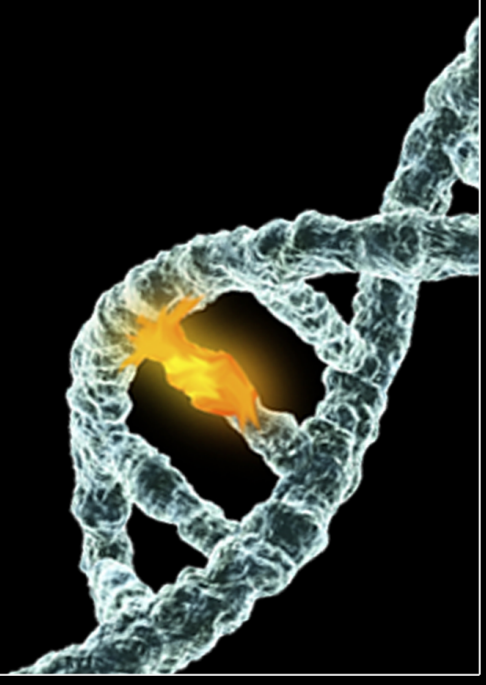
MutaFlow®

Pig-a Mutation Analysis



MutaFlow^{PLUS} (Mouse Thawing/Analysis) **Instruction Manual**

For research only. Not for use in diagnostic or therapeutic procedures.

Table of Contents

1. Materials Provided **(In order to use this kit, you must have already utilized a MutaFlow Rodent Blood Freezing Kit)**

Kit Component	Quantity[a]	Storage Conditions[b]
Thawing Solution A	5 mL	Ambient
Thawing Solution B	175 mL	Ambient
Anticoagulant Solution	10 mL	2 °C to 8 °C
Buffered Salt Solution	1 liter	Unopened: Ambient Opened: 2 °C to 8 °C
Anti-CD24-PE Solution[c]	650 µL	2 °C to 8 °C
Anti-CD45-PE Solution[c]	200 µL	2 °C to 8 °C
Anti-CD61-PE Solution[c]	380 µL	2 °C to 8 °C
Nucleic Acid Dye Solution (contains SYTO® 13)[d]	1.2 mL	–10 °C to –30 °C

a. Sufficient materials are provided to thaw and analyze 25 blood samples.
b. Please note that although some kit components are shipped at ambient temperature, they must be stored at the temperatures indicated above upon receipt.
c. Warning! Contains sodium azide. Irritant. See SDS.
d. SYTO® 13, trademark Life Technologies

2. Additional Materials Required

2.1. General Lab Equipment and Materials

- 15 mL polypropylene centrifuge tubes
- Microcentrifuge tubes or similarly sized tubes
- Micropipette and appropriate tips
- –10 °C to –30 °C freezer
- 2 °C to 8 °C refrigerator
- 37 °C incubator or water bath
- Flaked/chipped ice
- Flow cytometer capable of 488 nm excitation (with HTS if analysis of samples will take place in 96-well plate format)
- 8 or 12 Multi-channel pipettor
- Disposable reagent reservoirs
- Flow cytometry tubes
- Centrifuge with swinging bucket rotor
- 96-well plate holders for centrifuge
- Small volume 0.2 µm filters (e.g., Acrodisc®, PALL Life Sciences cat # 4192)
- Large volume 0.2 µm filters (e.g., 250 mL - 1000 mL)
- 96 well round bottom deep well plates, 2 mL (e.g., Axygen Scientific cat # P-DW-20-C)
- 96-well U-bottom standard assay plates (e.g., Falcon cat # 353910)
- 8 channel aspirator manifold and bridge, V&P Scientific cat # VP 180PC-3S

2.2. Materials for Separation and Analysis

- Heat-inactivated fetal bovine serum
- Lympholyte®-Mammal, sterile liquid, Cedarlane Laboratories cat # CL5110, CL5115, or CL5120
- LS Columns, Miltenyi Biotec cat # 130-042-401
- Anti-PE MicroBeads, Miltenyi Biotec cat # 130-048-801
- CountBright™ Absolute Counting Beads, Invitrogen cat # C36950
- MidiMACS™ Separator, Miltenyi Biotec cat # 130-042-302 or QuadroMACS™ Separator, Miltenyi Biotec cat # 130-090-976; the QuadroMACS™ Separator has the advantage of allowing four separations to occur simultaneously
- Post-column aspiration device (see Appendix C)

3. Technical Support

Litron Laboratories
3500 Winton Place
Rochester, New York 14623
Telephone: 585-442-0930
Fax: 585-442-0934
World Wide Web: www.LitronLabs.com
Order Toll Free: 877-4-LITRON (877-454-8766)
Email: pigatechsupport@LitronLabs.com

4. First-Time Users

We strongly recommend reading the entire instruction manual before performing these procedures. Using flow cytometry to score the incidence of extremely rare populations requires careful attention to detail; therefore, do not deviate from the procedures described in this manual. In order to achieve reliable results, it is important that these steps are followed using the reagents supplied with this kit. If you have questions, please contact Litron Laboratories by calling (585) 442-0930, faxing us at (585) 442-0934, or sending an email to **PIGATECHSUPPORT@LITRONLABS.COM.** Training videos are available on Litron's website: www.LitronLabs.com. Some additional important considerations for first-time users are listed below.

4.1. Data Acquisition Template Preparation

Flow cytometer data acquisition templates are available from Litron (www.LitronLabs.com or email pigatechsupport@litronlabs.com) but are specific to BD CellQuest™ Pro or FACSDiva™ software. If you are unable to use these data acquisition templates, prepare one PRIOR TO processing samples for analysis. Flow cytometry operators who are not using CellQuest™ Pro or FACSDiva™ software will find Appendix A of this manual valuable for constructing their own data acquisition and analysis template.

4.2. Flow Cytometer Requirements

This assay requires that at least one laser provides 488 nm excitation. Standard factory-installed filter sets are typically sufficient to achieve fluorescent resolution of the relevant cell populations. There is some advantage to a second red diode laser, as this provides better resolution of Counting Beads. A flow cytometer without a High Throughput Sampler (HTS) can be used if analysis of samples will take place individually in single tubes. A flow cytometer with a HTS is necessary if flow cytometric analysis will occur in 96-well plate format.

4.3. Daily Calibration with Instrument Calibration Standard

Optimal fluorescent resolution is critical when scoring rare events. Therefore, an *Instrument Calibration Standard* is crucial for consistently setting appropriate PMT voltages and compensation on a day-to-day basis, and for establishing the demarcation line that defines mutant and wild-type cells. This standard is created using "mutant-mimicking cells" and is described later in this manual.

First-time users of this kit should prepare and analyze an Instrument Calibration Standard before progressing to experimental blood samples. This will help ensure that fluorescent resolution is sufficient and that the data acquisition template is functioning properly. **Once you have analyzed an Instrument Calibration Standard, we highly encourage you to email or fax bivariates to Litron for evaluation and/or troubleshooting.**

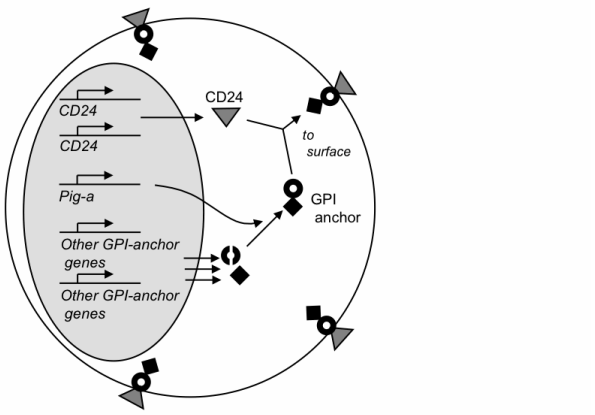
4.4. Other Important Considerations

- The stock reagents included in this kit and those purchased separately are sterile. Sterility needs to be maintained, therefore vials and bottles should only be opened in a laminar flow hood or similar sterile environment.
- Mutant phenotype cells are identified by their LACK of an anti-CD24-PE signal. Therefore, **spuriously high values will be generated if any cells do not come into direct contact with every reagent**, especially anti-CD24-PE. For this reason, follow the instructions in this manual carefully and when indicated, introduce cells DIRECTLY into the next reagent that has been pre-aliquoted into NEW wells of a 96 well plate.
- We recommend that you do not process and analyze more than 12 samples per day until you become thoroughly proficient with the method.
- It is very important to carefully control and standardize aspirations, especially the last aspiration (Section 10.2). To achieve this goal, we recommend fashioning an aspirator with a bridge that controls how far down the tip will reach. With this in place, aspiration will leave a consistent low volume of supernatant. Appendix C shows an example of how such an aspiration device can be made.
- Once blood is thawed, we recommend that the samples be analyzed on the same day.

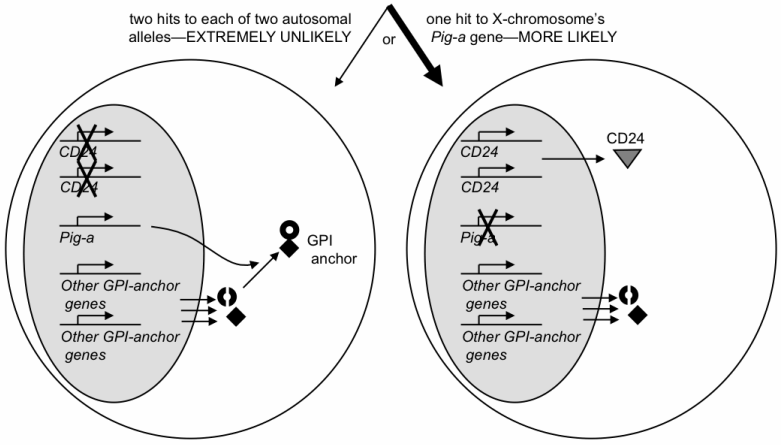
5. Introduction

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

Hematopoietic cells require GPI anchors to attach a host of proteins to their cell surface (for instance, CD24, CD59, and CD55). Importantly, of the genes required to form GPI anchors, only *Pig-a* is located on the X-chromosome. Mutations in the *Pig-a* gene can prevent functional anchors from being produced, resulting in cells lacking these proteins on their surface. Thus, cells without these cell surface markers represent a reliable phenotypic marker of *Pig-a* mutation.



