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Quality Counts!



**Micronucleus
Analysis
Kit**



MicroFlow (In Vitro, Standard)

Instruction Manual

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

READ ME FIRST

In order to assure high quality results, pay particular attention to:

- **The health of your cells**

Assay performance may be compromised unless your cell culture practices consistently provide optimally growing cultures with **minimal numbers of non-dividing or dying cells and consistent doubling times between routine passages**. For instance, strict attention to maximum allowable densities should be followed. We have found that a good rule of thumb is to maintain suspension cultures below 1×10^6 cells/ml and attachment cultures at less than 80 % confluency.

- **Cytotoxicity assessments**

In order to minimize the occurrence of false positive results, it is important to use a sensitive measure of cytotoxicity. The method of choice for flow cytometry-based micronucleus analyses is relative survival based on the use of Counting Beads, a reagent that is described in more detail herein. First-time users are strongly advised to read Sections 3.3.3 and 3.4 in order to choose the cytotoxicity assessment method that best fits their experimental designs.

Table Of Contents

1. Materials Provided.....	3
2. Additional Materials Required.....	3
3. Recommended Reading for First-Time Users.....	4
3.1. Introduction to MicroFlow.....	4
3.2. High Content Analysis	4
3.3. General Advice	5
3.4. Choosing a Cytotoxicity Method	7
4. Reagent Preparation	8
5. Suspension Cells.....	9
5.1. Basic Protocol.....	9
5.2. High Content Protocol 1.....	11
5.3. High Content Protocol 2.....	13
6. Attachment Cell Protocol	15
Appendices	
Appendix A: Template Preparation and Representative Plots	17
Appendix B: Flow Cytometric Analysis Recommendations	23
Appendix C: Tube Placement During Nucleic Acid Dye A Photoactivation	26
Appendix D: Mode of Action Signatures (CHO-K1 Cells).....	27
Appendix E: References, Ordering Information, and Technical Services	29

1. Materials Provided

50 Sample Kit Components	Quantity	Storage Condition
Incomplete Lysis Solution 1	30 ml	2 °C to 8 °C
Incomplete Lysis Solution 2	30 ml	2 °C to 8 °C
Nucleic Acid Dye A (Ethidium monoazide, EMA)	0.180 ml	–10 °C to –30 °C, light sensitive
Nucleic Acid Dye B (SYTOX® Green nucleic acid stain)	0.230 ml	–10 °C to –30 °C, light sensitive
RNase Solution	0.140 ml	–10 °C to –30 °C
10x Buffer	30 ml	Ambient

200 Sample Kit Components	Quantity	Storage Condition
Incomplete Lysis Solution 1	120 ml	2 °C to 8 °C
Incomplete Lysis Solution 2	120 ml	2 °C to 8 °C
Nucleic Acid Dye A (Ethidium monoazide, EMA)	0.720 ml	–10 °C to –30 °C, light sensitive
Nucleic Acid Dye B (SYTOX® Green nucleic acid stain)	0.920 ml	–10 °C to –30 °C, light sensitive
RNase Solution	0.560 ml	–10 °C to –30 °C
10x Buffer	120 ml	Ambient

400 Sample Kit Components	Quantity	Storage Condition
Incomplete Lysis Solution 1	240 ml	2 °C to 8 °C
Incomplete Lysis Solution 2	240 ml	2 °C to 8 °C
Nucleic Acid Dye A (Ethidium monoazide, EMA)	1.5 ml	–10 °C to –30 °C, light sensitive
Nucleic Acid Dye B (SYTOX® Green nucleic acid stain)	1.9 ml	–10 °C to –30 °C, light sensitive
RNase Solution	1.2 ml	–10 °C to –30 °C
10x Buffer	240 ml	Ambient

2. Additional Materials Required

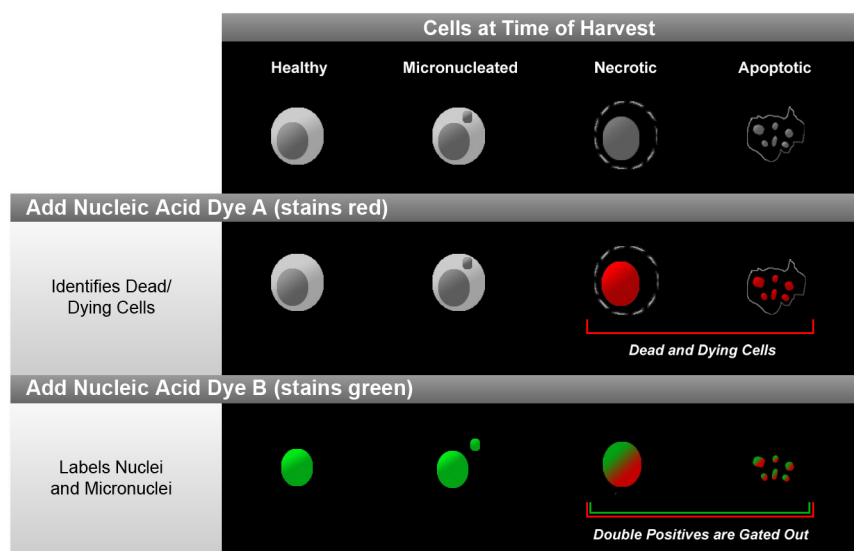
- **6 micron fluorescent latex microspheres are needed for High Content Protocols 1 and 2, as well as the Attachment Cell Protocol (Life Technologies, cat. no. C-16508, are recommended)**
- Cell line L5178Y, TK6, V79, or CHO-K1 (additional cell lines under review)
- Sterile fetal bovine serum (FBS)
- Filter-sterilized deionized water (dH₂O)
- Freezer (approximately –10 °C to –30 °C)
- Refrigerator (approximately 2 °C to 8 °C)
- Centrifuge
- CO₂-regulated, 37 °C incubator
- Flow cytometer capable of 488 nm excitation
- Flow cytometry tubes
- Disposable pipettes sized 5, 10, 25, and 50 ml
- Vortex Mixer
- Light source to photoactivate Nucleic Acid Dye A (fluorescent or incandescent; fluorescent is preferred because it is cooler)
- Polypropylene centrifuge tubes (for instance 15 ml)
- Micropipettors (20 µl - 1000 µl) and tips
- Cell culture flasks and/or microwell plates; additional materials for maintaining a cell line

3. Recommended Reading for First-Time Users

The *In Vitro* MicroFlow Kit was developed for the flow cytometric enumeration of micronuclei (MN) in cultured mammalian cells. It is an effective and rapid tool for evaluating the genotoxic activity of chemicals. This manual describes procedures for scoring MN using a 2-color labeling technique.

3.1. Introduction to MicroFlow

There has been a growing consensus that the *in vitro* MN assay offers significant benefits over traditional chromosome aberration (CA) assays. Whereas CA assays detect structural chromosome damage, the MN endpoint is responsive to both structural and numerical alterations. Furthermore, the MN assay has much higher throughput capacity than CA analysis – an advantage that is significantly enhanced now that MN scoring has been successfully automated using flow cytometry.



The advantage of the *In Vitro* MicroFlow method relative to other automated scoring procedures is the use of sequential staining that results in the differentiation of MN and the chromatin of apoptotic and necrotic cells. Therefore, reliable MN measurements are obtained even when appreciable numbers of dead cells are present.

A key component of the kit is Nucleic Acid Dye A (i.e., ethidium monoazide, or EMA), a reagent that crosses the compromised outer membrane of apoptotic and necrotic cells. A unique property of this dye is that it is covalently bound to DNA through photoactivation. Thereafter, cells are washed and the cytoplasmic membranes are digested with detergent to liberate nuclei and MN. During the lysis step, Nucleic Acid Dye B (i.e., SYTOX Green) is introduced which labels all chromatin. In this way, differential staining of healthy chromatin versus that of dead/dying cells is achieved. A diagram of this staining strategy is shown here.

3.2. High Content Analysis

The *In Vitro* MicroFlow kit is a high information content assay. That is, in addition to MN measurements, several valuable endpoints can be acquired simultaneously. These additional endpoints provide cytotoxicity information that is extremely valuable for setting appropriate top concentrations of test article, and also for interpreting the context in which MN induction occurs. These endpoints are:

- **Relative Survival:** 6 micron microspheres can be added at either of several stages of the cell processing steps. By scoring these "Counting Beads" on the flow cytometer, the number of healthy nuclei (i.e., EMA-negative) per bead can be determined. From these Nuclei-to-Bead ratios, relative survival values can be calculated. The advantage of these flow cytometry-derived relative survival measurements is that they represent a multi-parametric means of evaluating cell health. Indeed, this method has been found to reveal cytotoxicity that other cell scoring methods, such as Coulter counts, tend to underestimate.
- **Cell Cycle Information:** Test article-induced perturbations to the cell cycle are apparent by studying histograms of Nucleic Acid Dye B (SYTOX Green) fluorescence. For instance, expected G2/M blocks following treatment with alkylating agents are readily observed.
- **Dead/dying cells:** The health of treated cells can be inferred from the percentage of particles that are stained with Nucleic Acid Dye A (EMA-positive). Since the fragmented nuclei of apoptotic cells can each form many such particles, this statistic is particularly sensitive to apoptosis.

- **Aneugenic Signatures:** In addition to the information on micronucleus formation provided by the *In Vitro* MicroFlow methodology, the use of certain cell lines can provide the user with an indication of genotoxic mode of action. These signatures can be assessed simultaneously with the standard analysis and do not require the use of additional reagents or supplementary procedures. **(See appendix D for examples of aneugenic and clastogenic profiles.)**

We are currently validating this approach and more information will be available in upcoming versions of this manual. If you would like to discuss this sooner, please contact us directly at (585) 442-0930 or at info@litronlabs.com.

3.3. General Advice

Please read the entire instruction manual before performing these procedures. If you have any questions, please contact Litron by calling (585) 442-0930, faxing us at (585) 442-0934, or sending an email to info@litronlabs.com.

Deviating from the procedures described in this manual may adversely affect the results of your assay. Substitution of kit components, changes in temperature conditions, incubation times, reagent volumes, etc., is not advisable. While it is possible to scale up experiments as necessary, we recommend that no more than 30 samples are analyzed per day until you are proficient at performing the processing and analysis procedures.

