BD Rhapsody™ System

Single-Cell Labeling with BD® AbSeq Ab-Oligos for Intracellular CITE-seq

Protocol

23-24464(02)
2023-12

For Research Use Only

Regulatory information

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

History

Revision	Date	Change made
23-24464(01)	2023-10	Initial release.
23-24464(02)	2023-12	Added AbSeq Enhancer footnote to Table 3.

Contents

Introduction

This protocol details how to stain for intracellular antigens using BD® Intracellular AbSeq (IC-AbSeq) antibodies for profiling with the BD Rhapsody™ system. Each BD® IC-AbSeq antibody is conjugated to an antibody-specific oligonucleotide barcode for profiling alongside surface protein and mRNA expression. Note that this protocol should be used alongside *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-24262 or *BD Rhapsody™ System Single-Cell Labeling with the BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-21339 to support single-cell labeling with BD® AbSeq Ab-Oligos without and with sample multiplexing, respectively. This protocol has been tested for use with up to 40 surface markers. The following protocols are available for use with high-plex experiments (however, additional optimization may be required):

- *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol*, 23-22314
- *BD Rhapsody™ System Single-Cell Labeling with the BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos Protocol*, 23-22354

  See Procedure on page 14 for notes on how to use this protocol in conjunction.

Immediately following this protocol, users will proceed to a protocol for Single-Cell Capture and cDNA Synthesis using one of the protocols listed in Table 8. Protocols to Follow for Cell Capture and cDNA Synthesis Steps.

Note: The BD® IC-AbSeq protocol is not compatible with BD Rhapsody™ Targeted Assay or TCR BCR Assay Kits.

Required and recommended materials

BD Rhapsody™ Intracellular AbSeq Buffer Kit (Catalog no. 570742) contents

Material	Part number
Perm Buffer	51-9022686
Base Buffer	51-9022685
Proteinase K, Molecular Biology Grade	51-9022689
0.1M DTT, Molecular Biology Grade	51-9022688
Nuclease-free water	51-9022687

For best results, use required and recommended materials and equipment from the indicated supplier.

Required materials

Store the reagents at the storage temperature specified on the label.

Material	Step	Supplier	Catalog no.
20,000–1 million cells	–	–	–
BD Pharmingen™ Stain Buffer (FBS)	Surface staining or sample multiplexing	BD	554656
BD® Single-Cell Multiplexing Kit (if sample multiplexing)	Human sample multiplexing kit	BD	633781
BD® AbSeq Immune Discovery Panel Kit, (if staining for IDP) or custom Surface AbSeq panel	Surface AbSeq staining	BD	625970 (IDP) or various
BD® Intracellular AbSeq Panel	IC staining	BD	Various
BD® AbSeq Enhancer Kit Kit contains: BD® AbSeq Enhancer 1, PN: 51-9022717 BD® AbSeq Enhancer 2, PN: 51-9022718 BD® AbSeq Enhancer 3, PN: 51-9022719	IC staining	BD	570750
BD® RNase Inhibitor	Cell hydration, IC staining, IC washing, cell suspension, cell capture	BD	570751
BD® OMICS-Guard Sample Preservation Buffer Kit	Fixation	BD	570908
BD Rhapsody™ Enhanced Cartridge Reagent Kit	Cartridge loading	BD	664887
BD Rhapsody™ Cartridge Kit or BD Rhapsody™ 8-Lane Cartridge	Cartridge loading	BD	633733 or 666262
BD Rhapsody™ cDNA Kit	cDNA synthesis	BD	633773
Vybrant™ DyeCycle™ Green Stain	BD Rhapsody™ cartridge loading	Thermo Fisher Scientific	V35004
INCYTO disposable hemocytometer	BD Rhapsody™ cartridge loading	INCYTO	CN DHC-N01-5
100% Molecular Biology Grade Ethanol	BD Rhapsody™ cartridge loading	Major supplier	–
70% Ethanol or IPA	Sterilization	Major supplier	–

Where supplier is listed as "Major Supplier", products from any manufacturer or supplier are acceptable as long as they fit the material description.

Recommended materials

Supplies	Supplier	Catalog no.
Single Channel Pipettes (20, 200, 1000 µL)	Major supplier	–
Low-retention, filtered pipette tips for single channel pipettes	Major supplier	–
Serological Pipette (15 mL)	Major supplier	–
Cell Strainer Cap	Corning	352235
BD Human Fc Block™	BD	564220
Lint-Free Cloth (Kim-Wipes)	Major supplier	–
5 mL, 12×75 mm round-bottom polystyrene tubes.	Thermo Fisher Scientific	FB149563A
Eppendorf 5-mL DNA LoBind Tubes	Eppendorf	0030122348
Eppendorf 1.5-mL Protein and DNA LoBind Tubes	Eppendorf	022431021

Required equipment

Equipment	Supplier	Catalog no.
Temperature controlled centrifuge	Major supplier	–
37 °C incubator/water bath	Major supplier	–
Ice bucket	Major supplier	–
Sample vortexer	Major supplier	–
BD Rhapsody™ P1200M Pipette	BD	–
BD Rhapsody™ P5000M Pipette	BD	–
Thermoblock for 1.5-mL tubes, 37 °C, set to 1,200 rpm shaking	Major supplier	–
Thermoblock for 1.5-mL tubes, 80 °C	Major supplier	–
Cell counter	Major supplier	–
BD Rhapsody™ Scanner and/or BD Rhapsody™ Express System or BD Rhapsody™ HT Xpress Package	BD	633701 and/or 633707 or 666625
Digital timer	Major supplier	–

