PROTOCOL

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Cell Fractionation Kit - HT

MS862

Rev 0

DESCRIPTION

MS862 Cell Fractionation Kit - HT

Sufficient materials are provided for fractionation of adherent cells cultured in one 96-well or 48-well plate, or corresponding to approximately 1.5 x 10^6 cells.

Kit Contents:

Item	MS862
Buffer A	34 mL
Detergent HT I	7 µL
Detergent HT II	35 µL
Detergent HT III	700 µL
5X SDS Sample Buffer	1500 µL

Storage:

Store Buffer A, Detergent HT II, Detergent HT III and 5 x SDS Sample Buffer at -20°C, store Detergent HT I at -80°C.

INTRODUCTION

The MS862 Cell Fractionation Kit - HT provides a method and reagents for a simple and rapid preparation of cytosolic, mitochondrial and nuclear fractions from adherent cells. The kit is especially suitable for, but not limited to, high throughput fractionation of adherent cells in a 96-well plate format. The entire fractionation of 96 samples into the cytosolic, mitochondrial and nuclear fraction is achieved in one hour. The kit is based on sequential detergent extraction of cytosolic, mitochondrial and nuclear proteins without the need for mechanical disruption of cells, and thus fractionates adherent cells into cytosol-containing, mitochondria-containing and nuclei-containing fractions. These fractions are referred throughout the protocol as cytosolic, mitochondrial and nuclear fractions. The kit prepares sufficient sample material for subsequent Western blot analyses, dot blot analyses or for analyses by microplate ELISA assays.

MS862 is designed to allow the measurement of any proteins which are differentially represented in the cytosol, mitochondria and nuclei, and is particularly applicable to studies of proteins that translocate between these three cellular compartments. As an example, the use of the kit is described throughout this protocol in relation to the following of cytochrome *c* release from the mitochondria to the cytosol during apoptosis (see Figures 2, 3 and 4), as this is perhaps the best known mitochondrial protein translocation event and it is an important component of apoptosis research. Similarly, the kit was used to measure the release of Smac/Diablo from the mitochondria to the cytosol and the translocation of Bax from cytosol to mitochondria during apoptosis as well as cleavage on nuclear poly (ADP-ribose) polymerase (PARP), see Figures 2 and 3.

The movement of Bax from the cytosol to the mitochondria, the consequent permeabilization of the mitochondrial outer membrane followed by the release of apoptogenic proteins, including cytochrome c, Smac/Diablo, HtrA2/Omi, AIF and endonuclease G from mitochondrial intermembrane space into the cytosol or their further translocation into the nucleus (AIF and endonuclease G) are considered hallmarks of many apoptotic pathways leading to cell death. 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. Identification and quantitation all of these protein movements between the cellular compartments is necessary to fully understand and to be able to differentiate between the different pathways of cell death.

Most of the current biochemical methods of quantification of these proteins in cytosolic, mitochondrial and nuclear fractions involve mechanical disruption of cells followed by a complicated centrifugation scheme to obtain cytosolic, mitochondria-enriched and nuclei-enriched fractions. These methods are time-consuming and limited to small 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 which leads to losses of uncharacterized cell material which is difficult to account for.

The MS862 Cell Fractionation Kit HT provides a simple and rapid method to obtain cytosolic, mitochondrial and nuclear fractions, thus avoiding time consuming and inefficient cell disruption and differential centrifugation. The kit is based on sequential and selective extraction of cytosolic, mitochondrial and nuclear proteins with proprietary detergents that allow sequential release of cytosolic mitochondrial and nuclear proteins to the extracellular buffer. In the first step, the plasma membrane is selectively permeabilized with Detergent HT I and the cytosol-containing fraction is collected. In the second step, mitochondrial proteins are then extracted with Detergent HT II and the mitochondria-containing fraction is collected. In the third step, nuclear proteins are then extracted with Detergent HT III and the nuclei-containing fraction is collected.

In untreated cells, mitochondrial intermembrane space proteins including cytochrome *c* and Smac/Diablo remain in the mitochondrial fraction (Figures 1, 2, 3 and 4). However, if cytochrome *c* and Smac/Diablo are released from the mitochondrial intermembrane space into cytosol, as frequently occurs in apoptosis, the cytosolic cytochrome *c* and Smac/Diablo are found in the cytosolic fraction with other cytosolic proteins (Figures 2, 3 and 4). Similarly, in untreated cells, Bax is present with other cytosolic proteins in the cytosolic fraction (Figures 2 and 3). However, if Bax is translocated from the cytosol to the mitochondria, as frequently occurs in apoptosis, the mitochondrial Bax is found in the mitochondrial fraction with other mitochondrial proteins (Figures 2 and 3).

The three distinct fractions generated can be analyzed by Western blot, dot blot or by ELISA microplate. For Western blot analysis, MitoSciences' MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Cocktail is recommended (typical results shown below), which contains an antibody against cytochrome *c* (MSA06 Anti-cytochrome *c* Monoclonal Antibody) plus antibodies against key mitochondrial and cytosolic markers. For the analysis of cytochrome *c* by microplate ELISA assay, Mitosciences' MSA41 Cytochrome c Protein Quantity Microplate Assay Kit is highly recommended (see Figure 4 and product literature for typical results using this kit). These methods were verified on HeLa cells untreated or treated with a protein kinase inhibitor Staurosporine to undergo apoptosis. The proportion of cytochrome *c* found in the cytosol-containing fractions by this method correlated well with the results of immunocytochemical analysis using MitoSciences' MSA07 ApoTrack™ Cytochrome *c* Apoptosis ICC Antibody Kit.

ADDITIONAL REAGENTS AND EQUIPMENT REQUIRED

- Cell counting device such as hematocytometer
- Tissue culture-treated multi-well plate, collagen I-coated plate such as BD Biocoat Collagen I 96-well plate (BD Biosciences 354407) is recommended

- Plate shaker (optional)
- Centrifuge equipped with standard microplate holders (optional)
- Three 96-well plates with conical shaped wells for fraction collection

PROTOCOL

Note: This protocol contains detailed steps for preparation of subcellular fractions from cells grown in a 96-well plate and their analysis by Western blot or microplate ELISA. Be completely familiar with the protocol and protocol notes before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

1. *Grow cells.* Seed adherent cells into 96-well tissue culture-treated plate and allow them to attach. For example, seed 15,000 HeLa cells per well of 96-well plate and incubate overnight.

2. *Treat cells (optional).* Incubate cells under desired conditions. For example, treat cells with variable concentration of apoptosis inducer. In parallel, incubate the untreated control cells in another well(s).

3. Warm up Buffer A to room temperature (RT).

4. *Prepare Buffer B.* To prepare Buffer B, dilute Detergent HT I 1000-fold in Buffer A. For example, to 6 mL of Buffer A add 6 µL of Detergent HT I. Mix well by pipetting. Prepare only amount needed for immediate use.

5. *Buffer A wash.* If the treatment led to partial cell detachment, centrifuge the plate for 3 min at 300 x g at RT. Using a multi-channel pipette carefully remove and discard the media. Add 100 µL per well of Buffer A. Centrifuge the plate for 3 min at 300 x g at RT. Carefully remove and discard the wash.

