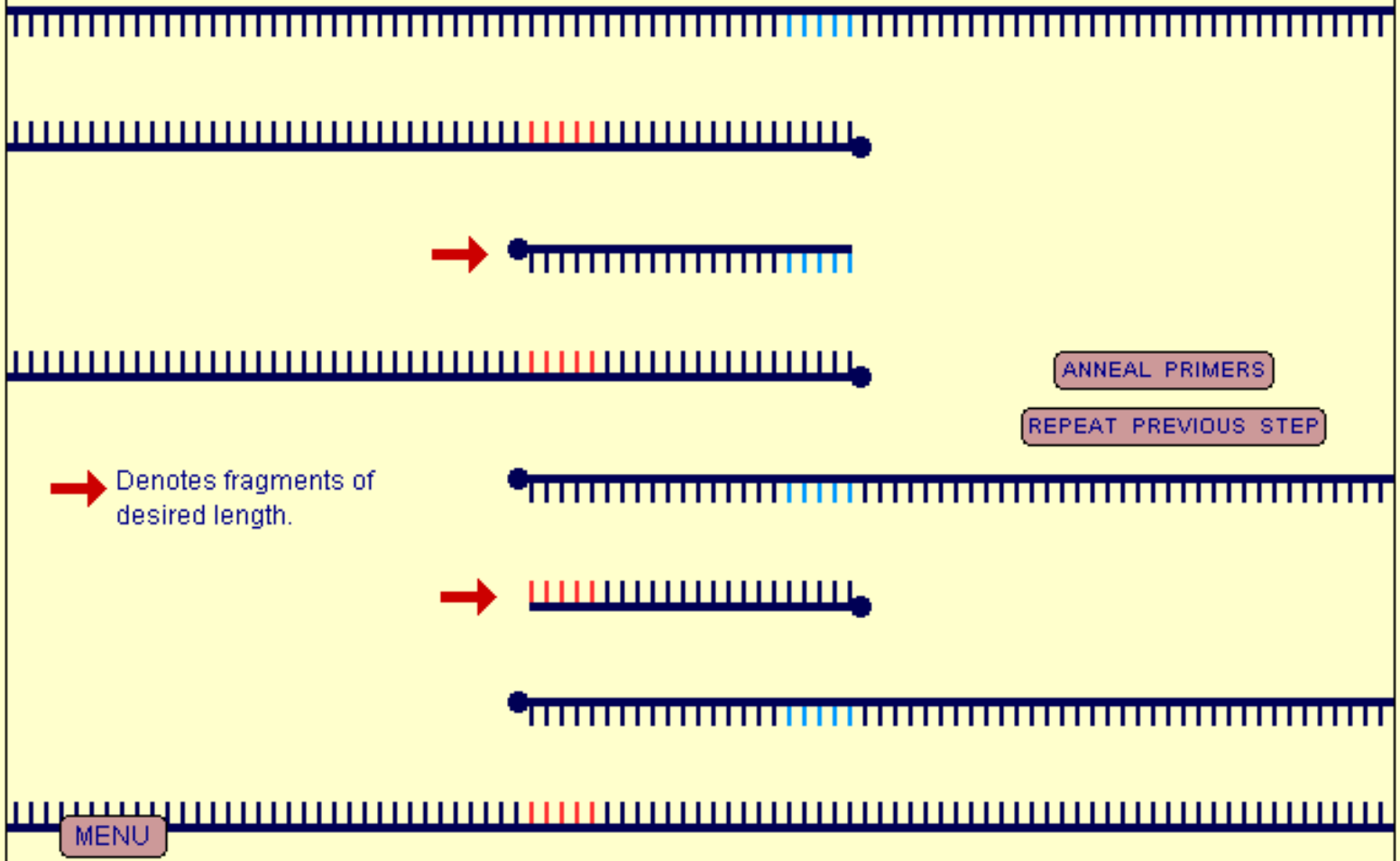
REPEAT PREVIOUS STEP

MENU

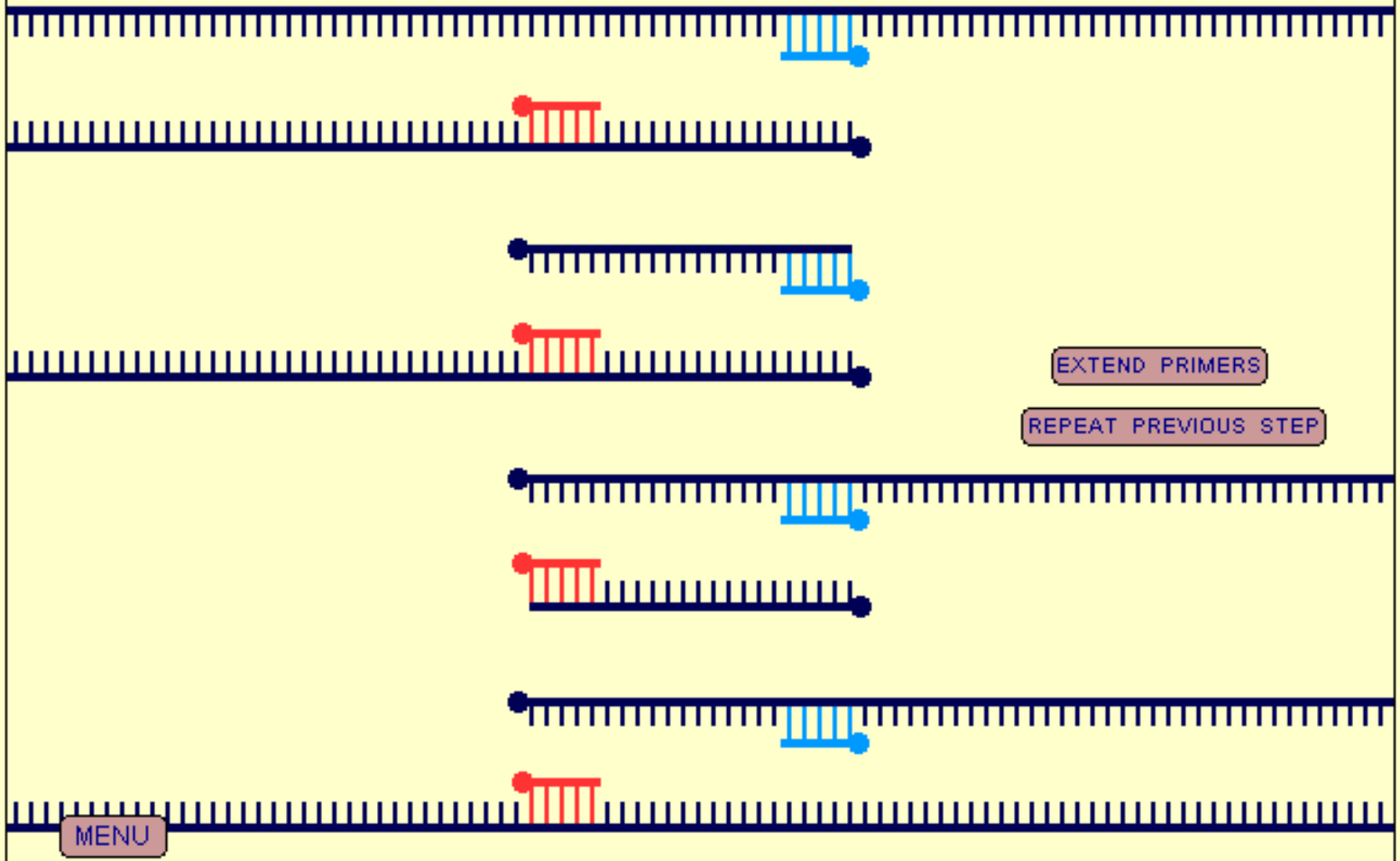
Polymerase Chain Reaction: Cycle Three



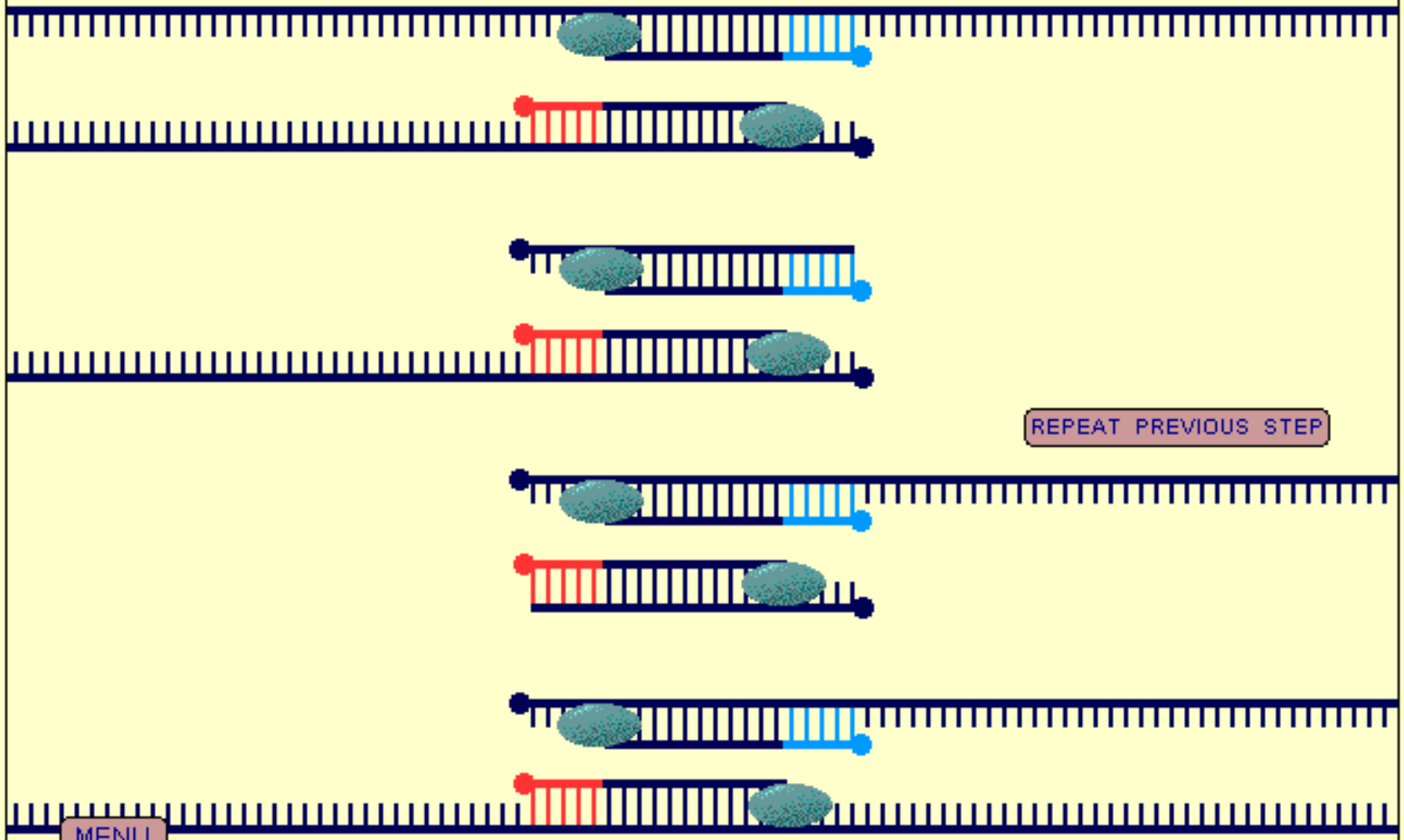
