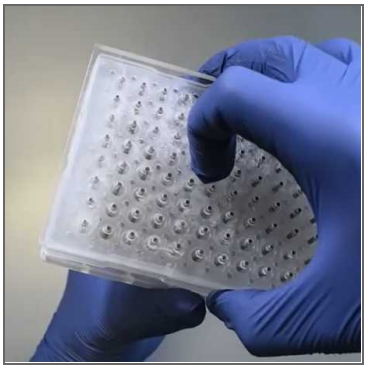


JAN 15, 2024

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1

Protocol Citation: Tomasz Suchan, Christophe Dufresnes, Robin Schmidt, Johanna Ambu, Sven Gippner, Miguel Vences 2024. ddRADseq for animal population genomics/phylogenomics. **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygx3nzwg8j/v1

MANUSCRIPT CITATION: Ambu, J., Martínez-Solano, Í., Suchan, T., Hernandez, A., Wielstra, B., Crochet, P. A., & Dufresnes, C. (2023). Genomic phylogeography illuminates deep cyto-nuclear discordances in midwife toads (*Alytes*). *Molecular Phylogenetics and Evolution*, *183*, 107783. https://doi.org/10.1016/j.ympev.2023.107783

ddRADseq for animal population genomics/phylogenomics

Forked from RAD-sequencing

Tomasz Suchan[1], Christophe Dufresnes[2,3], Robin Schmidt[4], Johanna Ambu[2], Sven Gippner[4], Miguel Vences[4]

[1]W. Szafer Institute of Botany, Polish Academy of Sciences;
[2]College of Biology and Environment, Nanjing Forestry University, Nanjing 210037, People's Republic of China;
[3]Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum national d'Histoire naturelle, CNRS, Sorbonne Université, EPHE, Université des Antilles, Paris, France;
[4]Zoological Institute, Technische Universität Braunschweig, Mendelssohnstraße 4, 38106 Braunschweig, Germany

Robin Schmidt
Zoological Institute, Technische Universität Braunschweig, M...

Protocol status: Working
So far, this protocol, or the previous version it derives from (Brelsford et al., 2016), has been successfully used in phylogeographic studies across various non-model animals, including amphibians, reptiles, mammals, and insects (e.g. Ambu et al., 2023).

Created: Oct 04, 2023

Last Modified: Jan 15, 2024

PROTOCOL integer ID: 88808

Keywords: ddRADseq, Restriction-site Associated DNA sequencing, RADseq, RAD library preparation, Genotyping by sequencing, RAD sequencing, double digest RAD, double digested restriction-site associated DNA

ABSTRACT

Restriction-site Associated DNA sequencing (RADseq) offers a simple, affordable, and versatile approach to swiftly genotype thousands of genetic markers without prior optimization for population genetic and phylogenetic analyses on any kind of organism (Baird et al. 2008). Although RADseq protocols have undergone significant advancements, they remain difficult to implement de novo for researchers inexperienced with the technique in labs that are not a priori equipped for NGS technologies.

The protocol presented here aims to facilitate such implementation. It consists of an adaptation of the double digest RADseq (ddRADseq) protocol proposed by Peterson et al. (2012) and further modified by Brelsford et al. (2016). Compared to the latter, it optimizes reagent usages and introduces a facilitated procedure of AMPure purification of an entire 96-well plate at once, by using a custom magnetic 96-pin device.

The procedure goes as follows. Genomic DNA of the samples undergoes enzyme restriction by SbfI, a 'rare'-cutter, and MseI, a more 'frequent'-cutter. The digested DNA fragments are then ligated to adapters on each end, with the SbfI adapter containing individual 4-8 bp barcodes for multiplexing samples in the library. The ligated fragments are then purified and amplified by replicate PCRs using primers that include TruSeq Illumina indices for sequencing as well as indexing. PCR products are then pooled and size selection (400-500bp) is performed either with a PippinPrep Instrument or through ad hoc migration/excision on an agarose gel. The size-selected library is purified, quantified, and controlled before Illumina sequencing.

So far, this protocol, or the previous version it derives from (Brelsford et al., 2016), has been successfully used in phylogeographic studies across various non-model animals, including amphibians, reptiles, mammals, and insects (e.g. Ambu et al., 2023).

Note

All steps can be visualized by a detailed video tutorial on How to double digest Restriction site Associated DNA sequencing (ddRADseq).

