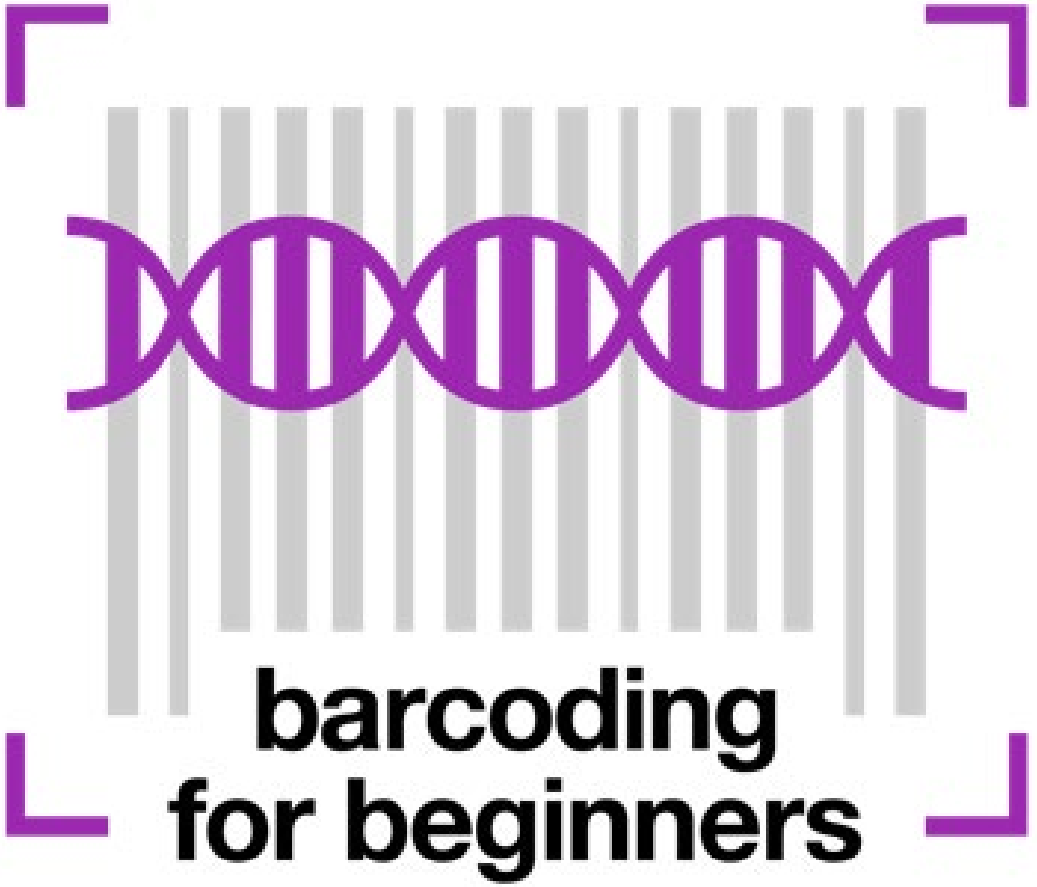
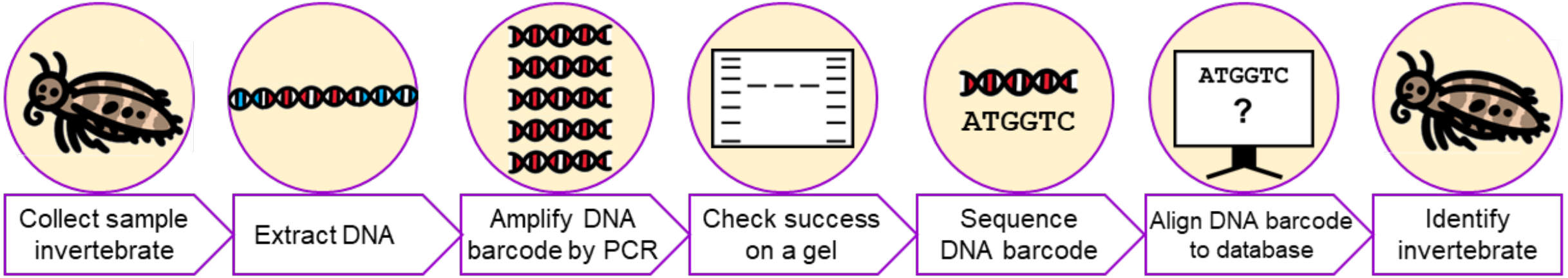
# DNA barcoding



# Overview of practical activities

After lunch you will be applying your skills in micropipetting and gel electrophoresis to extract DNA from invertebrates and amplify the DNA barcode by PCR.

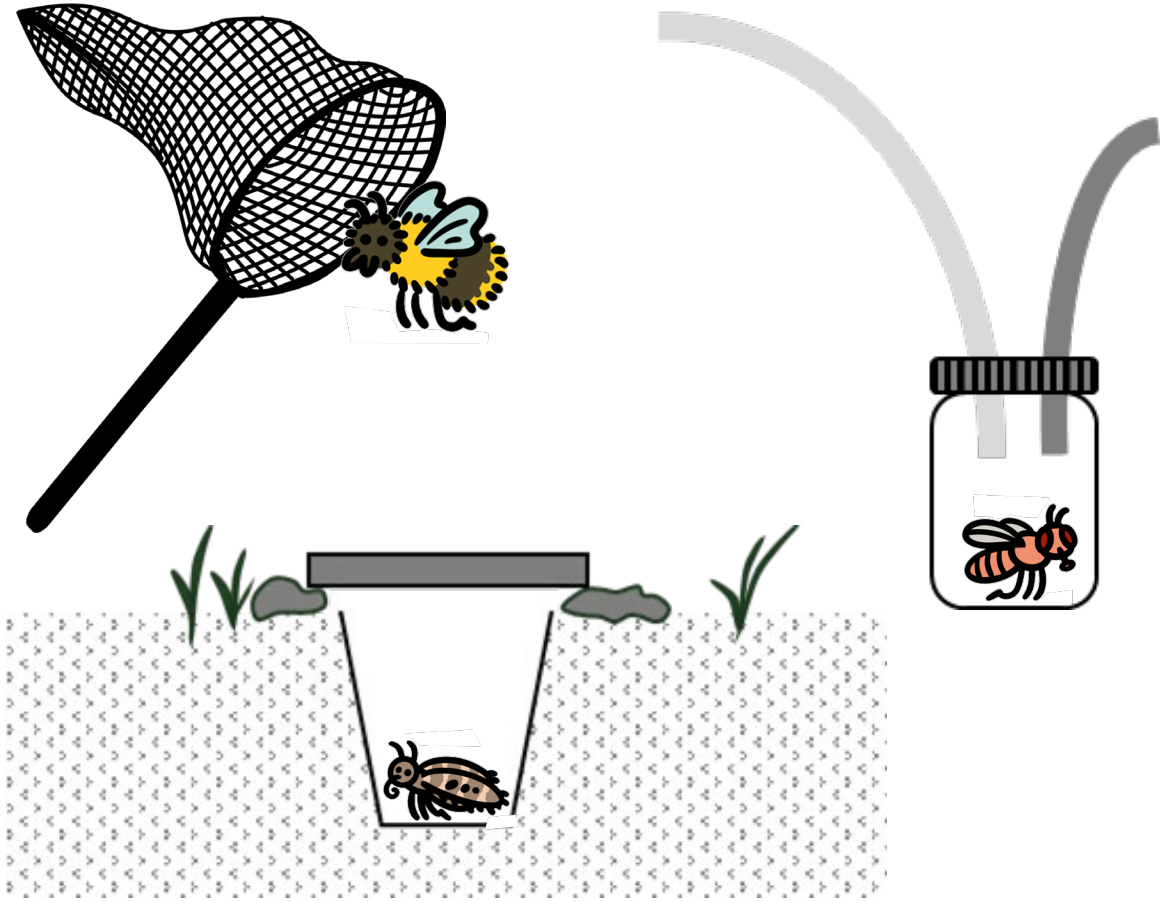


# Collect invertebrate sample

# Collect invertebrate sample

Choose the invertebrate that you wish to use in your DNA barcoding.

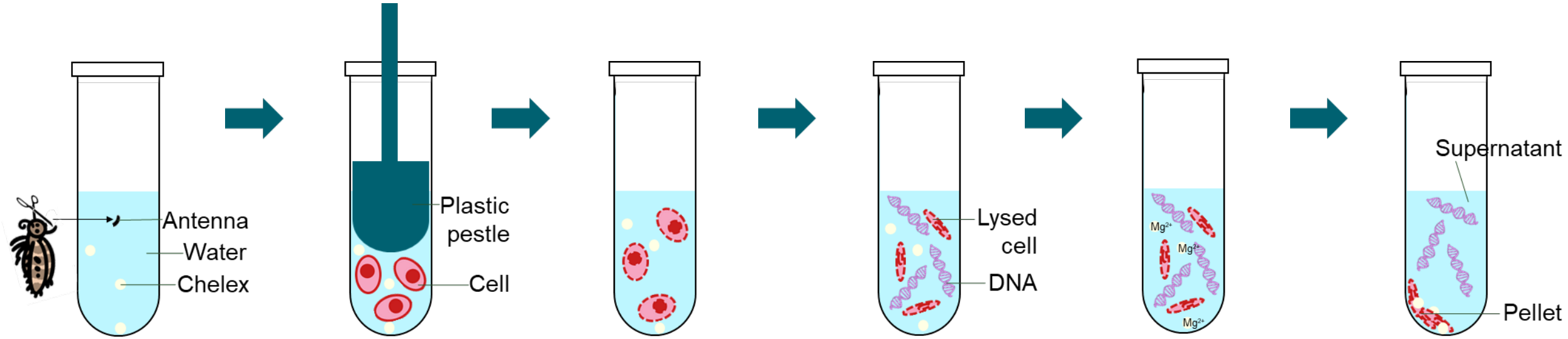
These are freshly deceased invertebrates from windowsills. They have been stored in a Ziplock bag in the freezer (at -20°C).