3.3.1. Flow Cytometer Considerations

The assay is performed with a flow cytometer capable of providing 488 nm excitation. Standard factory-installed filter sets are typically sufficient to achieve fluorescent resolution of the relevant cell populations. In most cases, the red (Nucleic Acid Dye A) fluorescence should be collected in the FL3 channel, and the green (Nucleic Acid Dye B) fluorescence should be collected in the FL1 channel.

Litron's support web pages (www.litronlabs.com/support.html) provide CellQuest™ 3.3 and CellQuest™ Pro 5.2 templates, as well as a FACSDiva™ 6.1 template for data acquisition and analysis. Alternately, see "Template Preparation" on page 17 to create a new template file. As this template is relatively complex, it is advisable to create it before you are ready for cell analysis.

3.3.2. Cell Lines

This method was developed using mouse and human lymphoblastoid cell lines (L5178Y and TK6). More recent studies support the use of V79 and CHO-K1 cells. In all cases, optimal cell health is critical for *in vitro* micronucleus assays. A good rule of thumb is to maintain suspension cultures below 1×10^6 cells/ml and attachment cultures at less than 80 % confluency.

3.3.3. Cytotoxicity Measurement Strategies

Relative survival is a common approach for assessing cytotoxicity and thus for setting top test article concentrations. For instance, a common strategy requires treated cell cultures to exhibit relative survival of 50 % or greater, otherwise the treatment is deemed too cytotoxic for the MN endpoint. However, it is known that the manner by which cell counts are made can significantly affect relative survival values. When relative survival measurements are accomplished using methods that do not thoroughly address the presence of dead and dying cells, test article-induced cytotoxicity can be significantly underestimated. This leads to test article concentrations that are inappropriately high for the MN assay, and may increase the occurrence of false positive results.

This manual describes four strategies for accomplishing cytotoxicity measurements: the Basic Protocol, High Content Protocol 1, High Content Protocol 2, and Attachment Cell Protocol. The advantage of the latter three protocols is that they incorporate Counting Beads into the analyses.

Section 3.4 provides information that will be helpful for choosing a cytotoxicity measurement strategy that best fits your needs. Briefly, we recommend a sensitive method that is accomplished simultaneously with the MN measurement. As described in High Content Protocol 1, High Content Protocol 2, and Attachment Cell Protocol, latex microspheres (Counting Beads) are added to cell cultures prior to flow cytometric analysis. Nuclei-to-Bead Ratios are then used to calculate relative survival values. This is a sensitive measure of cytotoxicity, as the nuclei must exhibit the light scatter characteristics and fluorescence profile of healthy cells. **This multi-parametric assessment often reveals toxicity that is not detected by other techniques.**

3.3.4. Criteria for Micronucleus Scoring

The *In Vitro* MicroFlow sequential staining procedures are used in conjunction with other cell culture and flow cytometry parameters to ensure that reliable micronucleus measurements are obtained. At a minimum, we recommend using the following criteria to guard against false positive results:

1. Relative survival should be greater than or equal to 50 %.
2. MN events must exhibit 1/100th to 1/10th the SYTOX Green fluorescent intensity of 2n nuclei (page 21, Plot H).
3. MN events must fall within a Forward Light Scatter versus SYTOX Green fluorescence region (page 20, Plot E).
4. MN events must fall within a Side Light Scatter versus SYTOX Green fluorescence region (page 20, Plot F).
5. MN events must be EMA-negative (see page 21, Plot G). As discussed in section 3.2, this helps to further exclude apoptotic and necrotic chromatin from analysis. Even so, caution should be exercised when interpreting MN data for test article concentrations that are associated with high percentages of EMA-positive events. Under these circumstances, the MN scoring region may be contaminated by apoptotic bodies.

3.3.5. Treatment Conditions

Please refer to the most recent guidance documents (OECD, Kirsch-Volders, *et. al.*) but in general:

Short-term treatment (with or without metabolic activation) is achieved by exposing logarithmically dividing cells to test article for 3 to 6 hours. After exposure, cells are washed and transferred to fresh growth medium for a duration of time that approximates **1.5 to 2 normal cell cycles**. At this time, cells are harvested and processed for MN scoring.

Long-term treatment (without metabolic activation) is accomplished by exposing logarithmically dividing cells to test article for a duration of time that approximates 1.5 to 2 normal cell cycles. At this time, cells are harvested and processed for MN scoring.

The use of cytochalasin B is advised when performing MN assays with mitogen-stimulated primary lymphocytes. For cell lines that divide continuously in culture, most guidance documents suggest that this reagent is not required. **For standard cell line work, we recommend avoiding the use of cytochalasin B.**

3.3.6. Limit Concentration

Please refer to the most recent guidance documents (OECD, Kirsch-Volders, *et. al.*), but a generally accepted principle is that test articles should be evaluated at concentrations that cause minimal toxicity, modest toxicity, and distinct toxicity.

Current recommendations suggest that distinct toxicity is indicated by approximately 50 % toxicity, for instance 50 % relative survival at the time of harvest (relative to concurrent solvent or negative control). The manner in which cell counts are made can affect relative survival values. We recommend using a sensitive method based on Counting Beads (see Sections 3.4.2 and 3.4.3).

For non-cytotoxic agents, the top concentration is usually specified by expert working groups and/or regulatory bodies. While *in vitro* genotoxicity assays have historically advised 0.01 M, 5 mg/ml or 5 µl/ml, which ever is the lowest, the reader should note that these values are currently under consideration by expert groups and therefore subject to change.

3.4. Choosing a Cytotoxicity Method

The *In Vitro* MicroFlow method is compatible with several different approaches for evaluating test article-related cytotoxicity. The information provided below is designed to give you the information necessary to select the cytotoxicity method that best suits your experimental design.

3.4.1. Suspension Culture: Basic Protocol

This protocol is appropriate for suspension cell lines. Since Counting Beads are not included in this protocol, it may be useful when one is multiplexing the *In Vitro* MicroFlow kit with other assays that are expected to be adversely affected by the presence of latex microspheres. Caution must be exercised when using this protocol, since the use of cytotoxicity assessments that are less sensitive than the Counting Bead approach can lead to an increased incidence of false positive results when inappropriately high test article concentrations are evaluated.

3.4.2. Suspension Culture: High Content Protocols

High Content Protocols provide an additional, sensitive cytotoxicity measurement that is not available with the Basic Protocol. With these methods, Nuclei-to-Bead Ratios are acquired simultaneously with MN values and are used to derive relative survival values. Compared to standard approaches for measuring relative survival, for instance via Coulter counts, this method has a higher sensitivity that guards against the selection of inappropriately high test article concentrations.

High Content Protocol 1

Latex microspheres (Counting Beads) are added to suspension cell cultures just prior to cell lysis step. Since Counting Beads are not present throughout all cell processing steps, this method enables the user to multiplex the micronucleus endpoint with other assays, for instance those that examine cytotoxicity by other means. Thus, High Content Protocol 1 may be valuable to first-time users who are interested in comparing Nuclei-to-Bead Ratios to cytotoxicity assessments that they have historically relied upon.

High Content Protocol 2

Latex microspheres (Counting Beads) are added to suspension cell cultures prior to treatment. These beads remain in place throughout the cell processing steps. As Counting Beads are present throughout the processing steps, this can be especially useful when centrifugation and aspiration of cells will occur before the ultimate cell harvest (e.g., when S9 metabolic activation is employed). Since Counting Beads are present throughout all processing steps, this protocol may be problematic for users that are multiplexing the micronucleus endpoint with other assays.

3.4.3. Attachment Cell Protocol

The use of attachment cell lines offer certain advantages, including the elimination of centrifugation steps. By culturing cells in multi-well plates, treatment and washing steps can occur in the same vessel by simply aspirating the various supernatants and replacing them with the appropriate reagents.

Likewise, the Attachment Cell Protocol that we recommend is very efficient, as it does not require trypsinization or centrifugation steps. Rather, *In Vitro* MicroFlow reagents are added sequentially to cells while they are still attached to the growth vessel. The Counting Beads are included at the final processing step (i.e., addition of Lysis Solution 2), therefore no extra steps are required. This supplements MN measurements with Nuclei-to-Bead ratios, thereby providing a means of deriving relative survival measurements.

4. Reagent Preparation

Working solutions should be prepared fresh each day cell harvest and staining/lysis is performed. It is most practical to prepare these solutions, as described below, before cell harvest begins.

4.1. 1x Buffer Solution

Number of samples	Volume of dH ₂ O	Volume of 10x Buffer	Volume of FBS
1	3.6 ml	0.4 ml	0.08 ml

1. Use the chart above to determine the volume of 1x Buffer Solution required. Scale up as necessary.
2. Add the required volumes of dH₂O, 10x Buffer and fetal bovine serum (FBS) to a clean vessel. Filter sterilize and refrigerate the resulting 1x Buffer Solution.

4.2. Nucleic Acid Dye A Working Solution

Number of samples	Volume of 1x Buffer Solution	Volume of Nucleic Acid Dye A
1	0.33 ml	3.3 µl

1. Use the chart above to determine the volume of Nucleic Acid Dye A Working Solution required. Scale up as necessary.
2. Combine the required volumes of 1x Buffer Solution and Nucleic Acid Dye A in a clean polypropylene vessel. Protect from light and store on ice until use.

4.3. Complete Lysis Solution 1

Number of samples	Volume of Incomplete Lysis Solution 1	Volume of Nucleic Acid Dye B	Volume of RNase Solution
1	0.55 ml	2.2 µl	2.75 µl

1. Use the chart above to determine the amount of Complete Lysis Solution 1 required. Scale up as necessary.
2. Combine the required volumes of Incomplete Lysis Solution 1 with Nucleic Acid Dye B and RNase Solution in a clean polypropylene vessel. Protect Complete Lysis Solution 1 from light and store at room temperature until use.

4.4. Complete Lysis Solution 2

Number of samples	Volume of Incomplete Lysis Solution 2	Volume of Nuclei Acid Dye B
1	0.55 ml	2.2 µl

1. Use the chart above to determine the amount of Complete Lysis Solution 2 required. Scale up as necessary.
2. Combine the required volumes of Incomplete Lysis Solution 2 with Nucleic Acid Dye B in a clean polypropylene vessel. Protect Complete Lysis Solution 2 from light and store at room temperature until use.