Acquisition of cell surface CD24-negative phenotype via gene mutation



The MutaFlow® method is illustrated on page 5 with magnetic separation shown on page 6. Briefly, blood samples are processed through Lympholyte®-Mammal Solution to remove the majority of leukocytes and platelets. Cells are then incubated with Anti-CD24-PE (to label wild-type (wt) RBCs), Anti-CD61-PE (to label remaining platelets) and Anti-CD45-PE (to label leukocytes). Antibody-labeled samples are incubated with Miltenyi 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 to capture Cell:Bead ratios.

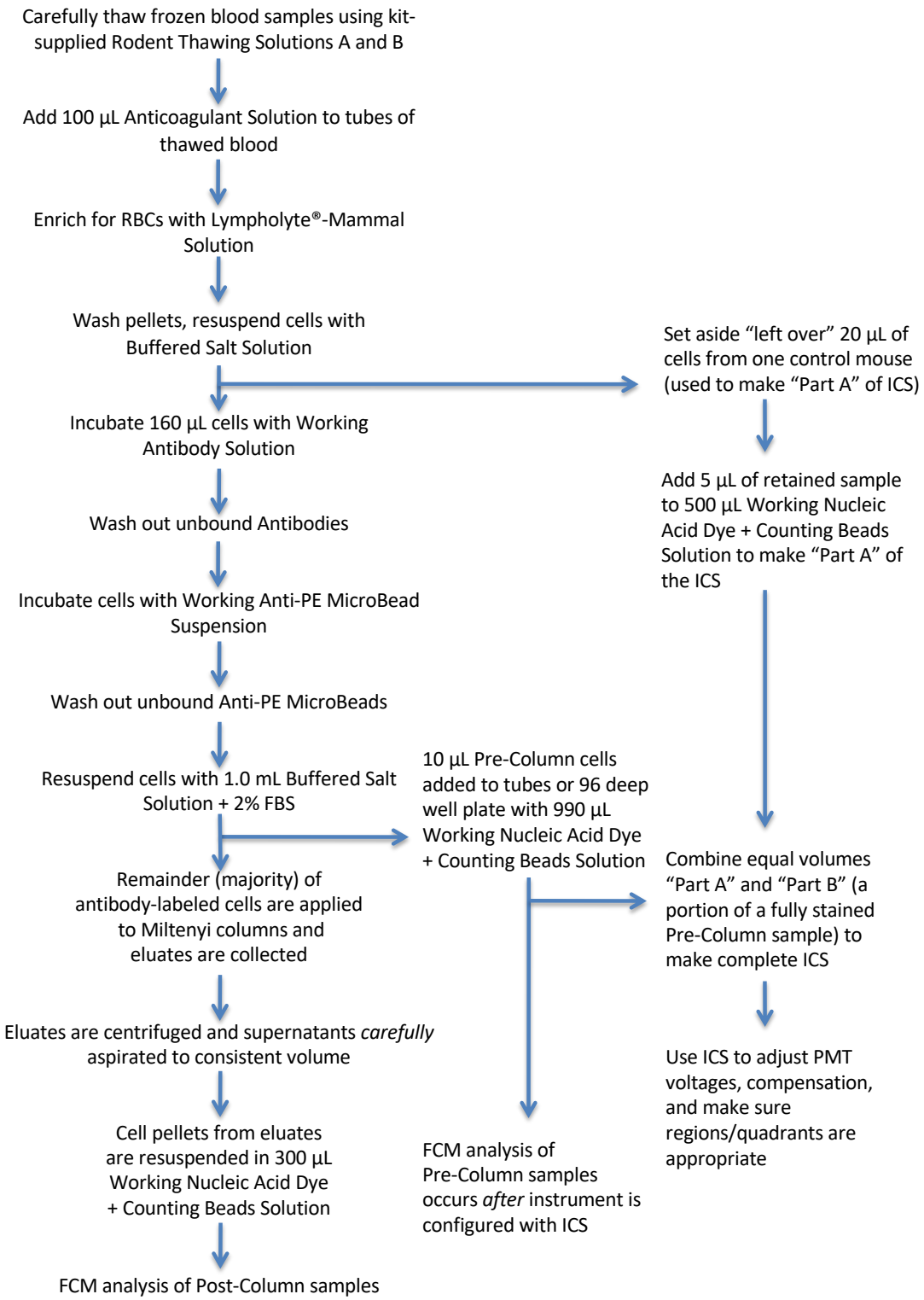
The remaining portion (majority) of the blood sample is applied to a Miltenyi LS Column that has been suspended in a magnetic field. These columns selectively retain wt cells, whereas *Pig-a* mutants (lacking CD24 on their surface) pass through the columns.

Eluates are collected, centrifuged, and stained with a nucleic acid dye (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.

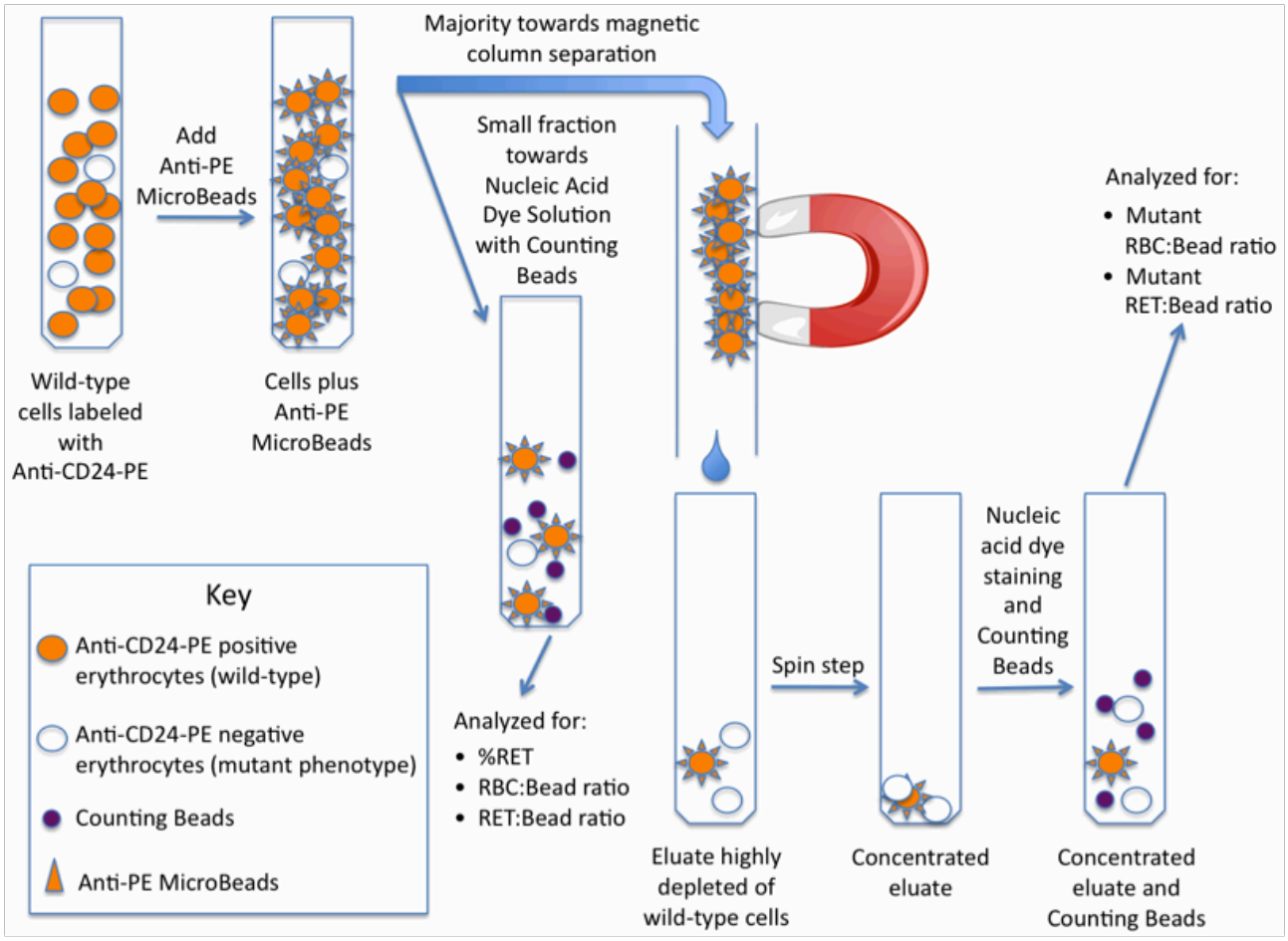
From the Pre- and Post-Column analyses, the following values are calculated:

- RET percentage, an index of bone marrow toxicity
- 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 30 days for mice).
  - This mutant cell value is typically based on greater than 150 x 10^6 RBC equivalents.
- Frequency of mutant-phenotype RETs
  - Note that upon acute mutagen exposure this index of genotoxicity reaches a maximal value faster than mutant phenotype RBCs (can be as soon as 2 weeks) since RETs are turned over at a much faster rate than the total RBC pool.
  - This mutant cell value is typically based on greater than 3 x 10^6 RET equivalents.

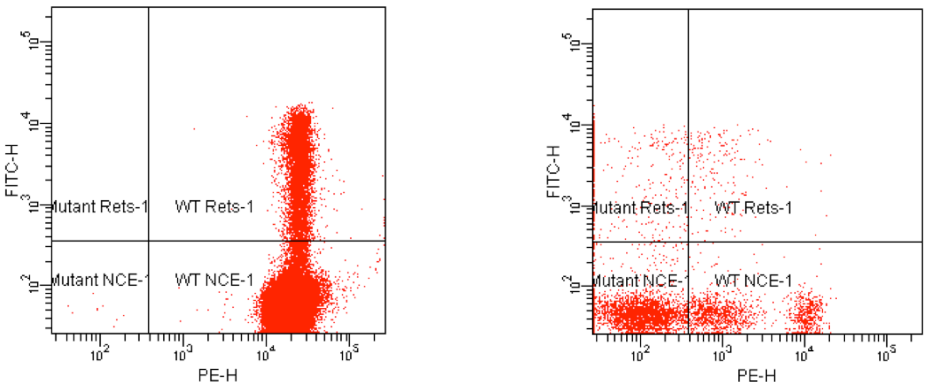
6. Overview of the Method



6.1. Overview of Magnetic Separation



The bivariate plot on the left shows data from a **Pre-Column** sample. The bivariate plot on the right shows data from the corresponding **Post-Column** sample. This blood was obtained twenty-five days after 3 consecutive days of dosing with the mutagen ENU. The **Post-Column** sample has become greatly enriched for mutant cells (i.e., upper left and lower left events).



