Jun 07, 2023

RADSeq protocol EFGL

Forked from BestRAD protocol

DOI
dx.doi.org/10.17504/protocols.io.kqdg39bjqg25/v1

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Mimulus

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DOI: https://dx.doi.org/10.17504/protocols.io.kqdg39bjqg25/v1

Protocol Citation: EagleFish GeneticsLab 2023. RADSeq protocol EFGL. **protocols.io https://dx.doi.org/10.17504/protocols.io.kqdg39bjqg25/v1**

Manuscript citation:

Protocol status: Working
We use this protocol and it's working

Created: February 16, 2023

Last Modified: June 07, 2023

Protocol Integer ID: 77120

Keywords: Massively parallel sequencing, MPS, restriction-site associated DNA, RAD, sequence capture, genotyping, population genetics, Eagle Genetics Lab, EFGL, Radseq, radseq protocol efgl modified, radseq protocol efgl modified from protocol, protocol

Abstract

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

Citation

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 LINK

Attachments

DOCX BestRAD protocol v1.... 460KB

XLSX BestRAD_library_prep... 75KB

Materials

MATERIALS

CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #**B7204S

NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns **New England Biolabs Catalog #**E7335S

PstI - 10,000 units **New England Biolabs Catalog #**R0140S

BfaI - 500 units **New England Biolabs Catalog #**R0568S

NEBNext Ultra II DNA Library Prep Kit for Illumina - 24 rxns **New England Biolabs Catalog #**E7645S

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

Troubleshooting

Safety warnings

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

Annealing TOP/BOTTOM BestRAD adapters

45m

1 **Preparing BestRAD adapters** (Skip section if BestRAD adapters have been previously annealed)

Note

We have used 3 restriction enzymes with 6 base pair recognition sites (PstI, BamHI, and HindIII) and 1 enzyme with an 8 bp recognition site (SbfI) in our protocol. Adapters should be compatible with the base pair overhang that each restriction enzyme leaves. Both PstI and SbfI leave the same overhangs so the same adapters can be used with either enzyme. Adapters are ordered from Integrated DNA Technologies as single stranded top adapters in one plate and single stranded bottom adapters in another plate. They have to be re-hydrated, diluted to the proper concentration, and annealed prior to beginning the BestRAD protocol. See attached documents below for the sequences of our top and bottom adapters that we use. Note that the file format is for importing into IDTdna's Custom DNA Oligo, single-stranded DNA, 96 well plate form (https://www.idtdna.com/pages/products/custom-dna-rna/dna-oligos/custom-dna-oligos). We've ordered adapters a variety of ways, but the best/easiest is to order the adapters at 25nmole scale, standard desalting purification, PCR plate type, dry ship option, normalized yield and nmol quantity of 10. All top adapters are biotinylated.

We typically make a 1µM working concentration of annealed adapters if the restriction enzyme we are using is a 6 base-pair cutter. We make a 50nM working concentration of annealed adapters if the restriction enzyme is an 8 base-pair cutter. A 6 bp cutter will cut more frequently so we need a higher concentration of it.

BamHI_adapter_bottom.txt
BamHI_adapter_top.txt
HindIII_adapter_top.txt
HindIII_adapter_bottom.txt
Sbf_Pst_adapter_top.txt
Sbf_Pst_adapter_bottom.txt

Note

If you already have a plate of diluted, annealed adapters, skip to Step 5

Equipment and supplies needed for steps 1-4:
thermalcycler (We have several different types of thermalcyclers. Here's an example of one we use often)

Equipment

Mastercycler Pro	NAME
thermalcycler	TYPE
Eppendorf	BRAND
No Longer Manufactured	SKU

Equipment

EPPENDORF SCIENTIFIC CENTRIFUGE 5804	NAME
plate centrifuge	TYPE
Eppendorf	BRAND
02-262-8153PM; discontinued	SKU

Equipment

Repeater-M4	NAME
pipette	TYPE
Eppendorf	BRAND
14-287-150	SKU

Equipment

ALPS 25 V	NAME
manual heated plate sealer	TYPE
Thermo Scientific	BRAND
AB-0384/110; discontinued	SKU

p10 multi-channel pipette and tips
benchtop centrifuge
1 mL Eppendorf combi-tips (Fisher Scientific; 13-683-703)
vortex
heat seals (Fisher Scientific; AB-0745)
unskirted 96 well PCR trays (Fisher Scientific; AB-0700)
Reagents needed:
Bottom adapter plate
Top adapter plate
Duplex Buffer **IDT Catalog #**11-01-03-01
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36

1.1 Making a **100µM** stock of bottom and top adapters

Assuming the bottom and top adapters were ordered dry and at a 25 nmole scale and normalized to 10nmol, first re-hydrate to **100µM** by adding 100 µL of nuclease-free water to each well with repeater pipette and combitip

Heat seal the plate

Expected result

Now you should have a plate of 100 µL top adapters at **100µM** concentration and a plate of 100 µL bottom adapters at **100µM** concentration.

1.2 Making a **10uM** stock of bottom and top adapters

Note

$C1 = 100\mu M$
$C2 = 10\mu M$
$V2 = 100\mu l$
$C1V1 = C2V2$
$V1 = (C2V2)/C1$
$V1 = (10 * 100)/100$
$V1 = 10$

1.3 Mix the **100µM** adapter plates by gently vortexing, then briefly spin the plates down

1.4 Use a p10 multi-channel pipette to transfer 10 µL of each top adapter to a new plate.

1.5 Using a repeater pipette and combitip, add 90 µL of nuclease-free water to the same wells of the new plate.

Heat seal the plate

Note

Even though you need to use the plates immediately in the next steps, you still need to heat seal them after every dilution so you can vortex the plate and spin it down

1.6 Repeat for the bottom adapters from step 1.4.

Heat seal the plate

Expected result

Now you should have a plate of 100 µL top adapters at **10µM** concentration and a plate of 100 µL bottom adapters at **10µM** concentration.

2 Choose between the following options:

- If using 6 base pair cutter (PstI, BamHI, HindIII), need to make **1µM** dilution of BestRAD adapters
- If using 8 base pair cutter (SbfI), need to make **50nM** dilution of BestRAD adapters

STEP CASE

6 base pair cutter, 1µM adapters

From 22 to 212 steps

3 Making a **1.0µM** working concentration of **COMBINED** bottom and top adapters

Note

$$C1 = 10\mu M$$
$$C2 = 1.0\mu M$$
$$V2 = 50\mu l$$
$$C1V1 = C2V2$$
$$V1 = (C2V2)/C1$$
$$V1 = (1 * 50)/10$$
$$V1 = 5$$

3.1 Mix the **10µM** adapter plates by gently vortexing, then briefly spin the plates down

3.2 Using a multi-channel pipette, add 2.5 µL of the **10µM** top adapters to a new plate. Discard tips.

3.3 Using a multi-channel pipette, add 2.5 µL of the corresponding **10µM** bottom adapters (the same well position as the top adapter plate) to the same well of the new plate. Discard tips.

