BioLegend®
Enabling Legendary Discovery™

Technical Protocols

TotalSeq™-A Antibodies and Cell Hashing with 10x Single Cell 3' Reagent Kit v3 or v3.1 Protocol

This is an updated version of our TotalSeq™-A antibodies and cell hashing with 10x Single Cell 3' Reagent Kit v3.1 protocol. For specific changes, please see the change table at the bottom of the protocol.

Buyer is solely responsible for determining whether Buyer has all intellectual property rights that are necessary for Buyer's intended uses of the BioLegend TotalSeq™ products. For example, for any technology platform Buyer uses with TotalSeq™, it is Buyer's sole responsibility to determine whether it has all necessary third-party intellectual property rights to use that platform and TotalSeq™ with that platform.

The protocol below is intended for customers who are using TotalSeq™-A antibodies and cell hashing reagents with the 10x Single Cell 3' Reagent Kit v3.1 kit.

For customers using TotalSeq™-B or TotalSeq™-C antibodies, please refer to our TotalSeq™-B or C protocol.

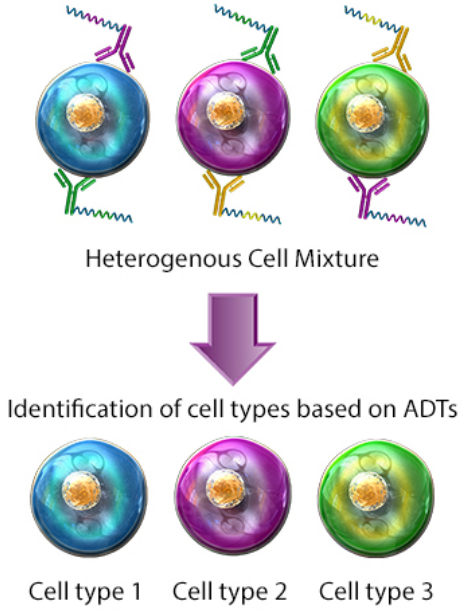
Please read the entire protocol below and the 10x Genomics user guide CG000185_Rev_D for v3 reagents and CG000204_Rev_D for v3.1 reagents, before starting your experiments.

Commonly used abbreviations and terminology:

- **ADT** - **A**ntibody **D**erived **T**ag. Refers to a TotalSeq™ DNA-barcoded oligonucleotide that is directly conjugated to a specific antibody clone of interest. Each antibody clone was raised against a specific extracellular protein epitope and can be used to characterize the expression of that surface antigen on cells. Thus, ADTs serve as DNA tags used to catalog and quantify distinct surface protein expression levels.
- **HTO** – **H**ash **T**ag **O**ligonucleotide. Refers to a TotalSeq™ DNA-Barcoded Oligonucleotide that is directly conjugated to a cocktail of two independent antibody clones that are specific for surface proteins known to be ubiquitously expressed on various cell types. The intention of an HTO is to enable researchers to load multiple samples onto a single lane of a 10x chip and maintain the ability to determine sample origin.
- **CITE-Seq** - **C**ellular **I**ndexing of the **T**ranscriptome and **E**pitopes by **Seq**uencing. This application was first described by Stoeckius *et al.* (*Nat Methods* 14, 865–868 (2017)) of the NY Genome Center as they used antibodies coupled with oligonucleotides to simultaneously measure proteins and RNA at a single-cell level.
- **Cell Hashing** – Application where oligo-tagged antibodies against ubiquitously expressed surface proteins uniquely label cells from distinct samples, which can be subsequently pooled. By sequencing these tags alongside the cellular transcriptome, users can assign each cell to its original sample.

