Journal of Visualized Experiments

Opsonophagocytic Killing Assay to Assess Immunological Responses Against Bacterial Pathogens

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59400R2
Full Title:	Opsonophagocytic Killing Assay to Assess Immunological Responses Against Bacterial Pathogens
Keywords:	Opsonophagocytosis, immune killing, HL-60, bacterial culture, complement, phagocytosis, bacterial infection, Streptococcus pneumoniae
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Athens, GA USA

The University of Georgia

Center for Molecular Medicine, Complex Carbohydrate Research Center

November 15, 2018

Dear Dr. Upponi:

We are excited to submit the enclosed manuscript for your consideration. In this protocol, we describe a simplified opsonophagocytic killing assay that utilizes basic culture conditions and cell counting to determine bacterial cell viability after co-culture with treatment conditions and HL-60 immune cells. We believe our study serves towards comparing the ability of phagocytic immune cells to respond to and kill bacteria based on different treatments and/or conditions.

We respectfully suggest as possible reviewers: Dr. Gerald Pier (Harvard Medical School), Dr. Liise-Anne Pirofski (Albert Einstein College of Medicine) or Dr. Jorge Vidal (Emory University), who are authorities in bacterial infectious diseases. We do not wish to exclude any reviewers.

Allow us to thank you in advance for your time and consideration. We hope that you are as excited editorially as we are scientifically about this work and its future implications. We eagerly look forward to hearing your comments in the coming weeks.

Sincerely,

Fikri Y. Avci, Ph.D.

Assistant Professor,
Department of Biochemistry and Molecular Biology

TITLE:

Opsonophagocytic Killing Assay to Assess Immunological Responses Against Bacterial Pathogens

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KEYWORDS:

opsonophagocytosis, immune killing, HL-60, bacterial culture, complement, phagocytosis, bacterial infection

SUMMARY:

This opsonophagocytic killing assay is used to compare the ability of phagocytic immune cells to respond to and kill bacteria based on different treatments and/or conditions. Classically, this assay serves as the golden standard for assessing effector functions of antibodies raised against a bacterium as opsonin.

ABSTRACT:

A key aspect of the immune response to bacterial colonization of the host is phagocytosis. An opsonophagocytic killing assay (OPKA) is an experimental procedure in which phagocytic cells are co-cultured with bacterial units. The immune cells will phagocytose and kill the bacterial cultures in a complement-dependent manner. The efficiency of the immune-mediated cell killing is dependent on a number of factors and can be used to determine how different bacterial cultures compare with regard to resistance to cell death. In this way, the efficacy of potential immune-based therapeutics can be assessed against specific bacterial strains and/or serotypes. In this protocol, we describe a simplified OPKA that utilizes basic culture conditions and cell counting to determine bacterial cell viability after co-culture with treatment conditions and HL-60 immune cells. This method has been successfully utilized with a number of different pneumococcal serotypes, capsular and acapsular strains, and other bacterial species. The advantages of this OPKA protocol are its simplicity, versatility (as this assay is not limited to antibody treatments as opsonins), and minimization of time and reagents to assess basic experimental groups.

INTRODUCTION:

The opsonophagocytic killing assay (OPKA) is a critical tool for linking alterations in bacterial

structure or function to subsequent changes in immune response and function. As such, it is frequently used as a complementary assay to determine immune-based efficacy of antibody treatments, vaccine candidates, enzyme optimization, etc. While in vivo assays are necessary to determine effective clearance or protection in a bacterial infection model, the OPKA can be used to assess immune contribution to bacterial cell death at the most basic components: bacteria, immune cells, and experimental treatments. Previous studies have shown that OPKAs can be modified and used for a variety of bacteria and serotypes, including *Streptococcus pneumoniae*[1], *Staphylococcus aureus*[2], *Pseudomonas aeruginosa*[3]. Furthermore, these optimized assays can be used to assess different experimental treatments, including the ability of an enzyme to make the bacterium more accessible to complement-mediated immune cells[4] and antibody treatments to improve opsonization[5]. Classically, OPKA assay has been successfully used in basic and clinical research settings as a powerful indicator for protection induced by pathogen-specific antibodies[6-9].

Different types of immune cells may be used for assessment of opsonophagocytic killing. One commonly used phagocytic population is the HL-60 human leukemic cell line. This cell line can be kept as inactivated promyelocytes in culture; however, they can be differentiated into various activated states via different drug treatments[10,11]. Treatment of HL60 with N,N-dimethylformamide differentiates the cell line into activated neutrophils with strong phagocytic activity[11]. While HL-60 cells have been optimized and are frequently used for these phagocytosis assays[10], other primary polymorphonuclear leukocytes can be used as the immune arm of the experiment[12].

Additionally, these assays can be simplified[13] or multiplexed[14] to look at multiple antibiotic-resistant strains of the bacteria to be tested. The multiplexed method has been made more feasible through the development of software that can efficiently count bacterial colony forming units (CFUs) per spot on an agar plate[15]. Here, we describe a streamlined method using one bacterial strain, HL-60 cells, baby rabbit complement, and blood agar plates. With this method, multiple treatments can be assessed quickly to address specific research questions on how the innate immune response to bacterial infection can be modulated.

PROTOCOL:

1. Culture, differentiation, and validation of HL-60 cells

1.1. Prepare HL-60 cell culture media composed of 500 mL RPMI with L-glutamine and 50 mL heat-inactivated fetal bovine serum. Do not add antibiotics as this may affect the differentiation of the HL-60 cells.

1.2. For propagation/maintenance of HL-60 cells, culture 5 x 10^6 cells in 10 mL of HL-60 cell culture media in 75 cm^2 vented flasks at 37 °C and 5% CO_2. Passage cells every 3–4 days to maintain optimal cell concentrations.

NOTE: The cell concentration should not exceed 5 x 10^6/mL.

