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Tools & Tips for Analyzing Apoptosis: A Kit Selection Guide

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Apoptosis: The Beginning of the End

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Over the past three decades, the study of apoptosis (programmed cell death) has gained significant importance in human disease and its clinical management. Failure to regulate apoptosis is a common feature in several diseases including autoimmune disorders, neurodegenerative diseases, cancer, and AIDS. Hence, it is important to understand the apoptotic processes in cells in order to devise therapeutic means to intervene and reset the balance between cell survival and death. It is now well recognized that there are two main pathways for the induction of apoptosis, the extrinsic or receptor-mediated pathway, and the intrinsic or mitochondrial pathway. Induction of either pathway can result in the activation of caspases, a class of intracellular cysteine proteases that are responsible for the cleavage of a variety of cellular substrates and the morphological changes attributed to apoptosis. Other less well-defined caspase activation pathways, such as autophagy, have recently been described, but will not be discussed here.

A very large beneficiary of apoptosis research is oncology, since most cancer cells exhibit defects in their suicidal machinery. By better understanding caspase activation pathways, new therapeutic agents may be developed to induce death in cancerous cells. On the other hand, pharmacologic interference with the induction or completion of apoptosis holds promise for the treatment of several neurodegenerative disorders.

The extrinsic pathway is activated by the interaction of a specific death ligand with its cell surface death receptors (DR), which are members of the tumor-necrosis factor (TNF) superfamily. This pathway plays an important role in the regulation of apoptosis in cells involved in the immune system. Fas, TNFα, or TNF-related apoptosis-inducing ligand (TRAIL) interact with their cognate receptor to induce a conformational change. For example, following FasL binding to its receptor, an intracellular death-inducing signaling complex (DISC) is formed via the stepwise recruitment of cytosolic proteins, such as procaspase-8 and the Fas-associated death domain protein (FADD). FADD is an adapter protein that acts as a bridge to link the death receptor to death effector domains (DED) of caspases-8 and 10. Formation of the DISC leads to the dimerization and activation of caspase-8, which in turn can activate caspase-3 and other downstream events. The extrinsic pathway can also crosstalk with the intrinsic pathway via caspase-8 mediated cleavage of Bid, which can trigger the release of proapoptotic mitochondrial proteins.

The intrinsic pathway is the most common pathway for cell death in vertebrates and can be activated by a variety of stimuli, including growth factor withdrawal, heat shock, oncogene activation, DNA-damaging agents, reactive oxygen species, excessive cytosolic calcium, and other cellular stresses. These agents cause the permeabilization of the mitochondrial outer membrane (MOMP) and release of cytochrome *c* and other proteins. The permeabilization of MOMP can occur as a result of either a change in the mitochondrial permeability transititon pore (PTP) or by the action of pro-apoptotic members of the Bcl-2 family of proteins. The PTP complex is composed of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenosine nuclear transporter (ANT) channel in the inner mitochondrial membrane, and the soluble matrix protein cyclophilin D. Opening of the PTP, triggered by higher levels of cytosolic calcium, allows water and solutes of 1.5 kDa to freely diffuse from the cytosol to the mitochondrial matrix leading to mitochondrial swelling and collapse in the transmembrane potential. The second mechanism of MOMP permeabilization involves pro-apoptotic members of the Bcl-2 family, whereby Bax and Bak oligomerize and insert into the outer mitochondrial membrane. BH3-only proteins, such as Bim and Bid contribute to the oligomerization of these proteins. In contrast, the Bcl-2 antiapoptotic members Bcl-2, and Bcl-X_L inhibit protein release. While significant progress has been made in our understanding of the regulation and interaction of Bcl-2 member proteins, the exact details remain to be elucidated.

Mitochondrial outer membrane permeabilization is the key event leading to caspase activation in the intrinsic pathway. Cytochrome *c* released into the cytosol from the intermembrane space binds to the apoptosis protease-activating factor (Apaf-1), which then oligomerizes in the presence of ATP. Pro-caspase-9 molecules can then bind to each of the Apaf-1 monomers via the caspase recruitment domain (CARD) forming a caspase-activating complex, the apoptosome. Active caspase-9 participates in activation of downstream caspases-3 and -7.

The extrinsic and intrinsic pathways for caspase activation converge on downstream effector caspases, which ultimately results in apoptotic cell death. Since caspases play a central role in regulation and execution of cell death, they must be tightly regulated. Regulation can occur by either inhibiting caspase activity, or by blocking its activation. The IAP family of proteins acts to inhibit caspase activity. Members of this family include XIAP, cIAP1, cIAP2, hILP-2, ML-IAP, NAIP, survivin, and apollon. A key domain present in all members is a baculovirus IAP repeat (BIR), a 65-residue domain rich in histidine and cysteine residues, which acts in concert with the flexible region preceeding the BIR domain to inhibit the activity of caspases. Also, present in some IAPs is a RING domain located at the carboxy terminus, which functions as a E3 ubiquitin ligase to provide specificity of transfer of ubiquitin moieties to the target protein.

The activity of IAPs can be regulated by the mitochondrial protein Smac/DIABLO, normally localized to the mitochondria. Upon its release from the mitochondria, Smac/DIABLO acts as an IAP antagonist to inhibit XIAP, thereby acting as a proapoptotic molecule. Omi/HtrA2 is another mitochondrial protein, that when released into the cytosol, can inhibit IAPs.

Caspases can also be regulated by blocking their activation. For example, the FLIP protein, a caspase-8 homolog lacking proteolytic activity, can block caspase-8 activation. FLIP possess DEDs at their N-termini and can be recruited to the DISC, and under conditions of overexpression, can prevent caspase-8 activation. However, when FLIP is present at lower concentrations in the DISC, it can aid in the cleavage of procaspase-8.

Cellular apoptosis is manifested by a number of distinctive biochemical and morphological changes to give the apoptotic phenotype. These changes provide measurable markers to indicate that apoptosis has occurred. At this point it is prudent to discuss the difference between apoptosis and necrosis. While necrosis and apoptosis can be distinguished in some situations, it is not so obvious in others because dying cells exhibit features of both apoptosis and necrosis. Since there are no clear biological markers to distinguish apoptosis from necrosis, morphological changes remain the most reliable method for differentiation. The following Table compares morphological features of apoptosis with necrosis.

Type of cell death:	Apoptosis	Necrosis
Feature		
Plasma Membrane	• Phosphatidylserine translocates to surface • Blebbed	• Ruptured, early lysis without formation of vesicles
Mitochondria	• Increased membrane permeability • Release of cytochrome *c* into cytosol	• Swelling
Cell Degradation	• Phagocytosis • No inflammation	• Release of proinflammatory molecules • Inflammation
Cell Shape	• Formation of apoptotic bodies	• Disrupted and swollen
DNA Fragmentation	• Internucleosomal DNA cleavage; free 3'-OH ends	• Diffuse and random
Cytoplasm	• Presence of apoptotic bodies	• Vacuolation of cytoplasm

Necrosis is characterized as a pathological or accidental cell death wherein the cell is rendered energetically incapable of surviving due to ATP depletion. On the other hand, apoptosis is a programmed form of cell death, which requires ATP.

A number of experimental methods and techniques are available to study cell death that take advantage of the morphological and biochemical changes during apoptosis. This brochure is designed to provide an overview of the techniques available to study apoptosis. Due to the often complex machinery of cell death, it is advised to always use more than two (separate) techniques to validate apoptosis in the experimental system. This brochure will also act as a guide and provide researchers with the tools and tips to measure apoptosis-induced changes that occur at the plasma membrane, mitochondria, activation of caspases in the cytoplasm, and DNA fragmentation in the nucleus.

References

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Cover Photo: The Small Milkweed Bug (*Lygaeus kalmii*) is a true bug in the order Hemiptera, and is related to the predatory assassin bugs. The Small Milkweed bug is found across much of the United States and Canada. Adults feed on nectar and milkweed seeds, but may also be predatory in the spring before the milkweeds set seed. More information may be found at http://bugguide.net/node/view/460. Photo credit: Scot Mitchell.

Induction of Apoptosis

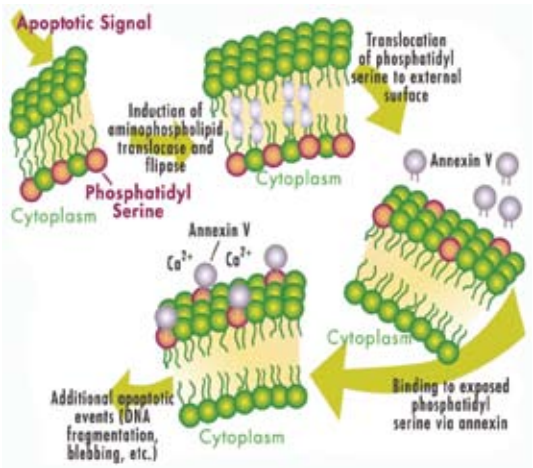
Although apoptosis is a normal cellular process, its study requires a more controlled experimental process. This can be accomplished by using a variety of agents that force cells to undergo apoptosis either by stimulating cell surface receptors or activation of the mitochondrial apoptotic pathway. The following table provides examples of more commonly used agents for inducing apoptosis. An extensive list is provided in the Appendix. (See Table III, page 25.)

Product	Cat. No.	Optimum Dose for Apoptosis Detection	Recommended Solvent for Preparing Stock Solution
Actinomycin D	114666	500 ng/ml	Methanol
Aphidicolin	178273	2 µg/ml	DMSO
A23187	100105	10 µg	DMSO
Caffeine	205548	16 mM	Boiling H_20
Camptothecin	208925	4 µg/ml	DMSO
Cycloheximide	239764	100 µg/ml	H_20
Dexamethasone	265005	1 µM	Ethanol
Doxorubicin	324380	0.2 µg/ml	H_20
5-Flurouracil	343922	25 µg/ml	DMSO, Hot H_20
Hydroxyurea	400046	500 nM	H_20
Paclitaxel (TAXOL)	580555	100-580 nM	DMSO
Staurosporine	569397	500 nM	DMSO
Thymidine	6060	2 nM	PBS
Vinblastine	677175	60 nM	Methanol

I. Measurement of Apoptosis-Induced Changes at the Plasma Membrane

In normal viable cells, phosphatidyl serine (PS) is located on the cytoplasmic side of the cell membrane. Upon induction of apoptosis, rapid alterations in the organization of phospholipids occur, leading to translocation of PS to the outer leaflet of the plasma membrane. PS translocation to the cell surface is one of the earliest events in apoptosis and precedes nuclear breakdown, DNA fragmentation, and membrane blebbing. Recognition of PS by phagocytes *in vivo* results in the removal of apoptotic cells, thus apoptosis is not associated with the local inflammatory response which generally accompanies necrosis. Annexin V binding to PS can be used as a marker of early-stage apoptosis. *In vitro* detection of externalized PS can be achieved through interaction with Annexin V, a Ca^{2+}-dependent protein. In the presence of calcium, rapid high-affinity binding of Annexin V to PS occurs. Annexin V is typically conjugated to a fluorochrome for easy indentification of apoptotic cells by flow cytometry or immunofluorescence microscopy.

Annexin/ Phosphatidyl Serine in Early Stages of Apoptosis



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Measurement of Changes at the Plasma Membrane: Annexin V Kits

Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Number of Tests/Samples	Principle of Assay/ Notes	Price
NEW Annexin V-Biotin*	PF036	IF, FC	Intact Cells	Yes	100 Tests	Useful for detection of membrane changes during early apoptosis. Also useful in conjunction with GFP or other fluorophores.	€399
Annexin V-Biotin Apoptosis Detection Kit II	CBA058	IF, FC	Intact Cells	No	20 Tests	Useful for detection of membrane changes during early apoptosis. Also useful in conjunction with GFP or other fluorophores.	€176
Annexin V-FITC *	PF032	IF, FC	Intact Cells	Yes	100 Tests	Useful for detection of membrane changes during early apoptosis.	€386
Annexin V-FITC Apoptosis Detection Kit II	CBA059	IF, FC	Intact Cells	Yes	20 Tests	A convenient kit for the identification of changes in the plasma membrane that occur during apoptosis.	€176
NEW Annexin V-PE Apoptosis Detection Kit	CBA060	IF, FC	Intact Cells	No	25 Tests 100 Tests	Useful for detection of membrane changes during apoptosis.	€176 €299

Note: **FC**: flow cytometry; **IF**: immunofluorescence
*Sold under license of U. S. Patent 5,834,196.

Measurement of Changes at the Plasma Membrane: Fas and Fas-ligand Kits

Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Number of Tests/Samples	Principle of Assay/ Notes	Price
Fas Ligand ELISA Kit	QIA27	ELISA	Cell Lysates Serum	Yes	96 Tests	Suitable for the *in vitro* quantitation of human FasL. Recognizes both membrane bound and soluble FasL.	€737
Fas/APO-ELISA Kit	QIA24	ELISA	Cell Lysates Serum	Yes	96 Tests	Rapid, precise assay for the *in vitro* quantitation of human Fas/APO-1/CD95 protein. Measures both membrane bound and soluble Fas protein.	€737

Measurement of Plasma Membrane Integrity Using Vital Dyes

Under normal conditions vital dyes are not capable of crossing the plasma membrane of intact non-apoptotic cells. When the membrane properties are compromised, during apoptosis or necrosis, these dyes cross the cell membrane and bind cellular components. This principle can be used as a simple way of assessing the integrity of the plasma membrane.

Useful Tools for Measuring Changes at the Plasma Membrane

Product Name	Cat. No.	Sample Type	Principle of Assay/ Notes	Size	Price
Actinomycin D, 7-Amino-	129935	Intact Cells	Useful in distinguishing early apoptotic cells (7-AAD negative), from late apoptotic/ dead cells (7-AAD positive), which have lost membrane integrity. Useful as a viability marker when using Annexin V-PE.	1 mg	€133
Propidium Iodide	537059	Intact Cells	Can be used to distinguish between early apoptotic cells (PI-negative) and late apoptotic/dead cells (PI-positive). Useful as a viability marker when using Annexin V-FITC.	50 mg 100 mg 250 mg 1 g	€66 €117 €282 €953
Propidium Iodide Solution	537060	Intact Cells	Can be used to distinguish between early apoptotic cells (PI-negative) and late apoptotic/dead cells (PI-positive). Useful as a viability marker when using Annexin V-FITC.	5 ml	€81
Live/Dead Double Staining Kit	QIA76	Intact Cells	This kit uses a cell-permeable green fluorescent Cyto-dye to stain live cells and propidium iodide to stain dead cells. Viable cells will stain only with the Cyto-dye, fluorescing green, whereas the dead cells will stain with both Cyto-dye (green) and propidium iodide (red), resulting in a yellow fluorescence. This kit can be used for immunofluorescence and flow cytometry.	100 Tests	€325

Technical Tips and Frequently Asked Questions

- **Why are all of my cells staining positive for Annexin V and PI?**
  Most cells in your prepartion are dead. Keep in mind that when cells are necrotic/dead, they will stain non-specifically for Annexin V: FITC. Perform a time-course experiment consisting of untreated versus treated cells (camptothecin).

