WG2 SOP-03 TBMNK Assay

1. Overview

This Standard Operating Procedure (SOP) describes the sample processing steps for the TBMNK Assay to be used for the Working Group 2 (WG2) Inter-Laboratory Study (ILS). The TBMNK Assay is a two-tube immunofluorescent panel that includes a three-color counting tube and an eight-color immunophenotyping tube (Tables 1A and 1B) . In addition to fluorescent conjugated monoclonal antibodies (mAb) each tube probes for excluding non-viable and apoptotic cells.

The scope of the WG2 ILS includes the evaluation of three Test Samples (cryopreserved PBMC) and quality control (QC) material (synthetic TBMNK cells).

This SOP describes all steps related to the processing of the Test Samples and QC materials.

NOTE 1: This procedure should be conducted only after the processes described in WG2 SOP-1, Flow Cytometer Setup and Performance Characterization has been successfully completed.

NOTE 2: Given that the compensation matrices will be applied post-acquisition, this procedure may be conducted before or after the processes described in WG2 SOP-2, Compensation Setup have been completed.

An overview of the workflow is shown in Figure 1. Briefly, Test Samples will first be stained with a viability dye (LIVE/DEAD™ Fixable Aqua). Next; an aliquot of cells will be stained in singlicate with CD45 and Annexin V in a Trucount™ tube using a No Wash method (Table 1A). The remaining cells will be stained in triplicate with fluorophore labeled mAb and Annexin V followed by a washing step (Table 1B). For all three Test Samples the Counting Tube will be prepared in singlicate while the Immunophenotyping Tube will be prepared in triplicate. Nine FMO tubes will be prepared in singlicate from a single Test Sample. It is not necessary to prepare FMO tubes for each Test Sample (Table 5).

NOTE 3*:* A single set of FMO tubes from one of the donor cryoPBMCs will be sufficient.

NOTE 4*:* Left-over cryoPBMC from the Test Samples can be used for preparation of cell-based compensation controls as described in the SOP-02.

The synthetic TBMNK cells (TruCytes TBMNK) will serve as the QC material and will be processed as described above with the exception that the viability and Annexin V staining and FMO controls will not be included; and the full panel will be stained in singlicate (Figure 1).

All samples may be processed on the same day or over a period of different testing days so long as the span of the testing days does not exceed two weeks. Please review the notes in SOP-01 for instrument gain settings across different experimental days.

A local data file naming scheme to be used for saving the .fcs files should be developed ahead of time. The process for naming files to for transfer to NIST and the file transfer process are described in Sections 4.6 and 4.7.

