Protocol for multiplexed opsonophagocytic killing assay (UAB-MOPA) for antibodies against Streptococcus pneumoniae

(Version E.02, December 2014)

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Procedures

Introduction

Opsonization assay has become an important tool in assessing pneumococcal vaccine immunogenicity (reference 1). To facilitate the widespread use of the opsonization assay, we have prepared a detailed protocol for 4-fold multiplexed opsonophagocytic killing assay (UAB-MOPA). Although this protocol describes the multiplexed assay, this protocol can be used as a single serotype assay with the minor changes described in Note 9 in Assay Notes section. Also, this protocol can be used with minor alterations (e.g., a different serial dilution factor or different number of wells per sample). Additional information on this assay can be found in publications (references 2 and 3) and at a web site (www.vaccine.uab.edu).

To facilitate data analysis, an Excel®-based data processing program was developed that converts colony counts to "opsonization index". This program, "opsotiter3", can be obtained from this reference laboratory by sending a written request to Rob Burton at robburton@uab.edu.

We have collaborated with the US National Institute of Standards and Technology (NIST) to develop software that can efficiently enumerate colonies in a digital image (i.e., a digital camera picture or scanned image). This software is called "NICE" (NIST's Integrated Colony Enumerator) and is available free of charge. For more information about NICE, contact: nice@nist.gov. To download the software and/or the software manual, go to: ftp://ftp.nist.gov/pub/physics/mlclarke/NICE/.

If academic investigators need MOPA target strains for non commercial uses, they can obtain the strains from BEI Resources (www.beiresources.org). For commercial uses, please contact Dr. Debbie Bidanset at the University of Alabama at Birmingham (Debbie@uab.edu). Please note that registration with BEI resources is required to obtain the strains.

BEI contact information:

BEI Resources
10801 University Boulevard
Manassas, VA 20110-2209
Email: contact@beiresources.org
Web site: www.beiresources.org

Telephone: 1-800-359-7370

The procedures outlined above are necessary to establish the assay. Afterwards, the UAB-MOPA procedure will be the primary protocol used, with the other procedures (bacteria working stock preparation/characterization, complement lot screening, etc) being used only occasionally.

This protocol is based on more than 10 years of continuous development work by many people in Nahm laboratory, to whom we are indebted. We are also indebted to many other individuals who have provided assistance and critical comments as well as to the NIH and WHO for financial support.

Pneumococci are human pathogens. As such, biosafety concerns apply. Check local guidelines for the proper handling of pneumococci.

If there are additional questions, please contact Dr. Moon H. Nahm (nahm@uab.edu) or Mr. Robert L. Burton (robburton@uab.edu).

Abbreviations: **OBB,** Opsonization Buffer B; **HI**, Heat-Inactivated; **THYA**, Todd-Hewitt Yeast Agar plates; **THYB,** Todd-Hewitt Yeast Broth; **NSK**, Non-Specific Killing; **TTC**, 2,3,5,-triphenyltetrazolium chloride; **OD,** Optical Density; **HBSS,** Hanks Balanced Salt Solution; **CFU,** Colony Forming Units; **QC,** Quality Control

Procedures

Title: Bacteria Stocks

Revision History: 2/1/08; 6/23/08; 9/20/12; 11/14/14

1. Bacteria Stocks

Pneumococci are human pathogens. As such, biosafety considerations apply. Check local guidelines for the proper handling of pneumococci.

A. Master Stock Maintenance

To maintain the integrity of the pneumococcal bacterial stocks, the master stock vials from BEI Resources should never be thawed. All master stock vials should be stored at -80°C. To produce assay stocks, retrieve the master stock vial from the freezer, quickly remove a fleck of ice from the vial, and streak it onto a blood agar plate (see below for more details). Immediately, return the master stock vial to the freezer.

B. Production of Assay Stocks

From the master stock tube, many aliquots of assay stocks can be produced and frozen. An aliquot of assay stock is thawed and used for only one experiment. The procedure below is for preparing 48 tubes of assay stock. If more aliquots are desired, scale up accordingly by increasing the number of 50 ml tubes used, i.e., do not remove more than 10 ml of culture from each tube.

1. Retrieve the master stock vial from the freezer, quickly remove a fleck of ice from the vial, and streak it onto a blood agar plate. **Immediately, return the master stock vial to the freezer.**
2. Incubate the plate upside down overnight at 37°C in a candle jar (Note 13). Pneumococci yield alpha-hemolytic colonies that can be identified by a green halo

surrounding the colony.

3. Transfer ~20 isolated colonies to a 50 ml tube containing 50 ml of THY broth (see Note 14). Incubate for 3-8 hours in a 37°C water bath until the top 150 microliters of the culture broth has an OD_{600} of ~0.6-0.9.
4. Harvest the top 10 ml of the broth and transfer to a fresh 50 ml tube. Add 5 ml of 80% sterile glycerol and 10 ml of fresh THY broth to the 10 ml of bacteria.
5. Mix well, and dispense 0.5 ml aliquots into sterile 1.5 ml microcentrifuge tubes (~ 48 tubes). Indicate the last aliquot prepared with an identifiable mark (this vial will be used to determine purity later).
6. Randomly select an aliquot (not the last aliquot prepared) that will **not** be frozen and will be used to compare the density pre- and post-freezing (see below). Transfer the remaining tubes to a labeled cardboard freezer box and place the box (without a lid) into a -70ºC freezer. After stocks have frozen completely (overnight is best), attach an appropriately labeled lid. Store the tubes frozen at -70°C until needed, up to ~18 months.

C. Characterization of Assay Stocks

Before an assay stock can be accepted, it must be characterized. If the assay stock meets the criteria defined below, the lot of stock will be accepted.

1. Confirm the serotype of the strain using standard serotyping protocols (Quellung, agglutination, latex bead-based, ELISA, etc).

2. Look for the presence of other microbial contamination by thawing the last aliquot prepared and streaking ~10 microliters onto a blood agar plate. After overnight growth at 37°C, only alpha-hemolytic pneumococcal colonies should be visible.

3. Determine the antibiotic sensitivity and resistance of the frozen assay stocks.
   a. Prepare OBB.
   b. Melt the overlay agar (you will need ~180 ml for each strain being tested) in a microwave using 50% power. Gently and carefully swirl overlay agar to ensure that all agar clumps have melted. Alternatively, overlay can be prepared and autoclaved the day of the assay to obviate the need for melting in a microwave. After the agar is melted, aliquot 13 ml to each of 13 sterile tubes. Keep tubes in 50ºC water bath until needed (do not use until agar temperature has equilibrated to 50°C).
   c. Dry small (10 cm x 10 cm) THYA plates (you will need 13 THYA plates for each strain being tested) by removing the lids and placing the plates in a laminar flow hood for 30-60 minutes (see Note 4). After plates are dry, replace lid to prevent over-drying, and keep at RT until needed. Plates should be labeled with strain name and antibiotic concentration (see below).
   d. Rapidly thaw a frozen assay stock tube prepared above in a 37°C water bath using gentle, constant agitation.
   e. Serial dilutions of bacteria (in triplicate) can be conveniently prepared in a microtiter plate as follows:
      i. Dilute the bacteria 10-fold by mixing 30 microliters of bacteria with 270 microliters of OBB in row A, columns 1 through 3 of a microtiter plate. Add 240 microliters OBB to rows B though H, columns 1 through 3.
      ii. Prepare 5-fold dilutions by mixing 60 microliters of dilute bacteria from previous dilution (row A) with 240 microliters of OBB in row B. Continue 5-fold serial dilutions for a total of 8 dilutions (10-fold to 7.8×10^5-fold).
   f. Spot 10 microliters from each dilution from each of the three columns onto THYA plates (13 total plates are needed).

g. Incubate the THYA plates at RT to allow the fluid to absorb into the agar (10-20 minutes).
h. Remove one of the overlay tubes from the water bath, add 13 microliters of TTC, and add 12 ml of this overlay to one of the 13 THYA plates. This is the "no antibiotic" control plate.
i. Remove three of the overlay tubes from the water bath, and add 13 microliters of TTC to each tube. To one tube, add 6.5 microliters of optochin, and add 12 ml of this overlay to one of the 13 THYA plates. This is the "0.5X optochin" plate. To the second tube, add 13 microliters of optochin, and add 12 ml of this overlay to one of the 13 THYA plates. This is the "1X optochin" plate. To the third overlay tube, add 26 microliters of optochin, and add 12 ml of this overlay to one of the 13 THYA plates. This is the "2X optochin" plate.
j. Remove three of the overlay tubes from the water bath, and repeat the above for trimethoprim.
k. Remove three of the overlay tubes from the water bath, and repeat the above for streptomycin.
l. Remove three of the overlay tubes from the water bath, and repeat the above for spectinomycin.
m. After all overlay has been added, there should be 13 total plates:

Plate #	Antibiotic
1	No antibiotic
2	optochin, 0.5X
3	optochin, 1X
4	optochin, 2X
5	spectinomyin, 0.5X
6	spectinomyin, 1X
7	spectinomyin, 2X

Plate #	Antibiotic
8	streptomycin, 0.5X
9	streptomycin, 1X
10	streptomycin, 2X
11	trimethoprim, 0.5X
12	trimethoprim, 1X
13	trimethoprim, 2X

n. After the overlay has set, incubate the plates overnight at 37°C in a candle jar (Note 13).
o. Count the colonies.
p. Calculate the average of each of the triplicates for a bacteria dilution that produces 50-150 CFU/spot in the no antibiotic plate. Determine the sensitivity of the strain to each concentration of antibiotic by comparing the CFU/spot in the presence of antibiotic to the CFU/spot without antibiotic. A strain should be resistant to the desired antibiotic at 2X assay concentration (with <20% reduction in CFU/spot compared to the no antibiotic plate), and sensitive to the other antibiotics at 0.5X assay concentration (with >95% reduction in CFU/spot compared to the no antibiotic plate).

4. Determine the optimal dilution factor for the assay stock. Prior to being used as targets in the UAB-MOPA assay, each assay stock must be titrated in assay conditions to determine the dilution necessary to yield about 120 CFU/spot on THYA plates.

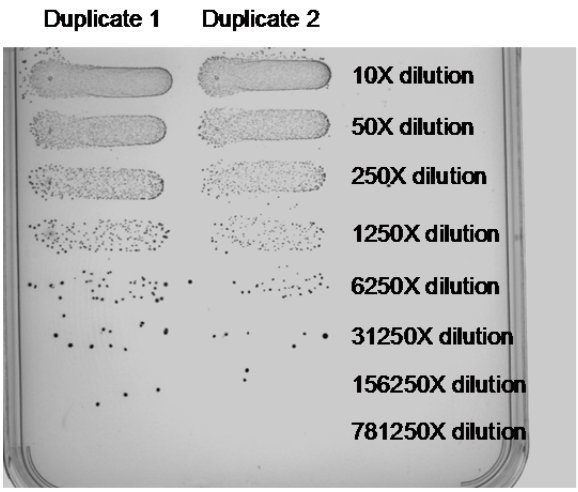
a. Prepare OBB.
b. Melt the overlay agar (~150 ml is sufficient for up to 12 strains) in a microwave using 50% power. Gently and carefully swirl overlay agar to ensure that all agar clumps have melted, and place bottle in 50ºC water bath until needed (do not use until agar temperature has equilibrated to 50°C). Alternatively, overlay can be prepared and autoclaved the day of the assay to obviate the need for melting in a microwave.
c. Dry small (10 cm x 10 cm) THYA plate (you will need 1 THYA plate for each

strain being tested) by removing the lid and placing the plate in a laminar flow hood for 30-60 minutes (see Note 4). After plate is dry, replace lid to prevent over-drying, and keep at RT until needed.

d. Add 20 microliters OBB to 16 wells (2 entire columns) of a fresh microtiter plate (in place of the 20 microliters of test serum that is used in the assay). This is the "assay plate".
e. Prepare the differentiated HL60 cells:
    i. Transfer the DMF-differentiated HL60 cells (differentiated for 5 or 6 days) from the culture flasks (1 flask will be sufficient to test up to 12 strains) to 50 ml centrifuge tubes.
    ii. Centrifuge the HL60 cells at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5 minutes at RT.
    iii. Remove the supernatant and combine all cell pellets in 50 ml of 1X HBSS (**without** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
    iv. Remove the supernatant and add 50 ml of 1X HBSS (**with** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
    v. Remove the supernatant, and suspend the cells at 1 x 10^7 cells/ml in Opsonization Buffer B (**store the cells at RT until needed**). See "Cell Counting" procedure for performing viable cell counts.
    vi. When counting the cells, determine the viability using trypan blue exclusion. Record the number of live cells and the number of dead cells. The cell viability must be ≥90% for the cells to be considered acceptable for use in the assay.
f. Rapidly thaw a frozen assay stock vial and wash bacteria:
    i. Gently swirl the tube in a 37°C water bath until thawed.
    ii. Centrifuge tube at 12,000*g* for 2 minutes in microcentrifuge.
    iii. Carefully remove supernatant and discard.
    iv. Add 1 ml Opsonization Buffer B to the tube, and mix.
    v. Centrifuge tube at 12,000*g* for 2 minutes.
    vi. Carefully remove supernatant and discard.
    vii. Suspend bacteria pellet in original volume of Opsonization Buffer B (i.e., 0.5 ml).
g. Dilutions of several bacterial cultures can be conveniently prepared in a fresh microtiter plate as follows (this is the "dilution plate"):
    i. Dilute the thawed bacteria 10-fold by mixing 15 microliters of bacteria with 135 microliters of Opsonization Buffer B in columns 1 and 2 of row A. Add 120 microliters Opsonization Buffer B to columns 1 and 2, rows B though H.
    ii. Prepare 5-fold dilutions by mixing 30 microliters of dilute bacteria from previous dilution (row A) with 120 microliters of Opsonization Buffer B in row B. Continue 5-fold serial dilutions for a total of 8 dilutions (10-fold to 7.8 x 10^5-fold).
h. Transfer 10 microliters of the diluted bacteria from column 1 of the dilution plate to column 1 of the assay plate. Repeat for column 2.
i. Incubate plate at RT and room air on a mini-orbital shaker (700 rpm) for 30 minutes.
j. During this time, remove a vial containing an adequate volume of complement (~0.3 ml for each strain tested) from the freezer and leave at RT to thaw (**immediately** after complement has thawed completely, incubate on ice until needed).
k. After the 30-minute incubation, prepare HL60/complement mixture by mixing 1 ml of HL60 cell suspension and 0.25 ml of complement (for each strain tested).
l. Add 50 microliters of the HL60/complement mixture to each well of the plate (16

wells/strain).

m. Incubate plate on a mini-orbital shaker (700 rpm) for 45 minutes at 37ºC with 5% CO_2 in a **single** layer, i.e., do not stack plates. In order to maintain a constant CO_2 percent during this incubation step, do not open the incubator door.
n. After incubation, place plates on ice to stop phagocytic process for ~20 minutes.
o. Using a multi-channel pipettor, mix the contents of each well, remove 10 microliters from each well in an 8 well column, and apply as eight-10 microliter spots to THYA plates on the left side. **Immediately**, tilt the plates to shape the spots into a small strip of fluid (~2-3 cm long). Apply the second 8 well column to the center of the THYA plate. **You must tilt plate immediately to prevent spots from running together.**
p. Incubate the THYA plates at RT for 10-20 minutes to allow the fluid to absorb into the agar.
q. Remove the overlay from the water bath, and add 150 microliters of TTC. Add 12 ml of this overlay to each plate.
r. Incubate the plates at RT ~20 minutes to allow the agar to solidify.
s. Incubate the plates upside down overnight at 37°C in a candle jar (Note 13).
t. Count the colonies and determine the average of the duplicates.
u. Determine the dilution factor required to yield the ideal number of colonies per spot (70-180 CFU/spot). In the example shown below, the optimal dilution factor is ~3000X (the 1250-fold dilution produced too many colonies and the 6250-fold dilution produced too few colonies).



