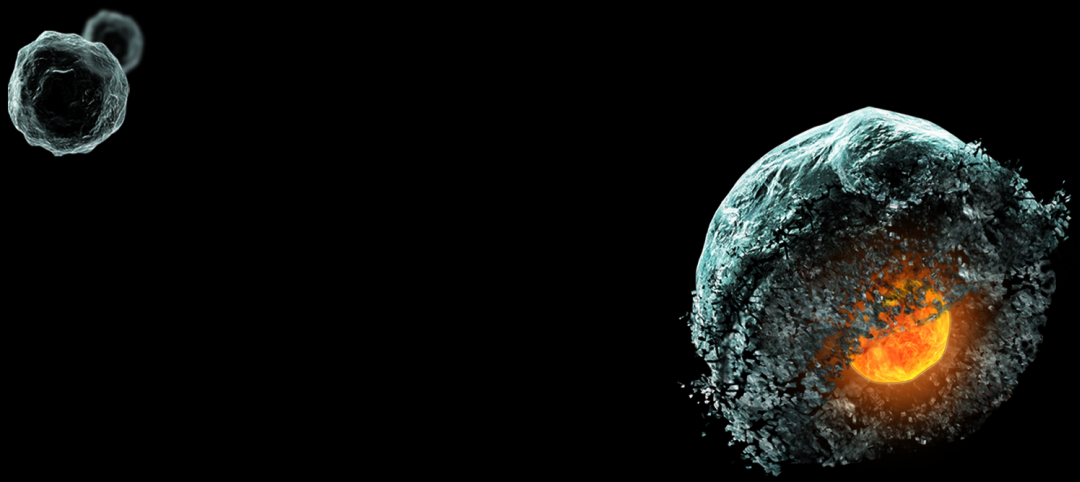
MultiFlow®

Multiple endpoints. One step.



MultiFlow DNA Damage Kit – p53, γH2AX, Phospho-Histone H3, Cleaved PARP

Instruction Manual

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

Table of Contents

1. Materials Provided

Kit Components	Quantity[a]	Storage Condition[b]
Nuclei Release Solution with Counting Beads[c]	22 mL	Ambient, light sensitive
DNA Stain	550 µL	2 °C to 8 °C, light sensitive
RNase Solution	110 µL	2 °C to 8 °C
p53 Antibody FITC (specific for human p53)[d]	110 µL	2 °C to 8 °C, light sensitive
γH2AX Antibody Alexa Fluor® 647 (cross-species compatible)[d]	110 µL	2 °C to 8 °C, light sensitive
Phospho-Histone H3 Antibody PE (cross-species compatible)[d]	44 µL	2 °C to 8 °C, light sensitive
Cleaved PARP Antibody Violet (cross-species compatible)[d, e]	220 µL	2 °C to 8 °C, light sensitive

a. Sufficient materials are provided to analyze up to 384 samples.
b. Please note that although kit components are shipped at ambient temperature, they must be stored at the temperatures indicated above upon receipt.
c. Warning! Irritant. See SDS (available on Litronlabs.com).
d. Warning! May contain sodium azide. See SDS (available on Litronlabs.com).
e. Uses Brilliant™ Violet 421 (BV421) fluorochrome.

2. Additional Materials Required

- Human cells, ideally with functional p53
- Flow cytometer capable of 405 nm, 488 nm and 647 nm excitation, optionally equipped with High Throughput Sampler (HTS) or similar autosampler
- Polypropylene centrifuge tubes (e.g., 15 mL)
- Non-cell-culture-treated, 96 well U-bottom plates for analysis (e.g., Falcon Cat. No. 353910)

For adherent cells:

- Accutase® cell detachment solution (Sigma Cat. No. A6964)
- Fetal bovine serum (FBS), heat-inactivated

Optional but recommended:

- Halt™ Protease and Phosphatase Inhibitor Cocktail (100X) (Thermo Fisher Cat. No. 78440)
- VWR® Rayon Films for Biological Cultures, from VWR®, cat. no. 60941-086
- Activated carbon filter, e.g., Honeywell "R" Replacement Carbon Pre-Filter, from Breathe Naturally, cat. no. HPA300, cut to size

3. Ordering Information and Technical Services

Litron Laboratories
3500 Winton Place
Rochester, New York 14623
Telephone: 585-442-0930
Order Toll Free: 877-4-LITRON (877-454-8766)
Fax: 585-442-0934
Ordering email: info@LitronLabs.com
Technical Support email: InVitroMultiFlowTechSupport@LitronLabs.com
World Wide Web: www.LitronLabs.com

4. First Time Users

We strongly recommend reading the entire instruction manual before performing these procedures. Using flow cytometry to accurately score genotoxic biomarker responses requires careful attention to detail; therefore, do not deviate from the procedures described in this manual. In order to achieve reliable results, it is important that these steps are followed using the reagents supplied with this kit. If you have questions, please contact Litron Laboratories by calling (585) 442-0930, or sending an email to InVitroMultiFlowTechSupport@LitronLabs.com. Training videos and App Notes are available on Litron's website: www.LitronLabs.com. Some additional important considerations for first-time users are listed below.

4.1. Flow Cytometer Templates

Flow cytometer data acquisition templates are available from Litron (www.LitronLabs.com or email InVitroMultiFlowTechSupport@LitronLabs.com) but are specific to BD FACSDiva™ or Miltenyi MACSQuantify™ software. If you are unable to use these data acquisition templates, prepare one PRIOR TO processing samples for analysis. Flow cytometry operators who are not using FACSDiva™ or MACSQuantify™ software will find the plots in this manual valuable for constructing their own data acquisition and analysis template.

4.2. Daily Calibration with Complete Labeling Solution

Optimal fluorescent resolution is critical when scoring biomarker responses and low frequency events. Therefore, daily calibration with Complete Labeling Solution (see Section 6) and its fluorescent beads is crucial for consistently setting appropriate PMT voltages on a day-to-day basis. First-time users of this kit should prepare and analyze Complete Labeling Solution before progressing to experimental samples.

It is preferred that all bead fluorescence values (along with FSC and SSC) be approximately 1/50th of the linear scale of the machine, and PerCP-Cy5 bead fluorescence value should be approximately 1/200th the linear scale of the machine. For BD FACSCanto™ and similar machines, the linear scale is 255,000, resulting in most bead fluorescence values (along with FSC and SSC) being approximately 5,100, and PerCP-Cy5 being approximately 1,275. For Miltenyi MACSQuant® machines, the linear scale is 1,000, resulting in most bead fluorescence values (along with FSC and SSC) being approximately 20, and PerCP-Cy5 being approximately 5.

4.3. Initial Experiments

When setting up the flow cytometer for the first time, or when making changes to the machine or method (e.g., aligning the laser or changing cell types), prepare a high-volume sample. This will help ensure there is sufficient volume to optimize voltages and other instrument settings without running out of sample. Generate this high-volume sample by adding 200 μL of Complete Labeling Solution to 100 μL of untreated cells in a flow cytometry tube. After a 60 minute incubation, place the tube on the flow cytometer and adjust settings as described in this manual. This step is important for correctly setting PE/PerCP compensation (see Section 8.6).

In order to become familiar with the performance of the MultiFlow assay, perform a set of initial experiments. See Appendix B for assay advice and suggested chemicals. These experiments are also suggested if you are switching from one cell type to another.

It is possible to use plates and/or tubes other than those recommended here as long as the ratio of Complete Labeling Solution to cell suspension is maintained at 2:1. This will enable users without a robotic sampling device, or those that wish to analyze more cells per sample, to employ the methods and biomarkers described here.

4.4. Treatment and Sampling Times

This kit enables sampling from microtiter plate wells, offers compatibility with a variety of human cell lines, and can be useful for studying DNA double-strand breaks, aneugenicity, and cytotoxicity, as well as aspects of DNA repair. As such, there is no single recommended human cell line, treatment schedule, or sampling time. Rather, these variables will need to be optimized by each user in consideration of their objectives. The following is some general advice that users may find helpful.

> Litron routinely performs MultiFlow DNA Damage assessments with continuous treatment using TK6 suspension cells, with samples taken at 4 and 24 hours (Avlasevich, et al., 2021).

