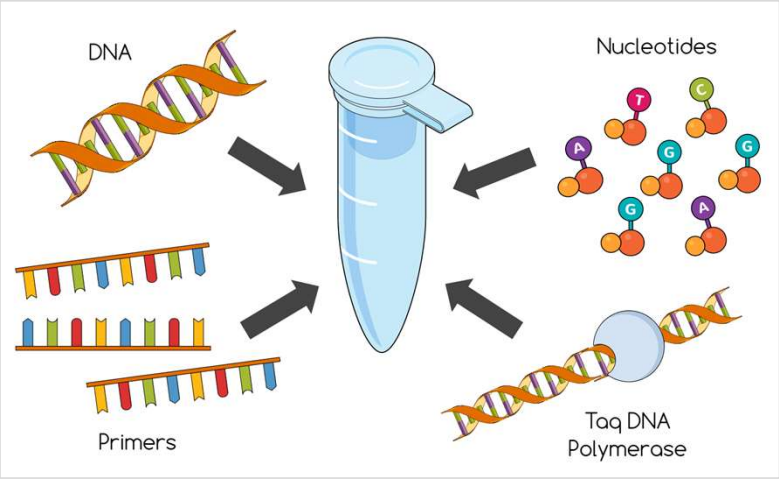
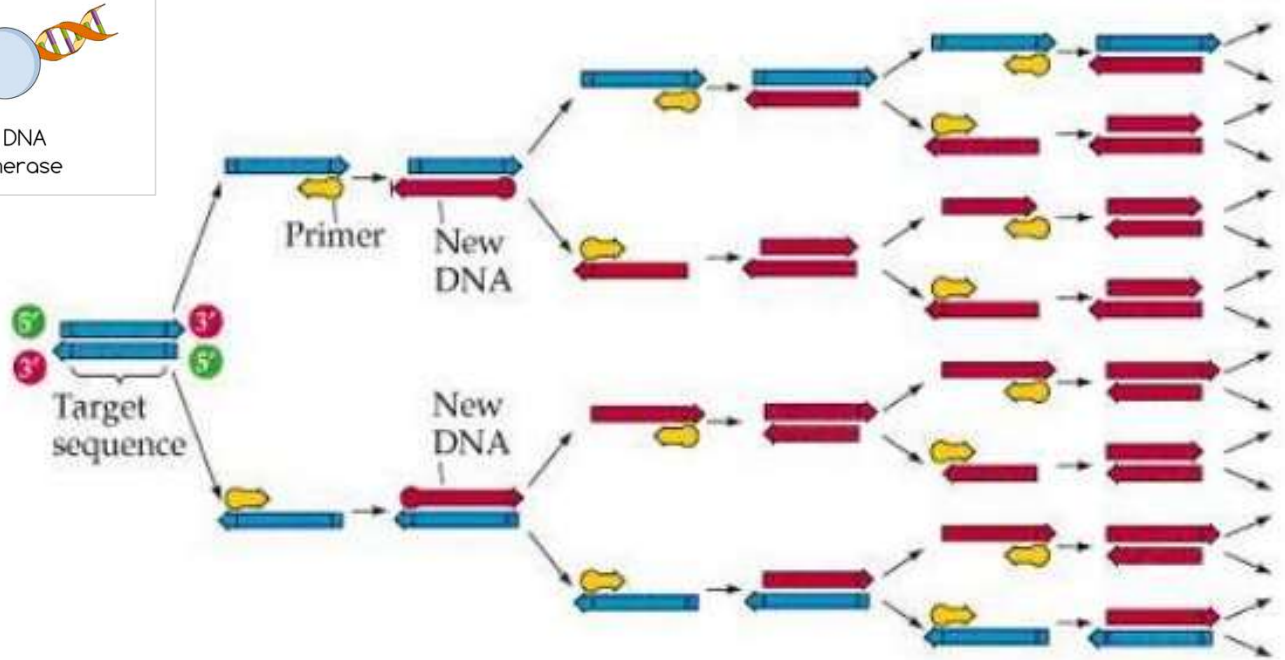
