

## In-Cell ELISA (ICE) Assay Platform

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Rev.0

### Monitoring apoptosis in cells: a high-throughput, quantitative cell-based assay.

#### Introduction:

##### *Apoptosis:*

Apoptosis is a self-killing mechanism of genetically-programmed cell death that is involved in normal development and homeostasis in the animal kingdom. The deregulation of apoptosis plays a major role in variety of pathologies, including cancer, autoimmunity, immunodeficiency and neurodegenerative disease (1-4). The apoptotic program of cell death is orchestrated by cysteine-dependent aspartate-directed proteases (caspases) through cleavage of variety proteins causing cell shrinkage, nuclear fragmentation, chromatin condensation and membrane blebbing, and leading to phagocytic recognition and engulfment of the dying cells (5).

Apoptosis typically proceeds through two different pathways, the extrinsic (receptor-mediated) pathway and the intrinsic pathway (6, 7). Both pathways converge by activation of executioner caspases (caspase-3 and caspase-7). In the intrinsic pathway Bax and/or Bak-dependent mitochondrial outer membrane permeabilization (MOMP) leads to release of cytochrome c and other pro-apoptotic factors from the mitochondrial intermembrane space (IMS) to the cytosol. The released cytochrome c forms with apoptosis protease-activating factor 1 (APAF1) apoptosome, a scaffold for activation of an initiator caspase (caspase-9). Active caspase-9 then activates the executioner caspases. The caspase function is inhibited by X-linked inhibitor of apoptosis protein (XIAP) which function is neutralized by two other proteins released from IMS, second mitochondrial-derived activator of caspase (Smac, also known as DIABLO) and OMI (also known as HTRA2).

In the receptor-mediated pathway specific ligands (or agonist antibodies) bind to CD95 or TRAIL-R1/-R2 receptors of the plasma membrane causing receptors' oligomerization and recruitment of Fas-associated protein with death domain (FADD) to their intracellular domain (death domain, DD). Subsequently, FADD recruits procaspase-8 and 10 and FADD-like interleukin -1 b converting enzyme (FLICE)-like inhibitory protein (cFLIP). This, so-called death-inducing signaling complex (DISC) promotes homodimerization and activation of initiator caspases (caspase-8 and 10), which are then processed to their mature forms (6).

The downstream pathway of activation of the executioner caspases is cell type-dependent. Some cells (Type II cells) process only small amounts of caspase-8 at the DISC complex and thus they require amplification of the apoptotic signal through mitochondria. In these cells, active caspase-8 cleave BH3-only member of Bcl-2 family Bid. The cleaved Bid (tBid) translocates to mitochondria and activates Bax and Bak, resulting in MOMP and ultimately activation of executioner caspases as described above. Other cells (Type I cells) process large amounts of caspase-8 at the DISC complex. In these cells the executioner caspases are directly activated by the active caspase-8 without involvement of mitochondrial-dependent signal amplification by caspase-9.

MOMP is highly regulated process, primarily controlled through the interactions between pro- and anti-apoptotic factors of the B cell lymphoma 2 (Bcl-2) protein family (7-9). These proteins can be divided into three groups based on their Bcl-2 homology domains (BH): (1) the pro-apoptotic effectors, members that contain BH1, BH2 and BH3 domains (Bax, Bak and Bok) and actually cause mitochondrial outer membrane permeabilization; (2) the pro-apoptotic BH3-only proteins (Bad, Bid, Bim, BMF, BNIP3, HRK, Noxa and Puma) that transduce signals to the effectors; and (3) the anti-apoptotic Bcl-2 proteins, members that contain all four (BH1-BH4) homology domains (Bcl-2, Bcl-XL, Bcl-W, A1 and Mcl-1). However, there is a little evidence that Bok is a functional effector. In healthy cells the function of Bax and Bak effectors is kept in check (neutralized) by the anti-apoptotic members of Bcl-2 family. There are currently two major model of Bax and Bak activation. According the indirect activation model, Bax and

Bak are in a constitutively active state and their function is neutralized by the anti-apoptotic Bcl-2 proteins. During apoptosis the competitive interaction of the BH3-only proteins with the anti-apoptotic Bcl-2 proteins frees the effectors. In the direct activator-derepressor model, Bax and Bak are directly activated by a subset of BH3 only Bcl-proteins (direct activators) and the anti-apoptotic Bcl-2 proteins inhibit MOMP by either neutralizing the BH3-only proteins or inhibiting the activated Bax and Bak. According this model, a second subset of BH3-only proteins (sensitizers) can not directly activate Bax and Bak, but neutralize the anti-apoptotic Bcl-2 proteins. Although the exact mechanisms of these interactions still need to be clarified, the propensity of cells to undergo apoptosis clearly depends on complex interplay of these regulators which is cell-type and trigger-dependent.

Assaying proteins participating in apoptosis, namely protein levels and post-translational modifications of Bcl-2 family proteins and other regulators, as well as measuring re-localization of cytochrome c, Smac, AIF and Bax, activation of caspases, and proteolytic cleavage of their downstream targets, such as poly-ADP-ribose 1 (PARP-1) or BAP31, is frequent in both basic and applied areas of research including molecular mechanism of apoptotic signaling and mechanism of drug-induced apoptosis both in toxicology and disease therapy. The full understanding of this complex biological process often requires measurement of many analytes, under variety of conditions, including time- and dose-dependent measurements in a variety of cells. This creates demands for a reliable yet simple high throughput multi-analyte protein assay platform. Traditional methods of analysis mainly include low throughput and semi-quantitative Western blotting or flow cytometry instrumentation. This application note demonstrates the utility of the In-Cell ELISA (ICE) technique to monitor levels of proteins participating in or being markers of apoptotic cascade in cultured cells.

ICE, also known as fixed-cell ELISA or In-Cell Western™, is a high-throughput, quantitative and very reproducible cell-based method to determine protein levels and their post-translational modifications in cultured cells. The sample preparation is very simple; adherent or suspension cells exposed to desired conditions are fixed in 96 or 384 well microplates. The fixation “freezes” biological processes and eliminates unwanted changes during sample processing, thus allowing measurements of in vivo levels of rapidly changing analytes. ICE is a quantitative version of immunocytochemistry utilizing primary and labeled secondary antibodies. In-well duplexing is possible with IRdye®-labeled secondary antibodies and a LI-COR Odyssey® Infrared imager. Alternatively, single analytes per well can be measured colorimetrically using a spectrophotometric plate reader. Inter-well normalization can be achieved using a control protein readout (e.g. tubulin) or with a whole cell stain (e.g. Janus Green). Given the flexibility, throughput and reproducibility, ICE is a powerful platform for characterizing the protein composition of cells and for examining alterations in protein levels and their modifications induced by variety of conditions including drug treatment and thus it is well suited to study apoptosis. More information and resources for ICE applications are available on the MitoSciences website ([www.mitosciences.com/in-cell-elisa.html](http://www.mitosciences.com/in-cell-elisa.html)).

In this experiment we investigate the relative steady-state levels of many pro- and anti- apoptotic regulators of Bcl-2 protein family in HeLa, HL-60 and HepG2 cells. We correlate their levels to the cell line-specific sensitivities to apoptosis by the protein kinase C inhibitor Staurosporine. In addition, we examined the time-dependent changes in these and other proteins which are well known components or markers of the apoptotic cascade. The measured proteins are listed in Table I.