1.3. Generate working stocks of HL-60 cells by aliquoting approximately 1 x 10^6 cells/mL in HL60 culture media with 10% dimethyl sulfoxide (DMSO) into 1 mL cryogenic tubes.

NOTE: Working stocks may be stored at -80 °C. The master stock should be stored at -120 °C.

1.4. Differentiate HL-60 cells by culturing 1.5 x 10^7 cells in 15 mL of HL-60 cell culture media with 0.6% N,N-dimethylformamide (DMF) at 37 °C and 5% CO_2 in sterile filter-capped 75 cm^2 flasks for 3 days prior to OPKA.

1.5. Validate that the HL-60 cells have been successfully differentiated and are appropriate for use in the OPKA assay by testing viability and cell surface markers according to established flow cytometry protocols[16-19]. After differentiation, harvest HL-60 cells and stain approximately 1 x 10^4 cells with fluorescently-conjugated antibodies/stains for CD71, CD35, annexin V, and propidium iodide.

NOTE: Differentiated cells should be ≥ 65% viable, ≥ 55% $CD35^+$, and ≤ 20% $CD71^+$ as determined through established validation protocols[14] (**Figure 1**).

2. Preparation of OPKA buffers and reagents

2.1. Prepare 50 mL of sterile opsonization buffer B (OBB) by mixing 42.5 mL of sterile 1x phosphate-buffered saline (PBS) with Ca^{2+}/Mg^{2+}, 5 mL of heat-inactivated fetal bovine serum, and 2.5 mL of 0.1% sterile gelatin. Store at 4 °C.

2.2. Obtain baby rabbit complement and store at -80 °C.

2.3. Obtain or prepare bacterial culture plates (i.e., 15 x 100 mm 5% sheep's blood agar plates).

3. Preparation of bacterial stock samples

3.1. Obtain a stock of the bacterial strain(s) to be tested.

NOTE: For this protocol, serotype 3 *Streptococcus pneumoniae* (WU2, generously provided by Dr. Moon Nahm) is used.

3.2. Grow the bacterial strain in an appropriate broth (i.e., Todd-Hewitt broth + 0.5% yeast extract for this WU2 strain) for approximately 2−4 h at 37 °C.

NOTE: The optical density at 600 nm (OD_{600}) of the culture should be between 0.6 and 0.8.

3.3. Pellet the bacteria by centrifugation at 6000 x *g* for 2 min and resuspend the cells in 10−30 mL of 15% glycerol in the appropriate broth. Aliquot the bacterial culture (500 µL per aliquot) into sterile 1.5 mL centrifuge tubes and store at -80 °C.

3.4. Thaw out one vial of bacterial stock in a 37 °C water bath. Pellet the bacterial cells and resuspend in 500 µL of OBB under sterile conditions.

3.5. Prepare different dilutions of the bacterial stock in OBB (i.e., 10 µL of no dilution, 10 µL of 1:10, 10 µL of 1:100, etc.). Perform the OPKA assay (sections 4–6, including HL-60/complement co-culture) as described below using various dilutions of the untreated bacterial stock. Culture the plates overnight at 30 °C (no CO_2).

NOTE: The temperature 30 °C is specific for WU2 to prevent overgrowth; other strains/serotypes may grow optimally at 37 °C.

3.6. Count the colonies for each dilution of untreated bacterial stock co-cultured with HL-60 cells and complement. Determine which dilution of bacteria yields the optimal number of countable colonies (approximately 80−120 CFUs for untreated bacteria co-cultured with HL-60 cells). Note this dilution for future OPKAs involving this bacterial stock.

4. Bacterial treatment and culture

4.1. Thaw one tube of bacterial stock prepared in step 3.3. Pellet bacteria (6000 x *g* for 2 min) and resuspend cell pellet in OBB at optimal dilution as determined in step 3.6.

4.2. Pipette 10 µL of resuspended bacterial dilution per well in a round-bottom 96-well cell culture plate.

4.3. Add 20 µL of appropriate antibody or drug treatment to each experimental well in duplicate.

NOTE: In this protocol, a serotype-specific antibody generated in mice is added as treatment X and a glycoside hydrolase enzyme known to degrade the serotype 3 polysaccharide capsule is added as treatment Y (**Figure 2**)[4,20]. For control wells, use 1x PBS or OBB, depending on the buffer used for treatment wells.

4.4. Shake the sample plate at approximately 90 rpm for 1 h at room temperature. Adjust these conditions depending on the optimal temperature or shaking conditions of the treatments being tested.

5. HL-60 bacterial co-culture

5.1. Prepare HL-60 cells by harvesting the HL-60 differentiated cells that are treated with DMF three days prior (see step 1.4) into 15 mL conical tubes. Pellet the cells (500 x *g*, 3 min), discard the supernatant, and wash with at least 10 mL of 1x PBS.

5.2. Pellet the washed cells (500 x *g*, 3 min), discard the supernatant, and resuspend the cells in OBB (start with 1 mL OBB and adjust for a final concentration of 1 x 10^7/mL after cell counting).

5.3. Add baby rabbit complement (sterile, undiluted baby rabbit serum, age 3–4 weeks) at a 1:5 final volume.

NOTE: The final concentration of the HL-60-complement mixture should be 1 x 10^7/mL. If testing complement dependency, a second solution containing active HL-60 cells with heat-inactivated complement may be used (complement may be inactivated by incubating in a water bath at > 55 °C for at least 30 min).

5.4. After one-hour bacterial culture is complete (step 4.4), divide each sample (i.e., 10 µL of each 30 µL sample well into two new wells) into duplicate wells for two groups (i.e., use only 20 µL of the original 30 µL co-culture to account for pipetting error): one set will be co-cultured with HL-60-complement and one will include bacteria only. Add 50 µL of the HL-60-complement mixture (from step 5.3) to each experimental set of wells (delegated +HL-60); add 50 µL of OBB alone to the wells of bacteria only (delegated -HL-60).

