PETRI-seq Detailed Protocol

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Some Notes Before Starting

- Before proceeding with the full protocol below, we recommend trying *in situ* reverse transcription and qPCR to confirm cell preparation is working. See paper for details ("qPCR quantification after in situ DNase or in situ RT" section in methods)
- PETRI-seq cell preparation and split-pool barcoding can be done in 2 days, as detailed below. Please be aware that day 2 takes about 12 hours and does not include any stopping points prior to lysis. We will update this protocol if we later find any feasible stopping points.
- Please go through the materials listed below, and prepare reagents as needed. Specifically, we recommend diluting and annealing the barcoding oligos in the first half of day 2. We usually do this during the many centrifugations.

Materials Needed

click name of item for order information

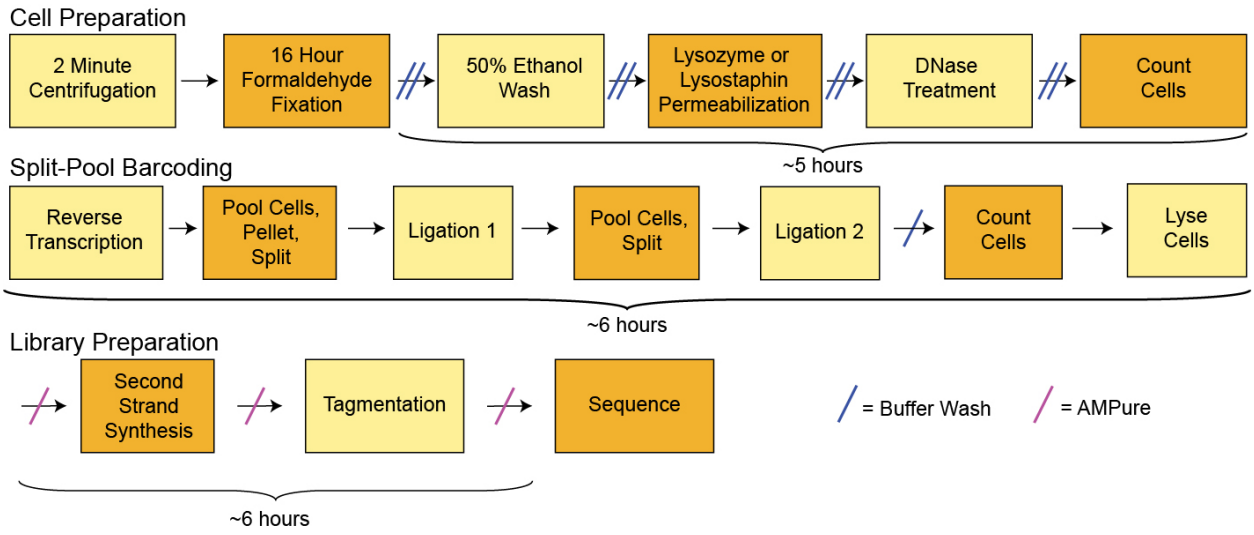
- 4% formaldehyde diluted in 1x PBS (ice-cold for use on Day 1)
- PBS-RI (ice-cold for use on Day 2): 1x PBS supplemented with 0.01 U/μL SUPERase In RNase Inhibitor
- Lysozyme and/or lysostaphin (depending on what kind of cells are being sequenced)
- 100% Ethanol (ice-cold for use on Day 2)
- TEL-RI (ice-cold for use on Day 2): 100 mM Tris pH 8.0, 50 mM EDTA, 0.1 U/μL SUPERase In RNase Inhibitor (10x more RNase inhibitor than PBS-RI)
- DNase-RI buffer (ice-cold for use on Day 2): 1.1x DNase I reaction buffer, 0.1 U/μL SUPERase In RNase Inhibitor
- DNase I
- Semen test hemocytometers
- Maxima H Minus Reverse Transcriptase
- dNTPs (10 mM each)
- 96-well PCR plates
- Foil seals for PCR plates
- 5% Tween-20
- T4 Ligase
- BSA
- 0.5 M EDTA
- 2x Lysis-T: 50 mM EDTA, 400 mM NaCl, 1% Triton X-100
- Proteinase K
- AMPure XP beads

