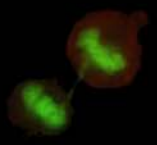


MicroFlow®

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

CRITICAL for the success of this assay:

- **Cell Culture Conditions**

  While optimal cell culture conditions are cell line specific, a useful rule of thumb is that suspension cell lines should be maintained below 1 x 10^6 cells/mL, and attachment cell lines should be maintained at less than 80 % confluency.

  It is critical to perform a growth curve with the cell line you intend to use before performing micronucleus (MN) assays. This is because the doubling time dictates the appropriate post-exposure cell harvest time. Also, if cells are not doubling in the expected time-frame, this is a strong signal that there are issue(s) with the culture conditions that must be resolved before MN experiments are performed.

- **Overtly Cytotoxic Conditions**

  Chemical treatment conditions that cause overly cytotoxic effects can result in "false positive" MN results (some literature refers to these as "irrelevant positive" results). Consult the most current Organisation for Economic Co-operation and Development (OECD) Test Guideline (No. 487) for specific advice about choosing an appropriate cytotoxicity measurement and cytotoxicity limits that should not be exceeded when conducting MN assays. Additional information regarding cytotoxicity measurements appears in Sections 3 and 11 of this instruction manual.

- **Flow Cytometer Considerations**

  Most flow cytometers that are capable of providing 488 nM excitation are compatible with this assay.

  Avoid excessive flow cytometric event acquisition rates as they compromise FITC channel fluorescence signals.

Table of Contents

1. Materials Provided

250/50 Sample Kit Components	Quantity[a]	Storage Condition[b]
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)	0.170 mL	–10 °C to –30 °C, light sensitive
Nucleic Acid Dye B (SYTOX® Green nucleic acid stain[c])	0.230 mL	–10 °C to –30 °C, light sensitive
RNase Solution	0.145 mL	–10 °C to –30 °C
10X Buffer	30 mL	Ambient

1000/200 Sample Kit Components	Quantity[a]	Storage Condition[b]
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)	0.685 mL	–10 °C to –30 °C, light sensitive
Nucleic Acid Dye B (SYTOX® Green nucleic acid stain[c])	0.925 mL	–10 °C to –30 °C, light sensitive
RNase Solution	0.580 mL	–10 °C to –30 °C
10X Buffer	120 mL	Ambient

2000/400 Sample Kit Components	Quantity[a]	Storage Condition[b]
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)	1.37 mL	–10 °C to –30 °C, light sensitive
Nucleic Acid Dye B (SYTOX® Green nucleic acid stain[c])	1.85 mL	–10 °C to –30 °C, light sensitive
RNase Solution	1.16 mL	–10 °C to –30 °C
10X Buffer	240 mL	Ambient

a. Sufficient materials are provided to analyze 250, 1,000, or 2,000 96-well cultures, or 50, 200, or 400 larger format cultures.
b. Please note that although some kit components are shipped at ambient temperature, they must be stored at the temperatures indicated above upon receipt.
c. SYTOX® Green, trademark Life Technologies.

2. Additional Materials Required

- 6 micron fluorescent microspheres (Life Technologies, cat. no. C-16508, or comparable)
- Cell line TK6, L5178Y, V79, or CHO-K1 (see Section 3.3; additional cell lines are compatible, but there are fewer published studies)
- 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 plate carriers for certain attachment cell lines)
- CO_2-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 and sonicator
- Light source to photoactivate Nucleic Acid Dye A (fluorescent- or LED-type are preferred)
- Polypropylene centrifuge tubes (e.g., 15 mL)
- Micropipettors (2 μL - 1000 μL) and tips
- Cell culture flasks and/or microwell plates; additional materials for maintaining a cell line
- Accutase® and 1X phosphate buffered saline (PBS) is required for certain attachment cell lines (see Section 9)

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. Inattention to culture conditions and overall cell health, substitution of kit components and changes to 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. Study Design

Historically, when the MN assay is performed in cell lines that are not metabolically competent, the experiment is performed under more than one condition (e.g., in the presence and absence of an exogenous metabolic activation system). It is beyond the scope of this instruction manual to provide guidance about this and other experimental design elements such as number of treatment groups, etc. When considering experimental details such as these, whether in the context of regulatory or non-regulatory studies, it is useful to consult the most recent OECD Test Guideline (No. 487) for general advice.

3.2. Flow Cytometer Considerations

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 efficiently performed when the flow cytometer is equipped to automatically analyze wells of 96 well plates (e.g., with Becton Dickinson [BD] High Throughput System [HTS]). If your intention is to use 96 well plates for treatment and analysis, you should consult the *In Vitro* MicroFlow instruction manual that is specific for 96 well plates (www.LitronLabs.com). The current instruction manual should be followed for those that **do not** intend to use 96 well plates for cell treatment or further processing.

3.3. Cell Lines

This method was developed using human lymphoblastoid cells (TK6), mouse lymphoma cells (L5178Y TK+/-) and an attachment cell line (CHO-K1). Additional cell lines that have been successfully studied with this method include: V79, HepG2, A549, A375, 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.

While optimal cell culture conditions are cell line specific, a useful rule of thumb is that suspension cell lines should be maintained below 1 x 10^6 cells/mL, and attachment cell lines should be maintained at less than 80 % confluency.

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, it may be due to cells that have not fully recovered from thawing, or suboptimal cell culture conditions that are causing sluggish growth and/or cell death.

3.4. Cell Culture Vessels

This instruction manual describes cell culture and treatment steps that utilize culture-treated 24 well plates. Other vessel sizes, especially for attachment-type cell lines, will require adjustments that scale with the different surface areas. If your intention is to use 96 well plates for treatment and analysis, you should consult the *In Vitro* MicroFlow instruction manual that is specific for 96 well plates (www.LitronLabs.com).

3.5. 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.

3.6. Cytotoxicity Measurements

Proper cytotoxicity measurements are critical for effectively evaluating chemicals for genotoxic potential, and for striking a balance between assay sensitivity and specificity. Even so, it is not the goal of this MicroFlow Kit instruction manual to make exact and highly prescriptive recommendations for measuring cytotoxicity, or for setting an MN assay cytotoxicity limit. That contradicts our view that there are a number of viable and potentially appropriate strategies for accomplishing these goals. Thus, each laboratory should develop a strategy that makes sense given their cell line of choice, laboratory equipment, and other considerations. Ultimately, a laboratory's cytotoxicity strategy should be well articulated and supported by experimental data generated at their own facility. To help with the development of a strategy, some general information is provided below.

The most recent MN OECD Test Guideline (no. 487) has a stated preference for cytotoxicity measurements that ensure a sufficient number of cells underwent division over the course of treatment. This explains the Test Guideline's preference for Relative Increased Cell Counts (RICC) or Relative Population Doubling (RPD) for cytotoxicity assessments when cytochalasin B is not used to arrest cells in a binucleated state.

Note that RICC and RPD both require cell (or nuclei) density measurements at two time points, one when treatment with test article is initiated, and one when cells (or nuclei) are harvested for MN analysis. As indicated in the OECD Test Guideline, the density of cells or the density of nuclei can be used for RICC and RPD calculations.

