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Construction of individuals ddRADseq libraries for macro-algae (Kelp) V.3 V.3

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Stéphane Mauger , Aurélien Baud , Gildas Le Corguillé , Gwenn Tanguy , Erwan Legeay , Emeline Creis , Myriam Valero , Philippe Potin , Christophe Destombe (2023).Genetic resources of macroalgae: Development of an efficient method using microsatellite markers in non-model organisms. *Algal Research - Biomass, Biofuels and Bioproducts*, 2023, 75, pp.103251. **⟨10.1016/j.algal.2023.103251⟩**

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We use this protocol and it's working

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Keywords: Kelp, SNPs marker, ddRAD-seq, Genomics population, Micro-algae, genomic dna purification step, algae sample, construction of individual ddradseq library, algae population, genomic dna, individual ddradseq library, associated dna, de novo snp discovery, genomic dna from the sample, sequencing method, dna, original ddradseq protocol, sequencing, restriction enzymes psti, ddradseq, digested restriction, purification, rad taq enrichment step, inhibitors of pcr, several genetic study, effectiveness in several genetic study, ligation to adaptor, final pcr amplification

Abstract

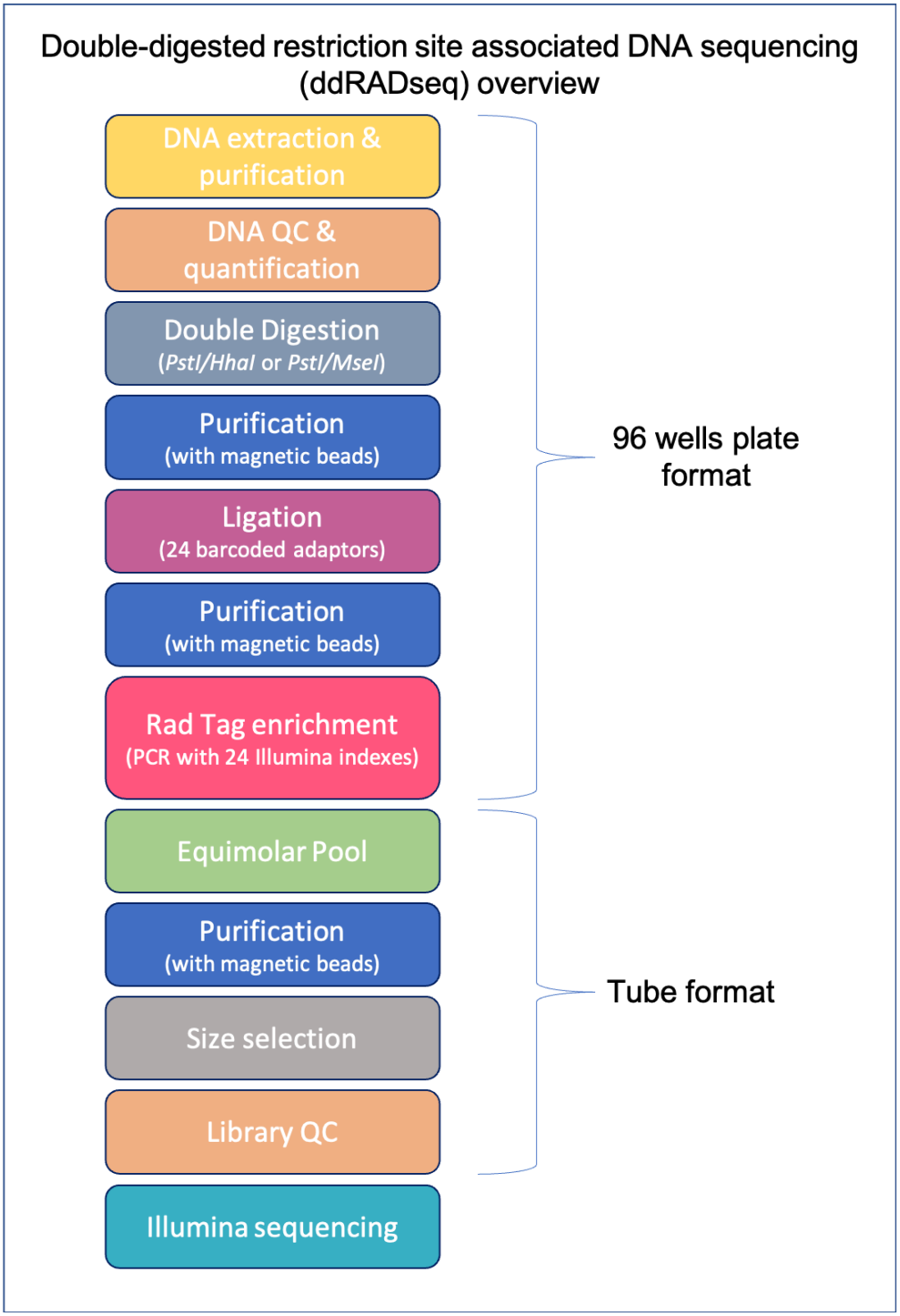
This protocol describes a double digested restriction-site associated DNA (ddRADseq) procedure, that is a variation on the original RAD sequencing method (**Davey & Blaxter 2011**), which is used for *de novo* SNP discovery and genotyping.

This protocol differs from the original ddRADseq protocol (**Peterson et al 2012**), in which the samples are pooled just after the ligation to adaptors (i.e. before size selection and PCR). This protocol is an update of the protocol from **Claire Daguin Thiebaut et al.** (dx.doi.org/10.17504/protocols.io.bv4tn8wn) adapted for macro-algae.

The following protocol is intended for the construction of individual ddRADseq libraries from genomic DNA of various macro-algae samples (Kelp). In the present protocol, we added a genomic DNA purification step to eliminate the inhibitors of PCR and Ligation present in macro-algae (polysaccharides). Moreover, all samples are treated separately until final PCR amplification (Rad Taq enrichment step) performed before pooling.
Despite being slightly more costly and time-consuming in the lab, it allows for fine adjustement of each sample representation in the final library pool ensuring similar number of reads between samples. Finally, we have defined new P1 adapters (barcodes) with variable sequences and variable sizes (6bp to 13bp) to increase the efficiency of the Illumina sequencing.

Briefly, purified genomic DNA from the samples are individually digested with 2 restriction enzymes ***PstI/HhaI*** or ***PstI/MesI*** (one rare-cutter and one more frequent cutter) then ligated to a barcoded adaptor (among 24 available) at one side, and a single adaptor at the other side, purified with magnetic beads, and PCR-amplified allowing the addition of a Illumina index (among 24 available) for **multiplexing a maximum of 576 samples per library**. Samples are then pooled in equimolar conditions after visualisation on an agarose gel. Purification and size selection is then performed before final quality control of the library and sequencing.

This protocol has proven its effectiveness in several genetic studies of marco-algae populations.



Overview of ddRADseq libraries preparation

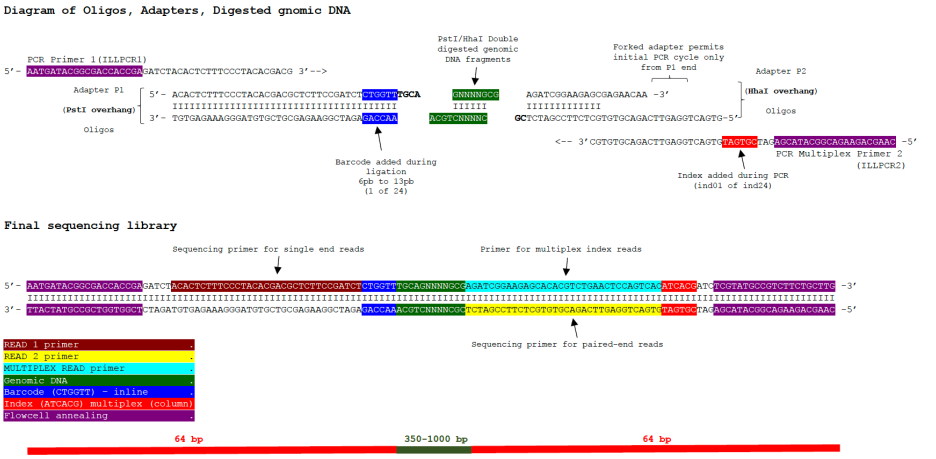
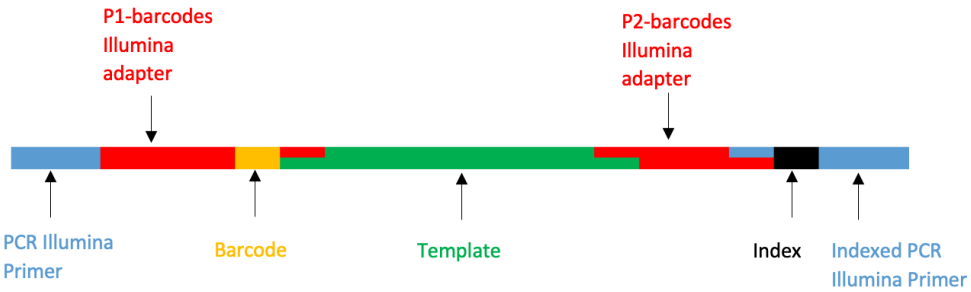


