Opsonophagocytic Killing Assay to Measure Anti-Group A Streptococcus Antibody Functionality in Human Serum.

Summary/Abstract

The opsonophagocytic killing assay (OPKA) is designed to measure the functionality of strain-specific antibodies and therefore assess protective immunity or the immunogenicity of Group A Streptococcus (GAS) (type A *Streptococcus pyogenes*) vaccines. Opsonisation of GAS for phagocytosis is an important mechanism by which antibodies protect against disease *in vivo.* The Opsonophagosytic or Opsonic Index (OI) determined is the estimated dilution of anti-sera that kills 50% of the target bacteria. Here we describe the protocol of the standardised GAS OPKA developed by Jones *et al* 2018.

Key Words

Antibody, Phagocytosis, Opsonophagocytic killing assay, *Streptococcus pyogenes*, HL-60 cells, Baby rabbit complement, In vitro, Functional assay.

1. Introduction

Group A Streptococcus (GAS), a Gram-positive bacteria, can cause asymptomatic infections, mild and severe disease and is the leading causative agent of pharyngitis in children and adolescents worldwide (1, 2). There is currently no licenced vaccine despite a large global burden of disease resulting in more than 0.5 million deaths per year (3). A lack of standardised immunoassays to measure post-vaccination GAS immunity has hindered vaccine development to date, the WHO GAS Vaccine Development Technology Roadmap highlighted this as a key priority activity for vaccine development in 2019 (3). The Lancefield Assay, widely used in the past, measures growth and survival of GAS in fresh human or animal blood (plus immune or non-immune serum) (4). Inter-donor variation in neutrophil and complement activity disqualify this type of assay from full standardisation and therefore use in large, multi-centre vaccine studies. The OPKA described in this chapter, first developed by Jones *et al* 2018 (5), is an important tool for the reliable detection and quantification of functional anti-GAS antibodies which is important for the continued development of GAS vaccines.

The GAS OPKA assay makes use of baby rabbit complement (BRC) as a standard source of complement and continuous human cell line promyelocytic leukemia cell (HL-60) as an exogenous source of phagocytic cells. This method removes the major sources of variation present in many current and past functional assays used to measure GAS vaccine immunogenicity. The assay was optimised for seven clinically relevant GAS strains of emm-type 1, 12 and 6 but other strains can be tested and optimised to be used in the assay.

2. Materials

Prepare all reagents at room temperature. Pyrogen free or deionised water is required for buffer preparations (unless otherwise stated). Volumes can be adjusted accordingly.

1. Tissue culture medium for HL-60 cell recovery from cryogenic storage (M1 medium), 500ml RPMI, 50ml foetal calf serum (FCS), 5ml L-glutamine (200mM). Optional additives; 5ml Penicillin-streptomycin stock (10,000 U/ml), 1ml Fungizone (Amphotericin B).
2. Tissue culture medium for HL-60 cell propagation (M2 medium), 500ml RPMI, 50ml FCS, 5ml L-glutamine (200mM).
3. Freezing medium, 9ml FCS, 1ml DMSO.
4. Todd-Hewitt-yeast extract broth (THY broth), 6g Todd-Hewitt broth, 1g yeast extract, 200ml water. Mix until all components are dissolved and filter sterilise using a 0.22μm bottle top filter into a sterile 200ml bottle.
5. Bacteria storage buffer, 3g Tryptone Soya Broth, 0.5g glucose, 10ml glycerol, 100ml sterile pyrogen free water. Mix until all components are dissolved and autoclave.
6. Todd-Hewitt-yeast extract agar (THY plates), 48g Todd-Hewitt broth, 8g yeast extract, 24g bacteriological agar, 1600ml water. Autoclave and bring to 50°C in a water bath. Pour 25ml agar into 100 x 100mm square agar plate. Leave on a flat surface to dry for ~20 minutes. Invert stacked plates and store at 4°C for up to 1 month.
7. Todd-Hewitt yeast extract overlay agar, 48g Todd- Hewitt broth, 8g yeast extract, 12g bacteriological agar, 1600ml water. Make fresh on day of assay. Autoclave and store in a 50°C water bath until required.

