

Technical Data Sheet

APO-BRDU™ Kit

Product Information

Material Number:	556405
Size:	60 Tests
Component:	51-6576AK
Description:	APO-BRDU™ Kit Part A
Component:	51-6576BK
Description:	APO-BRDU™ Kit Part B

Description

One of the later steps in apoptosis is DNA fragmentation, a process which results from the activation of endonucleases during the apoptotic program. These nucleases degrade the higher order chromatin structure into fragments of ~300 kb and subsequently into smaller DNA pieces of about 50 bp length. A method which is often used to detect fragmented DNA utilizes a reaction catalyzed by exogenous TdT, often referred to as "end-labeling" or "TUNEL" (terminal deoxynucleotidyltransferase dUTP nick end labeling). In the APO-BRDU™ assay, TdT catalyzes a template-independent addition of brominated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl (OH) termini of double- and single-stranded DNA. After incorporation, these sites are identified by flow cytometric means by staining the cells with a FITC-labeled anti-BrdU mAb. The APO-DIRECT™ assay (Cat. No. 51-6536KK) is a single-step method for labeling DNA breaks with FITC-dUTP, followed by flow cytometric analysis.

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. Sufficient reagents are provided to process 60 cell suspensions. The kit includes 5 ml of positive and 5 ml of negative control cell suspensions, approximately 1×10^6 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 in the Fixation Protocol.

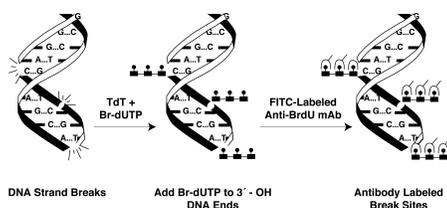
The APO-BRDU™ Kit consists of two parts (part A and B).

Part A (Comp. No. 51-6576AK) consists of:

- One 0.30 ml bottle of FITC-Labeled Anti-BrdU mAb [Isotype: mouse; 1 µg/test (contains 0.05% sodium azide)] (Comp. No. 51-6584AZ)
- One 30 ml bottle of PI/RNase Staining Buffer (5 µg/ml, 200 µg/ml RNase) [Comp. No. 51-6585AZ]
- One 0.6 ml vial of Reaction Buffer (containing cacodylic acid (dimethylarsenic)) [Comp. No. 51-6580AZ]
- One 126 ml bottle of Rinsing Buffer (containing 0.05% sodium azide) [Comp. No. 51-6583AZ]
- One 120 ml bottle of Wash Buffer (containing 0.05% sodium azide) [Comp. No. 51-6579AZ].

Part B (Comp. No. 51-6576BK) consists of:

- One 0.48 ml bottle of Br-dUTP (54.7 µg/ml) [Comp. No. 51-6582EZ]
- One 5 ml bottle of Negative Control Cells [contains 70% (v/v) ethanol] [Comp. No. 51-6578LZ]
- One 5 ml bottle of Positive Control Cells [contains 70% (v/v) ethanol] [Comp. No. 51-6577LZ]
- One 0.045 ml bottle of TdT Enzyme [200 µg/ml (S.A.= 100,000 U/mg) in 50% (v/v) glycerol solution; will not freeze at -20°C] [Comp. No. 51-6581EZ]

**Schematic Representation of APO-BRDU™ Labeling.**

The enzyme deoxynucleotidyl transferase (TdT) catalyzes a template dependent addition of brominated deoxyuridine triphosphates (Br-dUTP) to the 3'-hydroxyl ends of double- and single-stranded DNA. After Br-dUTP incorporation, DNA break sites are identified by a FITC-labeled anti-BrdU mAb.

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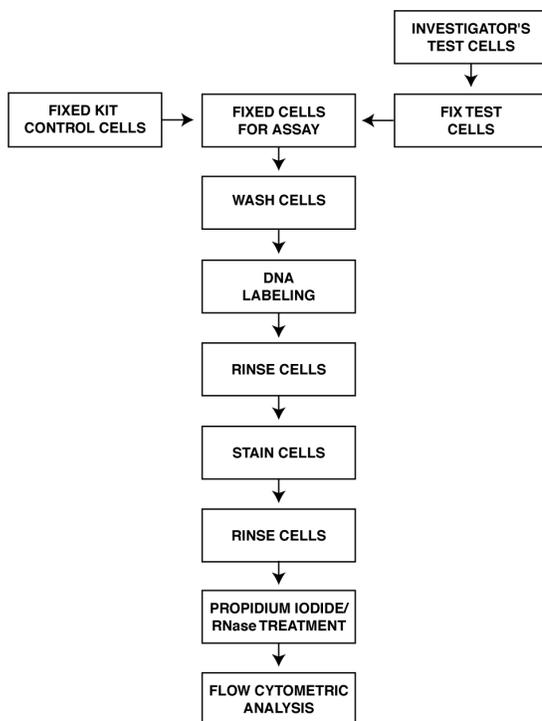
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The APO-BRDU™ Kit consists of two parts (A and B). Part A (Comp. No. 51-6576AK) is shipped at ambient temperature and should be stored at 4°C upon arrival. Part B (Comp. No. 51-6576BK) is shipped at -80°C on dry ice, and should be stored at -20°C upon arrival. BD Biosciences Pharmingen has determined that this shipping method is adequate to maintain the integrity of the kit components.



