Polymerase Chain Reaction Workshop

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- The PCR was conceptualized by Kary Mullis in 1983.
- **Kary Banks Mullis** (born December 28, 1944) is a Nobel Prize-winning American biochemist.
- PCR has subsequently revolutionized molecular analysis in the life sciences to the point that Dr. Mullis received the 1993 Nobel Prize in Chemistry for his discovery.


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REWIND TO THE PCR
Explained and demonstrated by Nobel Prize recipient Kary Mullis
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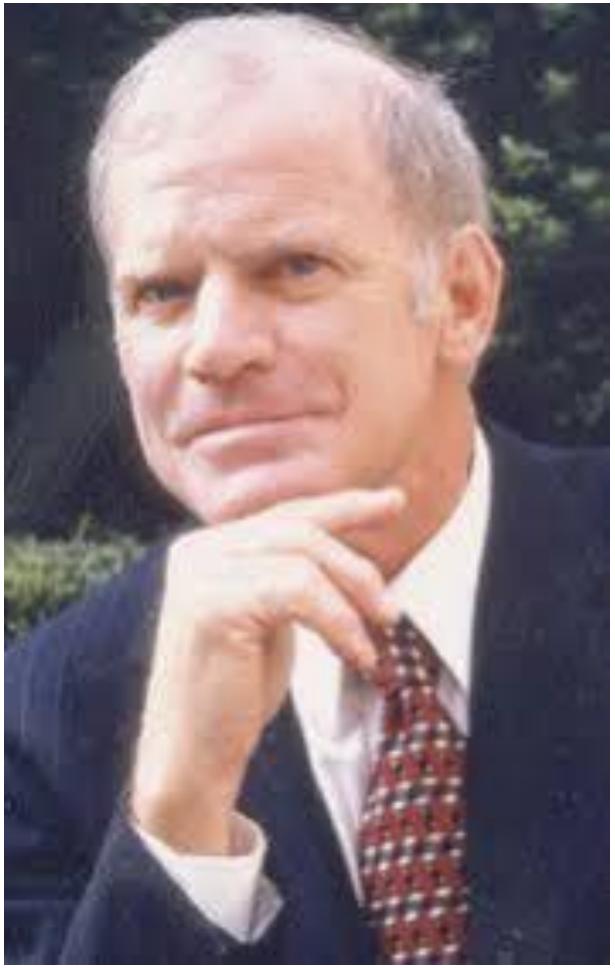
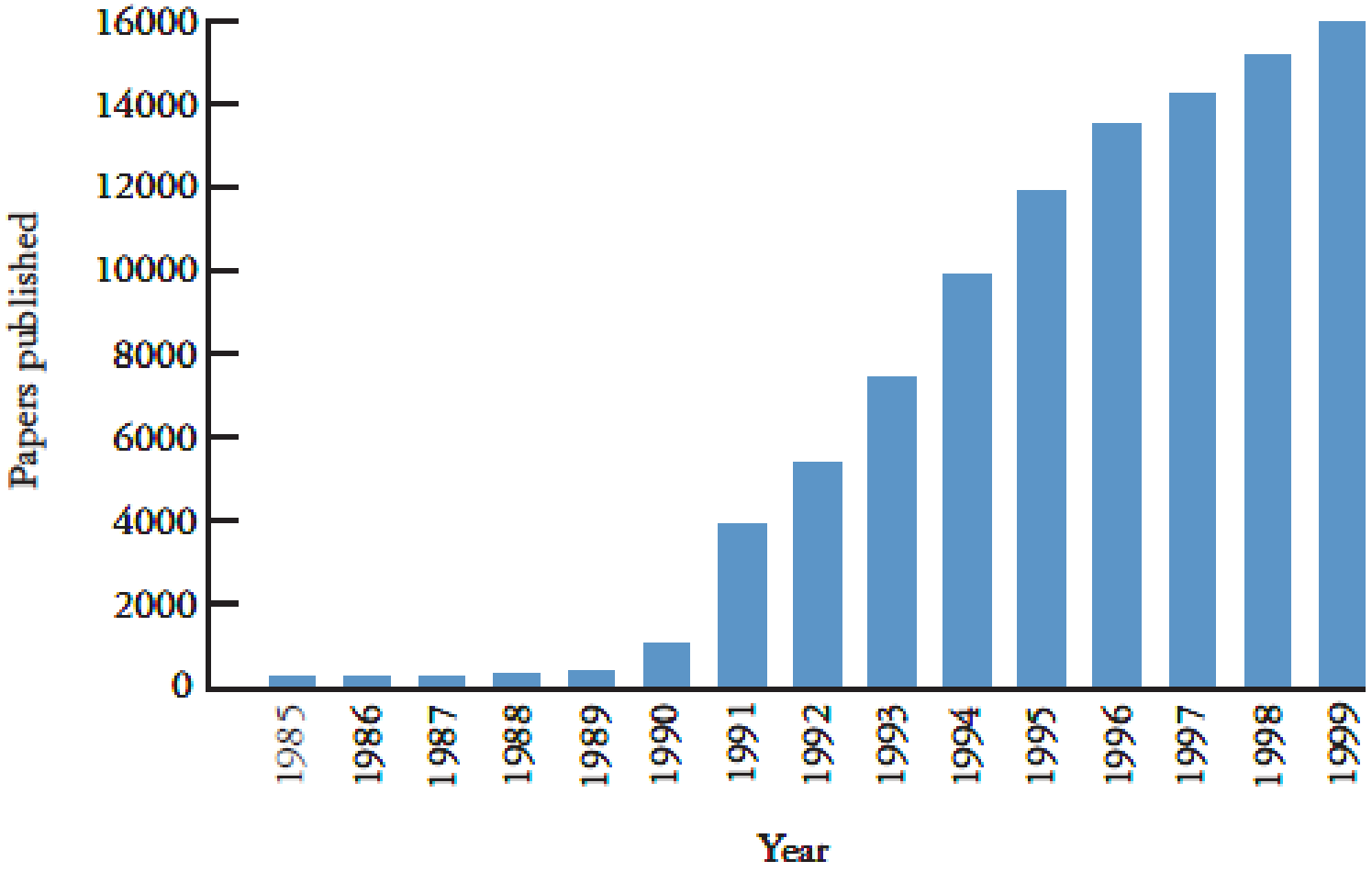




Number of publications citing the polymerase chain reaction from 1985 to 1999. This shows the rapid spread of the use of the PCR in research (2014-2105,56557).

- PCR is a technique to amplify a piece of DNA (target DNA) very rapidly outside of a cell.

- PCR Applications:

- Is a potent tool in forensic medicine.
- For detection of viral infections before they cause symptoms

- For prenatal diagnosis of a wide array of genetic diseases

- PCR can detect and amplify as little as one DNA molecule in almost any type of sample.

- Although DNA degrades over time , PCR has allowed successful cloning of DNA from samples more than 40,000 years old.