- **My cells no longer stain positive for Annexin V, or the percentage of cells staining positive has dropped dramatically.**
  Culturing certain cell lines for more than 20 passages eventually selects for cells that are resistant to apoptotic cell death. Thaw out a new vial of cells for use in your experiments.

- **Are all apoptotic signaling pathways responsible for PS exposure activated by active caspases?**
  No, cathepsin B can induce PS exposure in a caspase-independent manner.

- **I am using adherent cells; can I still use this technique?**
  Yes, but there may be a higher background than with suspension cells.

- **Cells stain positive for Annexin V, but do not stain positive with trypan blue or another vital dye.**
  Cells in early stages of apoptosis have an intact membrane and dyes such as trypan blue cannot cross the membrane, so cells remain negative for trypan blue staining.

- **I permeabilized my cells and everything is staining positive.**
  Annexin V staining is not suitable for examining cells when using any technique that disrupts the membrane (e.g., fixation or permeabilization).

- **I don't have a flow cytometer; can I still perform this assay?**
  Yes, this technique works well for immunofluorescence microscopy.

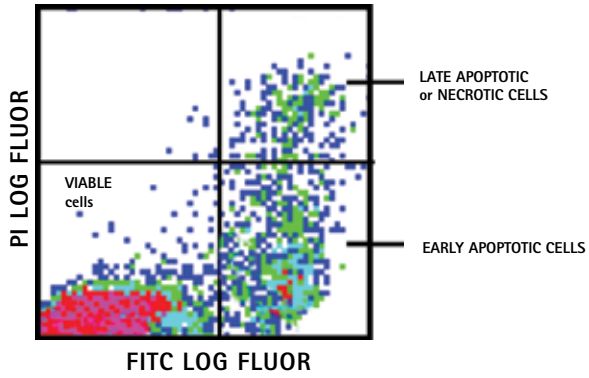
- **I had EDTA in my sample buffer and I did not get any positive staining. Why?**
  The binding of Annexin V to PS is calcium-dependent and removal of calcium by a chelating agent like EDTA will prevent Annexin V from binding to PS.

Annexin V-FITC Apoptosis Detection Kit
Cat. No. PF032

Viable cells do not bind Annexin V-FITC or Propidium Iodide (PI) as reflected in the lower left-hand quadrant of the dot plot. Early apoptotic cells with exposed PS but intact cell membranes bind Annexin V-FITC, but exclude PI. Fluorescence from this population is reported in the lower right-hand quadrant. Necrotic or apoptotic cells in terminal stages will be both Annexin V-FITC and PI positive and are reported in the upper right-hand quadrant. Note that a small percentage of normal cell death should be expected in routine cultures of untreated cells.

(see ordering information on page 4)

A sample dotplot



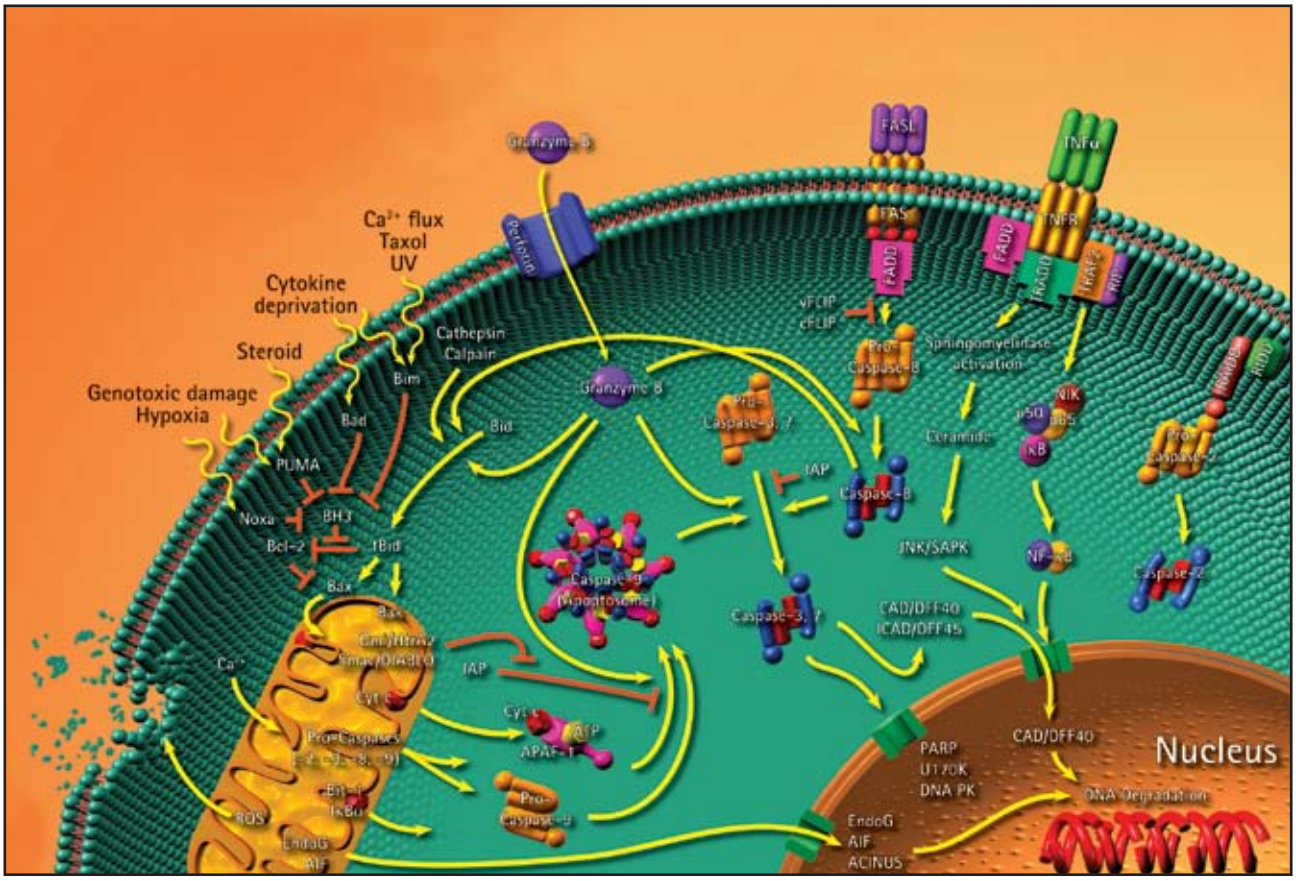
II. Measurement of Apoptosis-Induced Changes in the Mitochandria

One of the earliest measurable changes in apoptosis occurs when the outer membrane of the mitochondria becomes permeable and proteins located in the intermembrane space are released into the cytosol. These proteins include cytochrome *c*, Smac/DIABLO, HtrA2/OMI, Endonuclease-G (EndoG), and Apoptosis-inducing Factor (AIF). The loss of cytochrome *c* is accompanied by a loss in mitochondrial transmembrane potential ($\Delta\Psi_m$), disruption of the electron transport change, and release of Ca^{2+}. The regulation and implementation of mitochondrial outer membrane permeabilization (MOMP) involves a number of proteins, particularly those in the Bcl-2 family. The pro-apoptotic Bcl-2 family members, Bax and Bak, assist in MOMP, resulting in the eventual activation of procaspase-9. In contrast, the antiapoptotic proteins Bcl-2/Bcl-X_L help to suppress apoptosis by interacting with or preventing the pro-apoptotic proteins from carrying out their anti-apoptotic function. The exact mechanism by which MOMP occurs remains under active investigation.

Reference

Green, D.R. et al. 2004. *Science* **305**, 626.

Mitochondrial Pathway for Apoptosis



Measurement of Changes in the Mitochondria: Kits

Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Number of Tests/Samples	Principle of Assay/Notes	Price
Bcl-2 ELISA Kit	QIA23	ELISA	Cell Extracts	Yes	96 Tests	A quantitative colorimetric assay for the measurement of Bcl-2. Able to detect significant decreases in Bcl-2 protein levels before significant levels of apoptosis are detected.	€728
Cytochrome *c* ELISA Kit	QIA74	ELISA	Cell Extracts	Yes	96 Tests	Suitable for the *in vitro* quantitation of cytochrome *c*. Sensitivity: 0.3 ng/ml; Assay Range: 0.625 - 20 ng/ml	€743
Cytochrome *c* Release Apoptosis Assay Kit	QIA87	IB	Cell Extracts	No	100 Tests	Assay kit provides reagents for the isolation of a highly enriched mitochondrial fraction from the cytosol. Translocation of cytochrome *c* from the mitochondrial fraction to the cytosol is monitored by immunoblotting with the cytochrome *c* antibody provided with the kit.	€641
Cytosol/Mitochondria Fractionation Kit	QIA88	Extraction/ Fractionation	Intact Cells	No	1 Kit 100 Extractions	This kit provides reagents useful for the isolation of a highly enriched mitochondrial fraction from the cytosol. The enriched fractions can be used to study factors of interest using Western blotting, ELISA, or other assays.	€386
Mitocapture™ Apoptosis Detection Kit	475866	IF, FC	Intact Cells	No	100 Tests	This kit provides a simple, fluorescent-based method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial membrane potential.	€507
NEW InnoCyte™ Flow Cytometric Cytochrome *c* Release Kit	CBA077	IF, FC	Intact Cells	No	50 Tests	This kit provides a rapid method for inhibitor screening and assessing the regulation of apoptotic signaling in cells.	€266

Note: **ELISA**: enzyme linked immunosorbent assay; **FC**: flow cytometry; **IB**: immunoblotting; **IC**: immunocytochemistry; **IF**: immunofluorescence

Measurement of Changes in the Mitochondria: Related Kits

Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Number of Tests/Samples	Principle of Assay/Notes	Price
Glutathione Apoptosis Detection Kit	QIA89	Fluorometric	Cell Extracts	No	100 Tests	Diminished levels of glutathione (GSH) occur during early apoptosis. This assay detects *in vitro* changes in glutathione during apoptosis using monochlorobimane (MCB), a dye that fluoresces blue when bound to GSH.	€538
Hsp27 ELISA Kit	QIA119	ELISA	Intact Cells, Cell Extracts	Yes	96 Tests	A quantitative colorimetric assay for the measurement of Hsp27. Assay range 0.78-50 ng/ml.	€704
Cu/Zn Superoxide Dismutase ELISA	QIA97	ELISA	Intact Cells	No	96 Tests	A colorimetric sandwich ELISA method for detecting Cu/Zn superoxide dismutase (SOD) in different types of biological samples. Sensitivity: 70 pg/ml; Assay Range: 0.08–5 ng/ml.	€731

Measurement of Changes in the Mitochondria: Related Products

Product Name	Cat. No.	Application	Species Reactivity	Size	Price
NEW Anti-ANT Mouse mAb (5F51BB5AG7)	AP1034	IB, IC, IP	bovine, human, rat	50 µg	€294
NEW Anti-Cytochrome *c* Mouse mAb (7H8.2C12)	AP1029	IB, IC	horse, human, mouse, rat	50 µg	€183
NEW Anti-Cytochrome *c* Mouse mAb (6H2.B4)	AP1030	FC, IC, IP	human, mouse, rat	50 µg	€183
Anti-Cytochrome *c* (Ab-1) Sheep pAb	PC323	IB, IF, IP	canine, human, rabbit, rat	50 µg 100 µg	€231 €338
Cytochrome *c*, Equine Heart	250600	positive control microchondrial marker		100 mg 1 g	€60 €338
NEW Anti-Cyclophilin D Mouse mAb (E11AE12BD4)	AP1035	IB, IC	bovine, human, rat	50 µg	€294
NEW Anti-F_1F_0 α Mouse mAb (7H10BD4F9)	AP1036	IB, IC	bovine, human, mouse, rat	50 µg	€294
NEW Anti-F_1F_0-β Mouse mAb (3D5AB1)	AP1037	IB, IC, IP	bovine, human, mouse, rat	50 µg	€294

Note: **FC**: flow cytometry; **IB**: immunoblotting; **IC**: immunocytochemistry; **IF**: immunofluorescence; **IP**: immunoprecipitation

Measurement of Changes in the Mitochondria: Inhibitors/Modulators

Product Name	Cat. No.	Principle of Assay/Notes	Size	Price
Atractyloside, Dipotassium Salt	189300	Causes the release of cytochrome *c* from mitochondria. Acts as an ADP/ATP translocase inhibitor.	50 mg	€118
Bongkrekic Acid, Triammonium Salt	203671	Acts as a ligand of the adenine nucleotide translocator. A potent inhibitor of mitochondrial megachannel (permeability transition pore). Significantly reduces signs of apoptosis induced by nitric oxide. Prevents the apoptotic breakdown of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$), as well as a number of other phenomena linked to apoptosis.	500 µg	€348
Carbonyl Cyanide *m*-Chlorophenylhydrazone	215911	Protonophore. Uncoupling agent for oxidative phosphorylation that inhibits mitochondrial function. Approximately 100 times more effective than 2,4-dinitrophenol. Binds with cytochrome *c* oxidase with high affinity (K_d = 270 nM). Inhibits transport processes and depresses growth.	250 mg	€85
Carboxyatractyloside	216200	A highly selective inhibitor of the cytosolic site-specific mitochondrial ADP/ATP carrier (AAC; K_i <10 nM).	5 mg	€200
Mitochondrial Permeability Transition Pore Reagents Set	475876	Set containing four Mitochondrial Permeability Transition Pore Reagents	1 set	€465
Oligomycin	495455	A mixture of A, B, and *C* isomers. A macrolide antibiotic that inhibits membrane-bound mitochondrial ATPase (F_1), preventing phosphoryl group transfer. An inhibitor of predominantly F_1-type ATPases (IC_{50} = 50 µM). Induces apoptosis in cultured human lymphoblastoid and other mammalian cells.	10 mg	€85
Rotenone	557368	A mitochondrial toxin and a potent, reversible, and competitive inhibitor of complex I (NADH-CoQ reductase) of the respiratory chain.	1 g	€91
Ru360	557440	A cell-permeable oxygen-bridged dinuclear ruthenium amine complex that has been shown to bind to mitochondria with high affinity (K_d = 340 pM). Specifically blocks Ca^{2+} uptake into mitochondria *in vitro* (IC_{50} = 184 pM).	500 µg 1 mg 1 set	€170 €290 €390
Smac-N7-Peptide	567370	A peptide that contains the N-terminal seven residues of Smac (Second Mitochondria-derived Activator of Caspases, also known as DIABLO) and promotes procaspase-3 activation at around 10 µM.	1 mg 5 mg	€81 €322
Smac-N7-Peptide, Cell Permeable	567375	Cell-permeable version of Cat. No. 567370.	1 mg	€275
Valinomycin, *Streptomyces fulvissimus*	676377	A cyclododecadepsi-peptide ionophore antibiotic. Potassium ionophore of the mobile ion-carrier type that transports alkali metal ions across artificial or biological lipid membranes. Uncouples oxidative phosphorylation by binding to sites on membranes rich in sulfhydryl groups. Induces apoptosis in murine thymocytes. Also reported to inhibit NGF-induced neuronal differentiation.	25 mg 100 mg	€79 €267

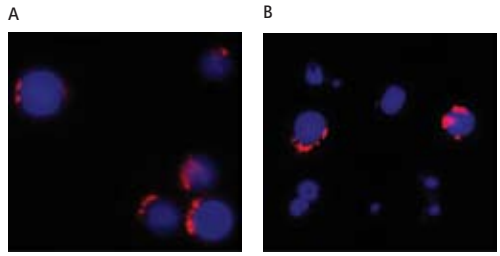
Use of Dyes to Measure Changes in the Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Detection of changes in $\Delta\Psi_m$ can be performed using cationic dyes that accumulate in the mitochondria. For example, the cationic dye JC-1 can be used to detect changes in the membrane potential associated with the mitochondrial permeability transition.

