



STANDARD OPERATING PROCEDURE

Multicolor Panel Antibody Staining of T Lymphocytes

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1.0 PURPOSE

1.1 To assess the presence of a panel of antibodies on the surface of CD3 Jurkat cells by flow cytometry. Cells will be stained with 4 different antigen-specific markers to assess the expression of CD3, CD4, CD8, and CD45RO on the surface of the cells.

2.0 SCOPE


2.1 This SOP applies to the use of multiple conjugated antibodies to understand the expression of surface markers on cells. Multiple antibody staining is referred to a panel. This can be done on most cell types, particularly suspension cells.

3.0 REFERENCES

Document #/Reference	Title
BD 23-18013-00	BD Accuri C6 Plus System Users Guide
BIOT222-01	SOP - Introduction, Maintenance, and Operation of the BD Accuri C6 Plus Cytometer

4.0 DEFINITIONS


Terms	Definitions
Fluorescein isothiocyanate (FITC)	A derivative of fluorescein used in a wide range of applications, including flow cytometry.
Allophycocyanin (APC)	A fluorochrome commonly used in flow cytometry.
Phycoerythrin (PE)	A fluorochrome commonly used in flow cytometry.
Cluster of Differentiation 3 (CD3)	A multimeric protein complex often associated with the T Cell Receptor (TCR). This protein is present on the surface of all T Cells, making it an important marker for differentiating lymphocytes.
Cluster of Differentiation 4 (CD4)	A multimeric protein complex often associated with the T Cell Receptor (TCR). This protein is present on the surface of T Helper Cells.
Cluster of Differentiation 8 (CD8)	A multimeric protein complex often associated with the T Cell Receptor (TCR). This protein is present on the surface of T Cytotoxic Cells.

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Cluster of Differentiation 45 RO (CD45RO)	An Isoform of CD45, this antigen represents activation status of T Cells
Fluorescence Minus One (FMO)	A flow cytometry control for data analysis. These controls use all fluorochromes in the panel except for one, allowing for a more accurate determination of positive versus negative staining.
Compensation	A flow cytometry control for the instrument. These controls use single fluorochrome stains to help the instrument differentiate true signal from spillover signal of other fluorochromes.
Fluorophore/Fluorochrome	A molecule with fluorescent properties.
Gating Strategy	In data analysis, the combination of scaffolding graphs to identify subsets of cells.
Forward Scatter (FSc)	A flow cytometer parameter to measure the size of cells.
Side Scatter (SSc)	A flow cytometer parameter to measure internal complexity of cells.

5.0 SAFETY

- 5.1 Use of BSL-1 Safety Measures and discard all waste in biohazard.
- 5.2 Users should always wear goggles, lab coats, and gloves when handling samples and the cytometer.
- 5.3 Operators must be aware of safety symbols located on and around the instrument, indicating specific hazards including:
 - Biological Hazard
 - Electrical Hazard
 - Laser Hazard


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6.0 RESPONSIBILITIES

Role	Responsibilities
Instructors / Lab Technicians	It is the responsibility of the course instructor/lab assistant to ensure that this SOP is performed as described and to update the procedure when necessary.
Students/Trainees	It is the responsibility of the students/technicians to follow the SOP as described and to inform the instructor about any deviations or problems that may occur while performing the procedure.

7.0 MATERIALS

- Jurkat Cells (or some CD3+ T Cells)
- Phosphate Buffered Saline (Gibco / 10010)
- BD CS&T RUO beads (BD Biosciences / 661414))
- BD FACSClean (BD Biosciences / 340345)
- DI Water
- 12 x 75-mm capped polystyrene tube (Falcon/Corning / 352058 or 352054)
- Phosphate Buffered Saline
- CD3 FITC (BioLegend / 300405/6 – 25 test or 100 test, depending on need)
- CD4 PE (BioLegend / 980804)
- CD8 APC (BD Biosciences / 555369)
- CD45RO PECy7 (BioLegend / 304229/30 - 25 test or 100 test, depending on need)
- BD Pharmingen Stain Buffer (BD Biosciences / 554656) or PBS + 1% FBS
- RPMI1640 Media (Gibco / 11875119)
- HI Fetal Bovine Serum (Gibco / 61640071)
- UltraComp eBeads Compensation Beads (ThermoFisher / 01-2222-42)

