Monitoring protein movements to and from the mitochondrion in apoptosis, a high throughput quantitative solution

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Abstract

Mitosciences have developed a simple, rapid cell fractionation method to obtain cytosol-, mitochondria- and nuclei-containing fractions. The method involves sequential and selective extraction of cytosolic and then mitochondrial proteins by detergents from a nucleus containing fraction. This cell fractionation procedure can be performed either on cells in suspension or in a high throughput microplate format on adherent cells.

Cell fractionations in a 96 well plate format are used to monitor translocation of Bax from the cytosol to the mitochondria and cytochrome c and Smac from the mitochondria into the cytosol in HeLa cells induced to undergo apoptosis by Staurosporine treatments. Cell fractionation followed by a cytochrome c sandwich ELISA assay, offers a complete quantitative high throughput approach to measure cytochrome c release from mitochondria in cells undergoing apoptosis.

Intoduction

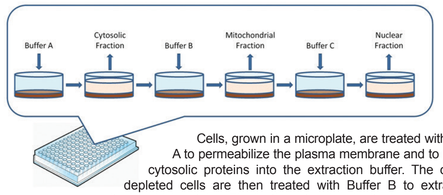
The movement of pro-apoptotic factors of the Bcl2-family from the cytosol to the mitochondria and the consequent permeabilization of the mitochondrial outer membrane to release cytochrome c and Smac from the mitochondrial intermembrane space into the cytosol or AIF to the nucleus are now well characterized steps in programmed cell death. However, there are concurrent movements of other proteins, including kinases and transcription factors (e.g. p53), to and from the organelle to both signal and modulate apoptosis.

The identification and quantification of these protein movements between cellular compartments is necessary to fully understand and to differentiate between different pathways of cell death. Biochemical cell fractionation approaches mostly utilize mechanical cell disruptions which are often difficult to standardize, require a relatively large amount of cell sample and the lack of multiple instruments often limits these methods to process one sample at a time making it time consuming and difficult to perform on a large number of samples. They also carry the risk of disrupting the mitochondrial membranes leading, in particular, to artificial release of mitochondrial intermembrane space pro-apoptotic proteins. The mechanical cell disruption is often incomplete and thus a removal of unbroken cells is frequently required. This leads to losses of uncharacterized cell material which is difficult to account for.

The **Mitosciences cell fractionation kit (ab109718)** is based on a selective and sequential extraction of cytosolic and mitochondrial proteins thus eliminating the need of mechanical cell disruption and differential centrifugation. This poster presents a new generation of this methodology specifically designed to fractionate adherant cells directly, without their detachment, in a high throughput microplate format. We demonstrate the utility of this high throughput fractionation on following the movement of Bax, cytochrome c and Smac in HeLa cells induced to undergo apoptosis by Staurosporine treatment using western blotting. When coupled to a microplate sandwich ELISA assay, these methodologies also present a complete high throughput solution, as shown on monitoring of the cytochrome c release in apoptosis.

Figure 1: Cell Fractionation Kits HT Method

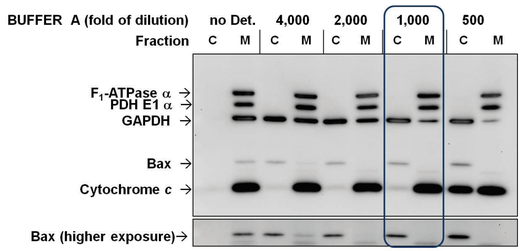
Cell Fractionation HT method (ab109718): showing fractionation of adherant cells into cytosol-, mitochondria- and nuclei-containing fractions.



Cells, grown in a microplate, are treated with Buffer A to permeabilize the plasma membrane and to release cytosolic proteins into the extraction buffer. The cytosol-depleted cells are then treated with Buffer B to extract the mitochondrial proteins. Finally, the cytosol and mitochondria-depleted cells are treated with Buffer C to extract nuclear proteins.

Figure 2: Method optimization for separation of cytosolic and mitochondrial fractions.

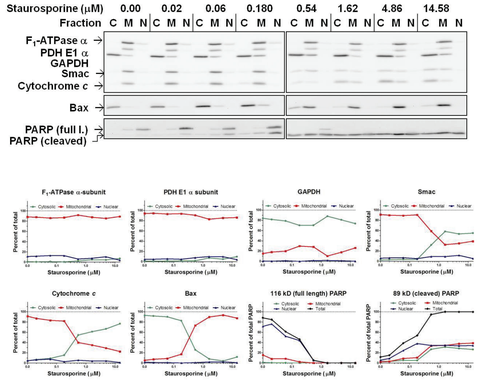
Optimization of cell permeabilization to separate cytosolic and mitochondrial fractions.



