Integrating CITE-seq with Seq-Well or Honeycomb single-cell RNA-seq

V1.6

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Purpose: This protocol guides a user through the steps to successfully prepare sequencing libraries for whole transcriptome and antibody-derived tags (ADTs) used in CITE-seq (for cell hashing or surface protein measurement).

What this protocol assumes: You are generally familiar with standard Seq-Well or Honeycomb protocols and can successfully navigate the sample capture and processing methods. It also assumes you are familiar with the appropriate steps to isolate and label your single cell populations of interest with oligo-linked antibodies. Because there are many flavors of CITE-seq antibody, and even more sample types, we urge the user to familiarize themselves with their specific antibody and sample prior to beginning this protocol. This includes obtaining the necessary primers which will be specific to the user's chosen CITE-seq antibody (in this protocol we use the **BD Human Single-Cell Multiplexing Kit (# 633781)**, and have designed the required primers – unique to Seq-Well or Honeycomb, accordingly).

Seq-Well

Where this protocol starts: This protocol begins after the user has dissociated and labeled their samples of interest with their CITE-seq panel of interest, and has followed the standard Seq-Well sample capture and processing steps up to **page 23** of the **Seq-Well** protocol.

START on **page 23** of **Seq-Well** at **step 1:**

WTA prep (50uL reactions):

WTA reaction	1 rxn	24 rxns	n SWs (x n x 1.1)
2x KAPA HiFi Hotstart Readymix	25 uL	600 uL	
Water	14.4 uL	345.6 uL	
SMART PCR Primer (100uM)	0.4 uL	9.6 uL	
BDsmk wta additive primer (10uM)	0.2 uL	4.8 uL	
Total volume	**40 uL**	**960 uL**	

Then continue Seq-Well PCR steps as normal

In Seq-Well purification of PCR products (page 24), make the following changes:

0. If performing cell hashing and "overloading" the Seq-Well array (e.g. 30e3 cells loaded), pool the 24 WTA reactions into 2 separate pools, thereby limiting the total number of bead barcodes per pool.
    a. Each pool will get a distinct Nextera index. This will help the dropseq workflow run faster and discovery more cells.
1. Ensure both pools of 12 WTA reactions are well-mixed.
2. SPRI at 0.6x volumetric ratio.
3. Let the tubes sit at room temperature for 5 minutes so that long PCR products bind to the beads.
4. Place tubes on the magnet for 5 minutes.
5. **Recover the 0.6x SPRI supernatant, which contains the short ADT amplicon.**
6. Continue the SPRI of the long PCR products as described in the standard Seq-Well protocol.
7. Pool the 2 0.6x SPRI supernatants. All ADTs originating from the same Seq-Well array should receive the same sequencing index.
8. To 160uL of pooled 0.6x SPRI supernatant, add 140uL SPRI beads.
    a. The total concentration of PEG is now equivalent to a 2x SPRI, so the short amplicons will now bind to the SPRI beads.
9. Ethanol wash, elute in 50uL water, and do a 2nd 2x SPRI. Final elution in 50uL water.

Quality check of short fraction of WTA products

1. Run a BioAnalyzer or TapeStation according to the manufacturer's instructions. Use 1uL of the purified short WTA product as input.
    a. Expect a hump centered around 300bp in length. This is a complex mixture of short products, including the amplified ADT. Step-out PCR will more selectively amplify the ADT.
2. Use Qubit to quantify the concentration of short WTA product.
    a. Expect 1-5 ng/uL DNA. Keep in mind that the target ADT amplicon is a small portion of this mixture.

3. Dilute short WTA product to 175 pg/uL DNA.

Step-out PCR of ADT amplicon (50uL reaction)

Hash amplification reaction	1 rxn
2x KAPA HiFi Hotstart Readymix	25 uL
Diluted short WTA product (175 pg/uL)	20 uL
P5-SMART PCR hybrid oligo (10uM)	2.5 uL
CITE-seq RPI index primer (10uM)	2.5 uL
Total volume	**50 uL**

Hash library amplification PCR program (modified HC WTA program):

Temp	Time	# of Cycles
95C	5 min	1
98C	20 sec	10
60C	45 sec	
70C	1 min	
72C	2 min	1
4C	Hold	

Final preparation of ADT sequencing library

1. SPRI 50uL reaction at 1.2x ratio. Elute in 30uL water
2. Run a BioAnalyzer or TapeStation according to the manufacturer's instructions. Use 1uL of the step-out PCR product as input.
    a. For BD cell hashing kit, expect a very sharp spike around 255bp.
    b. Other CITE-seq or cell hashing formats may have different lengths.
3. Use Qubit to quantify the concentration of short WTA product.
4. Dilute to 4nM and pool with mRNA sequencing libraries.
    a. Cell hashing requires relatively few reads (~10e6 per Seq-Well array) to achieve optimal cell classification. Therefore, the ADT sequencing library should be an accordingly small proportion of the final pooled sequencing library.