Polymerase Chain Reaction: Cycle Three



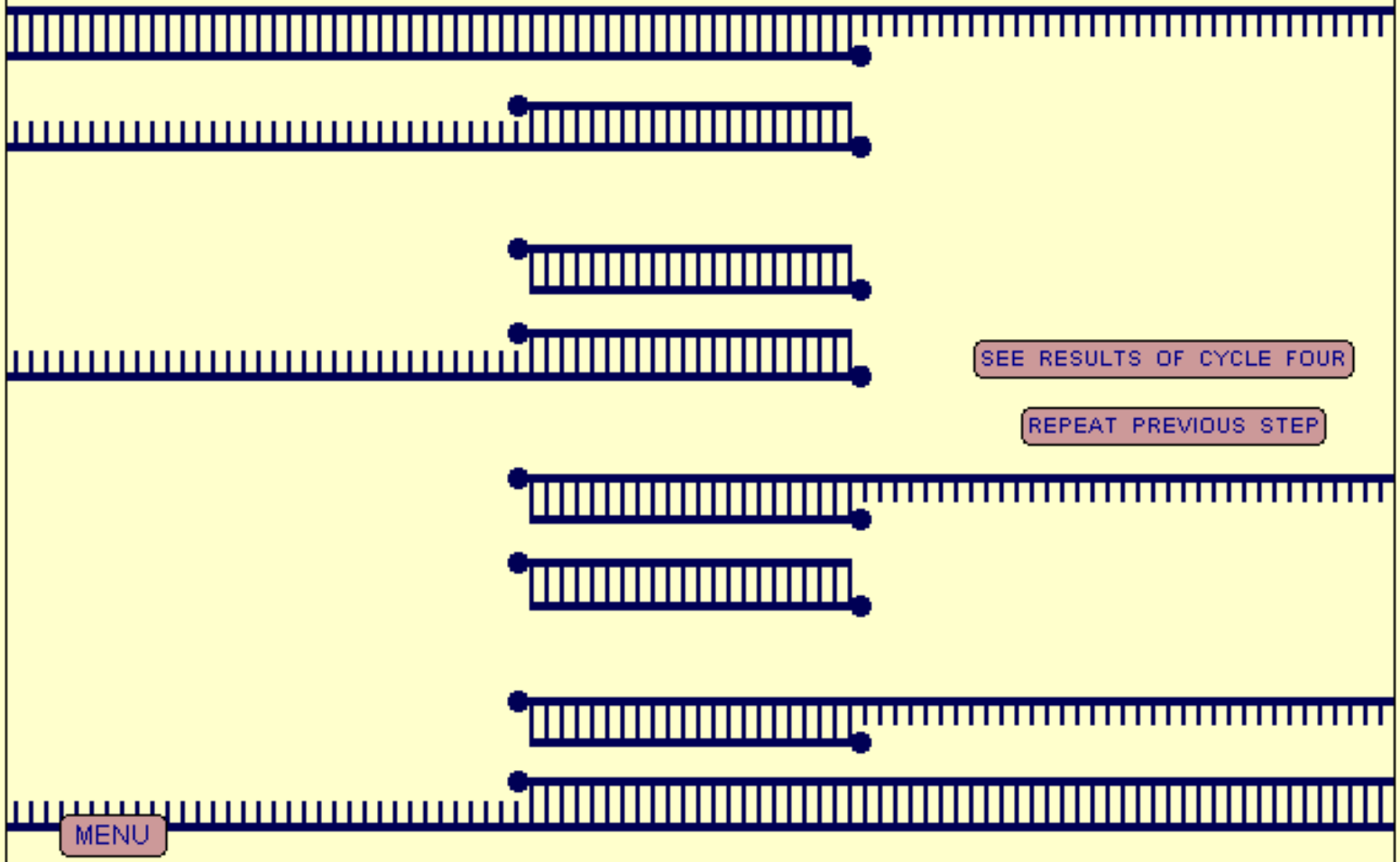
Polymerase Chain Reaction: Cycle Three



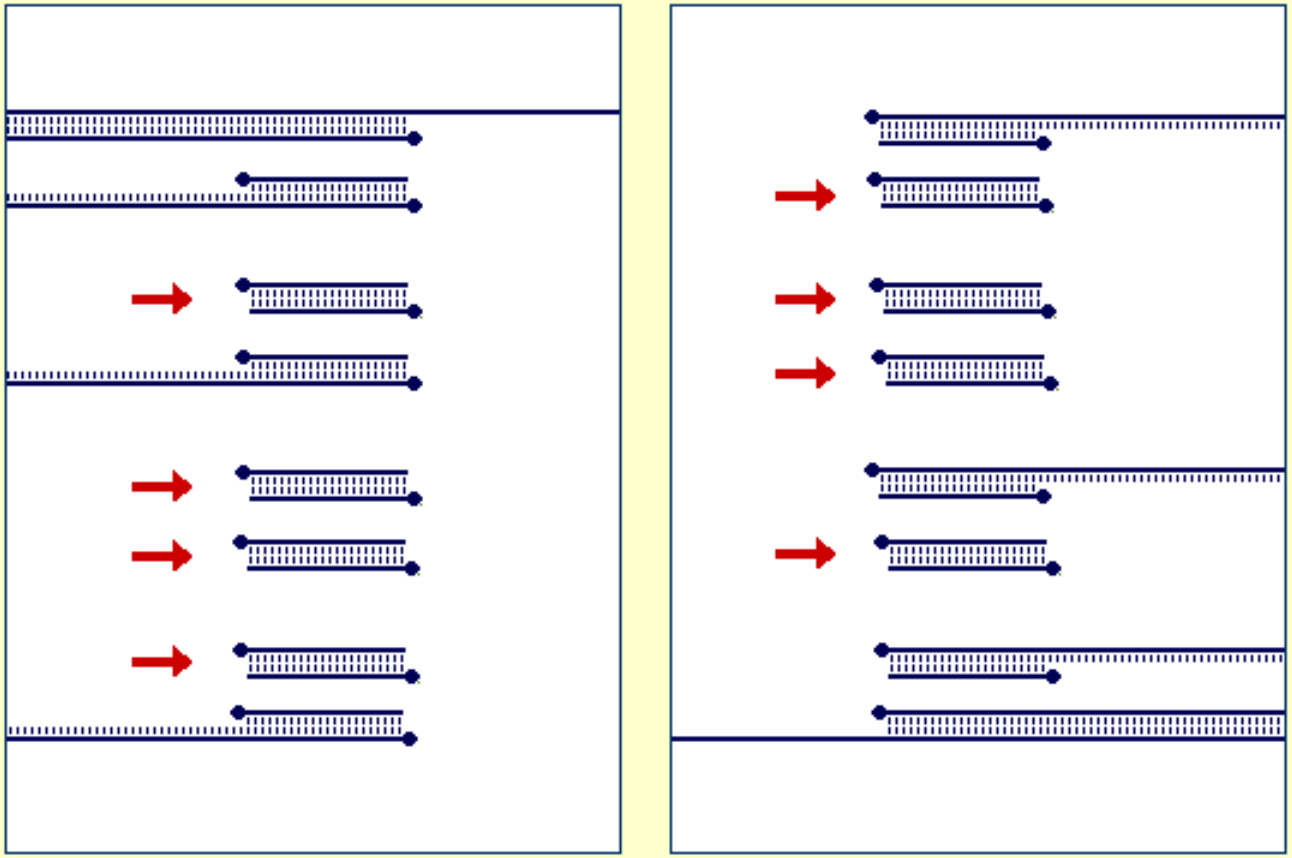
