Last edit: October 27, 2017

## SPLiT-seq Protocol, Version 3.0

Projected Experimental Time: 2 Days
Recommended time on day 1 to start: morning

## Addition of RNase inhibitor to buffers:

When any buffer has “+RI” next to it, this indicates that enzymatics RNase inhibitor should be added to a final concentration of 0.1 U/uL.

## Centrifugation Steps:

All centrifugation steps should be performed with a swinging bucket rotor. Using a fixed angle centrifuge may lead to more cell loss. Depending on the tissue type, centrifugation speeds may need to be changed to optimize cell retention (e.g. smaller cells = higher speeds).

## DNA Barcoding Plate Generation

What you need:

- Three 96 well plates from IDT - Reverse Transcription Barcode Primers, Ligation Round 1,and Ligation Round 2 Stock DNA Oligo plates (100 uM)
- Two linker oligos - BC_0215, BC_0060 (*Note: these are assumed to be in stock concentration of 1mM, be sure to correct for volume if only have 100 uM stocks)*
- Six 96 well PCR plates (3 stock plates that will last at least 10 experiments, and 3 plates for 1st experiment)

*Note: This will generate 100 uL of DNA barcodes for each well. Each SPLiT-seq experiment requires only 4 uL/well of the reverse transcription primer solution which will last for 25 experiments. Each SPLiT-seq experiment requires only 10 uL/well of the barcode/linker solutions, so these plates will last a total of 10 experiments.*

*Round 1 reverse transcription barcoded primers (final concentrations of 12.5 uM random hexamer and 12.5 uM 15dT primers in each of 48 wells)*

1. Using multichannel pipette, add 12.5 uL of rows A-D in the IDT Reverse Transcription Barcode Primers to rows A-D of the BC Stock 96 well PCR plate.
2. Using multichannel pipette, add 12.5 uL of rows E-H in the IDT Reverse Transcription Barcode Primers to rows A-D of the BC stock 96 well PCR plate (mixing polydT with random hexamer primer here)
3. Add 75ul of water to rows A-D of the BC stock 96 well PCR plate.

*Round 2 ligation round (Final concentrations of 12uM barcodes, 11uM linker-BC_0215)*

1. Using multichannel pipette, add 12uL of IDT Round 2 Barcodes to R1 Stock 96 well PCR plate
2. Add 138.6ul of BC_0215(1mM) to 10.9494mL water in a basin (BC_0215_dil)
3. Using multichannel pipette, add 88uL BC_0215_dil to each well of R2 Stock 96 well PCR plate

*Ligation Round 3 (Final concentrations of 14uM barcodes, 13uM linker-BC_0060)*

1. Using multichannel pipette, add 14uL of Round 3 Barcodes to R3 Stock 96 well PCR plate
2. Add 163.8ul of BC_0060(1mM) to 10.6722mL water in a basin (BC_0060_dil)
3. Using multichannel pipette, add 86uL BC_0060 to each well R3 Stock 96 well PCR plate

For each ligation plate (R2 and R3, not including reverse transcription barcodes), anneal the barcode and linker oligos with the following thermocycling protocol:

1. Heat to 95C for 2 minutes
2. Ramp down to 20C for at a rate of -0.1C/s
3. 4C

Aliquot out 10 uL of each barcode/linker stock plate into 3 new 96 well PCR plates. These are the plates that should be used for DNA barcoding in the split-pool ligation steps in the protocol.

