Supplemental Table 1. *List of populations that were quantified as a percentage of the indicated parent population in each of the 6 DuraClone panels.*

	PARENT POPULATION	% GATED
BASIC	CD45+/ PBMCs	lymphocytes
	lymphocytes	T cells (CD3+)
	lymphocytes	B cells (CD19+)
	lymphocytes	NK cells (CD3-CD56+)
	lymphocytes	NKT cells (CD3+CD56+)
	T cells	CD4+ T cells
	T cells	CD8+ T cells
	T cells	DN T cells
	NK cells	CD56++ NK cells
	PBMCs	CD14+ (monocytes)
	CD14+ monocytes	CD14++CD16+
	CD14+ monocytes	CD14++CD16-
	CD14+ monocytes	CD14+CD16+
	CD14+ monocytes	CD64++CD16+

	PARENT POPULATION	% GATED
TCR	CD45+/ PBMCs	CD3+
	CD3+	TCRαβ+
	TCRαβ+	CD4 Tαβ+
	CD4 Tαβ+	naive (CD45RO-)
	CD4 Tαβ+	memory (CD45RO+)
	TCRαβ+	CD8+ Tαβ+
	CD8 Tαβ+	naive (CD45RO-)
	CD8 Tαβ+	memory (CD45RO+)
	Tαβ+	DN Tαβ+ (CD4-CD8-)
	CD3+	Tγδ+ cells
	TCRγδ+	CD4+ Tγδ
	TCRγδ+	CD8+ Tγδ
	TCRγδ+	DN Tγδ cells

	PARENT POPULATION	% GATED
T-ACT	CD45+/ PBMCs	CD3+
	CD3+	CD4+
	CD3+CD4+	CD28+CD27+
	CD3+CD4+	CD28+CD27-
	CD3+CD4+	CD28-CD27+
	CD3+CD4+	CD28-CD27-
	CD3+CD4+	HLADR+CD45RA+
	CD3+CD4+	HLADR+CD45RA-
	CD3+CD4+	HLADR-CD45RA+
	CD3+CD4+	HLADR-CD45RA-
	CD3+CD4+	CD57+
	CD3+CD4+	CD28+CD57+
	CD3+CD4+	CD28+CD57-
	CD3+CD4+	CD28-CD57+
	CD3+CD4+	CD28-CD57-
	CD3+CD3+	CD8+
	CD3+CD8+	CD28+CD27+
	CD3+CD8+	CD28+CD27-
	CD3+CD8+	CD28-CD27+
	CD3+CD8+	CD28-CD27-
	CD3+CD8+	HLADR+CD45RA+
	CD3+CD8+	HLADR+CD45RA-
	CD3+CD8+	HLADR-CD45RA+
	CD3+CD8+	HLADR-CD45RA-
	CD3+CD8+	CD57+
	CD3+CD8+	CD28+CD57+
	CD3+CD8+	CD28+CD57-
	CD3+CD8+	CD28-CD57+
	CD3+CD8+	CD28-CD57-

	PARENT POPULATION	%GATED
T-MEM-REG	CD45+/ PBMCs	CD3+
	CD3+	CD4+
	CD3+CD4+	CCR7+CD45RA+
	CD3+CD4+	CCR7+CD45RA-
	CD3+CD4+	CCR7-CD45RA+
	CD3+CD4+	CCR7-CD45RA-
	CD3+CD4+	CD62L+CD45RA+
	CD3+CD4+	CD62L+CD45RA-
	CD3+CD4+	CD62L-CD45RA+
	CD3+CD4+	CD62L-CD45RA-
	CD3+CD4+	CD62L+CCR7+
	CD3+CD4+	CD62L+CCR7-
	CD3+CD4+	CD62L-CCR7+
	CD3+CD4+	CD62L-CCR7-
	CD3+CD4+	Tregs (CD25highCD127low)
	Tregs	naive (CD45RA+)
	Tregs	memory (CD45RA-)
	CD3+CD3+	CD8+
	CD3+CD8+	CCR7+CD45RA+
	CD3+CD8+	CCR7+CD45RA-
	CD3+CD8+	CCR7-CD45RA+
	CD3+CD8+	CCR7-CD45RA-
	CD3+CD8+	CD62L+CD45RA+
	CD3+CD8+	CD62L+CD45RA-
	CD3+CD8+	CD62L-CD45RA+
	CD3+CD8+	CD62L-CD45RA-
	CD3+CD8+	CD62L+CCR7+
	CD3+CD8+	CD62L+CCR7-
	CD3+CD8+	CD62L-CCR7+
	CD3+CD8+	CD62L-CCR7-

	PARENT POPULATION	% GATED
B CELL	CD45+/ PBMCs	CD19+
	CD19+	naive (IgD+CD27-)
	CD19+	transitional (IgM+CD27-CD24highCD38high)
	CD19+	MZB (enriched; IgD+CD27+)
	CD19+	IgD-CD27-
	CD19+	IgM memory (IgM+CD27+CD38low/negative)
	CD19+	CS memory (IgM-IgD-CD27+CD38low/negative)
	CD19+	plasmablasts (IgD-IgM-CD27highCD38high)
	CD19+	CD21low

	PARENT POPULATION	% GATED
DC	CD45+/ PBMCs	HLADR+LIN-
	CD45+/ PBMCs	pDC
	CD45+/ PBMCs	mDCs
	mDCs	BDCA3+ mDCs
	mDCs	CD16+ mDC
	mDCs	mDC1s

Supplementary Table 2. *Validation of automated analysis pipelines using an independent data set.* Data generated by the ONE study (Streitz et al, 2013) with 10 healthy volunteers were collected independently using standardized flow cytometry settings (see Methods). The data were analyzed either using automated pipelines or by three manual analyzers (Manual 1 from the ONE study and Manual 2 and the reference manual from the CNTRP). Data from the Automated, Manual 1 and Manual 2 were compared to those from the reference manual to determine Spearman's correlation coefficients (numbers shown in the Table below).

	Input Gate	Gated Population	Automated	Manual 1 (ONE study)	Manual 2 (CNTRP)
Basic Panel	PBMCs	lymphocytes	0.99	0.99	0.76
	lymphocytes	T cells	1.00	0.96	0.99
	lymphocytes	B cells	0.90	0.98	1.00
	lymphocytes	NK cells	0.99	0.99	0.99
	lymphocytes	NKT cells	0.97	0.98	0.98
	T cells	CD4+ T cells	0.99	0.99	0.99
	T cells	CD8+ T cells	1.00	1.00	1.00
	T cells	DN T cells	0.99	not done	0.99
	NK cells	CD56++NK cells	0.90	0.96	0.94
	PBMCs	monocytes	0.99	1.00	0.70
	monocytes	CD14++CD16+	0.83	0.93	0.83
	monocytes	CD14++CD16-	0.94	0.94	0.76
	monocytes	CD14+CD16+	0.81	0.71	0.71
	monocytes	CD64++CD16+	0.93	0.92	0.85
B cell panel	lymphocytes	CD19+	1.00	0.99	0.99
	CD19+	naive B	0.98	0.98	0.98
	CD19+	transitional B	0.60	0.60	0.53
	CD19+	MZB (enriched)	0.85	0.70	0.53
	CD19+	IgD-CD27-	0.85	0.88	0.55
	CD19+	IgM memory	0.97	not done	0.92
	CD19+	CS memory	0.93	0.98	0.98
	CD19+	plasmablasts	0.80	0.82	0.97
	CD19+	CD21low	0.80	not done	0.92

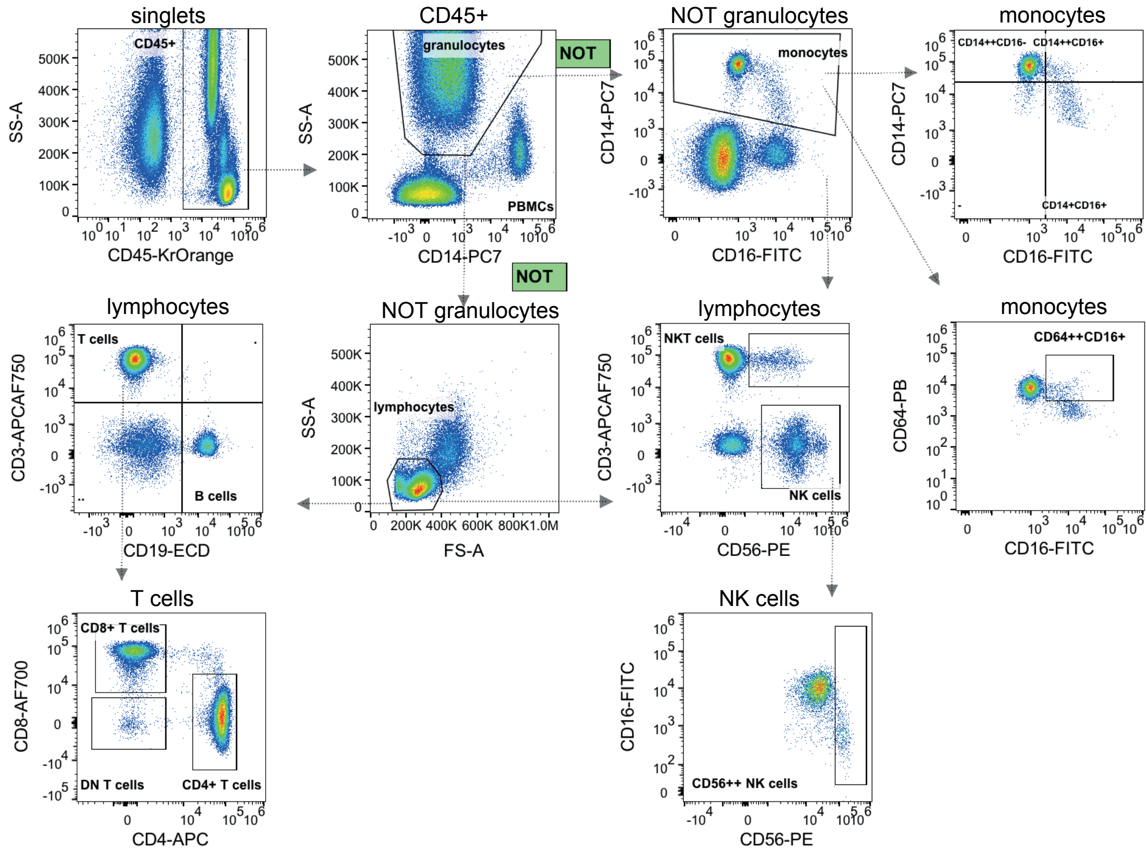
Supplemental Table 3. *List of antibody clones used in panel.*

