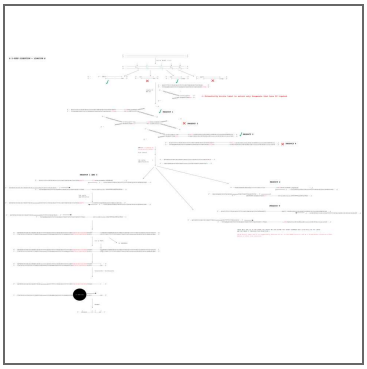

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hyRAD (Suchan et al. 2016; Grealy et al.)

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ABSTRACT

This bench protocol is based on the work of Tomasz Suchan, for performing hyRAD with RNA baits, with some changes.

GUIDELINES

Use at your own risk. You are responsible for double-checking that everything is correct!

Familiarise yourself with the relevent literature (Suchan et al. 2016, Peterson et al.) before attempting!

MATERIALS

MATERIALS

- HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2040S**
- DNase I (RNase-free) - 1,000 units **New England Biolabs Catalog #M0303S**
- MspI - 5,000 units **New England Biolabs Catalog #R0106S**
- EcoRI-HF - 10,000 units **New England Biolabs Catalog #R3101S**
- Tris-HCl, pH 8.0 (UltraPure) **Thermo Fisher Scientific Catalog #15568025**
- Superase-In RNase Inhibitor **Thermofisher Catalog #AM2694**
- 5 M Sodium chloride (NaCl) **Sigma Aldrich Catalog #S5150-1L**
- Mineral oil **Sigma Catalog #M5904**
- Cot1 - Chicken Hybloc DNA **Contributed by users Catalog #CHB**
- 10% SDS solution **Thermo Fisher Scientific Catalog #15553027**

PROTOCOL integer ID:
34631

- RNeasy® Mini Kit **Qiagen Catalog #74104**
- SUPERase• In™ RNase Inhibitor (20 U/µL) **Thermo Fisher Scientific Catalog #cat# AM2694**
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977023**
- UltraPure™ 0.5M EDTA, pH 8.0 **Thermo Fisher Catalog #15575020**
- UltraPure™ SSPE, 20X **Thermo Fisher Catalog #15591043**
- AmpliTaq Gold™ DNA Polymerase with Gold Buffer and MgCl2 **Thermo Fisher Catalog #4311806**
- Denhardt's Solution (50X) **Thermo Fisher Catalog #750018**
- TURBO™ DNase (2 U/µL) **Thermo Fisher Catalog #AM2238**
- SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO **Thermo Fisher Catalog #S7563**
- KAPA HiFi Hotstart PCR kit **Roche Catalog #KK2502**
- 20X SSC **Sigma Aldrich Catalog #S6639-1L**
- Bovine Serum Albumin (BSA) **Contributed by users Catalog #BSA-50**
- dNTPs 100 mM ea. **Bioline Catalog #BIO-39025**
- Salmon sperm DNA 10 mg/ml **Sigma Aldrich Catalog #D7656-1ML**
- Biotin-UTP **Sigma Aldrich Catalog #11388908910**

Note

EcoRI and MspI were chosen based on in-silico analysis of a subset of a bird genome. These enzymes produced approximatley 10,000-20,000 fragments ca. 180 bp (between 150-200 bp range).

	Oligos	Concentration	Working concentration	Synthesis scale	Purification	Supplier	Resuspension buffer	Sequence 5'-3'

	P1.1_EcoRI	100 uM	na	100 nmol	HPLC	IDT	TE	A*C*A*C*TCTTTCCCTACACGACGCTCTTCCGATCTAATACGACTCACTATAGG
	P1.2_EcoRI	100 uM	na	100 nmol	HPLC	IDT	TE	A*A*T*T*CCTATAGTGAGTCGTATTAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT
	P2.1_MspI	100 uM	na	100 nmol	HPLC	IDT	TE	G*T*G*A*CTGGAGTTCAGACGTGTGCTCTTCCGATCTC
	P2.2_MspI	100 uM	na	100 nmol	HPLC	IDT	TE	C*G*G*A*GATCGGAAGAGCGAGAACAA
	IS7	100 uM	10 uM	25 nmol	desalted	IDT	Water	ACACTCTTTCCCTACACGAC
	IS8	100 uM	10 uM	25 nmol	desalted	IDT	Water	GTGACTGGAGTTCAGACGTGT
	CL72_Custom_Seq_Primer	100 uM	na	25 nmol	desalted	IDT	Water	ACACTCTTTCCCTACACGACGCTCTTCC
	CL72_i5_index	100 uM	na	25 nmol	desalted	IDT	Water	GGA AGA GCG TCG TGT AGG GAA AGA GTG T
	CL105_CL106_Std	100 uM	Dilute to 10^11 - 10^2 copies/ul	4 nmol Ultramer	desalted	IDT	TET buffer	ACACTCTTTCCCTACACGACGCTCTTCCTCGTCGTTTGGTATGGCTTCTATCGUATCGATCGATCGACGATCAAGGCGAGTTACATGAAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
	P5-indexing primer	100 uM	10 uM	25 nmol	desalted	IDT	Water	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNACACTCTTTCCCTACACGACGCTCTT
	P7-indexing primer	100 uM	10 uM	25 nmol	desalted	IDT	Water	CAAGCAGAAGACGGCATACGAGATNNNNNNNNGTGACTGGAGTTCAGACGTGT
	BlockF	200 uM	na	100 nmol	HPLC	IDT	Water	
	BlockR	200 uM	na	100 nmol	HPLC	IDT	Water	CAAGCAGAAGACGGCATACGAGATIIIIIIIIGTGACTGGAGTTCAGACGTGTGCTCT/3InvdT /
	P5_F_primer	100 uM	10 uM	25 nmol	desalted	IDT	Water	AATGATACGGCGACCACCGAGATCTACAC

	P7_R_primer	100 uM	10 uM	25 nmol	desalted	IDT	Water	CAAGCAGAAGACGGCATACGAGAT

Equipment

Water bath or heat block
qPCR machine (QuantStudio 3)
Thermal cycler
Qubit 3.0 Fluorometer
LabChip GX II or equivalent fragment analyser
Magnetic rack (e.g., Dynamag)
Pippin HT

Consumables

0.5 ml microcentrifuge tubes for fluoresence
Qubit DNA HS Assay Kit
QIAquick PCR purification kit
Pippin HT cassette 2% Marker B (Sage Science)
0.2 ml Lo-bind PCR tubes
50 ml Falcon tubes
15 ml Falcon tubes
2.0 ml Lo-bind Safelock Eppendorf tubes
1.5 ml Lo-bind Safelock Eppendorf tubes
8-well qPCR tubes

BEFORE START INSTRUCTIONS

Read Suchan et al. (2016)
Also see: dx.doi.org/10.17504/protocols.io.mt2c6qe by George Olah

Store MyOne C1 Streptavidin beads at 4 deg C in a fridge.

Probe synthesis

1 Preparation

1.1

Note

Perform all reaction set-up steps in a reagent-only pre-PCR space inside a dedicated ultraclean environment. Add DNA and subsequent master-mixes to the reaction, and perform wash steps, in a separate pre-PCR space.

"Suit up" in this order: hair net, nitrile gloves, facemask, coveralls, gumboots, booties, second pair of gloves.

1.2 Prepare the space by decontaminating surfaces with 10% household bleach followed by 70% ethanol. UV irradiate pipettes and racks. Racks should be bleached between subsequent uses and UV irradiated.

1.3 Ensure ice is available. Thaw reagents on ice as needed. Keep enzymes on ice at all times. Do not vortex enzymes to mix but mix by flicking the tube gently. Pulse centrifuge all reagents before opening.

1.4 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-bind Safelock tube	1	10 mM Tris-HCl
	1.5 ml Lo-bind Safelock tube	1	10X Annealing Buffer
	0.5 ml Lo-bind Safelock tube	2	P1 and P2 adapter oligos
	0.2 ml Lo-bind PCR tube	1	Annealing P1 and P2 adapter oligos

1.5 Prepare all necessary buffers and UV decontaminate where appropriate.

Note

Aliquot 5X SYBR into 500-ul batches and store at -20 deg C in foil.

Aliquot dNTPs into 50-ul batches and store at -20 deg C.

	Buffer	Reagent	Volume to add	Final concentration in solution
	10 mM Tris-HCl	1 M Tris-HCl	10 ul	10 mM
		Ultrapure water	990 ul	na
	10 X Annealing Buffer	1 M Tris-HCl	100 ul	100 mM
		5 M NaCl	100 ul	500 mM
		0.5 M EDTA	20 ul	10 mM
		Ultrapure water	780 ul	na

1.6 Before resuspending oligos, pulse centrifuge to collect the pellet at the bottom of the tube. Add the appropriate buffer (see Materials) and vortex thoroughly. Store at -20 deg C. Dilute out the working concentrations (below) and store at -20 deg C when not in use. Thaw on ice. Vortex and pulse centrifuge after each thaw. Before beginning library preparation, make sure you have enough of each working stock prepared!

Note

Note: Do not store oligos and adapters in the same box as enzymes or reagents!

The standards should be diluted in a totally different space, such as a teaching lab to ensure it does not contaminate the lab at extremely high concentration.

Also take extreme care with the positive control oligo as it will become a template for library preparation!

	Working stock	Reagent	Volume to add
	10 uM P5_Indexing_Primer	100 uM Stock	50 ul
		Ultrapure water	450 ul
	10 uM P7_Indexing_Primer	100 uM Stock	50 ul
		Ultrapure water	450 ul

1.7 Pre-program the thermal cycler.

1.8 Combine the following in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)

	P1.1 adapter oligo (EcoRI)	100 ul	100 uM	10 uM	10 ul
	P1.2 adapter oligo (EcoRI)	100 ul	100 uM	10 uM	10 ul
	Annealing buffer	100 ul	10 X	1 X	10 ul
	Ultrapure water	100 ul	na	na	70 ul

1.9 Combine the following in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)
	P2.1 adapter oligo (MspI)	100 ul	100 uM	10 uM	10 ul
	P2.2 adapter oligo (MspI)	100 ul	100 uM	10 uM	10 ul
	Annealing buffer	100 ul	10 X	1 X	10 ul
	Ultrapure water	100 ul	na	na	70 ul

1.10 Incubate in a thermal cycler at:

95 deg C for 1 min
Cool at a rate of 0.1 deg C / sec until the solution reaches 20 deg C

Store at -20 deg C.

