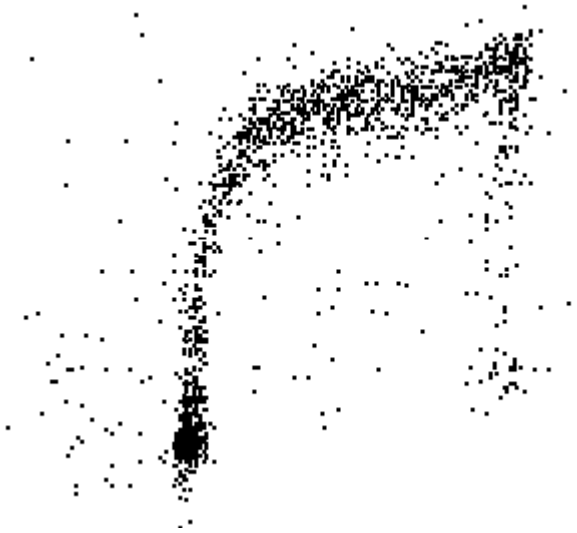
EZ-BrdU™

A Complete Kit for Measuring Cell Proliferation using BrdU by Flow or Image Cytometry



Catalog No. AC1001

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Description of Kit

The Phoenix Flow Systems, Inc. **EZ-BrdU™** assay kit is a two color staining method for measuring cell proliferation by multi-parameter analysis of DNA replication and cellular DNA content/cell cycle position with flow or image cytometry (2). The kit contains instructions and all reagents which include the BrdU solution for feeding to the cells before they are stained, washing, rinsing buffers for processing individual steps in the assay; denaturation, neutralization buffers for making the incorporated BrdU accessible for labeling, fluorescein labeled anti-BrdU antibody (F~PRB-1) for labeling incorporated DNA, and propidium iodide/RNase A solution for counter staining the total DNA. In addition, positive control cells are included to eliminate some of the variables of the assay. The positive control cells are fixed cultured cells which have BrdU incorporated into their DNA. Phoenix Flow Systems also offers other dye configurations for the assay including phycoerytherin, TRITC, and the Alexa dyes from Molecular Probes.

Contents of the EZ-BrdU™ Kit

The **EZ-BrdU™** Kit is shipped in one container which houses two packages containing different colored reagent bottles. One package (plastic bag) is shipped at ambient temperature and the contents should be stored at 2-8C upon arrival. The other package is a styrofoam box containing frozen ice packs and this reagent contents should be stored at -15 to -25C upon arrival. Phoenix Flow Systems, Inc. has determined this shipping method is adequate to maintain the integrity of the Kit components. **UPON ARRIVAL STORE THE REAGENTS AT THE APPROPRIATE TEMPERATURES.**

Precautions and Warnings

1. The components of this kit are for **Research Use Only** and are not intended for diagnostic procedures.
2. The components ACNC12, ACWB15, ACRB17, and ACPR18 contain 0.05% (w/v) sodium azide as a preservative. These materials are harmful if swallowed; avoid skin contact, wash immediately with water. See **Material Safety Data Sheets**.
3. Component ACDB16 contains 2N HCL. This material is harmful if swallowed, avoid skin contact, wash immediately with water. See **Material Safety Data Sheets.**

Reagents and Materials Required, but not Supplied

1. Flow Cytometer capable of measuring Red and Green Fluorescence.
2. Distilled water
3. 70% (v/v) ethanol
4. Ice Bucket
5. 12 x 75 mm flow cytometry polystyrene test tubes
6. Pipettes and Pipetting Aids

EZ-BrdU™ Kit Components:

COMPONENT	CAP COLOR	PART NUMBER	VOLUME (ml)	STORAGE CONDITIONS
Positive Control Cells	white cap	ACNC12	5.000	-15 to -25C
BrdUrd Photolyte	pink cap	ACBP13	2.000	-15 to -25C
Wash Buffer	blue cap	ACWB15	175.000	2 to 8C
Rinse Buffer	red cap	ACRB17	130.000	2 to 8C
Denaturation Buffer	clear cap	ACDB16	65.000	2 to 8C
Neutralization Buffer	green cap	ACNB14	65.000	2 to 8C
Fluorescein~anti-BrdU monoclonal antibody (PRB-1 clone)	orange cap	ACFM20	0.325	2 to 8C
Propidium Iodide/RNase Staining Buffer	amber bottle	ACPR18	32.500	2 to 8C