5. Determine the performance of the assay stock in the assay using the UAB-MOPA protocol described later. Using the optimal dilution factor determined above, each assay stock should be tested at least 3 times using at least 2 QC sera. The QC sera should have been tested multiple times (more than 30 independent runs is preferable) with a previously qualified assay stock.

D. Acceptance Criteria for Bacteria Assay Stocks

In order for an assay stock of bacteria to be accepted as targets in the UAB-MOPA, all of the following criteria must be met:

1. The assay stock is pure as determined by streaking the final aliquot on blood agar plates and overnight incubation.
2. The serotype is confirmed as determined by Quellung, multibead assay, ELISA, etc.

3. The stock is resistant to the appropriate antibiotic and sensitive to the other three antibiotics. The stock should be resistant to the appropriate antibiotic at 2X assay concentration and sensitive to the appropriate antibiotics at 0.5X assay concentrations.
4. The assay dilution factor is ≥200.
5. The mean opsonic indices of at least two QC sera (tested at least 3 times in the UAB-MOPA) using the new assay stock are within the range (mean ± 2SD) determined using the previous assay stock.

Title: HL60 Cells

Revision History: 2/1/08; 5/28/08

2. HL60 Cells

HL60 is a human cell line. As such, biosafety considerations apply. Check local guidelines for the proper handling of human cell lines. Before you begin culturing HL60 cells, and periodically thereafter, you should check your incubator to ensure proper CO_2 levels and humidity (Note 6). CO_2 levels should be confirmed using an outside reference (such as a FYRITE® Gas Analyzer produced by Bacharach, Pittsburgh, PA). Proper humidity is maintained by ensuring the humidity pan is always full. Proper humidity is vital for two reasons. First, it helps minimize evaporation in cell cultures and assay plates. Second, the CO_2 sensors of some incubators require proper humidity for proper function.

A. Production of Master Cell Banks

When you receive the stock vial from ATCC, you should thaw, expand, and freeze the cells to produce a master cell bank (~60 tubes) directly from this stock tube. During the expansion and production of the master cell bank, it is critical that the culture is properly maintained so as to ensure the quality of the cells. Proper maintenance includes monitoring of CO_2 levels (Note 6), keeping the cell density below 5 x 10^5 cells/ml, and proper humidity (Note 6). For freezing the cells, the use of a controlled-rate freezer system (Cryomed systems from Thermo-Forma, for example) is highly recommended. Whatever system is used, the sample temperature should decrease ~1°C/minute during the entire freezing process.

1. Add 10 ml CM3 to 15-ml centrifuge tube.
2. Thaw frozen HL60 cells (original tube obtained from ATCC) quickly in a 37ºC water bath, and add the cells to the centrifuge tube containing 10 ml CM3.
3. Centrifuge the cells at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5-10 minutes at RT. Remove the supernatant, as much as possible (Note 5).
4. Suspend the cell pellet in CM3 (the volume of medium is indicated in the lot-specific information received from ATCC), and transfer cells to the necessary number of 150 cm^2 flasks (no more than 70 ml/flask). Place the flask(s) (lying flat) in a tissue culture incubator (37ºC, 5% CO_2, See Note 6).
5. After 3 or 4 days, add ~50 ml fresh CM3 to the flask. Do not add more than 120 ml medium to each 150 cm^2 flask. When more than 120 ml of medium is needed, use multiple flasks.
6. Monitor the cell density using a hemacytometer. When cells reach a density of ~5 x 10^5 cells/ml, add fresh CM3 to adjust the cell density to ~2 x 10^5 cells/ml. Do not add more than 120 ml medium to each 150 cm^2 flask. When more than 120 ml of medium is needed, use multiple flasks.
7. When you have 10 flasks containing ~120 ml of medium per flask and a cell density of ~5 x 10^5 cells/ml (usually ~3 weeks), freeze the cells.
    a. Prepare fresh freezing medium:

35 ml FBS (heat inactivated at 56°C for 30 minutes)
7 ml DMSO
28 ml RPMI 1640

b. Transfer the contents of all flasks to an appropriate number of 50 ml centrifuge tubes (~24). Keep a small volume (~1-2 ml) of culture to test for sterility.
c. Centrifuge the tubes at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5 minutes.
d. Remove supernatant (as much as possible), and discard supernatant.
e. Add 2.5 ml of freezing medium to each 50 ml centrifuge tube and gently re-suspend the cell pellets. Combine the cells from all 24 tubes together in a 150 cm^2 flask (total volume should be ~60 ml).
f. Aliquot 1 ml of cells in freezing medium to each vial (each vial should contain ~10^7 cells), and place the vial on ice until all are ready.
g. Put all cryovials except one (that will be used to check for microbial contamination below) into controlled-rate freezer and begin freezing program. Sample temperature should decrease ~1°C per minute.
h. When freezing is completed, transfer cryovials to cryobiological storage system (liquid nitrogen storage system).

8. The small volume of culture collected above should be checked for mycoplasma contamination using standard tissue culture mycoplasma screening techniques. Also, it should be checked for other microbial contamination by streaking onto blood agar plates.

B. Initiation of Working Cell Cultures

To maintain effector cell integrity, a new vial of cells from the master cell bank should be thawed every 3-4 months.

1. Add 10 ml CM3 to 15-ml centrifuge tube.
2. Remove one master cell bank Cryovial from liquid nitrogen storage. Gently swirl the vial in a 37ºC water bath to rapidly thaw the frozen cells. Add the thawed cells to the centrifuge tube containing 10 ml CM3.
3. Centrifuge the cells at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5-10 minutes at RT. Remove the supernatant, as much as possible (Note 5).
4. Suspend the cell pellet in ~70 ml CM3. Transfer the cells to a 150 cm^2 flask. Place the flask (lying flat) in a tissue culture incubator (37ºC, 5% CO_2, See Note 6).
5. After 3-4 days of culture, add ~50 ml CM3.
6. Every 3-4 days, remove ~50 ml of cells and discard. Add 50 ml of fresh CM3.
7. After ~2 weeks of culture, begin feeding cells with CM1 (every 3-4 days, remove 50 ml of cells and discard, add 50 ml of fresh CM1).
8. After cells have been in culture for at least 3-4 weeks, begin regular propagation schedule below. We have found that this is easily begun on a Wednesday or a Friday. If this is done on Wednesday, count cells, adjust cell concentration to ~8 x 10^5 cells/ml in **old** culture medium, aliquot 40 ml of cells per T150 flask, and add 80 ml fresh CM1. If this is done on Friday, count cells, adjust cell concentration to ~8 x 10^5 cells/ml in **old** culture medium, aliquot 20 ml of cell per T150 flask, and add 60 ml fresh CM1.
9. Follow the propagation schedule below.

C. Propagation of Working Cell Cultures

This procedure is designed for differentiating cells up to two times per week. This helps to conserve the passage number of the culture. Cells differentiated on Wednesdays will be used in the assay the following Monday and Tuesday (days 5 and 6 of differentiation,

respectively). Cells differentiated on Fridays will be used the following Wednesday and Thursday (days 5 and 6 of differentiation, respectively). Cells not used after 6 days will be discarded.

Monday: Add 40 ml CM1 to each 150 cm^2 flask of cells (there should be ~80 ml in each flask prior to feeding) to produce a final volume of ~120 ml/flask. Cell concentration should now be ~3 x 10^5 cells/ml.

Wednesday: Mix contents of flask(s), and remove 80 ml from each flask (leaving ~40 ml). The removed cells can be pooled and used for differentiation (see below), or discarded if not needed. Add 80 ml CM1 to the remaining cells in each flask to produce a final volume of ~120 ml/flask, and return flask to incubator for continued propagation. Cell concentration should now be ~3 x 10^5 cells/ml.

Friday: Mix contents of flask(s), and remove all volume except 20 ml from each flask (i.e., remove ~100 ml/flask). The removed cells can be pooled and used for differentiation (see below), or discarded if not needed. Add 60 ml CM1 to the remaining cells in each flask to produce a final volume of ~80 ml/flask, and return flask to incubator for continued propagation. Cell concentration should now be ~2 x 10^5 cells/ml.

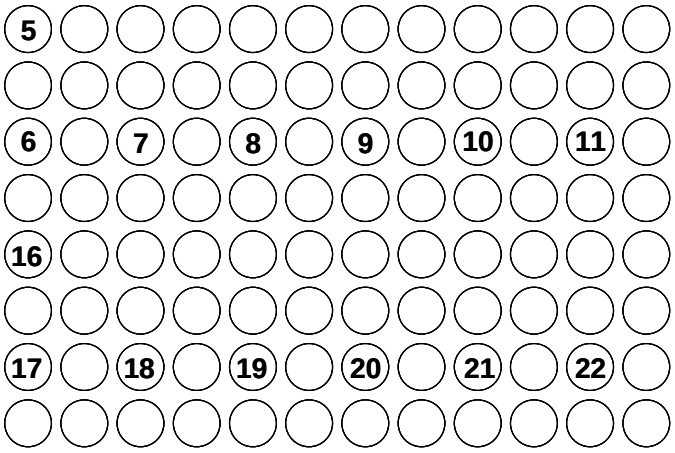
D. Differentiation of HL60 Cells

1. Centrifuge the HL60 cells at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5 minutes at RT. Remove ALL the supernatant (to completely remove any antibiotics).
2. Gently re-suspend the cell pellet in CM2 (containing 0.8% DMF), count the cells, and adjust the concentration to ~4 x 10^5 cells/ml.
3. Add 100 ml of the cell suspension to each 150 cm^2 flask needed.
4. Incubate the flask in an incubator (37ºC, 5% CO_2, See Note 6) for 5-6 days, lying flat. Do not feed the culture during this period. Cells not used within 6 days will be discarded.
5. Four flasks will usually yield enough differentiated cells to prepare 7 opsonization assay microtiter plates at ~200:1 (HL60:bacteria).

E. Determination of HL60 Cell Phenotype

We recommend determining the HL60 cell phenotype once every 2 to 4 weeks, more often if changes are noted in QC sera performance (low titers, incomplete killing, etc). We also recommend that both differentiated cells and undifferentiated cells are used for FACS analysis. The undifferentiated will be used as controls.

1. Label four 15 ml centrifuge tubes as “A”, “B”, “C”, or “D”. Label 22 FACS tubes “1” through “22”. Also, label 14 wells of a 96-well V-bottom plate as indicated below (the numbers will correspond to the FACS tube numbers when the cells are transferred from the plate to the FACS tubes):



2. Mix contents of flasks containing HL60 cells, and count cells. We recommend testing both differentiated cells and undifferentiated cells.
3. Transfer indicated number of cells to indicated tube (typically ~3 ml of differentiated and ~4 ml of undifferentiated cells contain 2 x 10^6 cells):

Tube	Sample	Intended Use	# Cells Needed
A	Undifferentiated Cells	Surface Markers	2 X 10^6
B	Undifferentiated Cells	Viability by FACS	1 X 10^6
C	Differentiated Cells	Surface Markers	2 X 10^6
D	Differentiated Cells	Viability by FACS	1 X 10^6

4. Keep Tubes B and D at room temperature until needed.
5. Centrifuge Tubes A and C at 350*g* for 5 minutes at 4°C.
6. While cells are centrifuging, prepare dilutions of antibodies, see Note 15 (store diluted antibodies at 4°C protected from light until needed):

	Volume (microliters)	
	Antibody	FACS buffer
CD11b PE	6	24
CD35 PE	3	27
CD71 PE	3	27
IgG1 Isotype PE (1/5)	6	24
IgG1 Isotype PE (1/80)	1	79
IgG2a Isotype PE	2	32

7. Remove supernatant from Tubes A and C.
8. Add 10 ml FACS Buffer to Tubes A and C, and centrifuge at 350*g* for 5 minutes at 4°C.
9. Remove supernatant from Tubes A and C, and suspend the cell pellets in 1 ml FACS Buffer.
10. Aliquot 50 microliters of cell suspension from Tube A to wells 5, 6, 7, 8, 9, 10, and 11 of the 96-well, V-bottom plate labeled above. Aliquot 50 microliters of cell suspension from Tube C to wells 16, 17, 18, 19, 20, 21, and 22 of the 96-well, V-bottom plate labeled above.
11. Add 10 microliters of diluted CD11b PE to wells 6 and 17 (changing tips between wells) and gently mix.
12. Add 10 microliters of diluted CD35 PE to wells 7 and 18 (changing tips between wells) and gently mix.

