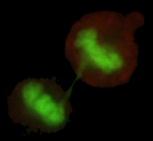


MicroFlow®

Quality Counts!

Micronucleus Analysis Kit

MicroFlow^{PLUS} (Rodent Liver)

Instruction Manual

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

Table of Contents

1. Materials Provided

Kit Components	Quantity[a]	Storage Condition[b]
10X Liver Rinse	150 mL	Ambient
EDTA Solution	40 mL	2 °C to 8 °C
EGTA Solution	600 µL	2 °C to 8 °C
Collagenase Solution	15 mL	–10 °C to –30 °C
Incomplete Liver Lysis Solution 1	15 mL	Ambient
Incomplete Liver Lysis Solution 2	15 mL	2 °C to 8 °C
Ki-67 Antibody eFluor® 660[c]	20 µL	2 °C to 8 °C, light sensitive
Liver Nucleic Acid Dye (SYTOX® Green nucleic acid stain)[d]	90 µL	–10 °C to –30 °C, light sensitive
RNase Solution	75 µL	2 °C to 8 °C

a. Sufficient materials are provided to collect and analyze 25 liver samples.
b. Please note that although some kit components are shipped at ambient temperature, they must be stored at the temperatures indicated upon receipt.
c. Warning! Contains sodium azide. Irritant. See SDS (on Litron's website).
d. Warning! Contains dimethyl sulfoxide. See SDS (on Litron's website).

2. Additional Materials Required

- Hanks' Balanced Salt Solution (HBSS: without calcium and magnesium; Cellgro Cat. No. 21-022-CM)
- Commercially-heat-inactivated, sterile fetal bovine serum (FBS)
- Deionized water (dH_2O)
- Dimethyl sulfoxide (DMSO)
- -10 °C to -30 °C freezer
- 2 °C to 8 °C refrigerator
- Centrifuge with swinging bucket rotor
- Shaking incubator (see Appendix B)
- T25 flasks
- Scale for weighing livers and liver slices
- Scissors that fit into a 50 mL conical centrifuge tube and can reach the bottom of the tube
- Flow cytometer capable of 488 nM and far red excitation (e.g., 633 nM).
- Disposable pipettes sized 5, 10, 25, and 50 mL
- Vortex mixer (preferably fitted with a vertical six 50 mL tube holder for isolating liver cells – see Appendix B)
- Conical centrifuge tubes (e.g., 15 mL, 50 mL)
- Micropipettors (20 µL - 1000 µL) and tips
- Flow cytometry tubes
- 0.2 micron filter units
- 5 mL Polystyrene round-bottom tubes with 35 micron cell strainer cap (BD Falcon Cat. No. 352235)
- Low-lint laboratory tissue (e.g., Kimwipe)

3. Aliquot Collagenase Solution

If using Collagenase Solution for more than one experiment, it is best to store it in small aliquots to prevent multiple freeze-thaw cycles. Collagenase Solution may be thawed upon receipt, so that would be an optimal time to aliquot it. Mix thoroughly before transferring to smaller containers and store at -10 °C to -30 °C.

4. Technical Support

Litron Laboratories
3500 Winton Place
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

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

5.2. Flow Cytometer Requirements

This assay requires two lasers: one that provides 488 nm excitation and one that provides far red excitation (e.g., 633 nm). Standard factory-installed filter sets are typically sufficient to achieve fluorescent resolution of the relevant cell populations. Using BD instruments, the green dye-associated fluorescence (i.e., SYTOX® Green) should be collected in the FITC channel, and the anti-Ki-67-associated fluorescence (i.e., eFluor® 660) should be collected in the APC channel.

6. Introduction

This kit is used to prepare rat or mouse liver samples for flow cytometric enumeration of micronuclei, a biomarker of chromosomal damage. Simultaneous with micronucleus frequencies, the assay also provides quantitative information related to the amount of proliferation the tissue has experienced.

6.1. The Micronucleus Test

The most widely used approach for studying *in vivo* chromosome damaging potential is to expose laboratory rodents to test material and score the incidence of micronucleated cells. The test is most often conducted with hematopoietic cells, especially reticulocytes [Matter and Schmid 1971; Heddle, 1973; MacGregor et al., 1980]. In fact for the last several decades the erythrocyte-based micronucleus assay has been a cornerstone of required safety testing for the registration of new pharmaceuticals and industrial chemicals [ICH, 2011; Hayashi et al., 2007].

While the erythrocyte-based micronucleus assay continues to represent a pivotal genetic toxicology endpoint, the most recent safety assessment guidance documents stress the importance of considering other tissues in addition to those of the hematopoietic compartment. For instance the S2(R1) document of the International Conference on Harmonisation states: "The inclusion of a second *in vivo* assay in the battery is to provide assurance of lack of genotoxicity by use of a tissue that is well exposed to a drug and/or its metabolites, " [ICH, 2011]. Also, in regard to certain pro-genotoxicants showing negative results in traditional chromosomal damage assays, it states: "…these examples likely reflect a lack of appropriate metabolic activity or lack of reactive intermediates delivered to the hematopoietic cells of the bone marrow."

