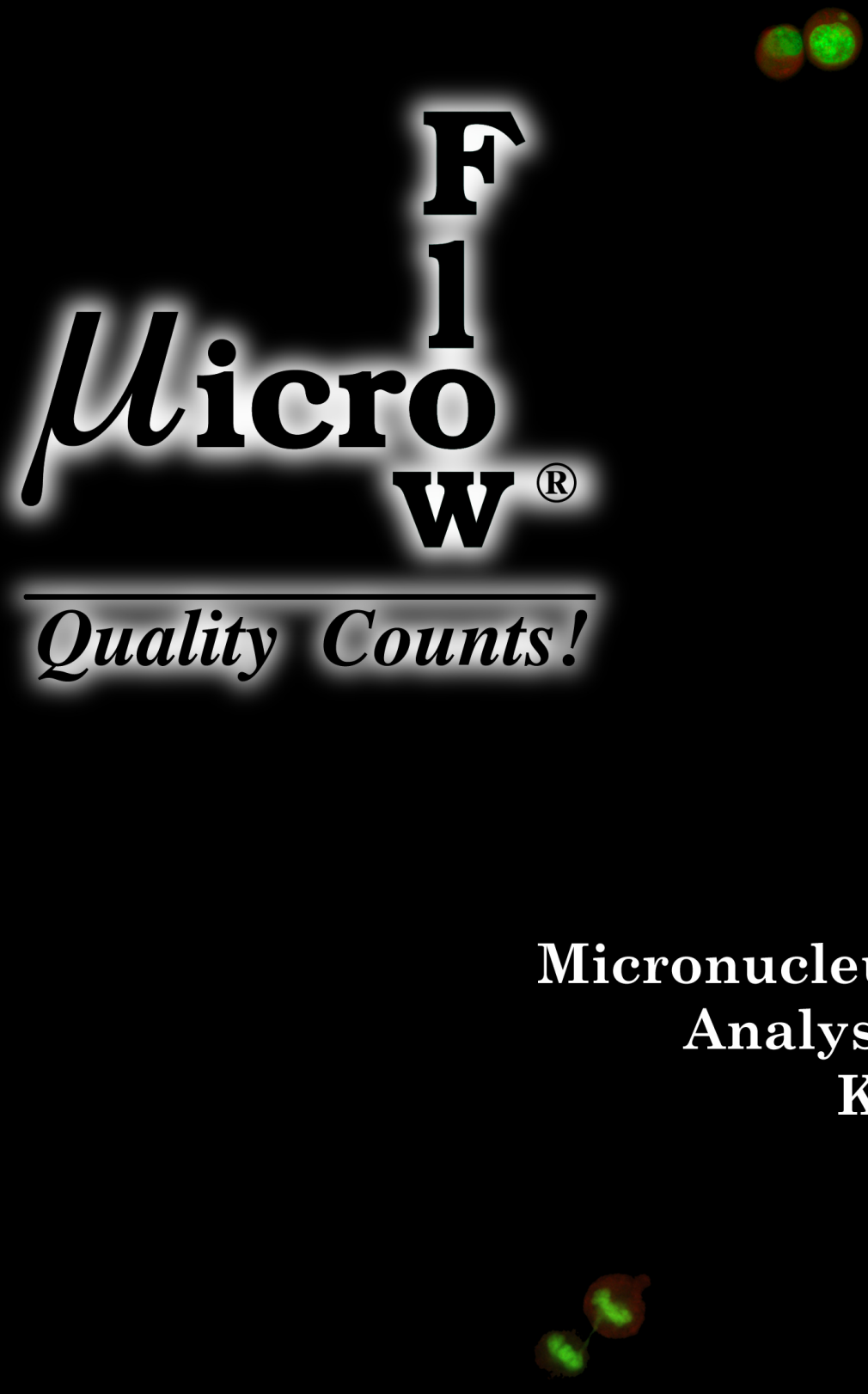


Micronucleus Analysis Kit

MicroFlow Archive Stage 1
Rodent Fixed Blood

Instruction Manual

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

Table Of Contents

1. Materials Provided

Kit Component	Quantity[a]	Storage Condition
Anticoagulant/Diluent	30 mL	2 °C to 8 °C
Long-Term Storage Solution (LTSS)	130 mL	2 °C to 8 °C
Buffer Solution	2 L	2 °C to 8 °C
Cryovials	120	Ambient
Cryovial Storage Boxes	6	Ambient

a. Each kit provides sufficient materials for the collection and fixation of up to 60 blood samples.

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)
- Refrigerator set at 2 °C to 8 °C
- Micropipettors and appropriate tips
- 15 mL polypropylene centrifuge tubes
- Blood collection supplies (may include K_2EDTA tubes, Litron recommends BD Catalog number 367861)
- Vials for collecting/diluting blood (capable of holding 0.5 mL)
- Labels compatible with ultracold storage (Litron recommends Cryo-Tags® labels)

3. 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 exactly 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.

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. Introduction

This kit is used for preparing mouse or rat blood samples for storage for subsequent flow cytometric enumeration of micronucleated erythrocyte populations. It is ideal for facilities that can collect blood samples and fix them within 24 hours after collection (preferably same day).