Antigen	Clone
CCR7	G043H7
CD11c	BU15
CD123	SSDCLY107D2
CD127	R34.34
CD14	RMO52
CD16	3G8
CD16	3G8
CD19	J3-119
CD21	BL13
CD24	ALB9
CD25	B1.49.9
CD27	1A4CD27
CD28	CD28.2
CD3	UCHT1
CD38	LS198-4-3
CD4	13B8.2
CD45	J.33
CD45RA	2H4LDH11
CD45RO	UCHL1
CD56	N901 (NKH-1)
CD57	NC1
CD62L	DREG56
CD64	22
CD8	B9.11
HLA-DR	IMMU357
IgD	IADB6
IgM	SA-DA-4
LIN_CD14	RMO52
LIN_CD19	J3-119
LIN_CD20	B9E9 (HRC20
LIN_CD3	UCHT1
LIN_CD56	N901 (NKH-1)
TCRab	IP26A
TCRgd	IMMU510
BDCA1	AD5-8E7
BDCA3	AD5-14H12

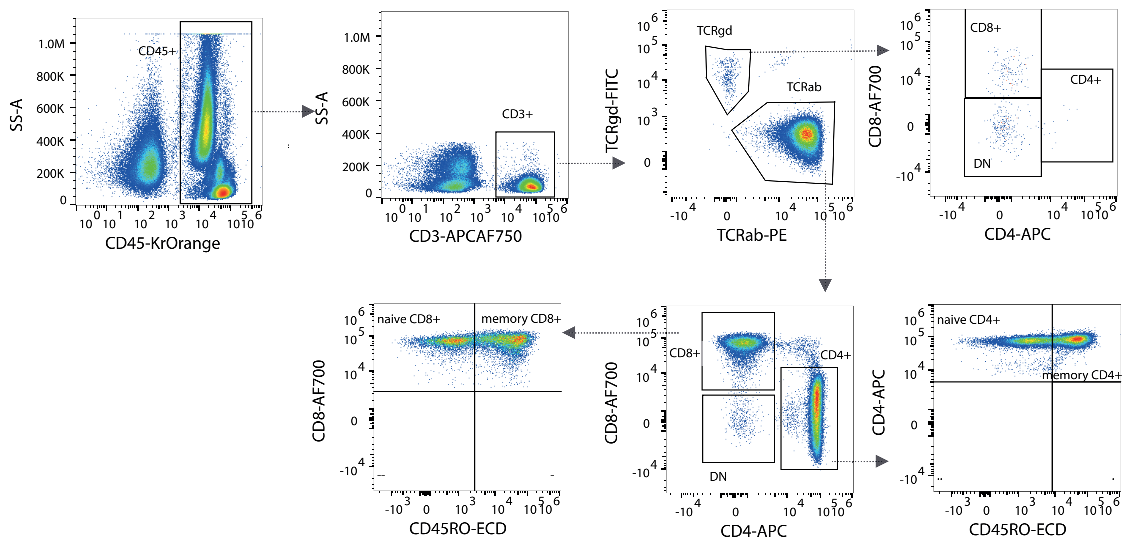
Supplemental Figure 1

Supplemental Figure S1. *The manual gating strategy.* Representative data showing the manual gating strategy for each of the panels and populations listed in Supplemental Table 1.