5. Suspension Cells

5.1. Basic Protocol

5.1.1. Cell Harvest

1. Remove treatment cultures from the incubator and determine cell density. NOTE: Only cultures that have not been treated with overly cytotoxic concentrations of test article should be processed for micronucleus scoring.
2. Aliquot volume corresponding to 5×10^5 cells into 15 ml centrifuge tubes.
3. Bring cells up to a consistent volume with fresh growth medium.
4. Collect cells via centrifugation at $300 \times g$ for 5 minutes.
5. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet.
6. Gently tap the tubes to resuspend the cells, being careful not to splash the cells too high in the tube.
7. Place samples on ice for 20 minutes before continuing to Section 5.1.2.

5.1.2. Nucleic Acid Dye A Staining

1. Add 300 μ l of Nucleic Acid Dye A Working Solution to each tube. Gently pipette up and down, making sure all cells come into contact with this solution. Leave caps off tubes.
2. Place samples in a small plastic or metal rack, taking care to submerge the tubes about 2 cm deep in ice. See Appendix C for a visual depiction.
3. Place source (cool white fluorescent light source is recommended) approximately 15 cm above the samples (i.e., this is about 5 cm above the top of standard 15 ml tubes; see Appendix C). Expose the samples to visible light for 30 minutes, with samples remaining on ice throughout this period of time.
4. Turn off the visible light source and add 3 ml of cold 1x Buffer Solution to each sample. From this point forward, limit the exposure of samples to light (e.g., cover tubes with foil).
5. Collect cells via centrifugation at $300 \times g$ for 5 minutes.
6. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet.
7. Gently tap the tubes to resuspend the cells, being careful not to splash the cells too high in the tube. Proceed immediately with section 5.1.3.

5.1.3. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Add 500 μ l of Complete Lysis Solution 1 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. (Care should be taken not to vortex at too high of speeds. Data indicates metaphase chromosomes can break loose at these higher speeds).
2. Repeat for each of the remaining samples.
3. Incubate the samples in Complete Lysis Solution 1 for one hour at room temperature protected from light.
4. Add 500 μ l of Complete Lysis Solution 2 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds.
5. Repeat step 4 for each of the remaining samples.
6. Incubate the samples in Complete Lysis Solution 2 for 30 minutes at room temperature, protected from light.
7. Stained samples should be protected from light until flow cytometric analysis occurs. These samples can be stored at either room temperature for up to 24 hours, or refrigerated for up to 72 hours before analysis.

5.1.4. 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 from the website, or create a template following recommendations found in Appendix A. If creating a template, do this prior to cell harvest.
2. Protect samples from light. If necessary, retrieve stained samples from the refrigerator and allow samples to equilibrate to room temperature (30 minutes is usually sufficient).
3. Ensure that the sample is a homogeneous suspension by VERY GENTLY pipetting up and down several times. Transfer one stained sample to a flow cytometry tube just prior to analysis. Note that this transfer step should be accomplished without vortexing and without vigorous mixing.
4. Place sample on flow cytometer. Set the threshold parameter (also referred to as the acquisition trigger) to FL1 fluorescence (SYTOX Green). Adjust the threshold so that some cells are collected that fall just to the left edge of the FL1 range that is defined in Appendix B Plot C. For those instruments that are capable of thresholding on two parameters, a second parameter (either SSC or FSC) is recommended. Note that when a light scatter secondary threshold is used, it is important not to set the value too high, otherwise micronuclei will be excluded. Use the lower bounds of the "Light Scatter" region shown in appendix A as a guide.
5. As described in more detail in Appendix B Plot C, it may be desirable to make modest adjustments to the FL1 PMT voltage (SYTOX Green-associated PMT) so that G1 nuclei fall in a consistent green fluorescence channel.
6. Acquire desired number of events and save. Setting the stop mode so that 10,000 to 20,000 "healthy" nuclei (SYTOX Green-positive and EMA-negative nuclei) are acquired per sample is recommended.
7. Repeat steps 3 through 6 for remaining samples.
8. Micronucleus values can be expressed as 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 and/or EMA-positive staining characteristic (described in more detail in Appendices A and B).

Prior to analyzing experimental samples, it is recommended that you analyze negative control and positive control samples first to verify that the template and instrument settings are appropriate.

5.2. High Content Protocol 1

5.2.1. Cell Harvest

1. Remove treated cultures from the incubator. Mix each culture to achieve a homogeneous suspension then transfer a consistent volume of each into separate 15 ml centrifuge tubes. This consistent volume should be one that is estimated to provide approximately 5×10^5 cells of the negative control cultures.
2. Collect cells via centrifugation at $300 \times g$ for 5 minutes.
3. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet.
4. Gently tap the tubes to resuspend the cells, being careful not to splash the cells too high in the tube.
5. Place samples on ice for 20 minutes before continuing to Section 5.2.2.

5.2.2. Nucleic Acid Dye A Staining

1. Add 300 μ l of Nucleic Acid Dye A Working Solution to each tube. Gently pipette up and down, making sure all cells come into contact with this solution. Leave caps off tubes.
2. Place samples in a small plastic or metal rack, taking care to submerge the tubes about 2 cm deep in ice. See Appendix C for a visual depiction.
3. Place a light source (cool white fluorescent light source is recommended) approximately 15 cm above the samples (i.e., this is about 5 cm above the top of standard 15 ml tubes; see Appendix C). Expose the samples to visible light for 30 minutes, with samples remaining on ice throughout this period of time.
4. Add Counting Beads to 1x Buffer Solution. Do this by briefly sonicating and vortexing a stock bead suspension, then add approximately 1 drop of bead suspension per 10 ml. Mix the bead/lysis solution well after addition of the beads and just prior to use.

Green fluorescent beads (6 micron) from Life Technologies, catalog number C-16508, are recommended.
5. Turn off the visible light source and add 3 ml of cold 1x Buffer Solution to each sample. Mix 1x Buffer Solution between samples. From this point forward, limit the samples exposure to light (e.g., cover tubes with foil).
6. Collect cells via centrifugation at $300 \times g$ for 5 minutes.
7. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet.
8. Gently tap the tubes to resuspend the cells, being careful not to splash the cells too high in the tube. Proceed immediately with section 5.2.3.

5.2.3. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Add 500 μ l of Complete Lysis Solution 1 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. (Care should be taken not to vortex at too high of speeds. Data indicates metaphase chromosomes can break loose at these higher speeds).
2. Repeat for each of the remaining samples.
3. Incubate the samples in Complete Lysis Solution 1 for one hour at room temperature protected from light.
4. Add 500 μ l of Complete Lysis Solution 2 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds.
5. Repeat step 4 for each of the remaining samples.

6. Incubate the samples in Complete Lysis Solution 2 for 30 minutes at room temperature, protected from light.
7. Stained samples should be protected from light until flow cytometric analysis occurs. These samples can be stored at either room temperature for up to 24 hours, or refrigerated for up to 72 hours before analysis.

5.2.4. 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 from the website, or create a template following recommendations found in Appendix A. If creating a template, do this prior to cell harvest.
2. Protect samples from light. If necessary, retrieve stained samples from the refrigerator and allow samples to equilibrate to room temperature (30 minutes is usually sufficient).
3. Ensure that the sample is a homogeneous suspension by VERY GENTLY pipetting up and down several times. Transfer one stained sample to a flow cytometry tube just prior to analysis. Note that this transfer step should be accomplished without vortexing and without vigorous mixing.
4. Place sample on flow cytometer. Set the threshold parameter (also referred to as the acquisition trigger) to FL1 fluorescence (SYTOX Green). Adjust the threshold so that some cells are collected that fall just to the left edge of the FL1 range that is defined in Appendix B Plot C. For those instruments that are capable of thresholding on two parameters, a second parameter (either SSC or FSC) is recommended. Note that when a light scatter secondary threshold is used, it is important not to set the value too high, otherwise micronuclei will be excluded. Use the lower bounds of the "Light Scatter" region shown in appendix A as a guide.
5. As described in more detail in Appendix B Plot C, it may be desirable to make modest adjustments to the FL1 PMT voltage (SYTOX Green-associated PMT) so that G1 nuclei fall in a consistent green fluorescence channel.
6. Acquire desired number of events and save. Setting the stop mode so that 10,000 to 20,000 "healthy" nuclei (SYTOX Green-positive and EMA-negative nuclei) are acquired per sample is recommended.
7. Repeat steps 3 through 6 for remaining samples.
8. For each sample, determine the ratio of Nuclei-to-Counting Beads. Note that these nuclei should exhibit all the light scatter and fluorescence profiles associated with Plot H, Appendix A. Use these ratios to derive relative survival values. For instance, if solvent control samples average 20,000 nuclei to 400 beads, then this ratio of 20,000/400, or 50 to 1, is set to 100 %. Then, if a treated culture is observed to have 20,000 nuclei and 800 beads, the ratio of 20,000/800 or 25 to 1 would represent a 50 % relative survival value compared to control. These measurements represent a sensitive cytotoxicity assessment that can be used to either qualify or exclude data associated with treatment conditions that are too cytotoxic for the MN assay.
9. 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 100. With properly defined gates, these measurements exclude debris and other spurious events that are identified by their anomalous light scatter profile and/or EMA-positive staining characteristic (described in more detail in Appendices A and B).

Prior to analyzing experimental samples, it is recommended that you analyze negative control and positive control samples first to verify that the template and instrument settings are appropriate.

5.3. High Content Protocol 2

5.3.1. Cell Treatment

Combine all exponentially growing cells that will be needed in one vessel. Add latex Counting Beads to the vessel. The recommended ratio of nuclei to beads for negative control cultures at the time of cell harvest is approximately 50 cells per bead. This can usually be achieved by briefly sonicating and vortexing a stock bead suspension, and adding approximately 1 drop of bead suspension for every 10 ml cell culture. Mix the cells/bead suspension well and dispense across flasks or wells for treatment.

Green fluorescent beads (6 micron) from Life Technologies, catalog number C-16508, are recommended.

5.3.2. Cell Harvest

1. Based on seeding density and known growth characteristics of the cells, determine the volume of cell/bead suspension that is equivalent to approximately 5×10^5 cells of the negative control cultures. Transfer this consistent volume from each treated culture vessel into separate 15 ml centrifuge tubes.
2. Collect cells/beads via centrifugation at $300 \times g$ for 5 minutes.
3. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet.
4. Gently tap the tubes to resuspend the cells/beads, being careful not to splash the cells too high in the tube.
5. Place samples on ice for 20 minutes before continuing to Section 5.3.3.