Application Notes

Recommended Assay Procedure:

METHODS FOR FIXATION, STAINING, AND ANALYZING APO-BRDU™ SAMPLES

Fixation Protocol

This is a standard protocol used at BD Biosciences Pharmingen for quality control testing of the APO-BRDU™ Kit. 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 provided with this kit. The positive and negative control cells provided in the APO-BRDU™ Kit are already fixed.

1. Suspend the cells in 1% (w/v) paraformaldehyde in PBS (pH 7.4) at a concentration of $1-2 \times 10^6$ cells/ml.
2. Place the cell suspension on ice for 30-60 minutes.
3. Centrifuge cells for 5 min at $300 \times g$ 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 $1-2 \times 10^6$ cells/ml in 70% (v/v) ice cold ethanol. Let cells stand for a minimum of 30 min on ice or in the freezer. See note below.
7. Store cells in 70% (v/v) ethanol at -20°C until use. Cells can be stored at -20°C several days before use.

Note: In some biological systems, storage of the cells at -20°C in 70% (v/v) ethanol for at least 12-18 hr prior to staining for apoptosis detection yields the best results. Cells can be stored at -20°C for several months before use.

Staining 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 (51-6577LZ) and negative (51-6578LZ) control cells by swirling the vials. Remove 1 ml aliquots of the control cell suspensions (approximately 1×10^6 cells/ml) and place in 12 x 75 mm flow cytometry centrifuge tubes. Centrifuge the control cell suspensions for 5 min at $300 \times g$ 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 1.0 ml of Wash Buffer (51-6579AZ) for each tube. Centrifuge as before and remove the supernatant by aspiration.

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- Repeat the Wash Buffer treatment (Step 2).
- 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
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 ₂ O	32.25 µl	161.25 µl	322.50 µl
Total Volume	51.00 µl	255.00 µl	510.00 µl

*The appropriate volume of DNA Labeling Solution to prepare for a variable number of assays is based upon multiples of the component volumes needed 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 hr at 4°C.

- Incubate the cells in the DNA Labeling Solution for 60 min 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 RT 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 requirements of the cells supplied by the researcher.
- At the end of the incubation time, add 1.0 ml of Rinse Buffer (51-6583AZ) to each tube and centrifuge each tube at 300 x g for 5 min. Remove the supernatant by aspiration.
- Repeat the cell rinsing (as in Step 6) with 1.0 ml of the Rinse Buffer, centrifuge, and remove the supernatant by aspiration.
- Resuspend the cell pellet in 0.1 ml of the Antibody Staining Solution (prepared as described below).
- Incubate the cells with the FITC-labeled anti-BrdU Antibody Solution in the dark for 30 min at RT.
- Add 0.5 ml of the PI/RNase Staining Buffer (51-6585 AZ) to the tube containing the 0.1 ml Antibody Staining Solution.

Antibody Staining Solution	1 Assay	5 Assays	10 Assays
FITC-Labeled Anti-BrdU (orange cap)	5.00 µl	25.00 µl	50.00 µl
Rinsing Buffer (red cap)	95.00 µl	475 µl	950.00 µl
Total Volume	100.00 µl	500.00 µl	1000 µl

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

- Incubate the cells in the dark for 30 min at RT.
- Analyze the cells in PI/RNase Staining Buffer by flow cytometry. Analyze the cells within 3 hr of staining to obtain optimal results. Cells may begin to deteriorate if left overnight before analysis.

Analyzing APO-BRDU™ Samples by Flow Cytometry

This assay is run on a flow cytometer equipped with a 488 nm Argon laser as the light source. Expected results using the positive and negative control cells are shown in Figs. 1-3. Propidium iodide (PI; total cellular DNA) and FITC (apoptotic cells) are the two dyes being used. PI fluoresces at about 623 nm and FITC 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 on the X-axis, (Fig. 1). 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-BrdU (Log Green Fluorescence) on the Y-axis. Two single parameter gated histograms, DNA and FITC-BrdU, can also be added but are not necessary. By using the dual parameter display method, not only are apoptotic cells resolved but their cell cycle stage is also determined.

