FlowCellect™ Bivariate Cell Cycle Kit for DNA Replication Analysis

25 Tests

Cat. No. FCCH025102

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Not for use in diagnostic procedures

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Application

Examination of the cell cycle has proven to be an accurate and effective method for assessing cell health by analyzing cell proliferation in great detail. Also recognized as the “Circle of Life”, the cell cycle can be divided into two distinct stages (figure 1). The first stage is Interphase which consists of the G1, S, and G2 phases where cells are active, growing, and DNA replication takes place. The second is M-phase, also known as the “mitotic phase”, where cell division takes place [3]. Researchers have employed various strategies to closely interrogate each of the critical steps at various phases in the cell cycle. For example, analysis of cells in S-phase will provide a direct measurement of newly synthesized DNA. Also, having the ability to discriminate cells between the G2 and M-phases can serve as a good indicator of cells undergoing mitosis. In all, a comprehensive understanding of cell cycle behavior can provide the researcher with useful information which will be important in interpreting the intrinsic nature of cell proliferation and assist in the development of anti-neoplastic agents.

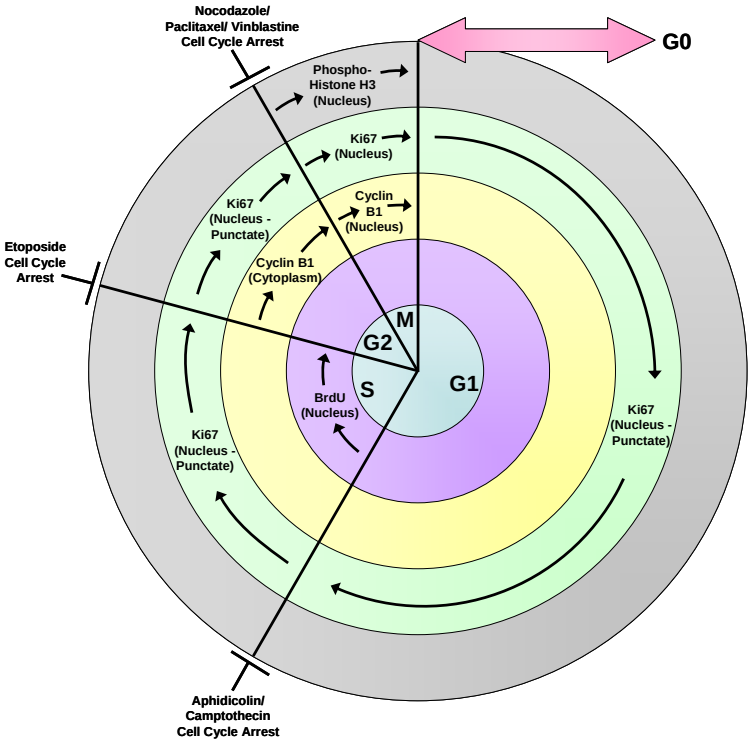

Figure 1. The “Circle of Life”, i.e.—the Cell Cycle The cell cycle can be divided up into two stages: Interphase and Mitosis. In interphase, cells are active and growing (G1 phase), DNA is replicated (S phase), and are preparing for mitosis as cells contain two copies of DNA (G2 phase). In mitosis, or M phase, cells undergo chromatin condensation eventually resulting in cell division.

Flow cytometric analysis of cell DNA content is widely used for the estimation of cell cycle phase distributions [1]. However, the limitation in evaluating by only one marker (such as a DNA dye only) is that cells within each phase cannot accurately be determined without the use of software modules. Studying the cell cycle is now made easier by using a cell cycle phase-specific antibody plus a DNA dye in flow cytometry applications. Bivariate analysis using flow cytometry will not only reveal the distribution of cells in particular phases of the cycle, but will also allow the researcher to elucidate the molecular and functional mechanisms associated within the cell cycle [2].

Millipore's FlowCellect™ Bivariate Cell Cycle Kit for DNA Replication Analysis allows the researcher to examine DNA replication in the S-phase by measuring the total amount of incorporated BrdU. The kit can be used to measure cell cycle distributions by discrimination all of cell cycle phases without the need of sophisticated software modules or algorithms. Having the ability to discriminate between cell cycle phases and to accurately measure DNA replication at S-phase, researchers are now provided with a powerful tool to closely monitor cell cycle activity which can have a huge impact in studying diseases associated with cell proliferation, apoptosis, and cancer.

