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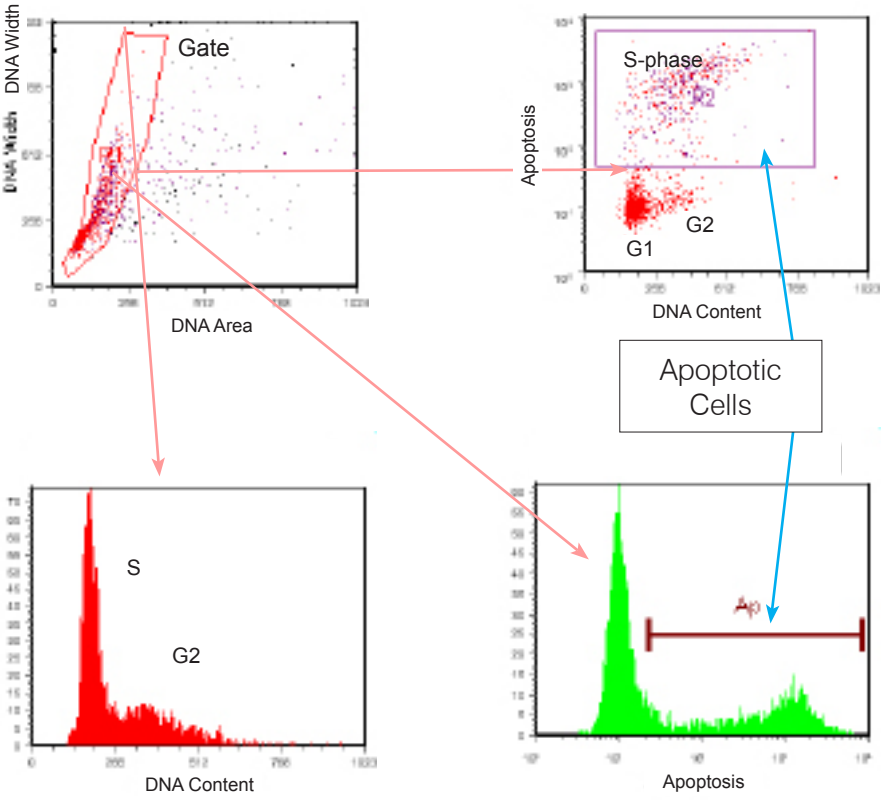
APO-BrdU™ Kit

Cat. No. TNB-6671-KIT

A Complete TUNEL Kit for Measuring Apoptosis by Dual Color Flow Cytometry



Technical Support

Web
www.tonbobio.com

Email
support@tonbobio.com

Phone
1-855-848-6626 toll-free
(858) 888-7300 local

This kit is developed for Research Use Only.

APO-BrdU™

A Complete Kit for Measuring Apoptosis
by Dual Color Flow Cytometry

Description

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. The kit contains 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 tranferase enzyme (TdT) and bromodeoxyuridine triphosphate (Br-dUTP); fluorescein labeled anti-BrdU antibody for labeling DNA breaks; and propidium iodide/RNase A solution for counter staining the total DNA.

Contents

The APO-BrdU Kit is shipped in one container and consists of two packages. One package is provided at ambient temperature and should be stored at 2-8° C upon arrival. The other is packaged in styrofoam containing frozen ice packs and the contents should be stored at -20° C upon arrival. We have determined the shipping method is adequate to maintain the integrity of the kit components. Upon arrival store the reagents at the appropriate 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 in this manual.

APO-BrdU Kit Components:

COMPONENT	COLOR CODE	PART NUMBER	VOLUME	STORAGE
Positive Control Cells	brown cap	TNB-6671-1002	5.0 mL	-20° C
Negative Control Cells	white cap	TNB-6671-1001	5.0 mL	-20° C
Wash Buffer	blue cap	TNB-6671-WB13	120.0 mL	2-8° C
Reaction Buffer	green cap	TNB-6671-XB14	600 µL	2-8° C
TdT Enzyme	yellow cap	TNB-6671-TD15	45 µL	-20° C
Br-dUTP	violet cap	TNB-6671-BU16	480 µL	-20° C
Rinse Buffer	red cap	TNB-6671-RB17	120.0 mL	2-8° C
FITC anti-BrdU mAb	orange cap	TNB-6671-FM18	300 µL	2-8° C
PI/RNase Staining Buffer	amber bottle	TNB-6671-PR19	30.0 mL	2-8° C

Precautions and Warnings

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

Component part numbers TNB-6671-1002 and TNB-6671-1001 contain 70% (v/v) ethanol as a preservative; TNB-6671-XB14 contains cacodylic acid (dimethylarsenic) as a buffer; TNB-6671-WB13, TNB-6671-RB17, and TNB-6671-PR18 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.

