BD Rhapsody™ System

Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos

(41 plex to 100 plex)

For Research Use Only

23-22354-00
5/2020

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Regulatory Information

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

History

Revision	Date	Change made
23-22354-00	5/2020	Initial release

Contents

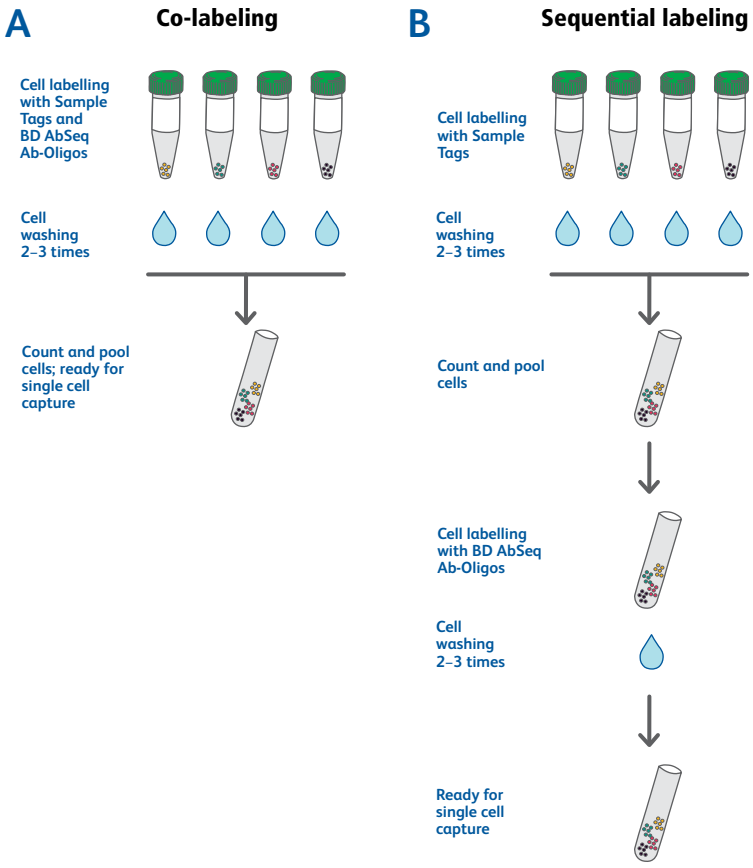
Introduction

This protocol describes the use of BD AbSeq Ab-Oligos (antibody-oligonucleotides) with the BD® Human Single-Cell Multiplexing Kit (Cat. No. 633781).

The BD AbSeq Ab-Oligos are used for antigen-expression profiling with BD Rhapsody™ single cell capture and downstream library preparation. Each BD AbSeq Ab-Oligo is an oligonucleotide-conjugated antibody that contains an antibody-specific barcode and poly(A)-tail for bead capture, PCR amplification, and library generation. The protocol supports BD® AbSeq labeling of 20,000 to 1 million cells. Up to 100 antibodies can be pooled together per staining reaction.

The BD Single-Cell Multiplexing Kit utilizes an innovative antibody-oligo technology to provide higher sample throughput for single cell library preparation. Every antibody-oligo in the kit, referred to as a Sample Tag, has a unique sample barcode conjugated to a human universal antibody. Up to 12 samples can be labeled and pooled prior to single cell capture with the BD Rhapsody™ Single-Cell Analysis system or other single cell analysis systems.

You can co-label cells with Sample Tags and BD AbSeq Ab-Oligos in a single tube (A), or you can sequentially label cells with Sample Tags and pool cells before labeling with BD AbSeq Ab-Oligos (B):



Sequential labeling is more economical than co-labeling, but you will save time by co-labeling. The biological effects of co-labeling versus sequential labeling might be different. These effects might depend on cell type and experimental condition. Consider potential effects in your experimental design.

Required materials

For a complete list of materials, see the appropriate BD Rhapsody instrument user guide.

NOTE Use only the tubes specified in the protocol. Use of other tubes could lead to sub-optimal results.