## Materials and Methods:

*Cell culture, treatment and fixation of adherent cells:* HeLa and HepG2 cells were cultured in standard DMEM media supplemented with 10% FCS. To prepare the samples for the ICE assay, 50,000 cells per 100 µL were seeded per well of 96 well Assay Plate supplied with In-Cell ELISA Support Pack (MitoSciences, MS922), and allowed to attach overnight. For the cell treatments media were removed and 100 µL per well of fresh media supplemented with a drug of interest (or as a control, with the drug vehicle, dimethylsulfoxide) was added. At the end of the treatment, the plates with cells were centrifuged

for 8 min at 500 x g and 100 µL of freshly prepared 8% paraformaldehyde in phosphate buffered saline (PBS) was gently added. The plates were re-centrifuged and incubated for additional 15 minutes at RT. The fixed cells were washed 3 x with PBS and stored refrigerated in PBS containing 0.02% sodium azide.

Protein Name	UniProt	Product	Manufacturer Cat. #
Bcl-2 homologous antagonist/killer (Bak)	<a href="#">Q16611</a>	ICE-validated antibody	MitoSciences MSR24
Apoptosis regulator Bax (Bax)	<a href="#">Q07812</a>	ICE-validated antibody	MitoSciences MSR25
Apoptosis regulator Bcl-2 (Bcl-2)	<a href="#">P10415</a>	ICE-validated antibody	MitoSciences MS776
Apoptosis regulator Bcl-X, isoform L (Bcl-XL)	<a href="#">Q07817</a>	ICE-validated antibody	MitoSciences MSR26
Induced myeloid leukemia cell differentiation protein (Mcl-1)	<a href="#">Q07820</a>	ICE-validated antibody	MitoSciences MSR28
BCL2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3)	<a href="#">Q12983</a>	ICE-validated antibody	MitoSciences MS778
Caspase-3 (cleaved)	<a href="#">P42574</a>	ICE-validated antibody	MitoSciences MSR27
Caspase-9 (cleaved)	<a href="#">P55211</a>	Fluorescence Microscopy Kit	Thermo 8402301
Cytochrome c	<a href="#">P99999</a>	WB, ICC, FC, ICE-validated antibody	MitoSciences MSA06
p53 up-regulated modulator of apoptosis (Puma)	<a href="#">Q9BXH1</a>	ICE-validated antibody	MitoSciences MSR29
Pyruvate dehydrogenase E2/E3 binding protein (PDH E2/E3BP)	<a href="#">P10515</a> <a href="#">O00330</a>	ICC, WB, FC-validated antibody	MitoSciences MSP06
Poly [ADP-ribose] polymerase 1 (PARP-1)	<a href="#">P09874</a>	WB, ICC, FC, ICE-validated antibody ICE kit	Mitosciences MS777 MSA43

**Table I. Analyzed proteins and antibodies used.** This table lists the assayed proteins and the primary antibodies or antibody kits used in ICE, immunocytochemistry (ICC) and/or Western Blot (WB) assays.

**Cell culture, treatment and fixation of suspension cells:** HL60 cells were cultured in standard RPMI1640 media supplemented with 10% FCS. To prepare the samples for the ICE assay, 120 µL per well of cell suspension at  $4 \times 10^6$  cells/ml was seeded in a round-bottom 96 well plate. The treatment was started immediately by an addition of 40 µL of drug (or as a control, the drug vehicle, dimethylsulfoxide) diluted in culture media. After the treatment 100 µL of cell suspension (about 300,000 cells) was transferred from each well into wells of the 96 well Assay Plate supplied with In-Cell ELISA Support Pack (MitoSciences, MS922). The plates with transferred cells were centrifuged for 8 min at 500 x g and fixed as described for the adherent cells.

**ICE assay:** The fixed cells were permeabilized for 30 min with 0.1% Triton X-100 in PBS, blocked for 2 hours in 20% Blocking Buffer (Sigma B6429) in PBS and incubated overnight at 4 C with 100 µL of primary antibody(ies) diluted in PBS supplemented with 10% Blocking Buffer. To determine the background signal, the cells were incubated in the absence of the primary antibody. All primary antibodies used in ICE were validated by the manufacturer by immunocytochemistry and Western blot analysis. The primary antibodies are listed in Table I. The following day cells were washed 3 x with 0.05% Tween 20 in PBS, incubated for 2 hours at RT with 100 µL of goat anti-rabbit IRdye®680LT and/or goat anti-mouse IRdye®800CW secondary antibody(ies) (as appropriate) diluted to 0.4 µg/mL in PBS supplemented with 10% Blocking Buffer and washed 4 x with 0.05% Tween 20 in PBS. The plates were then scanned using

the LI-COR Odyssey® infrared imager. Subsequently, plates were stained with 0.3% Janus Green, washed thoroughly with water. The cell-bound dye was released with 1 M HCl and read at OD595 on a SpectraMax plate reader.

*ICE analysis:* The average background intensity of each secondary antibody and experimental condition (treatment) used on each plate was determined from the signal of wells stained in the absence of the primary antibody(ies); this value was then subtracted from the signals of remaining wells that were stained with the primary antibody(ies). The resulting background-corrected signal was then divided by the Janus Green total cell stain signal to obtain normalized signal to account for differences in cellular amount (due to seeding imperfection or to effect of treatment). %CV was determined for each dose and time-point by dividing the standard deviation of the normalized signal by the mean normalized signal. In some experiments graphs were generated by plotting the normalized signal for each dose and time-point relative to the vehicle treated wells.

*Cell fractionation and Western blotting:* HeLa cells were fractionated into cytosolic, mitochondrial and nuclear fraction using the MitoSciences Cell Fractionation Kit –Standard (MS861). Equal volumes of cytosolic, mitochondrial and nuclear fractions were analyzed by Western blotting according MitoSciences protocol (<http://www.mitosciences.com/PDF/western.pdf>) with the use of HRP-conjugated secondary antibodies and chemiluminescence detection (ECL PLUS, GE Healthcare). The primary antibodies used are listed in Table I. The blots were imaged by UltraLum imaging system.

*Immunocytochemistry and cytochrome c re-localization assay.* The fixed cells were permeabilized, blocked and incubated with primary antibodies as described for the ICE method. For Bax and Bak antibody stained samples, Alexa Fluor® 488-conjugated goat anti-rabbit antibody (Life Technologies) was used. For pyruvate dehydrogenase subunit E2/E3 binding protein (PDH E2/E3BP) antibody stained samples, Alexa Fluor®647-conjugated goat anti-mouse antibody were used. Samples were imaged by confocal microscopy using Opera LX HCA (PerkinElmer). For the cytochrome c re-localization assay samples were prepared as above using cytochrome c antibody and Alexa Fluor488 conjugated goat anti-mouse antibody, and co-stained with DAPI. As cytochrome c is released from mitochondria in apoptosis, the released cytosolic cytochrome c diffuses also to nuclear region. This re-localization is the principal of the assay. The nuclear and cytoplasmic regions of every analyzed cell were determined based on their respective high intensity and low-intensity signals of DAPI staining. Then the medians of cytochrome c intensities in both the cytoplasmic and nuclear regions were determined in each sample. Finally, the nuclear minus cytoplasmic intensity median difference of cytochrome c was calculated.

