Protein and transcriptome quantitation using BD AbSeq™ Antibody-Oligonucleotide technology and the 10X Genomics Chromium™ Single Cell Gene Expression Solution

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Summary

The ability to measure gene and protein expression from the same single cell is a recent powerful new technological advance. This white paper describes a protocol to perform antibody-oligonucleotide labelling of cell suspensions with BD AbSeq™ Antibody-Oligonucleotide reagents in preparation for single-cell capture. BD AbSeq utilizes oligonucleotides conjugated to each antibody which contain an antibody-specific barcode (ABC), a poly (A)-tail that allows its capture along with mRNAs, as well as a PCR handle that allows PCR amplification and generation of an associated library. This protocol integrates BD AbSeq and the 10X Genomics Chromium single-cell RNA sequencing platforms to quantify gene and protein expression from the same single cells, using human immune cells from the peripheral blood as an example.

Preparing BD AbSeq Antibody Cocktail

1. Centrifuge BD AbSeq antibody tubes and place on ice. In this white paper, we used a BD AbSeq 20-plex cocktail (Appendix 1).
2. Into a new 1.5 mL microcentrifuge tube, pipet 2.6 μL per antibody per sample (2 μL + 30% overage) from each BD AbSeq antibody tube to create a 2X master mix (see below). Add BD Stain Buffer to bring final pool to a volume of 130 μL of 2X labeling master mix per sample.
3. Pipet-mix 15 times and centrifuge BD AbSeq antibody cocktail.
4. Place BD AbSeq antibody cocktail on ice until ready to use.

BD AbSeq Cell Labeling

Fc Block Staining for Myeloid and B Cells

- Notes: When working with cells that express Fc receptors, such as myeloid and B cells, Fc Block is recommended. Fc Block blocks non-specific binding in these cell types, thus reducing background noise. It also increases the specificity of antibody binding in cells with low expression of the protein target.
- Keep all reagents on ice and prepare all master mixes on ice.
- If processing more than one sample, include 20% overage.

Reagent	1 sample* (µL)	1 sample + 20% overage (µL)
BD Stain Buffer (FBS)	95.0	114.0
Human BD Fc Block	5.0	6.0
Total	**100.0**	**120.0**

1. In a new 1.5 mL microcentrifuge tube, pipet reagents with 20% overage for Fc Block mastermix on ice in the following order:

   **1 sample (uL) volume is for ~1x10^6 cells in 100 µL of Fc Block master mix. Adjust volumes accordingly for greater cell numbers.*
2. Pipet-mix 15 times and centrifuge the Fc Block mastermix, and then place on ice.
3. Centrifuge cell suspensions at 400xg for 5 minutes.
4. Remove the supernatant from the cell suspension tubes without disrupting the cell pellet and resuspend in 110 µL of the Fc Block mastermix. If a cell pellet is not seen, leave 20 µL of supernatant with the pellet prior to resuspending cells in 110 µL of the Fc Block mastermix.
5. Incubate at room temperature for 10 minutes.

Adding Antibody cocktail

6. Add 40 µL of the BD AbSeq antibody cocktail to a 1.5 mL microcentrifuge tube.
7. Add 60 µL of BD Stain Buffer to the antibody cocktail to bring the total volume to 100 uL.
8. Transfer 100 µL of cell suspension ***(from step 5 in Fc Block section)*** to the tube with the BD AbSeq antibody cocktail.
    - Note: If fewer than 20 antibodies are used or less than 100 uL of cell suspension is added, add BD Stain Buffer to bring the total volume up to 200 µL.
9. Pipet-mix 15 times.
10. Incubate the tubes on ice for 30-60 minutes.

Post-Labelling Washes

Note: Sufficient post-labelling washes are important for reducing noise that comes from residual unbound antibodies being captured onto 3' capture beads during single-cell capture. However, some cell loss occurs with each additional wash. Fewer washes can be performed if cell numbers are limiting.

