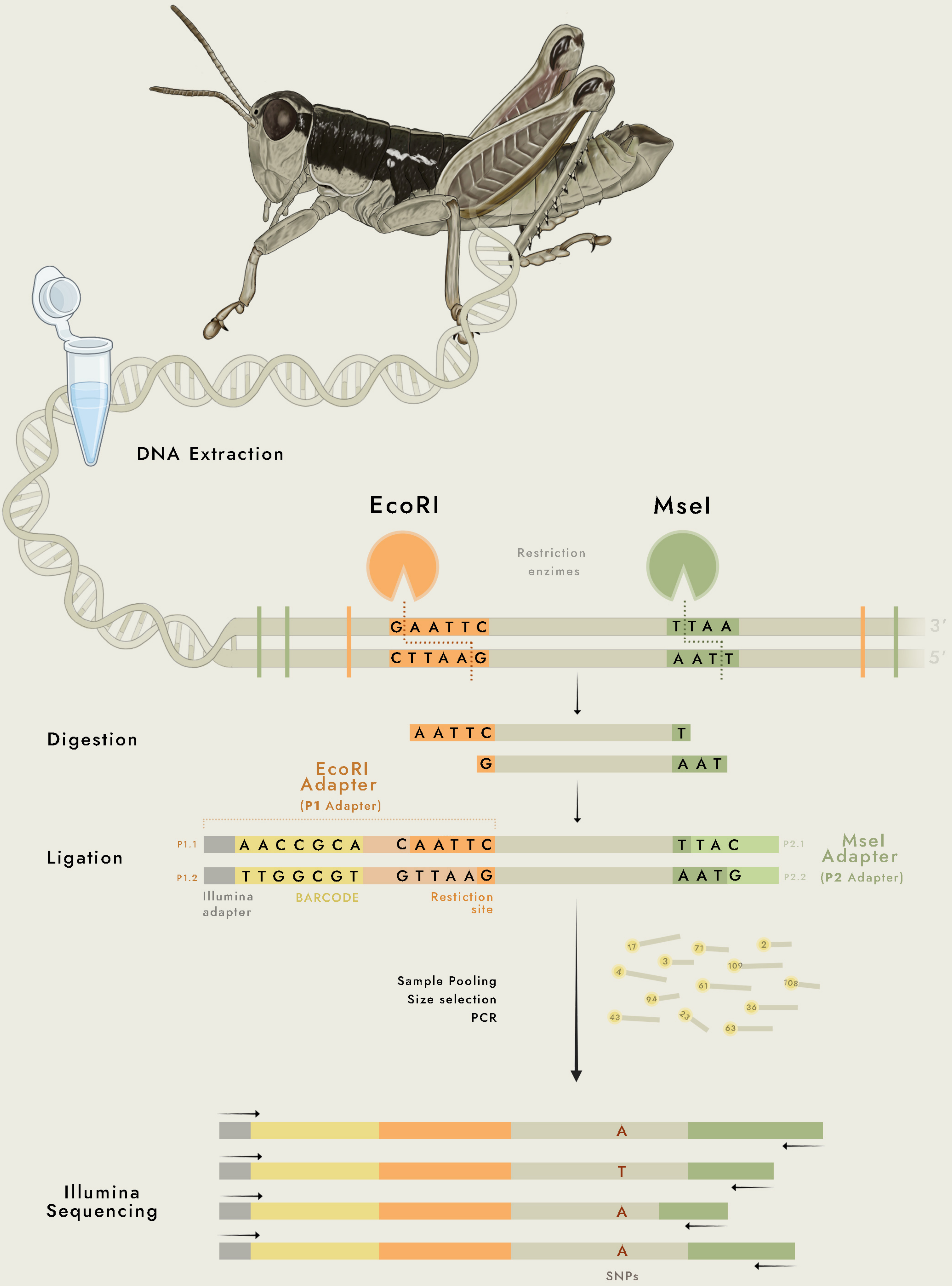
ddRADseq



DNA Extraction

EcoRI

MseI

Restriction enzimes

Digestion

EcoRI Adapter (**P1** Adapter)

Ligation

MseI Adapter (**P2** Adapter)

Illumina adapter

BARCODE

Restiction site

Sample Pooling
Size selection
PCR

Illumina Sequencing

SNPs

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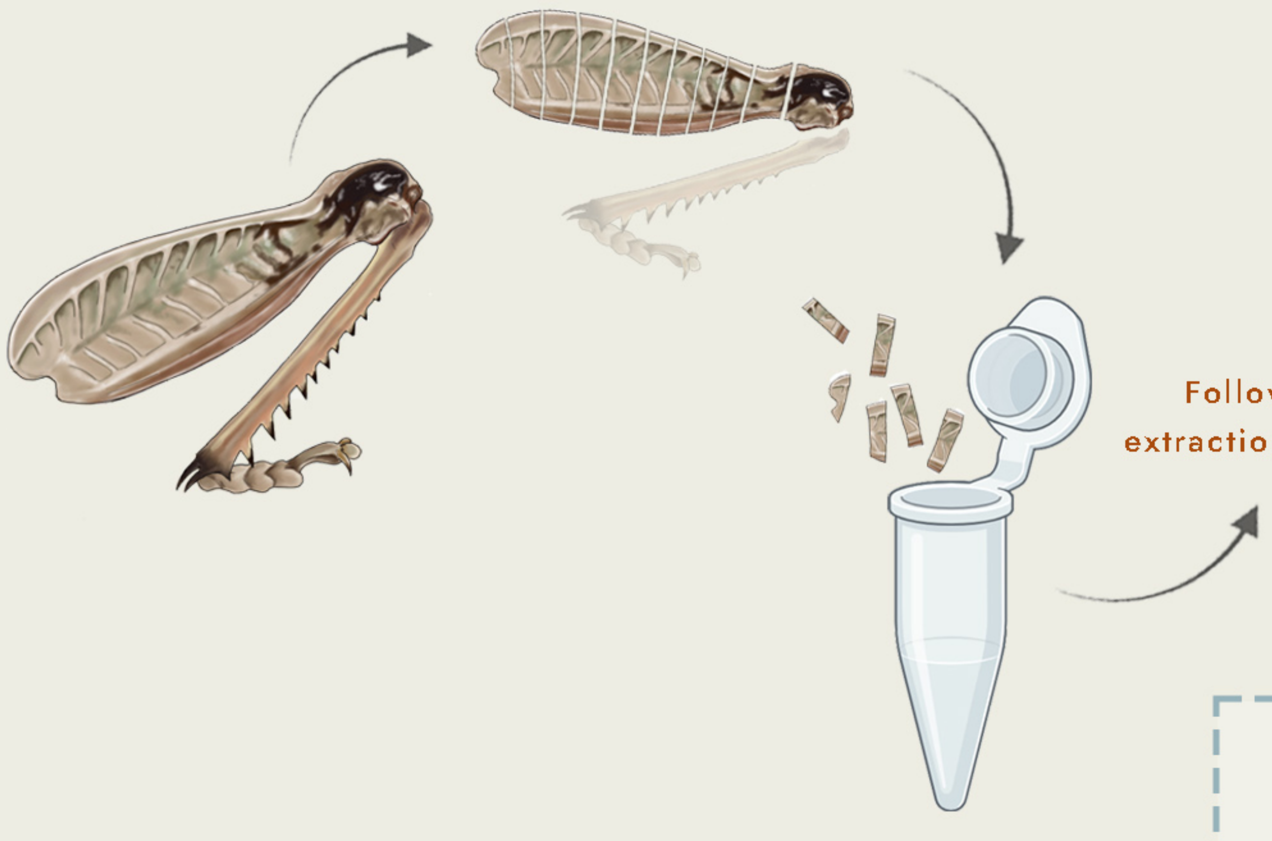
OrtegoLab, 2024

