

BD OneFlow™ Application Guide for Acute Leukemias

For BD OneFlow™ ALOT



23-18905-01
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Regulatory information

The BD FACSCanto II flow cytometer is a Class 1 Laser Product.

For In Vitro Diagnostic Use.

History

Revision	Date	Change made
23-18905-00	3/2017	Initial release
23-18905-01	11/2019	Removed CD from the installer description. Removed the cell range. Updated Australian and New Zealand addresses.

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Overview

This chapter covers the following topics:

- [Overview of the BD OneFlow system \(page 6\)](#)
- [Workflows for BD OneFlow ALOT \(page 7\)](#)

Overview of the BD OneFlow system

About the system The BD OneFlow™ system provides a comprehensive set of reagents and protocols to reproducibly set up the flow cytometer and stain patient specimens. The consistent instrument setup and sample staining enable you to acquire and analyze patient specimens for immunophenotyping of normal and aberrant cell populations in a manner compatible with that prescribed by the EuroFlow™ Consortium.

BD OneFlow™ ALOT (Acute Leukemia Orientation Tube) is used to stain patient peripheral blood (PB) or bone marrow (BM) specimens. The stained samples are acquired on the cytometer and then analyzed to identify aberrant immature hematopoietic cells.

-
- Materials needed**
- BD OneFlow™ ALOT
 - Catalog No. 660228
 - BD FACSDiva™ CS&T IVD beads (CS&T IVD beads)
 - Catalog No. 656046 or 656047
 - BD OneFlow™ Setup Beads
 - Catalog No. 658620
 - BD® FC Beads 8-color kit for BD OneFlow™ Assays (BD FC beads)
 - Catalog No. 658621
 - Templates installer for BD OneFlow Assays
 - Catalog No. 659305
 - BD FACSCanto™ II flow cytometer with a 3-laser, 8-color, 4-2H-2V BD default optical configuration, running BD FACSDiva™ software v8.0.1 or later
 - FIX & PERM® Cell Fixation & Cell Permeabilization kit
-

Workflows for BD OneFlow ALOT

Specimen preparation

Task	Reagents or materials	Template needed	Outcome
Staining the specimen	BD OneFlow ALOT FIX & PERM Wash buffer	None	Stained patient specimen is ready for acquisition.

Sample acquisition

Task	Reagents or materials	Template needed	Outcome
Import the OneFlow ALOT template.	None	OneFlow ALOT	The OneFlow ALOT template is imported into an experiment, and application settings are applied.
Acquiring the stained sample	Stained patient sample	The BD OneFlow ALOT Acquisition worksheet	The FCS file is generated.

Data analysis

Task	Reagents or materials	Template needed	Outcome
Analyzing the data using BD FACSDiva software	FCS file for patient sample	The BD OneFlow ALOT Analysis worksheet	Aberrant immature populations of hematopoietic cells are identified.

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Sample preparation

This chapter covers the following topic:

- [Staining the specimen \(page 10\)](#)

Staining the specimen

About the specimens

This procedure works for PB or BM specimens collected in EDTA-containing or heparin-containing blood collection tubes (for example, BD Vacutainer® tubes).

About the dried reagent

The BD OneFlow ALOT reagent is very sensitive to moisture. To avoid condensation, open the pouches only if they are at room temperature. Ensure that the pouch is completely resealed after removing a tube. Do not remove the desiccant from the reagent pouch.

Write the current date on the pouch label when it is first opened. Use the tubes from that pouch within one month before opening the next pouch.

About fixing and permeabilizing the cells

The volumes for the cell fixation and permeabilization steps are important. After using FIX & PERM Reagent A to fix the cells, wash them, centrifuge them, and remove the supernatant. We recommend that you measure the residual volume and then add wash buffer to give a final volume of 100 µL prior to adding the fixed cells to FIX & PERM Reagent B. This will ensure that the cells are completely permeabilized using FIX & PERM Reagent B.

Staining the specimen

1. If the pouches are stored refrigerated, allow them to reach room temperature before opening them.
2. For each patient specimen, remove a BD OneFlow ALOT (S) tube from its pouch. Do not remove the BD OneFlow ALOT (C) tube from its pouch at this time.
3. Place the tubes in a rack, protected from light.
4. Immediately reseat the pouch with any unused tubes.

Note: The reagent is very sensitive to light. Start staining the specimen immediately.

5. Write the patient ID on the tube label within the area provided.
6. Invert the specimen in the collection tube 10 times to mix well.
7. Add 50 μ L of wash buffer (filtered phosphate buffered saline (PBS) + 0.5% bovine serum albumin (BSA) + 0.09% or 0.1% sodium azide) and 50 μ L of unwashed specimen to the BD OneFlow ALOT (S) tube.