5.3.3. Nucleic Acid Dye A Staining

1. Add 300 μ l of Nucleic Acid Dye A Working Solution to each tube. Gently pipette up and down, making sure all cells come into contact with this solution. Leave caps off tubes.
2. Place samples in a small plastic or metal rack, taking care to submerge the tubes about 2 cm deep in ice. See Appendix C for a visual depiction.
3. Place a light source (cool white fluorescent light source is recommended) approximately 15 cm above the samples (i.e., this is about 5 cm above the top of standard 15 ml tubes; see Appendix C). Expose the samples to visible light for 30 minutes, with samples remaining on ice throughout this period of time.
4. Turn off the visible light source and add 3 ml of cold 1x Buffer Solution to each sample. From this point forward, limit the samples exposure to light (e.g., cover tubes with foil).
5. Collect cells via centrifugation at $300 \times g$ for 5 minutes.
6. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet. Leave 50 μ l – 100 μ l of supernatant per tube.
7. Gently tap the tubes to resuspend the cells, being careful not to splash the cells too high in the tube. Proceed immediately with section 5.3.4.

5.3.4. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Add 500 μ l of Complete Lysis Solution 1 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. Care should be taken not to vortex at too high of speeds. Data indicates metaphase chromosomes can break loose at these higher speeds.
2. Repeat for each of the remaining samples.
3. Incubate the samples in Complete Lysis Solution 1 for one hour at room temperature protected from light.
4. Add 500 μ l of Complete Lysis Solution 2 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds.

5. Repeat step 4 for each of the remaining samples.
6. Incubate the samples in Complete Lysis Solution 2 for 30 minutes at room temperature, protected from light.
7. Stained samples should be protected from light until flow cytometric analysis occurs. These samples can be stored at either room temperature for 24 hours or refrigerated for up to 72 hours before analysis.

5.3.5. 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 from the website, or create a template following recommendations found in Appendix A. If creating a template, do this prior to cell harvest.
2. Protect samples from light. If necessary, retrieve stained samples from the refrigerator and allow samples to equilibrate to room temperature (30 minutes is usually sufficient).
3. Ensure that the sample is a homogeneous suspension by VERY GENTLY pipetting up and down several times. Transfer one stained sample to a flow cytometry tube just prior to analysis. Note that this transfer step should be accomplished without vortexing and without vigorous mixing.
4. Place sample on flow cytometer. Set the threshold parameter (also referred to as the acquisition trigger) to FL1 fluorescence (SYTOX Green). Adjust the threshold so that some cells are collected that fall just to the left edge of the FL1 range that is defined in Appendix B Plot C. For those instruments that are capable of thresholding on two parameters, a second parameter (either SSC or FSC) is recommended. Note that when a light scatter secondary threshold is used, it is important not to set the value too high, otherwise micronuclei will be excluded. Use the lower bounds of the "Light Scatter" region shown in appendix A as a guide.
5. As described in more detail in Appendix B Plot C, it may be desirable to make modest adjustments to the FL1 PMT voltage (SYTOX Green-associated PMT) so that G1 nuclei fall in a consistent green fluorescence channel.
6. Acquire desired number of events and save. Setting the stop mode so that 10,000 to 20,000 "healthy" nuclei (SYTOX Green-positive and EMA-negative nuclei) are acquired per sample is recommended.
7. Repeat steps 3 through 6 for remaining samples.
8. For each sample, determine the ratio of Nuclei-to-Counting Beads. Note that these nuclei should exhibit all the light scatter and fluorescence profiles associated with Plot H, Appendix A. Use these ratios to derive relative survival values. For instance, if solvent control samples average 20,000 nuclei to 400 beads, then this ratio of 20,000/400, or 50 to 1, is set to 100 %. Then, if a treated culture is observed to have 20,000 nuclei and 800 beads, the ratio of 20,000/800 or 25 to 1 would represent a 50 % relative survival value compared to control. These measurements represent a sensitive cytotoxicity assessment that can be used to either qualify or exclude data associated with treatment conditions that are too cytotoxic for the MN assay.
9. 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 100. With properly defined gates, these measurements exclude debris and other spurious events that are identified by their anomalous light scatter profile and/or EMA-positive staining characteristic (described in more detail in Appendices A and B).

Prior to analyzing experimental samples, it is recommended that you analyze negative control and positive control samples first to verify that the template and instrument settings are appropriate.

6. Attachment Cell Protocol

6.1. Cell Harvest

1. Treatment of attachment cell lines takes place in standard cell culture well plates of various formats, depending on your requirements. Plate the cells at a density that will achieve approximately 80 % confluency for vehicle control cultures at time of cell harvest.
2. Rather than removing a known quantity of cells from the culture vessel for the staining procedure, the entire staining process can take place in the individual wells. The steps described below are for cells cultured in a 24-well plate.

6.2. Nucleic Acid Dye A Staining

1. Remove treatment plate from incubator. Place the well plate on ice, being sure that the ice is making direct contact with the bottom of the entire plate. Cover the well plate and incubate for 20 minutes on the ice.
2. Following the ice incubation, aspirate off treatment media and add 300 μ l of Nucleic Acid Dye A working solution per well. Make sure that the entire cell surface is covered with this solution. Leave the plate cover off.
3. Place a light source (cool white fluorescent light source is recommended) approximately 15 cm above the samples. Expose the samples to visible light for 30 minutes, with samples remaining on ice throughout this period of time.
4. Turn off the visible light source, remove plates from ice and aspirate off Nucleic Acid Dye A solution.
5. Add 1 ml of cold 1x Buffer Solution to each well. Aspirate off the 1x Buffer Solution from each well. Proceed immediately with section 6.3.

6.3. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Add 500 μ l of Complete Lysis Solution 1 to each well. Gently mix the plate for 5 seconds on a vortex mixer set low enough not to splash the solution out of the wells.
2. Incubate the samples in Complete Lysis Solution 1 for one hour in the dark at 37 °C.
3. Add Counting Beads to Complete Lysis Solution 2. Do this by briefly sonicating and vortexing a stock bead suspension, then add approximately 1 drop of bead suspension per 10 ml of Complete Lysis Solution 2. Mix the bead/lysis solution well after addition of the beads and just prior to use. Protect these solutions from light, and ensure they are at room temperature before adding the solutions to samples.

Green fluorescent beads (6 micron) from Life Technologies, catalog number C-16508, are recommended.
4. Vortex or resuspend Complete Lysis Solution 2 and add 500 μ l to each well. Gently rock the plate.
5. Incubate the samples in Complete Lysis Solutions 2 for 30 minutes in the dark at room temperature.
6. Stained samples should be protected from light until flow cytometric analysis occurs. These samples can be stored at either room temperature for 24 hours or refrigerated for up to 72 hours.

6.4. 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 from the website, or create a template following recommendations found in Appendix A. If creating a template, do this prior to cell harvest.
2. Protect samples from light. If necessary, retrieve stained samples from the refrigerator and allow samples to equilibrate to room temperature (30 minutes is usually sufficient).
3. Ensure that the sample is a homogeneous suspension by VERY GENTLY pipetting up and down several times. Transfer one stained sample to a flow cytometry tube just prior to analysis. Note that this transfer step should be accomplished without vortexing and without vigorous mixing.
4. Place sample on flow cytometer. Set the threshold parameter (also referred to as the acquisition trigger) to FL1 fluorescence (SYTOX Green). Adjust the threshold so that some cells are collected that fall just to the left edge of the FL1 range that is defined in Appendix B Plot C. For those instruments that are capable of thresholding on two parameters, a second parameter (either SSC or FSC) is recommended. Note that when a light scatter secondary threshold is used, it is important not to set the value too high, otherwise micronuclei will be excluded. Use the lower bounds of the "Light Scatter" region shown in appendix A as a guide.
5. As described in more detail in Appendix B Plot C, it may be desirable to make modest adjustments to the FL1 PMT voltage (SYTOX Green-associated PMT) so that G1 nuclei fall in a consistent green fluorescence channel.
6. Acquire desired number of events and save. Setting the stop mode so that 10,000 to 20,000 "healthy" nuclei (SYTOX Green-positive and EMA-negative nuclei) are acquired per sample is recommended.
7. Repeat steps 3 through 6 for remaining samples.
8. For each sample, determine the ratio of Nuclei-to-Counting Beads. Note that these nuclei should exhibit all the light scatter and fluorescence profiles associated with Plot H, Appendix A. Use these ratios to derive relative survival values. For instance, if solvent control samples average 20,000 nuclei to 400 beads, then this ratio of 20,000/400, or 50 to 1, is set to 100 %. Then, if a treated culture is observed to have 20,000 nuclei and 800 beads, the ratio of 20,000/800 or 25 to 1 would represent a 50 % relative survival value compared to control. These measurements represent a sensitive cytotoxicity assessment that can be used to either qualify or exclude data associated with treatment conditions that are too cytotoxic for the MN assay.
9. 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 100. With properly defined gates, these measurements exclude debris and other spurious events that are identified by their anomalous light scatter profile and/or EMA-positive staining characteristic (described in more detail in Appendices A and B).

Prior to analyzing experimental samples, it is recommended that you analyze negative control and positive control samples first to verify that the template and instrument settings are appropriate.

Appendix A – Template Preparation and Representative Plots

A CellQuest™ template file has been included with this kit. This template is used for all the protocols described in this manual and the following pages show actual screen images of the plots found on the template (seven bivariate graphs and two histograms). The various gates utilized for these analyses are described below. Flow cytometry operators who are not using CellQuest™ software should find these pages valuable for constructing their own data acquisition and analysis template.