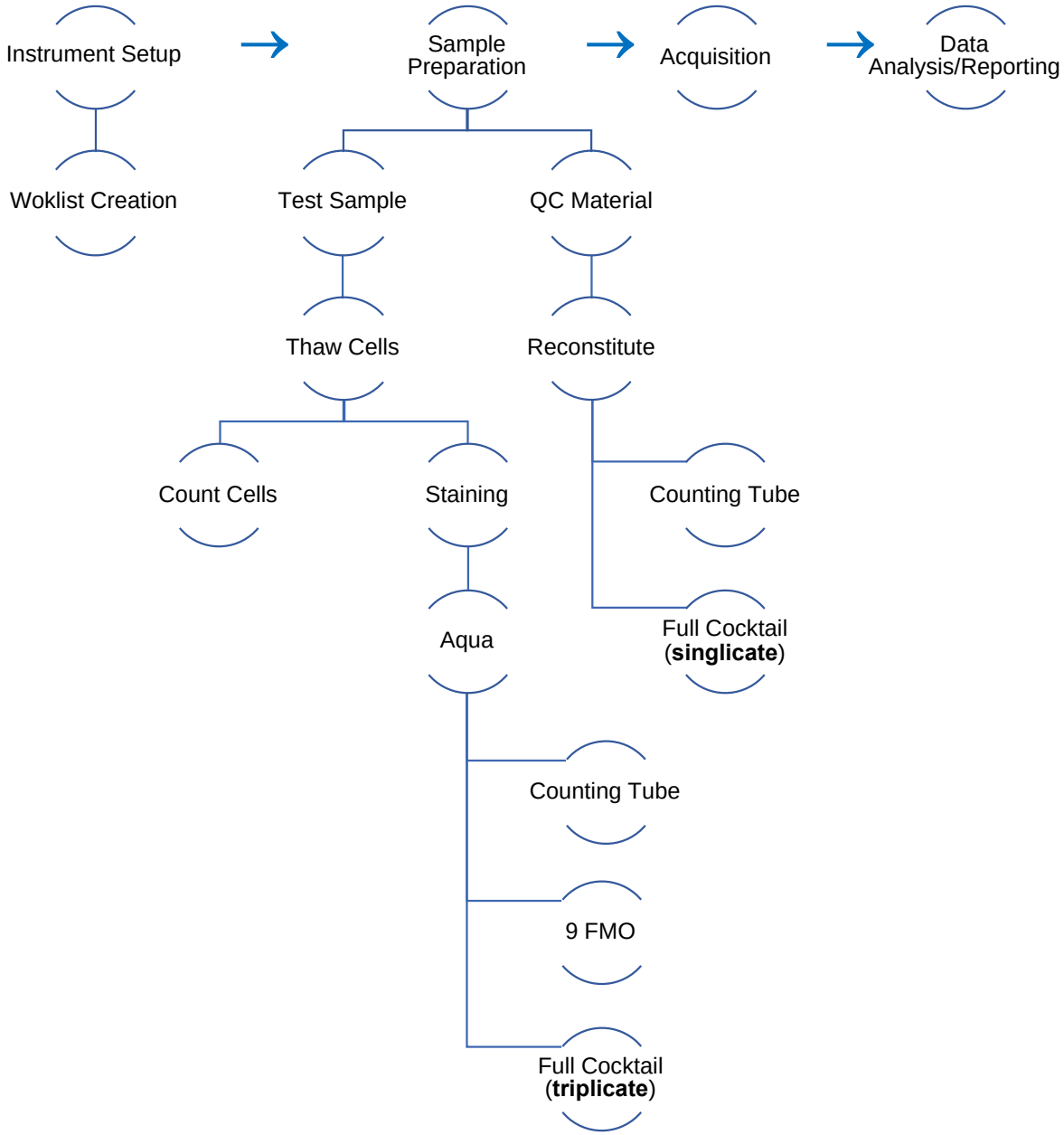


Figure 1. Working Group 2 Inter-Laboratory Study TBMNK Assay Workflow

Table 1A. TBMNK Panel Configuration- Counting Tube

Laser	Violet	Violet	Blue	Blue or Yellow Green	Blue	Blue or Yellow Green	Red	Red
	405mn	405mn	488nm	488nm	488nm	488nm	640nm	640nm
Approximate Filters	448/45	528/45	527/32	586/42	700/54	783/56	660/10	783/56
Probe	Pacific Blue/ Annexin V	Aqua/ cellular amines	-	-	PerCP-Cy5.5	-	APC	-
Marker	Phospha-tidylserine	Non-viable cells	-	-	CD45	-	Counting Beads*	-
Clone	N/Ap	N/Ap	-	-	2D1	-	N/Ap	-

*Counting beads can be detected in many different channels.

Table 1B. TBMNK Panel Configuration- Immunophenotyping Tube

Laser	Violet	Violet	Blue	Blue or Yellow Green	Blue	Blue or Yellow Green	Red	Red
	405mn	405mn	488nm	488nm	488nm	488nm	640nm	640nm
Approximate Filters	448/45	528/45	527/32	586/42	700/54	783/56	660/10	783/56
Probe	Pacific Blue/ Annexin V	Aqua/ cellular amines	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7
Marker	Phospha-tidylserine	Non-viable cells	CD3	CD16/CD56	CD45	CD4	CD19/CD14	CD8
Clone	N/Ap	N/Ap	SK7	3G8/HCD56	2D1	SK3	SJ25C1/M5E2	SK1

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.

3. Reagents and Materials

3.1. Reagents

Table 2. Test Samples and QC Material

Name	Description	Vendor	Cat #	Storage Conditions	Preparation	Supplied by
Cryopreserved PBMC*	Test Sample	AllCells	LP, CR, MNC, 10M Donor #1: 3063593 Donor #2: 3066774 Donor #3: 3069118	Liquid nitrogen vapor phase	See below	NIST
TruCytes TBMNK	QC	Slingshot	SSB-14-A	-20°C	See below	NIST

*Donor # of cryoPBMC shall be consistent between experiment(s) and study reporting spreadsheet.