Note: Make sure that all of the specimen is in contact with the reagent at the bottom of the tube.

Note: Do not wipe the outside of the tube with ethanol or isopropanol because the ink on the printed label can run.

8. Vortex vigorously 3–5 seconds to mix well.
9. Incubate for 30 minutes at 20°C–25°C, protected from light.
10. Add 1.5 mL of wash buffer. Vortex vigorously 3–5 seconds to mix well.
11. Add an additional 1.5 mL of wash buffer. Vortex gently to mix.
12. Centrifuge at 540g for 5 minutes at 20°C–25°C.
13. Remove the supernatant without disturbing the cell pellet, leaving approximately 50 μ L of residual liquid in the tube.
14. Vortex vigorously until the cell pellet is completely resuspended.
15. Add 100 μ L of FIX & PERM Reagent A (fixation solution) to the tube. Vortex vigorously 3–5 seconds to mix well.
16. Incubate for 15 minutes at 20°C–25°C, protected from light.
17. Add 1.5 mL of wash buffer. Vortex vigorously 3–5 seconds to mix well.
18. Add an additional 1.5 mL of wash buffer. Vortex gently to mix.
19. Centrifuge at 540g for 5 minutes at 20°C–25°C.

20. Remove the supernatant without disturbing the cell pellet, leaving approximately 50 µL of residual liquid in the tube.
21. Vortex vigorously until the cell pellet is completely resuspended.

Note: If you are unable to obtain a single-cell suspension, see [Troubleshooting](#).

22. Measure the volume in each tube using a pipet and add wash buffer to give a final volume of 100 µL in each tube. Vortex 3–5 seconds to mix well.

Note: It is important to have a final volume of 100 µL in each tube so that all of the cells will be completely permeabilized in steps 25–28.

23. Remove the appropriate number of BD OneFlow ALOT (C) tubes from the pouch and reseal the pouch immediately.
24. Write the patient ID on the BD OneFlow ALOT (C) tube label within the area provided.
25. Add 100 µL of FIX & PERM Reagent B (permeabilization solution) to the BD OneFlow ALOT (C) tube.
26. Transfer 100 µL of the sample from the BD OneFlow ALOT (S) tube to the corresponding BD OneFlow ALOT (C) tube.

Note: Make sure that the patient ID numbers on the two tubes are the same.

27. Vortex the BD OneFlow ALOT (C) tube vigorously 3–5 seconds to mix well.
28. Incubate for 15 minutes at 20°C–25°C, protected from light.
29. Add 1.5 mL of wash buffer. Vortex vigorously 3–5 seconds to mix well.
30. Add an additional 1.5 mL of wash buffer. Vortex gently to mix.
31. Centrifuge at 540g for 5 minutes at 20°C–25°C.

32. Remove the supernatant without disturbing the cell pellet, leaving approximately 50 μL of residual liquid in the tube.
33. Add 200 μL of wash buffer to the tube. Vortex vigorously 3–5 seconds to completely resuspend the cell pellet.

Next step

Specimens should be acquired immediately after staining. If a longer period of time is desired, each laboratory should validate that stained specimens acquired after being held under their storage conditions produce equivalent results to specimens acquired immediately after staining. Protect stained specimens from light until they are acquired.

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Sample acquisition

This chapter covers the following topics:

- [Setting up the experiment \(page 16\)](#)
- [Acquiring the stained sample \(page 22\)](#)

Setting up the experiment

About linking and unlinking compensation

When you create a new experiment, you must apply the correct application settings. Before applying the correct application settings, you should first link the appropriate compensation matrix to the experiment and then unlink the compensation matrix. Unlinking the compensation matrix allows updated application settings to be applied, thus giving photomultiplier tube voltages (PMTVs) that will result in correct target median fluorescence intensity (MFI) values, while retaining compensation values. When you apply the application settings, you keep the compensation values.

Before you begin

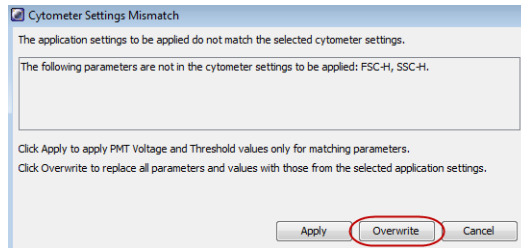
1. In BD FACSDiva software v.8.0.1 or later, ensure that cytometer warmup is complete, fluidics startup has been performed, and the cytometer is in the default 4-2H-2V configuration.
 2. Verify that the daily performance check was completed and passed for the default 4-2H-2V configuration using CS&T IVD beads within the past 24 hours. See the *Instrument Setup Guide for BD OneFlow™ Assays*.
 3. Recommended: confirm that the PMTVs are still within their daily target ranges. See the chapter for daily setup in the *Instrument Setup Guide for BD OneFlow™ Assays*.
 4. Make sure that you have installed the OneFlow ALOT template. See the *Instrument Setup Guide for BD OneFlow™ Assays* or the *BD OneFlow™ ALOT Instructions for Use*.
-

Setting up the experiment

1. Create a new experiment.
 - a. From the menu bar, select **Experiment > New Experiment > Blank Experiment**. Click **OK**.