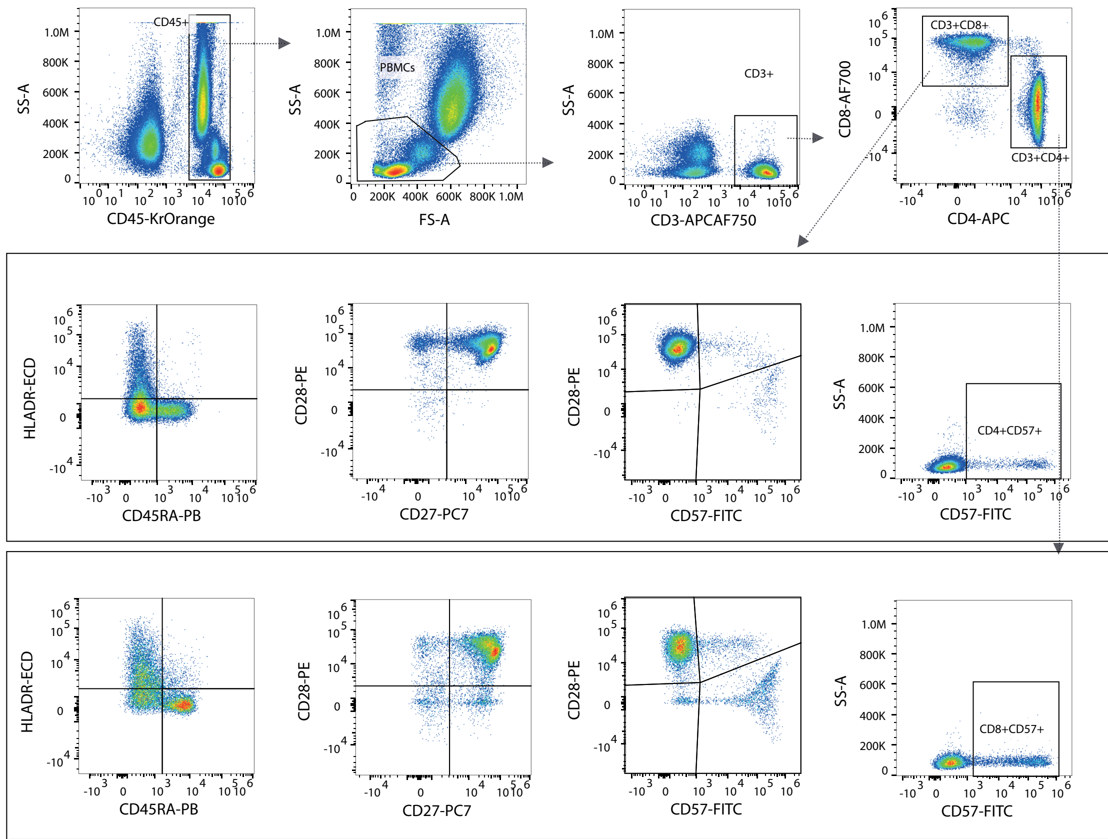
A. Basic Panel



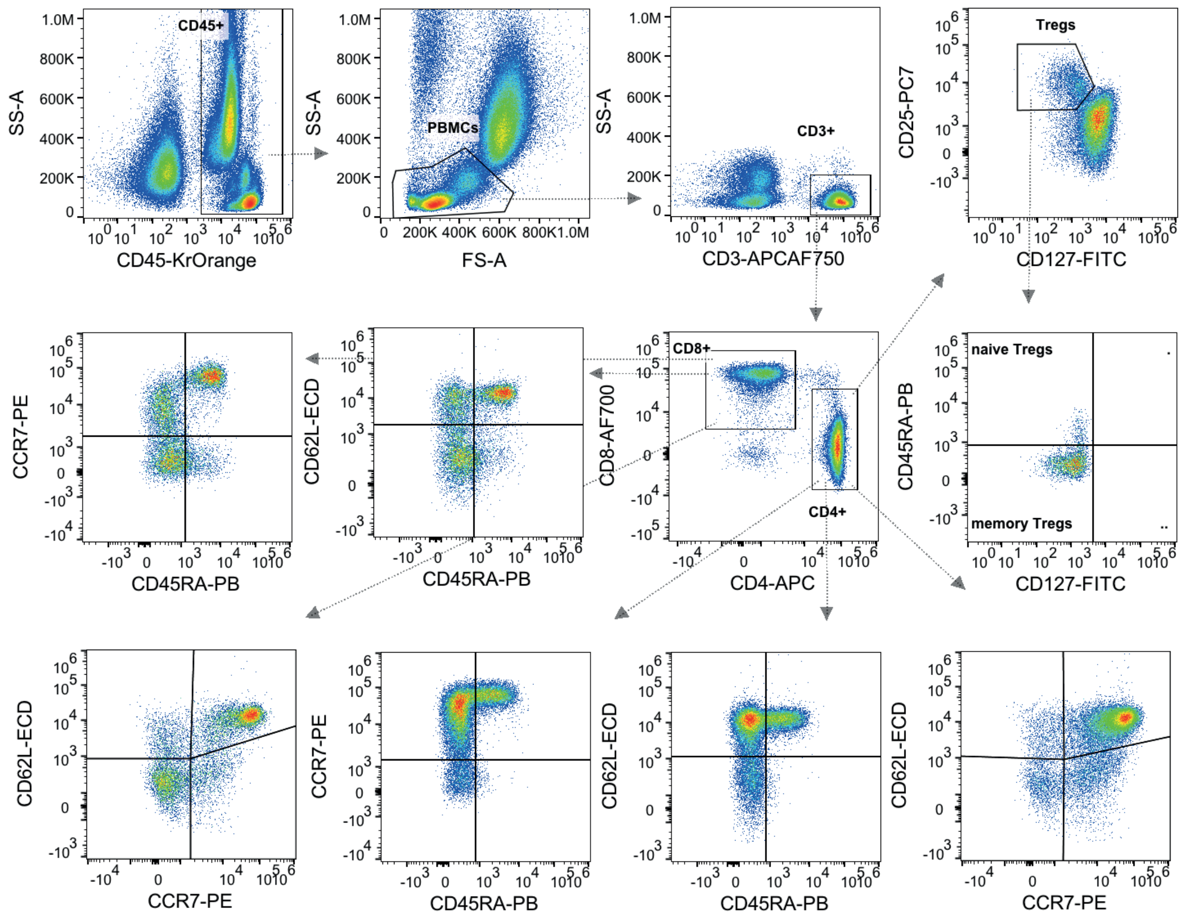
B. TCR Panel



C. T-ACT Panel



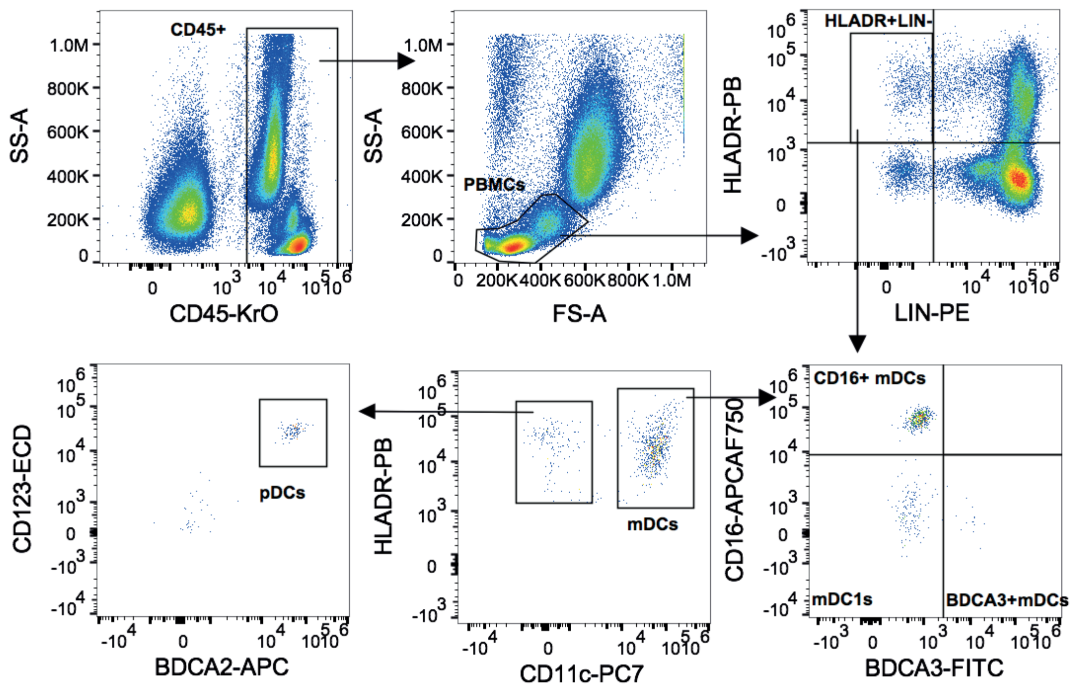
D. T-MEM-REG Panel



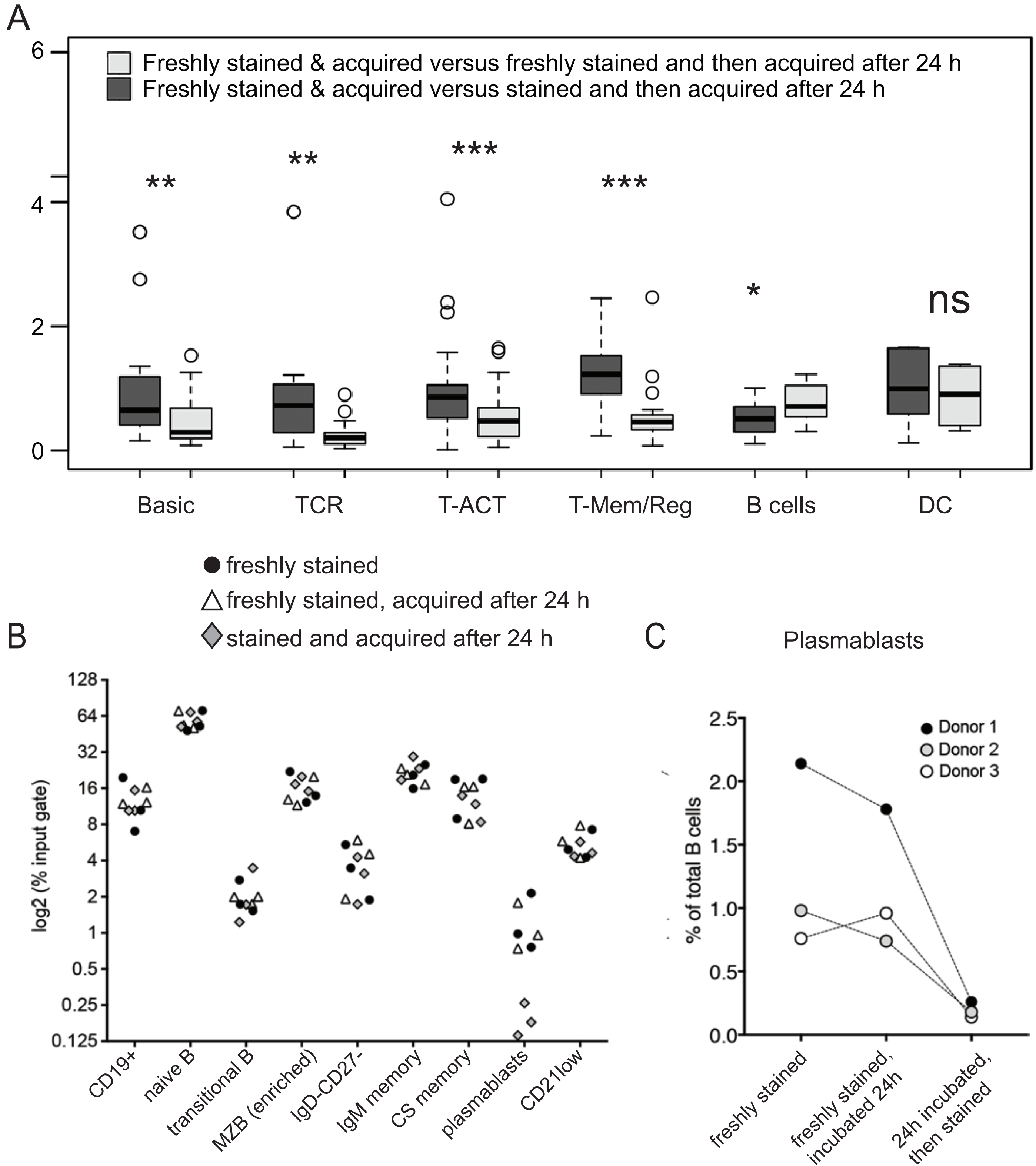
E. B cell Panel

PBMCs
SS-A
FS-A
CD19+
CD19-ECD
CD21low
CD21-PE
CD38-APCAF750
Gated on lymphocytes
how the CD21low gate was set
CD21-PE
plasmablasts
class-switched memory
CD27-PC7
IgM-PB
IgD-FITC
IgM-IgD-
naive B cells
MZB (enriched)
IgD-CD27-
CD27-PC7
Gated on lymphocytes
how the CD38 gates were set
CD38+ GATE
CD38++ GATE
IgM+
IgM memory
IgM+CD27-
CD24-APC
transitional B cell
Gated on PBMCs
how the CD27++ gate was set (CD27+ gate was set on IgD vs. CD27 plot)
CD27++ GATE
Gated on lymphocytes
how the CD24++ gates were set
CD24++ GATE

