HPAP CITEseq: Dual-index 3’ HT scRNAseq with Antibody Derived Tags and Hashtag Oligos

Compiled by Vincent Wu and Gregory Golden | Betts Lab
2023.05.03

Description

This assay simultaneously assesses mRNA and select surface antigen profiles at single-cell resolution. This protocol uses a combination of oligo-labeled antibodies (Biolegend TotalSeqA) and the 3’ scRNAseq chemistry (10X Genomics). Furthermore, samples are individually labeled with hash-tagging antibodies (oligo-labeled antibodies targeting β2m and CD298) for multiplexing and increased resolution for multiplet filtering upon superloading of the 10X chip. This protocol utilizes the HT system compatible with the Chromium X instrument, allowing double the output of emulsified cells compared to regular throughput systems. This protocol builds upon the following publications/protocols:

- Stoeckius et al., *Nature Methods* 2017
- Stoeckius et al., *Genome Biology* 2018
- BioLegend TotalSeq-A Antibodies and Cell Hashing with 10x Single Cell 3’ Reagent Kit v3.1 (Dual Index) Protocol (version from 10/22/21, Betts Lab)
- 10X Genomics Chromium Next GEM Single Cell HT Reagent Kits v3.1 (Dual Index) - RevB, September 10, 2021.

Reagents

Cell preparation

✓	Description	Vendor	Catalog #
	PBS (no Ca2+ or Mg2+)	Any	Any
	EasySep™ Dead Cell Removal (Annexin V) Kit	StemCell	17899
	Trypan Blue Stain (0.4%) for use with the Countess™ Automated Cell Counter	TF	T10282
	Fresh R10 (RPMI 1640, 10% FBS, 100 U/mL penicillin, 100 ug/mL streptavidin, 2 mM L-glutamine)		
	DNase I, 10 U/uL in glycerol	Roche Diagnostics	4716728001

Cell staining

✓	Description	Vendor	Catalog #
	TotalSeq™-A Human Universal Cocktail, V1.0	Biolegend	399907
	TotalSeq-A Hashtag Antibodies	Biolegend	Multiple
	Human TruStain FcX™ (Fc Receptor Blocking Solution)	Biolegend	422302
	Cell Staining Buffer	Biolegend	420201

	1.5ml DNA LoBind Tubes	Eppendorf	022431021
	1.5ml Protein LoBind Tubes	Eppendorf	0030108442
	Flowmi® Cell Strainers 40um (50 count)	Millipore/Sigma	BAH136800040-50EA

10X primary reagents

✓	Description	Vendor	Catalog #
	Chromium Next GEM Single Cell 3' HT GEM Kit v3.1 (8 rxns)	10X Genomics	1000373
	Dynabeads MyOne SILANE	10X Genomics	2000048
	Library Construction Kit (16 rxns)	10X Genomics	1000190
	Chromium Next GEM Single Cell 3' HT Gel Bead Kit v3.1 (8 rxns)	10X Genomics	1000372
	Dual Index Kit TT Set A, 96 rxns	10X Genomics	1000215
	Chromium Partitioning Oil (2X) (check volume)	10X Genomics	2000190
	Chromium Recovery Agent	10X Genomics	220016
	Chromium Next GEM Chip M	10X Genomics	2000417
	Chip Gasket, HT, 2-pack	10X Genomics	3000656
	10% Tween 20	BioRad	1662404
	Glycerin (glycerol), 50% (v/v) Aqueous Solution	Ricca Chemical Company	3290-32

Library preparation

✓	Description	Vendor	Catalog #
	Qiagen Buffer EB	Qiagen	19086
	Low TE Buffer (10mM Tris-HCl ph 8.0, 0.1 mM EDTA)	Thermo	12090015
	Molecular grade H2O	Any	Any
	Pure ethanol (200 proof)	Any	Any
	Eppendorf PCR tubes (check 10X protocol for specific catalog #s)		
	KAPA HiFi HotStart ReadyMix (2X)	Kapa Biosystems	KK2601
	SPRIselect Reagent (60 mL)	Beckman Coulter	B23318
	CHECK 10X protocol for additional reagents		

