Version 1

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Construction of individual ddRAD libraries V.1

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DYDIV

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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).
The present ddRAD protocol as been slightly adapted from Alan Brelsford's protocol published in the supplementary material of this paper:

Brelsford, A., Dufresnes, C. & Perrin, N. 2016. High-density sex-specific linkage maps of a European tree frog (*Hyla arborea*) identify the sex chromosome without information on offspring sex. *Heredity* **116,** 177–181 (2016). https://doi.org/10.1038/hdy.2015.83

In the present protocol, all samples are treated separately, in a microplate, until final PCR amplification 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 sequencing reads per sample in the final dataset.

Briefly, genomic DNA from the samples are individually digested with 2 restriction enzymes (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 12 available) for multiplexing a maximum of 288 sample 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.

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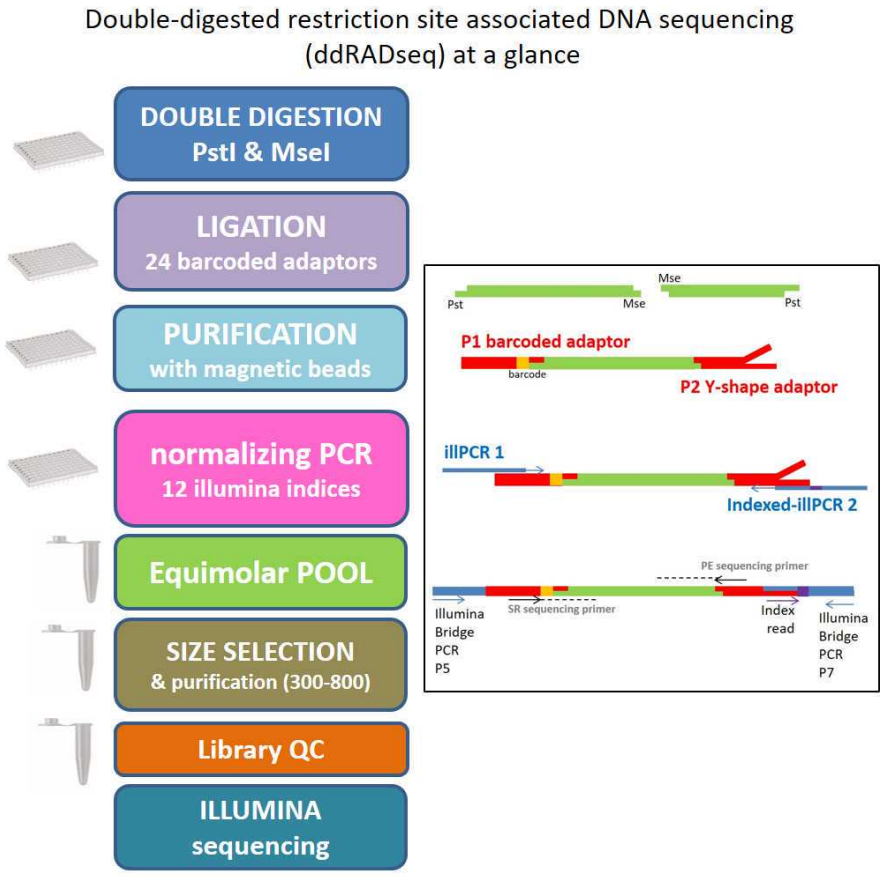
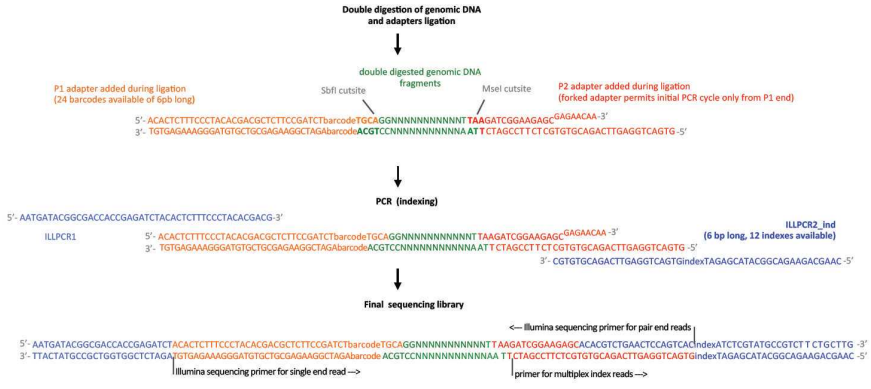


individual ddRAD at a glance



Sequences of adaptors used in the present protocol (Alicia Mastretta-Yanes, pers. comm.).

