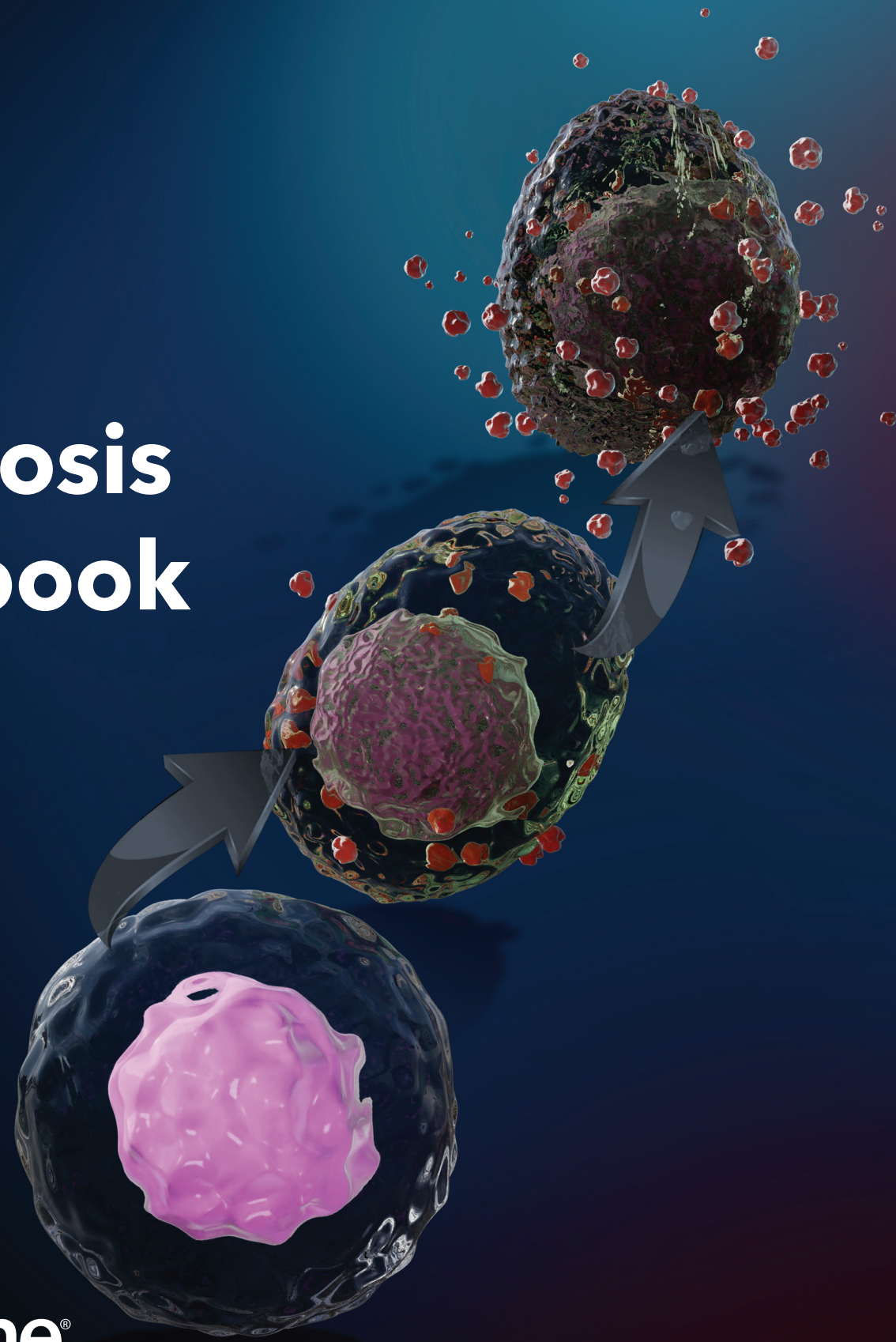
Apoptosis Handbook



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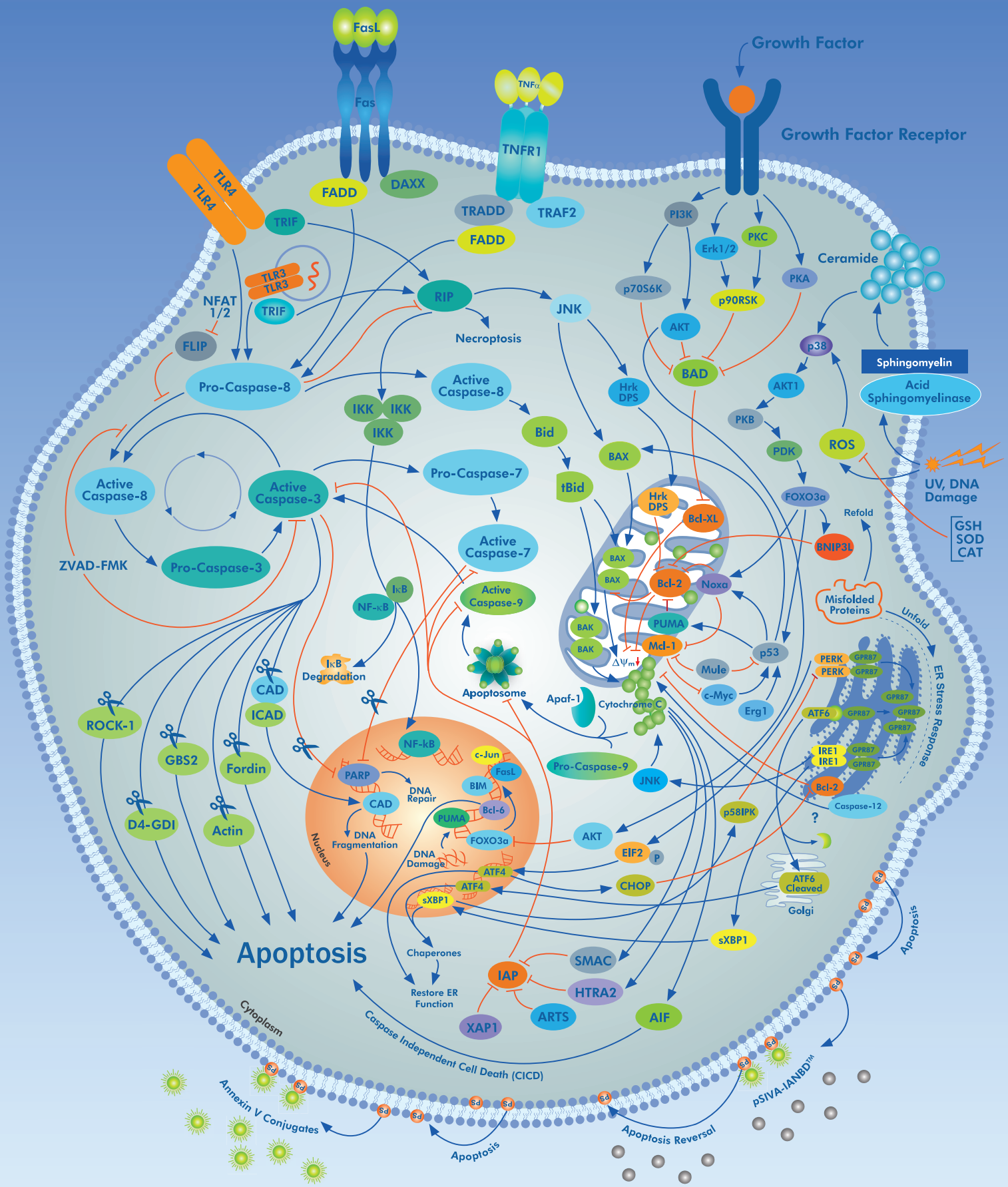

Apoptosis is derived from the Greek prefix apo-, meaning “away, off”, and the word ptosis, meaning “falling, a fall”.

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Introduction

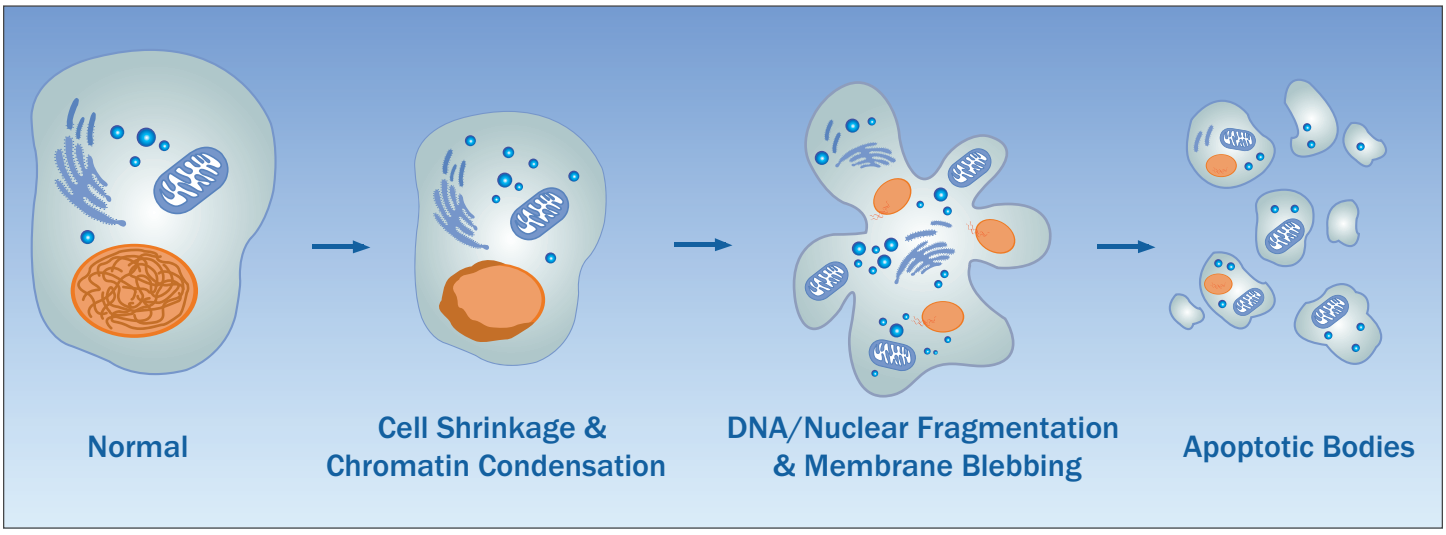
Apoptosis, a form of programmed cell death, is a coordinated and step-wise series of biochemical reactions resulting in the ordered disassembly of a cell from an organism. This normal biological process is required for proper organ development during embryogenesis and the removal of abnormal cells, such as the cells that are damaged by exposure to pathogens or undergo oncogenic transformation. The switch between cell survival and apoptosis is tightly regulated and critical to the development and well-being of an organism. For instance, defects in the apoptotic pathway that prevent cell death may lead to developmental abnormalities or unregulated tissue growth, as occurs in cancer. In contrast, unscheduled or premature apoptosis can result in the loss of functional cells, contributing to autoimmune, neurological and cardiovascular disorders. Consequently, manipulation of the apoptotic process is essential to better understand the development of various diseases and to discover potential therapeutic targets.

Induction of apoptosis evokes several significant biomolecular and morphological changes, some of which are commonly used as markers of apoptosis:

- **Activation of apoptotic signaling cascades**
- **Phosphatidylserine exposure on the outer leaflet of the plasma membrane**
- **Release of cytochrome c (Cyt c) from mitochondria**
- **Activation of caspases**
- **Cleavage of specific caspase substrates**
- **DNA fragmentation**

These events lead to major phenotypic alterations, highlighted in the figure below. Apoptosis concludes with the formation of apoptotic bodies, which are cleared by phagocytes or neighboring cells.