Diagram of oligos and adaptaters; final library; BRIEF GLOSSARY



Final ddRAD library construction

Protocol materials

- Tris-HCl 1M solution pH 8.0 Molecular Biology Grade Ultrapure **Thermo Scientific Catalog #** J22638.AP
- EDTA 0.5M **Fisher Scientific Catalog #**MRGF-1202
- Sodium Chloride **Fisher Scientific Catalog #**S271
- Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #**BP2818500
- PstI-HF **New England Biolabs Catalog #**RS3140RS
- HhaI - 10,000 units **New England Biolabs Catalog #**R0139L
- CutSmart® Buffer **New England Biolabs Catalog #**B7204S
- MseI - 500 units **New England Biolabs Catalog #**R0525S
- Quant-iT™ PicoGreen™ dsDNA Assay Kit **Invitrogen - Thermo Fisher Catalog #**P11496
- Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #**BP2818500
- Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #**M0493L
- Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #**BP2818500

Troubleshooting

Before start

1. Prepare all buffers and solutions in advance (see Step 1 to Step 5)
2. If not using Retsch © Mixer Mill MM 301 (or equivalente) and Grinding ball for the sample grinding, you can use Lysing Matrix H tube with FastPrep-24™ Classic or manual grinding as a last resort.

Solutions and buffers preparations

15m

1 **5 M sodium chloride solution (NaCl)** 5m

29.2 g NaCl (M.W. 58,44)

Dissolve the slat in MilliQ water and fill up to 100 mL .

Autoclave.

Store at Room temperature

Sodium Chloride **Fisher Scientific Catalog #**S271

2 **Annealing buffer stock (10x)** 10m

Annealing buffer composed 100 mM Tris-HCl, pH8; 500 mM NaCl and 10 mM EDTA

5 mL Tris-HCl, 1M solution, pH 8,0

5 mL NaCl, 5M solution

1 mL EDTA, 0.5 M solution

39 mL MilliQ water

Homogenize and autoclave.

Store at Room temperature

Tris-HCl 1M solution pH 8.0 Molecular Biology Grade Ultrapure **Thermo Scientific Catalog #** J22638.AP

EDTA 0.5M **Fisher Scientific Catalog #**MRGF-1202

Preparation of double-stranded barcoded P1 adaptors 4µM

1h 10m

3 Single-stranded oligos NGS grade P1 need to be annealed with their appropriate partner before ligation. We provide sequences for 48 uniquely barcoded adapter P1 oligo pairs (oligos P1_PstI_x.F and P1_PstI_x.R), **see the Barcoded_P1_adaptors.xlsx file below**.
To create Adapter P1, combine each oligo Forward with its complementary oligo Reverse in a 1:1 ratio in working strength annealing buffer (final buffer concentration 1x) for a total annealed adapter
concentration of 4µM.

In house barcoded P1 adaptors sequences (NGS grade needed) :

Barcoded_P1_adaptors.xlsx 13KB

3.1 In a PCR plate wells, combine each oligo P1_PstI_x.F with its complementary oligo P1_PstI_x.R : 30m

- 4 µL oligo Forward (100µM)
- 4 µL oligo Reverse (100µM)
- 10 µL Annealing buffer (10x)
- 82 µL nuclease free water

3.2

	A	B	C	D	E	F	G	H
	P1_PstI_01			P1_PstI_09			P1_PstI_17	
	P1_PstI_02			P1_PstI_10			P1_PstI_18	
	P1_PstI_03			P1_PstI_11			P1_PstI_19	
	P1_PstI_04			P1_PstI_12			P1_PstI_20	
	P1_PstI_05			P1_PstI_13			P1_PstI_21	
	P1_PstI_06			P1_PstI_14			P1_PstI_22	
	P1_PstI_07			P1_PstI_15			P1_PstI_23	
	P1_PstI_08			P1_PstI_16			P1_PstI_24	

Example of a plate map for barcoded P1 adaptors. Allow enough space betweeen the rows to avoid cross-contaminations between barcodes.

3.3 The reaction is performed in a thermocycler with the following PCR cycling conditions : 40m

	A	B	C	D
	Cycle step	Temperature	Time	Cycles
	Initial Denaturation	97.5°C	2.5 min	1

	A	B	C	D
	Annealing	96°C (-3°C per cycle)	1 min	25
	Hold	4°C		

PCR cycling conditions

Store at 4 °C (or at -20 °C for a long-term storage)

Preparation of double-stranded P2 adaptors 40µM

45m

4 Single-stranded oligos NGS grade P2 need to be annealed with their appropriate partner before PCR. We provide sequences for 4 uniquely adapter P2 oligo pairs (oligos P2_HhaI.F and P2_HhaI.R or P2_MseI.F and P2_MseI.R), **see the No-Barcoded_P2_adaptors.xlsx file below**.
To create Adapter P2, combine each oligo Forward with its complementary oligo Reverse in a 1:1 ratio in working strength annealing buffer (final buffer concentration 1x) for a total annealed adapter
concentration of 40µM.

No-barcoded P2 adaptors sequences (NGS grade needed) :

No-Barcoded_P2_adaptors.xlsx 10KB

4.1 In 1.5mL microtube, combine oligo P2_HhaI.F with its complementary oligo P2_HhaI.F (or P2_MseI.F and P2_MseI.R) 5m

400 µL oligo Forward (100µM)

400 µL oligo Reverse (100µM)

100 µL Annealing buffer (10x)

100 µL nuclease free water and mix by pipetting

Then aliquot this volume into 125 µL in each well of a 8- PCR tube strip.

4.2 The reaction is performed in a thermocycler with the following PCR cycling conditions : 40m

	A	B	C	D
	Cycle step	Temperature	Time	Cycles
	Initial Denaturation	97.5°C	2.5 min	1
	Annealing	96°C (-3°C per cycle)	1 min	25
	Hold	4°C		

PCR cycling conditions

Pool all reaction in a 1.5 mL tube.

Store at 4 °C (or at -20 °C for a long-term storage).

Preparation of Illumina indexed primers mix (5µM) 30m

5 30m

In 24 1.5 mL microtubes, combine each of the 24 Illumina indexed reverse primers ILLPCR2_ind01 to ILLPCR2_ind27 (no primer numbers ind17, ind24 and ind26) with the Illumina no-indexed forward primer ILLPRC1, **see the Illumina_indexed_primers.xlsx file below**.

5 µL ILLPCR1 oligo forward (100µM)

5 µL ILLPCR2 oligo reverse (100µM) ind01 to ind27 (one per tube)

90 µL nuclease free water and mix by pipetting

Store at 4 °C (or at -20 °C for a long-term storage)

Ilumina indexed primers sequences (NGS grade needed) :

Illumina_indexed_primers.xlsx 10KB

Genomic DNA extraction and purification 7h 47m

6

Genomic DNA extraction

Upon collection, a piece of tissue was cut out from a spot that was free of algal and animal epiphytes and stored in silica gel. Total genomic DNA was extracted from 15 to 20 mg of grinded dry tissue using the Nucleospin 96 plant kit (Macherey-Nagel, Germany).

