# Erythrocyte-Based *Pig-a* Gene Mutation Assay

Jeffrey C. Bemis, Nikki E. Hall and Stephen D. Dertinger

Litron Laboratories www.litronlabs.com

3500 Winton Place, Suite 1B Tele: 585-442-0930

Rochester, NY 14623 Fax: 585-442-0934

Corresponding author e-mail: sdertinger@litronlabs.com

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## Summary

In addition to chromosomal damage, assessment of gene mutation is an important part of genotoxicity testing employed during preclinical safety testing. The *Pig-a* gene mutation assay is based on the loss of function of the *Pig-a* gene, which results in a lack of cell surface expression of specific proteins that are targeted to the surface by GPI anchors. This cell surface phenotype is readily assessed by flow cytometric analysis of red blood cells. This unit describes a procedure for the collection, processing and

analysis of peripheral blood samples using materials supplied in MutaFlow® kits and a common benchtop flow cytometer.

# 1. Introduction

This protocol describes procedures for scoring the frequency of mutant phenotype erythrocytes (RBCs) and mutant phenotype immature erythrocytes (reticulocytes, or RETs) in the peripheral blood of rats using flow cytometry. The method is based on the endogenous *Pig-a* gene whose product is essential for the synthesis of glycosylphosphatidylinositol (GPI) anchors *(1,2)*.

Hematopoietic cells require GPI anchors to attach a host of proteins to their cell surface, e.g., CD24, CD59, and CD55 *(3)*. Importantly, of the genes required to form GPI anchors, only *Pig-a* is located on the X-chromosome. Thus, one inactivating mutation in the *Pig-a* gene can prevent functional anchors from being produced, resulting in cells lacking these proteins on their surface. This phenomenon is illustrated in **Fig. 1**. Thus, cells without these cell surface markers can be differentiated from wild-type cells by using fluorescent antibodies and can represent a reliable phenotypic marker of *Pig-a* mutation *(4-10)*. This is further supported by genetic sequencing experiments demonstrating in both cell culture (*11,12*) and whole animal models (*13-15*), that loss of function mutations in the *Pig-a* gene correspond to the GPI anchor null phenotype analyzed by the method described here.

Litron Laboratories developed a sample processing method that incorporates antibody/nucleic acid stain labeling of cells and immunomagentic separation followed by analysis using flow cytometry *(16)*. This methodology, commercially available as MutaFlow®, is illustrated in **Fig. 2**. Briefly, blood samples are processed through Lympholyte®-Mammal Solution to remove the majority of leukocytes and platelets. Cells are then incubated with Anti-CD59-PE (to label wild-type (wt) RBCs) and Anti-CD61-PE (to label remaining platelets). Antibody-labeled samples are incubated with Anti-PE MicroBeads, which bind to these antibodies.

A small fraction of each sample is stained with a nucleic acid dye (to differentiate leukocytes and RETs from mature RBCs). This dye solution also includes fluorescent Counting Beads and these “Pre-Column” samples are analyzed on a flow cytometer to capture Cell:Bead ratios.

The remaining portion (majority) of the blood sample is applied to a Miltenyi LS Column that has been suspended in a magnetic field. These columns selectively retain wt cells, whereas *Pig-a* mutants (without CD59 on their surface) pass through the columns.

Eluates are collected, concentrated by centrifugation and stained with a nucleic acid dye in order to differentiate leukocytes and RETs from mature RBCs. This dye solution also includes fluorescent Counting Beads and these “Post-Column” samples are analyzed on a flow cytometer to capture Mutant Cell:Bead ratios.

From the Pre-- and Post--Column analyses, the following values are calculated:

a. RET percentage, an index of bone marrow toxicity

b. Frequency of mutant-phenotype RBCs

Note that upon acute mutagen exposure this index of genotoxicity is not expected to reach a maximal response until the entire cohort of circulating RBCs has turned over (approximately 42-65 days for rats; *17-19*).

c. Frequency of mutant-phenotype RETs

Note that upon acute mutagen exposure this index of genotoxicity reaches a maximal value faster than the frequency of mutant phenotype RBCs (approximately 2 weeks), since RETs are turned over at a much faster rate than the total RBC pool.

A comprehensive collection of investigations on the *Pig-a* assay, including results from an international validation trial and discussion of the role of this assay for hazard identification and risk assessment *(20)* can be found in a special issue of *Environmental and Molecular Mutagenesis* dedicated to the *Pig-a* assay. The method described here utilizes standard flow cytometry tubes for sample processing and analysis. Information on a more efficient method using 96 well plates can be found at www.litronlabs.com. Additional information and video clips of specific processing steps can be accessed at www.litronlabs.com.

## 2. Materials

As noted, certain reagents and materials described below are from commercially available MutaFlow® kits (Litron Laboratories, Rochester, NY). Additional materials and supplies that are required, but not supplied with the kits, are specifically noted.

### *2.1 Reagents and solution preparation*

The working solutions are made up fresh, daily. The formulas are based on the volumes required for one sample and should be scaled up appropriately (*see* **Note 1**).

1. Anticoagulant Solution (MutaFlow$^{PLUS}$ kit)
2. Heat-inactivated fetal bovine serum (FBS)
3. Buffered salt solution (MutaFlow$^{PLUS}$ kit)

   Buffered salt solution + 2 % FBS: Combine 34.3 mL of Buffered salt solution with 0.7 mL of FBS. Filter sterilize with 0.2 µm filter and store at 2 °C to 8 °C.
4. CountBright$^{TM}$ Absolute Counting Beads (cat # C36950, Invitrogen)
5. Anti-Rat CD59-PE Antibody (MutaFlow$^{PLUS}$ kit) and Anti-Rodent CD61-PE Antibody (MutaFlow$^{PLUS}$ kit)

   Working antibody solution: Combine 65 µL of Buffered salt solution + 2 % FBS with 30 µL anti-rat CD59-PE antibody and 5 µL anti-rodent CD61-PE antibody. Pipette to mix and store protected from light, at 2 °C to 8 °C (*see* **Note 2**).
6. Nucleic Acid Dye Solution (MutaFlow$^{PLUS}$ kit)

   Working nucleic acid dye plus counting beads solution: Add 45 µL of stock nucleic acid dye solution and 45 µL of CountBright$^{TM}$ Beads to 1.41 mL of Buffered Salt Solution + 2 % FBS. Pipette to mix and store at room temperature, protected from light (*see* **Note 3**).

7. Lympholyte®-Mammal (Cedarlane Laboratories)
8. Anti-PE MicroBeads (cat # 130-048-801, Miltenyi Biotech)

   Working Anti-PE MicroBead suspension: Add 25 µL of Anti-PE MicroBeads to 75 µL of Buffered Salt Solution + 2 % FBS. Pipette to mix and store at 2 °C to 8 °C, protected from light.

### *2.2 Supplies and Specialized Equipment*

1. Centrifuge with swinging bucket rotor
2. 15 mL polypropylene centrifuge tubes
3. Microcentrifuge tubes
4. -10 °C to -30 °C freezer
5. 2 °C to 8 °C refrigerator
6. 37 °C incubator or water bath
7. Flaked/chipped ice
8. Flow cytometer capable of 488 nm excitation
9. Flow cytometry tubes
10. 0.2 µm filters - various types: syringe, flask, etc.
11. Heparin-coated capillary tubes (optional)
12. LS Columns (cat # 130-042-401, Miltenyi Biotech)
13. MidiMACS™ or QuadroMACS™ Separator (cat # 130-042-302; 130-090-976, Miltenyi Biotech)
14. Aspiration Device

Preparation: It is very important to carefully control and standardize aspirations, especially the last aspiration (**step 2 of section 3.9**). To achieve this, fashion an aspirator with a bridge that controls the depth to which the tip can reach when aspirating from a standard 15 mL centrifuge tube (**Fig. 3**). With this bridge, the aspirator will leave a consistent and low volume of supernatant across all tubes and not contact the bottom of the tube or disturb the cell pellet. The goal for the volume of supernatant left after the final aspiration is a consistent volume within the range of 20 µL to 50 µL. This value is required to make mutant cell frequency calculations (*see* **Section 3.12**).

15. Potassium EDTA Microtainer tubes (cat # 365974, Becton Dickinson, optional – for storage of blood samples and/or shipping samples off-site)
16. Exakt-Pak® shipping containers (e.g., cat # MD8204V20, EXAKT Technologies, optional – for shipping samples off-site)
17. Icepacks (e.g., Koolit® Foam Bricks, cat # 306F, Cold Chain Technologies, optional – for shipping samples off-site)
18. Preliminary Materials Preparation: Label all necessary tubes and vials with sample IDs. At least 2 flow cytometry tubes, 3 centrifuge tubes (15 mL), and 2 microcentrifuge tubes are required per sample.
19.

Template Preparation: Data acquisition template files are available from Litron (download from www.litronlabs.com or email invivopigatechsupport@litronlabs.com for

information), but are specific to CellQuest™ Pro or FACSDiva™ software. **Figure 4** shows actual screen images of the CellQuest™ Pro and FACSDiva™ template graphs. Flow cytometry operators who are not using CellQuest™ Pro or FACSDiva™ software should find the following instructions and graphics valuable for constructing their own data acquisition and analysis template.

We recommend that if you are using FACSDiva™ software, set the fluorescence parameter to "Height" rather than "Area" as the cell populations are tighter using this parameter. The Internal Calibration Standard (ICS) may be run using Single-Color Compensation controls and auto-compensation if available with your software package.

