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Title: Methylation Specific Multiplex Droplet PCR Using Polymer Droplet Generator Device for Hematological Diagnostics

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **Yes**, can you record movies/images using your own microscope camera?

Y

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

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

Protocol Length
Number of Shots: **48**

NOTE: The videographer included voice comments to the editor

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Lidija Malic**: This protocol presents a flexible mdPCR workflow that allows precise differential leukocyte counting based on epigenetic methylation markers. This flexibility may facilitate the translation of mdPCR toward clinical use **[1]**.

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

REQUIRED:

1.2. **Abdelrahman Elmanzalawy**: This method provides results that closely correlate to those obtained using immunofluorescence staining methods. Unlike immunofluorescence staining, however, this method doesn't require fresh blood samples or costly antibodies **[1]**.

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

OPTIONAL:

1.3. **Abdelrahman Elmanzalawy**: In hematological diagnostics, differential leukocyte counts serve as indicators for a spectrum of diseases, including infection, inflammation, anemia, and leukemia, and is being considered as an early prognostic cancer biomarker **[1]**.

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

Introduction of Demonstrator on Camera

1.4. **Lidija Malic**: Demonstrating the procedure with Abdelrahman Elmanzalawy will be Christina Nassif, a Technical Officer from our team at the NRC's Medical Devices Research Centre **[1][2]**.

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

Protocol

2. DNA Extraction

2.1. Begin by thoroughly mixing 20 microliters of Proteinase K and 400 microliters of Lysis-Binding buffer containing magnetic beads with freshly thawed human peripheral blood mononuclear cells in 100 microliters of PBS **[1-TXT]**.

2.1.1. WIDE: Talent mixing tube contents, with proteinase K and buffer containers visible in frame *Videographer: Important step* **TEXT: See text for PBMC preparation details**

2.2. After a 5-minute incubation at room temperature, place the tube on a magnetic rack for 1-2 minutes **[1]** before removing the supernatant **[2]**.

2.2.1. Talent placing tube onto magnet

2.2.2. Talent aspirating supernatant

2.3. With the tube removed from the magnet, resuspend the DNA-bead complex in 600 microliters of wash buffer 1 to remove any non-specifically bound beads **[1]**.

2.3.1. Talent adding wash buffer 1 to tube, with wash buffer container visible in frame

2.4. Place the tube back onto the magnet to allow removal of the supernatant **[1]** before washing the cells in 600 microliters of wash buffer 2 as just demonstrated **[2]**.

2.4.1. Talent aspirating supernatant

2.4.2. Talent adding wash buffer 2 to tube not on magnet, with wash buffer container visible in frame

2.5. After removing the supernatant, allow the tube to air dry on the magnet for 1 minute **[1]** before adding 100 microliters of elution buffer to the tube with thorough mixing **[2]**.

2.5.1. Talent setting timer, with tube on magnet visible in frame

2.5.2. Talent mixing tube contents, with buffer container visible in frame

2.6. Then place the tube on the magnetic rack for 1-2 minutes to separate the magnetic beads from the eluted DNA **[1]** and transfer the purified DNA solution to a new tube **[2-TXT]**.

2.6.1. Talent placing tube onto magnet *Videographer: Important step*

2.6.2. Talent adding DNA to tube *Videographer: Important step* **TEXT: Measure absorbance at 260 nm to assess DNA purity**

3. Bisulfite Conversion

3.1. For bisulfite conversion of the purified DNA, transfer 20 microliters of the eluted DNA sample to a PCR tube **[1-TXT]** and add 130 microliters of conversion reagent to the tube **[2]**.

3.1.1. WIDE: Talent adding DNA to tube **TEXT: *i.e.*, 200-500 ng DNA**

3.1.2. Talent adding conversion reagent to tube, with conversion reagent container visible in frame

3.2. After thorough mixing, briefly spin down the tube contents **[1]** and amplify the DNA on a thermal cycler **[2-TXT]**.

3.2.1. Talent placing tube into centrifuge

3.2.2. Talent placing tube into thermal cycler **TEXT: 98 °C 8 min; 54 °C 60 min, hold at 4 °C**

3.3. At the end of the cycle, add 600 microliters of binding buffer to an ion chromatography column placed in a collection tube **[1]** and add the DNA to the column **[2]**.

