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Measuring Erythrocyte Complement Receptor 1 Using Flow Cytometry

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Cover Letter

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Reims, November 25th 2019

Invited article

Dear Pr. Berard,
Please find enclosed the revised version of our manuscript entitled " Flow cytometry measurement of Complement Receptor 1 on Erythrocytes ", which we would like to re-submit for consideration in JoVE.
We would like to thank the reviewers for their constructive comments. We have responded to all the remarks raised, and made the necessary corrections. Our point-by-point responses are detailed in the enclosed file.
We fervently hope that this revised version will now be considered suitable for publication in your journal, and we thank you once again for your interest in our work.

Professor Jacques Henri Max Cohen

Our submission is followed by Aaron Berard who got in contact with us.

Professor Jacques Henri Max Cohen

TITLE:

Measuring Erythrocyte Complement Receptor 1 Using Flow Cytometry

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KEYWORDS:

CR1, CD35, complement C3b/C4b receptor, CR1 density polymorphism, flow cytometry, Alzheimer's disease, systemic lupus erythematosus, malaria

SUMMARY:

The aim of this method is to determine the CR1 density in the erythrocytes of any subject by comparing with three subjects whose erythrocyte CR1 density is known. The method uses flow cytometry after immunostaining of the subjects' erythrocytes by an anti-CR1 monoclonal antibody coupled to an amplified system using phycoerythrin (PE).

ABSTRACT:

CR1 (CD35, Complement Receptor type 1 for C3b/C4b) is a high molecular weight membrane glycoprotein of about 200 kDa that controls complement activation, transports immune complexes, and participates in humoral and cellular immune responses. CR1 is present on the surface of many cell types, including erythrocytes, and exhibits polymorphisms in length, structure (Knops, or KN, blood group), and density. The average density of CR1 per erythrocyte

(CR1/E) is 500 molecules per erythrocyte. This density varies from one individual to another (100–1,200 CR1/E) and from one erythrocyte to another in the same individual. We present here a robust flow cytometry method to measure the density of CR1/E, including in subjects expressing a low density, with the help of an amplifying immunostaining system. This method has enabled us to show the lowering of CR1 erythrocyte expression in diseases such as Alzheimer's disease (AD), systemic lupus erythematosus (SLE), AIDS, or malaria.

INTRODUCTION:

CR1 (complement receptor type 1, CD35) is a 200 kDa transmembrane glycoprotein present on the surface of many cell types, such as erythrocytes[1], B lymphocytes[2], monocytic cells, some T cells, follicular dendritic cells[3], fetal astrocytes[4], and glomerular podocytes[5]. CR1 interfering with its ligands C3b, C4b, C3bi[6-9], a subunit of the first complement component, C1q[10] and MBL (mannan-binding lectin)[11] inhibits the activation of complement and is involved in humoral and cellular immune response.

In primates, including humans, erythrocyte CR1 is involved in the transport of immune complexes to the liver and spleen, to purify the blood and prevent their accumulation in vulnerable tissues such as the skin or kidneys[12-14]. This phenomenon of immune adhesion between immune complexes and erythrocytes depends on the number of CR1 molecules[15]. In humans, the mean density of CR1/E is only 500 (i.e., 500 molecules of CR1 per erythrocyte). This density varies from one individual to another (100–1,200 CR1/E) and from one erythrocyte to another in the same individual. Some individuals of "null" phenotype express fewer than 20 CR1/E [16].

The density of CR1/E is regulated by two co-dominant autosomal alleles linked to a point mutation in intron 27 of the gene coding for CR1*1[17,18]. This mutation produces an additional restriction site for the HindIII enzyme. The restriction fragments obtained after digestion with HindIII in this case are 7.4 kb for the allele linked to a strong expression of CR1 (H: high allele) and 6.9 kb for the allele linked to low CR1 expression (L: low allele). This link is found in Caucasians and Asians but not in people of African descent[19].

The level of expression of erythrocyte CR1 is also correlated with the presence of point nucleotide mutations in exon 13 encoding SCR 10 (I643T) and in exon 19 encoding SCR16 (Q981H). It is high in homozygous 643I/981Q and low in homozygous 643T/981H individuals[20]. Thus, "low" individuals express around 150 CR1/E, "medium" individuals express around 500 CR1/E, and "high" individuals express around 1,000 CR1/E.

In addition to this erythrocyte density polymorphism, CR1 is characterized by a length polymorphism corresponding to four allotypes of different sizes: CR1*1 (190 kDa), CR1*2 (220 kDa), CR1*3 (160 kDa), and CR1*4 (250 kDa)[21] and an antigenic polymorphism corresponding to the blood group KN[22].

We present our method based on flow cytometry to determine the density of CR1/E. Using three subjects whose CR1/E density is known, expressing a low density level (180 CR1/E), a medium density level (646 CR1/E), and a high density level (966 CR1/E), it is easy to measure the mean

fluorescence intensity (MFI) of their erythrocytes or red blood cells (RBC), or RBC MFI, after anti-CR1 immunostaining using a flow cytometer. One can then plot a standard line representing the MFI as a function of CR1/E density. Measuring the MFI of subjects whose CR1/E density is not known and comparing it to this standard line, it is possible to determine the individuals' CR1/E density. This technique has been used for many years in the laboratory, and has enabled us to detect a reduction in the expression of erythrocyte CR1 in many pathologies such as systemic lupus erythematosus (SLE)[23], Acquired immunodeficiency syndrome (AIDS)[24], malaria[25], and recently Alzheimer's disease (AD)[26,27]. The development of drugs targeting CR1 to couple with erythrocytes, as in the case of anti-thrombotic drugs[28] requires the evaluation of CR1/E density, and the availability of a robust technique to quantify CR1.

The protocol presented runs in singlicate. It is adaptable to determine the density of CR1/E on many individuals using specific commercially available 96 well plates (see **Table of Materials**). To this end, it is easy to adapt our method to any 96 well plate. For each sample, a cell suspension of erythrocytes (0.5×10^6–1×10^6 erythrocytes) is distributed per well. For each well, first the primary anti-CR1 antibody is added, then streptavidin PE, the secondary anti-streptavidin antibody, and again streptavidin PE, using the same dilutions as those of our method, but by adapting volumes and respecting proportionality.

The blood samples from subjects of the range and from subjects to be quantified for CR1 should be drawn at the same time, stored in the refrigerator at 4 °C, and handled at 4 °C (on ice and/or in the refrigerator).

PROTOCOL:

The protocol for human blood collection and handling was reviewed and approved by the regional ethics committee (CPP Est II), and the protocol number is 2011-A00594-37. Because the following protocol describes the handling of human blood, institutional guidelines for disposing of biohazardous material should be followed. Laboratory safety equipment, such as lab coats and gloves, should be worn.

1. Erythrocyte washing

NOTE: The day before handling, prepare a PBS-BSA buffer with phosphate buffered saline (PBS) containing 0.15% of bovine serum albumin (BSA) and place it in the refrigerator at 4 °C. This buffer will be used as a washing buffer and as a dilution buffer.

1.1. Pipette 20 mL of PBS-BSA into a 50 mL tube.

1.2. Aspirate 250 µL of sodium ethylenediamine tetraacetic acid (EDTA) anticoagulated whole blood from blood storage tubes and add to the tube containing the 20 mL of PBS-BSA. Close the tube by screwing the cap. Mix gently by inverting the tube 2x.

1.3. Centrifuge the tube for 10 min at 4 °C at 430 x *g*. Remove and discard the supernatant using

a 10 mL pipette. Resuspend the pellet in the residual volume of supernatant by gentle and careful pipetting.

