Unlocking Value of Flow Cytometry During Drug Development: Establishment of Whole Blood Sample Stability for Extended Analysis

D. Adle, J. Thompson, D. Shea, C. Sheldon, and R. Islam
Celerion, Lincoln, Nebraska

celerion
Applied Translational Medicine

INTRODUCTION

Quantification of specific cell populations in whole blood by flow cytometry is routinely performed in clinical diagnostic laboratories and more recently has been implemented for assessing safety and efficacy in drug development. Immunophenotyping of T and B lymphocytes is used to monitor some forms of immunodeficiency and autoimmune disease. NK lymphocytes have been shown to mediate cytotoxicity against certain tumors and virus-infected cells. Hematopoietic stem cells (HSC) are relevant in a number of clinical settings including the treatment of various leukemias. Neupogen/ Filgrastim, a recombinant form of granulocyte colony stimulating factor (G-CSF), can be administered following chemotherapy to accelerate recovery by releasing and producing granulocytes and CD34$^+$ HSCs. Elevated circulating CD34$^+$ HSCs is a pharmacodynamic marker of G-CSF efficacy.

Current clinical protocols stipulate that samples be processed within 24-48 hours. However, these time constraints are not feasible for large clinical studies. Commercial fixatives for whole blood are available, but provide limited stability. Isolation and cryopreservation of PBMCs for downstream analysis by cell flow cytometry is an alternative, but is technically demanding and labor intensive. In addition, processing of PBMCs introduces variability and can deplete certain cell types, leading to inaccurate results. To overcome this hurdle, a long-term cryopreservation method was developed for direct processing of whole blood for enumeration of multiple markers of clinical relevance by cell flow cytometry.

METHODS

Fresh whole blood samples were analyzed within 24hrs of draw with the remainder being aliquoted with stabilizer immediately for cryopreservation to assess stability by comparing to original values. Human whole blood with or without stabilizer was processed and stained using FDA approved Trucount™ kits (BD Biosciences) and analyzed via cell flow cytometry. Cryopreserved whole blood was stored at -80°C, thawed, then immediately stained and lysed for direct acquisition. Human blood was donated from normal healthy adult subjects. Granulocyte colony stimulating factor (G-CSF) mobilized human blood was purchased from AllCells (Alameda, CA) for enumeration of CD34$^+$ hematopoietic stem cells.

RESULTS

Cryopreserved whole blood for enumeration of T-cells, B-cells and NK-cells (TBNK) was validated for long-term stability. All populations were clearly identifiable when comparing fresh to cryopreserved samples (Figure 1). A theoretical value was obtained from fresh samples analyzed within 24hrs. Absolute values of cyropreserved samples closely agreed with original fresh samples throughout all time points (% theoretical 80.4-102.6% for T-cell, 88.2-107.7% for B-cell and 79.1-113.8% for NK-cell) (Tables 1-3). Sample stability for TBNK analysis was demonstrated for at least 60 days. Long-term stability was also assessed for T Helper (CD4$^+$) and Cytotoxic (CD8$^+$) T-cell subsets with similar results (data not shown).

Long-term stability was also validated for CD34$^+$ HSCs. A theoretical value was obtained from a fresh G-CSF mobilized whole blood sample analyzed within 24hrs of the blood draw without stabilizer. The fresh sample included a viability stain to exclude dead cells to be consistent with clinical protocols. Sample integrity was largely unaffected by the stabilization process (Figure 2). Sample stability was demonstrated for CD34$^+$ HSCs for at least 90 days. Absolute CD34$^+$ cells/µL closely matched the theoretical value (89.8-100.9%) (Table 4).

Figure 1.
Gating strategy for TBNK panel from a representative individual analyzed fresh (within 24hrs) left panel and 37 days post-freezing with stabilizer, right panel.

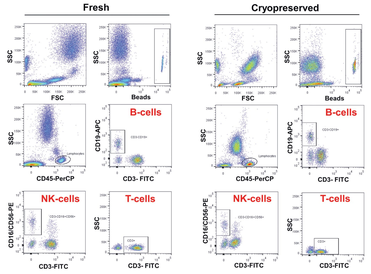

Figure 2.
Gating strategy for CD34$^+$ HSC enumeration of G-CSF mobilized whole blood analyzed within 24 hrs including viability stain (left panel) or with stabilizer analyzed 30 days post-freezing (right panel).

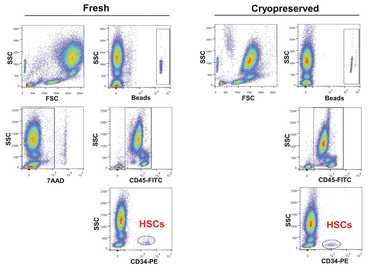

Table 1.
Absolute CD3$^+$ T lymphocytes per microliter from three individual subjects. A theoretical value was determined from Day 0 (fresh sample without stabilizer) and percent theoretical was calculated from days 7, 14, 37 and 60 (with stabilizer stored at -80°C). All samples were processed in triplicate.

