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Applied Translational Medicine

White Paper

# A Validated Method For Detection of CD34+ Hematopoietic Stem Cells With Extended Sample Stability


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## Introduction

Hematopoietic stem cells (HSC) are characterized by the expression of CD34, and are relevant in a number of clinical settings including the treatment of various leukemias. Neupogen/Filgrastim is a recombinant form of granulocyte colony stimulating factor (G-CSF) and may be administered following chemotherapy to accelerate recovery by releasing and producing granulocytes and CD34+ HSCs. Elevated circulating CD34+ HSCs is a pharmacodynamic (PD) marker of G-CSF efficacy.

CD34+ HSC enumeration by cell flow cytometry is routinely performed by clinical labs to monitor stem cell transplantation and mobilization with G-CSF. However, protocols mandate that samples be run fresh (within 24hrs) of draw which is not feasible for clinical studies with large sample volumes over extended periods of time. An accurate and reproducible method for enumeration of CD34+ HSCs by flow cytometry is described, which demonstrates that extended analysis beyond 30 days without compromising sample integrity is achievable.

## Methods

CD34+ cellular controls (Streck, Omaha, NE) or G-CSF mobilized blood (AllCells, Alameda, CA) fresh or stabilized were processed and stained using BD™ Stem Cell Enumeration Kit (BD Biosciences, San Jose CA). Analysis was modified from ISHAGE guidelines as viability staining was not included for stabilized samples. Stabilized whole blood samples were stored at -80ºC prior to analysis. Trucount™ tubes were used for determination of absolute numbers of cells 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

Assay performance was evaluated for precision and accuracy by analyzing three levels of CD34+ cellular controls in triplicate. A total of three analytical runs were performed with one on a different instrument by a different analyst on a different day *(Table 1)*. The mean value of all replicates for each level was within manufacturer claims.

*Table 1. Inter-batch Precision and Accuracy*

| Level | Mean | %CV | n | Expected Range | Pass/ Fail |
|---|---|---|---|---|---|
| Level 1 | 3.0 | 17.1 | 9 | 0.6-5.5 cells/µL | Pass |
| Level 2 | 35.2 | 6.1 | 9 | 27.8-41.8 cells/µL | Pass |
| Level 3 | 126.4 | 7.1 | 9 | 103.1-143.1 cells/µL | Pass |

Inter-instrument precision was assessed by analyzing G-CSF mobilized human blood in six replicates analyzed on two different instruments with the same configuration *(Table 2)*. Nearly identical results were obtained between instruments (0.4% difference).

*Table 2. Inter-instrument Precision*

| | Instrument 1 | Instrument 2 |
|---|---|---|
| AVG cells/µL | 73.5 | 73.2 |
| %CV | 8.2 | 10.3 |
| n | 6 | 6 |
| % Difference | 0.4% | |

Administration of G-CSF can cause elevation of WBCs above 40,000 cells/µL, which may necessitate dilution of the sample for accurate analysis. Evaluation of dilution integrity of a G-CSF mobilized sample demonstrated that a dilution factor may be applied for this assay *(Table 3.)*

*Table 3. Dilution Integrity*

| DF=1 | DF=2 77.7 cells/µL | DF=4 77.7 cells/µL |
|---|---|---|
| | 75.2 | 74.3 |
| | 80.0 | 95.2 |
| | 66.6 | 90.9 |
| | | |
| Mean | 73.9 | 86.8 |
| %CV | 9 | 12.7 |
| %Theoretical | 95.2 | 111.8 |

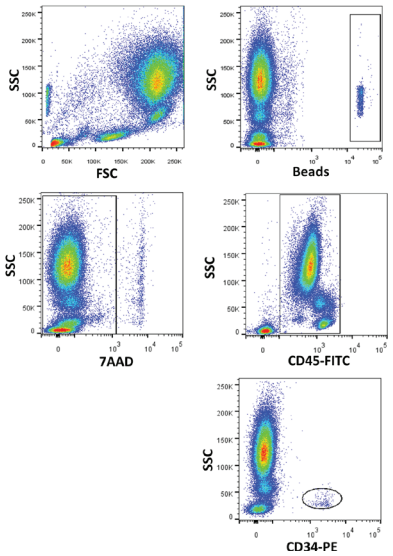
Long term stability was established up to 30 days post-freezing of stabilized whole blood. A theoretical value was obtained from a fresh G-CSF mobilized sample analyzed within 24hrs of the blood draw without stabilizer. The fresh sample included a viability stain to exclude dead cells which is performed in clinical protocols. Sample integrity was largely unaffected by the stabilization process *(Figure 1)*. Absolute CD34⁺ cells/μL closely matched the theoretical value *(Table 4)*.

*Table 4. Long-Term Stability*

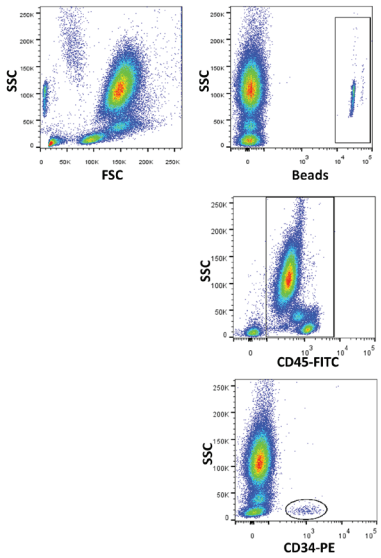
| **Mean CD34⁺ (Fresh Sample)** | **Day 7 73.5 cells/μL** | **Day 14 73.5 cells/μL** | **Day 30 73.5 cells/μL** |
|---|---|---|---|
| | 71.3 | 67.4 | 78.1 |
| | 75.3 | 71.9 | 70.6 |
| | 75.1 | 77.5 | 73.7 |
| | | | |
| Mean | 73.9 | 72.2 | 74.1 |
| %CV | 3.0 | 7.0 | 5.1 |
| %Theoretical | 100.6 | 98.4 | 100.9 |
| n | 3 | 3 | 3 |

*Figure 1. Gating Strategy for CD34⁺ HSC Analysis*

a. Fresh Sample (24hrs)




b. Stabilized Sample (Day 30)




Scatter plots of G-CSF mobilized whole blood analyzed within 24hrs including viability stain (*Figure 1a*) or with stabilizer analyzed 30 days post-freezing (*Figure 1b*).

## Conclusions

A validated method for CD34⁺ HSCs with extended sample stability is available for comparative analysis of biosimilars with Neupogen/Filgrastim. Importantly, integrity of stabilized samples is not compromised as values closely match those obtained under current clinical guidelines. Previously, reliable analysis of CD34⁺ HSCs required that samples be analyzed onsite due to time constraints of sample stability. Now, whole blood samples can be batched and shipped long distances to a central analytical lab. This novel method allows for analysis of large cohorts, with several collection time points and alleviates the burden of running freshly drawn samples in a short time period, thereby mitigating risk of dropped time points. It also provides the ability to re-evaluate samples post-analysis (ISR) per FDA guidance for a flow cytometry method. Lastly, it may be possible to "bank" samples for future analysis of additional cellular markers to further support the drug application process without the need to repeat costly clinical studies.