WG2 SOP-02
Compensation Setup

1. Overview

This Standard Operating Procedure (SOP) describes the creation of the spectral compensation matrices to be used for Working Group 2 (WG2) Inter-Laboratory Study (ILS) TBMNK Assay. Three different compensation matrices will be created as summarized in Figure 1. All three matrices will be run on each individual instrument enrolled in WG2 ILS. The single colors files acquired will be used to calculate compensation matrices that will be applied to the QC and test sample files generated per SOP-03.

Note 1: The TBMNK Assay spectral compensation will be applied post-sample acquisition, thus the creation of the compensation matrices (WG2 SOP-02) can be performed either before or after sample acquisition as described in WG2 SOP-03.

Note 2: Creation of the compensation matrices should be conducted only after the procedures described in WG2 SOP-01, Flow Cytometer Setup and Performance Characterization have been successfully completed.

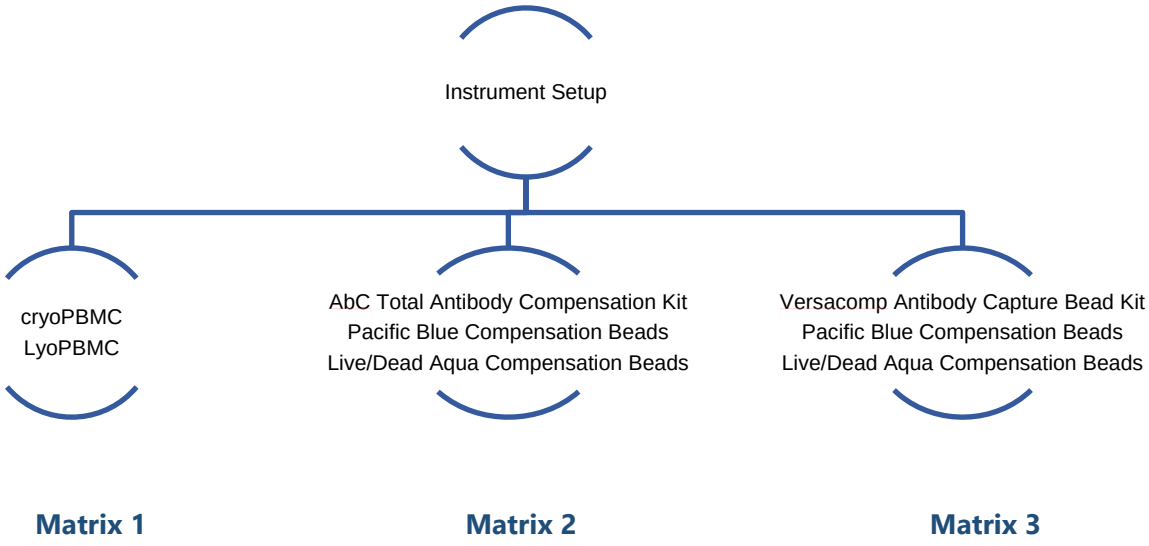

Figure 1. Compensation Workflow.

-Compensation Matrix 1 will use both cryopreserved PBMC (cryoPBMC) -and lyophilized leukocytes (lyoLeuk), (Reference Reagent CD4 T-cells, NIBSC). Note that the cryoPBMC can be from any source, e.g., cryoPBMC from AllCells provided for the study and cryoPBMC from other sources participants possess. It is not necessary that each laboratory use the same source and/or donor for the cryoPBMC. Note also, that it would be permissible to use leftover test samples from WG2 SOP-3 and based on expected yields, this would be feasible.

-Compensation Matrix 2 will use AbC™ Total Antibody Compensation Bead (Thermo Fisher) as well as Pacific Blue and Aqua compensation beads (Thermo Fisher). The AbC™ Total Antibody Compensation Bead Kit contains two types polystyrene microspheres- capture beads that bind all isotypes of mouse, rat, hamster, and rabbit immunoglobulin, and negative beads that have no antibody binding capacity. The Pacific Blue and Aqua Compensation Bead products were made exclusively for WG2 ILS and are ~6.0-micron microsphere surfaces directly functionalized with either Pacific Blue™ or Aqua dye. These microspheres will be used to setup compensation for the Annexin Pacific Blue™ and Aqua LIVE/DEAD products for Compensation Matrix 2 and Compensation Matrix 3.