# Extracting DNA

# Extracting DNA

An overview of the stages of DNA extraction from an invertebrate sample:




Add part of invertebrate to Chelex

Grind to disrupt tissue into single cells

Heat in alkaline Chelex to disrupt cell membranes

Cells lyse, DNA released

Chelex binds $Mg^{2+}$, stopping nucleases breaking down DNA

Centrifugation: cell debris in pellet with Chelex, DNA in supernatant

# Extracting DNA

To avoid liquid evaporating from the microfuge tube when heating:

- Close the lid firmly
- Stretch waterproof Parafilm around the top

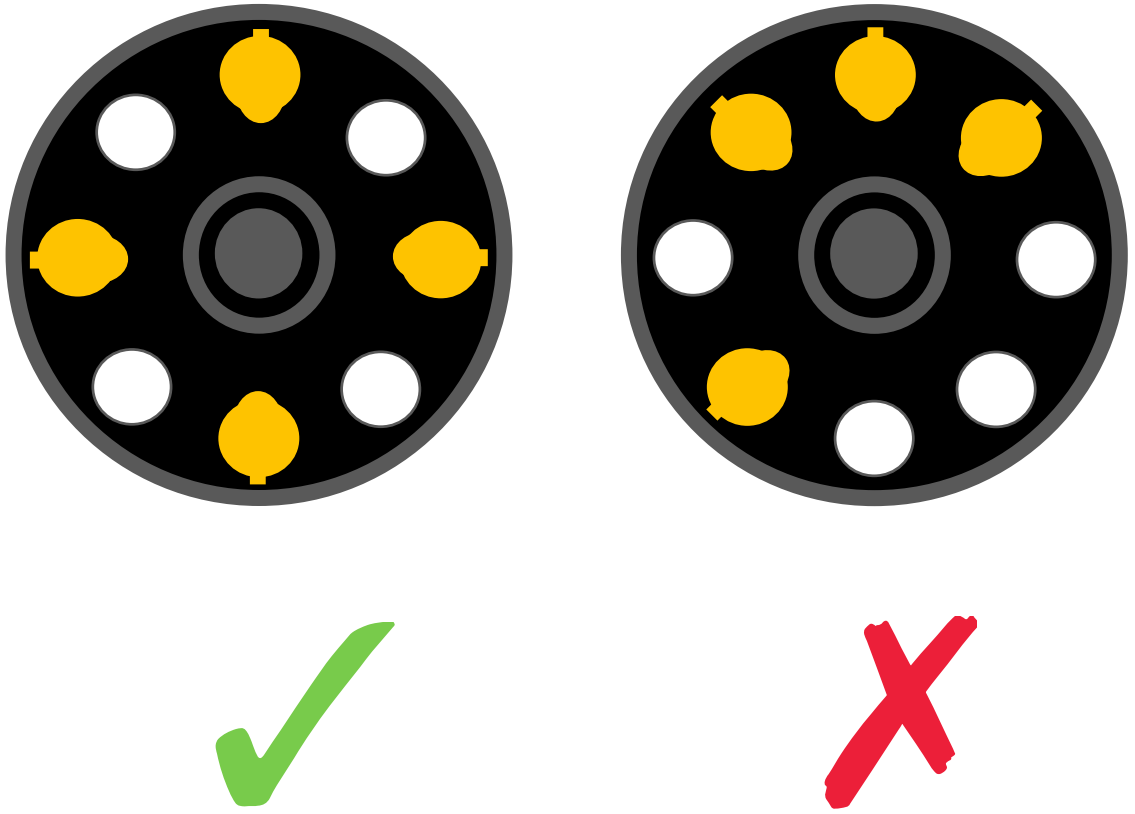
Take care not to touch the heating block as it is very hot and can cause burns

# Extracting DNA

Centrifugation spins the samples at a high speed (13,000 revolutions per minute / rpm).

If the rotor is unbalanced when spinning this fast, it will become misshapen.

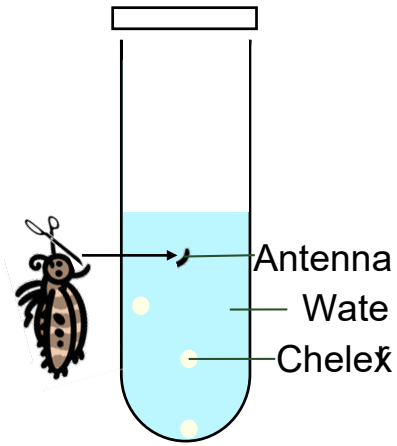
- Make sure that the rotor is balanced before starting centrifugation



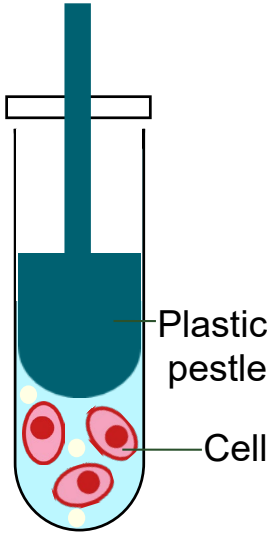
# Extracting DNA

## TOP TIPS

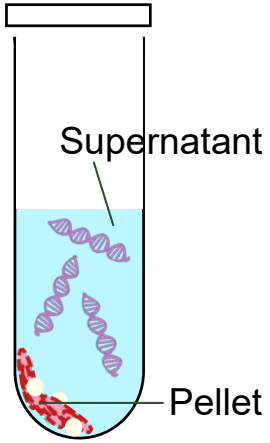
- Use a small amount of sample




- Thoroughly grind the tissue




- Make sure Chelex is not transferred after centrifugation

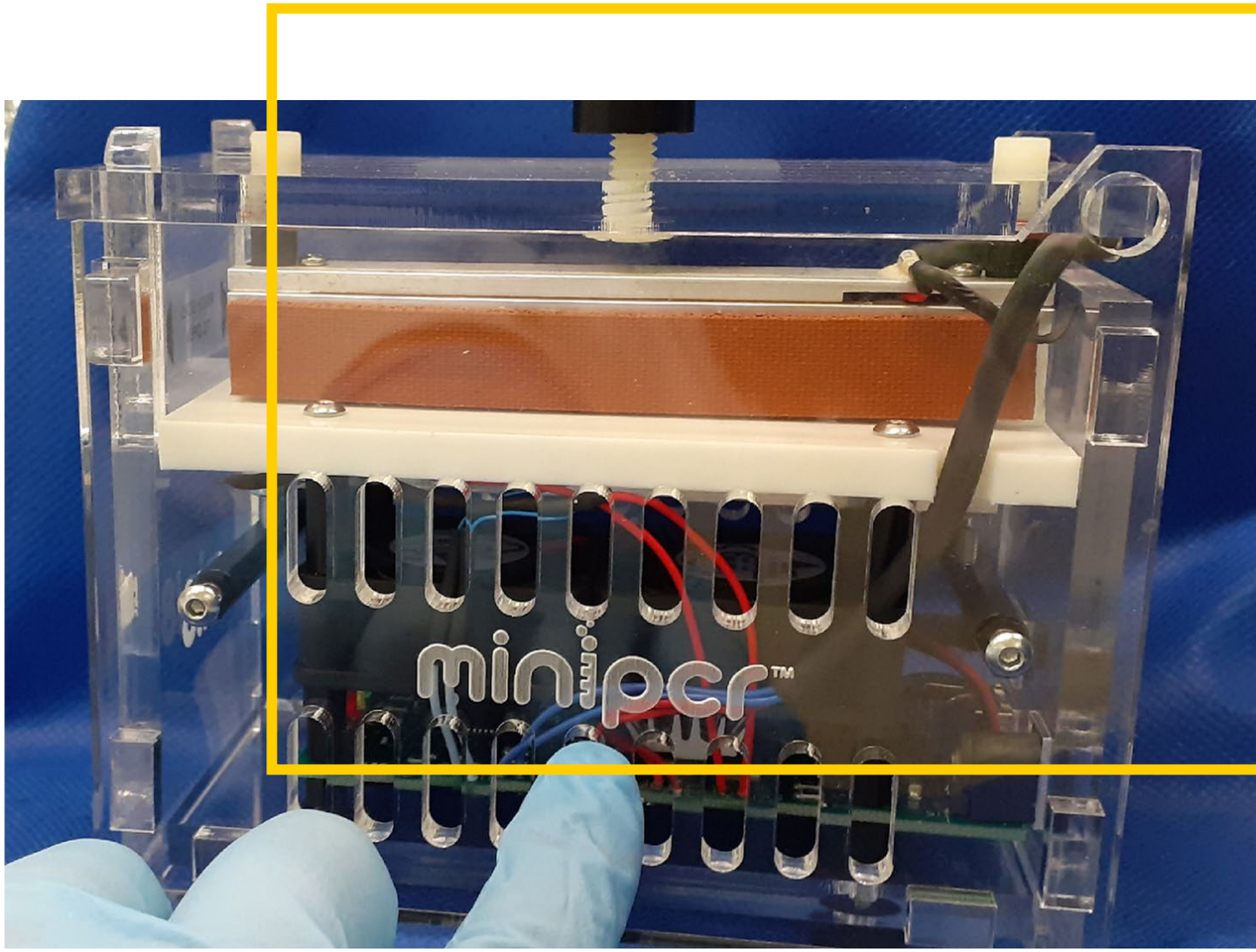


# Extracting DNA

## Health and Safety

- No chemical substances are classed as hazardous
- Wear gloves to avoid contaminating the sample with your DNA
- Take care when working with heating block to avoid burns

