TotalSeq™-A antibodies with 10X Single Cell 3' Reagent Kit v3 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 following protocol combines surface protein labeling with TotalSeq™-A antibodies and 10X Single Cell 3' Reagent Kit v3 with Feature Barcoding. The adjusted protocol is to provide guidance when NOT using TotalSeq™-B antibodies with the v3 kit, or when using a combination of TotalSeq™-A and TotalSeq™-B reagents.

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.

Reagent and Instument List:

- ☐ TotalSeq™-A antibody-oligo conjugates
- ☐ Human TruStain FcX™ (Fc Receptor Blocking Solution) (Cat# 422301/422302)
- ☐ 8-strip PCR tubes, emulsion safe (TempAssure PCR 8-strips, USA Scientific, Cat# 1402-4700)
- ☐ Nuclease-Free Pipette Tips (e.g. ThermoFisher Scientific AM12650, AM12660 or equivalent)
- ☐ Bioanalyzer chips and reagents (DNA High Sensitivity and small RNA kit, Agilent Cat# 5067-4626 and 5067-1548)
- ☐ SPRIselect reagent (Beckman Coulter, Cat# B23317)
- ☐ E-Gel™ EX Agarose Gels, 4% (ThermoFisher Scientific Cat# G401004)
- ☐ DNA LoBind Tubes (Eppendorf, Cat# 022431021)
- ☐ PCR Thermocycler (Bio-Rad, T100™ Thermal Cycler)
- ☐ KAPA HiFi HotStart ReadyMix (2X) (Kapa Biosystems, Cat# KK2601)
- ☐ TruSeq Small RNA Library Prep Kit (Illumina, suitable Cat#)
- ☐ Magnetic tube rack (*e.g.* ThermoFisher Scientific)
- ☐ Qubit (ThermoFisher Scientific, Cat# Q33226)
- ☐ Fuchs-Rosenthal Counting Chamber (Hemocytometer, VWR, Cat# 15170-230)
- ☐ Phosphate Buffered Saline (PBS) (BioLegend, Cat# 420501 or equivalent)
- ☐ TWEEN® 20 (Sigma-Aldrich, Cat# P9416-50ML)
- ☐ Cell Staining Buffer (BioLegend, Cat# 420201)
- ☐ 80% Ethanol
- ☐ Dextran Sulfate Sodium Salt (MP Biomedicals, Cat. No. 101516 or equivalent)
- ☐ Flowmi™ Cell Strainer (Bel-Art, H-B Instrument, Cat# H13680-0040)

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

I) Cell labeling

1. Carefully count all cells to ensure accurate quantitation.
    - 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. Resuspend 1-2 million cells in 50 µl Cell Staining Buffer.
3. Add 5 µl of Human TruStain FcX™ Fc Blocking reagent and 2 µl of Dextran Sulfate solution.
4. Incubate for 10 minutes at 4˚C.
5. While cells are incubating in Fc Block, prepare antibody-pool using 1 µg (or titrated amounts) of each TotalSeq™ 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. Wash cells 2 times with 1 mL PBS, spin 5 minutes 350*g* at 4˚C.
10. Resuspend cells in PBS at 500 cells/µl.
11. Filter cells through 40 µm strainers.
12. Verify cell concentration and viability by counting on hemocytometer after filtration.

II) 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:

TotalSeq-A only **OR** TotalSeq-A + TotalSeq-B

cDNA Amplification Reaction Mix	1X (µl)	4X + 10% (µl)	8X + 10% (µl)
Amp Mix	50	220	440
Feature cDNA Primers 2	14	61.6	123.2
ADT Additive Primer (0.2 µM stock)	1	4.4	8.8

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

III) ADT and mRNA library preparation

A) After cDNA amplification: Separate ADT-derived cDNAs (180bp) and mRNA-derived cDNAs (>300bp) as described in step 2.3 and proceed to step 2.3B (Supernatant Cleanup)

B) mRNA-derived cDNA >300bp (beads fraction). Proceed with standard 10x Genomics protocol for cDNA sequencing library preparation.

