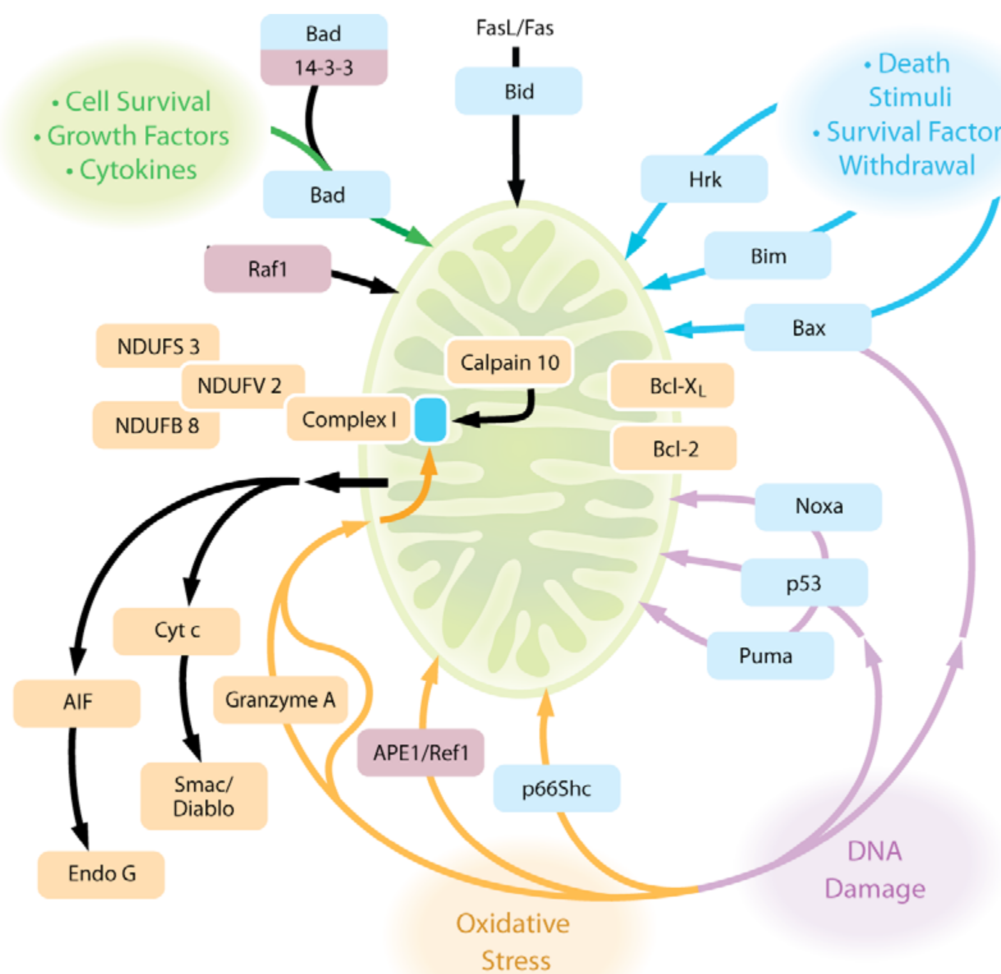
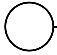
MitoSciences®

THE APOPTOSIS PLAYBOOK

Tips and Techniques for Researching Apoptosis Using Tools from MitoSciences





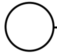
I. Introduction

Apoptosis, also known as programmed cell death, can be triggered by external stimuli or from within the cell. Mitochondria have been called the central executioner because of their key role in inducing apoptosis in response to cellular stresses such as DNA damage, oxidative stress and reduced efficiency in energy production.

Mitochondria also play a part in response to external stimuli by amplifying the orderly cell death response through processes in many cases similar to those found for the intra-cellular stress response. The key to the signaling and the execution of the cell death response is redistribution of, and alterations in the association of, a set of cytosolic and mitochondrially-localized pro-apoptotic and anti-apoptotic proteins. These include the Bax and Bcl-2 families of proteins.

Alterations in the ratio of Bax and Bcl-2-like proteins regulate the permeability of the mitochondrial outer membrane and can result in the release of several proteins from the inter-membrane space, including cytochrome *c*, SMAC/Diablo and AIF (apoptosis-inducing factor). Cytochrome *c* then reacts with a cytosolic protein APAF to induce an irreversible cascade of events involving caspases that lead to the orderly degradation of proteins and DNA.

MitoSciences provides a range of products for conducting research into apoptosis, all of which focus on measuring the movement of proteins into and out of mitochondria.



Two approaches are offered, one of which begins with the fractionation of cells into mitochondrial, cytoplasmic, and nuclear fractions, after which the fractions can then be probed with Western blotting antibodies or cocktails, or the target proteins can be quantitated with immunocapture microplate ELISA's or dipstick assays. The other approach uses 2-dye immunocytochemistry to image the release of cytochrome *c* in fixed cells.