All FlowCellect kits are optimized on the bench-top guava® flow cytometry systems, which saves valuable time and sample volume. All kits contain optimized fixation, permeabilization, wash, and flow buffers to provide researchers with a complete solution for bivariate analysis of total DNA content and cell cycle phase markers. With the guava platform and FlowCellect kits, researchers can finally have an easy, reliable and fully validated solution to study complex cell cycle distribution analysis right in the comfort of their own lab.

Test Principle

Detection of cell proliferation is a fundamental method for assessing cell health and evaluating anti-neoplastic agents. The most accurate method is to have a direct measurement of newly synthesized DNA [3]. However, using a DNA dye only has its limitations. An intercalating dye such as propidium iodide (PI) is an effective DNA staining reagent, however, PI alone will make discrimination of cell cycle phases and quantification of their content difficult. Hence, research tools using PI only require specialized software modules to differentiate and accurately measure cell cycle distributions.

Millipore's FlowCellect™ Bivariate Cell Cycle Kit for DNA Replication Analysis is designed to investigate cells undergoing replication in S-phase of the cell cycle with high accuracy and confidence in flow cytometry applications. The kit includes a directly conjugated Anti-BrdU Alexa Fluor® 488 conjugate plus a DNA dye (Propidium Iodide) which allows the researcher to perform bivariate analysis to distinguish cells in two dimensions without the need of software modules for data interpretation. Flow cytometry allows the simultaneous measurement of incorporated BrdU as well as the DNA content on a single cell level. In this way, the cohort of labeled cells can be followed through the cell cycle [1].

The kit includes an optimized protocol with all of the necessary components to provide the researcher a true "plug and play" design for their research when studying DNA replication and its relation to cell proliferation and cancer. The kit includes a BrdU precursor to allow for BrdU measurement by the conjugated antibody and an RNase reagent to ensure that the PI intensity is directly proportional to DNA content only. This immunocytochemical method is very sensitive, and it offers an assay to screen *in vivo* inhibitors involved in the S-phase transition by flow cytometry. It is important to determine the biological characteristics of certain cancers, not only to evaluate their malignant potentials but also to decide on the most effective anticancer therapies [6].

To validate the useful application of this kit by accurately measuring and quantitating the cells in S phase, a T lymphocyte cell line (Jurkat cells) were used for cell cycle analysis. Prior to cell staining, cells were incubated with a BrdU precursor for one hour at 37°C. Following BrdU pulse-labeling, the cells were fixed and permeabilized to ensure proper access for antibody entry into the cell. DNase was applied at 300 μg/mL final concentration for one hour at 37°C, followed by treatment with the anti-BrdU AF488 antibody for one hour on ice and in the dark. After a series of washing steps to remove any unbound antibody, the cells were stained with a propidium iodide/RNase solution. Cells were acquired using a guava® flow cytometer in which cell cycle distributions were determined using

bivariate analysis of plotting DNA content (PI staining) against BrdU staining. As noted in figure 3, by implementing this method DNA replication was accurately measured as indicated by the BrdU labeled cells in S phase. Moreover, cell cycle phases were accurately discriminated without the need of software modules.

BrdU incorporation has become the predominant methodology to assess DNA replication, and in particular, to study kinetics of cell cycle progression [5]. The percentage of BrdU labeled cells is a reliable estimate of the S phase compartment. If BrdU is administered as a short pulse (e.g. 30 minutes to one hour), BrdU measurement can provide information on the fraction of cells synthesizing DNA and the variation in the rate of DNA synthesis across the S phase. If BrdU is pulsed at a continuous level, the BrdU histogram gives data on the fraction of proliferating cells in the population [4]. In essence, BrdU/PI bivariate analysis by flow cytometry will make it possible to plot the DNA synthetic rate of cells against their DNA content, which will provide powerful information for the researcher in diagnosing various cancers.

Sufficient reagents are provided to perform 25 two-color tests. Detailed assay instructions are included to assist in analysis and to ensure the correct cell concentration is obtained during acquisition of sample data.