CUSTOM PRIMERS

These primers are specifically designed to work with the **BD Human Single-Cell Multiplexing Kit (# 633781)** and **Seq-Well**, and would need to be altered to accommodate alternate hashing kits.

BDsmk wta additive primer
5' CCTTGGCACCCGAGAATTCCAGTTGTCAAGATGCTACCGTTCAGAG 3'

CITE-seq RPI index primer
5' CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTCCA 3'
Index sequence – must be orthogonal to other Index 1 sequences in the pooled sequencing library

Honeycomb

Where this protocol starts: This protocol begins after the user has dissociated and labeled their samples of interest with their CITE-seq panel of interest, and has followed the standard Honeycomb sample capture and processing steps up to **page 18** of the **scRNAseq processing (PN 10347 v.0.3)** protocol.

START on **page 18** of **scRNAseq processing (PN 10347 v.0.3)** at **step 3:**

WTA prep (100uL reactions):

WTA reaction	1 HIVE	8 HIVEs (x8.8)	n HIVEs (x n x 1.1)
WTA Oligo	40 uL	352 uL	
PCR Enzyme	200 uL	1760 uL	
BD-HC WTA Linker primer (10uM)	5 uL	44 uL	
Total volume	**245 uL**	**2156 uL**	

FOLLOW Honeycomb protocol steps 4-16, with the following changes:

9. Add **155uL water** to each well

12. Add **245uL WTA Reaction** to each deepwell plate well

BEFORE INDEX PCR in Honeycomb protocol, do the following:
Post-WTA SPRI recovery of hash library & WTA library

0.6X Reverse SPRI prep:

0.6X SPRI Wash Solution	1 HIVE	8 HIVEs (x8.8)	n HIVEs (x n x 1.1)
Wash A	25 uL	220 uL	
SPRI Wash Concentrate	15 uL	132 uL	
Total volume	**40 uL**	**352 uL**	

1. From each WTA reaction (4x per HIVE), take 7.5uL and pool in new well, add 30uL water (60uL total vol.)

2. Add SPRI beads at 0.6X (volumetric), mix 10 times, incubate 3 min. @ room temp.
    a. For 60uL WTA product, add 36uL SPRI beads

3. Place on PCR plate magnet for 2 min.

4. Carefully transfer supernatant to a new **hash PCR plate** well, with **WTA PCR plate** on magnet
    a. For 60uL WTA product, transfer ~96uL

5. Add SPRI beads to supernatant **hash PCR plate** well, bringing final ratio to 2X, mix 10 times, incubate 3 min. @ room temp.
    a. SPRI to add = WTA product vol x 2 – SPRI vol already added
    b. For 60uL WTA product, add 84uL

6. While **hash PCR plate** is incubating in step (5), add 100uL 80% EtOH to the beads in the **WTA PCR plate**, mix ~6 times on plate magnet (by moving plate), let rest on magnet 2 min., aspirate and discard EtOH.

7. Place **hash PCR plate** on magnet for 2 min.

8. Repeat step (6), after aspirating EtOH from **WTA PCR plate** allow beads to air dry for 10 min.

9. While air drying in step (8), aspirate SPRI supernatant from **hash PCR plate** wells, and add 40uL of 0.6X SPRI wash solution, mix 10 times, incubate 3 min. @ room temp.

10. Place **hash PCR plate** PCR plate magnet for 2 min.

11. Carefully transfer **hash PCR plate** supernatant to a new **hash PCR plate** well.
    a. For 40uL SPRI wash, transfer ~40uL

12. Add SPRI beads to supernatant **hash PCR plate** well, bringing final ratio to 2X, mix 10 times, incubate 3 min. @ room temp.
    a. For 40uL SPRI wash, add 35uL

13. Elute library from beads on the **WTA PCR plate** by adding 20uL Wash A, removing from magnet, mixing ~10 times, and incubating 3 min. at room temp.

14. While waiting after elution in step (13), aspirate SPRI supernatant from **hash PCR plate** wells, and add 100uL 80% EtOH to the beads, mix ~6 times on plate magnet (by moving plate), let rest on magnet 2 min., aspirate and discard EtOH.

15. Repeat step (14) EtOH wash, after aspirating EtOH from **hash PCR plate** allow beads to air dry for 10 min.

16. Place the **WTA PCR plate** back on the magnet, wait 2 min. and transfer 18uL to new well, this is your recovered **WTA library** from which you will use 2uL in the **INDEX PCR Honeycomb protocol**

17. Elute library from beads on the **hash PCR plate** by adding 20uL Wash A, removing from magnet, mixing ~10 times, and incubating 3 min. at room temp.