## Nuclei Extraction (Optional):

1. Prepare the following items:
    - Keep dounce at 4C until use
    - 15ml of 1xPBS + 37.5 Superase-in + 19ul Enzymatics Rnase inhibitor. (kept on ice)
    - Precool centrifuge to 4C
2. Make **NIM1 buffer**:

| Reagent | Stock Concentration | Final Concentration | Volume (uL) |
|---|---|---|---|
| Sucrose | 1.5 M | 250mM | 2,500 |
| KCl | 1 M | 25mM | 375 |
| MgCl2 | 1 M | 5mM | 75 |
| Tris buffer, pH 8 | 1 M | 10mM | 150 |
| Water | NA | NA | 11,900 |
| Final Volume | | | 15,000 |

3. Make the **homogenization buffer:**

| Reagent | Stock Concentration | Final Concentration | Volume (uL) |
|---|---|---|---|
| NIM1 Buffer | 1.5 M | | 4,845 |
| 1 mM DTT | 1 mM | 1uM | 5 |
| Enzymatics RNase-In (40U/ul) | 40 U/uL | 0.4U/ul | 50 |
| Superase-In (20U/UL) | 20 U/uL | 0.2U/ul | 50 |
| 10% Triton X-100 | 10% | NA | 50 |
| Final Volume | | | 5,000 |

4. Dounce
    - Add tissue/cells sample to dounce. If cells, resuspend in 700ul of homogenization buffer.
    - Add homogenization buffer to ~700ul
    - Perform 5 strokes of loose pestle
    - Perform 10 - 15 of tight pestle
    - Add homogenization buffer  up to 1ml
    - Check cell lysis with 5ul trypan blue and 5ul cells on haemocytometer to see if nuclei have been released
5. Filter homogenates with 40um strainer into 5ml eppendorf tubes (or 15mL falcon). Tilting the filter 45° while straining over the tube ensures that the lysate passes through as intended.

    Note: This straining process is different from every other one below.
6. Spin for 4min at 600g (4C) and remove supernatant (can leave about 20uL to avoid aspirating pellet)
7. Resuspend in 1ml of 1x PBS + RI
8. Add 10ul of BSA
9. Centrifuge at 600g for 4min.
10. Resuspend in 200ul 1x PBS + RI.
11. Take 50ul of the resuspended cells from step 4 and add 150ul of 1xPBS + RI. Count sample on hemocytometer and/or flow-cytometer.
    - The volume of resuspended cells from the step 4 can be changed based on the considerations of the user.
12. Pass cells through a 40um strainer into a fresh 15mL Falcon tube and place on ice.
    - See note on step 4 of Fixation and Permeabilization.
13. Resuspend the desired number of nuclei (typically 2M) in 1mL 1x PBS + RI and proceed with step 5 in the following *Fixation and Permeabilization* protocol.

## Fixation and Permeabilization

1. Prepare the following buffers (calculated for two experiments):
    - A 1.33% formalin (360 uL of 37% formaldehyde solution (Sigma)+ 9.66 ml PBS) solution and store at 4C.
    - 6 mL of 1X PBS+RI (15 uL of SUPERase In and 7.5 uL of enzymatics rnase inhibitor)
    - 2 mL of  0.5X PBS+RI (5 uL of SUPERase in and 2.5 of enzymatics rnase inhibitor)
    - 500uL of 5% Triton X-100 + RI (2 uL of SUPERase In)
    - 500uL of 100mM Tris pH 8.0 + 2 uL SUPERase In
    - Set the centrifuge to 4C
2. Pellet cells by centrifuging at 500g for 3 mins at 4C. (Some cells may require faster centrifugation.)
3. Resuspend cells in 1mL of cold PBS+RI. Keep cells on ice between these steps.
4. Pass cells through a 40um strainer into a fresh 15mL Falcon tube and place on ice.
    Note: The cell resuspension is not likely to passively go through the strainer, which can cause cell loss. Instead, with a 1ml pipette filled with the resuspension, press the end of the tip directly onto the strainer and actively push the liquid through. The motion should take ~1 second.
5. Add 3 mL of cold 1.33% formaldehyde (final concentration of 1% formaldehyde). Fix cells on ice for 10 mins.
6. Add 160uL of 5% Triton-X100+RI to fixed cells and mix by gently pipetting up and down 5x with a 1mL pipette. Permeabilize cells for 3 mins on ice.
7. Centrifuge cells at 500g for 3 mins at 4C.
8. Aspirate carefully and resuspend cells in 500 uL of cold PBS+RI.
9. Add 500uL of cold 100 mM Tris-HCl, pH 8.0.
10. Add 20 uL of 5% Triton X-100.
11. Centrifuge cells at 500g for 3 mins at 4C.
12. Aspirate and resuspend cells in 300 ul of cold **0.5x** PBS+RI.
13. Run cells through a 40uM strainer into a new 1.7mL tube.
    - See note on step 4 of Fixation and Permeabilization.
14. Count cells using a hemacytometer or a flow-cytometer and dilute the cell suspension to 1,000,000 cells/mL. While counting cells, keep cell suspension on ice.
    *Note: This step will dictate how many cells enter the split-pool rounds. It will be possible to sequence only a subset of the cells that enter the split-pool rounds (can be done during sublibrary generation at lysis step). The total number of barcode combinations you will be using should be calculated to determine the maximum number of cells you can sequence with minimal barcode collisions. As a rule of thumb, the number of cells you process should not exceed more than 5% of total barcode combinations. We usually have a dilution between 500k to 1M cells/mL here (equates to 4-8k cells going into each well for reverse transcription barcoding rounds).*

