

Metabarcoding and Environmental DNA Workshop:

Assessing Biodiversity of a Local Pond using DNA Metabarcoding



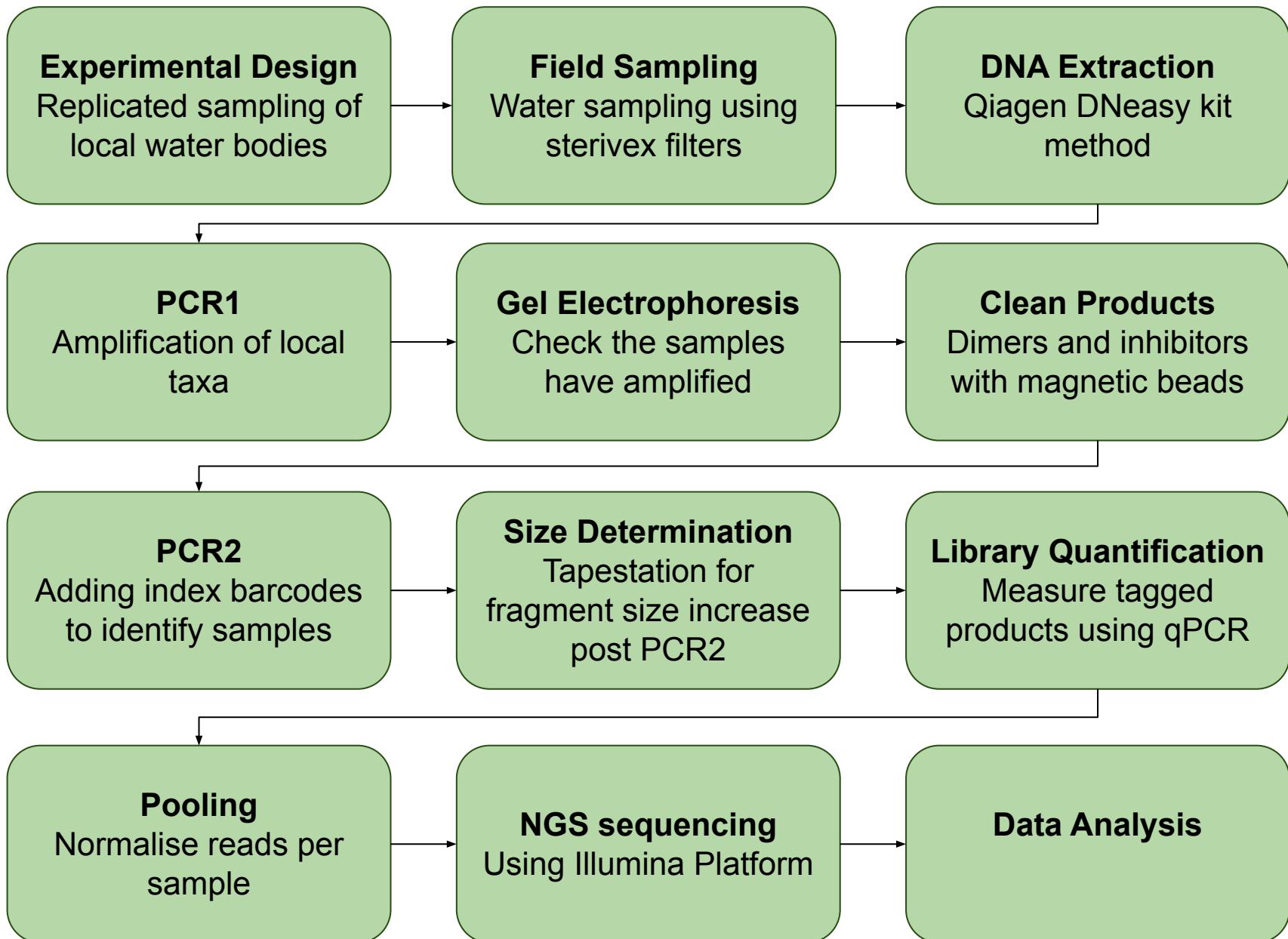
The University
Of Sheffield.

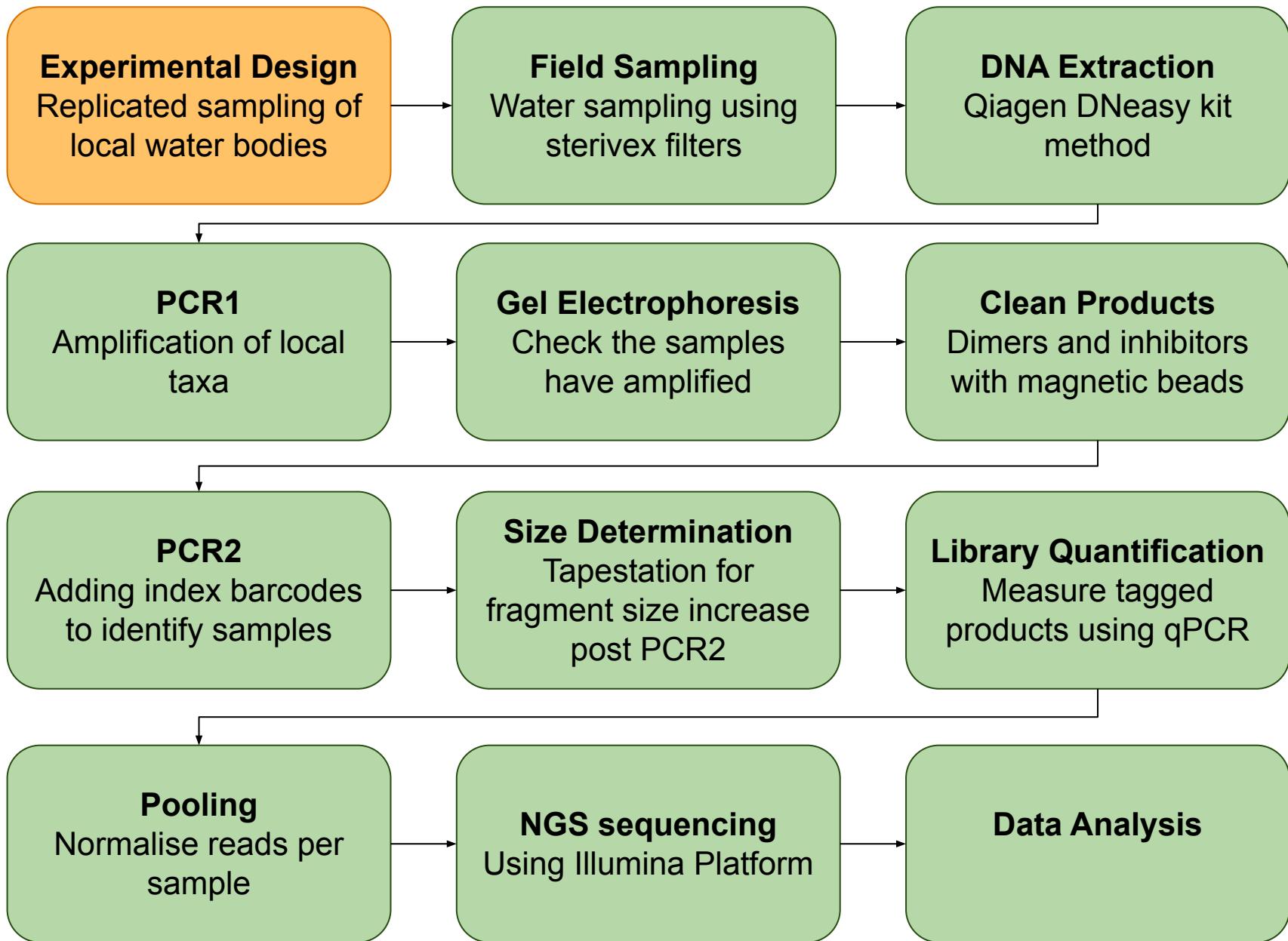


Natural
Environment
Research Council



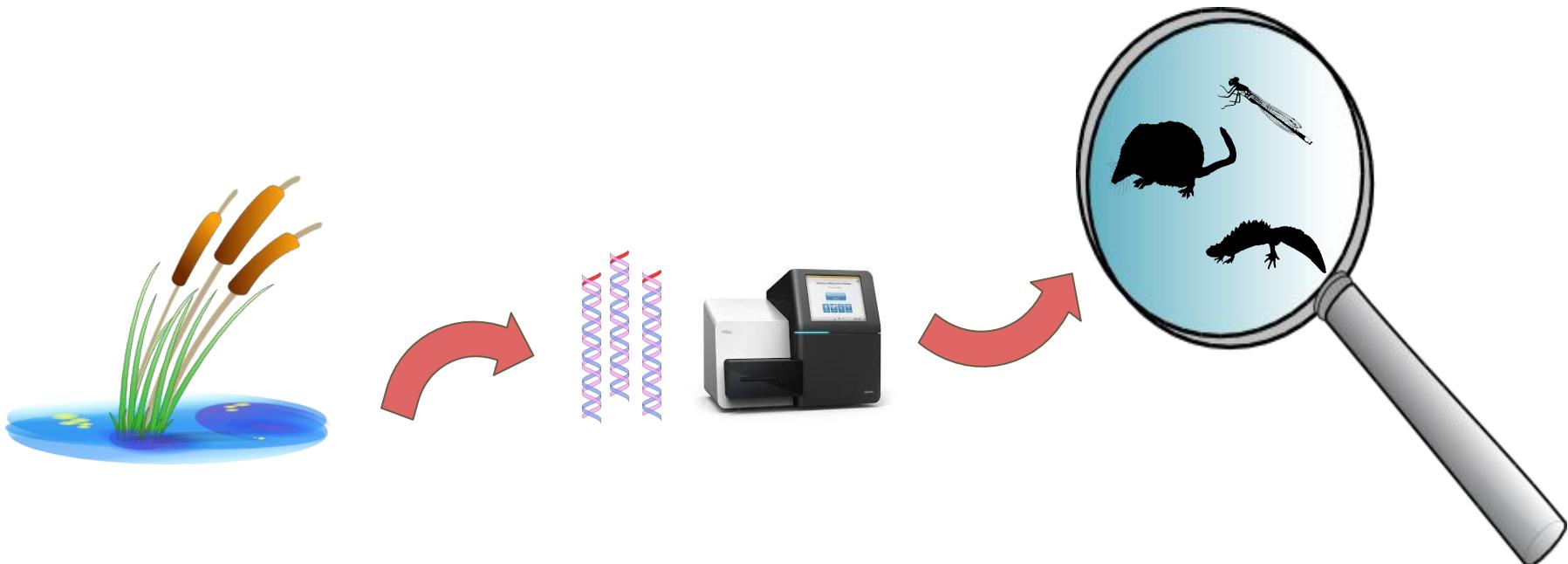
NEOF
NERC ENVIRONMENTAL
OMICS FACILITY





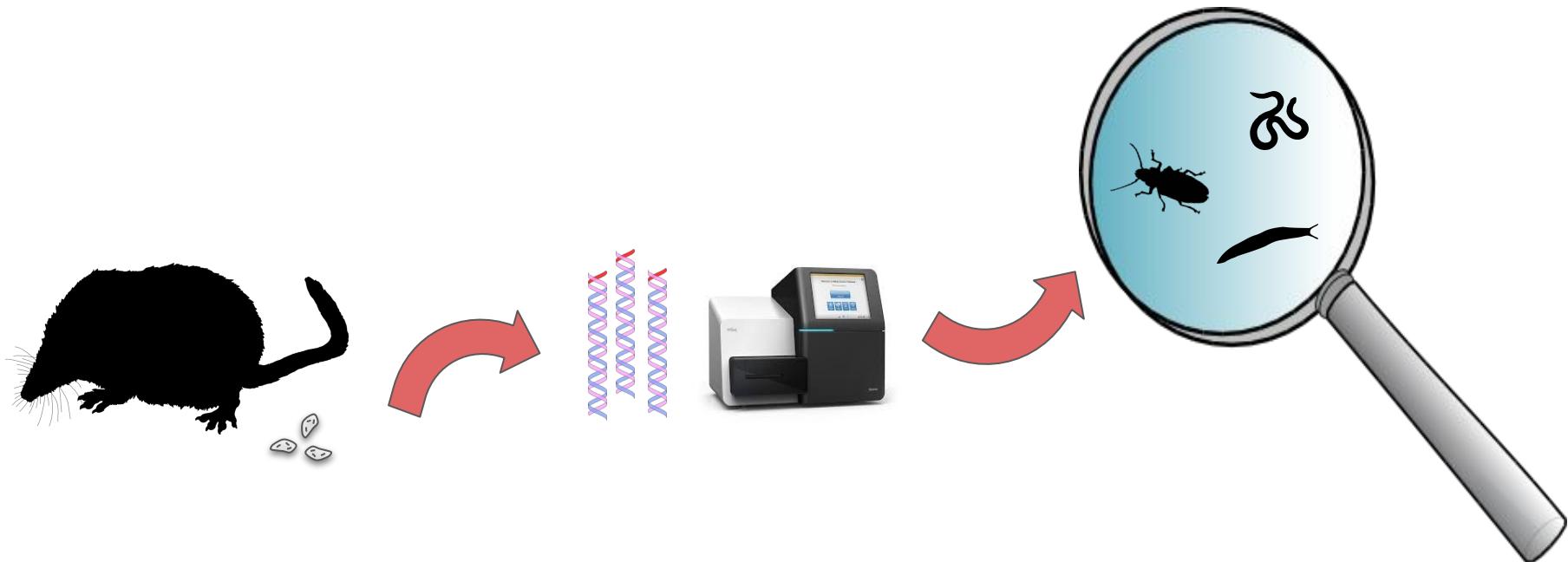
Why metabarcoding?

Identify a range of species in an environmental sample simultaneously, through genetic sequencing.



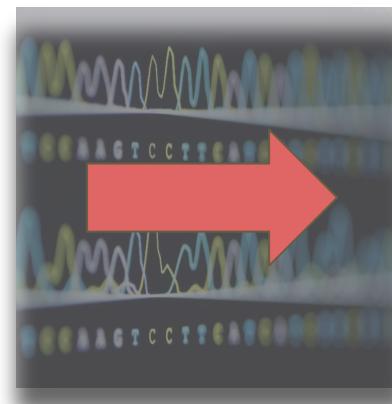
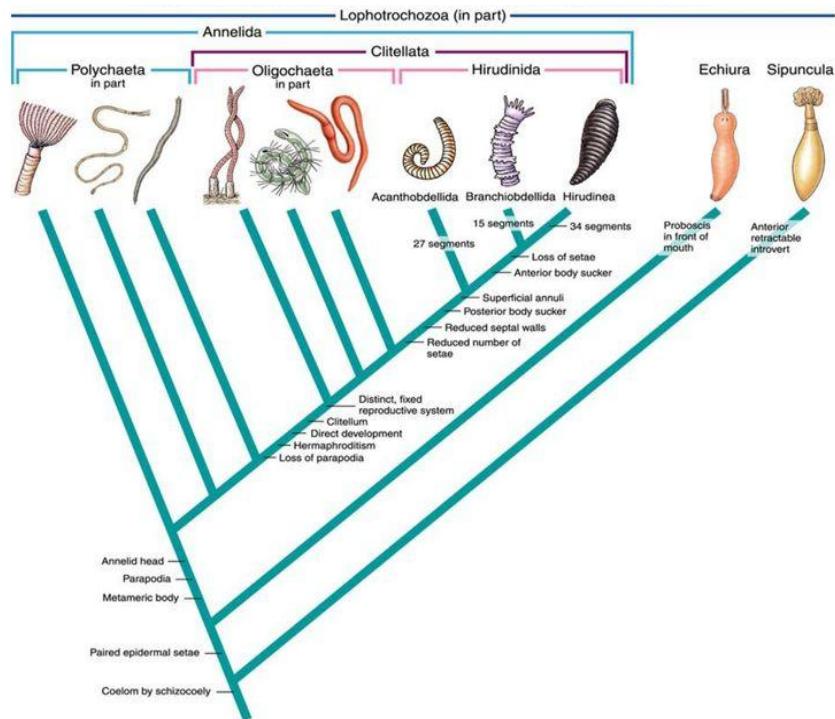
Why metabarcoding?

This allows scientists to carry out a range of studies including diet analysis and population surveys.

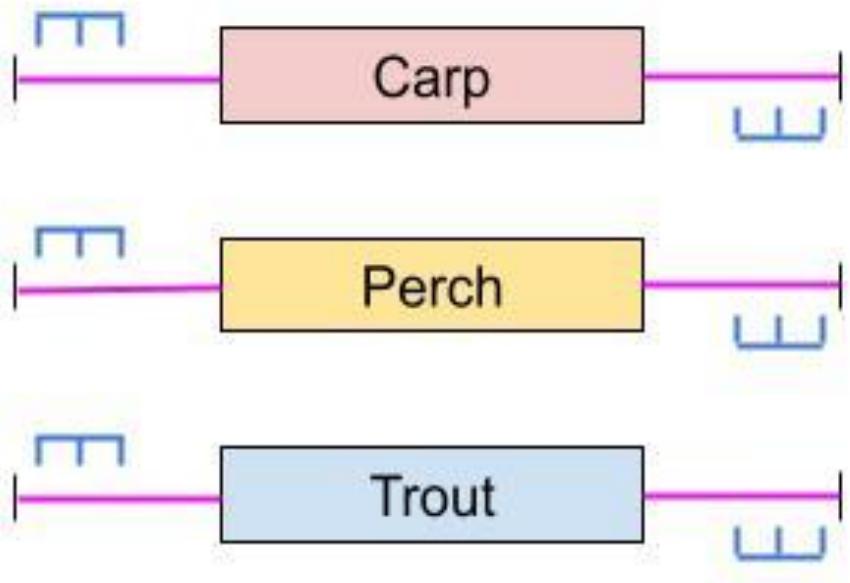


How does metabarcoding work?

Generic primers are used to target whole taxonomic groups, whilst bioinformatic analysis allows species identification



Metabarcoding genes allow species identification



— Conserved sequence for primer binding

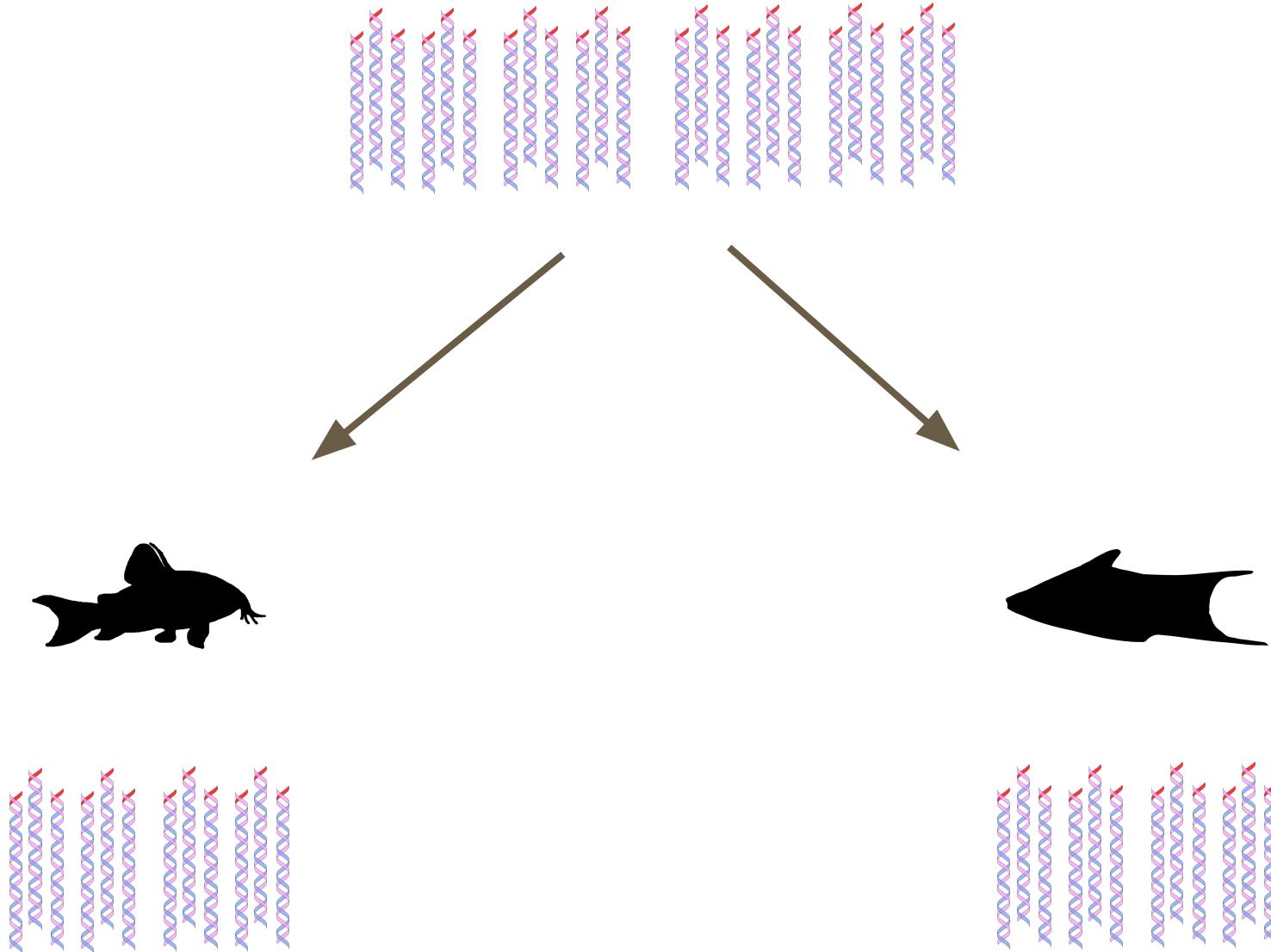
■ Fish primer

Barcode

The barcoding gene should be flanked by sequences that are conserved within the target phylogeny.

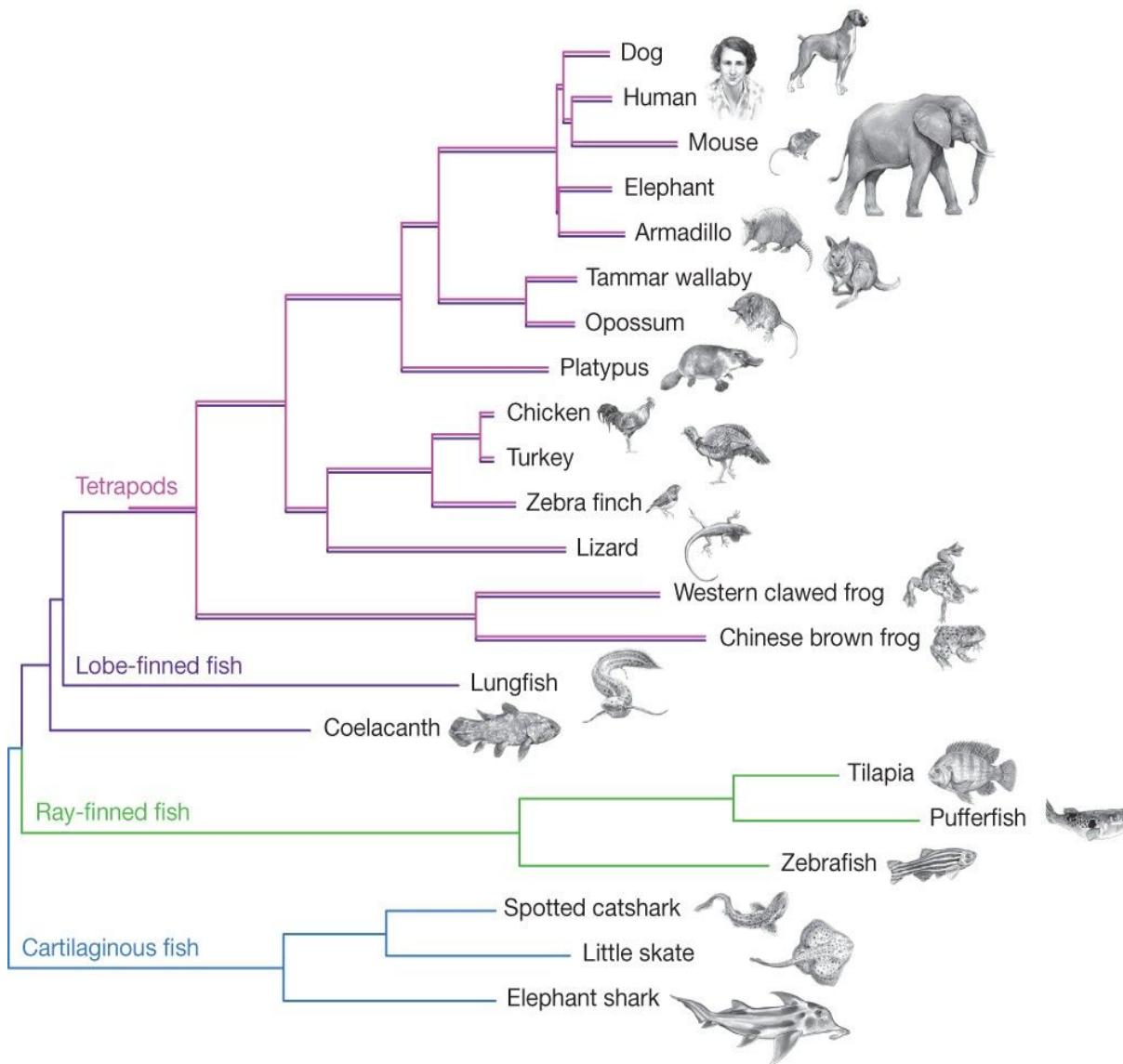
Primers are designed to target the conserved sequences.

