ABSOLUTE-S™ Kit

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Manufactured by Phoenix Flow Systems.

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NORTH AMERICA

AXXORA, LLC
6181 Cornerstone Court East
Suite 103
San Diego, CA 92121-4727
Phone: (858) 550-8828
Fax: (858) 550-8825
E-mail: axxora-usa@axxora.com

SWITZERLAND/REST OF THE WORLD

ALEXIS CORPORATION
Industriestrasse 17, Postfach
CH-4415 Lausen / Switzerland
Phone: +41 61 926 89 89
Fax: +41 61 926 89 79
E-mail: alexis-ch@alexis-corp.com

GERMANY

AXXORA DEUTSCHLAND GmbH
Gallusstrasse 10
DE-35305 Grünberg
Phone: (06401) 90077
Fax: (06401) 90078
E-mail: axxora-de@axxora.com

UK & IRELAND

AXXORA (UK) LTD.
P.O. Box 6757
Bingham, Nottingham NG13 8LS
Phone: +44 1949 836111
Fax: +44 1949 836222
E-mail: axxora-uk@axxora.com

For laboratory use only. Not for human or diagnostic use.

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ABSOLUTE-S™

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

Description of Kit

The Phoenix Flow Systems, Inc. **ABSOLUTE-S™** Kit is a two color staining method for measuring cell proliferation by multiparameter analysis of DNA replication and cellular DNA content/cell cycle position by flow or image cytometry (6). The kit contains instructions and all reagents which include BrdUrd Photolyte™ and Photolyte Enhancer™ solutions; washing, reaction and rinsing buffers for processing individual steps in the assay; terminal deoxynucleotidyl transferase enzyme (TdT), bromodeoxyuridine triphosphate (Br-dUTP), fluorescein labeled antiBrdUrd antibody (F~PRB-1) for labeling DNA breaks and propidium iodide/RNase A solution for counter staining the total DNA.

In addition, two types of control cells which address the two variables of this cell proliferation assay are included: “Reaction” and “Photolysis”. The “Reaction” control cells are fixed cultured cells which have BrdU incorporated into them and have been exposed to UV light to induce the breaks in the DNA. These cells should be stained without any other treatment. The “Photolysis” control cells are fixed cultured cells which have been incubated with BrdU but have not been exposed to UV light. These cells should be exposed to UV light using the same light source and exposure time as the experimental cells.

Precautions and Warnings

1. The components of this kit are for **Research Use Only** and are not intended for diagnostic procedures.

2. The component ABRXB16 contains cacodylic acid (dimethyl arsenic) as a buffer; ASPC11, ASNC12, ASWB15, ASRB19, and ASPR21 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. TdT Enzyme (ASTD17) will not freeze at -20C, because it is in a 50% (v/v) glycerol solution. Upon warming the TdT enzyme solution, microcentrifuge the tube for 30 seconds to force all the liquid to the bottom of the tube.

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. 37C Water Bath

5. Ice Bucket

6. 12 x 75 mm flow cytometry polystyrene test tubes

7. Pipets and Pipetting Aids

8. Dimethyl sulfoxide (DMSO)

9. UV light source that emits between 300-320 nm. A gel illumination box such as a Fotodyne UV21 Transilluminator Model No. 30-3025, 312 nm (Fotodyne Inc., New Berlin, WI phone: 800-362-3686) will work nicely if you have one in the laboratory. The average light intensity of the source is 4.5 mW/cm^2. The illumination area is 20 x20 cm. A low cost solution is a hand held 302 nm light from UVP products. Model No. UVM-57-302nm, part no 95-0104-01 and a J-129 stand for the light. 115V or 230 V models are available. http://www.uvp.com

Contents of the ABSOLUTE-S™ Kit

The **ABSOLUTE-S™** 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.**

The **Reagent Bottles** have colored caps to aid in their identification. Sufficient reagents are provided to process 50 cell suspensions and an additional 5 Reaction Controls and 5 Photolysis Controls. The Reaction Controls and Photolysis 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 have have been fixed as described on page 6.

