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Double digestion RADseq library

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We use this protocol and it's working

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Disclaimer

Protocol has been tested and used to successfully create libraries several times. Thus, it seems to be reliable and transferable. However, we recommend you to set it up and test it in your lab and system, before processing a large amount of samples.
Protocol is inspired in Peterson et al. 2012 (**10.1371/journal.pone.0037135**) and Parchman, Gompert and Buerkle 2011 rfseq protocol, with multiple modifications.

Abstract

A protocol to create libraries for Illumina to genotype through double digestion sequencing. The goal is to create a protocol that reduces costs and dedicated time at the expense of a slight reduction of quality, meaning that some of your samples may not meet depth minimum, but the specificity is of the tags is remarkable. Thus, you will probably have a low percentage of samples (in our case less than 10%) needed to be re-processed. The amount of library product should be more than enough to be run in an Illumina sequencer. This version of the protocol includes two clean-ups and no Inline tags in the adapters, making it more expensive and laborious than other versions.

Guidelines

The protocol is recommended to be done in three different days:
1- Digestion and first clean-up.
2- Ligation and second clean-up.
3- PCR and last clean-up.
Samples could be conserved for later use after every section, but we recommend to do so after clean-ups or PCR.

Protocol materials

EcoRI-HF - 10,000 units **New England Biolabs Catalog #**R3101S Step 10
MseI - 500 units **New England Biolabs Catalog #**R0525S Step 10
T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S Step 28
PHUSION PLUS DNA POLYMERASE 500 RXN **Thermo Scientific Catalog #**F630L Step 43

Safety warnings

Follow the common molecular biology lab safety procedures.

Ethics statement

Nothing to declare. No animals or personal data was used.

Before start

Before start, make sure you have at hand all the needed reagents, in particular the oligos (adapters and PCR primers). Other important reagents are: digestion enzymes with buffer, T4 ligase, DNA polymerase, Ampure beads, absolute ethanol, TE buffer, NaCl, and molecular grade water. Also a thermocycler, a centrifuge and a magnetic rack for 96-well plate are needed. Other lab equipment is necessary.

Oligo sequences

1 Make sure you have the required adapter oligos.

Note

	A	B
	MseI_P2.1_2N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNN
	MseI_P2.2_2N	/5Phos/TANNCTGTCTCTTATACGAGGACAA
	EcoRI_P1.1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
	EcoRI_P1.2	/5Phos/AATTCTGTCTCTTATACACATCTGACGCTGCCGACGA

Sequences of the adapter oligos. Note that two Ns have been added to MseI to be able to remove PCR duplicates in the bioinformatic pipeline (allowing a maximum of 16 combinations, enough for low-depth sequencing). These Ns can be removed (rendering no deduplication of reads). Also they can be reduced to one (only 4 combinations, thus not recommended). Also they might work if extended to three (allowing 64 combinations, enough for higher sequencing depth), however we have not tested it.

2 Make sure you have the required PCR primers oligos.

Note

	Step2_NXTi7_N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
	Step2_NXTi7_N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
	Step2_NXTi7_N703	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
	Step2_NXTi7_N704	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG
	Step2_NXTi7_N705	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG
	Step2_NXTi7_N706	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG
	Step2_NXTi7_N707	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG
	Step2_NXTi7_N708	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGG
	Step2_NXTi7_N709	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGG
	Step2_NXTi7_N710	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG
	Step2_NXTi7_N711	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG
	Step2_NXTi7_N712	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG
	Step2_NXTi5_S502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC
	Step2_NXTi5_S503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC
	Step2_NXTi5_S505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC
	Step2_NXTi5_S506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTC

	GGCAGCGTC
Step2_NXTi5_S507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC
Step2_NXTi5_S508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC
Step2_NXTi5_S510	AATGATACGGCGACCACCGAGATCTACACCGTCTAATTCGTCGGCAGCGTC
Step2_NXTi5_S511	AATGATACGGCGACCACCGAGATCTACACTCTCTCCGTCGTCGGCAGCGTC

Sequences of PCR primers, to allow pooling 96 samples. Those primers produce Nextera-like Illumina libraries. More primers can be created following Illumina scheme. These primers can be used to generate Metabarcoding libraries as well.