NOTE: For this example, approximately 800 bacterial CFUs are used for the initial co-culture with 5 x 10^5/50 µL HL-60 cells. If this multiplicity of infection is too high or too low as indicated by final colony numbers, adjust the initial bacterial dilution as opposed to the HL-60 cell count.

5.4. Shake the 96-well plate at 37 °C for 1 h (no CO_2).

6. Sample plating and overnight incubation

6.1. Dilute each well 1:5 with OBB, so that each sample has a volume of at least 50 µL.

6.2. Pipette 50 µL of each sample directly onto a designated area of a bacterial culture plate, ensuring adequate spacing between samples. For 15 x 100 mm round agar plates, pipet approximately 4 samples onto one plate.

6.3. Cover and allow samples to dry for approximately 15 min at room temperature.

6.4. Invert plates and culture overnight at 30 °C (no CO_2). Alternatively, culture plates in anaerobic jars to test whether anoxic conditions affect the bacterial growth or to control for morphology.

6.5. After overnight culture, count the colonies in each designated sample area. Analyze data by comparing the number of live cells in each set to the corresponding control and/or samples that do not receive HL-60 cell co-culture (indicative of 100% cell survival, 0% cell killing).

REPRESENTATIVE RESULTS:

Validation of HL-60 differentiation should be performed before starting the OPKA. This can be accomplished using flow cytometry to determine the extracellular expression of CD11b, CD35, CD71, and annexin V (**Figure 1**). Propidium iodide can also be used as a viability marker. After being treated with DMF for 3 days, expression of CD35 should be increased (≥ 55% of all cells)

and expression of CD71 should be decreased (≤ 20% of all cells). The percentage of annexin V+ and propidium iodide (PI+) cells together should be < 35% to ensure sufficient cell viability. If these percentages do not meet the minimum requirements, the culture conditions should be adjusted as described in the Discussion.

The number of CFUs obtained from step 6.5 can be used to compare the bacterial cell survival of different groups compared to the untreated control group (100% cell survival) as shown in **Figure 2**. For example, the average counts obtained from wells that received no treatment but were co-cultured with HL-60 should be relatively close in number to the cells that received no treatment and no co-culture of HL-60, which would be indicative of 100% cell survival, or 0% cell death. With an effective treatment, the numbers of colonies should be more different between HL-60 co-culture and no HL-60 cells (**Figure 2** and **Figure 3**). Larger differences between HL-60 and no HL-60 sets are indicative of more efficient phagocytosis. However, the treatment may actually improve bacterial cell growth in the set that is not co-cultured with HL-60 cells. This difference in treated and untreated samples should be noted. If the bacterial dilution is not optimized (step 3.6) or the colony growth is not carefully observed after plating (step 6.5 or see Discussion), overgrowth of the colonies may prevent accurate counting of colonies (**Figure 4**).

FIGURE LEGENDS:

Figure 1: Validation of HL-60 cell differentiation via flow cytometry. Differentiated HL-60 cells were harvested, washed, and resuspended in 1 x 10^5 cells/mL PBS. Cells were then aliquoted into 12 wells (100 µL/well) in a 96-well plate. Cells were then stained with fluorescently conjugated anti-CD35, anti-CD71, annexin V, and propidium iodide. Unstained cells or cells stained with fluorescently conjugated isotype antibodies were used as controls.

Figure 2: Treatment Y improves HL-60-mediated cell killing of bacteria. *S. pneumoniae* samples were treated with treatment X (antibody) or treatment Y (enzyme). OPKA was performed according to the protocol and bacterial CFUs were counted in duplicate. Samples that were not treated with HL-60 cells were used as a control (100% cell survival). Shown are the average percentages of bacterial CFUs in the HL-60 treated groups compared to the corresponding non-HL-60-treated groups. Bars represent standard error.

Figure 3: Bacterial CFUs after OPKA and overnight culture. Bacterial samples were treated and co-cultured without (**A**) or with (**B**) HL-60 cells for 1 h at 37 °C. Samples were diluted according to the protocol and plated on blood agar plates overnight at 30 °C (no CO_2).

Figure 4: Bacterial CFU overgrowth. Bacterial samples were treated and OPKA was performed. Samples were diluted and plated on blood agar plates overnight at 37 °C (no CO_2). Accurate assessment of colony numbers cannot be determined as overgrowth of colonies is shown. As 37 °C (no CO_2) led to bacterial overgrowth, the incubation temperature for future plates was lowered to 30 °C (no CO_2) to maintain countability of the colonies.

DISCUSSION:

OPKAs serve essential roles in assessing antibody mediated immune responses induced by

vaccinations[6,8]. The main significance of this simplified OPKA is the adaptability in the conditions to be tested (i.e., antibodies, enzyme treatments, etc.). In this sense, while this assay can be used to test the contribution of opsonins (i.e., antibodies) in phagocytosis, it can also be used to assess ways to overcome virulence factors (i.e., capsular polysaccharides) that normally inhibit phagocytic pathways. Minimizing the number of steps that are typically used in a multiplexed OPKA potentially minimizes the chances for technical errors that can affect the experimental results and reduces the amount of troubleshooting and optimization for obtaining usable data. As this protocol is suited for variations to treatment conditions, it allows for a great deal of versatility.

Pre-establishing the assay through culture of HL-60 and establishment of bacterial stocks is important to prevent extraneous optimization steps when performing the OPKA. Time must be dedicated to making sure all reagents and cell types are ready and functional before the experiment is performed. These steps include propagating the HL-60 cell line in culture (about two weeks), validating that specific concentrations of DMF effectively differentiate the HL-60 cells (about one week), and establishing contaminant-free and optimized bacterial stock dilutions (about two weeks).