2 Enzymatic digestion

2.1 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Step 2.2 Master mix
	0.2 ml Lo-Bind PCR tube	# of samples	Enzymatic digestion for samples

2.2 Combine the following in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)
	CutSmart Buffer (NEB)	50 ul	10 X	1 X	5 ul
	EcoRI-HF (NEB)	50 ul	20 U/ul	0.4 U/ul (20 U)	1 ul
	MspI (NEB)	50 ul	20 U/ul	0.4 U/ul (20 U)	1 ul
	Ultrapure water	50 ul	na	na	33 ul

Note

EcoRI-HF (NEB) is active for >8 hr.
MspI (NEB) is active between 2-4 hr.

Note: This reaction has been scaled up to a 50 ul reaction as NEB recommends not leaving 10 ul reactions longer than 1 hr due to evaporation. Also, 10-20 U fo enzyme is recommended to digest 1 ug of DNA in a reaction volume of 50 ul for 1 hr. So, to ensure complete digestion of 1 ug of genomic DNA, 20 U of each enzyme is used n a reaction volume of 50 ul for 4 hr. MspI is not active beyond 4 hr. Also, the enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol.

Consider performing several reactions to obtain enough DNA for quality control along the way. I have started with 4 ug of DNA (split into 4 reactions--two DNA extracts performed in duplicate).

The ddRAD protocol recommends NOT to heat-denature restriction enzymes as they will be removed ruing the SPRI bead cleanup that follows below.

2.3 Add 40 ul of master mix to 10 ul of DNA (at 100 ng/ul ca. 1000 ng total) in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge.

Note

Use high-quality DNA: quantify the concentration using the Qubit 3.0 fluorometer BR kit following the manufacturer's instructions:

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

Estimate the purity of the DNA using a NanoDrop spectrophotometer, following the manufacturer's instructions:

https://assets.thermofisher.com/TFS-Assets/CAD/manuals/NanoDrop-2000-User-Manual-EN.pdf

Note: do not pay attention to the DNA quanitity proveided by the NanoDrop or fragment analyser--the Qubit is much more accurate as it measures double-stranded DNA only.

Run the DNA on a fragment analyser or gel electrophoresis to determine the fragment length distribution:

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

Protocol

NAME

2% Agarose Gel Electrophoresis

CREATED BY

Alicia Grealy

PREVIEW

Expected result

DNA needs to be 100 ng/ul in 10 ul volume (i.e., 1 ug). Dilute to the sample to this concentration in Ultrapure water if needed.

e.g., I typically begin with samples that measure 1100 ng/ul in 100 ul (110 ug total):
In the example below, sample MD#033 was 1000 ng/ul in 100 ul
MD#034 was 1100 ng/ul in 100 ul

Pure DNA should have a 260/280 ratio of between 1.8-2.0 and a 230/260 ratio of 2.0-2.2. If the DNA is not pure, consider cleaning the neat extract using your method of choice (e.g., sodium-acetate/ethanol precipitation, etc.)

Fresh tissue should yield a high molecular weight band on an agarose gel (i.e., above 10 kb), with minimal smearing (as smearing indicates degradation).

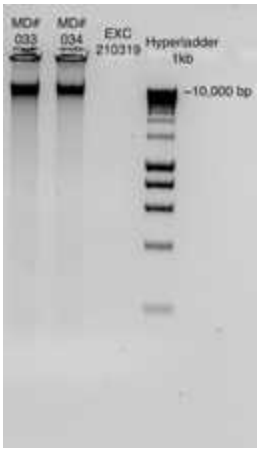


Figure 1. An example of decently high-quality DNA extracts run on a 2% agarose gel electrophoresis (@ 80V for 1 hr 10 min), though there is some degradation.

Note

Add the DNA in a physically separate space, suitable for 'modern' DNA (i.e., NOT inside an ultraclean environment).

2.4 Incubate in a thermal cycler at:

37 deg C for 4 hr
Hold at 4 deg C

2.5 Combine any replicates.

2.6 *SPRI bead clean-up*

Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 2X beads : reaction volume (i.e., 160 ul). Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 20 ul of 10 mM Tris-HCl.

Note

This step is to remove the enzymes only. To avoid losing product, use a ration of 1.8-2X beads, which should keep everything above 100 bp without too much yield loss.

2.7 *Qubit*

Measure the concentration using the Qubit HS kit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

Expected result

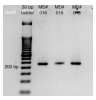
Expect a loss of around 3%.

e.g., MD#034 = 97 ng/ul in 20 ul (or 1940 ng total). 2 ug of this sample was input into the reaction, so the loss is approximately 3%.

2.8 *Agarose gel electrophoresis*

Electrophorese 2 ul of product (200-500 ng in 5-10 ul) on a 2% agarose gel.

Protocol



NAME

2% Agarose Gel Electrophoresis

CREATED BY

Alicia Grealy

PREVIEW

Expected result

Digested DNA should show a smear rather than a large band. However, some high-molecular weight fragments may remain but they won't be the same size as previously--it's just difficult to resolve these fragments on a gel (i.e., it is difficult to distinguish between 30,000 bp and 10,000 bp on this kind of gel).

Note: I have tested putting more enzyme into the reaction the results look the same.

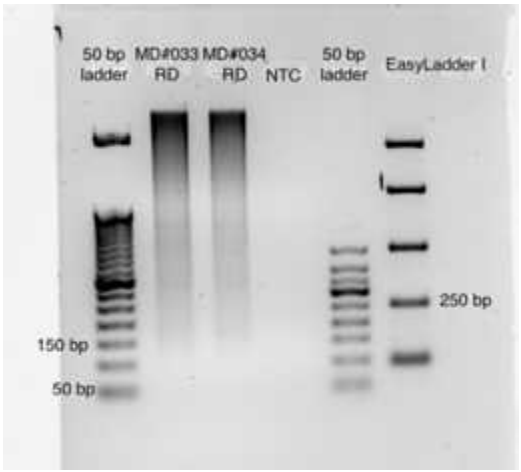


Figure 2. An example of DNA that has undergone the restriction digest with EcoRI-HF and MspI using the reaction conditions described above.

2.8.1 Make up 2 L of 1X TAE buffer:

50 ml of 40X TAE buffer
1950 ml of MilliQ water

Mix by inversion.

2.8.2 Using an electronic balance, weigh out 2.2 g of agarose powder on to a weigh boat using a spatula.

Note

Note that gel concentration can be adjusted. The more concentrated the gel, the greater the resolution of small fragment sizes.

2.8.3 Transfer the powder to a 200 ml conical flask.

2.8.4 Using the graduated measurng cylinder, measure out 110 ml of 1xTAE buffer. Add to the conical flask containing the agarose powder. Swirl the flask gently to mix.

2.8.5 Microwave the flask uncovered for 1 minute.

Safety information

A lid can loosly be placed over the flask but DO NOT tighten--allow steam to escape. Do not microwave for more than 1 minute at a time.

Do not fill flasks or beakers/Schott bottles more than half full with liquid!

2.8.6 Remove the flask from the microwave using oven mitts and swirl gently.

Safety information

The liquid is boiling! Use oven mitts to handle flask. Do not put your face over the opening to the flask as liquid can splash out!

2.8.7 Microwave the flask for a further minute but remove from the microwave if the agarose appears to boil excessively. Swirl to mix and examine near a light source to ensure the agarose has melted. Allow to cool for 5 minutes.

2.8.8 Place the gel casting tray into a rubber vice that will seal the ends tightly, or tape the ends with masking tape.

2.8.9 Place the assembly on a flat bench and use the spirit level to check it is level--adjust if needed.

2.8.10 Place a 20-well comb into the casting tray.

2.8.11 When the flask is cool to the touch, add 5 ul of SYBR Safe and swirl gently to mix. Avoid generating bubbles.

Safety information

Wait until the liquid is warm (not boiling) to cast the gel, or the tray may crack!

2.8.12 Pour the liquid gel slowly into the casting tray. Pop any bubbles that have formed using a clean pipette tip.

2.8.13 Let the gel set for 20-30 minutes at room temperature.

2.8.14 Allow residual gel to set in the flask, then scrape into the bin. Fill the flask half full with water and microwave until the water boils. Pour the water down the sink and clean the

flask using a bottle brush.

2.8.15 When the gel is set, remove the combs gently.

2.8.16 Place the casting try and gel in the electrophoresis tank.

2.8.17 Fill the electrophoresis tank with 1 X TAE buffer to the fill line indicated on the tank.

2.8.18 Pipette 3 ul of 50 bp DNA ladder into the first well of the gel.

Note

The recommended volume will depend on the concentration of the ladder. Check the manufacterer's recommendations. If the ladder is not pre-mixed with loading dye, be sure to add 1-2 ul of loading dye before loading into the gel.

2.8.19 Place some Parafilm across a 96-well PCR plate rack and press down firmly to create small wells.

2.8.20 For each sample, pipette 1-2 ul of loading dye onto the Parafilm, taking care not to pierce the Parafilm.

2.8.21 Mix 10 ul of PCR product with the loading dye by pipetting gently up and down.

2.8.22 Transfer the 12 ul of PCR product/loading dye to the wells of the gel, taking care not to pierce the bottom of the well with the pipette tip.

Note

The volume each well can take will depend on the size of the comb used. Be sure not to overload the wells or product will float out the top of the well.

2.8.23 Place the lid on the gel tank and plug the electrodes into the appropriate power slots. Ensure the positive electrode is at the base of the gel.

Safety information

Take care when working with electricity and water!

Check electrical cords of all equipment and ensure none are damaged and that cords are not a tripping hazard. Do not use if the electrical cord is damaged in any way. Tag the instrument with warnings, make the area safe, and notify your line manager and anyone else in the immediate area that may be affected.

Use electrical equipment indoors only in an area free of explosive material, corrosive gas, powerful vibrations, direct exposure to sunlight, and temperature fluctuations. Use in a space where cables will not come into contact with liquids, be manually damaged, or interfere with other workplace operations.

Do not use electrical equipment with any other power adapter or cord than the one supplied.

2.8.24 Switch the power pack on a set the voltage to 80 V and the time to 1 hr and 10 min.

Note

Note that the voltage and time can be adjusted to suit what you are running on the gel. For amplicons (one small product), I will run the gel at 96 V for 30-40 min. For shotgun libraries, I will run the gel as above. The lower the voltage and longer it is run, the greater the separation of fragments will be.

