Hib Colorimetric/Fluorometric Serum Bactericidal Assay

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Principle:
Serum bactericidal activity can be measured using a standardized assay where the dilution with 50% killing as compared to the complement control wells is given as the serum SBA titer.  This standard assay uses viable counts of CFU to determine the SBA titer.  This process is time consuming and laborious.  Alternatively a colorimetric compound such as Alamar Blue can be used to visualize bacterial survival.  Alamar blue is blue in its oxidized form.  When reduced by bacteria or tissue culture cells, Alamar Blue changes to a bright pink coloration that can be measured at 490 nm in the visible range or in the fluorometric UV at 530/590nm (excitation/emission).  The oxidized blue state can only be read at 400nm in the visible range and it is not fluorometric.  The fluorometric endpoint is preferred over the colorimetric endpoint.

Materials and reagents:
Round-bottom plates
diluent buffer:  Hanks with Ca$^{++}$ and Mg$^{++}$ buffer containing 2% Fildes Enrichment (FE)  
(49 ml of Hanks’ buffer and 1 ml of FE)
Alamar Blue buffer: 2.0 ml of BHI broth, 6.4 ml of Hanks + 2%FE, and 1.6 ml of Alamar blue
Overnight incubation (19-24 hrs) for colorimetric (read in spectrophotometer – Hib colorimetric)
6 hour-incubation for fluorometric (read in fluorometer program: Hib SBA)
Bacterial dilution scheme: 10$^{-3}$ plus 8 ml for current stock of strain GB3291

Procedure:
1. Prepare serum diluent buffer by mixing 49 ml of Hanks with 1 ml of F.E.
2. Remove 6.4 ml and place in separate 15 ml sterile tube
3. Prepare serum dilutions as follows:
   • Add 10 microliters (µl) of Hank’s (+) buffer to rows 2-12
   • Add 20 µl of buffer to rows 11 and 12 (total 20 µl)
   • Add 20 µl of sera to row 1, all sera in duplicate, no serum in columns 11 and 12
   • Serially dilute 10 µl of sera from rows 2-10 and discard the remaining 10 µl.
4. Prepare the working dilution of bacteria (10$^{-3}$ plus 8 ml) with the remaining buffer
5. Add 20 µl of bacterial suspension to all wells except column 12
6. Tap the sides of the plate(s) on each side 3 times.
7. Incubate in 5% CO2 incubator at 37°C for 15 minutes.
8. Prepare Alamar Blue buffer: 2.0 ml of BHI broth 6.4ml of Hanks+2%FE, 1.6 ml of AB
9. Add 25 µl of 3-4 week rabbit complement to all wells in plate
10. Add 25 µl Alamar blue buffer to all wells in plate
11. Incubate overnight (19-24 hours), 5% CO2, 37°C for colorimetric or 6 hours for fluorometric assay
12. Read plate(s) in spectrophotometer at 490 nm or in fluorometer at 530/590nm (excitation/emission).
Note: If viability is being performed plate a 5-μl aliquot of each well onto Chocolate agar (GCII agar base) after one hour of incubation (Step 11, above) instead of 6 or 19 hours. Incubate chocolate plates after spots have dried completely overnight (19-24 hours), 5% CO2, 37°C

Expected results:
Plates should turn to grades of pink or fluorescent, depending on the amount of cell growth per well. This in turn is an indicator of the amount of bacterial survival after the SBA reaction took place. The flurometric SBA is a one day assay. Assay plates should be read at 6 hours of incubation or when the complement controls are ≥ 20,000 fluorescence units (FU). Expected background in the blank controls is 3,000 + 500 FU. Blanks with Alamar blue alone should give the background signal to be subtracted from all other wells. Complement controls should give the maximum signal that it can be attained after appropriate incubation

Reference: Romero-Steiner et al. 2004. Measurement of Serum bactericidal activity specific for Haemophilus influenzae Type b by using a chromogenic and fluorescent metabolic indicator. CDLI, 11: 89-93