Some steps of this protocol are critical for obtaining countable colonies and adequate data. This protocol uses HL-60 cells as phagocytes due to the ease of using a human cell line that can be maintained in culture and differentiated with relatively few steps. Human peripheral blood mononuclear cells (PBMCs) may also be used; however, obtaining these cells and optimizing the conditions for their use may be more challenging. The HL-60 cells must be differentiated in order to function as phagocytes against the bacteria. To verify differentiation after the 3-day treatment with DMF, flow cytometry should be used to test for the expression of CD11b and CD35 on a majority of the cells before any OPKA is attempted, as discussed in step 1.5. Cell viability should also be verified (preferably with annexin V and propidium iodide staining). If a large number of cells are dead, apoptotic, or undifferentiated as observed with flow cytometry, the 3-day differentiation with 0.6% DMF in RPMI media can be modified (0.4%−0.8% DMF, 2−6 day culture time) until cell viability and differentiation markers are improved. This differentiation should be the first optimization of the OPKA as HL-60 function is critical for effective bacterial killing. We recommend validating HL-60 differentiation before every OPKA experiment.

The number of bacterial CFUs (step 3.6) initially dispensed into the 96-well plate (step 4.2) is also critical: dispensing too many cells will make counting difficult and inaccurate (step 6.5) and may decrease the cell death observed from HL-60 co-culture, whereas dispensing too few cells may increase the amount of deviation between duplicates and may not show any countable colonies after HL-60 co-culture. Optimizing the stock dilution is therefore critical, and must be tested with the full protocol, including HL-60/complement co-culture.

The importance of complement may also be tested with this protocol by including two sets of samples: one with active baby rabbit complement and one with heat-inactivated complement. HL-60 cells should be co-cultured with both sets, though a bacteria-only set should still be included as a 100% cell survival baseline.

For some bacterial serotypes, the morphology of the colonies may make cell counting or visibility problematic. Mucoid serotypes such as type 3 *Streptococcus pneumoniae*, for instance, can easily overgrow and reduce the CFU counts. This may prove especially problematic when testing treatments that affect the capsule, as overgrowth would be prevented in the treated group but greater number of smaller colonies would be counted. To prevent this discrepancy, control of cell growth is critical. Culturing the plated colonies at 30 °C overnight will likely allow for improved monitoring of bacterial growth and the plates can be removed when all colonies reach distinguishable, countable sizes. Additionally, different agar plates may be used to improve visibility of individual colonies. In this way, this protocol is advantageous as small changes to optimize conditions can thus be used to account for a number of bacterial strains or various treatment options.

ACKNOWLEDGMENTS:

We thank Dr. Moon Nahm (University of Alabama Birmingham) for his invaluable assistance in establishing OPKA assays in our laboratory. This work was supported by National Institutes of Health Grant 1R01AI123383-01A1 to FYA.

DISCLOSURES:

The authors have nothing to disclose.

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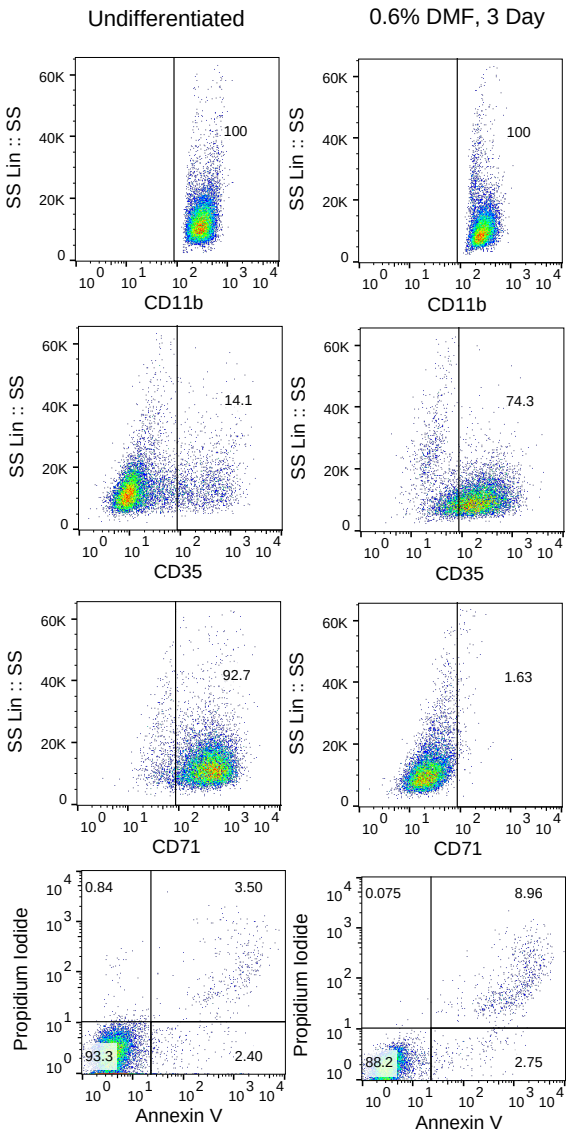