1.4. Add 20 mL of cold PBS-BSA (4 °C) into the tube containing the pellet. Centrifuge the tube for 10 min at 4 °C at 430 x *g*. Remove and discard the supernatant using a 10 mL pipette.

1.5. Add 20 mL of cold PBS-BSA (4 °C) into the tube containing the pellet. Centrifuge the tube for 10 min at 4 °C at 430 x *g*. Leave the tube in the centrifuge at 4 °C and go to section 2.

2. Erythrocyte dilution

2.1. Pipette 3 mL of cold PBS-BSA into a 50 mL tube and store it at 4 °C on a rack in ice.

2.2. Put the centrifuged tube containing the erythrocytes (step 1.4) on a rack placed in ice.

2.3. Pipette 8 µL of pelleted erythrocytes using the pipette and add to the 50 mL tube containing the 3 mL of PBS-BSA to obtain the erythrocyte dilution. Mix the tube gently by hand to obtain a homogeneous cell suspension of erythrocytes.

3. Erythrocyte immunostaining

3.1. Pipette 100 µL of erythrocyte dilution (obtained in section 4) and add to 1.4 mL tubes.

3.2. Centrifuge the tubes for 5 min at 4 °C at 430 x *g*. During centrifugation, prepare a dilution of biotinylated anti-CR1 J3D3 antibody at a concentration of 0.05 µg/µL in PBS-BSA buffer.

3.3. Once the centrifugation is done, remove and discard the supernatant.

3.4. Add 20 µL of biotinylated anti-CR1 J3D3 directly to the pellet. To prepare the negative control, add 20 µL of PBS-BSA buffer instead. Mix the tubes gently and incubate for 45 min at 4 °C.

3.5. After 45 min of incubation, add 750 µL of PBS-BSA to the tubes. Centrifuge the tubes for 5 min at 4 °C at 430 x *g*. Remove and discard the supernatant. Repeat.

3.6. In the meantime, prepare a 1:10 dilution of streptavidin-phycoerythrin diluted in PBS-BSA buffer. Pipette 20 µL of the 1:10 dilution of streptavidin-phycoerythrin and add to the tubes. Mix the tubes gently and incubate for 45 min at 4 °C.

3.7. Add 750 µL of PBS-BSA buffer into the tubes. Mix well and centrifuge for 5 min at 4 °C at 430 x *g*. Remove and discard the supernatant. Repeat.

3.8. During centrifugation, prepare a 1:100 dilution of biotinylated anti-streptavidin antibody diluted in PBS-BSA buffer.

3.9. Once the centrifugation is done, remove and discard the supernatant.

3.10. Pipette 20 µL of the 1:100 dilution of biotinylated anti-streptavidin into the tubes. Mix the tubes gently. Incubate the tubes for 45 min at 4 °C.

3.11. After 45 min of incubation, pipette 750 µL of PBS-BSA buffer and add into the tubes. Centrifuge the tubes for 5 min at 4 °C at 430 x *g*. Remove and discard the supernatant. Repeat.

3.12. Pipette 20 µL of the 1:10 dilution of streptavidin-phycoerythrin and add into the tubes. Mix the tubes gently. Incubate the tubes for 45 min at 4 °C.

3.13. Pipette 750 µL of PBS-BSA buffer and add into the tubes. Mix well and centrifuge the tubes for 5 min at 4 °C at 430 x *g*. Remove and discard the supernatant. Repeat this step 2x.

4. Immunostained erythrocyte fixation

4.1. During the last centrifugation, prepare the fixation buffer, a 1:100 dilution of 37% formaldehyde using the washing buffer PBS-BSA.

4.2. Pipette 450 µL of fixation buffer and add into immunostained erythrocyte tubes (from step 3.13) while vortexing for 5 s.

4.3. Pipette all fixed cells into 5 mL round bottom tubes and store in the refrigerator.

NOTE: The protocol can be paused here for up to 48 h.

5. Flow cytometry analysis of stained erythrocytes

NOTE: It is advisable to refer to the operator's manual for the cytometer (see **Table of Materials**) to know how to perform the cytometric readings. The suggested parameters below apply to the instrument used and must be optimized for each cytometer.

5.1. Turn on the flow cytometer, then turn on the computer. Let the optical system temperature stabilize by leaving it on for 30 min. Check the cytometer window in the software to ensure that the cytometer is connected to the workstation (the message **Cytometer Connected** is displayed).

5.2. Check that the buffer container is full and that the waste container is empty. Remove air bubbles in the buffer filter and the buffer line using the purge system. Prime the fluidics system by pressing the **Prime** button on the console of the cytometer. Wait until the indicator light changes from red to green.

5.3. To clean the fluidics, install a tube containing 3 mL of a cleaning solution on the sample injection port and allow the cleaning solution to run for 5 min with a high sample flow rate. Repeat this with the rinse solution with distilled water. Leave the tube containing water on the sample

injection port.

5.4. To prepare the calibrating beads, pipette 400 µL of PBS into the bottom of a round bottom tube. Mix the bead stocks strongly by vortexing for 30 s. Add a drop to the round bottom tube containing PBS. Mix carefully by vortexing for 30 s.

5.5. Run the performance check. Open the cytometer **Setup and Tracking** module in the software (**Figure 1A**). Verify that the cytometer configuration is correct for the experiment using PE immunostaining. Verify that the calibrating bead batch is correct with the configuration.

5.6. Install the bead tube on the sample injection port and let it run with a low sample flow rate. Run the performance check, which takes approximately 5 min to complete). Once the performance check is complete, verify that the cytometer performance is satisfactory (**Figure 1B**). Close the cytometer **Setup and Tracking** module in the software.

5.7. To set up an experiment and create application settings, click the **New Experiment** button on the browser toolbar and open the new experiment. Specify the parameters by selecting appropriate cytometer settings: **Forward Scatter** (FSC), **Side Scatter** (SSC), and **PE** from the drop-down menu of the experiment (**Figure 1C**). Select **Linear Mode** for FSC parameter and **Logarithm Mode** for SSC and PE parameters.

5.8. In the open experiment, select **Cytometer Settings** (**Figure 1D**), then select **Application Settings**, and create a global worksheet (**Figure 1E**). Use the gray boxes and crosshairs to guide the optimization.

5.9. Load the unstained control tube onto the cytometer and run **Acquisition**. Ensure that the population of interest (i.e., RBCs) is on scale by optimizing the FSC and SSC voltages. Optimize the FSC threshold value to eliminate debris without interfering with the population of interest.

5.10. Draw a gate around the RBCs on the FSC vs. SSC plot. Display the RBC population in the dot plot of PE fluorescence. If needed, increase the fluorescence of the photomultiplier tube (PMT) voltages to place the negative population within the gray boxes. Unload the unstained control tube from the cytometer.

5.11. Verify that the positive populations are on scale. Load the stained control tube onto the cytometer and run **Acquisition.** Lower the PMT voltage for the positive population if it is off scale until the positive population can be seen entirely on scale. Then unload the stained sample.

5.12. To record and analyze samples, on a new global worksheet, create the following plots for previewing the data: 1) FSC vs. SSC, and 2) PE fluorescence histogram. Load the first sample onto the cytometer and run **Acquisition**.

5.13. Draw an RBC gate around the erythrocytes on the FSC vs. SSC plot. Display the RBC population in the PE fluorescence histogram. In the **Statistics** view, select the mean for PE

fluorescence parameters on GR populations (**Figure 1F**).