Example of micro-algae stored in silica gel

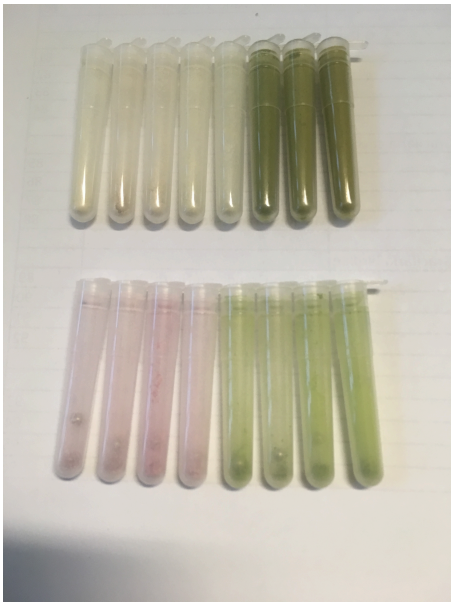
Silica gel drying agent, with moisture indicator (orange gel) **VWR International (Avantor) Catalog #**1.03806.0001

6.1 In a Rack of Tube Strips (consumable of NucleoSpin 96 Plant II kit) add 15 mg to 20 mg of dry tissue of each sample with one 3 mm grinding ball stainless steel. Close the Tubes Strips with Cap Strips. 3h

Example of dry algae with 3 mm steel ball before and after grinding process in individual tube

Grinding ball stainless steel **VWR International (Avantor) Catalog #**412-0254

6.2 Grind dry tissues using Mixer Mill MM400 Retch using 2 cycles of 00:02:00 at maximum frequency. 5m



Example of grinded samples in 96 wells plate format

MIXER MILL MM 400 **Retsch**

6.3 The extraction was performed according to the manufacturer's instructions using the PL1 lysis buffer except that we added one wash step with PW1 buffer (2 times PW1 washes in total) and one wash step with PW2 buffer (3 times PW2 washes in total). The extracted DNA was eluted into 120 µL (2 × 60 µL) of the supplied elution buffer. 2h

Instruction-NucleoSpin-96-Plant-II.... 912KB

Store at 4 °C (or at -20 °C for a long-term storage)

NucleoSpin 96 Plant II, 96-well kit for DNA from plants **Macherey-Nagel Catalog #**740663.4

7 Genomic DNA purification 3h 45m

The genomic DNA extracts were purified using the NucleoSpin gDNA Clean-up XS, Micro kit for DNA clean up and concentration (Macherey-Nagel, Germany).
The purifications were performed according to the manufacturer's instructions with elution into 30 µL (2 × 15 µL) of the supplied elution buffer.

7.1 Transferring the 120 µL of each samples into 1.5 mL microtubes. Add nuclease free water to fill up to 400 µL . 1h

7.2 The purifications were performed according to the manufacturer's instructions with elution into 30 µL (2 × 15 µL) of the supplied elution buffer. 2h

Instruction-NucleoSpin-gDNA-Clea... 805KB

7.3 Removal of residual ethanol and concentration were performed by incubation 00:15:00 at 70 °C 15m

7.4 The purified gDNA of each sample was transferred in a 96 wells PCR plate. 30m

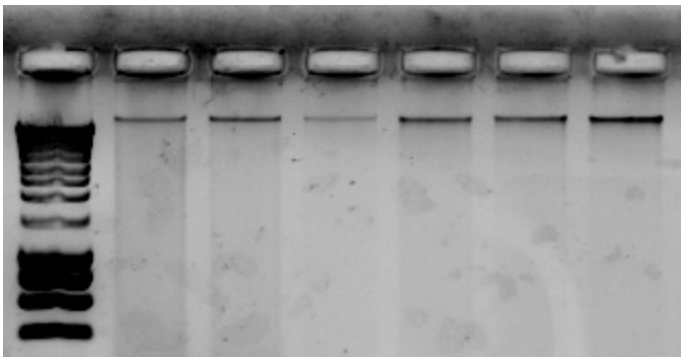
Store at 4 °C (or at -20 °C for a long-term storage)

NucleoSpin gDNA Clean-up XS, Micro kit for DNA clean up and concentration **Macherey-Nagel Catalog #**740904.250

Genomic DNA Quality and Quantification 4h

8 **Quality Control of Genomic DNA** 2h

Optional: load 1 µL to 3 µL of the Genomic DNA extract on an agarose gel to evaluate its quantity and quality using electrophoresis.



Example of agarose gel picture of genomic DNA

9 **Quantification of Genomic DNA (Preparation for one 96 wells PCR plate) using PicoGreen™** 2h

Quantify Genomic DNA extract using PicoGreen™.

Protocol

NAME

SOWA

Qant-iT™ PicoGreen® dsDNA Quantification

CREATED BY

Roey Angel

Preview

9.1 Take out all reagents from the fridge and bring them to room temperature.
Take out the DNA samples from the freezer. DNA samples should be slowly thawed on ice

Note

Quant-iT™PicoGreen® dsDNA reagent is dissolved in dimethylsulfoxide (DMSO), which freezes below 19 °C. The reagent must be completely thawed before using it by bringing it to room temperature. After the reagent thawed, it is advisable to briefly vortex the tube to make sure it is adequately mixed and to spin it down in a centrifuge or a mini centrifuge.

Note

Quant-iT™PicoGreen® dsDNA reagent is light sensitive and should be protected from light at all times.

Quant-iT™ PicoGreen™ dsDNA Assay Kit **Invitrogen - Thermo Fisher Catalog #**P11496

Greiner Bio-One 96-well sterile polystyrene plate, high binding, colour plate & bottom: black **Dutscher Catalog #**655077

9.2 **Preparation of** 11 mL **of 1X TE buffer**

In 15 mL sterile and nuclease-free tube

550 µL 20X TE (included in the kit)

10.450 mL nuclease-free water

Mix by inverting the tube several times.

9.3 **Preparation of DNA solution at 5000 pg/µl (for 3 ranges)**

In 0.5 mL nuclease-free tube

4 µL DNA-standard stock solution (λ DNA 100 ng/µl)

76 µL 1X TE buffer

Mix by inverting the tube several times.

9.4 **Preparation of the standard range 0 pg/µl to 1000 pg/µl**

Prepare the following standard mixture in 8 0.5 mL nuclease-free tubes

	A	B	C	D	E
	Tubes	Standard DNA solution concentration (pg/µL)	Standard DNA solution volume (µL)	1X TE (µL)	**Final DNA concentration (pg/µL)**
	1	5000	42	168	**1000**
	2	5000	21	189	**500**
	3	5000	9	171	**250**
	4	1000	21	189	**100**

	A	B	C	D	E
	5	500	21	189	**50**
	6	100	18	162	**10**
	7	50	18	162	**5**
	8	0	0	180	**0**

Standard DNA solutions preparation

Pipette 50 µL of each standard mixture in the first two columns of the black, sterile, 96-well plate :

	A	B	C	D	E	F	G	H
	1000 pg/µL	1000 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA
	500 pg/µL	500 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA
	250 pg/µL	250 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA
	100 pg/µL	100 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA
	50 pg/µL	50 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA
	10 pg/µL	10 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA
	5 pg/µL	5 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA
	0 pg/µL	0 pg/µL	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA	unknown DNA

Exemple of map plate for PicoGreen™ quantification

9.5 Pipette 49 µl of 1X TE buffer in the remaining wells.

49 µL 1X TE buffer

9.6 Pipette 1 µl of the unknown DNA samples in the remaining wells.
1 µL of DNA sample

9.7 **Prepare PicoGreen® work solution**
In 10 mL nuclease-free tube
25 µL picogreen® 200X solution (included in the kit)
4.975 mL 1X TE buffer
Mix and protect from light.

9.8 Pipette 50 µL of PicoGreen work solution in each well, including the standard and unknown sample wells.

9.9 Protect the 96-well plate from light and incubate for 00:05:00 at room temperature. 5m

9.10 Place the plate in a plate reader and measure the fluorescence according to the following parameters:

Excitation	~480 nm
Emission	~520 nm
Integration time	40 s
Lag time	0 s
Gain	Optimal
Number of flashes	10
Calculated well	highest standard
Shaking	5 s

Equipment	
Synergy 2	NAME
absorbance microplate reader	TYPE
BioTek	BRAND
Synergy2	SKU
https://www.biotek.com/products/detection/	LINK

Equipment	
SPARK	NAME
Microwell plate reader	TYPE
TECAN	BRAND
SPARK	SKU
https://www.tecan.com/blog/spark-multimode-microplate-reader-for-high-performance-cell-based-fluorescence-assays	LINK

9.11 Plot the measured fluorescent values of the standard samples against their known concentrations and fit a linear curve using linear regression. Make sure that the coefficient of determination (R2) is close to 1 (typically > 0.99). Calculate the DNA concentrations in the unknown samples using the slope and intercept parameters of the linear equation. Output values you obtained are in pg/µl, assuming 1 µl of each sample was used.