The **Reagent Bottles** have colored caps to aid in their identification. Sufficient reagents are provided to process 60 cell suspensions and an additional 5 Positive Controls. The Positive Controls are at approximately 1 x 10^6 cells per ml in 70% (v/v) ethanol. The control cells are derived from a human lymphoma cell line that has been fed BrdU and fixed as described on page 5.

Description of Cell Proliferation

Cell proliferation is that process wherein a single cell becomes committed to following a pathway of growth and the necessary duplication of specific cellular constituents (e.g., DNA), so that cell division may follow, resulting in the formation of two daughter cells. Each daughter cell must have all of the materials and information required to maintain viability and preserve the capability for further proliferation, as needed.

The proliferative process has been divided into several phases that are collectively termed the cell cycle, as they may be repeated in cyclical manner essentially indefinitely. The phases of the cell cycle are labeled G_1 (Gap 1), S (DNA Synthesis) and G_2/M (Gap 2/Mitosis). Each phase is characterized by certain activities that are required for proliferation to proceed to the next phase.

There is a pre-proliferative phase that is designated G_0 and it is the transition from G_0 to G_1 that signals the commitment to proliferation. In very basic terms, G_1 cells are characterized by increases in certain RNA and protein components (relative to G_0) as the cell begins preparing for the growth and replicative processes that must precede division.

When these preparations are completed, the cell enters the S-phase of the cell cycle, the phase in which the cellular DNA is duplicated, so that each daughter cell will receive a complete set of genetic information. The completion of DNA synthesis leads to the transition into the G_2 phase of the cycle, wherein the final preparations for division are made. Division itself comprises the M- (Mitosis) phase of the cycle, resulting in the formation of the two daughter cell progeny.

The phases of the cell cycle are often characterized according to the amount of DNA within the cell during each of the phases. Both G_0 and G_1 have what is described as the 2C complement of chromosomal DNA, leading to the oft-used G_0/G_1 designation for such cells. Total cellular DNA content steadily increases during the S-phase of the cell cycle, until the 4C total for cells with completely duplicated DNA is reached. This is the amount of DNA characteristic of cells in the G_2- and/or M-phase of the cell cycle (hence the common G_2/M designation).

Measurable Features of Cell Proliferation

One of the most easily measured features of cell proliferation is the changing amount of cellular DNA associated with each of the cell cycle phases. Cellular DNA may be labeled with any of a variety of DNA binding fluorochromes and the subsequent fluorescence measured to determine the relative DNA content and cell cycle position of the cells being studied. This type of measurement is inherently somewhat imprecise, though, with the cutoffs between the G_0/G_1-, S- and G_2/M-phases of the cell cycle not clearly delineated. It is often desirable to have a means of unequivocally identifying actively proliferating cells and the most straightforward manner for doing this is to some how specifically label newly replicated DNA. Though there are several approaches to accomplishing this, the methods of choice involve some how labeling the nucleotide building blocks that are incorporated during DNA replication. An ingenious method for allowing this has been developed, involving the use of 5-bromo-2-deoxyuridine (BrdU), a thymidine analog. As a thymidine analog, BrdU is preferentially incorporated into newly replicated DNA and can then be subsequently labeled, unequivocally identifying cells containing such DNA.