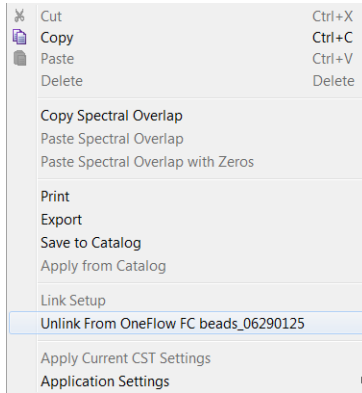
Note: You can also create an experiment directly from the **Browser** using the **Experiment** icon.

- b. If prompted by the **CST Mismatch** dialog, select **Use CST Settings**.
 - c. Rename the experiment according to your laboratory practice.
2. Link compensation.
 - a. In the **Browser**, right-click **Cytometer Settings**.
 - b. From the menu, select **Link Setup**.
 - c. Select the appropriate compensation matrix calculated using BD FC beads within the past 31 days. Click **Link**.
 - d. If prompted by the **Cytometer Settings Mismatch** dialog, select **Overwrite**.

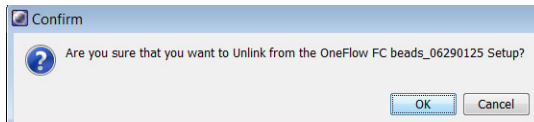


3. Unlink compensation.
 - a. In the **Browser**, right-click **Cytometer Settings**.

- b. From the menu, select **Unlink From** and select the previously linked compensation setup.



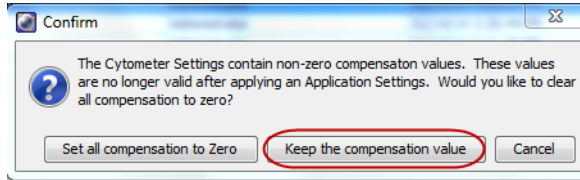
- c. From the **Confirm** dialog that opens, click **OK** to unlink from the previously linked compensation setup.



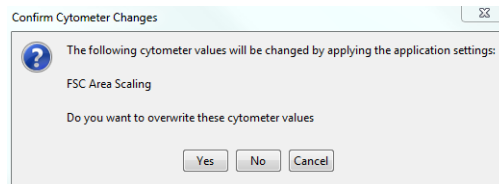
4. Apply application settings.
 - a. In the **Browser**, right-click **Cytometer Settings**.
 - b. From the menu, select **Application Settings > Apply**.
 - c. Select the most recent application settings. Click **Apply**.

Note: Confirm that the most recent application settings were created within the past 31 days using the BD OneFlow Setup beads. The application settings are created in the monthly setup as described in the *Instrument Setup Guide for BD OneFlow™ Assays*.

- d. When prompted by the **Confirm** dialog, select **Keep the compensation value**.

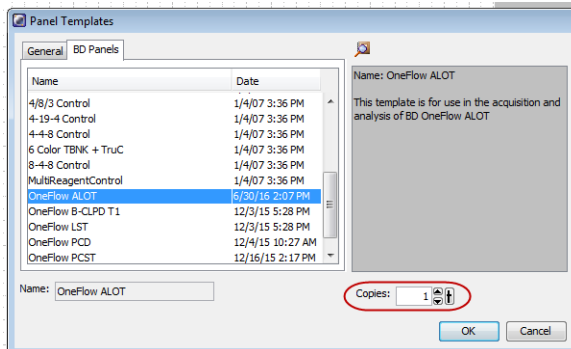


- e. If prompted by the **Confirm Cytometer Changes** dialog, click **Yes** to overwrite the cytometer values for **FSC Area Scaling**.