Total number of sequencing reads to be shared between target species



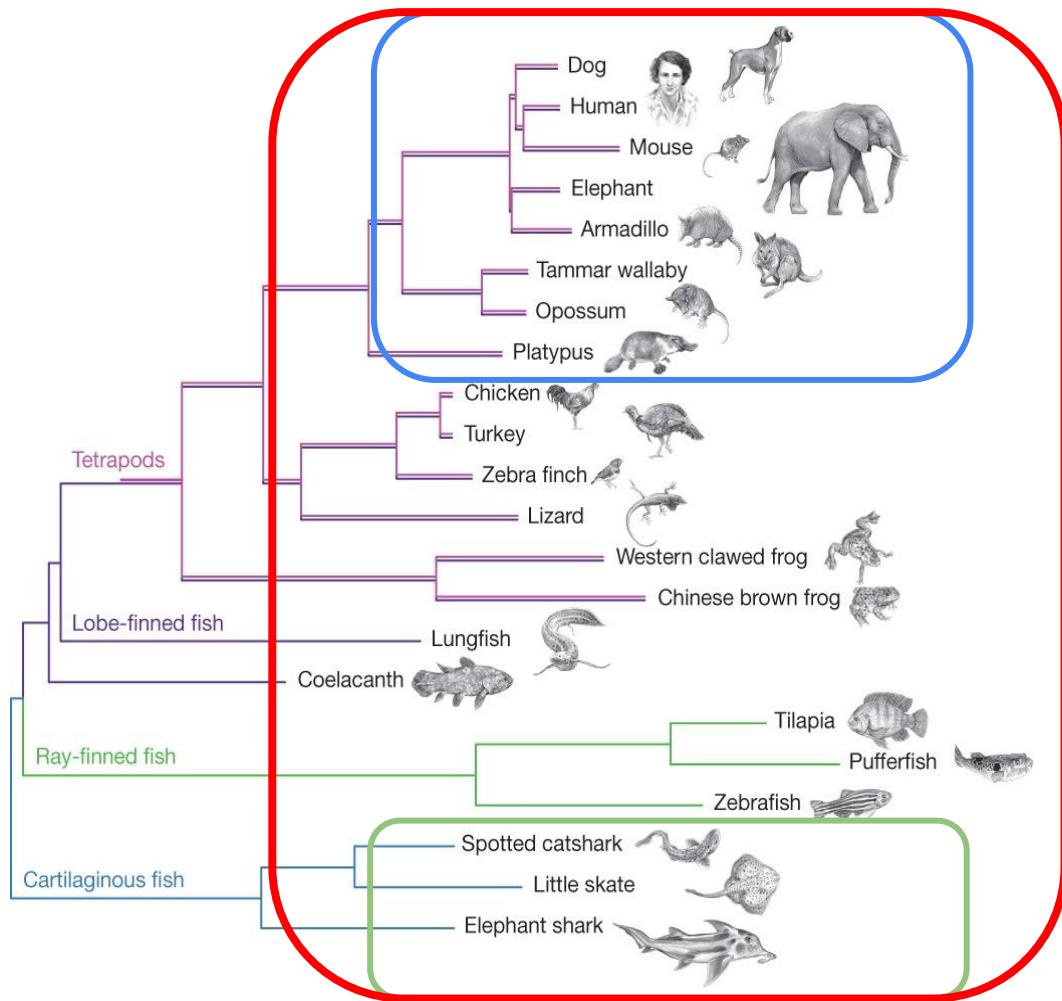


PCR 1 - Primer Selection



When choosing (or designing) primers it's important to have a clear focus on the scale of the phylogeny that is required to answer your question.

PCR 1 - Primer Selection



LCO1490

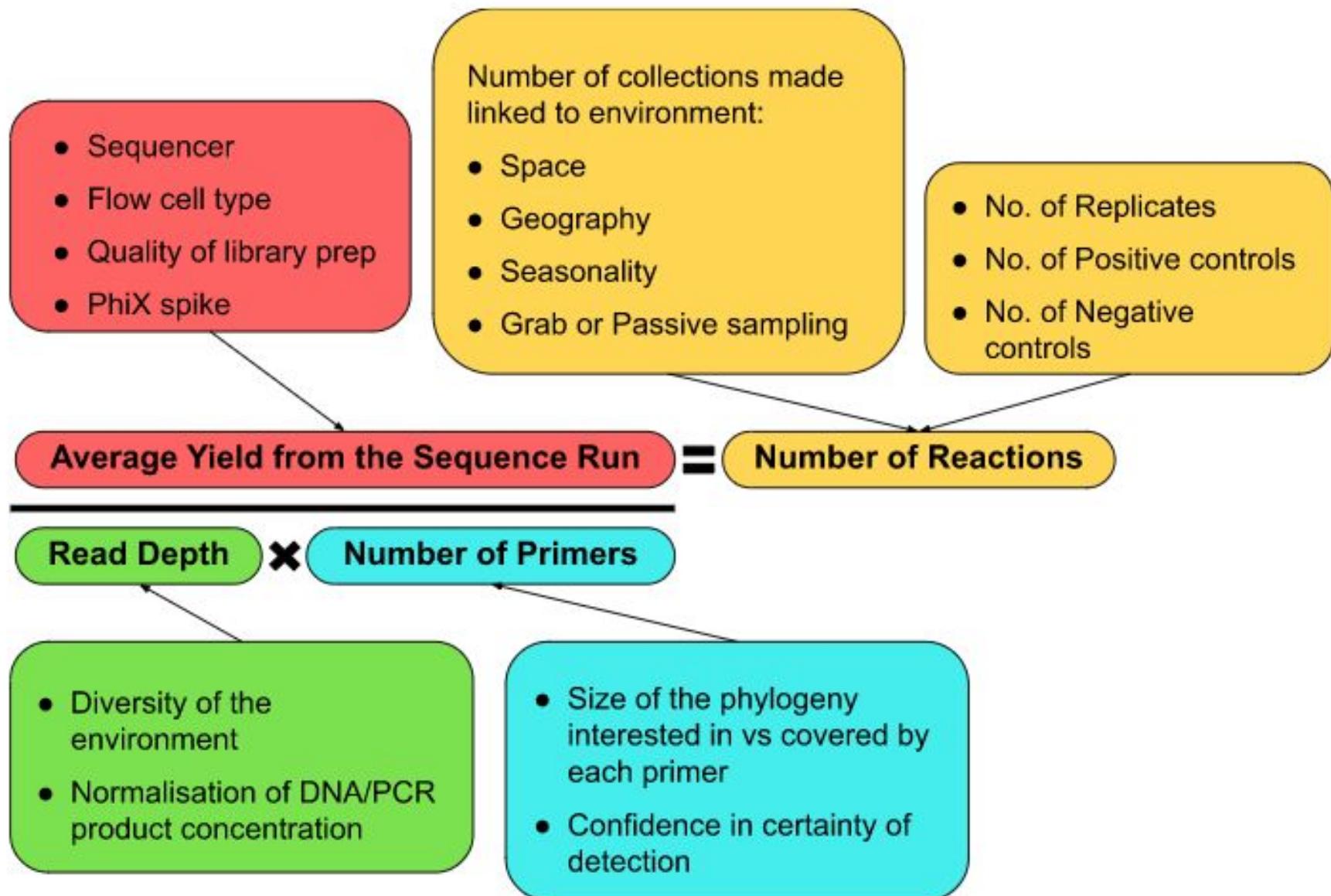
MiMammal-U

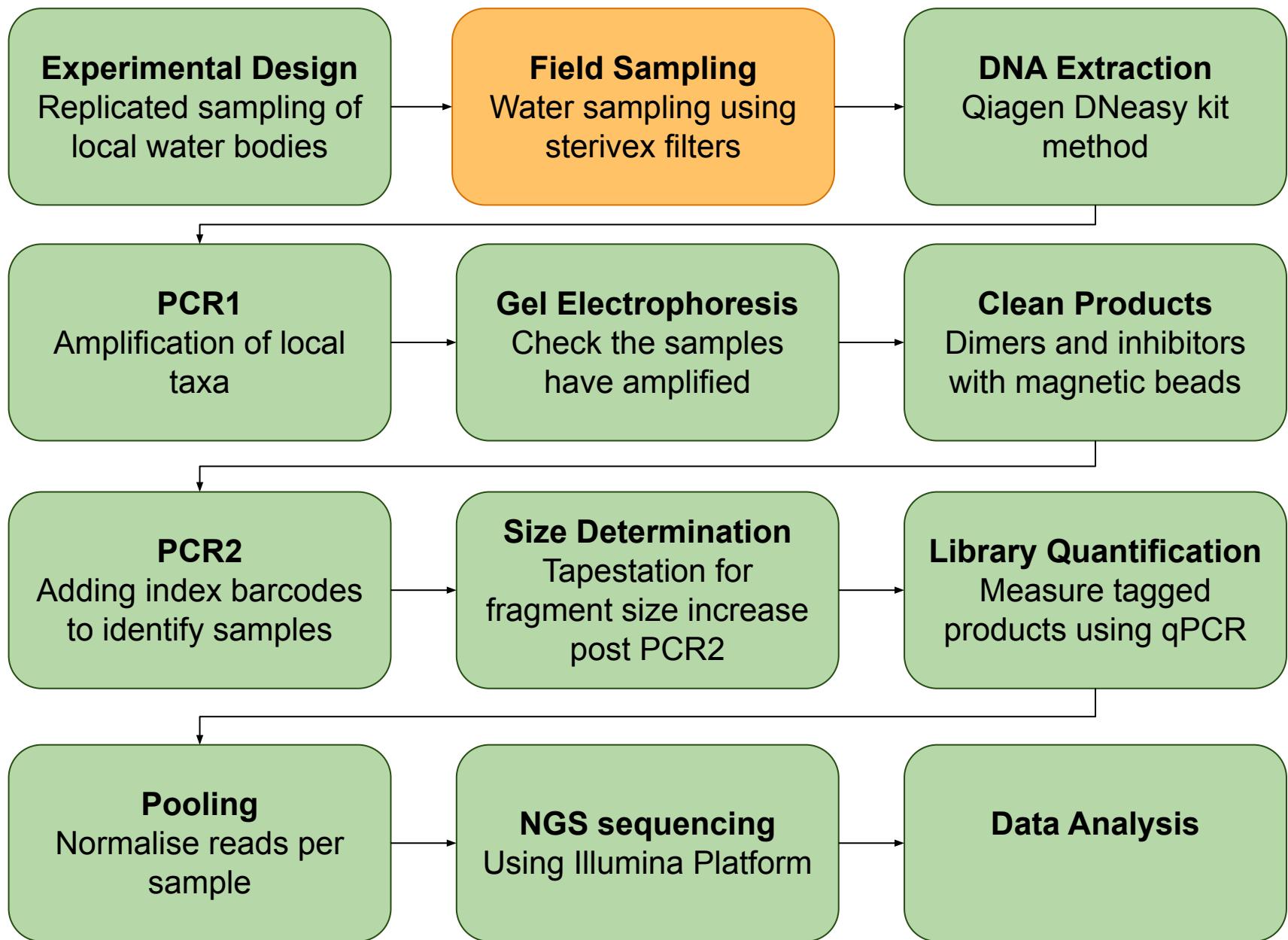
Elas02

The Diversity vs. Precision trade off

The more conserved the target region is across a phylogeny, the greater the number of potential species but the less read depth for each you will yield.

Experimental Design

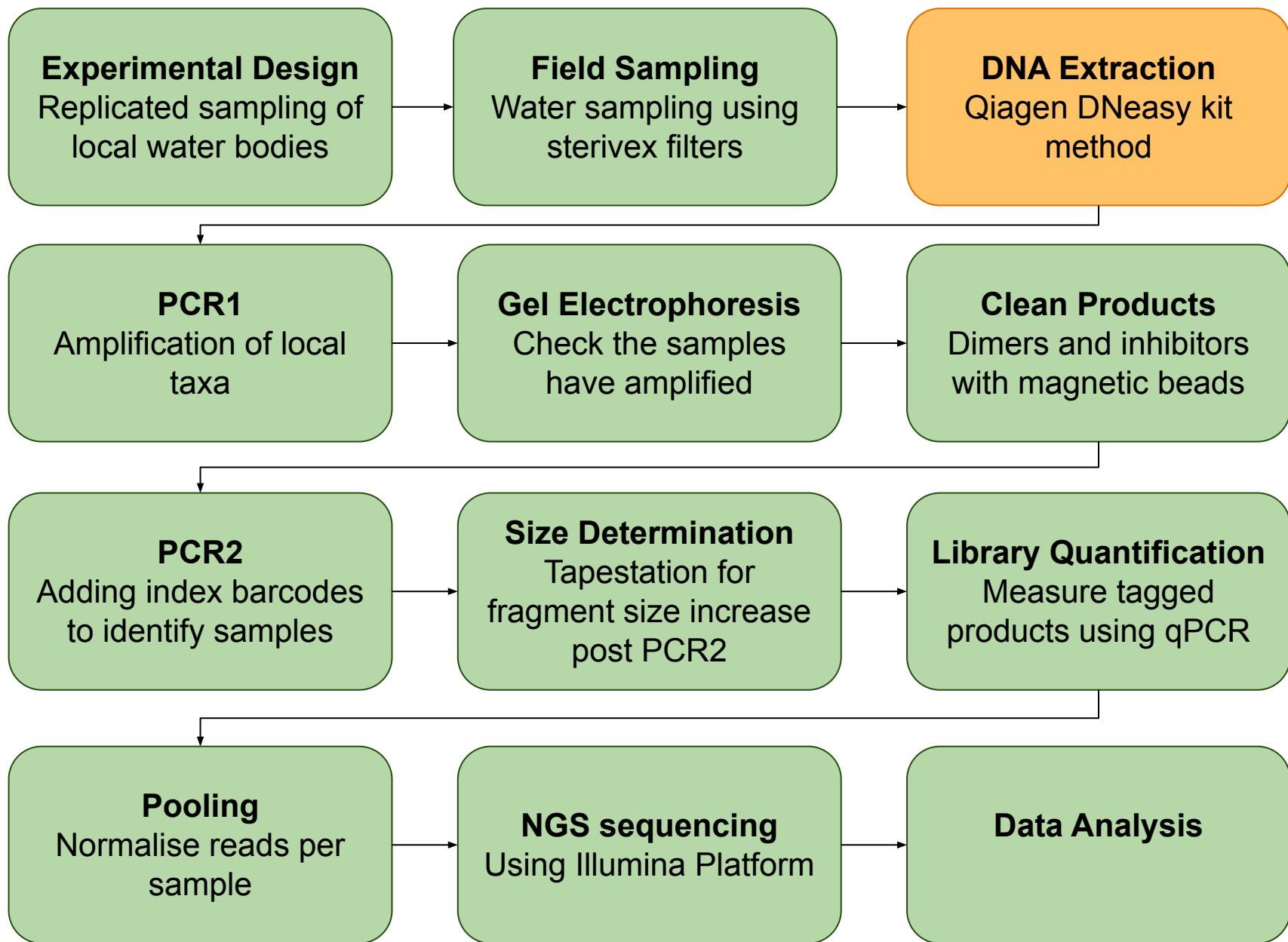




Sample Collection: Field Equipment



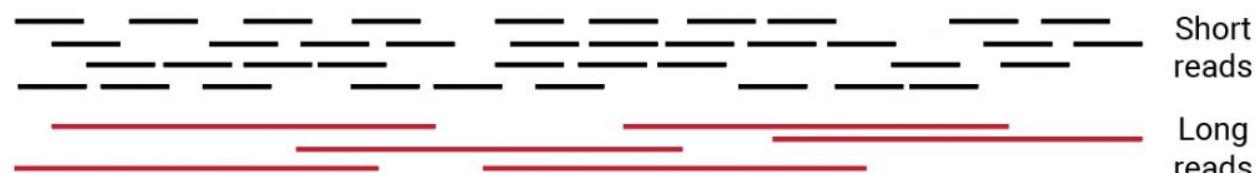
- Dipper
- Sterivex filter unit
- Syringe
- 250ml bottle
- Field negative bottle
- Caulking gun
- Box of ice
- 10% Bleach
- Tissue paper roll
- Gloves
- Permanent marker



Sample Type



Project Aim



Budget



Sample Type



Project Aim



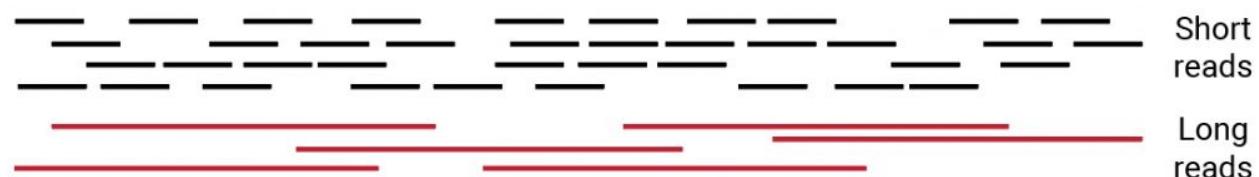
Budget



Sample Type



Project Aim



Budget



Sample Type



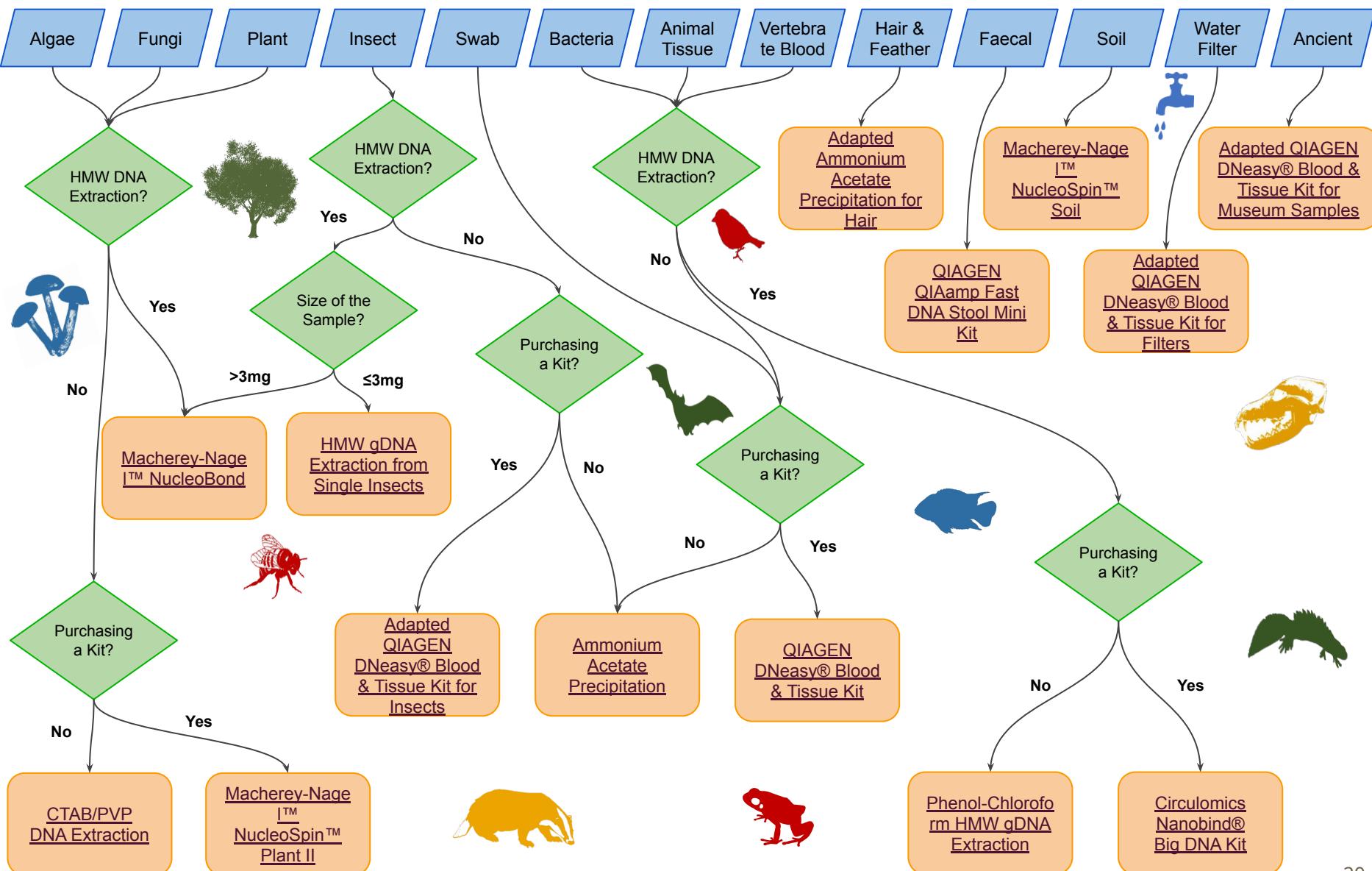
Project Aim



Budget



Recommended DNA Extraction Methods



Time to start the extractions



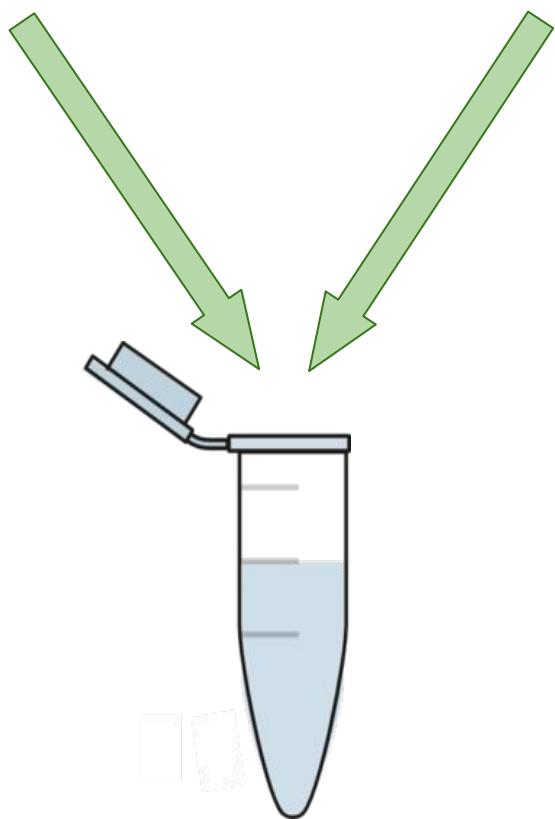
How to Pipette



DNA Extraction: Set-up

400 µl Buffer ATL

10 µl Proteinase K



Remember to label your tubes!

DNA Extraction: Remove Filter Paper from Unit



Sharp pipe cutter and scalpel - hold at inlet end.
Wear safety glasses.

