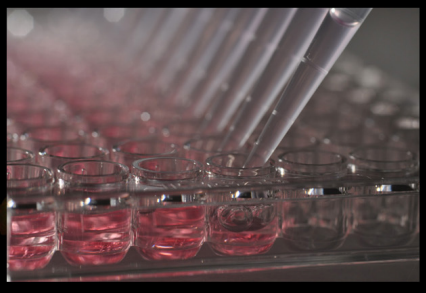
MicroFlow®

Quality Counts!

Micronucleus Analysis Kit



MicroFlow (In Vitro, 96 well)

Instruction Manual

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

READ ME FIRST

Critical for the success of this assay:

- **Cell Culture Conditions**

  It is critical to perform a growth curve with the cell line you intend to use before performing this assay. If cell cultures are not doubling in the expected ranges for the cell line, MN may not be expressed using standard treatment schedules.

  In order to prevent unhealthy cells from adversely impacting the assay (such as introducing false positives), cell cultures must be maintained below 1 x 10^6 cells/mL for suspension cells and less than 80 % confluency for attachment cultures. This is considered general advice; however, cell line-specific recommendations may supersede these suggestions.

- **Overtly Cytotoxic Conditions**

  It is well recognized that overly cytotoxic treatment conditions can lead to positive results in the micronucleus assay that are caused by the cytotoxic conditions rather than the treatment. Current recommendations state that your toxicity measurement should not be more than 55 % ± 5 %. Our current advice is to use concentrations that result in less than a 4-fold EMA-positive event increase over solvent controls as an additional cytotoxicity limit. This cytotoxicity measurement is <u>not</u> a part of an OECD Test Guideline so use of it should be discussed with regulatory officials prior to using as part of submission materials.

  Refer to information throughout this manual, especially Appendix B to ensure you employ the appropriate cytotoxicity method.

- **When Using Becton Dickinson (BD) High Throughput System (HTS) Instruments**

  When using BD's HTS-equipped instruments, BD Sheath Solution should be substituted with blood bank saline or filtered distilled water. This is because the use of certain specialized sheath compositions can compromise FITC (FL1) fluorescence stability.

  Additionally, excessive fluidics rates when using any flow cytometer can compromise FITC (FL1) fluorescence. Using the fluidics rates provided in the manual will help maintain stable FL1 fluorescence.

Table of Contents

1. Materials Provided

250/50 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

1000/200 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

2000/400 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 microspheres (Life Technologies, cat. no. C-16508) are recommended for certain cytotoxicity assessments**
- Cell line L5178Y, TK6, V79, or CHO-K1 (see Section 3.2; additional cell lines are under review)
- Commercially-heat-inactivated, sterile fetal bovine serum (FBS)
- Deionized water (dH_2O)
- -20 °C freezer
- 4 °C refrigerator
- Centrifuge with swinging bucket rotor to accommodate 96 well plate carriers
- CO_2-regulated, 37 °C incubator
- Flow cytometer capable of 488 nm excitation. A High Throughput Sampler (HTS) or similar device is recommended for 96 well plate-based work. See Section 3.1.
- Flow cytometry tubes (optional)
- Disposable pipettes sized 5, 10, 25, and 50 mL
- Vortex mixer and sonicator
- Light source to photoactivate Nucleic Acid Dye A (fluorescent or incandescent; fluorescent is preferred because it is cooler)
- Polypropylene centrifuge tubes (e.g., 15 mL)
- Micropipettors (20 μL - 1000 μL) and tips
- 96-well plates (U-bottom plates recommended for suspension cells; flat bottom plates recommended for attachment cells) and additional materials for maintaining cell lines
- 8 channel aspirator manifold and bridge (V&P Scientific, cat. nos. VP 180B and VP 180S). See Appendix F in the online supplementary material.
- Adhesive film to cover 96-well plates to prevent evaporation

3. Recommended Reading for First-Time Users

Please read the entire instruction manual before performing these procedures. Deviating from the procedures described in this manual may adversely affect the results of your assay. Substitution of kit components, changes in cell culture conditions, incubation times, reagent volumes, etc., is not advisable. If you have questions, please contact Litron by calling (585) 442-0930, faxing us at (585) 442-0934, or sending an email to info@litronlabs.com.

3.1. Flow Cytometer Requirements

The assay requires 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 PerCP channel, and the green (Nucleic Acid Dye B) fluorescence should be collected in the FITC channel.

The assay is most efficient when the flow cytometer is equipped to automatically analyze wells of 96 well plates (e.g., with Becton Dickinson [BD] High Throughput System [HTS]). Alternately, treated and stained specimens can be transferred from 96 well plates into standard flow cytometry tubes at time of analysis.

When using BD's HTS-equipped instruments, BD Sheath Solution should be substituted with blood bank saline or filtered distilled water. This is because the use of certain sheath compositions can also compromise FITC (FL1) fluorescence stability. Additionally, FITC (FL1) fluorescence stability can be compromised by excessive fluidics rates. Using the fluidics rates described in this manual will help maintain stable FL1 fluorescence.

3.2. Cell Lines

This method was developed using a human lymphoblastoid cell line (TK6), mouse lymphoma cell line (L5178Y TK+/-) and an attachment cell line (CHO-K1). Additional cell lines successfully used with this method include: HepG2, V79, A549, and AHH-1 cells. Other mammalian cell lines are expected to be compatible with this kit as long as cell division is occurring in a predictable manner.

Cell cultures must be maintained below 1 x 10^6 cells/mL for suspension cells and less than 80 % confluency for attachment cultures. Cell cultures above these values can result in positive results caused by the cytotoxic conditions rather than the treatment.

