A streamlined ddRAD tag protocol for use with the Ion Torrent sequencer, as a versatile probe for populations, genetics and genomics

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Abstract

Double digested Restriction-site Associated DNA (ddRAD) sequencing is a powerful approach for identifying and analyzing genome-wide SNP variation. Many studies have now used ddRAD protocols for population genetic studies. Here we have adapted the protocol from Peterson (2012) for Ion Torrent sequencing to produce a significantly streamlined workflow capable of having fully sequenced ddRAD libraries in two days. A reduced number of steps for producing a ddRAD library is achieved through the use of a unidirectional double digestion-ligation reaction with adaptors having fusion barcodes in the 5' and 3' ends. This also allows for the immediate pooling of sets of compatible barcoded samples for downstream processing. The A-adaptor contains the standard Ion Torrent barcodes and a key sequence, while the P1-adaptor contains a divergent Y-adaptor with a paired-end code for increased multiplexing. The described system and adaptors are compatible with the SbfI, PstI and NsiI restriction endonucleases in the A adaptor, while the P1-adaptor has compatible overhangs with NdeI or MseI, and, MspI or HpaII. The later two restriction enzymes were chosen as isoschizomers for their differentially sensitivity to CpG methylation, thus allowing the use of this protocol for epigenetic ddRAD profiling at genomic loci with 5-methylcytosine and 5-hydroxymethylcytosine modifications. This protocol takes an advantage in that the Ion Torrent platform has scalability with different sequencing chip sizes, and that the protocol has a range of compatible restriction endonucleases with different motif lengths. This ensures a versatile, cost-effective and flexible method to which you can tune the number of ddRAD loci being profiled, for both small and large genomes, with relative speed and with the ability to observe the performance from small pilot scale reactions.

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Guidelines

General guidelines:

Set up all ddRAD digestion-ligation reactions in an amplification free environment. Use appropriate pipetting practices to minimize barcode cross contamination. A post-amplification laboratory area should be used for processing all amplified libraries.

Tuning the number of genomic loci being sequenced:

An *in silico* digestion (eg. Rombauts et al., 2003; bioinformatics.org, or biopython.org using the Bio.Restriction package), of a suitable reference genome, can be used to guide and tune the number of genomic loci being sequenced and to examine allelic dropout. Plastid and mitochondrial sequences can also be used for predicting the number of organellar genome sequences also being sampled, so as to either include or minimize these sequences from the variant discovery. Thus an *in silico* digestion can be used to select enzymes that are most appropriate for ddRAD sequencing, and to scale and determine the expected frequency of cutting within the genome. Thus this utility can be used to fine tune the number of fragments, together with the number of individuals being sequenced for use on the different Ion Torrent chips. Note that the adapters comprise a total of 103 bp and this length must be added to total size of the targeted fragment length during an in silico size simulation (e.g. for sequence lengths of 100-300 bp, a range of 203-403 bp would be selected).

Magnetic bead-based DNA purification and separation:

This protocol requires the use of rare earth magnets for bead-based DNA purification and separation. Use individual 1.5 ml Eppendorf magnetic racks, a magnetic strip for PCR strips (8 well), and/or a 96-well PCR magnetic plate for separation. Care should be taken with the use of these magnets to prevent personal injury.

References:

1. **Dabney, J. and Meyer, M.** (2012). Length and GC-biases during sequencing library amplification: A comparison of various polymerase-buffer systems with ancient and modern DNA sequencing libraries. Biotechniques 52: 87–94.
2. **Peterson, B.K., Weber, J.N., Kay, E.H., Fisher, H.S., and Hoekstra, H.E.** (2012). Double Digest RADseq: An Inexpensive Method for De Novo SNP Discovery and Genotyping in Model and Non-Model Species. PLoS One 7: e37135.
3. **Rombauts, S., Van De Peer, Y., and Rouzé, P.** (2003). AFLPinSilico, simulating AFLP fingerprints. Bioinformatics 19: 776–7

Before start

Order single stranded oligo nucleotides:

Since the Y adaptors are used more frequently they can be ordered in the scale of 200 nmol (high

purity). Y adaptors are designated as P1 by Ion Torrent from Thermofisher.