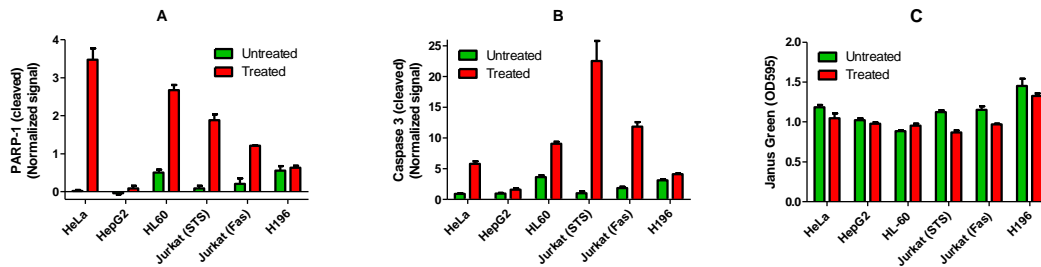
## Results:

### *I. ICE analysis of expression levels of Bcl-2 family proteins as an indicator of cell sensitivity to undergo apoptosis*

The sensitivity of a variety of cancer cell lines to undergo apoptosis by treatment with an inducer of the intrinsic pathway of apoptosis, protein kinase C inhibitor Staurosporine, or with an inducer of the receptor-mediated pathway of apoptosis, the Fas antibody, was tested (Figure 1). Cell lines analyzed included human cervical adenocarcinoma HeLa cells, human hepatocellular carcinoma HepG2 cells, human acute promyelocytic leukemia HL-60 cells, acute T cell leukemia Jurkat cells and small cell lung carcinoma H196 cells. The induction of apoptosis was measured using the PARP-1 (cleaved) ICE assay (MSA43, Figure 1A). Under these conditions, an increase of cleaved PARP-1 was detected in HeLa, HL-60 and Jurkat cells treated with Staurosporine and Jurkat cells treated with the Fas antibody. However, Staurosporine did not induce any significant PARP-1 cleavage in HepG2 and H196 cells. Using parallel samples, the levels of apoptosis were also measured using the caspase-3 (cleaved) ICE assay (MSR27, Figure 1B). As in the cleaved PARP-1 assay, an increase of cleaved caspase-3 was detected in HeLa, HL-60 and Jurkat cells treated with Staurosporine and Jurkat cells treated with Fas antibody. Staurosporine did not induce any significant caspase-3 cleavage in HepG2 and H196 cells. These experiments indicated that HepG2 and H196 cells are resistant to undergo apoptosis by treatment with

1 $\mu$ M Staurosporine for 6 hours. Note that the untreated cells had various cell line-dependent levels of spontaneous apoptosis as indicated by the levels of cleaved PARP-1 and cleaved caspase -3 (Figure 1 A and B).

The adherent cells undergoing apoptosis often detach from the culture plate. The cell detachment can lead to the cell loss and thus underestimating the proportion of apoptotic cells. Note that little/no loss of apoptotic cells was detected in the ICE assay, as indicated by measurement of Janus Green total cell stain after the ICE (Figure 1C). Thus the ICE assay performed as described in the Materials and Methods accurately reflects the relative levels of apoptosis in culture.

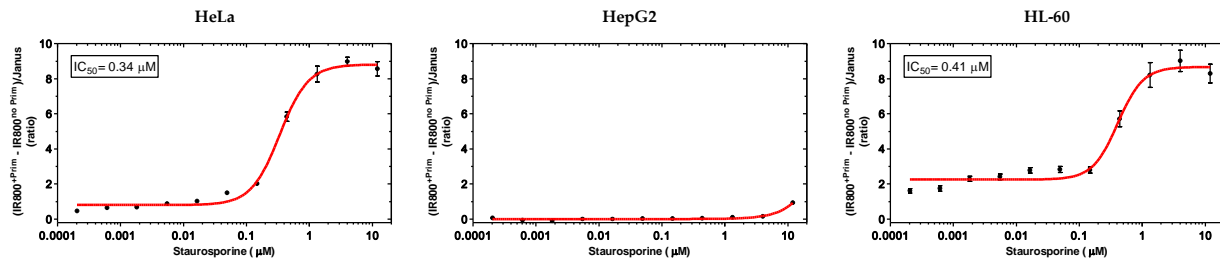


**Figure 1. Cell line-dependent sensitivity to undergo apoptosis.** Adherent cells were seeded (HeLa and HepG2 at 50,000 per well, H196 at 150,000 per well) directly in Assay Plate, allowed to attach overnight and treated with 1  $\mu$ M Staurosporine (STS) as indicated. Suspension cells were treated with 1  $\mu$ M STS or 50 ng/mL Fas antibody as indicated and transferred (HL-60 at 300,000 per well, Jurkat at 200,000 per well) in media containing 10% serum to the Assay Plate. Cells were fixed and parallel samples were analyzed by ICE using caspase-3 (cleaved) or PARP-1 (cleaved) antibodies as described in Materials and Methods. Mean and standard error of the mean (n=3) is shown. (A) Cleaved PARP-1 normalized to cell amount measured by Janus Green whole cell stain. (B) Cleaved caspase-3 normalized to cell amount measured by Janus Green whole cell stain. (C) Cell amount measured by Janus Green.

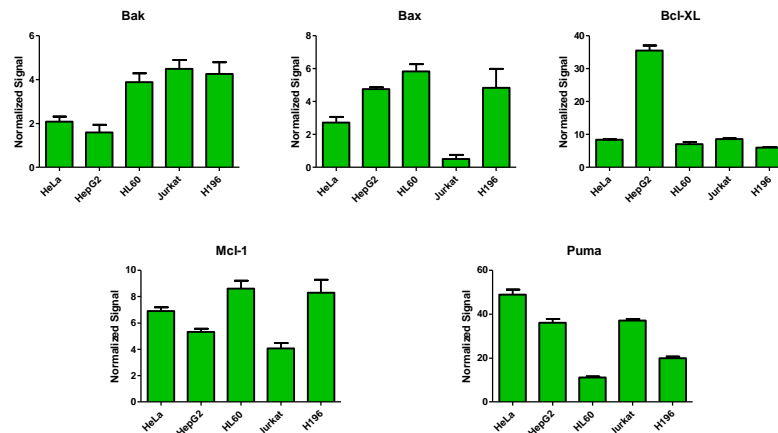
In an attempt to quantify the cell-type specific differences in sensitivity of cells to undergo apoptosis by Staurosporine treatment, Staurosporine IC<sub>50</sub> value of cleaved PARP-1 were determined in HeLa, HepG2 and HL-60 cells (Figure 2). In HeLa and HL-60 cells the levels of cleaved PARP-1 exhibited typical saturable sigmoid response to the amount of Staurosporine used with respective IC<sub>50</sub> values of 0.34  $\mu$ M and 0.41  $\mu$ M. However, no significant cleaved PARP-1 was detected in HepG2 cells if treated with up to 12  $\mu$ M Staurosporine, indicating a complete resistance of this cell line to undergo apoptosis under the experimental conditions.