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Kit Components

1. 20X Anti-BrdU - Alexa Fluor 488 Conjugated Antibody: (Part No. CS204818) One vial containing 150 μL.
2. Propidium Iodide Solution: (Part No. CS204836) One vial containing 400 μL DNA dye.
3. BrdU (5-Bromo-2'-deoxyuridine) Reagent: (Part No. CS204820) One vial containing 100 μL.
4. RNase A Reagent: (Part No. CS204821) One vial containing 25 μL.
5. Fixation Buffer: (Part No. CS202122) One bottle containing 13 mL.
6. 10X Wash Buffer: (Part No. CS202123) One bottle containing 13 mL.
7. 5X Assay Buffer: (Part No. CS202124) One bottle containing 55 mL.
8. 1X Permeabilization Buffer: (Part No. CS202125) One bottle containing 13 mL.

Materials Not Supplied

1. Test tubes for sample preparation and storage
2. 96-well, v-bottom plate (Costar: 3897). **Recommended but not required if performing assay in a plate method.*
3. Tissue culture reagents, i.e. HBSS, PBS w/o Ca^{2+} or Mg^{2+}, cell dislodging buffers, etc.
4. Pipettors with corresponding tips capable of accurately measuring 10 – 1000 μL
5. Tabletop centrifuge capable of exceeding 700 x g
6. Mechanical vortex
7. guava flow Cytometer
8. DNase I reagent (Sigma Aldrich: D4513)
9. Deionized water (for Buffer dilutions)
10. Dimethyl sulfoxide (DMSO)

Precautions

- The instructions provided have been designed to optimize the kit's performance. Deviation from the kit's instructions may result in suboptimal performance and may produce inaccurate data.
- Some assay components included in the kit may be harmful. Kit contains a paraformaldehyde solution (fixation solution). Please refer to the MSDS sheet for specific information on hazardous materials.
- The fluorochrome conjugated antibody is light sensitive and must be stored in the dark at 4°C.
- During storage and shipment, the product supplied in vials may condense within the vial. For maximum recovery of the product, centrifuge original vial prior to removing cap.
- Do not use reagents beyond the expiration date of the kit.

Storage

This kit must be stored at 2 - 8ºC. The 10X Wash Buffer (Part # CS202123) and Fixation Buffer (Part # CS202122) can be stored at either 2 - 8ºC or at room temperature upon receipt. **Caution:** The fluorochrome conjugated antibody should always be stored at 2 - 8ºC and stored in the dark. Propidium Iodide Solution can be stored at -20°C for long term storage, but avoid freeze/thaw.

All kit components are stable up to six (6) months from date of receipt if stored and handled correctly. **Please avoid repeated changes in temperature as this will affect the integrity of the product.**

Preparation of Reagents

1. **Wash Buffer**

   Wash Buffer is supplied at 10X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Wash Buffer is stable up to one year. Store at 2 - 8ºC.

   Note: 10X Wash Buffer can be stored at RT to avoid any potential precipitate formation

2. **Assay Buffer**

   Assay Buffer is supplied at 5X concentration and should be diluted to 1X with deionized water prior to use. Prepared 1X Assay Buffer is stable up to one year. Store at 2 - 8ºC.

3. **DNase I Reagent**

   DNase I can be purchased from Sigma Aldrich (Catalog #: D4513). Material is supplied as a lyophilized powder. Prior to use, reconstitute DNase I in 0.15M NaCl solution. It is advised to reconstitute DNase I at a high concentration (10 mg/mL) and store at -20ºC. Avoid freeze/thaw. Since DNase I will be used at 300 μg/mL final concentration in the assay procedure, creating a 10 mg/mL working stock solution will be a 1: 33 dilution in 1X Wash Buffer.

4. **Propidium Iodide/RNase solution**

   Propidium Iodide Solution is provided in the kit at a concentrated form. For every experiment, dilute Propidium Iodide Solution 1:12 in 1X Assay Buffer (one part PI solution to 11 parts 1X Assay Buffer). Following dilution of PI, directly add RNase solution at a 1:300 dilution (e.g. for 1 mL of diluted PI solution, add 3.3 μL RNase). Prepare fresh solution for each assay run. Keep fresh solution at room temperature prior to use.

