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Micro
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Quality Counts!

**Micronucleus
Analysis
Kit**

MicroFlow^{PLUS} (Rat Blood)

Instruction Manual

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

Table Of Contents

1. Materials Provided	1
2. Additional Materials Required	1
3. Store Kit-Supplied Calibration Standards	1
4. Ordering Information and Technical Services	1
5. First-Time Users	2
5.1. Template Preparation	2
5.2. Using Kit-Supplied Calibration Standards Samples	2
5.3. Using a CD71 Setup Sample	2
5.4. Flow Cytometer Requirements	2
5.5. Daily Calibration with Calibration Standards	2
6. Introduction	3
6.1. The Micronucleus Test	3
6.2. The MicroFlow [®] Method	3
6.3. Regulatory Acceptance	3
7. Overview of Method	4
8. Prepare Fixative Tubes and Anticoagulant/Diluent Vials	4
8.1. Prepare the Fixative Tubes (at least one day prior to blood collection)	4
8.2. Prepare the Anticoagulant/Diluent Vials (prior to blood collection)	5
9. Collect Blood Samples	5
10. Fix Blood Samples	5
11. Store Fixed Samples Until Analysis	7
11.1. Wash Samples out of Fixative	7
11.2. Transfer Samples Washed out of Fixative into Long Term Storage Solution (LTSS)	7
12. Wash Samples and Calibration Standards	8
12.1. Wash Samples and Calibration Standards out of LTSS	8
12.2. Wash Samples out of Fixative	8
13. Labeling Procedure	8
13.1. Prepare Labeling Solutions 1 and 2	8
13.2. Prepare DNA Staining Solution	9
13.3. Label Washed Blood Samples for Flow Cytometric Analysis	10
14. Flow Cytometric Analysis	11
14.1. Flow Cytometer Calibration with Calibration Standards	11
14.2. Analysis of Experimental Samples	14
15. Troubleshooting	16
16. References	18
17. License Agreement and Limited Product Warranty	18
Appendix A: Template Preparation	19

1. Materials Provided

Kit Component	Quantity ^a	Storage Conditions ^f
Anticoagulant/Diluent	10 mL or 30 mL	2 °C to 8 °C
Buffer Solution	0.5 L or 2 L	2 °C to 8 °C
Long Term Storage Solution (LTSS)	30 mL or 65 mL	2 °C to 8 °C
RNase Solution	50 μ L or 100 μ L	-10 °C to -30 °C
Anti-Rat CD71 Antibody ^b	50 μ L or 100 μ L	2 °C to 8 °C
DNA Stain	1 mL or 4 mL	2 °C to 8 °C
Anti-Rodent CD61 Antibody ^b	30 μ L or 50 μ L	2 °C to 8 °C
Calibration Standards in Long Term Storage Solution (LTSS)		
• Positive Control samples (fixed) ^c	Two or Three 1 mL aliquots	-75 °C to -85 °C
• Negative Control samples (fixed) ^d	Two or Three 1 mL aliquots	-75 °C to -85 °C
• Malaria Biostandard samples (fixed) ^e	Two or Three 1 mL aliquots	-75 °C to -85 °C

- a. Sufficient materials are provided to collect and analyze 15 (Trial Kit) or 60 blood samples (60 sample kit).
- b. Warning! Contains sodium azide. Irritant. See MSDS (available on website).
- c. Positive Control sample: Noninfectious, nonhazardous denatured rat blood sample with an elevated level of micronuclei.
- d. Negative Control sample: Noninfectious, nonhazardous denatured rat blood sample with a background level of micronuclei.
- e. Malaria Biostandard sample: Noninfectious, nonhazardous denatured rat blood sample used for calibration of flow cytometer.
- f. Please note that although some kit components are shipped at ambient temperature, they must be stored at the temperatures indicated above upon receipt.

2. Additional Materials Required

- Fixative must be methanol. Litron recommends at least 99.8 % purity, CAS # 67-56-1
- -75 °C to -85 °C freezer (a chest freezer is preferred)
- -10 °C to -30 °C freezer
- 2 °C to 8 °C refrigerator
- Micropipette and appropriate tips
- 25 mL pipettes
- Ice
- Blood collection supplies (may include K₂EDTA tubes, Litron recommends BD Catalog number 367861)
- Swinging bucket centrifuge (a 4 °C refrigerated centrifuge is preferred)
- Flow cytometer capable of 488 nm excitation
- Flow cytometry tubes
- Polystyrene flasks
- 15 mL polypropylene centrifuge tubes (VWR catalog number 21008-103 recommended)
- Heat-inactivated, freshly filter-sterilized fetal bovine serum

3. Store Kit-Supplied Calibration Standards

Please note: While it is acceptable that the MicroFlow^{PLUS} Kit-supplied Calibration Standards thaw during transport, it is important that they are immediately placed upright in a -75 °C to -85 °C freezer upon receipt.

4. Ordering Information and Technical Services

Litron Laboratories
 3500 Winton Place, Suite 1B
 Rochester, New York 14623
 Telephone: 585-442-0930
 Order Toll Free: 877-4-LITRON (877-454-8766)
 Fax: 585-442-0934
 email: info@LitronLabs.com
 World Wide Web: www.LitronLabs.com

5. First-Time Users

We strongly recommend reading the entire instruction manual before performing these procedures.

Please do not deviate from the procedures described in this manual. It is important that these steps are followed using the reagents supplied with this kit in order to achieve reliable results. If you have questions, please contact Litron Laboratories by calling (585) 442-0930, faxing us at (585) 442-0934, or sending an email to info@litronlabs.com.

5.1. Template Preparation

Template files are available on Litron's website (www.LitronLabs.com), but are specific to CellQuest™ or FACSDiva™ software. If you are unable to use these templates, please prepare one PRIOR to washing any samples for analysis. See the appendix for screen images of the CellQuest™ template's graphs and histogram. There is also a description of the gates utilized for each plot, and how a stop mode of 20,000 reticulocytes is set. Flow cytometry operators who are not using CellQuest™ software should find these pages valuable for constructing their own data acquisition and analysis template.

5.2. Using Kit-Supplied Calibration Standards Samples

Please note that all kit-supplied samples are in LTSS and must be washed out of LTSS (see Section 12.1) and labeled (see Section 13) prior to flow cytometric analysis (see Section 14.1).

First-time users of this kit are encouraged to analyze kit-supplied Positive Control and Negative Control samples after initial flow cytometer calibration with the Malaria Biostandard samples, and to calculate % RET and % MN-RET frequencies as described. These values should be similar to those indicated for that particular lot (see documentation on the website). If after performing the procedures as described, your results from the analysis of the Negative Control or Positive Control samples are not consistent with the data provided, fax (585-442-0934) or email representative plots (including Malaria Biostandard) and associated data to Litron Laboratories for troubleshooting. We recommend that you do not proceed with analyses of experimental samples until Litron has evaluated these plots.

5.3. Using a CD71 Setup Sample

For daily flow cytometer setup, a CD71 Setup sample is required. This should be a blood sample from an age-matched negative control or vehicle control rat collected and fixed at your laboratory. When using this kit for the first time, the kit-supplied Negative Control sample can serve as the CD71 Setup sample.

5.4. 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. For BD instruments (such as the FACSCalibur), the green (FITC) fluorescence should be collected in the FL1 channel, yellow (PE) fluorescence should be collected in the FL2 channel and the red (propidium iodide; PI) fluorescence should be collected in the FL3 channel. For other instruments, FL4 may provide better resolution of PI.

5.5. Daily Calibration with Calibration Standards

A CD71 Setup sample and Malaria Biostandard samples should be used for flow cytometer calibration on each analysis day prior to the analysis of experimental samples. Please note that these Calibration Standards must be washed out of Long Term Storage Solution (see Section 12.1) and labeled (see Section 13) prior to flow cytometric analysis (see Section 14.1).

6. Introduction

This kit is used for preparing rat blood samples for flow cytometric enumeration of micronucleated erythrocyte populations.

6.1. The Micronucleus Test

The *in vivo* micronucleus test was established as a means of analyzing chromosomal damage. The test is based on the observation that displaced chromatin, resulting from chromosomal loss or breakage, can form a secondary nucleus (micronucleus) outside the daughter nuclei of a dividing cell. Micronuclei (MN) occur spontaneously, but an elevation in the frequency of MN in a population of cells can be indicative of exposure to a genotoxic agent.