The growth of cells in the presence of BrdU has become an accepted method for monitoring DNA replication (1,2). The use of BrdU for assaying DNA replication has replaced methods utilizing radioisotope-labeled thymidine in both research and clinical laboratories (3,4). The incorporation of BrdU into cellular DNA is most commonly detected using anti-BrdU antibodies (1,5). This methodology requires that the cellular DNA be denatured to separate the duplex strands in order for the BrdU epitope to become accessible and reactive to the antibody. This denaturation process usually involves a heat treatment (>90C), acid (2-4N HCl) treatment, enzymatic (DNAse digestion) or UV light exposure (SBIP or Hammer). Each method has its proponents and detractors and no denaturation method has been proven to be clearly superior to any of the others. For this reason, PFS offers two kits presently. One uses the UV light exposure (SBIP) method (Absolute-S) and the other uses the acid denaturation method (EZ-BrdU). In the future we will be offering the other two methods as well. Proponents of the enzymatic treatment method maintain that the acid treatment results in the loss or denaturation of many cellular proteins. However, using the "low acid" method of the EZ-BrdU kit, we have not seen this limitation and have found it well suited for simultaneous staining of many

intracellular proteins. The EZ-BrdU method is definitely less labor intensive than either the enzymatic or SBIP method. We leave it up to the individual investigator to decide which method is best for his particular experimental settings. We only strive to provide the least confusing and least cumbersome protocols for each of the assays.

All BrdU incorporation methodologies first involve growing cells in the presence of BrdU (**BrdU Photolyte**™ solution in PFS kits) in order to label the synthesized cellular DNA with bromolated deoxyuridine. After the cells have been grown in the presence of the BrdU solution, they must be fixed to permeabilize the membranes of the cells so the anti-BrdU antibody can get into the nucleus of the cell where the DNA is located. A convenient method to accomplish this is with 70% ice cold ethanol. Once the cells are fixed in ethanol, they may be stored in the freezer until enough samples are collected to justify an experiment.

Flow Diagram of EZ-BrdU™ Cell Proliferation Assay

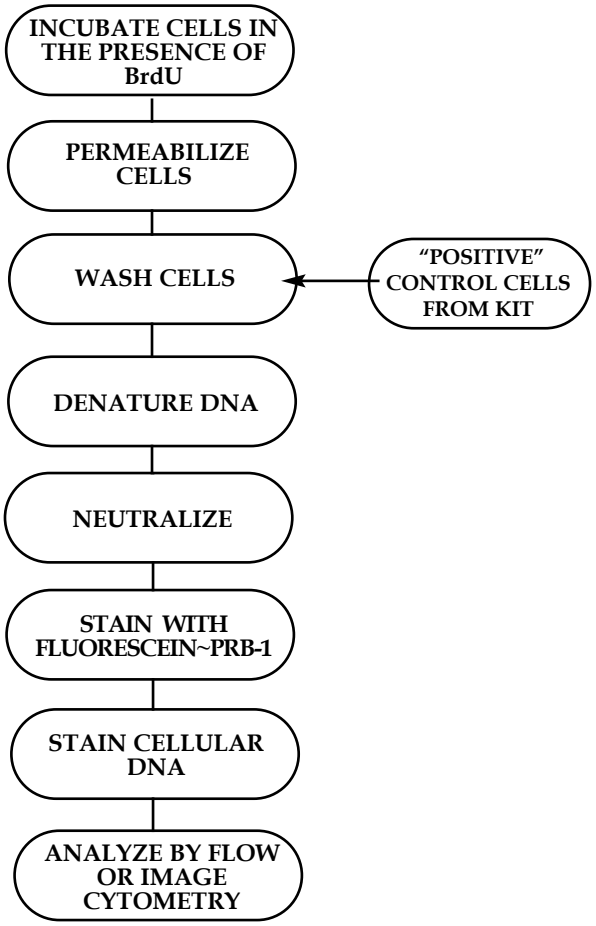


Figure 2: Flow diagram used in the EZ-BrdU™ Cell Proliferation Assay.

In Vitro Labeling of Cells with BrdU

Cells in Culture

Since the incorporation of BrdU into the cellular DNA is a requirement for the measurement of proliferating cells with this assay, the first step is to make sure the cells are growing and not confluent in culture. Determine the doubling time of the cell culture a few days prior to feeding the cells BrdU. On the day of the experiment, add the BrdU solution at a point prior to the completion of the synthesis phase (S-phase) of the cycle. Also, be careful when adding the BrdU solution not to disturb the cells so their cell cycling time is not disrupted.