-Compensation Matrix 3 will use VersaComp antibody capture beads (Beckman Coulter Life Sciences) as well as Pacific Blue and Aqua compensation beads (Thermo Fisher). The VersaComp Antibody Capture Bead Kit contains two vials of 3.0-3.4 µm beads in suspension at a concentration of approximately 1 x 10^7 particles/mL. VersaComp Antibody Capture Negative Beads act as a negative control that do not bind fluorochrome-conjugated antibodies. VersaComp Antibody Capture Positive Beads contains beads coated with an IgG-binding agent that bind all isotypes of mouse, rat, hamster, and rabbit immunoglobulin. The Pacific Blue™ and Aqua compensation beads products as described above will also be used with Compensation Matrix 3.

2. Safety

2.1. General

- Follow appropriate biosafety procedures per your institution's SOPs and Policies.
- Safety precautions for Universal Blood precautions should be used under Biological Safety Level-2 (BSL-2) conditions (see 29 CFR 1910.1030, Bloodborne Pathogens).
- Wear appropriate personal protective equipment at all times. Gloves, safety glasses or goggles, and a laboratory coat are required personal protective equipment when handling human biological fluids including human blood (see 29 CFR 1910.1030, Bloodborne Pathogens).

2.2. Liquid Nitrogen Handling

- Cryogloves/waterproof thermal insulated gloves: Hands should be protected with waterproof thermal insulated gloves that can be quickly removed if LN2 is spilled on them. Cryogloves/waterproof thermal insulated gloves are not intended for submerging hands into LN2.
- Body must be protected with pants, lab coats, and closed-toe shoes.
- Safety goggles: Eyes are sensitive to the extreme cold of LN2 and its vapors.
- Chemical splash goggles should be used when handling LN2 and when handling cryovials in LN2.

2.3. Sodium Azide

Some reagents contain sodium azide. Sodium azide under acid conditions yields hydrazoic acid, an extremely toxic compound. Azide compounds should be flushed with running water while being discarded. These precautions are recommended to avoid deposits in metal piping in which explosive conditions can develop. If skin or eye contact occurs, wash excessively with water.

3. Reagents and Materials

3.1. Reagents

Table 2. Compensation Materials

Compensation Matrix	Name	Vendor	Catalog #	Expiry	Storage Conditions	Reconstitution
1	Cryopreserved PBMC	Any source	NA	NA	Liquid nitrogen vapor phase	See WG2 SOP-03 section 4.3.1
	LyoLeuk (Reference Reagent CD4 T-cells)	NIBSC	15/270	Per certificate of analysis	-20°C or below	See section 4.3.1
2	Pacific Blue™ Compensation Bead	ThermoFisher Scientific	NA/Custom Product	6 months after receipt	2-8°C	Ready to use
	Aqua Compensation Bead	ThermoFisher Scientific	NA/Custom Product	6 months after receipt	2-8°C	Ready to use
	AbC™ Total Antibody Compensation Bead Kit	ThermoFisher Scientific	A10497	6 months after receipt	2-8°C	Ready to use
3	VersaComp Antibody Capture Bead Kit	Beckman Coulter Life Sciences	B22804	As indicated on product vial	2-8°C	Ready to use.

Table 3. Monoclonal Antibodies

Vendor	Catalog #	Marker	Format	Clone	Storage Conditions	Reconstitution	Supplied by
BioLegend	900005606	CD45	PCP-Cy5.5	2D1	2-8°C, protected from light	Ready to use	NIST
BioLegend	900005607	CD3	FITC	SK7	2-8°C, protected from light	Ready to use	NIST
BioLegend	900005609	CD14	APC	M5E2	2-8°C, protected from light	Ready to use	NIST
BioLegend	900005611	CD16	PE	3G8	2-8°C, protected from light	Ready to use	NIST
BioLegend	900005613	CD4	PE-Cy7	SK3	2-8°C, protected from light	Ready to use	NIST

BioLegend	900005606	CD8	APC-Cy7	SK1	2-8°C, protected from light	Ready to use	NIST

