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

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TITLE:

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

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SUMMARY:

Epigenetic markers are used for white blood cell (WBC) subtyping through the quantification of DNA methylation patterns. This protocol presents a multiplex droplet polymerase chain reaction (mdPCR) method using a thermoplastic elastomer (TPE)-based microfluidic device for droplet generation allowing for precise and multiplex methylation-specific target quantification of WBC differential counts.

ABSTRACT:

A multiplexed droplet PCR (mdPCR) workflow and detailed protocol for determining epigenetic-based white blood cell (WBC) differential count is described, along with a thermoplastic elastomer (TPE) microfluidic droplet generation device. Epigenetic markers are used for white blood cell (WBC) subtyping which is of important prognostic value in different diseases. This is achieved through the quantification of DNA methylation patterns of specific CG-rich regions in the genome (CpG loci). In this paper, bisulfite-treated DNA from peripheral blood mononuclear cells (PBMCs) is encapsulated in droplets with mdPCR reagents including primers and hydrolysis

fluorescent probes specific for CpG loci that correlate with WBC sub-populations. The multiplex approach allows for the interrogation of many CpG loci without the need for separate mdPCR reactions, enabling more accurate parametric determination of WBC sub-populations using epigenetic analysis of methylation sites. This precise quantification can be extended to different applications and highlights the benefits for clinical diagnosis and subsequent prognosis.

INTRODUCTION:

Analysis of white blood cells (WBCs) composition is among the most frequently requested laboratory tests in hematological diagnostics. Differential leukocyte count serves as an indicator for a spectrum of diseases including infection, inflammation, anemia, and leukemia, and is under investigation as an early prognostic biomarker for several other conditions as well. Gold standard in WBC subtyping involves immunostaining and/or flow cytometry both of which require costly, stability-prone fluorescent antibodies and are often highly dependent on operator proficiency in sample preparation. Moreover, this method is applicable to fresh blood samples only, such that the samples cannot be frozen for shipment or later analysis.

Epigenetic markers have recently emerged as powerful analytical tools for the study of phenotypic variations. Subsequently, human leukocyte populations have been shown to have cell-lineage DNA methylation patterns that allow for the precise characterization of WBC subsets. Subtyping based on epigenetic markers provides a promising alternative that does not depend on fresh blood sample collection or expensive antibodies and can be exploited as a biomarker for disease onset and susceptibility[1–5].

Genome-wide studies have been performed for extensive mapping of methylated specific CG-rich regions in the genome (CpG islands) in leukocyte subtypes to identify candidate epigenetic markers specific to leukocyte subtypes. PCR protocols have been developed because of this reason for methylated gene regions e.g., CD3Z and FOXP3, corresponding to $CD3^+$ T-Cells and $CD4^+$ $CD25^+$ Regulatory T-Cells (T-Regs), respectively. Wiencke et al. have demonstrated the utility of duplex droplet PCR for epigenetic subtyping of T-Cells, yielding results that highly correlate with flow activated cell sorting (FACS) analysis[6]. This quantitative genetic analysis method relies on partitioning the template nucleic acid molecules and PCR reagents into thousands of discrete, volumetrically defined, sub-nanoliter sized droplets containing zero, one or more target nucleic acid copies, using water-in-oil emulsions enabled by microfluidics[7,8]. The PCR amplification is performed within each individual droplet and the endpoint fluorescence intensity of each droplet is measured, allowing absolute quantification of targets present in the sample. Droplet PCR has been established to be more precise, accurate, and technically simpler than standard qPCR, making it a more favorable DNA methylation-based method for clinical evaluation of T-Cells. Although a rapidly emerging subtyping methodology, multiplexed epigenetic analysis to probe for various methylated CpG regions simultaneously is lacking. This is necessary for routine leukocyte differential counts.

Herein, a thermoplastic elastomer (TPE) droplet microfluidic device is presented and employed for methylation-specific multiplex droplet PCR (mdPCR). The technology has been used to delineate specific leukocyte subtypes, $CD3^+$ T-Cells and $CD4^+$ $CD25^+$ T-Regs, based on cell-lineage

DNA methylation patterns, i.e., epigenetic variation of CD3Z and FOXP3 CpG regions, respectively. A detailed protocol for DNA extraction, bisulfite conversion and mdPCR is described in concert with a fabrication method for a TPE droplet generation device. Representative results of the method are compared to those of immunofluorescence staining highlighting the utility of the proposed approach.

PROTOCOL:

All the experiments performed in this study involving human samples were approved by the NRC's Ethics Board and were done according to NRC's policies governing human subjects that follow applicable research guidelines and are compliant with the laws in Québec, Canada.

1. Cell preparation

1.1. Thaw the frozen human peripheral blood mononuclear cells (PBMCs) immediately by placing the cryovial in a water bath at 37 °C for 5 min.

1.2. Invert the cryovial twice to gently resuspend the cells and using a 1 mL pipette transfer the cell suspension into a 15 mL conical tube.

1.3. Add 10 mL of pre-warmed (37 °C) growth medium supplemented with 10% fetal bovine serum (RPMI-1640 + 10% FBS) to the 15 mL tube containing the PBMCs.

1.4. Centrifuge the cell suspension in a swinging bucket centrifuge at room temperature at a speed of 330 *x g* for 10 min with rapid acceleration and the brake on high.

1.5. Once the spin is over, carefully decant the supernatant. Resuspend the cell pellet in 3 mL of phosphate-buffered saline (PBS) pH 7.2, containing 2 mM ethylenediaminetetraacetic acid (EDTA) by tapping the side of the tubes.

1.6. Mix the cells by inverting the tube with the cap tightly closed.

1.7. Prepare two 1.5 mL of microtubes and using a pipette aliquot 1.5 mL of the cell suspension in each tube, one of which is used for subsequent immunofluorescence staining and one for the DNA extraction.

2. Immunofluorescence staining and imaging protocol

2.1. Resuspend cells (from 1.7) in 200 µL of PBS buffer containing 0.1% sodium azide and 2% FBS and adjust the final concentration of the cell suspension to a maximum of 2 x 10^7 cells/mL.

2.2. Divide the cell suspension by pipetting 100 µL volume in two separate 1.5 mL microtubes.

2.3. Add 20 µL volume of anti-Hu CD3/CD4 conjugated with Fluorescin isothiocyanate (FITC) and Phycoerythrin (PE) to one tube and anti-Hu CD4/CD25 conjugated with FITC and PE (see **Table of Materials**) to the second tube, respectively.

2.4. Add 1 drop of blue fluorescent live cell stain (see **Table of Materials**) to each tube.

2.5. Incubate at room temperature using a tube rotator for 2 h. Protect from light.

2.6. Centrifuge the cell suspension at room temperature at 330 *x g* for 10 min with rapid acceleration and the brake on high.

2.7. Decant the supernatant and carefully resuspend the cell pellet by tapping the tube. Add 1 mL of PBS, pH 7.2 containing 2 mM EDTA. Ensuring that the cap is tightly closed, mix the cells by inverting the tube 2x.

2.8. Repeat steps 2.6 and 2.7 for three times.

2.9. Resuspend cells in 20 µL of PBS pH 7.2 containing 2 mM EDTA.

2.10. Pipette 10 µL drop of the cell suspension onto a borosilicate microscope slide and wait for 2 min for cells to slowly sediment to the bottom of the drop.

2.11. Carefully place a glass cover slip on top of the microscope slide and place the slide on the stage of an inverted microscope.

2.12. Record images of the cells using a 10x objective and an EMCCD camera connected to the microscope for each of the fluorophores for both cell suspension samples.

2.13. Manually count fluorescently labeled cells (see **Supplementary Information** for raw data).

2.14. Take the ratio of anti-Hu CD3 and anti-Hu CD4/CD25 labeled-cells to DAPI-stained cells to obtain the proportions of $CD3^+$ T-Cells and $CD4^+$ $CD25^+$ T-Regs to total leukocyte.

3. DNA extraction and bisulfite conversion

3.1. DNA extraction

NOTE: Extract DNA from PBMCs prepared in section 1 using a magnetic DNA purification kit (see **Table of Materials**) following procedures provided by the manufacturer.

3.1.1. In a 1.5 mL tube suspend cells in 100 µL of PBS and add 20 µL of Proteinase K and 400 µL of Lysis/Binding buffer. Mix by pipetting up-down 10x, then perform incubation at room temperature for 5 min.

3.1.2. Capture the DNA-bead complex by placing the tube on a magnetic rack for 1-2 min, then carefully remove and discard the supernatant.

3.1.3. Remove the tube containing the DNA-bead complex from magnetic rack and resuspend the beads in 600 µL of Wash Buffer #1 to wash away any non-specific binding.

3.1.4. Place the tube again on the magnetic rack and carefully remove and discard the supernatant.

3.1.5. Repeat steps 3.1.3 and 3.1.4 with 600 µL of Wash Buffer #2.

3.1.6. Leave the tube open to air-dry for 1 min.

3.1.7. Remove the tube from the magnetic rack and elute the DNA by dispensing 100 µL of Elution Buffer and pipetting the DNA/bead complex up and down 20x.

3.1.8. Place the tube containing the eluted DNA again on the magnetic rack and incubate for 1-2 min to separate the magnetic beads from the eluted DNA.

3.1.9. Transfer the eluted purified DNA solution to a new clean tube.

3.1.10. Assess the concentration of the purified DNA sample by measuring the absorbance at 260 nm using a spectrophotometer.

3.2. Bisulfite conversion

NOTE: Perform the bisulfite conversion on purified DNA using a methylation kit (see **Table of Materials**) following procedures provided by the manufacturer.

3.2.1. To 20 µL of DNA sample (200-500 ng) in a PCR tube add 130 µL of Conversion Reagent. Mix well and spin down briefly.

3.2.2. Transfer the PCR tube to a thermal cycler and perform the cycling protocol as follows: 98 °C for 8 min; 54 °C for 60 min and hold at 4 °C.

3.2.3. Add 600 µL of the Binding buffer to an ion chromatography (IC) column placed into a collection tube.

3.2.4. Add the DNA sample to the IC column containing the binding buffer and mix by inverting the tube several times. Centrifuge for 30 s at full speed. Discard the collected flow-through.

