BestRAD

Dec 09, 2019

BestRAD protocol

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1 Works for me dx.doi.org/10.17504/protocols.io.6awhafe

Mimulus

Thom Nelson

ABSTRACT

Modified from protocol of Sean O'Rourke and Mike Miller published in:

Omar A. Ali, Sean M. O'Rourke, Stephen J. Amish, Mariah H. Meek, Gordon Luikart, Carson Jeffres and Michael R. Miller (2016). RAD Capture (Rapture): Flexible and Efficient Sequence-Based Genotyping. GENETICS.
https://doi.org/10.1534/genetics.115.183665

MATERIALS

NAME	CATALOG #	VENDOR
CutSmart Buffer - 5.0 ml	B7204S	New England Biolabs
NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns	E7335S	New England Biolabs
PstI - 10,000 units	R0140S	New England Biolabs
BfaI - 500 units	R0568S	New England Biolabs
NEBNext Ultra II DNA Library Prep Kit for Illumina - 24 rxns	E7645S	New England Biolabs

MATERIALS TEXT

Restriction enzymes:

PstI: NEB
BfaI-HF: NEB
CutSmart buffer

BestRAD plate adaptors:

BestRAD adaptors allow the addition of inline barcodes, and the isolation of RAD tags through purification by and enzymatic liberation of fragments from streptavidin beads. Well-specific Hamming barcodes (septamers in this case) are specified in an Excel spreadsheet and are not actually ordered as N's. Top oligos are 5'-biotinylated and contain the 3' PstI/SbfI overhang. Bottom oligos are 5'-phosphorylated to promote ligation.

Top oligo

```
Anatomy:   biotin spacer   SbfI   barcode overhang
Sequence: /5Biosg/GTACGT CCTGCAGG NNNNNNN TGCA
```

Bottom oligo

```
Sequence: /5Phos/NNNNNNN CCTGCAGG ACGTAC
```

Ordering Specs

Integrated DNA technologies

- Standard plate oligos
- 25 nmol standard desalting
- dry
- $1859.52 total for a set of 48

Streptavidin purification:

Dynabead M280 strepatividin, 2mL (GrizMart, ~ $500)

2X Binding and Wash Buffer:

1. 10 mM TrisHCl (pH 7.5)
2. 1 mM EDTA pH 8.0
3. 2 M NaCl
4. Concentrated (or dry) stocks should be available in chemical cabinet.

NextGen library prep:

NEBNext Ultra II

Kit

- includes reagents for end-repair, A-tail, ligation
- NEB E7645S 24 rxns, GrizMart, Fisher
- ~ $590

Oligos (12-plex)

- Indexed oligos containing Illumina sequencing primer sequences and required for annealing to flow cell. Added via PCR to NEBNext adaptor-ligated fragments.
- 12 barcoded i7 indexing primers
- 1 universal (i5) oligo
- NEBNext adaptor w/ USER enzyme
- NEB E7335S
- Grizmart
- ~$110

Universal primer with molecular barcode

- Modeled after i5 index primers from NEB #E7600 (p21 of manual)
- N's specify the equimolar addition of dATP, dTTP, dGTP, and dCTP during synthesis
  - Not truly random. Some GC-bias in addition
  - Should be sufficiently diverse to detect PCR duplicates
- Used in place of the universal i5 oligo in NEB #E7335S
- IDT, HPLC-cleaned - $90.50
- Primer with molecular barcode in bold:

```
5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACN NNN NNN NAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC*T-3'
```

- Compare to NEBNext i506 primer with indexing barcode in bold:

```
5´-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT AAT CTT AAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC*T-3´
```

Reaction purification and size selection:

- Ampure XP magnetic beads
- Polyethylene glycol (PEG-8000)
- NaCl

- Nice summary as of Dec 2017: http://core-genomics.blogspot.com/2012/04/how-do-spribeads-work.html

SAFETY WARNINGS

Please refer to the SDS (Safety Data Sheet) for safety warings and hazard information.