- 20,000–1 million cells
- BD® Stain Buffer (FBS) [Cat. No. 554656]
- BD AbSeq Ab-Oligos (various Cat. Nos.)
- BD® Human Single-Cell Multiplexing Kit (Cat. No. 633781)

  NOTE Never freeze BD AbSeq Ab-Oligos or Sample Tags.
- BD Rhapsody™ Targeted mRNA and AbSeq Reagent Kit (Cat. No. 633771)
- Latch Rack for 500-µL tubes (Thermo Fisher Scientific, Cat. Nos. 4900 or 4890)
- Falcon® Tubes, 5-mL round-bottom, polystyrene test tube (Corning, Cat. No. 352054)
- DNA LoBind Tubes, 1.5-mL (Eppendorf, Cat. No. 0030108051)

Suggested materials

- Human BD Fc Block™ (Cat. No. 564220)
- 8-Channel Screw Cap Tube Capper (Thermo Fisher Scientific, Cat. No. 4105MAT)
- Multi-channel pipette

Before you begin

- Use low-retention filtered pipette tips.
- Prime and treat the BD Rhapsody™ Cartridge. See the appropriate BD Rhapsody instrument user guide.
- Prepare a single-cell suspension. See *Preparing Single-Cell Suspensions Protocol* (Doc ID: 210964).
- If your biological sample contains red blood cell contamination, red blood cell lysis is required. See *Preparing Single-Cell Suspensions Protocol* (Doc ID: 210964).

Safety information

For safety information, see the *BD Rhapsody™ Single-Cell Analysis Instrument User Guide* (Doc ID: 214062) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID: 214063).

Preparing BD AbSeq labeling MasterMix

We recommend:

- Creating freshly pooled antibodies before each experiment.
- Creating pools with 30% overage to ensure adequate volumes for labeling. The reagents are viscous and can form bubbles easily.
- For high-plex, using an 8-Channel Screw Cap Tube Capper and multi-channel pipette to pipet BD AbSeq Ab-Oligos into 8-tube strips. Centrifuge the tube strip and pool BD AbSeq Ab-Oligos into a 1.5-mL LoBind tube.

1 Place all tubes of BD AbSeq Ab-Oligos to be pooled into a Latch Rack for 500-µL tubes (Thermo Fisher Scientific, Cat. No. 4890). Arrange the tubes so that they can be easily uncapped and re-capped with an 8-Channel Screw Cap Tube Capper (Thermo Fisher Scientific, Cat. No. 4105MAT) and aliquoted with a multi-channel pipette.

2 Centrifuge the BD AbSeq Ab-Oligo tubes in the Latch Rack in a tabletop centrifuge with a plate adapter at 400 × *g* for 30 seconds and place on ice.

3 Follow one of two workflows to label cells with Sample Tags and BD AbSeq Ab-Oligos:

- Co-labeling single-cell samples with Sample Tags and BD AbSeq Ab-Oligos on page 9. Ensures each sample is labeled independently with Sample Tags and Ab-Oligos.
- Sequential labeling of single-cell samples, first with Sample Tags, then with BD AbSeq Ab-Oligos on page 10. After labeling with Sample Tags, samples are pooled and stained together with AbSeq Ab-Oligos. This method provides AbSeq reagent cost savings.

4 In the pre-amplification workspace, pipet the reagents into a new 1.5-mL LoBind tube on ice.

BD AbSeq labeling MasterMix

For 87 plex and below, use the following volumes:

a Without optional Fc Block – 200 µL AbSeq labeling MasterMix

b With optional Fc Block – 175 µL AbSeq labeling MasterMix (25 µL Fc Block MasterMix will be prepared and added separately [see Fc Block MasterMix on page 9 for information on making Fc Block MasterMix])

For 88 plex and above, use the following volumes:

a Without optional Fc Block – 200 µL AbSeq labeling MasterMix

b With optional Fc Block – 200 µL AbSeq labeling MasterMix (25 µL Fc Block MasterMix will be prepared and added separately [see Fc Block MasterMix on page 9 for information on making Fc Block MasterMix])

NOTE When using Fc Block with >87 plex, the total volume will be over 200 µL. Incubation times longer than 30 minutes may increase sensitivity.

See the following examples.