TdT Enzyme (TNB-6671-TD15) 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.

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

Assay Principle

The fragmentation of genomic DNA by cellular nucleases during the later stages of apoptosis is also one of the most easily measured features of apoptotic cells. Nuclease activity generates DNA fragments ranging from ~300 bp to 50 bp in length, resulting in a typical DNA 'laddering' appearance when analyzed by agarose gel electrophoresis. These fragments have exposed 3'-hydroxyl (OH) ends which can be labeled with bromolated deoxyuridine triphosphates (Br-dUTP). An enzyme, terminal deoxynucleotidyl transferase (TdT), is used to catalyze the template-independent addition of Br-dUTP to the 3'-OH ends of double or single stranded DNA. This method is often called TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) or end labeling. Sites where the Br-dUTP is incorporated can then be detected with an antibody specific to BrdU. With the APO-BrdU Kit, cells are labeled with Br-dUTP using the method described, and then sites of incorporation are detected through staining with a FITC anti-BrdU antibody. Samples can then be analyzed via flow cytometry. Samples that are apoptotic will stain brightly with the anti-BrdU antibody due to the substantial number of exposed 3'-OH sites, while cells that are non-apoptotic will not have incorporated significant amounts of Br-dUTP and will stain dimly (see Figure 4).

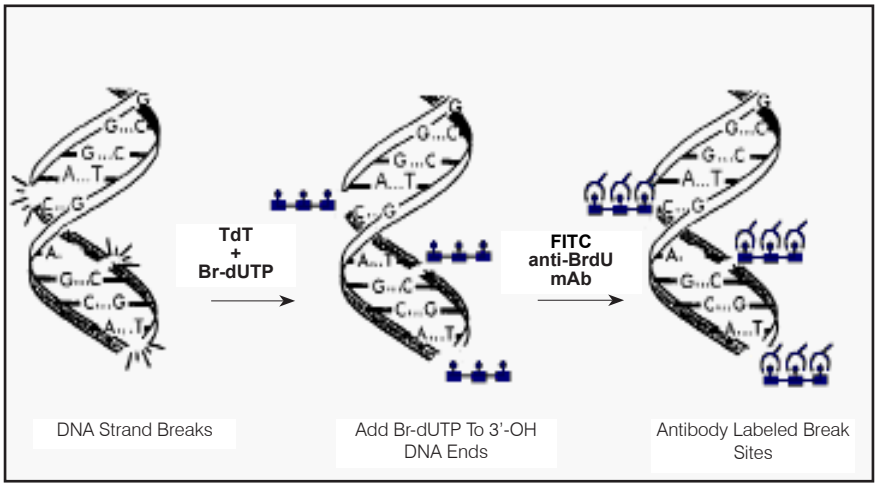


Figure 1: Diagrammatic representation of the addition of bromodeoxyuridine triphosphate (Br-dUTP) catalyzed by terminal deoxynucleotidyl transferase (TdT) to the 3'-OH sites of DNA strand breaks induced in the genome of apoptotic cells.