Part 1 - Digestion and BestRAD adaptor ligation

1 **I. Restriction Digest**

Dilute genomic DNA samples to a common concentration.

1. For multiplexes of ≥48, 5 - 10 ng/ul will suffice.
2. Add **10 µl** (50-100 ng) of genomic DNA to each well of a 96-well PCR plate.

2 Please select between the two following options:

- performing sheared, single-digest RAD
- performing double-digest

step case

IF performing sheared, single-digest RAD

Steps for performing sheared, single-digest RAD.

3 Into 1.5 ml tube, make restriction digest master mix (vols for 100 rxns).

- 76.2 µL water
- 134.4 µL 10X NEBuffer 3.1
- 13.4 µL PstI (NEB R0140L)

3.1 Add **76.2 µl water** .

3.2 Add **134.4 µl 10X NEBuffer 3.1** .

3.3 Add **13.4 µl PstI (NEB R0140L)** .

4 Pipette equal volumes of master mix into each tube of an 8-tube strip.

~ **27 µl** ea for full 96-well plate.

5 To each 96 plate well, pipet **2 µl PstI digestion master mix** .

6 In a thermal cycler:

a) 37 °C for 01:00:00 .

b) 80 °C for 00:20:00 .

c) Slow ramp to 4 °C — 0.1 °C /sec

7 **II. Adaptor Ligation**

This part assumes 20 µl ligation volume and and use of NEB3.1 in the previous step. If only CutSmart buffer was used for digestion, supplement ligation buffer with fullstrength NEB2.1 (2 µl per well).

Add 2 µl **annealed BestRad SbfI/PstI adaptors (50 nM)** .

8 Make ligation master mix (vols for 100 rxns):

- 347.2 µl **water**
- 56 µl **NEB2.1**
- 22.4 µl **rATP (100 mM, Fermentas R0441)**
- 22.4 µl **Ligase (NEB M0202L)**

From S. Bassham: NEB Buffer 2 is used in the ligation reactions in this protocol instead of ligase buffer because the salt it contains (50 mM NaCl) ensures the double-stranded adapters remain annealed during the reactions. T4 DNA Ligase is active in all 4 NEB Buffers if supplemented with 1mM rATP, but doesn't work at maximum efficiency in NEB 3.

8.1 Add 347.2 µl **water** .

8.2 Add 56 µl **NEB2.1** .

8.3 Add 22.4 µl **rATP (100 mM, Fermentas R0441)** .

8.4 Add 22.4 µl **Ligase (NEB M0202L)** .

9 Pipette 56 µl **master mix** into each tube of an 8-tube strip for multichannel distribution.

10 Into each 96 plate well, pipet 4 µl **ligation master mix** .

11 Incubate plate at 20 °C overnight.

12 **III. NEXT DAY**

Heat kill the ligation

1. Kill the ligation reactions by deactivating ligase at 65 °C .
2. In thermal cycler, incubate plate at 65 °C for 00:20:00 .

On Fishman Lab thermal cyclers, program available at THOM>LIGKILL.

12.1 Kill the ligation reactions by deactivating ligase at 65 °C .

12.2 In thermal cycler, incubate plate at 65 °C for 00:20:00 .

13 Multiplex 48 barcoded samples

1. Using an 8-channel pipettor:
   a) transfer 10 µl of each adaptor-ligated sample into an 8-tube PCR strip.
2. Combine pooled samples into a single 1.5 mL tube.
3. Split the multiplex into two or more aliquots.
   a) The following steps assume 200 µl **multiplexed DNA** .
4. Store plate at -20 °C for future multiplexing.

13.1 Using an 8-channel pipettor, transfer 10 µl of each adaptor-ligated sample into an 8-tube PCR strip.

13.2 Combine pooled samples into a single 1.5 mL tube.

13.3 Split the multiplex into two or more aliquots.

The following steps assume 200 µl **multiplexed DNA** .

