TECHNICAL BULLETIN

Purity Assessment by Flow Cytometry for Lineage-Specific Chimerism Analysis

The Importance of Purity Assessment in Chimerism Analysis

The EFI and ASHI now officially recognize purity assessment as an essential step in lineage-specific chimerism analysis, yet many chimerism labs are still unfamiliar with this procedure. **This Technical Bulletin reviews the importance of lineage-specific chimerism analysis, explains the need for purity assessment, and provides guidelines for assessing purity using flow cytometry.**

Isolating Cell Subsets Increases the Sensitivity and Utility of Chimerism Assays

Lineage-specific chimerism analysis is an important tool for monitoring the outcome of allogeneic hematopoietic cell transplantations (allo-HCT). Depending on engraftment success, the host may either achieve full donor chimerism or reach a state of **mixed chimerism**, in which host hematopoiesis persists alongside donor hematopoiesis in the recipient.[1] Chimerism status in transplant recipients can indicate the potential dynamics of disease relapse, graft-versus-host disease (GVHD), (GVT) effects and other outcomes.[2-9]

Investigating chimerism within specific cell subsets is an increasingly common practice that offers several advantages over analyzing the entire leukocyte population, particularly in recipients of non-myeloablative allo-HCT.[10,11] One such advantage is increased assay sensitivity. If a patient has mixed chimerism in only one or a few cell lineages, the overall proportion of host cells may be too low for detection by whole blood assays.[1] Furthermore, the significance of mixed chimerism differs between cell subsets. For instance, one recent study found that the degree of mixed chimerism within T and NK cells, but not myeloid cells, allowed patients to be classified into different risk groups for graft rejection.[12] In another study, full donor T cell chimerism consistently preceded GVT effects.[13] Recent literature suggests that this type of lineagespecific analysis is highly informative for chimerism labs.[14-18]

Purity Assessment Ensures the Reliability of Lineage-specific Analyses

Performing lineage-specific chimerism analysis can increase the sensitivity of assays and provide essential information. Both of these benefits depend upon the purity of isolated cell subsets, as contamination by non-target cells decreases the reliability of lineage-specific analysis. These issues are particularly common with pathological samples, in which leukocyte numbers and proportions can be variable and atypical.[19] Assessing the purity of isolated cell populations is therefore an essential quality control step. As of 2011, the official EFI and ASHI guidelines stipulate that the purity of sorted cell populations must be documented and taken in account when results are analyzed.

> "When HCE (Haemopoietic Chimaerism and Engraftment) testing is performed on cellular subsets isolated by cell sorting, the purity of the sorted population must be documented and taken into account in the analysis of the results. If this is not possible it must be clearly stated in the report."
>
> **EFI Standards version 5.6.1 (2011), Section I4.190**

> "Document the purity obtained if processing involves isolation of cell subsets. If purity is not assessed, this must be documented on the test report."
>
> **ASHI Standards 2011, Section D.5.3.4.1.8**

One of the most common and accurate ways to assess purity is with flow cytometric analysis. The following pages provide step-by-step instructions for performing purity assessments using flow cytometry.

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How to Perform Purity Assessments

Purity assessment is critically important in chimerism analysis to ensure that cell subsets are not contaminated by non-target cells. The most common method for purity assessment is flow cytometry, in which target cells are labeled with fluorescent markers and analyzed using a flow cytometer, allowing the proportion of each cell type in the sample to be calculated.

Choosing the Correct Antibodies for Staining

When preparing samples for purity assessment, the appropriate antibodies for staining depend on the cell type and mode of selection (i.e. positive or negative). **Table 1** shows the recommended staining reagents for specific cell types.

Some antibodies used in **positive selection** may fully or partially block the primary cell surface marker. Under these circumstances, there are three different methods of staining for purity assessment:

1. Use antibodies targeting alternative cell surface markers on the desired cell population.
2. Add a fluorochrome-conjugated antibody (e.g. PE-labeled) targeting the primary cell surface marker at the same time as the selection cocktail.
3. Use a fluorochrome-conjugated secondary antibody, such as a FITC-labeled goat anti-mouse IgG. These antibodies will bind to the antibodies used for positive selection.

Negative selection isolates unlabeled target cells. Samples separated by this method can be stained using antibodies against the primary cell surface marker (e.g. CD3 for T cells and CD19 for B cells).

Staining procedures for purity assessment are provided in the Product Information Sheets (PIS) for all EasySep™ and RoboSep™ isolation kits (see product list on final page). Consult the appropriate PIS for detailed instructions.