Polymerase Chain Reaction: Cycle Three



Polymerase Chain Reaction: Cycle Three



Polymerase Chain Reaction: Cycle Four Results

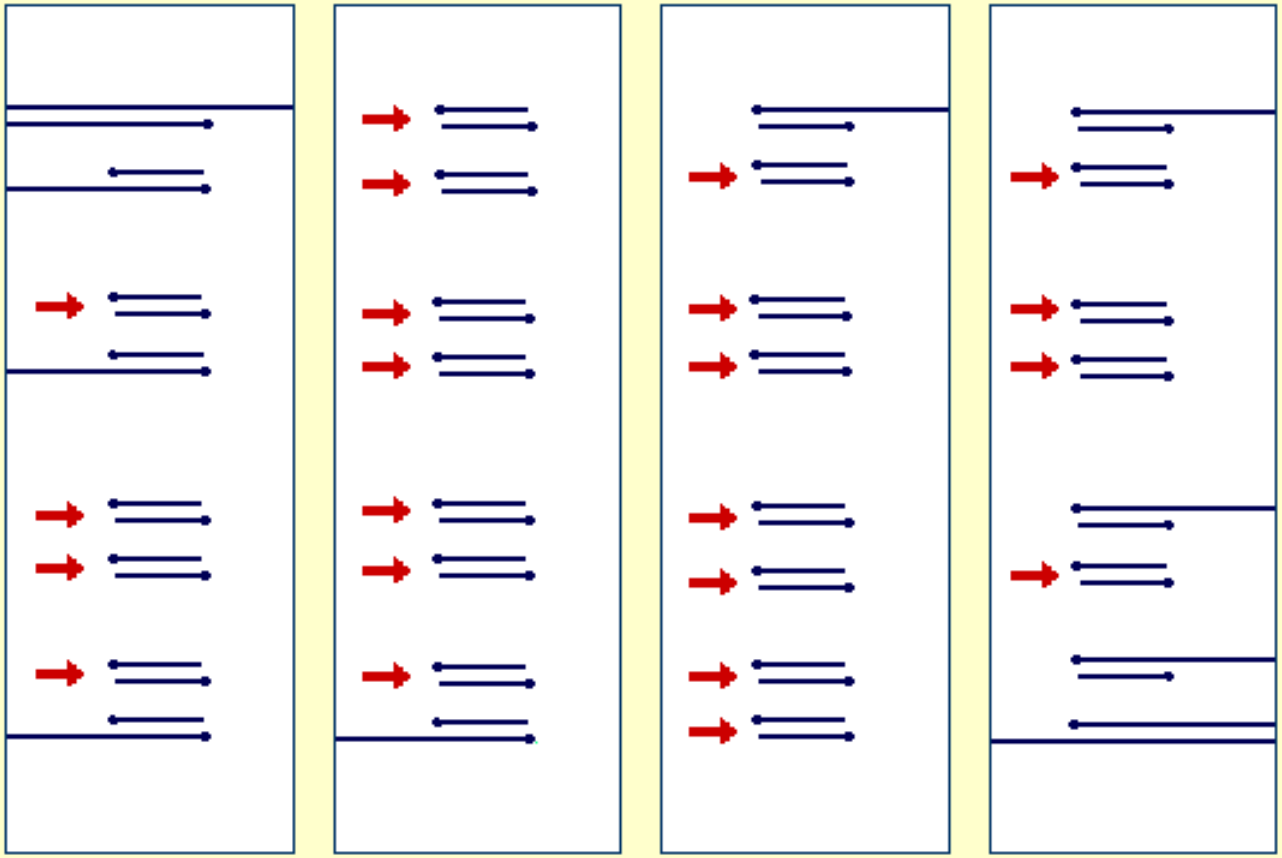


MENU

REPEAT PREVIOUS STEP