- Investigators have used the technique to clone DNA fragments from the mummified remains of humans and extinct animals as the woolly mammoth, creating the new fields of molecular archaeology and molecular paleontology

Components of PCR Reaction

1-Template DNA

2-Forward and reverse Primers

3-Thermo-stable polymerase

- Taq Polymerase

4-dNTP

- (dATP, dTTP, dCTP, dGTP)

5-PCR Buffer

6-Mgcl2

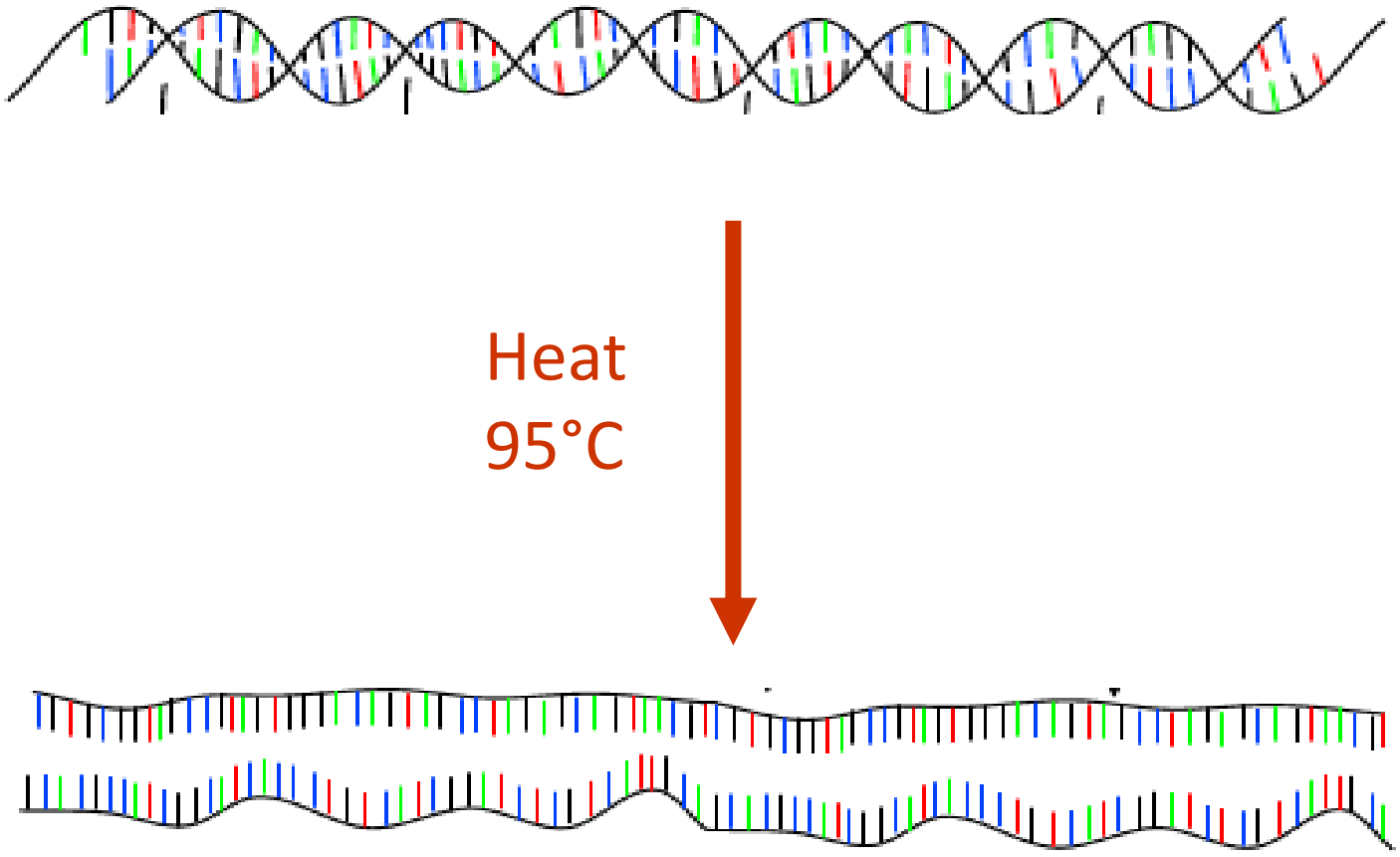
Thermocycler

Thermus aquaticus

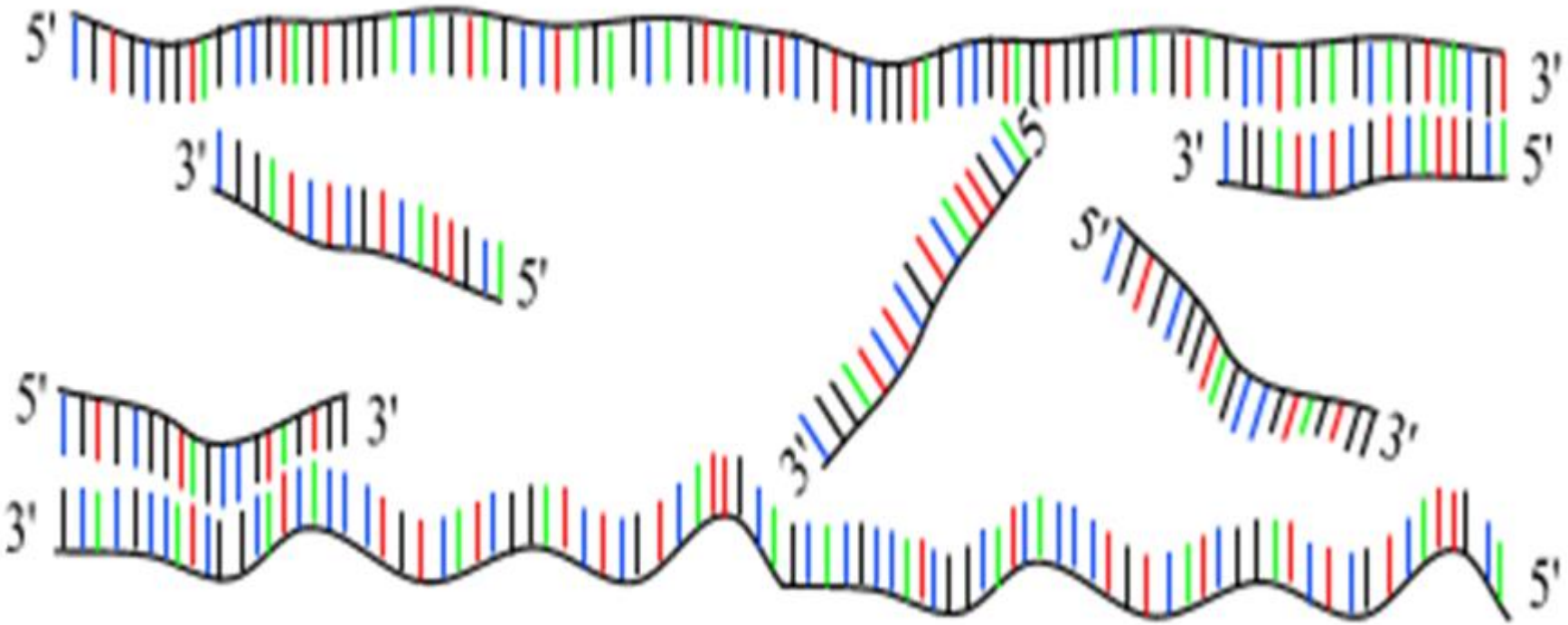
How does PCR work?