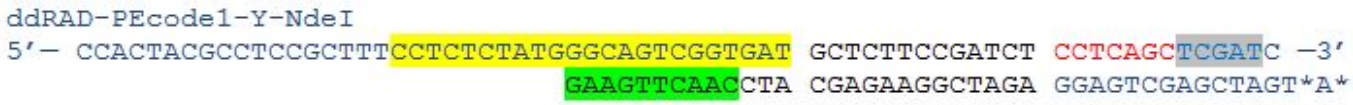
The following oligos permit the production of adaptors which have compatible overhangs for both *MspI and HpaII* (4 bp recognition sequences).

P1-Y adaptors for *MspI* and *HpaII* provided without a combinatorial multiplex code and paired-end sequencing (5' to 3'):

ddRAD-PE-Y-MspI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAG
ddRAD-PE-Y-MspI-bottom	C*G*CTGAGGAGATCGGAAGAGCATCCAACTTGAAG

Or P1-Y adaptors with a multiplex code and paired-end sequencing:

eg. a depiction of the ddRAD divergent Y adaptor used in this study.



Yellow/green - divergent Y adaptor

Yellow - P1 amplification primer binding site

Blue/grey - 5 nt combinatorial tag

Red - Nt.BbvCI nicking site for paired-end sequencing

* protected phosphorothioate bonds

ddRAD-PEcode-1-Y-MspI_top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCACGCAG
ddRAD-PEcode-1-Y-MspI_bottom	C*G*CTGCGTGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-2-Y-MspI_top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCTACGAG
ddRAD-PEcode-2-Y-MspI_bottom	C*G*CTCGTAGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-3-Y-MspI_top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCGTAGAG
ddRAD-PEcode-3-Y-MspI_bottom	C*G*CTCTACGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-4-Y-MspI_top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCGATACG
ddRAD-PEcode-4-Y-MspI_bottom	C*G*CGTATCGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG

ddRAD-PEcode-5-Y-MspI_top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCTATATG
ddRAD-PEcode-5-Y-MspI_bottom	C*G*CATATAGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-6-Y-MspI_top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCTCGTAG
ddRAD-PEcode-6-Y-MspI_bottom	C*G*CTACGAGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG

The following oligos permit the production of adaptors which have compatible overhangs for both *NdeI* (a 6 bp recognition sequence), and *MseI* (a 4 bp recognition sequence).

P1-Y adaptors for *NdeI* and *MseI* overhangs provided without a multiplex code and paired-end sequencing:

ddRAD-PE-Y-NdeI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGC
ddRAD-PE-Y-NdeI-bottom	T*A*GCTGAGGAGATCGGAAGAGCATCCAACTTGAAG

Or P1-Y adaptors for *NdeI* and *MseI* overhangs with a multiplex code and paired-end sequencing:

ddRAD-PEcode-1-Y-NdeI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCTCGCTC
ddRAD-PEcode-1-Y-NdeI-bottom	T*A*GAGCGAGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-2-Y-NdeI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCGACTAC
ddRAD-PEcode-2-Y-NdeI-bottom	T*A*GTAGTCGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-3-Y-NdeI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCGTCAGC
ddRAD-PEcode-3-Y-NdeI-bottom	T*A*GCTGACGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-4-Y-NdeI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCATACGC
ddRAD-PEcode-4-Y-NdeI-bottom	T*A*GCGTATGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-5-Y-NdeI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCTACATC
ddRAD-PEcode-5-Y-NdeI-bottom	T*A*GATGTAGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG
ddRAD-PEcode-6-Y-NdeI-top	CCACTACGCCTCCGCTTTCCTCTCTATGGGCAGTCGGTGATGCTCTTCCGATCTCCTCAGCATCGTC
ddRAD-PEcode-6-Y-NdeI-bottom	T*A*GACGATGCTGAGGAGATCGGAAGAGCATCCAACTTGAAG