In practice, *in vivo* non-hematopoietic cell assays most commonly focus on the liver. It is the main organ responsible for drug metabolism, and the local concentration of genotoxic intermediates is often highest in this tissue. Collaborative work conducted by Japanese scientists under the auspices of the JEMS/MMS Group has been addressing the need for a liver-based cytogenetic damage assay. This important research was summarized in several publications, including an IWGT report [Uno et al., 2015a], as well as papers published by the JEMS/MMS group [Uno et al., 2015b, Hamada et al., 2015].

This manual describes procedures for analyzing liver tissue for the frequency of micronuclei using a dual-laser flow cytometer. As described in more detail below, the assay simultaneously provides information related to the proliferative status of the liver tissue. Since DNA damage can only manifest as micronuclei upon cellular division, it is important to characterize the proliferative status of rodent liver samples when trying to interpret micronucleus frequencies. The requirement for hepatocyte proliferation also has implications for adequate experimental design, and this is described in more detail below.

6.2. The MicroFlow® Method

Litron Laboratories has developed and patented a flow cytometric method to measure micronuclei in the rodent liver hepatocyte population. The assay principle is based on detergent-liberation of nuclei and micronuclei. In conjunction with a nucleic acid dye and RNase, these particles exhibit characteristic light scatter and DNA-associated fluorescence that allow for enumeration via flow cytometry. Based on an antibody against the nuclear epitope Ki-67, it is also possible to simultaneously characterize recent hepatocyte proliferation. Other indices of proliferation can be generated by considering the proportions of nuclei with 2n, 4n, and 8n+ DNA content.

6.3. Experimental Design

This instruction manual focuses on describing the reagents and optimized rodent liver processing steps necessary to acquire reliable micronucleus and proliferation measurements via flow cytometric analysis. As alluded to above, the need for hepatocyte proliferation during the in-life treatment phase of a rodent liver micronucleus study is of critical importance. However, it is beyond the scope of this manual to teach or recommend appropriate experimental designs. Rather, the interested reader is directed to publications that describe this in detail. For example, publications by Uno *et al.,* 2015a and Hamada *et al.,* 2015 provide useful advice.

With this in mind, we have limited the discussion about appropriate experimental designs to some generalities, and to first-hand experiences that were observed over the course of developing the procedures found herein:

- Chromosomal damage will not result in micronucleus formation without cellular division.

- Hepatocyte division is pronounced in young rats, and slows down as the animals age. After about 8 weeks of age, the rate of division, and thus the generation of a potentially micronucleated population of hepatocytes, is usually quite low. Therefore rats at 8 weeks of age are too old and an incompatible age for starting a treatment regimen.

- Whereas certain chemical treatments or partial removal of liver tissue can promote hepatocyte proliferation, most groups have come to the conclusion these older strategies for making rodent liver compatible with micronucleus assays should not be employed for a variety of reasons, including animal welfare considerations.

- More recently, data have accumulated that suggest acute treatment studies can be performed with juvenile rats, for instance those at about 4 weeks of age [2015a]. In these cases, rats may be treated for 2 to 3 consecutive days, and several days thereafter liver tissue can be collected for micronucleus analyses.

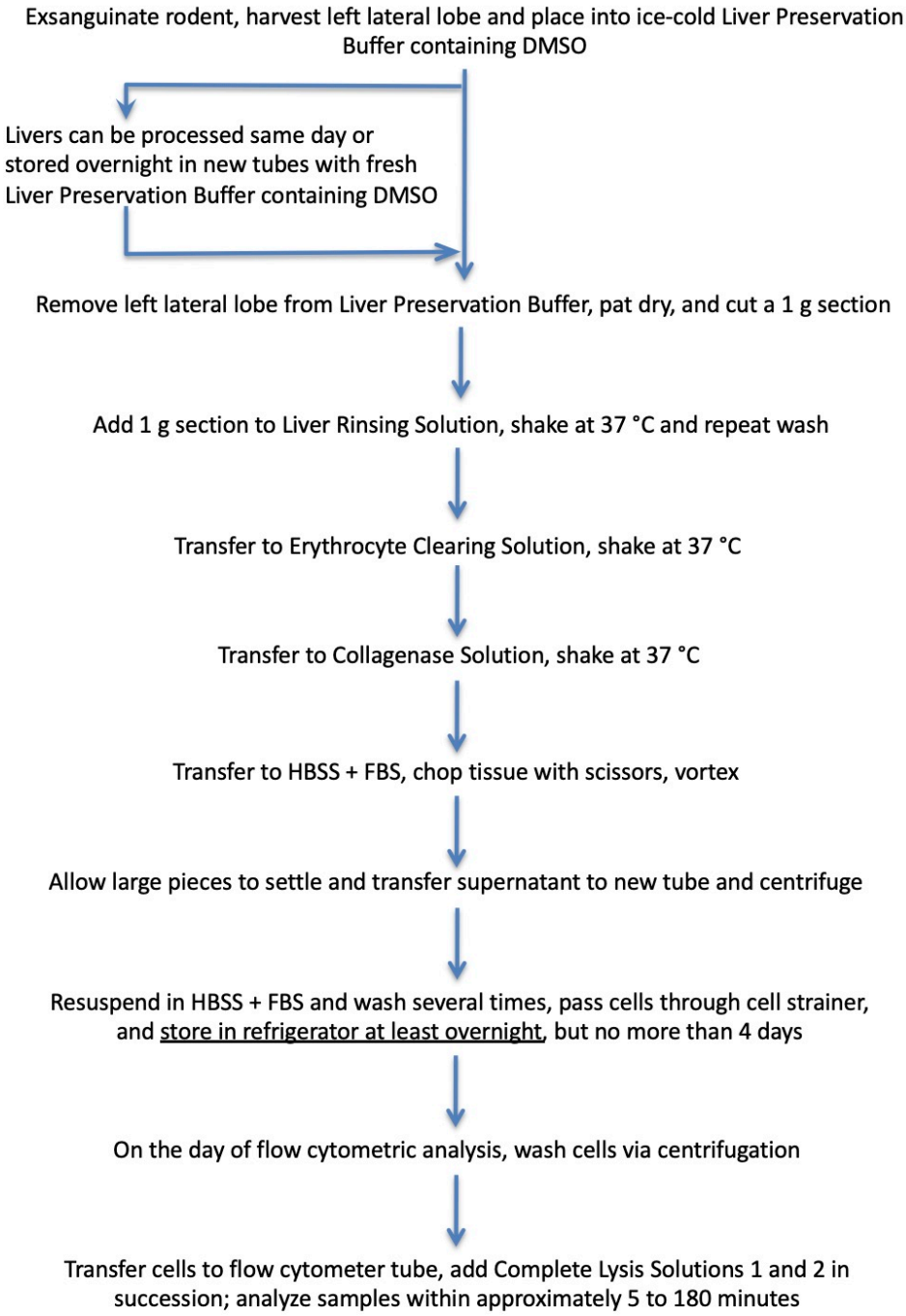
- Other data have accumulated to suggest that young rats of approximately 6 weeks old can be used effectively to study treatment-induced micronucleus formation, but in these cases assay sensitivity tends to benefit from repeat-treatment schedules, for example 14 to 28 consecutive days [Hamada *et al.,* 2015].

