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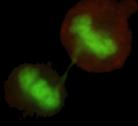
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*Quality Counts!*



**Micronucleus  
Analysis  
Kit**

**MicroFlow<sup>PLUS</sup> Archive Stage 2  
Mouse Blood**



**Instruction Manual**

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*For Research Use Only. Not for use in diagnostic procedures.*

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## 1. Materials Provided

Kit Component	Quantity <sup>a</sup>	Storage Conditions <sup>g</sup>
Buffer Solution	1 L	2 °C to 8 °C
RNase Solution	100 µL	-10 °C to -30 °C
Anti-Mouse CD71 Antibody <sup>b</sup>	100 µL	2 °C to 8 °C
DNA Stain	4 mL	2 °C to 8 °C
Anti-Rodent CD61 Antibody <sup>b</sup>	50 µL	2 °C to 8 °C
Calibration Standards in Long Term Storage Solution (LTSS)		
• Positive Control samples (fixed) <sup>c</sup>	Three 1 mL aliquots	-75 °C to -85 °C
• Negative Control samples (fixed) <sup>d</sup>	Three 1 mL aliquots	-75 °C to -85 °C
• CD71-Negative samples (fixed) <sup>e</sup>	Three 1 mL aliquots	-75 °C to -85 °C
• Malaria Biostandard samples (fixed) <sup>f</sup>	Three 1 mL aliquots	-75 °C to -85 °C

- a. Sufficient materials are provided to collect and analyze 60 blood samples.  
 b. Warning! Contains sodium azide. Irritant. See MSDS (available on website).  
 c. Positive Control sample: Noninfectious, nonhazardous denatured mouse blood sample with an elevated level of micronuclei.  
 d. Negative Control sample: Noninfectious, nonhazardous denatured mouse blood sample with a background level of micronuclei.  
 e. CD71-Negative sample: Noninfectious, nonhazardous denatured rodent blood sample used for calibration of flow cytometer.  
 f. Malaria Biostandard sample: Noninfectious, nonhazardous denatured mouse blood sample used for calibration of flow cytometer.  
 g. 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

- Fixed experimental blood samples processed using Litron's Archive Stage 1 MicroFlow kit
- -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
- 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<sup>PLUS</sup> 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 Standard Samples

Please note that all kit-supplied samples are in Long Term Storage Solution (LTSS) and must be washed out of LTSS (see Section 8.1) and labeled (see Section 9) prior to flow cytometric analysis (see Section 10.1).

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 CD71-Negative and 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. 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.

### 5.4. Daily Calibration with Calibration Standards

A CD71-Negative 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 LTSS (see Section 8.1) and labeled (see Section 9) prior to flow cytometric analysis (see Section 10.1).

## 6. Introduction

This kit is used for preparing mouse 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<sup>®</sup> 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.

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:

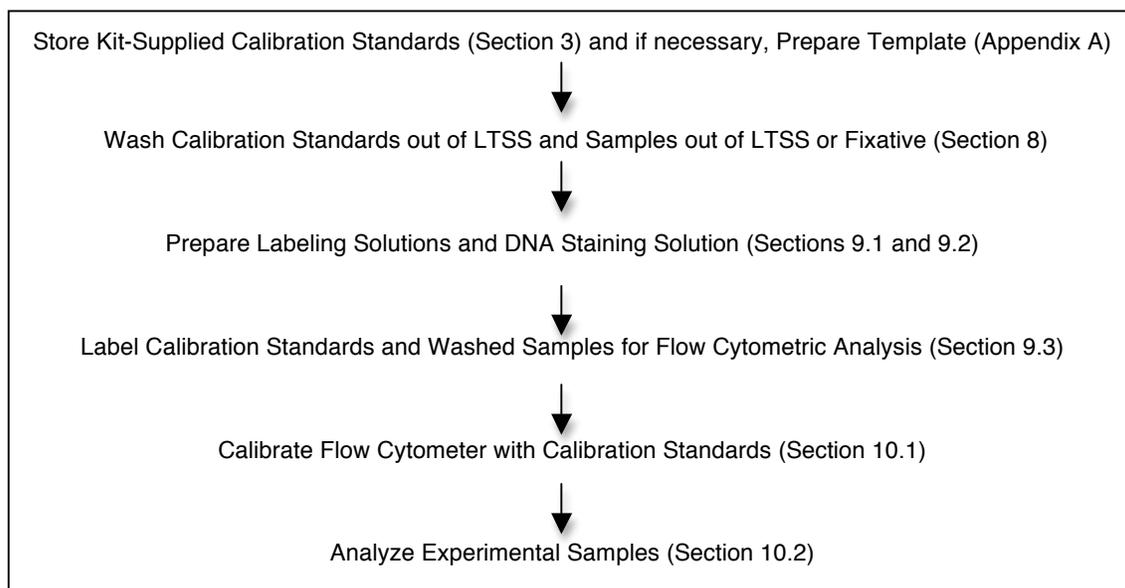
- Availability for many species of toxicological interest
- Anti-platelet antibody to ensure reliable data
- Calibration standards to ensure intra- and inter-laboratory reproducibility of data
- Ability to store samples for extended periods of time before analysis