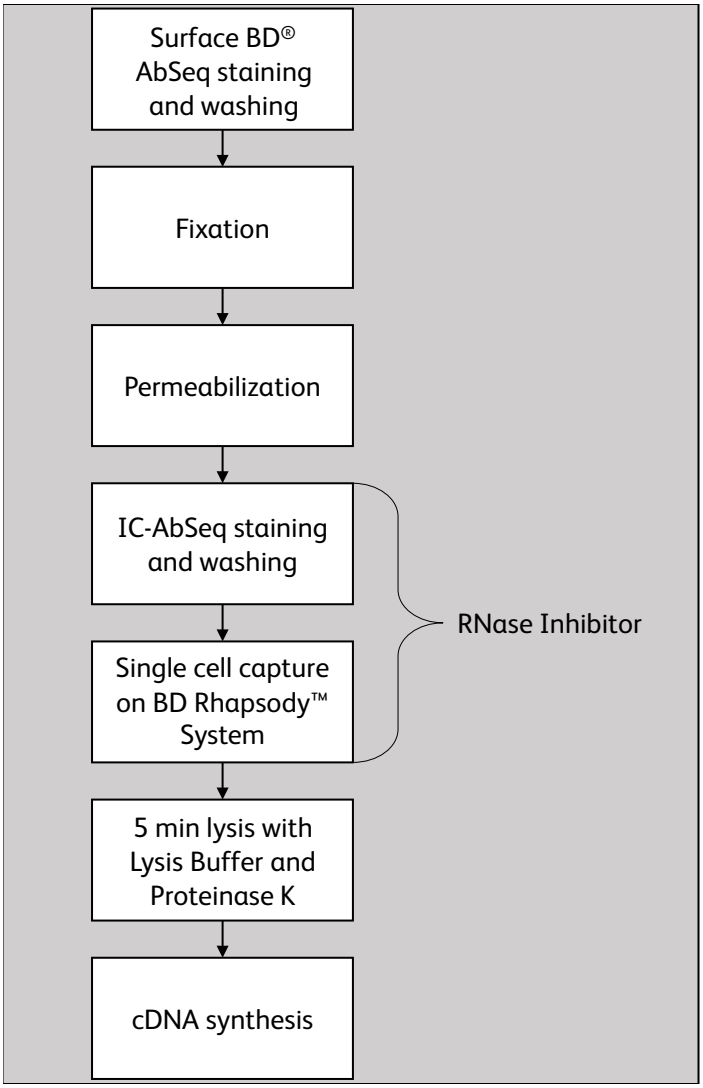
Best practices

- Use only the materials specified in this protocol. Use of other materials could impact results.
- Store all reagents at the recommended storage temperatures with tightly sealed caps when not in use.
- Use low retention filtered pipette tips.
- **Do not vortex** or **freeze** BD® AbSeq antibodies.
- Always use a swinging-bucket centrifuge for pelleting cells.
- It is important to keep cells and buffers on ice, especially after permeabilization.
- Keep centrifuge at 4 °C for all 800*g* centrifugation steps. Keep centrifuge lid closed between centrifugation steps to maintain temperature.
- For a complete list of materials for the BD Rhapsody™ System, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*, 23-21336, the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*, 23-21332, or the *BD Rhapsody™ HT Xpress System Instrument User Guide for Scanner-free Workflow*, 23-24256, or the *BD Rhapsody™ HT Single-Cell Analysis System Instrument User Guide*, 23-24257.
- The transient phosphorylation status of certain markers can result in dephosphorylation during surface AbSeq staining. If it is a concern, proceed immediately with 5 min fixation using BD® OMICS-Guard Sample Preservation Buffer and immediately permeabilize cells prior to surface staining. Once cells are permeabilized and treated with Cell Hydration Buffer, co-stain using surface and BD® Intracellular AbSeq (with BD Fc Block™ if required). For a list of compatible surface antibody clones, refer to *Antibodies to Human Cell-Surface Markers Tested for BD Phosflow™ Protocols*, 23-11080. Do not stop and store cells at the OMICS-Guard step. If you need further guidance, contact your local Field Application Specialist (FAS).

