T2234

A Validated Flow Cytometry Method for Absolute Quantification of CD34+ Hematopoietic Stem Cells with Extended Sample Stability

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Introduction

Neupogen/Filgrastim is a recombinant form of granulocyte colony stimulating factor (G-CSF) that may be administered following chemotherapy to accelerate recovery by stimulating production and mobilization of granulocytes and hematopoietic stem cells (HSCs) from the bone marrow into the blood stream. HSCs are characterized by expression of the CD34 protein on the cell surface by flow cytometry. HSC levels are very low in normal individuals however, become elevated following G-CSF administration. Thus, quantification of HSCs is a pharmacodynamic (PD) marker used to monitor G-CSF mobilization. Since G-CSF dramatically increases total white blood cell counts (WBC), HSCs are reported in absolute rather than relative values.

A major challenge with analysis of CD34+ HSCs in whole blood is sample instability. In clinical diagnostic labs, CD34+ HSC analysis is performed within 24 hr which is not feasible in large clinical studies over extended time periods. This time constraint does not allow for sample batching, shipping long distances or confirming assay reproducibility by incurred sample reanalysis (ISR), as recommended by FDA for bioanalytical methods.

Options are available to extend sample analysis but have their drawbacks. Commercial whole blood fixatives have limited stability, can over-fix cells and are limited to specific cell types. Cryopreservation of peripheral blood mononuclear cells (PBMCs) is an alternative for long-term stability but is labor intensive, can deplete certain cell types, and does not allow for accurate absolute quantitation as required for CD34+ HSC analysis. Due to these limitations, a novel method was developed for direct staining of cryopreserved whole blood for accurate and reproducible absolute quantitation of CD34+ HSCs extending analysis over 6 months without compromising sample integrity.

Purpose

- Develop and validate a flow cytometry method for absolute quantification of CD34+ Hematopoietic stem cells (HSC) as a pharmacodynamic (PD) marker for biosimilar comparison to Neupogen/ Filgrastim
- Overcome current sample instability issues to allow for batching, reanalysis of samples (ISR) and shipping long distances for large clinical studies

Methods

CD34+ cellular controls (Streck, Omaha, NE) or G-CSF mobilized blood (AllCells, Alameda, CA) were processed and stained identically using BD™ Stem Cell Enumeration Kit (BD Biosciences, San Jose CA). Viability staining was performed on unfrozen, fresh samples without stabilizer as recommended by ISHAGE guidelines. Stabilized whole blood samples were stored at -80°C prior to direct staining, processing and acquisition. Trucount™ tubes were used for determination of absolute cell numbers using the single platform method. Flow cytometry was performed on a Cytek DxP6 FACSCalibur flow cytometer using FlowJo Collectors' Edition software (Cytek, Fremont CA). 80,000 CD45+ events were collected during acquisitions. Post-acquisition, data was analyzed using FlowJo version 10.0 (TreeStar, Ashland OR).

Results

A "fit-for-purpose" approach was utilized for method validation. Assay performance was evaluated for accuracy, inter- and intra-batch precision, inter-instrument comparability, dilution integrity, and sample stability. Precision and accuracy were evaluated by analyzing three levels of FDA approved CD34+ clinical cellular controls. Three batches of three levels were run in triplicate with one batch by a different analyst on a different day. The means of all replicates were within the manufacturer's specification for all levels with a %CV less than 20% (Table 1).

Inter-instrument comparison was assessed by analyzing six sample preps of frozen G-CSF mobilized whole blood on two instruments with the same configuration and a percent difference between instruments was determined (Table 2). Fluorescent standard beads were used to determine mean fluorescent intensity target values for the assay to achieve inter-instrument and longitudinal comparability.

Evaluation of dilution integrity demonstrated that a dilution factor can be applied to the assay if required (Table 3). Frozen G-CSF mobilized whole blood was thawed and processed neat or diluted two and four fold prior to staining and acquisition. Percent recoveries with applied dilution factors were within 80-120% of the undiluted sample.

Robustness and benchtop stability were assessed by extending sample staining and instrument acquisition times. Assay performance met specifications with 80-120% recovery of control samples (Table 4).

Long-term stability of whole blood was established for at least six months post-freezing compared to the same sample analyzed within 24 hr following current clinical guidelines. All stability time points of cryopreserved whole blood met specifications (80-120%) of the fresh sample (Table 5).

Figure 1. Gating strategy for CD34+ HSC enumeration of G-CSF mobilized whole blood analyzed within 24 hr including viability stain (left panel) or same sample with addition of stabilizer analyzed post-freezing (right panel).