It is critical to perform a growth curve with the cell line you intend to use before performing this assay. If cell cultures are not doubling in the expected ranges for the cell line, MN may not be expressed using standard treatment schedules. If cells have non-standard doubling times, cells may not be fully recovered from thawing, or cell culture conditions may be causing sluggish growth and/or cell death.

3.3. Template Preparation

Template files are available on Litron's website (www.LitronLabs.com), but are specific to CellQuest™ 3.3, CellQuest™ Pro 5.2, FACSDiva™ 6.1, and MACSQuantify™ software. If you are unable to use these templates, please prepare one PRIOR to analysis. See Appendix A for screen images which can be used in preparation of a data acquisition and analysis template.

3.4. Cytotoxicity Measurements

Proper cytotoxicity measurements are critical for preventing irreverent positive results. Nuclei densities can be determined by adding counting beads to Lysis Solution 1 and obtaining an absolute bead count. This enables calculation of cytotoxicity metrics such as Relative Survival (RS) as well as Relative Increased Cell Counts (RICC) and Relative Population Doubling (RPD).

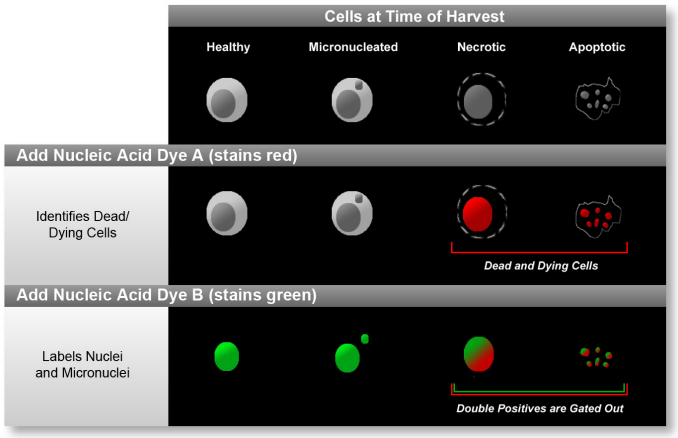
These values, along with the fold increase of percentage of EMA-positive nuclei*, can eliminate overly cytotoxic concentrations from analysis, and thereby reduce the incidence of false positive results. Relative Survival values greater than 45 % ± 5 %, and % EMA-positive nuclei* 4-fold and above over solvent controls indicate concentrations that should not be analyzed for micronuclei. For more information on other types of cytotoxicity measurements, see Appendix B.

*As noted in the "Read Me First" section of this manual, the 4-fold EMA criterion is not directly supported by OECD guidelines and therefore should only be applied to formal regulatory submissions following consultation with the appropriate agencies.

4. Introduction

The *In Vitro* MicroFlow Kit provides a highly efficient flow cytometric method for scoring micronuclei (MN) in cultured mammalian cells. Using a 2-color labeling technique, this is a fast, effective tool for evaluating the genotoxic activity of chemicals. This manual describes procedures required to process and score MN in 96-well plates.

4.1. The *In Vitro* MicroFlow Method



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 from 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 above.

4.2. High Content Analysis

The *In Vitro* MicroFlow kit provides the user with 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:

- **Cytotoxicity:** 6 micron microspheres can be added to the Lysis Solution 1. 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. This method has been found to reveal cytotoxicity that other cell scoring methods, such as Coulter counts, tend to underestimate (**Bryce et al., 2013)**.

  In addition, procedures that enable the determination of cytotoxicity metrics such as RICC and others are also compatible with *In Vitro* MicroFlow (See Appendix B). Once beads have been added to Lysis Solution 1, an absolute count of these particles can be readily obtained via manual counts using a hemocytometer, volumetric counting on a flow cytometer, or other instrument-based counting. The number of beads scored per sample can be used to calculate nuclei density on a per sample basis.

- **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 and 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.

5. Reagent Preparation

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

5.1. 1X Buffer Solution

Number of samples	Volume of dH_2O	Volume of 10X Buffer	Volume of FBS
96 (one plate)	18.0 mL	2.0 mL	0.4 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 dH_2O, 10X Buffer and FBS to a clean vessel. Filter sterilize and store on ice or refrigerate until use.

5.2. Complete Nucleic Acid Dye A Solution

Number of samples	Volume of 1X Buffer Solution	Volume of Nucleic Acid Dye A
96 (one plate)	6.5 mL	65 µL

1. Use the chart above to determine the volume of Complete Nucleic Acid Dye A 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 or refrigerate until use.

5.3. Complete Lysis Solution 1

Number of samples	Volume of Incomplete Lysis Solution 1	Volume of Nucleic Acid Dye B	Volume of Rnase Solution	Counting Beads (for cytotoxicity assessment)
96 (one plate)	11.0 mL	44 µL	55 µL	1 drop

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.

3. If desired, briefly sonicate and vortex a stock bead suspension. Add the bead suspension to the prepared Complete Lysis Solution 1 at 5 µL/mL (approximately 1 drop per 10 mL) and mix well.

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

4. Protect Complete Lysis Solution 1 from light and store at room temperature until use. At this point, perform an absolute bead count if interested in obtaining nuclei densities and density-dependent cytotoxicity measurements.

5.4. Complete Lysis Solution 2

Number of samples	Volume of Incomplete Lysis Solution 2	Volume of Nuclei Acid Dye B
96 (one plate)	11.0 mL	44 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.
3. Protect Complete Lysis Solution 2 from light and store at room temperature until use.

