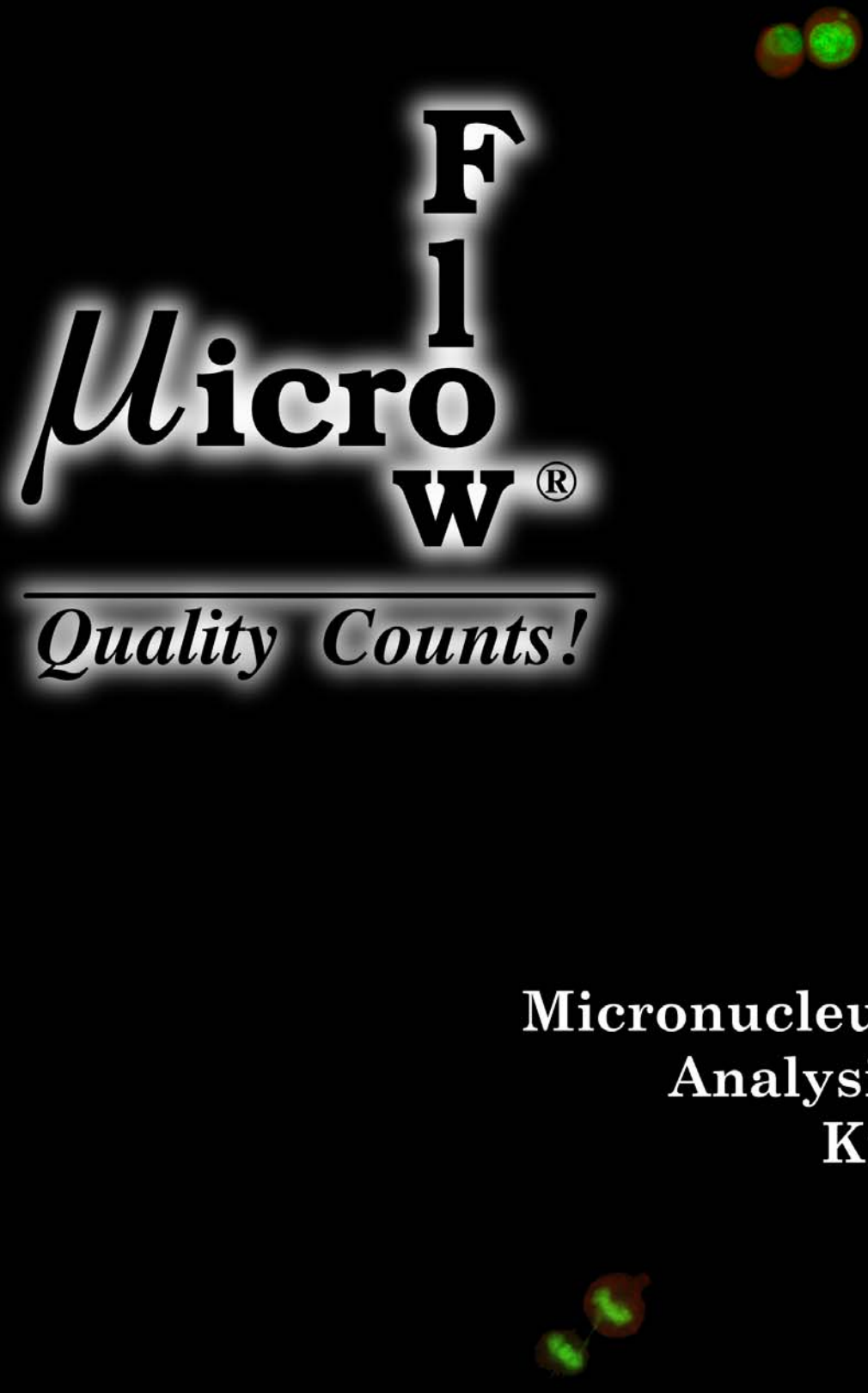

Micronucleus Analysis Kit

MicroFlow^{PLUS} (Mouse Bone Marrow)

Instruction Manual

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

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1. Materials Provided

Kit Component	Quantity[a]	Storage Conditions
Buffer Solution	3 liters	2 °C to 8 °C
Long-Term Storage Solution (LTSS)	65 ml	2 °C to 8 °C
RNase Solution	225 µl	2 °C to 8 °C
Mouse anti-CD71 Antibody[b]	100 µl	2 °C to 8 °C
DNA Stain	4 ml	2 °C to 8 °C
Platelet Antibody [b]	50 µl	2 °C to 8 °C
6 ml 12 x 75 mm tubes with 35 micron strainer cap	75	Ambient
Biological Standards – Malaria Biostandard blood samples (fixed)[c]	Two 1 ml aliquots	–75 °C to –85 °C

a. Sufficient materials are provided to collect and analyze 60 bone marrow samples.
b. Warning! Contains sodium azide. Irritant. See MSDS (available on website).
c. Malaria Biostandard blood sample: Noninfectious, nonhazardous denatured mouse blood sample used for calibration of flow cytometer.

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)
- 2 °C to 8 °C refrigerator
- 1 ml syringes and appropriately sized needles (for flushing bone marrow)
- Heat-inactivated, filter-sterilized fetal bovine serum
- 20 ml syringes (used to prepare columns)
- α-cellulose (Sigma Catalog Number C8002)
- Sigmacell® cellulose, type 50 (Sigma Catalog Number S5504)
- Fisher lens paper
- Microcentrifuge tubes
- Micropipette and appropriate tips
- 25 ml pipettes
- 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)
- Ice

3. Storing Kit-Supplied Biological Standards

Upon receipt of the MicroFlow^{PLUS} Kit, the container holding the Biological Standards must be placed upright in a –75 °C to –85 °C freezer.

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.

In this manual, polychromatic erythrocytes (PCEs) and reticulocytes (RETs) are used interchangeably.

5.1. Template Preparation

Template files are available on Litron's website (www.litronlabs.com/support.html), 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 a CD71 Setup Sample

For flow cytometer setup, a CD71 Setup sample is required. This is typically a bone marrow sample from an age-matched negative control or vehicle control mouse.

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) fluorescence should be collected in the FL3 channel.