With regard to treatment schedules, chemicals can be applied to cultured cells and treatment may be allowed to proceed in a "continuous" manner for one to two normal doubling times. This period of time allows for the study of late effects,

such as changes to relative nuclei counts and polyploidization. However, late time points such as this may not be optimal for studying other features of the DNA damage response pathway. For example, induction of γH2AX may be evident within hours after start of treatment, and for some chemicals, the effect may diminish over time as cells progress through apoptosis or DNA repair. Therefore, it is generally advisable to collect longitudinal data in order to understand the kinetics of appearance (and sometimes disappearance) of the biomarkers being measured by this multiplexed assay. One such treatment/harvest scheme is described in Avlasevich et al (2021), where TK6 suspension cells are assessed after 4 hours and 24 hours of continuous treatment.

Short-term treatments can be useful for some lines of investigation, such as studying the kinetics by which DNA damage occurs and is repaired. In these situations, it may be useful to treat cells for one to several hours. The cultures can be washed free of the test article via centrifugation and after resuspension in culture medium they can be re-incubated and analyzed with MultiFlow reagents over time. When washing cells out of test article and sampling at the same time point, it is generally advisable to obtain the specimen before centrifugation.

When using suspension cells, it is important to maintain them at an optimal concentration specific to your cell line. For example, it is best to maintain TK6 cells below 1 x 10^6 cells/mL. When using adherent cells, it is best to maintain cells at or below 80 % confluence at the time of harvest. If investigating multiple time points with adherent cells, satellite plates with different starting cell densities may be required to keep cells from becoming overgrown.

Some chemicals are not genotoxic until they are converted to reactive metabolites via metabolic activation. It is beyond the scope of this instruction manual to provide detailed information about metabolically competent cells and/or those that require an exogenous source of metabolic activation. For advice about using S9 with MultiFlow, see Tian, et al., 2020; Avlasevich et al., 2021.

A known concern when using microwell plates is the potential for volatile test articles to diffuse through the headspace and contaminate adjacent wells. To mitigate this risk when treating in plates, we have found that using breathable membranes in combination with activated carbon filters effectively mitigates cross-well contamination. These measures were validated in experiments using TK6 cells in 96 well plates. If using other cell types or plate formats, we recommend verifying that these materials do not adversely affect cell growth or viability. For additional information about using breathable mats and activated carbon filters, see Avlasevich et al., 2025 and Appendix C.

4.5. Cytotoxicity Limits

Genotoxicity assays must consider the extent to which exposure of cells to test articles affect the overall health of the treated cells. A balance needs to be struck. Too little exposure may not generate detectable levels of DNA damage, while high exposures that lead to cascading secondary effects and excessive cell death may inaccurately reflect a test article's genotoxic potential.

For this reason, genotoxicity assays use cytotoxicity limits to guide the maximal exposure considered by the assay. A cytotoxicity limit generally works in conjunction with precipitation advice and some prespecified concentration limit. For instance, in the absence of cytotoxicity, some groups will study the highest non-precipitating or lowest precipitating concentration. Also, depending on the industry, in the absence of cytotoxicity, some groups will limit the top concentration studied to 1 mM or 10 mM.

The recommendations found below are for TK6 cells. Other cells may require adaptations in order to find the best balance between assay sensitivity and specificity.

Continuous exposures, or short-term exposures with test article washout: When precipitation or a concentration limit are **not** factors, and test article-induced cytotoxicity drives the choice of top concentration, our best advice is to use 80 % post-treatment cytotoxicity as the upper limit. For the MultiFlow assay, this is readily measured alongside the various biomarkers as a reduction to relative nuclei counts (RNC; relative to concurrent solvent control). More information about this calculation can be found in **Section 9.2.**

Note that TK6 cells are often studied at two time points, for instance 4 and 24 hours of continuous exposure. In this case, it is the 24 hour cytotoxicity reading that dictates the top concentration evaluated at both time points.

Furthermore, we suggest that once two successive concentrations within the range 70 % to 80 % reduction to RNC are encountered, higher concentrations should not be evaluated, even if those higher concentrations have not surpassed the 80 % limit or have lower cytotoxicity values. It is also ideal to have several concentrations in the range of 50 % to 80 % reduction to RNC, as this is where most genotoxicity biomarkers are evident.

Short-term exposures **with metabolic activation** and test article washout: When precipitation or a concentration limit are **not** factors, and test article-induced cytotoxicity drives the choice of top concentration, our best advice is to use 70 % post-treatment cytotoxicity as the upper limit. As explained above, for the MultiFlow assay, this is readily measured alongside the various biomarkers as a reduction to RNC. Also, the cytotoxicity value that dictates the top concentration should be from a later (e.g., 24 hour) time point, not an early one.

Furthermore, we suggest that once two successive concentrations within the range 60 % to 70 % reduction to RNC are encountered, higher concentrations should not be evaluated, even if those higher concentrations have not surpassed the 70 % limit or have lower cytotoxicity values. It is also ideal to have several concentrations in the range of 50 % to 70 % reduction to RNC, as this is where most genotoxicity biomarkers are evident.

The cytotoxicity limit advice comes from a wealth of experience in TK6 cells at multiple laboratories. Depending on the particular cells being studied, and users' preference for sensitivity versus specificity, alternative cytotoxicity measurements and/or cytotoxicity limits may be useful, but that requires extensive work with many reference chemicals.

5. Introduction to the MultiFlow Family of Kits

MultiFlow kits were developed for rapid, multiplexed, flow cytometric enumeration of several biomarkers associated with mammalian cell nuclei. A common theme across this family of kits is the simultaneous liberation of nuclei, staining of nucleic acids, and immunological labeling of specific nuclear epitopes. Cells prepared via this one-step method are ready for flow cytometric analysis after a short incubation period. This simple, patented method for processing samples makes the screening of numerous compounds across many concentrations and/or at multiple time points both practical and efficient.

The MultiFlow DNA Damage Kit – p53, γH2AX, Phospho-Histone H3, Cleaved PARP employs a method for studying biomarkers that are associated with DNA damage response pathways. The first biomarker is human specific p53. Since this kit's reagents result in liberation and analysis of free nuclei, the p53 measurement can be thought of as a nucleus-specific measurement, i.e., a nuclear translocation event. The second biomarker is γH2AX as a measure of DNA double strand breaks. The third biomarker, specific phosphorylation of histone H3, serves as an indicator of mitotic cells. Whereas aneugenic chemicals often elevate the mitotic index, it tends to be reduced by cytotoxic conditions, including cytotoxicity associated with many DNA-reactive chemicals. Finally, the fourth biomarker, cleaved PARP, provides information on apoptosis.

In addition to a DNA stain, RNase, and antibody reagents, MultiFlow kits include fluorescent polystyrene "Counting Beads" at a known particle count. Using this information, it is possible to derive cell (nuclei) densities and thereby calculate commonly used cytotoxicity metrics such as Relative Nuclei Count (RNC), Relative Increased Nuclei Count (RINC), and Relative Population Doubling (RPD). See Section 9 for formulas.

This specific kit was designed as a basic research tool and has proven valuable as a genotoxic hazard ID assay, as well as for studying the kinetics of formation and disappearance of DNA repair foci. Furthermore, these specific biomarkers have demonstrated their usefulness for predicting chemicals' genotoxic mode of action. For more information about using the kit for genotoxic hazard ID assessment, see Bryce, et al., 2016, Bryce, et al., 2025.

6. Preparation of Complete Labeling Solution

Prepare Complete Labeling Solution (CLS) **the same day** as cell harvest and processing. HALT™ Protease and Phosphatase Inhibitor Cocktail is recommended to ensure stability of proteins and phosphorylated epitopes. HALT™ should be added to Complete Labeling Solution no more than 30 minutes before use. Use the chart below to determine the amount of Complete Labeling Solution required. Scale up as needed for the number of samples you plan to analyze. **Generally, it is useful to base your reagent requirement on 10 % to 15 % more samples than you plan to analyze** to accommodate for loss during transfers. Prepare Complete Labeling Solution before cell harvest begins.