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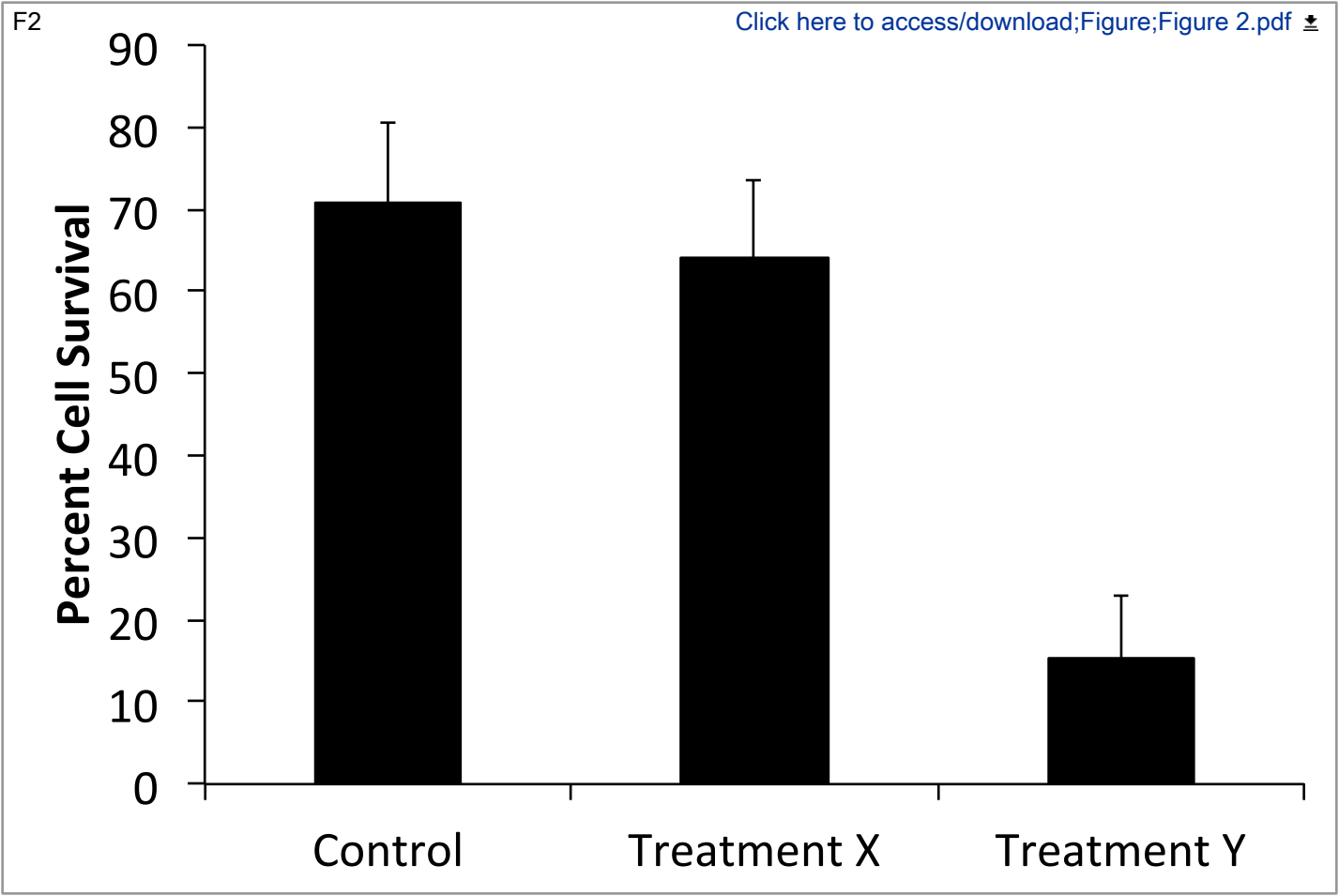