5.14. In the **Acquisition** dashboard, select all events in the stopping gate and 10,000 events to record (**Figure 1G**). Click **Record Data**. When the event recording has completed, remove the first tube from the cytometer. The global worksheet plots should look like those in **Figure 2**.

5.15. Load the following samples and record them.

6. Determination of the density of erythrocyte CR1

6.1. Take the values of the mean fluorescence intensities of the samples corresponding to the "low" subject (**Figure 3**, **Table I**, RBC MFI), "medium" subject (**Figure 4**, **Table D**, RBC MFI), "high" subject (**Figure 4**, **Table I**, RBC MFI), and to the negative control sample (**Figure 3**, **Table I**, RBC MFI).

6.2. On a graph representing the mean fluorescence intensity as a function of the density of CR1, place the four points corresponding to the negative control, "low" subject, "medium" subject, and "high" subject (blue points, **Figure 6**).

6.3. Draw the regression line to get the calibration line and its equation.

6.4. Take the values of the mean fluorescence intensity of the samples corresponding to the subjects whose density is to be determined. (**Figure 5**, **Tables D and I**, RBC MFI).

6.5. Obtain the equation by replacing "Y" using the values of the mean fluorescence intensities, and calculate the density of CR1/E (**Figure 6**).

6.6. Check on the graph that the mean fluorescence intensity values and the determined CR1/E density correspond to a point on the calibration line (**Figure 6**).

REPRESENTATIVE RESULTS:

The erythrocytes of three subjects whose density of CR1 is known ("low" subject [180 CR1/E], "medium" subject [646 CR1/E], and "high" subject [966 CR1/E]), and of two subjects whose CR1 density needed to be determined were immunostained by an anti-CR1 antibody coupled to an amplification system using the phycoerythrin fluorochrome. At the beginning, the CR1 density of the subjects from the low-high range was determined by the Scatchard method[29] using radiolabeled antibodies. The standards (low, medium, and high) determined were used for a calibration curve and made it possible to quantify new standards or substandards by our method of cytometry[30]. After passage of immunostained erythrocytes in the flow cytometer, the intensity of the labeling was observed and measured as the mean fluorescence intensity for each subject (RBC MFI) (**Figure 3F**,**I**; **Figure 4B**,**D**,**F**,**I**; **Figure 5B**,**D**,**F**,**I**). A curve was plotted using the values of the subjects with the known density of erythrocyte CR1 ("low" to "high") by reporting them as a function of the mean fluorescence intensity. Comparison of the regression line resulting from this curve to the values of the mean fluorescence intensity of the other subjects determined their

CR1/E density (**Figure 6**). **Figure 7** shows the overall workflow.

FIGURE AND TABLE LEGENDS:

Figure 1: Cytometer console and windows appearing during flow cytometry protocol. (**A**) Window appearing after the application of step 5.5 of the protocol. (**B**) Window appearing after the application of step 5.6 of the protocol. (**C**) Window appearing after the application of step 5.7 of the protocol. (**D**) Window appearing after the application of step 5.8 of the protocol. (**E**) Window appearing after the application of step 5.8 of the protocol. (**F**) Window appearing after the application of step 5.13 of the protocol. (**G**) Window appearing after the application of step 5.14 of the protocol

Figure 2: Appearance of the global worksheet analysis objects. Appearance of the global worksheet analysis objects after the application of step 5.14 of the protocol.

Figure 3: Results of flow cytometry analysis of anti-CR1 immunostaining of erythrocytes corresponding to negative control and to the subject from the range ("low" subject) who expressed LOW CR1 density (180 CR1/E). For each subject: (**A**,**E**) a dot blot showing the appearance of events acquired according to the size and granulometry parameters, (**G**) the gate selecting the erythrocyte population among the events, (**B**,**F**) a histogram representing the intensity of the labeling, (**C**,**H**) an associated statistical table presenting the number of events corresponding to the erythrocytes and their percentage, (**D**,**I**) an associated table giving the mean fluorescence intensity (RBC MFI).

Figure 4: Results of flow cytometry analysis of anti-CR1 immunostaining of erythrocytes corresponding to the subjects from the range: "medium" subject who expressed MEDIUM CR1 density (646 CR1/E) and "high" subject who expressed HIGH CR1 density (966 CR1/E). For each subject: (**A**, **E**) a dot blot showing the appearance of events acquired according to the size and granulometry parameters, (**G**) the gate selecting the erythrocyte population among the events, (**B**, **F**) a histogram representing the intensity of the labeling, (**C**, **H**) an associated statistical table presenting the number of events corresponding to the erythrocytes and their percentage, (**D**, **I**) an associated table giving the mean fluorescence intensity (RBC MFI).

Figure 5: Results of flow cytometry analysis of anti-CR1 immunostaining of erythrocytes corresponding to the subjects whose CR1 density was to be determined. For each subject: (**A**, **E**) a dot blot showing the appearance of events acquired according to the size and granulometry parameters, (**G**) the gate selecting the erythrocyte population among the events, (**B**, **F**) a histogram representing the intensity of the labeling, (**C**, **H**) an associated statistical table presenting the number of events corresponding to the erythrocytes and their percentage, (**D**, **I**) an associated table giving the mean fluorescence intensity (RBC MFI).

Figure 6: Calibration curve and regression line enabling determination of CR1 density. (**A**), (**B**) Calibration curve and regression line drawn according to the known CR1 density of the range subjects (negative control: 0 CR1, "low" subject [180 CR1/E], "medium" subject [646 CR1/E], and "high" subject [966 CR1/E]) and their respective values of mean fluorescence intensity obtained

by flow cytometry. (**C**), (**D**) From the equation of this regression line, we calculated the density of erythrocyte CR1 for subjects whose mean fluorescence intensity was quantified by flow cytometry: orange arrows, Subject 1 (mean fluorescence intensity = 1,334; CR1/E density = 459) and Subject 2 (mean fluorescence intensity = 2820; CR1/E density = 1,000).

Figure 7: Flowchart of the protocol to determine the erythrocyte CR1 density from human blood samples. Collect a human blood sample. Wash the human blood sample by centrifugation to obtain erythrocytes. Stain the erythrocytes using an anti-CR1 antibody. Use flow cytometry to determine the erythrocyte CR1 density according to a calibration curve.

DISCUSSION:

Several techniques are available to determine the density of erythrocyte CR1 (CR1/E). The first techniques used were the agglutination of red blood cells by anti-CR1 antibodies[31] and the formation of rosettes in the presence of erythrocytes coated with C3b[32]. These rudimentary techniques were rapidly replaced by immunostaining methods using radiolabeled anti-CR1 antibodies[1,33]. It is also possible to measure the concentration of CR1 in membrane extracts by enzyme-linked immunosorbent assay (ELISA)[34]. Although accurate, these techniques only provide an average value of the CR1/E density. The distribution of CR1/E density over the entire erythrocyte population is only available by flow cytometric analysis after immunostaining. This technique is difficult due to the low density of CR1/E. Nevertheless, an amplification method now makes it possible to easily measure the density of CR1/E[30].