DNA dilution

3h

3 Measure your DNA stock concentration. Recommended Nanodrop One.

1h

Equipment

NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer NAME

UV-Vis Spectrophotometer TYPE

Thermo Scientific BRAND

ND-ONE-W SKU

4 Dilute DNA stock to [M] 20 ng/µl at minimum volume of 7.5 µL

2h

Annealing of adapters

1h 50m

5 Dilute oligos EcoRI_P1.1, EcoRI_P1.2, MseI_P2.1_2N, and MseI_P2.2_2N to [M] 100 micromolar (µM) 10m

In case you have Tris EDTA (TE) ready, you can dilute the oligos in the buffer.

6 Create Annealing buffer stock (10x) (it is TE buffer (10x) plus the sodium chloride): 30m

[M] 100 millimolar (mM) Tris HCl, pH 8

[M] 10 millimolar (mM) EDTA

[M] 500 millimolar (mM) NaCl

In a 1L flask:

800 mL water

15.759 g Tris-HCl

2.922 g EDTA

29.22 g NaCL

Adjust pH to 8 and bring volume to 1L.

In case of TE buffer ready, you can just prepare a NaCl 0.5 M dilution on the TE.

7 Combine oligos EcoRI_P1.1 and EcoRI_P1.2 into the same stock to a final 5m

[M] 20 micromolar (µM) total dimer concetration

Therefore, add 5 µL EcoRI_P1.1 , 5 µL EcoRI_P1.2 , 2.5 µL 10x annealing buffer , and 12.5 µL water

In case of TE buffer ready: add 5 µL EcoRI_P1.1 , 5 µL EcoRI_P1.2 , 2.5 µL NaCl 0.5M , and 12.5 µL TE buffer

8 Combine oligos MseI_P2.1_2N and MseI_P2.2_2N, in the same fashion as with EcoRI_P1. 5m

9 Incubate each oligo combination in thermal cycler: 97.5 °C 00:02:30 followed by a touchdown of 1 °C 00:00:20 until reaching 21 °C . Hold afterwards at 4 °C 1h

Double digest 2h 15m

10 Prepare Digestion Mix adding 0.9 units EcoRI enzyme , 0.45 units MseI enzyme and 1 µL rCutSmart Buffer per sample. Then, add water to get a final volume of 2.5 µL Digestion Mix per sample. 15m

For example, for 48 samples, prepare mix in excess, so factor would be 110x:
5 µL EcoRI-HF at [M] 20000 units/ml total 100 units
5 µL MseI at [M] 10000 units/ml total 50 units
110 µL rCutSmart Buffer at [M] 10 X
155 µL water (you can add some extra water: such as 5 µL

EcoRI-HF - 10,000 units **New England Biolabs Catalog #**R3101S
MseI - 500 units **New England Biolabs Catalog #**R0525S

11 On a 96-well PCR plate, dispense 2.5 µL Digestion Mix in empty PCR tubes or plate wells, keeping it On ice 10m
Spin the plate, to bring to the bottom the digestion mix.

12 Transfer 7.5 µL genomic DNA at [M] 20 ng/µl to the wells and mix DNA and mix pipetting up and down a few times. Total volume should be 10 µL . It is fundamental to keep track of the layout of the DNA samples transferred into the plate. 20m

13 Incubate at 37 °C for 01:30:00 . Hold at 4 °C afterwards. Do not heat kill enzymes. 1h 30m
Reactions can be stored at 4 °C Overnight

Clean-up 2h 45m

14 Potentially size selection could be done here, but In this version we are proceeding with a simple clean-up. 30m

Take an aliquot of magnetics beads, out of the fridge to let them reach Room temperature before initiating the protocol (at least 00:30:00 at Room temperature)
For 96 samples, at least 1 mL magnetic beads

15 Add 10 µL magnetic beads (1X) to each digestion reaction. Mix well by pipetting up and down at least 10 times. If centrifuging samples after mixing, be sure to stop the centrifugation 20m

before the beads start to settle down (no more than 1000 rpm).

16 Incubate samples on bench top for at least 00:05:00 at room temperature. 5m

17 Place the plate on a magnetic stand, and wait 00:05:00 or until the solution is clear. 5m
While waiting, prepare 35 mL Ethanol 80% (28 mL ethanol absolute + 7 mL water). It has to be freshly prepared.

18 Carefully remove and discard the supernatant (approximately 17 µL), not disturbing the beads. 20m

19 Wash with 160 µL freshly prepared ethanol 80% , not removing the plate from the magnetic stand. Incubate beads in the ethanol for 00:00:30 . Remove and discard the ethanol, but do not disturbing the beads. Some ethanol can remain if so needed not to disturb the beads. 15m

Recommendation: remove 75 µL ethanol in two pipetting moves.

