BD Rhapsody™ System

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

Protocol

23-24312(01)
2022-12

For Research Use Only

Regulatory information

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

History

Revision	Date	Change made
23-24312(01)	2022-12	Initial release.

Contents

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

Introduction

This protocol describes the use of BD® AbSeq Ab-Oligos (antibody-oligonucleotides) with the BD® Flex Single-Cell Multiplexing Kits (Cat. no. 633849-633852).

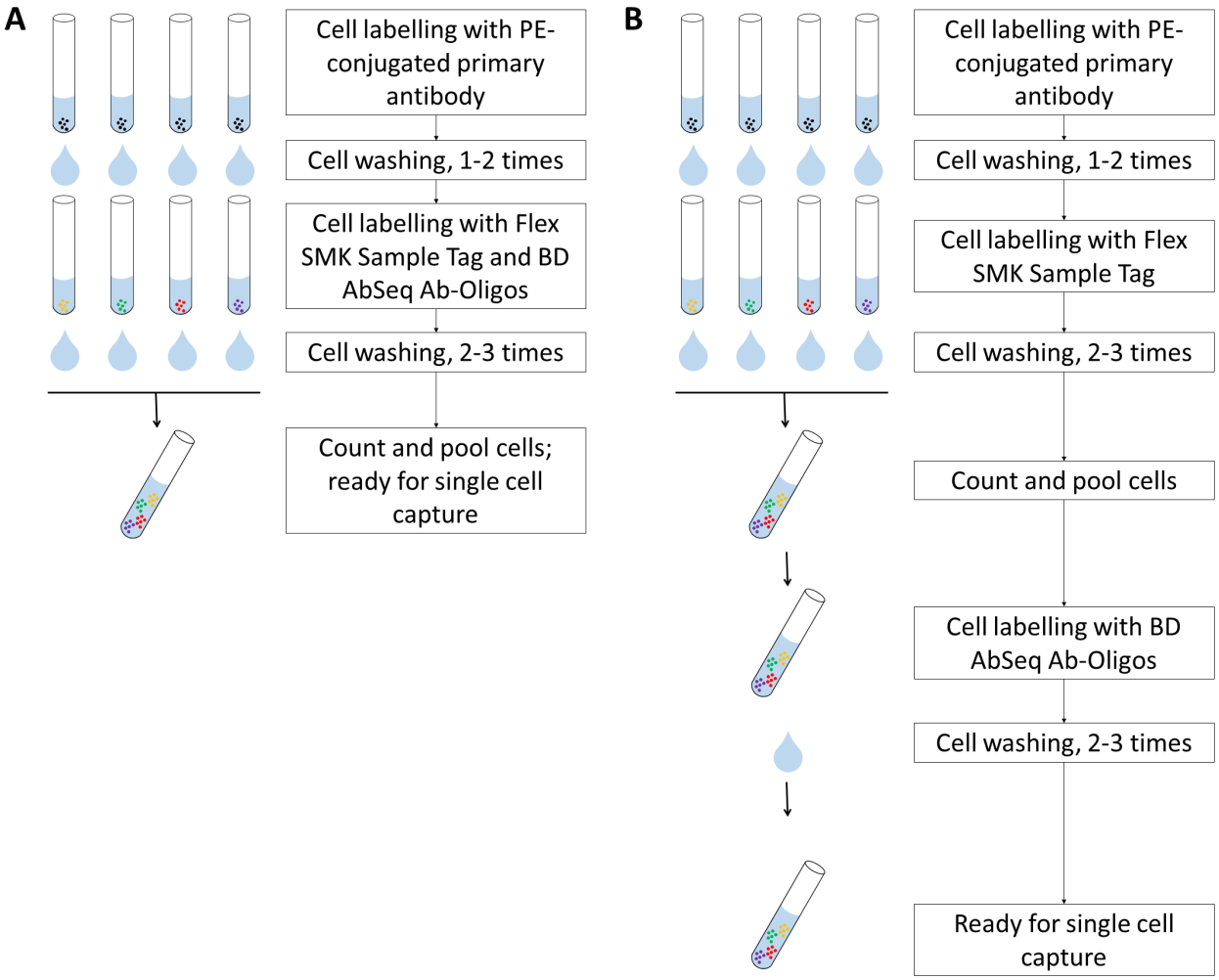
BD® AbSeq Ab-Oligos are used for antigen-expression profiling with BD Rhapsody™ single-cell capture and downstream library preparation. Each BD® AbSeq Ab-Oligos is an antibody conjugated to an oligonucleotide that contains an antibody-specific barcode, a poly(A)-tail for bead capture, and a primer binding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

BD® Flex Single-Cell Multiplexing Kits utilize an innovative antibody-oligo technology to provide higher sample throughput for single-cell library preparation, improve detection, and minimize multiplet data from single-cell experiments. Every antibody-oligo in the BD® Flex Single-Cell Multiplexing Kits, referred to as a Sample Tag, has a unique sample oligo barcode conjugated to an Anti-R-Phycoerythrin (PE), Clone E31-1459 antibody. This approach allows users to select any PE-conjugated primary antibody (or cocktail of antibodies) to tag their samples, regardless of species or cell type.