7. Reagent Preparation

IMPORTANT NOTE: Prepare fresh reagents on each analysis day.

7.1. Prepare Buffered Salt Solution + 2 % Fetal Bovine Serum (FBS)

This solution is used to prepare the Working Nucleic Acid Dye Plus Counting Beads Solution, Working Antibody Solution, Working Anti-PE MicroBead Suspension, as well as for washing and column elution steps. Prepare 20 mL per sample.

1. Determine the total number of samples that will be prepared that day. Use the chart below as a guide and scale up as needed.

Number of samples	Volume of Buffered Salt Solution	Volume of FBS
1	19.6 mL	0.4 mL

2. Combine the required volumes of Buffered Salt Solution and heat-inactivated FBS.

3. Filter sterilize through a 0.2 μm filter and store at 2 °C to 8 °C until needed.

7.2. Prepare Working Nucleic Acid Dye Plus Counting Beads Solution

This solution is used to stain the DNA and RNA of the various cell populations and to determine Cell:Bead and Mutant Cell:Bead ratios. Prepare 1.5 mL per sample.

1. Determine the total number of samples that will be analyzed that day (remember to include one extra sample for the Instrument Calibration Standard [ICS]). Use the chart below as a guide and scale up as needed.

Number of samples	Volume of Buffered Salt Solution + 2 % FBS	Volume of Stock Nucleic Acid Dye Solution	Volume of CountBright™ Absolute Counting Beads
1	1.41 mL	45 μL	45 μL

2. Thaw stock Nucleic Acid Dye Solution.

3. Place the required volume of Buffered Salt Solution + 2 % FBS into a sterile vessel (preferably a 50 mL tube). Aseptically add the required volume of stock Nucleic Acid Dye Solution to the tube containing Buffered Salt Solution + 2 % FBS. **Use a pipettor to mix (DO NOT VORTEX OR SONICATE).**

4. DO NOT vortex or sonicate the bottle of CountBright™ Absolute Counting Beads. Instead, resuspend with a pipettor to ensure a homogeneous suspension.

5. Aseptically add the required volume of CountBright™ Absolute Counting Beads to the vessel. Resuspend with a pipettor to mix. **Do not vortex or sonicate.**

6. Depending on the number of samples being analyzed on a particular day, the Working Nucleic Acid Dye Plus Counting Beads Solution should be divided among several vessels to ensure that the entire volume is not being mixed multiple times and possibly damaging the Counting Beads. For each set of up to 16 post-column samples, gently mix to homogeneously resuspend beads and aliquot approximately 4.8 mL Working Nucleic Acid Dye Plus Counting Beads Solution to a new 50 mL tube.

7. From the remaining volume left in the original tube from Step 5, aseptically transfer **990 μL** of Working Nucleic Acid Dye Plus Counting Beads Solution to one labeled flow cytometry tube for each Pre-Column sample. If using 96 well plate analysis, transfer this volume to the appropriate new well of a 96 well deep well plate. **Periodically resuspend the Working Nucleic Acid Dye Plus Counting Beads Solution with a pipettor to ensure the Counting Beads do not settle.**

8. From the remaining volume left in the original tube from Step 5, aseptically transfer **500 µL** of Working Nucleic Acid Dye Plus Counting Beads Solution to a flow cytometry tube or appropriate new well of the same 96 well deep well plate used in Step 7 above for the ICS sample (Part A).

9. Protect the tubes or plate from light and store at ambient temperature until needed.

10. Cover all vessels containing Working Nucleic Acid Dye Plus Counting Beads Solution with foil and store at ambient temperature until needed.

7.3. Prepare Working Antibody Solution

This solution is used to label wt RBCs and remaining platelets and leukocytes with PE-conjugated antibodies, and is light-sensitive. Prepare 100 µL per sample, but make at least 10 % extra to allow for loss during transfers.

1. Determine the total number of samples that will be prepared that day. Use the chart below as a guide and scale up as needed.

Number of samples	Vol of Buffered Salt Solution + 2 % FBS	Vol of Stock Anti-CD24-PE Solution	Vol of Stock Anti-CD45-PE Solution	Vol of Stock Anti-CD61-PE Solution
1	72 µL	7 µL	7 µL	14 µL

2. Place the required volume of Buffered Salt Solution + 2 % FBS into a sterile tube.

3. Quick spin the Anti-CD24-PE, Anti-CD45-PE and Anti-CD61-PE Solutions for best recovery of contents. Aseptically add the required volume of each to the tube containing Buffered Salt Solution + 2 % FBS. Resuspend with a pipettor to mix.

4. Using a reagent reservoir and a multi-channel pipettor, aseptically transfer 100 µL of this Working Antibody Solution to wells of a 96 well round bottom plate, one well for each sample. Protect the plate from light with foil and store at 2 °C to 8 °C or on ice until needed.

7.4. Prepare Working Anti-PE MicroBead Suspension

These MicroBeads bind to cells labeled with PE-conjugated antibodies. Prepare 100 µL per sample, but make at least 10 % extra to allow for loss during transfers.

1. Determine the total number of samples that will be prepared that day. Use the chart below as a guide and scale up as needed.

Number of samples	Volume of Buffered Salt Solution + 2 % FBS	Volume of Anti-PE MicroBeads
1	50 µL	50 µL

2. Place the required volume of Buffered Salt Solution + 2 % FBS into a sterile tube.

3. **Do not vortex or sonicate** the bottle of Anti-PE MicroBeads. Instead, resuspend with a pipettor to ensure a homogeneous suspension.

4. Aseptically add the required volume of Anti-PE MicroBeads to the Buffered Salt Solution + 2 % FBS. **Do not vortex or sonicate.** Instead, resuspend with a pipettor to ensure a homogeneous suspension.

5. Cover the tube with foil and store at 2 °C to 8 °C or on ice until needed.

8. Blood Thawing and Leukodepletion

8.1. Aliquot Lympholyte®-Mammal

1. On the morning of thawing and leukodepletion, gently shake the Lympholyte®-Mammal bottle and allow for air bubbles to disappear. Aliquot 1.5 mL into a deep well 96 well plate, one well for each blood sample. Protect from light and allow the aliquots to equilibrate to ambient temperature before use.

8.2. Thaw Frozen Blood Samples

Important Notes:

- Blood should be labeled and analyzed on the day it is thawed.
- Remove up to 10 cryovials from the -90 °C to -80 °C ultracold freezer. If 2 people are performing this step, more than 10 samples can be processed at one time.

1. Remove cryovials from the -90 °C to -80 °C ultracold freezer and thaw in a water bath set to 37 °C. Once thawed, transfer blood suspensions to new pre-labeled 15 mL centrifuge tubes.

2. Gently add **150 µL** Rodent Blood Thawing Solution A to each tube and vortex briefly on low speed. Incubate at ambient temperature for 5 minutes.

3. Gently add **750 µL** Rodent Blood Thawing Solution B to each tube and vortex briefly on low speed. Incubate at ambient temperature for 5 minutes.

4. Gently add **900 µL** Rodent Blood Thawing Solution B to each tube and vortex briefly on low speed. Incubate at ambient temperature for 5 minutes.

5. Gently add **4.8 mL** Rodent Blood Thawing Solution B to each tube and vortex briefly on low speed. Incubate at ambient temperature for 5 minutes.

6. Centrifuge samples at **1100 x g for 5 minutes**. Carefully aspirate the supernatants, removing as much as possible without disturbing the loosely packed pellets.

7. Add 100 µL Anticoagulant Solution to the pellet and mix. **PROCEED IMMEDIATELY TO SECTION 8.3, BELOW.**

8.3. Add Blood to Lympholyte®-Mammal

1. Using a pipettor, remove the entire contents of the centrifuge tube and gently layer on top of the pre-aliquoted, ambient temperature Lympholyte®-Mammal. Repeat for the remaining samples.

2. Centrifuge at **800 x *g* for 20 minutes** at ambient temperature. While samples are in the centrifuge, aliquot 1.5 mL Buffered Salt Solution + 2 % FBS into the appropriate wells of a deep well 96 well plate. Store at 2 °C to 8 °C until needed.

3. Remove samples from centrifuge. When removing, be aware that the resulting cell pellets will not be hard packed; therefore, avoid tapping or bumping the plates in a way that will cause the pellets to loosen or become dislodged from the bottom of the well. Maintain plates at ambient temperature.

8.4. Wash Cell Pellets out of Lympholyte®-Mammal

1. Holding the plate upright and using a multi-channel aspirator with a fixed bridge, carefully aspirate the supernatants, removing as much as possible without disturbing the loosely packed pellets.

2. Add **140 µL** cold Buffered Salt Solution (without FBS) directly to each pellet. Do not let the solution run down the side of the well, as that can re-introduce platelets and/or white blood cells that adhered to the side of the well. Gently pipette up and down to resuspend cells until there is no visual evidence of aggregation.

3. Transfer the entire contents of each well to the sample's corresponding deep well containing 1.5 mL of cold pre-aliquoted Buffered Salt Solution + 2 % FBS. Pipette at least 10 times to mix. Repeat for remaining samples.

4. Centrifuge at **235 x *g* for 10 minutes** at ambient temperature. After centrifugation, aspirate as much of the supernatant as possible without aspirating the pellet.

5. Add **100 µL** cold Buffered Salt Solution (without FBS) directly to each pellet. Gently pipette up and down to resuspend cells.

6. Proceed immediately to Section 9. Otherwise, store at 2 °C to 8 °C for up to 24 hours before proceeding with Section 9.

9. Sample Labeling

IMPORTANT NOTE

Once samples are fully labeled, it is advisable to analyze them within 3 hours. Therefore, it is important to proceed through Sections 9.1 to 10.2 in sequence; so careful timing of the samples and planning of the workday is critical. At least one of the wells (preferably from a vehicle control sample) from 9.1, below, will need to be saved. The cells remaining in this well will be used in Section 9.5, step 1 to make the Part A portion of the ICS. These unstained cells will become the "mutant mimicking cells".

