



# **Cell Fractionation Kit - Standard**

## **Instructions for Use**

For the rapid and simple separation of mitochondrial, cytosolic and nuclear fractions.

[View kit datasheet: www.abcam.com/ab109719](http://www.abcam.com/ab109719)

(use [www.abcam.cn/ab109719](http://www.abcam.cn/ab109719) for China, or [www.abcam.co.jp/ab109719](http://www.abcam.co.jp/ab109719) for Japan)

This product is for research use only and is not intended for diagnostic use.

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# 1. Introduction

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ab109719 (MS861) provides a method and reagents for a rapid preparation of cytosolic, mitochondrial and nuclear fractions. The kit is based on sequential detergent-extraction of cytosolic and mitochondrial proteins without the need for mechanical disruption of cells, and thus fractionates cells into cytosol-containing, mitochondria-containing and nuclei-containing fractions. These fractions are referred throughout the protocol as cytosolic, mitochondrial and nuclear fractions. With this kit sufficient sample material can be prepared for subsequent Western blot analysis, or for analysis by microplate ELISA or dipstick assay.

ab109719 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 successfully 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 Figure 4.

ab109719 provides a 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 and mitochondrial proteins with proprietary detergents that allow sequential release of cytosolic and mitochondrial proteins to the extracellular buffer. In the first step, the plasma membrane is selectively permeabilized with Detergent I. The cytosol-containing fraction is separated from the remainder of cells containing intact mitochondria and nuclei by a simple centrifugation step. In the second step, mitochondrial proteins are then extracted with Detergent II and separated from the nuclei-containing fraction by a second centrifugation step.

In control 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).

The three distinct fractions generated can be analyzed by Western blot or by ELISA microplate. For Western blot analysis, ApoTrack™ Cytochrome *c* Apoptosis WB Cocktail is recommended

(ab110415/MSA12) (typical results shown below), which contains an antibody against cytochrome c (ab110325/MSA06 Anti-cytochrome c monoclonal antibody) plus antibodies against key mitochondrial and cytosolic markers. For the analysis of cytochrome c by microplate ELISA assay, Abcam's ab110172 (MSA41) Cytochrome c Protein Quantity Microplate Assay Kit is recommended. These methods were verified on HeLa cells, 143B osteosarcoma cells, SHSY5Y neuroblastoma cells, HepG2 cells and HdFN fibroblast cells treated with Staurosporine or Jurkat TIB 152 cells incubated with Staurosporine or anti-Fas antibody to undergo apoptosis. The proportion of cytochrome c found in the cytosol-containing fractions by this method correlated with the results of immunocytochemical analysis using Abcam's ab110417/MSA07 ApoTrack™ Cytochrome c Apoptosis for Immunocytochemistry (see Figure 5).

## 2. Protocol Summary

- Grow cells in two 100 mm dishes, approximately  $2.5 \times 10^6$  cells.
- Induce apoptosis in one dish by a desired method
- Harvest cells by centrifugation at 300 x g for 5 min



- Re-suspend cells in 5 ml of 1X Buffer A
- Determine cell count and total cell number
- Centrifuge cells at 300 x g for 5 min



- Re-suspend cells in buffer A to  $6.6 \times 10^6$  cells/ml
- Prepare Buffer B by 1000-fold dilution of Detergent I in Buffer A
- Dilute the cell suspension with equal volume of Buffer B
- Incubate the tube with constant mixing for 7 min at RT



- Centrifuge the cell suspension at 5,000 x g for 1 min at 4°C
- Remove and save supernatant, save also pellet
- Centrifuge the supernatant at 10,000 x g for 1 min at 4°C
- Save the final supernatant; this is fraction C



- Re-suspend and combine both sequential pellets in Buffer A to the original volume of cell suspension prior the addition of Buffer B
- Prepare Buffer C by 25-fold dilution of Detergent II in Buffer A
- Dilute the suspension with equal volume of Buffer C
- Incubate the tube with constant mixing for 10 min at RT