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	900005608	CD19	APC	SJ25C1	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	900005610	CD56	PE	HCD56	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
RPMI-1640	Any	N/Ap	2-8°C	Ready to use	Testing Laboratory to purchase
PBS without Ca^{++}/Mg^{++}	GIBCO/ any	10010-023 or equivalent	Unopened RT (18-25°C) Opened 2-8°C	Ready to use	Testing Laboratory to purchase
FBS	Any	N/Ap	Unopened -20°C Opened at 2-8°C	Ready to use	Testing Laboratory to purchase
Penicillin-Streptomycin (10,000 U/mL)	Any	N/Ap	Unopened at -20°C Opened at 2-8°C	Ready to use	Testing Laboratory to purchase
Trypan Blue Solution 0.2%	Any	N/Ap	2-8°C	Ready to use	Testing Laboratory to purchase
Ethanol, e.g. 200 proof	Any	N/Ap	RT (18-25°C)	See below	Testing Laboratory to purchase
$CaCl_2$	Any	N/Ap	RT (18-25°C)	See below	Testing Laboratory 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
Trucount™ Tubes (# beads/tube: 46800)	BD Biosciences	340334	2-8 °C	Ready to use	NIST
Type II water, or better	N/Ap	N/Ap	Per Testing Laboratory's SOP	N/Ap	Testing Laboratory to purchase

3.2. Supplies

- Staining Tubes: 5 mL polystyrene round-bottom tubes w/o snap caps
- Pipet tips (P10, P20, P100, P200, P1000)
- 15 mL and 50 mL polypropylene conical tubes with screw caps
- 96-well plates, per your institution's SOP (for testing labs that stain in plates, only)
- Sterile serological pipettes, as required (e.g., 2 mL, 5 mL, 10 mL, 25 mL)

3.3. Equipment

- Pipettors P10, P20, P100, P200, P1000
- Pipette-aid
- Water Bath
- Cell counting equipment
- Centrifuge with swinging bucket rotor
- Biosafety Cabinet, class II, appropriate for BSL-2 per your institution's policies
- Your institution's cytometer(s), as described in Study Plan WG2-01, Appendix A

3.4. Reagent Preparation

70% Ethanol

Prepare per your institution's SOP.

RPMI-1640 Complete Cell Culture Media

Prepare a solution of RPMI-1640 containing a final volume of 10% FBS and 1% Penicillin-Streptomycin per your institution's SOP.

Staining Buffer

Prepare a solution of 1X PBS, 3.5 mM $CaCl_2$, containing a final volume of 2% FBS, pH 7.4, per your institution's SOP.

LIVE/DEAD™ Fixable Aqua Dead Cell Stain

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) of Aqua Live/Dead Staining kit to room temperature 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 the dye solution in dark at RT and use it within 2 hours of reconstitution.

FMO Cocktails

For Test Samples label nine polypropylene tubes FMO-TS-1, FMO-TS-2…. FMO-TS-9 as indicated in Table 5

NOTE 5: Note that FMO controls will NOT be used for the QC material.

The mAb have been titrated and bottled so that 5 µL of each mAb is used for optimal staining.

Calculate the total number of samples to be processed per analytical run (n).

Prepare enough cocktail for n+1 samples.

> ***Example***
>
> Number of samples = 3
> n+1=4
> Add 20 µL (i.e., 4 × 5 µL) of each mAb EXCEPT for one as indicated in Table 5 to the appropriate tube.

Keep at 2-8 °C protected from light. Use within 60 minutes of preparation.

Immunophenotyping Tube Cocktail

For the Test Sample, label a polypropylene tube FC-TS-# as indicated in Table 6.

For the QC material, label a polypropylene tube FC-QC as indicated in Table 8.

The mAbs have been titrated and bottled so that 5 µL of each mAb is used for optimal staining.

Calculate the total number of samples to be processed per analytical run (n).

Prepare enough cocktail for n+1 samples.

> ***Example***
> Number of samples = 3
> n+1=4
> Add 20 µL (i.e., 4 × 5 µL) of each mAb to a polypropylene tube labeled FC

Keep at 2-8 °C protected from light. Use within 60 minutes of preparation.

Counting Tube Cocktail

For the Test Sample, label a polypropylene tube TS-TC-# as indicated in Table 7.

For the QC material, label a polypropylene tube QC-TC as indicated in Table 9. The mAbs have been titrated and bottled so that 5 μL of each mAb is used for optimal staining.

Calculate the total number of samples to be processed per analytical run (n).

Prepare enough cocktail for n+1 samples.

Example

Number of samples = 3

n+1=4

Add 20 μL (i.e., 4 × 5 μL) of each mAb to a polypropylene tube labeled FC

Keep at 2-8 °C protected from light. Use within 60 minutes of preparation.

Table 5. Test Sample FMO Staining Map. NOTE: Cells are prelabeled with Aqua. EXCEPT for FMO-TS-1 which will use PBMC not prelabeled with Aqua. No additional Aqua will be added to the FMO Staining Cocktails.