GUIDELINES

Prevent cross-contamination by using low-retention filter tips for all pipetting procedures. If possible, we recommend working under a laminar flow bench to protect the samples and further avoid contamination. A non-template control on the plate may also be incorporated to stay tuned for possible contamination.

Handle the SbfI barcoded adapter plate with great care, as cross-contamination on this plate might invalidate your entire study and any future study using that plate. Moreover, barcode cross-contamination may only become apparent through inconsistent results after data analysis.

MATERIALS

Reagents:

- Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0493L**
- T4 DNA Ligase **New England Biolabs Catalog #M0202**
- ATP Solution (100 mM) **Thermo Fisher Scientific Catalog #R0441**
- Q5 Hot Start High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0493L**
- MseI - 2,500 units **New England Biolabs Catalog #R0525L**
- SbfI - 2,500 units **New England Biolabs Catalog #R0642L**
- Agencourt AMPure XP **Beckman Coulter Catalog #A63880**
- Water HPLC Plus **Merck MilliporeSigma (Sigma-Aldrich) Catalog #34877-2.5L-M**
- Ethanol, absolute **Merck MilliporeSigma (Sigma-Aldrich) Catalog #32205-2.5L-M**
- Monarch DNA Gel Extraction Kit **New England Biolabs Catalog #T1020S**
- Qubit 1X dsDNA BR Assay Kit **Invitrogen - Thermo Fisher Catalog #Q33230**
- Biozym LE Agarose **Biozym Catalog #840004**
- TAE (Tris-Acetate-EDTA) buffer, 1x **Contributed by users**
- GeneRuler 100 bp Plus DNA Ladder **Thermo Fisher Catalog #SM0321**

TriTrack DNA Loading Dye (6X) **Thermo Fisher Catalog #R1161**

dNTP Mix (25 mM each) **Thermo Scientific Catalog #R1121**

Midori Green Advance DNA Stain **Nippon Genetics Catalog #MG04**

Adapter:

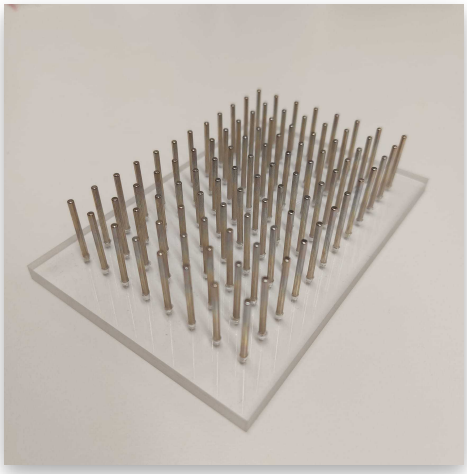
ddRAD_Adapter_Sequences.xlsx25KB

Consumables:

- 1.5 mL Microcentrifuge Tubes
- 5.0 mL Microcentrifuge Tubes
- 8-Cap Strips
- 8-Tubes Strips; Volume: 0.2 mL
- 96-Well PCR Plates (Non-Skirted)
- 96-Well Microplates (Well Shape: Round, V-bottom)
- Adhesive PCR Plate Seals
- Filter Tips (low retention quality)
- Qubit Microtubes

Equipment:

- Centrifuge (for plates and tubes)
- Gel Electrophoresis System
- Magnetic Stand-96 (Invitrogen AM10027)
- Magnetic 96-Pin Plate (custom made by Tomasz Suchan)



- Microwave
- Pipettes (10 µl, 200 µl, 1000 µl and multichannel)
- Personal protection equipment (lab coat, gloves, UV goggles)
- Qubit Fluorometer
- Racks (for plates and tubes)

- Thermocycler
- Vortex Mixer

SAFETY WARNINGS

Safety Warning for Wearing Protective Gear (Goggles) When Working with UV Light:
Caution: when working with UV light, it is imperative to prioritize your safety. Always wear appropriate protective gear, including safety goggles, to shield your eyes from potentially harmful UV radiation. Unprotected exposure to UV light can lead to serious eye damage. Protect your vision – safeguard your eyes.

