

BD Rhapsody™ Single-Cell Analysis System User Guide

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Becton, Dickinson and Company
BD Biosciences
2350 Qume Drive
San Jose, CA 95131 USA
Tel 1.877.232.8995

bdbiosciences.com
scomix@bdscomix.bd.com

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History

Revision	Date	Change made
Doc ID: 47395 Rev. 2.0	9/2017	Initial release.
Doc ID: 47395 Rev. 3.0	2/2018	<ul style="list-style-type: none">—Corrected volume used for the optional, diluted PCR2 panel supplement from 8.0 µL to 2.5 µL for 1 library.—Added protocol to pool single cell targeted libraries with the BD™ Single-Cell Multiplexing kits.—Updated contact information.—Rebranded from BD Genomics to BD Biosciences.

Revision	Date	Change made
Doc ID: 47395 Rev. 4.0	3/2018	<ul style="list-style-type: none"> —Updated screen captures and steps for running additional scans to latest BD Rhapsody™ Scanner software release. —Updated image analysis metrics table according to the BD Rhapsody Scanner software release. —Added option to filter low-abundance samples after pooling. —Changed dilution of PCR2 products for estimating its concentration from Elution Buffer to nuclease-free water. —Removed dilution of final libraries. —Changed the title of the user guide from the <i>Single Cell Targeted Library Preparation with the BD Rhapsody™ Single-Cell Analysis System User Guide</i> to the <i>BD Rhapsody™ Single-Cell Analysis System User Guide</i>. —Replaced single storage temperatures and room temperature incubation with temperature ranges: room temperature: 15°C to 25°C; 4°C: 2°C to 8°C; –20°C: –25°C to –15°C. —Replaced “BD Rhapsody™ Sample Loading Station” with “BD Rhapsody™ Express instrument.” The instrument remains the same.

Revision	Date	Change made
Doc ID: 47395 Rev. 5.0 23-21716-00	8/2019	<ul style="list-style-type: none"> —Updated analysis metrics, cell count, and sample result for BD Rhapsody™ Scanner. —Updated error and warning messages for BD Rhapsody™ Scanner software. —Added Mouse Immune Sample Tag sequences to Appendix C: Sequence Information. —Updated BD Biosciences technical support Email address to scomix@bdscomix.bd.com. —Removed BD Rhapsody™ Training Cells Kit information from the Required kits and storage conditions section. —Removed Preparing Jurkat and Ramos BD Rhapsody™ Training cells section from Appendix A: Guidelines to preparing single cell suspensions.

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Introduction

- [About this guide \(page 10\)](#)
- [Intended use \(page 10\)](#)
- [Safety symbols \(page 12\)](#)
- [Safety data sheets \(page 14\)](#)
- [Instrument technical support \(page 15\)](#)

About this guide

Introduction

This guide provides instructions on preparing mRNA sequencing libraries from single cells using the BD Rhapsody™ Single-Cell Analysis system. Single cell libraries can be prepared from single or multiplexed samples.

To use the BD Rhapsody Single-Cell Analysis system without the BD Rhapsody™ Scanner, see the *Single Cell Targeted Library Preparation with the BD Rhapsody™ Express Single-Cell Analysis System User Guide* (Doc ID: 76917).

Genomics technical publications are available for download from the BD Genomics Resource Library at bd.com/genomics-resources.

Intended use

Intended use

The BD Rhapsody Single-Cell Analysis system is intended for the preparation of single cell mRNA sequencing libraries. The BD Rhapsody Scanner uses brightfield and dual band fluorescence to count cells and measure cell viability. Proprietary BD™ Molecular Indexing technology is used to count individual mRNA molecules. Cells are entirely contained in the BD Rhapsody™ Cartridge, which is a single-use consumable.

The system is intended for use by professional scientific users, such as technicians and laboratory personnel, who are trained in the operation of the BD Rhapsody Express instrument and BD Rhapsody Scanner.

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

For more information on the purpose of the instruments, see the [BD Rhapsody Express instrument overview \(page 22\)](#) and the [BD Rhapsody Scanner overview \(page 24\)](#).

Restrictions

Any use of the BD Rhapsody Single-Cell Analysis system other than the procedures described in this user guide might result in damage to the instruments, loss of reagents or samples, or personal injury.

BD denies any responsibility for damage caused by the following:

- Any use of a BD Rhapsody instrument that does not comply with the procedures described in any guide used with the BD Rhapsody Single-Cell Analysis system
- Unauthorized alterations or adjustments to instrument hardware or software
- Any use of an instrument that violates locally applicable laws, rules, or regulations
- Evidence of any deviation from intended use voids the BD Rhapsody instrument warranty

Disclaimer

The instrument, external components, software, and consumables in the BD Rhapsody Single-Cell Analysis system are provided for research purposes only. BD disclaims all express and implied warranties, including, but not limited to, merchantability and fitness for use for a particular purpose.

Safety symbols



Introduction

This topic describes the safety symbols used in this guide.






For safety and limitations, see the *BD Rhapsody™ Express Instrument and BD Rhapsody™ Scanner Safety and Limitations Guide* (Doc ID: 42061).

Safety symbols

The following table lists the safety symbols used in this guide to alert you to potential hazards.

Symbol	Meaning
	Caution. Indicates the need for the user to consult the instructions for use for important cautionary information, such as warnings and precautions that cannot, for a variety of reasons, be presented on the device itself.
	Biological hazard

Other symbols

Symbol	Meaning
 The symbol consists of the letters 'REF' in a bold, sans-serif font, enclosed within a rectangular border.	Part number
 The symbol consists of the letters 'LOT' in a bold, sans-serif font, enclosed within a rectangular border.	Lot number
 A simple line drawing of a thermometer, showing a bulb at the bottom and a vertical scale with a horizontal line indicating a temperature level.	Storage temperature range
 A simple line drawing of an hourglass, consisting of two inverted triangles meeting at a central point.	Expiration date
 A simple line drawing of an open book with a lowercase letter 'i' on the right page.	Consult instructions for use

Safety data sheets

Introduction

This topic describes how to obtain safety data sheets (SDSs).

Obtaining SDSs

Before handling chemicals, read and understand the SDSs. To obtain SDSs for chemicals ordered from BD Biosciences, go to regdocs.bd.com or contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Instrument technical support

Introduction This topic describes how to get technical support for the BD Rhapsody Express instrument and BD Rhapsody Scanner.

Contacting technical support If technical assistance is required, contact BD Biosciences technical support at scomix@bdscomix.bd.com or 1.877.232.8995. You can contact technical support in Europe at help.biosciences@europe.bd.com or at these telephone numbers:

Location	Telephone number	Location	Telephone number
Worldwide	+32 2 40 09 895	—	
Austria	01 92 80 465	Netherlands	010 71 14 800
Belgium	02 40 17 093	Norway	800 18 530
Denmark	80 88 21 93	Portugal	800 86 01 76
Finland	800 11 63 17	South Africa	0800 98 10 08
France	01 70 70 81 93	Spain	91 41 46 250
Germany	069 22 22 25 60	Sweden	08 50 69 21 54
Greece	00800 12 75 06	Switzerland	044 58 04 373
Italy	02 36 00 36 85	United Kingdom	0207 07 53 226

Before contacting BD Biosciences, have the following information available:

- Product name, part number, and serial number or lot number
 - Any error messages
 - Details of recent system performance
 - For the BD Rhapsody Scanner, the version of the software that you are using
-

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Getting Started

- [BD Rhapsody™ targeted library workflow \(page 18\)](#)
- [Site requirements \(page 19\)](#)
- [Thermomixer settings \(page 20\)](#)
- [Thermal cycler programs setup \(page 20\)](#)
- [Pipette Settings \(page 21\)](#)
- [BD Rhapsody Express instrument overview \(page 22\)](#)
- [BD Rhapsody Scanner overview \(page 24\)](#)
- [Quick guide to the BD Rhapsody Scanner software \(page 27\)](#)
- [Best practices \(page 31\)](#)

BD Rhapsody™ targeted library workflow

Cartridge preparation (Optional) Multiplexing Cell preparation

Total time ~14 min

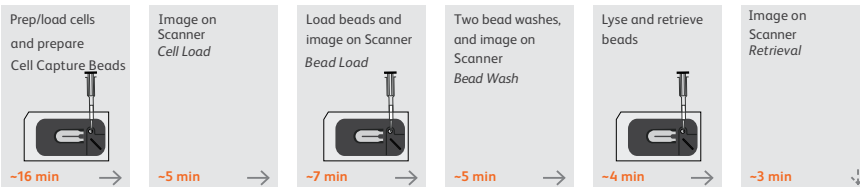
samples Time varies

Total time ~25 min



Capturing single cell mRNA with the BD Rhapsody™ Cartridge and Cell Capture Beads

Total time ~45 min

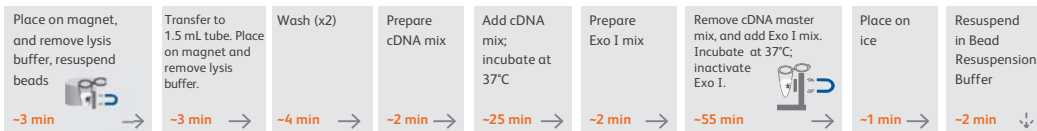


Proceed immediately to performing reverse transcription and Exonuclease I treatment.

Performing reverse transcription and Exonuclease I treatment on Cell Capture Beads

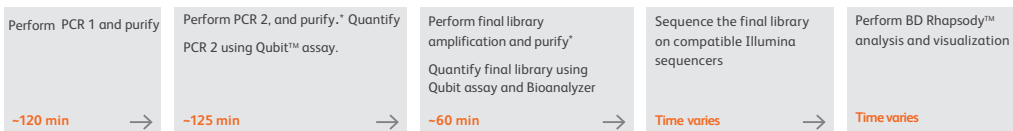
Total time ~1 hr 40 min

Set thermomixer to 37°C → Put bead collection tube on magnet → Prepare cDNA master mix



Preparing a single cell sequencing library with the BD Rhapsody™ Targeted Reagent Kit

Total time ~5 hrs



*Stopping point: May store sample at -20°C. See user guide for details.

Note: For use of two cartridges, see [Workflow with two BD Rhapsody™ Cartridges \(page 215\)](#).

Site requirements

Workspace designation

Dedicate two isolated workspaces in the laboratory to run high-sensitivity, single cell sequencing experiments:

- Pre-amplification workspace
- Post-amplification workspace

For detailed site requirements and technical specifications, see the *BD Rhapsody™ Express Instrument and BD Rhapsody™ Scanner Site Preparation Guide* (Doc ID: 47391) and [BD Rhapsody™ Scanner metrics \(page 201\)](#).

For installation of the BD Rhapsody™ Express instrument and the BD Rhapsody Scanner, see the *BD Rhapsody™ Single-Cell Analysis System Installation and Maintenance Guide* (Doc ID: 43084).

Genomics technical publications are available for download from the BD Genomics Resource Library at bd.com/genomics-resources.

Thermomixer settings

Settings Depending on the protocol, set the thermomixer from 16°C to 80°C and 0–1,200 rpm.

Thermal cycler programs setup

- Recommendations**
- Use a properly calibrated thermal cycler for 0.2 mL tubes with a maximum reaction volume of 50 μ L.
 - Use a heated lid set to $\geq 95^\circ\text{C}$.
 - For specific instrument operation, follow the instructions provided by the manufacturer.

Thermal cycler programs Add these thermal cycler programs to the post-amplification thermal cyclers:

Program (run time)	Steps	Cycles	Temperature	Time
Program 1 (~80 min): PCR1 and PCR2	1	1	95°C	3 min
	2	15	95°C	30 s
			60°C	3 min
			72°C	1 min
	3	1	72°C	5 min
4	1	4°C	∞	
Program 2 (~20 min): final amplification	1	1	95°C	5 min
	2	8	98°C	15 s
			60°C	30 s
			72°C	30 s
	3	1	72°C	1 min
4	1	4°C	∞	

Pipette Settings

Pipette programs BD Rhapsody™ pipettes are provided pre-programmed for use during single cell mRNA capture from the BD Rhapsody Cartridge. Do not change the settings, but confirm them before use. See [Retrieving single cell mRNA with the BD Rhapsody™ Cartridge and Cell Capture Beads \(page 93\)](#):

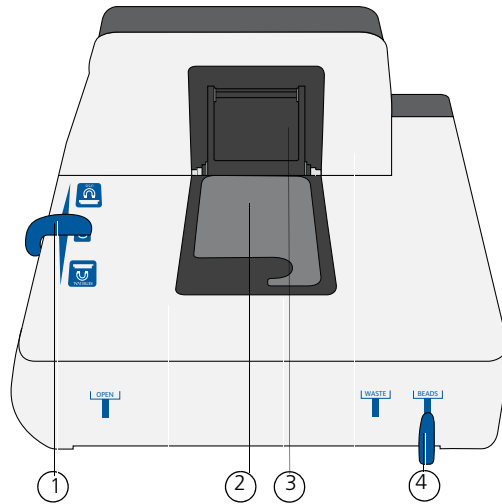
Pipette	Mode
P1200M	Prime/Treat
P1200M	Cell Load
P1200M	Bead Load
P1200M	Wash
P1200M	Lysis
P5000M	Retrieval

BD Rhapsody Express instrument overview

Introduction The BD Rhapsody Cartridge requires use of the BD Rhapsody Express instrument. The station is used to load reagents, cells, and Cell Capture Beads into the cartridge for bead capture and retrieval of single cell mRNA.

Safety For safety and limitations of the BD Rhapsody Express instrument, see the *BD Rhapsody™ Express Instrument and BD Rhapsody™ Scanner Safety and Limitations Guide* (Doc ID: 42061).

Components The following figure shows the main components of the BD Rhapsody Express instrument for operation. For maintenance of the Express instrument, see the *BD Rhapsody™ Single-Cell Analysis System Installation and Maintenance Guide* (Doc ID: 43084).



No.	Component
1	Left slider to position Retrieval (top) and Lysis (bottom) magnets. Slider shown in 0 (neutral) position: no magnets applied to BD Rhapsody Cartridge
2	Cartridge tray to install the BD Rhapsody Cartridge
3	Retrieval (top) magnet in up position
4	Front slider to position Waste Collection Container (WASTE) and 5 mL LoBind Tube for bead retrieval (BEADS) and for Waste Collection Container and 5 mL LoBind Tube access (OPEN)

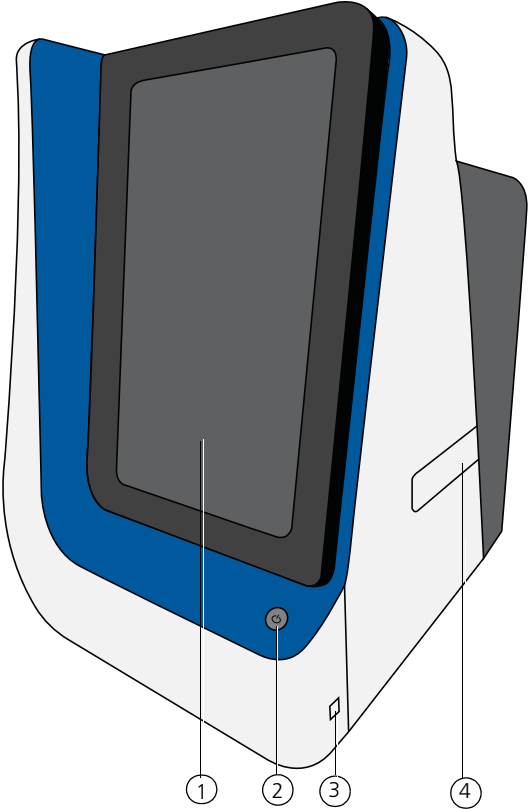
BD Rhapsody Scanner overview

Introduction The BD Rhapsody Scanner is used to count cells and measure cell viability by brightfield and dual band fluorescence imaging.

Safety For safety and limitations of the scanner, see the *BD Rhapsody™ Express Instrument and BD Rhapsody™ Scanner Safety and Limitations Guide* (Doc ID: 42061).

Installation and maintenance See the *BD Rhapsody™ Single-Cell Analysis System Installation and Maintenance Guide* (Doc ID: 43084) to review critical installation and periodic maintenance procedures on the scanner.

Components The following figure shows the main components of the scanner for operation:



No.	Front component
1	Scanner touchscreen
2	Scanner front power button and standby switch. Lit green: ON. Flashing: PC asleep.
3	USB 3.0 port
4	Cartridge loading door where a tray extends and retracts

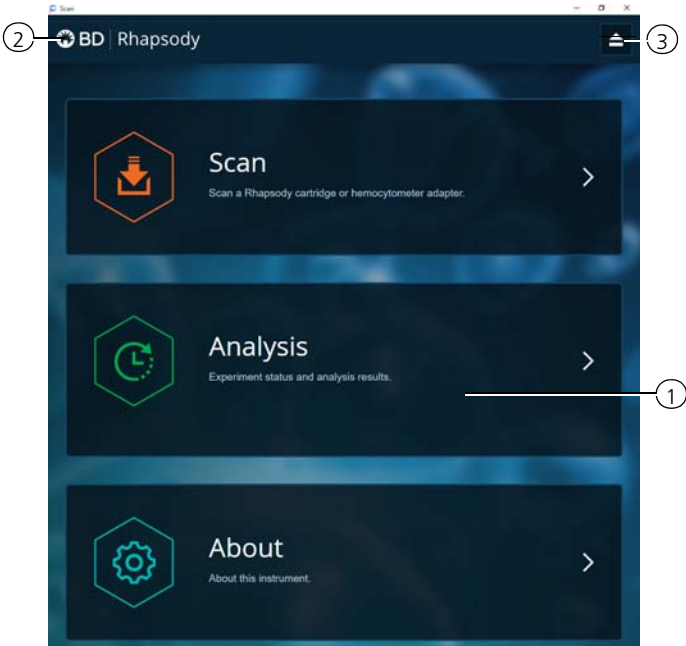


No.	Rear component
1	Master power switch
2	Fuse holder
3	24 VDC power input
4	USB 2.0 ports (2) ^a
5	Ethernet port

- a. The USB 2.0 ports on the rear of the instrument are primarily used by BD Biosciences technical support.

Quick guide to the BD Rhapsody Scanner software

Navigation



No.	Description
1	Tap an application (app) to navigate between scan, analysis, and about screens.
2	Tap Rhapsody on any screen to return to the main screen.
3	(Optional) Tap the eject icon to eject the tray from the sample loading door of the scanner. The scanner automatically ejects the tray.

The screenshot shows the 'Samples Calculator' interface in the BD Rhapsody software. The interface includes a top navigation bar with 'Scan', 'Experiment', and 'About' buttons. The main area is titled 'Samples Calculator' and contains several input fields and a table.

Callout 4 points to the 'Select Samples' button at the top left of the calculator area.

Callout 5 points to the 'Desired total volume' input field, which is set to 650 μl .

Callout 6 points to the table of samples, which has the following data:

Sample	Time	Concentration (cells/ μl)	Viable Cells (%)	Relative Amounts
Jurkat	2018-01-31 10:01:43.07	423.64	92.24	- 1 +
Ramos	2018-01-31 10:02:39.54	375.99	92.53	- 1 +

Callout 7 points to the 'Results' section, which displays the following information:

Sample	Stock Volume
Jurkat	15.3 μl
Ramos	17.2 μl

Additional results shown below the table are:

- Buffer volume: 617.5 μl
- Loading cell concentration: 19.9 cells/ μl
- Estimated cell doublet rate: 2.4%

Callout 4 also points to the 'Save' button at the bottom of the calculator area.

No.	Description
4	Tap Select Samples to display the window for selecting samples. Tap Calculate to calculate volumes for preparing cell suspensions. Tap Save on the Calculate tab to display saved calculations.
5	Enter the total volume and desired number of captured cells to prepare the cell suspension for loading into the BD Rhapsody Cartridge. See Loading cells in the BD Rhapsody Cartridge (page 94) .
6	(Optional) Enter the relative amounts of samples that comprise the cell suspension. The default is 1:1.
7	Obtain the volumes of buffer and stock cells required to prepare the cell suspension for cartridge loading.

**BD Rhapsody
Scanner software
workflows**

Scan application workflow

Step	Screen
1	Main menu
2	Hemocytometer/cartridge placement on tray
3	Scan
4	Cartridge removal

Analysis application workflow

Step	Screen
1	Main menu
2	Experiment details
3	Prepare sample
4	Select sample
5	Calculate cell suspension volumes
6	Save calculation
7	Analysis metrics

Best practices

Good laboratory practices

- Calibrate and service pipettes every 12 months to ensure accurate sample volume transfer at each step. To clean and calibrate the pipettes, see the *BD Rhapsody™ Single-Cell Analysis System Installation and Maintenance Guide* (Doc ID: 43084).
 - Unless otherwise specified, thaw reagents on ice. Store reagents at their specified storage conditions.
 - Collect small volumes by briefly centrifuging samples. Brief or pulse centrifugation is <1 second.
 - Gently vortex solutions containing enzymes. Minimize vortex duration and keep the vortex speed low. Do not vortex solutions containing Cell Capture Beads.
 - Gently pipet cells to avoid stressing or killing them.
 - Work in designated pre- or post-amplification workspaces according to the protocol.
 - Prepare reagent mixes in pre-amplification workspaces, and conduct amplification in post-amplification workspaces.
 - Wear suitable protective clothing, eyewear, and gloves.
-

RNase-free technique

Prevent the introduction of endogenous RNases into samples during processing by doing the following:

- Use low-retention, RNase-free pipette tips and low-binding reaction tubes when required for certain steps to prevent absorption to plastic surfaces and minimize bead loss.
 - Wear disposable gloves, and change them frequently.
 - Never reuse tips or tubes.
 - Keep tip boxes, reagent containers, and sample tubes closed when not in use.
 - Always maintain a clean laboratory bench, and if necessary, wipe work surface with a solution of 10% bleach.
-

Sterility

- Clean cell culture surfaces in the laminar flow hood with 70% ethyl alcohol, and appropriately sterilize the surfaces.
 - Use sterile serological pipettes to aseptically transfer media and cells.
 - Place a flask in a cell culture hood one at a time to prevent cross-cell contamination.
-

Cell Capture Beads

- Keep the Cell Capture Beads on ice before use.
Do not freeze Cell Capture Beads.
 - For maximum recovery, do not vortex samples containing Cell Capture Beads.
 - Gently mix suspensions with Cell Capture Beads by pipette only.
 - Use low retention tips and LoBind tubes when handling Cell Capture Beads.
-

BD Rhapsody Express instrument

- The Express instrument contains strong magnets. Avoid having metal pieces close to the station.
 - Wipe the Express instrument with 70% ethyl alcohol wipes after each use.
-

BD Rhapsody Cartridge

- Avoid pipetting bubbles into the cartridge. Before adding fluid to the cartridge, ensure that the pipette tip does not contain air.
 - To ensure an air-tight seal with the P1200M and P5000M pipettes, hold the pipette with one hand, and slightly twist the pipette to firmly seat a pipette tip on the pipette shaft.
 - Cells need to be prepared as close to cell loading in the cartridge as possible. Begin cell preparation during the prime or substrate treatment steps, and leave the cartridge in Cartridge Wash Buffer 2 (PN 650000061) until ready to proceed with cell loading.
-

**BD Rhapsody
Scanner software**

For easy access to analysis metrics, put the Rhapsody Data folder into the Quick Access folder:

1. Open a file explorer window.
 2. Enter **Public Documents** in the location box.
 3. Drag the **Rhapsody Data** folder to **Quick Access**.
 4. Open the **Rhapsody Data** folder.
 5. Open the folder for the appropriate experiment.
 6. Open the .csv files with Microsoft® Excel.
-

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3

Product Information

- [Required kit and storage conditions \(page 36\)](#)
- [Suggested kits \(page 45\)](#)
- [Reagents \(page 47\)](#)
- [Consumables \(page 50\)](#)
- [Equipment \(page 52\)](#)
- [Software \(page 55\)](#)

Required kit and storage conditions

Introduction

The BD Rhapsody™ Targeted Reagent Kit consists of four boxes:

- BD Rhapsody™ Cartridge Reagent Kit
- BD Rhapsody™ Cartridge Kit
- BD Rhapsody™ cDNA Kit
- BD Rhapsody™ Targeted Amplification Kit

The BD Rhapsody™ Targeted Reagent Kit—4 Pack (PN 633730) contains sufficient reagents and consumables to prepare four libraries. Each box can be purchased individually.

To perform sample multiplexing with the BD Rhapsody™ Single-Cell Analysis system, two additional components are required (PN 633780):

- BD® Single-Cell Multiplexing Kit—Human Sample Tag (12) Component
- BD® Single-Cell Multiplexing Kit Library Amplification Component

BD Rhapsody Targeted Reagent Kit—4 Pack (PN 633730)

- Store the four kit boxes at the specified storage temperatures. Use only non-frost free freezers for reagent storage.
- Keep the reagents on ice unless instructed otherwise.
- The BD Rhapsody™ Cartridge is single-use only.
- Limit preparation of mixes to $\leq 20\%$ overage.