Here, we present a method of quantifying CR1/E by flow cytometry based on amplification of the fluorescence signal of immunostained cells. The amplification system involves four successive layers of staining using the biotinylated anti-CR1 monoclonal antibody J3D3; phycoerythrin-streptavidin; a biotinylated goat anti-streptavidin antibody; and again phycoerythrin-streptavidin. J3D3 recognizes three antigenic sites on CR1[35], although no more than one at the same time. The biotinylated goat anti-streptavidin antibody is a polyclonal antibody that recognizes multiple epitopes on streptavidin and provides a better bridge between the two streptavidin layers than biotin-streptavidin alone. This process also benefits from the high fluorescence yield of phycoerythrin[36] and the low level of nonspecific binding of streptavidin[37]. With such a strong amplified signal, the low settings of the cytometer photomultiplier tubes enable perfect linearity. This method, which is characterized by excellent sensitivity and reproducibility, enables the detection of fewer than 100 CR1/cell.

However, this method requires samples from three subjects whose density of erythrocyte CR1 is known: one subject expressing a low level of erythrocyte CR1 (180 CR1/E), one subject expressing a medium level of erythrocyte CR1 (646 CR1/E) and one subject expressing a high level of erythrocyte CR1 (966 CR1/E). It is possible to take the first measurements of the erythrocyte CR1 density of several individuals, initially using the erythrocytes from the three subjects used in our study, which we can provide. The blood samples from subjects of the range and subjects to be quantified for CR1 should be drawn at the same time, stored in the refrigerator at 4 °C, and handled at 4 °C[38]. The blood drawn in EDTA tubes is easily routable and can be stored for 5 days at 4 °C, allowing time for quantification of erythrocyte CR1. After this, the density of erythrocyte

CR1 begins to decrease, and there is a collapse of the standard CR1 curve, especially at the point corresponding to the subject expressing a high level of erythrocyte CR1. Because the resulting regression line is distorted, the measure of CR1 density is no longer accurate. It should be noted that in vitro storage, handling conditions, and the multilayered staining lead to clustering of CR1 and a slight overestimation of the number of CR1 molecules. Nevertheless, the use of an anti-CR1 antibody targeting three epitopes such as J3D3 with the amplification system enables clustering to be fully performed, which enables correct measurement of CR1 density[39].

In fact, the density of CR1/E decreases during the life of the erythrocyte[40]. This would explain the heterogeneity of the density of CR1/E in the same individual. According to some authors, the intensity of catabolism of CR1 is not correlated with the initial density of CR1/E[41], whereas for other authors, the higher the initial density, the greater the intensity of catabolism[42]. The half-life of CR1 on the surface of erythrocytes is 11–32 days[42].

The method presented here has several advantages. The first is to be able to select, thanks to flow cytometry, the cell subpopulations to be studied within the same blood sample. By selecting the erythrocyte population using the gate function, the measurement of erythrocyte density is guaranteed exclusively. A bias in the measurement of erythrocyte CR1 caused by the presence of other cellular subpopulations such as white blood cells is avoided. The second advantage of this method is that it is adaptable to the quantification of other cellular receptors whose density is low by simply replacing the primary anti-CR1 antibody with an antibody specifically directed against an epitope of the receptor to be studied. It is also adaptable to using 96 well plates instead of tube racks, which requires lower blood and reagent volumes[25,38]. The third advantage of this method is that it is flexible. In studies concerning cells with a very high density of CR1, for example, human lymphocytes (10,000 CR1/cell), or nonhuman primate erythrocytes whose CR1 density is 10–100x greater than that of humans (10,000–100,000 CR1/cell)[43,44], it is possible to decrease the number of amplification system layers, using only biotinylated anti-CR1 monoclonal antibody, phycoerythrin-streptavidin or biotinylated anti-CR1 monoclonal antibody, biotinylated anti-mouse antibody, and phycoerythrin-streptavidin, thus adapting the fluorescence level to the higher density of CR1. The fourth advantage of this method is that it can be used for fixed or frozen erythrocytes, enabling blood samples to be collected in areas lacking the facilities for flow cytometry and stored for later accurate quantification of CR1[38].

More generally, with the new brighter fluorochromes, it no longer seems mandatory to use the system of indirect amplification. Besides, there are other methods using flow cytometry that make it possible to evaluate the density of the cellular receptors and to quantify it in units of measure (i.e. ABC, or antibody binding capacity). The ABC per cell can also be determined using saturating concentrations of antibody and calibrated beads. Several commercial systems are available. Some kits are precalibrated standard beads containing known levels of fluorochrome molecules such as PE bound per bead. The beads acquired on a flow cytometer on the same day at the same instrument settings as the individual patient specimens make it possible to draw a standard curve comparing the geometric mean of fluorescence to known PE content of the beads. The regression analysis, slope, intercept, and correlation coefficient are determined, and the ABC values are calculated from the measured geometric mean fluorescence of cells using the standard

curve[45,46].

A further type of bead test is based on binding of an antibody conjugated to beads with specific antibody binding capacity levels via the crystallizable portion of the fragment (Fc). Beads are labeled with the same antibody used to label the cells whose antigen density is to be measured. Thus, in a single experiment, any conjugated antibody can be used, as long as the same batch with the same fluorophore/protein ratio (F/P ratio) is used to stain both beads and cells[47]. Some kits are better for the quantitative determination of cell surface antigens by flow cytometry using indirect immunofluorescence assays[48,49].

In conclusion, our method has the advantage of providing very sensitive detection and being easy to implement on ordinary flow cytometry material.

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DISCLOSURES:
The authors have nothing to disclose.