3.2.5. To the column now add 100 µL of Wash Buffer. Perform centrifugation as described in step 3.2.4 and discard the flow-through.

3.2.6. Add 200 µl of Desulfonation Buffer and perform incubation at room temperature for 15-20 min. Centrifuge and discard the flow-through as described in steps above.

3.2.7. Add 200 µL of Wash Buffer to the column. Centrifuge at full speed for 30 s and discard the flow-through.

3.2.8. Repeat step 3.2.7.

3.2.9. Transfer the column to a new 1.5 mL collection tube and add 100 µL of PCR-grade water on the membrane of the column. Centrifuge at full speed for 1 min to elute the DNA.

3.2.10. Assess the concentration of bisulfite-converted DNA sample by measuring the absorbance at 260 nm using a spectrophotometer.

NOTE: Use a value of 40 µg/mL for absorbance at 260 nm = 1.0.

3.2.11. Store DNA at -20 °C for short term storage or at -70 °C for long term storage.

4. Droplet generation device fabrication

NOTE: A microfluidic device used for droplet generation (CAD file provided in the **Supplementary Information**) was fabricated in a clean room (class 1,000) environment in thermoplastic elastomer (see **Table of Materials**) using hot embossing generated by the following protocol**.**

4.1. SU-8 mold fabrication

4.1.1. Prepare an SU-8 mold on a 6" silicon wafer using standard photolithography as detailed below.

4.1.2. Clean a 6" silicon wafer using oxygen plasma at 500 W for 10 s.

4.1.3. Spin-coat SU-8 resist onto the silicon wafer at 900 rpm for 40 s to achieve a total film thickness of 100 µm.

4.1.4. Place the wafer on a hot plate and pre-bake for 15 min at 65 °C, followed by 2 h at 95 °C.

4.1.5. Expose to UV light at 365 nm (Hg i-line) through a high-definition transparency photomask using an exposure dose of 1,000 mJ/cm^2.

4.1.6. Place the wafer on a hot plate and post-bake for 15 min at 65 °C, followed by 40 min at 95 °C.

4.1.7. Develop by immersing in propylene glycol monomethyl ether acetate (PGMEA) for 5 min.

4.1.8. Rinse with PGMEA and isopropanol and dry with a stream of nitrogen gas.

4.1.9. Place the wafer on a hot plate and hard-bake for 2 h at 135 °C.

4.1.10. Silanize the wafer in the vacuum desiccator containing a drop of silanizing agent (tricholoro perfluoroctyl silane) placed on an adjacent glass microscope slide for 2 h.

NOTE: This is done to make the silanes form a monolayer on the surface of the SU-8 master. Tricholoro perfluoroctyl silane should be always handled in the fume hood and kept away from water sources.

4.2. Polydimethylsiloxane (PDMS) replica

4.2.1. Prepare liquid prepolymers of PDMS (see **Table of Materials**) at a 10:1 ratio w/w of elastomer base to curing agent in a plastic cup.

4.2.2. Place the cup in a planetary centrifugal vacuum mixer to mix and degas the PDMS mixture.

4.2.3. Pour PDMS mixture onto the mold placed in the custom metal holder that prevents resin leakage and cure at 65 °C for 2 h.

4.2.4. Using tweezers, carefully peel off the PDMS mold from the SU-8 master.

4.3. Epoxy mold

NOTE: An epoxy mold was fabricated from the SU-8/silicon master using an intermediate replication process with PDMS.

4.3.1. Prepare the epoxy resin (see **Table of Materials**) using a 100/83 w/w ratio of resin/hardener.

4.3.2. Degas the mixture under reduced pressure using a vacuum drying oven for 30 min.

4.3.3. Pour the resin over the PDMS replica and cure at 80 °C for 12 h.

4.3.4. Remove the cured epoxy mold from the PDMS replica, place on a hot plate and hard-bake for 2 h at 120 °C.

4.4. TPE device

4.4.1. Extrude pellets of TPE (see **Table of Materials**) at 165 °C in 2.0 mm thick and 7” wide sheets of several meters in length and store them as a roll for future use.

4.4.2. Cut the TPE sheet from the roll using scissors into a 7” square.

4.4.3. Place the TPE sheet between the epoxy mold and a non-patterned silanized silicon wafer (see step 4.1.10 for wafer silanization procedure).

4.4.4. Perform hot-embossing at a temperature of 125 °C, an applied force of 10 kN, and a pressure of 10^{-2} mbar for 10 min.

4.4.5. Demold carefully at room temperature using methanol spray to separate the embossed TPE from the silicon wafer and the epoxy mold.

4.4.6. Cut another 7" square sheet of TPE and place it between two non-patterned silanized silicon wafers.

4.4.7. Perform hot-embossing at a temperature of 140 °C, an applied force of 10 kN, and a pressure of 10^{-2} mbar for 10 min to form a planar surface for closing the channels and sealing the device.

4.4.8. Demold carefully at room temperature using methanol spray to separate the embossed TPE from the two silicon wafers.

4.4.9. Cut each of the embossed TPE sheets to device size using a doctor blade.

4.4.10. Punch the access holes for the inlet and outlet channels in the structured device using a 1 mm biopsy punch needle with a plunger.

4.4.11. Enclose the channels by placing a planar TPE device in direct contact with the channels at room temperature.

4.4.12. Optionally, place in an oven at 70 °C for 2 h to promote device bonding.

4.4.13. Fit the access holes with a disposable fluidic tubing (I.D. 0.25 mm, O.D. 0.8 mm). Seal the joints using an epoxy glue to ensure leak-proof manipulation.

5. Droplet generation and PCR

NOTE: **Table 1** outlines information on the forward and reverse primers along with the double-quenched hydrolysis probes for C-LESS, CD3Z and Foxp3 genes, which are required for the multiplex amplification of demethylated gene targets.

5.1. Prepare the master mix as described in **Table 2**.

5.2. Thaw all components of the master mix except for the enzyme mix. Mix the master mix thoroughly by pipetting up-down and spin down briefly.

5.3. Add the appropriate volume (1 µL) of bisulfite converted DNA (from section 3.2) to master mix in a PCR tube. Mix the reaction by pipetting up-down and spin down briefly.

5.4. Connect disposable fluidic tubing (I.D. 0.25 mm, O.D. 1.6 mm) to two precision glass syringes (250 µL volume) using PEEK fittings.

5.5. Prefill one precision glass syringe with 250 µL of carrier oil containing 5% fluoro-surfactant.

5.6. Prefill another precision glass syringe with 50 µL of carrier oil before loading 100 µL of the PCR mix to ensure dispensing of the entire sample volume during emulsification.

5.7. Set-up a droplet microfluidic device on a stage of an upright light microscope equipped with a high-speed camera to observe and record droplet formation in real-time.

5.8. Place the prefilled syringes onto the programmable syringe pump and using PEEK union with fittings (see **Table of Materials**), connect the tubing of the syringes to the tubing of the respective inlet channels of the droplet microfluidic device.

5.9. Place the tubing from the outlet of the droplet generator inside a 0.5 mL PCR tube.

5.10. Adjust the flow rate of the syringe pump to 2 µL/min and allow for the droplet size to stabilize before collecting the resulting emulsion.

5.11. Collect the emulsion and transfer 75 µL to a 0.2 mL PCR tube for thermal cycling.

5.12. Ensure that the oil content in the PCR tube closely matches the volume of the dispersed phase in order to prevent coalescence of the droplets during thermal cycling.

5.13. Place the 0.2 mL PCR tube in thermal cycler and perform the cycling protocol as follows: preheating at 95 °C for 5 min, then 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s.

5.14. Use the remaining emulsion to fill a borosilicate capillary tube (100 µm depth) with a rectangular profile in order to image droplets and assess the droplet diameter.

5.15. Place the borosilicate tube filled with the emulsion onto a microscope slide. Use an inverted microscope equipped with an EMCCD camera and 10x objective to record bright field images of the droplets.

5.16. Measure the droplet diameter using an image analysis software as detailed below.

5.17. Set the scale of known distance in microns corresponding to the number of pixels in the image by selecting the '**Analyze**' button and then '**Set Scale**'.

5.18. Convert the image to grayscale by selecting the '**Image**' button and then '**Type**'. Adjust brightness and contrast if needed. Set a manual threshold to delineate and fill the circles by selecting the '**Image**' button and then '**Adjust Threshold**'.

5.19. Analyze the particles by selecting the '**Analyze particles**' button and setting the circularity to 0.75 – 1.

5.20. Obtain the resulting area and the diameter of the measured droplets which is automatically displayed in the software.

5.21. Compute the mean droplet diameter and assuming a spherical droplet, estimate the partition volume, which will be used to calculate absolute target concentration.

5.22. Ensure that the droplets are monodisperse by analyzing the coefficient of variation (CV) which is taken as the ratio of the standard deviations to the mean values (k = 1), for the droplet diameter (< 3%).

6. Fluorescence imaging and image analysis

6.1. Fluorescence imaging

6.1.1. After amplification, transfer the PCR emulsion into borosilicate capillary tube (50 µm depth) with a rectangular profile in order to arrange droplets into a close-packed monolayer for imaging.

6.1.2. Fix the filled capillaries on a microscope slide and seal both sides using an UV acrylic adhesive. Apply a UV light source on the UV adhesive being careful not to illuminate the emulsion to avoid bleaching the sample.

6.1.3. Load the glass slide on an inverted microscope equipped with an EMCCD camera and a 10x objective.

6.1.4. Using microscope imaging software, select **Acquire | Live - Fast** to start real-time camera acquisition, observe the sample, and ensure that the width of the capillary is captured.

6.1.5. Set the bright field lamp for diascopic illumination at 3.5 V.

6.1.6. Set the broad-spectrum LED fluorescent lamp for episcopic illumination at 20% intensity.

6.1.7. Adjust the capture setting for all wavelengths (Bright field, FAM, HEX and Cy5) manually by selecting the **Calibration | Optical Configurations** command in the imaging software. For each fluorophore, the corresponding florescent filter cube needs to be manually switched. A filter cube for the bright field imaging must be used to keep the same optical path.

6.1.8. Adjust the exposure time manually prior to image acquisition using **Acquire | Camera Settings** command for each fluorophore as summarized in **Table 3**. Set the readout mode EM gain 17 MHz at 16-bit with a gain multiplier of 100 in the '**Capture Settings**' menu of the software.