Press 'Run' or 'Start' on the power pack and check to see that bubbles are rising from

2.8.25 electrodes.

2.8.26 When the run is over, switch of the power pack, remove the lid, and remove the gel from the tank, taking care not to let it slide off the tray.

Safety information

Do not remove the lid to the electrophoresis tank until the power pack is switched off.

2.8.27 

Place the gel on the UV transilluminator and photograph using the attached camera. Follow the manufactuerer's instructions to use the equipment.

Safety information

Take care working with UV. You should have UV safety training. Do not open the transilluminator while the UV is on! Use signage to warn others when the UV is on.

2.8.28 Discard the gel into a designated biohazard bin, and clean the UV dock with 70% ethanol.

2.8.29 Dispose of used tips into a designated sharps container.

2.8.30 Dispose of gel waste into a biohazard bag.

2.8.31 Used combs, beakers, flasks, and tray should be washed with warm water and placed on a rack to dry.

2.8.32 Gloves and chemical waste should be sealed in a biohazard bag for incineration.

3 Adapter ligation

3.1 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Step 3.2 Master mix
	0.2 ml Lo-Bind PCR tube	# of samples x 2	Reactions, two per sample

3.2 Combine the following in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)
	CutSmart Buffer (NEB)	20 ul	10 X	1 X	2 ul
	P1 adapter	20 ul	10 uM	0.5 uM	1 ul
	P2 adapter	20 ul	20 uM	1 uM	1 ul
	ATP	20 ul	100 mM	1 mM	0.2 ul
	T4 DNA ligase	20 ul	400 U/ul	20 U/ul (400 U)	1 ul

	Ultrapure water	20 ul	na	na	5.8 ul

Note

Peterson et al. suggests to use 2-10 fold adapters : sticky ends in a 40 ul reaction volume. Based on the approximate average fragment size and the # ng input of DNA, calculate the molarity of the DNA ends using:

https://nebiocalculator.neb.com/#!/dsdnaends

e.g., 500 ng of 275 bp DNA has 5.883 pmol DNA ends. So, for 10X adapters we would need ca. 30 pmol DNA ends per adapter, which, at ~45 bp, would be about 400 ng.

Molecular weight of P1 adapter = 32.7507 kDa. 10 uM P1 = 327.507 ng/ul
Molecular weight of P2 adapter = 18.3674 kDa. 20 uM P2 = 367.348 ng/ul

So using 1 ul of each at these concentrations should be enough for this quantity of input DNA.

e.g., if we have 14 ul of digested DNA at 97 ng/ul and we perform the ligation in duplicate, that would make the input 679 ng. If we estimate the average fragment length of the smear at 800 bp, this would be 2.7 pmol DNA ends. Even if we overestimated the fragment length (and it is actually 400 bp), the above amounts of adapter would still be in excess.

Adapter dimer will be removed with the size selection so it shouldn't be too bad if the adapters are in excess.

3.3 Add 11 ul of the above master mix to 9 ul of digested DNA in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge.

3.4 Incubate in a thermal cycler at:

16 deg C for ca. 20 hr (16 hr - overnight)

Combine any replicates.

Note

NEB suggest to perform the ligation overnight.

3.5 *SPRI bead clean-up*

Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 2X beads : reaction volume (i.e., 160 ul). Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 21 ul of 10 mM Tris-HCl.

Note

This step is to remove the T4 DNA ligase, adapter dimer (ca. 90 bp) and unligated adapter (55 and 35 bp).

At this stage we "could" remove some of the fragments that are not of interest. A right-sided selection followed by a left-sided selection can be sued to select fragments within a certain range. Potentially a simple right-sided selection may be beneficial here because it will remove larger fragments without such a loss of yield of smaller fragments that may be of interest. Trying to remove small fragments of litte interest (e.g. <100 bp) may result in a substantial loss of yield for those in the target range (180-300 bp).

For a right-sided selection, a ratio of 0.6 X beads should remove most fragments >500 bp without substantial reduction in yield in the 100-300 bp size range.

For a left sided-selection, a ratio of 1.6 X beads should remove most fragments <100 bp without a substantial reduction in yield in the 100-300 bp size range.

BUT this would only really be beneficial if doing the amplification BEFORE size-selection.

3.6 *Qubit*

Measure the concentration using the Qubit HS kit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

Expected result

Expect approximately 30% loss of yield.

e.g., MD#034 = 37.8 ng/ul (or 945 ng total), which is a 30% loss.

4 qPCR quant the RAD library

Note

This step is to make sure the ligation worked and to see what sized fragments would amplify. This step could be performed with alongside standards of known concentration to calculate the number of libarary molecules output in the RAD library.

You can also run more of a serial dilution of the library to ensure the quantitation is accurate. Here, I only input 1 ul of the neat library and 1 ul of a 1 in 20 dilution.

It could be possible to amplify the RAD library with indexes and THEN perform size selection. However, I am not sure how this may bias the probe set as it has not been tested, so I follow recommendations of Suchan et al. (2016), performing the size selection BEFORE the indexing PCR/library amplification.

After initial testing, I routinely skip this step

4.1 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Step 4.2 Master mix
	8-well strip qPCR tubes 0.1 ul profile	1	PCR amplification

4.2 Combine the following in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

	Reagent	V2	C1	C2	V1	x _____ rxn
	Ultrapure water	25 ul	na	na	15.9 ul	
	BSA	25 ul	10 mg /ml	0.4 mg/ml	1 ul	
	ABI Gold PCR Buffer	25 ul	10 X	1 X	2.5 ul	
	MgCl2	25 ul	25 mM	2.5 mM	2.5 ul	
	dNTPs	25 ul	25 mM	0.25 mM	0.25 ul	
	ABI Taq Gold DNA polymerase	25 ul	5 U/ul	0.05 U/ul	0.25 ul	
	SYBR Green	25 ul	5 X	0.12 X	0.6 ul	
	IS7	25 ul	10 uM	0.2 uM	0.5 ul	
	IS8	25 ul	10 uM	0.2 uM	0.5 ul	

4.3 Add 24 ul of master mix to the corresponding PCR tubes. Pulse centrifuge the tubes.

4.4 Dilute each library 1 in 20 (i.e., 1 ul of library in 19 ul of Ultrapure water). Vortex and pulse centrifuge.

4.5 Add 1 ul of DNA sample to the corresponding PCR tubes according to the scheme below. Pulse centrifuge the tubes.

e.g.,

	PCR NTC			
	PCR NTC			
	MD#033 Neat			

	MD#033 1 in 20			
	MD#034 Neat			
	MD#034 1 in 20			

4.6 Take the strip tubes to a post-PCR space. Place in thermal cycler and run the following program:

95 deg C for 10 min

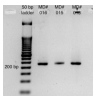
Followed by 50 cycles of:

95 deg C for 30 sec
60 deg C for 30 sec
72 deg C for 30 sec

4.7 *Agarose gel electrophoresis*

Electrophorese 2 ul of product (200-500 ng in 5-10 ul) on a 2% agarose gel.

Protocol



NAME

2% Agarose Gel Electrophoresis

CREATED BY

Alicia Grealy

PREVIEW

Expected result

The longest adapter dimer will be 112 bp but most should have been removed with the SPRI bead purification. Inserts of 50 bp will be 162 bp. If there are many fragments outside the target range (180 bp insert, i.e., 272 bp with adapters or say 250-300 bp), then definitely size select BEFORE indexing/amplification. If most amplified fragments are within the desired size range, it MAY be possible to size select after indexing/library amplification, but I have not tested this, and do not know whether it would bias the probe set in a different way. It is possible that size selecting after indexing/library amplification would deplete the yield so much as to require more amplification anyway.

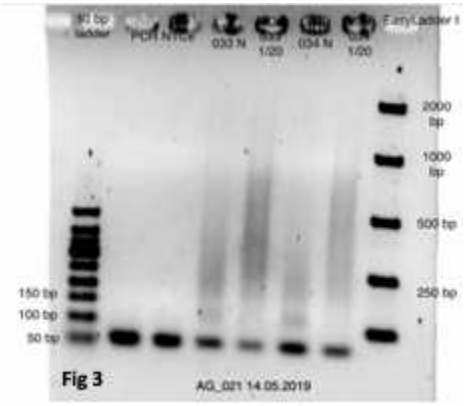


Figure 3. An example of the size of fragments that would be amplified from the RAD library BEFORE size selection. It does appear that ligation worked. Amplification of the neat extract does appears to be inhibited, so if quantitating the number of molecules via qPCR using a standard, perhaps run a small dilution series for each library.

4.7.1 Make up 2 L of 1X TAE buffer:

50 ml of 40X TAE buffer
1950 ml of MilliQ water

Mix by inversion.

4.7.2 Using an electronic balance, weigh out 2.2 g of agarose powder on to a weigh boat using a spatula.

Note

Note that gel concentration can be adjusted. The more concentrated the gel, the greater the resolution of small fragment sizes.

4.7.3 Transfer the powder to a 200 ml conical flask.

4.7.4 Using the graduated measurng cylinder, measure out 110 ml of 1xTAE buffer. Add to the conical flask containing the agarose powder. Swirl the flask gently to mix.

4.7.5 Microwave the flask uncovered for 1 minute.

Safety information

A lid can loosly be placed over the flask but DO NOT tighten--allow steam to escape. Do not microwave for more than 1 minute at a time.

Do not fill flasks or beakers/Schott bottles more than half full with liquid!

4.7.6 Remove the flask from the microwave using oven mitts and swirl gently.

Safety information

The liquid is boiling! Use oven mitts to handle flask. Do not put your face over the opening to the flask as liquid can splash out!

4.7.7 Microwave the flask for a further minute but remove from the microwave if the agarose appears to boil excessively. Swirl to mix and examine near a light source to ensure the agarose has melted. Allow to cool for 5 minutes.

4.7.8 Place the gel casting tray into a rubber vice that will seal the ends tightly, or tape the ends with masking tape.

4.7.9 Place the assembly on a flat bench and use the spirit level to check it is level--adjust if needed.

4.7.10 Place a 20-well comb into the casting tray.

4.7.11 When the flask is cool to the touch, add 5 ul of SYBR Safe and swirl gently to mix. Avoid generating bubbles.

Safety information

Wait until the liquid is warm (not boiling) to cast the gel, or the tray may crack!