Measurement of Changes in the Mitochondria: Dyes and Stains

Product Name	Cat. No.	Principle of Assay/Notes	Size	Price
3,3´-Dihexyloxacarbocyanine Iodide	305110	A cationic, cell-permeable, voltage-sensitive, lipophilic, and fluorescent carbocyanine dye that is used as a membrane potential $\Delta\Psi_m$ probe. Selectively stains mitochondria and the endoplasmic reticulum.	50 mg	€87
3,3´-Diethyloxadicarbocyanine Iodide	295600	A cationic, cell-permeable, voltage-sensitive, lipophilic, and fluorescent carbocyanine dye that recognizes hairpin quadruplex structures *in vivo* and *in vitro*. Also useful as a telomerase inhibitor and as an antitumor agent. Specifically stains mitochondria in living cells. Useful for the determination of mitochondrial content, localization, and oxidative capacity.	50 mg	€78
Rhodamine 123	555505	Membrane-permeable fluorescent dye for selectively staining mitochondria in living cells. Widely used for assessing mitochondrial membrane potential. Can be used to measure the efflux activity of P-glycoprotein in drug-resistant phenotypes in cancer cells.	50 mg	€108
JC-1	420200	A cationic carbocyanine that can be used as a ratiometric indicator of mitochondrial transmembrane potential ($\Delta\Psi_m$)	5 mg	€319

Anti-Cytochrome *c* Mouse mAb (6H2.B4) Cat. No. AP1030



Jurkat cells untreated (A) and treated (B) with Actinomycin D (Cat. No. 114666) at 1 µM for 7 h. Cells were fixed with 4% paraformaldehyde. Primary antibody was Anti-cytochrome c (Cat. No. AP1030) used at 1 µg/µl; secondary antibody was Goat anti-mouse IgG conjugated to Alexa Fluor® 546. Blue staining in the nucleus is DAPI (Cat. No. 268298) at 1 mg/ml. Cytochrome c (red) is localized to mitochondria but is released in dying (apoptotic) cells.

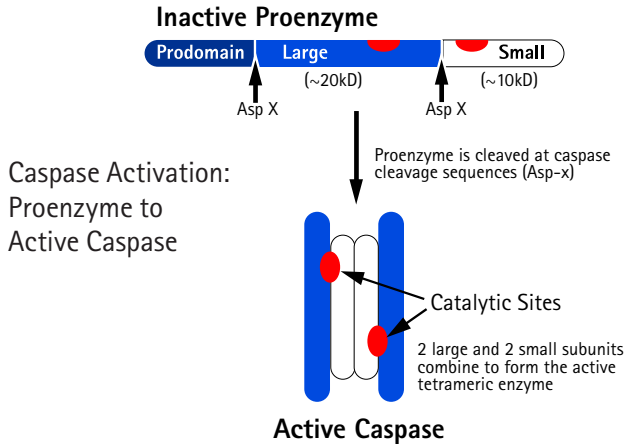
(see ordering information on page 7)

III. Measurement of Apoptosis-Induced Changes in the Cytoplasm

Activation of caspases is one of the most widely recognized features of apoptosis. Caspases are cysteine-dependent, aspartate-specific proteases. They exist as latent precursors in the cytoplasm which, upon activation, destroy key components of the cellular infrastructure. Thus far fourteen members of the caspase family have been identified, eleven of these are present in humans. Caspases can be subdivided into three groups. Upstream initiators include caspase-2, -8, -9, and –10. Downstream executioners include caspase-3, -6, and –7. A third group includes members involved in the inflammatory process are caspase-1, -4, -5, and –12. A distinctive feature of caspases is the absolute requirement of an aspartic acid residue in the substrate P1 position. The P4 residue is important in substrate recognition and specificity. Generally, catalysis involves a cysteine protease mechanism. Measurement of caspase activity is based on a tetrapeptide corresponding to substrate P4-P1 residues coupled either to a colorimetric (*p*NA) or fluorogenic (AFC, AMC) compound. Upon caspase-mediated peptide cleavage, the free colorimetric or fluorogenic group is released and can be measured by spectrophotometric or fluorometric methods. This procedure is useful for measurement of caspase activity in cell lysates.

Reference

S. J. Riedel and Y. Shi. 2004. *Nat. Rev. Molecular Cell Biol.* **5**,897.



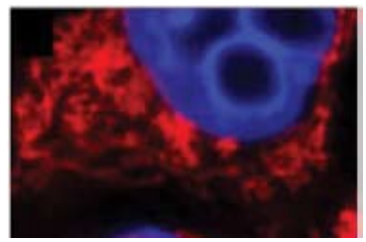
Measurement of Changes in the Cytoplasm: Fluorescent-Based Detection Kits

Kit Name	Cat. No.	Detection Method	Sample Type	Substrate Provided	Positive Control Provided	Inhibitor Provided *	Number of Tests/ Samples	Principle of Assay/Notes	Price
Caspase-3 Intracellular Activity Assay Kit I	235430	IF, FC	Intact Cells	(PhiPhiLux® G_1D_2)-Green	No	No	30 Tests	Detects intracellular caspase-3 activity using a specific caspase-3 substrate in living cells.	€610
Caspase-3 Intracellular Activity Assay Kit II	235432	IF, FC	Intact Cells	(PhiPhiLux® G_2D_2)-Red	No	No	30 Tests	Detects intracellular caspase-3 activity using a specific caspase-3 substrate in living cells.	€610

Note: **FC**: flow cytometry; **IF**: immunofluorescence

* Inhibitors available separately. For a complete list of inhibitors, see Table IV in Appendix (page 28).

Anti-Cyclophilin D Mouse mAb (E11AE12BD4) Cat. No. AP1035



Cultured fibroblasts were stained with Anti-Cyclophilin D Mouse mAb (E11AE12BD4) Cat. No. AP1035, at 5 µg/ml.
Detection by immunofluorescence.

(see ordering information on page 7)

Measurement of Changes in the Cytoplasm: Substrate-Based Caspase Detection Kits

Kit Name	Cat. No.	Detection Method	Sample Type	Substrate Provided	Positive Control Provided	Inhibitor Provided	Number of Tests/ Samples	Principles of Assay/Notes	Size	Price
Caspase-1 Assay Kit, Colorimetric	218790	Colorimetric	Cell Extracts	YVAD-*p*NA	No	No	100 Tests	Detects caspase-1 activity in cell lysates by cleavage of a caspase-1 specific substrate. *p*NA can be measured using a spectrophotometer or a 96-well plate reader at ~405 nm.	1 Kit	€581
Caspase-1 Assay Kit, Fluorometric	218791	Fluorometric	Cell Extracts	YVAD-AFC	No	No	100 Tests	Detects caspase-1 activity in cell lysates by cleavage of a caspase-1 specific substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€581
Caspase-1 Inhibitor Screening Assay Kit	218734	Colorimetric	Cell Extracts	YVAD-*p*NA	Yes	Yes	96 Tests	Detects caspase-1 activity in cell lysates by cleavage of a caspase-1 specific substrate. *p*NA can be measured using a spectrophotometer or a 96-well plate reader at ~405 nm.	1 Kit	€622
Caspase-2 Assay Kit, Colorimetric	218792	Colorimetric	Cell Extracts	VDVAD-*p*NA	No	No	100 Tests	Detects caspase-2 activity in cell lysates by cleavage of a caspase-2 substrate. *p*NA can be measured using a spectrophotometer or a 96-well plate reader at ~405 nm.	1 Kit	€653
Caspase-2 Assay Kit, Fluorometric	218793	Fluorometric	Cell Extracts	VDVAD-AFC	No	No	96 Tests	Detects caspase-2 activity in cell lysates by cleavage of a caspase-2 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€581
Caspase-3 Inhibitor Screening Assay Kit	235418	Colorimetric or Fluorometric	Cell Extracts	DEVD-*p*NA DEVD-AMC	Yes	Yes	96 Tests	Detects caspase-3 activity in cell lysates by cleavage of a caspase-3 substrate.	1 Kit	€616
Caspase-3 Cellular Activity Assay Kit	235419	Colorimetric or Fluorometric	Cell Extracts	DEVD-*p*NA DEVD-AMC	Yes	Yes	96 Tests	Detects caspase-3 activity in cell lysates by cleavage of a caspase-3 substrate. Cell lysis buffer provided.	1 Kit	€468
Caspase-3 Activity Assay	QIA70	Fluorometric	Cell Extracts	DEVD-AFC	Yes	Yes	96 Tests	Detects caspase-3 activity in cell lysates by cleavage of a caspase-3 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€514
Caspase-3 Activity Assay for HTS	HTS02	Fluorometric	Cell Extracts	DEVD-AFC	Yes	Yes	100 Tests 500 Tests	Detects caspase-3 activity in cell lysates by cleavage of a caspase-3 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	100 T 500 T	€514 €1950
Caspase-3 Immunoassay/ Activity Kit	QIA107	Fluorometric	Cell Extracts	DEVD-AFC	Yes	No	96 Tests	Immunocapture of active caspase-3 followed by detection of caspase-3 activity in cell lysates by cleavage of a caspase-3 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€803
Caspase-5 Assay Kit, Colorimetric	218804	Colorimetric	Cell Extracts	WEHD-*p*NA	No	No	100 Tests	Detects caspase-5 activity in cell lysates by cleavage of a caspase-5 substrate. *p*NA can be measured using a spectrophotometer or a 96-well plate reader at ~405 nm.	1 Kkit	€653
Caspase-6 Assay Kit, Colorimetric	218802	Colorimetric	Cell Extracts	VEID-*p*NA	No	No	100 Tests	Detects caspase-6 activity in cell lysates by cleavage of a caspase-6 substrate. *p*NA can be measured using a spectrophotometer or a 96-well plate reader at ~405 nm.	1 Kit	€653

Measurement of Changes in the Cytoplasm: Substrate-Based Caspase Detection Kits – (continued)

Kit Name	Cat. No.	Detection Method	Sample Type	Substrate Provided	Positive Control Provided	Inhibitor Provided	Number of Tests/ Samples	Principles of Asssay/ Notes	Size	Price
Caspase-7 Immunoassay/ Activity Kit, Fluorometric	QIA108	Fluorometric	Cell Extracts	DEVD-AFC	Yes	No	100 Tests	Immunocapture of active caspase-7 followed by detection of caspase-7 activity in cell lysates by cleavage of a caspase-7 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€803
Caspase-8 Assay Kit, Colorimetric	218770	Colorimetric	Cell Extracts	IETD-*p*NA	Yes	Yes	96 Tests	Detects caspase-8 activity in cell lysates by cleavage of a caspase-8 substrate. pNA can be measured using a spectrophotometer or a 96-well plate reader at ~405 nm.	1 Kit	€616
Caspase-8 Activity Assay Kit	QIA71	Fluorometric	Cell Extracts	IETD-AFC	Yes	Yes	96 Tests	Detects caspase-8 activity in cell lysates by cleavage of a caspase-8 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 kit	€514
NEW Caspase-8 Activity Assay Kit-HTS	HTS03	Fluorometric	Cell Extracts Intact Cells	IETD-AFC	Yes	Yes	100 Tests	Detects caspase-8 activity in cell lysates by cleavage of a caspase-8 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	100 T	€514
Active Caspase-8 Assay Kit	CBA046	Fluorometric	Cell Extracts	DEVD-AFC	Yes	No	182 Tests	Immunocapture of active caspase-8 followed by detection of caspase-8 activity in cell lysates by cleavage of a caspase-8 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€453
Caspase-9 Assay Kit, Colorimetric	218824	Colorimetric	Cell Extracts	LEHD-*p*NA	No	No	100 Tests	Detects caspase-9 activity in cell lysates by cleavage of a caspase-9 substrate. *p*NA can be measured using a spectrophotometer or a 96-well plate reader at ~405 nm.	1 Kit	€646
Caspase-9 Activity Assay Kit	QIA72	Fluorometric	Cell Extracts	LEHD-AFC	Yes	Yes	96 Tests	Detects caspase-9 activity in cell lysates by cleavage of a caspase-9 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€514
NEW Caspase-9 Activity Assay for HTS	HTS04	Fluorometric	Cell Extracts Intact Cells	LEHD-AFC	Yes	Yes	96 Tests	Detects caspase-9 activity in cell lysates by cleavage of a caspase-9 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	100 T	€514
Active Caspase-9 Assay Kit	CBA047	Fluorometric	Cell Extracts	DEVD-AFC	Yes	No	480 Tests	Immunocapture of active caspase-9 followed by detection of caspase-9 activity in cell lysates by cleavage of a caspase-9 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€453
Caspase-10 Assay Kit, Fluorometric	218811	Fluorometric	Cell Extracts	AEVD-AFC	No	No	96 Tests	Detects caspase-10 activity in cell lysates by cleavage of a caspase-10 substrate. (Ex at 400 nm and Em at 505 nm) can be measured using a fluorometer or a fluorescence microplate reader.	1 Kit	€581

Use of Fluorochrome - Inhibitor Conjugates for Active Caspase Detection

Caspase inhibitors act by binding to the active site of the caspase, either in a reversible or in an irreversible manner. Inhibitor design includes a peptide recognition sequence attached to a functional group such as an aldehyde (CHO), chloromethylketone (CMK), or fluoromethylketone (FMK). The peptide recognition sequence corresponding to that found in endogenous substrates determines the specificity of a particular caspase. Compounds with the Ac-YVAD-CHO sequence are potent inhibitors of caspase-1 (K_i~10 nM), and exhibit very weak inhibitory effect on capases-3 and 7 (K_i > 50 uM). Exclusion of the amino acid from the P4 position of the inhibitor peptide results in a potent, but less specific inhibitor like Z-VAD-FMK, which acts as a pan caspase inhibitor, and inhibits most caspases. The inhibitor can also be tagged with a fluorochrome, such as FITC, that can be used as a detection tool for activated caspases. For example, FITC conjugated to VAD-FMK provides a very useful, cell permeable, non-toxic inhibitor that binds irreversibly to activated caspases in apoptotic cells. The fluorescence intensity can be measured by fluorescence microscopy, fluorescence plate reader, or flow cytometry.