5.4. Daily Calibration with Biological 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.

5.5. Preliminary Analyses

First-time users of this kit are encouraged to analyze a CD71 Setup sample and Malaria Biostandard samples after initial flow cytometer calibration. If after performing the procedures as described, your results are not consistent with the plots in this manual, fax (585-442-0934) or email representative plots 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.

6. Introduction

This kit is used when preparing mouse bone marrow 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 micronuclei 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 hematopoetic stem cell differentiates into an erythroblast and eventually expels its nucleus to become a polychromatic erythrocyte (PCE). The newly formed PCE 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 PCE formation, MN may be retained in the PCE cytoplasm.

6.2. The MicroFlow® Method

Litron Laboratories has developed and patented a flow cytometric method to measure micronuclei in both the PCE and NCE populations. Unlike mature NCEs, immature PCEs 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 PCEs (MN-PCEs) can indicate acute genotoxicity associated with a recent cell division.

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:

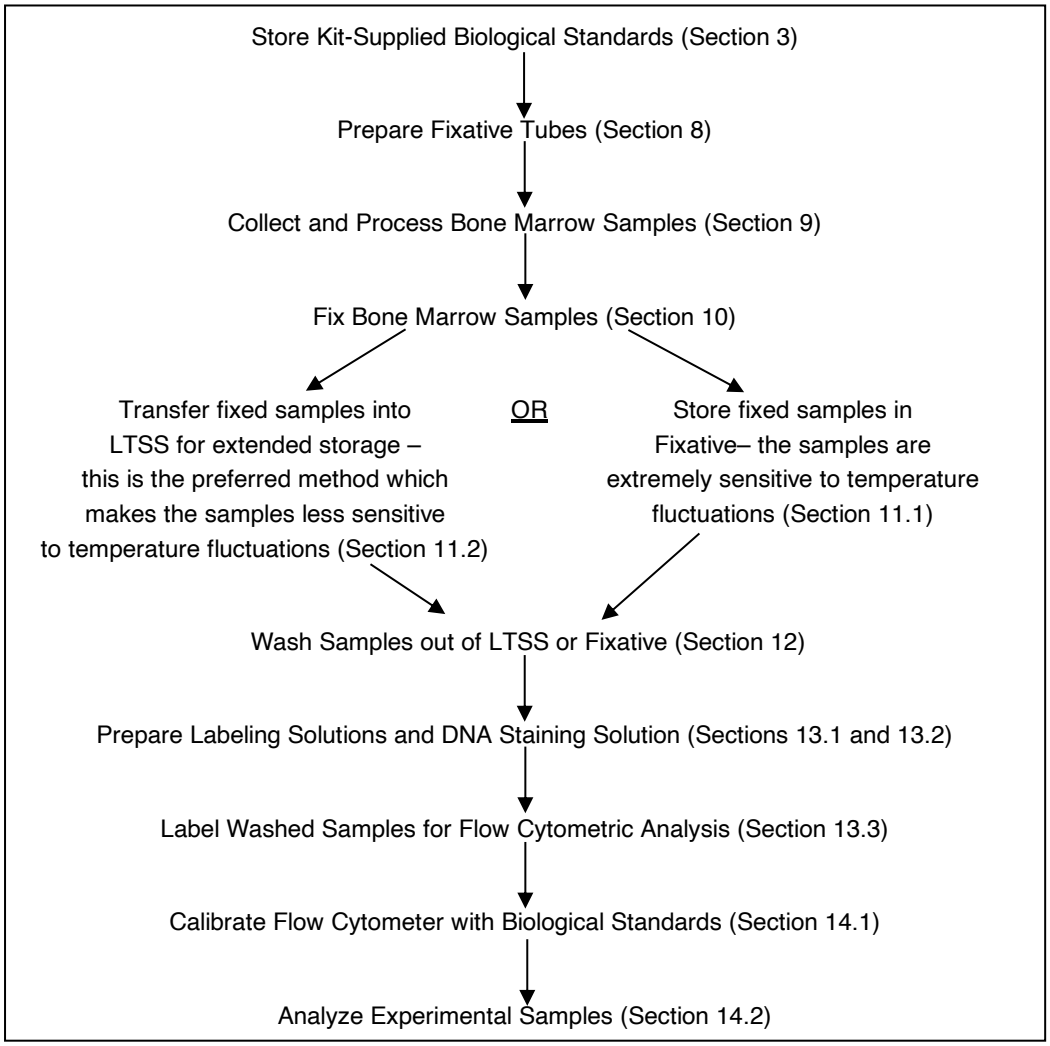
- Availability for many species of toxicological interest
- Anti-platelet antibody to ensure reliable data
- Biological standards to ensure intra- and inter-laboratory reproducibility of data
- Ability to store samples for extended periods of time before analysis
-

Crucial components of this method are the biological 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 biological standards, micronucleus analyses can be performed reliably and with minimal intra- and inter-experimental variation.

6.3. Regulatory Acceptance

The US FDA accepts 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 is an acceptable alternative to manual evaluation. Please note that the mouse bone marrow procedures described in this manual have not undergone the same rigorous validation as the mouse peripheral blood flow cytometric method.

7. Overview of Method



8. Prepare Columns and Fixative Tubes

8.1. Prepare Columns

Fractionation of mouse bone marrow through microcrystalline cellulose columns is helpful for reliable flow cytometric micronucleus analysis. Procedures are similar to those described by Romagna and Staniforth.

1. Prepare approximately 1.0 gram of cellulose mix per femur by mixing equal portions (by weight) of α-cellulose and Sigmacell® cellulose, type 50 into a screw cap bottle. Seal and mix vigorously by inverting and swirling for approximately five minutes.

2. Cut Fisher lens paper into circles, to use as filter discs, large enough to cover the bottom of a 20 ml syringe barrel, without gaps, but small enough to easily fit into the syringe.

3. Remove plungers from the 20 ml syringes and insert the circular filter discs into the bottom. Tare the syringe on a balance and add about 1.0 gram of matrix mix if one femur is to be fractionated and 1.2 grams of matrix mix if two femurs are to be fractionated. Tap the syringe upright to lightly pack the matrix material. Insert a modified syringe plunger (rubber tip removed and plastic shaved for easy fit) to very lightly pat down any remaining loose matrix material. Keep upright and cover with parafilm or foil until use.