1. Defining Gates:
    - G1 = R1 = "Single Cells"
    - G2 = R2 = "Total RBCs"
    - G3 = R3 = "Beads"
    - G4 = R1 and R2 and R3 = "Single Cells" and "Total RBCs" and NOT "Beads"

2. Gate and parameters for each Plot:

| | | |
|---|---|---|
| Plot A | No Gate | SSC-H vs. FSC-H |
| Plot B | G1 | FL1-H vs. FSC-H |
| Plot C | G4 | FL1-H vs. FL2-H |
| Plot D* | No Gate | FL4-H vs. FSC-A **or** FL3-H vs. SSC-H |

*If you have a second, red diode laser, use FL4 and either FSC or SSC for Plot D. Otherwise, use FL3. SSC is needed for single-laser analysis to provide optimal resolution when not using a red diode laser (and FL4).

3. Quadrant Key for Plot C:

   UL = mutant RETs

   UR = wild-type RETs

   LL = mutant mature RBCs (i.e., mutant normochromatic erythrocytes [NCEs])

   LR = wild-type mature RBCs (i.e., wild-type normochromatic erythrocytes [NCEs])

4. Alternate names for detectors:

| | | |
|---|---|---|
| Green | FL1 | FITC |
| Orange | FL2 | PE |
| Red | FL3 | PerCP-Cy5.5 |
| Far Red | FL4 | APC |

5. Save the template file. This template file should be suitable for all analyses. To ensure consistency of data, once the regions, compensation, etc have been set based on the ICS, it is preferable that no changes are made to the location and size of the regions between samples that are analyzed on the same day.

# 3. Methods

### *3.1 Blood Collection*

1. Aliquot 100 µL of kit-supplied Anticoagulant Solution into labeled microcentrifuge tubes, one for each blood sample. Refrigerate until use. This can be done before the day of blood collection.
2. On the morning of leukodepletion, gently shake the Lympholyte®-Mammal bottle and allow for air bubbles to disappear. Aliquot 3 mL into labeled 15 mL polypropylene centrifuge tubes, one for each blood sample. Protect from light and allow the aliquots to equilibrate to room temperature before use.
3. Obtain approximately 100 µL of blood per animal, taking care to collect free-flowing blood (*see* **Notes 4--6**).
4. Immediately upon collection of each blood sample, transfer 80 µL into the labeled microcentrifuge tube containing 100 µL of Anticoagulant Solution.
5. Refrigerate blood samples in Anticoagulant Solution as soon as possible, although they can be stored at room temperature for up to 4 hours before refrigeration. Process samples through Lympholyte®--Mammal (*see* **Section 3.2**) within 8 hours of collection.

### *3.2 Leukodepletion and Platelet Removal*

1. Using a pipettor, remove the entire contents of the microcentrifuge tube (80 µL blood plus 100 µL Anticoagulant Solution) and gently layer on top of the pre-aliquoted, room temperature Lympholyte®-Mammal (*see* **Fig. 5**). Repeat this step for the remaining samples (*see* **Note 7**).
2. Centrifuge the samples at 800 *x g* for 20 minutes at room temperature. Maintain tubes at room temperature (*see* **Note 8**).

3. Holding the tube upright, aspirate supernatants, removing as much as possible without disturbing the loosely packed pellet.
4. Add 150 µL cold Buffered Salt Solution (that does NOT contain FBS) directly to each pellet (*see* **Note 9**). Gently pipette up and down to resuspend cells until there is no visual evidence of aggregation.
5. Transfer the entire contents of each tube to a new corresponding 15 ml tube containing 5 ml cold pre-aliquoted Buffered Salt Solution + 2% FBS. Repeat for each sample.
6. Centrifuge at 235 x g for 10 minutes at room temperature. After centrifugation, aspirate as much of the supernatant as possible (*see* **Note 10**).
7. Add 150 µL cold Buffered Salt Solution (that does NOT contain FBS) directly to each pellet (*see* **Note 11**). Gently pipette up and down to resuspend cells until there is no visual evidence of aggregation.
8. Proceed immediately to **Section 3.3**. Otherwise store the sample at 4 (*see* **Note 12**).

***3.3 Sample Labeling***

Once the samples are fully labeled, it is advisable to analyze them within 3 hours otherwise loss of resolution of the signal in FL1 and/or FL2 channels may be experienced. Therefore, it is important to proceed through steps 1 of section 3.3. to step 7 of section 3.9 in sequence; so, a careful staging of the samples and of the workday is critical. See the work flow diagram at the end of the protocol for advice on personnel and time requirements for the processing steps in order to aid in experimental planning. At least one of the 15 mL tubes from step 1 of section 3.3 below, will need to be saved.

The cells remaining in this tube will be used in step 1 of section 3.7 to make the Part A portion of the ICS. These unstained cells will become the “mutant mimicking cells”.

1. Set a pipettor to 160 µL and pipette the first pellet (and Buffered Salt Solution previously added) up and down to resuspend the cells (*see* **Note 13**).
2. Carefully transfer 160 µL of the resuspended cells directly into the Working Antibody Solution in the labeled microcentrifuge tube, carefully pipetting up and down to mix. See the images in **Fig. 6** for an example (*see* **Note 14**).
3. Repeat steps 1 and 2 of section 3.3 for the remaining samples. Use a new pipette tip for each sample.
4. Incubate cells with Working antibody solution for 30 minutes at 2 °C to 8 °C, covered to protect from light.
5. Save at least one 15 mL tube and its remaining contents (preferably from a vehicle control) at 2 °C to 8 °C (*see* **Note 15**).
6. During the incubation, aliquot 10 mL cold Buffered Salt Solution + 2 % FBS to labeled 15 mL centrifuge tubes, one for each sample. Store at 2 °C to 8 °C until needed in **Section 3.4**.

### *3.4 Wash Labeled Cells out of Working Antibody Solution*

1. After the incubation, resuspend the cells by gently pipetting the contents up and down and transfer directly into the cold, pre-aliquoted Buffered Salt Solution + 2 % FBS prepared in step 6 of section 3.3 (*see* **Note 16**).
2. Centrifuge at 340 *x g* for 5 minutes at room temperature. After centrifugation, maintain at room temperature.

3. Holding the tube upright, aspirate supernatants, removing as much of the supernatant as possible without aspirating the pellet (*see* **Note 17**).

### *3.5 Incubate with Working Anti-PE MicroBead Suspension*

1. Pipette the Working Anti-PE MicroBead Suspension, to mix.
2. Add 100 µL to a sample tube by washing down the inside of the tube with MicroBead suspension, starting at about 0.5 cm above the pellet, carefully pipetting up and down to mix. Change the pipette tip and repeat for the remaining samples (*see* **Note 18**).
3. Incubate cells in a non-insulating rack for 30 minutes at 2 °C to 8 °C, protected from light.
4. After incubation, add 10 mL of cold Buffered Salt Solution + 2 % FBS to each tube. Cap tightly, invert each tube, and while inverted gently tap the bottoms to dislodge any settled cells. Repeat for all samples.
5. Centrifuge at 340 *x g* for 5 minutes at room temperature. After centrifugation, keep at room temperature.
6. Holding the tube upright, aspirate the supernatants, removing as much of the supernatant as possible without aspirating the pellet.

### *3.6 Stain Pre-Column Samples*

1. Add 1.0 mL cold Buffered Salt Solution + 2 % FBS directly to a pellet, carefully pipetting up and down to mix (*see* **Note 19**).
2. Transfer exactly 10 µL of this suspension to the labeled flow cytometry tube containing 990 µL of room temperature Working Nucleic Acid Dye Plus Counting Beads Solution. Shake gently or pipette to mix (*see* **Note 20**).

3. Repeat steps 1 and 2 for the remaining samples. Maintain them at room temperature until each sample has been processed to this point (*see* **Note 21**).
4. Maintain the cells remaining in the centrifuge tubes in the dark at 2 °C to 8 °C or on ice until proceeding to **Section 3.8** (*see* **Note 22**).

### *3.7 Prepare Instrument Calibration Standard (ICS) and Incubate Pre-Column Samples*

1. Prepare Part A of the ICS. Do this by retrieving the leukodepleted sample that was stored in step 5 of section 3.3 and gently resuspend cells by pipetting up and down. Transfer 5 µL of this sample to the labeled flow cytometry tube containing 500 µL of room temperature Working Nucleic Acid Dye Plus Counting Beads Solution. Pipette gently to mix the suspension .
2. Incubate the Pre-Column samples (including Part A of the ICS) in Working Nucleic Acid Dye Plus Counting Beads Solution for 30 minutes at 37 °C in the dark.
3. After incubation, transfer the samples to ice and protect from light. Ensure that the tubes are surrounded by flaked/chipped ice, not resting on top (*see* **Note 23**).

### *3.8 Column Separation*

Perform this section and the next (steps 1 of section 3.8 through step 7 of section 3.9) in batches of up to 8 samples (*see* **Note 24**).

1. Insert LS Columns into either a MidiMACS™ or QuadroMACS™ Separator. Place a reservoir or other appropriate vessel under the columns to catch the eluate.

2. Gently add 3 mL cold Buffered Salt Solution + 2 % FBS to each column reservoir to pre-wet it (*see* **Note 25**). Once the pre-wet volume has stopped dripping from the column, remove the reservoir and discard the collected rinse.
3. Place a clean, labeled 15 ml centrifuge tube under each column to collect the sample eluate (*see* **Note 26;** *see* **Fig. 7**).
4. Take the remainder of the Pre-Column sample (from step 4 of section 3.6) and carefully pipette up and down to resuspend the cells and MicroBeads without creating bubbles. Gently add the 1 mL of sample into the appropriate pre-wet LS Column reservoir. Repeat for additional column reservoirs (*see* **Note 27**).
5. When the sample has fully entered the column (e.g., when the sample cannot be seen above the column matrix), slowly add 5 mL cold Buffered Salt Solution + 2 % FBS, to the column reservoir as a column wash. Repeat for additional column reservoirs (*see* **Note 28**).
6. The eluates will appear clear or nearly so because the vast majority of the cells will be trapped in the column (*see* **Fig. 8**). Once an eluate has been collected, store it in the dark at 2 °C to 8 °C. Discard each LS Column after use – DO NOT reuse the columns.
7. Repeat steps 1 --5 until all Pre-Column samples in the batch of 8 have been through the column.