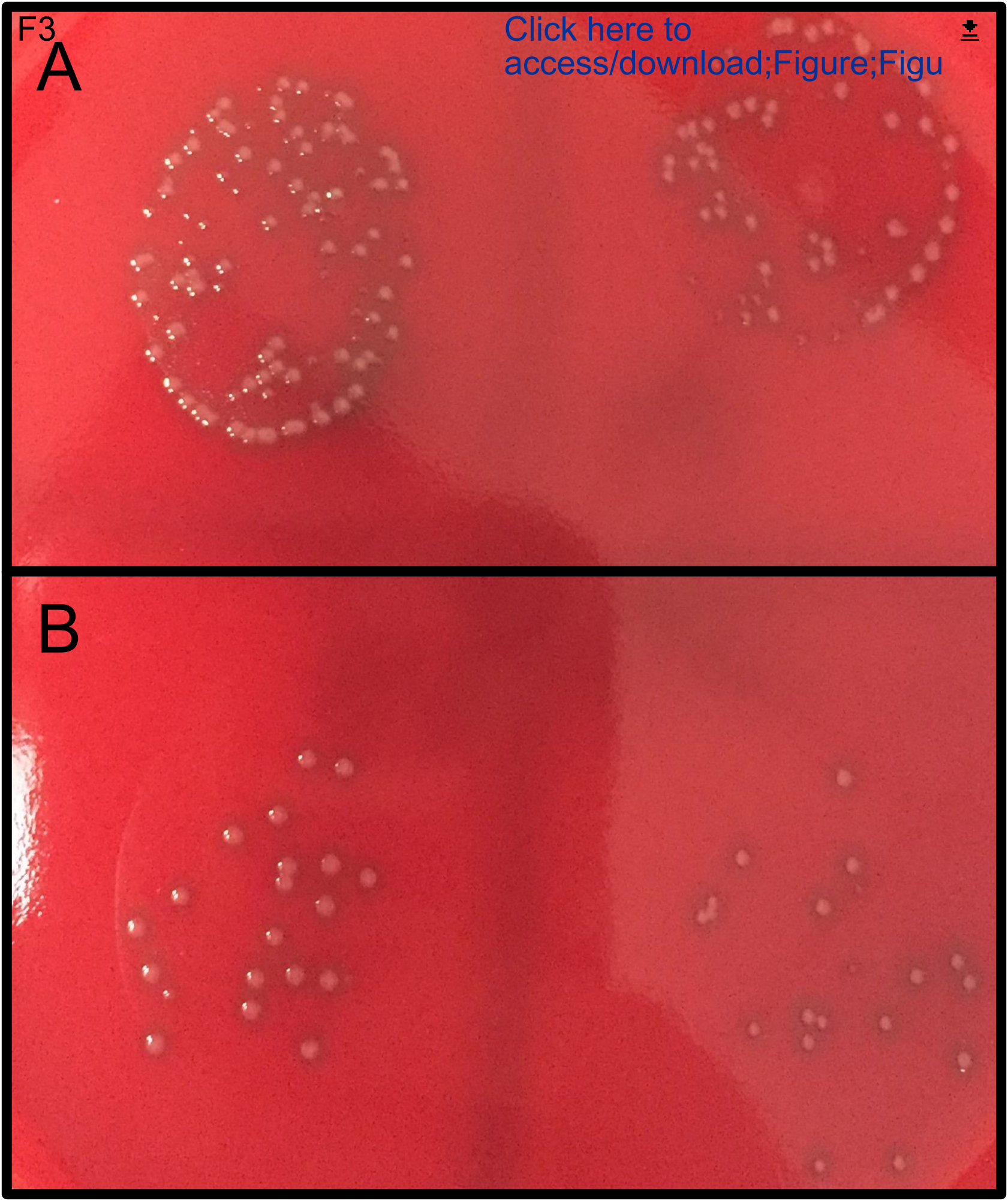
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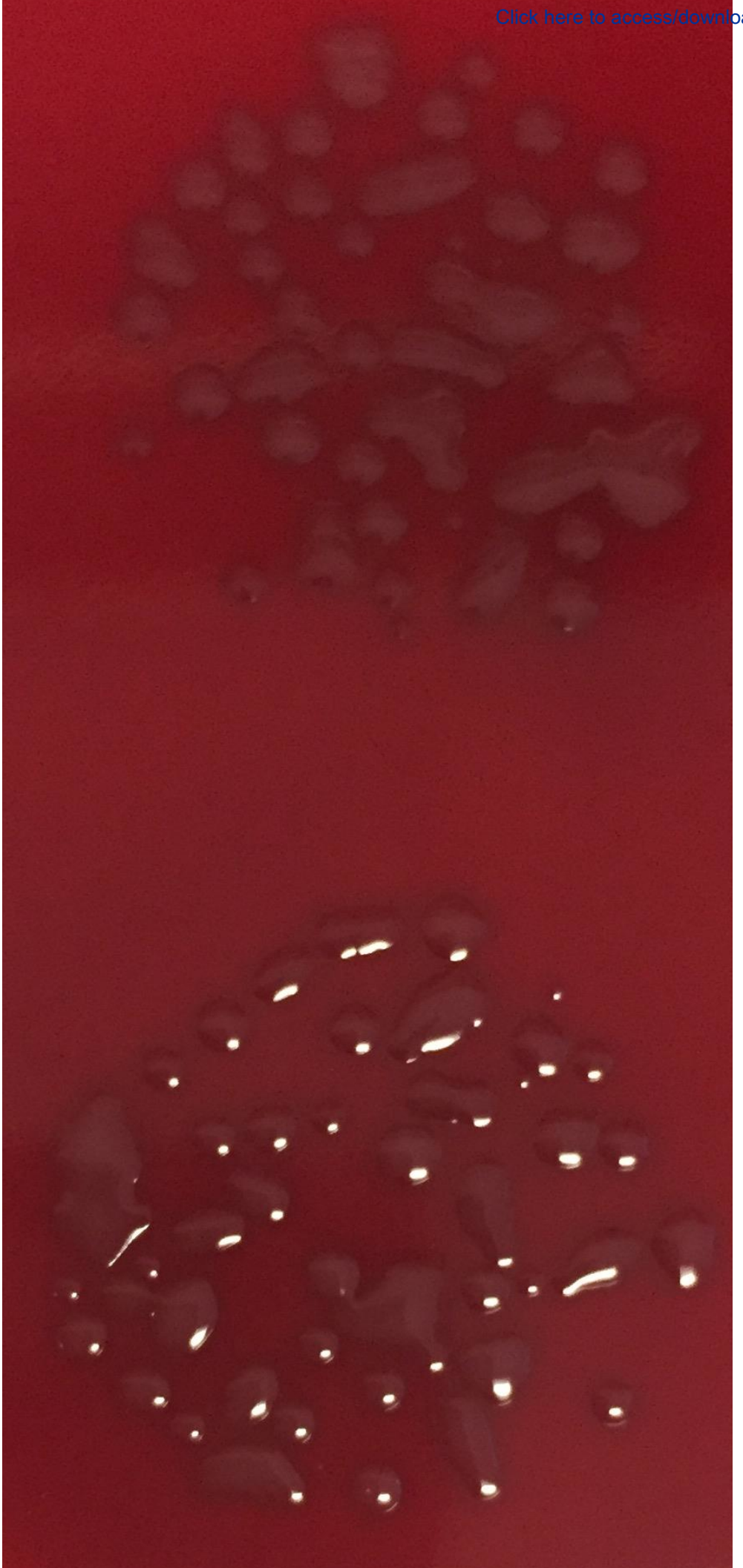

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Annexin V (APC conjugated)	BioLegend	640919	
anti-CD35, human (PE conjugated)	BioLegend	333405	
anti-CD71, human (PE conjugated)	BioLegend	334105	
bacterial strain to be used (ie, Streptococcus pneumoniae, WU2)	Bacterial Respiratory Reference Laboratory (Dr. Moon Nahm)		
blood agar plates	Hardy Diagnostic	A10	
Fetal Clone serum	HyClone	SH30080.03	
glycerol	Sigma	G9012-1L	
HL-60 cells	ATCC	CCL-240	
IgG Isotype Control (PE conjugated)	BioLegend	400907	
N,N-dimethylformamide (DMF)	Fisher Chemical	UN2265	
propidium iodide	Sigma	P4864	
RPMI media with L-glutamine	Corning	10-040-CV	

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Response to Reviewers' Comments:

We would first like to thank the editor and the reviewers for taking the time to review our manuscript carefully and constructively. We believe the revisions we have made to this protocol based on the reviewers' comments have greatly improved the structure and clarity of the work involved, and we appreciate the additional positive feedback that we received. Below we describe how we have addressed the reviewers' concerns.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We checked for grammar and spelling as advised.