Library QC reagents

✓	Description	Vendor	Catalog #
	PCR Tubes	Agilent	
	D5000 HS Tapestation Screentape	Agilent	
	D5000 HS Tapestation Buffers	Agilent	
	D5000 HS Tapestation Ladder	Agilent	
	D1000 HS Tapestation Screentape	Agilent	
	D1000 HS Tapestation Buffers	Agilent	

	D1000 HS Tapestation Ladder	Agilent	
	Qubit dsDNA HS Reagents	Invitrogen	
	Agilent Tech Loading Tips (for 2200 TapeStation)	Agilent	5067-5153

Primers (excluding 10X reagents)

✓	Description	Sequence (5' -> 3')	Vendor	Catalog #
	ADT cDNA PCR additive primer	CCTTGGCACCCGAGAATT*C*C		
	HTO cDNA PCR additive primer (v2)	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T		
	ADT Forward and Reverse Primers (1 per well)	variable		
	HTO Forward and Reverse Primers (1 per well)	variable		

- Indexing primers from Biolegend for compatibility with Dual Indexing for both ADT and HTO reactions. Order at least a 100nmole stock with standard desalting. No need for HPLC purification, per Smibert Lab recommendation though I imagine it would not hurt. Will cost a lot more money though. I have not used HPLC purification for these kind of primers for my other assays.
- Store all primers in -20C.

Library QC reagents

✓	Description	Vendor	Catalog #
	PCR Tubes	Agilent	
	D5000 HS Tapestation Screentape	Agilent	
	D5000 HS Tapestation Buffers	Agilent	
	D5000 HS Tapestation Ladder	Agilent	
	D1000 HS Tapestation Screentape	Agilent	
	D1000 HS Tapestation Buffers	Agilent	
	D1000 HS Tapestation Ladder	Agilent	
	Qubit dsDNA HS Reagents	Invitrogen	
	Agilent Tech Loading Tips (for 2200 TapeStation)	Agilent	5067-5153

Protocol

Section 1: Cell preparation

1. Prepare 500 mL fresh R10 media.
2. Prepare 50 mL of R10 + 10 U/mL DNAseI.
3. Procure cryopreserved vials of samples of interest, keep on dry ice until ready for thawing.
4. To a conical tube, add 14 mL of R10 media per 1 mL of cryopreserved sample and label the tube with the respective sample name.
5. To thaw the samples, partially submerge the cryopreserved sample tubes in a 37°C water bath and move the tubes to maintain a warm tube-water interface. Maintain movement and submergence until the sample is thawed enough to slide within the tube.
6. Immediately place the sample in its respective conical tube. Wash the cryotubes out with 200 µL R10 and place into the conical tube.
7. Cap the conical tubes and invert to mix the samples.
8. Spin the samples for 5 minutes at 500 xg.
9. Decant supernatant and resuspend the samples in R10 + DNaseI and count. Place cell counts below:

Sample				Live x _____ mL	For concentration: $2x10^6$ cells/mL
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		

10. Bring the concentration to $2x10^6$ live cells/mL for resting. If necessary, spin the samples at 500 xg for 5 min, and resuspend in the amount of R10 + DNAse1 to achieve $2x10^6$ live cells/mL.
11. Place samples in TC-treated flasks, and place samples in a 37°C incubator (5% CO_2) for 2 hours to rest.

After this point, when spinning live cells, set the centrifuge to 10C to help keep cells viable/slow transcriptional dynamics. KEEP SAMPLES COLD AT ALL TiMES!!!

12. After resting, spin the cells down at 500 xg for 5 minutes at 10°C.
13. Carefully pipette off supernatant, and resuspend cells in 1 mL ice cold Cell Staining Buffer for low-cell count samples and 3 mL for high-cell count samples. Count cells via trypan blue and automated cell counter (i.e. Countess II).
    a. Place cell counts below (pre-live cell enrichment):

Sample				Live x _____ mL	uL for 500k live cells
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		

14. If the viability of the sample is >= 90%, proceed to the next step.
    a. If the viability of the sample is <90% and you have **>**10 x10^6 cells per sample, use the Dead Cell Removal kit (StemCell; annexin V labeling to target phosphatidylserine which is exposed on the surface of dying/dead cells).
    b. If the viability of the sample is <90% and you have **<**10 x10^6 cells per sample,
        i. Filter the samples with 70 µm FACS filter tubes. Prime filter with 200 µL Cell Staining Buffer, followed by pipetting the sample through the filter. Then, pipette another 500 µL of cold Cell Staining Buffer onto the filter to flush through remaining cells.
    c. Do the same live cell enrichment for all samples in your experiment!