Cytology of Apoptosis

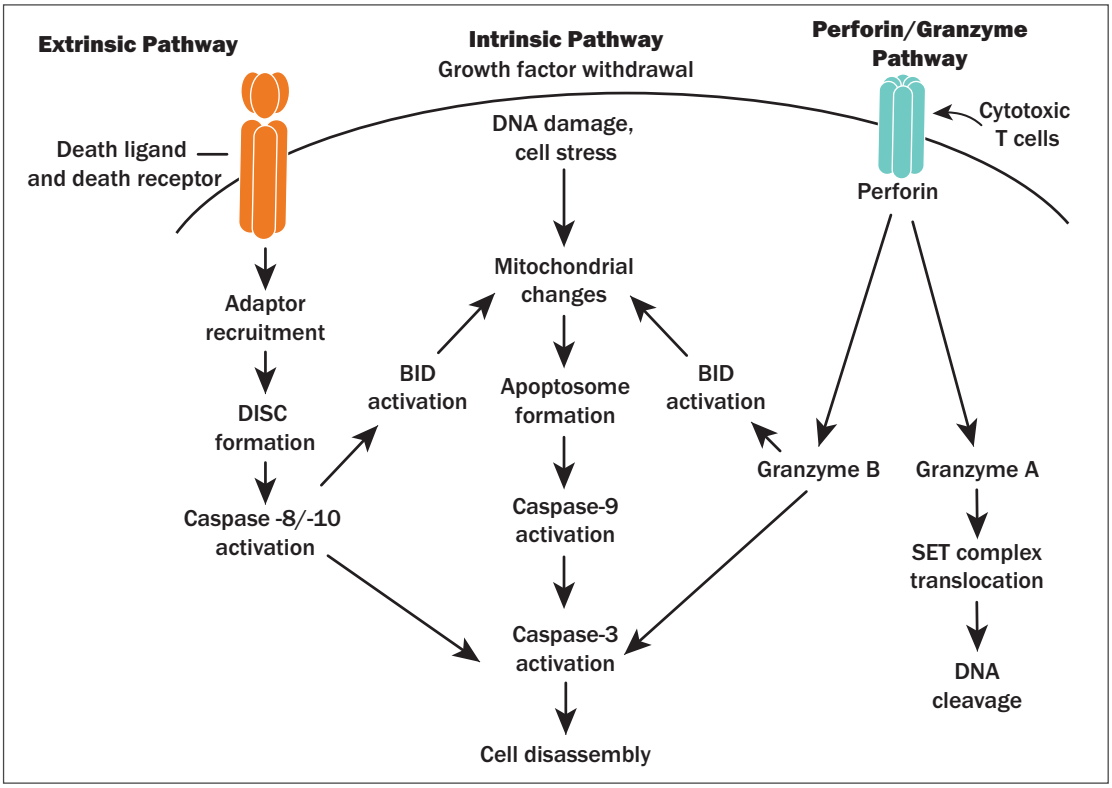


This guide will provide a brief introduction to apoptosis summarizing known signaling pathways and key molecular components. Researchers can also find technical tips and common tools to consider when choosing and optimizing assays for detecting apoptotic events.

Apoptosis Signaling Pathways

Apoptosis is executed by the extrinsic or intrinsic death signaling pathways, or in some cases by the perforin/granzyme B pathway and results in the activation of the caspase cascade. Multiple protein families are involved in the signaling pathways that promote or inhibit caspase activation.

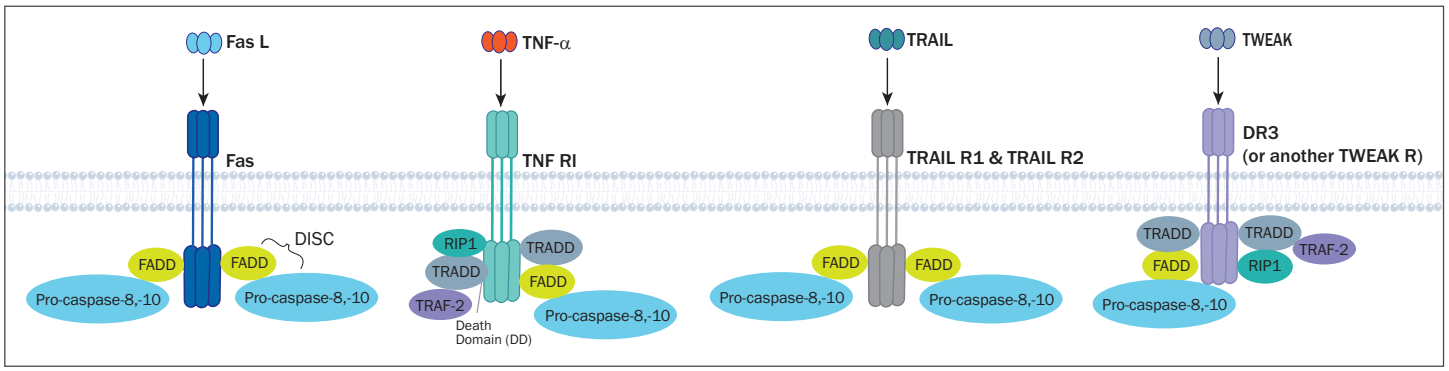
Initiation of Apoptosis Signaling Network



Extrinsic Pathway

The extrinsic pathway begins with the binding of a ligand to one of several death receptors, all members of the TNF receptor superfamily. This interaction triggers receptor oligomerization and the recruitment of adaptor proteins containing death domains (DD), such as TRADD and FADD. The resulting complexes bind and activate pro-caspases-8 and -10. The ligands including FASL, TNF-α, TRAIL and TWEAK may be anchored in the plasma membrane of neighboring cells or can act as soluble cytokines.

Death Receptors and Adaptor Proteins Involved in Apoptotic Signaling



Apoptosis Signaling Pathways

Perforin/Granzyme B Pathway

During an immune response to viruses and cellular transformation, cytotoxic cells including cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells identify affected cells and release serine proteases known as granzymes into the cytosol of targeted cells. Granzyme B triggers apoptosis either by cleaving BID to induce mitochondrial outer membrane permeabilization (MOMP) or by directly processing effector caspases.

Intrinsic Pathway

The intrinsic pathway of caspase activation can be initiated by a variety of unrelated factors, including DNA damage, growth factor withdrawal, loss of contact with the extracellular matrix, or exposure to glucocorticoids. These stimuli induce signaling cascades that result in the loss of mitochondrial integrity, release of Cyt c, and the subsequent activation of caspase-9.

Mitochondrial integrity is regulated by the family of Bcl-2 proteins, a group of more than 20 structurally related proteins that contain one to four Bcl-2 homology (BH) domains. Bcl-2 proteins are divided into 3 distinct subfamilies based on the presence of BH domains and their ability to either promote or inhibit apoptosis. The balance between pro- and anti-apoptotic family members determines whether the cell survives an apoptotic insult or undergoes cell death.

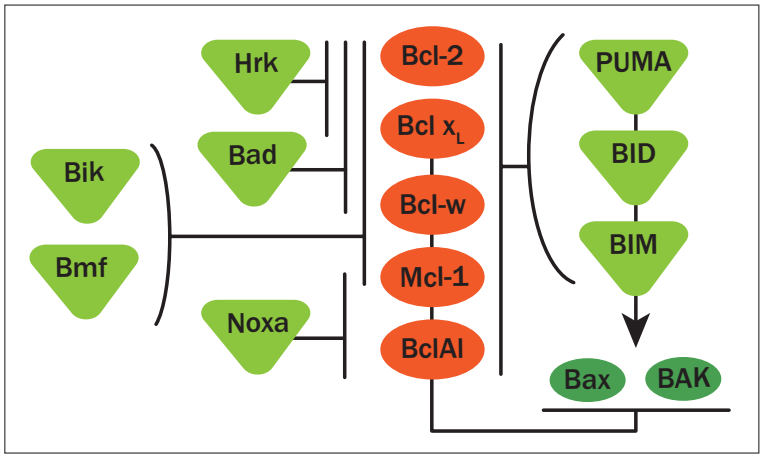
Classification of Mammalian Bcl-2 Members

Function	BH domain	Proteins*
Pro-apoptotic	BH multidomain	BAK, Bax, Bcl-rambo, Bcl-x_S, BOK
	BH3-only **	Bad, BID, Bik/Blk, BIM, Bmf, Hrk/DP5, Noxa, PUMA
Anti-apoptotic	BH multidomain	Bcl-2, Bcl-10, BclA1, Bcl-B, Bcl-w, Bcl-x_L, Mcl-1

* Other Bcl-2 interacting proteins that modulate apoptosis include Bag-1, BNIP3, and BNIP3L.

** BH3-only proteins induce apoptosis by interacting directly with Bax and Bak and/or by suppressing the activity of anti-apoptotic proteins in the mitochondria and endoplasmic reticulum.

Reported Interactions between Bcl-2 Family Members

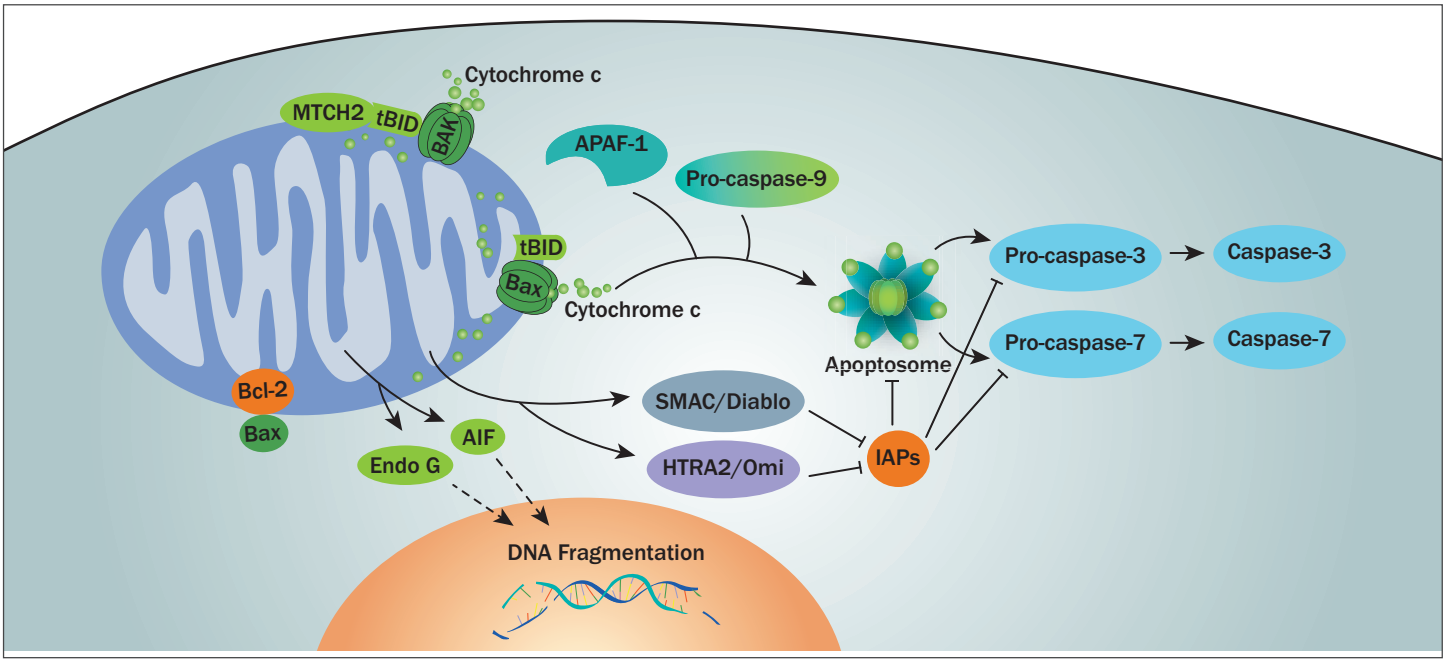


Protein quaternary structure, conformation, expression levels, post-translational modifications, and cellular localization of Bcl-2 proteins are frequently exploited in immunoassays to monitor apoptotic signaling pathways. For example, cleavage of cytoplasmic BID during apoptosis results in the migration and insertion of the C-terminal fragment, tBID (truncated BID), into the mitochondrial membrane. Likewise, the pro-apoptotic protein Bax is predominantly found as an inactive monomer in the cytosol in healthy cells, but oligomerizes and translocates to the mitochondria upon activation.