4. When ready to use, mount columns over 15 ml centrifuge tubes.

8.2. Prepare Fixative Tubes

1. One 15 ml polypropylene centrifuge tube is required per sample (two if fixing in duplicate). Add 2 ml of Fixative to each tube, and replace caps.

> Duplicate fixed samples can be prepared, if necessary.

2. 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.

3. 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.

4. Place the rack of tubes overnight (or longer) at –75 °C to –85 °C to allow for sufficient chilling of the Fixative.

9. Collect and Fractionate Bone Marrow Samples

9.1. Collect Bone Marrow Samples

1. Aliquot 2 ml to 3 ml heat-inactivated FBS into labeled centrifuge tubes (one for each femur).

2. Remove femur(s). Draw up FBS into a 3 ml syringe, insert needle and flush bone marrow from one femur into the corresponding centrifuge tube.

> If using two femurs for one column, combine the aspirated bone marrow before adding to the column.

3. Finely disperse the bone marrow into the FBS by repeatedly aspirating and discharging gently with the syringe into the centrifuge tube. Cap tube and process other femurs.

4. Centrifuge at approximately 100 *g* to 150 *g* for 5 minutes. Aspirate supernatant, leaving approximately 0.5 ml of serum. Resuspend pellet into residual serum. At this point, prepare slides, if necessary.

9.2. Fractionate Bone Marrow

1. Dilute the concentrated bone marrow with approximately 2 ml to 2.5 ml of Buffer Solution.

2. Holding the pipette tip just above the center of the column matrix, add the diluted bone marrow dropwise to pre-mounted columns.

3. Add 13 ml to 14 ml of Buffer Solution (dropwise at first so the column matrix is not disturbed) to elute the erythrocytes from the column and collect into 15 ml polypropylene centrifuge tubes. Note that the addition rate can gradually increase after a meniscus forms above the matrix.

4. Centrifuge the eluted erythrocyte fraction at approximately 200 *g* to 250 *g* for 10 minutes. Aspirate the supernatant, leaving approximately 300 µl.

5. Resuspend the cell pellet and fix within 4 hours.

10. Fix Bone Marrow Samples

It is extremely important that the tubes containing Fixative and fixed bone marrow remain ultracold (–75 °C to –85 °C) and do not come in contact with vapors from dry ice. CO_2 vapor causes carbonation and cellular aggregation. For this same reason, fixative should not be stored in a freezer containing dry ice, and fixation should not occur on dry ice. To avoid this problem, tubes containing Fixative should be taken directly from the freezer.

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

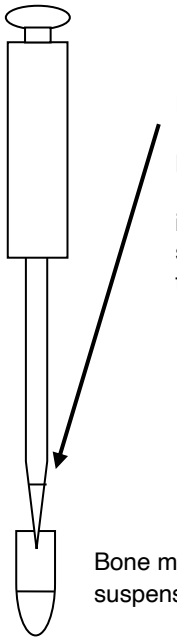
A video of the fixing procedure is available on our website (www.litronlabs.com).

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 bone marrow samples and the other removing the Fixative tubes from the freezer.

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

1. Immediately prior to fixing, invert the vial containing the bone marrow sample to ensure a homogeneous suspension.
2. Using a micropipettor, retrieve 150 µl of the bone marrow 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. Making sure that the pipette tip does not touch the side of the tube or the surface of Fixative, forcefully dispense directly into Fixative. See the diagram on the next page.
5. Cap the tube of fixed bone marrow 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).
6. Change the pipette tip and repeat steps 1 through 5 for the remaining 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 temperature returns to the required range before completing sample fixation. Fractionated bone marrow should be fixed within 4 hours of harvest. Again, 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.

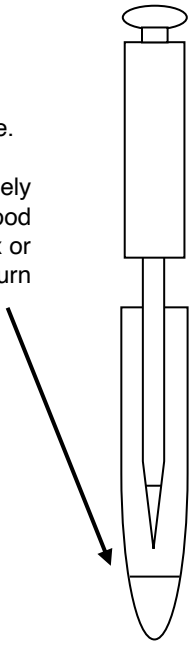


Bone marrow suspension

CORRECT FIXING TECHNIQUE

Remove 150 µl of bone marrow from the tube.

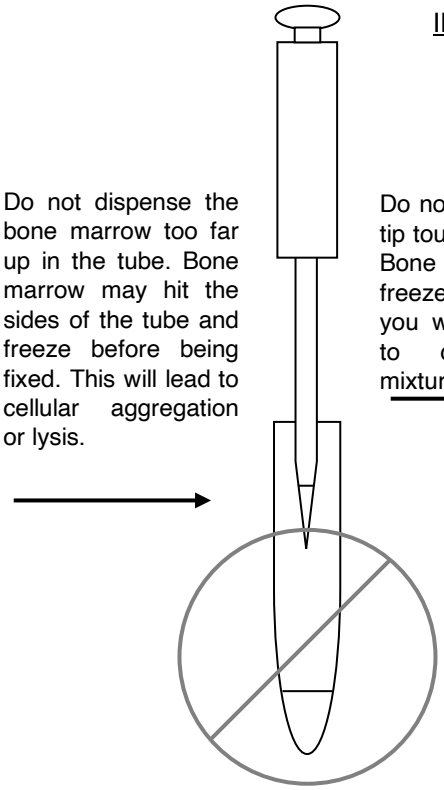
Hold the tip of the micropipettor approximately 1 cm above the Fixative. Forcefully inject blood into the ultracold Fixative. Secure cap, vortex or sharply strike the tube several times, and return to the ultracold freezer.



