Stability Assessment of Various Flow Cytometry Markers in Stabilized Whole Blood

Dominic Gagnon, Philippe Pouliot, Vicky Sgouroudis, Sylvia Pouvreau, Lucie Barblu, Bader Yassine Diab, Sandrina DaFonseca, Rose-Marie Brito, Valérie Hébert, Marylène Fortin, Audrey Gagnon, Jean-Francois Poulin, Yoav Peretz.

Caprion Biosciences, ImmuneCarta Division, Montreal, Canada

CAPRION BIOSCIENCES
ImmuneCarta Division

Introduction

- Multiparametric flow cytometry has become a tool of choice given its ability to assess cellular phenotypes and functions in complex mixtures of cells such as in peripheral blood and tissues. During drug development, particularly in the later stages, clinical trials are designed to include multiple investigative sites distributed globally requiring the need for specimens to be shipped for centralized analysis. The primary challenge is the delay between specimen collection and sample processing and analysis. Therefore, the assessment of sample stability becomes a requirement during assay development to ensure that the data collected is comparable to freshly isolated and analyzed samples. The data presented herein reports on the stability assessment of various phenotypic markers following blood collection in Cyto-Chex® collection tubes.
- Blood was collected in Cyto-Chex blood collection tubes from at least 3 volunteers and tested at various time points following venipuncture. Our assessment included various parameters such as time post-venipuncture, storage temperature, clones and fluorophore/antibody pairs from different providers. Main lineage cell subsets such as CD3 lymphocytes, CD4 T cells, CD8 T cells, Monocytes, and NK cells were analyzed along with other molecules of immune activation and maturation. Stability was calculated in three different donors in triplicate by determining the average % difference between the total frequencies detected over time compared to day 0 (freshly analyzed blood samples).
- Takentogether,thefrequenciesandfluorescenceintensitiesofthemarkersdetectedbyflowcytometryshowed varying stability profiles suggesting that Cyto-Chex® blood collection during clinical trial testing is a viable option for extending the stability of samples post-venipuncture. However, robust testing is required to validate the selection of clones and fluorochromes multiplexed in the panel along with the projected final readout.

Study Design and Methodology

Prior to evaluating stability, the following testing was done to assemble a full flow cytometry panel in whole blood collected in Cyto-Chex BCT:

1. Titration of all reagents for selection of titers with highest Signal-to-Noise (S/N) ratios. If markers were absent or Dim-expressing, whole blood was spiked with a cell line or with in-vitro stimulated PBMC. This was specifically done for immune activation/exhaustion markers.
2. FMO and isotype control testing for fluorescence spill over and specificity
3. Full panel testing for Intra assay precision on minimally 3 donors

Table I. List of Markers Tested in Cyto-Chex BCT.

Lineage Markers	Antibody Clone	Fluorophore
CD3	OKT3	BV711
CD3	UCHT1	PE-Cy5
CD4	RPA-T4	BV605
CD8	RPA-T8	BV650
CD14	M5E2	BV785
CD56	NCAM1	PE-CF594
Memory/Maturation Markers		
CD45RA	HI100	APC-Cy7
CD27	M-T271	PerCP-Cy5.5
CCR7	G043H7	PE-Cy7
CCR7	150503	Alexa 647
CD62L	DREG56	PE-Cy7
CD62L	SK11	Alexa 647
Immune Activation/Exhaustion Markers		
PD-1	EH12.2H7	BV785
ICOS	DX29	PE
TIM-3	344823	APC
CD69	FN50	FITC
HLA-DR	G46-6	Alexa 700
PD-L1	MIH1	PE
Granulocyte Markers		
CD15	MC-480	BV421
CD33	WM53	BB515
Macrophages and APC Markers		
CD16	3G8	BV711
CD16	eBioCB16	APC-efluor-780

Stability of Immune Markers (MdFI)

Tables II. Stability of immune markers analyzed in whole blood collected in Cyto-Chex BCT. Briefly, blood was collected in Cyto-Chex containing K3EDTA anticoagulant from 3 healthy volunteers and stored at 2-8°C (right) and ambient temperature (left). An aliquot of the sample was analyzed in triplicate at several time points post-venipuncture with panels containing the markers/clones/fluorophores listed in Table I. The MdFI for each marker was analyzed on the intended cell population and % differences to baseline were calculated to assess stability. To obtain positive signals for Dim expressing markers, Karpas cell line for PD-L1 and SEB stimulated PBMC for ICOS, PD-1, and TIM-3 detection were spiked at baseline. *% difference > 30% are highlighted*

