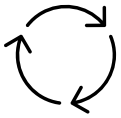
Polymerase Chain Reaction (PCR)

Supporting information for genetics module

This supporting information links to the case study research of crop varieties, including legumes (pea family). The steps below are general in nature to provide students with additional information as to how PCR works, and the components involved in this technology.

Background



Polymerase chain reaction (PCR) is a powerful, efficient and cost-effective way to copy or "amplify" small segments of DNA or RNA. The vast majority of PCR methods rely on 'thermal cycling', which involves exposing the reactants to cycles of repeated heating and cooling, permitting different temperature-dependent reactions—specifically, DNA melting and enzyme-driven DNA replication—to quickly proceed many times in sequence.

Primers (short DNA fragments) containing sequences complementary to the target region, along with a DNA polymerase, after which the method is named, enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the original DNA template is exponentially amplified. The simplicity of the basic principle underlying PCR means it can be extensively modified to perform a wide array of genetic manipulations.

- **Suggestion:** Show the **introductory PCR video –** find at the top of this page (Source: Genetics Learning Centre, US), which outlines the key process of PCR via an interactive video/slideshow.
    - Engaging & digestible content via animations.
        - Key concepts of PCR introduced, plus key terms.
        - Note: Video with slide-deck format (needs to be prompted to play next slide).
        - Interactive components throughout (dragging/ clicking). Opportunity for engagement from class or in small groups.

Protocol

The following shows the range components mixed for the PCR process in the crop research. More information the components are outlined later in this booklet (see Further Information – PCR Components).

Reaction setup:

Component	50 µl reaction	Final Concentration	X4
Template DNA	5 µl	<1,000 ng	
5x Standard *Taq* Reaction Buffer	10 µl	1x	
Magnesium Chloride ($MgCl_2$)	2 µl	400 µM	
10 mM dNTPs	1 µl	200 µM	
10 µM Forward Primer	1 µl	0.2 µM (0.05–1 µM)	
10 µM Reverse Primer	1 µl	0.2 µM (0.05–1 µM)	
Taq DNA Polymerase	0.2 µl	1.2 units/50 µl PCR	
Nuclease-free water	29.8 µl		

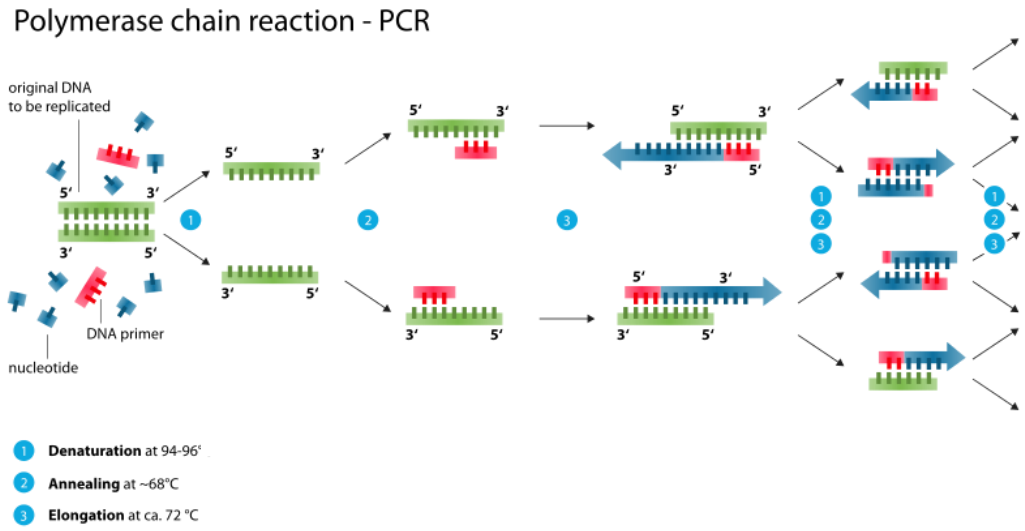
Protocol Notes:

- **Outline of combining & preparation:** All reaction components are combined in the correct quantities (outlined above), kept cool (on ice), with genetic material added & the combined mixture is transferred to the thermocycling PCR machine once ready.
- **Process:** Reaction mixture is added to each tube first, then sample DNA is added (from specific sample – e.g., an individual sample of a pea variety).
- **Centrifuges** are used throughout these processes for thorough mixing.
- Finally, transfer PCR tubes from being cooled on ice to a PCR machine once time for PCR process.
- **PCR Thermocycling:** The thermocycling (changing of temperatures to specific levels & stages of the PCR process) are outlined in the introductory PCR video/slideshow (source: Genetic Science Learning Centre).