- 80% ethanol (room temperature for AMPure washes)
- Magnetic rack for eppendorf or PCR tubes (we use eppendorf)
- NEBNext Second Strand Synthesis Module
- Nextera XT DNA Library Preparation Kit
- Nextera Index Kit v2 Set A
- If using EZ-Tn5 Transposase instead of Nextera, then the transposase must be loaded as follows:
    1. Anneal 5μL of 100μM SB117 (/5Phos/CTGTCTCTTATACACATCT) and 5μL of 100μM SB118 (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG).
    2. Dilute annealed primers 5x (to 10μM each).
    3. Take 10μL of annealed primers and add 10μL 100% glycerol.
    4. Combine 8μL of annealed primers in 50% glycerol with 2μL of EZ-Tn5.
    5. Incubate 30 minutes at room temperature to load the transposase. Store at -20°C until ready to use.
- NEBNext Multiplex Oligos for Illumina
    - Alternatively, i5 primers can be ordered from IDT based on the sequences in the NEB documentation
- Agilent Bioanalyzer HS DNA Kit
- Qubit HS dsDNA Kit
- Single-tube oligonucleotides (see Oligonucleotide table)
    - Resuspended and annealed oligos, 400μM: SB84 (~40μL), SB81 (~80μL)
        * To anneal each oligo (to form an intramolecular hairpin), heat to 95 °C for 3 minutes then decrease the temperature to 20°C at a ramp speed of -0.1°C/second.
    - Resuspended oligos, 400μM: SB85 (~40μL), SB82 (~80μL)
    - Resuspended oligos, 100μM: SB83, 70μM SB80
- 96 Round 1 RT oligonucleotides (see Oligonucleotide table)
    - Oligos can be ordered from IDT, resuspended in IDTE Buffer pH 8.0 at a concentration of 100μM.
    - Before use on Day 2, dilute the oligos to 10μM.
- 96 Round 2 ligation oligonucleotides, annealed (see Oligonucleotide table)
    - Oligos can be ordered from IDT, resuspended in IDTE Buffer pH 7.5 at a concentration of 100μM
    - To anneal the oligos, add the following to each well of a 96-well plate (scaled up or down as needed - only 2.24μL will be used per barcoding plate):
        * 3.52μL of 100μM linker SB83
        * 2.64μL RNase-free water
        * 3.84μL of 100μM barcode oligo
    - After combining the oligos, briefly spin down the plate then anneal them by heating the plate to 95°C for 3 minutes then decreasing the temperature to 20°C at a ramp speed of -0.1°C/second.
- 96 Round 3 ligation oligonucleotides, annealed (see Oligonucleotide table)
    - Oligos can be ordered from IDT, resuspended in IDTE Buffer pH 7.5 at a concentration of 70μM
    - To anneal the oligos, add the following to each well of a 96-well plate (scaled up or down as needed - only 3.49μL will be used per barcoding plate):

* 6.6μ of 70μM linker SB80
* 7.2μL of 70μM barcode oligo

– After combining the oligos, briefly spin down the plate then anneal them by heating the plate to 95°C for 3 minutes then decreasing the temperature to 20°C at a ramp speed of -0.1°C/second.

Overview of Workflow



Cell Preparation

Day 1

Note: Cells should be grown on Day 1 to the desired growth phase, which may require advanced preparation on preceding days depending on the experiment. For example, in our publication, we started *E. coli* and *S. aureus* overnights on the day before, then diluted cells into fresh media on "Day 1", and grew the cells to exponential or stationary phase. The following steps begin with cells that are in the growth phase and condition of interest.

1. Transfer ~9.6*10^8 cells (calculated roughly based on 1 OD_{600} = 8*10^8 cells/mL) to a centrifuge tube (room temperature) and spin at 5,525*g* for 2 minutes at 4°C.
    - The number of cells can be varied, but we recommend a corresponding adjustment of volumes used throughout the cell preparation protocol. For example, if double the number of cells are taken, then fix cells in 6 mL formaldehyde instead of 3 mL (and so on for the remaining steps). In our paper, we used ~2.2*10^9 cells fixed in 7 mL, which results in many more cells than needed for split-pool barcoding.
    - If combining multiple cell types for fixation (as we did with stationary-phase RFP *E. coli* and exponential-phase GFP *E. coli*), measure the OD600 of these cultures, then combine them to reach ~9.6*10^8 cells total (in the desired ratio of cell types).
2. Take out tubes immediately after spin, and keep cold on ice. Remove supernatant.
3. Resuspend cells in 3 mL of ice-cold 4% formaldehyde.
4. Rotate cell suspension for ~16 hours at 4°C.
    - Using something like a Thermo Scientific Lab Quake Rotator, for example.