Safety Warning for Handling Ethidium Bromide (if you are still working with EtBr):
Caution: ethidium bromide is a hazardous chemical commonly used in molecular biology. It is known to be a potential mutagen and should be handled with extreme care. To minimize risks to your health and the environment, follow these guidelines:

1. **Personal Protective Equipment:** wear appropriate personal protective equipment, including lab coats, gloves, and safety goggles, when working with ethidium bromide.
2. **Adequate Ventilation:** conduct all procedures involving ethidium bromide in a properly ventilated area or under a hood to minimize exposure to fumes.
3. **Avoid Skin Contact:** minimize skin contact with ethidium bromide. If skin contact occurs, wash the affected area immediately with copious amounts of water.
4. **Use Safer Alternatives:** whenever possible, consider replacing ethidium bromide with safer alternatives, such as Midori Green, which is non-hazardous and less harmful to human health and the environment.

BEFORE START INSTRUCTIONS

Before starting the ddRADseq protocol you should order and prepare all materials including reagents, consumables, and adapters in advance.

Note

You can access a comprehensive inventory of all reagents, consumables, and adapters within the Materials section of this protocol.

Adapter sequences

SbfI adapter p1.1
ACACTCTTTCCCTACACGACGCTCTTCCGATCT**NNNNNN**TGCA
SbfI adapter p1.2
[5phos]**nnnnnn**AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

where **NNNNNN** is a sample-specific 4-7 bp barcode, **nnnnnn** is a reverse complement; for instance, we use 96 barcoded adapters which allow barcoding a full PCR plate of samples. The barcode appears at the beginning of each read (i.e. "inline" barcode).

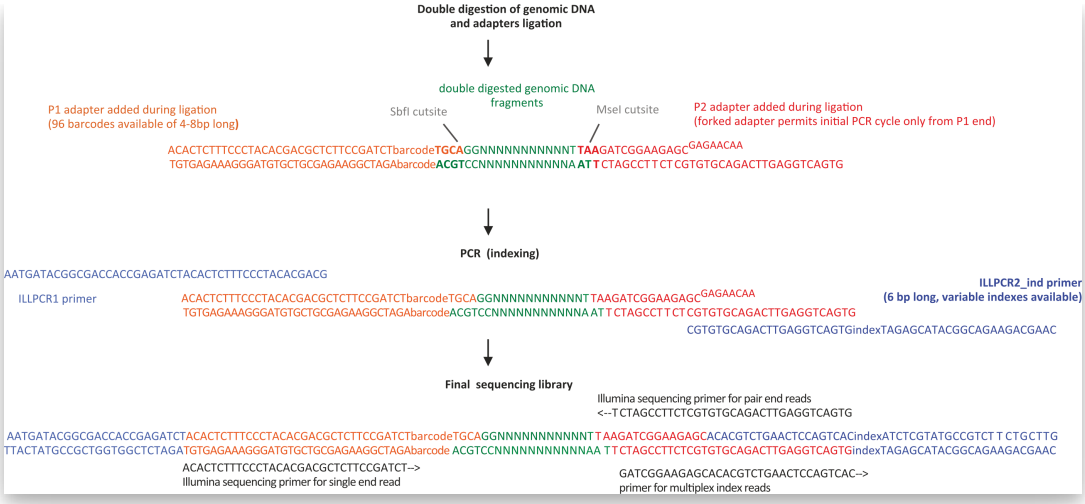
MseI adapter p2.1:
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
MseI adapter p2.2:
[5phos]TAAGATCGGAAGAGCGAGAACAA

MseI adapters are universal, the same is used for all the samples.

Primer sequences and preparation

Illumina PCR1 primer:
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG
Indexed PCR2 Primers:
CAAGCAGAAGACGGCATACGAGAT**nnnnnn**GTGACTGGAGTTCAGACGTGTGC

where **nnnnnn** is an Illumina index. These can be used to uniquely tag pools of samples barcoded with inline barcodes. In our case, we tag 96 samples with inline barcodes (in the P1 adapter) and then index these pools, each pool with a unique indexed primer.



Design of the adapters for the ddRADseq protocol.

Preparation

1 Before starting the protocol, it is necessary to prepare the SbfI and MseI adapters as follows:

1.1 Barcoded SbfI adapter annealing:

Anneal unique adapter pairs by mixing 2 µL SbfI adapter p1.1 (100 µM) and 2 µL SbfI adapter p1.2 (100 µM) with 196 µL nuclease-free water to make 200 µL double stranded SbfI adapter (2 µM).

Heat to 95 °C for 00:05:00 and slowly cool to Room temperature. Anneal the set of adapters in a plate format. It will be more convenient for later use in setting up reactions. Perform an additional 20-fold dilution to obtain a working solution at a concentration of 0.1 µM.