13.4 Store plate at -20 °C for future multiplexing.

14 Concentrate sample to 210 µL with Ampure 1X. Divide sample into two Bioruptor tubes.

15 Sonicate with BioRuptor NGS:
9 cycles: **00:00:30 on** , **00:01:30 off**

16 Run **2 µl** on the fragment analyzer NGS mode to assay shearing efficiency.

Part 2 - RAD-tag isolation

17 **I. Before beginning the following steps, set a wet or dry bath to 56 °C .**

18 **II. Prepare Dynabead M280 streptavidin magnetic beads.**

Adjust bead volume.

19 2X Binding and Wash (B+W) Buffer.

- **[M]10 undefined TrisHCl (pH 7.5)**
- **[M]1 undefined EDTA pH 8.0**
- **[M]2 undefined NaCl**

20 Transfer **30 µl Dynabeads** to a new 1.7 ml tube.

21 Place tube in magnetic rack and remove supernatant.

22 Wash the beads (1/2)

1. **100 µl 2X B+W buffer**
2. Mix **00:00:30**
3. Quick spin
4. Remove supernate

22.1 Add **100 µl 2X B+W buffer** .

22.2 Mix for **00:00:30** .

22.3 Quick spin.

22.4 Remove supernate.

23 Wash the beads (2/2)

1. **100 µl 2X B+W buffer**
2. Mix **00:00:30**
3. Quick spin
4. Remove supernate

23.1 Add **100 µl 2X B+W buffer** .

23.2 Mix for **00:00:30** .

23.3 Quick spin.

23.4 Remove supernate.

24 Resuspend beads in X µL 2X B+W buffer (X = multiplex volume).

25 **III. Bead Binding**

Add resuspended beads to ~ **200 µl multiplexed DNA** .

26 *While DNA fragments are binding, make a dilution BW buffer 2-fold in 10 mM Tris.*

1. Will need ~ **1 ml** per multiplex
2. Split dilution into 2x 1.5mL tubes and set one tube in a **56 °C** wet or dry bath.

27 Incubate at **Room temperature** for **00:20:00** .

Mix every **00:02:00** by inverting.

28 Quick spin

1. Remove liquid from cap of tube
2. Not enough to pellet beads

29 Place tube on magnetic rack - Wash (1/4)

1. Remove supernate.
2. Resuspend beads in **150 µl 1X B+W Buffer** .

30 Place tube on magnetic rack - Wash (2/4)

1. Remove supernate.
2. Resuspend beads in **150 µl 1X B+W Buffer** .

31 Place tube on magnetic rack - Wash (3/4)

1. Remove supernate.
2. Resuspend beads in **56 °C** **150 µl** **1X B+W Buffer** .

32 Place tube on magnetic rack - Wash (4/4)

1. Remove supernate.
2. Resuspend beads in **56 °C** **150 µl** **1X B+W Buffer** .

33 **IV. Liberate DNA from beads**

Dilute an aliquot of appropriate NEBuffer 10-fold to 1X.

34 Resuspend beads in **100 µl** **1X restriction digest buffer** (NEBuffer 3.1 for *PstI*).

35 Place on magnetic rack, remove supernate.

36 Resuspend beads in **40 µl** **1X digest buffer** .

37 Transfer to PCR tube.

38 Add **2 µl** **PstI** .

39 Incubate tube at **37 °C** for **01:00:00** .

On Fishman Lab thermal cyclers, program available at THOM>CUTSMART.

Because the next step removes the enzyme, there is no need to heat inactivate.

40 Quick spin.

41 Place tube on magnetic rack, **KEEP SUPERNATE!!**

42 **V. Bead cleanup of digestion reaction**

Allow an aliquot of Ampure XP beads to warm to **Room temperature** *for ~* **00:30:00** .

43 *Vortex prior to addition to resuspend.*

44 Add **40 µl Ampure XP beads** to ligation reaction.

1. Mix very well by pipetting
   a) slowly
   b) expel final volume slowly due to viscosity
2. Allow to incubate at **Room temperature** for **00:10:00** .