Barcoded A adaptors compatible with *SbfI*, *PstI* and *NsiI* overhangs

A nucleotide g is placed between the barcode adaptor and the overhang to ensure a unidirectional ligation permitting the use of *SbfI*, *PstI* or *NsiI* in a restriction and ligation reaction. This nucleotide can

be trimmed from the overhang tag for bioinformatic purposes.

```
>ddRAD-SbfI-13
----CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACGATgTGCA
T*T*GGTAGAGTAGGGACGCACAGAGGCTGAGTCAGATTGCCTGCTAc
```

* represents protected bonds. Overhangs and restriction sites (also appearing in the double stranded regions of adaptors), are protected internally with phosphorothioate bonds.

ddRAD-SbfI-13-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACGATgTGCA
ddRAD-SbfI-13-bottom	cATCGTCCGTTAGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-14-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTCGATgTGCA
ddRAD-SbfI-14-bottom	cATCGACACTCCAACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-15-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCGATgTGCA
ddRAD-SbfI-15-bottom	cATCGACCTCTAGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-16-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACGATgTGCA
ddRAD-SbfI-16-bottom	cATCGTCATCCAGATGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-17-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTCGTCGATgTGCA
ddRAD-SbfI-17-bottom	cATCGACGAATAGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-18-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCGATgTGCA
ddRAD-SbfI-18-bottom	cATCGCAATTGCCTCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-19-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACGATgTGCA
ddRAD-SbfI-19-bottom	cATCGTCCGACTAACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-20-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCAT*C*GATgTGCA
ddRAD-SbfI-20-bottom	cAT*C*GATGGATCTGCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-21-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCAATTACGATgTGCA
ddRAD-SbfI-21-bottom	cATCGTAATTGCGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-22-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCGATgTGCA
ddRAD-SbfI-22-bottom	cATCGCGTCTCGAACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-23-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACGATgTGCA
ddRAD-SbfI-23-bottom	cATCGTTCGTGGCACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-24-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTCATTCGATgTGCA
ddRAD-SbfI-24-bottom	cATCGAATGAGGTTCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-25-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACGATgTGCA
ddRAD-SbfI-25-bottom	cATCGTATCTCAGGCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-26-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCGATgTGCA
ddRAD-SbfI-26-bottom	cATCGAGGTTGTAACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-27-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCGATgTGCA
ddRAD-SbfI-27-bottom	cATCGCGGATGGTTCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-28-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGATC*C*GGAAT*C*GATgTGCA
ddRAD-SbfI-28-bottom	cAT*C*GATTC*C*GGATCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-29-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACCACTCGATgTGCA
ddRAD-SbfI-29-bottom	cATCGAGTGGTCGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-30-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGGTTAT*C*GATgTGCA
ddRAD-SbfI-30-bottom	cAT*C*GATAACCTCGCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-31-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCAAGCTGCGATgTGCA
ddRAD-SbfI-31-bottom	cATCGCAGCTTGGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T

ddRAD-SbfI-32-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACACGATgTGCA
ddRAD-SbfI-32-bottom	cATCGTGTGTAAGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-33-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTCATTGAACGATgTGCA
ddRAD-SbfI-33-bottom	cATCGTTCAATGAGAACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-34-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCATCGTTCGATgTGCA
ddRAD-SbfI-34-bottom	cATCGAACGATGCGACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-35-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCCATTGTCGATgTGCA
ddRAD-SbfI-35-bottom	cATCGACAATGGCTTACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-36-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGGAATCGTCGATgTGCA
ddRAD-SbfI-36-bottom	cATCGACGATTCCTTCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-37-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGAGAATGTCGATgTGCA
ddRAD-SbfI-37-bottom	cATCGACATTCTCAAGCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-38-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGGACGGACGATgTGCA
ddRAD-SbfI-38-bottom	cATCGTCCGTCCTCCACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-39-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAATCGGCGATgTGCA
ddRAD-SbfI-39-bottom	cATCGCCGATTGTTACTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-40-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACATAAT*C*GATgTGCA
ddRAD-SbfI-40-bottom	cAT*C*GATTATGTCAGCTGAGTCGGAGACACGCAGGGATGAGATGG*T*T
ddRAD-SbfI-41-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCACTTCGCGATgTGCA
ddRAD-SbfI-41-bottom	cATCGCGAAGTGGAACTGAGTCGGAGACACGCAGGGATGAGATGGT*T*
ddRAD-SbfI-42-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACGAAT*C*GATgTGCA
ddRAD-SbfI-42-bottom	cATC*G*ATTCGTGCTCTGAGTCGGAGACACGCAGGGATGAGATGGT*T*
ddRAD-SbfI-43-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGACACCGCGATgTGCA
ddRAD-SbfI-43-bottom	cATCGCGGTGTCAAGCTGAGTCGGAGACACGCAGGGATGAGATGGT*T*
ddRAD-SbfI-44-top	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGGCCAGCGATgTGCA
ddRAD-SbfI-44-bottom	cATCGCTGGCCTCCAACTGAGTCGGAGACACGCAGGGATGAGATGGT*T*