While some flow cytometers are capable of directly ascertaining cell (or nuclei) densities, others are not. In the latter case, there are two general approaches for performing these measurements:

- One approach is to use extra cells at time of treatment and again at time of harvest to make density measurements using a hemocytometer or other counting device. Depending on the device, there may or may not be enough cells in the wells of a post-treatment plate to perform both the cytotoxicity measurement and to process remaining cells for MN analysis. If the device requires too much volume or numbers of cells, then the use of a similarly-treated sister plate may provide the extra cells necessary for density measurements.
- A second approach makes use of fluorescent microspheres at a known density. In this manual, these fluorescent microspheres are referred to as "Counting Beads." By understanding the density of the Counting Beads, it is possible to calculate the density of cells or nuclei that are present in a Counting Bead-containing sample. In this scenario, one would simply make a Counting Bead density measurement using some reliable approach, and this can be used to infer cell (or nuclei) densities in experimental samples that contain Counting Beads. The advantage of this approach is that it is possible to determine nuclei densities in the same flow cytometric analyses that are used to score MN frequencies.

Finally, it should be pointed out that early in the development of the *In Vitro* MicroFlow assay, relative nuclei counts (RNC) at one time point (when cells are harvested for MN scoring) were used as the primary cytotoxicity endpoint. On initial consideration, this might be thought of as akin to relative cell counts, a cytotoxicity measurement that is not preferred by the current OECD Test Guideline due to the lack of proof of cell proliferation. However, there are several differences when using RNC with MicroFlow that should be appreciated. First, flow cytometric gating strategies omit potentially confounding unhealthy cells' nuclei from the analysis. These gating strategies consider forward scatter characteristics, side scatter characteristics, and permeability to the dye ethidium monoazide (EMA). Under these circumstances, RNC represent a more sensitive endpoint of cytotoxicity as compared to many methods that simply count whole cells, such as those that rely on Coulter counting. Second, qualitative information on cell cycle is simultaneously acquired with MN counts. Thus, expected cell cycle distributions in solvent-treated cultures can be confirmed to historical observations within the test facility, thereby providing evidence of cell proliferation.

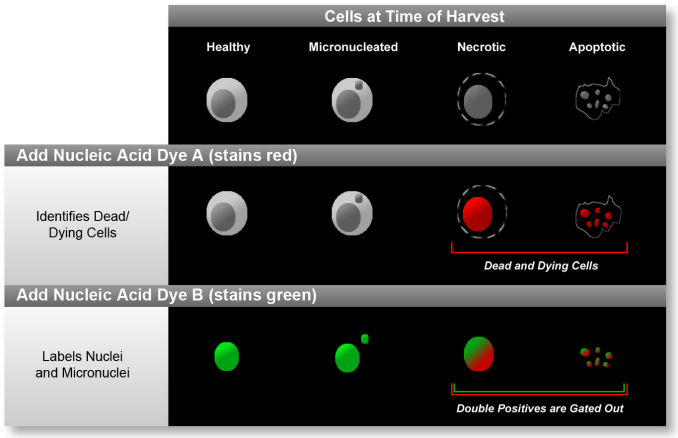
The use of EMA dye to stain the chromatin of dead and dying cells has been used throughout the development and commercialization of the *In Vitro* MicroFlow assay. It is therefore well documented that EMA-positive events provides a supplemental index of cytotoxicity, one that is especially sensitive to apoptosis. It follows then that MN increases that are accompanied by large increases in EMA-positive events should be interpreted with caution, as they may be the result of overt cytotoxicity as opposed to *bona fide* test article-induced genotoxicity.

Additional information regarding cytotoxicity measurements appears in Section 11.

4. Introduction

The *In Vitro* MicroFlow Kit provides a highly efficient flow cytometric method for scoring micronuclei 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 micronuclei in tubes rather than 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., 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 interpreting the context in which MN induction may have occurred. These endpoints are:

- **Cytotoxicity:** Cytotoxicity measurements such as RICC, RPD, and RNC can be accomplished directly and concurrently with MN scoring so long as the flow cytometer is capable of volumetric counting. Most modern flow cytometers are capable of this. In those cases where a flow cytometer is not equipped to directly measure nuclei density, one can make use of fluorescent microspheres at a known density, i.e., Counting Beads. By determining the density of Counting Beads, for instance via a Coulter counter, hemocytometer, or other measuring device, it is possible to calculate the density of nuclei that are present in a Counting Bead-containing sample. This represents another approach that one can use to simultaneously determine RICC or RPD or RNC along with MN frequency.

- **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 metric 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. Note: volumes indicated below are based on 24 well plates. Depending on your format and number of cells, it may be necessary to scale these volumes to accommodate different numbers of cells, and, in the case of attachment cells, different surface areas).

5.1. 1X Buffer Solution

Number of samples	Volume of dH_2O	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 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
1	0.33 mL	3.3 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)
1	0.55 mL	2.2 μL	2.75 μL	About 2.5 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.

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 ambient 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
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.

3. Protect Complete Lysis Solution 2 from light and store at ambient temperature until use.

6. Suspension Cell Protocol 1: Equal Number of Cells Processed

In this protocol, treated cells are harvested, counted, then the ***same number*** of cells in each culture are moved forward toward flow cytometric MN processing and scoring. In this scenario, cytotoxicity in the form of RCC, RICC, RPD, etc., must be derived from these analyses that are separate from MN scoring.

Depending on your cytotoxicity strategy and the volume of cell culture required for these analyses, it may be important to: i) collect cell (or nuclei) density measurements shortly before treatment with test chemical(s) takes place, and/or ii) seed cells into an extra vessel that will be treated with test chemical(s) in the very same way plates dedicated to MN analysis occurs. As described in Sections 3.6 and 11, these measurements can be useful for calculating certain cytotoxicity metrics.

Note that the volumes indicated below are based on 24 well plates. Depending on your format and number of cells, it may be necessary to scale these volumes to accommodate different numbers of cells.

6.1. Cell Harvest

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

2. Place plate(s) under an inverted microscope. Through visual examination, it may be possible to eliminate from MN scoring overly cytotoxic concentrations. At this time, it is also useful to examine wells for visible precipitate. While OECD Test Guideline 474 recommends analyzing the lowest precipitating concentration, precipitate can cause clogs and thereby interfere with reliable flow cytometer operation. Therefore, exercise caution if you decide to analyze wells at the lowest precipitating concentration. Generally speaking, it is best to eliminate any additional, higher concentration wells that exhibit visible precipitate.

3. Determine cell densities, and aliquot volumes corresponding to 5×10^5 cells into 15 mL centrifuge tubes.

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

5. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellets.

6. Loosen cells by gentle tapping, being careful not to splash the cells too high in the tube.

7. 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 300 µL of Complete Nucleic Acid Dye A Solution to each tube. Gently pipette up and down to mix, making sure all cells come into contact with this solution.

2. Place tubes 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 an LED or fluorescent light source above the tubes (see Appendix C). Expose the samples to visual 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.

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 pellets.

7. Loosen cells by gentle tapping, being careful not to splash the cells too high in the tube. 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 500 µL to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. (Do not use a high vortex speed, as metaphase chromosomes can break loose at these higher speeds.) Set the tube in a rack at ambient temperature; protected from light.

2. Repeat for each of the remaining samples.

3. Incubate the samples for one hour at ambient temperature while protected from light.

4. Add 500 µL Complete Lysis Solution 2 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. Set the tube in a rack at ambient temperature; protect from light.

5. Incubate the samples for 30 minutes at ambient temperature; protect from light.

6. After incubation, stained samples are ready for flow cytometric analysis. Samples can be stored in capped 15 mL centrifuge tubes for up to 24 hours at ambient temperature, protected from light, until flow cytometric analysis occurs. If samples cannot be analyzed within this time frame, it is best to refrigerate the samples (for up to 72 hours). In this case, allow samples to equilibrate to ambient temperature before flow cytometric analysis occurs.