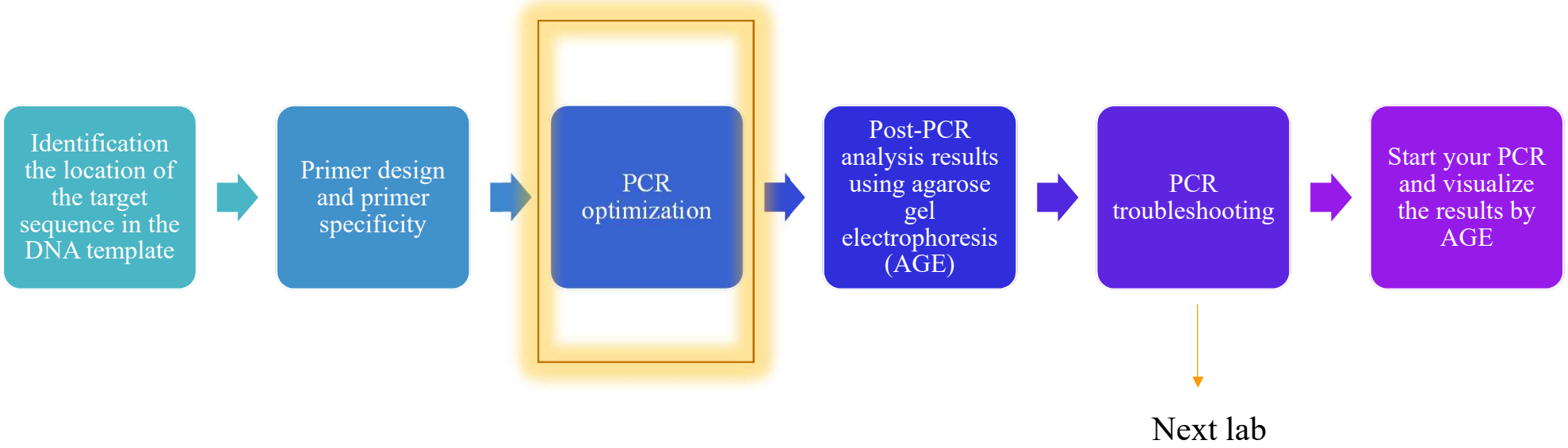
PCR- Optimization of Annealing Temperature

