Mitochondrial Fractionation Kit

For the isolation of mitochondrial & cytosolic fractions

(version C2)

Catalog No. 40015

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Overview

The Mitochondrial Fractionation Kit was designed to facilitate the study of apoptotic and signal transduction pathways. It provides unique formulations of buffers and reagents that enable isolation of highly enriched mitochondrial and cytosolic fractions from various mammalian cell lines, including both apoptotic and non-apoptotic cells. The fractionation procedure is simple and easy to perform. No ultra-centrifugations are required and no toxic chemicals are involved. The enriched fractions can be used to study translocation of a variety of proteins between the mitochondrial and cytosolic fractions by Western blot analysis, ELISA or other assays.

Lysis is performed in a pestle homogenizer using the gentle Cytosolic Buffer. This helps to eliminate accidental rupture of the mitochondria and the subsequent leakage of mitochondrial proteins (such as cytochrome c) into the cytosolic fraction. After lysis, the different cellular compartments are separated by centrifugation of the cellular lysate at different speeds to isolate, successively, the nuclei, mitochondria and cytosol.

product	format	catalog no.
Mitochondrial Fractionation Kit	100 rxns	40015

Example Experiment

To determine the mitochondrial and cytosolic cytochrome c levels, 5 x 10^7 HeLa cells were grown to 90-95% confluence. The cells were harvested and mitochondrial and cytosolic fractions were isolated using the Mitochondrial Fractionation Kit. Active Motif's FunctionELISA™ Cytochrome c Kit, which has since been discontinued, and Western blot analysis were used to monitor and quantify mitochondrial and cytosolic cytochrome c (Figure 1).

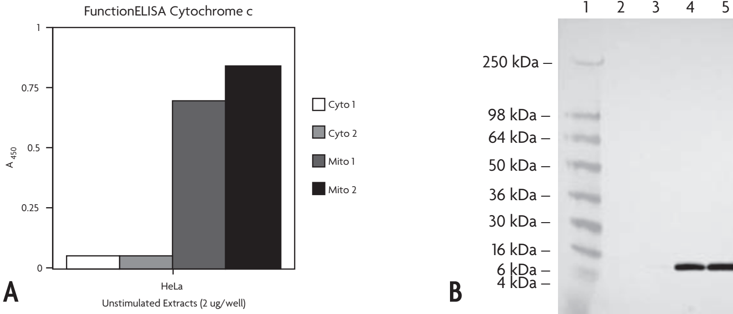

Figure 1: Location of cytochrome c in HeLa cells.

Mitochondrial and cytosolic extracts were isolated from HeLa cells grown to 90~95% confluence using the Mitochondrial Extract Kit. Five µg of each lysate was tested in duplicate using the FunctionELISA Cytochrome c Kit (Fig. 1A). 25 µg of each lysate was tested in duplicate by Western blot analysis using a 4-20% Tris-Glycine gel (Fig. 1B).

Lane 1: Protein standard
Lane 2: Cytosolic extract, sample 1
Lane 3: Cytosolic extract, sample 2
Lane 4: Mitochondrial extract, sample 1
Lane 5: Mitochondrial extract, sample 2

Kit Components and Storage

Please store each component at the temperature indicated in the table below. Do not re-freeze the Protein G Magnetic Beads.

Reagents	Quantity	Storage / Stability
1X Cytosolic Buffer	110 ml	4°C for short term -20°C for 6 months
Mitochondria Buffer	10 ml	4°C for 6 months
1 M DTT	100 µl	-20°C for 1 year
Protease Inhibitor Cocktail	100 µl	-20°C for 1 year

Note: The included buffers and reagents have been optimized for use with this kit. Substitution with other reagents may not give optimal results.

Additional materials required

- Cell scraper
- Phosphate buffered saline (PBS, pH 7.4)
- Pestle homogenizer
- Centrifuge
- Sterile, 15 ml centrifuge tubes
- Microcentrifuge
- Microcentrifuge tubes
- Pipettors and pipette tips
- Bio-Rad assay reagent and BSA standard, or similar
- 96-well, clear, flat-bottomed plate
- Microplate reader)

PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING!

General Considerations

- Store all kit components as recommended
- Keep buffers and sample on ice at all times during the procedure
- The number of strokes required to lyse the cells with a pestle homogenizer but leave the mitochondria intact depends on the cell line. For HeLa, PC3, DU145, JCA1 and ALVA31 cell lines, between 30 and 50 strokes seem to work well. In contrast, mitochondria from Jurkat and LNCaP cells appear to be more fragile and only 30 or fewer strokes should be applied. You may need to optimize the lysis procedure for your given cell line and/or treatment.

Buffer Preparation

Preparation of Complete Mitochondria Buffer

Prepare the amount of Complete Mitochondria Buffer required for your experiment by adding 2 µl Protease Inhibitor Cocktail and 1 µl DTT per 1 ml of Mitochondria Buffer. Some of the protease inhibitors lose their activity within 24 hours of dilution. Therefore, we recommend using the Complete Mitochondria Buffer immediately. Any remaining amount should be discarded if not used in the same day.

Cell Fractionation

This procedure is to be used for fractionation of ~5×10^7 cells.

1. Wash the cells 1X with 10 ml ice-cold PBS.
2. Add 10 ml ice-cold PBS and scrape the cells off the dish using a cell scraper. Transfer the cells to a pre-chilled 15 ml centrifuge tube and spin at 600 x *g* for 5 minutes at 4°C.
3. Remove supernatant and wash cell pellet 1X by gently resuspending it in 5 ml ice-cold PBS.
4. Spin at 600 x *g* for 5 minutes at 4°C and remove supernatant.