ddRAD library amplification and enrichment primers:

A-amp	CCATCTCATCCCTGCGTGTCTCCGACTCAG
P1-amp	CCACTACGCCTCCGCTTTCCTCTCTATG
paired-end_sequencing_primer	C*C*A*T*CTCATCCCTGCGTGTCTCCGAC

qPCR primers and probe:

Ion_left	CCACTACGCCTCCGCTTT
Ion_right	ATCTCATCCCTGCGTGTCTC
Ion_probe_5'FAM, 3'BHQ1	/56-FAM/CTCTATGGGCAGTCGGTGAT/3BHQ_1/

Materials

- PstI-HF RS3140RS by New England Biolabs
- SbfI-HF RS3642S by New England Biolabs
- NsiI-HF RS3127S by New England Biolabs
- MspI R0106S by New England Biolabs
- MseI R0525S by New England Biolabs
- HpaII R0171S by New England Biolabs
- T4 DNA ligase M0202S by New England Biolabs
- Nuclease free water B1500S by New England Biolabs

  2% Agarose Pippin Prep cassette - external marker B CSD2010 by sage science

  1.5% Agarose Pippin Prep cassette - external marker A CSD1510 by sage science

  Ion PGM™ Calibration Standard A27832 by Thermo Fisher Scientific
- 10 mM Adenosine 5-Triphosphate (ATP) PO756S by New England Biolabs
- NEBuffer™ 4 (NEB4) B7004S by New England Biolabs
- NEBuffer™ 2 (NEB2) B7002S by New England Biolabs
- BSA, Molecular Biology Grade 20 mg/ml B9000S by New England Biolabs
- NEBuffer™ 1 (NEB1) B7001S by New England Biolabs
- NEBuffer™ 3 (NEB3) B7003S by New England Biolabs

Protocol

Anneal the adaptors

Step 1.

Single-stranded oligos need to be annealed into adaptors by pairing with their appropriate partner oligo before ddRAD ligation.

Prepare annealing buffer stock (10X):

100 mM Tris HCl, pH 8

10 mM EDTA

500 mM NaCl

100X TE pH 8	2.5	ml
NaCl (58.44 g/mol)	0.73	g

nuclease free H_2O	Fill to	25	ml

REAGENTS

Nuclease free water B1500S by New England Biolabs

Tris-EDTA buffer solution (100X) pH 8.0 T9285-100ML by Sigma Aldrich

Anneal adaptors

Step 2.

Dilute oligos and create the adapters. Combine each complementary pair, as detailed in the materials section, in 1:1 ratios with1X annealing buffer in separate PCR tubes or strips.

Oligo top (100 µM)	40 µl
Oligo bottom (100 µM)	40 µl
10x annealing buffer (step 1)	10 µl
nuclease free H_2O	10 µl

NB. The Y adaptor is designated as the P1 adaptor by Ion Torrent. The A adaptors contain the barcodes and a 3' TGCA overhang. Barcoded adaptors should be pipetted carefully to avoid cross contamination.

REAGENTS

Nuclease free water B1500S by New England Biolabs

Anneal the adaptors

Step 3.

Use a thermocycler to anneal the adaptors by incubating complementary oligos:

- 97.5 °C for 2.5 minutes
- cool at a rate of not greater than 3 °C per minute
- when the solution reaches a temperature of 21°C, hold at 4 °C

Prepare working stocks of the adaptors

Step 4.

