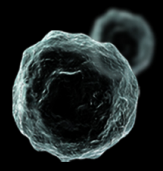
MultiFlow®

Multiple endpoints. One step.



MultiFlow – p53

Instruction Manual

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

Table Of Contents

1. Materials Provided 1
2. Additional Materials Required 1
3. Introduction to the MultiFlow® Family of Kits 1
4. General Advice About Treatment and Sampling Times 2
5. Preparation of Complete Labeling Solution 2
6. Sample Processing and Analysis 3
First Time Users 3
6.1. Processing Plates 3
6.2. Flow Cytometric Data Acquisition 4
7. Endpoint Gating Logic and Calculations 4
7.1. General Advice 4
7.2. Bead Collection 4
7.3. DNA Content Histogram 4
7.4. Polyploidy 5
7.5. Percent Polyploidy 6
7.6. Cycling Nuclei 6
7.7. Nuclear p53 6
7.8. Nuclear p53 Shift 7
7.9. Gating Logic Summary 7
8. Calculations 7
8.1. Cell Densities 7
8.2. Relative Nuclei Count (RNC) 7
8.3. Relative Increased Nuclei Count (RINC) 7
8.4. Population Doubling (PD) 8
8.5. Relative Population Doubling (RPD) 8
9. Disclaimer and Limited License Agreement 8
10. Ordering Information and Technical Services 8
Appendix A: Troubleshooting 9
Appendix B: Advice on Prototypical Chemicals and Initial Experiments 10

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)[c]	110 µL	2 °C to 8 °C, light sensitive

a. Sufficient materials are provided to analyze up to 384 samples.
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. Warning! See MSDS (available on website).

2. Additional Materials Required

- Human cells, ideally with functional p53
- Accutase® cell detachment solution if working with attachment cells (Sigma Cat. No. A6964)
- Fetal bovine serum (FBS), heat-inactivated, if working with attachment cells
- Flow cytometer capable of 488 nm and 647 nm excitation, optionally equipped with High Throughput Sampler (HTS) or similar autosampler
- Refrigerator
- Disposable pipettes
- Polypropylene centrifuge tubes (e.g., 15 mL)
- Micropipettors (20 µL - 1000 µL) and tips; multichannel preferred
- 96 well plates - U-bottom 96 well plates (e.g., BD Cat. No. 353910)

3. Introduction to the MultiFlow® Family of Kits

MultiFlow kits were developed for rapid, multiplexed, flow cytometric enumeration of several endpoints 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 protocol for processing samples makes screening of numerous compounds across many concentrations and/or at multiple time points both practical and efficient.

The MultiFlow – p53 kit, employs a method for studying endpoints that are associated with **DNA damage response pathways**. 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. 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 p53 activation.. Furthermore, this specific endpoint has demonstrated its usefulness for predicting chemicals' genotoxic mode of action. For more information about the genotoxic mode of action application, we encourage users to read the article by Bryce and colleagues: *Environ. Mol. Mutagen.* 57 (2016) 171-189.

In addition to DNA stain 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 8 for formulas.

4. General Advice About Treatment and Sampling Times

This kit enables serial sampling from microtiter plate wells, offers compatibility with a variety of human cell lines, and can be useful for studying genotoxic insult 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.

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 ©H2AX 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 else repair the damage. Therefore, it is generally advisable to collect longitudinal data in order to understand the kinetics of appearance (and sometimes disappearance) of the endpoints being measured by this multiplexed assay.

Short-term treatments can be useful for some lines of investigation, such as studying the kinetics by which DNA damage occurs and becomes repaired. In these situations, it may be useful to treat cells for up to one hour. 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 using attachment cells it is best to maintain cells at or below 80 % confluence at the time of harvest. If investigating multiple time points with attachment cells, satellite plates with different starting cell densities may be required to keep cells from becoming confluent.

Some chemicals are not genotoxic until they are converted to reactive electrophiles 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 users that are interested in studying promutagens it is advisable to review the literature on rat liver S9 preparations (and visit Molecular Toxicology, Inc.'s website: www.moltox.com). When a rat liver S9 metabolic activation system is employed with this kit, it is often necessary to limit the time cells are exposed to chemical plus activation system to approximately 3 to 4 hours, as is commonly done for many mammalian cell-based genotoxicity assays. After this exposure period, cells may be sampled for processing and analysis. Our current best advice is to first wash out chemical and rat liver S9 from the cells via centrifugation before combining them with kit reagents. If remaining cells are re-incubated, kinetics and endpoints that require greater manifestation times can be studied.