Mitochondrial Changes

The mitochondrial outer membrane permeability (MOMP) is a hallmark of the intrinsic pathway of caspase activation and often considered the point-of-no return. This is because MOMP typically leads to cell death, even in the absence of caspase activation. Activation of BH3-only proteins such as PUMA, BID, and BIM are necessary for the activation and oligomerization of Bax or Bak. These pro-apoptotic proteins form pores in the outer mitochondrial membrane, facilitating the release of multiple proteins into the cytosol.

Release of Pro-apoptotic Proteins from Mitochondria



1. Cyt c released from mitochondria, dATP, APAF-1, and pro-caspase-9 form the apoptosome, which results in the activation of caspase-9.
2. Smac/Diablo and HTRA2/Omi promote caspase activity through interactions with IAP proteins.
3. AIF (and possibly endonuclease G) translocate to the nucleus upon mitochondrial release and lead to chromatin condensation and DNA fragmentation.

Cytochrome c Release is an Indicator of MOMP

One of the most prevalent markers to analyze MOMP is the release of Cyt c into the cytosol. The extent of Cyt c translocation is often determined by Western blot analysis of cytosolic and enriched mitochondrial fractions (see the protocol on page 5). Immunofluorescence (IF) of fixed cells is an effective approach to visualize these changes at the single-cell level in which the distribution of Cyt c shifts from punctate mitochondrial staining to diffuse cytosolic staining during apoptosis. The localization of Cyt c that was once challenging to detect in suspension cells or for cells that have rounded up, for example, have been improved with the emergence of high-resolution fluorescence microscopy.

In addition, MOMP can be detected by measuring the mitochondrial transmembrane potential ($\Delta\Psi m$), which is disrupted once the outer membrane is permeabilized. The loss of $\Delta\Psi m$ can be observed using positively charged (cationic) dyes such as TMRE and JC-1 that accumulate in the mitochondrial matrix when $\Delta\Psi m$ is maintained. Accordingly, the mitochondria from healthy cells will fluoresce brighter than cells undergoing apoptotic death or mitochondrial depolarization, e.g. treated with the oxidative phosphorylation uncoupler FCCP.

Mitochondrial Changes

PROTOCOL: Western Blot Detection of Cytoplasmic Cytochrome c

A basic protocol and the required reagents, unless otherwise noted, are found in the Cytochrome c Apoptosis Detection Kit (catalog # KA0772).

MATERIALS

- PBS (not included in the kit)
- 1X Cytosol Extraction buffer, 1mM DTT and 1X Protease Cocktail (*prepare immediately before use*)
- Mitochondria Extraction buffer, 1mM DTT and 1X Protease Cocktail (*prepare immediately before use*)
- Cytochrome C Antibody (0.2mg/ml)
- Organelle Markers (not included in the kit)

METHOD

01 Induce apoptosis by desired method and include vehicle-treated cells/animal (negative control).

02

For cultured cells:

a. Collect ~5 x 10^7 cells by centrifugation at 200 x g for 5 minutes at 4°C.

b. Wash cells with 10 ml of ice-cold 1x PBS. Centrifuge at 600 x g for 5 minutes at 4°C. Remove supernatant.

c. Add 1 ml of 1X Cytosol Extraction Buffer Mix containing DTT and Protease Inhibitors to the cells. Resuspend the pellet by carefully pipetting up and down with a pipette. Incubate on ice for 15 minutes.

For tissue (fresh is recommended):

a. Isolate the tissue of interest using standard dissection procedures.

b. Wash with 10 ml of ice cold 1X PBS and mince the tissue with a scalpel or razor blade.

c. Resuspend each 10 mg of tissue in 1 ml of 1X Cytosol Extraction Buffer Mix containing DTT and Protease Inhibitors.

03 Homogenize cells in a pre-chilled Dounce tissue grinder (or pestle homogenizer). Perform the task with the grinder on ice. In general, 30-50 passes is suggested for cells and 20-30 passes for tissue. Efficient homogenization depends on the tissue and cell type.

Note: To check the efficiency of homogenization, pipette 2-3 µl of the homogenized suspension onto a coverslip and observe under a microscope. A shiny ring around the nuclei indicates that cells are still intact. If 70- 80% of the nuclei lack the shiny ring, proceed to step 4. Otherwise, perform 10-20 additional passes using the Dounce tissue grinder and check under a microscope again to confirm adequate homogenization. Excessive homogenization should also be avoided because it can damage the mitochondrial membrane and lead to the inappropriate release of mitochondrial components, comprising experimental results.

PROTOCOL: Western Blot Detection of Cytoplasmic Cytochrome c

04 Transfer homogenate to a microcentrifuge tube, and centrifuge at 700 x g for 10 minutes at 4°C. *The pellet contains the nuclei, cellular debris and intact cells, whereas the supernatant contains the cytosol and mitochondria.*

05 Transfer the supernatant into a fresh microcentrifuge tube, and centrifuge at 700 x g for another 10 minutes at 4°C to remove any residual nuclei.

06 Collect supernatant into a fresh microcentrifuge tube labeled Cytosolic Fraction, and centrifuge at 10,000 x g for 30 minutes at 4°C. *The resulting supernatant is the cytosolic fraction and the pellet is the mitochondrial fraction.* Centrifuge and collect the supernatant again to remove residual mitochondria.

Note: To remove any residual cytosolic components, consider washing the mitochondria pellet in 1ml of 1X Cytosol Extraction Buffer Mix containing DTT and Protease Inhibitors and centrifuge at 10,000 x g for another 15 minutes at 4°C.

07 Resuspend the pellet in 0.1 ml of Mitochondrial Extraction Buffer Mix containing DTT and protease inhibitors, vortex for 10 seconds and label tube as Mitochondrial Fraction.

Note: Collected fractions can be stored at -80°C until use. Avoid freeze/thaw cycles.

08 Load 10 µg each of the cytosolic and mitochondrial fractions isolated from uninduced and induced cells on a 12% SDS-PAGE. Then proceed with a standard Western blot procedure and probe with the cytochrome c antibody (recommended working concentration is 1 µg/ml) and the proper organelle markers.

Note: The appropriate organelle controls should be used to confirm the purity and integrity of each fraction. Beta-actin (catalog # NB600-501) is a recommended cytoplasmic marker whereas VDAC1 (catalog # NBP2-38163) is a recommended mitochondrial marker. For more information, see our Antibodies for Organelle Markers Handbook at novusbio.com/om-handbook.

To view a standard Western blotting protocol, download or request our Western Blot Handbook at novusbio.com/wb-handbook.

Caspase Activation

Caspases are a family of cysteine proteases found in the cytosol that act as the primary mediators of apoptosis. Based on their role in apoptosis or inflammation, caspases are subdivided into initiator and effector (executioner) groups. All caspases are synthesized as inactive zymogens containing a variable length pro-domain followed by a large and small subunit. Cleavage of caspases (arrow symbols) occurs at specific asparagine (Asn) residues located after the pro-domain and in between the large and small subunits, and leads to the formation of active heterotetramers.

Intrinsic or extrinsic death stimuli trigger the autocatalytic activation of apoptotic initiator caspases upon binding to dedicated protein complexes such as the apoptosome or the death-inducing signaling complex (DISC). Once activated, initiator caspases cleave pro-forms of executioner caspases to activate them, which in turn are responsible for the proteolytic processing of various cellular proteins. Caspases process close to 1000 substrates, some of which when cleaved are critical for the characteristic morphological and molecular changes associated with apoptosis. One of the first effector caspase substrates identified in apoptosis was PARP-1, which loses its ability to function in DNA repair upon cleavage.

Classification of Mammalian Caspases

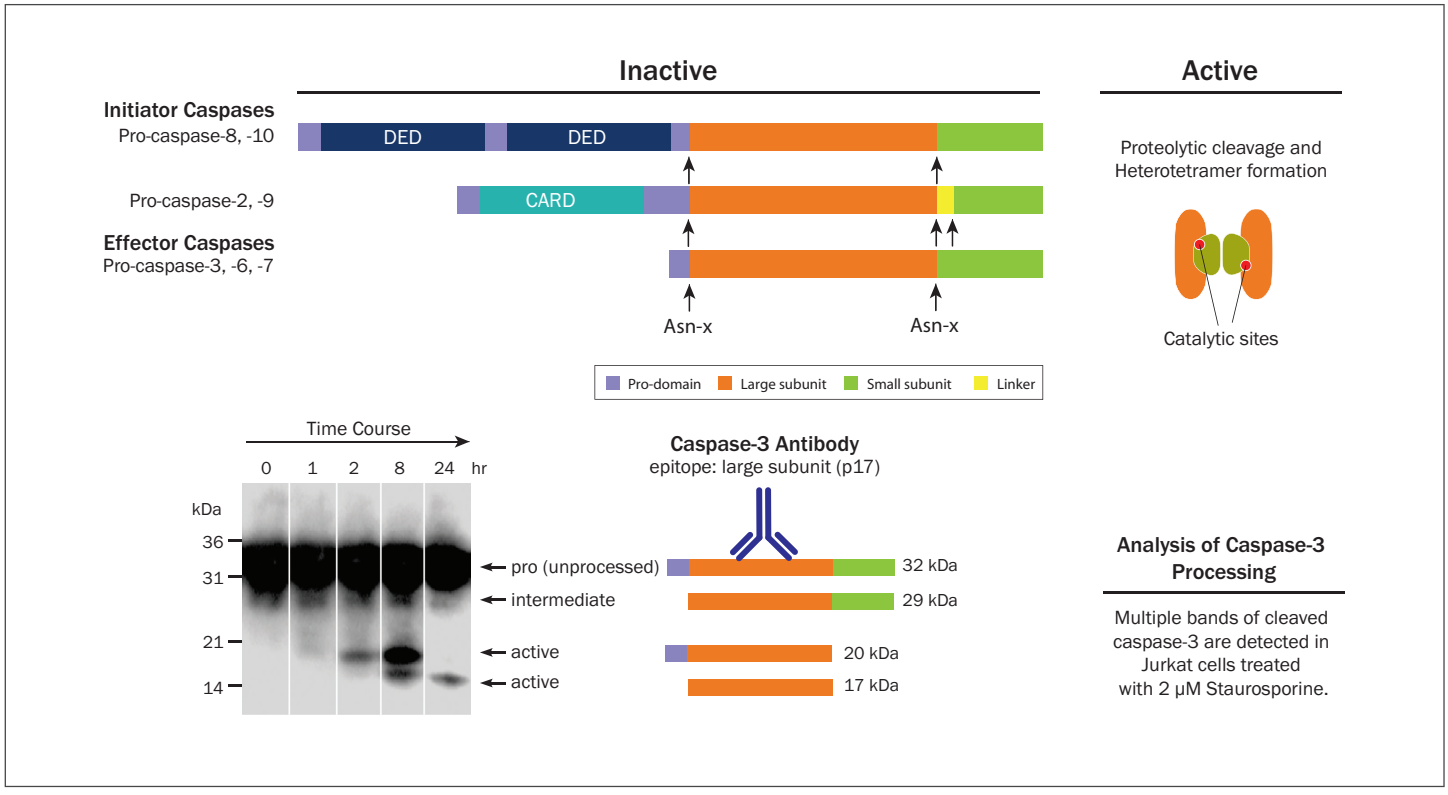
Function	Mammalian Caspase
Apoptotic Initiator Caspases	Caspase-2,-8,-9,-10
Apoptotic Effector Caspases	Caspase-3,-6,-7
Inflammatory Initiator Caspases	Caspase-1,-4,-5,-11, -12L
Keratinocyte Differentiation Effector Caspase	Caspase-14