Note

- The barcoded adapter stocks must be handled with extreme caution as cross-contamination would compromise all the studies using them. We recommend working with 8-cap strips or 96-well plates sealed with plastic caps (avoid adhesive PCR plate seals) and preparing aliquoted stocks.

1.2 **Non-barcoded MseI adapter annealing:**

Mix 100 µL MseI adapter p2.1 (100 µM) and 100 µL MseI adapter p2.2 (100 µM) with 800 µL nuclease-free water to make 1000 µL double stranded MseI adapter (10 µM).

Heat to 95 °C for 00:05:00 and slowly cool to Room temperature to anneal the single-stranded adapters into a double-stranded adapter.

Restriction Reaction

2 In this section of the protocol, the restriction digestion reaction of samples will be carried out.

2.1 Prepare the restriction reaction mastermix for all the samples using the amounts of reagents below (3 µL reaction mix per sample), mix by brief vortexing, and shortly centrifuge:

> **Note**
>
> We have found that preparing the reaction mix for 1.2x per sample is sufficient to avoid running out of mastermix due to high viscosity and/or pipetting errors.

1.9 µL nuclease-free water
0.9 µL CutSmart buffer (10x)
0.1 µL MseI (10,000 U/mL)
0.1 µL SbfI (HF) (20,000 U/mL)

2.2 Place 6 µL sample DNA in each well of a 96-well plate.

> **Note**
>
> Ideally, DNA should be at a minimum concentration of 5 ng/µL and a maximum concentration of 25 ng/µL, but lower concentrations may still work. We have obtained good results with some samples as low as 1 ng/µL.

Note

DNA should ideally be checked for integrity on an agarose gel before, so samples with large molecules can be preferentially chosen for library preparation (samples with degraded DNA might be less successful).

2.3 Add 3 µL restriction reaction mix to each well of the sample DNA plate.

2.4 The total reaction volume should now be 9 µL. Cover and seal the plate, centrifuge, and incubate at 37 °C for 03:00:00 on a thermal cycler with a heated lid. Inactivate the restriction enzymes for 00:20:00 at 65 °C. Store at 4 °C.

Ligation Reaction

3 In this section of the protocol, the prepared double-stranded SbfI and MseI adapters (as described in step 1 - Preparation) will be ligated to the restriction-digested DNA (see step 2 - Restriction Reaction).

3.1 Prepare the ligation reaction mastermix for all the samples using the amounts of reagents below (1.6 µL reaction mix per sample), mix by brief vortexing, and shortly centrifuge:

Note

We have found that preparing the reaction mix for 1.2x per sample is sufficient to avoid running out of mastermix due to high viscosity and/or pipetting errors.

- 0.26 µL CutSmart buffer (10x)
- 0.12 µL ATP (100 mM)
- 1 µL Non-barcoded MseI P2 adapter (10 µM)
- 0.17 µL T4 DNA ligase (400,000 U/ml)
- 0.06 µL nuclease-free water

3.2 Add 1.6 µL ligation reaction mix to each well of the restriction-digested DNA.

3.3 Add 1 µL barcoded SbfI P1 adapter (0.1 µM) to each well.

> **Note**
>
> Remember to use a unique SbfI P1 adapter for each sample/well.
> Use a multichannel pipet to increase efficiency and avoid confusion.

3.4 The total reaction volume should now be 11.6 µL. Cover and seal the plate, centrifuge, and incubate at 16 °C for 03:00:00 on a thermal cycler. Store at -20 °C if the library is not to be processed immediatly.

Purification (short fragment removal)

4 Purify the ligation reaction product using an AMPure XP protocol with a 1:1 ratio and elute in 45 uL of nuclease-free water.

We introduced the use of a custom-made magnetic 96-pin plate (see YouTube Tutorial, starting at 11:40) to increase the efficiency of the protocol.

4.1 Let the AMPure aliquot at Room temperature for 00:30:00 and then shake it gently to resuspend the magnetic beads.

> **Note**
>
> Do not vortex to protect the coating of the beads!

4.2 Add 8.4 µL nuclease-free water to each well to have 20 µL ligated product in each well.

4.3 Prepare one microplate with 20 µL AMPure beads in each well.

4.4 Prepare two microplates with 100 µL freshly prepared 70% ethanol in each well.

4.5 Prepare one microplate with 45 µL elution buffer / nuclease-free water in each well.

> **Note**
>
> Nuclease-free water + TRIS can alternatively be used, especially if the ligation products are not meant to be processed in the immediate future.