Assay Instructions

Note: This assay protocol has been optimized for human Jurkat cells. However, this kit is suitable for proliferation/cell cycle analysis of a variety of human cell types. Alternate species reactivity must be confirmed by the end user.

I. Labeling of Cells with BrdU

For adherent cells: Prior to cell seeding for assay, culture cells in growth media until ~70-80% confluent.

For suspension cells: The cell culture density should not exceed $2x10^6$ cells/mL.

To label cells *in vitro*, BrdU pulse-labeling time intervals that are optimal for each different cell line should be determined by end user. For example, an effective length of time for pulsing an actively proliferating human Jurkat cell line is 30-60 minutes. BrdU is provided at 1000X concentration, so add 1 μL of BrdU reagent per milliliter of cells directly into growth media in the tissue culture flask. The data provided is for 60 minutes of BrdU labeling at 37ºC prior to cell fixation.

II. BrdU and PI Staining Procedures

Note: During all steps in the assay procedure, keep all reagents on ice. After preparation of the cell cultures, follow the guidelines listed to ensure proper cell staining for optimal analysis.

1. Harvest Cells

For adherent cells, obtain cell culture by using a mild enzyme such as trypsin or an EDTA-based cell dissociation buffer.

For suspension cells, obtain cell culture by gently pipetting cell suspension up and down to ensure complete homogeneity. Remove cell culture media by centrifugation at 250 x g for 5 minutes.

*NOTE: It is recommended to prepare two extra samples for single color staining; one sample for BrdU staining only (using BrdU positive cells) and one sample for PI staining only. These samples will be used for compensation of test samples. Please refer to the compensation guide below for more details.

2. Fix and Permeabilize Cells

- For every $1x10^6$ cells, resuspend with 100 µL of 1X Wash Buffer and add 100 µL of Fixation Buffer. Incubate cells for 20 minutes on ice.
- Remove fixation buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.
- For every $1x10^6$ cells, resuspend with 100 µL ice cold 1X Permeabilization Buffer. Allow to incubate for 5 minutes on ice.
- Remove Permeabilization Buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.
- Wash cells once by adding 200 µL 1X Assay Buffer for every $1x10^6$ cells. Carefully disrupt cell pellet to homogeneity, and remove assay buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.

3. DNA Denaturation

- Add 100 µL of diluted DNase I (diluted to 300 µg/mL in 1X Assay Buffer) to each sample (~1x10^6 cells) for 1 hour at 37°C.
- Remove DNA denaturation buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.
- Wash cells once by adding 200 µL 1X Assay Buffer for every 1x10^6 cells. Again, carefully disrupt cell pellet to homogeneity and remove assay buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet.

4. BrdU Staining

- Add 5 µL of an optimized 20X stock Anti-BrdU direct conjugate to Alexa Fluor 488 to 95 µL of 1X Assay Buffer for every 1x10^6 cells stained. Allow to incubate on ice for one hour in the dark.
- Wash cells by adding 200 µL of 1X Assay Buffer for every 1x10^6 cells. Carefully disrupt cell pellet to homogeneity and remove assay buffer by centrifugation at 670 x g for 5 minutes. Aspirate and discard supernatant, only saving the cell pellet. Perform washing step twice to remove any excess Anti-BrdU antibody.

5. DNA Staining

- Add 200 µL of freshly prepared Propidium Iodide/RNase solution (cells in a final 0.5x10^6 cells per mL). Incubate for 30 minutes at room temperature in the dark.

