Study Title:	NIST Flow Cytometry Standards Consortium- WG1 Interlaboratory Study	
Study ID:	FCSC_WG1-001	Approval
Protocol ID:	p1	
Execution Date:		

SOP - WG1 Interlaboratory Study (ILS) One: Equivalent Reference Fluorophore (ERF) Beads Variability and Assignment of Unknowns

1. PURPOSE

Determine the variability of the ERF calibration scale between bead sets, instruments and laboratories.

2. SCOPE

Measure the mean fluorescence intensities for four different ERF-assigned bead sets from four different vendors and for a set of fluorescently-labeled PBMCs under the same gain settings for each fluorescence channel.

3. RESPONSIBILITIES

3.1 NIST

1. NIST will collect all materials from vendors/manufacturers and distribute to participants using the shipping address supplied by each participant.
2. NIST will collect all raw data files (e.g. FCS) from the participants to allow distribution and analysis of the ILS dataset.

3.2 Materials Vendors

Vendors who have agreed to provide materials for this ILS will send their materials to NIST with instructions for use.

3.3 Testing Laboratories

Testing labs will collect data according to this SOP using the flow cytometers they listed on the WG-1 Equipment Worksheet of the Study Spreadsheet found on the Consortium Google Drive.

4. MATERIALS

Hard-dyed, ERF-assigned multiwavelength emission and excitation beads include Daily Quality Control (DQC) beads from Beckman Coulter (sample of dyed beads with one intensity), Ultra-Rainbow Beads (URB) from Spherotech (sample mixture of 5 dyed beads and a blank bead with 6 different intensities) and AccuCheck (AC) beads from Thermo Fisher (sample mixture of 3 dyed beads with 3 different intensities). Fluorophore-specific, ERF-assigned FC beads are from BD (eight different fluorophore-

specific samples, each mixed with blank beads. The biological samples with unknown ERF values are lyophilized, prelabeled peripheral blood mononuclear cells (PBMCs) from BioLegend. See Table 1.

Table 1. Materials Supplied by NIST/Vendors

Name	Vendor	Material Code	Intensities	Samples	**User Instructions**
DQC hard-dyed beads	Beckman Coulter	DQC-bead	1	1	Appendix A
URB mix hard-dyed & blank beads	Spherotech	URBmix-bead	5 + blank	1	Appendix B
AC mix hard-dyed beads	Thermo Fisher	ACmix-bead	3	1	Appendix C
BD™ FC & blank beads	BD	V450-bead V500C-bead FITC-bead PE-bead PerCP-Cy5.5-bead PE-Cy7-bead APC-bead APC-Cy7-bead	1 + blank	8	Appendix D
lyophilized PBMCs (fluorophore-labeled)	BioLegend	V450-lyoPBMC-cell V500C-lyoPBMC-cell FITC-lyoPBMC-cell PE-lyoPBMC-cell PerCP-Cy5.5-lyoPBMC-cell PE-Cy7-lyoPBMC-cell APC-lyoPBMC-cell APC-Cy7-lyoPBMC-cell	1	8	Appendix E

5. PROCEDURES

Note : All ILS test samples (beads and cells) should be prepared and acquired on the same instrument in the sample analytical run (i.e. same instrument, same operator, same time frame) using the same laser power and gain settings for each fluorescence channel. Only the forward and side scattering settings will change between the bead and cell samples.

5.1 Instrument software, local filename scheme and acquisition software setup.

1. The acquisition software should be configured to show the actual wavelength values for each fluorescence channel of the instrument (e.g. laser/filter/bandwidth = 488/530/30). These values will likely be metadata in the saved FCS data file and ensures reliable metadata tracking.
2. All fluorescence channels and event features (height, area, width, geometric MFI, arithmetic MFI, etc.) from the instrument should be collected during a

sample run. This may require changing settings in the acquisition software that controls which fluorescence channels and event measurements are saved to a data file.

3. All data files should be written to a FCS file format that works with 3rd party analysis software (e.g. FlowJo, CytExpert, FCS express, etc). Please contact study organizers if other file formats will be collected.
4. Create a local data file naming scheme that can be used to save FCS data sets from this protocol. This naming scheme should be compatible with file naming limitations on the acquisition software. For example, <study>_<protocol ID>_<operator>_<material>_<replicate>.fcs (WG1_p1_je_DQC-bead_1.fcs) could be used for each instrument. Filenames can be short and tailored to the instrument filename workflow. Local filenames should be pre-determined for each FCS file that will be saved in this protocol (minimum of 57 files).

5.2 Bead and Cell Sample Preparation

1. Prepare each of the samples in triplicate per the manufacturers instructions located in the corresponding appendix (see Table 1). Sample preparation instructions are given for both test tubes and well plates formats.
2. Aliquot samples into a tube (i.e. 500 uL for 3 technical replicates from a single tube) or a plate (i.e. 200 uL into 3 different wells for 3 technical replicates) that is standard for the flow cytometer.
3. Protect from light (cover with Al foil) until ready to measure. Hold the samples at 4-8 °C if not in use within 2 hours after preparation.

5.3 Setting Threshold, Scattering and Fluorescence Gain for Beads

NOTE: This process does not generate data for the reporting spreadsheet.