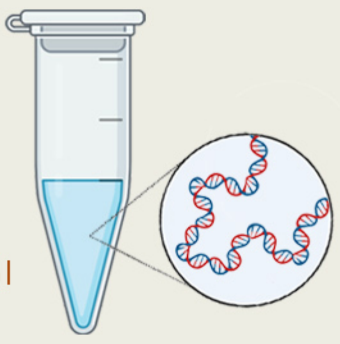
ddRADseq: *“Double digest restriction-site associated DNA sequencing”*

ddRADseq

1 DNA EXTRACTION



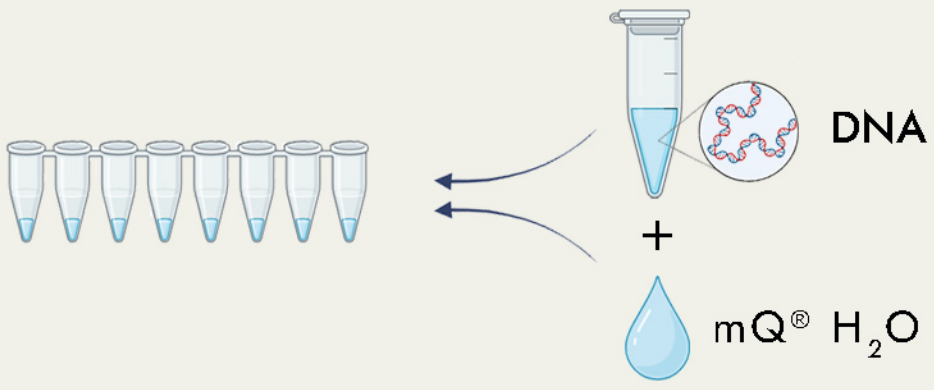
2 DNA QUANTIFICATION



Qubit™
Fluorometer
High Sensitivity Assay

STANDARDIZATION

300 ng DNA
in 17 µL



From this step onward, **briefly centrifuge** tube strips before opening

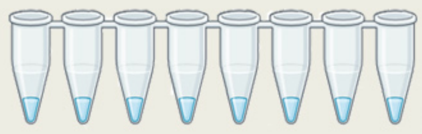
3 DOUBLE DIGEST

MASTERMIX DIGESTION	Per sample	X samples
Cutsmart Buffer	2 µL	
MseI	0.5 µL	
EcoRI	0.5 µL	

- Prepare **130 %** of the required volume*
- Use **tube strips**, not plates*

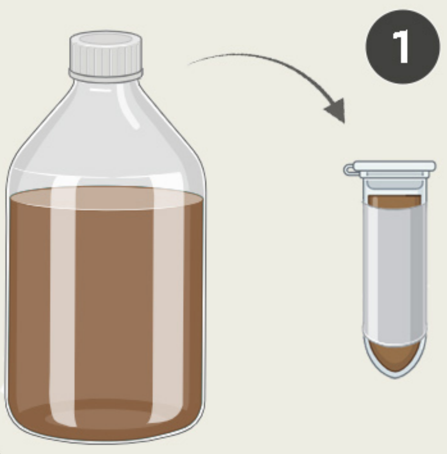
37° C → 3 hours
4° C → Hold (∞)

3 µL



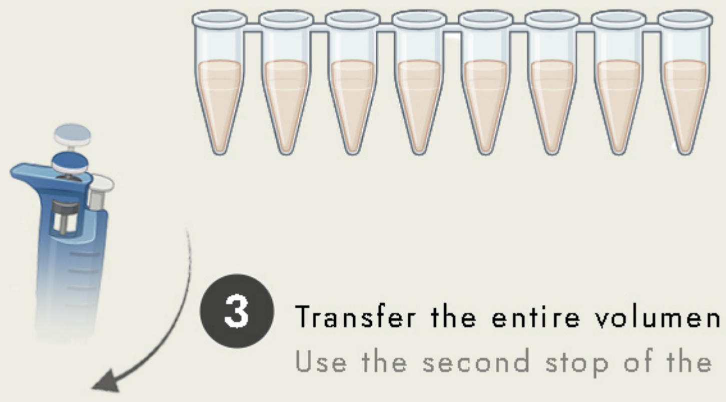
4 CLEAN-UP 1

Process samples in **two tube strips**
to ensure consistent timing across samples

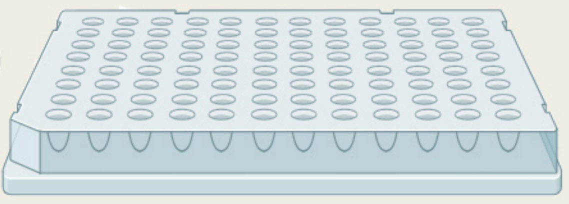


① Transfer **beads** to a 2 mL tube and store at 5°C until use

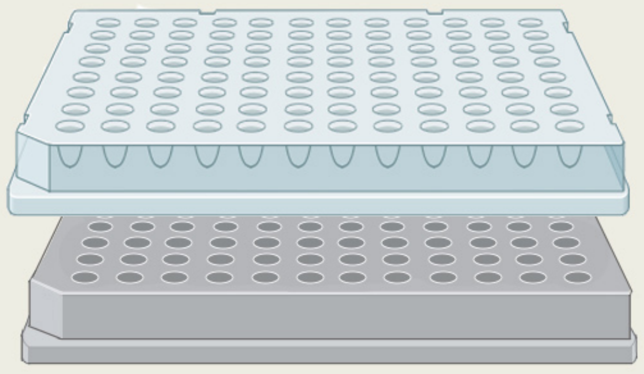
② Add 32 µL of beads per sample
(1.6 × volumen sample.
1.6 × 20 µL = 32 µL of beads)



③ Transfer the entire volumen of each sample to the plate
Use the second stop of the pipette to recover full volume



④ Incubate for **5 min** at room temperature 5′



⑤ Place on magnetic plate for **2 min** 2′

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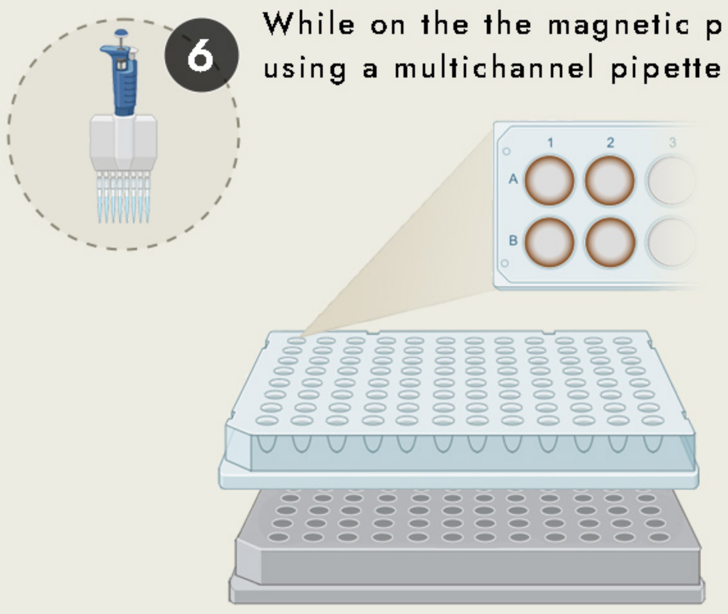
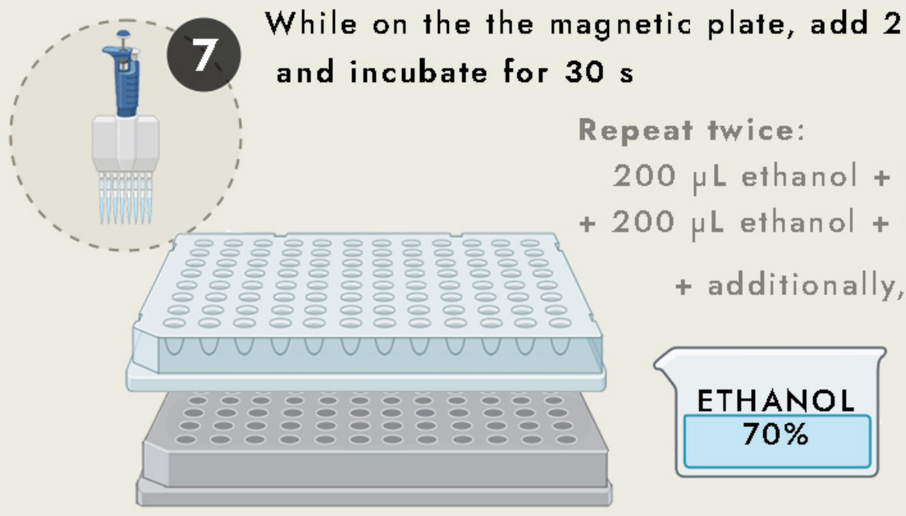


6 While on the the magnetic plate, **discard the supernatant** using a multichannel pipette

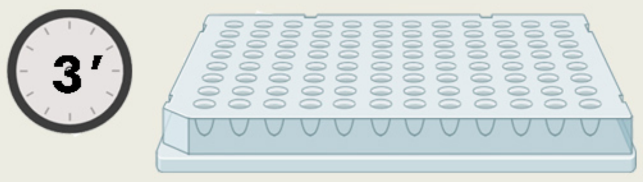
Avoid disturbing the bead pellet



7 While on the the magnetic plate, **add 200 µL of ethanol 70%** and incubate for 30 s

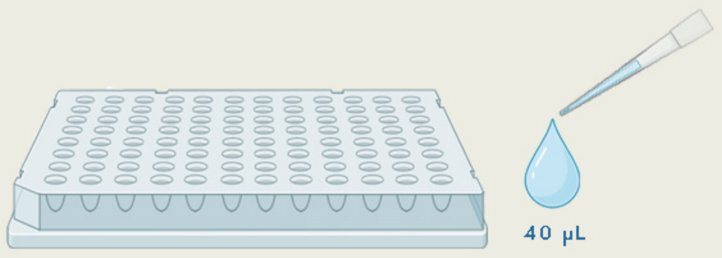
Repeat twice:
200 µL ethanol + 30″ + remove ethanol
+ 200 µL ethanol + 30″ + remove ethanol
+ additionally, remove ethanol

