




F
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Micro
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Quality Counts!



**Micronucleus
Analysis
Kit**

MicroFlow^{BASIC} (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 Condition
Long-Term Storage Solution (LTSS)	130 ml	2 °C to 8 °C
Buffer Solution	3 liters	2 °C to 8 °C
Vacuum-insulated shipping container ^b	1	Ambient
Bag containing icepacks ^c	1	-30 °C to -10 °C
Thin clear plastic bag for shipping SSF and SPP	1	Ambient
Cryovials	120	Ambient
Cryovial Storage Boxes	6	Ambient

- Each kit provides sufficient materials for the analysis of up to 60 bone marrow samples at Litron.
- Save the vacuum-insulated shipping container. This container is designed to ensure that up to 60 samples stored in LTSS will remain sufficiently cold for 48 to 72 hours.
- Upon receipt, place icepacks in a freezer set to -30 °C to -10 °C.

2. Additional Materials Required

- Fixative must be methanol; 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
- 15 ml polypropylene centrifuge tubes
- 3 ml syringes and appropriately sized needles (for flushing bone marrow)
- 20 ml syringes (used to prepare columns)
- Fisher lens paper
- α -cellulose (Sigma Catalog Number C8002)
- Sigmacell[®] cellulose, type 50 (Sigma Catalog Number S5504)
- Heat-inactivated, filter-sterilized fetal bovine serum (FBS)
- Micropipettors and appropriate tips
- Shipping forms for overnight delivery service
- Labels compatible with ultracold storage (Cryo-Tags[®] labels recommended)

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.

A signed Study Phase Plan is required at Litron before sample analysis is initiated. A Sample Submission Form is also required for each shipment of samples. These forms can be found on Litron's website (www.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 when preparing mouse bone marrow samples for flow cytometric enumeration of micronucleated erythrocyte populations. It is ideal for facilities that can collect bone marrow samples and fix them within 4 hours after collection.

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 hematopoietic 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.

5.2. 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

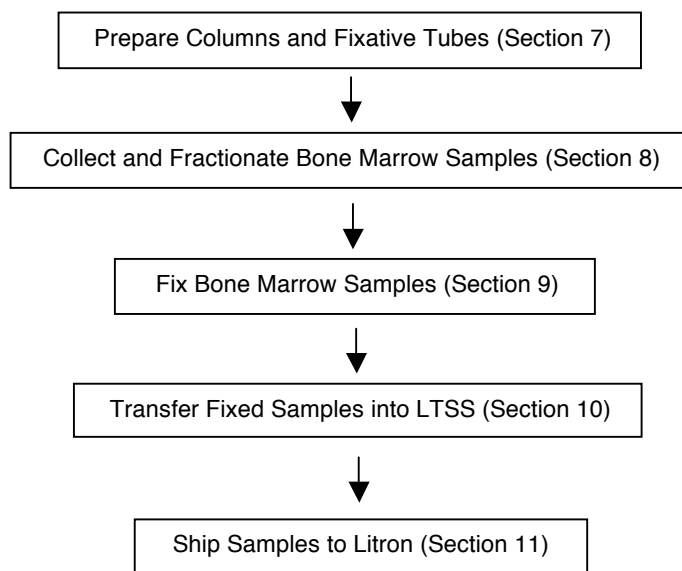
Crucial components of this method are the biological standards which aid flow cytometer configuration for the micronucleus scoring application. Fixed blood from animals infected with *Plasmodium berghei* are used to configure the flow cytometer before analysis. Whereas MN are relatively rare and exhibit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

5.3. 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.

6. Overview of Method

The following steps are performed when preparing bone marrow samples for shipment to Litron using this Kit.



7. Prepare Columns and Fixative Tubes

7.1. Prepare Columns

Fractionation of mouse bone marrow through microcrystalline cellulose columns is essential 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 approximately 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.

7.2. Prepare Fixative Tubes

1. One 15 ml polypropylene centrifuge tubes is required per sample (or two, if fixing in duplicate). Add 2 ml of Fixative (Solution A) to each tube, and replace caps. Duplicate fixed samples can be prepared.
2. Label (Cryo-Tags[®] labels recommended) each tube 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 (i.e., mouse) and Type (i.e., bone marrow). For OECD GLP, label samples with Unique ID and Sample ID. It is also helpful to label the cap of each tube.
3. If not using ultracold 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.