Four BD® Flex Single-Cell Multiplexing Kits (Cat. No. 633849-633852) are available. Each kit consists of six Sample Tags with unique sample oligo barcodes. These four kits are fully compatible, allowing up to 24 samples to be labeled and pooled prior to single-cell capture with the BD Rhapsody™ HT Single-Cell Analysis system.

Workflow

Cells are first labeled with a primary PE-conjugated antibody, followed by second-step labeling with the BD® Flex SMK Sample Tags. 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 and improve cell yield 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

- 50,000–1 million cells
- BD Pharmingen™ Stain Buffer (FBS) (Cat. No. 554656)
- PE-conjugated primary antibody (specific to sample of interest)
- BD® AbSeq Ab-Oligos (various)
- BD® Flex Single-Cell Multiplexing Kit (Cat. No. 633849-633852)

  Note: Never freeze BD® AbSeq Ab-Oligos or BD® Flex Single-Cell Multiplexing Kits.

- BD Rhapsody™ Cartridge Reagent Kit (Cat. No. 664887)
- 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)
- DNA LoBind Tubes, 1.5-mL (Eppendorf, Cat. No. 0030108051)

  Note: Use only the tubes specified in the protocol. Use of other tubes might lead to sub-optimal results.

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

Suggested materials

- BD Pharmingen™ Human BD Fc Block™ (Cat. No. 564219), BD Pharmingen™ Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™) (Cat. No. 553141), or BD Pharmingen™ Purified Mouse Anti-Rat CD32 (Rat BD Fc Block™) (Cat. No. 550270)
- 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*.
- If your biological sample contains red blood cell contamination, red blood cell lysis is required. See *Preparing Single-Cell Suspensions Protocol*.

Cell labeling with PE-conjugated primary antibody

1. For each sample, prepare single-cell suspension.
2. 50,000 – 1 million cells per sample in 5 mL Round Bottom Polystyrene Falcon® Test Tubes (Corning Cat. No. 352054).
3. Centrifuge cells at 400 × *g* for 5 minutes.
4. (Optional) For samples containing myeloid and B lymphocytes, we recommend blocking non-specific Fc Receptor mediated false-positive signal with Human BD Fc Block™, BD Pharmingen™ Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™), or BD Pharmingen™ Purified Mouse Anti-Rat CD32 (Rat BD Fc Block™).

   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 Pharmingen™ Stain Buffer (FBS)	95.0	114.0
BD Pharmingen™ Human BD Fc Block™[b]	5.0	6.0
Total	**100.0**	**120.0**

a. Sufficient for ≤1 x 106 cells. To block more cells, adjust volume.

b. Depending on the cell type, substitute BD Pharmingen™ Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block™), or BD Pharmingen™ Purified Mouse Anti-Rat CD32 (Rat BD Fc Block™).

   b. Pipet-mix BD Fc Block™ MasterMix, and briefly centrifuge. Place on ice.
   c. Remove supernatant from cells without disturbing pellet.
   d. Resuspend cells in 100 µL BD Fc Block™ MasterMix.
   e. Incubate at room temperature (15–25 °C) for 10 minutes.
   f. After BD Fc Block™, proceed to **step 6**.
5. Remove supernatant from cells without disturbing pellet and resuspend each sample in 100 µL of BD Pharmingen™ Stain Buffer (FBS). Pipet-mix.
6. Stain cells with PE-conjugated primary antibody following supplier's recommended staining protocol.

   Note: Selection of primary PE-conjugated antibody and staining concentration can impact the performance of BD® Flex SMK. Ideally the target(s) will be uniformly expressed at high levels on all of the cell types in your sample. Expression level and optimal titration of antibody on the sample of interest can be predetermined by flow cytometry.
7. Add 2 mL of BD Pharmingen™ Stain Buffer (FBS) to each tube and resuspend by pipet-mixing.
8. Centrifuge at 400 × *g* for 5 minutes.
9. 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.
10. (Optional) Repeat **steps 7–9** once more for a total of 2 washes.
11. Add 2 mL BD Pharmingen™ Stain Buffer (FBS) to each tube and resuspend by pipet-mixing and place on ice.
12. Follow one of the two workflows to label cells with Sample Tags and BD® AbSeq Ab-Oligos:
    - Co-labeling single cells with BD® AbSeq Ab-Oligos and Sample Tags.
    - Sequentially labeling single cells with Sample Tags and BD® AbSeq Ab-Oligos.

