

Development Report

Automated *S. pneumoniae* Opsonic Antibody detection Technology

U.S. Centers for Disease Control and Prevention
&
Flow Applications Inc.

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Opsonophagocytosis

The CDC and Flow Applications Inc. have developed a multiplexed, opsonophagocytic assay for the detection of antibodies against *S. pneumoniae* capsular antigens. This automated assay is applicable to the measurement of opsonic antibodies used as laboratory correlates of protection. Opsonophagocytic assays are more attractive than other measures of *in vitro* protective immunity because they more closely resemble models of *S. pneumoniae* clearance by the host, and appear to provide a closer correlation with serotype-specific vaccine efficacy than other assays such as ELISAs.

Background Information

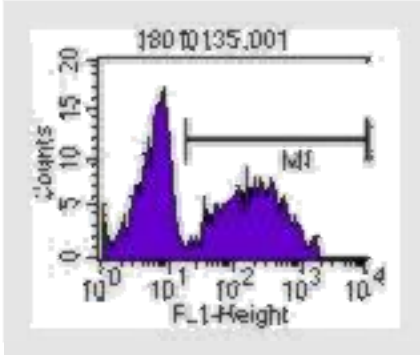
Host protection against pneumococcal disease is mediated by opsonophagocytosis. A variety of techniques have been used to determine opsonophagocytic activity (radioisotopes, chemiluminescence, flow cytometry, and viability assays). The presence of functional antibodies leads to complement activation,


effective opsonization, phagocytosis, and recovery from infection. The reproducible opsonophagocytic assay makes it possible to estimate the phagocytic titer of paired sera (pre- and post-vaccination) from vaccinated individuals who received either the polysaccharide vaccine or polysaccharide protein-conjugate vaccine. Analytical flexibility is inherent, and the assay is adaptable to following general protocols:

- Use of non-viable bacteria, labeled with a fluorescent dye, as the phagocytic target. The phagocytic titer is subsequently determined in a singular procedure.
- Use of non-viable bacteria, each serotype labeled with a different fluorescent dye, with the phagocytic titers subsequently determined in a multiplexed procedure.
- Use of fluorescent beads conjugated to specific polysaccharide. Each serotype polysaccharide is attached to a differently labeled fluorescent bead population so that measurement of opsonophagocytosis of each different fluorescent bead population results in a measure of the opsonic titers in a multiplexed procedure.

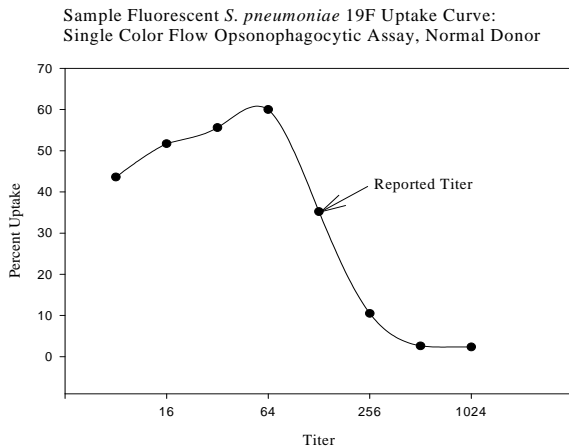
Single Color Bacteria Based Flow Cytometric Opsonophagocytic Assay

A reproducible, standardized flow cytometric opsonophagocytic assay has been developed that estimates the "functional" phagocytic activity of human sera against *S. pneumoniae* polysaccharide. This assay measures the complement dependent opsonic activity of serum using culturable phagocytic HL-60 cells. Phagocytic titers are reported as the reciprocal dilution of the dilution determined to have 50% of the maximal uptake (example shown in figure 2 on right) of fluorescently labeled, paraformaldehyde fixed *S. pneumoniae*. Opsonophagocytosis measures antibody function, as opposed to ELISA, which measures total antibody binding (functional and non-functional antibody). This assay, which uses non-viable bacteria, can be used for evaluating the human response to current and developing vaccines.