8. 1% gelatin solution, 4g gelatin, 400ml water. Dissolve gelatin in water and autoclave. Store at room temperature (RT) for ≤2 months.
9. 2, 3, 5-tetraphenyltetrazolium chloride (TTC) stock, 1.25g TTC, 50ml sterile pyrogen free water. Dissolve TTC in 40ml water and make up to a final volume of 50ml with the remaining water. Sterile filter with a 0.22µm filter. Liquid should have a yellowish tinge. Store at +4°C for ≤ 1 month.
10. Opsonisation buffer (OPS buffer), 5ml FCS, 40ml 1x HBSS (+Ca/Mg), 5ml 1% gelatin solution. Prepare on day of assay and discard after use.
11. HL-60 Cells, ATCC.
12. Baby rabbit complement, Pel-Freez.

3. Methods

3.1. HL-60 Cells.

HL-60 cells are promyelocytic leukaemia cells which are differentiated to a neutrophil-like cell with 0.8% dimethylformamide (DMF). All cell culture is to be undertaken in a culture hood under sterile conditions. Warm culture medium to 37°C before use.

3.1.1. HL60 Master Stock Propagation from ATCC Stock

1. Add 10ml M1 culture medium to a 15ml centrifuge tube. Thaw the master stock of HL-60 cells rapidly by swirling in a 37°C water bath. Transfer the cells into the M1 medium.
2. Centrifuge the tube at 1200rpm for 5 minutes at RT. Remove the supernatant.
3. Re-suspend the cells in M1 medium to a final concentration of $2x10^5$/ml and transfer to a culture flask. Incubate at 37°C/5% CO_2.
4. When the cell density reaches $5x10^5$cells/ml, add further fresh M1 medium to readjust the concentration to $2x10^5$/ml (see Note 1). To avoid risk of contamination, the medium must not reach the cap when the flask is horizontal.
5. When the concentration reaches $5x10^5$cells/ml in 10 flasks of 120ml. Transfer the contents of the flasks into 50ml centrifuge tubes and spin at 1200rpm for 5 minutes.
6. Extract and discard the supernatant being careful not to disturb the pellet.
7. Add 2.5ml freezing medium to each 50ml centrifuge tube and re-suspend each pellet.
8. Combine the contents of all tubes in a single culture flask and aliquot 1ml into cryovials.
9. Transfer cryovials into controlled rate freezing containers and into a -80°C freezer.
10. After a minimum of 2 hours, transfer the cryovials into a liquid nitrogen tank (maximum 1 week at -80°C).

3.1.2. HL-60 Working Stock preparation from master stock

1. Take a 1ml aliquot of HL-60 cells from the liquid nitrogen tank and transfer into a controlled rate freezing container.
2. Defrost cells quickly by swirling in a 37°C water bath.
3. Transfer into a 50ml centrifuge tube containing 50ml of fresh M1 medium.
4. Spin at 1200rpm for 5 minutes at RT. Pour off supernatant and re-suspend pellet in 10ml fresh M1 medium.
5. Transfer into a tissue culture flask and incubate at 37°C/5% CO_2 overnight.
6. Count cells and readjust to $2x10^5$/ml in warm M1 medium.
7. Repeat after 2-3 days.
8. Once the cell growth picks up, spin at 1200rpm for 5 minutes at RT, pour off supernatant and re-suspend pellet in warm M2 medium (no antibiotics or fungizone).

3.1.3. HL-60 working stock maintenance

- Every 3-4 days count cells and split the required number of flasks at $2x10^5$/ml with warm M2 medium. Cell density must remain $\leq 1.2x10^6$/ml.