Micronuclei are particularly apparent in red blood cells (erythrocytes), which otherwise lack DNA. During erythropoiesis, a hematopoietic stem cell differentiates into an erythroblast and eventually expels its nucleus to become a reticulocyte (RET). The newly formed RET is then released from the bone marrow into the circulating bloodstream, where it develops into a mature normochromatic erythrocyte (NCE). Although the main nucleus is lost during RET formation, MN may be retained in the RET cytoplasm. Peripheral blood is ideal for micronucleus analyses because samples can be obtained from an animal easily and at multiple time points.

6.2. The MicroFlow[®] Method

Litron Laboratories has developed and patented a flow cytometric method to measure micronuclei in both the RET and NCE populations. Unlike mature NCEs, immature RETs are still rich in RNA as well as certain surface proteins (e.g., transferrin receptor, also known as CD71), and can therefore be differentially stained based on these features. An increase in the frequency of micronucleated reticulocytes (MN-RETs) can indicate acute genotoxicity associated with a recent cell division. In mice, an increase in the frequency of micronuclei in the NCE population (MN-NCE) can indicate accumulated DNA damage associated with a sub-chronic or chronic treatment regimen. Elevated MN-NCE frequencies in rat blood need to be interpreted with caution, since splenic filtration function is the dominant factor that influences these values.

The MicroFlow method offers significant advantages compared to traditional microscopic scoring techniques, such as:

- Greater number of cells can be examined for MN
- Faster data acquisition
- Increased statistical power of the assay
- Objective analysis of samples

The MicroFlow method also offers advantages over other automated methods, including:

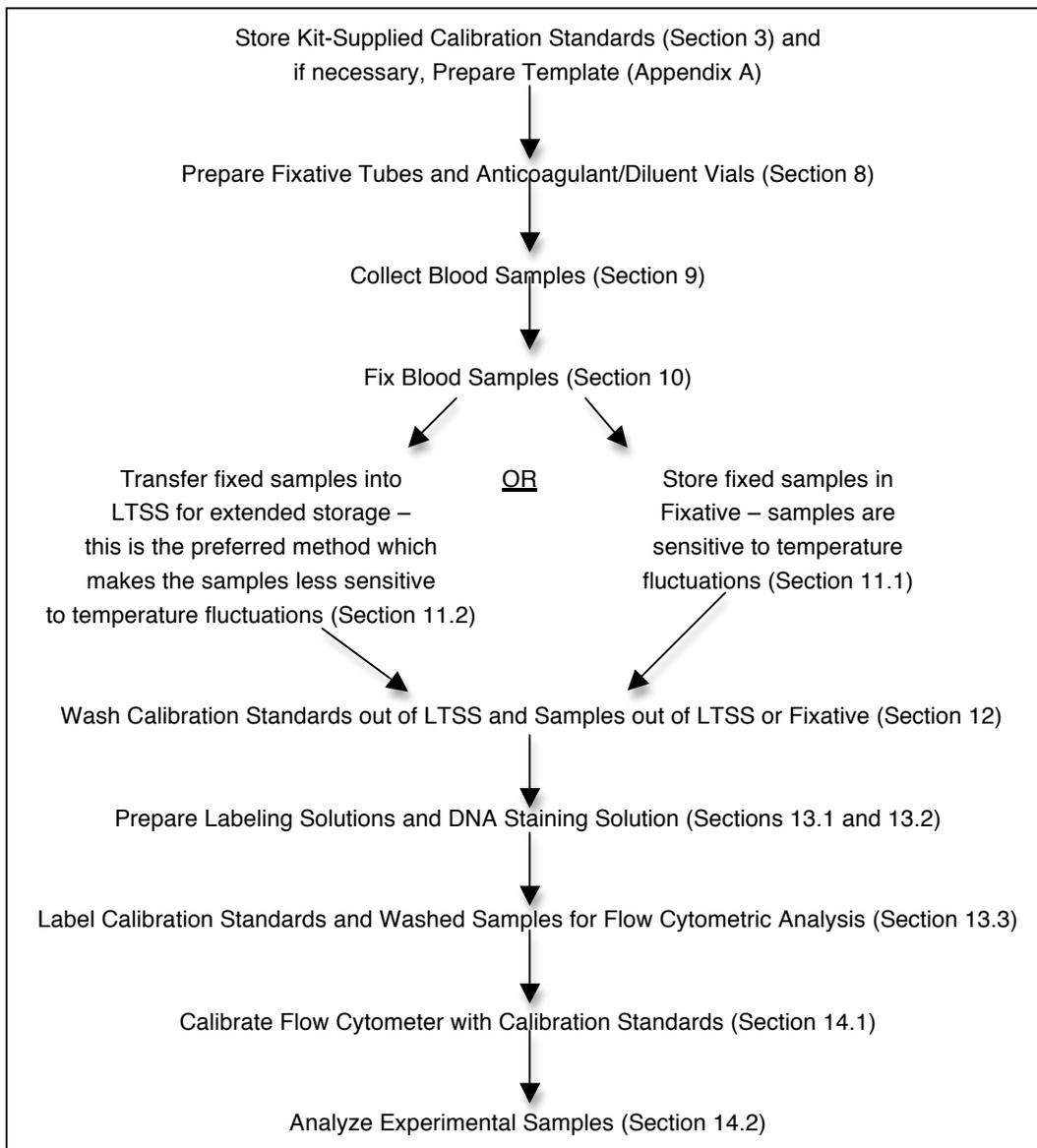
- Availability for many species of toxicological interest
- Anti-platelet antibody to ensure reliable data
- Calibration standards to ensure intra- and inter-laboratory reproducibility of data
- Ability to store samples for extended periods of time before analysis

Crucial components of this method are the Calibration Standards which aid flow cytometer configuration for the micronucleus scoring application. Fixed blood from animals infected with *Plasmodium berghei* are used to configure the flow cytometer before analysis. Whereas MN are relatively rare and exhibit a heterogeneous DNA content, parasitized cells are prevalent and have a homogenous DNA content. These characteristics make them ideal for calibrating the flow cytometer for the micronucleus scoring application. After optimizing the flow cytometer with the Calibration Standards, micronucleus analyses can be performed reliably and with minimal intra- and inter-experimental variation.

6.3. Regulatory Acceptance

The US FDA accepts preclinical MicroFlow data, and this method adheres to the necessary guidelines as stated by the International Workshop on Genotoxicity Test Procedures (IWGTP). Additionally, the most current Organization for Economic Co-Operation and Development (OECD) guidelines regarding micronucleus testing, Guideline 474, indicates that flow cytometry, using appropriate calibration standards, can provide better inter- and intra-laboratory reproducibility and sensitivity than manual microscopic scoring. It also states that “Commonly used laboratory strains of healthy young adult animals should be employed. Mice, rats, or another appropriate mammalian species may be used. When peripheral blood is used, it must be established that splenic removal of micronucleated cells from the circulation does not compromise the detection of induced micronuclei in the species selected. This has been clearly demonstrated for mouse and rat peripheral blood.”

7. Overview of Method



8. Prepare Fixative Tubes and Anticoagulant/Diluent Vials

8.1. Prepare the Fixative Tubes (at least one day prior to blood collection)

- One 15 mL polypropylene centrifuge tube is required per sample (or two, if fixing in duplicate). Add 2 mL of Fixative to each tube, and replace caps.
- Label each tube with the appropriate information. If labels are used, ensure they are compatible with ultra-cold storage. It is also helpful to label the cap of each tube.
- If not using ultra-cold freezer-compatible labels, it is helpful to cover the labels with clear tape, to protect them from splashed liquids and to prevent them from falling off in the freezer.
- Place the rack of tubes overnight (or longer) at $-75\text{ }^{\circ}\text{C}$ to $-85\text{ }^{\circ}\text{C}$ to allow for sufficient chilling of the Fixative.

Duplicate fixed samples should be prepared.

8.2. Prepare the Anticoagulant/Diluent Vials (prior to blood collection)

1. One clean vial is required for each sample. Aseptically aliquot 350 μ L Anticoagulant/Diluent into each vial.
2. Label vials with the appropriate information and refrigerate until needed.

9. Collect Blood Samples

Method: Collect blood using a method approved by the Institutional Animal Care and Use Committee (IACUC).

Volume: Collect 60 μ L to 120 μ L of blood from each animal into vials containing 350 μ L Anticoagulant/Diluent. Collecting more than 120 μ L of blood may compromise the flow cytometric analysis.