7. Suspension Cell Protocol 2: Equal Volume of Cells Processed

In this protocol, treated cells are harvested, optionally counted, and then the ***same volume*** of cells in each culture are moved forward toward flow cytometric MN processing and scoring. In this scenario, cytotoxicity in the form of RCC, RICC, RPD, etc., may be derived from analyses that are separate from MN scoring, or they may be derived from the same flow cytometric scoring that is performed to accomplish MN frequency measurements.

Depending on your cytotoxicity strategy and the volume of cell culture required for these analyses, it may be important to: i) collect cell (or nuclei) density measurements shortly before treatment with test chemical(s) takes place, and/or ii) seed cells into an extra vessel that will be treated with test chemical(s) in the very same way plates dedicated to MN analysis occurs. As described in Sections 3.6 and 11, these measurements can be useful for calculating certain cytotoxicity metrics.

Note that the volumes indicated below are based on 24 well plates. Depending on your format and number of cells, it may be necessary to scale these volumes to accommodate different numbers of cells.

7.1. Cell Harvest

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

2. Place plate(s) under an inverted microscope. Through visual examination, it may be possible to eliminate from MN scoring overly cytotoxic concentrations. At this time, it is also useful to examine wells for visible precipitate. While OECD Test Guideline 474 recommends analyzing the lowest precipitating concentration, precipitate can cause clogs and thereby interfere with reliable flow cytometer operation. Therefore, exercise caution if you decide to analyze wells at the lowest precipitating concentration. Generally speaking, it is best to eliminate any additional, higher concentration wells that exhibit visible precipitate.

3. Optionally, determine cell densities at this point (these can be used for RCC, RPD, and other cytotoxicity determinations). Then, transfer a consistent volume of cells into 15 mL centrifuge tubes. This consistent volume should equate to approximately 5 x 10^5 cells in the solvent control cultures.

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

5. Slowly and carefully aspirate the supernatants, taking care not to disturb the pellets.

6. Loosen cells by gentle tapping, being careful not to splash the cells too high in the tube.

7. Place samples on wet ice for 20 minutes before continuing to Section 7.2.

7.2. Complete Nucleic Acid Dye A Staining

1. Carefully add 300 μL of Complete Nucleic Acid Dye A Solution to each tube. Gently pipette up and down to mix, making sure all cells come into contact with this solution.

2. Place tubes 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 an LED or fluorescent light source above the tubes (see Appendix C). Expose the samples to visual 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.

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 pellets.

7. Loosen cells by gentle tapping, being careful not to splash the cells too high in the tube. 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 500 μL to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. (Do not use a high vortex speed, as metaphase chromosomes can break loose at these higher speeds.) Set the tube in a rack at ambient temperature; protected from light.

2. Repeat for each of the remaining samples.

3. Incubate the samples for one hour at ambient temperature while protected from light.

4. Add 500 μL Complete Lysis Solution 2 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. Set the tube in a rack at ambient temperature; protect from light.

5. Incubate the samples for 30 minutes at ambient temperature; protect from light.

6. After incubation, stained samples are ready for flow cytometric analysis. Samples can be stored in capped 15 mL centrifuge tubes for up to 24 hours at ambient temperature, protected from light, until flow cytometric analysis occurs. If samples cannot be analyzed within this time frame, it is best to refrigerate the samples (for up to 72 hours). In this case, allow samples to equilibrate to ambient temperature before flow cytometric analysis occurs.

7. Since an equal volume of cells were processed for each sample, it is possible to derive cytotoxicity metrics from these same samples that have been prepared for MN scoring (see Section 11).

8. Attachment Cell Protocol Number 1: No Accutase

Two protocols for collecting and preparing attachment-type cell lines for MN analysis are described. Protocol Number 1 (this section) simply relies on Complete Lysis Solutions 1 and 2 to completely liberate cells' nuclei for staining and analysis. So long as all cells are lifted and lysed effectively by these reagents, this is the simpler, recommended protocol. At Litron, we have found that this protocol is effective for preparing CHO-K1 and V79 cells for MN analysis. In this scenario, cytotoxicity in the form of RCC, RICC, RPD, etc., can either be derived from sister-plates with like-treated cells using any preferred and reliable counting device, or else simultaneous with MN scoring based on the density of liberated nuclei.

It is important for attachment cell lines to fully attach and recover after initially seeding. Therefore, it is usually best to seed vessels and then re-incubate them overnight to allow the cells to attach and reenter an exponential growth phase. When choosing a seeding density, 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.

Depending on your cytotoxicity strategy and the volume of cell culture required for these analyses, it may be important to: i) collect cell (or nuclei) density measurements shortly before treatment with test chemical(s) takes place, and/or ii) seed cells into an extra vessel that will be treated with test chemical(s) in the very same way plates dedicated to MN analysis occurs. As described in Sections 3.6 and 11, these measurements can be useful for calculating certain cytotoxicity metrics. For additional useful advice about the use of attachment cells, see the paper by Bemis et al., 2016.

Note that the volumes indicated below are based on 24 well plates. Depending on your format and number of cells, it may be necessary to scale these volumes to accommodate different numbers of cells and surface culture vessel surface areas.

8.1. Cell Harvest

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

2. Place plate(s) under an inverted microscope. Through visual examination, it may be possible to eliminate from MN scoring overly cytotoxic concentrations. At this time, it is also useful to examine wells for visible precipitate. While OECD Test Guideline 474 recommends analyzing the lowest precipitating concentration, precipitate can cause clogs and thereby interfere with reliable flow cytometer operation. Therefore, exercise caution if you decide to analyze wells at the lowest precipitating concentration. Generally speaking, it is best to eliminate any additional, higher concentration wells that exhibit visible precipitate.

3. Place samples on wet ice for 20 minutes before continuing to Section 8.2.

8.2. Complete Nucleic Acid Dye A Staining

1. Aspirate off treatment media and add 300 μL of Complete Nucleic Acid Dye A Solution to each well. Make sure that the entire well surface is covered with this solution. Leave the plate cover off.

2. Place an LED or fluorescent light source above the plate (see Appendix C). Expose the samples to visual light for 30 minutes, with samples remaining on ice throughout this period of time.

3. Turn off the visible light source, remove plates from ice, and aspirate off Complete Nucleic Acid Dye A Solution.

4. Add 3 mL of cold 1X Buffer Solution to each well. Aspirate off the 1X Buffer Solution from each well. Proceed immediately to section 8.3.

8.3. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Vortex or resuspend Complete Lysis Solution 1 and add 500 μL to each well. Gently mix the plate for 5 seconds on a vortex mixer set low enough to prevent the solution splashing out of the wells.

2. Incubate the samples for one hour at 37 °C; protect from light.

3. Add 500 μL Complete Lysis Solution 2 to each well. Gently rock the plate.

4. Incubate the samples for 30 minutes at ambient temperature; protect from light.

5. After incubation, stained samples are ready for flow cytometric analysis. Samples can be stored for up to 24 hours at ambient temperature, protected from light, until flow cytometric analysis occurs. It is best to cover plates with a mat during storage to prevent evaporation (see Appendix F). If samples cannot be analyzed within this time frame, it is best to refrigerate the samples (for up to 72 hours). In this case, allow samples to equilibrate to ambient temperature before flow cytometric analysis occurs.