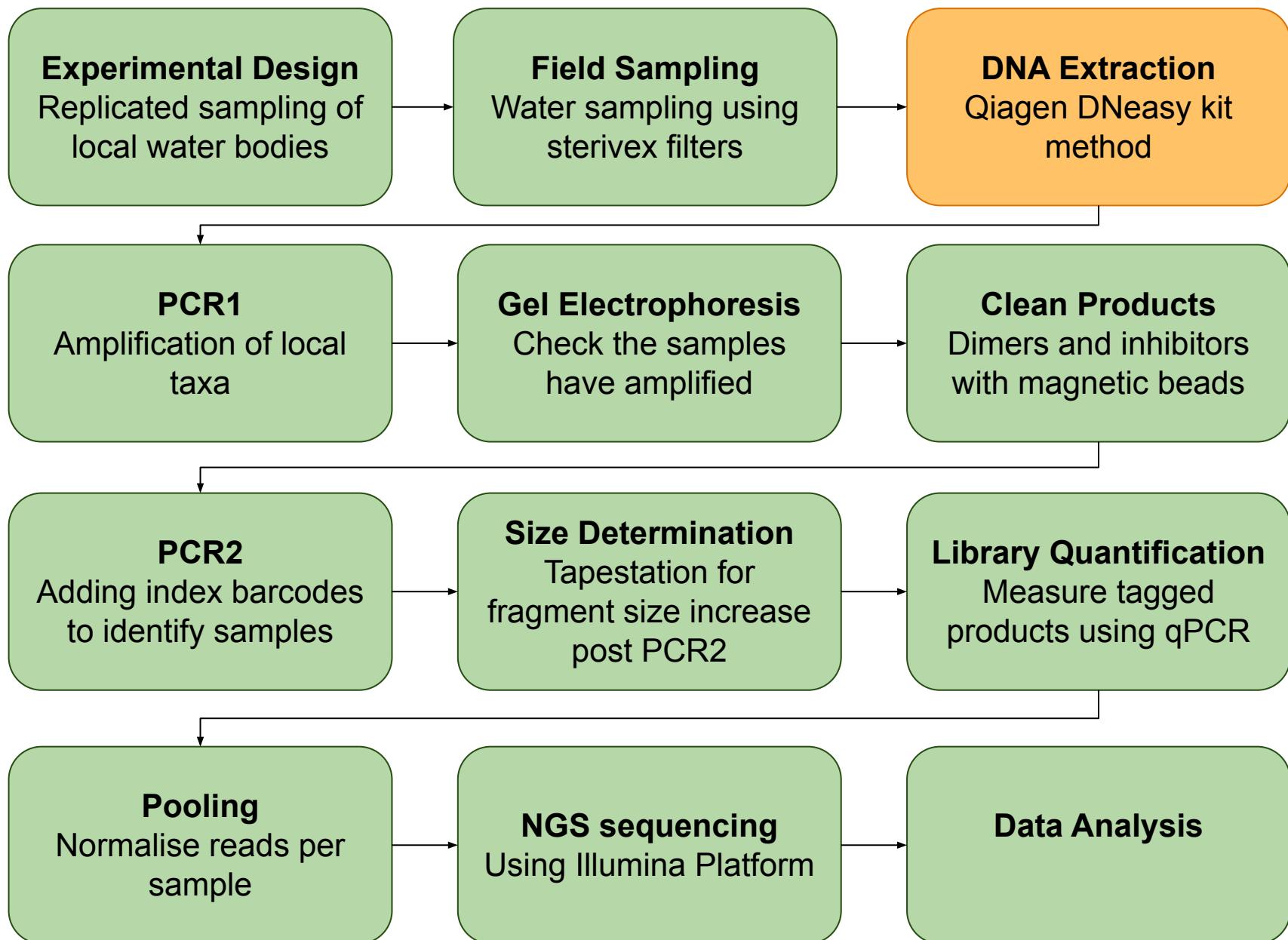
DNA Extraction: Overnight Incubation



Incubate the samples at 56°C overnight



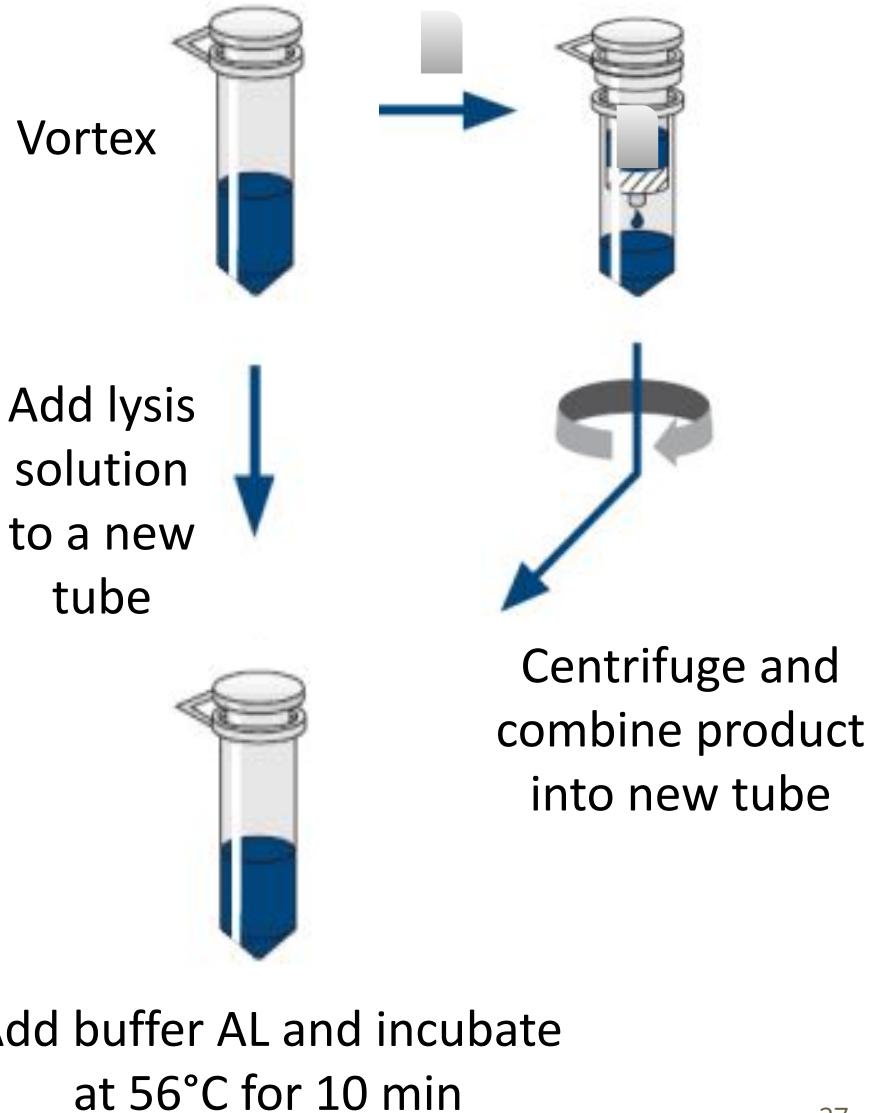
Hot surface



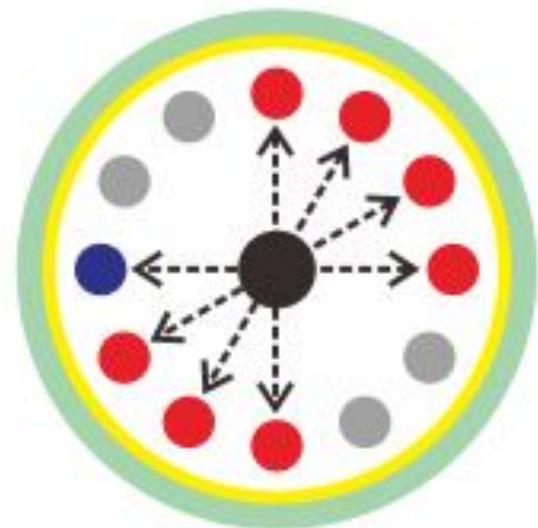
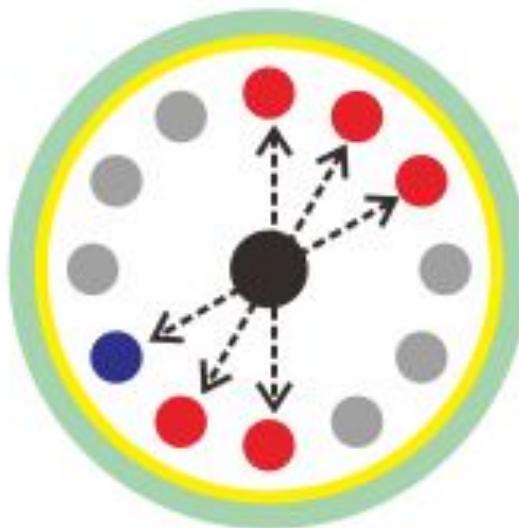
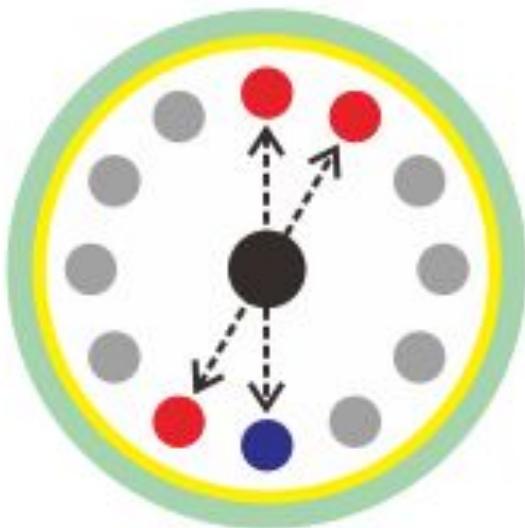
DNA Extraction: Qiagen DNA Purification Kit



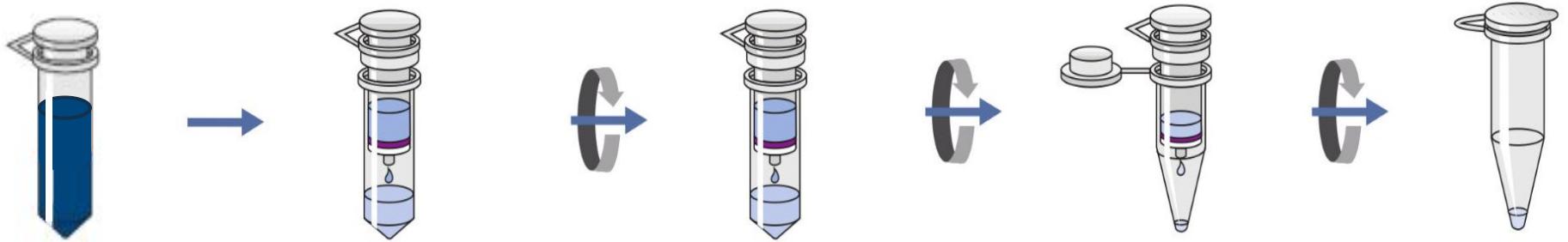
Add filter paper to
QIAshredder column



Balancing a Centrifuge



Load sample onto
spin column

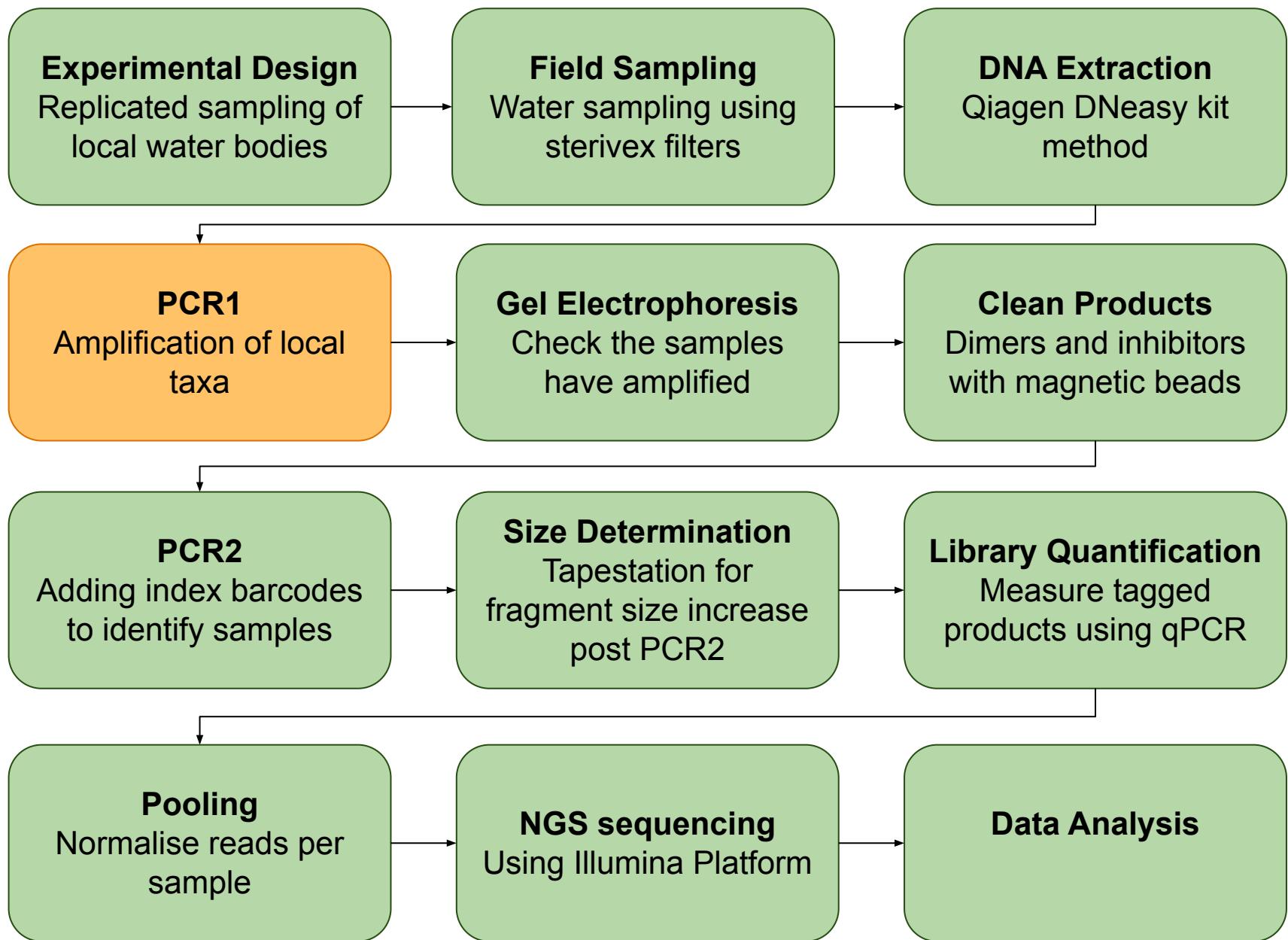


Add elution
buffer

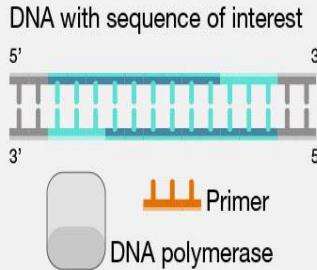
Add 100%
ethanol and
vortex

Wash the spin
column x2

Clean DNA

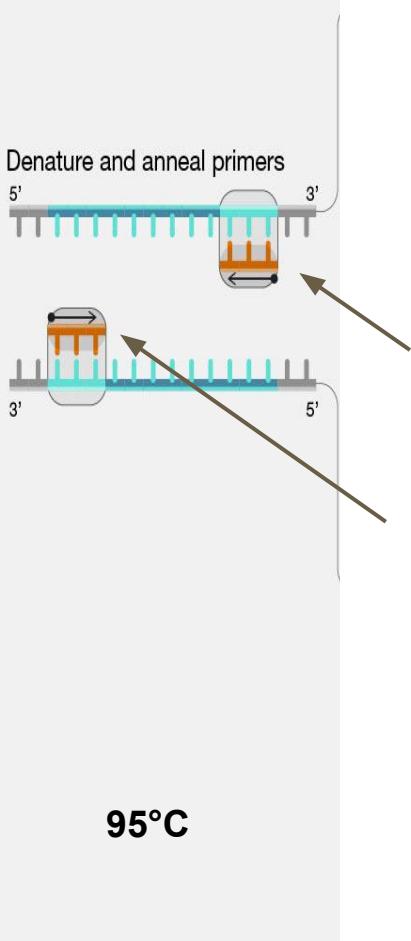


Polymerase Chain Reaction (PCR)

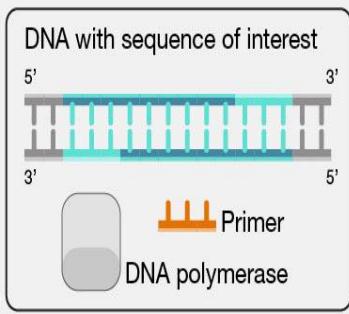


Step 1 - Denaturation

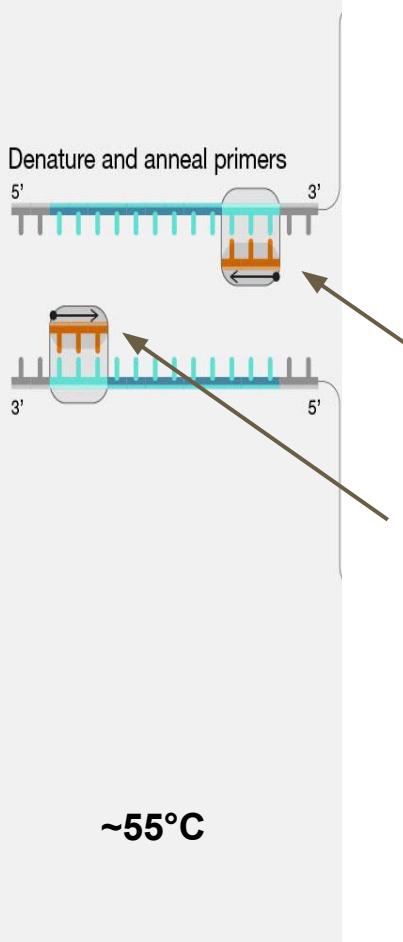
The double-stranded DNA is separated into single strands when heated



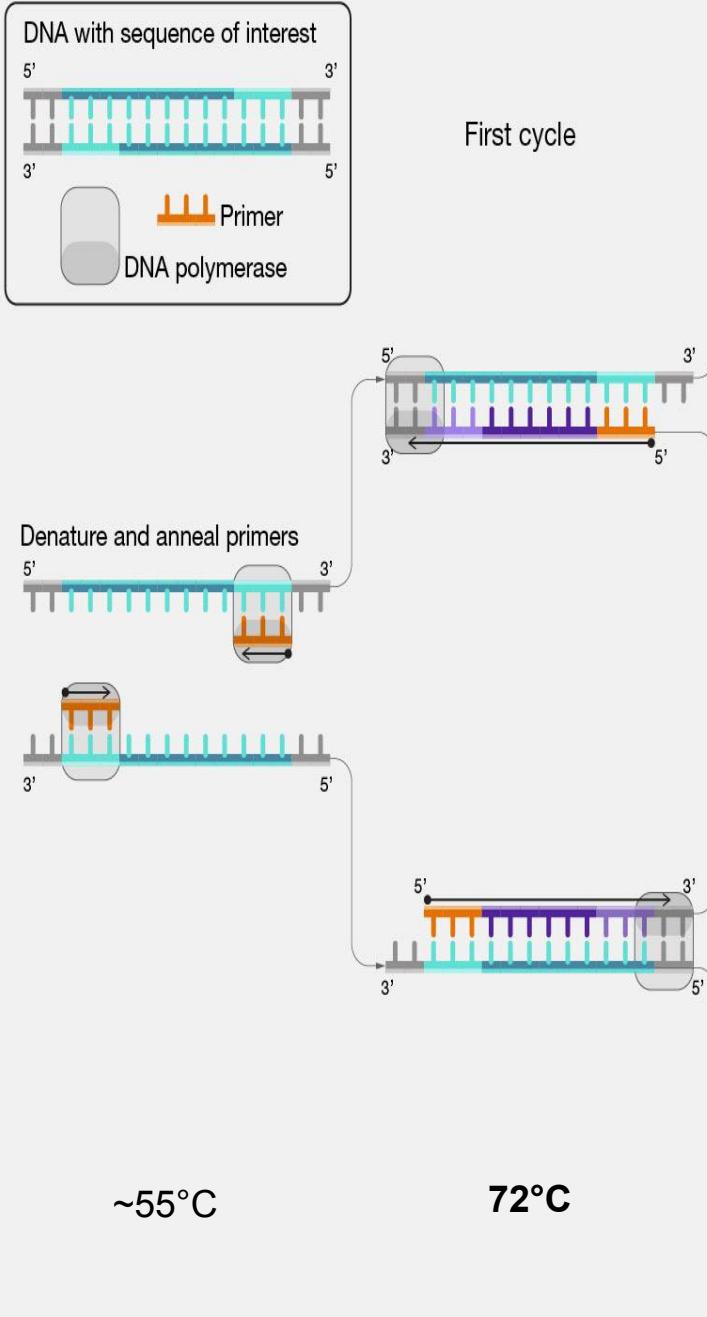
Step 2 - Annealing



- Primers bind
 - Determine melting temperature

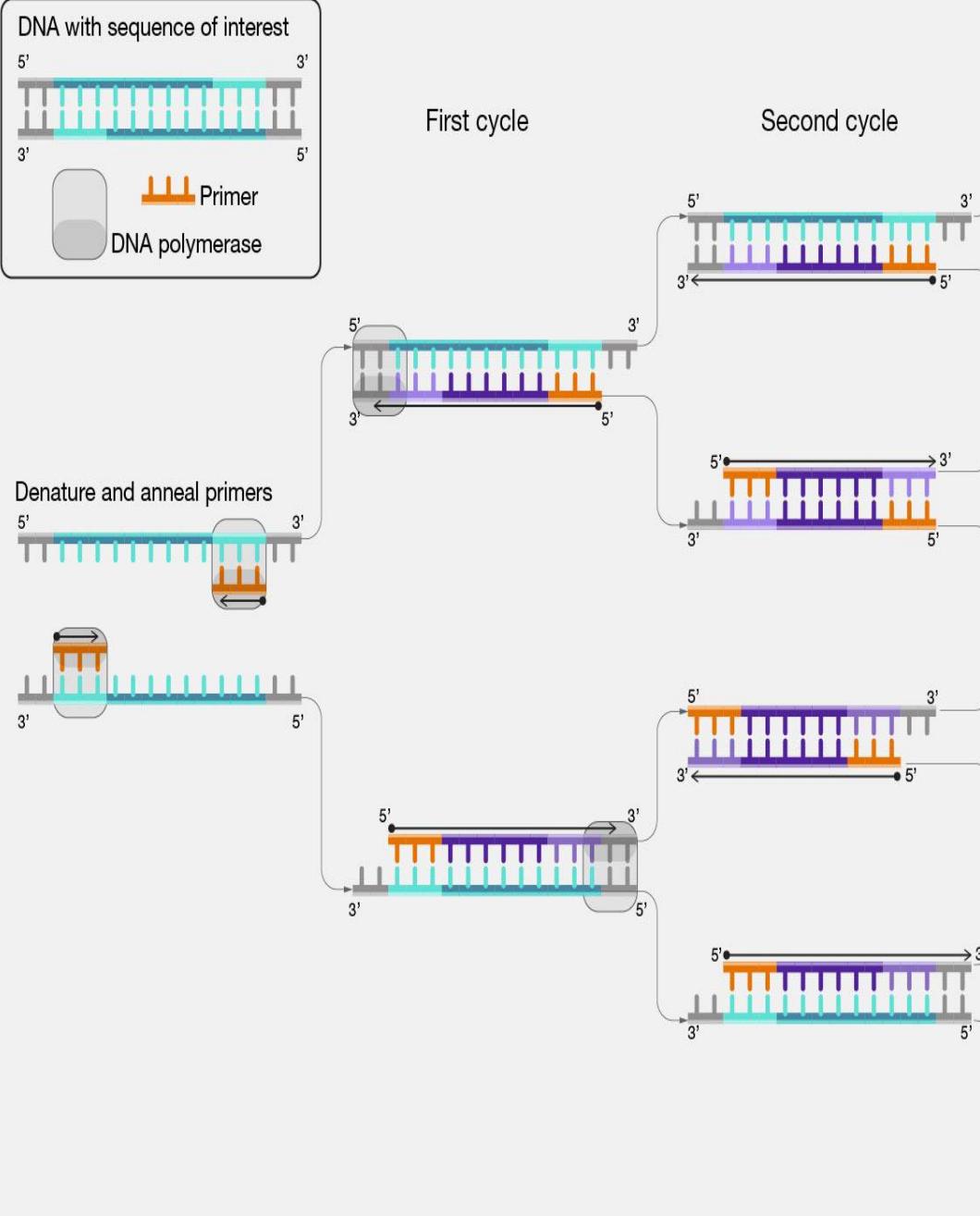


Step 3 - Extension

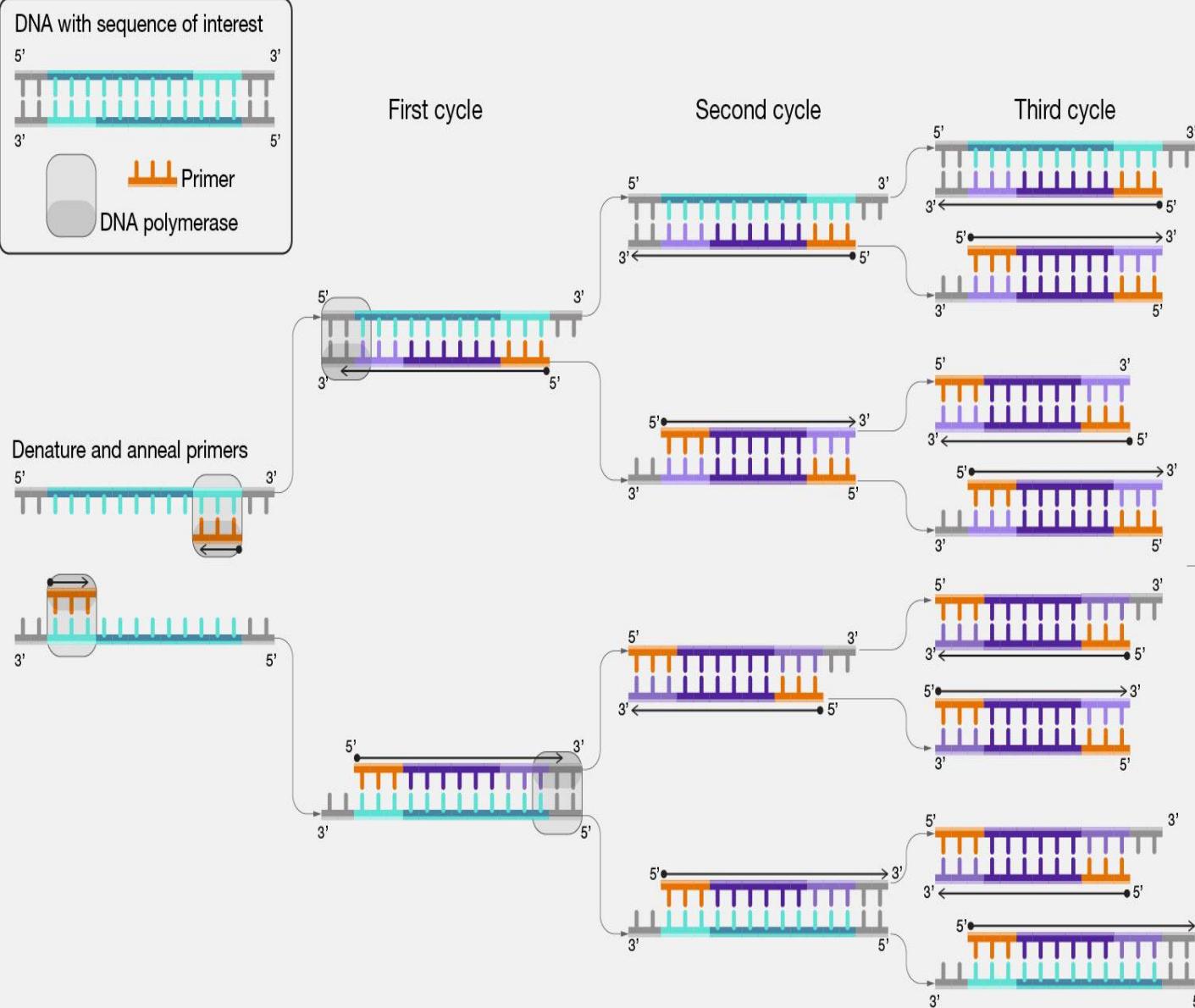


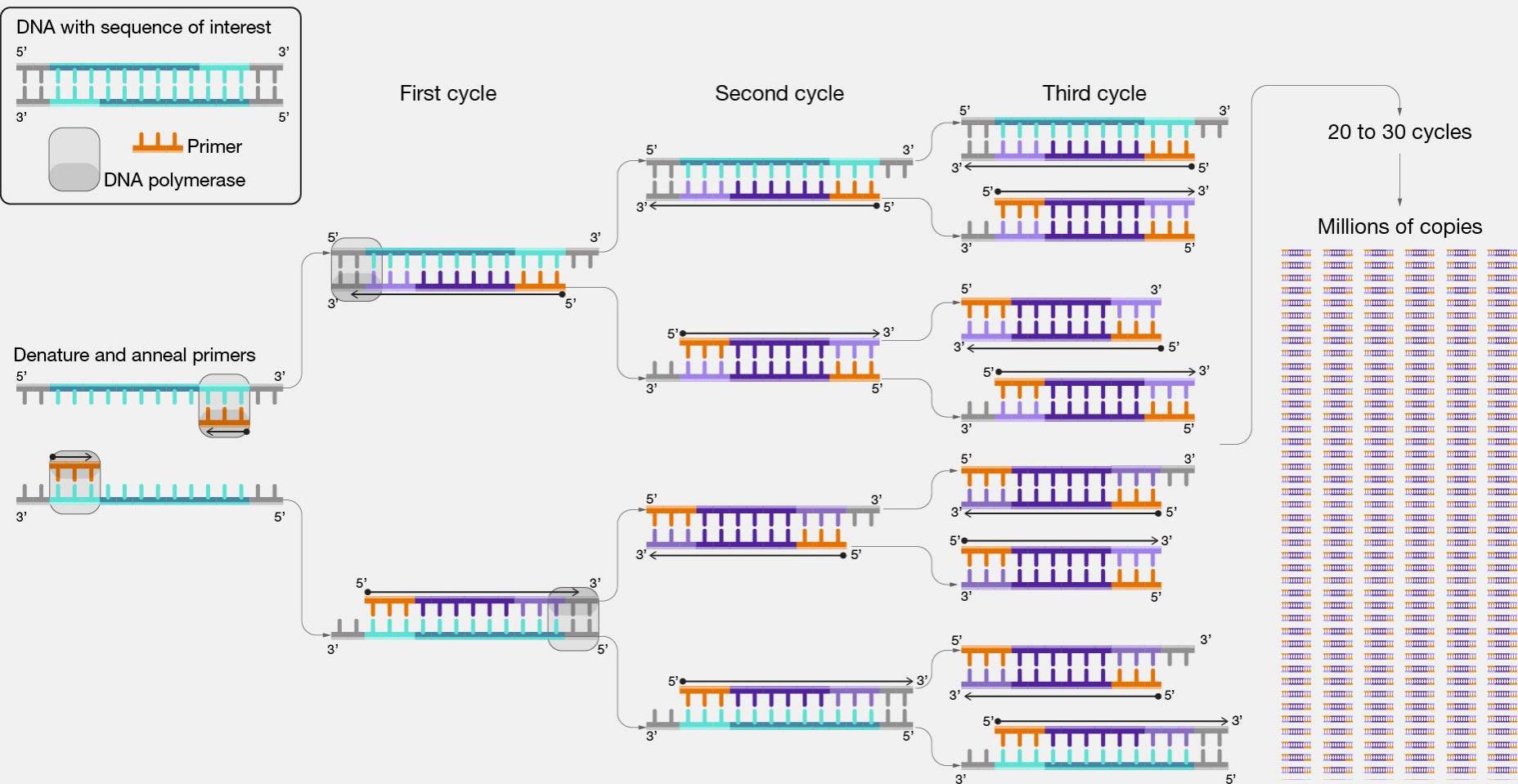
- Temperature increases
- Polymerase - amplicon extension
- Extension time

