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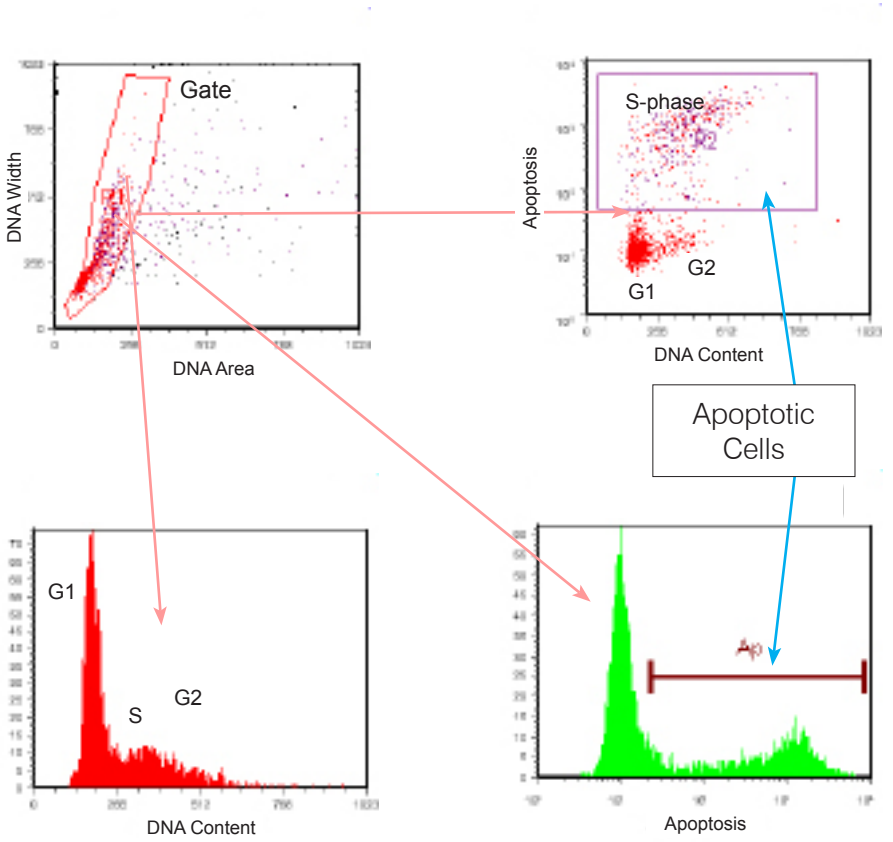
APO-DIRECT™ Kit

Cat. No. TNB-6611-KIT

A Complete Kit for Measuring Apoptosis by 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-DIRECT™

A Complete Kit for Measuring Apoptosis
by Flow Cytometry

Description

The APO-DIRECT™ reagent kit provides 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 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 tranferase enzyme (TdT), fluorescein isothiocyanate (FITC) deoxyuridine triphosphate (FITC-dUTP) for labeling DNA breaks and PI/RNase A solution for counter staining the total DNA.

Contents

The APO-DIRECT Kit is shipped in one container that houses two packages. One package is shipped at ambient temperature and should be stored at 2-8° C upon arrival. The other package is in styrofoam, containing frozen ice packs. The contents of this styrofoam container should be stored at -20° C upon arrival. We have determined this 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 50 cell suspensions. Included are 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 the fixation procedure.

APO-DIRECT Kit Components:

COMPONENT	COLOR CODE	PART NUMBER	VOLUME	STORAGE
Positive Control Cells	brown cap	TNB-6611-1002	5.0 mL	-20° C
Negative Control Cells	white cap	TNB-6611-1001	5.0 mL	-20° C
Wash Buffer	blue cap	TNB-6611-WB13	100.0 mL	2-8° C
Reaction Buffer	green cap	TNB-6611-XB14	500 µL	2-8° C
TdT Enzyme	yellow cap	TNB-6611-TD15	38 µL	-20° C
FITC-dUTP	orange cap	TNB-6611-BU16	400 µL	-20° C
Rinse Buffer	red cap	TNB-6611-RB17	100.0 mL	2-8° C
PI/RNase Staining Buffer	amber bottle	TNB-6611-PR18	25.0 mL	2-8° C