C) ADTs (supernatant fraction): Proceed with Step 4 (Cell Surface Protein Library Construction)

1) For TotalSeq-A ADTs, use the following reaction mix and cycling conditions (note that 5 µl DNA sample is sufficient for library amplification)

ADT Library amplification	1X (µl)
Purified ADT fraction	5
Truseq small RNA RPIx primer (containing i7 index) 10 µM stock	2.5
SI-PCR P5 oligo – 10 µM stock	2.5
Quantabio sparQ HiFi PCR Master Mix (2X)	50
RNAse-free water	40

Step	Temperature	Time	Cycles
1	98˚C	0:02:00	1
2	98˚C	0:00:20	6-10
3	60˚C	0:00:30	
4	72˚C	0:00:20	
5	72˚C	0:05:00	1
6	4˚C	HOLD	

2) Purify PCR product using 1.6X SPRI purification by adding 160 µl SPRI reagent.
    - Incubate 5 minutes at room temperature.
    - Place tube on magnet and wait 1 minute until solution is clear.
    - Carefully remove and discard the supernatant.
    - Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (first Ethanol wash).
    - Carefully remove and discard the ethanol wash.
    - Add 200 µl 80% Ethanol to the tube without disturbing the pellet and stand for 30 seconds (second Ethanol wash).
    - Carefully remove and discard the ethanol wash.
    - Centrifuge tube briefly and return it to magnet.
    - Remove and discard any remaining ethanol and allow the beads to air-dry for 2 minutes.
    - Resuspend beads in 20 µl water.
    - Pipette mix vigorously and incubate at room temperature for 5 minutes.
    - Place tube on magnet and transfer clear supernatant to PCR tube.
3) ADT libraries are now ready to be sequenced.
    - Quantify libraries by standard methods (QuBit, BioAnalyzer, qPCR).
    - ADT libraries will be around 180 bp (Figure 1).
4) **For TotalSeq-B ADTs, follow steps 4.1 – 4.3 in the 10X protocol.**

IV) Sequencing CITE-seq libraries:

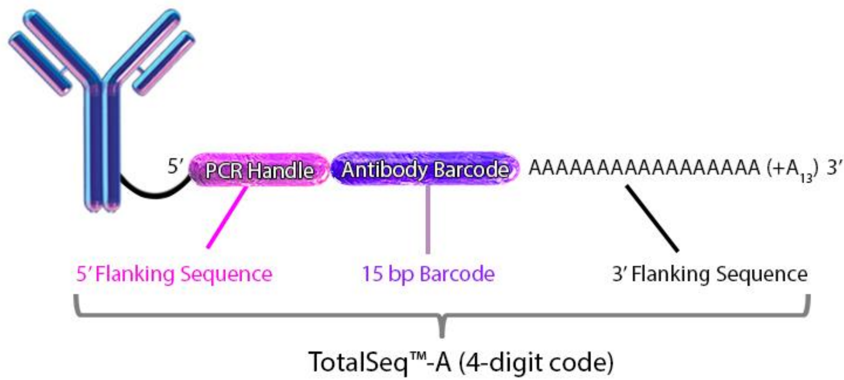
We estimate that an average of 100 molecules per ADT per cell is sufficient to achieve useful information. The number of reads required to obtain 100 molecules depends on the complexity of the sequencing library (*e.g.* duplication rate). ADT and mRNA cDNA sequencing libraries can be pooled at desired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

Notes:

Oligonuleotide 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)

CCTTGGCACCCGAGAATTCCA**AACAAGACCCTTGAG**BAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA*A*A.



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

Oligos required for ADT library amplification:

- 10x Genomics SI-PCR primer
  5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGC*T*C

- ADT cDNA PCR additive primer
  5'CCTTGGCACCCGAGAATT*C*C

- Illumina Small RNA RPI1 primer (for ADT amplification; i7 index 1, Oligonucleotide sequences, Illumina)
  5'CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCCTTGGCACCCGAGAATTC*C*A

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

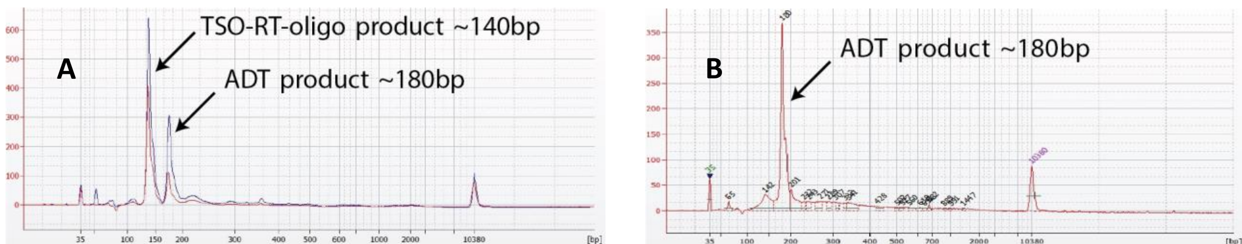


Figure 1. ADT library verification. (left graph) A TSO-RT-oligo product (~140 bp) can be amplified during the ADT PCR by carryover primers from cDNA amplification. The product will not cluster but will interfere with quantification. Sequential 2X SPRI purification of the ADT fraction after cDNA amplification 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.