Single Cell Labelling with the BD™ Single-Cell Multiplexing Kit and BD™ AbSeq Ab-Oligos

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

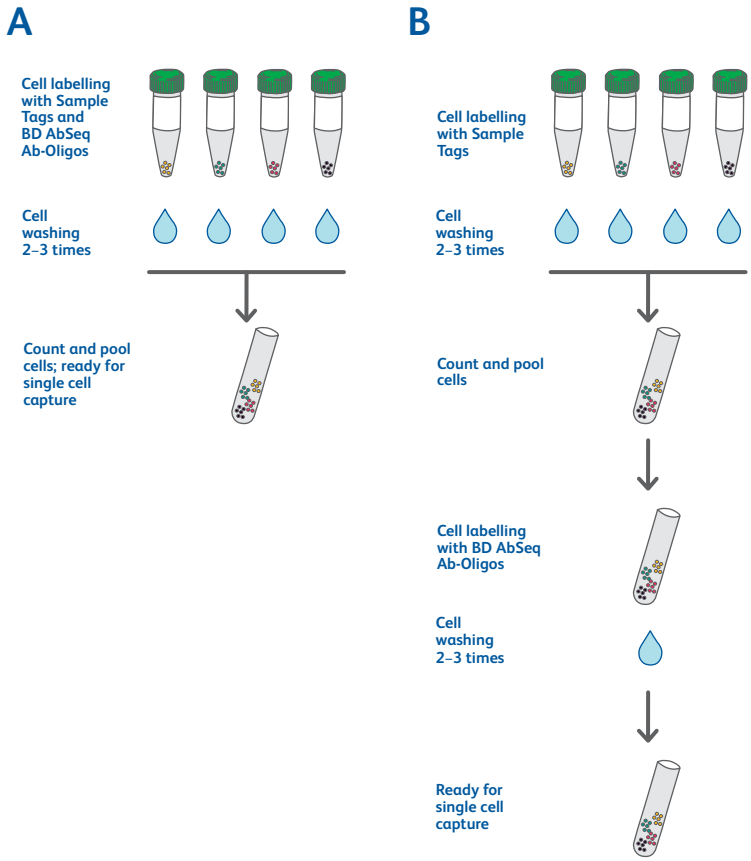
Introduction

This protocol describes use of BD AbSeq Ab-Oligos (antibody-oligonucleotides) with the BD 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 labelling of 20,000 to 1 million cells. Up to 40 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 labelled 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 labelling with BD AbSeq Ab-Oligos (B):



Sequential labelling is more economical than co-labelling, but you will save time by co-labelling. The biological effects of co-labelling 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

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

  Never freeze BD AbSeq Ab-Oligos or Sample Tags.
- BD Rhapsody™ Cartridge Reagent Kit (Cat. No. 633731)
- Latch Rack for 500 µL Tubes (Thermo Fisher Scientific Cat. No. 4900 or 4890)
- Falcon® tubes, 5 mL Round Bottom Polystyrene Test Tube (Corning Cat. No. 352054)

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

  Use only the tubes specified in the protocol. Use of other tubes might lead to increased cell loss.

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 BD Rhapsody™ Cartridge. See appropriate 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).

Preparing BD AbSeq Ab-Oligos

Note: BD Biosciences recommends:

- Creating freshly pooled antibodies before each experiment.
- Creating pools with 30% overage to ensure adequate volumes for labelling. **The reagents are viscous and 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 tube strip and pool BD AbSeq Ab-Oligos into a 1.5 mL LoBind Tube.

1 Place all 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 BD AbSeq Ab-Oligos in the Latch Rack in a tabletop centrifuge with a plate adapter tubes 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-labelling single cells with BD AbSeq Ab-Oligos and Sample Tags
- Sequentially labelling single cells with Sample Tags and BD AbSeq Ab-Oligos

Co-labelling single cells with BD AbSeq Ab-Oligos and Sample Tags

1 In pre-amplification workspace, pipet reagents into a new 1.5 mL LoBind Tube on ice:
2X BD AbSeq labelling master mix for co-labelling workflow

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
Per BD AbSeq Ab-Oligo	2.0	2.6	5.2
BD Stain Buffer (FBS) (Cat. No. 554656) (*N* = no. antibodies)	80 – (2.0 × *N*)	104 – (2.6 × *N*)	208 – (5.2 × *N*)
Total	**80.0**	**104.0**	**208.0**

Examples

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
10-plex BD AbSeq labelling			
Per BD AbSeq Ab-Oligo	2.0 (20.0 total)	2.6 (26.0 total)	5.2 (52.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	60.0	78.0	156.0
20-plex BD AbSeq labelling			
Per BD AbSeq Ab-Oligo	2.0 (40.0 total)	2.6 (52.0 total)	5.2 (104.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	40.0	52.0	104.0
40-plex BD AbSeq labelling			
Per BD AbSeq Ab-Oligo	2.0 (80.0 total)	2.6 (104.0 total)	5.2 (208.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	0.0	0.0	0.0

