NCL Method ITA-37.1

Immunophenotyping: Instrument Calibration and Reagent Qualification for Immunophenotyping Analysis of Human Peripheral Blood Mononuclear Cell Cultures

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Immunophenotyping is the use of antigen expression for the identification of distinct immune cell subsets (and their activation statuses) [1-4]. This technique can detect minute changes in cell populations and thus is used to characterize the cell makeup in many diseases as well as determine effects of treatments, such as nanoparticles [5]. It is important to develop a method that allows for immunological evaluation of nanoparticles because some nanoparticles are designed to modify the immune system while others cause immunotoxicity [4, 5]. Currently, the most common technique used to perform immunophenotyping is multicolor flow cytometry [2, 3].

NCL protocol ITA-37 covers two separate immunophenotyping panels (with 11-12 antibody-fluorophore conjugates):

Immunophenotyping Panel #1 (or Lymphocyte Panel):

This panel includes antibody-fluorophore conjugates that allow for analysis of different lymphocyte populations including B cells and T cells (CD8+ T cells, CD4+ T cells, regulatory T (Treg) cells, naïve T cells, and γδ TCR T cells). This panel also determines cellular CD25 and CD154 expression which are markers of proliferation and co-stimulation/presentation, respectively.

Immunophenotyping Panel #2 (or Monocyte, Dendritic cell (DC), Natural Killer (NK) Cell Panel):

This panel includes antibody-fluorophore conjugates that allow for analysis of different cell populations including CD14+ monocytes, DCs (plasmacytoid (p) and myeloid (m) DCs), and NK cells along with NK T cells. This panel also examines cellular CD69 and CD54 expression which are markers of early activation and adhesion, respectively.

When used in conjunction with other immunoassays, this protocol aids in establishing efficacy and safety profiles of engineered nanoparticles used for vaccine or drug delivery. This protocol has two parts, ITA-37.1 described in this document and intended for instrument calibration, and ITA-37.2, described in a separate document and intended for the analysis of nanoparticle-treated cells.

2. Principle

This protocol (ITA-37.1) uses compensation beads and peripheral blood mononuclear cells (PBMC) derived from the blood of healthy donor volunteers for instrument calibration and reagent qualification. Following this protocol is required before proceeding to the second part of the protocol, ITA-37.2, for the immunophenotyping of nanoparticle-treated cells.

Experimental steps described herein, allow for proper optimization and compensation of the antibody-fluorophore conjugates used in the panels. The antibody titrations allow for the optimization of antibody concentrations in which the optimal concentration/ dilution is defined as the concentration that leads to the greatest difference between the positive and negative mean fluorescence intensity. Single stain control experiments performed with compensation beads allow for the necessary compensation of the optimized antibody-fluorophores used in each panel. Lastly, fluorescence minus one (FMO) controls serve as a way to fine-tune the compensation obtained from the single stain control experiment to account for any minute changes in the protocol, including the presence of PBMC rather than compensation beads and fixation.

The variety and complexity of immune cell phenotypes that can be assessed during immunophenotyping depends on the available flow cytometry instrumentation. This protocol is optimized for the flow cytometer NovoCyte 3005 by Acea Biosciences Inc. (part of Agilent Technologies), which has the capacity to acquire data from 14 distinct fluorophores simultaneously. NovoCyte 3005 is equipped with 3 lasers (405 nm, 488 nm, and 640 nm) and 6 detectors (445/45 nm, 530/30 nm, 572/28 nm, 660/20 nm, 725/40 nm and 780/60 nm) [6].

If another cytometer is used, the procedure described herein would require an optimization according to the technical specifications of that instrument.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents for PBMC cultures

- 3.1.1 Human blood anti-coagulated with Li-heparin and obtained from healthy donors
- 3.1.2 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)
- 3.1.3 RPMI-1640 (GE Life Sciences, HyClone, SH30096.01)
- 3.1.4 Fetal bovine serum (GE Life Sciences, HyClone, SH30070.03)
- 3.1.5 Penicillin streptomycin solution (GE Life Sciences, Hyclone, SV30010)
- 3.1.6 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)
- 3.1.7 Ficoll Paque Premium (GE Healthcare, 17-5442-03)
- 3.1.8 Hank's balanced salt solution (HBSS) (Gibco, 14175-095)
- 3.1.9 ViaStain AOPI Staining solution (Nexcelom Biosciences, CS2-0106-5mL)

3.2 Controls

- 3.2.1 Phytohemagglutinin (PHA-M) (Sigma, L8902)
- 3.2.2 Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, P1585)
- 3.2.3 Ionomycin (STEMCELL Technologies, 73722)
- 3.2.4 Oligodeoxyribonucleotide, Human TLR9 ligand (ODN2216) (InvivoGen, tlrl-2216-1)

Table 1. Guidance on Concentrations and Purpose of Positive Controls.

Description/ Control	PMA	Ionomycin	PHA-M	ODN2216
Primary Purpose	Positive control for lymphocyte activation (CD154; CD25)	Positive control for lymphocyte activation (CD154; CD25)	Positive control for lymphocyte activation (CD69; CD54)	Positive control for monocyte and dendritic cell activation (CD69; CD54)
Final Concentration in Assay	50 ng/mL	1 µg/mL	10 µg/mL	5 µg/mL

Note: Other agents can be used as the assay positive control.

3.3 Reagents for Flow Cytometry

3.3.1 UltraComp eBeads Plus Compensation Beads (Invitrogen, 01-3333-42)

3.3.2 eBioscience Flow Cytometry Staining Buffer (Invitrogen, 00-4222-26)

3.3.3 Paraformaldehyde (PFA) 20% Solution (Electron Microscopy Science, 15713)

3.3.4 NovoRinse (Agilent Technologies, 872B603)

3.3.5 NovoClean (Agilent Technologies, 872B602)

3.3.6 NovoFlow (Agilent Technologies, 871B607)

3.3.7 Antibodies/Dyes (Tables 2-3)

Table 2. Anti-human Labeling Antibodies used in the Immunophenotyping Panel 1 (Lymphocyte Panel)