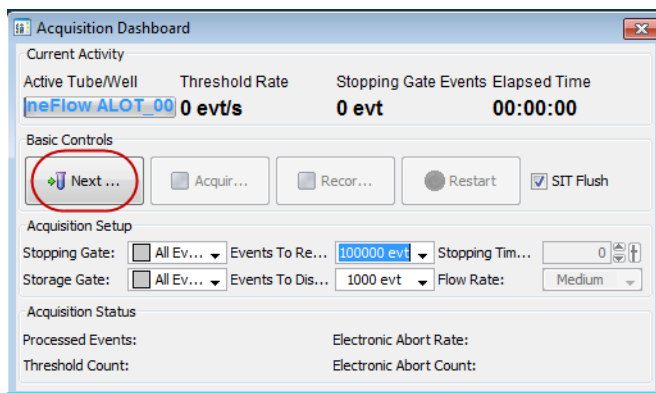
5. Import the OneFlow ALOT template.
- Select the experiment in the **Browser** and then select **Experiment > New Specimen** from the menu bar.
The **Panel Templates** dialog opens.
 - Navigate to the **BD Panels** tab and select the OneFlow ALOT template.
Note: Make sure that you select the template for the BD OneFlow tube that you are acquiring.

- c. Indicate the number of patient specimens you want to acquire using the **Copies** field.



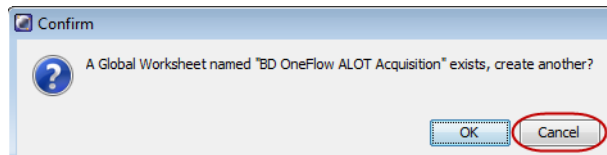
- d. Click **OK**.
- e. Rename each specimen, for example, with the appropriate patient ID in front of the specimen name.

Note: If you have to re-run a particular patient sample, set the current tube pointer to the tube you wish to re-run. Click **Next Tube** in the **Acquisition Dashboard** to create another tube for that patient. Do not select **Experiment > New Tube** from the menu bar or use the **New Tube** icon from the **Browser** menu bar to create the additional tube to be acquired because the labels and barcode fields will not be populated.



6. Confirm that all of the voltages are the same as those set as application settings.
 - a. In the **Browser**, select the application settings that you want to confirm.
 - b. In the **Inspector**, navigate to the **Parameters** tab to view the voltages in the application settings.
 - c. From the menu bar, select **Cytometer > Catalogs**.
The **Catalogs** dialog opens.
 - d. Navigate to the **Application Settings** tab.
 - e. Select the application settings used in the current experiment. Click **View**.
 - f. Confirm that the voltages in the catalog are the same as those in the application settings.
 - g. Click **Close** in the **Catalogs** dialog.

Note: If you want to acquire additional patient samples in the experiment, repeat [step 5](#) to add new specimens. Two **Confirm** dialogs will open asking if you want to create another Acquisition worksheet or another Analysis worksheet. Click **Cancel** in each dialog.



7. Scan the barcode on the tube label into the **Product ID** keyword field.
 - a. From the menu bar, select **Experiment > Experiment Layout** and navigate to the **Keywords** tab.
 - b. Highlight the **Product ID** keyword for the appropriate tube, and scan the barcode on the BD OneFlow ALOT tube label.

Note: If you cannot scan the barcode on the tube label, see [Troubleshooting](#).

The screenshot shows the 'Experiment Layout' window with the 'Acquisition' tab selected. It features a 'Quick Entry' section with a 'Value' field and a 'System Defined Keywords' checkbox. Below this is a table with columns for Name and Keyword, and rows for different tube types and their associated keywords.

Name	Keyword	Keyword	Keyword	Keyword	Keyword	Keyword	Keyword	Keyword
BD ALOT 063016								
BD ALOT 2817 BU								
OneFlow ALOT_001	SAMPLE ID	PATIENT ID	CASE NUMBER	PRODUCT ID 660226/000000,2	SPECIMEN TYPE	DOCTOR	TEMPLATE VERSION ALOTV1.0	TEMPLATE BUILD 033116
BD ALOT 9108 BU								
OneFlow ALOT_001	SAMPLE ID	PATIENT ID	CASE NUMBER	PRODUCT ID 660226/000000,2	SPECIMEN TYPE	DOCTOR	TEMPLATE VERSION ALOTV1.0	TEMPLATE BUILD 033116
BD ALOT 9077 BU								
OneFlow ALOT_001	SAMPLE ID	PATIENT ID	CASE NUMBER	PRODUCT ID 660226/000000,2	SPECIMEN TYPE	DOCTOR	TEMPLATE VERSION ALOTV1.0	TEMPLATE BUILD 033116
Tube_001	SAMPLE ID	PATIENT ID	CASE NUMBER					

- c. Manually add the appropriate information to the remaining keywords, as needed.
- d. Click **OK** to close the **Experiment Layout**.

Acquiring the stained sample

- Acquiring the tube**
1. In the **Browser**, expand the appropriate specimen and set the current tube pointer to that tube.
 2. Select the **BD OneFlow ALOT Acquisition** worksheet tab.
 3. Vortex the stained tube 3–5 seconds at low speed.
 4. Install the tube on the cytometer. Adjust the flow rate to **Medium** in the **Acquisition Dashboard**. Click **Acquire Data**.
 5. Verify that the population is on scale and adjust the gate in the first plot of the ALOT acquisition worksheet to exclude debris, if needed.
 6. Click **Record Data** in the **Acquisition Dashboard** to collect total events.

