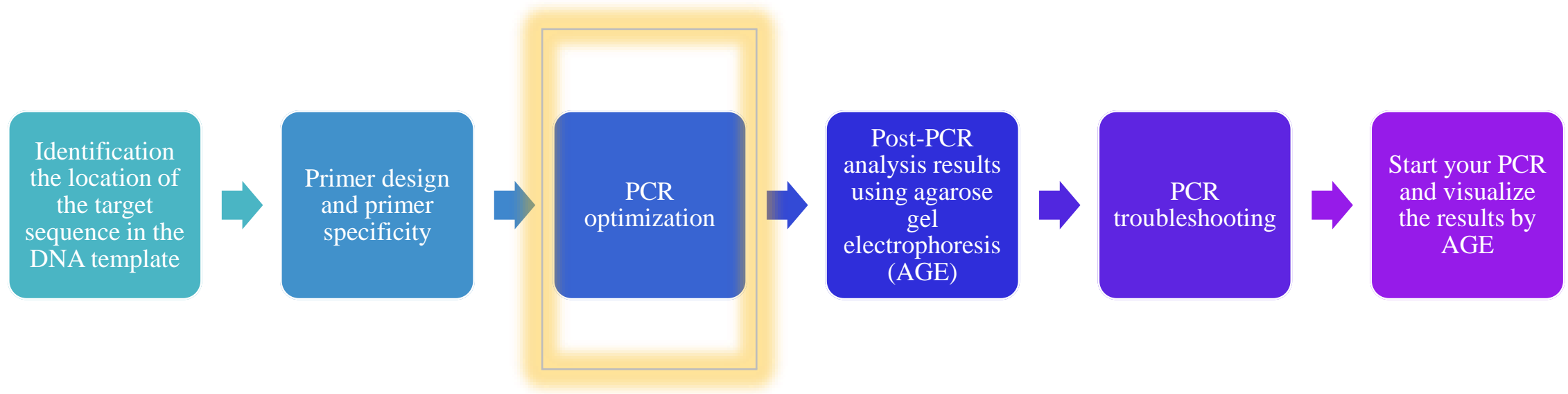


PCR- Optimization of Annealing Temperature

BCH361- Practical



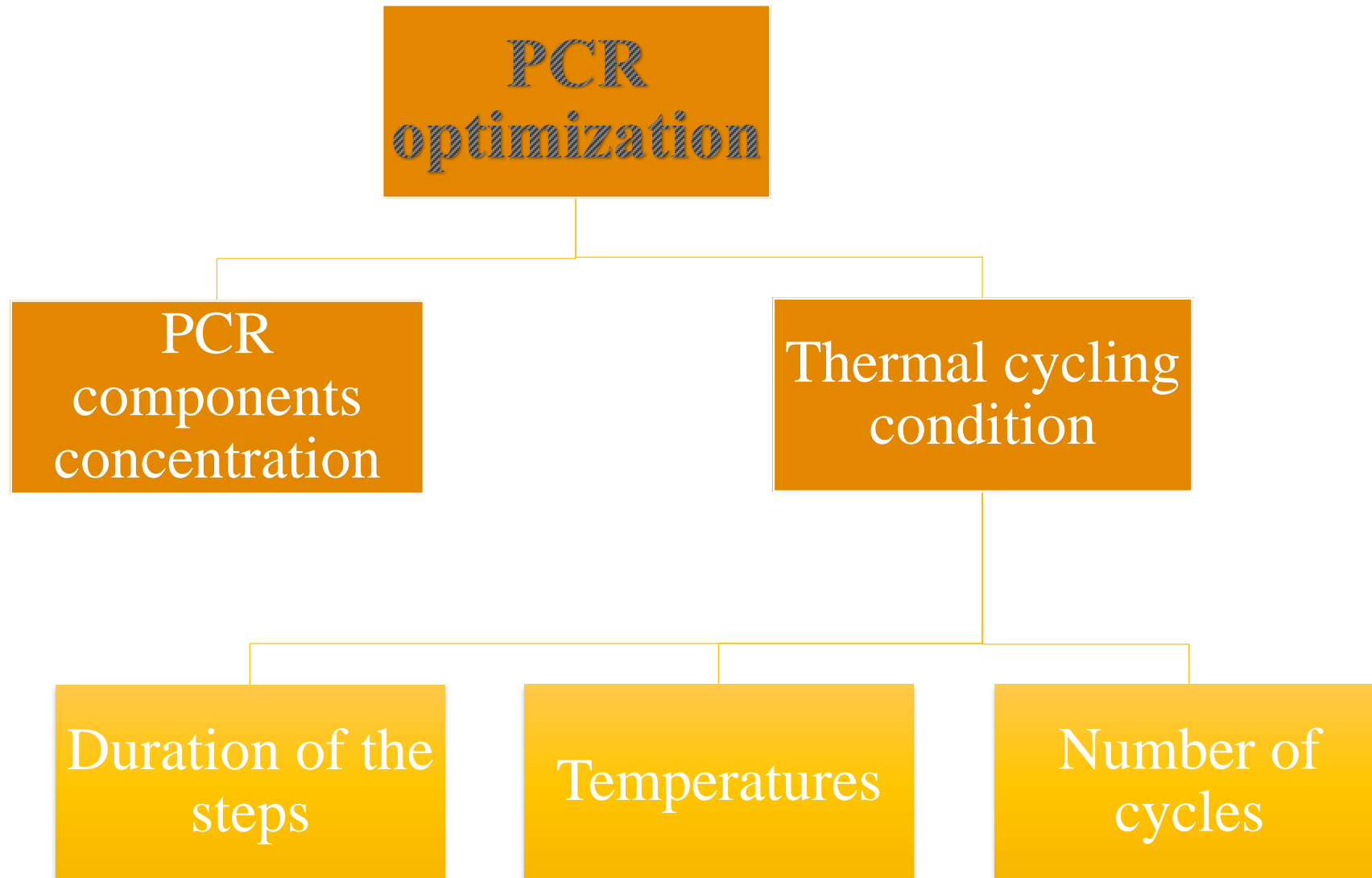
Performing PCR steps :



Next lab

PCR optimization:

- PCR optimization ?
- What if not?
- **REMEMBER:**
 - ➔ **There is no single set of conditions that is optimal for all PCR reactions. WHY?**



Standard concentrations of PCR components

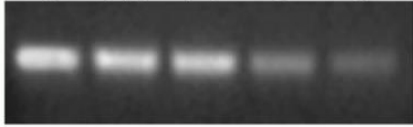
Component	Final concentration
Taq polymerase	0.5–2.0 units, ideally 1.25 units.
Deoxy-nucleotides (dNTPs)	Typical concentration is 200 μ M of each dNTP.
Magnesium Concentration	1.5-2.0 mM is optimal for Taq DNA Polymerase.*
Forward Primers	Typically 0.1-0.5 μ M .
Reverse Primer	Typically 0.1-0.5 μ M.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

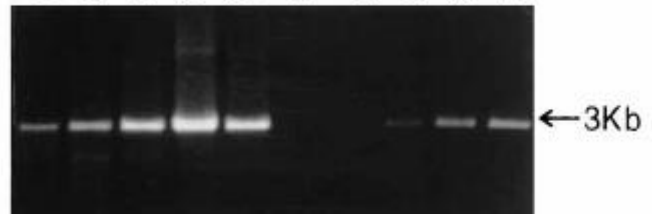
1 1.5 2 3 5 mM



Effect of [dNTP] (mM)