6. Guava Analysis

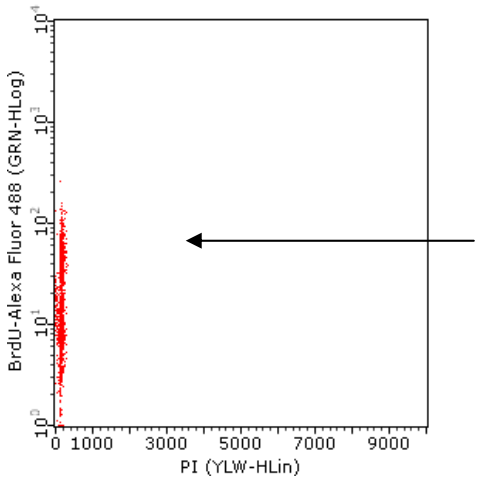
- All DNA measurements (PI staining plotted on the X-Axis) MUST be analyzed using linear scaling. Analysis using logarithmic scale will make it difficult to discern cellular distributions for each phase of the cell cycle.
- Voltage adjustment: In order to differentiate G1 and G2/M phase cell populations, increase the voltage accordingly until a good degree of cell separation is achieved.
- Performing cell analysis on a guava easyCyte instrument can be performed by using a 96-well plate (200 µL volumes) or single tube (500 µL volumes).
- If using alternative flow cytometry instrumentation, dilute cells according to instrument manufacturer's recommendations.

*NOTE for guava users: Since our initial cell density is high (e.g. 2000 cells/ µL), first test samples on the guava instrument to acquire accurate cells per µL density. If too many cells are present, dilute cell samples down in the well(s)/tube(s) accordingly with assay buffer to achieve < 500 cells/ µL acquired.

Compensation Guide

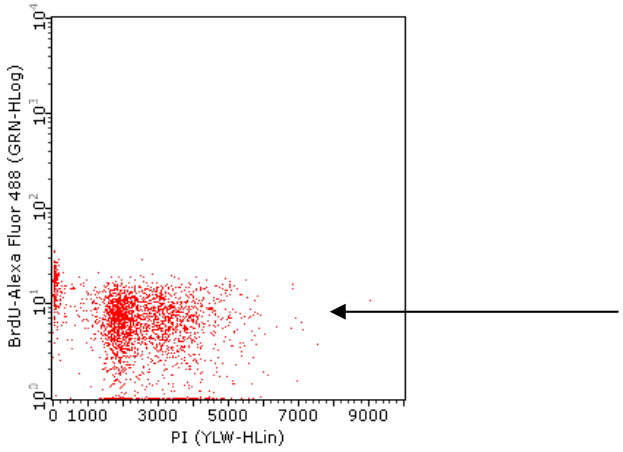
Use single color staining for compensation (Anti-BrdU and PI). Below are the typical parameters used when arranging compensation for guava flow cytometry systems. Please note that these settings must be fine tuned for every individual experiment provided that different cell models may be used. (Settings for other instrumentation may vary).

1. In one tube/well, use BrdU-AF488 single color stained sample. Using the compensation tools, adjust "Yellow to Green". Adjust until two cell populations are aligned along the Y-axis.



Anti-BrdU-AF488 single color staining (post compensation): The cell population must run parallel along the Y-axis. By adjusting the compensation ("yellow to green")

2. In the second tube/well, use PI single color stained sample. Using the compensation tools, adjust "Green to Yellow". Adjust until two cell populations are aligned along the X-axis.



Propidium Iodide single color staining (post compensation): The cell population must run parallel along the XY-axis. By adjusting the compensation ("green to yellow")