Effector Caspase Substrates

DNA Fragmentation
- ICAD
- Nuclear lamin (lamin B)

Membrane Blebbing
- Gelsolin
- ROCK-1
- PAK

Domain Organization and Activation of the Apoptotic Caspases



Caspase Activation

Detection of Processed Caspases

Antibodies are classic tools used to visualize caspase processing by Western blotting (Figure 1A). Applications such as flow cytometry and microscopy require antibodies that specifically recognize either the pro- or cleaved caspase forms because these techniques are unable to resolve multiple protein forms with a single antibody (Figure 1B). The bands observed on a Western blot to visualize caspase processing will depend on the epitope recognized by a given antibody and the caspase forms present. If the loss of pro-forms, e.g. of caspase-3, are not accompanied by or are proportional to cleaved caspase-3, the loss itself is considered an indicator of caspase processing. It is important to note that bands representing cleaved caspase fragments may be transient due to a short half-life and are not definitive markers of caspase activation. Confirmation of caspase activation can include immunodetection of processed physiological substrates such as PARP-1.

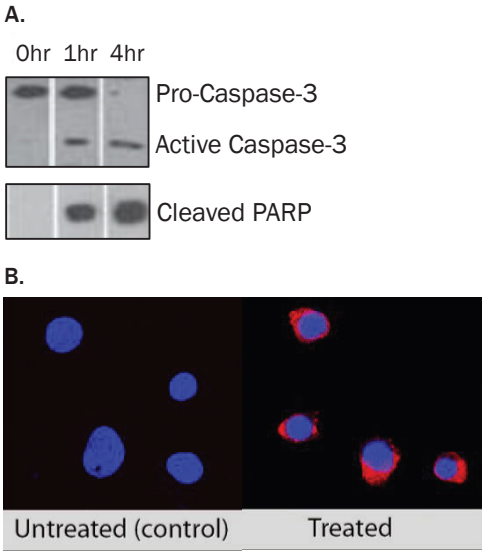


Figure 1. A) To induce apoptosis, Jurkat cells were treated with 2 uM staurosporine (STS) at the indicated times, and caspase-3 processing was monitored by Western blots. The pro-caspase (full length, ~32 kDa) and the large subunit of processed human caspase- 3 (~17 kDa) were detected using caspase-3 monoclonal antibody (catalog # NB100-56708). A fragment of cleaved PARP (~89 kDa) was detected using PARP monoclonal antibody (catalog # NB100-56599). Note that caspase-3 and PARP were processed only in cells treated with STS. B) Jurkat cells were treated as in A, but caspase-3 processing was visualized by IF using cleaved caspase-3 monoclonal antibody (catalog # MAB7071) followed by NorthernLights™ 557-conjugated secondary antibody (catalog # NL004), shown in red. Nuclei are stained with DAPI, shown in blue.

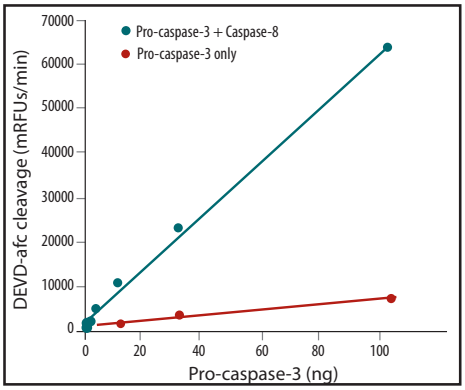
Detection of Caspase Activity

One approach to directly assess the activity of purified caspases or caspases enriched from cell extracts involves the use of peptide substrates conjugated to a colorimetric or a fluorescent reporter molecule (Figure 2A). When caspases cleave these peptide substrates, the reporter molecule is released and measured by a spectrophotometer or fluorometer, lending them to multi-well microplate based assays. These assays provide a quantitative analysis of caspase activation because the colorimetric or fluorometric signal is proportional to the enzymatic activity, and serve as an effective platform for screening caspase inhibitors. Typical formats to monitor caspase activity in live cells by flow cytometry and microscopy use fluorescent labeled peptide inhibitors that irreversibly bind the catalytic site of select caspases. For all individual caspases, peptide substrates and inhibitors provided in Novus kits have been developed for optimal enzyme kinetics and high affinity binding (Figure 2B).

While both methods have been widely used to examine the role of individual caspases, these peptides tools have limited specificity. Data generated from cell lysates or live cells, containing multiple caspases, should be interpreted with caution. For instance, given the promiscuity and catalytic efficiency of caspase-3, it will likely target additional peptides. Novus recommends performing complementary assays with siRNA/shRNA mediated gene silencing or using cells derived from caspase knockout animals to corroborate the contribution of individual caspases.

Caspase Activation

A.



B.

Apoptotic Caspases	Core Peptide Sequence
Caspase-2	VDVAD
Caspase-3	DEVD
Caspase-6	VEID
Caspase-7	DEVD
Caspase-8	IETD
Caspase-9	LEHD
Caspase-10	AEVD

Figure 2. A) Caspase-8 (catalog # NB705-C8) was preincubated with increasing amounts of pro-caspase-3 (catalog # NB731-C3) for 1 hour. Caspase-3 activity (blue) was determined by measuring the cleavage of peptide substrate, DEVD-afc in a fluorescence plate reader using the Caspase-3 Fluorometric Assay Kit (catalog # KA0741). Background activity of pro-caspase-3 (red) was measured by monitoring DEVD-afc cleavage in the absence of active caspase-8. B) The amino acids in each peptide substrate, or inhibitor, represent the preferred consensus sequence recognized by individual caspases.

Key Protein Inhibitors of Caspases

Caspase activity is inhibited by several families of proteins, including cellular FLICE-like inhibitory protein (cFLIP) and the Inhibitors of Apoptosis (IAP) family. cFLIP prevents activation of caspase-8 and -10 by competing for binding to DISC. IAPs are characterized structurally by the presence of one or three BIR (Baculovirus IAP domains) and act as caspase inhibitors by several mechanisms, including direct caspase binding. Eight IAPs have been identified in humans and some such as the cIAPs are ubiquitin ligases, targeting multiple proteins for proteasomal degradation. Not surprisingly, elevated levels of cFLIP and IAPs have been detected in cancer (see Figure 3 for an example).

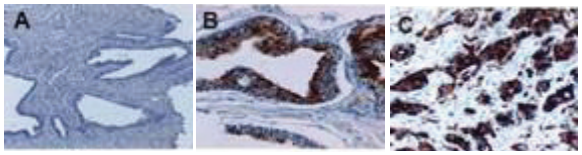
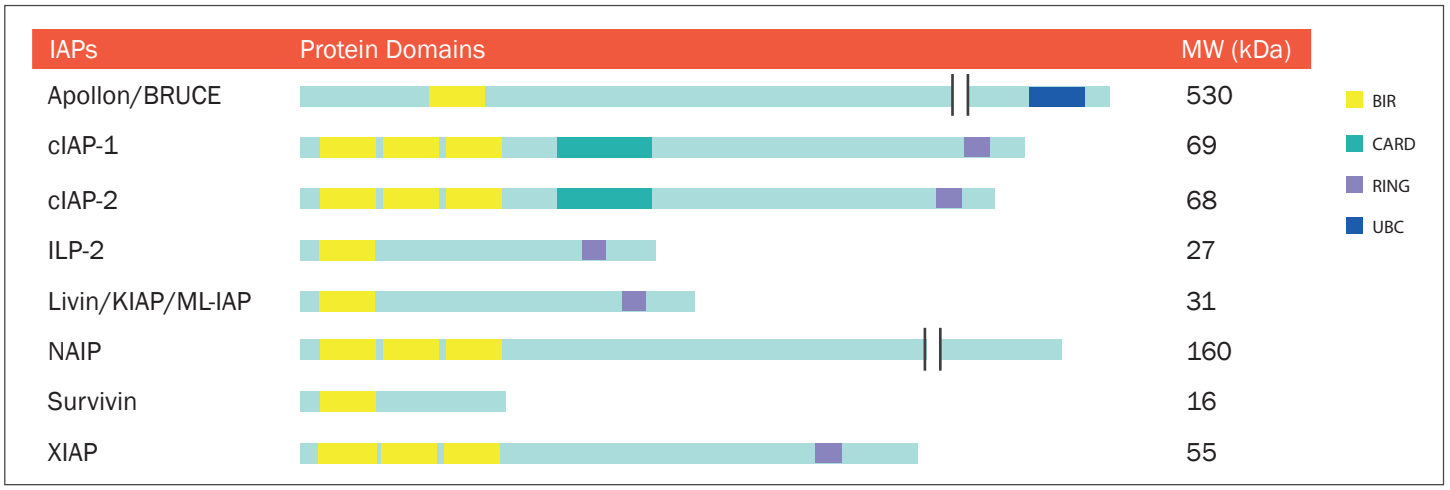


Figure 3. The presence of cIAP1 was assessed in (A) normal prostate, (B) prostate intraepithelial neoplasia (PIN) - a premalignant proliferation arising within the prostate and (C) prostate cancer. Formalin-fixed, paraffin-embedded (FFPE) sections were probed with human cIAP-1/HIAP-2 polyclonal antibody (catalog # NBP2-27190) for detection of cIAP1 (brown) and counterstained with hematoxylin (blue). Increased cIAP1 expression is observed in PIN and in prostate cancer compared to normal prostate.

