



Feb 16, 2024

A FAIR protocol of the Best-RAD sequencing approach

DOI

<https://dx.doi.org/10.17504/protocols.io.rm7vz3ok4gx1/v1>



Laure Benoit¹, Sabine Nidelet², Emeline Charbonnel¹, Marie-Pierre Chapuis¹

¹Centre de coopération Internationale en Recherche Agronomique pour le Développement;

²Institut National de la Recherche pour l'Agriculture, l'alimentation et l'Environnement



Laure Benoit

CIRAD

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.rm7vz3ok4gx1/v1>

Protocol Citation: Laure Benoit, Sabine Nidelet, Emeline Charbonnel, Marie-Pierre Chapuis 2024. A FAIR protocol of the Best-RAD sequencing approach. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.rm7vz3ok4gx1/v1>

License: This is an open access protocol distributed under the terms of the **[Creative Commons Attribution License](#)**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited



Protocol status: Working

We use this protocol and it's working

Created: March 27, 2021

Last Modified: March 21, 2024

Protocol Integer ID: 48598

Keywords: RAD sequencing, BestRAD, Sequence-Based Genotyping, PCR duplicate, multiplexing, adapters, biotinylated tag, SNP, high amount of target dna fragment, lower rate of pcr duplicate, low amounts of pcr duplicate, target dna fragment, dna concentration, pcr duplicate, sequencing, protocol on individual wild sample, sequencing approach, quantity of the dna input, oriental fruit fly, dna input, individual wild sample, dna, number of pcr cycle, pcr cycle, pcr, chimeric fragment, main improvements of the rad

Funders Acknowledgements:

Labex CEMEB

Grant ID: PROLAG project

ANR

Grant ID: DISLAND project



Abstract

The protocol is based on the Best-RAD sequencing approach developed in Ali et al. (2016) that allows two main improvements of the RAD-sequencing approach developed by Baird et al. (2008) : (1) a lower rate of PCR duplicates generated during the final enrichment and (2) an increase of the multiplexing capacity by a factor equal to the number of well barcode available (for a brief description, see Figure 1). We developed this protocol on individual wild samples of the Oriental fruit fly, *Bactrocera dorsalis*. We evaluated the library quality not only on a high amount of target DNA fragments but also on low amounts of PCR duplicates, chimeric fragments, and adaptor residues. To this aim, we tested and validated the following critical parameters : the quality and quantity of the DNA input, the ratio of the AMPure purifications and the number of PCR cycles of the final amplification. Consecutive recommendations and expectations (e.g., DNA concentration or fragment size) are indicated in notes throughout the protocol. Furthermore, special care has been taken to ensure the precision of each step (i.e. volumes, quantities, duration, materials with supplier references). Consequently, this protocol follows the FAIR principle and could be useful for an easy implementation of the Best-RAD sequencing approach in other laboratories and biological models.