3.1.4. Differentiation of HL60 Cells

This section describes the differentiation method in 200ml volumes, to differentiate other volumes, the concentration of cells and N,N-dimethylformamide (DMF) remains constant.

1. Count the cells and calculate the volume required to re-suspend at $4x10^5$cells/ml.
2. Cell viability must be ≥ 90% prior to differentiation.
3. Add 1.6ml DMF to 175ml fresh M2 medium (this will give a final concentration of 0.8% in 200ml).

4. Spin cells at 1200rpm for 5 minutes RT and pour off the supernatant.
5. Re-suspend cells in 25ml M2 medium and add to the 175ml of M2 containing DMF.
6. Incubate the cells at 37°C/5% CO_2 for 5 or 6 days. Do not feed the cells during this time.
7. Harvest cells for use in the assay (see section 3.5).

3.2. GAS bacterial stocks

All bacterial culture to be undertaken in a bacterial culture hood under sterile conditions.

3.2.1. Master Stock Preparation and Maintenance

Frozen vials of GAS strains are stored at -80°C. To maximise the integrity of the bacterial master stocks, the vials should remain frozen at all times. Vials can be stored for several years.

3.2.1.1. From clinical swabs

Swabs can be stored at +4°C for up to 4 months depending on the strain or for longer time if stored at -80°C.

1. Streak the swab along the edge of a horse blood agar plate.
2. Rotate the plate 90 degrees, streak using a sterile loop and repeat once more using the same loop (optional, use a new loop pass through the final streak and streak on clean zones towards the centre of the plate to ensure isolated colonies).
3. Incubate overnight at 37°C, 5% CO_2 until colonies appear.
4. To create a master stock, harvest enough colonies on a loop and mix well in 1ml of storage buffer before storing at -80°C.

3.2.1.2. From plated bacteria

Plated bacteria can be stored at +4°C for up to 4 weeks, depending on the storage condition. To create a master stock, harvest enough colonies on a loop and mix well in 1ml of storage buffer before storing at -80°C.

3.2.2. Expansion

Day 1,

1. Remove master vial from freezer and streak a fleck of the frozen bacteria onto 2 blood agar plates. Immediately return master vial to cold storage.
2. Label the plates with strain name and date and incubate at 37°C/5% CO_2 overnight.

Day 2,

1. Add 30ml THY broth to two 50ml falcon tubes, label them A and B plus the bacterial strain name.
2. Harvest a single colony from one blood agar plate using an inoculating loop.
3. Add harvested bacteria to tube A, repeat for tube B. Use 50ml THY broth alone to act as a blank.
4. Measure OD600 of the broth before and after inoculation, and of the blank. The ODs should be comparable.
5. Incubate tubes at 37°C/5% CO_2. The caps must be kept loose to allow gas exchange. Incubate for 2-3 hours.
6. Label the desired number of sterile 1.5ml Eppendorf tubes per serotype with the bacterial strain and date.

7. Check the OD of the tubes after 2 hours and then every hour/half-hourly until the OD reaches between 0.5 and 0.6 (bacteria are in exponential growth phase) (see Note 2). OD of blank must be ≤ 0.02.
8. On reaching required OD, harvest the top 10ml of broth (do not mix) and gently mix 1:1 with storage buffer.
9. Aliquot 0.5ml into the labelled Eppendorf's and transfer to -80°C freezer, this is the working bacterial stocks.

3.3. Human Serum Samples

Human serum must be handled in accordance with local guidelines and stored at -80°C. Prior to testing, the serum must be thawed at RT and heat-inactivated by incubating at 56°C for a minimum 30 minutes. Allow the samples to cool to RT before use in an assay. Samples can be stored at +4°C for up to one month during the testing process to avoid repeated freeze/thaw cycles (if sample requires retesting).