1. The DQC and AC beads are the brightest beads depending on the instrument.
2. The DQC peak from this sample will be used to set the gain for each channel.
3. Validate instrument operation per the manufacturer's instructions.
4. Run one of the DQC samples and start characterizing the beads on the instrument under initial conditions.
5. In the acquisition software, generate a dot plot that will contain side scatter (SSC) on the Y-axis and the forward scatter (FSC) on the X-axis.
6. Adjust the bead flow rate such that the detected event frequency is appropriate for the instrument. A typical event rate is 1000 events/sec.
7. With a suitable low threshold value on the forward scattering event, adjust the forward scattering (FSC) and side scattering (SSC) gains such that the main cluster of beads is centered at slightly less than half of full scale for both FSC and SSC.

8. Create a gate in the SSC vs. FSC dot plot around the main population as shown in the Appendices for each sample.
9. Optimize the threshold value on the forward scattering event, if necessary, to get a higher percentage of the gated main population relative to the total population. This should also minimize the amount of FSC background visible near the x-axis of the SSC vs. FSC dot plot.
10. For each fluorescence channel, make a histogram plot using main population gated data, to display fluorescence events on the X-axis. Adjust the channel gain such that the highest intensity signal peak is placed one order of magnitude (10^1) below the maximum on the scale, as shown in the Appendices for each sample.

    Note: One way to adjust the gain more precisely is to first create a gate around the signal peak and then generate a statistics pane to observe the mean inside that gate. Adjust the gain until the population has a mean around one order of magnitude (10^1) below the maximum on your scale.

    Note: Another way to adjust gains is to run a voltage/gain sweep with a set of multipeak hard-dyed beads and use the Separation Index (SI) between the two dimmest beads to determine the minimal gain for maximum low-end resolution and dynamic range.

11. Repeat the gain setting process (step 10) on all fluorescence channels used in this protocol. The fluorescence gain settings should not change during the execution of this protocol.

    Note: Some instruments may not have gain settings for fluorescence channels. In this case, collect data with manufacturer's settings.

12. Run one of the AC samples and start characterizing the beads on the instrument under the same conditions as set for the DQC beads.

    Note: You need to test whether or not the AC-H bead is significantly brighter than the DQC bead for any of the fluorescence channels. Only change the gain of the fluorescence channels that are significantly brighter.

13. In the acquisition software, generate a dot plot that will contain side scatter (SSC) on the Y-axis and the forward scatter (FSC) on the X-axis.
14. Create a gate in the SSC vs. FSC dot plot around the main population as shown in the Appendices for each sample.
15. For each fluorescence channel, make a histogram plot using main population gated data to display fluorescence events on the X-axis.
16. If the fluorescence intensity of the highest intensity peak is about one order of magnitude (10^1) below the maximum on the scale, as shown in the

Appendices for each sample, then Do Not change the gain for the fluorescence channel.

17. If the fluorescence intensity of the highest intensity peak is greater than the maximum on the scale or close to the maximum (well within one order of magnitude), then adjust the channel gain such that the highest intensity signal peak is placed one order of magnitude (10^1) below the maximum on the scale, as shown in the Appendices for each sample.

5.4 Data acquisition for Bead Samples (Experiment ID: e1)

NOTE: Using the thresholds, scattering and fluorescence gains established per section 5.3, an FCS file is generated for each bead sample. This includes 3 FCS files for each bead type, since 3 biological replicates are prepared for each bead type. Ensure all compensations are switched off. Record data from all channels of your instrument for all samples.

1. In the acquisition software, draw a SSC vs. FSC dot plot and for each fluorescence channel a fluorescence channel histogram using main population gated data (see Figures in Appendices for each sample). These plots will only be used to validate data collection. The reporting spreadsheet will be filled out after data collection is performed.
2. Remove a previously prepared bead sample from under the foil and vortex or mix with a pipette. For well plates, use an appropriate mixing protocol for the instrument, if available. If a mixing protocol is not available for the instrument, then manually mix samples using a pipette immediately prior to the measurement.
3. Initiate an acquisition cycle.
4. Check that the event rate is appropriate for the instrument. Adjust flow rate if required.
5. Draw a gate around the main population in the SSC vs. FSC dot plot and
6. Begin a new acquisition to acquire the bead sample data.
7. For each gated main population, record approximately
   a. 10,000 events for DQC beads
   b. 25,000 events for AC beads
   c. 50,000 events for URB beads
   d. 20,000 events for FC beads
8. Validate data collection by ensuring appropriate fluorescence event frequencies in the fluorescence histogram plot (For example, see Figures in Appendices for each sample).
9. Save the FCS data using the local filenaming scheme (see step 5.1.4) and record filename in a notebook or on the WG1 reporting spreadsheet. See process 6 below for instruction on entering the local filename in the reporting spreadsheet.

10. Run ~1 mL of dilution buffer for 30 seconds at a medium rate before acquiring the next sample to prevent carry-over from the last bead sample.
11. Repeat step 1-10 for each bead sample.

5.5 Scattering Threshold and Gain Settings for Cell Samples.

NOTE: This process does not generate data for the reporting spreadsheet. The fluorescence gain settings established per section 5.3 are not changed during this process.

1. In the acquisition software, generate a dot plot that will contain the side scatter (SSC) on the Y-axis and the forward scatter (FSC) on the X-axis.
2. Initiate data acquisition and start characterization on an aliquot (see Appendix E) of fluorophore-labeled cells. This process is independent of the fluorophore.
3. Adjust the cell flow rate such that the detected event frequency is appropriate for the instrument. A typical rate is 1000 events per second.
4. With a suitable low threshold value on the forward scattering event, adjust the forward scattering (FSC) and side scattering (SSC) gains such that the main cluster of cells is centered at slightly less than half of full scale for both FSC and SSC (see Figures in Appendix E).
5. Optimize the threshold value on the forward scattering event, if necessary, to get a higher percentage of the gated main population relative to the total population. This should also minimize the amount of FSC background visible near the x-axis of the SSC vs. FSC dot plot.