6. *Cytosol Extraction.* Add 50 µL per well of Buffer B. Incubate samples for 7 minutes at RT on a shaker with gentle agitation.

7. *Prepare Buffer C*. To prepare Buffer C, dilute Detergent HT II 200-fold in Buffer A. For example, to 6 mL of Buffer A add 30 µL of Detergent HT II. Mix well by pipetting. Prepare only amount needed for immediate use.

8. *Preparation of cytosolic fractions.* Centrifuge the plate for 3 min at 300 x g at RT. Carefully remove and transfer all the resulting supernatants containing cytosolic proteins into a 96-well collection plate. These are the cytosolic fractions (C).

9. *Mitochondria Extraction.* Add 50 µL per well of Buffer C. Incubate samples for 10 minutes at RT on a shaker with gentle agitation.

10. *Prepare Buffer D*. To prepare Buffer D, dilute Detergent HT III 10-fold in Buffer A. For example, to 5.4 mL of Buffer A add 600 µL of Detergent HT III. Mix well by pipetting. Prepare only amount needed for immediate use.

11. *Preparation of mitochondrial fraction.* Centrifuge the plate for 3 min at 300 x g at RT. Carefully remove and transfer all the resulting supernatants containing mitochondrial proteins into a 96-well collection plate. These are the mitochondrial fractions (M).

12. *Nuclei Extraction.* Add 50 µL per well of Buffer D. Incubate samples for 10 minutes at RT on a shaker with gentle agitation. The samples may become viscous due to the presence of DNA. To avoid pipetting errors careful pipetting is required.

13. *Preparation of nuclear fraction.* Centrifuge the plate for 3 min at 300 x g at RT. Carefully remove and transfer all the resulting supernatants containing mitochondrial proteins into a 96-well collection plate. These are the nuclear fractions (N).

PREPARATION OF SAMPLES FOR WESTERN BLOT ANALYSIS OF CYTOCHROME C RELEASE USING MSA12 APOTRACK™ CYTOCHROME C APOPTOSIS WB COCKTAIL:

1. Mix four volumes of fraction sample with one volume of 5X SDS-PAGE Sample Buffer (MSA12). For example, mix 36 µL of fraction sample with 9 µL of 5X SDS-PAGE Sample Buffer. Mix well by pipetting.

2. Incubate the samples containing SDS-PAGE Sample Buffer for 10 min at 37°C water bath.

3. Centrifuge to remove bubbles and load samples of equal volumes of fractions C, M and N side by side onto gel immediately.

4. Proceed with Western Blot analysis, we recommend following MitoSciences' guidelines, which can be found on line at http://www.mitosciences.com/PDF/western.pdf.

PREPARATION OF SAMPLES FOR CYTOCHROME C ELISA ANALYSIS USING MSA41 CYTOCHROME C PROTEIN QUANTITY MICROPLATE ASSAY KIT (REAGENTS ARE PROVIDED WITH MSA41):

1. *Dilute DETERGENT*. To dilute DETERGENT 4-fold, for example, to 750 µL of deionized dH_2O add 250 µL of DETERGENT (MSA41). Mix well by pipetting. Prepare only amount needed for immediate use.

2. *Prepare 1.25X Blocking Buffer.* To prepare 1.25X Blocking Buffer, dilute 10X Blocking Buffer 8-fold in SOLUTION I. For example, to 17.5 mL of SOLUTION I (MSA41) add 2.5 mL of 10X Blocking Buffer (MSA41). Mix well by pipetting. Prepare only amount needed for immediate use.

3. In a new 96-well plate, mix well four volumes (36 µL) of C or M fractions with one volume (9 µL) of 4-fold diluted DETERGENT. Mix four volumes (36 µL) of N fractions with one volume (9 µL) of deionized H_2O.

4. Incubate 10 min at RT.

5. Add 20 volumes (180 µL) of 1.25X Blocking buffer.

6. Proceed with Section B. PLATE LOADING in protocol provided with MitoSciences' MSA41 Rapid Microplate Assay Kit for Cytochrome *c*.

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PROTOCOL NOTES

i. *Scale.* The fractionation procedure was optimized for 96-well and 48-well plate. However, it can be utilized in a variety of cell culture formats. When scaling up or down, it is important to keep constant the ratio of the cell number to the plate surface so the cells form a monolayer. It is also important to keep constant the ratio of the amount of detergents to the cell number thus the ratio of detergents' volume to plate surface. Below are suggested parameters for various plate sizes for HeLa cells. It is however recommended to determine the optimal Detergent HT I and II dilution when changing the parameters, as further described below.

	Parameters are given per well						
Plate format	Surface (cm^2)	Cells seeding, optimum ($x\ 10^3$)	Cells seeding, range ($x\ 10^3$)	Wash Buffer A (μL)	Buffer B (μL)	Buffer C (μL)	Buffer D (μL)
96-well	0.32	15	12.5-18	100	50	50	50
48-well	0.75	33	29 - 40	230	115	115	115
24-well	2.0	90	76-110	600	305	305	305

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ii. *Buffer A thawing*. When Buffer A is thawed, the formation of white precipitate is normal. To dissolve the precipitate, incubate the samples 10 min in hot water bath with occasional inversion.

iii. If desired, Buffer A can be supplemented with protease inhibitors, such as Protease Inhibitor Cocktail (Sigma P8340) to minimize nonspecific proteolysis during the fractionation. The procedure was routinely performed at RT. If protein degradation is a concern, the fractionation can be performed at 4°C.

iv. *Pipetting.* Careful pipetting is required, especially to obtain the correct proportion of a protein in each fraction.

v. *Extracts collection.* The centrifugation steps prior the collection of any supernatant are not required if the cells are attached. Since the cells may partially detach during drug treatment as it often occurs in apoptosis, it is compulsory to sediment any detached cells by centrifugation prior the collection of any supernatant, *see Steps 5, 8, 11 and 13*, to avoid the loss of material and fraction cross-contamination. Thus, after a centrifugation step, proceed with supernatant collection immediately.

vi. *Detergent HT I and II extraction.* The appropriate extraction conditions depend on ratio of detergents to the total cellular mass, see DATA ANALYSIS section. Since cells vary in their size, the recommended dilutions of Detergent HT I and II were determined to be optimal for HeLa cells seeded at 15,000 cells per well of 96-well plate.

vii. *Optimalization of cytosol and mitochondria extraction.* To achieve optimal cytosol extraction, for other adherent cell types, we recommend initially titration of Detergent HT I. This can be easily achieved using a series of two-fold dilutions of Detergent HT I in buffer A and applying it into wells containing constant amount of cells in *Step 7* of the PROTOCOL. Then follow the remaining steps in the PROTOCOL. Determine the sample with the lowest Detergent HT I concentration in which GAPDH signal is present in C fraction and minimal/absent in the corresponding M fraction. When cytosol extraction is optimized, the extraction of mitochondrial proteins can be optimized similarly. The optimalization procedures are exemplified in Figures 5 and 6.

viii. If desired, mock-Detergent HT I extracted samples can be prepared by substituting Buffer B with Buffer A in Step 8. Similarly, mock-Detergent HT II or III extracted samples can be prepared by substituting Buffer C or Buffer D with Buffer A in *Step 11 or Step 13*, respectively.