Box	Component	Cap color	Quantity	Volume per unit	Storage
BD Rhapsody Cartridge Reagent Kit (PN 633731)	Cartridge Wash Buffer 1	Neutral	1 bottle	7 mL	2°C to 8°C
	Cartridge Wash Buffer 2	Neutral	1 bottle	4 mL	
	Sample Buffer	Neutral	1 bottle	28 mL	
	Lysis Buffer	Neutral	4 bottles	15 mL	
	Bead Wash Buffer	Neutral	1 bottle	10 mL	
	1 M DTT	White	1 vial	400 µL	
	Cell Capture Beads	Brown	4 vials	2 mL	
Waste Collection Container	Neutral	4 each	—		

Box	Component	Quantity	Storage
BD Rhapsody Cartridge Kit (PN 633733)	BD Rhapsody Cartridge	4 each	15°C to 25°C

Box	Component	Cap color	Quantity	Volume per unit	Storage
BD Rhapsody cDNA Kit (PN 633732)	Nuclease-Free Water	Neutral	2 vials	1 mL	-25°C to -15°C
	RT Buffer	Orange	1 vial	200 µL	
	RT 0.1M DTT	Orange	1 vial	50 µL	
	Reverse Transcriptase	Orange	1 vial	50 µL	
	dNTP	Orange	1 vial	100 µL	
	RNase Inhibitor	Orange	1 vial	50 µL	
	RT/PCR Enhancer	Black	1 vial	50 µL	
	10X Exonuclease I Buffer	Yellow	1 vial	100 µL	
	Exonuclease I	Yellow	1 vial	50 µL	
	Bead Resuspension Buffer	Black	1 vial	1 mL	

Box	Component	Cap Color	Quantity	Volume per unit	Storage
BD Rhapsody Targeted Amplification Kit (PN 633734)	Nuclease-Free Water	Neutral	1 vial	1 mL	-25°C to -15°C
	RT/PCR Enhancer	Black	1 vial	50 µL	
	PCR MasterMix	Blue	1 vial	800 µL	
	Elution Buffer	Blue	1 vial	400 µL	
	Universal Oligo	Blue	1 vial	110 µL	
	Library Forward Primer	Blue	1 vial	20 µL	
	Library Reverse Primer 1	Blue	1 vial	20 µL	
	Library Reverse Primer 2	Blue	1 vial	20 µL	
	Library Reverse Primer 3	Blue	1 vial	20 µL	
	Library Reverse Primer 4	Blue	1 vial	20 µL	
	Bead Resuspension Buffer	Black	1 vial	1 mL	

Kit	Components	Quantity	Volume per unit	Storage
BD Single-Cell Multiplexing Kit— Human Sample Tag (12) Component ^a	Sample Tag 1— Human	1 vial	20 µL	2°C to 8°C
	Sample Tag 2— Human	1 vial	20 µL	
	Sample Tag 3— Human	1 vial	20 µL	
	Sample Tag 4— Human	1 vial	20 µL	
	Sample Tag 5— Human	1 vial	20 µL	
	Sample Tag 6— Human	1 vial	20 µL	
	Sample Tag 7— Human	1 vial	20 µL	
	Sample Tag 8— Human	1 vial	20 µL	
	Sample Tag 9— Human	1 vial	20 µL	
	Sample Tag 10— Human	1 vial	20 µL	
	Sample Tag 11— Human	1 vial	20 µL	
	Sample Tag 12— Human	1 vial	20 µL	

- a. Required for multiplexing samples. Purchase by ordering PN 633780, which includes the BD Single-Cell Multiplexing Kit—Human Sample Tag (12) Component and the BD Single-Cell Multiplexing Kit Library Amplification Component.

Kit	Components	Quantity	Volume per unit	Storage
BD Single-Cell Multiplexing Kit Library Amplification Component (PN 633782) ^a	PCR MasterMix	1 vial	300 µL	-25°C to -15°C
	Elution Buffer	1 vial	500 µL	
	Universal Oligo	1 vial	20 µL	
	Library Forward Primer	1 vial	20 µL	
	Sample Tag PCR1 Primer	1 vial	20 µL	
	Sample Tag PCR2 Primer	1 vial	20 µL	

a. Required for multiplexing samples. This kit can be purchased as a standalone kit.

**BD Rhapsody™
targeted primer
panels**

Each panel includes a set of primers designed to target human (Hs) or mouse (Mm) genes. Each panel contains sufficient primers to prepare four libraries.

A supplement to a panel can be designed based on individual needs. The BD Rhapsody™ Panel Supplement contains primer pairs for additional targeted sequencing of selected genes. For more information on BD Rhapsody Panel Supplements, contact BD Biosciences technical support at scomix@bdscomix.bd.com and see [BD Rhapsody™ Panel Supplement \(PN 633742\) \(page 45\)](#).

Panel	Component	Cap color	Quantity	Vol. (µL)	Storage
BD Rhapsody™ Immune Response Panel Hs (PN 633750)	PCR1 Primers-Immune Response Panel Hs (tube label: PCR1-Immune Res. Hs)	Blue	1 vial	210	-25°C to -15°C
	PCR2 Primers-Immune Response Panel Hs (tube label: PCR2-Immune Res. Hs)	Blue	1 vial	50	
BD Rhapsody™ T Cell Expression Panel Hs (PN 633751)	PCR1 Primers-T Cell Expression Panel Hs (tube label: PCR1-T Cell Expression Hs)	Blue	1 vial	210	-25°C to -15°C
	PCR2 Primers-T Cell Expression Panel Hs (tube label: PCR2-T Cell Expression Hs)	Blue	1 vial	50	
BD Rhapsody™ Onco-BC Panel Hs (PN 633752)	PCR1 Primers-Onco-BC Panel Hs (tube label: PCR1-Onco-BC Hs)	Blue	1 vial	210	-25°C to -15°C
	PCR2 Primers-Onco-BC Panel Hs (tube label: PCR2-Onco-BC Hs)	Blue	1 vial	50	

Panel (continued)	Component	Cap color	Quantity	Vol. (µL)	Storage
BD Rhapsody™ Immune Response Panel Mm (PN 633753)	PCR1 Primers-Immune Response Panel Mm (tube label: PCR1-Immune Res. Mm)	Blue	1 vial	210	-25°C to -15°C
	PCR2 Primers-Immune Response Panel Mm (tube label: PCR2-Immune Res. Mm)	Blue	1 vial	50	

BD Rhapsody™ Targeted Training Kit—4 Pack (PN 633740)

For detailed information on the kit components, see [BD Rhapsody Targeted Reagent Kit—4 Pack \(PN 633730\) \(page 36\)](#).

Component	Quantity	Storage
BD Rhapsody Cartridge Reagent Kit (PN 633731)	1 kit	2°C to 8°C
BD Rhapsody Cartridge Kit (PN 633733)	1 kit	15°C to 25°C
BD Rhapsody cDNA Kit (PN 633732)	1 kit	-25°C to -15°C
BD Rhapsody Targeted Amplification Kit (PN 633734)	1 kit	
BD Rhapsody Immune Response Panel Hs (PN 633750)	1 kit	

Required kits from other vendors

Kit	Supplier	Catalog no.
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851

Suggested kits

BD Rhapsody™ Custom Panel Design (PN 633743) The BD Rhapsody Custom Panel Design contains a maximum of 500 custom primers. To order a BD Rhapsody Custom Panel Design, contact BD Biosciences scomix@bdscomix.bd.com.

Panel	Component	Cap color	Quantity	Vol.	Storage
BD Rhapsody Custom Panel Design (PN 633743)	BD Rhapsody™ 10X PCR1 Custom ID: xxxxxx ^a	Blue	1 vial	1,400 µL ^b	-25°C to -15°C
	BD Rhapsody™ 10X PCR2 Custom ID: xxxxxx ^a	Blue	1 vial	350 µL ^b	
	IDTE pH 8.0	Blue	1 vial	2.0 mL	

- Each design has a unique identifier that is provided with your BD Rhapsody Custom Panel primer design.
- To prepare a 1X dilution for use in the assay, dilute 1 part of PCR primer to 9 parts of IDTE buffer. Store the 1X dilution at -25°C to -15°C.

BD Rhapsody™ Panel Supplement (PN 633742) Add up to 100 additional supplemental primers to the BD Rhapsody targeted (predesigned) or custom panels for a maximum of 500 primers that can be used in an experiment. To order a BD Rhapsody Panel Supplement, contact BD Biosciences at scomix@bdscomix.bd.com.

Up to three BD Rhapsody Panel Supplements can be added per reaction provided that the total number of primers added is ≤500 (predesigned or custom panel plus panel supplement) and the primers have been designed to be compatible.

Panel	Component	Cap color	Quantity	Vol. (µL)	Storage
BD Rhapsody Panel Supplement (PN 633742)	BD Rhapsody™ 10X PCR1 Supp. ID: xxxxxx ^a	Blue	1 vial	120 µL ^b	-25°C to -15°C
	BD Rhapsody™ 10X PCR2 Supp. ID: xxxxxx ^a	Blue	1 vial	30 µL ^b	
	IDTE pH 8.0	Blue	1 vial	2.0 mL	

- Each design has a unique identifier that is provided with your BD Rhapsody Panel Supplement primer design.
- To prepare a 1X dilution for use in the assay, dilute 1 part of PCR primer to 9 parts of IDTE buffer. Store the 1X dilution at -25°C to -15°C.

Suggested kits from other vendors

Reagent	Supplier	Catalog no.
Agilent DNA High Sensitivity Kit	Agilent Technologies	5067-4626

Reagents

Required reagent for BD Rhapsody Cartridge workflow

Reagent	Supplier	Catalog no.
Ethyl alcohol, Pure (200 proof, molecular biology grade)	Sigma-Aldrich	E7023-500ML

Required reagents for sample multiplexing

Material ^a	Supplier	Catalog no.
BD® Stain Buffer (FBS)	BD Biosciences	554656
Nuclease-free water	Major supplier	—

- a. All reagents except for BD Stain Buffer (FBS) are required for the BD Rhapsody™ Single-Cell Analysis system.

Required reagents for cell preparation and staining

Reagent	Supplier	Catalog no.
Calcein AM cell-permeant dye ^a	Thermo Fisher Scientific	C1430
DRAQ7 TM ^a , 0.3 mM	BD Biosciences	564904
Dimethyl sulfoxide (DMSO)	Major supplier	—
70% ethyl alcohol or 70% isopropyl alcohol ^b	Major supplier	—

- a. Protect Calcein AM and DRAQ7 from light. Avoid multiple freeze-thaw cycles of Calcein AM. See manufacturer's storage recommendations.
- b. To clean the BD RhapsodyTM Express instrument and the BD RhapsodyTM Scanner, see the *BD RhapsodyTM Single-Cell Analysis System Installation and Maintenance Guide* (Doc ID: 43084). Instead of 70% alcohol, 10% (v/v) bleach may be used.

Required reagents for PCR product purification

Reagent	Supplier	Catalog no.
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
Ethyl alcohol, Pure (200 proof, molecular biology grade)	Sigma-Aldrich	E7023-500ML

**Suggested reagent
for cell suspension
preparation**

Reagent	Supplier	Catalog no.
1X RBC Lysis Buffer	Thermo Fisher Scientific	00-4333-57
Phosphate-buffered saline (calcium- and magnesium-free)	Major supplier	—

Consumables

Required consumables

Consumable	Supplier	Catalog no.
Falcon® Tube with Cell Strainer Cap	Thermo Fisher Scientific	352235
Improved Neubauer Hemocytometer	INCYTO	DHC-N01-5
DNA LoBind Tubes, 1.5 mL ^a	Eppendorf	0030108051
DNA LoBind Tubes, 5 mL Note: These are the Bead Retrieval Tubes to be used with the BD Rhapsody Express instrument.	Eppendorf	0030108310
Low retention filtered pipette tips, 10 µL ^a	Major supplier	—
Low retention filtered pipette tips, 200 µL ^a	Major supplier	—
Low retention filtered pipette tips, 1,000 µL ^a	Major supplier	—
Gilson™ PIPETMAN™ Tipack™ Filter Tips, 100–1,200 µL for BD Rhapsody™ P1200M pipette	Thermo Fisher Scientific	F171803G

Consumable (continued)	Supplier	Catalog no.
Gilson PIPETMAN Tipack Racked Pipet Tips, 500–5,000 µL for BD Rhapsody™ P5000M pipette	Thermo Fisher Scientific	F161370G
Qubit™ Assay Tubes	Thermo Fisher Scientific	Q32856
0.2 mL PCR 12-strip tubes ^a	Major supplier	—
10 mL sterile serological pipettes	Major supplier	—
Premoistened cleaning wipes with 70% ethyl alcohol or 70% isopropyl alcohol	Major supplier	—

- a. Provide material in both pre- and post-amplification workspaces.

Suggested consumables

Consumable	Supplier	Catalog no.
Lint-free wipers	Major supplier	—

Equipment

Required equipment

Supply pre- and post-amplification workspaces with the required equipment. You may need two sets of some equipment.

Equipment	Box	Components
BD Rhapsody™ Single-Cell Analysis system	BD Rhapsody Scanner (PN 633701)	<ul style="list-style-type: none"> • BD Rhapsody Scanner • Power supply and cable • Hemocytometer Adapter (PN 633703) • BD Rhapsody P1200M pipette (PN 633704) • BD Rhapsody P5000M pipette (PN 633705)
	BD Rhapsody Express instrument (PN 633702)	<ul style="list-style-type: none"> • BD Rhapsody Express instrument

Equipment	Supplier	Catalog no.
6-Tube Magnetic Separation Rack for 1.5 mL tubes ^a	New England Biolabs	S1506S
Large magnetic separation stand	V&P Scientific, Inc.	VP 772FB-1
Clear acrylic cylinder adapter for 15 mL tube magnet ^b	V&P Scientific, Inc.	VP 772FB-1A

Equipment (continued)	Supplier	Catalog no.
Low-profile magnetic separation stand for 0.2 mL, 8-strip tubes	V&P Scientific, Inc. OR	VP 772F4-1
	Clontech	635011
Thermomixer (16°C to 37°C, 1,200 rpm): <ul style="list-style-type: none"> • ThermoMixer® C • SmartBlock™ Thermoblock 1.5 mL 	Eppendorf	<ul style="list-style-type: none"> • 5382000023 • 5360000038
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
Heat block capable of 80°C Suggested: VWR® Advanced Mini Dry Block Heater with Heated Lid	VWR	10153-348
<ul style="list-style-type: none"> • 2100 Bioanalyzer OR • 4200 TapeStation Instrument 	Agilent Technologies	<ul style="list-style-type: none"> • G2940CA • G2991AA
Thermal cycler with heated lid	Major supplier	—
Water bath OR incubator at 37°C	Major supplier	—
Laminar flow hood	Major supplier	—
Digital timer ^a	Major supplier	—

Equipment (continued)	Supplier	Catalog no.
Pipettes (P2, P10, P20, P200, P1000) ^a	Major supplier	—
Multi-channel pipette, 2–20 µL OR 20–200 µL ^a	Major supplier	—
Microcentrifuge for 1.5–2.0 mL tubes ^a	Major supplier	—
Microcentrifuge for 0.2 mL tubes	Major supplier	—
Centrifuge and rotor for 15 mL tubes	Major supplier	—
Vortexer ^a	Major supplier	—
Pipet-Aid	Major supplier	—

- a. Provide material in both pre- and post-amplification workspaces.
- b. Holds 5 mL LoBind Tube in magnet.

Suggested equipment

Equipment	Supplier	Catalog no.
Logitech® Wireless Combo (keyboard and mouse)	Major supplier	MK270
Barcode reader with keyboard wedge, USB 2.0 OR 3.0 compatible ^a	Major supplier	—
Phase-contrast microscope	Major supplier	—

- a. The external barcode reader is optional. The scanner has an internal barcode reader. To purchase an external barcode reader, BD Biosciences recommends the USB Automatic Barcode Scanner Scanning Barcode Reader with Hands-Free Adjustable Stand (Brainydeal) or equivalent.

Software

BD Rhapsody™ Analysis pipeline

The BD Rhapsody Analysis pipeline takes the FASTQ read files and reference files for gene alignment. The pipeline filters by read quality, annotates R1 and R2 reads, annotates molecules, determines putative cells, determines the sample of origin (sample multiplexing only), generates expression matrices, generates a metrics summary, and performs clustering analysis.

For installation, see the *BD Single Cell Genomics Analysis Setup User Guide* (Doc ID: 47383).

For detailed information on the BD Rhapsody Analysis pipeline, see the *BD Single Cell Genomics Bioinformatics Handbook* (Doc ID: 54169).

BD® Data View

BD Data View is a software tool for visualization and exploratory analysis of output files generated following bioinformatics analysis. The software is included with the purchase of the BD Rhapsody Single-Cell Analysis system and is optional for use.

For installation, see the *BD Single Cell Genomics Analysis Setup User Guide* (Doc ID: 47383).

For detailed instructions on using BD Data View, see the *BD Single Cell Genomics Bioinformatics Handbook* (Doc ID: 54169).

4

Preparing the BD Rhapsody™ Cartridge

- [Priming the BD Rhapsody Cartridge \(page 58\)](#)
- [Treating the surface of the cartridge \(page 66\)](#)

Priming the BD Rhapsody Cartridge

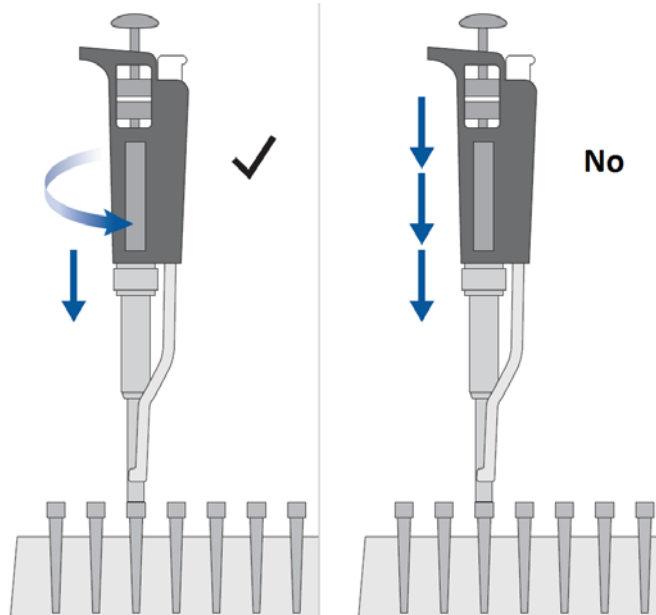
Before you begin After opening the cartridge packet, ensure that you prepare the cartridge in ≤ 1 day. If you leave the cartridge at 2°C to 8°C , equilibrate to 15°C to 25°C for 5 minutes.

If cell preparation takes ≥ 4 hours, begin preparing cells before cartridge preparation. See [Single sample: Preparing cells for the BD Rhapsody™ Cartridge \(page 67\)](#) or [\(Optional\) Multiplexed samples: Preparing cells for the BD Rhapsody™ Cartridge \(page 79\)](#).

- Equilibrate these reagents at 15°C to 25°C for ≥ 30 minutes before use:
 - Cartridge Wash Buffer 1 (PN 650000060)
 - Cartridge Wash Buffer 2 (PN 650000061)
 - Put these reagents on ice:
 - Sample Buffer (PN 650000062)
 - 1 M DTT (PN 650000063)
 - Lysis Buffer (PN 650000064)
 - DRAQ7 (protected from light)
 - Calcein AM (protected from light)
 - Once at 15°C to 25°C , resuspend Calcein AM (1 mg; Thermo Fisher Scientific cat. no. C1430) in $503.0\ \mu\text{L}$ of DMSO for a final stock concentration of 2 mM. Store the stock solution at -25°C to -15°C , and protect it from light.
 - Review pipette settings and operation. See [Pipette Settings \(page 21\)](#).
 - For the use of two cartridges, see [Workflow with two BD Rhapsody™ Cartridges \(page 215\)](#).
-

Procedure

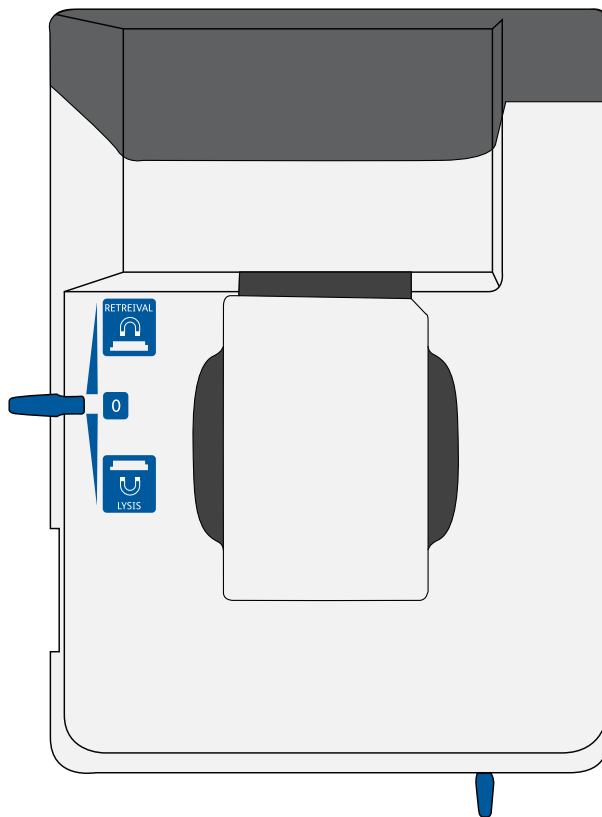
To ensure an air-tight seal with the BD Rhapsody™ P1200M or P5000M pipette, hold the pipette with one hand, and slightly twist the pipette to firmly seat a pipette tip on the pipette shaft:



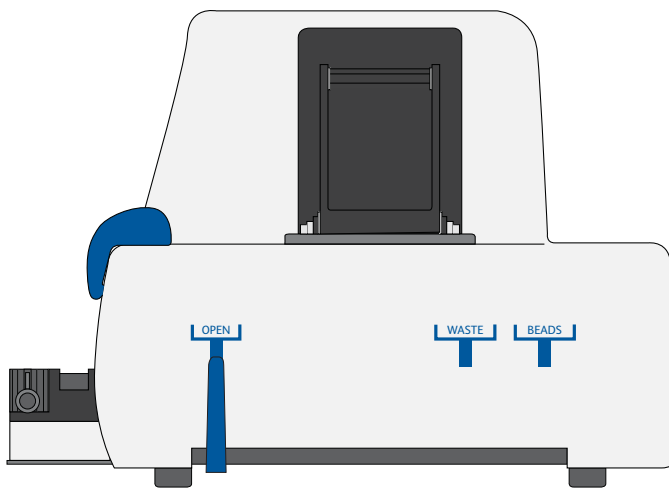
Avoid introducing bubbles while pipetting into the BD Rhapsody Cartridge.

Change pipette tips before every pipetting step.

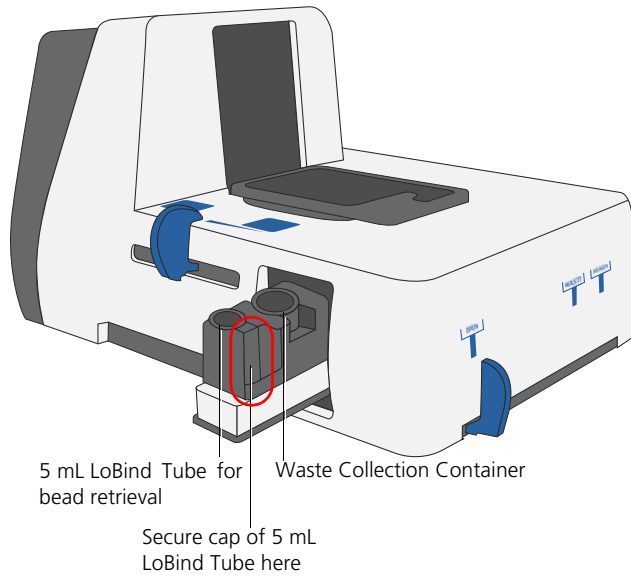
1. Move the left slider to the middle (0) position on the Express instrument. The Retrieval (top) magnet and Lysis (bottom) magnets are away from the cartridge tray:



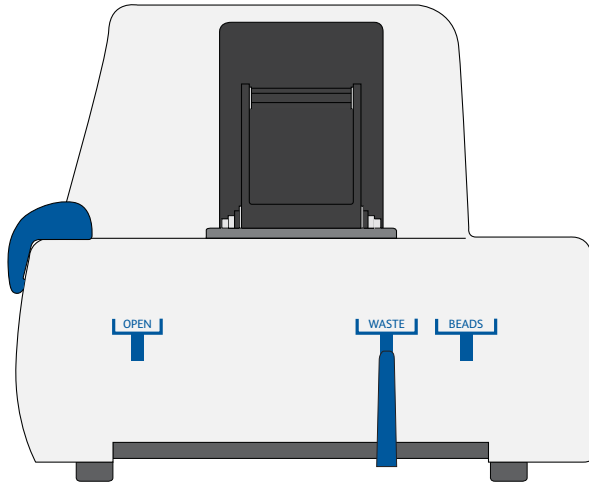
2. Move the front slider to **OPEN**:



3. Remove the cap of a Waste Collection Container (PN 650000090), and insert both the container and a new 5 mL LoBind Tube (Eppendorf cat. no. 0030108310) for bead retrieval into the appropriate slots in the drawer. Secure the cap of the 5 mL LoBind Tube to the holder:

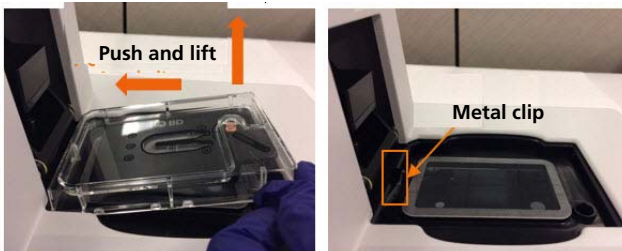


4. Move the front slider to **WASTE**:

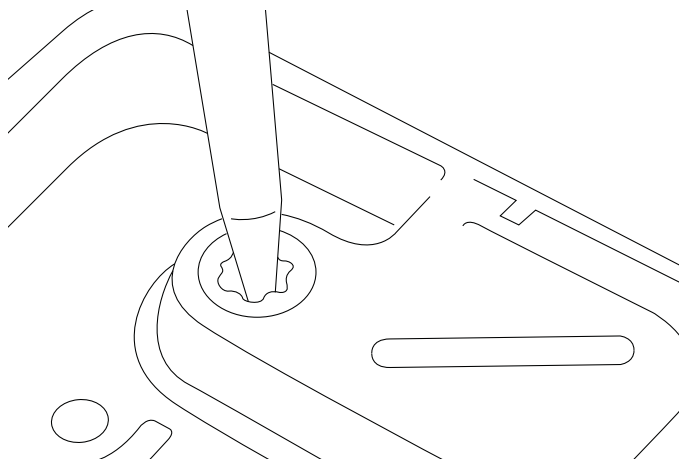


5. Push the cartridge into the far end of the tray to match cartridge and tray notches. Lay the cartridge flat, and release it. Ensure that the cartridge is flat in the tray and the barcode faces out.