5.6 Data Acquisition for Cell Samples (Experiment ID: e2)

NOTE: Using the established thresholds, scattering and fluorescence gains, an FCS file is generated for each cell sample.

1. In the acquisition software, draw a SSC vs. FSC dot plot and for each fluorescence channel a fluorescence channel histogram using main population gated data (see Figures in Appendix E). These plots will only be used to validate data collection. The reporting spreadsheet will be filled out after data collection is performed.
2. Remove a previously prepared cell sample from under the foil and vortex or mix with a pipette. For well plates, use an appropriate mixing protocol for the instrument, if available. If a mixing protocol is not available for the instrument, then manually mix samples using a pipette immediately prior to the measurement.
3. Initiate an acquisition cycle.
4. Check that the event rate is appropriate for the instrument. Adjust flow rate if required.

5. Draw a gate around the main cell population in the SSC vs. FSC dot plot and record approximately 10,000 events in the gated main population.
6. Validate data collection by evaluating the fluorescence event frequencies in the fluorescence histogram plot, made using main population gated data (For example, see Figures in Appendix E).
7. Save the FCS data using the local filenaming scheme (see step 5.1.4) and record file name in a notebook or on the WG1 reporting spreadsheet. See process 6 below for instruction on entering the local filename in the reporting spreadsheet.
8. Run ~1 mL of dilution buffer for 30 seconds at a medium rate before acquiring the next sample to prevent carry-over from the next bead sample.
9. Repeat steps 2-8 for each cell sample.

6. REPORTING

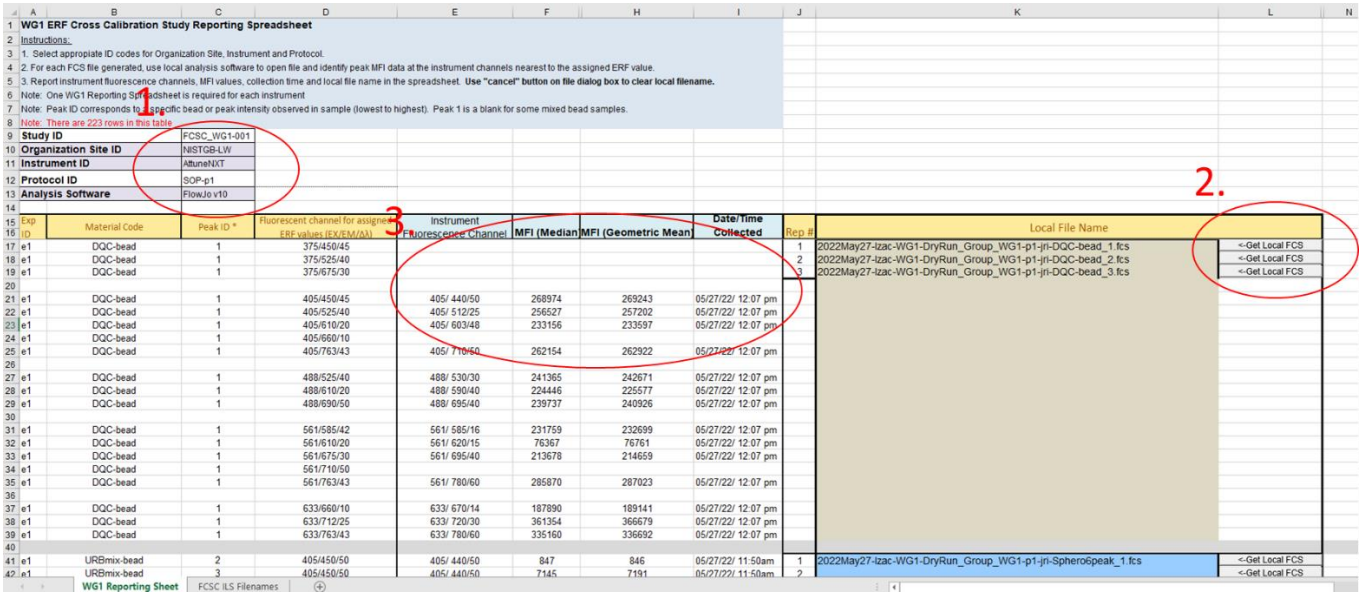
6.1 Data Analysis and Reporting Spreadsheet

An example of the WG1 reporting spreadsheet is shown in Figure 1. Some of the empty column values for each sample row should be filled out before data filename conversion and data submission. The prefilled columns are based on experimental details documented in the WG1 materials and instrument spreadsheet. There are a total of 57 FCS files (3 replicates, 19 samples) that are required to complete this protocol.