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ix. The nuclear (N) fraction is prepared using protein-denaturing detergent that also extracts the cellular DNA. Thus the obtained nuclear fractions may be viscous.

x. The cytosolic, mitochondrial and nuclear fractions prepared, respectively, in *Steps 8, 11 and 13* may be flash-frozen and stored at -80°C.

xi. Since a drug treatment may directly or indirectly alter the physical properties of biological membranes, the separation of cytosolic, mitochondrial and nuclear proteins into the separate fractions may be altered as it is true for any fractionation procedure.

xii. *Preparation of samples for cytochrome c ELISA analysis by MSA41* Cytochrome c Protein Quantity Microplate Assay Kit. The nuclear (N) fractions as they are generated by the MS862 already contain DETERGENT (MSA41) required to treat samples prior loading on the microplate. Thus the nuclear fractions are diluted only with dH_2O to keep the nuclear material in correct proportion to C and M fractions.

DATA ANALYSIS

1. *Control of fractionation.* The complete permeabilization of the plasma membrane by Detergent HT I and thus release of cytosolic proteins from the cells, as well as complete extraction of mitochondrial proteins by Detergent HT II, nuclear proteins by Detergent HT III, and thus separation of mitochondrial and nuclear compartments are prerequisite for assaying redistribution of cytochrome *c*, and others intermembrane-space localized pro-apoptotic proteins, from mitochondrial intermembrane space into cytosol or nucleus. The MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail allows monitoring, in addition to cytochrome *c*, of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate dehydrogenase E1 α,(PDH E1 α) a mitochondrial matrix protein of 44 kDa, to verify internally the permeabilization process and extraction of mitochondrial proteins. MS862 is optimized to deliver complete Detergent HT I-driven permeabilization of HeLa cells. When this cell line is used, the great majority of GAPDH, a cytosolic protein of about 38 kDa, is present in the C fraction, while little or no signal is present in the M or N fractions, indicating sufficient permeabilization by Detergent HT I to release cytosolic proteins out of the cells. In the untreated control cells, the great majority of cytochrome *c*, an intermembrane space protein of ~13 kDa, is present in the M fraction indicating intactness of mitochondrial outer membrane towards the Detergent HT I (Figures 1 and 2). In cells induced to undergo apoptosis, while cytochrome *c* redistributes from fraction M to fraction C, the great majority of PDH E1 α remains in the M fraction, indicating the intactness of the mitochondrial inner membrane (Figure 2). MS862 is also optimized to deliver complete Detergent HT II-driven extraction of mitochondrial proteins, while preserving majority of nuclear proteins in the Detergent HT II-resistant nuclear fraction. Thus in control HeLa cells the great majority of cytochrome *c* and PDH E1 α is present in the M fraction while little or no signal of these proteins is present in the N fraction. At the same time, the great majority of nuclear markers PARP and transcriptional factor SP1 are found in the nuclear fraction while little or no signal of these proteins is present in the C and M fractions (Figure 1).

2. *General mitochondrial marker*. The MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail allows comparison and normalization of the amounts of mitochondria among different cell types or treatments of cells by assaying for the mitochondrial inner membrane protein, Complex V α (~55 kDa).

3. *Determination of the distribution of a protein between cytosolic, mitochondrial and nuclear fractions.* The distribution of a protein between C, M and N fractions is calculated as percentage of the protein present in a fraction out of the sum of the protein present in C, M and N fractions. For example, the determination of cytosolic cytochrome *c* is indicated by the formula below.

$$\text{Cytochrome } c^{\text{fraction C}}\,(\%) = 100 \times \text{cytochrome } c^{\text{fraction C}} / (\text{cytochrome } c^{\text{fraction C}} + \text{cytochrome } c^{\text{fraction M}} + \text{cytochrome } c^{\text{fraction N}})$$

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If a drug or conditions change the distribution of a protein, the protein distribution before and after the treatment can be compared and protein translocation specific to the treatment can be calculated. For example, the release of cytochrome *c* caused by a drug treatment is indicated by the formula below.

Released Cytochrome *c* $^{\text{fraction C}}$ (%) = Cytochrome *c* $^{\text{fraction C}}$ of treated cell (%) - Cytochrome *c* $^{\text{fraction C}}$ of untreated cells (%)

FREQUENTLY ASKED QUESTIONS

1. **What is the minimum number of cells per well of 96 well plate needed for the fractionation?**

   The separation of cytosolic, mitochondrial and nuclear proteins to their appropriate fraction depends on the ratio of the amount of detergent to cell mass. Thus the amount of cells per well depends on the cell size and therefore may be cell type dependent. Standard separation with minimum fraction cross-contamination was obtained using 12,500 cells per well of 96-well plate and conditions given in the PROTOCOL.

2. **Can the fractionation be performed on cell lines of other species or primary cells?**

   The fractionation can be performed on any adherent cell type. Fractionation optimalization as described in the PROTOCOL NOTES and DATA ANALYSIS may be required.

3. **Can the fractionation be performed on suspension cells?**

   The fractionation was established for adherent cells. It may work, if optimized, properly also for suspension cells but we do not guarantee the results.

4. **What is the interpretation of a result showing that mitochondrial inner membrane protein markers (F_1-ATPase α-subunit) and nuclear markers (PARP) contaminating the cytosolic fraction?**

   Cells may partially detach. Make sure to centrifuge the cells as described in the PROTOCOL prior any collection of a supernatant. Centrifugation speed and time can be increased.

5. **What is the interpretation of a result showing that a mitochondrial intermembrane space protein or matrix protein (cytochrome c or PDH E1 α) but not mitochondrial inner membrane protein (F_1-ATPase α-subunit) is contaminating the cytosolic fraction?**

   Since soluble mitochondrial proteins were extracted, the concentration of Detergent HT I was too high. Decrease the Detergent HT I concentration when preparing Buffer B. Titration of Detergent HT I may be required as discussed in PROTOCOL NOTES and DATA ANALYSIS and shown in Figure 5.

6. **What is the interpretation of a result showing that a cytosolic protein (GAPDH) is contaminating the mitochondrial fraction?**

   This is caused by insufficient permeabilization of plasma membrane by the Detergent HT I. Increase the Detergent HT I concentration when preparing Buffer B. Titration of Detergent HT I may be required as discussed in PROTOCOL NOTES and DATA ANALYSIS and shown in Figure 5.

7. **What is the interpretation of a result showing that a mitochondrial inner membrane protein (F_1-ATPase α-subunit) is contaminating the nuclear fraction?**

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This is caused by insufficient extraction of mitochondrial proteins by the Detergent HT II. Increase the Detergent HT II concentration when preparing Buffer C. Titration of Detergent HT II may be required as discussed in PROTOCOL NOTES and DATA ANALYSIS and shown in Figure 6.