11. After incubation, transfer cell suspension to a 5 mL round-bottom polystyrene tube and add 2 mL of BD Stain Buffer to the tube and pipet mix to dilute unbound antibody oligo.
12. Centrifuge the cell labelling tube at 400xg for 5 minutes.
13. Uncap tube, invert, and pour supernatant from cell pellets into a 15 mL conical tube.
    - After wash steps are completed, the 15 mL conical tube can be spun down to ensure there was minimal cell loss to the supernatant during cell labelling washes.
14. Add 2 mL BD Stain Buffer to the tube and resuspend the pellet to further wash cells.
15. Centrifuge the tube at 400 x g for 5 minutes.
16. (*Optional*) Repeat steps 13-15 once more for a total of 3 washes.

17. Centrifuge the cell labelling tube at 400xg for 5 minutes.
18. Remove the supernatant without disturbing the pellet.
19. Resuspend in 1 mL of HBSS + 1% FBS and transfer to a 1.5 mL microcentrifuge tube.
20. Count cells. The antibody-oligonucleotide stained sample is now ready to be processed for surface antibody staining prior to FACS sorting.

Surface Staining prior to FACS sorting*

1. Centrifuge cells for 1 minute at 8,000 RPM.
2. Resuspend approximately $1x10^6$ cells in a 1.5 mL microcentrifuge tube in 100uL of HBSS + 1% FBS.
3. Stain with 5 ul of APC anti-human CD45 antibody in a 100 µL cell suspension (5:100 ratio). In this protocol, we isolate $CD45^+$ immune cells but any cell population of interest can be studied.
4. Incubate cells at room temperature for 15 minutes.
5. Wash once with 500 µL PBS, spin at 8,000 RPM for 1 minute.
6. Resuspend cells in ~350uL of HBSS + 1% FBS.
7. Filter the cell suspension with a Corning Falcon Test Tube with Cell Strainer Snap Cap.
8. Place on ice and sort cells into complete media.

* Although sequential staining was performed here, the sorting antibody can also be added into the Ab-Oligo staining cocktail to perform both stains simultaneously. This decreases the number of washing steps, minimizing potential cell loss.

Cell capture and getting started with 10X steps

1. Prepare cells for cell capture according to the 10x Chromium Single Cell 3' v2 User Guide.

- Note**:** Based on the Volume Calculator Table found on page 10 of the 10X protocol, cells should be suspended in 10.4 uL of PBS + 0.04% BSA. This suspension results in a cell stock volume of 1,000 cells/uL with a targeted cell recovery of 6,000 cells.

2. Proceed with GEM generation and Post GEM-RT Cleanup according to 10X Chromium Single Cell 3' v2 User Guide.
    - Note**:** Ensure that there is 35 µL of each GEM-RT product.

cDNA Amplification

1. Use the BD AbSeq protocol to carry out cDNA Amplification.
2. If BD AbSeq PCR1 primer is not already diluted, combine 2 µL of the BD AbSeq PCR1 primer with 18 µL water in a 1:10 dilution.
3. Prepare cDNA Amplification Reaction Mix as follows:

Reagent	**1X (µL)**	**4.4X (µL)**	**8.8X (µL)**
Nuclease-Free Water	6	26.4	52.8
Amplification Master Mix[a]	50	220	440
cDNA Additive[a]	5	22	44
cDNA Primer Mix[a]	2	8.8	17.6
BD AbSeq PCR1 primer (1:10)[b]	2	8.8	17.6
TOTAL	**65**	**286**	**572**

a. *From 10X Chromium Single Cell 3' Library & Gel Bead Kit v2, Reagents Module 1*

b. *Primer sequence:* CAGACGTGTGCTCTTCCGATCT

4. Add 65 µL cDNA Amplification Reaction Mix to each tube containing 35 µL purified GEM-RT product.
5. Pipet mix 15x and centrifuge briefly.