Fluorochrome	Marker	Cell Type	Purpose	Company	Concentration (µg/mL)	Catalog #
FITC	CD8a	T cells	Cytotoxic T cells	BioLegend	200	300905 or 300906
PE	CD4	T cells	T-helper	BioLegend	100	317409 or 317409
PE-Cy7	CD19	B cells	LPS co-receptor	BioLegend	100	302215 or 302216
APC	CCR4	T-reg	CD194	BioLegend	50	359407 or 359408
AF700	CD45RA	Naïve cells	Memory status	Invitrogen	50	56-0458-42
APC-Fire 750	TCR-γδ	TCR- γδ T cells	TCR- γδ receptor	BioLegend	400	331227 or 331228
PacBlue	CD45	All leukocytes	Pan-leukocyte	BioLegend	100	368539 or 368540
Zombie Aqua	Live/dead	Dead cells	Excludes dead cells	BioLegend	Not Available	423101 or 423102
BV570	CD3	T cells	T cells	BioLegend	80	300435 or 300436
BV650	CD25	T-reg and activation status of cells	IL-2 receptor α	BioLegend	100	302633 or 302634
BV711	CD154	lymphocytes	CD40L	BioLegend	100	310837 or 310838
BV785	CD127	T-reg	IL-7 receptor α	BioLegend	50	351329 or 351330

Table 3. Anti-human Labeling Antibodies for Immunophenotyping Panel 2 (Monocyte, DC, NK Cell Panel)

Fluorochrome	Marker	Cell Type	Purpose	Company	Concentration (µg/mL)	Catalog #
FITC	CD56	NK cells	Neural cell adhesion molecule	BioLegend	200	362545 or 362546
PE	CD14	Monocytes	LPS co-receptor	BioLegend	200	301805 or 301806
PE-Cy7	CD19	B cells	LPS co-receptor	BioLegend	100	302215 or 302216
APC	CD123	pDCs	Interleukin-3 receptor	BioLegend	100	306011 or 306012
AF700	CD54	Lymphocytes and monocytes	Adhesion	BioLegend	400	353125 or 353126
APC-Fire 750	CD20	B cells	B cells	BioLegend	200	302357 or 302358
PacBlue	CD45	All leukocytes	Pan-leukocyte	BioLegend	100	368539 or 368540
Zombie Aqua	Live/dead	Dead cells	Excludes dead cells	BioLegend	Not Available	423101 or 423102
BV570	CD3	T cells	T cells	BioLegend	80	300435 or 300436
BV650	CD69	Neutrophils/ monocytes	Early activation	BioLegend	50	310933 or 310934
BV785	CD11c	mDCs	Integrin α x	BioLegend	160	301643 or 301644

3.4 Equipment and Materials

3.4.1 Pipettes covering a range of 0.05 to 10 mL

3.4.2 96-well U-bottom plates

3.4.3 24-well round bottom plates

3.4.4 Polypropylene tubes, 15 and 50 mL

3.4.5 Microcentrifuge tubes

3.4.6 5 mL Polystyrene round bottom tubes; 12x75 mm

3.4.7 Centrifuge

3.4.8 Refrigerator, 2-8 ºC

3.4.9 Freezer, -20 ºC

3.4.10 Cell culture incubator with 5% CO_2 and 95% humidity

3.4.11 Biohazard safety cabinet approved for level II handling of biological material

3.4.12 Water bath

3.4.13 Vortex

3.4.14 Cellometer Auto2000

3.4.15 Acea Novocyte 3005

4. Preparation of Reagents and Controls

4.1 Reagent and Control Preparation for PBMC

4.1.1 Complete RPMI-1640 medium

The complete RPMI medium should contain the following reagents:

10% FBS (heat inactivated)

2 mM L-glutamine

100 U/mL penicillin

100 µg/mL streptomycin

Store at 2-8 °C protected from light for no longer than 1 month. Before use, warm the media in a water bath.

4.1.2 Heat inactivated fetal bovine serum

Thaw a 50 mL aliquot of fetal bovine serum and equilibrate to room temperature. Place the tube in a water bath set up to 56 °C and incubate

with mixing for 35 min. The heat inactivation takes 30 min and the initial 5 min is used to bring the entire content of the vial to 56 °C. Chill the serum and use to prepare complete culture media.

4.1.3 PMA (1 mg/mL stock)

Reconstitute in DMSO to a final concentration of 1 mg/mL. Prepare single use 20 µL aliquots and store at -20 °C. On the day of experiment thaw an aliquot at room temperature and dilute in culture media so the final concentration in the test sample is 50 ng/mL.

4.1.4 Ionomycin (10 mg/mL stock)

Ionomycin is supplied in ionomycin free acid 1%, ethyl alcohol 99%. Prepare single use 5 µL aliquots and store at -20 °C. On the day of experiment thaw an aliquot at room temperature and dilute in culture media so the final concentration in the test sample is 1 µg/mL.

4.1.5 ODN 2216 (1 mg/mL stock)

This oligonucleotide is supplied as lyophilized powder. Reconstitute in pyrogen-free, nuclease-free water to a final concentration of 1 mg/mL. Prepare single use 5 µL aliquots and store at -20°C. On the day of experiment thaw an aliquot at room temperature and dilute in culture media so the final concentration in the test sample is 5 µg/mL.

4.1.6 Phytohemagglutinin (PHA-M, 1 mg/mL stock)

Add 1 mL of sterile PBS or cell culture medium per 1 mg of PHA-M to the vial and gently rotate to mix. Store daily use aliquots at a nominal temperature of - 20ºC. Avoid repeated freezing/thawing. On the day of experiment dilute stock PHA-M solution in cell culture medium so the final concentration in the positive control sample is 10 µg/mL.

4.2 Collection and Handling of Whole Blood

This step requires approval by an Institutional Review Board (IRB) or another relevant board; please consult your organization for details on appropriate regulations within your research organization. Collect whole blood from healthy donor volunteers who have not been on medication and clear from infection for at least 2 weeks prior to the blood donation. Use Li-heparin tubes

and discard first 10 cc. For best results, whole blood should be used within 1 hour after collection. Prolonged storage (> 2 hr) of whole blood will lead to a decrease in cell function.

4.3 Preparation of PBMC

Note: PBMC isolated during this step are used in steps 6 (specifically, live: dead cell calibration described in 6.1.1.3 and 6.2.1.3), and 7 of this protocol. Procedures described in steps 6 and 7 may be performed on a separate day using PBMCs freshly isolated that day.