***3.9 Centrifuge and Stain Post-Column Samples***

1. Centrifuge a set of 8 eluate tubes from step 3.8.7 at 800 *x g* for 5 minutes (*see* **Note 29**).

2. Holding the tube upright, use the aspiration device constructed in Section 2.2 to carefully aspirate supernatants starting at the top (meniscus) and working downwards to prevent disturbing the pellet (*see* **Note 30**).

3. Gently tap the pellets loose (*see* **Note 31**).

4. Add 300 µL of Working Nucleic Acid Dye Plus Counting Beads Solution at room temperature, to each Post-Column sample (*see* **Note 32**).

5. Once a sample has been resuspended, transfer it to a flow cytometer tube. Incubate all the samples in the dark at 37 °C for 15 minutes.

6. After incubation, transfer the tubes to ice and protect from light. Store on ice for at least 5 minutes, but no more than 3 hours, before flow cytometric analysis (*see* **Note 33**).

7. Repeat the steps of sections 3.8 and 3.9, with the remaining batches of up to 8 samples.

***3.10 Flow Cytometric Analysis: Instrument Calibration***

1. Before analyzing samples, ensure that the flow cytometer is working properly. Follow the manufacturer's instructions for the appropriate setup and quality control procedures. Download the data acquisition template file from www.litronlabs.com or create your own based on instruction in Section 2.3.7 (see **Notes 34--37**).

2. Prepare the ICS by vigorously resuspending (pipetting) both Part A from step 1 of section 3.7 and Part B, which is obtained from any fully-stained, Pre-Column vehicle control sample. Combine equal volumes of each into a flow cytometry tube (e.g., 200 µL of Part A and 200 µL of Part B; *see* **Note 38**).

3. Immediately after creating the ICS, place it on the flow cytometer.

4. Threshold on FSC so that remaining platelets and other sub-cellular debris are eliminated. If your instrument is capable, threshold on both FSC and SSC, but be careful not to set the values so high that Counting Beads are thresholded out. In Plot A, adjust the “Single cells” region, so that it closely defines the major population of single, unaggregated erythrocytes. The resulting plot should look similar to the plot in **Fig. 9**.

5. Viewing Plot B, adjust the “Total RBCs” region, to eliminate contaminating leukocytes (those cells with high nucleic acid dye fluorescence). Together with the “Single Cells” region, this region is used to eliminate leukocytes from RBC-based measurements. The resulting plot should look similar to the plot in **Fig. 10**.

6. Viewing Plot D, adjust the FL4 (or FL3) PMT voltage so that Counting Beads fall within the “Beads” region. Adjust the position and size of the region as necessary. The resulting plot should look similar to one of the plots in **Fig. 11**.

7. Viewing Plot C, adjust PMT voltages so that mutant phenotype, mature RBCs (lower-left quadrant; LL) are in the first decade of FITC and PE fluorescence. The resulting plot should look similar to the plot in **Fig. 12**.

8. Viewing Plot C, adjust compensation so that the green (FITC) component of the PE label is eliminated. This is evident when the wild-type phenotype, mature RBCs (lower-right quadrant; LR) are at the same FITC fluorescence intensity as the mutant phenotype, mature RBCs (LL). See the before and after plots in **Fig. 13** (*see* **Note 39**).

9. Viewing Plot C, adjust compensation so that the orange (PE) component of the nucleic acid dye is eliminated. This is evident when the mutant-phenotype RETs (upper-left quadrant, UL) are positioned directly above the mutant-phenotype, mature RBCs (LL). It is appropriate for the cells with the highest FITC fluorescence to lean over to the

right, as shown in the center plot in **Fig. 14**. If using a digital instrument capable of biexponential scaling, it can be useful to temporarily view the PE fluorescence with biexponential scaling. This view can highlight overcompensation that may not be evident otherwise. The resulting plot should look similar to the one shown in **Fig. 15**.

10. Viewing Plot C, adjust the quadrant's position to ensure it is appropriate. Use a conservative approach, i.e. align the vertical line of the quadrant tight against the mutant cell population, for scoring cells as mutant phenotype RBCs (i.e., these cells need to exhibit very low PE fluorescence, similar to that of the mutant mimics). The resulting plot should look similar to the plot on the left, in **Fig. 16** (*see* **Note 40**).

### *3.11 Flow Cytometric Analysis: Experimental Samples*

1. After analyzing the ICS sample and before analyzing experimental samples, place a tube of water on the flow cytometer and run for approximately 5 minutes to clear the lines of mutant-mimicking cells.

2. Having determined the appropriate PMT voltages and compensation settings with the ICS, keep these parameters constant when proceeding to the analysis of experimental samples. To ensure consistency of data, it is preferable that no changes be made to the location and size of the regions/quadrants between samples.

3. Remove the first Pre--Column sample from ice and pipette up and down until the cells and Counting Beads are well resuspended. Immediately place on the flow cytometer and begin acquiring data. Repeat until all Pre--Column samples have been analyzed (*see* **Note 41**).

4. Once all Pre--Column samples are analyzed, run a tube of water for approximately 5 minutes to flush the system before continuing to the next step.

5. Remove the first Post--Column sample from ice and pipette vigorously up and down until well suspended. Place on the flow cytometer, and begin acquiring data. Repeat until all Post--Column samples have been analyzed (*see* **Note 42**).

### *3.12 Mutant Cell Frequency Calculations*

The data used to calculate % RET and mutant-phenotype cell frequencies are derived from both Pre-Column and Post-Column analyses.

Abbreviations:

- RETs = reticulocytes, RNA-positive fraction of total erythrocytes
- mature RBCs = RNA-negative fraction of total erythrocytes
- RBCs = total erythrocytes, includes both RNA--positive and negative fractions
- UL = number of gated events occurring in Plot C's upper left quadrant, defined as mutant RETs
- UR = number of gated events occurring in Plot C's upper right quadrant, defined as wild--type RETs
- LL = number of gated events occurring in Plot C's lower left quadrant, defined as mature mutant RBCs
- LR = number of gated events occurring in Plot C's lower right quadrant, defined as mature wild-type RBCs
- Counting Beads = number of events occurring in Plot D's Counting Bead region

Variables Related to Sample Volumes:

- *a* = Starting volume of antibody-labeled blood (µL), **Step 1 of section 3.6**; usually 1000 µL

- *b* = Volume of antibody-labeled blood added to Working Nucleic Acid Dye Plus Counting Beads Solution (µL), Step 2 of section 3.6.; usually 10 µL
- *c* = Volume of Working Nucleic Acid Dye Plus Counting Beads Solution used to prepare Pre-Column samples (µL), step 2 of Section 3.6.; usually 990 µL
- *d* = **LAB-SPECIFIC** value: the supernatant volume remaining in Post-Column samples following the final centrifugation and aspiration (µL), step 2 of Section 3.9; should be between 20 and 50 µL
- *e* = Volume of Working Nucleic Acid Dye Plus Counting Beads Solution added to each Post-Column sample (µL), step 4 of Section 3.9; usually 300 µL

Calculations Based on Sample Volume and Dilution Variables:

- *f* = Cell Dilution Factor = (b + c) / b
- *g* = Cell Concentration Factor = (a – b) / (d + e)
- *h* = Bead Dilution Factor = (e * 100) / (d + e)

Pre-Column Data:

- *i* = UL
- *j* = UR
- *k* = LL
- *l* = LR
- *m* = Counting Beads

Calculations Based on Pre-Column Data:

- *n* = Pre-Column RBC to Counting Bead Ratio = (*i* + *j* + *k* + *l*) / *m*
- *o* = Pre-Column RET to Counting Bead Ratio = (*i* + *k*) / *m*
- ***p* = %RET = (*i* + *j*) / (*i* + *j* + *k* + *l*) * 100**

Post-Column Data:

- *q* = UL
- *r* = LL
- *s* = Counting Beads

Calculations Based on Pre-- and Post--Column Data:

- *t* = Total RBC Equivalents = *n* * *s* * *f* * *g* * 100 / *h*
- *u* = Total RET Equivalents = *o* * *s* * *f* * *g* * 100 / *h*
- ***v* = Number of Mutant RBCs per $10^6$ Total RBCs = (*q* + *r*) / *t* * $10^6$**
- ***w* = Number of Mutant RETs per $10^6$ Total RETs = *q* / *u* * $10^6$**

*See* **Note 43**.

# 4. Notes

1. All solution preparation should be performed under sterile conditions in order to keep reagent vials from becoming contaminated. When working with any "bead" solution, be sure to sufficiently resuspend the particles before use and do not sonicate or vortex the stock or working solutions that contain beads.
2. Aseptically transfer 100 µL of the Working Antibody Solution to labeled microcentrifuge tubes, one for each sample. The Working Antibody Solution is light sensitive, therefore cover these tubes with foil and store at 2 °C to 8 °C until needed.
3. Aseptically transfer 990 µL of Working Nucleic Acid Dye Plus Counting Beads Solution to one labeled flow cytometry tube for each Pre-Column sample. Periodically pipette the Working Nucleic Acid Dye Plus Counting Beads Solution up and down to ensure the Counting Beads do not settle. Aseptically transfer 500

µL of Working Nucleic Acid Dye Plus Counting Beads Solution to a flow cytometry tube for the ICS sample (Part A). Cover the flow cytometry tubes containing the Working Nucleic Acid Dye Plus Counting Beads Solution with foil and store at room temperature until needed.