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. 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  $\mu$ 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  $\mu$ 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.

## 9. Labeling Procedure

### 9.1. 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 CD71-Negative 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-Mouse 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-Negative, and Negative Control and Positive Control samples). 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-Mouse CD71 Antibody
1	100 $\mu$ L	1 $\mu$ L	1 $\mu$ L

\* with or without 1 % heat-inactivated FBS

- Thaw RNase Solution. Quick spin RNase Solution and Anti-Mouse 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-Mouse CD71 Antibody to the tube containing Buffer Solution. Tap gently to mix. This is Labeling Solution 1.
- Remove 80  $\mu$ L of Labeling Solution 1 and place into a flow cytometry tube. This will receive 20  $\mu$ 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 $\mu$ L	0.5 $\mu$ L

- Aliquot 80  $\mu$ 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-Negative sample. Cover all tubes containing Labeling Solutions with foil, and store at 2 °C to 8 °C until needed.

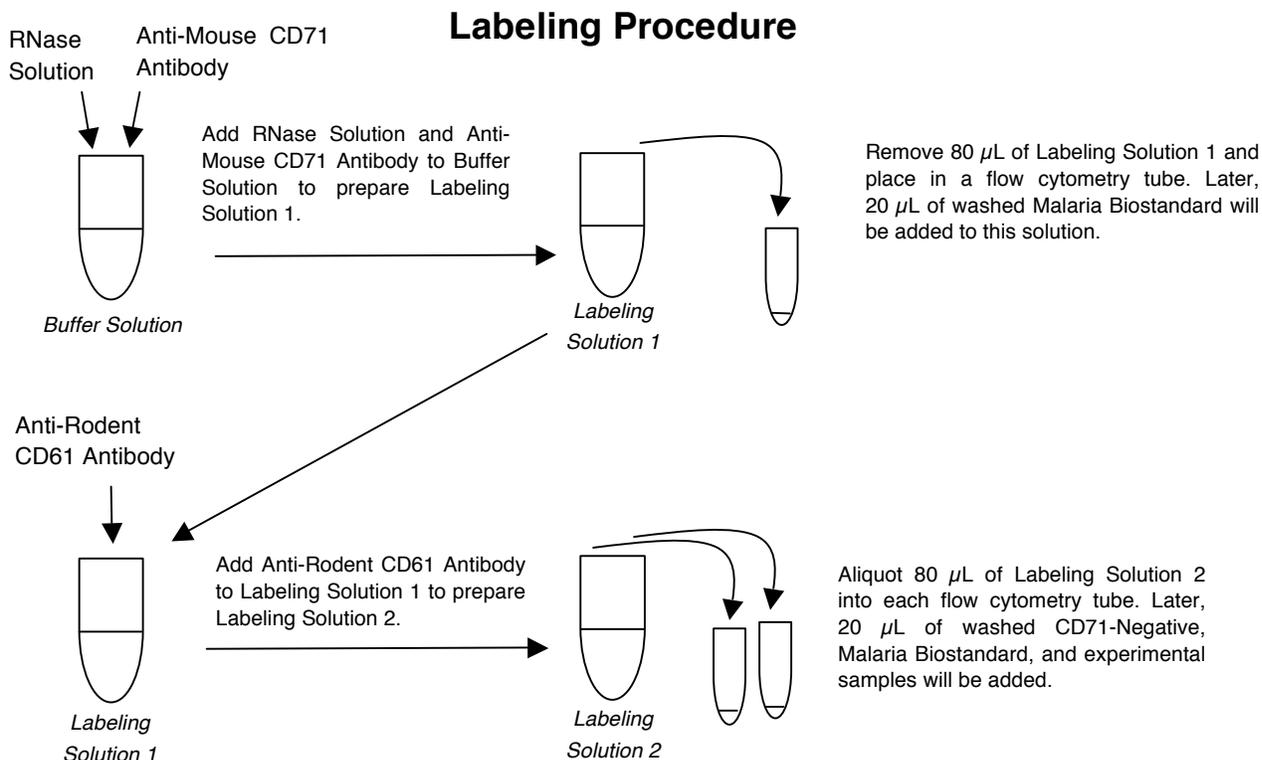
### 9.2. Prepare DNA Staining Solution

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-Negative 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.



### 9.3. 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  $\mu\text{L}$  of Malaria Biostandard to the FCM tube containing Labeling Solution 1. Gently tap to mix.
3. Add 20  $\mu\text{L}$  of Malaria Biostandard to an FCM tube containing Labeling Solution 2. Add 20  $\mu\text{L}$  of CD71-Negative sample to an FCM tube containing Labeling Solution 2. Gently tap to mix.