4.3.1 Place freshly drawn blood into 15- or 50-mL conical centrifuge tubes, add an equal volume of room-temperature PBS, and mix well.

4.3.2 Slowly layer the Ficoll-Paque solution underneath the blood/PBS mixture by placing the tip of the pipet containing Ficoll-Paque at the bottom of the blood sample tube. Alternatively, the blood/PBS mixture may be slowly layered over the Ficoll-Paque solution. Use 3 mL of Ficoll-Paque solution per 4 mL of blood/PBS mixture. For example, 15 mL Ficoll-Paque per 20 mL of diluted blood in a 50 mL tube.

Note: To maintain Ficoll-blood interface it is helpful to hold the tube at a 45° angle.

4.3.3 Centrifuge 30 min at 900 x g, 18-20ºC, without brake.

Note: Depending on the type of centrifuge, one may also need to set acceleration speed to minimum.

4.3.4 Using a sterile pipet, remove the upper layer containing plasma and platelets and discard it.

4.3.5 Using a fresh sterile pipet, transfer the mononuclear cell layer into another centrifuge tube.

4.3.6 Wash cells by adding an excess of HBSS and centrifuging for 10 min at 400xg, 18-20 ºC. The HBSS volume should be ~3 times the volume of mononuclear layer.

Note: Usually 4 mL of blood/PBS mixture results in ~ 2 mL of mononuclear layer and requires at least 6 mL of HBSS for the wash step. We use 10 mL of HBSS per each 2 mL of cells.

4.3.7 Discard supernatant and repeat wash step one more time.

4.3.8 Re-suspend cells in complete RPMI-1640 medium. Dilute cells 1:2 with AOPI, count cells and determine viability using AOPI exclusion. If viability is at least 90%, proceed to step 7.1.1.

Note: The cells isolated during this step can also be used to prepare live: dead cell controls for steps 6.1.1.3 and 6.2.1.3.

5. NovoCyte 3005 Instrument Settings

Important Note: The following experiments were optimized on the NovoCyte 3005 with the settings shown in Figure 1. If a different cytometer is used, then instrument calibration or fluorescent labels may need to be changed or adjusted accordingly.

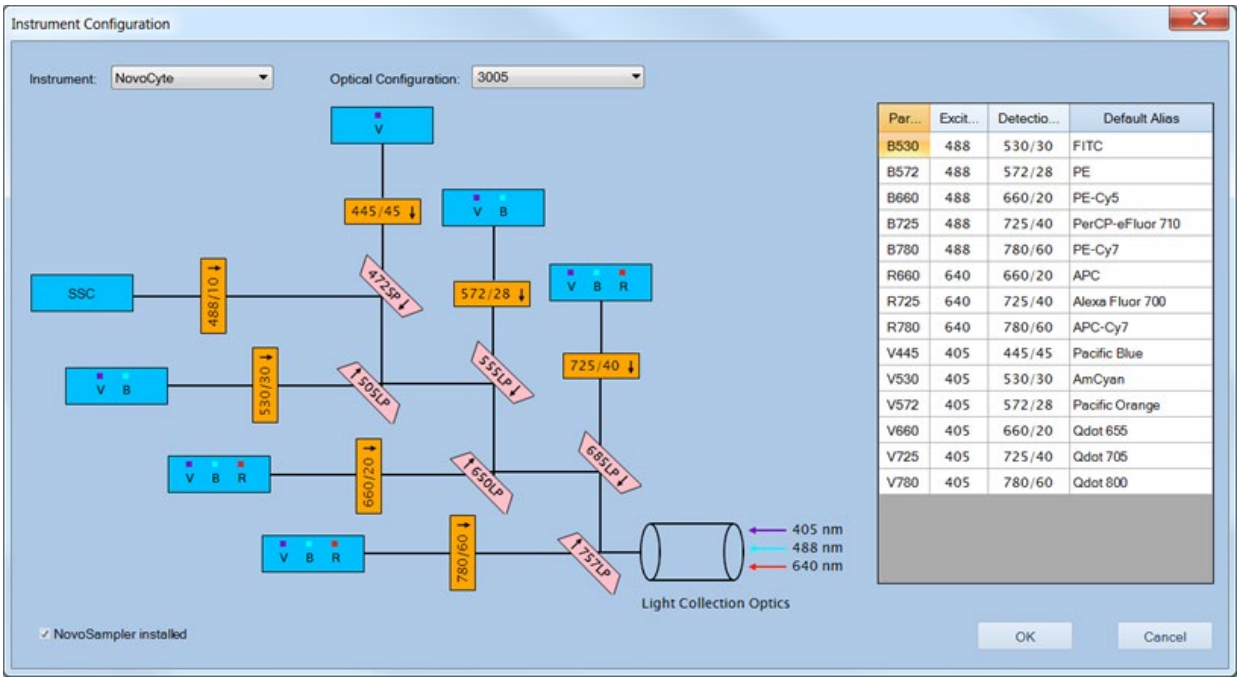

Figure 1. Settings on NovoCyte 3005 [7].

6. Flow Cytometry Control Experiments with Compensation Beads for Antibody Titration and Single Stain Controls

Important Note: The following procedures need to be completed for both Immunophenotyping Panel #1 and #2.

6.1 Antibody Titration (Optional)

Prepare a 6-point concentration curve that includes the antibody at the stock concentration (between 50 µg/mL to 400 µg/mL for a given antibody) and five 5-fold serial dilutions (dils) of the stock (Dils 5; 25; 125; 625; and 3125). Please check with manufacturer to determine that the concentration for a given antibody remains unchanged.

Note: Antibody titration needs to be performed to establish the antibody/dye dilutions for the panels that will be run. Please refer to Tables 2-3 for the antibody-fluorophore combinations used for Immunophenotyping Panels #1 and #2, respectively. Once the panel is established, the antibody titrations do not need to be run again unless there are issues encountered with a new antibody lot or the stock concentration changed. Antibody dilutions have already been determined for panels used herein.

6.1.1 Bead and Live: Dead PBMC Plate Preparation (without antibodies or dyes)

6.1.1.1 Make a 10-fold dilution of UltraComp eBeads Plus Compensation Beads using staining buffer as the diluent (making sure to vortex beads).