3.4. Baby Rabbit Complement

Caution must be exercised when handling BRC as its components are extremely heat-sensitive. When receiving BRC stock from the supplier, ensure that the contents are entirely frozen and transfer to -80°C storage immediately. There is the potential for high degrees of variation in performance between BRC lots; therefore, prospective lots need screening before use in an assay.

3.4.1. Preparing Working Aliquots of BRC

1. Defrost each bottle of BRC in cold water with constant agitation. E.g. inside an ice box containing a mixture of ice and cold water, placed on top of an orbital shaker.
2. Label tubes with the lot number and aliquot date. Place tubes on ice to cool. Place the bottle of BRC on ice as soon as it has thawed.
3. Quickly aliquot the BRC placing the aliquots in ice until finished (this can be done in a culture hood to ensure sterility).
4. Store all aliquots at -80°C until required.

3.5. Preparation of differentiated HL-60 cells (for use in the assay)

This procedure describes the process for 1x200ml flask, volumes can be altered accordingly. The concentration of cells required for use in the assay is $1x10^7$/ml, do not use cells $>1.5x10^6$/ml at initial count. See Note 3 for more detail on HL-60 cell acceptance criteria.

1. Re-suspend differentiated cells by shaking gently to ensure equal distribution throughout the flask. Count cells and check viability by trypan blue exclusion, cell viability must be ≥80%.
2. Decant cells into four 50 ml centrifuge tubes under sterile conditions. Spin tubes for 5 minutes at 1200rpm at RT.
3. Remove the supernatant and re-suspend each 50 ml of cells in 50 ml HBSS (-Ca/Mg). Centrifuge for 5 minutes at 1200rpm at RT.
4. Remove the supernatant and re-suspend each 50ml tube in 50ml HBSS (+Ca/Mg).
5. Centrifuge for 5 minutes at 1200rpm at RT.

6. Remove the supernatant and re-suspend cells at $1x10^7$ cells/ml in OPS buffer. Count the cells and assess the viability, cell viability must be ≥80%.
7. Store at room temperature until required.

3.6. Preparation of bacteria (for use in the assay)

1. Remove one vial of bacteria from the -80°C freezer and defrost.
2. Centrifuge the tube for 2 minutes at 12000rpm at RT.
3. Remove the supernatant using a pipette, being careful not to dislodge the pellet of cells.
4. Add 1ml of OPS buffer to each tube and vortex. Centrifuge the tube at 12000rpm for 2 minutes at RT.
5. Carefully remove the supernatant and re-suspend the pellet in 0.5ml OPS buffer.

3.7. Procedure for Determining Optimal Dilution of Bacteria (for One Serotype)

Optimal Dilution Experiment 1 (OD1) will determine a rough estimate for the dilution of bacteria required in the assay without the inclusion of serum and controls. Optimal Dilution Experiment 2 (OD2) includes human serum with associated control A and B to determine more accurately the optimal dilution of bacteria required. Procedure is carried out in 96-well round-bottom microtitre plates. OD assays are run in the presence of BRC and HL-60 cells as they can influence the bacterial growth.

3.7.1. Optimal Dilution Experiment 1

1. Prepare overlay agar.
2. Remove working aliquot of BRC (section 3.4) from the -80°C freezer and defrost on ice.
3. Dilute bacteria (working stock prepared as section 3.6) 2-fold by mixing 75µl bacteria with 75µl of OPS buffer in a single well (row A) of a microtitre plate (plate 1). Add 120µl of OPS buffer to rows B to H. 2 columns of the plate will be required per serotype.
4. Prepare 5-fold dilutions by diluting 30µl of diluted bacteria from row A with 120µl OPS buffer in row B. Repeat to column H (Figure 1).
5. In a second microtitre plate (plate 2), add 20µl OPS buffer to two columns for each serotype of bacteria to be tested.
6. Transfer 10µl of diluted bacteria prepared in the first plate to the appropriate wells in the second plate (in duplicate). Plate 1 can be discarded.
7. Incubate plate 2 at RT on a mini-orbital shaker for 30 minutes at 700rpm.
8. Following the incubation period, add 10µl of active BRC to each column in use on plate 2. BRC may be pre-diluted in OPS buffer prior to addition to the plate to ascertain a final in well pre-determined concentration. The optimal BRC concentration was determined for 7 different clinically relevant GAS strains (see Note 4).
9. Resuspend the HL-60 cells. Add 40µl to each column in use on plate 2.
10. Incubate on a mini-orbital shaker at 37°C/5% CO_2 for 90 minutes at 700rpm. Incubate multiple plates in a single layer to maintain equal CO_2 exposure.