1. Open the WG-1 reporting spreadsheet and click on the “WG1 reporting sheet” tab. Verify that the Study ID, Site ID, Instrument ID, Protocol ID and Analysis Software describe the origin of the FCS file and summary data. Use dropdown selections to change the site and instrument identifier. One WG1 reporting spreadsheet is required for each instrument at each site. See circle 1 in Figure 1.
2. For each FCS file for a bead or cell sample, use the “Get Local Filename” button in Column L to identify the local FCS file location and filename. See circle 2 in Figure 1.
3. Verify the date and time the file was collected. See circle 3 in Figure 1.
4. For each row, identify the fluorescence filter combinations on the flow cytometer that are closest to the assigned ERF values shown for the bead in Column D “Fluorescence channel for assigned ERF values”. Type the filter combination into the column E “Instrument Fluorescence Channel (laser/filter/bandwidth). Some rows may not be populated depending on the instrument and filter combinations. See circle 3 in Figure 1.
5. Open the FCS file with local flow cytometry analysis software. Generate a SSC vs. FSC dot plot and draw a gate around the main population. Generate a fluorescence channel histogram plot using main population gated data and the instrument fluorescence channels identified in Column E that are closest to the assigned ERF value.

6. Draw gates across the brightest fluorescence peak in the sample. If there are multiple peaks, draw gates across each of the peaks. (see Figures in Appendices for each sample). If you save these analysis parameters, do not write over the original FCS data file.
7. Report MFI median and MFI geometric mean values in column F and H. In the case of multiple peaks, use Column C “Peak ID” to identify the peak associated with the reported values.
8. Repeat steps 2-7 for each WG1 sample.
9. When completed successfully, one FCS file will be associated with one or more complete rows summarizing sample MFI measurements specific for instrument.

Figure 1 - WG1 Reporting Spreadsheet.



WG1 ERF Cross Calibration Study Reporting Spreadsheet

Instructions:

1. Select appropiate ID codes for Organization Site, Instrument and Protocol.
2. For each FCS file generated, use local analysis software to open file and identify peak MFI data at the instrument channels nearest to the assigned ERF value.
3. Report instrument fluorescence channels, MFI values, collection time and local file name in the spreadsheet. **Use "cancel" button on file dialog box to clear local filename.**

Note: One WG1 Reporting Spreadsheet is required for each instrument

Note: Peak ID corresponds to a specific bead or peak intensity observed in sample (lowest to highest). Peak 1 is a blank for some mixed bead samples.

Note: There are 223 rows in this table

Study ID	FCSC_WG1-001
Organization Site ID	NISTGB-LW
Instrument ID	AttuneNXT
Protocol ID	SOP-p1
Analysis Software	FlowJo v10

1.

2.

3

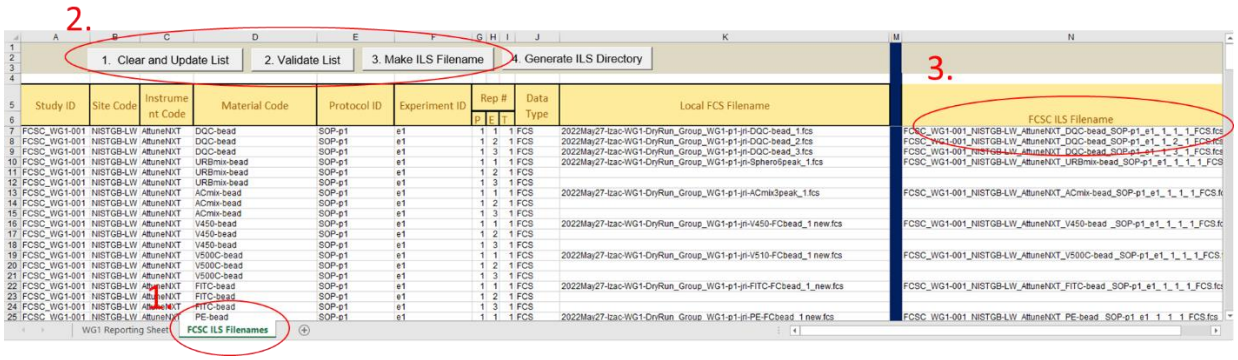
Exp ID	Material Code	Peak ID *	Fluorescent channel for assigned ERF values (EX/EM/Δλ)	Instrument Fluorescence Channel	MFI (Median)	MFI (Geometric Mean)	Date/Time Collected	Rep #	Local File Name	
e1	DQC-bead	1	375/450/45					1	2022May27-lzac-WG1-DryRun_Group_WG1-p1-jri-DQC-bead_1.fcs	<-Get Local FCS
e1	DQC-bead	1	375/525/40					2	2022May27-lzac-WG1-DryRun_Group_WG1-p1-jri-DQC-bead_2.fcs	<-Get Local FCS
e1	DQC-bead	1	375/675/30					3	2022May27-lzac-WG1-DryRun_Group_WG1-p1-jri-DQC-bead_3.fcs	<-Get Local FCS
e1	DQC-bead	1	405/450/45	405/ 440/50	268974	269243	05/27/22/ 12:07 pm			
e1	DQC-bead	1	405/525/40	405/ 512/25	256527	257202	05/27/22/ 12:07 pm			
e1	DQC-bead	1	405/610/20	405/ 603/48	233156	233597	05/27/22/ 12:07 pm			
e1	DQC-bead	1	405/660/10							
e1	DQC-bead	1	405/763/43	405/ 710/50	262154	262922	05/27/22/ 12:07 pm			
e1	DQC-bead	1	488/525/40	488/ 530/30	241365	242671	05/27/22/ 12:07 pm			
e1	DQC-bead	1	488/610/20	488/ 590/40	224446	225577	05/27/22/ 12:07 pm			
e1	DQC-bead	1	488/690/50	488/ 695/40	239737	240926	05/27/22/ 12:07 pm			
e1	DQC-bead	1	561/585/42	561/ 585/16	231759	232699	05/27/22/ 12:07 pm			
e1	DQC-bead	1	561/610/20	561/ 620/15	76367	76761	05/27/22/ 12:07 pm			
e1	DQC-bead	1	561/675/30	561/ 695/40	213678	214659	05/27/22/ 12:07 pm			
e1	DQC-bead	1	561/710/50							
e1	DQC-bead	1	561/763/43	561/ 780/60	285870	287023	05/27/22/ 12:07 pm			
e1	DQC-bead	1	633/660/10	633/ 670/14	187890	189141	05/27/22/ 12:07 pm			
e1	DQC-bead	1	633/712/25	633/ 720/30	361354	366679	05/27/22/ 12:07 pm			
e1	DQC-bead	1	633/763/43	633/ 780/60	335160	336692	05/27/22/ 12:07 pm			
e1	URBmix-bead	2	405/450/50	405/ 440/50	847	846	05/27/22/ 11:50am	1	2022May27-lzac-WG1-DryRun_Group_WG1-p1-jri-Sphero6peak_1.fcs	<-Get Local FCS
e1	URBmix-bead	3	405/450/50	405/ 440/50	7145	7191	05/27/22/ 11:50am	2		<-Get Local FCS