Note: To remove the cartridge from the Express instrument, push in the cartridge, and lift it from the tray:



Before loading the reagent into the cartridge, align the pipette tip with the inlet hole of the gasket, and then press down on the P1200M pipette to seal the pipette tip against the gasket and avoid leaks:



Note: In **Prime/Treat** mode, press the button once to aspirate 700 μ L, and press the button again to dispense 700 μ L.

6. Load the cartridge with 700 μ L of 100% (absolute) ethyl alcohol using the P1200M pipette in **Prime/Treat** mode.
7. Load the cartridge with 700 μ L of air using the P1200M pipette in **Prime/Treat** mode.

8. Load the cartridge with 700 μ L of Cartridge Wash Buffer 1 (PN 650000060) with the P1200M pipette in **Prime/Treat** mode.
 9. Leave the cartridge on the tray at 15°C to 25°C for 1 minute.
-

Treating the surface of the cartridge

Procedure

1. Load the cartridge with 700 μ L of air using the P1200M pipette in **Prime/Treat** mode.
2. Load the cartridge with 700 μ L of Cartridge Wash Buffer 1 (PN 650000060) using the P1200M pipette in **Prime/Treat** mode.
3. Leave the cartridge on the tray at 15°C to 25°C for 10 minutes.
4. Load the cartridge with 700 μ L of air using the P1200M pipette in **Prime/Treat** mode.
5. Load the cartridge with 700 μ L of Cartridge Wash Buffer 2 (PN 650000061) using the P1200M pipette in **Prime/Treat** mode.

Stopping point: The cartridge can be stored at 15°C to 25°C for ≤ 4 hours. You can leave the cartridge on the tray. The performance of the cartridge has not been validated at 15°C to 25°C storage for >4 hours.

6. Proceed according to single or multiplexed samples:
 - [Single sample: Preparing cells for the BD Rhapsody™ Cartridge \(page 67\)](#)
 - [\(Optional\) Multiplexed samples: Preparing cells for the BD Rhapsody™ Cartridge \(page 79\)](#)
-

5

Single sample: Preparing cells for the BD Rhapsody™ Cartridge

- [Staining cells with viability markers \(page 68\)](#)
- [Counting cells with the BD Rhapsody Scanner \(page 71\)](#)

Staining cells with viability markers

Introduction

Stain cells with Calcein AM to count live cells and DRAQ7™ to count dead cells.

To ensure accurate cell counting by the BD Rhapsody™ Scanner, stain cells with Calcein AM and DRAQ7 according to the protocol. If the cells are fluorescently labelled by other reagents or express fluorescent proteins, BD recommends proceeding with staining but testing for possible interference.

Cell viability <50% may impact experimental results. If possible, use cells of high viability.

Bead loading efficiency may be reduced in the BD Rhapsody Cartridge if the cell diameter is >20 µm.

If cell preparation takes ≥4 hours, begin preparing cells before cartridge preparation.

Before you begin

Follow sterility best practices. See [Sterility \(page 32\)](#).

Procedure**Protect Calcein AM and DRAQ7 from light until ready to use.**

1. Prepare a single cell suspension according to your laboratory protocol or the guidelines in [Guidelines to preparing single cell suspensions \(page 195\)](#).
2. Proceed as follows:
 - If you are using biological samples that contain red blood cell contamination, red blood cell lysis is required. See [Lysing red blood cells \(RBCs\) \(page 197\)](#), and then proceed with cell staining in step 3.
 - If the cells are not resuspended in cold Sample Buffer (PN 650000062), centrifuge the cell suspension at 300 x g for 5 minutes, aspirate the supernatant, and leave ~20 µL of the residual supernatant. Add up to 620 µL total volume of cold Sample Buffer, and then proceed with cell staining in step 3.
3. Add 3.1 µL of 2 mM Calcein AM and 3.1 µL of 0.3 mM DRAQ7 to the 620 µL volume of cell suspension (1:200 dilution).
4. Gently pipet the suspension up and down to mix well.
5. Incubate the suspension in the dark in a water bath, incubator, or heat block at 37°C for 5 minutes.
6. Pass the cells through a Falcon® Tube with Cell Strainer Cap (Thermo Fisher Scientific cat. no. 352235).

For low abundance or low volume samples, filtering is optional at this step. BD Biosciences recommends filtering the final sample before loading cells into the cartridge. See [Loading cells into the cartridge \(page 94\)](#).

7. Count cells immediately:

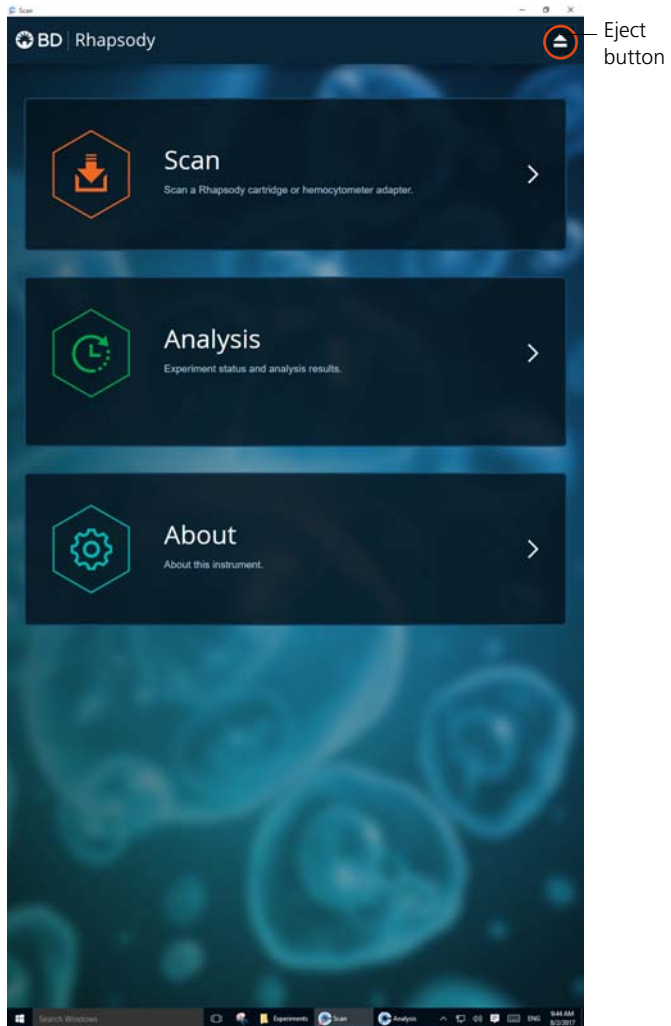
Keep cells on ice, and protect them from light.

- a. Gently mix cells well by pipette, and then gently pipet 10 μ L of the cell suspension into one chamber of the INCYTO™ disposable hemocytometer.
 - b. Insert the hemocytometer into the Hemocytometer Adapter so that the A and B sides of the hemocytometer align with A and B on the Hemocytometer Adapter.
 - c. Wait for 1 to \leq 5 minutes after loading.
 - d. Count the cells in the BD Rhapsody™ Scanner.
-

Counting cells with the BD Rhapsody Scanner

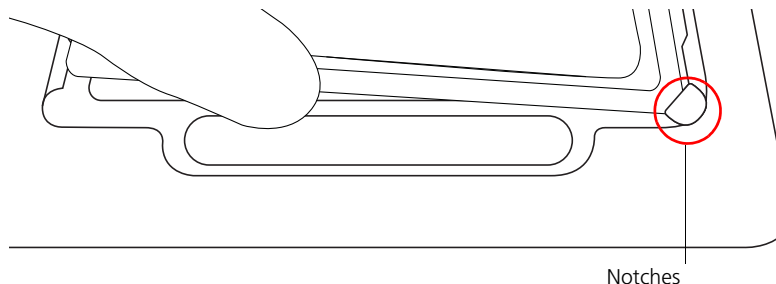
Procedure

1. Start the scanner software so that the main menu opens:



Notes:

- To return to the main menu on any screen, tap the BD logo in the upper left.
 - For updates to the BD Rhapsody Scanner software, contact BD Biosciences technical support at scomix@bdscmix.bd.com.
 - To troubleshoot scanner software error messages, see [BD Rhapsody Scanner software messages \(page 190\)](#).
2. Tap **Scan**. The tray door of the scanner opens automatically, and the tray is ejected.
 3. Place the Hemocytometer Adapter on the scanner tray so that the notch of the adapter matches the corner notch, and the barcode faces toward the front of the instrument:

**Note:**

- The scanner displays an alert if the adapter is in the wrong orientation.
 - You can manually enter the barcode in the application.
4. Tap **Continue**. The tray retracts, the door closes, and the scanner displays the hemocytometer setup screen.

5. Select the protocol from the drop-down menu. Enter the experiment name, sample name, protocol, and user. (If an experiment name was entered previously, select it from the drop-down menu.)

Do not use commas in the experiment name, sample name, or user.

For example:

Experiment: 20180131_SL_XH

Sample: Jurkat

Protocol: Hemocytometer

User: SL

Cartridge: 0107001033A

Side A

Start Side A Scan

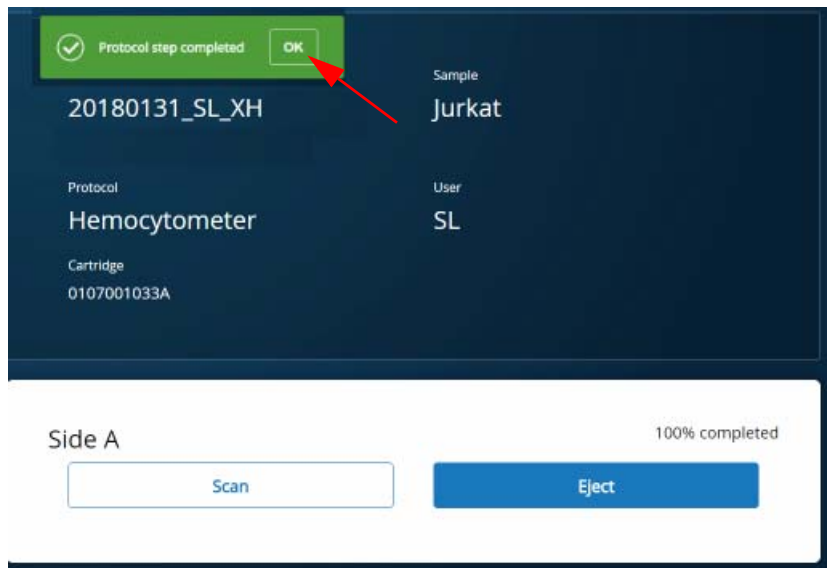
Side B

Note: Enter an experiment name that is appropriate for both the hemocytometer and cartridge scans. If necessary, tap the touch keyboard icon in the bottom right on the screen to enter information. There are no character or length limits.

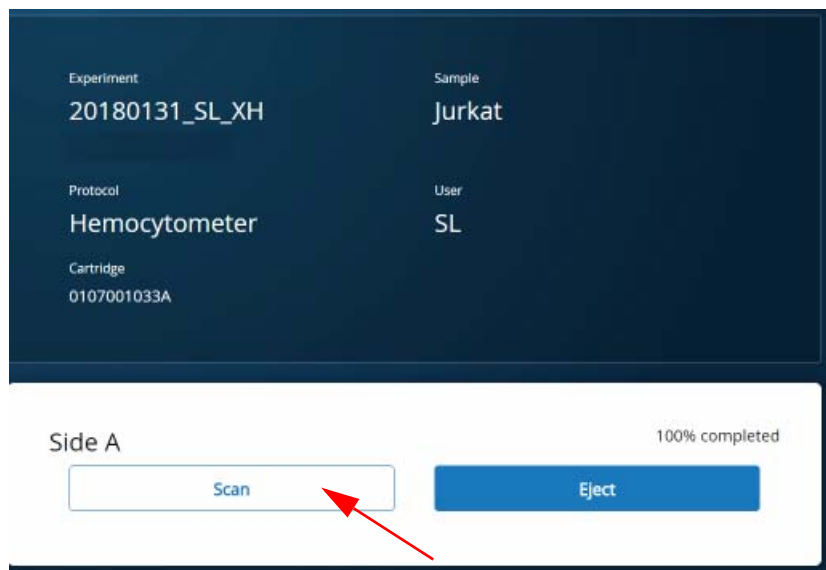
6. Tap **Side A** or **Side B**, depending on which side the cells were loaded, and then Select **Start Side A Scan** or **Start Side B Scan**, as required.

Note: To stop the scan, tap **Stop**, and then tap **Stop** again. To continue with the scan, tap **Continue**.

7. After the scan is complete, tap **OK**:



8. Scan the other side of the hemocytometer or eject it:
 - Scan the other side: Tap **Scan**, enter a new sample name, and then repeat steps 6–7. The hemocytometer remains in the scanner and saves time waiting for the tray to eject. For example:



- Eject the hemocytometer: Tap **Eject**. The Hemocytometer Adapter is ejected from the scanner, and the remove cartridge screen displays.
Note: The analysis runs in the background. For example, the cell scan step for Jurkat and Ramos cells is complete.
9. Remove the Hemocytometer Adapter from the tray, and then tap **Done** to retract the tray and return to the main menu.
 10. On the main menu, tap **Analysis**, and then tap the experiment. If no results are displayed, tap **Analyze**.

11. View the **total cell concentration** and **cell viability** on the results screen. For example, cell concentrations and viabilities are displayed for Jurkat and Ramos samples:

Jurkat
Hemocytometer 0107001033A

Scan Date	Sample	Step	Analysis Status
2018-01-31 10:01:43.07	Jurkat	Side A	✓ Completed

Analysis

Total cell concentration (live and dead)	423.64
Cell viability	92.24

Ramos
Hemocytometer 0107001033A

Scan Date	Sample	Step	Analysis Status
2018-01-31 10:02:39.54	Ramos	Side B	✓ Completed

Analysis

Total cell concentration (live and dead)	375.99
Cell viability	92.53

Note: If no value could be calculated, then **NaN** (not a number) is displayed. See [Troubleshooting \(page 177\)](#).

12. Proceed as follows:

- If the cell concentration is $\leq 1,000$ cells/ μ L, proceed to step [13](#).
- If the cell concentration is $> 1,000$ cells/ μ L, dilute the cell suspension in cold Sample Buffer (PN 650000062) to ~ 200 – 800 cells/ μ L. Recount the cells in the hemocytometer following steps [1–11](#), and then proceed to step [13](#).

13. Tap **Prepare** at the top of the results screen to display the Samples Calculator screen. See [Loading cells into the cartridge \(page 94\)](#).

14. Dispose of the hemocytometer according to local safety regulations.

15. Immediately proceed to Single cell capture on the BD Rhapsody Cartridge. [Retrieving single cell mRNA with the BD Rhapsody™ Cartridge and Cell Capture Beads \(page 93\)](#).

Minimize the time between cell pooling and single cell capture.

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6

(Optional) Multiplexed samples: Preparing cells for the BD Rhapsody™ Cartridge

- [Preparing single cell suspensions \(page 80\)](#)
- [Labelling samples with Sample Tags \(page 81\)](#)
- [Staining cells with viability markers \(page 82\)](#)
- [Counting cells with the BD Rhapsody Scanner \(page 85\)](#)

Preparing single cell suspensions

- Before you begin** Unless specified, perform the procedure in a pre-amplification workspace.
- Protect Calcein AM and DRAQ7 from light until ready to use.
- Some cell dissociation reagents, such as trypsin, may damage cell surface markers and decrease Sample Tag sensitivity. Use cell dissociation reagents suitable for cell surface staining.
- Cells may be lost during the wash steps (25–50%). For low-abundance samples (<100,000 cells), account for cell loss when preparing single cell samples.
- Cell viability <50% may impact experimental results. If possible, use cells of high viability.
- Bead loading efficiency may be reduced in the BD Rhapsody™ Cartridge if the cell diameter is >20 µm.
- If cell preparation takes ≥4 hours, begin preparing cells before cartridge preparation.
- Follow sterility best practices. See [Sterility \(page 32\)](#).
-

- Procedure**
1. Prepare a single cell suspension according to your laboratory protocol or the guidelines in [Guidelines to preparing single cell suspensions \(page 195\)](#).
 2. If you are using biological samples that contain red blood cell contamination, red blood cell lysis is required. See [Lysing red blood cells \(RBCs\) \(page 197\)](#), and then proceed with [Labelling samples with Sample Tags \(page 81\)](#).
-

Labelling samples with Sample Tags

1. Resuspend 12,000– 2×10^6 cells in 200 μL of BD Stain Buffer (FBS).
2. Briefly centrifuge the Sample Tag tubes to collect the contents at the bottom.
3. For each sample, transfer 180 μL of the cell suspension to a Sample Tag tube, and mix by pipette only.



Caution. Aqueous buffered solution (Sample Tag) contains BSA and $\leq 0.1\%$ 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 the cell suspension at 15°C to 25°C for 20 minutes.
5. Add 200 μL of BD Stain Buffer (FBS) to the cell suspension, and mix by pipette only.
6. Centrifuge the cells at $300 \times g$ for 5 minutes.
7. Remove the supernatant without disturbing the pellet, and resuspend the pellet in 500 μL of BD Stain Buffer (FBS).

For low-abundance samples, leave 50 μL of supernatant before resuspending the pellet in 500 μL of BD Stain Buffer (FBS).

8. Centrifuge the cells at $300 \times g$ for 5 minutes.
9. Remove the supernatant without disturbing the pellet, and resuspend the cells in 500 μL of **Sample Buffer** from BD Rhapsody™ reagents.

For low-abundance samples, leave 50 μL of supernatant. Resuspend the cells in Sample Buffer so that the total volume is 100 μL .

10. Proceed immediately to [Staining cells with viability markers \(page 82\)](#).

Staining cells with viability markers

Introduction

Stain cells with Calcein AM to count live cells and DRAQ7 to count dead cells.

To ensure accurate cell counting by the BD Rhapsody™ Scanner, stain cells with Calcein AM and DRAQ7 according to the protocol, even if the cells are fluorescently labeled by other reagents or express fluorescent proteins.

Before you begin

Follow sterility best practices. See [Sterility \(page 32\)](#).

Procedure**Protect Calcein AM and DRAQ7 from light until ready to use.**

1. Add 2 mM Calcein AM and 0.3 mM DRAQ7 at 1:200 dilution each to the total volume of cell suspension. For example, pipette 2.5 µL of each dye into 500 µL of cell suspension.
2. Gently pipet the suspension up and down to mix well.
3. Incubate the suspension in the dark at 37°C for 5 minutes.
4. Pass the cells through a Falcon® Tube with Cell Strainer Cap (Thermo Fisher Scientific cat. no. 352235).

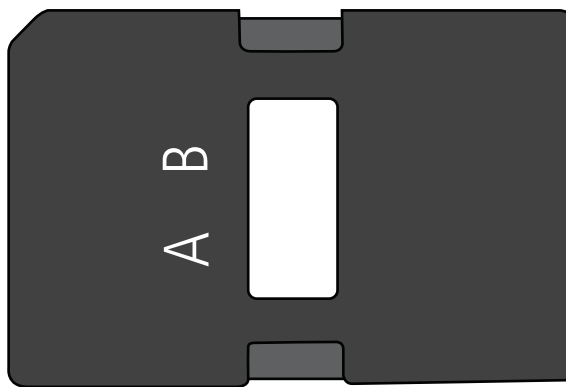
For low abundance or low volume samples, filtering is optional at this step. BD Biosciences recommends filtering the final pooled sample before loading cells into the cartridge. See [Loading cells into the cartridge \(page 94\)](#).

5. Count cells immediately:

Keep cells on ice, and protect them from light.

- a. Gently mix cells well by pipette, and then gently pipet 10 µL of the cell suspension into one chamber of the INCYTO™ disposable hemocytometer.

- b. Insert the hemocytometer into the Hemocytometer Adapter so that the A and B sides of the hemocytometer align with A and B on the Hemocytometer Adapter:

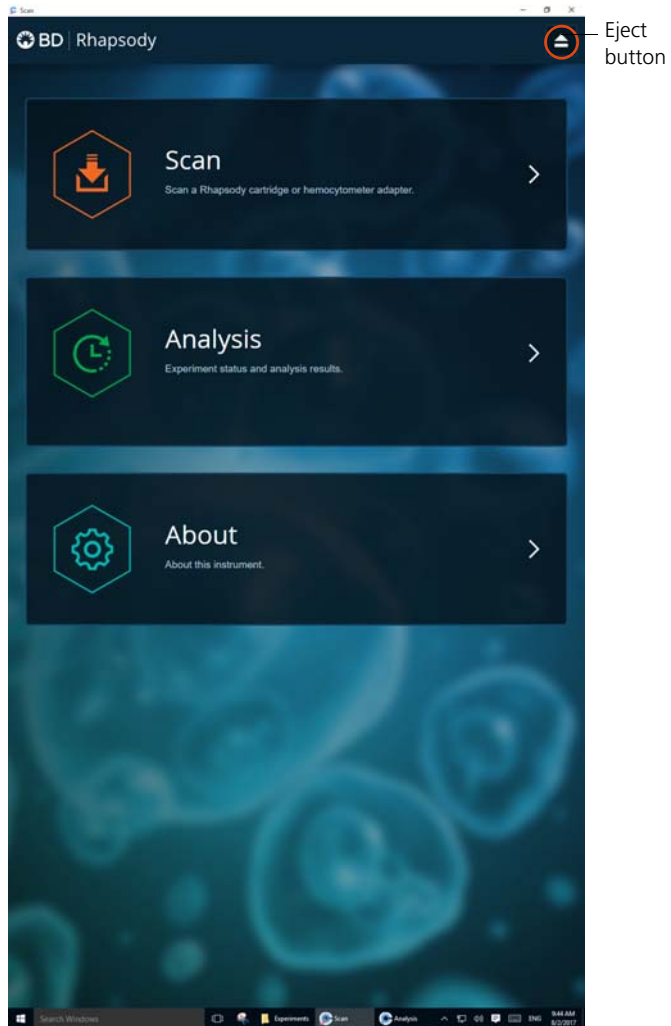


- c. Count the cells in the BD Rhapsody Scanner ≤ 5 minutes after loading.
-

Counting cells with the BD Rhapsody Scanner

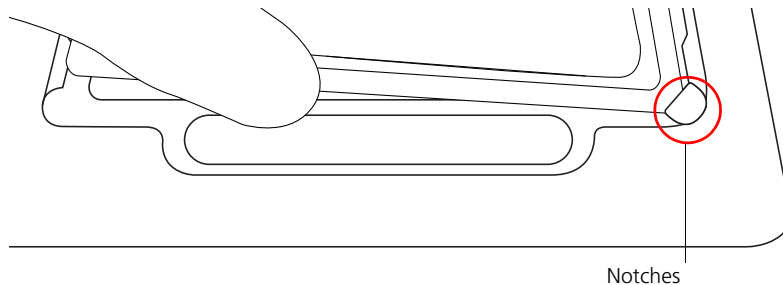
Procedure

1. Start the scanner software so that the main menu opens:



Notes:

- To return to the main menu on any screen, tap the BD logo in the upper left.
 - For updates to the BD Rhapsody Scanner software, contact BD Biosciences technical support at scomix@bdscmix.bd.com.
 - To troubleshoot scanner software error messages, see [BD Rhapsody Scanner software messages \(page 190\)](#).
2. Tap **Scan**. The tray door of the scanner opens automatically, and the tray is ejected.
 3. If necessary, wipe condensation from the top surface of the cartridge with a lint-free wiper.
 4. Place the Hemocytometer Adapter on the scanner tray so that the notch of the adapter matches the corner notch, and the barcode faces toward the front of the instrument:

**Note:**

- The scanner displays an alert if the adapter is in the wrong orientation.
 - You can manually enter the barcode in the application.
5. Tap **Continue**. The tray retracts, the door closes, and the scanner displays the hemocytometer setup screen.

6. Select the protocol from the drop-down menu. Enter the experiment name, sample name, protocol, and user. (If an experiment name was entered previously, select it from the drop-down menu.)

Do not use commas in the experiment name, sample name, or user name.

For example:

The screenshot displays the software interface for configuring a scan. It is divided into three main sections:

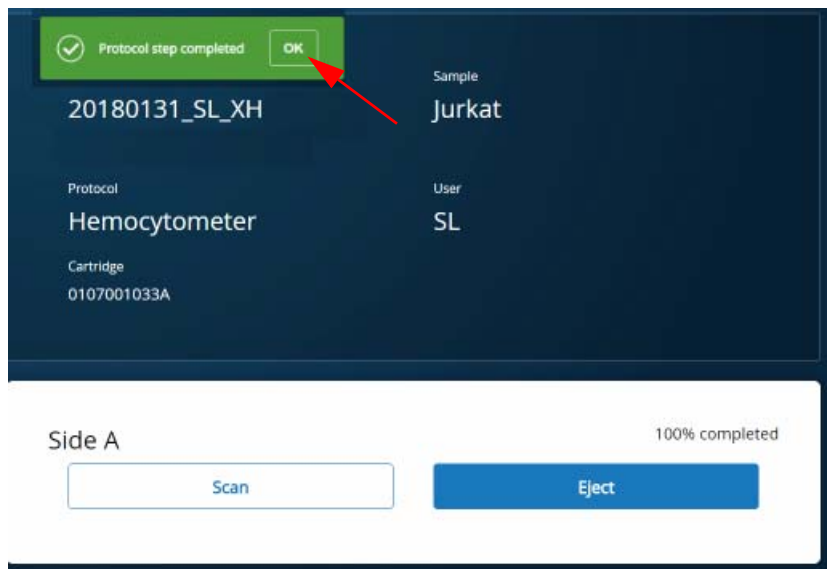
- Configuration Section:** Contains four input fields:
 - Experiment:** A dropdown menu with the value "20180131_SL_XH".
 - Sample:** A dropdown menu with the value "Jurkat".
 - Protocol:** A dropdown menu with the value "Hemocytometer".
 - User:** A text input field with the value "SL".
- Cartridge Section:** A text input field containing the value "0107001033A".
- Side A Section:** A white panel with the heading "Side A" and a blue button labeled "Start Side A Scan".
- Side B Section:** A dark blue panel with the heading "Side B".

