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Title: Measuring Naturally Acquired Phagocytosis-Inducing Antibodies to *Plasmodium falciparum* Parasites by a Flow Cytometry-Based Assay

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Author Questionnaire

1. Microscopy: Does your protocol demonstrate the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

Videographer: please film screen captures for step 6.2. as indicated

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **51**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Maria del Pilar Quintana**: This protocol allows measurement of the ability of antibodies to opsonize and induce the phagocytosis of *Plasmodium falciparum*-infected erythrocytes **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Maria del Pilar Quintana**: Antibodies present in the serum or plasma of individuals naturally exposed to parasite infection, as well as those induced by immunization with parasite antigens, can be tested **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

1.3. **Maria del Pilar Quintana**: Demonstrating the procedure with me will be Maiken Visti, a technician from my laboratory **[1][2]**.

1.3.1. INTERVIEW: Author saying the above
1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

Protocol

2. THP-1 and Infected Erythrocyte (IE) Culture Setup

2.1. To set up a THP-1 (T-H-P-one) cell culture for a phagocytosis assay, the day before the experiment, seed the cells in a 25 centimeters squared culture flask at a concentration of 2.5 x 10^5 cells per milliliter in THP-1 cell culture medium **[1-TXT]**.

2.1.1. WIDE: Talent adding cells to culture container, with medium container visible in frame **TEXT: THP-1**

2.2. On the day of the experiment, at least one hour before the experiment, block two, round-bottom, 96-well plates with 150 microliters of sterile 2% FBS in PBS per well **[1-TXT]**.

2.2.1. WIDE: Talent adding PBS + FBS to well(s), with PBS + FBS container visible in frame **TEXT: FBS: fetal bovine serum**

2.3. For mid- to late-stage trophozoite-infected erythrocytes harvest and purification, prepare around 1 to 2 milliliters of packed *P. falciparum* parasite culture in 10 milliliters of parasite culture medium with at least 5% parasitemia and add to a magnetic column **[1-TXT]**. Wash the column with 50 milliliters of parasite culture medium **[2-TXT]**.

2.3.1. Talent adding parasites to column on magnet *Videographer: Important/difficult step* **TEXT: See text for parasite culture details**

2.3.2. Talent adding medium to column, with medium container visible in frame *Videographer: Important/difficult step* **TEXT: Paramagnetic trophozoite-IEs will remain in column mesh**

2.4. To elute the infected erythrocytes, remove the column from the magnet **[1]** and flush it with 50 milliliters of parasite culture medium **[2-TXT]**.

2.4.1. Talent removing column from magnet

2.4.2. Talent adding medium to column with medium container visible in frame **TEXT: See text for all medium and solution preparation details** NOTE: please skip

the final part of the shot where a lot of bubbles were created (this should not happen)

2.5. Then collect the infected erythrocytes by centrifugation **[1-TXT]** and resuspend the pellet in **[2]** 1 milliliter of fresh parasite culture medium for counting **[3-added]**.

2.5.1. Talent placing tube(s) into centrifuge **TEXT: 10 min, 500 x g, RT**

2.5.2. Shot of pellet if visible

2.5.3. Added shot: Talent adding medium to the tube, with medium container and hemocytometer visible in frame

3. Parasite Staining and Opsonization Plate Preparation

3.1. Adjust the purified infected erythrocyte suspension to 3.3 x 10^7 cells per milliliter in parasite culture medium **[1]** containing ethidium bromide to a final concentration of 2.5 micrograms per milliliter **[2]**.

3.1.1. WIDE: Talent adding medium to cells, with medium container visible in frame

3.1.2. Talent adding EtBr to tube, with EtBr container visible in frame *Videographer: Important step*

3.2. Next, remove blocking solution from one 96-well plate by flicking the plate into a waste container **[1]**, then remove any excess over a paper towel **[1B-added]** and add 30 microliters of the ethidium bromide-labeled infected erythrocyte suspension to all but one well in the upper half of the plate **[2]**.

