***Haemophilus influenzae* opsono-killing assay* ^**
(from Robert Wilkinson, Jay Fishman, MGH, Boston)

1) Add 25uL dilution buffer to wells A through G of a sterile round bottom 96 well dilution microtiter dish (Costar 3799, Corning, NY) using one column for each sample to be tested.
2) For each heat inactivated sample serum add 25uL to well G and mix by pipeting up and down several times.
3) Continue 2-fold dilutions by transferring 25uL from well G to well F and mix as before
4) Continue for a total of 7 2-fold dilutions ending at well A
5) Add 10uL dilution buffer to columns 1 and 2 of a new sterile 96 well microtiter assay dish
6) Transfer 10uL of each dilution in duplicate to the new sterile round bottom assay microtiter dish, include undiluted aliquots in row H
7) Dilute stock H. flu to 1000 CFU/20uL in dilution buffer and add 20uL to each well
8) Incubate 15 minutes at 37oC on orbital shaker
9) Add 50uL heat inactivated complement diluted in dilution buffer to all 8 wells of column 1
10) Add 50uL active complement diluted in dilution buffer to all other wells
11) Briefly mix on orbital shaker and transfer to 37oC CO2 incubator for 45 minutes
12) Plate 5uL volumes of each well to a prewarmed chocolate agar plate (Remel Labs, Lenexa, KS) , allow inoculum to be absorbed before inverting plate
13) Incubate plates overnight at 37oC in CO2 incubator
14) Capture TIF images of each plate using an AlphaInotech FluorChem imager (AlphaImager (Cell Biosciences, Santa Clara, CA 95051)
15) Count colonies using the FluorChem analysis software
16) Determine opsonic index using Opsotiter 2.07 software developed at the University of Alabama at Birmingham.

Dilution Buffer

1X HBSS with Ca++ and Mg++ (Gibco, Life Technologies, Carlsbad, CA)
Fortified to 2% with Fildes Enrichment (BBL 211866, Beckton Dickinson Company, Sparks, MD)

Complement

Active and heat inactivated baby rabbit complement (Pel-Freez Biologicals, Rogers AR) is stored as undiluted aliquots at -80oC. Each lot is tested for nonspecific killing of H. flu in the absence of opsonizing antibody and titered appropriately.

Heat inactivation of complement

Test samples and control baby rabbit complement are heat inactivated by heating to 56oC for 30 minutes.

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

*Protocol modified from Sandra Romero-Steiner at CDC and Moon H. Nahm at University of Alabama

The NIH Bacterial Respiratory Pathogen Reference Laboratory
WHO Reference Laboratory for Pneumococcal Serology
Departments of Pathology and Microbiology
University of Alabama at Birmingham
Birmingham, AL 35294-2170
USA

http://www.vaccine.uab.edu/Hib%20Killing%20assay1%20-%20Colony%20counting.pdf

References:

1. Romero-Steiner, S., J. Fernandez, C. Biltoft, M. E. Wohl, J. Sanchez, J. Feris, S. Balter, O. S. Levine, and G. M. Carlone. 2001. Functional antibody activity elicited by fractional doses of Haemophilus influenzae type b conjugate vaccine (polyribosylribitol phosphate-tetanus toxoid conjugate). *Clin Diagn Lab Immunol 8:1115*.
2. Romero-Steiner, S., W. Spear, N. Brown, P. Holder, T. Hennessy, P. Gomez De Leon, and G. M. Carlone. 2004. Measurement of serum bactericidal activity specific for Haemophilus influenzae type b by using a chromogenic and fluorescent metabolic indicator. *Clin Diagn Lab Immunol 11:89*.
3. Bergeron, M. G., P. Simard, and P. Provencher. 1987. Influence of growth medium and supplement on growth of Haemophilus influenzae and on antibacterial activity of several antibiotics. *J Clin Microbiol 25:650*.
4. Nahm, M. H., K. H. Kim, P. Anderson, S. V. Hetherington, and M. K. Park. 1995. Functional capacities of clonal antibodies