- Although the procedure is compatible with mouse livers, very few mouse livers have been processed using these methods to date. Therefore, the entire left lateral lobe or entire liver may need to be processed and will likely be less than 1 gram.

- We recommend analyzing samples in single tube analysis and not in HTS mode as adjustment to the FL1 PMT voltage may be needed on a per sample basis.

7. Overview of the Method

A flow chart highlights key tissue processing steps associated with the MicroFlow kit-based liver micronucleus assay. Note that this schematic is only provided for a general overview of kit procedures; it lacks important details that will only be found in the pages that follow.



8. Reagent Preparation

Liver Preservation Buffer (minus the DMSO), Liver Rinse Solution, Erythrocyte Clearing Solution, and HBSS + FBS can be prepared one or two days before being used. Working Collagenase Solution must be prepared immediately before use. Complete Lysis Solution 1 and Complete Lysis Solution 2 must be prepared the day of sample analysis.

Use the charts below to determine the volumes required. Scale up as necessary and prepare more than indicated to allow for loss (10 % more works well).

8.1. Liver Preservation Buffer

Number of livers	Volume of HBSS	Volume of EDTA Solution	Volume of DMSO
1	12.9 mL	0.6 mL	1.5 mL
1 — (if storing unprocessed liver tissues in a refrigerator overnight)	25.8 mL	1.2 mL	3.0 mL

1. Add the required volumes of HBSS and EDTA Solution to a clean vessel.
2. Filter sterilize and store on ice or refrigerate until use.
3. Add the required volume of DMSO no more than one hour before obtaining livers and place on ice.

8.2. Liver Rinse Solution

Number of livers	Volume of dH_2O	Volume of 10X Liver Rinse
1	18.0 mL	2.0 mL

1. Combine the required volumes of dH_2O and 10X Liver Rinse in a clean vessel.
2. Filter sterilize and bring to ambient temperature before use.

8.3. Erythrocyte Clearing Solution

Number of livers	Volume of dH_2O	Volume of 10X Liver Rinse	Volume of EGTA Solution
1	17.98 mL	2.0 mL	20 µL

1. Combine the required volumes of dH_2O, 10X Liver Rinse, and EGTA Solution in a clean vessel.
2. Filter sterilize and bring to ambient temperature before use.

8.4. HBSS + FBS

Number of livers	Volume of HBSS	Volume of FBS
1	56.25 mL	6.25 mL

1. Combine the required volumes of HBSS and FBS in a clean vessel.
2. Filter sterilize and store at 2 °C to 8 °C, but bring to ambient temperature before use.

8.5. Working Collagenase Solution

Number of livers	Volume of HBSS + FBS	Volume of Collagenase Solution
1	9.5 mL	0.5 mL

1. Add the required volumes of HBSS + FBS to a clean vessel.
2. Thaw the Collagenase Solution and add the required volume *immediately* before use.

8.6. Complete Lysis Solution 1

Number of livers	Volume of Incomplete Liver Lysis Solution 1	Volume of Liver Nucleic Acid Dye	Volume of RNase Solution	Volume of Ki-67 Antibody eFluor® 660
1	400 µL	1.6 µL	2.0 µL	0.2 µL

1. Combine the required volumes of Incomplete Liver Lysis Solution 1, Liver Nucleic Acid Dye, RNase Solution and Ki-67 Antibody eFluor® 660 in a clean vessel.