4. Add 20  $\mu\text{L}$  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.

## 10. Flow Cytometric Analysis

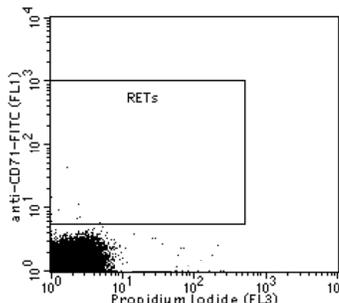
### 10.1. Flow Cytometer Calibration with Calibration Standards

Please note that the Calibration Standards must first be washed out of LTSS (Section 8.1) and labeled (Section 9.3) 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:

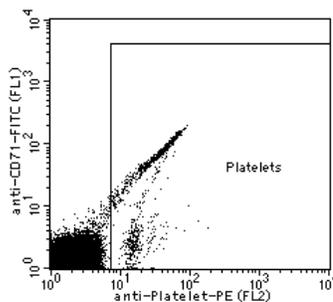
- Open the FASCDiva™ template (.xml file) for Mouse PLUS Blood (available for download from the website).
  - Open the following folders on your computer: My computer > New Volume(D) > BDExport > Templates > Experiment > General.
  - Drag the template into the General folder.
  - Close this window and 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-Negative 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 cells are located below the lower green demarcation on the Y-axis of Plot E. It is best to have some space between the cells and lower green line. Adjust the FL3 PMT voltage so the cells are within the first decade of red fluorescence. The resulting plot should look similar to the plot below.

**Plot E**

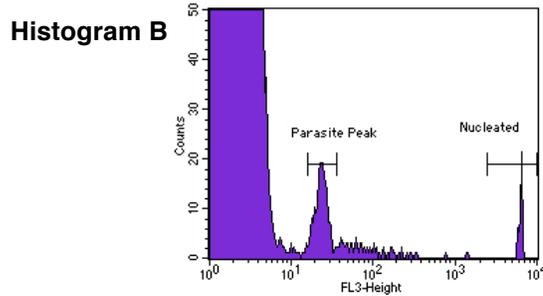


4. Place the Malaria Biostandard sample in Labeling Solution 1 on the flow cytometer. Refrigerate the CD71-Negative sample.
5. Viewing Plot D1, adjust the FL2 PMT voltage until the majority of cells in the lower left area are just to the left of the “Platelets” region. The resulting plot should look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