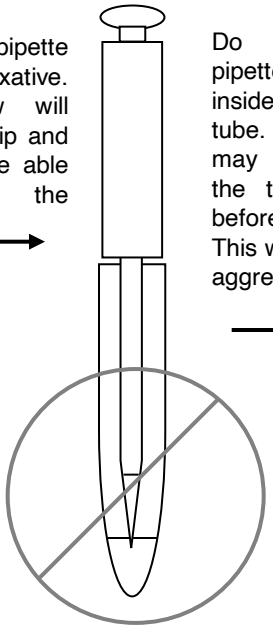
2 ml Ultracold Fixative

INCORRECT FIXING TECHNIQUES

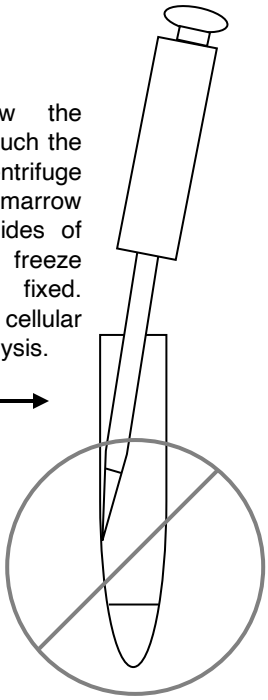
Do not dispense the bone marrow too far up in the tube. Bone marrow may hit the sides of the tube and freeze before being fixed. This will lead to cellular aggregation or lysis.



Do not let the pipette tip touch the Fixative. Bone marrow will freeze in the tip and you will not be able to dispense the mixture.



Do not allow the pipette tip to touch the inside of the centrifuge tube. Bone marrow may hit the sides of the tube and freeze before being fixed. This will lead to cellular aggregation or lysis.



11. Store Fixed Samples until Analysis

11.1. Maintain Fixed Samples in Ultracold Fixative

If you have access to a chest-style ultracold freezer that will maintain the fixed samples at the required temperature range consistently, samples can be maintained in Fixative until washing samples for subsequent analysis. If the freezer is not a chest freezer, is opened routinely, or the samples cannot be stored away from the freezer door, we recommend that the samples be transferred into LTSS, as described below.

11.2. Transfer into LTSS

Ideally samples should be transferred into LTSS at least 72 hours after fixing. 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.

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, a centrifuge tube rack and a 25 ml pipette ready for aliquoting Buffer Solution in step 5. 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 in the centrifuge tube rack 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.

   If a tube cracks, quickly transfer the fixed cells to another centrifuge 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 place 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 10 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. 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.

12. Wash Samples

12.1. Wash Samples out of LTSS

Ideally, samples 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).

It may be helpful for two individuals to perform this procedure, one tapping and opening tubes and the other adding Buffer Solution with 1 % FBS. 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. Until you are familiar and comfortable with the procedures described in this manual, it is recommended that no more than 20 samples are prepared for analysis on any particular day.

1. Prepare at least 12 ml of Buffer Solution with 1 % FBS for each sample to be washed. Filter through a 0.2 μm filter and place on ice.
   a. If samples are in cryovials, aliquot 12 ml of this Buffer Solution + 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 12 ml of this Buffer + FBS to each tube.

2. Remove the appropriate cells from the ultracold freezer and thaw.

3. Immediately upon thawing, either aliquot contents into tubes containing 12 ml Buffer + FBS or, add 12 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 μl 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.

12.2. Wash Samples out of Fixative

Follow the procedure described above for Washing Samples out of LTSS, but DO NOT add 1 % FBS to the Buffer Solution.

13. Labeling Procedure

13.1. Prepare Labeling Solutions I and II

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

Labeling solutions should be made fresh each analysis day and are prepared from Buffer Solution, RNase Solution, Mouse anti-CD71 Antibody, and Platelet 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, and use this in place of the “Volume of Buffer Solution” found in the table below.]*

1. Determine the total number of samples that will be prepared that day (including Malaria Biostandard and CD71 Setup 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 Mouse anti-CD71 Antibody
1	100 µl	2 µl	1 µl

* with or without 1 % heat-inactivated FBS

2. Quick spin RNase Solution and Mouse anti-CD71 Antibody for best recovery of contents.

3. Place the required volume of cold Buffer Solution into a sterile tube.

4. Aseptically add the required volumes of RNase Solution and Mouse anti-CD71 Antibody to the tube containing Buffer Solution. Tap gently to mix. This is Labeling Solution I.

5. Remove 80 µl of Labeling Solution I and place into a flow cytometry tube. This will receive 20 µl of Malaria Biostandard and will be used for calibrating the flow cytometer.

6. Quick spin Platelet Antibody for best recovery of contents.

7. Aseptically add the required volume of Platelet Antibody to the tube containing Labeling Solution I, using the chart below as a guide. Tap gently to mix. This is Labeling Solution II.

Number of samples	Approximate Volume of Labeling Solution I	Volume of Platelet Antibody
1	100 µl	0.5 µl

8. Aliquot 80 µl of Labeling Solution II 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 <u>not</u> be supplemented with FBS.