Number of wells	Nuclei Release Solution with Counting Beads	DNA Stain	RNase Solution	γH2AX Antibody Alexa Fluor® 647	Phospho-Histone H3 Antibody PE	p53 Antibody FITC	Cleaved PARP Antibody BV421	HALT™ Protease and Phosphatase Inhibitor Cocktail	FBS (Adherent cells only)
1	50 µL	1.25 µL	0.25 µL	0.25 µL	0.10 µL	0.25 µL	0.50 µL	.5 µL	1 µL
96 + 15 % = 110	5.5 mL	137.5 µL	27.5 µL	27.5 µL	11 µL	27.5 µL	55 µL	55 µL	110 µL

1. **Sufficiently mix (by tapping on the bottom of the bottle and inverting several times)** the Nuclei Release Solution with Counting Beads to ensure the beads are in homogeneous suspension. Bubble formation is normal.

2. Add the required volume of Nuclei Release Solution with Counting Beads to a clean polypropylene tube. Add the appropriate volumes of DNA Stain, RNase Solution, and antibodies. If processing adherent cells, add required volume of FBS. Mix well.

3. If using HALT™ Protease and Phosphatase Inhibitor Cocktail, add the required volume to the tube and mix well. Ensure the CLS will be used within 30 minutes.

4. Store at ambient temperature until use.

7. Sample Processing and Analysis

7.1. Suspension Cells

1. **Vortex or mix the CLS throughout aliquoting to keep the Counting Beads in homogenous suspension.** Add 50 µL of the CLS to individual wells of a clean 96 well non-cell-culture-treated U-bottom plate.

2. Gently resuspend cells to be analyzed by pipetting up and down several times. Immediately remove 25 µL of the cell suspension(s) and add to the well(s) containing the CLS.

3. Mix thoroughly by pipetting up and down **10 or more times**. Repeat for remaining wells.

4. Incubate 60 minutes at ambient temperature.

5. After incubation, proceed with analysis.

Ensure incubation temperatures do not go above 24 °C, as higher temperatures can degrade the phospho-histone H3 signal. Using HALT™ Protease and Phosphatase Inhibitor Cocktail can help counter that degradation.

7.2. Adherent Cells

Detachment procedures for adherent cells vary by cell line, and optimizing this step is critical for successful MultiFlow analysis. We recommend optimizing detachment conditions for your specific cell type and confirming compatibility with MultiFlow labeling (enzyme, volume, incubation time etc.). The following protocol provides an example for processing adherent cells. However, methods may need to be adjusted based on your cell line. For further assistance, please contact us at InVitroMultiFlowTechSupport@LitronLabs.com.

1. Gently aspirate away **all** growth medium from wells of a 96 well plate.

2. Add 25 µL of ambient temperature Accutase® to each well. Ensure that the entire cell layer is covered to promote detachment and formation of a single-cell suspension. For cell types that are dense or prone to clumping, consider increasing the volume of **Accutase®** to **50 µL to 100 µL** to achieve complete coverage and allow sufficient volume for gentle mixing after detachment.

3. Incubate for 5 to 15 minutes at 37 °C until cells begin to round and lift off the plate.

4. Gently pipette cells to ensure all have been detached and are in a single cell suspension.

5. **Vortex or mix the CLS throughout aliquoting to keep the Counting Beads in homogenous suspension.** Immediately after Accutase® incubation, add 50 µL of CLS **with FBS** to each well.

6. Mix thoroughly by pipetting up and down **10 or more times.**

7. Transfer the total volume of each well to a clean, non-cell-culture-treated 96 well U-bottom well plate for analysis.

8. Incubate 60 minutes at ambient temperature.

9. After incubation, proceed with analysis.

Ensure incubation temperatures do not go above 24 °C, as higher temperatures can degrade the phospho-histone H3 signal. Using HALT™ Protease and Phosphatase Inhibitor Cocktail can help counter that degradation.

7.3. Flow Cytometric Data Acquisition

1. Check Litron's website (www.LitronLabs.com) for supported flow cytometer templates or create a new one based on the plots shown in the following sections**.** If creating your own template, be sure to do this prior to processing samples.

2. Analyze samples on the flow cytometer within 1 to 2 hours of adding cells to the CLS. If needed, stagger the start times of cell treatments and CLS additions to maintain this time window. Prolonged incubation (greater than 1 to 2 hours) may cause bead settling and lead to inconsistent bead counts. If bead settling occurs, gently resuspend the sample by pipetting before acquisition, or adjust the cytometer's mixing settings to ensure uniform suspension.

3. Our current best advice is to collect at least 20 µL per sample at a rate of 1 µL/sec for BD machines. For Miltenyi machines, we recommend and uptake volume of 20 µL, fast mode (wash), and a flow rate of medium.

4. Samples should be mixed prior to analysis to ensure homogeneous distribution. For example, for BD HTS instruments, we recommend 4 mixing cycles with a volume of 40 µL. For Miltenyi machines, we recommend medium mixing, and 50 µL sample volume. If the cytometer does not have a mix function, we recommend mixing every 12 samples by manually pipetting up and down.

8. Biomarker Gating Logic

8.1. General Advice

This section is intended as guidance for creating and editing a flow cytometer data acquisition template for this assay. Templates for some flow cytometry platforms are available at www.LitronLabs.com or by contacting technical support. The gating logic and calculations described herein represent our current best advice. When creating templates, it can be helpful to have negative and positive control samples prepared (see Appendix B).

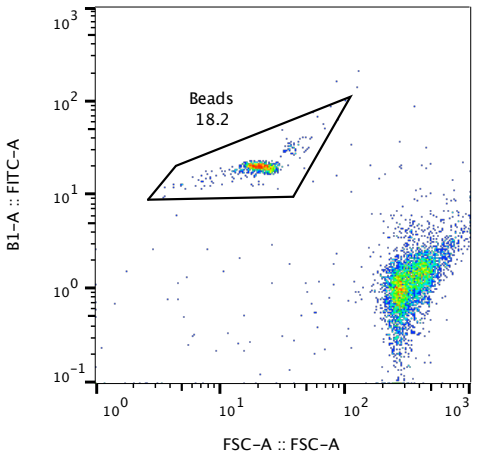
8.2. Beads

1. Counting Beads are provided in the Nuclei Release Solution at a known density that is specified on the label. The number of counting beads collected in a sample is used to derive cytotoxicity metrics (see Section 9).

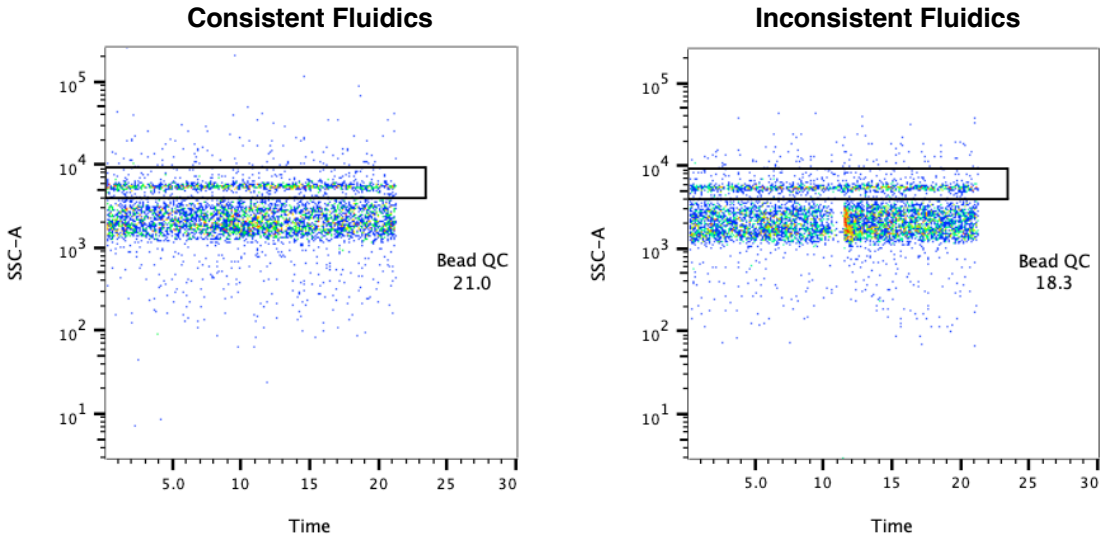
2. We recommend collecting Counting Bead events using a dot plot capturing a fluorescent channel vs. forward scatter. It is important to choose the channel that gives the best separation between the bead and nuclei populations to avoid overlap; typically, this is the FITC channel as seen in accompanying Bead Gate plot.