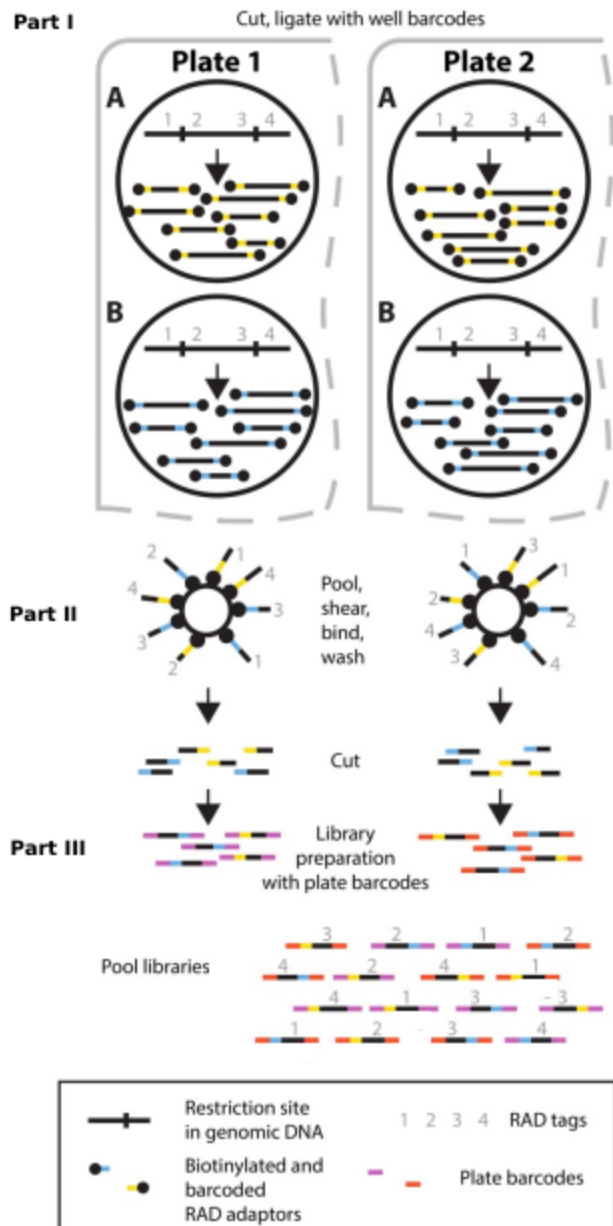


Figure 1. Schematic overview of the BestRAD method (slightly modified from Ali et al. 2016). **Part I:** Two wells are depicted in each of two different plates. Genomic DNA is digested with a restriction enzyme and ligated to biotinylated well barcode adapters (yellow and blue bars). DNA from each well is pooled platewise, mechanically sheared. **Part II:** Pools are incubated with streptavidin beads. Following washing, DNA is cleaved from the beads leaving the well barcodes. **Part III:** Library preparation is performed where a unique combination of plate barcode is added (red and purple bars). Multiple plate libraries can be pooled.



Guidelines

Enzymes

Both PstI and SbfI enzymes are compatible with this protocol.

DNA quality

Wherever possible, select input DNA samples of a molecular weight of at least 5kb. A more degraded DNA sample could be lost and should be avoided. Note that we worked on DNA extracted using a 96-Well Plate Animal Genomic DNA Miniprep Kit (Biobasic, Toronto, Canada, Cat.#: BS437) according to the manufacturer's protocol, except that lysis was made over-night and followed by an RNA degradation step using 4µL of RNase A (Qiagen, 100 mg/ml, Cat.#: 19101) during 2 min at room temperature.

Sample multiplexing

The protocol is designed to process samples in batches of 96 (i.e. to work in a 96-well plate format), but it is possible to work with fewer samples (e.g. batches of 24 samples).

Each sample must be associated to a unique ID and three multiplexing barcodes. These barcodes are :

- (1) a well biotinylated barcode as defined by Ali et al. (2016) that is unique to a sample within a batch (the list of barcode sequences is available in the Supplementary data FileS1; "New RAD" table).
- (2) the two P1 and P2 plate barcodes added to the Illumina adapters that are, in combination, unique to a batch (the list of sequences we used is available at the materials section). Any index compatible with illumina technology could be used, such as NEBNext Multiplex Oligos for Illumina (Cat.#: E7395S).

Control samples

Including one negative control (i.e. empty well) randomly located in each plate allows to easily identify possible technical errors (e.g. 96-well plate switch).

Including technical replicates (i.e. include twice the same DNA sample with different barcodes) allows to estimate genotyping error rate.

Materials

Equipments

Equipment	
Bioruptor Pico sonication device	NAME
Sonicator	TYPE
Diagenode	BRAND
B01060010	SKU

Equipment	
ThermoMixer	NAME
Shaking and temperature incubator	TYPE
Eppendorf	BRAND
ThermoMixer C F2.0	SKU
220 – 240 V/50 – 60 Hz (EU)	SPECIFICATIONS

Equipment	
Mini-Centrifuge	NAME
Centrifuge	TYPE
Fisherbrand	BRAND
16617645	SKU
100-240V, 50/60Hz	SPECIFICATIONS

Equipment	
Universal 320	NAME
Plate centrifuge	TYPE
Hettich	BRAND
1401/HET	SKU
https://www.hettichlab.fr/fr/produit/universal-320-320-r/	LINK
RPM MAX.: 16.000 min-1 POIDS: env. 31 kg 52 kg REFROIDISSEMENT: ventilé	SPECIFICATIONS

Equipment

Swing-out Rotor, 2- Places	NAME
Rotor	TYPE
Hettich	BRAND
1460/HET	SKU
https://www.hettichlab.fr/fr/produit/universal-320-320-r/	LINK
max capacity 10 plates	SPECIFICATIONS

Equipment

Vortex-Genie 2	NAME
Vortex	TYPE
Scientific Industries	BRAND
15547335	SKU

Equipment

Mastercycler

NAME

Thermocycler

TYPE

Eppendorf

BRAND

6332000010

SKU

Equipment

Bioanalyzer

NAME

Nanofluidic electrophoresis system

TYPE

Agilent

BRAND

G2991AA

SKU

Equipment

Qubit Fluorometer

NAME

Fluorometer

TYPE

Invitrogen

BRAND

Q33238

SKU

<https://www.thermofisher.com/order/catalog/product/Q33238#/Q33238>^{LINK}

Equipment	
DynaMag-2	NAME
Magnet	TYPE
Invitrogen	BRAND
12321D	SKU
https://www.thermofisher.com/order/catalog/product/12321D#/12321D ^{LINK}	