Multiomic Cytometry

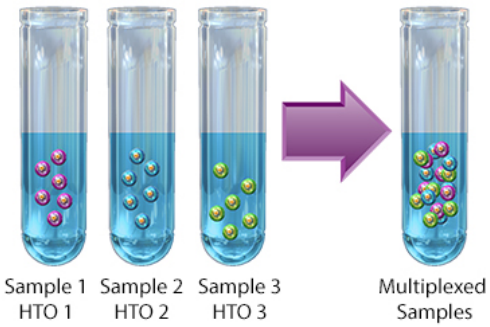
Also known as CITE-seq, is a single-cell analysis technique in which cells are stained with antibodies conjugated to short DNA oligos. These oligos contain a sequence barcode, also known as antibody derived tag (ADT), which is used to identify targeted cellular surface epitopes. Protein and RNA expression can be then characterized in single cells, simultaneously. CITE-Seq can be paired with cell hashing to allow sample multiplexing during sequencing.



Compatibility:
Cell Hashing
TotalSeq™– A
TotalSeq™– B
TotalSeq™– C

Cell Hashing

A technique used to stain cells for multiplexing single-cell partitioned samples during sequencing. Each cell hashing reagent contains a mixture of two distinct monoclonal antibodies targeting distinct ubiquitous cellular surface epitopes. Each monoclonal antibody is conjugated with a short DNA oligo containing the same sequence barcode, also known as hashtag oligo (HTO). HTOs are used to correlate tagged cells with their sample of origin, as they can be mixed together after staining. Cell hashing is compatible with multiomic cytometry and other single-cell analysis techniques.



Compatibility:
Multiomic Cytometry
TotalSeq™– A
TotalSeq™– B
TotalSeq™– C

Reagent and Instrument List

Primers used in sequencing library construction must be ordered from 3rd party vendors prior to starting the protocol:

ADT additive primer (0.2µM Stock) and/or HTO additive primer v2 (0.2µM Stock) (See notes at the end of the protocol for further details on primer sequences.)

TruSeq Small RNA RPIx (10µM Stock) and/or TruSeq D70x_LONG (10µM Stock) primers (See notes at the end of the protocol for further details on primer sequences.)

ADT additive primer v2 (0.2µM Stock) and/or HTO additive primer v2 (0.2µM Stock) (See notes at the end of the protocol for further details on primer sequences.)

For a full list of sequences, view the primer sequence table below.

For Cell Surface staining:
- TotalSeq™-A antibodies and/or TotalSeq™-A hashtag reagents
- Biotinylated antibody and oligo-barcoded streptavidin (optional)
- Human TruStain FcX™ (Fc Receptor Blocking Solution) (BioLegend, Cat# 422301/422302)
- TruStain FcX™ PLUS (anti-mouse CD16/32) (BioLegend, Cat# 156603/156604)
- Phosphate Buffered Saline (PBS) (BioLegend, Cat# 926201 or equivalent)
- Cell Staining Buffer (BioLegend, Cat# 420201)
- 12 x 75mm Falcon™ Round-Bottom Polystyrene Tubes (Fisher Scientific, Cat# 14-959-1A or equivalent)
- Flowmi™ Cell Strainer (Bel-Art, H-B Instrument, Cat# H13680-0040)

Optional
- Curiox Laminar Washing
  Some users may be using the Curiox Laminar Wash system, which has equivalent performance to centrifugation-based washing.
- Corning™ ThermalTray™ Thermo-conductive Platforms (Product Number 432074)

For library preparation:
- Quantabio sparQ HiFi PCR Master Mix (2X) (Quantabio, Cat# 95192-250) or KAPA HiFi HotStart ReadyMix (2X) (Kapa Biosystems, Cat# KK2601)
- Quantabio sparQ PureMag Beads (Quantabio, Cat# 951960) or SPRIselect reagent (Beckman Coulter, Cat# B23317)
- 4200 Tapesation (Agilent Technologies, Cat# G2991A)
- DNA High Sensitivity D1000 and High Sensitivity D5000 (Agilent, Cat# 5067-5584/5067-5592)
- Qubit™ 3 (Thermo Fisher Scientific, Cat# Q33226)
- Qubit™ dsDNA HS Assay Kit (Thermo Fisher, Cat# Q32854/Q32851)
- ADT additive primer (0.2µM Stock) and/or HTO additive primer v2 (0.2µM Stock) (See notes at the end of the protocol for further details on primer sequences.)
- TruSeq Small RNA RPIx (10µM Stock) and/or TruSeq D70x_s (10µM Stock) primers (See notes at the end of the protocol for further details on primer sequences.)

