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ProductInformation

APO-BRDU
A Flow Cytometry Kit for Apoptosis

Product Number **APO-BRDU**

TECHNICAL BULLETIN

Product Description

The APO-BRDU Kit is a two color staining method for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry.[1] The kit contains the instructions and reagents required for measuring apoptosis in cells including; positive and negative control cells for assessing reagent performance; washing, reaction, and rinsing buffers for processing individual steps in the assay; terminal deoxynucleotidyl transferase enzyme (TdT); bromodeoxyuridine triphosphate (Br-dUTP); and fluorescein-labeled anti-BrdU antibody for labeling DNA breaks and propidium iodide/ RNase A solution for counter staining the total DNA.

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]

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. The enzyme terminal 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 anti-BrdU monoclonal antibody. Non-apoptotic cells do not incorporate significant amounts of the Br-dUTP owing to the lack of exposed 3'-hydroxyl DNA ends.

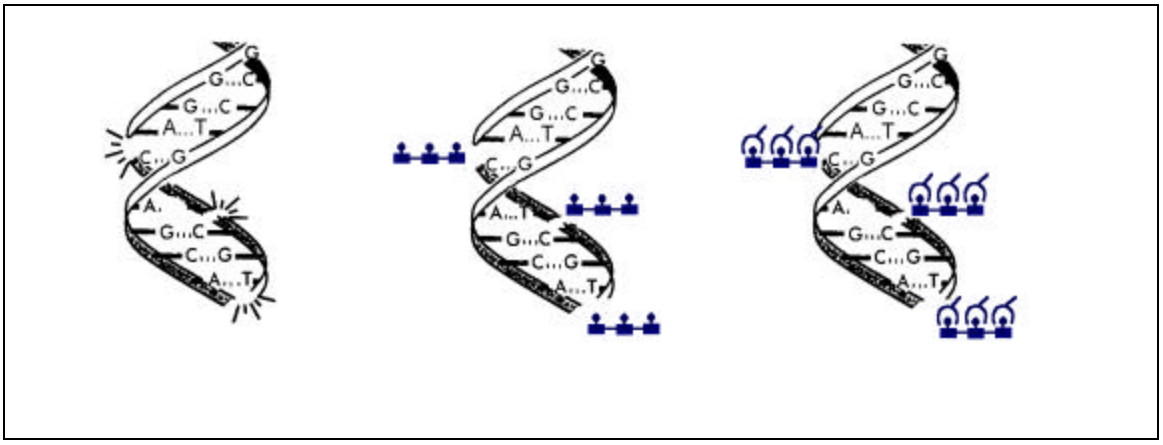
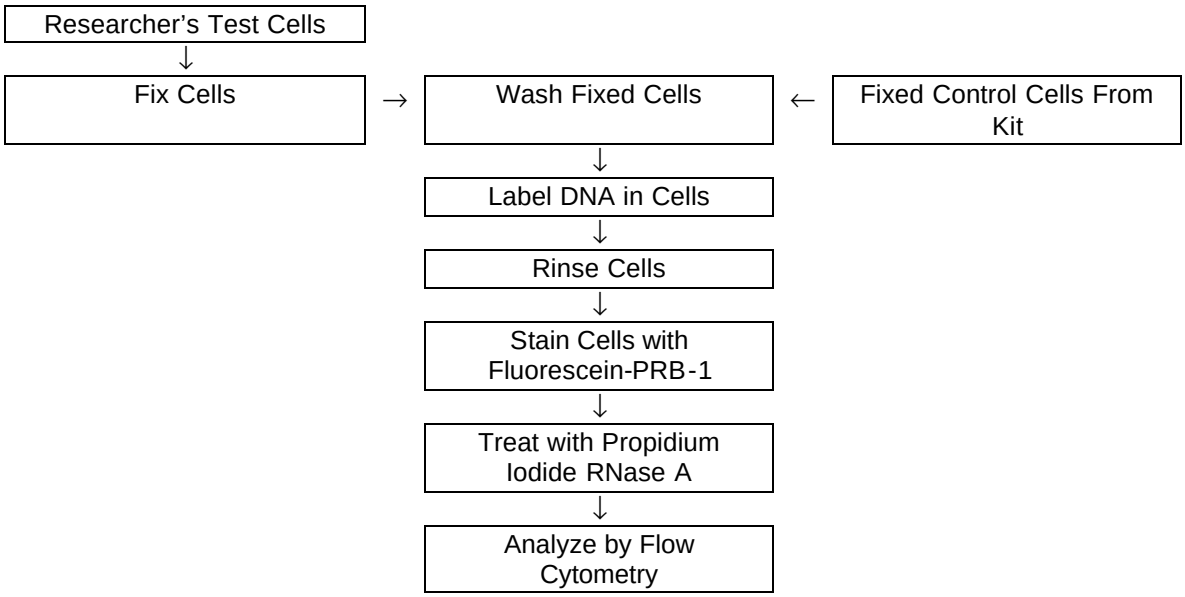


Figure 1: *Representation of the addition of bromodeoxyuridine triphosphate (Br-dUTP) catalyzed by terminal deoxynucleotidyl transferase (TdT) to the 3'-OH sites of DNA strand breaks induced in the genome of apoptotic cells.*

Flow Diagram of APO-BRDU Apoptosis Assay



Components

The APO-BRDU™ Kit is shipped in two packages. Both packages are shipped on wet ice packs. Upon arrival, APO-PART1 should be stored at –20 °C, and APO-PART2 should be stored at 2-8 °C.