Equipment	
Proline Pipette Pack	NAME
Pipette Pack	TYPE
Sartorius	BRAND
15673094	SKU
https://www.fishersci.fr/shop/products/proline-pus-mechanical-pipet-multipacks-8/15673094	LINK
Includes: 4 x Single-channel pipets (0.5 to 10µL, 10 to 100µL, 20 to 200µL, 100 to 1000µL), Tip Racks to 96 tips (0.1 to 10µL, 0.5 to 200µL, 10 to 1000µL), 1 x Linear Stand and 4 x Pipet Holders	

Equipment

Multipette® M4

NAME

Mechanical multi-dispenser pipette

TYPE

Eppendorf

BRAND

4982000012

SKU

<https://www.eppendorf.com/fr-fr/Boutique-en-ligne-et-Produits/Manipulation-des-liquides/Pipetage-manuel-distribution/Multipette-M4-p-4982000012>

LI
NK

1 canal 1 µL – 10 mL

SPECIFICATIONS

Supplies

- Filter tips (compatible with pipettes)
- Combitips advanced 0.1 mL (Eppendorf #0030089405)
- PCR plate (Thermo Scientific #AB0600)
- PCR Transparent adhesive film (4TITUDE #4TI-0500)
- Aluminium adhesive film (Axygen #PCR-AS-200)
- DNA LoBind Tubes (Eppendorf #0030108051)
- 0.65 ml Bioruptor Pico Microtubes (Diagenode #C30010011)

Reagents

☒ Nuclease-Free Water

☒ Buffer EB **Qiagen Catalog #19086**

☒ PstI (100,000 units/ml) - 50,000 units **New England Biolabs Catalog #R0140M**

☒ SbfI-HF - 2,500 units **New England Biolabs Catalog #R3642L**

☒ NEBuffer 4 - 5.0 ml **New England Biolabs Catalog #B7004S**

☒ NEBuffer 2 - 5.0 ml **New England Biolabs Catalog #B7002S**

⊗ ATP Solution (100 mM) **Thermo Fisher Scientific Catalog #R0441**

⊗ Quick Ligase **New England Biolabs Catalog #M0202**

⊗ Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

⊗ Ethanol 100%

⊗ Dynabeads M-280 Streptavidin **Thermo Fisher Scientific Catalog #11205D**

⊗ 1M Tris-HCl **Thermo Scientific Catalog #J22638**

⊗ 0.5M EDTA **Invitrogen - Thermo Fisher Catalog #AM9261**

⊗ 5M NaCl **Invitrogen - Thermo Fisher Catalog #AM9759**

Well barcode adapters

⊗ Duplex with /5Biosg/ and /5Phos/ modifications **Integrated DNA Technologies, Inc. (IDT) Catalog #100 nmole Duplex**

Well barcodes are composed of a duplex of two oligos:

Top oligo (well barcode in bold):

/5Biosg/GTACGTCCTGCAGG**XXXXXXXX**TGCA

Bottom oligo (well barcode in bold):

/5Phos/**XXXXXXXX**CCTGCAGGACGTAC

The list of **well barcode** sequences is available in the Supplementary data FileS1, "New RAD" table (<https://doi.org/10.1534/genetics.115.183665>)

Plate barcode adapters

P1 adapters **plate barcodes** are composed of a duplex of two oligos:

Top oligo (Illumina Universal Adapter + plate barcode in bold):

AATGATACGGCGACCAACGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT**XXXXX***T

Bottom oligo (plate barcode in bold + Illumina Universal Adapter):

/5Phos/**XXXXX**AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCAT*T

P2 adapters **plate barcodes** are composed of a duplex of two oligos:

Top oligo (plate barcode in bold + Illumina Universal Adapter):

/5Phos/**XXXXX**AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCAGAACAA

Bottom oligo (Illumina Universal Adapter + plate barcode in bold):

CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCT**XXXXX***T

List of 16 **P1 plate barcode** sequences :



AAGTG
ACAAT
CTAGA
GATAG
GCCTC
TGTGT
GTATT
CGGAC
AAACAA
ACGTCA
CGTCTC
CCCAAA
GTTTCGG
TGCCCA
TTGGGC
TACGAG

List of 8 **P2 plate barcode** sequences :

ACCTA
TAGGTC
CGTAC
GTACGA
AGTCA
GACTCG
CCGAT
TTAGGC

Kits

⊗ Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

⊗ Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

⊗ NEBNext Ultra II DNA Library Prep Kit for Illumina **New England Biolabs Catalog #E7645L**

⊗ KAPA Library Quantification Kits **Roche Catalog #07960140001**



Before start

DNA quality control

Beforehand, DNA quality should be checked with an electrophoresis on an agarose gel.