- **PCR proceeds in three distinct steps governed by temperature:**

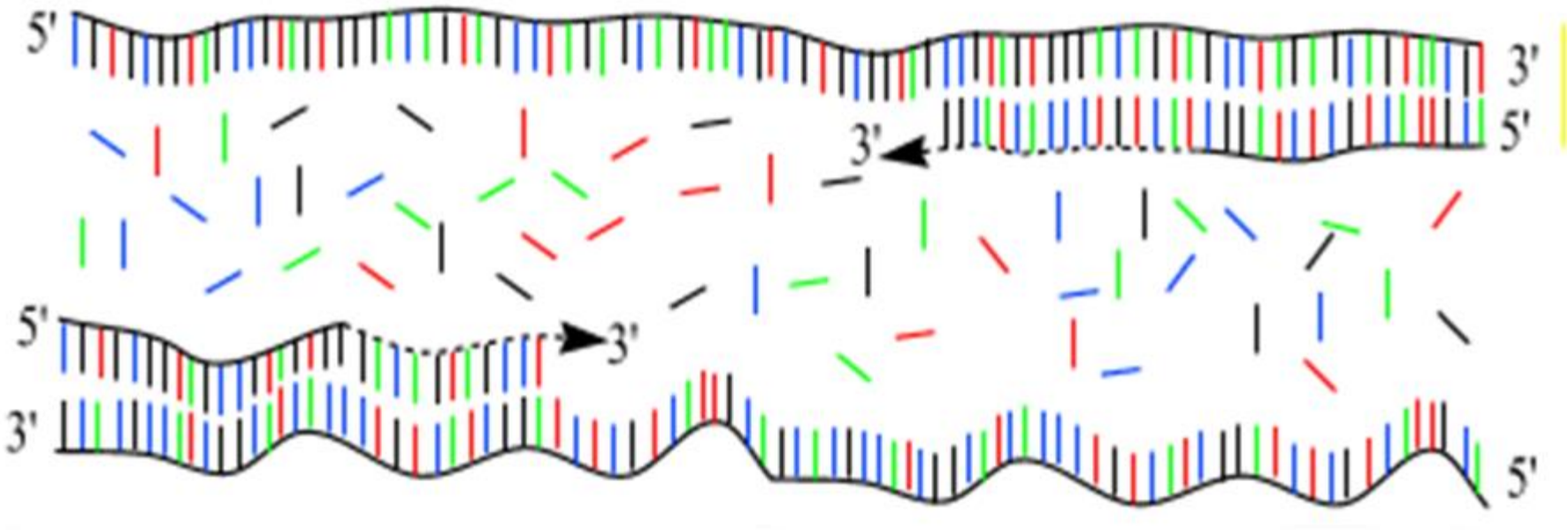
1- Denaturation



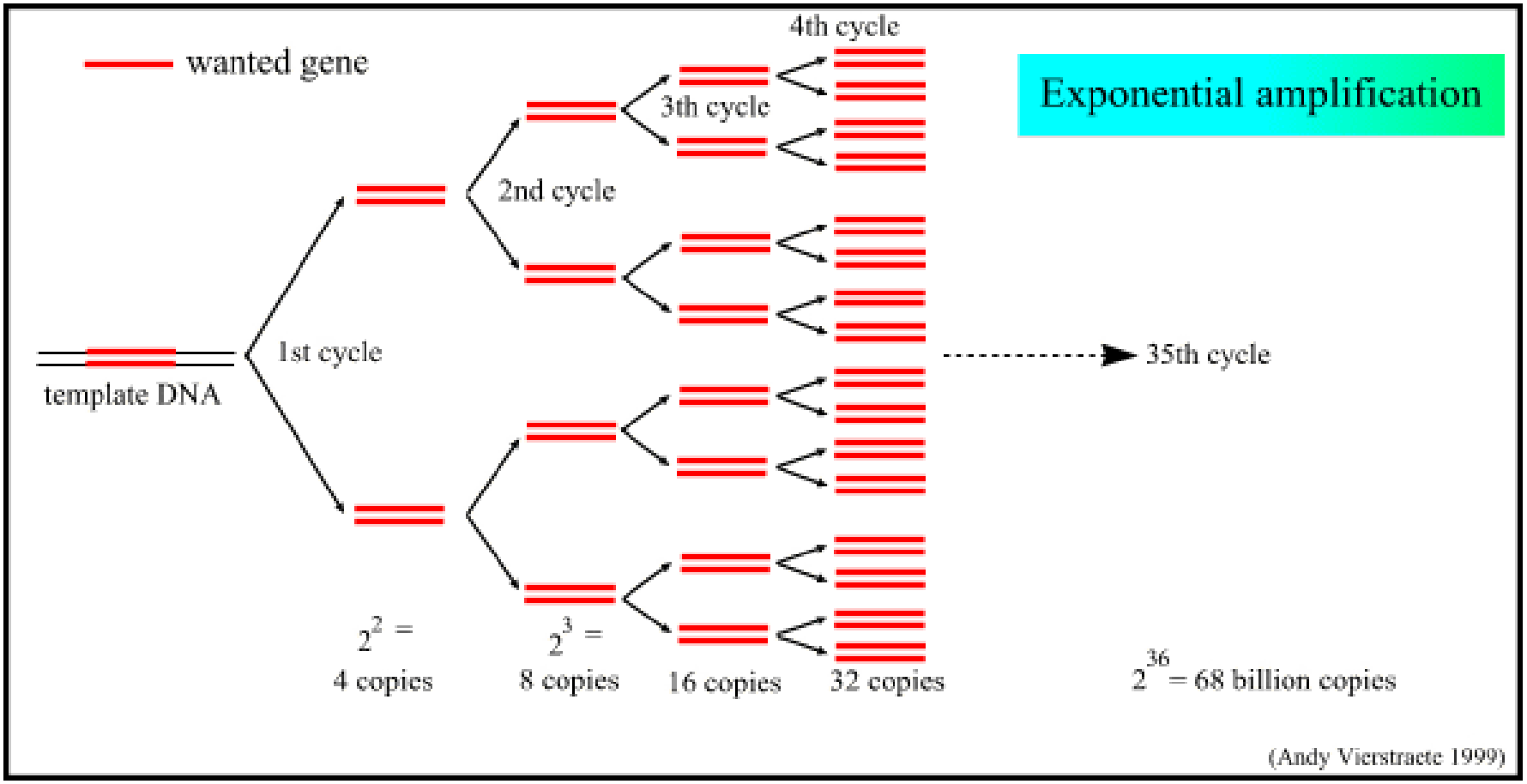
2-Annealing



3-Extension

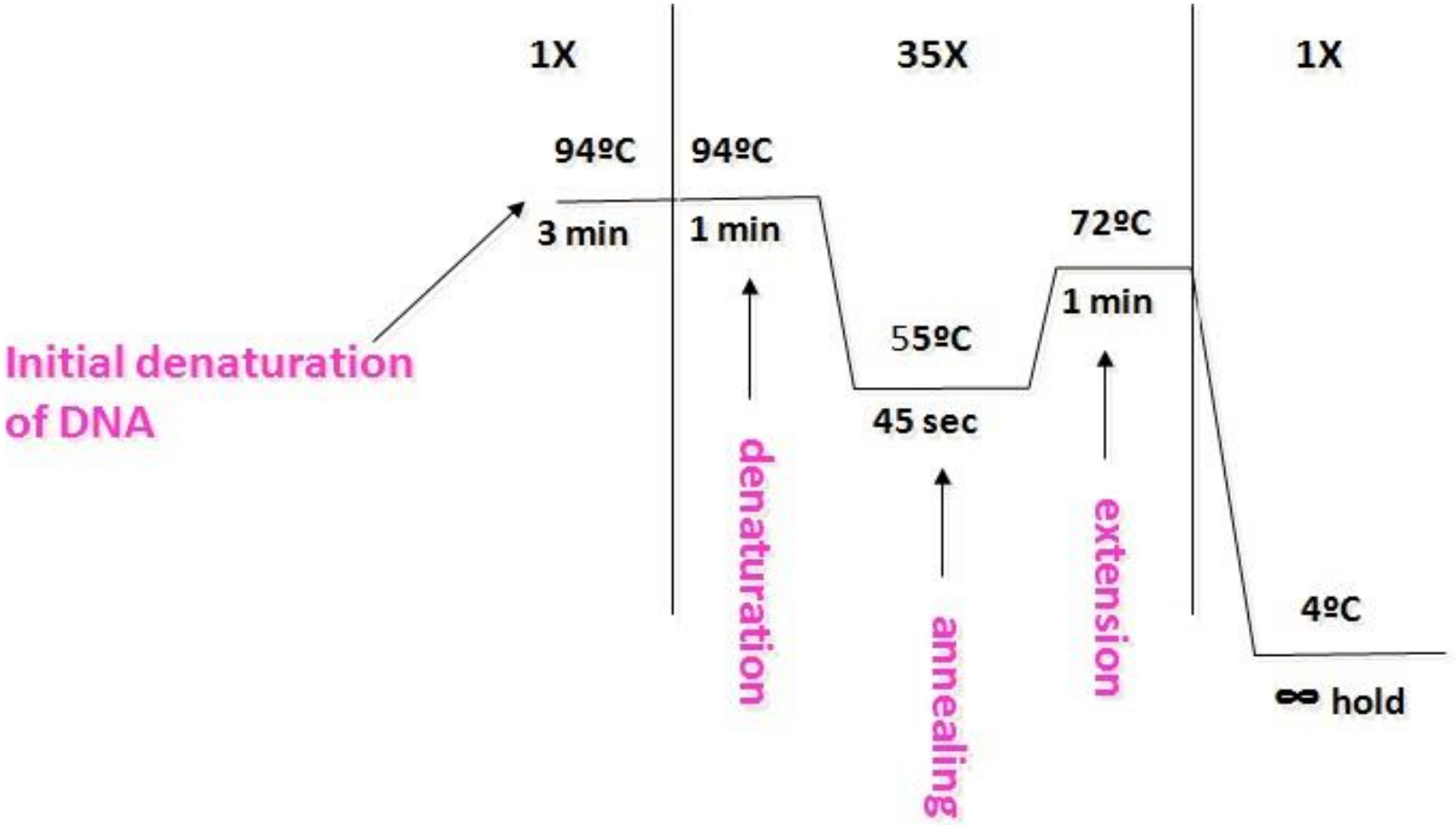


Exponential Amplification

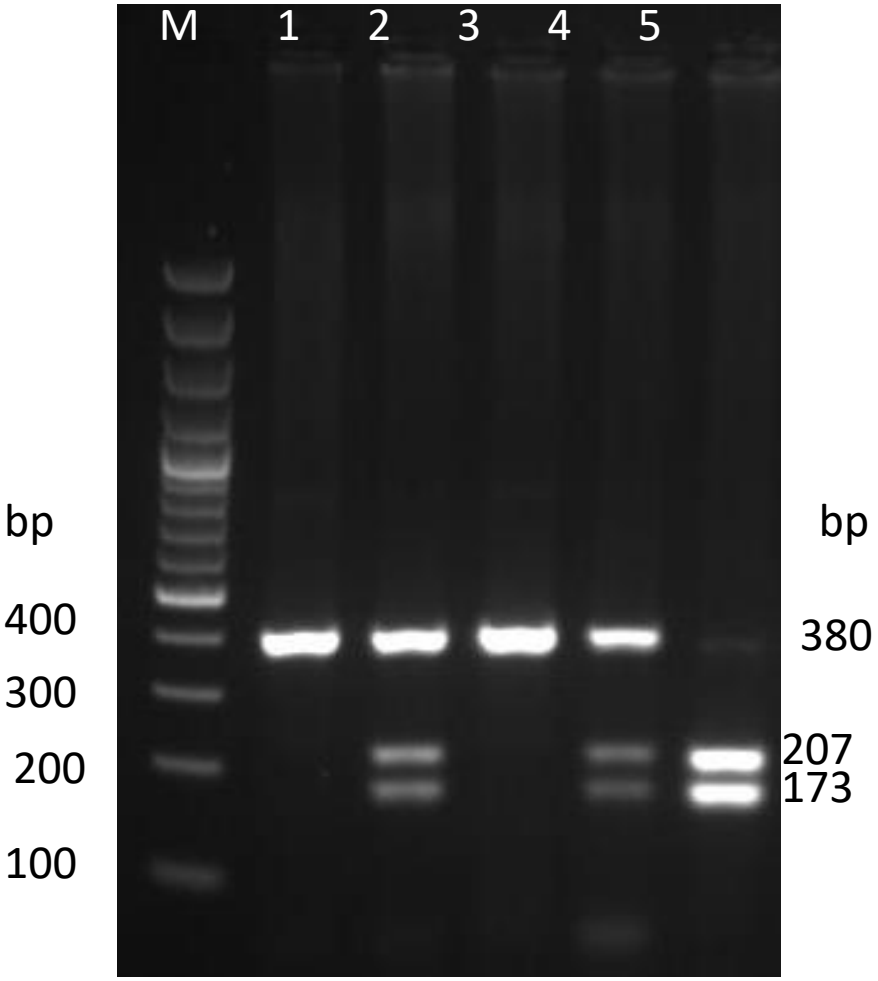


30 cycles --- 1 billion copies in theory

A simple thermocycling protocol



PCR product detection



Reagents and instrumentation

1-Template DNA

2-Forward and reverse Primers

3-Thermo-stable polymerase

- Taq Polymerase

4-dNTP

- (dATP, dTTP, dCTP, dGTP)

5-PCR Buffer

6-Mgcl2

7-Thermocycler

1-Template DNA

- The two main concerns regarding template are purity and amount.