**Reverse Transcription**

1. Aliquot out 4 uL of the RT barcodes stock plate into the top 4 rows (48 wells) of a new 96 well plate. Cover the this plate with an adhesive plate seal until ready for use.
2. Create the following reverse transcription (RT) mix on ice:

| Reagent | Stock Concentration | Desired Concentration | Per Reaction | Volume in Mix (48 wells + 10%) |
|---|---|---|---|---|
| 5X RT Buffer | 5x | 1x | 4 | 211.2 |
| Enzymatics Rnase Inhibitor | 40u/uL | 0.25u/uL | 0.125 | 6.6 |
| Superase In Rnase Inhibitor | 20U/uL | 0.25U/uL | 0.25 | 13.2 |
| dNTPs | 10mM (per base) | 500uM | 1 | 52.8 |
| Maxima H Minus Reverse Transcriptase | 200u/uL | 20u/ul | 2 | 105.6 |
| H2O | NA | NA | 0.625 | 33 |
| **Total Volume** | | | **8** | **422.4** |

3. Add 8uL of the RT mix to each of the top 48 wells. Each well should now contain a volume of 12uL.
4. Add 8uL of cells in 0.5x PBS+RI to each of the top 48 wells. Each well should now contain a volume of 20uL.
5. Add the plate into a thermocycler with the following protocol
    a. 50 C for 10 minutes
    b. Cycle 3 times:
        i. 8C for 12s
        ii. 15C for 45s
        iii. 20C for 45s
        iv. 30C for 30s
        v. 42C for 2 min
        vi. 50C for 3 min
    c. 50C for **5 min**
    d. 4C forever
6. Place the RT plate on ice.
7. Prepare 2 mL of 1x NEB buffer 3.1 with 20uL of Enzymatics RNase Inhibitor.
8. Transfer each RT reaction to a 15mL falcon tube (also on ice).
9. Add 9.6uL of 10% Triton-X100 to get a final concentration of 0.1%.
10. Centrifuge pooled RT reaction for 3 min at 500G.
11. Aspirate supernatant and resuspend into 2 mL of 1x NEB buffer 3.1 + 20uL Enzymatics RNase Inhibitor.