2. Protect from light and store at ambient temperature until use.

8.7. Complete Lysis Solution 2

Number of livers	Volume of Incomplete Liver Lysis Solution 2	Volume of Liver Nucleic Acid Dye
1	400 µL	1.6 µL

1. Combine the required volumes of Incomplete Liver Lysis Solution 2 and Liver Nucleic Acid Dye in a clean vessel.

2. Protect from light and store at ambient temperature until use.

9. Liver Harvest and Liver Cell Isolation

9.1. Liver Harvest

1. For this assay, approximately 1 g of left lateral lobe per rat or mouse is needed for processing. If your study will include additional liver assay(s) or analyses, you may need to excise the entire liver.

2. Aliquot 15 mL of **Liver Preservation Buffer** containing DMSO into the appropriate number of 50 mL tubes – one tube per liver. Keep on ice for the entire procedure.

3. For the following procedures, it is important to work fast, and to work on one animal at a time.

    3.1. First, use an approved method to anesthetize a single animal, for example via CO_2 overdose. Exsanguinate the animal (e.g., using a blind heart stick) in order to achieve the best liver MN results. No matter the method used to anesthetize or exsanguinate, it is important that the animal's heart is still beating in order to collect as much blood as possible. For 4 to 8 week old rats, it is usually possible to collect 4 to 9 mL of blood.

    3.2. Excise the left lateral lobe (or the entire liver, depending on your study's requirements).

    3.3. Transfer the left lateral lobe into a tube containing ice-cold **Liver Preservation Buffer**. Store on ice.

    3.4. If storing the livers overnight in the refrigerator, transfer each liver into 15 mL fresh **Liver Preservation Buffer** immediately before refrigeration. Otherwise proceed to step 9.2, below.

9.2. Rinse Livers

1. For each liver being processed, aliquot 10 mL **Liver Rinse Solution** into T25 flasks (1 per liver) and warm to 37 °C.

2. Remove a liver from **Liver Preservation Buffer** and gently pat dry with a laboratory tissue. Proceed with steps 3 and 4 before moving on to the next liver.

3. Using a razor blade or scalpel, cut a piece of liver approximately 1 g in size (0.8 g to 1.2 g). Place the liver piece into a T25 flask containing the warm **Liver Rinse Solution**.

4. Either discard the remaining liver tissue, or return it to the **Liver Preservation Buffer** and store on ice for up to 4 days.

5. Repeat steps 2 through 4 for all remaining livers. Incubate and shake for 10 minutes at 37 °C and 130 rpm. Ensure that all liver pieces are agitating and not stuck to the bottom of the flask. If a liver sticks, gently swirl the flask until the liver piece becomes suspended in the **Liver Rinse Solution**.

6. Remove the **Liver Rinse Solution** from each flask using a strip pipette.

7. Using a new, clean pipette, add 10 mL fresh, ambient temperature **Liver Rinse Solution** to each flask. Incubate and shake for 10 minutes at 37 °C and 130 rpm.

A separate strip pipette is needed for each liver piece to remove solutions. However, the same pipette can be used for the same liver piece throughout the various rinses if re-sheathed when not in use. We recommend writing the liver ID on the pipette for easy identification.

8. Remove the **Liver Rinse Solution** from each flask (using the saved strip pipettes). Using a new, clean pipette, add 10 mL ambient temperature **Erythrocyte Clearing Solution**. Incubate and shake for 10 minutes at 37 °C and 130 rpm.

9. Remove the **Erythrocyte Clearing Solution** from each flask (using the saved strip pipettes). Using a new, clean pipette, add 10 mL ambient temperature **Erythrocyte Clearing Solution**. Incubate and shake for 10 minutes at 37 °C and 130 rpm.

10. Remove the **Erythrocyte Clearing Solution** from each flask (using the saved strip pipettes). Using a new, clean pipette, add 10 mL ambient temperature **Working Collagenase Solution**. Incubate and shake for 30 minutes at 37 °C and 130 rpm.

9.3. Isolate Liver Cells

1. Remove the **Working Collagenase Solution** (using the saved strip pipettes). Using a new, clean pipette, add 13 mL **HBSS + FBS** to the flask and pour the contents of each into a 50 mL conical tube. If the liver piece gets stuck in the flask and does not transfer into the 50 mL tube, move the Buffer Solution + FBS back into the flask and swirl it around to help release it.

2. Chop the liver piece with scissors (approximately 30 times) to achieve approximately equal-size pieces (each piece is typically less than 0.5 mm). Vortex each tube at maximum speed for 3 minutes to break up the cells. Using a vortex mixer fitted with a vertical 50 mL tube holder allows you to vortex up to 6 tubes at one time. See Appendix B, Figure B-1.

3. Allow larger liver pieces to settle (approximately 10 to 20 seconds) and quickly pour the cell suspension (avoiding any large pieces of liver) to a new 15 mL centrifuge tube. Use a new, clean pipette (or a pipettor with a new pipette tip) to transfer any remaining cell suspension from the 50 mL tube to the 15 mL tube (still avoiding large pieces of liver). Repeat with remaining samples.

4. Centrifuge the tubes for 2 minutes at 200 x *g*. Aspirate the supernatants and tap the pellet to loosen the cells. Add 5 mL ambient temperature **HBSS + FBS** to the tubes to resuspend the cells and mix gently by pipetting up and down one or two times.