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Jun 25, 2021	Stephanie Ruault	Station Biologique de Roscoff, France
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51059

GUIDELINES

This protocol has been used sucessfully for several marine macro-algae (Guzinski et al 2018, Le Cam et al 2019) and marine vertebrate (ivory gull, Charbonnel et al, *in revision Heredity*), invertebrate species (isopods, tunicates (Le Moan et al, *in revision, Mol Ecol)*, mollusks (Tran Lu Y et al, *in revision, Mol Ecol*). Efficiency may rely on specific genome composition (GC rate, genome size, genome complexity). The main advantage of this protocol is to ensure homogeneous representation of the different samples in the final library pool.

The choice of restriction enzymes depends on the genome size and content. In the case of very large genomes, SbfI may replace PstI, using the same P1 adaptors set. For genomes with low GC content, use MspI instead of MseI.
The number of individual per library may be adjusted according to the expected yield of the sequencing run and the desired depth of sequencing per individual.
Starting DNA amount may also be adjusted to the number of individual multiplexed in a pool. This protocol has been successfully used with as little as 50 ng of genomic DNA for each individual. Strict normalization of all DNA extracts' concentrations is not required because of the normalizing PCR included at the end of the protocol.

Several individuals should be included in triplicates, from the digestion (or better, DNA extraction) step, allowing better adjustement of reads assembly parameters (for more explanations see Mastretta-Yanes et al 2015 and Paris et al 2017).

MATERIALS TEXT

Reagents :

- 100 % ethanol (molecular biology grade)
- Agarose
- TBE 1X buffer

- Ethidium Bromide Solution [10mg/ml]
- 100bp ladder
- Gel loading dye
- DNAse-Rnase free ultrapure water
- PstI-HF® ref NEB : R3140S
- MseI ref Neb : R0525S
- T4 DNA Ligase ref NEB : M0202L
- ATP 10mM ref NEB : P0756
- Annealing buffer stock (10X): 100 mM Tris HCl, pH 8, 500 mM NaCl, 10 mM EDTA
- dNTP set 4x0,25mlx100mM ref Neb : N0446S
- Q5® Hot Start High-Fidelity DNA Polymerase ref NEB : M0493L
- Ampure XP beads Beckman coulter (A63881) or NGS clean up and size selection (Macherey Nagel REF 744970.50)
- Elution solution/buffer for magnetic beads (Tris 10mM)
- Freshly diluted 70% ethanol
- Pippin Prep reagents and cassettes (1,5 % DF Marker K, ref SageScience CDF1510)
- NEBNext Library Quant kit for illumina ref Neb : E7630
- Qubit 1X dsDNA BR (Thermo, Q33266)

Primer and adapter sequences :

NB : All primers and adaptors are NGS grade oligonucleotides, HPLC purified, delivered at 100µM.

- **Adaptors**

Adapter P1: annealed oligos 1.1 and 1.2 (1 pair per barcode, 24 6-base barcodes) :
PstI adapter p1.1
ACACTCTTTCCCTACACGACGCTCTTCCGATCTnnnnnnTGCA
PstI adapter p1.2
nnnnnnAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

P1-PstI_sequences.pdf

Adapter P2: annealed oligos 2.1 and 2.2 :

MseI adaptor 2.1: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
MseI adaptor 2.2 : /5Phos/**TA**AGATCGGAAGAGCGAGAACAA

or for AT rich genomes:

MspI adaptor 2.1 : GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
MspI adaptor 2.2: /5Phos/**CG**AGATCGGAAGAGCGAGAACAA

- **PCR primer oligos:**

"ILLPCR1" : forward primer common to all reactions; "ILLPCR2" reverse primers contain unique 6-bases indices that allow for multiplexing in a single pool in an luumina lane. Each individual is tagged with one inline barcode, and one Illumina index. Therefore, with 24 barcodes and 12 indices, 288 individuals can be multiplexed in a single pool. One index read is necessary during the illlumina sequencing run (either single read or paired-end).