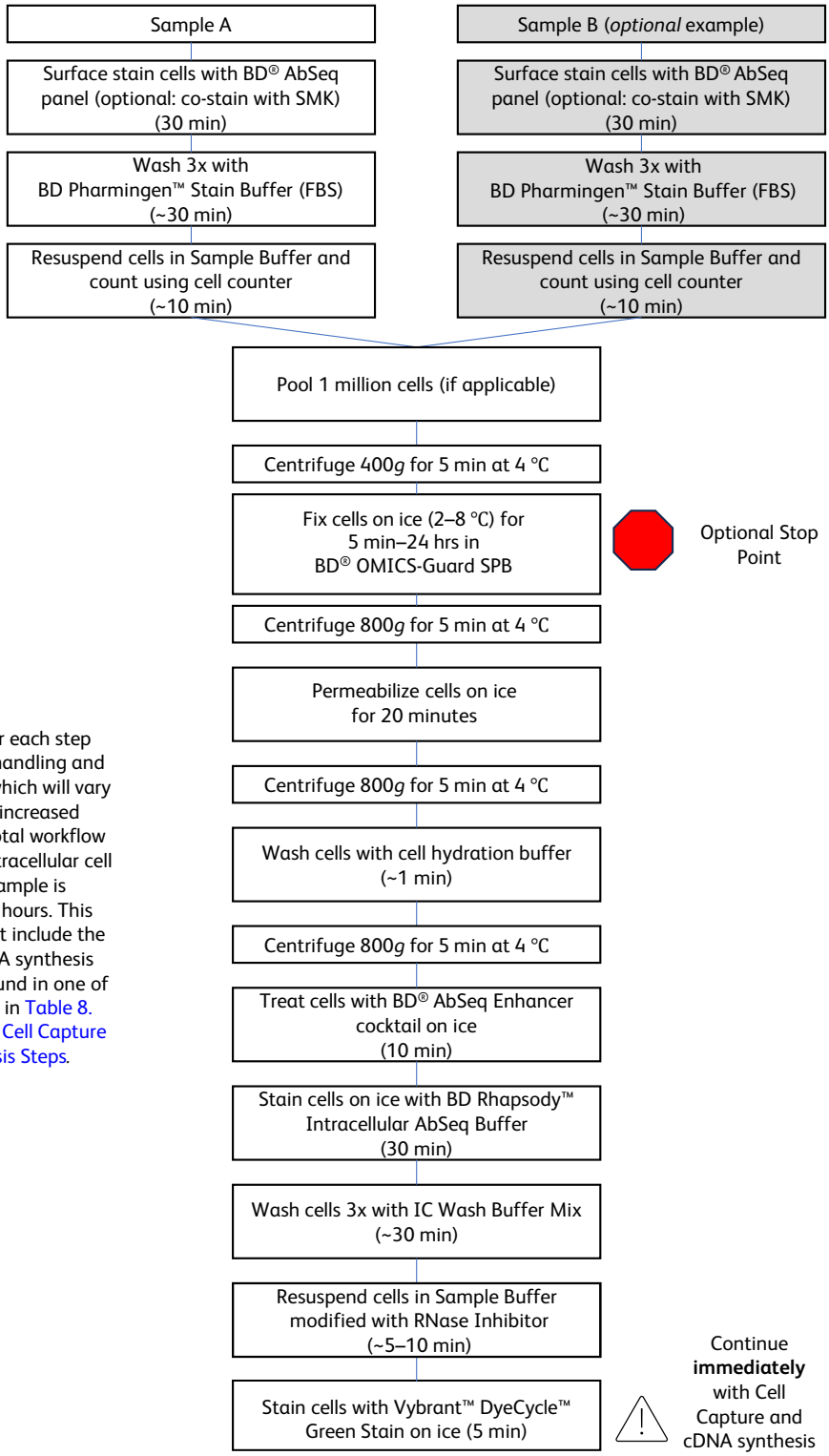
Additional documentation

- *BD Rhapsody™ System Single-Cell Labeling with the BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-21339 or *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol*, 23-22354.
- *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-24262 or *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (from 41 plex to 100 plex) Protocol*, 23-22314.
- *BD Rhapsody™ System Preparing Single-Cell Suspensions Protocol*, 23-24126.
- *BD Rhapsody™ Single Cell Capture and cDNA Synthesis with BD Rhapsody™ Single-Cell Analysis System Protocol*, 23-22951 or *BD Rhapsody™ Single-Cell Capture and cDNA Synthesis with BD Rhapsody™ Express Single-Cell Analysis System Protocol*, 23-22952 or *BD Rhapsody™ HT Single-Cell Analysis System Single-Cell Capture and cDNA Synthesis Protocol*, 23-24252 or *BD Rhapsody™ HT Xpress System Single-Cell Capture and cDNA Synthesis Protocol*, 23-24253.
- *BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA) and AbSeq Library Preparation Protocol*, 23-24118 or *BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA), AbSeq, and Sample Tag Library Preparation Protocol*, 23-24120.

Workflow overview



Time considerations



Time estimates in for each step include approximate handling and centrifugation times, which will vary between users and increased number of samples. Total workflow time for surface and intracellular cell staining for one sample is approximately 3.5–4 hours. This time estimate does not include the cell capture and cDNA synthesis steps, which can be found in one of the documents listed in Table 8. Protocols to Follow for Cell Capture and cDNA Synthesis Steps.