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8.0 EQUIPMENT

- Biological Safety Cabinet
- BD Accuri C6 Plus System
- Centrifuge
- 2-8C Refrigerator
- 37C, 5% CO₂, 70-80% Humidity incubator

9.0 PROCEDURE

9.1 Confirm the cytometer is on and appropriately calibrated. Follow the SOP for Introduction, Maintenance, and Operation of the BD Accuri C6 Plus Cytometer for further instruction

9.2 Sample Preparation

9.2.1 Prepare Stain Buffer:


9.2.1.1 Add 5mL of FBS to 495mL of PBS for a final concentration of 1%.

(Adjust volumes accordingly if preparing smaller volumes)

9.2.1.2 Or, if using BD Pharmingen Stain Buffer, remove from 2-8C and allow to room temperature for 15 to 30 minutes.

9.2.2 Sample preparation

9.2.2.1 Receive flask/well of Jurkat Cells.

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9.2.2.2 Take the full amount of volume in the flask and move to a 15mL conical tube. Spin down at 400 x g for 5 minutes and remove supernatant.

9.2.2.3 Resuspend the pellet in 10mL of media. Take out 250uL and dilute 1:2. Count on the automated cell counter. Record the viability and total viable cells per mL.

9.2.2.4 Remove cell suspension needed and place it into a new tube labeled "Jurkat FACS." Add enough Stain Buffer to bring the sample to 3mL.


9.2.2.5 Centrifuge the cells at 400 x g for 5 minutes. Discard supernatant and resuspend in 1200uL of Staining Buffer.

9.2.2.6 Prepare 6 tubes for sample acquisition and label accordingly. (1. Non-Stained, 2. FITC FMO, 3. PE FMO, 4. PECy7 FMO, 5. APC FMO, 6. Full Stain.)

9.2.2.7 Distribute 200uL in each tube. Store @ 2-8 until ready for use.
(Prepare Sample Stain, then remove cells from fridge)

9.2.2.8 Add 750uL of Staining Buffer to each tube, and centrifuge at 400 x g for 5 minutes. Discard the supernatant.

9.3 Compensation Control Preparation

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- 9.3.1 Compensation Samples (Comps) are used to help control Spillover from one Fluorochrome into another. We will perform 1 set of compensation tubes as a group.
- 9.3.2 Label 5 Falcon tubes with the individual Fluorochrome name or Compensation, including a negative control. (1. Non-Stained, 2. FITC, 3. PE, 4. PECy7, 5. APC)
- 9.3.3 Add 100 uL of Stain Buffer to each Compensation tubes.
- 9.3.4 Vortex on high speed the eBioscience UltraCompensation Beads.
- 9.3.5 Add one drop of Compensation beads to each tube.
- 9.3.6 Add 1.5uL of FITC CD3, PE CD4, PECy7 CD45RO and APC CD8 antibodies to the respective tube. NS should receive no antibody.
- 9.3.7 Mix tubes by vortex, and incubate at Room Temperature, protected from light for 15 – 30 minutes.
- 9.3.8 Centrifuge the tubes at 500 x g for 5 minutes at RT.
 - 9.3.8.1 Note: Check the tubes after centrifugation to see if there is a visible pellet. A pellet is not always noticeable with these beads due to their size, but it is good practice to check tubes/wells before removing the supernatant
- 9.3.9 Discard the supernatant, either by pipetting or inversion, and add 250 uL of Stain Buffer per tube.
- 9.3.10 Resuspend the beads by gentle vortex or pipetting and store at 2-8°C protected from light until ready to acquire.