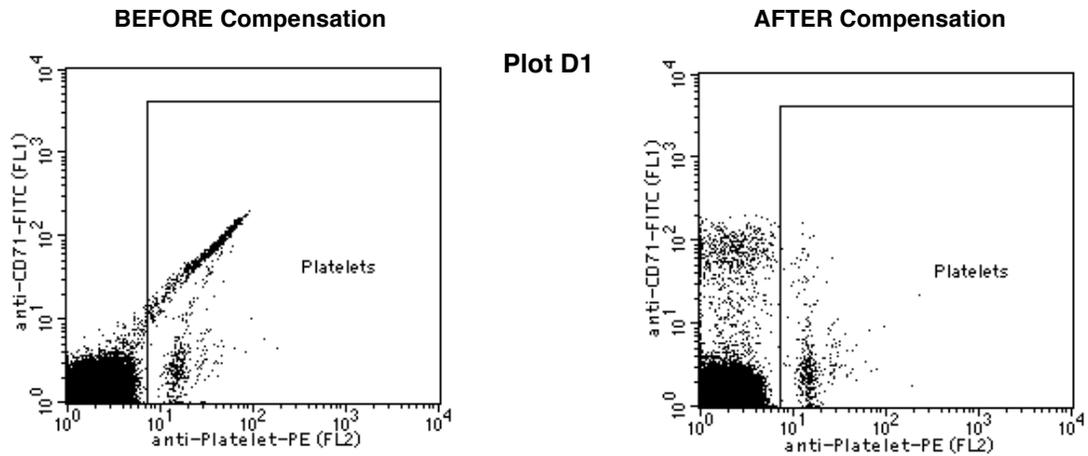
**Plot D1**



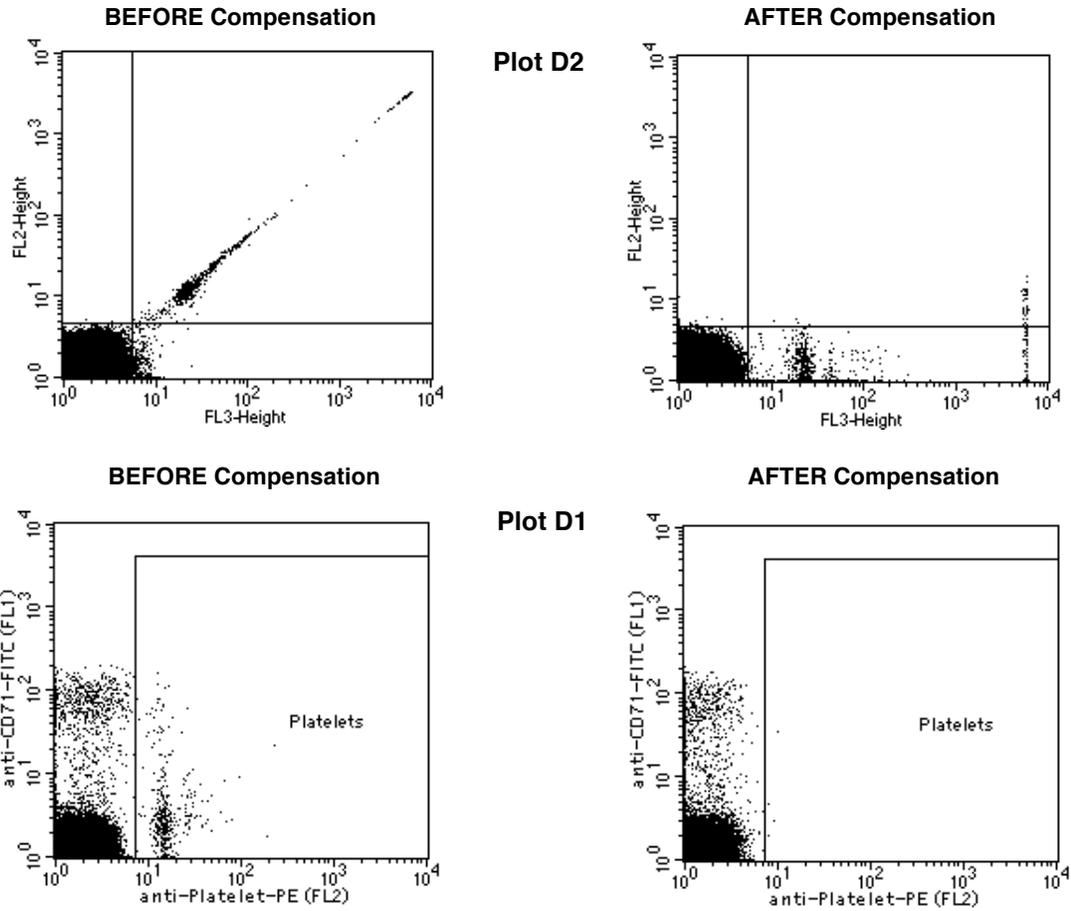
- Viewing Histogram B, adjust FL3 PMT voltage until the non-parasitized cells approach the first decade of FL3 fluorescence and the first parasite peak is well resolved. (BD CellQuest™ users are advised to place the first parasite peak at channel  $22 \pm 2$  on Histogram B.) The location of the nucleated 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.