The PCR process then replicates this sequence repeatedly over 20 to 30 cycles



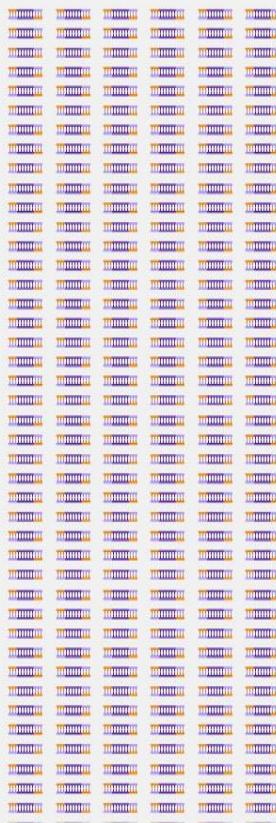
The number
of replicates
grows
exponentially





20 to 30 cycles

Millions of copies



The result - millions of exact copies of your genetic sequence, known as amplicons

The Thermocycler

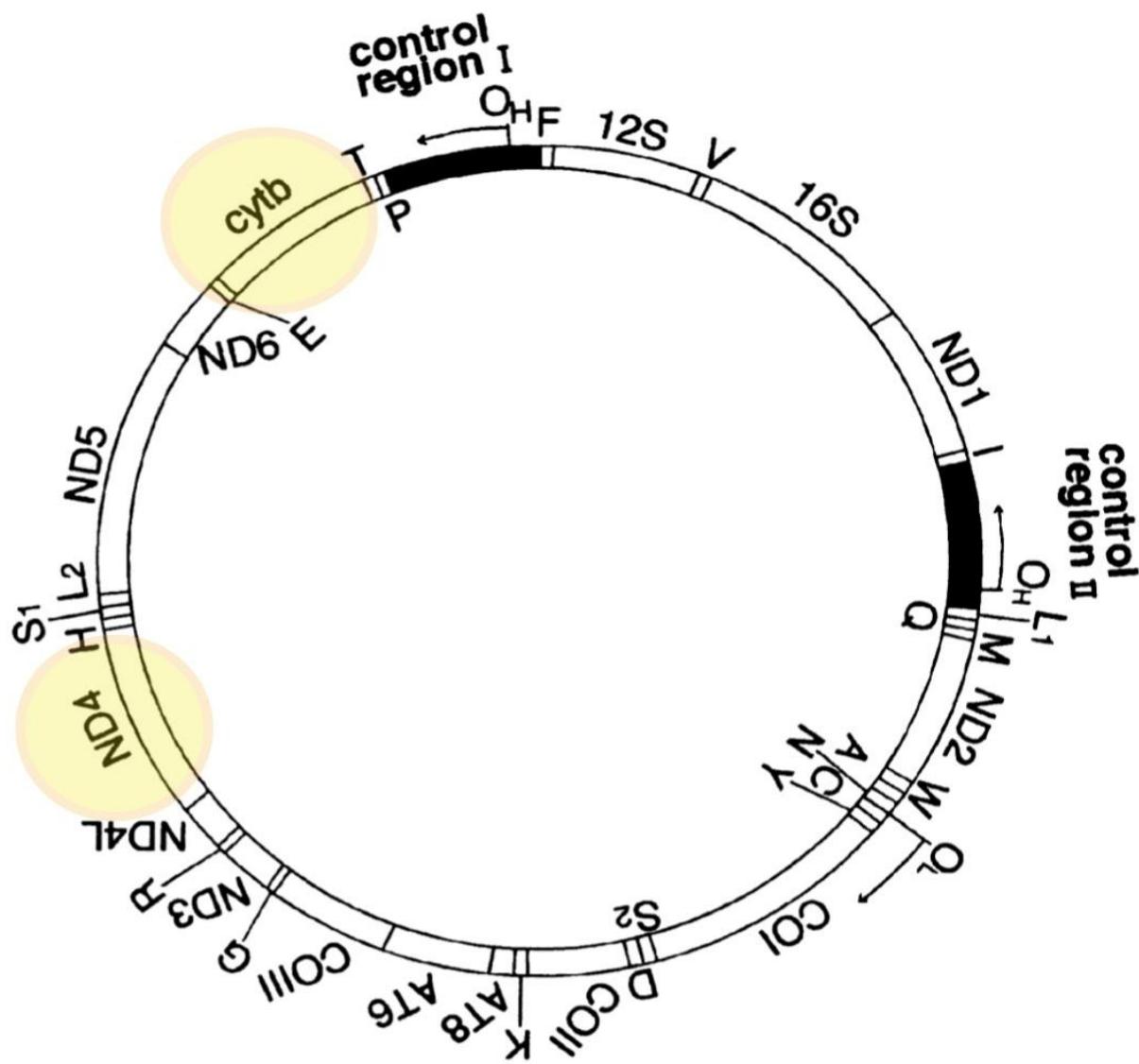


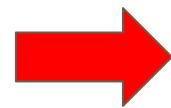
Easily
programmable.

Timings,
temperature,
cycles.

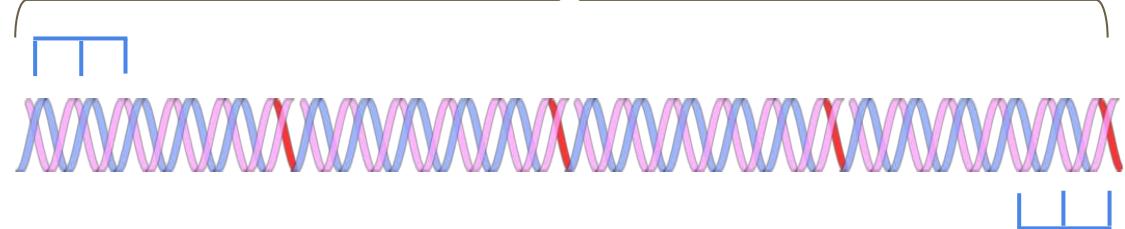
Tailor
conditions to
your sequence.

PCR 1 - Primer Selection

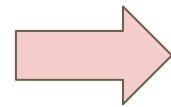


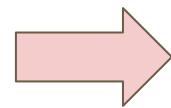


Amplicon

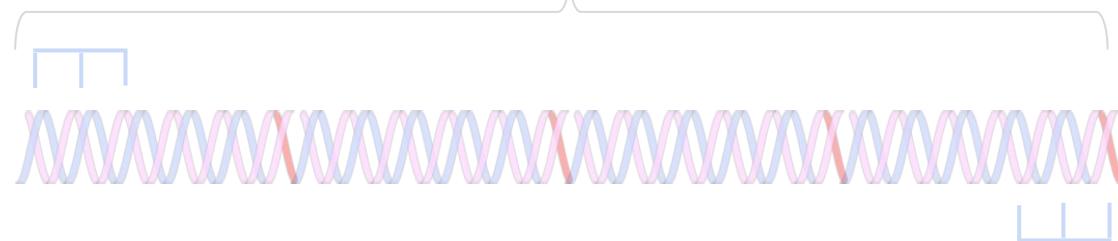


Amplicon length

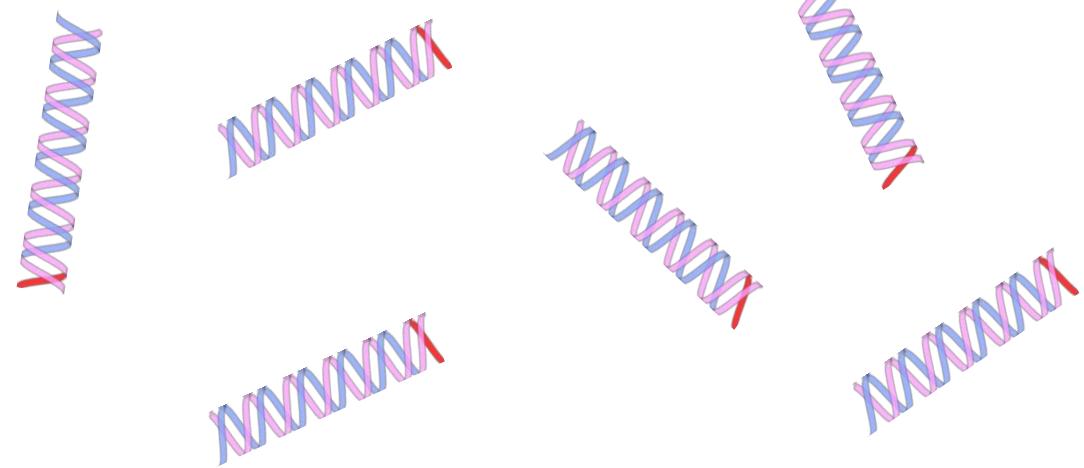
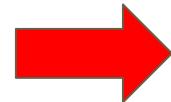




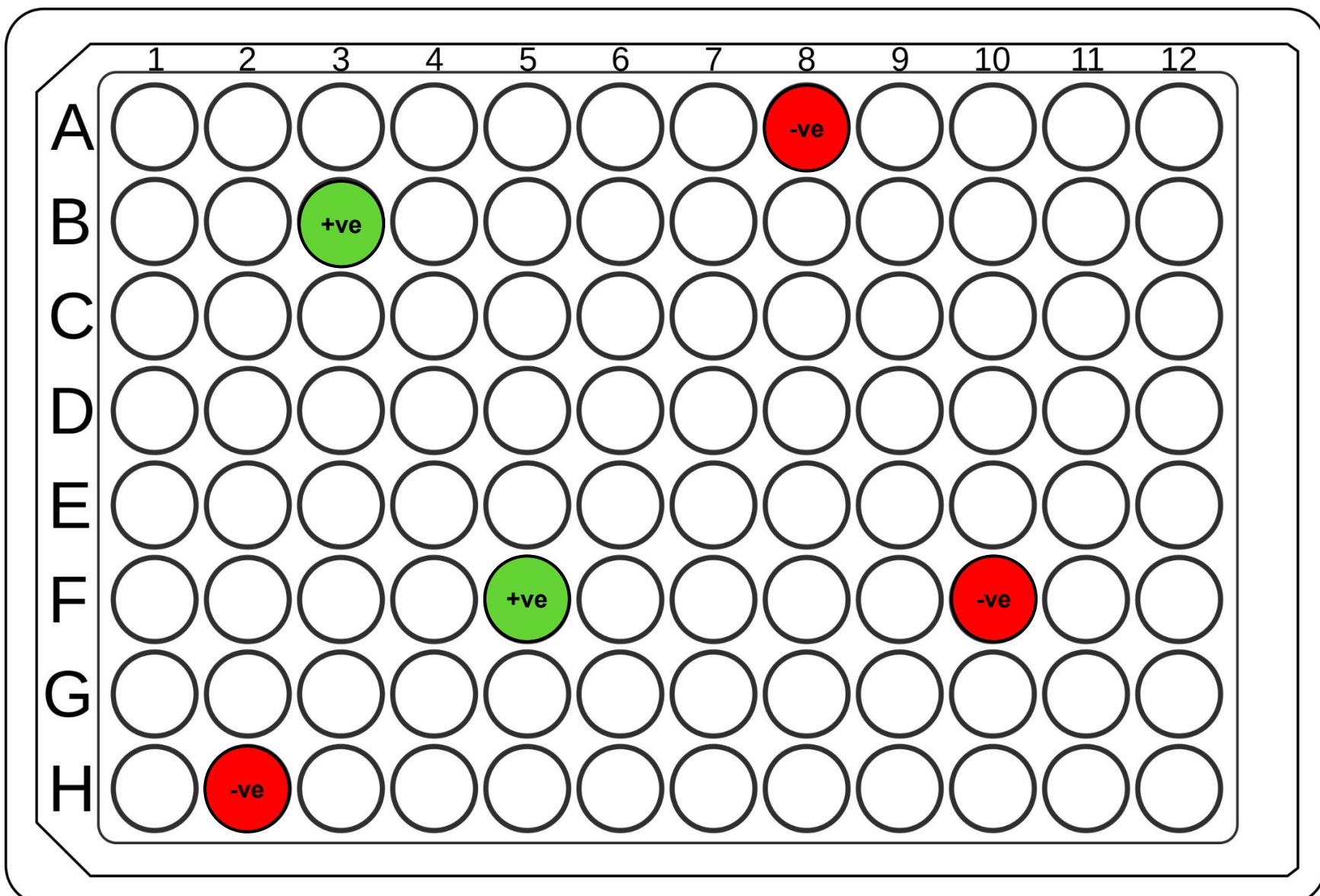
Amplicon



Amplicon length



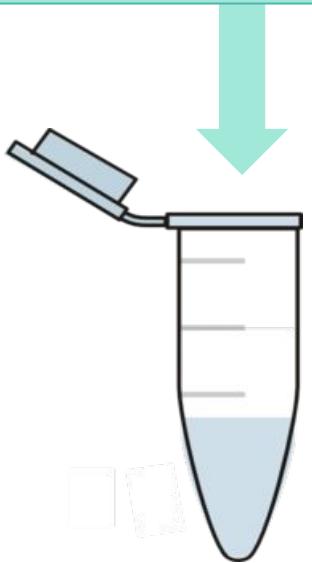
PCR 1 - Quality control



PCR 1 - Setting up a PCR

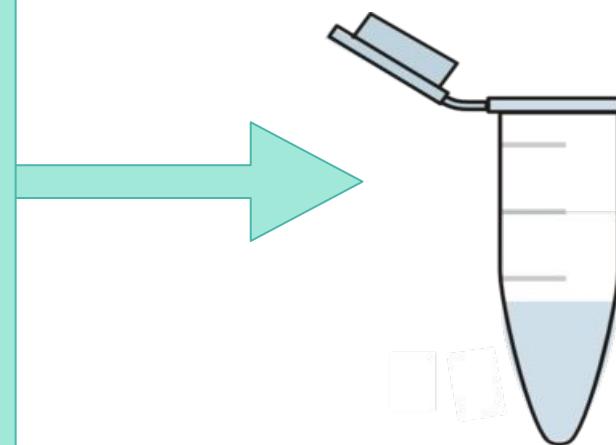
1 μ l extracted DNA

49 μ l ddH₂O

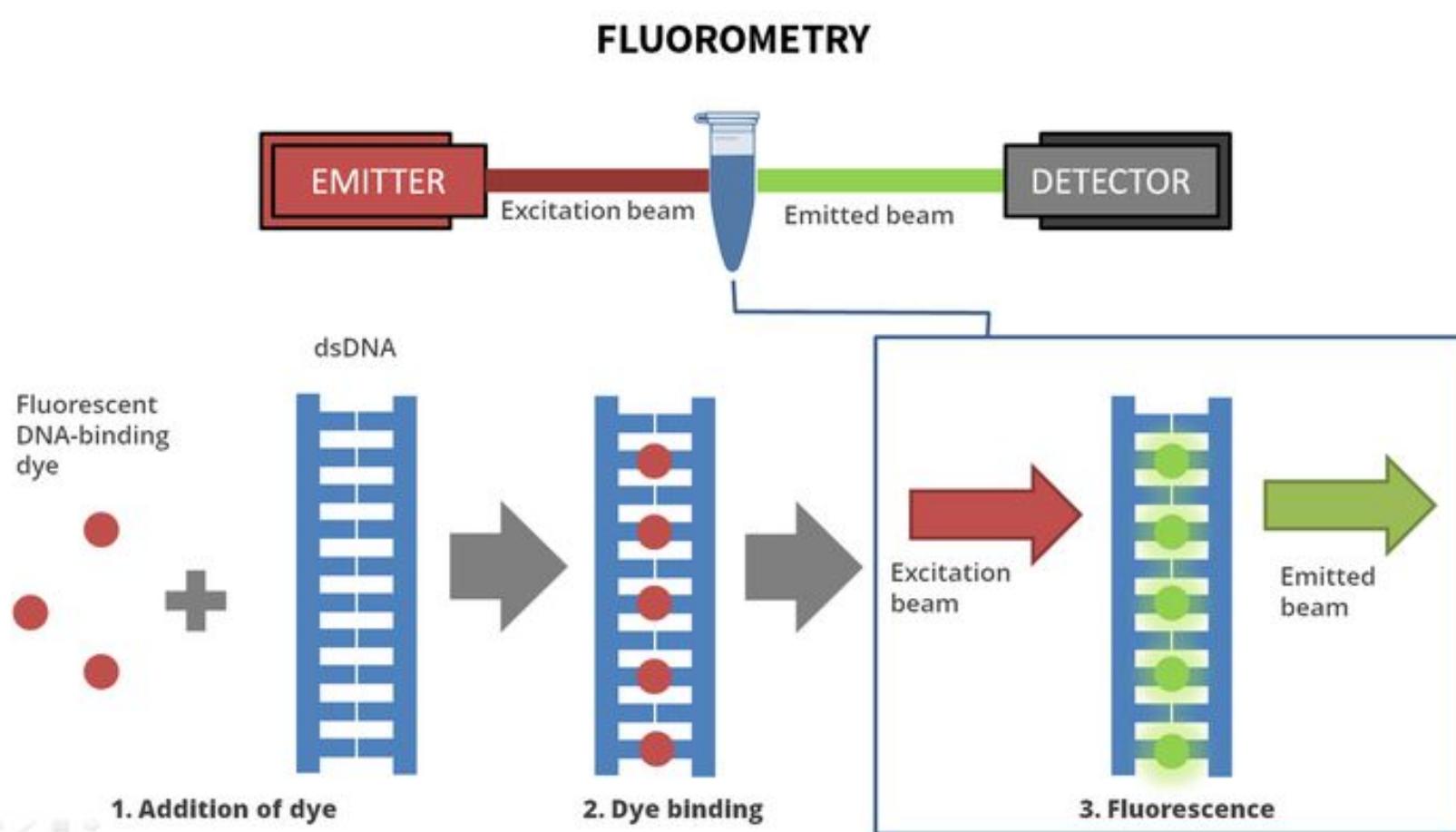


PCR 1 - Setting up a PCR

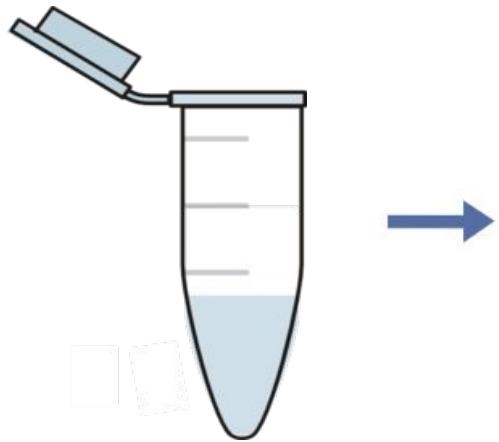
2 µl diluted DNA sample
1 µl 16s Reverse Primer
1 µl 16s Forward Primer
2 µl ddH₂O
4 µl MyTaq HS Mix



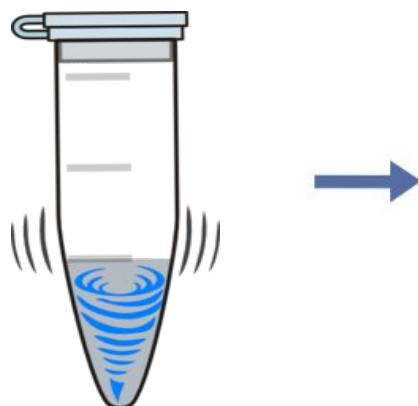
Quantification



Quantification

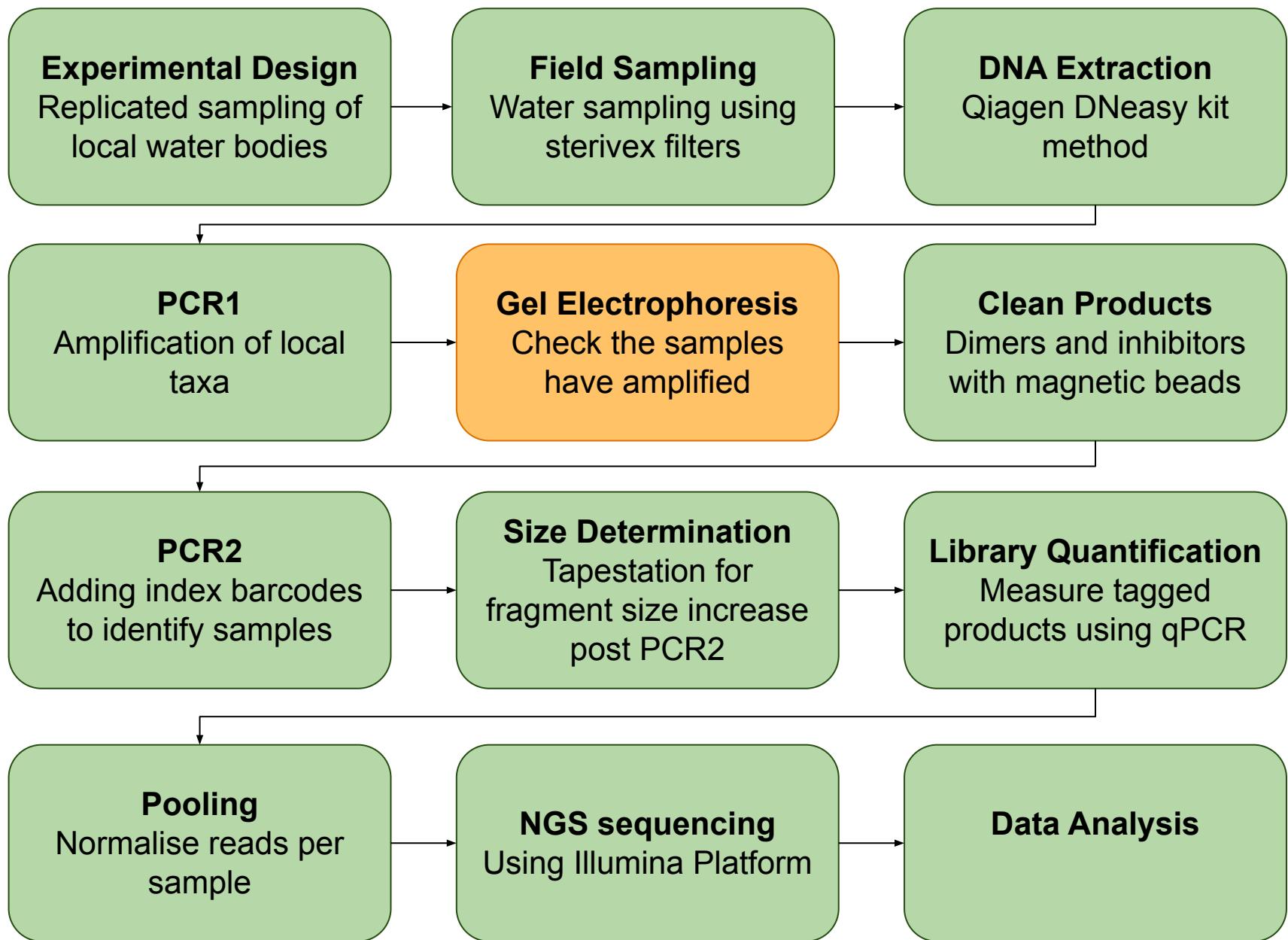


Add 1 μ l of
undiluted DNA to
Qubit Buffer

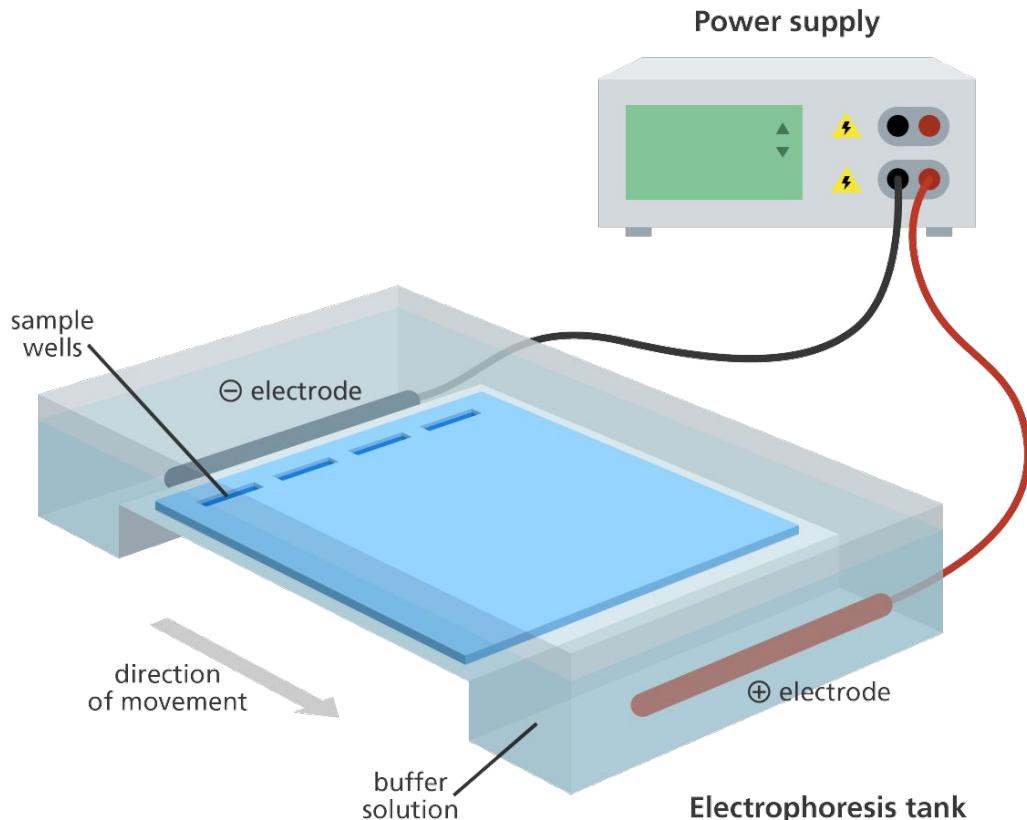


Vortex





Gel Electrophoresis

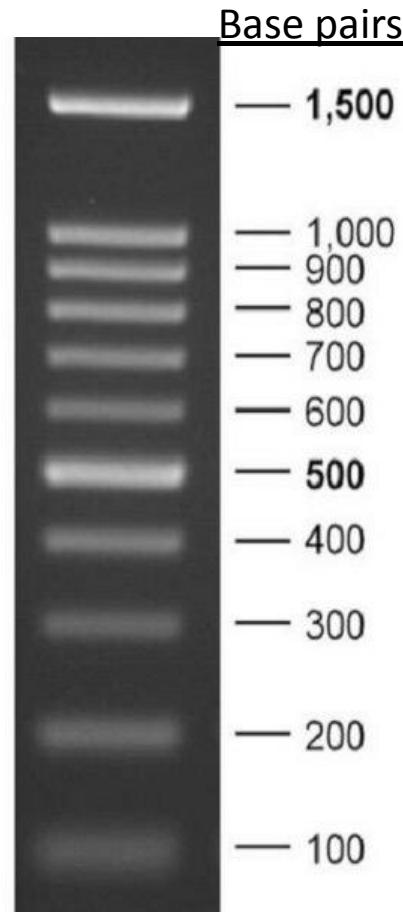


Visualise your products post-amplification.