F. DC Panel



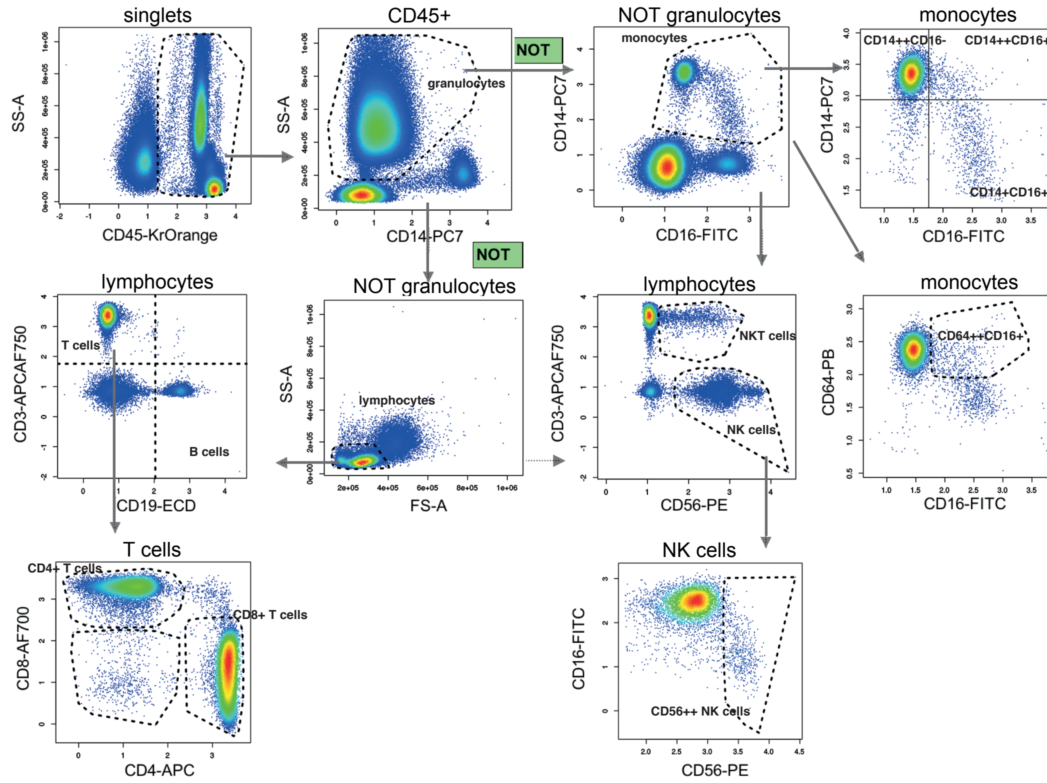
Supplemental Figure S2



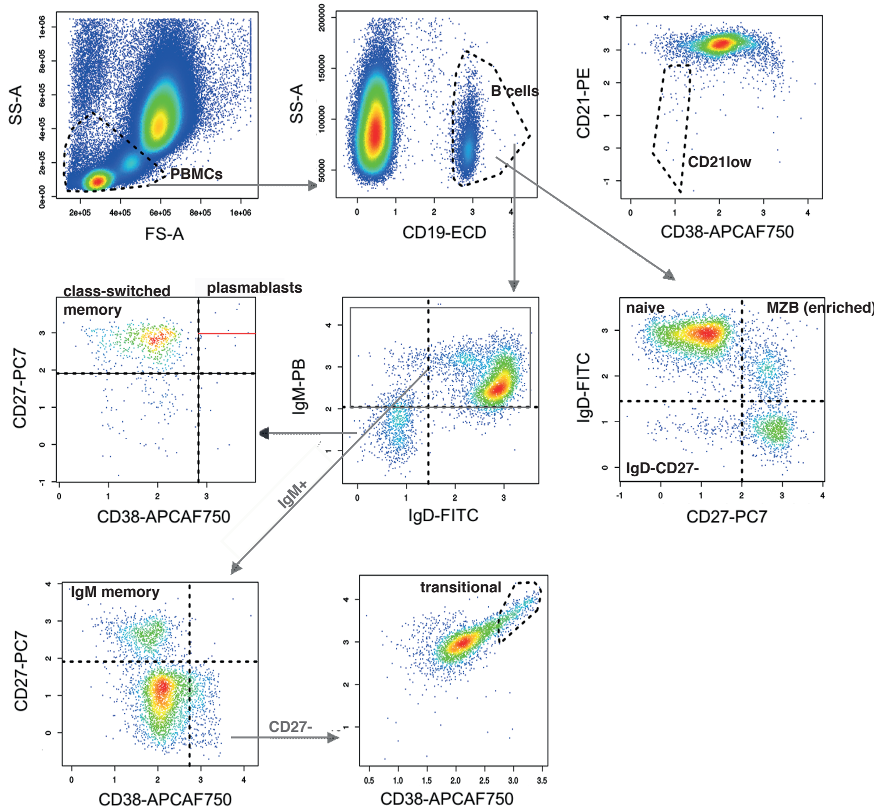
Supplementary Figure S2: *Effect of blood storage time on immune phenotyping.* Blood was collected and split into 3 samples which were: immediately stained and acquired (freshly stained blood); immediately stained and then stored at 4°C for 24h before acquisition (24 incubation of stained blood); or stored for 24h at room temperature and then stained and acquired (24 incubation of unmanipulated blood). (A) Box and whiskers plot showing the sum of all differences per panel between each storage condition and freshly stained blood; a value of 0 indicates no difference. (B) Individual % gated values from all three donors for the B cell panel. (C) Plasmablast detail showing effect of different staining conditions on individual samples.*$p<0.05$, **$p<0.005$, ***$p<0.0005$, ns= not significant, Wilcoxon signed rank-test.

Supplemental Figure S3

A. Basic Panel

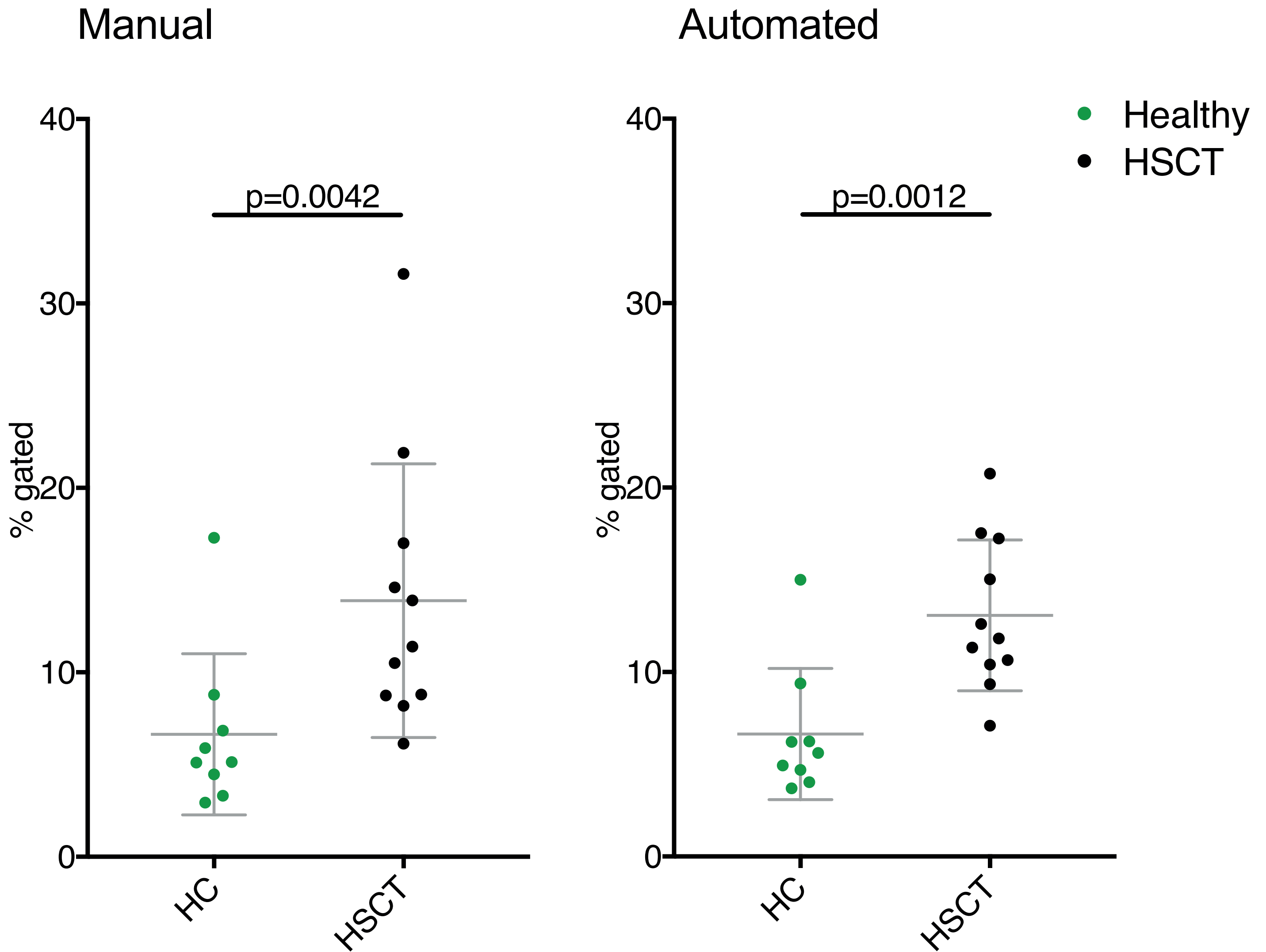


B. B cell Panel



Supplemental Figure S3. *Representative data showing automated gating for the Basic and B cell panels.* Quantified populations and their parent gates are indicated in Supplemental Table S1.