Useful Recent Reviews

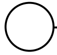
Cytochrome c: functions beyond respiration.
Ow YL, Green DR, Hao Z, Mak TW.
Nat Rev Mol Cell Biol. 2008 Jul;9(7):532-42. Review.

How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?
Chipuk JE, Green DR.
Trends Cell Biol. 2008 Apr;18(4):157-64. Epub 2008 Mar 7. Review.

Methods to dissect mitochondrial membrane permeabilization in the course of apoptosis.
Galluzzi L, Vitale I, Kepp O, Séror C, Hangen E, Perfettini JL, Modjtahedi N, Kroemer G.
Methods Enzymol. 2008;442:355-74.

The pathophysiology of mitochondrial cell death.
Green DR, Kroemer G.
Science. 2004 Jul 30;305(5684):626-9. Review.

Mitochondrial dynamics and apoptosis: a painful separation.
James DI, Martinou JC.
Dev Cell. 2008 Sep;15(3):341-3.



II. Cell Fractionation Analysis

A. Choice of Cell Type for Apoptosis Studies

Cell fractionation is a reliable and potentially high-content approach to studying the translocation of proteins in apoptosis, but there is an important point with respect to the appropriate choice of cell type that is emphasized by the data in Figure 1: namely that not all cells have equivalent levels of the various apoptotic factors, nor do they respond equally to apoptotic stimuli. Furthermore, the "steady state" distribution of some apoptotic factors varies between different fractions.

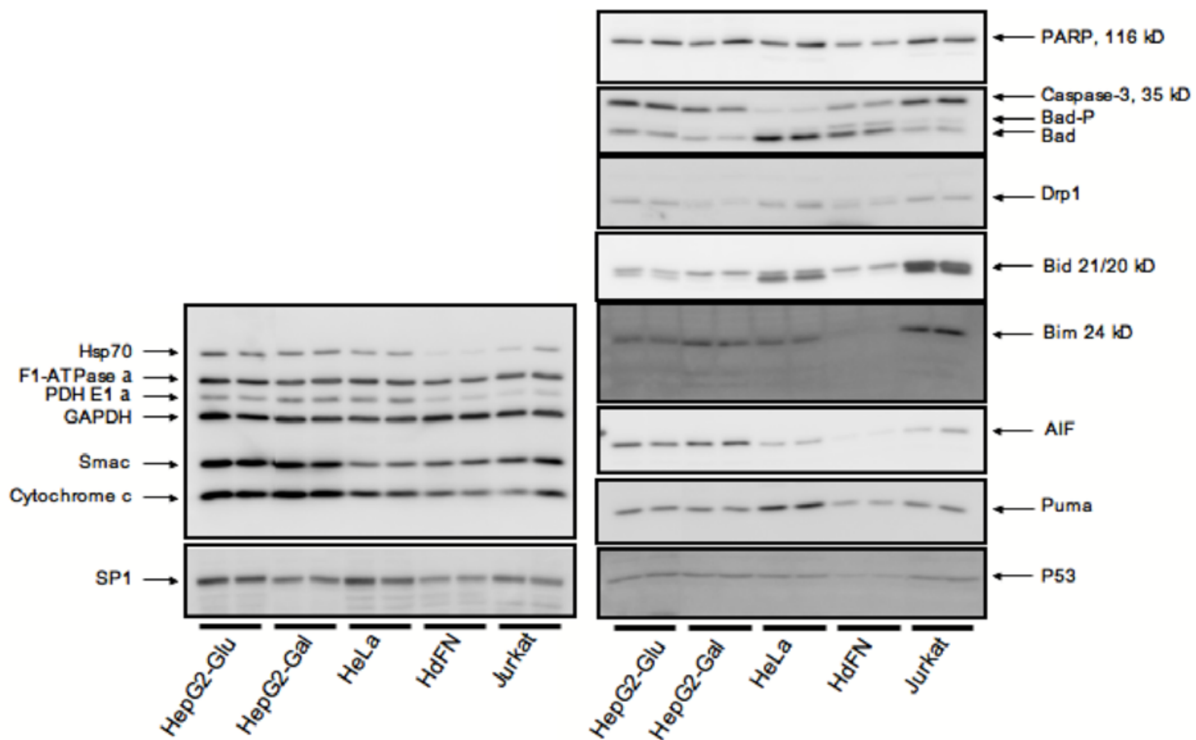
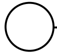


Figure 1. Steady-state levels of proteins involved in apoptosis and cellular compartment markers in various cell lines, analyzed by Western blotting analysis. The targets on the left were probed using the ApoTrack™ Cytochrome c Apoptosis Antibody Cocktail, to which antibodies against Smac/DIABLO and Hsp70 were added. SP1, a nuclear marker, was also probed separately. The targets in the gels on the right were probed using individual antibodies available from MitoSciences and from Cell Signaling Technology®.



