PRODUCT INFORMATION & MANUAL

APO-BRDU (TUNEL) Apoptosis Kit

NBP2-31161

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

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APO-BRDU™ *

A Complete Kit for Measuring Apoptosis by Dual Color Flow Cytometry & Microscopy

Catalog No. AU1001

Description of Kit

The **APO-BRDU™** Kit is a two color TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry and microscopy (1). The kit contains the instructions and reagents required for measuring apoptosis in cells includ-ing; positive and negative control cells for assessing reagent performance; washing, reaction and rinsing buffers for processing individual steps in the assay; terminal deoxynucleotidyl tranferase enzyme (TdT), bromodeoxyuridine triphosphate (Br-dUTP), and fluorescein labeled antiBrdU antibody for labeling DNA breaks and propidium iodide/RNase A solution for counter staining the total DNA.

Contents of the APO-BRDU™ Kit

The **APO-BRDU™** Kit is shipped in one container and consists of two packages. One package is shipped at ambient temperature and should be stored at 2-8C upon arrival. The other package is styrofoam containing frozen ice packs and the reagent contents should be stored at -20C upon arrival. Novus Biologicals has determined the ship-ping method is adequate to maintain the integrity of the kit components. **Upon arrival store the reagents at the appro-priate temperatures**.

Reagent bottles have color coded caps to aid in their identification. Sufficient reagents are provided to process 60 cell suspensions including 5 ml positive and 5 ml negative control cell suspensions of approximately 1 x 10^6 cells per ml in 70% (v/v) ethanol. The control cells are derived from a human lymphoma cell line and have been fixed as described on page 4.

APO-BRDU™ Kit Components:

COMPONENT	COLOR CODE	PART NUMBER	VOLUME (ml)	STORAGE CONDITIONS
Positive Control Cells	brown cap	CC1002	5.000	-15 to -25C
Negative Control Cells	white cap	CC1001	5.000	-15 to -25C
Wash Buffer	blue cap	ABWB13	120.000	2 to 8C
Reaction Buffer	green cap	ABRXB14	0.600	2 to 8C
TdT Enzyme	yellow cap	ABTD15	0.045	-15 to -25C
Br-dUTP	violet cap	ABBU16	0.480	-15 to -25C
Rinsing Buffer	red cap	ABRB17	120.000	2 to 8C
Fluorescein~PRB-1 mAb	orange cap	ABFM18	0.300	2 to 8C
PI/RNase Staining Buffer	amber bottle	ABPR19	30.000	2 to 8C

Precautions and Warnings

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

2. Component part numbers CC1001 and CC1002 contain 70% (v/v) ethanol as a preservative; ABRXB14 contains cacodylic acid (dimethylarsenic) as a buffer; ABWB13, ABRB17, and ABPR19 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 (ABTD15) will not freeze at -20C, because it is in a 50% (v/v) glycerol solution. Upon warming the TdT enzyme solution, centrifuge 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
2. Distilled water
3. 1% (w/v) paraformaldehyde (methanol free) in Phosphate Buffered Saline (PBS)
4. 70% (v/v) ethanol
5. 37C Water Bath
6. Ice Bucket
7. 12 x 75 mm flow cytometry test tubes
8. Pipets and Pipetting Aids

Description of Apoptosis

Apoptosis is the term that describes regulated cell death. It is believed to take place in the majority of animal cells. It is a distinct event that triggers characteristic morphological and biological changes in the cellular life cycle. It is common during embryogenesis (3,4), normal tissue and organ involution (5,6), cytotoxic immunological reactions (7,8) and occurs naturally at the end of the life span of differentiated cells (9,10). It can also be induced in cells by the application of a number of different agents including physiological activators, heat shock, bacterial toxins, oncogenes, chemotherapeutic drugs, ultraviolet and gamma radiation (11). When apoptosis occurs, the nucleus and cytoplasm of the cell often fragments into membrane-bound apoptotic bodies that are then phagocytized by neighboring cells. An alternative mode of cell death, necrosis, occurs as a result of gross injury to cells resulting in cellular lysing and release of cytoplasmic components into the surrounding environment, Necrosis often induces an inflammatory response in the tissue. A landmark of cellular self destruction by apoptosis is the activation of nucleases that degrade the higher order chromatin structure of the DNA into fragments of 50 to 300 kilobases and subsequently into smaller DNA pieces of about 200 base pairs in length (12). Numerous reviews of the events accompanying apoptosis are available and several well-researched model systems have been described (13,14,15).