3.3.1. Talent adding buffer to column, with buffer container visible in frame

3.3.2. Talent adding sample to column

3.4. Invert the tube several times to mix **[1]** and centrifuge for 30 seconds at full speed **[2]**.

3.4.1. Talent inverting tube

3.4.2. Talent placing tube into centrifuge

3.5. Discard the collected flow-through **[1]** and add 100 microliters of wash buffer to the column **[2]**.

3.5.1. Talent discarding flow through

3.5.2. Talent adding wash buffer to column

3.6. Centrifuge the column and discard the flow-through again as demonstrated **[1]**.

3.6.1. Talent adding tube(s) to centrifuge

3.7. Add 200 microliters of desulfonation buffer to the column for a 15-20-minute incubation at room temperature **[1]** before centrifuging the sample and discarding the flow-through as demonstrated **[2]**.

3.7.1. Talent adding buffer to column, with buffer container visible in frame

3.7.2. Talent adding tube(s) to centrifuge

3.8. Next, wash the column two times with 200 microliters of wash buffer per wash **[1]**.

3.8.1. Talent adding buffer to column, with buffer container visible in frame

3.9. After the second wash, transfer the column to a new 1.5-milliliter collection tube **[1]** and add 100 microliters of PCR-grade water to the membrane of the column **[2]**.

3.9.1. Talent placing column into tube

3.9.2. Talent adding water to membrane

3.10. Then elute the DNA by centrifugation of the column for 1 minute at full speed **[1]**.

3.10.1. Talent placing tube(s) into centrifuge

4. Droplet Generation and PCR

4.1. For droplet generation, mix 1 microliter of bisulfite-converted DNA with freshly prepared probe master mix in a PCR tube **[1-TXT]** and collect the sample with a brief centrifugation **[2]**.

4.1.1. WIDE: Talent mixing tube contents, with DNA and master mix container visible in frame **TEXT: See test for probe master mix preparation details**

4.1.2. Talent placing tube(s) into centrifuge

4.2. Use PEEK (peek) fittings to connect disposable fluidic tubing to two, 250-microliter-volume precision glass syringes **[1-TXT]** and prefill one precision glass syringe with 250 microliters of carrier oil containing 5% fluoro-surfactant **[2]** and one precision glass syringe with 50 microliters of carrier oil **[3]**.

4.2.1. Talent connecting tubing to syringe **TEXT: I.D. 0.25 mm, O.D. 1.6 mm tubing**

4.2.2. Talent filling syringe with carrier oil containing 5% fluoro-surfactant, with carrier oil containing 5% fluoro-surfactant container visible in frame

4.2.3. Talent filling syringe with carrier oil, with carrier oil container visible in frame

4.3. When both of the syringes have been loaded, load 100 microliters of the PCR mix into the syringe of carrier oil **[1]** and place a droplet microfluidic device onto the stage of an upright light microscope equipped with a high-speed camera **[2]**.

4.3.1. Talent adding PCR mix to syringe

4.3.2. Talent placing device onto stage

4.4. For the observation and recording of droplet formation in real-time, place the prefilled syringes onto a programmable syringe pump **[1]** and use PEEK union with fittings to connect the tubing of the syringes to the tubing of the respective inlet channels of the droplet microfluidic device **[2]**.

4.4.1. Talent placing syringe(s) onto pump *Videographer: Important step*

4.4.2. Talent connecting tubing to inlet channel(s) *Videographer: Important step*

4.5. Place the tubing from the outlet of the droplet generator to the inside of a 0.5-milliliter PCR tube **[1]** and adjust the syringe pump flow rate to 2 microliters/minute to allow the droplet size to stabilize before collecting the resulting emulsion **[2]**.

4.5.1. Talent placing tubing into tube

4.5.2. Talent adjust pump flow rate

4.6. Slowly and carefully collect the emulsion from the top of the tube **[1]** and transfer 75 microliters of the solution to a 200-microliter PCR tube for thermal cycling **[2]**.

4.6.1. Talent collecting emulsion *Videographer: Important step*

4.6.2. Talent adding solution to tube *Videographer: Important step*

4.7. Then confirm that the oil content in the PCR tube closely matches the volume of the dispersed phase to prevent coalescence of the droplets during thermal cycling **[1]** and place the 200-microliter tube into the thermal cycler **[2-TXT]**.