9.4 Sample Staining

- 9.4.1 Add 750uL of Staining Buffer to each prepared cell tube, and centrifuge at 400 x g for 5 minutes. Discard the supernatant.
- 9.4.2 Prepare the Master Mix antibody cocktail by adding the following reagents in a microcentrifuge tube. Label this Master Mix. Aliquot 100uL of the FITC MM to the tubes labeled CD3 FITC.



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FITC CD3	PE CD4	PECy7 CD45RO	APC CD8	Stain Buffer	Total Volume
5µL	5µl	5µl	5µl	80µl	100µl

9.4.3 Prepare the antibody Fluorescence Minus One (FMO) cocktails by adding the following reagents in individual microcentrifuge tubes.

Tube	Non Stained	FMO FITC	FMO PE	FMO PECy7	FMO APC
Stain Buffer	50	42.5	40	40	40
FITC CD3	-	-	5	5	5
PE CD4	-	2.5	-	2.5	2.5
PECy7 CD45RO	-	2.5	2.5	-	2.5
APC CD8	-	2.5	2.5	2.5	-

9.4.4 Aliquot 100uL Stain Buffer to the tube labeled Non-Stained.

9.4.5 Aliquot 50uL each FMO to the appropriately labeled tube.

9.4.6 Aliquot 100uL of the Master Mix to the tube labeled Full Stain.

9.4.7 Incubate at Room Temperature protected from light for 30 minutes +/- 5 minutes.

9.4.8 Add 1mL of Staining Buffer to each tube. Centrifuge at 400 x g for 5 minutes. Discard supernatant.


9.4.9 Resuspend pellet in 250uL of Staining Buffer. Store at 2-8 until ready for sample acquisition.

9.5 Set the Acquisition Parameters on the Cytometer

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9.5.1 Upon opening the program, a new workspace is automatically generated.

If you are not the first operator to read your sample, ensure that the previous workspace has been saved, then you select File > New Workspace to create a new one.


9.5.2 Set a Run Limit of 100,000 events. This indicates when the collection will end. This is completed by selecting **Run with Limits**, selecting the checkbox next to the events field, and typing in the number of events. You can choose to focus on a gated population or all cells.

9.5.3 Set a Fluidics Rate of Medium. This indicates how fast to collect the sample. Depending on the cell concentration and the size of the cells, this can be adjusted accordingly. A recommended flow rate is between 1,000 to 2,500 events per second.


9.5.4 Set a Threshold of 50,000 for FSC-H. The threshold sets the cut off for eliminating debris and noise from cell samples. By default, it is set to 80,000, but we will set it slightly lower.

9.6 Creating Sample Gating Strategy on the Cytometer


9.6.1 Confirm that the first graph displayed states “FSC-A” on the X-Axis for Forward Scatter Area, and “SSC-A” on the Y-Axis for Side Scatter Area. Rename plot to “Cells.”

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9.6.2 Select the oval gate icon  and add an oval gate to the bottom right of the graph.


9.6.3 Select the Dot Plot Icon  to add a new graph. To change the specifications, select the **Plot Spec** icon in the bottom right of the graph. Set “FSC-A” on the X-Axis for Forward Scatter Area, and “FSC-H” on the Y-Axis for Forward Scatter Height.




9.6.4 Rename the Plot to “Singlets.” Select the **Gate** icon at the top left. Select **Include** and select the gate from the Cells graph.


9.6.5 Select the Polygon gate icon.  Draw a diagonal, rectangular 4-point box. This will be used to separate single cells from clumps of cells that may register incorrectly.

9.6.6 Select the Dot Plot Icon to add a new graph. To change the specifications, select the **Plot Spec** icon in the bottom right of the graph. Set “FITC” on the X-Axis for GFP expression and “SSC-A” on the Y-Axis for Side Scatter Area. If not already, change the X-Axis to “Linear” instead of Log.

9.6.7 Rename the Plot to “CD3+.” Select the **Gate** icon at the top left. Select **Include** and select the gate from the Singlets graph.