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 below.

APO-BRDU Kit Components:

Component	Color Code	Catalog No.	Volume (ml)	Storage
APO-PART 1				**–20 °C**
Positive Control Cells	Brown cap	C 5601	5.0	–20 °C
Negative Control Cells	White cap	C 5726	5.0	–20 °C
TdT Enzyme	Yellow cap	T 7316	0.045	–20 °C
Br-dUTP	Purple cap	B 6679	0.480	–20 °C
APO-PART2				**2 – 8 °C**
Wash Buffer	Blue cap	W 1140	120	2 – 8 °C
Reaction Buffer	Green cap	R 4275	0.6	2 – 8 °C
Rinsing Buffer	Red cap	R 4150	120	2 – 8 °C
Anti-BrdU-Fluorescein Labeled	Orange cap	F 6175	0.3	2 – 8 °C
Propidium Iodide/RNase	Amber bottle	P 1607	30	2 – 8 °C

Precautions and Disclaimer

The components of this kit are for Research Use only and are not intended for diagnostic procedures.

Component part numbers C 5726 and C 5601 contain 70% (v/v) ethanol as a preservative; R 4275 contains cacodylic acid (dimethylarsenic) as a buffer; W1140, R 4150, and F 6175 contain 0.05% (w/v) sodium azide as a preservative. See Material Safety Data Sheets.

Preparation Instructions

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. 37 °C Water Bath
6. Ice bucket
7. 12 x 75 mm flow cytometry test tubes
8. Pipets and pipetting aids

TdT Enzyme (T 7316) will not freeze at –20 °C, 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.

Cell Fixation Procedure

Note: Cell fixation using paraformaldehyde is a required step in the APO-BRDU™ assay. 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.

1. Suspend 1-2 x 10^6 cells in 0.5 ml of 10 mM sodium phosphate pH 7.2, 150 mM sodium chloride (PBS).

2. Add the cell suspension into 5 ml of 1% (w/v) paraformaldehyde in PBS and place on ice for 15 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 cells in 0.5 ml of PBS.

6. Add cells to 5 ml of ice-cold 70% (v/v) ethanol. Let cells stand for a minimum of 30 min. in ice or the freezer. Note: In some biological systems storage of the cells at –20 °C in 70% (v/v) ethanol for at least 12-18 hours prior to staining for apoptosis detection yields the best results.

7. Store cells in 70% (v/v) ethanol at –20 °C until use. Cells can be stored at –20 °C several days before use.

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 (C 5601, brown cap) and negative (C 5726, white 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 (W1140, 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 in the table below).

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

NOTE: The DNA Labeling Reaction can also be carried out at 22-24°C overnight for the control cells. For samples other than the control cells provided in the kit, incubation times at 37°C 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 Rinsing Buffer (R 4150, 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 Rinsing Buffer (R 4150, red cap), centrifuge and remove the supernatant by aspiration.

8. Resuspend the cell pellet in 0.1 ml of the Antibody Solution (prepared as described in the table below).

DNA Labeling Solution	Catalog No. (Color code)	1 Assay	5 Assays	10 Assays
Reaction Buffer	R 4275 (green cap)	10.00 µl	50.00 µl	100.00 µl
TdT Enzyme	T 7316 (yellow cap)	0.75 µl	3.75 µl	7.50 µl
Br-dUTP	B 6679 (purple 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 Labeling 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 plus 1 (to account for pipetting error). The DNA Labeling Solution is active for approximately 24 hours.

Antibody Solution	Catalog No. (Color code)	1 Assay	5 Assays	10 Assays
Anti-BrDU-Fluorescein	F 6175(orange cap)	5.00 µl	25.00 µl	50.00 µl
Rinsing Buffer	R 4150 (red cap)	100.00 µl	500.00 µl	1000.00 µl
Total Volume		105.00 µl	525.00 µl	1050.00 µl

9. Wrap tubes with aluminum foil, to protect from light and incubate the cells with the Antibody Solution in the dark for 30 minutes at room temperature.

10. Add 0.5 ml of the Propidium Iodide/RNase A Solution (P 1607, 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 within 3 hours of staining.

Results

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 on the next page or DNA Peak/Integral (Coulter) signal on the X-axis, see Figure 5 on the next page. 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 Anti-BrdU-FITC (Log Green Fluorescence) on the Y-axis (see bottom display on Figure 5 on the next page). Two single parameter gated histograms, DNA and Anti-BrdU-FITC, can also be added but are not necessary. By using the dual parameter display method, not only are apoptotic cells resolved but their stage in the cell cycle is also determined. The Log Green Fluorescence histograms of the control cells should look like Figure 3 below.