9.1. Label Leukodepleted Blood with Working Antibody Solution

1. Set a pipettor to 160 µL and pipette the first set of wells containing leukodepleted blood (with Buffered Salt Solution previously added) up and down to resuspend the cells. Continue as necessary until there is no visual evidence of aggregation – 10 times is usually sufficient.

> Note: Steps 1 and 2 can be performed at ambient temperature as long as processing of all samples occurs in less than 10 minutes. If it will take longer, maintain plates on ice during processing.

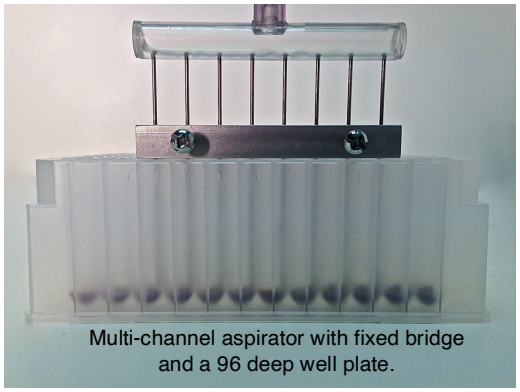
2. Carefully transfer 160 µL of the resuspended cells directly into the pre-aliquoted Working Antibody Solution in the corresponding sample's wells. Carefully pipette up and down to mix, taking care not to splash cells onto the side of the wells. Continue pipetting up and down to ensure adequate mixing – 10 times is usually sufficient. *Ensure that all the cells come into full contact with the Working Antibody Solution.*

3. Repeat steps 1 and 2 for the remaining samples. Use a new pipette tip for each sample. After all samples are labeled, use new pipette tips to move each sample to new wells of the same or a new round bottom 96 well plate.

4. Incubate cells with Working Antibody Solution, protected from light for **20 minutes** at 2 °C to 8 °C.

5. Save the remaining contents from at least one well from Step 2 above (preferably from a vehicle control) and place at 2 °C to 8 °C. This will be used to make Part A of the ICS sample. There should be approximately 20 to 30 µL remaining in the well after step 2, above. Add 150 µL of Buffer + 2 % FBS to this sample before using it to make Part A of the ICS sample.

6. During the incubation, aliquot 1.5 mL cold Buffered Salt Solution + 2 % FBS into wells in a new plate, one well for each sample. Store at 2 °C to 8 °C or on ice until needed in Section 9.2.



Multi-channel aspirator with fixed bridge and a 96 deep well plate.

9.2. Wash Labeled Samples 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 Section 9.1, step 6. 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 well. Carefully pipette up and down to mix. Continue pipetting up and down to ensure adequate mixing – 10 times is usually sufficient.

2. Centrifuge at **340 x *g* for 5 minutes** at ambient temperature.

3. After centrifugation, keep at ambient temperature. Aspirate supernatants using a multi-channel aspirator with a fixed bridge. Remove as much of the supernatant as possible without aspirating the pellet. The goal is to leave a minimal volume of supernatant behind.

4. Using a multi-channel pipettor, add 1.5 mL cold Buffered Salt Solution + 2 % FBS directly to the first set of samples. Carefully pipette up and down to mix. Change pipette tips and repeat for the remaining samples.

5. Centrifuge at **340 x *g* for 5 minutes** at ambient temperature.

6. After centrifugation, keep at ambient temperature. Aspirate supernatants using a multi-channel aspirator with a fixed bridge. Remove as much of the supernatant as possible without aspirating the pellet. The goal is to leave a minimal volume of supernatant behind.

9.3. Incubate Samples in Working Anti-PE MicroBead Suspension

1. Resuspend the Working Anti-PE MicroBead Suspension and place into a reagent reservoir.

2. Using a multi-channel pipettor, add 100 µL of the MicroBead suspension to each well of the first set of samples by washing it down the inside of the wells starting about 0.5 cm above the pellet. Carefully pipette up and down to mix, making sure not to splash cells high onto the sides of the wells. Continue as necessary until there is no visual evidence of aggregation – 10 times is usually sufficient. Change pipette tips and repeat for the remaining samples.

3. Incubate cells, protected from light, for **20 minutes** at 2 °C to 8 °C.

4. After incubation, add 1.5 mL of cold Buffered Salt Solution + 2 % FBS to each well. Gently pipette to mix thoroughly – 5 times is usually sufficient.

5. Centrifuge at **340 x *g* for 5 minutes** at ambient temperature.

6. After centrifugation, keep at ambient temperature. Using a multi-channel aspirator with a fixed bridge, aspirate supernatants, removing as much of the supernatant as possible without aspirating the pellet.

9.4. Stain Pre-Column Samples

1. Using a multi-channel pipettor, add 1.0 mL cold Buffered Salt Solution + 2 % FBS directly to the first set of samples. Carefully pipette up and down to mix, without splashing cells high onto the sides of the wells. Continue as necessary until there is no visual evidence of aggregation – 4 times is usually sufficient.

2. Transfer *exactly* **10 µL** of this suspension to the labeled flow cytometry tube or appropriate well of the 96 well deep well plate containing 990 µL of ambient temperature Working Nucleic Acid Dye Plus Counting Beads Solution (prepared in Section 7.2). Pipette gently to mix. This dilution is critical in determining the final mutation frequencies, so be careful to transfer this exact amount.

3. Repeat steps 1 and 2 for the remaining samples. These samples represent the **Pre-Column** samples. Maintain them at ambient temperature until each sample has been processed to this point. They will be incubated after Part A of the ICS is prepared (Section 9.5). One of these samples, preferably from a vehicle control, will also be used as "Part B" of the ICS (see Section 11.1, step 1).

4. The cells remaining in the plates will be processed further (Section 10) and are now referred to as **Post-Column** samples. Maintain these samples in the dark at 2 °C to 8 °C or on ice until proceeding to Section 10.

9.5. Prepare Part A of the Instrument Calibration Standard (ICS) and Incubate Pre-Column Samples

1. Prepare Part A of the ICS. Do this by retrieving the leukodepleted sample (preferably from a vehicle control animal) that was stored in Section 9.1 and had 150 µL of Buffered Salt Solution + 2 % FBS added. Gently resuspend cells by pipetting up and down. Transfer **5 µL** of this sample to the labeled flow cytometry tube or appropriate well of the 96 well plate containing 500 µL of ambient temperature Working Nucleic Acid Dye Plus Counting Beads Solution (prepared in Section 7.2). Pipette gently to mix.

2. Incubate the **Pre-Column** samples (including Part A of the ICS) in Working Nucleic Acid Dye Plus Counting Beads Solution for **10 minutes** at **ambient temperature** in the dark.

3. After incubation, transfer the samples to ice and protect from light. Ensure that the tubes (or plates) are surrounded by flaked/chipped ice, not resting on top. Store on ice for at least 5 minutes, but no more than 3 hours, before flow cytometric analysis.

4. Transfer to either flow cytometry tubes or appropriate wells of a standard 96-well plate immediately before analyzing on the flow cytometer.

10. Column Separation and Sample Staining

Important Note: Perform this section (steps 10.1 and 10.2) **in batches of up to 8 samples.**

10.1. Column Separation

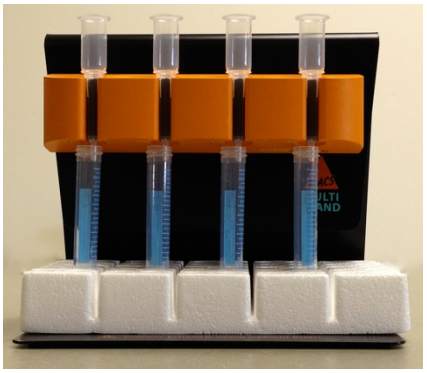
IMPORTANT NOTES
Avoid creating bubbles when adding samples or Buffered Salt Solution + 2 % FBS to the column. Air bubbles can block the column and prevent the eluate from passing through. Also, once the column has been "pre-wet" according to the directions below, it should be used immediately.

The entire elution process should occur **by the force of gravity only**; DO NOT force buffer or blood through the column with a plunger or other device.

1. Insert the appropriate number of LS Columns into either a MidiMACS™ or QuadroMACS™ Separator. Place a reservoir under the columns to collect the "pre-wet" rinse of the columns.

2. Gently add 3 mL cold Buffered Salt Solution + 2 % FBS to each column reservoir to pre-wet it. Be careful to avoid creating bubbles, and avoid disturbing the column matrix.

3. Once the pre-wet volume has stopped dripping from the column, remove the reservoir and **discard the collected rinse**. Place a clean, labeled 15 mL centrifuge tube under each column to collect the eluates for further processing. Ensure that the bottoms of the columns are inside the open tops of the centrifuge tubes (see the photo at right). Proceed immediately to the next step.

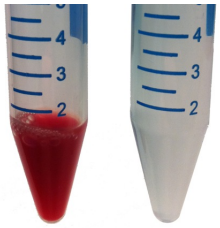


QuadroMACS™ Separator with LS Columns. Elution tubes placed directly underneath.

4. Take a sample (Section 9.4, Step 4) and carefully pipette up and down to resuspend the cells and anti-PE MicroBeads without creating bubbles. Gently add the entire 1 mL of sample into the appropriate pre-wet LS Column reservoir. Continue with additional samples, one per column.

5. When a 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. Be careful to avoid creating bubbles, and avoid disturbing the column matrix. Continue with remaining columns.

6. It takes approximately 5 minutes for the entire sample and wash to pass through the column and for the eluates to collect in the centrifuge tubes. The eluates will appear clear as the vast majority of the cells will be trapped in the column (see the picture).



Pre-column sample on left.
Post-column eluate on right.

7. Once eluates from this set are collected, store them in the dark at 2 °C to 8 °C or on ice and repeat steps 1 through 5 until all **Post-Column** samples in the batch of 8 have been through the column. Discard each LS Column after single use – **DO NOT reuse the columns**.

10.2. Centrifuge and Stain Post-Column Samples

1. Centrifuge a set of up to 8 eluate tubes from section 10.1 at **800 x *g* for 5 minutes at ambient temperature**.

2. After centrifugation, the pellet will be small and difficult to see. This is normal. Holding the tube upright, carefully aspirate supernatants starting at the top (meniscus) and working downwards to prevent disturbing the pellet. **It is critical that all samples have the same volume, and it is important to understand the average volume left in tubes.** See Appendix C for a description of an apparatus for consistent aspiration that guards against disturbing the cell pellets.