8. What is the interpretation of a result showing that a nuclear protein (PARP) is contaminating the mitochondrial fraction?

This is probably caused by too high concentration of Detergent HT II when extracting mitochondrial proteins. Decrease the Detergent HT II concentration when preparing Buffer C. Titration of Detergent HT II may be required as discussed in PROTOCOL NOTES and DATA ANALYSIS and shown in Figure 6.

9. What is the interpretation of a result showing that a protein is found in a fraction in untreated cells but found in different fraction of treated cells?

The protein shows treatment-specific re-localization from one compartment to another compartment. Please, see also PROTOCOL NOTE xii. It is always a good idea to confirm the re-localization by an independent assay, for example by immunocytochemistry.

10. Is the concentration of proteins in the fractions sufficient for Western blot analysis?

This is dependent on the Western blot sensitivity, mainly on the affinity of the primary antibody and detection method. We recommend to use appropriate HRP-conjugated secondary antibody and ECL detection (ECL Plus detection, GE Healthcare, # RPN2132). Using 15,000 HeLa cells per well and conditions as described in the PROTOCOL, fractions derived from 4-2 mg of total cellular protein per can be analyzed per lane. In our experience all the primary antibodies tested resulted in sufficient signal. If the protein amount to be analyzed is still a concern, we recommend the batch-based MS861 Cell Fractionation Kit yielding approximately 10 times higher total protein concentrations.

11. I can still see some crosscontamination of marker proteins even I optimized the extraction conditions. What can I do?

This method is adapted to a high throughput format using small extraction volumes; inevitably low levels of fraction crosscontamination are expected. To obtain better separation of cellular fractions we recommend the batch-based MS861 Cell Fractionation Kit.

12. Are the extracted proteins in native form?

This is a detergent based method. Detergent HT I and II are very mild and generally do not lead to protein denaturation. Detergent HT III is a strong denaturant. If the goal is to prepare nuclear proteins in a native state, please, use appropriate extraction method for your favorite protein instead Buffer D.

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EXAMPLE EXPERIMENT

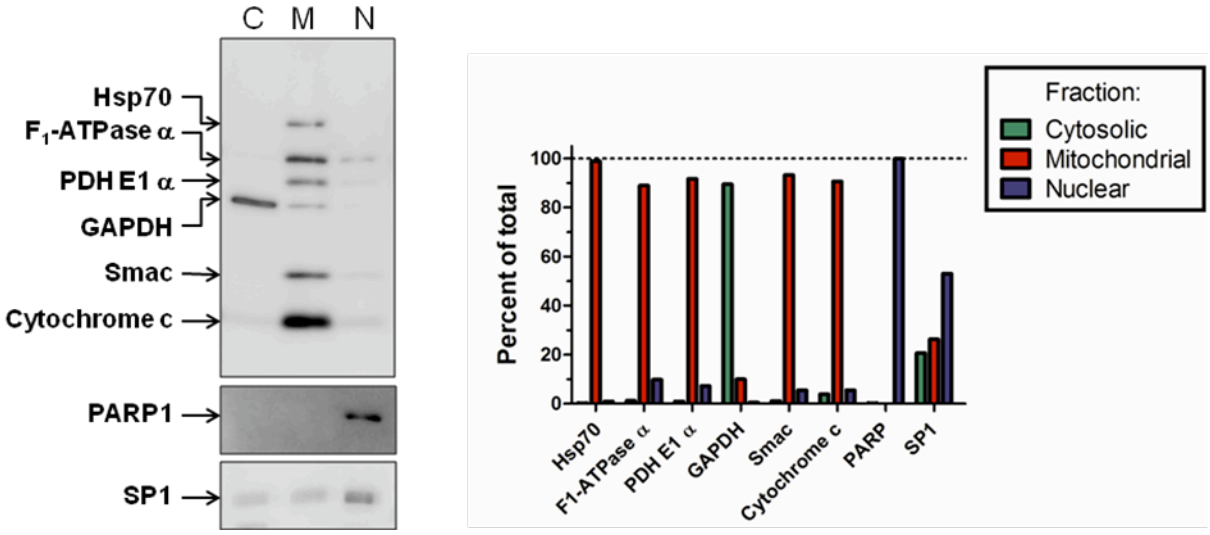

Figure 1. Characterization of cytosolic (C), mitochondrial (M) and nuclear (N) fraction of HeLa cells prepared as described in the Protocol. Fractions, each derived from one well of 48-well plate, were analyzed by Western blotting using MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (MitoSciences), containing antibodies against mitochondrial matrix (pyruvate dehydrogenase subunit E1 α, PDH E1 α), mitochondrial inner membrane (F_1-ATPase α), mitochondrial intermembrane space (cytochrome *c*) and cytosolic (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) markers, and supplemented with antibodies against additional mitochondrial intermembrane space (Smac) and mitochondrial matrix (Hsp70) markers, as well as with antibodies against nuclear (poly (ADP-ribose) polymerase, PARP and SP1) markers, followed by appropriate HRP-conjugated goat secondary antibodies and ECL Plus detection (Amersham, # RPN2132). Representative blots as well as the quantitative analysis, as described in DATA ANALYSIS, are shown.