Table 4. Other Reagents

Name	Vendor	Catalog #	Storage Conditions	Reconstitution	Supplied by
PBS without Ca^{++}/Mg^{++}	GIBCO/ any	10010-023 or equivalent	Unopened RT (18-25°C) Opened 2-8°C	Ready to use	Lab to purchase
FBS	Any	N/Ap	Unopened -20°C Opened at 2-8°C	Ready to use	Lab to purchase
Ethanol, e.g. 200 proof	Any	N/Ap	RT (18-25°C)	See below	Lab to purchase
$CaCl_2$	Any	N/Ap	RT (18-25°C)	See below	Lab to purchase
LIVE/DEAD™ Fixable Aqua Dead Cell Stain	Thermo Fisher	L34957	-20°C	See below	NIST
Pacific Blue™ Annexin V	Thermo Fisher	A35136	-2-8 °C	Ready to use	NIST

3.2. Supplies

- Staining Tubes: 5 mL Polystyrene Round-Bottom Tubes
- Pipet tips (P10, P20, P100, P200, P1000)
- 15 mL and 50 mL polypropylene conical tubes with screw cap lids
- 96-well plates, per your institution's SOP (for testing labs that stain in plates, only)
- Sterile serological pipettes (e.g., 2 mL, 5 mL, 10 mL, 25 mL)

3.3. Equipment

- Pipettors P10, P20, P100, P200, P1000
- Pipette-aid
- Water Bath
- Centrifuge
- Biosafety Cabinet
- Cytometer (as described in WG2 Study Plan)

3.4. Reagent Preparation

70% Ethanol

Prepare per your institution's SOPs.

Staining Buffer

Prepare a solution of 1X PBS ≥3.5 mM CaCl2, containing a final volume of 2% FBS, pH 7.4, per your institution's SOPs.

LIVE/DEAD™ Fixable Aqua Dead Cell Stain

Note this kit contains:

- Component A – Aqua dye
- Component B - Dimethylsulfoxide (DMSO), anhydrous

Bring 1 vial of fluorescent reactive dye (Component A) and 1 vial of anhydrous DMSO (Component B) to RT before removing the caps.
Add 50 µL of DMSO to the vial of reactive dye. Mix well and visually confirm that all of the dye has dissolved. Keep reconstituted dye solution in dark at room temperature and use it within 2 hours of reconstitution.

4. Procedure

4.1. Instrument Setup

Use the instrument setting from SOP-1 except that FSC and SSC will be adjusted as appropriate for different compensation matrices.

4.2. Worklist Creation

Create a worklist containing sample tubes and a local file naming scheme to save files described in the 'Compensation Controls' of the WG2 Reporting Spreadsheet.

4.3. Compensation Materials Preparation and Staining Procedure

Stained beads or cells will be acquired as compensation samples per Tables 5-8. In total 22 files will be generated.

4.3.1. Compensation Matrix 1

Lyophilized PBMC, CD4 T-cells (lyoLeuk) (Exp ID: e1)

Proper aseptic and biohazard technique should be used when handling and manipulating cells. Perform in biosafety cabinet.

- Remove 2 ampoules out of storage, place in a tube holder and allow to adjust to RT, break ampoule seal.
- Pipette 1 mL sterile distilled water in each of 2 lyophilized PBMC vials (each lyophilized PBMC vial contains 1.5 x 10^6 PBMC) and allow 15-30 min for rehydration.
- Mix cell suspension well by gently pipetting up and down and transfer 1 mL of lyophilized PBMC to a capped tube.
- Add 3 mL of PBS to each tube.
- Centrifuge at RT for 7 minutes at 350 × *g*.
- Aspirate the supernatant. Resuspend the cell pellet in 200 µL of PBS in one tube and in 200 µL of the staining buffer in the second tube.
- Add 100 µL cell suspension in PBS in previous step, to the Aqua compensation tube, comp tube 7 (Table 5) and then 900 µL PBS to make up 1 mL of cell suspension.
- Add 100 µL cell suspension in the staining buffer in previous step, to the Annexin V Pacific Blue compensation tube, comp tube 8 (Table 5).
- Add appropriate dilution of LIVE/DEAD™ Fixable Aqua Dead Cell Stain or Pacific Blue™ Annexin V (as indicated in Table 5). Take care to deposit the stain to the cell suspension. Vortex immediately.
- Incubate at RT for 30 min, protected from light.
- Add 3 mL of PBS to each tube.
- Centrifuge for 7 minutes at 350 × *g*.
- Aspirate the supernatant. Resuspend the cell pellet.
- Add 3 mL of PBS to each tube.