- Centrifuge the cell suspension at 5,000 x g for 1 min at 4°C
- Remove and save supernatant, save also pellet
- Centrifuge the supernatant at 10,000 x g for 1 min at 4°C
- Save the final supernatant; this is fraction M



- Re-suspend and combine both sequential pellets in Buffer A to the original volume of suspension after the addition of Buffer C; this is fraction N

## WESTERN BLOT ANALYSIS OF CYTOCHROME C RELEASE USING ANTIBODY COCKTAIL ab110415/MSA12:

- Mix four volumes of sample with one volume of 5X SDS-PAGE Sample Buffer
- Vortex thoroughly
- 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:  
$$\text{Cyt } c_c \text{ Released } (\%) = \text{Cyt } c_c \text{ Treated } (\%) - \text{Cyt } c_c \text{ Untreated } (\%)$$

### 3. Kit Contents

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Sufficient materials are provided for fractionation of  $1 \times 10^8$  cells or for preparation of 40 samples, each corresponding to one 100 mm plate at  $2.5 \times 10^6$  cells/plate.

- 2X Buffer A: 175 mL
- Detergent I: 25  $\mu$ L
- Detergent II: 1 mL
- 5X SDS Sample Buffer: 10 mL

### 4. Storage and Handling

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Store all components at  $-20^{\circ}\text{C}$ , except Detergent I stored at  $-80^{\circ}\text{C}$ . Ship on dry ice.

### 5. Additional Materials Required

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- Tube rotator for 1.5 ml tubes
- Cell counting device such as hemacytometer



## 6. Protocol

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***Note: This protocol contains detailed steps for preparation of subcellular fractions and 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 two 100 mm tissue culture plates at an equal density and grow them to semi-confluent density.
- 6.2. *Induce apoptosis.* Incubate cells in one dish in the presence of inducer of apoptosis at desired concentration and for desired time. In parallel, incubate the uninduced control cells in another dish.
- 6.3. Equilibrate 2X Buffer A to room temperature (RT, see note ii) and add equal volume of water to make 1X Buffer A.
- 6.4. *Collect cells.* For adherent cells, remove and save medium. Detach cells by treatment with 8 ml of 0.25% Trypsin-EDTA and add the detached cells into the saved medium. Rinse the plate with additional 4 ml of 0.25% Trypsin-EDTA and add the rinse to the pooled cells. Collect cells by centrifugation for 5 min at 300 x g at RT in a swinging bucket rotor centrifuge.

- 6.5. *1X Buffer A wash.* Re-suspend cell pellets in 5 ml of 1X Buffer A. Take a small aliquot (~25  $\mu$ L) of un-induced control cells for counting. Note the volumes of both cell suspensions. Collect cells by centrifugation for 5 min at 300 x g at RT.
- 6.6. *Count cells.* While centrifugation proceeds, count the uninduced cells using hemacytometer and determine the total cell number in the control sample.
- 6.7. *Prepare cell suspension in 1X Buffer A.* Discard supernatants and re-suspend control cell pellet in 1X Buffer A to  $6.6 \times 10^6$  cells/ml. Re-suspend the induced cell pellet in the same volume of 1X Buffer A. See Note iii.
- 6.8. *Prepare Buffer B.* To prepare Buffer B, dilute Detergent I 1000-fold in 1X Buffer A. For example, to 5 ml of 1X Buffer A add 5  $\mu$ l of Detergent I. Mix well by pipetting. Prepare only amount needed for immediate use.
- 6.9. *Cytosol Extraction.* Transfer a volume of the cell suspensions into a new set of tubes. Add the equal volume of Buffer B to the cell suspensions. Mix by pipetting. Incubate samples for 7 minutes on a rotator at RT.