5. Centrifuge the tubes for 2 minutes at 200 x *g*. Aspirate the supernatants and tap the pellet to loosen the cells. Add 5 mL ambient temperature **HBSS + FBS** to the tubes to resuspend the cells and mix gently by pipetting up and down one or two times.

6. Repeat step 5 one to three times, until the supernatants are clear in appearance. Once clear, aspirate the supernatants and tap the pellets to loosen the cells and mix gently by pipetting up and down one or two times.

7. Add 3 mL ambient temperature **HBSS + FBS** to the tubes to resuspend the cells.

8. Add the cells to the top of a 35 micron mesh cell strainer cap and filter into the round bottom tube. It might be necessary to gently tap the round bottom tube on a flat surface to start the flow through the filter cap.

9. Transfer the strained cells to a 15 mL centrifuge tube, place on ice and store in the refrigerator overnight or up to 3 days before analysis. Delaying analysis until one day after filtration represents a convenient way to stagger work associated with tissue collection, sample preparation, and data acquisition. In addition, delaying flow cytometric analyses until the following day, or even as many as 4 days after the mesh straining step described above, resulted in more reliable and less variable micronucleus frequencies.

10. Store the remaining **HBSS + FBS** in the refrigerator.

10. Simultaneous Cell Lysis and Nucleic Acid Dye Staining

1. On the day of flow cytometric analysis, gently tap the tubes to resuspend the cells. Add 5 mL cold **HBSS + FBS** to the tubes and mix gently by pipetting.

2. Centrifuge for 2 minutes at 200 x *g*. If the supernatant appears cloudy or dark in color, an additional rinse is recommended. Aspirate the supernatant and tap the tube to resuspend the pellet.

    2.1. If performing an additional rinse, add 5 mL cold **HBSS + FBS** to the tube and mix gently by pipetting. Centrifuge the tube for 2 minutes at 200 x *g*. Aspirate the supernatant and tap the tube to resuspend.

3. Add 2 mL cold **HBSS + FBS** to the tubes and mix gently by pipetting.

4. Transfer 100 µL of diluted cells to fresh flow cytometry tubes and keep at ambient temperature.

5. Slowly add 400 µL of ambient temperature **Complete Lysis Solution 1** to the first flow cytometry tube, rinsing down the sides of the tube to ensure that all cells come into contact with the solution. You can hold the tube in your hand and gently rotate while adding the solution over approximately 10 to 15 seconds.

6. Vortex the tube containing cells at a low speed for about 5 seconds and repeat with remaining samples.

7. Protect from light and incubate at ambient temperature for 30 minutes.

8. After incubation, forcefully add 400 µL of ambient temperature **Complete Lysis Solution 2** to the first flow cytometry tube and vortex at low speed for about 5 seconds. Repeat with remaining samples.

9. Protect from light and maintain at ambient temperature for at least 5 minutes, but not more than 3 hours, before flow cytometric analysis.

11. Flow Cytometric Analysis

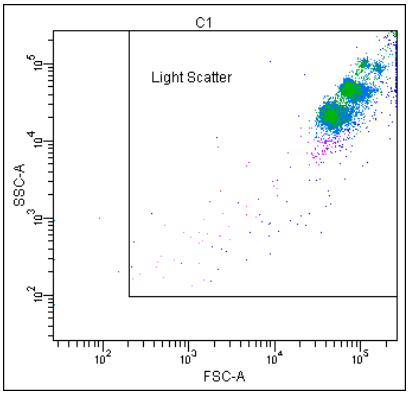
1. Before analyzing samples, ensure that the flow cytometer is working properly. Follow the manufacturer's instructions for the appropriate setup and quality control procedures. Open the template file or create one following the guidance in Appendix A.
    - If using FACSDiva™ software, perform the following steps:
        a. Locate the desired FACSDiva™ template (.xml file).
        b. Open the following folders on your computer: My computer > New Volume(D) > BDExport > Templates > Experiment > General.
        c. Drag the template into the General folder.
        d. Close this window and start the FACSDiva™ software.
        e. 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.
    - If using an alternative software, create a data acquisition and analysis template. See Appendix A, page 14.

2. Protect samples from light and maintain at ambient temperature.

3. Ensure that the sample is a homogeneous suspension by VERY GENTLY pipetting up and down several times.

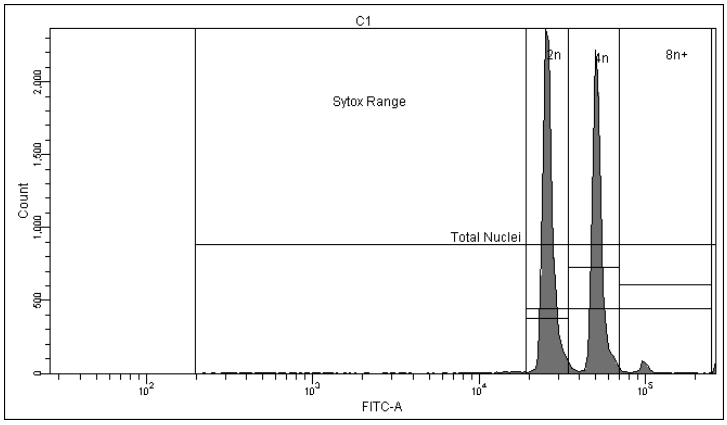
4. Place a sample on the flow cytometer. Adjust FSC and SSC voltages to bring nuclei into view in Plot A. The lower bounds of the region should be approximately 2 logs lower in FSC and SSC than the bottom left edge of the nuclei events, as shown here.