*Above image was supplied by the United States Centers for Disease Control and Prevention 

Example of fluorescence histogram illustrating the uptake of fluorescently labeled *S. pneumoniae* 19F by HL60 cells in the presence of opsonic antibodies (M1 region). The following graph illustrates the opsonophagocytic activity against *S. pneumoniae* 19F of a serum sample collected from a normal donor immunized with the 23-valent-polysaccharide vaccine. The expected titer was 128, and the observed titer was 128.



The data in the table below illustrates the correlation between the manual opsonophagocytic assay, live *S. pneumoniae*, and the single color flow cytometric assay using fluorescently labeled, fixed *S. pneumoniae*.

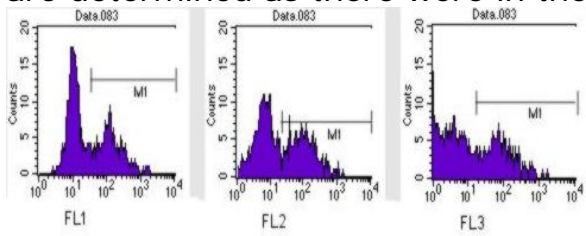
Correlation of Manual, and Single Color Flow Opsonophagocytic Assays for Anti-*Streptococcus pneumoniae* Antibodies


Serotype	Manual Assay vs. Single Color Bacterial Flow Assay
	r Values (p value)
4	0.88 (<0.001)
6B	0.77 (0.003)
9V	0.53 (0.08)
14	0.54 (0.04)
18C	0.77 (0.003)
19F	0.95 (<0.001)
23F	0.83 (<0.001)

Tri-Color Bacterial Assay: Multiplexed Serotype Opsonophagocytic Assay

The single color *S. pneumoniae* flow cytometric opsonophagocytic assay was the first step towards increased assay automation. The shift from a manual assay to the flow cytometric platform offered the potential for increases in automation and sample throughput, while at the same time reducing exposure to potentially infectious organisms, reducing sample volume used, and reducing cost associated with testing each serum sample individually for each serotype found in a particular vaccine. The tri-color flow cytometric bacterial opsonophagocytic assay represents the next step in this process. By combining three *S. pneumoniae* serotypes in a single assay, we have increased throughput threefold, reduced sample use by 2/3, and reduced cost by 2/3.

Opsonophagocytic activity against each serotype can be determined independently of the others. This is shown in the following histograms obtained on a single serum sample from a normal donor immunized with the 23-valent-polysaccharide vaccine. Percent uptakes for each serotype are determined as there were in the single color flow assay.



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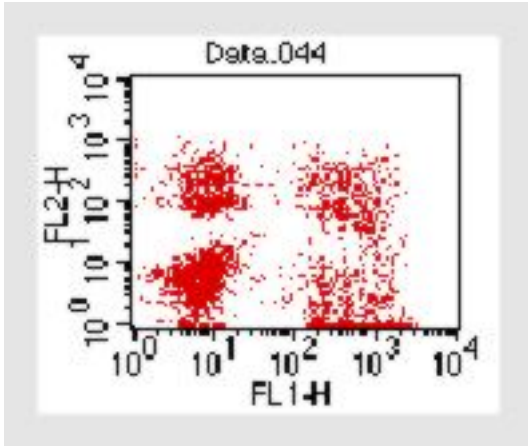
Resulting titers correlate very well with both the manual and single color *S. pneumoniae* opsonophagocytic assays. The table below lists the correlations obtained from 12 sera from normal donors immunized with the 23-valent pneumococcal polysaccharide vaccine.