Place cell counts below (post live cell enrichment):

Sample				**Live x _____ mL**	**uL for 500k live cells**
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		
	Total:		**x10^**		
	Live:		**x10^**		
	Viability:		**%**		

15. Take 500k live cells from each sample and place in separate 1.5ml DNA lobind tubes.
16. Spin down the cells at 200g for 10 min.
17. With a 1000ul tip inserted inside a 200ul tip, carefully remove supernatant and resuspend cells in 22.5ul Cell Staining Buffer. Keep cells on ice!
    a. Note: it's better to leave a 5 ul than lose cells!
    b. Keep cells on ice for all remaining steps.

Section 2: Cell staining

1. Take TotalSeqA v1.0 cocktail out of 4C and equilibrate at RT for 5 min. DO NOT OPEN VIAL.
    a. Use 1 cocktail vial per every two samples (ie 8 samples requires 4 vials)
    b. TotalSeqA Cocktail Lot#: _____
2. Spin down cocktail vial at 10k g for 30 seconds at RT.
3. Add 27.5ul of Cell Staining Buffer to each cocktail vial.
4. Vortex cocktail vials for 10 seconds. Briefly spin down.
5. Incubate at RT for 5 min.
6. Vortex again and spin down at 10k g for 30 seconds at RT.
7. Transfer the entire volume of each cocktail vial to an empty 1.5ml Protein LoBind tube (one per vial).
8. Spin down at 14k g for 10 min at 4C.
9. Add 2.5ul of TruStain FcX to each sample and gently pipet to mix.
10. Incubate for 10 min at 4C.
11. Add 12.5ul of Cell Staining Buffer to each sample tube.
12. Vortex and spin down hashing antibodies.
13. Add 0.5ug (1ul) of hashtag antibody to each sample tube. Be sure to take hashtag antibody from the top portion of the vial. Take note which HTO was added to each sample in the table below.

Sample	HTO Antibody

14. Add 12.5ul of antibody cocktail to each sample tube. Gently pipet to mix.
15. Incubate for 30 min at 4C.
    a. Make Template Switch Oligo (TSO) here, unless already reconstituted
    b. Run Readiness test on the Chromium X instrument
16. Add 1ml of Cell Staining Buffer to each sample tube.
17. Spin down at 400g for 5 min.
18. With a 1000ul tip inserted inside a 200ul tip, carefully pipette out supernatant and discard supernatant.
19. Repeat steps 16-18 for a total of 3 washes.
20. Resuspend cells in 200ul of Cell Staining Buffer. Count cells using trypan blue.

Place cell counts in table below:

Sample				Live x _____ mL	uL for Cell Pool
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		
	Total:		x10^		
	Live:		x10^		
	Viability:		%		

Pooled Cells/ul: ___________________

21. Keep cells on ice until ready to merge cells and load for 10X (i.e. GEM beads are thawed and master mix is prepared, refer to Section 3, Step 1).
22. In a new DNA lo-bind tube of 1ml Cell Staining Buffer, add cells from each sample to create a pooled sample. Read step 25 and Section 3, Step 2 below for more details before deciding how many cells to add.
    a. Use the "Cell Pooling Calculator (10X)" on the Betts Google Drive to calculate how many cells you should add per sample.
23. Spin down pooled cells at 400g for 5 min.
24. With a 1000ul tip inserted inside a 200ul tip, carefully pipette out the supernatant and discard.
25. Resuspend cell pellet in ____ ul of CSB (whatever is indicated on the Calculator) to get a final resuspension of 1500 cells/ul or higher (2000 cells/ul is recommended). I recommend at least a final volume of 150ul to assist with the next step.
26. Into a new DNA lo-bind tube, filter cells through a 40um strainer (FlowMi strainer) using a p1000 tip
    a. For cell pools >250ul, split the cell pool into 2 separate pools and use 2 FlowMi strainers. Account for the extra loss in your calculations.
    b. Uptake the cells with the p1000 tip, and push the tip into a FlowMi (with mild resistance) until the pipette won't go further into the filter. Into the new DNA lo-

bind tube, gently push the pipette plunger until you are to the second stop. You will then observe the cell pool sample passing through the filter over a period of ~10 seconds.