5.1. 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 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.

5.2. 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, 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

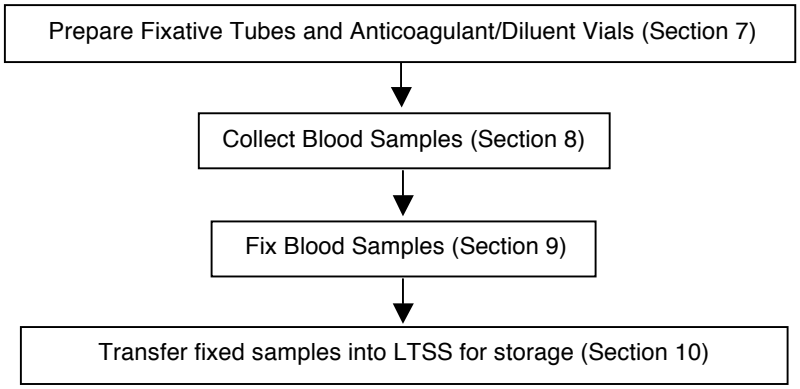
Crucial components of this method are the Calibration Standards, which aid flow cytometer configuration for the micronucleus scoring application. Fixed blood from animals infected with *Plasmodium berghei* are used to configure the flow cytometer before analysis. Whereas MN are relatively rare and exhibit 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.

5.3. 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.”

6. Overview of Method

The following steps are performed when preparing blood samples for storage using the MicroFlow Kit.



7. Prepare Fixative Tubes and Anticoagulant/Diluent Vials

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

1. 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.

Duplicate fixed samples should be prepared.

2. Label each tube with the appropriate information. 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.

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

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

1. One vial is required for each sample. Aseptically aliquot 350 µL Anticoagulant/Diluent into each vial.

2. Label vials with the appropriate information and refrigerate until needed.

8. Collect Blood Samples

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

Volume: Collect 60 µL to 120 µL of blood from each animal into vials containing 350 µL Anticoagulant/Diluent. Collecting much more than 120 µL 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 K_2EDTA-coated tubes (Litron recommends BD Catalog number 367861). Blood samples collected into K_2EDTA 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 µL of a sample and dilute into 350 µL Anticoagulant/Diluent and fix as described below.

9. Fix Blood Samples

It is extremely important that the tubes containing Fixative and fixed blood 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 blood samples DIRECTLY from the –75 °C to –85 °C freezer as described here, follow the alternate fixing procedure found on our 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 (in less than one minute) and work near the freezer. You can fix each sample in duplicate. The duplicate (backup) samples are important in the event of shipping complications or if flow cytometric analysis problems arise.

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.

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 .)

1. Immediately prior to fixing, invert the vial containing the blood/ Anticoagulant mixture to ensure a homogeneous suspension.

2. Using a micropipettor, retrieve 180 μL 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. Making sure that the pipette tip does not touch the side of the tube or the surface of fixative, forcefully dispense 180 μL of diluted blood sample directly into Fixative. See the diagram on the next page.

5. Cap the tube of fixed blood 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 diluted samples. There should be enough volume in each vial to fix each sample twice.

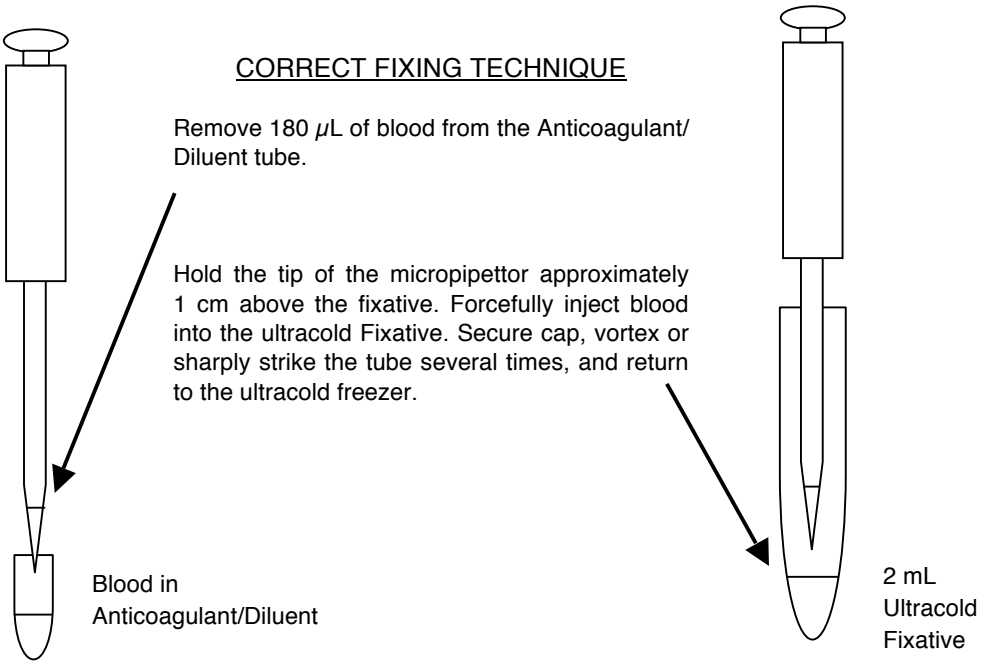
7. If the freezer temperature begins to warm up significantly (i.e., raises by 5 °C), stop processing samples. Wait until the freezer temperature returns to the required range before completing sample fixation. Again, due to their ability to maintain temperature, chest freezers are recommended.