6.1.9. In the LUT window of the software, set the LUTs scale such that the transmission signal is encompassed within the set range. In this experiment, the scale was set from 500 up to 12,000 approximately.

6.1.10. Automate the capture using a multipoint acquisition program using both XY scan and multiple wavelength options in that specific order. Ensure that the stage move to a position, capture all the different wavelengths, and then proceed to the next position.

6.1.11. First, set-up the XY scan by customizing the XY scan profile in the XY scan menu of the software. In '**Custom Multipoint Definition**', choose the large image definition box and set it to 40.0 x 1.0 mm approximately (the length of the emulsion filling). Use 1% overlap.

6.1.12. Enable the '**Use Focus Surface**' option and set up the focus surface curve by adjusting the focus plane at different points on the sample using the focus knob on the microscope.

6.1.13. Second, setup the multiple wavelength scan by selecting the scan tab. Add each of the optical configurations created in step 6.1.7 for each wavelength.

6.1.14. Click the option to close active shutter during stage movement and during filter change to avoid bleaching the sample and run the acquisition by pressing the '**Start Run**' button. Export each acquisition frame into tiff files and split each channel into a separated file using split multiple files option and by applying saved LUTs settings. Use point name and channel name option to easily differentiate each image file.

6.2. Image analysis

6.2.1. Sort all acquired images by brightfield and fluorescence filters to upload to an open source image analysis software.

6.2.2. Use image analysis software to create a pipeline in order to identify all droplets using brightfield images, then measure intensity of associated fluorescent droplets.

6.2.3. To pipeline, upload brightfield and fluorescent tiff images then add modules '**ColorToGray**', '**IdentifyPrimaryObjects**', '**MeasureObjectIntensity**', and '**ExportToSpreadsheet**'. Use brightfield droplet images to identify objects, then use objects as mask to measure intensity of fluorescent images.

6.2.4. Run pipeline with selected tiff images to extract average fluorescence intensity of fluorescent droplet images. Conduct the experiment in triplicate, with each set consisting of ~5,000 droplets for analysis.

6.2.5. Apply the '**definetherain**' algorithm (http://definetherain.org.uk/) to identify the positive and negative droplet clusters. The positives should be within 3 standard deviations of the mean. This determines the threshold intensity of positive droplets to be used for counting.

6.2.6. Again use the image analysis software to implement a new pipeline where the fluorescence threshold for each gene target is set as defined in the previous step.

6.2.7. Upload brightfield and fluorescent images to pipeline. Add modules '**ColorToGray**', '**RescaleIntensity**', '**Threshold**', '**IdentifyPrimaryObjects**', '**MeasureObjectSizeShape**', '**FilterObjects**', and '**ExportToSpreadsheet**'. Create unique modules for brightfield images and each fluorescent filter for droplets.

6.2.8. Rescale image intensity scale from 0 to 1 for each fluorescent image group. Then set the threshold to identify and count the objects above the established threshold. If necessary, add '**ExpandOrShrinkObjects**' module to shrink objects to facilitate the counting and identification of droplets in brightfield.

6.2.9. Only identify objects within selected size of 20-30 pixels and filter the counted objects to only retain those objects with a specific diameter (i.e., droplets in the 75 µm diameter range) and a round spherical eccentricity of 0.5 and below.

6.2.10. Export the results to a table that lists the total droplet count from brightfield images, as well as droplet counts of all the fluorescent channels used; namely Cy5 for C-LESS gene, HEX for methylated CD3Z gene, and FAM for methylated FOXP3 gene (see **Supplementary Information** for raw experimental data obtained for the presented mdPCR assay).

6.2.11. Calculate the ratio of negative droplets for each gene target and apply Poisson distribution to obtain the respective copies per droplet (CPD) using Equation 1

$$P(X = x) = (\lambda\text{^}x\ e\text{^}(-\lambda))/x,$$

where x represents the number of droplets containing 0, 1, 2 or more molecules, and λ represents the CPD value.

6.2.12. Calculate, the absolute target concentration by taking the ratio of the CPD value and the droplet volume obtained in step 5.20 with Equation 2.

$$\lambda = CPD = -ln(1 - p),$$

where the value (1-p) represents the fraction of negative droplets.

6.2.13. Calculate the percentage of $CD3^+$ T-Cells and $CD4^+$ $CD25^+$ T-Regs by dividing the respective CPD values of methylated CD3Z and FOXP3 genes by the C-LESS – or total cell – CPD value (see **Supplementary Information** for CPD calculations from raw data).

6.2.14. Compare these percent values to those obtained from immunofluorescence imaging using antibodies for $CD3^+$ T-Cell and $CD4^+$ $CD25^+$ T-Reg counting.

REPRESENTATIVE RESULTS:

The TPE-based microfluidic droplet generator device was fabricated using the described protocol as shown in **Figure 1**. A transparency mask was used in photolithography to obtain silicon (Si) master. Soft lithography was performed to obtain an inverse PDMS replica of the Si master which was then used to fabricate the epoxy mold. Epoxy precursor was poured onto the PDMS and cured to crosslink and harden. This mold, representing the exact replica of the Si master was more resilient for subsequent thermoforming of thermoplastics using hot embossing. Once the epoxy mold was obtained, thermoplastic elastomer was embossed. Following the embossing, the TPE was demolded, and devices were cut. A flat TPE substrate was used to seal the device. Holes were punched in the top cover, and the two substrates were bonded. Finally, the necessary tubing for world-to-chip connections were inserted and the device was ready for use. Sample images of fabricated master mold, TPE material and embossed devices are shown in **Figure 2**. Assembled device with tubing interconnects was operated with a pair of independent programmable syringe pumps to generate emulsions for mdPCR. Methylation-specific mdPCR was performed using bisulfite treated DNA extracted from frozen PBMCs. Appropriate primers and hydrolysis probes for CD3Z, FOXP3, and C-Less genes were added to the PCR mix for emulsification. Following droplet generation of appropriate size (approximately 72 µm diameter), the emulsion was subjected to a thermal cycling protocol. Finally, the droplets were introduced into a glass capillary having 1 mm width and 50 µm height for imaging. This results in a monolayer distribution of droplets ideal for fluorescence image acquisition (**Figure 3**). Images were recorded for each of the four wavelengths. An image analysis was then performed to identify all droplets (**Figure 4**) meeting the threshold criteria established through 'definetherain' algorithm. The fluorescence intensity of all droplets in their respective fluorophore is then plotted and the threshold of positive and negative droplets was established (**Figure 5**). Subsequently, identification and counting of these droplets were performed which were above the selected threshold. The CPD values were then calculated and the percentage of $CD3^+$ T-Cell and $CD4^+$ 25^+ T-Regs was established based on methylated CD3Z and FOXP3 copies, respectively, with respect to the CPD of total cells, or C-LESS gene (**Figure 6**). The percent values were then compared to those obtained through immunofluorescence imaging of T-Cell and T-Reg populations using appropriate antibodies.

FIGURE AND TABLE LEGENDS:

Table 1: Primer and hydrolysis probe design

Table 2: Master mix recipe for mdPCR

Table 3: Settings for optical configuration and image acquisition

Figure 1: Rapid prototyping of the droplet generator. Schematic illustration of the process used for the fabrication of (**A**) master mold and (**B**) TPE-based microfluidic emulsification device.

Figure 2: Components involved in the fabrication process. (**A**) Silicon master, (**B**) PDMS replica, (**C**) epoxy mold, (**D**) TPE material extruded into sheets and packaged in rolls, (**E**) embossed TPE and (**D**) assembled device.

Figure 3: Fluorescence image acquisition of droplets following thermal cycling. (**A**) Brightfield image of the droplets in a capillary that allowed monolayer image acquisition. (**B**) Cy5 filter image of C-LESS gene targets representing total cell count. (**C**) HEX filter image of methylated CD3Z gene target correlated to $CD3^+$ T-Cell count. (**D**) FAM filter image of methylated FOXP3 gene target correlated to $CD4^+$ $CD25^+$ T-Reg count. Scale bar is 100 µm.

Figure 4: Pipeline used for identifying droplets that meet threshold intensity. Images were first organized into appropriate fluorescent filters using file naming. The images were then converted to grayscale and relative intensities rescaled based on the minimum and maximum droplet fluorescent intensities. The threshold was then selected according to values obtained from 'definetherain' algorithm and the objects – or droplets – meeting the defined criteria were identified and quantified. Finally, the objects were filtered according to eccentricity and size to obtain the refined droplet count of spherical objects meeting 75 µm diameter size. The quantified objects and their intensities were then exported to a spreadsheet software for downstream analysis.

Figure 5: Intensity scatter plots for fluorescent intensity of droplets following mdPCR. (**A**) C-LESS gene amplification with Cy5 hydrolysis probe representing the total cell count as the target region was devoid of cytosine residues and, therefore, resistant to bisulfite treatment. (B) Methylated CD3Z gene amplification with HEX hydrolysis probe, indicative of $CD3^+$ T-Cell population. (**C**) Methylated FOXP3 gene amplification with FAM hydrolysis probe, representing the $CD4^+$ $CD25^+$ T-Reg population. All the scatter plots were subjected to 'definetherain' algorithm to set the appropriate threshold whereby the positives were within 3 standard deviations of the positive cluster mean. The amount of 'rain' was also established so that it does exceed 1% of the positive defined droplets. Each dataset for analysis consisted of ~5000 droplets and was run in triplicate (see Supplementary Information for raw data and analysis).

Figure 6: CPD values of all gene targets and determination of white blood cell subset percentage. (**A**) The calculated CPD values based on Poisson distribution of the positive droplet count as a ratio of total droplets. The input represents the theoretical CPD expected based on 740 ng of bisulfite treated DNA, 75 µm diameter droplets, assuming 6.6 pg for 1 gene copy. The input theoretical CPD was 0.25 and correlated with the C-LESS CPD representing the total cell count. (**B**) The ratio of CPD for CD3Z and FOXP3 with respect to C-LESS was then used to obtain the percent of $CD3^+$ T-Cells and $CD4^+$ $CD25^+$ T-Regs, respectively. These ratios as percent of total leukocytes were then compared with values obtained by immunofluorescence imaging. As

demonstrated, the values from mdPCR and immunofluorescence analysis correlate with no significant difference, with standard deviation errors from mdPCR being much less pronounced.