8. Collect and Fractionate Bone Marrow Samples

8.1. 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.
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.

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

8.2. 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 μ l.
5. Resuspend the cell pellet and fix within 4 hours.

9. Fix Bone Marrow Samples

It is extremely important that the tubes containing Fixative and fixed bone marrow remain ultracold ($-75\text{ }^{\circ}\text{C}$ to $-85\text{ }^{\circ}\text{C}$) and do not come in contact with vapors from dry ice. CO₂ 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 $-75\text{ }^{\circ}\text{C}$ to $-85\text{ }^{\circ}\text{C}$ 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 ($-75\text{ }^{\circ}\text{C}$ to $-85\text{ }^{\circ}\text{C}$) 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.

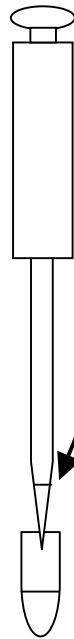
It may be helpful for two individuals to perform this procedure, one filling the micropipettors with bone marrow samples and the other removing the Fixative tubes from the freezer.

1. Immediately prior to fixing, invert the vial containing the bone marrow sample to ensure a homogeneous suspension.
2. Using a micropipettor, retrieve 150 μ l 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. Make sure that the pipette tip does not touch the side of the tube or the surface of fixative, and forcefully dispense the sample directly into Fixative. See the diagram on page 7.
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 ($-75\text{ }^{\circ}\text{C}$ to $-85\text{ }^{\circ}\text{C}$). If a vortexer cannot be placed right next to the freezer, hold the top of the tube with one hand, use your other hand to sharply strike the bottom of the tube several times before returning it to the freezer. (Steps 3 through 5 should take no more than 10 seconds.)

Remove only one tube of ultracold Fixative from the freezer at a time (unless two individuals are performing). After adding the 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 $-75\text{ }^{\circ}\text{C}$ to $-85\text{ }^{\circ}\text{C}$.)

6. Change the pipette tip and repeat steps 1 through 5 for the remaining samples. If necessary, 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 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.

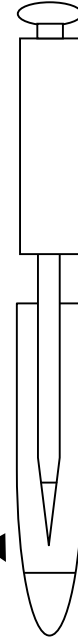
It can be helpful to separate duplicate fixed bone marrow samples by using two racks, one that will be sent to Litron for analysis, and the other that will be kept on site as a backup.



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 bone marrow into the ultracold Fixative. Secure cap, vortex or sharply strike the tube several times, and return to the ultracold freezer.

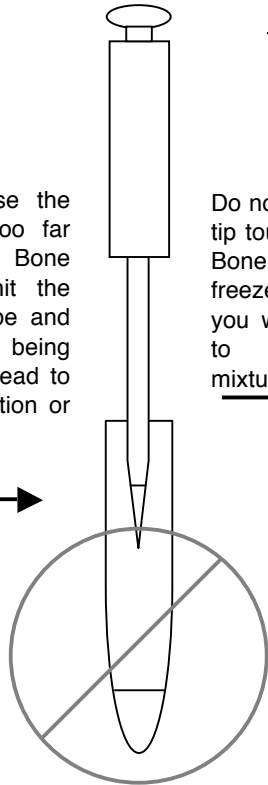
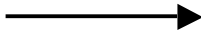