Measurement of DNA quantity

Beforehand, the amount of genomic DNA should be determined with a fluorescent method (as Qubit assay) in order to be normalized.

Sample metadata

Prepare a metadata file containing for each sample, a unique ID and the combination of the three barcodes used. This allows to avoid switching barcodes during the sample pooling and is required for sequence read demultiplexing.



Part I - Genomic DNA restriction, well barcode ligation, pooling and shearing

7h 50m

1 Genomic DNA enzymatic restriction

3h

- 1.1 In a 96-well microplate, normalize the DNA concentration of each DNA sample in a final volume of 10 μ L using EB buffer.

1h

Note

Ideally, use a total DNA quantity (i.e. for the batch of samples to be pooled) around 2000ng (e.g. 96 samples x 2ng/ μ L x 10 μ L, or 24 samples x 8ng/ μ L x 10 μ L).

At minimum, use a total DNA quantity of 700ng. We validated this minimal input DNA quantity by sequencing a library constructed from an input of 672ng.

It is possible to use a larger input DNA quantity, which allows a more reliable normalization. In this case, it is necessary in step 3 to use a volume that corresponds to around 2000ng. This also allows to keep the remaining volume in step 3 as a back-up.

It is possible to apply the protocol in parallel on several plates.

- 1.2 Into a 1.5 ml low bind tube on a cold block, prepare the master mix of enzymatic restriction (vols for 1 rxn) :

30m

 0.55 μ L H₂O

 1.2 μ L NEB buffer 4 10X

 0.25 μ L SbfI or PstI

1. Gently vortex and centrifuge the mix.
2. Using a new plate on a cold block, a Multipipette and a 0.5mL Combitip, distribute  2 μ L of mix into each well.
3. Using P10 multichannel pipet, add  10 μ L of normalized DNA.
4. Close the plate with self-adhesive film.
5. Vortex and centrifuge.

1.3

1h 30m

Apply the following restriction program using a thermocycler (heated lid on):



🔥 37 °C 1h

🔥 80 °C 20min

🔥 10 °C hold

2 **Ligation of well barcodes**

1h 30m

2.1 Into a 1.5 ml low bind tube, prepare the ligation master mix (vols for 1 rxn) :

40m

🧪 1.19 µL H₂O

🧪 0.4 µL NEB buffer 2 10X

🧪 0.16 µL rATP 100mM

🧪 0.25 µL Quick ligase

1. Gently vortex the mix and centrifuge.

2. Using a new plate on a cold block, a Multipipette and a 0.5mL Combitip, distribute

🧪 2 µL of mix into each well.

3. Using P10 multichannel pipet, add 🧪 12 µL of digested DNA

4. Using P10 multichannel pipet, add 🧪 2 µL of well barcodes (0.05µM if SbfI digestion or 1µM if PstI) according to the plate plan.

5. Close the plate with self-adhesive film

6. Gently vortex and centrifuge

2.2 Apply the following ligation program using a thermocycler (heated lid on):

50m

🔥 22 °C 30min

🔥 65 °C 10min (decrease the temperature slowly to 65 at 25°C, e.g. 2.7°C/min during 15min using the minimum ramp speed)

🔥 25 °C hold

3 **Pooling, purification and concentration**

1h 20m



3.1 Sample pooling

30m

Note

Choose the volume to be pooled according to the amount of DNA input and the number of samples. Total DNA quantity in the pool should be at least 700ng, and ideally 2000ng.

1. Using a multichannel pipet, pool each barcoded sample of a same batch and a same row of a plate in a 0.2mL 8-tubes PCR strip.
2. Transfer all the samples of a batch, i.e. all the volume of the 8-tubes PCR strip, in a 1.5mL low-bind tube.

3.2 Purification and concentration on AMPure beads (ratio 1X)

50m

Incubate AMPure beads at room temperature at least 00:30:00 .

Prepare 70% ethanol :

For one pool: 954 μ L H₂O + 2000 μ L Absolute Ethanol .

1. Measure the exact volume of the pool
2. Add an equivalent volume of AMPure beads and vortex
3. Incubate 00:05:00 at room temperature
4. Place on a magnetic holder for 00:05:00 .
5. Without removing the tube from the magnetic holder, remove supernatant, add 1000 μ L 70% ethanol , incubate 00:00:30 and discard the supernatant.
6. Repeat step 5 one time
7. Let dry on the magnetic holder for 00:08:00 .
8. Re-suspend in 100 μ L EB buffer (or in 102 μ L to do the optional 4.2 step)
9. Remove from the magnetic holder and vortex gently
10. Incubate 00:02:00 at room temperature
11. Centrifuge briefly and place on a magnetic holder for 00:05:00 .
12. Transfer 100 μ L (or 102 μ L for the optional 4.2 step) to Diagenode tubes for shearing
Vortex and centrifuge.