SEE RESULTS OF CYCLE FIVE

Polymerase Chain Reaction: Cycle Five Results

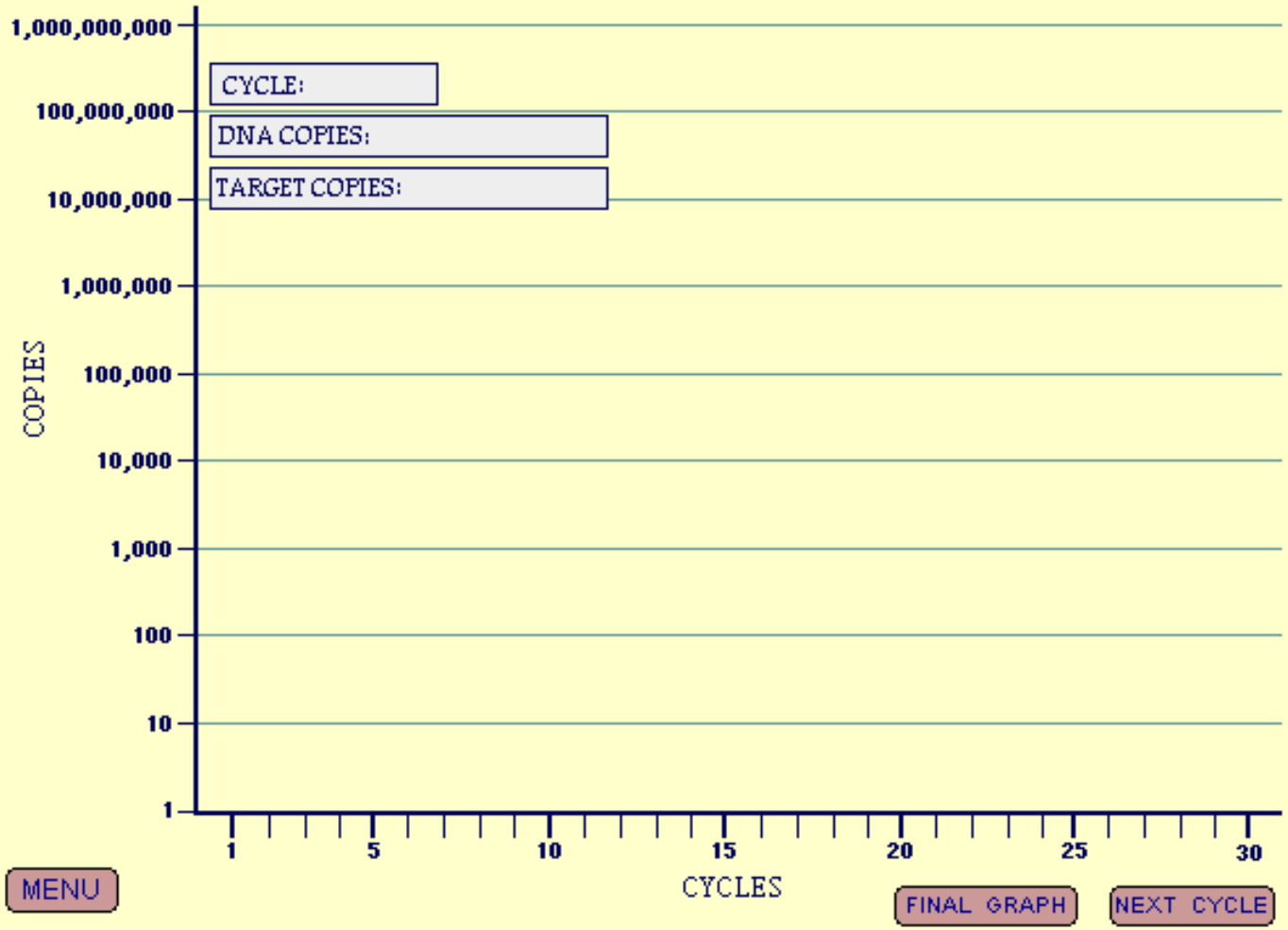


MENU

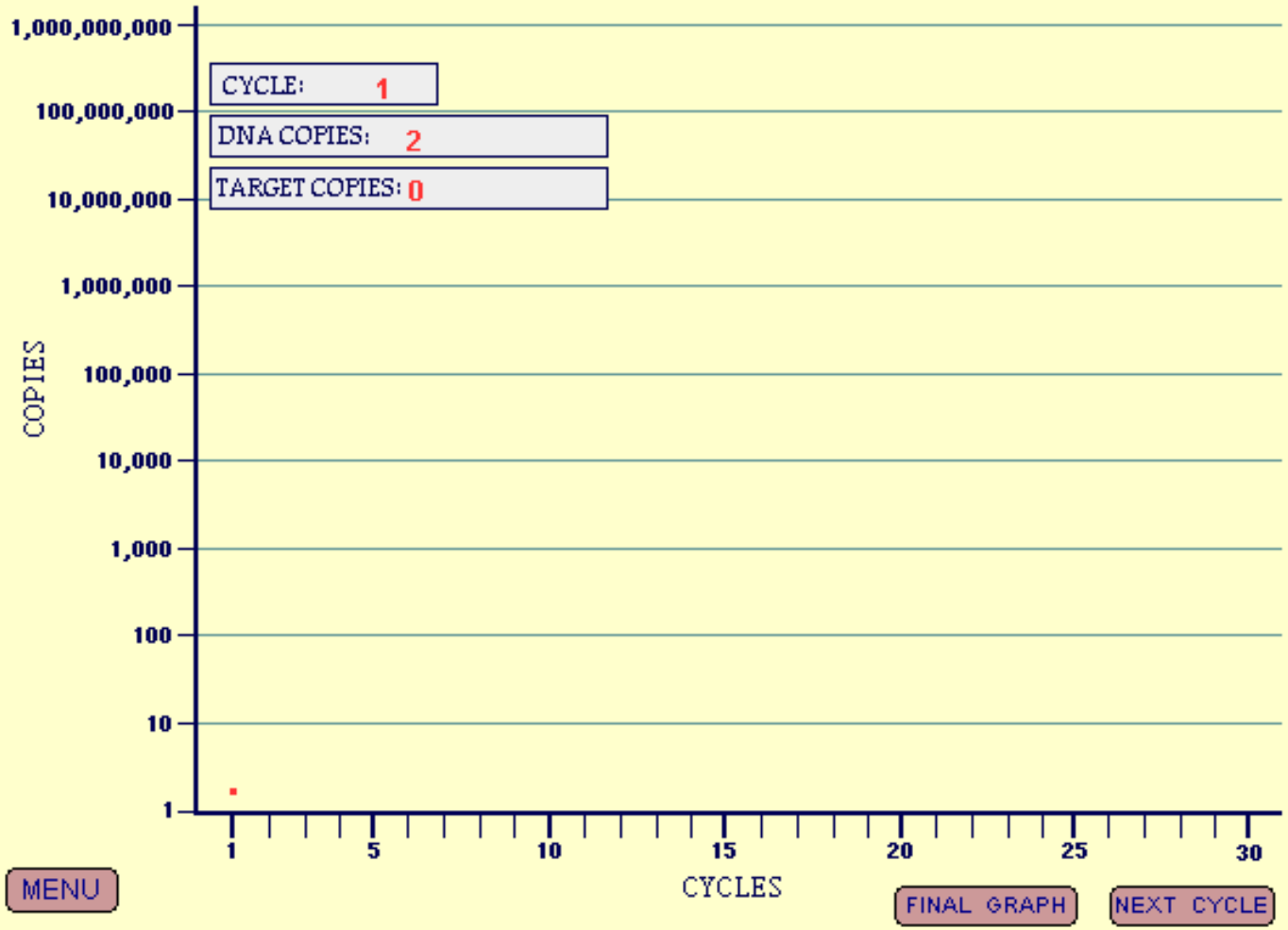
REPEAT PREVIOUS STEP