- Centrifuge for 7 minutes at 350 × *g*.
- Aspirate the supernatant. Resuspend the cell pellet.
- Add 400 µL staining buffer and then add 100 µL cells resuspended in either PBS or staining buffer from the step bullet #6 above, serving as unstained cells.
- Note: Lyophilized PBMCs (lyoLeuk) are dead cells and are stained with Aqua or Pacific Blue 100%, and therefore unstained cells are needed to serve as negative cell controls. During acquisition/analysis, participants need to identify more approporiate cell population, lymphocyte or monocyte, that gives better separation of the fluorescence peaks from positive and negative cell populations.

cryoPBMC (Exp ID: e1)

Proper aseptic and biohazard techniques should be used when handling and manipulating cells. Perform in biosafety cabinet.

- Refer to WG2 SOP-03 for instructions on cell thawing (In the cell thawing process, participants need to perform cell counting).
- Resuspend thawed cells in an appropriate volume of Staining Buffer to achieve cell concentration of 5 x 10^6/mL.
- Label tubes as indicated in Table 5.
- Add 100 µL cell suspension to each compensation tube.
- Add appropriate dilution of individual mAb (as indicated in Table 5). Take care to deposit the mAb to the cell suspension. Vortex immediately.
- Incubate at RT for 30 min, protected from light.
- Add 3 mL of PBS to each tube.
- Centrifuge for 7 minutes at 350 × *g*.
- Aspirate the supernatant. Resuspend the cell pellet.
- Add 3 mL of PBS to each tube.
- Centrifuge for 7 minutes at 350 × *g*.
- Aspirate the supernatant. Resuspend the cell pellet.
- Add 400 µL staining buffer and then add 100 µL unstained cells.
- Note: Only comp tube #2 (CD45 PerCP-Cy5.5) and tube #5 (CD14 APC) absolutely require the addition of unstained cells.

Acquire Compensation Matrix 1 Samples (cryoPBMC and lyoLeuk) on the flow cytometer as described in Section 4.5.

4.3.2. Compensation Matrix 2

AbC™ Total Antibody Compensation Bead (Exp ID: e2)

- Label tubes as indicated in Table 6.
- Completely resuspend the AbC™ Total Compensation capture beads (Component A) and negative beads (Component B) by gently vortexing for 10 seconds before use.
- Add 1 drop of AbC™ Total Compensation capture beads (Component A) to each tube.
- Add appropriate dilution of individual mAb (as indicated in Table 6). Take care to deposit the mAb to the cell suspension. Vortex immediately.
- Incubate for 15 minutes at RT, protected from light.
- Add 3 mL of the staining buffer to each tube.
- Centrifuge for 7 minutes at 250 × *g*.
- Decant the supernatant and resuspend the beads in 500 µL of staining buffer.
- Add one drop of negative beads (Component B) to each stained tube and mix well.
- Acquire on the instrument within 30 minutes of preparation.

- Vortex the thoroughly (at low speed) immediately prior to acquisition.

Acquire Compensation Matrix 2 AbC™ Total Antibody Compensation Bead on the flow cytometer as described in Section 4.5.

4.3.3. Compensation Matrix 3

VersaComp antibody capture beads (Exp ID: e3)

- Ensure proper resuspension of the individual kit components by vortexing vials prior to use.
- Label tubes as indicated in Table 7.
- Add one drop of negative beads and one drop of positive beads to each tube.
- Add mAb to the designated tube as indicated in Table 7. Take care to deposit the antibody directly to the bead suspension. Vortex immediately.
- Incubate at RT for 20 minutes, protected from light.
- Add 1 mL of the staining buffer to each test tube, vortex and centrifuge at 300 x *g* for 6 minutes.
- Decant the supernatant and resuspend the beads in 600 µL of staining buffer.

Acquire Compensation Matrix 3 VersaComp antibody capture beads on the flow cytometer as described in Section 4.5.

4.3.4. Pacific Blue™ and Aqua Compensation Bead Products (Exp ID: e4)

- Gently vortex the Pacific Blue™ or Aqua Compensation Bead products, and Component B from the AbC Total Antibody Compensation Bead kit (Component B) 10 seconds to completely resuspend before use.
- Label tubes as indicated in Table 8.
- Add 1 drop of the Pacific Blue™ or Aqua Compensation Bead product to the appropriate sample tube.
- Allow Pacific Blue™ or Aqua Compensation beads to sit in the tube for 5 minutes to warm to RT.
- Add 500 µL of the staining buffer to each tube.
- Add one drop of negative beads (Component B in the AbC™ Total Antibody Compensation Bead Kit) to sample tubes.
- Vortex the tubes before analyzing.
- Perform flow cytometry on the samples as described in Section 4.5.