BrdU Incorporation

a. Add 20 µl of the **BrdU Photolyte** stock solution (**ACBP13, pink cap**) per 10 ml of cell culture medium.
b. Incubate the cells at 37C (CO_2 incubator) for a minimum of 20 to 40 minutes. This time is dependent upon the doubling time of the particular cell culture. Some slow growing cells could require incubations time with the BrdU as high as 36 hours. For this reason, investigators should determine the optimal BrdU pulse duration for each cell system under investtigation.

Cells in a Mouse/Rat

There are two common methods for BrdU labeling of cells in a live mouse/rat: Intraperitoneal injection of a BrdU solution into the mouse and feeding the mouse with f BrdU that has been added to its drinking water. However, Phoenix Flow Systems is not experienced in either method.

BrdU Incorporation by injection

Inject the animal intraperitoneally with 334 ul (1mg) of BrdU Photolyte solution. Detection of the incorporated BrdU can occur in the bone marrow and elsewhere within as little as 1 hour post injection.

BrdU Incorporation by ingestion

Add 300ul of BrdU Photolyte per ml of drinking water. Water should be prepared fresh daily and feeding should extend over the course of a full week for best incorporation with little associated toxicity.

Cell Fixation Procedure for the EZ-BrdU™ assay

After the cells have been grown in the presence of the BrdU solution, they must be fixed to permeabilize the membranes of the cells so the anti-BrdU antibody can get into the nucleus of the cell where the DNA is located. A convenient method to accomplish this is with 70% ice cold ethanol. Once the cells are fixed in ethanol, they may be stored in the freezer until enough samples are collected to justify an experiment.

1. **Permeabilization and Fixation Procedure**

a. Centrifuge the cells for 5 minutes (300 x g) and remove the supernatant by aspiration.
b. Resuspend the cells at a concentration of 1 to 5 X 10^6 cells/ml in **Wash Buffer (blue cap)**.
c. Repeat Step a.
d. Resuspend the cell pellet in the tube in the residual **Wash Buffer (blue cap)** left after aspiration by gently vortexing the tube.
e. Adjust the cell concentration to 1 -2 X10^6 cells/ml in 70% (v/v) ice cold ethanol.
f. Store the cells over night or until ready to use (at least 18 hours).

EZ-BrdU™ STAINING PROTOCOL

The following protocol describes the method for measuring cell proliferation in the **Positive Control cells** that are provided in the **EZ-BrdU™** Kit. The same procedure should be employed for measuring cell proliferation in the cells that have undergone fixation and permeabilization by the researcher.

1. Resuspend the **positive (white cap)** control cells by swirling the vial. Remove a 1 ml aliquot of the control cell suspension (approximately 1 x 10^6 cells per 1 ml) and place in 12 x 75 mm centrifuge tubes. Centrifuge (300 x g) the control cell suspension for 5 minutes and remove the 70% (v/v) ethanol by aspiration being careful to not disturb the cell pellet.

2. Resuspend the tube of control cells with 1 ml of **Wash Buffer (blue cap)** for each tube. Centrifuge as before and remove the supernatant by aspiration.

3. Repeat the **Wash Buffer** treatment (step 2).

4. Resuspend the tube with the control cell pellet in 1 ml of the of the **Denaturation Buffer (clear cap)**.

5. Incubate 30 minutes at room temperature.

6. Centrifuge cells for 10 minutes at 400 x g.

7. Aspirate supernatant and immediately add 1 ml of **Neutralization Buffer** (green cap).

8. Centrifuge cells for 10 minutes at 400 x g.

9. Wash cells one time with 2 ml **Rinse buffer** (red cap).

10. Resuspend the cells pellet in 0.1 ml of the **Antibody Solution** (prepared as described below).

ANTIBODY SOLUTION	1 ASSAY	5 ASSAYS	10 ASSAYS
Fluorescein~PRB-1 (orange cap)	5.00 µl	25.00 µl	50.00 µl
Rinse Buffer (red cap)	95.00 µl	475.00 µl	950.00 µl
Total Volume	100.00 µl	500.00 µl	1000.00 µl

11. Incubate the cells with the **Fluorescein~PRB-1 Antibody Solution** in the dark for 60 minutes at room temperature.

12. Add 0.5 ml of the **Propidium Iodide/RNase A Solution (amber bottle)** to the tube containing the **Antibody Solution**.

