SIGNAL-seq: Barcoding Protocol (v1.0)

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Day 1 - Barcoding

Projected Experimental time: 1 full day (Barcoding)
Start the barcoding day by 09:00

Notes:

Plate Loading

Loading of the 48 RT barcoding wells in the sample loading table is dependent on the required sample concentrations and cell numbers required per sample.The RT plate design should be generated before running the barcoding experiment. After counting at the end of the SIGNAL-seq cell staining, use the cell concentrations to generate the final RT barcoding plate layout.

Cell Recovery

Due to the cells handling across lots of plasticware the cell yield at the lysis sublibrary generation stage is typically observed to be between 25%-50% of the total cells input into the barcoding RT plate. This is dependent on the cell type and starting cell input (more input usually leads to better retention proportion).

Refer to the original SPLiT-seq paper (Rosenberg & Rocco *et al.*, *Science,* 2018) for barcoding cell input guidelines. We typically load ~100-300k cells total into the RT plate depending on how many cells are intended to be sequenced.

Blocking 15 mL Falcon tubes

Blocking increases the cell recovery in the protocol. Make a solution of 0.5% Ultrapure BSA in ultrapure water, coat the inside of the tube with solution and leave at RT for 30 mins. Remove the BSA solution completely and air dry in a TC cabinet. Coated tubes be stored at 4C several weeks.

Addition of RNase Inhibitor to Buffers

When any buffer has “+RI” next to it, this indicates that Roche Protectorase (40 U/µL – 2.5 µL/mL) RNase inhibitor and Thermo Superase (20 U/µL – 5 µL/mL) should both be added to a final concentration of 0.1 U/µL.

Centrifugation Steps

All centrifugation steps should be performed with a swinging bucket rotor. All spinning speeds may need to be adjusted based on different sample types. When spinning tubes always mark the side of the tube perpendicular to the rotor with a marker, this will help you identify the pellet if it is small/invisible.

Decanting Supernatant
Always decant supernatant immediately as extended time can cause the pellet to become dislodged. When volume remaining is less than 1 mL always use a P200 and always leave ~50 µL left, this translates to the lowest graduation on a 15 mL Falcon tube (which spans the circumference of the tip).

Experiment Location
Performed all steps before cDNA amplification in a PCR-clean TC hood wiped down with RNAse-ZAP. After cDNA amplification move to a different PCR-clean post-PCR lab area.

Reagents to Generate on Previous Day

10% Triton X100 - Store at 4C

Reagent	Stock Concentration	Final Concentration	Volume (µL)
Triton X100	100 %	10 %	100
H_20	NA	NA	900
Total Volume			**1000**

2x Lysis Buffer - Store at 4C

Reagent	Stock Concentration	Final Concentration	Volume (µL)
Tris, pH 8.0	1 M	20 mM	30
NaCl	5 M	400 mM	120
EDTA, pH 8.0	0.5 M	100 mM	300
SDS	10 %	4.4 %	660
H_20	NA	NA	390
Total Volume			**1500**

BSA blocking mix (0.5% BSA) - Store at 4C

Reagent	Stock Concentration	Final Concentration	Volume (µL)
Ultrapure BSA	5 %	0.5 %	200
H_20	NA	NA	1800
Total Volume			**2000**

0.5x PBS + RI - Store at -20C

Reagent	Stock Concentration	Final Concentration	Volume (µL)
Protectorase RNAse	40 U/µL	0.1 U/µL	2.5
Superase	20 U/µL	0.1 U/µL	5
PBS	1x	0.5x	497
H_20	1x	0.5x	497
Total Volume			**1001.5**

Begin protocol at 1.2 - step 4 if continued from 1.1 Cell staining protocol if performing SIGNAL-seq ADT-Ab staining.

1.2 – Reverse Transcription Barcoding RT1

Thaw components: Maxima 5x Buffer, dNTPs.

1. Fastcool centrifuge to 4C and turn the thermomixer to 37C.
2. Thaw sample cell tubes at 37C for a minimal amount of time (~30 seconds, take tubes off just before ice is completely thawed) and place on ice.
3. Depending on the cell type sample, some cells are damaged and can clump by freezing. If unfamiliar with the sample type, check 5uL of the sample under the microscope. If there is significant clumping/damage >5 % cells are clumped, then filter the cell sample with a FlowMI 40 µm filter into a new 1.5 mL Eppendorf protein lo-bind tube.

