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A cost effective protocol for methylome and genome screen using ddRAD and high-throughput sequencing

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We use this protocol and it's working



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Abstract

The protocol describes the preparation of constructing high-throughput sequencing libraries that would allow scanning both the methylome and the genome of a targeted organism. The protocol is mainly designed in relation to breeding activities where one needs to screen a large number of animals. The protocol is a compilation of library construction demonstrated in the following:

Peterson et al 2012

Palaikostas et al 2015

Franchini et al 2017

Marconi et al 2019



Guidelines

Step-by-step procedure captured on these pages:

1. *Set up DNA digestions (96 well plate format)*. Prepare the restriction digest master mix and combine with DNA as indicated under Materials.
2. *Mix well and incubate @ 37°C for 60 minutes*.
3. *Cool to room temperature*.
4. *Add barcodes*. Add 3.000 µL barcode mix per sample (see Barcode Calcs and barcode table on next page).
5. *Mix well and incubate @ 22°C for 10 minutes*.
6. *Add ligase mastermix*. Use the ligation master mix composition listed under Materials (note: "nb double usual" flagged on sheet).
7. *Mix well and incubate @ RT for 2 hours*.
8. *Add 30 µL PB to inactivate*.
9. *Combine samples into library pools*. Use the plate map noted under Materials. Current pages show: Lib 1 wells 1A–6H and Lib 2 wells 7A–12H on Plate 1; Lib 3 wells 1A–6H on Plate 2; Lib 4 wells 7A–10H on Plate 2.

Materials

Reagents and supplies noted on these pages:

- DNA (30 ng/μL; 30 ng per sample)
- Nuclease-free water
- 10X CutSmart Buffer
- Restriction enzymes: Acil (10 U/μL) and NlaIII (10 U/μL)
- Barcode mix (see Barcode Calcs; barcode table provided on these pages)
- rATP (100 mM stock; used to 1 mM in ligation mix)
- T4 DNA ligase
- PB (add 30 μL per sample to inactivate after ligation)
- 96-well plate format for setup

Per-reaction mixes noted:

- Restriction digest master mix per sample (to 6 μL RE digestion volume): 10X CutSmart Buffer 0.600 μL; Acil 0.030 μL; NlaIII 0.030 μL; water 4.340 μL; plus DNA 1.000 μL.
- Ligation master mix per sample (3.000 μL): water 2.550 μL; 10X CutSmart 0.300 μL; rATP (100 mM) 0.120 μL; T4 DNA ligase 0.030 μL.

Barcode list (expanded with information from these pages):

- A full table of Barcode_Id, P1 Barcode, P2 Barcode, Index_i7, Index_i5 is provided across the barcode pages. The two pages shown here contain Ids 92–176.
- Index scheme across the table (as shown):
 - Ids 1–48: Indexi7 = *ATTACCTG*; Indexi5 = *ATAGAGGC*.
 - Ids 49–93: Indexi7 = *TCCGGAGA*; Indexi5 = *TATAGCCT*.
 - Ids 94–144: Indexi7 = *CGCTCATT*; Indexi5 = *CCTATCCT*.
 - Ids 145–176: Indexi7 = *GAGATTCC*; Indexi5 = *GGCTCTGA*.
- P2 Barcode by Id range (observed across pages):
 - Ids 1–24: P2 = AGTCAT.
 - Ids 25–48: P2 = GATCGT.
 - Ids 49–72: P2 = GCATTG.
 - Ids 73–93: P2 = TTAATG.
 - Ids 138–144: P2 = GATCGT. [from this page]
 - Ids 145–168: P2 = GCATTG.
 - Ids 169–176: P2 = TTAATG.
- Additional column noted: “emseq...” identifiers are listed for Ids 157–176 as follows:
 - 157 → emseq378
 - 158 → emseq379
 - 159 → emseq412
 - 160 → emseq414
 - 161 → emseq293
 - 162 → emseq294
 - 163 → emseq300



- 164 → emseq304
- 165 → emseq309
- 166 → emseq313
- 167 → emseq320
- 168 → emseq322
- 169 → emseq326
- 170 → emseq328
- 171 → emseq336
- 172 → emseq337
- 173 → emseq338
- 174 → emseq339
- 175 → emseq346
- 176 → emseq347
- Per-Id P1 Barcode sequences visible on this page (Ids 138–176):
 - 138 → CTAGGAC
 - 139 → AGAGT
 - 140 → ATGCT
 - 141 → GACTA
 - 142 → CAGTCAC
 - 143 → GCTAACA
 - 144 → ACACGAG
 - 145 → CCACGCT
 - 146 → CGTGCGA
 - 147 → GAACAAT
 - 148 → TCAGA
 - 149 → GATCG
 - 150 → CATGA
 - 151 → ATCGA
 - 152 → TCGAG
 - 153 → GTCAC
 - 154 → GCATT
 - 155 → CGATA
 - 156 → CGACAA
 - 157 → CGTATCA
 - 158 → ACTCGACA
 - 159 → TCTTCA
 - 160 → GTACACA
 - 161 → CTCTTCA
 - 162 → CTAGGAC
 - 163 → AGAGT
 - 164 → ATGCT
 - 165 → GACTA
 - 166 → CAGTCAC