Tube #	Material Code	Aqua	CD3	CD4	CD8	CD14	CD16	CD56	CD19	CD45	Annexin
FMO-TS-1	cryoPBMC-AqLD-fmo	-	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL
FMO-TS-2*	cryoPBMC-FITC-fmo	1 μL	-	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL
FMO-TS-3*	cryoPBMC-PE-Cy7-fmo	1 μL	5 μL	-	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL
FMO-TS-4*	cryoPBMC-APC-Cy7-fmo	1 μL	5 μL	5 μL	-	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL
FMO-TS-5*	cryoPBMC-APC-CD14-fmo	1 μL	5 μL	5 μL	5 μL	-	5 μL	5 μL	5 μL	5 μL	5 μL
FMO-TS-6*	cryoPBMC-PE-fmo	1 μL	5 μL	5 μL	5 μL	5 μL	-	-	5 μL	5 μL	5 μL
FMO-TS-7*	cryoPBMC-APC-CD19-fmo	1 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	-	5 μL	5 μL
FMO-TS-8*	cryoPBMC-PerCP-Cy5.5-fmo	1 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	-	5 μL
FMO-TS-9*	cryoPBMC-AxPB-fmo	1 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	5 μL	-

Table 6. Test Sample Immunophenotyping Tube Staining Map. Cells are prelabeled with Aqua. No additional Aqua will be added to the FC Staining Cocktail. Number sign is Test Sample number. Three replicates are prepared for each donor.

Tube #	Material Code	CD3	CD4	CD8	CD14	CD16	CD56	CD19	CD45	Annexin
FC-TS-#	panel1-cryoPBMC-#	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL

Table 7. Test Sample Counting Tube Staining Map. Cells are prelabeled with Aqua and added to a Trucount tube. No additional Aqua will be added to the Cell Counting Staining Cocktail. Number sign is Test Sample number. Two replicates are prepared for each donor.

Tube #	Material Code	CD45	Annexin
TS-TC-#	AqLD-TruCount-CD45-AxPB-cryoPBMC-#	5 µL	5 µL

Table 8. QC Immunophenotyping Tube Staining Map. No Aqua or Annexin will be used for the QC FC synthetic cell Trucytes Staining Cocktail. Number sign is QC material number. Three replicates are prepared, one replicate per vial of QC material.

Tube #	Material Code	CD3	CD4	CD8	CD14	CD16	CD56	CD19	CD45
FC-QC	panel1-TruCytes-#	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL	7.5 µL

Table 9. QC Counting Tube Staining Map. No Aqua or Annexin will be used for the QC FC synthetic cell TruCytes Staining Cocktail. Two replicates are prepared, one replicate per vial of QC material.

Tube #	Material Code	CD45
QC-TC	TruCount-CD45-TruCytes-#	7.5 µL

4. Procedure

4.1. Instrument Setup

Perform the daily instrument calibration per your institution's SOP.

4.2. Worklist Creation

Create a worklist containing sample tubes with appropriate local file names provided in the 'Controls' and 'Samples' of the WG2 Reporting Spreadsheet. Cell staining samples include a non-viable cell control and FMO controls in 'Controls' sheet as well as cell and synthetic cell samples in the 'Samples' sheet.

4.3. Test Sample Preparation

4.3.1. PBMC Thawing

Proper aseptic technique should be used when handling and manipulating cells. Perform in biosafety cabinet. Cells are to be thawed as rapidly as possible. A high concentration of DMSO can be toxic to the cells at room temperature or at 37°C.

- Warm RPMI-1640 Complete Cell Culture Media to room temperature (18-25°C).
- Removed vials from liquid nitrogen storage and keep on dry ice until ready for thawing.
- Check that the vial is tightly capped and place in a 37°C water bath.
- After 60 seconds at 37°C, remove the vial from the water bath every 15-20 seconds and gently invert 3-4 times to check the level of thawing.
- When the vial contents are 50-75% liquid, remove from the water bath and continue to gently invert the vial until the entire contents are liquid.
- Place the vial on wet ice and transport to a biosafety cabinet.
- Wipe the outside of the vial with an alcohol wipe and gently invert 5 times to mix the cells.
- Check to see if there is liquid caught up in the cap. If so, gently tap the upright vial on a benchtop to force the liquid into the vial.
- Transfer quickly the vial contents to a 15 mL tube at RT (18-25°C).
- Add 1 mL of media to rinse the vial and transfer any remaining cells to the 15 mL tube.
- Add 10 mL media dropwise (about 1 mL per second) to the cells. Securely cap the tube and gently invert 3-5 times after adding all the media.
- Centrifuge the tube at $200 \times g$ for 10 minutes at RT (18-25 C).
- Aspirate the supernatant without disturbing the pellet and resuspend the cells in 10 mL media, e.g., 1 mL first to resuspend cells followed by addition of 9 mL media.