3. The Counting Bead population should be removed from all other plots described herein. One way of accomplishing this is to incorporate gating logic that <u>excludes</u> events in the Beads region.

Bead Gate



4. A dot plot capturing a fluorescent channel (such as FITC) vs. time (below) can be used to assure consistent fluidics and data acquisition rates. Any breaks in the Bead QC region or in the nuclei outside of the Bead QC region could represent air uptake during sampling, as seen on the plot on the right, below. See Appendix A for more information.



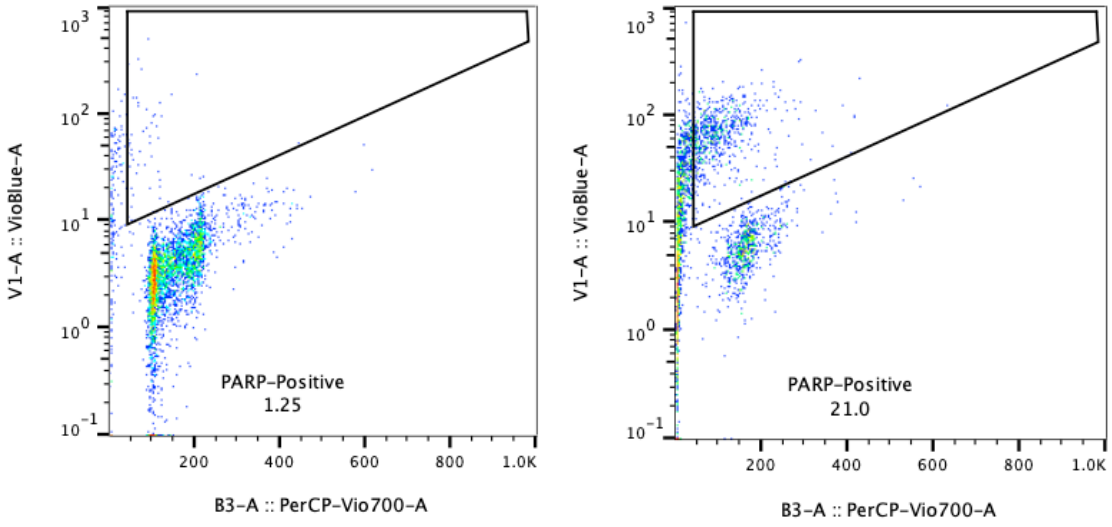
8.3. Cleaved PARP

1. Measurement of cleaved PARP can be performed either before or after applying the Nuclei gating step (Section 8.4). Excluding cleaved PARP positive events before applying the Nuclei gate helps refine the H2AX gate and ensures that cleaved PARP positive nuclei are not included in cytotoxicity calculations. Alternately, analyzing cleaved PARP after applying the Nuclei gate ensures the events included in the cleaved PARP gate have met all the requirements of Nuclei gate. Representative plots of each gating method are located at the end of this section.

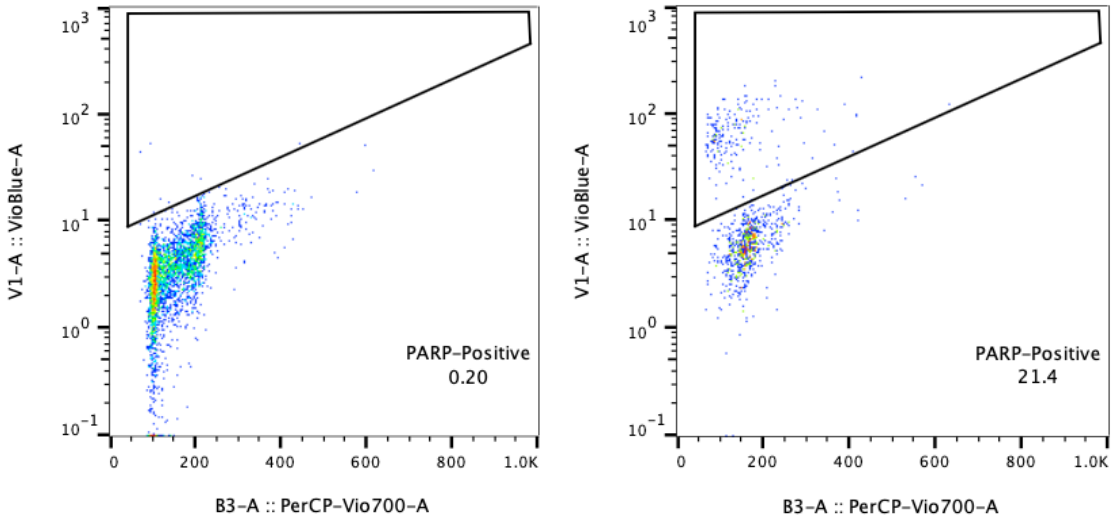
2. A dot plot utilizing Violet and PerCP should be used to quantify Cleaved PARP. This plot should exclude events falling into the Beads region created in Section 8.2. For cleaved PARP evaluation, the resolved Violet-positive population is enumerated based on all events other than Counting Beads, and includes nuclei with 2n and greater DNA content, as well as sub-2n chromatin. Some compounds, such as benzo(a)pyrene, autofluorescence in the Violet channel. For these compounds, the gate may need to be moved higher in Violet to accommodate the autofluorescence.

3. A positive control for cleaved PARP such as Brefeldin A or cyclophosphamide (in the presence of a liver S9 activation system) can be useful for setting the cleaved PARP region (see Appendix B). In the following example, cells were exposed to vehicle (left) or cyclophosphamide (right) for 24 hours. Treatment with cyclophosphamide induces a large fluorescence shift relative to the negative control.