DISCUSSION:

The presented experimental protocol and methods allow for in-house mdPCR using a fabricated TPE droplet generator, a thermal cycler, and fluorescence microscope. The fabricated device using soft TPE to TPE bonding affords hydrophobic surface properties that are uniform across all channel walls, such that the final device does not require any surface treatment for subsequent use as a droplet generator. This material has been routinely employed in point-of-care platforms that necessitate compatibility with high throughput manufacturing[9-13]. In addition, it is also optically clear and exhibits low fluorescence background in the visible spectrum which is an attractive feature for future integration of a complete sample-to-answer workflow using mdPCR. The PCR reagent recipes are also provided in a homebrew format to allow for facile multiplex gene quantification, while also maintaining stability throughout the thermal cycling protocol. As such one of the advantages of the presented devices and protocols is the flexibility in customizing the device design and reagents used for a particular application, which is difficult to achieve with commercial products and proprietary formulations. Furthermore, it removes the necessity of expensive instrumentation to perform the experiment.

Appropriate thermoplastic material selection of the microfluidic droplet generator is a critical parameter that can circumvent cumbersome surface treatment to render the device hydrophobic for efficient droplet formation. In addition, the selection of an optimized mdPCR buffer is also critical for maintaining droplet stability through the thermal cycling process – whilst not compromising the PCR efficiency and hydrolysis probe functionality. Further modification and optimization of the mdPCR buffer is, therefore, encouraged to arrive at highly specific and sensitive PCR amplification of selected gene targets, coupled with fluorescent based probe detection. This serves to increase signal-to-noise ratios, reducing the instances of 'rain' and simplifying the experimental analysis and identification of positive droplet populations. Troubleshooting of the experiment depends on assessing the critical steps from thermoplastic material selection to polymerase concentration to adapting the temperature ramp in the thermal cycling program in order to ensure droplet stability.

The current limitation of the described protocol is that it still necessitates manual sample transfer from the droplet generator to the thermal cycler and finally to a droplet imaging device and instrument such as a fluorescence microscope. Future efforts however are directed at the miniaturization of mdPCR and integration of these steps whereby droplet generation, PCR and imaging can be performed on a single automated platform.

The obtained results in **Figure 6** demonstrate the versatility of this mdPCR approach for performing differential white blood cell quantification. The proposed method is more precise and reproducible than immunofluorescence imaging, which is often affected by user manipulation, giving rise to unwanted measurement errors. This is also the case for flow cytometry techniques that also rely on an immune-based method of detection which is highly dependent on cell viability, as well as multiple protocol steps prone to user error. Alternative methods such as real-

time quantitative PCR (qPCR) for the quantification are often adversely affected by low copy number gene targets, a drawback that is remedied through mdPCR without the need for a dedicated calibration curve[11]. The presented mdPCR approach, therefore, presents a unique molecular approach to white blood cell differential counts that is based on methylation profiles of specific gene targets. Calibration curves for individual gene targets are also not necessary since mdPCR is based on an absolute quantification of binary positive and negative signals using Poisson distribution for CPD calculation.

The precise quantification of non-methylated gene islands, especially of low copy number genes such as FOXP3, presents a multitude of opportunities for clinical diagnosis. This is highlighted by the capacity of such an approach to identify known methylation patterns correlated to disease onset and progression. mdPCR, as a molecular approach with absolute quantification can be employed beyond methylation studies and can be applied to appropriately preserved samples, therefore removing the dependence on fresh clinical samples, and further expanding its applications. Future automation and miniaturization of this technique will be of great value for rapid and accurate clinical diagnosis and subsequent prognosis.

ACKNOWLEDGMENTS:

The authors acknowledge financial support from the National Research Council of Canada.

DISCLOSURES:

There are no conflicts to declare.