13. Incubate the cells in the dark for 30 minutes at room temperature.

14. Analyze the cells in the **Propidium Iodide/RNase A Solution** by flow cytometry.

15. Analyze the cells within three (3) hours of the staining.

Analyzing the EZ-BrdU™ Samples on the flow cytometer

This assay is run on a flow cytometer equipped with a 488 nm Argon laser as the light source. Propidium Iodide (total cellular DNA) and Fluorescein (Proliferating Cells) are the two dyes being used. Propidium Iodide (PI) fluoresces at about 623 nm and Fluorescein at 520 nm when excited at 488 nm. No fluorescence compensation is required. Two dual parameter and two single parameter displays are created with the flow cytometer data acquisition software. The gating display should be the standard dual parameter DNA doublet discrimination display with the DNA Area signal on the Y-axis and the DNA Width (Becton-Dickinson), see Figure 4 next page or DNA Peak/Integral (Beckman Coulter) signal on the X-axis, see Figure 5 on page 12. From this display, a gate is drawn around the non-clumped cells and the second gated dual parameter display is generated. The normal convention of this display is to put DNA (Linear Red Fluorescence) on the X-axis and the Fluorescein~PRB-1 (Log Green Fluorescence) on the Y-axis (see bottom display next page). Two single parameter gated histograms, DNA and Fluorescein~PRB-1, can also be added but are not necessary. By using the dual parameter display method, not only are cycling cells resolved but the total cell cycle is displayed. The dual parameter histograms of the control cells should look like Figure 3 below.

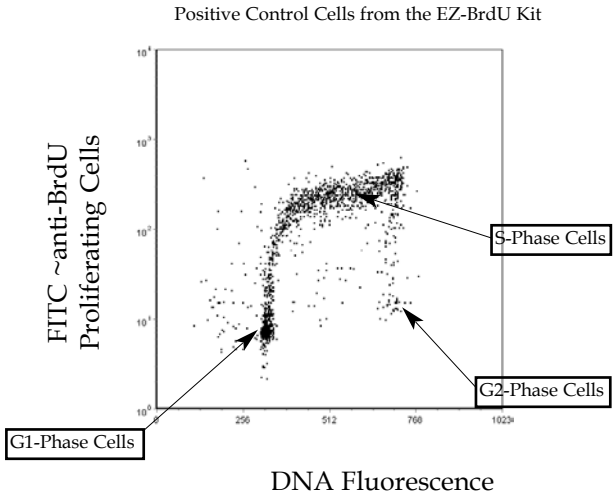
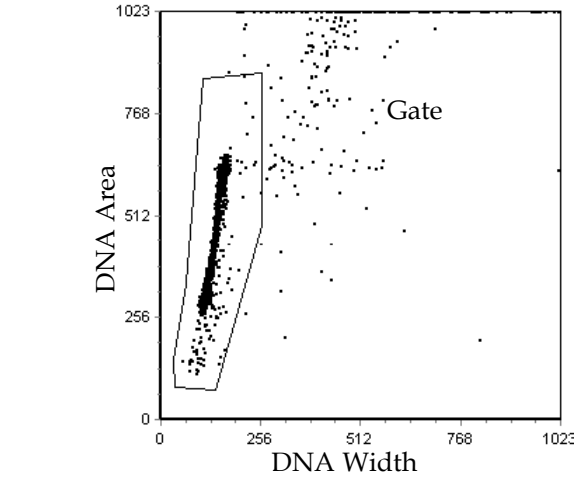
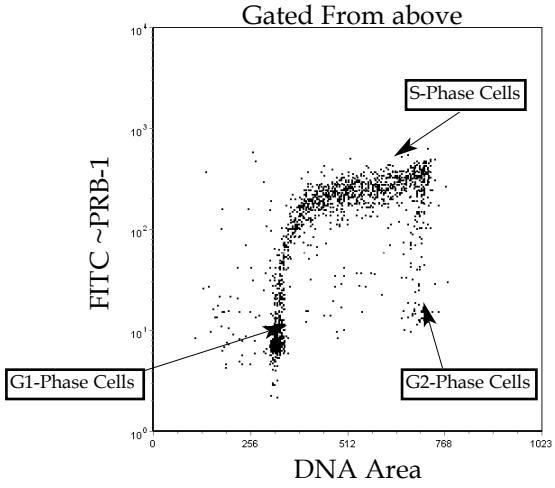