4.7.1. Shot of emulsion compared to dispersed phase *Videographer: Important step*

4.7.2. Talent placing tube into thermal cycler *Videographer: Important step* **TEXT: 95 °C 5 min; 45 cycles 95 °C 15 s; annealing/extension 60 °C 30 s**

5. Fluorescence Imaging

5.1. For fluorescence imaging of the emulsified sample, transfer the PCR emulsion into a 50-micrometer-deep borosilicate capillary tube **[1]** with a rectangular profile to allow arrangement of the droplets into a close-packed monolayer for imaging **[2]**.

5.1.1. WIDE: Talent loading sample into tube *Videographer: Important step*

5.1.2. Shot of loaded tube *Videographer: Important step*

5.2. After fixing and sealing the capillaries onto a microscope slide **[1]**, load the slide onto the stage of the inverted microscope **[2]** and, in the microscope imaging software, select **Acquire** and **Live – Fast** to start real-time camera acquisition **[3]**.

5.2.1. Shot of capillaries fixed and sealed onto slide

5.2.2. Talent placing slide onto microscope stage

5.2.3. Talent selecting acquire and live-fast, with monitor visible in frame

5.3. Then observe the sample under brightfield and fluorescence microscopy **[1]**.

5.3.1. LAB MEDIA: Figure 3

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

2.1., 2.6., 4.4., 4.6., 4.7., 5.1.

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

4.6., 4.7. Emulsion should be slowly and carefully collected from the top to prevent droplet coalescence during pipetting and transfer steps and ensure that the oil content in the PCR tube closely matches the volume of the dispersed phase, which aids in retaining emulsion stability during thermal cycling.

Results

6. Results: Representative Multiplex Droplet Identification and Characterization

6.1. Following droplet generation and thermal cycling, the droplets can be introduced into a glass capillary with a 1-millimeter width and a 50-micrometer height **[1]** to obtain a monolayer distribution of the droplets that is ideal for fluorescence image acquisition **[2]**.

6.1.1. LAB MEDIA: Figure 3
6.1.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize Figure 3A*

6.2. Images can then be recorded for each wavelength **[1]**.

6.2.1. LAB MEDIA: Figure 3 *Video Editor: please sequentially emphasize Figures 3B, 3C, and 3D*

6.3. After image analysis, the fluorescence intensity of all of the droplets in their respective fluorophores **[1]** can be plotted to establish the threshold **[2]** of the positive and negative droplets **[3]**.

6.3.1. LAB MEDIA: Figure 5 *Video Editor: please sequentially add/emphasize Figure 5A, 5B, and 5C*
6.3.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize pink, yellow, and green droplets*
6.3.3. LAB MEDIA: Figure 5 *Video Editor: please emphasize black droplets*

6.4. After identification and counting, the copies per droplet values can be calculated **[1]** and the percentage of CD3 (C-D-three)-positive T cells **[2]** and CD4-positive 25-positive regulatory T cells **[3]** can be determined based on the methylated CD3Z **[4]** and FoxP3 (fox-P-three) copies, respectively **[5]**, with respect to the copies per droplet of the total cells, or C-LESS (C-less), gene **[6]**.

6.4.1. LAB MEDIA: Figure 6
6.4.2. LAB MEDIA: Figure 6 *Video Editor: please emphasize black CD3+ T cells data bar*
6.4.3. LAB MEDIA: Figure 6 *Video Editor: please emphasize black CD4+ CD25+ T reg data bar*
6.4.4. LAB MEDIA: Figure 6 *Video Editor: please emphasize CD3Z data bar*
6.4.5. LAB MEDIA: Figure 6 *Video Editor: please emphasize Foxp3 data bar*
6.4.6. LAB MEDIA: Figure 6 *Video Editor: please emphasize C-LESS data bar*

6.5. The percent values can then be compared to those obtained through immunofluorescence imaging using the appropriate antibodies **[1]**.

6.5.1. LAB MEDIA: Figure 6 *Video Editor: please emphasize grey data bars in Figure 3B*

Conclusion

7. Conclusion Interview Statements

7.1. **Abdelrahman Elmazalawy**: Retaining the emulsion stability from the droplet generation through the thermal cycling and final droplet imaging steps is crucial for obtaining precise, reliable, and reproducible quantification results **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (4.6., 4.7., 5.1.)

7.2. **Lidija Malic**: mdPCR customization through tailored PCR mix formulation can be used for a number of applications, including cancer research, infectious disease diagnosis, and analytics at the single cell level **[1]**.

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