Further cautionary notes: 1) the levels of metabolic enzymes differ widely between different cell lines, and not always in accordance with the tissue type from which they were generated, 2) It is sometimes forgotten that the most commonly used cell lines e.g. HepG2, HeLa, etc., are transformed, and finally, 3) all cell lines are lazy, and if you give them enough glucose they will produce their energy by glycolysis rather than by oxidative phosphorylation. For many experiments it is preferable to grow cells in galactose plus glutamine to force them to use mitochondrial energy production.

B. Cell Fractionation Kits

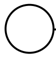
MitoSciences has developed two novel cell fractionation kits, neither of which require mechanical disruption nor complicated differential centrifugation to resolve the mitochondrial fraction from cytosol.

Additionally, the MitoSciences kits separate the mitochondrial fraction from one containing nucleus and other organelles, thereby allowing analysis of movements of proteins between mitochondria and nucleus, as well as movements of kinases and redistribution of transcription factors as part of the apoptotic process.

These kits have been validated HeLa, HepG2, Jurkat, and 143b cells, and are suitable for other adherent cell types. The overall procedure can be done in two formats, one for high-content analysis and the second for high-throughput applications.

Cell Fractionation Kit Standard (MS861)

For high-content analysis using Antibody Cocktails (see Section C ahead) this kit is used to fractionate cells which have been grown in 15 cm plates. The cell sample is released from the plate by trypsinization and collected before the



fractionation procedure. Fractions can then be probed with Antibody Cocktails which include apoptosis markers along with control markers for each fraction.

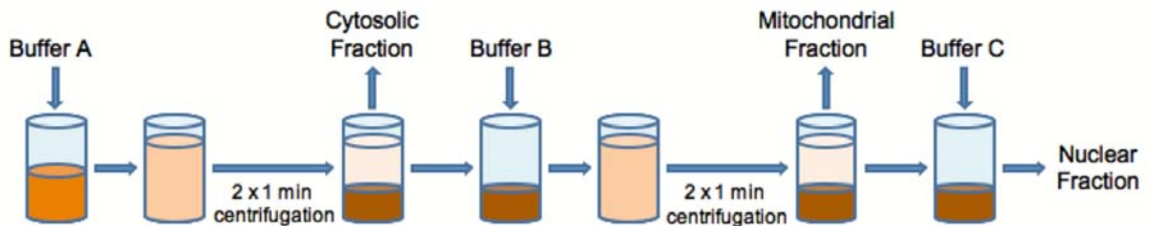


Figure 2. Schematic representation of fractionation of cell suspension into cytoplasmic, mitochondrial and nuclear fractions using Cell Fractionation Kit Standard (MS861).

Cell Fractionation Kit HT (MS862)

This high-throughput kit allows for the sequential release of the cytosol, mitochondrial and nuclear fractions from cells grown in a 96-well plate while the cells remain adherent to the plate. Protein detection using ELISA microplate, Western blot or dipstick assays can then used for analysis.

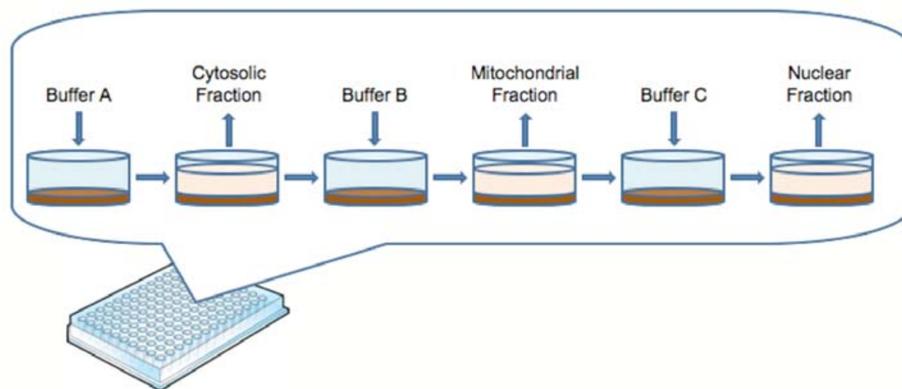
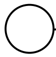


Figure 3. Schematic representation of fractionation of adherent cells into cytoplasmic, mitochondrial and nuclear fractions using the Cell Fractionation Kit HT (MS862).