4 Shearing

2h

Maintain tubes at 4°C if shearing is planned within 24 hours or freeze.



4.1

1h

1. Briefly vortex and centrifuge the tubes (maximum 12 tubes in parallel, i.e. 12 batches of 96 samples)
2. Check that there are no bubbles and place the tubes in the rotor (12 tubes of 0.5mL).
3. Place the rotor in the bath.
4. Run the following shearing program:

Four cycles of :

00:00:30 ON

00:01:30 OFF

Repeat the four previous steps one more time (for a total of 8 shearing cycles or a different total number of cycles, see note 4.2).

4.2 **Quality and quantity control (optional)**

1h

Run 1 μ L of each pool on an Agilent High Sensitivity (HS) chip (following manufacturer protocol).

Note

The desired fragment size profile depends on the sequencing read length. For a paired-end sequencing of 150 bp, the profile is expected to be centered around 350bp (200-500bp).

The size of the fragments obtained may depend on the biological model and the quality of the DNA input. It is cautious to test different numbers of fragmentation cycles in the bioruptor to adapt the shearing program to the experiment. The higher the number of cycles, the shorter the fragments.

Run 1 μ L of each pool on a Qubit High Sensitivity (HS) assay (following manufacturer protocol).

Note

At this step, the amount of DNA should be between 350ng and 1500ng. A DNA loss of about 25-50% is expected and mostly due to AMPure purification. The more degraded the input DNA, the larger the loss.

Part II - Selection of fragments carrying the restriction site

4h



5 Preparation of Dynabead M-280 magnetic streptavidin beads

40m

Since streptavidin beads adhere to tips, re-suspend by vortexing and not by pipetting.

Incubate the streptavidin beads at room temperature for at least 00:30:00 before using it.

1. For each pool, prepare 1 mL 2X Binding and Wash buffer (B&W 2X):

[M] 10 millimolar (mM) Tris-HCl (pH 8.0)

[M] 1 millimolar (mM) EDTA pH 8.0

[M] 2 Mass Percent NaCl

1. For each pool, transfer 30 μ L of Dynabeads into a new 1.5mL low bind tube.

2. Place the tube on a magnetic holder and discard the supernatant.

3. Without removing the tube from the magnetic holder, add 100 μ L B&W 2X

4. Remove the tube from the magnetic holder, vortex 00:00:30, centrifuge briefly.

5. Place the tube on the magnetic holder for 00:01:00 and discard the supernatant.

6. Repeat steps 3 to 5 twice for a total of 3 washes.

7. Re-suspend beads in 100 μ L B&W 2X.

6 Binding DNA to beads

1h 50m

- 6.1
1. Add shared DNA (~100 μ L) to the prepared beads.
 2. Briefly vortex and centrifuge the tubes.
 3. Apply the following binding program in a thermomixer:

22 °C 20min

900 rpm 15s every 2min

- 6.2 For each pool:

20m

1. Prepare B&W 1X: 250 μ L B&W 2X + 250 μ L H₂O . Incubate at

Room temperature

2. Prepare B&W 1X: 175 μ L B&W 2X + 175 μ L H₂O . Incubate at 56 °C in a thermomixer.



3. Prepare NEB buffer 4 1X: 30 μ L NEB buffer 4 10X + 270 μ L H₂O . Incubate at Room temperature .

6.3

1. Centrifuge briefly and transfer each pool in a 1.5mL low bind tube.
2. Place tubes on a magnetic holder for 00:01:00 and discard the supernatant.
3. Re-suspend the beads with 150 μ L 1X B&W at Room temperature .
4. Vortex gently and centrifuge briefly.
5. Place the tube on the magnetic holder for 00:01:00 and discard the supernatant.
6. Repeat steps 3 to 5 twice with B&W 1X buffer at Room temperature for a total of 3 washes.
7. Repeat steps 3 to 5 twice more with buffer B&W 1X at 56 °C for a total of 5 washes.

