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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](https://doi.org/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 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**

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

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

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

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	GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAGNN
	MseI_P2.2_2N	/5Phos/TANNCTGTCTCTTAT ACGAGGACAA
	EcoRI_P1.1	TCGTCGGCAGCGTCAGATGT GTATAAGAGACAG
	EcoRI_P1.2	/5Phos/AATTCTGTCTCTTAT ACACATCTGACGCTGCCGAC GA

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_N 701	CAAGCAGAAGACGGCATAACG AGATTCGCCTTAGTCTCGTG GGCTCGG	
Step2_NXTi7_N 702	CAAGCAGAAGACGGCATAACG AGATCTAGTACGGTCTCGTG GGCTCGG	
Step2_NXTi7_N 703	CAAGCAGAAGACGGCATAACG AGATTTCTGCCTGTCTCGTG GGCTCGG	
Step2_NXTi7_N 704	CAAGCAGAAGACGGCATAACG AGATGCTCAGGAGTCTCGTG GGCTCGG	
Step2_NXTi7_N 705	CAAGCAGAAGACGGCATAACG AGATAGGAGTCCGTCTCGTG GGCTCGG	
Step2_NXTi7_N 706	CAAGCAGAAGACGGCATAACG AGATCATGCCTAGTCTCGTG GGCTCGG	
Step2_NXTi7_N 707	CAAGCAGAAGACGGCATAACG AGATGTAGAGAGGTCTCGTG GGCTCGG	
Step2_NXTi7_N 708	CAAGCAGAAGACGGCATAACG AGATCCTCTCTGGTCTCGTG GGCTCGG	
Step2_NXTi7_N 709	CAAGCAGAAGACGGCATAACG AGATAGCGTAGCGTCTCGTG GGCTCGG	
Step2_NXTi7_N 710	CAAGCAGAAGACGGCATAACG AGATCAGCCTCGGTCTCGTG GGCTCGG	
Step2_NXTi7_N 711	CAAGCAGAAGACGGCATAACG AGATTGCCTCTTGTCTCGTG GGCTCGG	
Step2_NXTi7_N 712	CAAGCAGAAGACGGCATAACG AGATTCCTCTACGTCTCGTG GGCTCGG	
Step2_NXTi5_S5 02	AATGATACGGCGACCACCGA GATCTACACCTCTCTATTCGT CGGCAGCGTC	
Step2_NXTi5_S5 03	AATGATACGGCGACCACCGA GATCTACACTATCCTCTTCGT	



		CGGCAGCGTC
	Step2_NXTi5_S5 05	AATGATACGGCGACCACCGA GATCTACACGTAAGGAGTCG TCGGCAGCGTC
	Step2_NXTi5_S5 06	AATGATACGGCGACCACCGA GATCTACACACTGCATATCG TCGGCAGCGTC
	Step2_NXTi5_S5 07	AATGATACGGCGACCACCGA GATCTACACAAGGAGTATCG TCGGCAGCGTC
	Step2_NXTi5_S5 08	AATGATACGGCGACCACCGA GATCTACACCTAAGCCTTCG TCGGCAGCGTC
	Step2_NXTi5_S5 10	AATGATACGGCGACCACCGA GATCTACACCGTCTAATTCG TCGGCAGCGTC
	Step2_NXTi5_S5 11	AATGATACGGCGACCACCGA GATCTACACTCTCTCCGTCG TCGGCAGCGTC

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 [M] 20 micromolar (μM) total dimer concentration 5m
- Therefore, add EcoRI_P1.1 , EcoRI_P1.2 ,
 $\text{2.5 } \mu\text{L 10x annealing buffer}$, and $\text{12.5 } \mu\text{L water}$
- In case of TE buffer ready: add $\text{5 } \mu\text{L EcoRI_P1.1}$, $\text{5 } \mu\text{L EcoRI_P1.2}$,
 $\text{2.5 } \mu\text{L NaCl 0.5M}$, and $\text{12.5 } \mu\text{L TE buffer}$
- 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: $\text{97.5 } ^\circ\text{C}$ 00:02:30 followed by a touchdown of $\text{1 } ^\circ\text{C}$ 00:00:20 until reaching $\text{21 } ^\circ\text{C}$. Hold afterwards at $\text{4 } ^\circ\text{C}$ 1h

Double digest

2h 15m

- 10 Prepare Digestion Mix adding $\text{0.9 units EcoRI enzyme}$,
 $\text{0.45 units MseI enzyme}$ and $\text{1 } \mu\text{L rCutSmart Buffer}$ per sample. Then, add water to get a final volume of $\text{2.5 } \mu\text{L Digestion Mix}$ per sample. 15m
- For example, for 48 samples, prepare mix in excess, so factor would be 110x:
- $\text{5 } \mu\text{L EcoRI-HF}$ at [M] 20000 units/ml total 100 units
 $\text{5 } \mu\text{L MseI}$ at [M] 10000 units/ml total 50 units
 $\text{110 } \mu\text{L rCutSmart Buffer}$ at [M] 10 X
 $\text{155 } \mu\text{L water}$ (you can add some extra water: such as $\text{5 } \mu\text{L}$)
- 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 $\text{2.5 } \mu\text{L Digestion Mix}$ 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 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 magnetic 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 before the beads start to settle down (no more than 1000 rpm). 20m
- 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 μ L ethanol in two pipetting moves.