ILLPCR1 primer:
aaTGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG

Truseq Indexed illPCR2 Primers: each unique pcr2 primer lets you reuse the 24 barcodes in the same lane. If using two indexed primers, Illumina recommends to use 6 and 12. If three primers, use 4, 6, 12. If four primers, use 1, 4, 7, 9 or 6,8,11,12. If six primers, 2,4,5,6,7,12.
ILLPCR2_ind01
caAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind02
caAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind03
caAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind04
caAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind05
caAGCAGAAGACGGCATACGAGAT**CACTGT**GTGACTGGAGTTCAGACGTGTgc

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ILLPCR2_ind06
caAGCAGAAGACGGCATACGAGAT**ATTGGC**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind07
caAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind08
caAGCAGAAGACGGCATACGAGAT**TCAAGT**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind09
caAGCAGAAGACGGCATACGAGAT**CTGATC**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind10
caAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind11
caAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGACTGGAGTTCAGACGTGTgc
ILLPCR2_ind12
caAGCAGAAGACGGCATACGAGAT**TACAAG**GTGACTGGAGTTCAGACGTGTgc

small letters indicate PTO modifications during synthesis of primers.

Consumables :
PCR plates
8-tubes and 12-tubes *PCR* strips; Volume: 0.2mL
thermosealable films
2 mL low binding microcentrifuge tubes
Filter tips (better in low retention quality)
qPCR white plate 96
Agilent DNA High sensitivity DNA kit (5067-4626)
Qubit microtubes

Equipment :
Manual pipettors, monochannel and multichannel
Centrifuge for microcentrifuge tubes and plates
Vortex mixer
Personal protection equipment (lab coat, gloves, goggles)
Plate sealer
Microwave
Thermocycler
Gel Electrophoresis Systems
magnetic stand 96 (ref Thermo AM10027)
Pippin prep (Sage Science)
Qubit fluorimeter
qPCR equipment (e.g. LightCycler 480 Roche)
Bioanalyzer (Agilent)

SAFETY WARNINGS

Fully respect the strict separation between pre- and post-PCR labs. Use low retention filter tips for all pipeting steps.
Be very careful to avoid cross contamination between samples. Include non-template controls in each steps.

This protocol uses Ethidium Bromide (EtBr), which is a known mutagen. Wear a lab coat, eye protection and gloves when working with this chemical.

Preparation of double-stranded barcoded P1 adaptors 1µM 5m

1 5m

In a PCR plate wells, combine each oligo 1.1 with its complementary oligo 1.2 :

- **5 µl** adaptor P1-1 10µM
- **5 µl** adaptor P1-2 10µM
- **5 µl** annealing buffer 10X (100 mM Tris-HCl, pH 8, 500 mM NaCl, 10 mM EDTA Na_2)
- **35 µl** nuclease free water

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Seal the plate with a thermoseal film and incubate in a thermocycler or microplate incubator at **95 °C** for **00:05:00** , and then cool at a rate of 0.1°C / s down to 20°C.
Store at 4°C (or at -20°C for a long-term storage)

2

		25			9				17		
		2			10				18		
		3			11				19		
		4			26				20		
		5			13				21		
		6			14				22		
		7			15				23		
		8			16				24		

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

Preparation of double-stranded P2 Adaptor P2_Mse 10µM 5m

5m

3 in a microtube, combine oligo 2.1 with its complementary oligo 2.2

80 µl P2-1 100µM

80 µl P2-2 100µM

80 µl annealing buffer 10X (100 mM Tris-HCl, pH 8, 500 mM NaCl, 10 mM EDTA Na_2)

560 µl nuclease free water and mix by pipetting

Then aliquot this volume into **100 µl** in each well of a 8- *PCR* tube strip.

