**Streptococcus pneumoniae** Opsonophagocytic Assay* ^
(from Robert Wilkinson, Jay Fishman, MGH, Boston)

1) Add 20uL Opsonization Buffer (OB buffer) to rows A through G of a sterile, round bottom 96 well dish (Costar 3799, Corning, NY). Also add 20uL OB buffer to wells 1H and 2H.
2) Add 30uL of heat inactivated test serum to duplicate wells in row H.
3) Serially dilute test samples 1:3 from H to A by adding 10uL from H to G, mixing by pipetting up and down, and continuing to well A. Remove and discard excess 10uL from well A. All wells should contain 20uL of volume.
4) Add 10uL pneumococci containing ~500 viable cells to every well.
5) Place on orbital shaker set at 2000 RPM for 30 min at room temperature.
6) Place on orbital shaker set at 2000 RPM for 45 min at 37oC.
7) Move cells to wet ice.
8) Plate 10uL from each well to THYA plate (39 ml agar in 12cm x 12cm square petri dish, 30g/L Difco Todd Hewitt Broth, 5g/L Difco Yeast Extract, 15g/L Difco Agar) and incubate in CO2 incubator overnight at 37oC.
9) Capture TIF images of each plate using a FluorChem AlphaImager (Cell Biosciences, Santa Clara, CA 95051).
10) Count colonies using Integrated Colony Enumerator software (National Institute of Standards and Technology, Gaithersburg, MD).
11) Determine opsonic index using Opsotiter v2.07 software (University of Alabama at Birmingham, Birmingham, AL).

**Opsonization Buffer (OB buffer)**
- 10ml 10X HBSS with Ca++ and Mg++ (Gibco, Life Technologies, Carlsbad, CA)
- 1ml 10% gelatin (Gibco, Life Technologies, Carlsbad, CA)
- 5ml heat inactivated fetal calf serum (Atlanta Biologicals, Atlanta, GA)
- 84ml dHOH
- Filter sterilize to 0.22um (Millipore, Billerica, MA)

**S. pneumoniae**
- Frozen stocks must be pure and retain viability of >90% after freezing.
- Frozen stock is rapidly thawed in 37oC water bath and serially diluted as needed in OB buffer to reach a final concentration of 500 viable cells in 10uL.

**HL-60 Cells**
- HL-60 cells are grown in an undifferentiated state in CM1 media and kept between 200,000 and 400,000 cells per ml. Cells to be used in assay are induced to differentiate into phagocytic cells by culturing in DMF containing CM2 media without added antibiotics for 5 days or up to 6 days at a beginning concentration of 400,000 cells per ml. At time of assay differentiated HL-60 cells are harvested.
by centrifugation, washed in 1X HBSS without Ca++ or Mg++. Washed cells are washed again but this time in 1X HBSS with Ca++ and Mg++, counted, and adjusted to 17 million cells per ml in OB buffer.

CM1 media
RPMI 1640 (CellGrow, Mediatech Inc, Manassas, VA) supplemented with 10% heat inactivated-FCS (Atlanta Biologicals Atlanta, GA), 2mM Glutamine (MP Biomedicals LLC, Solon, OH), Pen-Strep (100IU/ml, 100ug/ml) (MP Biomedicals LLC, Solon, OH)

CM2 media
RPMI 1640 supplemented to 10% HI-FCS, 2mM Glutamine, 9% N,N-Dimethylformamide (BP-1160, Fisher Scientific, Fair Lawn, NJ)

Complement
Active and heat inactivated baby rabbit complement is stored as undiluted aliquots at -80°C. Each lot is tested for nonspecific killing of S.pneumoniae in the presence of differentiated HL-60 cells and in the absence of opsonizing antibody prior to use.

Heat inactivation of complement
Fetal calf serum used to cultivate HL-60 cells, test samples and control baby rabbit complement are heat inactivated by heating to 56°C for 30 minutes.

Agar plates
12cm x 12cm square petri dishes with 39ml of Todd Hewitt Agar supplemented with 4g/L Yeast Extract and 12g/L agar (THYA). Once inoculum has been absorbed overlay with 15 ml freshly prepared THYA with reduced agar content (7.5g/L) and supplemented with 1.25 mg TTC.

Colony counting
Colony counts are performed using Integrated Colony Enumerator software developed at the National Institute of Standards and Technology, Gaithersburg, MD.

Opsonic Index
Opsonic index is calculated using Opsotiter 2.07 software developed at the University of Alabama at Birmingham.

* Original methods from Protocol for multiplexed opsonophagocytic killing assay (UAB-MOPA) for antibodies against Streptococcus pneumoniae

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^ Note: Results are impacted by use of antimicrobial prophylaxis (e.g., trimethoprim-sulfamethoxazole, TMP-SMZ) in the post-transplant period. This may be corrected using a strain of Pneumococcus that is TMP-SMZ resistant.

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