TECHNICAL TIPS AND FREQUENTLY ASKED QUESTIONS

- 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 use in the assay prior to trypsinization of the adherent cell layer.
- Cell fixation using a DNA cross-linking chemical fixative (e.g. paraformaldehyde) is an important step in analyzing apoptosis by this method. Unfixed cells may lose smaller fragments of DNA that are not chemically fixed in place inside the cell prior to washing steps. The researcher may have to explore alternative fixation and permeabilization methods to fully exploit their systems.
- A cytospin or centrifugal cytology slide can be prepared from the APO-BRDU™ sample in the following manner: After completion of the FITC-labeled anti-BrdU antibody staining, but prior to the PI/RNase treatment, put a drop of the stained cells on a slide, spin it and observe the sample under a fluorescence microscope.
- To minimize cell loss during the assay, we recommend the use of 12 x 75 mm polystyrene tubes throughout the staining procedure and analysis. With other types of tubes or plastics (e.g. polypropylene), cells may build up on the sides of the tubes, resulting in significant loss of cell numbers, as well as inefficient staining of those cells which are not fully exposed to staining reagents. We also recommend that following wash steps, cells be resuspended by gentle mixing and not by pipetting, as plastic pipette tips can also contribute to cell loss throughout the assay.
- 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, when some cells become stuck to the side of the test tube and are not fully exposed to the Labeling Solution. This phenomenon can be overcome by washing all the cells from the side of the tube and making sure all cells are properly suspended at the beginning of the labeling reaction.

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6. If a low intensity of FITC staining is observed, try increasing the incubation time during the 50 µl DNA Labeling Reaction. Labeling times of up to 4 hr at 37°C may be required for certain cell systems.
7. If DNA cell cycle information is not required, it is not necessary to add the PI/RNase Staining Buffer to the samples.

Warning: Reaction Buffer (component 51-6580AZ) contains 0.2% Cacodylic acid (w/w).

Hazard Statements:

Suspected of causing cancer

Precautionary Statements:

Wear protective clothing / eye protection

Wear protective gloves

IF exposed or concerned: Get medical advice / attention

Dispose of contents / container in accordance with local / regional / national / international regulations

Danger: Negative Control Cells (component 51-6578LZ) and Positive Control Cells (component 51-6577LZ) contains 56.0% ethanol (w/w).

Hazard statements

Highly flammable liquid and vapor.

Causes serious eye irritation.

Precautionary statements

Keep away from heat/sparks/open flames/hot surfaces. No smoking.

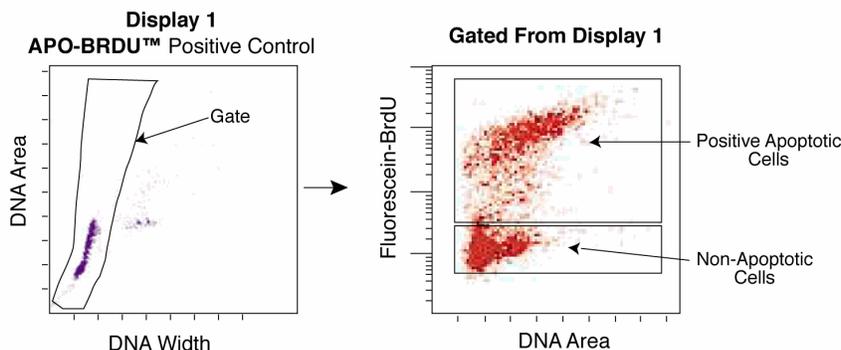
Wear protective gloves/protective clothing/eye protection/face protection.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Store in a well-ventilated place. Keep cool.

Dispose of contents/container in accordance with local/regional/national/international regulations.



Typical FACScan™ Gain Settings			
Parameter	Voltage	AMPS	Linear or Log
FSC	E00	2.57	Lin
SSC	475	1.00	Lin
FL-1	458		Log
FL-2	462		Log
FL-2A	1.10		
FL-2W	1.62		
If using FL-1 linear:			
FL-1	823		Lin
FL-2A	1.19		
FL-2W	1.72		
*Turn off all the compensation and put DDM@FL-2 if using FITC versus PI, DDM@FL-3 if using FITC versus 7-AAD.			

Flow Cytometer Setup for Becton Dickinson Hardware. Positive control cells were labeled with both PI (DNA) and FITC-BrdU mAb. Display 1: Non-clumped cells are gated. Gated From Display 1: Separate boxes are drawn around cells that stain positive (upper box) and negative (lower box) with the FITC-BrdU mAb. An example of FACScan™ gain settings is shown.