11. During this time remove THY agar plates from the fridge, remove lids and lay on the bench to dry for 30 minutes to 1 hour.
12. Place plate 2 on ice for ≥20 minutes to halt the phagocytic process.
13. Vortex the plate at a low speed. Using a manual 8-channel pipette, spot 10µl from each column of plate 2 side by side on a THY plate. Tilt the plate to the left and right so the spots measure approximately 1-1.5cm across, being careful not to allow the spots to merge. If there are multiple serotypes to be assessed, use a separate agar plate for each serotype.
14. Repeat this procedure for the next column, spot next to the previous column on the agar plate. Repeat until there are 3 columns of spots on the agar plate.
15. Leave agar plates at RT for ~20 minutes to allow the spots to dry. See note 5.
16. Remove the overlay agar from the water bath. Measure out amount of overlay and TTC (1µl/ml) required. Mix well and add 20ml to each THY agar plate, allow agar to solidify. TTC must be added after the agar has cooled to below 50°C as it turns red upon heating.
17. Invert the stacked plates and incubate at 37°C / 5% CO_2 for 16-18 hours. Bacteria will form red-coloured colonies.
18. After the incubation period, count the colonies using an automated colony counter (plates can be stored at +4°C for a maximum of 72 hours before reading).
19. Determine the average CFU count of the duplicate rows and identify the 2 dilutions between which the average CFU count yields 50-200 CFU.
20. Choose a number of dilutions (maximum of 6) between the range identified in OD1. For example, if the OD1 result was between 1 in 250 and 1 in 1250, dilutions chosen could be 1 in 250, 1 in 500, 1 in 750, 1 in 1000 1 in 1500 and 1 in 2000. Proceed to OD2.

