An Opsonophagocytic Killing Assay for the Evaluation of Group A Streptococcus Vaccine Antisera

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Abstract

Group A *Streptococcus* (GAS) is a major cause of global mortality yet there are no licenced GAS vaccines. Vaccine progress has been hampered, in part, by a lack of standardised assays able to quantify antibody function in test antisera. The most widely used assay was developed over 50 years by Rebecca Lancefield and relies on human whole-blood as a source of complement and neutrophils. Recently, an opsonophagocytic killing (OPK) assay has been developed for GAS by adapting the OPK methods utilised in *Streptococcus pneumonia* vaccine

testing. This assay uses dimethylformamide (DMF)-differentiated human promyelocytic leukemia cells (HL-60 cells) as a source of neutrophils and baby rabbit complement, thus removing the major sources of variation in the Lancefield assays. This protocol outlines methods for performing a GAS OPK assay including titering test sera to generate an opsonic index. This *in-vitro* assay could aid in selecting vaccine candidates by demonstrating whether candidate-induced antibodies lead to complement deposition and opsonophagocytic killing.

Key words: Complement, HL-60 cells, Phagocytosis, Group A Streptococcus, Vaccine, Antibody

1. Introduction

Group A *Streptococcus* (GAS) is a Gram-positive, human only, pathogen which causes a wide spectrum of disease, including superficial infections (such as pyoderma and pharyngitis), severe invasive GAS disease, acute rheumatic fever (ARF) and associated rheumatic heart disease (RHD) ***(1)***. Despite significant global morbidity and mortality caused by these clinical syndromes there is no currently licensed GAS vaccine ***(2)***. Development of a vaccine to GAS has been hampered, in part, by the lack of a reliable, standardised assay to measure a functional immune response. The most widely used assays, developed in the 1950s by Rebecca Lancefield (known as the "Lancefield" assay), measure the survival of GAS in fresh human blood ***(3)***. However, inter-individual differences in complement and neutrophils result in large data variability making comparisons between laboratories difficult ***(4)***. The need for improved assays capable of measuring functional GAS antibody responses was recently identified as an international priority for vaccine development ***(2, 5, 6)*** .

The development of an opsonophagocytic killing (OPK) assay for testing *Streptococcus pneumonia* antisera ***(7)*** has facilitated the advancement of vaccines for this bacterial pathogen ***(8)***. The assay readout is an "opsonic index", which is the reciprocal value of the serum dilution at which 50% killing is obtained. These values have been shown to correlate with the ability of vaccine candidates to generate protection in clinical trials and are now routinely used to evaluate the *Streptococcus pneumonia* vaccines ***(9)***. Recently, OPK assays have been developed for GAS by ourselves and others, with proof of principle using rabbit antisera and human intravenous immunoglobulin ***(10, 11)***. In order to overcome variability associated with traditional Lancefield assays, the GAS OPK uses an exogenous source of both complement (baby rabbit complement) and phagocytes (human promyelocytic leukemia cells (HL-60 cells)), in line with protocols developed for *S. pneumonia*. The HL-60 cells are differentiated to a neutrophil like cell by adding dimethylformamide (DMF) and the complement is sourced from baby rabbits meaning it has low heterophile antibody activity. When antibodies specific for GAS components on the bacterial surface are mixed with GAS bacteria, in conjunction with differentiated HL-60 cells and complement, the anti-GAS antibodies activate the classical complement pathway to stimulate opsonization of the bacteria with C4b and C3b complement factors. This leads to opsonophagocytosis and killing by the HL-60 phagocytic cell line.

This protocol describes a method for performing an OPK for GAS largely based on the *S. pneumonia* OPK ***(10)***. It makes use of the assay buffer utilized in the qualified *S. pneumonia* assays, and which we have found to be sufficient for measuring opsonophagocytosis against selected GAS strains. In a recent report Salehi *et al.,* ***(11)*** describe a modified GAS OPK in which additional reagents are included in the assay buffer. Given the growing momentum in

GAS vaccine development, harmonizing OPK assay protocols for selected GAS strain types (*emm*-types) between laboratories should now be a priority. Over and above vaccine development, the GAS OPK assay can be utilized to test natural immunity against GAS in human donors, as well as the efficacy of GAS specific monoclonal antibodies.