Before you begin

- Prepare a single cell suspension. See *BD Rhapsody™ System Preparing Single-Cell Suspensions Protocol*, 23-24126.
- If working with stimulated or cultured cells, it is recommended to strain cells through a 35 µm Cell Strainer Cap (Corning Catalog no. 352235) before beginning BD® AbSeq staining.
- For detailed information on surface labeling with BD® AbSeq Ab-Oligos, or co-labeling cells with Sample Tags and BD® AbSeq Ab-Oligos in a single tube, see *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-24262 or *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol*, 23-22314, or *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-21339 or *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol*, 23-22354, respectively.
- The BD® Single-Cell Multiplexing Kit can be co-stained with either surface AbSeq or IC-AbSeq. Plan to include it according to the experimental design.
- Create modified sample buffer by adding RNase inhibitor (25 µL/mL) to the sample buffer from the BD Rhapsody™ Enhanced Cartridge Reagent Kit (Catalog no. 664887). Users need 4 mL of modified sample buffer per single-lane cartridge or 3 mL of modified sample buffer per lane for 8-lane cartridge. See either Table 9. Modified Sample Buffer with RNase Inhibitor for Single-Lane Cartridge or Table 11. Modified Sample Buffer with RNase Inhibitor for HT Xpress Cartridge for the correct volume of modified sample buffer to prepare.

  Note: The concentration for BD® RNase Inhibitor is optimized for use with human peripheral blood mononuclear cells (PBMCs). If other cell types are used, we recommend that users test for optimal concentration. Use only BD® or New England Biolabs RNase Inhibitor (Catalog no. M0314L). Use of other RNase inhibitors will impact experiment.

Buffer mix tables for intracellular staining

All buffer mix tables make enough buffer for one intracellular staining test. Thaw reagents (except for BD® RNase Inhibitor and Proteinase K) at room temperature (15–25 °C) and place back on ice. Keep BD® RNase Inhibitor and Proteinase K at –25 °C to –15 °C. All components should be properly capped or sealed, and stored when not in use.

Prepare the buffer mixes on the same day you perform the intracellular staining and single-cell capture. We recommend adding RNase inhibitor just before use.

1. In a new 15-mL nuclease-free conical tube, pipet the reagents in Table 1. IC Wash Buffer Mix, and place back on ice.

Table 1. IC Wash Buffer Mix

IC Wash Buffer Component	Part number/Catalog no.	Amount (µL)
Base Buffer	51-9022685	3,000 (3 mL)
BD® RNase Inhibitor	570751	70
Nuclease-free water	51-9022687	4,000 (4 mL)
Total	–	**7,070 (7.070 mL)**

2. In a new 1.5-mL LoBind tube, pipet the reagents in Table 2. Cell Hydration Buffer Mix, and place back on ice.

Table 2. Cell Hydration Buffer Mix

Cell Hydration Buffer Component	Part number/Catalog no.	Amount (µL)
Base Buffer	51-9022685	465
BD® RNase Inhibitor	570751	30
Nuclease-free water	51-9022687	635
Total	–	**1,130**

3. In a new 1.5-mL LoBind tube, pipet the reagents in Table 3. BD® IC-AbSeq Stain Buffer Mix, and place back on ice.

Table 3. BD® IC-AbSeq Stain Buffer Mix

BD® IC-AbSeq Stain Buffer Component	Part number/Catalog no.	Volume (µL)
Base Buffer	51-9022685	48
BD® AbSeq Enhancer 1	51-9022717	12[a]
BD® AbSeq Enhancer 2	51-9022718	12[a]
BD® AbSeq Enhancer 3	51-9022719	12[a]
BD® RNase Inhibitor	570751	3.8
0.1M DTT	51-9022688	1.2
Nuclease-free water	51-9022687	31[a]
Total	–	**120[a]**

a. Certain cell type and target combinations may require the use of increased BD® AbSeq Enhancer concentrations to achieve better IC AbSeq signal to noise. We recommend optimizing for your sample type.

When increasing the BD® AbSeq Enhancer volume, decrease the amount of water. For example, if increasing the concentration of Enhancer by 1.5X, add 18 µL of each Enhancer and 13 µL of nuclease-free water. If increasing the concentration by 2X, add 24 µL of each Enhancer and 0 µL of nuclease-free water. The final volume may increase up to 125 µL.

Safety information

The Perm Buffer contains 87.68% Methanol (CAS number 67-56-1). This reagent is classified as hazardous according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) and Regulation (EC) No 1272/2008. Go to regdocs.bd.com/regdocs/sdsSearch to download the Safety Data Sheet.

	Danger
	H225: Highly flammable liquid and vapor. H301+H311+H331: Toxic if swallowed, in contact with skin or if inhaled. H370: Causes damage to organs.
Prevention	P210: Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. P233: Keep container tightly closed. P240: Ground and bond container and receiving equipment. P241: Use explosion-proof [electrical/ventilating/lighting] equipment. P242: Use non-sparking tools. P243: Take action to prevent static discharges. P260: Do not breathe dust/fume/gas/mist/vapors/spray. P264: Wash thoroughly after handling. P270: Do not eat, drink or smoke when using this product. P271: Use only outdoors or in a well-ventilated area. P280: Wear protective gloves/protective clothing/eye protection/face protection.
Response	P301+P310: IF SWALLOWED: Immediately call a POISON CENTER/doctor. P330: Rinse mouth. P303+P361+P353: IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water [or shower]. P363: Wash contaminated clothing before reuse. P312: Call a POISON CENTER or doctor/physician if you feel unwell. P304+P340: IF INHALED: Remove person to fresh air and keep comfortable for breathing. P311: Call a POISON CENTER or doctor/physician. P307+P311: IF exposed or concerned: Call a POISON CENTER/doctor. P370+P378: In case of fire: Use dry sand, dry chemical or alcohol resistant foam for extinction.
Storage	P403+P233: Store in a well-ventilated place. Keep container tightly closed. P405: Store locked up.
Disposal	P501: Dispose of contents/container to an approved facility in accordance with local, regional, national and international regulations.

