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Title: A Rapid, Multiplex Dual Reporter IgG and IgM SARS-CoV-2 Neutralization Assay for a Multiplexed Bead-based Flow Analysis System

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, done**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group?

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 15
Number of Shots: 46

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **SHERRY DUNBAR:** To our knowledge, this is the first example of a bead-based multiplexing protocol that uses two reporter signals to simultaneously measure two results per analyte.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.3.1.*

1.2. **SHERRY DUNBAR:** This technique allows the user to measure antigen-specific IgM and IgG at the same time, with less sample and a shorter time to results.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.3.2.*

OPTIONAL:

1.3. **SHERRY DUNBAR:** While this dual reporter method is specific to antibody isotyping, it could be adapted to measure other analyte pairs, such as post-translational modifications or free versus bound drug forms.

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

Introduction of Demonstrator on Camera

1.4. **SHERRY DUNBAR:** Demonstrating the procedure will be **STEVE ANGELONI**, a Senior Field Application Scientist from our laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.5. Procedures involving human subjects have been approved by the Institutional Review Board (IRB) at University of Rochester.

Protocol

2. Dual Reporter IgG and IgM Serological Assay

2.1. To begin the dual reporter IgG and IgM serological assay, prepare the required multiplex bead mixes **[1]** from the individual coupled bead and control bead stocks at a concentration of 1 x 10^6 beads per milliliter **[2-TXT]**. *Videographer: This step is important!*

2.1.1. WIDE: Talent at the bench with the bead stocks in view

2.1.2. Talent preparing the multiplex bead mixes. **TEXT: See text for full bead preparation details**

2.2. Next, dilute the serum samples 100-fold by adding 10 microliters of serum to 990 microliters of PBS-TBN *(P-B-S-T-B-N)* buffer **[1]**, then dilute the samples another 10-fold by adding 20 microliters of the 1 to 100 dilution to 180 microliters of PBS-TBN **[2]**. *Videographer: This step is important!*

2.2.1. Talent adding serum to buffer. Videographer NOTE: 2.2.1 and 2.2.2 shot together

2.2.2. Talent adding diluted sample to the buffer. Use 2.2.1

2.2.2 Added shot: 1 to 1000 dilutions.

2.3. When the bead mixes and serum samples are ready, add 50 microliters of the multiplex bead mixes to the assigned wells of a 96-well non-binding microtiter plate **[1]**, then add 50 microliters of the diluted serum samples to the appropriate wells **[2]**. *Videographer: This step is important!*

2.3.1. Talent adding multiplex bead mix to the 96-well plate.

2.3.2. Talent adding serum samples to the wells.

2.4. Once all samples have been added, cover the plate with a microplate foil seal **[1]** and incubate on a heated plate shaker at 37 degrees Celsius for 15 minutes **[2-TXT]**. Then, separate the beads from the reaction mixture by placing the plate onto a magnetic plate separator for 2 minutes **[3]**.

2.4.1. Talent covering the plate with foil.

2.4.2. Talent placing the plate on a shaker. **TEXT: 600 rpm** *Videographer: Obtain multiple usable takes because this will be reused in 2.6.4, 2.8.3, and 3.3.1.*

2.4.3. Talent placing the plate on magnetic plate separator. *Videographer: This step is important! Also, obtain multiple usable takes because this will be reused in 2.6.5. and 2.8.4.*

2.5. Keeping the plate on the magnet, remove the foil seal **[1]**, then carefully invert the plate over a waste container and gently flick the supernatant out of the wells **[2]**. While still holding the plate on the magnet, blot the plate on absorbent paper **[3]**. *Videographer: This step is important!*

2.5.1. Talent removing foil seal. Videographer NOTE: 2.5.1 to 2.5.3 shot together

2.5.2. Talent inverting plate over waste container and flicking the supernatant. *Videographer: Obtain multiple usable takes because this will be reused in 2.7.1.*