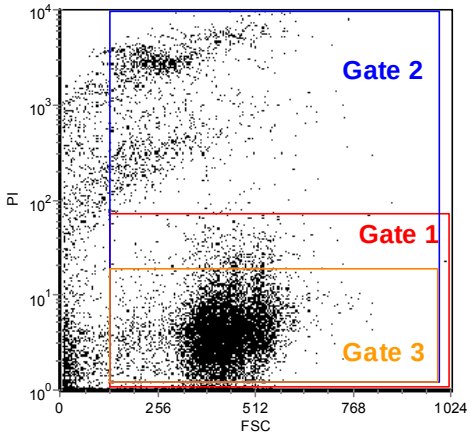
13. Add 10 microliters of diluted CD71 PE to wells 8 and 19 (changing tips between wells) and gently mix.
14. Add 10 microliters of diluted IgG1 PE Isotype (1/5 dilution) to wells 9 and 20 (changing tips between wells) and gently mix.
15. Add 10 microliters of diluted IgG1 PE Isotype (1/80 dilution) to wells 10 and 21 (changing tips between wells) and gently mix.
16. Add 10 microliters of diluted IgG2a PE Isotype to wells 11 and 22 (changing tips between wells) and gently mix.
17. Add nothing to wells 5 and 16. These wells are unstained control wells.
18. Incubate at 4°C protected from light for 30 minutes.
19. Add 150 microliters FACS buffer to each well, and centrifuge plate at 350*g* for 5 minutes at 4°C. Prepare FACS/PI buffer by adding 30 microliters PI stock solution to 6 ml FACS buffer (final PI concentration is ~5 ug/ml).
20. Remove supernatant from each well using separate pipet tips—do not cross-contaminate wells.
21. Suspend cell pellets in 250 microliters FACS/PI buffer.
22. Using separate pipet tips, transfer the contents of well 5 to FACS tube 5, well 6 to FACS tube 6, etc until all the contents of all wells have been transferred to FACS tubes. Store FACS tubes at 4°C protected from light until samples can be acquired (as soon as possible within 2 hours).
23. Begin processing Tubes B and D by adding cold 1X PBS up to 10 ml.
24. Centrifuge Tubes B and D at 350*g* for 5 minutes at 4°C. Prepare 50 micrograms/ml PI solution by adding 3 microliters of PI stock solution to 57 microliters 1X Annexin V Binding Buffer. Store at room temperature protected from light until needed.
25. Remove supernatant from Tubes B and D.
26. Add 10 ml cold 1X PBS to Tubes B and D, and centrifuge at 350*g* for 5 minutes at 4°C.
27. Remove supernatant from Tubes B and D, and suspend the cell pellet in 1 ml 1X Annexin V Binding Buffer.
28. Aliquot 100 microliters of cell suspension from Tube B to FACS tubes 1, 2, 3, and 4. Aliquot 100 microliters of cell suspension from Tube D to FACS tubes 12, 13, 14, and 15.
29. To FACS tubes 2 and 13, add 5 microliters Annexin V FITC.
30. To FACS tubes 3 and 14, add 10 microliters diluted PI.
31. To FACS tubes 4 and 15, add 5 microliters Annexin V FITC and 10 microliters diluted PI.
32. Add nothing to FACS tubes 1 and 12. They are unstained controls.
33. Incubate tubes at room temperature for 15 minutes, protected from light.
34. Add 300 microliters 1X Annexin V Binding Buffer to FACS tubes 1-4 and 12-15. Store FACS tubes protected from light at room temperature until samples can be analyzed with a flow cytometer (as soon as possible within 1 hour).

Assay tube summary:

Cell Type	Description	Buffer	FACS Tube #
Undifferentiated	Unstained	AV Binding Buffer	1
	Annexin V FITC	AV Binding Buffer	2
	PI	AV Binding Buffer	3
	Annexin V FITC, PI	AV Binding Buffer	4
	Unstained	FACS/PI Buffer	5
	CD11b PE	FACS/PI Buffer	6
	CD35 PE	FACS/PI Buffer	7
	CD71 PE	FACS/PI Buffer	8
	IgG1 PE Isotype, 1/5 (isotype control for CD11b)	FACS/PI Buffer	9
	IgG1 PE Isotype, 1/80 (isotype control for CD35)	FACS/PI Buffer	10
	IgG2a PE Isotype, 1/17 (isotype control for CD71)	FACS/PI Buffer	11
Differentiated	Unstained	AV Binding Buffer	12
	Annexin V FITC	AV Binding Buffer	13
	PI	AV Binding Buffer	14
	Annexin V FITC, PI	AV Binding Buffer	15
	Unstained	FACS/PI Buffer	16
	CD11b PE	FACS/PI Buffer	17
	CD35 PE	FACS/PI Buffer	18
	CD71 PE	FACS/PI Buffer	19
	IgG1 PE Isotype, 1/5 (isotype control for CD11b)	FACS/PI Buffer	20
	IgG1 PE Isotype, 1/80 (isotype control for CD35)	FACS/PI Buffer	21
	IgG2a PE Isotype, 1/17 (isotype control for CD71)	FACS/PI Buffer	22

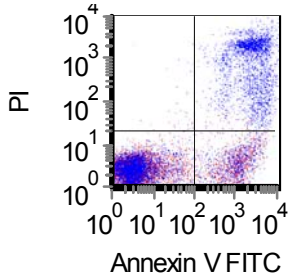
Data analysis

1. On an FSC vs FL3 (PI) dot plot, create three gates similar to the ones shown below. Gate 1 will be used for surface marker analysis of undifferentiated cells, Gate 3 will be used for surface marker analysis of differentiated cells, and Gate 2 will be used for viability analysis of both differentiated and undifferentiated cells.



2. For surface marker analysis:
   a. Create seven histograms of FL2 (one each for unstained, CD11b, CD35, CD71, IgG1 Isotype 1/5, IgG1 Isotype 1/80, and IgG2a Isotype) for undifferentiated cells and seven histograms for differentiated cells.
   b. Insert the appropriate data file into each histogram, and gate the histograms on Gate 1 for undifferentiated cells or Gate 3 for differentiated cells.
   c. Create a marker (M1) that results in ~1% of the cells being positive for the appropriate isotype.
   d. Record the percent positive cells (differentiated and undifferentiated) for each surface marker using M1.

3. For viability analysis:
   a. Create two FSC versus FL2 dot plots (one for unstained and one for AnnexinV/PI) for undifferentiated cells and two dot plots for differentiated cells. Note, the FACS tubes with cells stained **only** for Annexin V and **only** for PI are used for adjusting compensation during data acquisition, and are not used during analysis.
   b. Insert the appropriate data file into each dot plot, and gate the dot plots on Gate 2.
   c. Create quadrants as shown below.



   d. Record the percent positive cells (the unstained should be ~0%) for the upper right quadrant (AnV+/PI+ necrotic cells) and lower right quadrant (AnV+/PI- apoptotic cells). The percent in the upper left quadrant should be ~0%.

F. Acceptance Criteria for HL60 Cells

HL60 cell master cell bank must meet all of the following criteria:

1. There is no detectable microbial contamination including mycoplasma.
2. The cultures have the microscopic features described in reference 4 (R. Fleck, et al).
3. Proper documentation from the source is maintained.

Differentiated HL60 cells will be considered acceptable as effector cells in the UAB-MOPA if all of the following criteria are met:

1. The cultures have the microscopic features described in reference 4 (R. Fleck, et al).
2. Viability is ≥90% (trypan blue) or ≥65% (propidium iodide).
3. CD35 is expressed on ≥55% of the cells.
4. CD71 is expressed on ≤20% of the cells.
5. Apoptotic cells, defined as Annexin V+/PI-, represent ≤25% of the cells.

Title: Test Samples

Revision History: 2/1/08; 6/23/08; 9/20/12

3. Test Samples

Before being tested in the UAB-MOPA assay, samples need to be screened for bactericidal agents (most likely antibiotics) and also heat-inactivated (to destroy endogenous complement activity).

A. Sample Collection

Serum (not plasma) should be used in the UAB-MOPA. Check local regulations for handling human serum.

B. Sample Storage

For short-term storage (<1 month), samples may be kept at 4°C. Otherwise, samples should be stored at -80°C. Whenever samples are moved, make sure to enter this information into the sample logs.

C. Heat Inactivation

This can be done well in advance of the UAB-MOPA.

1. Remove samples from -80°C freezer and keep at room temperature.
2. Adjust temperature of water bath to 56°C.
3. After samples have thawed **completely**, mix samples thoroughly, and incubate samples in 56°C water bath for 30 minutes.
4. Remove samples from water bath and cool to room temperature.
5. Store samples at 4°C for short term storage (<30 days) or -80°C for long term storage.

D. Testing Samples for Bactericidal Agents (Antibiotics)

Since some individuals may be receiving antibiotic therapy at the time of serum collection, each test serum should be tested for the presence of antibiotics. Alternatively, if the MOPA testing involves multiple serotypes, only those samples that do not have an undetectable titer for at least one serotype need to be screened for antibiotics.

1. Prepare frozen stocks of R36A bacteria using the protocol described above for preparation of UAB-MOPA assay stocks. This can be done well in advance.
2. Melt the overlay agar (~13 ml is sufficient for up to 12 sera) in a microwave using 50% power. Gently and carefully swirl overlay agar to ensure that all agar clumps have melted, and place bottle in 50ºC water bath until needed. Alternatively, overlay can be prepared and autoclaved the day of the assay to obviate the need for melting in a microwave.
3. Dry THYA plate (you will need 1 THYA plate for up to 12 sera) by removing the lid and placing the plate in a laminar flow hood for 30-60 minutes (see Note 4). After plate is dry, replace lid to prevent over-drying, and keep at RT until needed.
4. Thaw vial of R36A stock in 37°C water bath with constant agitation.
5. Soak the tip of a cotton-tipped applicator in bacteria suspension and streak evenly onto a THYA plate (see Note 8): streak the entire surface of the plate, rotate the plate 45°, streak the entire surface of the plate, rotate the plate 45°, streak the entire surface of the plate.
6. Allow absorption of the fluid for 5-10 minutes.
7. Spot 5 microliters of undiluted test serum onto the THYA plate, leaving ~2-3 cm

between spots. If serum volume is limited, it may be pre-diluted 2- or 4-fold, and 5 microliters of the diluted serum is spotted. Up to 12 sera may be spotted onto each THYA plate, leaving at least 2 cm between spots.
8. Allow the serum to be completely absorbed.
9. Add 12 ml of overlay (~50°C) containing 25 micrograms/ml TTC.
10. Incubate the plate overnight at 37°C and 5% CO_2.
11. Inhibition of growth around where the serum was spotted is indicative of the presence of antibiotics (generally inhibition zone is ≥1 cm) if undiluted serum is tested. Samples that may contain antibiotics should be analyzed further after the inactivation or removal of antibiotics.

E. Production of QC Serum Pools

Ideally, multiple QC sera are included in each assay, and these sera have opsonization indices ranging from high to low for each serotype. When a pool is prepared, **no preservatives (including sodium azide) can be added.**

Serum pools must be aliquoted and stored frozen. Once an aliquot is thawed, it may be kept at 4°C up to one month, but should not be re-frozen.

In order to be considered for a pool, prospective single donor serum should:

- Not contain antibiotics
- Not display "irregular" killing curves for serotypes of interest
- If possible, be age-matched (toddlers, old adults, elderly adults, etc.) to the unknown test samples
- If possible, be vaccine-matched (polysaccharide, conjugate, etc.) to the unknown samples
- Have a sufficient volume that when a pool of ~5 donors is prepared, the total volume will be sufficient to last for 2-3 years
- Not contain sodium azide or other preservatives
- Be negative for infectious disease markers (HIV-1 and -2, HTLV-1 and -2, Hepatitis B and C, West Nile Virus, Chagas, and Syphilis).

Title: Complement

Revision History: 2/1/08; 5/28/08; 6/23/08; 9/1/09; 11/1/11

4. Complement

Complement components are extremely heat-sensitive. Therefore, great care should be taken when handling complement and preparing working aliquots. When stock bottles (100 ml) arrive from the manufacturer, ensure that they are still frozen and dry ice remains in the shipping container (if not, immediately contact the manufacturer). Quickly transfer the bottles to the designated area in the -80°C freezer for storage.

General tips:

- Thaw complement on ice or in a cold water bath—never thaw using warm/hot water
- Store complement in a -80°C freezer as far away from the door as possible
- When a working aliquot is thawed, do not re-freeze remaining complement—discard it

Complement lots show considerable lot-to-lot variation in their potency and non-specific

killing. Therefore, prospective lots need to be carefully screened. Prospective lots of complement may be screened using the CH50 assay, but ultimately must be tested in the UAB-MOPA (at least three times) against all serotypes of interest. The opsonization indices of QC sera as well as the non-specific killing (NSK) are calculated and compared to those obtained with previously qualified lots of complement.

A. Preparation of Working Aliquots of Complement

1. Remove 100 ml stock bottle of complement from -80°C.
2. Thaw stock bottle of complement under stream of cold water (~20°C), which provides constant agitation.
3. Label 12 tubes (sterile 15-ml centrifuge tubes) with "Complement for UAB-MOPA", lot#, batch#, and date. Place tubes in ice bucket.
4. Immediately after the complement has completely thawed, place bottle on ice.
5. Quickly aliquot 8 ml complement to each of the pre-cooled tubes, replacing them onto ice after aliquoting.
6. After the last aliquot has been prepared, remove ~10 microliters from this last aliquot and streak onto a blood agar plate to ensure sterility. Incubate plate overnight at 37°C and look for microbial growth. There should be no growth.
7. For control A in the UAB-MOPA, you will need heat-inactivated (HI) complement:
   a. Transfer ~10 ml of complement to a sterile 15-ml centrifuge tube.
   b. Incubate in a 56°C water bath for 30 minutes.
   c. Aliquot ~0.2 ml of HI complement to 1.5 ml sterile microcentrifuge tubes.
   d. Store at -80°C until needed.