   If performing SIGNAL-seq ADT-Ab staining, count cells here and generate the sample loading table.

4. Using the sample loading table generated the day before, dilute the sample cells into the appropriate concentration for **R1 RT barcoding plate** loading into a new 1.5 mL Eppendorf protein lo-bind tube, always keep tube cells on ice.
5. Thaw the **R1 RT barcoding plate** by placing it in a thermocycler and running the following program and briefly spin down the plate at 100g for 15 secs at 4C (note sample volume depends on the quantity of volume in your stock plate).

Run Time	**Lid Temp**	**Sample Volume**
10 min	**70C**	4 µL
Step	**Time**	**Temperature**
1	10 mins	25C
2	Hold	4C

While the plate is thawing, generate the following reverse transcription (RT) mix and place on ice:

N.B. concentration is calculated on a per well basis with a final volume of 20 µL per well.

Reagent	Stock Concentration	Desired Concentration (per well)	Per Reaction (per well)	Volume in Mix (48 wells + 15%)
5X RT buffer	5x	1x	4	220.8
Protectorase Rnase Inhibitor	40 uU/µL	0.25 U/µL	0.125	6.9
Superase Rnase Inhibitor	20 U/µL	0.25 U/µL	0.25	13.8
dNTPs	10 mM (per base)	500 µM	1	55.2
MHM RTranscriptase	200 U/µL	20 U/µL	2	110.4
H_20	NA	NA	0.625	34.5
Total Volume			**8**	**441.6**

6. Add **8 µL** of the RT mix to each of the top 48 wells of the **R1 RT barcoding plate**. Each well should now contain a volume of 12 µL. **N.B: DO NOT REUSE TIPS FOR STEPS 5 + 6 as this will cause barcode swapping.**
7. Add 8 µL of cells in 0.5x PBS+RI to each of the top 48 wells of the **R1 RT barcoding plate**. Each well should now contain a total volume of 20 µL. **Note:** mix cells gently by pipetting up and down exactly 3 times after dispensing cells to avoid cells from settling to the bottom of the tube. Spin plate at 100 g for 30 seconds to ensure reaction mixture is at the bottom of the plate.

8. Add the plate in a Thermocycler with the following protocol:

Run time	**Lid Temp**	**Sample Volume**
~40 min	70C	20 µL
Step	**Time**	**Temperature**
1	10	50C
Begin Cycling		
2	12 sec	8C
3	45 sec	15C
4	45 sec	20C
5	30 sec	30C
6	2 min	42C
7	3 min	50C
Go to step 2, repeat 2 times (3 cycles total)		
8	5 min	50C
9	Hold	4C

9. Thaw NEB buffer 3.1 and T4 Ligase 10X buffer.

10. Prepare 2 mL of 1x NEB buffer 3.1 (Buffer is 10x dilute in H_20), add an additional 20 µL of Protectorase RNase Inhibitor.

11. Transfer the **R1 RT barcoding plate** to ice.

12. Pool all the cells into a pre-cooled single 15 mL centrifuge tube on ice. Using a multichannel P200 pipette, pool each row into the top row (i.e B,C,D -> A) gently pipette up and down 3x at the front, back and middle of each well to resuspend cells that might have settled over the RT incubation. Use a P200 to move the pooled cells in row A into a 15mL Falcon tube.

13. Add 9.6 µL of 10% Triton-X100 to get a final concentration of 0.1 %, flick to mix.
14. Centrifuge pooled RT reaction in a swinging bucket centrifuge pre-cooled to 4C for 10 minutes at 250 g (acceleration 9, deceleration 5 to pellet better). Mark the vertical plane of the tube with a line where the pellet is expected to be on the 15 mL tube with a black lab marker based on its orientation with the axis rotor.

15. Aspirate supernatant with a P1000 for the first and then a P200 for the last 1000 µL of supernatant, so that ~50 µL of liquid remains above the pellet (This is marked by the lowest clear plastic line on the 15 mL falcon at the tip join). The pellet may often not be visible by eye, proceed slowly.

a. If you are really worried about the pellet, just leave a little more headroom ~50/60 µL. In order to check if your cells are there you can resuspend in ~1000 µL of NEB_3.1 buffer and take 10 µL to check/count on a microscope to validate the presence of cells. Keep the supernatant as a safety precaution!!!