Measurement of Changes in the Cytoplasm: Fluorochrome – Inhibitor Conjugates for Active Caspase Detection

Kit Name	Cat. No.	Detection Method	Sample Type	Substrate Provided	Positive Control Provided	Inhibitor Provided *	Number of Tests/ Samples	Principle of Assay/Notes	Price
Caspase Detection Kit	QIA90	IF, FC	Intact Cells	FITC-VAD-FMK	No	Yes	100 Tests	Detects caspase activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (FITC-VAD-FMK) to label caspases in the cell.	€598
Caspase Detection Kit	QIA92	IF, FC	Intact Cells	RED-VAD-FMK	No	Yes	100 Tests	Detects caspase activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (RED-VAD-FMK) to label caspases in the cell.	€598
Caspase-3 Detection Kit	QIA91	IF, FC	Intact Cells	FITC-DEVD-FMK	No	Yes	100 Tests	Detects caspase-3 activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (FITC-DEVD-FMK) to label caspases in the cell.	€598
Caspase-3 Detection Kit	QIA93	IF, FC	Intact Cells	RED-DEVD-FMK	No	Yes	100 Tests	Detects caspase-3 activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (RED-DEVD-FMK) to label caspases in the cell.	€610
NEW Active Caspase-3 ELISA Kit	CBA045	ELISA	Cell Extracts	Biotin-ZVKD-FMK	Yes	Yes	96 Tests	Solid phase ELISA designed to detect active caspase-3 in cell lysates. Detection is measured using a microplate reader at 450 nm.	€575
Caspase-8 Detection Kit	QIA113	IF, FC	Intact Cells	FITC-IETD-FMK	No	Yes	100 Tests	Detects caspase-8 activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (FITC-IETD-FMK) to label caspases in the cell.	€645
Caspase-8 Detection Kit	QIA114	IF, FC	Intact Cells	RED-IETD-FMK	No	Yes	100 Tests	Detects caspase-8 activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (RED-IETD-FMK) to label caspases in the cell.	€645
Caspase-9 Detection Kit	QIA115	IF, FC	Intact Cells	FITC-LEHD-FMK	No	Yes	100 Tests	Detects caspase-9 activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (FITC-LEHD-FMK) to label caspases in the cell.	€645
Caspase-9 Detection Kit	QIA116	IF, FC	Intact Cells	RED-LEHD-FMK	No	Yes	100 Tests	Detects caspase-9 activation using an irreversible, cell-permeable non-toxic, fluorescent caspase inhibitor (RED-LEHD-FMK) to label caspases in the cell.	€645

Note: **ELISA:** enzyme linked immunosorbent assay; **FC**: flow cytometry; **IF**: immunofluorescence

*Inhibitors also available separately. For a complete list of Caspase Inhibitors see Table IV in Appendix (page 28) or visit our Inhibitor Resource at www.calbiochem.com/inhibitors

Use of Immunoblot-Based Caspase Detection Kits

Caspases destroy key components of the cellular infrastructure and activating factors that mediate damage to the cells. Over 280 caspase targets have been identified to date. Proteins cleaved by these caspases are involved in cell cycle progression/regulation, cellular repair, cytoskeletal architecture and structure, DNA synthesis, cleavage, and repair, as well as cell detachment and cytokine precursors. The majority of substrates identified are cleaved by caspase-3 and some of these same substrates can also be cleaved by caspase-7. The demonstration of caspase substrate cleavage is an indirect way to measure caspase activity during apoptosis. Thus, analysis of changes in the molecular weight of caspase substrates can be measured by SDS-PAGE followed by immunoblot detection with specific substrate antibodies. Antibodies are available that are specific for the procaspase or activated form of certain caspases. Some of the antibodies recognize both the uncleaved (Pro) and cleaved (active) forms of the caspase. This procedure has made it possible to detect endogenous activated caspases or the resultant apoptosis-induced proteolysis of substrates.

Reference

Fischer, U. et al. 2003. *Cell Death and Differentiation* **10**, 76.

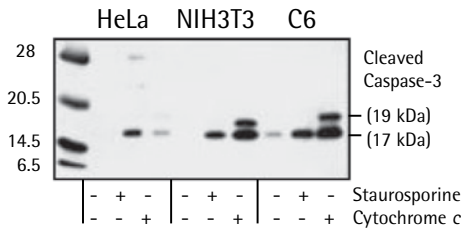
Measurement of Changes in the Cytoplasm: Immunoblot Kits

Kit	Cat No.	Application	Species Reactivity	Positive Control Provided	Inhibitor Provided *	Principle of Assay/Notes	Size	Price
NEW Cleaved Caspase Antibody Sampler Kit	AP1026	IB	See data sheet or website	No	No	Affinity purified antibodies against the following cleaved caspases: caspase-3 (Asp^{175}), caspase-6 (Asp^{162}), caspase-7 (Asp^{198}), caspase-8 (Asp^{384}), caspase-9 (Asp^{315}), caspase-9 (Asp^{330}), and anti-rabbit IgG, peroxidase conjugate for detection.	1 Kit	€653
Downstream Effector Caspase Sampler Kit	ASK23	IB	See data sheet or website	No	No	Detects caspases-3 ,-6 and -7 in cell lysates.	1 Kit	€406

Note: **IB**: immunoblotting

* For a list of Antibodies to Caspases see Table I in Appendix (page 22) or visit our Antibody Resource at www.calbiochem.com/antibodyresource.

Cleaved Caspase Antibody Sampler Kit
Cat. No. AP1026



Detection of cleaved caspase-3 by immunoblotting. Samples: Cell lysates from HeLa, NIH/3T3, and C6 cells, untreated, staurosporine-treated (1 µM *in vitro*) or cytochrome *c*-treated (0.25 mg/ml *in vitro*). Primary antibody: Anti-Cleaved Caspase-3 (Asp^{175}) (Cat. No. AP1027) (1:1000). Detection: chemiluminescence. Representative data from Cat. No. AP1026

Technical Tips and Frequently Asked Questions

A. Use of Fluorogenic or Colorimetric Substrates for Measuring Caspase Activity

In general, both colorimetric and fluorogenic substrates can be used to measure caspase activity in apoptotic cells, or to screen for activators and inhibitors of caspases.

Measurement of caspase activity is performed using synthetic caspase peptides conjugated to colorimetric substrates such as *p*NA (*p*-nitroaniline) or fluorogenic substrates such as AFC (7-amino-4-trifluoromethyl coumarin), or AMC (7-amino-4-methyl coumarin).

- **Caspase activity can be detected using either fluorogenic or colorimetric substrates; which is more sensitive?**

  Fluorogenic detection is more sensitive.

- **What is the relationship between the amount of AFC or AMC and caspase activity?**

  Caspase activity in the sample is proportional to the amount of free AFC or AMC released from the peptide.

- **What are the excitation and emission maximum wavelengths for the above substrates?**

Fluorogenic

	Excitation	Emission
-AFC	~400 nm	~505 nm
-AMC	~380 nm	~460 nm

AFC has an advantage over other fluorogenic labels. The larger Stoke's shift allows greater sensitivity.

Colorimetric	Absorbance
-*p*NA	~405 nm

- **There is very little difference in caspase activity when I compare uninduced versus induced samples.**

  Start with a population of healthy cells. Make sure that the agent you are using to induce apoptosis is effective in inducing apoptosis in your cell population (e.g., check literature). Use a positive control of apoptosis and evaluate using another detection method (e.g., immunoblot). Check to make sure that the fluorimeter has the correct filters or that the spectrophotometer is reading at the correct wavelength.

- **The peptide-based caspase substrate detects multiple caspase activities in my system.**

  Caspase substrates based on cleavage preferences are specific for a particular class of caspase and not necessarily a single caspase species.

- **I do not detect the expected caspase activity in my samples.**

  Caspases are cysteine proteases that require the presence of DTT for full activity. A freshly prepared solution of DTT should be used in all caspase assays.

Technical Tips and Frequently Asked Questions - continued

B. Use of caspase inhibitors

- **How can I determine if a caspase inhibitor is reversible or irreversible?**

  The C-terminal group determines the reversibility or the irreversibility of any caspase inhibitor. In general, caspase inhibitors with an aldehyde (CHO) group are reversible. The CMK, FMK, and FAOM groups are more reactive and form covalent bonds with the enzyme, creating an irreversible linkage. FMK is slightly less reactive than CMK and therefore is considered more specific for the enzyme site being inhibited.

- **What is the purpose of a methyl ester group on some inhibitors?**

  Sometimes the aspartic acid residue is esterified. This makes the inhibitors more hydrophobic and increases cell permeability of the peptide.

- **What are the advantages of using FMK-based caspase inhibitors and how do they differ from CHO-based inhibitors?**

  The FMK-based caspase inhibitors are cell-permeable because of the fact that the carboxyl group of aspartic or glutamic acid is esterified, making them more hydrophobic. These inhibitors covalently modify the thiol group of the enzyme, making them irreversible inhibitors. Generally, at the amine end of the inhibitor we have a Z, biotin, or Ac group. These groups also increase hydrophobicity of the molecule, which makes them more cell-permeable. Compared to the inhibitors with an Ac or a biotin group, those inhibitors with a Z-group are even more cell-permeable. Inhibitors with a biotin group can serve as a detection tool and are useful in tagging the enzyme-inhibitor site.

  The CHO-based inhibitors are reversible due to the fact that the thiol group of the enzyme forms a reversible adduct to the carbonyl group of the aldehyde. As a general rule CHO-based inhibitors are hydrated and hence are slow binding. The extent of their reversibility depends on the pH, metal ion concentration, and other conditions. When the aldehyde group is attached to the aspartic acid (D-CHO), the product exists as a pseudo acid aldehyde in equilibrium, making it somewhat cell-permeable.

- **The caspase inhibitor is not inhibiting apoptosis in my experimental system.**

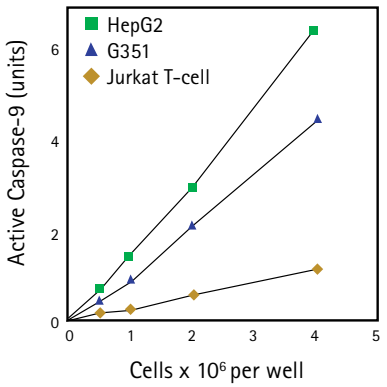
  Ensure that you are using a cell-permeable caspase inhibitor. Aldehyde-based inhibitors (ie., DEVD-CHO) will not cross the plasma membrane under normal culture conditions unless fused to a targeting sequence.

- **The peptide-based caspase inhibitor seems to be inhibiting multiple caspases in my system.**

  The peptide caspase inhibitors based on substrate preference are specific for a particular class of caspase and not necessarily a single caspase species. Additionally, caspase inhibitors must be used at several-fold higher concentrations than the recommended K_i or IC_{50} to ensure specificity of the targeted class of caspase. Please refer to the caspase inhibitor table for selection of a proper inhibitor. (See Table IV, page 28.)

Active Caspase-9 Assay Kit
Cat. No. CBA047

The Active Caspase-9 Assay Kit is useful for studying the effects of biochemical compounds on caspase-9 activity in cell lysates. The assay employs a monoclonal antibody specific for caspase-9 coated onto the wells of a 96-well plate. The immunocapture of active caspase-9 is followed by the detection of caspase-9 activity by cleavage of a fluorogenic substrate. Fluorescence is measured in a 96-well fluorescent plate reader.



Detection of active Caspase-9 in lysates from HepG2 hepatocytes, G361 melanoma cells and Jurkat T-cells. Caspase-9 was activated by incubating the indicated amount of cell lysates with cytochrome c and dATP. The data show that detection of Caspase-9 is proportional to the amount of activated cell extracts assayed.

IV. Measurement of Apoptosis-Induced Changes in the Nucleus

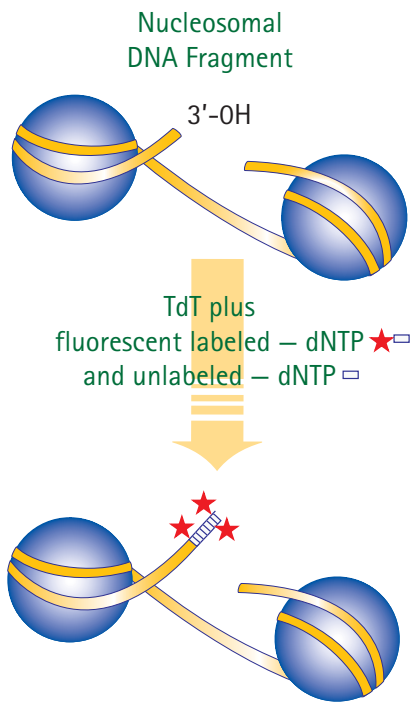
DNA fragmentation is one of the hallmarks of apoptosis. DNA is first cleaved into large fragments (50-200 kb), followed by cleavage into smaller fragments called nucleosomal units (180-200 bp). The primary DNase responsible for this DNA cleavage is CAD (caspase-activated DNase) or DFF40, (DNA fragmentation factor). CAD is maintained in an inactive state by forming a complex with ICAD (inhibitor of CAD). Upon receiving an appropriate apoptotic signal, caspase-3 cleaves ICAD, and CAD is released to cleave chromosomal DNA. CAD contains a nuclear localization signal, which allows it to cleave only DNA in the nucleus.

Reference

Nagata, S. 2005. *Ann. Rev. Immunol.* **23**, 853.

DNA Fragmentation

Principle of the Assay QIA39



FragEL™ DNA Fragmentation Detection Kit, Fluorescent-TdT Enzyme
Cat. No. QIA39

This kit is a fluorescein-conjugated version of the TdT Colorimetric FragEL™ DNA fragmentation Detection Kit (Cat. No. QIA33). Terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic events, and catalyzes the addition of fluorescein-labeled and unlabeled deoxynucleotides. When excited, fluorescein generates an intense signal that can be detected either by fluorescence microscopy or by flow cytometry. The mounting medium sustains the fluorescent signal from samples labeled on slides and aids in the morphological evaluation and characterization of normal and apoptotic cells. Non-apoptotic cells can be visualized using a DAPI filter.

(see ordering information on page 18)

Use of Agarose Gel Electrophoresis to Measure DNA Fragmentation.

Apoptotic nucleosomal fragments (180-200 bp) can be resolved by agarose gel electrophoresis to detect DNA ladders. DNA laddering may not be detectable in all cells undergoing apoptosis (e.g., nerve cells, hepatocytes, and embryonal-fibroblasts), or when the number of cells or the sample size is limited.

Reference

Nagase, H. et al. 2003. *Cell Death Differ.* **10**, 142.