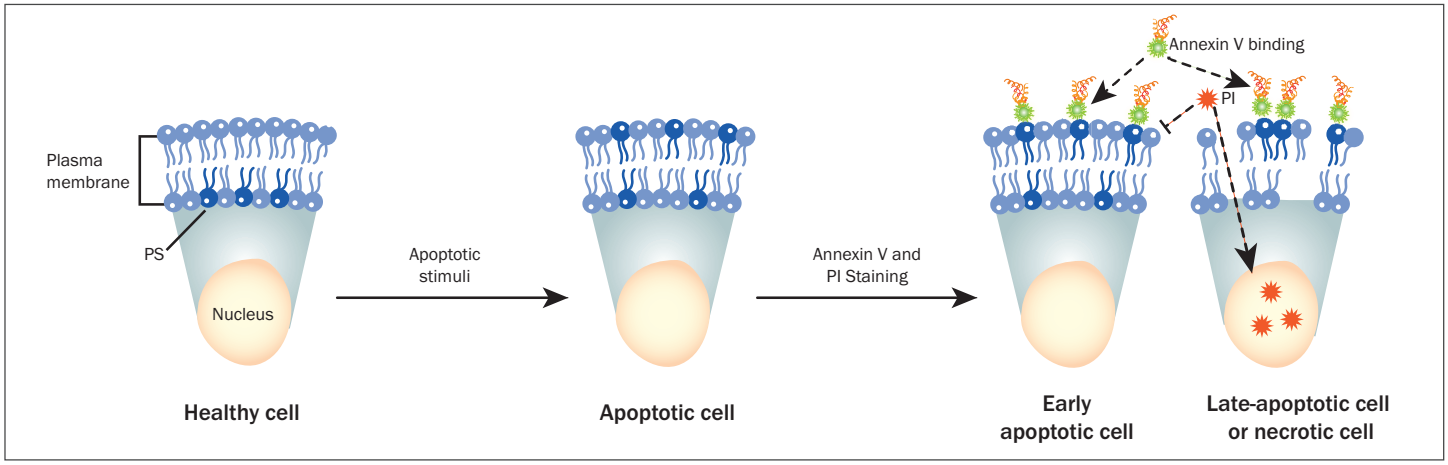
Domain Organization of the IAP Family



Cell Membrane Alterations

In healthy cells, phosphatidylserine (PS), an anionic phospholipid, is actively translocated to the inner leaflet of the cell membrane. During apoptosis, this distribution is randomized, resulting in the appearance of PS on the outer leaflet. Exposed PS can be detected by the calcium dependent binding of Annexin V. The anticoagulant protein, Annexin V, preferentially binds PS with the stoichiometry of ~50 PS monomers per protein molecule. When conjugated to a reporter molecule (FITC, Cy5, PE, EGFP, etc.), bound Annexin V can be detected by flow cytometry or by microscopy.

Principles of the Annexin V Apoptosis Assay and Dye Exclusion Method



Note: Due to the permeability of necrotic cells, labeled Annexin V may be able to penetrate the outer membrane and complex with PS in the inner leaflet. In this case, a positive signal of PS translocation in necrotic cells is an artifact. If false positive results from necrosis are of concern, consider including Necrostatin-1, a specific marker of necrosis (catalog # NB2324), in experiments.

Dye Exclusion Method

The dye exclusion method is a technique used to evaluate cell death by measuring the permeability of the cell membrane. Cells in necrosis, or in the late stages of apoptosis, have damaged plasma membranes that allow for the uptake of certain dyes including propidium iodide (PI) and trypan blue. Viable cells remain unstained because intact membranes exclude these dyes. Used in conjunction with the Annexin V assay, the dye exclusion method enables further discrimination of cells undergoing apoptosis (Figure 4).

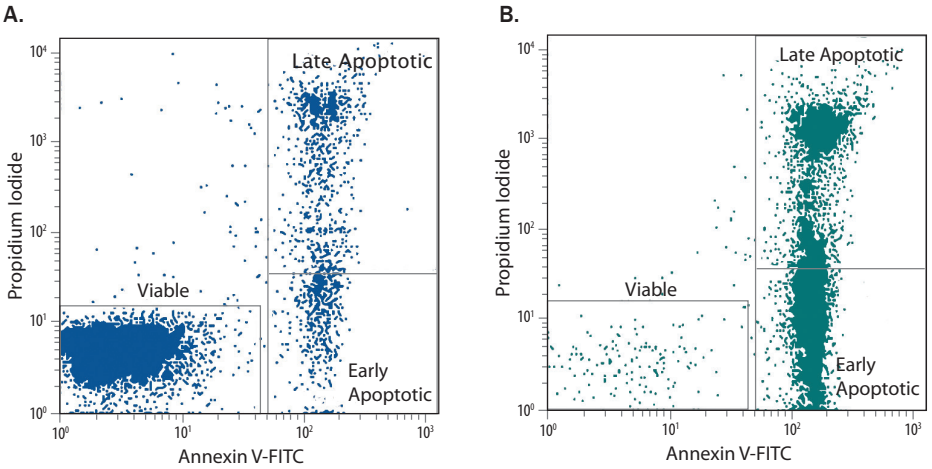


Figure 4. Detection of Apoptotic Dexamethasone-treated Thymocytes by Annexin V Staining. Thymocytes were left untreated (A) or treated with 100 nM dexamethasone for 15.5 hours (B), and then stained using Annexin V-FITC and PI provided in the Annexin V-FITC Apoptosis Detection Kit (catalog # NB4830-01-K). Analysis courtesy of Dr. C.M. Knudson, Howard Hughes Medical Institute, St. Louis, MO.

Cell Membrane Alterations

PROTOCOL: Annexin V and PI Staining by Flow Cytometry

A basic protocol and the required reagents, unless otherwise noted, are found in the Annexin V Apoptosis Kit - FITC (catalog # NBP2-29373)

MATERIALS

- PBS (not included in the kit)
- 1X Binding buffer: 10 mM Hepes pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$ (10X dilution before use)
- Positive control cells
- Annexin V-FITC staining solution
- Propidium iodine (PI) staining solution

METHOD

01 Induce apoptosis by desired method and include vehicle treated cells/animal (negative control).

02 Collect 1-5 x10^5 cells by centrifugation.

03 Wash cells 1X with cold 1X PBS and carefully remove the supernatant.

04 Re-suspend the cells in 1X Binding buffer at a concentration of ~1 × 10^6 cells/mL, preparing a sufficient volume to have 100 µL per sample.

05 Add 5 µl of Staining solution to tubes as indicated in below table and gently swirl to mix.

Vial #	Cells	Stain
1	Positive control cells (1x10^5)	none
2	Positive control cells (1x10^5)	5 µl of Annexin V-FITC
3	Positive control cells (1x10^5)	5 µl of PI
4	Uninduced experimental cells	5 µl of Annexin V-FITC + 5 µl of PI
5	Apoptosis induced experimental cells	5 µl of Annexin V-FITC + 5 µl of PI

06 Incubate the mixture for 20 minutes at room temperature in the dark.

07 Add 400 µl 1X Binding buffer to each tube, gently mix or flick the tube.

08 Analyze the cells immediately (within 1 hour) by flow cytometry.

Note: Use the positive control cells (positive for both Annexin V-FITC and PI) to set up compensation and quadrants. Apoptotic cells have a minimal uptake of PI and will appear dimly stained.

- Annexin V negative - PI negative populations are healthy cells.
- Annexin V positive - PI negative populations represent cells in early apoptosis.
- Annexin V positive - PI positive staining indicate cells are in necrosis (post-apoptotic necrosis or late apoptosis).

Advances in Detection of Apoptosis

Real-time Detection of Exposed PS

pSIVA-IANBD is an annexin-based probe conjugated to a polarity sensitive indicator that addresses the key limitation of traditional PS detection assays, background fluorescence. pSIVA-IANBD only fluoresces when bound to PS on the cell membrane (Figure 5), rendering it uniquely suitable for monitoring and quantifying PS exposure in real-time. Since binding is reversible, pSIVA™ can detect transient or permanent PS externalization, which facilitates the identification of cells rescued from apoptosis.

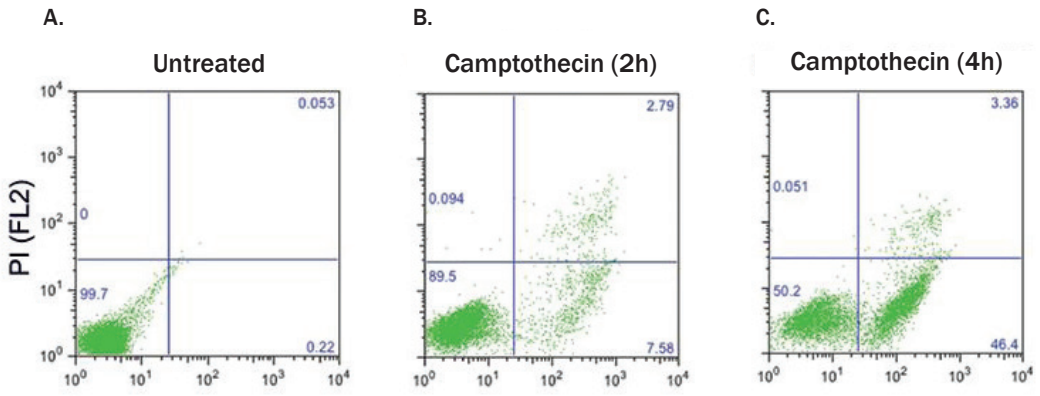


Figure 5. Apoptotic HL-60 cells were detected by pSIVA™. HL-60 cells were treated with vehicle (A), 45 µM camptothecin for 2 hours (B) or 4 hours (C), and stained with pSIVA™ and PI using the Polarity Sensitive Indicator of Viability Flow Cytometry Kit (catalog # NBP2-29611).

Simultaneous Detection of Multiple Changes in Apoptosis Pathways

High-throughput approaches enable the rapid measurement of multiple proteins in a single sample. With the Proteome Profiler Human Apoptosis Array Kit, 35 apoptosis-related proteins are simultaneously detected in a membrane-based sandwich immunoassay (Figure 6). Antibodies are spotted in duplicate on nitrocellulose membranes and bind to specific target proteins present in the sample. These proteins are detected using a cocktail of biotinylated secondary antibodies, streptavidin-HRP, and conventional chemiluminescent reagents without the need for specialized equipment.

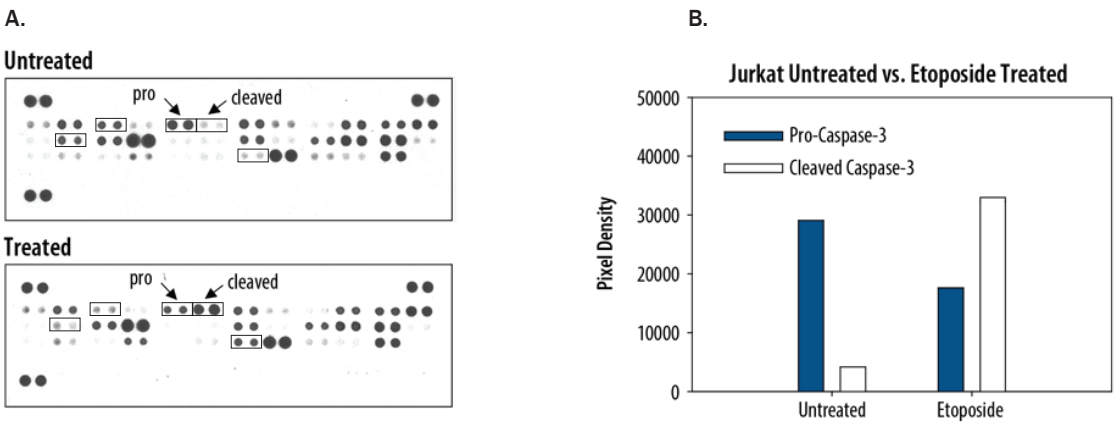
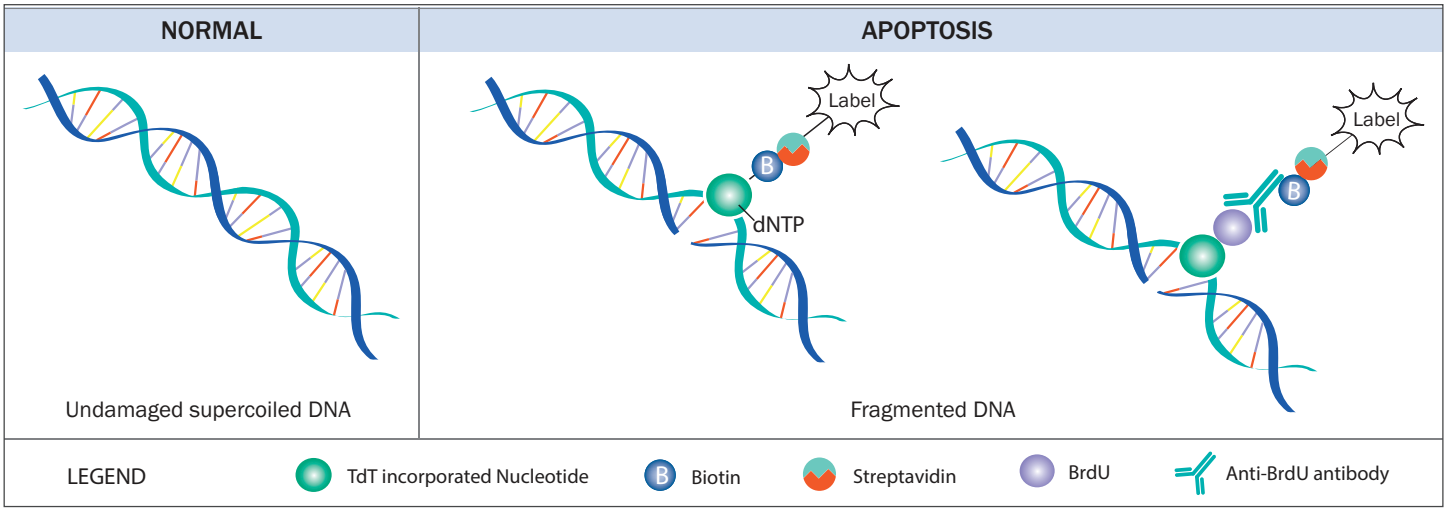