4. Use an IACUC approved method to collect blood. To prevent platelet activation and cellular aggregation, it is important that the blood is free flowing. For instance, if planning to collect blood by nicking the tail vein with a surgical blade, it is important to warm the animals under a heat lamp for several minutes. Once the blood starts flowing, use a heparin-coated capillary tube to collect approximately 100 µL. If planning to collect blood with a small gauge needle and syringe, it is important to first coat the inside of the needle/syringe with a small amount of Anticoagulant Solution. Do not overfill the needle/syringe with excessive Anticoagulant Solution such that blood is overly diluted at this point. Rather, a ratio of one part Anticoagulant Solution to 9 parts whole blood is ideal. If not planning to label and analyze blood on the same day it is collected, or if you plan to ship blood samples to an off--site facility, it is preferable to transfer whole blood into EDTA Microtainer tubes.
5. Blood Storage Guidance: It is possible to store refrigerated blood samples for up to 3 days after blood collection. If storing for subsequent labeling and analysis, collect blood as described in Section 3.1 and transfer each whole blood sample (approximately 100 µL) into Potassium EDTA Microtainer tubes (e.g., BD cat # 365974). Store at 2 °C to 8 °C until blood dilution, leukodepletion, and platelet removal (*see* **Section 3.1- – 3.2**). Following a recent update to the method, it is

now possible to freeze blood samples for longer-term storage (*21*). Instructions and supplies to perform this procedure can be obtained from Litron Laboratories.

6. Sample Shipping Guidance: It is also possible to transport blood for off-site labeling and analysis. Blood samples collected into Potassium EDTA Microtainer tubes can be shipped overnight, but they must be kept cold, not frozen. Litron recommends using Exakt--Pak® shipping containers (e.g., cat # MD8204V20) and icepacks (e.g., Cold Chain Technologies cat # 306F – Koolit® Foam Brick) frozen at -20 °C. To prevent freezing of the blood samples, make sure icepacks do not come into direct contact with the sample tubes.
7. Perform leukodepletion and subsequent washing steps at room temperature, and process all samples through steps 1 –8 of section 3.2, together.
8. When removing from centrifuge, be aware that the resulting cell pellets will not be hard packed, therefore avoid tapping or bumping the tubes in a way that will cause the pellets to loosen or become dislodged from the bottom of the tube.
9. Do not let the solution run down the side of the tube as that can re-introduce platelets and/or white blood cells that adhered to the inside of the tube.
10. Take care not to disturb the pellets.
11. Do not let the solution run down the side of the tube as that can re-introduce platelets and/or white blood cells that adhered to the inside of the tube.
12. These transferred cells can be stored at 2 °C to 8 °C up to 24 hours before proceeding with Section 3.3. Samples give similar results up to 24 hours, we have not tested beyond 24 hours.

13. Continue as necessary until there is no visual evidence of aggregation – 10 times is usually sufficient. Steps 3.3.1 and 3.3.2 can be performed at room temperature as long as processing of all samples occurs in less than 10 minutes. If it will take longer, maintain tubes on ice during processing.
14. Take care not to splash cells onto the side of the tubes. Continue pipetting up and down to ensure adequate mixing – 10 times is usually sufficient. Ensure that all cells come into full contact with the Working Antibody Solution. Keep the microcentrifuge tubes, both before and after addition of cells, protected from light as Working Antibody Solution is light sensitive.
15. This will become Part A of the ICS sample. There should be approximately 20--30 uL remaining in the tube after step 2 of section 3.3..
16. Be sure to only transfer cells that have been in contact with Working Antibody Solution for the entire incubation period. For instance, do not transfer blood that may have been on the side of the microcentrifuge tube. Cap the centrifuge tubes and invert to mix.
17. The goal is to leave a minimal amount of supernatant behind.
18. Make sure not to splash cells high onto the sides of the tube. Continue as necessary until there is no visual evidence of aggregation – 10 times is usually sufficient.
19. Be sure to not splash cells high onto the sides of the tube. Continue as necessary until there is no visual evidence of aggregation – 4 times is usually sufficient.

20. This dilution is critical in determining the final mutation frequencies, so be careful to transfer this exact amount.
21. These samples represent the **Pre-Column** samples. They will be incubated after preparing the ICS (see **Section 3.7**).
22. These samples will be processed further (see **Section 3.8**) and are now referred to as **Post-Column** samples.
23. Store on ice for at least 5 minutes, but no more than 3 hours, before flow cytometric analysis.
24. Avoid creating bubbles when adding samples or Buffered Salt Solution + 2 % FBS to the column. Air bubbles can block the column and prevent the eluate from passing through. For this same reason, once wet, the column should not be allowed to dry. That is why it is important to add the next solution/suspension as soon as the previous one has fully entered the column (i.e., is no longer in the column reservoir).
25. Be careful to avoid creating bubbles, and avoid disturbing the column matrix.
26. Ensure that the bottoms of the columns are inside the open tops of the centrifuge tubes.
27. Be careful not to disturb the top of the column matrix.
28. Be careful to avoid creating bubbles, and avoid disturbing the column matrix. The entire elution process should occur by the force of gravity only; DO NOT force the Buffer through the column with a plunger or other device. It takes approximately 5 minutes for the sample(s) and wash(es) to pass through the column and for the eluate(s) to collect in the centrifuge tube(s).

29. After centrifugation, the pellet will be small and difficult to see. This is normal.
30. It is critical that all samples have the same volume, and it is important to understand the average volume left in tubes.
31. Be careful that supernatants are not splashed high onto the sides of the tubes, as this may result in cells that do not come into contact with Working Nucleic Acid Dye Plus Counting Beads Solution.
32. Pipette to mix Working Nucleic Acid Dye Plus Counting Beads Solution prior to adding to the first sample, and to ensure a homogenous suspension of Counting Beads, pipette to mix after adding to every 4 or 5 samples. Carefully pipette up and down to resuspend the cells and Counting Beads, taking care not to splash onto the side of the tubes.
33. Ensure that the tubes are buried in the flaked/chipped ice, not resting on top. Samples can suffer from loss of cell integrity and resolution of signal if not analyzed within the recommended 3 hour time period. If you are processing a large number of samples, consider staggering the groups to allow sufficient time to process and analyze within stated time limits.
34. It is advisable to ensure the appropriate template is running on your flow cytometer prior to processing samples. Do not wait until samples are ready for analysis before downloading or creating the template.
35. For analog instruments, such as a BD FACS Calibur™, when analyzing ICS and Pre-Column Samples in tubes, an event rate of approximately 2,000 – 3,500 events per second is recommended. This is usually achieved with a High fluidics rate setting. Use a stop mode based on the length of time needed to acquire at

least 1,000 Counting Beads. Some initial experimentation may be required to determine the specific time for each flow cytometer, but 1 minute is usually sufficient when using a High fluidics rate.

36. For digital instruments, such as the BD FACS Canto™ II, when analyzing ICS and Pre-Column Samples in tubes, an event rate of approximately 2,000 – 3,500 events per second is recommended. This is usually achieved with a Medium fluidics rate setting. Use a stop mode based on the length of time needed to acquire at least 1,000 Counting Beads.
37. It is important to maintain the same fluidics rate setting for Post-Column samples that was used for the ICS and Pre-Column samples, even though the Post-Column samples will have lower cell densities (and therefore lower events per second).
38. Place the remaining Part A sample back on ice in case you need to prepare another ICS sample and return the tube part B was obtained from back with the other Pre-Column samples. This ICS now consists of adequate numbers of anti-CD59-PE positive and negative events to guide selection of PMT voltages and compensation settings.
39. One way that you can determine this is by looking at the “Y Geo Mean” values for the LL and LR quadrants. When these two values are approximately equal, compensation has been set correctly.
40. In the right-hand plot, below, the horizontal demarcation line that distinguishes mature RBCs from RETs is too low. This can lead to subtle variations in staining intensity causing greatly overestimated % RET values. Additionally, the vertical

demarcation line is positioned too far right. This can lead to subtle variations in staining intensity causing greatly overestimated frequencies of mutant phenotype cells.

41. It is VERY IMPORTANT that each sample is pipetted IMMEDIATELY before analysis.
42. It is VERY IMPORTANT that each sample is pipetted IMMEDIATELY before analysis; 10 times is usually sufficient. Use the same instrument settings as the Pre-Column samples. Do not adjust the location or size of regions. Use a stop mode based on the length of time needed to analyze nearly the entire volume of cells and Counting Beads. Some initial experimentation may be required to determine the specific time for each flow cytometer, but 5 to 6 minutes is usually sufficient when using a medium fluidics rate.
43. An Excel spreadsheet can be obtained from Litron (download from www.litronlabs.com/support.html or email invivopigatechsupport@litronlabs.com for more information). This spreadsheet can be used to make these calculations, and also provides examples of actual flow cytometric data.

    [Fig 17 near here]

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anchor-deficient erythrocytes as described herein and sells kits based on this technology (*In Vivo* MutaFlow®).