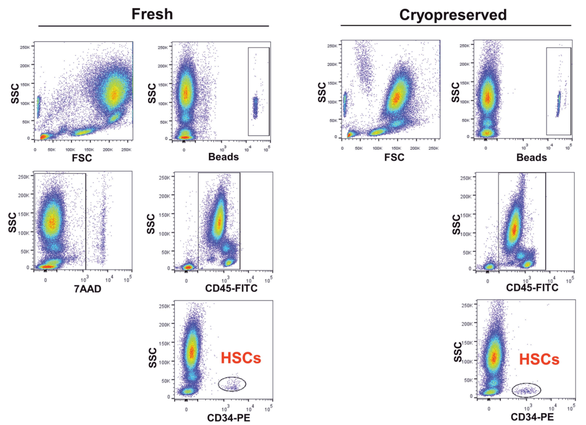


Table 1. Inter-Batch Precision and Accuracy. Results of three batches of three different levels of CD34+ cellular controls assayed in triplicate on different days by two analysts.

Level	Mean Absolute CD34+	% CV	n	Manufacturer Specification	Pass/Fail
Level 1	3.0	18.1	9	0.6 - 5.5	Pass
Level 2	35.2	6.5	9	27.8 - 41.8	Pass
Level 3	126.4	7.6	9	103.1 - 143.1	Pass

Table 2. Inter-Instrument Comparison. Six sample preps of G-CSF mobilized whole blood analyzed post-freezing on two different flow cytometers having the same configuration.

Instrument	Mean Absolute CD34+ (Cells/µL)	% CV	n	% Difference
1	68.0	8.22	6	0.15
2	67.9	10.3	6	0.15

Table 3. Dilution Integrity. G-CSF mobilized whole blood was analyzed in triplicate post-freezing processed neat or diluted two and four fold prior to staining and acquisition. A percent recovery was calculated after dilution factors were applied.

Dilution Factor	Mean Absolute CD34+ (Cells/µL)	% CV	n	% Recovery
1	77.7	15.0	3	100
2	73.9	9.2	3	95
4	86.8	12.7	3	112

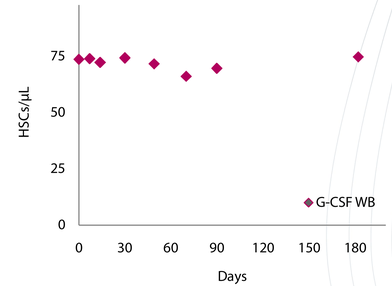
Table 4. Robustness and Benchtop Stability. Sample staining time and time to instrument acquisition were evaluated for effects on assay performance.

Parameter	Mean Absolute CD34+ (Cells/µL)	% CV	n	% Recovery
Control	79.1	11.3	3	100
2x Stain Time	65.6	14.7	3	82.9
4X Acquisition time	69.1	10.8	3	87.3

Table 5. Long-Term Stability. Stability was assessed by comparing absolute CD34+ values of stabilized frozen whole blood samples thawed and processed at different time points to the same fresh sample analyzed within 24 hr prior without stabilizer including viability stain.

Time Point	Day 0	Day 7	Day 14	Day 30	Day 49	Day 70	Day 90	Day 182
Mean Absolute CD34+ (Cells/µL)	68.1	71.3	67.4	78.1	71.8	62.3	67.9	76.9
	78.4	75.3	71.9	70.6	66.0	64.1	70.7	72.7
	73.9	75.1	77.5	73.7	76.6	71.5	70.2	74.6
	73.5	73.9	72.2	74.1	71.5	66.0	69.6	74.7
%CV	7.1	3.0	7.0	5.1	5.3	7.4	2.1	2.8
% of Fresh Sample	100	100.6	98.4	100.9	97.3	89.8	94.2	101.7
n	3	3	3	3	3	3	3	3

Figure 2. Long-Term Stability.



Conclusions

A validated CD34+ HSC flow cytometry method is available for comparative analysis of biosimilars with Neupogen/Filgrastim. Unique benefits made possible for the first time include:

- Greater than 6 month stability of preserved whole blood samples for absolute quantification of CD34+ HSCs
- Whole blood samples can be shipped from anywhere in the world to a central lab for analysis without compromising sample integrity
- Reduced personnel/instrument cost by batching samples for analysis during regular working hours
- Assay reproducibility can be confirmed by performing ISR according to FDA bioanalytical guidance