- Pure DNA solutions have OD260/OD280 values of 1.8 (1.7-2).

Amount of template DNA required

- 0.1-1 µg human genomic DNA
- 10 ng yeast genomic DNA
- 1 ng *E. coli genomic DNA*
- 1–2 pg of plasmid
- 20 pg of bacteriophage DNA

(for human genomic DNA, final concentration will not exceed 10 ng/µl)

	PCR inhibitor	Levels for inhibition
Co-purification contaminant	Hemoglobin	> 1 mg/ml
	Heparin	> 0.15 I.U./ml
Purification method contaminants	Phenol	> 0.2% (v/v)
	SDS	> 0.005% (w/v)
	Isopropanol	> 1%
	Sodium acetate	> 5 mM
	Sodium chloride	> 25 mM
	Ethanol	> 1%
	EDTA	> 0.5 mM

2-Primers

- Effective primer design is critical to the specificity and sensitivity of a PCR .

- Select your primers based on high impact factor articles.

- the most important in designing primers:

the relative sequence quality, length of the primer (20-30 nt),melting temperature (Tm 54-80°C), %GC content (40-60%), the 3 end of the sequence, and potential secondary structures.

Primer Dimers

- **Pair of Primers**

5’-ACGGATACGTTACGCTGAT-3’

5’-TCCAGATGTACCTTATCAG-3’

- **Complementarity of primer 3’ ends**

5’-ACGGATACGTTACGCTGAT-3’

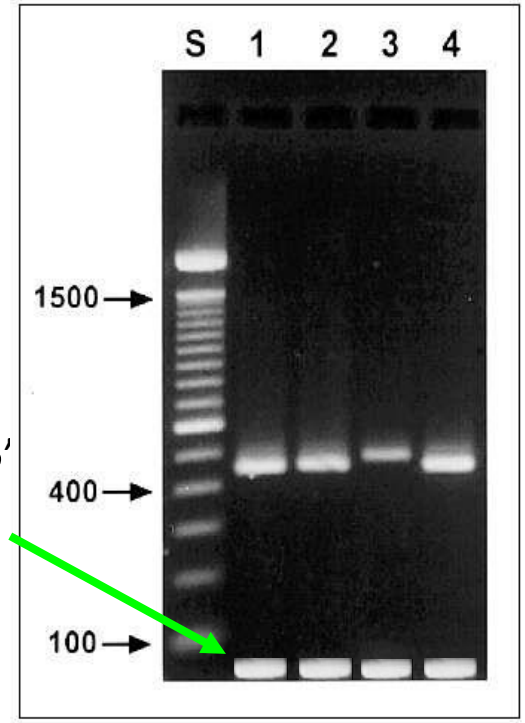
3’-GACTATTCCATGTAGACCT-5’

- **Results in PCR product**
- ***Primer 1***

5’-ACGGATACGTTACGCTGATAAGGTACATCTGGA-3’

3’-TGCCTATGCAATGCGACTATTCCATGTAGACCT-5’

Primer 2



Guidelines for the design and use of primers

	Standard PCR	Multiplex PCR	One-step RT-PCR
Length	18–30 nt	21–30 nt	18–30 nt
GC content	40–60%	40–60%	40–60%
T_m information	The T_m of all primer pairs should be similar	The T_m of all primer pairs should be similar. For optimal results, the T_m should be 60–88°C	The T_m of all primer pairs should be similar. The T_m should not be lower than the temperature of the reverse transcription (e.g., 50°C)
Estimating optimal annealing temperature	5°C below the calculated T_m	5–8°C below the calculated T_m (when greater than 68°C) or 3–6°C below the calculated T_m (when 60–67°C)	5°C below the calculated T_m
Location	–	–	To prevent detection of gDNA: Primer hybridizes to the 3' end of one exon and the 5' end of the adjacent exon. Alternatively, the primer hybridizes to a flanking region that contains at least one intron. If only the mRNA sequence is known, choose primer annealing sites that are 300–400 bp apart.
Concentration, A_{260} unit equivalence	20–30 µg	20–30 µg	20–30 µg

3-Taq polymerase

- Thermostable Taq DNA polymerase is capable to withstand the repeated heating and cooling inherent in PCR

- Taq is not infinitely resistant to heat, and for greatest efficiency it should not be put through unnecessary denaturation steps

Table 10.2.1 Characteristics of Common DNA Polymerases for PCR

	DNA Polymerase	
	Taq	Vent/*Tli* (*Pfu* is similar)
Half-life @ 95°C	~90 min	~420 min
3′→5′ Exonuclease	No	Yes
5′→3′ Exonuclease	Yes	No
Extension rate (nt/sec)	75	>80
Error rate (errors/bp)	2×10^{-5}	4×10^{-6}
Resulting ends	3′ A	>95% blunt
Example application	Standard amplification of target sequences	Target amplification for cloning blunt-ended fragments requiring DNA sequence fidelity

4-dNTP

- Stock solutions of dNTPs can be purchased from many commercial sources.

- The con. of dNTPs should be 50–200 μM.

- If the con. is higher the fidelity of the process will be adversely affected by driving Taq DNA polymerase to misincorporate at a higher rate than normal.
- If the con. is lower it may affect the efficiency of PCR
- Lower con. of dNTPs increase fidelity and specificity of the reaction

5-PCR Buffer

- Most suppliers of thermostable DNA polymerases provide 10× reaction buffer with the enzyme.