For additional safety information, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*, 23-21336 or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*, 23-21332.

Procedure

Note: Do not prepare buffers from Buffer Mix tables (Table 1. IC Wash Buffer Mix, Table 2. Cell Hydration Buffer Mix, and Table 3. BD® IC-AbSeq Stain Buffer Mix) until the day cartridge loading will be performed.

Perform the experiment using either of the following BD Rhapsody™ System Single-Cell Labeling protocols:

- *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-24262 or *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol*, 23-22314

  STOP after the section "Washing labeled cells" in step 7 and follow this protocol from Fixation and permeabilization of the cells on page 14 and subsequent steps.

or,

- *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol*, 23-21339 or *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos Protocol*, 23-22354

  STOP after the section "Washing the labeled cells" on step 7 and follow this protocol from Fixation and permeabilization of the cells on page 14 and subsequent steps. It is also possible to co-stain Human SMK with the IC Stain Mix (see instructions following Labeling cells with BD® IC-AbSeq Ab-Oligos on page 15 and the example in Table 7. Example of 10-plex BD® IC-AbSeq + Human SMK Labeling Master Mix).

Ensure that the intended total cell loading is between 20,000 to 40,000 single cells per cartridge lane for this protocol. Cell loading below or above this recommended range may not be suitable for current protocol configuration. Then proceed as described in the following procedure.

Fixation and permeabilization of the cells

Surface-stained cells should be washed three times with BD stain buffer with residual supernatant carefully removed. If cells have been sample tagged using the BD® Human Single-Cell Multiplexing Kit (SMK), we recommend resuspending in Sample Buffer (without BD® RNase Inhibitor) and pooling cells at this point, aiming for 250,000–1 million cells. The cell pool should be centrifuged again at 400*g* for 5 minutes and supernatant discarded. It is also possible to obtain cell counts and pool the cells while in BD® OMICS-Guard Sample Preservation Buffer and pool sample tagged cells before step 4.

The cell pellet is then ready for fixation, permeabilization, and intracellular staining, as described in the following steps:

1 Gently tap the bottom of the tube on the work bench to break the cell pellet, and add 1 mL of BD® OMICS-Guard Sample Preservation Buffer to the cell pellet. Gently pipette mix the samples 10 times to avoid bubble formation.

2 If cells are not already in a 5-mL 12×75 mm round-bottom polystyrene tube, transfer them at this point. Avoid using DNA LoBind tubes for fixation and permeabilization steps.

3 Incubate the cells in BD® OMICS-Guard Sample Preservation Buffer on ice for 5 minutes.

  Optional Stop Point: It is possible to store cells in the BD® OMICS-Guard Sample Preservation Buffer for up to 24 hours and resume step 4 the following day.

Note: BD® OMICS-Guard Sample Preservation Buffer has been tested for 24-hour storage using Human PBMCs. If using cells other than PBMCs, users are advised to test their sample type before storing cells longer than 40 minutes.

4 Using a swinging-bucket centrifuge, pellet cells at 800*g* for 5 minutes at 4 °C. Carefully decant supernatant into biohazardous waste by inverting tube. Keep the tube inverted and gently blot on a lint-free wipe without disturbing the cell pellet.

5 Gently tap the tube on the work bench at least 10 times to break the cell pellet.

6 Change vortex to slowest speed and continual vortexing mode (do not use touch mode).

IMPORTANT: Wear appropriate personal protective equipment, including eye protection, laboratory coat, and protective gloves to avoid methanol exposure.

7 While slowly vortexing the cells, add drops equaling 1 mL of ice-cold Perm Buffer.

Note: Vortexing is critical to prevent cell multiplets.

8 Permeabilize the cells on ice for 20 minutes.

Note: During this incubation, we recommend preparing the BD® IC-AbSeq Labeling Master Mix following Table 4. BD® IC-AbSeq Labeling Master Mix, Table 6. Example of 10-plex BD® IC-AbSeq Labeling Master Mix, or Table 5. BD® IC-AbSeq Labeling Master Mix + Human SMK Labeling Master Mix, Table 7. Example of 10-plex BD® IC-AbSeq + Human SMK Labeling Master Mix, in the section Labeling cells with BD® IC-AbSeq Ab-Oligos on page 15.

9 Using a swinging-bucket centrifuge, pellet the cells at 800*g* for 5 minutes at 4 °C.

10 Carefully remove and discard as much Perm Buffer as possible with a P1000 pipette without disturbing the cell pellet.

11 Add BD® RNase Inhibitor to Cell Hydration Buffer Mix, refer to Table 2. Cell Hydration Buffer Mix in section Buffer mix tables for intracellular staining on page 11. Gently vortex mix, briefly centrifuge, and place back on ice.

12 Gently tap the tube to break the cell pellet and add 1 mL of Cell Hydration Buffer Mix, then gently pipette mix
5–10 times and avoid bubble formation.

13 Centrifuge at 800*g* for 5 minutes at 4 °C. Continue this protocol from Labeling cells with BD® IC-AbSeq Ab-Oligos on page 15.