Cleaved PARP gating before Nuclei gate



Cleaved PARP gating after Nuclei gate

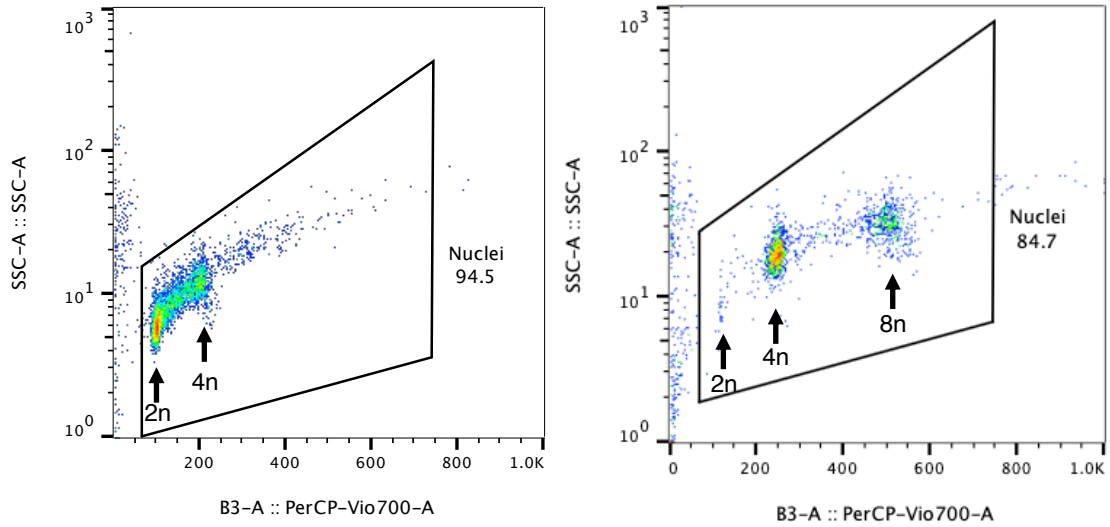


8.4. Nuclei

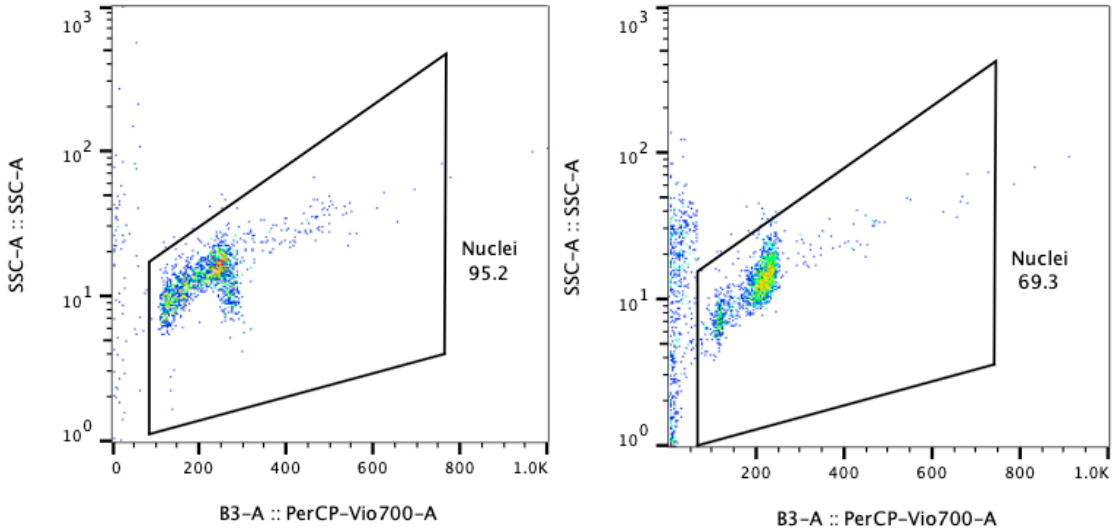
1. Many of the biomarkers studied in this assay will focus on nuclei with specific DNA content, e.g., 2n through 4n, or through 8n. A region capturing these events (2n through polyploidy) will provide nuclei numbers that will later be considered in cytotoxicity metrics (see Section 9).

2. A dot plot utilizing side scatter (SSC) and PerCP should be used to quantify nuclei. This plot should exclude events falling into the Beads region created in Section 8.2. This plot can either include or exclude events falling into the cleaved PARP-positive region created in Section 8.3, depending upon which gate you prefer to analyze first.

3. It is important to set the nuclei gate based on healthy nuclei, such as a negative control, as seen on the following plot (left). The nuclei gate should include 2n through 4n as well as polyploidy nuclei (8n), a seen in the following plot (right). All events that fall within the nuclei gate will be used for subsequent biomarker analyses.



4. The nuclei gate should exclude small debris that falls below 2n. It should also exclude nuclei with elevated SSC, above the main population. Events below and above the main 4n population should be included as seen in the two plots below. These nuclei indicate phospho-histone H3-positive and γH2AX-positive nuclei, respectively.

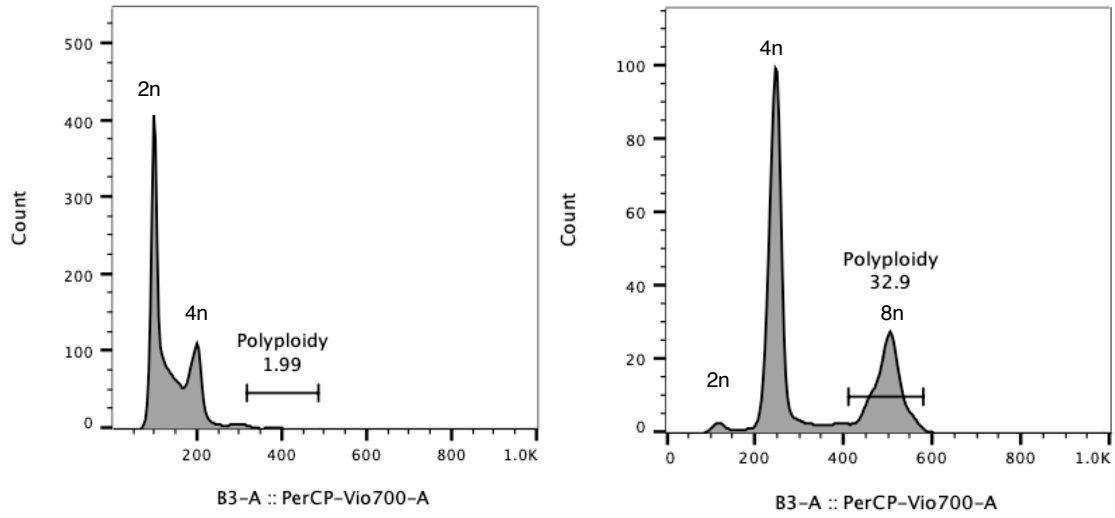


8.5. Polyploidy

1. Polyploidy can be a useful biomarker and is quantified on a PerCP histogram. This histogram should exclude events falling into the Beads region created in Section 8.2, and also exclude events falling outside the Nuclei region created in Section 8.4.

2. For polyploidy scoring, one may collect the number of nuclei that exhibit DNA-associated fluorescence corresponding to 8n DNA content, while other labs may wish to collect 8n and greater DNA content. The following examples demonstrate collecting only 8n DNA content. Note that the median numerical value of the 8n population (about 400 on the left plot and about 500 on the right plot, below) should be twice the numerical value of the 4n population (about 200 on the left plot and about 250 on the right plot, below, which should be twice the numerical value of the 2n population (about 100 on the left plot and about 125 on the right plot, below).

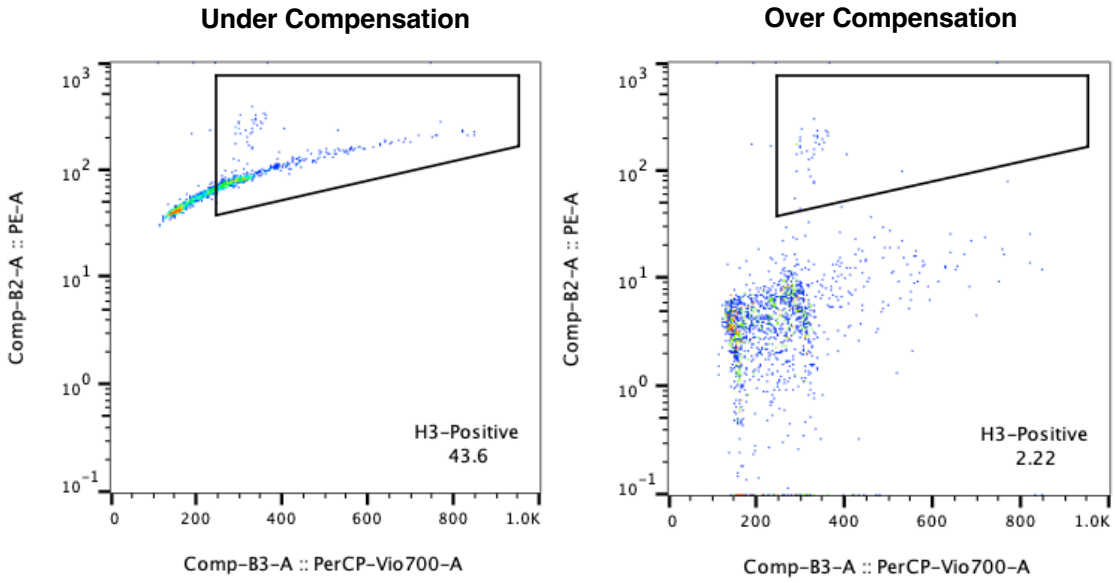
3. A positive control for polyploidy, such as the chemical AMG 900 or carbendazim, can be useful for setting the polyploidy region (see Appendix B). In the following example, 24 hours of exposure to carbendazim (right) induces a large degree of polyploidization relative to the negative control (left).