Genomic DNA preparation

1h

10 In a PCR plate, put around 100 ng of genomic DNA in a volume of 40 µL (in nuclease free water or Tris-HCl 5mM pH 8.5) for each sample. If possible, randomize the location of samples in the microplate. Keep a few empty wells for negative controls.

Double digestion

20h

11 Double digest around 100 ng of high quality genomic DNA with selected restriction enzymes 50 µL reaction volume. Use a digestion buffer appropriate for both enzymes. Here, we will do the protocol for the ***PstI*** and ***HhaI*** couple of enzymes but it's same with ***PstI*** and ***MseI*** couple. Both couple of enzyme woks well for micro-algae but it's possible to test double digestion on few sample to select the best couple of enzymes. The best couple given large smear with size range 100 bp to 1000 pb.

11.1 Vortex all reagents, except enzymes (stored at -20 °C), for approximately 00:00:05 10s

Spin down all reagents for approximately 00:00:05 and place On ice.

In a microtube, prepare the digestion mix, according to the following table for a total volume of 50 µL :

	A	B	C	D	E
		Initial concentration	Final concentration	n=1	**n=100 (1 plate)**
	Genomic DNA		~100 ng	40 µL	
	Cutsmart buffer	10X	1X	5 µL	**500 µL**
	Enzyme 1 (PstI HF)	20 u/µL	10U	0.5 µL	**50 µL**
	Enzyme 2 (HhaI or MseI)	20 u/µL	10U	0.5 µL	**50 µL**
	nuclease-free water			4 µL	**400 µL**
	TOTAL			50 µL	**1000 µL**

Digestion master mix composition

PstI-HF **New England Biolabs Catalog #**RS3140RS

HhaI - 10,000 units **New England Biolabs Catalog #**R0139L

MseI - 500 units **New England Biolabs Catalog #**R0525S

CutSmart® Buffer **New England Biolabs Catalog #**B7204S

11.2 Vortex the master mix and spin down. 5s

Aliquot 125 µL of the digestion master mix in each well of a 8-PCR tube strip.

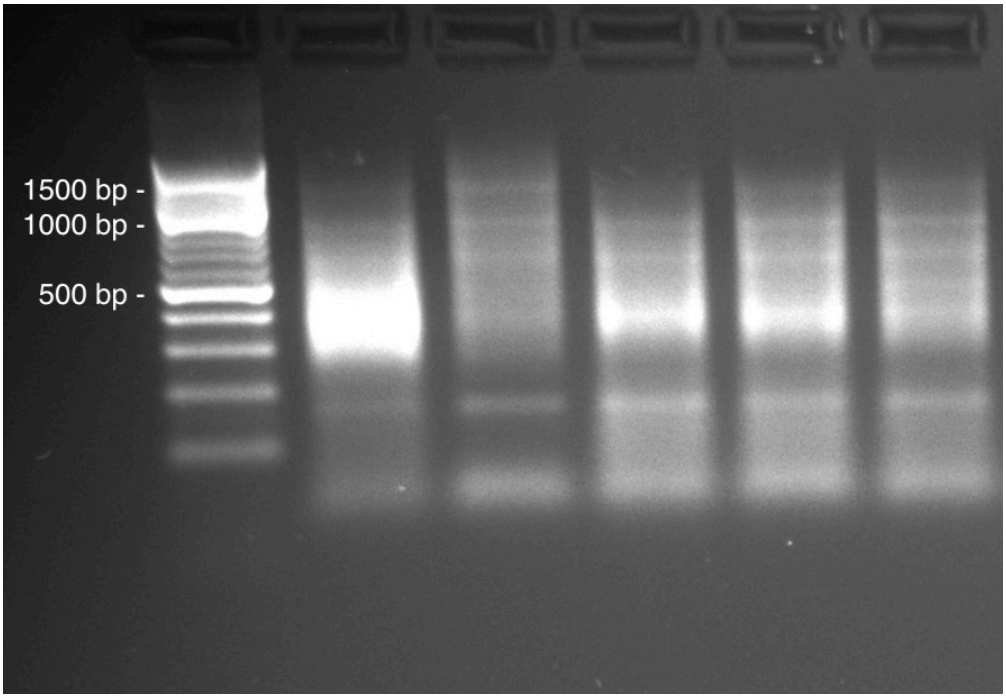
In the DNA plate (containing 40 µL per well), add 10 µL of digestion master mix with a x8 multichannel pipette and mix by pipetting, seal PCR plate and spin down.

Incubate at 37 °C Overnight

Then store at 4 °C

Check digestion on an agarose gel 2h

12 Check the efficiency of the digestion by electrophoresis of 5 µL of digested DNA in a 1.5 % agarose gel (standard quality). High molecular weigh DNA should no longer be visible.

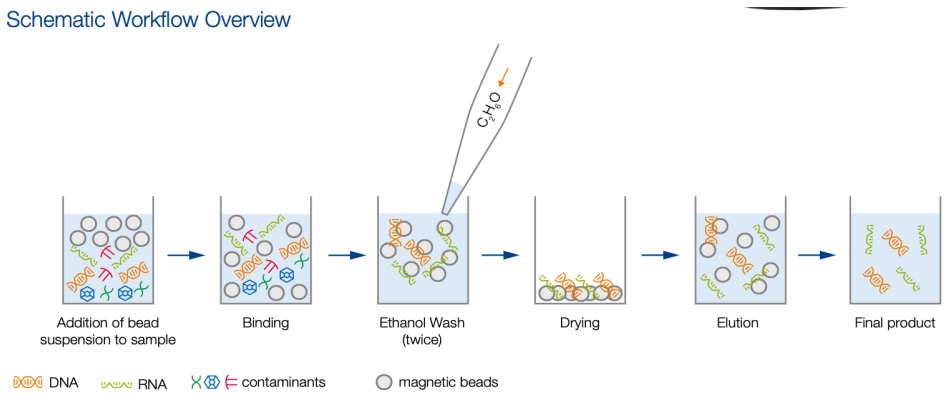


Example of agarose gel picture after digestion of genomic DNA. Size marker is a 100bp ladder.

Bead purification (96-well plate format)

1h

13 This protocol can be used to remove contaminants, unligated adapters, enzymes, buffer additives, salts... and short DNA fragments. The method utilizes a single-size selection step : After adding the appropriate volume of Bead Suspension to the DNA sample, beads will bind larger fragments. The supernatant contains smaller fragments and contaminants that are discarded. For most NGS sequencing applications it is optimal to remove all fragments below 100 bp. This can be achieved by using a volume ratio (bead suspension to sample) of 1:1, which is described in the following protocol.



NucleoMag kit for clean up and size selection Workflow (Macherey-Nagel)

NucleoMag kit for clean up and size selection of NGS library prep reactions **Macherey-Nagel Catalog #**744970.50

14 **Before starting**

Prepare 50 mL of fresh 80% Molecular Biology Grade Ethanol

40 mL Molecular Biology Grade Ethanol

10 mL nuclease-free water

Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #**BP2818500

Remove the NucleoMag® NGS Bead Suspension from the fridge. Let for approximately 30 min to bring the bead suspension to Room temperature .

Then, vortex this Bead Suspension stock solution carefully until homogenized and put in a reagent reservoir.

15 **Binding** 5m

This step binds DNA fragments 100 bp and larger to the magnetic beads.

Pipette 45 µL of NGS Beads suspension with x8 multichannel pipette and transfer in digestion plate (plate with 45 µL of digested template DNA for each sample), carefully mix by pipetting up and down 10 times.

Incubate 00:05:00 at Room temperature

16 **Separation** 5m

Place the purification plate onto the 96-well magnetic separator.