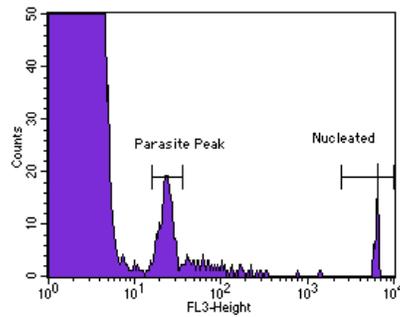


- Adjust FL2-%FL3 compensation to eliminate the yellow component of propidium iodide. Viewing Plot D2, increase compensation until the parasitized cells show low FL2-associated fluorescence which is on par with that observed for the NCEs at the origin. The nucleated cells' fluorescence should be low, 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.

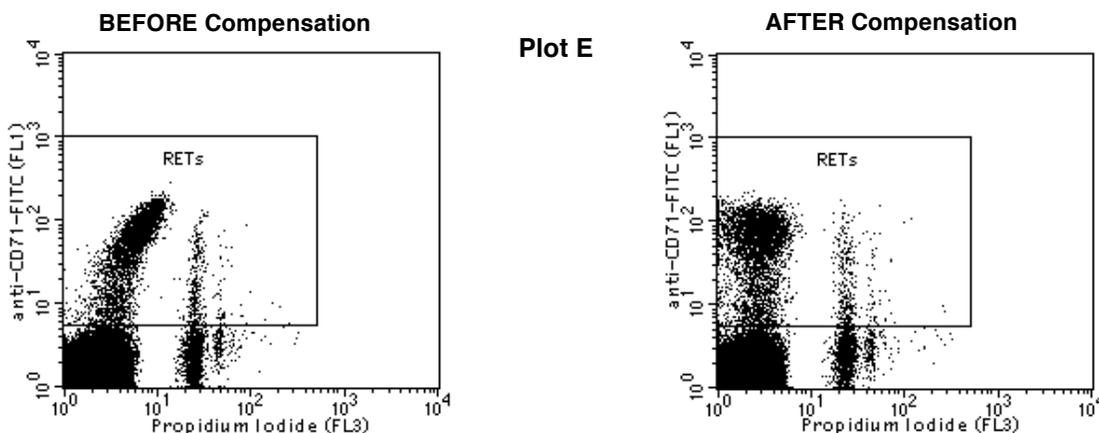


- Readjust the FL3 PMT voltage, if necessary. (BD CellQuest™ users are advised to place the first parasite peak at channel 22 ± 2 on Histogram B.) If this is performed, compensation may need slight adjustments.