3.4 Mix the duplex buffer by vortexing, then briefly spin down

3.5 Using a repeat pipette and a 1mL combi-tip, add 45 µL of duplex buffer to all wells.

Heat seal the plate

Expected result

Now you have a single plate with 50 µL of bottom and top adapters combined at **1µM** concentration.

4 Annealing adapters

3m

Run the plate of **COMBINED** adapters (50 µL) on a thermalcycler using the following program (In our lab, the program can be found using the menu options General → RAD → **duplexAssay**)

CHECK THE PROGRAM TO MAKE SURE IT'S CORRECT

1. 95 °C for 00:02:00
2. Reduce temperature by 1 °C for 00:01:00
3. repeat until temperature is down to 4 °C

Expected result

Now you have a single plate with 50 µL of bottom and top adapters combined at either **1µM** concentration if you were following the 6 bp cutter steps or **50nM** concentration if you were following the 8 bp cutter steps. These BestRad adapters include the barcodes for each sample

5 Check to see if there's sufficient stock made of **2X** B+W buffer (needed for step 15, size selection) and stored in 50mL conical tube at room temperature. If not, make it

Note

Equipment and supplies needed for this step:
50mL conical tube (Fisher Scientific; 12-565-271)

Reagents needed:
Tris-HCl 1.0M pH 7.5 **Fisher Scientific Catalog #**AAJ60636K2
EDTA 0.5M pH 8.0 **Fisher Scientific Catalog #**AAJ62786AP
NaCl **Fisher Scientific Catalog #**BP358-1
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36

5.1 Add the following to a 50mL conical tube:

1. 500 µL 1M Tris-HCl pH 7.5
2. 100 µL 0.5M EDTA pH 8.0
3. 5.844 g NaCl
4. Add enough water to obtain 50 mL final volume

Note

Final concentration is 2X: 10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 2M NaCl

6 Choose from the following options:
Quantification of <48 samples
Quantification of >48 samples

STEP CASE

<48 Samples 202 steps

DNA quantification <48 samples

7 **Quantify DNA with Qubit**

Note

Note that for sample sizes less than 48ish, we quantify each sample individually using a Qubit fluorometer. Otherwise, we use a plate reader.

Note

Equipment and supplies needed for this step:

Equipment

Qubit Fluorometer	NAME
Fluorometer to quantify DNA	TYPE
Invitrogen	BRAND
Q33226	SKU

Qubit assay tubes (Fisher Scientific; Q32856)
Eppendorf 2mL Snap-Cap Microcentrifuge Safe-Loc Tubes (Fisher Scientific; 05-402-7)
unskirted 96 well PCR trays (Fisher Scientific; AB-0700)

p10 pipette and tips
p200 pipette and tips

Reagents needed:
DNA tray(s)
Qubit dsDNA HS assay kit **Fisher Scientific Catalog #**Q32854
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36 if dilution necessary

7.1 Enter sample data info into “sampleData” tab of *BestRAD_library_prep_labData v1.2.xlsx* (attached in the DESCRIPTION tab of this protocol. It has example data for 20 samples.)

7.2 Qubit all samples with Qubit dsDNA HS assay kit.
Follow the manufacturer's protocol to make a working solution (1:200) of dye
https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

7.3 **Make sure you set the sample volume and the output to be read in ng/µl, not ng/mL**

Take **2** readings per sample and enter them into columns F and G in "qubitData" tab in *BestRAD_library_prep_labData v1.2.xlsx.* The spreadsheet has a column that will take the average. The target is **10ng/µl**.

7.4 If DNA concentration is off the Qubit scale,

1. perform a 1:5 dilution (1 µL of DNA, 4 µL of water) in a new PCR plate
2. Re-Qubit diluted samples

7.5 If DNA concentration is too low (less than 9ng/µl), reach out to lead biologist for replacement samples, or re-extract DNA then re-Qubit new samples

DNA Normalization

8 Normalize DNA to **10ng/µl**

Note

Equipment and supplies needed for this step:

manual heated plate sealer
plate seals (Fisher Scientific; AB-0745)
unskirted 96 well PCR trays (Fisher Scientific; AB-0700)
p10 pipette and tips
p200 pipette and tips
vortex
plate centrifuge
Results from "Norm" tab of *BestRAD_library_prep_labData v1.2.xlsx*
Reagents needed:
DNA tray(s)
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36

The table in "Norm" tab of *BestRAD_library_prep_labData v1.2.xlsx* has been formulated to normalize all samples to **10ng/µl** at a 30 µL final volume, and you'll need 10 µL for each sample in next step.

8.1 Label a new PCR plate: Normalized DNA for [insert RADseq project name], date, initials

8.2 Pipette the amount of DNA indicated in column F of "Norm" tab of *BestRAD_library_prep_labData v1.2.xlsx* from the DNA tray to corresponding well position in new Normalization plate

8.3 Pipette the amount of nuclease-free water indicated in column G of "Norm" tab to corresponding well position in new Normalization plate

8.4 Heat seal plate, Mix by gently vortexing, then briefly spin the plate down

Expected result

Tray(s) with 30 µL of your samples normalized to **10ng/µl**
Store at 4 °C if you plan to start the library in the next couple of days,
otherwise -20 °C

Day 1 - Digestion

9 **Digest the normalized DNA with a restriction enzyme**

Note

Equipment and supplies needed for this step:
thermal cycler
manual heated plate sealer
plate seals (Fisher Scientific; AB-0745)
p10 pipette and multi-channel pipette and tips
p200 pipette and tips
Repeater-M4 pipette
0.1mL combi-tip (Fisher Scientific; 13-683-700)
unskirted 96 well PCR trays (Fisher Scientific; AB-0700)
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific; 13-698-791)
vortex
plate centrifuge
timer

Reagents needed:
10µl of normalized DNA per sample
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36
CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #**B7204S

1 of the following restriction enzymes
PstI - 50,000 units **New England Biolabs Catalog #**R0140L
SbfI-HF - 2,500 units **New England Biolabs Catalog #**R3642L
BamHI - 10,000 units **New England Biolabs Catalog #**R0136S
HindIII - 10,000 units **New England Biolabs Catalog #**R0104S

NOTE: All restriction enzymes' concentrations are 20,000U/µl

9.1 Thaw reagents to prep for master mix
- Whichever restriction enzyme chosen for the project (PstI, SbfI, BamHI, or HindIII)
- CutSmart Buffer 10X
- Normalized DNA if it's been in the freezer

9.2 Add reagents from table below to a 1.5mL Lo-Bind tube to make a master mix. Add 5-10 to your sample size to account for pipette error. For example, if you have 20 samples, **multiply the volumes per sample by 25** .