B. Complement Screening 1 (CH50)

The CH50 assay is designed to measure total complement activity in a serum sample. Each test serum is co-incubated with either unmodified sheep red blood cells or with antibody-opsonized sheep red blood cells (opsonized with Hemolysin, rabbit anti-sera to sheep RBC). The Hemolysin antibodies should activate the complement cascade, ultimately resulting in the formation of membrane attack complexes that will puncture the membranes of the RBC and cause release of hemoglobin. There should be no significant lysis of the control RBC. After incubation, the reaction mixture is centrifuged, and the optical density (405 nm) of the supernatant is determined to measure the release of hemoglobin (protocol adapted from *Manual of Clinical Immunology*, 5th edition, Noel R. Rose, et al, ASM publication, 1997).

1. Prepare stock of working hemolysin:
   a. Add 2 ml of water to the vial containing the lyophilized hemolysin.
   b. Rotate the vial gently until all hemolysin has dissolved.
   c. Heat inactivate the hemolysin at 56°C for 30 minutes.
   d. Transfer the 2 ml of hemolysin to a 200 ml bottle and add 198 ml of 0.9% NaCl.
   e. Aliquot 25 ml of diluted hemolysin to each of eight labeled 50-ml centrifuge tubes.
   f. Store at -80°C until needed.
2. Prepare control sheep red blood cells (ctRBC) and sensitized (opsonized) sheep red blood cells (sRBC):
   a. Prepare 1X GVB and place in ice bath. Throughout the assay, return the unused buffer to the ice bath because cold buffer is needed near the end of the assay.
   b. Mix sheep blood well, and transfer 10 ml to each of two 50 ml centrifuge tubes (labeled "A" and "B"). Add 40 ml 1X GVB to each tube.
   c. Centrifuge tubes at 1300*g* (2500 rpm using Sorvall H1000B rotor), 2°C for 5 minutes.
   d. Aspirate supernatant and discard.

e. Continue washing RBC with centrifugation (1300*g*, 2°C, 5 minutes) and 50 ml 1X GVB until supernatant is clear (usually one or two more washes after first centrifugation is adequate).
f. Suspend cells in each tube in 40 ml 1X GVB and adjust cell concentration to ~1 x 10^9 cells/ml:
    i. Remove 150 microliters of cell suspension and add it to 2.1 ml of water.
    ii. Measure OD at 541 nm using spectrophotometer. A cell suspension containing 1 x 10^9 cells/ml should have an OD541 of ~0.7.
    iii. Adjust the volume of the cell suspension:

        final volume (in ml)=57.1*OD541

        The final volume should be ~50 ml and the cell concentration should be ~1 x 10^9 cells/ml. If the final volume is less than 40 ml, discard the cells and prepare new batch of cells.

g. Thaw one tube of working Hemolysin (25 ml), and add 25 ml of 1X GVB.
h. Place tubes containing RBC, tube with 50 ml 1X GVB, and tube containing diluted Hemolysin in 37°C water bath for at least 10 minutes to pre-warm.
i. Add the pre-warmed 1X GVB (volume added is equal to cell volume) to the cells in tube "A" (these are the control RBC, "ctRBC"). Add the pre-warmed Hemolysin (volume added is equal to cell volume) to the cells in tube "B" (these are the sensitized RBC, "sRBC") all at once, swirl, and place in 37°C water bath for 20 minutes, mixing tubes by gentle inversion every 5-10 minutes.
j. Centrifuge cells at 1,300*g*, 2°C for 5 minutes.
k. Aspirate and discard supernatant.
l. Suspend cells in each tube in 100 ml of ice cold 1X GVB (cell concentration should be ~5 x 10^8 cells/ml). Store cells at 4°C for up to two weeks.

3. CH50 assay:
   This protocol is designed to test up to 6 samples.
   a. Prepare 1X GVB and place in ice bath. Throughout the assay, return the unused buffer to the ice bath because cold buffer is needed near the end of the assay.
   b. Label assay plates (96 well, round bottom tissue culture treated plates):

Plate 1: Assay Control Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Replicate 1	Replicate 1	Replicate 1	Replicate 1	Replicate 1							
B	Replicate 2	Replicate 2	Replicate 2	Replicate 2	Replicate 2							
C	Replicate 3	Replicate 3	Replicate 3	Replicate 3	Replicate 3							
D	Replicate 4	Replicate 4	Replicate 4	Replicate 4	Replicate 4				Blank			
E	Replicate 5	Replicate 5	Replicate 5	Replicate 5	Replicate 5							
F	Replicate 6	Replicate 6	Replicate 6	Replicate 6	Replicate 6							
G	Replicate 7	Replicate 7	Replicate 7	Replicate 7	Replicate 7							
H	Replicate 8	Replicate 8	Replicate 8	Replicate 8	Replicate 8							
	0% Lysis	100% Lysis	0% Lysis	100% Lysis	Buffer Alone							
	ctRBC		sRBC									

Plate 2: Serum Control Plate (No RBC)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
C	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
D	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
E	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
F	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
G	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
H	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	

Plate 3: ctRBC Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
C	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
D	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
E	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
F	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
G	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
H	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	

Plate 4: sRBC Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
C	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
D	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
E	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
F	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
G	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
H	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	

c. Thaw test samples at room temperature. **Immediately** after samples have thawed, store them on ice.
d. Add 50 microliters of 1X GVB to plate 1, columns 1, 3, and 5, rows A-H.
e. Add 50 microliters of **water** to plate 1, columns 2 and 4, rows A-H.
f. Add 50 microliters of 1X GVB to plates 2, 3 and 4, columns 1 through 12, rows B-H.
g. Test samples can be tested neat or pre-diluted (2-fold or 4-fold) in 1X GVB if there is insufficient volume to test undiluted.
h. Add 100 microliters of sample 1 to plates 2, 3, and 4, columns 1 and 2, row A only. Add 100 microliters of sample 2 to plates 2, 3, and 4, columns 3 and 4, row A only. Continue through sample 6, if necessary.
i. Perform 2-fold serial dilutions of samples in plates 2, 3, and 4 by mixing contents of wells in row A, transferring 50 microliters to row B, mixing contents of wells in

row B, transferring 50 microliters to row C, etc. After transferring 50 microliters from row G to row H, mix contents of wells in row H, remove 50 microliters from row H and discard. Store plate on ice until needed.

j. Transfer 5 ml of ctRBC from storage bottle to a labeled 15-ml centrifuge tube. Transfer 5 ml of sRBC from storage bottle to a second labeled 15-ml centrifuge tube. Add 10 ml of cold 1X GVB to each tube, and centrifuge tubes at 1300*g* (2500 rpm using Sorvall H1000B rotor), 2°C for 5 minutes.
k. Remove and discard supernatant from each tube. Add 15 ml of cold 1X GVB to each tube, and centrifuge tubes at 1300*g*, 2°C for 5 minutes.
l. Suspend each cell pellet in 5 ml of cold 1X GVB and adjust cell concentration to ~2 x 10^8 cells/ml:
    i. Remove 150 microliters cell suspension and add it to 2.1 ml water.
    ii. Measure OD at 541 nm using spectrophotometer.
    iii. Adjust the volume of the cell suspension:

final volume (ml)=35.7*OD541

The final volume should be ~12 ml.

m. Add 50 microliters of ctRBC to plate 1, columns 1 and 2, rows A through H and to all wells of plate 3.
n. Add 50 microliters of sRBC to plate 1, columns 3 and 4, rows A through H and to all wells of plate 4.
o. Add 50 microliters of 1X GVB to plate 1, column 5, rows A through H and to all wells plate 2.
p. Incubate plates in warm room (37°C) on microtiter plate shaker at ~500 rpm (speed 5 on Lab-Line Instruments, model 4625) for 60 minutes.
q. Remove plates and add 150 microliters of **cold** 1X GVB to all wells **except** plate 1, columns 2 and 4, rows A-H which receive 150 microliters of **water**.
r. Centrifuge at 1300*g*, 2°C for 5 minutes.
s. Immediately remove 150 microliters of supernatant only (avoid disturbing cell pellet) to ELISA plate (flat-bottomed 96-well plate).
t. Read OD 405.
u. Data analysis:
    i. Open “CH50 Analysis Template V.02.xls”.
    ii. Copy and paste raw OD data into the indicated wells.
    iii. Enter required assay information (name, assay name, sample names, etc) into the indicated wells.
    iv. Save the file, and print the summary page and raw data page.

C. Complement Screening 2 (UAB-MOPA)

The protocol below can be used to screen one lot of complement against 4 serotypes using up to 5 QC sera.

1. Prepare OBB.
2. Dry large (~12 cm x ~12 cm) THYA plates by removing the lids and placing the plates in a laminar flow hood for 30-60 minutes (see Note 4). You will need 8 plates. After plates are dry, replace lid to prevent over-drying, and keep at RT until needed. Label the plates with the following information: assay plate identifier, row numbers, and strain name (or antibiotic added to overlay).
3. If not already done, prepare heat-inactivated (HI) complement for assay “Control A” (alternatively, many aliquots of heat-inactivated complement can be prepared ahead of time and kept at -80°C until needed):

a. Remove a vial of complement and allow to thaw completely at room temperature.
b. Incubate complement in 56°C water bath for 30 minutes.
c. Allow HI complement to cool to room temperature before using.

4. Melt the overlay agar (you will need ~210 ml) in a microwave using 50% power. Gently and carefully swirl overlay agar to ensure that all agar clumps have melted. Alternatively, overlay can be prepared and autoclaved the day of the assay to obviate the need for melting in a microwave. Aliquot 55 ml to each of four sterile bottles and place bottles in 50ºC water bath until needed (do not use until agar temperature has equilibrated to 50°C).
5. Prepare the microtiter plate following the plate layout diagram below.
   a. Add 20 microliters Opsonization Buffer B to columns 1 and 2, rows A through H. Add 20 microliters of Opsonization Buffer B to columns 3 through 12, rows A through G only.
   b. Add 30 microliters of heat-inactivated QC serum 1 to columns 3 and 4, row H only. Add 30 microliters of heat-inactivated QC serum 2 to columns 5 and 6, row H only. Continue for up to 5 total QC sera.
   a. Perform 3-fold serial dilutions in columns 3 through 12 of plate A and all columns of plates B through E. Make sure to change pipet tips between rows.
      i. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
      ii. Transfer 10 microliters of serum from row H to row G. Discard tips.
      iii. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
      iv. Using new pipet tips, transfer 10 microliters of serum from row G to row F. Discard tips.
      v. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
      vi. Continue through row A. After mixing plate, remove and discard 10 microliters from row A.

Plate layout diagram:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control A	Control B	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
B	Control A	Control B	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
C	Control A	Control B	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
D	Control A	Control B	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
E	Control A	Control B	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
F	Control A	Control B	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
G	Control A	Control B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
H	Control A	Control B	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
			QC Serum 1		QC Serum 2		QC Serum 3		QC Serum 4		QC Serum 5	

Control	Conditions	Use(s)
A	bacteria, complement (HI), HL60, t=75 minutes	Used to calculate non-specific killing
B	bacteria, complement, HL60, t=75 minutes	Used to calculate 0% killing in assay conditions Used to calculate non-specific killing

6. Prepare the differentiated HL60 cells:
   a. Transfer the DMF-differentiated HL60 cells from the culture flasks (1 flask should be sufficient for 2 assay plates) to 50 ml centrifuge tubes.
   b. Centrifuge the HL60 cells at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5 minutes at RT.
   c. Remove the supernatant and combine all cell pellets in 50 ml of 1X HBSS (**without** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
   d. Remove the supernatant and add 50 ml of 1X HBSS (**with** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
   e. Remove the supernatant, and suspend the cells at 1 x 10^7 cells/ml in Opsonization Buffer B (store the cells at RT until needed). See “Cell Counting”

procedure for performing viable cell counts.

f. When counting the cells, determine the viability using trypan blue exclusion. Record the number of live cells and the number of dead cells. The cell viability must be ≥90% for the cells to be considered acceptable for use in the assay.

7. Rapidly thaw a frozen working stock tube of each of the four bacteria strains and wash:
   a. Gently swirl the tubes in a 37°C water bath.
   b. Centrifuge the tubes at 12,000*g* for 2 minutes in microcentrifuge.
   c. Carefully remove supernatant and discard.
   d. Add 1 ml Opsonization Buffer B to each tube, and mix.
   e. Centrifuge the tubes at 12,000*g* for 2 minutes.
   f. Carefully remove supernatant and discard.
   g. Suspend bacteria pellet in original volume of Opsonization Buffer B (ie, 0.5 ml).
8. Prepare a bacterial mixture by adding the appropriate volume of each of the four strains of bacteria to one tube containing 10 ml of Opsonization Buffer B (should be ~50,000 CFU/ml for each bacteria strain). Add 10 microliters of the bacterial mixture to each well including all control wells.
9. Incubate the microtiter plate for 30 minutes at RT and room air on a mini-orbital shaker (700 rpm).
10. During this time, remove a prospective test lot of complement from the freezer (~1.3 ml of active complement and ~0.1 ml of heat-inactivated complement are needed). Leave the complement at RT to thaw (**immediately** after complement has thawed completely, keep on ice until needed).
11. During this incubation, transfer 4.8 ml of the HL60 cell suspension prepared above to a clean, sterile 15-ml tube. Also, transfer 400 microliters of the HL60 cell suspension to a clean, sterile 1.5 ml centrifuge tube. Keep both tubes at room temperature until needed.
12. Following the 30 minute incubation, add 100 microliters of inactivated complement to the tube containing the 400 microliters of HL60 cell suspension. Mix well, and add 50 microliters of the mixture to each well in column 1, rows A through H.
13. Add 1.2 ml of the active complement to the tube containing 4.8 ml of HL60 cell suspension. Mix well, and add 50 microliters of the mixture to each well of columns 2 through 12, rows A through H.
14. Incubate the microtiter plates on a mini-orbital shaker (700 rpm) for 45 minutes at 37ºC with 5% CO_2 in a **single** layer, i.e., do not stack plates. In order to maintain a constant CO_2 percent during this incubation step, do not open the incubator door.
15. After the incubation period, place the microtiter plates on ice for ~20 minutes (to stop the phagocytic process).
16. Spot 10 microliters of reaction mixture from each well onto **four** THYA plates. Starting with row H, mix the contents of each well and remove 10 microliters from each well in a 12-well row, and apply as twelve-10 microliter spots to THYA plates on the first (bottom) row of the THYA plate. **Immediately**, tilt the plates to shape the spots into a small strip of fluid (~2-3 cm long). Repeat this procedure for rows G, F, and E (for a total of 48 individual wells per plate). **You must tilt plate immediately to prevent spots from running together.** Rows D, C, B, and A are spotted onto a second set of four plates.
17. Leave the plates at RT for ~20 minutes to let the excess fluid seep into the agar (refer to Note 7).
18. Remove one bottle containing 52 ml overlay prepared above from the water bath, and add 52 microliters TTC stock and 52 microliters optochin stock. Mix well, and add 25 ml of this overlay per plate to each of the first of the four replicate plates spotted above. There should be 2 THYA plates that receive this overlay.
19. Remove the second bottle containing 52 ml overlay prepared above from the water

bath, and add 52 microliters TTC stock and 52 microliters streptomycin stock. Mix well, and add 25 ml of this overlay per plate to each of the second of the four replicate plates spotted above. There should be 2 THYA plates that received this overlay.