2. Keywords: Please provide at least 6 keywords or phrases.

We have added additional keywords.

3. Please define all abbreviations before use.

We believe we have corrected this.

4. Please use the micro symbol µ instead of u.

We now use the µ symbol.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

We have added steps in an attempt to improve the reproducibility of the protocol, and have included references for established protocols where necessary (i.e., for flow cytometry).

6. 1.2: Please specify the bacterial strain used in this protocol.

We have now specified that we use *Streptococcus pneumoniae serotype 3* WU2.

7. 1.3: Please describe how to pellet the bacteria; if centrifugation is used, please specify centrifugation parameter (force in x g and time).

We have added centrifugation details (now Step 3.3, Line 192).

8. 1.4: Please spell out OBB and provide its composition.

We now clarify this at Step 2.1, Line 186.

9. 2.2: Please describe how to validate the differentiation of HL-60 cells.

We now describe this in more detail in Step 1.4.

10. 4.1: Please indicate the specific step of section 1 where bacterial stock is prepared.

We now indicate this in Step 4.

11. 4.3: Please specify the appropriate antibody or drug treatment used in this protocol.

We now clarify what treatments could be represented by Treatments X and Y in Step 4.3.

12. 5.1: Please indicate the specific step of section 2 where the cells are obtained. Please describe how to pellet the cells and specify the volume of PBS/OBB used.

Please split into two or three steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
We now specify that the cells were prepared in Step 1.2. We have added details to the pellet/wash steps and have adjusted the number of steps in this section.
13. Please order the steps properly so that the protocol can be followed in chronological order.
We have reordered Steps 1 through 3 in the protocol to improve the chronological flow.
14. Figure 1: Please specify what treatments X and Y are. Please define error bars.
We now indicate potential treatments for X and Y.
15. Table of Equipment and Materials: Please sort the items in alphabetical order according to the name of material/equipment.
We have updated and alphabetized the materials used for this protocol.
16. References: Please do not abbreviate journal titles.
Updated.

Reviewers' comments:

Reviewer #1: Paschall et al. described a protocol for opsonophagocytic killing assay to determine the immunological responses against bacterial infection. In this in vitro assay, the bacteria of interest are cocultured with immune cells in the presence of complement proteins. Opsonized bacteria are killed by the phagocytic immune cells as measured by CFU count. Overall, the manuscript is well written and the experiments were carefully designed. A good amount of work was done in describing the protocol in depth. However, a minor revision is necessary to improve the overall quality of the manuscript and make the protocol easy to follow by other researchers in the field. Specific comments and suggestions are enumerated below:
1. Lane 75. Since it is a protocol journal, it is better to specify the model bacterial strain used in this project. It is important because the bacterial growth media and other assay conditions were specific to the bacterial strain used.
We now specify our model bacterial strain in Step 3.1, Line 198.
2. Lane 78: Specify speed (g) and time for centrifugation
We now specify centrifugation details (Lines 205 and 251).
3. What is the final concentration of baby rabbit complement in the assay? 1:5 volumetric ratio is not informative if the sources/vendors of complement are different.
We now revised this section by addressing that the complement source is undiluted baby rabbit serum and the actual concentration of the complement in the serum is not specified/measured by the manufacturer or the literature. In other words, a purified/ well-characterized mixture of complement proteins are not used in the assay.
4. Lane 83. Use full form of any abbreviations when used in the text for the first time, such as in the case of "OBB".
We now clarify OBB at Line 186 (as well as other abbreviations).
5. Lane 102. Specify the markers of HL-60 differentiation so that the readers don't need to wait until the discussion section.

We now describe the validation steps starting at Step 1.4, Line 173.
6. HL-60 cells remained the gold standard for OPA. However, it remains unclear why phagocytic immune cells like macrophages or neutrophils were not used in such assays. Are HL-60 cells more potent phagocytes than macrophages? It should be included in the discussion.
We now describe how HL-60 represents an easy to culture and optimize option for phagocytic function within our discussion (starting at Line 356), though other phagocytes may be used.
7. Does HL-60 culture or co-culture require incubation in CO2-incubator?
We now clarify the use of 5% CO2 for HL-60 cells in Step 1.3, Line 91.
8. Lane 88 and 162. Why 30C instead of 37C? What is the rationale?
We now note that 30C incubation times are specific for our type 3 strain of Strep pneumoniae in Step 3.5, Line 222, to avoid overgrowth of the mucoid strain, but that other strains may grow optimally at 37C.
9. Lane 76. The optimum OD at 600 nm should be reported. A culture time of 2-4 hrs is not informative since the amount of inoculum is unknown.
We now indicate an optimal range for the OD600 in Step 3.2, Line 203.

Reviewer #2: Manuscript Summary:

In "Opsonophagocytic killing assay to assess immunological responses against bacterial pathogens", Paschall et al describe an experimental method commonly used to measure the effector functions of antibodies and killing of bacterial cells.

Major Concerns:
There are many protocols for OPA including from the University of Alabama Birmingham. How does this protocol contribute?
As we have acknowledged in the manuscript and also mentioned by the reviewer, there are published protocols, which we also utilized to establish our own. As revised in response to Reviewer 4, this simplified, flexible and visualized/recorded protocol will aid researchers intending to establish the protocol for the first time. In sum, we were approached by JoVE to contribute a visual process for developing a simplified OPKA. Published protocols developed in the UAB will continue to be used as golden standards for high throughput, [pre]clinical studies.