2. Materials

2.1. HL-60 cell culture

1. HL-60 cells (from American Type Culture Collection (ATCC)
2. T-75 tissue culture flasks, filtered-cap.
3. Trypan Blue cell viability stain.
4. M1 medium: RPMI 1640, 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 100 U/mL Penicillin, 100 U/mL Streptomycin, 0.5 μg/mL Amphotericin-B.
5. M2 medium: RPMI 1640, 10% FBS, 1% L-Glutamine.
6. Freezing medium: 9 mL FBS, 1 mL DMSO.
7. Dimethylformamide (DMF).

2.2. Materials for bacterial growth, optimum dilution and OPK assay

1. GAS glycerol stock.
2. Horse blood agar plate.
3. Baby Rabbit Complement (3– 4 – week baby rabbit complement, Pel-Freez Biologicals) (*see* **Note 1)**.
4. Hank's balanced salt solution containing calcium chloride and magnesium chloride without phenol red (HBSS++) (1x) (ThermoFisher Scientific or similar).

5. Hank's balanced salt solution without calcium chloride, magnesium chloride or phenol red (HBSS--) (1x) (ThermoFisher Scientific or similar).
6. 100 x 100mm square petri-dishes.
7. Automated colony counter (ProtocCOL3, Synbiosis or similar).

2.3. Plates and medium preparation

1. 5 % 2,3,5-tetraphenyltetrazolium chloride (TTC): make up in sterile pyrogen free water and sterile filter, store at 4°C.
2. 1 % gelatin: prepare in sterile pyrogen free water and autoclave.
3. Todd-Hewitt-yeast (THY) agar plates: 3 % Todd-Hewitt broth, 0.5 % yeast, 1.5% agar. Prepare in sterile pyrogen free water. Autoclave and pour into 100 x 100mm square plates.
4. Todd-Hewitt-yeast (THY) overlay agar: 3 % Todd-Hewitt, 0.5 % yeast, 0.75 % agar. Make up on day of assay, autoclave and keep at 55°C.
5. Opsonisation buffer (OPS buffer): 5% heat inactivated FBS, 10% 10x HBSS (++), 0.1% gelatin. Make up in sterile pyrogen free water, prepare on day of assay.
6. Todd-Hewitt Broth (THB). Autoclave.
7. Tryptone Glucose Glycerol medium (TGG): 3% Tryptic Soy Broth, 0.5% glucose, 10% glycerol. Make up in sterile pyrogen free water and autoclave.

3. Method

3.1. Preparation of GAS TGG working stocks

1. Streak from GAS glycerol stock onto a horse blood agar plate.
2. Incubate plate overnight at 37℃, 5% CO_2 or until colonies appear (*see* **Note 2**).

3. Inoculate a single GAS colony into 10 mL of THB.
4. Incubate overnight at 37℃ in 5% CO_2 without agitation.
5. Dilute GAS overnight culture 10-fold in THB, pre-warmed to 37℃ in, a 50 mL falcon tube and incubate at 37℃in 5% CO_2 without agitation.
6. Once an absorbance at 600nm of 0.5–0.7 is reached place the tube on ice, and mix the bacterial suspension with TGG medium at 1:1 ratio, vortex and aliquot 500 µl per vial and store at -80˚C.

3.2. Preparation of bacteria for use in optimum dilution and OPK assays

1. Remove one vial of bacteria from the -80℃freezer and defrost at 37°C.
2. Centrifuge the tube for 2 min at 13,000–15,000 × g at room temperature (RT) and remove supernatant being careful not to disturb the pellet.
3. Wash once with 1 mL of OPS buffer and centrifuge the tube at 13,000–15,000 × g at RT, remove the supernatant and re-suspend the pellet in 0.5 mL OPS buffer.
4. Proceed making the pre-determined dilution (see section 3.6).

3.3. HL-60 cell expansion from ATCC master stock (*see* Note 3 and Note 4).

1. Seed the HL-60 cells at a concentration of 2 x 10^5/mL in a T-75 culture flask in M1 medium.
2. Grow the HL-60 cells in M1 medium suspension without agitation at 37℃ with 5 % CO_2 until they reach a density of 5 x 10^5 cells/mL. Maintain cells between 2 x 10^5cells/mL and 5 x 10^5cells/mL with M1 medium. The typical growth culture schedule requires feeding every 2–3 d (*see* **Note 5**).

3. When the concentration reaches 5 x 10^5 cells/mL in the desired number of flasks, pellet the HL-60 cells by centrifugation at 350 x g for 5 min and add freezing medium to obtain a concentration of 1 x 10^7 cell/mL.
4. Aliquot 1 mL, of HL-60 cells in freezing medium, into cryovials and transfer cryovials to liquid nitrogen.