44.1 Add **40 µl Ampure XP beads** to ligation reaction.

44.2 Mix very well by pipetting.
- *slowly*
- *expel final volume slowly due to viscosity*

44.3 Allow to incubate at **Room temperature** for **00:10:00** .

45 Make fresh 80% ethanol. ≥ 1 mL per reaction.

Use aliquoted ethanol in 50 ml conical tubes to limit evaporation/hydration of ethanol.

46 Place on magnetic rack and allow beads to pellet.

47 Slowly remove supernate.

48 Ethanol wash (1/2)
1. Add **200 µl 80% ethanol** .
2. Incubate for **00:00:30** with beads still on magnet.

48.1 Add **200 µl 80% ethanol** .

48.2 Incubate for **00:00:30** with beads still on magnet.

49 Ethanol wash (2/2)
1. Add **200 µl 80% ethanol** .
2. Incubate for **00:00:30** with beads still on magnet.

49.1 Add **200 µl 80% ethanol** .

49.2 Incubate for **00:00:30** with beads still on magnet.

50 Remove all ethanol and allow beads to dry ~ **00:05:00** with the lid open.

51 Elute DNA

1. Add **55 µl** **10 mM Tris-HCl** to bead pellet.
2. Wash buffer over beads and pipette to resuspend.
   a) Suspension will turn a pale, even brown.
3. Incubate at **Room temperature** for **00:10:00** .
4. Place tube on magnet to separate beads.
5. Pipette supernate and place in a clean PCR tube.

51.1 Add **55 µl** **10 mM Tris-HCl** to bead pellet.

51.2 Wash buffer over beads and pipette to resuspend.

Suspension will turn a pale, even brown.

51.3 Incubate at **Room temperature** for **00:10:00** .

51.4 Place tube on magnet to separate beads.

51.5 Pipette supernate and place in a clean PCR tube.

52

VI. Now is an okay time to stop if need be. Store eluted DNA at -20 °C overnight.

Part 3 - NextGen library prep

53

Use full reactions NEBNext Ultra DNA Library Prep Kit for Illumina (NEB E7370S/L) with no modifications **except**
A. Use 1:10 diluted adaptor.
B. Especially if performing double-digest, use a molecular barcoded i5 adaptor (see *Materials*)

I. End-repair and A-tailing

This step blunts single-stranded DNA ends and adds a single A overhang.

To the liberated DNA, add:

1. **3 µl NEBNext Ultra II End Prep Enzyme Mix**
2. **7 µl NEBNext Ultra II End Prep Reaction Buffer**

53.1 Add **3 µl NEBNext Ultra II End Prep Enzyme Mix** to the liberated DNA.

53.2 Add **7 µl NEBNext Ultra II End Prep Reaction Buffer** to the liberated DNA.

54 . Mix by pipetting 10x w/ a pipette set to 50 µl.

55 In a thermal cycler:

- **00:30:00** at **20 °C**
- **00:30:00** at **65 °C**
- Hold at **4 °C**

On Fishman Lab thermal cyclers, use THOM>NEB_EP.

56 **II. Adaptor ligation**

1. This part adds universal Illumina adaptors onto all end-prepped fragments. NEB NEXT adaptors form hairpins and contain a single uracil in the loop. The USER enzyme mix has endonuclease activity that cleaves the adaptor at the U, creating a Y-shaped adaptor for subsequent addition of oligos and amplification by PCR.

2. From NEB: "If input DNA ≤ 100 ng, use 1:10 diluted adaptor (diluted in 10 mM Tris, 10 mM NaCl)". Efficient RAD preps will isolate ~1%-15% of genomic DNA. Good luck getting anything greater than 100 ng total from 48 samples!

Make sure Ligation Master Mix is well-mixed prior to addition.

- Vortex quickly, follow with quick spin.