**Ligation Barcoding**

Make the following ligation master mix on ice:
*Note: Final concentration takes added volume of DNA barcodes into account. Concentrations of this mix is not the final concentration at time of barcoding*

| Reagent | Stock Concentration | Final Concentration | Volume (uL) |
|---|---|---|---|
| Water | NA | NA | 1337.5 |
| T4 Ligase Buffer 10x | 10X | 1X | 500 |
| Enzymatics Rnase Inhibitor | 40 U/uL | 0.32 U/uL | 40 |
| Superase In | 20 U/uL | 0.05 U/uL | 12.5 |
| BSA | 20 mg/mL | 0.2 mg/mL | 50 |
| T4 DNA Ligase | 400 U/uL | 8 U/uL | 100 |
| **Total Volume** | | | **2040** |

1. Add the 2mL of cells in NEB buffer 3.1 into the ligation mix. The mix should now have a volume of 4.04 mL
2. Add the mix into a basin
3. Using a multichannel pipet, add 40 uL of ligation mix (with cells) into each well of the round 1 DNA barcode plate.
4. Cover the round 1 DNA barcode plate with an adhesive plate seal and incubate for **30 minutes at 37C** with gentle rotation (50 rpm).
5. Make the round 1 blocking solution and add it to a new basin

| Reagent | Stock Concentration | Final Concentration | Volume (uL) |
|---|---|---|---|
| BC_0216 | 100 uM | 26.4 uM | 316.8 |
| 10x Ligase Buffer | 10X | 2.5X | 300 |
| Water | NA | NA | 583.2 |
| **Final Volume** | | | **1200 uL** |

6. Remove the round 1 DNA barcoding plate from the incubator and remove the cover.
7. Using a multichannel pipet, add 10 uL of the round 1 blocking solution to each of the 96 wells in the round 1 DNA barcoding plate.
8. Cover the round 1 DNA barcode plate with an adhesive plate seal and incubate for **30 minutes at 37C** with gentle rotation (50 rpm).

9. Remove round 1 DNA barcoding plate from the incubator, remove cover, and pool all cells into a new basin.
10. Pass all the cells from this basin through a 40 um strainer into another basin.
    - See note on step 4 of Fixation and Permeabilization.
11. Add 100 uL of T4 DNA ligase to the basin and mix by pipetting ~20 times.
12. Using a multichannel pipette, add 50 uL of cell/ligase solution into each well of the round 2 DNA barcode plate.
13. Cover the round 2 DNA barcode plate with an adhesive plate seal and incubate for **30 minutes at 37C** with gentle rotation (50 rpm).
14. Make the round 2 blocking solution and add it to a new basin

| Reagent | Stock Concentration | Final Concentration | Volume (uL) |
|---|---|---|---|
| BC_0066 | 100 uM | 11.5 uM | 369 |
| EDTA | 0.5 M | 125 mM | 800 |
| Water | NA | NA | 2031 |
| **Final Volume** | | | **3200 uL** |

15. Remove the round 2 DNA barcoding plate from the incubator and remove the cover.
16. Using a multichannel pipet, add 20 uL of the round 2 blocking and termination solution to each of the 96 wells in the round 2 DNA barcoding plate.
17. Pool all cells into a new basin. (no incubation for the final blocking step)
18. Pass all the cells from this basin through a 40 um strainer into a 15 mL falcon tube.
    - See the note for step 4.
19. Count cells on a flow cytometer. Make sure cells are well mixed before aliquoting sample for counting.

**Lysis**

1. Make the 2X lysis buffer:

| Reagent | Stock Concentration | Final Concentration (2X) | Volume (mL) |
|---|---|---|---|
| Tris, pH 8.0 | 1 M | 20 mM | 0.5 |
| NaCl | 5 M | 400 mM | 2 |
| EDTA, pH 8.0 | 0.5 M | 100 mM | 5 |
| SDS | 10% | 4.4 % | 11 |
| Water | NA | NA | 6.5 |
| **Final Volume** | | | **25** |

2. If white precipitate appears, warm at 37C until precipitate is back in solution (roughly 10-15 min).
3. Make the following wash buffer:

| Reagent | Volume (uL) |
|---|---|
| 1X PBS | 4000 |
| 10 % Triton X-100 | 40 |
| Superase In Rnase Inhibitor | 10 |
| **Final Volume** | **4050** |