	Ambient Temperature					2-8°C				
	Day3	Day7	Day14	Day21	Day28	Day3	Day7	Day14	Day21	Day28
CD3	14%	11%	26%	35%	20%	-3%	-5%	-4%	NT	-11%
CD4	-5%	-1%	2%	-3%	-7%	-29%	-31%	-27%	NT	-23%
CD8	-35%	-37%	-35%	-41%	-42%	-48%	-55%	-59%	NT	-62%
CD14	-27%	-44%	-51%	-54%	-56%	-14%	-9%	-12%	11%	23%
CD15	-12%	-15%	-15%	-17%	-5%	-31%	-35%	-37%	-35%	-28%
CD16	-46%	-54%	-63%	-63%	-62%	-30%	-34%	-50%	-51%	-52%
CD33	-41%	-49%	-53%	-53%	-51%	-22%	-30%	-44%	-46%	-44%
CD56	11%	4%	-9%	7%	-3%	-1%	1%	2%	NT	6%
PD-L1	-22%	-25%	-16%	-35%	-35%	-5%	-12%	-12%	-13%	-11%
CD27	-27%	-34%	-35%	-34%	-32%	-16%	-25%	-29%	NT	-33%
CD45RA	-12%	-14%	-14%	-15%	-17%	-16%	-10%	-10%	NT	-13%
CD62L	-66%	-66%	-66%	-67%	-65%	-54%	-60%	-64%	NT	-64%
CD69	-21%	-27%	-30%	-41%	-43%	-8%	-16%	-31%	NT	-49%
HLA-DR	40%	32%	42%	45%	45%	-7%	-7%	-6%	NT	-12%
ICOS	18%	17%	10%	1%	0%	-4%	-13%	-24%	NT	-32%
PD-1	8%	3%	-1%	-8%	-6%	10%	4%	-6%	NT	-17%
TIM-3	-14%	-21%	-25%	-30%	-31%	1%	0%	-13%	NT	-26%

Stability of Immune Markers (Frequency)

Table III. Stability of immune markers analyzed in whole blood collected in Cyto-Chex BCT. Briefly, blood was collected in Cyto-Chex containing K3EDTA anticoagulant from 3 healthy volunteers and stored at 2-8°C (right) and ambient temperature (left). An aliquot of the sample was analyzed in triplicate at several time points post-venipuncture with panels containing the markers listed in Table I. The frequency for each marker was analyzed on the intended cell population and % differences to baseline were calculated to assess stability. To obtain positive signals for Dim expressing markers, Karpas cell line for PD-L1 and SEB stimulated PBMC for ICOS, PD-1, and TIM-3 detection were spiked at baseline. *% difference > 30% are highlighted*

	Ambient Temperature					2-8°C				
	Day3	Day7	Day14	Day21	Day28	Day3	Day7	Day14	Day21	Day28
CD3	-77%	-67%	0%	3%	3%	-5%	-10%	-9%	NT	-5%
CD4	-1%	0%	0%	0%	1%	-5%	-2%	0%	NT	0%
CD8	-8%	-12%	-15%	-24%	-32%	-13%	-15%	-18%	NT	-21%
CD14	-87%	-71%	-49%	-54%	-52%	-2%	16%	-4%	-20%	-28%
CD15	-86%	-68%	-36%	-42%	-37%	3%	7%	-6%	-17%	-15%
CD16	-87%	-71%	-50%	-53%	-49%	-7%	-6%	-16%	-24%	-27%
CD33	-88%	-72%	-51%	-55%	-52%	0%	11%	-7%	-26%	-36%
CD56	-78%	-69%	-3%	-9%	-10%	-9%	-1%	17%	NT	9%
PD-L1	-77%	-32%	62%	191%	261%	-26%	-17%	-17%	NT	NT
CD27	-43%	-54%	-59%	-59%	-44%	-24%	-24%	-34%	NT	-54%
CD45RA	-11%	-6%	-7%	-6%	-6%	-11%	-3%	1%	NT	7%
CD62L	-92%	-93%	-93%	-93%	-93%	-68%	-77%	-80%	NT	-83%
CD69	36%	29%	59%	180%	278%	15%	6%	-11%	NT	-27%
HLA-DR	95%	59%	84%	92%	107%	19%	7%	-14%	NT	-41%
ICOS	19%	6%	12%	13%	15%	1%	-12%	-20%	NT	-32%
PD-1	6%	-8%	-9%	-19%	-14%	2%	-7%	-18%	NT	-31%
TIM-3	22%	3%	-6%	-10%	-10%	42%	27%	7%	NT	-13%