27. Count cells with trypan blue staining and get the cell concentration in cells/ul.

Sample			Total x _____ mL	
	Total:		x10^	
	Live:		x10^	
	Viability:		%	

28. Keep cells on ice until use and proceed directly to loading the Chromium Chip M.
    a. First: take GEM Beads out of -80C and incubate at RT 30 minutes before use.
    b. Note: Loading is based upon <u>total</u> cell concentration, not live cell, since dead cells count as cells!
    c. For cells + H_2O: 86.4ul
    d. ul and number of cells loaded per well:
        i. ul of cells: _____ # of cells: _____ ul of mH_2O: _____

Section 3: Modifications to 10X Chip M protocol

1. Follow the Chip M v3.1 protocol until Step 1.2c.
2. To superload the chip, we need to respect the volume limit in the chip. For rows 2A and 2B of Chip M, the final volume must be 140ul. As such, the 10X protocol prepares an initial combined mixture of 150ul (10X builds in extra volume into the initial mixture) before having the user load 140ul, thus ensuring 140ul is loaded without having to worry about volumes. For the initial combined mixture, 63.6ul of Master Mix is used per well, granting 86.4ul of space for cells.
    a. Utilize the Cell Suspension Volume Calculator Table to decide how many cells to use.
        i. When referring to your cell pool, <u>use total cell count</u>, not live cell count, as dead cells are emulsed as well as alive.
        ii. The Cell Suspension Volume Calculator Table only accounts for one well of Chip M (ie 2A, *not* 2A + 2B)
    b. For example, if we want a targeted cell concentration of 17,500 cells/well and our cell stock concentration is at 1500 cells/ul. We would then put in 24.1ul of cells and add 62.3ul of nuclease-free water to the Master Mix for Step 1.2b.
        i. If there are 16 reactions being run, that would require 385.6 ul of cell suspension
        ii. Expect a loss in cell concentration during washing and FlowMi filtration; i.e. a drop from 2000 cells/ul to 1200 cells/ul. So, expect to need a larger number of cells. Refer to the “Cell Pooling Calculator (10X)” on the Betts Google Drive to calculate how many cells you should add per sample.
3. Proceed with the rest of 10X Protocol Step 1 through Step 1.4.
4. At the end of Step 1.4, the GEMs must be evenly distributed between shared samples tubes (ie 1A and 1B) to ensure equal library representation downstream. For example, if there is equal GEM creation between 1a and 1b, then move forward through 10X