APO-BrdU Apoptosis Assay Workflow

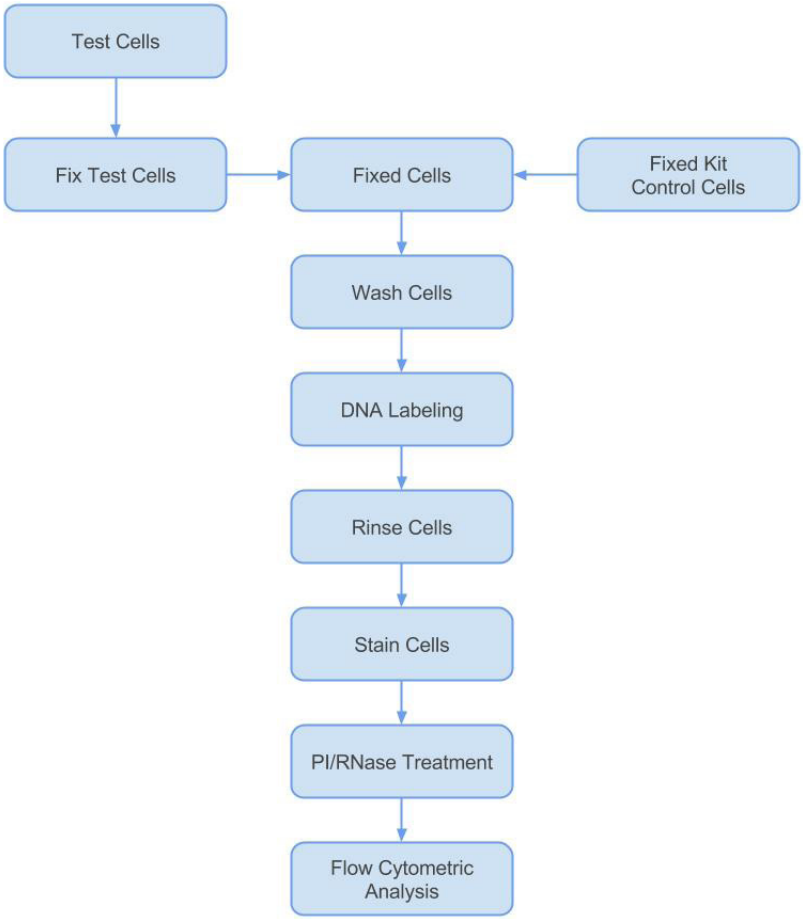


Figure 2: Flow diagram used in the APO-BrdU Apoptosis Assay. The positive and negative control cells are supplied in the kit and are already fixed. The cells supplied by the researcher should be fixed according to the suggested protocol.

Cell Fixation Procedure

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 provided 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 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 -20° C until use. Cells can be stored at -20° C for 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 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 (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) 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.

Note: It is very important to remove all ethanol from the reaction tube since it will inactivate the TdT enzyme. Do not skip these washes.

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
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 37° C in a temperature controlled waterbath. Shake cells every 15 minutes to resuspend.

APO-BrdU Protocol

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 depending on the characteristics of the cells supplied by the researcher.

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

7. Repeat the cell rinsing (step 6) with 1.0 mL of the Rinse Buffer (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 below).

ANTIBODY SOLUTION	1 ASSAY	5 ASSAYS	10 ASSAYS
FITC anti-BrdU(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 FITC anti-BrdU Antibody Solution in the dark for 30 minutes at room temperature. Wrap tubes with aluminum foil or store in the dark to protect from light.

10. Add 0.5 mL of the PI/RNase A Solution (amber bottle) to each tube.

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

11. Incubate cells in the dark for 15 minutes at room temperature.

12. Analyze the cells in PI/RNase Solution by flow cytometry. Analyze the cells within 3 hours of staining.

Flow Cytometric Analysis

This assay is run on a flow cytometer equipped with a 488 nm Argon laser. 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 (FITC) 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 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) or DNA Peak/Integral (Coulter, see Figure 5) signal on the X-axis. 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 anti-BrdU (Log Green Fluorescence) on the Y-axis (see bottom plot, Figure 4). Two single parameter gated histograms, DNA and FITC anti-BrdU, can also be added but are not necessary. By using the dual parameter display method, not only are apoptotic cells resolved but the cell cycle stage is also determined. The Log Green Fluorescence histograms of the control cells should look like Figure 3 below.