Bone marrow suspension

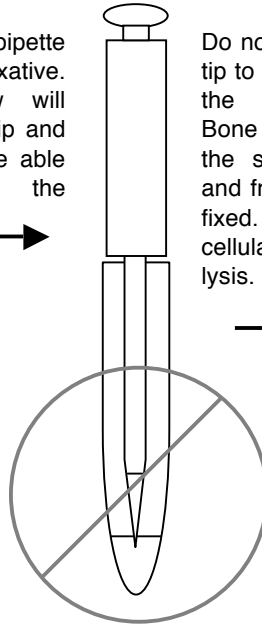
2 ml Ultracold Fixative

INCORRECT FIXING TECHNIQUES

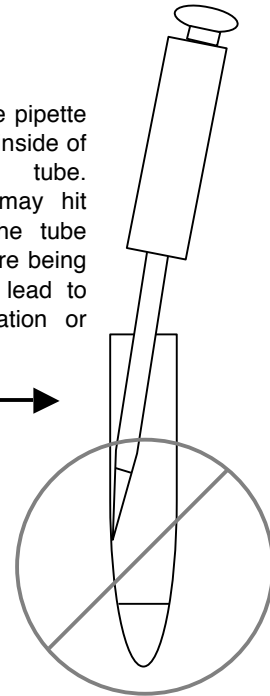
Do not dispense the bone marrow too far up in the tube. Bone marrow may hit the sides of the tube and freeze before being fixed. This will lead to cellular aggregation or lysis.



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.



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., bone marrow). 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 μ l 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.

It can be helpful to separate duplicate bone marrow samples by using separate storage boxes. One set of duplicates will be sent to Litron for analysis, and the other will be kept on site as a backup.
11. Place the cryovials in the cryo storage boxes, and place the boxes into the bags they came in, and store at -75 °C to -85 °C until shipping to Litron.
12. Ship samples to Litron for flow cytometric analysis as described below.

11. Ship Samples to Litron

1. Before preparing shipment, verify that all icepacks have been frozen in a freezer set to approximately -20 °C. Please use the shipping container that was provided by Litron. It is imperative that all icepacks are frozen and that the Litron-provided vacuum-insulated shipping container is used in order to maintain correct temperatures during transit.
2. **Complete Study Paperwork.** Complete and sign the appropriate documents and place them inside the thin clear plastic bag. Sample analysis cannot begin until the Sample Submission Form and Study Phase Plan are received.

3. **Prepare.** Position the following items close to the freezer where samples are stored:
 - Vacuum-insulated shipping container (supplied with kit)
 - Shipping bag (supplied with kit)
 - Frozen Ice packs (supplied with kit)
 4. **Place the bagged cryovial storage boxes upright at the bottom of the shipping box** (up to four boxes should fit in one layer on the bottom of the box). Do not ship more than eight cryovial storage boxes in any one box. Fill with frozen ice packs. Place the cardboard piece, silver panel, and foam piece on top of the icepacks (in this order). If you need additional shipping boxes or icepacks, contact Litron.
 5. **Seal Box.** Place the thin clear plastic bag containing the applicable forms on top of the foam piece. Close the cardboard flaps of the outer box and use shipping tape to secure the box top. *It is very important to get a tight seal in order to maintain temperatures during transit.*
 6. **Ship To Address.** *DO NOT Identify the samples as Category A or B biological substances.* Please verify that the shipper you use guarantees overnight delivery, and ship to the following address:

Litron Laboratories
 3500 Winton Place, Suite 1B
 Rochester, New York 14623
 585-442-0930
- Unexpected shipping delays may occur at any time. Therefore, it is best to ship samples on Monday or Tuesday and avoid shipping during holidays.
7. **Send Confirmation.** Immediately after shipping, send an email to info@litronlabs.com including your name, telephone number, date of shipment, and the shipper's tracking number.
 8. **Importing to USA.** Track international shipments often to ensure that the samples are not held up at Customs. Several copies of specific Customs forms are usually required. Information regarding importing materials into the United States should be available from your courier, or the United States National Center for Import and Export. As the nature of the samples is defined as a test kit, an import permit is not required, however a USDA inspection may be required. Please contact Litron or the USDA for further information (www.aphis.usda.gov).

12. Results

Preliminary results will be emailed and a hard copy of the final results will be provided, if requested. Once the analysis has been completed, backup samples may be discarded. Please inform Litron before disposal.

13. 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 during analysis at Litron.	Fixative is carbonated by CO ₂ vapor from dry ice .	Do not store Fixative in a freezer that is also used for storing dry ice. Do not store Fixative on dry ice during fixation. Instead, take each tube directly from the freezer and forcefully dispense the sample into the fixative.

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15. 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).

MicroFlow[®]. All rights reserved. *MicroFlow*[®] is a registered trademark of Litron Laboratories. U.S. Patent Nos. 5,229,265, 5,858,667 and 6,100,038. Copyright 2003-2013, Litron.

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.