Prepare appropriate working stock concentrations of annealed adapters

- dilute the P1-Y adaptors 10-fold to 8 µM
- dilute the barcoded A adaptors 4-fold to 20 µM

Store diluted A adaptors arranged in strips (aliquoted) at -20 °C. P1-Y adaptors can be stored in 1.5 ml Eppendorfs.

Digestion-ligation

Step 5.

Calculate quantity and quality of the DNA.

Use a fluorometric assay to quantitate the DNA samples (eg. Qubit Broad Range Assay; Thermofisher). Sample concentrations above 5 ng μl^{-1} are preferred.

NB. Accurate measurements of DNA quantities are important for sample normalization since the barcoded samples are pooled together at an early stage. Fluorometric assays are preferred for DNA quantitation since nanodrop values are prone to variability.

REAGENTS

Qubit™ dsDNA BR Assay Kit Q32853 by Thermo Fisher Scientific

Digestion-ligation

Step 6.

Prepare the DNA samples in PCR strips with quantities of 200 ng in a maximum volume of 40 µl nuclease free H_2O.

NB. Input amounts of DNA can be varied from 50 ng to 500 ng. Adjust volumes as necessary.

AMOUNT

200 ng Additional info: sample DNA

Digestion-ligation

Step 7.

Digestion-ligation is performed in a volume of 51 µl.

Calculate a master mix to dispense the digestion-ligation mixture to each sample tube containing the sample DNAs. Use PCR strips or a PCR plate to perform the individual digestion-ligation reactions. Please also see the NEB buffer compatibility table below (eg. use NEB4 for digests utilizing both *PstI*-HF and *NdeI*).

- 5.1 µl of 10X NEB buffer
- 0.8 µl ATP (10 mM)
- 1 µl P1-Y adapter
- 1 µl - 100 U T4 DNA Ligase (NEB)
- 1 µl - 20 U Restriction enzyme 1 (eg. *SbfI*-HF, *PstI*-HF or *NsiI*-HF from NEB)
- 1 µl - 20 U Restriction enzyme 2 (eg. *NdeI*, *MspI*, *MseI* or *HpaII* from NEB)
- 0.25 µl BSA (20 mg ml^{-1}; **necessary only for reactions with *MseI***; final concentration 100 µg ml^{-1} BSA)
- Enough water to ensure a final volume of 50 µl for each sample

Vortex the master mix and dispense 10 µl of the master mix to each tube. Briefly mix the sample, and then spin down the PCR strips before proceeding to add the barcoded A-adaptor.

Information on the compatibility of enzymes and the NEB buffer systems:

Enzyme	NEB Catalog #	cutter	site	adaptor	NEB1	NEB2	NEB3	NEB4	CutSmart [1]	BSA	inactivation
SbfI-HF	R3140S	8 bp	CC_TGCA^GG	A	50%	NR	NR	**100%**	100%	no	80 ºC
PstI-HF	R3140S	6 bp	C_TGCA^G	A	10%	75%	50%	**100%**	100%	no	not inactivated
NsiI-HF	R3127S	6 bp	A_TGCA^T	A	NR	NR	NR	NR	**100%**	no	80 ºC
NsiI	R0127S	6 bp	A_TGCA^T	A	10%	**75%**	**100%**	25%	25%	no	65 ºC
NdeI	R3140S	6 bp	CA^TA_TG	P1-Y	75%	**100%**	75%	**100%**	100%	no	65 ºC
MseI	R0525S	4 bp	T^TA_A	P1-Y	75%	**100%**	75%	**100%**	100%	yes	65 ºC
MspI	R0106S	4 bp	C^CG_G	P1-Y	75%	**100%**	50%	**100%**	100%	no	not inactivated
HpaII	R0171S	4 bp	C^CG_G	P1-Y	**100%**	50%	10%	**50-100%***	**100%**	no	80 ºC

NR - not recommended; * NEB4 buffer activity is either reported at 50% or 100%. [1] NEB changed the name of NEBuffer 4 to CutSmart® Buffer after BSA was added and DTT was removed.