4.7.12 Pour the liquid gel slowly into the casting tray. Pop any bubbles that have formed using a clean pipette tip.

4.7.13 Let the gel set for 20-30 minutes at room temperature.

4.7.14 Allow residual gel to set in the flask, then scrape into the bin. Fill the flask half full with water and microwave until the water boils. Pour the water down the sink and clean the flask using a bottle brush.

4.7.15 When the gel is set, remove the combs gently.

4.7.16 Place the casting try and gel in the electrophoresis tank.

4.7.17 Fill the electrophoresis tank with 1 X TAE buffer to the fill line indicated on the tank.

4.7.18 Pipette 3 ul of 50 bp DNA ladder into the first well of the gel.

Note

The recommended volume will depend on the concentration of the ladder. Check the manufacterer's recommendations. If the ladder is not pre-mixed with loading dye, be sure to add 1-2 ul of loading dye before loading into the gel.

4.7.19 Place some Parafilm across a 96-well PCR plate rack and press down firmly to create small wells.

4.7.20 For each sample, pipette 1-2 ul of loading dye onto the Parafilm, taking care not to pierce the Parafilm.

4.7.21 Mix 10 ul of PCR product with the loading dye by pipetting gently up and down.

4.7.22 Transfer the 12 ul of PCR product/loading dye to the wells of the gel, taking care not to pierce the bottom of the well with the pipette tip.

Note

The volume each well can take will depend on the size of the comb used. Be sure not to overload the wells or product will float out the top of the well.

4.7.23 Place the lid on the gel tank and plug the electrodes into the appropriate power slots. Ensure the positive electrode is at the base of the gel.

Safety information

Take care when working with electricity and water!

Check electrical cords of all equipment and ensure none are damaged and that cords are not a tripping hazard. Do not use if the electrical cord is damaged in any way. Tag the instrument with warnings, make the area safe, and notify your line manager and anyone else in the immediate area that may be affected.

Use electrical equipment indoors only in an area free of explosive material, corrosive gas, powerful vibrations, direct exposure to sunlight, and temperature fluctuations. Use in a space where cables will not come into contact with liquids, be manually damaged, or interfere with other workplace operations.

Do not use electrical equipment with any other power adapter or cord than the one supplied.

4.7.24 Switch the power pack on a set the voltage to 80 V and the time to 1 hr and 10 min.

Note

Note that the voltage and time can be adjusted to suit what you are running on the gel. For amplicons (one small product), I will run the gel at 96 V for 30-40 min. For shotgun libraries, I will run the gel as above. The lower the voltage and longer it is run, the greater the separation of fragments will be.

4.7.25 Press 'Run' or 'Start' on the power pack and check to see that bubbles are rising from electrodes.

4.7.26 When the run is over, switch of the power pack, remove the lid, and remove the gel from the tank, taking care not to let it slide off the tray.

Safety information

Do not remove the lid to the electrophoresis tank until the power pack is switched off.

4.7.27 Place the gel on the UV transilluminator and photograph using the attached camera. Follow the manufactuerer's instructions to use the equipment.

Safety information

Take care working with UV. You should have UV safety training. Do not open the transilluminator while the UV is on! Use signage to warn others when the UV is on.

4.7.28 Discard the gel into a designated biohazard bin, and clean the UV dock with 70% ethanol.

4.7.29 Dispose of used tips into a designated sharps container.

4.7.30 Dispose of gel waste into a biohazard bag.

4.7.31 Used combs, beakers, flasks, and tray should be washed with warm water and placed on a rack to dry.

4.7.32 Gloves and chemical waste should be sealed in a biohazard bag for incineration.

5 Size selection using Pippin HT

Note

The maximum input into the Pippin HT is 1 ug /lane sheared genomic DNA (i.e. 75 ng/ul in 20 ul)
The minimum input into the Pippin HT is 15 ng /lane sheared genomic DNA

5.1

Depending on the total yield and concentration determined using the Qubit (see Step 3.6 above), run approximately 1 ug of DNA in 20 ul (ca. 75 ng/ul) of each samples across a lane of a PippinHT electrophoresis system (2% gel, Marker 20B), selecting fragments 272 bp in size (272 bp peak with tight range 212-332 bp--this equates to an insert size of 180 bp) and following the manufacturer's instructions:

http://www.sagescience.com/wp-content/uploads/2015/10/PippinHT-Operations-Manual-Rev-B_460005.pdf

Note

180 bp insert size / probe size was used by Suchan et al. (2016) and Schmitt et al. (2018?). It is possible to make probes smaller than this, which may be beneficial for caputuring ancient DNA, but be sure to check the restriction enzymes used will generate enough loci within this size range.

5.2 *SPRI cleanup*

Combine any replicates. Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 2X beads : reaction volume. Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 21 ul of Ultrapure water.

Note

This cleanup is for the purpose of buffer exchange, but also will concentrate any replicates into a smaller working volume.

Qubit

5.3

Measure the concentration using the Qubit HS kit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

Expected result

Expect approximately 99% loss of yield.

e.g., 996 ng was input into the PippinHT lane for MD#033 and 9.724 ng came out (1%)

6 qPCR quant the size-selected RAD library

Note

This step is to make sure the size-selection worked. This qPCR could be performed alongside standards of known concentration to quantify the number of library molecules present in the RAD library after size selection.

After initial testing, I routinely skip this step.

Follow Steps 4.1 - 4.7 above to perform the qPCR if so desired.

Expected result

If the average insert size is 180 bp, then there should be a tight smear around ...

7 Index / amplify the RAD library

7.1 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Step 7.2 Master mix

	8-well strip qPCR tubes 0.1 ul profile	1	PCR amplification

7.2 Combine the following in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge. Ensure to prepare enough master mix for multiple reactions per library plus pipetting error.

Safety information

If you are not interested in sequencing the probes at all, then you can replace the indexing primers with IS7 and IS8 primers that will just amplify the library. If you aren't interested in knowing which probes came from which extract, then all reactions may be amplified with the same indexing primers.

Note

I have typically performed the indexing / library amplification in quadruplicate reactions, using 5 ul of library per reaction (using the up whole library). You can input less DNA into each reaction and perform more replicates if you desire more DNA at the end. This will mean you have more clonal probes, but that is not too much of an issue here because we do want several copies of each probe. You can also perform more cycles, though having little DNA and increasing the number of cycles can generate amplification artefacts. Typically I will perform 30 cycles, just enough to take the reaction to plateau.

Note

Remember that each library will have it's own unique combination of forward and reverse indexing primers. **Do not add these to the master mix**, but add each to each reaction individually! Take great care not to cross-contaminate primers: only have one tube open at a time. Use qPCR tubes with individual capped lids (not strip lids!).

Note

Ideally, indexing combinations should never be reused in the lab. Be sure to follow Illumina's recommendations when chosing primer combinations (e.g., ensure adequate diversity in the bases, ensure each is at least 3 bp different from each other, don't use indexes that will begin with two dark cycles, etc.). For instance, the NextSeq cannot read "GG" a the start of an index (so indexes should not end in "CC" as they are sequenced in the reverse complement).

	Reagent	V2	C1	C2	V1	x ____ rxn
	Ultrapure water	50 ul	na	na	29.5 ul	
	KAPA High Fidelity Buffer	50 ul	5 X	1 X	10 ul	
	P5_indexing_primer	50 ul	5 uM	0.2 uM	2 ul	Do not add to master mix
	P7_indexing_primer	50 ul	5 uM	0.2 uM	2 ul	Do not add to master mix
	KAPA HiFi Hot Start DNA Polymerase	50 ul	1 U/ul	0.02 U/ul	1 ul	
	dNTPs	50 ul	25 uM	0.25 uM	0.5 ul	

7.3 Add 41 ul of master mix to the corresponding PCR tubes. Pulse centrifuge the tubes.

7.4 Add 2 ul of the corresponding forward indexing primer to the appropriate reaction tube. Pulse

centrifuge the tubes.

7.5 Add 2 ul of the corresponding forward indexing primer to the appropriate reaction tube. Pulse centrifuge the tubes.

7.6 Add 5 ul of purified size-selected RAD library to the corresponding PCR tubes according to the scheme below. Pulse centrifuge the tubes.

> **Note**
>
> The number of ng of library input into each reaction for me is typically 1.25 - 2.21 ng. This is perhaps too little DNA for the number of cycles given, and may have generated an artefact. Potentially aim for 5-10 ng if possible.

e.g.,

	MD#033			
	MD#033			
	MD#033			
	MD#033			
	MD#034			
	MD#034			
	MD#034			
	MD#034			

7.7 Take the strip tubes to a post-PCR space. Place in thermal cycler and run the following program:

98 deg C for 10 min

Followed by 30 cycles of:

985 deg C for 20 sec
60 deg C for 30 sec
72 deg C for 40 sec

Then a final extension of:

72 deg C for 10 min

7.8 Pool replicate reactions into a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

7.9 *SPRI bead clean-up*

Purify the libraries using SeraMag Speed Beads or SeraMag Select using a1.4X beads : reaction volume. Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 40 ul of Ultrapure water.

Note

This cleanup is to remove PCR reagents and primer dimer, and to concentrate the replicates. Dimer should be around 160 bp. Fragments of interest are approximately 340 bp. This ratio of beads should remove almost everything below 200 bp.

7.10 *Qubit*

Measure the concentration using the Qubit HS kit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

Expected result

Expect amplification to increase the DNA about 250-300 X.

e.g., Approximately 9 ng of MD#033 input across 4 PCR reactions turned into 2392 ng total, so yield increased 265-fold.

Approximately 5 ng of MD#034 input across 4 PCR reactions turned into 1520 ng total, so yield increased 304-fold.

7.11 *Agarose gel electrophoresis*

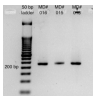
Electrophorese 5 ul of product (200-500 ng in 5-10 ul) on a 2% agarose gel.

Note

For any gel steps, a fragment analyser platform may alternatively be used, but I have found it to be much more straight forward to perform a gel.