- 167 → GCTAACA
- 168 → ACACGAG
- 169 → CCACGCT
- 170 → CGTGCGA
- 171 → GAACAAT
- 172 → TCAGA
- 173 → GATCG
- 174 → CATGA
- 175 → ATCGA
- 176 → TCGAG

Plate/well map (from the two images on these pages):

- Columns shown: Lib, Well, Plate.
- First four entries explicitly include P2 barcode text with Plate number:
 - Lib 1, Well 1A → Plate "1 AGTCAT"
 - Lib 1, Well 1B → Plate "1 GATCGT"
 - Lib 1, Well 1C → Plate "1 GCATTG"
 - Lib 1, Well 1D → Plate "1 TTAATG"
- Subsequent rows for Lib 1 and Lib 2 list Plate as "1"; beginning with Lib 3 rows the Plate value switches to "2".
- Library-to-well coverage visible (updated with these pages):
 - Lib 1: Wells 1E–6H → Plate 1. (This page explicitly shows 6F–6H.)
 - Lib 2: Wells 7A–12H → Plate 1. (This page shows 12D–12H, all Plate 1.)
 - Lib 3: Wells 1A–6H → Plate 2. (This page shows 1A–6H complete.)
 - Lib 4: Wells 7A–10H → Plate 2.
- Plate/well assignments (last page; all entries show Plate 2):
 - Lib 3 → Wells 6B, 6C, 6D, 6E, 6F, 6G, 6H → Plate 2.
 - Lib 4 → Wells 7A, 7B, 7C, 7D, 7E, 7F, 7G, 7H → Plate 2.
 - Lib 4 → Wells 8A, 8B, 8C, 8D, 8E, 8F, 8G, 8H → Plate 2.
 - Lib 4 → Wells 9A, 9B, 9C, 9D, 9E, 9F, 9G, 9H → Plate 2.
 - Lib 4 → Wells 10A, 10B, 10C, 10D, 10E, 10F, 10G, 10H → Plate 2.

Before start

Setup parameters noted on the sheet:

- Reaction Vol. (All): 12 μ L
- Reaction Vol. (RE digestion): 6 μ L
- DNA concentration: 30 ng/ μ L
- DNA per sample: 30 ng
- Restriction enzyme units per μ g DNA: 10
- Enzyme 1: Acil (10 U/ μ L)
- Enzyme 2: NlaIII (10 U/ μ L)
- Number of samples: 192
- % extra for digest: 5%



Barcode annealing

- 1 Resuspend the oligos in 0.25x TE pH 8 to the supplier-defined 100 μ M.

The oligos used can be found in the attached spreadsheet. Those oligos are compatible for digestion with the Acil and MseI restriction enzymes (enzymes with compatible recognition sites can be used as well).



Prepare the annealing buffer as follows:

	A	B
	Stock	mL
	1M Tris pH7.6	1,00
	2M NaCl	3,75
	H ₂ O	5,25
	Total	10,00

10X AB buffer.

For each top and bottom barcode, annealing uses the volumes specified below. PCR 200 μ l volume tubes are used for each barcode.

	A	B
	Reagent	Volume (μL)
	10X AB buffer	20
	100 μ M top oligo	25
	100 μ M bottom oligo	25
	MilliQ H ₂ O	130
	Total	200

Annealing reaction for each barcode.



Insert the PCR tubes in a PCR cycle set up at 96°C. The temperature should gradually decline to RT over a time period of 8 hours.

Those should comprise a stock 10µM. For library constuction it is advised to prepare 1 µM working solutions.

Untitled section

2

Untitled section

3

Barcode combinations

4 The barcode combinations are designed to digest ~ 20ng of template DNA.

A	B
Reagent	Volume (µl)
10X NEB Buffer 2	0,3
Barcode P1 Acil (1 uM)	0.216
Barcode P2 Msel (1.0 uM)	0,216
MilliQ H2O	2,268
Total:	3,0000

Barcode combinations for one reaction

The above should be repeated for each unique combination of P1 and P2 adaptors.

The above volumes should be multiplied by the number of reactions needed. It is advised to prepare for more than one reaction.



As those barcodes have to be kept for long storage in the freezer and thawing/freezing cycles can negatively affect them setting up for e.g. 20 reactions is a good compromise. This would allow for 20 batches of libraries.

It is more efficient to set up the P1 and P2 combinations in the form of a PCR 96-well plate.

Template DNA

- 5 Dilute the template DNA from each sample to a concentration of 20 ng/μl.

Add 1 μl of the diluted DNA to a separate well of a PCR plate.

Set up master mix for the DNA digestions (96 well plate format)

- 6 The protocol has tested with reagents from New England Biolabs. been Prepare the following master mix for 96 samples.