Correlation of Manual Opsono, Single Color Flow and Tri-Color (Three Serotype) Flow Opsono Assays for Anti-*Streptococcus pneumoniae* Antibodies

Serotype	Manual Assay vs. Tri-Color Bacterial Flow Assay r Values (p value)	Single Flow vs. Tri-Color Bacterial Flow r Values (p value)
4	0.61 (0.04)	0.80 (0.002)
6B	0.87 (<0.001)	0.86 (<0.001)
9V	0.79 (0.006)	0.73 (0.008)
14	0.75 (0.005)	0.63 (0.03)
18C	0.91 (<0.001)	0.74 (0.005)
19F	0.91(<0.001)	0.91 (<0.001)
23F	0.78 (0.003)	0.88 (<0.001)

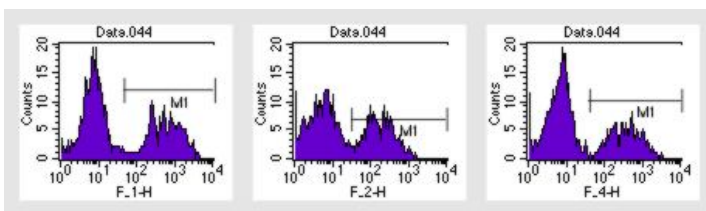
Tri-Color Bead Based Assay: Multiplexed Serotype Opsonophagocytic Assay

The next step in the development of multiplexed flow cytometric *S. pneumoniae* opsonophagocytic assays consists of making the transition to manufactured targets, which are amenable to standardization. Polysaccharide coated fluorescent beads are the most viable candidate for this. Our polysaccharide conjugated fluorescent micro spheres replace fluorescently labeled *S. pneumoniae* in the flow opsonophagocytic assay. The advantages of fluorescent micro spheres lie in the control of manufacture and the ability to design bead sets to cover specific serotype populations. As with the Tri-color bacteria based assay, the bead based flow opsonophagocytic assay reduces the amount of sera used to generate results, reduces cost per serotype, and increases relative sample throughput.



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The dot plot above shows the relative distribution of HL60 cell populations with ingested fluorescent polysaccharide conjugated beads. In this example, serotype 18C (FL1) is plotted against serotype 4 (FL2). The populations in the lower left quadrant are double negative HL60 cells; the lower right quadrant contains only serotype 18C beads; the upper right quadrant population contains both serotype 18C and serotype 4 beads; the upper left quadrant cells contain only serotype 4 beads. Percent uptakes of each serotype can be quantified independently from histograms displaying data from each detector in properly compensated instrumentation.



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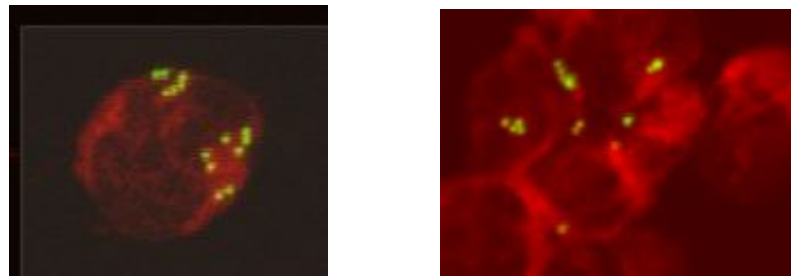
We have shown the ability of the assay to do three serotypes simultaneously, however, current clinical flow cytometers are capable of doing 4 serotypes. We are currently completing development of 4 serotype kits. The multiplexed *S. pneumoniae* opsonophagocytic assay results correlated well with the manual assay.

Correlation of a Manual Opsonophagocytic Assay and a Tri-Color (Three serotype) Flow Opsonophagocytic Assay

Serotype	Manual Assay vs.	
	Tri-color Bead Assay	Flow
	r Values (<i>p</i> value)	
4	0.76 (0.004)	
14	0.89 (<0.001)	
18C	0.93 (<0.001)	

HL60 Cells Phagocytosis of Ingested Fluorescent Beads Conjugated with *S. pneumoniae* Serotype 4 Polysaccharide

Active effector cell phagocytosis is shown as a final demonstration of opsonic activity. This depiction completes the model for correlate of immunity by a functional clearance mechanism. The following confocal micrographs clearly demonstrate the ingestion of labeled particles by effector cells as opposed to surface adherence.



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