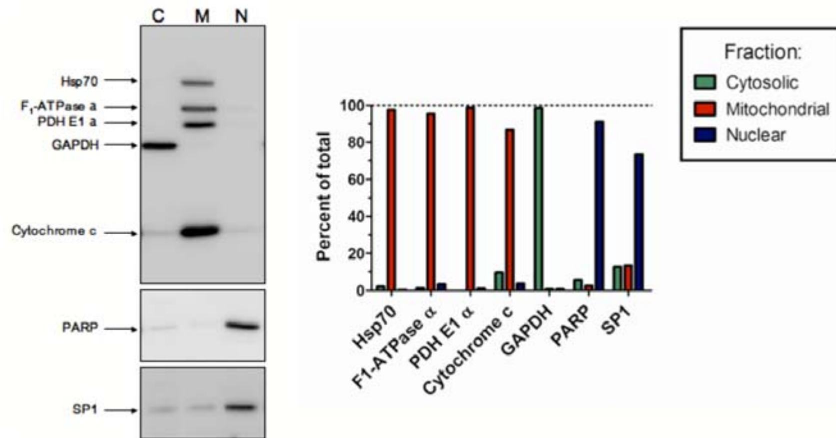


Figure 4. Characterization of cytosolic (C), mitochondrial (M) and nuclear (N) fractions prepared using Cell Fractionation Kit Standard from HepG2 cells. Fractions were analyzed by western blotting using the ApoTrack™ Cytochrome c Apoptosis Antibody Cocktail.

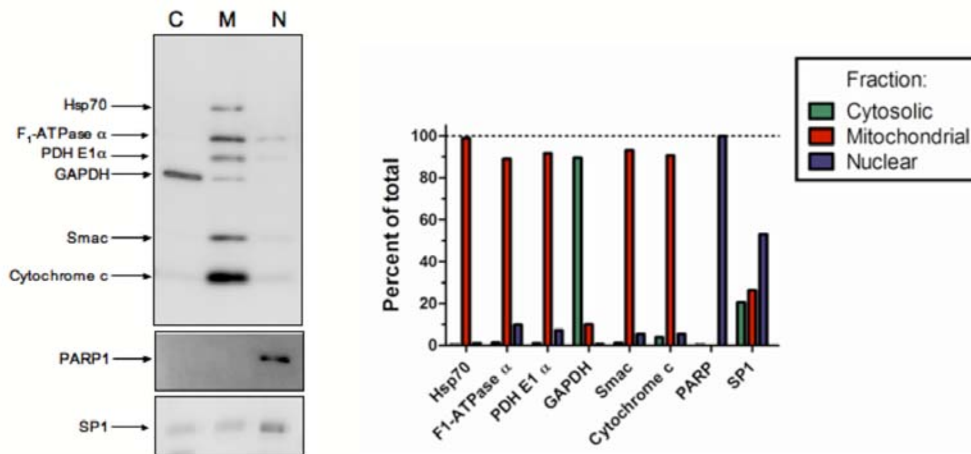
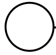


Figure 5. The same targets were probed as in Figure 4 above (with the addition of Smac/DIABLO), but in HeLa cells fractionated using the Cell Fractionation Kit HT.

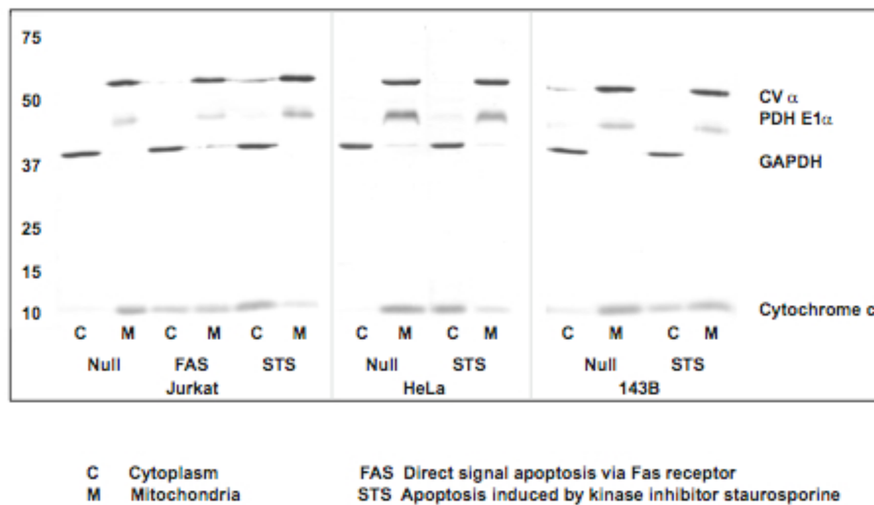
Cat. No.	Product Name	Format	Price
MS861	Cell Fractionation Kit Standard	15 cm	$195
MS862	Cell Fractionation Kit HT	96 well	$195



C. Antibody Cocktails for High-Content Analysis

Any evaluation of the movements of proteins involved in apoptosis requires both the detection of the various protein players in the cell death process along side the appropriate markers for the different compartments (cytoplasm, mitochondria, and nucleus). MitoSciences has simplified and made cost-effective the overall analysis by combining antibodies into cocktails that monitor several key proteins at once.