Note: The template automatically collects 100,000 total events. Use the menu in the **Acquisition Dashboard** to select a different number of events to acquire, if needed.

Inspecting the BD OneFlow ALOT Acquisition worksheet

1. Select the **BD OneFlow ALOT Acquisition** worksheet tab.
2. Inspect the dot plots on the ALOT acquisition worksheet, and adjust the gates as needed.

Some of the dot plots might look different from those in other experiments. The initial FSC-A vs SSC-A dot plot to identify cells and eliminate debris may appear compressed. This is a consequence of the target values used to create the application settings. The values are specified by the EuroFlow Consortium.

Note: Enlarge the dot plots while adjusting the gates so you can more readily see the populations of interest.

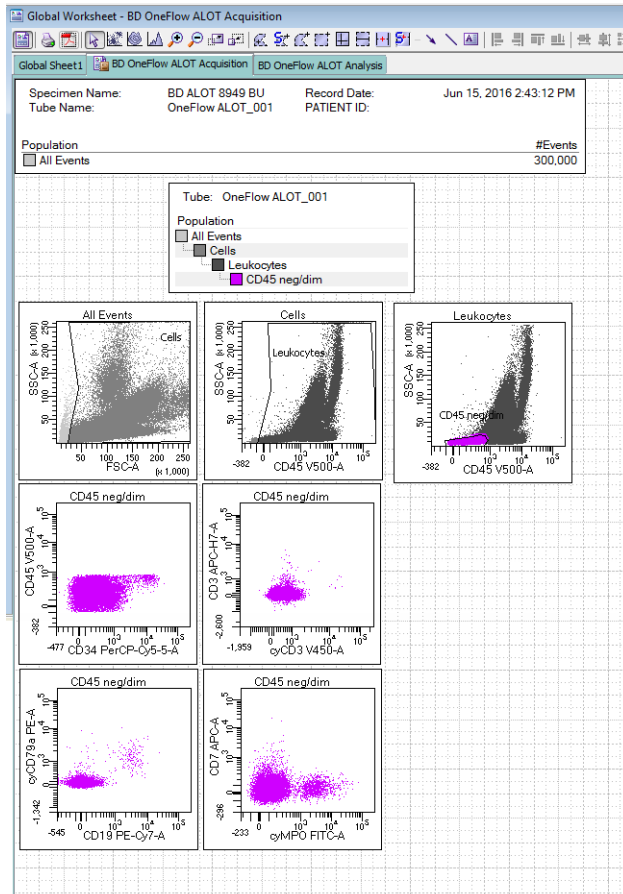
The FSC-A vs SSC-A dot plot is used to identify cells.

The CD45 V500-A vs SSC-A dot plot from the Cells population is used to identify leukocytes.

The CD45 V500-A vs SSC-A dot plot from the Leukocytes population is used to identify the CD45^{neg/dim} population.

The remaining dot plots do not contain gates and are included to assess staining of the CD45^{neg/dim} population with all of the

antibodies, therefore serving as an internal quality control for the tube.



3. Continue until all of the tubes have been acquired.
4. From the menu bar, select **File > Export > Experiments**, and select the **Directory Export** option. Click **OK**.

4

Data analysis

This chapter covers the following topic:

- [Analyzing the data using BD FACSDiva software \(page 26\)](#)

Analyzing the data using BD FACSDiva software

About the dot plots Some of the dot plots might look different from those in other experiments. The initial FSC-A vs SSC-A dot plot to identify cells and eliminate debris may appear compressed. This is a consequence of the target values used to create the application settings. The values are specified by the EuroFlow Consortium.

Analyzing BD OneFlow ALOT

1. From the menu bar, select **File > Import > Experiments**.
2. Select the experiment that you want to analyze. Click **Import**.
The experiment with the associated acquisition and analysis worksheets opens.
3. Select the **BD OneFlow ALOT Analysis** worksheet tab.
4. Inspect the dot plots on page 1 of the ALOT analysis worksheet, and adjust the gates as needed.

Note: Enlarge the dot plots while adjusting the gates so you can more readily see the populations of interest.