- Remove a small aliquot of cell suspension (≤ 50 µL) to perform cell counting and viability assessment per your institution's SOP, for example using a hemocytometer and trypan blue or automated cell counting/viability instrument.
- Centrifuge at 350 × *g* for 5 minutes at RT (18-25°C) and resuspend in an appropriate volume of PBS to achieve cell concentration of 1 x 10^6/mL.
- **NOTE 6:** Aqua Dead Cell must not be added to FMO-TS-1. Reserve 0.5x10^6 cells for FMO-TS-1 before the last step of centrifugation and cell suspension in PBS.
- **NOTE 7:** If cells are to be used for compensation controls as described in WG2 SPO-2, reserve appropriate number of cells prior to the last step, centrifuge and resuspend cells in the staining buffer instead of PBS.
- **NOTE 8: Participants need to re-assess the cell number/volume for Section 4.3.2.**

4.3.2. Test Sample Aqua Dead Cell Stain

- Add 10 mL of cells (1 x 10^6/mL) from Section 4.3.1 to a 15 mL polystyrene tube.
- Add 10 µL reconstituted Aqua dye (see Section 4). Incubate for 30 minutes at RT, protected from light.
- Centrifuge at 350 × *g* for 5 minutes. Aspirate the supernatant. Resuspend the cell pellet. Add 3 mL Staining Buffer.
- Centrifuge at 350 × *g* for 5 minutes.
- Aspirate the supernatant. Resuspend the cell pellet. Add 1 mL Staining Buffer.

4.3.3. Test Sample FMO Staining (exp ID=e1)

- For ONE of the Test Samples, label 9 tubes with the Test Sample ID and FMO-TS-1, FMO-TS-2…. FMO-TS-9 (see Table 5)
- **For tube FMO-TS-1 add 100 µL of thawed PBMC which were not labeled with Aqua (see Section 4.3.1).** **Note 9**: The rest of thawed PBMC in PBS from Section 4.3.2 shall be centrifuged at 350 × *g* for 5 minutes at RT (18-25°C) and resuspended in the staining buffer to achieve cell concentration of 1 x 10^6/mL for other usages such as FMO-TS-1 and cryoPBMC compensation tubes in SOP-02.

- For FMO-TS-2, FMO-TS-3…. FMO-TS-9 tubes, add 50 µL cells (prepared in Section 4.3.2, i.e. Aqua labeled) and 50 µL staining buffer to each tube.
- Add required volumes of antibodies provided in Table 5 or 35-45 µL of the appropriate FMO-TS cocktail cocktail to each tube.
- Mix well by gentle vortex.
- Incubate at RT (18-25°C) protected from light for 30 minutes.
- Add 2 mL of Staining Buffer.
- Centrifuge at 350 × g for 5 minutes at RT (18-25°C). Aspirate the supernatant. Resuspend the cell pellet. Add 2 mL Staining Buffer.
- Centrifuge 350 × g for 5 minutes at RT (18-25°C). Aspirate the supernatant. Resuspend the cell pellet.
- Add 500 µL of Staining Buffer.
- Hold at 2-8 °C, protected from light if not acquired on the cytometer immediately.
- Acquire on the instrument with 60 minutes of preparation.
- Vortex the thoroughly (at low speed) immediately prior to acquisition.

4.3.4. Test Sample Immunophenotyping Tube Staining (exp ID=e1)

NOTE 10: The Test Sample Immunophenotyping Tube will be stained in triplicate.

- For EACH Test Sample, label 3 tubes with the Test Sample ID and FC-TS-1, FC-TS-2, FC-TS-3 (see Table 6).
- For FC-TS-1, FC-TS-2, FC-TS-3 tubes, add 100 µL cells (prepared in Section 4.3.2, i.e. Aqua labeled) to each tube.
- Add required volumes of antibodies provided in Table 6 or 45 µL of the appropriate FC-TS cocktail to each tube.
- Mix well.
- Incubate at RT (18-25°C) protected from light for 30 minutes.
- Add 2 mL of Staining Buffer.
- Centrifuge at 350 × g for 5 minutes at RT (18-25°C). Aspirate the supernatant. Resuspend the cell pellet. Add 2 mL Staining Buffer.
- Centrifuge 350 × g for 5 minutes at RT (18-25°C). Aspirate the supernatant. Resuspend the cell pellet.