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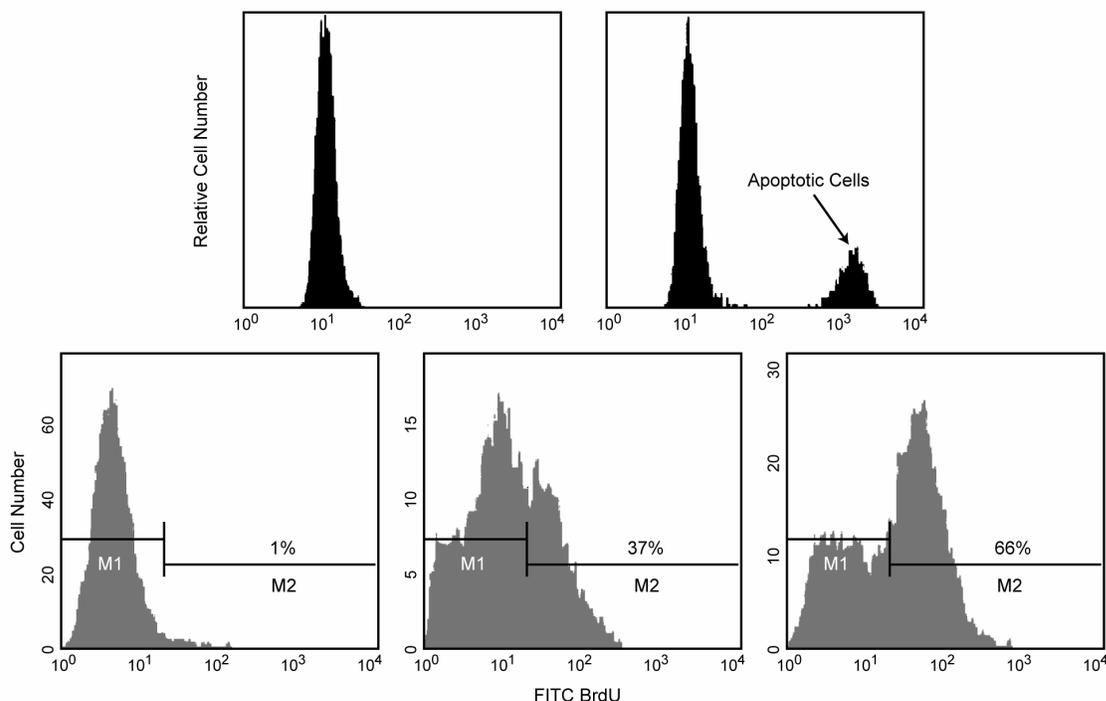
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Flow Cytometry Data of APO-BRDU™ Negative and Positive Control cells (top row). Negative and Positive control cells were incubated with Br-dUTP in the presence of TdT enzyme in order to incorporate Br-dUTP into exposed 3'-OH DNA ends. Br-dUTP sites are detected with a FITC-labeled anti-BrdU mAb. Non-apoptotic cells do not incorporate significant amounts of Br-dUTP due to the lack of exposed 3'-OH ends, and consequently have relatively little fluorescence compared to apoptotic cells which have an abundance of 3'-OH ends.

Flow Cytometric Analysis of HPB-ALL Human Leukemia Cells Using APO-BRDU™ (bottom row). HPB-ALL human leukemia cells were left untreated (left panel) or treated with anti-human Fas mAb, clone DX2 (Cat. No. 33450D) and Protein G for 2 hr (middle panel) or 12 hr (right panel). Cells were fixed and incubated with Br-dUTP in the presence of TdT enzyme in order to incorporate Br-dUTP into exposed 3'-OH DNA ends. Br-dUTP was detected with a FITC-labeled anti-BrdU mAb. Non-apoptotic cells (M1 gates) do not incorporate significant amounts of Br-dUTP due to lack of exposed 3'-OH ends, and consequently have relatively little fluorescence compared to apoptotic cells which have an abundance of 3'-OH end (M2 gates). DX2-induced, Fas-mediated apoptosis is shown by increases in the number of cells staining with anti-BrdU-FITC mAb (M2 gates) after 2 and 12 hr. The M1 and M2 gates demarcate non-apoptotic and apoptotic populations, respectively.

Product Notices

- Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

References

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- Walker PR, Kokileva L, LeBlanc J, Sikorska M. Detection of the initial stages of DNA fragmentation in apoptosis. *Biotechniques*. 1993; 15(6):1032-1040. (Biology)

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