6. Place in the thermal cycler, and run the program as follows:

Lid Temperature			
105°C			
Step	**Cycles**	**Temperature**	**Time**
Hot start	1	98°C	3 min
Denaturation	See below for total cycles	98°C	15 sec
Annealing		67°C	20 sec
Extension		72°C	1 min
Final extension	1	72°C	1 min
Hold	1	4°C	Hold

Targeted cell recovery <2,000 - 14 cycles total

Targeted cell recovery 2,000-6,000 - 12 cycles total

Targeted cell recovery 6000-10,000 - 10 cycles total

Targeted cell recovery >10,000 - 8 cycles total

7. After PCR is complete, cells can be stored at 4°C ≤72h or proceed to the next step.

Post cDNA Amplification Reaction Cleanup

Note: *Retain both the beads and the supernatant that contain cDNA and AbSeq oligos, respectively.*

1. Bring the SPRIselect Reagent to room temperature.
2. Vortex the SPRIselect Reagent until the beads are fully suspended.
3. Pipet 60 µL of SPRIselect Reagent magnetic beads (0.6X) to each sample in the strip tube containing cDNA amplification products, and pipet-mix 15 times.

4. Incubate the suspensions at room temperature for 10 minutes, and then place the strip tube in a 10x Magnetic Separator in the “High” position until the solution is clear.
    a. During this incubation period, label a new 0.2 mL 8-strip tube with your sample name and “ABC” to identify this tube as containing the AbSeq products.
5. While leaving the strip tube in a magnetic separator, taking care not to disturb the beads, carefully pipet the supernatant (which has the AbSeq oligos) into the strip tube labelled “ABC.**”** Do not dispose of the beads that contain the cDNA products.

    Note: *the cDNA and AbSeq products will proceed through separate workflows from this point forward.*
6. Set the ABC strip tube aside.
7. At this point, retrieve the strip tube on the magnet that contains the cDNA bound to the SPRIselect beads. Complete the cDNA purification and elution steps as outlined in the 10X Single Cell 3’ Reagent Kits v2.
8. To the ABC strip tube, add 120 µL (1.8X) of SPRIselect Reagent to each supernatant, and then pipet-mix 15 times.
9. Incubate the suspensions at room temperature for 5 minutes, and then place the strip tube into a 10x Magnetic Separator in the High position until the solution is clear.
10. Leaving the strip tube in the magnet separator, carefully remove and discard only the supernatant without disturbing the beads.
11. Keeping the strip tube in a magnetic separator, gently pipet 200 µL of fresh 80% ethyl alcohol to the side of the strip tube opposite each pellet.
12. Incubate the samples for 30 seconds on the magnetic separator.
13. While leaving the strip tube in the magnet separator, carefully remove and discard only the supernatant without disturbing the beads.
14. Repeat steps 12-14 once for a total of 2 washes.
15. Quickly spin strip tube to pull any residual ethanol to the bottom of the tubes.

16. Return strip tubes to the magnet at “Low” position.
17. Keeping the strip tube on the magnetic separator, remove any residual supernatant from each tube.
18. Leave the strip tube open on the magnetic separator for 2 minutes at room temperature to dry the beads.
19. Remove the strip tube from the magnetic separator, and then pipet 26 µL of Buffer EB into each tube and pipet-mix 15 times to completely resuspend the beads.
20. Briefly centrifuge the strip tube to collect the contents at the bottom, and then incubate the samples at room temperature for 2 minutes.
21. Place the strip tube on the magnetic separator on “High” for 1 minute until the solution is clear.
22. Transfer 25 µL of eluate into a new strip tube. These are the purified AbSeq products.

AbSeq Sample Index PCR

1. Prepare AbSeq Sample Index PCR Master Mix as follows:

Reagent	1X (µL)	4.4X (µL)	8.8X (µL)
PCR MasterMix	25	110	220
SI-PCR Primer[a]	1	4.4	8.8
TOTAL	**26**	**114.4**	**228.8**

[a.] Use from 10X Chromium Single Cell 3’ Library & Gel Bead Kit v2, Reagents Module 2.

2. Pipet-mix 15 times, and place on ice until use.
3. For each AbSeq library, pipet 5 µL of an individual Chromium i7 Sample Index to 26 µL of the AbSeq Sample Index PCR Master Mix in a new 8-strip tube.
4. Bring the AbSeq Sample Index PCR Master Mix to the post-amplification workspace.