- 6.10. *Centrifugation.* Centrifuge samples at 5,000 x g for 1 min at 4°C. Carefully remove all supernatants and transfer them to a new set of tubes. Save pellets on ice. Re-centrifuge the supernatant fractions at 10,000 x g for 1 min.
- 6.11. *Preparation of cytosolic fractions.* Transfer the resulting supernatants containing cytosolic proteins into a new set of tubes. These are the cytosolic fractions (C).
- 6.12. *Prepare suspensions of the cytoplasm-depleted “cells” (containing mitochondria and nuclei) in 1X Buffer A.* Re-suspend and combine the sequential cytoplasm-depleted “cell” pellets, generated in Steps 10 and 11, in 1X Buffer A. Use the same volume of 1X Buffer A as was used to suspend the intact control cells, to  $6.6 \times 10^6$  cells/ml in Step 9 prior to addition of Buffer B.
- 6.13. *Prepare Buffer C. To prepare Buffer C, dilute Detergent II 25-fold in 1X Buffer A.* For example, to 4.8 ml of 1X Buffer A add 0.2 ml of Detergent II. Mix well by pipetting. Prepare only amount needed for immediate use.
- 6.14. *Mitochondria Extraction.* Transfer a volume of the cytosol-depleted samples generated in Step 12 into a new set of tubes, for example 9/10 of the re-suspended sample. Add exactly the same volume of Buffer C to the suspensions.

Mix by pipetting. Incubate samples for 10 minutes on a rotator at RT.

- 6.15. *Centrifugation.* Centrifuge samples at 5,000 x g for 1 min at 4°C. Carefully remove all supernatants and transfer them to a new set of tubes. Save pellets on ice. Re-centrifuge the supernatant fractions at 10,000 x g for 1 min.
- 6.16. *Preparation of mitochondrial fraction.* Transfer the resulting supernatants containing mitochondrial proteins into a new set of tubes. These are the mitochondrial fractions (M).
- 6.17. *Preparation of nuclear fraction.* Re-suspend and combine the sequential cell pellets, generated in *Steps 15 and 16*, in 1X Buffer A to the original volume of suspensions after the addition of Buffer C in *Step 14*. These fractions contain re-suspended cytosol- and mitochondria-depleted remainder of cells containing nuclei and thus represent the nuclear fractions (N).
- 6.18. *Analysis by ELISA.* For analysis of fractions by Abcam's Cytochrome c Protein Quantity Microplate Assay Kit (ab110172/ MSA41), follow the provided protocol.

*Note: Analysis by SDS-PAGE/Western blotting.* Mix four volumes of sample with one volume of 5X SDS-PAGE Sample Buffer. The samples prepared from nuclear fractions (N) may become very viscous due to the presence of DNA. To avoid pipetting errors it is important to shear the DNA. This is best done using a probe sonicator; alternatively the DNA may be sheered by very vigorous vortexing for about two minutes. Incubate the samples containing SDS PAGE Sample Buffer for 10 min in 60°C water bath and vortex again briefly. Load samples of equal volumes of fractions C, M and N side by side onto gel immediately.

6.19. *Western Blotting.* Proceed with Western Blot analysis.

## **7. Protocol Notes**

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- i. *Cell collection.* Since cell detachment during apoptosis is a common phenomenon, it is compulsory to collect, in the case of adherent cells, any cells floating in the medium in addition to the cells attached to the dish *see Step 4*.
- ii. *2X Buffer A thawing.* After 2X Buffer A is thawed or brought to 1X with water, the formation of white precipitate is normal. To dissolve the precipitate, incubate the samples 10 min in hot water bath with occasional inversion.