In a thermocyler or microplate incubator, incubate at **95 °C** for **00:05:00** , and then cool at a rate of 0.1°C / s down to 20°C.
Store at 4°C (or -20°C for a long-term storage)

Genomic DNA preparation

4 In a PCR plate, put around 50-100ng of genomic DNA in a volume of 30µl (in nuclease free water or Tris-HCl 5mM pH 8.5) for each sample. See guidelines for more details. Ideally, DNA should be free from RNA. For this, include a RNAse treatment in the DNA extraction procedure. If possible, randomize the location of samples in the microplate. Keep a few empty wells for negative controls.

Double digestion 10s

10h 20m 10s

5 Thaw 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 a microtube, prepare the master mix, according to the following table for a total volume of 35µL:

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A	B	C	D	E
	concentration	final quantity	N=1	N=104 (1 plate)
genomic DNA		50-200ng	30	
Cutsmart buffer	10X	1X	3.5	364
Enzyme 1 Pst HF	20u/µL	8U	0.4	41.6
Enzyme 2 MseI	10u/µl	2U	0.2	20.8
H2O			0.9	93.6
TOTAL			35	

Digestion mix composition

Vortex mix the master mix and spin down.

Aliquot **65 µl** of the master mix in each well of a 8-PCR tube strip.

In the DNA plate (containing 30µL per well), add **5 µl** of Master mix with a multichannel pipette and mix by pipetting, seal PCR plate and spin down.

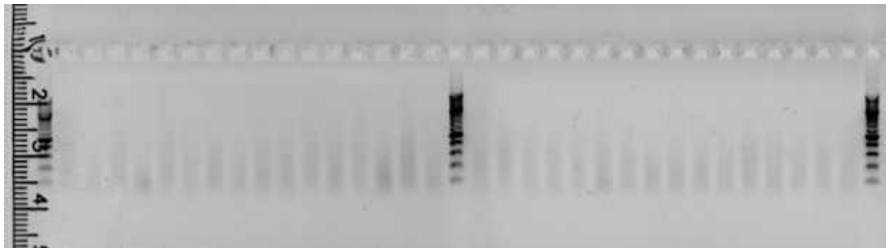
Incubate at **37 °C** **10:00:00**

then **65 °C** **00:20:00** in order to inactivate enzymes

4 °C **Overnight**

Check digestion on an agarose gel

6 Check the efficiency of the digestion by electrophoresis of 5µl 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 100pb ladder.

Adaptor ligation 10s

10s

7 Vortex mix 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.

Setup the ligation reaction according to the following table (total volume of 40µL):

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A	B
	N=1 / µl
digest DNA	30
ds barcoded adaptor P1 1µM	2.8
ligation mix (see below)	7.2

In a microtube, prepare the ligation mix according to the following table :

A	B	C	D	E
	concentration stock	final concentration	vol 1x µl	Vol µl plate
ds nonbarcoded adaptor P2	10µM		1.8	187.2
Cutsmart buffer	10X	1X	1	104
ATP	10mM	1 mM	4	416
T4 DNA ligase	400u/µl		0.4	41.6
TOTAL			7.2	

8 6h

Vortex the master mix and spin down.

Aliquot **93.5 µl** of the master mix in each well of a 8-PCR tube strip.

In the DNA digestion plate (30µL per well), add **2.8 µl** of Adapt P1 according to your plate map, from the adaptor plate (see step 1), with a multichannel pipette.

Then in all wells, add **7.2 µl** of ligation mix and mix by pipetting, seal PCR plate and spin down.

Incubate at **16 °C** **06:00:00** .

then

4 °C **Overnight**

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

Bead purification

9 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 ca. 200 bp. This can be achieved by using a volume ratio (bead suspension to sample) of 1.5, which is described in the following protocol.

From AMPure XP 's user guide (Beckman)

10 **Before starting**

- Prepare **50 mL** fresh 80% Molecular Biology grade Ethanol for MN beads (or 70% for AMPure XP beads).

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- Remove the NucleoMag® NGS Bead Suspension (or Beckman Ampure XP) 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 homogeneization and fill all wells of a 8-tube PCR strip with this solution, or use a reagent reservoir.
- Add **60 µl** of well-homogeneized bead suspension into the first 4 columns of a new PCR microplate with a multichannel pipette.