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

Note: When co-labeling, the total volume will be over 200 µL. Incubation times longer than 30 minutes may increase sensitivity.

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

BD® AbSeq labeling MasterMix for co-labeling 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 Pharmingen™ Stain Buffer (FBS) (N = no. antibodies)	200 – (2.0 x N)	260 – (2.6 x N)	520 – (5.2 x N)
Total	**200**	**260**	**520**

2. Pipet-mix the BD® AbSeq labeling MasterMix, and place back on ice.
3. Briefly centrifuge Sample Tag tubes to collect the contents at the bottom.
4. For each sample, in a new 1.5-mL LoBind tube, add 200 µL BD® AbSeq labeling MasterMix and 20 µL of Sample Tag.
5. Pipet-mix, and place on ice.
6. Centrifuge cells at 400 × *g* for 5 minutes.
7. Add 220 µL AbSeq/Sample Tag labeling mix to each sample for co-labeling workflow. Pipet-mix.

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

8. Incubate on ice for 30–60 minutes.
9. Proceed to Washing labeled cells.

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

Labeling with Sample Tags

1. Centrifuge cells at 400 × *g* for 5 minutes.
2. Resuspend cells in 180 µL BD Pharmingen™ Stain Buffer (FBS) and place on ice.
3. Briefly centrifuge Sample Tag tubes to collect the contents at the bottom.
4. For each sample, transfer 20 µL Sample Tag to cell suspension. Pipet-mix.

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

5. Incubate at room temperature (15–25 °C) for 20 minutes.
6. Add 2 mL BD Pharmingen™ Stain Buffer (FBS) to labeled cells and pipet-mix.
7. Centrifuge each tube at 400 × *g* for 5 minutes.
8. 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.
9. Add 2 mL BD Pharmingen™ Stain Buffer (FBS) to each tube and resuspend by pipet-mixing.
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. Add 2 mL BD Pharmingen™ Stain Buffer (FBS) to each tube and resuspend by pipet-mixing and place on ice.

Note: We recommend 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® Flex Single-Cell Multiplexing Kit and BD® AbSeq labeling and washing.

Labeling with BD® AbSeq Ab-Oligos

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

BD® AbSeq labeling MasterMix for sequential labeling 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 Pharmingen™ Stain Buffer (FBS) (Cat. No. 554656) (N = no. antibodies)	200 – (2.0 x N)	260 – (2.6 x N)	520 – (5.2 x N)
Total	**200**	**260**	**520**

2. Pipet-mix the BD® AbSeq labeling master mix, and place back on ice.
3. Centrifuge cells at 400 × *g* for 5 minutes.
4. Remove supernatant without disturbing pellet, and resuspend in 200 µLBD® AbSeq labeling MasterMix. Pipet-mix.
5. Incubate on ice for 30–60 minutes.
6. Proceed to Washing the labeled cells.

Washing labeled cells

Note: Sufficient post-labeling 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 or their sample.

1. Add 2 mL BD Pharmingen™ Stain Buffer (FBS) (3 mL for higher than 40 plex BD® AbSeq staining) to labeled 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 Pharmingen™ Stain Buffer (FBS) (3 mL for higher than 40 plex BD® AbSeq staining) 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 pellet in 620 µL cold Sample Buffer from the BD Rhapsody™ Cartridge Reagent Kit. Perform viability staining and count cell using the appropriate single-cell capture and cDNA synthesis protocol.

   Note: For low-abundance samples (<50,000), resuspend the cells in 200 µL of cold Sample Buffer.

   Note: We recommend 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 labeling and washing.
9. Place tube on ice, and proceed to single-cell capture. See the *Single-Cell Analysis Workflow with BD Rhapsody™ Systems* to find the appropriate protocol to follow.

Appendix A: Sample Tag sequences

Each Flex Sample Tag is an anti-PE 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

BD® Flex Single-Cell Multiplexing Kit A (Cat. No. 633849)

Note: Not Compatible with Hu SMK Tags 1-6 (Cat. No. 633781)

Sample Tag	Sample Tag Sequence	Notes
Sample Tag 1 – Flex	ATTCAAGGGCAGCCGCGTCACGATTGGATACGACTGTTGGACCGG	Barcode sequence is the same as human SMK Sample Tag 1
Sample Tag 2 – Flex	TGGATGGGATAAGTGCGTGATGGACCGAAGGGACCTCGTGGCCGG	Barcode sequence is the same as human SMK Sample Tag 2
Sample Tag 3 – Flex	CGGCTCGTGCTGCGTCGTCTCAAGTCCAGAAACTCCGTGTATCCT	Barcode sequence is the same as human SMK Sample Tag 3
Sample Tag 4 – Flex	ATTGGGAGGCTTTCGTACCGCTGCCGCCACCAGGTGATACCCGCT	Barcode sequence is the same as human SMK Sample Tag 4
Sample Tag 5 – Flex	CTCCCTGGTGTTCAATACCCGATGTGGTGGGCAGAATGTGGCTGG	Barcode sequence is the same as human SMK Sample Tag 5
Sample Tag 6 – Flex	TTACCCGCAGGAAGACGTATACCCCTCGTGCCAGGCGACCAATGC	Barcode sequence is the same as human SMK Sample Tag 6