- iii. *Detergent I permeabilization.* The appropriate permeabilization conditions depend on ratio of Detergent I to the total cellular mass, see **Data Analysis** section. Since cells vary in their size, the recommended cell concentration ( $3.3 \times 10^6$  cells/ml) during the treatment with Detergent I was determined to be optimal for 143B osteosarcoma cells, HeLa cells, HepG2 cells, HdFN cells and SHSY5Y cells. To keep the ratio of detergent to total cellular mass constant, small cells, like Jurkat cells, need to be re-suspended to  $20 \times 10^6$  cells/ml at Step 7, to obtain  $10 \times 10^6$  cells/ml during the treatment with Detergent I. The same rules apply to extraction of mitochondrial proteins by Detergent II.
- iv. For other cell types, we recommend to initially optimize the ratio of Detergent I to the total cellular protein. Prepare a two-fold dilution set of cell suspensions in 1X Buffer A. To each sample add equal volume of diluted detergent as in Step 9. Then follow the steps in the protocol. Determine the sample with the lowest ratio of detergent to cell number in which GAPDH signal is present in C fraction and absent in the corresponding M fraction. Use this ratio to analyze translocation of proteins between mitochondria and cytosol in a drug-treated sample of this particular cell line.

- v. The cytosolic, mitochondrial and nuclear fractions prepared, respectively, in Steps 11, 16 and 17 may be flash-frozen and stored at -80°C.
- vi. *Protein assay (optional)*. Save a small aliquot of cell suspension from Step 5 for subsequent determination of total protein in the whole cell suspension. Subsequently, when fractions are analyzed, load fractions derived from untreated and treated cells in amounts proportional, respectively, to the equal amount of protein in the untreated and treated whole cell suspension.
- vii. If desired, mock-Detergent I treated samples can be prepared by dilution of cell suspension prepared in Step 7 with equal volume of the 1X Buffer A. Similarly, mock-Detergent II treated samples can be prepared by dilution of suspension of cytosol-depleted remainder of cells prepared in Step 12 with equal volume of the 1X Buffer A.
- viii. If desired, 1X Buffer A can be supplemented with protease inhibitors, such as Protease Inhibitor Cocktail to minimize nonspecific proteolysis during the fractionation.

## 8. Data Analysis

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1. *Control of fractionation.* The complete permeabilization of the plasma membrane by Detergent I and thus release of cytosolic proteins from the cells, as well as complete extraction of mitochondrial proteins by Detergent II 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 ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (ab110415/ MSA12) allows monitoring, in addition to cytochrome c, of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate dehydrogenase E1  $\alpha$ , (PDH E1  $\alpha$ ) a mitochondrial matrix protein of 44 kDa, to verify internally the permeabilization process. ab109719 is optimized to deliver complete Detergent I-driven permeabilization of HeLa, 143B, HepG2, SHSY5Y, HepG2, HdFN and Jurkat cells. When these cells are 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 fraction, indicating sufficient permeabilization by Detergent 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 I. In cells induced to undergo apoptosis, while cytochrome *c* redistributes from fraction M to fraction C, the great majority of PDH E1  $\alpha$  remains in the M fraction, indicating the intactness of the mitochondrial inner membrane. ab109719 is also optimized to deliver complete Detergent II-driven extraction of mitochondrial proteins, while preserving majority of nuclear proteins in the detergent-resistant nuclear fraction. Thus in control HeLa or HepG2 cells the great majority of cytochrome *c* and PDH E1  $\alpha$  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.

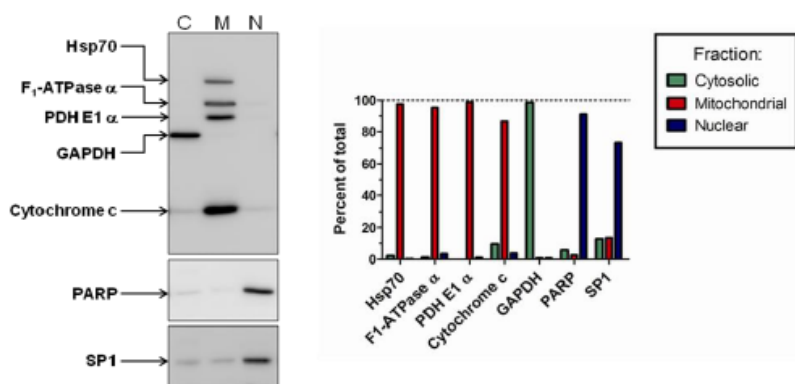
2. *General mitochondrial marker.* The kit allows comparison and normalization of the amounts of mitochondria among different cell types or treatments of cells by assaying for the mitochondrial inner membrane protein, Complex V  $\alpha$  (~55 kDa).
3. *Determination of the distribution of a protein 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.