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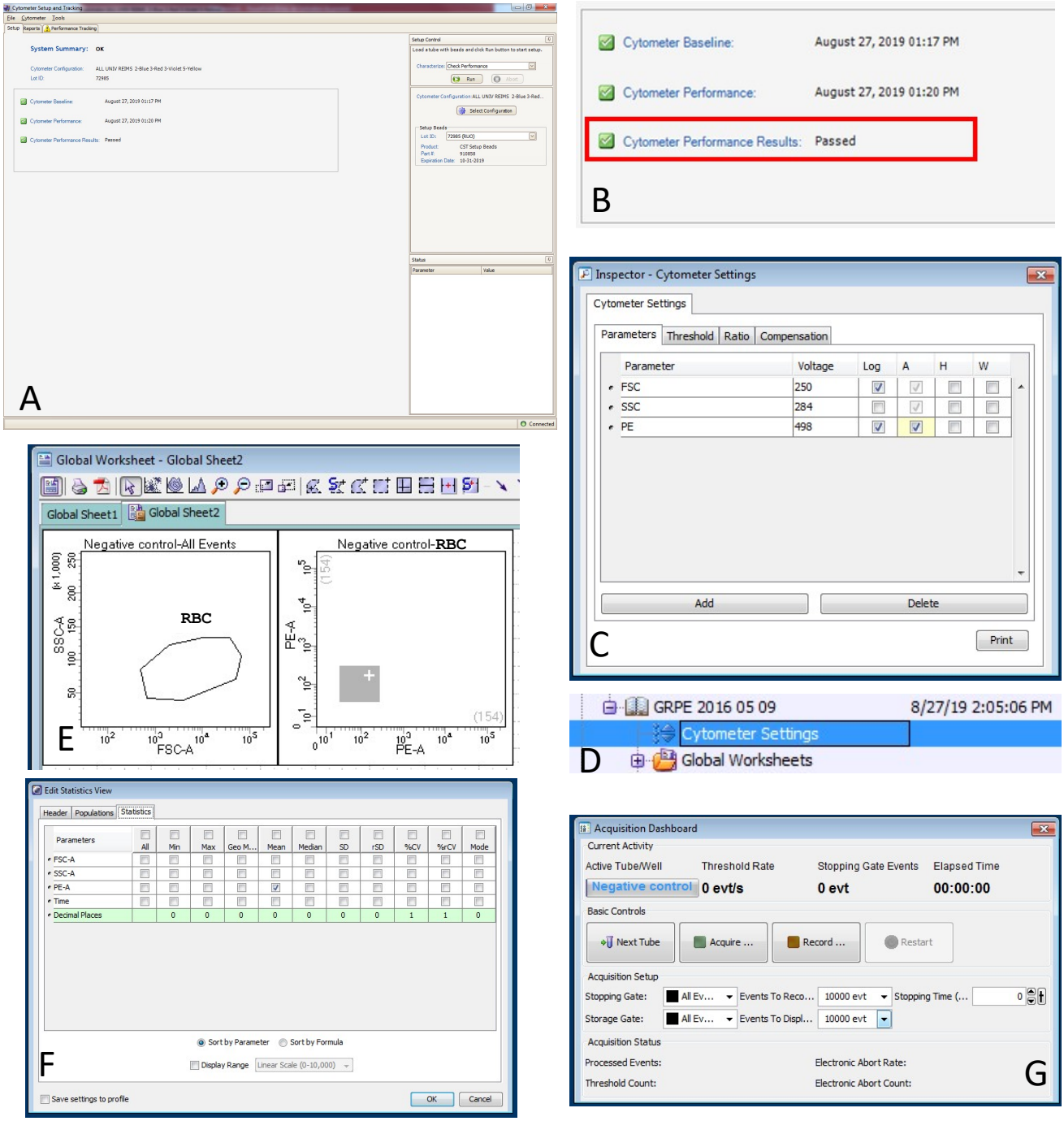
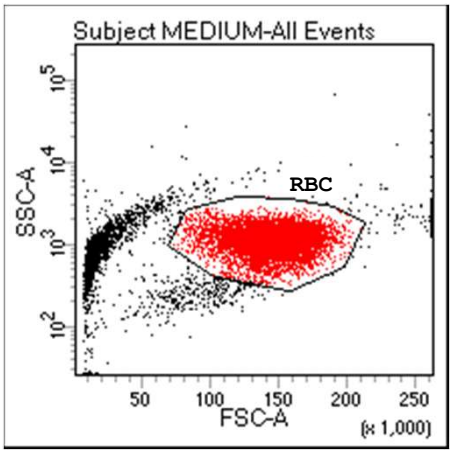
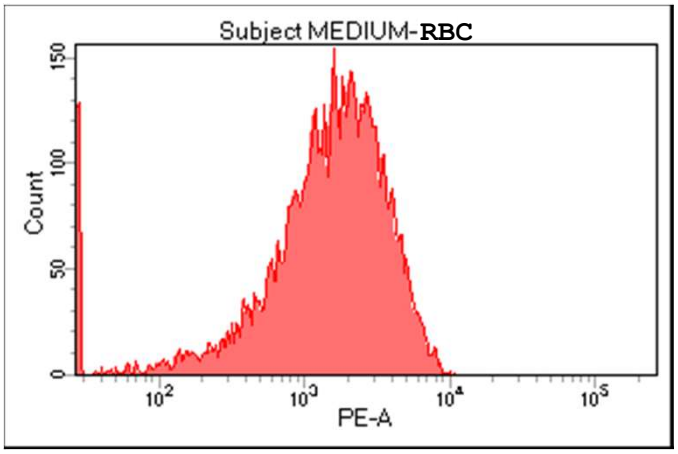


Figure 1





Tube: Subject MEDIUM

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
RBC	7,565	75.6	75.6

Experiment Name:	GRPE 2016 05 09
Specimen Name:	CR1
Tube Name:	Subject MEDIUM
Record Date:	May 9, 2016 12:26:50 PM

Population	#Events	%Parent	PE-A Mean
All Events	####	####	1,765
RBC	7,565	75.6	1,918