Component	For 1 sample (µL)	For 1 sample + 30% overage (µL)	For 2 samples + 30% overage (µL)
Per BD AbSeq Ab-Oligo	2.0	2.6	5.2
Total	2.0 * *N*	2.6 * *N*	5.2 * *N*
BD Stain Buffer (FBS) (Cat. No. 554656)	200.0 – (2.0 * *N*)	260 – (2.6 * *N*)	520 – (5.2 * *N*)
Total	**200.0**	**260**	**520**

N = no. of antibodies

Examples of different pools of AbSeq Ab-Oligos are described in the following table.

Component	For 1 sample (µL)	For 1 sample + 30% overage (µL)	For 2 samples + 30% overage (µL)
60 plex BD AbSeq labeling without Fc Block			
Per BD AbSeq Ab-Oligo	2.0 (120.0 total)	2.6 (156.0 total)	5.2 (312.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	80	104	208
Total	**200**	**260**	**520**
60 plex BD AbSeq labeling with Fc Block			
Per BD AbSeq Ab-Oligo	2.0 (120.0 total)	2.6 (156.0 total)	5.2 (312.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	55	71.5	143
Total	**175**	**227.5**	**455**

Component	For 1 sample (µL)	For 1 sample + 30% overage (µL)	For 2 samples + 30% overage (µL)
90 plex BD AbSeq labeling with or without Fc Block			
Per BD AbSeq Ab-Oligo	2.0 (180.0 total)	2.6 (234.0 total)	5.2 (468.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	20	26	52
Total	**200**	**260**	**520**

5 Pipet-mix the AbSeq labeling MasterMix and place back on ice.

Co-labeling single-cell samples with Sample Tags and BD AbSeq Ab-Oligos

1 To each Sample Tag tube containing 20 µL of Sample Tag, add 200 µL BD AbSeq labeling MasterMix (175 µL if <87 plex and Fc block is being performed). This is the "AbSeq/Sample Tag labeling MasterMix."

2 Centrifuge the cells at 400 × *g* for 5 minutes.

3 (Optional) For samples containing myeloid and B lymphocytes, we recommend blocking non-specific Fc Receptor–mediated false-positive signals with Human BD Fc Block (Cat. No. 564220).

To perform blocking:

a Pipet the reagents into a new 1.5-mL LoBind tube on ice:

Fc Block MasterMix

Component	For 1 sample (µL)[a]	For 1 sample + 20% overage (µL)
BD Stain Buffer (FBS) (Cat. No. 554656)	20.0	24.0
Human BD Fc Block (Cat. No. 564220)	5.0	6.0
Total	**25.0**	**30.0**

a. Sufficient for ≤1 × 10^6 cells. To block more cells, adjust the volume.

b Pipet-mix the Fc Block MasterMix and briefly centrifuge. Place on ice.

c Remove the supernatant from the cells without disturbing the pellet.

d Resuspend the cells in 25 µL of Fc Block MasterMix.

e Incubate the cells at room temperature (15°C to 25°C) for 10 minutes.

f After Fc Block, add BD AbSeq/Sample Tag labeling MasterMix into the cell suspension. Pipet-mix and proceed to step 5.

4 Remove the supernatant from the cells without disturbing the pellet and resuspend with the BD AbSeq/ Sample Tag labeling MasterMix. Pipet-mix.

5 Transfer the cells and the labeling MasterMix into a new 5-mL polystyrene Falcon tube.

6 Incubate on ice for 30–60 minutes.

NOTE If the staining volume exceeds 200 µL, incubation times longer than 30 minutes may increase sensitivity.

Aqueous buffered solution (Sample Tag) contains BSA and ≤0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

7 Proceed to Washing labeled cells on page 12.

Sequential labeling of single-cell samples, first with Sample Tags, then with BD AbSeq Ab-Oligos

Labeling with Sample Tags

1 Resuspend 20,000–1 million cells in 190 µL BD Stain Buffer (FBS) (Cat. No. 554656).

2 Briefly centrifuge the Sample Tag tubes to collect the contents at the bottom.

3 To each Sample Tag tube containing 20 µL of Sample Tag, transfer 180 µL of cell suspension. Pipet-mix.

Aqueous buffered solution (Sample Tag) contains BSA and ≤0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

4 Incubate at room temperature (15°C to 25°C) for 20 minutes.

5 Transfer each labeled cell suspension to a 5-mL polystyrene Falcon tube (Corning Cat. No. 352054).

6 Add 2 mL of BD Stain Buffer to the labeled cells and pipet-mix.

7 Centrifuge each tube at 400 × *g* for 5 minutes.

8 Uncap each tube and invert to decant the supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wiper to remove residual supernatant from the tube rim.