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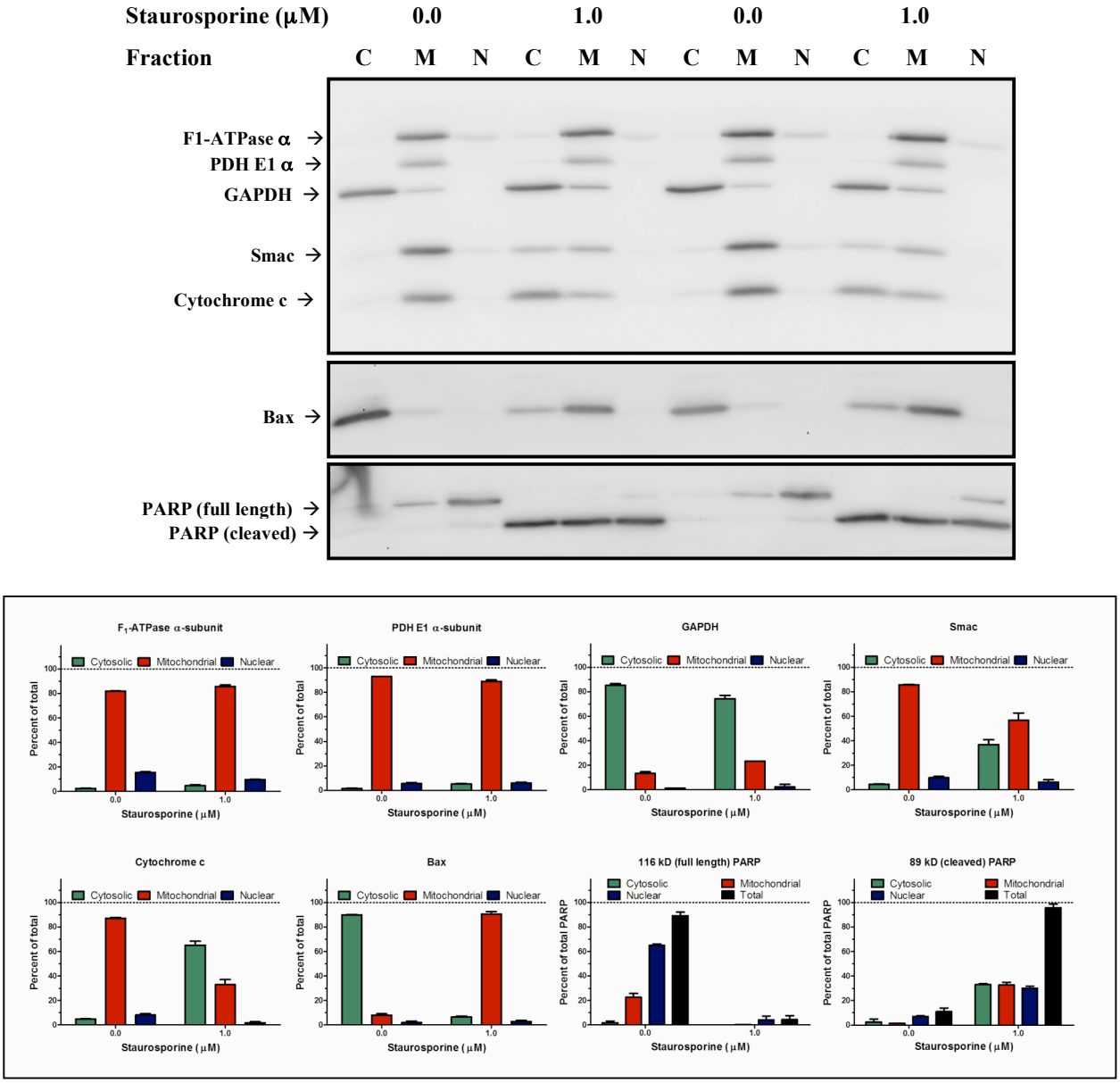

Figure 2. Western blot analysis of cytochrome c and Smac release from the mitochondria into the cytosol and Bax relocalization from the cytosol to mitochondria in HeLa cells induced to undergo apoptosis by Staurosporine treatment. HeLa cells were treated for 4 hrs with 1.0 µM Staurosporine or were left untreated. Cytosolic (C), mitochondrial (M) and nuclear (N) fractions were prepared as described in the PROTOCOL. Fractions, each derived from one well of 96-well plate, were analyzed by Western blotting using MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (MitoSciences), containing antibodies against F_1-ATPase α, PDH E1 α, GAPDH and cytochrome *c*, and supplemented with antibody against Smac, as well as with antibodies against Bax and PARP, followed by appropriate HRP-conjugated goat secondary antibodies and ECL Plus detection (Amersham, # RPN2132). Representative blots as well as the quantitative analysis (mean +/- standard error of the mean, n=2), as described in DATA ANALYSIS, are shown.

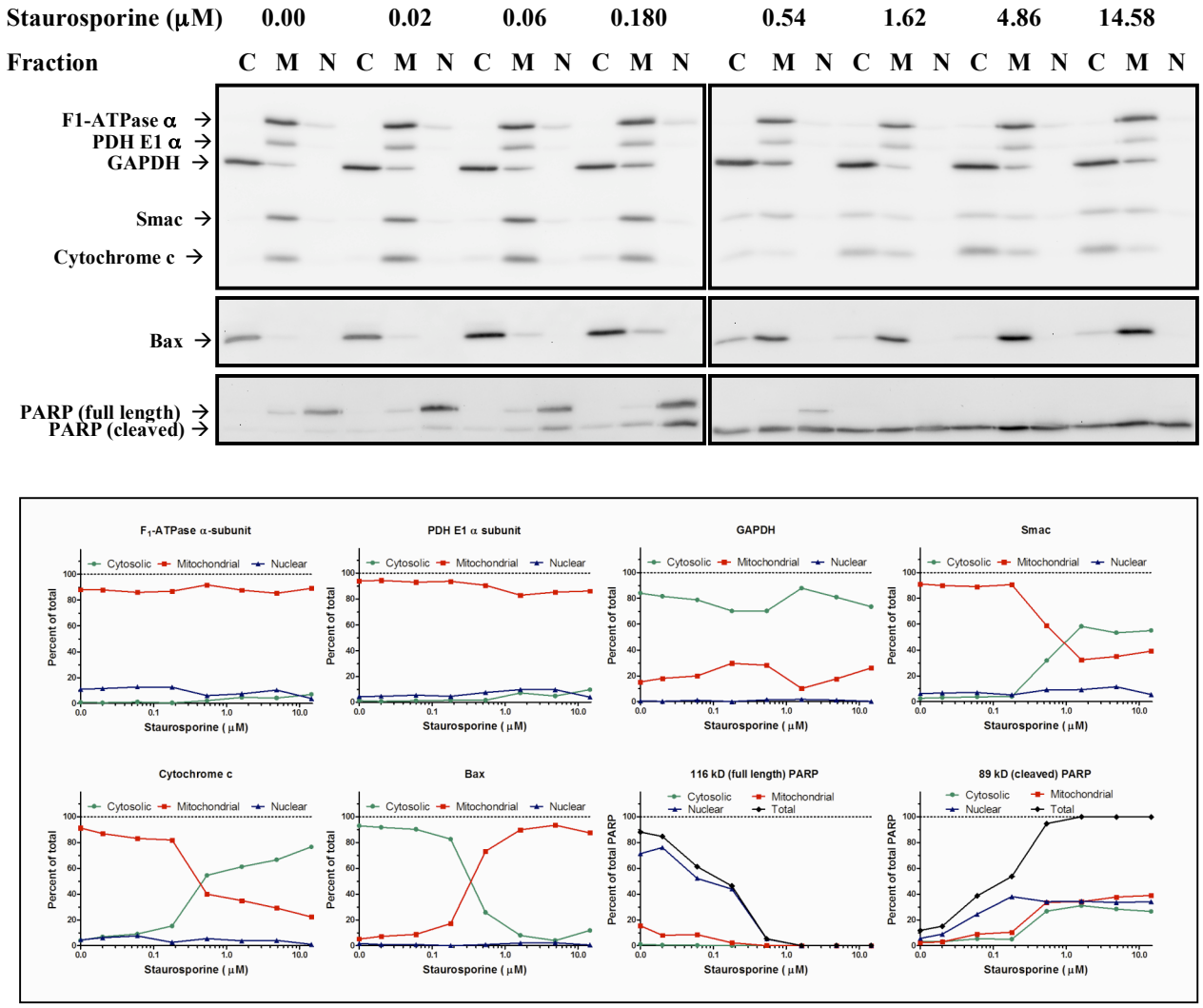

Figure 3. Western blot analysis of cytochrome c and Smac release from the mitochondria into the cytosol and Bax relocalization from the cytosol to mitochondria in HeLa cells induced to undergo apoptosis by Staurosporine treatment. Cytosolic (C), mitochondrial (M) and nuclear (N) fractions of HeLa cells treated for 4 hrs with 0.00, 0.02, 0.06, 0.18, 0.54, 1.62, 4.86 and 14.58 µM Staurosporine were prepared as described in the PROTOCOL. Fractions, each derived from one well of 96-well plate, were analyzed by Western blotting using MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (MitoSciences), containing antibodies against F_1-ATPase α, PDH E1 α, GAPDH and cytochrome *c*, and supplemented with antibody against Smac, as well as with antibodies against Bax and PARP, followed by appropriate HRP-conjugated goat secondary antibodies and ECL Plus detection (Amersham, # RPN2132). Representative blots as well as the quantitative analysis, as described in DATA ANALYSIS, are shown.