6. Since an equal volume of cells were processed for each sample, it is possible to derive cytotoxicity metrics from these same samples that have been prepared for MN scoring (see Section 11).

9. Attachment Cell Protocol Number 2: Using Accutase

Two protocols for collecting and preparing attachment-type cell lines for MN analysis are described. Protocol Number 2 (i.e., this Section) relies on Accutase® to lift cells from the culture plate prior to liberating cells' nuclei with Complete Lysis Solutions 1 and 2. At Litron, we have found that this protocol is more effective than Protocol Number 1 for preparing HepG2 cells for MN analysis. In this scenario, cytotoxicity in the form of RCC, RICC, RPD, etc., can either be derived after the Accutase step using any preferred and reliable counting device, from sister-plates with like-treated cells using any preferred and reliable counting device, or else simultaneous with MN scoring based on the density of liberated nuclei.

It is important for attachment cell lines to fully attach and recover after initially seeding. Therefore, it is usually best to seed plates and then re-incubate them overnight to allow the cells to attach and reenter an exponential growth phase. When choosing a seeding density, 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.

Depending on your cytotoxicity strategy and the volume of cell culture required for these analyses, it may be important to: i) collect cell (or nuclei) density measurements shortly before treatment with test chemical(s) takes place, and/or ii) seed cells into an extra plate that will be treated with test chemical(s) in the very same way plates dedicated to MN analysis occurs. As described in Sections 3.6 and 11, these measurements can be useful for calculating certain cytotoxicity metrics. For additional useful advice about the use of attachment cells, see the paper by Bemis et al., 2016.

Note that the volumes indicated below are based on 24 well plates. Depending on your format and number of cells, it may be necessary to scale these volumes to accommodate different numbers of cells.

9.1. Cell Harvest

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

2. Place plate(s) under an inverted microscope. Through visual examination, it may be possible to eliminate from MN scoring overly cytotoxic concentrations. At this time, it is also useful to examine wells for visible precipitate. While OECD Test Guideline 474 recommends analyzing the lowest precipitating concentration, precipitate can cause clogs and thereby interfere with reliable flow cytometer operation. Therefore, exercise caution if you decide to analyze wells at the lowest precipitating concentration. Generally speaking, it is best to eliminate any additional, higher concentration wells that exhibit visible precipitate.

3. Remove the treatment media from the wells. Some groups use an aspirating device, others empty plate contents by flicking onto an absorbent pad. In the latter case, it is important to use an impenetrable plastic-backed pad, and to immediately dispose of it properly.

4. Rinse the cells with 500 μL of 1X PBS. Remove the PBS from the wells, as in step 3, above.

5. Add 500 μL of Accutase® and incubate cells for 10 minutes in the dark at 37 ºC.

6. After incubation, add 1.5 mL of media, pipette cells up and down 6 to 8 times and transfer to new 15 mL centrifuge tubes. Optionally, determine cell densities at this point (these can be used for RCC, RPD, and other cytotoxicity determinations).

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

8. Slowly and carefully aspirate the media, taking care not to disturb the pellets.

9. Add 5 mL of media, mix cells by pipetting a few times.

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

11. Slowly and carefully aspirate the media, taking care not to disturb the pellets.

12. Loosen cells by gentle tapping, being careful not to splash the cells too high in the tube .

13. Place samples on wet ice for 10 minutes before continuing to Section 9.2.

9.2. Complete Nucleic Acid Dye A Staining

1. Carefully add 300 μL of Complete Nucleic Acid Dye A Solution to each tube. Gently pipette up and down to mix, making sure all cells come into contact with this solution.

2. Place tubes 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 an LED or fluorescent light source above the tubes (see Appendix C). Expose the samples to visual 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.

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 pellets.

7. Loosen cells by gentle tapping, being careful not to splash the cells too high in the tube. Proceed immediately to Section 9.3.

9.3. Simultaneous Cell Lysis and Nucleic Acid Dye B Staining

1. Vortex or resuspend Complete Lysis Solution 1 and add 500 μL to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. (Do not use a high vortex speed, as metaphase chromosomes can break loose at these higher speeds.) Set the tube in a rack at ambient temperature; protected from light.

2. Repeat for each of the remaining samples.

3. Incubate the samples for one hour at ambient temperature while protected from light.

4. Add 500 μL Complete Lysis Solution 2 to the first sample. Immediately vortex the sample on low to medium speed for 5 seconds. Set the tube in a rack at ambient temperature; protect from light.

5. Incubate the samples for 30 minutes at ambient temperature; protect from light.

6. After incubation, stained samples are ready for flow cytometric analysis. Samples can be stored in capped 15 mL centrifuge tubes for up to 24 hours at ambient temperature, protected from light, until flow cytometric analysis occurs. If samples cannot be analyzed within this time frame, it is best to refrigerate the samples (for up to 72 hours). In this case, allow samples to equilibrate to ambient temperature before flow cytometric analysis occurs.

7. Since an equal volume of cells were processed for each sample, it is possible to derive cytotoxicity metrics from these same samples that have been prepared for MN scoring (see Section 11).

10. 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.
- Prior to analyzing experimental samples, it is recommended that you analyze a solvent control sample and a positive control sample first to verify that the template and instrument settings are appropriate.
- Protect samples from light. Ensure samples have equilibrated to ambient temperature before data acquisition occurs.

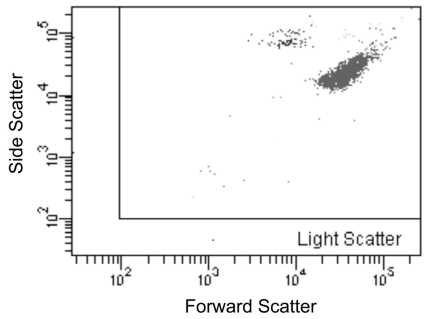
1. Before analyzing samples, ensure that the flow cytometer is working properly. Follow the manufacturer's instructions for the appropriate setup and quality control procedures.

2. 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.

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.

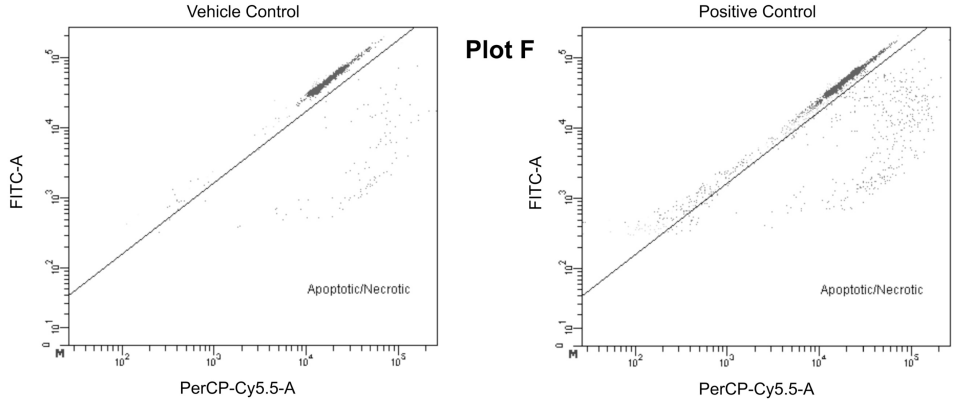
4. Place the plate on the flow cytometer. Using a solvent control culture for setup, acquire sample in "set-up mode". Adjust FSC and SSC voltages to bring nuclei into view as shown in Plot A, right. The bottom left edge of the nuclei events should be approximately 2 logs higher in FSC and SSC than the lower bounds of the region.