6.1.1.2 Add 50 µL of 10-fold diluted beads to each of the necessary 96-well U-bottom wells of the plate labeled “Bead and Live: Dead PBMC Plate” (6 wells of beads needed per antibody). (Please see Tables 2-3 for antibodies used in Immunophenotyping Panels #1 and #2 and Plate 1 in the Appendix for an example of plate setup.)

6.1.1.3 Add 50 µL of PBMC suspended in 1X PBS to each of the necessary wells of the 96-well plate labeled “Bead and Live: Dead PBMC Plate” (6 wells per dye). Ideally, this sample should contain 1:1 mixture of

live and dead PBMC at a total concentration of approximately $5x10^5$ cell/mL. See Plate 1 setup in the Appendix for an example.
Note: Freshly isolated or freeze thawed PBMC work well. If necessary, cells can be heat shocked at 70˚C to obtain about 50% cell death.

6.1.2 Antibody/Dye Titration Plate Preparation

6.1.2.1 Add 20 µL of staining buffer to each appropriate well in a 96-well U bottom plate labeled "Antibody/Dye Titration Plate" for preparation of the antibody dils (5 wells in a column prepared for each antibody—Wells B-F). Please see Plate 2 in Appendix for an example.

6.1.2.2 Add 20 µL of 1X PBS to each appropriate well in the 96-well U bottom plate labeled "Antibody/Dye Titration Plate" for preparation of the Zombie Aqua dye dils (5 wells in a column—Wells B-F). Please see Plate 2 in Appendix for an example.

6.1.2.3 Prepare each antibody/dye titration down a single column of the plate as follows:

a. Add 5 µL of appropriate antibody/dye to well B1; mix well.
b. Transfer 5 µL of diluted antibody/dye from B1 and add it to well C1; mix well.
c. Transfer 5 µL of diluted antibody/dye from C1 and add it to well D1; mix well.
d. Transfer 5 µL of diluted antibody/dye from D1 and add it to well E1; mix well.
e. Transfer 5 µL of diluted antibody/dye from E1 and add it to well F1; mix well.
f. Repeat steps a-e for each antibody in appropriate wells.
   Please see Plate 2 in Appendix for an example.

6.1.3 Combination of Antibody/Dye Titrations to Bead and Live: Dead PBMC Plate

6.1.3.1 Add 5 µL of appropriate stock antibody/dye to corresponding well in row A of the "Bead and Live: Dead PBMC Plate."

6.1.3.2 Transfer 5 µL of appropriate antibody/dye dilution from "Antibody/Dye Titration Plate" to corresponding well of the "Bead and Live: Dead PBMC Plate."

Note: Another ~10 fold-dilution applied to each dilution from the titration (5 µL antibody into 50 µL diluent) that will be taken into account in subsequent calculations.

6.1.3.3 Incubate the "Bead and Live: Dead PBMC Plate" (now with beads + antibody and PBMC + dye) in the dark at room temperature for 30 minutes.

6.1.3.4 Centrifuge plate for 1-5 minutes at 300xg.

6.1.3.5 Manually aspirate 40 µL of supernatant from each well.

6.1.3.6 Add 40 µL of staining buffer to each well. Resuspend pellets.

6.1.3.7 Plate ready to read.

6.1.4 Plate Reading with NovoCyte 3005

6.1.4.1 Turn on computer > NovoExpress > username and password

6.1.4.2 Turn on flow cytometer

6.1.4.3 Check default parameters: all parameters

6.1.4.4 Set up the experimental parameters (see Table 4.)

a. Plate Manager

i. Mode: custom; 96 well u-bottom

ii. Click on appropriate squares to create sample. Rename each well accordingly in work list.

iii. Indicate to run samples vertically.

iv. Mix every 12 wells; 1500 rpm, 10sec; No rinse

b. Experimental Run. Stop conditions: Events 12,000; 30 µL. Flow rate: Fast.

c. Cytometer Settings: Parameters, see Table 4.

6.1.4.5 Insert plate.

6.1.4.6 Click run plate. Highlight all boxes that are to be run (all). Click run; Click okay

6.1.4.7 Save file.

Table 4. Cytometer Parameters. Left: Panel #1; Right: Panel #2

Note: After samples are created, make sure that the experimental settings for each specimen are the same. Whenever a change is made, apply change to all and save file.

Parameter	Alias	Gain	A	H	Parameter	Alias	Gain	A	H
FSC	FSC	-	☒	☒	FSC	FSC	-	☒	☒
SSC	SSC	-	☒	☒	SSC	SSC	-	☒	☒
B530	CD8-FITC	460	☒	☒	B530	CD56-FITC	460	☒	☒
B572	CD4-PE	397	☒	☒	B572	CD14-PE	397	☒	☒
B660	PE-Cy5	537	☐	☐	B660	PE-Cy5	537	☐	☐
B725	PerCP-eFluor 710	648	☐	☐	B725	PerCP-eFluor 710	648	☐	☐
B780	CD19-PE-Cy7	478	☒	☒	B780	CD19-PE-Cy7	478	☒	☒
R660	CCR4-APC	537	☒	☒	R660	CD123-APC	537	☒	☒
R725	CD45RA-Alexa Fluor 700	648	☒	☒	R725	CD54-Alexa Fluor 700	648	☒	☒
R780	TCR-g/d-APC-Fire750	478	☒	☒	R780	CD20-APC-Fire750	478	☒	☒
V445	CD45-Pacific Blue	425	☒	☒	V445	CD45-Pacific Blue	425	☒	☒
V530	L/D-Zombie Aqua	460	☒	☒	V530	L/D-Zombie Aqua	460	☒	☒
V572	CD3-BV570	397	☒	☒	V572	CD3-BV570	397	☒	☒
V660	CD25-BV650	537	☒	☒	V660	CD69-BV650	537	☒	☒
V725	CD154-BV711	648	☒	☒	V725	Qdot 705	648	☐	☐
V780	CD127-BV785	478	☒	☒	V780	CD11c-BV785	478	☒	☒

6.1.5 Data Analysis in NovoExpress

6.1.5.1 Make a forward scatter (FSC) vs side scatter (SSC) plot. Draw an oval region of interest (ROI) around the beads.

6.1.5.2 Make a histogram by double-clicking on the bead population. Change the axes of the histogram (right click). X-axis: appropriate fluorophore; Y-axis count. Drag the analysis tab to each sample of corresponding fluorophore.