GRAPH

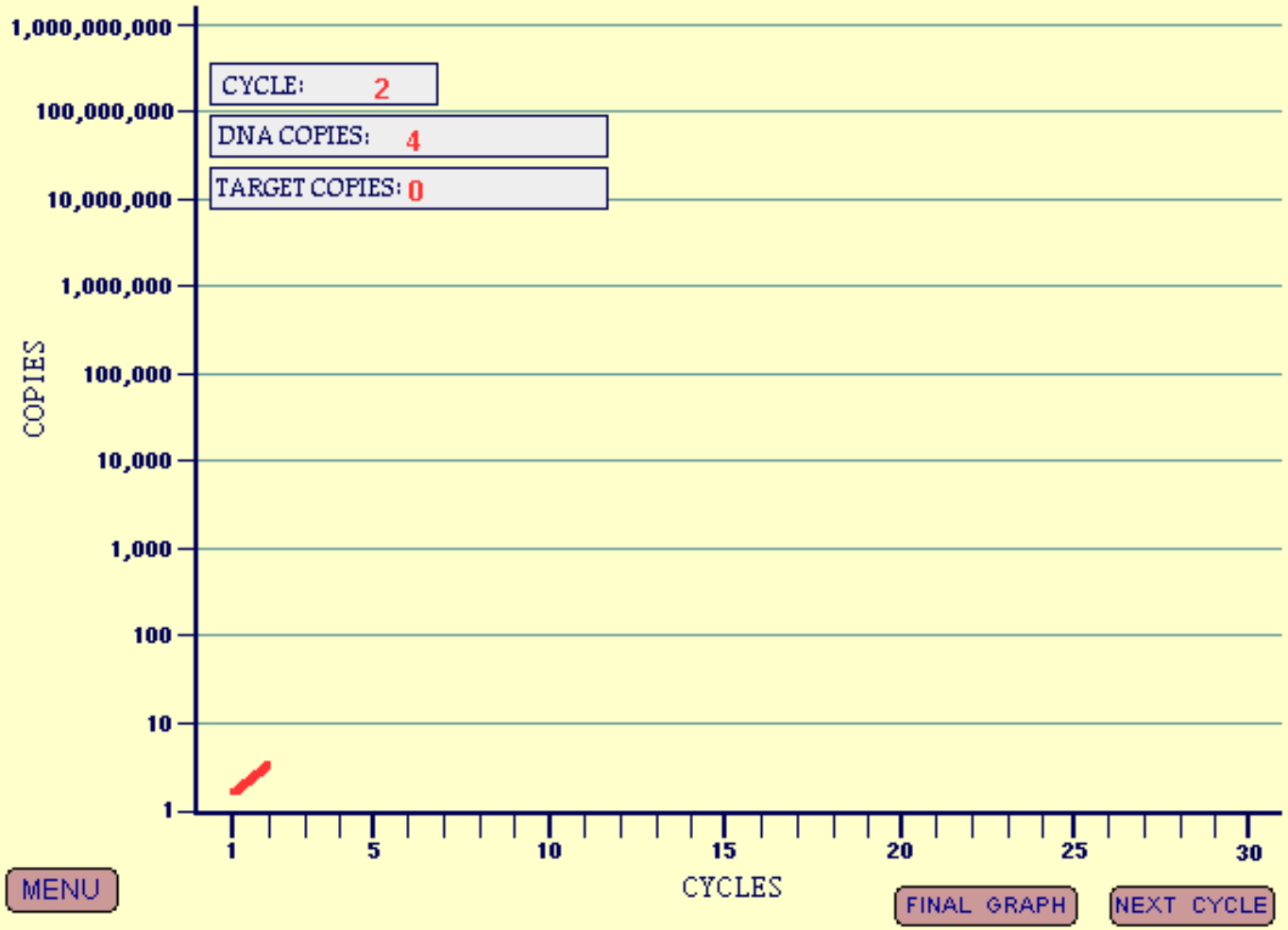
Polymerase Chain Reaction: Amplification Graph



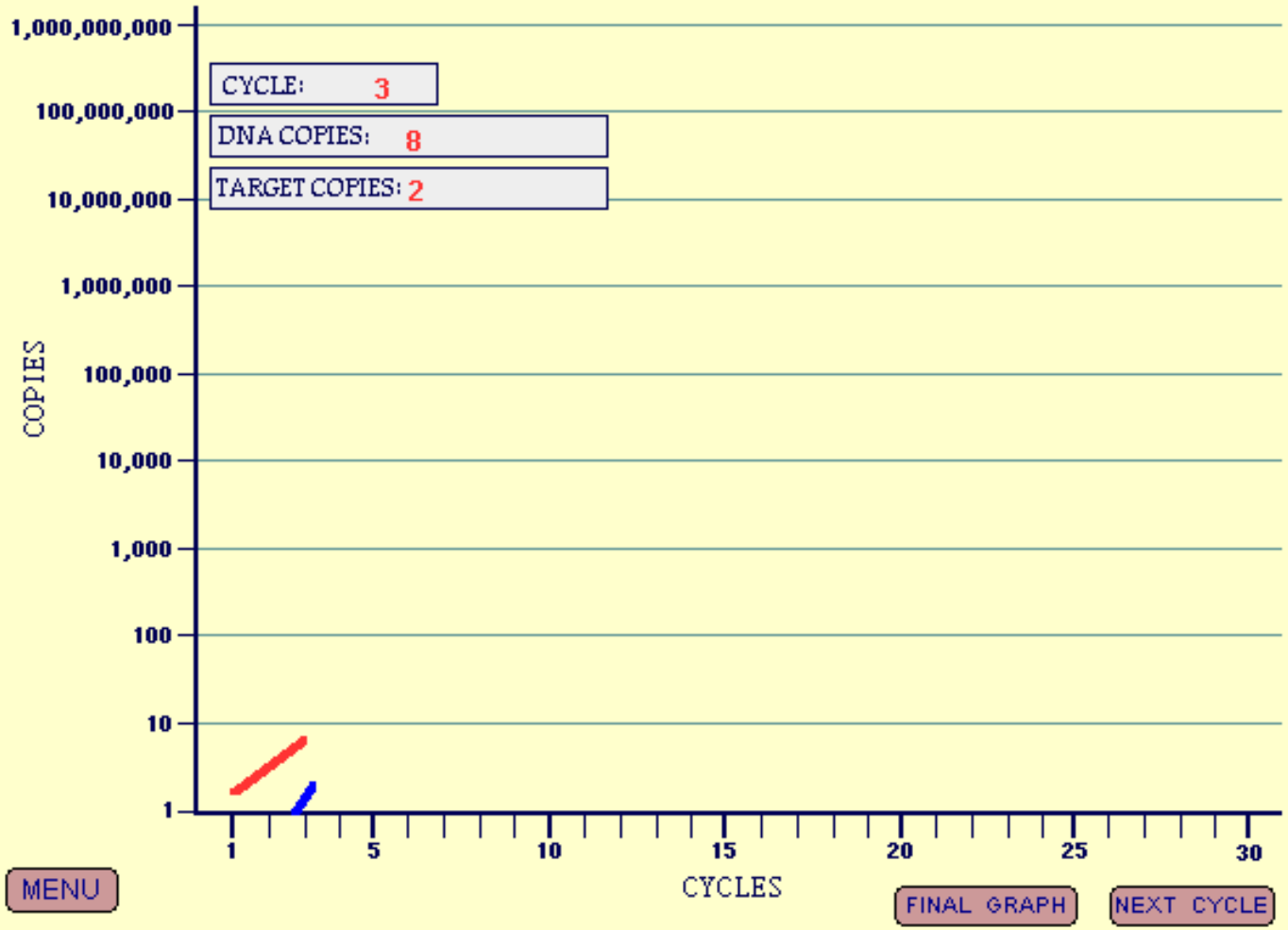
Polymerase Chain Reaction: Amplification Graph