ABSOLUTE-S™ Kit Components:

COMPONENT	CAP COLOR	PART NUMBER	VOLUME (ml)	STORAGE CONDITIONS
Reaction Control Cells	brown cap	ASPC11	5.000	-15 to -25C
Photolysis Control Cells	white cap	ASNC12	5.000	-15 to -25C
BrdUrd Photolyte	pink cap	ASBP13	1.000	-15 to -25C
Photolyte Enhancer	black cap	ASPE14	1.000	-15 to -25C
Wash Buffer	blue cap	ASWB15	200.000	2 to 8C
Reaction Buffer	green cap	ASRXB16	0.500	2 to 8C
TdT Enzyme	yellow cap	ASTD17	0.037	-15 to -25C
Br-dUTP	violet cap	ASBU18	0.400	-15 to -25C
Rinsing Buffer	red cap	ASRB19	100.000	2 to 8C
Fluorescein~PRB-1 mAb	orange cap	ASFM20	0.250	2 to 8C
PI/RNase Staining Buffer	amber bottle	ASPR21	30.000	2 to 8C

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 somehow specifically label newly replicated DNA. Though there are several approaches to accomplishing this, the methods of choice involve somehow 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 (BrdUrd), a thymidine analog. As a thymidine analog, BrdUrd 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 BrdUrd has become an accepted method for monitoring DNA replication (1,2). The use of BrdUrd for assaying DNA replication has replaced methods utilizing radioisotope-labeled thymidine in both research and clinical laboratories (3,4). The incorporation of BrdUrd into cellular DNA is most commonly detected using anti-BrdUrd antibodies (1,5). This methodology, although proven

effective, is limited when it comes to providing a complete analysis of the cells and/or tissues under investigation. This limitation arises, as a result of the techniques required to make the incorporated BrdUrd available for measurement. It requires that the cellular DNA be denatured to separate the duplex strands in order for the BrdUrd epitope to become accessible and reactive to the antibody. This denaturation process usually involves either a heat treatment (>90C) or acid (2-4N HCl) treatment. Such harsh cell treatment results in the loss or denaturation of many cellular proteins. This fact makes it difficult to combine additional simultaneous assays and probes for cell function and immunophenotyping when utilizing the heat/acid denaturation steps.

The **ABSOLUTE-S**™ Kit utilizes the **SBIP**™ (**S**trand **B**reak **I**nduced **P**hotolysis) methodology that does not require DNA denaturation and, therefore, is applicable in studies where preservation of antigens or other features of the cells is desired (6). The **SBIP**™ methodology first involves growing cells in the presence of the **BrdUrd Photolyte**™ in order to label the cellular DNA with bromolated deoxyuridine. Then an **Enhancer Photolyte**™ is added to the cell culture to sensitize the BrdUrd incorporated in the genome to subsequent photolysis. Next, the cells are harvested and the growing media is removed and replaced with Wash Buffer. The cells are then irradiated with UV light to induce strand breaks at the sites where BrdUrd is incorporated in the genome. From this point the cells can be immediately subjected to immunocytochemical detection of the presence of proliferation and other cellular proteins of interest or stored and stained at a later time.

The second part of the **ABSOLUTE-S**™ Assay involves labeling the UV light induced break-sites with bromolated deoxyuridine triphosphate (**Br-dUTP**) through a reaction catalyzed by terminal deoxynucleotidyl transferase (**TdT**). The **Br-dUTP** sites are then identified using a fluorescein labeled anti-BrdUrd antibody (**F~PRB-1**). These latter steps are the same protocol employed in the **APO-BRDU**™ Kit (Apoptosis

Measurement Kit). For simultaneous measurements of apoptosis and proliferation refer to the Phoenix Flow Systems, Inc. **LIFE and DEATH™** Assay. The **ABSOLUTE-S™** Assay provides the single most powerful technique for studies of both cell proliferation and cell death.