20. Remove the third bottle containing 52 ml overlay prepared above from the water bath, and add 52 microliters TTC stock and 52 microliters spectinomycin stock. Mix well, and add 25 ml of this overlay per plate to each of the third of the four replicate plates spotted above. There should be 2 THYA plates that received this overlay.
21. Remove the fourth bottle containing 52 ml overlay prepared above from the water bath, and add 52 microliters TTC stock and 52 microliters trimethoprim stock. Mix well, and add 25 ml of this overlay per plate to each of the fourth of the four replicate plates spotted above. There should be 2 THYA plates that received this overlay.
22. Incubate the plates at RT for ~20 minutes. After the overlay has solidified, place the plates, upside down, in a candle jar and incubate them for 16-18 hours at 37ºC (Note 13). Colonies that grow on the THYA plates with the optochin overlay are bacteria of the "optochin resistant" serotype, those that grow on the THYA plates containing streptomycin are bacteria of the "streptomycin resistant" serotype, etc.
23. Count colonies manually or using an automated counter (see Note 11 for colony counting options).
24. Calculate the opsonic indices and non-specific killing (Note 18). Opsonization indices can be calculated using linear interpolation of the serum dilution killing the indicated percentage (usually 50%) of the bacteria. Since each bacteria strain likely has different values for control B, each strain must be analyzed separately. Therefore, when the analysis is completed, you should have 4 files (1 for each serotype).
    a. Open analysis template "opsotiter3".
    b. Select the "RawData" worksheet of the template file.
    c. Enter raw data/assay information (cells requiring input are highlighted yellow):
        i. Enter the raw data (CFU/spot) into the corresponding cells, noting the positions for control A and control B. This can be done manually, or by copying and pasting data from another file. If data is entered electronically from another file, make sure the format is the same as the format indicated.
        ii. Enter the assay information into the indicated cells.
        iii. Make necessary changes to the adjustable assay parameters. Typically, these do not change from assay to assay.
        iv. Enter the sample information (name and pre-dilution factor) into the indicated cells.
    d. Save the file, and print the "RawData" and "Report" worksheets.
    e. To analyze another set of data, close the file, and re-open the template, i.e., do not use one analyzed file as a template to analyze another set of data.
25. After each QC serum has been tested at least twice with the prospective complement lot, calculate the average opsonization index for each serum for each serotype. Also, calculate the non-specific killing (NSK) for each run.

D. Complement Lot Acceptance Criteria

A prospective lot of complement will be considered acceptable if:

1. The CH50 value of the new complement is in the middle 70th percentile, i.e. do not use lots with CH50 values in the lower 15th or upper 15th percentile.
2. The CH50 value of the new complement for the control (unsensitized) RBC is <4. From our experience, most lots of baby rabbit complement (from 3-4 week old rabbits) do not have a detectable CH50 value for the control RBC.

3. For most serotypes, the NSK is ≤30%. For some serotypes (e.g. 6A, 6B, and 7F), the NSK may be higher. NSK up to 70% is acceptable for these serotypes.
4. For each QC sera, the mean opsonic index (tested at least twice) for the new complement is within the accepted range determined using the previously qualified complement lot (mean ± 2 SD).

Title: Fetal Bovine Serum
Revision History: 2/1/08; 5/28/08; 6/23/08; 11/1/11; 9/20/12

5. Fetal Bovine Serum (FBS)

We have recently discovered that the high NSK against serotype 14 that we saw with most lots of complement was due at least in part to the bovine serum that we used in the assay. This high NSK of serotype 14 was seen with all lots of FetalClone I that we tested. However, when other grades of FBS from HyClone and Atlanta Biologicals were used, the NSK against type 14 was negligible for most complement lots. Thus, we have discontinued use of FetalClone I in the assay (although we still use it for HL60 cultivation) and began using FBS in the OBB. Due to NSK of other serotypes (6B and 7F for examples), lots of FBS should be screened.

A. Heat Inactivation and Preparation of Assay Aliquots

1. Remove bottle of FBS from -20°C storage and place in 37°C water bath. Once the serum has **completely** thawed, remove from water bath and cool to room temperature.
2. In an equal-sized and equal–shaped bottle, add 500 ml of water and equilibrate to room temperature. This bottle will be used to monitor the temperature of the liquid in the bottle ("temperature monitor bottle"). Old empty FBS bottles work well for this purpose.
3. Place all bottles into 56°C water bath and position a thermometer into the temperature monitor bottle. The water level in the water bath should be ~1 cm above the liquid level in the bottles, but below the tops of the bottles.
4. When the temperature inside the temperature monitor bottle reaches 56°C, begin timing.
5. After 30 minutes at 56°C, remove FBS bottles from water bath. Allow to cool to room temperature.
6. Aliquot ~45 ml of heat-inactivated FBS to sterile 50-ml centrifuge tubes. For short-term storage (<1 month), aliquots can be stored at 4°C. For longer storage, aliquots should be stored at ≤ -20°C.

B. FBS Screening

The protocol below can be used to screen one lot of FBS against 4 serotypes using up to 5 QC sera.

1. Prepare OBB using the prospective lot of FBS.
2. Dry large (~12 cm x ~12 cm) THYA plates by removing the lids and placing the plates in a laminar flow hood for 30-60 minutes (see Note 4). You will need 8 plates. After plates are dry, replace lid to prevent over-drying, and keep at RT until needed. Label the plates with the following information: assay plate identifier, column numbers, and strain name (or antibiotic added to overlay).
3. If not already done, prepare heat-inactivated (HI) complement for assay "Control A" (alternatively, many aliquots of heat-inactivated complement can be prepared ahead of time and kept at -80°C until needed):

a. Remove a vial of complement and allow to thaw completely at room temperature.
b. Incubate complement in 56°C water bath for 30 minutes.
c. Allow HI complement to cool to room temperature before using.

4. Melt the overlay agar (you will need ~210 ml) in a microwave using 50% power. Gently and carefully swirl overlay agar to ensure that all agar clumps have melted. Alternatively, overlay can be prepared and autoclaved the day of the assay to obviate the need for melting in a microwave. Aliquot 55 ml to each of four sterile bottles and place bottles in 50ºC water bath until needed (do not use until agar temperature has equilibrated to 50°C).
5. Prepare the microtiter plate following the plate layout diagram below.
   a. Add 20 microliters Opsonization Buffer B to columns 1 and 2, rows A through H. Add 20 microliters of Opsonization Buffer B to columns 3 through 12, rows A through G only.
   b. Add 30 microliters of heat-inactivated QC serum 1 to columns 3 and 4, row H only. Add 30 microliters of heat-inactivated QC serum 2 to columns 5 and 6, row H only. Continue for up to 5 total QC sera.
   c. Perform 3-fold serial dilutions in columns 3 through 12 of plate A and all columns of plates B through E. Make sure to change pipet tips between rows.
      i. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
      ii. Transfer 10 microliters of serum from row H to row G. Discard tips.
      iii. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
      iv. Using new pipet tips, transfer 10 microliters of serum from row G to row F. Discard tips.
      v. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
      vi. Continue through row A. After mixing plate, remove and discard 10 microliters from row A.

   Plate layout diagram:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control A	Control B	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
B	Control A	Control B	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
C	Control A	Control B	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
D	Control A	Control B	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
E	Control A	Control B	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
F	Control A	Control B	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
G	Control A	Control B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
H	Control A	Control B	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
			QC Serum 1		QC Serum 2		QC Serum 3		QC Serum 4		QC Serum 5	

Control A	Contains bacteria + complement (HI) + HL60, t=75 minutes	Used to calculate non-specific killing.
Control B	Contains bacteria + complement + HL60, t=75 minutes	Used to calculate maximum CFU/spot in assay conditions. Used to calculate non-specific killing.

6. Prepare the differentiated HL60 cells:
   a. Transfer the DMF-differentiated HL60 cells from the culture flasks (1 flask should be sufficient for 2 plates) to 50 ml centrifuge tubes.
   b. Centrifuge the HL60 cells at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5 minutes at RT.
   c. Remove the supernatant and combine all cell pellets in 50 ml of 1X HBSS (**without** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
   d. Remove the supernatant and add 50 ml of 1X HBSS (**with** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
   e. Remove the supernatant, and suspend the cells at 1 x 10^7 cells/ml in Opsonization Buffer B (store the cells at RT until needed). See “Cell Counting” procedure for performing viable cell counts.

f. When counting the cells, determine the viability using trypan blue exclusion. Record the number of live cells and the number of dead cells. The cell viability must be ≥90% for the cells to be considered acceptable for use in the assay.

7. Rapidly thaw a frozen working stock tube of each of the four bacteria strains and wash:
   a. Gently swirl the tubes in a 37°C water bath.
   b. Centrifuge the tubes at 12,000*g* for 2 minutes in microcentrifuge.
   c. Carefully remove supernatant and discard.
   d. Add 1 ml Opsonization Buffer B to each tube, and mix.
   e. Centrifuge the tubes at 12,000*g* for 2 minutes.
   f. Carefully remove supernatant and discard.
   g. Suspend bacteria pellet in original volume of Opsonization Buffer B (ie, 0.5 ml).
8. Prepare a bacterial mixture by adding the appropriate volume of each of the four strains of bacteria to one tube containing 10 ml of Opsonization Buffer B (should be ~50,000 CFU/ml for each bacteria strain). Add 10 microliters of the bacterial mixture to each well including all control wells.
9. Incubate the microtiter plate for 30 minutes at RT and room air on a mini-orbital shaker (700 rpm).
10. During this time, remove complement from the freezer (~1.3 ml of active complement and ~0.1 ml of heat-inactivate complement are needed). Leave the complement at RT to thaw (**immediately** after complement has thawed completely, keep on ice until needed).
11. During this incubation, transfer 4.8 ml of the HL60 cell suspension prepared above to a clean, sterile 15-ml tube. Also, transfer 400 microliters of the HL60 cell suspension to a clean, sterile 1.5 ml centrifuge tube. Keep both tubes at room temperature until needed.
12. Following the 30 minute incubation, add 100 microliters of inactivated complement to the tube containing the 400 microliters of HL60 cell suspension. Mix well, and add 50 microliters of the mixture to each well in column 1, rows A through H.
13. Add 1.2 ml of the active complement to the tube containing 4.8 ml of HL60 cell suspension. Mix well, and add 50 microliters of the mixture to each well of columns 2 through 12, rows A through H.
14. Incubate the microtiter plates on a mini-orbital shaker (700 rpm) for 45 minutes at 37ºC with 5% CO_2 in a **single** layer, i.e., do not stack plates. In order to maintain a constant CO_2 percent during this incubation step, do not open the incubator door.
15. After the incubation period, place the microtiter plates on ice for ~20 minutes (to stop the phagocytic process).
16. Spot 10 microliters of reaction mixture from each well onto **four** THYA plates. Starting with row H, mix the contents of each well and remove 10 microliters from each well in a 12-well row, and apply as twelve-10 microliter spots to THYA plates on the first (bottom) row of the THYA plate. **Immediately**, tilt the plates to shape the spots into a small strip of fluid (~2-3 cm long). Repeat this procedure for rows G, F, and E (for a total of 48 individual wells per plate). **You must tilt plate immediately to prevent spots from running together.** Rows D, C, B, and A are spotted onto a second set of four plates.
17. Leave the plates at RT for ~20 minutes to let the excess fluid seep into the agar (refer to Note 7).
18. Remove one bottle containing 52 ml overlay prepared above from the water bath, and add 52 microliters TTC stock and 52 microliters optochin stock. Mix well, and add 25 ml of this overlay per plate to each of the first of the four replicate plates spotted above. There should be 2 THYA plates that received this overlay.
19. Remove the second bottle containing 52 ml overlay prepared above from the water bath, and add 52 microliters TTC stock and 52 microliters streptomycin stock. Mix

well, and add 25 ml of this overlay per plate to each of the second of the four replicate plates spotted above. There should be 2 THYA plates that received this overlay.