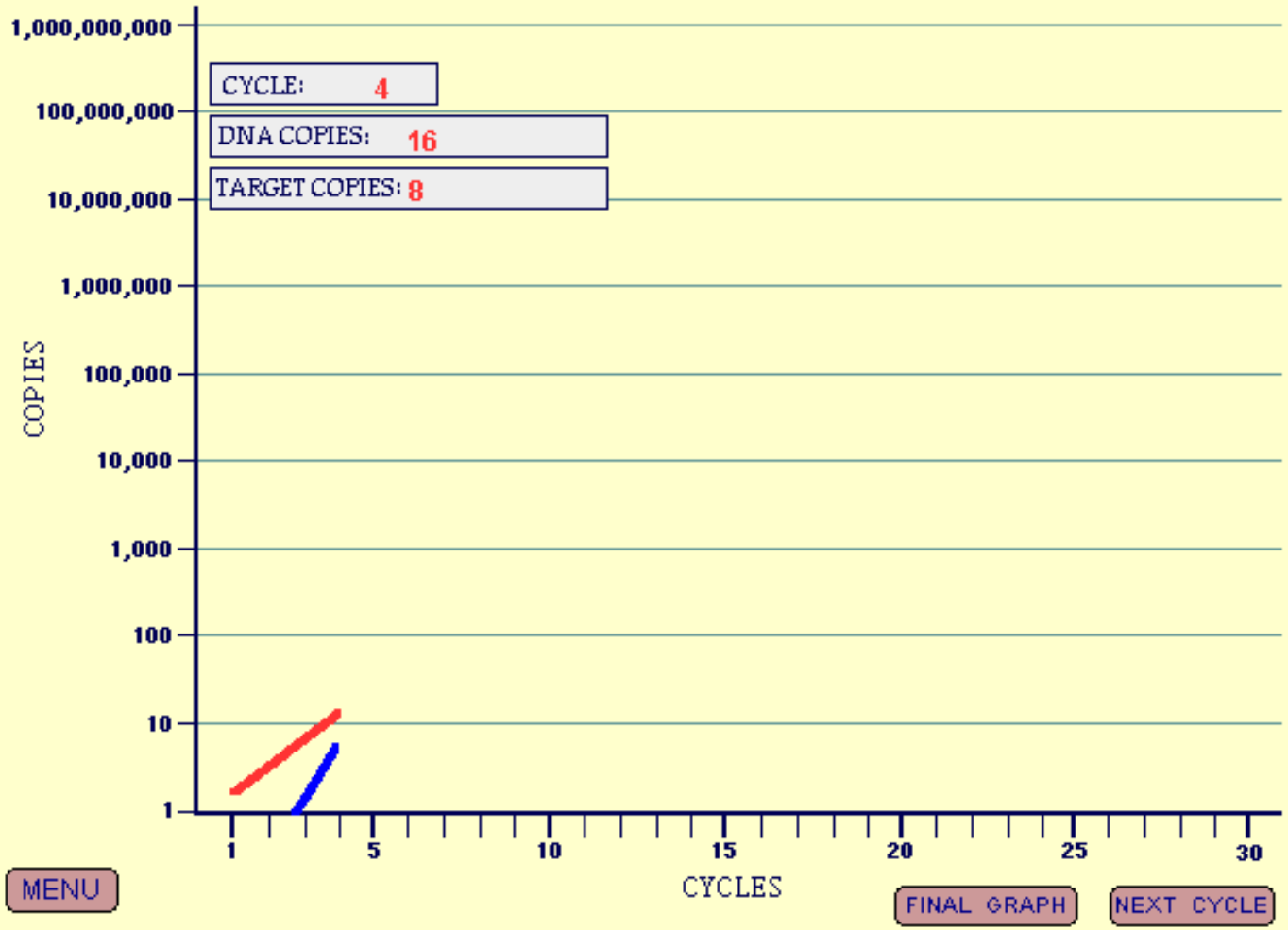
Polymerase Chain Reaction: Amplification Graph



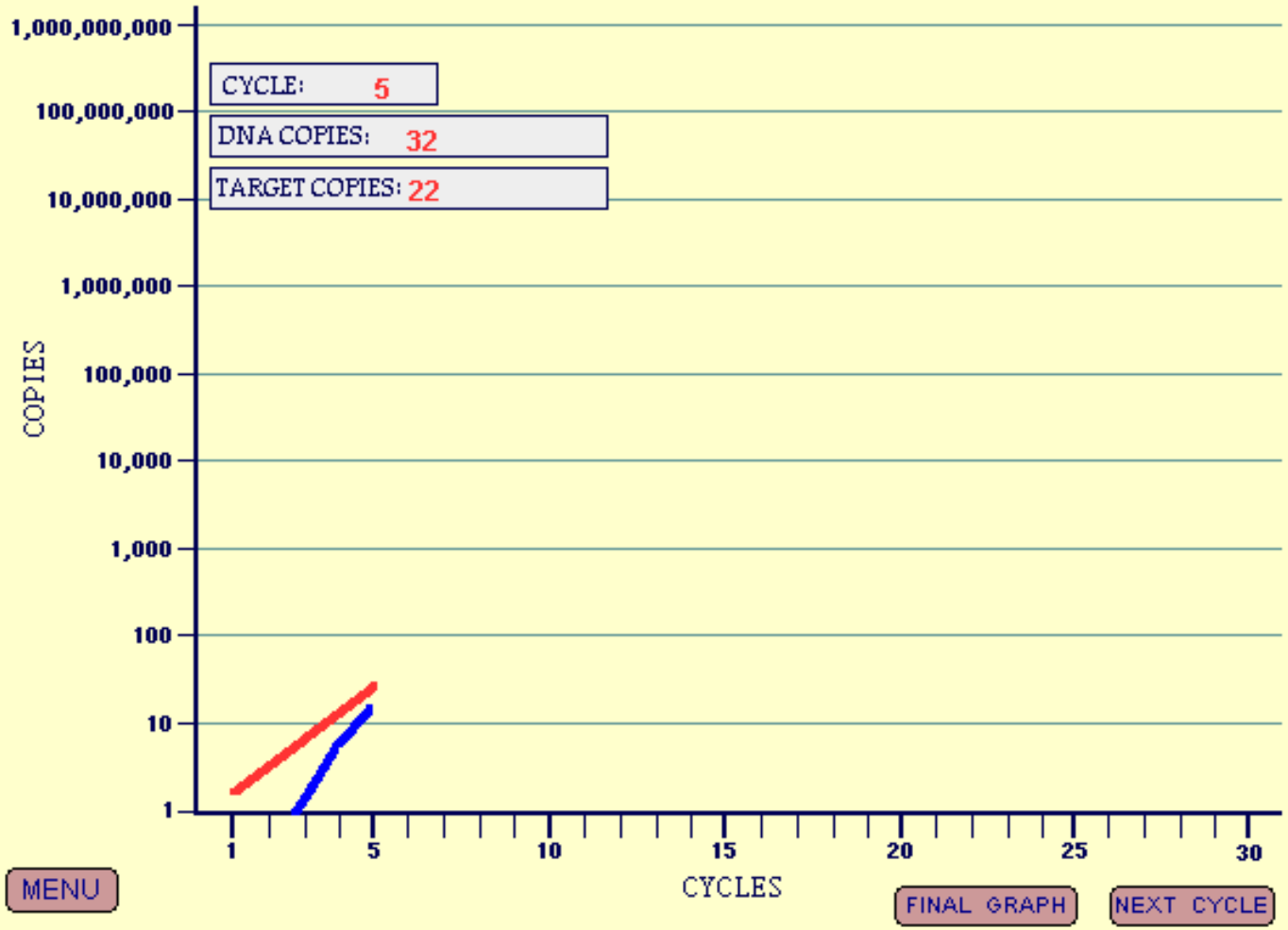
Polymerase Chain Reaction: Amplification Graph