Acquire Compensation Matrix 3 VersaComp antibody capture beads on the flow cytometer as described in Section 4.5.

4.4. Compensation Staining Maps

Table 5. Compensation Matrix-1 Staining

Experiment ID	Tube ID	Material /Bead	Antibody (fluorochrome)	Material Code	Volume of antibody to be added
e1	comp tube 1	cryoPBMC	CD3 (FITC)	FITC-cryoPBMC	5 µL
e1	comp tube 2	cryoPBMC	CD45 (PerCP-Cy5.5)	PerCP-Cy5.5-cryoPBMC	5 µL

e1	comp tube 3	cryoPBMC	CD16 (PE)	PE-cryoPBMC	5 µL
e1	comp tube 4	cryoPBMC	CD4 (PE-Cy7)	PE-Cy7-cryoPBMC	5 µL
e1	comp tube 5	cryoPBMC	CD14 (APC)	APC-cryoPBMC	5 µL
e1	comp tube 6	cryoPBMC	CD8 (APC-Cy7)	APC-Cy7-cryoPBMC	5 µL
e1	comp tube 7	lyoLeuk	live/dead fixable Aqua	AqLD-lyoLeuk	1 µL
e1	comp tube 8	lyoLeuk	Annexin V Pacific Blue	AxPB-lyoLeuk	5 µL

Table 6. AbC Total Antibody Beads Compensation Tubes and Staining volumes

Experiment ID	Tube ID	Material /Bead	Antibody (fluorochrome)	Material Code	Volume of antibody to be added
e2	comp tube 1	AbC total antibody compensation bead	CD3 (FITC)	FITC-AbCTotal	5 µL
e2	comp tube 2	AbC total antibody compensation bead	CD45 (PerCP-Cy5.5)	PerCP-Cy5.5-AbCTotal	5 µL
e2	comp tube 3	AbC total antibody compensation bead	CD16 (PE)	PE-AbCTotal	5 µL
e2	comp tube 4	AbC total antibody compensation bead	CD4 (PE-Cy7)	PE-Cy7-AbCTotal	5 µL
e2	comp tube 5	AbC total antibody compensation bead	CD14 (APC)	APC-AbCTotal	5 µL
e2	comp tube 6	AbC total antibody compensation bead	CD8 (APC-Cy7)	APC-Cy7-AbCTotal	5 µL

Table 7. VersaComp antibody capture bead Compensation Tubes and Staining volumes

Experiment ID	Tube ID	Material /Bead	Antibody (fluorochrome)	Material Code	Volume of antibody to be added
e3	comp tube 1	VersaComp antibody capture bead	CD3 (FITC)	FITC-Versacmp	5 µL
e3	comp tube 2	VersaComp antibody capture bead	CD45 (PerCP-Cy5.5)	PerCP-Cy5.5-Versacmp	5 µL

e3	comp tube 3	VersaComp antibody capture bead	CD16 (PE)	PE-Versacmp	5 µL
e3	comp tube 4	VersaComp antibody capture bead	CD4 (PE-Cy7)	PE-Cy7-Versacmp	5 µL
e3	comp tube 5	VersaComp antibody capture bead	CD14 (APC)	APC-Versacmp	5 µL
e3	comp tube 6	VersaComp antibody capture bead	CD8 (APC-Cy7)	APC-Cy7-Versacmp	5 µL

Table 8. Aqua and Pacific Blue Beads Compensation Tubes and Staining volumes

Experiment ID	Tube ID	Material /Bead	Antibody (fluorochrome)	Material Code	Volume of antibody to be added
e4	comp tube 7	Live/Dead Aqua compensation bead	live/dead fixable Aqua	AqBead	Per section 4.3.4
e4	comp tube 8	Pacific Blue compensation bead	Annexin V Pacific Blue	PBBead	Per section 4.3.4

4.5. Acquisition

- Create an acquisition template for the Compensation Matrices
- Use the gain settings for all fluorescence detectors finalized in the SOP-01. The gain settings for both FSC and SSC can be adjusted for different compensation control types to place them on scale if needed.
- Note: Because VersaComp antibody capture beads are 3-3.4 µm and both Aqua and Pacific blue compensation beads are 6 µm in diameter, the FCS and SSC gains must be adjusted for the size differences prior to acquisition of the Compensation Matrix 3.
- Acquire samples without compensation and with 15 000-20 000 events in the singlet gate
- Verify the gains setup in SOP-01 are appropriate for compensation controls. If the signal is too bright, re-stain the comp matrices with less antibody (e.g., 3-4 µL of antibody instead of 5 µL of antibody).