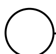
The **ApoTrack™ Cytochrome *c* WB Antibody Cocktail (MSA12)** is used to track the movement of cytochrome *c* out of mitochondria during apoptosis. The cocktail contains a mouse monoclonal antibody against cytochrome *c*, plus 3 monoclonal antibodies against control targets, including: 1) the α subunit of the F_1ATPase as a marker of the mitochondrial inner membrane, 2) the E1α subunit of the pyruvate dehydrogenase complex as a marker of the mitochondrial matrix, and 3) GAPDH as a marker of the cytosol.



C Cytoplasm
M Mitochondria

FAS Direct signal apoptosis via Fas receptor
STS Apoptosis induced by kinase inhibitor staurosporine

Figure 6. In this experiment, apoptosis was induced in Jurkat and 143B osteosarcoma cells by FAS and also by treatment with staurosporine (HeLa cells were also treated, but only with STS). Mitochondrial and cytoplasmic fractions were isolated (using kit Cell Fractionation Kit MS861) and probed using MSA12. As is clear from the gels, cytochrome c has translocated partially in FAS-induced cells and STS-treated osteosarcoma cells, and almost completely in STS-treated Jurkat and HeLa cells. The three control targets allow for verification of the "cleanness" of the cell fractionation.

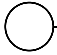


Cat. No.	Product Name	Apps.	Species Reactivity	Price
MSA12	ApoTrack™ Cytochrome *c* WB Antibody Cocktail	WB	human, mouse, rat, bovine	$425

D. Individual Antibodies

All of the antibodies offered in the aforementioned Antibody Cocktails are also available individually, as are several antibodies against other important apoptosis targets.

Cat. No.	Product Name	Apps.	Species Reactivity	Price
MSA09	AIF monoclonal antibody	WB	human, mouse, rat	$325
MS502	ATP synthase subunit α monoclonal antibody	WB, ICC	human, mouse, rat, bovine, zebrafish	$325
MSA06	Cytochrome c monoclonal antibody	WB, ICC	human, mouse, rat, bovine	$325
MSA92	GAPDH monoclonal antibody	WB	human	$325
MSP03	PDH E1α monoclonal antibody	WB, ICC	human, mouse, bovine	$325
MS715	Smac monoclonal antibody	WB, ICC	human	$325



E. Microplate Assays for High-Throughput Analysis

For studies that require the simultaneous analysis of many different samples, MitoSciences provides microplate ELISA assays for key apoptosis targets. Included among such studies are those that focus on cytochrome *c* as a circulating biomarker for head injury, hepatitis, and various other diseases. MitoSciences' ELISA tests can detect concentrations of cytochrome *c* in the range reported to be present in the different biological fluids under these circumstances.

MitoSciences has 96-well sandwich ELISA assay kits that use a capture mAb to isolate the protein being evaluated and then a second detector mAb to quantitate the amount of target protein present.

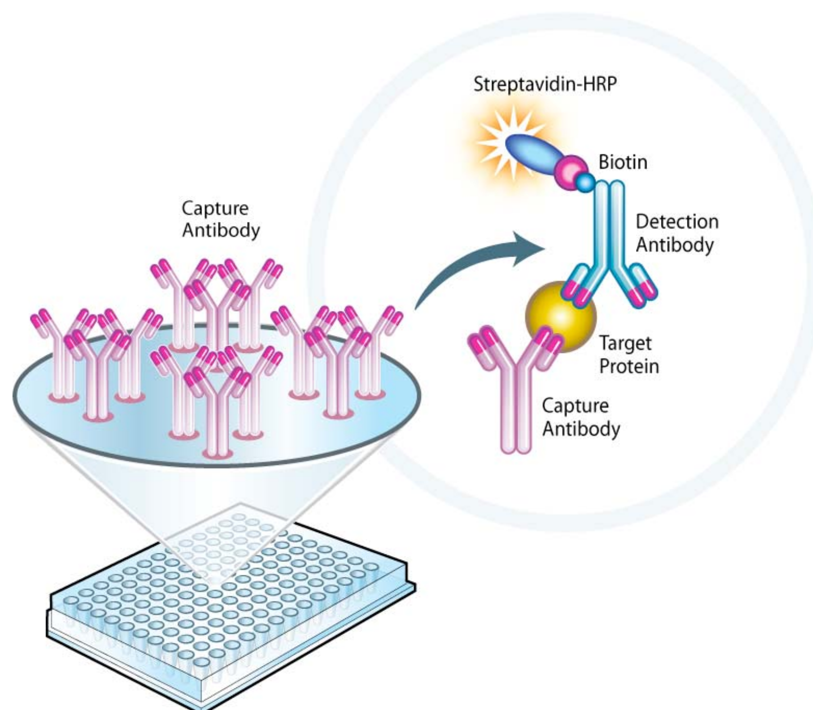
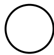


Figure 8. Schematic representation of sandwich ELISA assay concept.



