

ABSTRACT:

Functional immune cell based assays have been routinely used in basic research stages of the development of vaccines and biologics. However, their utility in clinical trials as surrogate markers of efficacy has not been fully recognized due to their highly variable nature. The variability arises from a variety of factors ranging from choice of assay, source of the cells, the sample processing methodology, sample preparation for the chosen assay and ultimately data analysis, and data reduction.

With a view to reducing variability and standardizing targeted steps of functional cell-based assays, an automated methodology for simultaneous staining and analysis of T cell function as determined by the presence of intracellular cytokines via flow cytometry was developed and validated. A 5-color flow cytometry assay (2-3 surface markers; 2-3 intracellular markers) was developed to measure antigen specific (peptide pool) dual cytokine profile responses in human PBMCs. Modifications to available sample preparation instruments were performed that enabled the automated pipetting, incubation, and staining of intracellular and surface molecules of stimulated human peripheral blood mononuclear cell populations (PBMC) for flow cytometric analysis.

The automated sample staining and analysis greatly reduced variability between specimens as compared to the manual methodology in the evaluation of the "rare event" cell-based functional assay. For a 0.5% population of cells, the manual method %CV=39, while the automated method %CV=15. Additionally, the automation enabled a significant reduction of "hands-on" sample preparation and analysis time. The complex nature of the cytokine profile and inter- and intra-subject variability was revealed on evaluation of the multiparametric T cell responses. The use of automation thus provides a greater degree of standardization in these functional assays, allowing for a correlation of variability and complex immune response profiles as surrogate markers of vaccine efficacy.

MATERIALS:

- RPMI-1640 pen/strep/glutamine with 10% FCS (in house)
- Staphylococcus Enterotoxin B (SEB) – List Biological Laboratories
- CD28 purified (in-house)
- CEF peptide pool (Panatecs)
- PBS (Beckman Coulter)
- Monoclonal Antibodies Beckman Coulter catalog items used at recommended doses
- IntraPrep Kit (Fixation, Lysis, and Permeabilization. Beckman Coulter)

METHODS:

In vitro Stimulations:

Cultures were 10×10^6 frozen PBMC in RPMI 1640-10% FCS [in-house]. The stimulated tubes received either: 2mg/ml Staphylococcus Enterotoxin-B (SEB) [Sigma] and 0.03mg/ml Co-stimulator anti-CD28, or 2ug/ml of the CEF peptide pool. Non-Stimulated Control tubes were designed for thaw/culture condition controls. All culture tubes were placed on a slant and incubated 1.5 hours in 37°C, 5% CO₂ incubator. 5ug/ml Brefeldin-A was added to all cultures which were replaced in the incubator for a total time of 18 hrs.

Sample Preparation Methods:

The **conventional manual** method of lysing, fixing, and permeabilizing cells was performed according to the IntraPrep™ Permeabilization Reagent kit package insert (Beckman Coulter, Inc.) for simultaneous surface and intracellular antigen detection. See "Manual Method" figure.

The **automated method** is a methodology which performs all pipetting, timing, fixing, lysing, permeabilizing, and washing steps on 32 tubes per carousel. This method is possible due to an in-house modification made to existing PrepPlus™2 and CellPrep™ instruments. The carousel is transferred directly (either with a robotic arm or manually) to a CellPrep instrument for a non-centrifugal wash and then to the FC500 flow cytometer for acquisition and analysis.

*Patent Pending

Beckman Coulter/Immunotech Antibodies:

Fluorochromes were chosen for optimum resolution of populations: IgG1-FITC / IgG1-PE / CD4-ECD / CD8-PC5 / CD3-PC7
IFN γ -FITC / IL2-PE / CD4-ECD / CD8-PC5 / CD3-PC7

Flow Cytometry:

Analysis was performed on the 5-color, one-laser, Cytoomics™ FC500 flow cytometer utilizing the CXP Cytometer Acquisition and Analysis software. Instrument alignment and performance was verified daily using the automated QC methods and FlowCheck and FlowSet beads. Pre-established target channels of fluorescence intensity were used for these assays.

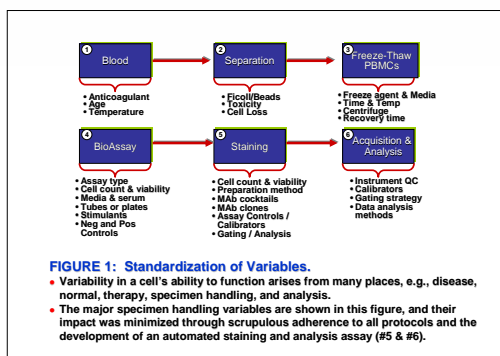


FIGURE 1: Standardization of Variables.

- Variability in a cell's ability to function arises from many places, e.g., disease, normal, therapy, specimen handling, and analysis.
- The major specimen handling variables are shown in this figure, and their impact was minimized through scrupulous adherence to all protocols and the development of an automated staining and analysis assay (#5 & #6).