Varieties of cancer cells have decreased sensitivity to undergo apoptosis or acquire resistance after a prolonged drug treatment. Deregulation of Bcl-2 protein family often contributes to cancer progression or drug resistance (9-13). The levels of each individual pro- and anti-apoptotic members of Bcl-2 protein family and their ratio (e.g. ratio of Bax/Bcl-2) are predominantly assayed by Western blotting. Here we have utilized the ICE as being a highly reproducible and high throughput technique to measure levels of several Bcl-2 proteins (Figure 3). The ICE signal measured with a Bcl-X antibody was nearly four-fold higher in HepG2 compared to Bcl-X signal in HeLa, HL-60, Jurkat and H196 cells. The Bcl-X antibody used in this assay has the propensity to recognize both the anti-apoptotic L form (of 26 kDa) and the pro-apoptotic S form (of 19 kDa). Since this Bcl-X-based ICE assay can't distinguish between these two isoforms, Western blotting was used to determine the proportion of the L and S isoforms of Bcl-X (Figure 4). The Western blot analysis revealed that the only detectable form of Bcl-X in HepG2 and HeLa (healthy or apoptotic) cells is the anti-apoptotic L isoform, indicating that the signal measure by ICE corresponds to the Bcl-XL. This result is consistent with previous study reporting that Staurosporine-induced switching of the L isoform into the S isoform described in 293 cells does not occur in cancer cells (14). As in the ICE platform the Western blotting confirmed that Bcl-XL is highly expressed in HepG2 cells compared to HeLa cells (Figure 4). The levels of Mcl-1 were not dramatically increased in HepG2 cells when compared to other cell lines analyzed (Figures 3 and 4) and the levels of Bcl-2 protein were extremely low in this cell

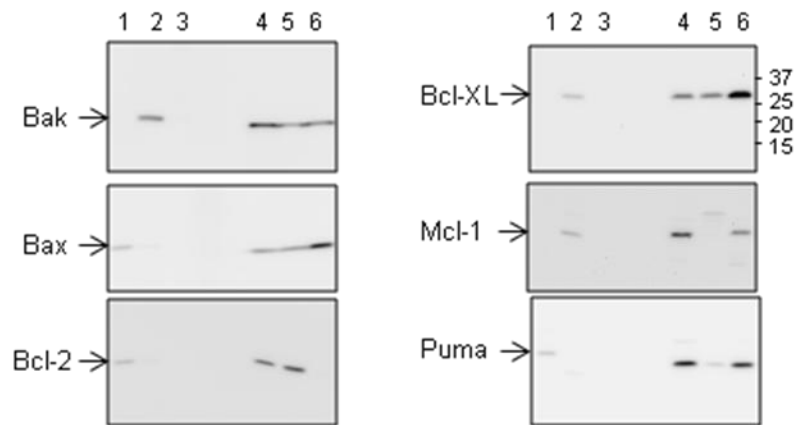
line (Figure 4). Also there was no marked reduction of both pro-apoptotic effector proteins Bak and Bax and of the BH3-only factor Puma in HepG2 cells when compared to their levels in other cell lines (Figures 3 and 4). Taking these results together, the resistance of HepG2 to undergo apoptosis correlates well with the increased levels of the Bcl-XL protein. This is consistent with previous report finding that down-regulation of Bcl-XL in HepG2 cells activated apoptosis induced by Staurosporine (15).



**Figure 2. Cell line-dependent sensitivity to undergo apoptosis induced by Staurosporine treatment.** Adherent cell lines (HeLa and HepG2) were seeded at 50,000 per well directly in Assay Plate, allowed to attach overnight and treated for 6 hours with Staurosporine as indicated. Suspension cells (HL-60) were treated for 6 hours with Staurosporine as indicated in a 96 well plate, and about 300,000 of treated cells was directly transferred (in media containing 10% serum) from each well into wells of the Assay Plate. The treated cells were then fixed and the plate was analyzed by ICE to measure the cleaved PARP-1 as described in Materials and Methods using MSA43. Mean and standard error of the mean ( $n=3$ ) of the cleaved PARP-1 normalized to cell amount is shown. The calculated Staurosporine  $EC_{50}$  of PARP-1 cleavage are shown.



**Figure 3. Cell line-dependent expression levels of Bcl-2 proteins.** Adherent cell (HeLa and HepG2 at 50,000 per well, H196 at 150,000 per well) were seeded directly in Assay Plate and allowed to attach overnight. Suspension cells (HL-60 at 300,000 per well, Jurkat at 200,000 per well) were seeded in media containing 10% serum to the Assay Plate. Cells were fixed and parallel samples were analyzed by ICE using Bak, Bax, Bcl-X, Mcl-1 and Puma antibodies, as described in Materials and Methods. Mean and standard error of the mean ( $n=3$ ) of the antibody-specific signal normalized to cell amount is shown.