1h

7 Release DNA from streptavidin beads

20m

1. Re-suspend the beads in 100 μ L NEB buffer 4 1X .
2. Remove the tube from the magnetic holder, vortex gently and centrifuge briefly
3. Place the tube on the magnetic holder for 00:01:00 and discard the supernatant.
4. Repeat step 1 to 3 for a total of 2 washes.
5. Re-suspend the beads in 40 μ L NEB buffer 4 1X .
6. Remove the tube from the magnetic holder, vortex gently and centrifuge briefly.
7. Add 2 μ L SbfI-HF .
8. Vortex gently.
9. Run the following release program in a thermomixer:

37 °C 60min

600 rpm 30s every 6 min

1. Vortex gently and centrifuge briefly.
2. Place the tube on the magnetic rack for 00:01:00 .
3. Transfer 40 μ L supernatant in a 1.5mL low bind tube.

8 Purification on AMPure beads (1X)

50m

Incubate AMPure beads at room temperature at least 00:30:00 .

Prepare 70% ethanol :

For one pool: 133 μ L H₂O + 467 μ L Absolute Ethanol .



1. Add  40 μL of AMPure beads to each pool.
2. Incubate  00:05:00 at room temperature
3. Place on a magnetic holder for  00:05:00 .
4. Without removing the tube from the magnetic holder, remove supernatant, add  200 μL 70% ethanol , incubate  00:00:30 and discard the supernatant.
5. Repeat step 5 one time
6. Let dry on the magnetic support for  00:08:00 .
7. Re-suspend in  51 μL EB buffer (or 52 μL for the optional step 9)
8. Remove from the magnetic holder and vortex gently
9. Incubate  00:02:00 at room temperature
10. Centrifuge briefly and place on magnetic holder for  00:05:00 .
11. Transfer  50 μL (or 51 μL for the optional step 9) to a 0.2mL PCR tube.
12. Vortex and centrifuge.

9 Quantity control (optional)

20m



Run  1 μL of each pool on a Qubit HS assay (following manufacturer protocol).

Note

At this step, the amount of DNA should be between 8ng and 30ng. The large loss of DNA is expected and mainly due to step 6, in which the fragments of interest (which carry the restriction site) are bound to the streptavidin beads, while the rest of the genome is washed out.

Part III - Library construction with NEBNext Ultra II kit

8h 30m

10

1h 20m

Note

The following steps are described in the NEB Next Ultra II kit's manual, except for two modifications concerning the AMPure purification ratios (see steps 12 and 15) and several recommendations (see notes and optional quality controls).

NEBNext End Prep



Directly into the  50 μL from step 8, add :

 3 μL NEBNext Ultra II End Prep Enzyme Mix

 7 μL NEBNext Ultra II End Prep Reaction Buffer

1. Vortex gently and centrifuge briefly (It is important to mix well. The presence of a small amount of bubbles will not interfere with performance)
2. Place in a thermocycler, with the heated lid set to $\geq 75^{\circ}\text{C}$
3. Run the following program:

 20 $^{\circ}\text{C}$ 30min

 65 $^{\circ}\text{C}$ 30min

 4 $^{\circ}\text{C}$ hold

Warning: move to the next step immediately.

11 Plate barcode adapter Ligation

40m

Directly into the  60 μL from step 10, on a cold block, add in order:

 30 μL NEBNext Ultra II Ligation Master Mix

 1 μL NEBNext Ligation Enhancer

 1.25 μL P1 adapters  1.5 micromolar (μM)

 1.25 μL P2 adapters  1.5 micromolar (μM)

1. Vortex gently and centrifuge briefly (It is important to mix well. The presence of a small amount of bubbles will not interfere with performance)
2. Place in a thermocycler, with the heated lid off
3. Run the following program:

 20 $^{\circ}\text{C}$ 15min

 10 $^{\circ}\text{C}$ hold

Samples can be stored overnight at -20°C .

12 Cleanup of Adaptor-ligated DNA (ratio 0.65X)

55m 30s

**Note**

The ratio recommended in the supplier manual is 0.9X. We suggest a more drastic library sizing with 0.65X ratio, which we have found to be the best for removing free adapters.

Incubate AMPure beads at room temperature at least 00:30:00 .

Prepare 70% ethanol :

For one pool: 133 μL H₂O + 467 μL Absolute Ethanol .

1. Transfer 93.5 μL from step 11 in a 1.5mL low-bind tube.
2. Add 61 μL Ampure beads
3. Incubate 00:05:00 at room temperature
4. Place on magnetic holder for 00:05:00 .
5. Without removing the tube from the magnetic holder, remove supernatant, add 200 μL 70% ethanol , incubate 00:00:30 and discard the supernatant.
6. Repeat step 5 one time.
7. Let dry on the magnetic support for 00:08:00 .
8. Re-suspend in 51 μL EB buffer (52 μL for the optional step 13).
9. Remove from the magnetic holder and vortex gently.
10. Incubate 00:02:00 at room temperature.
11. Centrifuge briefly and place on magnetic holder for 00:05:00 .
12. Transfer 17 μL (18 μL for the optional step 13) to a 0.2mL PCR tube.
13. Vortex and centrifuge.