6.1.5.3 To overlay graphs, duplicate histogram by right clicking. Right click on the duplicated histogram → Edit overlay → Add → Select the diluted samples to add → Add and close

6.1.5.4 Right click on graph → style → partial overlap

6.1.5.5 Copy image and make a PowerPoint with serial dilution.

6.1.5.6 Optimal dilution is determined by the antibody/dye concentration that has the largest difference between positive and negative stain. > 2 log differences needed. Need negative to be as low as possible without large shift in positive population.

6.1.5.7 Repeat analysis for each serial dilution.

6.1.5.8 Save files.

6.2 **Single Stain Controls with Compensation Beads**

Important Note: Must be completed each time a new antibody lot is obtained. One replicate per condition. Please see Tables 2-3 for antibody-fluorophore combinations used in single stain controls for Immunophenotyping Panels #1 and #2, respectively.

6.2.1 Plate Preparation with Beads and Cells

6.2.1.1 Make a 10-fold dilution of UltraComp eBeads Plus Compensation Beads using staining buffer as the diluent (making sure to vortex beads).

6.2.1.2 Add 50 µL of diluted beads to appropriate wells of a 96-well U-bottom plate (1 well/antibody).

6.2.1.3 Add 50 µL of cells prepared in 1X PBS (1:1 live: dead PBMC; approximately $5x10^5$ cell/mL) to 2 wells. At the end of this step, one should have 2 wells total: 1 well prepared for Zombie Aqua dye and 1 well prepared for unstained PBMC.

Note: Freshly isolated or freeze thawed PBMC work well. If necessary, cells can be heat shocked at 70˚C for 2 hours to obtain about 50% cell death.

6.2.2 Antibody Dilution Preparation

6.2.2.1 Prepare antibody dilutions (at least 5 µL each) with staining buffer as previously determined by antibody titration (Step 6.1) and indicated in Table 5.

Important note: Zombie Aqua dye dilution is to be prepared in 1X PBS.

6.2.2.2 Transfer 5 µL of antibody dilution/dye to corresponding well of the plate with the beads/cells.

6.2.2.3 Incubate plate in the dark for about 30 minutes.

6.2.2.4 Centrifuge plate for 5 min at 300xg.

6.2.2.5 Manually aspirate 30 µL of supernatant from the well.

6.2.2.6 Add 50 µL of staining buffer to the well and resuspend pellet.

6.2.2.7 Plate ready to read on the cytometer.

Table 5. Human Immunophenotyping Panel Antibody Dilutions

Immunophenotyping Panel #1		**Immunophenotyping Panel #2**	
Antibody	**Dilution**	**Antibody**	**Dilution**
CD8-FITC	Dil 25	CD56-FITC	Dil 125
CD4-PE	Dil 125	CD14-PE	Dil 125
CD19-PE/Cy7	Dil 125	CD19-PE/Cy7	Dil 125
CCR4-APC	Dil 125	CD123-APC	Dil 25
CD45RA-AF700	Dil 25	CD54-AF700	Dil 125
TCR-gamma/delta APC-Fire750	Dil 50	CD20-APC/Fire750	Dil 25
CD45-PacBlue	Dil 25	CD45-PacBlue	Dil 25
CD3-BV570	Dil 25	CD3-BV570	Dil 25
CD25-BV650	Dil 100	CD69-BV650	Dil 25
CD154-BV711	Dil 50	CD11c-BV785	Dil 25
CD127-BV785	Dil 25	Zombie Aqua	Dil 125
Zombie Aqua	Dil 125		

6.2.3 Plate Reading with NovoCyte 3005

6.2.3.1 Turn on computer > NovoExpress > username and password

6.2.3.2 Turn on flow cytometer

6.2.3.3 Save file.

6.2.3.4 Instrument → Auto compensation

6.2.3.5 In pop-up, de-select unused parameters. Rename other channels to the fluorophores used → Okay

Note: Panel #1 de-select Area and Height Parameters for PE-Cy5 and PerCP-eFluor710; Panel #2 de-select PE-Cy5, PerCP-eFluor710, and QDot705

6.2.3.6 Set up the experimental parameters

a. Plate Manager

i. Mode: custom; 96 well u-bottom

ii. Select wells and rename and reposition each well accordingly in work list.

iii. Indicate to run samples vertically.

iv. Mix every 2 wells; 1500 rpm, 10sec; Rinse every 3 wells

b. Experimental Run. Stop conditions: Events 25,000; 50 µL. Flow rate: Fast.

c. Cytometer settings: Parameters, please see Table 4 in Section 6.1.4.4.

Note: Need 2500 positive events for software to compensate correctly.

6.2.3.7 Insert plate into flow cytometer.

6.2.3.8 Click run plate. Highlight all boxes that are to be run (all) → run → Okay

6.2.3.9 Can adjust main cell gate for cell samples.

6.2.3.10 Save file.

6.2.3.11 Export FCS files and save compensation matrix.

7. Flow Cytometry Control Experiments with PBMC (FMO Controls) (2 Day procedure per panel)

Important Note: This step must be completed each time a new antibody lot is obtained. Please see Tables 2-3 for antibody-fluorophore combinations used in single stain controls for Immunophenotyping Panels #1 and #2, respectively. An unstained sample and a fully stained labeling antibody sample with all antibodies/dyes are also required. One replicate per condition. FMOs for panels may be processed concurrently or separately.

7.1 Experimental procedure for PBMC (Day 1)

Note: This procedure is optimized for 24 well plate. If other plates are used, the volumes may need to be adjusted accordingly.

7.1.1 Adjust concentration of PBMC from step 4.3.8 to 1.25 x 10^6 viable cells/mL using complete RPMI medium.

7.1.2 Dispense 800µL of PBMC per well in 24 well plate. Gently shake plates to allow all components to mix.

7.1.3 Dispense 200 µL of positive control samples into corresponding wells of 24 well plate containing 800 µL of PBMC for a total of 1 mL per well.

a. Immunophenotyping Panel #1: Positive Control = PMA/Ionomycin

b. Immunophenotyping Panel #2: Positive Control = ODN2216/PHA-M

7.1.4 Incubate for about 24 hours in a humidified 37˚C, 5% CO_2 incubator.

7.2 Experimental PBMC staining procedure (FMO controls) (Day 2)

7.2.1 Heat Shocked PBMC Preparation

7.2.1.1 Obtain needed quantity of treated PBMC from the 24-well plate and transfer to a 15 mL conical tube.

Note: 0.25x10^6 heat shocked PBMC needed per sample; rest of PBMC will be used in step 7.2.2.1.