Day 2

** Keep cells on ice as much as possible (unless otherwise indicated) throughout Day 2 and chill all reagents on ice before use
** For large volumes, we centrifuge at 5,525*g* using Allegra 25R centrifuge (Beckman Coulter); for small volumes, we centrifuge at 7,000*g* using 5415D centrifuge (Eppendorf)

1. Centrifuge cells at 5,525*g* for 10 minutes at 4°C.
2. Remove supernatant, and resuspend cells in 3 mL PBS-RI.
3. Centrifuge cells at 5,525*g* for 10 minutes at 4°C.
4. Remove supernatant and resuspend cells in 300μL PBS-RI.
5. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
6. Remove supernatant and resuspend cells in 150μL PBS-RI. Add 150μL 100% ethanol and invert tube to mix.
7. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
8. Remove supernatant and resuspend cells in 300μL PBS-RI (wash #1).
9. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
10. Remove supernatant and resuspend cells in 300μL PBS-RI (wash #2).
11. Resuspend cells in the appropriate cell wall degrading enzyme.
    - Lysozyme: 45μL of 100μg/mL lysozyme in TEL-RI
    - Lysostaphin: 45μL of 40μg/mL lysostaphin in TEL-RI
    - *Notes:* We have validated lysozyme for *E. coli* permeabilization and lysostaphin for *S. aureus* permeabilization. We have only used the enzymes separately (i.e. *E. coli* prepared alone or *S. aureus* prepared alone). The lysozyme concentration was set after titrating from 4 to 500μg/mL with little change in cDNA yield (measured by qPCR).
12. Permeabilize cells for 15 minutes at room temperature.
13. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
14. Remove supernatant and resuspend cells in 75μL PBS-RI (wash #1).
15. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
16. Remove supernatant and resuspend cells in 75μL PBS-RI (wash #2).
17. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
18. Resuspend cells in 30μL DNase-RI buffer.
19. Add 3μL DNase I
20. Incubate cells at room temperature for 30 minutes
21. Add 3μL DNase I stop solution
22. Heat cells to 50°C for 10 minutes with shaking at 500 rpm
    - We use a multi-therm shaker for this
    - DNase I is typically inactivated at 70°C, but we inactivate at 50°C to preserve cell integrity
23. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
    - The cell pellet usually looks different after DNase inactivation, specifically more pale and smaller than before DNase. While the pellet should remain visible, this difference in appearance is to be expected.
24. Remove supernatant and resuspend cells in 75μL PBS-RI (wash #1).

25. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
26. Remove supernatant and resuspend cells in 75μL PBS-RI (wash #2).
27. Centrifuge cells at 7,000*g* for 8 minutes at 4°C.
28. Remove supernatant and resuspend cells in 75μL 0.5x PBS-RI.
29. Count cells using a semen test hemocytometer.
    - Determine volume, v_c, of 3*10^7 cells
    - We have also used a Neubauer Improved hemocytometer, but the larger chamber depth makes it much more difficult to focus the cells in a single plane.

Split-Pool Barcoding

Day 2 (contd.)