3.4. HL-60 Working stock preparation (*see* note 6).

1. Seed a HL-60 aliquot ,prepared in section 3.3, at 2 x 10^5/mL in a T-75 culture flask.
2. Grow the HL-60 cells in suspension without agitation at 37°Cwith 5 % CO_2 maintaining a density of 2 x 10^5 –1 x 10^6 /ml by splitting every 3–4 d in M1 medium.
3. Once the cell growth accelerates (*see* **Note 7**), centrifuge at 350 x g for 5 min at RT, discard supernatant and re-suspend pellet in warm M2 medium maintaining cells at 2 x 10^5–1 x 10^6 /mL.
4. After approximately 2 weeks of maintenance in M2 medium, HL-60 cells can be differentiated for use in OPK assays (*see* **Note 8**).

3.5. HL-60 cell differentiation and preparation for use in assays (*see* note 9).

1. To differentiate HL-60 cells, centrifuge at 350 x g for 5 min RT and discard the supernatant.
2. Re-suspend HL-60 cells in 0.8% DMF prepared in M2 medium pre-warmed to 37°C.
3. Incubate the HL-60 cells at 37°C with 5 % CO_2 for 3–4 d (*see* **Note 10**). Do not feed the cells during this time (*see* **Note 11)**.
4. After 3– or 4– d differentiation decant HL-60 cells into 50mL falcon tubes and centrifuge tubes for 5 min at 350 x g at RT.

5. Discard the supernatant and re-suspend HL-60 ells in equal volume HBSS--. Centrifuge for 5 min at 350 × g at RT.
6. Remove the supernatant and re-suspend cells in equal volume HBSS++.
7. Centrifuge for 5 min at 350 x g at RT.
8. Remove the supernatant and re-suspend HL-60 cells at 1 x 10^7cells/mL in OPS buffer. Count the cells and assess the viability. (*see* **Note 12** and **Note 13**)
9. Store at RT until required (maximum of 6 h).

3.6. Optimum dilution assay

This assay will determine the dilution of bacteria required for input in the OPK assay, see **Figure 1** for diagrammatic representation.

1. Remove the required number of baby rabbit complement aliquots from the -80℃ freezer and thaw on ice.
2. Thaw one GAS TGG stock (prepared as in section 3.1) by placing tube at 37°C.
3. Following wash (see section 3.2) dilute the bacteria in OPS buffer 10-fold in well A1 of a 96 well plate (**Figure 1, Step 1**).
4. Serially dilute the bacteria 5-fold down a column of the 96 well plate (**Figure 1, Step 2**).
5. In a second 96 well plate, add 20 µl OPS buffer to two columns for each serotype of bacteria to be tested (**Figure 1**, **Step 3**).
6. Transfer 10 µl of diluted bacteria prepared in steps 1–4 to the appropriate wells in the second plate (in duplicate) (**Figure 1**, **Step 4**).
7. Incubate 96 well plate at RT on a mini-orbital shaker for 30 min at 700 rpm.

8. Following the incubation period, add 10 µl of active complement prediluted 2-fold in OPS buffer to each well in use in the 96 well plate (*see* **Note 14**).
9. Add 40 µl of differentiated HL-60 cells (prepared as in section 3.5) to each well in the 96 well plate, covered with lid that allows gas exchange.
10. Incubate on a mini-orbital shaker at 37°C/5 % CO_2 for 45–90 min at 700 rpm.
11. Keep the plates on ice for 20 min to halt the phagocytic process.
12. Spot 10 µl from each column of 96 well plate onto a THY plate. Tilt the plate so the spots run and measure approximately 1 cm across. Allow spot to dry at RT (**Figure 1**, **Step 5**).
13. Add 20 mL THY overlay agar (with TTC added at 1000-fold dilution) to each THY agar plate and wait for overlay agar to solidify.
14. Incubate plates for 16–18 h in a 37°C/5 % CO_2 incubator, in this time bacteria should form red-coloured colonies.
15. Read plates using an automated colony-counter (Protocol 3, Synbiosis or similar).
16. Determine the dilution of bacteria that yields 50–200 CFU's for input into OPK assay (section 3.7).

3.7. Opsonophagocytic killing assay (OPK assay)

See **Figure 2** for diagrammatic representation of OPK assay using one GAS strain and 5 serum samples. Columns 1 and 2 are used as controls to calculate non-specific killing (NSK) induced by complement alone, and maximum bacterial growth in the absence of test sera. These values are crucial for the assay acceptance criteria and subsequent calculation of the "opsonic index" (*see* **Note 16**).