Polymerase Chain Reaction (PCR)=DNA Photocopier





Performing PCR steps :



PCR optimization:

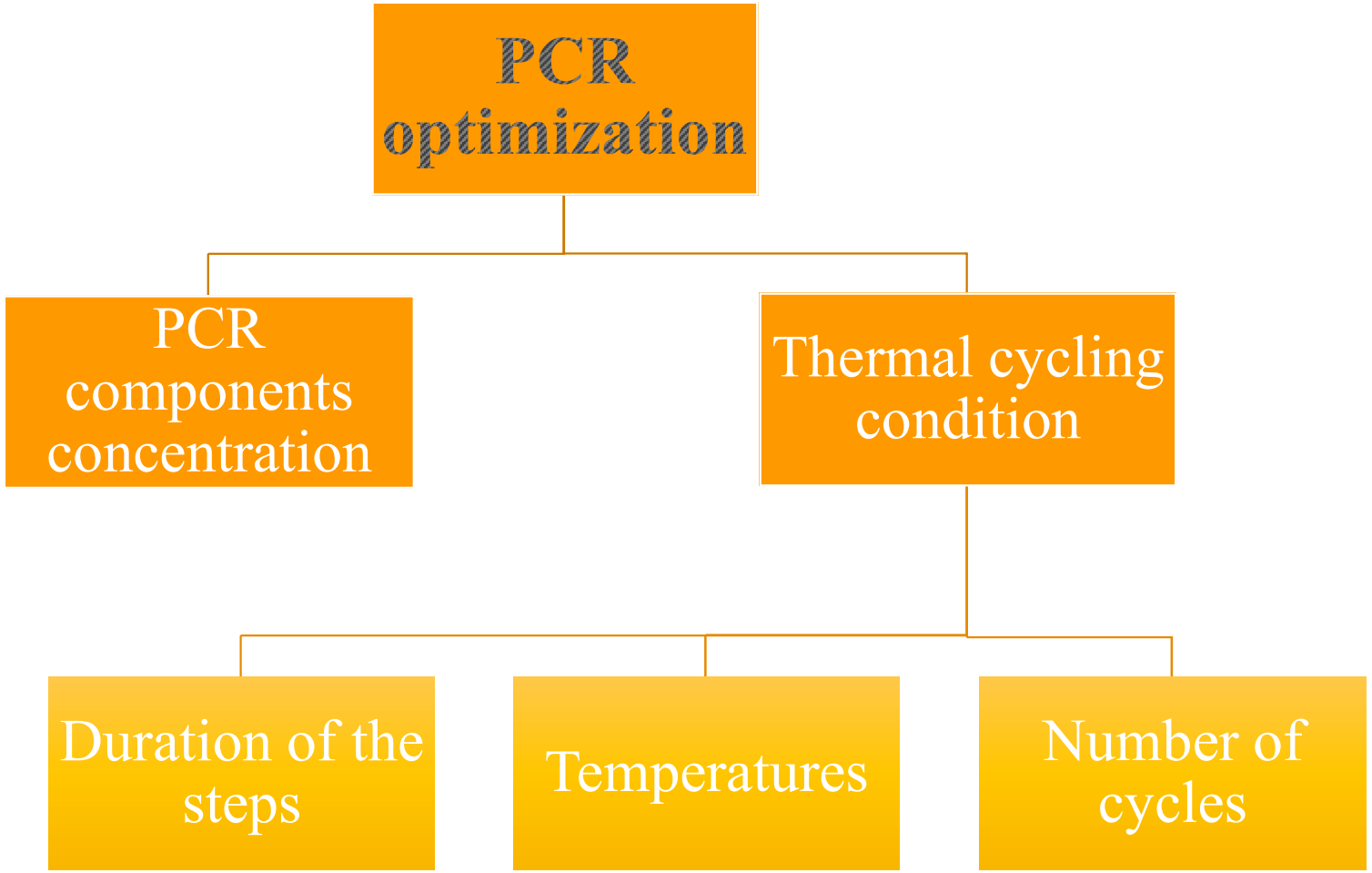
- PCR optimization ?

- What if not?

- **REMEMBER:**

➔**There is no single set of conditions that is optimal for all PCR reactions.**

WHY?


PCR optimization
PCR components concentration
Thermal cycling condition
Duration of the steps
Temperatures
Number of cycles

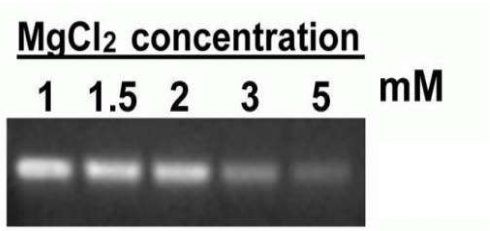
Standard concentrations of PCR components

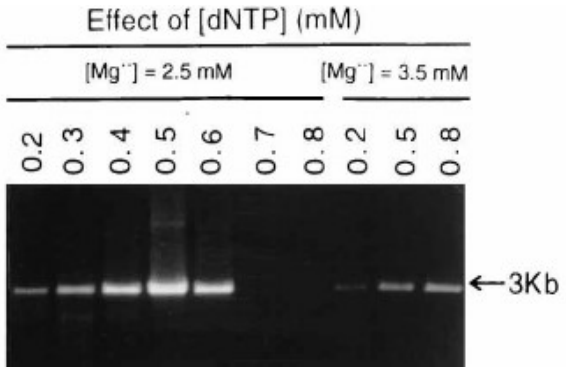
Component	Final concentration
Taq polymerase	0.5–2.0 units, ideally 1.25 units.
Deoxy-nucleotides (dNTPs)	Typical concentration is 200 µM of each dNTP.
Magnesium Concentration	1.5-2.0 mM is optimal for Taq DNA Polymerase.*
Forward Primers	Typically 0.1-0.5 µM .
Reverse Primer	Typically 0.1-0.5 µM.
DNA Template	1ng–1µg of genomic templates.