Plot A



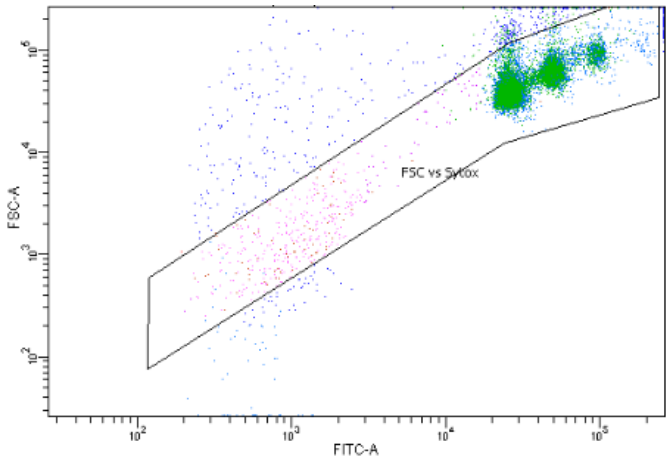
5. Adjust the FL1 PMT voltage so that 2n, 4n and 8n+ nuclei fall within the markers set in Histogram D (that is, within a consistent green fluorescence channel). Set the threshold parameter (also referred to as the acquisition trigger) to FL1 fluorescence (SYTOX® Green: FITC). Adjust the threshold so that the cells collected fall within the FL1 range that is defined below (i.e., nuclei as well as sub-2n chromatin that exhibit up to 1/100th the SYTOX® fluorescence signal of 2n nuclei should be included in the Sytox Range region).

Histogram D



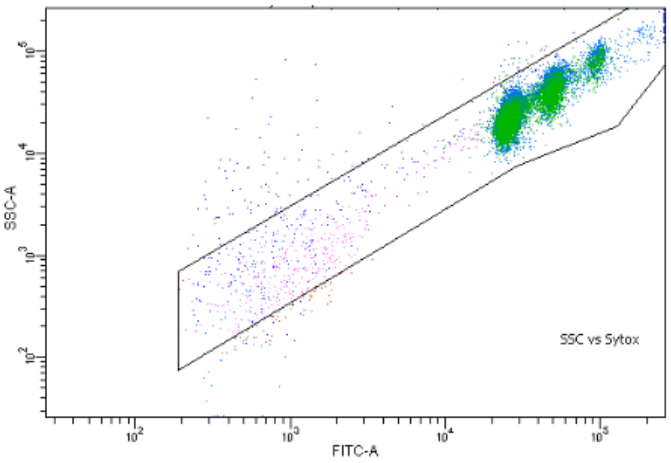
6. Much of the debris will fall above an appropriately located “FSC versus Sytox” region in Plot B. Adjust the location of the region if necessary to include nuclei but exclude debris. This is best observed with a positive control sample, as seen below.

Plot B



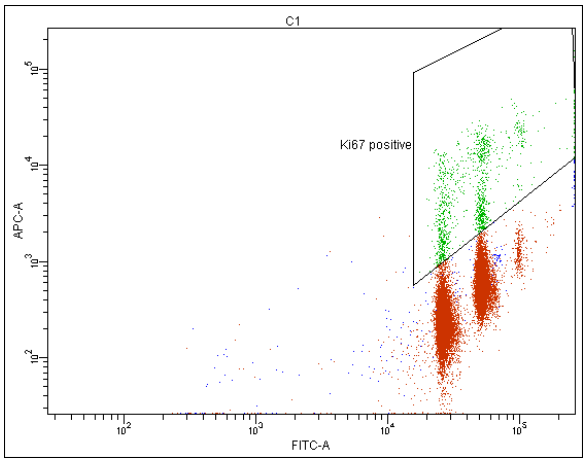
7. Much of the debris will fall above an appropriately located “SSC versus Sytox” region in Plot C. Adjust the location of the region if necessary to include nuclei but exclude debris. This is best observed with a positive control sample, as seen below.

Plot C



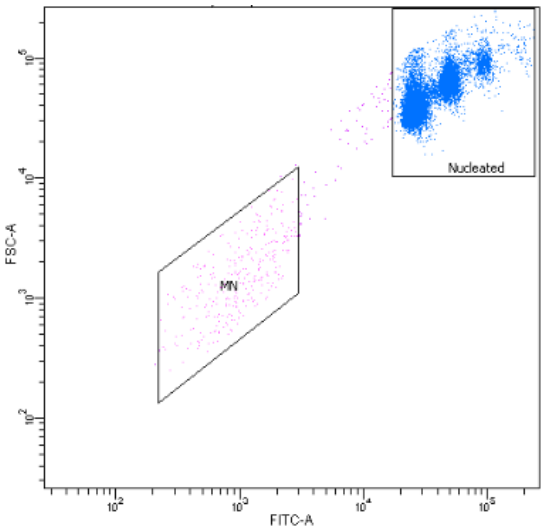
8. Adjust the APC PMT voltage so that the Ki-67 positive nuclei fall within the region set in Plot F. Ensure the majority of the population is below the region.