2.5.3. Talent blotting the plate on absorbent paper. *Videographer: Obtain multiple usable takes because this will be reused in 2.7.2. and 2.9.2.*

2.6. To wash the reaction wells, remove the plate from the plate magnet **[1]** and add 150 microliters of PBS-TBN to each well **[2]**. Cover the plate with a fresh foil seal **[3]** and incubate on the heated shaker for 2 minutes **[4]** before placing it back on the plate magnet for another 2 minutes **[5].** *Videographer: This step is important!*

2.6.1. Talent removing the plate from the plate magnet. Videographer NOTE: 2.6.1 and 2.6.2 shot together

2.6.2. Talent adding PBS-TBN to the wells. *Videographer: Obtain multiple usable takes because this will be reused in 2.9.3. and 3.3.2.*

2.6.3. Talent covering the plate with foil. Videographer NOTE: Use previous shot

2.6.4. *Use 2.4.2 Talent placing the plate on a shaker* Videographer NOTE: Use previous shot

2.6.5. *Use 2.4.3 Talent placing the plate on magnetic plate separator* Videographer NOTE: Use previous shot

2.7. While keeping the plate on the magnet, invert the plate to discard the supernatant **[1]**, and blot the plate on absorbent paper **[2-TXT]**.

2.7.1. *Use 2.5.2. Talent inverting plate over waste container and flicking the supernatant.*

2.7.2. *Use 2.5.3 Talent blotting the plate on absorbent paper.* **TEXT: Repeat the wash once more**

2.8. After the second PBS-TBN wash, remove the plate from the magnet **[1]** and add 100 microliters of fresh Detection Reagent mix to each well **[2-TXT]**. After covering the plate with a microplate foil seal, place it on a heated plate shaker for 15 minutes **[3]** and then on a magnetic plate separator for 2 minutes **[4]**.

2.8.1. Talent removing the plate from the magnet. Videographer NOTE: 2.8.1 and 2.8.2 shot together

2.8.2. Talent adding Detection Reagent mix to the plate. **TEXT: See text for Detection Reagent mix preparation** *Videographer: Obtain multiple usable takes because this will be reused in 3.3.3*

2.8.3. *Use 2.4.2 Talent placing the plate on a shaker.*

2.8.4. *Use 2.4.3 Talent placing the plate on magnetic plate separator*

2.9. While the plate is on the magnet, discard the Detection Reagent mix by inverting the plate over a waste container **[1]** and blotting it on absorbent paper **[2]**. Wash the reaction wells with PBS-TBN twice as previously demonstrated **[3]**, then remove the plate from the magnet **[4]**.

2.9.1. Talent inverting the plate to discard the detection reagent mix. Videographer NOTE: Use previous shot

2.9.2. *Use 2.5.3 Talent blotting the plate on absorbent paper*

2.9.3. *Use 2.6.2. Talent adding PBS-TBN to the wells*

2.9.4. Talent removing the plate from the magnet. Videographer NOTE: Use previous shot

2.10. Add 100 microliters of PBS-TBN to each well **[1]**, then cover the plate with foil seal and shake for 2 minutes at 37 degrees Celsius **[2]**. Remove the foil seal **[3]** and proceed to reading 50 microliters of the sample from each well in the flow analyzer **[4]**.

2.10.1. Talent adding 100 µL PBS-TBN to the wells.

2.10.2. Plate on the shaker. *Videographer: Obtain multiple usable takes because this will be reused in 3.2.1.*

2.10.3. Talent removing foil seal.

2.10.4. Talent at the flow analyzer computer, opening software for plate analysis, monitor visible in frame.

2.11. To read the plate, select the drop-down menu in the upper left corner, navigate to **Plate Configuration**, and select **Run Plate** **[1]**.