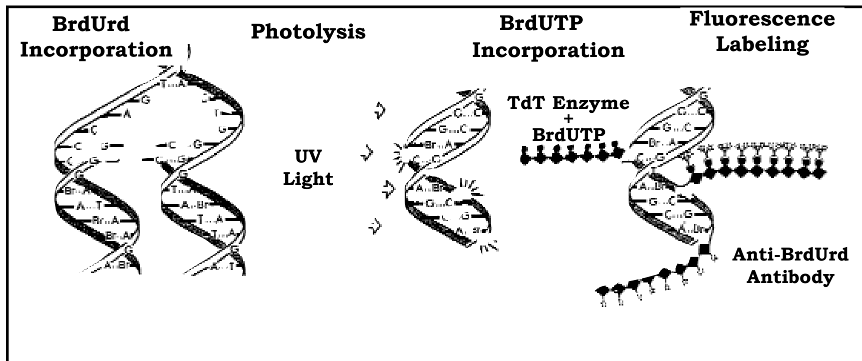


Figure 1: Schematic illustration of the principle of the ABSOLUTE-S™ methodology. Incorporation of BrdUrd during DNA replication is followed by cell exposure to UV light. High energy photons of UV light absorbed by the BrdUrd cause DNA photolysis at the sites adjacent to the incorporated BrdUrd. The 3′ OH ends in the photolysis-induced DNA breaks are then labeled with Br-dUTP using TdT as a catalyst. Fluorescein conjugated antiBRDU antibody (F-PRB-1) is used to label the Br-dUTP incorporation .

Flow Diagram of ABSOLUTE-S™ Cell Proliferation Assay

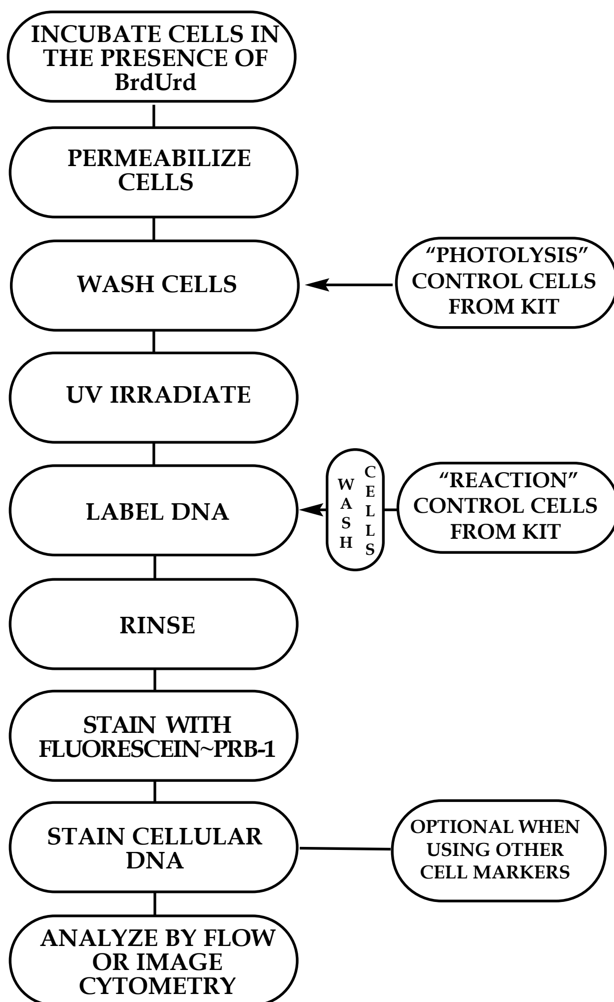


Figure 2: Flow diagram used in the ABSOLUTE-S™ Cell Proliferation Assay. The "Photolysis" and "Reaction" control cells are supplied with the kit and are already labeled with BrdUrd. The researcher must irradiate the experimental and "Photolysis" cells with UV light to introduce strand breaks in the DNA.

ABSOLUTE-S™ Procedure for Preparation and UV Irradiation of Cells

IMPORTANT CONSIDERATION FOR ACCOMPLISHING THE ABSOLUTE-S™ PROTOCOL
All steps of this protocol should be done in a manner to **MINIMIZE EXPOSURE** of the cells to **LIGHT** until **AFTER** the treatment of the cells with the UV light box has been accomplished.