8 Remove from magnetic plate and air-dry for 3 min until ethanol evaporates

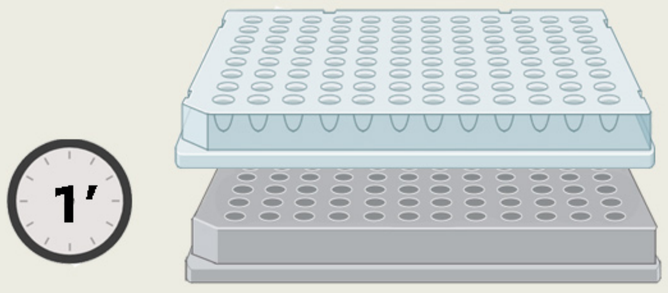


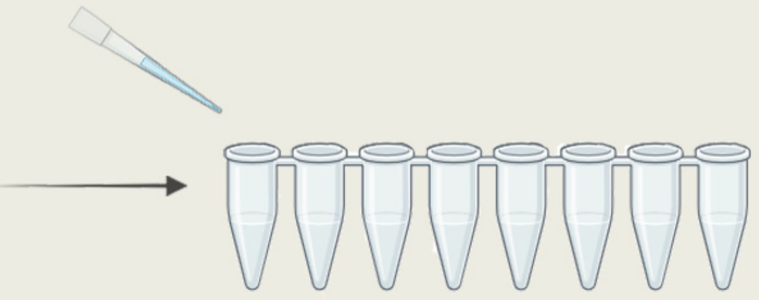
9 Add **40 µL of mQ® H_2O** per well using monocanal pipette

Resuspend by pipetting and gently scraping the tube walls



10 Place on magnetic plate for **1 min**.





Transfer eluate to new tube strip
while on the magnetic plate

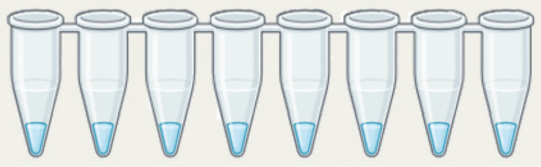
11 Quantify DNA

Qubit™
Fluorometer
High Sensitivity Assay

STANDARDIZATION

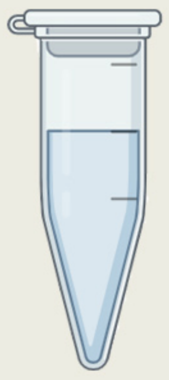
DNA concentration:
Lowest value obtained from Qubit
(never below 2 ng/µL)

Complete excel sheet



Final volume: 33 µL

5 LIGATION

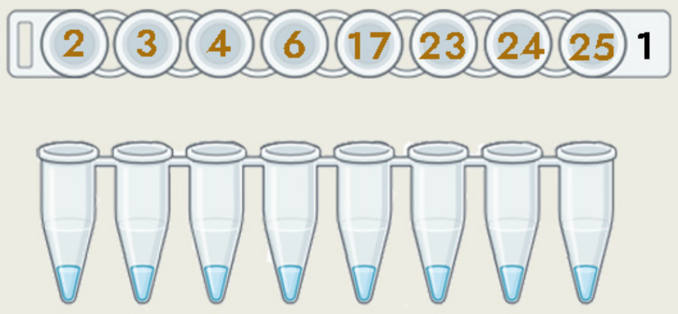


MASTERMIX LIGATION	Per sample	X samples
Ligase Buffer (10×)	4 µL	
Adapter MseI - P2 Working solution	1 µL	
Ligase	1 µL	

- Prepare **120 %** of the required volume*
- Use **tube strips**, not plates*

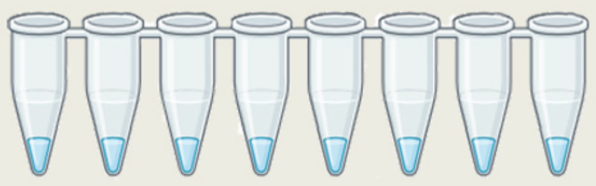
BARCODES

(Adapter EcoRI - P1)
(Working Solution)



1 µL

6 µL



40 µL
(33 µL + 6 µL + 1 µL)

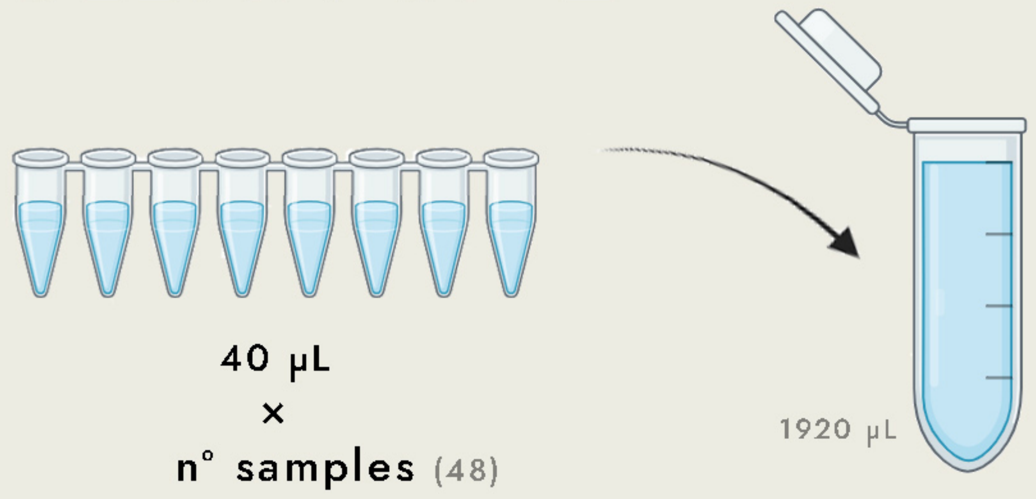
23° C → 2 hours
65° C → 10 min
63° C → 1:30 min
Decrease 2°C every 1:30 min until 23°C (i.e. 21 cycles in total)
10° C → Hold (∞)

For adapter preparation
(EcoRI-P1 barcodes and MseI-P2)
see **Appendix 1 and 2**

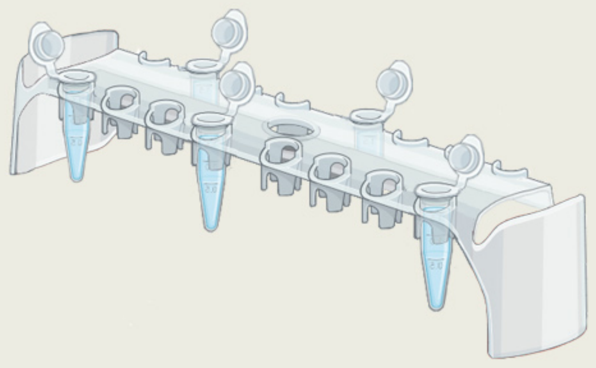
6 CLEAN-UP 2

1 **POOL samples**

Combine all tube strips (1920 µL total) into a single 2 mL tube
(Use two tubes if library > 48 samples)



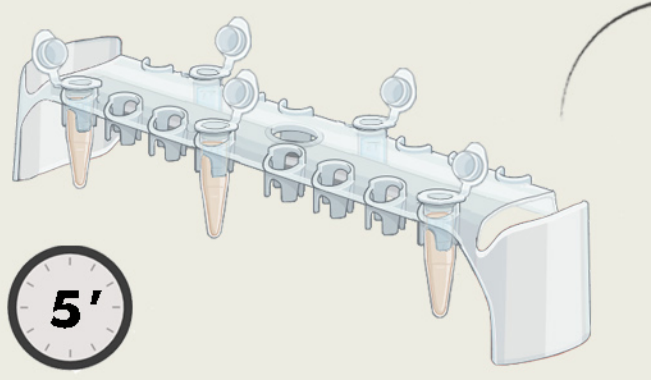
2 Place aliquots in a rack without the magnet