- Final con. In PCR reaction: 1x

6-Mgcl2

- Mg^{2+} is one of the most critical components in the PCR as its con.can affect the specificity and efficiency of the reaction.
- Act as a cofactor for *Taq DNA* polymerase

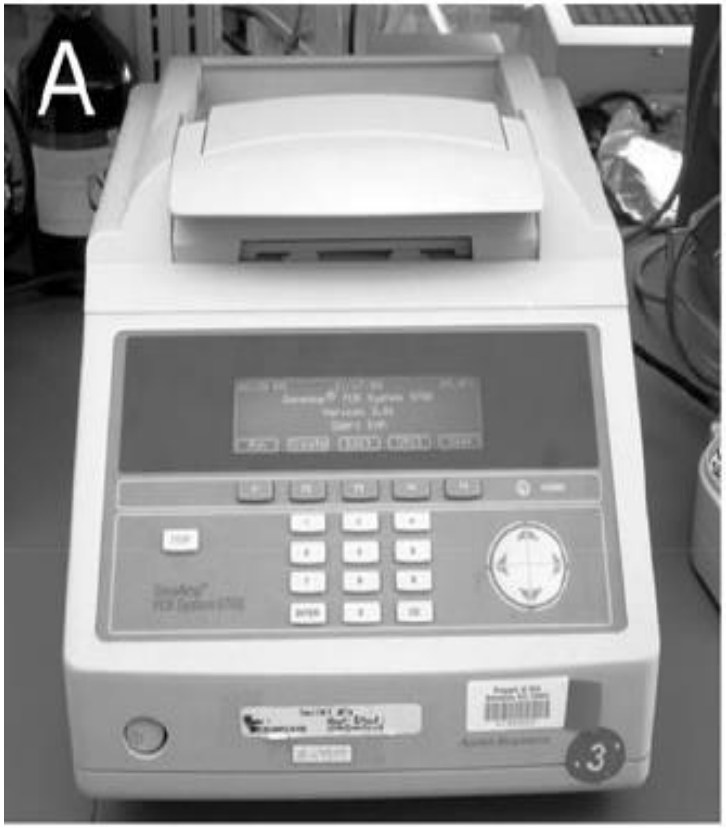
- Mg complexes with the negatively charged dNTPs, primers, and DNA template

PCR Master mix

- When performing multiple PCRs at the same time it is advisable to prepare a “master mix” of reaction ingredients.

- This process significantly reduces the number of pipetting actions that need to be performed and as such greatly contributes to the reproducibility and reliability of the PCR test protocol.

8-Thermocycler

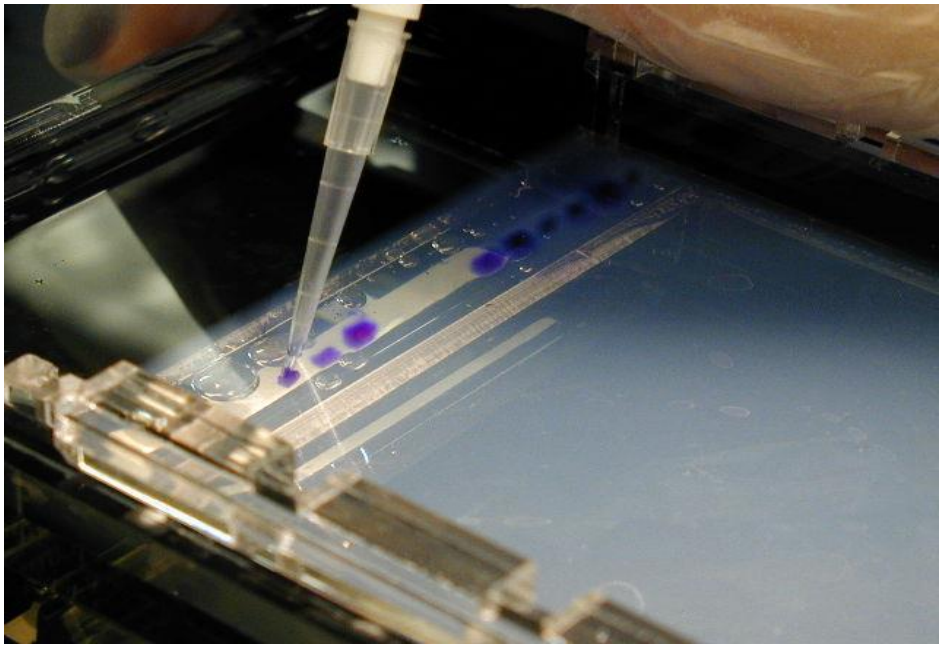


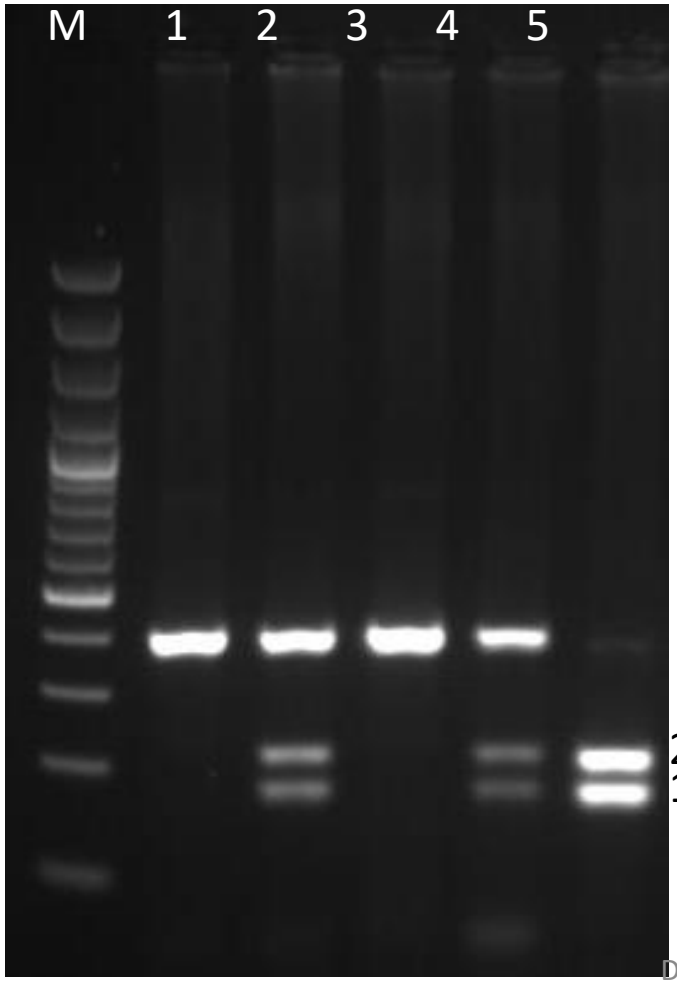
The PCR thermocycling parameters

- Program the thermal cycler with the desired PCR profile. The following is a generic profile that can be modified.

Initial step: (denaturation)	2 min	95◦C	
35 cycles: (denaturation)	15 sec	95◦C	
	30 sec	55◦C	(annealing)
	60 sec	72◦C	(extension)
Final elongation:	5 min	72◦C	(extension)
Final step:	indefinite	4◦C	(hold).

Gel electrophoresis of PCR products





PCR optimization

- If no bands are detected then the stringency may be too high whilst if several bands are seen then the stringency should be increased.

- How do you set about this optimization task?

Annealing Temprature

- No PCR product

  Decrease Ann. Tem.

Nonspecefic products

  increase ann. Tem

1-Temprature gradient PCR

- Determining the proper annealing temperature for a new set of PCR primers is a key component to successful and selective amplification of the target DNA sequence.

- Many thermal cyclers have a temperature gradient function that allows the researcher to designate a range of temperatures for the annealing step to be run in one experiment.