We routinely use the individual NEB buffers with excellent outcome. Less frequently we have used the NEB CutSmart buffer. Some users report difficulties in preparing ddRAD libraries with this buffer. Thus we tend to avoid the use of this universal buffer system. Note that the T4 DNA ligase (NEB M0202L) is optimally active in all buffers, provided that there is the inclusion of ATP into the reaction. Check buffer compatibilities and observe guidelines when using restriction endonucleases other than those used above, so as to ensure reduced star activity.

HpaII has sensitivity to methylation. Prepare suitable control reactions with sample DNAs. Two examples are (1) either the use of Whole Genome Amplified DNAs taken from a split of the original sample, and amplified to remove all methylation, or (2) alternatively prepare a comparative sample reaction using the *MspI* restriction nuclease instead of *HpaII*.

Please note:

- ATP is labile. We aliquot into convenient user amounts and store these at -20 ºC (eg. into 16-18 µl amounts in separate PCR tubes) to prevent freeze-thaw cycles.
- Digestion-ligation reactions can be alternatively carried out in smaller volumes (eg. 20-40 ul). Adjust the above volumes as necessary

REAGENTS

- T4 DNA ligase M0202S by New England Biolabs
- 10 mM Adenosine 5-Triphosphate (ATP) PO756S by New England Biolabs
- BSA, Molecular Biology Grade 20 mg/ml B9000S by New England Biolabs

Digestion-ligation

Step 8.

Add 1 ul of the barcoded adaptor to each tube. Carefully cap the PCR strip or seal the PCR plate (ensuring no sample cross-contamination), and mix for 10-15 secs. Spin the PCR strips or PCR plate down in a benchtop centrifuge.

Digestion-ligation

Step 9.

Incubate in a thermocycler

Unidirectional digestion-ligation is performed in a thermal cycler with digestion with times ranging from 30 minutes to 3 hours at 37 ºC.

- 30 minutes digestion-ligation at 37 ºC (this step can be up to 3 hours)
- 10 minutes at 65 ºC (to kill the T4 DNA ligase activity)
- Cool slowly at 2 ºC every 1.5 minutes
- Keep samples at 4 ºC or store at -20 ºC (for longer periods)

Pool barcoded samples and clean the digestion-ligation

Step 10.

Pool the differently barcoded samples in appropriate or equal volumes.

Pooling samples with compatible barcodes into a 1.5 ml Eppendorf provides a quicker and cheaper option than indivudally cleaning each reaction.

Optional - split a portion of the pooled digestion-ligations and store that portion at -20 °C. This option provides a backup point for the pooled or individual samples. This is especially handy if you are performing the ddRAD protocol the first time, or if you should experience difficulties in the subsequent Ampure XP cleanup (step 11), or in the size selection (step 12).

Clean the pooled library with Ampure XP

Step 11.

Make a fresh batch of 70% ethanol (for the wash steps) and follow the protocol below to use Ampure XP in cleaning the library.

Note that the ratio of Ampure XP to sample can be varied to suit the amplicon target length. Shorter fragments can be retained with higher ratios of Ampure XP to sample. A 1.1X volume of Ampure XP to sample will remove the majority of DNA fragments smaller than 150 bp.

Clean the size selected library with Ampure XP (one-sided clean up protocol for removal of short fragments and adaptors):

- Make sure the Ampure XP beads (Agencourt) are at room temperature and are thoroughly mixed together

- If your sample volume is large, split into two tubes
- Add 1.1X volumes of Agencourt AMPure XP beads to the sample and mix by pipetting up and down
- Incubate for 5 minutes at room temperature
- Pulse-spin the tube and place in a magnetic rack for 3 minutes, or until the beads have collected to the tube wall and the solution is clear
- Carefully remove and discard the supernatant without disturbing the beads
- Keep the tube on the magnet and add 500 µl of freshly prepared 70% ethanol (NB. keep the stock ethanol air tight)
- Incubate at room temperature for 30 seconds, and make the magnetic particles swim by rotating the tube on the rack
- Let them accumulate to the magnet and then carefully discard the supernatant
- Repeat the wash with another 500 µl of 70% ethanol, swim the beads and let them collect again
- Pulse-spin the tube, return to the magnet, let the beads collect again, and remove any residual ethanol with a 200 µl pipette tip
- Keeping the tube in the magnetic rack, with the cap open, let the beads air dry for 5 minutes at room temperature (or wait till evaporation is complete)
- Resuspend the beads in 25 µl of nuclease free water or 1X low TE Buffer
- Pulse-spin the tube or PCR strip down, return it to the magnet, and wait till the solution clears
- Collect the supernatant into a new tube (combine the supernatants of the same pool)
- ***Optional -*** repeat the magnetic bead capture on the magnetic rack to remove any residual beads, and remove the sample to a new tube