Plot F



9. Set a stop mode based on the number of events in the “Nucleated” region defined in Plot E. This number is typically set for at least 20,000 SYTOX® Green-positive nuclei per specimen. Adjust the locations of the regions if necessary. It is best to set the position of the regions with a positive control sample, as seen below. Acquire the desired number of events and save.

Plot E



10. Repeat steps 4 through 10 for remaining samples. Adjustment of the FL1 PMT voltage may be needed for each sample, but the position of the regions should stay the same.

11. Micronucleus values can be expressed as frequency percent by dividing the number of events that fall within the "MN" region by the number of events that fall within the "Nucleated" region and multiplying by 100. With properly defined gates, these measurements exclude debris and other spurious events that are identified by their anomalous light scatter profile described in more detail in Appendix A.

12. Calculations

1. Percent MN

$$\%\ MN = \frac{\#Events\ MN}{\#Events\ Nucleated} \times 100$$

2. Percent Ki-67-positive nuclei

$$\%\ Ki\text{-}67\text{-}positive = \frac{\#Events\ Ki\text{-}67\ positive}{\#Events\ Nucleated} \times 100$$

3. Hepatocyte Proliferation Index (HPI)

$$HPI = \frac{(\#Events\ 2n) + (2 \times \#Events\ 4n) + (3 \times \#Events\ 8n)}{\#Events\ Nucleated}$$

13. References

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14. 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® PLUS-RL Liver Micronucleus Kit for the preparation and analysis of 25 samples.

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. This product is for *in vitro* research purposes only and is not intended for clinical or diagnostic use.

Appendix A – Template Preparation and Representative Plots

A FACSDiva™ v6.1 template file can be downloaded from Litron's website (www.LitronLabs.com). The following pages show actual screen images of the plots found on the FACSDiva™ v6.1 template (five bivariate graphs and one histogram). Flow cytometry operators who are not using BD software should find these pages valuable for constructing their own data acquisition and analysis template.

1. Create plots and regions as shown on the following pages.

2. Define the following regions:
   - "Light Scatter" in Plot A
   - "FSC vs. Sytox" in Plot B
   - "SSC vs. Sytox" in Plot C
   - "Sytox Range" and "Total Nuclei" in Plot D
   - "MN" and "Nucleated" in Plot E
   - "Ki67 Positive" in Plot F

3. Specify the following gates based on the following regions:

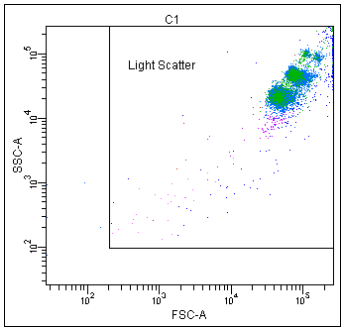
Tube: C1

Population	#Events	%Parent	%Total
All Events	20,411	####	100.0
Light Scatter	20,406	100.0	100.0
Sytox Range	20,411	100.0	100.0
Light Scatter AND Sytox Range	20,406	100.0	100.0
FSC vs Sytox	20,122	98.6	98.6
SSC vs Sytox	20,114	100.0	98.5
Nucleated	20,000	99.4	98.0
MN	26	0.1	0.1
Ki67 positive	1,250	6.1	6.1
2n	10,418	51.0	51.0
4n	9,260	45.4	45.4
8n+	414	2.0	2.0
Total Nuclei	20,093	98.4	98.4

4. Set a stop mode based on the number of events in the "Nucleated" region defined in Plot E. This number is typically set for at least 20,000 SYTOX® Green-positive nuclei per specimen.

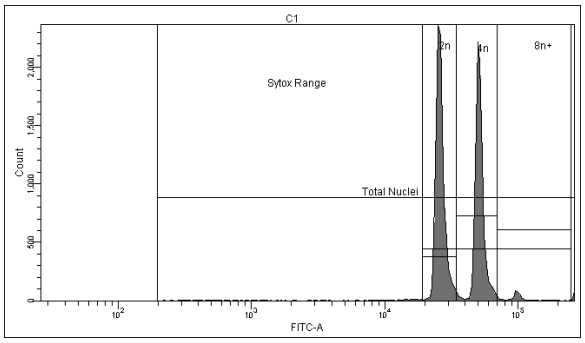
5. It is important not to be too restrictive with the "Light Scatter" region in Plot A, as MN could be excluded based on their small size. Therefore, the lower bounds of the region should be approximately 2 logs lower in FSC and SSC than the bottom left edge of the nuclei events, as shown here.

Plot A



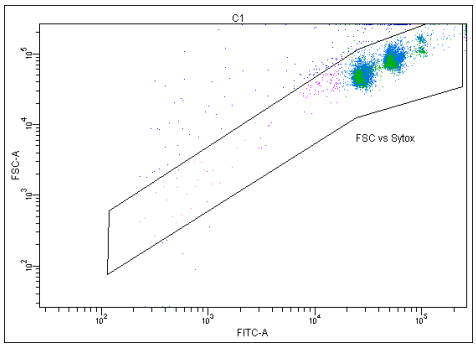
6. The "Sytox Range" region on Histogram D should include nuclei as well as sub-2n chromatin that exhibit up to 1/100th the SYTOX® fluorescence signal of 2n nuclei.