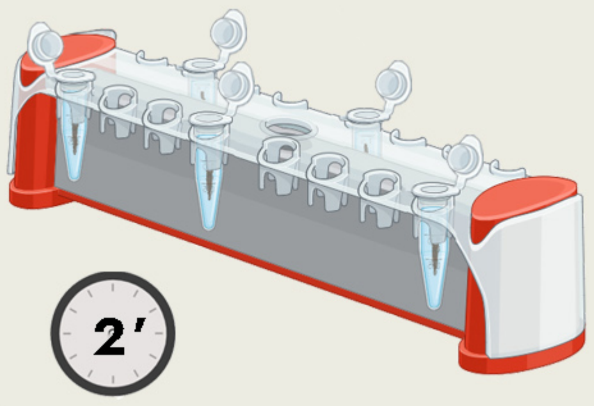
3 Add 1.6x volume of **beads (614.4 µL per aliquot*)**

1.6 × 384 µL (aliquot) = 614.4 µL of beads

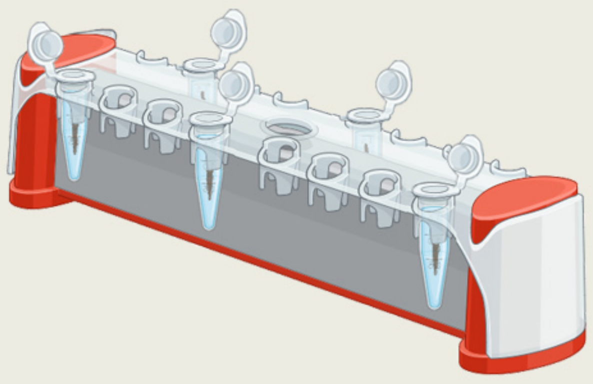
4 Incubate for **5 min** at room temperature



5 Place on magnetic rack for **2 min**

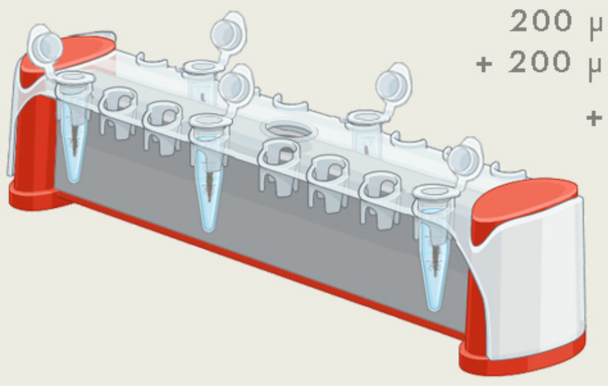


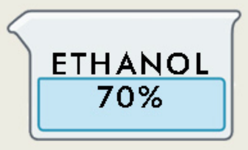
6 **Keep on the magnetic rack and discard the supernatant without disturbing the bead pellet**



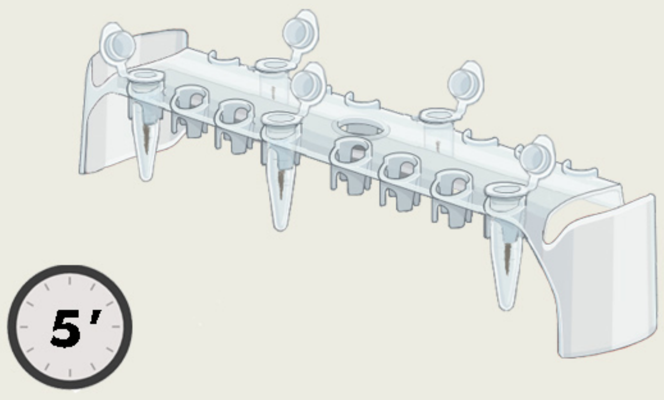
7 **While on the magnetic rack, add 200 µL of ethanol 70% and incubate for 30 s**

Repeat twice:
200 µL ethanol + 30” + remove ethanol
+ 200 µL ethanol + 30” + remove ethanol
+ aditionally, remove ethanol



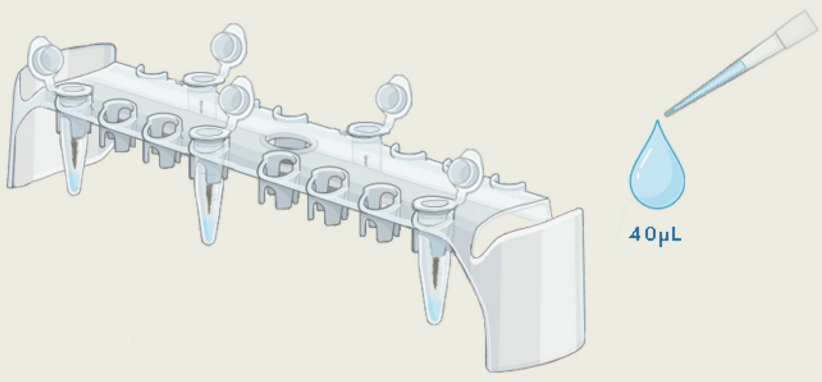


8 **Remove from magnetic rack and air-dry for 5 min until ethanol evaporates**

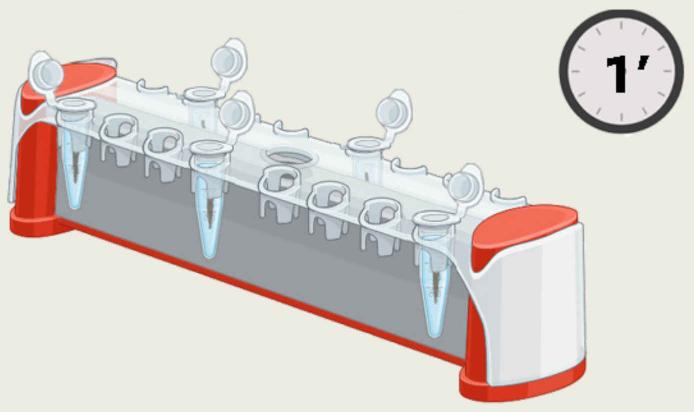


9 Add **40 µL of mQ® H_2O** per aliquot

Resuspend bead pellet by pipetting up and down

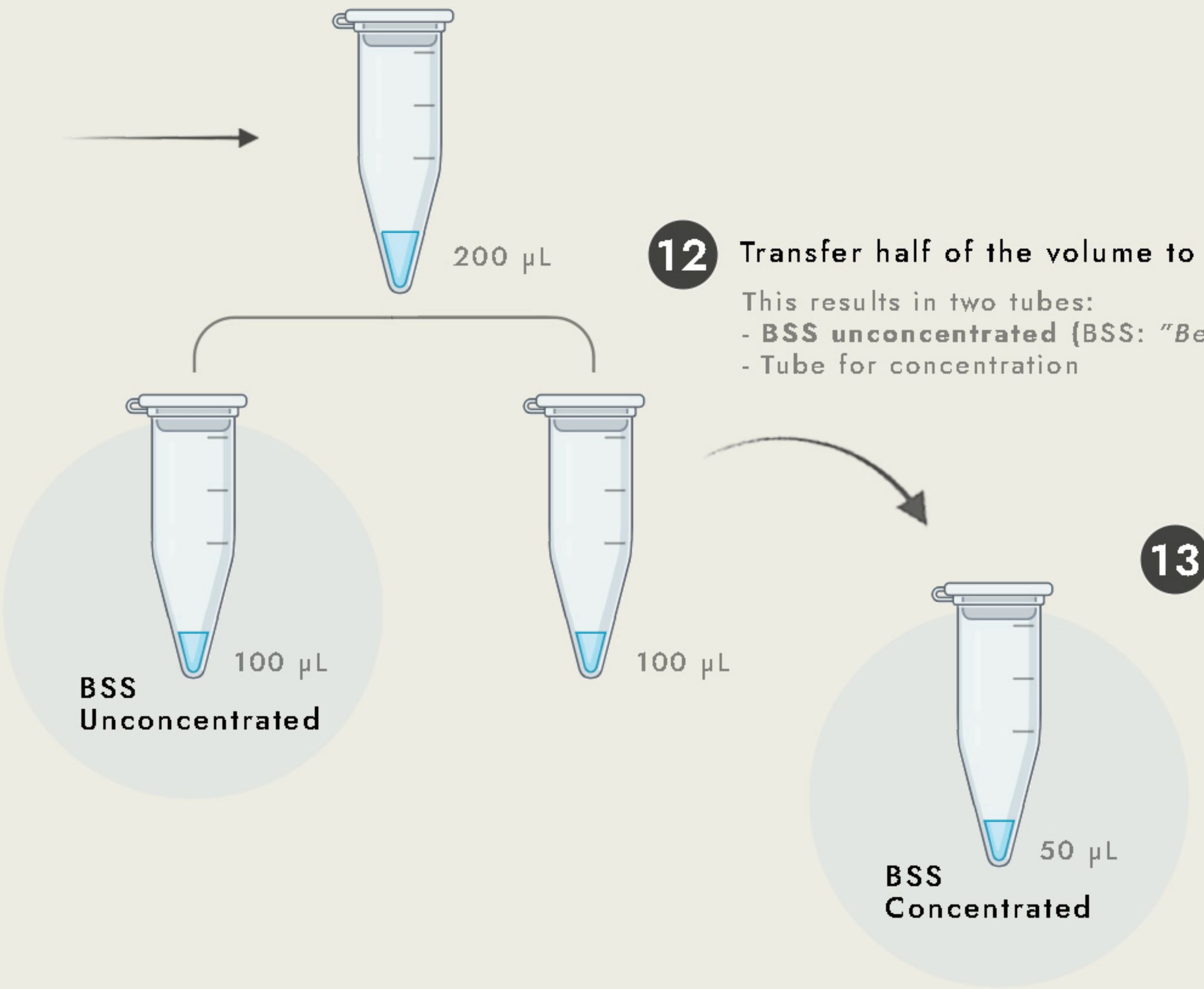


10 Place on magnetic rack for 1 min.



11 Combine all aliquots into a single tube

This clean-up step yields **40** μL per aliquot
Combine all aliquots into one tube
In this case: **5** aliquots × **40** μL = **200** μL