The PCR products are drawn through the gel by an electrical current.

Gel Electrophoresis

100bp ladder

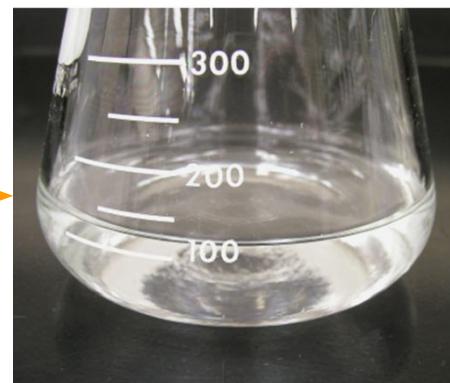
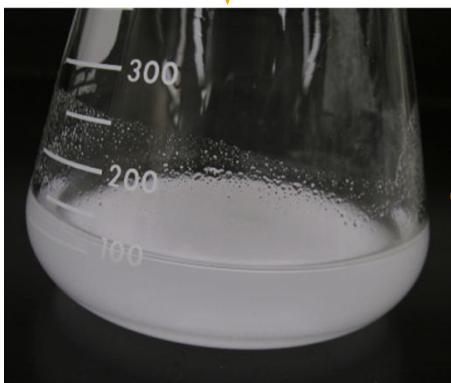


A ladder is usually added to the first lane in order to visualise the length of your PCR products, measured in base pairs.

Making a 1% Agarose Gel

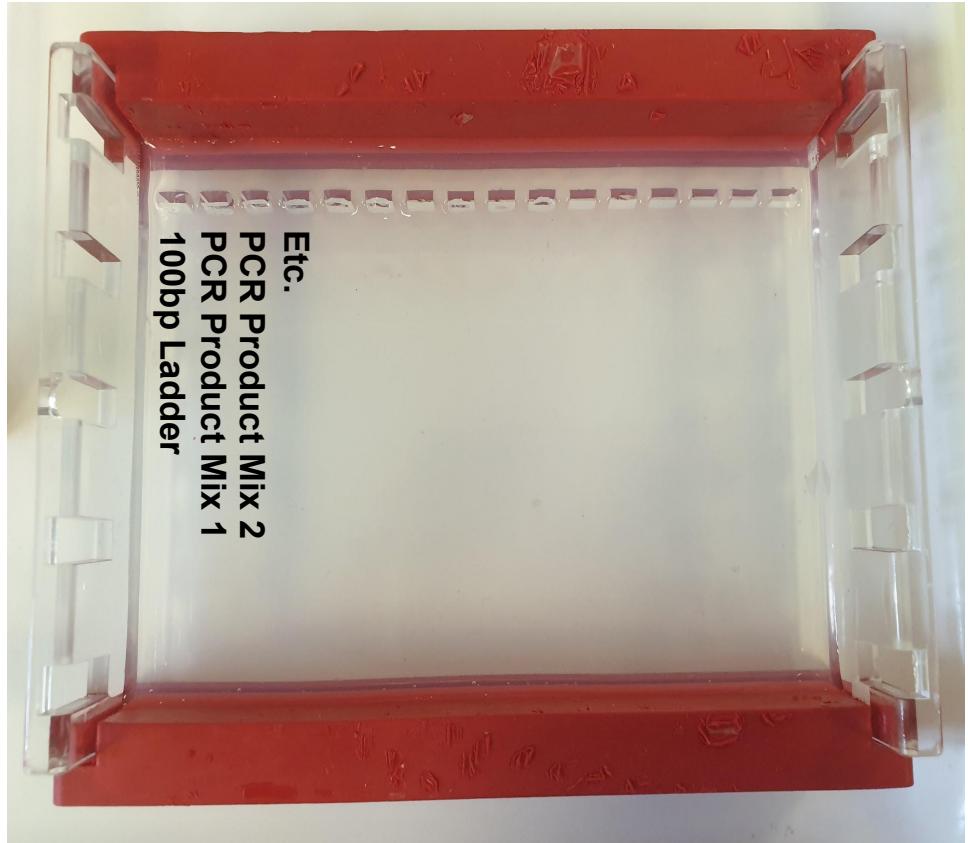
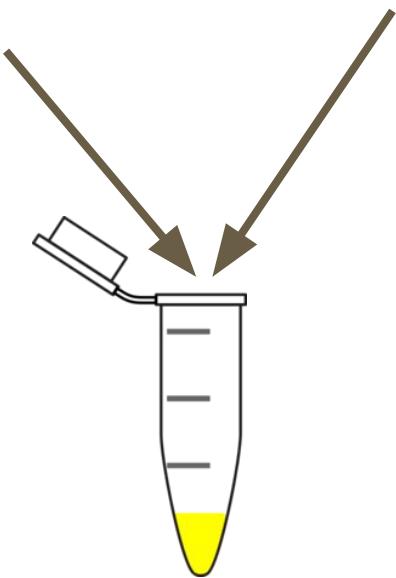


Goggles and heat proof gloves



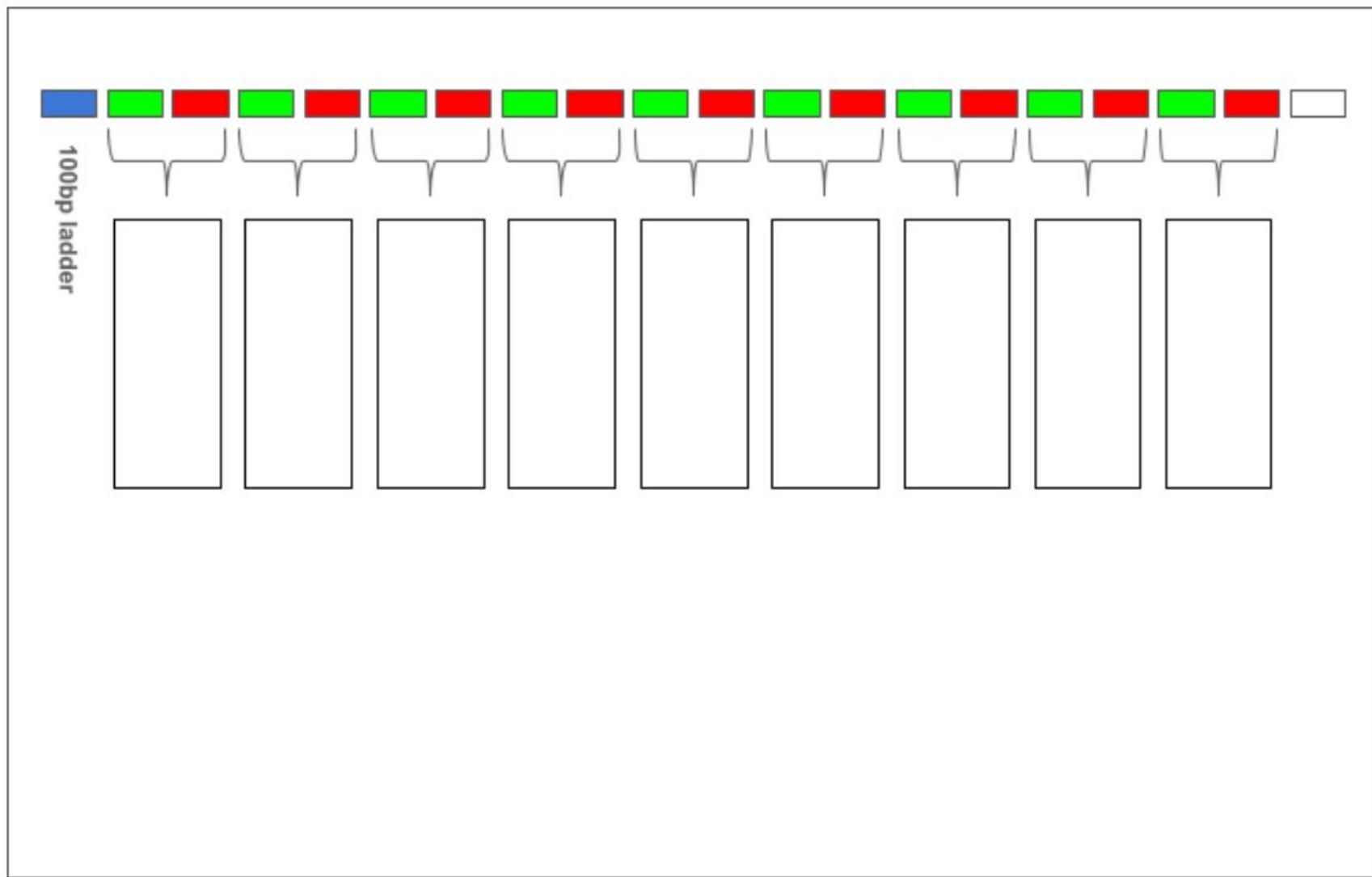
Loading the Gel

4 μ l PCR product
6 μ l Orange G



Be careful not to pierce the gel!

Gel Lane Map

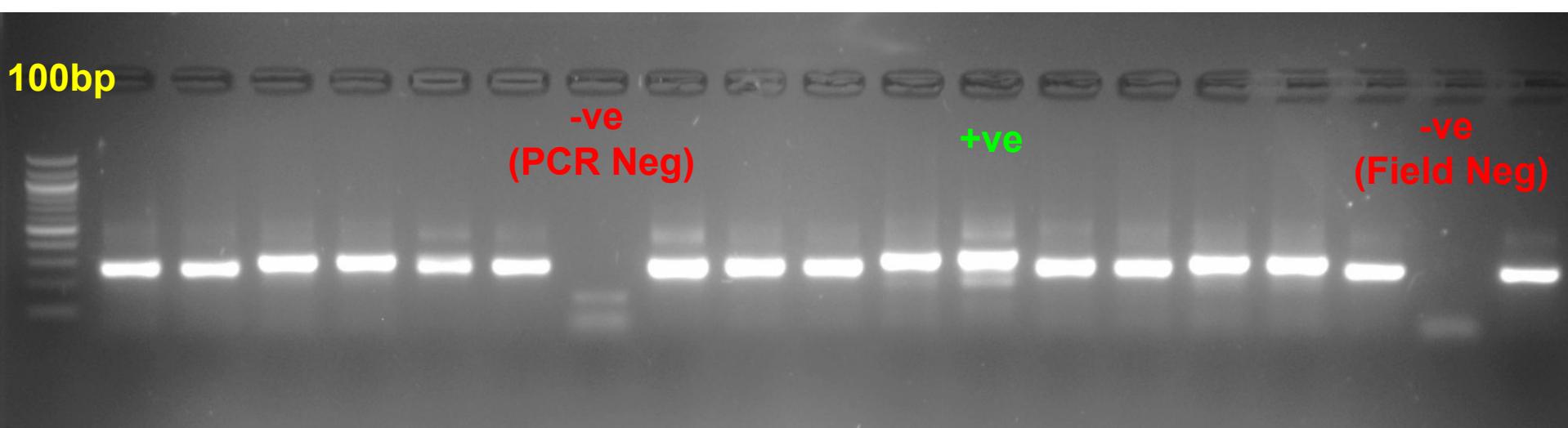


PCR 1 - Visualising Products



A gel imaging dock like this uses UV light to illuminate the PCR products in the gel