20 Wash AGAIN with 160 µL freshly prepared ethanol 80% , not removing the plate from the magnetic stand. Incubate beads in the ethanol for 00:00:30 . Remove and discard the ethanol, but do not disturbing the beads. In this step, ethanol remaining should be as little as possible. 25m

Recommendation: remove 80 µL ethanol with 200 µL tips , and afterwards use 10 µL tips to remove the last drops of ethanol.

21 After removing all visible liquid, let the beads air dry approximately 00:03:00 , but DO NOT over-dry (losing the glossy shine, turning lighter brown and starting to crack). Beads may overdry really quickly with these volumes. If necessary, do not remove ethanol from all wells at once. Do it in a manner that the first columns of the plate do not overdry, so removing ethanol and adding elution buffer (next step) might intercalate. 3m

22 Remove from stand, add 13 µL elution buffer and completely dissolve beads pipetting up and down. Use a pipette that can dispense the complete amount in a single maneuver. Make a very short spin (no more than 1000 rpm) to bring down the elution and let it rest at least 00:02:00 at Room temperature . Elution buffer could be water, Tris or TE buffers. 20m

23 Place back on the magnetic stand and, after 00:05:00 or when the solution is clear, transfer 10 µL DNA elution to a new plate or tube. Only if possible, use a pipette that can transfer the whole volume in a single maneuver. 20m

24 Quantify all or a random subset of samples. Fluorometric (such as Qubit) is recommended, but not essential. Nanodrop can be used as well. If so, you will have to scale up the volumes in the overall protocol to allow for the extra amount needed for quantification, or dilute and recover DNA at two steps above with more volume.
Using amounts of the current protocol, concentration after elution is down to [M] 5 ng/µl , having lost approximately half of the DNA in the digestion and clean-up.

Ligation 2h

25 Estimate proper amounts of T4 ligase.

Note

Example for elm:
Based on estimations on simRAD R package, there are 17.5 million cut sites per elm genome 2.1 Gbp.
Considering that 1 Gbp is approximately 1 pg, then we have 8.3 billion cut sites per ng.
For our digestion output we have 420 billion cut sites per sample (50 ng in 10 ul), that translates in 0.66 pmol (in 15 ul will be 0.044 uM).
One unit is defined as the amount of enzyme required to give 50% ligation of HindIII fragments of λ DNA (5´ DNA termini concentration of 0.12 µM, 300- µg/ml) in a total reaction volume of 20 µl in 30 minutes at 16°C in 1X T4 DNA Ligase Reaction Buffer.
Given that T4 Ligase is 400 units/ul, 12 units is 0.03 ul per sample should suffice.

26 Prepare Adapter Mix. 10m
For EcoRI dilution, mix:
0.9 µL of [M] 20 micromolar (µM) annealed adapters
4.91 µL of [M] 10 X annealing buffer or NaCl
44.19 µL water or TE buffer

For MseI dilution, mix:
30.55 µL of [M] 20 micromolar (µM) annealed adapters
11.95 µL of [M] 10 X annealing buffer or NaCl
107.5 µL water or TE buffer

Finally, mix:

50 µL EcoRI dilution

150 µL MseI dilution

27 In the plate with the clean-up digested DNA, dispense 2 µL Adapter Mix into each well. 10m

You can use same pipette tip, leaving the drop in the upper portion of the well. Spin down the liquids.

28 Prepare Ligation Mix. Per sample: 10m

1.5 µL of [M] 10 X T4 ligation buffer

0.03 µL of [M] 400000 units/ml T4 ligase

1.47 µL water

For 96 samples (approx. 110x,or even 105x):

165 µL of [M] 10 X T4 ligation buffer

161.7 µL of [M] 400000 units/ml T4 ligase

52.8 µL water

T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #**M0202S

29 With the plate On ice , quickly dispense 3 µL Ligation Mix into each well. Mix by pipetting up and down a few times and spinning down. 20m

Alternatively, you can use same pipette tip, leaving the drop in the upper portion of the well. Spin down the liquids. Perhaps, vortexing the covered plate in a plate vortexer. Spin down again. However, we have not try this approach.