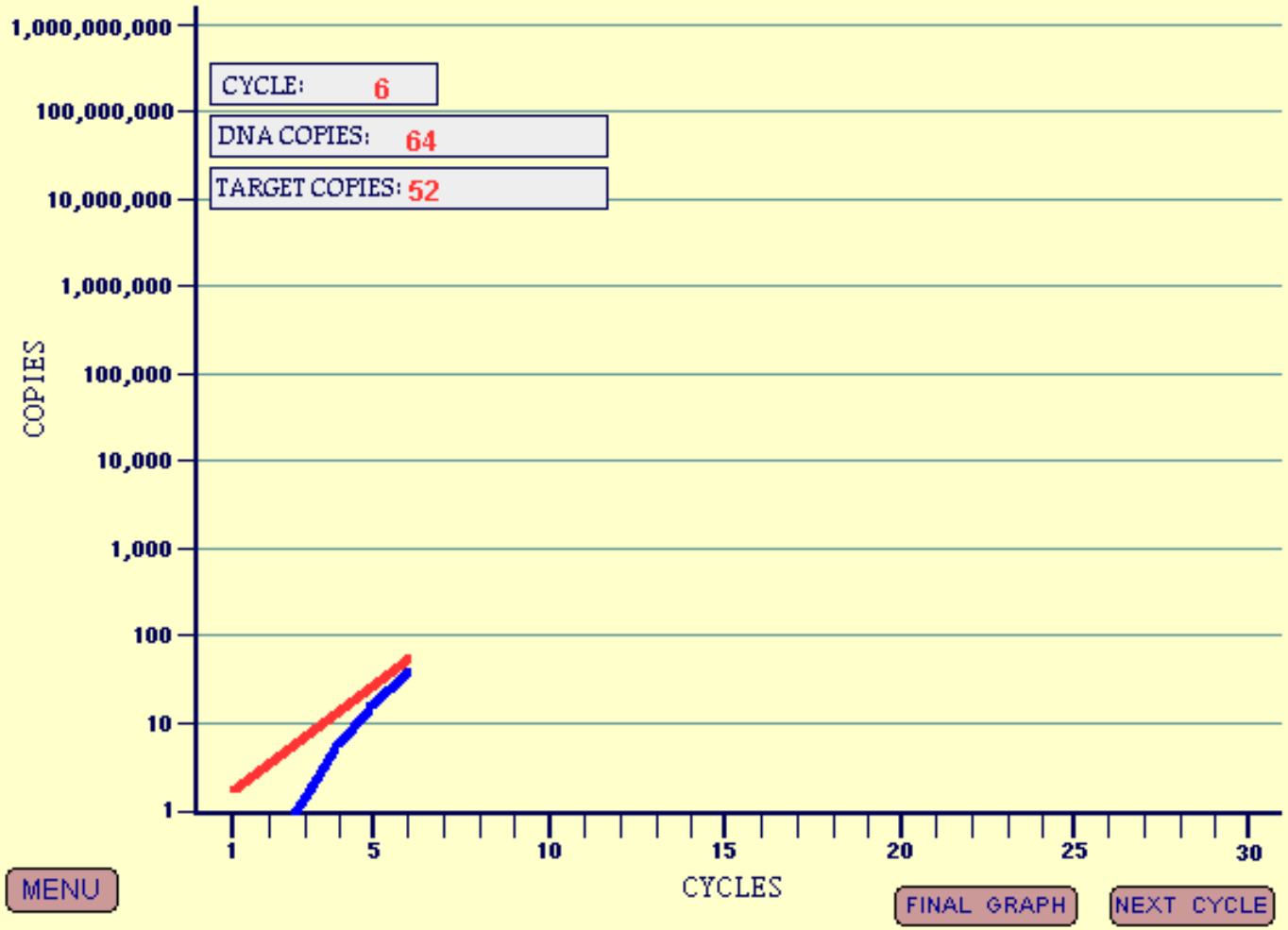
Polymerase Chain Reaction: Amplification Graph



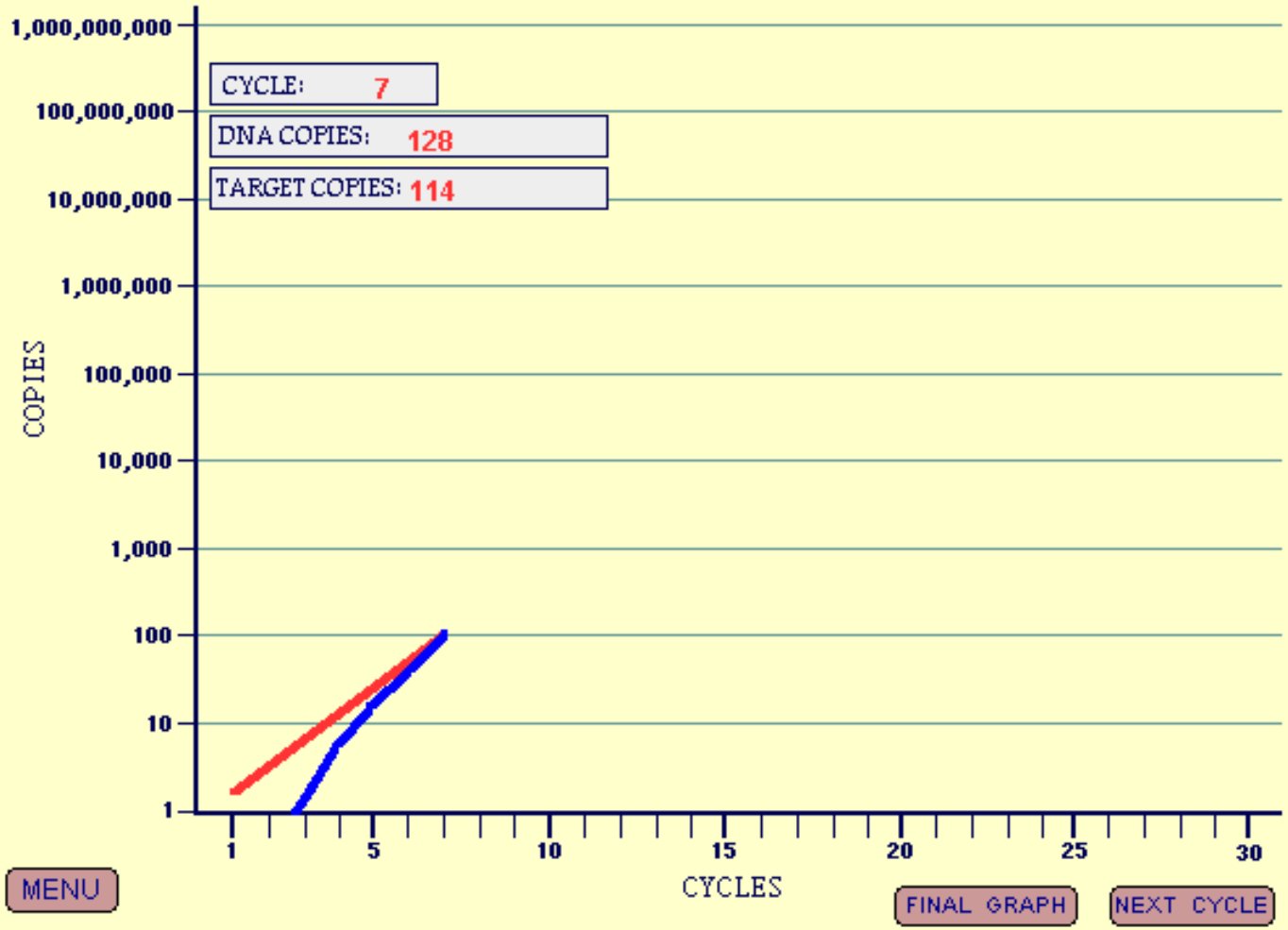
Polymerase Chain Reaction: Amplification Graph