Plot A

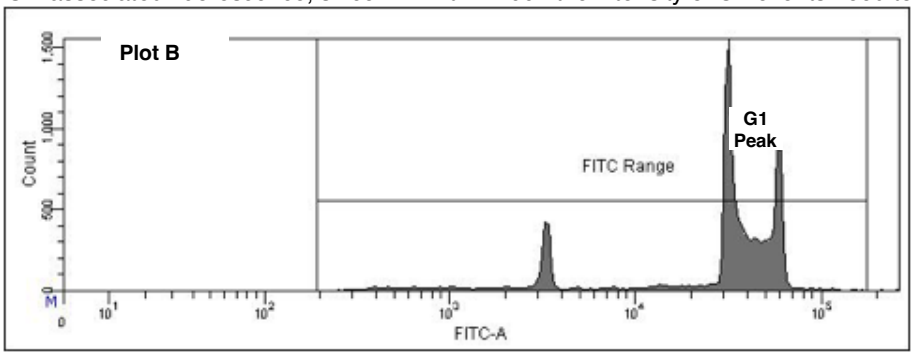


5. Adjust PerCP PMT voltage (EMA fluorescence) until the majority of the nuclei are above the Apoptotic/Necrotic region, as shown below. There should be nearly a log of fluorescent resolution between nuclei from healthy and dead cells. Representative plots showing EMA staining characteristics of a vehicle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

Plot 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.



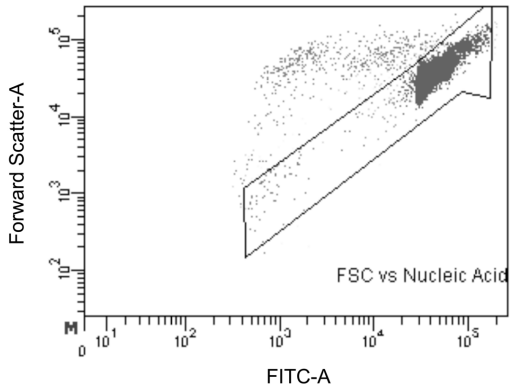
7. Set the threshold parameter (also referred to as the acquisition trigger) to FITC (FL1) fluorescence (i.e., SYTOX Green). Adjust the threshold so that some events are collected that fall just to the left edge of the FITC (FL1) range

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.

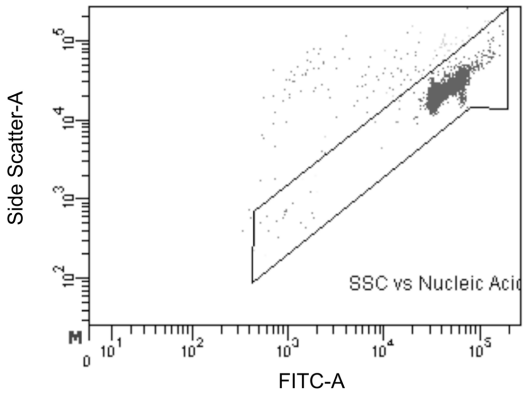
9. Adjust the position of the "FSC vs. Nucleic Acid Dye B" region until nuclei are positioned as shown in Plot D.

Plot D



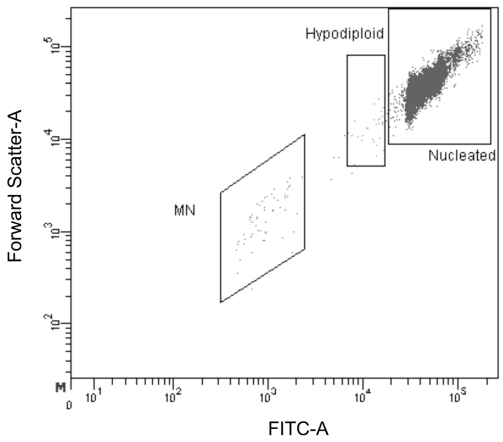
10. Adjust the position of the "SSC vs. Nuclei Acid Dye B" region until nuclei are positioned as shown in Plot E.

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: Some BD users may want to consider including Time in their gating logic

so that the first several seconds of analysis for each well are omitted from the data files. This can help in those cases where the FITC signal requires several seconds per sample to stabilize. See Appendix A.

14. Acquire data for the sample in its entirety and proceed to the next sample.

11. Calculations

1. Percent MN

$$\%\ MN = \frac{\text{No. MN Events*}}{\text{No. Nucleated Events*}} \times 100$$

*Note: these are Nucleic Acid Dye A (EMA) negative events that have been appropriately gated on light scatter and Nucleic Acid Dye B (SYTOX Green) fluorescence characteristics, as shown below.

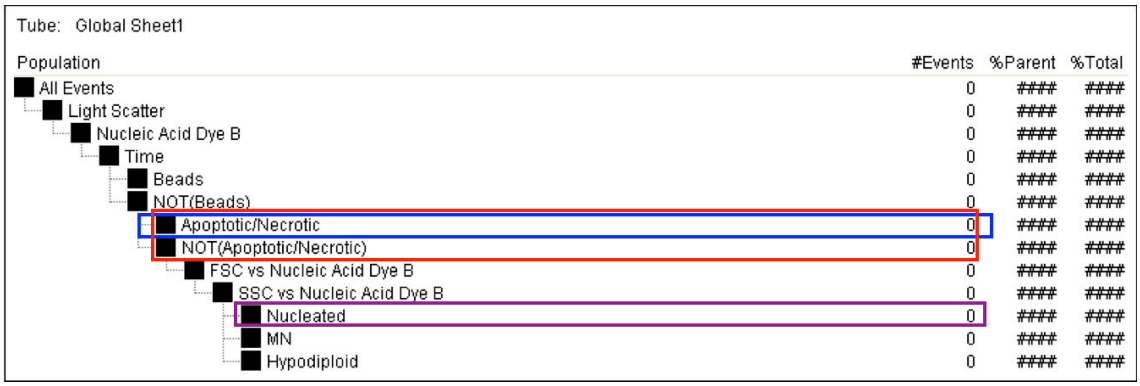
Examples:

$$\%\ MN\ (Solvent) = \frac{27\ \text{MN Events}}{9242\ \text{Nucleated Events}} \times 100 = 0.29\ \%$$

$$\%\ MN\ (Pos.\ control) = \frac{73\ \text{MN Events}}{9097\ \text{Nucleated Events}} \times 100 = 0.80\ \%$$

2. Percent EMA-Positive

The fraction of EMA-positive chromatin is responsive to cell death - both necrosis but especially apoptosis (since a single apoptotic cell can generate numerous EMA-positive chromatin events). There are a number of ways one could potentially devise a biomarker of cytotoxicity based on EMA staining. In general, we recommend using a numerator that equates to the number of EMA-positive chromatin events, irrespective of whether these events fall within the two regions “FSC vs Nucleic Acid B” and “SSC vs Nucleic Acid Dye B”. See blue rectangle encompassing “Apoptotic/Necrotic” events, below.