# PCR



# Explaining PCR

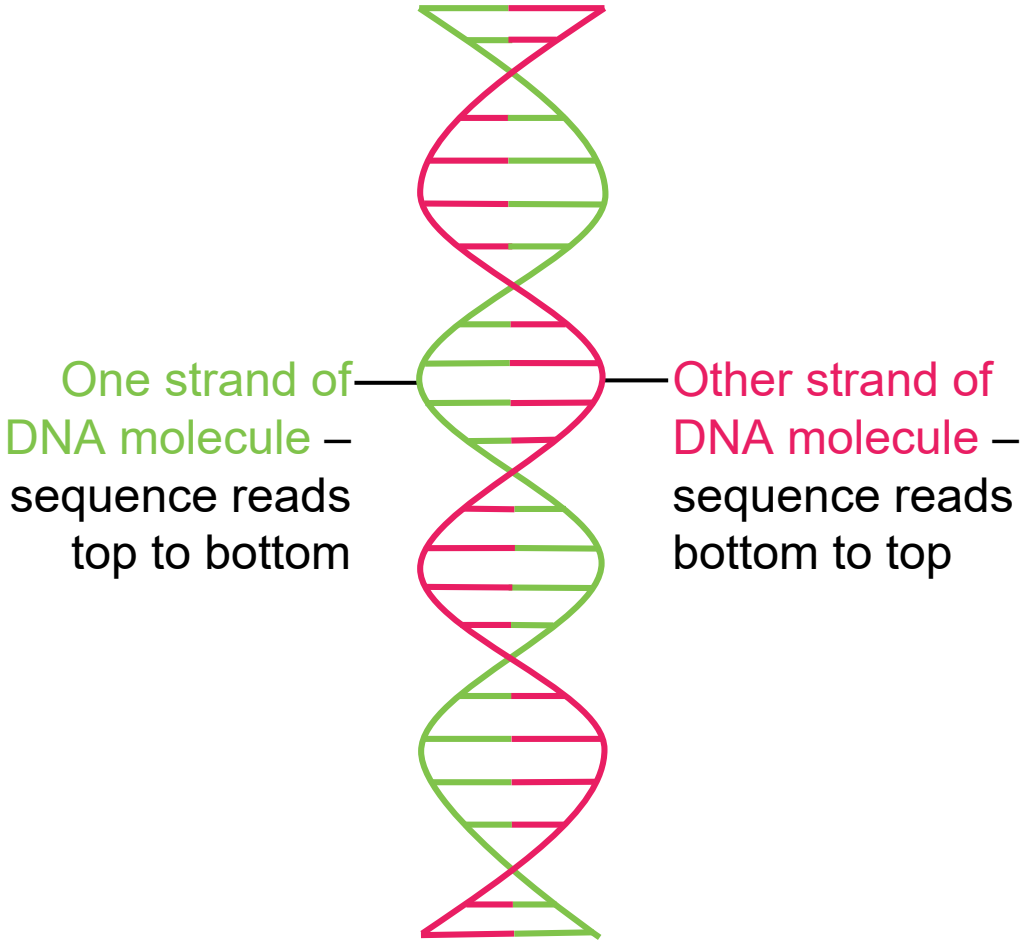
**PCR** stands for **P**olymerase **C**hain **R**eaction

A PCR is able to make copies of DNA, much as a photocopier makes copies of an image.

To understand how PCR works you need to know the basic structure of DNA.

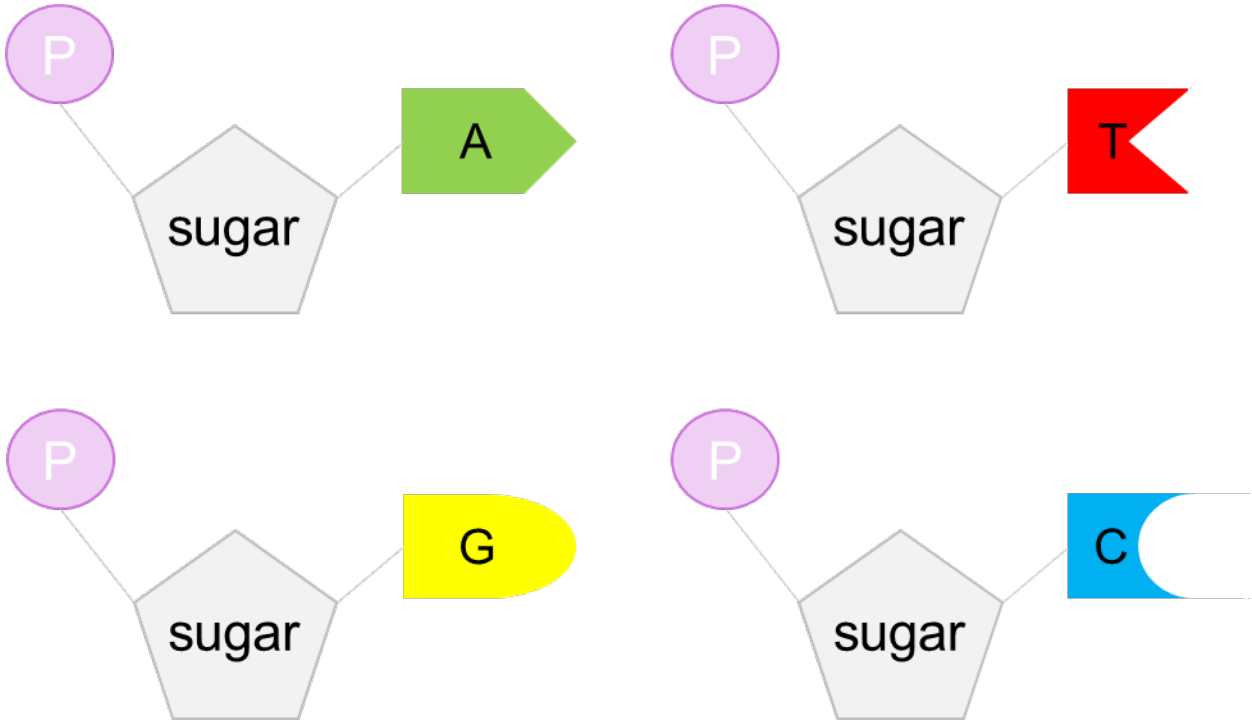
# Explaining PCR

- DNA is a polymer made of 2, antiparallel strands



# Explaining PCR

- DNA is a polymer made of 2, antiparallel strands
- Nucleotides are the monomers which make up the DNA polymer



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- DNA is a polymer made of 2, antiparallel strands
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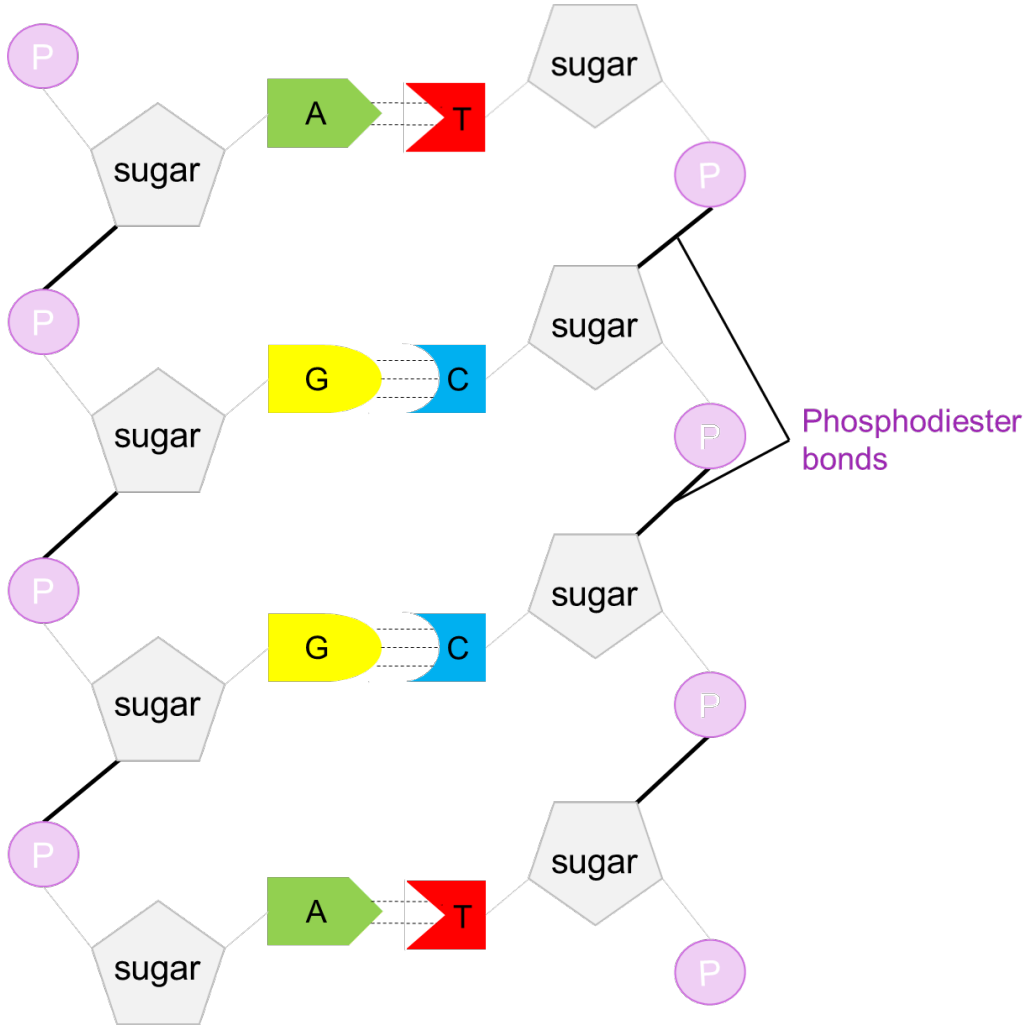
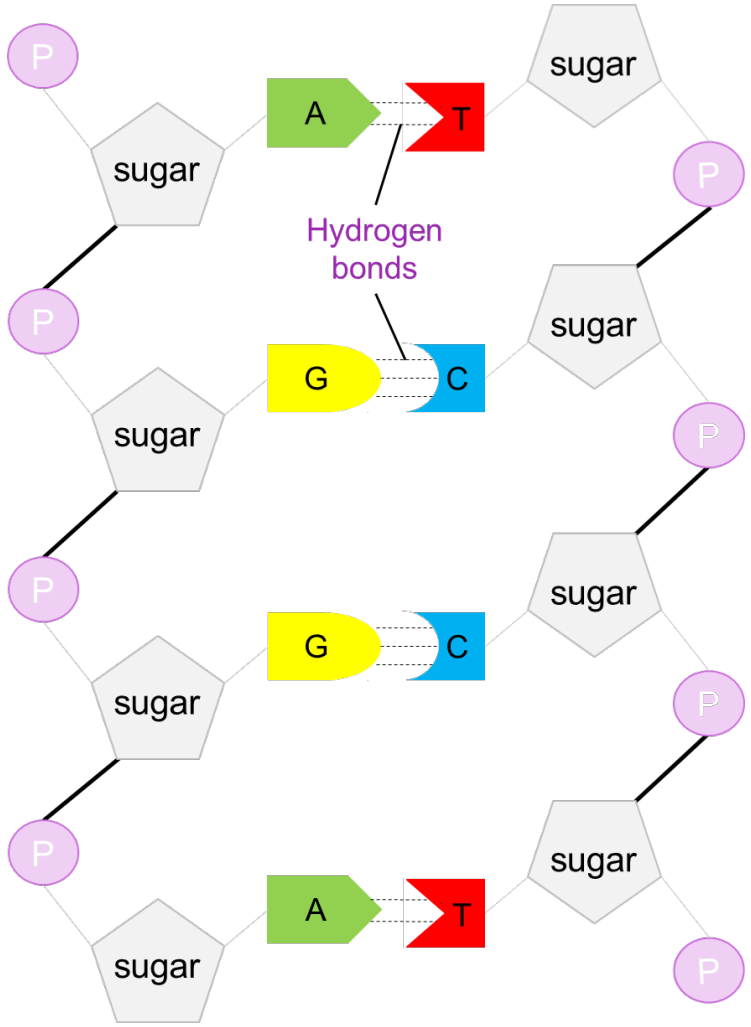
- DNA has a sugar phosphate backbone, with nucleotides joined by phosphodiester bonds