20. Remove the third bottle containing 52 ml overlay prepared above from the water bath, and add 52 microliters TTC stock and 52 microliters spectinomycin stock. Mix well, and add 25 ml of this overlay per plate to each of the third of the four replicate plates spotted above. There should be 2 THYA plates that received this overlay.
21. Remove the fourth bottle containing 52 ml overlay prepared above from the water bath, and add 52 microliters TTC stock and 52 microliters trimethoprim stock. Mix well, and add 25 ml of this overlay per plate to each of the fourth of the four replicate plates spotted above. There should be 2 THYA plates that received this overlay.
22. Incubate the plates at RT for ~20 minutes. After the overlay has solidified, place the plates, upside down, in a candle jar and incubate them for 16-18 hours at 37ºC (Note 13). Colonies that grow on the THYA plates with the optochin overlay are bacteria of the "optochin resistant" serotype, those that grow on the THYA plates containing streptomycin are bacteria of the "streptomycin resistant" serotype, etc.
23. Count colonies manually or using an automated counter (see Note 11 for colony counting options).
24. Calculate the opsonic indices and non-specific killing (Note 18). Opsonization indices can be calculated using linear interpolation of the serum dilution killing the indicated percentage (usually 50%) of the bacteria. Since each bacteria strain likely has different values for control B, each strain must be analyzed separately. Therefore, when the analysis is completed, you should have 4 files (1 for each serotype).
    a. Open analysis template "opsotiter3.XX.xls" ("XX" refers to version number that will change over time).
    b. Select the "RawData" worksheet of the template file.
    c. Enter raw data/assay information for a single plate (cells requiring input are highlighted yellow):
        i. Enter the raw data (CFU/spot) into the corresponding cells, noting the positions for control A and control B. This can be done manually, or by copying and pasting data from another file. If data is entered electronically from another file, make sure the format is the same as the format indicated.
        ii. Enter the assay information into the indicated cells.
        iii. Make necessary changes to the adjustable assay parameters. Typically, these do not change from assay to assay.
        iv. Enter the sample information (name and pre-dilution factor) into the indicated cells.
    d. Save the file, and print the "RawData" and "Report" worksheets.
    e. To analyze another set of data, close the file, and re-open the template, i.e., do not use one analyzed file as a template to analyze another set of data.
25. After each QC serum has been tested at least twice with the prospective FBS lot, calculate the average opsonization index for each serum for each serotype. Also, record the non-specific killing for each run.

C. FBS Lot Acceptance Criteria

A prospective lot of FBS will be considered acceptable if:

1. For each QC sera, the mean opsonic index (n≥2) for the FBS is within the accepted range determined using the previously qualified FBS lot (mean ± 2 SD).
2. For most serotypes, the NSK is ≤30%. For some serotypes (e.g. 6A, 6B, and 7F), the NSK may be higher. NSK up to 70% is acceptable for these serotypes.

6. UAB-MOPA Procedure

The protocol below is used for a single "run", with "run" defined as 7 microtiter plates. For experiments involving more than 41 samples, multiple "runs" should be performed (i.e., the first 7 microtiter plates are completely finished before beginning the next 7 microtiter plates). Test serum samples must be heat-inactivated as described previously prior to being tested in the UAB-MOPA.

Target strains are grouped according to the table below:

	Strain 1	Strain 2	Strain 3	Strain 4
Assay Cassette A	OREP4	SPEC6B	STREP14	EMC23F or TREP23F
Assay Cassette B	OREP18C	SPEC19F	EMC9V or STREP9V	TREP6A
Assay Cassette C	OREP3	SPEC1	STREP5	TREP19A
Assay Cassette D	OREP7F	SPEC6C	STREP33F	TREP22F
Assay Cassette E	OREP17F	SPEC9N	STREP8	TREP11A
Assay Cassette F	OREP10A	SPEC6D	STREP2	TREP12F
Assay Cassette G	empty*	SPEC20B	empty*	TREP15B

*To maintain the E:T ratio, two irrelevant strains of the proper antibiotic resistance should be included. For example, OREP3 and STREP8 could be included.

1. Prepare OBB (100 ml should be sufficient).
2. Dry large (~12 cm x ~12 cm) THYA plates by removing the lids and placing the plates in a laminar flow hood for 30-60 minutes (see Note 4). You will need 56 plates. After plates are dry, replace lid to prevent over-drying, and keep at RT until needed. Label the plates with the following information: assay plate identifier, row numbers, and strain name (or antibiotic added to overlay). For example, a plate labeled "C, H-E, OP" represents rows H, G, F, and E of microtiter plate C containing the optochin resistant strain.
3. Melt the overlay agar (you will need ~1500 ml for a seven microtiter plate assay) in a microwave using 50% power. Gently and carefully swirl overlay agar to ensure that all agar clumps have melted. Alternatively, overlay can be prepared and autoclaved the day of the assay to obviate the need for melting in a microwave. Aliquot 370 ml to each of four sterile bottles and place bottles in 50ºC water bath until needed .
4. If not already done, prepare heat-inactivated (HI) complement for assay "Control A" (alternatively, many aliquots of heat-inactivated complement can be prepared ahead of time and kept at -80°C until needed):
    a. Remove a vial of complement and allow to thaw completely at room temperature.
    b. Incubate complement in 56°C water bath for 30 minutes.
    c. Allow HI complement to cool to room temperature before using.
5. Prepare the microtiter plates following the plate layout diagram below. The procedure described below utilizes 8 dilutions of serum serially diluted 3-fold, starting at a 4-fold dilution. For alternative assay formats see Note 12.
    a. Add 20 microliters Opsonization Buffer B to rows A through H, columns 1

and 2 of plate A.

b. Add 20 microliters Opsonization Buffer B to rows A through G, columns 3 through 12 of plate A. Also, add 20 microliters Opsonization Buffer B to rows A through G, columns 1 through 12 of plates B, C, D, E, F and G.
c. Add 30 microliters of serum sample #1 to plate A, row H, columns 3 and 4. Add 30 microliters of serum sample #2 to plate A, row H, columns 5 and 6. Continue with remaining samples according to template below.
d. Perform 3-fold serial dilutions in columns 3 through 12 of plate A and all columns of plates B through E. Make sure to change pipet tips between rows.
    i. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
    ii. Transfer 10 microliters of serum from row H to row G. Discard tips.
    iii. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
    iv. Using new pipet tips, transfer 10 microliters of serum from row G to row F. Discard tips.
    v. Using a plate shaker, mix plate (800-1000 rpm) for ~5 seconds.
    vi. Continue through row A. After mixing plate, remove and discard 10 microliters from row A.

Plate layout:

Plate A

	1	2	3	4	5	6	7	8	9	10	11	12
A	Control A	Control B	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
B	Control A	Control B	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
C	Control A	Control B	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
D	Control A	Control B	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
E	Control A	Control B	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
F	Control A	Control B	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
G	Control A	Control B	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
H	Control A	Control B	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
			Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	

Plates B, C, D, E, F, and G

	1	2	3	4	5	6	7	8	9	10	11	12
A	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8	Dilution 8
B	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7	Dilution 7
C	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6	Dilution 6
D	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5	Dilution 5
E	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4	Dilution 4
F	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3	Dilution 3
G	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2	Dilution 2
H	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1	Dilution 1
	Sample 6, 12, 18, etc		Sample 7, 13, 19, etc		Sample 8, 14, 20, etc		Sample 9, 15, 21, etc		Sample 10, 16, 22, etc		Sample 11, 17, 23, etc	

Control A	Contains bacteria + complement (HI) + HL60, t=75 minutes	Used to calculate non-specific killing.
Control B	Contains bacteria + complement + HL60, t=75 minutes	Used to calculate maximum CFU/spot in assay conditions. Used to calculate non-specific killing.

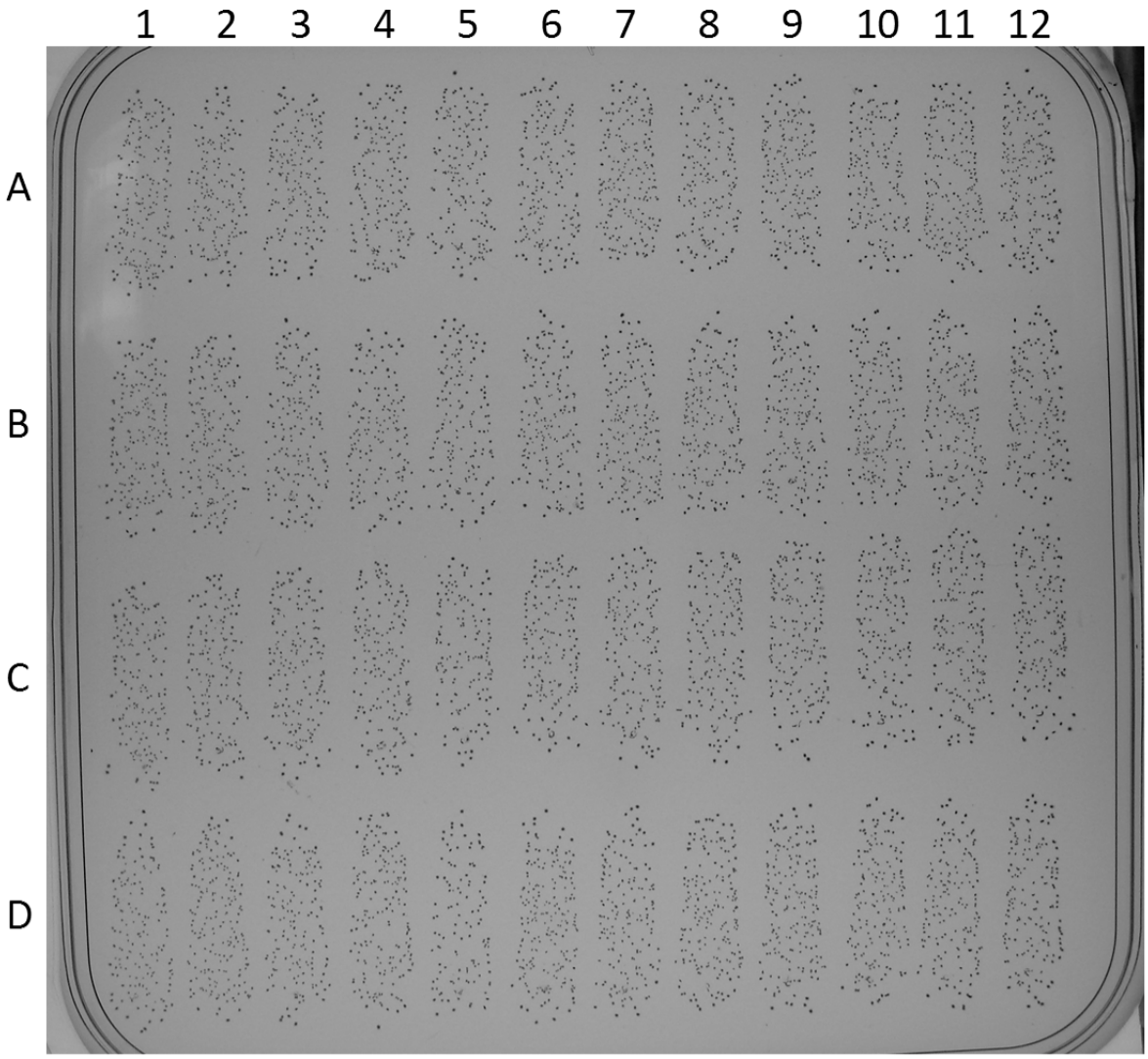
6. Prepare the differentiated HL60 cells:
    a. Transfer the DMF-differentiated HL60 cells from the culture flasks (5 flasks should be sufficient for 7 assay plates) to 50 ml centrifuge tubes.

b. Centrifuge the HL60 cells at ~350*g* (1200 rpm using a Sorvall RT7 with RTH-250 rotor) for 5 minutes at RT.
c. Remove the supernatant and combine all cell pellets in 50 ml of 1X HBSS (**without** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
d. Remove the supernatant and add 50 ml of 1X HBSS (**with** Ca^{++}/Mg^{++}). Centrifuge at ~350*g* for 5 minutes at RT.
e. Remove the supernatant, and suspend the cells at 1 x 10^7 cells/ml in Opsonization Buffer B (**store the cells at RT until needed**). See "Cell Counting" procedure for performing viable cell counts. You will need ~32 ml of cells.
f. When counting the cells, determine the viability using trypan blue exclusion. Record the number of live cells and the number of dead cells. The cell viability must be ≥90% for the cells to be considered acceptable for use in the assay.

7. Rapidly thaw a frozen working stock tube of each of the four bacteria strains and wash:
   a. Gently swirl the tubes in a 37°C water bath.
   b. Centrifuge the tubes at 12,000*g* for 2 minutes in microcentrifuge.
   c. Carefully remove supernatant and discard.
   d. Add 1 ml Opsonization Buffer B to each tube, and mix.
   e. Centrifuge the tubes at 12,000*g* for 2 minutes.
   f. Carefully remove supernatant and discard.
   g. Suspend bacteria pellet in original volume of Opsonization Buffer B (ie, 0.5 ml).
8. Prepare a bacterial mixture by adding the appropriate volume of each of the four strains of bacteria (using the dilution factors calculated in the Bacteria Stocks section above) to one tube containing 10 ml of Opsonization Buffer B (should be ~50,000 CFU/ml for each bacteria strain). Add 10 microliters of the bacterial mixture to each well including all control wells.
9. Incubate the microtiter plates for 30 minutes at RT and room air on a mini-orbital shaker (700 rpm).
10. During this time, remove required number of complement vials (~0.2 ml of heat inactivated complement and ~8 ml of active complement are needed) from the freezer. Leave the complement vials at RT to thaw (**immediately** after complement has thawed completely, incubate on ice until needed).
11. Following the 30 minute incubation, prepare HL60/complement (**HI**) mixture by mixing 0.4 ml of HL60 cell suspension with 0.1 ml of heat-inactivated complement. Add 50 microliters/well of mixture containing heat inactivated complement to each well of plate A, column 1.
12. Prepare HL60/complement (active) mixture by mixing 30.4 ml of HL60 cell suspension with 7.6 ml of complement. Add 50 microliters/well of mixture containing active complement to each well of all plates except column 1 of plate A.
13. Incubate the microtiter plates on a mini-orbital shaker (700 rpm) for 45 minutes at 37ºC with 5% CO_2 in a **single** layer, i.e., do not stack plates. In order to maintain a constant CO_2 percent during this incubation step, do not open the incubator door.
14. After the incubation period, place the microtiter plates on ice for ~20 minutes (to stop the phagocytic process).
15. Spot 10 microliters of reaction mixture from each well onto **four** THYA plates. Starting with row H, mix the contents of each well and remove 10 microliters from each well in a 12-well row, and apply as twelve-10 microliter spots to THYA plates on the first (bottom) row of the THYA plate. **Immediately**, tilt the plates to

shape the spots into a small strip of fluid (~2-3 cm long). Repeat this procedure for rows G, F, and E (for a total of 48 individual wells per plate). **You must tilt plate immediately to prevent spots from running together.** Rows D, C, B, and A are spotted onto a second set of four plates.