1. Using a multi-channel pipette, aliquot 2μL of each Round 1 RT oligo (10μM) across a 96-well plate.
    - We typically seal the plate with a foil seal at this point and centrifuge the plate to ensure oligos go to bottom of tubes.
2. Prepare an RT reaction mix (total volume 960μL) by combining the following:
    - 240μL 5x Maxima H Minus RT buffer
    - 24μL dNTPs
    - 12μL SUPERase In RNase Inhibitor
    - 24μL Maxima H Minus Reverse Transcriptase
    - (660 - v_c) μL RNase-free water
    - v_c μL cells (3*10^7 cells)
        - If multiple cell types are to be combined at this stage, add a total of 3*10^7 cells (e.g. 1.5*10^7 *S. aureus* and 1.5*10^7 *E. coli*)
3. Add 8μL of the RT reaction mix to each well of the 96-well plate containing the Round 1 RT oligos (already containing 2μL oligos)
    - We typically aliquot 106μL of the RT reaction mix across an 8-strip PCR tube then use a multi-channel pipette to aliquot 8μL into each well of the 96-well plate. To avoid cells pelleting in the PCR plate, we do not centrifuge the plate after adding cells. Instead, we try to add cells directly to the bottom of the plate and pipette up and down a few times.
4. Incubate the PCR plate for RT as follows:
    - 50°C 10 minutes
    - 8°C 12 seconds
    - 15°C 45 seconds
    - 20°C 45 seconds
    - 30°C 30 seconds
    - 42°C 6 minutes
    - 50°C 16 minutes
    - 4°C hold
5. Immediately after RT finishes and reaches 4°C, pool reactions into one eppendorf tube. Be sure to pipette up and down in cell well to get all of the cells.
6. Add 5% Tween-20 to the pooled cells, reaching a final concentration of 0.04% (125x dilution)
    - The volume of 5% Tween-20 to add should be calculated after measuring the volume of recovered pooled cells. For example, if the volume of pooled cells after RT is 830μL, add 6.6μL 5% Tween-20. We split the pooled cells (before adding Tween-20) into a few tubes so the volume in each is less than ~350μL. This is important for the dilution in the next step.

7. Incubate cells in 0.04% Tween-20 on ice for 3 minutes.
8. Add PBS-RI to bring the final concentration of Tween-20 to 0.01%.
    - For example, add a total of 2510μL PBS-RI to 836.6μL of 0.04% PBS-RI. If 836.6μL is split into 3 tubes of 279μL, then add 836.6μL PBS-RI to each of these tubes.
9. Centrifuge cells at 7,000*g* for 10-20 minutes at 4°C. A small pellet should be visible.
    - We were routinely centrifuging for 20 minutes (and sometimes at 10,000*g*), but we have noticed that cells pellet sooner than 20 minutes, so it should be fine to centrifuge for less time if a pellet is visible. We also often remove most of the supernatant and then spin an additional few minutes before removing the rest of it.
10. *During centrifugation,* aliquot 2.24μL of annealed Round 2 Ligation Oligos into a new 96-well plate. Briefly centrifuge the plate to collect volume at the bottom.
11. *After centrifugation,* remove supernatant and resuspend cells in 600μL 1x T4 ligase buffer supplemented with 0.1 U/μL SUPERase In RNase Inhibitor.
12. Prepare a ligation master mix as follows:
    - 7.5μL RNase-free water
    - 37.5μL 10x T4 ligase buffer
    - 16.7μL SUPERase In RNase Inhibitor
    - 5.6μL BSA
    - 27.9μL T4 ligase
    - 600μL resuspended cells
13. Add 5.76μL of the RT reaction mix to each well of the 96-well plate containing the Round 2 ligation oligos (already containing 2.24μL oligos)
    - We typically aliquot 76μL of the ligation reaction mix across an 8-strip of PCR tubes then use a multi-channel pipette to aliquot 5.76μL into each well of the 96-well plate. To avoid cells pelleting in the PCR plate, we do not centrifuge the plate after adding cells. Instead, we try to add cells directly to the bottom of the plate and pipette up and down a few times.
14. Incubate ligations for 30 minutes at 37°C.
15. After the ligation, add 2μL Round 2 blocking mix to each well.
    - Round 2 blocking mix includes:
        - 37.5μL 400μM SB84 blocking oligo (annealed to self, see explanation in materials)
        - 37.5μL 400μM SB85 blocking oligo
        - 25μL 10x T4 ligase buffer
        - 150μL RNase-free water
    - We typically aliquot 27μL of blocking mix across an 8-strip of PCR tubes then use a multi-channel to aliquot 2μL into each well
16. Incubate the blocking reaction for 30 minutes at 37°C.
17. *During blocking,* aliquot 3.49μL of annealed Round 3 Ligation Oligos into a new 96-well plate. Briefly centrifuge the plate to collect volume at the bottom.
18. Pool cells after blocking into a single eppendorf tube.
19. Add the following to the pooled cells for the Round 3 ligation:
    - 46μL 10x T4 ligase buffer
    - 12.65μL T4 ligase

- 115μL RNase-free water
  - In earlier iterations of PETRI-seq, we did not add a substantial amount of water to the round 3 mix. The 115μL recommended here amounts to only 1μL per 12μL in each well. We decided to add it, effectively diluting the reactions a bit, in order to facilitate further optimizations to the protocol.