6. Suspension Cell Protocol

6.1. Cell Harvest

1. Remove 96 well plate(s) containing treated cells from the incubator.

2. Place 96 well plate(s) under an inverted microscope. Through visual examination, eliminate from MN scoring overly cytotoxic concentrations. Also examine wells for visible precipitate and eliminate if necessary. Although OECD recommends analyzing the lowest precipitating concentration, precipitate can clog flow cells, therefore exercise caution if wells with precipitate are analyzed.

3. Collect cells via centrifugation at 300 x *g* for 5 minutes.

4. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet (see Appendix F in the online supplemental material).

5. Loosen cells by gentle tapping or place plates on a plate shaker on a low speed.

6. Place samples on wet ice for 20 minutes before continuing to Section 6.2.

6.2. Complete Nucleic Acid Dye A Staining

1. Carefully add 50 µL of Complete Nucleic Acid Dye A Solution to each well. Gently pipette up and down to mix, making sure all cells come into contact with this solution.

2. Place plates on wet ice (see Appendix G in the online supplemental material for a visual depiction).

3. Leave the plate cover off and place a light source approximately 6 inches above the plates (see Appendix G in the online supplemental material). With plates on ice, expose the samples to visible light for 30 minutes.

4. Turn off the visible light source and add 0.15 mL of cold 1X Buffer Solution to each sample. From this point forward, limit the exposure of samples to light.

5. Collect cells via centrifugation at 300 x *g* for 5 minutes.

6. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellet (see Appendix F in the online supplemental material).

7. Loosen cells by gentle tapping or place plates on a plate shaker on a low speed. Proceed immediately to Section 6.3.

6.3. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Vortex or resuspend Complete Lysis Solution 1 and add 100 µL to the first row using a multi-channel pipette. Immediately pipette the samples up and down to make sure all cells come into contact with the reagents.

2. Change tips and repeat Step 1 for all remaining rows.

3. Incubate the samples for one hour in the dark at 37 °C.

4. Vortex or resuspend Complete Lysis Solution 2 immediately before addition and add 100 µL to each well. Gently rock the plate to mix.

5. Incubate the samples for 30 minutes in the dark at room temperature.

6. Store samples at room temperature and protect from light for up to 24 hours before flow cytometric analysis. Use of an adhesive film that prevents evaporation should be used if samples will be stored more than 4 hours.

7. Attachment Cell Protocol

Regarding treatment of attachment cells, we suggest first seeding the appropriate plates and allowing the cells to attach overnight. The following day prepare the test article in growth medium at the chosen top concentration and perform serial dilutions to achieve the desired concentration range. Be sure to factor in the additional growth time for the overnight attachment phase to ensure that your cells do not over grow by the end of the experiment. For additional useful advice on the application of attachment cells see the paper by Bemis et al., (2016).

7.1. Cell Harvest

1. Remove 96 well plate(s) containing treated cells from the incubator.

2. Place 96 well plate(s) under an inverted microscope. Through visual examination, eliminate from MN scoring overly cytotoxic concentrations. Also examine wells for visible precipitate and eliminate if necessary. Although OECD recommends analyzing the lowest precipitating concentration, precipitate can clog flow cells, therefore exercise caution if wells with precipitate are analyzed.

3. Place plates on wet ice for 20 minutes (see Appendix G in the online supplemental material for a visual depiction).

4. Slowly and carefully aspirate the treatment media from the wells, taking care not to disturb the cell surface (see Appendix F in the online supplemental material). Immediately proceed to Section 7.2.

7.2. Complete Nucleic Acid Dye A Staining

1. Carefully add 50 μL of Complete Nucleic Acid Dye A Solution to each well. Make sure that the entire cell surface is covered with this solution.

2. Place plates on wet ice (see Appendix G in the online supplemental material for a visual depiction).

3. Leave the plate cover off and place a light source approximately 6 inches above the plates (see Appendix G in the online supplemental material). With plates on ice, expose the samples to visible light for 30 minutes.

4. Turn off the visible light source and add 0.15 mL of cold 1X Buffer Solution to each sample. From this point forward, limit the exposure of samples to light.

5. Slowly and carefully aspirate supernatants (see Appendix F in the online supplemental material). Proceed immediately to Section 7.3.

7.3. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Vortex or resuspend Complete Lysis Solution 1 and add 100 μL to each well. Gently mix the plate for 5 seconds on a plate shaker set low enough not to splash the solution out of the wells.

2. Incubate the samples for one hour in the dark at 37 ºC.

3. Vortex or resuspend Complete Lysis Solution 2 immediately before addition and add 100 μL to each well. Gently rock the plate to mix.

4. Incubate the samples for 30 minutes in the dark at room temperature.

5. Store samples at room temperature and protect from light for up to 24 hours before flow cytometric analysis. Use of an adhesive film that prevents evaporation should be used if samples will be stored more than 4 hours.

8. Flow Cytometric Setup and Data Acquisition

Important Notes:

- The following setup and compensation instructions are specific for FACSDiva™ software, but should be useful with other software packages. **For BD-brand HTS systems**, BD-brand sheath solutions should be replaced with blood bank saline solution or filtered distilled water in order to prevent FITC-channel shift.
- Prior to analyzing experimental samples, it is recommended that you analyze solvent control and positive control samples first to verify that the template and instrument settings are appropriate.
- Protect samples from light. Ensure samples have equilibrated to room temperature before data acquisition occurs.

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.