Measurable Features of Apoptosis

One of the most easily measured features of apoptotic cells is the break-up of the genomic DNA by cellular nucleases. These DNA fragments can be extracted from apoptotic cells and result in the appearance of "DNA laddering" when the DNA is analyzed by agarose gel electrophoresis (12). The DNA of non-apoptotic cells which remains largely intact does not display this "laddering" on agarose gels during electrophoresis. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3'-hydroxyl ends in the DNA. This property can be used to identify apoptotic cells by labeling the 3'-hydroxyl ends with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl ends of double- or single-stranded DNA with either blunt, recessed or overhanging ends (16). A substantial number of these sites are available in apoptotic cells providing the

basis for the method utilized in the **APO-BRDU™** Kit (1,17). Recent evidence has demonstrated that Br-dUTP is more readily incorporated into the genome of apoptotic cells than are the deoxynucleotide triphosphates complexed to larger ligands like fluorescein, biotin or digoxigenin (1). This greater incorporation gives rise to a stronger flow cytometry signal when the Br-dUTP sites are identified by a fluorescein labeled antiBrdU monoclonal antibody. Non-apoptotic cells do not incorporate significant amounts of the Br-dUTP owing to the lack of exposed 3′-hydroxyl DNA ends.

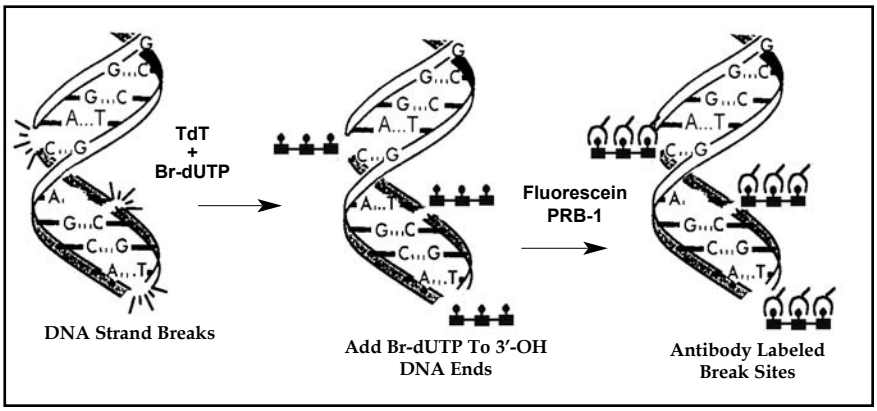


Figure 1: Diagrammatic representation of the addition of bromodeoxyuridine triphosphate (Br-dUTP) catalyzed by terminal deoxynucleotidyl transferase (TdT) to the 3′-OH sites of DNA strand breaks induced in the genome of apoptotic cells.