Sample Results

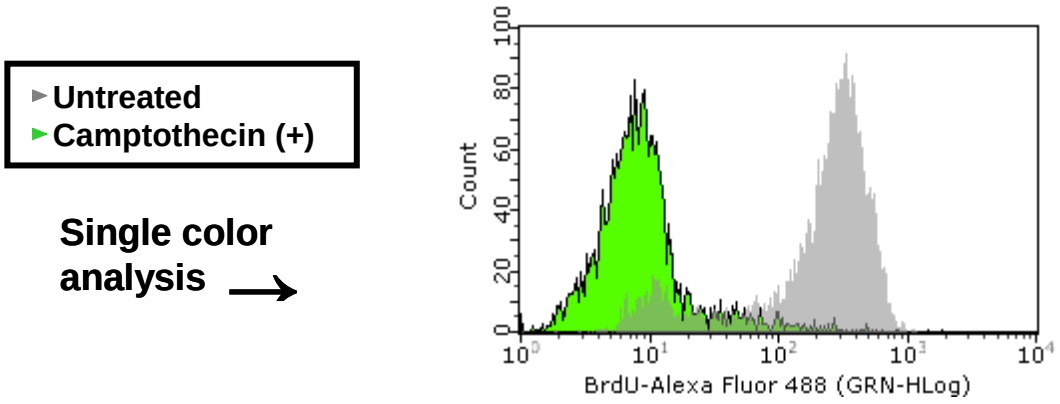
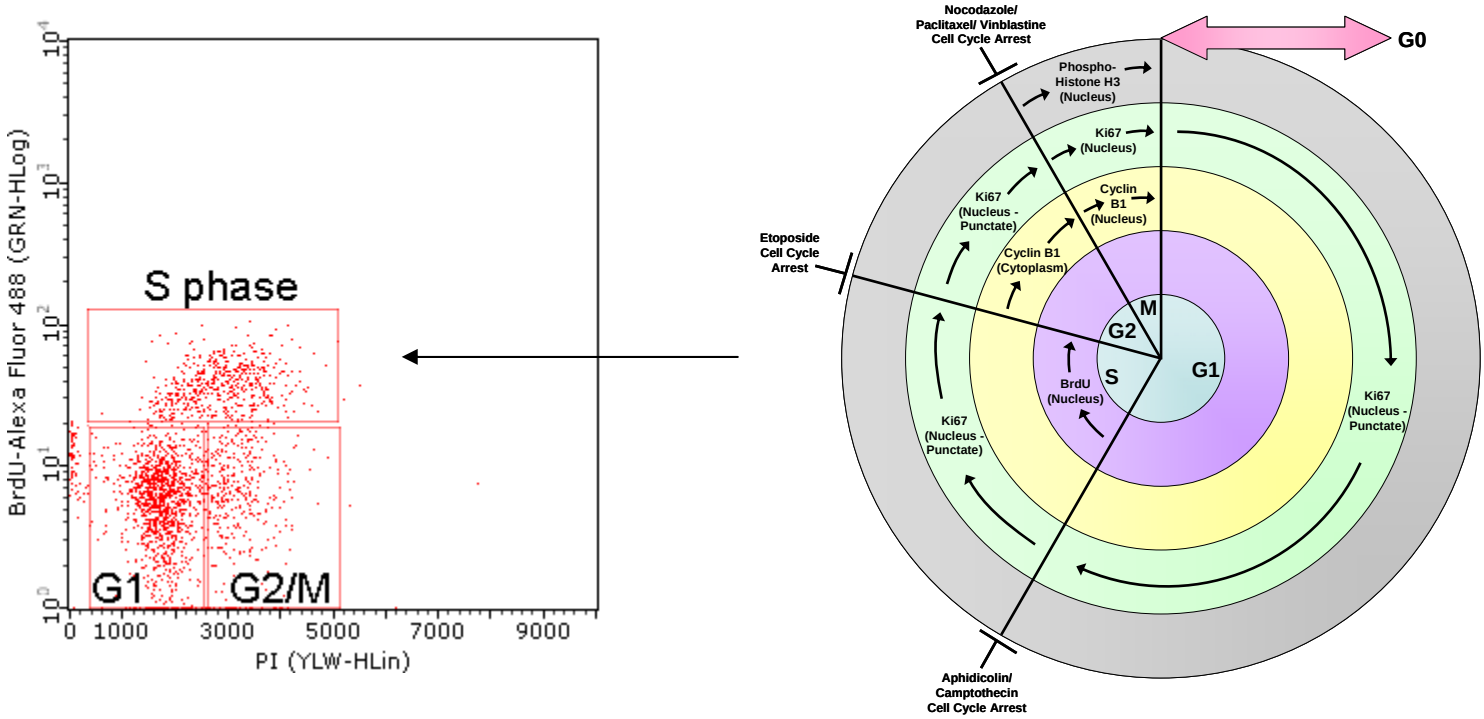


Figure 2. Anti-BrdU antibody validation using Camptothecin By treating Jurkat cells with cell cycle arrest agent Camptothecin, circulating cells are trapped at the G1/S phase transition. As a result, the BrdU precursor will not have the ability to incorporate into newly synthesized DNA indicated by a loss of signal when treated with the BrdU antibody (green). However, the untreated sample is allowed to incorporate the BrdU precursor freely, resulting in a high level of cell staining as detected by the BrdU-AF488 conjugated antibody (grey).