Precautions and Warnings

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

Components part numbers TNB-6611-1002 and TNB-6611-1001 contain 70% (v/v) ethanol as a preservative; TNB-6611-WB13 and TNB-6611-XB14 contain sodium cacodylate (dimethylarsinic) as a buffer; TNB-6611-RB17, and TNB-6611-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-6611-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 deoxyuridine triphosphates (dUTP). An enzyme, terminal deoxynucleotidyl transferase (TdT), is used to catalyze the template-independent addition of fluorescent tagged dUTP to the 3'-OH ends of double or single stranded DNA (Figure 1). This method is often called TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) or end labeling. With the APO-DIRECT Kit, cells are labeled with FITC-dUTP in a single step using the method described. Samples can then be analyzed via flow cytometry. Samples that are apoptotic will stain brightly due to the substantial number of exposed 3'-OH sites, while cells that are non-apoptotic will not have incorporated significant amounts of FITC-dUTP and will stain dimly (see Figure 6).

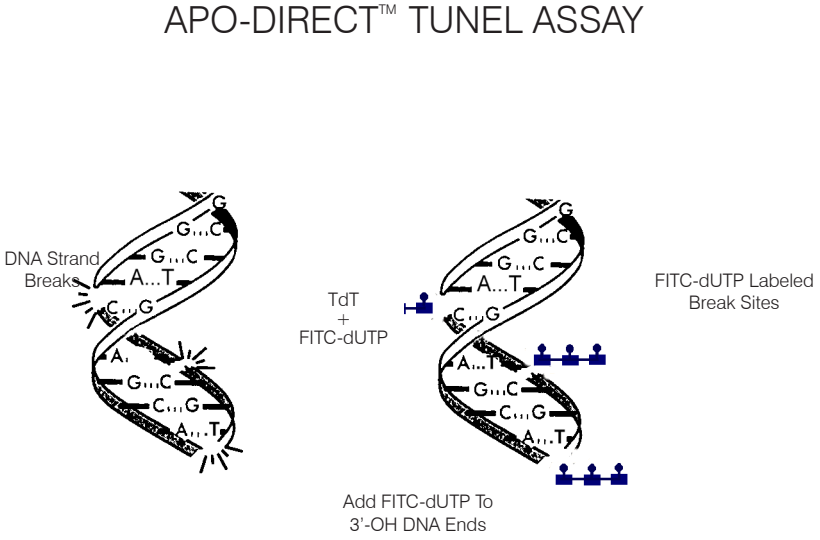


Figure 1: Diagrammatic representation of the APO-DIRECT TUNEL assay. Addition of FITC-dUTP at the 3'-OH sites of DNA strand breaks of the apoptotic cells is catalyzed by terminal deoxynucleotidyl transferase (TdT).

APO-DIRECT Apoptosis Assay Workflow

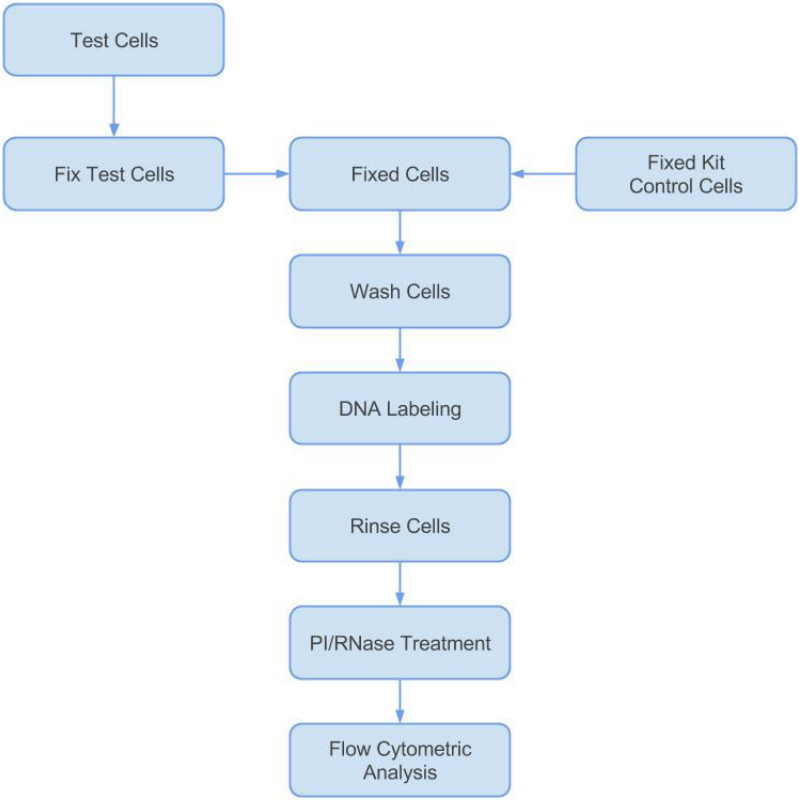


Figure 2: Flow diagram used in the APO-DIRECT Apoptosis Assay. The positive and negative control cells are supplied in the kit and are already fixed. The cells supplied by the researcher should be fixed according to the protocol provided.