Regarding the denominator, one can use the number of "Nucleated" events, as shown by the purple rectangle, above. Alternately, one can use the number of "EMA-positive" and "EMA-negative" events. This is shown as a red rectangle encompassing both "Apoptotic/Necrotic" events and "NOT (Apoptotic/Necrotic)" events, above.

$$\%\ \text{EMA-Positive} = \frac{\text{No. Apoptotic/Necrotic Events}}{\text{No. Nucleated Events}} \times 100$$

or

$$\%\ \text{EMA-Positive} = \frac{\text{No. Apoptotic/Necrotic Events}}{\text{No. Apoptotic/Necrotic Events} + \text{No. NOT (Apoptotic/Necrotic) Events}} \times 100$$

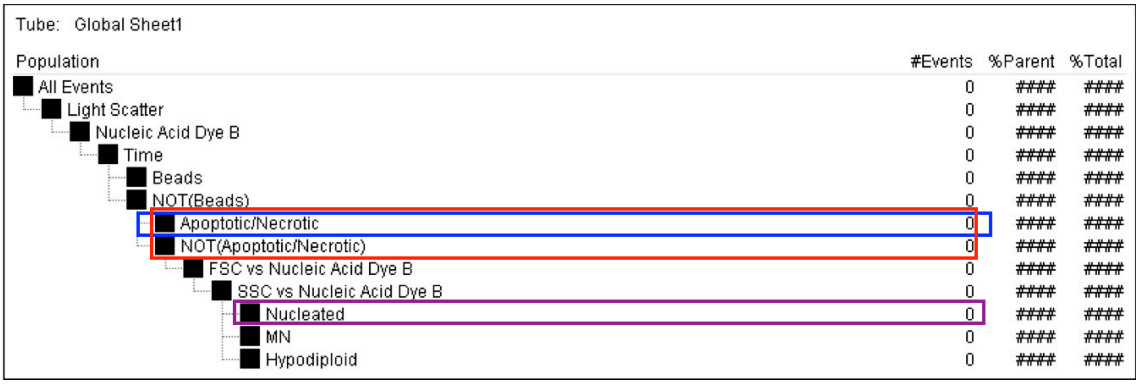
Example:

$$\%\ \text{EMA-Positive} = \frac{\text{353 Apoptotic/Necrotic Events}}{\text{8982 Nucleated Events}} \times 100 = 3.93\ \%$$

3. Percent EMA-Positive Fold Change

This fold change value is for an individual culture (numerator) relative to the mean concurrent solvent control cultures (denominator). Since an apoptotic cell can generate many EMA-positive events, this metric is particularly responsive to test article-induced apoptosis.

$$\text{EMA-Positive Fold Change} = \frac{\%\ \text{EMA-Positive}}{\text{Solvent Control Mean \% EMA-Positive}}$$



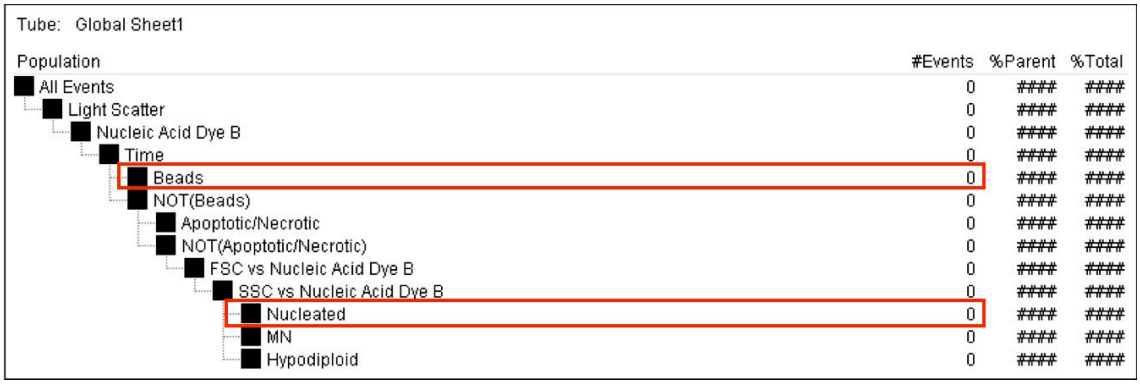
Example:

$$\text{EMA-Positive Fold Change} = \frac{\text{10.02 \% EMA-Positive}}{\text{3.40 \% EMA-Positive Solvent Control Mean}} = 2.95 \text{ fold}$$

4. Nuclei to Bead Ratio (NBR)

$$\text{NBR} = \frac{\text{No. Nucleated Events*}}{\text{No. Beads Events}}$$

*Note: these are Nucleic Acid Dye A (EMA) negative events that have been appropriately gated on light scatter and Nucleic Acid Dye B (SYTOX Green) fluorescence characteristics, as shown below.



Example:

$$\text{NBR} = \frac{\text{9097 Nucleated Events}}{\text{835 Beads Events}} = 10.89$$

5. Percent Relative Nuclei Count (% RNC)

This value is derived from NBR and considers an individual culture relative to the concurrent solvent control cultures mean value. This measurement is generally taken at time of harvest, i.e., when MN are being scored.

$$\text{\% RNC} = \frac{\text{NBR}}{\text{Solvent Control Mean NBR}}$$

Example:

$$\text{\% RNC} = \frac{\text{9.89 NBR}}{\text{12.58 Solvent Control Mean NBR}} \times 100 = 78.6 \text{ \%}$$

6. Percent Cytotoxicity, Based on % RNC

This value is derived from % RNC and considers an individual culture relative to the concurrent solvent control cultures mean value. This measurement is generally taken at time of harvest, i.e., when MN are being scored. For this metric values ≤ 0 % represent no cytotoxicity, and values ≥ 100 % represent maximal cytotoxicity.

$$\text{\% Cytotoxicity} = 100 - \text{\% RNC}$$

Example:

$$\text{\% Cytotoxicity} = 100 - 78.6 \text{ \%} = 21.4 \text{ \%}$$

7. Percent Relative Increased Cell Count (RICC)

   This formula comes from OECD Test Guideline 487. As noted in the Guideline, "when using automated systems, for instance flow cytometry, laser scanning cytometry or image analysis, the number of cells in the formula can be substituted by the number of nuclei".

   It should be apparent that when using number of cells (or nuclei) as suggested by this formula, the volume of cells (or nuclei) must be held constant.

   Another way to approach this calculation is to substitute a cell (or nuclei) number with a cell (or nuclei) to Counting Bead ratio. Note that this is only valid if the same Counting Bead solution has been used throughout the experiment.

$$\% \text{ RICC} = \frac{\textit{Final} \text{ Number of Cells} - \textit{Starting} \text{ Number of Cells}}{\text{Mean } \textit{Final} \text{ Number of Cells for Solvent Controls} - \text{Mean } \textit{Starting} \text{ Number of Cells for Solvent Controls}} \times 100$$

   Example:

$$\% \text{ RICC} = \frac{7.5 - 2.0}{8.0 - 2.0} \times 100 = 84.6\ \%$$

8. Percent Cytotoxicity, Based on RICC

   This value is derived from RICC and considers an individual culture relative to the concurrent solvent control cultures mean value. This measurement is generally taken at time of harvest, i.e., when MN are being scored. For this metric values ≤ 0 % represent no cytotoxicity, and values ≥ 100 % represent maximal cytotoxicity.

$$\% \text{ Cytotoxicity} = 100 - \text{RICC}$$

   Example:

$$\% \text{ Cytotoxicity} = \times 100 - 84.6 = 15.4\ \%$$

9. Number of Population Doublings (PD)

   This formula comes from OECD Test Guideline 487. As noted in the Guideline, "when using automated systems, for instance flow cytometry, laser scanning cytometry or image analysis, the number of cells in the formula can be substituted by the number of nuclei".