RE Digestion Master Mix	Per Sample
Water	0.68µl
CutSmart 10X buffer	1.2µl
Restriction Enzyme	0.12µl
Total	**2.0µl**

Note

Recipe above assumes 20units/µl of enzyme and 2.4 units per reaction

9.3 Vortex and briefly spin down

9.4 Label PCR plate "Digestion"

9.5 Using a p10 pipette or 0.1mL combitip, add 2 µL of master mix to each well

9.6 Using a multi-channel p10 pipette, add 10 µL of normalized DNA to corresponding wells

9.7 Heat seal PCR plate, vortex gently and quickly spin down.

9.8 Run the following thermalcycler program (In our lab, the program is "**1 RE-digest**" and can be found using the menu options General → RAD) 1h 30m

1. 37 °C for 01:00:00
2. 85 °C for 00:30:00

CHECK THE PROGRAM TO MAKE SURE IT'S CORRECT

9.9 Set a timer for 01:15:00 1h 30m

Prep next step, about 00:15:00 prior to end of thermalcycler program

Expected result

Tray labeled "Digestion" with 12 µL of ~ 100 ng of DNA per sample that has been digested with a restriction enzyme

Day 1 - Ligation

10 **Ligate adapters onto digested DNA**

Note

Equipment and supplies needed for this step:
thermal cycler
plate centrifuge
manual heated plate sealer
plate seals (Fisher Scientific; AB-0745)
p10 pipette and multi-channel pipette and tips
p200 pipette and tips
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific; 13-698-791)
vortex
Reagents needed:
plate of annealed adapters
plate of digested DNA
CutSmart Buffer 10X (comes with restriction enzyme) **New England Biolabs**
100mM ATP Solution **Thermofisher Catalog #**R0441
T4 ligase **New England Biolabs Catalog #**M0202M
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36

10.1 1. Thaw BestRAD adaptors (prepared in steps 1-4) **and** reagents for Ligation Master Mix (listed above)

10.2 gently vortex and spin down all reagents

10.3 Make Ligation master mix in a 1.5mL Lo-Bind tube by combining reagents below.
Multiply by the number of samples you have + 5-10 extra to account for pipette error.

Ligation Master Mix	Per sample
Water	1.28 µl
CutSmart Buffer 10X	0.4 µl
ATP	0.16 µl
T4 ligase	0.16 µl
Total	**2 µl**

10.4 Remove Digestion PCR plate from thermocycler (Step 9.9), quick spin

10.5 With a multi-channel p10 pipette, add 2 µL of annealed adaptors to each well in Digestion PCR plate, corresponding to the same well setup in the "library prep" tab of *BestRAD_library_prep_labData v1.2.xlsx*. Discard tip after each use.

10.6 Using a p10 pipette, add 2 µL of Ligation master mix to each well. Discard tip after each use.

10.7 Heat seal PCR plate, gently vortex, quick spin

10.8 Overnight 16h 30m

Run thermalcycler program: (In our lab, the program is called "**2 Ligation**" and can be found using the menu options : General → RAD)

1. 20 °C for 16:00:00
2. 80 °C for 00:30:00

Note

Do not let plate sit at 4°C hold for too long, recommended to proceed to next step as soon as program ends.

Expected result

16 µL of digested DNA per sample with BestRad adapters attached

Day 2 - Sonication

11 **Prepping the Sonicator**

Note

Equipment and supplies needed for this step:

Equipment

QSonica SONICATOR SYSTEM Q800R1	NAME
Sonicator	TYPE
QSonica	BRAND
discontinued	SKU

Two 1 Liter bottles for de-ionized water

Reagents needed:
de-ionized water (From Fish Health Lab)

11.1 Fetch ~ 2 L of de-ionized water from the Fish Health Lab. Take containers with you

11.2 Connect all corresponding tubes to the machine as labeled and screw on the filter

11.3 Empty 1 L of de-ionized water into sonicator and start the machine.

11.4 When the filter is fully saturated, and water cycle is complete, add more de-ionized water until the level is just below the top white spout.

11.5 Proceed to next step, and let machine cool to 4 °C

Day 2 - Pool samples

12

Note

Equipment and supplies needed for this step:
p200 pipette and tips
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific; 13-698-791)
OR
2.0mL Lo-Bind microcentrifuge tube (Fisher Scientific; 13-698-792)

Reagents needed:
"Digestion" plate of digested samples with BestRad adapters attached

12.1 **Pooling samples**

- Using a p200 single channel pipette, pool all 16 µL of each well from Digestion PCR plate into a new 1.5mL Lo-Bind tube
- If library contains more than **93** samples, pool into a 2.0mL Lo-Bind tube instead

Expected result

A library tube with pooled samples, volume = 16 µL X number of samples

Day 2 - Sonicate, precipitate, ligate library

13 **purifying pooled library**

Note

Equipment and supplies needed for this step:

Equipment

DynaMag Spin	NAME
Magnet for microcentrifuge tubes	TYPE
Invitrogen	BRAND
12320D	SKU

p200 pipette and tips
p1000 pipette and tips
1.5mL Lo-Bind microcentrifuge tubes (Fisher Scientific; 13-698-791)
Bioruptor Plus TPX microtubes (Fisher Scientific; C30010010-50)
5.0mL Axygen Polypropylene Snaplock non-sterile tube (Fisher Scientific; 14-568-100)
OR
15mL conical tube (Fisher Scientific; 14-959-53A)
vortex
benchtop centrifuge
Reagents needed:
tube with pooled library
Ampure XP beads **Beckman Coulter Catalog #**A63881
low TE **Fisher Scientific Catalog #**AAJ75793AP
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36
100% ethanol (laboratory grade, non-denatured!)

Note

Ampure XP beads have been aliquoted into 2 mL tubes to prevent contamination of the stock. In general, whenever they are used in the protocol, they should be vortexed thoroughly to ensure mixing

Note

if library contains >**18** samples

- Rule of thumb: Each tube should have 200-300µl of pooled samples to allow adequate room for 80% ethanol wash step
- At the end, the combined total low TE volume of all tubes must be 210 µL

Using the table below, calculate the respective volumes required, or enter in number samples in "Pooling_calculator" tab in library prep spreadsheet to have volumes calculated

		N = number of samples			
No. Samples	Tubes needed	Vol. of pooled samples	Vol. of AMPure XP Beads	Vol. of 80% EtOH	Vol. of Low TE in each tube
0-18	1	16*N	16*N	4 *(16*N)	210
19-37	2	(16*N) ÷ 2	(16*N) ÷ 2	4 * ((16*N) ÷ 2)	105
38-56	3	(16*N) ÷ 3	(16*N) ÷ 3	4 * ((16*N) ÷ 3)	70
57-74	4	(16*N) ÷ 4	(16*N) ÷ 4	4 * ((16*N) ÷ 4)	52.5
75-93	5	(16*N) ÷ 5	(16*N) ÷ 5	4 * ((16*N) ÷ 5)	42
94-100	6	(16*N) ÷ 6	(16*N) ÷ 6	4 * ((16*N) ÷ 6)	35