Measurement of Changes in the Nucleus: Agarose Gel Electrophoresis to Measure DNA Fragmentation

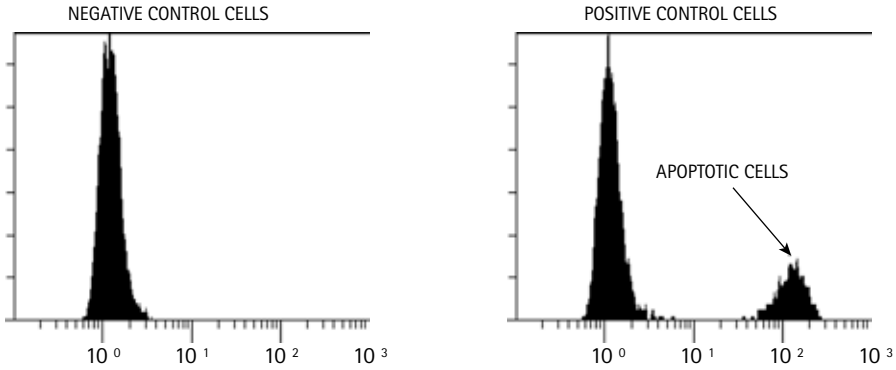
Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Negative Control Provided	Number of Tests/Samples	Principle of Assay/Notes	Price
Suicide Track™ DNA Ladder Isolation Kit	AM41	DNA electrophoresis	Intact Cells	Yes	Yes	25 Tests	Designed for the purification and visualization of DNA ladder fragments only or both fragments and intact DNA.	€228

Measurement of Changes in the Nucleus: Use of Vital Dyes

Product Name	Cat. No.	Principle of Assay/Notes	Size	Price
Acridine Orange	113000	A cell-permeable, cationic fluorescent dye that interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, excitation maximum at 502 nm and an emission maximum at 525 nm; for RNA, the excitation maximum shifts to 460 nm and the emission maximum shifts to 650 nm (red).	1 g	€83
Bisbenzimide H 33258 Fluorochrome	382061	Membrane-permeable, adenine-thymine-specific fluorescent stain. Useful for staining DNA, chromosomes, and nuclei. Excitation max.: 346 nm, Emission max.: 460 nm	100 mg 250 mg 1 g	€44 €69 €190
Bisbenzimide H 33342 Fluorochrome	382065	Cell-permeable, adenine-thymine-specific fluorescent stain. Useful for staining DNA, chromosomes, and nuclei for fluorescence microscopy and flow cytometry applications. Excitation max.: 346 nm, Emission max.: 460 nm	100 mg	€69
DAPI	268298	Cell-permeable DNA-binding dye. Binds preferentially to DNA rich in adenine and thymine. Useful for microscopic detection of nuclei and nuclear DNA in normal and apoptotic cells. Excitation max.: ~359 nm, Emission max.: ~461 nm	10 mg	€131
Propidium Iodide	537059	Membrane impermeable DNA intercalator. Has red fluorescence at 488 nm. Useful for flow cytometry. Can be used to differentiate between apoptotic and necrotic cell death while staining only necrotic cells.	50 mg 100 mg 250 mg 1 g	€66 €117 €282 €953
Propidium Iodide Solution	537060	Membrane impermeable DNA intercalator. Has red fluorescence at 488 nm. Useful for flow cytometry for staining apoptotic cells and nuclei. Can be used to differentiate between apoptotic and necrotic cell death while staining only necrotic cells. A convenient form of propidium iodide useful for flow cytometry studies.	5 ml	€81

Apo-BrdU™ Kit
Cat. No. CBA040

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 or laser scanning cytometry. The kit contains both positive and negative controls. Flow cytometry data is indicated below.



Log Green Fluorescence
Flow Cytometry Data of Apo-BrdU Negative & Positive Control Cells

Use of Terminal Deoxyuridine Nucleotide End Labeling (TUNEL) to Measure DNA Fragmentation

DNases generate free 3′-hydroxyl termini, which in turn can be labeled with bromolated deoxyuridine triphosphate nucleotides (Br-dUTP). The reaction is catalyzed by deoxynucleodidyl transferase (TdT) and Br-dUTP sites can be detected with a BrdU antibody conjugated to a fluorophore. Since non-apoptotic cells lack exposed 3′-hydroxyl ends, little or no Br-dUTP is incorporated.

Use of TUNEL to Measure DNA Fragmentation: DNA Fragmentation Based Kits

Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Negative Control Provided	Number of Tests/Samples	Principle of Assay/Notes	Price
FragEL™ DNA Fragmentation Detection Kit, Colorimetric-Klenow Enzyme	QIA21	Light-microscopy	Frozen and Paraffin Tissue Sections; Fixed Cell Preparations	Yes	Yes	50 tests	Detects DNA fragmentation by labeling ends of DNA breaks. Colorimetric detection.	€489
FragEL™ DNA Fragmentation Detection Kit, Colorimetric-TdT Enzyme	QIA33	Light-microscopy	Frozen and Paraffin Tissue Sections; Fixed Cell Preparations	Yes	Yes	50 tests	Detects DNA fragmentation by labeling ends of DNA breaks. Colorimetric detection.	€491
FragEL™ DNA Fragmentation Detection Kit, Fluorescent-TdT Enzyme	QIA39	Fluorescent microscopy, FC	Frozen and Paraffin Tissue Sections; Fixed Cell Preparations	Yes	Yes	50 Tests	Detects DNA fragmentation by labeling ends of DNA breaks. Fluorescent detection.	€386
NEW Apo-BrdU™	CBA040	FC IF	Intact Cells	Yes	Yes	60 Tests	A two color TUNEL assay for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry and microscopy.	€465
NEW Apo-Direct™	CBA041	FC	Intact Cells	Yes	Yes	50 Tests	A two color TUNEL assay for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry or laser scanning cytometry.	€465

Note: **FC**: flow cytometry; **IF**: immunofluorescence

For more information or to place an order, contact your local distributor (see back cover).

Use of TUNEL to Measure DNA Fragmentation: Other Related Kits

Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Negative Control Provided	Number of Tests/Samples	Principle of Assay/Notes	Price
Nucleosome ELISA Kit	QIA25	ELISA	Cell Lysate	Yes	Yes	96 Tests	This kit allows the quantitation of apoptotic cells *in vitro* by DNA affinity-mediated capture of free nucleosomes followed by their anti-histone-facilitated detection.	€351
Cell Death Detection (Nuclear Matrix Protein) ELISA Kit	QIA20	ELISA	Culture Super-natant	Yes	Yes	96 Tests	Designed for the quantitative detection of NMP41/7. The specific detection of soluble human NMPs afforded by the Cell Death Detection (Nuclear Matrix Protein) ELISA allows quantitation of cell death.	€737

Technical Tips and Frequently Asked Questions

- **The percentage of apoptotic cells in my sample seems lower than expected. Why?**
  Induction of death should be carried out on subconfluent healthy growing cells for the most robust and reproducible cell death response. When assessing the death of adherent cells one needs to consider the fact that dying cells will lose their adherence properties. If analysis requires harvesting of cells, the culture supernatant should also be collected to ensure complete analysis of the cell population. Additionally, apoptotic cells are more buoyant and thus more difficult to spin down in a centrifuge. This may lead to underestimation of dead cells in an experiment. It is advisable to increase time and speed during centrifugation of apoptotic samples.

- **The background staining is higher than expected in my samples.**
  When assessing apoptotic cell death with antibody staining, it is advisable to use negative isotype controls since some dying cells tend to become both "sticky" and autofluorescent. The resulting higher background can be controlled by including at least 1% BSA in all staining procedures.

- **I do not detect the apoptosis-associated oliogonucleosomal DNA laddering on agarose gels.**
  DNA laddering is difficult to detect in some cell types, especially embryonic fibroblastic cells, such as NIH/3T3. Please consider using alternative assays such as the FragEL™ DNA Fragmentation Kit.

- **All of my cells are staining brown with the FragEL DNA Fragmentation Detection Kit.**
  Overdevelopment of the slide with the DAB reagent is the most common cause of high background with this type of kit. Decrease the length of incubation of the DAB reagent to lower background staining.

V. Measurement of Cell Proliferation

In multicellular organisms, a delicate balance between cell proliferation and cell death is maintained for normal cellular homeostasis, failure of this balance can lead to disease states. For example, on an average day humans produce ~60 x 10^9 new cells and at the same time eliminate roughly the same number of mature cells. Hence, a coupled relationship exists between proliferation and apoptosis. While certain morphological and biochemical changes serve as markers of apoptosis, cell proliferation can be used as another criteria for assessing apoptosis.

Incorporation of the thymidine analog, bromodeoxyuridine (BrdU), into newly synthesized DNA strands of actively proliferating cells can be used as an alternative to radioisotopic methods for measuring cell proliferation. Following partial denaturation of double-stranded DNA, BrdU is detected immunochemically using an anti-BrdU antibody, allowing the assessment of the population of cells, which are actively synthesizing DNA.

Reference

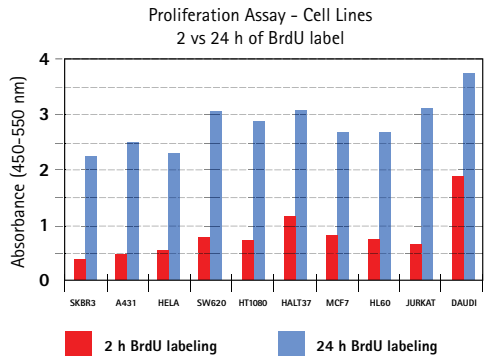
Reed, J.C. 2002. *Nat. Rev. Drug Discov.* 1, 111.

Measurement Of Cell Proliferation: Kits

Kit Name	Cat. No.	Application	Sample Type	Positive Control Provided	Number of Tests/Samples	Principle of Assay/Notes	Price
Rapid Cell Proliferation Kit	QIA127	Colorimetric	Intact cells	No	500 Tests	Assay measures the increased activity of cellular mitochondrial dehydrogenases that can cleave the tetrazolium dye WST-1 to formazan. The activity of mitochondrial dehydrogenases is proportional to cell number. Measure absorbance at 450 nm in a microplate reader.	€259
BrdU IHC System	HCS30	Colorimetric	Intact cells, Paraffin embedded Tissue sections	Yes	50 Tests	Assay is based upon incorporation of BrdU (thymine analog) into newly synthesized DNA of actively proliferating cells. Utilizes biotinylated anti-BrdU antibody to detect incorporated BrdU. Staining is visualized using streptavidin-peroxidase and diaminobendizine (DAB).	€515
BrdU Cell Proliferation Assay	QIA58	Colorimetric	Intact cells	No	200 Tests 1000 Tests	Assay is based upon incorporation of BrdU (thymine analog) into newly synthesized DNA of actively proliferating cells. Utilizes anti-BrdU antibody to detect incorporated BrdU. Measure absorbance at 450 nm in a microplate reader.	€278 €641
BrdU Cell Proliferation Assay, HTS	HTS01	Fluorometric	Intact cells	No	200 Tests 1000 Tests	High-throughput screening version of Cat. No QIA58. Assay is based upon incorporation of BrdU (thymine analog) into newly synthesized DNA of actively proliferating cells. Utilizes anti-BrdU antibody to detect incorporated BrdU. Use a fluorescent plate reader capable of measuring wavelengths between 315 and 340 nm for excitation and 370 and 470 nm for emission can be used for detection.	€282 €682

BrdU Cell Proliferation Assay No. QIA58

BrdU Cell Proliferation Assay is a non-isotopic immunoassay for quantification of BrdU incorporation into newly synthesized DNA of actively proliferating cells. It is sensitive, rapid, and easy to perform.



Example of 2 h versus 24 h BrdU labeling of adherent and nonadherent cell lines.

Appendix: Useful Tables and Technical Protocols

Table I: Characterization of Antibodies to Caspases

Antibody	Cat. No.	Size of Pro-caspase* (kDa)	Size of Active Subunits* (kDa)	Size of Molecules Recognized (kDa)	Species Reactivity	Application	Size	Price
NEW Anti-Caspase-1 Mouse mAb (14F468)	AP1043	45	20/10	Reacts with the 45 kDa pro-caspase 1 and cleaved forms of caspase-1 that retain amino acids 371-390.	human, mouse	IB, PS	50 µg	€198
NEW Anti-Caspase-1 (31-45) Rabbit pAb	AP1044	45	20/10	Reacts with the 45 kDa pro-caspase 1 and cleaved forms of caspase-1 that retain amino acids 31-45.	human	IB, PS	50 µg	€176
Anti-Pan Caspase-1 (390-404) Rabbit pAb	PC84	45	20/10	Reacts with 35 kDa (strongly) and 10 kDa form moderately.	human	IB, PS	100 µg	€319
Anti-Caspase-2 (1-14) Rabbit pAb	PC107	51	19/12	Reacts with ~52 kDa pro-caspase-2.	human	IB	100 µg	€319
Anti-Caspase-3 Rabbit pAb	235412	32	17/12	Reacts with ~32 kDa pro-caspase-3.	bovine, canine, hamster, human, monkey, mouse, porcine, rabbit, rat, yeast	IB	100 µl	€404
Anti-Caspase-3 (Ab-2) Mouse mAb (10C1.C9)	AM34	32	17/12	Reacts with the 32 kDa pro-caspase-3 and the 17 kDa active subunit.	human	IB	100 µg	€338
Anti-Caspase-3 (Ab-3) Mouse mAb (AM1.4.1-1B)	AM39	32	17/12	Reacts with 32 kDa pro-caspase-3.	human	IB	100 µg	€248
Anti-Caspase-3 (Ab-4) Mouse mAb (AM1.31-11)	AM65	32	17/12	Reacts with 11 kDa active subunit.	human, mouse	IB, PS	100 µg	€338
Anti-Cleaved Caspase-3 (Asp^{175}) Rabbit pAb	AP1027	32	17/12	Reacts with the ~17 kDa active subunit of caspase-3.	human, mouse, rat	FC, IB, IC, PS	50 µl	€231
Anti-Caspase-3, Cleaved (Ab-2) Rabbit pAb	PC679	32	17/12	Reacts with 17 kDa active subunit of caspase-3.	human, mouse, rat	FS, IB, IC, PS	50 µg	€319
Anti-Caspase-4 (Ab-1) Rabbit pAb	PC109	43	20/10	Reacts with ~42 kDa pro-caspase-4	human	IB	100 µl	€319
Anti-Caspase-6 (250-264) Rabbit pAb	218774	34	18/11	Reacts with 34 kDa pro and active forms of caspase-6.	bovine, canine, hamster, human, monkey, mouse, porcine, rabbit, rat, sheep	IB	100 µg	€384
Anti-Cleaved Caspase-6 (Asp^{162}) Rabbit pAb	AP1012	34	18/11	Reacts with 18 kDa subunit of the active caspase-6.	human, mouse, rat	IB	50 µl	€278
Anti-Caspase-7 (Ab-1) (186-198) Rabbit pAb	PC334	35	20/12	Reacts with pro and active (20-23) kDa subunits of caspase-7.	human	IB	100 µg	€338
Anti-Active Caspase-7 Rabbit pAb	PC520	35	20/12	Reacts with 20 kDa subunit of the active caspase-7.	human, mouse, rat	IB, IF, IP	50 µl	€338
Anti-Caspase-8 (Ab-3) Mouse mAb (1-3)	AM46	55	18/11	Reacts with 55 kDa pro-caspase-8 and 28 kDa intermediate subunit.	human	IB	100 µg	€338
Anti-Caspase-8 (Ab-1) (236-247) Rabbit pAb	PC335	55	18/11	Reacts with 18 kDa subunit (active) of caspase-8.	human	IB	100 µg	€338
Anti-Cleaved Caspase-8 (Asp^{384}) Mouse mAb (11G10)	AP1013	55	18/11	Reacts with the ~10 kDa subunit (active) of Caspase-8.	human	IB	100 µl	€413

Characterization of Antibodies to Caspases – (continued)