PCR 1 - Visualising Products

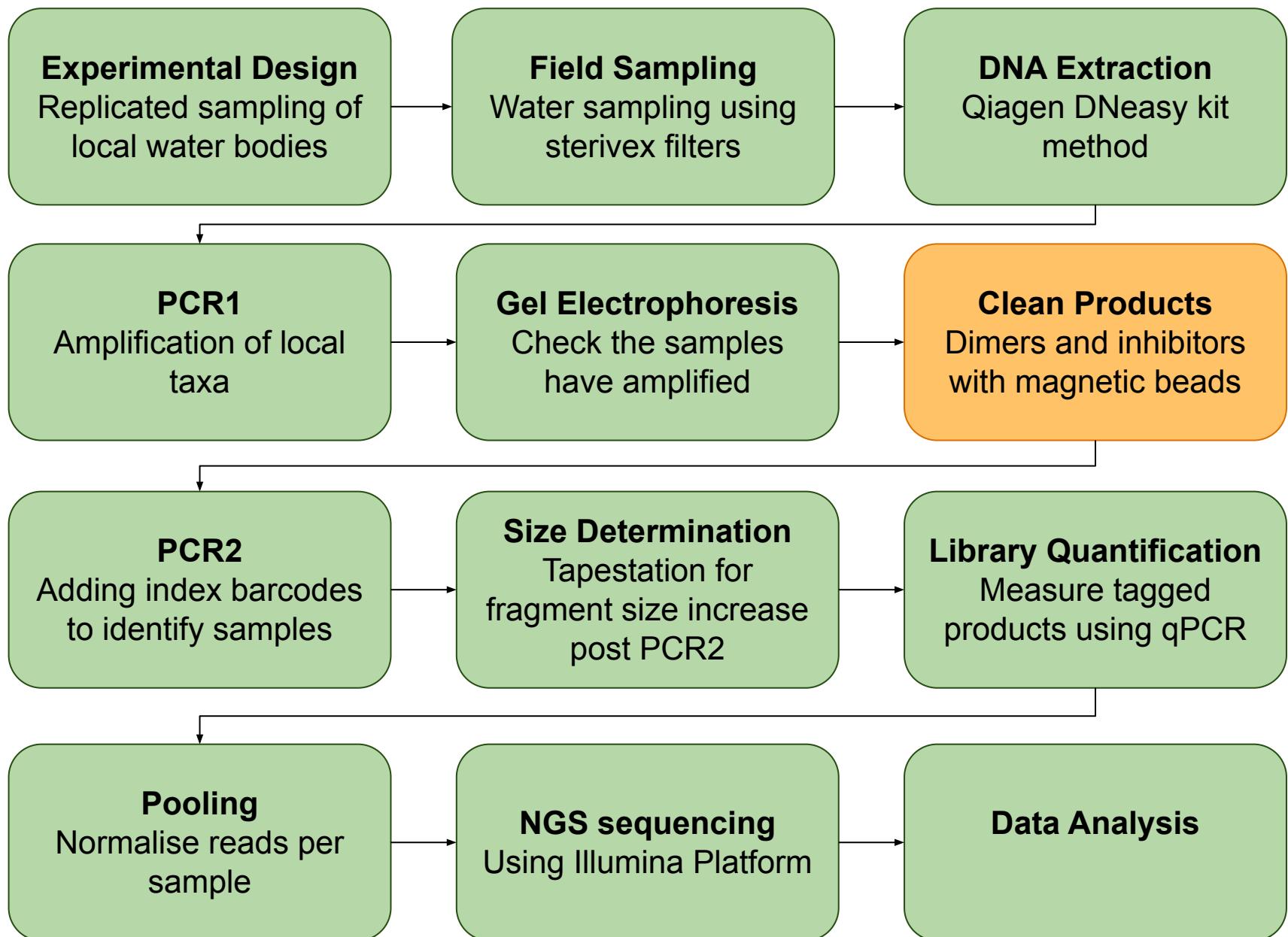


Gel viewings

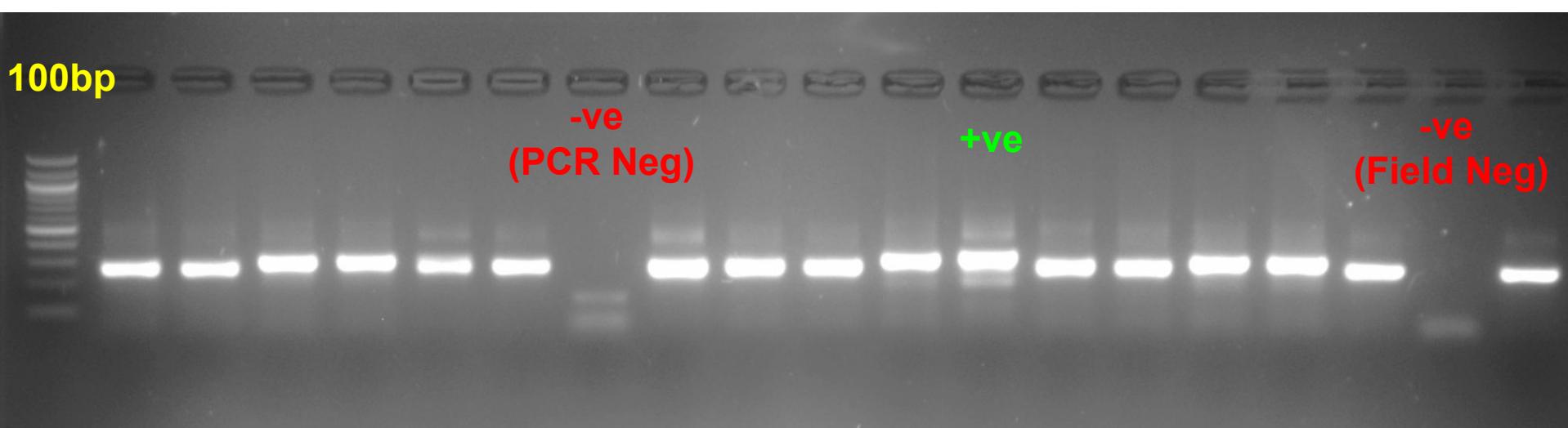


Lab tours

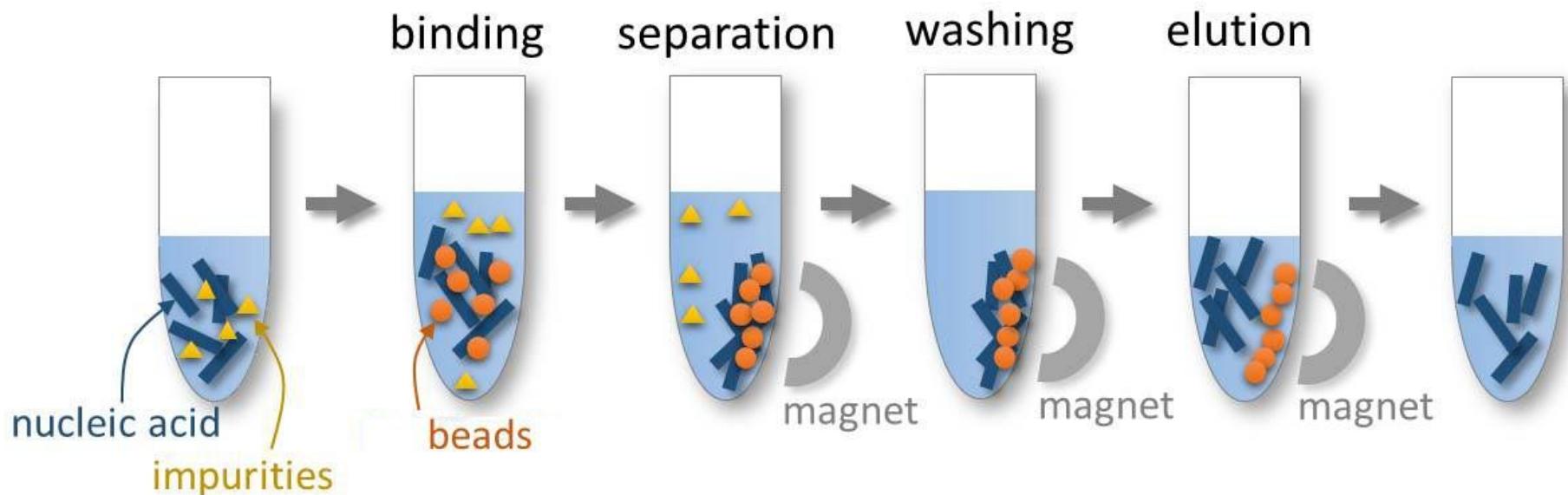




PCR 1 - Visualising Products

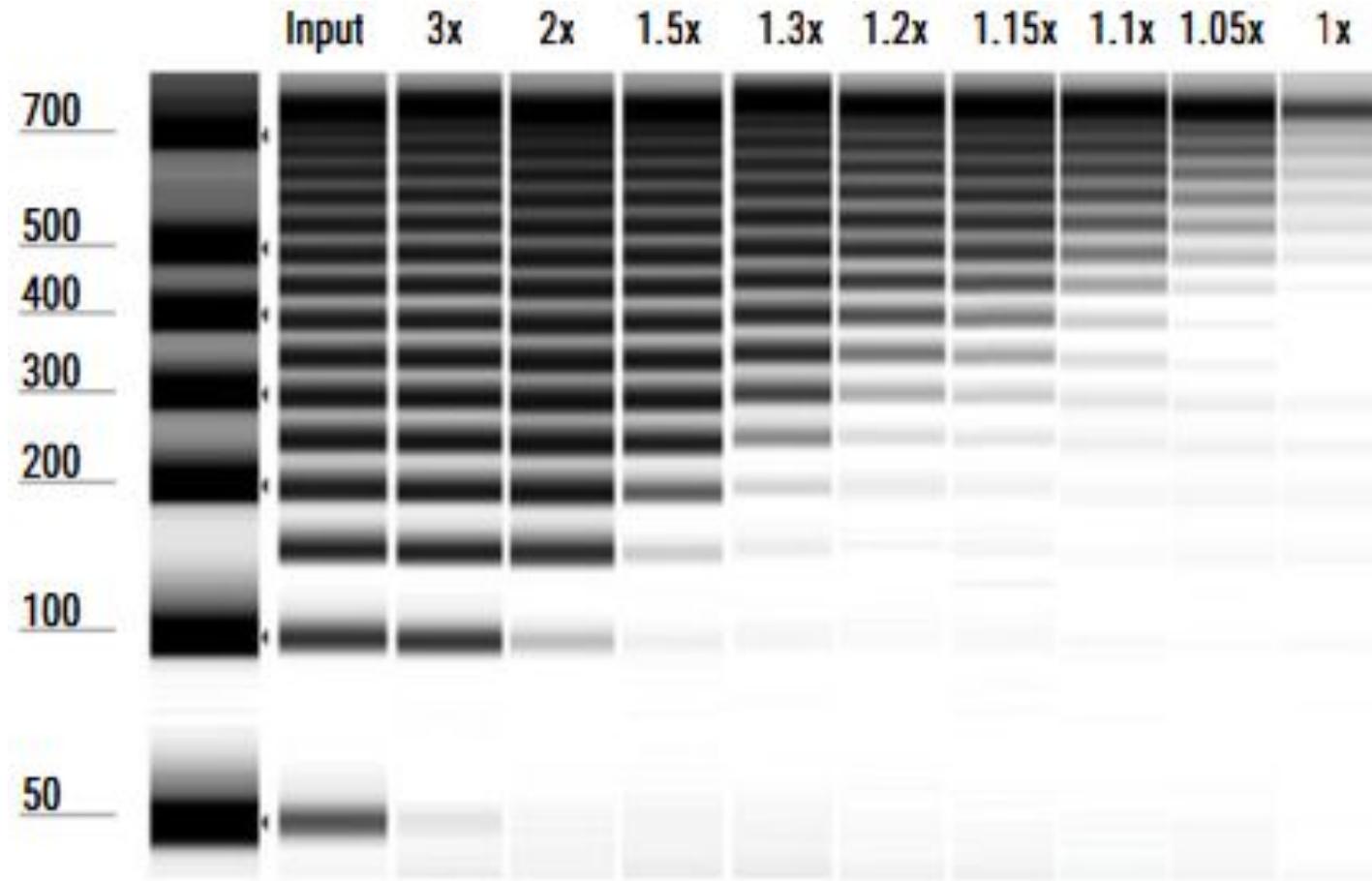


PCR 1 - Bead cleaning

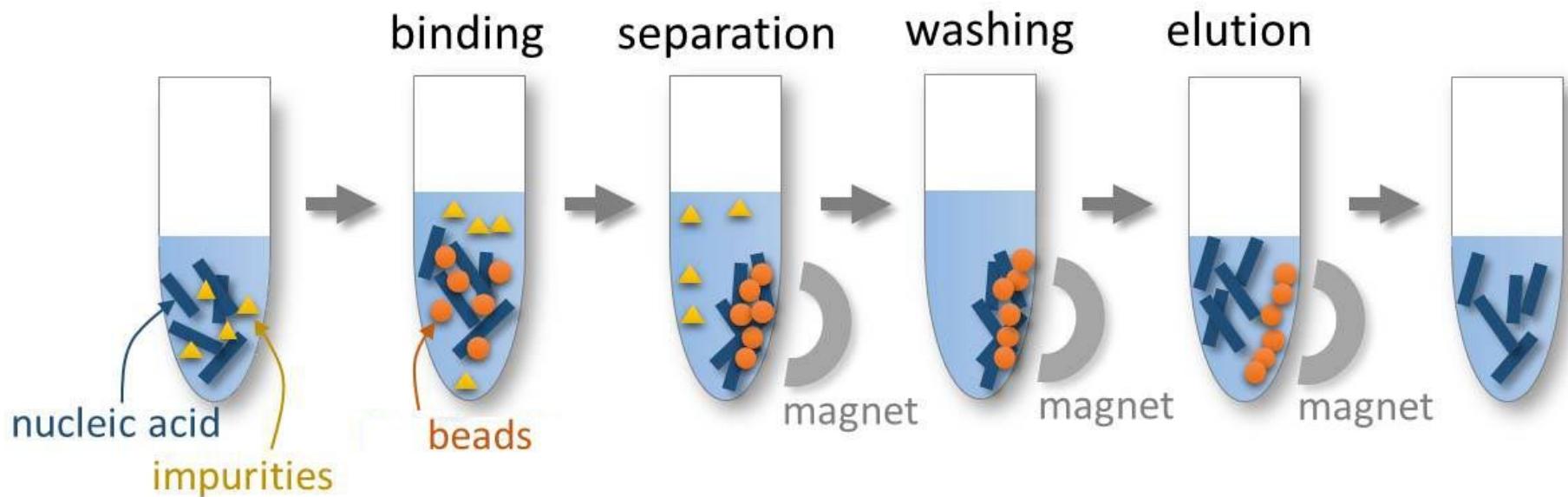


Strong magnet

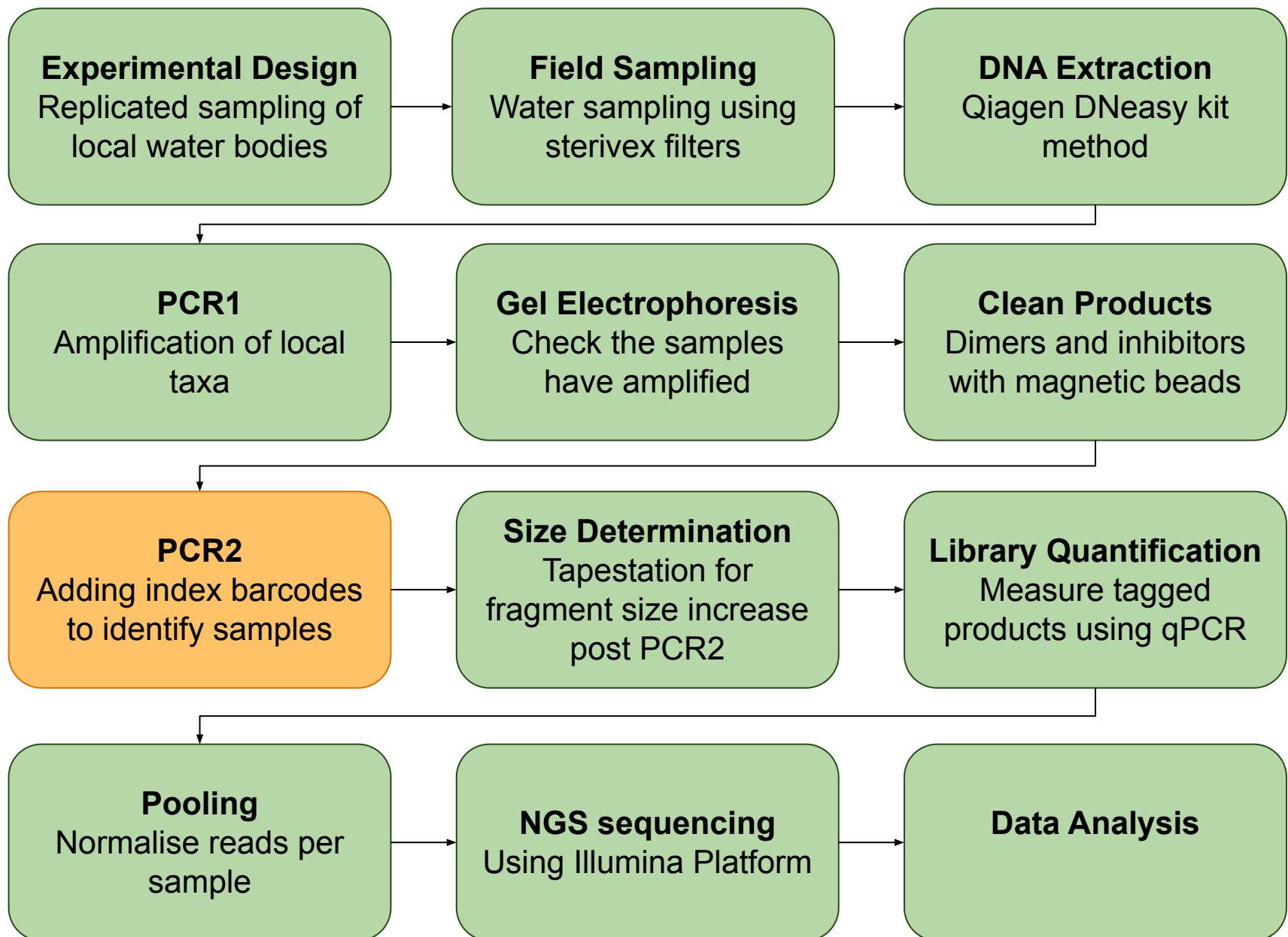
PCR 1 - Bead cleaning



PCR 1 - Bead cleaning



Strong magnet



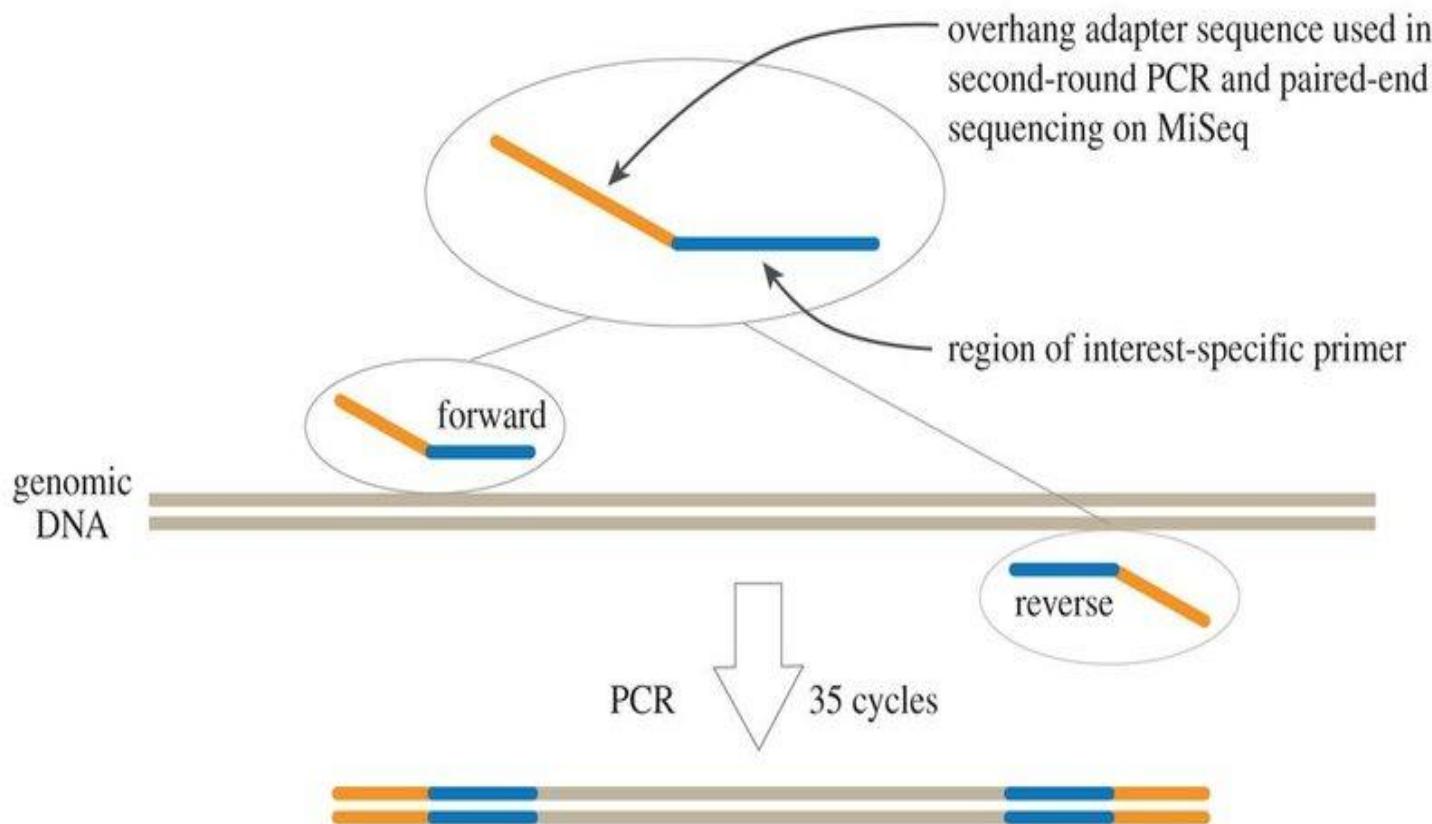
PCR 1:

Primers (Blue) incorporate an overhang adapter sequence (Orange) at the ends of our amplicons

F: 5'ACACTTTCCCTACACGACGCTTCCGATCTNNNNNNGTGCCAGCMGCCGCGGTAA3'

R: 5'GTGACTGGAGTTCAGACGTGTGCTCTCCGATCTGGACTACHVGGGTWTCTAAT3'

first-round tailed PCR to amplify regions of interest

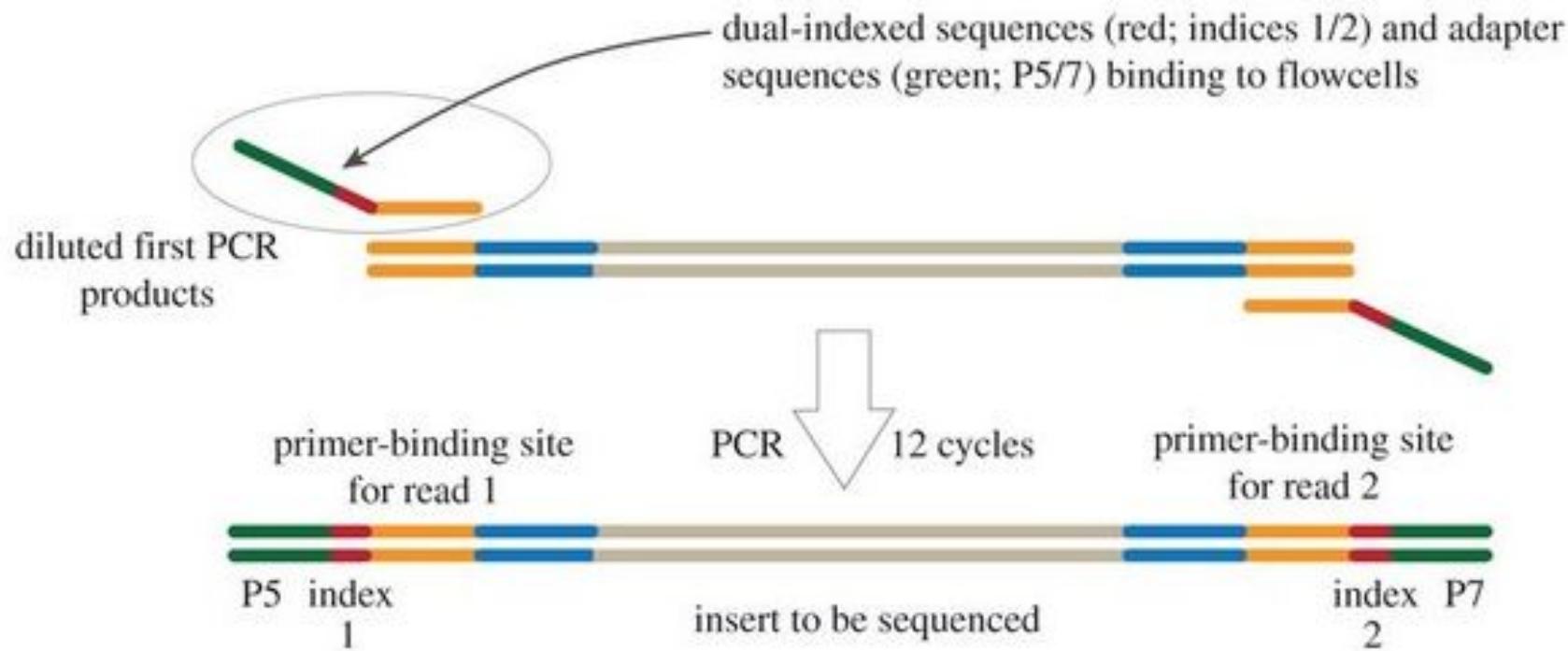


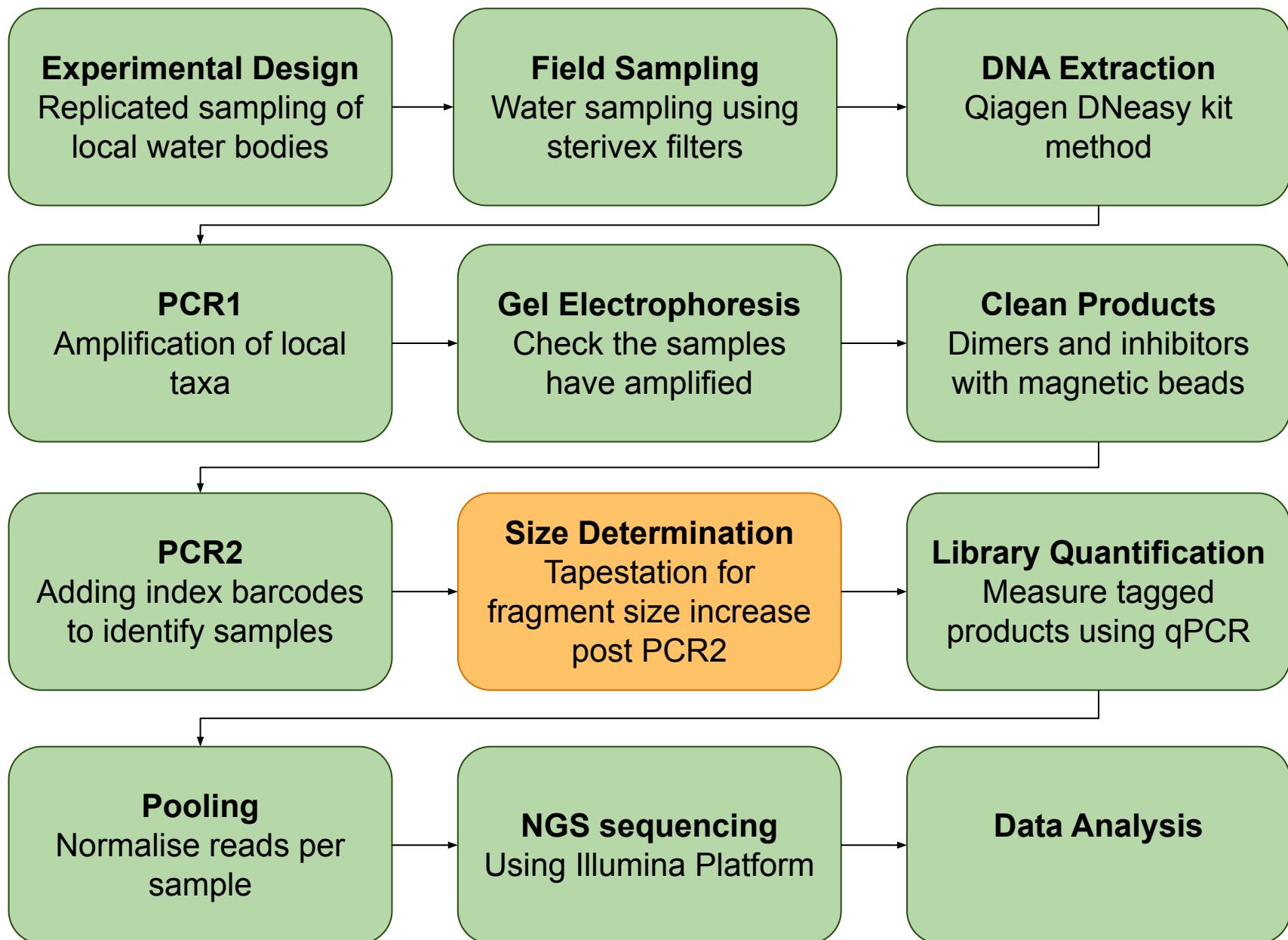
PCR 2:

Unique identifier sequences are attached to our PCR 1 products

i5 index seq AATGATAACGGCGACCACCGAGATCACACATGCTTACTGACACTCTTCCCTACACGACGCTTCCGATCT

i7 index seq CAAGCAGAAGACGGCATACGAGATACTGGTCCGGTACTGGAGTTCAGACGTGTGCTTCCGATCT





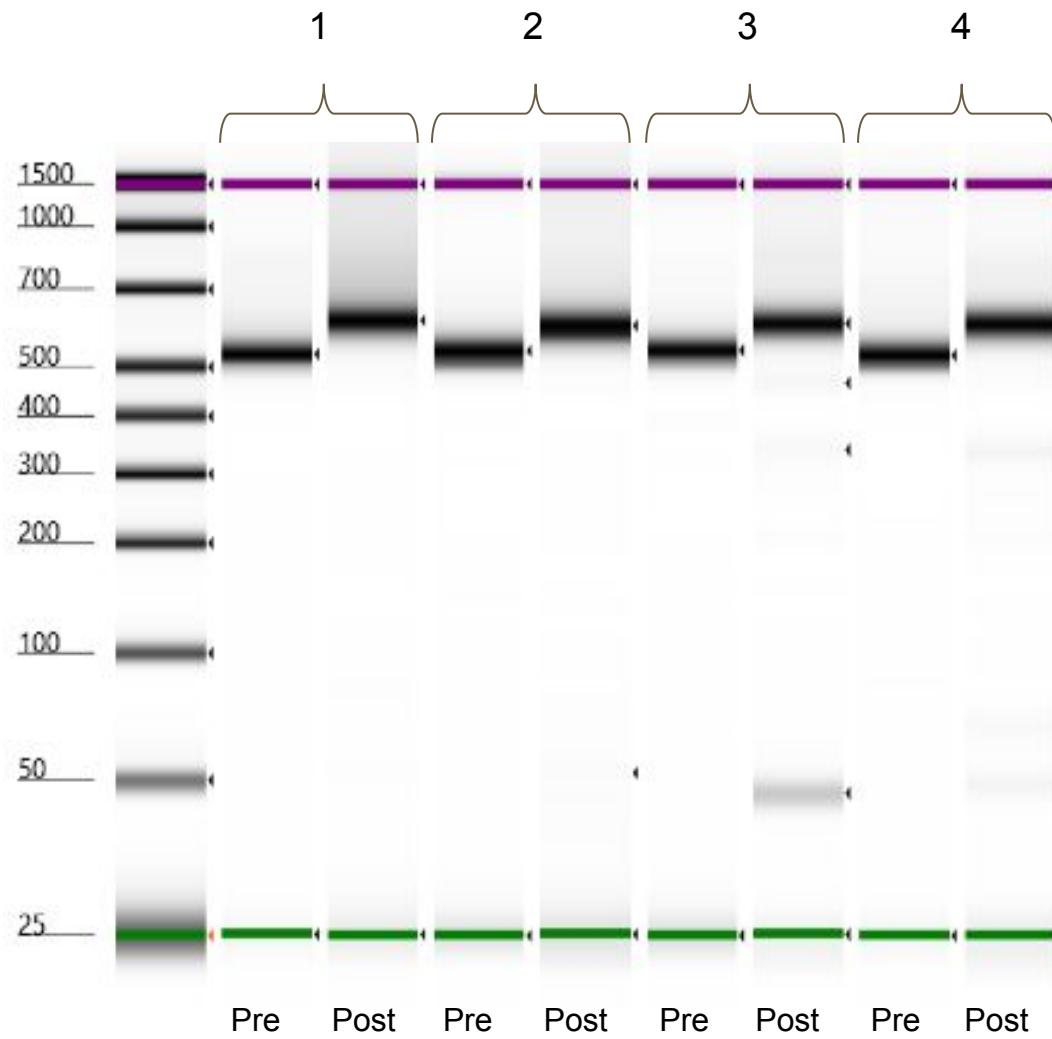
PCR 2 - TapeStation



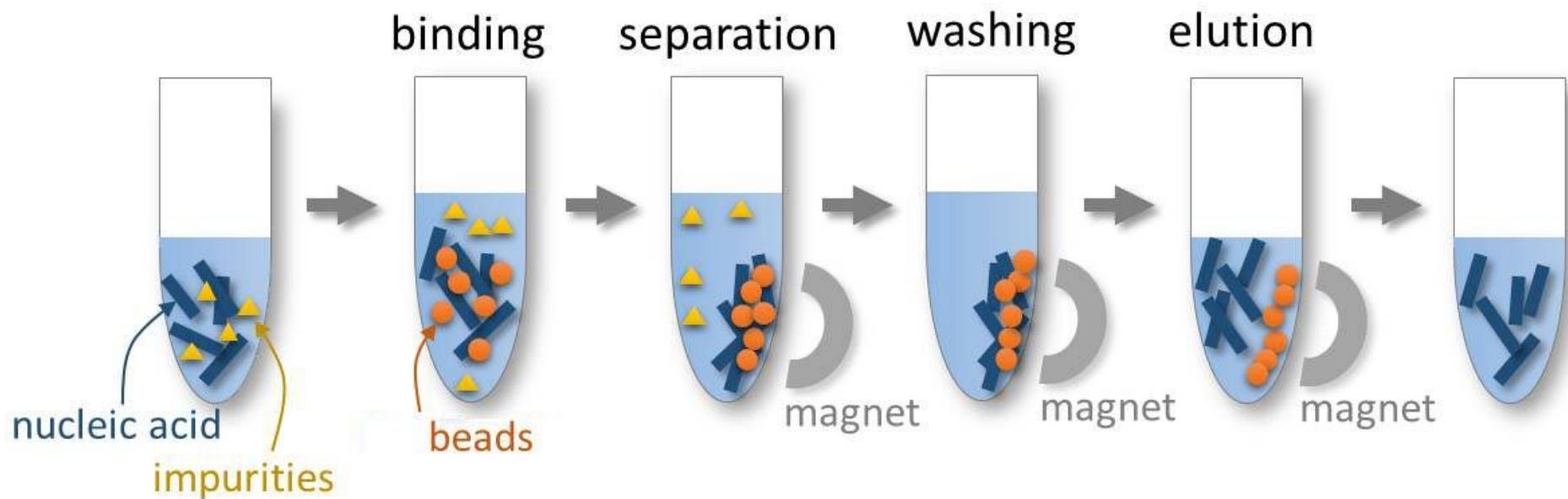
Works like a
miniature gel.

Compare
samples pre
and post-PCR 2.