3. Gently tap pellets loose. 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.

4. Gently resuspend Working Nucleic Acid Dye Plus Counting Beads Solution with a pipettor prior to adding to the first sample. To ensure a homogenous suspension of Counting Beads in all samples, resuspend gently after adding to every 6 to 8 samples.

5. Add 300 μL ambient temperature Working Nucleic Acid Dye Plus Counting Beads Solution to each Post-Column sample. Carefully pipette up and down to resuspend the cells and Counting Beads, taking care not to splash onto the side of the tubes.

6. **Incubate in the dark at ambient temperature for 10 minutes.**

7. After incubation, transfer the tubes to ice and protect from light. Ensure that the tubes are buried in the flaked/chipped ice, not resting on top. Store on ice at least 5 minutes, but no more than 3 hours, before flow cytometric analysis.

8. Transfer to either flow cytometry tubes or appropriate wells of a standard 96-well plate immediately before analyzing on the flow cytometer.

9. Repeat sections 10.1 and 10.2 with the remaining batches of up to 8 samples.

11. Flow Cytometric Analysis – 96 Well Plate Based Analysis

Flow cytometric analysis can also be performed in single tubes (see Appendix E).

Important Notes

- Part A of the ICS is prepared from a non-antibody-labeled leukodepleted blood sample, preferably from a negative control animal, in Working Nucleic Acid Dye Plus Counting Beads Solution.
- Part B of the ICS is part of an antibody-labeled and stained Pre-Column sample, preferably from a negative control animal.
- Once the complete ICS is made, it should be analyzed immediately.
- Maintain a consistent fluidics rate throughout the analysis (for ICS and experimental samples).
- For digital instruments, such as a FACSCanto™, when analyzing **ICS and Pre-Column** samples in a 96 well plate, a Sample Flow Rate of approximately 2,000 to 6,500 events per second is recommended. This is usually achieved by running samples at 1.0 µL/sec Sample Flow Rate. 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 1.0 µL/sec Sample Flow Rate on a FACSCanto™. **The same Loader settings used for the ICS should be used for experimental samples except: Sample Volume = 60 µL for Pre-Column and 180 µL for Post-Column when using 1.0 µL/sec.**
- 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).

11.1. Instrument Calibration

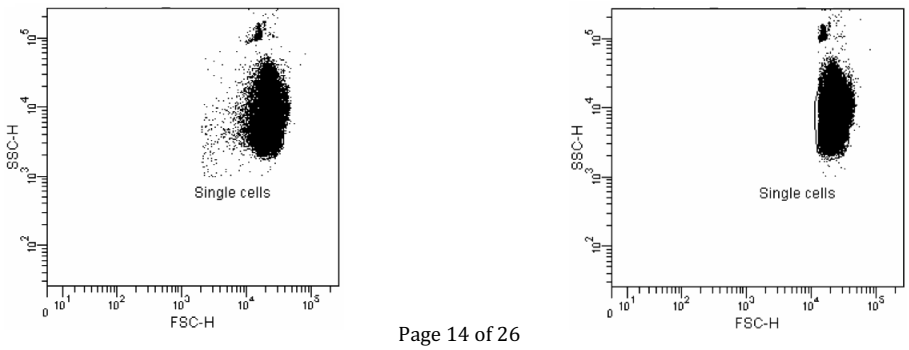
1. Before analyzing samples, ensure that the flow cytometer is working properly. Follow the manufacturer's instructions for the appropriate setup and quality control procedures. Open the data acquisition template file.

2. Prepare the ICS by combining equal volumes (e.g., 150 µL) of Part A (from Step 1 in Section 9.5) and the retained experimental Pre-column sample "Part B" (preferably from a vehicle control animal, see Section 9.4, Step 3) in a designated well in a 96 well plate. Pipette each part to resuspend before combining. Place the remaining Part A sample back on ice in case you need to prepare another ICS sample. The remaining sample that Part B was taken from should be placed with the other Pre-Column samples waiting for analysis. This ICS now consists of adequate numbers of anti-CD24-PE positive and negative events to guide selection of PMT voltages and compensation settings.

3. Immediately after creating the ICS, place it on the flow cytometer. The following Loader settings are a good place to start:
    - Throughput = Standard
    - Sample Flow Rate = typically 1.0 µL/sec
    - Sample Volume = 200 µL when using 1.0 µL/sec
    - Mixing Volume = 100 µL
    - Mixing Speed = 200 µL
    - Number of Mixes = 4
    - Wash Volume = 250 µL

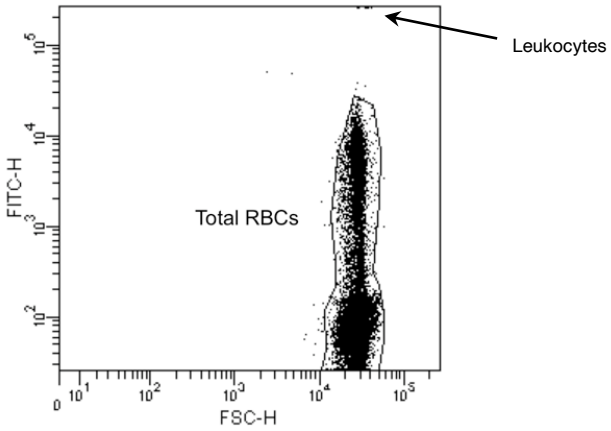
4. Threshold on FSC so that remaining platelets and other sub-cellular debris are eliminated. If your instrument is capable, threshold on both FSC and SSC, but be careful not to set the values so high that Counting Beads are thresholded out. In Plot A, adjust the "Single cells" region so that it closely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

Plot A **Recommended** **NOT Recommended**



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 below.

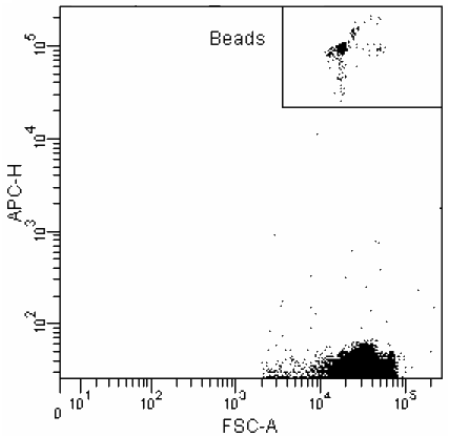
Plot B



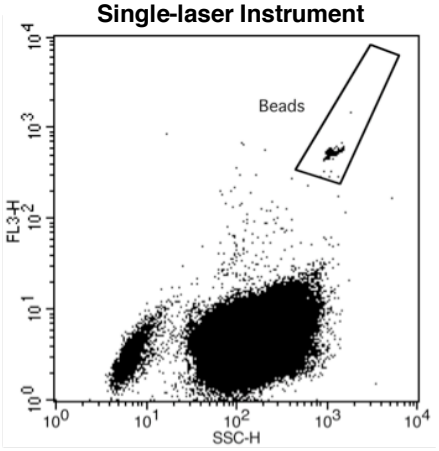
6. Viewing Plot D, adjust the FL4 (or FL3) PMT voltage so that Counting Beads fall within the "Beads" region. Adjust the position and size of the region as necessary. The resulting plot should look similar to one of the plots below.

Plot D

Dual-laser Instrument

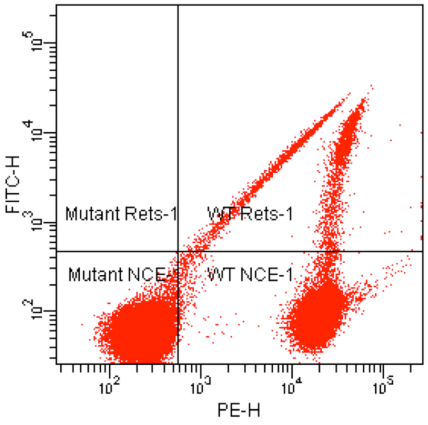


Single-laser Instrument

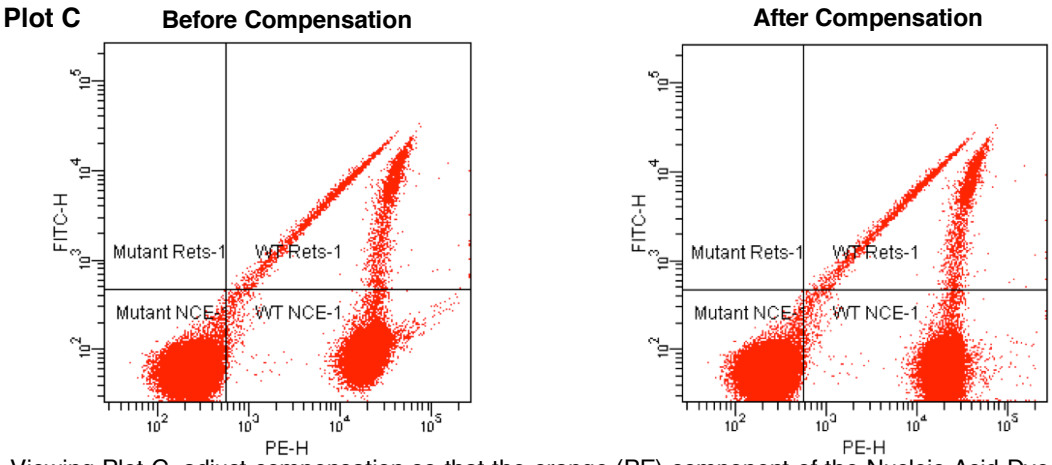


7. Viewing Plot C, adjust PMT voltages so that mutant phenotype, mature RBCs (lower-left quadrant; LL) are in the first to second decade of FITC and PE fluorescence. The resulting plot should look similar to the plot below.