# Explaining PCR

- DNA is a polymer made of 2, antiparallel strands
- Nucleotides are the monomers which make up the DNA polymer
- DNA has a sugar phosphate backbone, with nucleotides joined by phosphodiester bonds
- Nucleotides from antiparallel strands form complementary base pairs (Adenine with Thymine, Cytosine with Guanine), joined by hydrogen bonds



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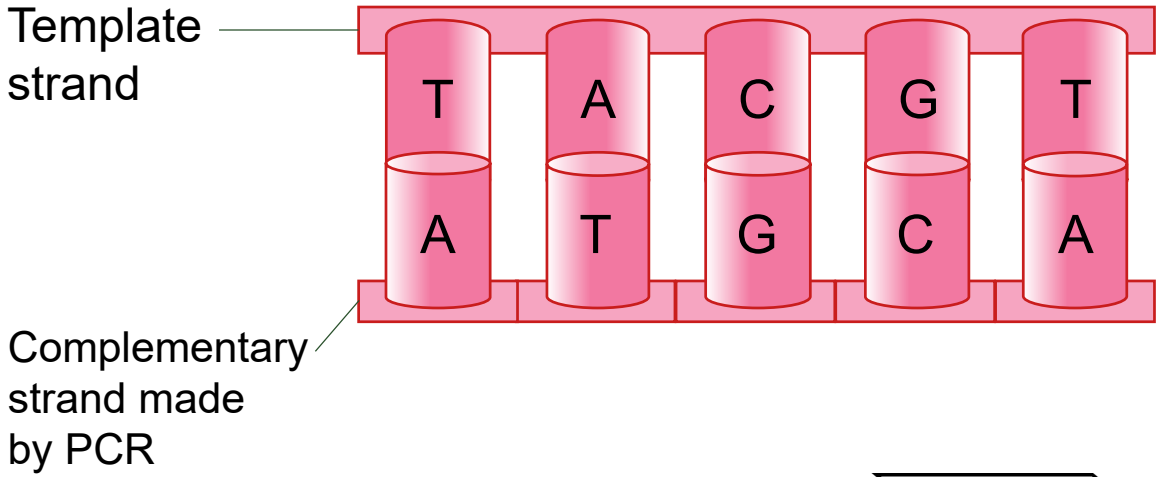


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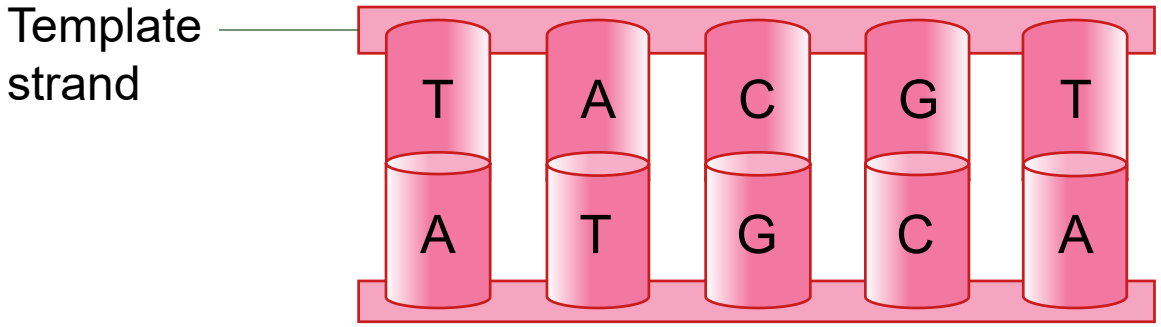


Template strand

# Explaining PCR

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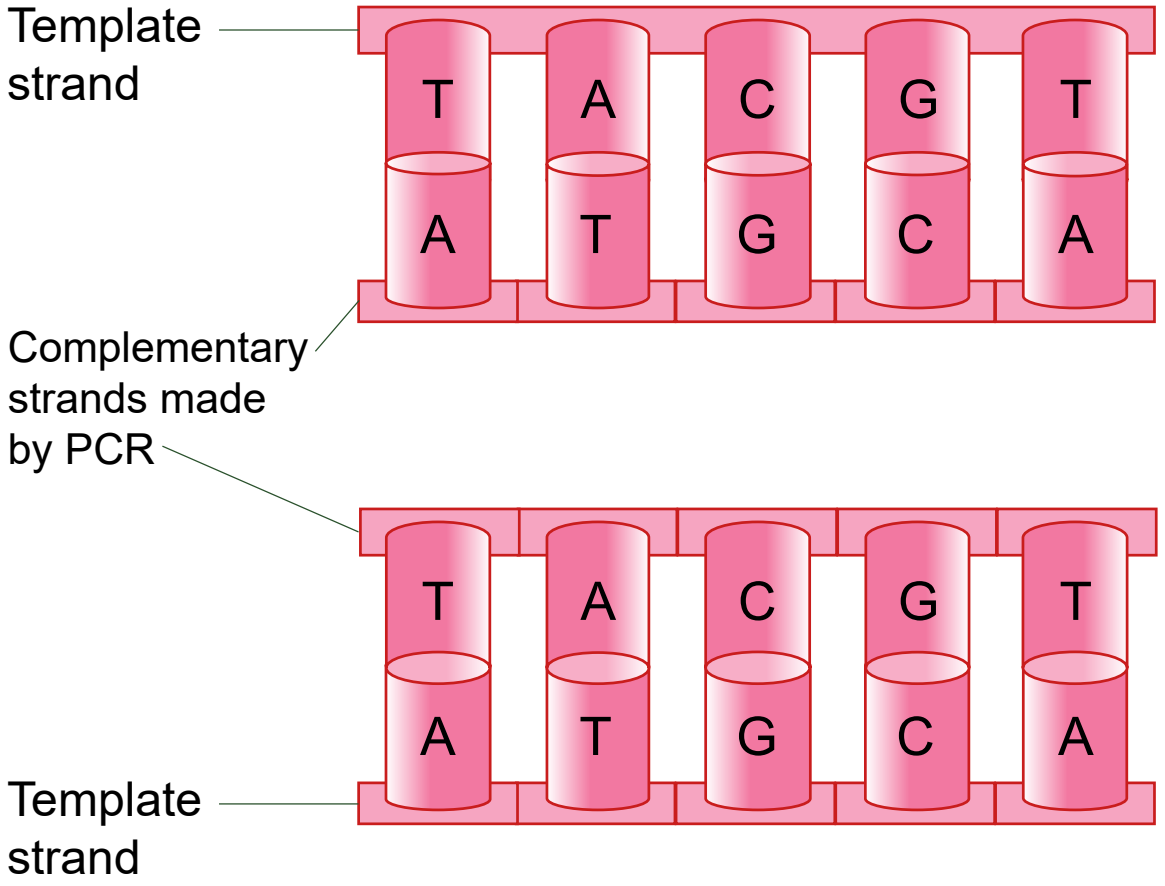
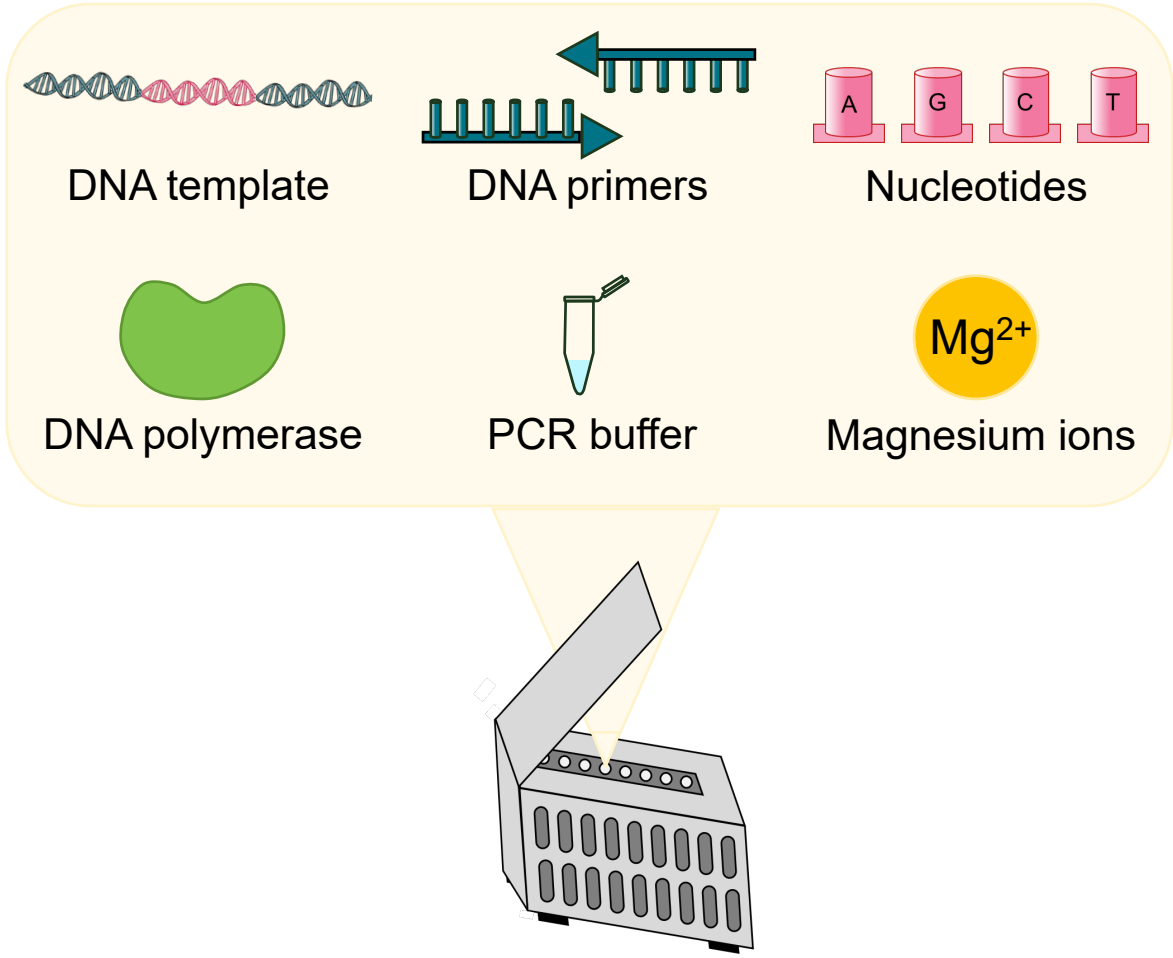