5. Pipet 19 µL of the amplified AbSeq product from post-cDNA amplification reaction clean up, step 23, to each well of the 8-strip tube. Pipet-mix 15 times, and briefly centrifuge the tube.
6. Run the mix in the thermal cycler:

Step	Cycles	Temperature	Time
Hot start	1	95°C	5 min
Denaturation	8-12*	98°C	15 sec
Annealing		54°C	30 sec
Extension		72°C	30 sec
Final extension	1	72°C	1 min
Hold	1	4°C	Hold

Stopping point: the PCR can run overnight.

- Cycle number depends on cell type and number and may require optimization.

  1,000 - 5,000 cells: 10-12 cycles

  5,000 - 10,000 cells: 8-10 cycles

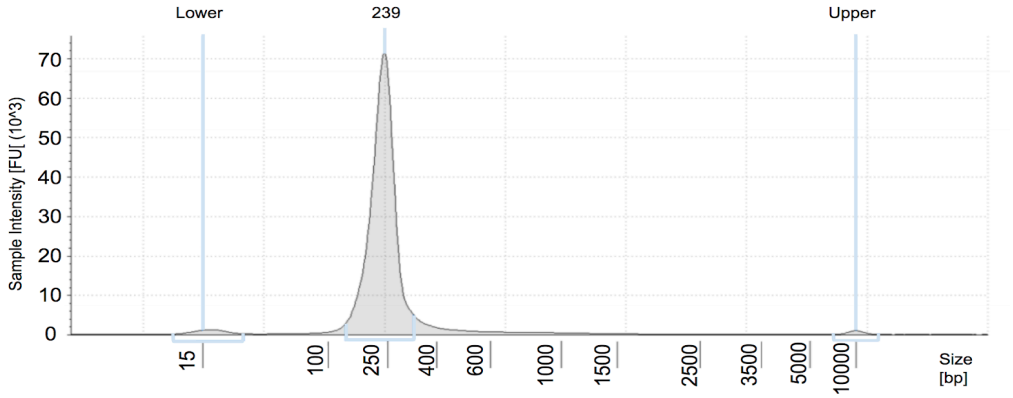
Library Cleanup

1. Vortex the SPRIselect Reagent at high speed until the beads are fully suspended.
2. Pipet 30 µL of SPRIselect Reagent magnetic beads (0.6X) to each sample in the 8-strip sample tube, and pipet-mix 15 times.
3. Incubate the suspensions at room temperature for 5 minutes, and then place the strip tube in a 10x Magnetic Separator until the solution is clear.
4. Without disturbing the beads and while leaving the strip tube in a magnetic separator, carefully pipet the supernatant, which has the AbSeq products, into a new 8-strip tube. Dispose of the old 8-strip tube containing the SPRIselect beads.
5. To the strip tube containing supernatant, pipet 20 µL (1.0X) of SPRIselect Reagent into each sample, and then pipet-mix 15 times.

6. Incubate the suspensions at room temperature for 5 minutes, and then place the strip tube for 2 minutes in a 10x Magnetic Separator until the solution is clear.
7. Carefully remove and discard only the supernatant without disturbing the beads and while leaving the tube in the magnetic separator.
8. Keeping the strip tube in the magnetic separator, gently pipet 200 µL of fresh 80% ethyl alcohol to the side of the strip tube opposite each pellet. Leave the tube open.
9. Incubate the samples for 30 seconds on the magnetic separator.
10. Carefully remove and discard only the supernatant without disturbing the beads and while leaving the tube in a magnetic separator.
11. Repeat steps 8-10 once for a total of 2 washes.
12. Keeping the strip tube on the magnetic separator, use a small-volume pipet to remove any residual supernatant from each tube.
13. Leave the strip tube open on the magnetic separator to dry the beads at room temperature for 2 minutes.
14. Remove the strip tube from the magnetic separator, and then pipet 32 µL of Buffer EB into each tube, pipet mix 15 times to completely resuspend the beads.
15. Briefly centrifuge the strip tube to collect the contents at the bottom, and then incubate the samples at room temperature for 2 minutes.
16. Place the strip tube on the magnetic separator for 30 seconds or until the solution is clear.
17. Transfer 30 µL of eluate into a new strip tube. These are the purified AbSeq libraries. Dispose of the 8-strip tube containing the SPRIselect beads.
18. AbSeq libraries can be stored at -4°C for <72 h or -20°C for long-term storage.
19. There are now two libraries ready to be quality checked and sequenced.
    a. mRNA library for each of the samples
    b. AbSeq library for each of the samples