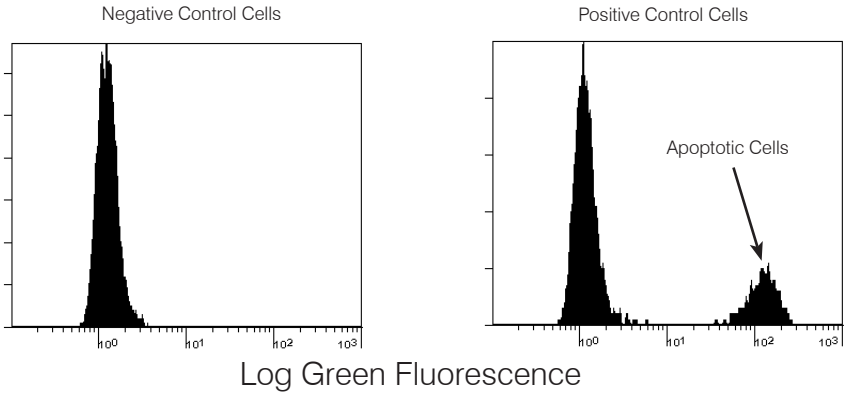
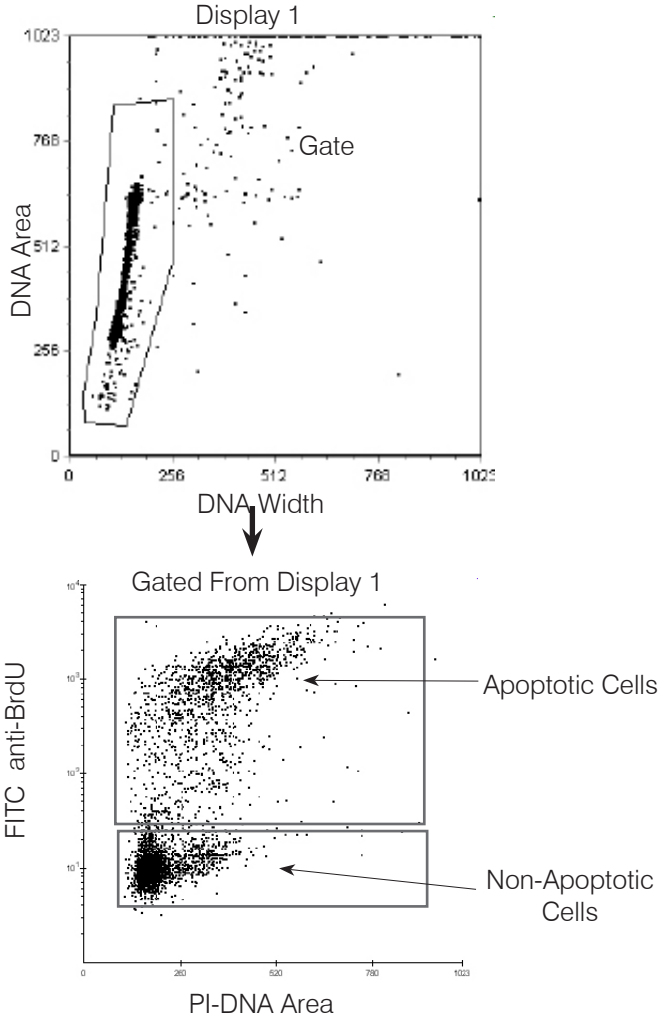


Figure 3: Representative Negative & Positive Control Cell Histograms

Flow Cytometer Setup for Becton Dickinson Hardware

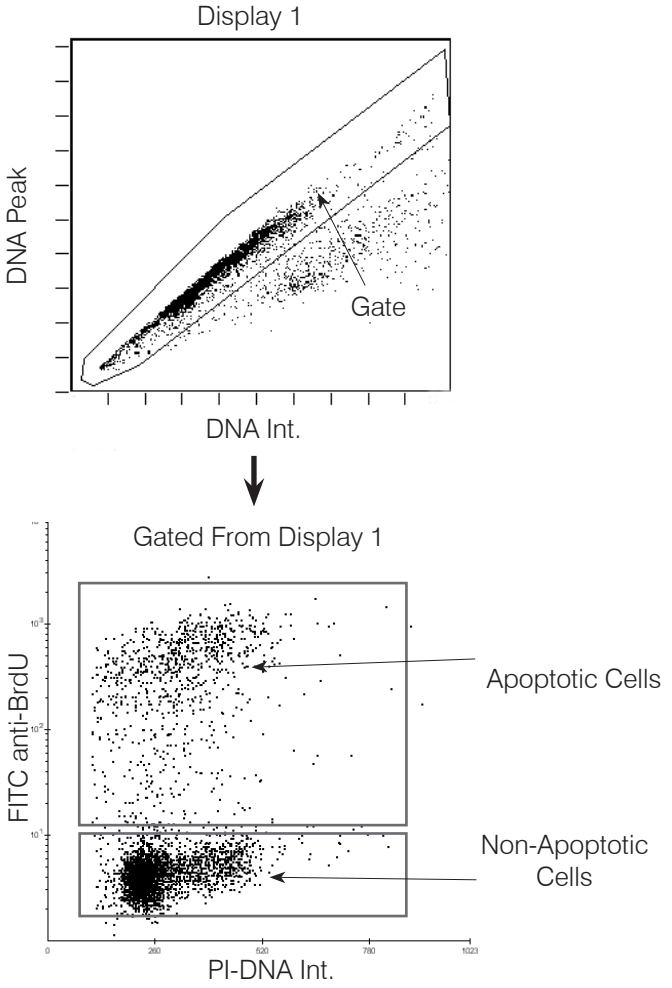


Typical FACScan™ or FACSCaliber™ Gain Settings

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™-FC-500™ 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

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.