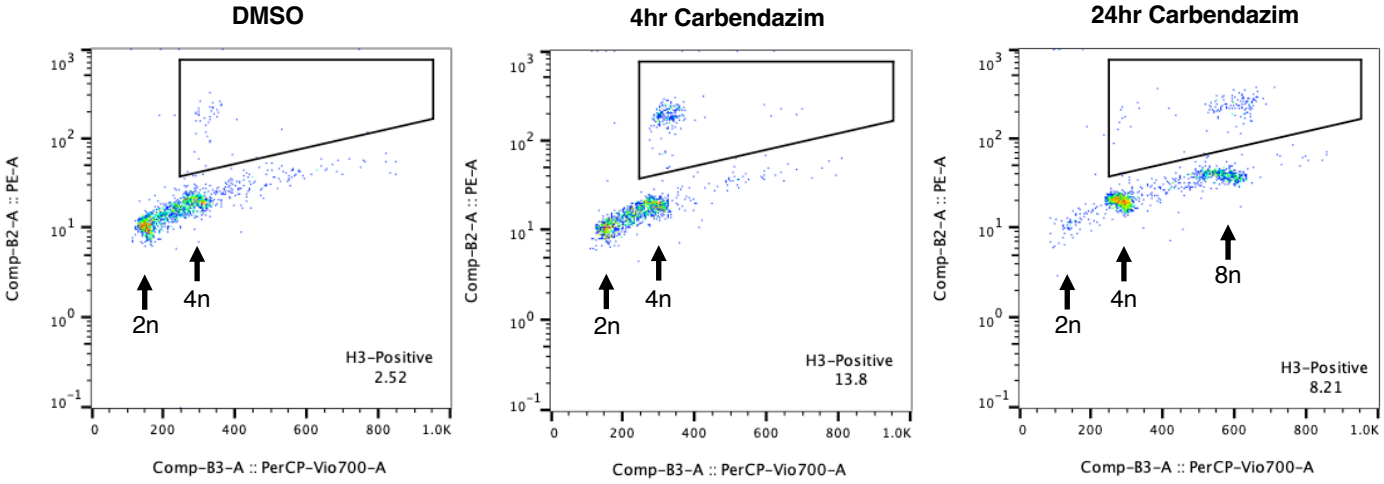
8.6. Phospho-Histone H3

1. To measure the number of phospho-histone H3-positive events, create a dot plot of PE vs. PerCP. On this plot phospho-histone H3-positive events will be resolved as a PE-positive population(s) for the 4n and greater DNA content. This plot should exclude events falling into the Beads region created in Section 8.2 and also exclude events falling into the Nuclei region created in Section 8.4.

2. Due to the spectral overlap of the DNA-associated fluorescence and PE fluorescence, compensation to eliminate nucleic acid dye spillover into the adjacent PE channel is usually necessary. Note that when PMT voltages and compensation are optimally set, phospho-histone H3-positive events should resolve from the negative population by approximately 1 log. The plots below demonstrate no compensation (left) and over compensation (right), whereas the plots at the end of this section demonstrate proper compensation.



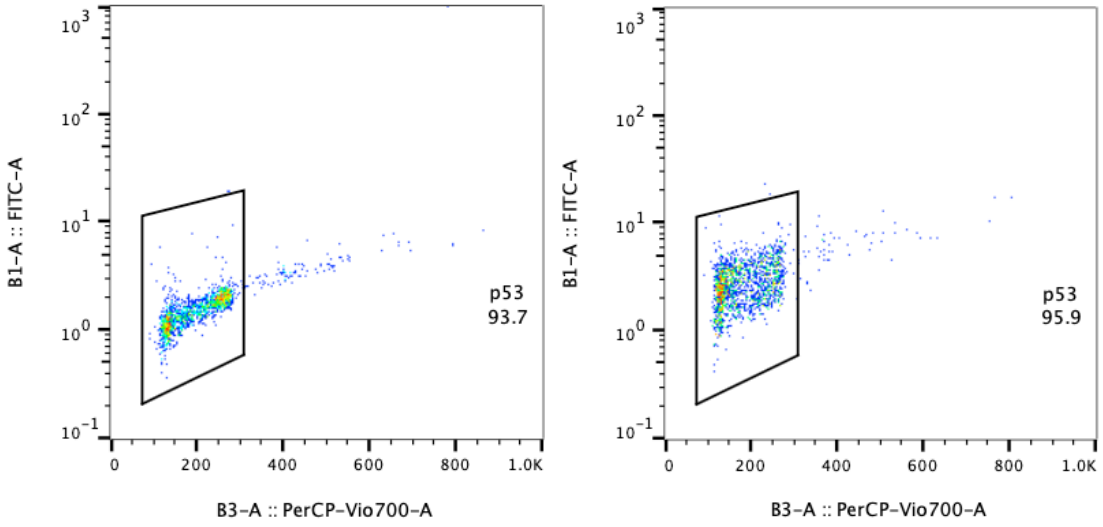
3. While phospho-histone H3-positive events can be seen in negative control cells, a tubulin binder-type aneugenic positive control for these events, such as vinblastine or carbendazim, can be useful for setting the phospho-histone H3 positive region (see Appendix B). In the following example, 4 hours of exposure to carbendazim (middle) induces a large number of phospho-histone H3-positive events relative to the negative control (left). 24 hours of exposure to carbendazim (right), induces the formation of an 8n polyploid population.



8.7. Nuclear p53

1. To capture the median channel fluorescence (MCF) shift of p53-positive nuclei, create a FITC vs. PerCP dot plot that excludes phospho-histone H3-positive nuclei identified in Section 8.6, capturing nuclei with 2n through 4n DNA content only.

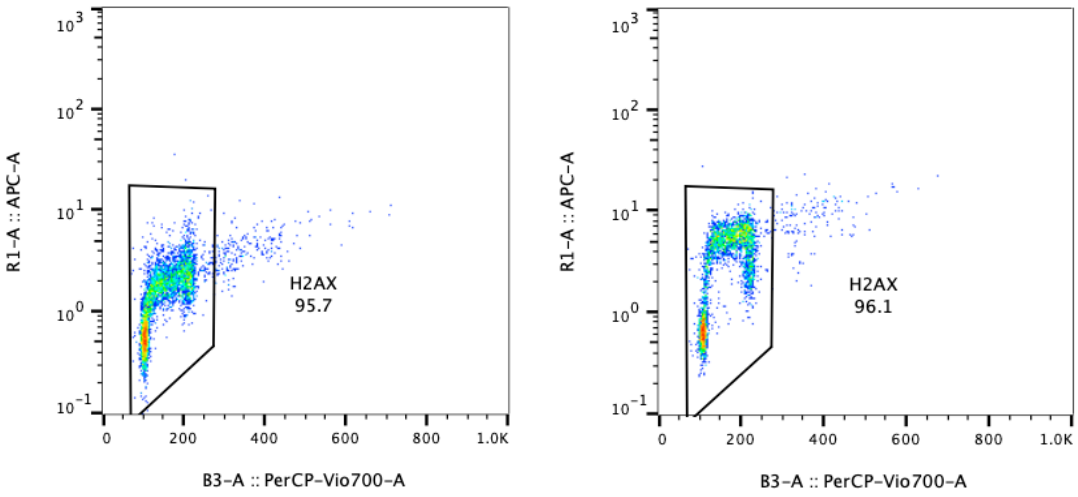
2. A positive control for p53, such as MMS or 5-fluorouracil, can be useful for setting the p53 region (see Appendix B). In the example below, 24 hours of continuous exposure to 5-fluorouracil (right) induces a large fluorescence shift relative to the negative control (left)



8.8. γH2AX

1. To capture the MCF shift of γH2AX positive nuclei, create an APC vs. PerCP dot plot that excludes phospho-histone H3-positive nuclei identified in Section 8.6., capturing nuclei with 2n through 4n DNA content only.