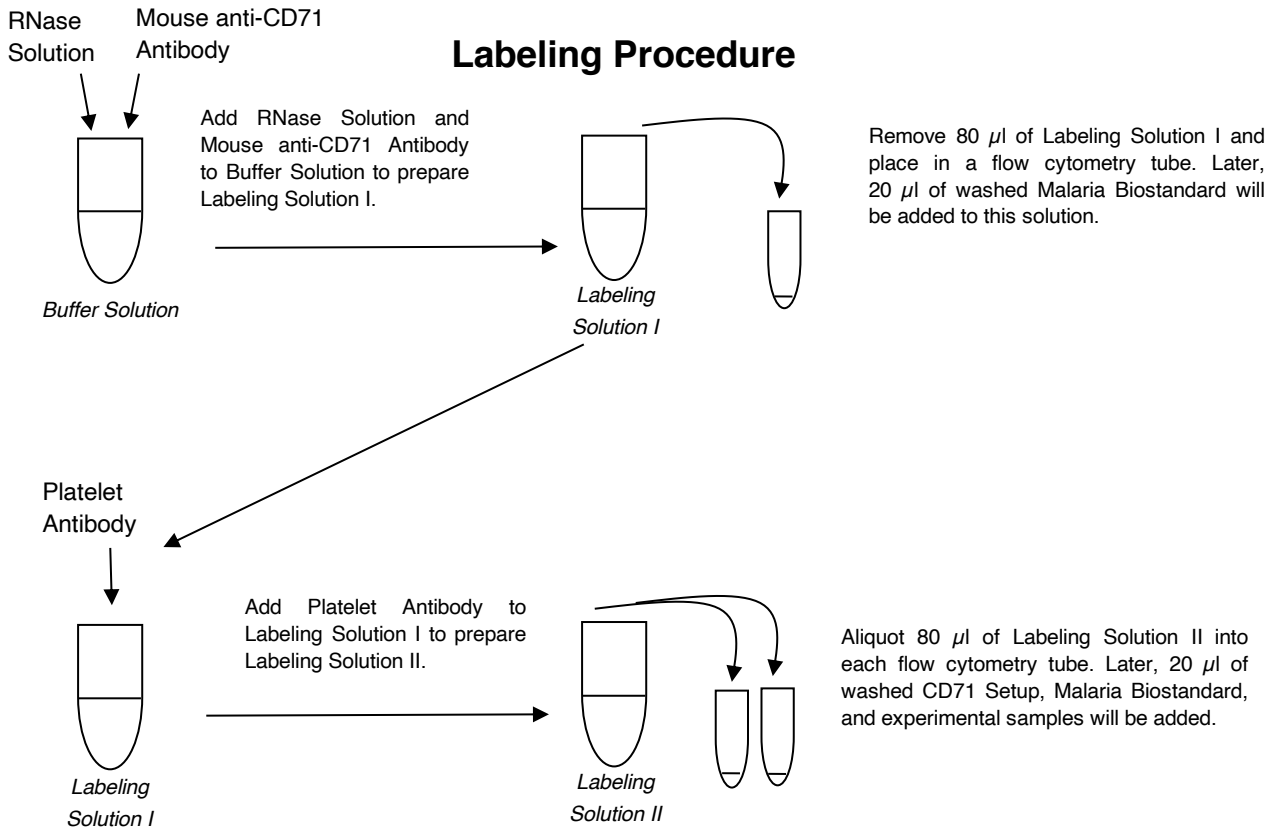
1. Determine the number of samples that will be analyzed that day (including Malaria Biostandard and CD71 Setup samples), and use the chart below as a guide to preparing DNA Staining Solution. You'll need approximately 2 ml DNA Staining Solution per sample.

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

2. Place the required volume of Buffer Solution into a sterile flask, and add the necessary volume of DNA Stain.

3. Cap the flask and shake gently to mix.

4. Store DNA Staining Solution in the dark at 2 °C to 8 °C (not on ice) until needed.



13.3. Label Washed 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 cells to the Labeling Solution, all of the sample comes into contact with the Labeling Solution. If a drop of cells is on the side of the tube, wash it down with the solution already in that tube.

1. With washed 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 I. Gently tap to mix.
3. Add 20 µl of Malaria Biostandard to an FCM tube containing Labeling Solution II. Add 20 µl of CD71 Setup sample to an FCM tube containing Labeling Solution II. Gently tap to mix.
4. Add 20 µl of each experimental bone marrow sample to the appropriately labeled FCM tubes containing Labeling Solution II. Tap the tubes gently to mix.
5. Return all washed bone marrow samples to 2 °C to 8 °C for storage.
6. Cover FCM tubes with foil to protect from light, and incubate in a 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. Protect from light and incubate the tubes containing the experimental samples at approximately 37 °C for 30 minutes, **and the Malaria Biostandard samples at room temperature for 30 minutes**, to ensure complete degradation of cellular RNA.
8. After the incubation, return all tubes to 2 °C to 8 °C until analysis.

14. Flow Cytometric Analysis

14.1. Flow Cytometer Calibration with Biological Standards

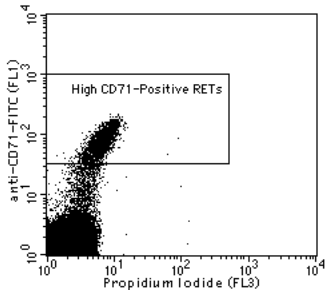
Please note that the following setup and compensation instructions are specific for CellQuest software, but should be useful with other software packages.

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 add to the top of a cell strainer. It might be necessary to gently tap the tube on a flat surface to start the flow through the filter cap. Remove the cap and place the sample on the flow cytometer. 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.

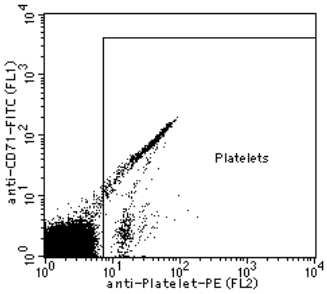
Plot E



4. Add 1.5 ml to 2.0 ml of DNA Staining Solution to the Malaria Biostandard sample that had been incubated in Labeling Solution I. Place on the flow cytometer. Refrigerate the CD71 Setup sample.

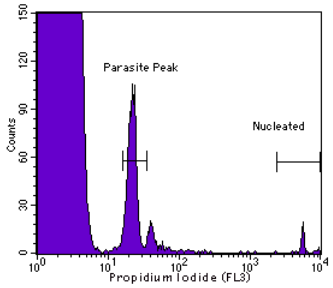
5. Viewing Plot D1, adjust the FL2 PMT voltage until the majority of cells in the lower left area are just to the left of the “Platelets” region. The resulting plot should look similar to the one below.

Plot D1

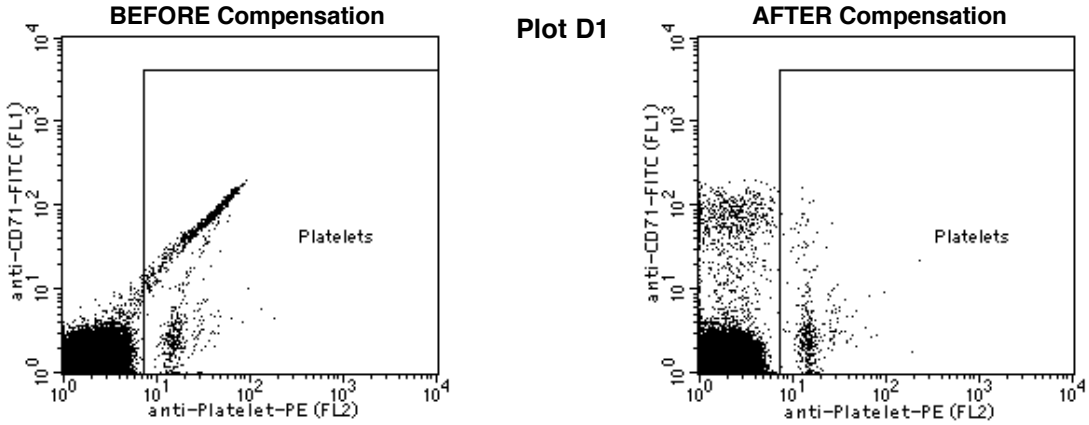


6. Viewing Histogram B, adjust FL3 PMT voltage until the non-parasitized cells approach the first decade of FL3 fluorescence and the first parasite peak is well resolved. (BD CellQuest™ users are advised to place the first parasite peak at channel 22 ± 2 on Histogram B.) The location of the nucleated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