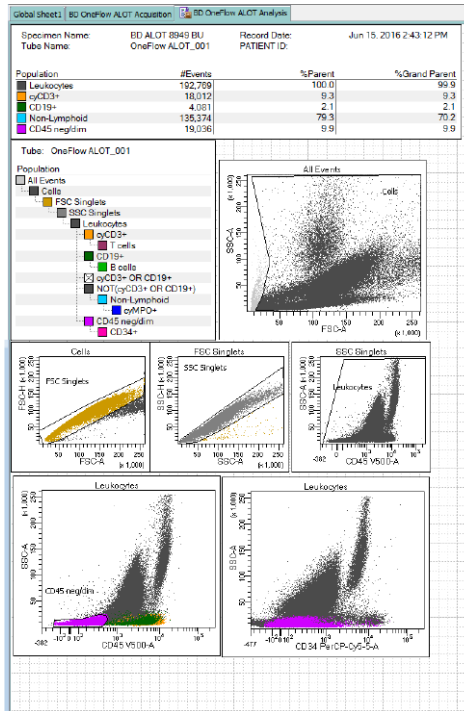
The first three dot plots on page 1 of the ALOT analysis worksheet identify cells, FSC singlets, and SSC singlets. Debris and doublets are excluded by adjusting the gates.

Examine the Leukocytes population in the CD45 V500-A vs SSC-A dot plot from the SSC Singlets gate.

Examine the CD45^{neg/dim} population in the CD45 V500-A vs SSC-A dot plot from the Leukocytes gate.

The CD34 PerCP-Cy5.5-A vs SSC-A dot plot does not have a gate and is used for informational purposes.

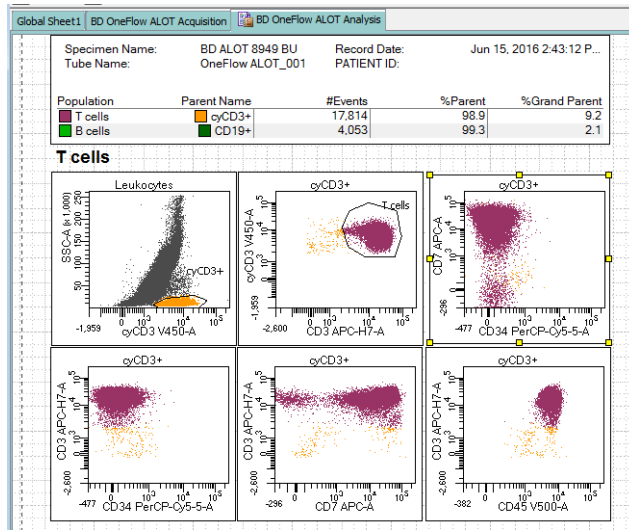
Note: These are examples of normal BM. Patient samples may look different.



- Inspect the dot plots identifying T cells on the top half of page 2 of the ALOT analysis worksheet, and adjust the gates as needed.

Cells expressing cytoplasmic CD3 (cyCD3⁺) are identified in the cyCD3 V450-A vs SSC-A dot plot from the Leukocytes population.

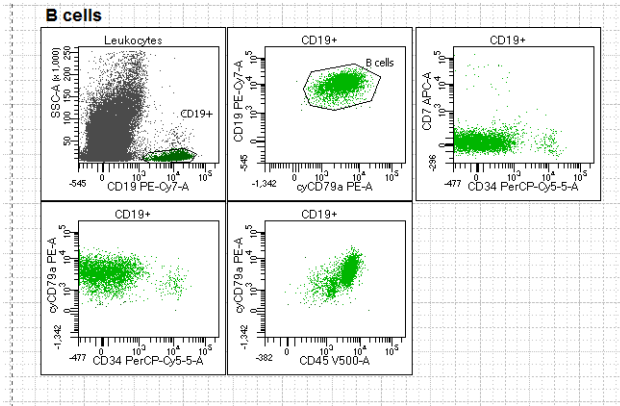
T cells are identified in the CD3 APC-H7-A vs cyCD3 V450-A dot plot from the cyCD3⁺ population. The cyCD3⁺ cells are characterized in the remaining dot plots.



- Inspect the dot plots identifying B cells on the bottom half of page 2 of the ALOT analysis worksheet, and adjust the gates as needed.

CD19⁺ cells are identified in the CD19 PE-Cy7-A vs SSC-A dot plot from the Leukocytes population.

B cells are identified in the cyCD79a PE-A vs CD19 PE-Cy7-A dot plot from the CD19⁺ population. The CD19⁺ cells are characterized in the remaining dot plots.