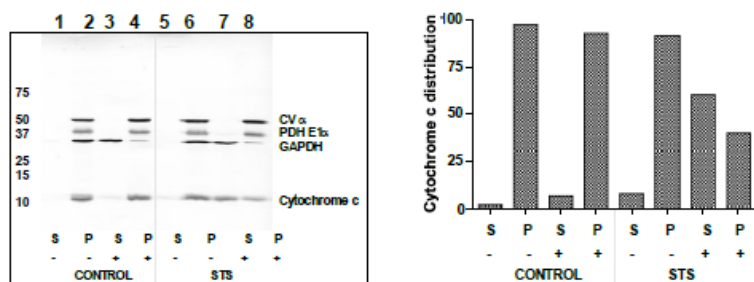
Cytochrome c fraction C (%) =  $100 \times \text{cytochrome c fraction C} / (\text{cytochrome c fraction C} + \text{cytochrome c fraction M} + \text{cytochrome c fraction N})$

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.

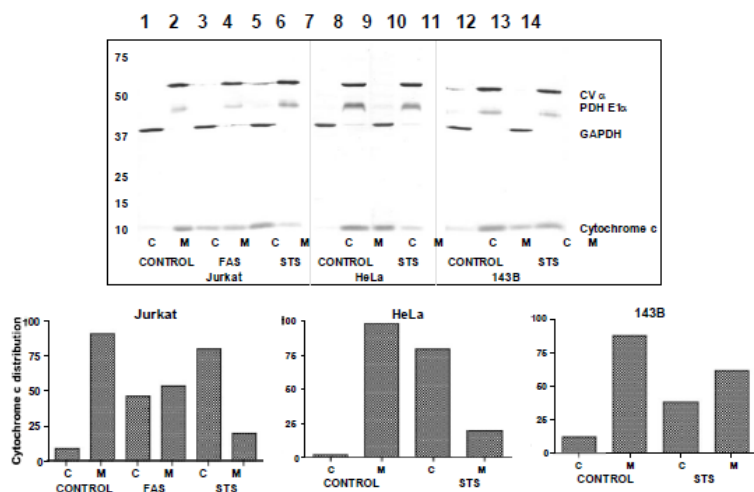
Released Cytochrome c fraction C (%) =  $\text{Cytochrome c fraction C of treated cell (\%)} - \text{Cytochrome c fraction C of untreated cells (\%)}$