Anticoagulant: Use only kit-supplied Anticoagulant/Diluent as the liquid anticoagulant, and collect blood directly into it. If a capillary tube or syringe is used to draw blood, pre-fill it with Anticoagulant/Diluent. Have extra vials containing Anticoagulant/Diluent ready in case of a spill. After collecting blood into Anticoagulant/Diluent, mix gently. Samples can be stored at room temperature for up to 6 hours before fixing. Refrigerated samples can be stored for up to 24 hours before fixing.

Warning: Some capillary tubes include a clot activator that will cause aggregation and make the samples unanalyzable.

An alternative method is to collect blood into K₂EDTA-coated tubes (Litron recommends BD Catalog number 367861). Blood samples collected into K₂EDTA tubes are stable at 2 °C to 8 °C for 2 days. They should be diluted into Anticoagulant/Diluent immediately before fixing as follows: invert to ensure a homogeneous suspension, remove 100 μ L of sample and dilute into 350 μ L Anticoagulant/Diluent and fix as described below.

10. Fix Blood Samples

It is extremely important that the tubes containing Fixative remain ultracold (–75 °C to –85 °C) and do not come in contact with vapors from dry ice. CO₂ vapor causes carbonation and cellular aggregation. Therefore, Fixative should not be stored in a freezer containing dry ice.

If you are unable to fix blood samples DIRECTLY from the –75 °C to –85 °C freezer as described here, follow the alternative fixing procedure found on the website.

A video of the fixing procedure is available on our website (www.litronlabs.com).

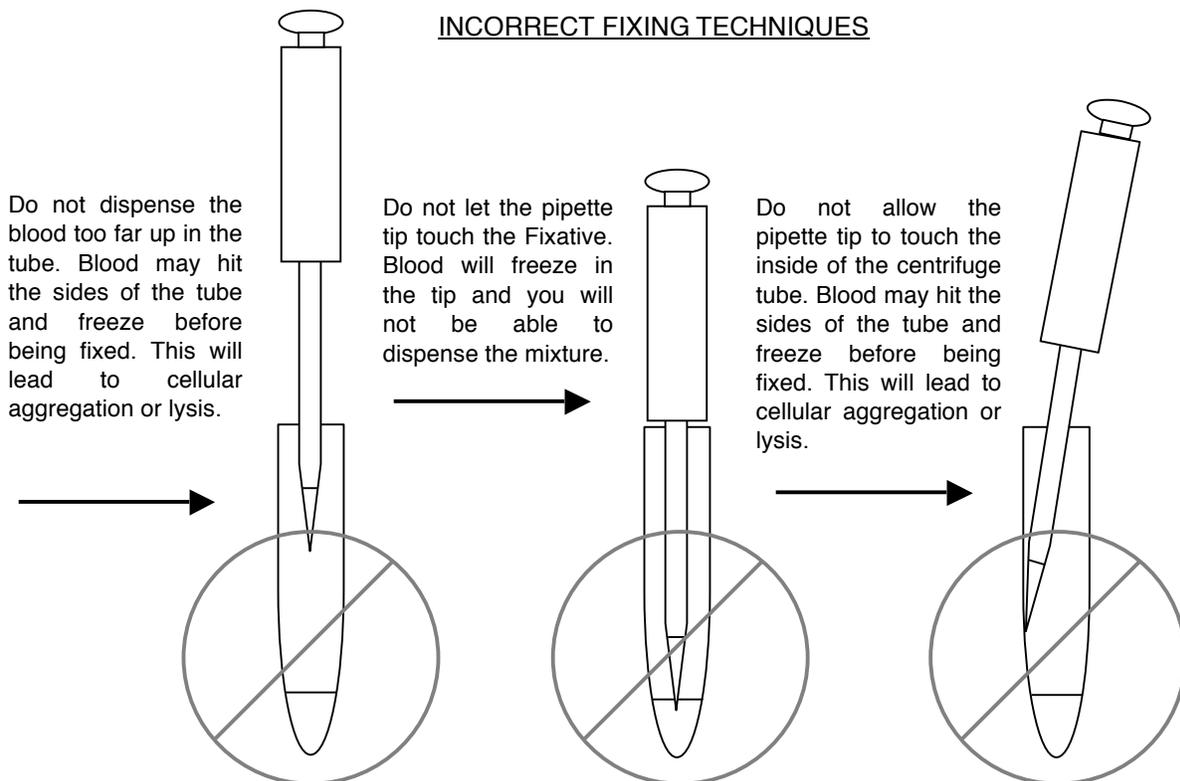
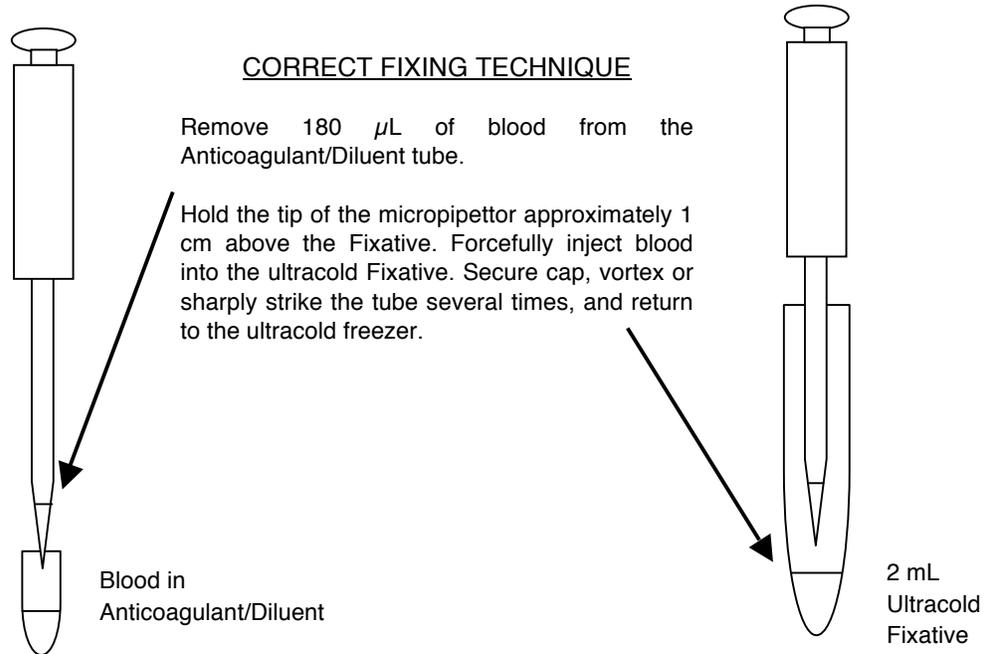
Keep Fixative in an ultracold (–75 °C to –85 °C) freezer (a chest freezer is preferred because they maintain temperature better than upright freezers). Perform the following steps very quickly and work near the freezer. Samples can be fixed in duplicate.

It may be helpful for two individuals to perform this procedure, one filling the micropipettors with diluted blood samples and the other removing the Fixative tubes from the freezer.

1. Immediately prior to fixing, invert the vial containing the diluted blood to ensure a homogeneous suspension.
2. Using a micropipettor, retrieve 180 μ L of the diluted blood sample.
3. Remove the corresponding labeled 15 mL tube containing Fixative from the freezer, uncap and position the pipette tip approximately 1 cm above the surface of the ultracold Fixative.
4. Make sure that the pipette tip does not touch the side of the tube or the surface of Fixative, and forcefully dispense directly into Fixative. See the diagram on the next page.
5. Cap the tube of fixed blood VERY tightly and vortex briefly (only 3 to 5 seconds) and return it to the ultracold freezer (–75 °C to –85 °C). If a vortexer cannot be placed right next to the freezer, hold the top of the tube with one hand, use your other hand to sharply strike the bottom of the tube several times before returning it to the freezer. (Steps 3 through 5 should take no more than 10 seconds.)

Remove only one tube of ultracold Fixative from the freezer at a time (unless two individuals are performing). After adding the blood sample and mixing, return this tube to the freezer before working with another tube. (As stated, the Fixative and fixed blood must be maintained at –75 °C to –85 °C .)

6. Change the pipette tip and repeat steps 1 through 5 for the remaining diluted samples. There should be enough volume in each vial to fix each sample twice, if necessary.
7. If the freezer temperature begins to warm up significantly (i.e., raises by 5 °C), stop processing samples. Wait until the freezer temperature returns to the required range before completing sample fixation. Again, due to their ability to maintain temperature, chest freezers are recommended.
8. Store the samples at –75 °C to –85 °C for at least 3 days before washing to store in LTSS or analyzing on the flow cytometer.