Note: Enter an experiment name that is appropriate for both the hemocytometer and cartridge scans. If necessary, tap the touch keyboard icon in the bottom right on the screen to enter information. There are no character or length limits.

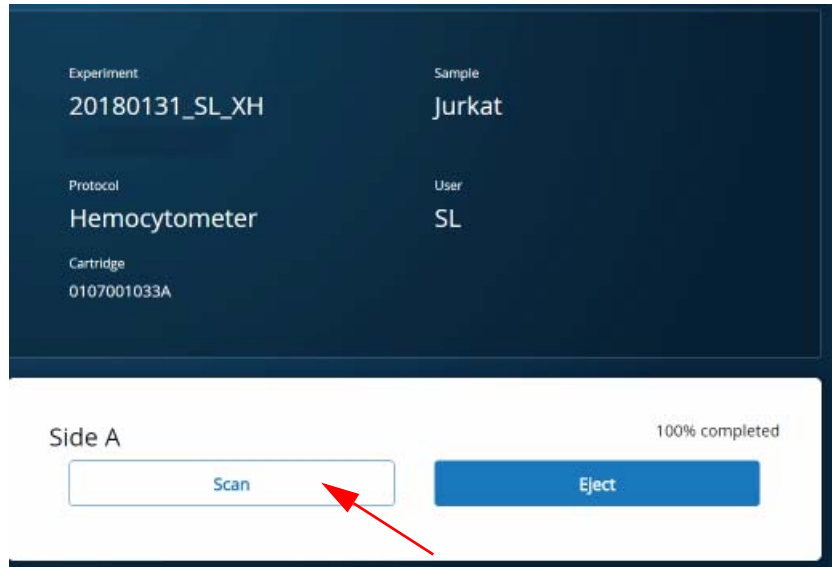
7. Tap **Side A** or **Side B**, depending on which side the cells were loaded, and then Select **Start Side A Scan** or **Start Side B Scan**, as required.

Note: To stop the scan, tap **Stop**, and then tap **Stop** again. To continue with the scan, tap **Continue**.

8. After the scan is complete, tap **OK**. For example:



9. Scan the other side of the hemocytometer, or eject it:
 - Scan the other side: Tap **Scan**, enter a new sample name, and then repeat steps 7–8. The hemocytometer remains in the scanner and saves time waiting for the tray to eject. For example:



- Eject the hemocytometer: Tap **Eject**. The Hemocytometer Adapter is ejected from the scanner, and the remove cartridge screen displays.

Note: The analysis runs in the background. For example, the cell scan step for Jurkat and Ramos cells is complete.
10. Remove the Hemocytometer Adapter from the tray, and then tap **Done** to retract the tray and return to the main menu.
 11. On the main menu, tap **Analysis**, and then tap the experiment. If no results are displayed, tap **Analyze**.

12. View the **total cell concentration** and **cell viability** on the results screen. For example, cell concentrations and viabilities are displayed for samples labelled with Sample Tags. For example:

The screenshot displays the results for three sample tags. Each tag's results are shown in a separate panel. The analysis results for Tag1 and Tag2 are highlighted with red boxes.

Tag1
Hemocytometer 0107001032A

Date	Sample	Step	Analysis Status
2017-12-08 09:29:17.42	Tag1	Side A	✓ Completed

Analysis

Total cell concentration (live and dead)	246.72
Cell viability	100.00

Tag2
Hemocytometer 0107001032A

Date	Sample	Step	Analysis Status
2017-12-08 09:29:49.34	Tag2	Side B	✓ Completed

Analysis

Total cell concentration (live and dead)	21.79
Cell viability	100.00

Tag3

Note: If no value could be calculated, then NaN (not a number) is displayed. See [Troubleshooting \(page 177\)](#).

13. Proceed as follows:

- If the cell concentration is $\leq 1,000$ cells/ μL , proceed to step 14.
 - If the cell concentration is $> 1,000$ cells/ μL , dilute the cell suspension in cold Sample Buffer (PN 650000062) to ~ 200 – 800 cells/ μL . Recount the cells in the hemocytometer following steps 1–12, and then proceed to step 14.
14. Tap **Prepare** at the top of the results screen to display the Samples Calculator screen. [Loading cells into the cartridge \(page 94\)](#).
15. Dispose of the hemocytometer according to local safety regulations.
16. Immediately proceed to Single cell capture on the BD Rhapsody™ Cartridge. [Retrieving single cell mRNA with the BD Rhapsody™ Cartridge and Cell Capture Beads \(page 93\)](#).

Minimize the time between cell pooling and single cell capture.

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7

Retrieving single cell mRNA with the BD Rhapsody™ Cartridge and Cell Capture Beads

- [Loading cells in the BD Rhapsody Cartridge \(page 94\)](#)
- [Imaging cells in the cartridge \(page 103\)](#)
- [Preparing Cell Capture Beads \(page 108\)](#)
- [Loading Cell Capture Beads and imaging \(page 109\)](#)
- [Lysing cells and retrieving Cell Capture Beads \(page 110\)](#)

Loading cells in the BD Rhapsody Cartridge

- Before you begin**
- Prepare cells as close to cell loading as possible. See [Single sample: Preparing cells for the BD Rhapsody™ Cartridge \(page 67\)](#) or [\(Optional\) Multiplexed samples: Preparing cells for the BD Rhapsody™ Cartridge \(page 79\)](#).
 - Keep the other reagents, including Sample Buffer (PN 650000062), on ice, unless instructed otherwise.

Loading cells into the cartridge To ensure an air-tight seal with the BD Rhapsody™ P1200M and P5000M pipettes, hold the pipette with one hand, and slightly twist the pipette to firmly seat a pipette tip on the pipette shaft.

Change pipette tips before every pipetting step.

1. Refer to the following table to determine an acceptable multiplet rate for the number of captured cells on retrieved Cell Capture Beads:

Estimated multiplet rate based on the number of captured cells on retrieved Cell Capture Beads

Number of captured cells on retrieved Cell Capture Beads (target) ^{a,b}	Estimated multiplet rate (%)
100	0.0
500	0.1
1,000	0.2
2,000	0.5
3,000	0.7
4,000	1.0
5,000	1.2
6,000	1.4

Number of captured cells on retrieved Cell Capture Beads (target) ^{a,b} (continued)	Estimated multiplet rate (%)
7,000	1.7
8,000	1.9
9,000	2.1
10,000	2.4
11,000	2.6
12,000	2.8
13,000	3.1
14,000	3.3
15,000	3.5
16,000	3.8
17,000	4.0
18,000	4.2
19,000	4.5
20,000	4.7

- a. The number of cells sequenced may be less than the number of cells captured due to bead loss during handling, panel choice, and sample composition. The validated range of cells sequenced is 100–10,000 cells.
- b. This sample calculator gives loading calculations based on *total cell count*, which does not consider cell viability. The viable cells captured in the cartridge may be less than the targeted number of captured cells if the viability of the sample is <100%.

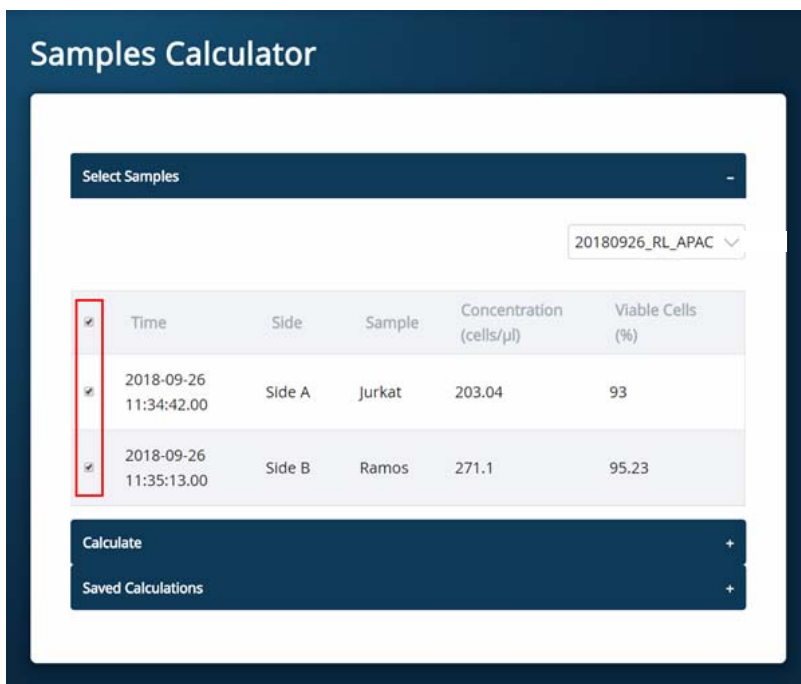
2. According to the number of captured cells chosen in step 1, obtain the volume(s) of stock cell and volume of cold Sample Buffer (PN 650000062) to prepare a cell suspension of $\geq 650 \mu\text{L}$ ($\geq 610 \mu\text{L}$ for low cell number samples) for loading into a BD Rhapsody Cartridge:

Note: To troubleshoot BD Rhapsody™ Scanner software error messages, see [BD Rhapsody Scanner software messages \(page 190\)](#).

- a. In the BD Rhapsody Scanner software, navigate to the **Analysis** screen, and proceed to the next step. If you are on the results screen that displays the viability and concentration of cells, tap **Prepare** at the top of the screen, and skip to step **d**.
- b. On the Analysis screen, tap the experiment.
- c. On the results screen, tap **Prepare**.
- d. On the Samples Calculator screen, select the experiment from the drop-down menu.

Note: You can search an experiment on the current screen by entering keywords in the search box.

- e. If necessary, tap the checkboxes to clear samples individually or tap the checkbox in the header column to clear them at once. For example, two samples are selected:



- f. Tap Calculate.
- g. Select the cartridge type from the drop-down menu. The cartridge type is the first four digits on the barcode label of the cartridge.

- h. Enter the **Desired total volume** and the **Desired number of captured cells**. For example, a desired total volume of 650 μL of a mixture of two samples and 10,000 cells captured are entered:

Samples Calculator

Select Samples +

Calculate -

Cartridge type
0109 575 μl

Desired total volume - 650 + μl

Desired number of captured cells - 10000 + cells

Sample	Time	Concentration (cells/ μl)	Viable Cells (%)	Relative Amounts
--------	------	---------------------------------------	------------------	------------------

- i. If necessary, enter the relative amount of each sample below the desired number of captured cells. For example, equal amounts of two samples are entered:

Desired number of captured cells cells

Sample	Time	Concentration (cells/ μ l)	Viable Cells (%)	Relative Amounts
Jurkat	2018-01-31 10:01:43.07	423.64	92.24	<input type="button" value="-"/> <input type="text" value="1"/> <input type="button" value="+"/>
Ramos	2018-01-31 10:02:39.54	375.99	92.53	<input type="button" value="-"/> <input type="text" value="1"/> <input type="button" value="+"/>

Results

- j. Obtain the calculated stock cell and buffer volumes to prepare the cell suspension for loading into the cartridge.

For example, two stock volumes and the buffer volume are calculated:

Results	
Sample	Stock Volume
Jurkat	15.3 μ l
Ramos	17.2 μ l
Buffer volume	617.5 μ l
Loading cell concentration	19.9 cells/ μ l
Estimated cell doublet rate	2.4%

Save

- k. (Optional) Tap **Save**. You can view the calculation by clicking the **Rhapsody Data** shortcut on the scanner screen and navigating to the appropriate folder.

1. (Optional) Click **Saved Calculations**. You can click saved calculations at any time. For example, the saved calculations from one experiment with two samples are displayed:

Samples Calculator

Select Samples +

Calculate +

Saved Calculations -

Cartridge type 0109	575 μ l	Buffer volume	617.5 μ l
Desired total volume	650 μ l	Loading cell concentration	19.9 cells/ μ l
Desired number of captured cells	10000 cells	Estimated cell doublet rate	2.4 %

Sample	Operator	Scan Time	Concentration (cells/ μ l)	Viable Cells (%)	Relative Amounts	Stock Volume
Jurkat		2018-01-31 10:01:43 .07	423.6	92.24	1	15.3
Ramos		2018-01-31 10:02:39 .54	376	92.53	1	17.2

3. Prepare the cell suspension:

Stock cell volume or cell number	Cell suspension preparation
Calculated Stock Volume is <610 μL	In a new 1.5 mL LoBind Tube, prepare the cell suspension in cold Sample Buffer (PN 650000062) according to the volumes obtained in step 2.
<ul style="list-style-type: none"> • Calculated Stock Volume \geq610 μL, or • Buffer volume is negative, or • Cell concentration too low warning is displayed 	Use 610 μL of the cold cell suspension for cartridge cell loading. ^a

- a. If the ratio of cells and targeted number of cells cannot be achieved, mix cells to prepare the maximum input of cells in a total volume of 610 μL . For example, if the Samples Calculator specifies mixing 700 μL of Jurkat cells and 30 μL of Ramos cells, mix 580 μL of Jurkat cells with 30 μL of Ramos cells.

Ensure the stock solution is well suspended by gently pipetting cells up and down before transferring the appropriate volume for dilution.

4. If the samples were not filtered before counting cells, pass the final dilution of the prepared single sample or pooled sample through a Falcon® Tube with Cell Strainer Cap (Thermo Fisher Scientific cat. no. 352235) before loading the sample into the cartridge.
5. Load the cartridge on the tray with 700 μL of air using the P1200M pipette in **Prime/Treat** mode.
6. Change the mode of the P1200M pipette to **Cell Load**.
7. With a manual pipette, gently pipet the cell suspension up and down to mix.

8. On the P1200M, press the pipette button once to aspirate 40 μ L of air, immerse the pipette tip in cell suspension, and then press the button again to aspirate 575 μ L of cold cell suspension.
9. Insert the tip of the pipette perpendicular to the port, seal the pipette tip against the gasket, and then press the button a third time to dispense 615 μ L of air and cells.

Air bubbles that may appear at the inlet or outlet of the cartridge do not affect cartridge performance.

10. Incubate the cells by one of these methods:
 - On the BD Rhapsody™ Express instrument: Leave the cartridge with loaded cells protected from light on the tray at 15°C to 25°C for 15 minutes. During incubation on the laboratory bench, prepare the Cell Capture Beads (PN 650000089). See [Preparing Cell Capture Beads \(page 108\)](#). After preparing the Cell Capture Beads, proceed to [Imaging cells in the cartridge](#), or
 - In the scanner: Proceed immediately to [Imaging cells in the cartridge](#) to install the cartridge and enter a 15 minute run delay.

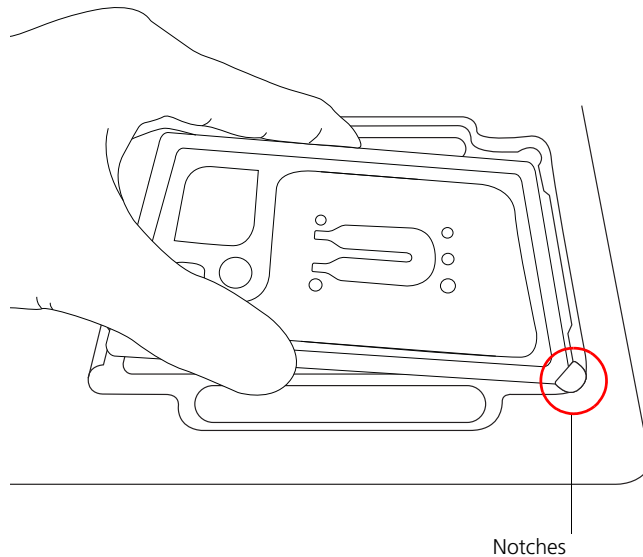
Imaging cells in the cartridge

Procedure

1. Navigate to the **Scan** application. The tray door of the scanner opens automatically, and the tray is ejected.

Note: If the tray is not ejected, tap the eject button in the upper right of the cartridge insertion screen.
2. Push the cartridge on the tray toward the back of the Express instrument, and then pull up on the cartridge to remove it.
3. If necessary, wipe condensation from the top surface of the cartridge with a lint-free wiper.

4. Place the cartridge on the scanner tray so that the cartridge and tray notches match and the barcode faces toward the front of the instrument:



Notes:

- The scanner displays an alert if the cartridge is in the wrong orientation.
 - You can manually enter the barcode in the application.
5. Tap **Continue**. The tray retracts, the door closes, and the scanner displays the experiment setup screen.
 6. Select from the drop-down menu or enter the experiment name, sample name, protocol, and user.

7. Tap Cell Load:

The screenshot shows a mobile application interface for a scan. At the top, there is a 'Scan' title bar with a back arrow and a home icon. Below this is a form with the following fields:

- Experiment: dropdown menu with 'example' selected.
- Sample: dropdown menu with 'fb' selected.
- Protocol: dropdown menu with 'Cartridge Scan' selected.
- User: text input field.
- Cartridge: text input field with '0109003002L' entered.

Below the form is a section titled '1 CellLoad'. It contains the question 'Do you want to set a time delay before the scan starts?' with a help icon. Underneath is a 'Minutes' label and a numeric input field with a minus sign, '0', and a plus sign. At the bottom of this section is a blue button labeled 'Start CellLoad Scan'. Below the 'CellLoad' section is a dark blue section titled '2 BeadLoad'.

8. Proceed according to incubation location:
 - Express instrument (15 minute incubation completed): Proceed to step 9.
 - Incubation in scanner: Enter a 15 minute time delay to delay the start of the scan and incubate the cartridge in the scanner by proceeding to step 9. During incubation in the scanner, prepare the Cell Capture Beads (PN 650000089). See [Preparing Cell Capture Beads \(page 108\)](#). After preparing the Cell Capture Beads, proceed to step 10.
9. Tap **Start Cell Load Scan**. The tray retracts into the scanner, and the door closes. If entered, the 15 minute countdown time displays in the Cell Load window. The scan proceeds after the countdown completes. The scan takes an additional ~4 minutes to complete.

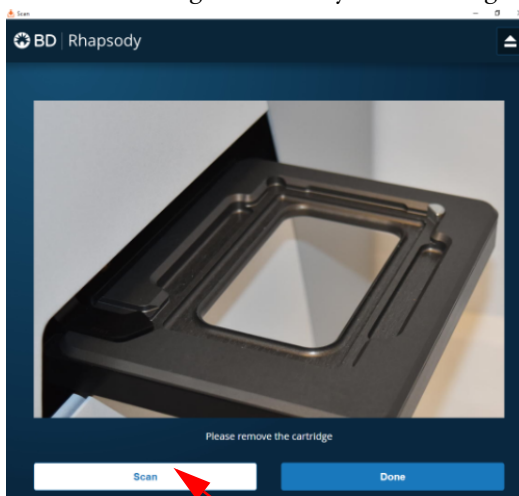
Note: To stop the scan, tap **Stop**, and then tap **Stop** again. You can then scan the cartridge with a different step or rerun the scan. To continue with the current scan, tap **Continue**.

10. After the scan is complete, tap **OK**, and then **Eject**. The cartridge is ejected from the scanner, and the remove cartridge screen opens.

Note: The analysis runs in the background.

11. Scan another cartridge or retract the tray:

- Scan another cartridge: Tap **Scan**, and proceed to step 4 to place a new cartridge on the tray for scanning:



- Retract the tray: Tap **Done**

- To confirm that the analysis is running, navigate to the **Analysis** screen, and then tap the experiment to view the ongoing analysis on the results screen. Upon completion, the results are listed:

a1-anchor1: max width=3.75 in. (360 px)

Ctrl-85pct

Cartridge 0109033258A

Scan Date	Sample	Step	Analysis Status
2018-01-31 10:26:00.49	Ctrl-85pct	Cell Load	✓ Completed

Note: If the analysis is not running, tap **Analyze**. To re-analyze a scan, tap the row for the scan, and then tap **Re-Analyze**.

Preparing Cell Capture Beads

- Before you begin**
- Prepare the pre-amplification workspace for preparation of the Cell Capture Beads for the BD Rhapsody Cartridge.
 - Keep the Cell Capture Beads on ice before use.
 - For maximum recovery, do not vortex samples containing Cell Capture Beads.
 - Gently mix suspensions with Cell Capture Beads by pipette only.
-

Preparing Cell Capture Beads

Use low retention pipette tips and LoBind Tubes when handling Cell Capture Beads.

1. Place the tube with Cell Capture Beads (PN 650000089) (beads) on the 1.5 mL tube magnet for 1 minute.
 2. Carefully remove and appropriately discard the storage buffer without disturbing the beads and while leaving the tube on the magnet.
 3. Remove the tube from the magnet, and then pipet 750 μ L of cold Sample Buffer (PN 650000062) into the tube of beads.
 4. Pipet the bead suspension up and down to mix.
 5. Keep the beads on ice.
 6. After the Cell Load scan, and after you have confirmed that the analysis is running, proceed to [Loading Cell Capture Beads and imaging \(page 109\)](#).
-

Loading Cell Capture Beads and imaging

Loading Cell Capture Beads on to the BD Rhapsody Cartridge

1. Push the cartridge into the far end of the Express instrument tray to match the cartridge and tray notches. Lay the cartridge flat, and release it. Ensure that the cartridge is flat in the tray and the barcode faces out.
2. Change the mode of the BD Rhapsody P1200M pipette to **Prime/Treat**.
3. Load the cartridge with 700 μL of air using the P1200M pipette in **Prime/Treat** mode.
4. Change the mode of the BD Rhapsody P1200M pipette to **Bead Load**.
5. Use a P1000 standard pipette to gently pipet the Cell Capture Beads in cold Sample Buffer (PN 650000062) up and down to mix, and, using the BD Rhapsody P1200M pipette in **Bead Load** mode, immediately load the cartridge with 630 μL of beads.
6. Let the beads settle in the cartridge on the tray at 15°C to 25°C for 3 minutes.
7. Image the cartridge with the scanner (**Bead Load** step). For detailed instructions, see [Imaging cells in the cartridge \(page 103\)](#).
8. Push the cartridge into the far end of the tray to match cartridge and tray notches. Lay the cartridge flat, and release it. Ensure that the cartridge is flat in the tray and the barcode faces out.
9. Change the mode of the BD Rhapsody P1200M pipette to **Wash**.

Note: In **Wash** mode, press the button once to aspirate 720 μL of air or reagent. After aspiration, insert the tip into the cartridge, and then press the button once to dispense 700 μL of air or liquid. After removing the pipette tip from the cartridge inlet, press the button once to dispense the remaining 20 μL of air or liquid before ejecting the pipette tip.

10. Load the cartridge with 700 μL of air using the BD Rhapsody P1200M pipette in **Wash** mode.
 11. Load the cartridge with 700 μL of cold Sample Buffer (PN 650000062) using the BD Rhapsody P1200M pipette in **Wash** mode.
 12. Repeat steps 10–11 once for a total of two washes.
 13. Image the cartridge with the scanner (**Bead Wash** step). For detailed instructions, see [Imaging cells in the cartridge \(page 103\)](#).
-

Lysing cells and retrieving Cell Capture Beads

Introduction

In the BD Rhapsody Cartridge, lyse the single cells to release the mRNA for capture by the beads.

Lysing the cells

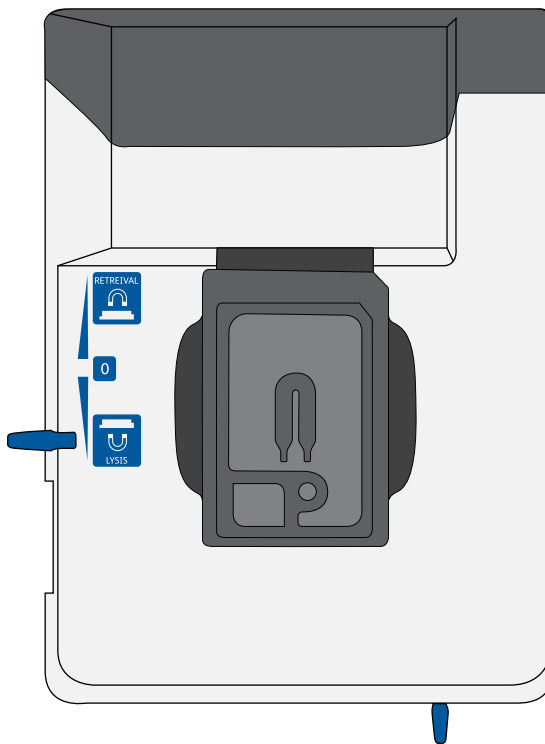
Avoid bubbles.

1. Add 75.0 μL of 1 M DTT (PN 650000063) to one bottle of 15 mL Lysis Buffer (PN 650000064), and then check the **Add DTT** box on the Lysis Buffer label.

Use the Lysis Buffer with DTT ≤ 24 hours and then discard.

2. Briefly vortex the lysis mix, and place it on ice.
3. Push the cartridge into the far end of the Express instrument tray to match cartridge and tray notches. Lay the cartridge flat and release it. Ensure that the cartridge is flat in the tray and the barcode faces out.