1. Remove the required number of complement aliquots from the -80℃freezer and thaw on ice.
2. Add 20 µl OPS buffer to columns 1 and 2, rows A–H of a 96 well plate (**Figure 2**, **Step 1**).
3. Add 20 µl OPS buffer to columns 3–12, rows A–G (not row H) of the 96 well plate (**Figure 2**, **Step 2**).
4. Heat-inactivate the required volume of test serum at 56℃ for 30 min.
5. Make an initial dilution of test serum (2-fold to 10-fold) in OPS buffer and add 30 µl of this diluted serum to row H, columns 3–12 of plate in duplicate. Continue adding up to 5 samples in column H (**Figure 2**, **Step 3**).
6. Perform a 3-fold serial dilution of serum by transferring 10 µl from row H columns 3–12, through row A (**Figure 2**, **Step 4**).
7. Thaw one GAS TGG stock (prepared as in section 3.1) by placing tube at 37°C.
8. Following wash (see section 3.2) and re-suspension in the appropriate volume of OPS buffer, add 10 µl of bacterial mixture to all wells (*see* **Note 15**) (**Figure 2**, **Step 5**).
9. Incubate the 96-well plates for 30 min at RT on a mini-orbital shaker at 700rpm (**Figure 2**, **Step 6**). This allows time for antibodies to opsonise the bacteria.
10. Heat inactivate required volume of complement by incubating at 56℃ for 30 min.
11. Following the 30 min incubation period, add 10 µl of heat inactivated complement pre-diluted 2-fold in OPS buffer to, column 1 (**Figure 2**, **Step 7**).
12. Add 10 µl of active complement pre-diluted 2-fold in OPS buffer to all other wells (columns 2–12) (*see* **Note 16**) (**Figure 2**, **Step 8**).
13. Add 40 µl of differentiated HL-60 cells (prepared as in section 3.5) to all wells and cover with lid that allows gas exchange (**Figure 2**, **Step 9**).

14. Incubate on a mini-orbital shaker at 700 rpm at 37°C/5% CO_2 for 45–90 min (*see* **Note 17**) (**Figure 2**, **Step 10**).
15. Put plates on ice for 20 min to halt the phagocytosis process.
16. Spot 10 µl from each column of 96 well plate onto a THY plate. Tilt the plate so spots run and measure approximately 1 cm across. Allow to dry at RT (**Figure 2, Step 11**).
17. Add 20 mL overlay agar (with added TTC at 1000-fold dilution) to each THY agar plate and wait for overlay agar to solidify.
18. Incubate plates for 16–18 h in a 37°C/5% CO_2 incubator, in this time bacteria should form red-coloured colonies.
19. Read plates using an automated colony-counting software (*see* **Note 18**).

4. Notes

1. Baby rabbit complement is extremely sensitive to temperature fluctuations. When received from supplier, complement should be thawed on ice and aliquoted into 1–5 mL aliquots for use in assay. Once frozen, aliquots should only be thawed once for use in assays and the remainder can be heat-inactivated for use as control.
2. Colonies are beta haemolytic and may vary in morphology between strain types depending on level of hyaluronic acid encapsulation.
3. All cell culture should be undertaken in a culture hood under sterile conditions. It is highly recommended to have tissue culture facilities in an area physically separate from the bacterial work lab space.
4. Cell viability is assessed by diluting HL-60 cells in trypan blue followed by counting on a haemocytometer.
5. Cell concentrations must not exceed 5 x 10^5/mL at this stage.

6. It is highly recommended that HL-60 cells are tested regularly for mycoplasma contamination using a commercial PCR detection kit (Universal Mycoplasma Detection Kit, ATCC or similar). Mycoplasma detection should be carried out before freezing down master aliquots and monthly for working stocks.
7. Following reconstitution from liquid nitrogen, HL-60 cells take some time to recover and for cell growth to accelerate. In the first 2 weeks following reconstitution cells will typically only need splitting every 5–7 d in order to maintain cell density below 1 x 10^6 cells/mL. After this initial phase splitting of HL-60 cells will be required every 3–4 d in order to maintain cell density below 1 x 10^6 cells/mL. Once splitting is required every 3–4 d HL-60 cells can be propagated in M2 medium.
8. It is recommended that HL-60 cells only be used for differentiation up to passage 35-40.
9. HL-60 cell viability must be ≥90% prior to differentiation.
10. The day HL-60 cells should be used following differentiation must be experimentally defined using flow cytometry and is typically day 3–6. Cells should be used when they show maximum expression of CD35, minimum expression of CD71 and fewest dead cells (*see* **Note 9** for details of flow cytometry analysis).
11. To determine whether the HL-60 cells are correctly differentiated, flow cytometry analysis is strongly recommended. Typically, the differentiated cultures should show 80% viability estimated using Trypan blue. The cell surface markers that confirm the acquisition of the differentiated phenotype are maturation marker CD35 (Antibody clone DF1513, Bio-Rad) and proliferation markers CD71 (Antibody clone E11, Bio-Rad) (**Figure 3).** More than 55% of the cells in the cultures should express CD35 and less than 12% of the cells should maintain CD71 expression. The cultures should not have