11. Store Fixed Samples until Analysis

IMPORTANT NOTE

We strongly recommend that methanol fixed samples be transferred into LTSS as described in Sections 11.1 and 11.2 below. If you have access to a chest-style ultracold freezer that will maintain the fixed samples at the required temperature range consistently, and where the samples can be stored toward the bottom of the freezer, samples can be maintained in Fixative.

If the freezer is not a chest-style freezer, is opened routinely, or the samples cannot be stored away from the freezer door, samples should be washed out of Fixative and transferred into LTSS, as described below. Ideally, samples should be transferred into LTSS after they have been in fixative for at least 3 days. It is possible to transfer samples into LTSS many days or even weeks after fixation in Fixative, but during this time they are extremely sensitive to temperature fluctuations.

11.1. Wash Samples out of Fixative

It may be helpful for two individuals to perform this procedure, one tapping and opening tubes and the other adding Buffer Solution. Once two people have become proficient with this procedure, it is possible to remove as many as 3 tubes from the freezer at a time.

1. Pack Buffer Solution on ice to achieve ice-cold, but not freezing, temperature (approximately 45 minutes).
2. Have a container of ice and a 25 mL pipette ready for aliquoting Buffer Solution in step 4. Perform the following steps as quickly as possible (within approximately 20 seconds); therefore plan to work at a location adjacent to the freezer.
3. Remove up to three tubes of fixed experimental samples from the ultracold freezer. Quickly place the capped tubes on ice and close the freezer. Tap each tube sharply 3 or 4 times (or vortex for 3 to 5 seconds) to resuspend the cells and loosen the cap on each tube.
4. Immediately add 12 mL of ice-cold Buffer Solution to each tube. (Be careful not to touch the tube with the pipette tip to prevent transfer of sample from one tube to another.) Tighten the caps, invert the tubes once to mix the solutions, and immediately replace on ice until all are processed.
5. Repeat steps 3 and 4 for additional samples. **Note that once Buffer Solution has been added to the fixed cells, it is important that the tubes remain on ice or at 2 °C to 8 °C unless otherwise specified.**
6. Centrifuge the tubes at approximately 300 x *g* to 400 x *g* for 5 minutes. When centrifugation is complete, quickly remove the tubes and immediately replace them on ice.
7. Aspirate the supernatant from each tube, leaving less than 50 µL of supernatant in which to resuspend cells. Recap the tubes and immediately return to ice.
8. Working with one sample at a time, quickly resuspend the cells in the remaining supernatant by tapping the bottom of the tube or by vortexing. Put the tube back on ice and continue to resuspend the remaining samples.
9. At this point, either transfer cells into LTSS for further storage (**Section 11.2**) or proceed to labeling (**Section 13**) and analysis (**Section 14**).

If a tube cracks, quickly transfer the fixed cells to another centrifuge tube.

11.2. Transfer Samples Washed out of Fixative into Long Term Storage Solution (LTSS)

1. After washing out of Fixative and resuspending pellets (**Section 11.1, Steps 1-8**), add 1 mL of LTSS to each tube and store at –75 °C to –85 °C. Samples can also be transferred into cryo-vials to save freezer space.

It can be helpful to separate duplicate blood samples by using separate storage boxes.

12. Wash Samples and Calibration Standards

12.1. Wash Samples and Calibration Standards out of LTSS

Ideally, samples and Calibration Standards should be washed on the day they will be analyzed, but once washed out of LTSS, they are stable for approximately three days (stored at 2 °C to 8 °C). *[Note: To conserve Calibration Standard samples, thaw and remove 0.5 mL for washing and refreeze the rest.]*

1. Prepare at least 5 mL of Buffer Solution with 1 % FBS for each sample in LTSS and Calibration Standard to be washed. Filter through a 0.2 μ m filter and place on ice. (If all of this solution is not used, refrigerate and filter again on the day of use, or discard and prepare fresh when washing samples on another day.)
 - a. If samples are in cryovials, aliquot 5 mL of this Buffer Solution + 1 % FBS into the appropriate number of labeled 15 mL centrifuge tubes (one tube per sample) and keep on ice.
 - b. If samples are in 15 mL tubes, you will add 5 mL of this Buffer + 1 % FBS to each tube.
2. Remove the appropriate cells from the ultracold freezer and thaw at ambient temperature.
3. Immediately upon thawing, either aliquot contents into tubes containing 5 mL Buffer + 1 % FBS, or add 5 mL Buffer Solution + 1 % FBS to each tube. Invert once to mix, then place back on ice. Repeat with the remaining samples.
4. Centrifuge the tubes at approximately 300 x *g* to 400 x *g* for 10 minutes. When centrifugation is complete, quickly remove the tubes and immediately replace them on ice.
5. Aspirate the supernatant from each tube, leaving less than 50 μ L of supernatant in which to resuspend cells. Recap the tubes and immediately return to ice.
6. Working with one sample at a time, quickly resuspend the cells in the remaining supernatant by tapping the bottom of the tube or by vortexing. Put the tube back on ice and continue to resuspend the remaining samples.
7. After all pellets are resuspended, store the samples at 2 °C to 8 °C, or on ice until labeling for flow cytometric analysis.

12.2. Wash Samples out of Fixative

Follow the procedure described in section 11.1.

13. Labeling Procedure

13.1. Prepare Labeling Solutions 1 and 2

Labeling Solution 1 degrades RNA and labels RETs. Labeling Solution 2 degrades RNA, and labels RETs and platelets. Prepare one aliquot of Malaria Biostandard in Labeling Solution 1. Prepare one aliquot of Malaria Biostandard and one aliquot of a CD71 Setup sample (see “First Time Users” section) in Labeling Solution 2. Prepare all experimental samples in Labeling Solution 2.

Labeling solutions should be made fresh each analysis day and are prepared from Buffer Solution, RNase Solution, Anti-Rat CD71 Antibody, and Anti-Rodent CD61 Antibody, in the proportions indicated in the tables below. *[Note that heat-inactivated, filter-sterilized fetal bovine serum (FBS), has been found to subtly improve sample staining characteristics. If using FBS, create a mixture of 1 % FBS in Buffer Solution, filter on the day of use, and use this in place of the “Volume of Buffer Solution” found in the table below.]*

- Determine the total number of samples that will be prepared that day (including Malaria Biostandard, CD71 Setup, Negative Control and Positive Control samples), and use the chart below as a guide to preparing the Labeling Solutions and scale up as needed.

Number of samples	Volume of Buffer Solution*	Volume of RNase Solution	Volume of Anti-Rat CD71 Antibody
1	100 μ L	1 μ L	1 μ L

* with or without 1 % heat-inactivated FBS

- Thaw RNase Solution. Quick spin RNase Solution and Anti-Rat CD71 Antibody for best recovery of contents.
- Place the required volume of cold Buffer Solution into a sterile tube.
- Aseptically add the required volumes of RNase Solution and Anti-Rat CD71 Antibody to the tube containing Buffer Solution. Tap gently to mix. This is Labeling Solution 1.
- Remove 80 μ L of Labeling Solution 1 and place into a flow cytometry tube. This will receive 20 μ L of Malaria Biostandard and will be used for calibrating the flow cytometer.
- Quick spin Anti-Rodent CD61 Antibody for best recovery of contents.
- Aseptically add the required volume of Anti-Rodent CD61 Antibody to the tube containing Labeling Solution 1, using the chart below as a guide. Tap gently to mix. This is Labeling Solution 2.

Number of samples	Approximate Volume of Labeling Solution 1	Volume of Anti-Rodent CD61 Antibody
1	100 μ L	0.5 μ L

- Aliquot 80 μ L of Labeling Solution 2 into labeled flow cytometry tubes. Prepare one tube for each experimental sample to be analyzed that day, and also one tube each for the Malaria Biostandard and CD71 Setup sample. Cover all tubes containing Labeling Solutions with foil, and store at 2 °C to 8 °C until needed.