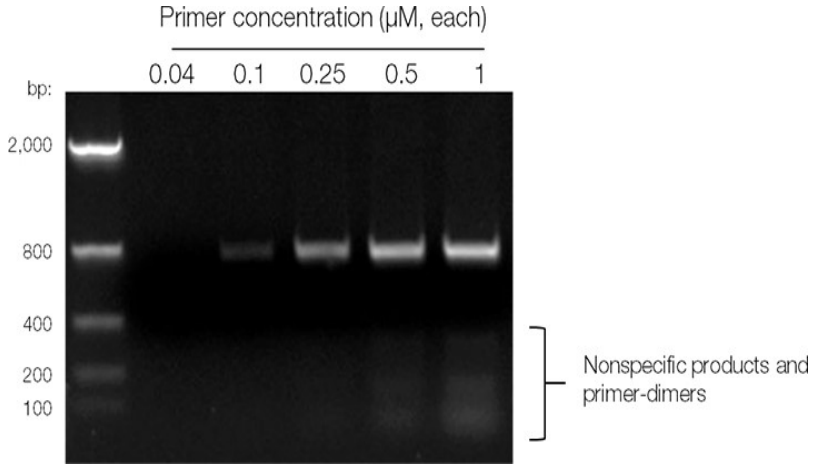
PCR optimization:

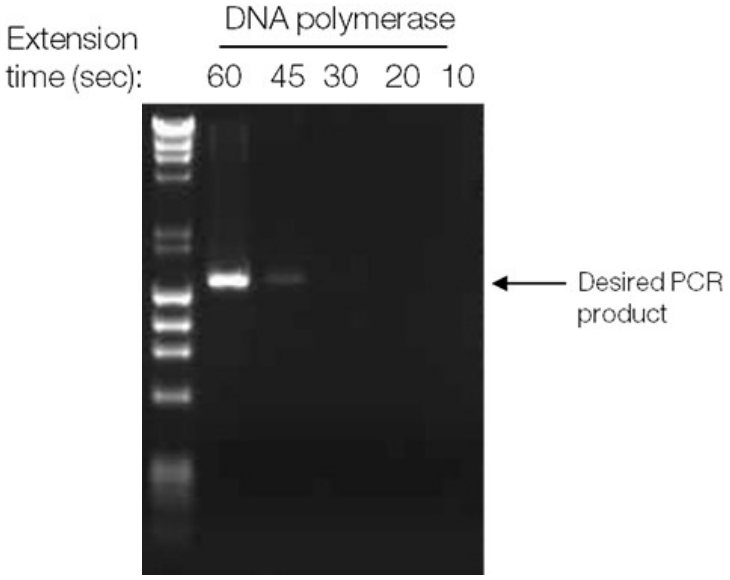
- It is important to note that while optimization of one parameter, other parameters should be fixed and not changed. WHY ?

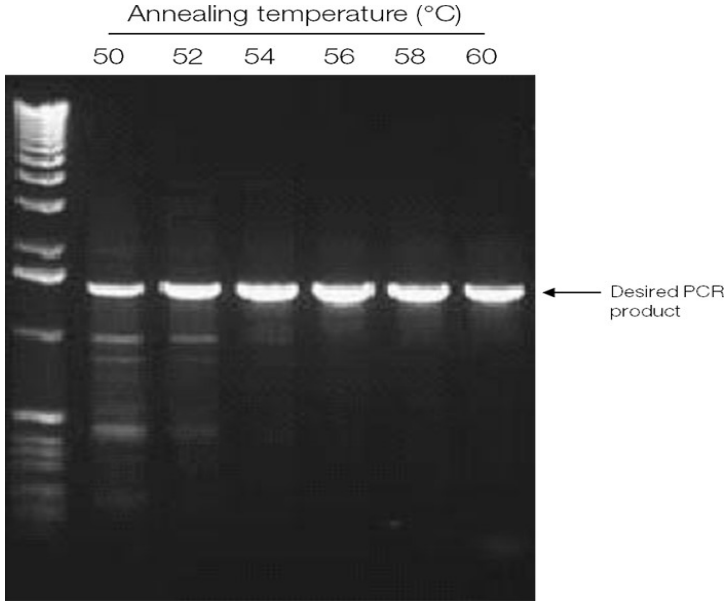
➔ How you will know that you reached to the optimum conditions?