# Explaining PCR

**PCR** stands for **P**olymerase **C**hain **R**eaction

To amplify a region of DNA, a PCR must include:

- DNA template
- DNA primers
- DNA polymerase
- nucleotides
- PCR buffer
- magnesium ions

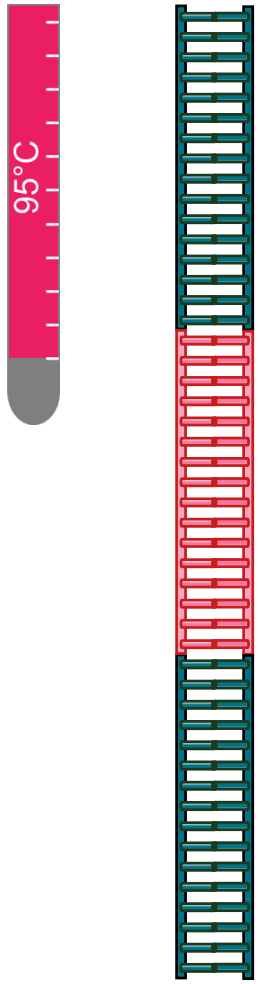


# Explaining PCR

**PCR** stands for **P**olymerase **C**hain **R**eaction

Copying DNA occurs in 3 stages:

- Denaturation
- Annealing
- Extension




**Denaturation**

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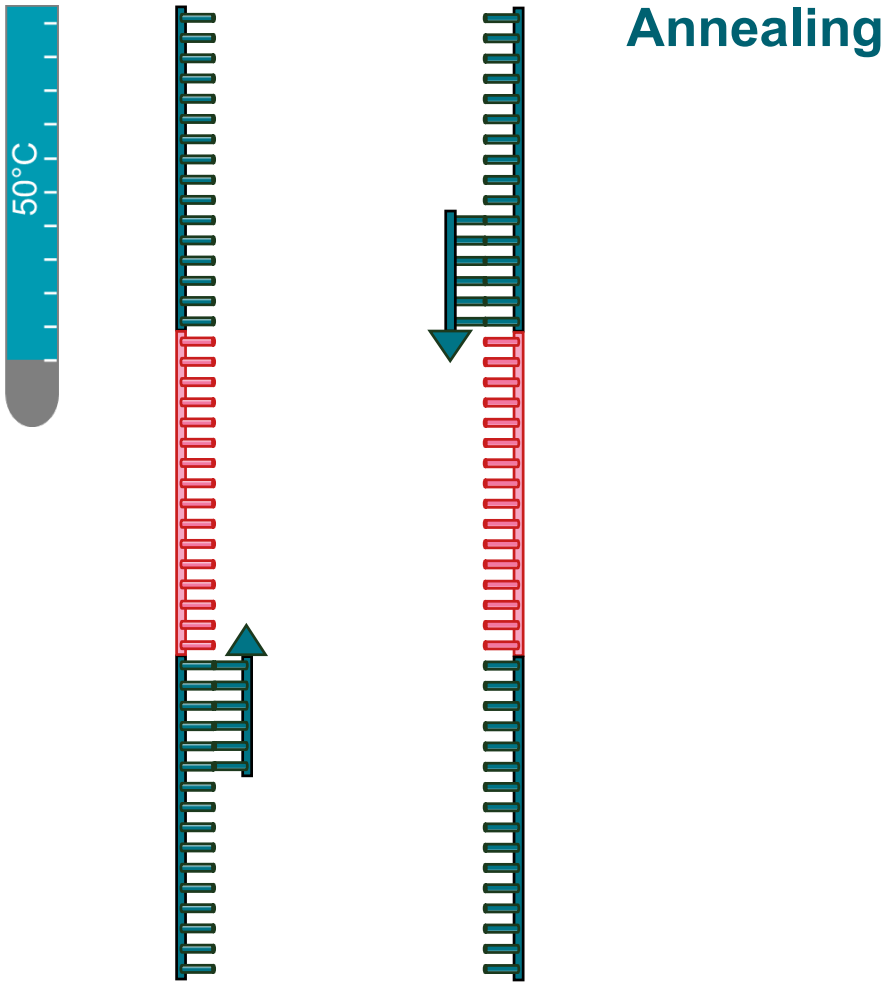

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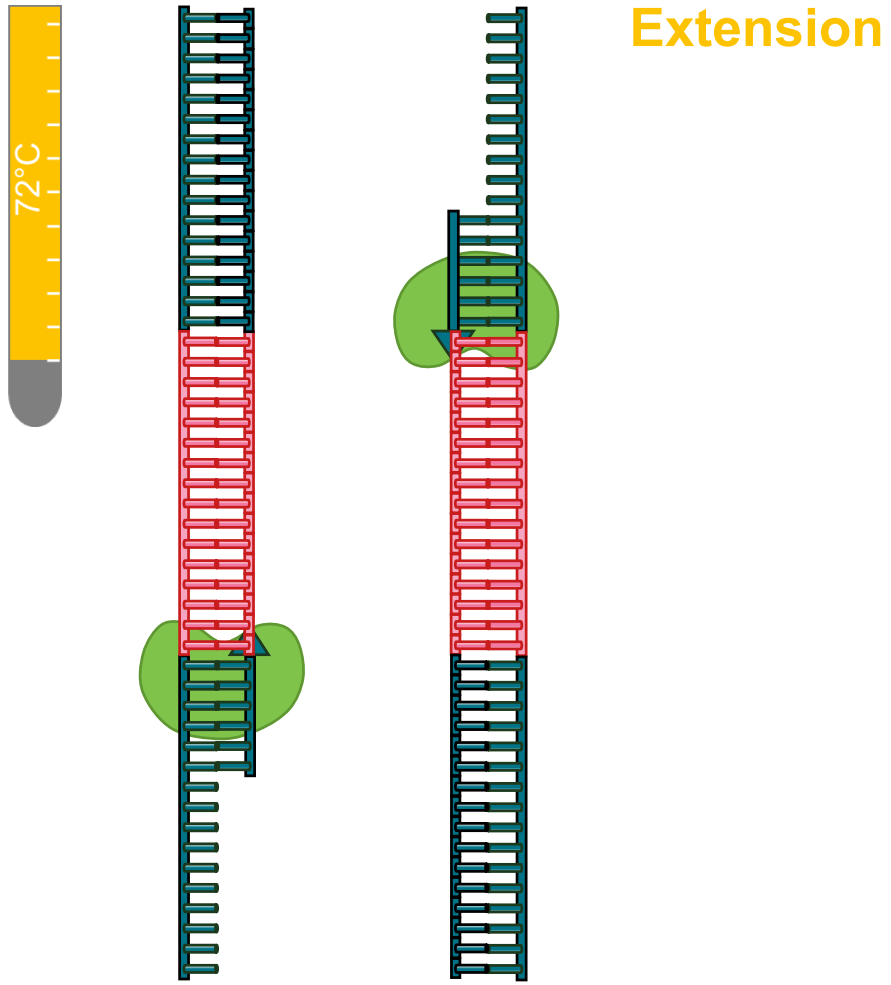