- 20 Wash AGAIN with  160 μ L 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 μ L ethanol with  200 μ L tips , and afterwards use  10 μ L 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 μ L 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 μ L 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  5 ng/ μ L , 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 μ l), that translates in 0.66 pmol (in 15 μ l will be 0.044 μ M).

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 μ M, 300- μ g/ml) in a total reaction volume of 20 μ l in 30 minutes at 16°C in 1X T4 DNA Ligase Reaction Buffer. Given that T4 Ligase is 400 units/ μ l, 12 units is 0.03 μ l per sample should suffice.

26 Prepare Adapter Mix.

10m

For EcoRI dilution, mix:

0.9 μ L of [M] 20 micromolar (μ M) annealed adapters

4.91 μ L of [M] 10 X annealing buffer or NaCl

44.19 μ L water or TE buffer

For MseI dilution, mix:

30.55 μ L of [M] 20 micromolar (μ M) annealed adapters

11.95 μ L of [M] 10 X annealing buffer or NaCl

107.5 μ L water or TE buffer

Finally, mix:

50 μ L EcoRI dilution

150 μ L MseI dilution

27 In the plate with the clean-up digested DNA, dispense 2 μ L Adapter Mix into each well. You can use same pipette tip, leaving the drop in the upper portion of the well. Spin down the liquids.

10m

28 Prepare Ligation Mix. Per sample:

10m

1.5 μ L of [M] 10 X T4 ligation buffer

0.03 μ L of [M] 400000 units/ml T4 ligase

1.47 μ L water

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

165 μ L 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 20m

by pipetting up and down a few times and spinning down.
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 $^{\circ}\text{C}$ for 1h 11m 30s
⌚ 01:00:00 , then heat-kill at 🌡️ 65 $^{\circ}\text{C}$ for ⌚ 00:10:00 After the heat-kill, cool the solution at 🌡️ 2 $^{\circ}\text{C}$ per ⌚ 00:01:30 until it reaches room temperature.

Clean-up

2h 45m

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

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 20m
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).

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 20m
the beads.

36 Wash with 🧪 160 μL freshly prepared ethanol 80% , not removing the plate from the 15m
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.

So removing  150 μ L ethanol would be enough.

- 37 Wash AGAIN with  160 μ L 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 μ L ethanol with  200 μ L tips , and afterwards use  10 μ L 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 μ L 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 μ L DNA elution to a new plate or tube. Only if possible, use a pipette that can transfer the whole volume in a single maneuver. In this step, after transferring  8 μ L DNA elution to a plate for storage, the remaining  8 μ L DNA elution can be transferred straight to the plate for PCR reaction, with Primers already dispensed (see next section). 20m

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:
 1.5 μ L of [M] 5 micromolar (μ M) 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. 20m



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 $^{\circ}\text{C}$ for 00:00:30

12 cycles of: 98 $^{\circ}\text{C}$ for 00:00:10 ; 55 $^{\circ}\text{C}$ for 00:00:10 ;

72 $^{\circ}\text{C}$ for 00:00:20

72 $^{\circ}\text{C}$ 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 .
- 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 magnetic 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 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).
- 50 Incubate samples on bench top for at least 00:05:00 at room temperature.
- 51 Place the plate on a magnetic stand, and wait 00:05:00 or until the solution is clear.
- 52 Transfer all the supernatant to a new 1.5 mL microtube , discarding the beads. Try not to transfer beads since they are attached to large fragments of DNA.
- 53 Add 64 μL magnetic beads (0.2X) to the PCR pool supernatant, just transferred 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).
- 54 Incubate samples on bench top for at least 00:05:00 at room temperature.

10m

30m

3m

5m

5m

3m

3m

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 μ L 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 μ L 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 μ L 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 μ L 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 μ L DNA elution to a tube. 5m
- 62 Quantify in Qubit. Following the protocol, we obtained a concentration higher than 15 ng/ μ L . Normally, Illumina services require 20 μ L at 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 3.5 ng/ μ L . 20m



Protocol references

Protocol is inspired in Peterson et al. 2012 ([10.1371/journal.pone.0037135](https://doi.org/10.1371/journal.pone.0037135)) and Parchman, Gompert and Buerkle 2011 rfseq protocol, with multiple modifications.