4. Add 70ul of 10% triton to the cells. (~0.1% final conc.)
5. Centrifuge for 5 min at 1000G in 15ml tube.
   Note: The pellet for the steps below will be very small and it may not be visible.
6. Aspirate supernatant, leave ~30ul to avoid removing pellet.
   a. If possible, remove as much supernatant as possible with 20uL pipet.
7. Resuspend with 4 mL of wash buffer.
8. Centrifuge for 5 min at 1000G.
9. Aspirate supernatant and resuspend in 50ul 1x PBS + RI.
10. Dilute 5ul into 195uL of 1x PBS and count via flow cytometry.
    - Or take 5ul into 5ul of 1x PBS and count on hemocytometer (it can be hard to distinguish debris from cells).
11. Determine how many sublibraries you would like to generate (# sublibraries= # tubes needed), and how many cells you would like to have for each of these sublibraries.
12. Aliquot the desired number of cells for each sublibrary into new 1.7mL tubes. Add 1x PBS to each tube to a final volume of 50uL.
13. Add 50uL of 2x Lysis buffer to each tube.
14. Add 10uL of Proteinase K (20mg/mL) to each lysate.
15. Incubate at 55C for 2 hrs with shaking at 200rpm.
16. Stopping point: Freeze lysate(s) at -80C.

**Prepare buffers**
First make the following stock solutions:

100mM PMSF (resuspended in isopropanol)

| **2x B&W** | |
|---|---|
| **Reagents** | **Volume** |
| 1M Tris-HCl pH 8.0 | 500uL |
| 5M NaCl | 20ml |
| EDTA, 0.5M | 100ul |
| Nuclease Free Water | 29.4ml |
| **Total** | **50mL** |

| **1x B&W-T** | |
|---|---|
| **Reagents** | **Volume** |
| 1M Tris-HCl pH 8.0 | 100uL |
| 5M NaCl | 4ml |
| EDTA, 0.5M | 20ul |
| Tween 20 10% | 100ul |
| Nuclease Free Water | 15.78ml |
| **Total** | **20mL** |

Then make the following smaller aliquots (with added RNase inhibitor):

**1x B&W-T + RI:**

| | **Volume per Number of Samples (uL)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Reagent** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| 1xB&W-T | 3600.0 | 4200.0 | 4800.0 | 5400.0 | 6000.0 | 6600.0 | 7200.0 | 7800.0 |
| SUPERase In | 5.0 | 5.8 | 6.7 | 7.5 | 8.3 | 9.2 | 10.0 | 10.8 |
| **Final Volume** | **3605.0** | **4205.8** | **4806.7** | **5407.5** | **6008.3** | **6609.2** | **7210.0** | **7810.8** |

**2x B&W + RI:**

| | **Volume per Number of Samples (uL)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Reagent** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| 2xB&W | 110.0 | 220.0 | 330.0 | 440.0 | 550.0 | 660.0 | 770.0 | 880.0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| SUPERase In | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 12.0 | 14.0 | 16.0 |
| **Final Volume** | **112.0** | **224.0** | **336.0** | **448.0** | **560.0** | **672.0** | **784.0** | **896.0** |

**Tris-T + RI:**

| | Volume per Number of Samples (uL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Reagent** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| 10mM Tris-HCl (pH 8.0) | 600.0 | 1200.0 | 1800.0 | 2400.0 | 3000.0 | 3600.0 | 4200.0 | 4800.0 |
| Tween-20 (10%) | 6.0 | 12.0 | 18.0 | 24.0 | 30.0 | 36.0 | 42.0 | 48.0 |
| SUPERase In | 1.5 | 3.0 | 4.5 | 6.0 | 7.5 | 9.0 | 10.5 | 12.0 |
| **Final Volume** | **607.5** | **1215.0** | **1822.5** | **2430.0** | **3037.5** | **3645.0** | **4252.5** | **4860.0** |