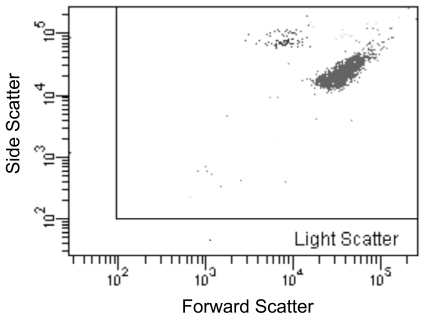
2. Users of BD-brand HTS equipment should set their loader settings as follows:
   a. Throughput = Standard
   b. Sample Flow Rate = 0.5 µL/sec
   c. Sample Volume = 75 µL
   d. Mixing Volume = 100 µL
   e. Mixing Speed = 100 µL/sec
   f. Number of Mixes = 5
   g. Wash Volume = 400 µL

3. The system should be primed at least three times before proceeding with analysis.

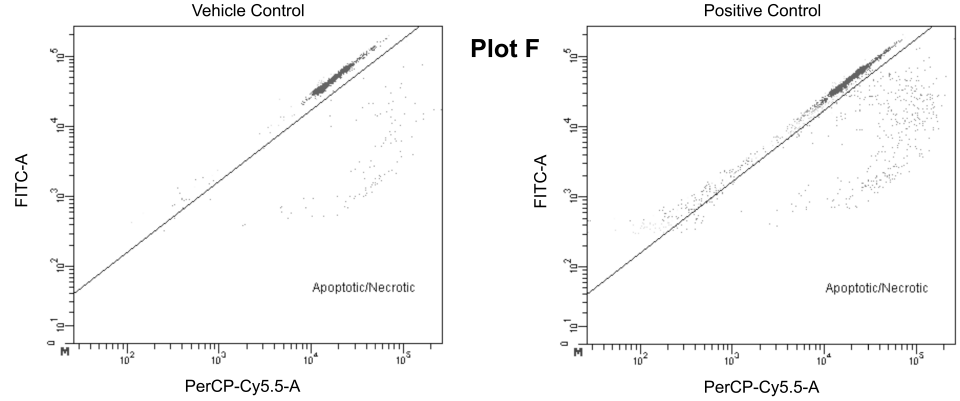
4. Place the plate on the flow cytometer. Using a solvent control well for setup, acquire sample in "set-up mode". Adjust FSC and SSC voltages to bring nuclei into view as shown in Plot A, right.

5. Adjust PerCP PMT voltage (EMA fluorescence) until the majority of the nuclei are above the Apoptotic/Necrotic region, as shown below. There should be nearly a log of fluorescent resolution between nuclei from healthy and dead cells. Representative plots showing EMA staining characteristics of a Vehicle Control and a Positive Control are shown below.

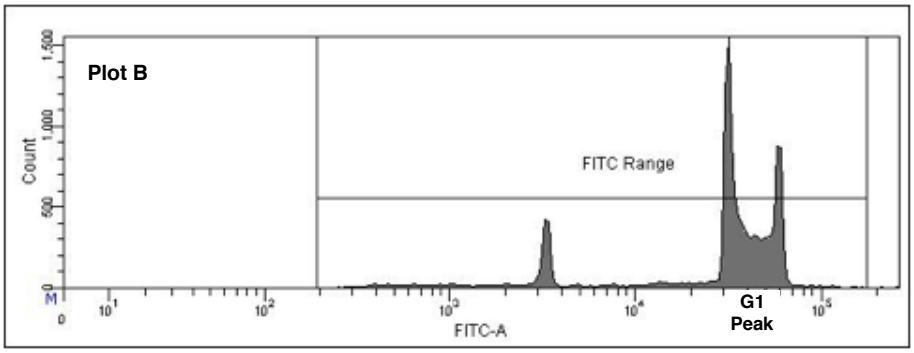
Plot A



Plot F



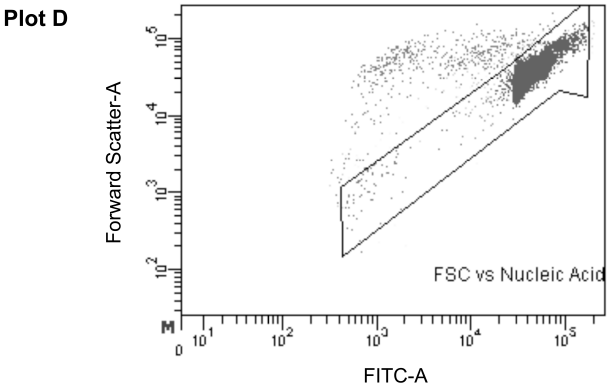
6. Locate the nuclei G1 peak in PLOT B and adjust the FITC (FL1) PMT voltage until the peak is positioned at a high enough FITC (FL1) channel so that 1/100th of this fluorescence signal will still fall on scale. It is important to set nuclei high in SYTOX-associated fluorescence, since MN with 1/100th the intensity of G1 events need to fall on scale.



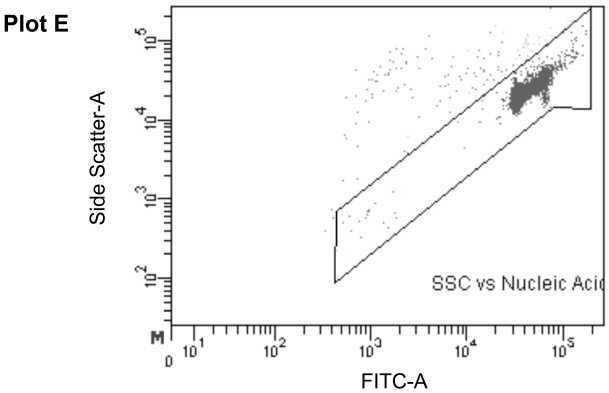
7. Set the threshold parameter (also referred to as the acquisition trigger) to FITC (FL1) fluorescence (i.e., SYTOX Green). Adjust the threshold so that some events are collected that fall just to the left edge of the FITC (FL1) range that is defined in Plot B, above. For those instruments that are 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 Plot A (step 4) as a guide.