Other essential reagents:
- Nuclease-free Water (Thermo Fisher, Cat# AM9937)
- Ethanol (Sigma, Cat# E7023-500ML)
- Nuclease-Free Pipette Tips (e.g. Thermo Fisher Scientific AM12650, AM12660 or equivalent)
- TempAssure PCR 8-strips (USA Scientific, Cat# 1402-4700)
- PCR Thermocycler (Bio-Rad, T100™ Thermal Cycler)
- Countess™ II FL Automated Cell Counter (ThermoFisher, Cat# AMQAF1000)

Researchers are advised to validate equivalent products when substituting for the above recommendations.

Protocol

---

1) Cell labeling

1. Prepare cell suspensions.
   - This protocol has been optimized using fresh human PBMCs isolated using Ficoll gradients and mouse splenocytes prepared using mechanical dissociation. If using cells isolated from whole lysed blood or other sample types, users may need to optimize staining concentrations.
   - BioLegend has not tested this protocol using single cell suspensions derived from enzymatically digested tissue. Enzymatic digestion may result in cleavage of epitopes and result in reduced staining with TotalSeq™ antibodies. Optimization of staining conditions and concentrations may be required.

2. Assess Cell Viability.
   - Carefully count all cells to ensure accurate quantitation and assess cell viability.
   - Ideal cell viability is ≥ 95%.

- Low cell viability is associated with generation of poor data and is not ideal for single cell experimentation.
- If low cell viability is observed, users may need to enrich live cells or repeat cell suspension preparation.

- Contact Technical Services with any questions regarding cell viability. BioLegend uses Countess II for counting and assessing cell viability using the following protocol, however other methods for assessing cell viability are suitable. For more information about the protocol used by BioLegend, see the following link, details can be found under "PBMC viability assessment—general methods".

3. Dilute cells in appropriate volume prior to staining.
   - If working with human cells, dilute 1 million cells in 45µL of Cell Staining Buffer in 12 x 75mm flow cytometry tubes.
   - If working with mouse cells, dilute 1 million cells in 49.5µL of Cell Staining Buffer in 12 x 75mm flow cytometry tubes.

4. Block cells.
   - Add 5 µl of Human TruStain FcX™ Fc Blocking reagent or 0.5 µl of TruStain FcX™ PLUS (anti-mouse CD16/32) antibody. The final blocking volume should be 50 µl.
     Note: We no longer recommend the use of dextran or monocyte blocker during blocking/staining. If you have any questions please contact BioLegend Technical Support.
   - Incubate for 10 min at 4°C.
   - While cells are incubating in Fc Block, proceed to step 5.

5. Prepare antibody pool using 1 µg (or titrated amounts) of each TotalSeq™, Cell Hashing, and/or biotinylated antibody. For more information regarding TotalSeq™ antibody concentrations, please reach out to BioLegend Tech Services. We recommend adding Cell Hashing antibodies into each respective sample's antibody pool. We do not recommend staining cells with non-hashtag antibodies in one large cell suspension and then aliquoting cells for hashtag antibody staining due to observed poor performance.
   - If using biotinylated antibodies, we recommend staining with your primary antibody first followed by staining with streptavidin TotalSeq™ conjugates. Do not stain with more than 1 unique biotinylated antibody to use for detection.

6. If antibody cocktail volume is less than 50 µL, add Cell Staining Buffer up to 50 µL, then centrifuge the antibody pool at 14,000 x *g* at 2 – 8°C for 10 minutes before adding to the cells. If volume of pool is above 50 µL, no volume adjustment is necessary.
   - If using an antibody cocktail larger than 50 µL, contact Tech Services or local Technical Applications Scientists for protocol guidance before proceeding with this protocol.