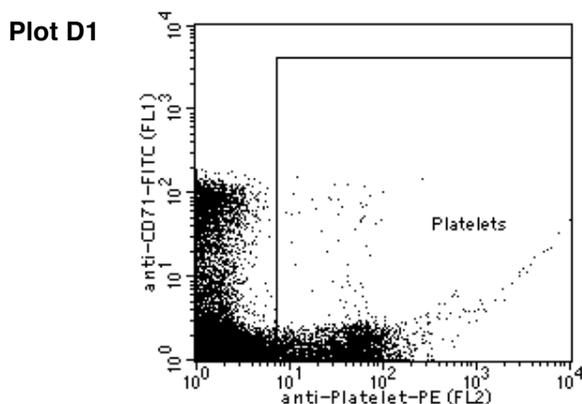
**Histogram B**



- 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-Negative 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-Negative 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-Negative and Malaria Biostandard samples each day, analyze experimental samples.

## 10.2. 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:

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

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

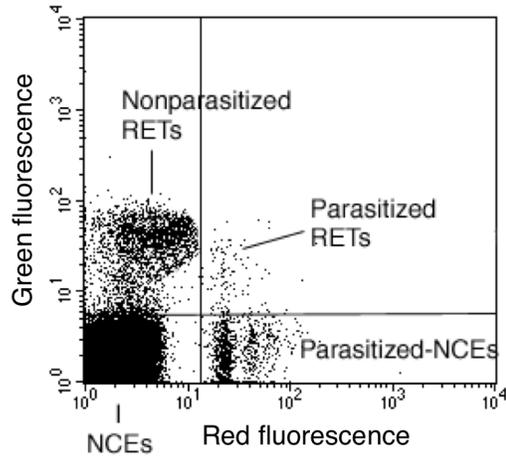
$$\% \text{ MN-RET} = (\text{UR}) / (\text{UR} + \text{UL}) * 100$$

## 11. Troubleshooting

Observation	Possible cause	Suggestion
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 10.1, step 10.
	Incubation with Labeling Solution 1s too short or room is too cold	Follow the instructions in Label Washed Blood (Section 9.3); 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 10.1, step 10.
Wide FSC or cell aggregates (see Troubleshooting Figure 3)	Fixative is carbonated by CO <sub>2</sub> 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	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.  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.  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).
		Washed cells are stable at 2 °C to 8 °C for up to three days.
NCEs too high in green fluorescence (see Troubleshooting Figure 4)	Green PMT voltage too high when setting up with CD71-Negative sample	Lower voltage so that NCEs are below the green fluorescence boundary.
	Cell density may be too high, resulting in an acquisition rate > 6,000 cells per second	Decrease cell density to < 6,000 cells per second by adding an additional 0.5 to 1.0 mL of DNA Staining Solution to the sample.

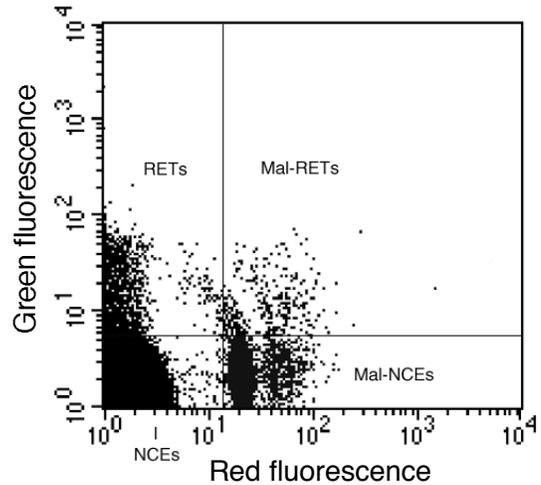
### Troubleshooting Figure 1

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.