2 Pipet-mix the 2X AbSeq labeling master mix, and place back on ice.

3 To each Sample Tag tube containing 20 µL of Sample Tag, add 80.0 µL 2X AbSeq labeling master mix.

4 Pipet-mix, and place on ice.

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

6 (Optional) For samples containing myeloid and B lymphocytes, BD Biosciences recommends blocking non-specific Fc Receptor-mediated false-positive signal 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 master mix

Component	For 1 sample (µL)[a]	For 1 sample + 20% overage (µL)
BD Stain Buffer (FBS) (Cat. No. 554656)	95.0	114.0
Human BD Fc Block (Cat. No. 564220)	5.0	6.0
Total	**100.0**	**120.0**

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

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

c. Remove supernatant from cells without disturbing pellet.

d. Resuspend cells in 110 µL Fc block master mix.

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

f. After Fc Block, proceed to step 8.

7 Remove supernatant from cells without disturbing pellet and resuspend each sample in 110 µL BD Stain Buffer (Cat. No. 554656). Pipet-mix.

8 Pipet 100 µL of each cell suspension into the corresponding Sample Tag tube that contains 2X BD AbSeq and Sample Tag labeling master mix for co-labelling workflow. Pipet-mix.

	**Caution**. 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.

9 Incubate on ice for 30–60 minutes.

10 Proceed to Washing the labelled cells.

Sequentially labelling single cells with Sample Tags and BD AbSeq Ab-Oligos

Labelling with Sample Tags

1 Resuspend 20,000–1 million cells in 200 µL BD Stain Buffer (FBS) (Cat. No. 554656).

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

3 For each sample, transfer 180 µL cell suspension to a Sample Tag tube. Pipet-mix.

Caution. 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 labelled cell suspension to a 5 mL polystyrene Falcon tube (Corning Cat. No. 352054). Add 2 mL BD Stain Buffer to labelled cells and pipet-mix.

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

7 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.

8 Add 2 mL BD Stain Buffer to each tube and resuspend by pipet-mixing.

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

10 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.

11 (Optional) Repeat steps 8–10 once more for a total of 3 washes.

12 Resuspend pellet in 500 µL cold BD Sample Buffer from the BD Rhapsody Cartridge Reagent Kit (Cat. No. 633731). Perform viability staining and count cell 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.

BD Biosciences recommends pooling more cells (up to one million cells) than you want to be captured in the BD Rhapsody Cartridge, because there can be cell loss during BD AbSeq labelling and washing.

Labelling with BD AbSeq Ab-Oligos

1 In pre-amplification workspace, pipet reagents into a new 1.5 mL LoBind Tube on ice:
2X BD AbSeq labelling master mix for sequential labelling workflow

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
Per BD AbSeq Ab-Oligo	2.0	2.6	5.2
BD Stain Buffer (FBS) (Cat. No. 554656) (*N* = no. antibodies)	100 – (2.0 × *N*)	130 – (2.6 × *N*)	260 – (5.2 × *N*)
Total	**100.0**	**130.0**	**260.0**

Examples

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
10-plex BD AbSeq labelling			
Per BD AbSeq Ab-Oligo	2.0 (20.0 total)	2.6 (26.0 total)	5.2 (52.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	80.0	104.0	208.0
20-plex BD AbSeq labelling			
Per BD AbSeq Ab-Oligo	2.0 (40.0 total)	2.6 (52.0 total)	5.2 (104.0 total)

Component	1 sample (µL)	1 sample + 30% overage (µL)	2 samples + 30% overage (µL)
BD Stain Buffer (FBS) (Cat. No. 554656)	60.0	78.0	156.0
40-plex BD AbSeq labelling			
Per BD AbSeq Ab-Oligo	2.0 (80.0 total)	2.6 (104.0 total)	5.2 (208.0 total)
BD Stain Buffer (FBS) (Cat. No. 554656)	20.0	26.0	52.0

2 Pipet-mix the 2X AbSeq labeling master mix, and place back on ice.

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

4 (Optional) For samples containing myeloid and B lymphocytes, BD Biosciences recommends blocking non-specific Fc Receptor-mediated false-positive signal 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 master mix

Component	For 1 sample (µL)[a]	For 1 sample + 20% overage (µL)
BD Stain Buffer (FBS) (Cat. No. 554656)	95.0	114.0
Human BD Fc Block (Cat. No. 5642220)	5.0	6.0
Total	**100.0**	**120.0**

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

b. Pipet-mix Fc Block master mix, and briefly centrifuge. Place on ice.

c. Remove supernatant from cells without disturbing pellet.

d. Resuspend cells in 110 µL Fc block master mix.

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

f. After Fc Block, proceed to step 6.