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

Protocol



NAME

2% Agarose Gel Electrophoresis

CREATED BY

Alicia Grealy

PREVIEW

Expected result

There should be a tight smear around a peak of 340 bp with little product below 200 bp. Insert sizes of 0 bp will be at 160 bp. Indexing dimer should be at 115 bp, but the purification should have removed most of this.

e.g.,

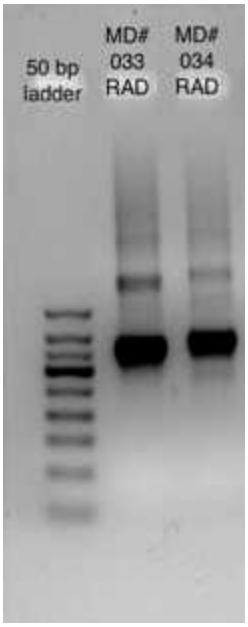


Figure 4. A large band can be seen around 350 bp, however, there is also a larger fragment above 450 bp present. This may be PCR artefact from too many cycles given a small input of DNA (see Belt and Demarini, 1991). This was not investigated further.

7.11.1 Make up 2 L of 1X TAE buffer:

50 ml of 40X TAE buffer
1950 ml of MilliQ water

Mix by inversion.

7.11.2 Using an electronic balance, weigh out 2.2 g of agarose powder on to a weigh boat using a spatula.

Note

Note that gel concentration can be adjusted. The more concentrated the gel, the greater the resolution of small fragment sizes.

7.11.3 Transfer the powder to a 200 ml conical flask.

7.11.4 Using the graduated measurng cylinder, measure out 110 ml of 1xTAE buffer. Add to the conical flask containing the agarose powder. Swirl the flask gently to mix.

7.11.5 Microwave the flask uncovered for 1 minute.

Safety information

A lid can loosly be placed over the flask but DO NOT tighten--allow steam to escape. Do not microwave for more than 1 minute at a time.

Do not fill flasks or beakers/Schott bottles more than half full with liquid!

7.11.6 Remove the flask from the microwave using oven mitts and swirl gently.

Safety information

The liquid is boiling! Use oven mitts to handle flask. Do not put your face over the opening to the flask as liquid can splash out!

7.11.7 Microwave the flask for a further minute but remove from the microwave if the agarose appears to boil excessively. Swirl to mix and examine near a light source to ensure the agarose has melted. Allow to cool for 5 minutes.

7.11.8 Place the gel casting tray into a rubber vice that will seal the ends tightly, or tape the ends with masking tape.

7.11.9 Place the assembly on a flat bench and use the spirit level to check it is level--adjust if needed.

7.11.10 Place a 20-well comb into the casting tray.

7.11.11 When the flask is cool to the touch, add 5 ul of SYBR Safe and swirl gently to mix. Avoid generating bubbles.

Safety information

Wait until the liquid is warm (not boiling) to cast the gel, or the tray may crack!

7.11.12 Pour the liquid gel slowly into the casting tray. Pop any bubbles that have formed using a clean pipette tip.

7.11.13 Let the gel set for 20-30 minutes at room temperature.

7.11.14 Allow residual gel to set in the flask, then scrape into the bin. Fill the flask half full with water and microwave until the water boils. Pour the water down the sink and clean the flask using a bottle brush.

7.11.15 When the gel is set, remove the combs gently.

7.11.16 Place the casting try and gel in the electrophoresis tank.

7.11.17 Fill the electrophoresis tank with 1 X TAE buffer to the fill line indicated on the tank.

7.11.18 Pipette 3 ul of 50 bp DNA ladder into the first well of the gel.

Note

The recommended volume will depend on the concentration of the ladder. Check the manufacterer's recommendations. If the ladder is not pre-mixed with loading dye, be sure to add 1-2 ul of loading dye before loading into the gel.

7.11.19 Place some Parafilm across a 96-well PCR plate rack and press down firmly to create small wells.

7.11.20 For each sample, pipette 1-2 ul of loading dye onto the Parafilm, taking care not to pierce the Parafilm.

7.11.21 Mix 10 ul of PCR product with the loading dye by pipetting gently up and down.

7.11.22 Transfer the 12 ul of PCR product/loading dye to the wells of the gel, taking care not to pierce the bottom of the well with the pipette tip.

Note

The volume each well can take will depend on the size of the comb used. Be sure not to overload the wells or product will float out the top of the well.

7.11.23 Place the lid on the gel tank and plug the electrodes into the appropriate power slots. Ensure the positive electrode is at the base of the gel.

Safety information

Take care when working with electricity and water!

Check electrical cords of all equipment and ensure none are damaged and that cords are not a tripping hazard. Do not use if the electrical cord is damaged in any way. Tag the instrument with warnings, make the area safe, and notify your line manager and anyone else in the immediate area that may be affected.

Use electrical equipment indoors only in an area free of explosive material, corrosive gas, powerful vibrations, direct exposure to sunlight, and temperature fluctuations. Use in a space where cables will not come into contact with liquids, be manually damaged, or interfere with other workplace operations.

Do not use electrical equipment with any other power adapter or cord than the one supplied.

7.11.24 Switch the power pack on a set the voltage to 80 V and the time to 1 hr and 10 min.

Note

Note that the voltage and time can be adjusted to suit what you are running on the gel. For amplicons (one small product), I will run the gel at 96 V for 30-40 min. For shotgun libraries, I will run the gel as above. The lower the voltage and longer it is run, the greater the separation of fragments will be.

7.11.25 Press 'Run' or 'Start' on the power pack and check to see that bubbles are rising from electrodes.

7.11.26 When the run is over, switch of the power pack, remove the lid, and remove the gel from the tank, taking care not to let it slide off the tray.

Safety information

Do not remove the lid to the electrophoresis tank until the power pack is switched off.

7.11.27 Place the gel on the UV transilluminator and photograph using the attached camera. Follow the manufactuerer's instructions to use the equipment.

Safety information

Take care working with UV. You should have UV safety training. Do not open the transilluminator while the UV is on! Use signage to warn others when the UV is on.

7.11.28 Discard the gel into a designated biohazard bin, and clean the UV dock with 70% ethanol.

7.11.29 Dispose of used tips into a designated sharps container.

7.11.30 Dispose of gel waste into a biohazard bag.

7.11.31 Used combs, beakers, flasks, and tray should be washed with warm water and placed on a rack to dry.

7.11.32 Gloves and chemical waste should be sealed in a biohazard bag for incineration.

7.12 Use a LabChip GXII or equivalent fragment analyser (HiSense kit) to measure the molarity of the libraries.

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

Alternatively, use the Qubit concentration and the average fragment size seen on the gel to estimate the molarity of the libraries:

http://www.endmemo.com/bio/dnamolarity.php

8 Pool amplified libraries

8.1 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Pooled RAD library
	1.5 ml Lo-Bind Safelock tube	1	Aliquot of RAD library for sequencing

8.2 Pool libraries in equimolar concentrations such that the total volume is is approximately 100 ul.

Expected result

e.g., Combine 35 ul of MD#034 with 31.01 ul of MD#033. The total ng will be 3184.4 ng, the concentration will be 48.24 ng/ul.

8.3 Aliquot 20 ul of the pooled RAD library into a new 1.5 ml Lo-bind Safelock tube for future sequencing. Store at -20 deg C. See Steps 58-63 in the following protocol to quantitate and

sequence the final RAD library.

9 Adapter removal

9.1 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Step 9.2 master mix
	0.2 ml Lo-Bind PCR tube	1 per 1 ug DNA	Adapter removal reaction

9.2 Combine the following in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

> **Note**
>
> Perform enough replicate reactions for each 1 ug of DNA.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)	x _____ rxn
	CutSmart Buffer (NEB)	50 ul	10 X	1 X	5 ul	
	MspI	50 ul	20 U/ul	0.4 U/ul (20 U)	1 ul	
	Ultrapure water	50 ul	na	na	25.4 ul	

9.3 Aliquot 31.4 ul of the above master mix into 0.2 ml Lo-bind PCR tubes.

9.4 Add 18.6 ul (1 ug) of pooled purified RAD library to each tube. Vortex and pulse centrifuge.

Note

Remember to adjust the water volume in the reactions to accomodate the volume of library added to the reaction.

9.5 Incubate the reactions in a thermal cycler at:

37 deg C for 4 hr

Combine the replicates.

9.6 *SPRI bead clean-up*

Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 2X beads : reaction volume. Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 30 ul of Ultrapure water.

Note

This cleanup is simply to remove the cut-off adapters (68 bp).

9.7 *Qubit*

Measure the concentration using the Qubit HS kit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

Expected result

Expect a loss of about 56%.

e.g., After adapter removal, MD#033/MD#034 RAD library was 59.8 ng/ul or 1794 ng total. Before we had 3184 ng--so about 56% was lost.

9.8 *Agarose gel electrophoresis*

Electrophorese 5 ul of product (200-500 ng in 5-10 ul) on a 2% agarose gel.

Protocol

NAME

2% Agarose Gel Electrophoresis

CREATED BY

Alicia Grealy

PREVIEW

Expected result

50-53 bp are being cut-off during the adapter removal process, so the peak should be between 296-299 bp.

e.g.,

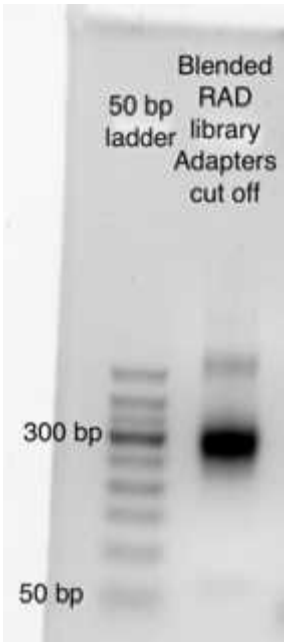


Figure 5. The RAD library after adapter removal. It is the right size.

9.8.1 Make up 2 L of 1X TAE buffer:

50 ml of 40X TAE buffer
1950 ml of MilliQ water

Mix by inversion.

9.8.2 Using an electronic balance, weigh out 2.2 g of agarose powder on to a weigh boat using a spatula.

Note

Note that gel concentration can be adjusted. The more concentrated the gel, the greater the resolution of small fragment sizes.

9.8.3 Transfer the powder to a 200 ml conical flask.

9.8.4 Using the graduated measurng cylinder, measure out 110 ml of 1xTAE buffer. Add to the conical flask containing the agarose powder. Swirl the flask gently to mix.

9.8.5 Microwave the flask uncovered for 1 minute.

Safety information

A lid can loosly be placed over the flask but DO NOT tighten--allow steam to escape. Do not microwave for more than 1 minute at a time.

Do not fill flasks or beakers/Schott bottles more than half full with liquid!