protocol step 2.1. If there is unequal distribution between 1a and 1b, then from the sample with more GEMs slowly withdraw GEMs over 30 seconds and dispense in the opposite sample tube over 20 seconds.
    a. If 1a has complete/partial GEM emulsions and 1b has no GEMs, then move forward with a blank 1b.
5. Proceed with the rest of 10X Protocol Step 1 through Step 2.1.
6. For Step 2.2a, add in the additional reagents to cDNA Amplification Mix as such:
    a. Add in 1ul of 0.2uM ADT PCR additive primer (to create 1X primer concentration)
    b. Add in 1ul of 0.1uM HTO PCR additive primer (to create 1X primer concentration)
        i. Just add 1ul of each 10um primer to the final reaction.
7. Add 65ul of this amplification mix to the sample for Step 2.2b.
    a. Number of cDNA amplification cycles: _____
        i. For HT reactions, consider starting at 8 cycles
8. Proceed with the rest of Step 2.2.
9. For step 2.3, follow these instructions with the below modifications exactly.
    a. In step 2.3d, DO NOT DISCARD THE SUPERNATANT. Reserve the supernatant in new strip tubes. Clearly label the tubes! This supernatant contains your ADT and HTO libraries. Meanwhile, your beads are binding your full length cDNAs. **After the supernatant has been saved, proceed with the rest of the 10X protocol for the bead cleanup and preparation of your cDNA fraction. Follow the below steps for the supernatant.**
    b. With this supernatant, add 140ul SPRIselect (thus doing a 2X SPRI cleanup) to the supernatant.
        i. Add 2500ul of SPRI beads to mutlichannel reservior just prior to use (for 16 rxns worth)
    c. Pipet to mix and incubate for 10 min at RT with caps open and no bubbles.
        i. During the incubation, create new 80% EtOH using molecular grade H_2O.
        ii. If planning on running Tapestation/Qbit, bring out enough TapeStation strips and allow to come to RT.
        iii. Near end of incubation, create 8mL of 80% EtOH (1.6 mL of H_2O, 6.4 mL of EtOH) just prior to use (for 16 rxns worth)
    d. Centrifuge briefly and place on magnet in High position.
    e. After beads have separated, remove and discard supernatant.
    f. Add 200ul of 80% EtOH and wait 30 secs.
    g. Discard EtoH.
    h. Repeat steps f-g one more time.
    i. Centrifuge the tube briefly to collect beads and place tube on magnet in Low position.
    j. Once beads have moved, use pipet to remove any remaining EtOH. Dry for 1 minute.
        i. Add 1mL of EB to mutlichannel reservior just prior to use (for 16 rxns worth)
    k. Resuspend beads in 46.5ul Buffer EB. Pipet to mix.
    l. Incubate for 2 min at RT.

m. Place on magnet in Low position.
n. After beads have separated, collect 46ul of the volume into a strip tube.
o. For each paired sample, combine the two fractions of 46ul eluent (ie from 1a and 1b) into a 92 ul pool.
p. Quantify DNA concentration and quality of the RNA and ADT/HTO libraries using the Qbit and Tapestation D5000.
    i. Tapestation (start first). Label enough tapestation-compatible 8 strip tubes for all samples +1. Add 2ul of D5000 buffer to each tube. Add 2 ul D5000 ladder to the ladder tube. Add 2 ul of each sample to their respective tube. Save photos of each electropherogram to ascertain sample quality.
    ii. Qbit (during Tapestation run): On the top of each tube, label enough Qbit tubes for all samples + 2 standards (Standard 1 and Standard 2) + 1 blank. Create enough Qbit working buffer (1ul dye + 199ul Qbit buffer) for all tubes + 1. Add 190ul of buffer to the Standard tubes, and 199ul to all other tubes. Add 1ul of sample to their respective sample tube, or 1ul EB to the Blank. Mix all tubes well via vortex and run on the Qbit.

10. At Step 2.3m, pool samples from corresponding tubes to form cDNA samples for downstream analyses.
11. Proceed with the rest of the 10X Chip M protocol as specified for the RNA fraction, see protocol below for ADT/HTO fraction.
    a. Do not freeze RNA/ADT/HTO if quantifying the next day!

Section 4: ADT amplification

1. Take 45ul of the volume from Section 3, Step 7 into a new strip tube.
2. Add 50ul of Kapa Hifi Master Mix to each sample.
3. To each sample, add 2.5ul (10uM stock) of each primer in a DI_ADTx primer pair. This will uniquely index that sample. RECORD WHICH PRIMER PAIRS ARE USED BY WHICH SAMPLE. Do not intermix primers from different pairs.

10X Chip Well/Sample	ADT Primer Pair	10X Chip Well/Sample	ADT Primer Pair

4. Pipet to mix sample (final volume should be 100ul).

5. Amplify with the following settings
    - 2min @ 98°C
    - Cycle 6-10x (depending upon yield)
        i. 20sec @ 98°C
        ii. 30sec @ 60°C Cycles Done: _____ (suggested 8)
        iii. 20sec @ 72°C
    - 5 min @ 72°C
    - Hold @ 4°C

Section 5: HTO amplification

1. Take 45ul of the volume from Section 3, Step 7n into a new strip tube.
2. Add 50ul of Kapa Hifi Master Mix to each sample.
3. To each sample, add 2.5ul (10uM stock) of each primer in a DI_HTOx primer pair. This will uniquely index that sample. RECORD WHICH PRIMER PAIRS ARE USED BY WHICH SAMPLE. Do not intermix primers from different pairs.