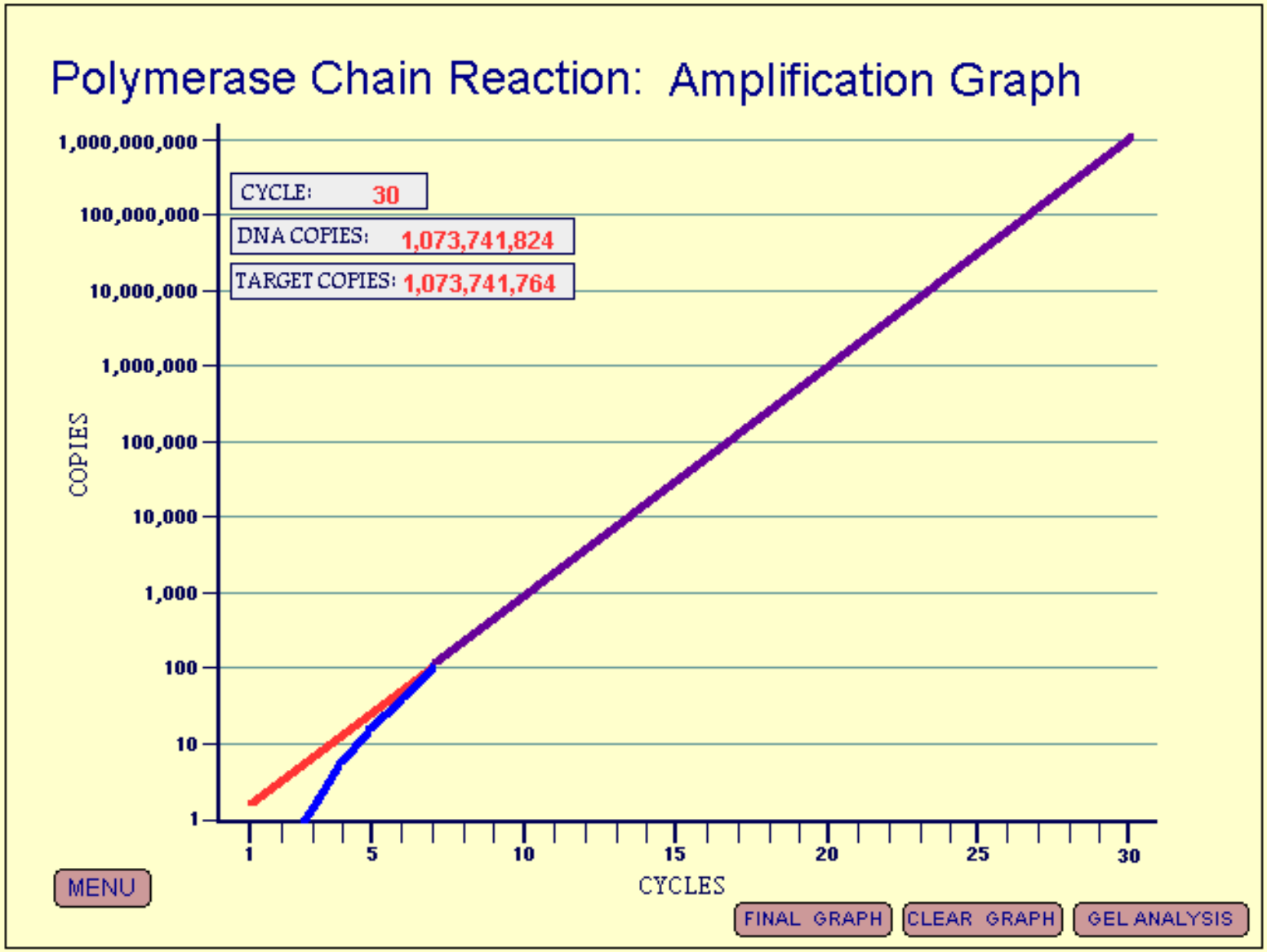


Polymerase Chain Reaction: Amplification Graph

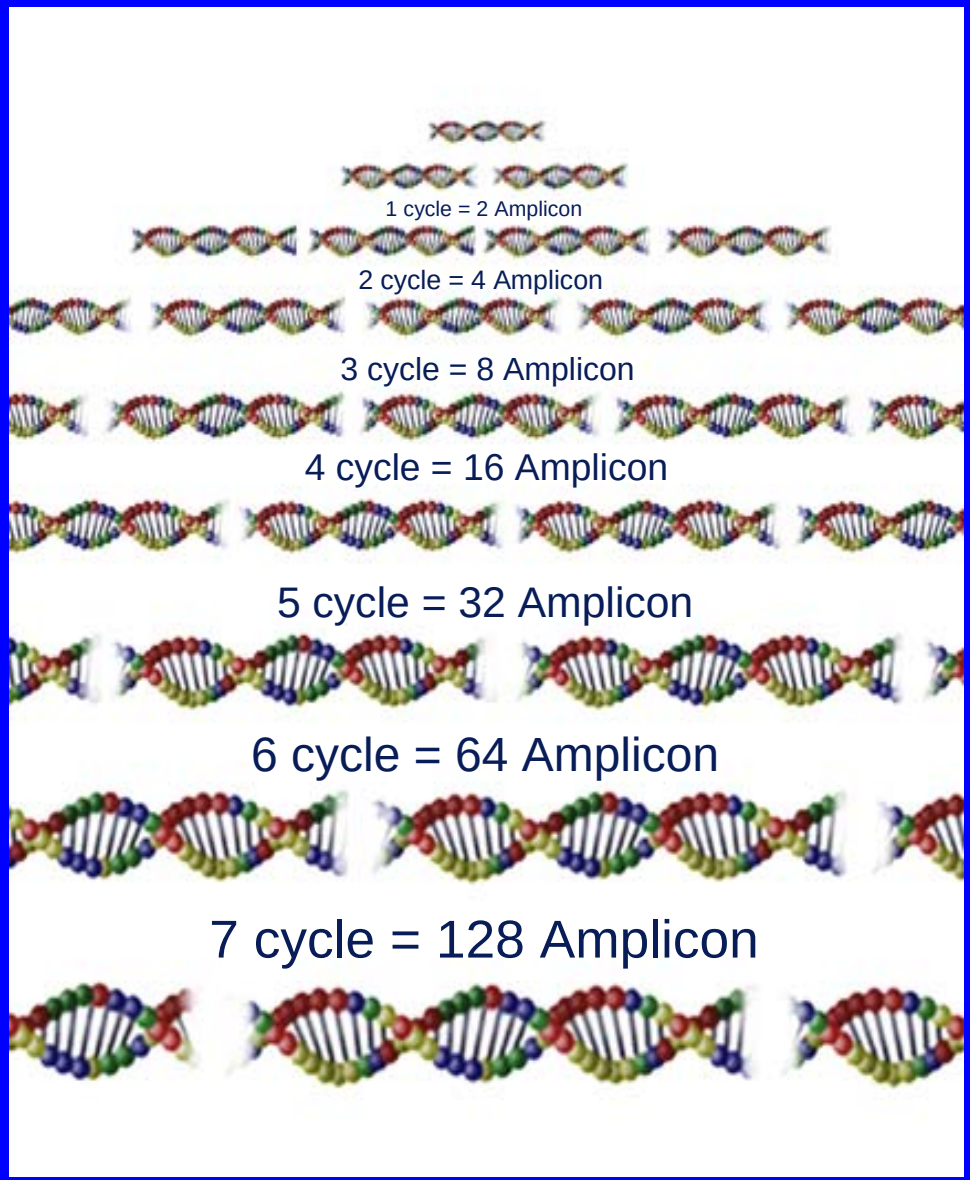


Polymerase Chain Reaction: Amplification Graph




Polymerase Chain Reaction: Amplification Graph
1,000,000,000
100,000,000
10,000,000
1,000,000
100,000
10,000
1,000
100
10
1
COPIES
CYCLE: 30
DNA COPIES: 1,073,741,824
TARGET COPIES: 1,073,741,764
1
5
10
15
20
25
30
CYCLES
MENU
FINAL GRAPH
CLEAR GRAPH
GEL ANALYSIS

Target Amplification



No. of Cycles	No. Amplicon Copies of Target
1	2
2	4
3	8
4	16
5	32
6	64
20	1,048,536
30	1,073,741,764