Figure 2

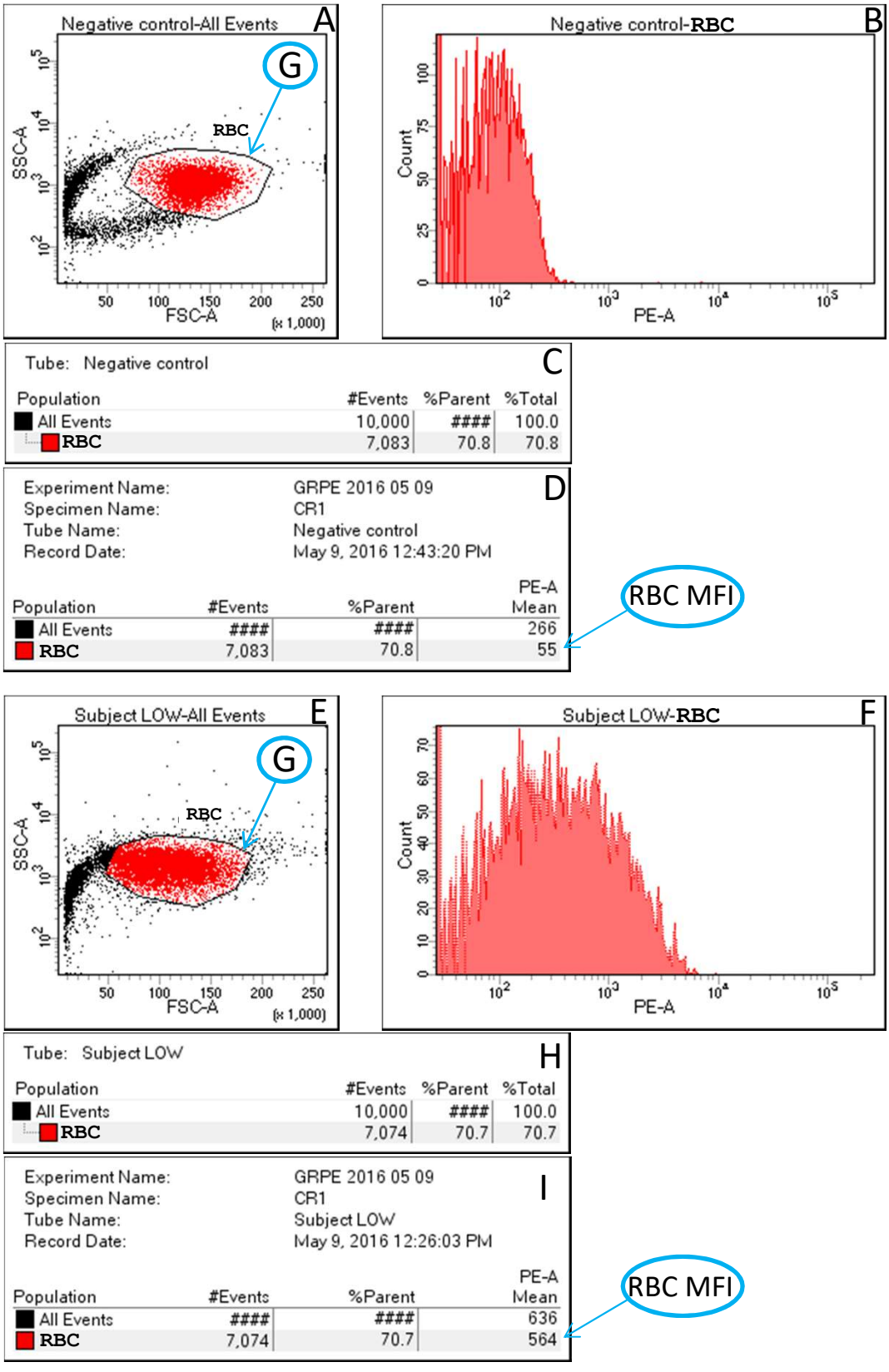


Figure 3

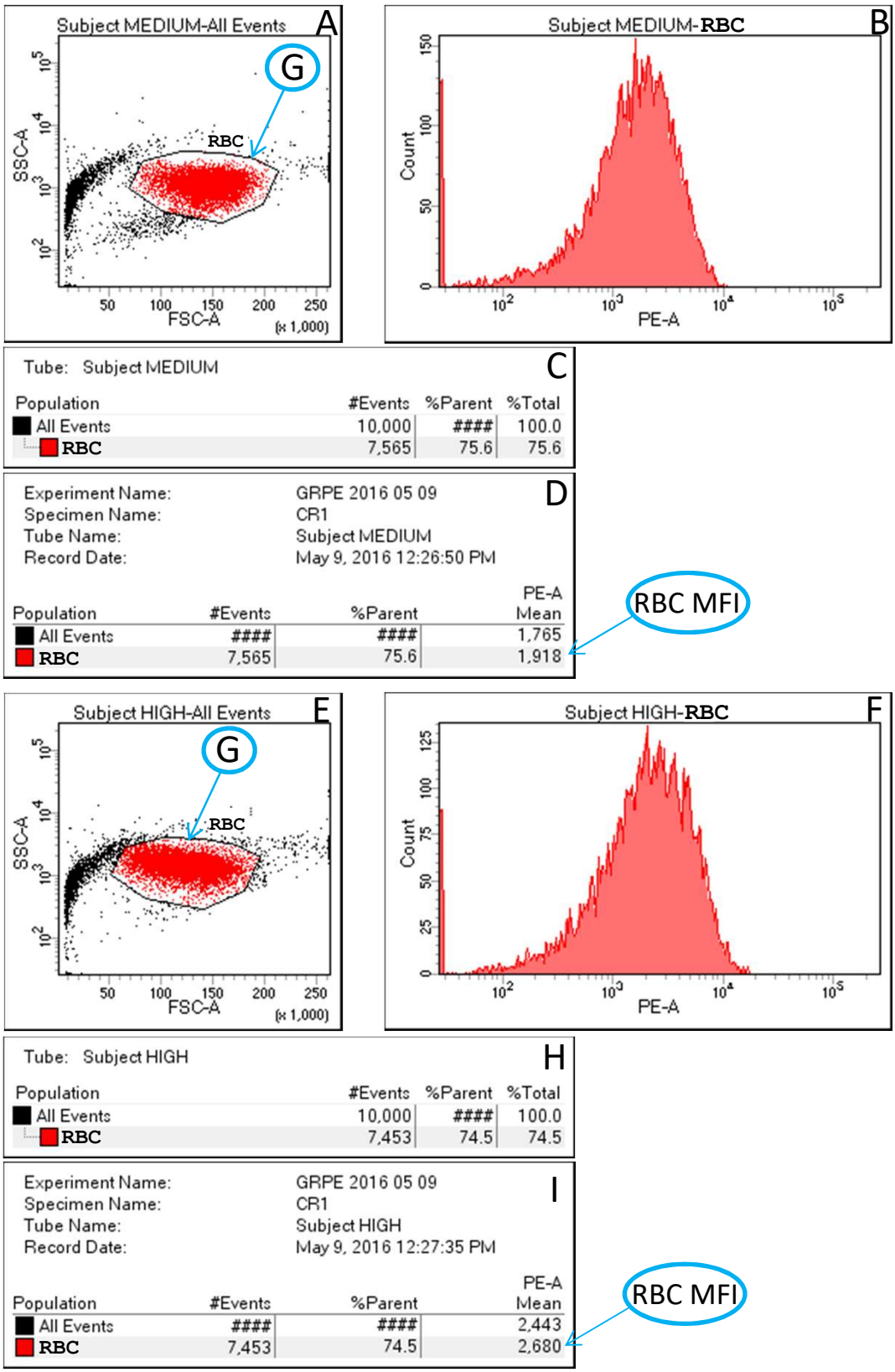
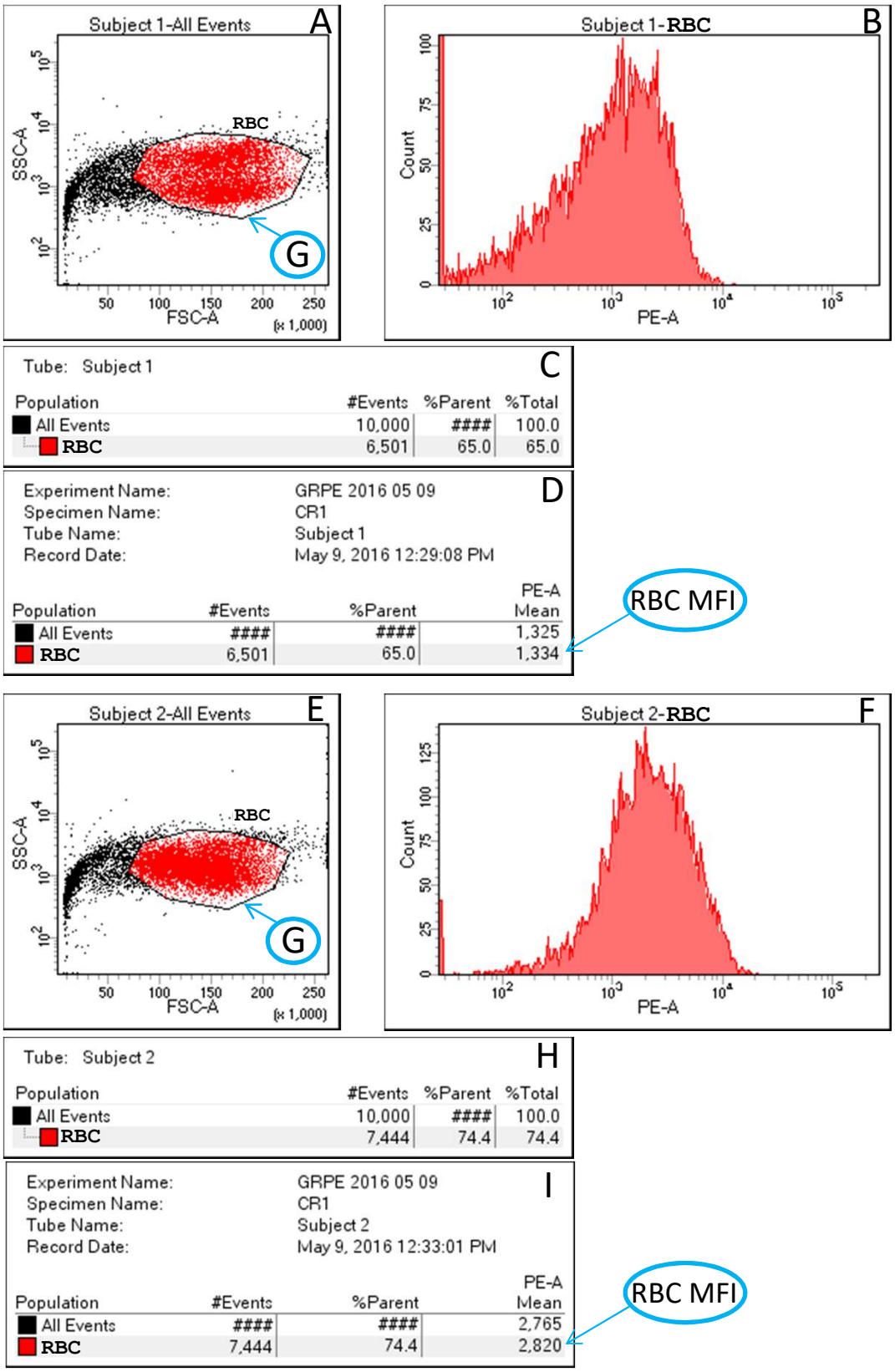