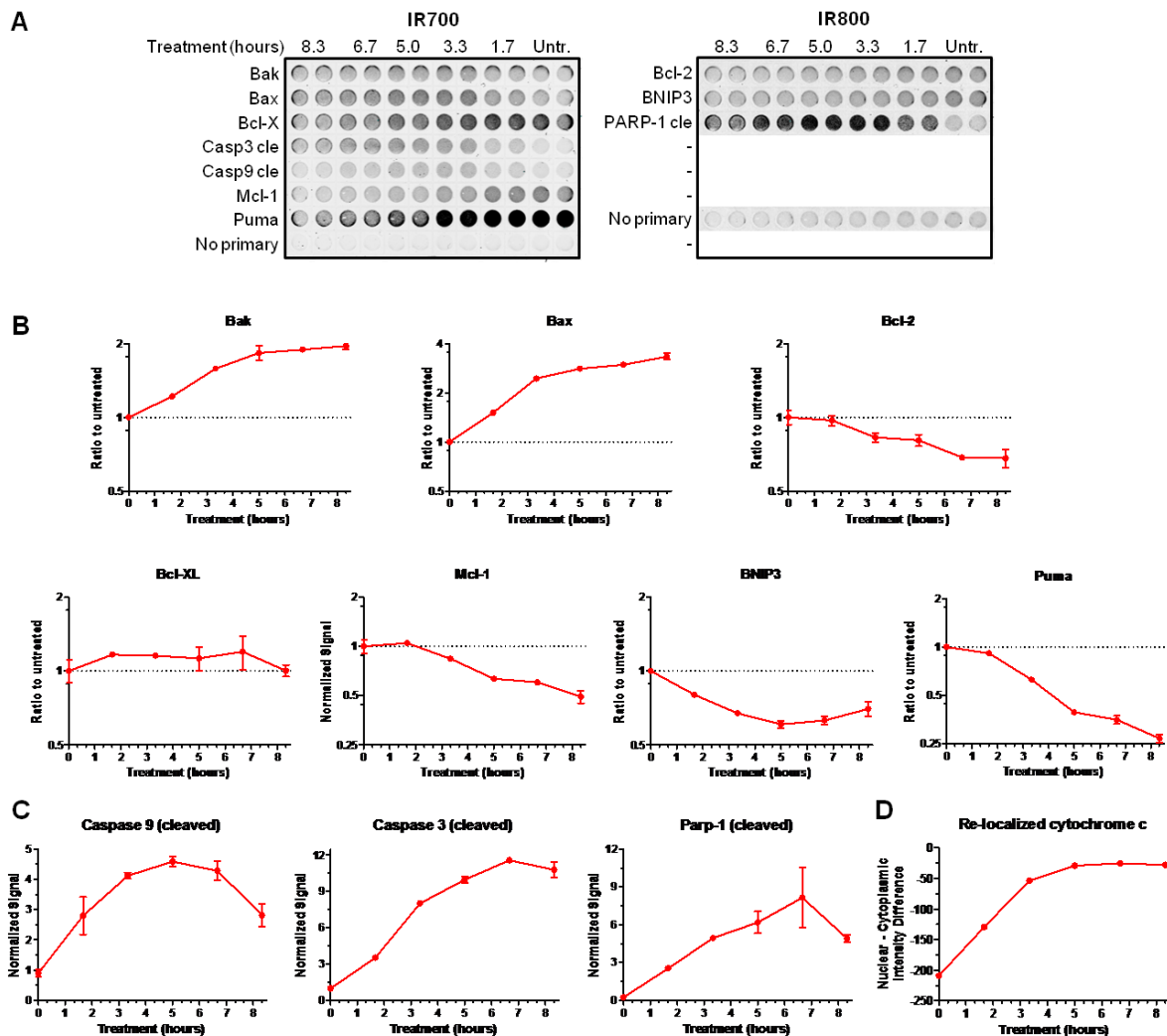


**Figure 4. Western blot analysis of Bcl2 family proteins in whole HeLa and HepG2 cells and in cytosolic, mitochondrial and nuclear fractions of HeLa cells.** HeLa cells were fractionated into cytosolic (lane 1), mitochondrial (lane 2) and nuclear (lane 3) fractions as described in Materials and Methods. Equal proportions of each fraction corresponding to 10  $\mu$ g of the whole cells as well as 25  $\mu$ g of whole cell lysates of untreated HeLa cells (lane 4), HeLa cells treated for 4 hours with 1  $\mu$ M Staurosporine (lane 5) and untreated HepG2 cells (lane 6) were analyzed by Western blotting with indicated antibodies. Note that Bax, Bcl-2 and Puma are mainly in the cytosolic fraction, and Bak, Bcl-XL and Mcl-1 are in mitochondrial fraction of untreated cells. Also note that Bax is in the mitochondrial fraction of Staurosporine-treated cells.

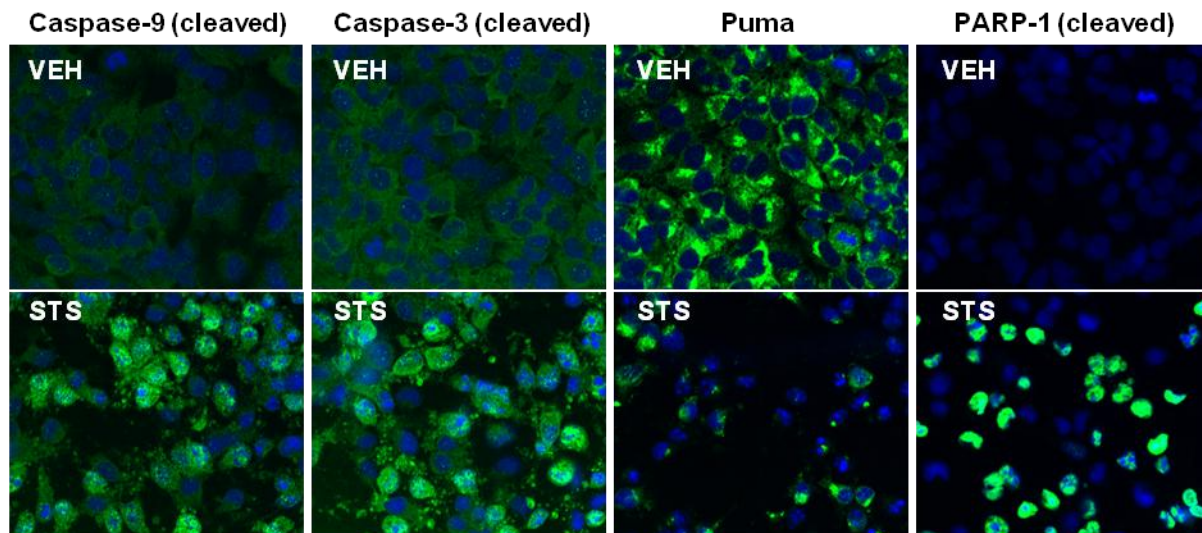
## II. Monitoring the progression of apoptosis by ICE

Here we document assaying time-dependent changes in the Bcl-2 protein family, activation of caspases and proteolysis of their downstream target PARP-1 using the ICE technique. In general, saturating amounts of the inducers, about 2-3 fold higher concentrations comparing to the inducer's IC<sub>50</sub> value of cleaved PARP-1, were used for the time-course ICE experiments. Based on the Staurosporine IC<sub>50</sub> value of PARP-1 cleavage of 0.34  $\mu$ M in HeLa cells (see Figure 2), 1  $\mu$ M Staurosporine treatment was chosen for the multi-analyte time-course ICE analysis (Figure 5A-C). Cytochrome c re-localization was measured on parallel samples by quantitative fluorescence microscopy as described in Materials and Methods (Figure 5D). As expected, the early release of cytochrome c from mitochondria was accompanied by the cleavage of caspase-9 reaching its maximal levels around 5 hours after the induction (Figure 5A and C). The caspase-9 cleavage was closely followed by the cleavage of its substrate, caspase-3, peaking around 6.7 hours of the treatment. The target of caspase-3, PARP-1 was cleaved nearly concomitantly with the activation (cleavage) of caspase-3; the levels of the cleaved PARP-1 reached its maximum also at the 6.7 hours time point. The antibodies specificities towards the cleaved proteins formed in cells with typical apoptotic nuclei were confirmed by immunocytochemistry (Figure 6). Interestingly, Bak and Bax ICE signals started to increase significantly already at 1.7 hours after the induction and they leveled off after 5 hours of treatment (Figure 5 A and B). These unexpected changes in Bak and Bax ICE signals were followed by Western blot and immunocytochemical analysis (Figures 4 and 7). The Western blot analysis revealed that there is no significant change in the levels of Bak and Bax proteins of untreated and 4 hour Staurosporine-treated HeLa cells (Figure 4). It is well known that while only Bax translocate to mitochondria in apoptosis (Bax is already mitochondria-localized in healthy cells), both Bak and Bax undergo conformational changes and form oligomeric structures in the mitochondrial outer membrane (16). Our ICE results suggested that the antibodies used in the Bak and Bax ICE assays have likely higher affinity towards the oligomeric forms of Bak and Bax in the mitochondrial outer membrane. Samples prepared as those used in ICE time course experiment were further analyzed by immunocytochemistry (Figure 7). The vehicle-treated cells stained with Bak or Bax antibodies exhibited very weak cytosolic signals. After 4 hours of treatment with Staurosporine, most of the cells exhibited predominantly mitochondrial Bak and Bax signals as these signals co-localized with mitochondrial marker (pyruvate dehydrogenase subunit E2/E3 binding protein, PDH E2/E3BP). Most