5. Preparation of Complete Labeling Solution

Prepare Complete Labeling Solution **the same day** as cell harvest and processing. 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.

1. **Sufficiently vortex (or mix by inversion)** 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. Mix well.

3. If using attachment cells add the specified volume of filtered FBS to the tube. Mix well.

4. Protect the Complete Labeling Solution from light and store at room temperature until use.

Number of wells	Volume of Nuclei Release Solution with Counting Beads	Volume of DNA Stain	Volume of RNase Solution	Volume of p53 Antibody FITC	Volume of FBS (attachment cells only)
1	50 µL	1.25 µL	0.25 µL	0.25 µL	1.00 µL
96 + 15 % = 110	5.5 mL	137.5 µL	27.5 µL	27.5 µL	110.0 µL

6. Sample Processing and Analysis

****First time users**:** As a means to appropriately set up the flow cytometer for MultiFlow analyses for the first time, it is recommended to generate an additional volume of sample derived from untreated cells. This process should also be performed after making changes to the machine or method, e.g. aligning the laser, changing cell types, but it should not be necessary for every set of samples analyzed.

Generate a high volume sample by scaling up the volumes used such that 200 µL of complete labeling solution is combined with 100 µL of cell suspension in a standard sample tube. Once the extra sample is processed according to the manual, it can be applied to the machine and analyzed using the instrument parameters described below. This approach should provide a sufficient amount of sample to optimize voltages and other instrument settings according to the provided gating schemes and example plots.

See Appendix B for advice and example chemicals for the conduct of initial experiments to become familiar with the performance of the MultiFlow assay. Such set-up experiments may also be useful 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 endpoints described herein.

6.1. Processing Plates

6.1.1. Suspension Cells

1. **Vortex or mix the Complete Labeling Solution throughout aliquoting to keep the Counting Beads in homogenous suspension.** Add 50 µL of the Complete Labeling Solution to individual wells of a clean 96 U-bottom well plate.

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

3. Add the cell suspension(s) to the well(s) containing the Complete Labeling Solution and mix thoroughly by pipetting up and down **10 or more times**. Repeat for remaining wells.

4. Incubate 30 minutes at room temperature.

5. After incubation, proceed with analysis.

6.1.2. Attachment Cells

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

2. Add 25 µL of cold (4 °C) Accutase® to each well.

3. Incubate for 10 minutes at room temperature.

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

5. Mix thoroughly by pipetting up and down **15 or more times or until cells are lifted.**

6. Transfer the total volume of each well to a clean 96 well U-bottom well plate for analysis.

7. Incubate 30 minutes at room temperature.

8. After incubation, proceed with analysis.

6.2. 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 the samples on the flow cytometer within 4 hours of adding cells to Complete Labeling Solution. If necessary, stagger cell treatment start times and addition of cells to of Complete Labeling Solution to keep within this 4 hour window.

   - Our current best advice is to collect at least 20 µL per sample at a rate of 1 µL/sec.

   - Samples should be mixed prior to analysis. For example, for HTS instruments, we recommend 4 cycles with 40 µL volumes. If the cytometer does not have a mix function, we recommend mixing every 12 samples by manually pipetting up and down.

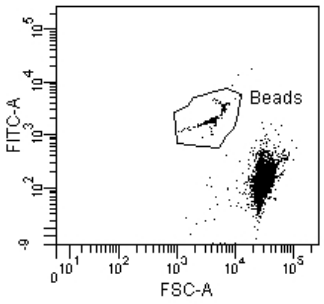
7. Endpoint Gating Logic and Calculations

7.1. General Advice

This section is intended as guidance for creating and editing a flow cytometer data acquisition template. 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).

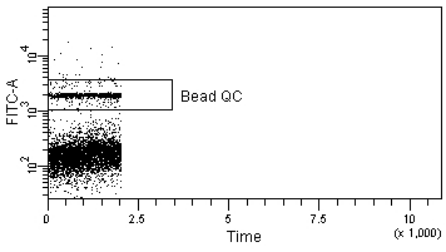
7.2. Bead Collection

- 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 8).