# Explaining PCR

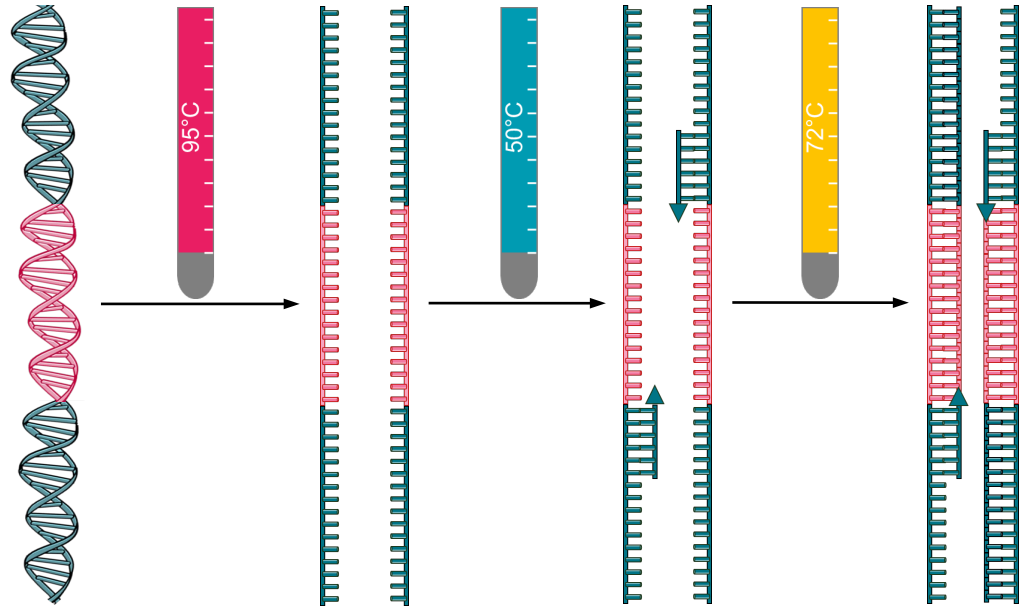
**First PCR cycle**

The double-stranded DNA is heated and denatures

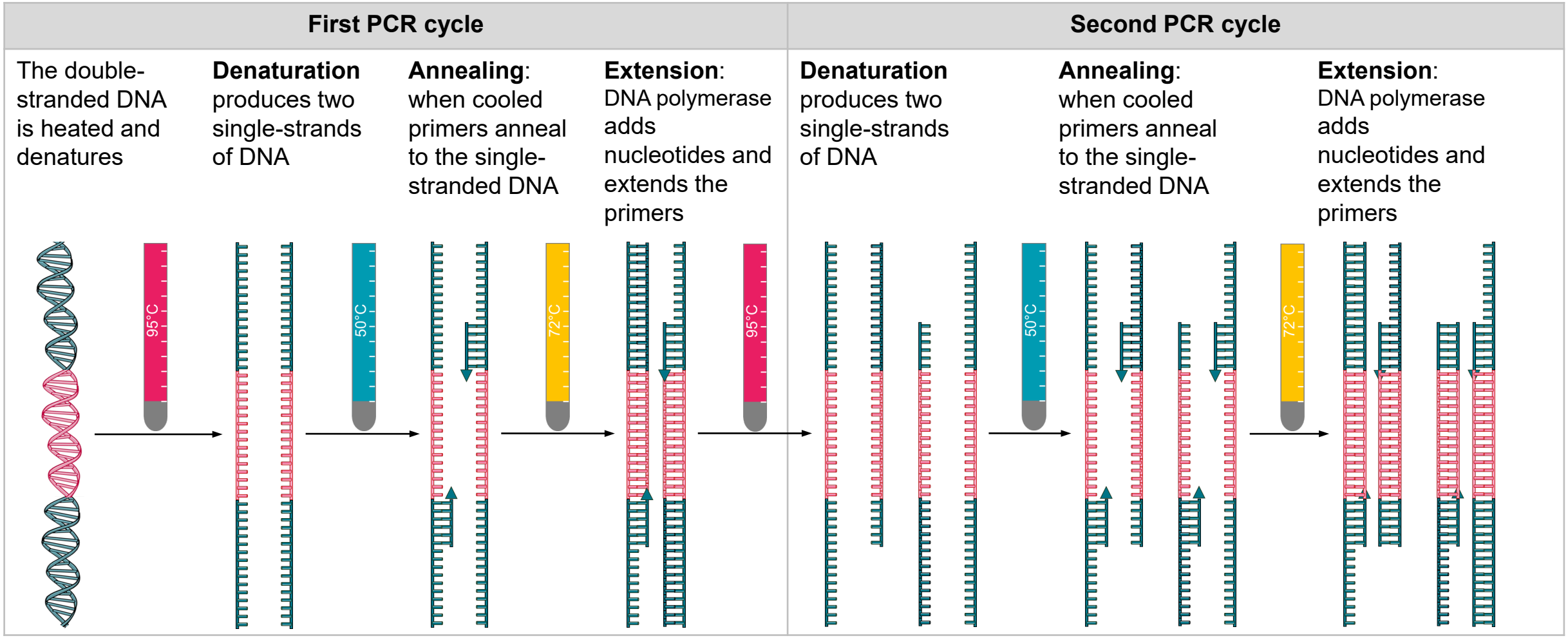
**Denaturation** produces two single-strands of DNA

**Annealing**: when cooled primers anneal to the single-stranded DNA

**Extension**: DNA polymerase adds nucleotides and extends the primers



# Explaining PCR



# Explaining PCR

**PCR** stands for **P**olymerase **C**hain **R**eaction

A PCR is able to make copies of DNA, much as a photocopier makes copies of an image.

DNA is copied exponentially, with the number of lengths of double-stranded DNA doubling for each cycle of PCR.


| Number of PCR cycles | Lengths of dsDNA |
|---|---|
| 1 | 2 |
| 2 | 4 |
| 5 | 32 |
| 10 | 1,024 |
| 20 | 1,048,576 |
| 35 | 34,359,738,368 |

# Amplifying the DNA barcode

No universal region of DNA that remains constant with a species, but shows variation between species, has been identified. Instead different DNA barcodes are used in different taxonomic groups.

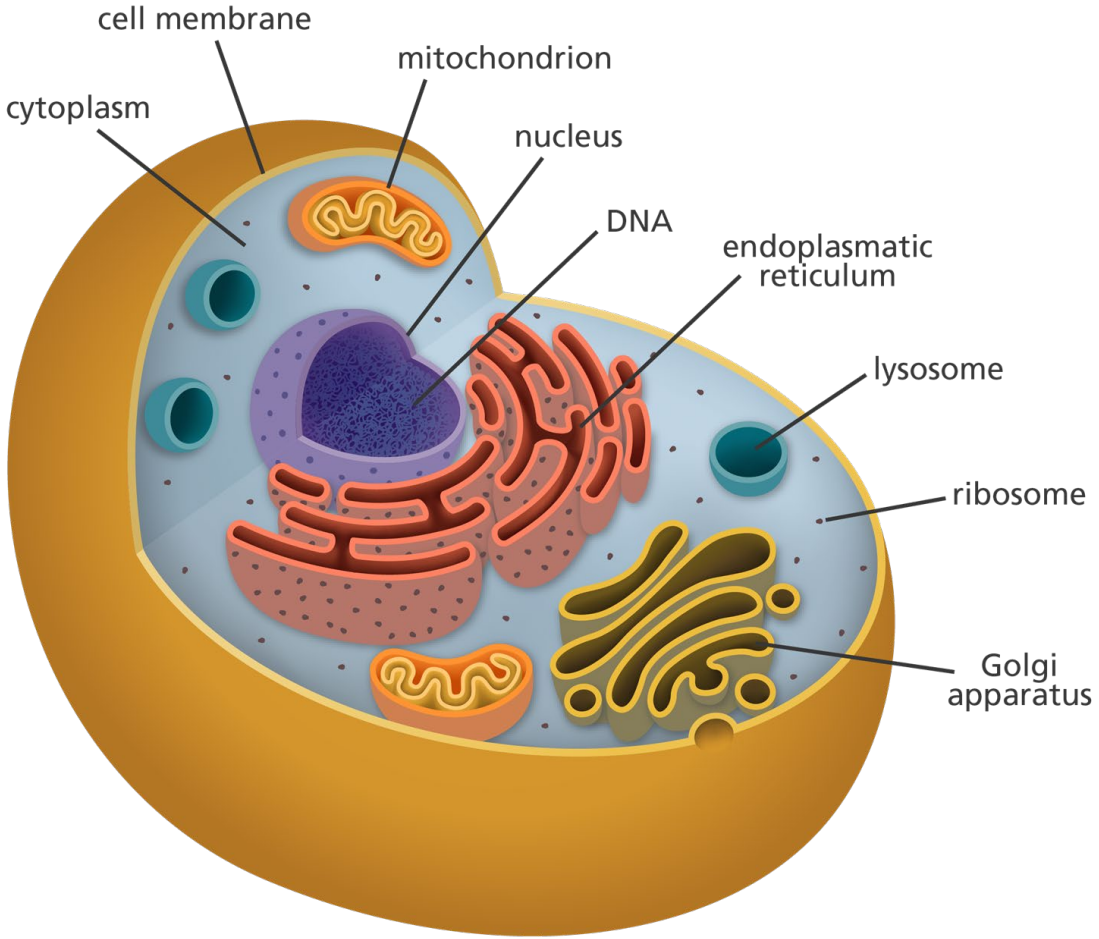
Paul Hebert proposed the mitochondrial cytochrome c oxidase subunit 1 (COI) gene as an unique DNA barcode region for animals. Sequence diversity in a 650 bp region near the 5' end of the COI gene provides strong species level resolution for different animal groups.

Hebert, P. D. N., Ratnasingham, S. and deWaard, J. R. (2003) 'Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species.' *Proceedings of the Royal Society B 270*; pp S96-S99.

# Amplifying the DNA barcode

The mitochondrial cytochrome c oxidase subunit 1 (COI) gene is a good DNA barcode because:

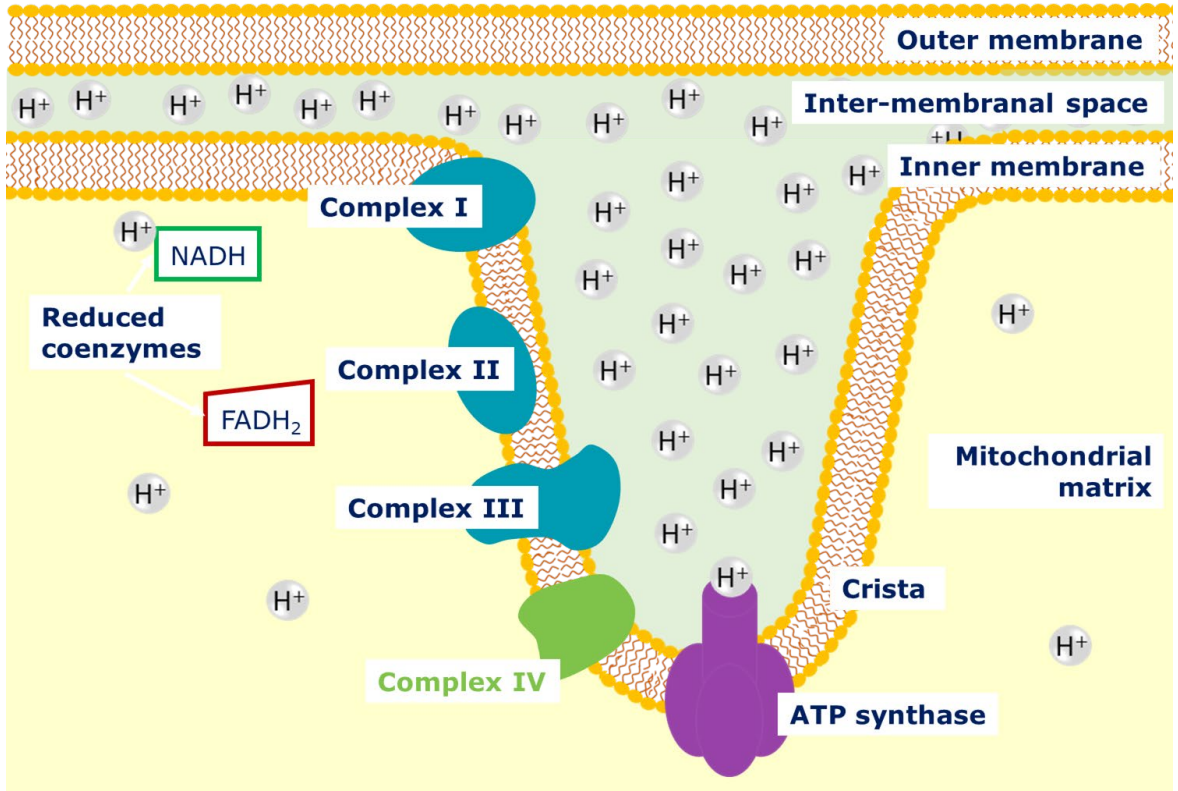
- It is part of the mitochondrial DNA.
  *There are multiple mitochondria per cell, but only one nucleus, so it is easier to obtain DNA barcode sequence from a small sample using mitochondrial DNA than nuclear DNA.*