Flow Diagram of APO-BRDU™ Apoptosis Assay

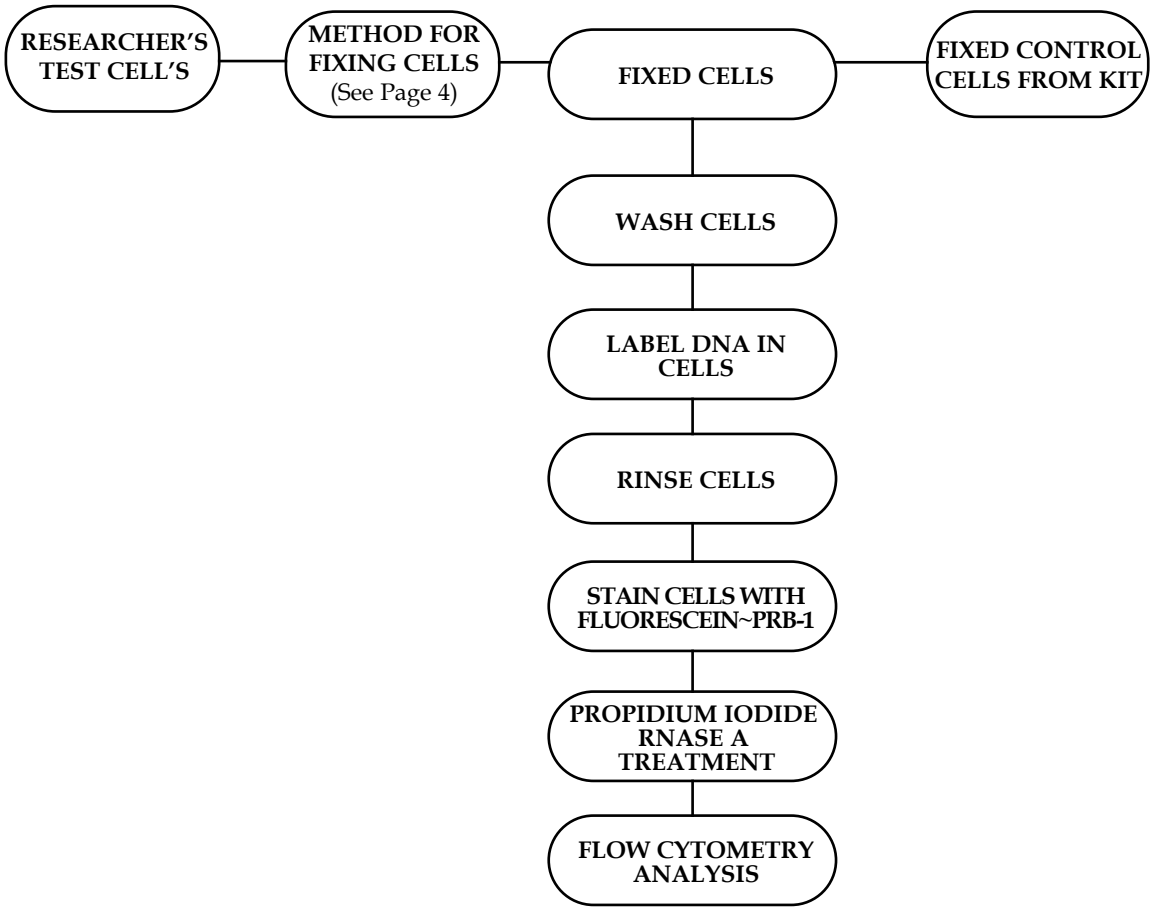


Figure 2: Flow diagram used in the APO-BRDU™ Apoptosis Assay. The positive and negative control cells are supplied in the kit and are already fixed. The cells supplied by the researcher should be fixed by the researcher according to a protocol suggested on page 4.

Cell Fixation Procedure for APO-BRDU™ Assay

NOTE: Cell fixation using paraformaldehyde is a **required** step in the **APO-BRDU**™ assay to cross link the DNA in the cells. Ethanol treatment is required to permeabilize the cells. The following cell fixation procedure is a suggested method. Variables such as cell origin and growth conditions can affect the results. The fixation conditions provided below should be considered as guidelines. Additional experimentation may be required to obtain results comparable to the control cells provide with this kit. The **positive** and **negative control cells** provided in the **APO-BRDU**™ **KIT** are already fixed as outlined below.

1. Suspend the cells in 1%(w/v) paraformaldehyde in PBS, pH 7.4 at a concentration of 1-2 x 10^6 cells/ml.
2. Place the cell suspension on ice for 30-60 minutes.
3. Centrifuge cells for 5 minutes at 300 x g and discard the supernatant.
4. Wash the cells in 5 ml of PBS then pellet the cells by centrifugation. Repeat the wash and centrifugation.
5. Resuspend the cell pellet in the residual PBS in the tube by gently vortexing the tube.
6. Adjust the cell concentration to 1-2 x 10^6 cells/ml in 70% (v/v) ice cold ethanol. Let cells stand for a minimum of 30 minutes on ice or in the freezer. See note below.
7. Store cells in 70% (v/v) ethanol at -20C until use. Cells can be stored at -20°C several days before use.

Note: In some biological systems storage of the cells at -20C in 70% (v/v) ethanol for at least 12-18 hours prior to staining for apoptosis detection yields the best results.

APO-BRDU™ PROTOCOL

The following protocol describes the method for measuring apoptosis in the **positive** and **negative control** cells that are provided in the **APO-BRDU™** kit. The same procedure should be employed for measuring apoptosis in the cell specimens provided by the researcher.

1. Resuspend the **positive (brown cap)** and **negative (natural cap)** control cells by swirling the vials. Remove 1 ml aliquots of the control cell suspensions (approximately 1 x 10^6 cells per 1 ml) and place in 12 x 75 mm flow cytometry centrifuge tubes. Centrifuge (300 x g) the control cell suspensions for 5 minutes and remove the 70% (v/v) ethanol by aspiration being careful to not disturb the cell pellet.

2. Resuspend each 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 each tube of the control cell pellets in 50 µl of the **DNA Labeling Solution** (prepared as described below).

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.

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

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.