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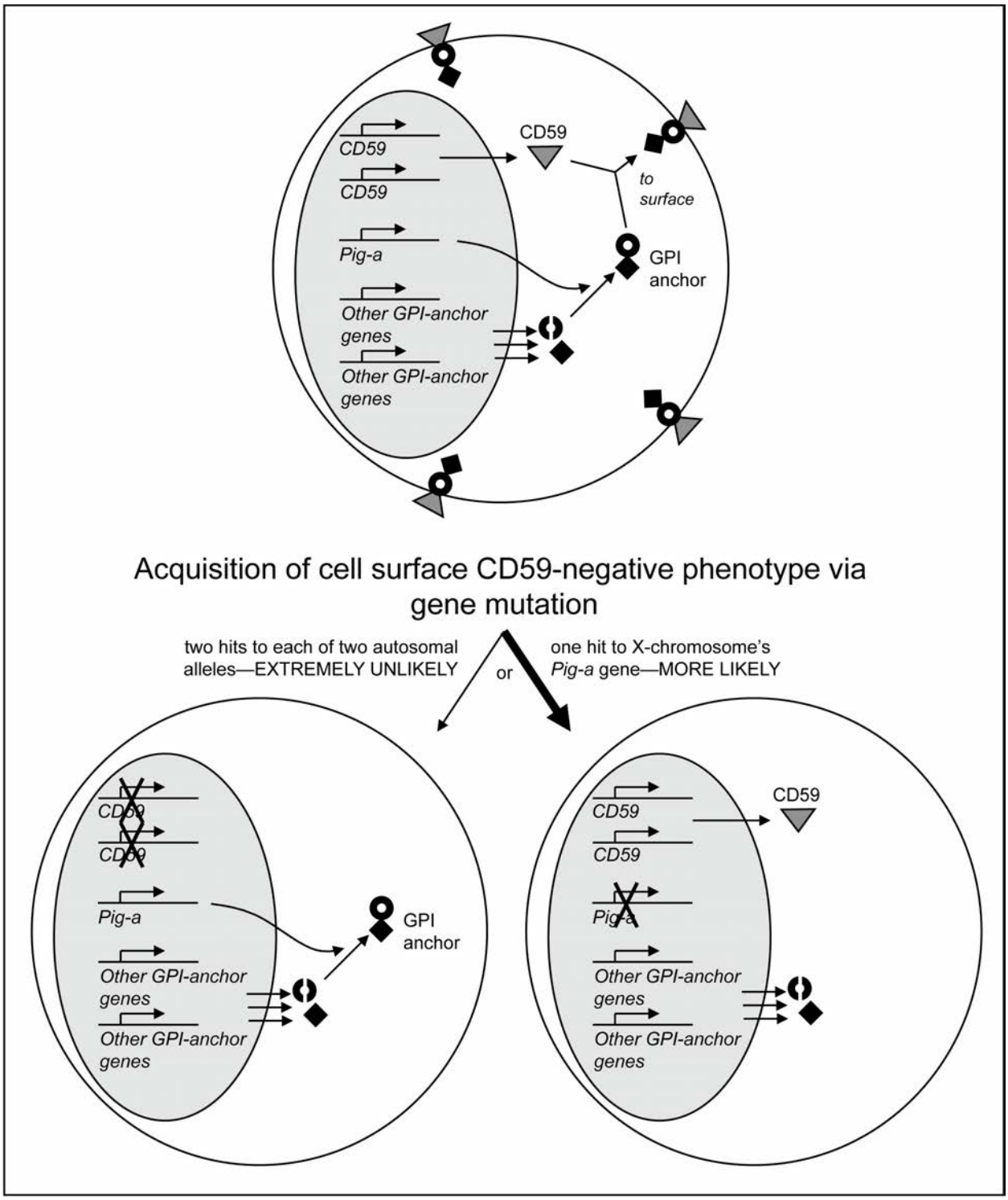


**Fig. 1** Cartoon shows a normal, wild type cell at the top and describes the acquisition of the *Pig-a* mutant phenotype.

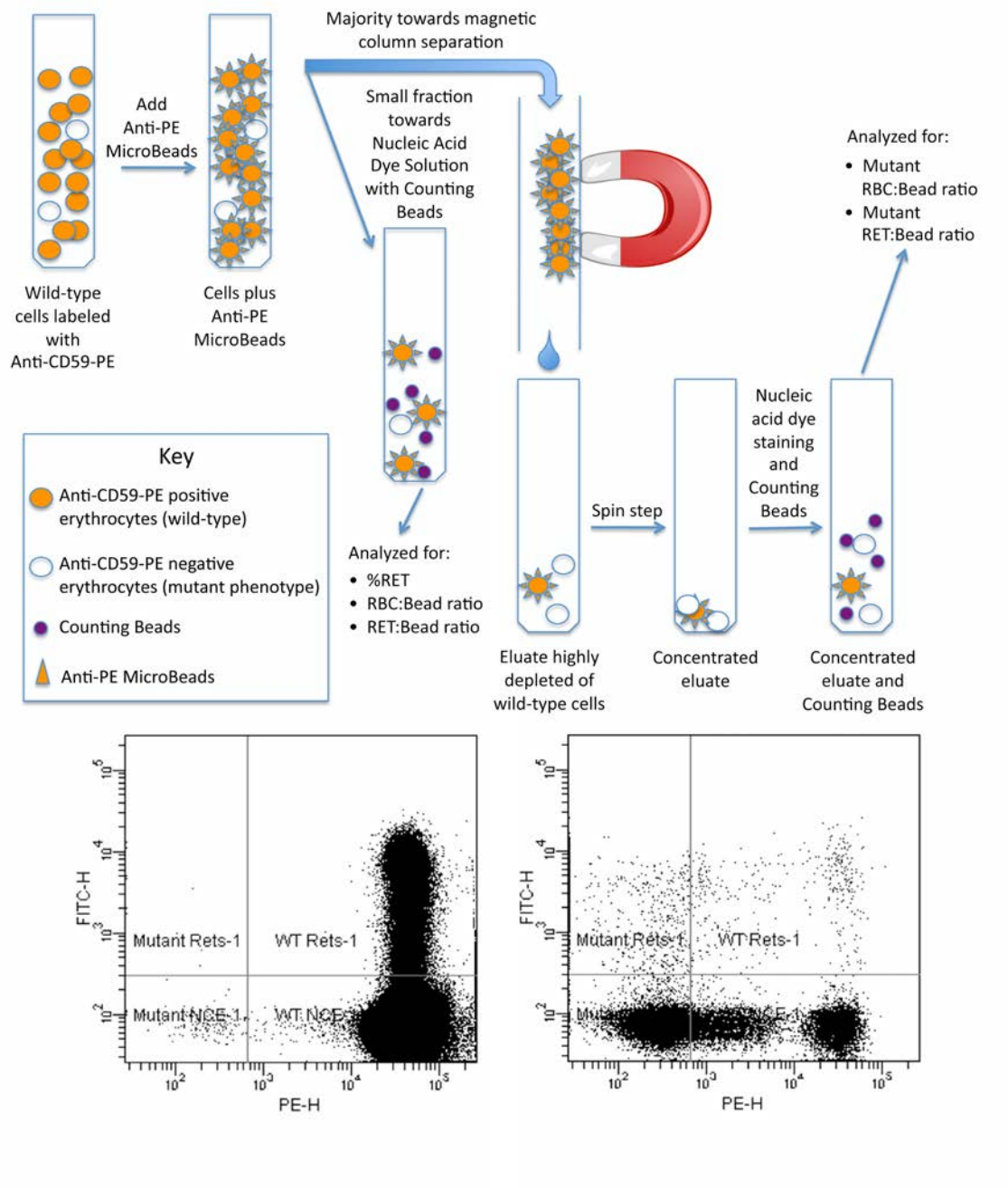


**Fig. 2** The schematic at the top, shows the strategy for immunomagnetic separation and enumeration of the Pre-- and Post--Column samples for quantification of *Pig--a* mutant cell frequency. The bottom plots show data for a Pre--Column sample (left) and a Post--Column sample (right) from a blood sample obtained 25 days after 3 consecutive days of exposure to the mutagen ethyl nitrosourea. Notice how the Post--Column sample has become greatly enriched for mutant cells.