E.g. If N=48, then you need 3 tubes, the volume of pooled samples = 256 µL, volume of AMPure XP beads = 256 µL, volume of 80% EtOH = 1.024 mL, volume of low TE = 70 µL

13.1 Determine how many 1.5mL Lo-Bind tubes you need based on table above.

13.2 Aliquot the pooled library into the tubes equally

13.3 Add an equal volume of AMPure XP beads to pooled library amount in a 1:1 ratio

13.4 Gentle vortex, quick spin.

13.5 Incubate tube(s) at room temperature for 00:10:00 10m

13.6 During the 10 min incubation, prep **80%** ethanol in 5mL tube(s) or 15ml conical

Note

e.g. to make 5 mL of 80%, mix 4 mL of 100% ethanol and 1 mL of nuclease-free water
For 100 samples: need 12 mL 100% ethanol, 3 mL water
For 74 samples: need 9.6 mL 100% ethanol, 2.4 mL water
For 56 samples: need 8 mL 100% ethanol, 2 mL water
For 37 samples: need 5.6 mL 100% ethanol, 1.4 mL water

The volumes above take into account the extra ~2mL of 80% ethanol needed in the rest of the protocol

13.7 Place tube(s) on DynaMag Spin, and allow the magnetic beads to stick to the side of the tube(s) for 00:05:00 5m

13.8 While the tube(s) are still on the magnet, using a p200 pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard supernatant

13.9 While tube(s) are still on the magnet, add 2X total volume of 80% ethanol to the beads and incubate for 00:00:30 . 30s

Note

e.g. If 48 samples: 256 µL of library in 3 tubes: 2X total volume = (256 µL pooled library + 256 µL beads)*2 = 1024 µL 80% ethanol **per tube**

13.10 Discard supernatant as in step 13.8

13.11 repeat step 13.9 one more time

13.12 Discard supernatant as in step 13.8

13.13 Leave tube(s) on magnet and allow any residual ethanol to evaporate by letting stand for 00:05:00 uncovered 5m

13.14 Take tube(s) off the magnet
Add low TE to each tube, refer to table at the beginning of this section for how much
Pipette up and down a few times to completely resuspend the bead pellet

13.15 Incubate tubes(s) at room temperature for 00:05:00 . 5m

13.16 Place tube(s) in magnetic stand for 00:05:00 5m

Note

THE SUPERNATANT HAS THE DNA IN IT NOW. YOU WANT TO KEEP THE SUPERNATANT

13.17 Using a p200 pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove the supernatant and **put into a TPX tube**.

Repeat for all library tubes on the magnet. Deposit all supernatants into **same TPX tube**

13.18 Pipette 210 µL of water into another TPX tube

LABEL BOTH TPX TUBES TO AVOID MIXUP!!

Expected result

1 TPX tube with 210 µL of pooled, digested DNA fragments
1 TPX tube with 210 µL of water to use as a balance in the sonicator

14 Sonication

Note

Equipment and supplies needed for this step:
Sonicator (prepped in step 7)
Reagents needed:
TPX tube with library
TPX tube with water as balance

14.1 Sonicate library for 00:04:30 at 20% capacity, and at 4 °C 4m 30s

14.2 Remove tubes after sonication has completed

OK to sit in room temperature for next step.
Discard TPX vial containing water
Turn off sonicator and coolant pump

Expected result

210 µL of pooled, sonicated library in tube.

Note

DNA fragments should be between 200-500bp long. Can check this on gel or Bioanalyzer here if troubleshooting needed

Some fragments now lack BestRad adapters after sonication. The next step gets rid of these

Day 2 - Bead Selection

15 **Prep buffers for bead binding in Step 17**

Note

Equipment and supplies needed for this step:
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
p200 pipette and tips
p1000 pipette and tips
heat block
Reagents needed:
2X B+W buffer (directions on how to prepare in step 5)
CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #**B7204S
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36

15.1 Thaw 10X CutSmart Buffer at room temperature

15.2 Check heat block to make sure it's on and set to 56 °C

15.3 To a 1.5mL Lo-Bind tube add:
- 25 µL 10X CutSmart Buffer
- 225 µL water

15.4 To a 1.5mL Lo-Bind tube add:
- 250 µL 2X B+W buffer
- 250 µL water

15.5 To a 1.5mL Lo-Bind tube add:
- 175 µL 2X B+W buffer
- 175 µL water

15.6 Place tube from step 15.5 in heat block set to 56 °C

Expected result

1 tube of 250 µL 1X CutSmart Buffer
1 tube of 500 µL 1X B +W Buffer
1 tube of warm 350 µL 1X B +W Buffer in heat block

16 **Prep Dynabead M280 streptavidin magnetic beads**

Note

Equipment and supplies needed for this step:
p200 pipette and tips
vortex
benchtop centrifuge
DynaMag Spin
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
Reagents needed:
2X B+W buffer made in Step 5
Dynabeads M-280 Streptavidin **Fisher Scientific Catalog #**11-206-D

16.1 Vortex stock bottle of Dynabeads and transfer 20 µL to new 1.5mL Lo-Bind tube

Note

Dynabeads are temperature sensitive, place beads back in fridge immediately after use

16.2 Place tube on the DynaMag Spin for 00:00:10 10s

16.3 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 20 µL supernatant

16.4 Remove tube from magnet and add 100 µL **2X** B+W buffer

16.5 Gently vortex for 00:00:30, then quick spin 30s

16.6 Place tube on the magnet for 00:00:30 30s

16.7 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 100 µL supernatant

16.8 Repeat steps 16.4 through 16.7

16.9 Take tube off magnet, re-suspend beads in 200 µL of **2X** B+W buffer

Vortex

Expected result

200 µL of Dynabead M280 Streptavidin beads prepped for binding

Day 2 - binding DNA to beads 22m

17 **Bind DNA to beads**

Note

Equipment and supplies needed for this step:
p1000 pipette and tips
p200 pipette and tips
benchtop centrifuge
DynaMag Spin
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
Reagents needed:
sonicated library from Step 14.2
prepped beads from 16.9
2X B+W buffer made in Step 5
1X B+W buffer from Step 15.4
warm 1X B+W buffer from Step 15.6

17.1 Transfer all 210 µL of sonicated library to freshly prepped 200 µL Dynabeads tube from Step 16.9

17.2 Incubate for 00:20:00 at room temperature while gently vortexing every 2 minutes for 10 seconds (e.g 20:00 minute mark, vortex till 19:50. At 18 minutes, vortex till 17:50, etc) 20m

17.3 Quick spin then place tube on the DynaMag Spin for 00:00:30 30s

17.4 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 410 µL supernatant

17.5 Take tube off magnet, re-suspend beads in 150 µL of **1X** B+W buffer from Step 15.4

Mix by pipetting

17.6 Place tube on the DynaMag Spin for 00:00:30 30s

17.7 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 150 µL supernatant

17.8 Repeat steps 17.5 - 17.7 2 more times

17.9 Take tube off magnet, re-suspend beads in 150 µL of warm **1X** B+W buffer from Step 15.6

Mix by pipetting

Note

For following steps, keep warm **1X** B+W buffer in heat block at 56 °C when not in use

17.10 Place tube on the DynaMag Spin for 00:00:30 30s

17.11 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 150 µL supernatant

17.12 Repeat steps 17.9 - 17.11 1 more time

17.13 Take tube off magnet, re-suspend beads in 200 µL of **2X** B+W buffer

Mix by pipetting

Expected result

A tube with 200 µL of DNA fragments with BestRad adapters bound to the beads.

