Experiment (6): Optimization of Annealing Temperature

Aim:

- To optimize different parameters that effects PCR results/performance.
- Optimization of PCR annealing temperature.
- Be familiar with PCR technique and thermal cycler device.

Introduction:

PCR optimization means to find the most effective and optimum conditions. Failure to amplify under optimum conditions can lead to the generation of multiple undefined and unwanted products, even to the exclusion of the desired product.[1] When developing a protocol for PCR amplification of a new target, it may be important to optimize all parameters including reagent concentrations, cycling temperatures, and cycle number.

PAUSE AND THINK ➔ There is no single set of conditions that is optimal for all PCR reactions. Why?

PCR Optimization :

In PCR optimization, you need to optimize:

(**1**) PCR components (reagents) concentration.
(**2**) Thermal cycling condition.

In this lab, we will briefly review the conditions that should optimized and the focus will be on the annealing temperature optimization.

1. PCR components concentration:

Optimization of PCR reagents aim to find out the most optimum concentration of all PCR component including, primer concentration, $MgCl_2$, DNA template…etc. It is important to note that while optimization of one parameter, other parameters should be fixed and not changed (one factor at a time). Generally, the concentration of PCR components should be as shown in the **table.1**.

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

Table.1. Standard concentrations of PCR components.

Component	Concentration
Taq polymerase	0.5–2.0 units, ideally 1.25 units.
dNTPs	Typical concentration is 200 µM of each dNTP.
Magnesium	1.5-2.0 mM is optimal for Taq DNA Polymerase.
Forward and reverse primers	Typically 0.1-0.5 µM of each primer.
DNA Template	1ng–1µg of genomic templates.

2. Thermal cycling condition:

Optimization of thermal cycling condition aims to reach optimum cycling temperatures, duration of each step in PCR and number of cycles. Sitting up the thermal cycling conditions is divided to three stages. **First stages** is initial denaturation, a typical reaction will start with a three minutes denaturation at 94-97°C, this stages aim to denature the template and activate “hot-start” DNA polymerase. **Next stage** is the three PCR steps (denaturation, annealing and elongation), which will repeated from 25 to 35 cycles. **Last stage** is the final elongation phase; a period of 5 minutes or longer to allows synthesis of many uncompleted amplicons to finish. **Table.2** shows the general PCR thermal cycling condition.

Table.2. General PCR thermal cycling condition.

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

* Depend on the primer annealing temperature.
◊ Depend on DNA polymerase optimum temperature.

From all, optimizing the annealing temperature of your PCR assay is one of the most critical parameters for reaction specificity.

Optimization of annealing temperature (Ta):

The purity yield of the reaction products depend on several parameters, one of which is the 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. [2] 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. For example if your primer Tm is 58 °C, you will start from 53 °C and you will increase the temperature for 8 degree, so the Ta is often fall in the range of 53 - 60 °C.

PAUSE AND THINK ➔ When optimizing Ta what you should do with other PCR component?

Materials:

PCR buffer, DNA Taq polymerase, dNTPs, $MgCl_2$, primers, DNA template, Nuclease free water.

Protocol:

1. Start by applying the standard concentration of PCR component that work with majority of PCR reaction. Use the below 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**

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**

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	∞	

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

Results:

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

References:

1. Roux KH. Optimization and troubleshooting in PCR. PCR Methods Appl, 1995;5:185-94.
2. Rychlik W, Spencer WJ, Rhoads R. Optimization of the annealing temperature for DNA amplification in vitro. Nucleic Acids Res, 1990;21: 6409–12.