- Add 500 µL of Staining Buffer.
- Hold at 2-8 °C, protected from light if not acquired on the cytometer immediately.
- Acquire on the instrument with 60 minutes of preparation.
- Vortex the thoroughly (at low speed) immediately prior to acquisition.

4.3.5. Test Sample Counting Tube Staining (exp ID=e2)

NOTE 11: The Test Sample Counting Tube will be stained in singlicate.

– For each Test Sample, label 1 Trucount™ tube with the Test Sample ID and TS-TC (Table 7).

– Add 100 µL cells (prepared in Section 4.3.2) to the appropriate Trucount™ tube.

– Add 5 µL of CD45 PerCP-Cy5.5 and 5 µL of Annexin V Pacific Blue (or 10 µL counting tube cocktail).

– Vortex gently to mix.

– Incubate at RT protected from light for 30 minutes.

– Add 500 µL of Staining Buffer. Cap the tube and vortex gently to mix.

– Hold at 2-8 °C, protected from light if not acquired on the cytometer immediately.

– Acquire on the instrument with 60 minutes of preparation.

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

4.4. QC/Synthetic cell TruCytes Preparation

4.4.1. TruCytes TBMNK Synthetic Cell Reconstitution

– Add 105 µL of Type II sterile water to the vial. Let sit at RT (18-25°C) for 15 minutes. Mix by gentle pipetting just prior to use.

– After reconstitution, store at 2-8°C for up to seven days.

4.4.2. QC Synthetic Cell Counting Tube Staining (exp ID=e3)

NOTE 12: The QC material Counting Tube will be stained in singlicate. PBS buffer is preferred for handling QC material instead of the staining buffer that might cause aggregation around QC material. Additionally, the detection threshold could be lowered to ensure the inclusion of all lymphocytes due to the low refractive index of the lymphocytes.

- Label 1 Trucount™ tube as QC-TC (Table 9).
- Add 100 µL synthetic cells (prepared in Section 4.4.1) to the Trucount™ tube.
- Add 7.5 µL of CD45 PerCP-Cy5.5.
- Vortex gently to mix.
- Incubate at RT protected from light for 30 minutes.
- Add 500 µL of PBS. Cap the tube and vortex gently to mix.
- Hold at 2-8 °C, protected from light if not acquired on the cytometer immediately.
- Acquire on the instrument within 60 minutes of preparation.
- Vortex the thoroughly (at low speed) immediately prior to acquisition.

4.4.3. QC Synthetic Cell Immunophenotyping Tube Staining (exp ID=e4)

NOTE 13: The QC material Immunophenotyping Tube will be stained in singlicate. PBS buffer is preferred for handling QC material instead of the staining buffer that might cause aggregation around QC material. Additionally, the detection threshold could be lowered to ensure the inclusion of all lymphocytes due to the low refractive index of the lymphocytes.

- Label a single tube as QC-FC (Table 8). Note that the QC synthetic cells from each vial will be stained in singlicate.
- Add 100 µL synthetic cells (prepared in Sections 4.4.1).
- Add required volumes of antibodies provided in Table 8.
- Mix well.
- Incubate at RT (18-25°C) protected from light for 30 minutes.
- Add 2 mL of PBS.
- Centrifuge at 500 × g for 8 minutes at RT (18-25°C). Aspirate the supernatant. Resuspend the cell pellet. Add 2 mL PBS.

- Centrifuge 500 × *g* for 8 minutes at RT (18-25°C). Aspirate the supernatant. Resuspend the cell pellet.
- Add 500 µL of PBS.
- Hold at 2-8 °C, protected from light if not acquired on the cytometer immediately.
- Acquire on the instrument with 60 minutes of preparation.
- Vortex the thoroughly (at low speed) immediately prior to acquisition.

4.5. Acquisition

- Create an acquisition template for the TBMNK Test Samples and a second acquisition template for the TBMNK QC. Note: Plots from the gating hierarchy provided in SOP-04 can serve as acquisition templates.
- 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 sample types if needed.
- The threshold should be set on the PerCP-Cy5.5 channel so that most of the debris is excluded from the CD45+ events.
- Acquire samples WITHOUT compensation and with a minimum of 50,000 events in the CD45+ gate. Note that compensation will be applied post-acquisition.
- **Note 14**: For acquisition of QC material, carefully adjust gains for FSC and SSC to minimize the loss of synthetic cells due to the low refractive index of the synthetic lymphocyte population (See examples provided in Appendix C and D in SOP-04).