3.2.1. Talent flicking plate on waste container

3.2.1.B. Added shot: Talent flicking plate over paper towels

3.2.2. Talent adding IEs to well(s), with IE container visible in frame

3.3. Add 30 microliters of parasite culture medium to the empty well **[1]** and incubate the plate for 10 minutes at room temperature protected from light **[2]**.

3.3.1. Talent adding medium to well, with medium container visible in frame

3.3.2. Talent covering plate

3.4. At the end of the incubation, add 170 microliters of parasite culture medium to each well **[1]** and sediment the IEs at the bottom of the plate by centrifugation **[2-TXT]**.

3.4.1. Talent adding medium to well(s), with medium container visible in frame

3.4.2. Talent placing plate into centrifuge **TEXT: 3 min, 500 x g, RT**

3.5. Remove the supernatant by flicking the plate into a waste container **[1]** and wash the ethidium bromide-labeled IEs two more times with 200 microliters of parasite culture medium per well as just demonstrated **[2]**.

3.5.1. Plate being flicked into a waste container

3.5.2. Talent adding medium to well(s), with medium container visible in frame

3.6. After the second wash, use a multichannel pipette to carefully remove the entire volume of supernatant from each well without disturbing the pellets **[1]** and resuspend the ethidium bromide-labeled infected erythrocytes in 30 microliters of antibody solution, including the appropriate controls and test samples previously prepared in parasite culture medium **[2]**.

3.6.1. Supernatant being removed *Videographer: Important step*

3.6.2. Talent adding solution to well(s), with solution container visible in frame *Videographer: Important step*

~~3.7. **[1]**.~~

~~3.7.1. Control(s) being added, with control tubes visible in frame *Video Editor: please emphasize each control tube when mentioned*~~

3.8. Then, place the plate at 37 degrees Celsius for 45 minutes protected from light **[1]**.

3.8.1. Talent placing plate at 37 °C

4. THP-1 Cell and Phagocytosis Plate Preparation

4.1. While the IEs are being opsonized **[0-added]**, collect the THP-1 cells by centrifugation **[1]** and re-suspend the pellet **[2]** in 12 milliliters of fresh pre-warmed THP-1 cell culture medium **[3-added]**.

4.1.0. Added shot: Talent transferring cells from culture flask into tube

4.1.1. WIDE: Talent placing tube(s) into centrifuge

4.1.2. Shot of pellet

4.1.3. Added shot: Talent adding medium to the cells, with medium container visible in frame

4.2. After a second centrifugation **[1]**, resuspend the pellet in 1 milliliter of THP-1 cell culture medium for counting **[1B-added]** and adjust the cells to a 5 x 10^5 cells per milliliter of medium concentration **[2]**.

4.2.1. Talent placing tube into centrifuge

4.2.1.B. Added shot: Talent adding 1 milliliter of medium to the cells

4.2.2. Talent adding extra medium to tube, with medium container visible in frame

4.3. Remove the blocking solution from the second 96-well plate **[1]** and add 100 microliters of THP-1 cells to each well **[2]**.

4.3.1. Talent flicking plate over waste container and paper towels *Videographer: Important step*

4.3.2. Talent adding cells to well(s) *Videographer: Important step*

4.4. Then place the plate in the cell culture incubator **[1]**.

4.4.1. Talent placing plate into incubator

5. Phagocytosis Assay

5.1. At the end of the opsonization incubation, add 170 microliters of parasite culture medium to each well of the opsonization plate **[1]** and sediment the IEs to the bottom of the plate by centrifugation **[2]**.

5.1.1. WIDE: Talent adding medium to well(s), with medium container visible in frame

5.1.2. Talent placing plate into centrifuge

5.2. Wash the opsonized IEs two times with 200 microliters of parasite culture medium per well **[1]**, using a multichannel pipette to carefully remove the supernatant after the second wash **[2]**.