Minor Concerns:
1. line 186: It should be treatment Y that imporves HL-60 mediated cell killing of bacteria, correct?
We have now corrected the figure title to reflect this.
2. What is the source of bacteria used in the experiment?
The bacteria we used were a generous gift from Dr. Moon Nahm at the NIH Bacterial Respiratory Pathogen Reference Laboratory. We now indicate this in Step 3.1, Line 198.

Reviewer #3: The manuscript by Paschall et al describe an Opsonophagocytic killing assay using HL60 differentiated cells.
Main concerns:
1. The title is opsonophagocytosis killing assay and many authors use (OPKA) whereas in this manuscript the authors use OPA. I suggest use OPKA in the entire manuscript because in their protocol they are really measuring killing as they plate viable bacteria on agar plates. OPA may be more accurate when measuring phagocytosis these cells by a flow cytometry assay as other authors do.
We thank the reviewer for this correction and have now updated OPA to OPKA throughout the manuscript.
2. In the abstract is mentioned that this assay has been successfully used for testing pneumococcal strains of serotypes 3, 4 and 19A whereas many serotypes have been tested indeed if you do a search at the literature. This paragraph should be modified because it seems that only works or at least is only validated against the serotypes mentioned above
We agree that this statement should be revised and have updated the abstract accordingly.
3. The introduction is too short. The authors do not explain the type of cells driven by differentiation of HL60 using DMF.
We have updated the Introduction with more information about HL-60 cells and their differentiation.
4. The authors explain in different parts of the manuscript that the temperature used for bacteria is 30 degrees Celsius (lines 88, 162 and 195). The authors explain that incubating the agar plates at 30 degrees to avoid overgrowth of serotype 3. I agree that 30 degrees without 5% CO2 may be optimal for serotype 3 but if you are measuring OPKA of other pneumococcal serotypes, the use of 37 degrees Celsius in the presence of 5% CO2 is optimal. This should be explained in the manuscript that 30 degrees without CO2 is just for serotype 3 whereas for other serotypes, the 37ºC with CO2 may be optimal. This should be explained in the process 1.5.
We now note this stipulation in Step 3.5, Line 208.
5. The optimal growth of HL60 cells is 37ºC with CO2 as recommended by ATCC http://www.lgcstandards-atcc.org/products/all/CCL-240.aspx?geo_country=es#culturemethod
Why the authors do not use CO2 for HL60 cells?
We now clarify the use of 5% CO2 for HL60 cell culture in Steps 1.2 and 1.4.
6. The authors should explain in point 2 (line 94,) the concentration of HL60 in the propagation flask, the limit that should not be exceeded and the concentration of the differentiated flask. These data is required if a new person starting from the beginning try to replicate this protocol.
We now discuss these details for propagation and differentiation within Step 1 (Step 1.2, 1.4, 1.5).
7. The authors should indicate in point 5.1 the optimal MOI (multiplicity of infection) or at least explain why they recommend 10to7 cells/ml
We now indicate multiplicity of infection in Step 5.3, Lines 263-266.
8. In line 141 change C by Celsius as they write Celsius in the entire manuscript

We have changed this accordingly.

9. White colonies in figure 3 are not S. pneumoniae. If authors want to show overgrowth of S. pneumoniae which is the bacteria mentioned in this manuscript, the authors should show a figure with pneumococcal overgrowth not with other bacteria specie because from my perspective, colonies in figure 3 suggest contamination of the assay.

We have now replaced Figure 3 depicting pneumococcal overgrowth with no other contaminating bacterial species.

Reviewer #4: Manuscript Summary:

Robust and clear protocol for an OPK assay. Generally, this assay appears simple and stratightforward when described but can be very difficult to establish. The manuscript is worthy of publication but needs more details and specific ranges for variables.

Major Concerns:

The assay is difficult to establish and details around the validation of differentiation are not discussed in sufficient detail. Passage number of the original culture of HL60 is important as is the maintenance of the master and working stocks of HL60.

We now discuss generation of master/working stocks in Step 1.3, Line 87.

The "cell count" in HL60 cultures prior to differentiation, normal growth rate for the HL60 line?

We have provided more details regarding HL-60 cell concentrations and passage times (Step 1.2).

Some additional discussion around the assessment of differentiation by flow cytometry would be helpful (a figure?). Additional discussion around quality control of the assay would be helpful (how many passages the HL60 are used for, selection and testing of the complement source, conformation of differentiation.

We have now added details for HL-60 validation (Step 1.5) and passage recommendations (Discussion).

The authors also comment on "adjustments" to the differentiation protocol.... a little longer or more DMF. This isn't anywhere near detailed enough to guide a new laboratory through the establishment of the assay.

We now indicate an optimal range to try in the Discussion (0.4% to 0.8% DMF, 2 to 6 day culture times, Line 352).

A trouble shooting/ tricks and tips component would significantly enhance the manuscript.

We have added details for troubleshooting to the Discussion accordingly.

The cells are almost certainly cultured without antibiotics, this is not stated.

We now clarify this in Step 1.1, Line 80.

Additional components are normally present in HL60 RPMI media (L-Glut).

We now clarify this in Step 1.1, Line 78, and within the Materials List.

Cultured at 5% CO2/ 37C?

We now clarify temperatures and CO2 usage throughout the manuscript.

The authors only state RPMI + FCS. Further details on the culture and expansion of the HL60 are required. I would ask that a "first/ pre-establishing the assay" checklist/ steps section is added. a simple comment as to how long it took the authors to establish the assay for the first time would be useful together with a comment on how frequently the assay is performed in their laboratory and how often it fails/ the range within which they consider the assay to be "successful"/ "failing".
We have now added these suggestions to Step 1 and to the Discussion section.