10X Chip Well/Sample	HTO Primer Pair	10X Chip Well/Sample	HTO Primer Pair

4. Pipet to mix sample (final volume should be 100ul).
5. Amplify with the following settings
    - 2min @ 98°C
    - Cycle 8-12x (depending upon yield)
        i. 20sec @ 98°C
        ii. 30sec @ 64°C Cycles Done: _____ (suggested 7-8)
        iii. 20sec @ 72°C
    - 5 min @ 72°C
    - Hold @ 4°C

Section 6: ADT and/or HTO cleanup

1. Ensure that libraries have equilibrated to RT.
2. From amplified products in Section 4 and/or Section 5, proceed with a 1.2X SPRI purification by adding 120ul SPRI reagent to the library.

    a. Add 2300ul of vortexed SPRI beads to mutlichannel reservior just prior to use (for 16 rxns worth)
3. Pipet to mix and incubate for 10 min at RT, no bubbles.
    a. If planning on running Tapestation/Qbit, bring out enough TapeStation strips and allow to come to RT.
    b. Near end of incubation, create 8mL of 80% EtOH (1.6 mL of H_2O, 6.4 mL of EtOH) just prior to use (for 16 rxns worth)
4. Place tubes on the magnet in High position.
5. After beads have separated, remove and discard supernatant.
6. Add 200ul of 80% EtOH and wait 30 secs.
7. Discard EtoH.
8. Repeat steps f-g one more time.
9. Centrifuge the tube briefly to collect beads and place tube on magnet in Low position.
10. Once beads have moved, use pipet to remove any remaining EtOH. Dry for 1 minute.
    a. Add 1mL of EB to mutlichannel reservior just prior to use (for 16 rxns worth)
11. Resuspend beads in 31ul Buffer EB. Pipet to mix.
12. Incubate for 2 min at RT.
13. Place on magnet in Low position.
14. After beads have separated, collect 30ul into a new 8 strip tube. Label this carefully as this is your final library for ADT and/or HTO!.
15. Quantify DNA concentration and quality of the RNA and ADT/HTO libraries using the Qbit and Tapestation D5000.
    a. Tapestation (start first). Label enough tapestation-compatible 8 strip tubes for all samples +1. Add 2ul of D5000 buffer to each tube. Add 2 ul D5000 ladder to the ladder tube. Add 2 ul of each sample to their respective tube. Save photos of each electropherogram to ascertain sample quality.
    b. Qbit (during Tapestation run): On the top of each tube, label enough Qbit tubes for all samples + 2 standards (Standard 1 and Standard 2) + 1 blank. Create enough Qbit working buffer (1ul dye + 199ul Qbit buffer) for all tubes + 1. Add 190ul of buffer to the Standard tubes, and 199ul to all other tubes. Add 1ul of sample to their respective sample tube, or 1ul EB to the Blank. Mix all tubes well via vortex and run on the Qbit.
16. Libraries can be stored at -20°C in DNA lo-bind tubes until ready to sequence.

Section 7: ADT and/or HTO quality checks

1. Check library concentration using 1ul with Qubit and a HS dsDNA assay.
2. Check library fragments with a D1000 HS Tape. Both libraries should consist of one peak at 230bp. Refer to the original multiplexing cell hashing CITEseq and Biolegend protocols for troubleshooting if other peaks are observed.

Section 8: RNA Library Index Primers

1. Record below which indexing primer pair (or the well on the Primer Plate) was used for each 10X well (to enable pooling for sequencing).

10X Chip Well/Sample	Primer Pair (Well on Plate)	10X Chip Well/Sample	Primer Pair (Well on Plate)

Index primer Plate Type: ____ PN: _____ LN: _____

cDNA Sample Index PCR amplification cycles done: _____ (11 recommended)