13 Quantity control (optional)

20m



Run 1 μL of each pool on a Qubit HS assay (following manufacturer protocol).

Note

The amount of DNA is expected to be the same or slightly lower as in step 9. If the quantity is significantly lower as in the step 9 (loss of more than 20%), you could go back with the remaining DNA from step 3 if available.

14 Final PCR enrichment

1h 30m

**Note**

The number of final enrichment cycles depends on the quality and quantity of the input DNA (i.e. step 10 of this protocol) and also on the final quantity of library desired depending of the sequencing platform and flowcell format used. For a standard application (Illumina sequencing, which may require around 100ng of library), the NEBNext Ultra II manual recommends 6-7 cycles for 10-30ng of input DNA. For applications requiring a larger library quantity (e.g. RAD-capture by probe hybridization, which may require around 300ng of library), it is recommended to increase the number of cycles (otherwise, it comes with the risk of increasing the rate of PCR duplicates).

We tested 6, 9 and 12 PCR cycles for 12ng of NEB input DNA and found an optimal amplification with 9 cycles (i.e. 105ng and 11nM of library for an Illumina NovaSeq sequencing). Furthermore, 9 cycles led to good sequencing results for several libraries constructed from a range of 8-30ng of input DNA.

Into a PCR tube, prepare PCR master mix (vols for 1 rxn) :

🧴 25 µL NEBNext Ultra II Q5 Master Mix

🧴 5 µL RAD Lib F [M] 10 micromolar (µM)

🧴 5 µL RAD Lib R [M] 10 micromolar (µM)

🧴 15 µL library

1. Vortex gently and centrifuge briefly
2. Place in a thermocycler, with the heated lid on, and run the following program:

🔥 98 °C 30s

6-12 cycles of :

🔥 98 °C 10s

🔥 65 °C 1m15s

🔥 65 °C 5m

🔥 10 °C hold

15 Cleanup of amplified library (ratio 0.65X)

50m

**Note**

The ratio recommended in the supplier manual is 0.9X. We suggest a more drastic library sizing with 0.65X ratio, which we have found to be the best for removing free adapters.

Incubate AMPure beads at room temperature at least 00:30:00 .

Prepare 70% ethanol :

For one pool: 133 μL H₂O + 467 μL Absolute Ethanol .

1. Verify the 50 μL from step 13 and transfert in a new low bind tube.
2. Add 32.5 μL Ampure beads
3. Place on a magnetic holder for 00:05:00 .
4. Without removing the tube from the magnetic holder, remove supernatant, add 200 μL 70% ethanol , incubate 00:00:30 and discard the supernatant.
5. Repeat step 4 one time
6. Let dry on the magnetic support for 00:08:00 .
7. Re-suspend in 22 μL EB buffer
8. Remove from the magnetic holder and vortex gently
9. Incubate 00:02:00 at room temperature
10. Centrifuge briefly and place on magnetic holder for 00:05:00 .
11. Transfer 21 μL to a 0.2mL PCR tube.
12. Vortex and centrifuge.

16 Final quality controls

3h

Run 1 μL of each library on a Qubit HS assay (following manufacturer protocol).

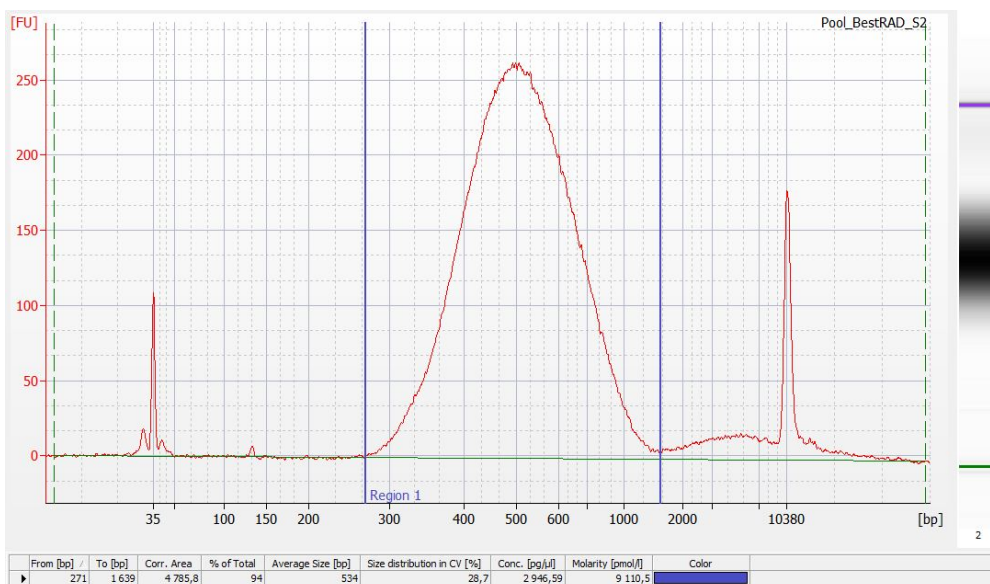
Note

At this step, the amount of DNA should be 3-10 times higher than in step 13.