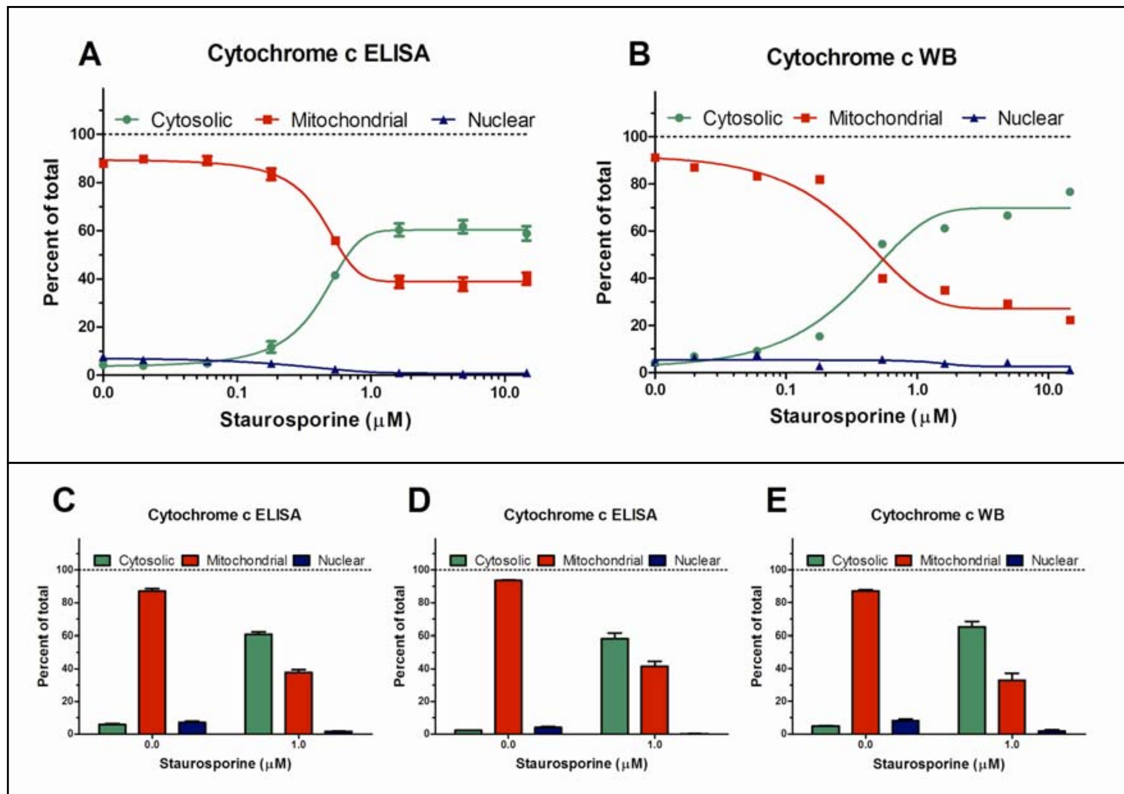
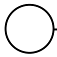


Figure 9. Qantitative ELISA analysis of cytochrome c release from the mitochondria into the cytosol in HeLa cells induced to undergo apoptosis by Staurosporine treatment. Cytosolic (C), mitochondrial (M) and nuclear (N) fractions of HeLa cells treated for 4 hrs with 0.00, 0.02, 0.06, 0.18, 0.54, 1.62, 4.86 and 14.58 μM Staurosporine (**A** and **B**) or with 0.0 and 1.0 μM Staurosporine (**C**, **D**, **E**) were prepared with Cell Fractionation Kit HT (MS862). Fractions, each derived from one well of 96-well plate, were analyzed by Cytochrome c Protein Quantity Microplate Assay Kit (MSA41) (**A**, **C** and **D**). Parallel analyses of fractions prepared independently and thus representing inter-assay variation of the Cell Fractionation Kit HT are shown in **C** and **D**. Western blot analysis of cytochrome *c* using ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (MSA12) is shown for comparison (**B** and **E**). Data represent mean +/- standard error of the mean, n=4 (A and C), n=3 (D), n=2 (E), n=1 (B).

Cat. No.	Product Name	Apps.	Species	Price
MSA42	Apoptosis-Inducing Factor (AIF) Protein Quantity Microplate Assay Kit	ELISA	human	$425
MSA41	Cytochrome *c* Protein Quantity Microplate Assay Kit	ELISA	human, mouse, rat. bovine	$425



F. Dipstick Assay for Rapid and Simple Analysis

Sometimes detection of AIF levels is a part of a larger project and a quick but quantitative measurement is needed. When the desire for instant gratification takes hold and you want immediate results, MitoSciences provides an assay for AIF in dipstick form. Results are quantitative and yet are obtained in as little as 30 minutes.