Tables IV. Stability of CCR7 and CD62L clone/fluorochrome combinations. Briefly, blood was collected in Cyto-Chex containing K3EDTA anticoagulant from 3 healthy volunteers and stored at 2-8°C (right). An aliquot of the sample was analyzed in triplicate at several time points post-venipuncture. The MdFI and frequency for each marker was analyzed on the intended cell population and % differences to baseline were calculated to assess stability. *% difference > 30% are highlighted*

Antibodies tested			Frequency			MdFI		
Marker	Fluorochrome	Clone	Day3	Day5	Day7	Day3	Day5	Day7
CCR7	Alexa 647	150503	21%	16%	23%	-15%	-22%	-23%
CCR7	PE-Cy7	3D12	80%	116%	120%	27%	57%	42%
CCR7	PE-Cy7	G043H7	7%	25%	37%	-13%	180%	209%
CD62L	Alexa 647	SK11	-54%	-51%	-45%	-68%	-70%	-71%
CD62L	PE-Cy7	DREG-56	0%	3%	15%	-57%	-68%	-72%

Dot plots for the Analysis of a Subset of Markers

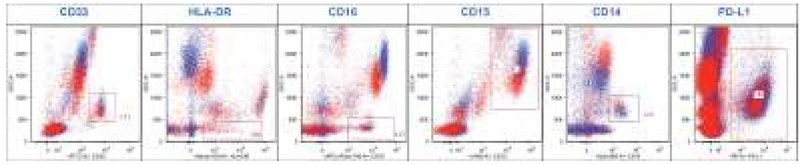
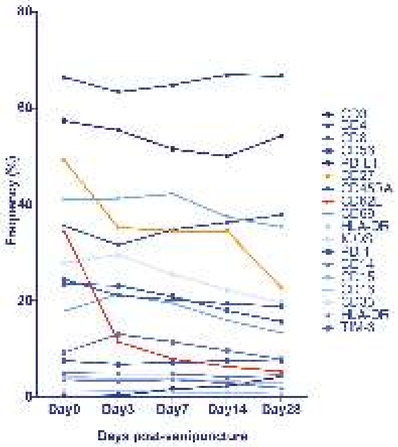


Figure 1. Example of gating used to compare timepoints. Visual Comparison Day 0 (D0) (in blue) and Day 7 (D7) (in red). All markers (CD33, HLA-DR, CD16, CD15, CD14, and PD-L1) were analyzed on lymphocytes, monocytes or granulocytes. Data shown is for blood stored at 2-8°C.



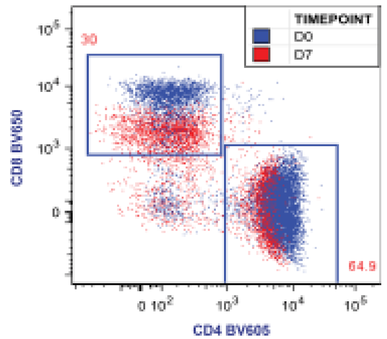


Figure 2. Stability of immune cell subsets analyzed in whole blood collected in Cyto-Chex BCT. Briefly blood was collected in Cyto-Chex containing K3EDTA anticoagulant from 3 healthy volunteers and stored at 2-8°C. An aliquot of the sample was analyzed in triplicate at several time points post-venipuncture with panels containing the markers listed in Table I. The frequencies for each cell subset was analyzed and presented over time.

Figure 3. Dot plot depicting the variation of the MFI within the positive gate. Visual comparison of D0 and D7 showing a decreased MFI while maintaining a stable frequency of CD4 and CD8 T cell subsets.

Conclusions

The following important points are to be considered when assessing stability using Cyto-Chex blood collection tubes:

- The distinction in the assessment of the stability of protein expression versus the stability of an ongoing biological process must be made. Both have to be carefully evaluated using a combination of cell lines expressing the marker of interest and activated samples mimicking the natural upregulation of the protein.
- The minimal amount of time that blood should be kept at ambient temperature prior to storing at 2-8°C in order to maximally fix the sample (simulating sample processing at the clinical site).
- In the stability design, spiking heterologous samples for a positive signal might induce some activation within the Cyto-Chex tube.
- Post-processing and pre-acquisition stability should also be evaluated particularly for large batch analyses.
- Assessing stability on the intended reported readouts, i.e. MdFI, frequency of subsets.

Contact: Caprion Biosciences Inc. • 201 President-Kennedy • Montreal, Quebec H2X 3Y7
dgagnon@caprion.com or yperetz@caprion.com