an abundance of dead cells which can be determined using commercial viability staining kits (such as the LIVE/DEAD cell stain kits from ThermoFisher Scientific). Typically, more than 85% of the cells should show negative staining with viability stain, indicating the cells are alive.

12. Do not use HL-60 cells that exceed 1.5 x 10^6 cells/mL at initial count as this may indicate a problem during differentiation.
13. HL-60 cell viability before and after HBSS washes should be greater than 80% as assessed by trypan blue staining.
14. For some GAS strains, dilution of baby rabbit complement will need to be optimised by carrying out the optimum dilution assay with several different dilutions of complement. Some strains may require a higher concentration of complement, and it is recommended the highest concentration of complement, which gives a maximum of 35% Non-Specific Killing (NSK), is used for the OPK assay.
15. GAS bacteria are added at the concentration determined during the optimal dilution assay (section 3.6).
16. Columns 1 and 2 are used as controls to calculate NSK induced by complement alone (average counts of column 2 containing active complement, compared to average counts of column 1 containing heat-inactivated complement). It is recommended this does not exceed 35 % for GAS OPK assay. In addition the maximum bacterial growth with all assay components aside from serum is calculated from average counts of column 2. It is from this maximum that the 50 % killing criteria is calculated and thus is important to have numbers of colonies countable by automated colony counter**.**
17. The incubation time of GAS with HL-60 cells is another parameter that can be adjusted depending on the GAS strain type. Incubation times of between 45–90 min are likely

to enable phagocytosis. For longer incubation times the dilution of complement added to the assay will need to be adjusted accordingly in order to prevent high NSK of >35 %.

18. To facilitate data analysis, Opsotiter, an Excel®-based template, has been developed by researchers at the Bacterial Respiratory Pathogen Reference Laboratory at the University of Alabama at Birmingham (UABRF, Birmingham, AL, USA). A license to use this software can be requested from UABRF ***(12)***. Data obtained from the colony counter can be directly inserted into the Opsotiter software, which converts colony counts to an "opsonic index" (OI) as well as calculating NSK for the OPK assay and maximum killing for each serum sample tested. The OI is defined as the dilution of serum that kills 50% of bacteria and is determined using linear interpolation. For GAS OPK assays, the following acceptance criteria for determining an OI is recommended:
    a. NSK of less than 35 % (see **Note 14**)
    b. Maximum killing of at least 70 % for test serum. This enables reliable linear interpolation of the 50th centile and determination of an accurate OI.

    OI's can be compared between sera in assays to the same GAS strain type. Direct comparison of OI's between different GAS strain types is not advised. See **Figure 4** for an example result of a GAS OPK assay.