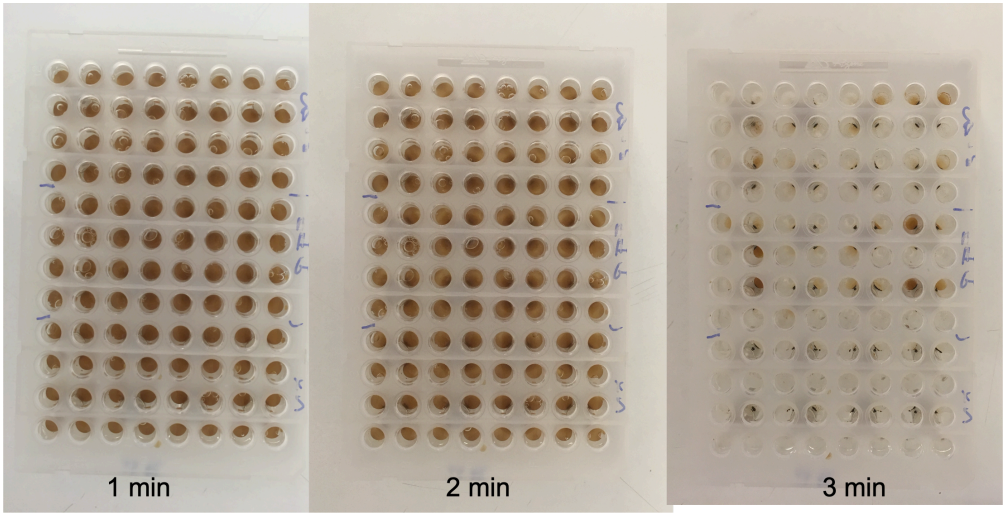
Wait at least 00:05:00 until all the beads have been attracted by the magnets or until the liquid appears clear.

The supernatant contains unwanted low molecular weight contaminants and unwanted smaller DNA fragments.

Remove and discard the supernatant (~90 µl) by pipetting.

Note

Do not disturb the attracted beads while aspirating the supernatant. Remove the supernatant with the multichannel from the opposite side of the well.



Example of separation process where beads have been attracted by the magnets

NucleoMag® SEP **Macherey-Nagel Catalog #744900**

17 **1st wash with 80 % ethanol** 30s

Place 80% ethanol in a reagent reservoir.

With a x8 multichannel pipette, dispense 200 µL of 80% ethanol into the purification plate without disturbing the bead pellet.

Incubate the purification plate at room temperature for at least 00:00:30

Carefully and completely remove and discard ethanol by pipetting.

18 **2nd wash with 80 % ethanol**

With a x8 multichannel pipette, dispense 200 µL of 80% ethanol into the purification plate without disturbing the bead pellet.

Incubate the purification plate at room temperature for at least 00:00:30

Carefully and completely remove and discard ethanol by pipetting.

19 **Dry the beads** 5m

Let the purification plate on the magnetic separator and incubate at Room temperature for **maximum** 00:05:00 in order to allow the remaining traces of ethanol to evaporate.

Note

Take care not to over dry the bead pellet (bead pellet appears cracked in this case) as this will significantly decrease elution efficiency.

20 **Elute DNA fragments** 10m

Take the purification plate from the magnetic stand, and add 40 µL of nuclease-free water with a x8 multichannel pipette to resuspend the bead pellet by pipetting up and down 10 times.

Incubate the purification plate at Room temperature for 00:05:00 .

Separate the magnetic beads against the side of the wells by placing the 96-well plate on the magnetic separator.

Wait at least 00:05:00 until all the beads have been attracted by the magnets or until the liquid appears clear.

Transfer 35 µL of the supernatant containing the digested purified template DNA to a new 96-well plate. **Be careful to avoid pipeting beads during this step**.

Seal the plate and store at 4 °C (or store -20 °C for a long-term storage) until adaptor ligation.

Adaptor ligation

18h

21 For each sample of one line of the digested purified plate (with 35 µL of digested purified template DNA) add 5 µL of double-stranded barcoded P1 adaptors at 4 µM.
Use one double-stranded barcoded P1 adaptors per line.

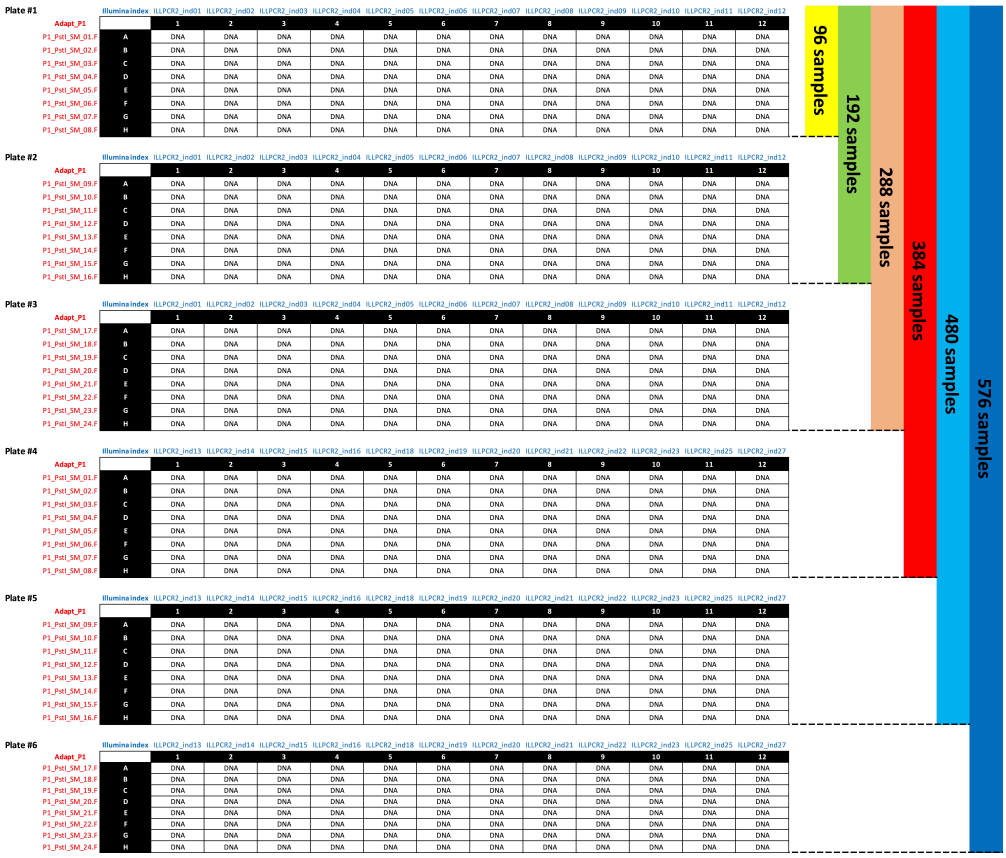
One P1 adapter per line.

	1	2	3	4	5	6	7	8	9	10	11	12
P1_PstI_01→ A	dna-01	dna-02	dna-03	dna-04	dna-05	dna-06	dna-07	dna-08	dna-09	dna-10	dna-11	dna-12
P1_PstI_02→ B	dna-13	dna-14	dna-15	dna-16	dna-17	dna-18	dna-19	dna-20	dna-21	dna-22	dna-23	dna-24
P1_PstI_03→ C	dna-25	dna-26	dna-27	dna-28	dna-29	dna-30	dna-31	dna-32	dna-33	dna-34	dna-35	dna-36
P1_PstI_04→ D	dna-37	dna-38	dna-39	dna-40	dna-41	dna-42	dna-43	dna-44	dna-45	dna-46	dna-47	dna-48
P1_PstI_05→ E	dna-49	dna-50	dna-51	dna-52	dna-53	dna-54	dna-55	dna-56	dna-57	dna-58	dna-59	dna-60
P1_PstI_06→ F	dna-61	dna-62	dna-63	dna-64	dna-65	dna-66	dna-67	dna-68	dna-69	dna-70	dna-71	dna-72
P1_PstI_07→ G	dna-73	dna-74	dna-75	dna-76	dna-77	dna-78	dna-79	dna-80	dna-81	dna-82	dna-83	dna-84
P1_PstI_08→ H	dna-85	dna-86	dna-87	dna-88	dna-89	dna-90	dna-91	dna-92	dna-93	dna-94	dna-95	dna-96

Map of plate with the P1 adaptors lines

Note

Depending on the number of samples you want to put into the library (maximum 576 samples), you can mix the number of barcoded P1 adaptors (one per lines) and the number of Illumina indexes (one per columns).



Number and map of plates with combinaison of barcoded P1 adaptors and Illumina Indexes depending of the number of samples required into the library.

22 Vortex all reagents, except enzymes (stored at -20 °C), for approximately 00:00:05 20m

Spin down all reagents for approximately 00:00:05 and place On ice.