Tip - when pipetting the final supernatant into a new Eppendorf, withdraw the sample into the pippette tip and keep the pipette against the magnet in the tube. Slowly pipette out the supernatant, being carefull to discard the remaining 1 µl, together with the tip containing any residual magnetic beads. Also note these video guidelines for working with Ampure XP beads:

Optional - if the Ampure XP causes excessive sample clumping that does not separate into the smaller magnetic particles in the ethanol washes, utilize a spin column purification kit to purify the pooled library instead of the Ampure XP method (eg. the QIAquick PCR Purification Kit; a capacity of 10 µg; Catalogue # 28104). Excessive Ampure-DNA clumping is observed in samples with high molecular weight DNAs, or with samples that have excessive polysaccharides and impurities. The later two are detrimental to the efficient purification and elution of the DNA libraries.

NB. Since pure ethanol is hygroscopic, prepare fresh 70% ethanol daily for daily use.

REAGENTS

Agencourt Ampure XP A63880 by Beckman Coulter

Nuclease free water B1500S by New England Biolabs

Ethanol (molecular biology grade, ≥99.8%) 51976-500ML-F by Sigma Aldrich

Size select the library with the Pippin Prep

Step 12.

Size select the library with the Pippin Prep/Blue Pippin

The adapters are a total 103 bp long and this length must be added to the total size of the targeted fragment length (e.g. for sequence lengths of 100-300 bp, a range of 203-403 bp should be selected with the appropriate separation cassette). Follow the Pippin Prep manual and load your samples onto a 2% gel cassette with marker B, or a 1.5% cassette with marker A. Seal the collection ports with a plastic seal to prevent overflow and prevent sample wicking (score a few millimeters around the perimeter of the elution port with a scalpel blade). If necessary program the Pippin Prep for multiple size selections, or alternatively combine togther a manual size selection to make more than 3 library size fractions.

Caution - wear protective gloves and clothing since, and dispose of these cassettes appropriately since they contain ethidium bromide).

- When the run is completed or paused, unseal the ports and remove the fractionated sample to a new Eppendorf Lo-Bind DNA tube.
- Wash the elution port with 40 µl of fresh 1X TE buffer containing 0.1% Tween 20. Ensure the inside of the port is thoroughly rinsed.
- Leave the 1X TE Tween solution in the port for 1 minute and then combine the port washes into the sample.
- Repeat the rinsing of the port with 40 µl, and combine all the sample elution washes from that port.
- Measure and record the total wash-eluted volume on the Eppendorf tube.

Optional - reseal the port, continue the Pippin Prep run, and collect the second or third sample size fractionation into new Eppendorf tubes.

Tip - use narrow pipette tips to load/unload/wash the Pippin Prep ports (eg. Biotix 100 µl filter tips) .

Alternative - size selection can also be performed with alternative agarose gel size selection (eg. Clonewell II system; Thermofisher Scientific).

REAGENTS

2% Agarose Pippin Prep cassette - external marker B CSD2010 by sage science

1.5% Agarose Pippin Prep cassette - external marker A CSD1510 by sage science

Tween 20 P9416-50ML by Sigma Aldrich

Tris-EDTA buffer solution (100X) pH 8.0 T9285-100ML by Sigma Aldrich

Clean the size selected library

Step 13.

Clean the size selected library with Ampure XP.

Use the combined wash-eluted volume to determine the Ampure XP volume for purification.

Follow the procedure **as in step 11**, and elute the purified DNAs in 25 µl nuclease free H_2O.