5. References

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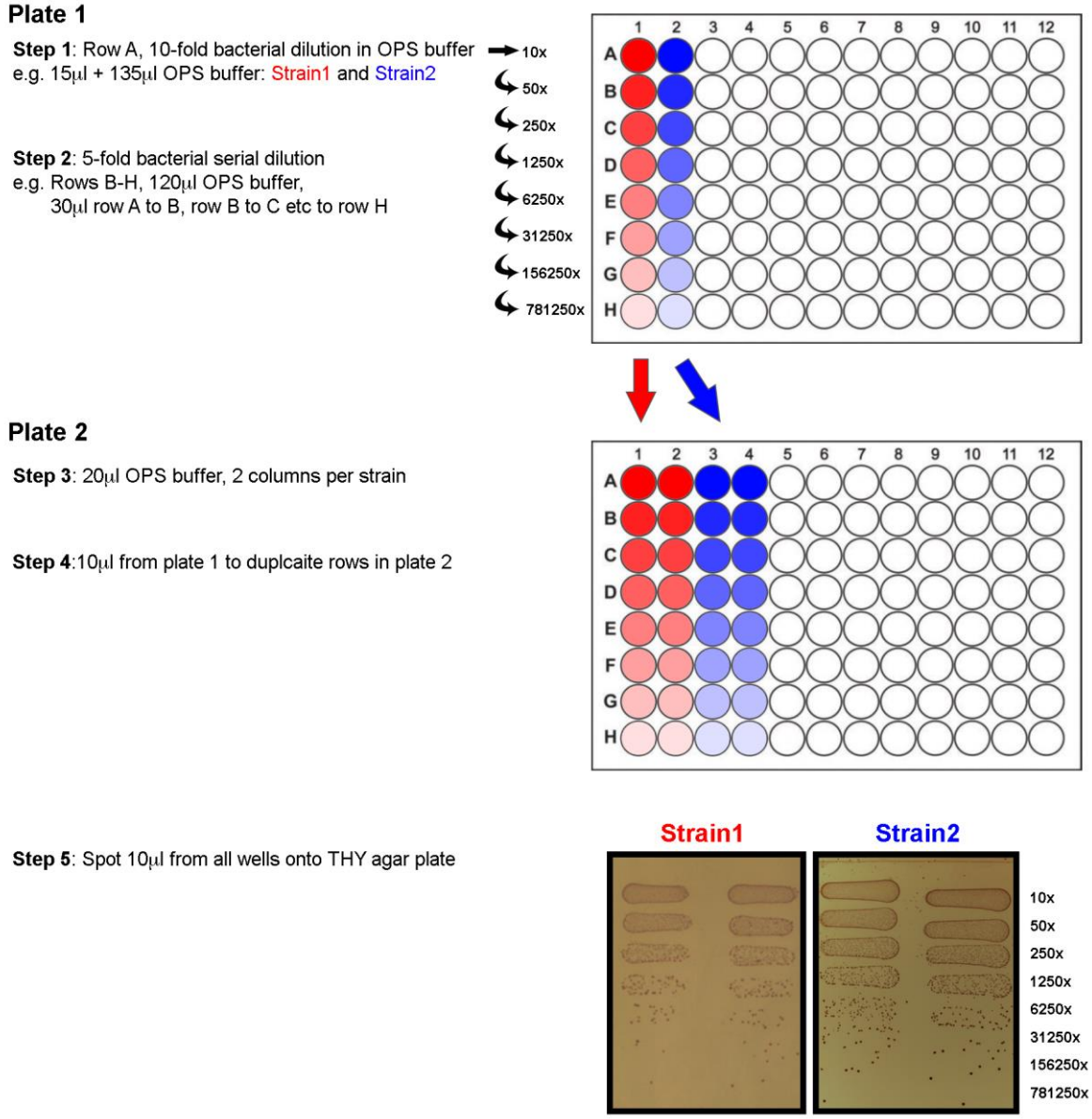

Figure 1| **Optimum dilution assay procedure** | In this example two GAS strain-types are assessed for optimum dilution represented in red and blue. OPS = opsonisation, THY = Todd-Hewitt Yeast.