20. Add 8.51μL of the RT reaction mix to each well of the 96-well plate containing the Round 3 ligation oligos (already containing 3.49μL oligos)
    - We typically aliquot 110μL of the ligation reaction mix across an 8-strip of PCR tubes then use a multi-channel pipette to aliquot 8.51μL into each well of the 96-well plate. To avoid cells pelleting in the PCR plate, we do not centrifuge the plate after adding cells. Instead, we try to add cells directly to the bottom of the plate and pipette up and down a few times.
21. Incubate ligations for 30 minutes at 37°C.
22. After the ligation, add 10μL Round 3 blocking mix to each well.
    - Round 3 blocking mix includes:
      - 72μL 400μM SB81 blocking oligo (annealed to self, see explanation in materials)
      - 72μL 400μM SB82 blocking oligo
      - 120μL 10x T4 ligase buffer
      - 336μL RNase-free water
      - 600μL 0.5 M EDTA
    - We typically aliquot 133μL of blocking mix across an 8-strip of PCR tubes then use a multi-channel to aliquot 10μL into each well
23. After adding the blocking mix, pool cells (total volume ~2.1 mL) and split into 2 eppendorf tubes.
24. Measure the volume of pooled cells, and add Tween-20 to a final concentration of 0.01%.
25. Centrifuge cells at 7,000*g* for 10 minutes at 4°C.
26. Remove supernatant and resuspend cells in 500μL TEL-RI + 0.01% Tween-20.
    - The cells in the two tubes can be combined here in a total of 500μL.
27. Centrifuge cells at 7,000*g* for 10 minutes at 4°C.
28. Remove supernatant and resuspend cells in 30μL TEL-RI.
29. Count cells using a semen test hemocytometer.
30. Transfer ~10,000 cells into eppendorf tubes for lysis.
    - Up to 40,000 cells can be taken for each lysate, but we typically take 10,000 cells in case cell counts are underestimated. We usually prepare 10-20 lysates from the pooled cells at this stage to be safe in case anything goes wrong in downstream steps or to be able to sequence more cells as needed.
    - We recommend counting cells and aliquoting lysates *quickly*, as cell lysis may occur prior to making lysates and result in higher levels of cross-contamination in the final library. We also may later recommend more washes, but at this point we have only tested a single wash before lysis.
31. Add TEL-RI to the 10,000 cells to bring the volume to 25μL.
32. Add 25μL 2x lysis-T and 5μL proteinase K (20 mg/mL) to the cells in TEL-RI.
33. Lyse cells for 1 hour at 55°C with shaking at 750 rpm.
    - We use a multi-therm shaker for this.
34. Save lysates at -80°C.

- We typically proceed to library preparation the following day.

Library Preparation

Day 3

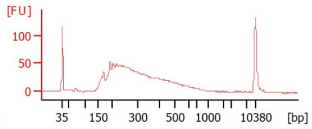
Note that during library preparation, it is critical to carry over the entire library at each step before amplification. Any lost cDNA prior to amplification will correspond to a reduction in UMIs captured per cell.

1. Thaw lysates on ice
2. Purify lysates by adding AMPure XP beads at a 1.8x ratio (approximately 99μL of beads, but measure the volume of lysate to be precise). Elute in 20μL RNase-free water after following the purifications steps below.
3. We use the following AMPure purification protocol:
   (a) Incubate the lysate with beads for at least 5 minutes
   (b) Place tubes on a magnetic stand.
   (c) Let sit for about 2 minutes to allow beads to separate.
   (d) Once the solution is clear, remove the liquid, leaving behind the beads and being *very* careful not to remove any beads
   (e) Add 200μL 80% ethanol and let sit for at least 30 seconds, keeping tubes on magnetic rack (wash #1).
   (f) Remove ethanol.
   (g) Add 200μL 80% ethanol and let sit for at least 30 seconds, keeping tubes on magnetic rack (wash #2).
   (h) Remove ethanol.
   (i) Keeping tubes on magnetic rack with lids open, allow to dry for ~10 minutes, watching to be sure beads are not over-dried.
   (j) Take tubes off magnetic rack, and resuspend beads in 20μL RNase-free water.
   (k) Incubate beads in water for ~10 minutes to elute cDNA.
   (l) Place tubes on the magnetic stand.
   (m) Let sit for ~1 minutes, until solution is clear.
   (n) Remove the entire volume, leaving behind all of the beads, and transfer the volume to a PCR tube. We find that it is possible to recover the entire volume while excluding beads if pipetting carefully.
4. Add the following mix to each purified lysate:
   - 14μL RNase-free water
   - 4μL NEBNext Second Strand Synthesis Reaction Buffer
   - 2μL NEBNext Second Strand Synthesis Enzyme Mix
5. Incubate the 40μL reaction for 2.5 hours at 16°C with heated lid off.
6. Purify the reaction by adding 72μL AMPure XP beads and following the purification protocol above. Elute in 20μL water.
7. Proceed to tagmentation or store the double-stranded cDNA at -20°C.
8. For tagmentation, we use a slightly modified version of the Nextera XT protocol.
9. Add 25μL TD buffer and 5μL ATM to the purified lysate.