Cell Fixation Procedure for APO-DIRECT Assay

NOTE: Cell fixation using paraformaldehyde is a required step in the APO-DIRECT 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-DIRECT™ 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 the 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 -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 hours prior to staining for apoptosis detection yields the best results in this assay.

APO-DIRECT PROTOCOL

The following protocol describes the method for measuring apoptosis in the positive and negative control cells that are provided in this 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 cell per 1 mL) and place in 12 x 75 mm 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.

   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).

STAINING SOLUTION COMPONENT	1 ASSAY	6 ASSAYS (2 controls+4 unknown)	12 ASSAYS (2 controls+10 unknown)
Reaction Buffer (green cap)	10.00 μL	60.0 μL	120.0 μL
TdT Enzyme (yellow cap)	0.75 μL	4.5 μL	9.0 μL
FITC-dUTP (orange cap)	8.00 μL	48.0 μL	96.0 μL
distilled H2O	32.25 μL	193.5 μL	387.0 μL
Total Volume	51.00 μL	306.0 μL	612.0 μL

The appropriate volume of DNA 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. 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 bath. Shake cells every 15 minutes 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 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 with 1.0 mL of the Rinse Buffer (red cap), centrifuge and remove the supernatant by aspiration.

8. Resuspend the cell pellet in 0.5 mL of the PI/RNase solution (amber bottle).

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

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

Analyzing APO-DIRECT Samples

This assay is run on a flow cytometer equipped with a 488 nm Argon Laser as the light source. Propidium Iodide (DNA) and FITC (Apoptotic Cells) are the two dyes being used. Propidium Iodide (PI) fluoresces at about 623 nm and FITC at 520 nm. Two dual parameter and two single parameters displays are created with the cytometer data acquisition software. The instrument settings and acquisition protocols for the different cytometers are included in this manual. The gate 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), (Figure 5A) or DNA Integral (Coulter) signal on the X-axis, (Figure 5B). A region 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 d-UTP (Log Green Fluorescence) on the Y-axis, (Figure 6). Two single parameter gated histograms, DNA and d-UTP, 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.

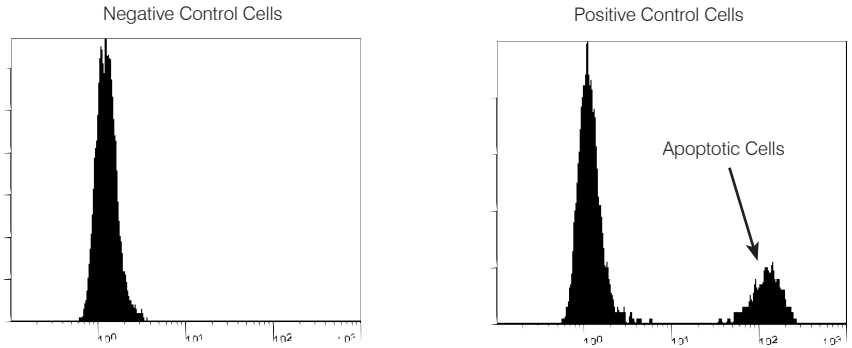


Figure 3: Cytometry Data of APO-DIRECT™
Negative & Positive Control Cells

Cytometer Setups
Typical Gain Settings

BD FACScan and FACSCaliber Flow Cytometers

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	380 Volts
FL 2	1.46	414 Volts
FL 2 Width	.87	
FL 2 Area	3.25	
	Threshold- FL2, 40	

BeckmanCoulter XL, FC-500 Flow Cytometer

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 4

Technical Tips

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.

Occasionally a mirror image population of cells at lower intensity is observed in the PI vs FITC-dUTP 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 were 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.

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 permeabilization methods to fully exploit their systems.

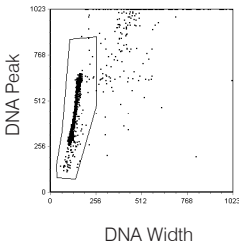
If a low intensity of FITC staining is observed, try increasing the incubation time during the 50 μL DNA Labeling Reaction. Some researchers have found labeling times of up to four hours at 37° C may be required for certain cell systems.