56.1 Vortex quickly.

56.2 Quick spin.

57 To end-prepped sample, add:

1. **30 µl NEBNext Ultra II Ligation Master Mix**
2. **1 µl NEBNext Ligation Enhancer**
3. **2.5 µl NEBNext Adaptor for Illumina (diluted 1:10 from stock)**

57.1 Add **30 µl NEBNext Ultra II Ligation Master Mix** .

57.2 Add **1 µl NEBNext Ligation Enhancer** .

57.3 Add **2.5 µl NEBNext Adaptor for Illumina (diluted 1:10 from stock)** .

58 Mix sample by pipetting 10X with a pipette set to 80 µl.

59 Quick spin to collect any liquid from the side of the tube.

60 In a thermal cycler:

1. **Either**
a) **keep the thermal cycler lid open OR**
b) **manually turn off heated lid**
2. **00:15:00** at **20 °C**

On Fishman Lab thermal cyclers, use THOM>NEB_LIG.

61 Add **3 µl USER enzyme** to the ligation mixture.

62 Mix sample by pipetting 10X with a pipette set to 80 µl.

63 In a thermal cycler with heated lid:

1. **00:15:00** at **37 °C**

On Fishman Lab thermal cyclers, use THOM>NEBUSER.

64 **III. Bead cleanup of ligation reaction**

Allow an aliquot of Ampure XP beads to warm to **Room temperature** *for ~* **00:30:00** .

65 *Vortex prior to addition to resuspend.*

66 *Make fresh 80% ethanol. ≥ 1 mL per reaction.*

67 Add **87 µl** **Ampure XP beads** to ligation reaction.

1. Mix very well by pipetting.
   a) slowly
   b) expel final volume slowly due to viscosity
2. Allow to incubate at **Room temperature** for **00:10:00** .

67.1 Add **87 µl** **Ampure XP beads** to ligation reaction.

67.2 Mix very well by pipetting.
   a) slowly
   b) expel final volume slowly due to viscosity

67.3 Allow to incubate at **Room temperature** for **00:10:00** .

68 *Make fresh 80% ethanol. ≥ 1 mL per reaction.*

69 Place on magnetic rack and allow beads to pellet.

70 Slowly remove supernate.

71 Ethanol wash (1/2)

1. Add **200 µl** **80% ethanol** .
2. Incubate for **00:00:30** with beads still on magnet.

71.1 Add **200 µl 80% ethanol** .

71.2 Incubate for **00:00:30** with beads still on magnet.

72 Ethanol wash (2/2)

1. Add **200 µl 80% ethanol** .
2. Incubate for **00:00:30** with beads still on magnet.

72.1 Add **200 µl 80% ethanol** .

72.2 Incubate for **00:00:30** with beads still on magnet.

73 Remove all ethanol and allow beads to dry ~ **00:05:00** with the lid open.

74 Elute DNA

1. Add **17 µl 10 mM Tris-HCl** to bead pellet.
2. Wash buffer over beads and pipette to resuspend.
3. Incubate at **Room temperature** for **00:10:00** .
4. Place tube on magnet to separate beads.
5. Pipette supernate and place in a a clean PCR tube.

74.1 Add **17 µl 10 mM Tris-HCl** to bead pellet.

74.2 Wash buffer over beads and pipette to resuspend.

74.3 Incubate at **Room temperature** for **00:10:00** .

74.4 Place tube on magnet to separate beads.

74.5 Pipette supernate and place in a a clean PCR tube.

75 **IV. PCR enrichment of adaptor-ligated fragments**

To the purified DNA, add:

1. **25 µl NEBNext Ultra II Q5 Master Mix**
2. **5 µl i7 index primer**
3. **5 µl i5 universal primer** — USE i5 WITH MOLECULAR BARCODE

75.1 Add **25 µl NEBNext Ultra II Q5 Master Mix** to the purified DNA.

75.2 Add **5 µl i7 index primer** to the purified DNA.

75.3 Add **5 µl i5 universal primer** to the purified DNA. — USE i5 WITH MOLECULAR BARCODE

76 Mix by pipetting 40 µl 10X.

77 *Split reaction into 2 x 25 µl reactions and run separately to reduce PCR bias.*

78 In a thermal cycler:

1. **98 °C** for **00:00:30** — Denaturation Cycle
2. Amplification Cycles — 8-12 cycles depending on input
   a) **98 °C** for **00:00:10**
   b) **65 °C** for **00:01:15**
3. **65 °C** for **00:05:00** — Final extension cycle
4. **4 °C** forever

79 **V. Bead cleanup of PCR**

Allow an aliquot of Ampure XP beads to warm to **Room temperature** *for ~* **00:30:00** .