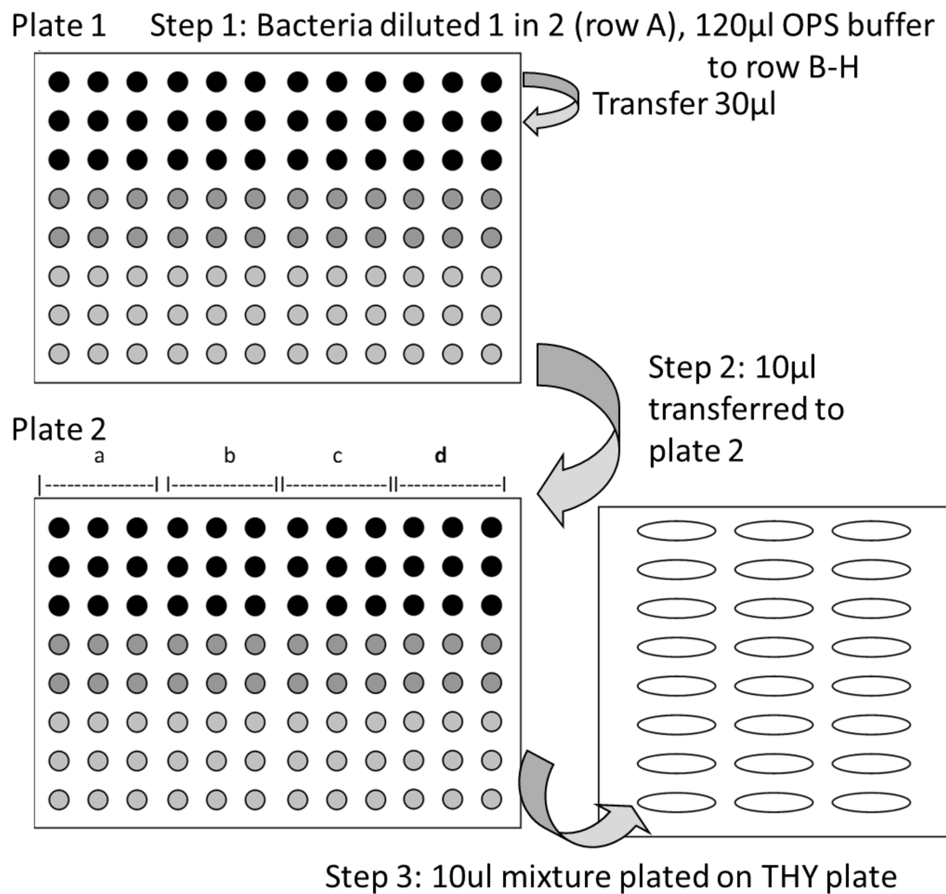

Figure 1: OD1 assay procedure. Bacteria is serially diluted in plate 1 then transferred to plate 2 when BRC and HL-60 cells are added. After incubation, the mixture is then spotted onto THY agar and incubated overnight.

3.7.2. Optimal Dilution Experiment 2

1. Make up each dilution of bacteria working stock determined in OD1 required for the OD2 experiment.
2. Assay a known positive quality control serum, Control A (heat inactivated BRC, no serum) and Control B (active BRC, no serum) as detailed in section 3.9 of this method (each dilution of bacteria will be added to four columns of a plate) (Figure 2).

3. Select the dilution that yields ~100 CFU in Control B and a QC within the pre-determined accepted range.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Heat inactivated complement	Active complement	Dilution 8		Heat inactivated complement	Active complement	Dilution 8		Heat inactivated complement	Active complement	Dilution 8	
B			Dilution 7				Dilution 7				Dilution 7	
C			Dilution 6				Dilution 6				Dilution 6	
D			Dilution 5				Dilution 5				Dilution 5	
E			Dilution 4				Dilution 4				Dilution 4	
F			Dilution 3				Dilution 3				Dilution 3	
G			Dilution 2				Dilution 2				Dilution 2	
H			Dilution 1				Dilution 1				Dilution 1	
	Control A	Control B	Positive serum		Control A	Control B	Positive serum		Control A	Control B	Positive serum	
	Bacteria dilution 1				Bacteria dilution 2				Bacteria dilution 3			

Figure 2: OD1 assay layout. One dilution of bacteria is assayed with a dilution series of known positive QC serum, Control A and Control B.

3.8. OPKA Procedure

The following procedure details the quantities required for four 96-well plates (controls, QCs and 16 unknown sera). Unknown sera can also be used pre-diluted in OPS buffer e.g. at 1:4 or 1:30 dilution.

1. Prepare 400ml overlay agar. Store in a 50°C water bath until required. Ensure a minimum 16 THY agar plates are available for use.
2. Remove the BRC from the -80°C freezer and defrost on ice.
3. Label each round-bottomed 96-well microtitre plate with plate ID (A, B, C or D) and sample layout. The procedure described below utilises 8 dilutions of serum serially diluted 3-fold, in duplicate.
4. Add 20µl of OPS buffer to columns 1 and 2 of each plate, rows A-H inclusive.