16. Resuspend in 1 mL of 1x NEB buffer 3.1 + RI, resuspend slowly with a P1000. Once cells are fully resuspended, add an additional 1 mL of 1x NEB buffer 3.1 + RI. Keep the solution in ice and proceed to **1.3 – Ligation barcoding.**

1.3 – Ligation Barcoding round L2 and L3

Thaw components: Blocking Oligos BC_0340, BC_0066 at RT, then place on ice.

1. Thaw **L2 Ligation barcoding plate** with thermocycling protocol and spin down plate 100 g, 15 secs, 4C:

Run Time	**Lid Temp**	**Sample Volume**
10 min	**70C**	10 µL
Step	**Time**	**Temperature**
1	10 mins	25C
2	Hold	4C

Make the following ligation master mix on ice (briefly centrifuge components with tabletop centrifuge):

Reagent	**Stock Concentration**	**Final Concentration**	**Volume**
T4 Ligase Buffer 10x	10x	1x	500
Protectorase Rnase Inhibitor	40 U/µL	0.32 U/µL	40
Superase In	20 U/µL	0.05 U/µL	12.5
Mol. Bio. BSA	20 mg/mL	0.2 mg/mL	50
T4 DNA Ligase	400 U/µL	8 U/mL	100
H20	NA	NA	1337.5
Total volume			**2040**

2. Add the 2.04 mL of ligation mix to the cells in NEB buffer 3.1. The mix should now have a volume of 4.08 mL.

3. Add the mix into a BSA-blocked basin with a P1000 and mix 10x slowly with the pipette.

4. Using a multichannel pipet, add 40 µL of the combined ligation mix + cells into each well of the **L2 Ligation barcoding plate**, mix 3x time by gently pipetting up and down in each well while distributing cells in the ligation mix. **N.B: It is critical to use a different pipette tip for each well !!!** For the last column you will need to tilt the buffer dam to pool the ligation cell suspension in the corner and aliquot into the L2 ligation plate using a single P200. If you run out of ligation cell suspension before the end of the 12th column it is not a problem. The assay will work fine just make a note of skipped wells.

5. Cover the **L2 Ligation barcoding** plate with a PCR cover seal and incubate in the thermocycler with the following protocol:

Run Time	**Lid Temp**	**Sample Volume**
30 min	**50C**	50 µL
Step	**Time**	**Temperature**
1	30 mins	37C
2	Hold	4C

6. Whilst the ligation continues, make the round 2 blocking solution in a new basin:

Reagent	**Stock Concentration**	**Final Concentration**	**Volume (µL)**
BC_0340	100 µM	26.4 µM	316.8
T4 Ligase Buffer 10x	10x	2.5x	300
Water	NA	NA	583.2
Total Volume			**1200uL**

7. Remove the round **L2 Ligation barcoding plate** from the thermocycler and remove the cover.

8. Using a multichannel pipette, add 10 µL of the round 2 blocking to each of the 96 wells in the round 2 DNA barcoding plate. **N.B Use individual tips for each well and mix by pipetting 5x times as the ligation mix is viscous.**

9. Cover the **L2 Ligation barcoding plate** with an adhesive plate seal and incubate the plate in the thermocycler with the following protocol:

Run Time	Lid Temp	Sample Volume
30 min	**50C**	60 µL
Step	**Time**	**Temperature**
1	30 mins	37C
2	Hold	4C

10. Remove the **L2 Ligation barcoding plate** from the thermocycler and thaw the **L3 Ligation barcoding plate** in the thermocycler and spin down plate 100g, 15 secs, 4C:

Run Time	Lid Temp	Sample Volume
10 min	**70C**	10 µL
Step	**Time**	**Temperature**
1	10 mins	25C
2	Hold	4C

11. Remove the cover and pool all the cells into a new **BSA blocked basin**, when pooling the cells pipette up and down at the back, front and middle of each of the wells 3x in order to resuspend any cells that might have settled at the bottom/sides of the wells during the ligation reaction.

    Note: Set multichannel pipette to 65 µL and use the same tips.

12. Pass all cells through a 40 µm strainer using a P1000 gently pressed against the filter held in the other hand directly **into a new BSA-blocked basin**.