30 Incubate the total volume (15 µL) at Room temperature or 23 °C for 01:00:00 , then heat-kill at 65 °C for 00:10:00 After the heat-kill, cool the solution at 2 °C per 00:01:30 until it reaches room temperature. 1h 11m 30s

Clean-up 2h 45m

31 Take an aliquot of magnetics beads, out of the fridge to let them reach Room temperature before initiating the protocol (at least 00:30:00 at Room temperature) 30m

For 96 samples, at least 1.5 mL magnetic beads

32 Add 15 µL magnetic beads (1X) to each digestion reaction. Mix well by pipetting up and down at least 10 times. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle down (no more than 1000 rpm). 20m

33 Incubate samples on bench top for at least 00:05:00 at room temperature. 5m

34 Place the plate on a magnetic stand, and wait 00:05:00 or until the solution is clear. 5m
While waiting, prepare 35 mL Ethanol 80% (28 mL Ethanol absolute + 7 mL Water). It has to be freshly prepared.

35 Carefully remove and discard the supernatant (approximately 27 µL), not disturbing the beads. 20m

36 Wash with 160 µL freshly prepared ethanol 80% , not removing the plate from the magnetic stand. Incubate beads in the ethanol for 00:00:30 . Remove and discard the ethanol, but do not disturbing the beads. Some ethanol can remain if so needed not to disturb the beads. 15m
So removing 150 µL ethanol would be enough.

37 Wash AGAIN with 160 µL freshly prepared ethanol 80% , not removing the plate from the magnetic stand. Incubate beads in the ethanol for 00:00:30 . Remove and discard the ethanol, but do not disturbing the beads. In this step, ethanol remaining should be as little as possible. 25m

Recommendation: remove 160 µL ethanol with 200 µL tips , and afterwards use 10 µL tips to remove the last drops of ethanol.

38 After removing all visible liquid, let the beads air dry approximately 00:03:00 , but DO NOT over-dry (losing the glossy shine, turning lighter brown and start to crack). Beads may overdry really quick with these volumes. If necessary, do not remove ethanol from all wells at once. Do it in a manner that the first columns of the plate do not overdry, so removing ethanol and adding elution buffer (next step) might intercalate. Normally, half plate can be done with no need of intercalation. 3m

39 Remove from stand, add 20 µL elution buffer and completely dissolve beads pipetting up and down. Use a pipette that can dispense the complete amount in a single maneuver. Make a very short spin (no more than 1000 rpm) to bring down the elution and let it rest at least 00:02:00 at Room temperature . Elution buffer could be water, Tris or TE buffers. 20m

40 Place back on the magnetic stand and, after 00:05:00 or when the solution is clear, transfer 16 µL DNA elution to a new plate or tube. Only if possible, use a pipette that can 20m

transfer the whole volume in a single maneuver. In this step, after transferring 8 µL DNA elution to a plate for storage, the remaining 8 µL DNA elution can be transferred straight to the plate for PCR reaction, with Primers already dispensed (see next section).

Amplification

3h

41 In an empty half-plate, dispense to the bottom of each well, avoiding touching the walls, or touching consistently the same side wall: 20m

1.5 µL of [M] 5 micromolar (µM) Primer R 5xx

All the wells of ROW A must have the same Primer, same for B, and the rest. In this way, you will have deployed 8 different primers, each one 12 times.

42 Now transfer 8 µL cleaned-up ligation product , to the bottom of the wells. That would be no more than 10 ng of non-size-selected DNA. 10m

43 For a total volume 15 µL , prepare PCR mix: 30m

Per sample, the final volumes :

3 µL of [M] 5 X Buffer

0.3 µL of [M] 10 millimolar (mM) each dNTPs

0.55 µL water

0.15 µL polymerase

1.5 µL of [M] 5 micromolar (µM) Primer F 7xx (to dispense in aliquoted mix)

1.5 µL of [M] 5 micromolar (µM) Primer R 5xx (already dispensed)

PHUSION PLUS DNA POLYMERASE 500 RXN **Thermo Scientific Catalog #**F630L

For half a plate (102x), first mix:

306 µL of [M] 5 X Buffer

30.6 µL of [M] 10 millimolar (mM) dNTPs

56.1 µL water

15.3 µL polymerase

Aliquot the mix in 12 microtubes 34 µL each and add to each 12.75 µL of a different Primer F 7xx. Dispense at the well rims 5.5 µL specific PCR Mix to each well, in the following manner:

Dispense aliquot number 1 to all the wells of the COLUMN 1; aliquot number 2 to COLUMN 2; and so on. Spin down.
If you really want to avoid possible traces of cross-contamination, you should use different pipette tips per well.