4.6. Local Filename Entry on the WG2 Test and QC Samples Reporting Spreadsheet

- Open the WG2 Reporting Workbook and select the "Test and QC Samples" 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.
- For each FCS file collected (29 total files) find the corresponding material code row using the terms in "Column D" and description in "Column C". See Figure 2.
- Identify the associated "Get Local FCS Filename" button in Column H to select the local FCS file location for Column G (directory is stored in hidden Column I). See Figure 2.

- Press the button and use the file dialog box to identify the local FCS filename corresponding to the files collected in Section 4.5.

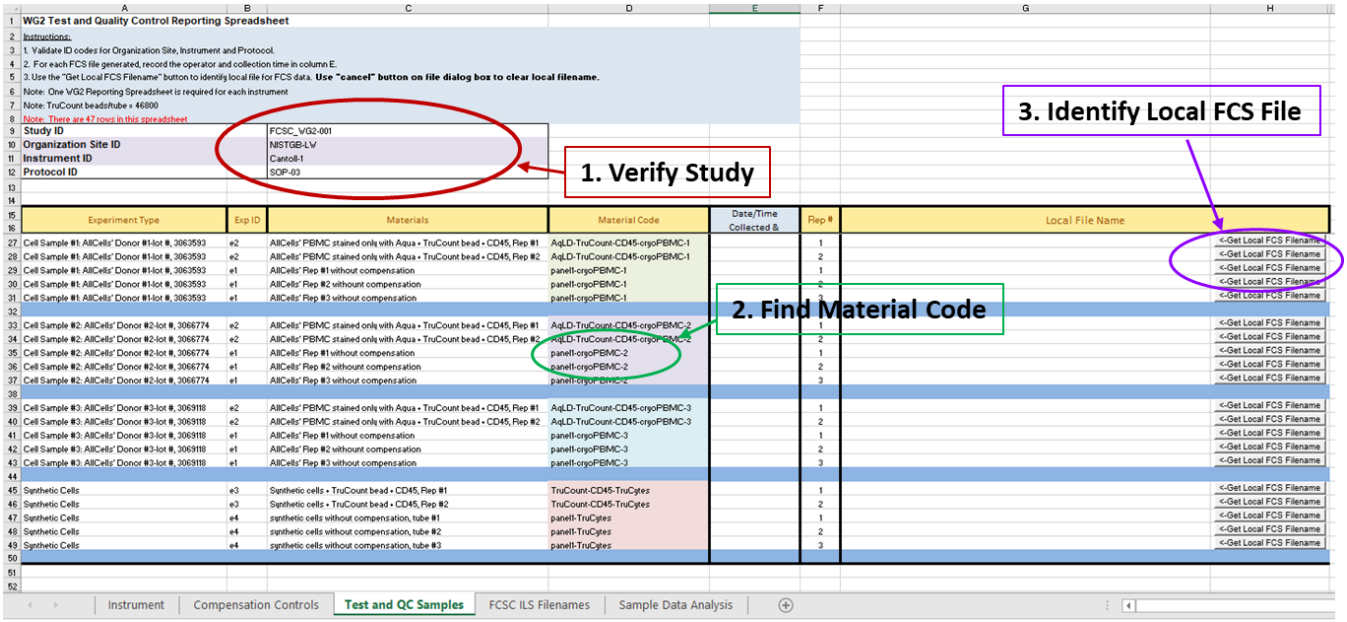


Figure 2. Test and QC Samples Reporting Spreadsheet.