Histogram D



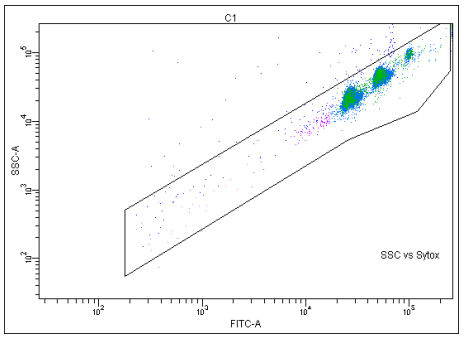
7. Much of the debris will fall above an appropriately located “FSC versus Sytox” region in Plot B.

Plot B



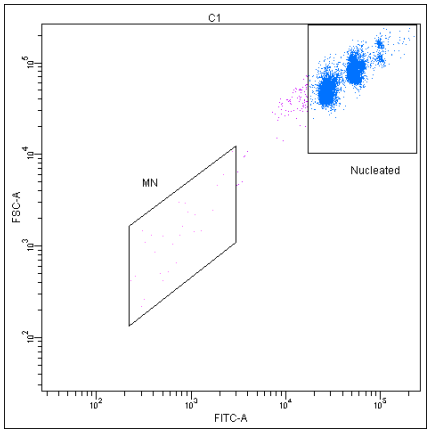
8. Much of the debris will fall above an appropriately located “SSC versus Sytox” region in Plot C.

Plot C



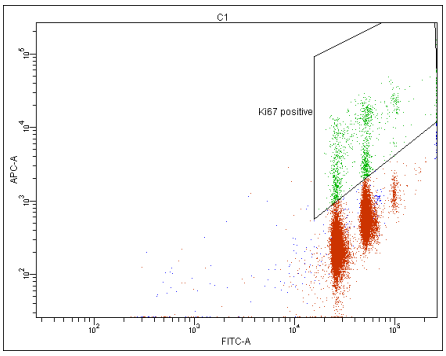
9. On Plot E, only nuclei and micronuclei that fall within the gating strategy described are used to calculate MN frequency. Ensure the micronuclei gate contains events that are 1/10th to 1/100th of the 2n population, both in FSC and FITC.

Plot E



10. Plot F shows the region used to enumerate the proportion of nuclei that exhibit anti-Ki-67-associated fluorescence as an indicator of cell proliferation.

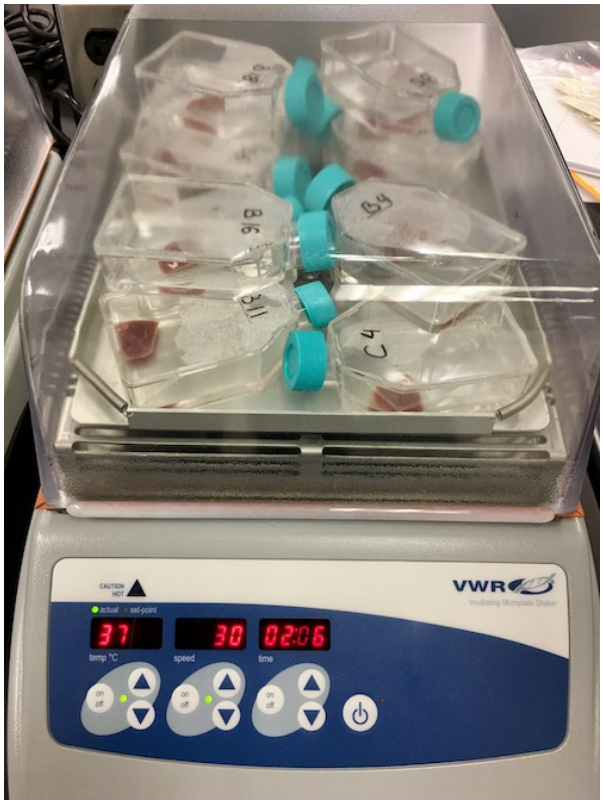
Plot F



Appendix B – Equipment Needed

Figure B-1: A Vortex Genie 2 vortex mixer fitted with a vertical 50 mL tube holder with a 6 50 mL tube capacity.

Figure B-2: A VWR incubating microplate shaker incubating 12 livers in individual T25 flasks at 37 °C and 130 rpm.



Appendix C – Representative Data

Representative data are shown for 6 week old male Sprague Dawley rats exposed to the liver hepatocarcinogen diethylnitrosamine (DEN) at 0, 10, 20 and 40 mg/kg/day. N = 6 per group. Treatments occurred for three consecutive days, and left lateral lobes were collected 4 days after the last exposure. Tissue processing occurred according to MicroFlow instructions described herein.

The top graph shows a DEN-dependent increase in micronuclei. The lower two graphs show that DEN treatment resulted in a compensatory proliferative response, as indicated by the elevated frequency of Ki-67-positive nuclei, and also a shift to nuclei with higher ploidy status.

Note that not all micronucleus-inducing agents cause this type of proliferative response. In fact, some chemicals will reduce the frequency of Ki-67-positive nuclei. This reinforces the value of making these liver proliferation assessments in conjunction with micronucleus frequency determinations, as they help with interpretation of results.