Figure 6. Jurkat cells were either treated with 25 µM etoposide or vehicle for 6 hours and the expression profile of apoptosis-related proteins in cell lysates were assessed using the Human Apoptosis Array (A, R&D Systems catalog # ARY009). Cells treated with etoposide had more processed caspase-3 and less caspase-3 precursor than vehicle treated cells, which is indicative of caspase-3 activation (B). The amount of Bcl-2 and Trail R2/Dr5 was lower in cells treated with etoposide, while phospho-Rad17 (S635) was higher, indicating that the induction of apoptosis was associated with changes in levels of these proteins.

DNA Fragmentation

A characteristic feature of cells undergoing late stage apoptosis is DNA fragmentation. CAD, an endonuclease that is activated by caspases, cleaves chromosomal DNA into 180-bp nucleosomal fragments. These fragments give an appearance of DNA laddering when run on an agarose gel. Other methods for detecting DNA fragments resulting from apoptosis, particularly for intact tissues, are *in situ* nick translation and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL).

Principle and Variations of the TUNEL Assay



Variations of the TUNEL method involve incorporating different modified nucleotides (dNTP) into DNA nicks that are detected by fluorescence or a chromogenic reaction (Figure 7). One version uses the highly purified TdT enzyme along with a cation to incorporate biotinylated nucleotides into the 3'-OH ends of double-or single-stranded DNA with either blunt, recessed or overhanding ends. The biotinylated nucleotides can then be detected by bright field microscopy using streptavidin - horseradish peroxidase (SA-HRP) and colorimetric substrates including diaminobenzidine (DAB) or TACS Blue Label®.

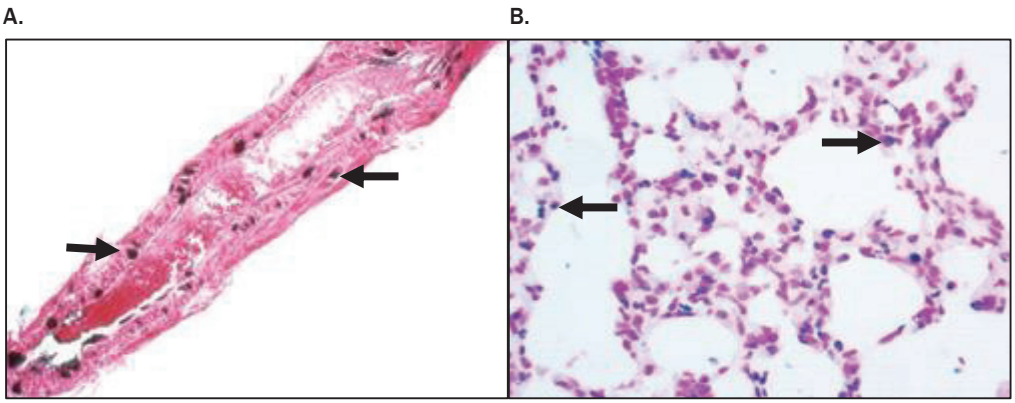


Figure 7. A) Detecting DNA fragmentation in a FFPE section of apoptotic rat small artery using biotinylated nucleotides from the VasoTACS in situ Apoptosis Detection kit (catalog # NB4826-30-K) and staining with DAB (brown). Nuclei were stained with Red Counterstain C (red). Data was provided courtesy of Dr. Jun Zhang, FDA. B) DNA fragmentation of a FFPE section of mouse apoptotic lung parenchyma tissue was detected by TACs-XL (catalog # NB4828-30-BK) based on the incorporation of BrdU and using the substrate TACS Blue Label® (blue). Nuclei was stained with Nuclear Fast Red counterstain (red). Data was provided courtesy of F. D'Agostini, University of Genova Via A. Pastore, Genova, Italy.

DNA Fragmentation

In another variation, bromodeoxyuridine (BrdU) is incorporated into the 3'-OH ends of DNA fragments and then probed with an anti-BrdU antibody. Typically, the antibody is either labeled with a fluorochrome such as FITC for fluorescence detection or detection involves consecutive binding of a biotinylated anti-BrdU antibody and SA-HRP, followed by the development of a chromogenic reaction. The incorporation of BrdU into DNA breaks is more efficient than the biotinylated equivalent and tends to offer greater sensitivity in comparison.

TUNEL staining is not always a specific indicator of apoptosis because cells actively repairing DNA damage or undergoing necrosis can also incorporate labeled nucleotides. This problem can be alleviated by using dual labeling, such as detecting DNA breaks with TUNEL and processing of caspase-3 with an appropriate antibody.

PROTOCOL: TUNEL and Active Caspase-3 Detection by IHC/ICC

This protocol is modified from the following kits: Steps 2-16 pertinent reagents are from Apo-BrdU-IHC Kit (catalog # NBP2-31164). Steps 17-29 (except Caspase 3 antibody) and pertinent reagents are from anti-rabbit HRP-AEC IHC Detection Kit (catalog # CTS006).

MATERIALS

NOT supplied in kits:

- 1X PBS
- 1X PBS, 1mM MgSO4
- 10mM Tris pH 8.0
- DNase I
- 3% H_2O_2 – in methanol
- anti-active caspase-3 antibody (catalog # AF835)
- Aqueous Mounting Medium (catalog # CTS011)

Apo-BrdU-IHC Kit

- Proteinase K – in 10mM Tris pH 8.0 (100x dilution before use)
- 1X Reaction Buffer (10x dilution before use)
- Blocking Buffer
- DAB in H_2O_2/Urea (prepare immediately before use)
- Methyl Green

HRP-AEC detection kit:

- Avidin Blocking Reagent
- Biotin Blocking Reagent
- Biotinylated Secondary Antibody
- HSS-HRP
- AEC Chromogen and Buffer (prepare immediately before use)

Optional: Preparation of positive TUNEL control - Nuclease treated sample

- Cover the entire specimen with 1 µg/ml DNase I in 1X PBS, 1mM $MgSO_4$ and incubate at room temperature (RT) for 20 min
- Rinse with 1X PBS

Complete Labeling Reaction Mixture	1 Sample
5x Reaction Buffer	10 µl
TdT Enzyme	0.75 µl
Br-dUTP	8 µl
diH20	32.25 µl
Total volume	51 µl

Antibody Solution	1 Sample
Biotin~PRB-1	5 µl
Blocking Buffer	95 µl
Total volume	100 µl

1x Conjugate Solution	1 Sample
200x Conjugate	0.5 µl
Blocking Buffer	100 µl
Total volume	100.5 µl

PROTOCOL: TUNEL and Active Caspase-3 Detection by IHC/ICC

METHOD

01 Induce apoptosis by desired method and include vehicle-treated cells/animal (negative control).

02 Following standard procedures to prepare cells or tissue sections for ICC/IHC. Wash the specimen in 1X PBS.
Do not let cells/tissue sections dry out during or between any step! For help on preparing IHC samples, see our IHC handbook at novusbio.com/ihc-handbook.

03 Cover the entire specimen with 100 µl Proteinase K solution and incubate in a humidity chamber at RT at noted below. *Do not overincubate.*

Paraffin-embedded tissue section	20 minutes
Frozen tissue section	10 minutes
Cells	5 minutes

04 Rinse slide with 1X PBS for 5 minutes.

05 Block endogenous peroxidase by incubating specimen with 100 µl of 3% H_2O_2 for 10 minutes at RT.

06 Rinse slide with 1X PBS for 5 minutes.

07 Cover specimen with 100 µl 1X Reaction Buffer and incubate for 10-30 minutes at RT. Then carefully aspirate or blot solution. Prepare Complete Labeling Reaction Mixture during the incubation.

08 Immediately apply 50 µl of Complete Labeling Reaction and cover the specimen with a piece of parafilm, cut slightly larger than the specimen. Incubate in a humidity chamber for 1 to 1.5 hours at 37 °C.
The parafilm prevents evaporation and folding up one corner aids in its application/removal.

Note: Depending on the tissue and fixation conditions, the incubation period of the DNA End Labeling Reaction may need to be adjusted (shortened or lengthened).

09 Remove parafilm and rinse slide with 1X PBS for 5 minutes.

10 Cover specimen with 100 µl with Blocking Buffer for 10 minutes at RT. Then carefully aspirate or blot solution.

11 Immediately apply 100 µl of Antibody Solution onto the specimen and incubate for 1 to 1.5 hours in the dark at RT. Cover slides with aluminum foil.

12 Rinse slide with 1X PBS for 5 minutes.

PROTOCOL: TUNEL and Active Caspase-3 Detection by IHC/ICC

13 Cover specimen with 100 µl with Blocking Buffer for 10 minutes at RT. Then carefully aspirate or blot solution.

14 Immediately cover specimen with 100 µl of 1X Conjugate Solution and incubate for 30 minutes at RT.

15 Rinse slide with 1X PBS for 5 minutes.

16 Incubate tissue section with DAB solution for up to 15 minutes at RT. *Monitor nuclei staining (dark brown color) under the microscope.*

17 Wash slide in 1X PBS for 20 minutes.

18 Block endogenous peroxidase by incubating specimen with 100ul of 3% H_2O_2 for 10 minutes at RT.

19 Rinse slide in 1X PBS and block endogenous biotin. Incubate slide with Avidin Blocking Reagent for 15 minutes, followed by a 15 minute incubation with Biotin Blocking Reagent.