Supplemental Figure S4



Supplemental Figure S4. *Comparison of CD14++CD16+ monocytes in healthy versus HSCT using manual or automated gating*. The percentages of CD14++CD16+ monocytes for 9 healthy volunteers and 11 post-HSCT subjects are shown as the propotion of total CD14+ monocytes. Group medians were compared by Mann-Whitney.

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CNTRP Standardization Study SOPs

Version August 10, 2018

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NOTE TO THIS SOP: this was created by fusing two documents for Phase I (samples from healthy volunteers, protocol version June 2014) and Phase II (samples from post HSCT patient, protocol version September 2016). The cell handling and acquisition is the same for both protocols. The method of cytometer standardization differs: in phase I machine calibration was done using the Navios 'Autosetup'function (see section X); while in phase II we moved to a manual calibration based on FSP-bead target region (see section X). All Phase II-specific protocol parts are described in section 10.

SECTION 1: IMPORTANT INFORMATION

- **Storage and handling of Duraclone tubes**: Each site will be sent 5-8 sets of Duraclone tubes (30 to 48 individual tubes) in sealed Ziploc bags containing a dessicant. Please ensure the bag remains sealed and is stored in the dark at room temperature.

- **Traces of blood**: When working with whole blood, try to avoid smearing on the tube walls. This can cause RBC contamination of the final sample. As the Navios can only record 1.8 million events in one file, RBC contamination can cut off acquisition before the end of the sample. If blood does contaminate the tube walls, remove immediately with a moist Q-tip.

- **Use of FACs tube inserts:** The Navios is configured to leave a large residual volume in the FACs tubes. In order to minimize this dead volume, we are transferring the stained cells into 1.2 ml FACs tube inserts. . Before running, add the small inserts into a 5 mL FACs tube.

Table 1: Duraclone panels (custom formulation from Beckman)

Channel	Fluorochrome	BASIC	TCR	T-ACT	T-MEM/REG	B CELL	DCs
FL1	FITC	CD16	TCRgd	CD57	CD127	IgD	BDCA3
FL2	PE	CD56	TCRab	CD28	CCR7	CD21	LIN
FL3	ECD	CD19	CD45RO	HLA-DR	CD62L	CD19	CD123
FL5	PC7	CD14		CD27	CD25	CD27	CD11c
FL6	APC	CD4	CD4	CD4	CD4	CD24	BDCA2
FL7	A700	CD8	CD8	CD8	CD8		
FL8	APC-A750	CD3	CD3	CD3	CD3	CD38	HLA-DR
FL9	PacBlue	CD64		CD45RA	CD45RA	IgM	CD16
FL10	KrOrange	CD45	CD45	CD45	CD45	CD45	CD45
Custom lot #		14BDC0**46**A	14BDC0**47**A	14BDC0**48**A	14BDC0**50**A	14BDC0**50**A	14BDC0**51**A

*Antibodies to be added manually.

SECTION 2: Staining whole blood with Duraclone panels

Before starting, read section 1: Important Information.

2.1. Drawing peripheral blood

- **3 mL** whole blood is to be drawn into 1 x purple-topped K_2EDTA tube for analysis of fresh whole blood
- **50 mL** is to be drawn into 5 x 10 mL green-topped NaHeparin tubes for isolation of PBMCs.
- Be sure to fill the tubes as much as possible and mix immediately to prevent clotting.
- The blood should stay at room temperature until processed**.**
- **Staining of whole blood and processing of PBMCs should be done within 4 hours of accessioning.**

2.2. Removal of plasma for staining of Duraclone panel #5 (B CELL)

This panel requires that the cells be resuspended in PBS before starting the staining. *The Duraclone tube #5 is a B cell panel containing anti-IgM which would be blocked by the IgM contained in plasma.*

- Briefly vortex the whole blood in the 3 ml EDTA tube.
- Transfer 800 μL of this blood to a fresh 15 mL falcon tube labeled with Donor ID and 'plasma-free'.
- Add 10 mL of cold PBS and vortex briefly.
- Centrifuge at 300 x g for 10 min at 4°C.
- Aspirate supernatant with a pipette. Pellet will be 'fluffy' and easily disturbed.
- Vortex briefly.
- Repeat the wash with 10 mL cold PBS.
- When aspirating pellet after the second wash, try to remove as much supernatant as possible.
- Ensure that there is about 800 μL of cells in the tube. Top up with PBS if necessary.

2.3. Staining with Duraclone panels

- Identify one set of six Duraclone tubes for each sample of whole blood (18 tubes for 3 samples) and label with Donor ID-Panel ID
- Briefly vortex blood.
- Add 100 μL of whole blood into the bottom of each of the 5 Duraclone tubes **except the #5 (B CELL) tubes.** Be careful not to get blood on the tube walls.
- Add 10 μL of of BDCA3-FITC and 8 μL of BDCA2-APC to all #6 tubes.
- Add 100 μL of plasma-free cells into the bottom of all Duraclone #5 tubes.
- Vortex all tubes for **10 s**.
- With a Q-tip, remove any blood from the sides of the tubes.
- Incubate for 15 min at RT in the dark
- Proceed to 2.4: Fixing and Lysing the RBCs

2.4. Fixing and Lysing the RBCs

Before starting: Prepare enough VersaLyse-Fix for all tubes (2 mL per tube). To run all 9 panels on 3 donor samples, prepare 61.5 mL VersaLyse-Fix (60 mL VersaLyse + 1.5 mL 10 x IOTest 3).

- Vortex blood for 2 s.
- To each tube, add 2 mL VersaLyse-Fix, vortex IMMEDIATELY before adding to the next tube. *Even just a few seconds delay in vortexing will reduce the efficiency of RBC lysis- which leads to all kinds of downstream problems such as acquiring too few events!*
- Incubate for 15 min in the dark at room temperature.
- Vortex for2 s.
- Add 2.0 mL cold IFN-buffer to all tubes.
- Centrifuge at 300 x g for 5 min at 4°C.
- Aspirate or decant supernatant.
- Vortex for 2 s.
- Repeat wash in 3 mL cold IFN buffer, aspirate and vortex pellet.
- Resuspend pellet in 300 µL IFN buffer. The final volume should be about 400 µL.
- Transfer entire volume to small FACs tube inserts.
- Store at 4°C in the dark until running on the Navios (within 12 h).

==

SECTION 3: PBMC ISOLATION AND FREEZING

NOTES:

- **RT until freezing:** The blood and blood derivatives should be maintained at room temperature until frozen in the Mr. Frosty (which allows gradual cooling). BEFORE STARTING: make sure all reagents are at room temperature and that enough freezing containers (Mr. Frosties) have been removed from the freezer to warm up to room temperature; ensure that the isopropanol in the Mr. Frosties is changed after five freeze/thaw cycles.
- **FP buffer:** This section refers to FP buffer which is 2% FCS in PBS without sodium azide.
- **Maintaining good cell viability:** When resuspending cell pellets, first resuspend in a small volume (1-2 mLs) and then top up to desired volume. NEVER ALLOW THE CELLS TO SIT IN A PELLET.
- **Labeling cryovials:** Label cyrovials with Donor ID (donor ID-Site Code, for example 2E) and # million cells per vial.
- **Simplified isolation protocol:** This protocol uses the SepMate tubes from StemCell. It may be helpful to watch the following video:

http://www.stemcell.com/en/Business-Catalog/Technical-Resources/D/E/D/B/8/SepMate-Hassle-Free-PBMCs-in-Just-15-Minutes.aspx

PROCEDURE:

1) **Dilute blood.** Starting with 50 mL whole blood: transfer blood from collection tubes into 2 x 50 mL Falcon tubes, 25 mL/tube. Add an equal volume of PBS to each tube of blood to dilute 2 x. Mix gently by inversion.

2) **Prepare SepMate tubes.** Add **15 mL** of Lymphoprep into the bottom of a SepMate tube through the hole in the separation wall- disregard bubbles. Do this 4 x to create 4 tubes. Add 25 mL diluted blood to each SepMate tube, allowing it to run rapidly down the side of the tube. The wall in the SepMate tube will prevent the layers from mixing. *The Lymphoprep should cover the tube insert*

3) **Isolate PBMCs**. Centrifuge at 1200 x g for 10 min at room temperature. *No special settings are required for acceleration or braking.* After spinning, pour off the entire top layer of the SepMate tube into a new 50 mL falcon tube. *Pour with one smooth motion and do not keep the tube inverted for more than 2 s.*

4) **Combine cells**. Top up to 50 mL with FP buffer. Spin down cells at 1000 x g for 5 min and carefully remove supernatant. Combine the pellets from the four SepMate tubes from the same donor. Repeat wash with 50 mL of FP buffer; reduce the centrifugation speed to 453 x g. *The cells are now in a low viscosity buffer and do not need to be spun so hard.*

5) **Remove platelets.** Re-suspend the cells in 30 mL FP buffer. Spin for 10 min at 129 x g and carefully discard supernatant. *Platelets will not pellet at this low speed; however the PBMC pellet will not be very firm so remove supernatant immediately after the spin and leave a bit (≈ 1 mL) of FP buffer in the tube covering the pellet*

6) **Count live cells.** After the supernatant is discarded, re-suspend the pellet in FACs buffer equal to the original blood volume. Count live cells with automated counter or with a haemocytometer as follows: Transfer 50 µL of cells into a 1.5 mL eppendorf tube and add 50 µL of trypan blue. Determine live cell count using a haemocytometer, excluding cells which have taken up the blue dye. *There should be about 50 live cells/large square in the haemocytometer.* Make sure all cryovials are labelled before embarking on the final spin. ***Never leave cells sitting in a pellet.***

7) **Resuspend in freezing medium.** Centrifuge for 453 x g for 5 min at room temperature. Gently resuspend the cell pellet in sterile freezing medium (10% DMSO in FBS; this should be at room temperature) to get a final concentration of 10 x 10^6 cell/mL.