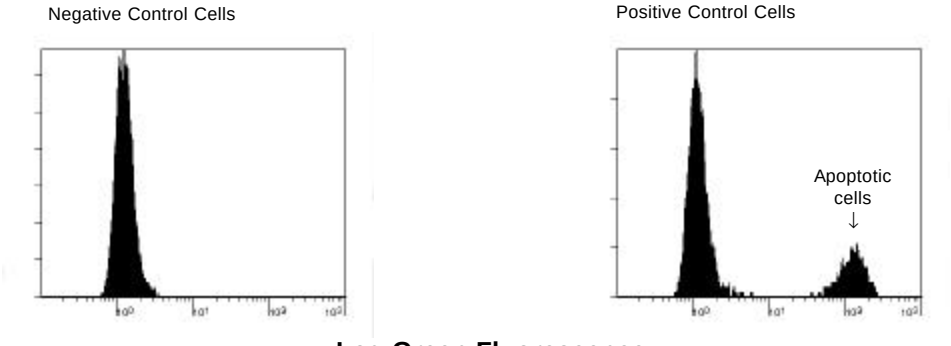
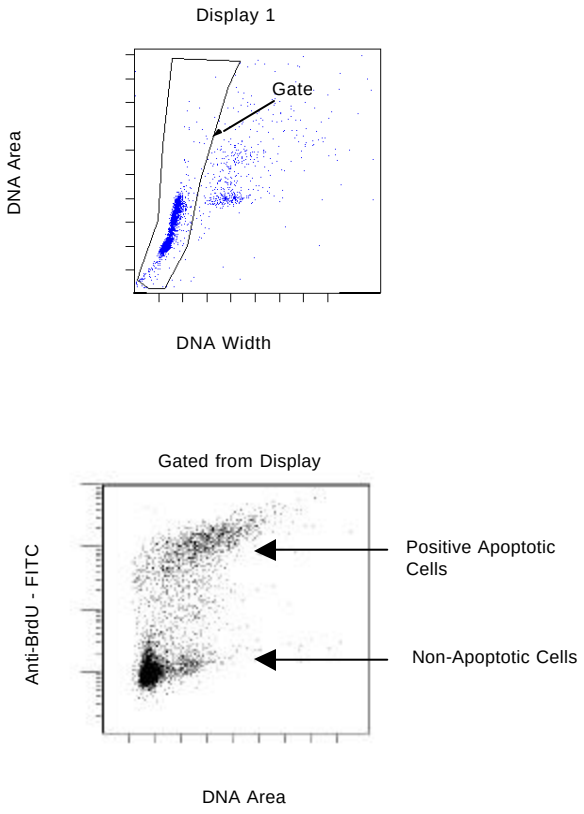


Figure 3: Flow Cytometry Data of APO-BRDU™ Negative & Positive Control Cells

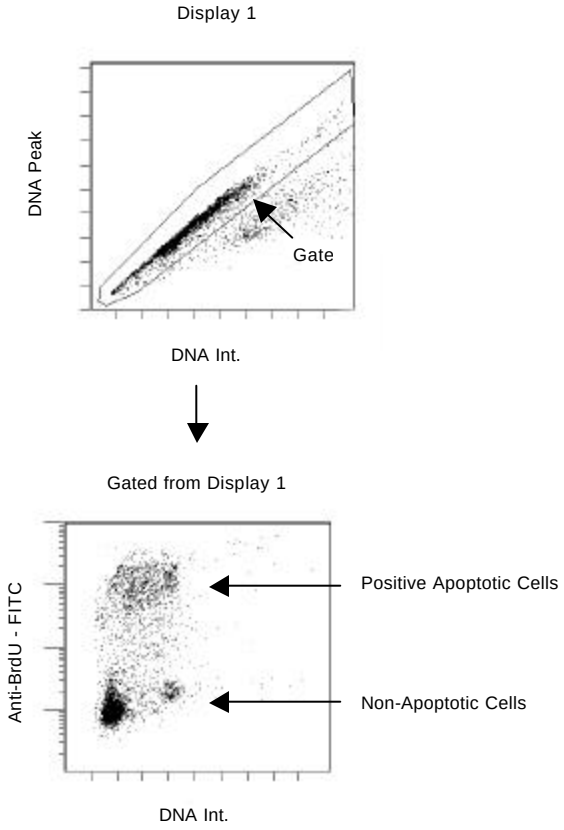
Flow Cytometer Setup for Becton Dickinson Hardware



Typical 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 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

Specificity

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

1. For those researchers using adherent cell lines, 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 cross-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 Anti-BrdU-FITC 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 Phoenix Flow Systems' QPF Solution (Cat.No. QPF0001) 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 during the 50 µl DNA Labeling Reaction because 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 37 °C 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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