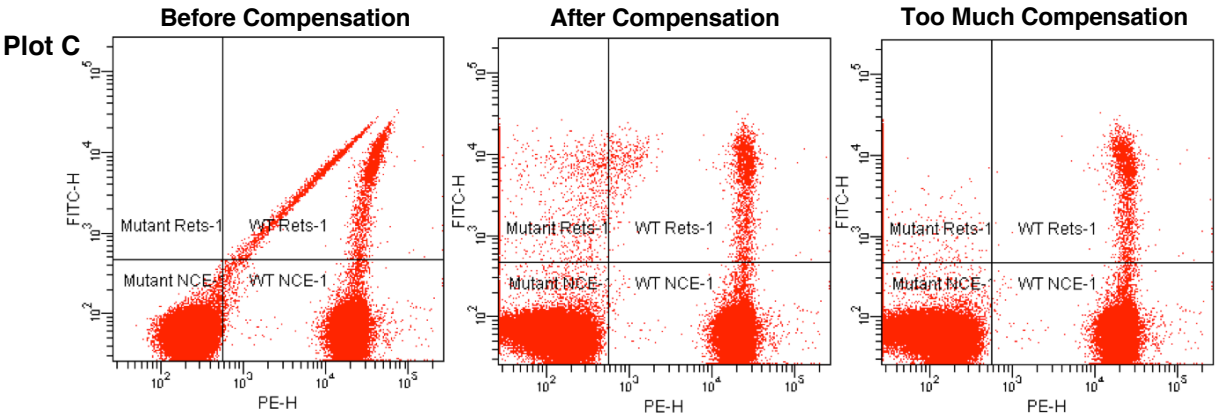
Plot C



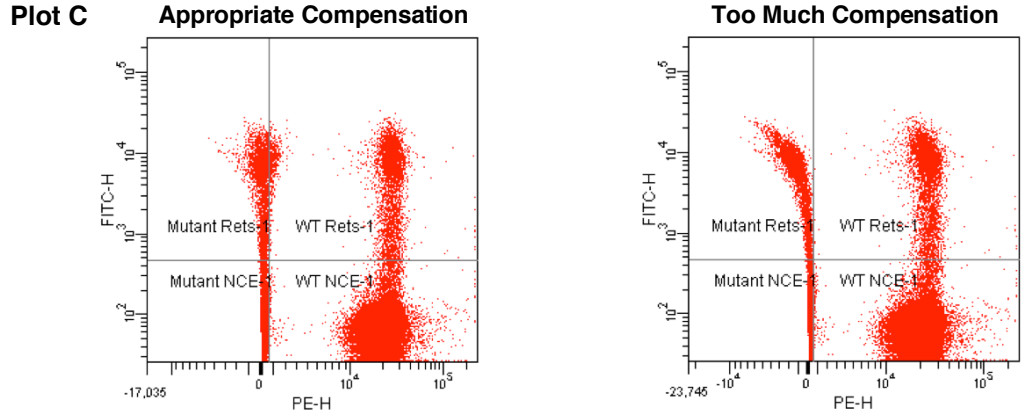
8. Viewing Plot C, adjust compensation so that the green (FITC) component of the PE label is eliminated. This is evident when the wild-type phenotype, mature RBCs (lower-right quadrant; LR) are at the same FITC fluorescence intensity as the mutant phenotype, mature RBCs (LL). One way that you can determine 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. See the before and after plots below



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 slightly to the right, as shown in the center plot below.



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 plot on the left, below.

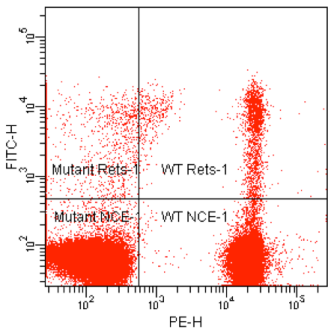


10. Viewing Plot C, adjust the quadrant's position to ensure it is appropriate. Use a conservative approach for scoring cells as mutant phenotype RBCs (i.e., these cells need to exhibit low PE fluorescence, similar to that of the mutant mimics). The resulting plot should look similar to the plot on the left, below.

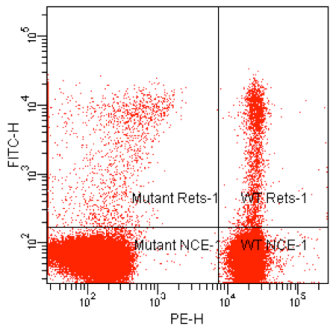
    In the right-hand plot, below, the horizontal demarcation line that distinguishes mature RBCs from RETs is too low. This can lead to subtle variations in staining intensity causing greatly overestimated % RET values. Additionally, the vertical demarcation line is positioned too far right. This can lead to subtle variations in staining intensity causing greatly overestimated frequencies of mutant phenotype cells.

Plot C

Recommended



NOT Recommended



11.2. Pre-Column Analysis of Experimental Samples in a 96 Well Plate

1. After analyzing the ICS sample and before analyzing experimental samples, add fresh distilled or deionized water to one or more wells and run for approximately 3 minutes to clear the lines of mutant-mimicking cells.

2. Maintain the same settings (except the Sample Volume should be 60 μL for Pre-Column samples), regions and Sample Flow rate used for the ICS sample and resuspend a set (typically the first row) of **Pre-Column** samples by pipetting up and down until the cells and Counting Beads are well resuspended; 10 times is usually sufficient. Do not vortex.

3. Transfer 200 μL to the corresponding wells in a new round bottom 96 well plate. Repeat for the remaining samples, using the same plate. Immediately place the plate with the **Pre-Column** samples on the flow cytometer, with well A1 at the top right. Select the wells you want to analyze and press "Run Wells".

4. Use a stop mode based on the length of time needed to acquire at least 1,000 Counting Beads - 1 minute is usually sufficient when using a 1.0 μL/sec Sample Flow Rate on a FACSCanto™.

5. Repeat until all **Pre-Column** samples have been analyzed.

11.3. Post-Column Analysis of Experimental Samples in a 96 Well Plate

1. After analyzing the Pre-Column samples and before analyzing the Post-Column samples, add fresh distilled or deionized water to one or more wells and run for approximately 3 minutes to clear the lines.

2. Maintain the same settings, regions and fluidics rate setting used for the Pre-Column samples, although a Sample Volume of 180 μL should be used. Use a stop mode based on the length of time needed to analyze nearly the entire volume of cells and Counting Beads - 3 minutes is usually sufficient when using a 1.0 μL/sec Sample Flow Rate on a FACSCanto™.

3. Remove the first **Post-Column** sample tube from ice and pipette up and down until well suspended; 4 to 5 times is usually sufficient. Do not vortex. Transfer the entire content to the appropriate well of a new round bottom 96 well plate. Repeat for the next seven samples. Immediately place the plate with the **Post-Column** samples on the flow cytometer, with well A1 at the top right. Select the wells you want to analyze and begin acquiring data.

4. Repeat until all **Post-Column** samples have been analyzed.

11.4. 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, or normochromatic erythrocytes (NCEs)
- RBCs = total erythrocytes, includes both RNA-positive and negative fractions
- UL = number of gated events occurring in Plot C's upper left quadrant, that is mutant RETs
- UR = number of gated events occurring in Plot C's upper right quadrant, that is wild-type RETs
- LL = number of gated events occurring in Plot C's lower left quadrant, that is mature mutant RBCs
- LR = number of gated events occurring in Plot C's lower right quadrant, that is mature wild-type RBCs
- Counting Beads = number of events occurring in Plot D's Counting Bead region

Sample Volume Variables:

- a = Starting Volume of antibody-labeled blood (μL), Section 9.4, step 1; usually 1000
- b = Volume of antibody-labeled blood added to Working Nucleic Acid Dye Plus Counting Beads Solution (μL), Section 9.4, step 2; usually 10
- c = Volume of Working Nucleic Acid Dye Plus Counting Beads Solution used to prepare Pre-Column samples (μL), Section 7.2, step 6; usually 990
- d = **LAB-SPECIFIC value**: the supernatant volume remaining in Post-Column samples following the final centrifugation and aspiration (μL), Section 10.2, step 2; should be between 20 and 50
- e = Volume of Working Nucleic Acid Dye Plus Counting Beads Solution added to each Post-Column sample (μL), Section 10.2, step 5; usually 300

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 + j) / 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**

An Excel spreadsheet can be obtained from Litron's website: www.LitronLabs.com (or email pigatechsupport@litronlabs.com). This spreadsheet can be used to make these calculations and also provides examples of actual flow cytometric data.

12. References

An updated list of journal articles utilizing this method can be found at www.LitronLabs.com/Resources/Publications/In-Vivo-MutaFlow-Kits.

- OECD (2002) Test No. 470: Mammalian Erythrocyte Pig-a Gene Mutation Assay, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/4faea90e-en.

13. License Agreement and Limited Product Warranty

By utilizing this kit, your company is agreeing to be bound by the terms of this License. This License allows the use of the MutaFlow® Kit for the analysis of 25 samples (MutaFlow® Kit).

By accepting these products, you acknowledge that they will be used in accordance with their intended labeling. For *in vitro* research use only. Not for human or animal diagnostic or therapeutic use. Uses other than the labeled intended use may be a violation of local laws.

This warranty limits our liability to replacement of this product. Litron shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Appendix A: Template Preparation

Data acquisition template files are available from Litron's website: www.LitronLabs.com (or email pigatechsupport@litronlabs.com) but are specific to CellQuest™ or FACSDiva™ software. The next pages show actual screen images of the CellQuest™ and FACSDiva™ template graphs. Flow cytometry operators who are not using CellQuest™ or FACSDiva™ software should find these pages 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". The 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 NOT 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.

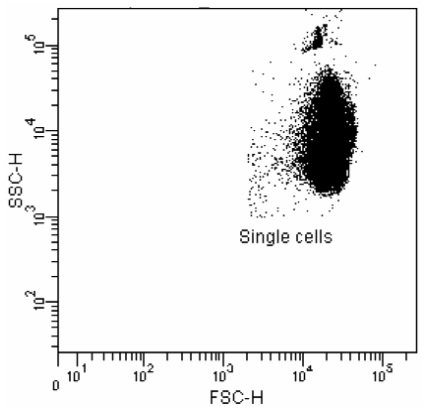
3. Quadrant Key for Plot C:
   UL = mutant RETs
   UR = wild-type RETs
   LL = mutant mature RBCs (i.e., mutant normochromatic erythrocytes [NCEs])
   LR = wild-type mature RBCs (i.e., wild-type normochromatic erythrocytes [NCEs])

4. Alternate names for detectors:

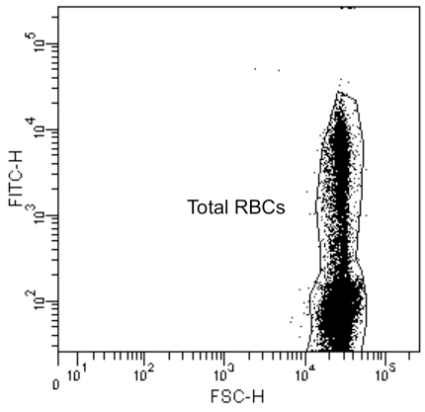
Green	FL1	FITC
Orange	FL2	PE
Red	FL3	PerCP-Cy5.5
Far red	FL4	APC

5. Save the template file. This template file should be suitable for all analyses. To ensure consistency of data, it is preferable that no changes be made to the location and size of the regions between samples.