8) **Aliquot and freeze.** Aliquot 1 mL cell volumes into pre-labelled cryovials; there should be 10 x 10^6 cells in each vial. Place in a Mr. Frosty freezing container which has been acclimatized to room temperature and store at -80°C overnight.

9) **Transfer to liquid N_2.** Move cells from the Mr. Frosty to liquid nitrogen after they have been at -80°C overnight.. *Do not allow the cells to sit at -80°C for more than 3 days.*

==

SECTION 4: THAWING FROZEN PBMC ALIQUOTS

PROCEDURE:

1) Prepare thawing solution. For 10 mL: 1.5 mL FBS, 250 ul 1 M HEPES, 50 ul 7.5% sodium bicarbonate, 8.2 mL RPMI. For each vial of cells to be thawed, add 10 mL thawing solution into a 14 mL falcon tube. Warm to 37degC.

2) **Thawing of cells:** Take cells from liquid nitrogen and immediately thaw at 37°C until the frozen cells detach from the sides of the vial. Using a transfer pipette, add warm thawing solution dropwise into the cryovial until full. Then gently transfer the diluted cells into 14 ml falcon tube with thawing solution.

3) **Washing cells:** Centrifuge for 10 min at 453 x g. *This is a serum-rich solution so spin time is increased to 10 min.* Resuspend pellet in 10 mL of thawing solution.

4) **Counting cells and determining viability:** remove 50 μL of cells into a 1.5 mL tube and add 50 μL of trypan blue. Determine live cell count using a haemocytometer, excluding cells which have taken up the blue dye. Each vial should contain about 10 x 10^6 cells; we expect to count about 50 live cells/large square. Then determine the count of the dead cells and record % viability {(live cells/live + dead cells) x 100%}. *Any reliable automated cell counter which allows differentiation of live and dead cells can be used for this step*.

5) **Preparing cells for Duraclone panel staining:**

- Transfer 1 x 10^6 cells per panel to a 14 mL falcon tube.
- Top up with FP buffer to 10 mL.
- Centrifuge for 10 min at 453 x g and discard supernatant
- Resuspend pellet in FP buffer to a final concentration of 10^7 cells/mL.

==

SECTION 5: STAINING PBMCS WITH DURACLONE PANELS

PROCEDURE:

1) **Ensure adequate volume**. Each sample of PBMCs should be 1.0 x 10^6 cells in 110 ul of FP buffer per panel in a 15 mL falcon tube.

2) **Cell staining.**
   - Add 100 ul of PBMCs into the bottom of each of the Duraclone tubes and vortex for **10 s**.
   - To Duraclone DC tube: add 10 μL of of BDCA3-FITC and 8 μL of BDCA2-APC and vortex for 2 s.
   - Incubate for 15 min in the dark at room temperature.

3) **Removing antibodies.** Add 3 mL of FP buffer to all tubes, centrifuge for at RT for 5 min at 453 x g. Discard supernatant and vortex for 2 s.

4) **Fixing.** Add 1 mL of 1 x IO Test 3 Fixative solution to each tube (dilute 10 x IO Test 3 Fixative solution 1/10 with PBS to get 1 x solution). Vortex briefly and incubate for 15 min in the dark at room temperature.

5) **Removing fixative**. Add 3 mL of cold IFN buffer to all tubes, centrifuge for 5 min at 453 x g at 4°C. Discard supernatant and vortex for 2 s.

6) **Resuspending cells**. Add 300 μL of cold IFN buffer to all tubes and transfer entire volume to small FACs tube inserts. The final volume should be about 400 μL.

7) Store at 4°C in the dark until running on the Navios. *The samples must be run within 12 h.*

==

SECTION 6: BUFFERS AND REAGENTS

FP	2% heat inactivated FBS in PBS
IFN	2% heat inactivated FBS in Isoflow buffer + 0.1%NaN_3
1 x IOTest 3 Fix	1 mL 10 X IOTest 3 Fix + 9 mL PBS
VersaLyse-Fix	10 mL VersaLyse + 250 ul 10 x IOTest 3 Fix
Freezing solution	10% DMSO in FBS. (Make this fresh)
Thawing solution	15% FBS, 25 mM HEPES, 0.0375% $NaHCO_3$ in RPMI *(For 10 mL: 1.5 mL FBS, 250 ul 1 M HEPES, 50 ul 7.5% sodium bicarbonate, 8.2 mL RPMI)*

TABLE 2: Reagents to be sent to each lab from Vancouver

Product	Supplier	Cat #	Store	NOTE	Needed/ sample	A sites (9 sets)	B sites (5 sets)	Ship.t# *
SepMateTM	StemCell	5450	RT		4 tubes	15 tubes	none	1
FACs tube inserts (1.2 ml Library tubes)	VWR	83009-678	RT		9 tubes	100 tubes	60 tubes	1
FVD-eF780	eBioscience	65-0865-14	-80°C	keep freeze/thaw to a minimum	1 ul	20 ul	10 ul	2
K_2EDTA Vacutainer tubes, 3 ml	Fisher	265732	RT		1 tube	6 tubes	none	1
NaHeparin Vacutainer tubes, 10 ml	Fisher	0268399B	RT		5 tubes	20 tubes	none	1
Duraclone tubes	Beckman	see table 2	RT	dark and dry!	1 x set of 6	10 sets of 6	6 sets of 6	1
BDCA3-FITC**	Miltenyi	130-090-513	4°C	store in the dark	10 ul	*250 ul*	80 ul	2
BDCA2-APC**	Miltenyi	130-090-905	4°C	store in the dark	8 ul	*250 ul*	80 ul	2
CD4-fluorochromes for compensation	Beckman	varied	4°C	store in the dark	10 ul per test x 9 colours	30 ul x 9	15 ul x 9	2

***Shipment# 1- room temperature**, should arrive at all sites on Monday, June 30th or Wednesday July 2.
Shipment# 2- cold, sent out to arrive at all A sites on Friday, July 4 and all B sites on Thursday, July 10th.
**The Miltenyi Abs have already been set to the A sites.

Extra reagents have been provided in case of mistakes. Please save all unused reagents at the correct storage temperature for return to the Levings lab.

TABLE 3: Regents to be supplied by individual labs or directly from Beckman to the individual labs

Product	Supplier	Cat #	Sites	Provided by
Lymphoprep™	StemCell	07851	A	not provided
DMSO	Fisher	D128-500	A	not provided
PBS*	Life Technologies	1419-144	A & B	not provided
FBS, heat inactivated**	NorthBio	NBSF-701	A & B	not provided
RPMI 1640	Life Technologies	11875-093	A & B	not provided
1 M HEPES	Life Technologies	15630-080	A & B	not provided
7.5% $NaHCO_3$	Life Technologies	25080094	A & B	not provided
Trypan Blue	Life Technologies	15250-061	A & B	not provided
10% NaN_3	Sigma	S8032	A & B	not provided
Isoflow buffer	Beckman	8546859	A & B	Beckman
10 x IO Test3 Fixative	Beckman	A07800	A & B	Beckman
VersaLyse	Beckman	A09777	A	Beckman

* needs to be Ca^{2+} and Mg^{2+}-free
**inactivated at 56°C for 30 min.

NOTES TO REAGENTS:

- The suppliers and catalogue numbers for the reagents supplied by the labs are suggestions only; for instance, Ficoll can be used in place of Lymphoprep.
- It is assumed that each site will have Falcon tubes, cryovials, pipettes and other basic lab reagents as well as the reagents listed in the table above.
- FlowCheck-Pro and FlowSet-Pro beads, as well as FlowClean, will also be provided by Beckman to the labs running the samples on the Navios machines (either clinical labs or A sites).

