PCR

Expect to set up between 5-20 PCR reactions per 100M cell sample, depending on the yield of the gel extraction. The recommended input of 500ng gDNA per 100uL reaction can be adjusted, although higher inputs (>1ug) can lead to inhibition of the PCR reaction or higher background. If you find you will be setting up a very high number of PCRs based on the 500ng input, it's recommended that you process an unimportant stock of sgRNA library-containing cells for optimizing this step.

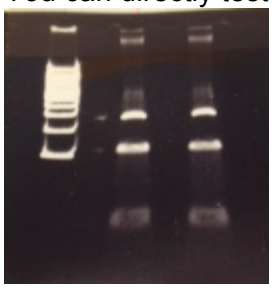
PCR Reaction	**100 uL reaction (vol uL)**
Water	bring final vol to 100 uL
5XBuffer	**20**
DMSO (100%)	**3**
P(Common) 100 uM	**0.4**
P(Index) 100 uM	**0.4**
dNTP (10 mM)	**2**
Phusion	**1**
gDNA	500 ng

PCR conditions

- **98ºC, 30s**
- **23 cycles**
  - **98ºC, 30s**
  - **56ºC, 15s**
  - **72ºC, 15s**
- **72ºC, 10min**
- **7ºC hold**

After the PCR is completed, pool all your reactions for a given sample in a 5mL or 15 mL tube. Mix reaction well. All of the sgRNAs in the pool should now be represented at much greater abundance, so you may proceed with only a fraction of the sample for PCR purification. Remember that this material represents a significant contaminant to pre-PCR samples, so be sure to keep them separate.

You can directly test the PCR by running an aliquot on a 20% TBE gel with 100bp ladder:



Enriched PCR product @ ~274bp
Primer dimer @ ~150bp

Step 4a: PCR purification

There are several possible ways to remove the gDNA and primer dimer background from the PCR. The recommended method is listed below, but two other options are provided at the end of this protocol.

SPRI bead selection. Proceed with 100-300 uL of pooled PCR reaction

You will perform a double SPRI purification using SPRIselect Reagent (Beckman and Coulter) to select for your 270 bp sample. Double SPRI allows you to exclude anything above and below your target size. You will need: low retention/nonstick tubes, magnetic stand, 80% EtOH (make fresh), and Elution Buffer.

Some references if you are curious about how SPRI beads work:

Fisher S, *et al.*: A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries. *Genome Biol* 2011

DeAngelis MM, Wang DG, Hawkins TL: Solid-phase reversible immobilization for the isolation of PCR products. *Nucleic Acids Res* 1995

Lis JT. Size Fractionation of double stranded DNA by precipitation with polyethylene glycol. *Nucleic Acids Research* (1975)

You can also purchase Seramag speed beads (Thermo Scientific) if you want to make your own cost efficient SPRI beads: https://ethanomics.files.wordpress.com/2012/08/serapure_v2-2.pdf

Amounts listed below are for 300uL of PCR reaction, but you can adjust volumes according to the input. Since you likely have far more sgRNA amplicon from the PCR than will be needed for sequencing, you can be conservative with the starting material for this step if you set up fewer than 6 PCRs in case an error occurs in the PCR clean-up.

1) Add 0.65X SPRI beads.

- 300 uL * 0.65= 195uL SPRIselect. At this ratio, fragments >300 will bind to the beads
- Mix well. You want a homogenous mixture. If you splash any of the mixture onto the sides of your tubes or cap spin down briefly.
- Incubate 10 min at RT
- Place tubes on magnetic stand for 5 min or until supernatant clears
    - I recommend Dyna mag 2 (Life Tech)
- Transfer the supernatant to a new tube. **KEEP supernatant**. Your sample is here.

2) Add 1X SPRI beads

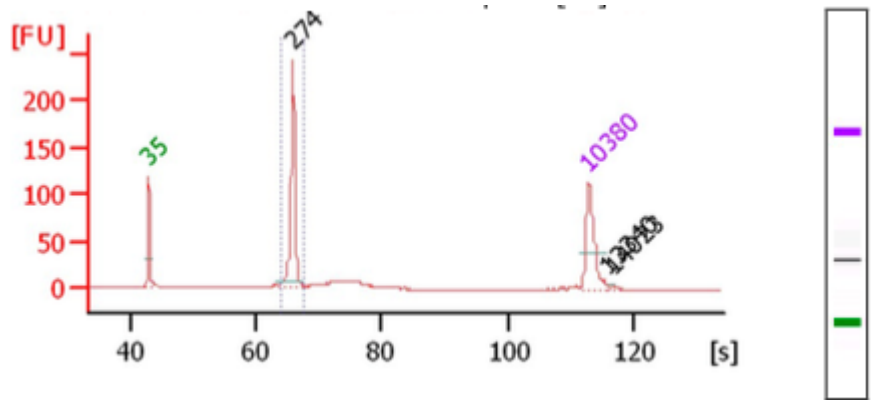
- 300 uL * 1= 300 uL SPRIselect. At this ratio, fragments >150 will bind to the beads.
    - Note this is based on the original PCR input volume, not the volume of the supernatant obtained from the first SPRI selection.
- Mix well and Spin tubes briefly.
- Incubate 10 min at RT
- Place tubes on magnetic stand for 5 min or until supernatant clears
- Remove supernatant. **KEEP beads**. Your sample is bound to the beads.
- Rinse beads with 1mL (make sure all you beads are covered) fresh 80% EtOH. Wait 2 min. Remove EtOH. Repeat for a total of 2 washes.
- Air Dry 5-15 min. The beads will look glossy after your remove the EtOH. The texture will turn from glossy (wet) to matte (dry).
- Once dry, elute in 20 uL of EB.

