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

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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 is 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 catalyzed reaction to bind a fluorescent azide 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-tested against the traditional LSTIM assay performed at National Jewish Health. Validation criteria included accuracy, precision, specificity, sensitivity, stability, and determination of reference ranges.

Results

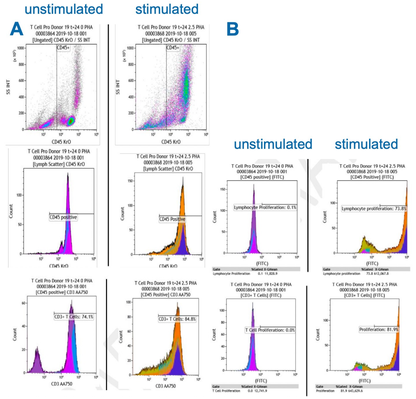

Figure 1: Gating Strategy – Identification of proliferating CD3+ T cells and total lymphocytes (CD45+). A: Histograms of unstimulated and stimulated cells. B: Proliferating lymphocytes and T cells identified by EdU staining.

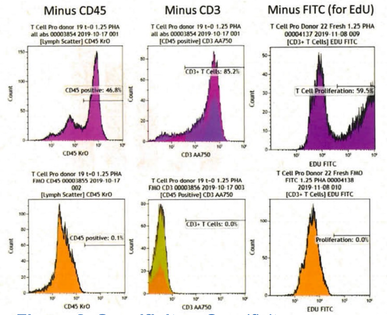

Figure 2: Specificity – Specificity was determined by fluorescence-minus-one (FMO) approach in which individual fluorescent antibodies are dropped to determine level of non-specific staining.

Sample	Flow Cytometry Assay Result	LSTIM Assay Result
Donor One	Normal	Normal
Donor Two	Normal	Normal
Donor Three	Normal	Normal
Donor Four	Normal	Normal
Donor Five	Normal	Normal
Donor Six	Normal	Normal
Donor Seven	Normal	Low
Donor Eight	Normal	Low
Donor Nine	Normal	Normal
Donor Ten	Normal	Normal
Donor Eleven	Normal	Normal
Donor Twelve	Normal	Normal
Donor Thirteen	Normal	Normal
Donor Fourteen	Normal	Normal
Donor Fifteen	Normal	Normal
Donor Sixteen	Normal	Normal
Donor Seventeen	Normal	Normal
Donor Eighteen	Normal	Normal
Donor Nineteen	Normal	Low
Donor Twenty	Normal	Low

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

	Percent CV of %EdU+ CD3+ T cells					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average
0 PHA	7.1	0.0	0.0	0.0	0.0	1.4
1.25 PHA	0.5	0.3	0.3	0.4	1.2	0.6
2.5 PHA	0.5	0.6	0.8	0.8	1.9	0.9
5 PHA	0.4	1.1	1.0	0.3	1.3	0.8
10 PHA	0.1	0.8	0.8	0.5	1.3	0.7

Table 2: Intra-assay precision: CV of samples run in triplicate on the same day. Predetermined acceptance level of a CV <25%

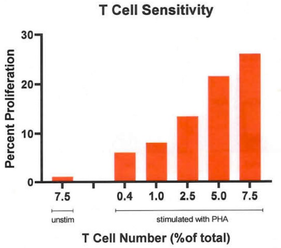

Figure 3: Sensitivity – Analysis of sensitivity through T cell depletion and ability to detect proliferation compared to background.

Reference Range % of Proliferating Cells		
	Lymphocytes	T Cells
0.0 PHA	0.8	0.7
0.3 PHA	22.3	29.1
0.6 PHA	39.8	53.5
1.25 PHA	53.3	66.2

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

(A) % CD3 Proliferation Following Stimulation															
	Donor 5			Donor 15			Donor 16			Donor 19			Donor 21		
	T=0	T=24	T=48	T=0	T=24	T=48	T=0	T=24	T=48	T=0	T=24	T=48	T=0	T=24	T=48
0.0 PHA	2.0	2.0	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.3 PHA	60.5	61.8	62.1	21.5	25.8	18.9	56.3	63.4	52.3	29.1	31.4	29.3	55.6	53.6	57.2
0.6 PHA	75.1	76.1	77.1	56.6	69.6	34.9	77.6	72.8	74.8	62.1	68.0	43.4	75.5	76.7	74.8
1.25 PHA	83.5	84.2	84.6	77.8	79.5	62.6	83.7	82.2	79.1	74.3	77.8	59.6	78.3	77.2	83.1
2.5 PHA	83.1	84.0	84.4	79.2	82.1	60.2	83.6	78.4	78.5	75.4	81.9	65.7	80.4	78.1	82.2
(A) Percent Difference from Day Zero															
	Donor 5			Donor 15			Donor 16			Donor 19			Donor 21		
0.0 PHA		0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0		0.0	0.0
0.3 PHA		2.1	1.3		18.2	6.4		11.9	3.7		3.8	0.3		3.6	1.5
0.6 PHA		1.3	1.3		20.6	23.7		6.4	1.8		4.5	17.7		1.6	0.4
1.25 PHA		0.8	0.7		2.2	10.8		1.8	2.8		2.3	11.0		1.5	2.9
2.5 PHA		1.1	0.8		3.6	13.6		6.4	3.1		4.1	6.9		2.9	1.1

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

Conclusions

The new flow cytometry assay performs well in terms of accuracy, sensitivity, specificity, stability, and precision. It was able to detect normal proliferation in samples deemed low proliferation in the LSTIM assay, possibly due to it's ability to select specifically select for 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 are that reference ranges were determined using adult populations, not pediatric populations. Sample size is also small, and continued analysis of data will be need to continue to assess the assay. Moving forward this assay could be used for confirmatory testing of SCID, and also potentially used as demonstration of reconstitution of immune function following bone marrow transplant.

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