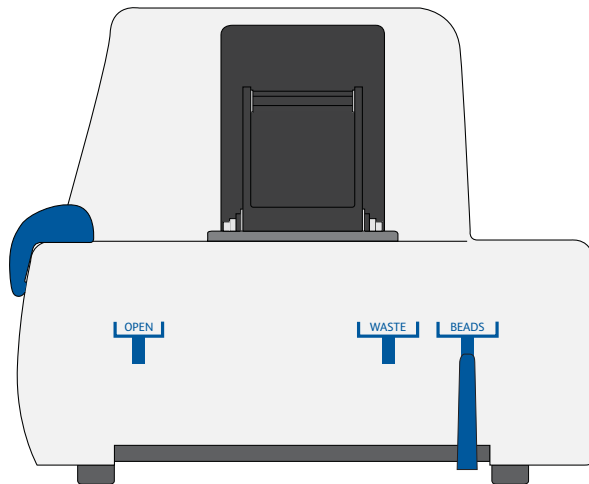
4. Move the left slider to **LYSIS**. The (bottom) magnet is in the up position and is in contact with the cartridge:



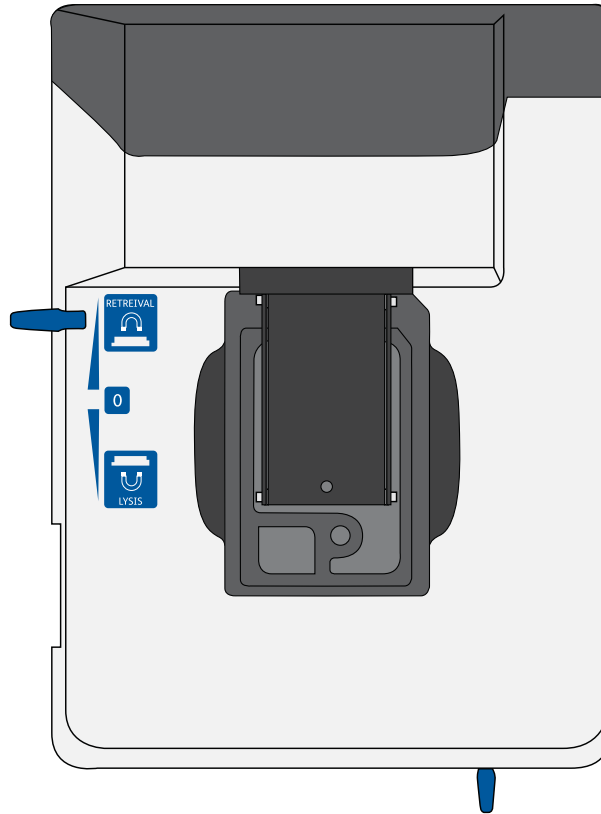
5. Change the mode on the BD Rhapsody P1200M pipette to **Lysis**.
 6. Load the cartridge with 550 μL of Lysis Buffer with DTT using the BD Rhapsody P1200M pipette in **Lysis** mode.
 7. Leave the cartridge on the tray at 15°C to 25°C for 2 minutes.
Maintain recommended lysis time for best performance.
-

Retrieving the Cell Capture Beads from the cartridge

1. Ensure that a 5 mL LoBind Tube (Eppendorf cat. no. 0030108310) was inserted into the drawer for bead retrieval.
2. Confirm that the mode on the BD Rhapsody P5000M pipette is **Retrieval**. The pipette is locked into this single mode.
3. Move the front slider to **BEADS**:



4. Move the left slider to **RETRIEVAL**. The (top) magnet is in the down position and is in contact with the cartridge:



5. Leave the Retrieval magnet in the down position for 30 seconds.
6. Use the BD Rhapsody P5000M pipette to aspirate 5,000 μL of Lysis Buffer with DTT.
7. Press down on the BD Rhapsody P5000M pipette to seal the pipette tip against the gasket of the cartridge to avoid leaks.

8. Move the left slider to the middle (0) position, and *immediately* load the cartridge with 4,950 μ L of Lysis Buffer with DTT using the BD Rhapsody P5000M pipette. The Retrieval (top) magnet is in its full up position and is away from the cartridge.

The Cell Capture Beads (beads) are collected in the 5 mL LoBind Tube.

9. Remove the pipette tip from the inlet gasket of the cartridge before pressing the dial button once to purge the tip. Discard the pipette tip.
10. Move the front slider to **OPEN**, and then remove and cap the 5 mL LoBind Tube.
11. Uncap the tube and place it on the large magnetic separation stand fitted with the 15 mL tube adapter for 1 minute.

During the 1 minute incubation, proceed to imaging the cartridge in step 12.

12. Image the cartridge with the scanner (**Retrieval** step). For detailed instructions, see [Imaging cells in the cartridge \(page 103\)](#).
13. Proceed immediately to [Performing reverse transcription and Exonuclease I treatment on the Cell Capture Beads \(page 117\)](#) to process the beads and begin reverse transcription.
14. Appropriately dispose of the BD Rhapsody Cartridge according to biosafety level (BSL):



Biological hazard.

- BSL-1. Discard the cartridge in a recycle container.
- BSL-2. Discard the cartridge in a biosafety waste container.

Dispose of waste using proper precautions and in accordance with local regulations. For more information, see [Waste \(page 220\)](#).

15. Appropriately dispose of the waste in the Waste Collection Container.
 16. Appropriately dispose of the Lysis Buffer with DTT.
 17. Wipe the Express instrument with 10% bleach or 70% ethyl alcohol. See the *BD Rhapsody™ Single-Cell Analysis System Installation and Maintenance Guide* (Doc ID 43084).
-

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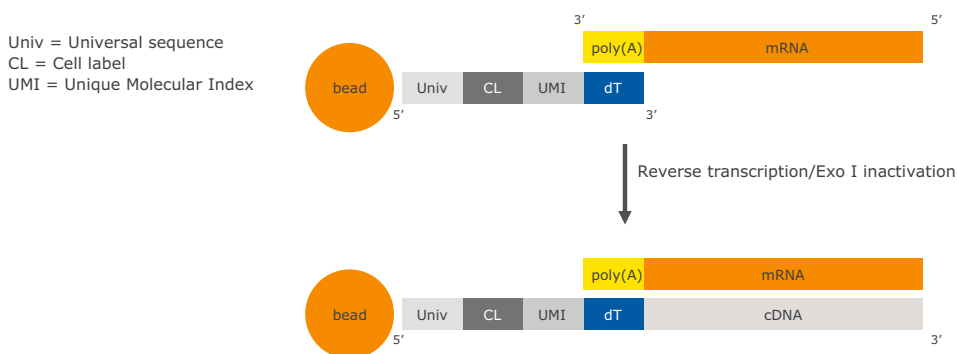
Performing reverse transcription and Exonuclease I treatment on the Cell Capture Beads

- [Performing reverse transcription on the Cell Capture Beads \(page 118\)](#)
- [Treating the sample with Exonuclease I \(page 123\)](#)

Performing reverse transcription on the Cell Capture Beads

Introduction

Prepare the reverse transcription mix, wash the Cell Capture Beads, and then perform reverse transcription on the beads with captured poly(A) mRNA with or without a Sample Tag:



Before you begin

- Obtain the 5 mL LoBind Tube of retrieved beads. See [Retrieving the Cell Capture Beads from the cartridge \(page 112\)](#).
- Start reverse transcription ≤ 30 minutes after washing retrieved beads with Bead Wash Buffer.
- Prepare the cDNA mix in the pre-amplification workspace.
- Thaw reagents in the BD Rhapsody™ cDNA Kit at 15°C to 25°C, and then put them on ice. Keep enzymes at -25°C to -15°C.
- Ensure that the SmartBlock™ Thermoblock 1.5 mL or equivalent is installed on the thermomixer and is set to 37°C and 20 minutes.

Washing the Cell Capture Beads

Keep the Cell Capture Beads cold during washes.

Use low retention tips to handle Cell Capture Beads.

Start reverse transcription ≤ 30 minutes after washing retrieved beads with Bead Wash Buffer.

1. After the 1 minute incubation on the magnet [see [Retrieving the Cell Capture Beads from the cartridge \(page 112\)](#)] and while leaving the 5 mL LoBind Tube on the magnet, use a pipette to carefully remove all but ~1 mL of supernatant without disturbing the beads.
2. Remove the tube from the magnet, resuspend the ~1 mL beads by gently pipetting the suspension up and down, and then transfer the bead suspension to a new 1.5 mL LoBind Tube.
3. If beads remain in the 5 mL LoBind Tube, pipet an additional 0.5 mL of Lysis Buffer with DTT into the tube, rinse the tube, and transfer the suspension to the 1.5 mL LoBind Tube of beads.
4. Place the tube on the 1.5 mL tube magnet for ≤ 2 minutes, and then carefully remove and appropriately discard the supernatant without disturbing the beads and while leaving the tube on the magnet.

Avoid leaving Lysis Buffer or bubbles in the tube. Lysis Buffer may cause the reverse transcription reaction to fail.

5. Remove the 1.5 mL LoBind Tube from the magnet, and then pipet 1.0 mL of cold Bead Wash Buffer (PN 650000065) to the tube. Gently mix the suspension by pipette only. Do not vortex.

6. Place the tube on the 1.5 mL tube magnet for ≤ 2 minutes, and then carefully remove and appropriately discard the supernatant without disturbing the beads and while leaving the tube on the magnet.
7. Remove the 1.5 mL LoBind Tube from the magnet, and then pipet 1.0 mL of cold Bead Wash Buffer (PN 650000065) to the tube. Gently mix the suspension by pipette only. Do not vortex.
8. Pipet the entire suspension to a new 1.5 mL LoBind Tube, and put the tube on ice.

Performing reverse transcription

When working with Cell Capture Beads (beads), use only low retention tips and LoBind Tubes.

Limit preparation of mixes to $\leq 20\%$ overage.

Prepare the cDNA mix on ice.

1. In the pre-amplification workspace, in a new 1.5 mL LoBind Tube that is on ice, pipet the components in the following order to prepare the cDNA mix:

cDNA mix

Component	1 library (μL)	1 library + 20% overage (μL)	2 libraries + 10% overage (μL)
Nuclease-Free Water (PN 650000076)	106.7	128.0	235.0
RT Buffer (PN 650000067)	40.0	48.0	88.0
dNTP (PN 650000077)	20.0	24.0	44.0
RT 0.1 M DTT (PN 650000068)	10.0	12.0	22.0
RT/PCR Enhancer (PN 650000070)	3.3	4.0	7.3
RNase Inhibitor (PN 650000078)	10.0	12.0	22.0
Reverse Transcriptase (PN 650000069)	10.0	12.0	22.0
Total	200.0	240.0	440.0

2. Gently vortex and centrifuge the mix, and then put it back on ice.

3. Place the tube of washed beads [see [Washing the Cell Capture Beads \(page 119\)](#)] on the 1.5 mL tube magnet for ≤ 2 minutes, and then carefully remove and appropriately discard the supernatant without disturbing the beads and while leaving the tube on the magnet.
4. Use a low retention tip to pipet 200 μL of the cDNA mix to resuspend the beads. Gently mix the suspension by pipette only. Do not vortex.

Prepared cDNA mix with beads should be kept on ice until ready for transfer.

5. Transfer the bead suspension to a new 1.5 mL LoBind Tube.
6. Ensure that the SmartBlock Thermoblock 1.5 mL or equivalent is installed on the thermomixer.
7. Incubate the suspension on the thermomixer at 1,200 rpm and 37°C for 20 minutes.

Shaking is critical for this incubation.

During reverse transcription incubation, view the image analysis to determine if the analysis metrics have passed. See [Reviewing the analysis metrics \(page 206\)](#).

8. After incubation, put the tube on ice.
-

Treating the sample with Exonuclease I

- Before you begin**
- Ensure that the SmartBlock Thermoblock 1.5 mL or equivalent is installed on the thermomixer and is set to 37°C and 30 minutes.
 - Set a second thermomixer or heat block to 80°C.

Preparing the Exonuclease I mix

When working with Cell Capture Beads, use only low retention tips and LoBind Tubes.

Limit preparation of mixes to $\leq 20\%$ overage.

Prepare the Exonuclease I mix on ice.

1. In the pre-amplification workspace, prepare the Exonuclease I mix in a new 1.5 mL LoBind Tube that is on ice by adding the components in the following order:

Exonuclease I mix

Component	1 library (µL)	1 library + 20% overage (µL)	2 libraries + 10% overage (µL)
Nuclease-Free Water (PN 650000076)	170.0	204.0	374.0
10X Exonuclease I Buffer (PN 650000071)	20.0	24.0	44.0
Exonuclease I (PN 650000072)	10.0	12.0	22.0
Total	200.0	240.0	440.0

2. Gently vortex and centrifuge the mix, and then put it back on ice.

**Treating the Cell
Capture Beads with
Exonuclease I**

1. Place the tube of beads with cDNA mix on the 1.5 mL tube magnet for ≤ 2 minutes, and then carefully remove and appropriately discard the supernatant without disturbing the beads and while leaving the tube on the magnet.
2. Remove the tube from the magnet, and then use a low retention tip to pipet 200 μL of Exonuclease I mix to the tube, Gently resuspend the beads by pipette only. Do not vortex.
3. Incubate the suspension on the thermomixer at 1,200 rpm and 37°C for 30 minutes.
4. If the thermomixer or heat block needs to preheat to a different temperature (80°C thermomixer or heat block), put the samples on ice until that temperature is reached.
5. Immediately proceed to [Inactivating Exonuclease I](#).

**Inactivating
Exonuclease I**

1. Transfer the bead suspension with Exonuclease I to the thermomixer in the pre-amplification workspace at 80°C (no shaking) for 20 minutes, or place the bead suspension in a heat block at 80°C for 20 minutes.
2. Put the bead suspension on ice for ~1 minute.
3. Place the tube on the 1.5 mL tube magnet until the solution is clear (≤ 1 minute).
4. Carefully remove and appropriately discard the supernatant without disturbing the beads and while leaving the tube on the magnet.
5. Remove the tube from the magnet, and with a low retention tip, pipet 200 μL of cold Bead Resuspension Buffer (PN 650000066) to gently resuspend the beads. Do not vortex.

Stopping point: The Exonuclease I-treated beads can be stored at 2°C to 8°C for ≤ 3 months.

6. Proceed according to single or multiplexed samples:
 - Single sample: [Single sample: preparing a single cell targeted sequencing library with the BD Rhapsody™ Targeted Reagent Kit \(page 127\)](#).
 - Multiplexed samples: [\(Optional\) Multiplexed samples: preparing a single cell targeted sequencing library with the BD Rhapsody™ Targeted Reagent Kit \(page 153\)](#)
-

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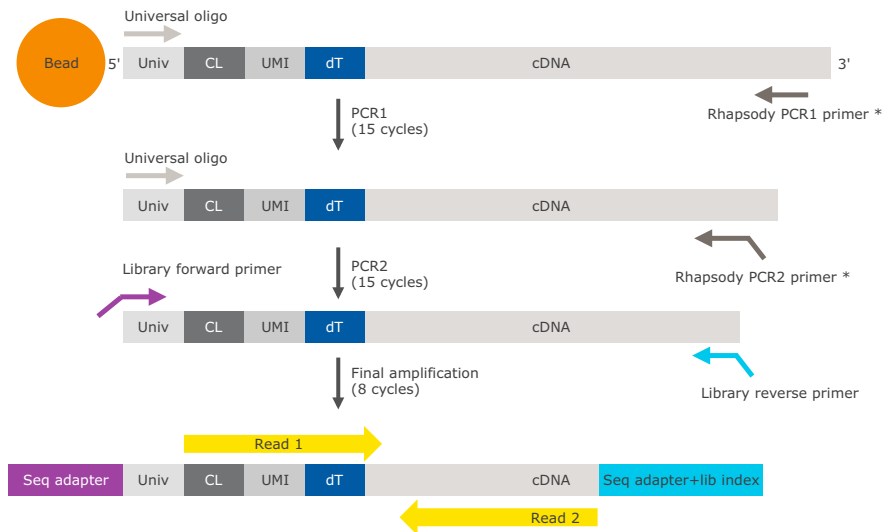
9

Single sample: Preparing a single cell targeted sequencing library with the BD Rhapsody™ Targeted Reagent Kit

- [Workflow \(page 128\)](#)
- [Performing PCR1 on the Exonuclease I-treated sample \(page 129\)](#)
- [Purifying the PCR1 products \(page 135\)](#)
- [Performing PCR2 on the PCR1 products \(page 137\)](#)
- [Purifying the PCR2 products \(page 140\)](#)
- [Quantifying the PCR2 products \(page 142\)](#)
- [Performing the final amplification for generating the sequencing library \(page 142\)](#)
- [Purifying the final amplification products \(page 145\)](#)
- [Performing quality control on the final sequencing library \(page 147\)](#)
- [Sequencing the final library \(page 149\)](#)

Workflow

Perform PCR1 (targeted amplification), PCR2 (nested targeted amplification), and then a final amplification to generate a library that is ready for sequencing on Illumina systems:



* Panel-specific

Univ = universal sequence; UMI = Unique Molecular Index;
CL = cell label; dT = oligo(dT).

Performing PCR1 on the Exonuclease I-treated sample

- Before you begin**
- Obtain the Exonuclease I-treated and inactivated Cell Capture Beads. When working with Cell Capture Beads, use low retention tips and LoBind Tubes. See [Inactivating Exonuclease I \(page 124\)](#).
 - PCR1 primers and PCR2 primers are components of the BD Rhapsody™ targeted primer panels. Order the primer panel specific to your application. See [BD Rhapsody™ targeted primer panels \(page 42\)](#).
 - PCR1 Supplement primers and PCR2 Supplement primers are custom add-on primers to specific BD Rhapsody targeted primer panels. Contact BD Biosciences technical support for more details at scomix@bdscomix.bd.com.
 - The BD Rhapsody™ Targeted Amplification Kit (PN 633734) contains sufficient reagents for four amplifications. Sub-sampling of the Exonuclease I-treated Cell Capture Beads may require purchasing additional BD Rhapsody Targeted Amplification kits.
 - Thaw reagents in the BD Rhapsody Targeted Amplification Kit at 15°C to 25°C, and then put it on ice.
 - Keep enzymes at –25°C to –15°C.
 - Prepare the pre-amplification workspace for preparation of the PCR1 reaction mix.
 - Prepare the post-amplification workspace to run and purify PCR1 products.
-

**Preparing the PCR1
reaction mix**

Limit preparation of mixes to $\leq 20\%$ overage.

Prepare the mixes on ice.

1. In the pre-amplification workspace, in a new 1.5 mL LoBind Tube that is on ice, add these components in the following order to prepare the PCR1 reaction mix with or without the PCR1 panel supplement:

Before use of BD Rhapsody™ 10X PCR1 Custom primers and/or BD Rhapsody™ 10X PCR1 Supplement primers, dilute 1 part of the 10X PCR primer stock to 9 parts of IDTE buffer to prepare a 1X primer solution. BD Rhapsody™ targeted (predesigned) primer panels are provided at 1X concentration and should not be diluted.

PCR1 reaction mix

Component	1 library (μ L)	1 library + 20% overage (μ L)	2 libraries + 10% overage (μ L)
Nuclease-Free Water (PN 650000076)	Up to 30.0	Up to 36.0	Up to 66.0
PCR MasterMix (PN 650000073)	100.0	120.0	220.0
Universal Oligo (PN 650000074)	20.0	24.0	44.0
RT/PCR Enhancer (PN 650000070)	10.0	12.0	22.0
PCR1 primer panel ^a	40.0	48.0	88.0
(Optional) PCR1 panel supplement ^b	(10.0)	(12.0)	(22.0)
Total	200.0	240.0	440.0

- a. To order Custom Primers, see [Suggested kits \(page 45\)](#).
- b. Order from BD Biosciences. Up to two panel supplements can be added per reaction provided that the total number of primers added is ≤ 500 (predesigned or custom panel plus panel supplement) and the primers have been designed to be compatible. Substitute water so that the total reaction volume per library remains 200.0 μ L. To order panel supplements, see [Suggested kits \(page 45\)](#).

2. Gently vortex and centrifuge the mix, and then put it back on ice.
-

Amplifying the PCR1 reaction mix with Cell Capture Beads

1. Choose between using the entire sample or a subsample of the PCR1 reaction:
 - Entire sample of Exonuclease I-treated beads: Skip to step 3.
 - Sub-sampling: Proceed to the step 2.
2. Sub-sample the Exonuclease I-treated beads:
 - a. Based on the number of viable cells in the sample volume detected in imaging analysis, determine the volume of beads to subsample for targeted sequencing. For example, if image analysis reveals 10,000 viable cells in the sample volume, use 1/10 the volume of beads for a 1,000 cell subsample.
 - b. Completely resuspend the beads by pipetting the bead suspension up and down, and then pipet the calculated volume of bead suspension into a new 1.5 mL LoBind Tube.

The remaining beads can be stored at 2°C to 8°C for ≤3 months.
3. Place the tube of Exonuclease I-treated beads in cold Bead Resuspension Buffer (PN 650000066) from step 1 or the subsample from step 2 on the 1.5 mL tube magnet. Carefully remove and appropriately discard the supernatant without disturbing the beads while leaving the tube on the magnet.

4. Remove the tube from the magnet, and then use a low retention tip to pipet 200 μL of PCR1 reaction mix to gently resuspend the beads [see [Preparing the PCR1 reaction mix \(page 130\)](#)]. Do not vortex.
5. Ensuring that the beads are fully suspended, pipet 50 μL of the PCR1 reaction mix with beads into each of four 0.2 mL PCR tubes. Transfer any residual mix to one of the tubes.
6. Bring the PCR1 reaction mix into the post-amplification workspace.
7. On the post-amplification thermal cycler, start the PCR1 thermal cycler program to ramp the heated lid and heat block of the thermal cycler to $\geq 95^{\circ}\text{C}$, and then pause the thermal cycler.
8. For each 0.2 mL PCR tube, gently mix the suspension of beads by pipette only, and then immediately put the tube in the thermal cycler that has been ramped to 95°C . Do not vortex.

Do not proceed to thermal cycling until each tube is gently mixed by pipette to ensure uniform bead suspension.

9. Unpause **Program 1** on the PCR1 reaction mix with beads to run the thermal cycler program:

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	15	95°C	30 s
Annealing		60°C	3 min
Extension		72°C	1 min
Final extension	1	72°C	5 min
Hold	1	4°C	∞

Do not let the PCR1 reaction run overnight.

10. Briefly centrifuge the tubes to collect the contents at the bottom.
11. In the post-amplification workspace, resuspend the beads by pipetting, and combine bead suspension from the four tubes into a new 1.5 mL LoBind Tube. Use a small-volume pipette to transfer any residual suspension to the 1.5 mL tube.
12. Place the tube of beads with PCR1 reaction on the 1.5 mL tube magnet for ≤ 2 minutes.

Retain the supernatant in the next step.

13. Carefully pipet the **supernatant** (PCR1 products) into a new 1.5 mL LoBind Tube without disturbing the beads while leaving the tube on the magnet.

Retain the beads and the supernatant (PCR1 products) in the two tubes.

14. Pipet 200 μ L of cold Bead Resuspension Buffer (PN 650000066) to the beads. Gently resuspend the beads by pipette only. Do not vortex.
 15. Store the beads at 2°C to 8°C in the post-amplification workspace until all image analysis metrics pass. See [Reviewing the analysis metrics \(page 206\)](#).
-

Purifying the PCR1 products

- Before you begin**
- Prepare the post-amplification workspace for the purification.
 - Bring the Agencourt® AMPure® XP magnetic beads (AMPure XP beads) to 15°C to 25°C.
-

Purifying the sample with AMPure XP beads

1. In a new 5.0 mL LoBind Tube, prepare 80% (v/v) ethyl alcohol by pipetting 1,600 µL of 100% (absolute) ethyl alcohol to 400.0 µL of nuclease-free water. Cap and vortex the tube for 10 seconds.

Make fresh 80% ethyl alcohol, and use it in ≤24 hours.

2. Vortex the AMPure XP beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
3. Pipet 140 µL of AMPure XP beads (0.7X volume of amplification volume) to the tube containing the 200 µL of supernatant (PCR1 products) from the amplification. See [Amplifying the PCR1 reaction mix with Cell Capture Beads \(page 132\)](#).
4. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample.
5. Incubate the suspension at 15°C to 25°C for 5 minutes, and then place it on the 1.5 mL tube magnet for 5 minutes.
6. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tube on the magnet.
7. Keeping the tube on the magnet, gently pipet 500 µL of 80% ethyl alcohol to the side of the tube opposite the pellet. Leave the tube open.
8. Incubate the sample for 30 seconds on the magnet.
9. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tube on the magnet.

10. Repeat steps 7–9 once for a total of two washes.
 11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
-

**Drying the
AMPure XP beads**

1. Leave the tube open on the magnet to dry the AMPure XP beads at 15°C to 25°C for 5 minutes. Remove the tube from the magnet.
2. Pipet 30 µL of Elution Buffer (PN 650000075) into the tube, and then repeatedly pipet the suspension up and down until the solution appears suspended and is uniform in color. You may see small clumps. Clumps do not affect performance.
3. Incubate the sample at 15°C to 25°C for 2 minutes, and then briefly centrifuge the tube to collect the contents at the bottom.
4. Place the tube on the magnet until the solution is clear, usually ≤30 seconds.
5. Pipet the entire eluate (~30 µL) into a new 1.5 mL LoBind Tube. These are the purified PCR1 products.

Stopping point: The purified PCR1 products can be stored at 2°C to 8°C before proceeding in ≤24 hours or at –25°C to –15°C for ≤6 months.

Performing PCR2 on the PCR1 products

- Before you begin**
- Prepare the pre-amplification workspace for preparation of the PCR2 reaction mix.
 - Prepare the post-amplification workspace to run and purify PCR2 products.
-

Preparing the PCR2 reaction mix Limit preparation of mixes to $\leq 20\%$ overage.

Prepare the mixes on ice.

1. In the pre-amplification workspace, in a new 1.5 mL LoBind Tube that is on ice, add the following components in this order to prepare the PCR2 reaction mix with or without PCR2 panel supplement:

Before use of BD Rhapsody 10X PCR2 Custom primers and/or BD Rhapsody 10X PCR2 Supplement primers, dilute 1 part of the 10X PCR primer stock to 9 parts of IDTE buffer to prepare a 1X primer solution. BD Rhapsody™ targeted (predesigned) primer panels are provided at 1X concentration and should not be diluted.