**Purification of cDNA**

*Note: We performed agitation steps on a vortexer with a foam 1.7mL tube holder on a low setting (2/10).*

*Wash MyOne C1 Dynabeads*

1. For each lysate to be processed, add 44uL of MyOne C1 Dynabeads to a 1.5 mL tube (eg, 1 lysate=44uL, 2 lysates = 88uL, 3 lysates = 132ul etc)
2. Add 800uL of 1xB&W-T buffer
3. Place sample against a magnetic rack and wait until liquid becomes clear (1-2 min).
4. Remove supernatant and resuspend beads in 800uL of 1xB&W-T buffer.
5. Repeat steps 3-4 two more times for a total of 3 washes.
6. Place sample against a magnetic rack and wait until liquid becomes clear.
7. Resuspend beads in 100uL (per sample) 2xB&W buffer + RI.

**Sample Binding to Streptavidin:**

1. Add 5uL of 100uM PMSF (resuspended in isopropanol) to each sample and leave at room temperature for 10 min.
2. Add 100ul of resupended C1 beads to each tube.
3. To bind cDNA to C1 beads, agitate at room temperature for 60 min.
4. Place sample against a magnetic rack and wait until liquid becomes clear (1-2 min).
5. Remove supernatant and resuspend beads in 250uL of 1xB&W
6. Agitate beads for 5 min at room temperature.
7. Repeat steps 5 and 6.
8. Remove supernatant and resuspend beads in 250 uL of 10mM Tris+T
9. Agitate beads for 5 min at room temperature.
10. Leave beads in final wash solution on ice.

*Template Switch*

Prepare the following mix depending on the number of samples:

| | Volume per Number of Samples (uL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Reagent** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| Water | 88.0 | 176.0 | 264.0 | 352.0 | 440.0 | 528.0 | 616.0 | 704.0 |
| Maxima RT Buffer | 44.0 | 88.0 | 132.0 | 176.0 | 220.0 | 264.0 | 308.0 | 352.0 |
| Ficoll PM-400 (20%) | 44.0 | 88.0 | 132.0 | 176.0 | 220.0 | 264.0 | 308.0 | 352.0 |
| 10mM dNTPs (each, total is 40mM) | 22.0 | 44.0 | 66.0 | 88.0 | 110.0 | 132.0 | 154.0 | 176.0 |
| RNase Inhibitor | 5.5 | 11.0 | 16.5 | 22.0 | 27.5 | 33.0 | 38.5 | 44.0 |
| TSO (BC_0127) | 5.5 | 11.0 | 16.5 | 22.0 | 27.5 | 33.0 | 38.5 | 44.0 |
| Maxima RT RnaseH Minus Enzyme | 11.0 | 22.0 | 33.0 | 44.0 | 55.0 | 66.0 | 77.0 | 88.0 |
| **Total** | **220.0** | **440.0** | **660.0** | **880.0** | **1100.0** | **1320.0** | **1540.0** | **1760.0** |

1. Place sample against a magnetic rack and wait until liquid becomes clear.
2. With sample still on magnetic rack, remove supernatant and wash with 250uL of water (do not resuspend beads this time).
3. Resuspend sample in 200ul of Template Switch Mix.
4. Incubate at room temp for 30 min with agitation or rolling.
5. Incubate at 42C for 90 min with agitation or rolling (we shook in incubator at 100 rpm).
6. Potential Stopping Point. If stopping perform the following (otherwise skip to next section):
    a. Place sample against a magnetic rack and wait until liquid becomes clear.
    b. Resuspend in 250uL Tris-T.