MgCl2 concentration
1 1.5 2 3 5
mM


Effect of [dNTP] (mM)
[Mg++] = 2.5 mM
[Mg++] = 3.5 mM
0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.2 0.5 0.8
3Kb


Primer concentration (μM, each)
0.04 0.1 0.25 0.5 1
bp:
2,000
800
400
200
100
Nonspecific products and primer-dimers


Extension time (sec):
DNA polymerase
60 45 30 20 10
Desired PCR product


Annealing temperature (°C)
50 52 54 56 58 60
Desired PCR product

General PCR thermal cycling condition

	Step	Temperature	Duration	Cycle
Stage 1	**Initial denaturation**	94–97 °C	3 min	x1
Stage 2	**Denaturation**	94–97 °C	30 sec	x (25-35)
	Annealing	50-65 °C	30 sec	
	Elongation	72-80 °C	30-60 sec	
Stage 3	**Final elongation**	75-80 °C	5-7 min	x1

PCR optimization of annealing temperature (Ta):

- Reaching the optimum Ta is critical for **reaction specificity**, as non-specific products may be formed as a result of **non-optimal Ta.**

- **HOW?**

➔Optimization done by applying temperature gradient PCR, where **PCR carried with different Ta starting at 5 °C** below the lowest calculated melting temperature (Tm) of the primer pair.

- Example.

PCR optimization of annealing temperature (Ta):

- When optimizing Ta what you should do with other PCR component?

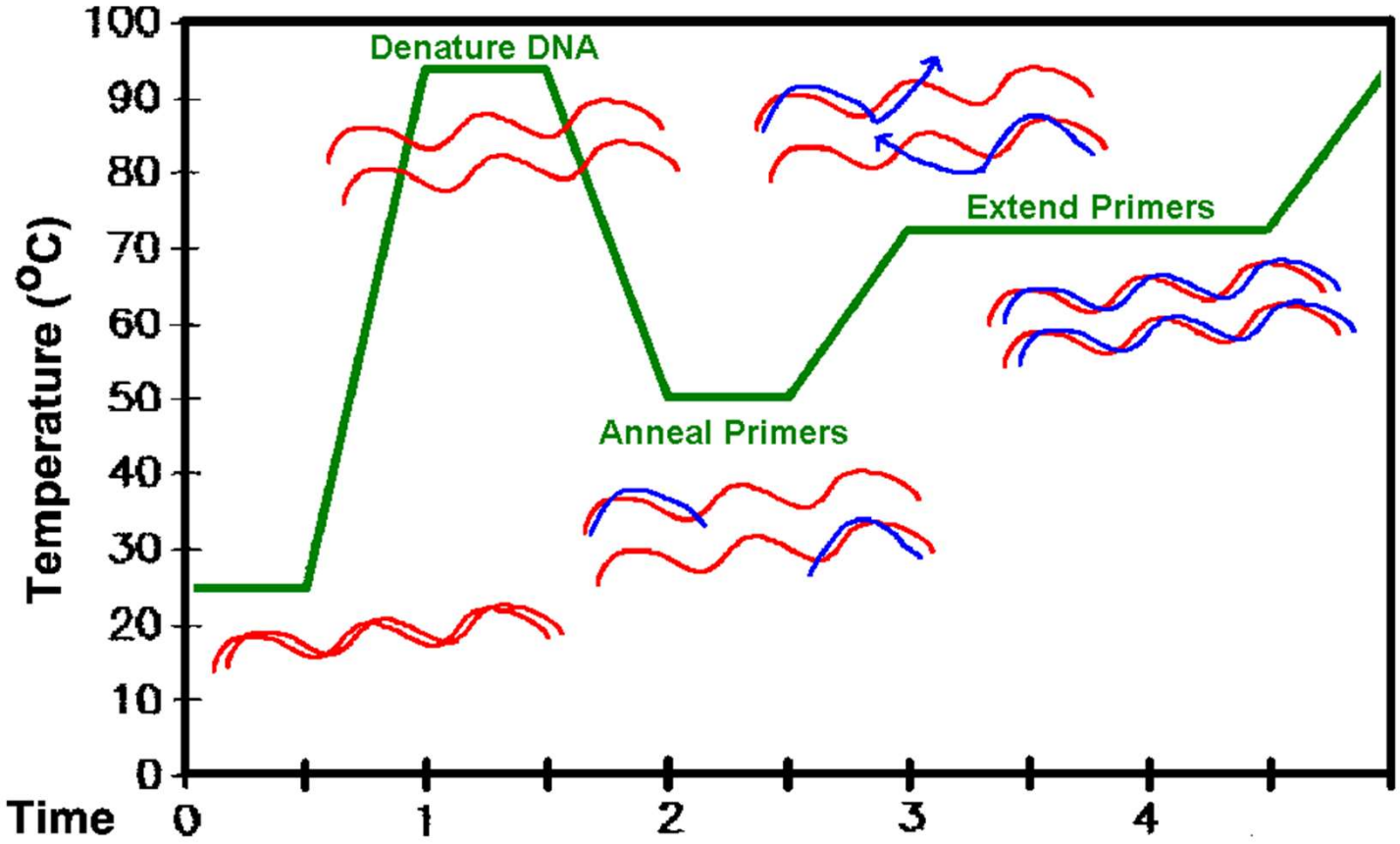
➔ Start by applying the standard concentration of PCR component that work with majority of PCR reaction.