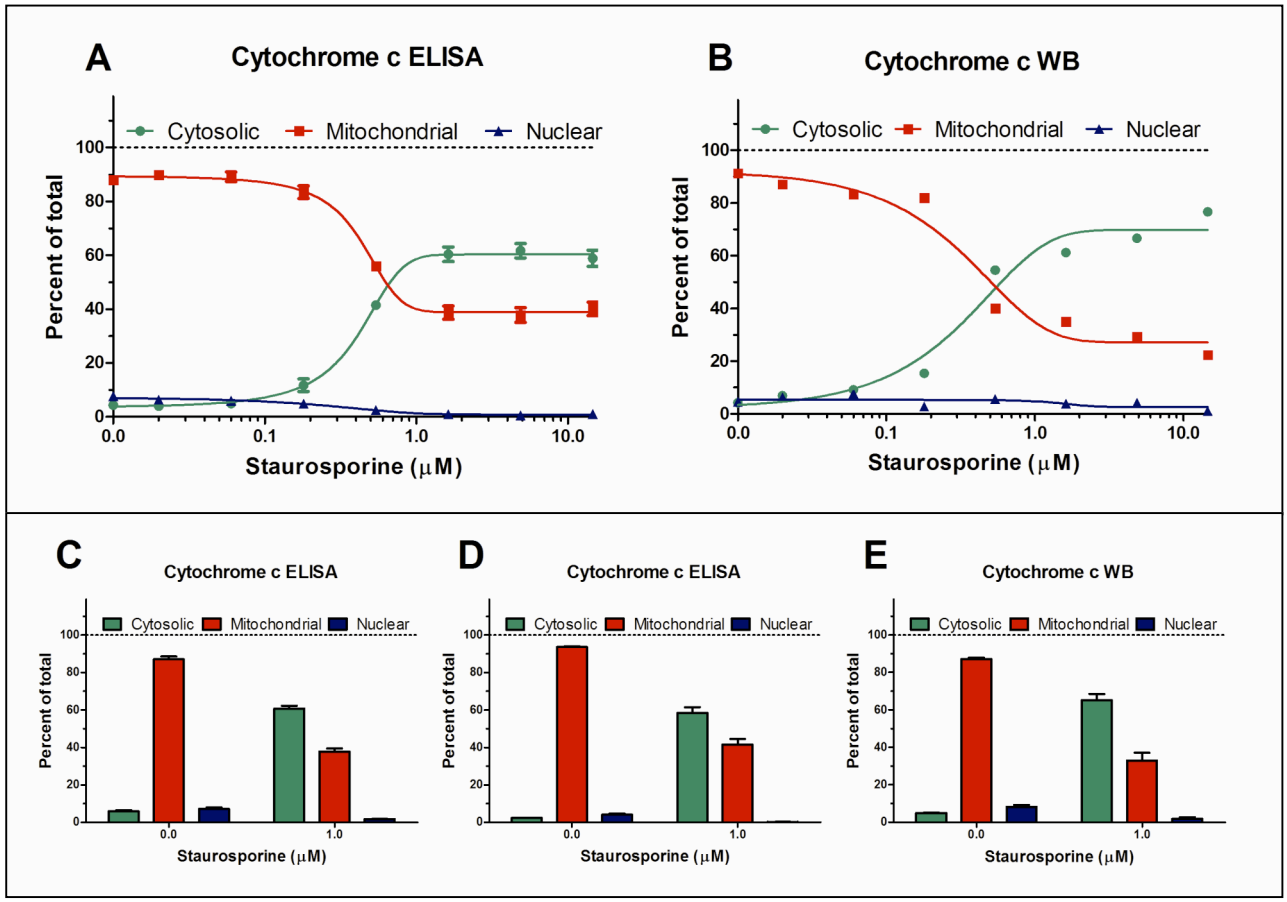

Figure 4. Qantitative ELISA analysis of cytochrome c release from the mitochondria into the cytosol in HeLa cells induced to undergo apoptosis by Staurosporine treatment. Cytosolic (C), mitochondrial (M) and nuclear (N) fractions of HeLa cells treated for 4 hrs with 0.00, 0.02, 0.06, 0.18, 0.54, 1.62, 4.86 and 14.58 µM Staurosporine (**A** and **B**) or with 0.0 and 1.0 µM Staurosporine (**C**, **D**, **E**) were prepared as described in the PROTOCOL. Fractions, each derived from one well of 96-well plate, were analyzed by Cytochrome c Protein Quantity Microplate Assay Kit (**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 MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (MitoSciences), described in Figure 2 and 3, 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).

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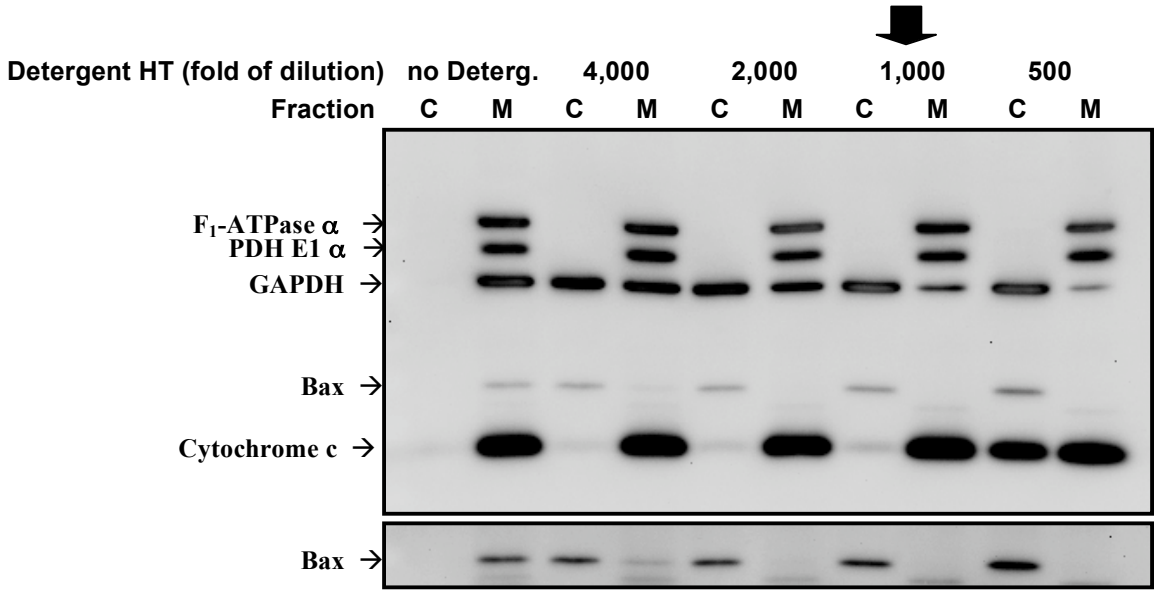