- Each column of wells on the block will have a different annealing temperature.

- The annealing temperature gradient should be set up to flank the *Tm of the primers (e.g.* ±5*)*

- 2- Denaturation of the template before *Taq* polymerase is added to the reaction provides a dramatic improvement in specificity and sensitivity (Hot-Start).

Table 10.2.6 $MgCl_2$ Titration Mixture Set Up

Components	Final concentration	Per tube volume
10× PCR buffer $MgCl_2$-free	1×	5 µl
Titration:	1.0 mM	1 µl
25 mM $MgCl_2$	1.5 mM	3 µl
	2 mM	4 µl
	3 mM	6 µl
	4 mM	8 µl
	5 mM	10 µl
40 mM dNTP mix	0.2 mM each dNTP	1 µl
10 µM forward primer	1 µM	5 µl
10 µM reverse primer	1 µM	5 µl
Sterile, nuclease-free H_2O		*X* µl (to a final volume of 50 µl)
5 U/µL hot-start *Taq* DNA polymerase	0.025 U/µl	0.25 µl
>100 ng/µl template	2 ng/µl	1 µl

DNA polymerase

- If you have no product band(s) or weak band(s) you may have too little or old DNA polymerase.

- Taq will become inactivated at high temperatures.
- So, try to limit the time the enzyme spends above 90°C by using a short denaturation time at 94°C, stay 15 s.

PCR contamination

- In PCR small quantities of contaminating DNA may be amplified.

- Major sources of DNA contamination:

  bench surfaces, laboratory equipment, pipettors, airborne particles such as microbes and debris such as skin or hair, contaminated solutions

Contamination Detection

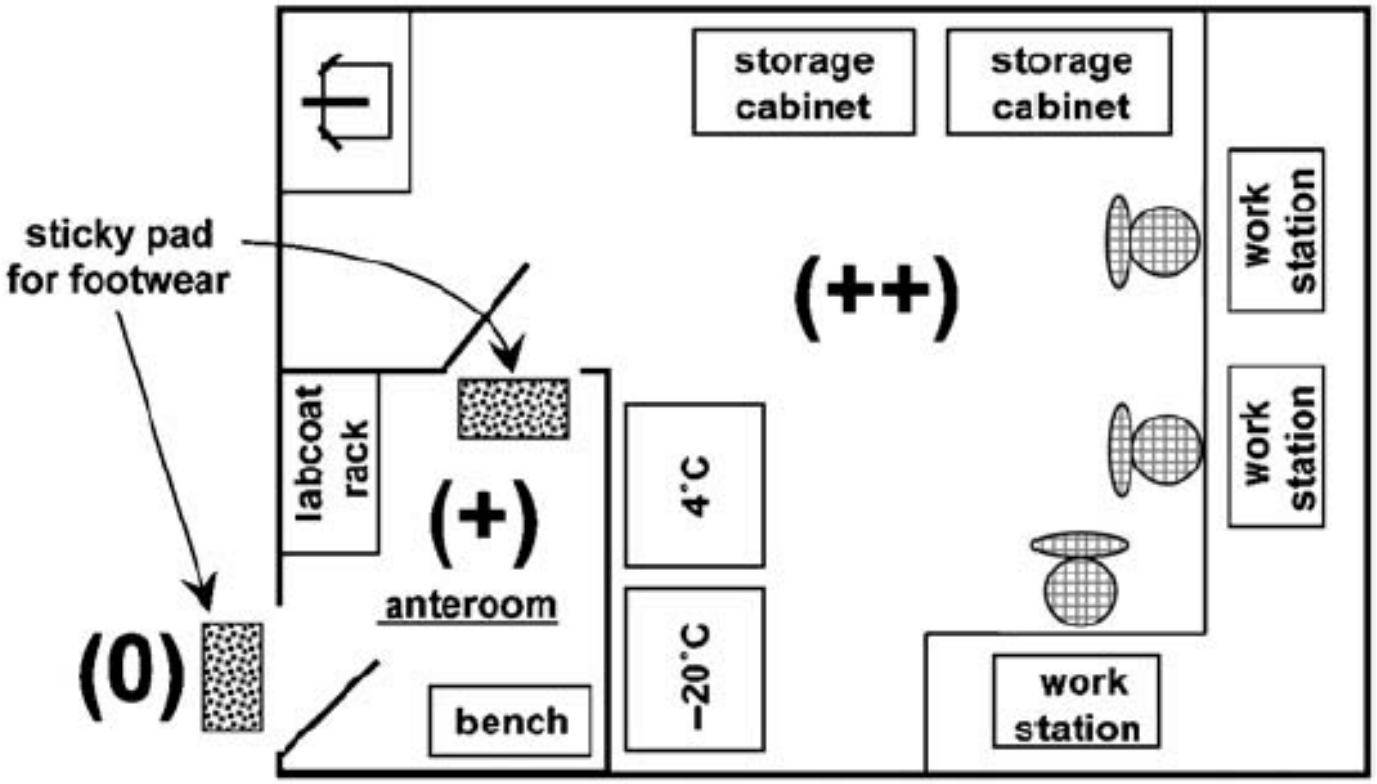
- Negative control: helps to verify that any specific PCR product amplified is not the result of contamination.
- Negative controls comprise a PCR mix without DNA template.
- All negative controls should contain no amplification products (apart from perhaps primer dimers) and the finding of PCR products within any negative control means that the whole test batch should be repeated.

- It should be emphasized that negative control samples are absolutely essential for PCR assays performed in the molecular diagnostic clinical laboratory