18 **Liberate DNA from beads**

Note

Equipment and supplies needed for this step:
thermal cycler
p10 pipette and tips
p200 pipette and tips
p1000 pipette and tips
plate centrifuge
DynaMag Spin
magnet stand for plate
unskirted PCR plate (Fisher Scientific; AB-0700)
manual heated plate sealer
plate seal (Fisher Scientific, AB-0745)
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
Reagents needed:
DNA and beads from Step 17.13
1X CutSmart buffer from Step 15.3
SbfI-HF - 2,500 units **New England Biolabs Catalog #**R3642L

18.1 Re-suspend beads in 100 µL of 1X CutSmart buffer from Step 15.3

Mix by pipetting

18.2 Place tube on the DynaMag Spin for 00:00:30 30s

18.3 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 300 µL supernatant

18.4 Repeat steps 18.1 - 18.3. Note that this time around the volume of the supernatant to remove will be 100 µL

18.5 Take tube off magnet, re-suspend beads with bound DNA in 40 µL 1X CutSmart

Mix by pipetting

18.6 Transfer all 40 µL beads mix into well C3 of PCR plate

> Note
>
> In our lab, we don't use any PCR tubes, only plates. This step can be done in a tube that would fit a thermalcycler.

18.7 Pipette 2 µL SbfI-HF (NEB R3642L) into the well

18.8 Use a p200 pipette set to 20µl to mix the solution

DO NOT VORTEX or SPIN DOWN PLATE

18.9 Heat seal PCR plate

18.10 Run thermalcycler program: 3 37hold (General → RAD → 3 37hold) 1h

- 37 °C for 01:00:00

18.11 After program has completed, give the plate a quick spin

18.12 Place plate on magnet in plate format for 00:00:30 30s

Note

In our lab, we rigged a magnet to work on a plate. However, there are commercially available plate magnets. If you performed the incubation in a PCR tube, transfer the liquid to a tube that will fit the tube magnet stand.

18.13 transfer all 42 µL supernatant to new 1.5mL Lo-Bind tube

Expected result

42 µL of DNA fragments with BestRad adapters attached in tube

19 **Size select DNA fragments**

Note

Equipment and supplies needed for this step:

p200 pipette and tips
DynaMag Spin
unskirted PCR plate (Fisher Scientific; AB-0700)
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
Reagents needed:
80% ethanol from Step 13.6
DNA and beads from Step 18.13
low TE **Fisher Scientific Catalog #**AAJ75793AP
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

19.1 Add 42 µL of AMPure XP beads to 42 µL DNA tube

Mix by pipetting

19.2 Incubate 84 µL mixture at room temperature for 00:10:00 10m

19.3 Place tube on the DynaMag Spin for 00:05:00 5m

19.4 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 84 µL supernatant

19.5 While still on magnet, add 168 µL of 80% ethanol (made the same day) to beads and incubate for 00:00:30 30s

19.6 While the tube is still on the magnet, using a pipette and tip, insert the tip into the tube away from the bead pellet and slowly remove and discard the 168 µL supernatant

19.7 Repeat wash steps 19.5 - 19.6

19.8 Leave tube on magnet and allow any residual ethanol to evaporate by letting stand for 00:05:00 uncapped 5m

19.9 Resuspend beads with 55 µL low TE

Mix by pipetting

19.10 Incubate at room temperature for 00:05:00 5m

19.11 Place on DynaMag Spin for 00:05:00 5m

Note

NOW THE SUPERNATANT HAS THE DNA IN IT

19.12 Transfer 55 µL supernatant to PCR plate (well C3 is preferred)

Note

If stopping is necessary, transfer supernatant to a new 1.5mL Lo-Bind tube and store in -20 °C

Expected result

55 µL purified DNA fragments with BestRad adapters attached

Day 2 - Ligation

20 **Ligating NEBNext adapters to DNA fragment**

Note

Equipment and supplies needed for this step:
thermal cycler
p10 pipette and tips
p200 pipette and tips
manual heated plate sealer
plate seals (Fisher Scientific, AB-0745)
plate centrifuge
vortex
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
timer
Reagents needed:
NEBNext Ultra II DNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #**E7645L
1M Tris-HCl pH7.5 **Fisher Scientific Catalog #**AAJ60636K2
purified DNA from Step 19.12

Note

The protocol calls for 10mM Tris-HCl pH 7.5, so make a dilution if needed
The NEBNext kit has 2 components: End Prep reagents and Ligation reagents

20.1 Thaw reagents from NEBNext End prep kit
- Ultra II End Prep Enzyme Mix
- Ultra II EP Buffer

20.2 Add 3 µL of Ultra II End Prep Enzyme Mix and 6.5 µL of Ultra II EP Buffer to the well of the tray with the 55 µL of DNA

Mix by pipetting (set p200 to 40µl)

20.3 Heat seal the plate and quick spin

20.4 Run thermalcycler program: 4 End Prep (General → RAD → End Prep) 1h
- 20 °C for 00:30:00
- 65 °C for 00:30:00

Set a timer for 45 min

20.5 While program is running, drain sonicator.
1. Disconnect the tube labeled “coolant supply” from the sonicator and remove the white nozzle end completely.
2. Turn on the pump, and tip the sonicator backwards to drain as much water as possible, then turn pump off.
3. Use paper towels to absorb all remaining water in sonicator
4. Leave lid open for a day or 2 to air dry. Unscrew the filter, empty out any remaining water and let it sit to air dry.

20.6 When timer goes off, thaw the following reagents for ligation:
- Ultra Prep II Ligation master mix
- NEBNext Adaptor
- Ligation Enhancer

20.7 Dilute NEBNext adapter 1:10 in a new 1.5mL Lo-Bind tube
1 µL of NEBNext Adaptor + 9 µL 10mM Tris HCl pH 7.5

Note

The manufacturer's protocol says to dilute the NEBnext adapter IF the DNA input is ≤ 100 ng. We don't check this and assume the adapter always needs diluting. Also, the protocol says to mix the 10mM Tris HCl pH 7.5 with 10mM NaCl, but we haven't been doing this (May, 2023).