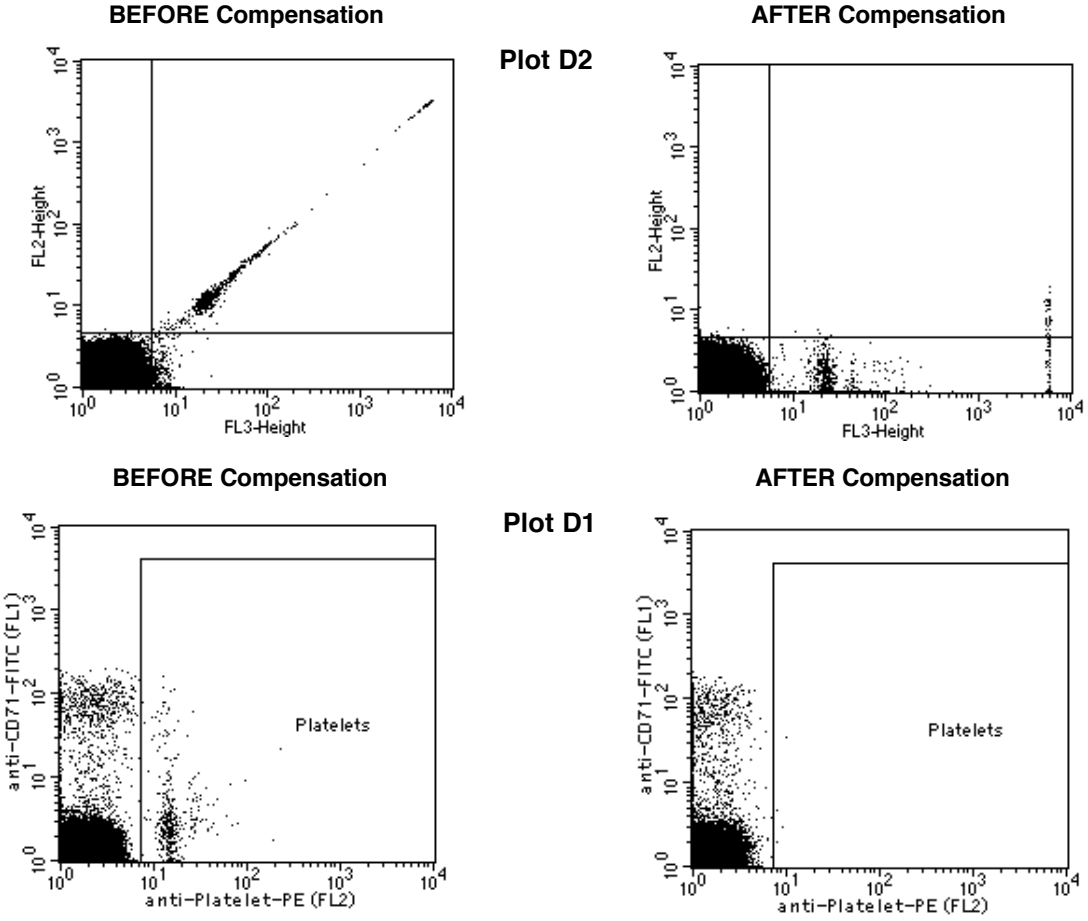
Histogram B



7. 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.

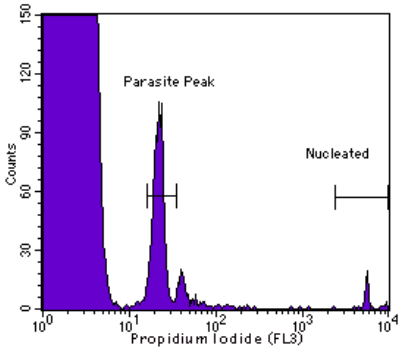


8. Adjust FL2-%FL3 compensation to eliminate the yellow component of propidium iodide. Viewing Plot D2, increase compensation until the parasitized cells show low FL2-associated fluorescence which is on par with that observed for the NCEs at the origin. The nucleated cells' fluorescence should be low,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



9. 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.

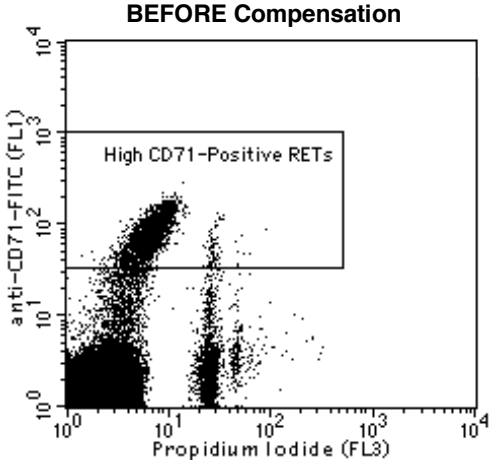
Histogram B



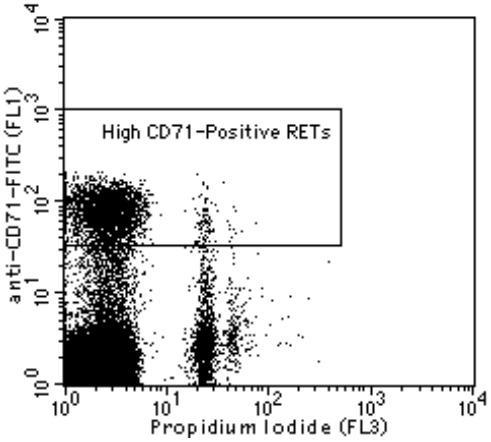
10. The green fluorescent antibody (FITC-conjugated) has a slight far-red (FL3) component. Use the FL3-%FL2 compensation to eliminate the red component of FITC. Viewing Plot E, increase compensation until the young, FITC-positive cells are positioned directly above the mature, FITC-negative cells. Correct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