Prepare amplification reactions for enrichment of the ddRAD library

Step 14.

Create reactions for the ddRAD library amplification with Platinum PCR SuperMix High Fidelity polymerase. The library is amplified to produce sufficient quantities and enrich the number of fragments with a A and Y-P1 end.

Split the sample and in a single PCR tube add together:

- 12.5 µl DNA from the purified sample from the size selection
- 100 µl **Platinum® PCR SuperMix High Fidelity**
- 10 µl primer mix (or 5 µl of A and P1)
- 7.5 µl of H_2O to make the total volume 130 µl

NB. The Phusion HF polymerase, which has high fidelity, is not a recommended alternative in this step. AccuPrime Pfx SuperMix, which has low and stable GC bias, is however an alternative (Dabney and Meyer, 2012).

REAGENTS

Platinum PCR SuperMix 11306-016 by Thermo Scientific

Amplify the ddRAD library

Step 15.

Amplify both reactions in a thermocycler with the following PCR conditions:

- 95 °C for 5 minutes; 1 cycle; then 12 cycles of
- 95 °C for 15 sec;
- 58 °C for 15 sec;
- 70 °C for 1 min;
- then hold at 4 °C

The number of cycles can be varied (8 - 16 cycles), due to the numbers of fragments generated. This is dependent on the genome size, the use of different enzyme sites and the resulting size fractionation (ie. tuning). The number of cycles should be sufficient to generate the library, and be measurable on a Bioanalyzer, but not overly cycled.

Clean the amplified ddRAD library

Step 16.

Clean the amplified library with Ampure XP

Combine the respective split reactions and determine the Ampure XP volume ratio for purification.

Follow the procedure **as in step 11**, and elute the purified DNAs in 25 µl nuclease free H_2O.

Quantify and qualify the amplified ddRAD library

Step 17.

Quantify and qualify the amplified ddRAD library, and calculate the Template Dilution Factor (TDF).

- Dilute a portion of the sample 1:5 and also 1:10.
- Run 1 µl of each of these on a Bioanalyser HS DNA chip or a suitable fragment analyser (eg. provide brand)
- Alternatively use a qPCR with a standard curve (with qPCR primers provide in our Materials section), or digital PCR, to quantitate the molarity of ddRAD library.
- Follow the quidelines as set out in the Ion Torrent user manual library quantitation.

- Calculate the TDF from the peak area as determined by the Bioanalyzer on a HS DNA chip, or with digital PCR/qPCR, and follow the Ion Torrent One Touch, Chef or Isothermal Amplifcation guidelines for determining the targeted TDF prior to templating ISPs.

Library quality and quantity should be assessed by a fragment analyzer like the Agilent Bioanalyzer. This can provide valuable information on library size selection and the presence of adaptors.

Sequence the ddRAD library

Step 18.

Sequence the ddRAD library.

If you do not have a reference genome, include the Ion Calibration Standard when sequencing (catalogue # A27832) provided by Thermofisher Scientific. Spike this into a designated portion of the sample immediately prior to preparing the ISPs for sequencing.

Consult the Ion Torrent mannuals and use the recommended TDF as input (eg. Ion Chef; 40-80 pM using the Ion Chef with the HiQ chemistry). Use lower levels of the targeted TDF first (eg. 40 pM). The TDF and input volumes can vary between instrumentation (ie. the Ion Chef, Ion One Touch or Ion Isothermal Amplification kit for 500 bp reads). The anticipated TDF can also have dependencies on the chemistry type being used. Therefore please consult the appropriate Ion Torrent manual for sequencing.

REAGENTS

Ion PGM™ Calibration Standard A27832 by Thermo Fisher Scientific

Warnings

Ethidium Bromide is an intercatalating agent of double stranded DNA and is a potential mutagen. Some types of Pippin Prep and Blue Pippin cassettes may contain ethidium bromide. Therefore please use appropriate protection and dispose of contaminated materials appropriately.

Magnetic separation is required during this protocol. Care should be taken with the use of these magnets to prevent high impact fragmentation and personal injury.

The ddRAD method is not to be used for diagnostic purposes.