In a microtube, prepare the ligation mix, according to the following table for a total volume of 60 µL:

	A	B	C	D	E
		Initial concentration	Final concentration	n=1	**n=100 (1 plate)**
	Digested purified template DNA + P1 adaptor			40 µL	
	P2 adaptor (HhaI or MseI)	40 µM	330 nM	0.5 µL	**50 µL**
	T4 ligase buffer	10X	1X	6 µL	**600 µL**

	A	B	C	D	E
	T4 ligase	400 u/µL	160U	0.4 µL	**40 µL**
	nuclease-free water			13.1 µL	**1310 µL**
	TOTAL			60 µL	**2000 µL**

Ligation master mix composition

23 Vortex the master mix and spin down. 17h

Aliquot 125 µL of the ligation master mix in each well of two 8-PCR tube strip.

In the digested purified plate (containing 35 µL of digested purified template DNA and 5 µL of barcoded P1 adaptors), add 20 µL of ligation master mix with a x8 multichannel pipette and mix by pipetting, seal PCR plate and spin down.

Incubate at 16 °C Overnight

Then store at 4 °C or at -20 °C if not performing the bead purification the day after.

Bead purification (96-well plate format) 1h

24 **Before starting**

Prepare 50 mL of fresh 80% Molecular Biology Grade Ethanol

40 mL Molecular Biology Grade Ethanol

10 mL nuclease-free water

Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog** #BP2818500

Remove the NucleoMag® NGS Bead Suspension from the fridge. Let for approximately 30 min to bring the bead suspension to Room temperature .

Then, vortex this Bead Suspension stock solution carefully until homogenized and put in a reagent reservoir.

NucleoMag kit for clean up and size selection of NGS library prep reactions **Macherey-Nagel Catalog #**744970.50

25 **Binding**

This step binds DNA fragments 100 bp and larger to the magnetic beads.

Pipette 60 µL of NGS Beads suspension with x8 multichannel pipette and transfer in adaptor-ligated plate (plate with 60 µL of digested and adaptor-ligated template DNA for each sample), carefully mix by pipetting up and down 10 times.

Incubate 00:05:00 at Room temperature

26 **Separation**

Place the purification plate onto the 96-well magnetic separator.

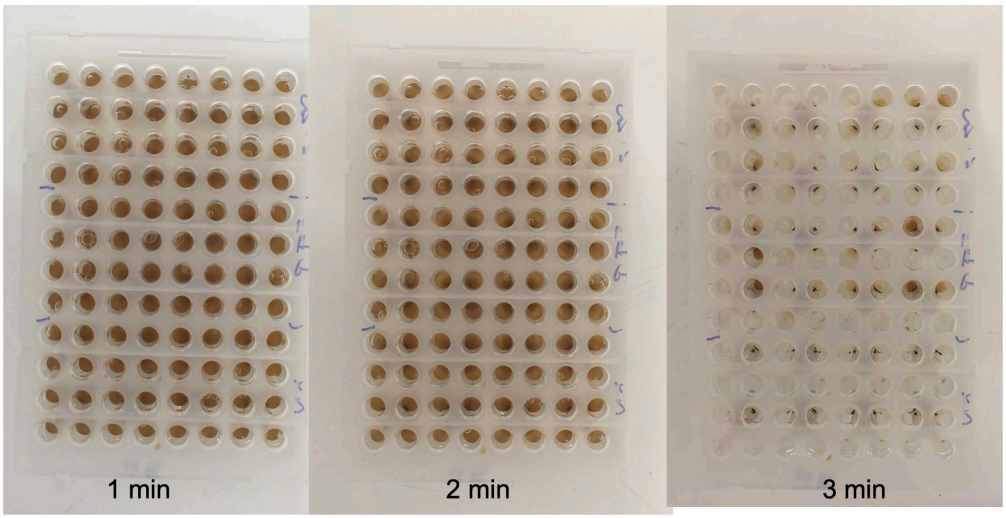
Wait at least 00:05:00 until all the beads have been attracted by the magnets or until the liquid appears clear.

The supernatant contains unwanted low molecular weight contaminants and unwanted smaller DNA fragments.

Remove and discard the supernatant (~120 µl) by pipetting.

Note

Do not disturb the attracted beads while aspirating the supernatant. Remove the supernatant with the multichannel from the opposite side of the well.



Example of separation process where beads have been attracted by the magnets

NucleoMag® SEP **Macherey-Nagel Catalog #**744900

27 **1st wash with 80 % ethanol**

Place 80% ethanol in a reagent reservoir.

With a x8 multichannel pipette, dispense 200 µL of 80% ethanol into the purification plate without disturbing the bead pellet.

Incubate the purification plate at room temperature for at least 00:00:30

Carefully and completely remove and discard ethanol by pipetting.

28 **2nd wash with 80 % ethanol**

With a x8 multichannel pipette, dispense 200 µL of 80% ethanol into the purification plate without disturbing the bead pellet.

Incubate the purification plate at room temperature for at least 00:00:30

Carefully and completely remove and discard ethanol by pipetting.

29 **Dry the beads**

Let the purification plate on the magnetic separator and incubate at Room temperature for maximum 00:05:00 in order to allow the remaining traces of ethanol to evaporate.

Note

Take care not to over dry the bead pellet (bead pellet appears cracked in this case) as this will significantly decrease elution efficiency.

30 **Elute DNA fragments** 10m

Take the purification plate from the magnetic stand, and add 40 µL of nuclease-free water with a x8 multichannel pipette to resuspend the bead pellet by pipetting up and down 10 times.

Incubate the purification plate at Room temperature for 00:05:00 .

Separate the magnetic beads against the side of the wells by placing the 96-well plate on the magnetic separator.

Wait at least 00:05:00 until all the beads have been attracted by the magnets or until the liquid appears clear.

Transfer 35 µL of the supernatant containing the adaptor-ligated purified template DNA to a new 96-well plate. **Be careful to avoid pipeting beads during this step**.

Seal the plate and store at 4 °C (or store -20 °C for a long-term storage) until PCR amplification.

Rad Tag enrichment (PCR) 3h

31 PCR amplification to generate Illumina sequencing indexed libraries :

In this PCR, Illumina indexed primers are incorporated in order to produce fragments compatible with Illumina sequencing, and to insert an index allowing multiplexing of barcoded samples. This index will be read during the sequencing run.

This PCR is expected to have a homogenizing effect. Primers are thus included in limiting quantity, in order to produce equalized amounts of PCR fragments among samples. The number of cycles is limited to a maximum of 15 (optimal with 12). After those cycles, a final PCR cycle is then performed after addition of primers in large excess.

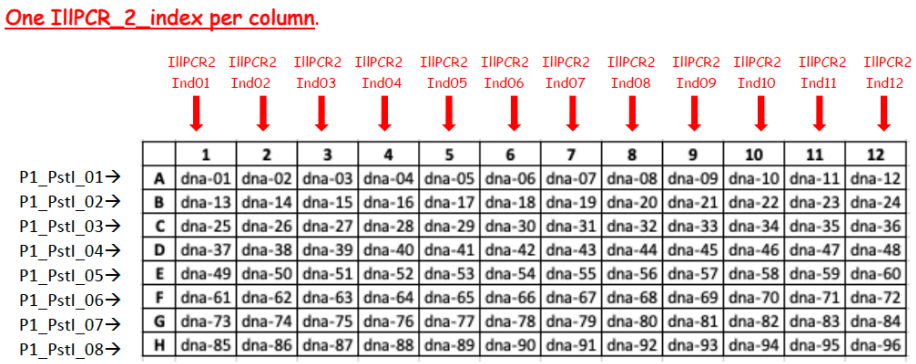
The Reaction mixture for a total volume of 40 µL is :

	A	B	C	D
		Initial concentration	Final concentration	n=1
	Adaptor-ligated purified template DNA			10 µL
	Primer mix (ILLPCR1 and ILLPCR2ind)	5 µM each	0.17 µM	1.36 µL
	Q5 buffer	5X	1X	8 µL

	A	B	C	D
	High GC enhancer	5X	1X	8 µL
	dNTP mix	25mM each	0.20 µM	0.32 µL
	Q5 hotstart hifi polymerase	2 u/µL	0.8 U	0.40 µL
	nuclease-free water			11.92 µL
	Total mix			30 µL
	TOTAL reaction			40 µL

PCR mixture composition

We need to prepare one PCR mixture per index, i.e. 12 PCR mixtures for one plate.