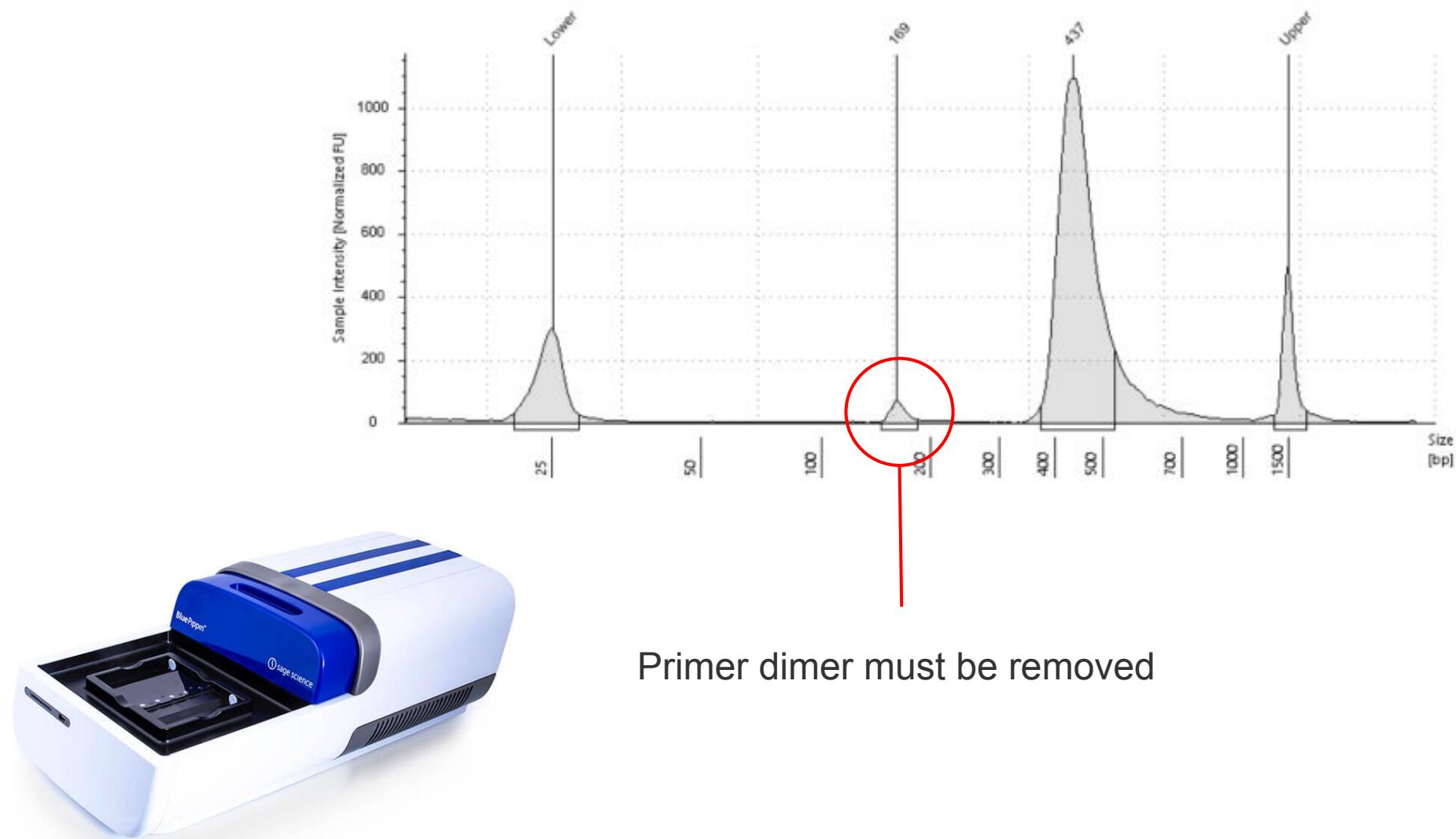
PCR 2 - Tapestation



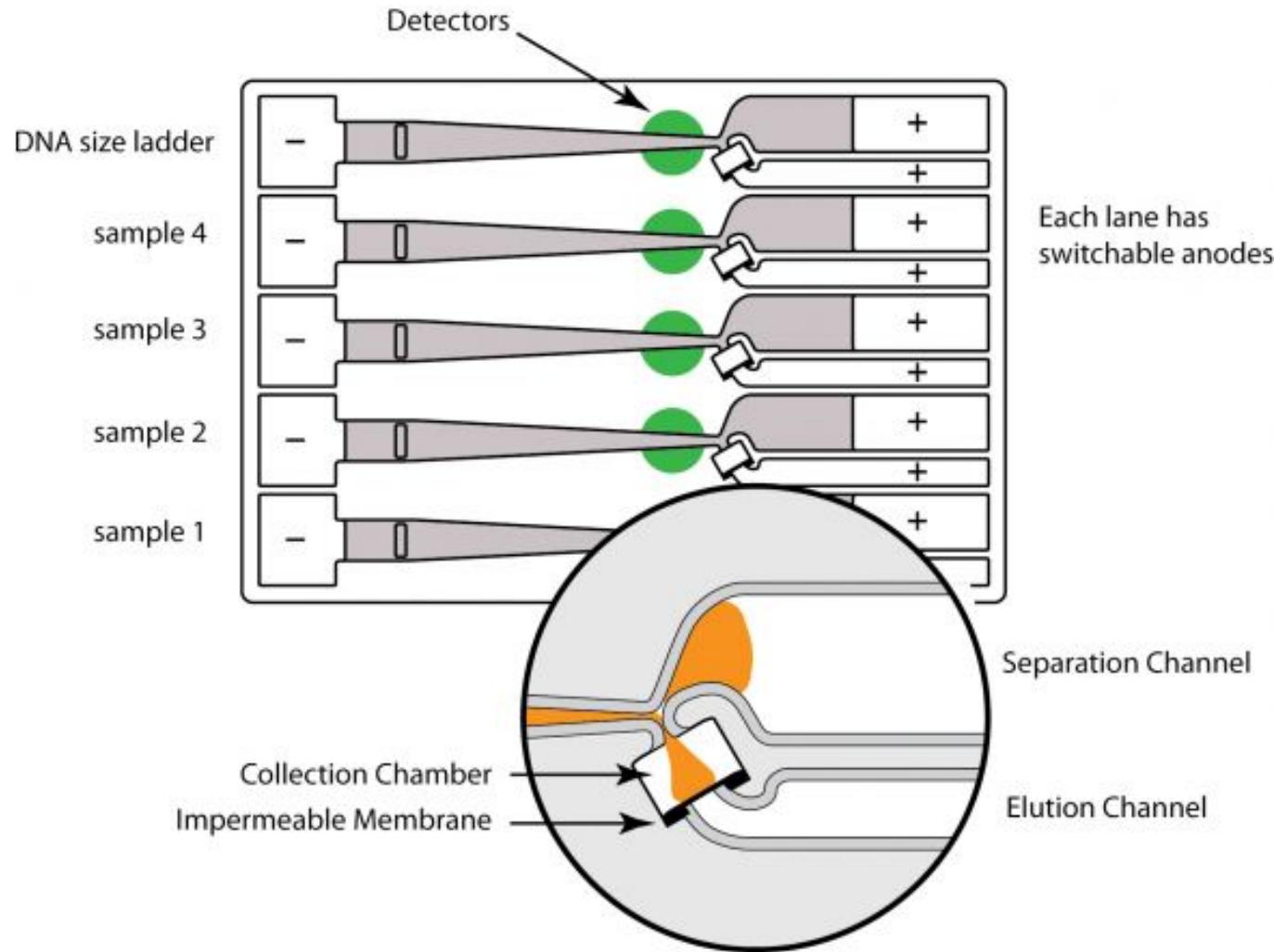
PCR 2 - Another Bead Clean

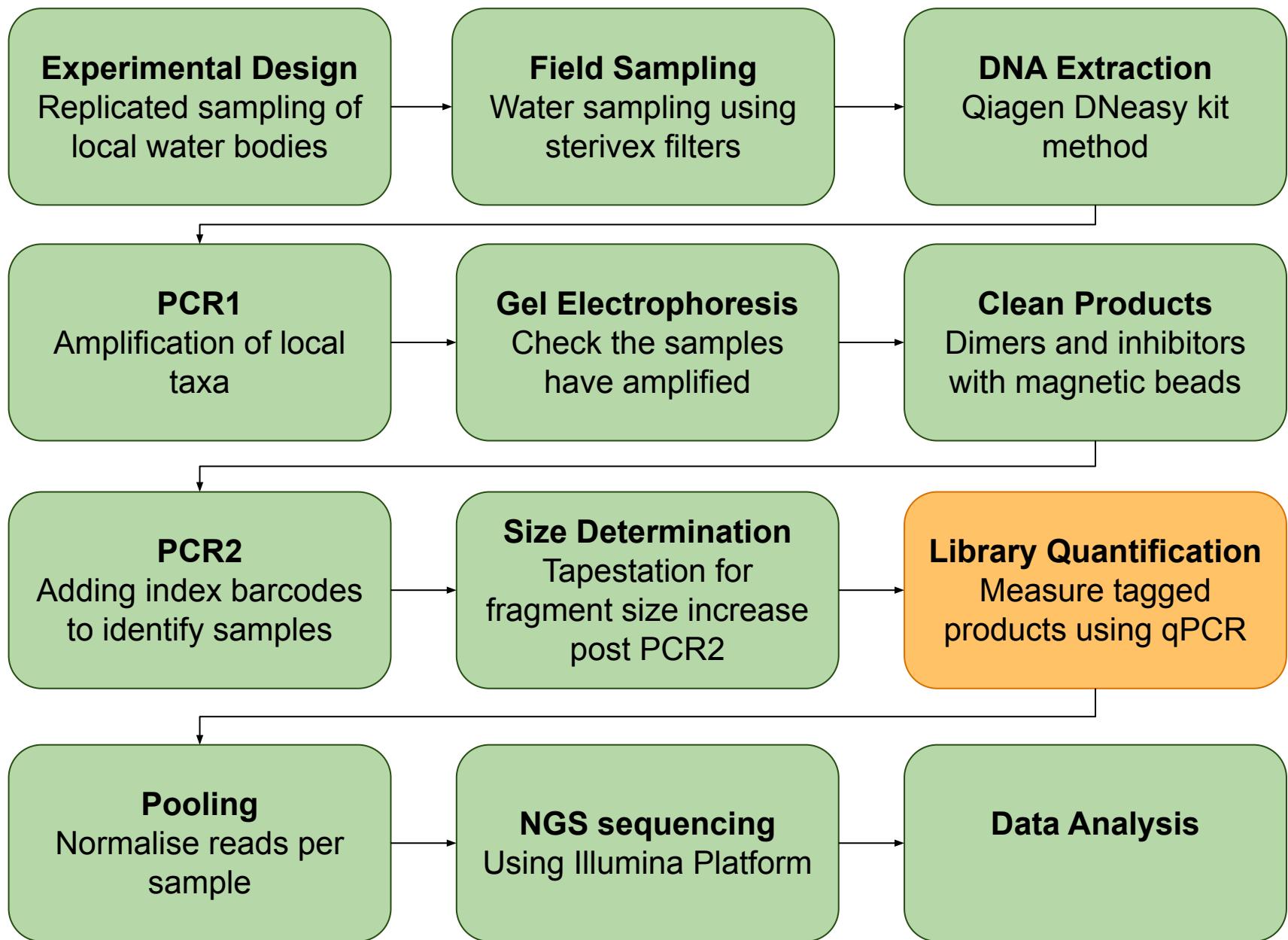


Blue Pippin - When Bead Cleaning Falls Short



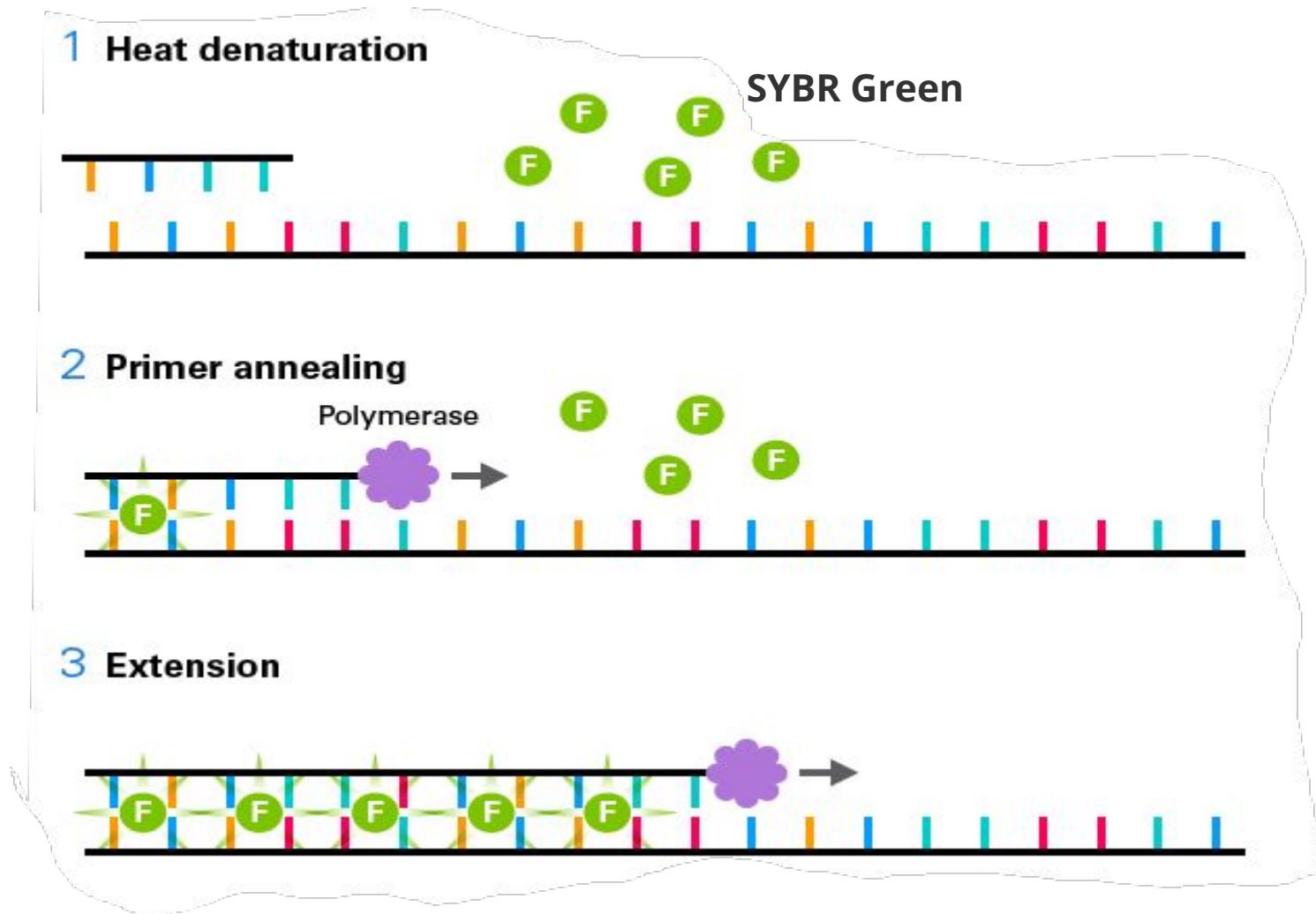
Blue Pippin - When Bead Cleaning Falls Short





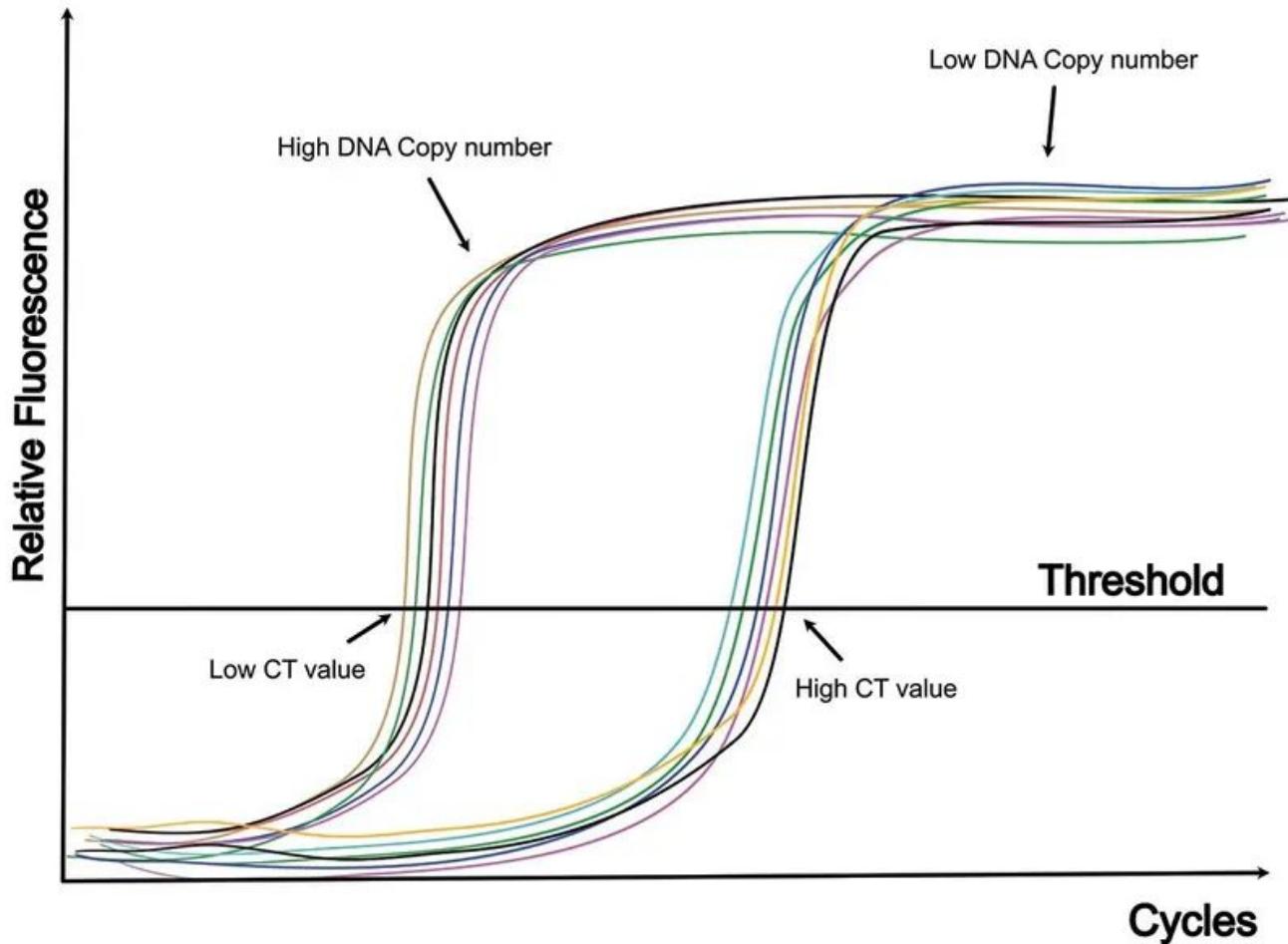
Quantification - qPCR

SYBR Green Assay

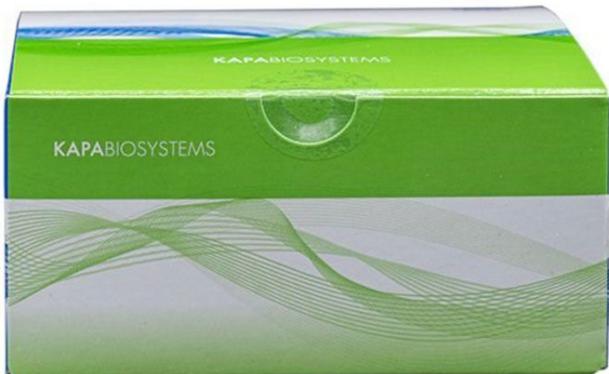


Quantification - qPCR

Amplification curve
real-time PCR

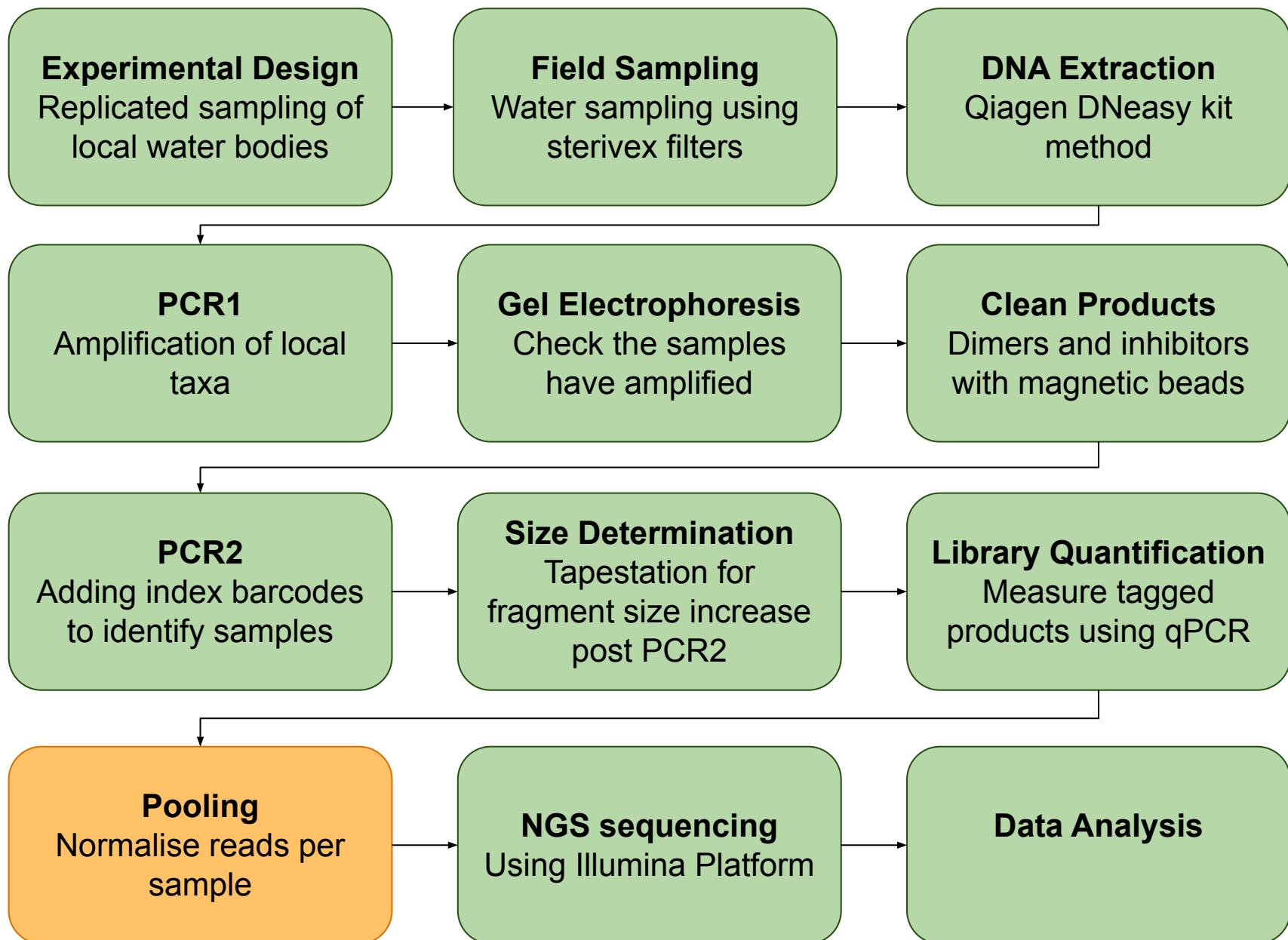


Quantification - qPCR



Unlabelled DNA
still present after
PCR2.

Products that only
have unique
identifier
sequences
attached can be
quantified using
qPCR.



Pooling - Balancing Sample Concentrations

$$\frac{(\text{Final Volume } (\mu\text{l}) \times \text{Final Concentration } (\text{ng}/\mu\text{l}))}{(\text{Number of Samples} \times \text{Initial Concentration } (\text{ng}/\mu\text{l}))} = \text{Volume to Pool } (\mu\text{l})$$

X02 = 13.39

Total Volume to Pool (μ l) = 42.87

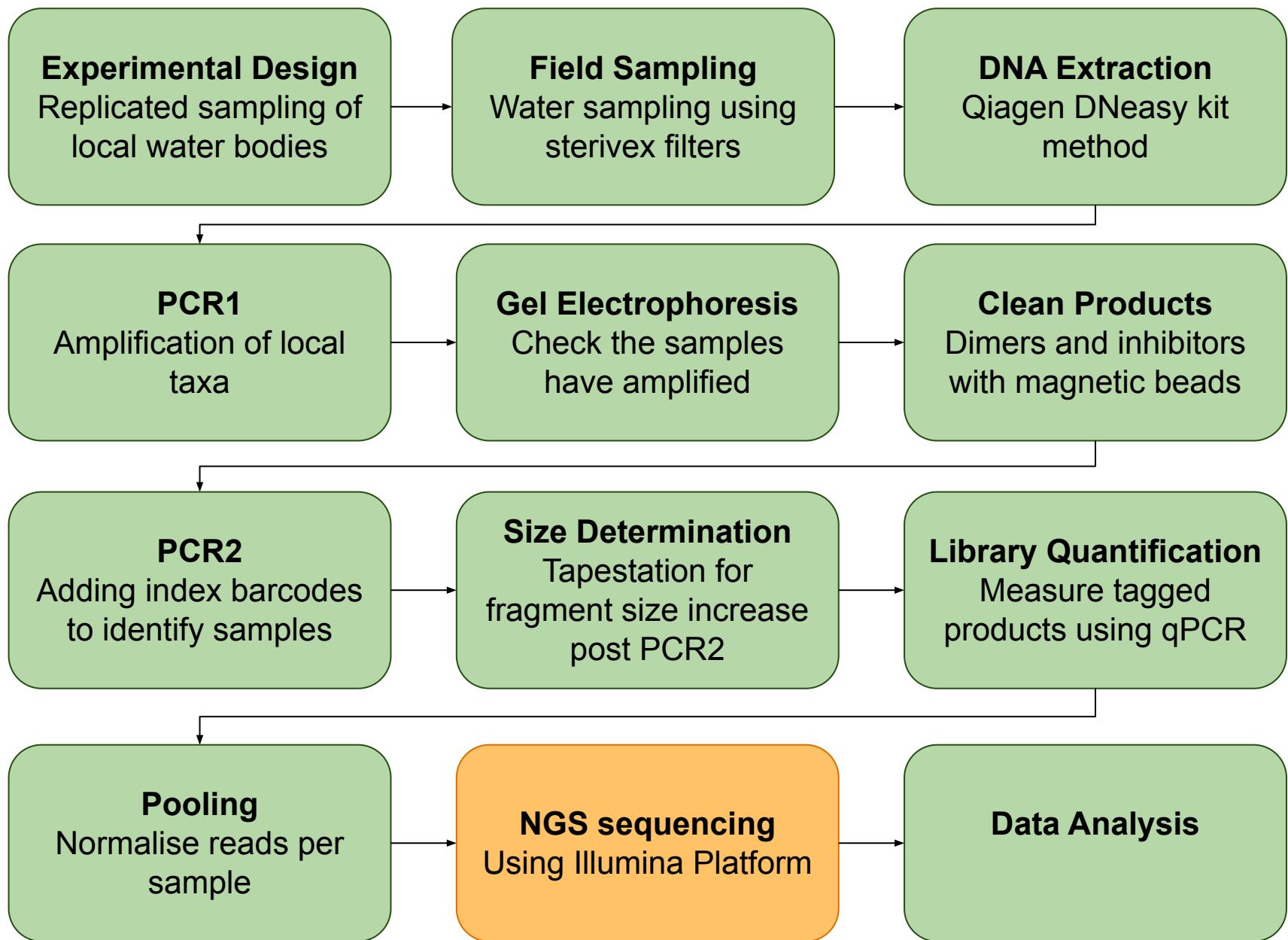
X03 = 6.87

X04 = 8.31

Volume of H₂O (μ l) = 7.13

$$X_{05} = 3.66$$

$$X_{06} = 5.81$$



Sequencing - Next Generation Sequencing

illumina®

Oxford
NANOPORE
Technologies

PacBio

Sequencing - illumina®



Sequencing - illumina®

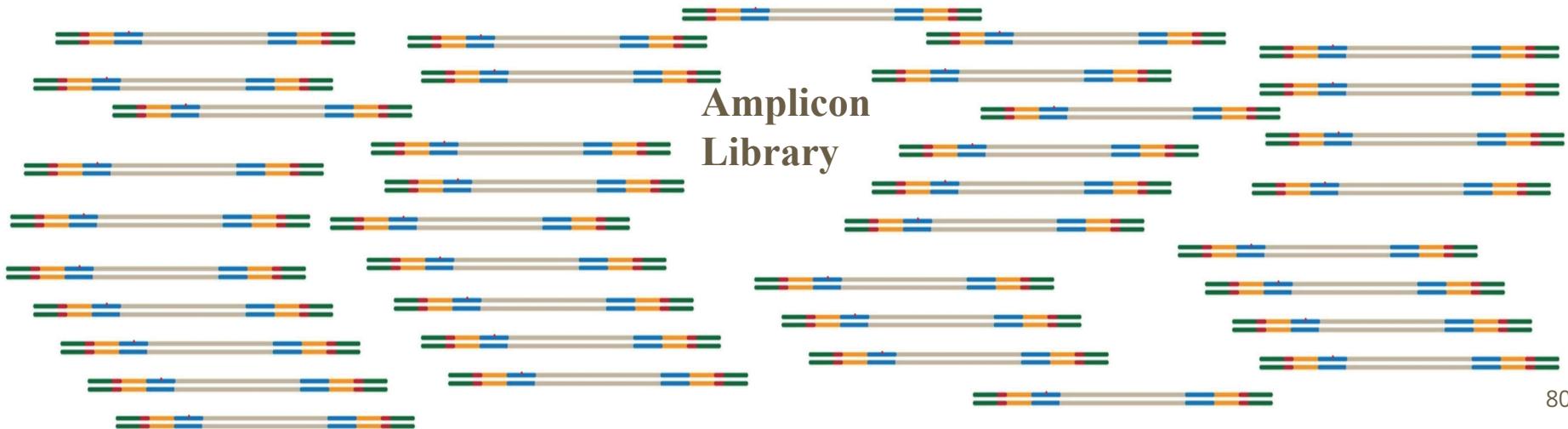
Platform	Maximum Read Length	Maximum Output	Estimated Cost (2024)*
iSeq 100	2 x 150bp	1.2 Gb	£600
MiniSeq	2 X 150bp	7.5 Gb	£700 - £1,600
MiSeq	2 x 300bp	15 Gb	£1,200 - £1,700
NovaSeq X	2 x 150bp	8 Tb	£2,300 - £3,800

* Based on quotes from February 2024.