7. Carefully pipette out the prepared antibody pool, avoiding the bottom of the tube, and add the TotalSeq™ antibody cocktail to the 50 µL blocked-cell suspension.
   Note for Curiox Laminar Wash system users: Transfer 80µL* of cell suspension to one well of the Curiox Wash plate and place on the Thermal Tray** on ice.
   *80µL is the maximum volume of cells that can be used with Curiox Laminar Wash system.
   **Either ThermalTray or traditional incubation methods can be used

8. Incubate for 30 minutes at 4°C.
   Note for Curiox Laminar Wash system users: Perform the following after step 8.
   - Transfer Curiox Wash Plate to the Curiox Laminar Wash System and wash using the following parameters (Flow Rate: 10, # of cycles: 25)
   - Remove Curiox Wash Plate from the system and add 40µL of wash buffer to the well containing the washed cells.
   - Resuspend by pipetting gently.
   - Proceed to step 11.

9. Add 3 mL of Cell Staining Buffer and spin at 4°C for 5 minutes at 400 x *g* -600 x *g* depending on your sample type. Repeat wash 2 more times for a total of 3 washes.
   - BioLegend recommends the use of a swing bucket centrifuge as centrifuging with fixed angle rotors may lead to "smearing" of the cell pellet, which may result in cell loss. Contact Tech or local field representative if you have any questions.
   - BioLegend recommends manually pouring out supernatant being careful not to disrupt the cell pellet. Between 50-150 µL of residual supernatant will remain in the tube after decanting, which is taken into consideration in step 11 of this protocol. Do not try to forcefully remove any remaining liquid as this will disrupt the cell pellet and result in cell loss.

10. If using a biotinylated primary antibody, incubate the stained cells with the appropriate oligo barcoded streptavidin at the recommended amount specified on the product technical datasheet for 20 minutes. Repeat step 9, then proceed to step 11.

11. Add 200µL of Cell Staining Buffer to the cells for an approximate final volume of 250-350 µL.

12. Slowly filter cells through 40 µm Flowmi™ Cell Strainer.
    Note: 40 µm Flowmi™ Cell Strainer may be too small for some sample types.

13. Verify cell concentration and viability after filtration.
    Note: If using 10x Genomics Chromium Controller for single cell partitioning, we highly recommend determining cell viability. Ideally the viability should be >90% after filtration for optimal capture rate. The presence of a large number of non-viable cells can decrease the efficiency of cell partitioning and recovery within the 10x Genomics Chromium chip.

14. Adjust cell concentration using PBS according to the input requirements of your single cell partitioning platform.

II) Run 10x Genomics single cell 3' v3 assay as described through Post Gem-RT Cleanup – Dynabeads (step 2.1). 10x Genomics Document CG000204, Rev D.

At cDNA amplification step (Step 2.2), use the following table:

	ADT 1 rxn (µl)	HTO 1rxn (µl)	ADT + HTO 1 rxn (µl)
Amp Mix	50	50	50
cDNA Primers*	15	15	15
ADT Additive Primer (0.2 µM stock)	1	0	1
HTO Additive primer v2 (0.2 uM stock)	0	1	1
Total	**66**	**66**	**67**

* included with 10x Genomics 3' kit, different from Feature cDNA primers 2.

Notes:

Follow steps 2.3A and 2.3B exactly to separate ADTs/HTOs from cDNA. **Continue to use 70 µL of sparQ or SPRI beads in step 2.3B.**

See notes at the end of the protocol for further details on primer sequences.

III) ADT and mRNA library preparation

A) Prepare Sample Index PCR Mix.