7.2.1.2 Centrifuge sample for 10 min at 400xg. Aspirate the supernatant and resuspend the pellet in 1 mL 1X PBS.

7.2.1.3 Transfer sample to microcentrifuge tube and place sample in digital heat block set to 70˚C for at least 1.5 hours.

7.2.2 PBMC Preparation

7.2.2.1 Transfer 750 µL of cell sample (0.75x10^6 cells/well) to appropriately labeled microcentrifuge tubes.

Note: Samples needed = unstained control, FMO control for each labeling antibody, fully stained control containing all labeling antibodies/dyes

7.2.2.2 Centrifuge samples at 400xg for 7 min. Aspirate each supernatant and resuspend each pellet in 1 mL 1X PBS.

7.2.2.3 Add 62.5 µL heat shocked PBMC (0.25x10^6 dead PBMC) to each sample.

Note: Total of 1x10^6 PBMC in each sample (~0.75x10^6 live cells and ~0.25x10^6 dead cells)

7.2.3 Staining Procedure

7.2.3.1 Centrifuge each sample at 400xg for 7 min. Aspirate each supernatant and resuspend each pellet in 49.2 µL of 1X PBS.

7.2.3.2 Make 5-fold dilution of Zombie Aqua dye for staining samples with 1X PBS as the diluent.

Note: One will need 0.8 µL of 5-fold diluted Zombie Aqua dye x sample number (n) = 0.8 (n+2) µL of the 5-fold dilution.

7.2.3.3 Add 0.8 µL of a 5-fold diluted Zombie Aqua dye to all samples except unstained sample and Zombie Aqua FMO sample.

7.2.3.4 Incubate samples for 30 minutes at room temperature in the dark.

7.2.3.5 Wash each sample 2x with 500 µL staining buffer.

a. Add 500 µL staining buffer to each tube.
b. Centrifuge samples for 7 min at 400xg.
c. Aspirate off each supernatant.

7.2.3.6 Resuspend each sample in 40 µL staining buffer.

7.2.3.7 Prepare 5-fold antibody dilutions in staining buffer in labeled microcentrifuge tubes (one dilution per antibody needed; see Table 6).

7.2.3.8 Make a master mix (MM) for each FMO control in an appropriately labeled microcentrifuge tube.

Note: Add 5-fold dilution antibodies to the appropriate microcentrifuge tube as indicated in Tables 7-8 (Single column indicates a single tube). Add necessary amount of staining buffer to make final volume 60 µL.

7.2.3.9 Add appropriate FMO MM (60 µL) to appropriate cell sample (40 µL) (prepared in step 7.2.3.6).

7.2.3.10 Incubate samples for 30 min at room temperature in the dark.

7.2.3.11 Wash each sample 2x with 500 µL staining buffer.

7.2.3.12 Fix cells by resuspending each cell sample in 100 µL 2% PFA and incubating for 15 minutes at room temperature in the dark.

7.2.3.13 Wash each sample 2x with 500 µL staining buffer.

7.2.3.14 Resuspend each sample in 500 µL staining buffer and transferred samples to appropriately labeled flow tubes.

7.2.3.15 Briefly vortex samples.

Table 6. Volumes of 5-fold Antibody Dilutions Needed for FMOs

Antibody dilution determined to be optimal in Step 6.1	Final Antibody Dilution	Volume of 5-fold dilution Antibody needed (µL) per 100 µL sample
Dil 25	Dil ~250	8
Dil 50	Dil ~500	4
Dil 100	Dil ~1000	2
Dil 125	Dil ~1250	1.6

Note: Prepare enough diluted antibodies for n + 2. Each antibody is only needed at one previously determined optimal dilution (see Table 5). I.e., for Immunophenotyping Panel #1, the antibody dilution that was determined to lead to the optimal concentration of CD8-FITC was Dil 25; therefore, 8 µL of the 5-fold dilution of CD8-FITC is needed per FMO control.

Table 7. Immunophenotyping Panel #1 FMO Control Samples

		FMO controls												Fully stained labeling Ab sample	Unstained
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
		CD8-FITC FMO MM	CD4-PE FMO MM	CD19-PE/Cy7 FMO MM	CCR4-APC FMO MM	CD45RA-AF700 FMO MM	TCR-γδ APC-Fire750 FMO MM	CD45-PacBlue FMO MM	L/D Zombie Aqua FMO MM	CD3-BV570 FMO MM	CD25-BV650 FMO MM	CD154-BV711 FMO MM	CD127-BV785 FMO MM	MM	MM
Antibodies added to each sample	**1**—CD8-FITC Dil 5	**X**	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**
	2—CD4-PE Dil 5	1.6µL	**X**	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	**X**
	3—CD19-PE/Cy7 Dil 5	1.6µL	1.6µL	**X**	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	**X**
	4—CCR4-APC Dil 5	1.6µL	1.6µL	1.6µL	**X**	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	**X**
	5—CD45RA-AF700 Dil 5	8µL	8µL	8µL	8µL	**X**	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**
	6—TCR- γδ APC Fire750 Dil 5	4µL	4µL	4µL	4µL	4µL	**X**	4µL	4µL	4µL	4µL	4µL	4µL	4µL	**X**
	7—CD45-PacBlue Dil 5	8µL	8µL	8µL	8µL	8µL	8µL	**X**	8µL	8µL	8µL	8µL	8µL	8µL	**X**
	8—L/D Zombie Aqua Dil 5	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
	9—CD3-BV570 Dil 5	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**	8µL	8µL	8µL	8µL	**X**
	10—CD25-BV650 Dil 5	2µL	2µL	2µL	2µL	2µL	2µL	2µL	2µL	2µL	**X**	2µL	2µL	2µL	**X**
	11—CD154-BV711 Dil 5	4µL	4µL	4µL	4µL	4µL	4µL	4µL	4µL	4µL	4µL	**X**	4µL	4µL	**X**
	12—CD127-BV785 Dil 5	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**	8µL	**X**
Total volume (µL)		**46.8**	**53.2**	**53.2**	**53.2**	**46.8**	**50.8**	**46.8**	**54.8**	**46.8**	**52.8**	**50.8**	**46.8**	**54.8**	**0**
Staining Buffer (µL)		**13.2**	**6.8**	**6.8**	**6.8**	**13.2**	**9.2**	**13.2**	**5.2**	**13.2**	**7.2**	**9.2**	**13.2**	**5.2**	**60**