WG1 Reporting Sheet | FCSC ILS Filenames

6.2 Generating FCSC Files

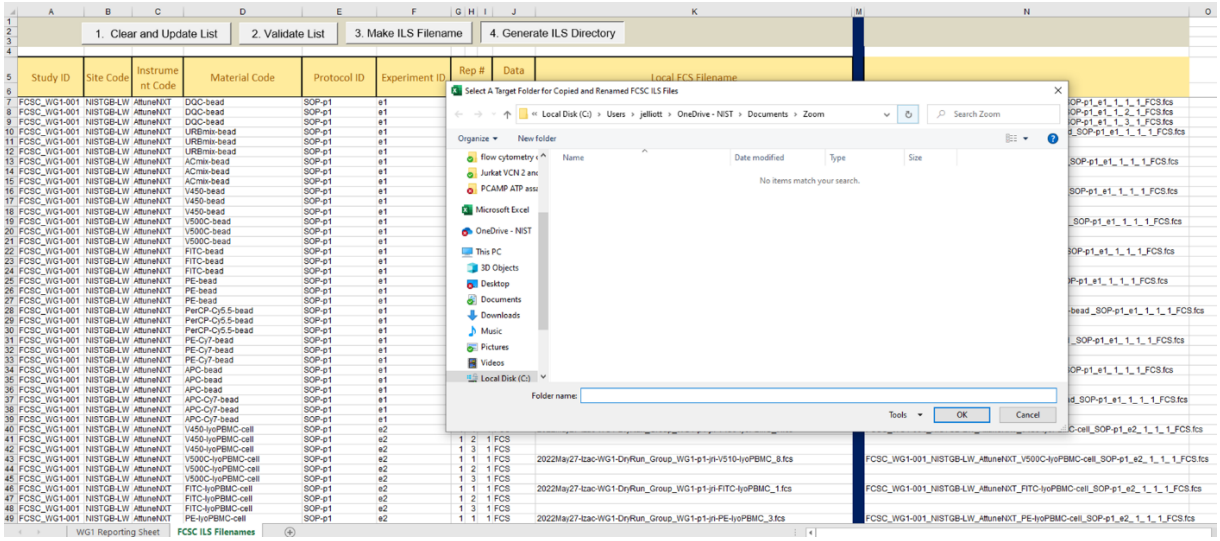
1. Once the WG1 reporting spreadsheet is completed, click on the “FCSC ILS Filenames” tab. This is shown in Circle 1, Figure 2.

Figure 2 - The FCSC ILS Filename Tab



2. Press the “1. Clear and Update List” Button. This will populate the spreadsheet with the local filenames and the relevant metadata to generate the standardized FCSC ILS filename. This is shown in Circle 2, Figure 2.
3. Press the “2. Validate List” Button. This checks that the data will generate unique ILS filenames with the appropriate parsers for each FCS file. Rows will be colored depending on metadata issues. Check the highlighted metadata cells to ensure accidental changes in data quality have not occurred. Colors in the local FCS file name in Column K) will indicate the present of improper parsers, duplicate or missing filenames. Functional standardized ILS filenames can still be generated after ensuring the spreadsheet values are correct. Repeat step 3 if re-validation is required.
4. Once data has passed validation (i.e. color highlights have been assessed), press the “3. Make ILS filenames” button. This will concatenate each the metadata terms and generate a standardized FCSC ILS filename for each row. The filename will be observed in column L “FCSC ILS filename”. Each of these names will be unique.
5. Press the “4. Generate ILS directory” button to generate a directory with a copy of the WG1 FCS files renamed with the standardized ILS filename. Pressing this button brings up a folder save dialog box (Figure 3). Identify where the ILS directory should be saved and press “OK”.
6. A new window will be opened showing the saved directory. Inside the directory, each FCS file and the corresponding reporting spreadsheet will be saved in the directory. This directory will be uploaded to NIST for bulk analysis by WG3.