   Note: For apoptosis-induced cells, detachment of dead cells during the wash step will reduce the number of cells (relative to a plate of “normal” cells) that are obtained by scraping the plate. Therefore, to obtain fractions of an adequate concentration, buffer volumes may need to be reduced to coincide with the reduced number of cells being fractionated.

5. Add 1 ml ice-cold 1X Cytosolic Buffer. Resuspend the cell pellet by pipetting up and down gently with a pipette. Incubate on ice for 15 minutes, then transfer to a pre-chilled pestle homogenizer.
6. On ice, homogenize the cells using 30-50 strokes with the homogenizer. Transfer the supernatant to a pre-chilled microcentrifuge tube.
7. Spin the lysate at 800 x *g* (~3000 rpm) for 20 minutes at 4°C. The resulting pellet is the nuclei, cellular debris and intact cells, whereas the supernatant contains the cytosol, including the mitochondria.
8. Transfer the supernatant to a fresh, pre-chilled microcentrifuge tube.
9. Spin the supernatant a 2nd time at 800 x *g* (~3000 rpm) for 10 minutes at 4°C to remove any residual nuclei.
10. Transfer the supernatant to a fresh, pre-chilled microcentrifuge tube.
11. Spin the supernatant at 10,000 x *g* (~11,000 rpm) for 20 minutes at 4°C to pellet the mitochondria. The supernatant is the cytosolic fraction.
12. Transfer the supernatant to a fresh pre-chilled microcentrifuge tube.
13. Wash the mitochondrial pellet 1X with 100 µl 1X Cytosolic Buffer and spin at 10,000 x *g* for 10 minutes at 4°C. Remove and discard the supernatant.
14. Lyse the mitochondrial pellet by adding 100 µl Complete Mitochondria Buffer and incubating on ice for 15 minutes. After incubation, vortex for 10 seconds to mix thoroughly. This is the **Mitochondrial Fraction**.
15. Centrifuge the cytosolic supernatant obtained in step 12 at 16,000 x *g* (~14,000 rpm) for at least 20 minutes at 4°C to remove any residual mitochondria. Transfer the cytosolic supernatant to a fresh, pre-chilled microcentrifuge tube. This is the **Cytosolic Fraction**.
16. Measure the protein concentration of each fraction using a Bio-Rad assay with a BSA standard curve, or similar.
17. Aliquot the fractions and store at -80°C. Avoid freeze/thaw cycles.

Appendix

Section A. Troubleshooting Guide

Problem/question	Possible Cause	Recommendation
Low protein concentration in cytosolic fraction	Cell lysis was not efficient	Increase the number of strokes performed with the homogenizer
Mitochondrial protein leakage	Cell lysis was too vigorous	Reduce the number of strokes with the homogenizer to 30 strokes
Low protein concentration in mitochondrial fraction	Complete Mitochondria Buffer not made properly	Add 2 µl Protease Inhibitor Cocktail and 1 µl DTT per 1 ml of buffer

Section B. Related Products

Transcription Factor ELISAs	Format	Catalog No.
TransAM™ c-Myc	1 x 96 rxns	43396
TransAM™ MAPK Family	2 x 96 rxns	47296

For a complete list of the over 40 TransAM Kits available, please visit **www.activemotif.com/transam**

in-cell Western Phospho ELISAs	Format	Catalog No.
FACE™ Bad	1 x 96 rxns	48165
FACE™ FAK	1 x 96 rxns	48145
FACE™ JNK	1 x 96 rxns	48110

For a complete list of the over 20 FACE Kits available, please visit **www.activemotif.com/face**

Cell Extracts	Format	Catalog No.
HeLa nuclear extract	200 µg	36010
HeLa nuclear extract (TNF-α stimulated)	200 µg	40210
HeLa whole-cell extract (TNF-α stimulated)	200 µg	40200
Nuclear Extract Kit	100 rxns	40010

Apoptosis Assays	Format	Catalog No.
DNA Damage Assay (Fluorescent)	2 x 96 rxns	18030
Mitochondrial Fractionation Kit	100 rxns	48015

Apoptosis-related Antibodies	Application	Format	Catalog No.
Bcl-10 mAb (Clone 151)	WB	200 µg	39393
Caspase-3 mAb	WB	100 µg	40924
Caspase-7 mAb	WB	100 µg	40929
Caspase-8 mAb	FACS, IHC, WB	100 µg	40930
Caspase-9 pAb (Pro-form)	WB	100 µg	40931
Caspase-14 mAb	WB	100 µg	40932
D4-GDI mAb	IP, WB	100 µg	40941
DAP-3 mAb	WB	100 µg	40963
DAP-5 mAb	WB	100 µg	40964
DcR3 mAb	WB	100 µg	40933
DR4 mAb	FACS, WB	100 µg	40934
DR6 mAb	WB	100 µg	40927
DRAK1 pAb	WB	100 µg	40923
DRAK2 pAb	WB	100 µg	40926
Livin/ML-IAP mAb	WB	100 µg	40958
MyD88 pAb	WB	100 µg	40914
PARP-1 N-terminal pAb	WB	200 µl	39559
PARP-1 C-terminal pAb	WB	200 µl	39561
Rad21 mAb (Clone 52A311)	WB	200 µg	39383
STAT3 phospho Tyr705 pAb	WB	200 µl	39595
TRAF2 mAb	WB	100 µg	40919
TRAF5 mAb	WB	100 µg	40920
TRAIL mAb	FACS, WB	100 µg	40966

For an up-to-date list of Active Motif's nearly 400 antibodies, please visit **www.activemotif.com/abs**

Application Key: ChIP = Chromatin Immunoprecipitation; **EMSA** = Electrophoretic Mobility Shift Assay; **IF** = Immunofluorescence; **IHC** = Immunohistochemistry; **IP** = Immunoprecipitation; **WB** = Western blot

Technical Services

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