Step-by-Step Protocol: Purity Assessment by Flow Cytometry

Staining Cells for Flow Cytometry

1. After cell separation, place 100 µL of enriched cells into two separate 5 mL FACS tubes (cells should be at a concentration between 1 x 10^6 and 1 x 10^7 cells/mL).
2. To the first tube, add the appropriate fluorescently-conjugated monoclonal antibody or antibodies (see **Table 1**) according to the antibody manufacturer's instructions. The volume will typically be 5-20 µL of antibody per test.
3. To the second tube, add the appropriate fluorescently-conjugated isotype control antibody (e.g. FITC mouse IgG_1).
4. If desired, add a viability stain such as propidium iodide (PI) or 7AAD to each sample. This step allows dead cells to be gated out for more accurate flow cytometry analysis.
5. Incubate at 2 – 8°C or on ice for 30 minutes in the dark.
6. Wash cells with 1 mL of PBS. Pour off supernatant and resuspend the pellet in 100-500 µL of PBS or FACS sheath fluid. If samples cannot be analyzed immediately, fix with 1% paraformaldehyde and store for up to two weeks at 2 – 8° protected from light.

Table 1. Primary markers and recommended staining reagents for specific cell lineages

CELL TYPE	PRIMARY MARKERS	RECOMMENDED STAINING REAGENTS FOR ISOLATED CELLS	
		If Positively Selected	If Negatively Selected
T Cells	CD3	CD5 and CD20 (T cells = $CD5^+CD20^-$) or CD2 (note: NK cells are also $CD2^+$)	CD3
B Cells	CD19	CD20 or CD22	CD19
Myeloid Cells	CD15 and/or CD33	CD14 and CD66b	CD33
Granulocytes	CD66b	CD66b clone G10F5	CD66b
Monocytes	CD14	CD14 clone UCHM1 or MOP9, or CD36	CD14
NK Cells	CD56	CD56 clone NCAM16.2	CD3 and CD56 (NK cells are $CD3^-CD56^+$)
Hematopoietic Progenitors	CD34	CD34 clone 8G12 or 581, AC136, or BirmaK3	CD34

Gating Sample for Target Cells

In order to obtain an accurate assessment of purity, it is important to gate out cellular debris and dead cells during analysis of the sample. The purpose of purity assessment in chimerism analysis is to ensure that enriched cells are not contaminated by other nucleated cells. Since RBC and debris do not contain nuclei and will not affect downstream DNA-based assays, they should be excluded from the flow cytometry analysis.

To gate the target cell population:

1. Create a dot plot of the data displaying FSC vs. SSC, and place a gate around all leukocytes. RBC and debris will be visible in the bottom left corner of the plot, and should be excluded by the gate. (**Figure 1**, left panel).
2. Create a second dot plot of the data displaying FSC vs. the viability stain. Dead cells will be positive for the viability marker and should be excluded by the gate. **Figure 1** (right panel) provides an example of gating out dead cells using propidium iodide (PI), a common viability stain.

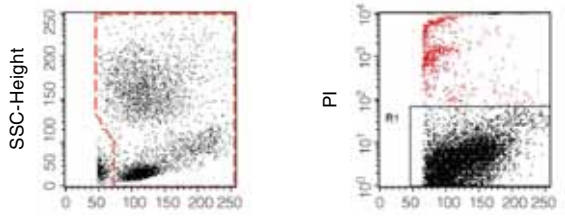

Figure 1. Examples of gates excluding RBC and debris (left) and dead cells (right)

Assessing Sample Purity

Collect 10,000 - 50,000 events for each sample. Sample purity is the percentage of cells positive for the relevant staining antibody in the gated population (**Figure 2**). The procedure for obtaining purity data will depend on the flow cytometry software used.

In keeping with EFI and ASHI guidelines, document the purity assessment results clearly in your report.

For more details, please contact techsupport@stemcell.com. Guidelines for purity assessment are provided in the Product Information Sheets for all EasySep™ and RoboSep™ kits.