STEP 4b: Sequencing library QC and submission for sequencing

Once Your PCR Product is Purified:

1) Measure PCR clean-up yield by Nanodrop and/or Qubit, and dilute a small portion of your sample to 400 pg/uL based on this measurement.

2) Run samples on a Bioanalyzer (Agilent) with High Sensitivity DNA chip. The enriched PCR product is at 274bp, but the bioanalyzer peak will run in around 276bp-280bp. You can pool multiple samples together prior to analyzing, or analyze each sample in a separate channel to obtain a more accurate concentration of the sequencing sample prior to pooling.



Samples with higher background than shown here, in the form of a broad, low smear above or below the sgRNA amplicon, have been run without problems. Your sequencing facility may request these traces, and also request or perform additional QC in the form of qPCR.

3) Submit samples for sequencing, along with the mix of the two custom sequencing primers listed in the PCR section. The exact input volume and molar concentration will depend on the preferences of your sequencing facility. Our facility requests 15uL of 3-10nM (1-3ng/uL) pooled sample, with an additional 5uL for each lane.

The sequencing parameters are:

- **Run type: Single-end 50**
    - In the event you are sharing a sequencing run with other types of samples, longer sequencing formats will provide extraneous information but the sgRNA amplicons should be compatible
- **Index length: 1x6**
- **Sequencing primers: Read 1, custom primers; Read 2, standard Illumina index primer**
- **PhiX Spike-in: Depending on how evenly your samples are divided up into A and B sets, there should be sufficient diversity to request "standard 1% PhiX"; if the sequencing lane contains only A or B, there will be no diversity after the 19bp sgRNA protospacer is read, so request higher (up to 20%) spike-in**

Appendix. Other PCR Clean-up methods
Option 1: Proceed with 1mL of your pooled PCR reaction

1) Add: 5X vol PB and 1/100 vol 3M NaOAc, pH 5.3. Mix well.
2) Set up vaccum manifold. Use 1 column per 4mL of PB.

3) Wash 2X, PE and dry spin.
4) Elute 30 uL

5) Load 1ug-4ug (I loaded everything) on 20% TBE gel(0.5X). Run @200V, 1h

6) Stain gel with sybrsafe.

7)Prepare electroelution tubes:
- a) label tubes (w/EtOH resistant water proof marker)
- b) pre wet tubes, 5 min (fill tubes with 0.5X TBE)
- c) decant

8) Cut out PCR band (270 bp). Use a new blade for each sample, or fill a beaker with EtOH, and clean blades between sample.

9) Place PCR band in prewet electroelution tubes, and fill tubes with 0.5X TBE. Make sure tubes are tightly capped. Check for air bubbles.

10) Place tubes in a clean gel box. There is a golden rack for midi tubes, but micro tubes will need to be taped.

11)Fill chamber with new 0.5X TBE, and run @160V for 90 min. Reverse current for 2 min.

12) Your PCR should be in the medium. Remove ALL the medium. You will not recover your entire volume as the gel absorbs some of the TBE.

13) Add 3.2mL PB, 40uL NaOAc. Mix well. Apply to gel extraction columns. 2, PE washes, and 1 dry spin. Elute in 35uL EB.

Option 2: Rapid Extraction

1. Follow Steps 1-6 from Option 1 (listed above)

2. While gel is running prepare tubes and tips for rapid extraction. Pierce the bottom (tip) of a 0.5 ml nonstick tube with an 18.5 gauge needle and place it inside a 1.5 ml nonstick tube. Use a clean blade or clean scissors to cut the tips off of p1000 pipette tips.

3. Excise 264 bp PCR product, and place excised gel piece inside 0.5 mL tube

4. Spin tubes at 20,000 x g for 3 min. This will force the gel through the hole, and gel will crush into tiny pieces. Check for gel pieces in small tube. Residual pieces should be transferred to large tube. If you cannot tap the residual pieces use a pipette tip.

5. Add 200 uL of water to gel pieces, and incubate for 10 min at 70ºC

6. Vortex gel slurry for 30s and use pre-cut p1000 tips to transfer gel mixture to Costar Spin-X columns.

7. Spin tubes for 3 min at 20,000 x g to recover the elution mixture free of gel debris.

8. Transfer eluate to a new 1.5 mL nonstick tube

9. Isopropanol Precipitation
a. 200 uL of eluate (insert is here)
b. 1.5 uL-2uL of glycoblue (this will help you visualize your pellet)
c. 25 uL 3M NaOAc or 3M NaCl
i. Mix Well (invert 10-15X)

10. Add 0.75 mL Isopropanol, mix well

11. -30ºC, 30 min to 18h or -80ºC, 30 min

12. Pellet: Spin 30 min at 20,000 x g at 4ºC , remove supernatant

13. Wash pellet 2X with ice cold 80% EtOH

14. Air Dry

15. Resuspend pellet in 15 uL water or Qiagen EB