Practical Part

Aim:

- Optimization of PCR annealing temperature.
- Be familiar with PCR technique and thermal cycler device.

Principle:


Denature DNA
Extend Primers
Anneal Primers
Temperature (°C)
100
90
80
70
60
50
40
30
20
10
0
Time
0
1
2
3
4

Method:

1. You will be using GoTaq® Green Master Mix (Promega).
2. prepare a master mix that contains everything except the DNA template by multiplying the volume per reaction of each component by (number of desired reaction +1 for pipetting error):

Components	Volume per reaction (µl)	Master mix (Volume per reaction X..)
GoTaq® Green Master Mix, 2X	6.25	
upstream primer, 10µM	0.25	
downstream primer, 10µM	0.25	
DNA template	2	----
Nuclease-Free Water to	3.75	
Final volume	12.5	

Method:

3. Using special PCR tubes, distribute the master mix by pipetting --- µl to each tube.
4. Add 2 µl DNA template for each template.
5. Centrifuge the tubes briefly.
6. Set the thermal cycling condition as following:

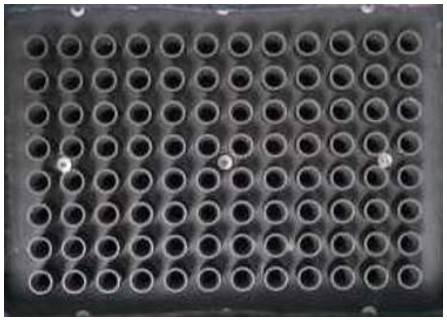
Step	Temperature	Duration	Cycle
Initial denaturation	95 °C	2 min	x1
Denaturation	95 °C	30 sec	x 35
Annealing	___ - __ °C *	30 sec	
Elongation	72 °C	1 min	
Final elongation	72 °C	5 min	x1
Storage	4 °C	∞	

* The Tm for both primers is 57

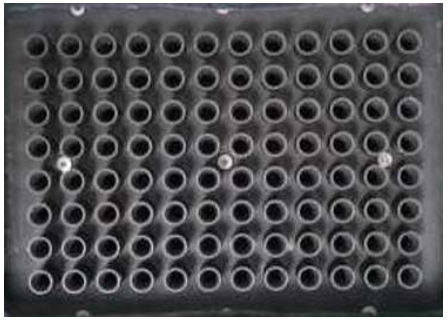
7. Try different 8 annealing temperatures depending on your primer pair Tm.
8. Set the final volume in the thermal cycler to be 12.5 µl.
9. Start PCR !!