4.6 Transfer your 20 µL ligation product to the AMPure plate and (optionally) mix by pipetting 10 times up and down.

> **Note**
>
> Make sure that you have 20 µL of AMPure beads in each well to get a 1:1 DNA/beads ratio. Pipetting AMPure can pose challenges at times so it may be advantageous to trim the pipette tip with a sterile razor blade.

4.7 Incubate for 00:05:00 at Room temperature.

4.8 Equip a 96-pin magnet device (custom-made by Tomasz Suchan) with a clean PCR plate as a cover and apply it to the beads+DNA plate. Wait for 00:10:00 to separate the beads from the solution.

Note

Be sure that the solution is clear before proceeding and do not rotate the plates and microplates during the following steps.

4.9 Rinse the attached beads twice for 00:00:30 each in the ethanol microplates, then hold the plate/device for a couple of minutes to dry the beads. Cracks on the beads indicate drying.

Note

Do not overdry the beads! As soon as the beads lose their shine and cracks appear you should proceed with step 4.10.

4.10 Elute the beads by placing the dried plate/device on the elution plate and remove the 96-pin magn 30m plate slowly. Then gently shake/move the PCR cover plate to solubilize the beads. Wait 00:10:00 to 00:20:00 until the solution homogenizes and the DNA fully detaches from the beads.

4.11 Introduce the 96-pin magnet plate again on the PCR plate to attract the (now DNA-free) beads. Wait at least one minute, then remove the plate/device and throw the PCR plate with the used AMPure beads away.

Note

Some beads may remain but should be avoided in subsequent molecular reactions. To achieve this, place the elution plate on an Ambion RNA Magnetic stand-96 magnet (which will attract the beads in the corners of each well) to transfer the DNA for the upcoming library amplification.

Library Amplification

5 The library amplification/PCR step employs Illumina PCR primers to amplify restricted fragments from their ligated adapters.

Note

To ameliorate stochastic differences in PCR production of fragments and obtain larger DNA quantity for the size-selection stage, we run two separate 10 µL reactions per restriction-ligation product, which are later pooled together.

5.1 Prepare the PCR reaction mastermix for all samples using the amounts of reagents below (7 µL per sample but remember to prepare enough mastermix to run 2 PCR reactions per sample!):

- 1.48 µL nuclease-free water
- 2.00 µL Q5 buffer (5x)
- 0.08 µL dNTPs (25 mM)
- 0.67 µL Forward primer (5 uM)
- 0.67 µL Reverse primer (5 uM)
- 0.10 µL Q5 Hot Start High-Fidelity polymerase
- 2.00 µL High GC enhancer

5.2 Add 7 µL PCR reaction mix to each well of two PCR plates.

5.3 Add 3 µL ligated DNA purified in step 4.

5.4 PCR Reaction:

Denaturation
00:00:30 at 98 °C
20 cycles
00:00:20 at 98 °C,
00:00:30 at 60 °C,
00:00:40 at 72 °C
Final Elongation
00:02:00 at 72 °C

Hold at 4 °C

5.5 Pool the two replicate plates together to obtain a single plate with 20 uL of amplicons in each well.

5.6 Run 3 µL of each PCR product on a 1.5 % agarose gel at [M] 120 V for 00:30:00. You should see a smear of PCR product from 200 bp to 1000 bp, sometimes with a bright band of adapter dimer at 120 bp.

5.7 Pool the PCR products of all samples into one tube. Samples that failed to amplify, or amplified only the adapter dimer, as revealed by the gel, can be excluded from the pool.

Size selection

6 Conduct size selection within the range of 400 to 500 base pairs. You can achieve this using a PippinPrep instrument, following the provided guidelines from the manufacturer. Another method involves a prolonged migration on an agarose gel, excising the desired region, and purifying it using a gel extraction kit, as outlined below.

6.1 Prepare a 2.0 % agarose gel using clean TAE buffer and a large comb, with the exact volume adjusted as needed.

Note

Please note that the size of the gel must be sufficient to perform a 3-hour migration, and the amount of library (400 µL library + 100 µL loading dye) loaded should fit within the gel. We usually load 12 large wells, each with ~40 µL of library + dye. There are two methods to achieve this:

1. Using a gel comb with large comb teeth specifically designed for obtaining wider wells in the gel.
2. Using duct tape to create larger teeth on a regular gel comb. This requires some testing, however, to make sure to obtain properly shaped wells.