For ADT	For HTO	Volume (µl)
Purified ADT/HTO fraction	Purified ADT/HTO fraction	5
SI PCR primer (10uM stock)	SI PCR primer (10uM stock)	2.5
TrueSeq Small RNA RPIx (10uM stock)	TruSeq D70x_s (10uM stock)	2.5
2X QuantaBio or Kapa Hifi Master Mix	2X QuantaBio or Kapa Hifi Master Mix	50
RNAse-free water	RNAse-free Water	40
	Total	**100**

Note: For samples that contain both ADT and HTO, perform two separate reactions and add 5 µl of "purified ADT/HTO fraction" from the same sample to ADT or HTO reaction.

B) Incubate in a thermal cycler with the following protocol:

ADT		**HTO**	
98°C	2min	98°C	2min

98°C	20sec		98°C	20sec	
60°C	30sec	14 - 15 Cycles	64°C	30sec	13 - 15 Cycles
72°C	20sec		72°C	20sec	
72°C	5min		72°C	5min	
4°C	hold		4°C	hold	

C) Post ADT/HTO library amplification clean-up

1. Add 120 µl sparQ or SPRIselect Reagent (1.2X) to each sample.
2. Incubate 5 min at room temperature.
3. Place on the magnet in its High position until the solution clears.
4. Carefully remove and discard the supernatant.
5. Place tubes on magnet in its High position. Wash pellet twice with 200 µl 80% ethanol.
6. Centrifuge briefly. Place on the magnet Low. Remove remaining ethanol.
7. Remove from the magnet. Add 40.5 µl water.
8. Incubate 2 min at room temperature.
9. Place on the magnet in its Low position until the solution clears.
10. Transfer 40 µl to a new tube strip. Store at 4°C for up to 72 h or at −20°C for long-term storage.
11. ADT/HTO libraries are now ready to be sequenced
    Quantify libraries by standard methods (QuBit, BioAnalyzer, qPCR).
    ADT libraries will be around 180 bp (Figure 1B).

Sequencing CITE-seq libraries:

To obtain sufficient read coverage for both libraries, we typically sequence ADT libraries in 5-10% of a lane and cDNA library fraction at 90% of a lane (HiSeq2500 Rapid Run Mode Flow Cell). See table below for sequencing depth recommendations

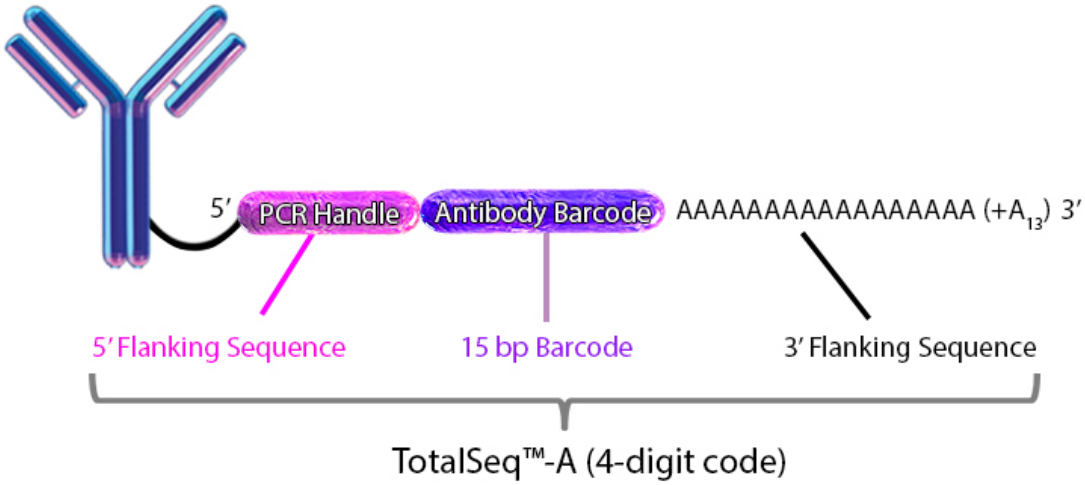
Library Type	Minimum Sequencing Depth (reads/cell)
3' Gene Expression Library	20,000-50,000
Cell Surface Protein Library <100 ADT panel	5,000
Cell Surface Protein Library ≥100 ADT panel	10,000
Cell Hashing Libraries	500

Notes:

Oligonucleotide sequences:

TotalSeq™ antibodies. Each clone is barcoded with a unique oligonucleotide sequence. These contain standard small TruSeq RNA read 2 sequences and can be amplified using Illumina's Truseq Small RNA primer sets (RPIx – primers, see example RPI1 below)

CCTTGGCACCCGAGAATTCCAAACAAGACCCTTGAGBAAAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A.