Table 8. Immunophenotyping Panel #2 FMO Control Samples

		FMO controls											Fully stained labeling Ab sample	Unstained
		1	2	3	4	5	6	7	8	9	10	11	12	13
		CD56-FITC FMO MM	CD14-PE FMO MM	CD19-PE/Cy7 FMO MM	CD123-APC FMO MM	CD54-AF700 FMO MM	CD20 APC-Fire750 FMO MM	CD45-PacBlue FMO MM	L/D Zombie Aqua FMO MM	CD3-BV570 FMO MM	CD69-BV650 FMO MM	CD11c-BV785 FMO MM	MM	MM
Antibodies added to each sample	**1**—CD56-FITC Dil 5	**X**	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	**X**
	2—CD14-PE Dil 5	1.6µL	**X**	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	**X**
	3—CD19-PE/Cy7 Dil 5	1.6µL	1.6µL	**X**	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	**X**
	4—CD123-APC Dil 5	8µL	8µL	8µL	**X**	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**
	5—CD54-AF700 Dil 5	1.6µL	1.6µL	1.6µL	1.6µL	**X**	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	1.6µL	**X**
	6—CD20 APC-Fire750 Dil 5	8µL	8µL	8µL	8µL	8µL	**X**	8µL	8µL	8µL	8µL	8µL	8µL	**X**
	7—CD45-PacBlue Dil 5	8µL	8µL	8µL	8µL	8µL	8µL	**X**	8µL	8µL	8µL	8µL	8µL	**X**
	8—L/D Zombie Aqua Dil 5	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
	9—CD3-BV570 Dil 5	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**	8µL	8µL	8µL	**X**
	10—CD69-BV650 Dil 5	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**	8µL	8µL	**X**
	11—CD11c-BV785 Dil 5	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	8µL	**X**	8µL	**X**
Total volume (µL)		**52.8**	**52.8**	**52.8**	**46.4**	**52.8**	**46.4**	**46.4**	**54.4**	**46.4**	**46.4**	**46.4**	**54.4**	**0**
Staining Buffer (µL)		**7.2**	**7.2**	**7.2**	**13.6**	**7.2**	**13.6**	**13.6**	**5.6**	**13.6**	**13.6**	**13.6**	**5.6**	**60**

7.3 Data Acquisition with NovoCyte 3005 for FMO controls

7.3.1 Turn on computer > NovoExpress > username and password

7.3.2 Turn on flow cytometer.

7.3.3 Save file.

7.3.4 Check the experimental parameters.

a. Plate Manager
   i. Mode: custom; 24 tube rack
   ii. Indicate samples to run horizontal.
   iii. Click on proper squares to create sample. Rename each sample accordingly in work list.
   iv. Mix: 1 cycle every, 2 wells (1500 rpm; 10 sec); Rinse: 1 cycle, every 3 wells
b. Experimental Run. Stop conditions: Events 300,000; 300 µL. Fast (Apply changes to all samples)
c. Cytometer Settings: Parameters, please see Table 4 in Section 6.1.4.4. (Make sure channels and laser intensity are the same as the single stain control experiment.)

7.3.5 Insert samples in tube rack.

7.3.6 Click run plate. Highlight all boxes that are to be run (all). → run → okay

7.3.7 Save file.

7.3.8 Export FCS files.

7.4 Data Analysis of FMO controls with NovoExpress

Overview: The necessary data from this analysis is the compensation/spillover matrices. The FMO control samples need to be compensated with the compensation matrix from the single stain control samples. The compensation matrix may then need to be adjusted to account for the differences between beads (single stain controls) and cells (FMO controls).

7.4.1 Application of compensation matrix to FMO controls

Note: There are multiple ways to apply the compensation matrix of the single stain controls to the FMO controls. Below is one method.

7.4.1.1 Make a copy of the single stain control file (.ncf) that correlates to the FMO control file.

7.4.1.2 Upload the FCS files from the appropriate FMO control experiment into the single stain control file (.ncf).

a. Right click on file name in Experimental Manager.
b. Click on Import FCS files.
c. Browse for the folder with appropriate FCS files.

7.4.1.3 Apply the compensation matrix from the single stain controls to the FMO samples.

a. Under "Compensation Specimen" click on the "+" for any sample.
b. Right click on Compensation and select Copy.
c. Right click on overarching FMO control experimental sample and click on Paste.
d. "Are you sure you want to paste Compensation to all samples in *experimental sample name*?" Yes.
e. Can then delete the single stain Compensation Specimen.

7.4.1.4 Rename the file with the compensated FMO samples and save.

7.4.2 Analysis Method/Compensation Adjustment of FMO controls

7.4.2.1 Make plots on each FMO sample; Refer to Figure 2 for examples of gates

a. Make a plot of FSC-H vs. SSC-H and gate main the cell population "P1".

b. Make a plot of FSC-A vs. FSC-H from "P1" and gate the single cells "P2".

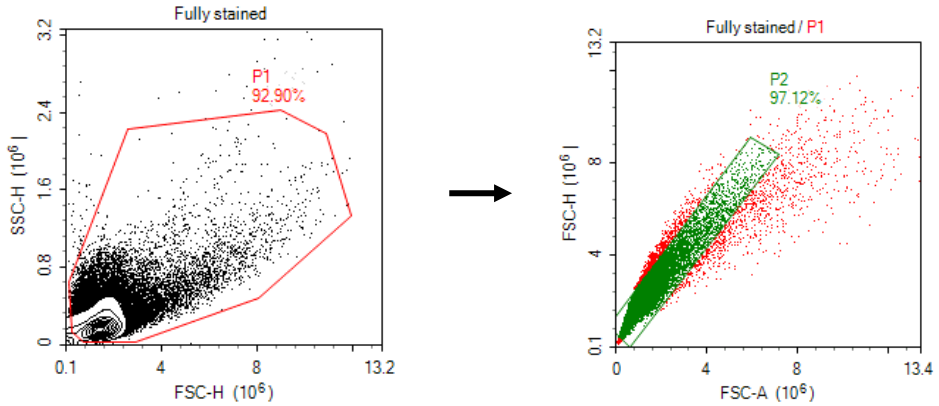

Figure 2. Gating FMO samples. Left: Gate of the cell population; Right: Gate of the singlet population.