Figure 3 - Folder Save Dialog Box for WG1 ILS Files



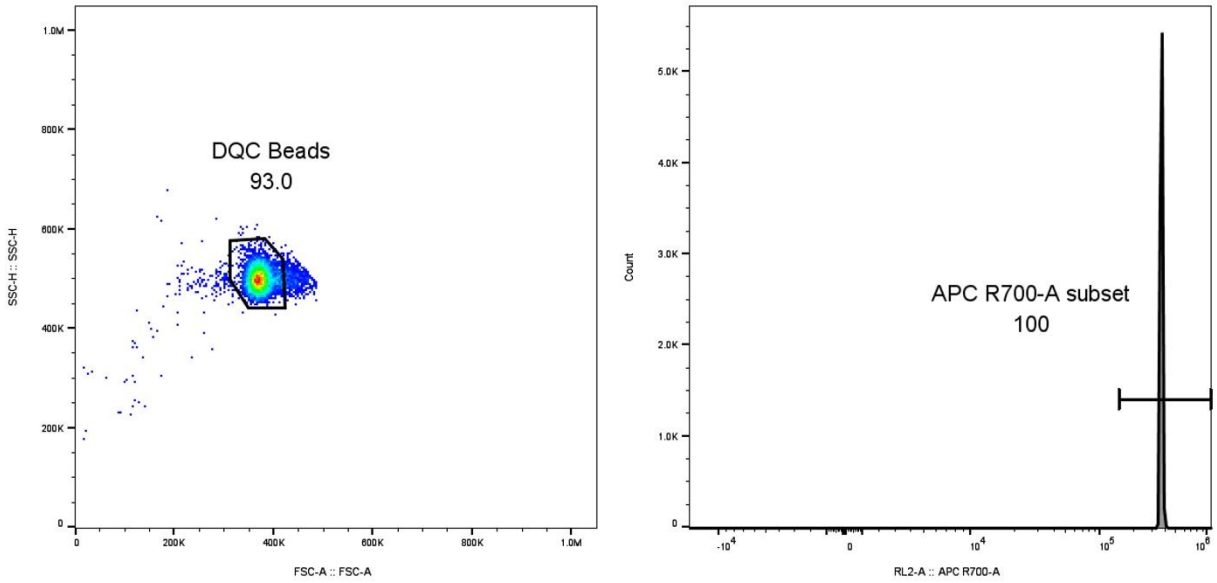
Appendix A - **CytoFLEX Ready to Use Daily QC (DQC) Fluorospheres**

Vendor: Beckman Coulter **Lot #** AL03 **Storage**: 2-8°C
Diluent: Milli Q Water – not supplied

Product Description: CytoFLEX Ready to Use Daily QC Fluorospheres is a suspension of ~3 μm fluorescent microspheres with a uniform size and fluorescence intensity, which may be used for daily verification of a flow cytometer's optical alignment and fluidics system. Fluorescence emission between 355 nm and 800 nm can be detected from the fluorospheres when excited at 355 nm, 375 nm, 405 nm, 488 nm, 561 nm and 638 nm. The DQC beads are suspended in an aqueous medium containing surfactants and stabilizers.

Experimental Procedure

1. Centrifuge the vial to allow the 250 μL of sample to collect at the bottom of the vial.
2. Vigorously vortex the DQC vial for 2-3 seconds.
3. Pipette 50 μL of DQC fluorospheres (~10^7 mL^{-1}) to a tube.
4. Add 450 μL of Milli Q Water to the tube (~10^6 mL^{-1}).
5. Vigorously vortex the tube for 2-3 seconds.
6. Run particle suspension on your flow cytometer using the tube as a sample holder or transfer the appropriate amount of sample to a well, depending on the sample format of your flow cytometer.
7. Repeat steps 2 – 6 to produce a total of 3 biological replicates (not from the same sample).



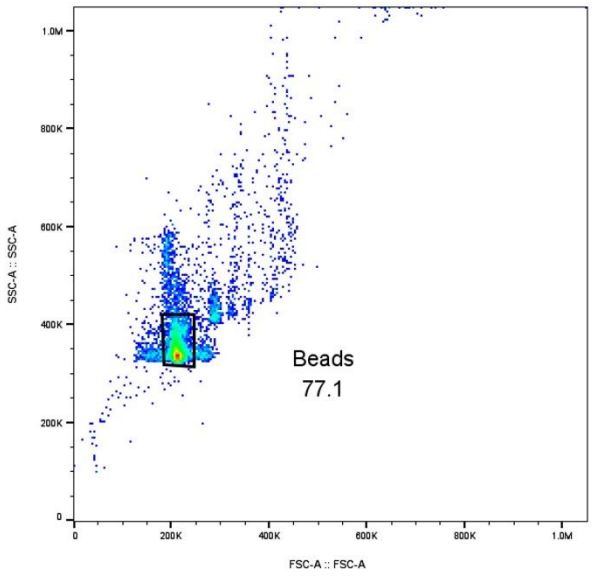
Appendix B - **SPHERO™ Ultra Rainbow Quantitative Particle (URQP) Kit**