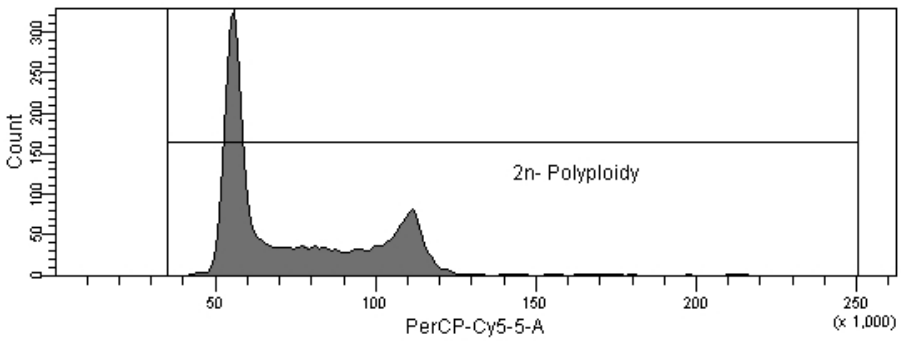
- We recommend collecting Counting Bead events using a dot plot capturing a fluorescence channel vs. forward scatter (right). It is important to choose the channel that gives the best separation between the bead and nuclei populations to avoid overlap; typically this is the FITC channel.

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



- A dot plot capturing a fluorescence channel (such as FITC) vs. time (right) can be used to ensure consistent fluidics and data acquisition rates. Any breaks in the "Bead QC" region could represent air uptake during sampling. See Appendix A for more information.

7.3. DNA Content Histogram



- For this plot, we recommend thresholding on PerCP and Forward Scatter to eliminate small debris below the 2n population. Ensure that these thresholds are not so high as to cut into the Counting Beads region described in Section 7.2.

- Many of the endpoints studied in this assay will focus on nuclei with specific DNA content, e.g,. 2n-4n, or 8n. A region capturing these events (2n through Polyploidy) will provide nuclei numbers that will later be considered in cytotoxicity metrics. A DNA content histogram on the PerCP channel capturing DNA associated flouresecence will facilitate examination of the DNA content of interest.

- This plot should exclude events falling into the Counting Beads region created in Section 7.2.

- PMT voltage should be set to ensure that the left most peak of the DNA content histogram (corresponding to 2n DNA content) is position such that 8n DNA content will remain on the x-axis scale. It can be helpful to view DNA content in linear space, as shown above.

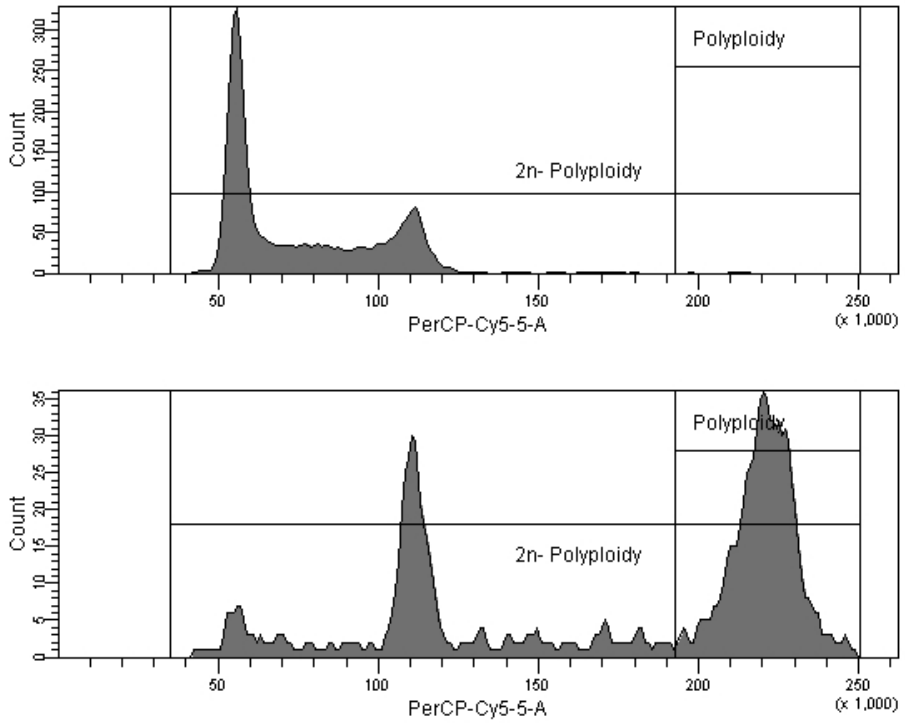
- Some users may wish to collect cell cycle-specific information. This can be accomplished by applying the appropriate regions to the histogram plots that correspond to the endpoint of interest to obtain the numbers that are G1, S or G2/M specific.