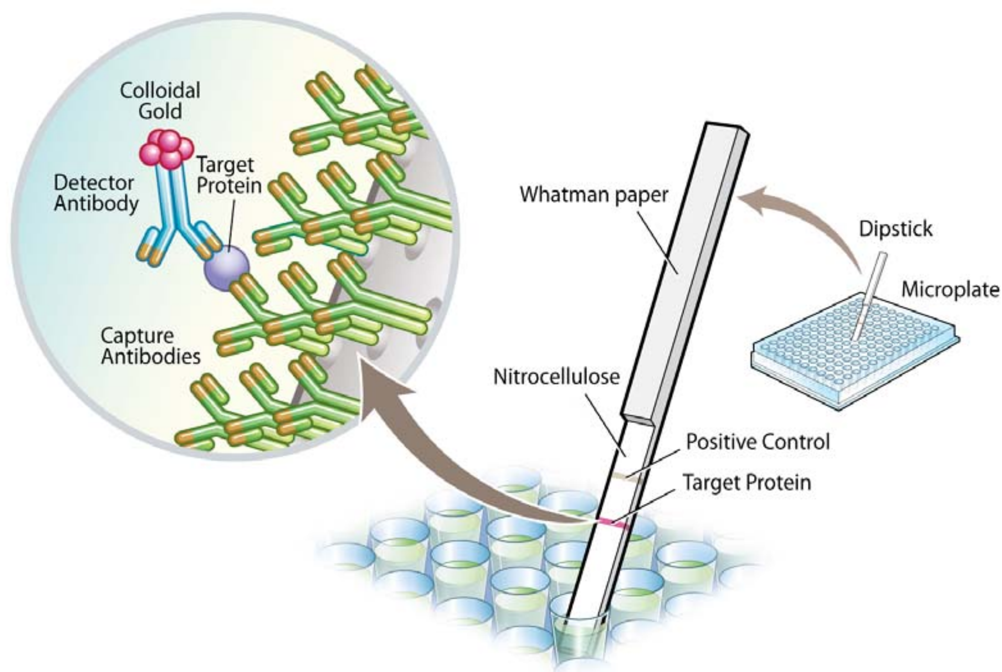
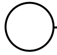


Figure 10. Schematic representation of protein quantity dipstick assay.

Cat. No.	Product Name	Apps.	Species	Price
MSA31	Apoptosis-Inducing Factor (AIF) Protein Quantity Dipstick Assay Kit	ELISA	human	$325



III. Fixed Cell Analysis

ApoTrack™ Cytochrome c Apoptosis ICC Antibody Kit

Apoptotic cells which have released mitochondrial cytochrome *c* into the cytosol can be differentiated from non-apoptotic cells which still retain cytochrome *c* in their mitochondria by fluorescence microscopy.

These kits contain a cytochrome *c* monoclonal antibody and an isotype-specific secondary antibody which has a fluorescein fluorophore conjugated to it. The kit also contains an ATP synthase V subunit α monoclonal antibody and the appropriate isotype-specific secondary antibody which has a Texas Red fluorophore conjugated to it.

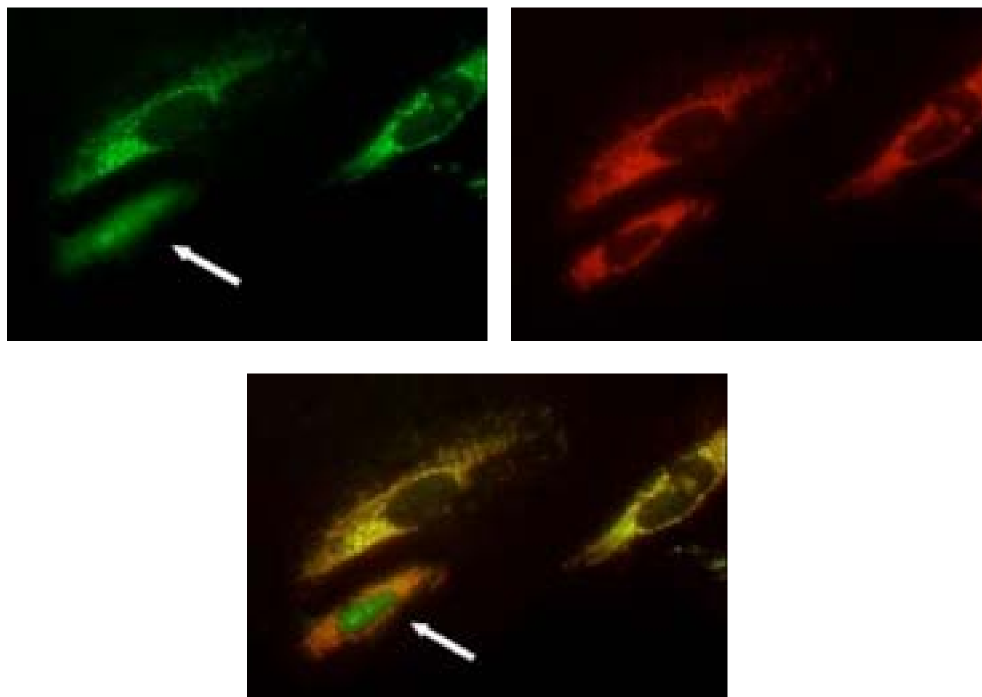
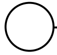