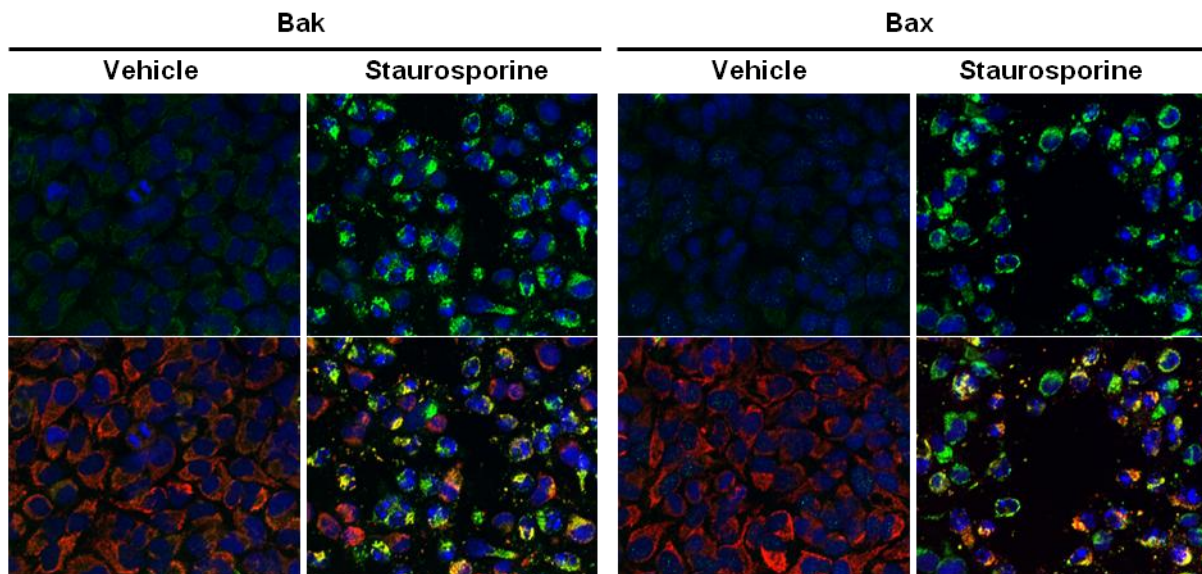
importantly the signal intensities of mitochondria-localized Bak and Bax were markedly increased when compared to the diffused signals of untreated cells or of the small fraction of treated cells that apparently did not respond to the inducer.



**Figure 5. Time course of apoptosis progression in HeLa cell treated with Staurosporine using multi-analyte ICE assay.** HeLa cells (50,000 per well) were seeded directly in Assay Plate, allowed to attach overnight and treated with 1  $\mu$ M Staurosporine for variable time periods as indicated. Cells were fixed and parallel samples were analyzed in duplicates by ICE using Bak, Bax, Bcl-2, Bcl-X, Mcl-1, BNIP3, Puma, caspase-9 (cleaved), caspase-3 (cleaved), and PARP-1 (cleaved) antibodies (A-C), or for cytochrome c relocalization by fluorescence microscopy (D), as described in Material and Methods. Images of a plate analyzed by ICE using indicated antibodies scanned at the 700 channel (IR700) and 800 channel (IR800) are shown in A. Note: whenever possible, two analytes were duplexed (measured in the same well) taking advantage of mouse and rabbit origin of the primary antibodies used. Mean and standard error of the mean ( $n=2$ ) is shown (B-D). The data in B are further normalized as a ratio of a treatment-time point sample to the untreated control.



**Figure 6. Immunocytochemical analysis of cleaved caspase-9, cleaved caspase-3, cleaved PARP-1 and Puma in cells treated with Staurosporine.** HeLa cells were treated for 4 hours with 1  $\mu$ M Staurosporine (STS) or drug vehicle (VEH). Samples were processed for immunocytochemistry using caspase-9 (cleaved), caspase-3 (cleaved), PARP-1 (cleaved) or Puma antibodies (all shown in green) and the nuclei were co-stained with DAPI (in Blue).



**Figure 7. Immunocytochemical analysis of Bak and Bax in cells treated with Staurosporine.** HeLa cells were treated for 4 hours with 1  $\mu$ M Staurosporine or drug vehicle (DMSO). Samples were processed for immunocytochemistry using Bak or Bax antibody (both in green) and mitochondrial marker (PDH E2/E3BP, in red) and nuclei were co-stained with DAPI (in blue). Representative images of Bak or Bax signals overlaid with DAPI are shown in the upper panels. The same images of Bax or Bak overlaid with PDH E2/E3BP and DAPI are shown in the lower panels. Note very low Bak and Bax staining in vehicle treated cells and strong mitochondrial Bak and Bax staining in most of Staurosporine treated cells.

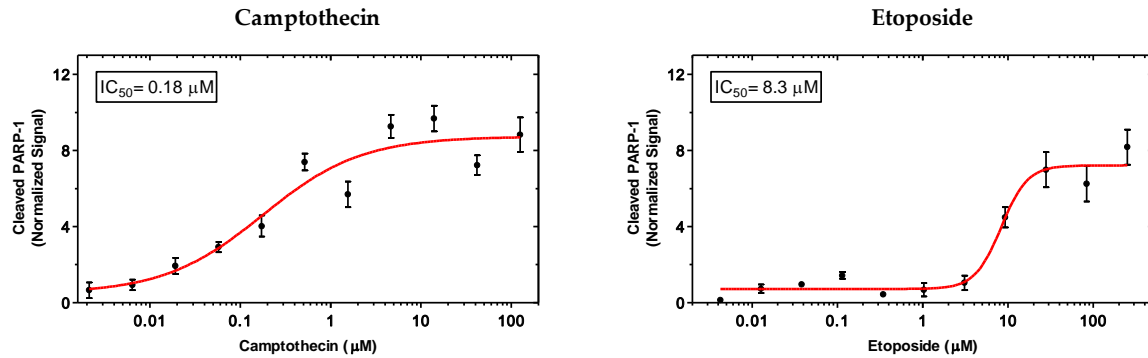
The levels of the anti-apoptotic proteins of the Bcl-2 family Bcl-2, Bcl-XL and Mcl-1 were measured as well using the ICE assay (Figure 5 A and B). The levels of Bcl-XL were constant through the entire time

course. The levels of Bcl-2 and Mcl-1 declined steadily after 3.3 hours of the treatment, with Bcl-2 having decreased only 25% at 8.3 hours, while levels of Mcl-1 decreased by 50% at this time-point. These results, mainly the marked decrease of Mcl-1 measured by ICE, were confirmed by Western blotting (Figure 4). These results are in agreement with findings that Mcl-1 is targeted by ubiquitination for degradation by proteasome early in apoptosis (17). In the ICE experiment the levels of BH3-only pro-apoptotic factors of Bcl-2 family, BNIP3 and Puma were monitored as well (Figure 5A and B). BNIP3 levels decreased modestly reaching a minimum at 5 hours of the treatment. The levels of Puma protein decreased steadily starting at 3.3 hours after the induction, declining by 75% at the 8.3 hours time point. The marked decrease of Puma measured by ICE was confirmed by Western blotting and immunocytochemistry (Figure 4 and 6). The decrease of Puma is consistent with latest findings reporting caspase-dependent proteolysis of this protein (18).