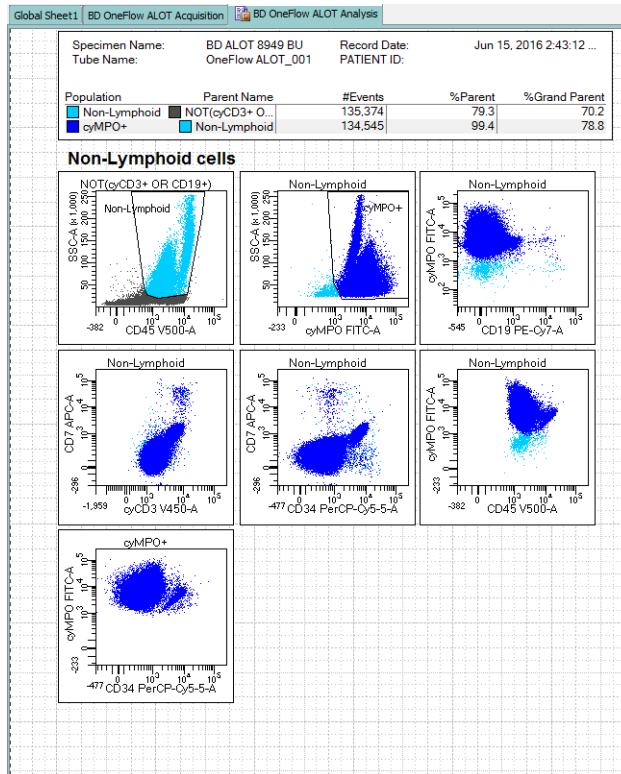


7. Inspect the dot plots identifying non-lymphoid cells on page 3 of the ALOT analysis worksheet, and adjust the gates as needed.

Non-lymphoid cells are identified in the CD45 V500-A vs SSC-A dot plot from the NOT(cyCD3⁺ OR CD19⁺) population.

Cells expressing cytoplasmic myeloperoxidase (cyMPO⁺) are identified in the cyMPO FITC-A vs SSC-A dot plot from the

Non-Lymphoid population. The non-lymphoid cells are characterized in the remaining dot plots.

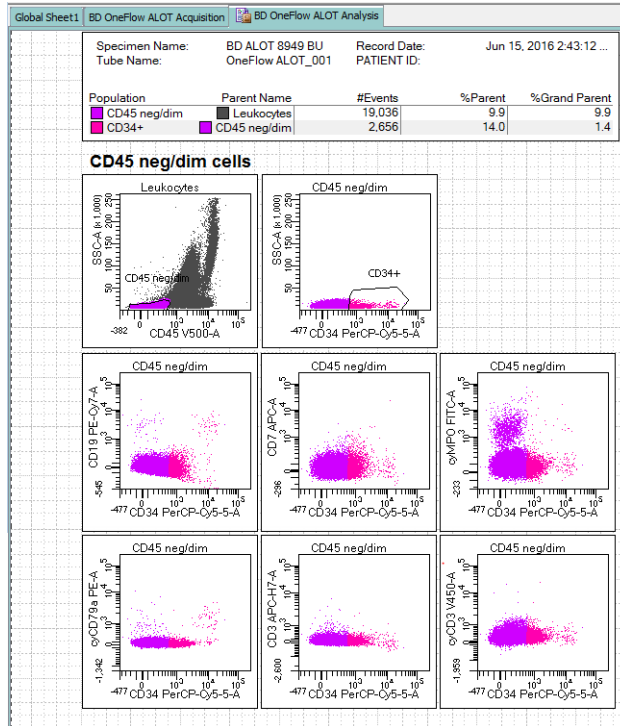


- Inspect the dot plots identifying $CD45^{\text{neg/dim}}$ cells on page 4 of the ALOT analysis worksheet, and adjust the gates as needed.

$CD45^{\text{neg/dim}}$ cells are identified in the CD45 V500-A vs SSC-A dot plot from the Leukocytes population.

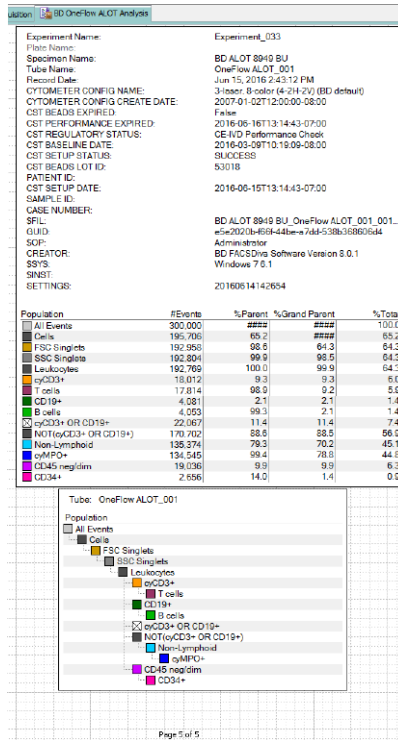
$CD45^{\text{neg/dim}}CD34^+$ cells are identified in the CD34 PerCP-Cy5.5-A vs SSC-A dot plot from the $CD45^{\text{neg/dim}}$ population.