20.8 Make Ligation master mix in a 1.5mL Lo-Bind tube by combining reagents below.
Multiply by the number of samples you have + 5-10 extra to account for pipette error.

Adaptor ligation	Vol. per sample
Ultra Prep II Ligation master mix	15 µl
*NEBNext Adaptor **(diluted)**	2.5 µl
Ligation Enhancer	1 µl
Total	**18.5 µl**

Note

The NEBNext adapter is a double stranded hairpin that will ligate to both sides of the DNA fragments. It contains the priming sites for the PCR primers in Step _____

20.9 Add master mix to well of plate with DNA (the volume should be 83 µL)
Mix by pipetting (set p200 to 40µl and mix)

20.10 Heat seal the plate, quick spin

20.11 Run thermal cycler program: 5 Adaptor Ligation (General → RAD → 5 Adapter Ligation 15m
20 °C for 00:15:00

20.12 After program has ended add 3 µL of NEBNext USER enzyme

Mix by pipetting as above

Note

The NEBNext adapter has a uracil base in it. The USER enzyme cleaves the hairpin adapter at the uracil so now the DNA fragments are linear and have the PCR priming sites on both sides needed in Step 22

20.13 Heat seal the plate, quick spin

20.14 Run thermalcycler program: 6 USER enzyme (General → RAD → 6 USER enzyme) 15m

37 °C for 00:15:00

Expected result

A well in a PCR plate with 86 µL of DNA fragments that have BestRad adapters on one side with the barcodes and NEBNext adapters on both sides with the PCR priming sites

Day 2 - Ampure Size Selection and Clean-Up 20m

21 **Size selection and cleanup of adaptor-ligated DNA**

Note

Equipment and supplies needed for this step:
p10 pipette and tips
p200 pipette and tips
1.5mL Lo-Bind microcentrifuge tubes (Fisher Scientific, 13-698-791)
DynaMag Spin
unskirted 96 well PCR tray (Fisher Scientific; AB-0700)
Reagents needed:
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
low TE **Fisher Scientific Catalog #**AAJ75793AP
80% ethanol

21.1 Transfer all 86 µL solution from 96-well unskirted PCR plate to a new 1.5mL Lo-Bind tube

21.2 Add 13.5 µL of nucelase-free water and 44 µL AMPure XP beads

mix by pipetting

21.3 Incubate at room temperature for 00:05:00 5m

21.4 Place tube on DynaMag Spin, and allow the magnetic beads to stick to the side of the tube for 00:05:00 5m

21.5 Transfer all 143.5 µL supernatant to new 1.5mL Lo-Bind tube

21.6 Add 26 µL AMPure XP beads

mix by pipetting

21.7 Incubate at room temperature for 00:05:00 5m

21.8 Place tube on DynaMag Spin, and allow the magnetic beads to stick to the side of the tube for 00:05:00 5m

21.9 Remove and discard supernatant (~ 170 µL)

21.10 While still on magnet, add 200 µL of fresh 80% ethanol to beads and incubate for 00:00:30 30s

21.11 Repeat 21.9 - 21.10

21.12 Leave tube on magnet and allow any residual ethanol to evaporate by letting stand for 00:05:00 uncovered 5m

21.13 Take the tube off the magnet and re-suspend the beads in 17 µL low TE

Mix by pipetting

21.14 Incubate for 00:02:00 at room temperature 2m

21.15 Put tube on DynaMag Spin for 00:05:00 5m

21.16 Transfer 15 µL into a new unskirted PCR plate

OR

If stopping for the day, transfer into a new 1.5mL Lo-Bind tube, store at -20 °C

Expected result

15 µL of purified DNA fragments with BestRAD and NEBNext adapters

22 PCR amplification

Note

Equipment and supplies needed for this step:
thermal cycler
p10 pipette and tips
200 pipette and tips
manual heated plate sealer
plate seals (Fisher Scientific, AB-0745)
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
vortex
plate centrifuge
Reagents needed:

NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns **New England Biolabs Catalog #**E7335S

15 µL of DNA with ligated adapters from Step 21.16

22.1 Thaw reagents from NEBNext Multiplex Oligo kit (orange box) labeled: “RADseq PCR Amp”

22.2 Select an Index (i7) from the NEBNext Multiplex Oligo kit to use and record it in the "Final Library" tab in *BestRAD_library_prep_labData v1.2.xlsx.*

Note

The i7 MUST BE unique per library. If you are preparing more that 1 library, use different i7s.

22.3 Add the following reagents to the 15 µL of DNA:

PCR Amplification	Volume
Ultra II Q5 Hot Start HiFi PCR Master Mix	25 µl
Index Primer/i7 Primer	5 µl
Universal PCR Primer/i5 Primer	5 µl

Total volume is 50 µL

22.4 Heat seal the plate, gently vortex, quick spin

22.5 Run thermal cycler program: General → RAD→ 7 NEBNext PCR 11x 28m

Runs for about 00:28:00

Cycle Step	Temp	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	11
Annealing/Extension	65°C	75 s	
Final Extension	65°C	5 min	1
Hold	4°C	∞	1

Note

The BestRAD protocol says to perform 4-12 cycles of PCR. Currently we use 11.

22.6 Transfer 50 µL PCR product to new 1.5mL Lo-Bind tube

Note

Do NOT stop here. Complete PCR purification below

23 **PCR purification**

Note

Equipment and supplies needed for this step:
p200 pipette and tips
1.5mL Lo-Bind microcentrifuge tube (Fisher Scientific, 13-698-791)
DynaMag Spin
vortex
Reagents needed:
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
low TE **Fisher Scientific Catalog #**AAJ75793AP
80% ethanol

23.1 Add 45 µL AMPure XP beads to the 50 µL of DNA from Step 22.6

mix by pipetting

23.2 Incubate at room temperature for 00:05:00

23.3 Place tube on DynaMag Spin, and allow the magnetic beads to stick to the side of the tube for 00:05:00

23.4 Remove and discard supernatant (~ 95 µL)

23.5 While still on magnet, add 200 µL of fresh 80% ethanol to beads and incubate for 00:00:30

23.6 Repeat 21.9 - 21.10

23.7 Leave tube on magnet and allow any residual ethanol to evaporate by letting stand for 00:05:00 uncovered

23.8 Take the tube off the magnet and re-suspend the beads in 33 µL low TE

Mix by pipetting

23.9 Incubate for 00:02:00 at room temperature

23.10 Put tube on DynaMag Spin for 00:05:00

23.11 Transfer 28 µL into a new 1.5mL Lo-Bind tube

Label the tube with species "Radseq", library number, date

Store at -20 °C if stopping for the day

Expected result

28 µL of final, undiluted library

Day 3 - quantify library

1h 3m

24 **Qubit Rad library**

Note

Equipment and supplies needed for this step:
Qubit fluorometer
Qubit assay tubes (Fisher Scientific, Q32856)
1.5mL or 2.0mL microcentrifuge tube
p10 pipette and tips
p200 pipette and tips
vortex
benchtop centrifuge
Reagents needed:
DNA library from Step 23.11
Qubit dsDNA HS assay kit **Fisher Scientific Catalog #**Q32854

24.1 Thaw library to room temperature

24.2 Make 1:200 diluted dye mix in a microcentrifuge tube
199 µL Qubit buffer
1 µL Qubit dye

24.3 Pipette 198 µL of diluted dye mix from Step 24.2 and 2 µL of the library into a qubit assay tube

Vortex thoroughly, quick spin

24.4 Incubate at room temperature for 00:03:00 3m

24.5 Put qubit assay tube in Qubit Fluorometer and take **2** readings per sample and enter them into BestRAD_library_prep_labData v1.1 spreadsheet in “Final Library” tab. The spreadsheet has a column that will take the average. The target is **4-20 ng/µl**.