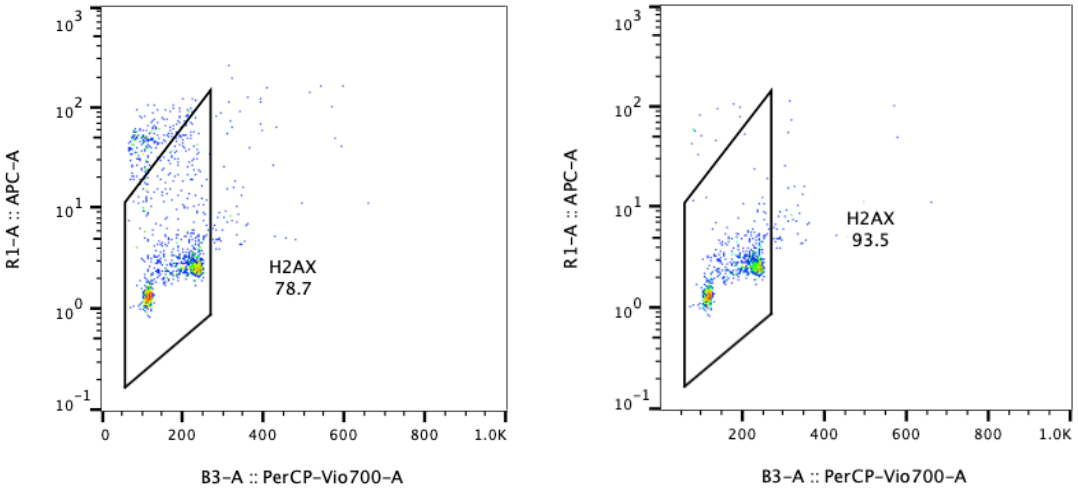
2. A positive control for γH2AX, such as MMS or stavudine, can be useful for setting the γH2AX region (see Appendix B). In the example below, 4 hours of continuous exposure to stavudine (right) induces a large fluorescence shift relative to the negative control (left).



3. Some compounds at cytotoxic concentrations will produce highly fluorescent γH2AX-positive nuclei, e.g., those that approximate greater than one log of separation. Since these events represent apoptotic nuclei, we recommend

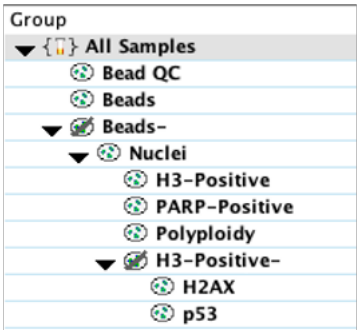
adjusting the γH2AX region to avoid scoring this population. As mentioned in Section 8.3, excluding cleaved PARP-positive events from the Nuclei gate can prevent many of these highly fluorescent γH2AX-positive nuclei from appearing on this plot.

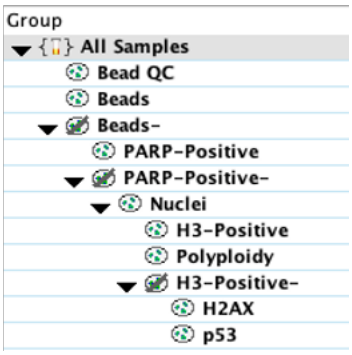
4. The plots below represent γH2AX plots for two gating strategies, gating PARP positive nuclei before or after applying the nuclei gate. For this example, cells were treated for 24 hours with a high concentration of Brefeldin A, inducing highly fluorescent apoptotic nuclei. The plot on the left demonstrates a gate position that includes γH2AX-positive nuclei but excludes those caused by apoptosis. The plot on the right has similar gate placement, but many of the highly fluorescent γH2AX-positive apoptotic nuclei were removed with the cleaved PARP gate.



8.9. Gating Logic Summary

The gating logic on the left measures cleaved PARP-positive events after applying the Nuclei gate, and the gating logic to the right measures cleaved PARP-positive events before applying the Nuclei gate.





9. Calculations

9.1. Cell Densities

1. Based on the absolute number of counting beads/mL provided on the Nuclei Release Solution Plus Counting Beads bottle, one can derive cell density values that will be used to calculate relative cytotoxicity. Be sure to refer directly to the stock bottle used to make the Complete Labeling Solution, as the absolute bead count will change across lots. This number is used directly in the formula below (number of beads in Nuclei Release Solution Plus Counting Beads) but is corrected for the volume of additional reagents added during preparation of the Complete Labeling Solution. This means dividing by 1.052 if using suspension cells without HALT (1.062 if using HALT) and dividing by 1.072 if using adherent cells without HALT (1.082 if using HALT).

2. The other values are obtained from the flow data for each sample. An Excel® spreadsheet is available on Litronlabs.com that is formatted to accept bead and nuclei frequency data from the flow cytometer and automatically calculate cytotoxicity information.

$$\text{Cell Density} = \frac{\#\text{Events Nuclei}}{\#\text{Events Beads}} \times \frac{\text{Number of beads/mL in Nuclei Release Solution Plus Counting Beads}}{\text{1.052 mL or 1.062 mL or 1.072 mL or 1.082 mL}} \times \text{dilution factor (2)}$$

Note – the following formulas are commonly used cytotoxicity indicators based on cell density. They are equivalent to those used for cell-based cytotoxicity assessment, where “nuclei” values are substituted for “cells”.

9.2. Relative Nuclei Count (RNC)

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

9.3. Relative Increased Nuclei Count (RINC)

$$\text{RINC} = \frac{\text{Increase in the number of nuclei in treated culture (final – starting)}}{\text{Mean increase in the number of nuclei in solvent control culture(s) (final – starting)}} \times 100$$

9.4. Population Doubling (PD)

$$\text{PD} = [\log(\text{Post-treatment nuclei number}/\text{Pre-treatment nuclei number})] / \log 2$$

9.5. Relative Population Doubling (RPD)

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

9.6. Percent Cleaved PARP

$$\text{Percent Cleaved PARP} = \frac{\#\text{Events Cleaved PARP-Positive}}{\#\text{Events Nuclei}} \times 100$$

9.7. Cleaved PARP Fold Change

$$\text{Cleaved PARP Fold Change} = \frac{\text{Percent Cleaved PARP of treated culture}}{\text{Mean Percent Cleaved PARP of solvent control culture(s)}}$$

9.8. Percent Polyploidy

Note that while it is often useful to express these values as fold-increase relative to mean solvent control, when the spontaneous rate of polyploidization is low, percent polyploidy and fold-increase values will be subject to appreciable counting error unless large numbers of events are scored.

$$\text{Percent Polyploidy} = \frac{\#\text{Events Polyploidy}}{\#\text{Events Nuclei}} \times 100$$

9.9. Polyploidy Fold Change

$$\text{Polyploidy Fold Change} = \frac{\text{Percent Polyploidy of treated culture}}{\text{Mean Percent Polyploidy of solvent control culture(s)}}$$

9.10. Percent Phospho-Histone H3

$$\text{Percent Phospho-Histone H3} = \frac{\text{\#Events Phospho-Histone H3-positive}}{\text{\#Events Nuclei}} \times 100$$

9.11. Phospho-Histone H3 Fold Change

$$\text{Phospho-Histone H3 Fold Change} = \frac{\text{Percent Phospho-Histone H3 of treated culture}}{\text{Mean Percent Phospho-Histone H3 of solvent control culture(s)}}$$

9.12. Nuclear p53 Fold Change

Values for nuclear p53 are obtained from the MCF of the FITC channel.

$$\text{Nuclear p53 fold change} = \frac{\text{FITC MCF of treated culture}}{\text{Mean FITC MCF of solvent control culture(s)}}$$

9.13. γH2AX Fold Change

Values for γH2AX are obtained from the MCF of the APC channel.

$$\text{γH2AX fold change} = \frac{\text{APC MCF of treated culture}}{\text{Mean APC MCF of solvent control culture(s)}}$$

10. Disclaimer and Limited License Agreement

By purchasing, taking receipt of, or utilizing this kit, the user/user's company agrees to be bound by the terms of this License. This License allows the use of this kit for the preparation and analysis of up to 384 samples. Purchase and/or use of this kit does not relieve the user from any limitations set forth by other existing or future patents or licensing agreements. The user assumes any and all responsibility and/or liability associated with how they combine or commercialize these kits, products or methods.

Any discoveries, modifications or improvements to the methodology made during the use of this kit are the property of Litron Laboratories.

This warranty limits our liability to replacement of this product only. 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.

11. References

- Avlasevich, S. L., Briggs, E., Tichenor, K., Conrad, A., Hall, N., Bryce, S. M., Bemis, J. C., & Dertinger, S. D. (2025). Mitigation of Volatile Chemicals' Effect on Adjacent Microtiter Plate Wells. *Environmental and Molecular Mutagenesis*, *66*(3), 134–141.

- Avlasevich, S., Pellegrin, T., Godse, M., Bryce, S., Bemis, J., Bajorski, P., & Dertinger, S. (2021). Biomarkers of DNA damage response improve *in vitro* micronucleus assays by revealing genotoxic mode of action and reducing the occurrence of irrelevant positive results. *Mutagenesis*, *36*(6), 407–418.