- Spin down the plate containing the digested-ligated products. Fill each well of a 8-PCR tube strip with Tris-Hcl pH8 10mM.
- In the DNA digestion plate, add **5 µl** of Tris 10mM with a multichannel pipette, to be sure that you will be able to take 40µl of digested-ligated DNA.

11 Binding

This step binds DNA fragments 200bp and larger to the magnetic beads.

- For the first 4 columns of the digestion-ligation plate, Transfer **40 µl** of the DNA samples in the first 4 columns of the purification plate containing beads and carefully mix by pipetting up and down 10 times.
- Incubate the purification plate at room temperature for 5 min.

12 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 (ca 100 µl) by pipetting.
- Do not disturb the attracted beads while aspirating the supernatant. Remove the supernatant with the multichannel from the opposite side of the well.

13 1st wash with 80 % ethanol

- Place 80% (or 70%) ethanol in a 25ml reservoir.
- With a multichannel pipette, dispense **200 µl** 80% (or 70%) ethanol into the purification plate without disturbing the bead pellet.
- Incubate the purification plate at room temperature for at least 30 s.
- Carefully and completely remove and discard ethanol by pipetting.

14 2nd wash with 80 % ethanol

- With a multichannel pipette, dispense **200 µl** 80% (or 70%) ethanol into the purification plate without disturbing the bead pellet.
- Incubate the purification plate at room temperature for at least 30 s.
- Carefully remove and discard ethanol by pipetting.

15 Dry the beads

Let the purification plate on the magnetic separator and incubate at room temperature **for maximum 5 min** 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.

16 Elute DNA fragments library

10m

- Take the purificaation plate from the magnetic stand, and add **40 µl** of Tris 10mM pH 8 with a 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.

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- 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 purified DNA fragments to a new 96-well plate. Be careful to avoid pipeting beads during this step.
- Store the plate at 4°C until and proceed to purification of columns 5-8 and 9-12 similarly (steps 11-15).
- Seal the plate and store at 4°C (or store -20°C for a long-term storage) until PCR amplification.

PCR amplification to generate Illumina sequencing indexed libraries

17 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 (see ligation step for details). 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.

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

A	B	C	D
	conc initiale	conc final	vol 1X µl
Q5 buffer NEB	5X	1x	8
dNTP mix	25mM each	0.2 mM	0.3
primer mix (illPCR1 and illPCR2index)	5µM each	0.17 µM	1.4
Q5 hotstart hifi DNA polymerase NEB	2u/µl		0.4
High GC enhancer NEB	5X	1X	8
nuclease-free water			11.9
Adaptor-ligated purified template DNA			10
total mix			30
TOTAL reaction			40

PCR mixture composition

10s

19 **1st mix preparation**
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 a 5ml microtube, prepare the 1st mix according to the following table :

A	B	C
	Vol µl 1X µl	Vol µl for 1 plate (104 reactions)
Q5 buffer 5X	8	832
dNTP mix 25mM	0.3	31.2
Q5 hot start DNA polymerase 2u/µl	0.4	41.6
High GC enhancer 5X	8	832
nuclease free water	3.3	343.2
total	20	2080

First mix composition

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Vortex mix all reagents in the mix and spin down.

Aliquot **130 µl** of the 1st mix into each well of a 8-tube *PCR* strip.

In a new PCR plate , dispense **20 µl** of 1st mix with a multichannel in each column.

20 2nd mix preparation (primer PCR mix) :

prepare 1 mix for each index in a 1.5 ml microtube, (4 mix for a full a plate).
for 1 index

A	B	C
	vol 1x µl	Vol µl for 3 columns
illPCR Primer mix 5µM	1.4	39.2
nuclease-free water	8.6	240.8
total	10	280

Second mix composition (primer PCR mix)

Vortex mix all reagents and spin down.

In a 12-tubes *PCR* strip, aliquot **93 µl** of the primer PCR mix into 3 consecutive wells according to the following scheme :

ind 1 Ind 4 ind 7 ind 9

In the PCR plate , dispense **10 µl** of 2nd mix in each line of the plate with a 12 multichannel pipette, onto the 20µl of the first mix.