Cytosolic (C) and mitochondrial (M) fractions of HeLa cells, seeded at 30,000 per well of a 48-well plate were prepared using the Cell Fractionation HT method and variable concentrations of Buffer A . Fractions were analyzed by western blotting using **MitoSciences ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (ab110415)** and supplemented with antibodies against an additional cytosolic (Bax) marker, followed by appropriate HRP-conjugated goat secondary antibodies and ECL detection. The Blue box indicates the dilution of Buffer A for the optimal separation of cytosolic and mitochondrial proteins

Figure 3: Movement of proteins following Staurosporine treatment in HeLa cells.

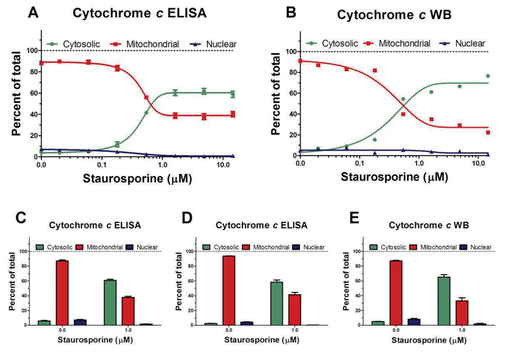
Cytochrome c and Smac are released from the mitochondria into the cytosol and Bax re-localizes from the cytosol to mitochondria during apoptosis induced by Staurosporine treatment in HeLa cells.



HeLa cells were treated for 4 hrs with concentrations of 0.00, 0.02, 0.06, 0.18, 0.54, 1.62, 4.86 and 14.58 μM Staurosporine. Cytosolic (C), mitochondrial (M) and nuclear (N) fractions, were each derived from one well of a 96-well plate, prepared using the Cell Fractionation HT method. Mobilization of proteins was analyzed by western blotting using **MitoSciences ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (ab110415)** and supplemented with an antibody against Smac, as well as with antibodies against Bax and PARP. Representative blots as well as the quantitative analysis (mean +/- standard error, n=2) are shown.

Figure 4: Quantitative ELISA analysis of cytochrome *c* release following Staurosporine treatment in HeLa cells.

Quantitative 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 mM Staurosporine (C, D, E) were prepared using the **Cell Fractionation HT method (ab109718)**. Fractions, each derived from one well of a 96-well plate, were analyzed by **MitoSciences ELISA Cytochrome c Protein Quantity Microplate Assay Kit (ab110172)** (panels A, C and D). Parallel analyses of fractions prepared independently and thus showing the inter-assay variation of the Cell Fractionation HT method are shown in C and D. Western blot analyses of cytochrome c using **MitoSciences ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (ab110415)** are 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).

Conclusions

- The HT method is designed for parallel fractionation of a large number of small samples of adherant cells in a microplate format. It allows a preparation of cytosolic, mitochondrial and nuclear fractions from 96 cell samples in one hour.
- The fractions prepared by this method are particularly suitable for high throughput sandwich ELISA microplate assays and also for Western blotting.
- Validation of the Cell Fractionation HT methodology on the proteins known to translocate between cytosol and mitochondria in cells undergoing apoptosis:
- Staurosporine concentration-dependent release of cytochrome c (EC_{50} = 0.40 mM) and Smac (EC_{50} = 0.52 mM) from the mitochondria into the cytosol.
  - Staurosporine concentration-dependent translocation of Bax (EC_{50} = 0.37 mM) from the cytosol into the mitochondria.
  - Cleavage of nuclear-localized full length PARP.

Other possible uses of this methodology

- Monitoring movements of other proteins (e.g. AIF, Bad, Bid, Endo G, HtrA2) involved in apoptosis.
- Monitoring movements of kinases and transcription factors (e.g. p53) during apoptosis.
- Other signaling events that involve protein re-localization.
- Separation of isoforms of enzymes, that have differential distribution, for activity assays.