Figure 5. Optimalization of cell permeabilization to separate cytosolic and mitochondrial fractions. Cytosolic (C), mitochondrial (M) fractions of HeLa cells, at 30,000 per well of 48-well plate, were prepared as described in the PROTOCOL using variable concentrations of Detergent HT I diluted in Buffer A. Fractions were analyzed by Western blotting using MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (MitoSciences), containing antibodies against F_1-ATPase α, PDH E1 α, GAPDH and cytochrome *c*, and supplemented with antibody against Bax, followed by appropriate HRP-conjugated goat secondary antibodies and ECL Plus detection (Amersham, # RPN2132). Arrow indicates the dilution of Detergent HT I for optimal separation cytosolic and mitochondrial proteins.

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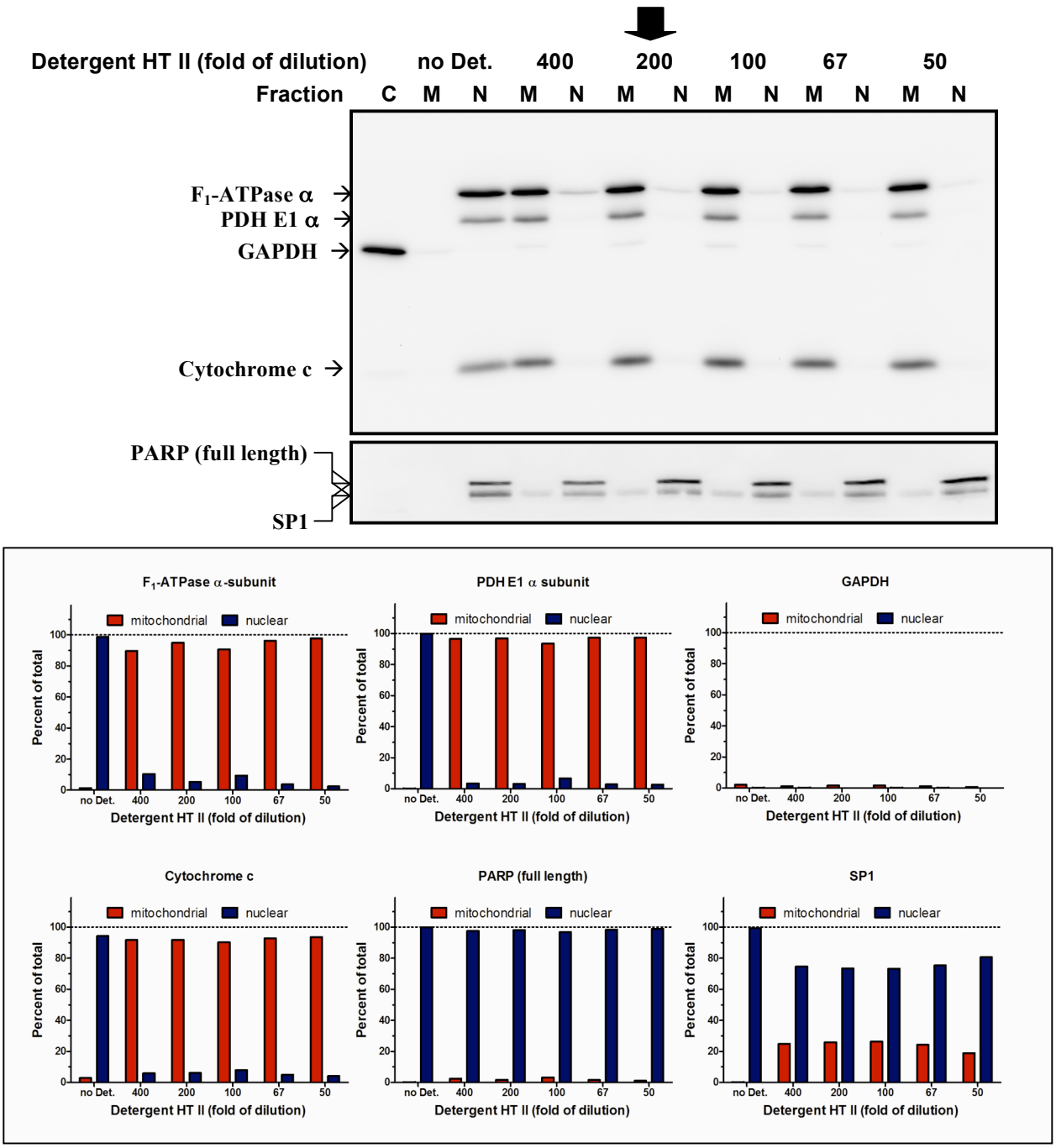

Figure 6. Optimalization of separation mitochondrial and nuclear fractions. Cytosolic (C), mitochondrial (M) and nuclear (N) fractions of HeLa cells, at 18,000 per well of 96-well plate, were prepared as described in the PROTOCOL using variable concentrations of Detergent HT II diluted in Buffer A. Fractions were analyzed by Western blotting using MSA12 ApoTrack™ Cytochrome *c* Apoptosis WB Antibody Cocktail (MitoSciences), containing antibodies against F_1-ATPase α, PDH E1 α, GAPDH and cytochrome *c*, as well as with antibodies against PARP and SP1, followed by appropriate HRP-conjugated goat secondary antibodies and ECL Plus detection (Amersham, # RPN2132). Arrow indicates optimal dilution of Detergent HT II to for separation cytosolic and mitochondrial proteins. Representative blots as well as the quantitative analysis, as described in DATA ANALYSIS, are shown.

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FLOW CHART

(For quick reference only. Be completely familiar with previous details of this document before performing the assay.)