T-Cell

Day	Subject 1			Subject 2			Subject 3		
	Mean T-Cell	% Theoretical	%CV	Mean T-Cell	% Theoretical	%CV	Mean T-Cell	% Theoretical	%CV
0	1875.1	100.0	7.8	2008.8	100.0	6.5	1218.7	100.0	2.7
7	1710.4	91.2	5.5	1799.3	89.6	2.4	1063.7	87.3	0.6
14	1692.8	90.3	2.3	1890.7	94.1	3.7	1089.4	89.4	1.9
37	1735.2	92.5	12.1	2060.2	102.6	8.6	1116.2	91.6	1.9
60	1722.5	91.9	6.2	1948.9	97.0	5.5	980.0	80.4	9.2

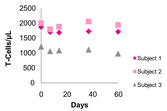

Table 2.
Absolute CD3$^-$CD19$^+$ B lymphocytes per microliter from three individual subjects. A theoretical value was determined from Day 0 (fresh sample without stabilizer) and percent theoretical was calculated from days 7, 14, 37 and 60 (with stabilizer stored at -80°C). All samples were processed in triplicate.

B-Cell

Day	Subject 1			Subject 2			Subject 3		
	Mean B-Cell	% Theoretical	%CV	Mean B-Cell	% Theoretical	%CV	Mean B-Cell	% Theoretical	%CV
0	373.5	100.0	9.7	498.9	100.0	9.6	145.9	100.0	7.1
7	348.6	93.3	3.9	466.6	93.5	5.8	128.7	88.2	5.6
14	348.0	93.2	4.6	508.8	102.0	4.2	129.4	88.7	6.1
37	347.5	93.0	14.9	537.1	107.7	11.3	132.6	90.9	4.1
60	347.5	93.0	8.2	533.2	106.9	5.1	129.6	88.8	7.7

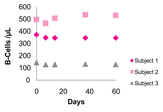

Table 3.
Absolute CD3$^-$CD16$^+$CD56$^+$ NK lymphocytes per microliter from three individual subjects. A theoretical value was determined from Day 0 (fresh sample without stabilizer) and percent theoretical was calculated from days 7, 14, 37 and 60 (with stabilizer stored at -80°C). All samples were processed in triplicate.

NK-Cell

Day	Subject 1			Subject 2			Subject 3		
	Mean NK-Cell	% Theoretical	%CV	Mean NK-Cell	% Theoretical	%CV	Mean NK-Cell	% Theoretical	%CV
0	187.7	100.0	7.8	172.2	100.0	10.9	124.5	100.0	9.9
7	158.8	84.6	7.8	158.2	91.9	8.6	110.9	89.0	4.3
14	166.9	88.9	6.8	173.8	100.9	5.3	115.2	92.5	7.5
37	164.3	87.6	8.5	196.0	113.8	4.3	103.3	83.0	10.7
60	158.3	84.3	9.7	183.5	106.5	9.1	98.5	79.1	10.2

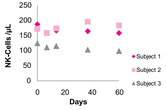

Table 4.
Absolute CD34$^+$ HSCs of G-CSF mobilized whole blood. A theoretical value was determined from Day 0 (fresh sample without stabilizer) and percent theoretical was calculated from days 7, 14, 30, 49, 70 and 90 (with stabilizer stored at -80°C). Day 0 included viability stain for analysis. All samples were processed in triplicate.

Mean CD34$^+$ (Fresh Sample)	Day 7 73.5 cells/µL	Day 14 73.5 cells/µL	Day 30 73.5 cells/µL	Day 49 73.5 cells/µL	Day 70 73.5 cells/µL	Day 90 73.5 cells/µL
	71.3	67.4	78.1	71.8	62.3	67.9
	75.3	71.9	70.6	66.0	64.1	70.7
	75.1	77.5	73.7	76.6	71.5	70.2
Mean	73.9	72.2	74.1	71.5	66.0	69.6
%CV	3.0	7.0	5.1	5.3	7.4	2.1
%Theoretical	100.6	98.4	100.9	97.3	89.8	94.2
n	3	3	3	3	3	3

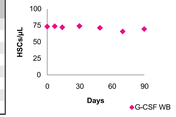

Table 5.
Cell types and CD markers evaluated for stability

Cell type	Cell Surface Marker	Stability
T Cell	CD45, CD3, CD4, CD8	> 60 days
B Cell	CD45, CD19	> 60 days
NK Cell	CD45, CD16, CD56	> 60 days
Hematopoietic Stem Cell	CD45, CD34	> 90 days

CONCLUSIONS

A method has been developed and validated for long-term stabilization of whole blood for the detection of several specific cell populations by flow cytometry without compromising sample integrity (Table 5). This provides the foundation for analysis of stabilized whole blood for the determination of many more specific subsets which may have applications to drug development. The benefits of whole blood stability for flow cytometric analysis are:

- Reduced personnel/instrument cost by batching samples for analysis during regular working hours
- Samples may be shipped from a clinical CRO to specialized flow cytometry labs for analysis without impacting quality
- Assay reproducibility can be confirmed by performing incurred sample reanalysis as required by FDA bioanalytical guidance
- New opportunities to analyze banked samples for additional phenotypic markers without need to repeat studies will significantly reduce client costs