6. At the end of the incubation time add 1.0 ml of **Rinse Buffer (red cap)** to each tube and centrifuge each tube (300 x g) for five minutes. Remove the supernatant by aspiration.

7. Repeat the cell rinsing (as in step 6) with 1.0 ml of the **Rinse Buffer (red cap)**, centrifuge and remove the supernatant by aspiration.

8. 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

9. Incubate the cells with the **Fluorescein~PRB-1 Antibody Solution** in the dark for 30 minutes at room temperature. Hint: Wrap tubes with aluminum foil.

10. Add 0.5 ml of the **Propidium Iodide/RNase A Solution (amber bottle)** to the tube containing the 0.1 ml **Antibody Staining Solution.**

Note: If the cell density is low, decrease the amount of PI/RNase A solution to 0.3 ml.

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

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

13. Analyze the cells within 3 hours of staining.

Analyzing the APO-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 (Apoptotic 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 parameters 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 8. 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 FITC~PRB-1 (Log Green Fluorescence) on the Y-axis (see bottom display next page). Two single parameter gated histograms, DNA and FITC~PRB-1, can also be added but are not necessary. By using the dual parameter display method, not only are apoptotic cells resolved but at which stage of the cell cycle they are in is also determined. The Log Green Fluorescence histograms of the control cells should look like Figure 3 below.

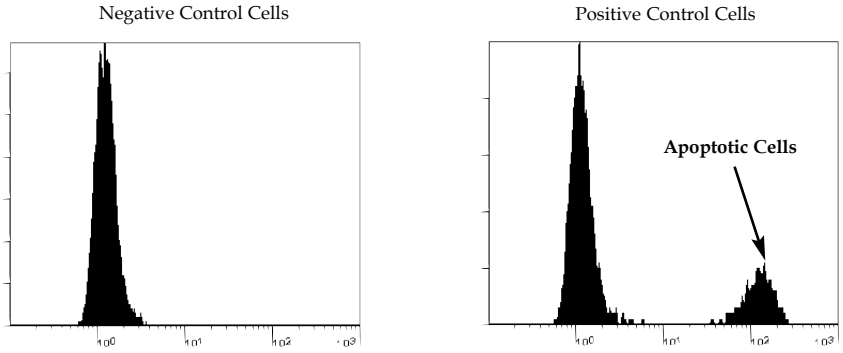
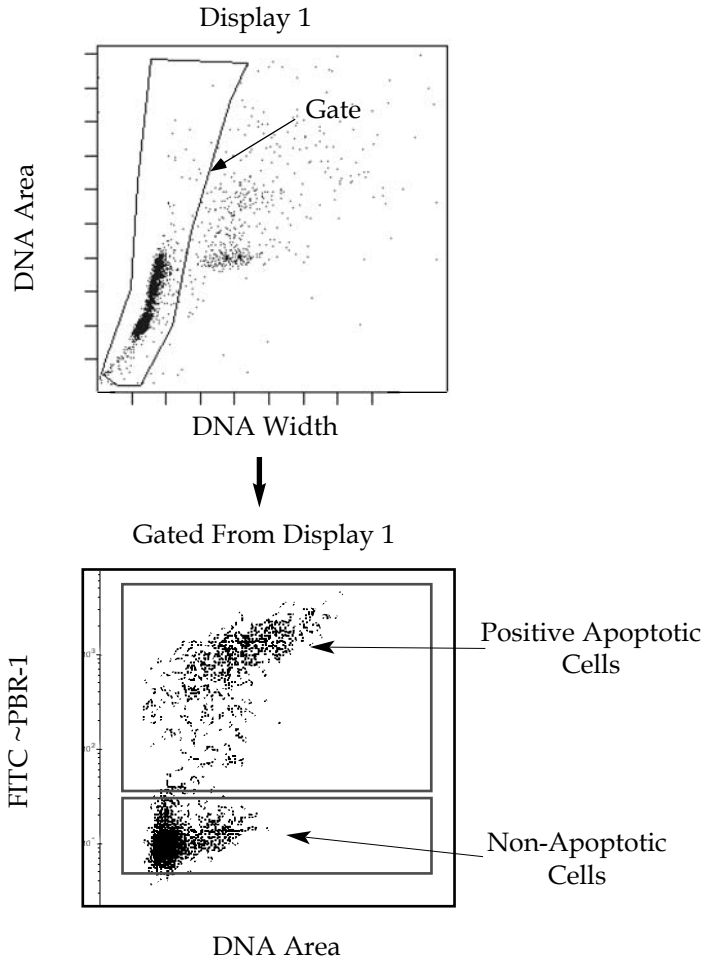