How Thermal Cycler will control the temperature during temperature gradient PCR?

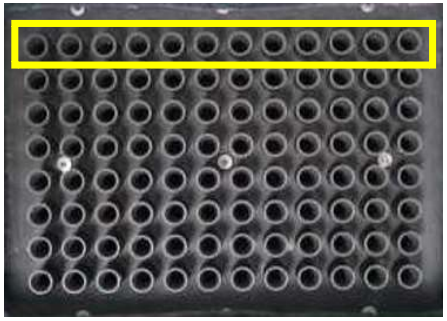
Initial denaturation
All the rows have the same temperature (94 -97 °C)



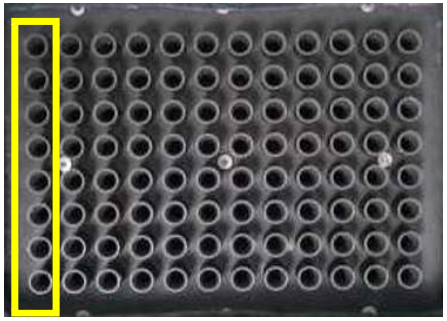
Denaturation
All the rows have the same temperature (94 -97 °C)



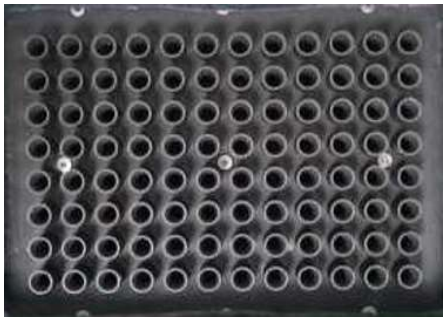
Annealing
Each raw/column will have different annealing



OR



Extension
All the rows have the same temperature (72 -80 °C)



Results:

- Analyse the results using 2% agarose gel, and determine the optimum Ta.

→ Known that the amplicon size is 350 bp, and the primer Tm is 60°C.

Method:

1. Start by applying the standard concentration of PCR component that work with majority of PCR reaction. Use the table to calculate the needed volume of each PCR component:

Components	Stock concentration	Final concentration	Volume per reaction (µl)
PCR buffer	10X	1X	
Taq polymerase	5 U/µl	0.05 U/µl	
dNTPs	10 mM	200 µM	
$MgCl_2$	25 mM	1.5 mM	
Forward primer	10 µM	0.4 µM	
Reverse primer	10 µM	0.4 µM	
DNA Template	45 ng/ µl	90 ng	
Water			
Total volume			**50 µl**

$C_1 \times V_1 = C_2 \times V_2$

Method:

2. Prepare a master mix that contains everything except the DNA template by multiplying the volume per reaction of each component by (number of desired reaction +1 for pipetting error):

Volume per reaction (µl)	Master mix (Volume per reaction x ….)
50 µl	**- µl**

Method:

3. Using special PCR tubes, distribute the master mix by pipetting --- µl to eah tube.
4. Add the DNA template for each template.
5. Centrifuge the tubes briefly.
6. Set the thermal cycling condition as following:

Step	Temperature	Duration	Cycle
Initial denaturation	94 °C	3 min	x1
Denaturation	94 °C	30 sec	x 25
Annealing	____ - ___ °C	30 sec	
Elongation	72 °C	30 sec	
Final elongation	72 °C	5 min	x1
Storage	4 °C	∞	

Method:

3. Try different 8 annealing temperatures depending on your primer pair Tm.
4. Set the final volume in the thermal cycler to be 50 µl.
5. Start PCR !!