Antibody	Cat. No.	Size of Pro-caspase* (kDa)	Size of Active Subunits* (kDa)	Size of Molecules Recognized (kDa)	Species Reactivity	Application	Size	Price
Anti-Caspase-9 (1-134) Rabbit pAb	218794	45	17/10	Reacts with the 45 kDa pro-caspase-9 and the ~10 kDa active subunit. Intermediate forms of ~37 kDa may also be recognized.	bovine, human, mouse, rat, sheep	IB	100 µl	€413
Anti-Cleaved Caspase-9 (Asp^{315}) Rabbit pAb	AP1014	45	17/10	Reacts with the ~35 kDa intermediate of caspase-9.	human	IB, IP	50 µl	€278
Anti-Cleaved Caspase-9 (Asp^{353}) Rabbit pAb	AP1015	45	17/10	Reacts with the 37 kDa intermediate and 17 kDa active subunit caspase-9 .	rat	FFS, IB, IC	50 µl	€278
Anti-Cleaved Caspase-9 (Asp^{353}) Rabbit pAb	AP1028	45	17/10	Reacts with the 37 kDa intermediate of caspase-9	mouse	IB, IC	50 µl	€231
Anti-Caspase-9, Cleaved (Ab-2) Rabbit pAb	PC680	45	17/10	Reacts with the 37 kDa intermediate of caspase-9	human	IB, IC	50 µg	€319
Anti-Caspase-10 (Ab-1) (221-234) Rabbit pAb	PC332	55	17/12	Reacts with the ~58 kDa proform and the ~23 kDa (active form of caspase-10.	human	IB	100 µg	€324
Anti-Caspase-12 (Ab-1) (100-116) Rabbit pAb	PC557	50	20/10	Reacts with the ~53 kDa proform of caspase-12.	human, mouse, rat	IB	100 µg	€338
Anti-Caspase-12 (Ab-2) (2-17) Rabbit pAb	PC558	50	20/10	Reacts with the ~53 kDa proform of caspase-12.	human, mouse, rat	IB	100 µg	€338
Anti-Caspase-14 (Ab-1) Mouse mAb (70A1426)	AM64	30	20/10	Reacts with the ~30 kDa proform of caspase-14	mouse	IB	100 µg	€346

Note: **ELISA**: enzyme-linked immunosorbent assay; **FC**: Flow cytometry; **FFS**: Free-floating section; **FS**: Frozen sections; **IB**: immunoblotting; **IC**: immunocytochemistry; **IP**: immunoprecipitation; **PS**: paraffin sections

*Size of the Pro-caspase and size of active subunits are the predicted molecular weight. However, the observed molecular weight in immunoblots may vary due to experimental conditions (degree of apoptosis, molecular weight markers, gel percentage, etc).

To view our complete antibody portfolio visit our Antibody Resource at www.calbiochem.com/antibodyresource

Table II: Classification of Bcl-2 Members

Pro-Apoptotic Bcl-2 Family Members

Antibody Description	Cat. No.	Species Reactivity	Application	Size	Price
Anti-Bak (Ab-1) Mouse mAb (TC-100)	AM03	human	IB	20 µg 100 µg	€113 €338
Anti-Bak (Ab-2) Mouse mAb (TC-102)	AM04	human, mouse	IB, IF	20 µg 100 µg	€86 €338
Anti-Bak (2-14) Rabbit pAb	196150	hamster, human, porcine	ELISA, IB, IP	100 µg	€378
Anti-Bax (44-62) Rabbit pAb	196821	bovine, human, mouse, porcine, rat	IB	100 µl	€378
Anti-Bax (Ab-1) (150-165) Rabbit pAb	PC66	human, mouse, opossum, rat	FS, IB, PS	100 µg	€319
Anti-Bax (Ab-2) Mouse mAb (ID3)	AM13	human	IB, IF, IP	100 µg	€338
Anti-Bax (Ab-3) Mouse mAb (2D2)	AM32	human	IB	100 µg	€338
Anti-Bax (Ab-4) (98-117) Rabbit pAb	PC103	human	IB	100 µg	€319
Anti-Bax (Ab-6) Mouse mAb (6A7)	AM44	human, mouse, rat	IP	100 µg	€338

Pro-Apoptotic Bcl-2 Family Members – BH3 Only Members

Antibody Description	Cat. No.	Species Reactivity	Application	Size	Price
Anti-Bad (19-35) Rabbit pAb	195872	bovine, canine, hamster, human, monkey, mouse, ovine, porcine, rabbit, rat	IB, PS	100 µg	€378
PhosphoDetect™ Anti-Bad (pSer^{112}) (Ab-3) Rabbit pAb	PC636	human, mouse, rat	IB	50 µg	€384
PhosphoDetect™ Anti-Bad (pSer^{136}) (Ab-4) Rabbit pAb	PC637	human, mouse, rat	IB	10 T	€373
PhosphoDetect™ Anti-Bad (pSer^{112}) (Ab-1) Rabbit pAb	PC518	human, mouse, rat	IB, IP	50 µl	€282
PhosphoDetect™ Anti-Bad (pSer^{136}) (Ab-2) Rabbit pAb	PC519	human, mouse, rat	IB	50 µl	€282
Anti-Bid (Ab-1) (76-85) Rabbit pAb	PC562	human	PS	100 µg	€294
Anti-Cleaved Bid (Ab-1) Rabbit pAb	PC645	mouse	IB	10 T	€457
Anti-Bim Rat mAb (14A8)	AM53	human, mouse	IB, IF, IP	50 µg	€384
Anti-Bim (22-40) Rabbit pAb	202000	human, mouse, rat	IB, IC	100 µg	€350
Anti-Bmf (2-14) Rabbit pAb	PC685	human, mouse	IB	100 µg	€319
Anti-Bnip3L (77-92) Rabbit pAb	PC525	human	IB	100 µg	€338
Anti-Noxa Mouse mAb (114C307)	OP180	human	IB	100 µg	€351
Anti-PUMA (Ab-1) (2-16) Rabbit pAb	PC686	human	IB	100 µg	€350

Anti-Apoptotic Bcl-2 Family Members

Antibody Description	Cat. No.	Species Reactivity	Application	Size	Price
Anti-Bcl-2 (Ab-2) Rabbit pAb (20-34)	PC68	human, mouse, opossum	FS, IB, PS	100 µg	€319
PhosphoDetect™ Anti-Bcl-2 (pSer^{87}) Rabbit pAb	PC502	human	PS	25 µg	€282
Anti-Bcl-2 (Ab-1) Mouse mAb (100)	OP60	human	FS, IB, PS	100 µg	€294
Anti-Bcl-2 (Ab-2) Mouse mAb (8C8)	AM59	human, monkey	FS, IB, IP, PS	100 µg	€338
Anti-Bcl-2 (Ab-3) Mouse mAb (4D7)	OP91	human	FC, IB, IF, IP, PS	100 µg	€294
Anti-Bcl-2 (Ab-4) Mouse mAb (10C4)	AM43	mouse, rat	IB	100 µg	€338
Anti-Bcl-2α (Ab-1) Mouse mAb (100/D5)	AM58	human	FS, IB, IP, PS	100 µg	€338
Bcl-2 Family Antibody Sampler Kit	ASK12	human		1 each	€461
Anti-Bcl-X_L (Ab-2) Mouse mAb (2H12)	AM05	human	IB	100 µg	€338
Anti-Mcl-1 (Ab-1) Mouse mAb (RC13)	AM50	human	IB, IP	100 µg	€338
Anti-Mcl-1 (121-139) Rabbit pAb	444206	canine, human, monkey, porcine, rabbit	ELISA, IB	100 µg	€384

Note: **ELISA**: enzyme-linked immunosorbent assay; **FC**: Flow cytometry; **FFS**: Free-floating section; **FS**: Frozen sections; **IB**: immunoblotting; **IC**: immunocytochemistry; **IP**: immunoprecipitation; **PS**: paraffin sections

Table III: Inducers of Apoptosis

Product Name	Cat. No.	M.W.	Size	Price
A23187, Free Acid, *Streptomyces chartreusensis*	100105	523.6	1 mg 5 mg 10 mg 50 mg	€35 €91 €146 €472
A23187, Mixed Calcium-Magnesium Salt	100106		10 mg	€132
N-Acetyl-L-cysteine	106425	163.2	5 g	€58
Actinomycin D, *Streptomyces* sp.	114666	1255.5	1 set 5 mg	€152 €89
Actinomycin D, 7-Amino-	129935	1270.4	1 mg	€133
AG 17	658425	282.4	5 mg	€58
AG 82	658400	202.2	5 mg	€63
AG 490	658401	294.3	5 mg	€49
Anandamide	172100	347.5	5 mg	€105
Anisomycin, *Streptomyces griseolus*	176880	265.3	10 mg	€58
Aphidicolin	178273	338.5	1 mg	€105
Apoptosis Inducer Set I	178486		1 set	€388
Apoptosis Inducer Set II	178489		1 set	€417
Bafilomycin A1, *Streptomyces griseus*	196000	622.8	10 µg	€159
Bak BH3 Fusion Peptide, Cell-Permeable	196350	4404.2	500 µg	€282
Bak BH3 Fusion Peptide, Cell-Permeable, Negative Control	196355	4362.2	500 µg	€282
Bcl-2 Binding Peptide, Cell-Permeable	197220	3399.9	1 mg	€289
Bcl-2 Binding Peptide, Cell-Permeable, Negative Control	197225	3357.8	1 mg	€289
Betulinic Acid	200498	456.7	5 mg	€129
Bleomycin Sulfate, *Streptomyces verticillus*	203401		15 U	€468
CAFdA	205500	303.7	1 mg	€143
Calphostin C, *Cladosporium cladosporioides*	208725	790.8	50 µg 100 µg	€106 €186
Camptothecin, *Camptotheca acuminata*	208925	348.4	50 mg	€82
CAPE	211200	284.3	25 mg	€105
Chelerythrine Chloride	220285	383.8	5 mg	€115
2-Chloro-2′-deoxyadenosine	220467	285.7	10 mg	€68
Colcemid	234109	371.4	5 mg	€106
Colchicine, *Colchicum autumnale*	234115	399.4	1 g 5 g	€72 €282
Corticosterone	235135	346.5	1 g	€117
Cyclophosphamide Monohydrate	239785	279.1	1 g	€97
Cyclosporin A, *Tolypocladium inflatum*	239835	1202.6	100 mg	€89
Daunorubicin, Hydrochloride	251800	564	5 mg	€82
Dexamethasone	265005	392.5	100 mg	€87
2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone	287805	259.1	50 mg	€51
Dolastatin 15	320900	837.1	1 mg	€227
Doxorubicin, Hydrochloride	324380	580	10 mg	€197
(–)-Epigallocatechin Gallate	324880	458.4	10 mg	€55

Table III: Inducers of Apoptosis - (continued)

Product Name	Cat. No.	M.W.	Size	Price
Etoposide	341205	588.6	25 mg	€58
Etoposide Phosphate	341206	668.6	5 mg	€79
ET-18-OCH3	341207	523.7	5 mg	€112
5-Fluorouracil	343922	130.1	1 g	€45
Folimycin, *Streptomyces* sp.	344085	866.1	10 µg	€91
Forskolin, *Coleus forskohlii*	344270	410.5	10 mg 25 mg 50 mg	€171 €384 €673
H-7, Dihydrochloride **Not for sale in Japan*	371955	364.3	1 mg 5 mg	€100 €353
Genistein	345834	270.2	20 mg 50 mg	€87 €173
[6]-Gingerol, *Zingiber officinale*	345868	294.4	5 mg	€153
Glycodeoxycholic Acid, Sodium Salt	361311	471.6	5 g	€128
H-89, Dihydrochloride **Not for sale in Japan*	371963	519.3	1 mg	€106
HA14-1	371971	409.2	1 set 2 mg	€329 €74
4-Hydroxynonenal	393204	156.2	1 mg	€91
4-Hydroxyphenylretinamide	390900	391.6	5 mg	€137
Hydroxyurea	400046	76.1	5 g	€72
Indanocine	402080	339.4	1 mg	€166
Ionomycin, Free Acid, *Streptomyces conglobatus*	407950	709	1 mg 5 mg 10 mg	€146 €506 €894
Ionomycin, Calcium Salt, *Streptomyces conglobatus*	407952	747.1	1 mg 5 mg 10 mg 25 mg	€116 €430 €683 €1369
Kaempferol	420345	286.2	25 mg	€82
KN-93	422708	501	1 mg 5 mg	€141 €424
Licochalcone-A, Synthetic	435800	338.4	10 mg 50 mg	€87 €321
Methotrexate	454125	454.5	100 mg	€98
Mitomycin C, *Streptomyces caespitosus*	47589	334.3	2 mg	€148
Mitomycin C, *Streptomyces caespitosus*, Carrier-Free	475820	334.3	10 mg	€145
MT-21	475952	281.4	10 mg	€112
Muristerone A, *Ipomoea* sp.	475946	496.6	1 mg	€230
(±)-S-Nitroso-N-acetylpenicillamine	487910	220.2	1 set 20 mg	€209 €90
S-Nitrosoglutathione	487920	336.3	1 set 10 mg 50 mg	€181 €61 €242
Okadaic Acid, *Prorocentrum concavum*	495604	805	10 µg 25 µg 100 µg	€51 €82 €275
Oligomycin	495455		10 mg	€85
p53 Activator, Cell-Permeable	506131	4434.1	500 µg	€304
Paclitaxel, *Taxus* sp.	580555	853.9	5 mg 25 mg 100 mg	€94 €365 €1301
Phorbol-12-myristate-13-acetate	524400	616.8	1 mg 5 mg 10 mg 25 mg	€58 €164 €294 €518

Table III: Inducers of Apoptosis - (continued)

Product Name	Cat. No.	M.W.	Size	Price
Puromycin, Dihydrochloride	540222	544.4	25 mg 100 mg	€54 €181
1-Pyrrolidinecarbodithioic Acid, Ammonium Salt	548000	164.3	100 mg	€44
Quercetin, Dihydrate	551600	338.3	100 mg	€35
Rapamycin	553210	914.2	100 µg 1 mg	€54 €266
Scriptaid	565730	326.4	5 mg	€137
Smac-*N*7 Peptide	567370	725.9	1 mg 5 mg	€81 €322
Smac-N7 Peptide, Cell-Permeable	567375	3051.7	1 mg	€275
Sodium Butyrate	567430	110.1	250 mg	€56
Sodium 4-Phenylbutyrate	567616	186.2	100 mg	€62
Spermine, Tetrahydrochloride	5677	348.3	5 g	€163
D-*erythro*-Sphingosine, Free Base, Bovine Brain	567725	299.5	10 mg	€81
D-*erythro*-Sphingosine, N-Acetyl-	110145	341.5	5 mg	€101
D-*erythro*-Sphingosine, N,N-Dimethyl-	310500	327.6	5 mg	€93
D-*erythro*-Sphingosine, N-Hexanoyl-	376650	397.6	5 mg	€89
D-*erythro*-Sphingosine, N-Octanoyl-	219540	425.7	5 mg	€89
Staurosporine, *Streptomyces* sp.	569397	466.5	100 µg 250 µg 1 mg	€176 €343 €938
Sulfasalazine	573500	398.4	100 mg	€38
Tamoxifen Citrate	579000	563.7	100 mg	€46
Tamoxifen, 4-Hydroxy-, (Z)-	579002	387.5	5 mg	€129
Sulindac Sulfide	574102	340.4	5 mg	€76
Thapsigargin	586005	650.8	1 mg	€114
α-Toxin, *Staphylococcus aureus* **Not for sale outside of U.S.*	616385	33,000	250 µg	*
Trichostatin A, *Streptomyces* sp.	647925	302.4	1 mg	€212
Valinomycin, *Streptomyces fulvissimus*	676377	1111.3	25 mg 100 mg	€79 €267
(±)-Verapamil, Hydrochloride	676777	491.1	100 mg	€51
Veratridine	676950	673.8	5 mg	€67
Vitamin D_3, 1α, 25-Dihydroxy-	679101	416.7	50 µg	€258
Vitamin E Succinate	679130	530.8	100 mg	€38