1. Create plots and regions as shown on pages 18 through 22.

2. Define gates based on the following regions:

Gate Region Name(s)

- G1 = "Light Scatter"
- G2 = "Doublet Discrimination"
- G3 = "FL1 Range"
- G4 = "SSC vs SYTOX"
- G5 = "FSC vs SYTOX"
- G6 = "Light Scatter" and "Doublet Discrimination" and not "Beads"
- G7 = "Light Scatter" and "Doublet Discrimination" and "FL1 Range" and not "Beads"
- G8 = "Light Scatter" and "Doublet Discrimination" and "FL1 Range" and "FSC vs SYTOX" and "SSC vs SYTOX" and not "Beads"
- G9 = "EMA Positive"
- G10 = "Light Scatter" and "Doublet Discrimination" and "FL1 Range" and "FSC vs SYTOX" and "SSC vs SYTOX" and not "EMA Positive" and not "Beads"
- G11 = "Nucleated" and "Light Scatter" and "Doublet Discrimination" and "FL1 Range" and "FSC vs SYTOX" and "SSC vs SYTOX" and not "EMA Positive" and not "Beads"
- G12 = "Beads"
- G13 = not "Beads"

Please note, the following terms are used interchangeably:

Nucleic Acid Dye A = EMA
Nucleic Acid Dye B = SYTOX

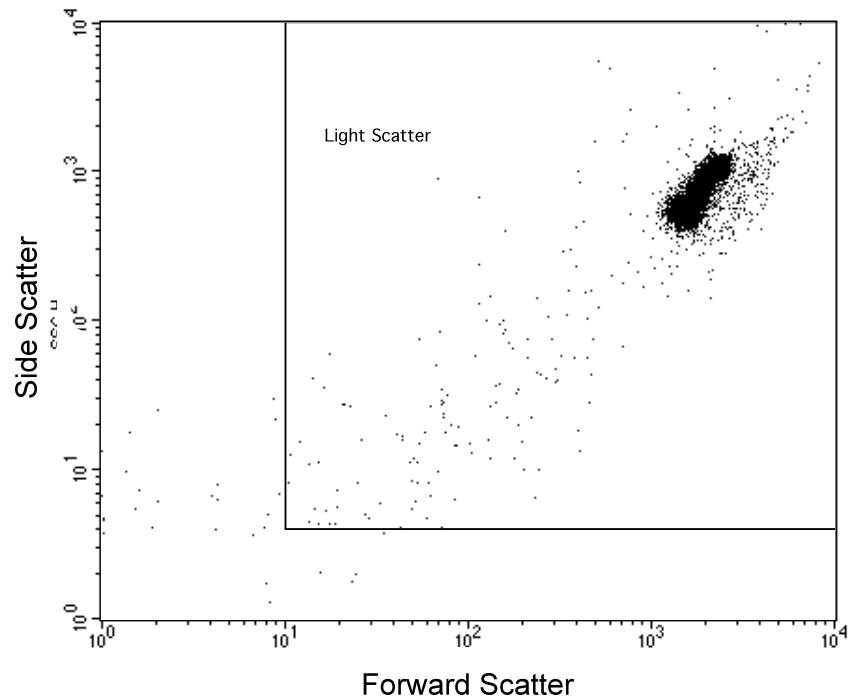
3. Specify the following gates for each plot:

Plot	Gate	Region Stats
Plot A	G13	
Plot B	No Gate	
Plot C	G6	
Plot D	G11	
Plot E	G7	
Plot F	G7	
Plot G	G7	Obtain EMA-positive frequency from Plot G
Plot H	G10	Obtain MN and Nuclei numbers from Plot H
Bead Count Plot	No Gate	Obtain Bead number from Bead Count Plot

4. Use Gate G11 to set the stop mode. This will allow you to stop data acquisition at a specified number of nuclei. For instance, we routinely acquire 10,000 to 20,000 SYTOX-Positive/EMA-negative nuclei per sample.
5. Save this template file. This file should be a good first approximation of a template that you will find useful for quantifying MN in suspension cell lines.
6. Note that data collected in the Bead Count Plot is used only in the High Content Protocols 1 and 2, as well as the Attachment Cell Protocol. This plot provides the bead number that is used to calculate cytotoxicity via the Nuclei-to-Bead Ratio method.

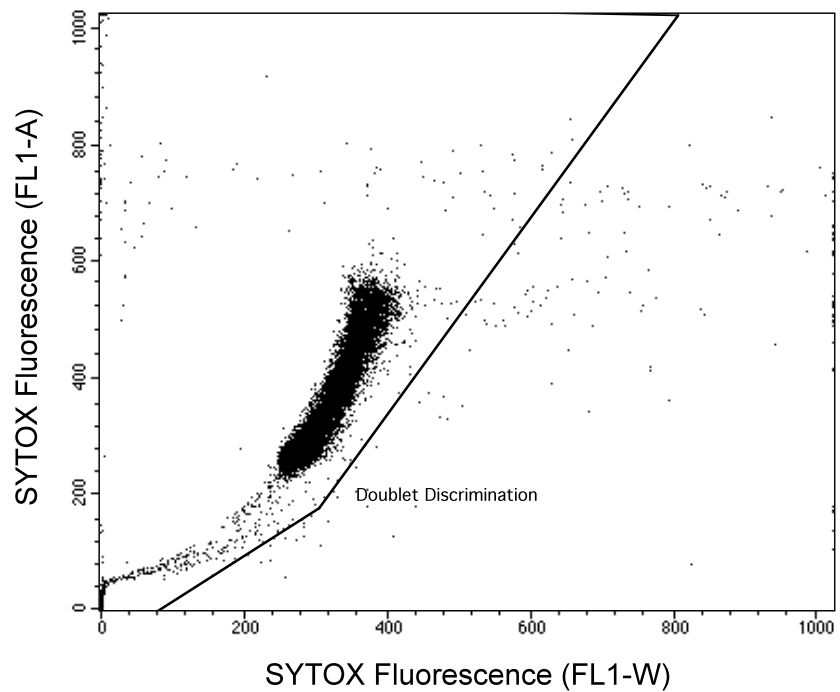
PLOT A

NOTE: It is important not to be too restrictive with the “Light Scatter” region, 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 shown here.

**PLOT B**

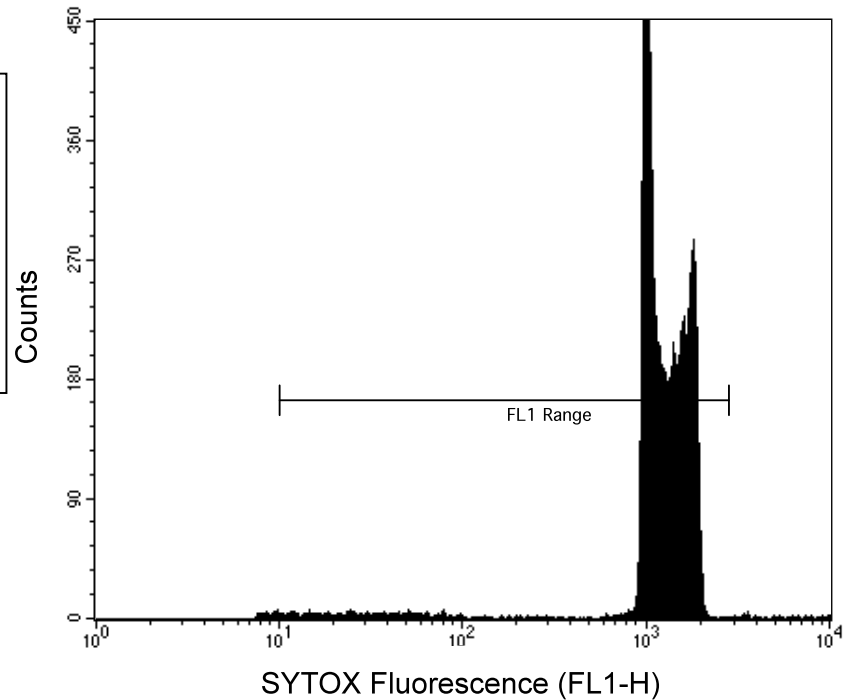
NOTE: Doublet Discrimination may not be available for all flow cytometer models.

NOTE: Make sure the Doublet Discrimination region extends all the way to the zero channel of the X- and Y-axis or MN events may be excluded.

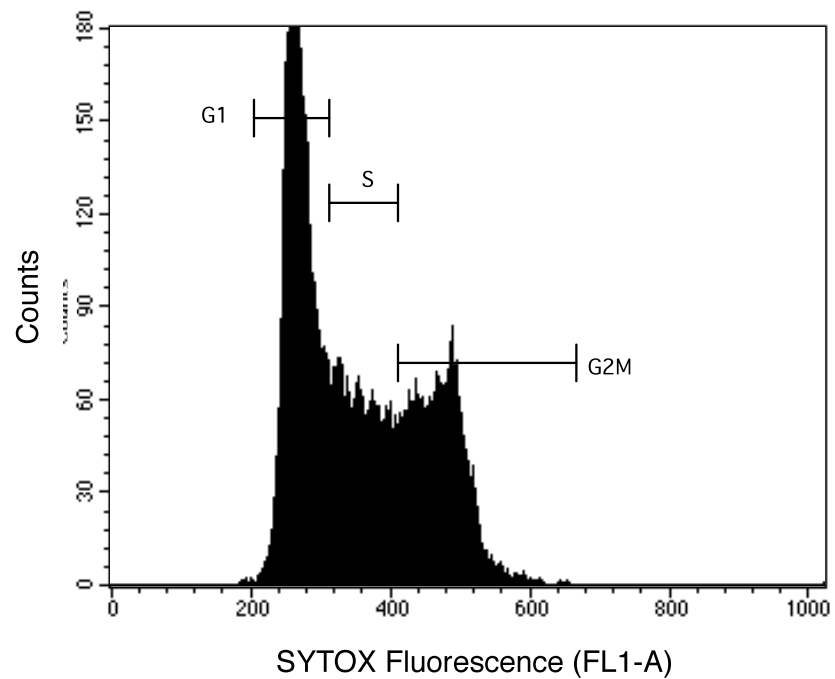


PLOT C

NOTE: SYTOX fluorescence (FL1) PMT voltage is adjusted so that G1 nuclei peak falls at approximately the 10^3 log line. Based on this position, the "FL1 Range" region will include nuclei as well as sub 2n chromatin that exhibit up to $1/100^{\text{th}}$ the SYTOX fluorescence signal as 2n nuclei.