Thermocycling conditions for a routine PCR

Specific to the pea variety (legume crop) that was prepared for PCR thermocycling, the below conditions were used. These can be adapted as required:

STEP	TEMP	TIME
Initial Denaturation	95°C	5 mins
40 Cycles	95°C 55-60°C 72°C	45 seconds 45 seconds 1 minute/kb
Final Extension	72°C	5 minutes
Hold	12°C	∞



The introductory animation provides an overview of the PCR process & steps in an engaging format. For further details on the general method, see below:

The individual steps common to most PCR methods are as follows:

- **Initialization**: This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96 °C.

- Denaturation: This step is the first regular cycling event and consists of heating the reaction chamber to 94–96 °C for 45 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.

- Annealing: In the next step, the reaction temperature is lowered to 55–65 °C for 45 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3' end of each strand. It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind *only* to a perfectly complementary part of the strand, and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. During this step, the polymerase binds to the primer-template hybrid and begins DNA formation.

- Extension/elongation: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for Taq polymerase is approximately 75–80 °C, although a temperature of 72 °C is commonly used with this enzyme. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that are complementary to the template in the 5'-to-3' direction. The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. As a general rule-of-thumb, at their optimal temperature, most DNA polymerases polymerize a thousand bases per minute. Under optimal conditions, at each extension/elongation step, the number of DNA target sequences is doubled. With each successive cycle, the original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential amplification of the specific DNA target region. The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies.

- Final elongation: This single step is optional but is performed at a temperature of 70–74 ° (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.

- Final hold: The final step cools the reaction chamber to 4–15 °C for an indefinite time and may be employed for short-term storage of the PCR products.

Further Information - PCR Components:

The polymerase chain reaction (PCR) was developed to mimic nature's own way of replicating DNA. DNA is a repeating sequence of nucleotides, and each nucleotide contains three parts. The backbone of DNA is a repeating sugar and phosphate unit, and each sugar has a nitrogenous base attached to it. There are four nitrogenous bases; guanine, cytosine, adenine and thymine. DNA consists of two

sugar phosphate strands running parallel to one another with two nitrogenous bases joining in between every two sugars. These nucleotide bases are required to be present in the PCR mixture to grow the DNA chains.

Magnesium Chloride

Thermostable polymerase requires the presence of magnesium to act as a cofactor during the reaction process. Its role is similar to that of a catalyst: the magnesium is not actually consumed in the reaction, but the reaction cannot proceed without the presence of the magnesium. Essentially, Mg^{2+}increases the Tm of two interacting DNA strands by shielding the negatively charged phosphodiester backbone and decreasing the electrostatic repulsion between DNA strands, i.e., it stabilizes base pairing. This way, primers that might not bind stably to a certain sequence under "normal" conditions -due to mis-paired bases- may do so in the presence of higher Mg^{2+} ion concentrations.

dNTPs

The purpose of the deoxynucleotide triphosphates (dNTPs) is to supply the “bricks.” Since the idea behind PCR is to synthesize a virtually unlimited amount of a specific stretch of double-stranded DNA, the individual DNA bases must be supplied to the polymerase enzyme.

PCR buffer

This is necessary to create optimal conditions for activity of Taq DNA polymerase. Buffers often contain Tris-HCl, KCl, and sometimes $MgCl_2$.

Taq polymerase

It is isolated from a heat-loving bacterium that is naturally found in hot springs, so the enzyme doesn't break down at the high temperatures necessary for copying DNA using a polymerase chain reaction. At high temperatures, Taq polymerase attaches nucleotides to a DNA template, thereby copying the DNA.

Primers

DNA sequencing is used to determine the nucleotides in a DNA strand. The Sanger chain termination method of sequencing uses a primer to start the chain reaction. In PCR, primers are used to determine the DNA fragment to be amplified by the PCR process.