Figure 3: Flow Cytometry Data of APO-BRDU™ Negative & Positive Control Cells

Flow Cytometer Setup for Becton Dickinson Hardware

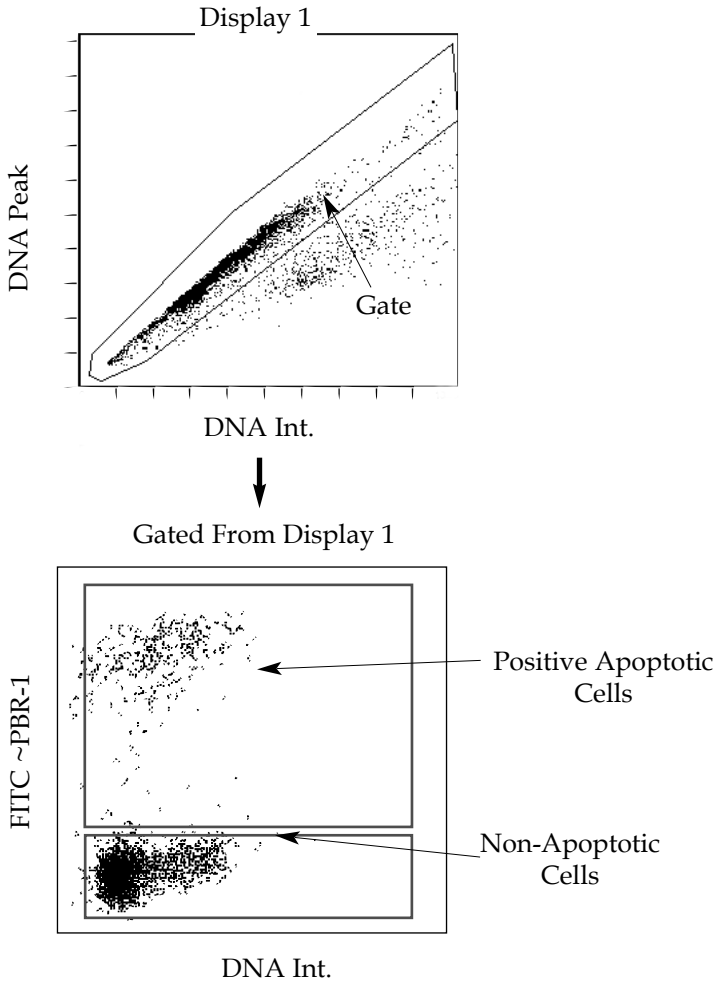


Typical Caliber/FACScan™ Gain Settings

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	380 Volts
FL 3	1.46	414 Volts
FL 3 Width	.87	
FL 3 Area	3.25	
	Threshold- FL 3, 40	

Figure 4: APO-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: APO-BRDU™ Positive Control Cells

Technical Tips and Frequently Asked Questions About the APO-BRDU™ Assay

1. For those researchers using adherent cell line systems, the cells in the supernatant have a higher probability of being apoptotic than do the adherent cells. Save cells in the supernatant for assay prior to trypsinization of the adherent cell layer.

2. Cell fixation using a DNA crossing linking chemical fixative is an important step in analyzing apoptosis. Unfixed cells may lose smaller fragments of DNA that are not chemically fixed in place inside the cell during washing steps. The researcher may have to explore alternative fixation and permeablization methods to fully exploit their systems.

3. A cytospin or centrifigal cytology slide can be prepared from **APO-BRDU™** sample in the following manner. After completion of the **Fluorescein~PRB-1** antibody staining , but prior to the **Propidium Iodide/RNAse A** treatment, put a drop of the stained cells on a slide, spin it and observe the sample under a fluorescence microscope.

4. Surface marker staining of cellular antigens can be accomplished by first incubating the cells with the fluorescent labeled antibody and then using a comercially available fix and perm solution to rapidly fix and permeabilize the cells in preparation for the **APO-BRDU™ Assay.**

5. 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 during the assay.

6. 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 have become stuck to the side of the test tube and are not fully exposed to the reaction solution. This phenomenon can be overcome by washing all the cells from side of the tube and making sure all cells are properly suspended at the beginning of the labeling reaction.

7. If a low intensity of fluorescein staining is observed, try increasing the incubation time during the 50 µl **DNA Labeling Reaction**. Some researchers have found labeling times of up to four hours at 37C may be required for certain cell systems.

8. If the 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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