BD® Flex Single-Cell Multiplexing Kit B (Cat. No. 633850)

Note: Not Compatible with Hu SMK Tags 7-12 (Cat. No. 633781)

Sample Tag	Sample Tag Sequence	Notes
Sample Tag 7 – Flex	TGTCTACGTCGGACCGCAAGAAGTGAGTCAGAGGCTGCACGCTGT	Barcode sequence is the same as human SMK Sample Tag 7
Sample Tag 8 – Flex	CCCCACCAGGTTGCTTTGTCGGACGAGCCCGCACAGCGCTAGGAT	Barcode sequence is the same as human SMK Sample Tag 8
Sample Tag 9 – Flex	GTGATCCGCGCAGGCACACATACCGACTCAGATGGGTTGTCCAGG	Barcode sequence is the same as human SMK Sample Tag 9
Sample Tag 10 – Flex	GCAGCCGGCGTCGTACGAGGCACAGCGGAGACTAGATGAGGCCCC	Barcode sequence is the same as human SMK Sample Tag 10
Sample Tag 11 – Flex	CGCGTCCAATTTCCGAAGCCCCGCCCTAGGAGTTCCCCTGCGTGC	Barcode sequence is the same as human SMK Sample Tag 11
Sample Tag 12 – Flex	GCCCATTCATTGCACCCGCCAGTGATCGACCCTAGTGGAGCTAAG	Barcode sequence is the same as human SMK Sample Tag 12

BD® Flex Single-Cell Multiplexing Kit C (Cat. No. 633851)

Note: Not Compatible with Ms SMK Tags 1-6 (Cat. No. 633793)

Sample Tag	Sample Tag Sequence	Notes
Sample Tag 13 – Flex	AAGAGTCGACTGCCATGTCCCCTCCGCGGGTCCGTGCCCCCCAAG	Barcode sequence is the same as mouse SMK Sample Tag 1
Sample Tag 14 – Flex	ACCGATTAGGTGCGAGGCGCTATAGTCGTACGTCGTTGCCGTGCC	Barcode sequence is the same as mouse SMK Sample Tag 2
Sample Tag 15 – Flex	AGGAGGCCCCGCGTGAGAGTGATCAATCCAGGATACATTCCCGTC	Barcode sequence is the same as mouse SMK Sample Tag 3
Sample Tag 16 – Flex	TTAACCGAGGCGTGAGTTTGGAGCGTACCGGCTTTGCGCAGGGCT	Barcode sequence is the same as mouse SMK Sample Tag 4
Sample Tag 17 – Flex	GGCAAGGTGTCACATTGGGCTACCGCGGGAGGTCGACCAGATCCT	Barcode sequence is the same as mouse SMK Sample Tag 5
Sample Tag 18 – Flex	GCGGGCACAGCGGCTAGGGTGTTCCGGGTGGACCATGGTTCAGGC	Barcode sequence is the same as mouse SMK Sample Tag 6

BD® Flex Single-Cell Multiplexing Kit D (Cat. No. 633852)

Note: Not Compatible with Ms SMK Tags 7-12 (Cat. No. 633793)

Sample Tag	Sample Tag Sequence	Notes
Sample Tag 19 – Flex	ACCGGAGGCGTGTGTACGTGCGTTTCGAATTCCTGTAAGCCCACC	Barcode sequence is the same as mouse SMK Sample Tag 7
Sample Tag 20 – Flex	TCGCTGCCGTGCTTCATTGTCGCCGTTCTAACCTCCGATGTCTCG	Barcode sequence is the same as mouse SMK Sample Tag 8
Sample Tag 21 – Flex	GCCTACCCGCTATGCTCGTCGGCTGGTTAGAGTTTACTGCACGCC	Barcode sequence is the same as mouse SMK Sample Tag 9
Sample Tag 22 – Flex	TCCCATTCGAATCACGAGGCCGGGTGCGTTCTCCTATGCAATCCC	Barcode sequence is the same as mouse SMK Sample Tag 10
Sample Tag 23 – Flex	GGTTGGCTCAGAGGCCCCAGGCTGCGGACGTCGTCGGACTCGCGT	Barcode sequence is the same as mouse SMK Sample Tag 11
Sample Tag 24 – Flex	CTGGGTGCCTGGTCGGGTTACGTCGGCCCTCGGGTCGCGAAGGTC	Barcode sequence is the same as mouse SMK Sample Tag 12

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