SECTION 7: SAMPLE ACQUISITION ON THE NAVIOS

7.1. Start-up procedure

- Make sure a carousel is loaded
- Check that there is enough sheath and that waste cube is not too full
- Check cleanse tank fill level (FlowClean is stable for 3 months once bottle is opened)
- Turn on PC, login (password = password), wait for PC to totally finish booting up
- Double click on the “Navios Software” icon
    - Login = CNTRP
    - Password = password
- If fail password attempt more than 5 times, will lock out that user
- Check Status Bar (at the bottom of the screen)

Run a cleanse panel:

- Load a tube of freshly diluted bleach (position 1) and three tubes of water (positions 2, 3, 4) on the carousel
- Clear the worklist
- From the “Panels” tab, open the “Common” folder, drag the Cleanse Panel into the Acquisition Manager
- Enter the carousel number
- Click “Play”

7.2. Quality control: FlowCheck-Pro and FlowSet-Pro

Run FlowCheck-Pro:

- Clear the worklist
- From the “Protocols” tab, open the “Common Acquisition folder”, drag the “Alignment Blue Red Violet_ALIGN.pro” on the workspace
- In the Acquisition Manager, enter:
    - Carousel number
    - FlowCheckPro for Sample ID1
    - Today’s Date for Sample ID2 (eg. 2014-05-23)
    - City code ad User’s inials for Sample ID3 (eg. YVR_SJ)
    - Run number for Sample ID4 (eg. Run1)
- Load a tube of FlowCheck-Pro (DO NOT DILUTE)
- Click “Play”
- Make sure beads run at least 150 events/s initially (though this may slow down during acquisition) and that CVs are below the number stated on each region. If beads have settled, can vortex the tube by clicking “Pause”, then “Play”
- When sample acquisition is complete, click “Finish”

Check Levey-Jennings plot

- Click Levey-Jennings Icon
- Template → Select Template →Click on “Alignment Blue Red Violet_ALIGN” → Open
- Click on “Data Table”
- Any peaks that had CVs above the specified limit will show in red in the data table – so if any parameters “failed”, follow troubleshooting suggestions:
  - Re-run the beads
  - Prime (will restart acquisition and erase any acquired data)
  - Cleanse (will have to re-initialize – click the “ZZZ” icon)
  - If that doesn’t work, run a cleanse panel

Daily FlowSet-Pro (Phase I only):

- Clear the worklist
- From the “Protocols” tab, open the “CNTRP Acquisition folder”, drag the “Daily FlowSet-Pro” protocol on the workspace
- In the Acquisition Manager, enter:
  - Carousel number
  - Daily FSP for SampleID1
  - Today’s Date for Sample ID2 (eg. 2014-05-23)
  - City code and User’s initials for Sample ID3 (eg. YVR_SJ)
  - Run number for Sample ID4 (eg. Run1)
- Load a tube of **Lot 3143650F** of FlowSet-Pro (DO NOT DILUTE)
- Click “Play”
- After sample is finished acquiring, check Levey-Jennings:
  - Click Levey-Jennings Icon
  - Template → Select Template →Click on “Daily FlowSet-Pro” → Open
  - Click on “Data Table”
- If X-Means are out of range, AutoSetup may need to be re-done

7.3. Acquiring samples

- Clear the worklist
- Drag “6 Duraclone Tube” Worklist down to the Acquisition Manager
- **Delete any entries that are not required**
- Enter the following information:
  - Sample ID1: Patient ID
  - Sample ID2: CNTRP_ONE_Phase1
  - Sample ID3: Panel Tube Number (eg. “ONE_01” for Tube #1 the Duraclone tubes; “Custom_01” for the first of the custom liquid antibody tubes)
  - Sample ID4: City name-User Initals (eg. YVR-SJ)
- Click “Play”
- “Prime” if there appears to be a clog (but this will restart the acquisition)

- Stop conditions for each tube (sample will stop at which ever comes first)
    - 1,800,000 total events
    - 300 seconds
- Make a copy of your LMD files and transfer to your shared drive; you are responsible for backing up your files

7.4. Shut-down procedure

- Run a cleanse panel
- Put a Kimwipe under the probe, then click on "cleanse" icon
    - Wait for instrument to depressurize
    - But don't click the "cleanse" icon if the instrument will not be used for many days – the FlowClean can form deposits in the lines over time; alternatively, could run another cleanse panel after this step
- Fill sheath cube and onboard cleanse tank if necessary; empty waste cube if necessary
    - If need to do this in the middle of the day, click the 'Idle" icon, then click it again to re-pressurize
- Close Navios software
- **Double click "Turn Off Cytometer" icon on desktop**
- **LOG OUT of Windows (Do not forget to do this)**
- Turn off PC overnight (unless IT needs it to be on for virus scanning?)
- Swab sample probe with water

SECTION 8: WEEKLY INSTRUMENT MAINTENANCE

If the instrument has not been used for one week:

- Start-Up procedure
- Cleanse panel
- FlowCheck-Pro
- FlowSet-Pro
- Shut-Down Procedure

SECTION 9: AUTOSETUP (phase I only)

The purpose of Autosetup is to standardize the instrument to yield consistent signals intensities (brightness) over time, by using FlowSet-Pro beads. Autosetup does this by adjusting the voltages of each PMT to place the FlowSet-Pro bead in the same channel every time. Autosetup will also calculate a compensation matrix based on single-stained samples.

You may need to follow this procedure if:

- The Daily FlowSetPro protocol shows deviations that are out of range (see the Levey-Jennings plots)
- The lasers have been aligned by a Service Engineer
-

9.1. Procedure for the 'AS_CNTRP_ONE Study' application

Prepare single-stained Versacomp beads using the SAME fluorochomes as in the Duraclone Panel:

- Label 9 tubes with each fluorochrome name
- Add one drop of negative Versacomp beads and one drop of positive Versacomp beads into each tube
- Add 10uL of the appropriate CD4 antibody into each tube and vortex immediately
- Incubate at room temp in the dark for 20min
- Add 1mL of PBS, vortex, and centrifuge at 300 X G for 6 minutes
- Decant supernatant, resuspend pellet, and add 1mL of PBS
- Prepare a "Verify" tube – use Duraclone Tube #1
- Tools → AutoSetup Scheduler
- Choose "AS_CNTRP_ONE_Study" and enter the carousel number, Click "Schedule", then close
- Enter information in the SampleID fields
- Load tubes according to the list on the carousel
  - Load a tube of **Lot 3143650F** of FlowSet-Pro for first tube position
  - Load each single-stained Versacomp tube for the following 9 positions
  - Load the Verify tube in Position 11
- Click "Play"
- Once the tubes have been acquired, print the Status Page (Cytometer → Print Status Page)

Adjust compensation in Kaluza:

- Use previous normal PB samples as a reference (i.e. a sample acquired with the previous version of a "good" comp matrix)
- Use the previous comp matrix as a guide – new comp matrix should be very similar
- Adjust the compensation on the Verify tube

- Enter the new compensation matrix into the settings file:
  - In the Navios software, drag the "AS_CNTRP_ONE_Study_settings.pro" into the Workspace (no plots should appear)
  - In the Cytometer Control window, click on the "Compensation" tab
  - Enter the values from the adjusted matrix from Kaluza
    - Be careful – in the Cytometer Control's comp matrix, there is a row and column for FL4 that will all show 0.0, but FL4 is not present in the comp matrix that comes from Kaluza
    - Save Protocol As – same name
  - Update the voltages in the Daily FlowSet-Pro protocol:
    - Drag the "Daily FlowSet-Pro.pro" to the Workspace
    - Cytometer → Get cytosettings from protocol: choose "AS_CNTRP_ONE_Study_settings.pro"
    - Cytometer control → Compensation tab → Clear compensation matrix
    - Lower discriminator to 50
    - Change flow rate to "Medium"
    - Save Protocol As – same name

Note: the "AS_CNTRP_ONE_Study_settings.pro" file contains the following hardware settings: voltages, gains, compensation matrix, flow rate, and acquisition time limit

SECTION 10: PHASE II -SPECIFIC SOPS

10. 1 Patient Cohorts

Subjects

- Adult (>18 y) HSCT patients 3 months post transplant (100 d +/- 10 d).

Inclusion criteria

- HSCT patients who have undergone fully ablative transplants for lymphoid or myeloid malignancies.

Exclusion criteria

- Treatment with systemic steroids at time of sample (so this would exclude patients with active GVHD)
- Relapse at or prior to time of sampling

Analysis cohorts

- Montreal and Vancouver will recruit 10-20 patients (in total). Blood will be analysed immediately and PBMCs isolated and frozen after 24 h. Samples will be thawed from both Montreal and Vancouver and analysed post thaw for comparison with fresh. Aliquots of these samples only will be sent to Edmonton for inter-site comparisons.

10.2 Patient Samples

A total of 3 ml + 28 ml peripheral blood is to be drawn from each donor as follows:

- SECTION **3 mL** whole blood is to be drawn into 1 x purple-topped K_2EDTA tube for analysis of fresh whole blood
- **~28 mL** is to be drawn into 4 x 7 mL green-topped NaHeparin tubes for isolation of PBMCs
- Be sure to completely fill the tubes and mix immediately to prevent clotting.
- The blood should stay at room temperature until processed**.**
    - **Staining of whole blood should be done within 4 hours of accessioning, PBMCs should be processed 24 h after accessioning.**

10.3 Phase 2-specific Navios procedures

Daily Flow Check pro has not changed, follow instructions in section 7.2

10.3.1 Daily Flow-Set Pro

'Daily Flow Set Pro' is run every time there is a sample; these are project-specific bead lots ***and all study sites must be using the same lot (LOT#9001011) until May 2016 for the CARE Trial and then LOT#9001019 until December 2016).*** FSP beads provide the target values for fluorescent intensities. The daily FSP quality control check ensures that the voltages for the DuraClone assay produce the same signal intensities (MFI) for the FSP

beads every time the cytometer is used. The target MFIs (= 'Channels' in Beckmanese) are listed in table 4 in Section 11.1. *If the machine fails FlowSet-Pro and all attempts at troubleshooting (the same as those listed for a failed FlowCheck-Pro above), the voltages will need to be re-calibrated (Section 11).*

- Clear the Worklist
- From the "Protocols" tab, open the "CNTRP Acquisition folder", drag the "Daily FlowSet-Pro" protocol on the workspace.
- In the Acquisition Manager, enter:
    - Carousel number (1 or 2, depending where the samples are loaded)
    - Sample ID1: FSP
    - Sample ID2: Date (format 2014-05-23)
    - Sample ID3: City code and User's initials (format. YVR_RG)
    - Sample ID4: Run number (eg. Run1)
- Add 3 drops of undiluted FlowSet-Pro to a flow tube **(WITH INSERT!)**, load the carrousel and click "Play"
- After sample is finished acquiring, check Levey-Jennings plot by clicking on the icon: select "Template", "Daily FlowSet-Pro" and "Data Table"
- If any values are out of range (turn an alarming shade of red), then follow the troubleshooting procedures outlined in 10.3.2. If these don't work then you may need to recalibrate voltages (see section 11).
- If anything is unusual or you need to run the protocol again, click on the last point in the graph and add a comment.