4.6. Local Filename Entry on the WG2 Compensation Control Reporting Spreadsheet

1. Open the WG2 Reporting Workbook and select the “Compensation Control” Tab. Verify that the Study ID, Site ID, Instrument ID and Protocol ID at the top of the Spreadsheet describe the current execution of the protocol. See Figure 2 for example. One Workbook is required for each instrument.
2. For each file collected (22 total files) find the corresponding material code row using the terms in “Column D”. See Figure 2.
3. Identify the material associated “Get Local Filename” button in Column F to identify the local file location. See Figure 2
4. Press the button and use the file dialog box to identify the local FCS filename corresponding to the files collected in Section 4.5.

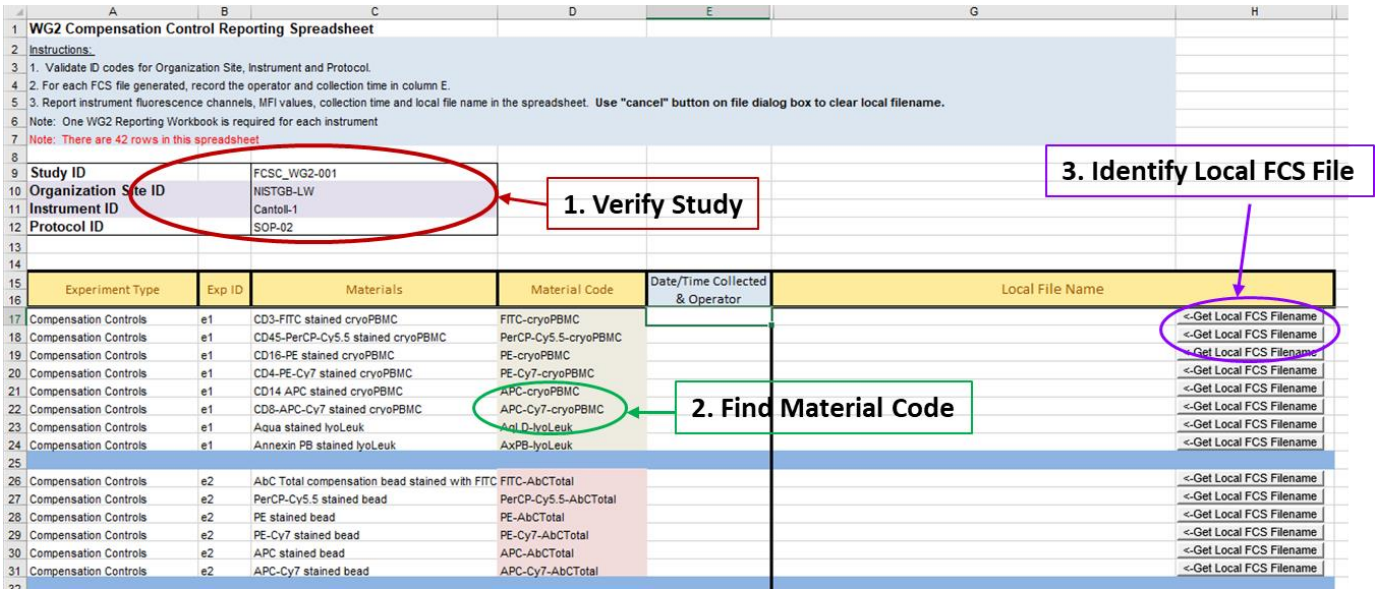

Figure 2- WG2 Compensation Control Reporting Spreadsheet.

5. Attachments

1. Information for Lyophilized Leuokcytes (Reference Reagent CD4 T-cells, NIBSC)
2. Information for Annexin Pacific Blue
3. Information for Aqua LIVE/DEAD Stain
4. Information for Pacific Blue and Aqua Coated Compensation Beads
5. Information for AbC Total Product
6. Information for VersaComp Beads