ab109719 Cell Fractionation Kit - Standard

For the rapid and simple separation of mitochondrial, cytosolic and nuclear fractions. This product is for research use only and is not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab109719
(use abcam.cn/ab109719 for China, or abcam.co.jp/ab109719 for Japan)

Materials Supplied and Storage

Store all components at -20°C, except Detergent I stored at -80°C. Ship on dry ice.
Sufficient materials are provided for fractionation of 1 x10^8 cells or for preparation of 40 samples, each corresponding to one 100 mm plate at 2.5 x 10^6 cells/plate.

- 2X Buffer A: 175 mL
- Detergent I: 25 µL
- Detergent II: 1 mL
- 5X SDS Sample Buffer: 10 mL

Materials Required, Not Supplied:

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

1. Assay Procedure

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.

1.1 ***Grow cells.*** Seed two 100 mm tissue culture plates at an equal density and grow them to semi-confluent density.

1.2 ***Optional: 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.

1.3 Equilibrate 2X Buffer A to room temperature (RT, see note 2.2) and add equal volume of water to make 1X Buffer A.

1.4 ***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.

1.5 ***1X Buffer A wash.*** Re-suspend cell pellets in 5 ml of 1X Buffer A. Take a small aliquot (~25 µL) 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.

1.6 ***Count cells.*** While centrifugation proceeds, count the uninduced cells using hematocytometer and determine the total cell number in the control sample.

1.7 ***Prepare cell suspension in 1X Buffer A.*** Discard supernatants and re-suspend control cell pellet in 1X Buffer A to 6.6 x 10^6 cells/ml. Re-suspend the induced cell pellet in the same volume of 1X Buffer A. See Note 2.3.

1.8 ***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 µl of Detergent I. Mix well by pipetting. Prepare only amount needed for immediate use.

1.9 ***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.

1.10 ***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.

1.11 ***Preparation of cytosolic fractions.*** Transfer the resulting supernatants containing cytosolic proteins into a new set of tubes. These are the cytosolic fractions (C).

1.12 ***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 1.10 and 1.11, in 1X Buffer A. Use the same volume of 1X Buffer A as was used to suspend the intact control cells, to 6.6 x 10^6 cells/m in Step 1.9 prior to addition of Buffer B.

1.13 ***Prepare Buffer C.*** *To prepare Buffer C, dilute Detergent II 25-fold in 1X Buffer A*. For example, to 4.8 m of 1X Buffer A add 0.2 ml of Detergent II. Mix well by pipetting. Prepare only amount needed for immediate use.

1.14 ***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.

1.15 ***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.

1.16 ***Preparation of mitochondrial fraction.*** Transfer the resulting supernatants containing mitochondrial proteins into a new set of tubes. These are the mitochondrial fractions (M).

1.17 ***Preparation of nuclear fraction.*** Re-suspend and combine the sequential cell pellets, generated in *Steps 1.15 and 1.16*, in 1X Buffer A to the original volume of suspensions after the addition of Buffer C in *Step 1.14*. These fractions contain re-suspended cytosol- and mitochondria-depleted remainder of cells containing nuclei and thus represent the nuclear fractions (N).

1.18 ***Optional: 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.

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

2. Protocol Notes

2.1 *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 1.4*.

2.2 *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.

2.3 *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 x 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 x 10^6 cells/ml at Step 7, to obtain 10 x10^6 cells/ml during the treatment with Detergent I. The same rules apply to extraction of mitochondrial proteins by Detergent II.

2.4 *Other cell types.* 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 1.9. 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.

2.5 The cytosolic, mitochondrial and nuclear fractions prepared, respectively, in Steps 1.11, 1.16 and 1.17 may be flash-frozen and stored at -80°C.

2.6 *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.

2.7 If desired, mock-Detergent I treated samples can be prepared by dilution of cell suspension prepared in Step 1.7 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 1.12 with equal volume of the 1X Buffer A.

2.8 If desired, 1X Buffer A can be supplemented with protease inhibitors, such as Protease Inhibitor Cocktail to minimize nonspecific proteolysis during the fractionation.

3. Data Analysis

3.1 ***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 a,(PDH E1 a) 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 a 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 a 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.

3.2 ***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 a (~55 kDa).

3.3 ***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 x cytochrome *c* fraction C/ (cytochrome *c* fraction C + cytochrome *c* fraction M + 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 (%) = Cytochrome *c* fraction C of treated cell (%) - Cytochrome *c* fraction C of untreated cells (%)

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