16. Leave the plates at RT for ~20 minutes to let the excess fluid seep into the agar (refer to Note 7).
17. Remove one bottle containing 350 ml overlay prepared above from the water bath, and add 350 microliters TTC stock and 350 microliters optochin stock. Mix well, and add 25 ml of this overlay per plate to each of the first of the four replicate plates spotted above. There should be 14 THYA plates that receive this overlay.
18. Remove the second bottle containing 350 ml overlay prepared above from the water bath, and add 350 microliters TTC stock and 350 microliters streptomycin stock. Mix well, and add 25 ml of this overlay per plate to each of the second of the four replicate plates spotted above. There should be 14 THYA plates that receive this overlay.
19. Remove the third bottle containing 350 ml overlay prepared above from the water bath, and add 350 microliters TTC stock and 350 microliters spectinomycin stock. Mix well, and add 25 ml of this overlay per plate to each of the third of the four replicate plates spotted above. There should be 14 THYA plates that receive this overlay.
20. Remove the fourth bottle containing 350 ml overlay prepared above from the water bath, and add 350 microliters TTC stock and 350 microliters trimethoprim stock. Mix well, and add 25 ml of this overlay per plate to each of the fourth of the four replicate plates spotted above. There should be 14 THYA plates that receive this overlay.
21. Incubate the plates at RT for ~20 minutes. After the overlay has solidified, place the plates, upside down, in a candle jar and incubate them for 16-18 hours at 37ºC (Note 13). Colonies that grow on the THYA plates with the optochin overlay are bacteria of the “optochin resistant” serotype, those that grow on the THYA plates containing streptomycin are bacteria of the “streptomycin resistant” serotype, etc.
22. After overnight incubation, plates should look like this:



23. Count colonies manually or using an automated counter (see Note 11 for colony counting options).
24. After data is analyzed, THYA plates should be discarded after sterilization (Note 10).

Title: Data

Revision History: 2/1/08; 11/1/11

7. Data Handling

A. Data Conversion

Opsonization indices can be calculated using linear interpolation of the serum dilution killing the desired percentage (usually 50%) of the bacteria using analysis template "opsotiter3" (Note 18).

Since each bacteria strain likely has different values for control B, each strain must be analyzed separately. Therefore, when the analysis is completed, you should have 4 files (1 for each serotype).

1. Open analysis template "opsotiter3".

2. Select the “RawData” worksheet of the template file.
3. Enter raw data/assay information (cells requiring input are highlighted yellow):
4. Enter the raw data (CFU/spot) into the corresponding cells, noting the positions for control A and control B. This can be done manually, or by copying and pasting data from another file. If data is entered electronically from another file, make sure the format is the same as the format indicated.
5. Enter the assay information into the indicated cells.
6. Make necessary changes to the adjustable assay parameters. Typically, these do not change from assay to assay.
7. Enter the sample information (name and pre-dilution factor) into the indicated cells.
8. Save the file, and print the “RawData” and “Report” worksheets. All analyzed data files must be stored in a secure, backed-up location.
9. To analyze another set of data, close the file, and re-open the template, ie do not use one analyzed file as a template to analyze another set of data.
10. Give the data to the laboratory supervisor for review to determine which, if any, samples should be repeated (see Note 16).

B. Assay Acceptance Criteria

Data from an entire run may be accepted if all of the following criteria are met (note that the data from individual samples may still be rejected, see below):

1. No micro-colonies are present. Micro-colonies are bacteria colonies that are ~5- to 10-fold smaller than regular colonies. Micro-colonies are usually an indication of incomplete antibiotic sensitivity.
2. The maximum CFU/spot is ≥ 70 and ≤180.
3. The NSK for all serotypes is ≤ 70%.
4. The titers for QC sera are within the acceptable ranges (mean ± 2SD). If three QC sera are included, the assay data will be accepted if two of the three are in range.

C. Acceptance Criteria for Individual Sample Data

For individual serum samples, the OI will be accepted if all of the above criteria are met and:

1. No bactericidal agents (antibiotics) were detected.
2. The replicate precision is acceptable as determined by the laboratory supervisor.
3. The sample’s killing curve is not irregular. We are currently developing criteria to define regular (and irregular) curves. See Note 16 for tentative acceptance scheme.

Title: Assay Notes

Revision History: 2/1/08; 6/23/08; 11/1/11; 9/20/12

8. Assay Notes

Note 1: Baby rabbit complement and/or HL60 cells can influence the number of viable bacterial cells. Therefore, complement and HL60 cells should be present when determining the dilution factor for each strain of bacteria used in the OPKA.

Note 2: Brief description of the UAB-MOPA method:

Serum	20 microliters	Neat or diluted
Bacteria	10 microliters	2000 total CFU/well (500 CFU/well each serotype

		X 4 serotypes)
HL60 cells/Complement (4:1 mixture)	50 microliters	400,000 HL60/well; final complement conc. is 12.5%
Total volume	80 microliters	

Note 3: TTC turns red upon heating and should **not** be added before autoclaving, or reheating in a microwave oven. Add TTC to the agar after it cools to ~50ºC.

Note 4: Drying the THYA plates for the correct amount of time is very important. Usually 30-60 minutes is sufficient, although drying times vary depending on the temperature and humidity in the air (our laboratory is usually ~21°C with a relative humidity of ~70%). Under-drying the plates results in an excess number of colonies around the perimeter of the spot. This can affect the counting of the colonies. Over-drying the plates can cause the spots to run together when the plates are tilted.

Note 5: It is important to remove as much of the DMSO-containing freezing medium as possible because DMSO causes the HL60 cells to differentiate.

Note 6: It is important to maintain the CO_2 concentration at 5% as HL60 cells are sensitive to subtle $\%CO_2$ changes. It is recommended the $\%CO_2$ be checked regularly with an outside reference (such as a FYRITE® Gas Analyzer produced by Bacharach, Pittsburgh, PA). Also, proper humidity is important. Make sure that water level within the incubator is maintained at levels specified by the manufacturer.

Note 7**:** Usually, for absorption of excess liquid, plates can be incubated on the bench top in room air. However, if excessive contamination occurs (for example, due to poor air quality), plates may need to be incubated in a laminar flow hood. In this case, absorption times may need to be shortened.

Note 8: Blood agar plates may also be used. If blood agar plates are used, do not add an overlay.

Note 9: With the following modifications, this protocol can be used for a **single serotype** assay:

a. For the assay, bacteria should be diluted to ~100,000 CFU/ml (for UAB-MOPA, bacteria is diluted to ~50,000 CFU/ml). Adding 10 microliters of diluted bacteria to each well yields an effector:target ratio of 400:1 (UAB-MOPA uses a 200:1).
b. After the assay is complete, spot 5 microliters of the final reaction product onto a single THYA plate (for UAB-MOPA, 10 microliters is spotted on four THYA plates).
c. Since only one serotype is added to each well, antibiotics are not needed and should not be added to the overlay (TTC is still added to the overlay).

Note 10: *Streptococcus pneumoniae* is a human pathogen that must be handled in biosafety level BSL2 conditions. All items that come in contact with bacteria are considered biohazardous, and should be disposed of in accordance with local regulations.

Note 11: You can take a picture of your THYA plates with a digital camera or scanner and email the images to someone with colony counting software (see reference 6). We have also collaborated with the US National Institute of Standards and Technology to develop software that can enumerate colonies in a digital image. This software is called

"NICE". For more information about NICE contact: nice@nist.gov. To download the software, go to: ftp://ftp.nist.gov/pub/physics/mlclarke/NICE/.

Note 12: Alternate assay formats include the use of 11 serum dilutions, different serial dilution factor (i.e., 2 instead of 3), and/or 8-fold sensitivity.

Note 13: We use 10 gallon glass fish tanks as candle jars. After positioning plates in tank, light candle, place aluminum foil over the top of the tank, and seal foil tightly with tape. To ensure consistent, adequate growth, tanks should not be filled to more than 50% capacity.

Note 14: We have obtained **inconsistent** results with autoclaved THY broth. Therefore, we recommend filter sterilization.

Note 15: Because different antibody clones and different lots of the same clone may provide different results, the optimal antibody concentrations should be determined by the individual users. The concentrations that are provided are clone- and lot-specific.

Note 16: Tentative acceptance criteria for individual samples:

Good curves
Currently, the best definition of a good curve is one that has the general sigmoid shape killing curve and does not fit into one of the categories below.

U curves
Samples that display a U-shaped killing curve with maximum killing between 40% and 70% (inclusive) will be repeated. If the second run data matches the first run data, the first run data will be accepted. If the second run data differs by more than 3-fold from the first run data, the sample will be tested a third time. If the data from the third run matches (within 3-fold) the first or the second run data, the first or second run data will be used, respectively. If the third run does not match either of the first two runs, the OI will be assigned as "TND".

N curves
Samples that display an N-shaped killing curve with maximum killing of the right-most titer point between 40% and 70% will be repeated. If the second run data matches the first run data, the first run data will be accepted. If the second run data differs by more than 3-fold from the first run data, the sample will be tested a third time. If the data from the third run matches (within 3-fold) the first or the second run data, the first or second run data will be used, respectively. If the third run does not match either of the first two runs, the OI will be assigned as "TND".

Extraneous data
In the UAB-MOPA, quite often a sample needs to be repeated for only one serotype in that bacteria group. Sometimes, we can mix/match the group partners to re-test only the serotypes that need to be re-tested. However, most of the time, we get extraneous repeat data for serotypes that did not need to be repeated. In these situations, follow the rules above for repeat data (first run, second run, third run, etc).

Note 17: Barbital is a schedule IV controlled substance, and thus requires a prescription. The barbital should be stored at room temperature in a secure location.

Note 18: Opsotiter3 is an Excel®-based program that was developed to analyze data from opsonophagocytic killing assays, specifically assays following the UAB MOPA format. After raw colony count data is pasted into the program, opsotiter3 estimates the opsonic index for each sample by determining the dilution of serum that kills 50% of the bacteria using a linear interpolation algorithm. Opsotiter3 helps to manage all of the assay information by tabulating the opsonic indices for all of the test samples, as well as other user-entered information and assay-specific information into a concise report sheet. Opsotiter3 also generates the point-to-point dose-response curves for each sample.

Note 19: We have found lot-to-lot variations in the activity of optochin. Therefore, each new batch should be tested for adequate potency, and the concentration should be adjusted accordingly. The concentration used for the assay should kill the sensitive strains at ½ the assay concentration but have no effect on the resistant strains at twice the assay concentration. From our experience, the concentration used for the assay varies from 2 mg/L to 8 mg/L. Optochin is soluble in water up to at least 8 mg/ml, so a 1000X solution can be prepared. Click here for more information.

Title: References

Revision History: 2/1/08; 8/1/13

9. References

1) Romero-Steiner S, Frasch CE, Carlone G, Fleck RA, Goldblatt D, and Nahm MH. Use of opsonophagocytosis for serological evaluation of pneumococcal vaccines. Clin. Vacc. Immuno. 2006 Feb; 13(2):165-169.

2) Kim KH, Yu J, Nahm MH. Efficiency of a pneumococcal opsonophagocytic killing assay improved by multiplexing and by coloring colonies. Clin. Diag. Lab. Immunol. 2003 Jul; 10(4):616-21.

3) Burton RL and Nahm MH. Development and validation of a fourfold multiplexed opsonization assay (UAB-MOPA) for pneumococcal antibodies. Clin. Vacc. Immuno. 2006 Sep; 13(9):1001-1009.

4) Fleck RA, Romero-Steiner S, and Nahm MH. Use of HL-60 cell line to measure opsonic capacity of pneumococcal antibodies. Clin. Diag. Lab. Immunol. 2005 Jan; 12(1):19-27.

5) Romero-Steiner S, et al. Multilaboratory evaluation of a viability assay for measurement of opsonophagocytic antibodies specific to the capsular polysaccharides of streptococcus pneumoniae. Clin. Diag. Lab. Immunol. 2003 Nov ; 10(6) :1019-1024.

6) Putman M, Burton RL, and Nahm MH. Simplified method to automatically count bacterial colony forming unit. J. Immunol. Meth. 2005 July ; 302(1-2):99-102.

7) Burton RL, and Nahm MH. Development of a fourfold multiplexed opsonophagocytosis assay for pneumococcal antibodies against additional serotypes and discovery of serological subtypes in *Streptococcus pneumoniae* serotype 20. Clin Vaccine Immunol 2012;19:835-41.

10. Materials, Reagents, Solutions, and Equipment

Note that the items listed below that have specific manufacturers and catalog numbers represent those used at UAB. Comparable items from other manufacturers may work as well, but have not been tested by UAB.