- Users may also track sub-2n DNA content to describe levels of DNA fragmentation resulting from apoptosis. Please contact technical support for advice on how to obtain these types of information.

7.4. Polyploidy

- Polyploidy can be a useful endpoint that is quantified on the same DNA content histogram introduced in Section 7.3. For polyploidy analysis, one may collect the number of nuclei that exhibit DNA-associated fluorescence corresponding to 8n DNA content, however other labs may wish to collect 8n and greater DNA content. The examples described below demonstrate collecting only 8N DNA content.

- A positive control for polyploidy such as the chemical AMG 900 can be useful for setting the polyploidy region (see Appendix B). In the example below, 24 hours of exposure to AMG 900 (bottom) induces a large degree of polyploidization relative to the negative control (top).



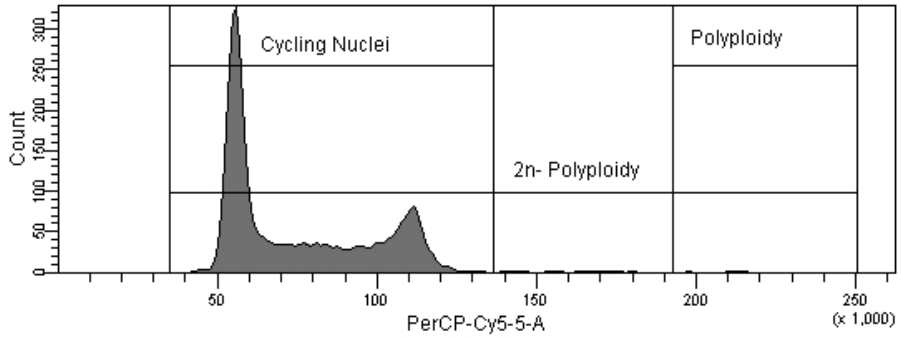
7.5. Percent Polyploidy

Use the formula below to calculate percent polyploidy for each treated and solvent control well. 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.

$$\text{Percent Polyploidy} = \frac{\#\text{Events of Polyploidy (8n) DNA content events}}{\#\text{Events of 2n-Polyploidy DNA content events}} \times 100$$

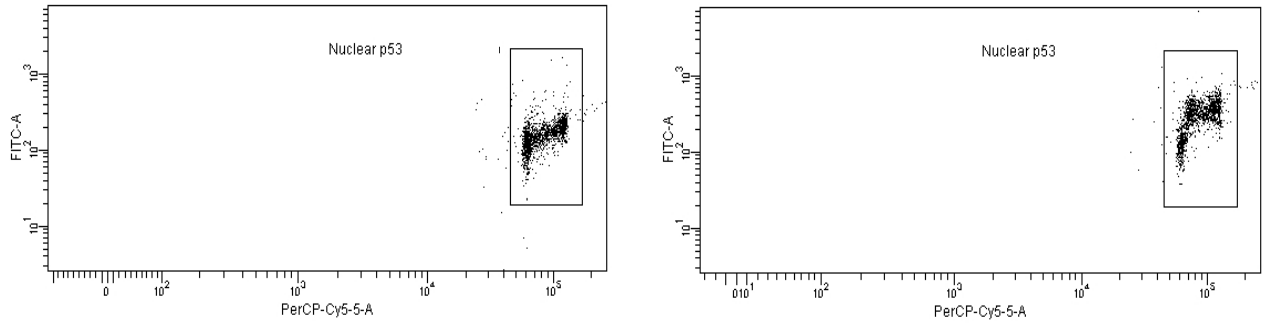
7.6. Cycling Nuclei

- The remaining endpoints described herein are focused on events with 2n-4n DNA content only.
- Create a “Cycling Nuclei” region capturing 2n-4n DNA content on the DNA content histogram as shown below.
- If cell number changes appreciably across experiments, subtle shifts in DNA associated fluorescence may be observed. If this happens, adjust regions and/or PMT voltages to ensure all of the 2n-4n DNA content events fall within the “Cycling Nuclei” region.