1. **BrdUrd Incorporation and Permeabilization**
   a. Add 20 µl of the **BrdUrd Photolyte™** stock solution (**ASBP13, pink cap**) per 10 ml of cell culture medium.
   b. Incubate the cells at 37C (CO_2 incubator) for 20 to 40 minutes.*
   c. Following the incubation with the **BrdUrd Photolyte™,** add dimethyl sulfoxide (DMSO) to the cell culture medium to achieve a final concentration of 2% (v/v) DMSO and then immediately add 20 µl of **Photolyte Enhancer™** (**ASPE14, black cap**) per 10 ml of cell culture medium. Incubate an additional 20 minutes in the dark at 37C (CO_2 incubator).
   d. Centrifuge the cells for 5 minutes (300 x g) and remove the supernatant by aspiration.
   e. Resuspend the cells at a concentration of 1 to 5 X 10^6 cells/ml in **Wash Buffer (blue cap)**.
   f. Repeat Step D.
   g. Resuspend the cell pellet in the tube in the residual **Wash Buffer (blue cap)** left after aspiration by gently vortexing the tube.
   h. Adjust the cell concentration to 1 -2 X10^6 cells/ml in 70% (v/v) ice cold ethanol**.
   i. Store the cells protected from light in the freezer over night or until ready to use (at least 18 hours).

***Note:** The time of incubation with the **BrdUrd Photolyte™** may vary with the cell type and whether a pulse-chase experiment is being performed.

2. **Photolysis of DNA of the Experimental cells and ABSOLUTE-S™ control cells**
The Photolysis(white cap) control cells supplied with the Kit should be photolyzed at the same time as the experimental cell sample.
   a. Swirl the bottles containing the **Photolysis control cells (white cap)** and the **Reaction control cells (white cap)** to resuspend the cells. Transfer 1ml of the cell suspension to 12 x 75 mm polystyrene flow cytometer test tubes.
   b. Transfer 1 ml of the BrdUrd incorporated experimental cells obtained in part 1 (from page 7) to appropriately labeled 12x75 mm polystyrene test tubes.
   c. Centrifuge tubes (300 x g) for 5 minute. Remove the supernatent by aspiration being careful not to disturb the cell pellet.
   d. Add 2 ml of **Wash Buffer (blue cap)** and centrifuge (300 x g) for 5 minutes. Remove the supernatant by aspiration being careful to not disturb the cell pellet.
   e. Resuspend the pellet in 0.5 ml of **Wash Buffer (blue cap)**. Be sure to free any cells that may have adhered to the tube sides during washing.
   f. Place the test tube containing the **experimental cells** and the tubes containing the **Photolysis control cells** (the **Reaction control cells** do **not** need to be irradiated) on the irradiating surface of the light box. (If large numbers of cells need to be irradiated, this step may be carried out in a 60x15 mm polystyrene petri dish.) Illuminate the cells for 5*** minutes on high setting using a Fotodyne UV21 DNA transilluminator or equivalent light source. Twenty minutes with the UVP hand held light.
   g. After illumination, add 1ml of **Wash Buffer (blue cap)** to each tube(including the non-irradiated **Reaction control cells**) and centrifuge (300 x g) for 5 minutes. Remove the supernatant by aspiration being careful not to disrupt cell pellets.

The cells are now ready for staining. Proceed to the next page.

Note: If multiparameter analysis, i.e. surface receptors and proliferation markers, is to be performed cell "fixation" at this point may not be desirable. Please contact Phoenix Flow Systems, Inc. for further information on their **Stick-It™** fixing and permeabilization cell treatment solutions.

***Note:** Time of UV irradiation may vary depending upon the light source used, as well as, the experimental samples. Optimum irradiation times (1 to 10 minutes) must be determined experimentally for each cell type.

ABSOLUTE-S™ STAINING PROTOCOL

The following protocol describes the method for measuring cell proliferation in the **Photolysis** and **Reaction control cells** that are provided in the **ABSOLUTE-S**™ Kit. The same procedure should be employed for measuring cell proliferation in the cells that have undergone **SBIP**™ by the researcher.

If a negative control for the **ABSOLUTE-S**™ assay is desired, wash one extra tube of **Photolysis** or **Reaction control cells** out of alcohol and follow the procedure below except, **do not** add TdT to the tube. See figure 3 on page 11.