8. After positioning the G1 peak on Plot B, make sure that PerCP (EMA fluorescence) PMT voltage is still appropriate (Plot F). See step 5.

9. Adjust the position of the "FSC vs. Nucleic Acid Dye B" region until nuclei are positioned as shown in Plot D.

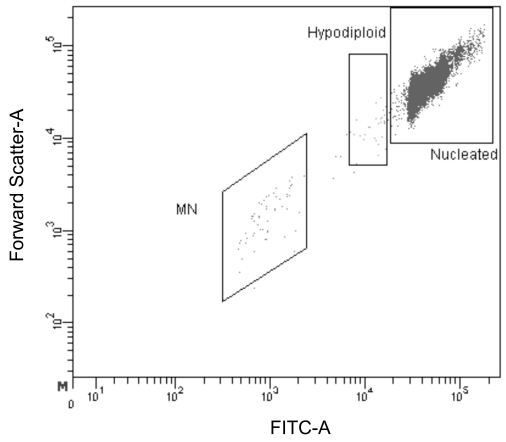


10. Adjust the position of the "SSC vs. Nuclei Acid Dye B" region until nuclei are positioned as shown in Plot E.



11. Ensure that nuclei fall within the “Nucleated” region, as shown in Plot G.

Plot G



12. It is preferable that the regions and instrument settings are not changed between experimental samples. Therefore, carefully consider PMT voltage and threshold settings during setup.

13. Set a stop mode based on the number of events in the Nucleated region defined in Plot G. This number is typically set for at least 5000 healthy cells’ nuclei. Note: BD HTS users should include Time in their gating logic so that the first several seconds of analysis for each well are omitted from the data files.

14. Acquire data for the plate in its entirety or select the wells you wish to process.

9. Calculations

Cytotoxicity measurements are discussed in Appendix B.

1. Percent MN

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

2. Fold EMA

$$\text{Fold EMA} = \frac{\%\text{Parent Apoptotic/Necrotic}}{\text{Mean \%Parent Apoptotic/Necrotic of solvent control culture(s)}} \times 100$$

10. Troubleshooting

Observation	Possible Cause	Suggestion
Solvent control wells have high MN or EMA-positive values.	Doubling time of cell culture is different than expected.	Establish a growth curve to confirm the doubling time of your culture both after thaw and during routine cell passage. Use the doubling time to determine the correct time to harvest cells.
	Cells may not be fully recovered from thaw.	Wait at least one week (2 weeks for TK6 cells) before treatment to allow cells to equilibrate.
	Media, serum, antibiotics, or other growth factors may not be appropriate for your cell line.	Ensure that you are using cell culture media and supplements that are appropriate for your cell line.
	The environment is causing sluggish growth.	Maintain an incubation temperature of 37 °C, CO_2 levels at 5 %, and humidity near 88 %.
	Cells are overgrown.	Ensure that cell cultures do not exceed the following: Suspension cells: 1 x 10^6 cells/mL. Attachment cells: 80 % confluence.
Positive controls not showing a dose-related response.	Cells may have been harvested too early, resulting in too few cell divisions for micronuclei to be expressed.	Use the doubling time you calculated for your cell culture to determine the appropriate harvest time. If positive controls do not respond as expected, it is almost always due to reduced expression time (MN require 1.5 to 2 normal cell cycles to be expressed; see Appendix D in the online supplemental materials). If positive controls do not respond, perform a new growth curve experiment to measure the current doubling time of the cell culture. If, after the growth curve experiment and subsequent altering of expression times, positive controls still do not respond as expected, contact Litron for assistance.
FITC channel values shift over time when using the HTS on a BD flow cytometer.	Sheath is not appropriate.	Ensure that blood bank saline or filtered distilled water is used in place of BD sheath fluid.

11. References

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12. License Agreement and Limited Product Warranty

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Appendix A – Template Preparation and Representative Plots

CellQuest™ v3.3, CellQuest™ Pro v5.2, FACSDiva™ v6.1, and MACSQuantify™ template files 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 (seven bivariate graphs and two histograms). Flow cytometry operators who are not using BD or Miltenyi software should find these pages valuable for constructing their own data acquisition and analysis template.

1. Create plots and regions as shown on pages 14 through 16.

2. Define the following regions:
   - "Light Scatter" in Plot A
   - "FITC Range" in Plot B
   - "SSC vs. Nucleic Acid Dye B" in Plot D
   - "FSC vs. Nucleic Acid Dye B" in Plot E
   - "Apoptotic/Necrotic" in Plot F
   - "MN", "Nucleated" (and optional "Hypodiploid") in Plot G
   - "Beads" in Bead Plot
   - "Time" in Time Histogram