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Figure 1

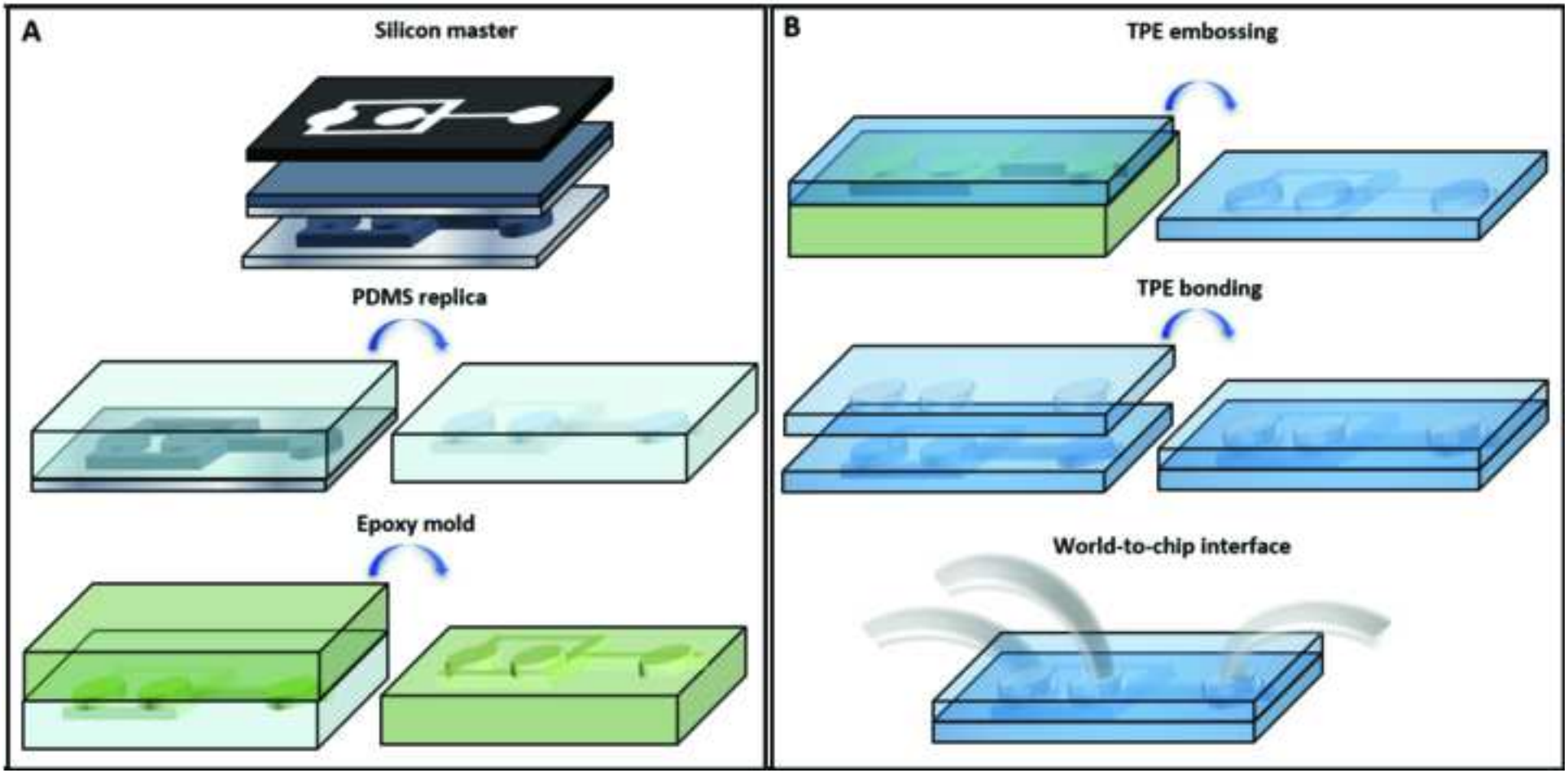

Figure 2

Click here to access/download;Figure;Figure 2.psd

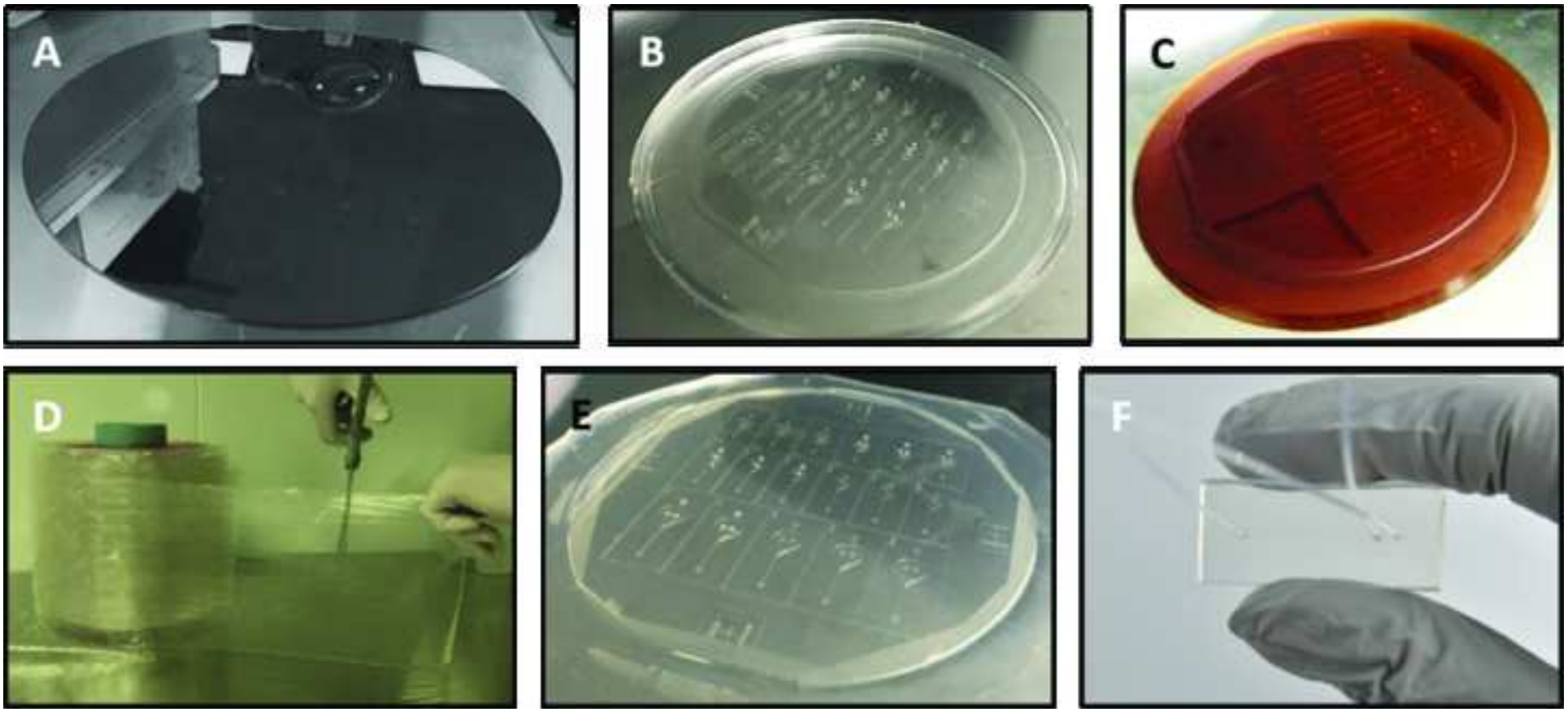

Figure 3

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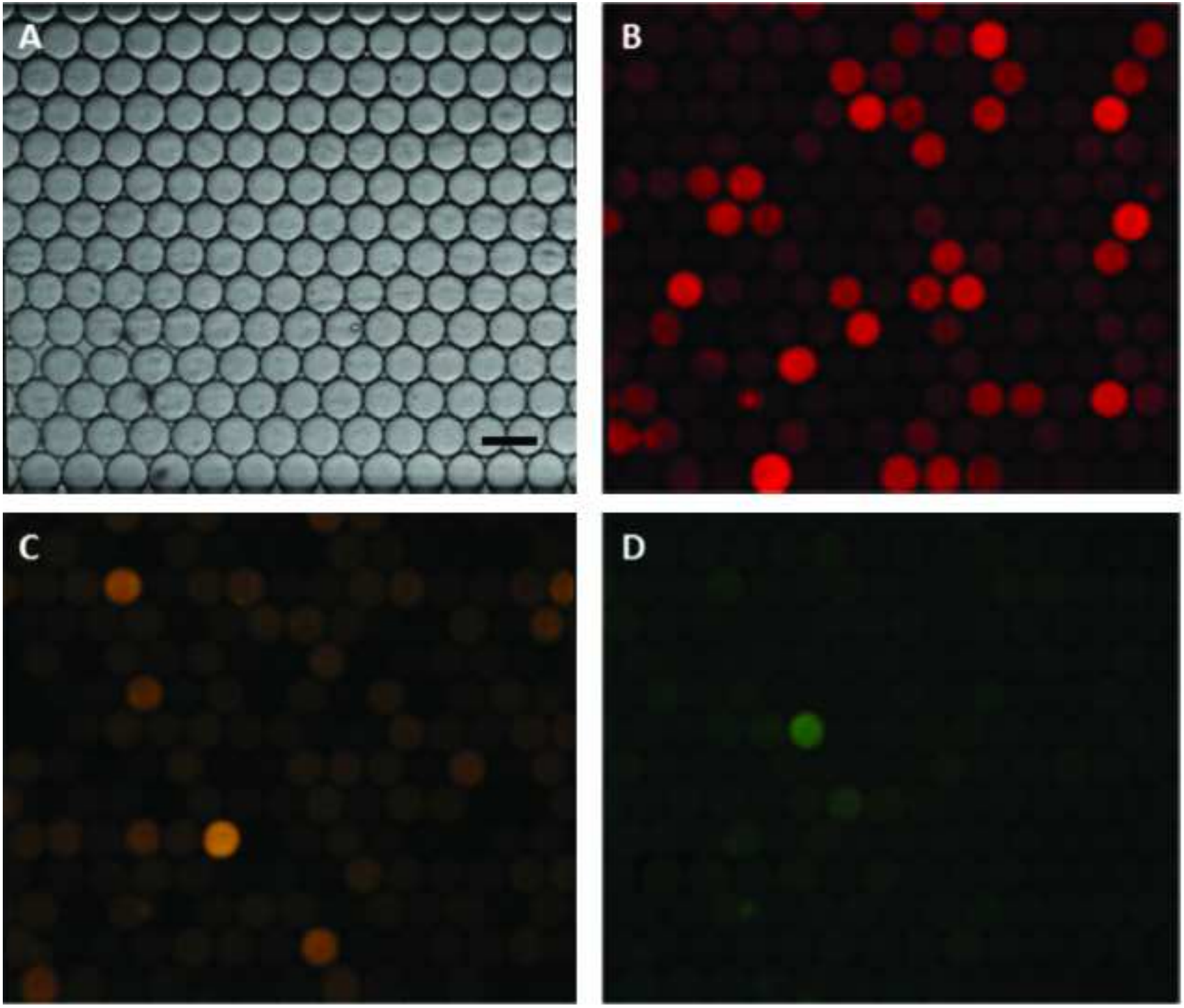

Figure 4

Click here to access/download;Figure;Figure 4.psd

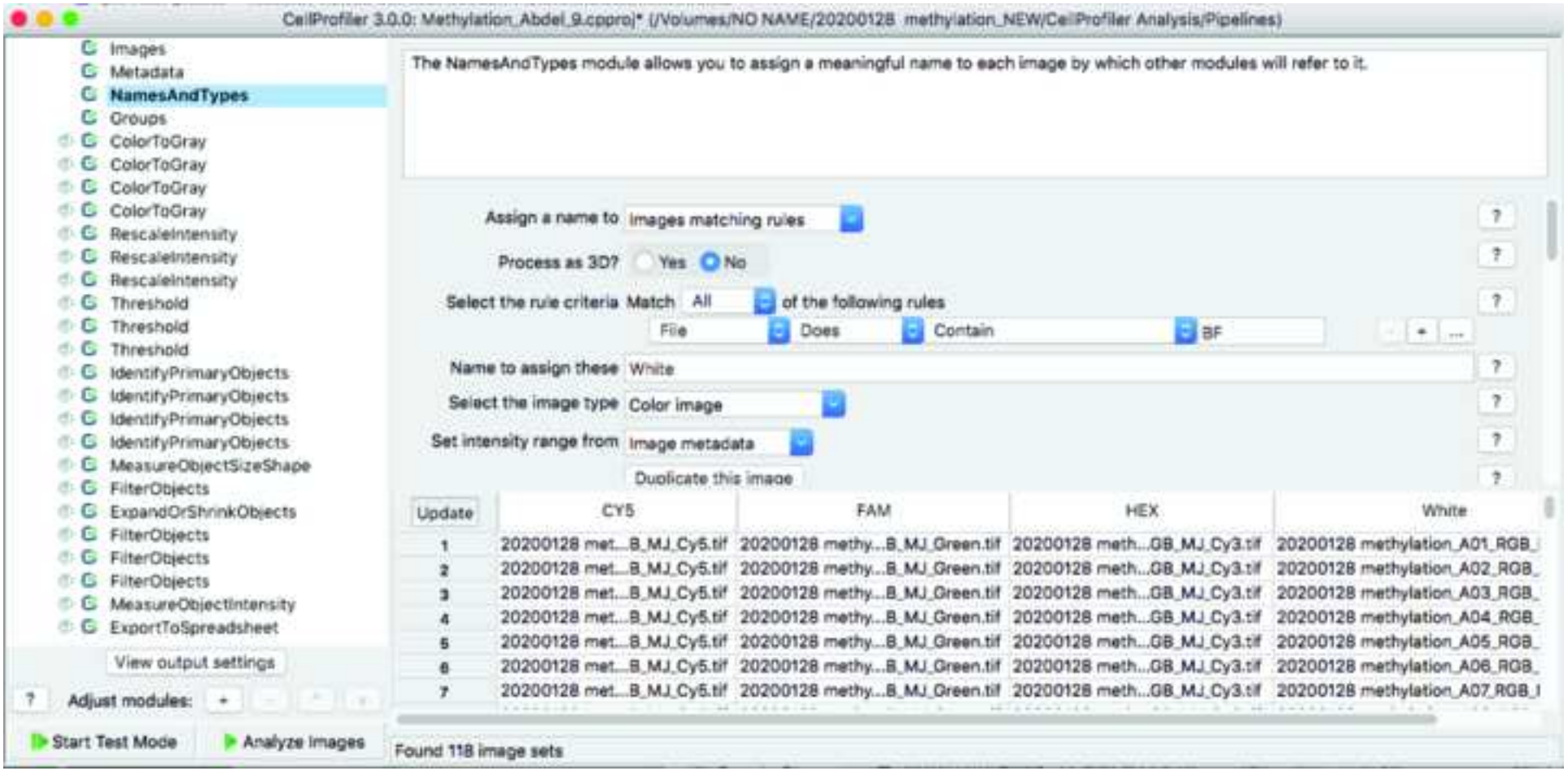

Figure 5

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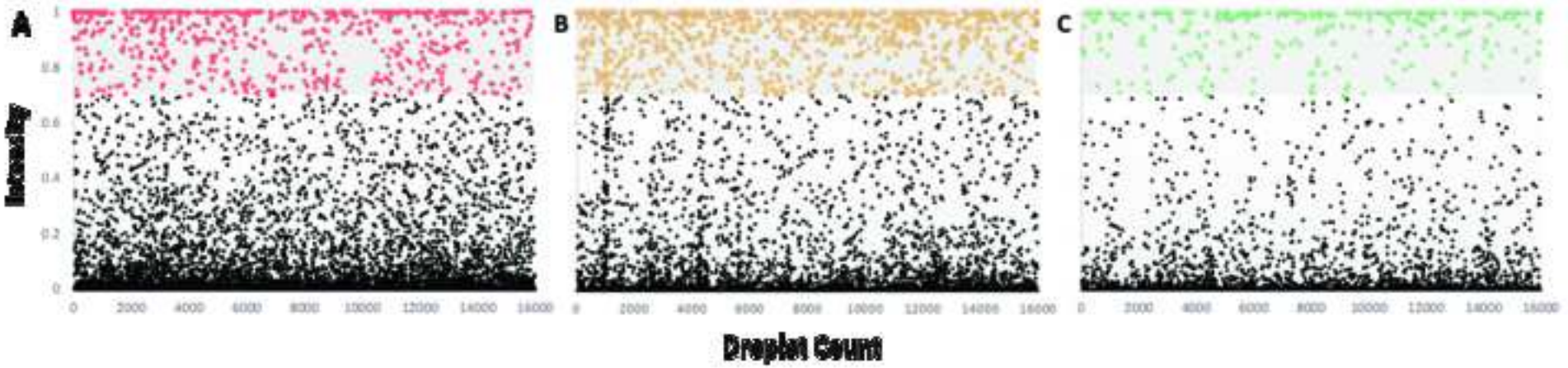