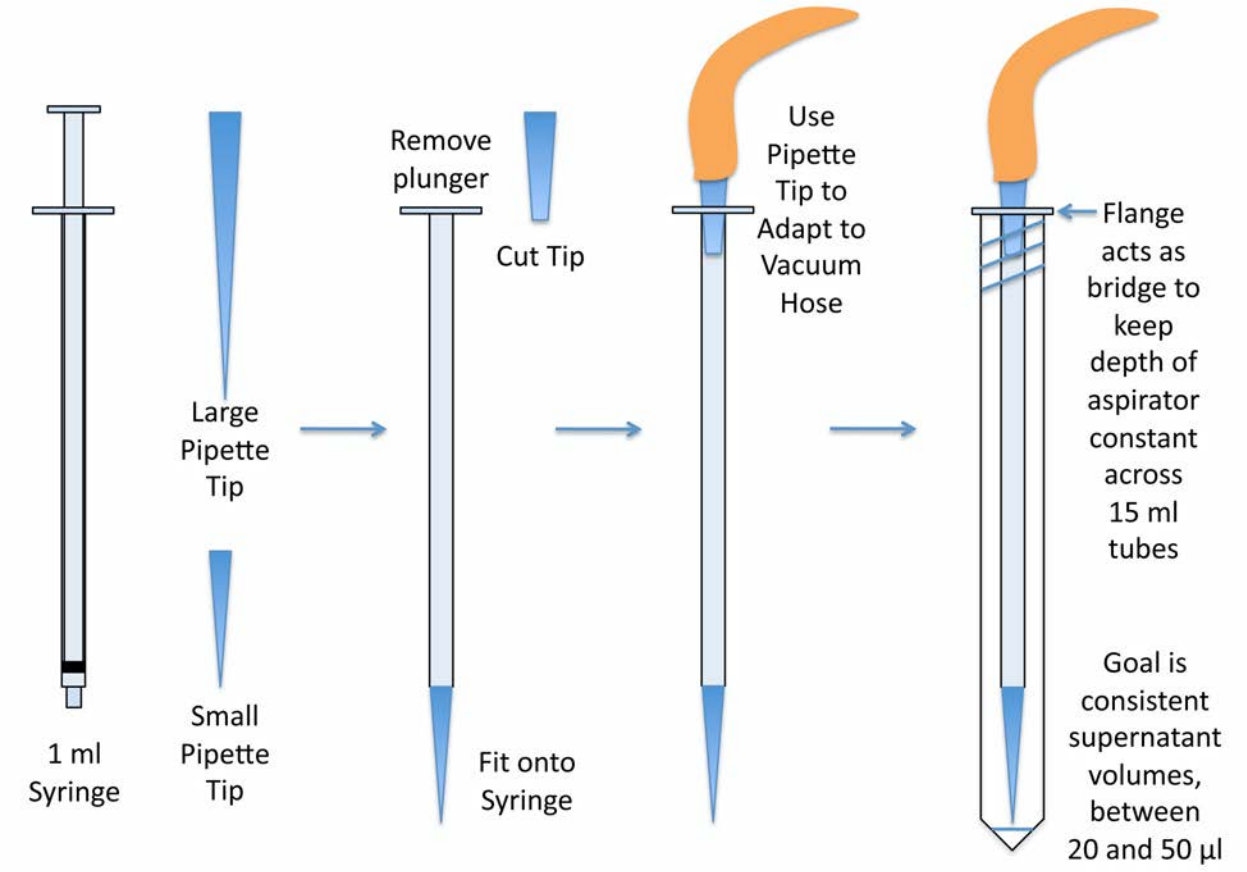


**Fig. 3** Schematic for assembly of the aspiration device described in step 14 of Section 2.2.

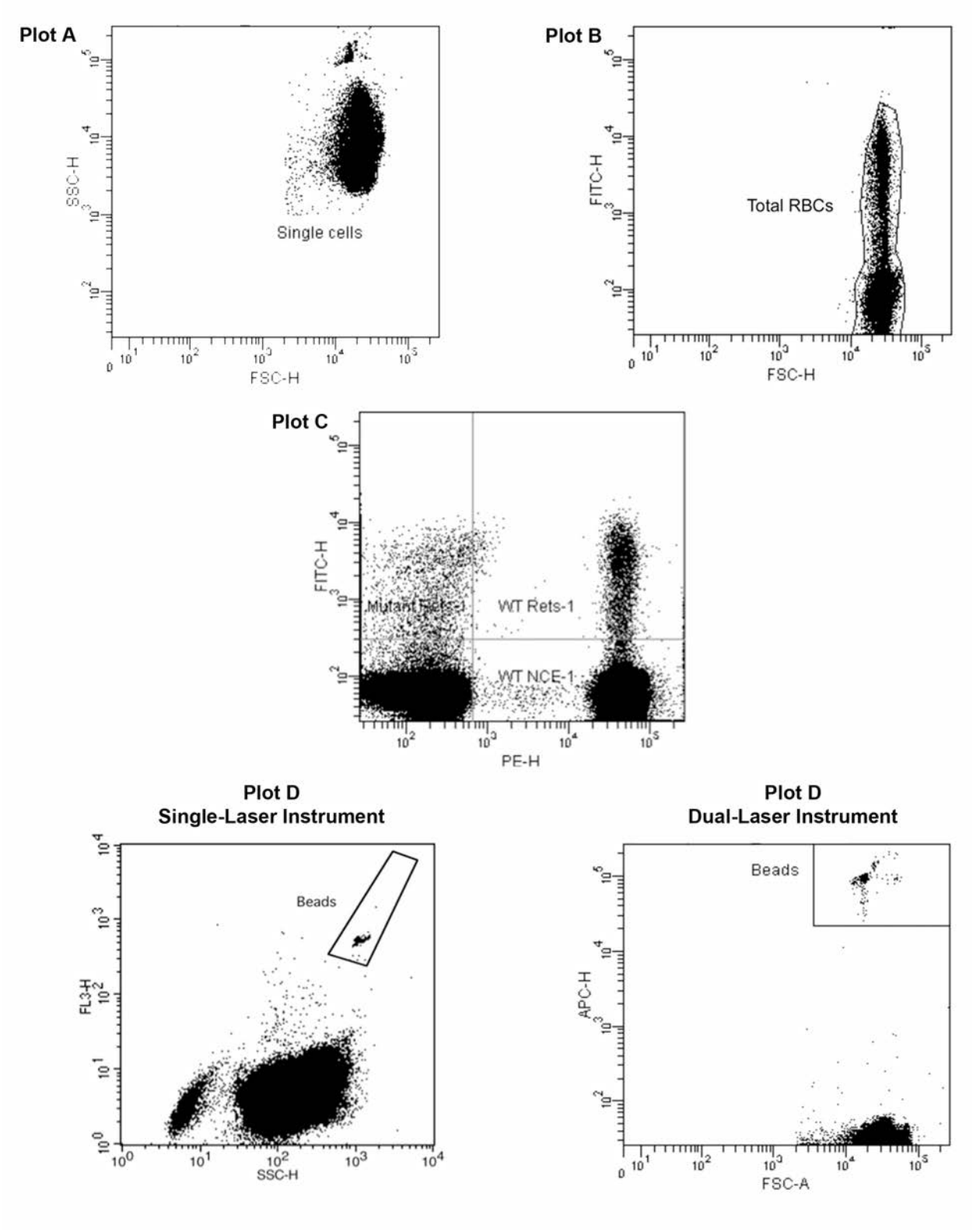


**Fig. 4** Examples of the bivariate plots used in the analytical template.


**Fig. 5** Blood layered onto Lympholyte®-Mammal.

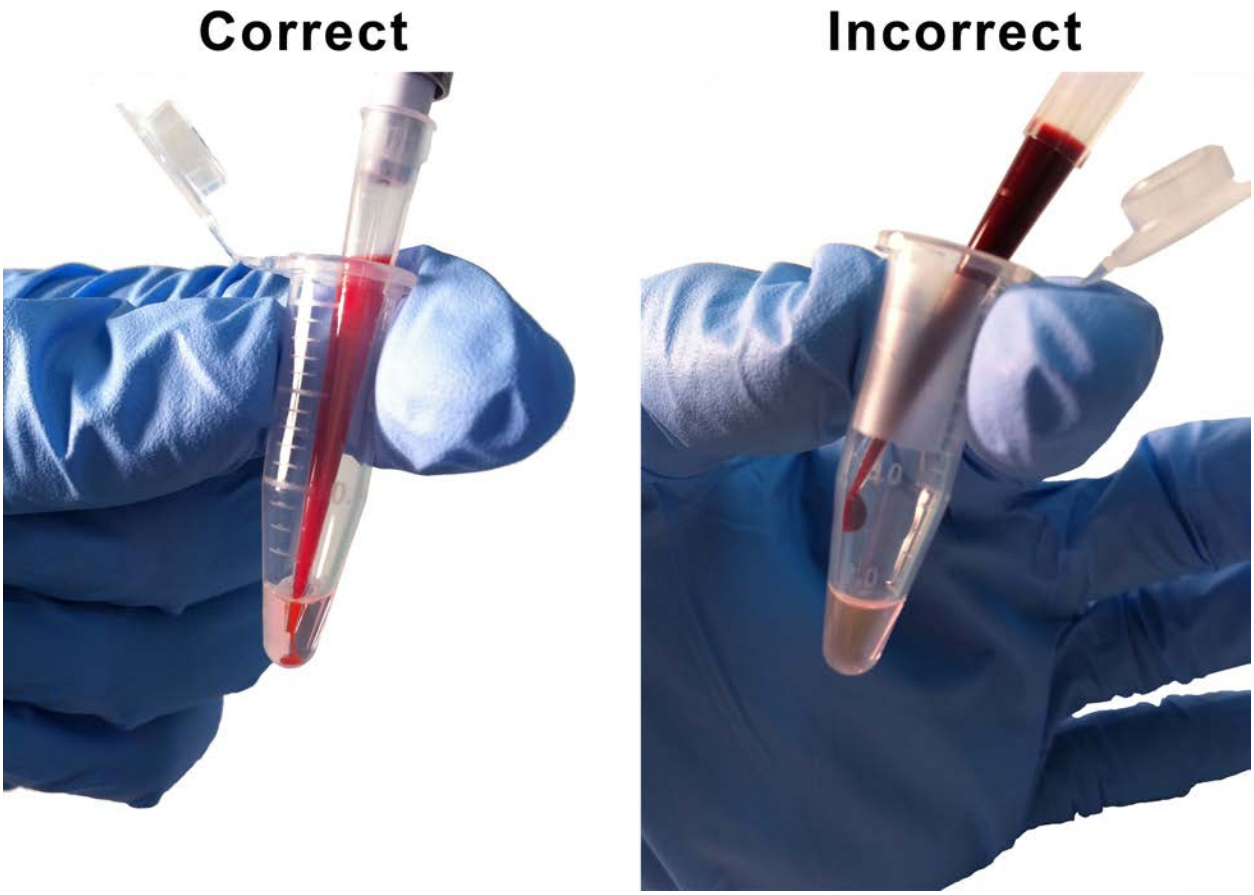


**Fig. 6** Correct versus incorrect introduction of sample to Working Antibody Solution.

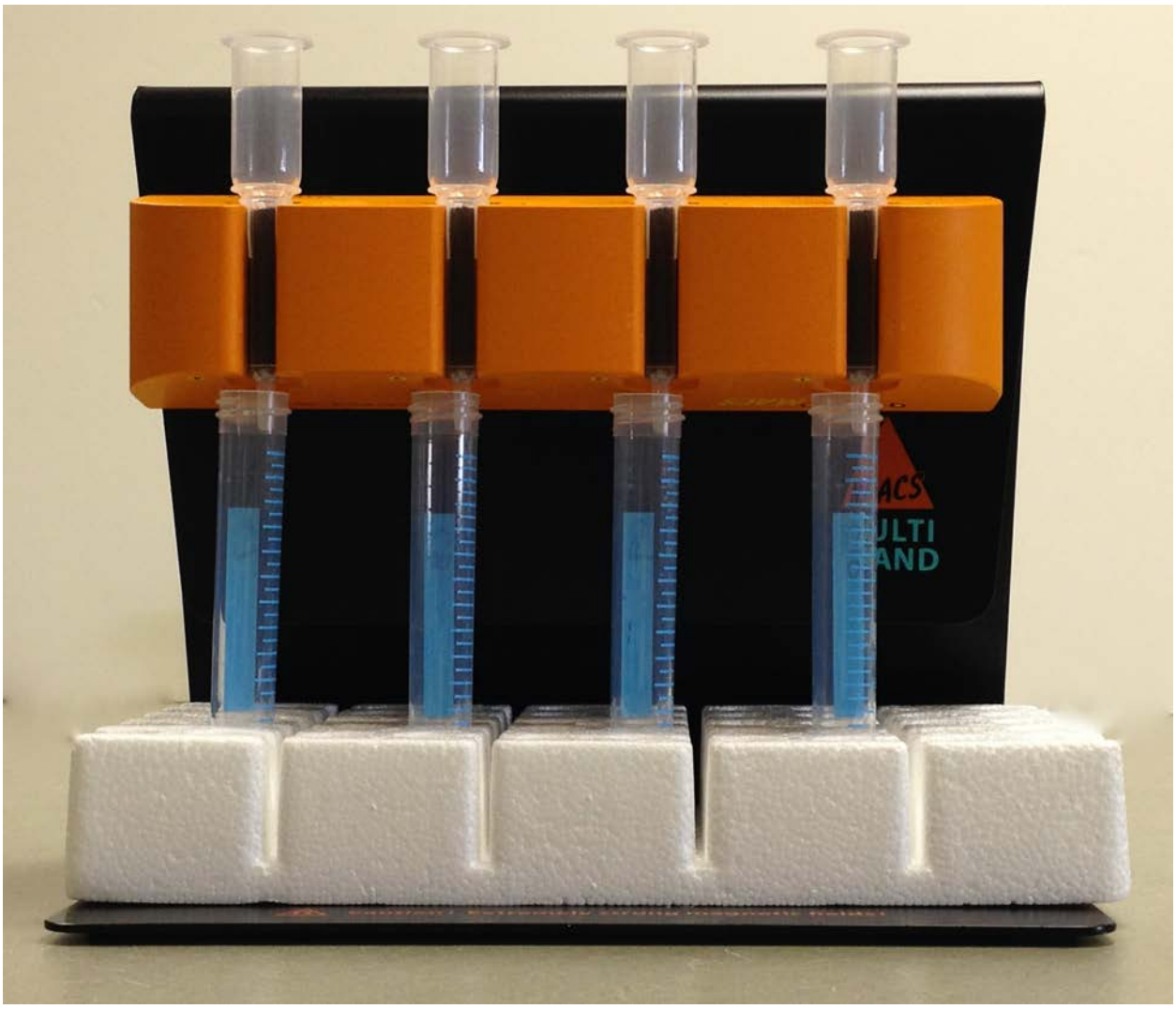