A. Plasticware and Glassware

Description	Manufacturer	Catalog Number
Tissue culture flask, vent cap (T150, 150 cm^2)	Falcon	355000
Microtiter plate (round bottom, tissue culture treated, for UAB-MOPA protocol)	CoStar	3799
Microtiter plate (ELISA plate, flat bottom, for CH50 assay)	CoStar	9017
Small square Petri dish (10 cm x 10 cm x 1.5 cm)	Nunc	4021
Large square Petri dish (12 cm x 12 cm x 1.5 cm)	VWR (Greiner Bio-One #688102)	82051-066
Sterile reagent reservoir*	CosStar	4870
Cryovial (2 ml, self standing)	VWR	66008-284
Microcentrifuge tubes (1.5 ml)	Fisher	05-406-16
Centrifuge tubes (50 ml)	Fisher	05-539-6
Centrifuge tubes (15 ml)	Corning	430790
Pipets (50 ml)	Falcon	357550
Pipets (25 ml)	Falcon	357535
Pipets (10 ml)	Falcon	357551
Pipets (5 ml)	Falcon	357543
Filter, 0.22 micrometer, syringe top	Millipore	SLGP033RS
Assorted pipet tips	Any	Any
Cotton-tipped applicators	General Medical Corp	24-806-2S
Inoculation loops, disposable	Nunc	254437
Filter, 0.2 micrometer, bottle top	Millipore	SCGPT05RE
Glass bottles (1L, 500 ml, 250 ml, 100 ml)	Any	Any
Fiberboard boxes for freezing (2 inch)	Fisher	11-678-24A

B. Solutions and Reagents

Penicillin/Streptomycin stock (100X)	Invitrogen	15140-148
GlutaMax-1 (100X)	Invitrogen	35050-061
RPMI 1640	CellGro	MT 10-040-CM
Bovine Serum (Fetalclone I, for	HyClone	SH30080.03

HL60 cells)		
Fetal Bovine Serum (defined FBS or premium FBS, for OBB)	HyClone or Atlanta Biologicals	SH30070.03 or 11150
10X HBSS (without Ca, Mg, phenol red)	Invitrogen	14185-052
10X HBSS (with Ca and Mg, without phenol red)	Invitrogen	14065-056
Baby Rabbit Complement (3-4 week, see complement section for lot acceptance criteria)	Pel-freez Biologicals (Rogers, AR, USA)	31061
Sheep blood (in alsevers solution)	Colorado Serum Company	CS1113

C. Bacterial Culture Reagents

Description	Manufacturer	Catalog Number
Todd Hewitt Broth	Becton-Dickinson	249240
Yeast Extract	Becton-Dickinson	212750
Bacto Agar	Becton-Dickinson	214010
Blood Agar Plates	Remel	1202

D. Chemicals

Description	Manufacturer	Catalog Number
Gelatin	Sigma	G-9391
Glycerol	Sigma	G-7893
N,N-dimethylformamide (DMF)	Fisher	D131-1
2,3,5,-triphenyltetrazolium chloride (TTC)	Sigma	T-8877
Streptomycin sulfate	Sigma	S-6501
Optochin (Ethylhydrocupreine HCl)	Sigma	E-9876
Spectinomycin	Sigma	S-9007
Trimethoprim	Sigma	T-7883
Dimethylsulfoxide (DMSO)	Sigma	D-2650
Sodium azide	Sigma	S-2002
Calcium chloride	Sigma	C-4901
Magnesium chloride, 1M solution	Fluka	63020
Sodium chloride	Fisher	S271
Barbital sodium C-IV (see Note 17)	Sigma	B-0500
Hemolysin	Sigma	S-1389
Hydrochloric acid	Fisher	A144
Trypan blue solution (0.4%)	Sigma	T-8154

E. Equipment and Software

Description	Manufacturer	Catalog Number
Assorted pipettors	Any	Any
Mini-orbital shaker (with rpm display). You will need two: one at room temperature and one at 37ºC with 5% CO_2)	Bellco Biotechnology	7644-20115

CO_2 incubator, 37ºC, 5% CO_2, humidified	Thermo-Fisher	13-255-25
Water bath (56ºC)	Any	Any
Water bath (50ºC)	Any	Any
Water bath (37ºC)	Any	Any
Microwave oven (1.65 kW)	General Electric	Model JES1358WJ01
Controlled-rate freezer	Thermo Forma	Cryomed Systems
Cryobiological storage system	Thermo Forma (or equivalent)	8031 (or equivalent)
Microcentrifuge	Kendro (or equivalent)	Biofuge fresco (or equivalent)
Water purification system	Millipore	Synergy 185
Laminar flow hood (biological safety cabinet)	Any	Any
Colony counting software	Synbiosis or NIST (See Note 11)	ProtoCOL or NICE (See Note 11)
Calculation programs	www.vaccine.uab.edu	www.vaccine.uab.edu
Autoclave	Any	Any
Computer (PC) with MS Excel®	Any	Any
Microplate reader (with 405 nm filter)	Bio-Tek Instruments	ELx808
Spectrophotometer (with 541 nm filter)	Bio-Rad	Smart Spec 3000
Centrifuge (with 15-ml and 50-ml tube adaptors and microplate carriers)	Kendro	RT7 Plus
Flow cytometer (with Cell Quest)	Becton Dickinson	FACS Caliber
Flow cytometry analysis software	DeNovo Software	FCS Express

F. Cell Lines

Description	Manufacturer	Catalog Number
HL60 cell line	ATCC	CCL-240

G. Bacteria

BEI Catalog #	Strain Name	Description	Reference
NR-13388	SPEC1	Spectinomycin resistant variant of L82006 (serotype 1)	2
NR-31700	STREP2	Streptomycin resistant variant of DBL2 (serotype 2)	7
NR-13389	OREP3	Optochin resistant variant of Wu2 (serotype 3)	2
NR-13390	OREP4	Optochin resistant variant of DS2382-94 (serotype 4)	2
NR-13391	STREP5	Streptomycin resistant variant of DBL5 (serotype 5)	2
NR-13392	TREP6A	Trimethoprim resistant variant of EF6796 (serotype 6A)	2
NR-13393	SPEC6B	Spectinomycin resistant variant of BG25-9 (serotype 6B)	2
NR-20805	SPEC6C	Spectinomycin resistant variant of BGO-2197 (serotype 6C)	7
NR-20806	SPEC6D	Spectinomycin resistant variant of MNZ920	7

		(serotype 6D)	
NR-13394	OREP7F	Optochin resistant variant of DS2617-97 (serotype 7F)	2
NR-31701	STREP8	Streptomycin resistant variant of DS5675-06 (serotype 8)	7
NR-31702	SPEC9N	Spectinomycin resistant variant of DS1398-00 (serotype 9N)	7
NR-13395	EMC9V	Streptomycin resistant variant of 1081748 (serotype 9V)	2
NR-31703	OREP10A	Optochin resistant variant of DS3032-06 (serotype 10A)	7
NR-31705	TREP11A	Trimethoprim resistant variant of DS3160-06 (serotype 11A)	7
NR-31704	TREP12F	Trimethoprim resistant variant of DS4031-06 (serotype 12F)	7
NR-13396	STREP14	Streptomycin resistant variant of DS2214-94 (serotype 14)	2
NR-33666	TREP15B	Trimethoprim resistant variant of DS0556-97 (serotype 15B)	7
NR-31706	OREP17F	Optochin resistant variant of DS3022-06 (serotype 17F)	7
NR-13397	OREP18C	Optochin resistant variant of GP116 (serotype 18C)	2
NR-13398	TREP19A	Trimethoprim resistant variant of DS3519-97 (serotype 19A)	2
NR-13399	SPEC19F	Spectinomycin resistant variant of 2217-94 (serotype 19F)	2
NR-33664	SPEC20B	Spectinomycin resistant variant of DS3014-06 (serotype 20B)	7
NR-31707	TREP22F	Trimethoprim resistant variant of DS3433-06 (serotype 22F)	7
NR-13400	EMC23F	Clinical isolate (1212458), naturally resistant to trimethoprim (serotype 23F)	2
NR-33665	STREP33F	Streptomycin resistant variant of DS3052-06 (serotype 33F)	7

H. Flow Cytometry Reagents

Description	Manufacturer	Catalog Number
Anti-human CD11b PE	BDIS	555388
Anti-human CD35 PE	BDIS	559872
Anti-human CD71 PE	BDIS	555537
IgG1 PE Isotype	BDIS	556650
IgG2a PE Isotype	BDIS	555574
Propidium Iodide Solution	Sigma	P4864
Annexin V FITC	BD Pharmingen	51-65874X
10X Annexin V Binding Buffer	BD Pharmingen	51-66121E
Microtiter plate (V-bottom)	Nunc	249570
FACS tubes	Falcon	352008

I. Recipes for Prepared Solutions

Water
Only ultrapure water should be used for preparation of solutions.

CM1 (tissue culture medium for HL60 propagation)
To 1 liter of RPMI 1640, add 114 ml FetalClone I (heat inactivated at 56°C for 30 minutes), 11.4 ml GlutaMax-1, and 11.4 ml penicillin/streptomycin stock. If desired, penicillin/streptomycin may be omitted.

CM2 (tissue culture medium for HL60 differentiation)
To 1 liter of RPMI 1640, add 114 ml FetalClone I (heat inactivated at 56°C for 30 minutes), 11.4 ml GlutaMax-1, and 9.1 ml DMF (Dimethylformamide). **Do not add penicillin/streptomycin.**

CM3 (tissue culture medium for recovering HL-60 after cryogenic storage)
To 1 liter of RPMI 1640, add 256 ml FetalClone I (heat inactivated at 56°C for 30 minutes), 12.8 ml GlutaMax-1, and 12.8 ml penicillin/streptomycin stock. If desired, penicillin/streptomycin may be omitted.

Todd-Hewitt-Yeast broth (THYB)
In a 1L glass, sterile bottle, add 30 g of Todd-Hewitt broth and 5 g of yeast extract to 1000 ml water. Mix solution until all components have completely dissolved. Sterile filter using a 0.22 micrometer bottle top filter into a sterile 1L glass bottle. Store at 4ºC. See Note 14.

Todd-Hewitt-yeast extract agar plate (THYA plate)
In a 500 ml glass, autoclavable bottle, add 12 g of Todd-Hewitt broth, 2 g of yeast extract and 6 g of Bacto agar to 400 ml of water. Autoclave and let agar cool to 56ºC in a water bath. On a level surface, use a 25 ml pipet to add 25 ml to a square Petri dish (~12 cm x ~12 cm) and incubate at RT for ~20 minutes. Store in sealed plastic bags at 4ºC for up to one month.

Overlay agar
In a 1L glass, autoclavable bottle, add 24 g of Todd-Hewitt broth, 4 g of yeast extract and 6 g of Bacto agar to 800 ml of water. Autoclave and store at RT. Alternatively, overlay agar can be made the day of the assay, autoclaved, and stored in a 50°C water bath until needed for the assay (agar should be kept in water bath at least 1-2 hours to ensure cooling to 50°C).

TTC stock
Prepare a 25 mg/ml (1000X) stock solution in water by adding 1.25 g of TTC (2,3,5,-triphenyltetrazolium chloride) to 40 ml water. After dissolution, adjust volume to 50 ml with additional water and sterile filter using 0.2 micrometer filter. Store at 4ºC. Solution should have slight yellow color. If solution develops red color, discard and prepare new. TTC colorizes the bacterial colonies making them much easier to count.

Streptomycin stock
Prepare a 300 mg/ml (1000X) stock solution in water by adding 3 g of antibiotic to 5 ml of water. After dissolution, adjust volume to 10 ml with additional water, and sterile filter using 0.2 micrometer filter. Make 1 ml aliquots and store at -20ºC for up to 3 months.

Optochin stock
See Note 19 (the current lot of optochin is used at 8 mg/L). Prepare an 8 mg/ml (1000X) stock solution in water by adding 80 mg of antibiotic to 5 ml of water. After dissolution, adjust volume to 10 ml with additional water, and sterile filter using 0.2 micrometer filter. Make 1 ml aliquots and store at -20ºC for up to 3 months.

Trimethoprim stock
Prepare a 25 mg/ml (1000X) stock solution in DMSO by adding 250 mg of antibiotic to 5 ml of DMSO. After dissolution, adjust volume to 10 ml with additional DMSO. Make 1 ml aliquots and store at -20ºC for up to 3 months.

Spectinomycin stock
Prepare a 300 mg/ml (1000X) stock solution in DMSO by adding 3 g of antibiotic to 5 ml of DMSO. After dissolution, adjust volume to 10 ml with additional DMSO. Make 1 ml aliquots and store at -20ºC for up to 3 months.

1% sterile gelatin solution
Add 1 g of gelatin to 100 ml of water. Autoclave and store at RT.

80% (approximate) glycerol
Mix 20 ml of water and 100 g glycerol. Autoclave and store at RT.

Opsonization Buffer B (OBB)
Mix 80 ml of sterile water, 10 ml of 10X HBSS (with Ca^{++}/Mg^{++}), 10 ml of 1% gelatin, and 5.3 ml of **FBS** (inactivated for 30 minutes at 56°C). **This buffer is used only for 1 day.**

1X HBSS (Hanks' balanced salt solution)
Add 50 ml 10X HBSS stock to 450 ml sterile water, and mix.

10% (w/v) Sodium Azide
Add 5 g NaN_3 to 40 ml of water. After complete dissolution, add water to 50 ml.

10X PBS (with azide)
To 800 ml of water, add the ingredients in the table below. After complete dissolution, add water to 1L. This PBS is used only for FACS analysis—do not use for bacteria culture because it contains azide.

Dry chemical	Weight (grams)
NaCl	80.00
KH_2PO_4	3.14
$Na_2HPO_4 \cdot 7H_2O$	20.61
KCl	1.60
NaN_3	10.00

1X PBS (with azide)
Add 100 ml of 10X PBS (with azide) to 900 ml of water.

FACS Buffer (with azide)
Combine 100 ml of 10X PBS (with azide), 30 ml of FBS (heat-inactivated), and 870 ml of water.

Calcium Chloride (0.3M)
Add 1.66 g of $CaCl_2$ to 40 ml of water. After complete dissolution, add water to 50 ml.

Sodium Chloride (0.9%)
Add 2.25 g NaCl to 200 ml water. After complete dissolution, add water to 250 ml.

5X Gelatin veronal buffer (GVB)
In 1L glass bottle, add 750 ml of water, 41.5 g of NaCl, 5.1 g Barbital sodium C-IV (see Note 1), and 5 g gelatin. Insert stir bar, and place bottle on heated magnetic stirrer set at ~90°C. After all reagents dissolve (~30-60 minutes), remove from heat and add 2 ml of 10% NaN_3, 1 ml of 0.3M $CaCl_2$, and 2 ml of 1M $MgCl_2$. After solution has cooled to room temperature, mix solution and check pH, which should be ~9. Adjust pH to 7.35 ± 0.05 with 6M HCl (should take about 2.5 ml). Add water up to 1L. Store at RT for up to 1 month.

1X Gelatin veronal buffer (GVB)
In glass bottle, add 100 ml 5X GVB and 400 ml water.

11. Cell Counting

1. Prepare 5-fold dilution of sample by mixing 10 microliters of sample with 40 microliters of trypan blue solution.
2. Load one counting chamber of the hemacytometer with the diluted sample allowing capillary action to fill the chamber. Do not over-fill the chamber.
3. Count the number of viable cells (cells that exclude the trypan blue) and the number of nonviable cells (cells that take up the trypan blue dye) in the four corner counting fields and the middle counting field (there are 9 defined counting fields on the hemacytometer). Enter the cell counts onto the assay cover sheet.
4. Calculate the cell concentration:

Number of cells/ml = (# of cells total in 5 counting fields) x 10^4