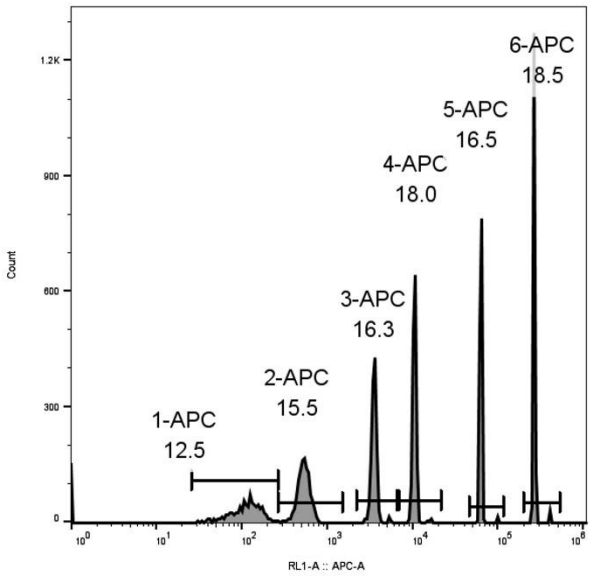
Vendor: Spherotech **Lot #** AP01 **Storage**: 2-8°C
Diluent: Milli Q Water – not supplied

Product Description: The kit contains 6 intensities of Ultra Rainbow Beads (URBs) with various intensities that have been assigned ERF (Equivalent Number of Reference Fluorophores) units by NIST. These beads are designed to accurately measure comparable data from different types of instruments at different locations. In addition, the URBs can be used for routine calibration of flow cytometers.

Experimental Procedure

1. Shake vigorously or vortex each URB bottle for 10 seconds before use.
2. Add 2 drops (~80 μL) from each dropper vial of beads (6 vials, each ~2 x 10^6 mL^{-1}) to a tube.
3. Then add 0.4 mL of Milli Q Water to the tube. (producing ~1.8 x 10^5 mL^{-1} for each intensity bead or ~1.1 x 10^6 mL^{-1} for the mixture of six beads)
4. Vortex briefly.
5. Run particle suspension on your flow cytometer using the tube as a sample holder or transfer the appropriate amount of sample to a well, depending on the sample format of your flow cytometer.
6. Repeat steps 1 – 5 to produce a total of 3 biological replicates (not from the same sample).





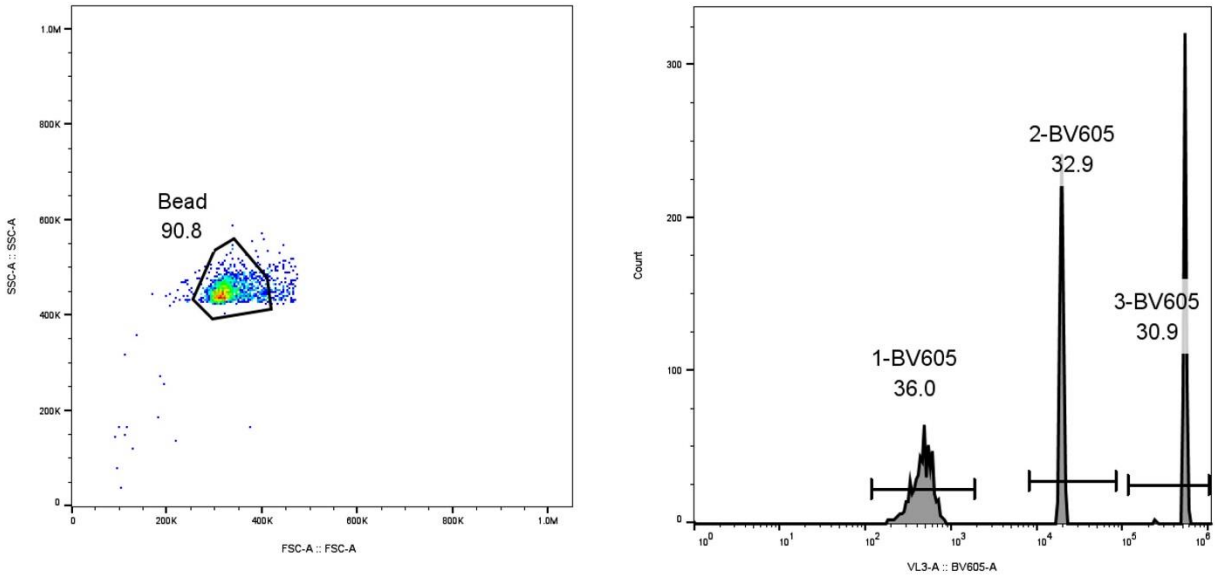
Appendix C - **AccuCheck (AC) ERF Reference Particles**

Vendor: Thermo Fisher Scientific **Lot #** 2335550 **Storage**: 2-8°C
Diluent: Milli Q Water – not supplied

Product Description: The Invitrogen™ AccuCheck ERF Reference Particles (Cat. No. A55950) are a mixture of three particles (all with ~3 μm diameters) with 3 different fluorescence intensities (low, medium and high) and assigned values in Equivalent Reference Fluorophores (ERF) units. The NIST-traceable standards are for individual channels within a fluorophore's selected bandpass filter resulting in accurate, quantitative and comparable flow cytometry fluorescence intensity measurements.

Experimental Procedure

1. Briefly vortex AccuCheck ERF Reference Particles to resuspend. Add one drop (~40 μL) of AccuCheck ERF Reference Particles (~6.7 x 10^5 mL^{-1} for each intensity bead) to a flow cytometry tube.
2. Add 1 mL of Milli Q Water. (producing ~2.7 x 10^4 mL^{-1} for each intensity bead or ~8.0 x 10^4 mL^{-1} for the mixture of three beads)
3. Vortex briefly to mix.
4. Run particle suspension on your flow cytometer using the tube as a sample holder or transfer the appropriate amount of sample to a well, depending on the sample format of your flow cytometer.
5. Repeat steps 1 – 4 to produce a total of 3 biological replicates (not from the same sample).