**Fig. 7** QuadroMACS™ separator with LS columns and rack with tubes placed directly underneath.


**Fig. 8** Pre--Column sample on left, Post--Column eluate on right.

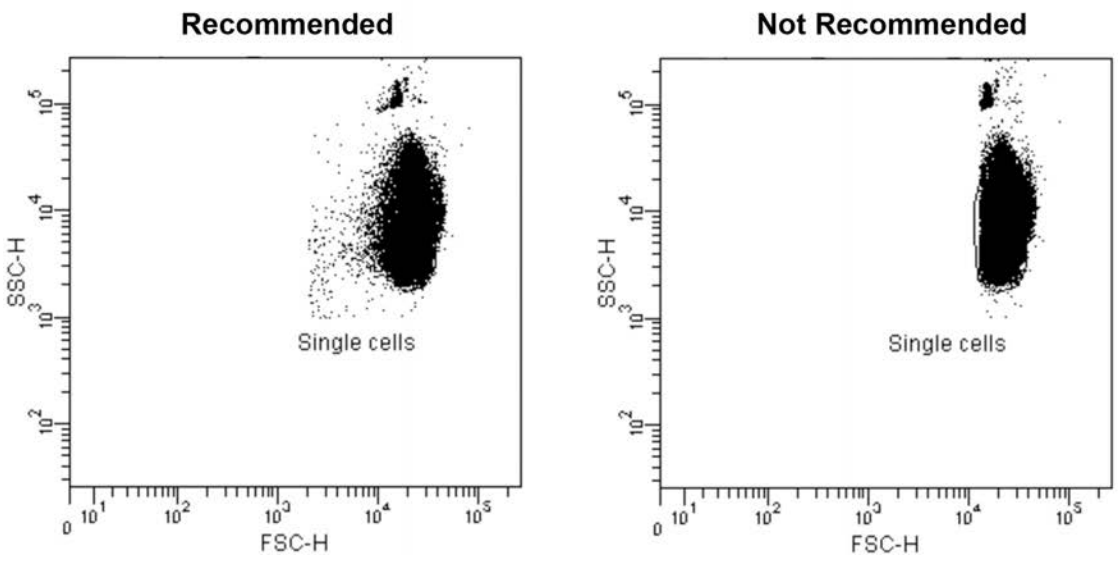


Fig. 9 Plot A: Recommended versus not recommended threshold setting.

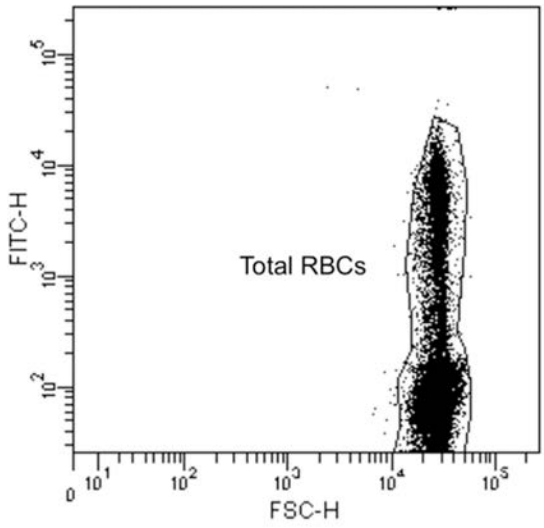


**Fig. 10** Plot B: Placement of the “Total RBCs” gate.

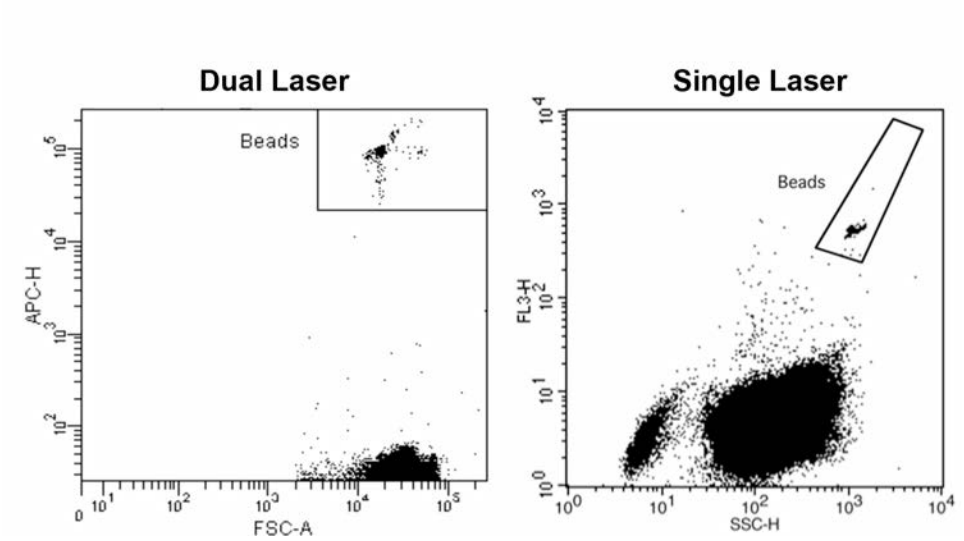


Fig. 11 Plot D: Capturing counting beads on a dual laser versus single laser instrument.