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

Tube: Global Sheet1

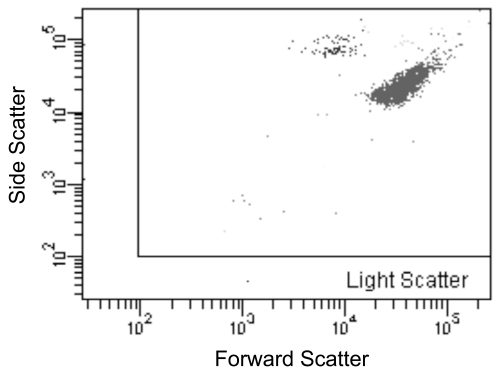
Population	#Events	%Parent	%Total
All Events	0	####	####
Light Scatter	0	####	####
Nucleic Acid Dye B	0	####	####
Time	0	####	####
Beads	0	####	####
NOT(Beads)	0	####	####
Apoptotic/Necrotic	0	####	####
NOT(Apoptotic/Necrotic)	0	####	####
FSC vs Nucleic Acid Dye B	0	####	####
SSC vs Nucleic Acid Dye B	0	####	####
Nucleated	0	####	####
MN	0	####	####
Hypodiploid	0	####	####

4. Set a stop mode based on the number of events in the "Nucleated" region defined in Plot G. This number is typically set for at least 5,000 healthy cells' nuclei.

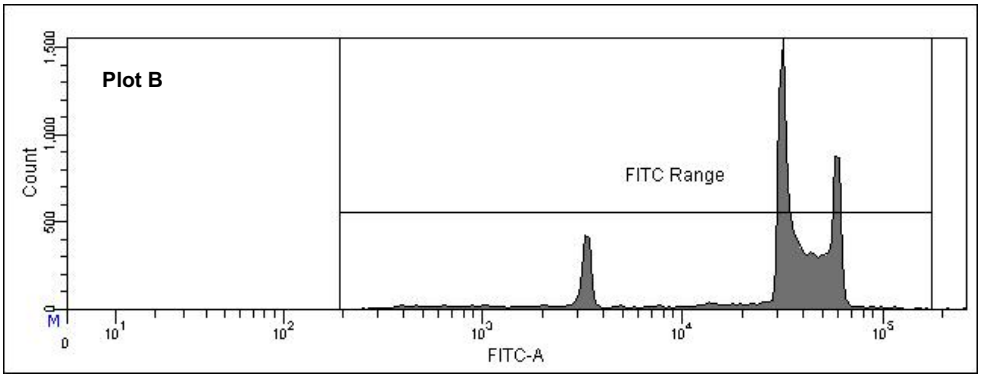
5. **Set the Storage Gate to Nucleated in Plot G.** In conjunction with the Time Histogram shown in step 14, this gating logic excludes the first few seconds of data from each well. This strategy is important for BD-brand HTS users, as fluorescence signals tend to require several seconds before they stabilize.

6. 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.

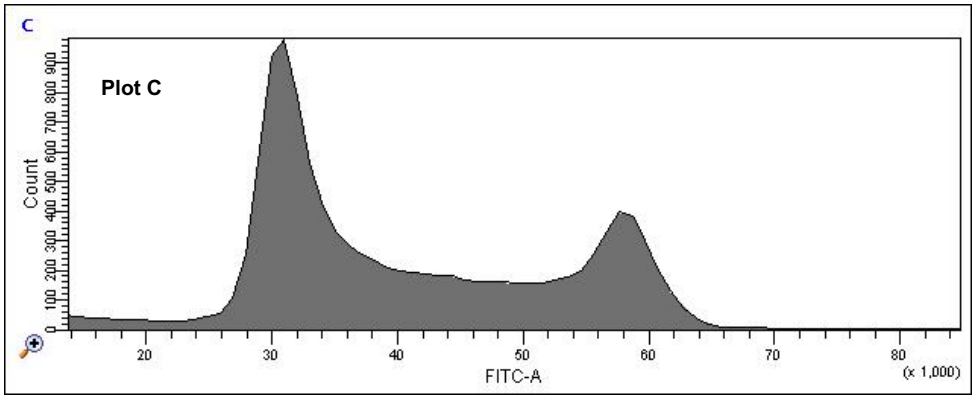
Plot A



7. The “FITC Range” region should include nuclei as well as sub-2n chromatin that exhibit up to 1/100th the SYTOX fluorescence signal of 2n nuclei.

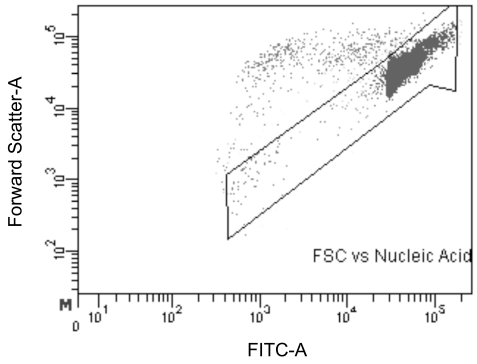


8. By examining the Nuclei Acid Dye B range with a linear FITC-A scaling in Plot C, one can obtain cell-cycle data.



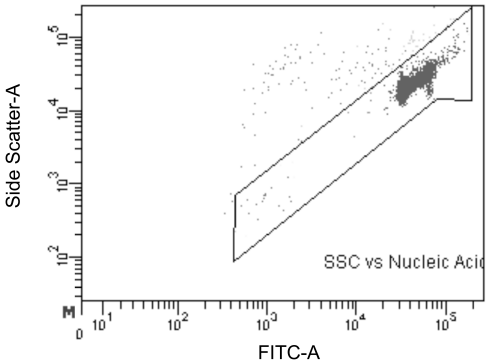
9. Much of the chromatin associated with dead/dying cells falls above an appropriately located “FSC versus Nucleic Acid Dye B” region in Plot D.