- Grow adherent cells overnight in a 96-well plate, approximately 1.5 x10^3 cells per well.
- Treat cells (induce apoptosis) as desired

↓

- Prepare Buffer B by 1000-fold dilution of Detergent HT I in Buffer A
- Centrifuge cells at 300 x g for 3 min (optional)
- Remove media
- Add 100 µL per well of Buffer A
- Centrifuge cells at 300 x g for 3 min (optional)
- Remove buffer A wash

↓

- Add 50 µL per well of Buffer B
- Incubate the plate with gentle agitation for 7 min at RT
- Prepare Buffer C by 200-fold dilution of Detergent HT II in Buffer A
- Centrifuge cells at 300 x g for 3 min (optional)
- Remove and save the extract. This is cytosolic (C) fraction

↓

- Add 50 µL per well of Buffer C
- Incubate the plate with gentle agitation for 10 min at RT
- Prepare Buffer D by 10-fold dilution of Detergent HT III in Buffer A
- Centrifuge cells at 300 x g for 3 min (optional)
- Remove and save the extract. This is mitochondrial (M) fraction

↓

- Add 50 µL per well of Buffer D
- Incubate the plate with gentle agitation for 10 min at RT
- Centrifuge cells at 300 x g for 3 min (optional)
- Remove and save the extract. This is nuclear (N) fraction

WESTERN BLOT ANALYSIS OF CYTOCHROME C RELEASE USING MSA12 APOTRACK™ CYTOCHROME C APOPTOSIS WB COCKTAIL:

- Mix four volumes (36 μL) of fraction sample with one volume (9 μL) of 5X SDS-PAGE Sample Buffer (MSA12)
- Incubate 10 minutes at 37°C
- Load the samples on the gel

↓

- Incubate the blocked membrane with provided antibody cocktail diluted 250-fold in PBS containing 5% non-fat milk powder for 2 hrs at RT

↓

- Calculate the cytosolic cytochrome *c* in both untreated and treated cells:

$$\text{Cyt } c^{C}\ (\%) = 100 \times \text{Cyt } c^{C} / (\text{Cyt } c^{C} + \text{Cyt } c^{M} + \text{Cyt } c^{N})$$

- Calculate the treatment-specific release of cytochrome c into the cytosol:

$$\text{Cyt } c^{C} \text{ Released } (\%) = \text{Cyt } c^{C} \text{ Treated } (\%) - \text{Cyt } c^{C} \text{ Untreated } (\%)$$

PREPARATION OF SAMPLES FOR CYTOCHROME C ELISA ANALYSIS USING MSA41 RAPID MICROPLATE ASSAY KIT FOR CYTOCHROME *C* (REAGENTS ARE PROVIDED WITH MSA41):

- Dilute DETERGENT (MSA41) 4-fold with deionized H_2O
- Prepare 1.25X Blocking Solution by diluting 10X Blocking Solution (MSA41) 8-fold with SOLUTION 1 (MSA41)

↓

- Mix four volumes (36 μL) of C or M fractions with one volume (9 μL) of 4-fold diluted DETERGENT
- Mix four volumes (36 μL) of N fractions with one volume (9 μL) of deionized H_2O
- Incubate 10 minutes at RT
- Add twenty volumes (180 μL) of 1.25X Blocking Solution
- Proceed with section B. Plate Loading in protocol provided with MitoSciences' MSA41 Rapid Microplate Assay Kit for Cytochrome *c*

↓

- Calculate the cytosolic cytochrome *c* in both untreated and treated cells:

$$\text{Cyt } c^{C}\ (\%) = 100 \times \text{Cyt } c^{C} / (\text{Cyt } c^{C} + \text{Cyt } c^{M} + \text{Cyt } c^{N})$$

- Calculate the treatment-specific release of cytochrome c into the cytosol:

$$\text{Cyt } c^{C} \text{ Released } (\%) = \text{Cyt } c^{C} \text{ Treated } (\%) - \text{Cyt } c^{C} \text{ Untreated } (\%)$$

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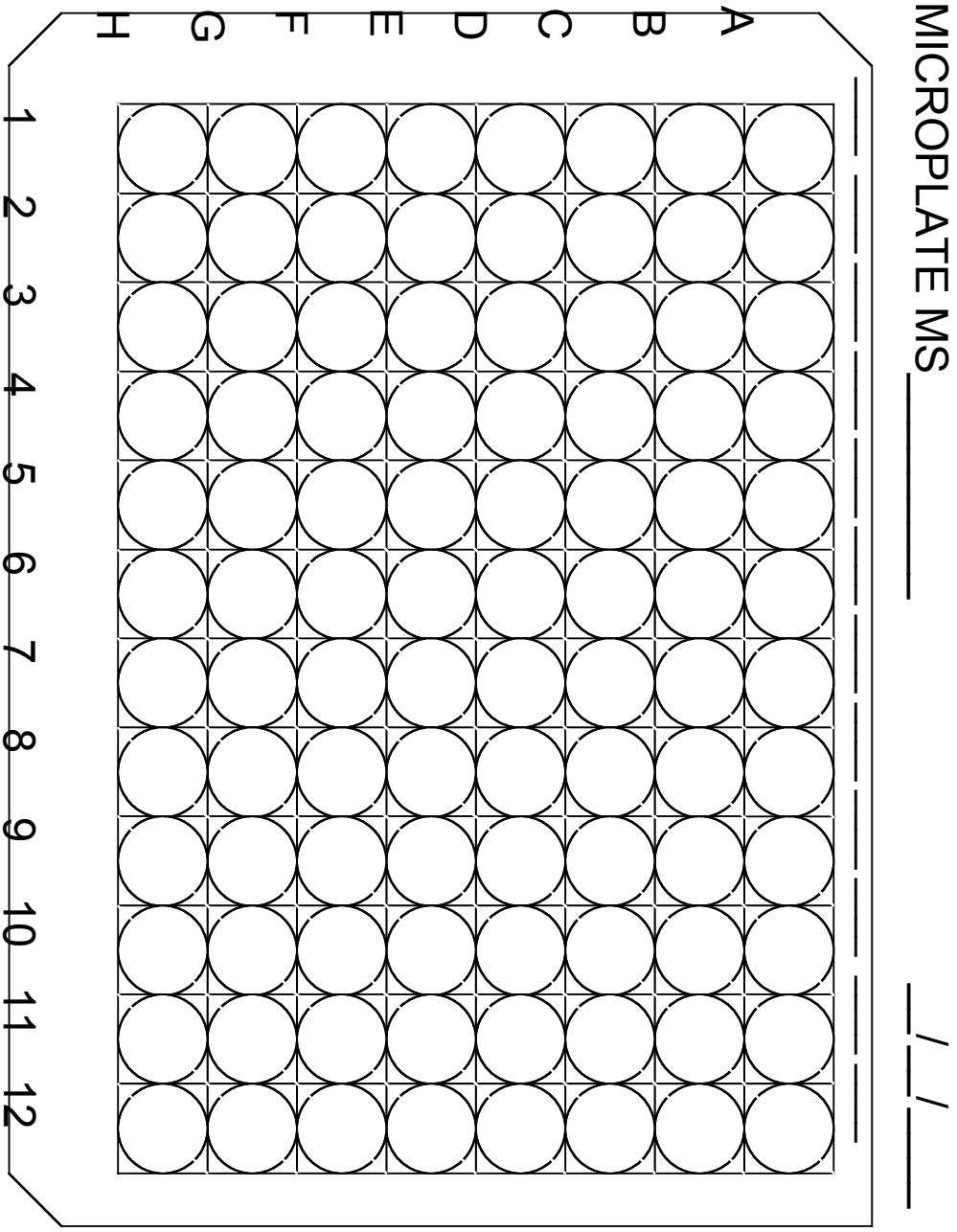
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