13. Add **100uL of T4 DNA ligase** to the basin and mix by pipetting ~20x times gently with a wide-bore P1000.

14. Using a multichannel pipette, add 50 µL of cell/ligase solution into each well of the **L3 Ligation barcoding plate**, mix 3x times whenever retrieving suspension from the buffer dam to make sure that the cells are not settling onto the bottom of the buffer dam and mix 3x times when distributing cells into the L3 ligation plate to make sure that reaction mixture is adequately mixed. **N.B: It is critical to use a different pipette tip for each well !!!**

15. Cover the **L3 Ligation barcoding plate** with an adhesive plate seal and incubate in the thermocycler with the following protocol:

Run Time	Lid Temp	Sample Volume
30 min	**50C**	60 µL
Step	**Time**	**Temperature**
1	30 mins	37C
2	Hold	4C

16. Make the round 3 blocking solution and add it to a new basin:

Reagent	Stock Concentration	Final Concentration	Volume (µL)
BC_0066	100 µM	11.5 µM	369
EDTA	0.5 M	125 mM	800
Water	NA	NA	2031
Total Volume			**3200**

17. Remove the **L3 Ligation barcoding plate** from the thermocycler and remove the cover.

18. Using a multichannel pipette, add 20uL of the round 3 blocking solution to each of the 96 wells in the **L3 Ligation barcoding plate**.

19. Pool the cells into a BSA blocked basin as before with 3x pipetting at front middle and back of wells to dislodge cells that might be settled on the bottom/sides of the wells.

20. Pass the cells from this basin through a 40 µm strainer (with a wide-bore P1000) **into a BSA blocked 15mL falcon tube.**

1.4 Sublibrary Generation and Lysis

1. Make the following wash buffer and warm the 2x Lysis buffer at 37C (in the incubator), the lysis buffer will appear cloudy before being pre-warmed. Mix after warming until all precipitate dissolves:

Reagent	Stock Concentration	Final Concentration	Volume (µL)
10% TX100	10 %	0.1 %	40
Superase	40 U/µL	0.1 U/µL	10
1X PBS	NA	NA	4000
Total Volume			**4050**

2. Add 70 µL of 10% triton to the cells (to get to ~0.1 % final conc.)

3. Centrifuge for 5 mins at 800 g in 15 mL tube, mark the prospective pellet position on the size with a line or dot.

4. Aspirate supernatant – N.B.: **BE VERY CAREFUL TO LEAVE ~50 µL,** in order to avoid removing the cell pellet which typically cannot be seen at this stage.

5. Resuspend in 4 mL of wash buffer, resuspend carefully with a P1000.

   a. If you are worried about the pellet during these spins you can perform a similar process as before, resuspend the pellet in a partial volume depending on the cell input (i.e. 1 mL for ~200k cells) and take 10 µL of cells to check under the microscope.

6. Centrifuge for 5 min at 800 g.

7. Carefully aspirate supernatant, take as much as possible while preserving the pellet, if worried about the pellet use a P10 instead of the P200.

8. Resuspend in 60 µL 1x PBS+RI to give a total volume of around 100 µL, count this volume with a P200 pipette so that the total cell recovery can be accurately measured.

9. Take 5 µL and count on a Haemocytometer N-chip with DiYO or Trypan (usually 2x dilution but can dilute as necessary) using the GFP channel and filter, the DiYO will fluoresce. Use the BF light to align the counting grid (Depending on the sample there can be too much cell debris for an automated cell counter to accurately count). (Trypan also works fine if you don't have access to DiYO).

10. Determine how many sublibraries you want to generate at a specific cell number using the sample loading table. The number of sublibraries is equal to the number of tubes required.

11. Aliquot the desired number of cells into each sublibrary tube in a PCR strip tube. Add 1x PBS+RI to each tube in order to generate a final total volume of 25 µL, if the cell solution volume is low (i.e. for a ~250 cell sublibrary), add the PBS first.

12. Check the 2x Lysis buffer for precipitate, vortex until solution is clear, warming further can help. Create a Lysis master mix by adding 25 µL of 2x Lysis buffer and 5 µL of Proteinase K (20 mg/µL) for each sublibrary to be generated (30 µL of Lysis master mix per tube; total volume in tube 55 µL).

13. Add 30 µL of Sublibrary master mix to each sublibrary PCR tube and briefly vortex and then spin down ~1 sec on the tabletop centrifuge.

14. Incubate on the thermocycler with the following program:

Run Time	**Lid Temp**	**Sample Volume**
60 min	80C	55 µL
Step	**Time**	**Temperature**
1	60 mins	65C
2	Hold	4C

15. Remove tubes from thermocycler and freeze lysates at -80C. Lysates can be stored for up to 6 months before processing with **Library Preparation.**