Plot D



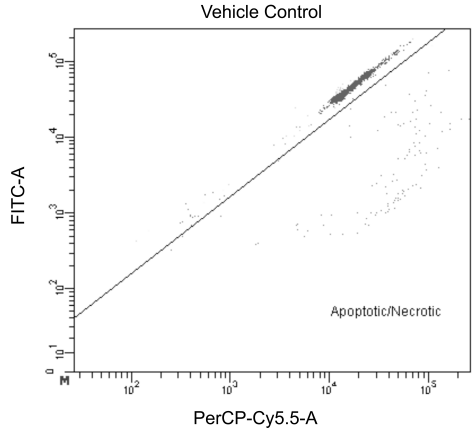
10. Much of the chromatin associated with dead/dying cells falls above an appropriately located “SSC versus Nucleic Acid Dye B” region in Plot E.

Plot E



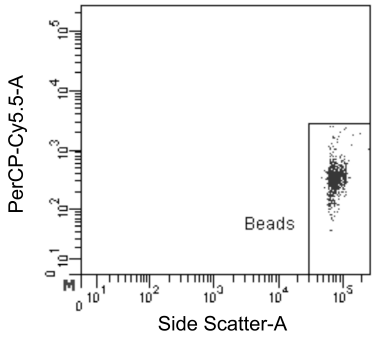
11. A gate based on an appropriately positioned “Apoptotic/Necrotic” region in Plot F is used to exclude the chromatin of dead/dying cells.

Plot F



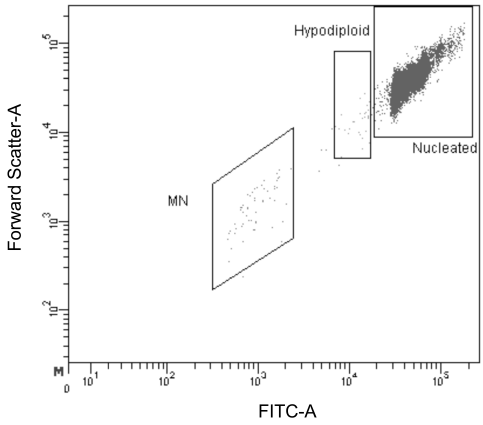
12. The Bead Count Plot allows for the resolution of the counting beads used for the Nuclei-to-Bead Ratio calculation or other cytotoxicity metrics.

Bead Plot



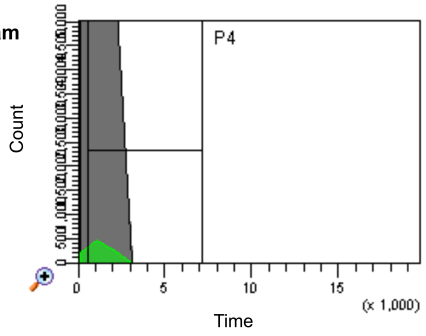
13. Only nuclei and micronuclei that meet the multiple characteristics associated with “healthy cells” are used to calculate MN frequency.

Plot G



14. When analyzing 96 well plates using BD-brand HTS equipment, a “Time” marker should be set and used in the Data Acquisition logic so that approximately the first 10 seconds of data are not saved. Over this initial period of time, FL1 fluorescence is not stable.

Time Histogram



Appendix B – Cytotoxicity Measurement Strategies

1. Methods to Obtain Cytotoxicity Measurements

1.1. Values Obtained From the Template

The #Events for the Nucleated region and %Parent Apoptotic/Necrotic region values provide data that can be used to calculate Relative Survival (RS) and Fold EMA values respectively.

To obtain data for the calculation of Relative Increased Nuclei Count (RINC) and Relative Population Doubling (RPD), pre-treatment measurements of #Events for the Nucleated region and %Parent Apoptotic/Necrotic region can be performed as follows:

- For suspension cells cultures, process several wells of cells with the *In Vitro* MicroFlow reagents before treatment.
- For attachment cells cultures, seed several additional wells and allow cells to attach. Before treatment, process these wells with the *In Vitro* MicroFlow reagents as described in this manual.

1.2. Addition of Beads to Complete Lysis Solution 1

Determining Nuclei-to-Bead ratios from #Events of the Nucleated and Bead regions can provide data to calculate RS.

By performing an absolute count on the beads in Complete Lysis Solution 1 before addition to the treated cultures, Nuclei Density (ND) and commonly used cytotoxicity measurements based on density, such as Relative Nuclei Count (RNC) can be obtained. Although the number should be relatively consistent over time, be sure determine the bead number for each batch of Complete Lysis Solution 1 prepared.

By obtaining pre-treatment measurements as described in 1.1 (and using Nuclei-to-Bead ratios or absolute bead count), data for the calculations of RINC and RPD can be obtained.

1.3. Addition of Beads to Cell Cultures

Measuring cell density before and after treatment provides data to calculate Relative Increased Cell Count (RICC) and RPD values.

- For suspension cells cultures:
    - Obtain an initial cell and bead count before treatment. This can be accomplished via flow cytometry by using a commercially available “absolute counting bead” added at a known concentration to an aliquot of cells obtained at the start of exposure. This sample is then analyzed on the flow cytometer and a cell density value is determined based on comparison of the number of cells analyzed to the known bead count per unit volume in the sample.
    - Obtain a cell and bead count at the time of harvest and before lysis. Aliquots from the control and treated cultures can be mixed with a known concentration of counting beads (ideally use the same bead solution as was used for the initial cell counts) and the final cell density can be determined.
    - Other approaches to obtain cell count/density information, such as Coulter Counter, can also be applied. **Does this go here or should it go under volumetric measuring (since that’s what it is)?**
- For attachment cells cultures:
    - On the day of seeding, prepare several additional wells that will be harvested at the time of treatment to provide data on initial cell density.
    - Once the cells have been harvested, they can be analyzed immediately or held at refrigerated temperatures and processed along with the remaining wells from the treated cultures. This will yield nuclei to bead ratios (or absolute counts if a known bead count is employed) that can then be applied to the appropriate cytotoxicity calculations (see below).