7.4.2.2 Adjust compensation using the fully stained sample.

a. Make a plot of FSC-H vs. SSC-H and gate main the cell population "P1".

b. Make a plot of FSC-A vs. FSC-H from "P1" and gate the single cells "P2".

c. Make a plot of each fluorophore pair (example FITC vs. PE; FITC vs. PE-Cy-7; FITC vs. APC; etc.) from "P2".

Note: Plots can auto-populate by clicking on the dot plot symbol until all pairwise fluorophore plots are plotted.

d. Right click on pairwise plots and click Edit overlay. Add FMO sample that corresponds to the fluorophore on the x-axis. Apply.

e. For plots that have large compensation, check the compensation. Adjust the compensation of the parameters using the quick

compensation adjustments on the fully stained sample. (Adjust plots to eliminate or minimize the slanting in the FMO overlay)

f. Once compensation of the fully stained sample is complete, apply the compensation to all the FMO samples.

7.4.2.3 Save compensated file and export compensated FCS files.

8. References

1. Oughton, J.A. and Kerkvliet, N.I. Immune cell phenotyping using flow cytometry. *Current protocols in toxicology*. **2005**, 23(1), 18.18. 11-18.18. 24.
2. McCoy, J., J Philip, *Immunophenotyping*. 2019: Springer.
3. McKinnon, K.M. Flow Cytometry: An Overview. *Curr Protoc Immunol*. **2018**, 120, 5 1 1-5 1 11. DOI: 10.1002/cpim.40. PMID: 29512141.
4. Newton, H.S. and Dobrovolskaia, M.A. Immunophenotyping: Analytical approaches and role in preclinical development of nanomedicines. *Adv Drug Deliv Rev*. **2022**, 185, 114281. DOI: 10.1016/j.addr.2022.114281. PMID: 35405297.
5. Dobrovolskaia, M.A. and McNeil, S.E. Immunological properties of engineered nanomaterials. *Nat Nanotechnol*. **2007**, 2(8), 469-478. DOI: 10.1038/nnano.2007.223. PMID: 18654343.
6. Acea BioScience Inc., *NovoCyte® Flow Cytometer Technical Specifications*. 2017.
7. Agilent Technologies, *NovoCyte 3 Lasers 3005 Model 3005-default configuration*. 2022.

9. Abbreviations

PBMC	peripheral blood mononuclear cells
Treg	regulatory T cells
DC	dendritic cell
NK	natural killer
pDC	plasmacytoid DC
mDC	myeloid DC
FMO	fluorescence minus one
FBS	fetal bovine serum
PBS	phosphate buffered saline
PHA-M	phytohemagglutinin

ODN	oligodeoxyribonucleotide
PFA	paraformaldehyde
QC	quality control
FSC	forward scatter
SSC	side scatter
ROI	region of interest
MM	master mix
Ab	antibody

10. Appendix

Plate 1—Bead and Live: Dead PBMC Plate, Panel #1 Example

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Live/Dead PBMC
B	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Live/Dead PBMC
C	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Live/Dead PBMC
D	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Live/Dead PBMC
E	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Live/Dead PBMC
F	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Dil 10 Beads	Live/Dead PBMC
G												
H												

Plate 2—Antibody/Dye Titration Plate, Panel #1 Example

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	CD8-FITC Ab Dil 5	CD4-PE Ab Dil 5	CD19-PE/Cy7 Ab Dil 5	CCR4-APC Ab Dil 5	CD45RA-AF700 Ab Dil 5	TCR-γδ-APC/Fire750 Ab Dil 5	CD45-PB Ab Dil 5	CD3-BV570 Ab Dil 5	CD25-BV650 Ab Dil 5	CD154-BV711 Ab Dil 5	CD127-BV785 Ab Dil 5	Zombie Aqua dye Dil 5
C	CD8-FITC Ab Dil 25	CD4-PE Ab Dil 25	CD19-PE/Cy7 Ab Dil 25	CCR4-APC Ab Dil 25	CD45RA-AF700 Ab Dil 25	TCR-γδ-APC/Fire750 Ab Dil 25	CD45-PB Ab Dil 25	CD3-BV570 Ab Dil 25	CD25-BV650 Ab Dil 25	CD154-BV711 Ab Dil 25	CD127-BV785 Ab Dil 25	Zombie Aqua dye Dil 25
D	CD8-FITC Ab Dil 125	CD4-PE Ab Dil 125	CD19-PE/Cy7 Ab Dil 125	CCR4-APC Ab Dil 125	CD45RA-AF700 Ab Dil 125	TCR-γδ-APC/Fire750 Ab Dil 125	CD45-PB Ab Dil 125	CD3-BV570 Ab Dil 125	CD25-BV650 Ab Dil 125	CD154-BV711 Ab Dil 125	CD127-BV785 Ab Dil 125	Zombie Aqua dye Dil 125
E	CD8-FITC Ab Dil 625	CD4-PE Ab Dil 625	CD19-PE/Cy7 Ab Dil 625	CCR4-APC Ab Dil 625	CD45RA-AF700 Ab Dil 625	TCR-γδ-APC/Fire750 Ab Dil 625	CD45-PB Ab Dil 625	CD3-BV570 Ab Dil 625	CD25-BV650 Ab Dil 625	CD154-BV711 Ab Dil 625	CD127-BV785 Ab Dil 625	Zombie Aqua dye Dil 625
F	CD8-FITC Ab Dil 3125	CD4-PE Ab Dil 3125	CD19-PE/Cy7 Ab Dil 3125	CCR4-APC Ab Dil 3125	CD45RA-AF700 Ab Dil 3125	TCR-γδ-APC/Fire750 Ab Dil 3125	CD45-PB Ab Dil 3125	CD3-BV570 Ab Dil 3125	CD25-BV650 Ab Dil 3125	CD154-BV711 Ab Dil 3125	CD127-BV785 Ab Dil 3125	Zombie Aqua dye Dil 3125
G												
H												