Table IV: Caspase Inhibitors

Product	Cat. No.	Sequence	Cell - Permable	Reversible?	Known Target Caspases (or Granzyme B)	Size	Price
Caspase Inhibitor I	627610*	Z-VAD(OMe)-FMK[a]	Yes	No	General	1 mg	€231
Caspase Inhibitor I, Biotin Conjugate	218742	Biotin-X-VAD(OMe)-FMK[a]	–	No	General	1 mg	€468
Caspase Inhibitor II	218735	Ac-VAD-CHO	No‡	Yes	General	1 mg	€81
Caspase Inhibitor II, Cell Permeable	218830	Ac-AAVALLPAVLLALLAPVAD-CHO	Yes	Yes	General	1 mg	€231
Caspase Inhibitor III	218745	Boc-D(OMe)-FMK[a]	Yes	No	General	250 µg 1 mg	€81 €204
Caspase Inhibitor IV	218784	Boc-D(OBzl)-CMK[b]	Yes	No	General	5 mg	€81
NEW Caspase Inhibitor VI	219007	Z-VAD-FMK[a]	–	No	General	250 µg 1 mg	€87 €260
NEW Caspase Inhibitor X	218723	BI-9B12	No	Yes	3, 7, 8	5 mg	€167
Caspase Inhibitor VIII	218729	Ac-VDVAD-CHO	No	Yes	2, 3, 7	1 mg	€129
Caspase Inhibitor, Negative Control	342000*	Z-FA-FMK	Yes	No	1	1 mg 5 mg	€89 €373
Caspase-1 Inhibitor I	400010	Ac-YVAD-CHO	No‡	Yes	1, 4	1 mg 5 mg	€81 €324
Caspase-1 Inhibitor I, Cell-Permeable	400011	Ac-AAVALLPAVLLALLAP-YVAD-CHO	Yes	Yes	1, 4	1 mg	€204
Caspase-1 Inhibitor II	400012	Ac-YVAD-CMK[b]	Yes	No	1, 4	5 mg	€283
Caspase-1 Inhibitor II, Biotin Conjugate	400022	Biotin-YVAD-CMK[b]	–	No	1, 4	5 mg	€365
Caspase-1 Inhibitor IV	400015	Ac -YVAD-AOM[c]	Yes	No	1, 4	1 mg	€109
Caspase-1 Inhibitor V	400019	Z-Asp-CH2-DCB[d]	Yes	No	1, 4	5 mg	€422
Caspase-1 Inhibitor VI	218746	Z-YVAD(OMe)-FMK[a]	Yes	No	1, 4	250 µg 1 mg	€116 €343
Caspase-2 Inhibitor I	218744	Z-VD(OMe)VAD(OMe)-FMK[a]	Yes	No	2	250 µg 1 mg	€148 €412
Caspase-2 Inhibitor II	218814	Ac-LDESD-CHO	No	Yes	2, 3	1 mg 5 mg	€93 €371
Caspase-3 Inhibitor I	235420	Ac-DEVD-CHO‡	No‡	Yes	3, 6, 7, 8, 10	1 mg 5 mg	€90 €343
Caspase-3 Inhibitor I, Biotin Conjugate	235422	Biotin-DEVD-CHO	–	Yes	3, 6, 7, 8, 10	1 mg	€145
Caspase-3 Inhibitor I, Cell-Permeable	235423	Ac-AAVALLPAVLLALLAP-DEVD-CHO	Yes	Yes	3, 6, 7, 8, 10	1 mg	€201
InSolution™ Caspase-3 Inhibitor I, Cell-Permeable	235427	Ac-AAVALLPAVLLALLAP-DEVD-CHO	Yes	Yes	3, 6, 7, 8, 10	1 mg	€222
Caspase-3 Inhibitor II	264155*	Z-D(OMe)E(OMe)VD(OMe)-FMK[a]	Yes	No	3, 6, 7, 8, 10	250 µg 1 mg	€102 €293
InSolution™ Caspase-3 Inhibitor II	264156	Ac-AAVALLPAVLLALLAP-DEVD-CHO	Yes	Yes	3, 6, 7, 8, 10	250 µg	€109
Caspase-3 Inhibitor II, Biotin Conjugate	218747	Biotin-X-D(OMe)E(OMe)VD(OMe)-FMK[a]	–	No	3, 6, 7, 10	1 mg	€461
Caspase-3 Inhibitor III	218750	Ac-DEVD-CMK[b]	Yes	No	3, 6, 7, 8, 10	1 mg 5 mg	€90 €343
Caspase-3 Inhibitor IV	235421	Ac-DMQD-CHO	No‡	Yes	3	1 mg 5 mg	€109 €353

Key: a: FMK = Fluoromethyl ketone; b: CMK = Chloromethyl ketone; c: AOM = 2,6-dimethylbenzoyloxy ketone; d: DCB = 2,6 dichlorobenzoyloxy.
‡: These aldehyde-based inhibitors may be cell-permeable, albeit to a lesser extent.
*Sold under license of U. S. Patents 5,210,272 and 5,344,939.

Table IV: Caspase Inhibitors - (continued)

Product	Cat. No.	Sequence	Cell - Permeable	Reversible?	Known Target Caspases (or Granzyme B)	Size	Price
Caspase-3 Inhibitor V	219002	Z-D(OMe)QMD(OMe)-FMK	Yes	No	3	1 mg	€337
NEW Caspase-3 Inhibitor VII	219012		Yes	Yes	3	1 mg	€145
Caspase-3/7 Inhibitor I	218826	5-[(S)-(+)-2-(Methoxymethyl) pyrrolidino]sulfonylisatin	Yes	Yes	3, 7	1 mg	€129
NEW Caspase-3/7 Inhibitor II	218832	Ac-DNLD-CHO	No‡	Yes	3, 7, 8, 9	1 mg	€123
Caspase-4 Inhibitor I	218755	Ac-LEVD-CHO	No‡	Yes	4	1 mg	€70
Caspase-4 Inhibitor I, Cell-Permeable	218766	Ac-AAVALLPAVLLALLAP-LEVD-CHO	Yes	Yes	4	1 mg	€222
Caspase-5 Inhibitor I	218753	Z-WE(OMe)HD(OMe)-FMK[a]	Yes	No	1, 4, 5	250 µg 1 mg	€150 €390
Caspase-6 Inhibitor I	218757	Z-VE(OMe)ID(OMe)-FMK[a]	Yes	No	6	250 µg 1 mg	€116 €324
Caspase-6 Inhibitor II, Cell-Permeable	218767	Ac-AAVALLPAVLLALLAP-VEID-CHO	Yes	Yes	6	1 mg	€212
Caspase-8 Inhibitor I, Cell-Permeable	218773	Ac-AAVALLPAVLLALLAP-IETD-CHO	Yes	Yes	8, Granzyme B	1 mg	€212
Caspase-8 Inhibitor II	218759	Z-IE(OMe)TD(OMe)-FMK[a]	Yes	No	8, Granzyme B	250 µg 1 mg	€123 €351
InSolution™ Caspase-8 Inhibitor II	218840	Z-IE(OMe)TD(OMe)-FMK[a]	Yes	No	8, Granzyme B	250 µg	€116
Caspase-9 Inhibitor I	218761	Z-LE(OMe)HD(OMe)-FMK[a]	Yes	No	9	250 µg 1 mg	€160 €397
InSolution™ Caspase-9 Inhibitor I	218841	Z-LE(OMe)HD(OMe)-FMK[a]	Yes	No	9	250 µg	€153
Caspase-9 Inhibitor II, Cell-Permeable	218776	Ac-AAVALLPAVLLALLAP-LEHD-CHO	Yes	Yes	9	1 mg	€242
Caspase-9 Inhibitor III	218728	Ac-LEHD-CMK[b]	Yes	No	9	1 mg	€116
Caspase-13 Inhibitor I	219005	Ac-LEED-CHO	No‡	Yes	13	1 mg	€56
Caspase-13 Inhibitor II	219009	Z-LE(OMe)E(OMe)D(OMe)-FMK[a]	Yes	No	13	250 µg 1 mg	€102 €307
CrmA, Recombinant	PF122	—	No	—	1, Granzyme B	100 µg	€684
Granzyme B Inhibitor I	368050	Z-AAD-CMK[b]	Yes	No	Granzyme B	1 mg	€81
Granzyme B Inhibitor II	368055	Ac-IETD-CHO	No‡	Yes	8, Granzyme B	1 mg	€70
Granzyme B Inhibitor IV	368056	Ac-IEPD-CHO	No	Yes	8, Granzyme B	1 mg	€109
Group III Caspase Inhibitor I	368620	Z-A-E(OMe)-V-D(OMe)-FMK[a]	Yes	No	6, 8, 9, 10	1 mg	€324
InSolution™ Q-VD-OPh, Non-O-methylated	551476	Q-Val-Asp-CH2-Oph	Yes	No	3, 8, 9, 10, 12	1 mg	€199
XIAP, Human, Recombinant, *E. coli*	PF137	—	No	—	3, 7	50 µg	€567

Key: a: FMK = Fluoromethyl ketone; b: CMK = Chloromethyl ketone.
‡: These aldehyde-based inhibitors may be cell-permeable, albeit to a lesser extent.

Technical Protocols:

I. Induction of Apoptosis

PROTOCOL FOR DNA DAMAGE–INDUCED APOPTOSIS (48 h)

The following protocol is based on p53-dependent G1-arrest that occurs in response to DNA damage by chemical agents such as doxorubicin, 5-fluorouracil, paclitaxel, and vinblastine. A typical time course for p53 and $p21^{WAF1}$ induction is 40 to 48 hours treatment with a DNA-damaging agent. Other proteins involved in apoptosis are also induced (although not all proteins involved in apoptosis will be induced by a particular agent in a given cell type). We recommend taking several time points (i.e., 24, 48, and 72 hours). Maximal induction of $p21^{WAF1}$ requires wild-type p53 activity. In the absence of wild-type p53, p21WAF1 can also be induced by serum stimulation of G1-arrested cells or by treatment with agents such as dexamethasone, albeit at significantly lower levels than that seen upon p53-dependent induction.

Day 1: Inoculate 2 or more 10-cm tissue culture dishes for adherent cells 1 x 10^6 cells/dish or T-75 flasks for non-adherent cells with approximately (1-5 x 10^5 cells/ml). One dish or flask will be used as a negative control for uninduced or basal level expression.

Day 2: Confirm that cells are growing by visual inspection of tissue culture dishes or by viable cell counts on non-adherent cells in T-75 flasks. Add DNA damaging agents at the indicated final concentration. Add appropriate volume of buffer or solvent to the uninduced control.

Day 3: Check cells to determine if cells have begun to die. If too few cells are dead, incubate for additional time and check again. Harvest cells if greater than 75% of the cells appear to have died.

Day 4: Harvest cells and prepare lysates for either immunoblotting or immunoprecipitation. For any agent used, a time course of induction can be performed by inoculating additional dishes or flasks and harvesting at various times after addition of the DNA damaging agent.

Day 5: Resolve proteins on SDS-PAGE. Visualize the protein of interest from total lysates by immunoblotting using chemiluminescent detection.

Always compare levels of p53 or $p21^{WAF1}$ from treated cells with those from untreated controls to confirm induction. For γ irradiation treatment to induce p53 and $p21^{WAF1}$. (See El-Deiry, et al. 1994. *Cancer Res.* **54**, 1169 or Deng, et al. 1995. *Cell* 82, **675**.)

II. Caspase Assays (Colorimetric and Fluorometric)

The colorimetric caspase substrates can be used to measure the induction of caspase activity in apoptotic cells, or to screen for activators and inhibitors of caspases.

This is a general protocol designed for use with a microplate reader. The amounts of cell extract, substrate, inhibitor, and p-nitroaniline (pNA) used are for an assay volume of 140 µl. The researcher may scale up this procedure to use in a spectrophotometer. Optimization is recommended.

SOLUTIONS, REAGENTS, AND EQUIPMENT

- Caspase Substrate
- Caspase inhibitor
- Cell Lysis Buffer: 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT, 100 µM EDTA, pH 7.4
- Assay Buffer: 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 µM EDTA, 10% glycerol, pH 7.4
- PBS (phosphate buffered saline): dissolve 8 g of NaCl, 200 mg of KCl, 1.44 g of Na_2HPO_4 in 800 ml of distilled water; adjust the pH to 7.4 with HCl; add distilled water to 1 L

• 96-well plate and 96-well plate reader

Recommended Additional Materials

• Purified Caspase

• *p*-Nitroaniline (*p*NA)

PREPARATION OF CELL EXTRACTS

Induce apoptosis in cells by using an appropriate apoptosis inducing agent (see previous protocol). Controls may include untreated cells, cells treated with an inactive analog of the apoptosis inducer (if available), or a "time-zero" sample from the apoptosis induction time course. A sufficient number of cells must be used to assay caspase activity in duplicate, and must include an inhibitor control to determine protein concentration. In general, the number of cells described below will give an adequate protein concentration for the 96-well plate assay; however, differences in cell size, volume, and protein concentration may necessitate increased plating densities. When 2 x 10^7 cells/ml are used for lysis, the protein concentration will be about 1-3 mg/ml and a 10 µl assay sample will contain 10-30 µg of protein. Depending on the cell line, a minimum of 10^6 cells in 50 µl lysis buffer are required.

- Count cells and harvest by centrifugation. Wash cells one time with PBS. If the cells have been treated with a potential caspase inhibitor, it may be necessary to wash more thoroughly to prevent any adverse effects.
- Resuspend cells to the desired concentration using ice-cold lysis buffer. Incubate 5 minutes on ice.
- Centrifuge at 10,000 x g, 10 minutes at 4°C.
- Save the supernatant (cytosolic extract) and hold on ice until use. Extracts can be flash-frozen in an acetone/ethanol bath and stored at -70°C for later use.

ASSAY PROCEDURE

Preparation of stock solutions of caspase substrate and inhibitor.