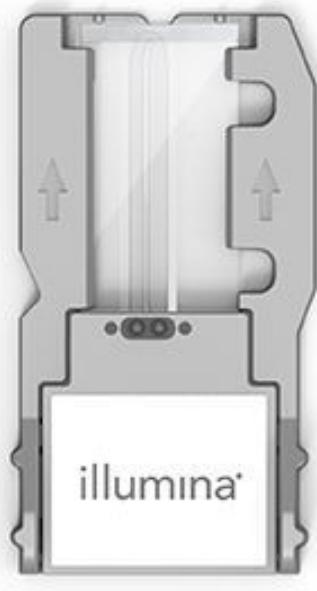
Sequencing - illumina®

Library Preparation recap

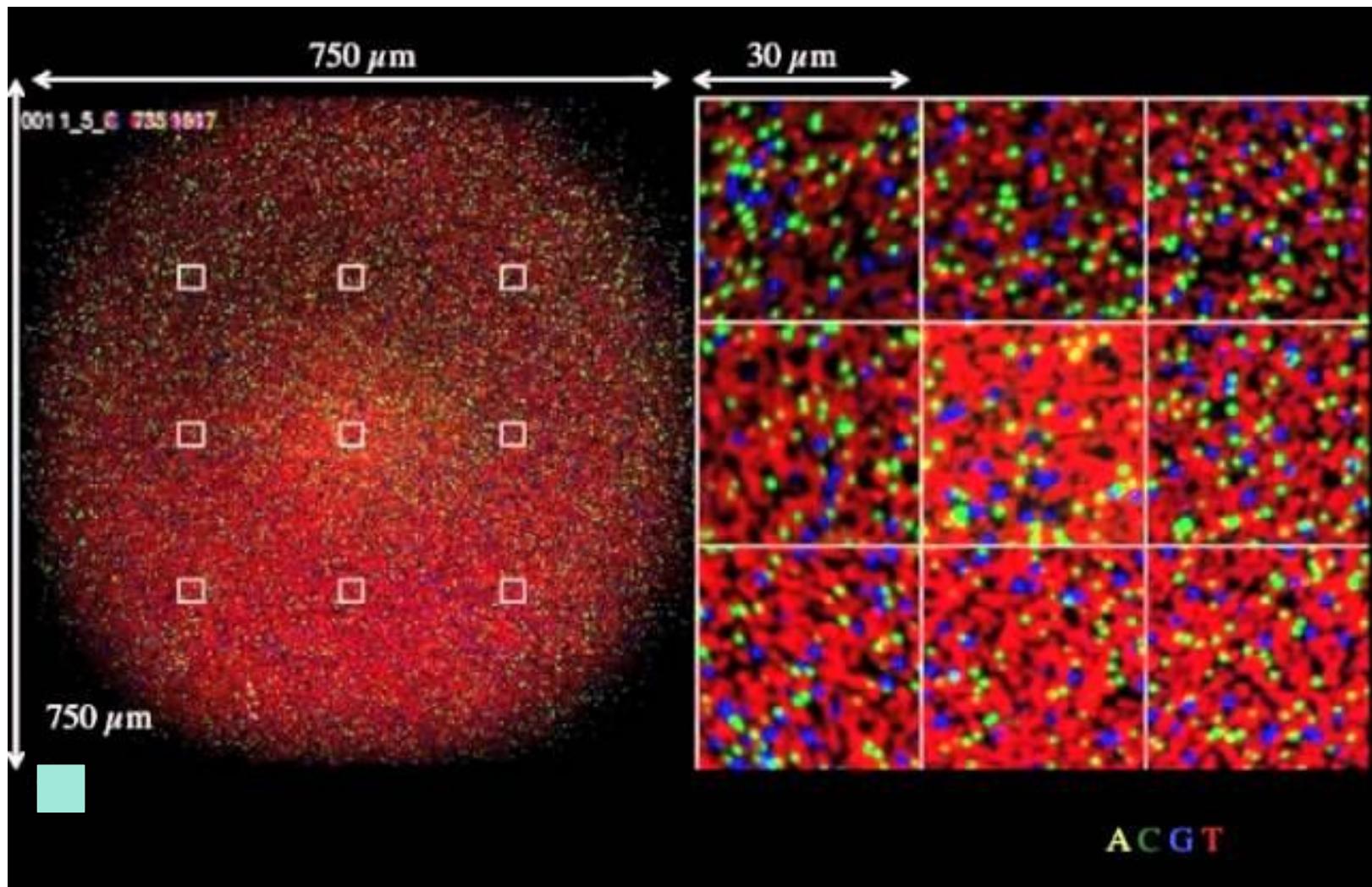
- In our case we have PCR amplified amplicons with specific primers
- Adapters were added to both ends using PCR.
- The result is a library of DNA fragments ready for sequencing.



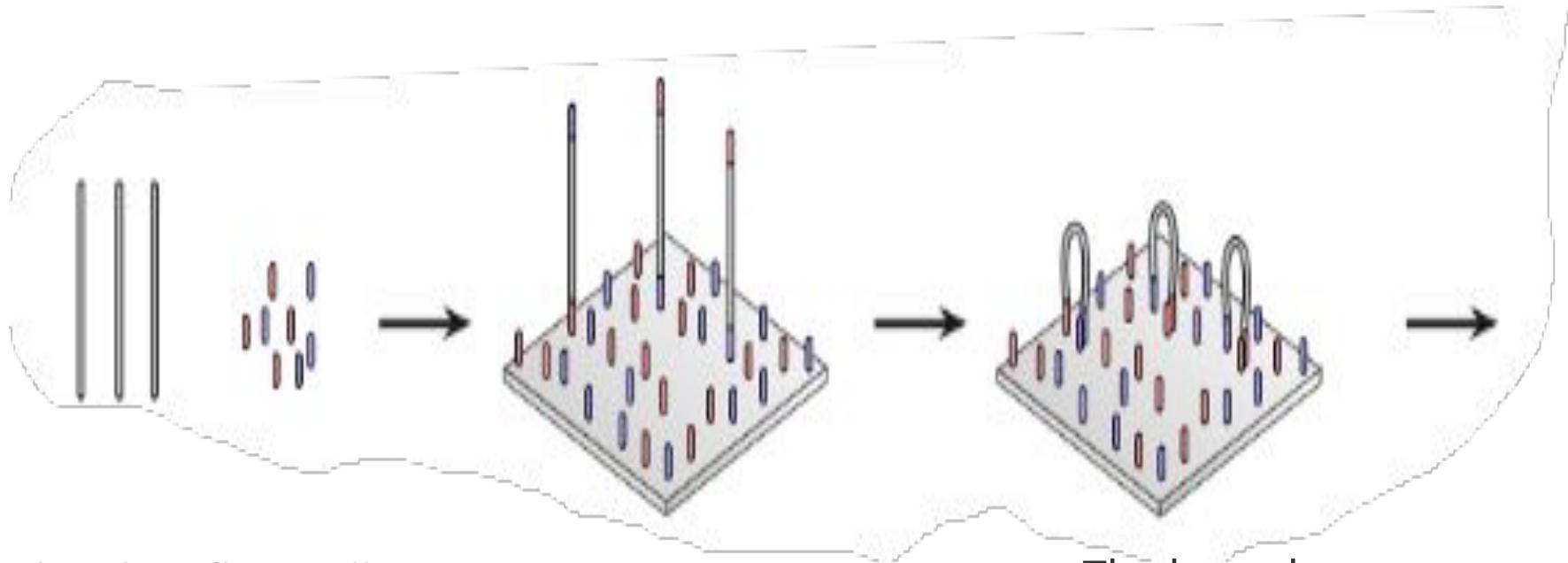
Sequencing - illumina®



Sequencing by Synthesis (SBS)



Cluster Generation (Bridge Amplification)

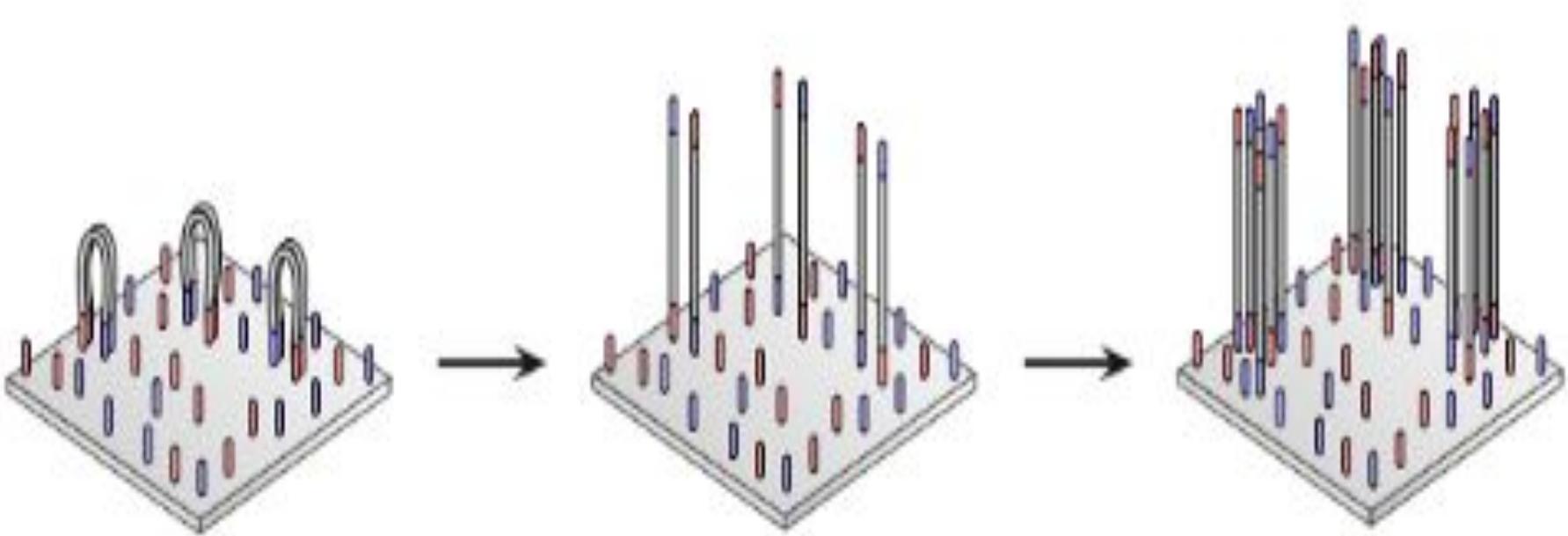


The glass flow cell is coated with two types of primers. DNA is denatured to form single strands

DNA fragments are added to the flow cell and hybridize with one of the primers on the flow cell surface.

The bound fragments bend over and hybridize to nearby primers.

Cluster Generation (Bridge Amplification)

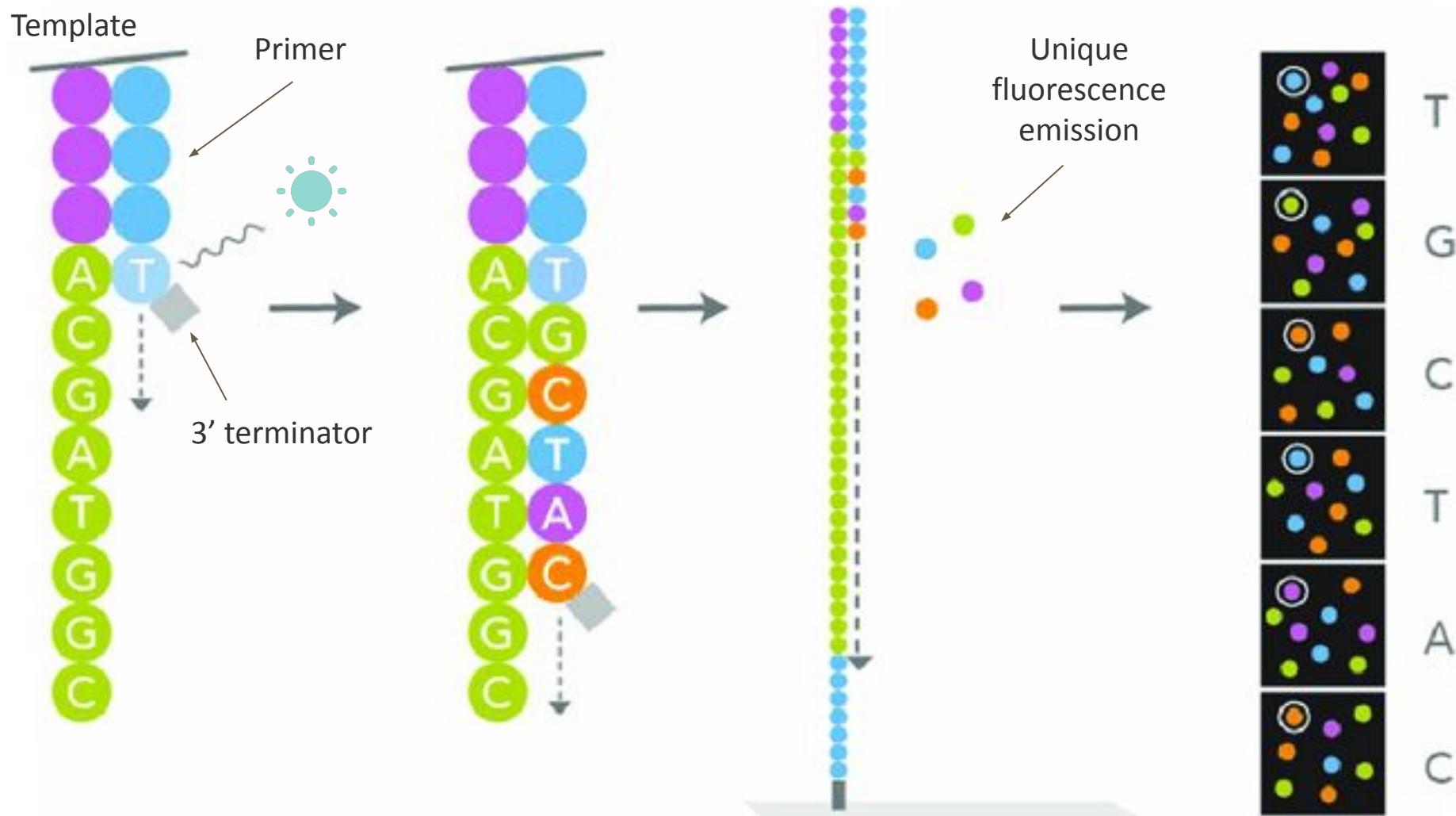


DNA polymerase synthesizes the complementary strand.

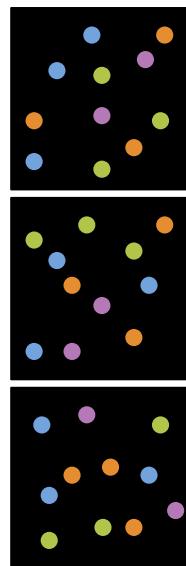
Denaturation forms two separate DNA fragments

Repetition forms clusters of identical strands

Sequencing by Synthesis (SBS)



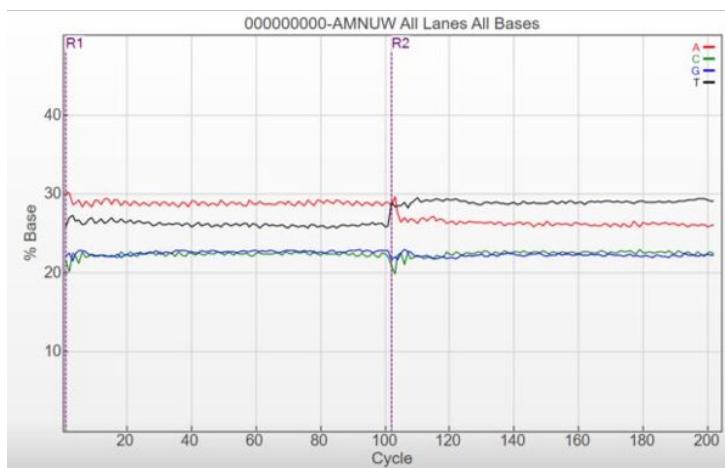
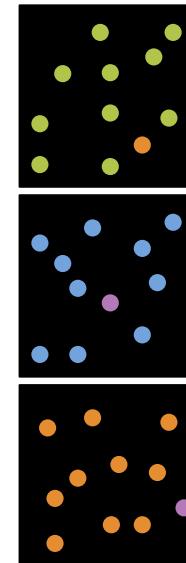
PhiX - Balancing Diversity



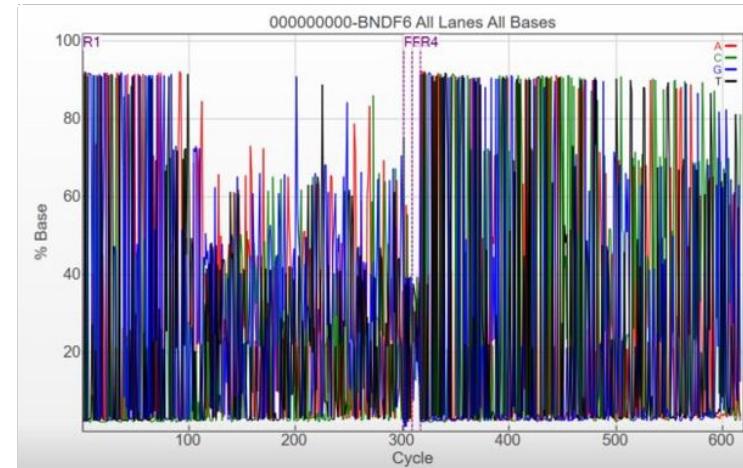
Cycle 1

Cycle 2

Cycle 3

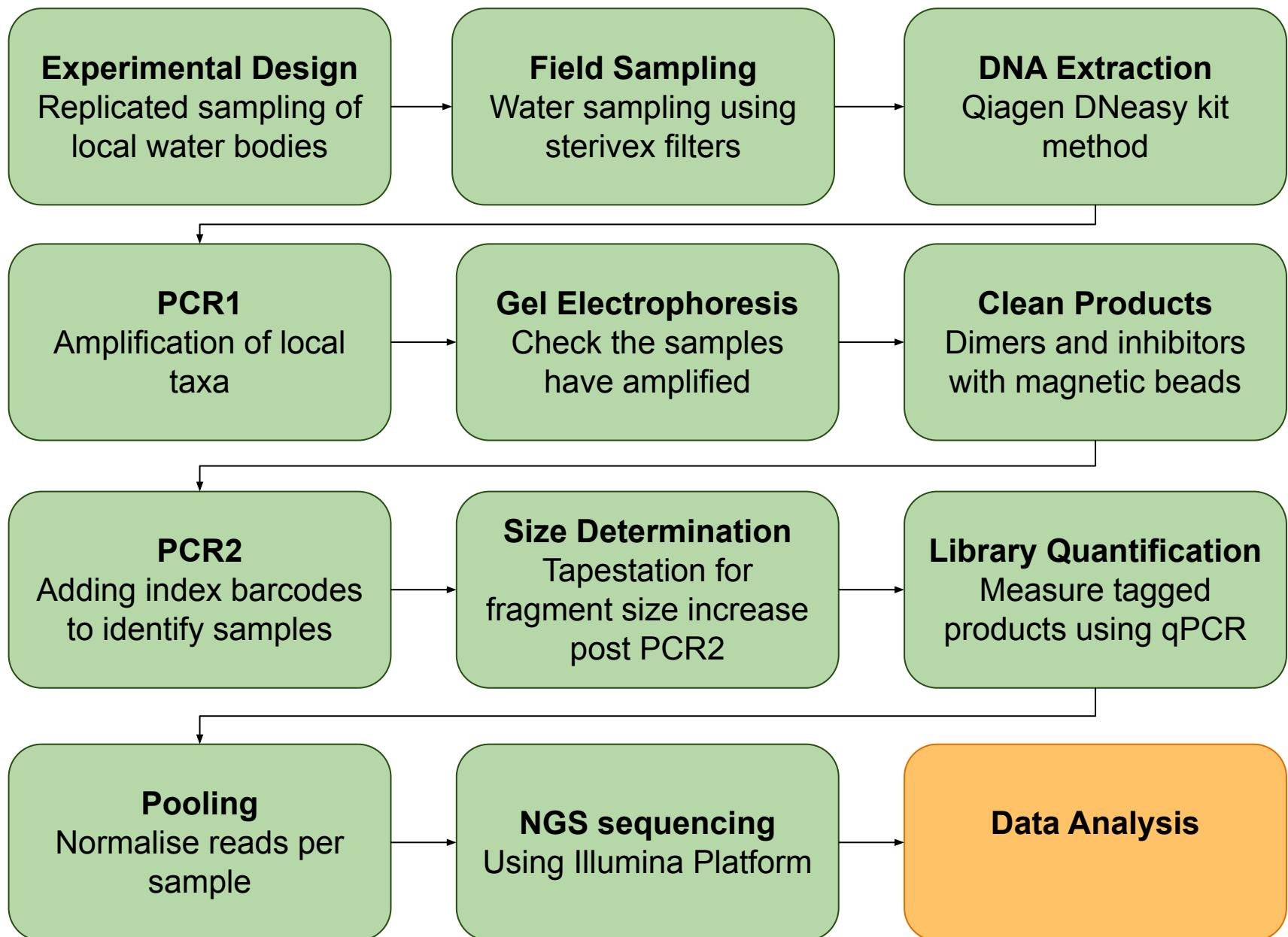


Well-balanced



Low diversity

Data output



Previous eDNA study

Samples:

- Weston Park November (**WP_N**)
- Weston Park October (**WP_O**)
- Rother Valley (**RotV_O**)
- Crookes Valley (**CRV_O**)
- Positive controls - Mexican river samples, African Cichlid stomachs (**Pos**)
- Negatives
 - Field (**N_fld**)
 - Extraction (**N_ex**)
 - PCR (**N_w**)

Riaz 12S-V5 primer set

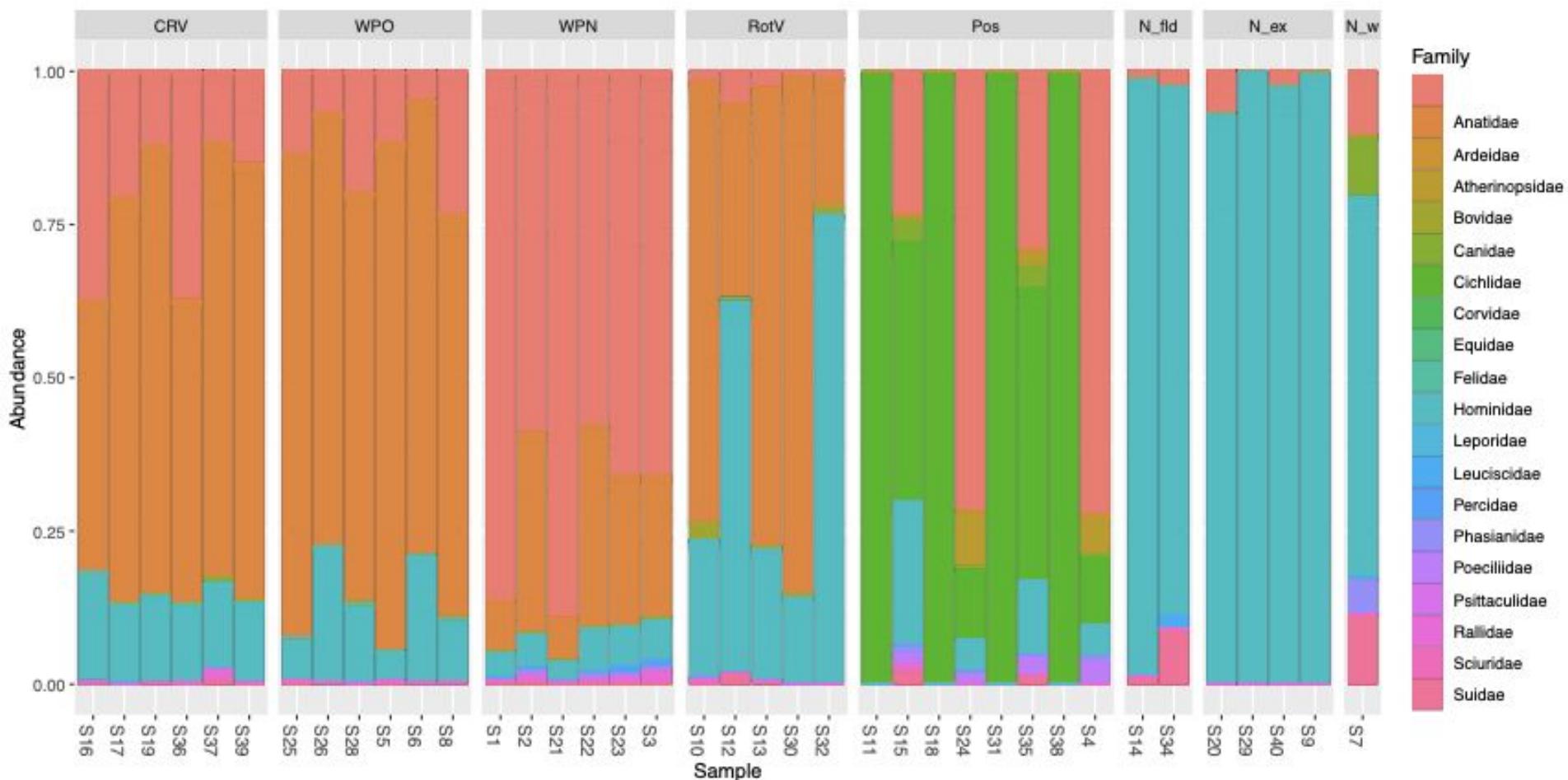
(<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3241669/>)



What did we find?



What did we find?



Take Home Message

Average Yield from the Sequence Run

=

Number of Reactions

Read Depth

×

Number of Primers