Labeling cells with BD® IC-AbSeq Ab-Oligos

Note: Before pooling intracellular antibodies for staining, we recommend centrifuging them at 400*g* for 30 seconds prior to uncapping and place on ice.

14 Add BD® RNase Inhibitor to BD® IC-Abseq Stain Buffer Mix, refer to Table 3. BD® IC-AbSeq Stain Buffer Mix in section Buffer mix tables for intracellular staining on page 11. Gently vortex mix, briefly centrifuge, and place back on ice.

15 Prepare BD® IC-AbSeq Labeling Master Mix, by pipetting the following reagents into a new 1.5-mL DNA LoBind tube. All components should be properly capped/sealed and stored when not in use.

Note: It is important to retain the remaining 50 µL of the BD® IC-AbSeq Stain Buffer for the blocking step.

Table 4. BD® IC-AbSeq Labeling Master Mix

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
Per BD® IC-AbSeq Ab-Oligo	2.0	2.6	5.2
BD® IC-AbSeq Stain Buffer (N = number of antibodies)	50.0 – (2.0 × N)	65.0 – (2.6 × N)	130.0 – (5.2 × N)
Total	**50.0**	**65.0**	**130.0**

Table 5. BD® IC-AbSeq Labeling Master Mix + Human SMK Labeling Master Mix

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
Per BD® IC-AbSeq Ab-Oligo	2.0	2.6	5.2
BD® IC-AbSeq Stain Buffer (N = number of antibodies)	30.0 – (2.0 × N)	45.0 – (2.6 × N)	90.0 – (5.2 × N)
Total	**30.0**	**45.0**	**90.0**

Table 6. Example of 10-plex BD® IC-AbSeq Labeling Master Mix

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
Per BD® IC-AbSeq Ab-Oligo	2.0 (20.0 total)	2.6 (26.0 total)	5.2 (52.0 total)
BD® IC-AbSeq Stain Buffer	30.0	39.0	78.0
Total	**50.0**	**65.0**	**130.0**

Table 7. Example of 10-plex BD® IC-AbSeq + Human SMK Labeling Master Mix

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% Overage (µL)
Per BD® IC-AbSeq Ab-Oligo	2.0 (20.0 total)	2.6 (26.0 total)	5.2 (52.0 total)
BD® IC-AbSeq Stain Buffer	10.0	19.0	38.0
Total	**30.0**	**45.0**	**90.0**

To each Sample Tag tube containing 20 µL of Sample Tag, add 30 µL BD® IC-AbSeq Labeling Master Mix.

Note: We recommend:

– Creating freshly pooled antibodies before each experiment.

– Creating pools with 30% overage to ensure adequate volumes for pipetting. The reagents are viscous and form bubbles easily. Avoid creating bubbles by pipetting slowly.

– For high-plex panels, using an 8-Channel Screw Cap Tube Capper and multi-channel pipette to pipette BD® IC-AbSeq Ab-Oligos into 8-tube strips. Centrifuge tube strip and pool BD® IC-AbSeq Ab-Oligos into a 1.5-mL DNA LoBind tube.

16 Pipet the BD® IC-AbSeq Labeling Master Mix and place on ice.

17 Carefully decant supernatant into biohazardous waste by inverting tube from step 13. Keep the tube inverted and blot on a lint-free wipe without disturbing the cell pellet.

18 Gently tap the tube on the work bench at least 10 times to break the cell pellet. Resuspend cells with the 50 µL of retained BD® IC-AbSeq Stain Buffer with BD® AbSeq Enhancer and incubate on ice for 10 minutes. This incubation step is critical to allow BD® AbSeq Enhancer into cells.

19 Add 50 µL of BD® IC-AbSeq Labeling Master Mix to the tube containing 50 µL of cell suspension plus BD® IC-AbSeq Stain Buffer with BD® AbSeq Enhancer, to make a final volume of 100 µL cell staining mix. Gently pipette mix to avoid bubble formation and incubate on ice for 30 minutes. It is critical to not extend the incubation more than 30 minutes to limit RNase activity.

Note: During this incubation, we recommend priming and treating BD Rhapsody™ Cartridge(s).

Note: See Table 8. Protocols to Follow for Cell Capture and cDNA Synthesis Steps following to find the correct protocol to follow for the BD Rhapsody™ System cartridge priming and treating steps.

Washing labeled cells

20 Add BD® RNase Inhibitor to IC Wash Buffer Mix, refer to Table 1. IC Wash Buffer Mix in section Buffer mix tables for intracellular staining on page 11. Vortex mix and place back on ice.

21 Add 2 mL of IC Wash Buffer to the labeled cells and pipet mix.

22 Using a swinging-bucket centrifuge, pellet cells at 800*g* for 5 minutes at 4 °C.

23 Invert to decant supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wipe to remove residual supernatant from the tube.

24 Add 2 mL of IC Wash Buffer to cell pellets and resuspend by pipet mixing for the second wash.

25 Centrifuge with a swinging-bucket centrifuge at 800*g* for 5 minutes at 4 °C.

26 Invert to decant supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wipe to remove residual supernatant from the tube.

27 (Optional) Repeat steps 24–26 for a total of 3 washes.