10.3.2 Troubleshooting if Navios QC (FlowCheck- or FlowSet-Pro) fails

If either FCP or FSP fails, follow these troubleshooting suggestions in the following order:

1) Re-run the beads
2) Prime
3) Cleanse (click the "ZZZ" icon afterwards to re-pressurize).
4) If none of this works, re-run a new cleanse panel. Make sure bleach has been freshly diluted.
5) Ensure also that all beads are within their closed and open vial expiry dates (beads should be used within 65 days of opening the vial). If the vial is reaching its open-vial expiry date, try running a freshly-opened bottle of beads.
6) IF FCP continues to fail, call Beckman to get expert help. If FSP continues to fail, the voltages will need to be recalibrated. See section 11.

10.3.3 Acquiring patient samples

- Clear the Worklist
- Drag the "6 Tube" Worklist down to the Acquisition Manager. This allows all samples from one donor to be run automatically in the following order: Duraclone 01-06
- Note that the Worklist is constructed for ~1 full carousel (6 x 3 patients), so if you have less than three donors, delete all rows you don't need.
- Enter the following information: (titles in italics should already be saved in the Worklist)
    - Sample ID1: Patient ID – FILL IN
    - Sample ID2: *CNTRP_Phase2*
    - Sample ID3: *Tube number* (eg. ONE_01)- will auto-populate
    - Sample ID4: City name-User Initials (eg. YVR-RG)- FILL IN

- Click “Play”
- Acquisition of each sample will stop at which ever comes first: 1,800,000 total events or 300 seconds. *THIS MEANS RBC LYSIS MUST BE COMPLETE or the machine will acquire a bunch of RBCs and stop.*
- Once acquisition is complete, an LMD file is created and It can be retrieved from C: /Beckman Coulter/ User Data / Users / CNTRP /LMD

11: MANUAL RECALIBRATIION OF ONE-STUDY SPECIFIC VOLTAGES USING CURRENT FSP LOT (Phase 2)

This is procedure replaces ‘Autosetup’. The purpose of re-calibrating voltages is to standardize the instrument to yield consistent signal intensities (brightness) over time using FlowSet-Pro beads. This is done by adjusting the voltages of each PMT to place the fluorescent peaks from the beads in the same intensity region (known in Beckmanese as ‘channels’) every time. These target intensities can vary with each lot of FSP beads; see table 4 in this section for current FSP lot target regions. Any change in the Navios (eg. hardware, fluidics) that causes the FSP bead peaks to be out of range in the “Daily FSP” protocol, means that voltage re-calibration needs to be performed.

Voltages may need to be re-calibrated if:

- during the daily FSP procedure, any of the peaks do not land within the boundaries set in the Levey-Jennings graph even after troubleshooting measures have been attempted
- the Navios lasers have been re-aligned by a Service Engineer

11.1 Instructions for voltage recalibration

- Clear the Worklist
- From the Worklist tab, open the “CNTRP” folder. Drag the “Daily Flow Set Pro.WLS” into the Acquisition Manager
- In the Acquisition Manager, enter all fields that are not auto-populated:
    - Carousel number (1 or 2, depending where the samples are loaded)
    - Sample ID1: FSP
    - Sample ID2: Date (format 2014-05-23)
    - Sample ID3: City code and User’s initials (format. YVR_RG)
    - Sample ID4: Run number (eg. Run1)
    -
- Add at least 6 drops of undiluted FlowSetPro (make sure there is an insert in the tube!), load the carousel
    - Ensure FlowSetPro vial is not expired! Should have been open for 2 months or less
- Enable Setup mode and QuickSet (this will show the voltage slider bars)
- Click “Play”
- Adjust voltages to match those in table 4 below (make sure the lot number referred to in table 4 is the same lot number as the beads you are using!!)
- Save Protocol
- Uncheck Setup mode and QuickSet
- Allow an LMD file to be acquired

- Check the X-MODEs of each parameter (click on the lower left-hand side of the graph to see the statistics; use the value from the specific parameter, not from 'ALL'). Compare all values to those in table 4 (from the same bead lot). If necessary put back in setup mode, adjust voltage up or down and repeat the acquisition of an LMD file and checking of the X-MODEs.
- Repeat until all of the values are within 1-2 of the target values (sometimes it is not possible to get within 1- although mostly it is).
- Print status page- **this will be used to update the voltages in the cytosettings**.
- Check the Levey-Jennings plot for the Daily Flow Set Pro: the most recent data point should be in the middle of each plot. (If not continue adjusting voltages as described above).

Parameter	FOR Levey-Jennings of Daily FlowSet-Pro.WLS	
	X-MODE	Range
FS	69.75	+/-10%
SS	309.5	+/-10%
FL1	119.32	+/-10%
FL2	301.34	+/-10%
FL3	151.78	+/-10%
FL4	NA	NA
FL5	39.56	+/-10%
FL6	9.53	+/-10%
FL7	219.96	+/-10%
FL8	41.47	+/-10%
FL9	35.83	+/-10%
FL10	119.32	+/-10%

Table 4: FSP Target regions for lot# 9001019 (set on April 13, 2016 and adjusted on June 13, 2016 to capture X-MODEs instead of X-MEANs).

11.2 Updating Cytosettings for Phase 2 protocols

NOTE: This step is VERY important; all patient samples will be run using these settings. This should be done immediately after adjusting voltages and printing status page. As this procedure now requires manual entry, be very careful to do this without mistakes and double-check the inputted values. IF POSSIBLE, ALWAYS DO THIS WITH TOGETHER WITH ONE OTHER PERSON!

- From the Protocols tab, pull the "AS_CNTRP_ONE_Study_settings.PRO" to the workspace
- Open the Cytometer Control window and change the voltages to the newly determined voltages (use the Status Page as a guide).
- Save the protocol
- Re-drag the protocol to the Workspace to double-check that voltage changes were properly saved.

11.3 Lot Crossover Protocols for FlowCheck-Pro and FlowSet-Pro

This is to be done every time a new lot of beads are to be used, before the current beads expire!

FlowCheck-Pro Lot Crossover (This is not specific for phase II)

- Run old lot (soon to be expired lot) of FCP using the *Alignment Blue Red Violet_ALIGN.pro* protocol as described in section 7.2. to ensure machine is optimal.
- Run the new lot of FCP beads.
- In Cytometer control, put into setup mode and adjust all three gates in FS histogram if necessary to make sure singlet beads are being captured (voltage can also be adjusted if necessary)
- Put in quickset mode to create slider bars for voltage adjustment at the top of each histogram. Then look at the histograms FL1-FL10. Click on blue bar or on grey arrows to increase or decrease voltages to put the coloured peaks into the middle of the gate.
- FILE→SAVE AS → save over old protocol. Name does not change. **Protocol is now updated.**
- Click the red "x" to abort the current sample
- Update lot number and date: Icon tube with pipette=Product Editor. Update all information. *Careful the lot numbers on the bottle may be different than that on the box. USE LOT NUMBERS ON THE BOTTLE!!!*
- Take out of quickset mode and out of setup mode. Create an LMD file from the FCP beads by running the usual protocol.
- Open the Levey-Jennings plot; one point will appear; this is from the tube of the new lot that was just acquired.
- Check Levy Jennings to make sure all values are within acceptable range. If not, follow troubleshooting procedures described in 10.3.2.

Flow Set Pro Lot Crossover

- Follow instructions in 10.3.1 (Run Flow Set Pro: Project-specific machine calibration) and run the **new lot** of FSP beads using phase I/II-specific 'daily flow set pro.WLS'.
- Updating manual means in the Levey-Jennings template for daily Flow Set Pro
    - After running daily Flow Set Pro with the **new** lot of FSP beads, click on the Levey-Jennings icon.
    - In the menu: Template → Select Template → 'Daily Flow Set Pro'.
    - One point will appear (this is from the new lot of beads that was just acquired).
    - On the right-hand side of the plot for each parameter, click on "Mean" and then select 'manual mean'. This will allow you to enter a value into the box. Enter the value from the corresponding parameter as provided in table 4 (check lot number in the table caption- it should be the same as your **new** lot of FSP beads). This table will be updated in the latest version of the immune monitoring protocol, which should have been sent to you with the new lot of FSP beads.
    - Once the mean values for all 11 parameters have been updated, check that the data point is close to the mean (as indicated by the dotted white line in each plot).
    - Close the Levey-Jennings window and click yes when prompted to save the template. (DO NOT CHANGE THE NAME OF THE TEMPLATE).
    - Note: It is no longer necessary to run the old FSP bead lot; old lot vials can be discarded.