- Bryce, S. M., Avlasevich, S. L., Conrad, A., Hall, N. E., Tichenor, K., Briggs, E., Dertinger, S. D., & Bemis, J. C. (2025). Application of Biological Modifiers to a Multiplexed, Human Cell-Based DNA Damage Assay Provides Mechanistic Information on Genotoxicity and Molecular Targets. *Environmental and Molecular Mutagenesis*, *66*(5), 280–290.

- Bryce, S. M., Bernacki, D. T., Bemis, J. C., & Dertinger, S. D. (2016). Genotoxic mode of action predictions from a multiplexed flow cytometric assay and a machine learning approach. *Environmental and Molecular Mutagenesis*, *57*(3), 171–189.

- Lagunas, T., Melnikov, F., Cole, G., Niu, S., Esparza, E., Davies, J., Hasselgren, C., Fullerton, A., & Zhong, Y. (2025). Analysis of Multiplexed Flow Cytometric Assays and Toxicogenomic Signatures for Genotoxicity Prediction: A Model Performance and Case Study Approach. *Environmental and Molecular Mutagenesis*.

- Tian, S., Cyr, A., Zeise, K., Bryce, S. M., Hall, N., Bemis, J. C., & Dertinger, S. D. (2020). 3Rs-friendly approach to exogenous metabolic activation that supports high-throughput genetic toxicology testing. *Environmental and Molecular Mutagenesis*, *61*(4), 408–432

Appendix A: Troubleshooting

Issue	Potential Causes	Solutions
Inappropriate or sporadic bead count	Solutions not properly mixed prior to use	We recommend vortexing any solutions containing beads immediately before use.
	Threshold(s) too high	Be sure that thresholds are below the bead region.
	Nuclei moving into bead region	On some cytometers responses can cause nuclei to move into the bead region. Consider changing your template to capture beads on the channel that provides the best separation between nuclei and beads on your flow cytometer.
	Inadequate mixing function on flow cytometer	If beads number decreases over time your cytometer may not have a sufficient mixing function. Orbital shakers are typically not satisfactory for this assay. Consider manual mixing between rows or columns if performing analysis in multi-well assay plates.
	Clogged/dirty flow cytometer	Be sure to perform routine cleaning cycles on your flow cytometer.
	Air moving through flow cell	Air can cause skips in beads that can be observed in the "Bead QC" region. Be sure that your cytometer is purged of air before analysis. Also, observe that the sampling probe is calibrated adequately to the well/tube so that it is not sampling air.
Unhealthy cell cycle for negative control samples	Unhealthy cells	Check the health of your cells. Be sure doubling time is appropriate for your cell line and that passage number is not too high. Ensure suspension cells are cultured at the recommended density and adherent cells are maintained below 80 % confluency.
	Incomplete lysis	Complete lysis of cells requires sufficient mixing of cells into CLS. We recommend pipetting up and down 10 to 15 times when mixing cells into CLS, taking care to avoid generating bubbles.
	Clogged/dirty flow cytometer	Be sure to perform routine cleaning cycles on your flow cytometer.
	Air moving through flow cell	Air can cause skips in beads that can be observed in the "Bead QC" region. Be sure that your cytometer is purged of air before analysis. Also, observe that the sampling probe is calibrated adequately to the well/tube so that it is not sampling air.
	Event rate too high	Lowering your acquisition rate can improve the quality of the data collected.
Sporadic / inconsistent nuclei number amongst controls	Inappropriate cell plating technique	Mix cells well before plating.
	Clogged/dirty flow cytometer	Be sure to perform routine cleaning cycles on your flow cytometer.
	Air moving through flow cell	Air can cause skips in beads that can be observed in the "Bead QC" region. Be sure that your cytometer is purged of air before analysis. Also, observe that the sampling probe is calibrated adequately to the well/tube so that it is not sampling air.
Fluorescence shift over time or between samples	Insufficient incubation time	Our best advice is to incubate samples for 60 minutes at ambient temperature.
	Significant differences in cell density	Cell density can cause modest shifts in cell cycle peaks. We recommend adjusting cell cycle regions to accommodate shifts resulting from significant changes in cell density (i.e., 4 hour vs. 24 hour time points).
Insufficient antibody resolution	Inappropriate antibody or DNA stain concentration	Consult Section 6 to confirm that Complete Labeling Solution was prepared correctly.
	Inappropriate voltages or compensation	Compensation and voltage can affect resolved populations. For help adjusting these settings please contact Litron's technical support team.
	Event rate too high	Lowering your acquisition rate can improve the quality of the data collected.
Too few cells sampled	Insufficient starting cell density	We recommend samples of at least 200,000 cells/mL.
	Insufficient sample volume	Optimize sample volume to maximize events collected while avoiding collecting air.

Appendix B: Advice on Prototypical Chemicals and Initial Experiments

Experiments with the following reference chemicals are useful for initial work aimed at ensuring that sample processing and flow cytometer settings at your laboratory are optimal, and for confirming that the cell line(s) you intend to study are compatible with the kit reagents.

The following information is for the human lymphoblastoid cell line TK6. To perform the experiments summarized below as an initial pilot experiment, treat cells with 20 concentrations with square root 2 dilution schemes (where each dose is 70.7 % of the previous dose and every other dose decreases by 50 %). Example starting concentrations are listed below. Treat for 24 continuous hours and perform MultiFlow analyses at both 4 hours and 24 hours. Only examine non-cytotoxic doses (see Section 4.5).

We encourage you to send example plots and/or data to the Litron technical support team in order to receive assistance in review for quality of the sample processing and analysis. Also contact Litron technical support for advice about treatment and handling of other cell lines.

Chemical	Cas. No.	Starting Conc.* (μM)	Expected Cleaved PARP Response	Expected p53 Response	Expected γH2AX Response	Expected Phospho-Histone H3 Response	Expected Polyploidy Response	Predominant Genotoxic Mode of Action
AMG 900	945595-80-2	0.1	Modest effect at 24 hours	Increase at 24 hours	No effect	Decrease at 4 and 24 hours	Robust increase at 24 hours	Prototypical aneugen (aurora kinase inhibitor phenotype)
Carbendazim	10605-21-7	1000	Large effect at 24 hours	Increase at 24 hours	Decrease at 24 hours	Increase at 4 and 24 hours	Increase at 24 hours	Prototypical aneugen (atypical tubulin binder phenotype)
Carbonyl cyanide m-chlorophenyl hydrazone (CCCP)	555-60-2	100	Large effect at 24 hours	No effect	Decrease at 24 hours	No effect	No effect	Non genotoxic apoptogen
Cyclophosphamide**	50-18-0	100	Large effect at 24 hours	Increase at 24 hours	Large effect at 24 hours	Decrease at 4 and 24 hours	No effect	Prototypical clastogen; requires metabolic activation
5-Fluorouracil	51-21-8	1000	Large effect at 24 hours	Increase at 4 and 24 hours	No effect	Decrease at 4 and 24 hours	No effect	Atypical clastogenic response (certain clastogens do not have a γH2AX increase)
Methyl methane-sulfonate (MMS)	66-27-3	1000	Modest effect at 24 hours	Increase at 24 hours	Modest effect at 4 hours; large effect at 24 hours	Decrease at 4 and 24 hours	No effect	Prototypical clastogen
Stavudine	3056-17-5	1000	Modest effect at 24 hours	Increase at 24 hours	Large effect at 4 and 24 hours	Decrease at 4 hours	No effect	Prototypical clastogen
Vinblastine Sulfate	143-67-9	0.01	Little to no effect	No effect	No effect	Increase at 4 and 24 hours	No effect	Prototypical aneugen (tubulin binder phenotype)

* These concentrations are appropriate starting ranges for TK6 cells. Other cell lines may require different concentrations.
**Metabolic activation needed for biomarker responses.

Appendix C – Strategies for Dealing with Volatile Chemicals

The volatility of some test articles can negatively affect MultiFlow data in adjacent wells. When dealing with volatile chemicals, or test articles that have not been characterized in terms of volatility, it may be useful to treat cells in 96 well 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 96-well plate cover is placed on top to hold it in place.