12 Transfer half of the volume to a new tube

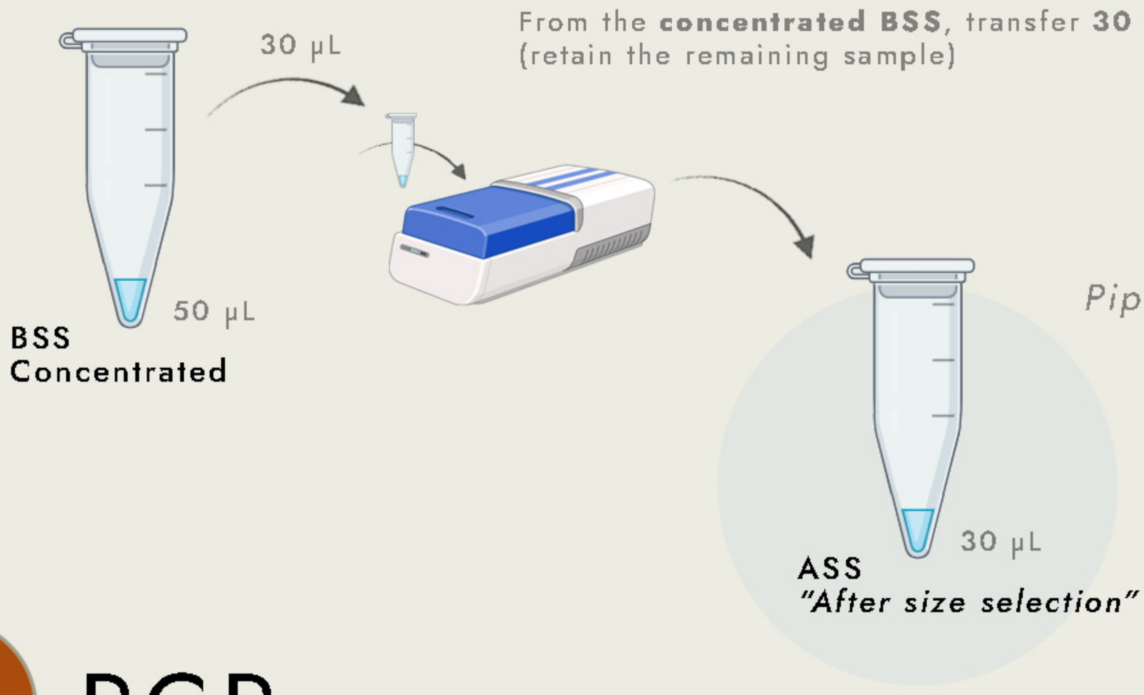
This results in two tubes:
- **BSS unconcentrated** (BSS: *"Before size selection"*)
- Tube for concentration

13 Concentrate to half of the original volume

Using a vacuum concentrator (SpeedVac) with the following settings:
- Manual mode
- Heater on
- Drying rate: High

7 Size Selection - Pippin-prep

From the **concentrated BSS**, transfer **30 μL** to a new tube
(retain the remaining sample)



Pippin-Prep Size Selection
475 - 580 bp
(for **Orthoptera**)

8 PCR

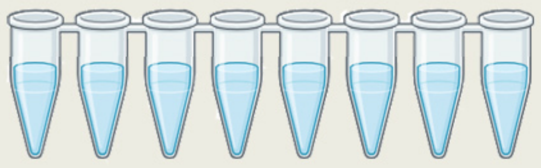
PCR Mastermix

mQ® H_2O	160.8 μL
Buffer HF (10×)	48 μL
dNTP (10 mM)	4.8 μL
$MgCl_2$ (50 mM)	4.8 μL
DMSO	2.4 μL
Primer F (PCR-1)	2.4 μL
Primer R (PCR-2) Index	2.4 μL
Taq HF	2.4 μL
DNA (ASS)	2 μL

Control (-): No DNA (add 19 μL PCR mastermix only)

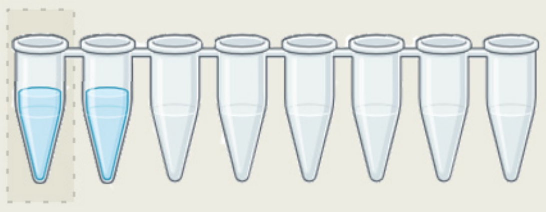
Important:
Record the **index** (PCR-2) used for each library

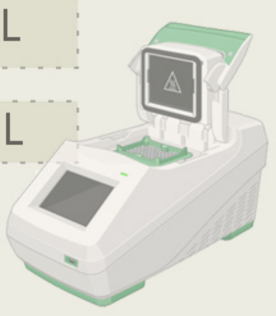
12 CYCLES



19 μL PCR Mastermix
2 μL DNA (ASS)

20 CYCLES Control +/-

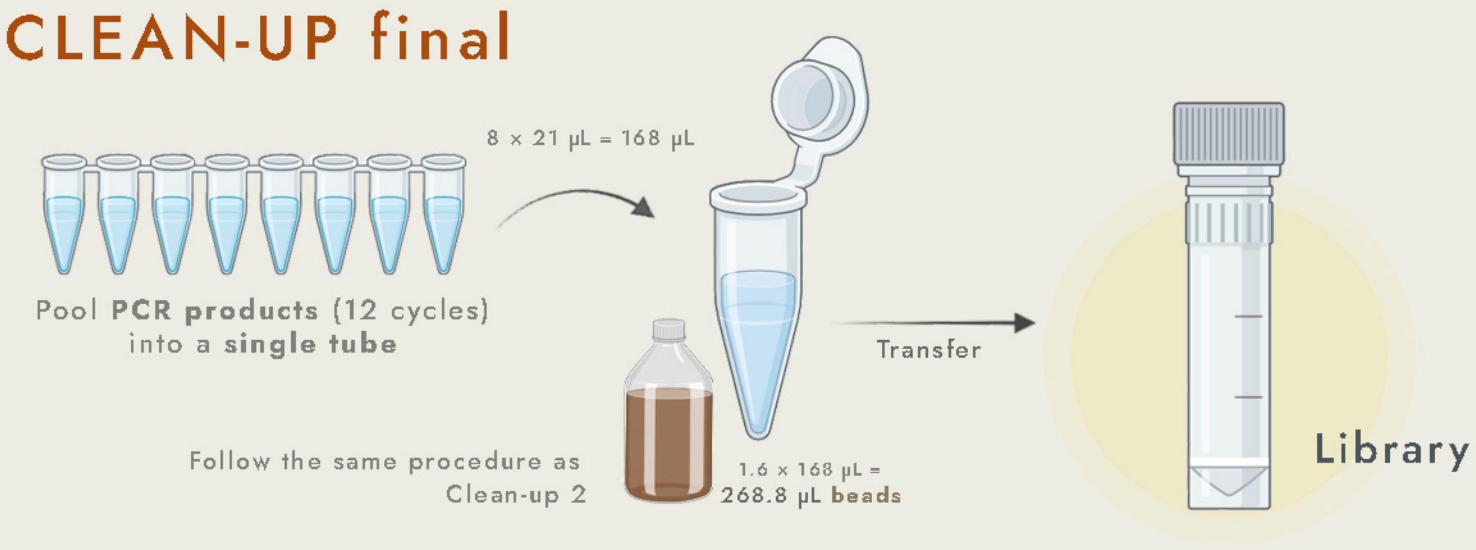




98° C → 0:30 min
12 Cycles
98° C → 0:20 min
65° C → 0:30 min
72° C → 0:40 min
72° C → 10 min
4° C → Hold (∞)