1.4. Time Histogram

It is also possible to obtain cytotoxicity information based on the “Time” Histogram (see Appendix A). If care is taken during sample processing such that the final volumes of the samples for analysis are very consistent across wells, then the number of events collected per unit time can provide RS values. Users who choose the employ this method should ensure that the region set to collect count information in the “Time Histogram” plot is not impacted by variations in the length of total analysis time some samples require to meet established stop gates.

1.5. Volumetric Counting

Many flow cytometers are equipped with volumetric sampling that enables accurate cell density determination to be made for all samples. This capability can provide data for the calculation of the same cytotoxicity measurements as described above.

Measuring cell density before treatment also provides data to calculate RINC and RPD values.

2. Cytotoxicity Measurement Calculations

2.1. Fold EMA

The health of cells can be inferred from the percentage of EMA-positive events (%Parent Apoptotic/Necrotic). This statistic is particularly sensitive to apoptosis. Applying a specific EMA fold-induction criteria for exclusion of overly toxic concentrations has been shown an effective means of limiting genotoxicity assessment to relevant concentrations in conjunction with other cytotoxicity parameters.

Fold EMA is calculated as follows:

$$\text{Fold EMA} = \frac{\%\text{Parent Apoptotic/Necrotic}}{\text{Mean \%Parent Apoptotic/Necrotic of solvent control culture(s)}} \times 100$$

2.2. Relative Survival (RS)

RS is a common approach for assessing cytotoxicity and for setting top test article concentrations. A common strategy requires treated cell cultures to exhibit RS 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. While this approach is commonly used for non-regulatory testing, it should be noted that RS is not supported by the current OECD 487 Test Guideline relating to *in vitro* micronucleus testing.

RS is calculated as follows:

$$\text{RS} = \frac{\#\text{Events of Nucleated (or Cell-to-Bead ratio, or other nuclei or cell count)}}{\text{Mean \#Events of Nucleated (or Cell-to-Bead ratio, or other nuclei or cell count) of solvent control culture(s)}} \times 100$$

2.3. Cell Density (CD) or Nuclei Density (ND)

CD or ND can be provided by the device measuring the cells or nuclei (flow cytometer or Coulter Counter), or can be calculated by dividing the number of cells or nuclei counted by the volume measured.

If using an absolute count of beads added to Complete Lysis Solution 1, ND is calculated as follows:

$$\text{ND} = \frac{\#\text{Events Nucleated}}{\#\text{Events Beads}} \times \text{Number of beads/mL in Complete Lysis Solution 1} \times \text{Dilution Factor (2)}$$

2.4. Relative Nuclei Count (RNC)

Once ND is calculated, RNC can be calculated as follows:

$$\text{RNC} = \frac{\text{Density of nuclei in treated culture}}{\text{Mean density of nuclei in solvent control culture(s)}} \times 100$$

2.5. Relative Increased Nuclei Count (RINC) or Relative Increased Cell Count (RICC)

The cytotoxicity assessment metrics defined for the *in vitro* MN method in the current OECD 487 Test Guideline includes RICC. This proliferation-based calculation is believed to provide more relevant information on cell death than other previously used approaches. The In Vitro MicroFlow method can provide data for the calculation of RINC (where nuclei are substituted for cells), and if certain methods are used, RICC.

RINC or RICC is calculated as follows:

$$\text{RINC or RICC} = \frac{\text{Final \#Events of Nucleated (or other nuclei or cell count)} - \text{Starting \#Events of Nucleated (or other nuclei or cell count)}}{\text{Mean Final \#Events of Nucleated (or other nuclei or cell count) of solvent control culture(s)} - \text{Mean Starting \#Events of Nucleated (or other nuclei or cell count) of solvent control culture(s)}} \times 100$$

2.6. Relative Population Doubling (RPD)

The cytotoxicity assessment metrics defined for the *in vitro* MN method in the current OECD 487 Test Guideline includes RPD. This proliferation-based calculation is believed to provide more relevant information on cell death than other previously used approaches.

Before RPD can be calculated, Population Doubling (PD) for each culture must be calculated:

$$PD = [\log(\text{Final number of nuclei/Starting number of nuclei})] / \log 2$$

RPD is calculated as follows:

$$\text{RPD} = \frac{\text{PD of treated culture}}{\text{Mean PD of solvent control culture(s)}} \times 100$$

Appendix H – Criteria for Scoring Micronuclei

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. The maximum concentration examined should be based on cytotoxicity or established limit concentrations (see Section 3.4). Treatment conditions should be sufficient to induce 55 % ± 5 % cytotoxicity.

2. MN events must exhibit 1/100th to 1/10th the SYTOX Green fluorescent intensity of 2n nuclei (Appendix A, Plot G).

3. MN events must fall within a FCS vs. Nucleic Acid Dye B fluorescence region (Appendix A, Plot D).

4. MN events must fall within a SSC vs. Nucleic Acid Dye B fluorescence region (Appendix A, Plot E).

5. MN events must be outside the Apoptotic/Necrotic region (Appendix A, Plot F). 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 values may be artificially high. Inclusion of an EMA fold-induction cut-off value may be useful to eliminate overly toxic conditions from interfering with genotoxicity assessment. Our current advice is to only examine concentrations where the EMA Fold is less than or equal to 4 times the average solvent control.