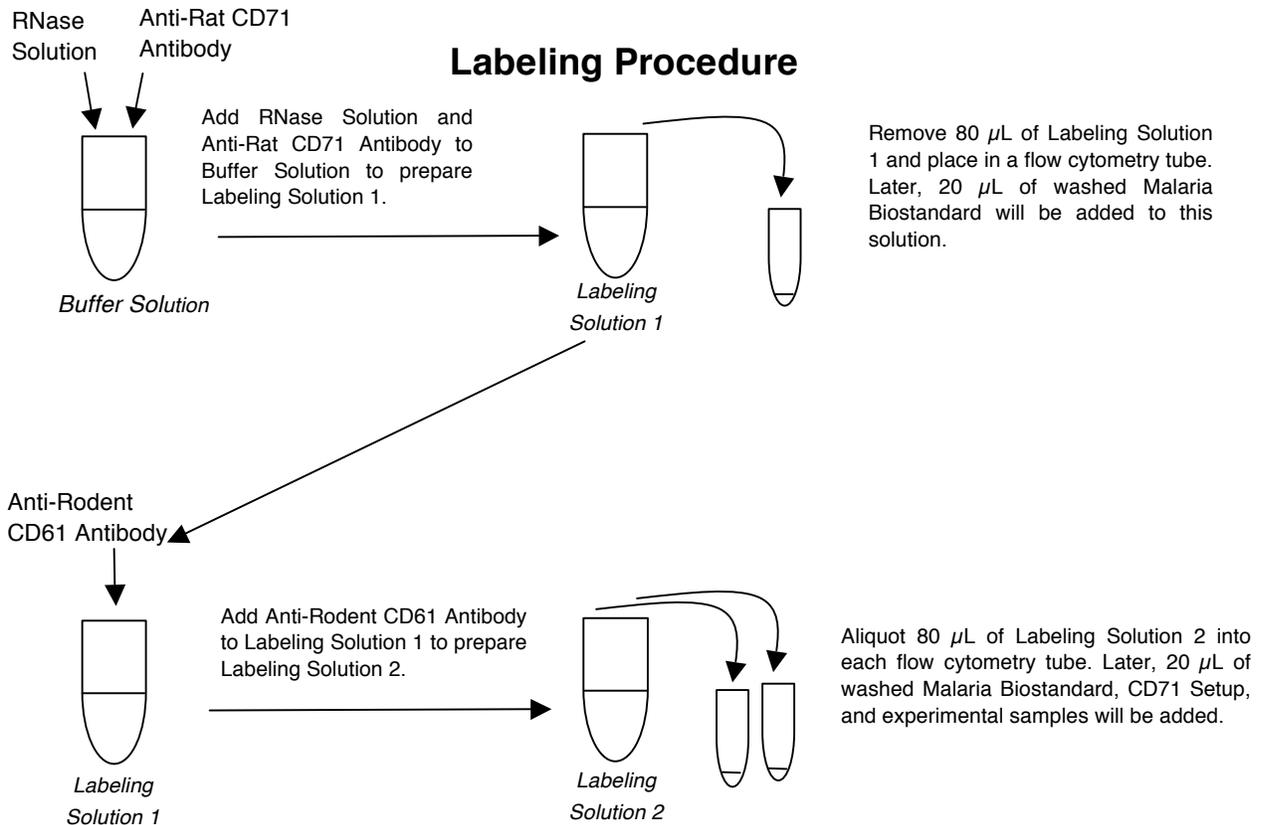
13.2. Prepare DNA Staining Solution

The DNA Staining Solution is used to stain the DNA of malaria parasites and micronuclei. This solution should be prepared fresh each analysis day and is prepared from Buffer Solution and DNA Stain in the proportions indicated in the table, below. Note that Buffer Solution should not be supplemented with FBS.

- Determine the number of samples that will be analyzed that day (including Malaria Biostandard and CD71 Setup samples), and use the chart below as a guide to preparing DNA Staining Solution and scale up as necessary.

Number of samples	Volume of Buffer Solution	Volume of DNA Stain
1	2 mL	0.05 mL

- Place the required volume of Buffer Solution into a sterile container, and add the necessary volume of DNA Stain.
- Cap the container and shake gently to mix.
- Store DNA Staining Solution in the dark at 2 °C to 8 °C (not on ice) until needed.



13.3. Label Washed Blood Samples for Flow Cytometric Analysis

To ensure an accurate reading, it is important that all cells are labeled and the cellular RNA is degraded. Therefore, make sure that when adding the blood sample to the Labeling Solution, all of the sample comes into contact with the Labeling Solution. If a drop of blood is on the side of the tube, wash it down with the solution already in that tube.

1. With washed blood samples on ice, tap the tubes to resuspend the cell pellets (if not recently tapped out).
2. Add 20 µL of Malaria Biostandard to the FCM tube containing Labeling Solution 1. Gently tap to mix.
3. Add 20 µL of Malaria Biostandard to an FCM tube containing Labeling Solution 2. Add 20 µL of CD71 Setup to an FCM tube containing Labeling Solution 2. Gently tap to mix.
4. Add 20 µL of each experimental blood sample to the appropriately labeled FCM tubes containing Labeling Solution 2. Tap the tubes gently to mix.
5. Return any remaining washed blood samples to 2 °C to 8 °C for storage.
6. Cover FCM tubes with foil to protect from light, and incubate at 2 °C to 8 °C refrigerator (preferred) or on ice for 30 minutes.
7. After 30 minutes at 2 °C to 8 °C, tap each tube very gently to resuspend cells and place in new racks (that are not cold). Protect from light and incubate the tubes at room temperature for 30 minutes to ensure complete degradation of cellular RNA. If room temperature is below 20 °C, the second 30 minute incubation can be modified so that the samples are incubated at approximately 37 °C for 15 minutes and then room temperature for 15 minutes.
8. After the incubation at room temperature, return the tubes to 2 °C to 8 °C until analysis.

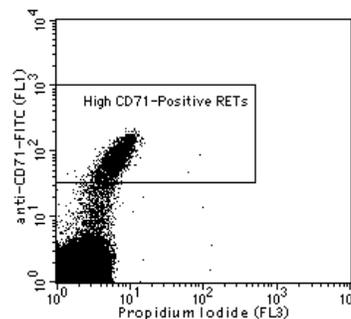
14. Flow Cytometric Analysis

14.1. Flow Cytometer Calibration with Calibration Standards

Please note that the Calibration Standards must first be washed out of LTSS (Section 12.1) and labeled (Section 13.3) before being used to calibrate the flow cytometer. The following setup and compensation instructions are specific for CellQuest™ software, but should be useful with other software packages. If using FASCDiva™ software, perform the following steps:

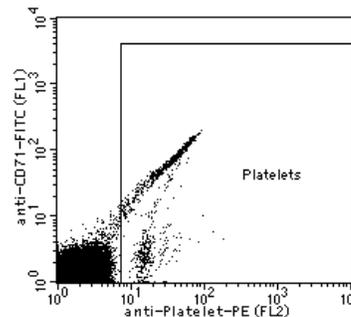
- Open the FASCDiva™ template (.xml file) for Rat PLUS Blood (available for download from the website).
 - Open the following folders on your computer: My computer > New Volume(D) > BDEExport > Templates > Experiment > General.
 - Drag the template into the General folder.
 - Close this window and start the FASCDiva™ software.
 - Click on “Experiment” in the menu bar and create a new folder. Select the new folder and click the “New Experiment” button on the Browser toolbar. The Experiment Template dialog appears. Click the “General Tab” and select your template.
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 template file.
 2. Gently tap the bottom of the tube containing the washed and labeled CD71 Setup sample to loosen settled cells. Add 1.5 mL to 2.0 mL of DNA Staining Solution and place the sample on the flow cytometer. (While this sample is being analyzed, add DNA Staining Solution to the Malaria Biostandard samples, and place at 2 °C to 8 °C until analysis.) In Plot A, adjust the “Single Cells” region to include the cells of interest and exclude aggregates (see Appendix A).
 3. Adjust FL1 PMT voltage so the High CD71-Positive RETs are located just above the lower green demarcation on the Y-axis of Plot E. Adjust FL3 PMT voltage so the NCEs are within the first decade of red fluorescence. The resulting plot should look similar to the plot below.