- We have preliminary results using EZ-Tn5 Transposase instead of the Nextera kit. EZ-Tn5 gives a higher yield (theoretically 2x) and is less expensive. To use EZ-Tn5, instead add:
  - 23μL TD buffer
  - 2μL loaded EZ-Tn5 **diluted 1:16** (see materials for how to load)
  - 4.9μL water

10. Briefly spin down the reaction contents.
11. Incubate the reaction on a preprogrammed thermocycler: 5 minutes at 55°C, hold at 10°C.
12. Once the reaction reaches 10°C, immediately add 12.5μL NT.
13. Briefly spin down the reaction contents.
14. Incubate at room temperature for 5 minutes.
15. Add the following to the reaction:
    - 2.5μL i50x primer (NEB kit E7600S) or 2.5 μL of the same primer from IDT (10μM)
    - 2.5μL N70x primer (Nextera Index Kit v2)
    - 20μL water
    - 37.5μL NPM
      - In this reaction, NPM is used at a much higher rate than the other Nextera reagents. We recognize that this will lead to extra expense that we think can be avoided by using another PCR mix. We have had success using Q5 (with GC enhancer) and phusion. With Q5, we first diluted the tagmentation reaction (after NT) 4x and set up the PCR in a total volume of 500μL (because Q5 is sensitive to the SDS in NT). With Phusion, we first diluted the tagmentation reaction (after NT) 2x and set up the PCR in a total volume of 250μL. We set up the Q5 reaction by adding the following after tagmentation and following the same thermocycling protocol as with NPM:
        * 187.5μL water
        * 100μL Q5 5x buffer
        * 10μL 10 mM dNTPs
        * 5μL Q5 enzyme
        * 85μL GC enhancer
        * 25μL i50x primer (NEB kit E7600S) or 2.5μL 100μM primer from IDT + 22.5 μL water
        * 25μL N70x primer (Nextera Index Kit v2)
16. Thermocycle until early-exponential phase of amplification:
    - 72°C 3 min
    - 95°C 30 sec
    - ~16x [until early-exponential]:
      - 95°C 10 sec
      - 55°C 30 sec
      - 72°C 30 sec
    - 72°C 5 min
    - 4°C hold
      - To determine the number of cycles to amplify, we first run 8 PCR cycles and then remove 5μL from the reaction. We add EvaGreen dye and ROX low reference dye to the 5μL and then amplify on our qPCR. Alternatively, SYBR could be added to the initial reaction, and the entire reaction could be run on a qPCR.
17. Purify the products with AMPure beads at a 1x ratio, as described above. Elute in 20-40μL.

18. Quantify the library concentration using the Qubit dsDNA HS kit and Agilent Bioanalyzer HS DNA assay.
    - We usually see a concentration ~1-5 ng/μL on the Qubit.
    - Purify again if there is a spike in the bioanalyzer trace below ~200 bp. The empty construct is 185 bp.
    - The Bioanalyzer size distribution does usually skew towards short fragments. Here is an example of a library that gave good sequencing results:



    - We use the molarity given by the bioanalyzer to determine the amount of sample to load for sequencing.
19. Sequence libraries using at least 75 cycles paired-end (58 cycles read 1, 17 cycles read 2). We recommend about 50 million reads per PETRI-seq library.