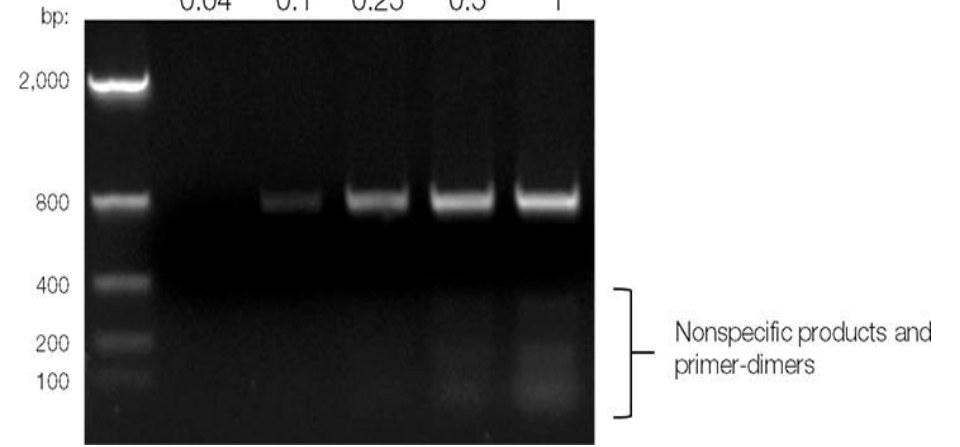
[Mg²⁺] = 2.5 mM [Mg²⁺] = 3.5 mM

0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.2 0.5 0.8



Primer concentration (μM, each)

0.04 0.1 0.25 0.5 1



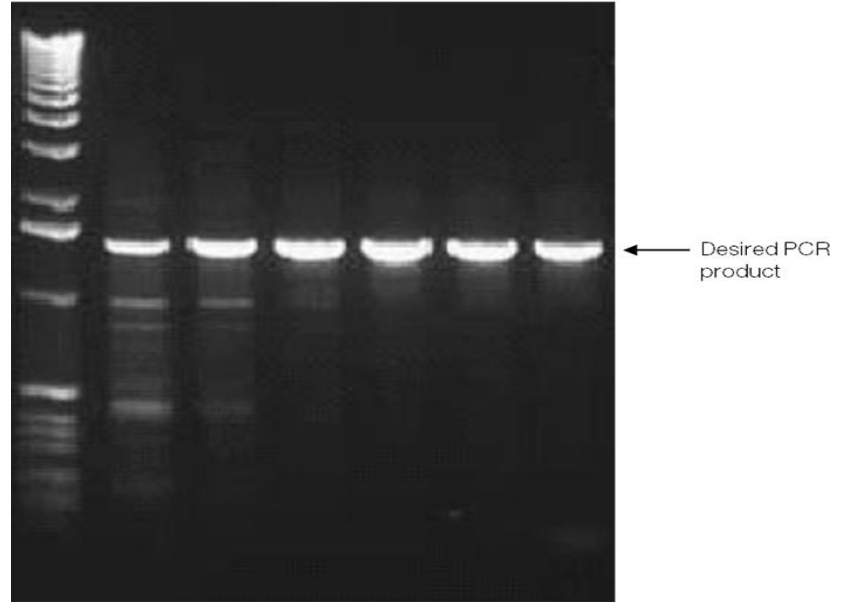
Extension time (sec):

**DNA polymerase
60 45 30 20 10**



Annealing temperature (°C)

50 52 54 56 58 60



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 (T_a):