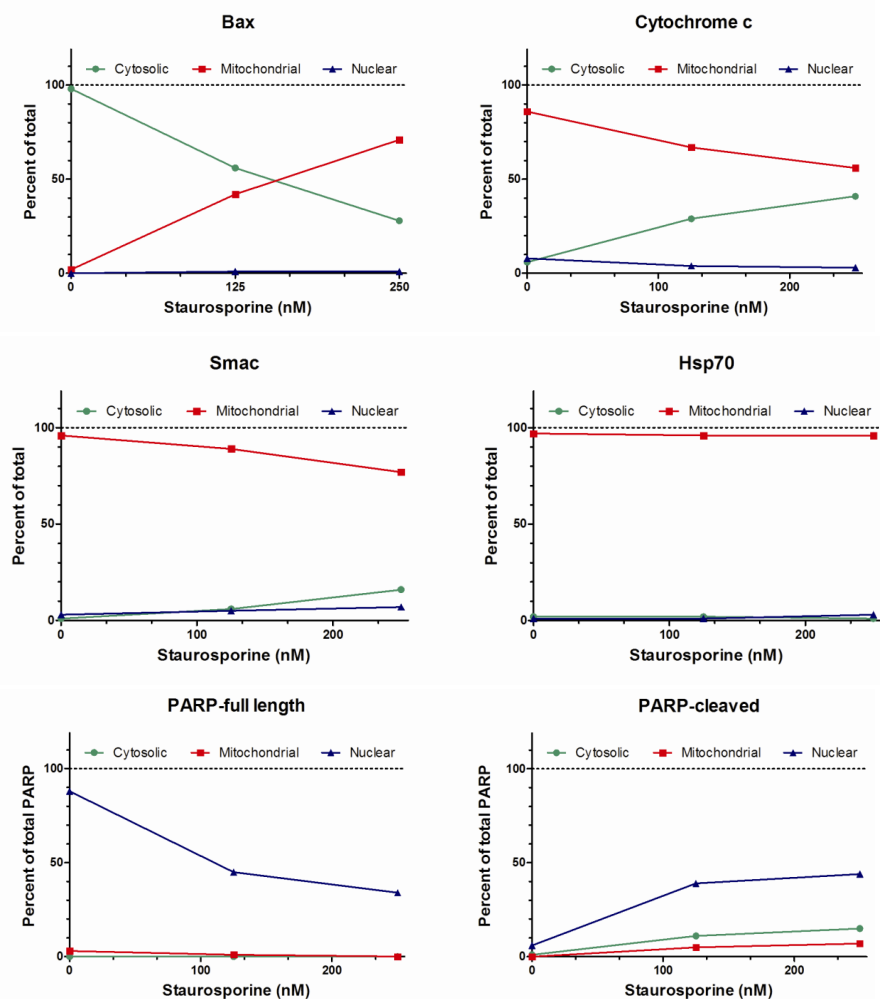
**Figure 1.** Cytosolic (C), mitochondrial (M) and nuclear (N) fractions of HepG2 cells were prepared as described in the Protocol. Fractions were analyzed by Western blotting using ab110415 ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (MSA12) containing antibodies against mitochondrial matrix (pyruvate dehydrogenase subunit E1 $\alpha$ , PDH E1 $\alpha$ ), mitochondrial inner membrane (F1-ATPase  $\alpha$ ), mitochondrial intermembrane space (cytochrome c) and cytosolic (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) markers as well as with antibodies against additional mitochondrial matrix (Hsp70) and nuclear (poly (ADP-ribose) polymerase, PARP and SP1) markers, followed by appropriate HRP-conjugated goat secondary antibodies and ECL detection. Representative blots as well as the quantitative analysis, as described in Data Analysis, are shown.



**Figure 2.** HeLa cells were treated for 4 hrs with 1  $\mu$ M Staurosporine (STS) or were left untreated (CONTROL). The cytosolic fraction (S) and mitochondria-containing reminder of the cells (P) were prepared by Detergent I extraction (“+”, 1X Buffer A containing Detergent I, as described in the Protocol), or by mock-extraction (“-”, 1X Buffer A without Detergent I). The samples derived from 12  $\mu$ g of whole cells protein were analyzed by Western blotting ab110415 ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (MSA12), an alkaline phosphatase-conjugated goat anti-mouse secondary antibody, and AP Conjugate Substrate Kit. A representative blot as well as the quantitative analysis, as described in Data Analysis, is shown.