2.11.1. SCREEN: 20210415_1649_05. 0:00-0:07. *Video Editor: Emphasize the Run Plate icon being clicked at 0:05*

2.12. Eject the plate carrier by selecting the **Eject** icon **[1]**, then load the plate onto the plate carrier **[2]** and select the **Retract** icon to retract the plate carrier **[3]**. Once the plate carrier has retracted into the analyzer **[4]**, select the **Run** icon to begin reading the plate **[5]**.

2.12.1. SCREEN: 20210415_1649_05. 0:07-0:10. *Video Editor: Emphasize the Eject icon (triangle with line at the bottom) being clicked at 0:08. The Eject and Play icons get grayed out once the button is clicked and the tray starts ejecting.*

2.12.2. Talent loading the plate onto the plate carrier. *Obtain multiple usable takes because this will be reused in 3.3.4*

2.12.3. SCREEN: 20210415_1649_05. 0:14-0:17. *Video Editor: Emphasize the Retract icon (triangle with line above) being clicked at 0:15. The Retract and Play icons get grayed out while the tray is retracting.*

2.12.4. SCREEN: 20210415_1649_05. 0:17-0:20. Videographer NOTE: 2.12.4 and 2.12.5 are shot together. Mislabeled as 2.12.3

2.12.5. SCREEN: 20210415_1649_05. 0:20-0:40, then skip to 15:30. *Video Editor: Emphasize the Run icon being pressed between 0:20-0:21, speed up footage after the Run icon is pressed up until 0:40*

3. Dual Reporter Neutralization Assay

3.1. For the dual reporter neutralization assay, add 50 microliters of the multiplex bead mixes to assigned wells of a 96-well non-binding microtiter plate **[1]**, then add 25 microliters of 2 micrograms per milliliter ACE2 *(ace-two)* to each well **[2-TXT]** and cover the plate with a foil seal **[3]**.

3.1.1. WIDE: Talent adding multiplex bead mix to the wells.

3.1.2. Talent adding ACE-2 to the wells. **TEXT: ACE2: Angiotensin-Converting Enzyme-2**

3.1.3. Talent covering the plate with foil.

3.2. Following a 2-minute incubation on a heated plate shaker **[1]**, add 25 microliters of 1 to 500 serum dilutions to the assigned wells **[2-TXT]**.

3.2.1. *Use 2.10.2. Plate on the shaker.*

3.2.2. Talent adding diluted serum to the wells. **TEXT: Add PBS-TBN to the control wells**

3.3. Once all samples have been added, perform the incubation **[1]**, wash **[2]**, detection **[3]**, and analysis steps as previously demonstrated for the serological assay **[4]**.

3.3.1. *Use 2.4.2. Talent placing the plate on a shaker*

3.3.2. *Use 2.6.2. Talent adding PBS-TBN to the wells*

3.3.3. *Use 2.8.2. Talent adding Detection Reagent mix to the plate*

3.3.4. *Use 2.12.2. Talent loading the plate onto the plate carrier*

Results

4. Results: Optimization of the Dual Reporter IgG and IgM Serological Assay and Neutralization Assay

4.1. A test of anti-IgM *(eye-G-M)* conjugated to the DyLight 405 *(die-light-four-oh-five)* reporter dye **[1]** using serum samples within 5 to 60 days from symptom onset **[2]** and an IgG *(eye-G-G)*-stripped serum sample **[3]** did not produce a high signal **[4]**.

4.1.1. LAB MEDIA: Figure 7A, 7B, 7C

4.1.2. LAB MEDIA: Figure 7A. *Video Editor: Emphasize the 11 serum samples in the legend.*

4.1.3. LAB MEDIA: Figure 7A. *Video Editor: Emphasize the stripped serum sample in the legend.*

4.1.4. LAB MEDIA: Figure 7A, 7B, 7C

4.2. For samples with a high IgM median fluorescence intensity **[1]**, the highest signals were seen for the receptor binding domain **[2]** and nucleocapsid antigens **[3]**. While the IgM titers should have been elevated in some samples, the observed median fluorescence intensity levels did not exceed 140 MFI *(M-F-I)* units **[4].**