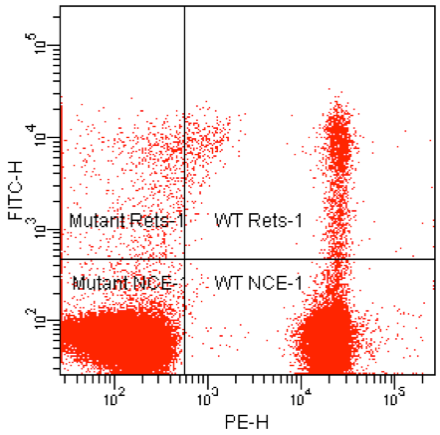
Plot A



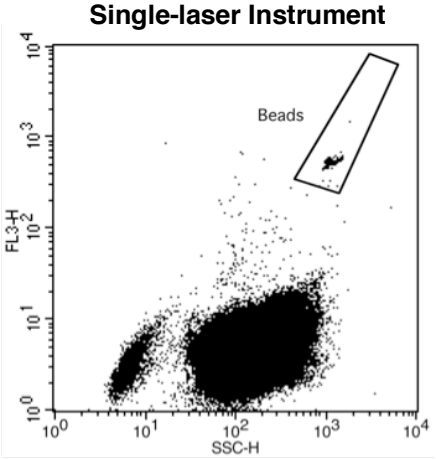
Plot B



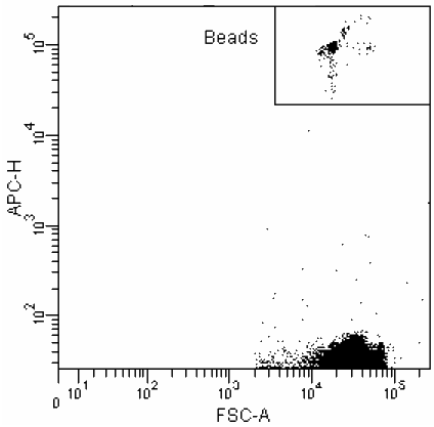
Plot C



Plot D
Single-laser Instrument



Plot D
Dual-laser Instrument

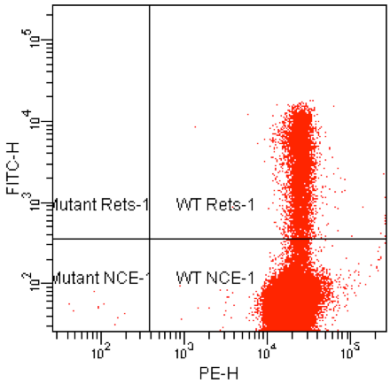


Appendix B: Example Plots

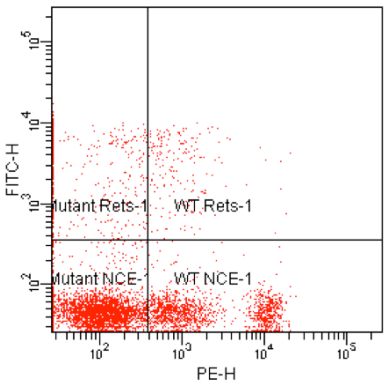
Example Plots

The plot on the left shows data from a **Pre-Column** sample that was acquired in 1 minute. This blood sample was obtained twenty-five days after three consecutive days of dosing with the mutagen ENU.

The plot on the right shows data from a **Post-Column** sample collected in 3 minutes. This blood sample was obtained twenty-five days after 3 consecutive days of dosing with the mutagen ENU.

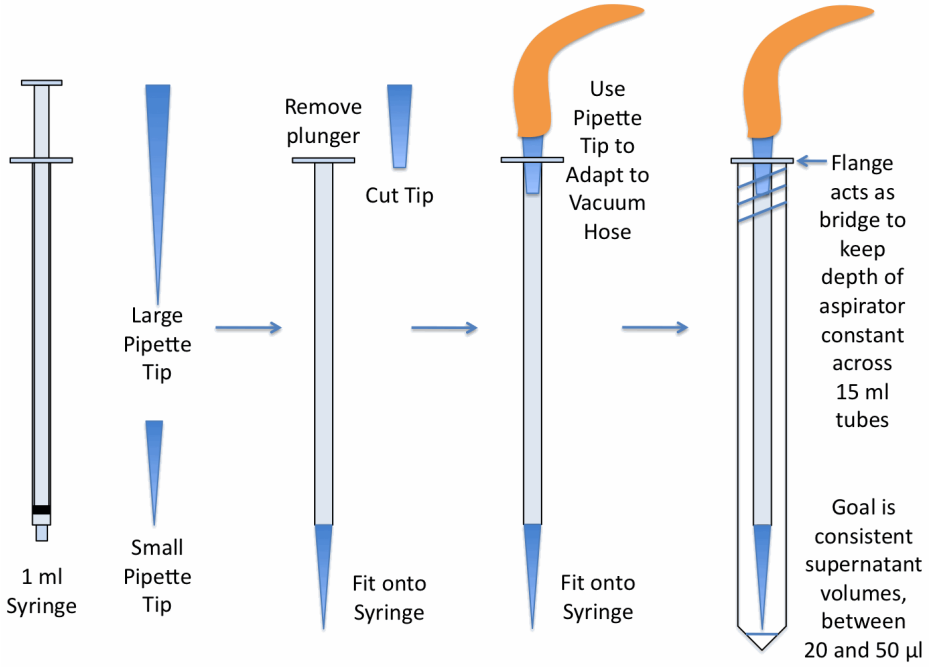


Plot C



Appendix C: Aspirating Samples Post Column

It is very important to carefully control and standardize aspirations, especially the last aspiration (Section 10.2). To achieve this, we recommend fashioning an aspirator with a bridge that controls the depth that the tip will reach. With this bridge, the aspirator will leave a consistent and low volume of supernatant that is the same across all tubes. The goal for the volume of supernatant left after the final aspiration is a *consistent value* within the range of 20 µL to 50 µL in each tube. Note that a consistent volume that ensures cells are not aspirated off is required to make mutant cell frequency calculations (see Section 11.4).



Appendix D: Flow Cytometric Analysis – Tube Based Analysis

Important Notes

- Part A of the ICS is prepared from a non-antibody-labeled leukodepleted blood sample, preferably from a negative control animal, in Working Nucleic Acid Dye Plus Counting Beads Solution.
- Part B of the ICS is part of an antibody-labeled and stained Pre-Column sample, preferably from a negative control animal.
- Once the complete ICS is made, it should be analyzed immediately.
- Maintain a consistent fluidics rate throughout the analysis (for ICS and experimental samples).
- For analog instruments, such as a FACSCalibur™, when analyzing **ICS and Pre-Column** samples in tubes, an event rate of approximately 2,000 to 6,500 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.
- For digital instruments, such as a FACSCanto™, when analyzing **ICS and Pre-Column** samples in tubes, an event rate of approximately 2,000 to 6,500 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.
- 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).

1.1 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. Open the data acquisition template file.

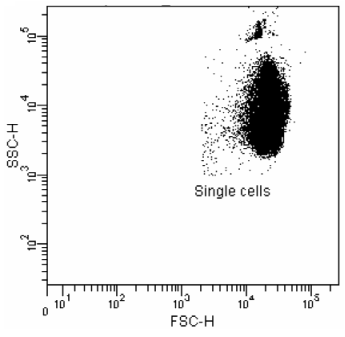
2. Prepare the ICS by combining equal volumes (e.g., 150 μL) of Part A (from Step 1 in Section 9.5) and the retained experimental Pre-column sample "Part B" (preferably from a vehicle control animal, see Section 9.4, Step 3) in a flow cytometry tube. Pipette each part to resuspend before combining. Mix by gentle pipetting. Place the remaining Part A sample back on ice in case you need to prepare another ICS sample. The remaining sample from which Part B was taken should be placed with the other Pre-Column samples waiting for analysis. This ICS now consists of adequate numbers of anti-CD24-PE positive and negative events to guide selection of PMT voltages and compensation settings.

3. Immediately after creating the ICS, place it on the flow cytometer. Set the fluidics rate appropriate for the flow cytometer (see Important Notes, above).

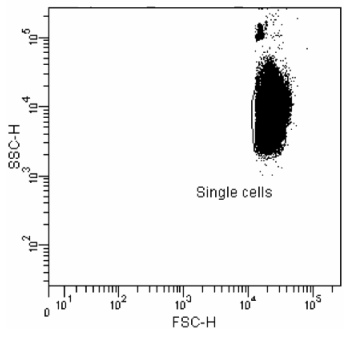
4. Set a FSC threshold so that remaining platelets and other sub-cellular debris are eliminated. If your instrument is capable, use FSC and SSC thresholds, but be careful not to set the values so high that Counting Beads are excluded out. In Plot A, adjust the "Single cells" region so that it closely defines the major population of single, unaggregated erythrocytes. The resulting plot should look similar to the plot on the left.

Plot A

Recommended

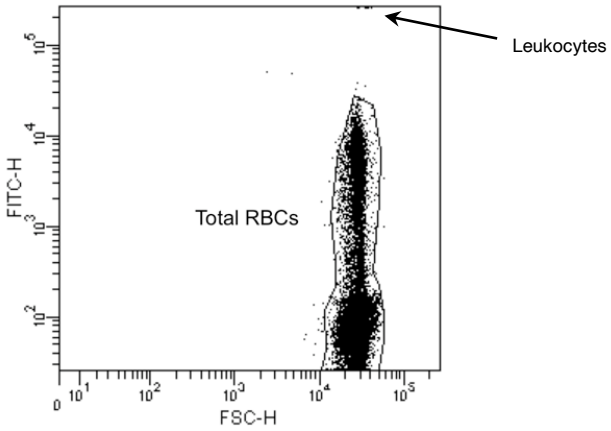


NOT Recommended



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 below.