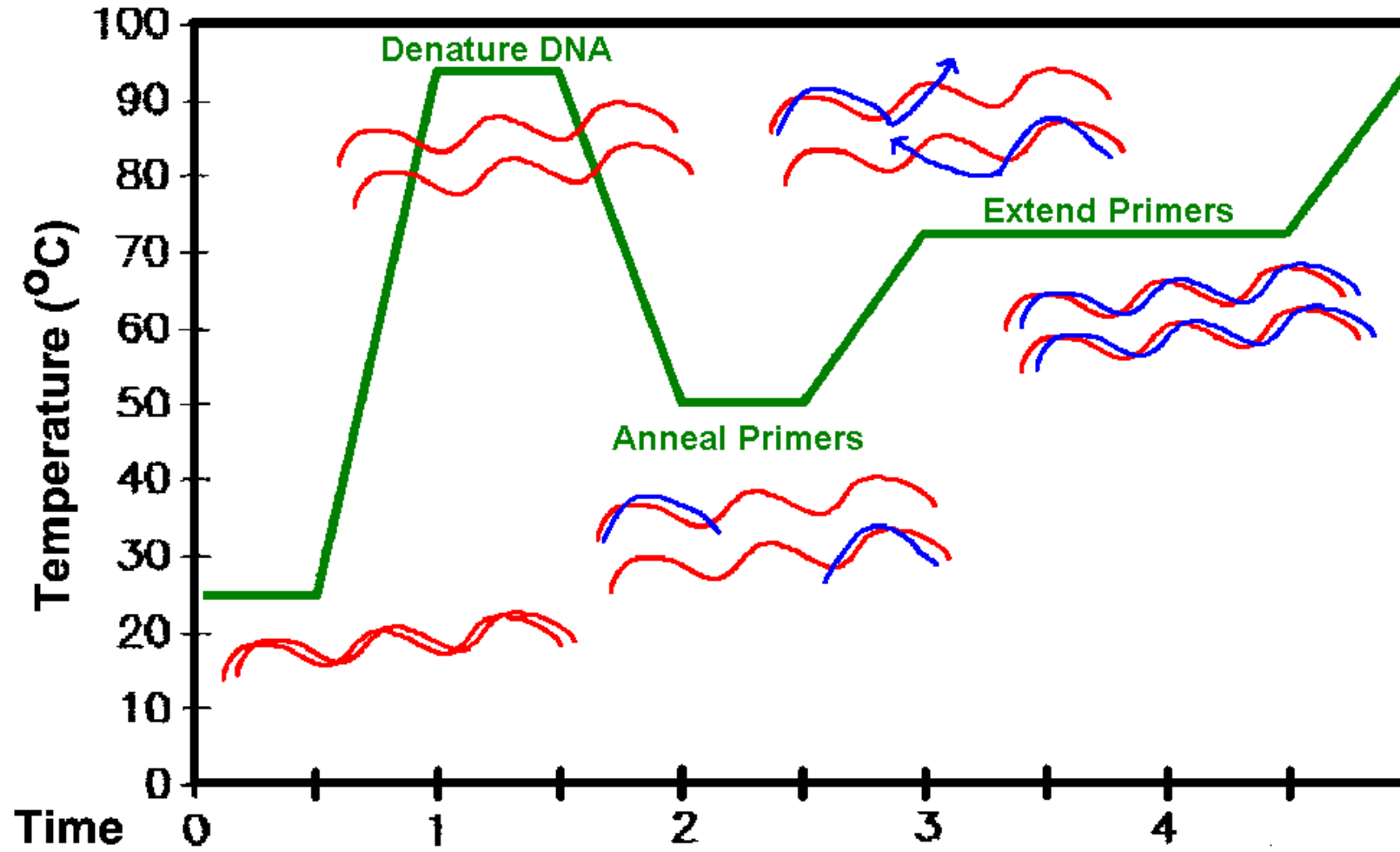
- When optimizing T_a what you should do with other PCR component?
 - ➔ Start by applying the standard concentration of PCR component that work with majority of PCR reaction.

Practical Part

Aim:

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

Principle:



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 each 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 T_m .
4. Set the final volume in the thermal cycler to be 50 μ l.
5. Start PCR !!

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

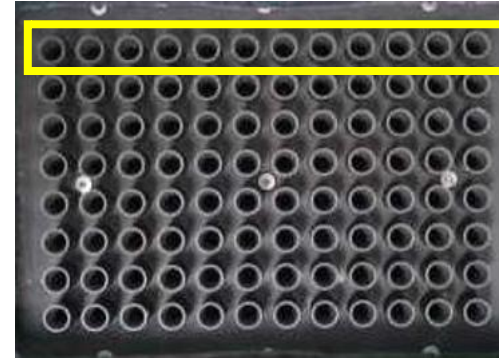
All the rows have the same temperature
(94 -97 °C)



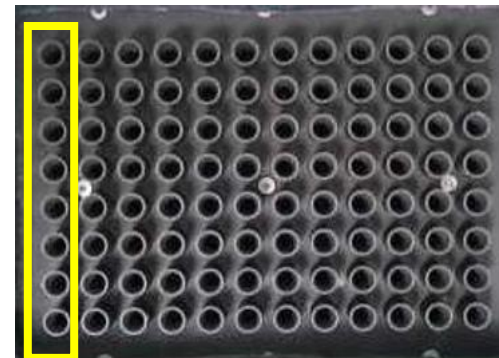
All the rows have the same temperature
(94 -97 °C)



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



OR



Results:

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