Note

Don't use any tube other than the qubit assay tubes in the Qubit.

Make sure you set the sample volume and the output to be read in ng/µl, not ng/mL

Expected result

26 µL of your Radseq library at a concentration >= **4 ng/µl**.

25 **E-gel of library**

Note

Equipment needed
p10 pipette and tips
p200 pipette and tips
parafilm
vortex
benchtop centrifuge
timer
thumb drive

Equipment

E-Gel Power Snap Electrophoresis System	NAME
e-gel electrophoresis	TYPE
Invitrogen	BRAND
G8300	SKU

Reagents needed

Invitrogen E-Gel EX Agarose Gels 1% **Fisher Scientific Catalog #**G402021

Invitrogen E-Gel 1 Kb Plus Express DNA ladder **Fisher Scientific Catalog #**10-488-091

6X loading dye (comes with ladder)

Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36

25.1 Thaw 1 Kb ladder, loading dye, and RAD library if needed

Gentle vortex, quick spin

25.2 Cut strip of parafilm, tape to benchtop if necessary

25.3 Onto parafilm, pipette 15 µL of 1 Kb ladder. Add 5 µL of nuclease free water to the ladder. Mix by pipetting

25.4 Onto parafilm, pipette 5 µL of the RAD library. Add 3 µL of loading dye and 12 µL of nuclease free water to the library

RETURN LIBRARY TO FREEZER

25.5 Unwrap e-gel, remove comb, remove camera (top) from the Power Snap unit and load e-gel into machine

25.6 Pipette all 20 µL of diluted ladder from parafilm into lane 1 of e-gel

25.7 Pipette all 20 µL of diluted RAD library from parafilm into lane 2 of e-gel

25.8 Pipette 20 µL of water into remaining empty wells of e-gel

25.9 Close lid, turn Power Snap on, select "E-gel 0.8-2%" program, push start, put camera back on Power Snap unit

Set timer for 15 min

Note

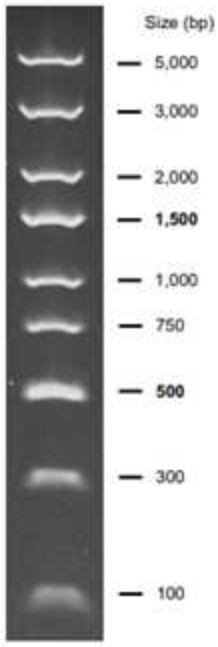
The program's duration is 26 min but the gel only needs to be run for 10-15min

25.10 When timer goes off, tap "view gel" → adjust brightness to max →Take picture → Tap "Export"→ insert thumb drive into usb port → Tap "Export"

25.11 Paste e-gel picture in the BestRAD_library_prep_labData v1.1 spreadsheet under “Final Library” tab

Crop picture to include only lane1 and lane2

Use this image to determine approximate size of library and enter it into “Final Library” tab



size of library should be ~300-500bp

Expected result

21 µL of RAD library at >= 4 ng/µl and a size ~ 300- 500bp

26 **qPCR library quantification**

Note

Equipment needed
p10 pipette and tips
p200 pipette and tips
repeater M-4 pipette
1.5mL Lo-Bind microcentrifuge tubes (Fisher Scientific, 13-698-791)
Applied Biosystems MicroAmp Optical 7500 Plate (Fisher Scientific 43-168-13)
MicroAmp Optical Adhesive Film (Fisher Scientific 43-119-71)
vortex
benchtop centrifuge
0.1 mL Eppendorf combi-tips (Fisher Scientific; 13-683-700)
KimWipes
thumb drive

Equipment

7500 Real Time PCR system	NAME
qPCR	TYPE
Thermo Scientific	BRAND
discontinued	SKU

Reagents needed
NEBNext Library Quant Kit for Illumina - 500 rxns **New England Biolabs Catalog #**E7630L
Invitrogen Nuclease-Free water **Fisher Scientific Catalog #**43-879-36
TE pH 8.0 (1X TE Solution) **IDT Technologies Catalog #**11-01-02-05

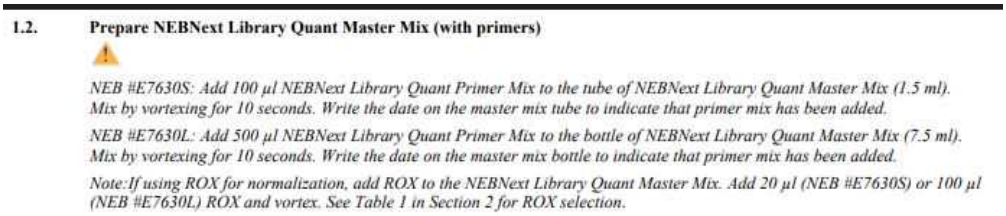
26.1 Thaw reagents in NEBNext library quantification kit and the RAD library

Gently vortex the library, quick spin

26.2 Check small bottle in fridge for 1X dilution of qPCR dilution buffer from NEBNext Library Quant kit. If low, make more:

150 µL 10X Buffer
1350 µL nuclease-free water

26.3 Make sure the master mix in the qPCR kit has been prepped. Look for the "Prep checkbox". If it hasn't, follow instructions below



1.2. Prepare NEBNext Library Quant Master Mix (with primers)

⚠

NEB #E7630S: Add 100 µl NEBNext Library Quant Primer Mix to the tube of NEBNext Library Quant Master Mix (1.5 ml). Mix by vortexing for 10 seconds. Write the date on the master mix tube to indicate that primer mix has been added.

NEB #E7630L: Add 500 µl NEBNext Library Quant Primer Mix to the bottle of NEBNext Library Quant Master Mix (7.5 ml). Mix by vortexing for 10 seconds. Write the date on the master mix bottle to indicate that primer mix has been added.

Note:If using ROX for normalization, add ROX to the NEBNext Library Quant Master Mix. Add 20 µl (NEB #E7630S) or 100 µl (NEB #E7630L) ROX and vortex. See Table 1 in Section 2 for ROX selection.