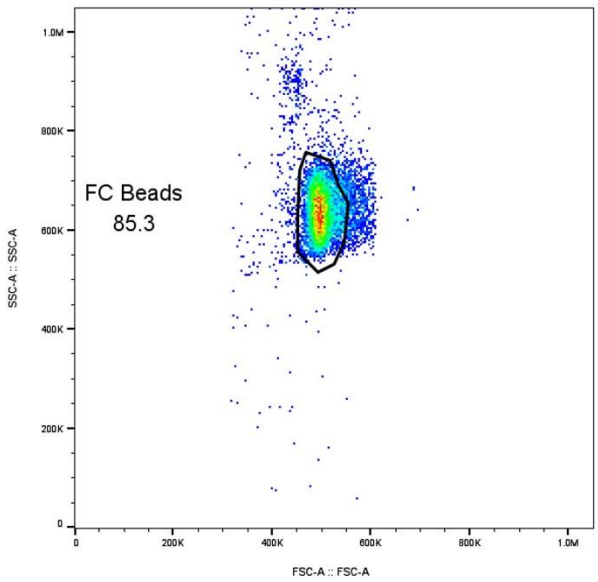
Appendix D - BD™ FC Beads

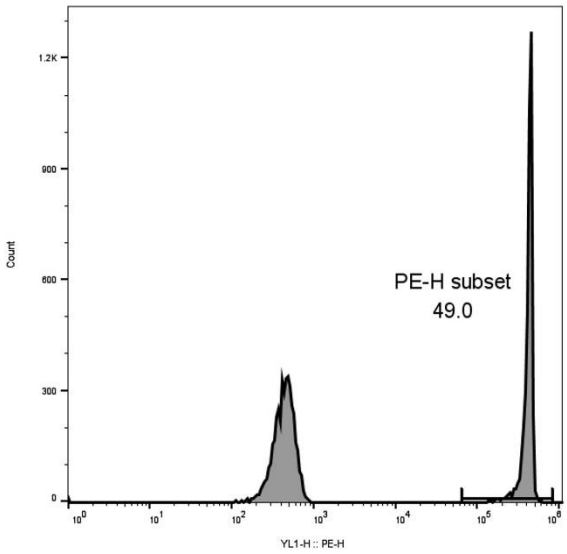
Vendor: BD **Lot #** 661623(V-450), 661624(V-500C), 661615(FITC), 661616(PE), 661619 (PerCP-C5.5), 661617(PE-Cy7), 661620(APC), 661622(APC-Cy7) **Storage**: 2-8°C
Diluent: buffer – supplied

Product Description: BD FC Beads are 3-µm polystyrene beads coupled to fluorochromes and dried down in single-use 12 × 75-mm tubes. Each tube comprises a mixture of positive beads and negative beads.

Experimental Procedure

1. Allow the 3 vials for each fluorophore to reach room temperature.
2. Place the 3 vials in a rack, protected from light.
3. Add 0.5 mL of BD FC Beads Dilution Buffer to each tube.
4. Vortex the tubes vigorously for 3 to 5 seconds to rehydrate the beads.
   Note: When rehydrated and protected from light, the beads are stable for
   - 1 hour at 18°C to 25°C
   - 4 hours at 2°C to 8°C
5. Vortex each tube of beads for 3 to 5 seconds just before sampling.
6. Run particle suspension on your flow cytometer using the tube as a sample holder or transfer the appropriate amount of sample to a well, depending on the sample format of your flow cytometer.
7. Repeat steps 5 and 6 to produce a total of 3 biological replicates (not from the same sample).





Appendix E - **Lyophilized Pre-stained Veri-Cells™ Plate**

Vendor: BioLegend **Lot #** **Storage**: 2-8°C
Diluent: reconstitution buffer – supplied, cell staining buffer – not supplied

Product Description: Lyophilized pre-stained Veri-Cells™ are provided as single use controls for use in flow cytometric analysis. Unopened pouches should be stored at 2°C - 8°C with desiccant pack, until expiration date listed on the package. After opening, we recommend using the plate immediately. Do not open sealed pouches until ready to use. We do not recommend storing opened packages for prolonged periods of time due to possibility of lyophilized pellets being exposed to moisture, which may affect product performance

Experimental Procedure

1. Remove unopened pouches from the refrigerator, and warm to ambient temperature for 10-15 minutes.
2. Open the pouch, remove the plate and centrifuge the plate at 500 × g for 1 minute at ambient temperature to allow the pellets to collect at the bottom of the wells before reconstituting.
3. Reconstitute the lyophilized pellet in 100ul of Lyo-PBMC Reconstitution Buffer (supplied) by puncturing through the easy-pierce seal with a pipette tip. Wait 5-10 minutes before proceeding to the next step.
4. Check visually that the pellet is completely dissolved then centrifuge plate at 500 x g for 1 minute at ambient temperature to ensure reconstituted reagent is at bottom of well before removing the pierced seal.
5. Carefully remove the seal by firmly grasping the plate on the bench top and slowly peeling back the pierced seal.
6. Samples can be acquired directly from the plate using an instrument with an HTS attachment or transferred to tubes for acquisition.
7. Before acquiring samples add 400 µL of cell staining buffer (not provided, 1x PBS with 2% fetal bovine serum, pH 7.4) to each sample.