1. Resuspend each tube of the cell pellets (from page 8) in 50 µl of the **DNA Labeling Solution** prepared as described below.

2. Incubate the cells in the **DNA Labeling Solution** for 60 minutes at 37C in a temperature controlled water bath. Shake the cells every 15 minutes to resuspend****.

3. At the end of the incubation time add 2 ml of **Rinse Buffer (red cap)** to each tube and centrifuge each tube (300 X g) for five minutes. Remove the supernatant by aspiration.

DNA LABELING SOLUTION	1 ASSAY	5 ASSAYS	10 ASSAYS
TdT Reaction Buffer (green cap)	10.00 µl	50.00 µl	100.00 µl
TdT Enzyme (yellow cap)	0.75 µl	3.75 µl	7.50 µl
Br--dUTP (violet cap)	8.00 µl	40.00 µl	80.00 µl
Distilled H_2O	32.25 µl	161.25 µl	322.50 µl
Total Volume	51.00 µl	255.00 µl	510.00 µl

The appropriate volume of Staining Solution to prepare for a variable number of assays is based upon multiples of the component volumes combined for 1 Assay. **Mix only enough DNA Labeling Solution to complete the number of assays prepared per session.** The DNA Labeling Solution is active for approximately 24 hours.

****Note: The DNA Labeling Reaction can also be carried out at 22-24C overnight for the control cells. For samples other than the control cells provided in the kit, incubation times at 37C may need to be adjusted to longer or shorter periods depending on the characteristics of the cells supplied by the researcher.

ABSOLUTE-S™ STAINING PROTOCOL
(cont.)

4. Resuspend the cell pellets in 100 µl of **Fluorescein~PRB-1 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)	100.00 µl	500.00 µl	1000.00 µl
Total Volume	105.00 µl	525.00 µl	1050.00 µl

5. Incubate the cells with the **Fluorescein~PRB-1 Antibody Solution** in the dark for 30 minutes at room temperature.

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

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

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

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

*******Note:** If surface receptors have been stained prior to the DNA labeling, it may be desirable to omit the **PI/RNase A Solution** step. The propidium iodide component may interfere with the fluorophores used to stain other molecules in the cells.

Analyzing the ABSOLUTE-S™ 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 (Coulter) signal on the X-axis, see Figure 5 on page 13. 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.

Photolysis or Reaction Control Cells <u>without</u> TdT | Photolysis or Reaction Control Cells with TdT

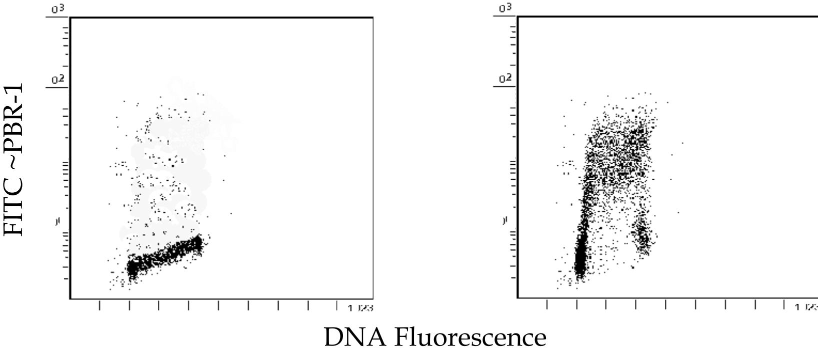
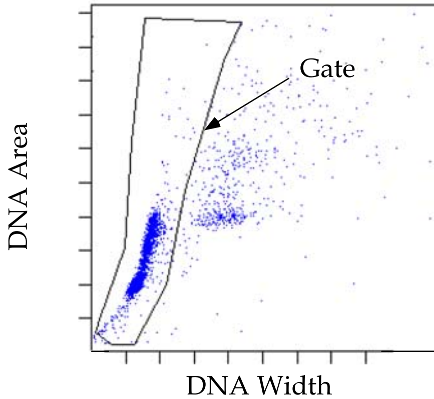
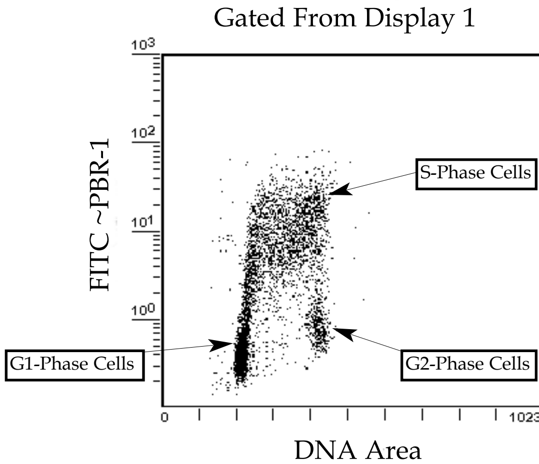