Plot E

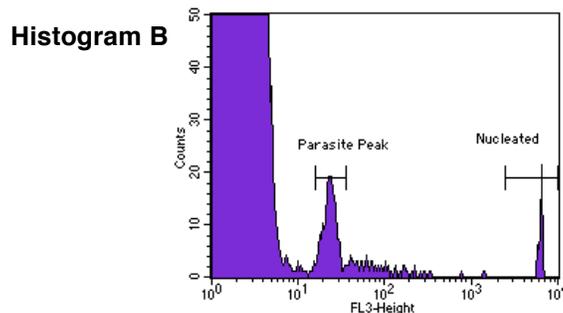


4. Place the Malaria Biostandard sample in Labeling Solution 1 on the flow cytometer. Refrigerate the CD71 Setup sample.
5. Viewing Plot D1, adjust the FL2 PMT voltage until the majority of cells in the lower left area are just to the left of the “Platelets” region. The resulting plot should look similar to the one below.

Plot D1

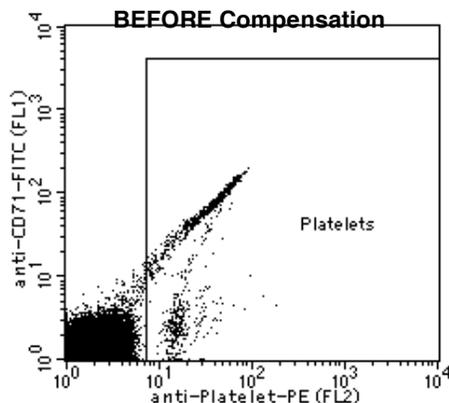


- Viewing Histogram B, adjust FL3 PMT voltage until the non-parasitized cells approach the first decade of FL3 fluorescence and the first parasite peak is well resolved. (BD CellQuest™ users are advised to place the first parasite peak at channel 22 ± 2 on Histogram B.) The location of the nucleated cells should fall in the last half of the last decade of red fluorescence. The resulting plot should look similar to the one below.

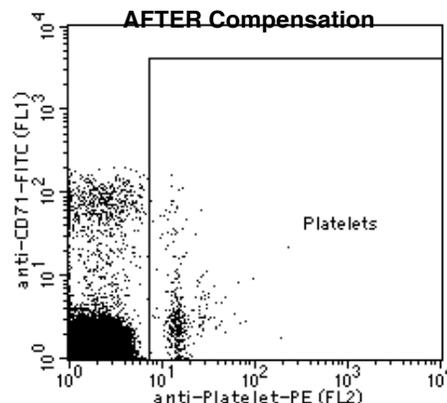


- Eliminate the yellow (FL2) component of the FITC signal. Adjust FL2-%FL1 compensation to eliminate the yellow component of FITC. Viewing Plot D1, increase compensation until the young, FITC-positive cells are positioned directly above the mature, FITC-negative cells. Correct compensation is set when cells containing a single parasite are aligned in a vertical plane. See the before and after plots below.

Plot D

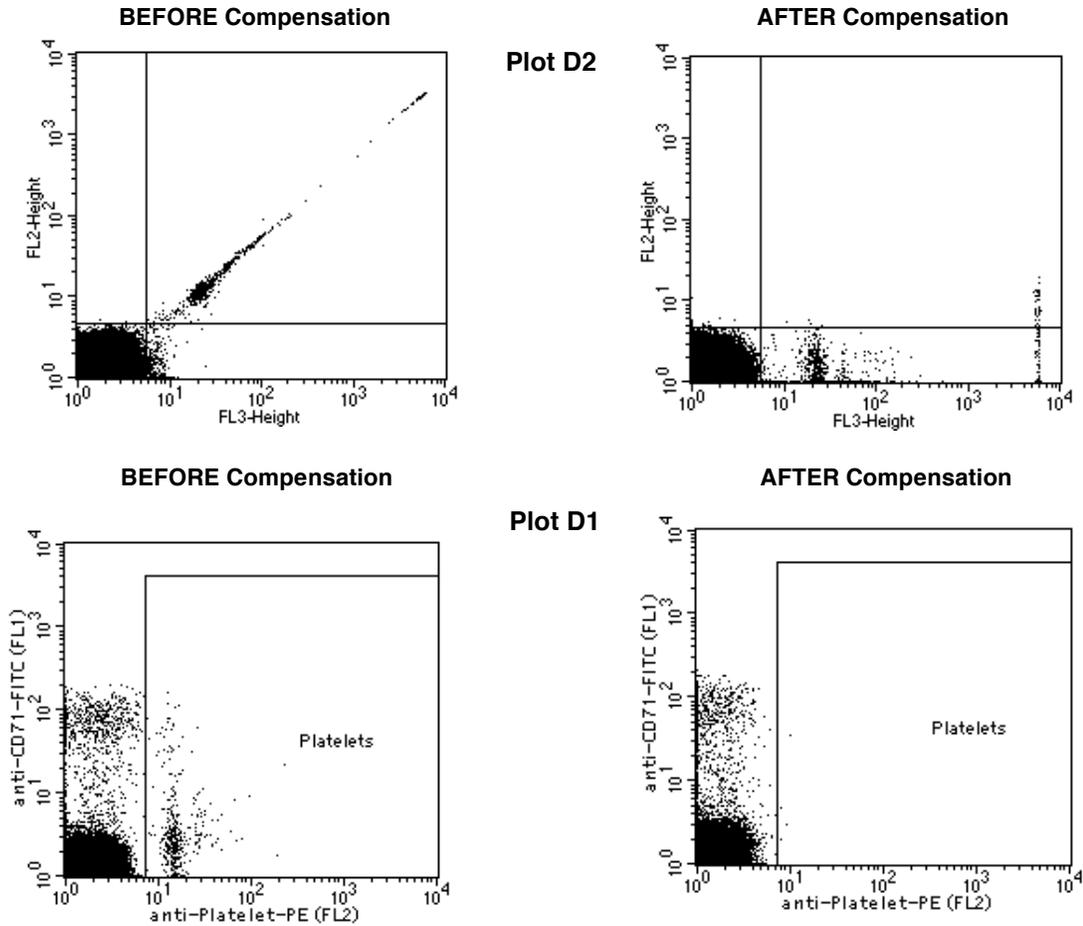


AFTER Compensation

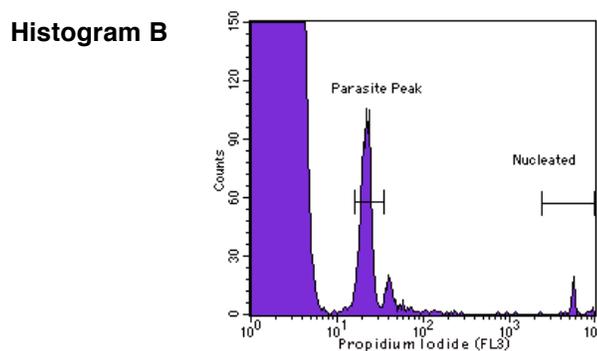


Plot D1

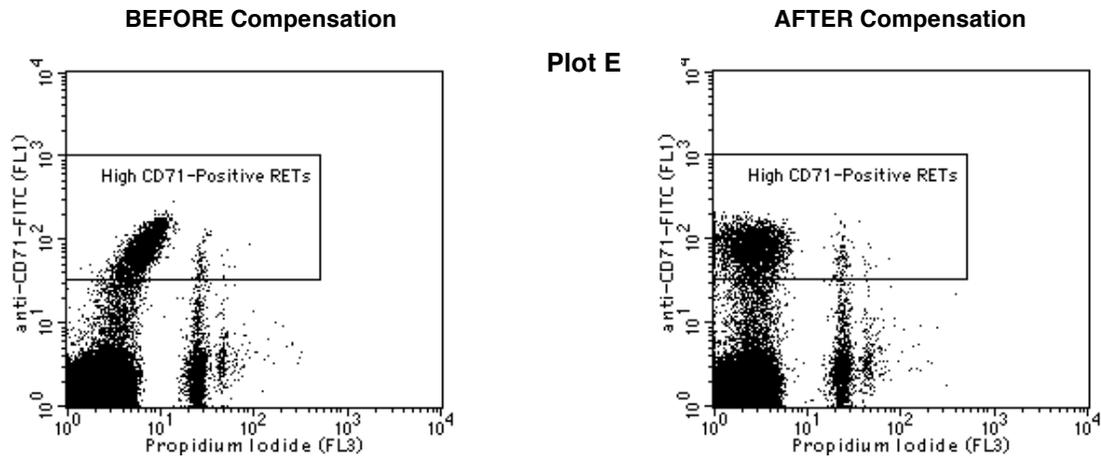
- Adjust FL2-%FL3 compensation to eliminate the yellow component of propidium iodide. Viewing Plot D2, increase compensation until the parasitized cells show low FL2-associated fluorescence which is on par with that observed for the NCEs at the origin. The nucleated cells' fluorescence should be low, no more than the first decade of FL2. When compensation is set appropriately, the parasitized cells should not be evident in the "Platelets" region of Plot D1. See the before and after plots of D2 and D1, below.