9.8.6 Remove the flask from the microwave using oven mitts and swirl gently.

Safety information

The liquid is boiling! Use oven mitts to handle flask. Do not put your face over the opening to the flask as liquid can splash out!

9.8.7 Microwave the flask for a further minute but remove from the microwave if the agarose appears to boil excessively. Swirl to mix and examine near a light source to ensure the agarose has melted. Allow to cool for 5 minutes.

9.8.8 Place the gel casting tray into a rubber vice that will seal the ends tightly, or tape the ends with masking tape.

9.8.9 Place the assembly on a flat bench and use the spirit level to check it is level--adjust if needed.

9.8.10 Place a 20-well comb into the casting tray.

9.8.11 When the flask is cool to the touch, add 5 ul of SYBR Safe and swirl gently to mix. Avoid generating bubbles.

Safety information

Wait until the liquid is warm (not boiling) to cast the gel, or the tray may crack!

9.8.12 Pour the liquid gel slowly into the casting tray. Pop any bubbles that have formed using a clean pipette tip.

9.8.13 Let the gel set for 20-30 minutes at room temperature.

9.8.14 Allow residual gel to set in the flask, then scrape into the bin. Fill the flask half full with water and microwave until the water boils. Pour the water down the sink and clean the flask using a bottle brush.

9.8.15 When the gel is set, remove the combs gently.

9.8.16 Place the casting try and gel in the electrophoresis tank.

9.8.17 Fill the electrophoresis tank with 1 X TAE buffer to the fill line indicated on the tank.

9.8.18 Pipette 3 ul of 50 bp DNA ladder into the first well of the gel.

Note

The recommended volume will depend on the concentration of the ladder. Check the manufacterer's recommendations. If the ladder is not pre-mixed with loading dye, be sure to add 1-2 ul of loading dye before loading into the gel.

9.8.19 Place some Parafilm across a 96-well PCR plate rack and press down firmly to create small wells.

9.8.20 For each sample, pipette 1-2 ul of loading dye onto the Parafilm, taking care not to pierce the Parafilm.

9.8.21 Mix 10 ul of PCR product with the loading dye by pipetting gently up and down.

9.8.22 Transfer the 12 ul of PCR product/loading dye to the wells of the gel, taking care not to pierce the bottom of the well with the pipette tip.

Note

The volume each well can take will depend on the size of the comb used. Be sure not to overload the wells or product will float out the top of the well.

9.8.23 Place the lid on the gel tank and plug the electrodes into the appropriate power slots. Ensure the positive electrode is at the base of the gel.

Safety information

Take care when working with electricity and water!

Check electrical cords of all equipment and ensure none are damaged and that cords are not a tripping hazard. Do not use if the electrical cord is damaged in any way. Tag the instrument with warnings, make the area safe, and notify your line manager and anyone else in the immediate area that may be affected.

Use electrical equipment indoors only in an area free of explosive material, corrosive gas, powerful vibrations, direct exposure to sunlight, and temperature fluctuations. Use in a space where cables will not come into contact with liquids, be manually damaged, or interfere with other workplace operations.

Do not use electrical equipment with any other power adapter or cord than the one supplied.

9.8.24 Switch the power pack on a set the voltage to 80 V and the time to 1 hr and 10 min.

Note

Note that the voltage and time can be adjusted to suit what you are running on the gel. For amplicons (one small product), I will run the gel at 96 V for 30-40 min. For shotgun libraries, I will run the gel as above. The lower the voltage and longer it is run, the greater the separation of fragments will be.

9.8.25 Press 'Run' or 'Start' on the power pack and check to see that bubbles are rising from electrodes.

9.8.26 When the run is over, switch of the power pack, remove the lid, and remove the gel from the tank, taking care not to let it slide off the tray.

Safety information

Do not remove the lid to the electrophoresis tank until the power pack is switched off.

9.8.27  Place the gel on the UV transilluminator and photograph using the attached camera. Follow the manufactuerer's instructions to use the equipment.

Safety information

Take care working with UV. You should have UV safety training. Do not open the transilluminator while the UV is on! Use signage to warn others when the UV is on.

9.8.28 Discard the gel into a designated biohazard bin, and clean the UV dock with 70% ethanol.

9.8.29 Dispose of used tips into a designated sharps container.

9.8.30 Dispose of gel waste into a biohazard bag.

9.8.31 Used combs, beakers, flasks, and tray should be washed with warm water and placed on a rack to dry.

9.8.32 Gloves and chemical waste should be sealed in a biohazard bag for incineration.

10 *In-vitro* transcription and biotinylation

10.1 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Step 10.3 master mix
	0.2 ml Lo-Bind PCR tube	# reactions	Transcription/biotinylation reaction
	0.2 ml Lo-Bind PCR tube	2	Aliquots of TURBO DNase and SuperaseIn RNAse inhibitor

	1.5 ml Lo-Bind Safelock tube	1	Combine probes for clean up
	RNeasy mini spin column	1	Purification
	1.5 ml Lo-Bind Safelock tube with the lid cut off	1	Elution
	1.5 ml Lo-Bind Safelock tube	1	Final tube for probes
	0.5 ml Lo-Bind Safelock tube	Depends on total ug of probes	6-ul aliquots of probes

10.2 Aliquot 10 ul of TURBO DNAse and 10 ul of SuperaseIn RNAse inhibitor into separate 0.2 ml Lo-bind tubes.

10.3 Combine the following in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

Note

Perform enough replicate reactions to transcribe all the library left into probes (inputting 500 ng of library per reaction).

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)	x _____ rxn
	Reaction Buffer (HiScribe kit)	20 ul	10 X	0.75 X	1.5 ul	
	dATP	20 ul	100 uM	7.5 uM	1.5 ul	
	dCTP	20 ul	100 uM	7.5 uM	1.5 ul	
	dGTP	20 ul	100 uM	7.5 uM	1.5 ul	

	dUTP	20 ul	100 uM	5 uM	1 ul	
	biotin-UTP	20 ul	10 mM	2.5 mM	5 ul	
	T7 RNA polymerase mix	20 ul	?	?	1.5 ul	
	Ultrapure water	20 ul	na	na	Adjust depending on the volume of library added	

Note

Typically I do not add water to the reaction.

10.4 Aliquot 13.5 ul of the above mater mix to 0.2 ml Lo-bind PCR tubes. Bring all tubes to a post-PCR space, including teh aliquots made in Step 10.2.

10.5 Concentrate the "probe set" (i.e., the pool RAD library with the adapters cut off) such that roughly 500 ng in 6.5 ul can be added per reaction using a SpeedVac, following the manufacturer's instructions:

https://assets.thermofisher.com/TFS-Assets/LED/manuals/DNA130%20User%20Manual%20final%20versionpdf.pdf

Expected result

e.g., After quanting and the gel, I had 1495 ng of MD#033/MD#034 in 25 ul. I concentrated this to 16.5 ul (i.e., 90.6 ng/ul). Then I added 6.5 ul into two replicate transcription/biotinylation reactions (i.e., 589 ng was input into dupllicate reactions).

10.6 Add 6.5 ul of the "probe set" (ca. 500 ng) to each reaction. Vortex and pulse centrifuge.

10.7 Incubate in a thermalcycler at:

37 deg C for 16 hr

In the last 30 min, add 2 ul of TURBO DNase (2 U/ul, or 4 U for up to 10 ug). Pipette up and down to mix.

10.8 Combine replicate reactions in a 1.5 ml Lo-bind tube. Top up to 100 ul with RNase-free water.

10.9 *Cleanup using an RNeasy Mini Kit*

> **Note**
>
> This step is to remove reagents.

Follow the manufacturer's instructions to purify the probes using an RNeasy Mini Kit:

http://www.bea.ki.se/documents/EN-RNeasy%20handbook.pdf

Elute in 60 ul RNase-free water.

10.10 Add 2.5 ul of SUPERase-IN RNAse Inhibitor (20 U/ul) to the eluate. Flick to mix and pulse centrifuge.

10.11 *Qubit*

Measure the concentration of the probes using the Qubit RNA kit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

Expected result

The HiScribe kit suggests that 1 ug DNA --> 10 ug, but after inputting 1.2 ug DNA I got ~40 ug:

e.g., Measuring the concentration of a 1 in 2 and a 1 in 10 dilution of the probes (MD#033/MD#034) I estimate the neat to be either 23.7 or 55.8 ug of RNA, respectively. Averaging those values gives approximately 39.75 ug of RNA, or enough for about 40 hybridisation capture reactions (giving each reaction 1 ug of probes).

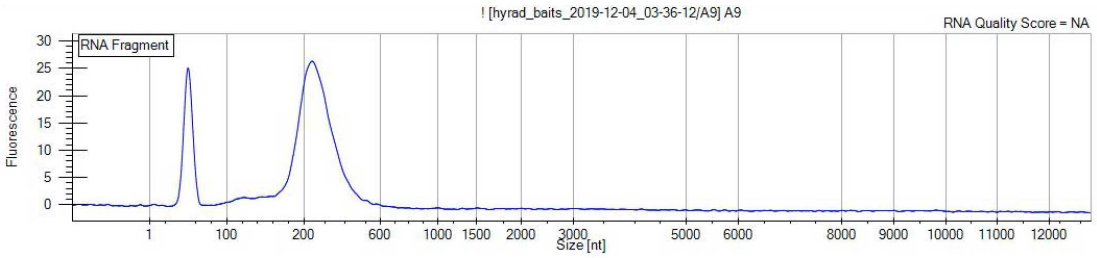
10.12 *Fragment analyse*

Examine the fragment length distribution of the probes using a fragment analyser such as the LabChip GXII RNA kit (or equivalent fragment analyser) following the manufacturer's instructions:

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

Expected result

The probes should have a peak at about 210-239 bp (with a tight distribution perhaps from 160-500 bp length).



10.13

Dilute the RNA probes to 200 ng/ul with RNAse-free water. Aliquot into 6 ul batches in 0.5 ml Lo-bind Safelock tubes and store at -80 deg C.

Hybridisation

11 Label tubes.

	Tube	Qty	For ...
	1.5 ml Lo-Bind Safelock tube	1	Mineral oil aliquot
	0.2 ml Lo-Bind PCR tube	# capture reactions x 2	Hybridisation
	1.5 ml Lo-Bind Safelock tube	2	Step 14 and 15 master mixes

12 Ensure all primer stocks are pepared in the correct buffer and that enough aliquots of the working concentrations are diluted out. Vortex and pulse centrifuge.