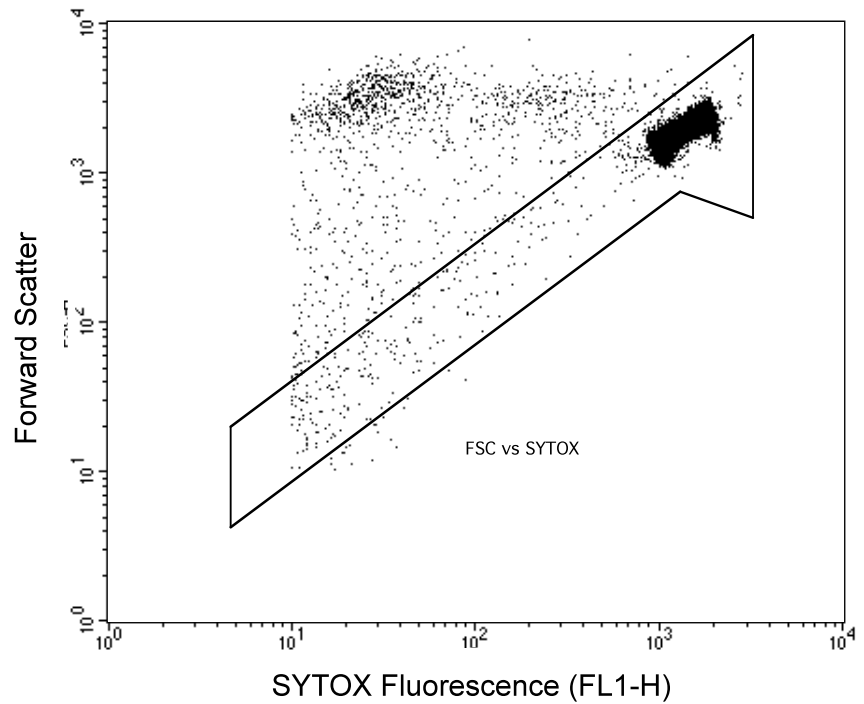
**PLOT D**

NOTE: BD instrument users, when FL1 is set to Doublet Discrimination mode, it is possible to represent SYTOX-associated fluorescence in linear scale (in addition to log scale). This is useful for evaluating cell cycle effects.

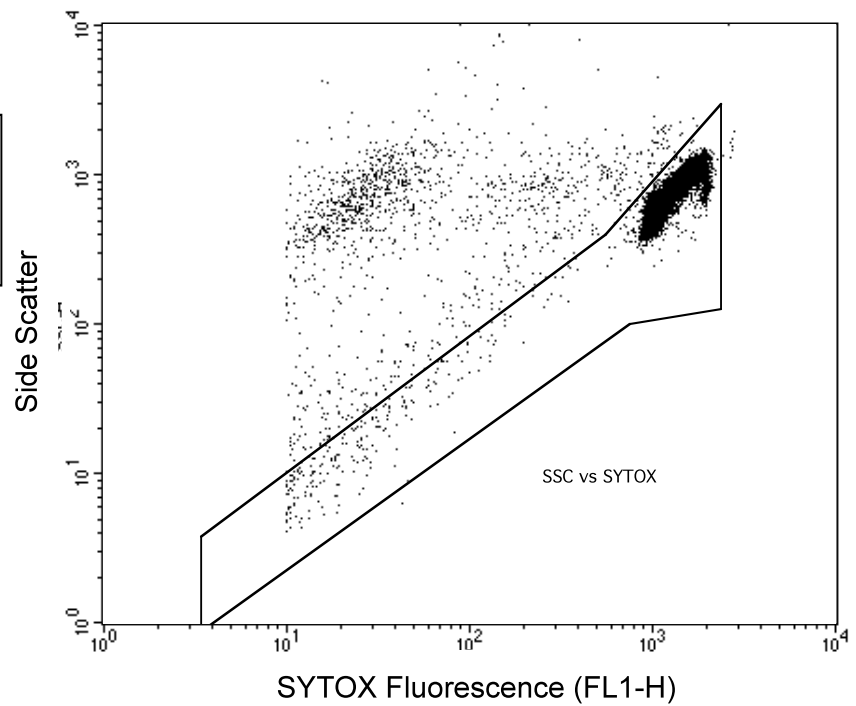


PLOT E

NOTE: Much of the chromatin associated with dead/dying cells falls above an appropriately located “FSC versus SYTOX” region.

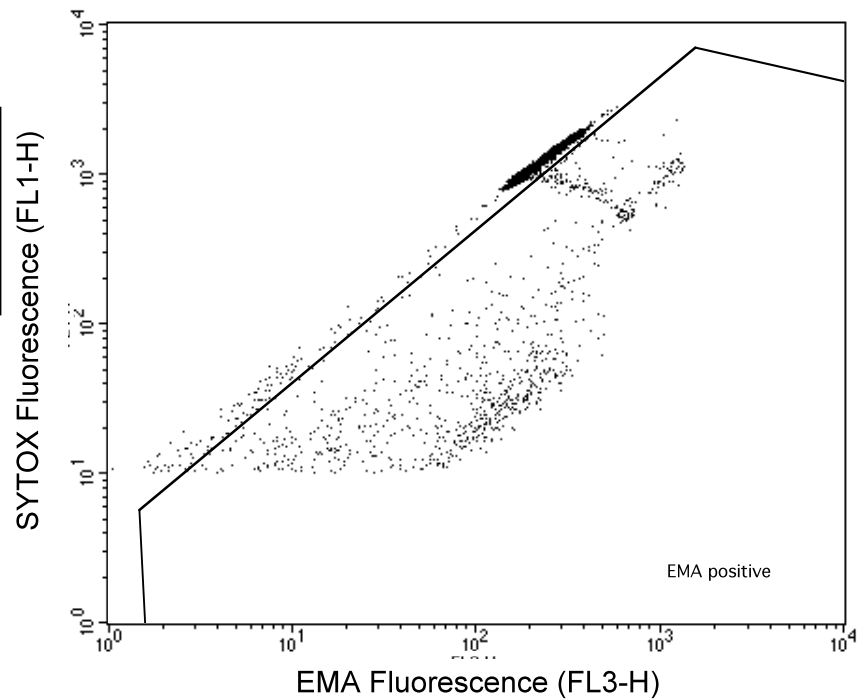
**PLOT F**

NOTE: Much of the chromatin associated with dead/dying cells falls above an appropriately located “SSC versus SYTOX” region.

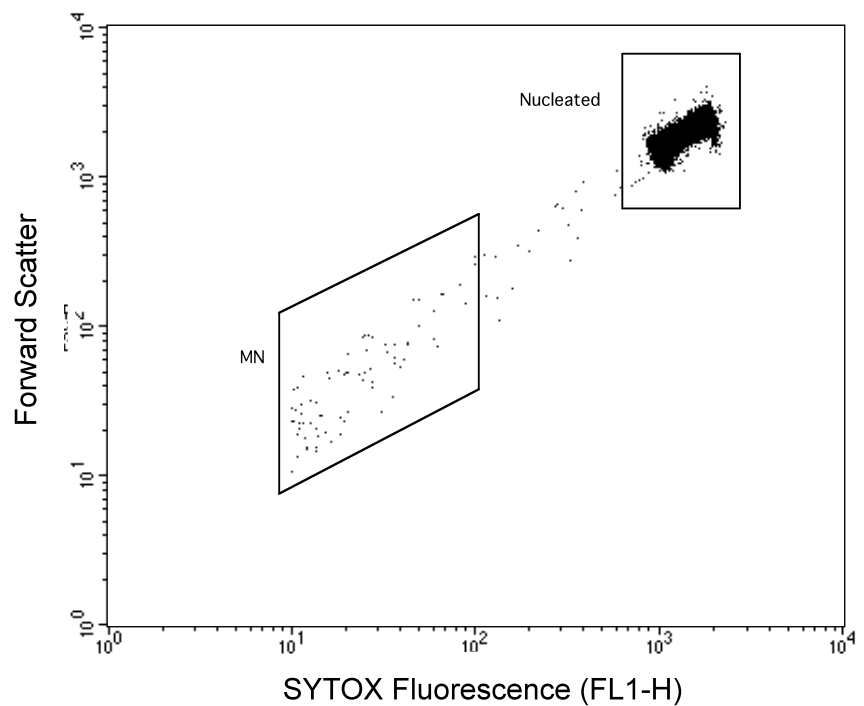


PLOT G

NOTE: A gate based on an appropriately positioned “EMA positive” region is used to exclude the chromatin of dead/dying cells (page 17 describes gates in more detail).

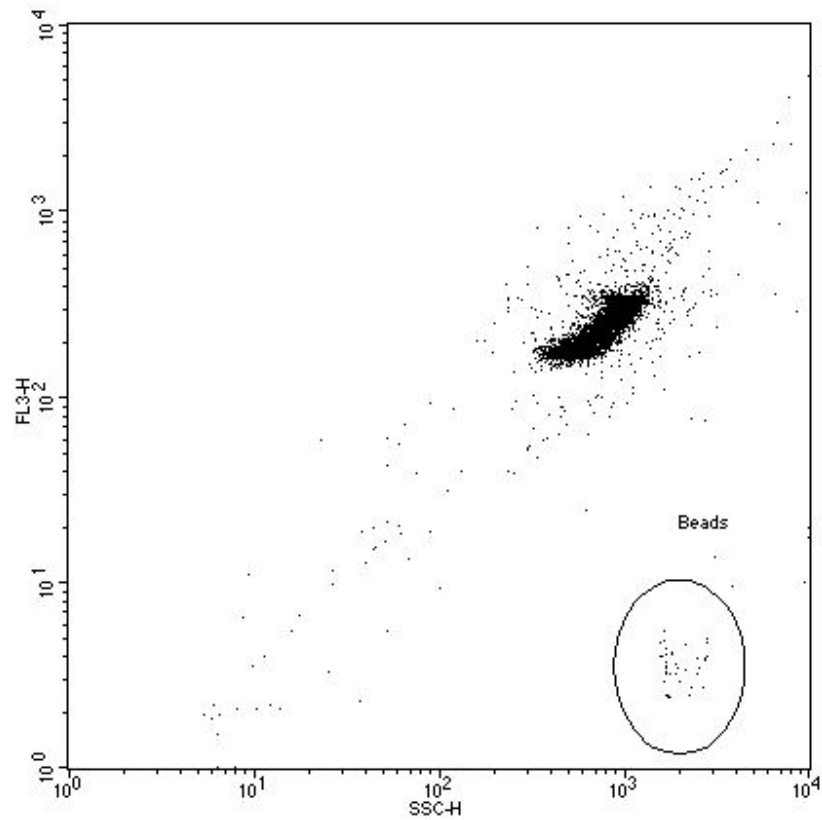
**PLOT H**

NOTE: Only nuclei and micronuclei that meet the 6 characteristics associated with Gate G10 (page 17) are used to calculate MN frequency.