9 Add 2 mL of BD Stain Buffer to each tube and pipet-mix to resuspend.

10 Centrifuge at 400 × *g* for 5 minutes.

11 Uncap each tube and invert to decant supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wiper to remove residual supernatant from tube rim.

12 (Optional) Repeat step 9 through step 11 once more for a total of two washes.

13 Count and pool cells to desired ratios. For subsequent AbSeq staining ensure the total number of pooled cells is within the range of 20,000–1 million cells.

Labeling with BD AbSeq Ab-Oligos

1 Centrifuge cells at 400 × *g* for 5 minutes.

2 (Optional) For samples containing myeloid and B lymphocytes, we recommend blocking non-specific Fc Receptor–mediated false-positive signals with Human BD Fc Block (Cat. No. 564220).

To perform blocking:

a Pipet reagents into a new 1.5-mL LoBind tube on ice:

Fc Block MasterMix

Component	For 1 sample (µL)[a]	For 1 sample + 20% overage (µL)
BD Stain Buffer (FBS) (Cat. No. 554656)	20.0	24.0
Human BD Fc Block (Cat. No. 564220)	5.0	6.0
Total	**25.0**	**30.0**

a. Sufficient for ≤1 × 10^6 cells. To block more cells, adjust the volume.

b Pipet-mix the Fc Block MasterMix and briefly centrifuge. Place on ice.

c Remove the supernatant from the cells without disturbing the pellet.

d Resuspend the cells in 25 µL of Fc block MasterMix.

e Incubate the cells at room temperature (15°C to 25°C) for 10 minutes.

f After Fc Block, add 175 µL (87 plex and below) or 200 µL (88 plex and above) of BD AbSeq labeling MasterMix into the cell suspension. Pipet-mix and proceed to step 4.

3 Remove the supernatant from the cells without disturbing the pellet and resuspend with 200 µL BD AbSeq labeling MasterMix. Pipet-mix.

4 Transfer the cells and AbSeq labeling MasterMix (200–225 µL) into a new 5-mL polystyrene Falcon tube.

5 Incubate on ice for 30–60 minutes.

NOTE If the staining volume exceeds 200 µL, incubation times longer than 30 minutes may increase sensitivity.

Washing labeled cells

Sufficient post-labeling washes are important for reducing noise that comes from residual unbound Ab-Oligos being captured onto 3' capture beads during single-cell capture. However, some cell loss occurs with each additional wash. You can choose to perform more or fewer washes depending on the cell abundance.

1 Add 3 mL of BD Stain Buffer to labeled cells and pipet-mix for dilution.

2 Centrifuge each tube at 400 × *g* for 5 minutes.

3 Uncap each tube and invert to decant the supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wiper to remove the residual supernatant from the tube rim.

4 Add 3 mL of BD Stain Buffer to each tube and resuspend by pipet-mixing for the first wash.

5 Centrifuge at 400 × *g* for 5 minutes.

6 Uncap each tube and invert to decant the supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wiper to remove the residual supernatant from the tube rim.

7 (Optional) Repeat step 4 through step 6 once or twice more for a total of two to three washes.

8 Resuspend the pellet in 620 µL of cold Sample Buffer (Cat. No. 650000062) from the BD Rhapsody Cartridge Reagent Kit (Cat. No. 633731). Perform viability staining and count the cells using the appropriate single-cell capture and cDNA synthesis protocol.

NOTE For low-abundance samples (<20,000), resuspend the cells in 200 µL of cold BD Sample Buffer. For other 3' single-cell capture platforms, resuspend in the recommended buffer and volume according to the manufacturer.

9 Place the tube on ice and proceed to single-cell capture. See the *Single-Cell Analysis Workflow with BD Rhapsody™ Systems* (Doc ID: 220524) to find the appropriate protocol to follow.