Map of plate with the Illumina indexes columns

Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #**M0493L

32 **First PCR mix preparation (with primers in limiting quantity)** 30m

Defreeze and vortex all reagents, except enzymes (stored at -20°C), for approximately 00:00:05

Spin down all reagents for approximately 00:00:05 and place On ice.

In 12 0.5 mL microtubes, prepare the 1st mix according to the following table (one mix per column) :

	A	B	C	D	E
		Initial concentration	Final concentration	n=1	**n=10 (one column of 1 plate)**
	Adaptor-ligated purified template DNA			10 µL	
	Primer mix (ILLPCR1 and ILLPCR2ind)	5 µM each	0.17 µM	1.36 µL	**13.6 µL**
	Q5 buffer	5X	1X	8 µL	**80 µL**
	High GC enhancer	5X	1X	8 µL	**80 µL**
	dNTP mix	25mM each	0.20 µM	0.32 µL	**3.2 µL**
	Q5 hotstart hifi polymerase	2 u/µL	0.8 U	0.40 µL	**4 µL**
	nuclease-free water			11.92 µL	**119.2 µL**
	TOTAL			30 µL	**300 µL**

PCR mix composition

Vortex mix all reagents in the mix and spin down.
In a new PCR plate, dispense 30 µL of 1st mix in each column.

33 **DNA and mix combination** 1h 30m

Spin down the adaptor-ligated purified template DNA plate.
With a multichannel pipette, transfer 10 µL of adaptor-ligated purified template DNA into the PCR plate and mix by pipetting.
Finally, aliquot the 40 µL of the total mix by dispensing 20 µL into 1 additional new empty PCR plates.
Seal the 2 PCR plates and spin down.

The 2 PCR will be performed in parallel in 2 different thermal cyclers, in order to reduce the PCR bias.

	A	B	C	D
	Cycle step	Temperature	Time	Cycles
	Hot start initial denaturation	98°C	30 sec	1
	Denaturation	98°C	20 sec	15
	Annealing	60°C	30 sec	15
	Extension	72°C	40 sec	15
	Final extension	72°C	10 min	1
	Hold	4°C		

PCR program for the Illumina indexing PCR

After PCR, pool back the 2 PCR plates into a single plate with a multichannel

34 **Final cycle (with primers in large excess)** 1h

In a 12-tube PCR strip, prepare the 2nd mix according to the following table (one mix per column) :

	A	B	C	D	E
		Initial concentration	Final concentration	n=1	**n=10 (one colomn of 1 plate)**
	Primer mix (ILLPCR1 and ILLPCR2ind)	5 µM each	3.35 µM	2.68 µL	**26.8 µL**
	Q5 buffer	5X	1X	0.80 µL	**8 µL**
	dNTP mix	25mM each	0.20 µM	0.32 µL	**3.2 µL**
	nuclease-free water			0.20 µL	**2 µL**
	TOTAL			4 µL	**40 µL**

Final cycle PCR mix composition

Mix all reagents by pipetting and spin down.

Dispense 4 µL of final cycle mix in each line of the PCR plate with a 12 multichannel pipette.
Seal the PCR plate and spin down.

In a thermocycler, run the final cycle as follows :

	A	B	C	D
	Cycle step	Temperature	Time	Cycles
	Denaturing	98°C	3 min	1
	Annealing	60°C	2 min	1
	Extension	72°C	12 min	1
	Hold	12°C		

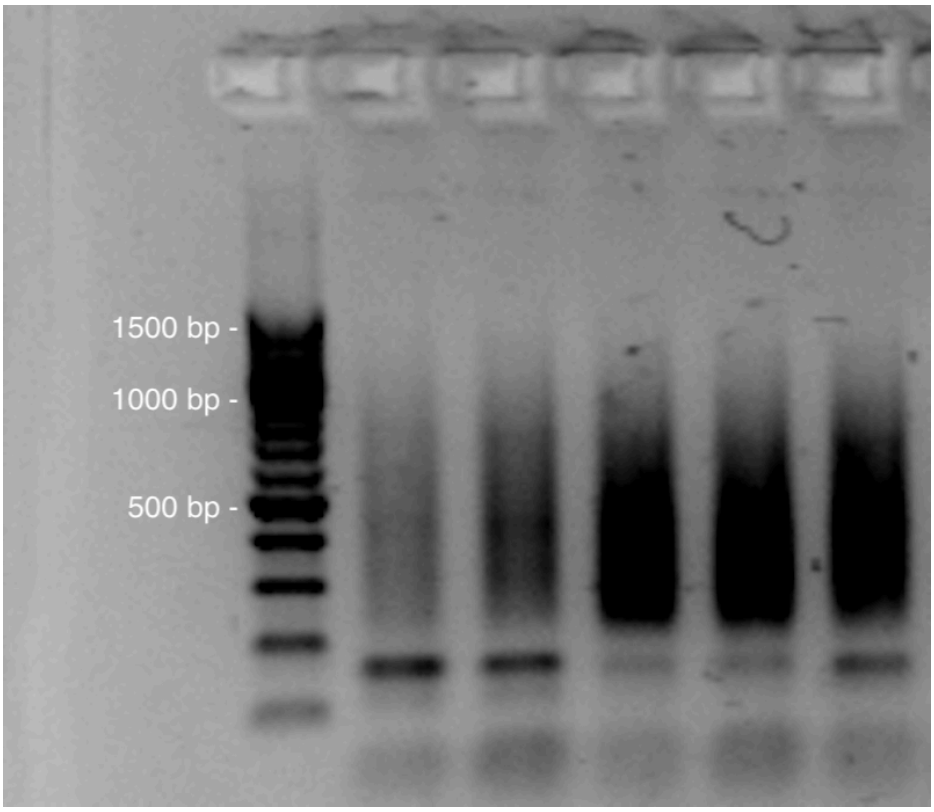
PCR program for the final cycle of the illumina PCR

After PCR, place the plate at 4 °C (or -20 °C for a long-term storage).

Check PCR on an agarose gel

1h

35 Check the efficiency of the PCR by electrophoresis of 5 µL of digested DNA in a 1.5 % agarose gel (standard quality).



Example of smears obtained after the Illumina PCR.
Size marker is a 100bp ladder.

Sample pooling (equimolar)

30m

36 Each barcoded and indexed individual can now be pooled in a single tube, in equimolar conditions.

After the normalizing PCR, all smears should have similar intensity on the agarose gel. In this case, pool 5 µL of all individuals in a single low binding 1.5 mL microtube.

If not, normalization can be made at this step. For this, roughly estimate the concentration of fragments from the gel picture, and pools accordingly. It can be efficient to make intermediate pools for example, one pool for the low, one for the medium, and another one for the high intensity samples in 3 low binding 1.5 mL microtubes .

Vortex mix and spin down.

Store at 4 °C (or -20 °C for a long-term storage) until bead purification.

Bead purification (microtube format) 30m

37 **Before starting**

Prepare 10 mL of fresh 80% Molecular Biology Grade Ethanol

8 mL Molecular Biology Grade Ethanol

2 mL nuclease-free water

Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #**BP2818500

Remove the NucleoMag® NGS Bead Suspension from the fridge. Let for approximately 30 min to bring the bead suspension to Room temperature.

Then, vortex this Bead Suspension stock solution carefully until homogenized and put in a reagent reservoir.

NucleoMag kit for clean up and size selection of NGS library prep reactions **Macherey-Nagel Catalog #**744970.50

38 **Binding**

This step binds DNA fragments 100 bp and larger to the magnetic beads.

Pipette a volume of NGS Beads suspension to have a ratio 1:1 with the sample pooling volume, and transfer in the pooling sample tube(s).

Carefully mix by pipetting up and down 10 times.

Incubate 00:05:00 at Room temperature

39 **Separation**

Place the purification tube(s) onto the magnetic microtube stand.

Wait at least 00:05:00 until all the beads have been attracted by the magnets or until the liquid appears clear.

The supernatant contains unwanted low molecular weight contaminants and unwanted smaller DNA fragments.

Remove and discard the supernatant by pipetting.

Note

Do not disturb the attracted beads while aspirating the supernatant. Remove the supernatant with the pipette from the opposite side of the well.

Rack de séparation MagJET, 12 tubes de 1,5 ml **Thermo Fisher Scientific Catalog** #MR02

40 **1st wash with 80 % ethanol**

Dispense 1 mL of 80% ethanol into the purification tube(s) without disturbing the bead pellet.