Single-cell capture and cDNA synthesis

Have you added RNase inhibitor to your sample buffer? (25 µL RNase Inhibitor/mL Sample Buffer. See Table 9. Modified Sample Buffer with RNase Inhibitor for Single-Lane Cartridge or Table 11. Modified Sample Buffer with RNase Inhibitor for HT Xpress Cartridge for details on how to prepare the Modified Sample Buffer.)

28 Resuspend the pellet in 620 µL of **cold modified sample buffer**.

- **a** For low-cell concentrations (below 500,000 cells starting amount), we recommend to resuspend cells in 310 µL cold modified sample buffer and halve the volume of Vybrant™ DyeCycle™ Green Stain to 1.6 µL in the next step. If unsure about concentration, use 310 µL.

29 Add 3.1 µL of Vybrant™ DyeCycle™ Green Stain to the cells (1.6 µL for 310 µL cell suspension) and gently pipet mix 8–10 times. Incubate on ice for 5 minutes while protecting from light.

30 Filter cells through a Falcon tube with Cell Strainer Cap.

31 Count cells immediately using the BD Rhapsody™ Scanner by gently pipetting 10 µL of cells into an INCYTO disposable hemocytometer.

32 Keep remaining cells on ice until single-cell capture steps have completed.

Note: Since these are fixed cells, cell viability is not applicable.

- For use with the BD Rhapsody™ Express single-lane cartridge workflow, proceed to the section Single-lane cartridge protocol on page 19.
- For use with BD Rhapsody™ HT Xpress 8-lane cartridge workflow, skip to the section HT Xpress protocol on page 20.
- See Table 8. Protocols to Follow for Cell Capture and cDNA Synthesis Steps for information on the necessary protocol for cell capture and cDNA synthesis.

Table 8. Protocols to Follow for Cell Capture and cDNA Synthesis Steps

Cartridge Type	Protocol	Step
BD Rhapsody™ Single-Lane Cartridge	With BD Rhapsody™ Scanner: *BD Rhapsody™ System Single-Cell Capture and cDNA Synthesis with BD Rhapsody™ Single-Cell Analysis System Protocol*, 23-22951 or Without BD Rhapsody™ Scanner: *BD Rhapsody™ System Single-Cell Capture and cDNA Synthesis with BD Rhapsody™ Express Single-Cell Analysis System Protocol*, 23-22952	Priming and loading BD Rhapsody™ Single-Lane Cartridge
BD Rhapsody™ 8-Lane Cartridge	With BD Rhapsody™ Scanner: *BD Rhapsody™ HT Single-Cell Analysis System Single-Cell Capture and cDNA Synthesis Protocol*, 23-24252 or Without BD Rhapsody™ Scanner: *BD Rhapsody™ HT Xpress System Single-Cell Capture and cDNA Synthesis Protocol*, 23-24253	Priming and loading BD Rhapsody™ 8-Lane Cartridge

Single-lane cartridge protocol

For the following section, proceed using the appropriate Single-Cell Capture and cDNA synthesis protocol listed in Table 8. Protocols to Follow for Cell Capture and cDNA Synthesis Steps to find the correct protocol. However, be sure to include the following modifications outlined in steps 33-36 of this section.

Note: Once loaded with cells, the cartridge should be kept on ice for the duration of the cell and bead loading incubation steps.

33 Replace sample buffer with the cold Modified Sample Buffer by adding RNase inhibitor (25 µL/mL). This is critical for RNA yield. See Table 9. Modified Sample Buffer with RNase Inhibitor for Single-Lane Cartridge. Use the Modified Sample Buffer for Single Cell Capture protocol which requires sample buffer, including preparing cell suspension, preparing cell capture beads, and washing cell capture beads.

Table 9. Modified Sample Buffer with RNase Inhibitor for Single-Lane Cartridge

Cartridge Type	Vol. of Cold Sample Buffer (µL)	Vol. of RNase Inhibitor (µL)	Step
BD Rhapsody™ Single-Lane Cartridge	4,000	100	Preparing cell-suspension, Preparing cell capture beads, Washing cell capture Beads

34 For the cell and bead loading steps, **place entire cartridge on ice for the duration of the cell loading incubation (~15 minutes). Likewise, keep cartridge on ice during the bead capture incubation (~3 minutes)**. Try to keep cartridges flat and wipe the top and bottom of the cartridge with a lint-free wipe before performing the scanner steps.

35 Use Lysis Buffer with 1 M DTT (5 mM final DTT concentration) and Proteinase K for the cell lysis step.

- **a** Right before lysis step, add Proteinase K (40 units/mL, add 50 µL/mL) to Lysis Buffer with 1 M DTT. See Table 10. Modified Lysis Buffer with Proteinase K for Single-Lane Cartridge. Keep on ice.
- **b** Lyse the cells 5 minutes (instead of 2 minutes) at room temperature (15–25 °C).
- **c** Use Lysis Buffer with 1 M DTT (no Proteinase K) for the retrieval step.

Table 10. Modified Lysis Buffer with Proteinase K for Single-Lane Cartridge

Cartridge Type	Vol. of Lysis Buffer with DTT (µL)	Vol. of Proteinase K (µL)	Step
BD Rhapsody™ Single-Lane Cartridge	1,000	50	Lysis

36 Use *BD Rhapsody™ System Single-Cell Capture and cDNA Synthesis with BD Rhapsody™ Single-Cell Analysis System Protocol*, 23-22951 for instructions on cDNA synthesis. After cDNA synthesis, exonuclease-treated beads can be stored at 2–8 °C for up to 3 months before proceeding to library preparation using the BD Rhapsody™ WTA Amplification Kit (Catalog no. 633801).