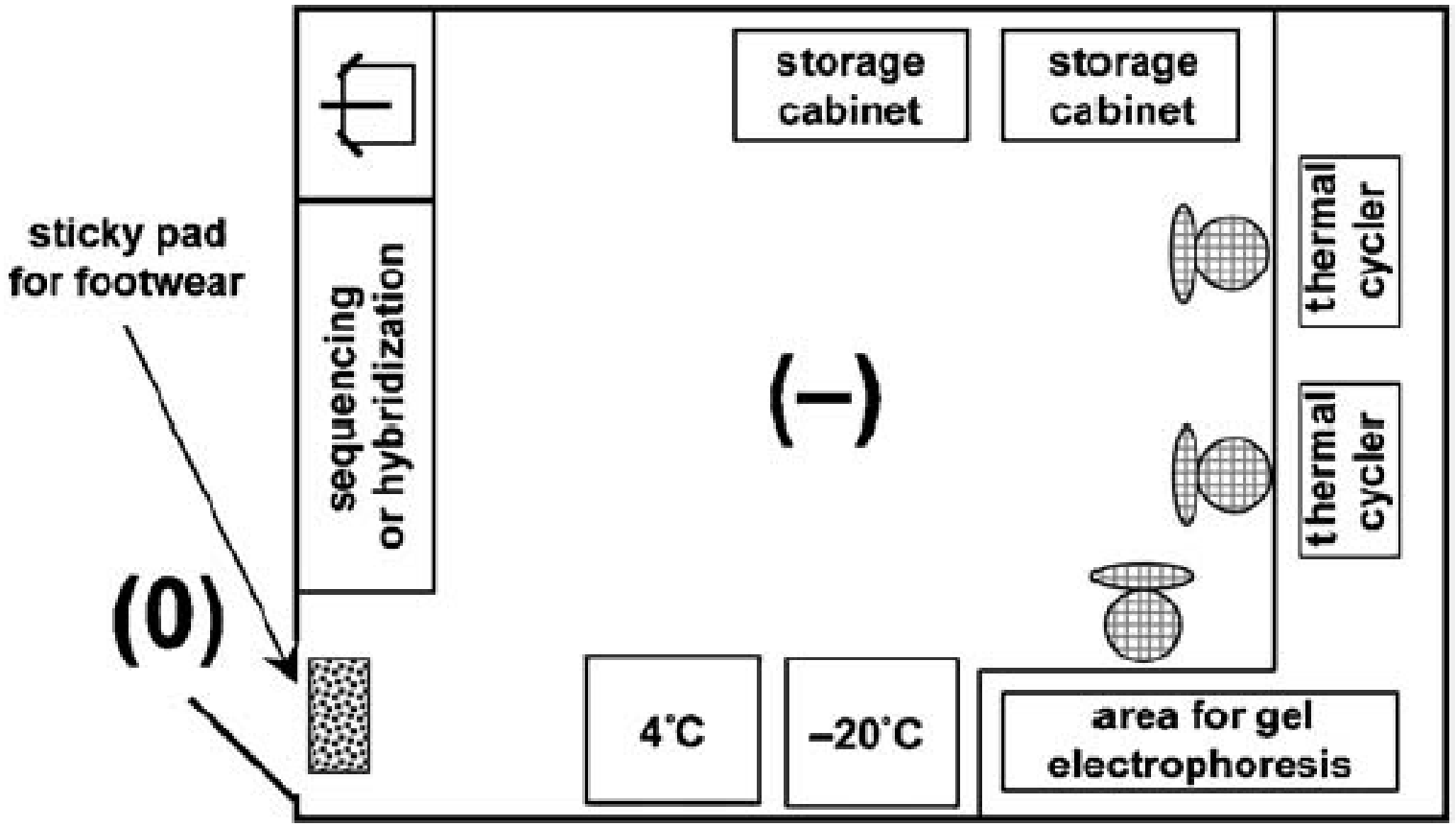
Prevent contamination by

1-It is best to avoid setting up PCRs in the same area as you process post-PCR samples.

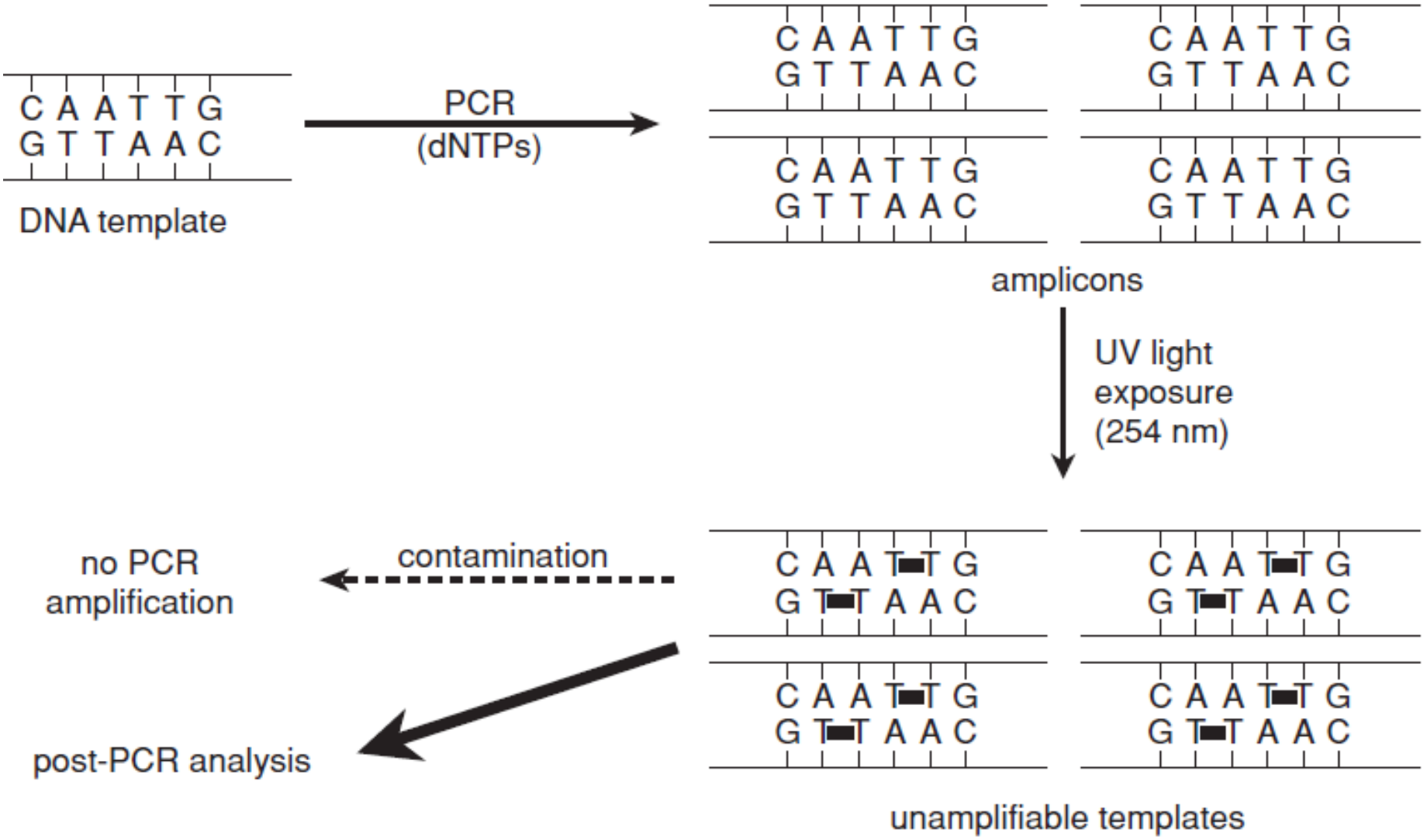
2-Clinical specimens and PCR amplification products are ready sources of contamination, and should not “back-track” into any of the previous PCR laboratories.



Pre-PCR set up laboratory



Thermocycling and Post-PCR set up laboratory



Thymidine dimer contamination. Standard PCR is performed. Prior to post-PCR analysis, the reaction mix is exposed to short-wave UV light to form thymidine (T-T) dimers which cannot participate in future PCRs should these amplicons contaminate a new PCR reaction.