PCR2 reaction mix

Component	1 library (μL)	1 library + 20% overage (μL)	2 libraries + 10% overage (μL)
Nuclease-Free Water (PN 650000076)	Up to 8.0	Up to 9.6	Up to 17.6
PCR MasterMix (PN 650000073)	25.0	30.0	55.0
Universal Oligo (PN 650000074)	2.0	2.4	4.4
PCR2 primer panel ^a	10.0	12.0	22.0
(Optional) PCR2 panel supplement ^b	(2.5)	(3.0)	(5.5)
Total	45.0	54.0	99.0

- a. To order Custom Primers, see [Suggested kits \(page 45\)](#).
- b. Order from BD Biosciences. Up to three panel supplements can be added per reaction provided that the total number of primers added is ≤ 500 (predesigned or custom panel plus panel supplement) and the primers have been designed to be compatible. Substitute water so that the total reaction volume per library remains 45.0 μL . To order panel supplements, see [Suggested kits \(page 45\)](#).

2. Gently vortex and centrifuge the mix, and then put it back on ice.
3. Bring the PCR2 reaction mix to the post-amplification workspace.
4. In a new 0.2 mL PCR tube, pipet 5.0 μ L of the purified PCR1 products to 45 μ L of the PCR2 reaction mix for a total of 50 μ L of mix.
5. Gently vortex and centrifuge the tube.

Amplifying the PCR2 reaction mix

Run **program 1** on the PCR2 master mix in the post-amplification thermal cycler:

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	15	95°C	30 s
Annealing		60°C	3 min
Extension		72°C	1 min
Final extension	1	72°C	5 min
Hold	1	4°C	∞

Stopping point: The PCR can run overnight.

Purifying the PCR2 products

Before you begin

- Prepare the post-amplification workspace for the purification.
 - Bring the Agencourt AMPure XP magnetic beads to 15°C to 25°C.
 - Obtain fresh 80% ethyl alcohol. See [Purifying the sample with AMPure XP beads \(page 135\)](#).
-

Purifying the sample with the AMPure XP beads

1. Vortex the AMPure XP beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Briefly centrifuge the tube of PCR2 products to collect contents at the bottom.
3. Pipet 35.0 μL of AMPure XP beads (0.7X of PCR2 product volume) into the tube containing 50 μL of the PCR2 products.
4. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample (<1 second).
5. Incubate the suspension at 15°C to 25°C for 5 minutes, and then place it on the strip tube magnet for 3 minutes.
6. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tube on the magnet.
7. Keeping the tube on the magnet, gently pipet 200 μL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. Leave the tube open.
8. Incubate the sample for 30 seconds on the magnet.
9. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tube on the magnet.

10. Repeat steps 7–9 once for a total of two washes.
 11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
-

**Drying the
AMPure XP beads
and eluting the
sample**

1. Leave the tube open on the magnet to dry the AMPure XP beads at 15°C to 25°C for 3 minutes. Remove the tube from the magnet.
2. Pipet 30 µL of Elution Buffer (PN 650000075) into the tube, close the tube, and then pulse vortex to completely resuspend the AMPure XP beads.
3. Briefly centrifuge the tube to collect the contents at the bottom, and then incubate the sample at 15°C to 25°C for 2 minutes.
4. Place the tube on the strip tube magnet until the solution is clear, usually ≤30 seconds.
5. Pipet the entire eluate (~30 µL) into a new 1.5 mL LoBind Tube. These are the purified PCR2 products.

Stopping point: The purified PCR2 products can be stored at 2°C to 8°C before proceeding on the same day or at –25°C to –15°C for ≤6 months.

Quantifying the PCR2 products

Procedure

1. Estimate the concentration by quantifying 2 μL of the PCR2 products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay Kit. Follow the manufacturer's instructions.
 2. If the estimated concentration of the PCR2 products is:
 - ≥ 10 ng/ μL : Dilute the products to 10 ng/ μL in nuclease-free water (major supplier). Proceed to [Performing the final amplification for generating the sequencing library](#).
 - < 10 ng/ μL : See [Troubleshooting \(page 177\)](#).
-

Performing the final amplification for generating the sequencing library

Before you begin

- Prepare the pre-amplification workspace for the preparation of final amplification.
 - Prepare the post-amplification workspace to run and purify final amplification products.
-

Preparing the final amplification mix

Limit preparation of the mix to $\leq 20\%$ overage.

Prepare the mix on ice.

1. In the pre-amplification workspace, in a new 1.5 mL LoBind Tube that is on ice, add the following components in this order to prepare the final amplification mix:

Final amplification mix

Component	1 library (μL)	1 library + 20% overage (μL)	2 libraries + 10% overage (μL)
Nuclease-Free Water (PN 650000076)	18.0	21.6	39.6
PCR MasterMix (PN 650000073)	25.0	30.0	55.0
Library Forward Primer (PN 650000079)	2.0	2.4	4.4
Library Reverse Primer 1–4 (PN 650000080, 650000091–93)	2.0	2.4	— ^a
Total	47.0	56.4	99.0

- a. To prepare the final amplification mix for two or more libraries, prepare the master mix without library reverse primer. With a new 0.2 mL tube for each library, combine:
- 45.0 μL of final amplification mix without reverse primer
 - 2.0 μL of reverse primer.

Note: Bring the mix to the post-amplification workspace.
Add 3.0 μL of 10 ng/ μL of PCR2 products.

Gently vortex the mix, and proceed to [Amplifying the final amplification mix \(page 144\)](#).

2. Gently vortex and centrifuge the mix, and then put it back on ice.
3. Bring the final amplification mix into the post-amplification workspace.

4. In a new 0.2 mL PCR tube, pipet 3.0 μL of 10 ng/ μL of PCR2 products to 47.0 μL of the final amplification mix for a total of 50 μL of the final amplification master mix.
5. Gently vortex and centrifuge the tube.

Amplifying the final amplification mix

Run **program 2** on the final amplification master mix in the post-amplification thermal cycler to prepare the final amplification products:

Step	Cycles	Temperature	Time
Hot start	1	95°C	5 min
Denaturation	8	98°C	15 s
Annealing		60°C	30 s
Extension		72°C	30 s
Final extension	1	72°C	1 min
Hold	1	4°C	∞

Stopping point: The PCR can run overnight.

Purifying the final amplification products

Before you begin

- Prepare the post-amplification workspace for the purification.
 - Bring the Agencourt AMPure XP magnetic beads to 15°C to 25°C.
 - Obtain fresh 80% ethyl alcohol. See [Purifying the sample with AMPure XP beads \(page 135\)](#).
-

Purifying the sample with the AMPure XP beads

1. Vortex the AMPure XP beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Briefly centrifuge the tube of final amplification products to collect the contents at the bottom.
3. Pipet 30.0 μL of AMPure XP beads (0.6X volume of amplification volume) into the tube containing 50.0 μL of the final amplification products.
4. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample.
5. Incubate the suspension at 15°C to 25°C for 5 minutes, and then place it on the strip tube magnet for 3 minutes.
6. Carefully remove and appropriately discard only the clear liquid supernatant without disturbing the AMPure XP beads and while leaving the tube on the magnet.
7. Keeping the tube on the magnet, pipet 200 μL of fresh 80% ethyl alcohol to the side of the tube opposite the pellet. You can leave the tube open.
8. Incubate the sample for 30 seconds on the magnet.
9. Carefully remove and appropriately discard the supernatant without disturbing the AMPure XP beads and while leaving the tube on the magnet.
10. Repeat steps 7–9 once for a total of two washes.
11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

**Drying the
AMPure XP beads
and eluting the
sample**

1. Leave the tube open on the magnet to dry the AMPure XP beads at 15°C to 25°C for 3 minutes. Remove the tube from the magnet.
2. Pipet 30 µL of Elution Buffer (PN 650000075) into the tube, close the tube, and then pulse vortex to completely resuspend the AMPure XP beads.
3. Briefly centrifuge the tube to collect the contents at the bottom, and then incubate the sample at 15°C to 25°C for 2 minutes.
4. Place the tube on the magnet until the solution is clear, usually ≤30 seconds.
5. Pipet the entire eluate (~30 µL) into a new 1.5 mL LoBind Tube. This is the final sequencing library.

Stopping point: The final sequencing library can be stored at -25°C to -15°C for ≤6 months until sequencing.

Performing quality control on the final sequencing library

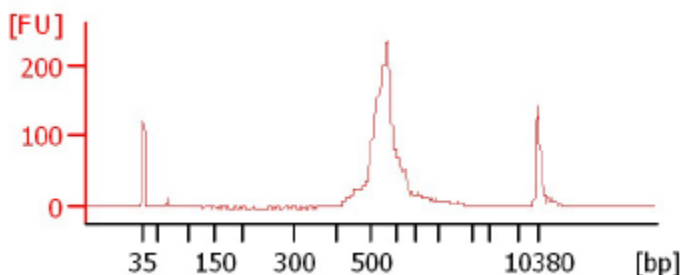
Before you begin Perform quality control in the post-amplification workspace.

Procedure

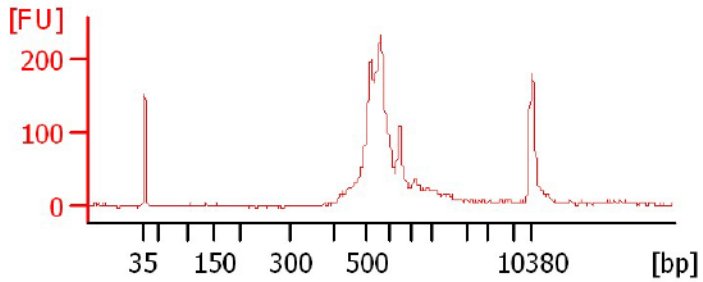
1. Estimate the concentration by quantifying 2 μL of the final amplified library with a Qubit Fluorometer using the Qubit dsDNA HS Kit. Follow the manufacturer's instructions. The expected library concentration is $>1.5 \text{ ng}/\mu\text{L}$.
2. Set the size range to 200–2,000 bp. Measure the size range of the library using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent cat. no. 5067-4626) for 50–7,000 bp, 5–1,000 $\text{pg}/\mu\text{L}$.

The library should show a fragment distribution within a size range of 350–600 bp. For example:

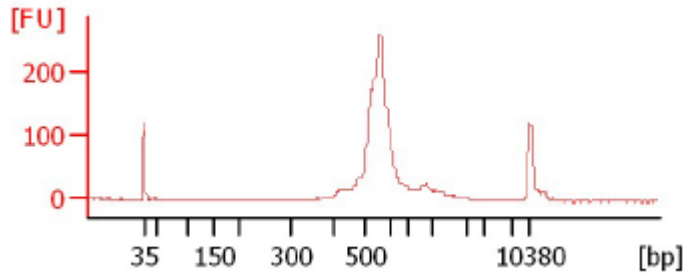
BD Rhapsody™ Immune Response Panel Hs (human)



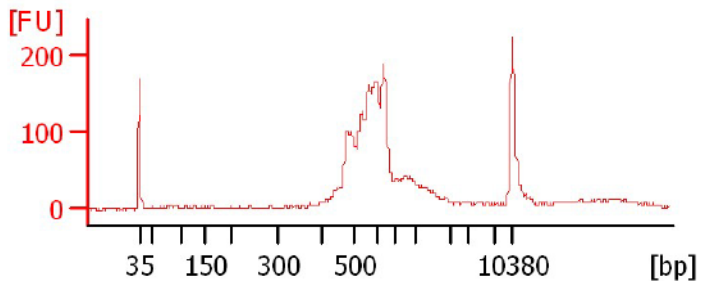
BD Rhapsody™ T Cell Expression Panel Hs (human)



BD Rhapsody™ Onco-BC Panel Hs (human)



BD Rhapsody™ Immune Response Panel Mm (mouse)



3. If the concentration or size of the library is outside of the expected range, see [Troubleshooting \(page 177\)](#) or contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Sequencing the final library

Introduction

This section provides requirements and recommendations to achieve meaningful sequencing results.

Requirements

Parameter	Requirement
Platform	Illumina ^a
Paired-end reads	Minimum of 75 x 75 paired read length
PhiX	Required ^b
Analysis	See the <i>BD Single Cell Genomics Bioinformatics Handbook</i> (Doc ID: 54169)

- a. To review Illumina Index 1 (i7) sequences, see [Sequence information \(page 211\)](#).
 - b. BD Rhapsody™ targeted assays produce low-diversity libraries, so the addition of PhiX is required to provide high-quality sequencing results. For specific recommendations, see [Loading densities and PhiX concentrations \(page 151\)](#).
-

Recommendations Sequencing depth

Sequencing depth is dependent on application. For cell type clustering, shallow sequencing is sufficient. For in-depth analysis, such as comparison across multiple libraries, deep sequencing is recommended. BD Biosciences recommends meeting the requirement for recursive substitution error correction (RSEC) sequencing depth of ≥ 6 in order to reach the threshold of sequencing saturation where most molecules of the library have been recovered. RSEC sequencing depth is reported by the analysis pipeline. The actual sequencing reads/cell required to achieve this depth can vary, because it depends on the chosen gene panel, number of cells, and sequencing run quality.

The table below is a recommended starting point for sequencing amount per cell with primary cells:

Gene panel	Reads/cell for clustering by cell type	Reads/cell for deep sequencing (RSEC depth ≥ 6)
BD Rhapsody™ Immune Response Hs	~2,000	~20,000
BD Rhapsody™ T Cell Expression Hs		
BD Rhapsody™ Onco-BC Hs		
BD Rhapsody™ Immune Response Mm		

For detailed information on RSEC, contact BD Biosciences technical support at scomix@bdscomix.bd.com, and inquire about Fan J, Tsai J, Shum E. Technical Note: *Molecular Index counting adjustment methods*.

Loading densities and PhiX concentrations

ILLUMINA SYSTEM	LIBRARY LOADING DENSITY	PHIX CONCENTRATION
MiSeq V2	6–10 pM	10%
MiSeq V3	6–10 pM	10%
MiniSeq High or Mid Output	1–1.5 pM	20%
NextSeq High or Mid Output	1–1.5 pM	20%
HiSeq 2500	7–15 pM	10%
HiSeq 3000/4000	3 nM	15%

- First-time users are encouraged to start at the low end of the loading density recommendation to avoid over-clustering.
 - Dilute PhiX to the same concentration as your library before combining samples to achieve the desired final concentration of PhiX. See Illumina instructions for detailed preparation and storage of PhiX and optimal cluster density ranges.
 - Quantify sequencing libraries as recommended above [see [Performing quality control on the final sequencing library \(page 147\)](#)] or according to Illumina or service provider instructions.
-

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10

(Optional) Multiplexed samples: Preparing a single cell targeted sequencing library with the BD Rhapsody™ Targeted Reagent Kit

- [Workflow \(page 154\)](#)
- [Performing PCR1 on the Exonuclease I-treated sample \(page 154\)](#)
- [Purifying the PCR1 products \(page 160\)](#)
- [Performing PCR2 on the PCR1 products \(page 162\)](#)
- [Purifying the PCR2 products \(page 165\)](#)
- [Quantifying the PCR2 products \(page 167\)](#)
- [Performing final amplification of pooled samples \(page 168\)](#)
- [Purifying the final amplification products \(page 171\)](#)
- [Performing quality control on the final sequencing libraries \(page 173\)](#)
- [Sequencing \(page 174\)](#)

Workflow

Perform PCR1 and PCR2 on the multiplexed samples and then a final amplification to generate single cell targeted and Sample Tag libraries for sequencing on an Illumina system.

Performing PCR1 on the Exonuclease I-treated sample

- Before you begin**
- Obtain the Exonuclease I-treated and inactivated Cell Capture Beads. When working with Cell Capture Beads, use low retention tips and LoBind Tubes. See [Inactivating Exonuclease I \(page 124\)](#).
 - PCR1 primers and PCR2 primers are components of the BD Rhapsody™ targeted primer panels. Order the primer panel specific to your application. See [BD Rhapsody™ targeted primer panels \(page 42\)](#).
 - PCR1 Supplement primers and PCR2 Supplement primers are custom add-on primers to specific BD Rhapsody targeted primer panels. Contact BD Biosciences technical support for more details at scomix@bdscomix.bd.com.
 - Sample Tag PCR1 Primer and Sample Tag PCR2 Primer are components of the BD™ Single-Cell Multiplexing Kit Library Amplification Component (PN 633782).
 - The BD Rhapsody Targeted Amplification Kit (PN 633734) contains sufficient reagents for four amplifications. Sub-sampling of the Exonuclease I-treated Cell Capture Beads may require purchasing additional BD Rhapsody Targeted Amplification kits.
 - Thaw reagents in the BD Rhapsody Targeted Amplification Kit at 15°C to 25°C, and then store on ice until use.
 - Keep enzymes at -25°C to -15°C.

- Prepare the pre-amplification workspace for preparation of the PCR1 reaction mix.
 - Prepare the post-amplification workspace to run and purify PCR1 products.
-

Preparing the PCR1 reaction mix

1. In the pre-amplification workspace, add these components in the following order to prepare the PCR1 reaction mix in a new 1.5 mL LoBind Tube that is on ice with or without the PCR1 panel supplement:

Before use of BD Rhapsody™ 10X PCR1 Custom primers and/or BD Rhapsody™ 10X PCR1 Supplement primers, dilute 1 part of the 10X PCR primer stock to 9 parts of IDTE buffer to prepare a 1X primer solution. BD Rhapsody™ targeted (predesigned) primer panels are provided at 1X concentration and should not be diluted.

PCR1 reaction mix

Component	1 library (µL)	1 library + 20% overage (µL)
Nuclease-Free Water (PN 650000076) ^a	Up to 28.8	Up to 34.6
PCR MasterMix (PN 650000073) ^a	100.0	120.0
Universal Oligo (PN 650000074) ^a	20.0	24.0
RT/PCR Enhancer (PN 650000070) ^a	10.0	12.0
PCR1 primer panel ^b	40.0	48.0
(Optional) PCR1 panel supplement ^c	(10.0)	(12.0)
Sample Tag PCR1 Primer (PN 91-1060) ^d	1.2	1.4
Total	200.0	240.0

- a. Use from the BD Rhapsody Targeted Amplification Kit (PN 633734).
- b. To order Custom Primers, see [Suggested kits \(page 45\)](#).

- c. Order from BD Biosciences. Up to two panel supplements can be added per reaction provided that the total number of primers added is ≤ 500 (predesigned or custom panel plus panel supplement) and the primers have been designed to be compatible. Substitute water so that the total reaction volume per library remains 200.0 μL . To order panel supplements, see [Suggested kits \(page 45\)](#).
 - d. Use from the BD Single-Cell Multiplexing Kit Library Amplification Component (PN 633782).
2. Gently vortex and centrifuge the mix, and place it back on ice until ready to use.

Amplifying the PCR1 reaction mix with Cell Capture Beads

-
1. Choose between using the entire sample or a subsample of the PCR1 reaction:
 - Entire sample of Exonuclease I-treated beads: Skip to step 3.
 - Sub-sampling: Proceed to the step 2.
 2. Sub-sample the Exonuclease I-treated beads:
 - a. Based on the number of viable cells in the sample volume detected in imaging analysis, determine the volume of beads to subsample for targeted sequencing. For example, if image analysis reveals 10,000 viable cells in the sample volume, use 1/10 the volume of beads for a 1,000 cell subsample.
 - b. Completely resuspend the beads by pipetting the bead suspension up and down, and then pipet the calculated volume of bead suspension into a new 1.5 mL LoBind Tube.

The remaining beads can be stored at 2°C to 8°C for ≤ 3 months.
 3. Place the tube of Exonuclease I-treated beads in cold Bead Resuspension Buffer (PN 650000066) from step 1 or the subsample from step 2 on the 1.5 mL tube magnet. Carefully remove and appropriately discard the supernatant without disturbing the beads while leaving the tube on the magnet.

4. Remove the tube from the magnet, and then use a low retention tip to pipet 200 μ L of PCR1 reaction mix to gently resuspend the beads [see [Preparing the PCR1 reaction mix \(page 155\)](#)]. Do not vortex.
5. Ensuring that the beads are fully suspended, pipet 50 μ L of the PCR1 reaction mix with beads into each of four 0.2 mL PCR tubes. Transfer any residual mix to one of the tubes.
6. Bring the PCR1 reaction mix into the post-amplification workspace.
7. On the post-amplification thermal cycler, start the PCR1 thermal cycler program to ramp the heated lid and heat block of the thermal cycler to $\geq 95^{\circ}\text{C}$, and then pause the thermal cycler.
8. For each 0.2 mL PCR tube, gently mix the suspension of beads by pipette only, and then immediately put the tube in the thermal cycler that has been ramped to 95°C . Do not vortex.

Do not proceed to thermal cycling until each tube is gently mixed by pipette to ensure uniform bead suspension.

9. Unpause **Program 1** on the PCR1 reaction mix with beads to run the thermal cycler program:

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	15	95°C	30 s
Annealing		60°C	3 min
Extension		72°C	1 min
Final extension	1	72°C	5 min
Hold	1	4°C	∞

Do not let the PCR1 reaction run overnight.

10. Briefly centrifuge the tubes to collect the contents at the bottom.
11. In the post-amplification workspace, resuspend the beads by pipetting, and combine the bead suspension from the four tubes into a new 1.5 mL LoBind Tube. Use a small-volume pipette to transfer any residual suspension to the 1.5 mL tube.
12. Place the tube of beads with PCR1 reaction on the 1.5 mL tube magnet for ≤ 2 minutes.

Retain the supernatant in the next step.

13. Carefully pipet the **supernatant** (PCR1 products) into a new 1.5 mL LoBind Tube without disturbing the beads and while leaving the tube on the magnet.

Retain the beads and the supernatant (PCR1 products) in the two tubes.

14. Pipet 200 μL of cold Bead Resuspension Buffer (PN 650000066) to the beads. Gently resuspend the beads by pipette only. Do not vortex.
 15. Store the beads at 2°C to 8°C in the post-amplification workspace until all image analysis metrics pass. See [Reviewing the analysis metrics \(page 206\)](#).
-

Purifying the PCR1 products

- Before you begin**
- Prepare the post-amplification workspace for the purification.
 - Bring the Agencourt® AMPure® XP magnetic beads (AMPure XP beads) to 15°C to 25°C.
-

Purifying the sample with AMPure XP beads

1. In a new 5.0 mL LoBind Tube, prepare 80% (v/v) ethyl alcohol by pipetting 1,600 µL of 100% (absolute) ethyl alcohol to 400.0 µL of nuclease-free water. Cap and vortex the tube for 10 seconds.

Make fresh 80% ethyl alcohol, and use it in ≤24 hours.

2. Vortex the AMPure XP beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
3. Pipet 200 µL of AMPure XP beads (1X volume of amplification volume) to the tube containing the 200 µL of supernatant (PCR1 products) from the amplification. See [Amplifying the PCR1 reaction mix with Cell Capture Beads \(page 157\)](#).

Use the ratio (volume) of Agencourt AMPure XP magnetic beads specified in this step. Due to the smaller fragment size of the Sample Tags, the ratio in this step differs from the ratio specified for the purification of PCR1 products of a single sample.

4. Vortex the suspension at high speed for 5 seconds, and then briefly centrifuge the sample.
5. Incubate the suspension at 15°C to 25°C for 5 minutes, and then place it on the 1.5 mL tube magnet for 5 minutes.
6. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tube on the magnet.
7. Keeping the tube on the magnet, gently pipet 500 µL of 80% ethyl alcohol to the side of the tube opposite the pellet. Leave the tube open.

8. Incubate the sample for 30 seconds on the magnet.
9. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tube on the magnet.
10. Repeat steps 7–9 once for a total of two washes.
11. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.

**Drying the
AMPure XP beads**

1. Leave the tube open on the magnet to dry the AMPure XP beads at 15°C to 25°C for 5 minutes. Remove the tube from the magnet.
2. Pipet 30 µL of Elution Buffer (PN 650000075) into the tube, and then repeatedly pipet the suspension up and down until the solution appears suspended and is uniform in color. You may see small clumps. Clumps do not affect performance.
3. Incubate the sample at 15°C to 25°C for 2 minutes, and then briefly centrifuge the tube to collect the contents at the bottom.
4. Place the tube on the magnet until the solution is clear, usually ≤30 seconds.
5. Pipet the entire eluate (~30 µL) into a new 1.5 mL LoBind Tube. These are the purified PCR1 products.

Stopping point: The purified PCR1 products can be stored at 2°C to 8°C before proceeding in ≤24 hours or at –25°C to –15°C for ≤6 months.

Performing PCR2 on the PCR1 products

Preparing the PCR2 reaction mix

1. In the pre-amplification workspace, add these components in the following order to prepare the **two** reaction mixes, targeted PCR2 and Sample Tag PCR2, in separate, new 1.5 mL LoBind Tubes that are on ice with or without the PCR2 panel supplement:

Before use of BD Rhapsody 10X PCR2 Custom primers and/or BD Rhapsody 10X PCR2 Supplement primers, dilute 1 part of the 10X PCR primer stock to 9 parts of IDTE buffer to prepare a 1X primer solution. BD Rhapsody targeted (predesigned) primer panels are provided at 1X concentration and should not be diluted.

Targeted PCR2 reaction mix

Component	1 library (μL)	1 library + 20% overage (μL)
Nuclease-Free Water (PN 650000076) ^a	Up to 8.0	Up to 9.6
PCR MasterMix (PN 650000073) ^a	25.0	30.0
Universal Oligo (PN 650000074) ^a	2.0	2.4
PCR2 primer panel ^b	10.0	12.0
(Optional) PCR2 panel supplement ^c	(2.5)	(3.0)
Total	45.0	54.0

- a. Use from the BD Rhapsody Targeted Amplification Kit (PN 633734).
- b. To order Custom Primers, see [Suggested kits \(page 45\)](#).