Product table

Antibodies	Applications	Species	Datasheet abcam.com/...
Anti-AIF antibody [7F7AB10]	Flow Cyt, ICC/IF, IP, In-Cell ELISA, WB	Hu	ab110327
Anti-Cleaved PARP antibody [4B5BD2]	Flow Cyt, ICC/IF, In-Cell ELISA, WB	Hu	ab110315
Anti-Cytochrome C antibody [37BA11]	Flow Cyt, ICC/IF, In-Cell ELISA, WB	Hu, Ms, Rat, Ce, Cow	ab110325
Anti-Smac / Diablo antibody [8H5AA3]	Flow Cyt, ICC	Hu	ab110288
Anti-Smac / Diablo antibody [Y12]	Flow Cyt, ICC/IF, IHC-P, IP, WB	Hu, Ms, Rat	ab32023
Anti-Bcl2 antibody	ICC, IHC-Fr, IHC-P, IP, WB	Hu, Ms, Rat, Cow	ab7973
Anti-Bax antibody	IHC-Fr, IHC-P, IP, WB	Hu, Ms, Rat	ab7977
Anti-PARP antibody	IHC-Fr, IHC-P, IP, WB	Hu, Ms, Rat	ab6709
Anti-Endo G antibody	ICC/IF, IHC-P, WB	Hu, Ms, Rat	ab64668
Anti-Bak antibody [Y164]	Flow Cyt, ICC, IHC-P, WB	Hu, Ms, Rat	ab32371
Anti-Bid antibody [3C5]	ELISA, Flow Cyt, ICC/IF, IHC-P, WB	Hu	ab114051
Anti-Bad antibody [Y208]	Flow Cyt, ICC/IF, IHC-P, IP, WB	Hu, Ms, Rat	ab32445
Anti-Bim antibody	IHC-P, WB	Hu	ab15184
Anti-MCL1 antibody [8C6D4B1]	ELISA, Flow Cyt, ICC/IF, IHC-Fr, IHC-P, WB	Hu, Ms	ab31948
Anti-BNIP3L antibody	ICC/IF, IHC-P, WB	Hu, Ms	ab8399
Anti-HtrA2 / Omi antibody	ICC/IF, IHC-FoFr, WB	Hu, Ms	ab64111
Anti-PUMA antibody	ICC/IF, IHC-P, WB	Hu, Ms	ab9643
Anti-p53 antibody [PAb 240]	ELISA, Flow Cyt, ICC/IF, IHC (Methanol fixed), IHC-Fr, IHC-P, IP, WB	Hu, Ms, Rat	ab26

Western blot antibody panels and ICC antibody kits	Applications	Species	Datasheet abcam.com/...
ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail	WB	Hu	ab110415
ApoTrack™ Cytochrome c Apoptosis ICC Antibody Kit	In-Cell ELISA	Hu, Ms, Rat	ab110417

Kits	Tests	Species	Datasheet abcam.com/...
Cytochrome c Protein Quantity Microplate Assay Kit	1 x 96 tests	Hu, Ms, Rat, Cow	ab110172
p53 Human ELISA Kit	1 x 96 tests	Hu	ab117995
Mitochondrial Aldehyde Dehydrogenase (ALDH2) Activity Assay Kit	1 x 96 tests	Hu	ab115348

Cell Fractionation Kits	Datasheet abcam.com/...
Cell Fractionation Kit Standard	ab109719
Cell Fractionation Kit - HT	ab109718
Mitochondria Isolation Kit for Tissue	ab110168
Mitochondria Isolation Kit for Tissue (with Dounce Homogenizer)	ab110169
Mitochondria Isolation Kit for Cultured Cells	ab110170
Mitochondria Isolation Kit for Cultured Cells (with Dounce Homogenizer)	ab110171

Lysates	Amounts	Applications	Species	Datasheet abcam.com/...
Brain mitochondrial lysate	50 µg	SDS-Page, WB	Ms	ab110345
Brain mitochondrial lysate	2 mg	Immunocapture, BNPage	Ms	ab110351
Brain mitochondrial lysate	50 µg	SDS-Page, WB	Rat	ab110342
Brain mitochondrial lysate	2 mg	Immunocapture, BNPage	Rat	ab110348
Heart mitochondrial lysate	2 mg	Immunocapture, BNPage	Cow	ab110338
Heart mitochondrial lysate	50 µg	SDS-Page, WB	Hu	ab110337
Heart mitochondrial lysate	50 µg	SDS-Page, WB	Ms	ab110344
Heart mitochondrial lysate	2 mg	Immunocapture, BNPage	Ms	ab110350
Heart mitochondrial lysate	50 µg	SDS-Page, WB	Rat	ab110341
Heart mitochondrial lysate	2 mg	Immunocapture, BNPage	Rat	ab110347
Liver mitochondrial lysate	50 µg	SDS-Page, WB	Hu	ab110339
Liver mitochondrial lysate	50 µg	SDS-Page, WB	Ms	ab110343
Liver mitochondrial lysate	2 mg	Immunocapture, BNPage	Ms	ab110349
Liver mitochondrial lysate	2 mg	Immunocapture, BNPage	Rat	ab110346
Liver mitochondrial lysate	50 µg	SDS-Page, WB	Rat	ab110340

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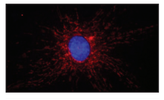
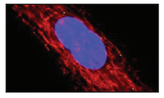


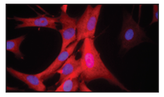
Anti-MTCO1 antibody [1D6E1A8]- Mitochondrial Loading Control (ab14705)

Applications: Flow Cyt, ICC/IF, IHC-Fr, WB
Species: Hu, Ms, Rat, Ce, Cow, Zfsh



Anti-VDAC1 / Porin antibody [20B12AF2] - Mitochondrial Loading Control (ab14734)

Applications: Flow Cyt, ICC/IF, IHC-P, WB
Species: Hu, Ms, Rat, Dm



Anti-GAPDH antibody [3E8AD9] (ab110305)

Applications: Flow Cyt, ICC/IF, IP, In-Cell ELISA, WB
Species: Hu

160_11_MBS