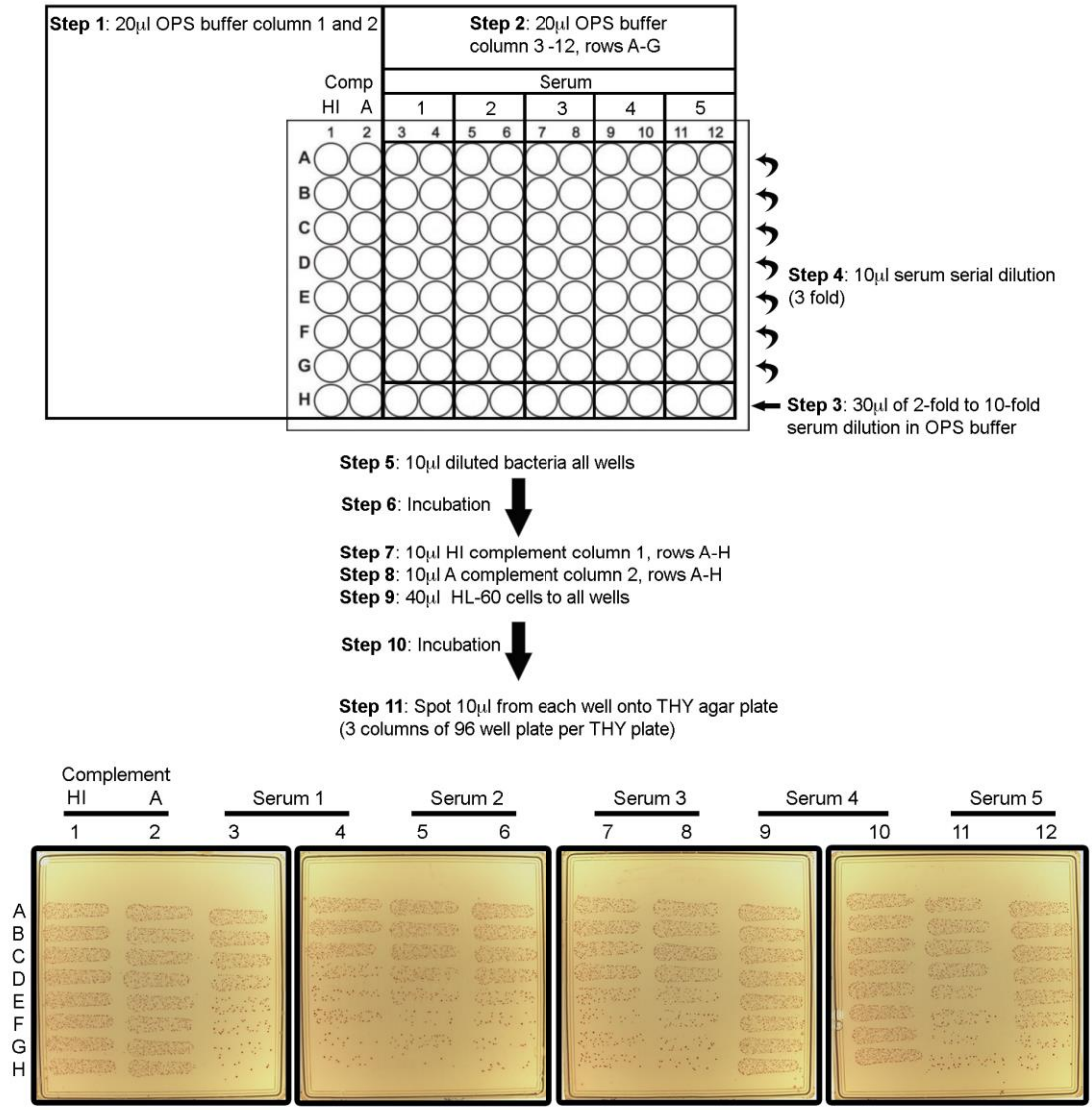

Figure 2| **OPK assay procedure setup**| In this setup one 96 well plate is spotted onto 4 THY plates. In this example killing can be observed for all serum except serum 4. Note the difference in bacterial number between column 1 and 2 is used to calculate the non-specific killing. Comp = Complement, A = Active complement, HI = Heat Inactivated Complement, OPS = opsonisation, THY = Todd-Hewitt Yeast.

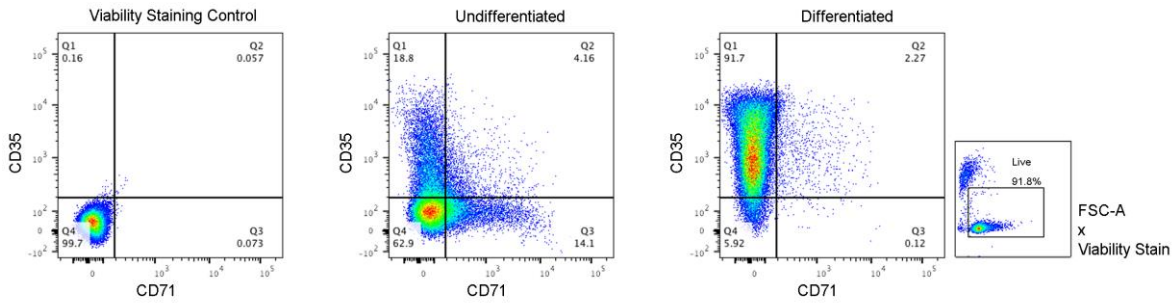

Figure 3 |**Undifferentiated compared to differentiated HL-60 phenotype**| The viability staining control shows a sample stained only with the viability stain and is used as a negative staining control to set gates for CD71 and CD35. In this example the undifferentiated HL-60 cells have 18.8% CD35+ and 14.1% CD71+, while the differentiated have 91.7% CD35+ and 0.12% CD71+. In this example the differentiated HL-60 cells show 91.8% live cell staining.