7.7. Nuclear p53

- In order to capture the median channel fluorescence (MCF) shift of p53-positive nuclei, create a FITC vs. PerCP dot plot derived from the “Cycling Nuclei” (2n-4n DNA content) region of the DNA histogram. A region capturing nuclei with room to accommodate vertical shifts in fluorescent intensity will be used to derive the MCF values.
- A positive control for p53 such as camptothecin can be useful for setting the p53 region (see Appendix B). In the example below, 4 hours of continuous exposure to camptothecin (right) induces a large fluorescence shift relative to the negative control (left).

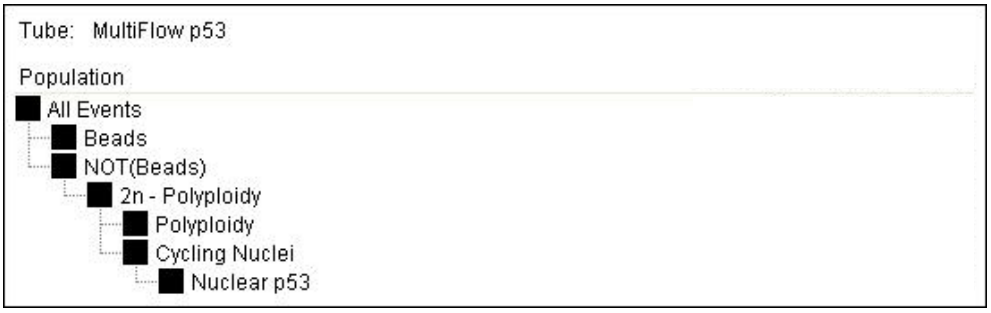


7.8. Nuclear p53 shift

Values for nuclear p53 shift are obtained from the MCF of the FITC channel. Use the formula below to calculate nuclear p53 shift for each treated and solvent control well.

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

7.9. Gating Logic Summary



8. Calculations

8.1. Cell Densities

- 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 (divide by 1.035 mL for suspension cells and 1.055 mL for attachment cells).

- The other values are obtained from the flow data for each sample.

$$\text{Cell Density} = \frac{\text{\#Events of 2n-Polyploidy nuclei}}{\text{\#Events of beads}} \times \frac{\text{Number of beads/mL in Nuclei Release Solution Plus Counting Beads}}{\text{1.035 mL or 1.055 mL}} \times \text{dilution factor (2)}$$

> 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”.

8.2. Relative Nuclei Count (RNC)

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

8.3. Relative Increased Nuclei Count (RINC)

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

8.4. Population Doubling (PD)

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

8.5. Relative Population Doubling (RPD)

$$RPD = \frac{\text{Number of Population Doublings in treated cultures}}{\text{Number of Population Doublings in mean solvent control culture(s)}} \times 100$$

9. 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.

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10. 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
email:	info@LitronLabs.com
World Wide Web:	www.LitronLabs.com

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 (e.g., FITC) 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.
	Incomplete lysis	Complete lysis of cells requires vigorous mixing of cells into Complete Labeling Solution. We recommend pipetting up and down 10 to 15 times when mixing cells into Complete Labeling Solution.
	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 30 minutes at ambient conditions.
	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 5 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 the 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 for 24 continuous hours, and perform MultiFlow analyses at both 4 hrs and 24 hrs. We encourage you to send example plots and/or data to Litron in order to receive assistance in review for quality of the sample processing and analysis. Also contact Litron for advice about treatment and handling of other cell lines.

Chemical	Cas. No.	Conc. Range* (μM)	Predominant Genotoxic Mode of Action	Expected p53 Response	Expected Polyploidization Response	Notes
Methyl methane-sulfonate	66-27-3	250-1000	Clastogen	Increase at 4 and 24 hrs	No appreciable effect	Prototypical clastogen response
Vinblastine Sulfate	143-67-9	0.001-0.01	Aneugen	Little to no effect at 4 hrs, increase at 24 hrs	No appreciable effect at 4 hrs, increased at 24 hrs	Prototypical aneugen response
5-Fluorouracil	51-21-8	50-175	Clastogen	Increase at 4 and 24 hrs	No appreciable effect	Atypical clastogen response (certain clastogens do not show the typical γH2AX increase)
AMG 900	945595-80-2	0.01-0.1	Aneugen	Increase at 4 and 24 hrs	No appreciable effect at 4 hrs, robust increase at 24 hrs	Atypical aneugen response (certain protein kinase inhibitors do not show the typical phospho-histone H3 increase)

* These concentrations are appropriate starting ranges for TK6 cells. Other cell lines may require different concentrations.