Troubleshooting

Observation	Possible causes	Recommended solutions
Do not have the recommended buffer for labeling with Sample Tags or BD AbSeq Ab-Oligos	Various	Labeling with Sample Tags and BD AbSeq Ab-Oligos has been optimized in BD Stain Buffer (FBS) (Cat. No. 554656). Use of other staining buffers could result in less than optimal staining.
Total stain volume exceeds 245 µL	Co-Staining Sample Tags with ≥100 BD AbSeq Ab-Oligos	Co-Staining Sample Tags with ≥100 BD AbSeq Ab-Oligos requires that no BD Stain Buffer (FBS) is used in order to keep the staining volume at between 220-245 µL. Co-Staining Sample Tags with ≥100 BD AbSeq Ab-Oligos results in >220-245 µL (Fc Block) stain volume may require incubation up to 60 minutes on ice for optimal results.
Cells require labeling with Sample Tags and/or BD AbSeq Ab-Oligos at a different temperature	Physiological requirement	Protocols for Sample Tag and/or BD AbSeq Ab-Oligo labeling have been optimized for staining on ice. Use of other staining temperatures has not been tested and requires user optimization. For certain cytokine receptors, staining at room temperature can increase the sensitivity. However, incubation at room temperature for a long period of time might negatively impact the cell viability and RNA quality.
Cell loss	Wrong tube used in washes	Use Falcon polystyrene flow tubes and centrifuge cells using a benchtop centrifuge with swing bucket rotor. This centrifugation method reduces cell loss.
	Excessive washing or loss during washing	We recommend washing cells at least twice before loading on to the Rhapsody cartridge to decrease noise caused by unbound Ab-Oligos. Fewer washes can cause higher levels of noise. Therefore, for certain cell types, user optimization is required for the washing step to minimize cell loss and to avoid high levels of noise.
Cell loss during sorting	Various	• Sort more cells than needed for cartridge loading. • Label Sample Tags and/or BD AbSeq with fluorescent antibody together to reduce cell loss during multiple washing steps, and proceed to cartridge loading right after sorting.

Appendix A: Sample Tag sequences

Each Sample Tag is a human universal antibody conjugated with a unique oligonucleotide sequence to allow for sample identification. Each Sample Tag has common 5' and 3' ends and the Sample Tag sequence:

GTTGTCAAGATGCTACCGTTCAGAG[Sample Tag sequence]AAAAAAAAAAAAAAAAAAAAAAAA

Sample Tag	Sample Tag sequence
Sample Tag1 – Human	ATTCAAGGGCAGCCGCGTCACGATTGGATACGACTGTTGGACCGG
Sample Tag2 – Human	TGGATGGGATAAGTGCGTGATGGACCGAAGGGACCTCGTGGCCGG
Sample Tag3 – Human	CGGCTCGTGCTGCGTCGTCTCAAGTCCAGAAACTCCGTGTATCCT
Sample Tag4 – Human	ATTGGGAGGCTTTCGTACCGCTGCCGCCACCAGGTGATACCCGCT
Sample Tag5 – Human	CTCCCTGGTGTTCAATACCCGATGTGGTGGGCAGAATGTGGCTGG
Sample Tag6 – Human	TTACCCGCAGGAAGACGTATACCCCTCGTGCCAGGCGACCAATGC
Sample Tag7 – Human	TGTCTACGTCGGACCGCAAGAAGTGAGTCAGAGGCTGCACGCTGT
Sample Tag8 – Human	CCCCACCAGGTTGCTTTGTCGGACGAGCCCGCACAGCGCTAGGAT
Sample Tag9 – Human	GTGATCCGCGCAGGCACACATACCGACTCAGATGGGTTGTCCAGG
Sample Tag10 – Human	GCAGCCGGCGTCGTACGAGGCACAGCGGAGACTAGATGAGGCCCC
Sample Tag11 – Human	CGCGTCCAATTTCCGAAGCCCCGCCCTAGGAGTTCCCCTGCGTGC
Sample Tag12 – Human	GCCCATTCATTGCACCCGCCAGTGATCGACCCTAGTGGAGCTAAG