Note: This *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos for intracellular CITE-seq Protocol* is **not compatible** with BD Rhapsody™ System Targeted Assay or BD Rhapsody™ System TCR BCR Assay Kits.

- For sequencing guidance, see *BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA) and AbSeq Library Preparation Protocol*, 23-24118 or *BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA), AbSeq, and Sample Tag Library Preparation Protocol*, 23-24120. Use the latest whole transcriptome sequencing pipeline for analysis. See the *BD® Single-Cell Multiomics Bioinformatics Handbook*, 23-21713 or your local Field Applications Specialist (FAS) for more information.

HT Xpress protocol

For the following section, proceed using the appropriate Single-Cell Capture and cDNA synthesis protocol listed in Table 8. Protocols to Follow for Cell Capture and cDNA Synthesis Steps to find the correct protocol. However, be sure to include the following modifications outlined in steps 37–40 of this section.

Note: Once loaded with cells, the cartridge should be kept on ice during the duration of the cell loading and bead loading incubation steps.

37 Replace sample buffer with cold Modified Sample Buffer by adding RNase inhibitor (25 µL/mL). This is critical for RNA yield. See Table 11. Modified Sample Buffer with RNase Inhibitor for HT Xpress Cartridge.

- **a** Use 96-deep well plate to dispense the Modified Sample Buffer for the wash steps.
- **b** Keep 96-deep-well plate with modified Sample Buffer and cells on ice while working.
- **c** Use cold Modified Sample Buffer for all HT Xpress protocol steps which require sample buffer including: preparing cell suspension, preparing cell capture beads, and washing cell capture beads (see Table 11. Modified Sample Buffer with RNase Inhibitor for HT Xpress Cartridge).

38 For the cell loading step, **do not** transfer the cartridge to the BD Rhapsody™ Scanner after 7 minutes. Instead, **place entire cartridge on ice during the entire cell loading incubation (~15 minutes) before transferring to the scanner. Begin cell load scan immediately upon transfer. Likewise, keep cartridge on ice during the bead capture incubation (~3 minutes)**. Try to keep cartridges flat and wipe the top and bottom of the cartridge with a lint-free wipe before performing the scanner steps.

Table 11. Modified Sample Buffer with RNase Inhibitor for HT Xpress Cartridge

Cartridge Type	Vol. of Cold Sample Buffer (µL)	Vol. of RNase Inhibitor (µL)	Step
BD Rhapsody™ 8-Lane Cartridge (1 lane)	2,400	60	Preparing cell-suspension, Preparing cell capture beads, Washing cell capture beads
BD Rhapsody™ 8-Lane Cartridge (8 lanes)	19,200	480	Preparing cell-suspension, Preparing cell capture beads, Washing cell capture beads

39 Use Lysis Buffer with 1 M DTT (5 mM final DTT concentration) and Proteinase K for the lysis step.

- **a** Right before lysis step, add Proteinase K (40 units/mL, add 50 µL/mL) to Lysis Buffer with 1 M DTT. See Table 12. Modified Lysis Buffer with Proteinase K for HT Xpress Cartridge. Keep on ice.
- **b** Lyse the cells for 4 minutes at room temperature (15–25 °C).
- **c** Place the retrieval magnet down for 1 minute before retrieval.
- **d** During retrieval: Use Lysis Buffer with 1 M DTT (no Proteinase K).

Table 12. Modified Lysis Buffer with Proteinase K for HT Xpress Cartridge

Cartridge Type	Vol. of Lysis Buffer with DTT (µL)	Vol. of Proteinase K (µL)	Step
BD Rhapsody™ 8-Lane Cartridge (1 lane)	250	13	Lysis
BD Rhapsody™ 8-Lane Cartridge (8 lanes)	2,000	100	Lysis

40 Use *BD Rhapsody™ HT Single-Cell Analysis System Single-Cell Capture and cDNA Synthesis Protocol*, 23-24252 for instructions on cDNA synthesis. After cDNA synthesis, exonuclease-treated beads can be stored at 2–8 °C for up to 3 months before proceeding to library preparation using the BD Rhapsody™ WTA Amplification Kit (Catalog no. 633801).

Note:

- This *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos for intracellular CITE-seq Protocol* is **not compatible** with BD Rhapsody™ System Targeted Assay or BD Rhapsody™ System TCR BCR Assay Kits.
- For sequencing guidance, see *BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA) and AbSeq Library Preparation Protocol*, 23-24118 or *BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA), AbSeq, and Sample Tag Library Preparation Protocol*, 23-24120. Use the latest whole transcriptome sequencing pipeline for analysis. See the *BD® Single-Cell Multiomics Bioinformatics Handbook*, 23-21713 or your local Field Applications Specialist (FAS) for more information.

Becton, Dickinson and Company
BD Biosciences
155 North McCarthy Boulevard
Milpitas, California 95035 USA

bdbiosciences.com
scomix@bdscomix.bd.com