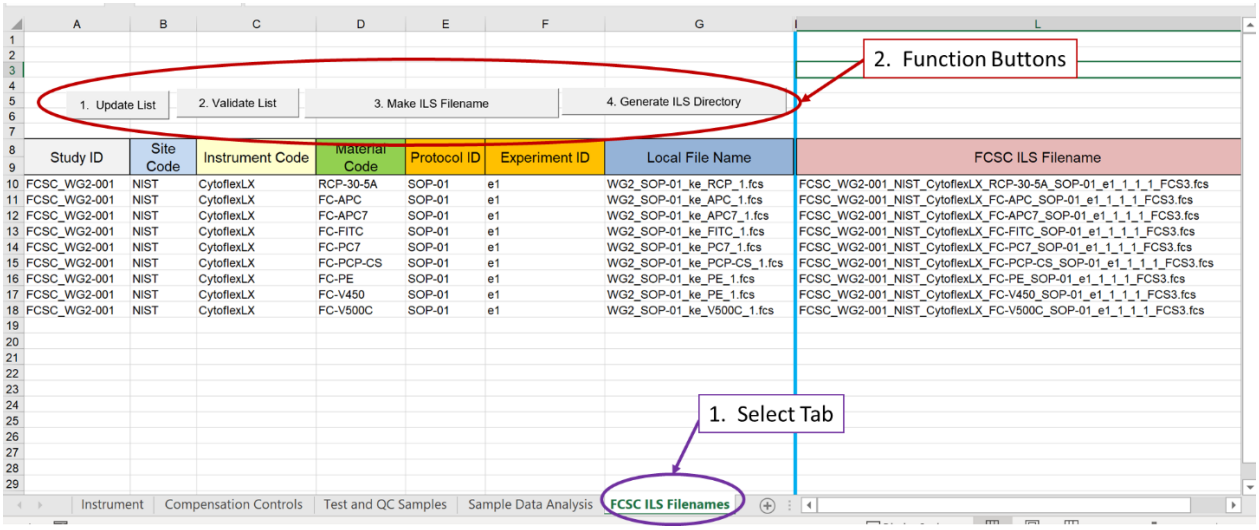
4.7. Generate FCSC Files with Standardized File Names

- Once the files have been collected during SOP-01, SOP-02 and SOP-03 have been recorded in the WG2 reporting spreadsheets (Instrument, compensation control, Test and QC Samples Tabs), select the "FCSC ILS Filenames" tab. This is shown in Figure 3.
- Press the "1. Clear and Update List" Button. This will clear the spreadsheet and transfer the local FCS filenames and the relevant metadata from each of the data collection worksheets to the FCSC ILS Filenames spreadsheet.
- Press the "2. Validate List" Button. This checks that the data transferred will result in the generation of unique ILS filenames for each FCS file. Rows will be highlighted if non-unique FCSC ILS filename or missing metadata are detected. Cells will also be highlighted if they have space or underline characters. Metadata in the highlighted cells will need to be manually checked and corrected in the spreadsheets.

  NOTE 15: If other FCS files from protocol or technical replicates are generated, additional instructions are required for file inclusion. See study organizers.
- Once data has passed validation, press the "3. Make ILS filename" button. This will concatenate each of the metadata terms and generate a standardized FCSC

ILS filename for each row. The filename will be observed in column N "FCSC ILS filename". Each of these names will be unique and in the FCSC ILS filenaming format.

- Press the "4. Generate ILS Directory" button to generate a directory that contains copies of the WG2 FCS files renamed with the standardized ILS filename. Pressing this button brings up a filesave dialog box populated with a predefined directory generated from the study metadata. Identify where the ILS directory should be saved and press "OK".
- A directory window will automatically appear with the new FCS data with the standardized filenames. Verify the correct FCS files with the new filenames were written to the directory.
- ***NOTE 16:*** This directory and included files will be used for bulk raw data analysis in WG3.



Study ID	Site Code	Instrument Code	Material Code	Protocol ID	Experiment ID	Local File Name	FCSC ILS Filename
FCSC_WG2-001	NIST	CytoflexLX	RCP-30-5A	SOP-01	e1	WG2_SOP-01_ke_RCP_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_RCP-30-5A_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-APC	SOP-01	e1	WG2_SOP-01_ke_APC_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-APC_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-APC7	SOP-01	e1	WG2_SOP-01_ke_APC7_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-APC7_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-FITC	SOP-01	e1	WG2_SOP-01_ke_FITC_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-FITC_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-PC7	SOP-01	e1	WG2_SOP-01_ke_PC7_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-PC7_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-PCP-CS	SOP-01	e1	WG2_SOP-01_ke_PCP-CS_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-PCP-CS_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-PE	SOP-01	e1	WG2_SOP-01_ke_PE_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-PE_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-V450	SOP-01	e1	WG2_SOP-01_ke_PE_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-V450_SOP-01_e1_1_1_1_FCS3.fcs
FCSC_WG2-001	NIST	CytoflexLX	FC-V500C	SOP-01	e1	WG2_SOP-01_ke_V500C_1.fcs	FCSC_WG2-001_NIST_CytoflexLX_FC-V500C_SOP-01_e1_1_1_1_FCS3.fcs

Figure 3. FCSC ILS Filename generation spreadsheet

5. Attachments

- AllCells Thawing Protocol
- Slingshot TDS for TruCytes TBMNK