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.

A cytospin or centrifigal cytology slide can be prepared from an APO-BrdU sample in the following manner. After completion of the FITC anti-BrdU antibody staining step, but prior to the PI/RNAse A 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 using a single 12 x 75 mm polystyrene flow cytometry tube per sample throughout the staining procedure and analysis. An electrostatic charge can build up on the sides of the tube causing cells to adhere to the tube wall. The sequential use of multiple tubes can result in significant cell loss during the assay. We also recommend that care is taken throughout the staining procedure to wash cells from the side of the tube. Cells can also be lost through the use of pipetting for mixing steps, as cells can adhere to the plastic pipette tips.

Occasionally a mirror image population of cells at lower intensity is observed in the 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.

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.

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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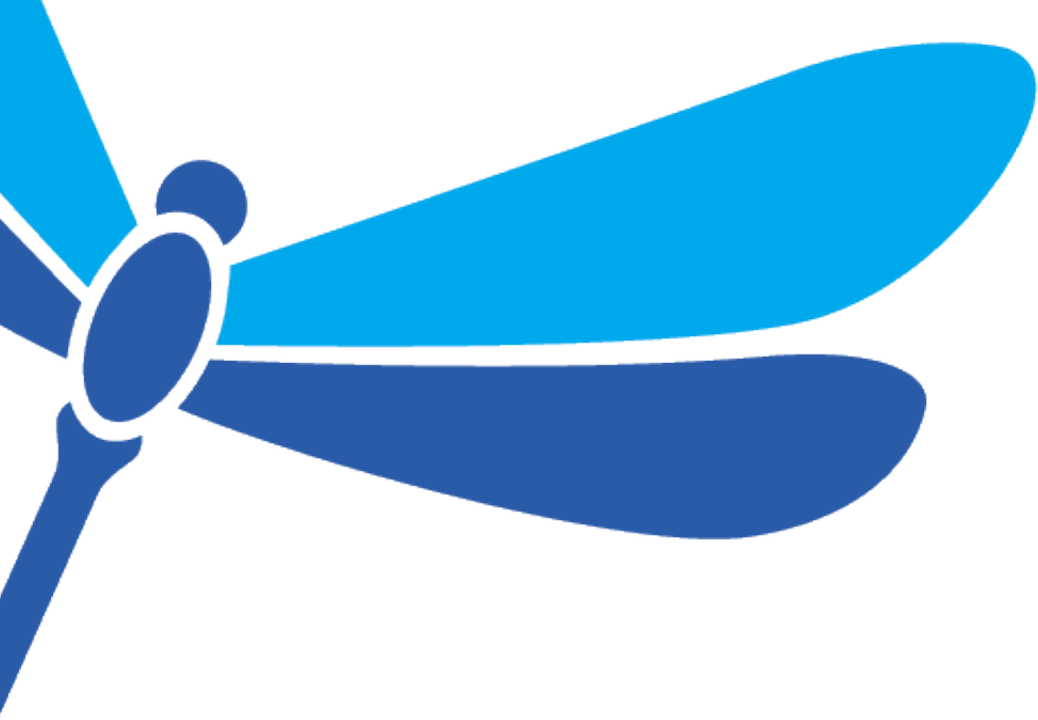
Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P and Traganos F. 1992. Cytometry. 13(8): 795-808.

Also available from Tonbo:

Product Name	Cat. No.
EZ-BrdU™ Kit	TNB-6600-KIT
APO-DIRECT™ Kit	TNB-6611-KIT
Flow Cytometer Sheath Fluid (30X)	TNB-4600-L600
Lymphocyte Separation Medium	TNB-4700-L100
RBC Lysis Buffer (10X)	TNB-4300-L100
Caspase Inhibitors	
Z-VAD(OMe)-FMK (General Caspase Inhibitor)	TNB-1001-M001
Q-VD-OPH (General Caspase Inhibitor)	TNB-1002-M001
Z-DEVD-FMK (Caspase-3 Inhibitor)	TNB-1003-M001
Z-IETD-FMK (Caspase-8 Inhibitor)	TNB-1004-M001



Tonbo Biosciences

6335 Ferris Square
Suite A
San Diego, CA 92121

(855) 848-6626 toll-free
(858) 888-7300 local

orders@tonbobio.com
www.tonbobio.com

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