6.2 Fill the gel rig with clean gel buffer (e.g., TAE).

6.3 Prepare a gel template with 400 µL pooled amplicons + 100 µL clean loading dye (5x).

6.4 Load 3 µL 100 bp ladder in a few wells, leaving a regular interspace.

> **Note**
>
> With the 12-well design mentioned above, we would typically load the ladder in the first and every fifth well, so the wells in between consist of 3 series of 4 wells in which to load the library in step 6.5.
>
> Note that a larger volume of ladder than usual is needed due to the wide wells and the explicit need to clearly see the ladder for gel excision.

6.5 Load as much library as possible in the remaining wells, but without overflowing the wells.

6.6 Migrate the gel at low voltage and for a sufficiently long time to ensure a high-resolution migration. We typically go for [M] 80 V during 03:00:00.

6.7 Check for proper migration by very quick UV exposure and prepare tubes for the gel pieces.

6.8 Cut the 400 - 500 bp region on a UV table using sterile scalpel/razor blades. Minimize UV exposure by only cutting a few gel pieces at a time and individualize each piece in the prepared tubes. Try to remove the empty agarose to limit the amount of gel to be purified and be careful not to include ladder fragments in your excisions.

Safety information

Protect yourself from UV light by wearing appropriate protective clothing and UV protection glasses.

6.9 Purify each gel slice separately using a gel extraction kit (e.g., Monarch DNA Gel Extraction Kit from New England Biolabs). Elute in ~15 - 20 µL of elution buffer and pool elution products into a single tube.

6.10 Accurately measure the total elution volume (in µL, with a pipet) and its concentration (in ng/µL, with a fluorometer, e.g., Qubit, using 1 µL of sample). Multiply both numbers to obtain the amount of DNA in your library (in ng). You can then divide this number by 25 to calculate the elution volume needed to concentrate the library to an optimal 25 ng/µL in the next step. Keep in mind that you might lose 10 - 15 % of your DNA yield during this concentration step and that it is best to have at least 10 µL of library (down to 3-4 ng/µL is still ok).

Purification & Concentration

7 Purify the ligation reaction product using the AMPure XP protocol with a 1:1 ratio and elute in the calculated volume of nuclease-free water.

7.1 Let the AMPure aliquot at Room temperature for 00:30:00 and then gently shake it to resuspend the magnetic beads.

Note

Do not vortex to protect the coating of the beads!

7.2 Add AMPure to your elution following a 1:1 volume ratio. Mix by inverting the tube gently several times and wait 00:05:00. Put the tube on a magnet rack for tubes.

7.3 Wait a couple of minutes until the beads migrate to the tube wall. Then, remove the supernatant by

pipetting.

7.4 Rince the beads twice using 1000 µL freshly prepared 70% ethanol, wait 00:00:30, and remove the ethanol.

7.5 After the second rinsing step, let the beads dry. This may take up to 30-40 minutes, especially in 1.5 mL tubes and without air conditioning. The beads will progressively lose their shine, which will indicate drying.

7.6 Elute the DNA by pipetting the desired volume of water on the beads, to detach them from the tube wall. You should remove the tube from the rack and re-pipet the elution liquid on the beads until all of them are detached.

7.7 Wait a few minutes and put the tube back on the magnet rack. Then wait a few more minutes until the beads get stuck again. Carefully pipet the elution volume (avoiding the beads) in a clean final and properly labeled 1.5 ml tube.

Final quantification

8 Check size selection and library fragment size using gel electrophoresis or a TapeStation/Bioanalyzer/Fragment Analyzer.

9 Quantify the amount of DNA using a fluorimeter (e.g., Qubit).

Illumina Sequencing

10 Proceed to Illumina sequencing or equivalent. DNA quantity and mean fragment length can be used to calculate the molarity of the library.

Note

The sequencing strategy depends on the need of your study, but also on the genome size of your study organism. The bigger the genome, the higher number of reads will be needed to obtain a proper coverage for enough loci across samples included in the library. We have typically used one lane of NextSeq 550 (which yields ~400M reads) for 96-sample libraries in species of 5-10 Gb genomes. The bigger the genomes, the higher the number of reads needed, or the fewer samples to be included in the sequenced library.