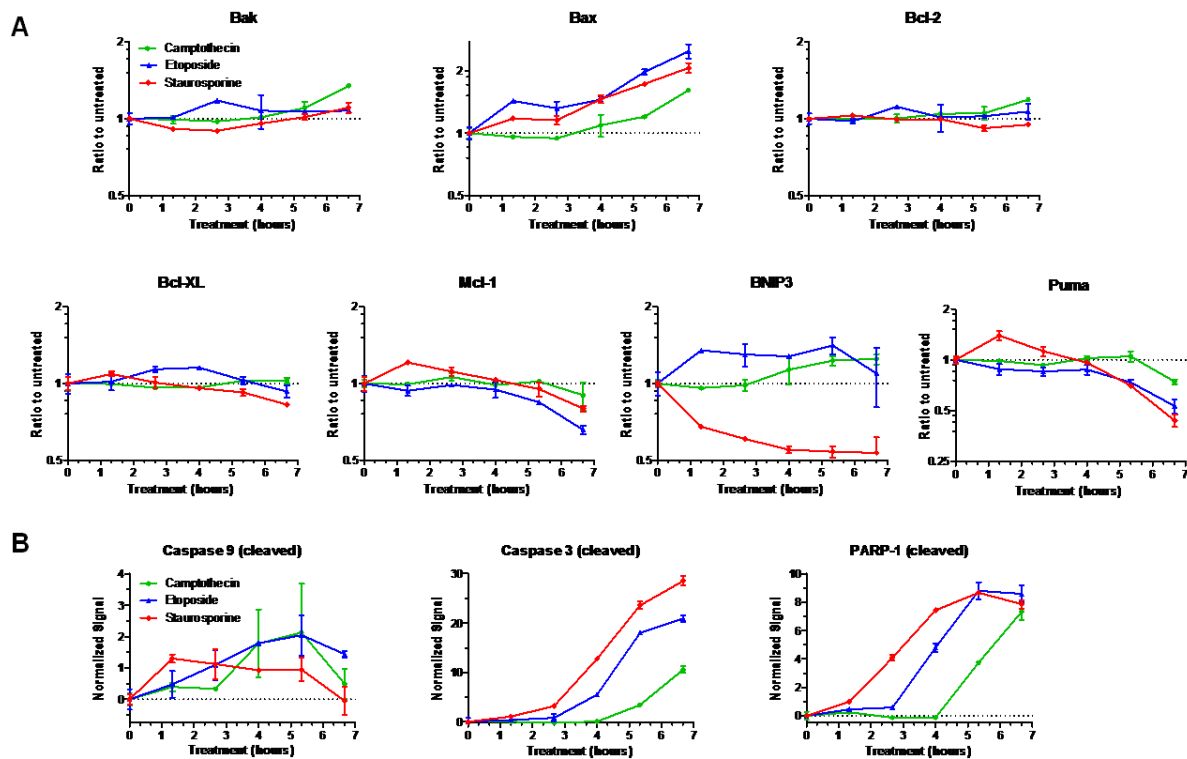
As for the HeLa cells, a similar time-course experiment was performed for HL-60 cells induced to undergo apoptosis with Staurosporine, topoisomerase I inhibitor camptothecin and topoisomerase II inhibitor etoposide. To determine the appropriate concentration of camptothecin and etoposide for this experiment, IC50s of PARP-1 cleavage were determined for these two drugs on HL-60 cells (Figure 8). PARP-1 cleavage exhibited typical saturable sigmoid response to camptothecin or etoposide dilutions with respective IC50 values of 0.18  $\mu$ M and 8.3  $\mu$ M. As described above, the Staurosporine IC50 value of PARP-1 cleavage in HL-60 cells was 0.41  $\mu$ M (Figure 2). Thus for the time course experiment, 0.4  $\mu$ M camptothecin, 20  $\mu$ M etoposide and 1  $\mu$ M Staurosporine were used (Figure 9). All three drugs induced the mitochondrial-mediated pathway of apoptosis as evidenced by cleavage of caspase-9. The cleaved caspase-9 reached its maximal levels 4 to 5 hours after the induction, depending on the inducer used. The cleavage of caspase-9 was followed by the cleavage of caspase-3 and cleavage of PARP-1, with Staurosporine inducing the earliest response in all three analytes described above, followed by etoposide and camptothecin (Figure 9 B). As in HeLa cells, the Bax ICE signals were increasing steadily with the time of the induction also in HL-60 cells treated with Staurosporine, etoposide or camptothecin (Figure 9 A). This suggests that Bax oligomerization in the mitochondrial outer membrane can be measured by ICE in cells treated with variety of apoptotic stimuli including kinase and topoisomerase inhibitors. On the contrary to HeLa cells treated with Staurosporine, none of the tested drugs induced significant increase in Bak signal in HL-60 cells (Figure 9A). The differential Bak activation in HeLa and HL-60 to the Staurosporine treatment may suggest that the pro-apoptotic effectors are utilized in a cell type specific fashion to trigger mitochondrial outer membrane permeabilization. The ICE analysis of the anti-apoptotic factors revealed no changes in the levels of Bcl-2 and Bcl-XL within the time span of the experiment. As in HeLa cells treated with Staurosporine, the treatments with camptothecin, etoposide and Staurosporine induced decrease of Mcl-1 levels also in HL-60 cells, results consistent with the degradation of Mcl-1 protein in apoptosis. Also, as in HeLa cells treated with Staurosporine, all three drugs induced decrease of the pro-apoptotic BH3 only factor Puma, but unlike in HeLa cells, the decrease was initiated in much later phase. BNIP3 was decreased in Staurosporine treated HL-60, similarly as in Staurosporine-treated HeLa. Unlike the response to Staurosporine, there was no change in the levels of BNIP3 in HL-60 cells treated with camptothecin or etoposide, pointing to differential response of HL60 cells to the tested drugs in the levels of this protein.

## Conclusions:

1. *HepG2 resistance to apoptosis through high levels of Bcl-XL.* The ICE platform proved useful in determining cell line sensitivity to apoptosis by measuring the cleaved PARP-1 IC50 of a drug. ICE platform revealed cell-type specific resistance to apoptosis induced by Staurosporine. Combination of ICE and Western blotting of pro-apoptotic Bax and Bak and anti-apoptotic Bcl-2, Bcl-XL and Mcl-1 showed that the resistance of HepG2 cells to apoptosis by Staurosporine treatment correlated with



**Figure 8. Determination of EC<sub>50</sub> of the PARP-1 cleavage in HL-60 cells treated with topoisomerase inhibitors.** HL-60 were treated for 6 hours with camptothecin or etoposide as indicated in a 96 well plate, and about 300,000 of treated cells was directly transferred (in media containing 10% serum) from each well into wells of the Assay Plate. The treated cells were then fixed and the plate was analyzed by ICE to measure the cleaved PARP-1 using MSA43 as described in Materials and Methods. Mean and standard error of the mean (n=4) of the cleaved PARP-1 normalized to cell amount is shown. The calculated EC<sub>50</sub> of PARP-1 cleavage are shown.



**Figure 9. Time-course of apoptosis progression in HeLa cell treated with camptothecin, etoposide and Staurosporine using multi-analyte ICE assay.** HL-60 were treated as indicated for variable time periods with 0.4 µM camptothecin, 10 µM etoposide, or 1 µM Staurosporine in a 96 well plate, and about 300,000 of treated cells was directly transferred (in media containing 10% serum) from each well into wells of the Assay Plate. Cells were fixed and parallel samples were analyzed by ICE using Bak, Bax, Bcl-2, Bcl-X, Mcl-1, BNIP3, Puma, caspase-9 (cleaved), caspase-3 (cleaved), and PARP-1 (cleaved) antibodies, as described in Materials and Methods. Whenever possible, two analytes were duplexed (measured in the same well) taking advantage of mouse and rabbit origin of the primary antibodies used. Mean and standard error of the mean (n=2) is shown. The data in A are further normalized as a ratio of a treatment-time point sample to the untreated control.

high expression of Bcl-XL. These experiments demonstrated a utility of ICE platform in assaying expression levels of regulators of apoptosis in cancer cells.