21 DNA and mix combination

Spin down the ligation purified DNA plate.

With a multichannel pipette, transfer **10 µl** of purified ligated DNA into the PCR plate and mix by pipetting.

Finally, aliquot the **40 µl** by dispensing **10 µl** into 3 additional new empty PCR plates.

Seal the 4 PCR plates and spin down.

The 4 PCR will be performed in parallel In 4 different thermal cyclers, in order to reduce the PCR bias.

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A	B	C	D
hot start initial denaturation	98°C	30s	
amplification cycles			
denaturation	98°C	20s	
annealing	60°C	60s	15 cycles
extension	72°C	40s	
final extension	72°C	10min	
hold	12°C		

PCR program for the Illumina indexing PCR

Seal the 4 PCR plates and spin down.

The 4 PCR will be performed in parallel In 4 different thermal cyclers, in order to reduce the PCR bias.

After PCR, pool back the 4 PCR plates into a single plate with a multichanel.

22 Final cycle (with pirmers in large excess)

Prepare 1 mix for each index in a 1.5 ml microtube

A	B	C	D	E
	conc stock	final concentration	vol µl 1X	vol µl for 3 columns
Q5 buffer	5X	1X	0.2	24
dNTP mix	25mM each	0.2mM	0.08	9.6
illPCR primer mix	5µM each	0.2µM	0.4	48
nuclease-free water			0.32	38.4
total			4	120

Vortex mix all reagents and spin down. In a 12-tube *PCR* strip, aliquot **40 µl** of the mix into 3 consecutive wells according to the following scheme:

ind 1 Ind 4 ind 7 ind 9

Dispense **4 µl** of final cycle mix in each line of the plate with a 12 multichannel pipette.

Seal the PCR plate and spin down.

In a thermocycler, run the final cycle as follows:

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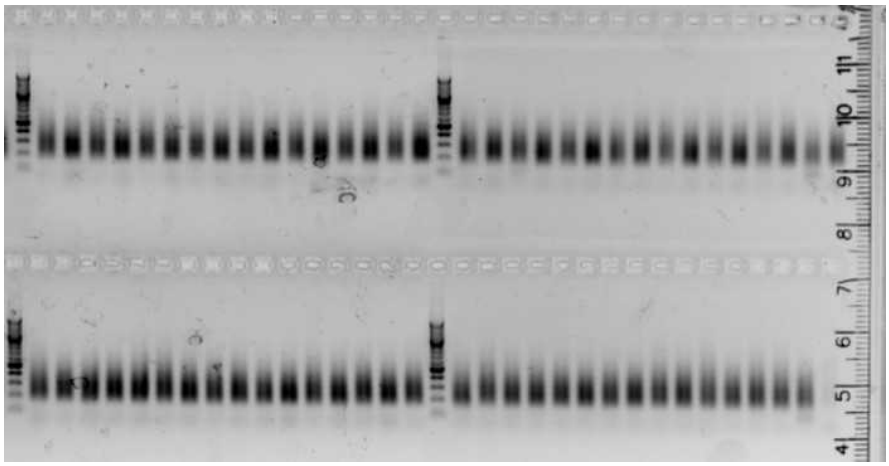
A	B	C
denaturing	98°C	3 min
annealing	60°C	2 min
extension	72°C	12 min
hold	12°C	

PCR program for the final cycle of the illumina PCR

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

Agarose gel electrophoresis

23 Check the efficiency of the PCR on an agarose 1.5% gel . Load 5µl of each PCR product.



Example of smears obtained after the Illumina PCR. Size marker is a 100bp ladder. In this case, 5µl of each sample were pooled in the final library. Ding so, homogeneous number of reads per sample were indeed obtained after demultiplexing the raw sequences dataset.

Samples pooling

24 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. Proceed to next step.

25 Estimate the double strand DNA concentration in the pool by fluorimetry with a Qubit equipment. If the concentration is too low (less than 20 nM), perform a bead clean-up (ratio 1:1, see above steps 9-16) on a partial volume of the pool to increase the final concentration. Work in a microtube instead of a microplate, with a magnetic microtube stand. Adapt the elution volume to the desired final concentration. Elution should be done in at least 50µl, for subsequent steps.