- c. Order from BD Biosciences. Up to three panel supplements can be added per reaction provided that the total number of primers added is ≤ 500 (predesigned or custom panel plus panel supplement) and the primers have been designed to be compatible. Substitute water so that the total reaction volume per library remains 45.0 μL . To order panel supplements, see [Suggested kits \(page 45\)](#).

Sample Tag PCR2 reaction mix

Component	1 library (μL)	1 library + 20% overage (μL)
Nuclease-Free Water (PN 650000076) ^a	15.0	18.0
PCR MasterMix (PN 91-1052) ^b	25.0	30.0
Universal Oligo (PN 91-1054) ^b	2.0	2.4
Sample Tag PCR2 Primer (PN 91-1061) ^b	3.0	3.6
Total	45.0	54.0

- a. Use from the BD Rhapsody Targeted Amplification Kit (PN 633734).
 - b. Use from the BD Single-Cell Multiplexing Kit Library Amplification Component (PN 633782).
2. Gently vortex and centrifuge the mixes, and place them back on ice until ready to use.
 3. Bring the PCR2 reaction mixes to the post-amplification workspace.
 4. In two separate and new 0.2 mL PCR tubes:

- a. Targeted: Pipet 5.0 μL of the purified PCR1 products to 45 μL of the targeted PCR2 reaction, and mix by pipette for a total of 50 μL .
 - b. Sample Tag: Pipet 5.0 μL of the purified PCR1 products to 45 μL of the Sample Tag PCR2 reaction, and mix by pipette for a total of 50 μL .
5. Gently vortex and centrifuge the tubes.

Amplifying the PCR2 reaction mix

Run **program 1** on the PCR2 master mix in the post-amplification thermal cycler:

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	15	95°C	30 s
Annealing		60°C	3 min
Extension		72°C	1 min
Final extension	1	72°C	5 min
Hold	1	4°C	∞

Stopping point: Targeted and Sample Tag PCR2 can run overnight.

Purifying the PCR2 products

- Before you begin**
- Prepare the post-amplification workspace for the purification.
 - Bring the Agencourt AMPure XP magnetic beads to 15°C to 25°C.
 - Obtain fresh 80% ethyl alcohol. See [Purifying the sample with AMPure XP beads \(page 160\)](#).
-

Purifying the sample with the AMPure XP beads

1. Vortex the AMPure XP beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Briefly centrifuge the tube of PCR2 products to collect contents at the bottom.
3. Pipet AMPure XP beads according to the specified ratio (volume) into each tube containing 50 μ L products:
 - Targeted PCR2 products: Use **0.7X (35 μ L)** of Agencourt AMPure XP magnetic beads.
 - Sample Tag PCR2 products: Use **1X (50 μ L)** of Agencourt AMPure XP magnetic beads.
4. Vortex the suspensions at high speed for 5 seconds, and then briefly centrifuge the sample (<1 second).
5. Incubate the suspensions at 15°C to 25°C for 5 minutes, and then place them on the strip tube magnet for 3 minutes.
6. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tubes on the magnet.
7. Keeping the tubes on the magnet, gently pipet 200 μ L of fresh 80% ethyl alcohol to the side of each tube opposite the pellet. Leave the tubes open.
8. Incubate the samples for 30 seconds on the magnet.
9. Carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP beads while leaving the tubes on the magnet.

10. Repeat steps 7–9 once for a total of two washes.
 11. Keeping the tubes on the magnet, use a small-volume pipette to remove any residual supernatant from the tubes.
-

**Drying the
AMPure XP beads
and eluting the
sample**

1. Leave the tubes open on the magnet to dry the AMPure XP beads at 15°C to 25°C for 3 minutes. Remove the tubes from the magnet.
2. Pipet 30 µL of Elution Buffer (PN 650000075) into each tube, close the tubes, and then pulse vortex to completely resuspend the AMPure XP beads.
3. Briefly centrifuge the tubes to collect the contents at the bottom, and then incubate the samples at 15°C to 25°C for 2 minutes.
4. Place the tubes on the strip tube magnet until the solution is clear, usually ≤30 seconds.
5. For each tube, pipet the entire eluate (~30 µL) into a new 1.5 mL LoBind Tube. These two tubes are the purified targeted and Sample Tag PCR2 products.

Stopping point: The purified PCR2 products can be stored at 2°C to 8°C before proceeding on the same day or at -25°C to -15°C for ≤6 months.

Quantifying the PCR2 products

Procedure

1. Estimate the concentration by quantifying 2 μL of the targeted and Sample Tag PCR2 products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay Kit. Follow the manufacturer's instructions.
2. Dilute an aliquot of the PCR2 products to the following concentrations in nuclease-free water (major supplier):
 - Targeted PCR2 products: 10 ng/ μL .
 - Sample Tag products: 0.5 ng/ μL .

If the estimated concentration of the targeted PCR2 products is <10 ng/ μL : See [Troubleshooting \(page 177\)](#).

3. Proceed to [Performing final amplification of pooled samples \(page 168\)](#).
-

Performing final amplification of pooled samples

Preparing the final targeted and Sample Tag amplification mixes

1. In the pre-amplification workspace, prepare 1 library + 20% overage of the final amplification mix for each of the two products. Add these components in the following order to prepare the mix in a new 1.5 mL LoBind Tube that is on ice:

Note: Using the same Library Reverse Primer for both final amplification mixes is recommended but not required.

Targeted final amplification mix

Component	1 library (μL)	1 library + 20% overage (μL)
Nuclease-Free Water (PN 650000076) ^a	18.0	21.6
PCR MasterMix (PN 650000073) ^a	25.0	30.0
Library Forward Primer (PN 650000079) ^a	2.0	2.4
Library Reverse Primer 1–4 (PN 650000080, 650000091–93) ^a	2.0	2.4
Total	47.0	56.4

- a. Use from the BD Rhapsody Targeted Amplification Kit (PN 633734).

Sample Tag final amplification mix

Component	1 library (μL)	1 library + 20% overage (μL)
Nuclease-Free Water (PN 650000076) ^a	18.0	21.6
PCR MasterMix (PN 91-1052) ^b	25.0	30.0
Library Forward Primer ^b (PN 91-1055)	2.0	2.4
Library Reverse Primer 1–4 (PN 650000080, 650000091–93) ^a	2.0	2.4
Total	47.0	56.4

- a. Use from the BD Rhapsody Targeted Amplification Kit (PN 633734).
 - b. Use from the BD Single-Cell Multiplexing Kit Library Amplification Component (PN 633782).
2. Gently vortex and centrifuge the mixes, and place them back on ice until ready to use.
 3. Bring the final amplification mixes into the post-amplification workspace.

4. In two separate and new 0.2 mL PCR tubes:
 - a. Targeted library master mix: Pipet 3.0 μL of 10 ng/ μL of targeted PCR2 products to 47.0 μL of the targeted final amplification mix for a total of 50 μL . Gently vortex and centrifuge the mix.
 - b. Sample Tag library master mix: Pipet 3.0 μL of 0.5 ng/ μL of Sample Tag PCR2 products to 47.0 μL of the Sample Tag final amplification mix for a total of 50 μL . Gently vortex and centrifuge the mix.

Amplifying the final targeted and Sample Tag amplification mixes

Run **program 2** on the final amplification master mix in the post-amplification thermal cycler to prepare the final amplification products:

Step	Cycles	Temperature	Time
Hot start	1	95°C	5 min
Denaturation	8	98°C	15 s
Annealing		60°C	30 s
Extension		72°C	30 s
Final extension	1	72°C	1 min
Hold	1	4°C	∞

Stopping point: The PCR can run overnight.

Purifying the final amplification products

Before you begin

- Prepare the post-amplification workspace for the purification.
 - Bring the Agencourt AMPure XP magnetic beads to 15°C to 25°C.
 - Obtain fresh 80% ethyl alcohol. See [Purifying the sample with AMPure XP beads \(page 160\)](#).
-

Purifying the samples with the AMPure XP beads

1. Vortex the AMPure XP beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
2. Briefly centrifuge the tubes of final amplification products to collect the contents at the bottom.
3. Pipet AMPure XP beads according to the specified ratio (volume) into each tube containing 50 μ L products:
 - Final targeted library: Use **0.6X (30 μ L)** of Agencourt AMPure XP magnetic beads.
 - Final Sample Tag library: Use **1X (50 μ L)** of Agencourt AMPure XP magnetic beads.
4. Vortex the suspensions at high speed for 5 seconds, and then briefly centrifuge the sample.
5. Incubate the suspensions at 15°C to 25°C for 5 minutes, and then place them on the strip tube magnet for 3 minutes.
6. Carefully remove and appropriately discard only the clear liquid supernatant without disturbing the AMPure XP beads and while leaving the tube on the magnet.
7. Keeping the tube on the magnet, pipet 200 μ L of fresh 80% ethyl alcohol to the side of each tube opposite the pellet. You can leave the tube open.
8. Incubate the samples for 30 seconds on the magnet.
9. Carefully remove and appropriately discard the supernatant without disturbing the AMPure XP beads and while leaving the tubes on the magnet.

10. Repeat steps 7–9 once for a total of two washes.
 11. Keeping the tubes on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
-

**Drying the
AMPure XP beads
and eluting the
sample**

1. Leave the tube open on the magnet to dry the AMPure XP beads at 15°C to 25°C for 3 minutes. Remove the tubes from the magnet.
2. Pipet 30 µL of Elution Buffer (PN 650000075) into the tubes, close the tubes, and then pulse vortex to completely resuspend the AMPure XP beads.
3. Briefly centrifuge the tubes to collect the contents at the bottom, and then incubate the samples at 15°C to 25°C for 2 minutes.
4. Place the tubes on the magnet until the solution is clear, usually ≤30 seconds.
5. For each tube, pipet the entire eluate (~30 µL) into a new 1.5 mL LoBind Tube. These two tubes are the final sequencing libraries.

Stopping point: The final sequencing library can be stored at –25°C to –15°C for ≤6 months until sequencing.

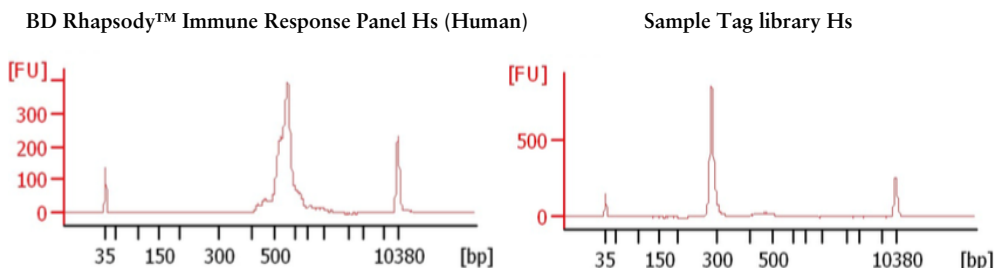
Performing quality control on the final sequencing libraries

Before you begin Perform quality control in the post-amplification workspace.

Procedure

1. Estimate the concentration by quantifying 2 μL of the final amplified libraries with a Qubit Fluorometer using the Qubit dsDNA HS Kit. Follow the manufacturer's instructions. The expected library concentration is $>1.5 \text{ ng}/\mu\text{L}$.
2. Set the size range to 200–2,000 bp. Measure the size ranges of the libraries using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent cat. no. 5067-4626) for 50–7,000 bp, 5–1,000 $\text{pg}/\mu\text{L}$.

The Sample Tag library should show a fragment distribution of 200–350 bp. The BD Rhapsody targeted library shows a fragment distribution that depends on the panel used. For example:



3. If the concentration or size of a library is outside of the expected range, see [Troubleshooting \(page 177\)](#) or contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Sequencing

Introduction

This section provides requirements and recommendations to achieve meaningful sequencing results.

Requirements

- Illumina® BaseSpace and sample sheet sequencing run setup: Unless different Library Reverse primers were used for the targeted library and Sample Tag library, enter the pooled targeted and Sample Tag library as one sample.

Parameter	Requirement
Platform	Illumina ^a
Paired-end reads	Minimum of 75 x 75 paired read length
PhiX	Required ^b
Analysis	See the <i>BD Single Cell Genomics Bioinformatics Handbook</i> (Doc ID: 54169)

- a. To review Illumina Index 1 (i7) sequences, see [Sequence information \(page 211\)](#).
- b. BD Rhapsody™ targeted assays produce low-diversity libraries, so the addition of PhiX is required to provide high-quality sequencing results. For specific recommendations, see [Loading densities and PhiX concentrations \(page 151\)](#).

Recommendations **Sample Tag library**

- Pooling samples of the same type: ≥ 120 reads/cell. For example: combining different donor peripheral blood mononuclear cells.
- Pooling different sample types: ≥ 600 reads/cell. For example: combining Jurkat cells with peripheral blood mononuclear cells.

Note: To determine the ratio of BD Rhapsody targeted library to Sample Tag library to pool for sequencing, there is a Sample Tag sequencing calculator available. Contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Loading densities and PhiX concentrations

Consider these recommendations for any Illumina system used:

Library	Illumina system	Library loading density (pM)	PhiX concentration
Sequencing targeted library and Sample Tag library together	MiniSeq High or Mid Output	1–1.5	20%
	NextSeq High or Mid Output	1–1.5	20%
	Other Illumina systems that have not been validated with the libraries	For recommendations, see Loading densities and PhiX concentrations (page 151) .	
Sequencing Sample Tag library alone	MiniSeq High or Mid Output	1.0	50%
	NextSeq High or Mid Output	1.0	50%

Note: BD Biosciences recommends sequencing targeted libraries with Sample Tag libraries rather than sequencing the Sample Tag library alone. Only a limited number of reads are required to call the Sample Tags.

For additional recommendations on sequencing depth, see [Recommendations \(page 150\)](#).

11

Troubleshooting

- [Single sample: cell preparation troubleshooting \(page 178\)](#)
- [Multiplexed samples: cell preparation troubleshooting \(page 179\)](#)
- [Scanning troubleshooting \(page 180\)](#)
- [Cartridge loading troubleshooting \(page 182\)](#)
- [Single sample: library preparation troubleshooting \(page 186\)](#)
- [Multiplexed samples: library preparation troubleshooting \(page 188\)](#)
- [Sequencing troubleshooting \(page 189\)](#)
- [BD Rhapsody Scanner software messages \(page 190\)](#)

Single sample: cell preparation troubleshooting

Introduction

This topic describes possible problems and recommended solutions for cell preparation issues.

Doublets or clumps of adherent cells

Possible causes	Recommended solutions
Cell samples that tend to clump	Filter the cell suspension through an appropriately sized cell strainer multiple times to remove clumps and doublets.

Reported viability from the BD Rhapsody™ Scanner suspected to be too high

Possible causes	Recommended solutions
DRAQ7 staining in the current protocol is optimized for cell lines. The optimal concentration of DRAQ7 may be higher.	Before the BD Rhapsody experiment, optimize the DRAQ7 concentration for your cell types according to the manufacturer's protocol. See bdbiosciences.com/ds/pm/tds/564904.pdf .

Multiplexed samples: cell preparation troubleshooting

Observation	Possible causes	Recommended solutions
No pellet after centrifuging cells or very few cells	Rare or dilute sample	After each centrifugation step, leave 50 μ L of supernatant.
Do not have the recommended buffer for labelling with Sample Tags	Various	Label with Sample Tags in BD Stain Buffer (FBS) (PN 554656) or 1X PBS with 1% FBS or 0.1% BSA.
Cells require labelling with Sample Tags at a different temperature	Physiological requirement	Use protocols for Sample Tag 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.

Scanning troubleshooting

Introduction This topic describes possible problems and recommended solutions for scanning issues.

Incorrect alignment

Possible causes	Recommended solutions
Cartridge or hemocytometer not properly inserted on BD Rhapsody™ Express instrument tray	Ensure that the notched corner of the cartridge or hemocytometer is aligned with the notch of the tray, and the barcode is facing toward the front of the instrument.

Tray not ejected from BD Rhapsody Scanner

Possible causes	Recommended solutions
Cartridge in incorrect orientation or no cartridge	Tap the eject button at the right top corner of the cartridge insertion screen. Reinsert the cartridge on the tray.

BD Rhapsody Scanner not analyzing all images

Possible causes	Recommended solutions
Masked or incompletely processed wells	The software automatically corrects for masked or incompletely processed wells. No manual extrapolation is necessary.

Persistent scanning artifacts

Artifacts may include apparent cells in an empty hemocytometer, consistently low capture rate warnings, and auto-exposure errors.

Possible causes	Recommended solutions
Dirty optics	Contact BD Biosciences technical support at scomix@bdscomix.bd.com .

Cartridge loading troubleshooting

Introduction

This topic describes possible problems and recommended solutions for BD Rhapsody™ Cartridge issues. These issues arise during image analysis. Also see [BD Rhapsody™ Scanner metrics \(page 201\)](#).

Number of cells captured too high or cell doublet rate too high (out of range)

Possible causes	Recommended solutions
Too many cells loaded	<ul style="list-style-type: none"> • Confirm that the calculation of cell dilution is correct. • Reduce the number of cells loaded in the cartridge.
Improper counting with hemocytometer	<ul style="list-style-type: none"> • Recount cells with the BD Rhapsody Scanner. • Follow Staining cells with viability markers (page 68).
Incorrect calculation of cell concentration	<ul style="list-style-type: none"> • Use the BD Rhapsody Scanner for cell counting and analysis. • Use the Analysis app Sample Calculator for preparing cell suspensions.

Number of cells captured lower than anticipated

Possible causes	Recommended solutions
Incorrect calculation of cell concentration	<ul style="list-style-type: none"> Use the BD Rhapsody Scanner for cell counting and analysis. Use the Analysis app Sample Calculator for preparing cell suspensions.
Cell size larger than recommended range	Cell capture efficiency is reduced in the BD Rhapsody Cartridge if the cell diameter is >20 μm .

Bead loading density out of range

Possible causes	Recommended solutions
Insufficient number of Cell Capture Beads loaded	<ul style="list-style-type: none"> Ensure that all of the beads are pelleted before removing storage buffer and are resuspended in Sample Buffer (PN 650000062). Use the Bead Load mode on the pipette. Ensure the beads are well suspended before loading into the cartridge.

Image analysis fails at Bead Wash, indicating insufficient removal of excess Cell Capture Beads

Possible causes	Recommended solutions
Pipette mode incorrect	Ensure that the correct pipette mode is used for the step.
Pipette step missed	Review protocol, and repeat, if necessary.

Cell retention out of range, indicating cell loss during cartridge workflow

Possible causes	Recommended solutions
Pipette mode incorrect	Ensure that the correct pipette mode is used for the step.

Bead retrieval efficiency out of range, indicating poor retrieval

Possible causes	Recommended solutions
Retrieval (top) magnet not positioned properly	Check magnet position.
Pipette mode incorrect	Ensure that the correct pipette mode is used for the step.

Image analysis does not start

Possible causes	Recommended solutions
Software does not initiate analysis after a scan	<ol style="list-style-type: none"> 1. On the BD Rhapsody Scanner main menu, tap Analysis. 2. Tap the experiment. 3. Tap Analyze to start the analysis. You can analyze multiple scan steps at the same time.

Dropped the cartridge or hit it against object

Possible causes	Recommended solutions
Various	<ul style="list-style-type: none"> • If the cartridge was dropped, BD recommends using a new cartridge; otherwise, carefully review the imaging metrics before proceeding. • If the cartridge was struck, proceed at your own risk, and carefully review the imaging metrics before proceeding.

Air bubble in cartridge

Possible causes	Recommended solutions
Air bubble present in pipette tip while dispensing buffer	<p>Confirm that there is an air bubble in the cartridge by examining an image of the cartridge.</p> <p>Ensure that the pipette tip contains only buffer and no air bubble is trapped in the cartridge at the end of aspiration.</p>
Re-used pipette tip	Use a new pipette tip at every pipetting step.

Image analysis fails Contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Installation or mechanical issues See the *BD™ Rhapsody Single-Cell Analysis System Installation and Maintenance Guide* (Doc ID: 43084).

Single sample: library preparation troubleshooting

PCR2 product yield too low

Possible causes	Recommended solutions
PCR1 and PCR2 primers might have been swapped by mistake	Ensure that the correct primer tools are used for each step.
cDNA synthesis may have failed due to incomplete washing of Lysis Buffer. cDNA master mix combines with the Lysis Buffer to form precipitates.	Avoid leaving behind Lysis Buffer or bubbles after removing Lysis Buffer from the tube during bead wash after retrieval from the cartridge. Use new tubes for each wash step, as described in the protocol.
cDNA may have failed due to thermomixer not shaking during reverse transcription	<ul style="list-style-type: none">• Samples need to be on the thermomixer in shake mode.• Where applicable, ensure that a SmartBlock™ Thermoblock is installed on the thermomixer for 1.5 mL tubes so that the reaction can proceed at the designated temperature.

Concentration of final sequencing library too low

Possible causes	Recommended solutions
Issue with PCR2 product yield or quality	<ol style="list-style-type: none"> Determine the product purity: <ul style="list-style-type: none"> Load 1 μL of purified PCR2 products at 1 ng/μL in a High Sensitivity DNA Chip on the Agilent Bioanalyzer. Follow the manufacturer's instructions. Confirm that the PCR2 products should show an average size range of 350–600 bp. If the products pass quality control, proceed to Performing the final amplification for generating the sequencing library (page 142). Repeat the final amplification. If the products do not pass quality control, contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Final sequencing product size too high

Possible causes	Recommended solutions
<ul style="list-style-type: none"> Over-amplification Input amount of PCR2 products too high 	Repeat the final amplification with a lower input of PCR2 products.

Multiplexed samples: library preparation troubleshooting

Observation	Possible causes	Recommended solutions
Yield of Sample Tag Library is too low after final amplification (<1 ng/ μ L)	Various	<ul style="list-style-type: none"> • Ensure that the cells were labelled with Sample Tags correctly during sample preparation. • During library preparation, Sample Tag PCR1 and PCR2 Primers may have been swapped. Ensure that correct primers are used for each step. • AMPure® purification of <1X concentration leads to significant loss of Sample Tag products. Re-amplify and purify the product with 1X AMPure Beads.
Low sequencing quality	Insufficient PhiX	<ul style="list-style-type: none"> • Sequencing targeted library and Sample Tag library together: Use 20% PhiX and a loading concentration of 1.2 pM. • Sequencing Sample Tag library alone: Use 50% PhiX and a loading concentration of 1.0 pM.
Low sensitivity of Sample Tags (<95%)	Insufficient sequencing of the Sample Tag library	<ul style="list-style-type: none"> • Pooled samples of the same cell type: 120 reads/cell. • Pooled samples of different cell types: 600 reads/cell.

Sequencing troubleshooting

Over-clustering on the Illumina flow cell due to under-estimation of the library

Possible causes	Recommended solutions
Inaccurate measurement of the library concentration	Quantitate library according to this user guide.

Low sequencing quality

Possible causes	Recommended solutions
Insufficient PhiX	Use the recommended concentration of PhiX with the library to be sequenced. See Loading densities and PhiX concentrations (page 151) .

BD Rhapsody Scanner software messages

Introduction

This topic lists error and warning messages that you may encounter while using the BD Rhapsody Scanner software.

If the recommended solutions do not resolve the problem, contact BD Biosciences tech support at scomix@bdscomix.bd.com or 1.877.232.8995.

Error messages

Error messages require you to perform an action, according to a displayed dialog, before you can proceed.

Error message	Possible causes	Recommended solutions
“Failed to open door. Ensure nothing is blocking the door.”	Obstruction	Remove obstruction, and retry opening the scanner cartridge door.
“Failed to open door.”	Various; not an obstruction	Restart scanner.
“Failed to close door. Ensure nothing is blocking the door.”	Obstruction	Remove obstruction, and retry closing the scanner cartridge door.
“Failed to close door.”	Various; not an obstruction	Restart the scanner.
“Obstruction detected while ejecting.”	Obstruction or calibration update required	Remove obstruction, and retry ejecting the cartridge.
“Obstruction detected while retracting stage.”	Obstruction	<ol style="list-style-type: none"> 1. Remove obstruction, and retry retracting cartridge into the scanner. 2. Click Cancel. 3. Restart the scanner.

Error message (continued)	Possible causes	Recommended solutions
“Available disk space is extremely low.”	Low disk space	Contact BD tech support scomix@bdscmix.bd.com or 1.877.232.8995.
“Failed to initialize...”	Various	Contact BD tech support scomix@bdscmix.bd.com or 1.877.232.8995.
“Requested move not safe.”	Various	Contact BD tech support scomix@bdscmix.bd.com or 1.877.232.8995.
“Unknown cartridge barcode.”	Unsupported barcode scanned	Contact BD tech support scomix@bdscmix.bd.com or 1.877.232.8995.
“Failed to read file.”	Various	Contact BD tech support scomix@bdscmix.bd.com or 1.877.232.8995.
“Cartridge alignment feature not found.”	<ul style="list-style-type: none"> ● Incorrect cartridge ● Light failed 	Rescan the cartridge.
“Failed to start analysis messaging because: Exception of type 'NetMQ.Address Already In Use Exception' was thrown.”	The last time Analysis software was run, it failed to shut down completely.	Restart the computer.

Warnings

Warnings display in the Warnings box on the scanner display and are mostly informative messages that require no action from you.

Warning message	Possible causes	Recommended solutions
“Barcode scan failed. Please make sure the cartridge is inserted and aligned correctly.”	Barcode not read	<ol style="list-style-type: none"> 1. Reinsert the cartridge, and rescan the cartridge. 2. If necessary, restart the scanner.
“Available disk space is running low.”	Limited disk space	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.
“The protocol was not found.”	Protocol missing	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.
“Auto-exposure is not configured for image type. Using default value instead.”	Method not found	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.
“Auto-exposure resulted in images darker than normal.”	<ul style="list-style-type: none"> • No cell in image tile • Light failed 	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.
“Auto-exposure resulted in images brighter than normal.”	Various	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.
“Auto-exposure adjustment unsuccessful. Using nearest value.”	Algorithm calculation	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.