Incubate the purification tube(s) at room temperature for at least 00:00:30

Carefully and completely remove and discard ethanol by pipetting.

41 **2nd wash with 80 % ethanol**

Dispense 1 mL of 80% ethanol into the purification tube(s) without disturbing the bead pellet.

Incubate the purification tube(s) at room temperature for at least 00:00:30

Carefully and completely remove and discard ethanol by pipetting.

42 **Dry the beads**

Let the purification tube(s) on the magnetic separator and incubate at Room temperature for maximum 00:05:00 in order to allow the remaining traces of ethanol to evaporate.

Note

Take care not to over dry the bead pellet (bead pellet appears cracked in this case) as this will significantly decrease elution efficiency.

43 **Elute DNA fragments** 10m

Take the purification tube(s) from the magnetic stand, and add 100 µL of nuclease-free water with a pipette to resuspend the bead pellet by pipetting up and down 10 times.

Incubate the purification tube(s) at Room temperature for 00:05:00 .

Separate the magnetic beads against the side of the tube by placing the tube(s) on the magnetic separator.

Wait at least 00:05:00 until all the beads have been attracted by the magnets or until the liquid appears clear.
Transfer 90 µL of the supernatant in a new(s) low binding 1.5 mL microtube(s). **Be careful to avoid pipeting beads during this step**.

Store at 4 °C (or store -20 °C for a long-term storage) until size selection.

Intermediate pools quantification and final pooling (Optional) 30m

44 If you made intermediate pools (low, medium and high intensity on agarose gel), estimate the double strand DNA concentration in the pools by fluorimetry with a Qubit equipment.

Qubit_dsDNA_HS_Assay_UG.pdf 229KB

Pool in equimolar concentration the 3 intermediate pools in a single low binding 1.5 mL microtube with a minimum concentration of [M] 20 nanomolar (nM) and minimum volume of 30 µL .

Vortex mix and spin down.

Store at 4 °C (or -20 °C for a long-term storage) until size selection.

Size selection with sage science Pippin-Prep 2h

45 Perform the size selection of fragments between 300 and 800 bp using a 1,5% DF marker K agarose gel cassette, according to the Pippin prep manufacturer's instructions :

Pippin-prep-Quick-Guide-CDF1510... 636KB

Note

In the case you do not have access to a Pippin prep, you can alternatively perform a double size-selection with beads (see the bead manufacturer's instructions for details), or by smear excision and purification from an agarose gel. From our experience, size selection with the Pippin prep is the most accurate and repeatable method.

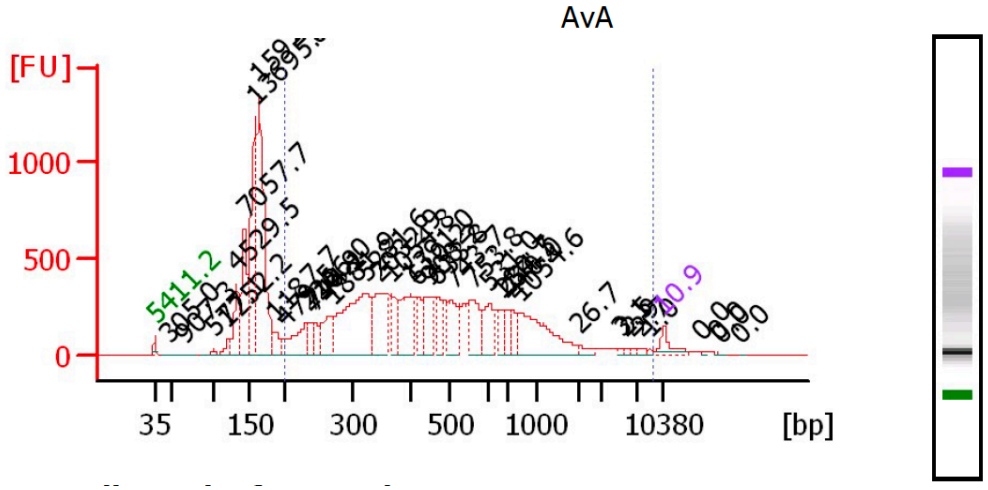
Quality control of the libraries

3h 30m

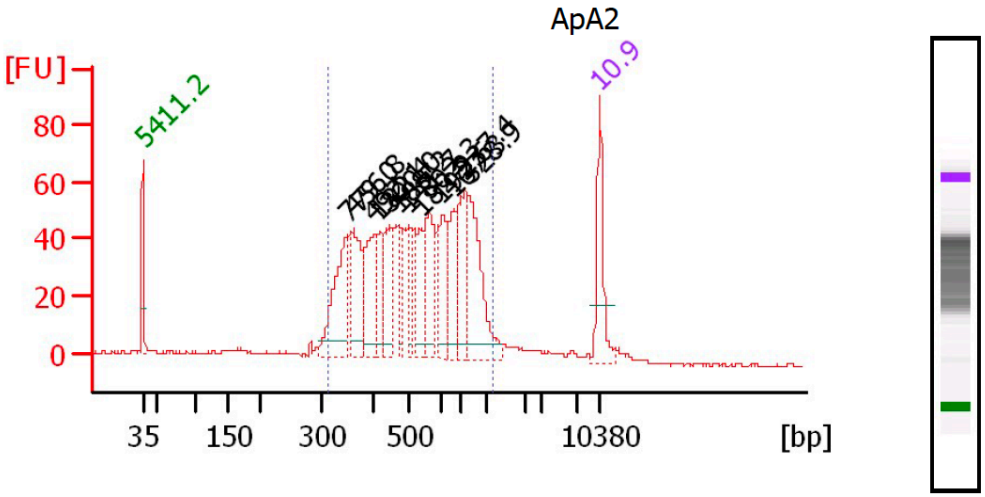
46 **Control the quality of the library** with a Bioanalyzer (Agilent) (or equivalent equipment) in a High Sensitivity DNA chip. Dilute your pool 1:2 or more and load 1µl of the pool before and after size selection, according to the manufacturer's instructions :

1h

Agilent_high_SensitivityDNA_KG_E... 6MB



Example of a Bioanalyzer profile obtained for a ddRADseq library **before size selection** (DNA HS kit)



Example of a Bioanalyzer profile obtained for a ddRADseq library **after size selection** (DNA HS kit)

47 **Fluorimetric estimation of the dsDNA concentration in the library.** 30m

Perform a quick estimation of the DNA concentration with a fluorimetric assay, in a QubitTM apparatus or equivalent, with the Qubit ds 1X DNA HS assay kit, according to the manufacturer's instructions:

Qubit_dsDNA_HS_Assay_UG.pdf 229KB

48 **qPCR quantification** 2h

In the case you need an accurate estimation of the DNA concentration in your library, perform a qPCR quantification with the NEBNext Library Quant Kit for Illumina, or equivalent, which uses P5 and P7 illumina primers to target the double stranded DNA fragments in the library. Follows the kit's user guide and perform your quantitative qPCR in a qPCR thermocycler (e.g. LightCycler 480, Roche).

NEBNext_manualE7630.pdf 2.6MB

Contrarily to the fluorimetric method (Qubit), the qPCR estimation will only consider dsDNA fragments starting with P5 and ending with P7 illumina sequences, that will be effectively amplified onto the flowcell of the Illumina sequencer.

48.1 **Suggestions to prepare library dilutions for qPCR**

In 0.5 mL low binding microtube, prepare 1:1 000 dilution of library with buffer supplied in the qPCR kit. Then, prepare the 3 library dilutions (1:10 000 to 1:30 000) to be used on triplicate for qPCR analysis.

1:100 : 1 µL of library + 99 µL of 1X buffer

1:1 000 : 10 µL of library + 90 µL of 1X buffer

1:10 000 : 20 µL of 1:1 000 dilution + 180 µL of 1X buffer

1:20 000 : 50 µL of 1:10 000 dilution + 50 µL of 1X buffer

1:30 000 : 50 µL of 1:10 000 dilution + 100 µL of 1X buffer

You should get more than 10 nM, that is the library concentration usually required by the sequencing platform facilities.

Use the average size of the library size range as estimated from the Bioanalyzer profile to convert DNA concentration from nM to ng/µL using the attach file below :

nM_ngµL_Conversion_Calculator.xlsx 14KB

49 **Library ready for sequencing**

The library is now ready for sequencing in single read or paired-end 150 bases in an Illumina sequencer, with one index read.