20 Wash slide in 1X PBS for 5 minutes.

21 Cover specimen with active caspase-3 Antibody Solution (5-15 µg/ml) and incubate overnight at 2-8 °C.

22 Wash slide 3 times in 1X PBS for 15 minutes.

23 Cover specimen with anti-rabbit secondary Antibody Solution for 30-60 minutes at RT.

24 Wash slide 3 times in 1X PBS for 15 minutes.

25 Incubate tissue section with HSS-HRP for 30 minutes at RT.

26 Wash slide 3 times in 1X PBS for 2 minutes.

27 Incubate tissue section for 2-5 minutes with AEC Chromogen. *Monitor red color staining development under the microscope.*

28 Rinse slide with diH_2O and counterstain with Methyl Green.

29 Rinse slide with PBS, mount using Aqueous Mounting Medium, and then dry the slide.

30 Image with a bright field microscope.

Activators and Inhibitors of Apoptosis

The use of chemical modulators to initiate or prevent programmed cell death are crucial tools to investigate the complex components of molecular pathways. As illustrated in the table below, apoptosis inducers are grouped based on their mechanism of action and include several examples of classic chemotherapeutic agents that interfere with DNA synthesis. Novel compounds such as AZD 5585 dihydrochloride and MSC 2032964A allow for more selective targeting and activation of apoptotic pathways.

Common Chemical Modulators of Apoptosis

	Chemical	How it works*	Catalog #
Activators	Actinomycin D	Inhibits RNA polymerase	NB1229
	AZD 5582 dihydrochloride	Dimeric Smac mimetic; potent IAP inhibitor	NB5141
	AT 101	Downregulates Bcl-2 and Mcl-1	NB3367
	Camptothecin	Topoisomerase I Inhibitor	NB1100
	Cisplatin	Platinum agent inducing DNA damage	NB2251
	Doxorubicin hydrochloride	Antitumor antibiotic agent. Inhibits DNA topoisomerase II	NB2252
	Etoposide	Topoisomerase II Inhibitor	NB1226
	Mitomycin C	DNA cross-linking antitumor agent	NB3258
	MSC 2032964A	Potent and selective ASK1 inhibitor; orally bioavailable	NB5641
	Nutlin 3	MDM2 antagonist; inhibits MDM2-p53 interaction	NB3984
	Paclitaxel (Taxol)	Promotes assembly and inhibits disassembly of microtubules	NB1097
	PRIMA-1^{MET}	Restores mutant activity p53 activity	NB3710
	Staurosporine	Broad spectrum protein kinase inhibitor	NB1285
	Vinblastine	Disrupts microtubules	NB1256
Inhibitors	Z-VAD-FMK	Irreversible pan caspase inhibitor	NB2163
	Q-VD-OPH	Less toxic irreversible pan caspase inhibitor, for *in vivo* studies	NBP2-29391
	Z-DEVD-FMK	Irreversible caspase 3 inhibitor	NB2166

* Effective doses are dependent on the cellular context and should be determined empirically.

Activators and Inhibitors of Apoptosis

Blocking the progression of apoptosis frequently involves either a cell-permeable, broad-spectrum caspase or caspase-specific inhibitor. For instance, if measuring cytochrome c release from mitochondria, pan caspase inhibitors will prevent the down-stream activation of the caspase cascade. While caspase-mediated cell death is blocked, cell survival may not be long term. Similarly, cells treated with caspase inhibitors and death receptor ligands (e.g. TNF-α) are diverted to other cell death pathways (i.e. necroptosis).

Determining an Effective Drug Concentration

The suitable drug concentration required to initiate or inhibit an apoptotic response in the majority of targeted cells can be determined by performing a dose titration experiment. For a good starting point, consult the literature to identify potential working concentrations. It is also important to understand the relationship between concentration and exposure times. If inducing apoptosis at a low dose, or with a mild stimulus, then a longer treatment time may be necessary.

PROTOCOL: Induction of Death Receptor - Mediated Apoptosis

Apoptosis in Fas or TNF receptor positive cells can be induced by the appropriate ligands or by using an agonist antibody. A basic protocol using the anti-Fas monoclonal antibody (EOS9.1 catalog # NBP2-00463) is described below:

01 Apply 0.05-0.1 µg/ml anti-Fas antibody to 0.5 × 10^5 cells/ml in fresh media (e.g. Jurkat in fresh RPMI-1640 medium containing 10% fetal bovine serum). *Cells should be in the exponential (log) phase prior to treatment. For a negative control incubate untreated cells under identical conditions.*

02 Incubate for 3–16 hours in a 37 °C incubator. The optimal time should be determined empirically.

03 Harvest the cells by centrifugation.

04 Wash in 1X PBS and resuspend in 1X PBS.

05 Proceed to apoptosis detection.

Experimental Controls

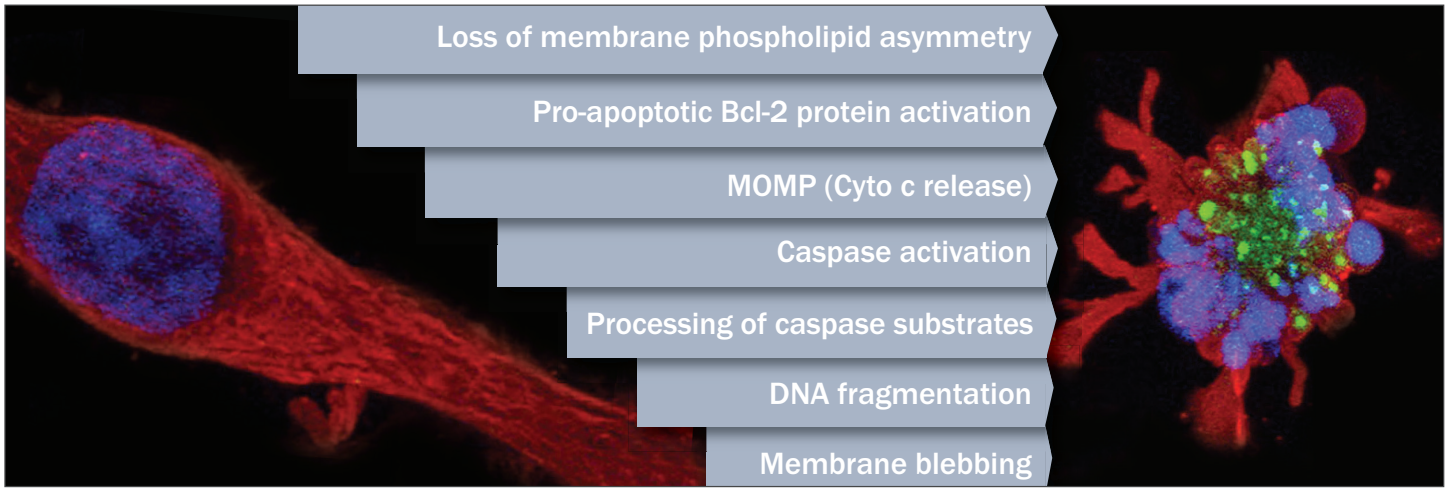
All experiments measuring apoptosis should include both positive and negative controls. During data analysis, negative and positive controls are key to identify and separate healthy and apoptotic populations in experimental samples. Negative controls are also important to assess the health of vehicle-treated cells and serve as baseline measurements crucial for determining the magnitude of the apoptotic response. Samples treated with a caspase inhibitor can serve as additional negative controls. If cells lacking treatment with apoptosis inducers or treated with caspase inhibitors already show significant signs of cell death, then the activity and effect of the drug will be difficult to establish.

Additional Considerations for Assay Development

Timeline for Observing Apoptotic Events

Apoptosis is a dynamic process and determining the optimal time to measure the incidence of apoptosis or the activity of molecules involved is critical for understanding the underlying mechanisms. Generally, cultured cell lines undergo apoptosis at a much faster rate in comparison to tissues (from several hours to days). Several factors can influence the timing, including cell type, apoptotic stimulus, the specific apoptotic mechanism involved, and experimental conditions. Another complication to consider is that cells have a heterogeneous response to a death stimulus. This varied response may be caused by an asynchronous cell cycle position, differences in transcriptional activity, the abundance of Bcl-2 proteins, and many others. Thus, the design of early experiments should factor in a time course, even if replicating previously published results.

General Sequence of Apoptotic Progression



Apoptotic Mechanisms May Vary with Cell Types

Several factors can affect how cells respond to an apoptosis-inducing agent and so it is important to understand the biological system under study. For example, primary cells may respond to death stimuli differently compared to immortalized cells. The human breast adenocarcinoma cell line, MCF-7, lacks caspase-3, while primary neurons do not express Bak, depending almost exclusively on Bax for mitochondria-mediated apoptosis. Likewise, cancer cell lines may respond differently from primary tumors, or freshly explanted cells, if only because these cells may have unique sets of genetic or epigenetic alterations that lead to the activation of oncogenes or the inactivation of tumor suppressor genes.

Even the same cell line can respond differently over time, as cultured cancer cells evolve, accumulating mutations that may affect drug resistance. These changes may account for some of the discrepancies reported in the literature for the same cell line, not to mention the possibility of cell line misidentification and the contamination with other cell lines, or mycoplasma. As a result, a response to a death stimulus may depend on how long the cell line has been passaged. Cell lines with higher passage numbers may respond differently, or even be insensitive, to a death stimulus that was once potent in lower passage cells. Good cell practice is to limit the number of acceptable passages used in an experiment.

Finally, differences in tissues can further complicate the response of individual cells to an apoptotic stimulus. The diverse composition of cells in a tumor microenvironment facilitate complex and dynamic molecular signals that inhibit apoptosis and promote tumor progression.

Additional Considerations for Assay Development

Assay Formats

Apoptosis can be detected by a number of different methods, each of which has their advantages and disadvantages, both practical and conceptual. This is why it is a good practice to use more than one assay for the same experiment. Selecting the optimal assay to use will depend on equipment availability, sample type, downstream applications, assay sensitivity and whether or not quantitative results are needed. Also, techniques enabling single cell measurements are highly recommended considering all cells do not respond the same to apoptotic stimuli.

Summary of Apoptosis Assays

Feature	Detection Method	Technique*	Sample Type	Catalog #**
MOMP	Cytochrome c release	WB (subcellular fractions)	Cell/tissue lysates	NB100-56503
		ICC (confirm with organelle markers)	Fixed cells	
ΔΨm Dissipation	Staining with ΔΨm-sensitive probe	Flow, Fluorescence Microscopy	Live cells	NBP2-54877 (kit)
PS externalization	Annexin V binding	Flow, Fluorescence Microscopy	Live cells	NBP2-29373 (kit)
	pSIVA binding	Flow, Fluorescence Microscopy (in real-time)	Live cells	NBP2-29611, NBP2-29382 (kits)
Caspase Activation	Processed caspase-3	WB (recognizes pro- and cleaved caspase forms)	Cell/tissue lysates	NB100-56112
	Processed caspase-7			NBP1-19230
	Processed caspase-8			NB100-56527
	Cleaved caspase-3	ICC	Fixed cells	MAB835
		Flow, IHC	Fixed cells, fixed tissues	NB100-56113
	Cleaved caspase-7	IHC	Fixed tissues	NB100-56115
	Caspase-2 activity assay	Func	Purified/enriched lysate	NBP2-54820 (kit)
	Caspase-2 inhibition	Flow	Live cells	KA0738 (kit)
	Caspase-8 activity assay	Func	Purified/enriched lysate	NBP2-54816 (kit)
	Caspase-8 inhibition	Flow	Live cells	KA0760 (kit)
	Caspase-9 activity assay	Func	Purified/enriched lysate	NBP2-54822 (kit)
	Caspase-9 inhibition	Flow	Live cells	KA0765 (kit)
	Caspase-10 activity assay	Func	Purified/enriched lysate	NBP2-54828 (kit)
Caspase Processing of Physiological Substrates	Cleaved PARP	WB (pro- and cleaved forms)	Cell/tissue lysates	NB100-56599
	Cleaved ICAD	WB (pro- and cleaved forms)	Cell/tissue lysates	NBP1-77034
	Cleaved gelsolin	WB (pro- and cleaved forms)	Cell/tissue lysates	NB110-10067
	Cleaved ROCK1	WB	Cell/tissue lysates	NB100-56596
		ICC	Fixed cells	
DNA Fragmentation	DNA laddering	DNA Gel Electrophoresis	Cell/tissue lysates	NBP2-54824 (kit)
	TUNEL	IHC/ICC	Fixed tissues/cells	NBP2-31164 (kit)
		Flow	Live cells	NBP2-31161 (kit)
Nuclear Condensation	Staining of condensed chromatin with Hoechst 33342	Flow, Microscopy	Live or fixed cells	NB5117

* Flow = Flow Cytometry, Func = Functional - fluorescence microplate reader, ICC = Immunocytochemistry, IHC - Immunohistochemistry, WB = Western blot

**For a comprehensive list of available reagents and kits, please visit Novus Biological's website

Various Forms of Cell Death: Apoptosis, Autophagy, and Necrosis

Historically, the processes of cell death including apoptosis, autophagy and necrosis have been studied and viewed as mutually exclusive events. However, these processes have also been shown to have overlapping mechanisms that can work sequentially or in parallel. Apoptosis and autophagy may be dually activated in response to cell stress, but autophagy more often acts as a cell survival mechanism. Necrotic cell death can be induced by inhibiting specific regulatory proteins of apoptosis or autophagy, while for some chemotherapeutic drugs, increasing the dosage redirects apoptotic signaling to necrosis.