Please visit https://www.biolegend.com/totalseq for detailed information:

Oligos required for ADT library amplification:

- PAGE purification is the preferred method when ordering primers.
- The phosphorothioate bonds in the primer renders the oligonucleotide resistant to nuclease degradation.
- A unique Illumina primer (index) should be used for each 10x Genomics sample lane used within one sequencing lane.

1. 10x Genomics SI-PCR primer 5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC*T*C
2. ADT cDNA PCR additive primer 5'CCTTGGCACCCGAGAATT*C*C
3. HTO cDNA PCR additive primer v2 5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T
4. Illumina Small RNA RPI1 primer (for ADT amplification; i7 index 1, Oligonucleotide sequences, Illumina)
   5'CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A
5. Illumina TruSeq D701_LONG primer (for HTO amplification; i7 index 1)
   5'CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T

* indicates a phosphorothioate bond
B indicates C or G or T; not A nucleotide

Primers Used for Sequencing Library Construction:

Name	Sequence 5' -> 3'	Do Not Use with 10x Index
SI-PCR primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC*T*C	
HTO additive primer v2	GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
ADT additive primer	CCTTGGCACCCGAGAATT*C*C	
D701_LONG	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D702_LONG	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D703_LONG	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D704_LONG	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D705_LONG	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	

D706_LONG	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D707_LONG	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D708_LONG	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D709_LONG	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D710_LONG	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	
D711_LONG	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	SI-GA-E7
D712_LONG	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT*C*T	SI-GA-A10

RPI1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	
RPI2	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	
RPI3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	
RPI4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	
RPI5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	
RPI6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	
RPI7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	SI-GA-A11
RPI8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A	

Representative Data and Troubleshooting

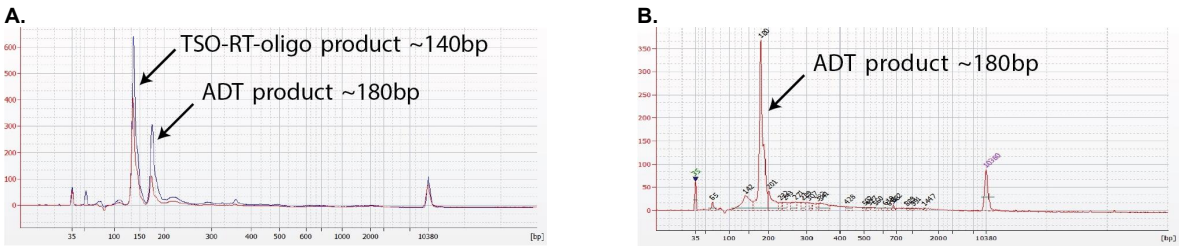


Figure 1. ADT library verification.
(Left graph) A TSO-RT-oligo product (~140 bp) can be amplified during the ADT PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. Sequential 2X sparQ or SPRI purification of the ADT fraction after cDNA amplification reduces carryover of primers from cDNA amplification, and minimizes the amplification of this product during ADT-library amplification. To further enrich for ADT specific product the purified ADT library can be reamplified for 3 additional cycles with ADT specific primer sets or P5/P7 generic primers. (Right graph) A clean ADT library will contain a predominant single peak at around 180 bp.