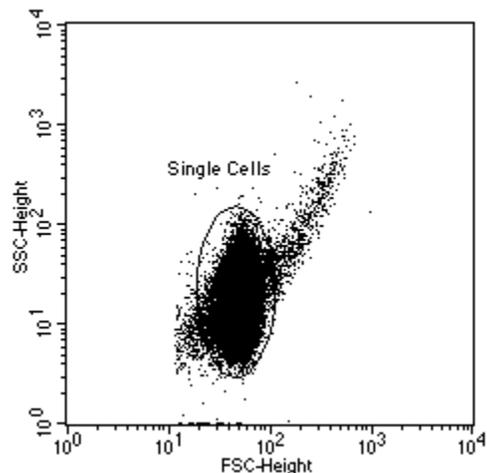
### Troubleshooting Figure 2

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 10.1, step 10).



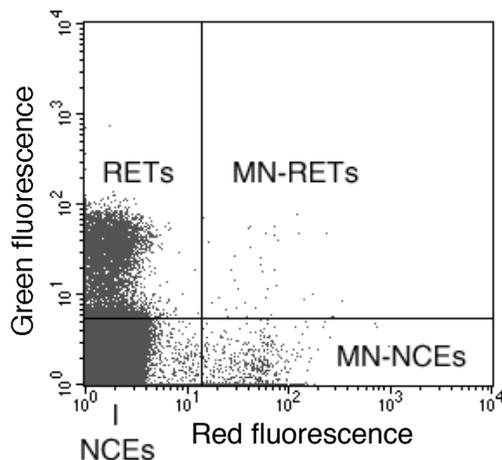
### Troubleshooting Figure 3

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.



### Troubleshooting Figure 4

If the major NCE population of a sample (i.e., population in lower left quadrant) is above the quadrant’s green fluorescent boundary, an artificially high number of RETs will be scored. This may be due to an inappropriate green PMT voltage setting (ensure that this setting is carefully determined with the CD71-Negative sample at the beginning of each analysis day). Alternately, NCEs may exhibit high green fluorescence when sample acquisition rates are too high (> 6,000 cells per second). If necessary, adjust sample density to < 6,000 cells per second by adding an additional 0.5 to 1 mL of cold DNA Staining Solution. Immediately place the sample back on the cytometer and re-analyze.



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## Appendix A: Template Preparation

Template files can be found on Litron's website ([www.LitronLabs.com](http://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.

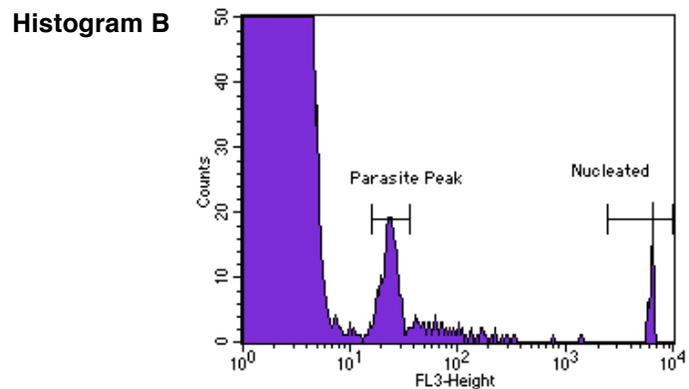
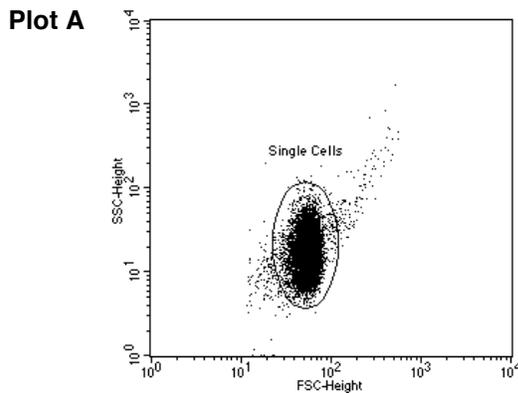
### Defining Gates:

- G1 = R1 = "Single Cells"
- G2 = R2 = "Total RBCs"
- G3 = R1 and R2 = "Single Cells" AND "Total RBCs"
- G4 = R3 = "RETs"
- G5 = R1 and R2 and R3 = "Single Cells" AND "Total RBCs" AND "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 "RETs" AND NOT "Platelets"