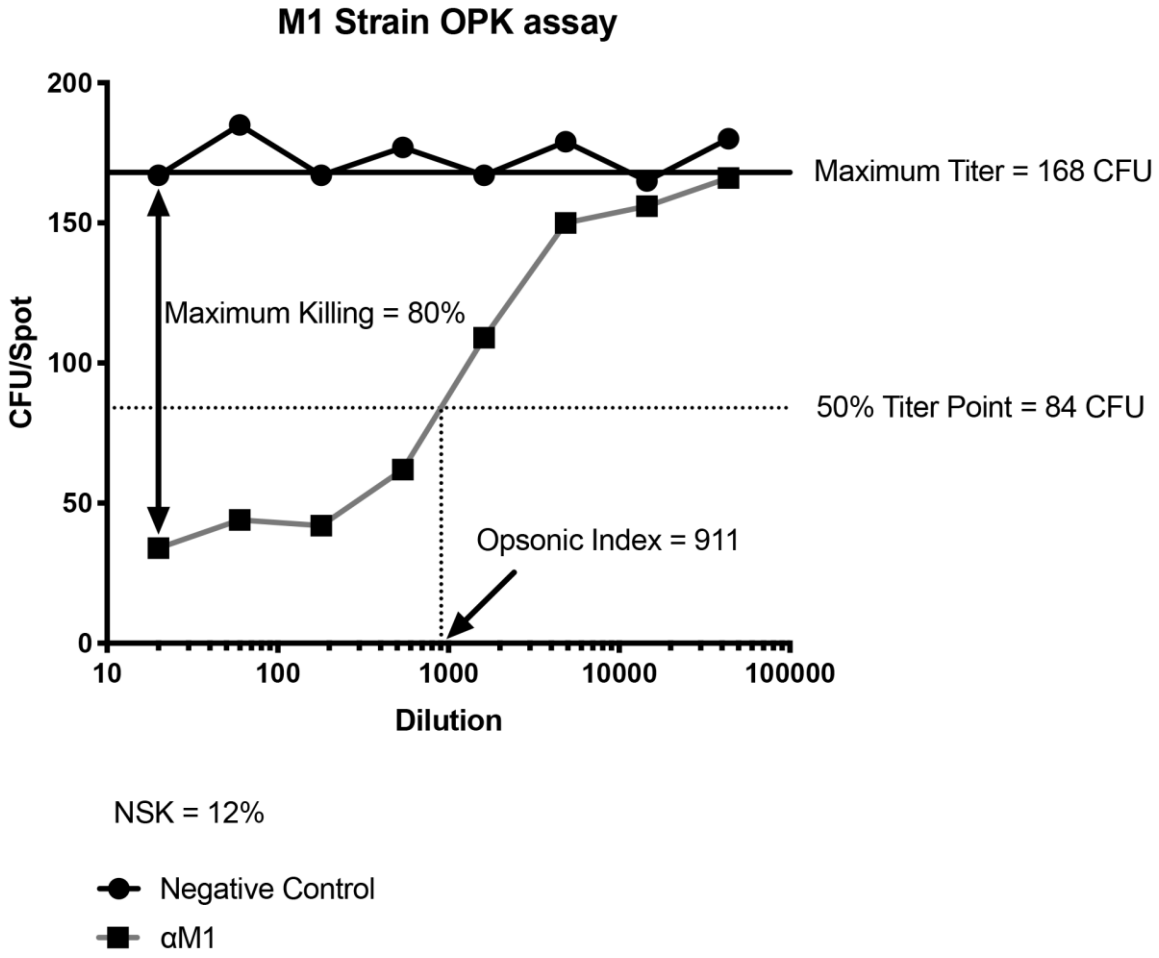

Figure 4|**Example output of M1 OPK assay**| OPK assay with M1 GAS using vaccinated rabbit serum targeting the M1 protein (squares) and a negative control (circles). Indicated on graph are maximum average growth (solid horizontal line, calculated from column 2), 50% titer Point (dashed horizontal line, half of maximum average growth), maximum killing (vertical double headed arrow, lowest CFU achieved by immune serum as percentage of maximum average growth) and Opsonic Index (OI; arrow, dilution of serum that kills 50% of bacteria). Non-Specific Killing (NSK; average counts of column 2, compared to average counts of column 1) is also calculated on Opsotiter spreadsheet and is indicated below the graph.