A	B
Reagent	Volume (μl)
10X Cutsmart Buffer	60,5
Acil (10U/μl)	2
MseI (10U/μl)	2
MilliQ H2O	439,5
Total	504

The protocol has been tested with reagents from New England Biolabs (NEB)

DNA digestion (96 well plate format)

- 7 Add 5 μl of the above mastermix to each well of the PCR plate that contains the template DNA.



Mix well and incubate @ 37°C for 60 minutes.

It is advised to use a PCR cycle for the digestion.



Before proceeding leave the plates to room temperature for 5min.

Barcode ligation

8 Add 3 μ l of a unique barcode mix (check step 3 for preparing the barcode combinations) to each well of previously digested DNA



9 Mix well and incubate at room temperature for 10 minutes.

10 Add 3 μ l to each well of the following ligation mastermix

	A	B
	Reagent	Volume
	10X CutSmart	30,2
	rATP (100mM)	12,1
	T4Ligase	2,0
	MilliQ H2O	258
	Total	302,4

11 Mix well and incubate at room temperature for 2 hours.

Template purification

12 Purify the teplate with a QIAquick PCR Purification Kit for PCR Cleanup



Add 30 μ l of the PB buffer to each well to stop the ligation

Combine samples to library pools

Before following the QIAquick PCR Purification Kit protocol add 5 μ l 3M NaAce pH5.2 to each library pool

Add 17 μ l of EB to each library pool (EB can be preheated to 55 °C before elution).



Centrifuge at 14,000 g for 1min.

Repeat the process by adding another 17µl of EB.

13 Further purification with AMPure XP beads

Add 1.1X volume of beads (33 ul) to each library

10 min in the bench

2 min in magnet

Remove supernatant

Add 250ul fresh 70% ethanol (2 times)

Elute each library in 20ul EB

It is possible to perform purification only with beads. However, since those are typically more expensive, using the QIAquick PCR Purification Kit for PCR Cleanup is a more economical option.

Gel electrophoresis

14 Prepare a 1.1% agarose gel using 1X TAE buffer.

It is advised to prepare the gel in advance.

We have best results when gel apparatus and buffer has been kept overnight in the fridge.

Electrophoresis is run with the apparatus fitted in a box containing ice to minimize template loss.

During electrophoresis the following voltages are used:

45V for 3min

60V for 3min

75V for 3min

90V for 3min

100V for 60min

15 Cut gel slices from each library corresponding to 300 - 600 bp of the template. This size range can be adjusted depending on the used restriction enzymes. It is advised, though, to be within 200-700 bp.

16 Purify each gel slice with the QIAquick Gel Extraction Kit

Each library is eluted with 40 µl of EB buffer

PCR amplification

17 PCR primers with unique i5 and i7 indexes are used for the amplification.



The indexed primers that have been tested can be seen in the attached spreadsheet



Indexes.xlsx 9.5KB

Amplification can be tested at various ranges of PCR cycles.

Even though the library construction protocol allows for removing the PCR duplicates bioinformatically it is best to use as few cycles as possible. This highly depends on the amount of templated DNA that was used.

In our case using 48 samples per library (20ng each) we usually conduct 12-13 cycles.

The amplification reaction contains:

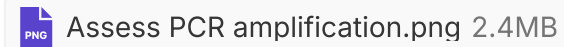
A	B
Reagent	Volume
Phusion High-Fidelity Master Mix	12,5
Primer mix (10 µM)	0,5
MilliQ H2O	11,0
Library template	1,0
Total	25

18 It is important to assess the amplified product in comparison to the template library.

A gel electrophoresis can be used for this.

In a 1.5% agarose gel with 1X TAE, the amplified libraries and the corresponding templates can be run. An amplified product at least twice as bright as the template should be observed.

An example can be seen in the attached image



- 19 Following the determination of the optimal number of PCR cycles a large volume PCR is conducted using the reaction shown under step 17.

The only difference is that multiple reactions per library are prepared. Commonly, 12-24 replicate reactions per library are prepared.

- 20 Following PCR amplification, all reactions corresponding to the same library are pooled.

As long as the constructed libraries have unique combinations of inner barcodes and indices can be pooled and sequenced in a single lane. This is a major determinant of the overall cost per sample.

The pool is initially cleaned with the QIAquick PCR Purification Kit for PCR Cleanup as shown in step 12

Typically the pool is eluted to 60 μ L with the EB buffer.

Following purification with AMPure XP beads is performed as in step 13.

It is possible to perform purification only with beads. However, since those are typically more expensive, using the QIAquick PCR Purification Kit for PCR Cleanup is a more economical option.

Typically, the final pool is eluted to 30 μ L with the EB buffer.

Sequencing library

- 21 It is advised to quantify the library with a Qubit fluorometer using HS assay kit.

In addition to make sure that the pool doesn't contain primer-dimers the library can be checked through either the Agilent TapeStation or with gel electrophoresis.



An example of a gel image from a successfully sequenced library can be seen in the attached



Library_for_sequencing.png 1MB