Manual ICS Method

Hands-On Time = 60-90 min

Summary:

- 6-8 reagent additions
- 3-4 incubation times
- 2-3 centrifugal washes
- Total Time = 90-120 min for ~30 tubes at one time

Automated ICS Method

Hands-On Time = 15 min

Summary:

- 5 reagent additions
- 3 incubation times
- 1 NON-centrifugal wash
- Total Time = 120-150 min for 32 tubes, however, 2nd batch can begin 90 minutes into the 1st batch

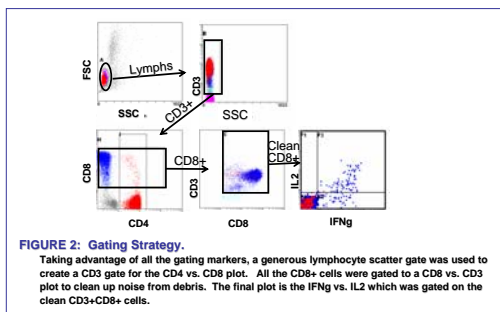


FIGURE 2: Gating Strategy.

Taking advantage of all the gating markers, a generous lymphocyte scatter gate was used to create a CD3 gate for the CD4 vs. CD8 plot. All the CD8+ cells were gated to a CD8 vs. CD3 plot to clean up noise from debris. The final plot is the IFN γ vs. IL2 which was gated on the clean CD3+CD8+ cells.

RESULTS:

		MANUAL			AUTOMATED		
		% positive of CD3+CD8+ cells	MFI of the IFN γ	MFI of the IL2	% positive of CD3+CD8+ cells	MFI of the IFN γ	MFI of the IL2
IL2+IFN γ -	Average	0.10		1.06	0.52		1.94
	SD	0.07		0.35	0.09		0.17
	%CV	71		33	17		9
IL2+IFN γ +	Average	0.35	12.43	3.94	0.78	14.99	4.92
	SD	0.12	2.75	0.73	0.10	2.07	0.60
	%CV	33	22	18	12	14	12
IL2-IFN γ +	Average	0.42	2.53		0.40	6.72	
	SD	0.08	1.50		0.06	0.89	
	%CV	20	59		15	13	

TABLE 1: Precision.

The basic statistics in this table were created from 19 replicate staining tubes from a single culture. Both the manual and automated methods had 19 replicates of the CEF stimulated cultures, many controls, isotypes, and color compensation tubes for a total of 32 tubes processed by each method on the same day.

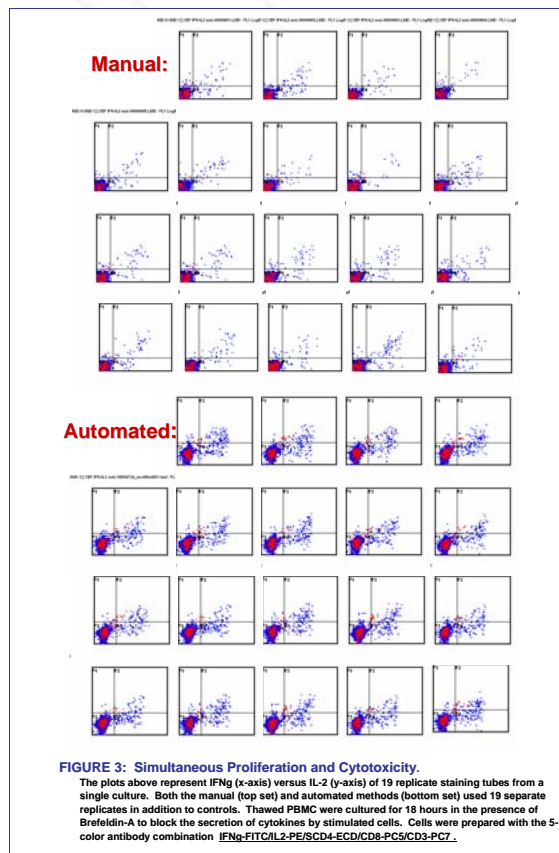


FIGURE 3: Simultaneous Proliferation and Cytotoxicity.

The plots above represent IFN γ (x-axis) versus IL-2 (y-axis) of 19 replicate staining tubes from a single culture. Both the manual (top set) and automated methods (bottom set) used 19 separate replicates in addition to controls. Thawed PBMC were cultured for 18 hours in the presence of Brefeldin-A to block the secretion of cytokines by stimulated cells. Cells were prepared with the 5-color antibody combination IFN γ -FITC/IL2-PE/SCD4-ECD/CD8-PC5/CD3-PC7.

SUMMARY:

- Complex functional immune-cell assays utilizing a wash, can be totally automated.
- Precision is up to 4 times better using the standardized automated method.
- Hands-on time is reduced from 60-90 minutes to 15 minutes due to automation.
- Other sources of variability in assays result can now be addressed specifically (e.g. specimen handling, anti-coagulant, freezing, thawing, culturing, or biological).
- This Standardized Functional Assay Consists of:
 - Automated reagent and specimen pipetting, and incubation timings.
 - Automated cell washing (non-centrifugal).
 - Automated flow cytometer setup for voltage and color compensation.
 - Automated acquisition of data.
 - Automated data analysis region adjustments during acquisition.
 - Automated data export.
- The automated QC and QA of the Flow Cytometer allowed total hands-off assay analysis - - color compensation and voltages NEVER NEEDED TWEAKING.
- Complex biological response profiles can be correlated with vaccine efficacy or vaccine progression due to consistent assay results.

References:

Maecker et al. *BMC Immunology* 2005, 6:13, doi:10.1186/1471-2172-6-13
Cox et al. *AIDS Research and Retroviruses*, 21 (1), 2005