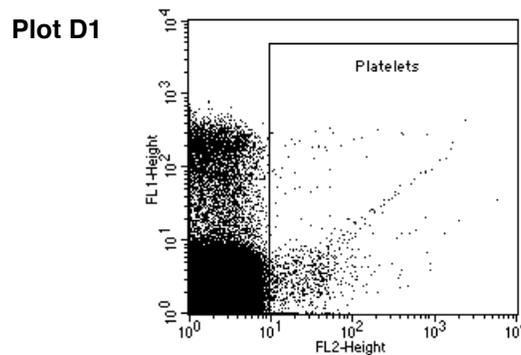
- Readjust the FL3 PMT voltage, if necessary. (BD CellQuest™ users are advised to place the first parasite peak at channel 22 ± 2 on Histogram B.) If this is performed, compensation may need slight adjustments.



- The green fluorescent antibody (FITC-conjugated) has a slight far-red (FL3) component. Use the FL3-%FL2 compensation to eliminate the red component of FITC. Viewing Plot E, increase compensation until the young, FITC-positive cells are positioned directly above the mature, FITC-negative cells. Correct compensation is set when cells containing a single parasite are aligned in a vertical plane. See the before and after plots, below.



- Refrigerate the Malaria Biostandard sample in Labeling Solution 1, and place the Malaria Biostandard sample in Labeling Solution 2 on the flow cytometer. This sample includes the anti-platelet antibody, therefore it should have FL2-associated fluorescence (PE). Without adjusting voltages or compensation, the resulting plot should look similar to the one below. Refrigerate the Malaria Biostandard sample in Labeling Solution 2.



- Once instrumentation and software settings are configured with the CD71 Setup and Malaria Biostandard samples, continue to monitor the location of the nucleated cell peak of the experimental blood samples throughout the course of an analysis session. This measurement is useful for guarding against instrument drift. If the nucleated cells drift out of position, use the CD71 Setup and Malaria Biostandard samples stored at 2 °C to 8 °C to readjust the instrument as necessary.
- After calibrating the flow cytometer with the CD71 Setup and Malaria Biostandard samples each day, analyze experimental samples.

14.2. Analysis of Experimental Samples

- It is preferable that the regions and quadrants are not changed between experimental samples. This is why it is worth taking some extra time to carefully consider PMT voltages and compensation settings initially. The region “Single Cells” on Plot A may need to be adjusted between samples (to accurately define the light scatter characteristics of unaggregated cells).
- If this is your first time using this process, analyze the kit-supplied Positive Control and Negative Control Samples. Otherwise, proceed to the analysis of experimental samples.

3. It is important to maintain a consistent flow rate, therefore, do not change the flow rate after calibrating your instrument with the Calibration Standards.
4. Analyze experimental samples by adding 1.5 mL to 2.0 mL of DNA Staining Solution to a sample. Place the sample on the flow cytometer and analyze. It is important not to change the FL3 PMT voltage (Malaria Biostandard assured this was appropriate), although FL3-%FL2 compensation may need slight adjustment so that RETs sit directly above the NCEs.
5. While one sample is being analyzed, add 1.5 mL to 2.0 mL of DNA Staining Solution to the next sample and place at 2 °C to 8 °C.
6. To obtain meaningful micronucleus statistics, it is important to use a spreadsheet rather than CellQuest's "% Gated" or "% Total" statistics. A sample spreadsheet is available from the website. Within this spreadsheet, input the number of "Events" observed in the Quadrant Statistics to calculate the following:

$$\% \text{ RET} = (\text{UL} + \text{UR}) / (\text{UL} + \text{UR} + \text{LL} + \text{LR}) * 100$$

$$\% \text{ MN-NCE} = (\text{LR}) / (\text{LR} + \text{LL}) * 100$$

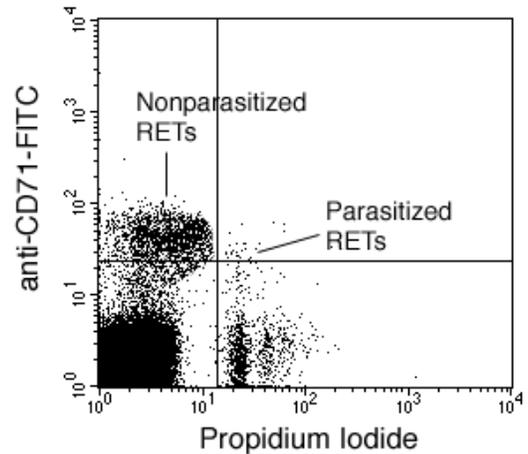
$$\% \text{ MN-RET} = (\text{UR}) / (\text{UR} + \text{UL}) * 100$$

15. Troubleshooting

Observation	Possible cause	Suggestion
Blood clots	Blood touches the side of the tube before reaching Anticoagulant/Diluent	Shake the blood collection tube immediately before bleeding each animal to coat the inside of the tube with Anticoagulant/Diluent.
Clumps of cells in Fixative	Blood touches the side of the tube and aggregates before reaching Fixative	Make sure the pipette tip is positioned straight down the middle of the tube and is not touching the side.
Carbonation observed upon addition of Buffer Solution to fixed blood samples	Fixative is carbonated by CO ₂ vapor from dry ice	Do not store Fixative on dry ice during fixation unless following the Alternate Fixing Procedure supplied by Litron. Do not store Fixative in a freezer containing dry ice.
Nonparasitized reticulocytes are pulled out in red fluorescence (see Troubleshooting Figure 1)	Compensation is insufficient	Increase compensation until the nonparasitized reticulocyte population looks like that in Section 14.1, step 10.
	Incubation with Labeling Solution 1s too short or room is too cold	Follow the instructions in Labeling Washed Blood (Section 13.3); increase incubation time in Labeling Solution, or modify temperature.
Populations of malaria-infected cells form arches rather than vertical planes (see Troubleshooting Figure 2)	Compensation is too high	Decrease compensation until the populations of malaria-infected cells form vertical planes as in Section 14.1, step 10.
Wide FSC or cell aggregates (see Troubleshooting Figure 3)	Fixative is carbonated by CO ₂ vapor from dry ice	Do not store Fixative on dry ice during fixation unless following the Alternate Fixing Procedure supplied by Litron.
	Samples are not maintained at the appropriate temperatures	<p>Blood cells collected in Anticoagulant/Diluent can be maintained for up to 6 hours at room temperature or for up to 24 hours at 2 °C to 8 °C.</p> <p>Blood cells fixed in Fixative must be maintained at –75 °C to –85 °C at all times until the addition of Buffer Solution or being transferred into LTSS.</p> <p>Fixed cells placed in Buffer Solution must be maintained at 2 °C to 8 °C or on ice at all times (except during centrifugation if a refrigerated centrifuge is not available).</p> <p>Washed cells are stable at 2 °C to 8 °C for up to three days.</p>

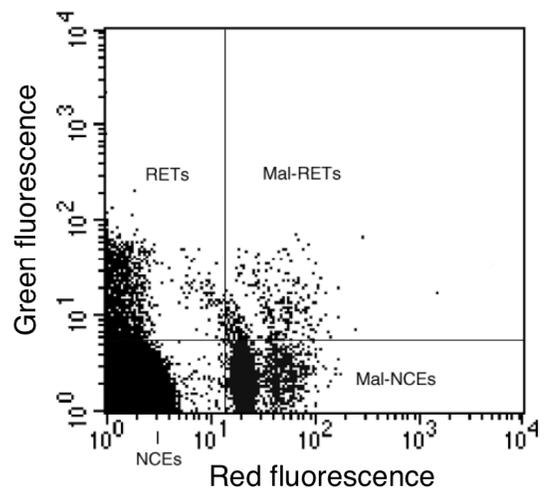
Troubleshooting Figure 1

If nonparasitized RETs are not well resolved from parasitized RETs, the FL3-%FL2 may not be set high enough. Another explanation for poor resolution between nonparasitized RETs and parasitized RETs is that cellular RNA may not be completely degraded. Incubate in the Labeling Solution for a longer period of time, or incubate for 30 minutes at 2 °C to 8 °C, 15 minutes at approximately 37 °C, and 15 minutes at room temperature.