Figure 3: Flow Cytometry Data of EZ-BrdU™

Flow Cytometer Setup for Becton Dickinson Hardware



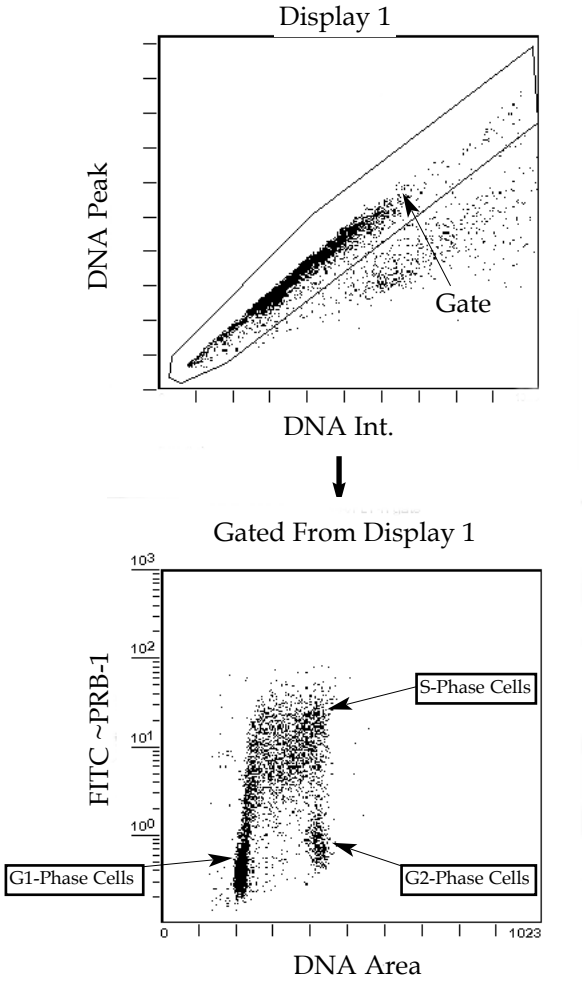


Typical Caliber™ Gain Settings

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	400 Volts
FL 3	1.46	430 Volts
FL 3 Width	.87	
FL 3 Area	3.25	
	Threshold- FL 3, 40	

Figure 4: EZ-BrdU™ Positive Control Cells

Flow Cytometer Setup for Beckman Coulter Hardware



Typical XL™ Gain Settings

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	589 Volts
FL 3	2.00	698 Volts
AUX(FL3 Peak)	1.00	250 Volts

Discriminator-AUX (FL3 Peak)

Figure 5: EZ-BrdU™ Positive Control Cells

Technical Tips and Frequently Asked Questions About the EZ-BrdU™ Assay

1. To minimize cell loss during the assay, restrict the assay to the use of a single 12 x 75 mm test tube. If polystyrene plastic test tubes are used an electrostatic charge can build up on the sides of the tube. Cells will adhere to the side of the tube and the sequential use of multiple tubes can result in significant cell loss.

2. If there is a low intensity of green fluorescence, i.e. lack of S-phase cells, adjust the cell concentra tion in culture prior to the time of **BrdU Photolyte™** addition to ensure that cells are indeed in an exponential phase of growth. The cells can not be confluent when the **BrdU Photolyte™ is** added. The cells should be in an exponential growth phase for a minimum of two passages before attempting this assay. If this is not the problem, try lowering the concentration of PI/RNase A solution to reduce quenching of the BrdU signal.

3. If DNA cell cycle information is not required, it is not necessary to add the PI/RNase A solution to each tube.

References

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