Figure 6

Click here to access/download;Figure;Figure 6.psd

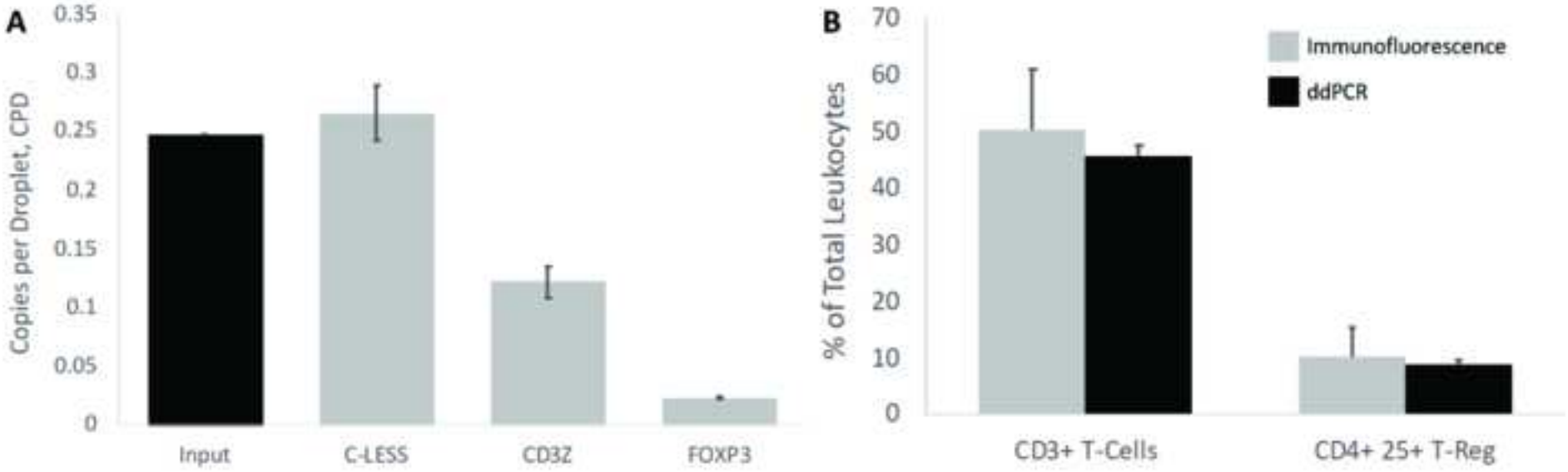

Table 1

FOXP3 Forward	GGG TTT TGT TGT TAT AGT TTT TG
FOXP3 Reverse	TTC TCT TCC TCC ATA ATA TCA
CD3Z Forward	GGA TGG TTG TGG TGA AAA GTG
CD3Z Reverse	CAA AAA CTC CTT TTC TCC TAA CCA
C-LESS Forward	TTG TAT GTA TGT GAG TGT GGG AGA GA
C-LESS Reverse	TTT CTT CCA CCC CTT CTC TTC C
FOXP3 Probe	/56-FAM/CA ACA CAT C/ZEN/C AAC CAC CAT /3IABkFQ/
CDZ3 Probe	/56-HEX/CC AAC CAC C/ZEN/A CTA CCT CAA /3IABkFQ/
C-Less Probe	/56-CY5/CT CCC CCT C/ZEN/T AAC TCT AT/3IABkFQ/

Table 2

Reagent	Stock Solution	Master Mix Volume
Tris-HCl	1 M	2 µL
KCl	1 M	10 µL
MgCl2	25 mM	16 µL
C-LESS Primers	10 µM	10 µL
CD3Z Primers	10 µM	10 µL
FOXP3 Primers	10 µM	10 µL
C-LESS probe	10 µM	5 µL
CD3Z probe	10 µM	5 µL
FOXP3 probe	10 µM	5 µL
HotStarTaq DNA Polymerase	5 Units/µL	8 µL
Bisulfite-treated DNA Target	Variable	Variable
Nuclease-Free Water		Variable
Total Volume		***100 µL***

Working Concentration
20 mM
100 mM
4 mM
1 µM each
1 µM each
1 µM each
500 nM
500 nM
500 nM
0.4 Units/µL
Variable

Table 3

Optical configuration	Exposure	DIA lamp	Florescent light
Bright field	50 ms	ON	OFF
FAM	300 ms	OFF	20%
HEX	300 ms	OFF	20%
Cy5	400 ms	OFF	20%

Name of Material/ Equipment	Company
0.2 ml PCR Tubes with Flat Caps	Bio-Rad, Mississauga, ON
008-FluoroSurfactant	RAN Biotechnologies, Beverly, MA
6" silicon wafer	Silicon Quest International, Santa Clara, CA
Andor iXon Ultra 897 EMCCD camera	Oxford Instruments, Abingdon, UK
Anti-Hu CD3/CD4 Antibody Cocktail FITC, PE	Thermo Fisher Scientific, Waltham, MA
Anti-Hu CD4/CD25 Antibody Cocktail FITC, PE	Thermo Fisher Scientific, Waltham, MA
CellProfiler 3.0	CellProfiler
CFI Plan Fluor 10X	Nikon, Japan
Commercial peripheral blood mononuclear cells (PBMCs)	American Type Culture Collection (ATCC), Manassas, VA
Contact angle goniometer (Model 200-F1)	Ramé-Hart Instrument Co. (Netcong, NJ)
Binder Series VD Vacuum Drying Chambers	Fisher, Canada
VitroTubes	Vitrocom, NJ, USA
Definetherain' algorithm	(http://definetherain.org.uk/)
LIGHTNINGCURE UV-LED spot light source	Hamamatsu, Japan
FEP tubing (I.D. 0.25mm, O.D. 1.6 mm)	Dolomite
FEP tubing (I.D. 0.25mm, O.D. 0.8 mm)	Dolomite
Double-quenched PrimeTime probes	IDT, Coralville, IA
Eclipse LV150N microscope	Nikon, Melville, NY
Epoxy resin (Conapoxy FR-1080)	Cytec Industries, Woodland Park, NJ
EVG 520 system	EV Group, Schärding, Austria
EZ DNA Mehtylation-Lightning Kit	Zymo Research, Irvine, CA
FASTCAM Mini AX200 high-speed camera	Photron, San Diego, CA
Forward/Reverse Primers	IDT, Coralville, IA
GM1070 photoresist	Gersteltec, Pully, Switzerland
High-definition transparency photomask	Fineline Imaging, Colorado Springs, CO
HotStar Taq *Plus* DNA Polymerase	Qiagen, Hilden, Germany
Image J analysis software	Image J
Isopropanol	Anachemia, Montreal, QC
LED fluorescent lamp X-cite Xylis	Excelitas, MA, USA
magnetiQ Blood & Cell DNA Extraction	Galenvs Sciences Inc., Montreal, QC

Mediprene OF 400M	Hexpol TPE, Åmål, Sweden
NanoDrop One Spectrophotometer	Thermo Fisher Scientific, Waltham, MA
NIS-Elements Advanced Research software	Nikon, Japan
Novec 7500 Engineered Fluid	3M, St Paul, MN
NucBlue Live Cell Stain	Thermo Fisher Scientific, Waltham, MA
MicroTight ZDV Adapter for 1/16” to 1/32” OD tubing with	IDEX Health & Science, Oak Harbor, WA
PBS (pH 7.4)	Sigma-Aldrich, Oakville, ON
PDMS (Sylgard 184)	Dow Corning, Midland, MI
Planetary centrifugal vacuum mixer	ThinkyUSA, CA, USA
Biopsy Punch Needle with Plunger	Ihc world, Maryland, USA
Precision glass syringe (250 µL)	Zinsser NA, Northridge, CA
Programmable syringe pump (neMESYS)	Cetoni GmbH, Korbussen, Germany
Propylene glycol monomethyl ether acetate (PGMEA)	Sigma-Aldrich, Oakville, ON
T100 Thermal Cycler	Bio-Rad, Mississauga, ON
Tabletop Microscope TM3030Plus	Hitachi High-Technologies, Mississauga, ON
Ti Eclipse inverted microscope	Nikon, Melville, NY
Ti-PS 1000W lamp	Nikon, Japan
UV acrylic adhesive	Loctite

Catalog Number	Comments/Description
TFI0201	PCR tube
008-FluoroSurfactant	Fluoro-surfactant
	EMCCD camera
MA5-16728	
22-8425-71	
	Used for fluorescence image analysis
	10x objective
PCS-800-011	
p/n 200-U1	
5015 and 5010	Borosilicate capilary tube
LC-L1V5	DEL UV light source
3200063	Disposable fluidic tubing
3200302	Disposable fluidic tubing
	Upright light microscope
D5030	
	SU-8 photoresist
203603	
	Used to assess droplet diameter
	Broad-spectrum LED fluorescent lamp
DE1010	

	Thermoplastic elastomer (TPE)
13-400-518	
	Used for image acquisition
	Carrier Oil
R37605	Blue fluorescent live cell stain (DAPI)
P-881	PEEK fittings
806552	
ARV 310	
IW-125-0	
2607808	
484431	
1861096	
	Inverted microscope
AA 352	

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Dr. Vineeta Bajaj
Review Editor - JoVE

April 30, 2020

Dear Dr. Bajaj,

We thank the Editor and Reviewers for their time and comments. We found the suggestions and comments valuable and helpful in our revision, allowing us to improve the quality of the article. Our responses to the Reviewers' comment are described below in a point-to-point manner. Appropriated changes, suggested by the Editor and the Reviewers, have been introduced to the manuscript submitted in tracked changes format. We hope you find them appropriate and the manuscript suitable for publication.