26.4 Make serial dilutions in 1.5 Lo-Bind tubes as follows:

Note

The accuracy of the qPCR depends on the accuracy and precision of the preparation of the serial dilutions below. In the following steps, each time a dilution is made, it should be thoroughly mixed but you also want to avoid bubbles forming. Annoying, yes.

Note

If the Qubit score from Step 24.5 was > 25 ng/µl, use dilutions starting from 1:100,000 or 1:200,000 in qPCR

If the Qubit score was > 30 ng/µl, make addition dilutions up to 1:2,000,000 dilution

If the Qubit score was high, you may not want to run the more concentrated dilutions (1 and 2).

1. 1:1000 dilution: 1 µL RAD library + 999 µL 1X Buffer
2. 1:10,000 dilution: 10 µL of 1:1000 dilution + 90 µL 1X Buffer
3. 1:100,000 dilution: 10 µL of 1:10,000 dilution + 90 µL 1X Buffer
4. 1:200,000 dilution: 50 µL of 1:100,000 dilution + 50 µL 1X Buffer
5. 1:500,000 dilution: 20 µL of 1:100,000 dilution + 80 µL 1X Buffer
6. 1:1,000,000 dilution: 10 µL of 1:100,000 dilution + 90 µL 1X Buffer
7. 1:2,000,000 dilution: 50 µL of 1:1,000,000 dilution + 50 µL 1X Buffer

26.5 Using a 0.1mL combitip, dispense 8 µL of Quant Master Mix to each well of a MicroAmp Optical 7500 Plate according to plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	STANDARD 2 10pM	STANDARD 2 10pM	STANDARD 2 10pM	LIBRARY 1:100,000	LIBRARY 1:100,000	LIBRARY 1:100,000						
B	STANDARD 3 1pM	STANDARD 3 1pM	STANDARD 3 1pM	LIBRARY 1:200,000	LIBRARY 1:200,000	LIBRARY 1:200,000						
C	STANDARD 4 0.1pM	STANDARD 4 0.1pM	STANDARD 4 0.1pM	LIBRARY 1:500,000	LIBRARY 1:500,000	LIBRARY 1:500,000						
D	STANDARD 5 0.01pM	STANDARD 5 0.01pM	STANDARD 5 0.01pM	LIBRARY 1:1,000,000	LIBRARY 1:1,000,000	LIBRARY 1:1,000,000						
E	NTC	NTC	NTC									
F												
G												
H												

Example of plate layout - all standards and library dilutions are in triplicate

26.6 Using a p10 pipette, add 2 µL of standards (use standards 2-5) from qPCR kit, library dilutions and no template control (NTC; 1X Buffer) to each well according to plate layout. Discard tip after each use.

> Note
>
> Add to side of well. **To avoid bubbles, DO NOT pump**!

26.7 Use MicroAmp Optical Adhesive Film to seal plate (this isn't heat sealed). Use a KimWipe to press down on the seal to avoid smudges

Quick spin **DO NOT VORTEX**

Check underside of plate for dirt/lint, check wells for bubbles

26.8 Turn on 7500 qPCR system. Push to eject tray, load plate, push to close

26.9
1. Open 7500 v2.3 software
2. User name: GUEST
3. Click "Template" → Open “E7630 slow_v2” (screen will be on “Plate setup”)

26.10
1. In left “Setup” column: Click on “Experiment Properties”

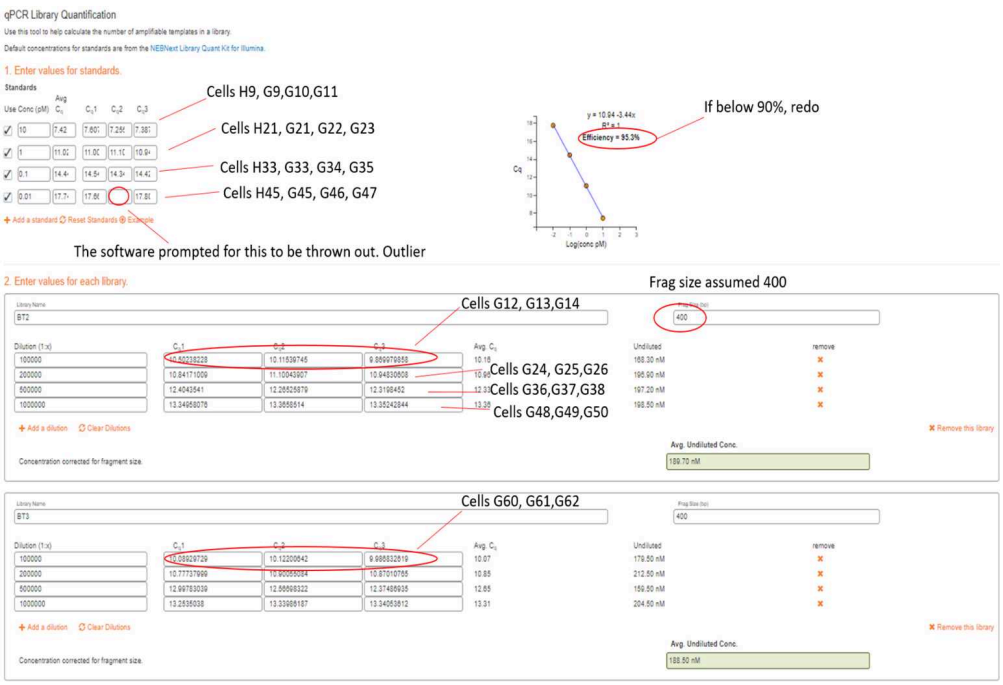
2. Enter in Experiment name (e.g. CarpRADseq_library1_09292022)
3. Leave everything else as is

26.11
1. In left “Setup” column: Click on “Plate Setup”
2. Click on “Assign Targets and Samples” tab
3. Highlight all wells that you’re not using à Right click and select “Clear”

26.12 Click on “Save as” and save to project folder on computer
Start Run (~ 01:00:00)

1h

26.13 Export data.xls onto flash drive, and enter in results on NEB website to get the undiluted concentration of your RAD library
https://nebiocalculator.neb.com/#!/qPCRlibQnt



Example of Nebiocalculator – Efficiency needs to be as close to 100% as possible (+/- 10%), and $R^2 = 1$

Note

Fragment size is the approximate band size obtained from E-gel

26.14 Dilute RAD library to 4nM using V1 = (C2*V2/V1) using 1X TE

Note

C1 = concentration of library according to qPCR
C2 = 4nM
V2 = 50 µL (This may change if you have a very high or low concentrated library. 50µl is appropriate if your library was ~150-250nM)

Keep original and diluted libraries at -20 °C

Expected result

Refer to "BESTRAD on NextSeq Protocol" for how to load the library

Citations

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