- II. *ICE platform in monitoring progression of apoptosis.* Time-dependent changes in activation of an initiator caspase, an executioner caspase and the cleavage of the executioner caspase target were demonstrated using the ICE platform by measuring protein levels of cleaved caspase-9, cleaved caspase-3 and cleaved PARP-1 in HeLa and HepG2 cells induced to undergo apoptosis by variety of treatments. In addition, the time-dependent degradation of Mcl-1 and Puma was demonstrated using the ICE assay in cells undergoing apoptosis.
- III. *ICE to monitor changes immediately preceding mitochondrial outer membrane permeabilization.* Importantly, Bak and Bax antibodies were identified that are useful to detect changes of Bak and Bax related to their conformation and/or oligomerization. Thus using the Bak and Bax ICE assays, early apoptotic changes immediately upstream mitochondrial outer membrane permeabilization and cytochrome c release can be measured in high throughput format.
- IV. *ICE as a robust and flexible assay system to monitor apoptosis.* The ICE platform is an ideal high-throughput microplate assay, capable of in-well duplexing, to detect apoptosis and measure changes of many proteins participating in this process using both adherent and suspension cells. For example, up to 14 protein targets can be measured under six different conditions (time points) in duplicates using a single microtiter plate, as shown in this study for HeLa and HL-60 cells treated with several drugs. ICE assays are reliable, for example for HeLa cells treated with Staurosporine shown in Figure 2, the coefficient of variation (CV) value for signals 2-fold over background averaged 3%. ICE assays are inherently flexible as readouts can be selected from a large offering of MitoSciences-validated antibodies not limited to apoptosis ([www.mitosciences.com/in-cell-elisa.html](http://www.mitosciences.com/in-cell-elisa.html)).

## References:

1. Pettigrew, C. A. and T. G. Cotter (2009). "Deregulation of cell death (apoptosis): implications for tumor development." *Discov Med* **8**(41): 61-3.
2. Nagata, S. (2010). "Apoptosis and autoimmune diseases." *Ann N Y Acad Sci* **1209**: 10-6.
3. Su, H. C. and M. J. Lenardo (2008). "Genetic defects of apoptosis and primary immunodeficiency." *Immunol Allergy Clin North Am* **28**(2): 329-51, ix.
4. Kermer, P., J. Liman, et al. (2004). "Neuronal apoptosis in neurodegenerative diseases: from basic research to clinical application." *Neurodegener Dis* **1**(1): 9-19.
5. Danial, N. N. and S. J. Korsmeyer (2004). "Cell death: critical control points." *Cell* **116**(2): 205-19.
6. Verbrugge, I., R. W. Johnstone, et al. "SnapShot: Extrinsic apoptosis pathways." *Cell* **143**(7): 1192, 1192 e1-2.
7. Brenner, D. and T. W. Mak (2009). "Mitochondrial cell death effectors." *Curr Opin Cell Biol* **21**(6): 871-7.
8. Tait, S. W. and D. R. Green (2010). "Mitochondria and cell death: outer membrane permeabilization and beyond." *Nat Rev Mol Cell Biol* **11**(9): 621-32.
9. Leibowitz, B. and J. Yu "Mitochondrial signaling in cell death via the Bcl-2 family." *Cancer Biol Ther* **9**(6): 417-22.
10. Yip, K. W. and J. C. Reed (2008). "Bcl-2 family proteins and cancer." *Oncogene* **27**(50): 6398-406.
11. Adams, J. M. and S. Cory (2007). "The Bcl-2 apoptotic switch in cancer development and therapy." *Oncogene* **26**(9): 1324-37.
12. Biswas, S., Q. Shi, et al. (2010) "A role for proapoptotic Bax and Bak in T-cell differentiation and transformation." *Blood* **116**(24): 5237-46.
13. Fernandez-Luna, J. L. (2008). "Regulation of pro-apoptotic BH3-only proteins and its contribution to cancer progression and chemoresistance." *Cell Signal* **20**(11): 1921-6.
14. Revil, T., J. Toutant, et al. (2007). "Protein kinase C-dependent control of Bcl-x alternative splicing." *Mol Cell Biol* **27**(24): 8431-41.

15. Takehara, T., X. Liu, et al. (2001). "Expression and role of Bcl-xL in human hepatocellular carcinomas." *Hepatology* **34**(1): 55-61.
16. Dewson, G. and R. M. Kluck (2009). "Mechanisms by which Bak and Bax permeabilise mitochondria during apoptosis." *J Cell Sci* **122**(Pt 16): 2801-8.
17. Zhong, Q., W. Gao, et al. (2005). "Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis." *Cell* **121**(7): 1085-95.
18. Hadji, A., C. Clybouw, et al. (2010) "Caspase-3 triggers a TPCK-sensitive protease pathway leading to degradation of the BH3-only protein puma." *Apoptosis*.

## In-Cell ELISA product offerings from MitoSciences for Apoptosis studies:

### ICE-validated primary antibodies:

#### Primary Antibodies








✓ Cat. No.	Description	Pathway
<input type="checkbox"/> MSA09	Apoptosis-inducing factor (AIF) monoclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MSR24	Bak monoclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MSR25	Bax polyclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MS776	Bcl-2 mouse monoclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MSR26	Bcl-X monoclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MS778	BNIP3 monoclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MSR27	Caspase 3 (cleaved) monoclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MSA04	Cyclophilin D monoclonal antibody	Permeability Transition Pore
<input type="checkbox"/> MSA06	Cytochrome c monoclonal antibody	<a href="#">Apoptosis</a> <a href="#">OXPHOS</a>
<input type="checkbox"/> MSR28	Mcl-1 monoclonal antibody	<a href="#">Apoptosis</a>
<input type="checkbox"/> MS777	Poly [ADP-ribose] polymerase 1 (PARP-1) (cleaved) monoclonal antibody	<a href="#">Apoptosis</a> <a href="#">NAD+ NADH Cycling</a>
<input type="checkbox"/> MSR29	PUMA monoclonal antibody	<a href="#">Apoptosis</a>

### ICE Kits:


#### In-Cell ELISA Kits

Cat. No.	Name	Reactivity	Amount
<a href="#">MSA43</a>	<a href="#">PARP-1 (cleaved) In-Cell ELISA Kit (IR)</a>	human	2 x 96 tests

**ICE support products:****Secondary Antibodies**

Data	Cat. No.	Name	Amount
	MS923	Goat anti-mouse IRDye® 800CW, IgG (H+L)	12 x 96 tests
	MS924	Goat anti-rabbit IRDye® 680LT, IgG (H+L)	12 x 96 tests
	MS925	Goat anti-mouse IRDye® 680LT, IgG1 isotype specific	12 x 96 tests
	MS926	Goat anti-mouse IRDye® 800CW, IgG2a isotype specific	12 x 96 tests
	MS927	Goat anti-mouse IRDye® 800CW, IgG2b isotype specific	12 x 96 tests
	MS928	Goat anti-mouse HRP, IgG (H+L)	12 x 96 tests
	MS929	Goat anti-rabbit HRP, IgG (H+L)	12 x 96 tests

**Whole Cell Stain**

Data	Cat. No.	Name	Amount
	MS930	Janus Green cell normalization stain	2 x 96 tests

**Support Pack**

Cat. No.	Name	Amount
<a href="#">MS921</a>	<a href="#">In-Cell ELISA (In-Cell Western) Support Pack w/o Plates</a>	5 x 96 tests
<a href="#">MS922</a>	<a href="#">In-Cell ELISA (In-Cell Western) Support Pack (Includes 5x tissue-culture treated 96-well black/clear imaging plates.)</a>	5 x 96 tests

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