To verify PCR success,
see **Appendix 3**

CLEAN-UP final

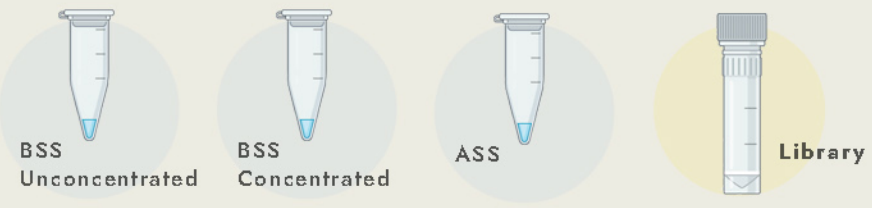


Pool **PCR products** (12 cycles)
into a **single tube**

Follow the same procedure as
Clean-up 2

Finally, **cuantify** using
Qubit ™ (3 μL) and Nanodrop (2 μL)

At the end of each library, 4 tubes should be obtained



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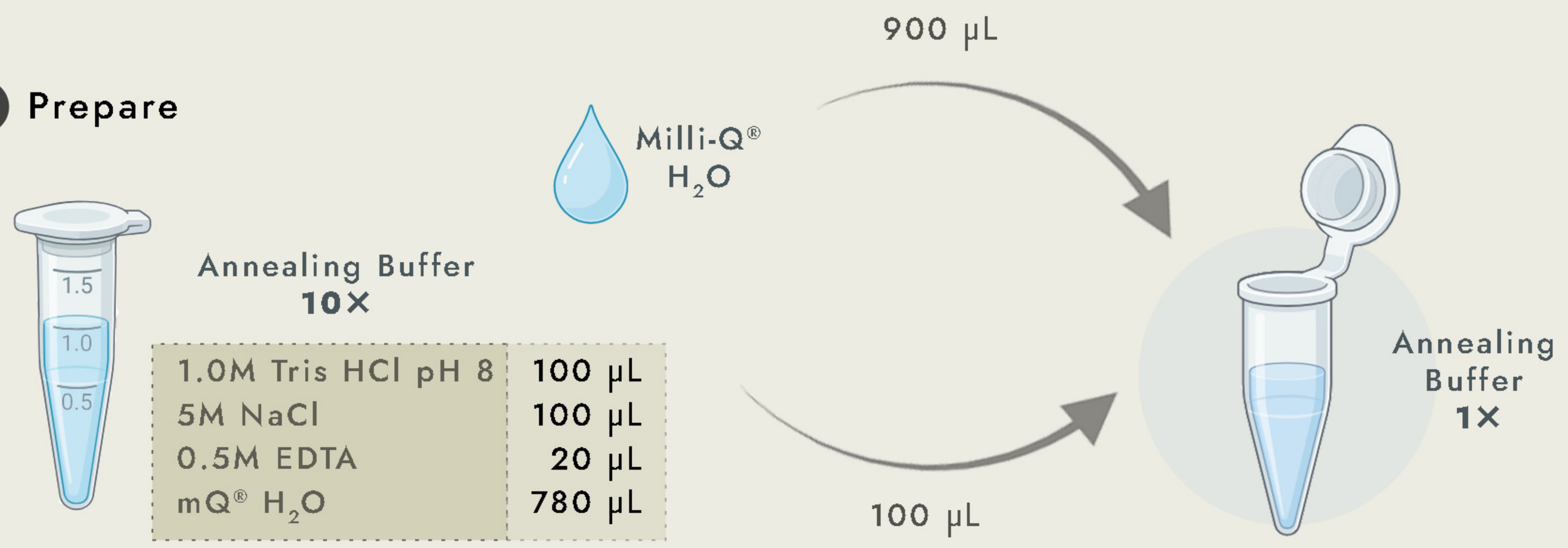
OrtegoLab, 2024

Appendix 1

ANNEALING ADAPTERS:

MseI ADAPTER (P2)

① Prepare



Milli-Q® H_2O

900 µL

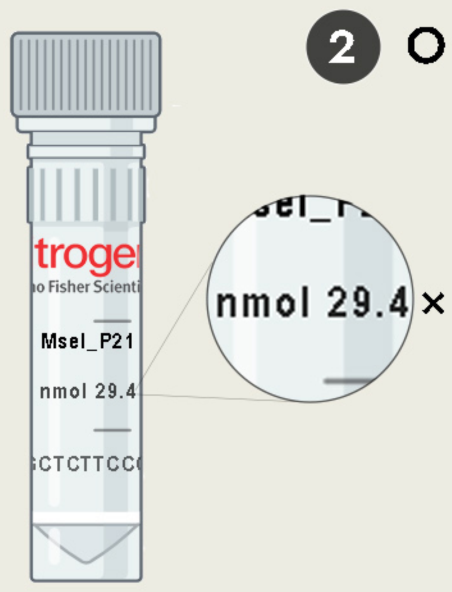
Annealing Buffer
10×

1.0M Tris HCl pH 8	100 µL
5M NaCl	100 µL
0.5M EDTA	20 µL
mQ® H_2O	780 µL

100 µL

Annealing Buffer
1×

② Oligo dilution: P2.1 and P2.2 adapters



nmol 29.4 × 10 = 294 µL H_2O

H_2O volume (µL) :
nmol × 10

③ Prepare Annealed Adapter Stock 40 µM

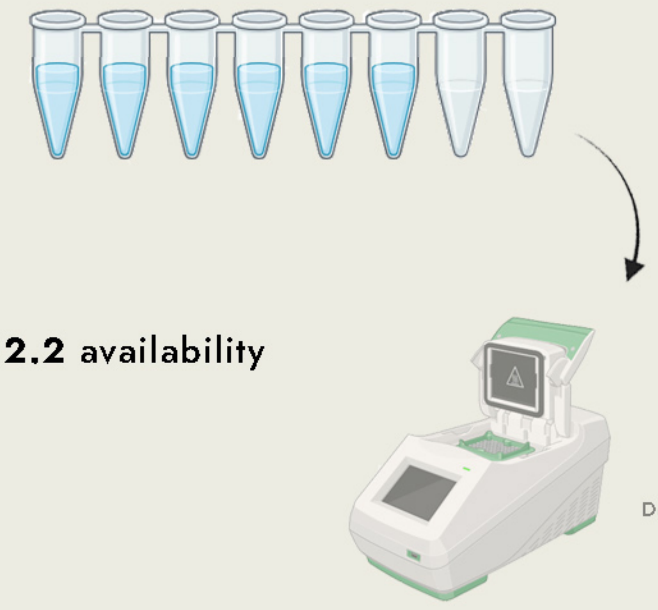
MseI Annealed Adapter Stock **40 µM**

Adaptador **P2.1** (100 µM)	40 µL
Adaptador **P2.2** (100 µM)	40 µL
Annealing Buffer **10**×	10 µL
mQ® H_2O	10 µL

- Preparae the mix in **tube strips**
- Prepare as **many tubes** as required based on **P2.1** and **P2.2** availability

P2.1 Adapter: 294 µL
294 µL / 40 µL × tube = 7.35 tubes

P2.2 Adapter: 260 µL
260 µL / 40 µL × tube = 6.5 tubes



Prepare mix
for 6 tubes

97.5° C → 2:30 min
94.5° C → 1 min
93° C → 0:30 min
Decrease 3°C every min until 23°C (i.e. 24 cycles in total)
4° C → Hold (∞)

④ Prepare MseI (P2) Adapter

Annealing
Buffer
1×

8.96 µL

Annealed Adapter Stock **40 µM**

96.04 µL

Adapter MseI
MseI (Working Solution)