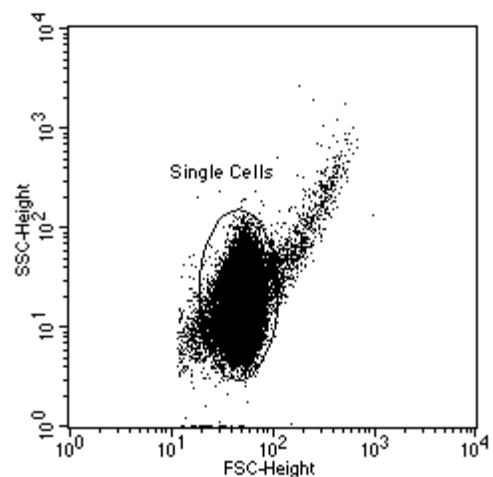
Troubleshooting Figure 2

Excessive FL3-%FL2 compensation can result in poor resolution as parasitized RETs arch back towards the RET population. It is important that the parasitized cells exhibit a vertical profile (i.e., consistent FL3 fluorescence as seen in Section 14.1, step 10).



Troubleshooting Figure 3

A large shoulder to the right of the main population of cells (SSC vs. FSC) is usually a sign of cellular aggregation. When cells are aggregated to a modest level (as shown), this problem can be addressed by restricting the “Single Cells” region to the dimensions close to those illustrated in Plot A, Appendix A. In the bivariate to the right, the region is set correctly, and excludes aggregated cells.



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Appendix A: Template Preparation

Template files can be found on Litron's website (www.LitronLabs.com), but are specific to CellQuest™ or FACSDiva™ software. The next pages show actual screen images of the CellQuest™ template's graphs and histogram, the gates used for each plot, and how a stop mode of 20,000 reticulocytes is set. Flow cytometry operators who are not using CellQuest™ software should find these pages valuable for constructing their own data acquisition and analysis template.

It may be helpful to run Single-Color Compensation controls and use auto-compensation if this is available with the software you are using.

We recommend that if you are using FACSDiva™ software, have parameters in "Height" rather than "Area". When analyzing the Single-Color control for the DNA stain, adjust PMT voltage so that nucleated cells fall in the last decade of red fluorescence.

Defining Gates:

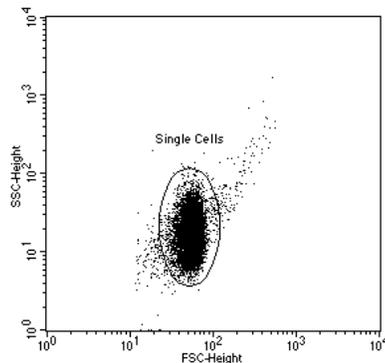
- G1 = R1 = "Single Cells"
- G2 = R2 = "Total RBCs"
- G3 = R1 and R2 = "Single Cells" AND "Total RBCs"
- G4 = R3 = "High CD71-Positive RETs"
- G5 = R1 and R2 and R3 = "Single Cells" AND "Total RBCs" AND "High CD71-Positive RETs"
- G6 = R1 and R2 and not R4 = "Single Cells" AND "Total RBCs" AND NOT "Platelets"
- G7 = R1 and R2 and R3 and not R4 = "Single Cells" AND "Total RBCs" AND "High CD71-Positive RETs" AND NOT "Platelets"

1. Gate for each Plot:	Plot A	No Gate
	Plot B	G1
	Plot C	G1
	Plot D1	G3
	Plot D2	G1
	Plot E	G3
	Plot F	G6

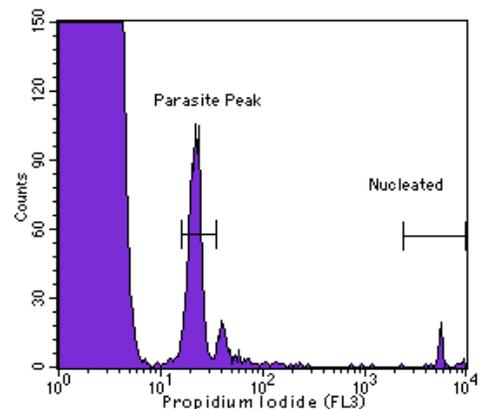
2. Use Gate G7 to set the stop mode. This will allow you to stop data acquisition at a specified number of RETs (typically 20,000). For this gate to work accurately, the lower green demarcation line for region "High CD71-Positive RETs" on Plot E and the horizontal line of the quadrant in Plot F need to be at the exact same Y-value.

3. Save this template file. This template file should be appropriate for all rat blood analyses. To ensure consistency of data, it is preferable that no changes be made to the location and size of the regions between samples. The exception is the location and size of the Single Cells region on Plot A which may require minor adjustments.

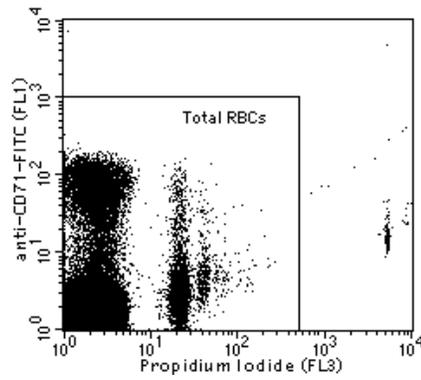
Plot A



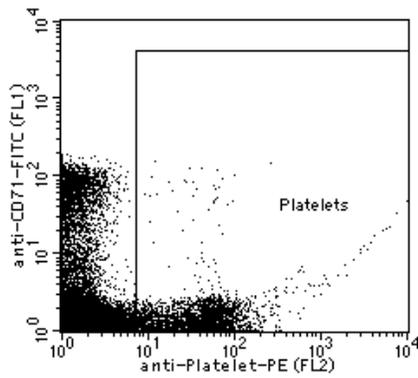
Histogram B



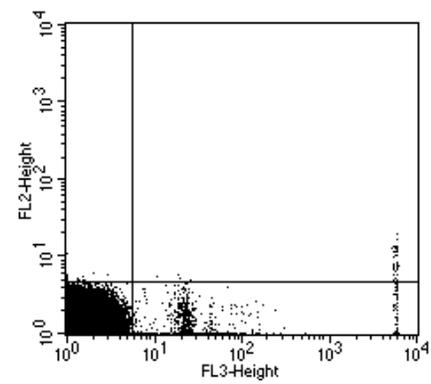
Plot C



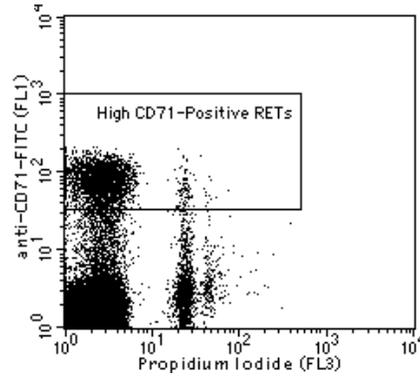
Plot D1



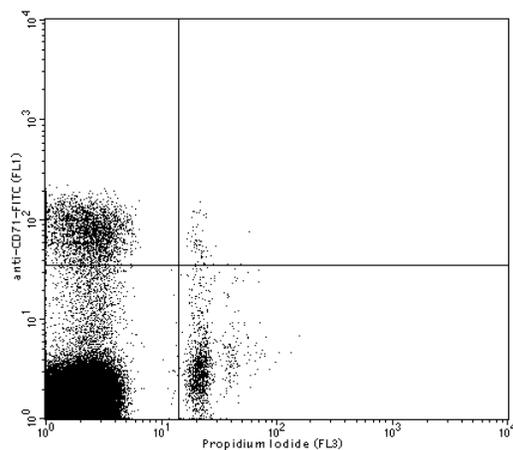
Plot D2



Plot E



Plot F



Quadrant Statistics

File: Sample ID:
 Patient ID: Tube:
 Acquisition Date: Gate: G6
 Gated Events: 199291 Total Events: 221400
 X Parameter: FL3-H Propidium Iodide (Log) Y Parameter: FL1-H anti-CD71-FITC (Log)
 Quad Location: 14, 5

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	7472	3.75	3.37	2.48	2.27	62.28	45.80
UR	599	0.30	0.27	26.23	24.52	25.24	16.39
LL	188946	94.81	85.34	2.11	1.99	1.59	1.49
LR	2274	1.14	1.03	23.45	22.62	2.83	2.62