5. Add 20µl OPS buffer to rows A-G, columns 3-12 inclusive.
6. Add 30µl of serum sample 1 into row H, columns 3 and 4 of plate A. Add 30µl of serum sample 2 into row H, columns 5 and 6. Continue adding a maximum of 5 samples per plate (4 test samples and a QC sample).
7. Perform 3-fold serial dilutions in columns 3-12 by transferring 10µl from row H to row G making sure no bubbles form while mixing. Then transfer 10µl from row G to row F and continue up the plate. Once 10µl is transferred from row B to row A and mixed, remove 10µl from row A and discard (see Figure 3).
8. Add 10µl of bacterial mixture (prepared as section 3.7 and pre-diluted to optimal dilution determined in section 3.8) to each well including all control wells. When adding bacteria, pipette 10ul by non-reverse pipetting directly into the liquid at the bottom of the well and 'bubble' in to ensure contact.
9. Incubate the 96-well plates for 30 minutes at room temperature on a mini-orbital shaker at 700rpm.
10. Incubate an aliquot of BRC in a 56°C degree water bath to heat inactivate for minimum 30 minutes.
11. Following the incubation period, add 10µl of heat inactivated BRC (pre-diluted in OPS buffer if indicated) column 1 of all plates. Add 10µl of active BRC (also pre-diluted) to all other wells (columns 2–12). When adding BRC, reverse pipette onto the side of the well, then tap the plate to mix in the well.
12. Resuspend the HL-60 cells and add 40µl to all wells.
13. Incubate on a mini-orbital shaker at 700 rpm at 37°C/5% CO_2 for 90 minutes. Incubate the plates in a single layer to maintain equal CO_2 exposure.
14. During this time remove THY agar plates from the fridge, remove lids and lay on the bench to dry for 30 minutes to 1 hour.

15. Place plates on ice for ≥20 minutes to halt the phagocytosis process.
16. Replace the covers on the dried agar plates and stack in piles of 4 (1 stack per 96 well plate). Label the side of each plate with the 96 well plate ID, the section of 96 well plate (1, 2, 3 or 4), technician initials and the date.
17. Vortex the plate at a low speed. Using multichannel pipette, remove 10µl from a single column and spot 10µl on agar plate by reverse pipetting to minimise bubbles. Tilt the plate to the left and right so the spots measure approximately 1-1.5cm across, being careful not to allow the spots to merge.
18. Repeat this procedure for the next column, spot next to the previous column on the agar plate. Repeat until there are 3 columns of spots on the agar plate.
19. Leave all plates at RT for ~20 minutes to allow the reaction mixture to soak into the agar (or until completely dry).
20. Remove the overlay agar from the water bath. Measure out amount of overlay and TTC (1µl/ml) required. Mix well and add 20ml to each THY agar plate, allow to solidify.
21. Invert the stacked plates and incubate at 37°C / 5% CO_2 for 16-18 hours. Bacteria will form red-coloured colonies.
22. After the incubation period, count the colonies using an automated colony counter (plates can be stored at +4°C for a maximum of 72 hours before reading).

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<tr><td>H</td><td colspan="2">Dilution 1</td><td colspan="2">Dilution 1</td><td colspan="2">Dilution 1</td><td colspan="2">Dilution 1</td><td colspan="2">Dilution 1</td></tr>
<tr><td></td><td>Control A</td><td>Control B</td><td colspan="2">Test serum 1</td><td colspan="2">Test serum 2</td><td colspan="2">Test serum 3</td><td colspan="2">Test serum 4</td><td colspan="2">QC sample</td></tr>
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Figure 3: OPKA plate layout.

3.9. Data analysis

1. The Opsonic Index (OI) is the estimated dilution of serum that kills 50% of the target bacteria.
2. The OI is calculated using the Opsotiter software, which is an Excel based program developed to analyse data from OPKAs.
3. Raw colony counts generated from the automated colony counter are copied and pasted into the program. The program tabulates the opsonic indices for the individual samples as well as determining the percentage of non-specific killing (NSK).
4. Each bacterial strain must be analysed separately giving 1 file for each 96 well plate.

3.9.1. Limits of Detection

The following applies when samples are run neat.