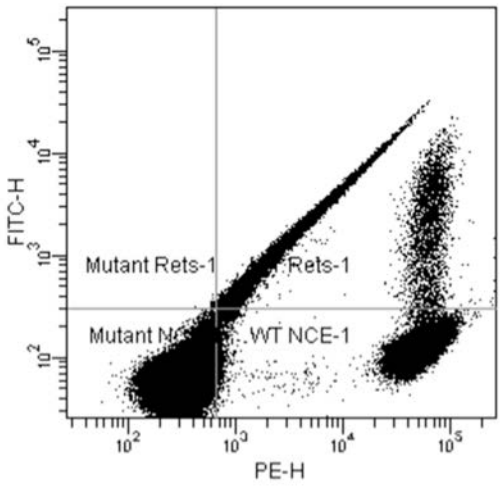


**Fig. 12** Plot C: Adjusting PMT voltage to place mature RBCs in the first decade of FITC and PE fluorescence.

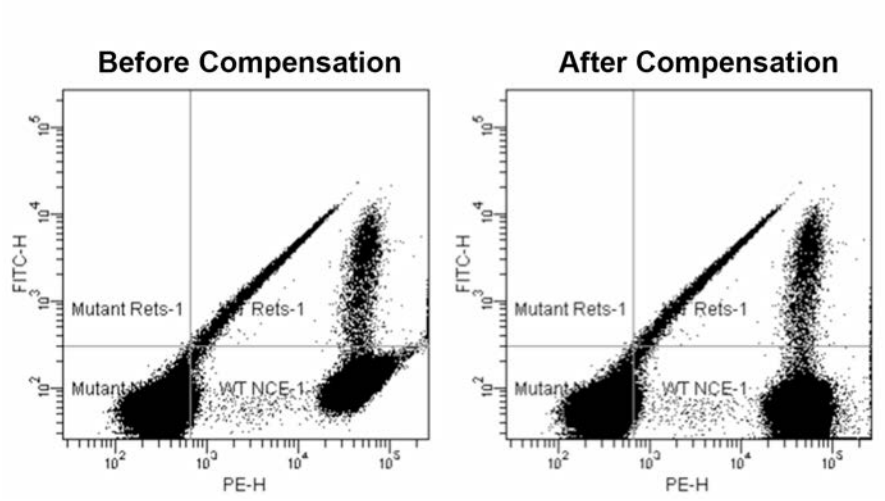


**Fig. 13** Plot C: Adjust compensation to eliminate FITC spillover into the PE channel.

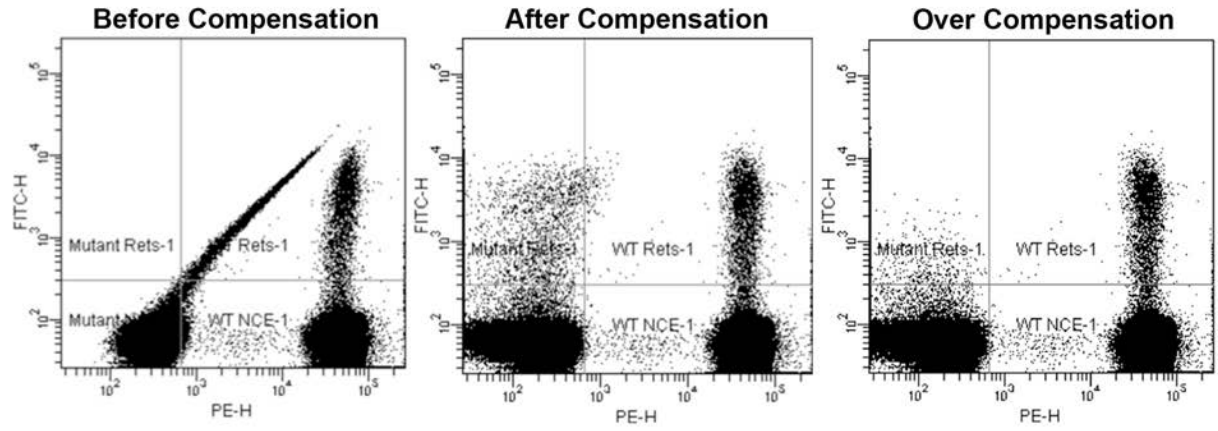


**Fig. 14** Plot C: Adjust compensation to eliminate PE spillover into the FITC channel.

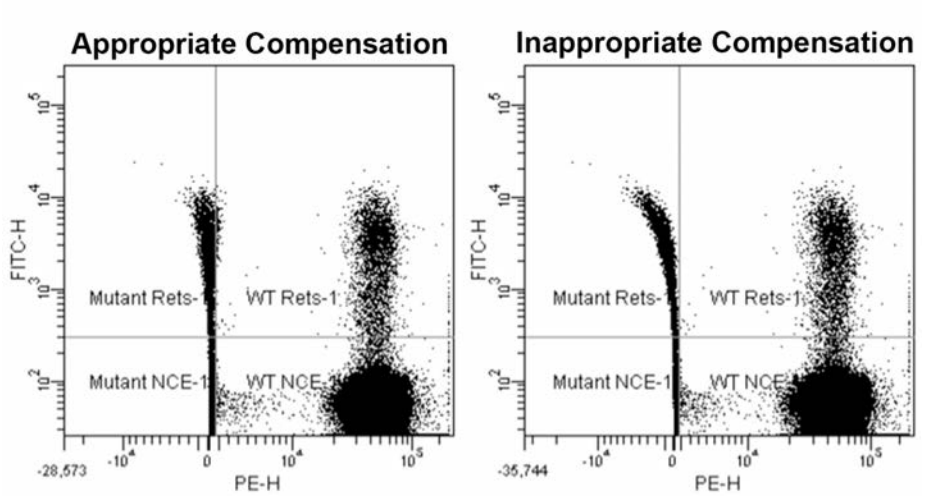


**Fig. 15** Plot C: Appropriate versus inappropriate compensation on a digital machine.

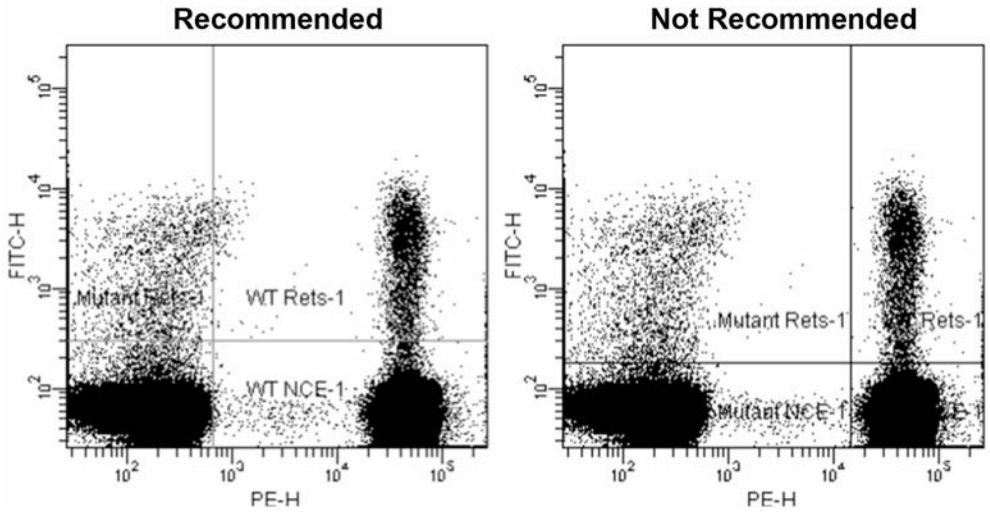


Fig. 16 Plot C: Recommended versus not recommended position of the vertical demarcation line

## Fig. 17 Overview of work flow for a typical processing day

It is helpful to have 2 or 3 people available when many samples (≥ 16) will be collected and analyzed. Below is a recommended outline for a day of collecting and analyzing samples.

| | | |
|---|---|---|
| Day(s) before blood collection | Label all necessary tubes and vials with sample IDs. At least 2 flow cytometry tubes, 3 centrifuge tubes (15 ml), and 2 microcentrifuge tubes are required per sample. **Step 18 of section 2.2.** Aliquot the anticoagulant into labeled vials prior to blood collection. **Step 1 of section 3.1.** | |
| Day of collection, early morning (3 people) | Two people collect blood samples. **Section 3.1**. | A third person prepares reagents and aliquots into pre-labeled tubes and vials. **Section 2.1 & 2.2.** |
| Day of collection, early morning (2 or 3 people) | Two people process all samples through Lympholyte, washing cells, and adding to Working Antibody Solution. **Sections 3.2 & 3.3.**<br><br>If there are more than about 24 samples, it can be helpful for one person to aspirate after centrifugation (**Step 3 of section 3.2**), while one or two people perform the first wash (**Step 4 of section 3.2**) for all samples before moving to the next step. Repeat this process for the additional rinses. | |
| Day of collection, late morning (2 or 3 people) | Two people add all samples to Buffered Salt Solution + 2 % FBS. (**Step 1 of section 3.4**). After centrifugation (**Step 2 of section 3.4**), one person aspirates (**Step 3 of section 3.4**) while another adds Working Anti-PE MicroBead Suspension (**Steps 1 & 2 of section 3.5**).<br><br>If working up more than 24 samples at a time, have one person aspirate (**Step 3 of section 3.4**), while one or two people add Working Anti-PE MicroBead Suspension (**Steps 1 & 2 of section 3.5**). | |
| Day of collection, late morning (2 or 3 people) | After aspiration (**Step 6 of section 3.5**), one person adds Buffered Salt Solution + 2 % FBS and resuspends the cells (**Step 1 of section 3.6**) while another removes 10 $\mu$l for Pre-Column samples (**Step 2 of section 3.6**).<br><br>If working up more than 24 samples at a time, have one person aspirate (**Step 6 of section 3.5**), while one person adds Buffered Salt Solution + 2 % FBS and resuspends cells (**Step 1 of section 3.6**). A third person could be removing 10 $\mu$l for Pre-Column samples (**Step 2 of section 3.6**). | |
| Day of collection, late morning to early afternoon (2 people) | After incubation, one person performs instrument calibration and analyzes Pre-Column samples on the flow cytometer. **Sections 3.10 through 3.11.** | One person performs column separation (in batches of up to 8 samples) and cell staining. **Sections 3.8 & 3.9**. |
| Day of collection, early to late afternoon (1 person) | One person analyzes Post-Column samples on the flow cytometer. **Section 3.11.** | |