On behalf of all authors,

Lidija Malic

Canada

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The authors have thoroughly proofread the manuscript for spelling and grammar issues.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

The manuscript has been formatted as per requirements.

3. Please provide an email address for each author.

Email addresses of each author have been added to the manuscript.

4. Please define all abbreviations during the first time use.

All abbreviations have been defined during first time use.

5. Please ensure the Short Abstract/Summary clearly describes the protocol and its applications in complete sentences between 10-50 words: “This protocol presents…”

Summary has been reduced to 49 words and described in complete sentences.

6. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

The abstract is within the word limit and states the goal of the protocol.

7. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 48-53, 544-57, 65-68,

Lines 48-53 were changed to: “Epigenetic markers have recently emerged as powerful analytic tools for the study of phenotypic variations. Subsequently, human leukocyte populations have shown to have cell-lineage DNA methylation patterns that allow for the precise characterization of white blood cell (WBC) subset. Subtyping based on epigenetic markers provides a promising alternative that does not depend on fresh blood sample collection or expensive antibodies and can be exploited as a biomarker for disease onset and susceptibility.”

Lines 65-68 were changed to: "mdPCR has been established to be more precise, accurate, and technically simpler than standard qPCR, making it a more favorable DNA methylation based method for clinical valuation of T-Cells."

Lines 544-57 correspond mostly to Figure 5 caption which does not share any overlap with published work.

8. ddPCR is trademarked, please reword this to be generic.

A generic term has been used to describe the technique, multiplex droplet PCR abbreviated by mdPCR.

9. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Thermo Fisher Scientific, Waltham, MA, NucBlue Live Cell Stain, Ti Eclipse inverted microscope (Nikon), Andor iXon Ultra EMCCD camera (Oxford Instruments, Abingdon, UK), Magnetic Blood and Cell DNA extraction kit (Galenvs, Canada), Lightening Conversion Reagent, EZ DNA Methylation-Lightening Kit (Zymo Research, US), NanoDrop, GM1070 (Gersteltec, Pully, Switzerland), (Silicon Quest International, Santa Clara, CA), (Silicon Quest International, Santa Clara, CA), photomask (Fineline Imaging, Colorado Springs, CO), PGMEA; Sigma-Aldrich, Oakville, ON, (Anachemia, Montreal, QC), S (Sylgard 184; Dow Corning, Midland, MI), Conapoxy FR-1080; Cytec Industries, Woodland Park, NJ, Binder Series VD 275 Vacuum Drying Chambers, Fisher, Canada, Hexpol TPE, Åmål, Swed, EV Group, Schärding, Austria, Mediprene sheet, Cell Profiler, etc.

Commercial language has been removed and referenced in the Table of Material and Reagents.

10. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

An ethics statement has been included before the numbered protocol steps.

11. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Protocol section has been written in the imperative tense.

12. The Protocol should contain only action items that direct the reader to do something.

Only action words have been used in the Protocol section.

13. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

The protocol steps have been modified to only contain 2-3 action sentences.

14. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

The protocol steps have been modified to include more details.

15. 1: Source of PBMCs?

Supplier of the PBMCs is listed in the list of materials.

16. 2.13: How is the imaging performed? Please include all button clicks in the software, knob turns, etc.

All the detailed steps for imaging have been included.

17. 3: Is this performed from fluorescently labelled cells?

This was not done on the fluorescently labelled cells from step 2 but rather the remaining unstained cells from the same frozen PBMCs used for step 2. This is clarified in the revised protocol.

18. 4: Please include the AUTOCAD design files as supplementary materials. 4.2.8: What is the volume of the solutions used? How do you perform the hard bake?

Autocad design file of the device has been included as supplementary information and the design section has been removed from the protocol to avoid redundancy.

“Rinsing with PGMEA and IPA is performed using a squirt bottle (volume is not measured).” has been added to the 4.2.8.

“The hard bake was performed by heating on a hot plate.” was added to the protocol.

19. 5.3: What is the appropriate volume?

The appropriate volume is 1 µL. This has been added to the protocol.

20. 5:15: For all steps involving software usage, please include all the button clicks, knob turns etc.

All the detailed steps for software usage have been included.

21. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

In order to tell the most cohesive story of the protocol, sections that describe the assay workflow from DNA extraction, bisulfite treatment, droplet generation, thermal cycling, and droplet imaging have been selected in a 2.75-page limit.

22. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The data and figures presented in this article have been generated for the purpose of this article and are new. There was no reused material that requires explicit copyright permission.

23. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols

All figure legends have a title and short descriptions of data with relevant symbols.

24. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Discussion has been modified to address the points above.

25. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al.

All the references have been changed to the correct format.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In this study M. Malic et al. describe an alternative method for the ddPCR. The study is carefully performed and well-written. However, raw data about the quantification of WBC using the new ddPCR device at least as supplementary informations are necessary.

Major Concerns:
Without the inclusion of raw data received using the new ddPCR device the performance of the ddPCR can not be determined and reproduced.

We would like to thank the reviewer for this observation. In order to provide the audience with complete information allowing them to easily reproduce the assay and data analysis, we have included in the revised manuscript as supplementary information experimental raw data from ddPCR and immunofluorescence experiments in two separate Excel Documents. In addition to the raw data, all the appropriate calculations are included to facilitate data analysis.

Reviewer #2:
The authors developed a thermoplastic microfluidic droplet generator device and described a digital droplet PCR-based protocol able to distinguish and count WBC subpopulations based on specific epigenetic characteristics. DNA from blood samples was bisulfite-converted and encapsulated in water-in-oil droplets, as microreactors of the PCR reaction. In each droplet are also included primers and TaqMan fluorescent probes specific for CpG loci correlated with WBC sub-population types. This type of analysis is feasible not only from fresh blood but also from frozen sample. Results from the custom workflow were then compared to immunofluorescence staining which is the gold standard for WBC subtyping.

Minor concerns:
The authors should mention the critical steps and the possible pitfalls of the submitted protocol and underline the advantages/disadvantages of this custom device compared to other commercialized digital droplet PCR devices……
As useful information for the reader, instruments/reagents catalogue numbers used in the protocol should be also provided.

We thank the reviewer for his time and encouraging comments. In the discussion section, we have highlighted the critical steps of the protocol, including device material selection, PCR mix optimization, including polymerase concentration and inclusion of a ramp rate in thermal cycling program to ensure droplet stability. We have also highlighted that the advantage of the presented devices and protocols is the cost reduction as well as flexibility in customizing the device design and reagents used to a particular application, which is difficult to achieve with commercial products and proprietary formulations. A disadvantage of this approach, as well as some existing ddPCR commercial instruments is that both still require manual sample preparation as well as three separate instruments. As such, it

necessitates manual sample transfer from droplet generator to PCR tube for thermal cycling and to droplet imaging device for fluorescence microscopy. Our future work is directed towards integration of these steps into an automated format. A paragraph describing these disadvantages has also been included in the discussion section.

Reviewer #3:
The manuscript by malic et al. is very interesting at both the technical level and at the molecular genetics level.
Though I cannot judge the merits of the thermoplastic device generation it appears effective. Moreover, it embodies a means to disclose the ddPCR field that is dominated by one company. Access to more platforms would increase the innovation in the field and a diverse custom design market. The blood cell counting is a nice example of such a custom design that is not covered by the dominant parties in the field. Epigenetics is not a major subject and cell counting neither. By combining those two niches, the paper by Malic et al. serves a new public by providing both a custom design and platform. The robustness of this approach is not evident from the provided results and requires more extensive validation. Calibration curves of diluted cells would be an option as well as the comparison to facs analyzed clinical samples.

We thank the reviewer for his observation. Indeed, the main goal of this manuscript is to provide the framework for the average bench scientist to conduct ddPCR – from TPE device fabrication/assembly, to operation, as well as 'home-brew' mix of PCR reagents. It is therefore our motivation to de-mystify the current 'black box' of ddPCR operation, by further simplifying the parameters inherent to droplet generation, PCR reagent formulations, as well as downstream analysis.

We do agree that a more comprehensive quantitative measure using multiplexing may be better served by performing the ddPCR experiments using a standard curve of known WBC subset populations. To accomplish this, a very pure cell population (for CD4+ CD25+ Tregs, for example) would need to be isolated efficiently. However, when using commercial kits for Treg isolation, the sample purity was very questionable. This was the case for magnetic based purification of Tregs with ThermoFisher kits, as well as bead-based filtration approaches from PluriSelect. Given that the isolation efficiency and purity was always variable at best, we felt that performing such standard curves would not be very effectual in delineating the quantitative capacity of our approach. Instead, using various samples from frozen PBMCs provided enough variation in WBC subset cell ratios and quantities that were confirmed with immunofluorescence (IF). Given that we were able to correlate (using IF and ddPCR) varying ratios of WBC subsets from PBMCs and Whole Blood samples, we were confident in assessing the quantitative efficacy through this approach. It is also important to note that various concentrations for total cell number (C-LESS gene) were also verified with excellent correlation to IF quantification, in addition to the frequency of the other 2 genes of interest (FOXP3, CD3Z).

In terms of IF vs FACS, we opted for IF evaluation especially for CD4+ CD25+ Tregs for several reasons. First, in order to perform ddPCR and IF using the same sample, we were

limited by the cell number for either IF or FACS analysis. This is especially true for Tregs that – due to their low quantity – would not register reliable signals using FACS. Secondly, Tregs require 2 different stains (CD4 and CD25), in addition to the fact that discriminating between bright and dim CD25 signals is quite unreliable using FACS (coupled with low cell numbers). The resulting artifacts and high noise/signal ratio renders FACS unsuitable for this particular application – while using IF, a simple imaging threshold can be used to deduce the resulting cell ratios. And finally, the fluorescent antibodies used for IF were also the same FACS grade products. All staining and IF analysis was performed in parallel with the ddPCR experiments within the same day – circumventing the need to optimize any FACS protocols for later sample analysis that may be influenced by sample freshness.