4.2.1. LAB MEDIA: Figure 7B, 7C.

4.2.2. LAB MEDIA: Figure 7B, 7C. *Video Editor: Emphasize the title (RBD IgM RP2 signal) and brown and yellow bars in 7B.*

4.2.3. LAB MEDIA: Figure 7B, 7C. *Video Editor: Emphasize the title (Nc IgM RP2 signal) and brown, yellow, and blue bars in 7C*

4.2.4. LAB MEDIA: Figure 7B, 7C. *Video Editor: Emphasize the brown bar reaching 140 MFI for the first dilution in 7B.*

4.3. Furthermore, the control bead for IgM **[1]** lacked a significant dynamic range for median fluorescence intensity when using DyLight 405 conjugated to anti-IgM **[2]** compared to a phycoerythrin-labeled anti-IgM at the same concentration **[3]**.

4.3.1. LAB MEDIA: Figure 7D.

4.3.2. LAB MEDIA: Figure 7D. *Video Editor: Emphasize all blue bars.*

4.3.3. LAB MEDIA: Figure 7D. *Video Editor: Emphasize all orange bars.*

4.4. For IgG detection **[1]**, the brilliant violet conjugate had higher median fluorescence intensity signals **[2]** than the streptavidin conjugate of fluorophore Super Bright 436 **[3]**. However, the signal intensity for the brilliant violet conjugate varied across the ACE-2 titrations **[4].**

4.4.1. LAB MEDIA: Figure 9C, 9E. *Video Editor: Emphasize all blue columns in 9C and 9E*

4.4.2. LAB MEDIA: Figure 9C, 9E. *Video Editor: Emphasize the blue columns in the middle section (Brilliant Violet rows) in 9C and 9E*

4.4.3. LAB MEDIA: Figure 9C, 9E. *Video Editor: Emphasize the orange columns in the upper and lower sections (Biotin Anti-IgG-SA-Superbright rows) in 9C and 9E*

4.4.4. LAB MEDIA: Figure 9C, 9E. *Video Editor: Emphasize the blue columns and ACE2 concentration column in the middle section (Brilliant Violet rows) in 9C and 9E*

4.5. This signal fluctuation by the brilliant violet conjugate across ACE2 concentrations also interfered with determining the percent inhibition by ACE2 across the range of IgG titers **[1]**.

4.5.1. LAB MEDIA: Figure 9D, 9F. *Video Editor: Emphasize the blue columns and ACE2 concentration column in the middle section (Brilliant Violet rows) in 9D and 9F*

4.6. For IgM detection **[1]**, the phycoerythrin anti-IgM displayed higher signals **[2]** than those generated by DyLight 549 *(die-light-five-four-nine)* anti-human IgM **[3]**. While determining ACE2 percent inhibition of IgM binding, there was a slight but insignificant difference between the two IgM detection reagents **[4]**.

4.6.1. LAB MEDIA: Figure 9C, 9E. *Video Editor: Emphasize all orange columns in 9C and 9E*

4.6.2. LAB MEDIA: Figure 9C, 9E. *Video Editor: Emphasize the orange columns in the upper section (PE-anti-IgM rows) in 9C and 9E.*

4.6.3. LAB MEDIA: Figure 9C, 9E. *Video Editor: Emphasize the orange columns in the middle and lower sections (DyLight 549 rows) in 9C and 9E.*

4.6.4. LAB MEDIA: Figure 9D, 9F. *Video Editor: Emphasize the orange columns for PE-anti-IgM rows and DyLight 450 rows differently in 9D and 9F*

Conclusion

5. Conclusion Interview Statements

5.1. **STEPHEN ANGELONI:** One of the most important steps in this procedure is to prepare the multiplex bead mix from the individual bead stocks correctly.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested b-roll: 2.1.2.*

5.2. **STEPHEN ANGELONI:** This procedure could also be used to measure efficacy of vaccines and monitoring immune responses to other pathogens.

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