13 Thaw reagents. Flick all reagents to mix and pulse centrifuge where possible.

	Reagent	Stored at...	Thaw at...
	20 X SSPE	4 deg C	Room temperature
	500 mM EDTA	4 deg C	Room temperature
	50 X Denardt's Solution	-20 deg C	Room temperature
	10% SDS	4 deg C	Room temperature
	Chickent Cot-1 (HyBloc)	-20 deg C	On ice

	FWD_blocking_primer	-20 deg C	On ice
	REV_blocking_primer	-20 deg C	On ice
	SUPERas-In RNAse inhibitor	-20 deg C	On ice
	Salmon Sperm DNA	-20 deg C	On ice

14 Combine the following "**BLOCKS**" master mix in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)	x _____ rxn
	Chicken Cot-1 (HyBloc)	3.25 ul	1 ug/ul	0.77 ug/ul	2.5 ul	
	Salmon Sperm DNA	3.25 ul	10 ug/ul	0.77 ug/ul	0.25 ul	
	FWD_blocking_primer	3.25 ul	200 uM	15.4 uM	0.25 ul	
	REV_blockg_primer	3.25 ul	200 uM	15.4 uM	0.25 ul	

"BLOCKS" master mix

15 Aliquot 3 ul of the **BLOCKS** master mix into 0.2 ml Lo-bind tubes, one for each capture reaction.

16 Combine the following "**HYBS**" master mix in a 1.5 ml Lo-bind Safelock tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)	x _____ rxn
	SSPE	20 ul	20 X	9 X	9 ul	
	EDTA	20 ul	0.5 M	0.0125 M	0.5 ul	
	Denhardt's solution	20 ul	50 X	8.75 X	3.5 ul	
	SDS	20 ul	10 %	0.25 %	0.5 ul	
	SUPERase-In RNase inhibitor	20 ul	20 U/ul	1 U/ul	1 ul	

"HYBS" master mix

17 Aliquot 14.5 ul of the **HYBS** master mix into 0.2 ml Lo-bind tubes, one for each capture reaction.

18 Bring all the tubes to the post-PCR space.

19 Pre-program the thermal cycler:

95 deg C for 5 min
60 deg C for 5 min
60 deg C for hold

Note

Recommendations for the hybridisation temperature

20 Thaw a 6-ul aliquot of the probes from Step 10.13 on ice.

21 Add 5.5 ul of baits (ca. 500-1000 ng at 100-200 ng/ul) to each **HYBS** reaction. Flick to mix and pulse centrifuge. Place on ice.

22 Add 7 ul (100-500 ng) of shotgun library to the corresponding **BLOCKS** tube. Flick to mix and pulse centrifuge.

Note

Shotgun libraries can contain a single sample or a pool of samples...

Ideally the library should be double-indexed and purified (but not size-selected--a lot of the dimer should be washed away in the capture but it will be size-selected after the capture anyway).

The input amount of DNA is 100-500 ng in 7 ul (i.e., 14-72 ng/ul). The range is 1 ng input to up to 2 ug input DNA.

23 Transfer the **BLOCKS** tubes to a thermalcycler and start the program. Allow it to proceed through Step 1 (i.e., 95 deg C for 5 min).

24 When the thermalcycler reaches Step 2, transfer the **HYBS** tubes to the thermalcyler. Allow it to proceed through Step 2 (i.e., 60 deg C for 5 min).

25 When the thermalcycler reaches Step 3, transfer 18 ul from the **HYBS** tubes to the corresponding **BLOCKS** tubes. Pipette to mix. Discard the **HYBS** tubes.

26 Add 15 ul of mineral oil to the top of each reaction.

27 Allow the thermalcycler to proceed through Step 3 for 42 hr (i.e., 60 deg C for 42 hr).

Enrichment

28 Label tubes.

	Tube	Qty	For ...
	50 ml Falcon tubes	3	Wash buffer 2, Wash buffer 2.2, Binding buffer
	15 ml Falcon tubes	3	Aliquots for Wash buffer 2.2 and Binding buffer, and 10 mM Tris-HCl/0.05% Tween-20
	1.5 ml Lo-bind Safelock tube	1	Aliquot for MyOne C1 Streptavidin beads
	1.5 ml Lo-bind Safelock tube	3 x # hybridisation reactions	Enrichment

29 Prepare all necessary buffers and UV decontaminate.

	Buffer	Reagent	Volume	C2
	Wash buffer 2	20 X SSC	100 ul	0.1 X
		10% SDS	200 ul	0.1 %
		Ultrapure water	19.7 ml	na

	Wash buffer 2.2	10% SDS	400 ul	0.08%
		Wash buffer 2	10 ml	na
		Ultrapure water	39.6 ml	na
	Binding buffer	5 M NaCl	10 ml	1 M
		1 M Tris-HCl	500 ul	10 mM
		0.5 M EDTA	100 ul	1 mM
		Ultrapure water	39.4 ml	na
	10 mM Tris-HCl, 0.05% Tween-20	1 M Tris-HCl	500 ul	10 mM
		100% Tween-20	25 ul	0.05%
		Ultrapure water	49.475 ml	na

30 Aliquot reagents for to take to the post-PCR space.

	700 ul * # reactions	Binding buffer
	30 ul * # reactions	MyOne C1 Streptavidin beads
	35 ul * # reactions	10 mM Tris-HCl/0.05% Tween-20
	1600 ul * # reactions	Wash Buffer 2.2

31 Set a water bath or thermalshaker to 55 deg C. Warm Wash buffer 2.2 to 55 deg C for 45 min.

32 Set a heat block to 95 deg C.

33 Pellet the MyOne C1 Streptavidin beads for 2 min with the magnetic rack. Discard the supernatant.

34 Add 200 ul * # reactions of Binding buffer to the beads. Vortex and pulse centrifuge.

35 Pellet the beads with the magnetic rack and discard the supernatant.

36 Repeat Step 34-35 two more times for a total of 3 washes.

37 Resuspend the beads in 70 ul * # reactions of Binding buffer.

38 Aliquot 70 ul of beads into a 1.5 ml Lo-bind Eppendorf tube (1 per reaction).

39 Warm the bead aliquots to 55 deg C in the thermoshaker/water bath for 2 minutes.

40 Transfer the hybridised libraries at 60 deg C to the bead aliquots. Pipette to mix.

41 Incubate the hybridised libraries and beads in the thermoshaker/waterbath for 30 min at 55 deg C. Agitate every 5 minutes by flicking or gently continuously shake.

42 Pulse centrifuge. Pellet the beads with the magnetic rack. Discard the supernatant.

43 Add 500 ul heated Wash Buffer 2.2 to the beads. Vortex and pulse centrifuge.

44 Incubate 10 min at 55 deg C in the thermoshaker/water bath. Agitate every 2 min by flicking.

45 Pulse centrifuge. Pellet the beads with the magnetic rack. Discard the supernatant.

46 Repeat the wash steps above (Steps 43-45) two more times for a total of 3 washes.

47 Add 30 ul of 10 mM Tris-HCl/0.05% Tween-20 to the washed beads. Resuspend by pipetting.

48 Incubate at 95 deg C in a heat block for 5 min.

49 Pellet the beads with a magnetic rack and transfer the supernatant to a clean 1.5 ml tube.

50 At this point you can treat with RNAseA to remove any RNA or put RNAse A in the PCR but it is not necessary as the RNA will not amplify.

51 Dilute the captured libraries 1 in 10 in Ultrapure water (i.e., 1 ul in 9 ul Ultrapure water). Vortex and pulse centrifuge.

Library amplification

52 Label tubes.

53 Thaw reagents on ice.

54 Ensure that all primer stocks are prepared in the correct buffer and that enough aliquots of the working concentrations are diluted out. Vortex and pulse centrifuge.

	Oligo	Reagent	Volume
	10 uM P5_primer	100 uM P5	50 ul

		Ultrapure water	450 ul
	10 uM P7_primer	100 uM P7	50 ul
		Ultrapure water	450 ul

55 Make up the following master mix in a 1.5 ml Lo-bind tube.

	Reagent	V2	C1	C2	V1	x _____ rxn
	Ultrapure water	25 ul	na	na	10.9	
	BSA	25 ul	10 mg/ml	0.4 mg/ml	1 ul	
	ABI Gold PCR Buffer	25 ul	10 X	1 X	2.5 ul	
	MgCl2	25 ul	25 mM	2.5 mM	2.5 ul	
	dNTPs	25 ul	25 mM	0.25 mM	0.25 ul	
	ABI Taq Gold DNA polymerase	25 ul	5 U/ul	0.05 U/ul	0.25 ul	
	SYBR Green	25 ul	5 X	0.12 X	0.6 ul	
	P5	25 ul	10 uM	0.4 uM	1 ul	
	P7	25 ul	10 uM	0.4 uM	1 ul	

Master mix for end-point PCR

56 Add 22.5 ul of master mix to each PCR tube following the schematic below. Pulse centrifuge the tubes.

	Capture001 Neat	
	Capture001 1in10	

	Capture002 Neat	
	Capture002 1in10	
	Capture003 Neat	
	Capture003 1in10	
	PCR NTC	
	PCR NTC	

57 Make up the following master mix in a 1.5 ml Lo-bind tube.

	Reagent	V2	C1	C2	V1	x ____ rxn
	Ultrapure water	25 ul	na	na	10.9	
	BSA	25 ul	10 mg/ml	0.4 mg/ml	1 ul	
	ABI Gold PCR Buffer	25 ul	10 X	1 X	2.5 ul	
	MgCl2	25 ul	25 mM	2.5 mM	2.5 ul	
	dNTPs	25 ul	25 mM	0.25 mM	0.25 ul	
	ABI Taq Gold DNA polymerase	25 ul	5 U/ul	0.05 U/ul	0.25 ul	
	SYBR Green	25 ul	5 X	0.12 X	0.6 ul	
	P5	25 ul	10 uM	0.4 uM	1 ul	
	P7	25 ul	10 uM	0.4 uM	1 ul	

Master mix for final PCR

58 Add 20 ul of master mix to each PCR tube following the schematic below. Pulse centrifuge the tubes.

	Capture001 Neat	Capture003 Neat
	Capture001 Neat	Capture003 Neat
	Capture001 Neat	Capture003 Neat
	Capture001 Neat	Capture003 Neat
	Capture002 Neat	
	Capture002 Neat	
	Capture002 Neat	
	Capture002 Neat	

59 Pulse centrifuge the 8-well qPCR strip tubes containing the master mix. Bring to the post-PCR space.

60 To the end-point PCR, add 2.5 ul of both neat and 1in10 captured library to the corresponding tubes. Add 2.5 ul nuclease free water to the remaining wells as PCR no-template controls. Vortex and pulse centrifuge.