Figure 11. Staurosporine-treated HeLa cells analyzed with anti-cytochrome *c* and anti-ATP synthase subunit α antibodies. The white arrow indicates cytochrome *c* release from mitochondria, making the identification of apoptotic cells particularly easy when the images are merged.



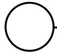
ATP synthase is an inner mitochondrial membrane protein and serves as a mitochondrial marker since it is not released into the cytoplasm during apoptosis, unlike cytochrome *c*. Thus one is able to observe cytochrome *c* and a mitochondrial marker (ATP synthase subunit alpha) simultaneously by fluorescence microscopy using MSA07. Buffers and blocking solution for immunocytochemical analysis are also included in the kit.

Cat. No.	Product Name	Apps.	Species	Price
MSA07	ApoTrack™ Cytochrome *c* Apoptosis ICC Antibody Kit	ICC	human	$425

V. Other Apoptosis Research Areas

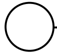
A. The Emerging Role of Complex I in Apoptosis

In the last couple of years a key role of the respiratory chain Complex I has been uncovered. This enzyme connects electron transfer rates and thus respiratory chain efficiency to oxidative free radical production, which in turn, induces apoptosis. It appears that certain cellular stress events lead to translocation of proteases including the granzymes or to activation of intra-mitochondrial proteases such as calpain, which cleave subunits of complex I. The result is reduced enzyme turnover or less efficient electron transfer through the complex which leads to increased production of superoxide and other free –radicals. The end point of this cascade is apoptosis.



MitoSciences provides a kit (MS101) that can immunocapture Complex I, as well as mAbs with which to evaluate which subunits have been cleaved by the various proteases. In fact, the original publication showing granzyme A cleave of Complex I used MitoSciences mAbs to demonstrate the event.

Cat. No.	Product Name	Apps.	Species Reactivity	Price
MS101	Complex I Immunocapture Kit	IP	human, mouse, rat, bovine	$375
MS109	Complex I subunit 8 kDa monoclonal antibody	WB	human, mouse, rat, bovine	$325
MS103	Complex I subunit GRIM-19 monoclonal antibody	WB, ICC	human, mouse, rat, bovine	$325
MS111	Complex I subunit NDUFA9 monoclonal antibody	WB	human, mouse, rat, bovine	$325
MS107	Complex I subunit NDUFB4 monoclonal antibody	WB, ICC	human, rat, bovine	$325
MS108	Complex I subunit NDUFB6 monoclonal antibody	WB	human, mouse, rat, bovine	$325
MS105	Complex I subunit NDUFB8 monoclonal antibody	WB	human, mouse, rat, bovine	$325
MS110	Complex I subunit NDUFS3 monoclonal antibody	WB, ICC	human, mouse, rat, bovine zebrafish	$325
MS104	Complex I subunit NDUFS4 monoclonal antibody	WB	human, mouse, rat, bovine	$325



B. The Curious Case of the Disintegrating Permeability Transition Pore

It took many years after the original description of a mitochondrial permeability transition that led to cell death for this concept to be taken seriously. After this work of the 1970's the mystique of the PTP grew rapidly and was the explanation for everything adverse that happen to mitochondria. A pore was identified of ever increasing complexity, which at one time contained hexokinase of the cytosol, porin and the peripheral benzodiazipine binding protein of the outer membrane, adenylate kinase of the inter-membrane space, the adenine nucleotide translocase of the inner membrane, and cyclophilin d of the matrix space.

Then, knockouts (in mice) knocked out these components one by one. Porin went, the adenine nucleotide translocase went, cyclophilin d went. So what is left? Clearly new thinking and novel experiments are needed. MitoSciences may not be able to help with the former but we have excellent mAbs against porin, adnenine nucleotide translocase and cyclophilin d with which you can test your ideas.

Cat. No.	Product Name	Apps.	Species Reactivity	Price
MSA02	ANT monoclonal antibody	WB, ICC	human, rat, bovine, C. elegans	$325
MSA04	cyclophilin d monoclonal antibody	WB, ICC	human, mouse, rat, bovine	$325
MSA03	porin (VDAC) monoclonal antibody	WB	human, mouse, rat, bovine, Drosophila	$325

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