Figure 4

Figure 5

Click here to access/download;Figure;Figure 5 R2 ARTICLE JOVE RBC pdf.pdf



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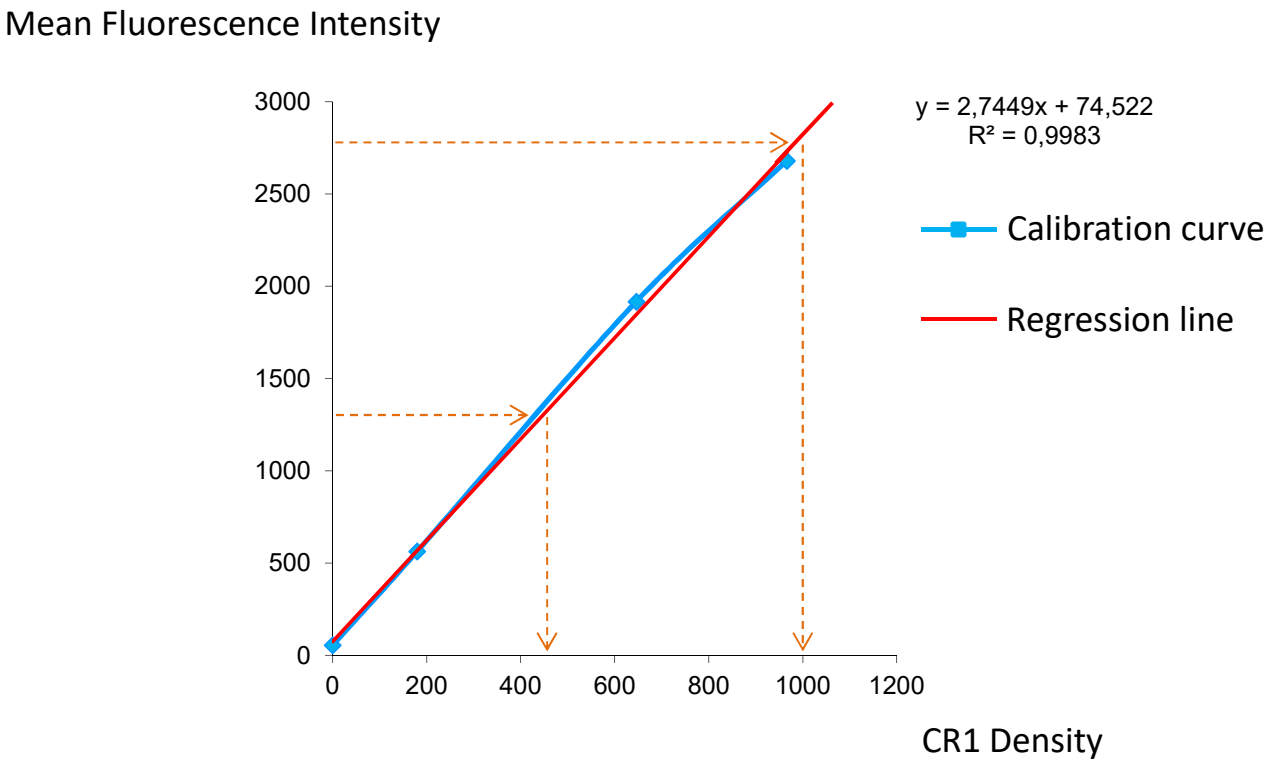

Figure 6

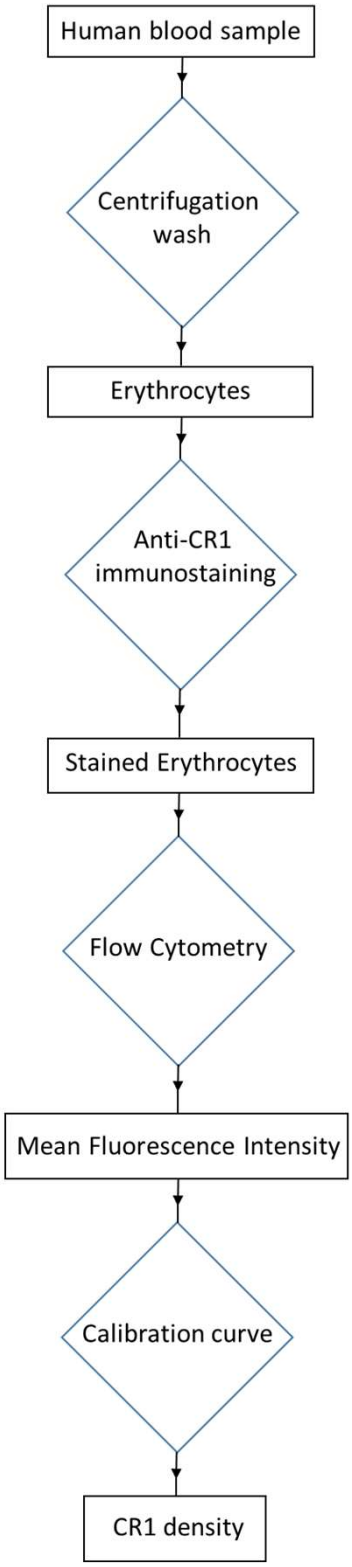

Figure 7

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1000E Barrier Tip	Thermo Fischer Scientific , F-67403 Illkirch, France	2079E	sample pipetting
1-100 µL Bevelled, filter tip	Starlab GmbH, D-22926 Ahrenburg, Germany	S1120-1840	sample pipetting
Biotinylated anti-CR1 monoclonal antibody (J3D3)	Home production of non-commercial monoclonal antibody, courtesy of Dr J. Cook		immunostaining
Blouse			protection
Bovin serum albumin (7,5%)	Thermo Fischer Scientific , F-67403 Illkirch, France	15260037	cytometry
Centrifuge	Thermo Fischer Scientific , F-67403 Illkirch, France	11176917	centrifugation
Clean Solution	BD, F-38801 Le Pont de Claix, France	340345	cytometry
Comorack-96	Dominique DUTSCHER SAS, F-67172 Brumath	944060P	rack
Cytometer Setup & Tracking Beads Kit	BD, F-38801 Le Pont de Claix, France	655051	cytometry
Formaldehyde solution 36.5 %	Sigma Aldrich, F-38070 Saint Quentin Fallavier, France	F8775-25ML	Fixation
10 µL Graduated, filter tip	Starlab GmbH, D-22926 Ahrenburg, Germany	S1121-3810	sample pipetting
LSRFORTESSA Flow Cytometer	BD, F-38801 Le Pont de Claix, France	647788	cytometry
Microman Capillary Pistons	Dominique DUTSCHER SAS, F-67172 Brumath	067494	sample pipetting
Micronic 1.40 mL round bottom tubes	Dominique DUTSCHER SAS, F-67172 Brumath	MP32051	mix