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- 9.6.8 Select the Box gate icon.  Draw a box in the right half of the graph.
- 9.6.9 Select the Dot Plot Icon to add a new graph. To change the specifications, select the **Plot Spec** icon in the bottom right of the graph. Set “PE” on the X-Axis for CD4 expression and “APC” on the Y-Axis for CD8 expression.
- 9.6.10 Rename the Plot to “CD4 vs CD8.” Select the **Gate** icon at the top left. Select **Include** and select the gate from the CD3+ graph.
- 9.6.11 Select the Quadrant gate icon.  Place the center of the gate at the 10^3 for both X and Y axes.
- 9.6.12 Select the Dot Plot Icon to add a new graph. To change the specifications, select the **Plot Spec** icon in the bottom right of the graph. Set “PECy7” on the X-Axis for CD45RO expression and “SSC-A” on the Y-Axis for Side Scatter Area.
- 9.6.13 Rename the Plot to “CD4+/CD45RO+.” Select the **Gate** icon at the top left. Select **Include** and select the Q3 quadrant of the CD4/CD8 graph.
- 9.6.14 Select the Box gate icon.  Draw a box in the right half of the graph.
- 9.6.15 Select the Dot Plot Icon to add a new graph. To change the specifications, select the **Plot Spec** icon in the bottom right of the graph. Set “PECy7” on

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the X-Axis for CD45RO expression and “SSC-A” on the Y-Axis for Side Scatter Area.

9.6.16 Rename the Plot to “CD8+/CD45RO+.” Select the **Gate** icon at the top left. Select **Include** and select the Q1 quadrant of the CD4/CD8 graph.

9.6.17 Select the Box gate icon.  Draw a box in the right half of the graph.


9.7 Sample Acquisition

9.7.1 Now that your acquisition setting and gating strategy are complete, we can begin acquiring samples. Read samples in the following order. (1. Compensation Controls, 2. Non-Stained Controls, 3. FMO Controls, 4. Full Stain Sample)

9.7.2 Select empty sample well A1 and enter the first sample’s name “Negative Comp_Initials” in the text box above the 96-well grid. You can name samples at any point, but it is highly suggested you record the name before as to not forget.

9.7.3 Resuspend your sample in the sample tube and place tube on the SIP.

9.7.4 Click **Run** to start the sample collection. Save your workspace. Adjust all gates as needed.

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9.7.5 The selected Well will flash blue during acquisition. Once the 100,000 events are recorded, the run will finish and the selected Well will stop flashing. Remove the tube from the SIP. (Comps only need 5,000 to 10,000 events)

9.7.6 Repeat for all comps. (Through A4)

9.7.7 Select the next empty row sample well B1 and enter the next sample's name "NonStained_Initials" in the text box above the 96-well grid.

9.7.8 Resuspend your cells in the sample tube and place tube on the SIP.

9.7.9 Click **Run** to start the sample collection.


9.7.10 The selected Well will flash blue during acquisition. Once the 100,000 events are recorded, the run will finish and the selected Well will stop flashing. Remove the tube from the SIP. Repeat for all samples. (Through B5)

9.8 Data Analysis

9.8.1 Select the Batch Analysis tab to compare the expression of markers from every sample.

9.8.2 Click the **Batch Analysis** tab. Select the **Show Plots from Collect** checkbox.

9.8.3 Select all gates by marking the checkbox under that plot.

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9.8.4 Select the samples to analyze (all).

9.8.5 Select the **Show Statistics with All Plots** check box to show statistics.

9.8.6 Further Analyses can be completed in a software analysis package such as FlowJo or FCS Express.

9.9 Export the Data.

9.9.1 Select **Export to PowerPoint** and include statistics.

9.9.2 Navigate to the destination folder, record the file name and document the full file pathway.

9.9.3 Print out the PowerPoint and attach to this Lab Workbook.

10.0 ATTACHMENTS

- SOP - Introduction, Maintenance, and Operation of the BD Accuri C6 Plus Cytometer (For Startup and Calibration instructions)
 - Document Number: SOP-BIOT222-01, 10Oct2023, Bryan Pelkey