TotalSeq™-A Antibodies and Cell Hashing with 10x Single Cell 3' Reagent Kit v3 3.1 Protocol

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Abstract

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.

TotalSeq™-B antibodies are designed to be used with 10x Single Cell 3′ Reagent Kit v3 with Feature Barcoding. The adjusted protocol below is for customers who are utilizing TotalSeq™-A antibodies with the v3 kit instead. Note: Step 4 from the 10x Genomics user guide document number CG000185, Rev B only applies to the cDNA library and NOT to the TotalSeq™-A library.

Please read the entire protocol below and the 10x Genomics user guide for the Chromium Single Cell 3' Reagent Kits v3 with Feature Barcoding technology for Cell Surface Protein before starting the experiments. 10x Genomics user guide document number CG000185, Rev B.

Commonly used abbreviations:

- ADT: Antibody derived tags, the oligo sequence conjugated to regular TotalSeq™-A antibodies
- HTO: Hashtag oligonucleotides, the oligo sequence conjugated to TotalSeq™-A Hashtag antibodies

Materials

MATERIALS

Human TruStain FcX™ (Fc Receptor Blocking Solution) **BioLegend Catalog #**422301, 422302

TruStain FcX™ PLUS (anti-mouse CD16/32) **BioLegend Catalog #**156603, 156604

Phosphate Buffered Saline (PBS) **BioLegend Catalog #**926201

Cell Staining Buffer **BioLegend Catalog #**420201

For Cell Surface staining:

- TotalSeq™-A antibody-oligo and/or TotalSeq™-A Cell Hashing conjugates
- Biotinylated antibody and oligo **barcoded** streptavidin
- 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 × 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)

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 (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 the above recommendations.

I) Cell labeling

1
- Prepare single cell suspension following a suitable protocol.
- Resuspend cells at a concentration of 2×10^7cells/mL in staining buffer.
- Make note of cell viability (>95%) and also include dead cells in the total cell count.
- If high cell death is observed, live cell enrichment (e.g. by Flow Cytometry) is recommended.

2 Aliquot 50 µl of cells into 12 × 75mm tubes.

3 Add 5 µl of Human TruStain FcX™ Fc, or 0.5 µl of TruStain FcX™ PLUS (anti-mouse CD16/32) Blocking reagent.
Note: We no longer recommend the use of dextran during blocking/staining. If you have any questions please contact BioLegend **Technical Support**.

4 Incubate for 10 min at 4°C.

5 While cells are incubating in Fc Block, prepare antibody pool using 1 µg (or titrated amounts) of each TotalSeq™ or Biotinylated antibody.

6 To maximize performance, centrifuge the antibody pool at 14,000xg at 2 – 8°C for 10 minutes before adding to the cells. **Note:** If antibody cocktail volume is less than 50 µl, add Cell Staining Buffer up to 50 µl, then centrifuge.

7 Carefully pipette out the liquid, avoiding the bottom of the tube, and add the TotalSeq™ antibody cocktail to the cell suspension.

8 Incubate for 30 minutes at 4°C.

9 Add 2 mL of cell staining buffer and spin 5 minutes 350g at 4°C. Repeat wash 2 more times for a total of 3 washes.

10 If using biotinylated antibodies, incubate with the appropriate oligo**barcoded** streptavidin at the recommended amount specified in the product technical datasheet for 20 minutes.

11 Add 2 mL of cell staining buffer and spin 5 minutes 350g at 4°C. Repeat wash 2 more times for a total of 3 washes.

12 Resuspend cells in 500 µL of PBS to get a concentration of 1×10^6 cells/mL.

13 Filter cells through 40 µm Flowmi™ Cell Strainer.

14 Verify cell concentration by counting on hemocytometer after filtration.

15 Dilute cells as necessary for appropriate input into the 10X Chromium controller.

II) Run 10x Genomics single cell 3' v3 assay

16 Run 10x Genomics single cell 3' v3 assay as described through Post Gem-RT Cleanup – Dynabeads (step 2.1). 10x Genomics Document CG000185, Rev B.

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 (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.**
ADT: Antibody derived tags, it refers to regular TotalSeq™-A antibodies
HTO: TotalSeq™-A Hashtag antibodies
See notes at the end of the protocol for further details on primer sequences.

III) ADT and mRNA library preparation

17 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.

18 Incubate in a thermal cycler with the following protocol:

For ADT:

	98°C	2 min	
	98°C	**20 sec**	**14 - 15 Cycles**
	60°C	**30 sec**	
	72°C	**20 sec**	
	72°C	5 min	
	4°C	hold	

For HTO:

	98°C	2 min	
	98°C	**20 sec**	**12 - 13 Cycles**
	64°C	**30 sec**	
	72°C	**20 sec**	
	72°C	5 min	
	4°C	hold	

19 Post ADT/HTO library amplification clean-up

19.1 Add 120 µl sparQ or SPRIselect Reagent (1.2X) to each sample.

19.2 Incubate 5 min at room temperature.

19.3 Place on the magnet in its High position until the solution clears.

19.4 Carefully remove and discard the supernatant.

19.5 Place tubes on magnet in its High position. Wash pellet twice with 200 µl 80% ethanol.

19.6 Centrifuge briefly. Place on the magnet Low. Remove remaining ethanol.

19.7 Remove from the magnet. Add 40.5 µl water.

19.8 Incubate 2 min at room temperature.

19.9 Place on the magnet in its Low position until the solution clears.

19.10 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.

19.11 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 1).

Note: Step 4 from the 10x Genomics user guide document number CG000185, Rev B only applies to the cDNA library and NOT to the TotalSeq™-A library.

20 **Sequencing CITE-seq libraries:**

We estimate that an average of 100 molecules per ADT per cell is sufficient to achieve useful information. The number of reads required to obtain 100 molecules depends on the complexity of the sequencing library (e.g. duplication rate). ADT and mRNA cDNA sequencing libraries can be pooled at desired proportions. 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 Flowcell) (Approximately 5k reads/cell for ADTs).

Please visit our **website** for more important notes and troubleshooting regarding this protocol.