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

Different Gates Used for Different Cytometers

A

Becton-Dickinson



B

Coulter

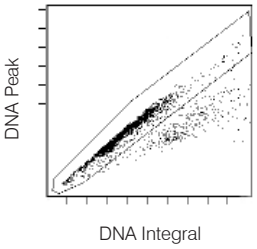


Figure 5

Common Gated Display

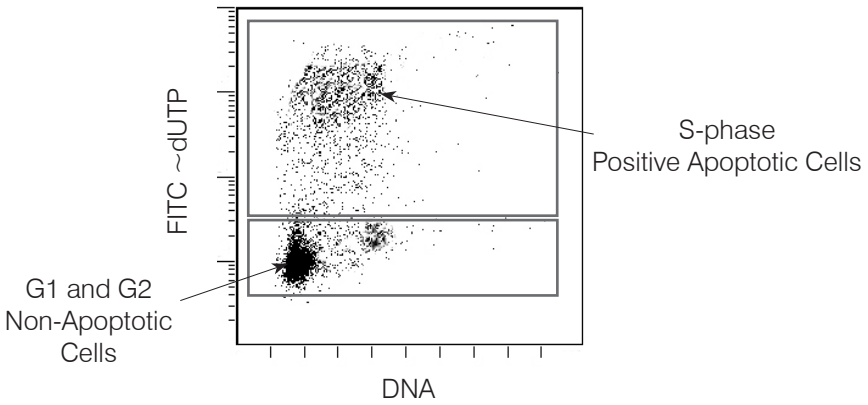


Figure 6

Figures 5 and 6 illustrate how the data should be acquired on the respective cytometer. No fluorescence compensation or gating from a scatter parameter is required.

References

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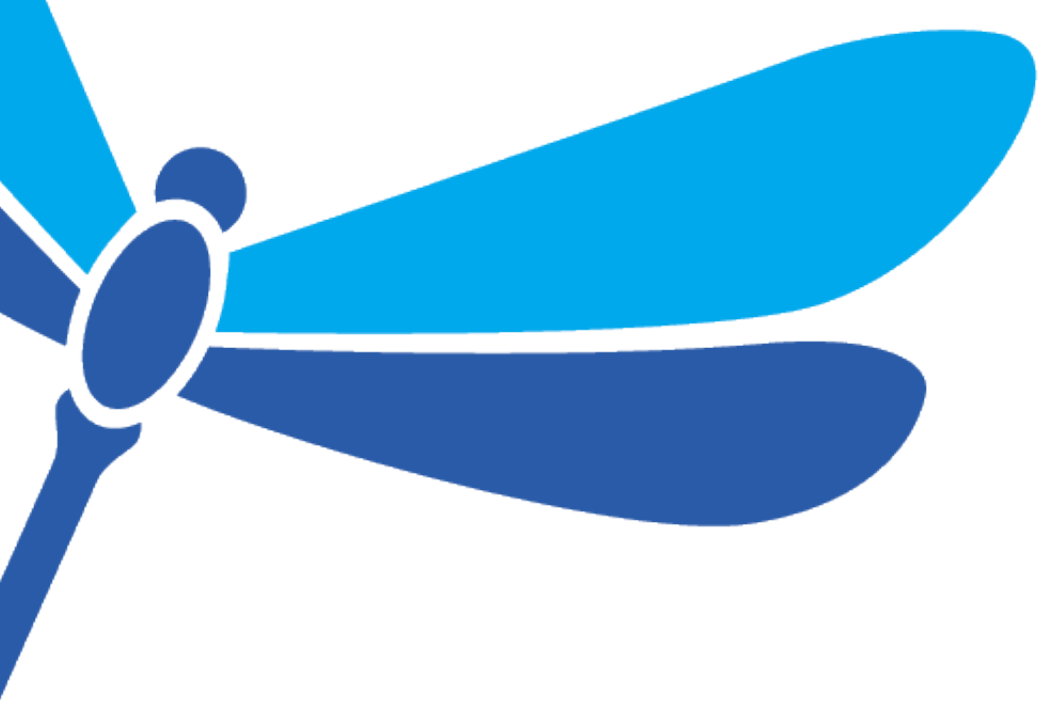
Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P and Traganos F. 1992. Cytometry. 13(8): 795-808.

Also available from Tonbo:

Product Name	Cat. No.
EZ-BrdU™ Kit	TNB-6600-KIT
APO-BrdU™ Kit	TNB-6671-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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