Size selection with sage science Pippin-Prep

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

Quick-Guide-CDF1510-marker-K3.pdf

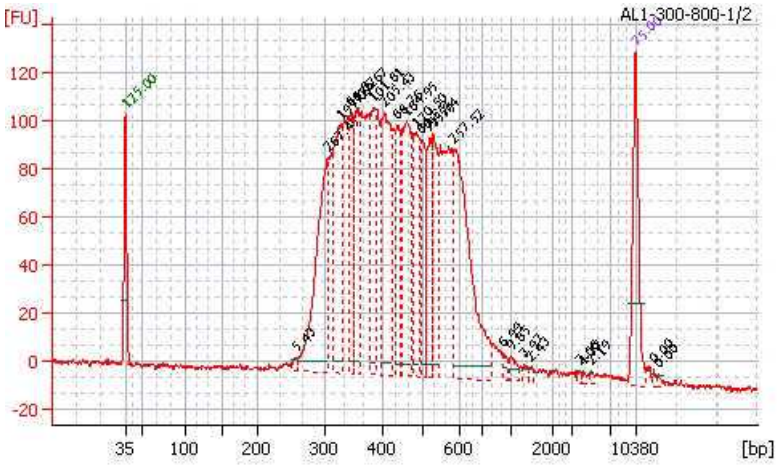
Citation: Claire Daguin Thiebaut, Stephanie Ruault, Charlotte Roby, Thomas Broquet, FrÃÂ©dÃÂ©rique Viard, Alan Brelsford (09/21/2021). Construction of individual ddRAD libraries. https://dx.doi.org/10.17504/protocols.io.bv4tn8wn

Warning :
If you perform the size selection on the pool without prior bead clean-up, you may observe a shift between observed and expected size ranges of the smear (approximately 250-750 obtained instead of 300-800).
If you see adaptor dimers on the gel after PCR, it is also preferable to perform a bead clean-up before size selection in the Pippin Prep.
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, and has to be prefered if you have serveral pools of libraries (for example 3 pools of 192 samples) in order to increase the number of common loci between samples in the ddRAD sub sampling of the genome.

Quality control of the libraries

27 **Control the quality of the library** with a Bioanalyzer (Agilent) 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 :

HighSensitivity_DNA_KG.pdf.pdf



Example of a Bioanalyzer profile obtained for a ddRADseq library (DNA HS kit)

28 **Fluorimetric estimation of the dsDNA concentration** in the library.

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

Qubit_dsDNA_BR_Assay_UG.pdf

29 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).

manualE7630.pdf

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.

Citation: Claire Daguin Thiebaut, Stephanie Ruault, Charlotte Roby, Thomas Broquet, FrÃÂ©dÃÂ©rique Viard, Alan Brelsford (09/21/2021). Construction of individual ddRAD libraries. https://dx.doi.org/10.17504/protocols.io.bv4tn8wn

29.1 *Suggestions to prepare library dilutions :*

in a 8-tube PCR strip:
Prepare intermediate dilutions (1:10 and 1:100) of the library with the dilution buffer supplied in the qPCR kit.

Then prepare the 4 library dilutions to be used in triplicate for qPCR analysis :
1:1000 : 10µl of 1/100 + 90µl buffer 1X
1:2000 : 50µl of 1/1000 + 50µl buffer 1X
1:4000 : 50µl of 1/2000 + 50µl buffer 1X
1:8000 : 50µl of 1/4000 + 50µl buffer 1X

You should get more than 10 nM, that is the library concentration usually required by the sequencing platform facilities.
The library is now ready for sequencing in single read or paired-end 150 bases in an Illumina sequencer, with one index read. Use the average size of the library size range as estimated from the Bioanalyzer profile to convert DNA concentration from nM to ng/µl.

Citation: Claire Daguin Thiebaut, Stephanie Ruault, Charlotte Roby, Thomas Broquet, FrÃÂ©dÃÂ©rique Viard, Alan Brelsford (09/21/2021). Construction of individual ddRAD libraries. https://dx.doi.org/10.17504/protocols.io.bv4tn8wn