Bead Count Plot

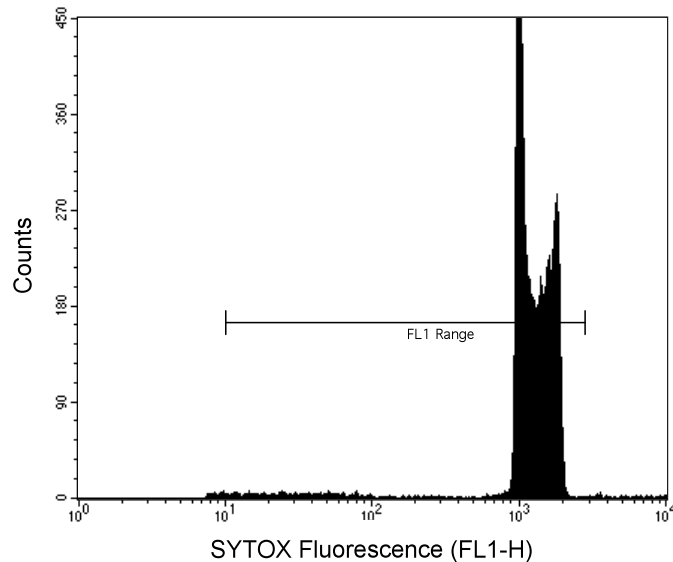
NOTE: The Bead Count Plot allows for the resolution of the counting beads used for the Nuclei-to-Bead Ratio calculation.



Appendix B – Flow Cytometric Analysis Recommendations

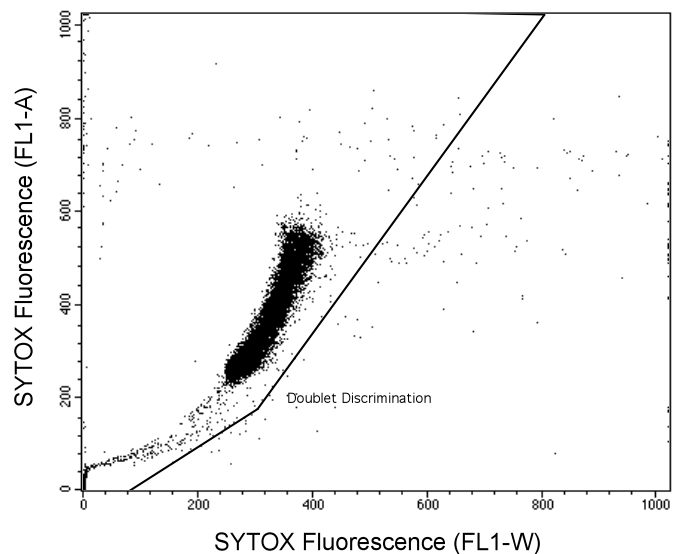
1. Transfer a stained, room temperature sample into a flow cytometry tube, very gently pipetting the suspension several times prior to the transfer. Place the sample on the flow cytometer and begin running in setup mode.
2. Locate the G1 peak in PLOT C and adjust the FL1 PMT voltage until the peak is positioned at the 10^3 channel of FL1 fluorescence. It is important to set nucleated cells high in SYTOX-associated fluorescence, since MN with $1/100^{\text{th}}$ the intensity of G1 events need to fall on scale.

PLOT C

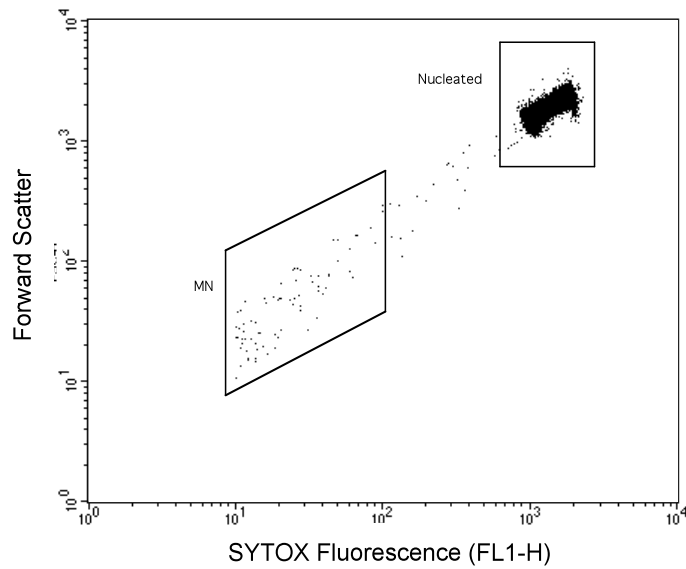


3. Once the G1 peak is approximately positioned, make sure the doublet discrimination region (if available) appropriately eliminates aggregates. This is available on Becton Dickinson Instruments by setting FL1 to the Doublet Discrimination mode. The bivariate graph should show SYTOX Green associated fluorescence (area on the Y-axis and width on the X-axis).

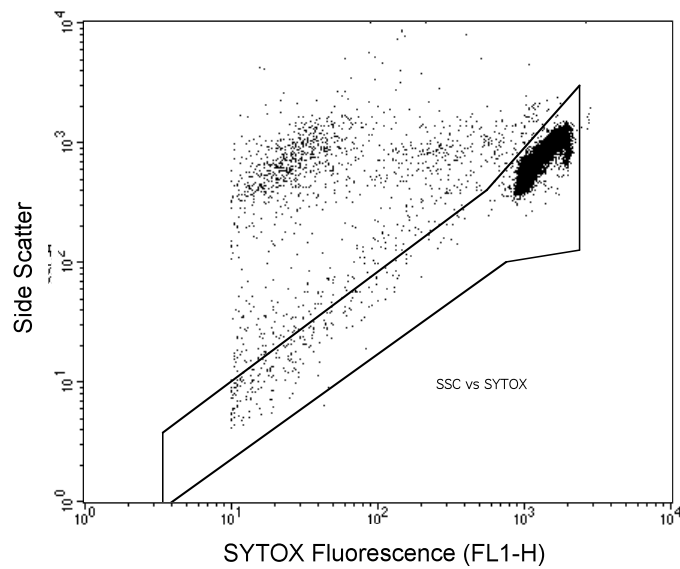
PLOT B



- After positioning the G1 peak, ensure that the free nuclei appropriately fall within the “Nucleated” region, as shown below. Depending on the Forward Scatter position of events for your particular instrument, the regions shown below may need to be adjusted vertically.

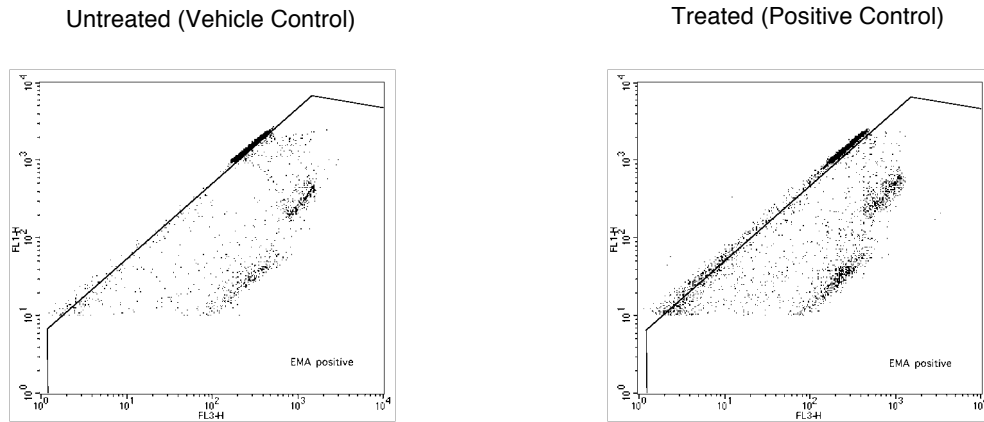
PLOT H

- Adjust the SSC voltage and/or the vertical position of the “SSC vs SYTOX” region until free nuclei are positioned as shown below.

PLOT F

6. Appropriate FL3 PMT voltage (EMA fluorescence) should result in nearly a log of fluorescent resolution between healthy and dead cells. Representative plots showing EMA staining characteristics are shown below.

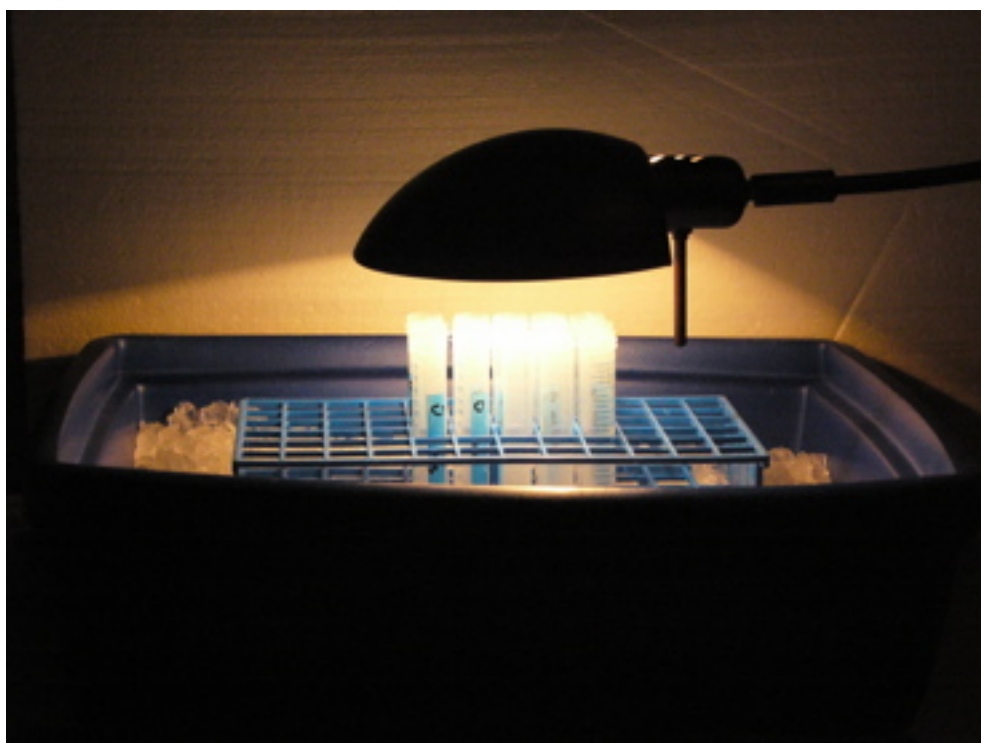
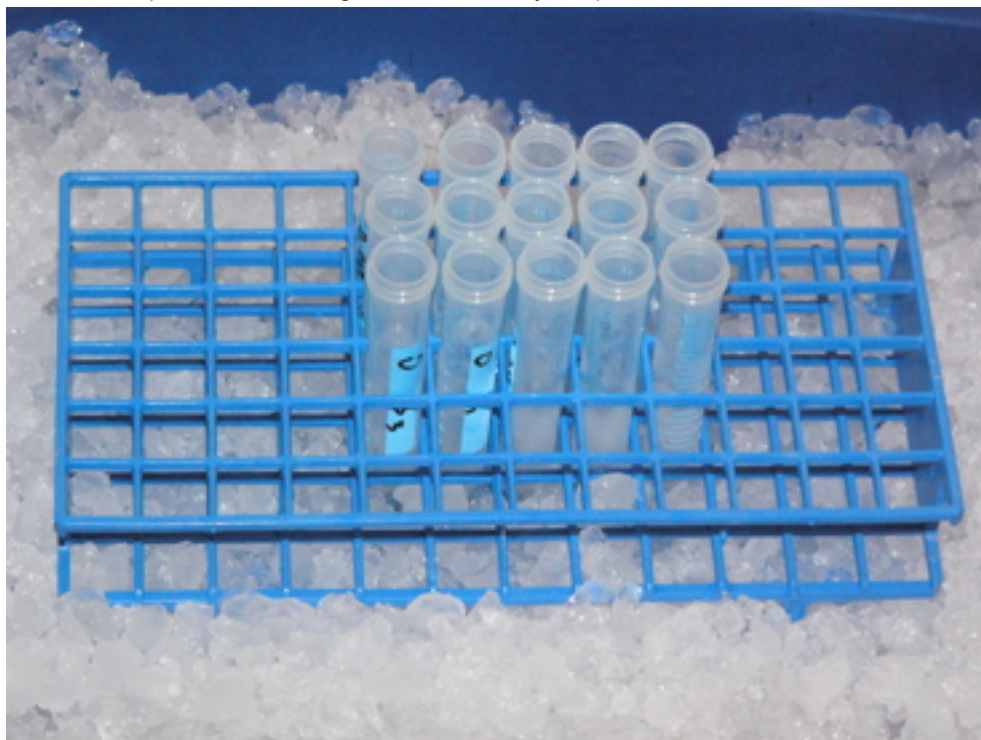
PLOT G