8. Store the samples at –75 °C to –85 °C for at least 3 days before washing to store in LTSS.

CORRECT FIXING TECHNIQUE

Remove 180 μL of blood from the Anticoagulant/ Diluent 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.

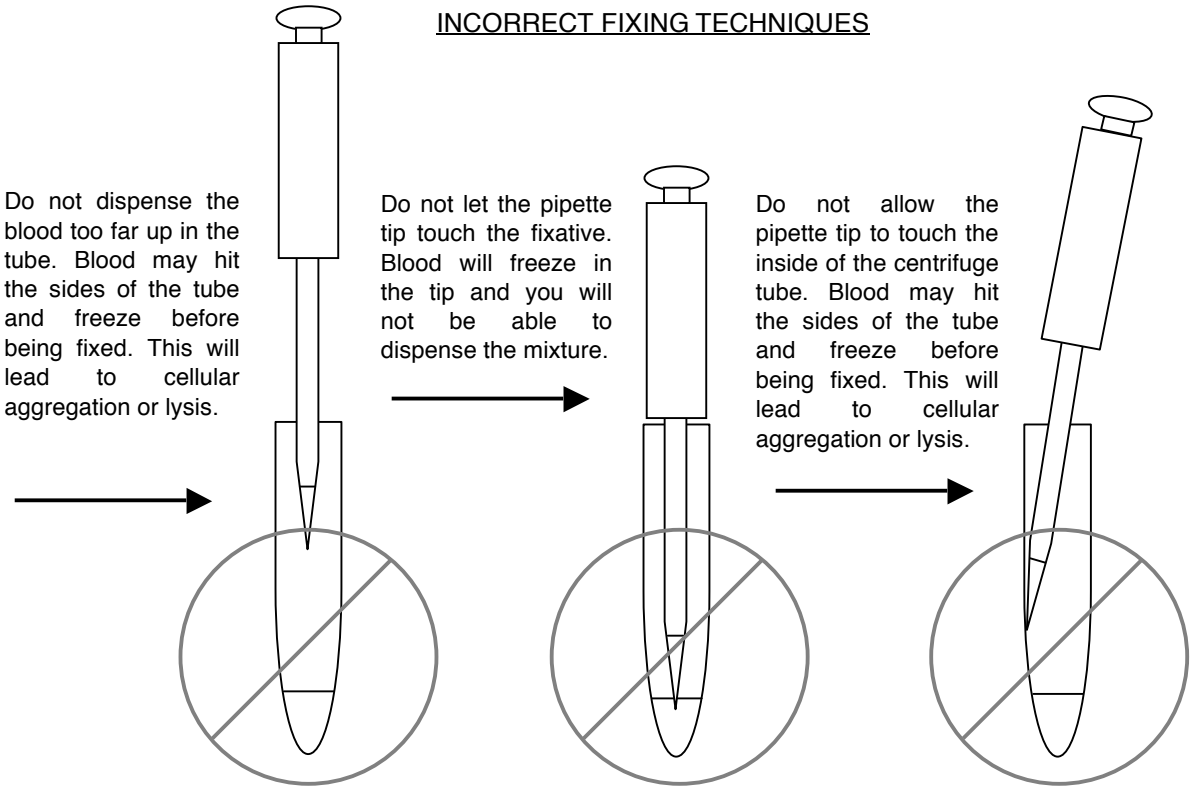


INCORRECT FIXING TECHNIQUES

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



10. Transfer Fixed Samples into LTSS

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.

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. Label each cryovial with the animal identification number. For FDA GLP analyses, individual samples must be labeled with the following information: Sample ID, Study ID, Date Collected, Source (e.g., mouse or rat) and Type (i.e., Blood). For OECD GLP, label samples with Unique ID and Sample ID.

2. Pack Buffer Solution on ice to achieve ice-cold, but not freezing, temperature (approximately 45 minutes).

3. 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.

4. 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.

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

5. 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.

6. Repeat steps 4 and 5 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.**

7. 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.

8. 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.

9. 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.

10. Add 1 mL of LTSS to each tube, and transfer contents into the appropriate cryovial and tighten caps.

11. Place the cryovials in the cryovial storage boxes and store at –75 °C to –85 °C.

> It can be helpful to separate duplicate blood samples by using separate storage boxes. One set of duplicates can be analyzed in-house or sent to Litron for analysis, and the other can be kept on site as a backup.

11. 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.

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13. License Agreement and Limited Product Warranty

By utilizing this kit, your company is agreeing to be bound by the terms of this License. This License allows the use of the MicroFlow® Kit for the collection and storage of 60 samples.

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

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