44 Run a PCR amplification following the polymerase recommended program: 30m
98 °C for 00:00:30
12 cycles of: 98 °C for 00:00:10 ; 55 °C for 00:00:10 ; 72 °C for 00:00:20
72 °C for 00:05:00

45 It is highly recommended to quantify in Qubit and/or agarose gel. Empirically, we obtain [M] 10 ng/µl with 12 cycles . 1h 30m

Pooling and size selection 1h 55m

46 Combine 4 µL of each successful reaction, into a single 1.5 mL microtube . You may use same pipette tip. Different volumes per sample can be pooled, if yields have been uneven. Let's say total volume of the pool is 384 µL . Transfer 320 µL to a new 1.5 mL microtube . 10m

47 Choosing fragments between 330bp and 410bp. Adding adapter lengths (140bp total) that would be 470bp to 550bp. In our experience, size selection with Ampure beads should follow then these ratios: 0.5X in the first step and 0.2X in the second; to achieve these sizes. You can try different ratios to evaluate selection ranges.

48 Take an aliquot of magnetics beads, out of the fride to let them reach Room temperature before initiating the protocol (at least 00:30:00 at Room temperature) 30m
For 96 samples, at least 0.24 mL magnetic beads

49 Add 160 µL magnetic beads (0.5X) to the PCR pool . Mix well by pipetting up and down at least 10 times. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle down (no more than 1000 rpm). 3m

50 Incubate samples on bench top for at least 00:05:00 at room temperature. 5m

51 Place the plate on a magnetic stand, and wait 00:05:00 or until the solution is clear. 5m

52 Transfer all the supernatant to a new 1.5 mL microtube, discarding the beads. Tried not to transfer beads since they are attached to large fragments of DNA. 3m

53 Add 64 µL magnetic beads (0.2X) to the PCR pool supernatant, just transfered in a new tube. Mix well by pipetting up and down at least 10 times. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle down (no more than 1000 rpm). 3m

54 Incubate samples on bench top for at least 00:05:00 at room temperature. 5m

55 Place the plate on a magnetic stand, and wait 00:05:00 or until the solution is clear. While waiting, prepare 2 mL Ethanol 80% (1.6 mL Ethanol absolute + 0.8 mL Water). It has to be freshly prepared. 5m

56 Carefully remove and discard the supernatant, not disturbing the beads. 3m

57 Wash with 800 µL freshly prepared ethanol 80% , not removing the plate from the magnetic stand. Incubate beads in the ethanol for 00:00:30 . Remove and discard the ethanol, but do not disturb the beads. Some ethanol can remain if so needed not to disturb the beads. 3m

58 Wash AGAIN with 800 µL freshly prepared ethanol 80% , not removing the plate from the magnetic stand. Incubate beads in the ethanol for 00:00:30 . Remove and discard the ethanol, but do not disturb the beads. In this step, ethanol remaining should be as little as possible. Use 10 µL tips to remove the last drops of ethanol. 3m

59 After removing all visible liquid, let the beads air dry approximately 00:05:00 , but DO NOT over-dry (losing the glossy shine, turning lighter brown and start to crack). 5m

60 Remove from stand, add 28 µL elution buffer and completely dissolve beads pipetting up and down. Use a pipette that can dispense the complete amount in a single maneuver. Make a very short spin (no more than 1000 rpm) to bring down the elution and let it rest at least 00:02:00 at Room temperature . Elution buffer could be water, Tris or TE buffers. 5m

61 Place back on the magnetic stand and, after 00:05:00 or when the solution is clear, transfer 24 µL DNA elution to a tube. 5m

62 Quantify in Qubit. Following the protocol, we obtained a concentration higher than [M] 15 ng/µl . 20m

Normally, Illumina services require 20 µL at [M] 10 nanomolar (nM) .

Following the conversion formula at:
https://knowledge.illumina.com/library-preparation/dna-library-prep/library-preparation-dna-library-prep-reference_material-list/000001240

For a mean library size of 510bp, it is needed less than [M] 3.5 ng/µl .

Protocol references

Protocol is inspired in Peterson et al. 2012 (**10.1371/journal.pone.0037135**) and Parchman, Gompert and Buerkle 2011 rfseq protocol, with multiple modifications.