5.2.1. Talent adding medium to plate, with medium container visible in frame

5.2.2. Medium being removed from well(s), with medium container visible in frame

5.3. Resuspend the opsonized IEs in 100 microliters of pre-warmed THP-1 cell culture medium per well **[1]** and transfer 50 microliters of each opsonized IE suspension to a corresponding well in the phagocytosis plate **[2]**.

5.3.1. Talent adding medium to well(s), with medium container visible in frame *Videographer: Important step*

5.3.2. Talent adding IEs to well(s), with IE plate visible in frame *Videographer: Important step*

5.4. When all of the cells have been added, place the plate in the cell culture incubator for no more than 40 minutes protected from light **[1]**.

5.4.1. Talent placing plate into incubator

5.5. At the end of the incubation, stop the phagocytosis by centrifugation at 4 degrees Celsius **[1]** remove the supernatant **[1B-added]** and resuspend the pellets with 150 microliters of room-temperature ammonium chloride lysing solution per well **[2]**.

5.5.1. Talent placing plate into centrifuge

5.5.1.B. Added: Plate being flicked into a waste container

5.5.2. Pellet(s) being resuspended, with lysis solution container visible in frame

5.6. After exactly three minutes, add 100 microliters of ice cold 2% FBS in PBS to stop the lysis **[1]** and sediment the intact cells by centrifugation **[2]**.

5.6.1. Talent adding PBS + FBS to well(s), with PBS + FBS container visible in frame *Videographer: Important step*

5.6.2. Talent placing plate into centrifuge

5.7. Next, wash the wells three time with 200 microliters of fresh ice cold 2% FBS in PBS per well per wash **[1]**.

5.7.1. Talent flicking plate ~~with ice cold 2% FBS + PBS container visible in frame~~

5.8. After the final wash, re-suspend the cell pellet in 200 microliters of fresh ice cold 2% FBS in PBS per well **[2]**.

5.8.1. Ice cold 2% FBS in PBS being added to well(s), with ice cold 2% FBS in PBS container visible in frame

6. Flow Cytometry Acquisition and Analysis

6.1. For flow cytometric acquisition and analysis, immediately load the cells onto a flow cytometer **[1]** and use the linear forward- versus linear side-scatter plot for the wells with no IEs to gate the THP-1 cells **[2]**.

6.1.1. WIDE: Talent loading plate onto cytometer

6.1.2. Talent at cytometer, gating cells, with monitor visible in frame

6.2. Acquire 10,000 events on this gate **[1]** and use the THP-1 cells with no IEs wells to set up a histogram plot to measure ethidium bromide fluorescence intensity **[2]**.

6.2.1. SCREEN: Events being acquired *Videographer: please film*

6.2.2. SCREEN: Histogram plot being created *Videographer: please film*

6.3. For analysis, open the linear forward- versus linear side-scatter plot for the wells with no IEs and gate the THP-1 cells **[1]**.

6.3.1. SCREEN: screenshot_1: 00:14-00:30 *Video Editor: please speed up*

6.4. Then set up a positive gate in a FL3 histogram **[1]** and copy these gates onto all of the other samples wells to determine the percentage of ethidium bromide-positive THP-1 cells **[1]**.

6.4.1. SCREEN: screenshot_1: 00:40-01:14 *Video Editor: please speed up*

6.4.2. SCREEN: screenshot_1: 01:24-01:51

6.5. For each sample tested, phagocytosis can be reported as the absolute value **[1]** or as the relative phagocytosis calculated as a percentage using the positive control as the maximum **[2]**.

6.5.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize Figure 4A*

6.5.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize Figure 4B*

Protocol Script Questions

A. Which 4-6 steps from the protocol are the most important for viewers to see?
2.3., 3.1., 3.6., 4.3., 5.3., 5.6.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

2.3. Obtaining highly pure and synchronous trophozoite IEs is probably the most difficult aspect. To facilitate this, we always maintain parasite synchronicity by regular sorbitol treatment during the early ring stage. We also perform magnetic purification only with parasite cultures with at least 5% parasitemia.