Micropipette Microman - type M25 -	Dominique DUTSCHER SAS, F-67172 Brumath	066379	sample pipetting
Phosphate buffered Saline (PBS)	Thermo Fischer Scientific , F-67403 Illkirch, France	10010031	cytometry
Pipette PS 325 mm, 10 mL	Dominique DUTSCHER SAS, F-67172 Brumath	391952	sample pipetting
powder-free Nitrile Exam gloves	Medline Industries, Inc, Mundelein, IL 60060, USA	486802	sample protection
Reference 2 pipette, 0,5-10 µL	Eppendorf France SAS, F-78360 Montesson, France	4920000024	sample pipetting
Reference 2 pipette, 20-100 µL	Eppendorf France SAS, F-78360 Montesson, France	4920000059	sample pipetting
Reference 2 pipette, 100-1000 µL	Eppendorf France SAS, F-78360 Montesson, France	4920000083	sample pipetting
Rinse Solution	BD, F-38801 Le Pont de Claix, France	340346	cytometry
Round bottom tube	Sarstedt, F-70150 Marnay, France	55.1579	cytometry
Safe-Lock Tubes, 1.5 mL	Eppendorf France SAS, F-78360 Montesson, France	0030120086	mix
streptavidin R-PE	Tebu Bio, F-78612 Le Perray-en-Yvelines, France	AS-60669	immunostaining
Tapered Centrifuge Tubes 50 mL	Thermo Fischer Scientific , F-67403 Illkirch, France	10203001	mix
Vector anti streptavidin biotin	Eurobio Ingen, F-91953 Les Ulis, France	BA-0500	immunostaining
Vortex-Genie 2	Scientific Industries, Inc, Bohemia, NY 111716, USA	SI-0236	mix

Answer to editor

A1 Title reworded to make it concise. Please check.

OK for reworded title

A2 Corresponds to which exon ?
I643T corresponds to exon 13 of *CR1* gene. We have added this to the manuscript as follows:
"in exon 13 encoding SCR 10 (I643T) and in exon 19 encoding SCR16 (Q981H)"

A3 Corresponds to which exon ?
Q981H corresponds to exon 19 of *CR1* gene. We have added this to the manuscript as follows:
"in exon 13 encoding SCR 10 (I643T) and in exon 19 encoding SCR16 (Q981H)"

A4 How do you know the CR1 density ? How do you figure out that these 3 individuals will have three different values ? Is this available along with the kit as control blood ?
At the beginning, the CR1 density of the subjects of the range (standards : low, medium and high) was determined by the Scatchard method using radiolabelled antibodies, studying the free ligand / bound ligand ratio after separation of the erythrocytes in dibutyl phthalate cushion tubes. The standards (low, medium and high) thus determined were used for the calibration curve and made it possible to quantify new standards or sub-standards by our method of cytometry. They correspond to healthy volunteers whose CR1 density is thus known. We did not use a kit.

A5 Please expand
GR corresponds to erythrocyte and GR MFI corresponds to the mean fluorescence intensity of the erythrocytes. GR is the abbreviation for red cells ("**G**lobule **R**ouge" in French).
"GR" has been replaced by "RBC" in the manuscript and in the figures 1, 2, 3, 4 and 5; "or red blood cells (RBC)" has been added to the manuscript.

A6 How do you confirm just with a single run ?
The samples are usually run singly, because the handling error rate is extremely low and when a reagent is missing, the result is abnormal enough to be immediately suspected. In our hands, there is less than 1% handling errors when distributing reagents at the tube level, as the tubes are spaced.
During the development of the method, the use of triplicates did not yield additional accuracy.

A7 How do you ensure that these people will be in the required range ?
We know the density of erythrocyte CR1 of the 3 subjects of the range (standards) expressing respectively a low level (180 CR1 / E), a medium level (646 CR1 / E) and a high

level (966 CR1 / E) thanks to the experiments previously mentioned in point A4. If a problem occurs with an abnormal level of CR1, due to a physiological or technical problem, cytometry fluorescence measurements will not yield a linear calibration curve (R^2 not around 0.99) to quantify CR1 properly.
If a standard is not satisfactory, we consider that the experiment has to be redone.
For subjects whose density is to be quantified, we determine the value according to the equation of the regression line established from the calibration curve. For off-range subjects, we extrapolated the value thanks to the equation, assuming that curve is a straight line.

A8 Some of the shorter steps of the protocol can be combined to show 2-3 action per step. Please see section 1,2, 3 and rearrange the others accordingly.
Section 5 has been rearranged.

We can have a minimum of 1 page and up to 2.75 pages of highlighting including headings and spacings. Please adjust the highlight if needed.
The highlighting has been checked.

A9 Do you add this to the pellet directly ? Do you first disperse the pellet ?
The 20 µL of biotinylated anti-CR1 J3D3 are added to the pellet directly. Then by mixing the tube gently, the pellet is dispersed in the antibody solution.
At step 3.4, “Add 20 µL of biotinylated anti-CR1 J3D3 “ was replaced by “Add 20 µL of biotinylated anti-CR1 J3D3 to the pellet directly”.

A10 Do u place it in rocking ?
The tubes are not placed in rocking.

A11 From here on, please reformat as shown for above steps.
Step : « 3. Erythrocyte immunostaining » was reformated.

A12 New or the one with sample ? Please bring out this clarity throughout the protocol

It is not a new tube. It is the one with sample.

“1.4 mL tube” has been replaced by ”the same tube”.

A13 Is this the wash step ?
Step 3.13 corresponds to the wash step.

A14 Added this for clarity. Please ensure that the protocol is a cohesive story from one step to the next.
“immunostained erythrocyte tubes” is added for clarity.

A15 Do you incubate with the fixation buffer for some time?
The immunostained erythrocytes are fixed by placing them in 450 µl of fixation buffer. They stay there all the time. It is in this buffer that the cytometer reading is done. They can then

be stored in the refrigerator for up to 48 hours to be read again on the cytometer if necessary.

A16 Any particular number of cells ?
The entire cell suspension (450 µL) is transferred to 5 mL round bottom tubes.

A17 Please ensure that the cytometer is added to the table
The cytometer is present in the table at line 12 : LSRFORTESSA Flow Cytometer (catalog number 647788).

A18 Prime the fluidic system How ?
We press the prime button on the console located at the front of the cytometer and wait until the indicator light changes from red to green.
This part has been reworded.

A19 Please reword this part as it matches with previously published literature.
This part has been reworded.

A20 Please check the new step number.
Removed in the new version of the manuscript.

A21 SIP Please expand.
The sample injection port (SIP) is a support (rack) on the cytometer on which the tube containing the cell suspension is placed. It enables the needle of the cytometer to suck up the cell suspension in order to analyze the immunostaining.

A22 Please reword as it matches with the previously published literature.
This part concerning : step 5. Flow cytometry analysis of stained erythrocytes, has been reworded.

A23 Please reword as it matches with previously published literature.
This part concerning : step 5. Flow cytometry analysis of stained erythrocytes, has been reworded.

A24 Please reword as it matches with previously published literature.
This part concerning : step 5. Flow cytometry analysis of stained erythrocytes, has been reworded.

A25 Citation ?
The reference : Scatchard, G. The attractions of proteins for small molecules and ions. *Annals of the New York Academy of Sciences*. **51**(4), 660-672 (1949). has been added to the manuscript.

A26 Please revise the step number.
The step numbers have been revised concerning the legend to figure 1.

A27 Please include the legend here.
The legend to figure 7 has been included in the manuscript.

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Author(s):	Aymric Kisserli 1,2, Sandra Audonnet 3, Valérie Duret 2,4, Brigitte Reveil 2, Thierry Tabary 2,4, Rachid Mahmoudi 5,6 , Jacques Henri Max Cohen 2.

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