Library QC and Sequencing

1. Estimate the concentration of each AbSeq library by quantifying 2 µL with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. The expected library concentration should be at least 2 ng/µL.
2. Run 1 µL of ~1 ng of each AbSeq library on an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit.
3. AbSeq libraries should show a sharp peak at ~226 bp. For example:



4. Pool libraries for next-generation sequencing, e.g. on an Illumina HiSeq4000.

Data Analysis

2,127 cells were sequenced, with a mean number of 34,175 reads per cell from the mRNA library (median genes/cell = 802), and 28,465 reads per cell from the AbSeq library (median Ab-Oligos/cell = 20). The cDNA and AbSeq libraries were processed separately through the standard Cell Ranger pipeline. FASTQ files for the cDNA library were processed using Cell Ranger, aligning R2 reads to the human reference genome GRCh38, and generating a gene-cell matrix. For the AbSeq library, R2 reads from the FASTQ files were trimmed to 60 bases and aligned to a custom reference containing the 20-plex antibody-specific barcodes (ABC) using

STAR. Next, AbSeq read counts were extracted from the BAM alignment file generated by Cell Ranger. AbSeq read counts were combined with the gene-cell matrix from the cDNA library. The resulting gene-cell matrix contained both mRNA and AbSeq expression for each filtered cell.

The gene-cell matrix was then imported into BD Data View for data visualization and analysis. A subset of highly variable genes was selected based on the z-score of the dispersion of the log-10 transformed gene expression data. Next, the selected variable genes together with AbSeq counts were log-10 and principle component analysis (PCA) transformed. The top 50 PCs were used as input for t-Distributed Stochastic Neighbor Embedding (tSNE) to visualize cells in 2D.

Using combined AbSeq and single-cell RNA-seq data, we observed that the addition of protein measurements to transcriptome data can improve clustering and the ability to discern heterogeneity. In the example shown in **Fig. 1**, lymphocytes were analyzed using AbSeq and scRNA-seq. tSNEs using combined AbSeq/scRNA-seq data (right panel) compared to scRNA-seq data alone (left panel) show distinct clustering of two $CD4^+$ T cell subsets.

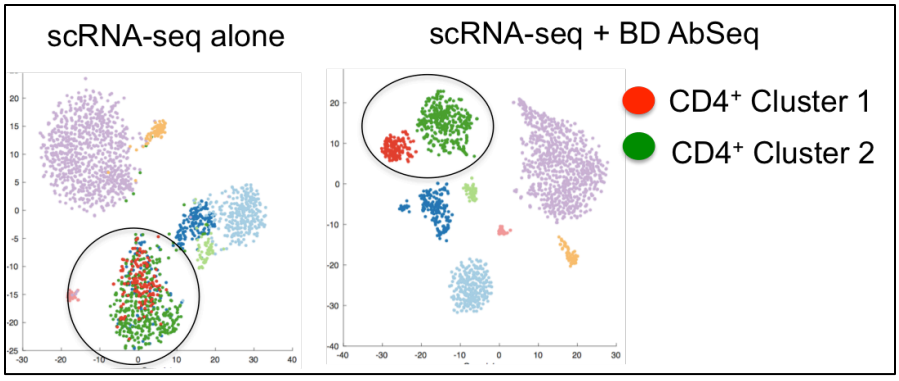


Fig. 1. tSNE plots incorporating scRNA-seq data alone (left) vs scRNA-seq and AbSeq protein data (right). Note superior ability to distinguish two $CD4^+$ T cell subsets (depicted in red and green) with combined protein and RNA data.

Appendix 1: List of AbSeq Antibody-Oligos used

CD127	CD27
CD14	CD28
CD161	CD3
CD183	CD38
CD185	CD4
CD19	CD45RA
CD194	CD45RO
CD196	CD8
CD197	CD95
CD25	HLA-DR