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) Add 1 volume of active complement to 4 volumes of washed HL-60 cells. ~5mls needed per plate
7) Add 100uL heat inactivated complement to 400 uL washed HL-60 cells
8) Add 50uL HL-60 cells (ATCC, Manassas, VA) with active baby rabbit complement to all wells in columns 2-12
9) Add 50uL HL-60 cells with heat inactivated baby rabbit complement to all wells in column 1
10) Place on orbital shaker set at 2000 RPM for 45 min at 37oC
11) Move cells to wet ice
12) 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) tilt dish to allow inoculum to run 2 to 3 cm. (Difco Microbiology Systems, Sparks, MD 21152)
13) Once inoculum volume has been absorbed by the agar overlay with 15ml THYA (30g/L Difco Todd Hewitt Broth, 5g/L Difco Yeast Extract, 7.5g/L Difco Agar, 83ug/ml TTC) Overlay with TTC-containing overlay agar (Note: TTC is a vital dye, 2,3,5- Triphenyltetrazolium chloride, available from Sigma T8877, Sigma Aldrich Cheme GmbH, St. Louis, MO). Note: Original methods use TTC at a final concentration of 25ug/ml while the above methods result in TTC at 83ug/ml or ~ 3x the original assay. The higher concentration provided better colony staining. The use of 39 ml agar per plate extends the shelf life of the plates for counting (although less can be used 25ml).
14) Once overlay agar has solidified, invert plate and incubate in CO2 incubator overnight at 37oC
15) Capture TIF images of each plate using a FluorChem AlphaImager (Cell Biosciences, Santa Clara, CA 95051)
16) count colonies using *Integrated Colony Enumerator* software (National Institute of Standards and Technology, Gaithersburg, MD)
17) 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 -80oC. 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 56oC 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-sulfametoxazole, 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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