Warning message (continued)	Possible causes	Recommended solutions
“Autofocus did not find perfect focus. Using nearest value.”	<ul style="list-style-type: none"> ● Bubble in cartridge ● Calibration needs update 	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.
“Attempted to use exposure time greater than maximum. Using the maximum instead.”	Light failed	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.

Warning message (continued)	Possible causes	Recommended solutions
“No alignment marks found. Continuing without cartridge specific calibration.”	Cartridge misaligned	Reinsert cartridge, and rescan.
“Not enough local alignment marks detected.”	<ul style="list-style-type: none"> ● Bead is covering an alignment mark ● Bad image 	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.
“Not enough local fiducials detected.”	<p>Image is out of focus, or bubbles, or clumps of beads have accumulated in the cartridge. After reprocessing with Analysis, review the number of tiles processed in the cartridge results CSV file located in the Public Documents\Rhapsody Data folder of your computer.</p> <p>If the number of tiles is the same as the total number in the cartridge results CSV file, reprocessing is successful. Otherwise, results are extrapolated.</p>	Contact BD tech support scomix@bdscomix.bd.com or 1.877.232.8995.

A

Guidelines to preparing single cell suspensions

- [Finding guidelines for preparing single cell suspensions \(page 196\)](#)
- [Isolating peripheral blood mononuclear cells \(PBMCs\) using cell preparation tubes \(CPTs\) \(page 196\)](#)
- [Lysing red blood cells \(RBCs\) \(page 197\)](#)
- [Preparing cryopreserved leukocytes or PBMCs \(page 199\)](#)

Finding guidelines for preparing single cell suspensions

Introduction

Proceed to the appropriate guideline according to cell type:

- [Isolating peripheral blood mononuclear cells \(PBMCs\) using cell preparation tubes \(CPTs\) \(page 196\)](#)
 - [Lysing red blood cells \(RBCs\) \(page 197\)](#)
 - [Preparing cryopreserved leukocytes or PBMCs \(page 199\)](#)
 - [Preparing cryopreserved leukocytes or PBMCs \(page 199\)](#)
-

Before you begin

Follow sterility best practices. See [Sterility \(page 32\)](#).

Isolating peripheral blood mononuclear cells (PBMCs) using cell preparation tubes (CPTs)

Procedure

1. Centrifuge the CPTs (BD Vacutainer® CPT™ tubes) within 2–3 hours of blood collection for best results.
2. Upon receipt of the tubes, immediately remix the blood sample by gently inverting the CPTs 8–10 times.
3. Centrifuge the CPTs at 1,700 x g at 15°C to 25°C for 20 minutes at acceleration 9 and deceleration 9 using an appropriate centrifuge rotor for glass blood tubes.
4. Aspirate ~1/2 of the plasma without disturbing the whitish mononuclear cell layer.

5. Label a new 15 mL conical tube with the sample name, and then gently collect the mononuclear cell layer with a 5 mL pipette. Transfer the cells to the labelled 15 mL tube.

Avoid vigorous pipetting that may dislodge the gel plug in the CPT.

6. Add phosphate-buffered saline (PBS) to the 15 mL tube to a final volume of 10 mL, and gently pipet the suspension to mix.
 7. Centrifuge the 15 mL tube at 300 x g for 15 minutes.
 8. Aspirate as much supernatant as possible without disturbing the pellet.
 9. Resuspend the pellet by gently tapping the 15 mL tube with an index finger.
 10. Repeat steps 6–9 once for a total of two washes.
 11. Proceed to [Lysing red blood cells \(RBCs\)](#).
-

Lysing red blood cells (RBCs)

Procedure

1. To the resuspended pellet of RBCs from [Isolating peripheral blood mononuclear cells \(PBMCs\) using cell preparation tubes \(CPTs\) \(page 196\)](#), pipet ≤ 1 mL of PBS, and then transfer the suspension to a new 50 mL conical tube.
2. Pipet 10 mL of 1X RBC Lysis Buffer (Thermo Fisher Scientific cat. no. 00-4333-57) to the suspension, and then pipet up and down to mix.
3. Incubate the suspension at 15°C to 25°C for 5 minutes with occasional mixing by tapping the 50 mL tube with an index finger.
4. Pipet 20 mL of PBS to the 50 mL tube to neutralize the RBC lysis.

5. Centrifuge the tube at 120 x g for 5 minutes, and then aspirate the supernatant without disturbing the pellet.
 6. Resuspend the pellet by gently tapping the 50 mL tube with an index finger.
 7. Pipet 20 mL of PBS to the 50 mL tube, and then centrifuge the tube at 120 x g for 5 minutes.
 8. Remove the supernatant without disturbing the pellet, and pipet ≤ 1 mL of PBS to the pellet.
 9. Repeat steps 2–7 once for a total of two lyses and washes.
 10. Remove the supernatant without disturbing the pellet.
 11. Gently resuspend the pellet in 620 μ L of cold Sample Buffer (PN 650000062).
 12. Proceed to [Preparing the BD Rhapsody™ Cartridge \(page 57\)](#).
-

Preparing cryopreserved leukocytes or PBMCs

- Before you begin**
- Follow sterility best practices. See [Sterility \(page 32\)](#).
 - Warm media to 37°C.
-

Procedure

1. Remove the vial of cells from a -80°C freezer or liquid nitrogen tank, and manually shake the vial in a 37°C water bath until the cells are thawed (~1–2 minutes).
 2. Add 9 mL of warm RPMI medium with 10% FBS and 1% antimycotic/antibiotic to a new 15 mL conical tube.
 3. Add 1 mL of the warm RPMI medium with 10% FBS and 1% antimycotic/antibiotic slowly one drop at a time to the thawed cells.
 4. Gently pipet the entire volume of cells to 9 mL of warm RPMI medium in a new 15 mL conical tube.
 5. Centrifuge the cells at 180 x g for 5 minutes.
 6. Aspirate 9 mL of supernatant, and resuspend the pellet in the remaining 1 mL of media. Pipet gently to mix.
 7. Gently pipet the suspended cells to a new 1.5 mL LoBind Tube.
 8. Centrifuge the cells at 300 x g for 5 minutes.
 9. Remove and discard the supernatant, and then resuspend the cells in 620 µL of cold Sample Buffer (PN 650000062).
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B

BD Rhapsody™ Scanner metrics

- [Image analysis metrics \(page 202\)](#)
- [Reviewing the analysis metrics \(page 206\)](#)

Image analysis metrics

Understanding analysis metrics The chart lists the steps and metrics measured during image analysis of the hemocytometer or BD Rhapsody™ Cartridge.

Step	Metric	Definition	Use	Passing threshold
Hemocytometer	Total cell concentration (cell/ μ L)	Number of viable (Calcein AM-positive) and non-viable (DRAQ7-positive) cells/ μ L	Calculates concentration of the cell suspension to determine the volume of cell suspension to use for loading onto the cartridge.	Value
Hemocytometer	Cell viability (%)	Percent viable (Calcein AM-positive) cells	Assesses cell viability to determine if the cell sample meets the quality threshold.	Value
Cell Load	Number of wells with viable cells at Cell Load	Number of wells containing viable (Calcein AM-positive) cells	Provides a preliminary estimate of the number of wells with viable cells captured. ^a	—
Cell Load	Number of viable cells captured in wells at Cell Load	Number of viable (Calcein AM-positive) cells captured in a well	Provides a preliminary estimate of the total number of viable cells captured in the cartridge. ^b	—

Step (continued)	Metric	Definition	Use	Passing threshold
Cell Load	Cell multiplet rate at Cell Load ^d (%)	Number of wells containing multiple viable (Calcein AM-positive) cells versus the total number of wells containing one or more viable cells	Provides a measure of cell clumping.	—
Bead Wash	Number of wells with viable cells and a bead	Total number of wells with one bead and ≥ 1 viable (Calcein AM-positive) cells	Provides an estimate of the number of wells with viable cells captured with beads. ^c	Value
Bead Wash	Number of viable cells captured in wells with a bead	Total number of viable (Calcein AM-positive) cells captured in a well with a bead	Estimates the total number of viable cells captured on a Cell Capture Bead at cell lysis. ^c	—
Bead Wash	Cell multiplet rate ^d (%)	Number of wells containing one bead and multiple viable (Calcein AM-positive) cells versus the total number of wells containing one bead and one or more viable cells	Provides an estimate of the occurrence of multiple cells captured by the same bead in a well.	Value

Step (continued)	Metric	Definition	Use	Passing threshold
Bead Wash	Bead loading efficiency (%)	Percent wells with one bead	Indicates if the cartridge is significantly underloaded with beads.	≥80%
Bead Wash	Cell retention rate (%)	Number of viable (Calcein AM-positive) cells captured in wells prior to lysis versus number of viable cells captured in wells prior to bead loading	Indicates if a significant number of cells initially loaded into wells are lost during the workflow prior to lysis.	≥60%
Retrieval	Bead retrieval efficiency (%)	Percent of wells with beads removed	Indicates if the number of beads retrieved is significantly lower than expected.	≥90%

- a. The number of wells with viable cells is less than the total number of viable cells captured in wells because of wells containing cell multiplets.
- b. Cell capture efficiency at Cell Load (%) = $\frac{\text{Number of viable cells captured in well at Cell Load}}{\text{Total number of viable cells imaged}} * 100$.
- c. The metric, the number of wells with viable cells and a bead, is less than the metric, the number of viable cells captured in wells with a bead, due to wells containing cell multiplets. Multiple cells captured with one bead will appear as one cell in sequencing data. Because of this, the appropriate metric to use for an estimation of the number of cells that could be recovered in sequencing is the metric, number of wells with viable cells and a bead.
- d. The estimated multiplet rate is calculated by Poisson distribution using the number of cells loaded into the cartridge. See [Loading cells into the cartridge \(page 94\)](#). The cell multiplet rate reported by the BD Rhapsody Scanner is determined by imaging. If the cell multiplet rate >> estimated multiplet rate, this may indicate cell clumping.

**Viewing image
analysis metrics**

To view image analysis metrics in a spreadsheet, click the **Rhapsody Data** shortcut on the BD Rhapsody Scanner screen. The Rhapsody Data folder contains ExperimentName_SampleName.csv, PrepareSampleResults.csv, and Cell Count.csv.

Reviewing the analysis metrics

1. Navigate to the Analysis application.
2. Tap the experiment to ensure that the analyses are completed.
For example:

Dispense1-1

Cartridge 0109034055A

Scan Date	Sample	Step	Analysis Status
2018-02-21 10:29:27.56	Dispense1-1	Cell Load	✓ Completed
2018-02-21 10:57:04.44	Dispense1-1	Bead Load	✓ Completed
2018-02-21 11:11:49.59	Dispense1-1	Bead Wash	✓ Completed
2018-02-21 11:26:42.94	Dispense1-1	Retrieval	✓ Completed

3. Review the analysis metrics. For PASS criteria, see [Image analysis metrics \(page 202\)](#). For example:

Analysis	
Number of wells with viable cells at cell load	9118
Cell multiplet rate at cell load	2.4 %
Number of wells with viable cells and a bead	7999
Cell multiplet rate	2.0 %
Bead loading efficiency	✓ PASS
Excess bead rate	✓ PASS
Cell retention rate	✓ PASS
Bead retrieval efficiency	✓ PASS

4. (Optional) View quantitative analysis metrics for each experiment in a spreadsheet. For example, the .csv files from the analysis of the Jurkat and Ramos cells are displayed:

Note: To set up the shortcut to the Rhapsody Data folder in Quick Access, see [BD Rhapsody Scanner software \(page 33\)](#).

- (1) [Analysis metrics \(page 208\)](#)
- (2) [Cell count \(page 209\)](#)
- (3) [Sample result \(page 209\)](#)

(1) Analysis metrics

Barcode	0109003003A
Experiment	Dispense 1-1
Sample	Dispense 1-1
Scan System ID	R-1016
Scan start date and time	6/5/2019 10:20
Operator	PC
Analysis Software Version	1.3.0.12
Cell load analysis last updated	6/5/2019 11:10
Total number of tiles	26
Cell load number of tiles processed	26
Number of wells with viable cells at cell load	4001
Number of viable cells captured in wells at cell load	4052
Cell multiplet rate at cell load	1.30%
Bead wash analysis last updated	6/5/2019 11:37
Bead wash number of tiles processed	26
Number of wells with viable cells and a bead	3665
Number of viable cells captured in wells with a bead	3710
Cell multiplet rate	1.20%
Bead loading efficiency	94.70%
Bead loading acceptance	PASS
Excess bead rate	0.00%
Excess bead acceptance	PASS
Cell retention rate	96.90%
Cell retention acceptance	PASS
Bead retrieval analysis last updated	6/5/2019 11:47
Bead retrieval number of tiles processed	26
Bead retrieval efficiency	95.90%
Bead retrieval acceptance	PASS
Bead retrieval efficiency relative to bead wash	95.70%
Number of retrieved beads with 1+ viable cells	3507

(2) Cell count

Analysis software version	1.3.0.12
Experiment	20190528_PC_CartridgeSWValidation
Sample	Jurkat
Barcode	0107001031A
Protocol step	Side A
Scan System ID	RESOLVE-1017
Operator	PC
Scan start date and time	5/28/2019 10:59
Analysis last updated	6/5/2019 9:46
Total cell concentration (live and dead)	1645.89
Cell viability	94.98

Analysis software version	1.3.0.12
Experiment	20190528_PC_CartridgeSWValidation
Sample	Ramos
Barcode	0107001031A
Protocol step	Side B
Scan System ID	RESOLVE-1017
Operator	PC
Scan start date and time	5/28/2019 11:00
Analysis last updated	6/5/2019 9:47
Total cell concentration (live and dead)	1697.5
Cell viability	94.65

(3) Sample result

Experiment	20190528_PC_CartridgeSWValidation					
Cartridge type	109					
Cartridge volume(uL)	575					
Total volume to prepare (uL)	1500					
Desired number of captured ce	10000					
Sample buffer volume (uL)	1479.6					
Loading cell concentration	22.8					
Cell doublet rate (%)	2.4					
Sample	Operator	Scan Date and Time	Concentration	Viable Cells	Relative Amount	Stock Volume (uL)
Jurkat	PC	57:49.7	1668.4	95.14	1	10.2
Ramos	PC	58:38.2	1686.4	94.7	1	10.1

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C

Sequence information

- [Illumina library Index 1 \(i7\) sequences \(page 212\)](#)
- [Sample Tag sequences \(page 213\)](#)

Illumina library Index 1 (i7) sequences

Primers supplied in the BD Rhapsody™ Targeted Reagent Kit

Library Reverse Primer	Sequence
1	GCTACGCT
2	CGAGGCTG
3	AAGAGGCA
4	GTAGAGGA

Sample Tag sequences

Sequences

Each Human 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]AAAAAAAAAAAAAAAAAAAAAAAAAAAA

Sample Tag	Sample Tag sequence
Sample Tag 1— Human	ATTCAAGGGCAGCCGCGTCACGATTGGATACGACTGTTGGACCGG
Sample Tag 2— Human	TGGATGGGATAAGTGCGTGATGGACCGAAGGGACCTCGTGGCCGG
Sample Tag 3— Human	CGGCTCGTGCTGCGTCTCAAGTCCAGAACTCCGTGTATCCT
Sample Tag 4— Human	ATTGGGAGGCTTTCGTACCGCTGCCGCCACCAGGTGATACCCGCT
Sample Tag 5— Human	CTCCCTGGTGTTCAATACCCGATGTGGTGGGCAGAATGTGGCTGG
Sample Tag 6— Human	TTACCCGCAGGAAGACGTATACCCCTCGTGCCAGGCGACCAATGC
Sample Tag 7— Human	TGTCTACGTCCGACCGCAAGAAGTGAGTCAGAGGCTGCACGCTGT
Sample Tag 8— Human	CCCCACCAGGTTGCTTTGTTCGGACGAGCCCGCACAGCGCTAGGAT
Sample Tag 9— Human	GTGATCCGCGCAGGCACACATACCGACTCAGATGGGTTGTCCAGG

Sample Tag	Sample Tag sequence
Sample Tag 10— Human	GCAGCCGGCGTCGTACGAGGCACAGCGGAGACTAGATGAGGCCCC
Sample Tag 11— Human	CGCGTCCAATTTCCGAAGCCCCGCCCTAGGAGTTCCTGCGTGC
Sample Tag 12— Human	GCCCATTCATTGCACCCGCCAGTGATCGACCCTAGTGGAGCTAAG

Each Mouse Immune Sample Tag is an Anti-Mouse CD45, Clone 30-F11 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]AAAAAAAAAAAAAAAAAAAAAAAAA

Sample Tag	Sample Tag sequence
Sample Tag 1— Mouse Immune	AAGAGTCGACTGCCATGTCCCCTCCGCGGGTCCGTGCCCCCA AG
Sample Tag 2— Mouse Immune	ACCGATTAGGTGCGAGGCGCTATAGTCGTACGTTCGTTGCCGTGC C
Sample Tag 3— Mouse Immune	AGGAGGCCCGCGTGAGAGTGATCAATCCAGGATACATTCCTCGT C
Sample Tag 4— Mouse Immune	TTAACCGAGGCGTGAGTTTGGAGCGTACCGGCTTTGCGCAGGG CT
Sample Tag 5— Mouse Immune	GGCAAGGTGTCACATTGGGCTACCGCGGGAGGTCGACCAGATC CT
Sample Tag 6— Mouse Immune	GCGGGCACAGCGGCTAGGGTGTTCGGGTGGACCATGGTTCAG GC
Sample Tag 7— Mouse Immune	ACCGGAGGCGTGTGTACGTGCGTTTCGAATTCCTGTAAGCCCAC C

Sample Tag	Sample Tag sequence
Sample Tag 8— Mouse Immune	TCGCTGCCGTGCTTCATTGTCGCCGTTCTAACCTCCGATGTCTC G
Sample Tag 9— Mouse Immune	GCCTACCCGCTATGCTCGTCGGCTGGTTAGAGTTTACTGCACGC C
Sample Tag 10— Mouse Immune	TCCCATTCGAATCACGAGGCCGGGTGCGTTCTCCTATGCAATCC C
Sample Tag 11— Mouse Immune	GGTTGGCTCAGAGGCCCCAGGCTGCGGACGTCGTCGGACTCGC GT
Sample Tag 12— Mouse Immune	CTGGGTGCCTGGTCGGGTTACGTCGGCCCTCGGGTCGCGAAGG TC

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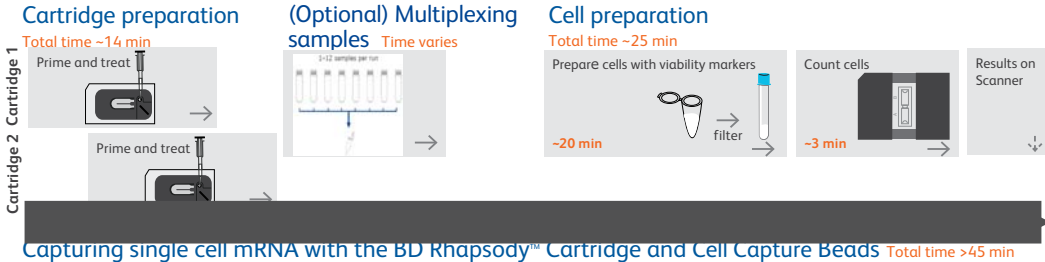
D

Workflow with two BD Rhapsody™ Cartridges

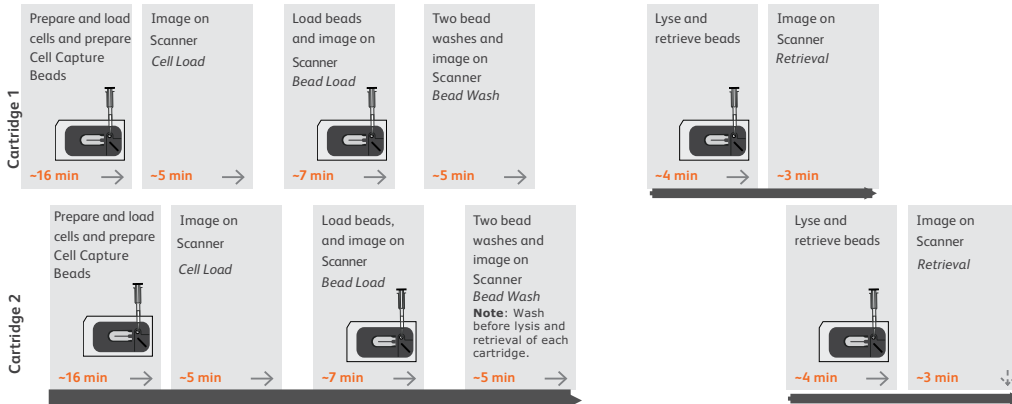
- [Workflow with two cartridges \(page 216\)](#)
- [Best practices with a two-cartridge workflow \(page 217\)](#)

Workflow with two cartridges

Staggered boxes indicate staggering the start of like steps.



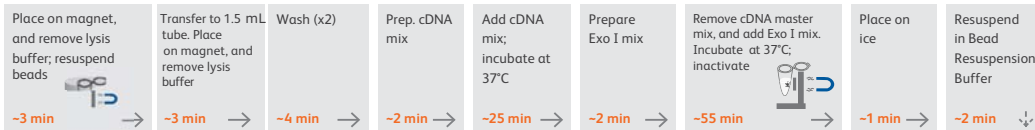
Capturing single cell mRNA with the BD Rhapsody™ Cartridge and Cell Capture Beads Total time >45 min



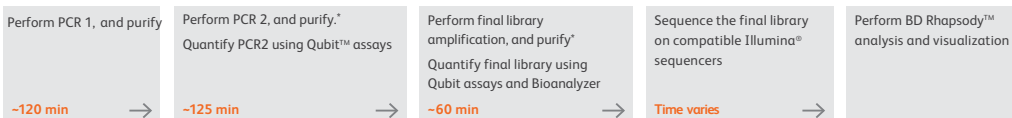
Proceed immediately to performing reverse transcription and Exonuclease I treatment.

Performing reverse transcription and Exonuclease I treatment on Cell Capture Beads Total time ~1 hr 40 min

Set thermomixer to 37°C → Put bead collection tube on magnet → Prepare cDNA master mix



Preparing a single cell sequencing library with the BD Rhapsody™ Targeted Reagent Kit Total time ~5 hrs



*Stopping point: May store sample at -20°C. See user guide for details.

Best practices with a two-cartridge workflow

Reagent preparation

To prepare a master mix of sufficient volume for two cartridges, follow the volumes for two libraries plus 10% overage listed for preparing a master mix.

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E

Safety

- [General safety and limitations \(page 220\)](#)
- [Chemical safety \(page 220\)](#)
- [Physical safety \(page 222\)](#)
- [Instrument waste disposal \(page 222\)](#)

General safety and limitations

For instrument safety, see the *BD Rhapsody™ Express Instrument and BD Rhapsody™ Scanner Safety and Limitations Guide* (Doc ID: 42061).

Genomics technical publications are available for download from the BD Genomics Resource Library at bd.com/genomics-resources.

Chemical safety

Requirements

- Read and comprehend all safety data sheets (SDSs) by chemical manufacturers before you use, store, or handle any chemicals or hazardous materials.
 - Wear personal protective equipment (gloves, safety glasses, fully enclosed shoes, lab coats) when handling chemicals.
 - Do not inhale fumes from chemicals. Use adequate ventilation, and return caps to bottles immediately after use.
 - Check regularly for chemical spills or leaks. Follow SDS recommendations for cleaning up spills or leaks.
-

Waste

The BD Rhapsody™ system has two waste types or streams. Each waste stream requires individual consideration for safe and responsible disposal:

Waste	Description
Stream 1: Waste Collection Container	<ul style="list-style-type: none"> ● Frequency of Handling: every BD Rhapsody experiment ● Content: ethanol (11%), polymer microparticles (<1%), cells (trace) ● Main Risk Constituent: cells (trace) ● Collect and dispose of all waste in the Waste Collection Container using proper precautions and according to local safety regulations.
Stream 2: BD Rhapsody™ Cartridge	<ul style="list-style-type: none"> ● Frequency of Handling: every BD Rhapsody experiment ● Content: polymer (99%), polymer microparticles (<1%), lysis buffer (<1%) ● Main Risk Constituent(s): lysis buffer ● Collect and dispose of all used BD Rhapsody Cartridges using proper precautions and according to local safety regulations.

Physical safety

See the *BD Rhapsody™ Express Instrument and BD Rhapsody™ Scanner Safety and Limitations Guide* (Doc ID: 42061).

Genomics technical publications are available for download from the BD Genomics Resource Library at bd.com/genomics-resources.

Instrument waste disposal

Disposal of the instrument

Contact BD Biosciences technical support at scomix@bdscomix.bd.com before moving the BD Rhapsody™ Scanner or disposing of the BD Rhapsody™ Express instrument or the BD Rhapsody Scanner. For more information, see [Instrument technical support \(page 15\)](#).

Glossary

B

BD Rhapsody™ Express instrument Mechanical station used for loading Cell Capture Beads and cells into the BD Rhapsody™ Cartridge.

BD Rhapsody™ Scanner Instrument used for counting cells on a hemocytometer and counting viable cells in the cartridge workflow.

BD Rhapsody™ Single-Cell Analysis system The system includes:

- BD Rhapsody™ Targeted Reagent Kit
- BD Rhapsody™ Express instrument
- BD Rhapsody™ P1200M pipette
- BD Rhapsody™ P5000M pipette
- BD Rhapsody Scanner
- Hemocytometer Adapter
- Bioinformatics software and sequencing analysis

L

load To add a reagent to the BD Rhapsody Cartridge.

P

protocol The protocol files for the BD Rhapsody Scanner describe all instrument settings for a cartridge scan and the order in which the scans occur.

S

scan

A series of images taken of the Hemocytometer Adapter or BD Rhapsody Cartridge by the BD Rhapsody Scanner at multiple predetermined locations.

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