80 *Vortex prior to addition to resuspend.*

81 *Make fresh 80% ethanol. ≥ 1 mL per reaction.*

82 Add **45 µl Ampure XP beads** to ligation reaction.

1. Mix very well by pipetting.
   a) slowly
   b) expel final volume slowly due to viscosity
2. Allow to incubate at **Room temperature** for **00:10:00** .

82.1 Add **45 µl Ampure XP beads** to ligation reaction.

82.2 Mix very well by pipetting.
a) slowly
b) expel final volume slowly due to viscosity

82.3 Allow to incubate at **Room temperature** for **00:10:00** .

83 *Make fresh 80% ethanol. ≥ 1 mL per reaction.*

84 Place on magnetic rack and allow beads to pellet.

85 Slowly remove supernate.

86 Ethanol wash (1/2)

1. Add **200 µl 80% ethanol** .
2. Incubate for **00:00:30** with beads still on magnet.

86.1 Add **200 µl 80% ethanol** .

86.2 Incubate for **00:00:30** with beads still on magnet.

87 Ethanol wash (2/2)

1. Add **200 µl 80% ethanol** .
2. Incubate for **00:00:30** with beads still on magnet.

87.1 Add **200 µl 80% ethanol** .

87.2 Incubate for **00:00:30** with beads still on magnet.

88 Remove all ethanol and allow beads to dry ~ **00:05:00** with the lid open.

89 Elute DNA

1. Add **33 µl 10 mM Tris-HCl** to bead pellet.
2. Wash buffer over beads and pipette to resuspend.
3. Incubate at **Room temperature** for **00:10:00** .
4. Place tube on magnet to separate beads.
5. Pipette supernate and place in a a clean PCR tube.

89.1 Add **33 µl 10 mM Tris-HCl** to bead pellet.

89.2 Wash buffer over beads and pipette to resuspend.

89.3 Incubate at **Room temperature** for **00:10:00** .

89.4 Place tube on magnet to separate beads.

89.5 Pipette supernate and place in a a clean PCR tube.

90

VI. (Optional) Size selection of library

A. Inefficient digestion or shearing of genomic DNA can result in large fragments (≥ 1000 bp) making it through library prep.

B. In addition, size selection of double-digest RAD libraries can allow for customization of genomic coverage because all fragments originating from a specific RAD locus should be equal in length across samples but different from other RAD loci.

C. Use agarose gel or Blue Pippin to size select libraries. At the very least clipping out any fragments greater than 1000 bp.

Part 1 - Digestion and BestRAD adaptor ligation

step case

IF performing double-digest

Steps for performing double-digest.

3

For optimal enzyme activity and minimal star activity, BfaI and PstI need to be used sequentially. PstI has lower activity and star activity in low-salt buffers like CutSmart, while BfaI has very reduced activity in high-salt buffers like NEB 3.1. Other enzyme combinations may be combined into a single digest step.

Perform first digestion:

BfaI master mix (add components to a 1.5 mL tube)

For full 96-well plate:

- **76.2 µl water**
- **134.4 µl 10X CutSmart Buffer**
- **13.4 µl BfaI (NEB R0568S)**

For 1/2 plate (48):

- **38.1 µl water**
- **67.2 µl 10X CutSmart Buffer**
- **6.7 µl BfaI**

4 For full 96-well plate: Add **76.2 µl water** .

For 1/2 plate (48): Add **38.1 µl water** .