Plot E

BEFORE Compensation

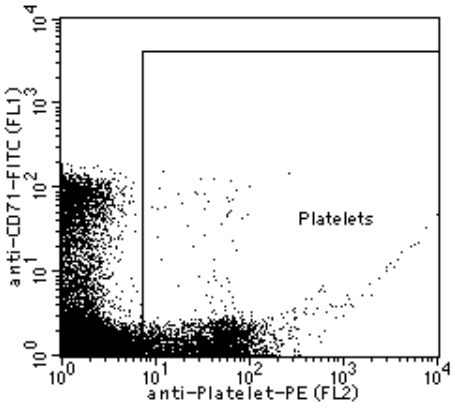


AFTER Compensation



11. Refrigerate the Malaria Biostandard in Labeling Solution I. Add 1.5 ml to 2.0 ml of DNA Staining Solution to the Malaria Biostandard sample incubated in Labeling Solution II, and place 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 in Labeling Solution II.

Plot D



12. 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 bone marrow 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 stored CD71 Setup and Malaria Biostandard samples at 2 °C to 8 °C to readjust the instrument readjusted as necessary.

13. After calibrating the flow cytometer with the CD71 Setup and Malaria Biostandard samples each day, analyze experimental samples.

14.2. Analysis of Experimental Samples

1. 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).

2. It is important to maintain a consistent flow rate, therefore, do not change the flow rate after calibrating your instrument with the Biological Standards.

3. Analyze experimental samples by adding 1.5 ml to 2.0 ml of DNA Staining Solution to a sample and adding it to the top of a cell strainer. It might be necessary to gently tap the tube on a flat surface to start the flow through the filter cap. Remove the cap and 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.

4. While one sample is being analyzed, add 1.5 ml to 2.0 ml of DNA Staining Solution to the next sample, add it to the cell strainer, filter and place at 2 °C to 8 °C.

5. 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:

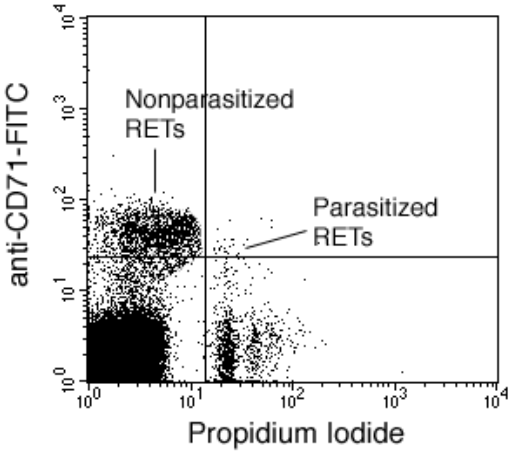
   % RET = (UL+UR)/(UL+UR+LL+LR) * 100
   % MN-NCE = (LR)/(LR+LL) * 100
   % MN-RET = (UR)/(UR+UL) * 100

15. Troubleshooting

Observation	Possible cause	Suggestion
Clumps of cells in Fixative	Bone marrow 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 bone marrow samples	Fixative is carbonated by CO_2 vapor from dry ice	Do not store Fixative on dry ice during fixation; take each tube directly from the freezer and forcefully dispense the sample into the fixative. 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 FL3-%FL2 compensation until the nonparasitized reticulocyte population looks like that in Section 14.1, step 10.
	Incubation with Labeling Solution is too short	Follow the instructions in Labeling Washed Samples for Flow Cytometric Analysis (Section 13.3); increase incubation time in Labeling Solution.
Populations of malaria-infected cells form arches rather than vertical planes (see Troubleshooting Figure 2)	Compensation is too great	Decrease FL3-%FL2 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; take each tube directly from the freezer.
	Samples are not maintained at the appropriate temperatures	Cells fixed in Fixative must be maintained at –75 °C to –85 °C at all times until the addition of Buffer Solution.
		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.

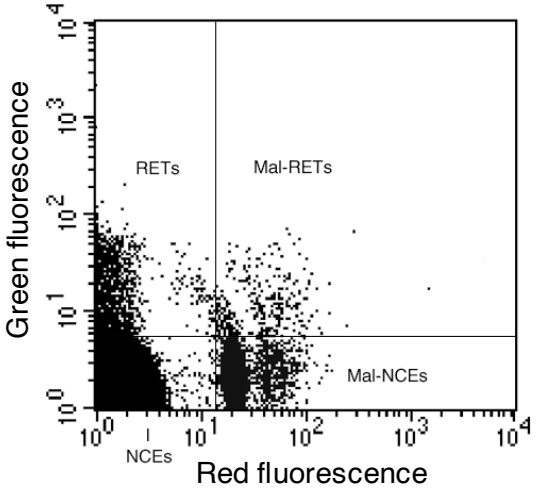
Troubleshooting Figure 1

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.



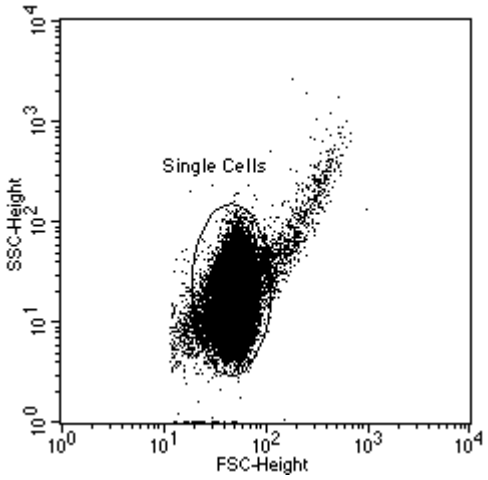
Troubleshooting Figure 2

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 14.2, 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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17. 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 MicroFlow® Kit for the analysis of 60 samples, either in-house (MicroFlow^{PLUS} Kit), or at Litron's facility (MicroFlow^{BASIC} Kit).