   It should be apparent that when using number of cells (or nuclei) as suggested by this formula, the volume of cells (or nuclei) must be held constant.

   Another way to approach this calculation is to substitute a cell (or nuclei) number with a cell (or nuclei) to Counting Bead ratio. Note that this is only valid if the same Counting Bead solution has been used throughout the experiment.

$$PD = \frac{1}{\text{Log}_{10}2} \times \text{Log10} \frac{\text{Final Number of Cells}}{\text{Starting Number of Cells}}$$

   Example:

$$PD = \frac{1}{\text{Log}_{10}2} \times \text{Log10} \frac{8}{2} = 2$$

10. Percent Relative Population Doublings (RPD)

    This value is derived from RPD and considers an individual culture relative to the concurrent solvent control cultures mean value. This measurement is generally taken at time of harvest, i.e., when MN are being scored.

$$\%\ \text{RPD} = \frac{\text{PD}}{\text{Mean Solvent Control PD}} \times 100$$

Example:

$$\%\ \text{RPD} = \frac{1.5}{2} \times 100 = 75\ \%$$

11. Percent Cytotoxicity, Based on RPD

    This value is derived from RPD and considers an individual culture relative to the concurrent solvent control cultures mean value. This measurement is generally taken at time of harvest, i.e., when MN are being scored. For this metric values ≤ 0 % represent no cytotoxicity, and values ≥ 100 % represent maximal cytotoxicity.

$$\%\ \text{Cytotoxicity} = 100 - \text{RPD}$$

Example:

$$\%\ \text{Cytotoxicity} = 100 - 75 = 25\ \%$$

12. Troubleshooting

Observation	Possible Cause	Suggestion
Solvent control cultures exhibit unusually high MN or EMA-positive frequencies.	Cells may not be fully recovered from thaw.	Wait at least one week (2 weeks for TK6 cells) before treatment to allow cells to fully recover.
	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.
	Cells are overgrown.	Ensure that optimal cell growth conditions are maintained before and during treatment.
Positive controls not showing a dose-related response.	Cells may have been harvested too early, resulting in too few cell divisions for MN to be expressed.	Lack of an expected response can sometimes be attributed to a suboptimal cell harvest time. Generally speaking, harvest should occur within 1.5 to 2 normal cell cycles. If positive controls still do not respond after ensuring harvest time is correct, contact Litron for assistance.
FITC channel values shift over time.	Some instruments' FITC channel does not stabilize until several seconds of analysis have occurred.	Use a "Time gate" to omit data collected over the first several seconds of data acquisition.
Solvent and positive control cultures exhibit unusually low MN frequencies.	MN events are being thresholded out.	Ensure that threshold setting(s) are not eliminating MN events from data acquisition. For instance, it is important that a FITC channel threshold setting does not exclude events with ≥ 1/100 the DNA-dye associated fluorescence of 2N nuclei.

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14. License Agreement and Limited Product Warranty

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This warranty limits our liability to replacement of this product. Litron shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product. This product is for research purposes only and is not intended for clinical or diagnostic use.

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 below.

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

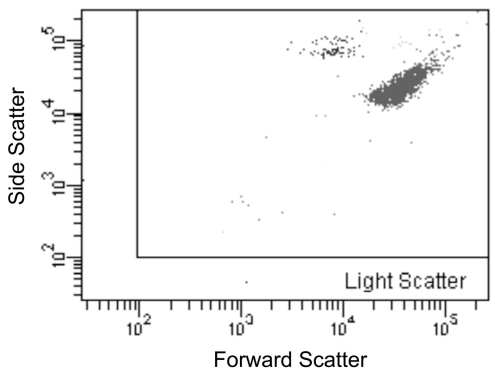
Population	#Events	%Parent	%Total
All Events	0	####	####
Light Scatter	0	####	####
Nucleic Acid Dye B	0	####	####
Time	0	####	####
Beads	0	####	####
NOT(Beads)	0	####	####
Apoptotic/Necrotic	0	####	####
NOT(Apoptotic/Necrotic)	0	####	####
FSC vs Nucleic Acid Dye B	0	####	####
SSC vs Nucleic Acid Dye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

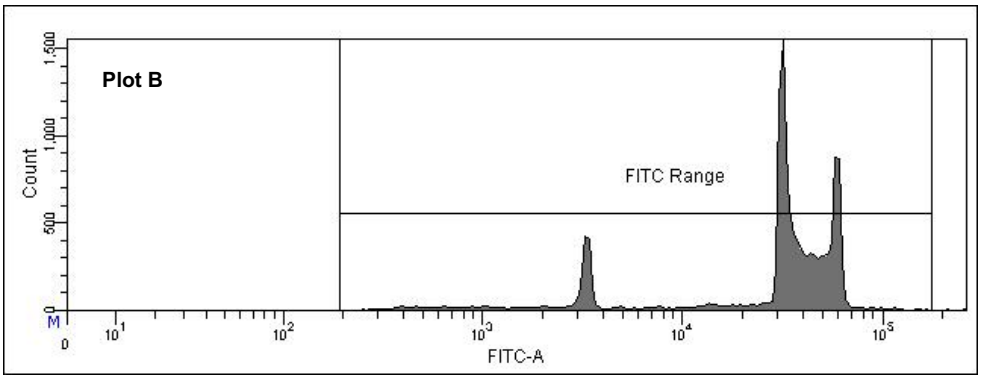
5. Set the Storage Gate to Nucleated in Plot G. In conjunction with the Time Histogram shown in step 14, this gating logic excludes the first few seconds of data from each culture. This strategy is important for BD-brand HTS users, as fluorescence signals tend to require several seconds before they stabilize.

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.

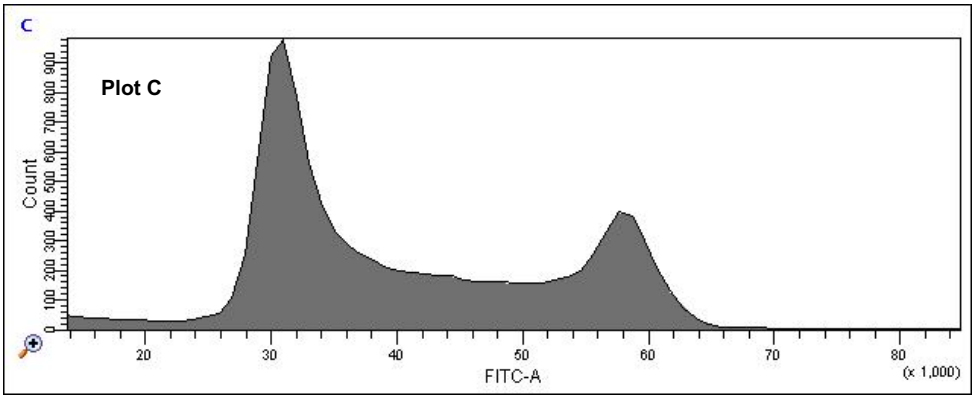
Plot A



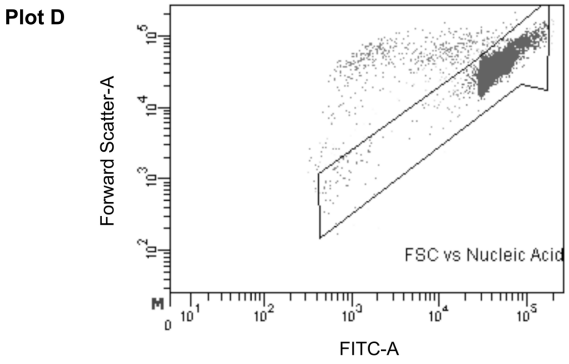
7. The "FITC Range" region should include nuclei as well as sub-2n chromatin that exhibit up to 1/100th the SYTOX fluorescence signal of 2n nuclei.