Comparison of Apoptosis, Autophagy, and Necrosis

	Process of Cell Death	Apoptosis Type I	Autophagy Type II	Necrosis Type III
Cellular Events	General Induction	External and Internal stimuli such as death ligands, growth factor depletion and different cellular stresses	Nutrient starvation and other cellular stresses such as hypoxia and growth factor depletion	External stimuli such as injuries, infection, radiation, hypoxia, heat, toxins
	Energy Dependent	ATP-dependence (high ATP requirement)	ATP-dependence (ATP generation for survival)	Limited ATP dependence (low ATP requirement)
	Externalized PS	Yes	No	No
	BH3-only proteins	Bad, BID, Bik/Blk, BIM, Bmf, Hrk/DP5, Noxa, Puma	Beclin, EGL-1, BAD, BID, BNIP3, BIK NIX, NOXA, PUMA	*Likely for necroptosis*
	Key Enzymatic Activity*	Caspase cascade, Cathepsins, and Calpain	Lysosomal Proteases and Hydrolases	Calcium-dependent proteases including Cathepsins, and Calpain
	DNA fragmentation	Specific Digestion (DNA ladder on agarose gel)	None	Random Digestion (DNA smear on agarose gel)
	Inflammatory Response	No	No	Yes
Cell Morphology	Mitochondria	MOMP - Fragmented network, cristae remodeling	Damaged mitochondria degraded within autophagosomes	Swelling
	Chromatin Condensation (pyknosis)	Yes	No	No
	Outer Membrane	Membrane blebbing, no loss of integrity	Weak membrane blebbing, no loss of integrity	Loss of membrane integrity
	Final Stage	Apoptotic bodies	Self-Digestion	Total Cell Lysis

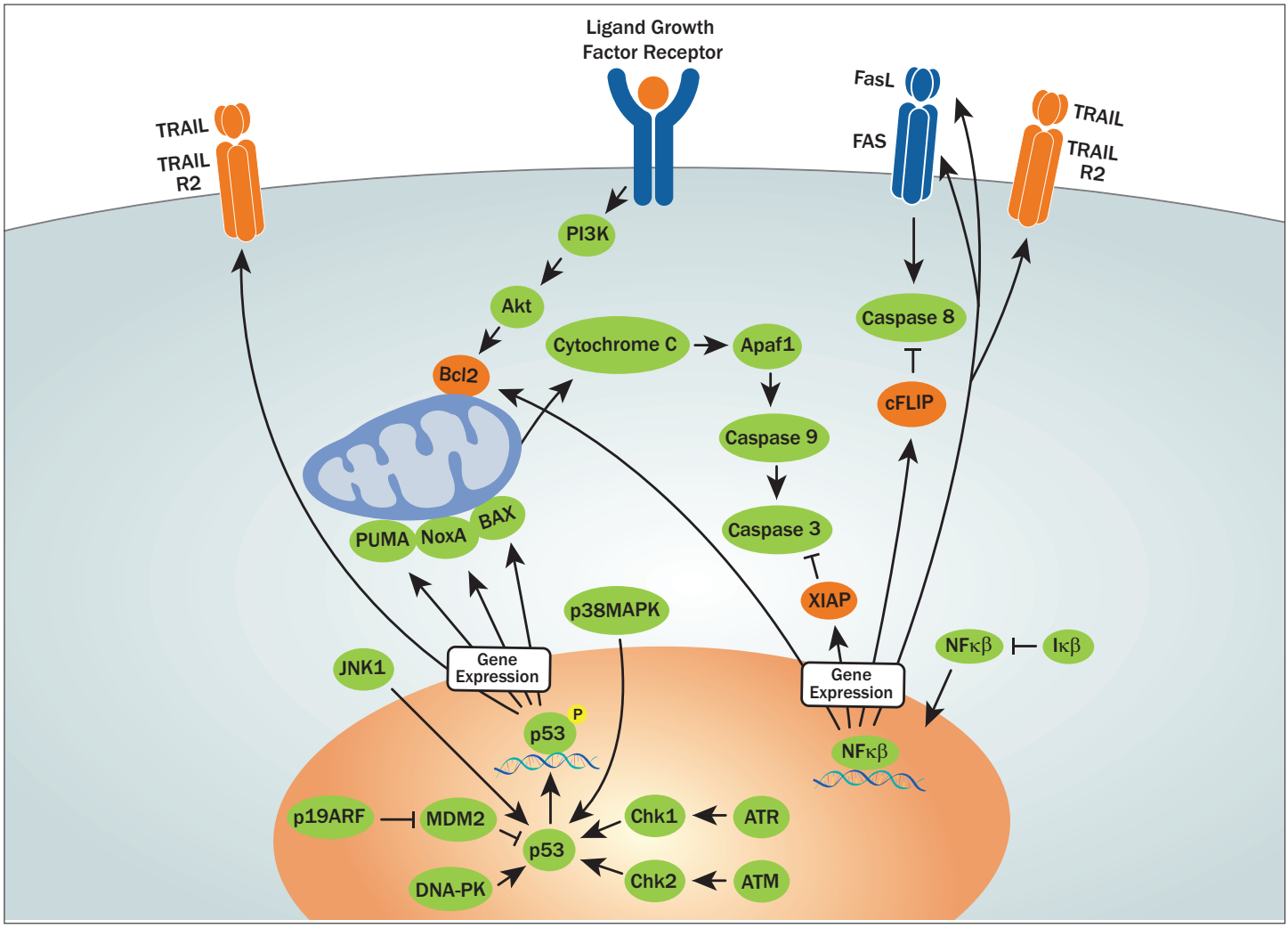
*Caspases may block the autophagic pathway

What is Necroptosis?

Necrosis was originally considered an unregulated form of cell death, a view that has been retired with the discovery of necroptosis, pyroptosis, and other forms of regulated necrosis. Similar to apoptosis, necroptosis can be induced through death receptors, but signaling is mediated by the activation of the receptor interacting protein kinase-3 (RIPK3) and its substrate, mixed lineage kinase like (MLKL).

Other Important Molecular Components of Apoptosis

One of the best-known tumor suppressor proteins, **p53**, is a multi-functional transcription factor that regulates cellular processes affecting proliferation, cell cycle checkpoints, senescence, and apoptosis. Defects in the p53 tumor suppressor gene or pathways signaling to p53 have been linked to more than 50% of human cancers. In response to genotoxic stress signals or DNA damage, p53 becomes phosphorylated which inhibits its interaction with the negative regulator, MDM2. p53 induces cell cycle arrest to enable repair of DNA damage or, for irreparable damage, triggers apoptosis.



Another crucial family of transcription factors, **Rel/NF-κB**, is also activated by apoptotic stimuli involved in DNA damage and mediates up-regulation of pro-apoptotic genes. NF-κB is predominantly found in the cytoplasm associated with inhibitory IkB proteins. When IκB is phosphorylated, NF-κB is released from the complex and translocates to the nucleus. In addition, NF-κB has a role in the cell survival response by inhibiting p53-dependent apoptosis, as well as up-regulating anti-apoptotic members of the Bcl-2 family and specific caspase inhibitors.

Besides NF-kB and p53, the STAT family of transcription factors along with Mad, Max, and c-Myc are involved in regulating proliferative and pro-apoptotic gene expression. Cell survival signals are also activated by growth factors along with other anti-apoptotic ligands that bind to cell surface receptors and activate intracellular kinases including PI3K, Akt/PKB, PKA, PKC, MEKs, ERK1/2, p90RSK, and p38 MAP kinases.

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Acronyms and Abbreviations

AIF	apoptosis inducing factor
APAF1	apoptotic protease activating factor 1
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and rad3 related
BCL-2	B-cell lymphoma 2
BAD	bcl-2-associated death promoter
BAK	bcl-2 antagonist killer 1
BAX	bcl-2 associated X protein
BIK	bcl-2 interacting killer
BIM	bcl-2-like protein 11
BIR	baculovirus IAP repeat
BNIP3	bcl-2 interacting protein 3
BOK	bcl-2 related ovarian killer
cFLIP	cellular FLICE-like inhibitory protein
CHK1/2	checkpoint kinase 1/2
CIAP	cellular inhibitor of apoptosis
DD	death domains
DED	death effector domains
DISC	death-inducing signaling complex
ERK1/2	extracellular signal–regulated kinases
FADD	fas-associated protein with death domain
FAS	apoptosis-stimulating fragment
FCCP	trifluorocarbonylcyanide phenylhydrazone
FFPE	formalin-fixed, paraffin-embedded
FITC	fluorescein isothiocyanate
IAP	inhibitor of apoptosis
ICAD	inhibitor of caspase-activated DNase
Iκβ	inhibitor of kappa beta
ILP-2	(inhibitor of apoptosis)-like protein 2
MCL-1	induced myeloid leukemia cell differentiation protein
MDM2	mouse double minute 2 homolog
MLKL	mixed lineage kinase like
MOMP	mitochondrial outer membrane permeability
MTCH2	mitochondrial carrier homolog 2
NAIP	neuronal apoptosis inhibitor protein
NF-κβ	nuclear factor kappa-light-chain-enhancer of activated b cells
P38MAPK	mitogen-activated protein kinase
P90RSK	(p90) ribosomal S6 kinase
PAK	p21-activated protein kinase
PARP-1	poly(ADP-ribose) polymerase-1
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
pSIVA-IANBD	polarity sensitive indicator of viability and apoptosis - N,N′-dimethyl-N-(iodoacetyl)-N′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediamine
RIPK3	receptor interacting protein kinase-3
ROCK-1	rho-associated protein kinase 1
SMAC	second mitochondria-derived activator of caspase
STAT	signal transducer and activator of transcription
tBID	truncated BH3-interacting domain death agonist
TNF-α	tumor necrosis factor alpha
TRAF-2	(tumor necrosis factor) receptor-associated factor 2
TRADD	(tumor necrosis factor) receptor type 1 -associated death domain
TRAIL	(tumor necrosis factor)-related apoptosis-inducing ligand
TWEAK	(tumor necrosis factor) related weak inducer of apoptosis
XIAP	X-linked inhibitor of apoptosis

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