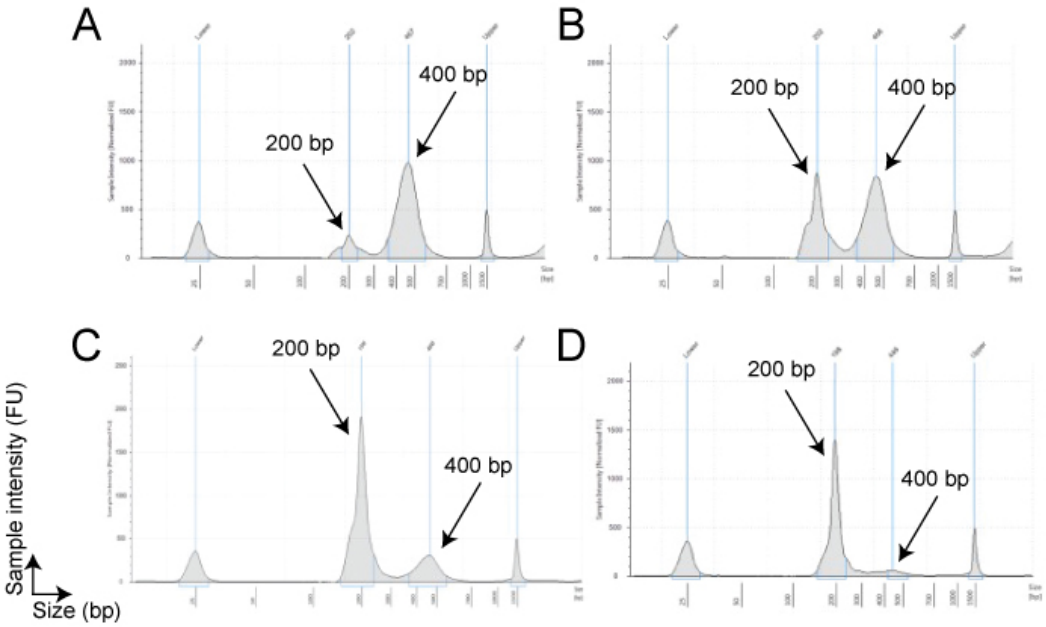


Figure 2. My ADT/HTO library contains a large peak at ~400bp
Overamplification of a library can lead to depletion of available primers and/or dNTPs resulting in self-priming of PCR products by their P5 and/or P7 adapters. This can lead to the production of “daisy-chains” or “bubble products”. These products consist of essentially 2 ADT or HTO barcode sequences attached to one another in 1 long oligo tag that is twice as long as the original oligo tag; these products appear as peaks at approximately 400 bp. These peaks can be more or less pronounced (panel A and B respectively).

The larger peak is perfectly acceptable to sequence. However, it is difficult to quantify these libraries to titrate for sequencing. This error can be corrected by performing another PCR reaction using generic P5/P7 primers (not used in the protocol) for one or two cycles on the 1.2x sparQ or SPRI cleaned up product. The PCR reaction will convert the larger product to the desired size product partially (panel C) or completely (panel D) as the major peak.

Version Change Table

Section	Changes
Section 1 step 1	Added general information regarding types of cells and cell isolation methods used during testing. Also, added information regarding cells isolated via enzymatic digestion of whole tissues.
Section 1 step 2	Added information regarding methods used to assess cell viability
Section 1 step 5	Added “Contact Technical Services” regarding antibody staining concentrations.
Section 1 step 8	Added protocol information for the use of the Curiox Laminar Washing System
Section 1 step 9	Reduced wash volumes from 3.5mL to 3mL
Section 1 step 9	Changed centrifugation speed recommendation from 400 x *g* to 400 x *g* -600 x *g*
Section 1 step 11	Changed resuspension volume from 500 µL to 250-350 µL
Section 3 PCR conditions	Changed conditions from 12-13 cycles to 13-15 cycles when amplifying HTO libraries
Section 3 Sequencing depth	Added recommended sequencing depths

recommendations	
Primer sequences table	Provided longer D70X primer sequences to help reduce the occurrence of HTO drop outs.