Results

7. Results: Representative Evaluation of Parasite-IE Opsonization and Phagocytosis

7.1. The THP-1 cells should be periodically checked for Fc gamma-receptor surface expression by flow cytometry **[1]**. The cells should be negative for CD16 (C-D-sixteen) **[2]** and positive for CD32 and CD64 **[3]**.

7.1.1. LAB MEDIA: Figure 1
7.1.2. LAB MEDIA: Figure 1 *Video Editor: please emphasize red histogram in Figure 1A*
7.1.3. LAB MEDIA: Figure 1 *Video Editor: please emphasize green and yellow histograms in Figures 1B and 1C*

7.2. In this opsonization experiment **[1]**, the THP-1 cells were gated first according to their forward and side scatter profiles **[2]** to allow quantification of the percentage of ethidium bromide-labeled cells, indicative of cells that have phagocytized at least one antibody-opsonized IE **[3]**.

7.2.1. LAB MEDIA: Figure 2
7.2.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize cells in gate*
7.2.3. LAB MEDIA: Figure 2 *Video Editor: please emphasize red histogram*

7.3. The negative control samples should all generate a single negative peak in the FL3 (F-L-three) channel **[1]** with few events observed in the ethidium bromide marker **[2]**.

7.3.1. LAB MEDIA: Figure 3A *Video Editor: please emphasize blue, cyan and green peaks to left of gate*
7.3.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize bottom of blue, cyan and green histogram in right side of gate*

7.4. Accordingly, the mean phagocytosis values **[1]**, both as absolute ethidium bromide-positive THP-1 cells **[2]** and as relative phagocytosis percentages, should be very low **[3]**.

7.4.1. LAB MEDIA: Figures 3B and 3C
7.4.2. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize black, blues, and green data clusters in both graphs*

7.5. In contrast, the positive controls should generate traces with two peaks **[1]** - a negative peak **[2]** and a clearly positive and well-separated peak - located within the ethidium bromide marker **[3]**.

7.5.1. LAB MEDIA: Figure 3A
7.5.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize red and yellow histograms to left of gate*
7.5.3. LAB MEDIA: Figures 3A *Video Editor: please emphasize red, yellow, and pink histograms*

7.6. The mean phagocytosis values **[1]** are normally highest for the positive control **[2]**, followed by the malaria-exposed female pool **[3]** and the single malaria-exposed woman controls **[4]**.

7.6.1. LAB MEDIA: Figures 3B and 3C
7.6.2. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize red data cluster in Figure 3B*
7.6.3. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize yellow data clusters in Figures 3B and 3C*
7.6.4. LAB MEDIA: Figures 3B and 3C *Video Editor: please emphasize pink data clusters in Figures 3B and 3C*

7.7. Although this methodology generates consistent results **[1]**, deviations in the slope coefficient of the adjusted lines have been observed **[2]**. Therefore, the use of relative values is recommended, especially if it is not possible to run all of the samples in a single experiment **[3]**.

7.7.1. LAB MEDIA: Figure 4
7.7.2. LAB MEDIA: Figure 4 *Video Editor: please sequentially add/emphasize Slope text in Figure 4A and 4B*
7.7.3. LAB MEDIA: Figure 4 *Video Editor: please emphasize Figure 4B*

Conclusion

8. Conclusion Interview Statements

8.1. **Maria del Pilar Quintana**: Be sure to carefully plan the experiment in advance, to prepare both the THP-1 cells and parasites accordingly, and to only use trophozoites with a greater than 80% purity **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.3.)

8.2. **Maria del Pilar Quintana**: In addition, make sure to always include the appropriate controls to allow the quality of the experiment to be assessed and to facilitate data analysis **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (3.7., 6.4.) *Videographer: Can cut for time*