Development and Validation of a New Clinical Assay: T-cell Proliferation with PHA Mitogen Stimulation Detected by Flow Cytometry

Samuel Maltby, MSIV 1; Vijaya Knight, MD/PhD1 2
1University of Colorado, School of Medicine, 2Children’s Hospital Colorado

Background

Severe Combined Immunodeficiency (SCID) is a collection of adaptive immunodeficiencies with many gene variations and various clinical presentations. It is traditionally characterized by a low T cell population resulting in immune dysfunction. New born screening for SCID is done in all 50 states and requires confirmation testing if positive. This is typically done with a PHA stimulation assay. Older assays like the Lymphocyte Stimulation Assay (LSTIM) use tritiated thymidine to detect proliferation through incorporation in newly synthesized DNA. These lab assays are useful in detecting overall lymphocyte proliferation, but are limited in determining additional information about the cells. A new assay using flow cytometry allows for additional information and better selection of cells that are proliferating and can be useful in situations like extreme lymphopenia. When developing a new assay, it is important to ensure the results are accurate and repeatable demonstrated through appropriate validation.

Methods

This assay uses EdU, a nucleoside analogue, that can undergo a copper mediated reaction to label a fluorescent acid that can be detected by flow cytometry. Cells are also surfaced stained with CD45 and CD3 for selection of T cells. Prior to staining, peripheral Blood mononuclear cells (PBMCs) are separated from whole blood through centrifugation and stimulated with PHA and incubated for 3 days. Cells are pulsed with EdU on the final day. Samples collected for normal donors were cross validated. Cells are also surfaced stained with CD45 and CD3 for selection of T cells identified by EdU staining.

Figure 1: Gating Strategy – Identification of proliferating CD3+ T cells and total lymphocytes (CD45+) A: Histograms of unstimulated and stimulated cells. B: Proliferating lymphocytes and T cells identified by EdU staining.

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Figure 2: Specificity – Specificity was determined by fluorescence-minus-one (FMO) approach in which individual fluorescent antibodies are dropped to determine level of non-specific staining.

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Table 1: Accuracy results – Using 20 normal donor samples, the flow assay detected 20 normal results, while the LSTIM detected 4 low stimulation samples.

Table 2: Intra-assay precision: CV of samples run in triplicate on the same day. Predetermined acceptance level of a CV <0.25%.

Table 3: Reference Ranges – Determined by 31 adult normal donor patients. Shows lower limits of normal.

Table 4: Sample stability – % of CD3+ T cells proliferating at 0, 24, and 48 hr

Conclusions

The new flow cytometry assay performs well in terms of accuracy, specificity, sensitivity, stability, and precision. It was able to detect normal proliferation in samples deemed low proliferation in the LSTIM assay, possibly due to its ability to detect specifically labeled T cells. While gathering data, it was also demonstrated that T cell proliferation was able to be detected in an extremely lymphopenic patient (chart not shown) and further demonstrated by the sensitivity of the assay. Some limitations in our validation ranges were determined using adult populations, not pediatric populations. Sample size is also small, and continued analysis of data will be needed to continue to assess the assay. Moving forward the assay could be useful for confirmatory testing of SCID, and also potentially used as demonstration of reconstitution of immune function following bone marrow transplant.

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