Cell Cycle Phases
G1=52%
S=24%
G2/M=18%

*Cell Cycle distributions

Figure 3. Detection of DNA Replication by analysis of S phase cells As described in Darzynkiewicz et al. [2], bivariate analysis of DNA content versus immunofluorescence of BrdU Alexa Fluor 488 conjugate can distinguish S phase cells with great accuracy, not only based on their difference in DNA content from G1, or G2/M cells but also as having incorporated the BrdU.

Technical Hints

- Kit antibody, 5X Assay Buffer, and 1X Permeabilization Buffer should always remain at 2 - 8ºC, both prior and during use. For long term storage, Propidium Iodide can be stored at -20ºC. If keeping at -20ºC, avoid multiple freeze/thaw cycles.
- Mix samples thoroughly before use; avoid excessive bubbling.
- If minor precipitate is detected in the 10X Wash Buffer place the bottle in a warm water bath for 30 minutes, followed by mixing the contents on a mechanical vortex.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- For certain cell cultures, cell pellets may become hazy or transparent following the fixation step, making it difficult to see. If sampling a small collection of cells for flow analysis, it is recommended to perform all steps in a smaller collection tube (e.g. micro-centrifuge tube)
- Do not mix or interchange reagents from various kit lots.

Troubleshooting

Assay Step	Potential Problem	Experimental Suggestions
Reagent Preparation	Precipitation found in 10X Wash buffer	• If storing at -20ºC, precipitate can form in the 10X wash buffer. Prior to use, place bottle in a 37ºC water bath, swirling the contents occasionally. If this does not remove the precipitate completely, allow 10X Wash Buffer to sit at room temperature overnight.
Acquisition	Acquisition rate decreases dramatically	This usually indicates that the fluid pathway on the instrument may be blocked. This can be alleviated by the following: • Decreasing number of cells for analysis. The guava easyCyte™ Plus has the capacity of analyzing a steady stream of 300 – 500 cells per microliter. Any cell densities in excess can essentially block the normal flow, causing disruption during the assay. Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per milliliter. • Adherent or sticky cells can result in cellular clumping. Use a more aggressive enzyme for dissociation such as trypsin during cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping if needed (Catalog No. SCNY00060; 60 μM) • After many uses, it is possible that the fluid system on the guava easyCyte™ Plus requires cleaning. Run a Quick Clean procedure to clean the fluid system during or after an assay. This will prevent any material from forming within the glass capillary walls.
Cellular Analysis	A loss or lack of signal	• Cell numbers may need to increase. Cell loss is common during washing steps in the assay procedure. A substantial decrease in cell numbers can lead to a loss of signal. Make sure that cell density remains at approximately 0.5 million cells per milliliter during analysis. • Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. A lack of signal may indicate that excess antibody will need to be used during the staining procedure.
Cellular Analysis	Background and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure.
Cellular Analysis	Variability in day to day experiments	• Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any drop in cell numbers or viability can influence experimental results. • When using the guava easyCyte™ Plus instrument for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use. (**See Analytical Sensitivity and Detection Limits Section for Guava Check standards*)

For further support, please contact Millipore's Technical services at +1(800) 437-7500

References

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2. Darzynkiewicz, Z. *et al.* (2001) Flow Cytometry in Analysis of Cell Cycle and Apoptosis. Semin Hematol. Apr;38(2):179-93.
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4. Lacombe, F. *et al.* (1996) Flow cytometry study of cell cycle, apoptosis and drug resistance in acute leukemia. *Hematol Cell Ther.* Dec;38(6):495-504.
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Related Products

1. FlowCellect™ Bivariate Cell Cycle Kit for G2/M Analysis (Catalog No. FCCH025103)
2. FlowCellect ™ Multi-Color DNA Damage Response Kit (Catalog No. FCCH025104)
3. FlowCellect™ EGFR/MAPK Pathway Activation Kit (Catalog No. FCCS025101)
4. FlowCellect™ PI3K-mTOR Signaling Cascade Mapping Kit (Catalog No. FCCS025210)
5. FlowCellect™ Multi-STAT Activation Profiling Kit (Catalog No. FCCS025550)
6. FlowCellect™ PI3K/MAPK Dual Pathway Activation and Cancer Marker Detection kit (Catalog No. FCCS025100)

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