# Amplifying the DNA barcode

The mitochondrial cytochrome c oxidase subunit 1 (COI) gene is a good DNA barcode because:

- COI encodes a protein involved in the electron transport chain of respiration. *Use of a gene involved in a key reaction for the cell, means the rate of change in gene sequence will be slow enough to remain identical in the same species, but fast enough to vary between species.*




Schematic diagram showing the mitochondrial membranes with complexes I-IV of the electron transport chain and ATP synthase. Complex IV is the cytochrome c oxidase, of which subunit 1 is a part.

# Amplifying the DNA barcode

Mitochondria have circular DNA located in the mitochondrial matrix. Much of the mitochondrial DNA encodes proteins and RNA involved in cellular respiration and cell reproduction.

The length of mitochondrial DNA varies between invertebrates. Mitochondrial DNA from the fruit fly *Drosophila melangoster* is shown as an example, with the position of the cytochrome c oxidase subunit 1 gene, used as a DNA barcode, shown.

The cytochrome c oxidase subunit 1 gene from *Drosophila melangoster* mitochondrial DNA

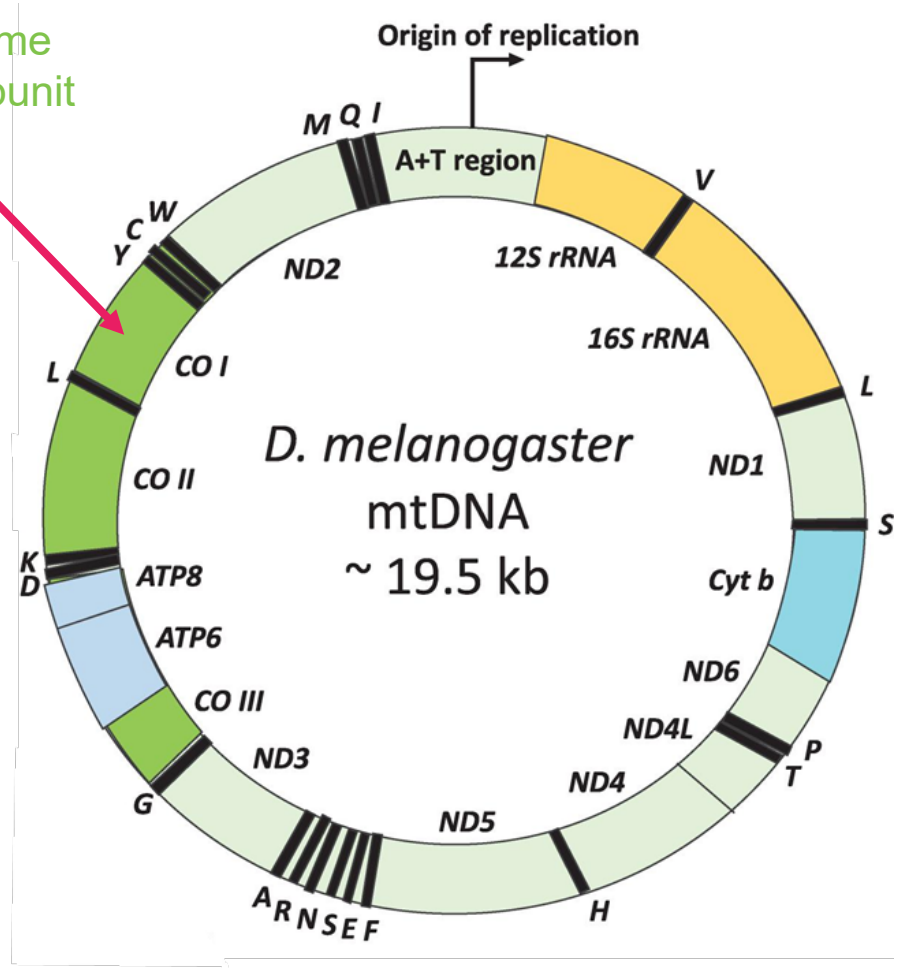
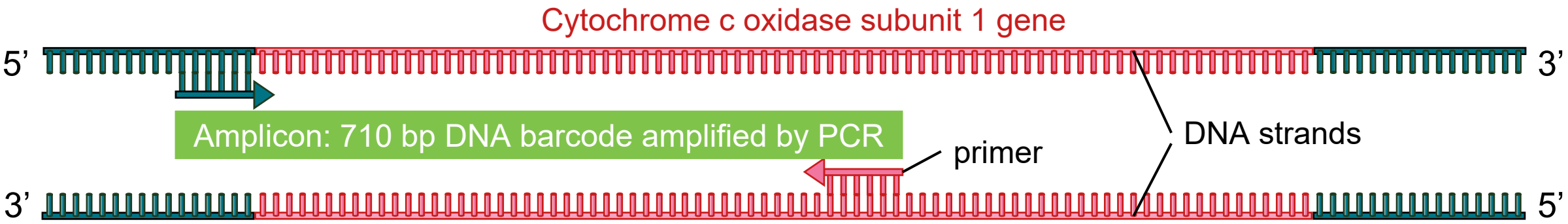



Image from: Salminen, T. S. and Vale P. F. (2020) '*Drosophila* as a Model System to Investigate the Effects of Mitochondrial Variation on Innate Immunity'. *Frontiers in Immunology 11*; pp521-533.

# Amplifying the DNA barcode

Primers have been designed that amplify a 710 base pair region near the 5’ end of the cytochrome c oxidase subunit 1 gene.




NOTE: This image is not to scale!

# Amplifying the DNA barcode

The sequence of the primers used to amplify the 710 base pair region near the 5’ end of the cytochrome c oxidase subunit 1 gene is shown.

| | |
|---|---|
| Invertebrate Forward primer | TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG |
| Invertebrate Reverse primer | CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA |

The sequence complementary to the cytochrome c oxidase subunit 1 gene is shown in red, the additional sequence added to the primer to help with DNA sequencing is shown in black.

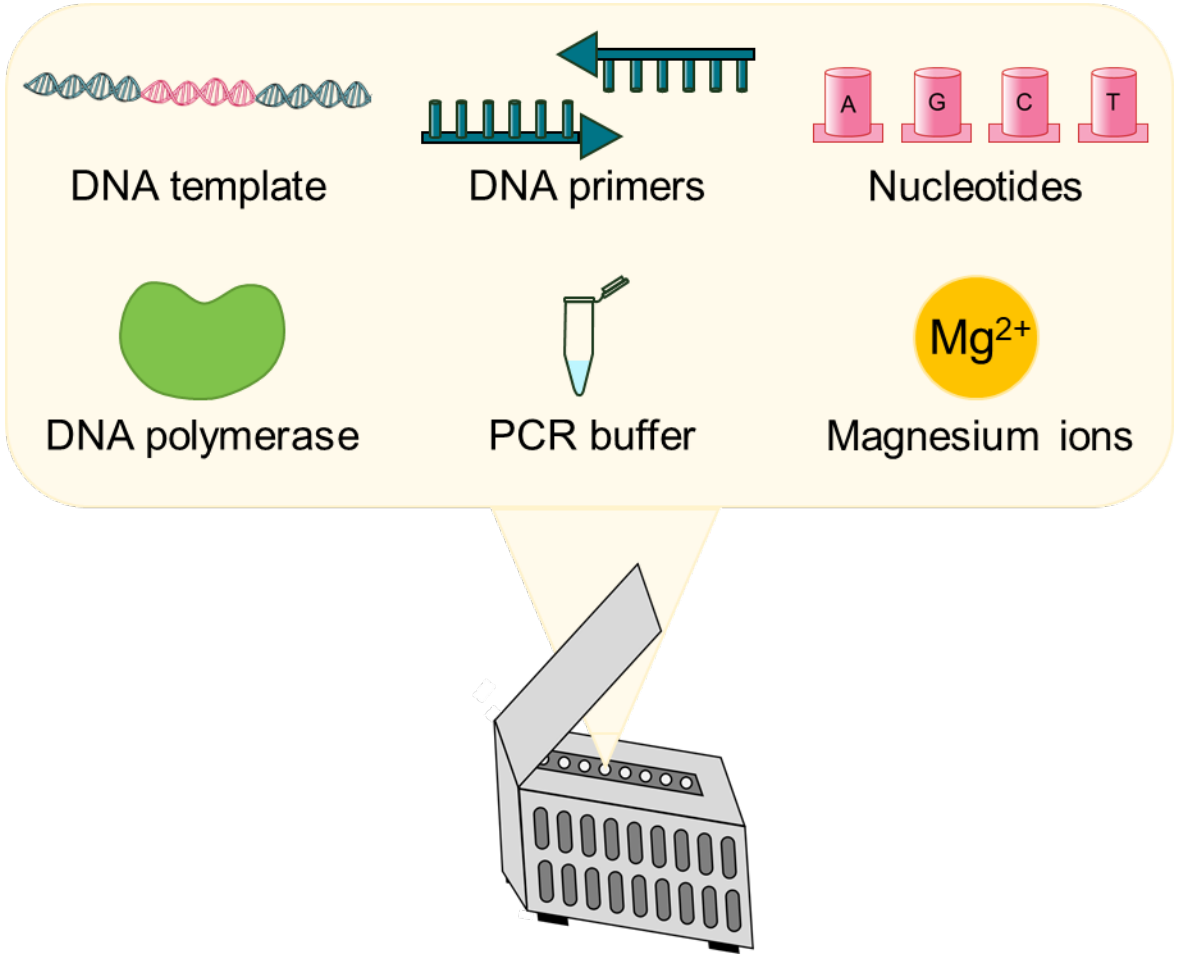
Primers designed by: Folmer, O., Black, M., Hoeh, W., Lutz, R. and Vrijenhoek, R. (1994) ‘DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates.’ *Molecular Marine Biology and Biotechnology 3 (5)*; pp 294-299.