1. Prepare a 100 mM stock solution of substrate in DMSO. Prepare a 1:50 dilution (2 mM) of the stock solution in assay buffer. Aliquot and freeze the remaining stock solution at -20°C.
2. Prepare a 100 mM stock solution of inhibitor in DMSO. Prepare a 1:1000 dilution (100 µM) in assay buffer and then prepare a working (500 nM) stock by further diluting 5 µl into 1 ml assay buffer. Aliquot and freeze the remaining stock solution at -20°C.
3. Add the appropriate amount of Assay Buffer to each well of the 96-well plate (see table below). Blank and cell extract samples are essential for determining cellular activity. To measure non-specific hydrolysis of caspase substrate, preparation of an inhibitor-treated cell extract is recommended. Using a positive-control sample which includes purified caspase enzyme is also recommended. See table on next page.
4. Allow the 96-well plate to equilibrate to 37°C. The assay may also be performed at room temperature; however, the rate of substrate cleavage will be higher at 37°C.
5. Add 10 µl of cell extract to the appropriate wells. Do not add cell extract to the blank (control) wells.
6. Add 20 µl known inhibitor (final concentration 100 nM) or test inhibitor to the appropriate wells.
7. Pre-incubate the plate at the assay temperature for 10 minutes (or as desired) to allow enzyme/inhibitor interaction.
8. Start the reaction by addition of 10 µl pNA-conjugated substrate that has been pre-equilibrated to the assay temperature. The final concentration will be 200 µM.
9. Read the absorbance (A) at 405 nm in a 96-well plate reader. Record data at 1-10 minute intervals for 30–120 minutes (as desired, depending on your caspase activity and the amount of protein).

DATA ANALYSIS

1. Plot data as A_{405} versus time for each sample.
2. For each sample, determine the initial time period over which the plot of absorbance versus time remains linear, and if there is sufficient change in absorbance (ΔA) to obtain an accurate slope. The initial substrate concentration (200 µM) is saturating. For most samples, the rate of substrate cleavage will remain constant for up to 2 hours or more. However, highly active samples can reduce the substrate to sub-saturating levels during the course of the experiment. Therefore, choose the data from the early, linear portion of the curve for use in the slope calculation.
3. Obtain the slope of the line fitted to the linear portion of the data, using a suitable linear regression program.
4. Average the slopes of replicate samples.
5. If the blank has a significant slope, subtract this number from all the samples.
6. The above data will give a qualitative indication of caspase activity. To quantify the caspase activity in the samples, express as pmol substrate hydrolyzed/min.
7. Determine the 96-well plate reader conversion factor:
   A. Prepare a 50 µM stock of *p*NA-conjugated standard in assay buffer. Add 100 µl to 2 96-well plate wells.
   B. Determine the average A_{405} using 100 µl assay buffer as a blank.
   C. Calculate the conversion factor. This calculation is based on the concentration of *p*NA in the calibration standard (50 µM). The extinction coefficient for pNA in the assay buffer is 10,000 $M^{-1}cm^{-1}$.
   D. Conversion factor (µM/absorbance) = 50 µM ÷ Average A^{405}.
8. Calculate the activity as pmol substrate hydrolyzed/min: Activity (pmol/min) = slope (absorbance/min) x conversion factor (µM/absorbance) x assay volume (µl).

Assay Mixture Examples

Sample	Assay Buffer	Cell Extract	Purified Caspase (~2U/ml)	Inhibitor	Substrate
Blank	90 µl	0	0	0	10 µl
Cell Extract	80 µl	10 µl	0	0	10 µl
Inhibitor-Treated Extract	60 µl	10 µl	0	20 µl	10 µl
Purified Caspase	75 µl	0	15 µl	0	10 µl
Test Sample/Cell Extract	60 µl	10 µl	0	20 µl	10 µl
Test Sample/Purified Caspases	55 µl	0	15 µl	20 µl	10 µl
Cell Extract/Purified Caspase	65 µl	10 µl	15 µl	0	10 µl

III. General Guidelines for Using Fluorometric Substrates for Measuring Caspase Activity

SUBSTRATE PRINCIPLE

A synthetic peptide substrate is labeled with AFC (7-amino-4- trifluoromethylcoumarin), a fluorescent molecule, to form a fluorogenic compound that can be used for measuring caspase activity. Alternatively, the substrate may be conjugated to other fluorescent molecules, such as AMC (7-amino-4-methylcoumarin).

When AFC is attached to the substrate, it produces a blue fluorescence upon exposure to light (excitation max.: ~400 nm). Caspase enzymatically cleaves the AFC-substrate and releases free AFC. Free AFC produces a yellow fluorescence (emission max.: ~505 nm).

AFC has several advantages over other fluorogenic labels. The larger Stoke's shift between bound and free AFC enables the substrate to be both chromogenic (yellow-green color is visible to the naked eye) and fluorogenic (detection of emission at ~505 nm with a fluorimeter). With a larger Stoke's shift, greater sensitivity can be achieved.

ASSAY PRINCIPLE

A fluorimeter is first calibrated with known amounts of free AFC or AMC. The release of AFC or AMC in the reaction mixture is monitored with a fluorimeter. Caspase activity in the sample is proportional to the amount of free AFC or AMC produced. A unit is defined as the amount of caspase required to produce 1 pmol of AFC (or AMC)/min at 25°C at saturating substrate concentrations.

EXAMPLE

Purified or partially purified caspase preparations (~15 ng enzyme). If the sample is not purified, a negative control containing a specific caspase inhibitor should be assayed.

GENERAL FLUOROMETRIC ASSAY PROCEDURE

1. Prepare a 500 µM stock solution of caspase substrate in DMSO.
2. Prepare a 500 µM stock solution of caspase inhibitor in DMSO.
3. Buffer: 100 mM HEPES, 10% sucrose, 10 mM DTT, 500 M EDTA. Adjust pH to 7.5 using 0.1 N NaOH or HCl.
4. Prepare several dilutions of sample using the caspase buffer (see 3 above).
5. Ideally, each sample dilution should be tested in three different reaction mixtures:
   - Substrate only (blank)
   - Sample + inhibitor + substrate (negative control)
   - Sample + substrate (sample)
6. Prepare a calibration curve by measuring known amounts of AFC (excitation max: ~400 nm; emission max: ~505 nm) or AMC (excitation max: ~380 nm; emission max: ~460 nm) in a fluorometer.
7. Reaction with inhibitor should be started first because of the time required for the inhibitor to react with the sample before substrate addition. A preliminary time course for maximum effect should be determined. Example: Mix 440 µl of caspase buffer with 20 µl of inhibitor in a tube, add 20 µl sample. Mix gently. Incubate at 30°C for 30 min to 12 h.
8. To blank tubes add 480 µl of buffer, 20 µl of substrate.
9. Add 20 µl substrate to negative control tubes.
10. To sample tubes add 460 µl of caspase buffer, 20 µl of substrate. Mix well then add 20 µl of sample.
11. Incubate all tubes at 30°C for 60 minutes and measure fluorescence for time zero.
12. Measure fluorescence after another 60 minutes (t1).
13. Calculate change in fluorescence (ΔFU) for each sample at t1 as follows:
14. ΔFU = (sample FU at t1 – blank FU at t1) – (Sample FU at time zero – Blank FU at time zero)
15. Calculate enzyme activity for t1. If the activity is low, assay should be allowed to proceed for a longer time (up to 24 h).
16. For final results, use sample dilution that gives highest sample reading and lowest negative control reading.

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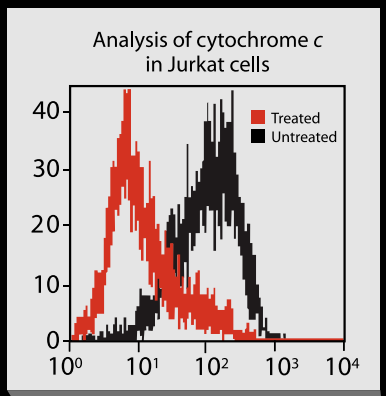

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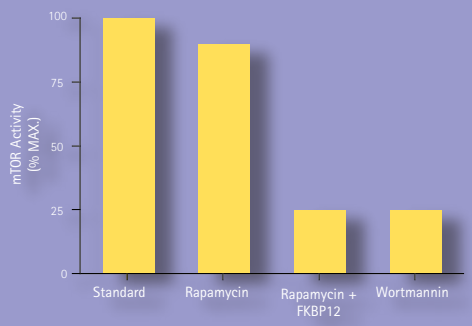

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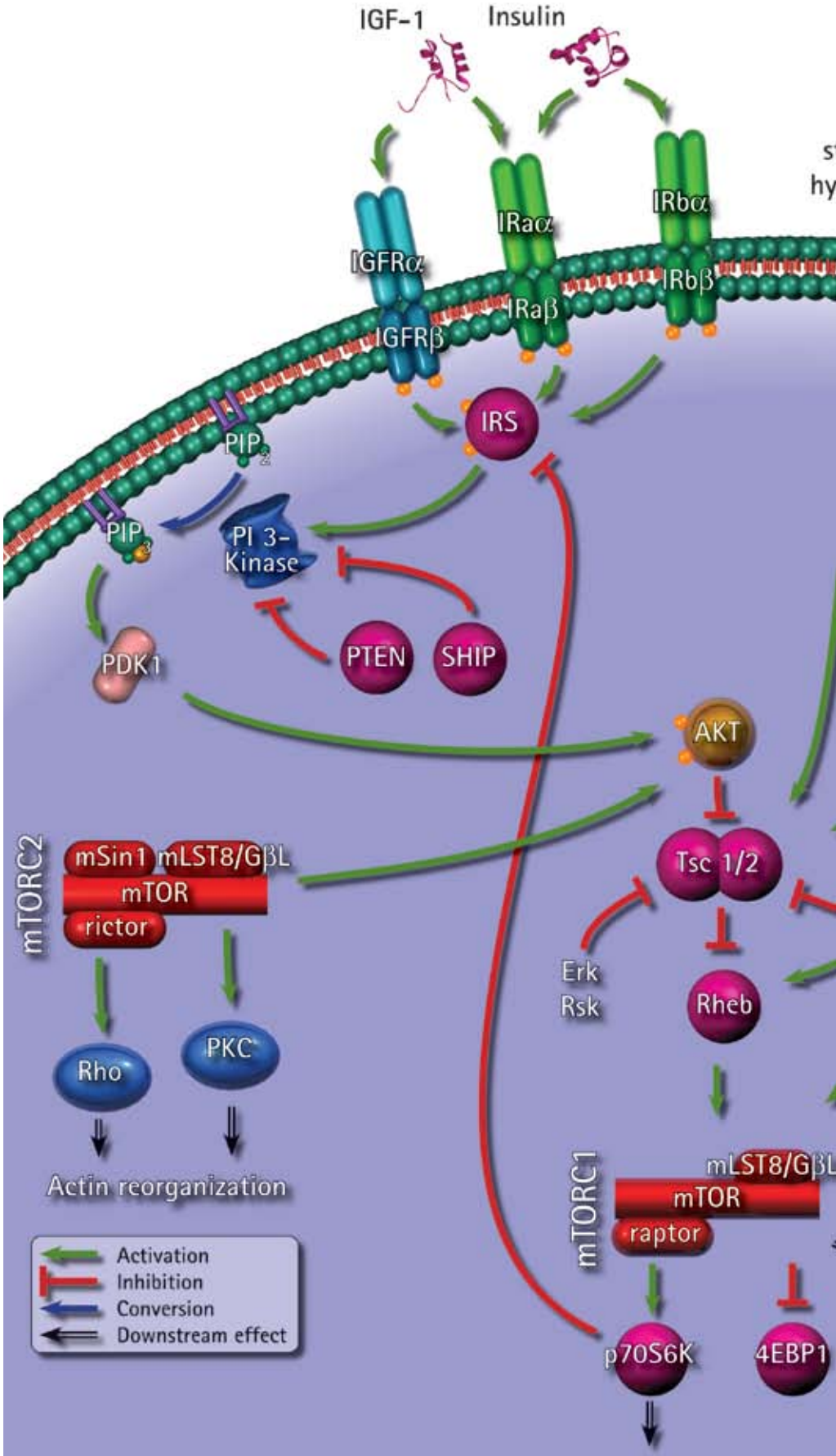

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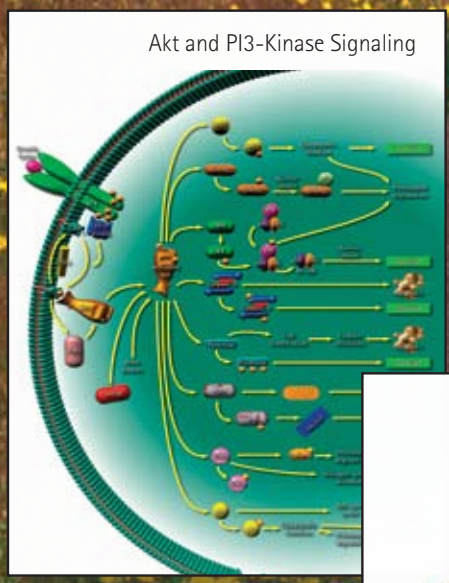

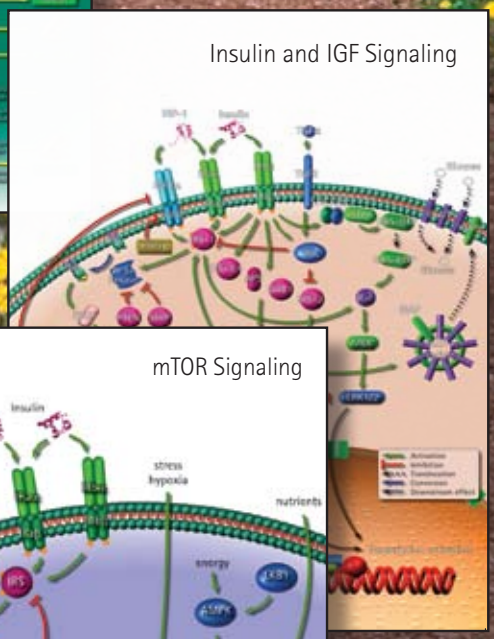

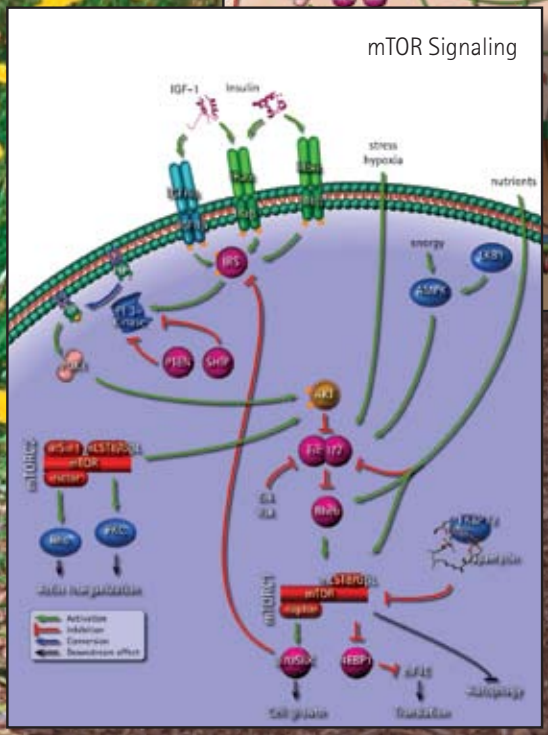

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