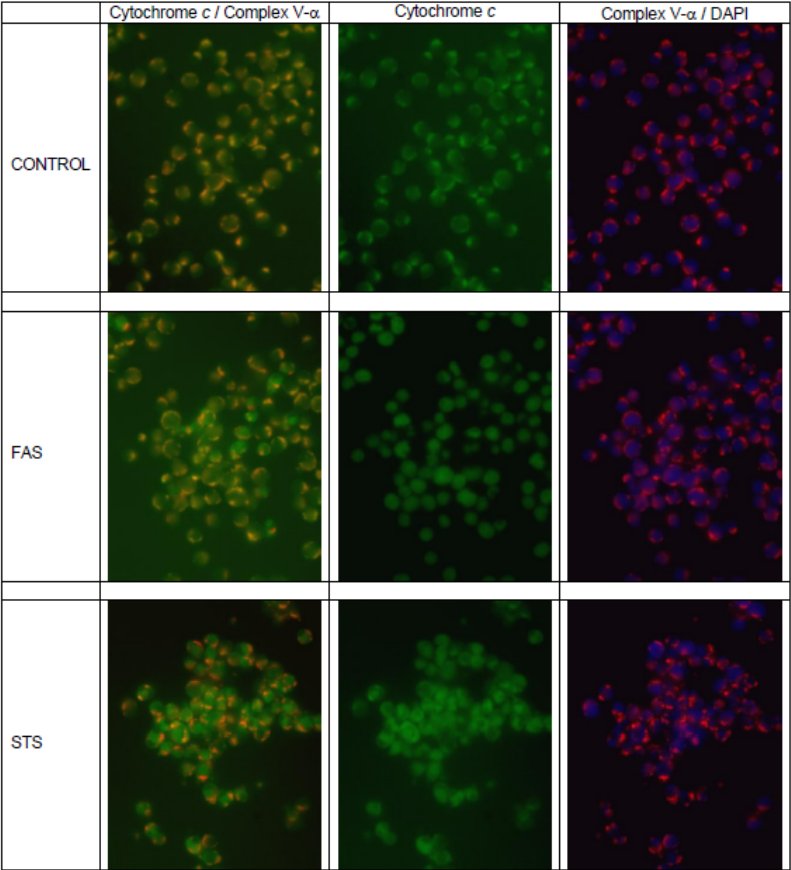


**Figure 3.** Jurkat cells were treated for 4 hrs with 50 ng/ml Fas antibody (clone CH11) or 1  $\mu$ M Staurosporine (STS) or were left untreated (CONTROL). HeLa cells and 143 B cells were treated, respectively, for 4 hrs and 5 hrs with 1  $\mu$ M STS, or were left untreated (CONTROL). The cytosolic fraction (C) and mitochondria-containing reminder of the cells (M) were prepared as described in the Protocol. The samples were analyzed by Western blotting using ab110415 ApoTrack™ Cytochrome c Apoptosis WB Antibody Cocktail (MSA12), an alkaline phosphatase-conjugated goat anti-mouse secondary antibody and AP Conjugate Substrate Kit. Representative blots as well as the quantitative analysis, as described in Data Analysis, are shown.



**Figure 4.** HeLa cells were treated with 0, 125 or 250 nM Staurosporine. Cytosolic (C), mitochondrial (M) and nuclear (N) fractions were prepared as described in the Protocol. Fractions were analyzed by Western blotting using antibodies against Bax, cytochrome c, Smac, Hsp70 (ab2799) and PARP (following with appropriate HRP-conjugated goat secondary antibodies and

ECL detection. Quantitative analyses of representative blots, as described in Data Analysis, are shown.



**Figure 5.** Jurkat cells were treated for 4 hrs with 1  $\mu$ M Fas antibody (clone CH11) or 1  $\mu$ M Staurosporine (STS) or were left untreated (CONTROL) and processed for immunocytochemistry using Abcam's ApoTrack™ Cytochrome c Apoptosis Kit (ab110417/MSA07) for Immunocytochemistry. Overlays of cytochrome c (in green) and Complex V-α (in red) staining are shown in left panels, cytochrome c only staining in middle panels and overlays of Complex V-α and DAPI (in blue) staining in right panels.

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