Figure 3: Flow Cytometry Data of ABSOLUTE-S™ Photolysis or Reaction Control Cells without and with TdT added to reaction

Flow Cytometer Setup for Becton Dickinson Hardware



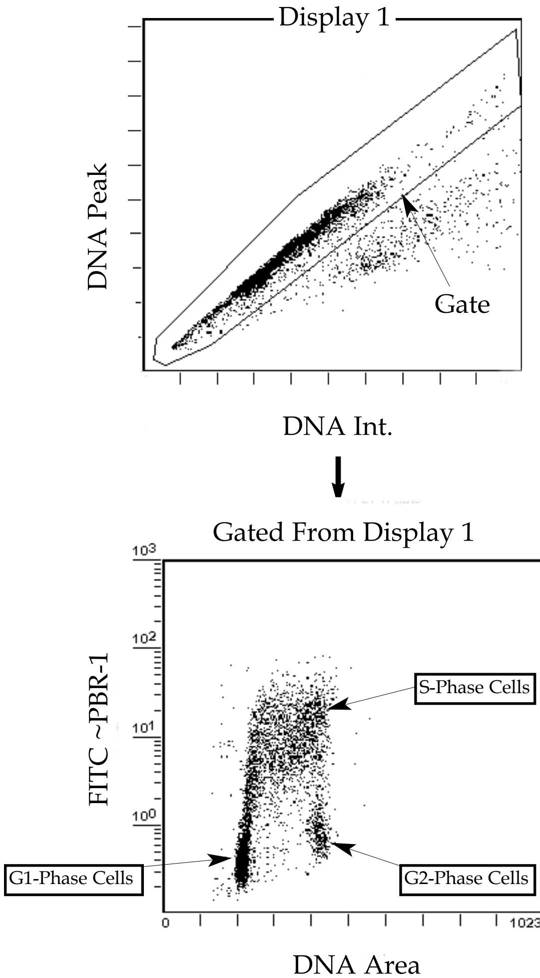


Typical FACScan™ Gain Settings

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	380 Volts
FL 3	1.46	386 Volts
FL 3 Width	.87	
FL 3 Area	3.25	
	Threshold- FL 3, 40	

Figure 4: ABSOLUTE-S™ Positive Control Cells

Flow Cytometer Setup for 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: ABSOLUTE-S™ Control Cells

Technical Tips and Frequently Asked Questions About the ABSOLUTE-S™ Assay

1. To minimize cell lose 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. Occasionally a mirror image population of cells at lower intensity is observed in the flow cytometry dual parameter display. This population arises because during the 50 µl DNA Labeling Reaction some cells became stuck to the side of the test tube and were not fully exposed to the reaction solution. This phenomenon can be overcome by washing all cells from the side of the tube and making sure all cells are properly suspended at the beginning of the labeling reaction.

3. If there is a low intensity of green fluorescence, i.e. lack of S-phase cells, adjust the cell concentration in culture prior to the time of **BrdUrd Photolyte™** addition to ensure that cells are indeed in an exponential phase of growth. The cells can not be confluent when the **BrdUrd 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.

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

5. Make sure that your cells are not wearing sunglasses during the UV light treatment. If the UV light treatment is given to the cells while in growth media, substantially higher levels of radiation will be required to cause the photolysis of the BRDU labeled DNA. This increased radiation is required due to UV absorbing components of the growth media that act like sun glasses or sun block. Be sure to remove the media and place the cells in the **Wash Buffer (blue cap)**.

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