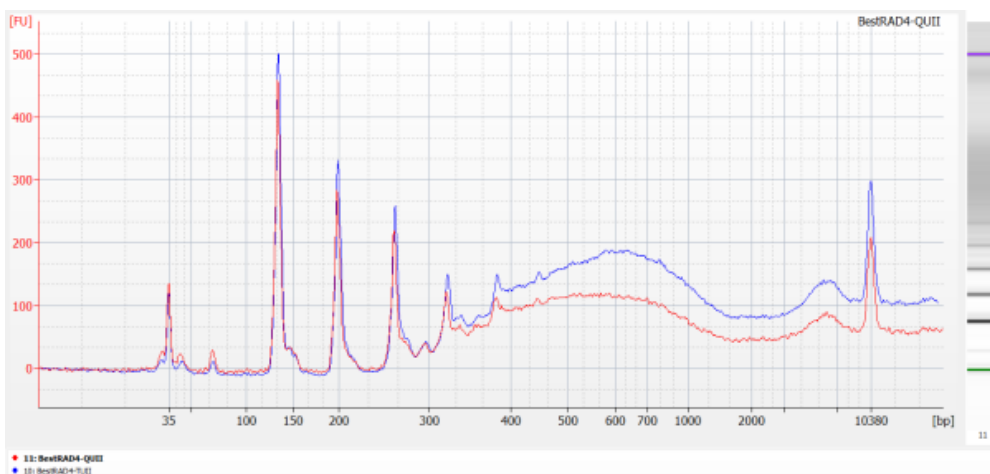
Run 1 μL of each library on an Agilent HS chip (following manufacturer protocol).

Note

Calculate the average size of the fragments. It should be around 120bp more than the average of the sheared DNA fragments (see Step 4.2).



Profile of a high-quality library on an Agilent HS chip (NEB input of 12ng, 0.65X AMPure purifications, 9 PCR cycles, final library of 180ng). Fragment sizes are tightly centered around the mean (534 bp), without unexpectedly large fragments.



Profile of two over-amplified libraries with free adapters on an Agilent HS chip (NEB input 50ng, 0.9X AMPure purifications, 12 PCR cycles). Low molecular weight peaks form a scale pattern. Over-amplification is visible around 6000bp.

Unexpectedly low molecular weight peaks (about 120bp, 240bp) are likely free adapters and adapter doublets. If peak heights are relatively elevated, the amount of adapters used was too large and/or the AMPure purification from step 12 and 15 was not efficient



enough. You can apply once more cleanup of step 15 in order to attempt to remove this unexpected peaks.

Unexpectedly high molecular weight peaks (e.g., above 6000bp) are likely artificial concatenation, i.e. artifact sequences formed by two or more biological sequences. These fragments are caused by over-amplification in step 14, because of a too large number of cycles. Over-amplification will also produce more PCR duplicates. See the note of step 14 to optimize the number of PCR cycles.

Small fragments are favored by Illumina sequencing. Thus, unexpected low molecular weight peaks are more problematic than high ones.

Run  2 μ L of each library on a Kapa assay (following manufacturer protocol).

Note

At this step, the concentration should ideally reach a minimum of 10nM. However, when input DNA is of low quality or quantity, 2nM may be sufficient (depending on the sequencing facility).

KAPA library concentration can be used to normalize several batches of samples aimed to be pooled altogether before Illumina sequencing. Don't use concentration values obtained by a Qubit assay, because it represents all DNA fragments and not only those with both Illumina adapters which will be sequenced.

Protocol references

Ali, O. A., Jeffres, C., & Miller, M. R. (2016). **RAD Capture (Rapture): Flexible and Efficient Sequence-Based Genotyping**. *Genetics*, 202(2), 16. <https://doi.org/10.1534/genetics.115.183665>

Baird, N. A., Etter, P. D., Atwood, T. S., Currey, M. C., Shiver, A. L., Lewis, Z. A., Selker, E. U., Cresko, W. A., & Johnson, E. A. (2008). **Rapid SNP Discovery and Genetic Mapping Using Sequenced RAD Markers**. *PLOS ONE*, 3(10), e3376. <https://doi.org/10.1371/journal.pone.0003376>