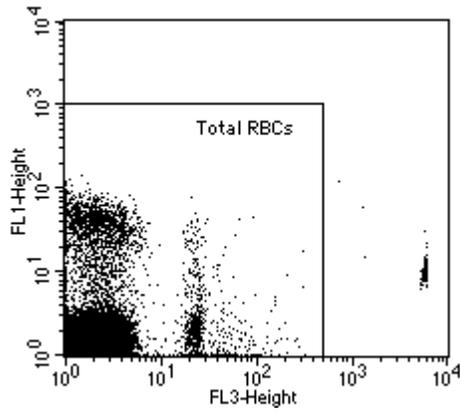
1. Gate for each Plot:	Plot A	No Gate
	Plot B	G1
	Plot C	G1
	Plot D1	G3
	Plot D2	G1
	Plot E	G3
	Plot F	G6

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 "RETs" on Plot E and the horizontal line of the quadrant in Plot F need to be at the exact same Y-value.

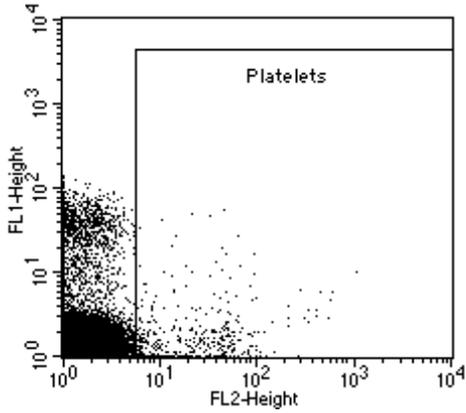
3. Save this template file. This template file should be appropriate for all mouse blood analyses. To ensure consistency of data, it is preferable that no changes be made to the location and size of the regions between samples. The exception is the location and size of the Single Cells region on Plot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



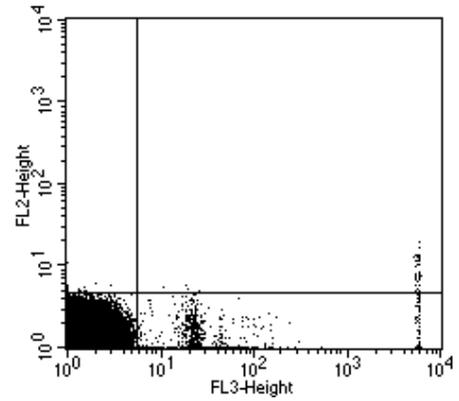
Plot C



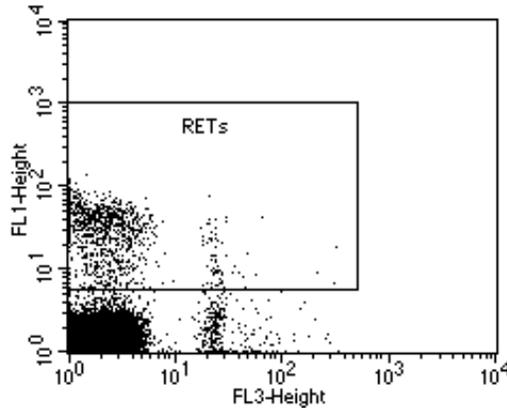
Plot D1



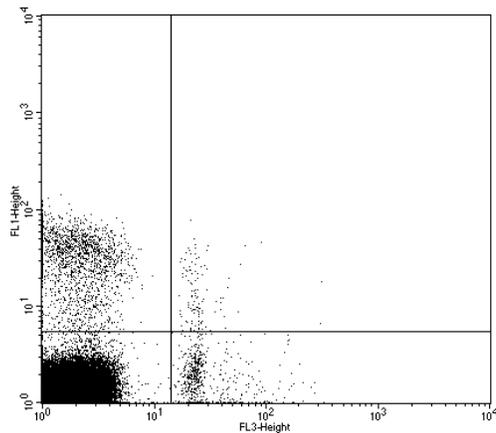
Plot D2



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