7. Allow the samples to equilibrate on the FCM instrument for about 30 seconds before acquiring data. Do not change the flow rate during data acquisition, or between samples.
8. It is preferable that the regions and instrument settings are not changed between experimental samples. This is why it is worth taking some extra time to carefully consider PMT voltage and Threshold settings initially. One possible exception to this rule is that SYTOX (FL1) PMT may be adjusted modestly between samples with good rationale. That is, the G1 peak can be used as a guide: adjust FL1 PMT voltage until this peak falls in a consistent FL1 channel (or at least into a narrow range of channels). This is best determined by considering Plots C and D.

Appendix C – Tube Placement During Nucleic Acid Dye A Photoactivation

Ideal tube placement during Nucleic Acid Dye A photoactivation is shown below.

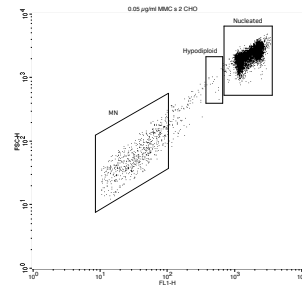


Appendix D – Mode of Action Signatures (CHO-K1 Cells)

There are some advantages to the use of certain cell lines. As with other mammalian cell lines, MN induction can be observed in CHO-K1 cells when exposed to aneugenic or clastogenic agents. When using this specific cell line, it is possible to discriminate between aneugenic and clastogenic modes of action. Two characteristics of the MicroFlow data can help investigators differentiate between these activities. These responses are referred to herein as clastogenic and aneugenic signatures.

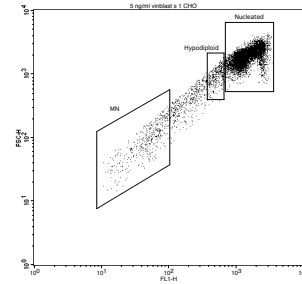
Clastogenic Signature:

- Significant MN induction
- No shift in median fluorescence intensity of MN events
- No robust increase in the frequency of hypodiploid nuclei



Aneugenic Signature:

- Significant MN induction
- Increased median fluorescence intensity of MN events
- Robust increase in the frequency of hypodiploid nuclei



1. Advantages of Using CHO-K1 Cells

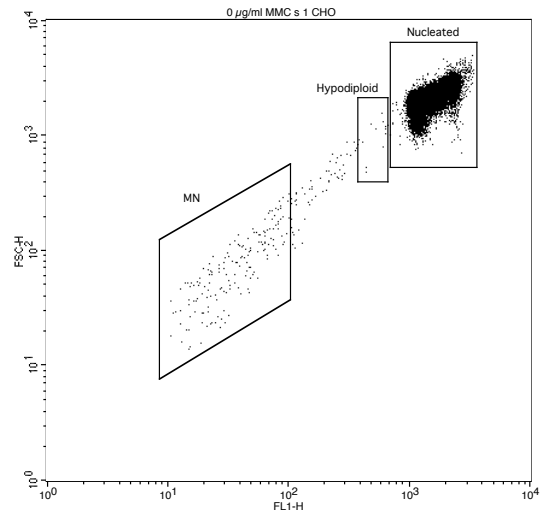
- The shift in median fluorescence intensity of MN events is likely related to the whole chromosome nature of aneugen-induced MN. It is interesting that this shift has not been evident in Litron's work with L5178Y or TK6. We attribute this to the larger average size of hamster chromosomes as compared to mouse and human chromosomes. This characteristic would be expected to enhance the difference in MN size generated by aneugens versus clastogens.
- Robust increases in the frequency of hypodiploid nuclei upon aneugen treatment was first noted with TK6 cells, although more recent work with that cell line suggest that it does not discriminate between modes of action as reliably as when hamster cell lines such as CHO-K1 are studied. We attribute induction of hypodiploidy to aneuploidization, possibly via failed cytokinesis and/or extra centrosome content. These are situations that lead to multipole division, and this is a mechanism that promotes rapid acquisition of aneuploidy.

We hypothesize that the lysis procedure used for flow cytometric MN scoring is capable of revealing aneuploidization, as hypodiploid nuclei are apparent as sub-2n DNA events with the light scatter and DNA staining characteristics of healthy cells' nuclei: EMA-negative nuclei with SYTOX-intensity slightly lower than 2n.

2. Comparison of Bivariates

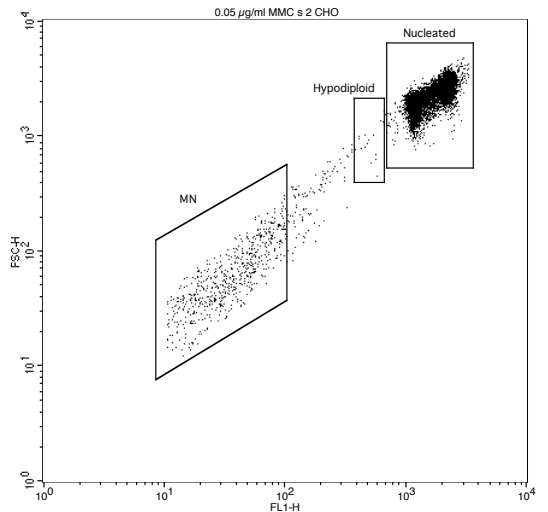
Examples of clastogenic and aneugenic signatures are shown below. In these cases, CHO-K1 cells were treated with the clastogen mitomycin C or the aneugen vinblastine. In order to enumerate hypodiploid nuclei, a region should be created on PLOT H that is adjacent to the Nucleated region.

Vehicle Control



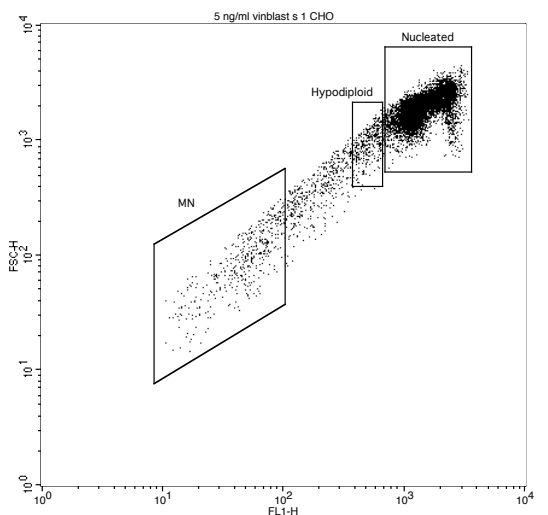
Clastogen

- MN induction
- No shift in MN fluorescence
- Lack of robust hypodiploid response



Aneugen

- MN induction
- Shift in MN fluorescence
- Robust hypodiploid response



Appendix E – References, Ordering Information, and Technical Services

- M. Kirsch-Volders, T. Sofuni, M. Aardema, S. Albertini, D. Eastmond, M. Fenech, M. Ishidate Jr., S. Kirchner, E. Lorge, T. Morita, H. Norppa, J. Surrallés, A. Vanhauwaert, A. Wakata, Report from the in vitro micronucleus assay working group, *Mutat. Res.* 540 (2003) 153-163.
- S.L. Avlasevich, S.M. Bryce, S.E. Cairns, S.D. Dertinger, In vitro micronucleus scoring by flow cytometry: differential staining of micronuclei versus apoptotic and necrotic chromatin enhances assay reliability, *Environ. Molec. Mutagen.* 47 (2006) 56-66.
- S.M. Bryce, J.C. Bemis, S.L. Avlasevich, S.D. Dertinger, *In vitro* micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity, *Mutat. Res.* 630 (2007) 78-91.
- S.M. Bryce, S.L. Avlasevich, J.C. Bemis, M. Lukamowicz, A. Elhajouji, F. Van Goethem, M. De Boeck, D. Beerens, H. Aerts, J. Van Gompel, J.E. Collins, P.C. Ellis, A.T. White, A.M. Lynch, S.D. Dertinger, Interlaboratory Evaluation of a Flow Cytometric, High Content *In Vitro* Micronucleus Assay, *Mutat. Res.* 650 (2008) 181-195.
- Organisation for Economic Co-operation and Development (OECD) Guidelines for the testing of chemicals, Draft Guideline 487: *In vitro* micronucleus test, December 13, 2007 (3rd version).

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