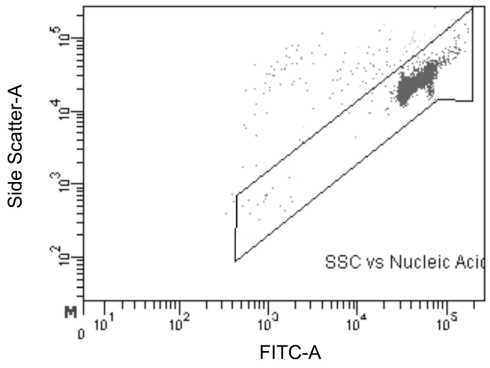
8. By examining the Nuclei Acid Dye B range with a linear FITC-A scaling in Plot C, one can observe cell-cycle positions.



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.

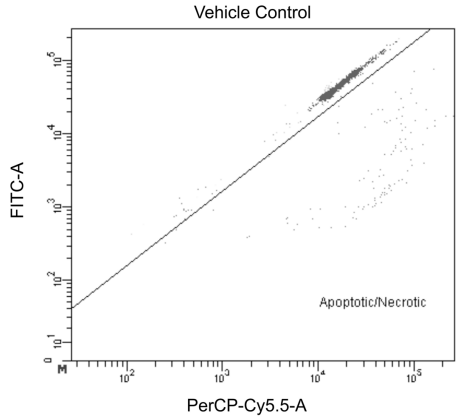


10. Much of the chromatin associated with dead/dying cells falls above an appropriately located "SSC versus Nucleic Acid Dye B" region in Plot E.



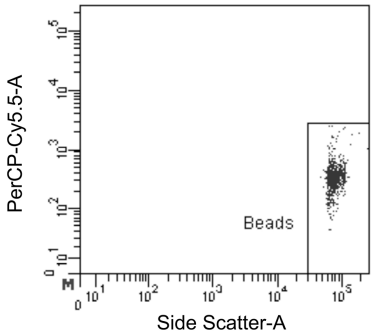
11. A gate based on an appropriately positioned “Apoptotic/Necrotic” region in Plot F is used to exclude the chromatin of dead/dying cells.

Plot F



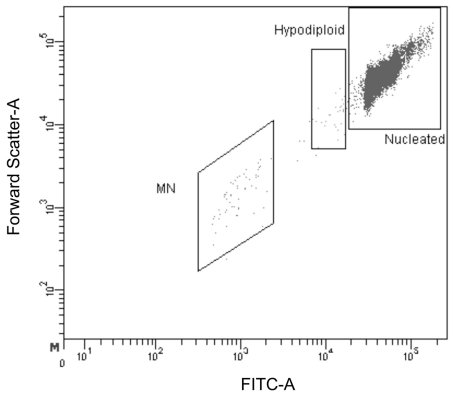
12. The Bead Count Plot allows for the resolution of the counting beads used for the Nuclei-to-Bead Ratio calculation or other cytotoxicity metrics.

Bead Plot



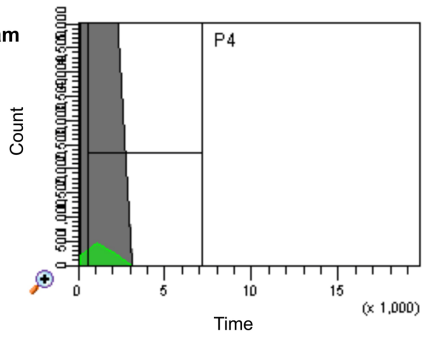
13. Only nuclei and micronuclei that meet the multiple characteristics associated with “healthy cells” are used to calculate MN frequency.

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: 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 3.6). 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 percent EMA-positive 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 percent EMA-positive fold change is less than or equal to 4.

Appendix C: Placement During Nucleic Acid Dye A Photoactivation

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

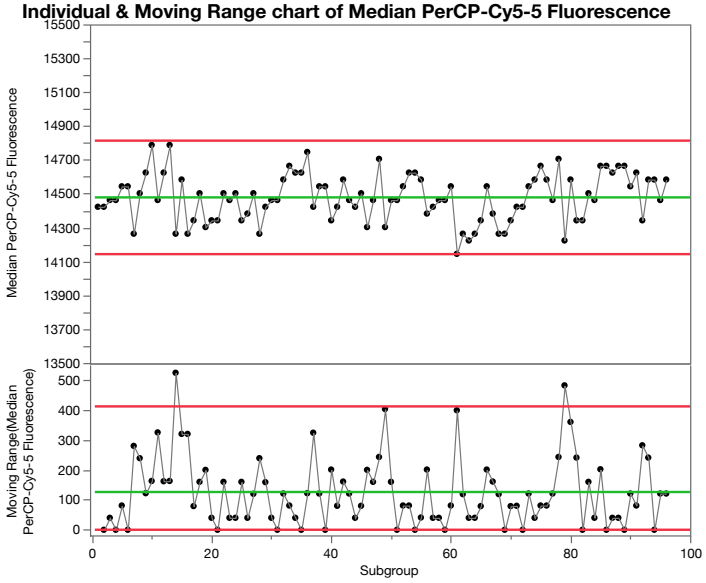
Appendix D: Counting Beads as Quality Control Particles

Throughout this instruction manual we have referred to fluorescent latex microspheres as “Counting Beads.” It is true that they can serve an important role facilitating cell (or nuclei) density measurements, especially for flow cytometers that are not equipped to make volumetric counts.

However, it should also be appreciated that these particles can also serve important quality control functions. Since they are highly uniform in terms of size, light scatter, and fluorescence intensities, they can give kit users important insights into the behavior of the flow cytometer, as well as elements of benchtop processing.

One instructive example is provided below. The top panel shows an “I-type control chart.” The Y-axis displays median bead fluorescence intensity (PerCP-Cy5-5) for each in vitro MicroFlow sample analyzed on a particular day. The X axis shows each of the individual samples analyzed on a particular day, in order of acquisition, left to right. In this example, there is no drift with respect to time, and the level of variation is within the expected range. Together, this information provides evidence that the instrumentation and other elements of sample processing were “under control.”

Thus, Counting Beads should be viewed as a powerful quality control tool, one that is incorporated into each well that is analyzed.



Median PerCP-Cy5-5 Fluorescence Limit Summaries

Points plotted	LCL	Avg	UCL	Limits Sigma	Subgroup Size
Individual	14143.27	14478.41	14813.54	Moving Range	1
Moving Range	0	126.0526	411.7549	Moving Range	1

Appendix E – Strategies for Dealing with Volatile Chemicals

The volatility of some test chemicals can negatively affect MicroFlow data in adjacent wells. When dealing with volatile chemicals, or test substance that have not be characterized in terms of volatility, it may be useful to treat cells in plates that have a specialized mat applied, or in more extreme cases, to also cover the wells with activated carbon.

Two examples are shown below. Top panel, VWR® Rayon Films for Biological Cultures, from VWR®, cat. no. 60941-086. Bottom panel, the rayon film in combination with Honeywell "R" Replacement Carbon Pre-Filter, from Breathe Naturally, cat. no. HPA300, cut to size. When using the carbon filter, the plate cover is placed on top to hold it in place.