# Performing PCR

Set up your PCR following the experimental procedure.

| Label | Reagent | Volume in PCR |
|---|---|---|
| MM | 2x PCR Master mix | 12.5 µl |
| Initials | DNA sample | 5.0 µl |
| FOR | Forward primer | 2.0 µl |
| REV | Reverse primer | 2.0 µl |
| $H_2O$ | Nuclease-free water | 3.5 µl |
| | **Total volume** | **25.0** µl |

The master mix contains the PCR buffer, magnesium ions, nucleotides and DNA polymerase.

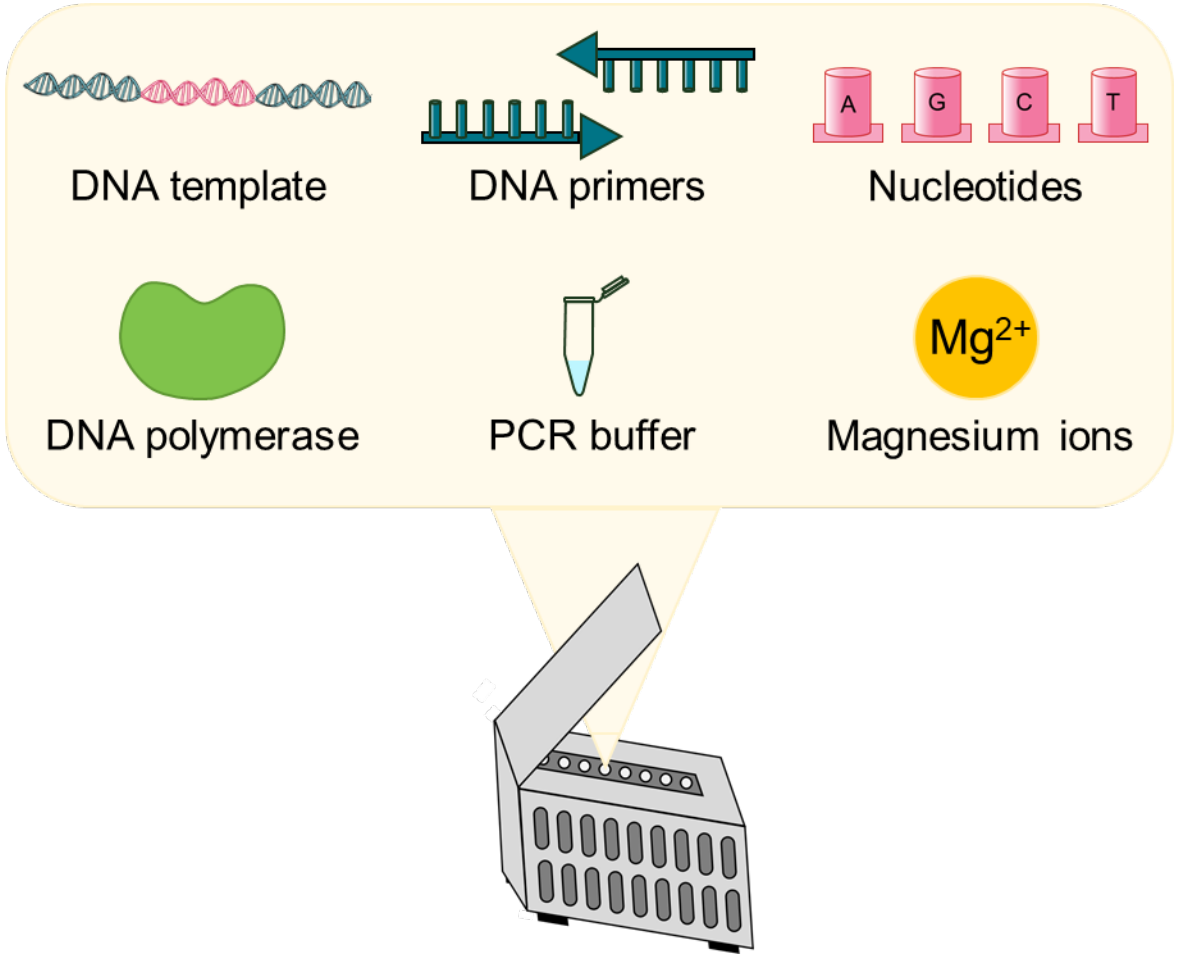


# Performing PCR

Make sure one group also sets up a negative control.

| Label | Reagent | Volume in PCR |
|---|---|---|
| MM | 2x PCR Master mix | 12.5 µl |
| FOR | Forward primer | 2.0 µl |
| REV | Reverse primer | 2.0 µl |
| $H_2O$ | Nuclease-free water | 8.5 µl |
| | **Total volume** | **25.0** µl |

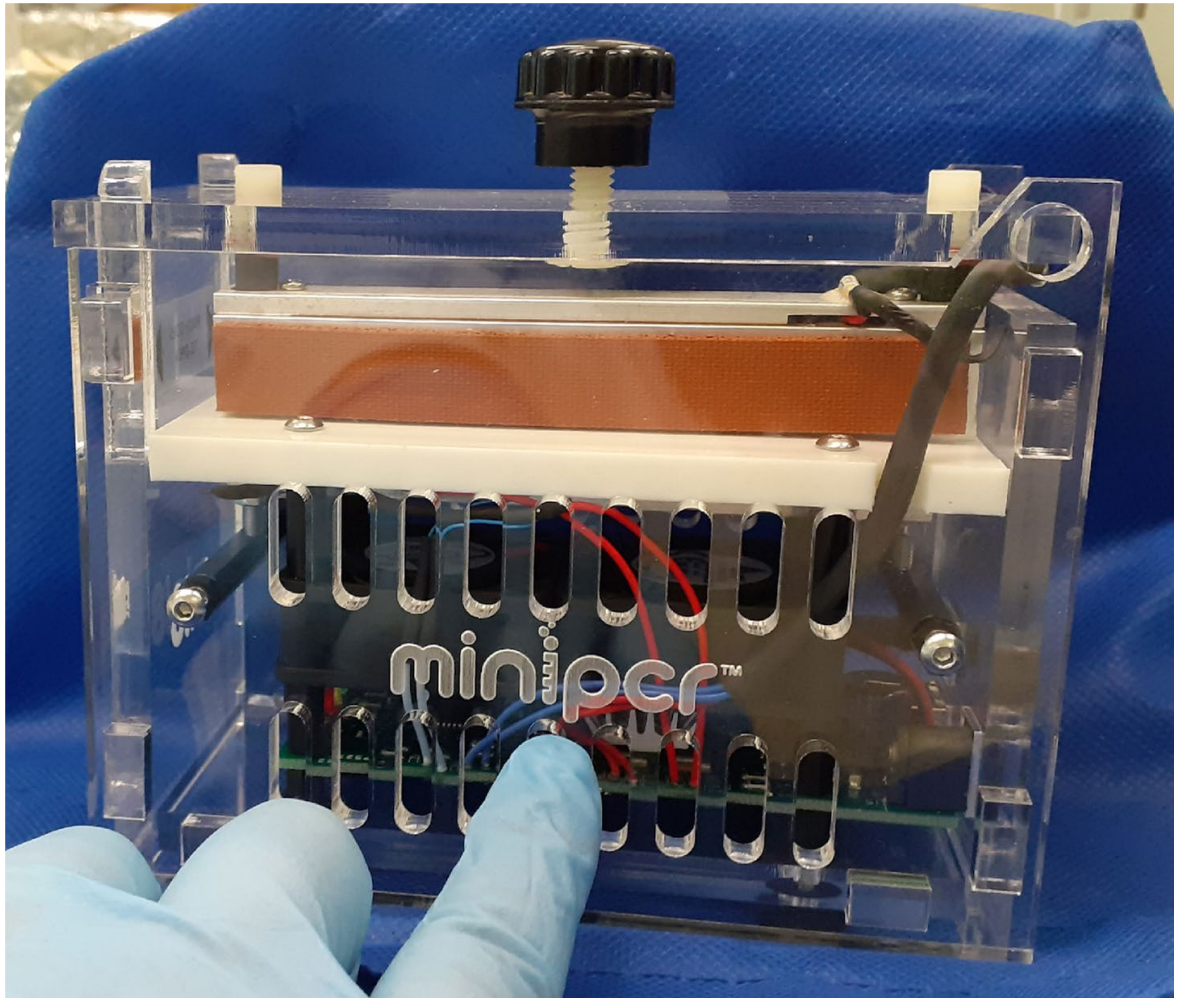
The master mix contains the PCR buffer, magnesium ions, nucleotides and DNA polymerase.



# Performing PCR

Programme the MiniPCR machine to carry out the thermal cycling.

| Phase | Temperature | Time | |
|---|---|---|---|
| Initial denaturation | 94°C | 60 seconds | |
| Denaturation | 95°C | 30 seconds | 35 cycles |
| Annealing | 50°C | 30 seconds | |
| Extension | 72°C | 45 seconds | |
| Final extension | 72°C | 180 seconds | |



# Performing PCR

Start the PCR.

You can monitor the progress of thermal cycling and how many DNA copies have been made on the computer screen.

When the PCR is finished, remove tubes to the fridge (4°C) until you are ready for electrophoresis.

# Next practical activities

Tomorrow you will check your success using gel electrophoresis and use some pre-sequenced invertebrate DNA to develop skills in bioinformatics.