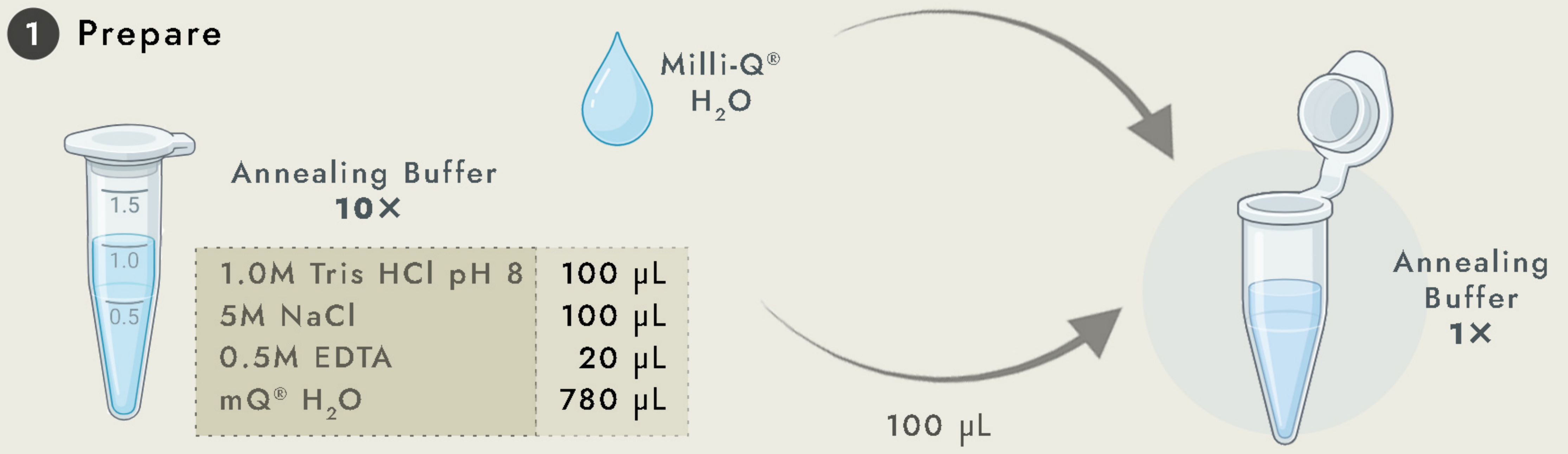
Marina Trillo©
OrtegoLab, 2024

Appendix 2

ANNEALING ADAPTERS:

EcoRI ADAPTER (P1) - BARCODES

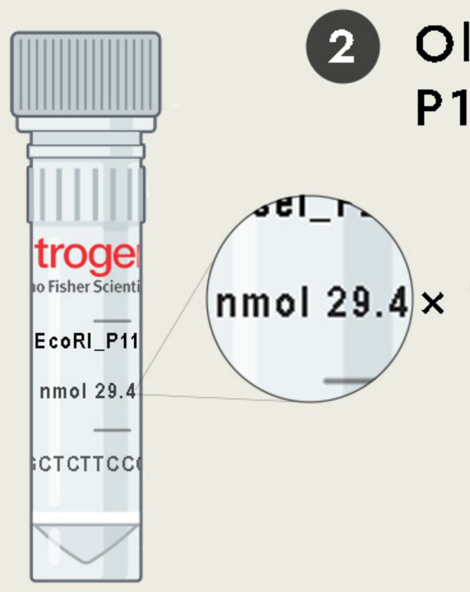
① Prepare



Annealing Buffer **10×**

1.0M Tris HCl pH 8	100 μL
5M NaCl	100 μL
0.5M EDTA	20 μL
mQ® H_2O	780 μL

② Oligo dilution for each barcode: P1.1 and P1.2 adapters



nmol 29.4 × 10 = 294 μL H_2O

H_2O Volume (μL):

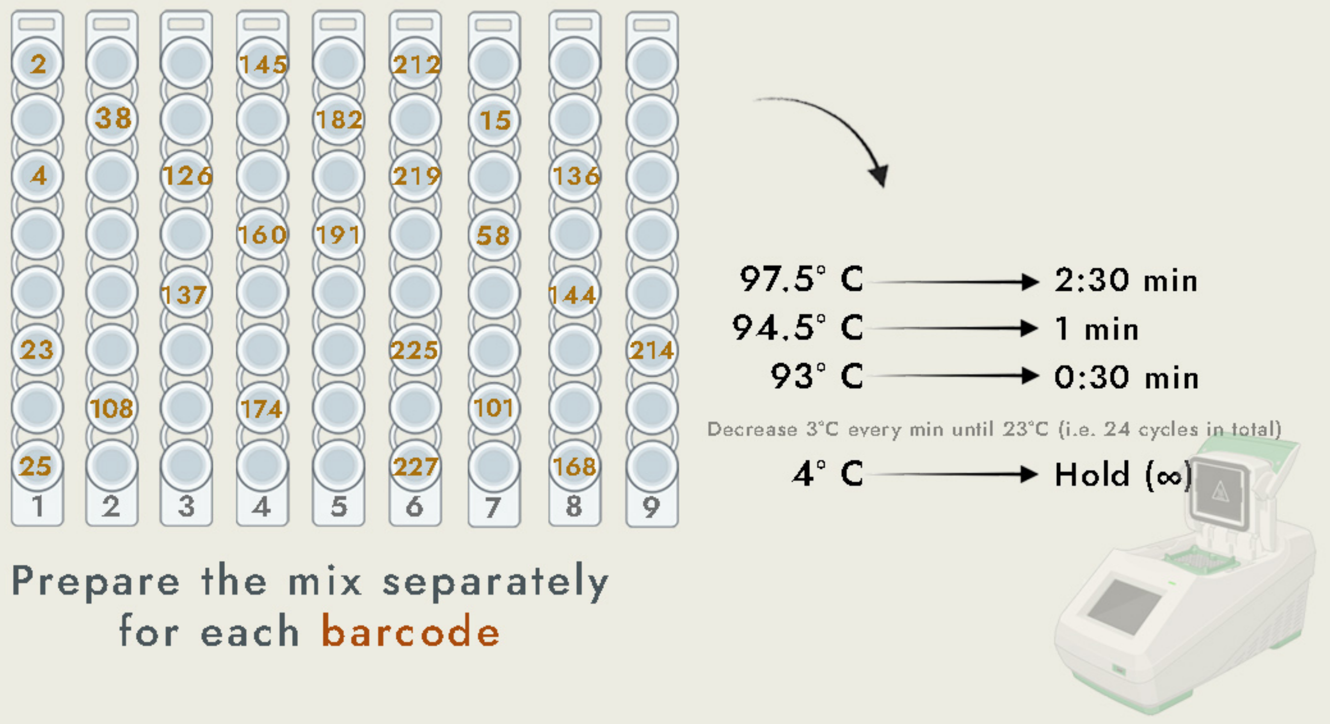
nmol × 10

③ Prepare Annealed Adapter Stock 40 μM

EcoRI Annealed Adapter Stock **40 μM**

P1.1 Adapter (100 μM)	40 μL
P1.2 Adapter (100 μM)	40 μL
Annealing Buffer 10×	10 μL
mQ® H_2O	10 μL

- Prepare the mix in **tube strips**

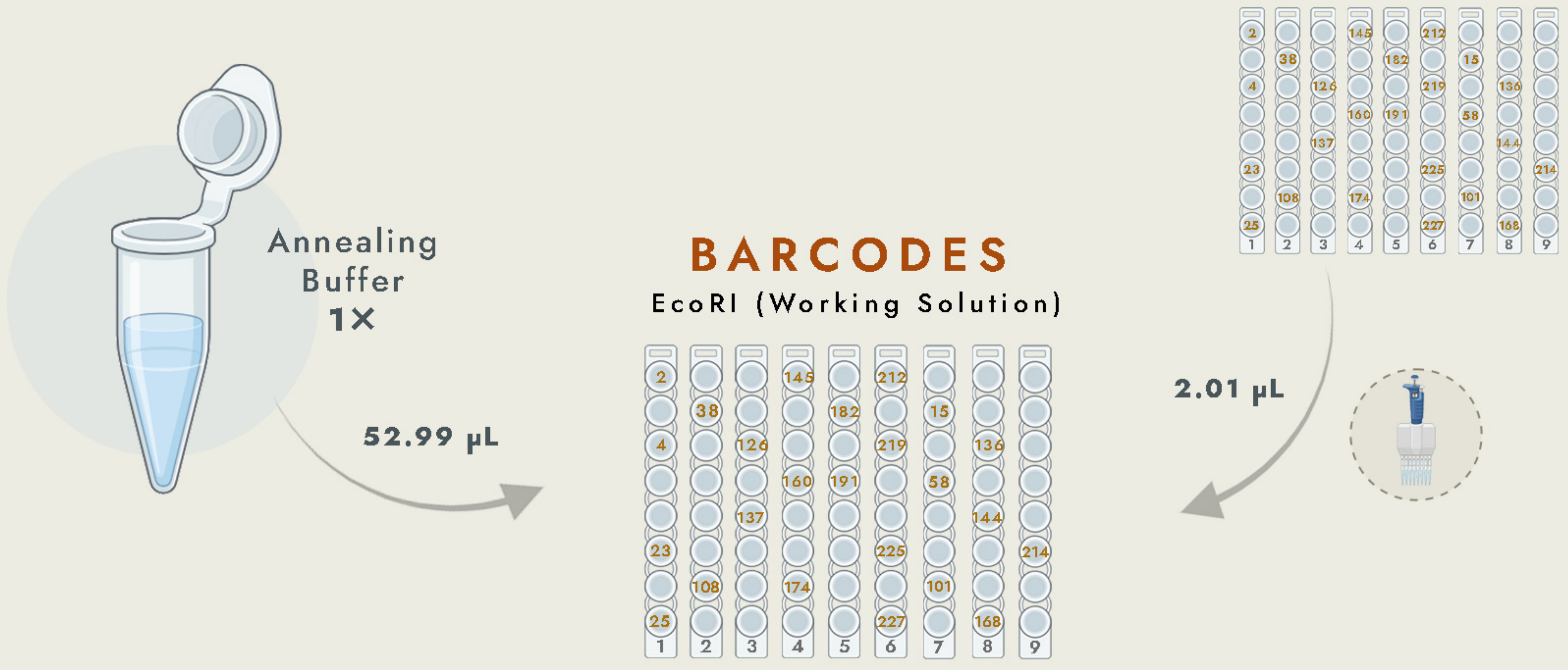


Prepare the mix separately for each barcode

97.5° C → 2:30 min
94.5° C → 1 min
93° C → 0:30 min
Decrease 3°C every min until 23°C (i.e. 24 cycles in total)
4° C → Hold (∞)