**cDNA Amplification**

Prepare the following PCR mix depending on the number of samples:

| | Volume per Number of Samples (uL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Reagent** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| Kapa Hifi 2x Master Mix | 121.00 | 242.00 | 363.00 | 484.00 | 605.00 | 726.00 | 847.00 | 968.00 |
| BC_0108 (10uM) | 9.68 | 19.36 | 29.04 | 38.72 | 48.40 | 58.08 | 67.76 | 77.44 |
| BC_0062 (10uM) | 9.68 | 19.36 | 29.04 | 38.72 | 48.40 | 58.08 | 67.76 | 77.44 |
| Water | 101.64 | 203.28 | 304.92 | 406.56 | 508.20 | 609.84 | 711.48 | 813.12 |
| **Total** | **242.0** | **484.0** | **726.0** | **968.0** | **1210.0** | **1452.0** | **1694.0** | **1936.0** |

1. Place sample against a magnetic rack and wait until liquid becomes clear.
2. With sample against magnet wash with 250uL nuclease-free water (do not resuspend).
3. Resuspend sample with 220uL PCR mix and split equally into 4 different PCR tubes.
4. Run the following thermocycling program:
    a. 95C 3 min
    b. 98C 20s
    c. 65C 45s
    d. 72C 3min
    e. Repeat (b-d) 4x (5 total cycles)
    f. 4C hold.
5. Combine all 4 reactions into a single 1.7mL tube. Make sure to resuspend any beads that may be stuck to the bottom or sides of the PCR tubes before combining reactions.
6. Place sample against a magnetic rack and wait until liquid becomes clear.
7. Transfer 200uL of supernatant to 4 optical grade qPCR tubes (50uL in each tube).
8. Add 2.5uL of 20x evagreen to each qPCR tube.
9. Run the following qPCR program (make sure to remove samples, once signal starts to leave exponential phase to prevent overamplification).
    a. 95C 3 min
    b. 98C 20s
    c. 67C 20s
    d. 72C 3min
    e. Repeat (b-d) until signal plateaus out of exponential amplification
    f. 72C 5 min
    g. 4C hold
10. Optional: Run an agarose gel or bioanalyze resulting qPCR. There will likely be a combination of cDNA and dimer present.

*SPRI size selection (0.8x)*

1. Combine qPCR reactions into a single tube.
2. Take out 180 uL of the pooled qPCR reaction and place in new 1.7 mL tube
3. Add 144uL of Kapa Pure Beads to tube and vortex briefly to mix. Wait 5 min to bind DNA.
4. Place tube against magnetic rack and wait until liquid becomes clear.
5. Remove the supernatant.
6. With tubes still on magnetic rack, wash with 750uL 85% ethanol. Do not resuspend beads.
7. Repeat step 6.
8. Remove ethanol and air dry bead (~5min). To not let beads overdry and crack.
9. Resuspend beads from each tube in 20uL of water. Once beads are fully resuspended in the water, incubate the tube at 37C for 10 min.
10. Bind tubes against magnetic rack and wait until liquid becomes clear.
11. Transfer 18.5uL of elutant into a new optical grade PCR tube.

12. Run a bioanalyzer trace on 10 uL of the elutant
13. If no dimer is present after size selection, jump directly to “Tagmentation and Illumina Amplicon Generation” section. If dimer is still present, proceed to step 14 to perform a second amplification and size selection step. This may be necessary for cells with low RNA content, but should not be necessary for cells with high RNA content (eg, HeLa-S3, NIH/3T3, etc.).

*Optional: Second qPCR:*

14. Make the following qPCR mix depending on the number of samples:

| | Volume per Number of Samples (uL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Reagent** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
| Kapa Hifi 2x Master Mix | 27.50 | 55.00 | 82.50 | 110.00 | 137.50 | 165.00 | 192.50 | 220.00 |
| BC_0062 (10uM) | 2.20 | 4.40 | 6.60 | 8.80 | 11.00 | 13.20 | 15.40 | 17.60 |
| BC_0108 (10uM) | 2.20 | 4.40 | 6.60 | 8.80 | 11.00 | 13.20 | 15.40 | 17.60 |
| Evagreen 20x | 2.75 | 5.50 | 8.25 | 11.00 | 13.75 | 16.50 | 19.25 | 22.00 |
| Sample | 20.35 | 40.70 | 61.05 | 81.40 | 101.75 | 122.10 | 142.45 | 162.80 |
| **Total** | **55.00** | **110.00** | **165.00** | **220.00** | **275.00** | **330.00** | **385.00** | **440.00** |