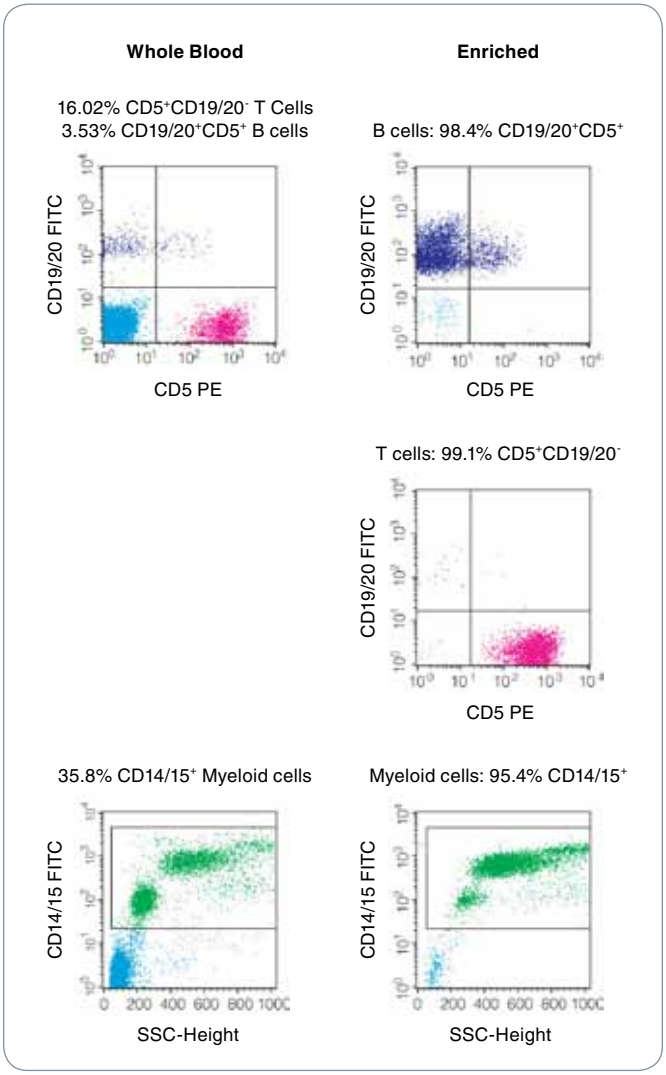

Figure 2. Typical FACS plots before and after enrichment of selected cells (plots show viable (PI^-) cells gated on $CD45^+$)

Purity Assessment by Flow Cytometry for Lineage-Specific Chimerism Analysis

EasySep™ Kits for Isolating Cells for Chimerism Analysis and Recommended Antibodies for Purity Assessment

CELL TYPE	MARKER	EASYSEP™ REAGENT KIT		RECOMMENDED STAINING ANTIBODIES	
		Tissue	Catalog#	Antigen, Clone	Catalog#
T cells	CD3	WB[1,2] PBMC, BM, SPLEEN, LN[3]	18081HLA 18051HLA	Anti-Human CD5, UCHT2 and Anti-Human CD2, RPA-2.10 or Anti-Human CD20, 2H7	60082 60007 60008
B cells	CD19	WB PBMC, BM, SPLEEN, LN	18084 18054	Anti-Human CD20, 2H7 or Anti-Human CD22, HIB22	60008 60083
	CD19/CD20	WB PBMC, BM, SPLEEN, LN	18184HLA 18454HLA	Anti-Human CD22, HIB22 or Anti-Human CD45, HI30 and Anti-Human CD43, CD43-10G7	60083 60018 60085
Myeloid Cells	CD15	WB	18681HLA	Anti-Human SSEA-1 (CD15), MC-480	60060
	CD33	WB PBMC, BM, SPLEEN, LN	18287HLA 18257	Anti-Human CD14, M5E2 and Anti-Human CD66b, G10F5	60004 60086
	CD33/66b	WB	18683HLA	Anti-Human CD14, M5E2 and Anti-Human CD66b, G10F5	60004 60086
Granulocytes	CD66b	WB	18682	Anti-Human CD66b, G10F5	60086
Monocytes	CD14	WB PBMC, BM, SPLEEN, LN	18088 18058	Anti-Human CD14, M5E2 or Anti-Human CD36, FA6-152	60004 60084
NK Cells	CD56	WB PBMC, BM, SPLEEN, LN	18085HLA 18055	Anti-Human CD56 (NCAM), HCD56	60021
Hematopoietic Progenitor Cells	CD34	WB PBMC, BM, SPLEEN, LN	18086 18056RF	Anti-Human CD34, 581	60013

1. Anti-Human CD45, HI30 (Catalog# 60018) is also recommended for all whole blood purity assessments.
2. WB = Whole Blood; Whole blood kits also work on other red blood cell-containing samples (ie. buffy coat, cord blood, bone marrow).
3. PBMC = Peripheral Blood Mononuclear Cell; BM = Bone Marrow; LN = Lymph Node

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Antibodies from STEMCELL Technologies

Ensure consistent downstream cell analysis, including purity assessments, by using high-quality primary and secondary antibodies. These antibodies are verified to work with our cell isolation reagents in specific applications.