The remaining dot plots present the expression of each of the markers in combination with CD34 for the CD45^{neg/dim} population.



- Examine the results in the statistics box on page 5 of the ALOT analysis worksheet.

Confirm that all of the keywords are present in the statistics box. If any of the keywords are missing, see [Troubleshooting](#).



10. Perform further analyses as needed.

Note: The gates in the dot plots of the ALOT analysis worksheet are provided for analyzing normal and aberrant cell populations in the specimen. Aberrant cell populations will require further analysis.

11. Save the ALOT analysis worksheet as a PDF.

Note: The ALOT analysis worksheet is a global worksheet. Any gates that are adjusted when analyzing a sample on a global worksheet will be changed in previously analyzed files. Previously saved PDFs will not change, but if you go back to a

previously analyzed global worksheet, you will have to readjust the gates so they match what they were before.

12. (Optional) Click **Print** to print the ALOT analysis worksheet.
 13. Analyze the next sample.
-

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5

Troubleshooting

This chapter covers the following topics:

- [Problems using BD OneFlow ALOT \(page 36\)](#)
- [Problems using the OneFlow ALOT template \(page 39\)](#)

**Problems using
BD OneFlow ALOT**

Problem	Possible cause	Solution
The resolution between debris and cells is poor.	Specimen was poorly lysed.	Repeat staining; vortex tubes until no cell aggregates remain before adding FIX & PERM Reagent A.
	Specimen is of poor quality.	Check cell viability.
	Specimen is too old.	Obtain a new specimen and stain it immediately.
	Instrument settings are inappropriate.	Follow proper instrument setup procedures. See the <i>Instrument Setup Guide for BD OneFlow™ Assays</i> .
The cytoplasmic staining (cyMPO, cyCD79a, cyCD3) is dim.	The cells were not completely permeabilized.	Repeat staining; carefully measure the specimen volumes in the cell fixation and permeabilization steps such that the ratio of fixed sample to FIX & PERM Reagent B is 1:1.

Problem	Possible cause	Solution
Cells clump after being fixed.	Cells were not completely resuspended before fixing them.	Vortex tubes until no cell aggregates remain before adding FIX & PERM Reagent A.
	Cells were not thoroughly washed after fixing them.	<ol style="list-style-type: none"> 1. Fix the cells using FIX & PERM Reagent A. 2. Add wash buffer and incubate the tubes for 2 minutes, protected from light. 3. Centrifuge the tubes and proceed to the permeabilization step.
Staining is dim or fading.	Cell concentration was too high at the staining step.	Check the cell concentration and adjust as needed.
	The BD OneFlow tube was exposed to light for too long.	Repeat staining with a new BD OneFlow tube.
	Stained cells were stored too long before acquiring them.	Repeat staining with a fresh specimen and acquire it promptly.
Few or no cells are recorded.	Cell concentration was too low.	Resuspend fresh specimen at a higher concentration. Repeat staining and acquisition.
	Cytometer is malfunctioning.	Troubleshoot the instrument. See the cytometer instructions for use for more information.

Problem	Possible cause	Solution
Some of the dot plots are dimmed.	FSC-H and SSC-H were not selected when the application settings were created.	Check that FSC-H and SSC-H are selected on the Parameters tab of the Inspector .
The barcode on the BD OneFlow ALOT (C) tube label cannot be scanned.	The barcode on the tube label has been compromised.	Scan the barcode on the BD OneFlow ALOT (C) pouch label into the Product ID keyword field in the Experiment Layout . Next, after the last digit of the barcode, manually enter a semicolon (;) followed by the six-digit tube-specific ID, found adjacent to the barcode on the tube label.

Problems using the OneFlow ALOT template

Problem	Possible cause	Solution
The dot plots on the worksheets are missing or the dot plots do not have gates.	The template did not import correctly.	<ol style="list-style-type: none"> 1. Close the current experiment. 2. Create a new experiment. 3. Re-import the OneFlow ALOT template.
Some of the keywords are missing from the statistics box in the analysis worksheet.	BD FACSDiva software did not import all of the keywords into the panel template.	<ol style="list-style-type: none"> 1. In the Browser, set the current tube pointer to the tube that you are analyzing. 2. Navigate to the analysis worksheet. 3. Right-click the statistics box and select Edit Stats View. 4. In the Header tab, select the All checkbox. 5. Click OK.
The statement For in vitro diagnostic use does not appear in the footer of the analysis worksheet when it is printed.	The paper margins in the printer settings were changed.	<ol style="list-style-type: none"> 1. From the BD FACSDiva software menu bar, select File > Page Setup. 2. Ensure that all of the margins are set to 2.54 cm or 1 inch, depending on your default standards. 3. Click OK.