18. Place the **hash PCR plate** back on the magnet, wait 2 min. and transfer 18uL to new well, this is your recovered **hash library** from which you will use 2uL in the **hash library amplification**

Hash library amplification

1. Follow PCR prep to prepare hash PCR mastermix, on ice.

PCR prep (50uL reactions):

Hash amplification reaction	1 HIVE	8 HIVEs (x8.8)	n HIVEs (x n x 1.1)
Water	18 uL	158.4 uL	
PCR Enzyme (KAPA)	25 uL	220 uL	
BD-HC linker-UPS primer (10uM)	2.5 uL	22 uL	
Bead-specific WTA primer (10uM)	2.5 uL	22 uL	
Total volume	**48 uL**	**422.4 uL**	

2. Ensure the below PCR program is ready to go

Hash library amplification PCR program (modified HC WTA program):

Temp	Time	# of Cycles
95C	5 min	1
98C	20 sec	10
60C	45 sec	
70C	1 min	
72C	2 min	1
4C	Hold	

3. Add 48 uL of hash amplification PCR mastermix to a new PCR plate for each HIVE, and then from the **hash PCR plate** add 2uL of **hash library** in its respective well, seal the plate, briefly spin down and run the **Hash library amplification PCR program**

NOW run INDEX PCR per Honeycomb Protocol (page 20), with following **changes:**

4-6. **DO NOT** pool WTA reactions (you already did this – you should simply use **4uL per HIVE of WTA library**)

7. Include an index PCR reaction for each **hash library** (using **4uL of final PCR product per reaction**) in addition to one for each **WTA library**, with every library receiving a unique dual index well (record which library gets which index well!) – **REDUCE H2O added per rxn by 2uL**

FOR WTA library follow 0.8X SPRI CLEAN-UP Honeycomb Protocol (page 22)

FOR hash library do the following:

0.6X Reverse SPRI prep:

0.6X SPRI Wash Solution	1 HIVE (2 washes)	8 HIVEs (x8.8)	n HIVEs (x n x 1.1)
Wash A	50 uL	440 uL	
SPRI Wash Concentrate	30 uL	264 uL	
Total volume	**80 uL**	**704 uL**	

1. Add SPRI beads at 1.6X (volumetric), mix 10 times, incubate 3 min. @ room temp.
    a. For 50uL INDEX PCR product, add 80uL SPRI beads
2. Place on PCR plate magnet for 2 min.
3. Carefully aspirate supernatant and add 40uL of 0.6X SPRI wash solution, mix 10 times, incubate 3 min. @ room temp.
4. Place PCR plate on magnet for 2 min.
5. Carefully transfer supernatant to a new well.
    a. For 40uL SPRI wash, transfer ~40uL
6. Add SPRI beads to supernatant well, bringing final ratio to 2X, mix 10 times, incubate 3 min. @ room temp.
    a. For 40uL SPRI wash, add 35uL
7. Repeat steps (3-6)
8. Carefully aspirate supernatant and add 100uL 80% EtOH to the beads, mix ~6 times on plate magnet (by moving plate), let rest on magnet 2 min., aspirate and discard EtOH.
9. Repeat step (8), after aspirating EtOH from plate allow beads to air dry for 10 min.
10. Elute library from beads by adding 30uL Wash A, removing from magnet, mixing ~10 times, and incubating 3 min. at room temp.
11. Place the plate back on the magnet, wait 2 min. and transfer 28uL to new well, this is your recovered **indexed hash library** from which you will create your sequencing library pools (including WTA libraries)

SEQUENCING NOTES

Quant and tape **indexed hash library**. Tape should be a very sharp spike at ~250bp.

Sequence on either dual indexed NextSeq or NovaSeq R1 25 / R2 50, I1 8 /I2 8, with Honeycomb custom index 1 & 2 and custom read 1 & 2.

While short, the **indexed hash library** is compatible with the **indexed WTA library**. There's no reason to sequence the **hash library** anywhere near as deeply as the **WTA library** (and due to smaller size, the **hash library** will bind the flow cell preferentially) – I typically aim for 1/20th of the reads of a **WTA library** (e.g. 1.25% of the pool mass is **hash library** for every 23.75% **WTA library**, if you wanted to put 4 HIVEs on 1 sequencing run).

CUSTOM PRIMERS

These primers are specifically designed to work with the **BD Human Single-Cell Multiplexing Kit (# 633781)** and **Honeycomb Hives**, and would need to be altered to accommodate alternate hashing kits.

BD-HC WTA Linker:
5' GTTGTCAAGATGCTACCGTTCAGAG 3'

BD-HC Linker-UPS:
5' GAGGAAGCAGTGGTATCAACGCACCGCAGAGTTGTCAAGATGCTACCGTTCAGAG 3'

Bead-specific WTA primer:
5' CAGTGGTATCAACGCAGAGT*A*C 3'