5 Remove supernatant without disturbing pellet, and resuspend in 110 µL BD Stain Buffer (Cat. No. 554656). Pipet-mix.

6 In new 5 mL polystyrene Falcon tube (Corning Cat. No. 352054), combine 100 µL pooled cell suspension labelled with Sample Tags and 100 µL 2X BD AbSeq labelling master mix for sequential labelling workflow. Pipet-mix.

7 Incubate on ice for 30–60 minutes.

8 Proceed to Washing the labelled cells.

Washing the labelled cells

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. Users can choose to perform more or fewer washes depending on the abundance of their sample.

1 Transfer each labelled cell suspension to a 5 mL polystyrene Falcon tube (Corning Cat. No. 352054), if the cells are in a different tube type. Add 2 mL BD Stain Buffer to labelled cells and pipet-mix.

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

3 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.

4 Add 2 mL BD Stain Buffer to each tube, and resuspend by pipet-mixing.

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

6 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.

7 (Optional) Repeat steps 4–6 once more for a total of 3 washes.

8 Resuspend the pellet in 620 µL cold Sample Buffer (Cat. No. 650000062). Perform viability staining and count cell 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 recommended buffer and volume according to manufacturer.

9 Place 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 labelling with Sample Tags or BD AbSeq Ab-Oligos	BD Stain Buffer not used	Labelling with Sample Tags and BD AbSeq Ab-Oligos is optimal in BD Stain Buffer (FBS) (Cat. No. 554656). Label Sample Tags and BD AbSeq Ab-Oligos in BD Stain Buffer (FBS).
Cells require labelling with Sample Tags and/or BD AbSeq Ab-Oligos at a different temperature	Physiological requirement	Use protocols for Sample Tag and/or BD AbSeq Ab-Oligos labelling that have been optimized for the specific sample type.
Accidentally resuspended cells in BD Stain Buffer (FBS) rather than Sample Buffer before cell counts	Various	BD Biosciences recommends centrifuging the samples and resuspending the cells in Sample Buffer after labelling with Sample Tags. This ensures optimal performance of cell loading in the BD Rhapsody Cartridge.
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.

Observation (continued)	Possible causes	Recommended solutions
Cell loss after sorting	Various	• Sort more cells than needed for cartridge loading. • Sort cells into 5 mL polystyrene Falcon tube. Use the same 5 mL polystyrene Falcon tube that was used for sorting for cell labelling by following these steps: 1. Prepare: – For co-labelling: Pipet 100 µL into each Sample Tag tube containing 20 µL Sample Tag and 80 µL 2X BD AbSeq labelling master mix. – For sequential labelling: Pipet 180 µL BD Stain Buffer into each Sample Tag tube containing 20 µL Sample Tag. 2. Pipet-mix, and place on ice. 3. Sort cells into a 5 mL polystyrene Falcon tube. 4. Centrifuge the sorted cell suspension at 400 × *g* for 5 minutes. 5. Uncap the tube and invert to decant supernatant into biohazardous waste. 6. Keep the tube inverted and gently blot on a lint-free wiper to remove residual supernatant from tube rim. 7. Resuspend cell pellet with the 1X cell labelling master mix (step 1), and proceed with cell labelling.

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 Tag 1—Human	ATTCAAGGGCAGCCGCGTCACGATTGGATACGACTGTTGGACCGG
Sample Tag 2—Human	TGGATGGGATAAGTGCGTGATGGACCGAAGGGACCTCGTGGCCGG
Sample Tag 3—Human	CGGCTCGTGCTGCGTCGTCTCAAGTCCAGAAACTCCGTGTATCCT
Sample Tag 4—Human	ATTGGGAGGCTTTCGTACCGCTGCCGCCACCAGGTGATACCCGCT
Sample Tag 5—Human	CTCCCTGGTGTTCAATACCCGATGTGGTGGGCAGAATGTGGCTGG
Sample Tag 6—Human	TTACCCGCAGGAAGACGTATACCCCTCGTGCCAGGCGACCAATGC
Sample Tag 7—Human	TGTCTACGTCGGACCGCAAGAAGTGAGTCAGAGGCTGCACGCTGT
Sample Tag 8—Human	CCCCACCAGGTTGCTTTGTCGGACGAGCCCGCACAGCGCTAGGAT
Sample Tag 9—Human	GTGATCCGCGCAGGCACACATACCGACTCAGATGGGTTGTCCAGG
Sample Tag 10—Human	GCAGCCGGCGTCGTACGAGGCACAGCGGAGACTAGATGAGGCCCC
Sample Tag 11—Human	CGCGTCCAATTTCCGAAGCCCCGCCCTAGGAGTTCCCCTGCGTGC
Sample Tag 12—Human	GCCCATTCATTGCACCCGCCAGTGATCGACCCTAGTGGAGCTAAG

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