15. Add 31.5 uL of the qPCR master mix to each optical PCR tube with the previous PCR sample. Gently mix by flicking and spin tubes briefly in a table centrifuge to remove air bubbles.
16. Run the following qPCR program (make sure to remove samples, once signal starts to leave exponential phase to prevent overamplification).
    a. 95C 3 min
    b. 98C 20s
    c. 67C 20s
    d. 72C 3min
    e. Repeat (b-d) until signal plateaus out of exponential amplification
    f. 72C 5 min
    g. 4C hold
17. Run an agarose gel or bioanalyze resulting qPCR. While there may still be dimer, amplified cDNA should be clearly visible between 500bp to 2500bp. See lane 2 on figure 1 for expected size distribution.

*Second SPRI size selection (0.8x)*

18. Combine qPCR reactions into a single tube.
19. Take out 40uL of the qPCR reaction and place in new 1.7 mL tube
20. Add 32uL of Kapa Pure Beads to each tube and vortex briefly to mix. Wait 5 min to bind DNA.

21. Place tubes against magnetic rack and wait until liquid becomes clear.
22. Remove the supernatant.
23. With tubes still on magnetic rack, wash with 750uL 85% ethanol.
24. Repeat step 5.
25. Remove ethanol and air dry bead (~5min). To not let beads overdry and crack.
26. Resuspend beads from each tube in 20uL of water and wait 5 min.
27. Bind tubes against magnetic rack and wait until liquid becomes clear.
28. Transfer 18.5uL of elutant into a 1.7mL tube.
29. Run an agarose gel or bioanalyze resulting qPCR. There should be almost no dimer at this point. If dimer remains, perform another round of qPCR followed by another 0.8x SPRI size selection.

**Tagmentation and Illumina Amplicon Generation**

1. Quibit amplified cDNA and dilute to 0.12ng/uL.
2. Preheat a thermocycler to 55 degrees.
3. For each sample, combine 600 pg of purified cDNA with H2O in a total volume of 5 ul.
4. To each tube, add 10 ul of Nextera TD buffer and 5 ul of Amplicon Tagment enzyme (the total volume of the reaction is now 20 ul). Mix by pipetting ~5 times. Spin down.
5. Incubate at 55 C for 5 minutes.
6. Add 5 ul of Neutralization Buffer. Mix by pipetting ~5 times. Spin down. Bubbles are normal.
7. Incubate at room temperature for 5 minutes.
8. Add to each PCR tube in the following order:
    1. 15 ul of Nextera PCR mix
    2. 8 ul H2O
    3. 1 ul of 10 uM (N7 indexed primer, one of BC_0076-BC_0083)
    4. 1 ul of 10 uM Nextera (BC_0118) N501 oligo
9. Run the following thermocycling program:
    1. 95 C 30 sec
    2. 12 cycles of:
        1. 95 C 10 seconds
        2. 55 C 30 seconds
        3. 72 C 30 seconds
    3. Then: 72 C 5 minutes 4 C forever
10. Transfer 40ul out of the 50uL reaction to a 1.7mL tube.
11. Add 28uL of Kapa Pure beads to do a 0.7x cleanup. Elute in 20ul.
12. Bioanalyze resulting sample and quibit before sequencing. See lane 1 on figure 1 for expected size distribution.

**Illumina Sequencing**

1. Use a paired-end sequencing run with a 150 bp kit.
2. Set read1 to 66 nt (transcript sequence)
3. Set read2 to 94 nt (cell-specific barcodes and UMI)
4. Include a 6nt read 1 index to ready sublibrary indices (this is 4th round of barcodes).