④ Prepare EcoRI adapter (P1) (EcoRI - Working Solution)

EcoRI Annealed Adapter Stock **40 μM**

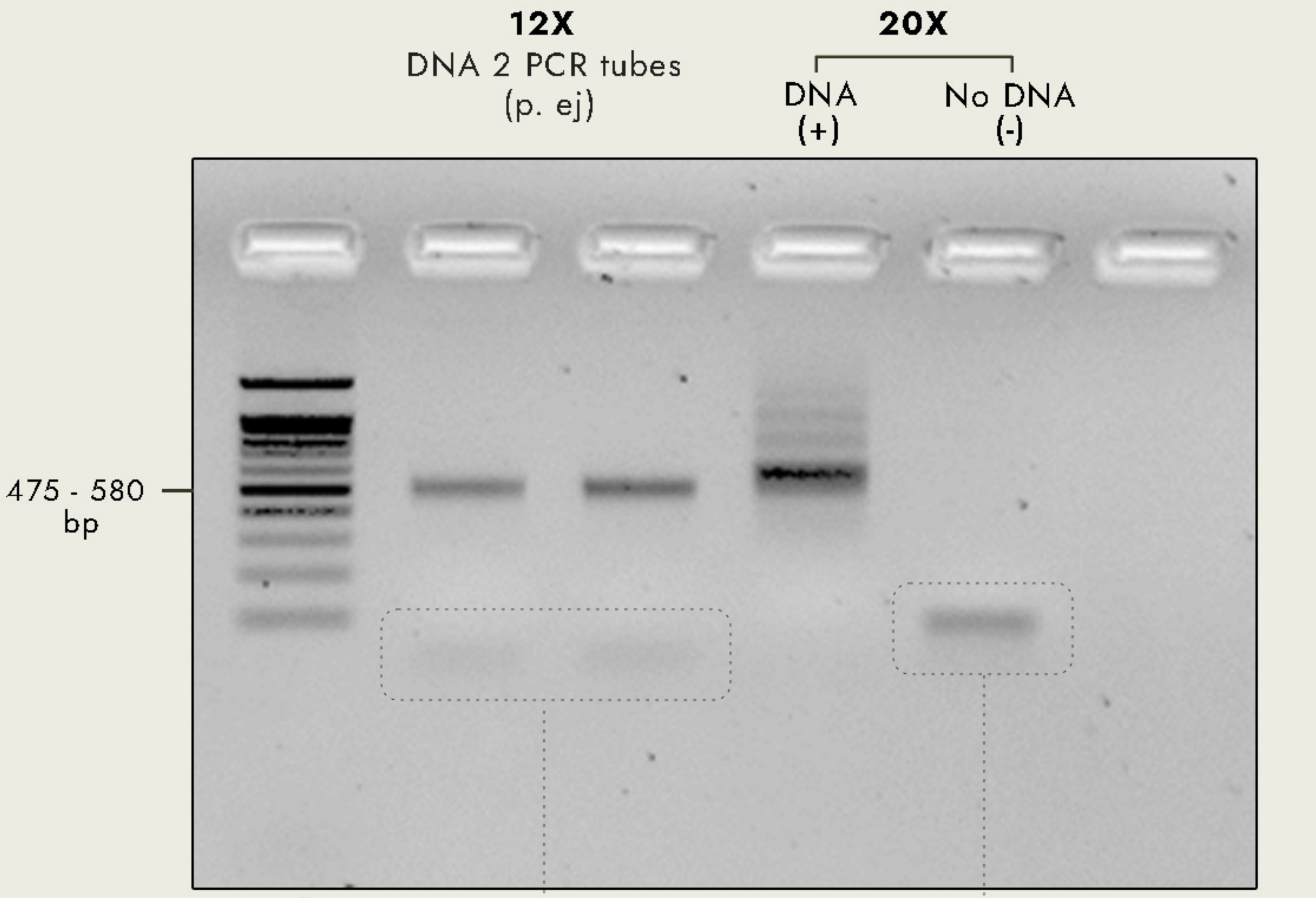


BARCODES
EcoRI (Working Solution)

(Adapter EcoRI - P1)

PCR:

Gel Electrophoresis

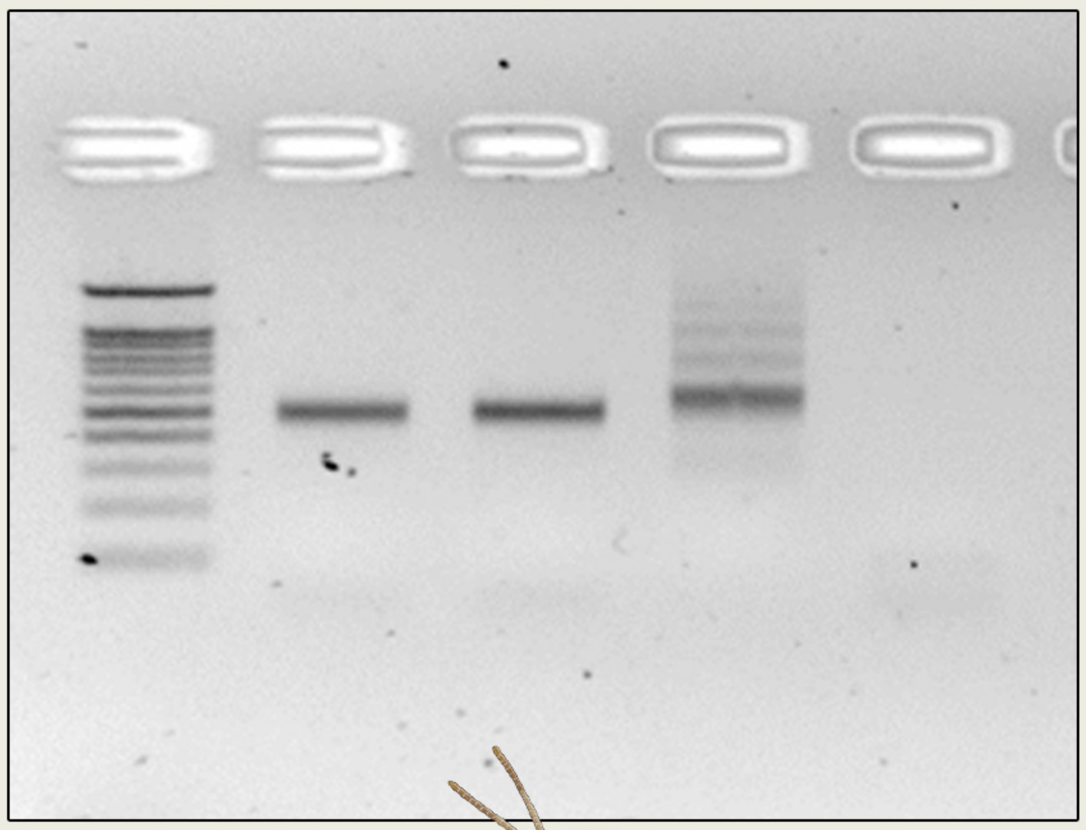


Oropodisma_L5

i18

Dimers
Minimize as much as possible

Dimers
In the absence of DNA, dimers are more abundant as they are not incorporated by Taq polymerase



Oropodisma_L6

i20

Quantification

Oropodisma_L5	**Qubit:**	9.47 ng/µL
	Nanodrop:	17.3 ng/µL
Oropodisma_L6	**Qubit:**	15.5 ng/µL
	Nanodrop:	25.1 ng/µL

Cargamos:

3 µL ladder

2 µL loading dye
2 µL PCR product

Agarose gel 1.5 %

For small gel tray: 50 mL

TBE: 50 mL
Agarose: 0.75 g
SybrSafe: 2.5 µL