- The upper limit of detection (ULD) is 8748. This is calculated by taking the fold dilution of serum (3) to the power of 7 (number of fold dilutions), and multiplying by 4 (total volume in well (80µl) divided by volume of serum in well (20µl)).
- However, Opsotiter automatically multiplies all 8748 results by 2 for statistical purposes. The ‘OI final’ on the results sheet is therefore displayed as 17,496.
- Samples that have an opsonic index higher than the upper limit of detection may be repeated at a pre-dilution of 1 in 30.
- The lower limit of detection (LLD) is 4, as this is the initial dilution of serum once all components have been added to the plate. However Opsotiter automatically divides all

results of 4 by 2 for statistical purposes. The' OI final' on the results sheet is therefore displayed as 2.

- The lower limit of quantification (LLOQ) was determined for 7 different clinically relevant GAS strains (see Note 6). Samples below LLOQ can be reported as half LLOQ for statistical purposes.

3.9.2. Tentative Plate Acceptance Criteria

- Non Specific Killing (NSK) ≤35%; (1-average Control B CFU/average Control A CFU)*100. See Note 4.
- Colony counts in Control B between 50 and 150.
- QC sample OI measurement between the pre-determined acceptability range.

3.9.3. Tentative Sample acceptance criteria

- Sample replicates must cross the 50% killing line within one 3 fold dilution. If they do not then the sample will be retested.
- Good killing curves are those that show a sigmoid shape. Curves may be irregular and 'N' or 'U' shaped curves may be identified. Irregular curves may be treated individually as they may not conform to acceptance criteria.
- For a sample to be considered positive, the maximum killing must be greater than 70%. If the sample falls between 40–70% maximum killing, the sample must be repeated.

4. Notes

1. HL-60 cell concentrations must not exceed $5x10^5$/ml at master stock propagation stage, during working stock maintenance, cell density must remain ≤$1.2x10^6$/ml. Cell viability must remain ≥80%. HL-60 cell viability tends to decline over 25-26 passages when a decrease in viability occurs and irregular cell shapes are seen under the microscope.

2. Different strains will grow at different rates. Inoculation from a single isolated colony will ensure that the broth culture is pure. To avoid contamination from other bacteria that may be present in the swab and increase purity of the strain of interest it is recommendable to inoculate single colonies from the blood agar plates and streak them onto a new blood agar plate and incubate overnight at 37°C, 5% CO_2 until colonies appear.

3. HL-60 cells must be mycoplasma free. On the day of assay, cell viability must be ≥80%. Phenotype of HL-60 cells is determined by flow cytometry using mouse anti-human CD35 FITC conjugated antibody and mouse anti-human CD71 PE-conjugated antibody. Differentiated cells are accepted for use in the assay if the up-regulation of CD35 was ≥55% of the cell population and CD71 expressing was down-regulated by ≤12% when compared to the working stock preparation.

4. The concentration of BRC can be optimised to reduce NSK to below an acceptable level. ≤35% NSK is commonly used as acceptance criteria in an optimised assay, however, levels up to 60% can also be accepted. The optimal concentration determined for strains emm1 (43, 02-12 and GAS05134), emm12 (611020, 611025 and GAS 09437) was 2.1%, for emm6 (GASOPA6_02) was 3.1% (5). Other factors that can be optimised to ensure maximal killing but acceptable levels of NSK are plate shaking speed and the incubation time.

5. For the spots to dry plates can be left to air dry on a bench top, however if excessive contamination occurs, plates can be dried in a laminar flow hood with a shortened length of time.

6. The LLOQ was determined for strains emm1 43; 9, 02-12; 15, GAS05134; 8, emm12 611020; 7, 611025; 5, GAS 09437; 7 and emm6 GASOPA6_02; 6 (5). The average LLOQ across all strains was 8. The LLOQ can be determined by spiking low concentrations of known positive serum into heat inactivated non-immune (antibody depleted) serum to produce a sample of OI 4-12. Generate each sample individually and run four times in duplicate.

5. References

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