61 Place tubes in the thermal cycler and run the following PCR program:

95 deg C for 10 min

Followed by 50 cycles of:

95 deg C for 30 sec
60 deg C for 30 sec

72 deg C for 30 sec

62 When the PCR is finished, determine the optimal number of cycles to give in the final PCR to ensure libraries are not over-amplified. i.e., stop the PCR during the linear phase of amplification.

Note

This is particularly important if you performed a capture that contained a pool of samples, and why if you DO capture a pool you ensure that both forward and reverse indexes are unique (not just the combination)--over-amplification can cause tag-jumping, so if the indexes are completely unique to a sample, even if tag jumping occurs they will be thrown out becuase the erroneous combination can be identified.

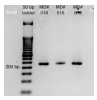
This step could be performed with alongside standards of known concentration to calculate the number of library molecules output from the capture library. This could be compared to to the number of library molecules input into the capture--if the capture worked, you would expect much fewer molecules OUT of the capture than went in.

The Neat and 1in10 dilution of libraries should show be approximately 3.33 cycles apart. If they are not, there might be too much input DNA in the qPCR. Dilute futher and run the qPCR to get a more accurate estimate of library molecules.

63 *Agarose gel electrophoresis*

Run 10 ul of PCR product from the first PCR on a 2% agarose gel electrophoresis.

Protocol



NAME

2% Agarose Gel Electrophoresis

CREATED BY

Alicia Grealy

PREVIEW

63.1 Make up 2 L of 1X TAE buffer:

50 ml of 40X TAE buffer

1950 ml of MilliQ water

Mix by inversion.

63.2 Using an electronic balance, weigh out 2.2 g of agarose powder on to a weigh boat using a spatula.

Note

Note that gel concentration can be adjusted. The more concentrated the gel, the greater the resolution of small fragment sizes.

63.3 Transfer the powder to a 200 ml conical flask.

63.4 Using the graduated measurng cylinder, measure out 110 ml of 1xTAE buffer. Add to the conical flask containing the agarose powder. Swirl the flask gently to mix.

63.5 Microwave the flask uncovered for 1 minute.

Safety information

A lid can loosly be placed over the flask but DO NOT tighten--allow steam to escape. Do not microwave for more than 1 minute at a time.

Do not fill flasks or beakers/Schott bottles more than half full with liquid!

63.6 Remove the flask from the microwave using oven mitts and swirl gently.

Safety information

The liquid is boiling! Use oven mitts to handle flask. Do not put your face over the opening to the flask as liquid can splash out!

63.7 Microwave the flask for a further minute but remove from the microwave if the agarose appears to boil excessively. Swirl to mix and examine near a light source to ensure the agarose has melted. Allow to cool for 5 minutes.

63.8 Place the gel casting tray into a rubber vice that will seal the ends tightly, or tape the ends with masking tape.

63.9 Place the assembly on a flat bench and use the spirit level to check it is level--adjust if needed.

63.10 Place a 20-well comb into the casting tray.

63.11 When the flask is cool to the touch, add 5 ul of SYBR Safe and swirl gently to mix. Avoid generating bubbles.

Safety information

Wait until the liquid is warm (not boiling) to cast the gel, or the tray may crack!

63.12 Pour the liquid gel slowly into the casting tray. Pop any bubbles that have formed using a clean pipette tip.

63.13 Let the gel set for 20-30 minutes at room temperature.

63.14 Allow residual gel to set in the flask, then scrape into the bin. Fill the flask half full with water and microwave until the water boils. Pour the water down the sink and clean the flask using a bottle brush.

63.15 When the gel is set, remove the combs gently.

63.16 Place the casting try and gel in the electrophoresis tank.

63.17 Fill the electrophoresis tank with 1 X TAE buffer to the fill line indicated on the tank.

63.18 Pipette 3 ul of 50 bp DNA ladder into the first well of the gel.

Note

The recommended volume will depend on the concentration of the ladder. Check the manufacterer's recommendations. If the ladder is not pre-mixed with loading dye, be sure to add 1-2 ul of loading dye before loading into the gel.

63.19 Place some Parafilm across a 96-well PCR plate rack and press down firmly to create small wells.

63.20 For each sample, pipette 1-2 ul of loading dye onto the Parafilm, taking care not to pierce the Parafilm.

63.21 Mix 10 ul of PCR product with the loading dye by pipetting gently up and down.

63.22 Transfer the 12 ul of PCR product/loading dye to the wells of the gel, taking care not to

pierce the bottom of the well with the pipette tip.

Note

The volume each well can take will depend on the size of the comb used. Be sure not to overload the wells or product will float out the top of the well.

63.23 Place the lid on the gel tank and plug the electrodes into the appropriate power slots. Ensure the positive electrode is at the base of the gel.

Safety information

Take care when working with electricity and water!

Check electrical cords of all equipment and ensure none are damaged and that cords are not a tripping hazard. Do not use if the electrical cord is damaged in any way. Tag the instrument with warnings, make the area safe, and notify your line manager and anyone else in the immediate area that may be affected.

Use electrical equipment indoors only in an area free of explosive material, corrosive gas, powerful vibrations, direct exposure to sunlight, and temperature fluctuations. Use in a space where cables will not come into contact with liquids, be manually damaged, or interfere with other workplace operations.

Do not use electrical equipment with any other power adapter or cord than the one supplied.

63.24 Switch the power pack on a set the voltage to 80 V and the time to 1 hr and 10 min.

Note

Note that the voltage and time can be adjusted to suit what you are running on the gel. For amplicons (one small product), I will run the gel at 96 V for 30-40 min. For shotgun libraries, I will run the gel as above. The lower the voltage and longer it is run, the greater the separation of fragments will be.

63.25 Press 'Run' or 'Start' on the power pack and check to see that bubbles are rising from electrodes.

63.26 When the run is over, switch of the power pack, remove the lid, and remove the gel from the tank, taking care not to let it slide off the tray.

Safety information

Do not remove the lid to the electrophoresis tank until the power pack is switched off.

63.27 Place the gel on the UV transilluminator and photograph using the attached camera. Follow the manufactuerer's instructions to use the equipment.

Safety information

Take care working with UV. You should have UV safety training. Do not open the transilluminator while the UV is on! Use signage to warn others when the UV is on.

63.28 Discard the gel into a designated biohazard bin, and clean the UV dock with 70% ethanol.

63.29 Dispose of used tips into a designated sharps container.

63.30 Dispose of gel waste into a biohazard bag.

63.31 Used combs, beakers, flasks, and tray should be washed with warm water and placed on a rack to dry.

63.32 Gloves and chemical waste should be sealed in a biohazard bag for incineration.

64 To the second PCR add 5 ul of the neat library (as long as amplification was NOT inhibited in the first PCR; if amplification efficiency was poor, amplify a dilution and do more replicates). Perform enough replicates to amplify the entire captured library.

65 Perform Step 61 above, but stop the PCR during the linear phase of amplification as determined by the end-point PCR above.

Expected result

The number of cycles needed may be greater than was needed for the initial library amplification (before capture) but is usually between 15-20 cycles.

66 Combine replicates in a clean 1.5 mL Lo-bind Safelcok tube. Vortex and pulse centrifuge.

67 *Purify*

Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 1.6X beads : reaction volume (i.e., 160 ul). Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 40 ul of Ultrapure water.

Size select enriched libraries

68 Run each enriched library in duplicate across two lanes (20 ul each) of a PippinHT electrophoresis system (2% gel, Marker 20B), selecting fragments between 160-500 bp and following the manufacturer's instructions:

http://www.sagescience.com/wp-content/uploads/2015/10/PippinHT-Operations-Manual-Rev-B_460005.pdf

69 *Purify*

Combine replicates. Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 2X beads : reaction volume (i.e., 160 ul). Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 25 ul of Ultrapure water.

Pool enriched libraries

70 *Qubit*

Measure the concentration of the neat library and these dilutions in duplicate on the Qubit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

71 Dilute the libraries to 5 ng/ul in Ultrapure water in a total volume of 5-10 ul.

72 *Fragment analyse*

Use a LabChip GXII or equivalent fragment analyser (HiSense kit) to measure the molarity of the libraries between 160-500 bp.

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

Expected result

Libraries will be insert size + 136 bp, so the smallest fragments of interest will be ca. 166 bp (30 bp insert).

73 Pool enriched libraries in equimolar concentrations.

Quantitate the final sequencing library

74 Dilute the libraries 1/2, 1/5, 1/10 in Ultrapure water (i.e., create a serial dilution in 10 ul volume).

75 *Qubit*

Measure the concentration of the neat library and these dilutions in duplicate on the Qubit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

76 *Fragment analyse*

Measure the molarity of the neat library and dilutions on a LabChip GXII Hisense kit (or equivalent fragment analyser) following the manufacturer's instructions:

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

77 Based on the average fragment length and Qubit measurement, calculate the molarity of the library dilutions. Create a standard curve to check that the concentrations are linear. If they can be "trusted", extrapolate the neat concentration based on the dilutions. Average all the measurements of the neat concentration to get the best estimate of the library molarity.

Sequence

78 Dilute the library to between 2-4 nM in Ultrapure water.

Note

Note that if your libraries were built using the single-stranded protocol (Gansauge and Meyer 2013; Gansauge et al. 2017), you will need CL72_custom_sequencing_primer to sequence. This can be spiked into well 12 (but select 'no custom primer' in the run set up) or into well 18 (select 'custom primer' in the run set up). Spiking the custom primers into the run is preferable so that the remaining Illumina primers are present and can sequence PhiX.

You do not need custom i5_indexing_primer to sequence off a MiSeq or NovaSeq because these instruments prime off P5. You do not need a custom i7 indexing primer because it uses primers already included in the kit. Note that for the NextSeq you **will** need custom i5_indexing_primer in addition to the CL72_custom_sequencing_primer.

79 Follow the manufacturer's instructions to perform the sequencnig run on your Illumina platform of choice.