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Appendix A: Template Preparation

Template files have been included with this kit, but are specific to CellQuest™ or FACSDiva™ software. The next three pages show actual screen images of the CellQuest template's graphs and histogram. The fourth page describes the gates which are utilized for each plot, and describes how a stop mode of 20,000 reticulocytes was 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 cell fall in the last decade of red fluorescence.

1. 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"

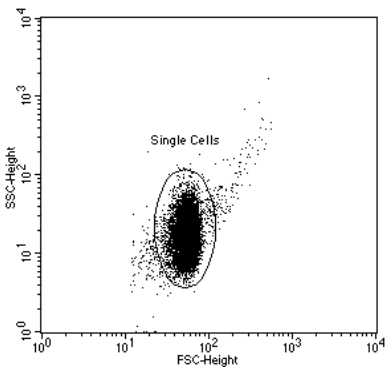
2. Gate for each Plot:

Plot A	No Gate
Plot B	G1
Plot C	G1
Plot D1	G3
Plot D2	G1
Plot E	G3
Plot F	G6

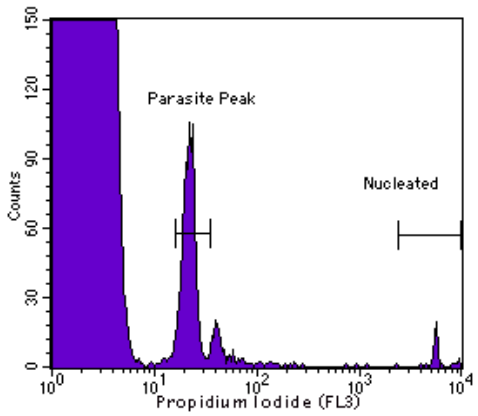
3. Use Gate G7 to set the stop mode. This will allow you to stop data acquisition at a specified number of RETs (typically 20,000). For this gate to work accurately, the lower green demarcation line for region "High CD71-Positive RETs" on Plot E and the horizontal line of the quadrant in Plot F need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

4. Save this template file. This template file should be appropriate for all mouse bone marrow 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.

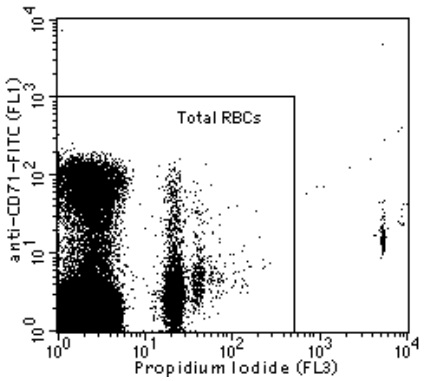
Plot A



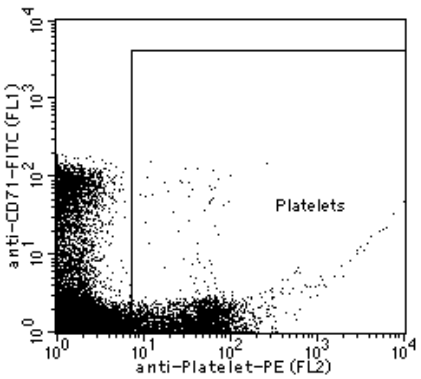
Histogram B



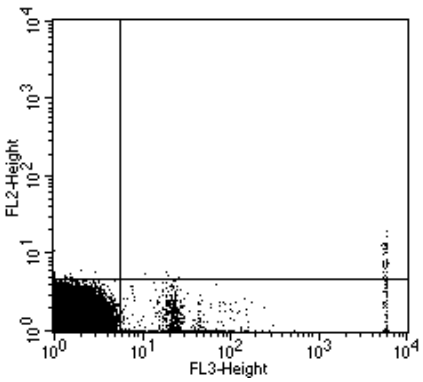
Plot C



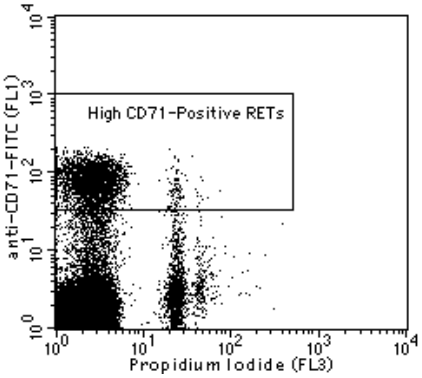
Plot D1



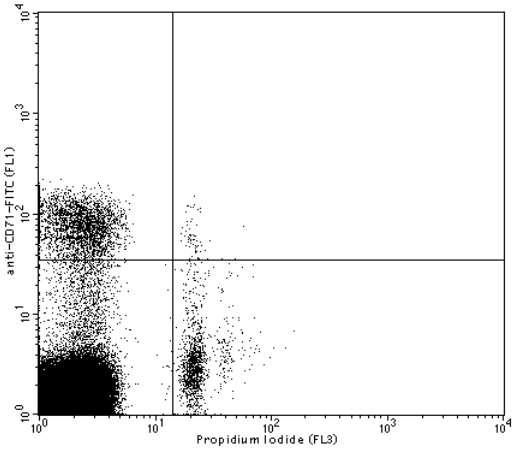
Plot D2



Plot E



Plot F



Quadrant Statistics

File:	Sample ID:
Patient ID:	Patient Name:
Tube:	Acquisition Date:
Gate: G6	Gated Events: 202291
Total Events: 221400	X Parameter: FL3-H Propidium Iodide (Log)
Y Parameter: FL1-H anti-CD71-FITC (Log)	Quad Location: 14, 34

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	4721	2.33	2.13	2.40	2.19	79.63	74.07
UR	132	0.07	0.06	25.47	23.25	61.34	57.10
LL	194178	95.99	87.70	2.11	1.98	1.74	1.53
LR	3260	1.61	1.47	25.34	23.58	4.41	3.36