CD3/CD4				%CD3+	
Image	*Count (DAPI)*	*Count (CD3-FITC)*	*Count (CD4-PE)*		*Image*
1	216	81	18	37.5	1
2	43	24	8	55.81395349	2
3	36	18	7	50	3
4	28	17	8	60.71428571	4
5	19	13	11	68.42105263	5
6	20	9	3	45	6
7	107	37	29	34.57943925	7
8	89	47	13	52.80898876	**Total**
9	53	25	5	47.16981132	%PBMC
Total	611	271	102		%CD4
%PBMC		44.35%	16.69%		
%CD3			37.64%		

IF

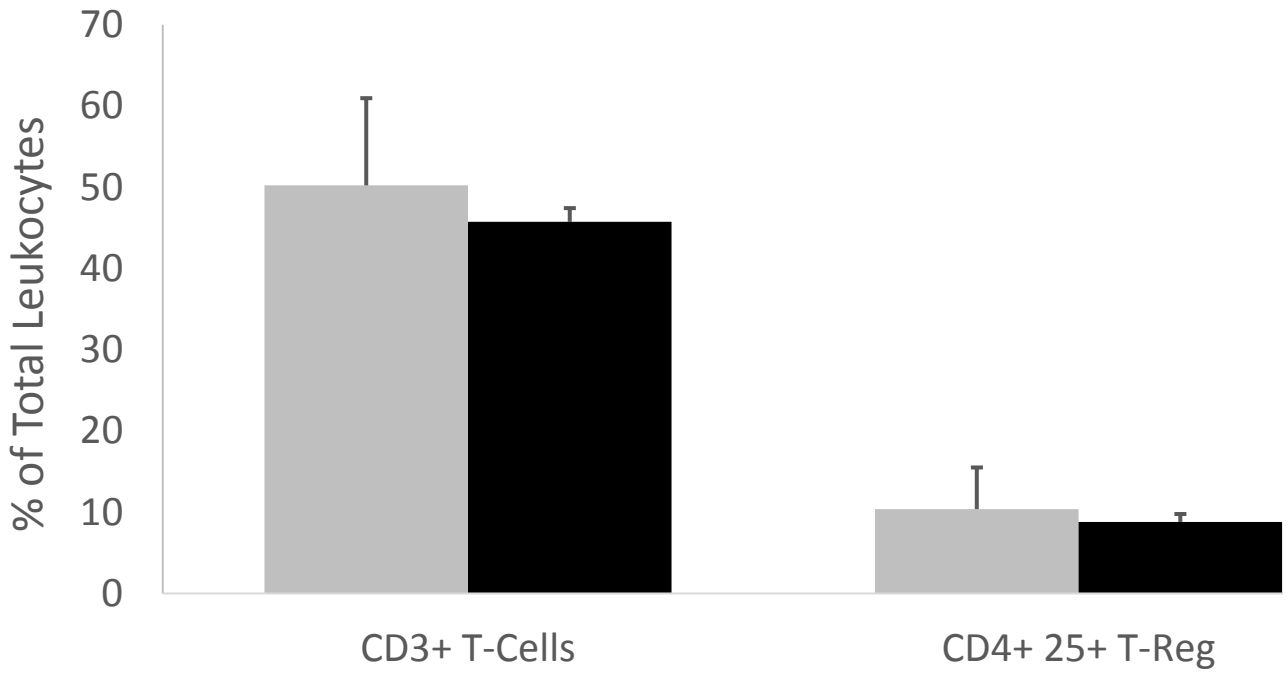
	%	STD	
CD3Z	50.22305902	10.73795509	*CD3+ T-Cells*
FOXP3	10.3831135	5.126160282	*CD4+ 25+ T-Reg*

ddPCR*

	%	STD	
CD3Z	45.7346453	1.693574642	*CD3+ T-Cells*
FOXP3	8.806667515	0.974237773	*CD4+ 25+ T-Reg*

**Values extracted from CPD Calculation Excel Sheet*

CD4/CD25			%CD4+ 25+
Count (DAPI)	*Count (CD4-FITC)*	*Count (CD25-PE)*	
44	12	4	9.090909091
74	18	7	9.459459459
64	16	4	6.25
47	12	3	6.382978723
32	6	2	6.25
28	9	5	17.85714286
23	9	4	17.39130435
312	82	29	
	26.28%	9.29%	
		35.37%	



Imported from Cell	Count_CY5_FilterObjects	Count_FAM_FilterObjects
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	34	3
	32	4
	32	5
	31	5
	44	2
	46	4
	36	6
	35	3
	41	2
	24	1
	23	2
	32	1
	53	8
	27	1
	51	4
	31	3
	70	4
	26	2
	42	6
	36	4
	66	5
	28	0
	40	1
	8	4
	42	4
	48	3
	42	2
	39	4
	39	2
	25	3
	22	0
	6	1
	25	0
	38	4
	44	7
	21	6
	41	6
	26	3
	31	2

24	4
49	8
22	4
23	3
35	1
34	2
31	0
40	3
36	5
39	1
24	1
10	3
19	1
27	3
22	1
33	4
16	2
45	4
23	2
23	4
24	2
8	1
21	5
28	6
36	3
36	3
33	2
54	4
35	4
27	3
23	2
25	6
18	1
10	2
17	2
25	2
40	10
21	1
44	4
37	1
36	4

23	2
42	4
5	0
18	4
23	3
52	5
41	5
58	6
37	6
43	3
42	2
40	2
25	5
13	2
35	1
21	1
28	3
44	6
39	2
36	2
28	5
39	3
22	4
32	3
19	2
25	3
10	1
30	7
28	3
29	2
42	4
34	3
41	2
26	3
19	1
39	1
16	4

Total All Replicates

	C-LESS	FOXP3
Count	3720	367

% Empty Droplets	0.766785781	0.976992038
CPD	0.265547811	0.023276776
% of C-LESS		8.765568893

Replicate Set1

	C-LESS	FOXP3
Count	1305	117
% Empty Droplets	0.748651772	0.977465331
CPD	0.289481328	0.022792454
% of C-LESS		7.873549105

Replicate Set2

	C-LESS	FOXP3
Count	1154	126
% Empty Droplets	0.785780583	0.976610358
CPD	0.241077682	0.023667521
% of C-LESS		9.817383649

Replicate Set3

	C-LESS	FOXP3
Count	1261	124
% Empty Droplets	0.765264334	0.976917349
CPD	0.267533971	0.023353227
% of C-LESS		8.72906979

Experimental CPD

	Average
CLESS	0.266030993
CD3Z	0.121740025
FOXP3	0.023271067

Theoretical CPD

Diameter	75
Volume	0.000220781
DNA	740
1 Copy	6.6
Copies	112121.2121
Volume	100
Copies/uL	1121.212121

CPD	0.247542614

Summary For Plot

	CPD	Standard Deviation
Input	0.247542614	0
C-LESS	0.266030993	0.024236799
CD3Z	0.121740025	0.013249054
FOXP3	0.023271067	0.000443281

Count_HEX_FilterObjects	Count_White_FilterObjects	ImageNumber
8	109	1
17	152	2
20	167	3
16	163	4
15	153	5
24	160	6
74	154	7
18	137	8
25	169	9
19	176	10
14	131	11
10	126	12
9	142	13
19	213	14
9	90	15
23	145	16
19	143	17
30	152	18
11	115	19
14	165	20
10	128	21
20	118	22
15	135	23
14	175	24
24	169	25
21	157	26
27	137	27
13	139	28
28	131	29
14	98	30
15	124	31
7	77	32
2	20	33
14	147	34
13	136	35
12	207	36
20	132	37
23	216	38
15	150	39
16	150	40

22	157	41
21	156	42
6	99	43
13	125	44
16	217	45
17	173	46
13	149	47
20	157	48
19	151	49
14	164	50
10	66	51
3	19	52
6	101	53
16	109	54
6	126	55
21	156	56
8	121	57
14	141	58
16	142	59
17	91	60
13	150	61
4	48	62
11	122	63
12	123	64
17	176	65
15	142	66
22	171	67
22	168	68
12	159	69
15	154	70
18	107	71
4	78	72
10	56	73
7	96	74
6	116	75
13	125	76
24	149	77
10	111	78
20	159	79
14	134	80
17	157	81

15	149	82
21	131	83
7	42	84
11	94	85
14	98	86
19	197	87
22	143	88
23	163	89
14	150	90
11	130	91
22	134	92
10	172	93
16	98	94
8	53	95
11	111	96
12	102	97
12	130	98
18	184	99
17	131	100
18	199	101
14	151	102
21	191	103
19	102	104
16	96	105
11	65	106
13	157	107
2	41	108
18	161	109
12	135	110
20	179	111
10	168	112
21	156	113
17	180	114
11	140	115
11	141	116
9	127	117
18	121	118

CD3Z	Total Droplets
1825	15951

0.885587111	
0.121504452	
45.75614911	

CD3Z	Total Droplets
663	5192
0.872303544	
0.136617815	
47.193999	

CD3Z	Total Droplets
567	5387
0.894746612	
0.111214716	
46.13231496	

CD3Z	Total Droplets
595	5372
0.889240506	
0.117387544	
43.87762194	

Standard Deviation
0.024236799
0.013249054
0.000443281

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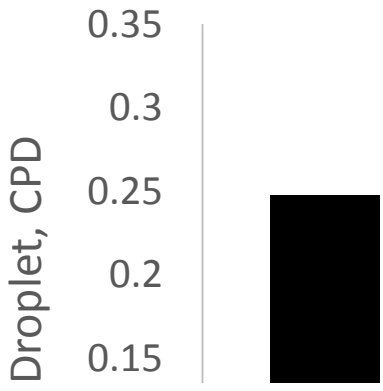

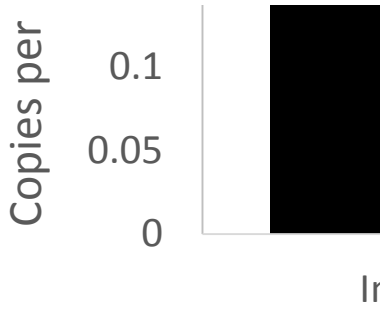
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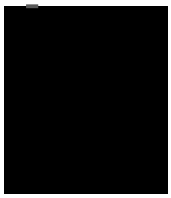

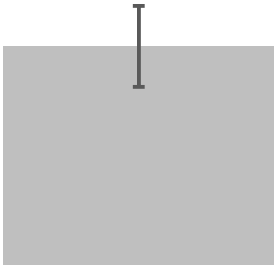


ɳput
C-LESS
CD3Z
FOX

P3

Device CAD file