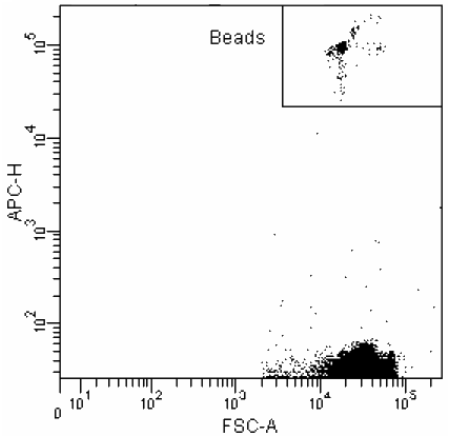
Plot B



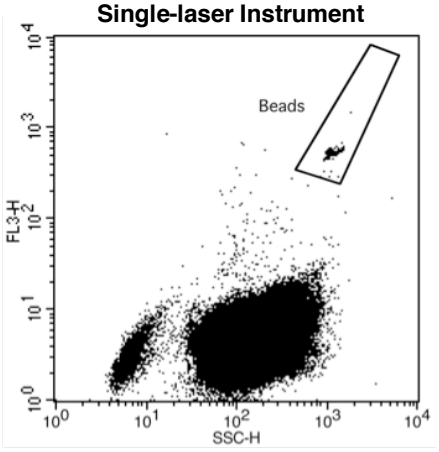
6. Viewing Plot D, adjust the FL4 (or FL3) PMT voltage so that Counting Beads fall within the "Beads" region. Adjust the position and size of the region as necessary. The resulting plot should look similar to one of the plots below.

Plot D

Dual-laser Instrument

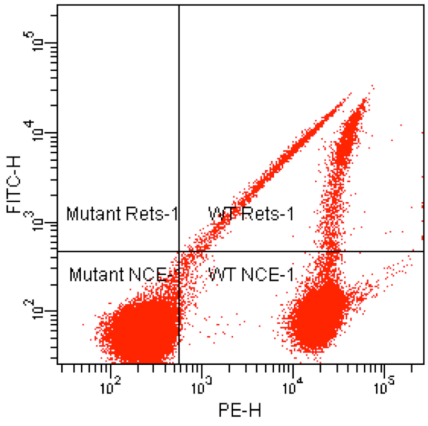


Single-laser Instrument

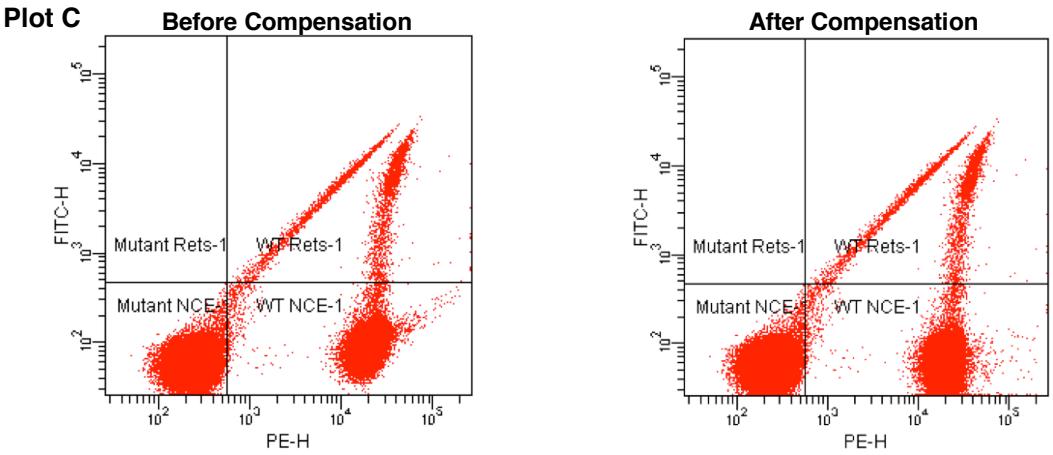


7. Viewing Plot C, adjust PMT voltages so that mutant phenotype, mature RBCs (lower-left quadrant; LL) are in the first to second decade of FITC and PE fluorescence. The resulting plot should look similar to the plot below.

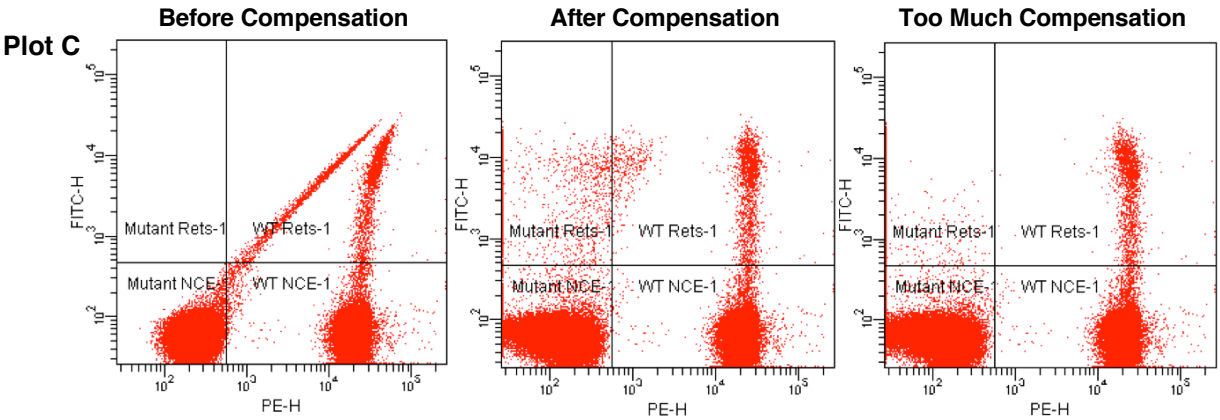
Plot C



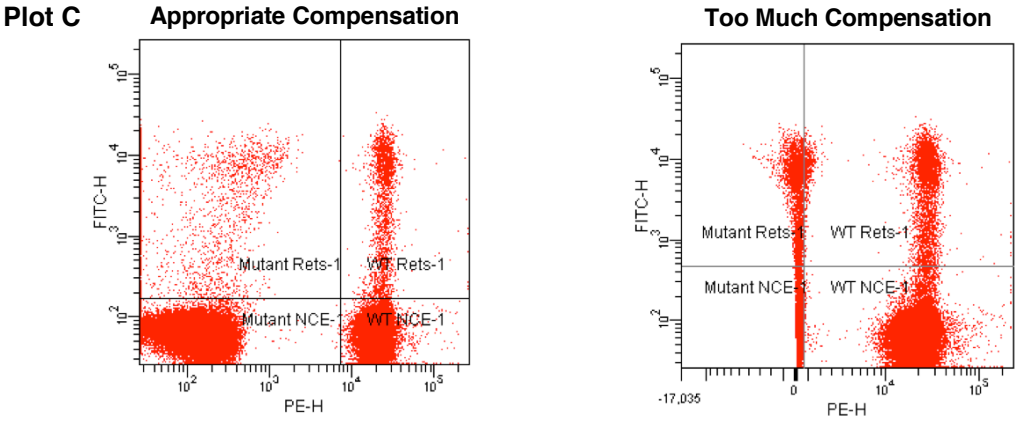
8. Viewing Plot C, adjust compensation so that the green (FITC) component of the PE label is eliminated. This is evident when the wild-type phenotype, mature RBCs (lower-right quadrant; LR) are at the same FITC fluorescence intensity as the mutant phenotype, mature RBCs (LL). One way that you can determine 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. See the before and after plots below



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 slightly to the right, as shown in the center plot below.



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 plot on the left, below.

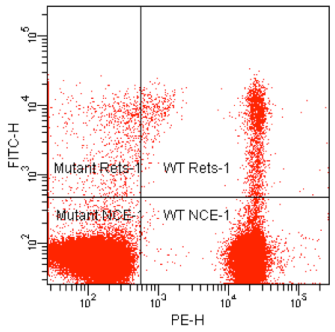


10. Viewing Plot C, adjust the quadrant's position to ensure it is appropriate. Use a conservative approach for scoring cells as mutant phenotype RBCs (i.e., these cells need to exhibit low PE fluorescence, similar to that of the mutant mimics). The resulting plot should look similar to the plot on the left, below.

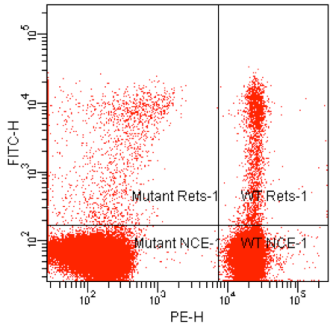
    In the right-hand plot, below, the horizontal demarcation line that distinguishes mature RBCs from RETs is too low. This can lead to subtle variations in staining intensity causing greatly overestimated % RET values. Additionally, the vertical demarcation line is positioned too far right. This can lead to subtle variations in staining intensity causing greatly overestimated frequencies of mutant phenotype cells.

Plot C

Recommended



NOT Recommended



1.2 Pre-Column Analysis of Experimental Samples in Tubes

1. After analyzing the ICS sample and before analyzing experimental samples, add filtered water to a new clean flow tube, place it on the flow cytometer and run for several minutes to clear the lines of mutant-mimicking cells.

2. Maintain the same settings, regions and fluidics rate setting used for the ICS sample and remove the first **Pre-Column** sample from ice and pipette up and down until the cells and Counting Beads are well resuspended; 10 times is usually sufficient; do not vortex.

3. Immediately place on the flow cytometer and begin acquiring data. Use a stop mode based on the length of time needed to acquire at least 1,000 Counting Beads - 1 minute is usually sufficient when using a High fluidics rate.

4. Repeat until all **Pre-Column** samples have been analyzed.

1.3 Post-Column Analysis of Experimental Samples in Tubes

1. After analyzing the Pre-column samples add water to a new clean flow tube, place it on the flow cytometer and run for approximately 3 minutes.

2. Maintain the same settings, regions and fluidics rate setting used for the Pre-Column samples, although the events per second will be lower. Use a stop mode based on the length of time needed to analyze nearly the entire volume of cells and Counting Beads - 3 minutes is usually sufficient when using a High fluidics rate setting (for analog machines) and 3 minutes is usually sufficient when using a Medium fluidics rate setting (for digital machines).

3. Remove the first **Post-Column** sample from ice and pipette up and down until well suspended; 4 to 5 times is usually sufficient; do not vortex or sonicate.

4. Immediately place on the flow cytometer, and begin acquiring data. Repeat until all **Post-Column** samples have been analyzed.