ELISA PRODUCT INFORMATION & MANUAL

Cytochrome c Releasing Apoptosis Assay Kit

NBP2-54881

Enzyme-linked Immunosorbent Assay for quantitative detection. For research use only.
Not for diagnostic or therapeutic procedures.

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

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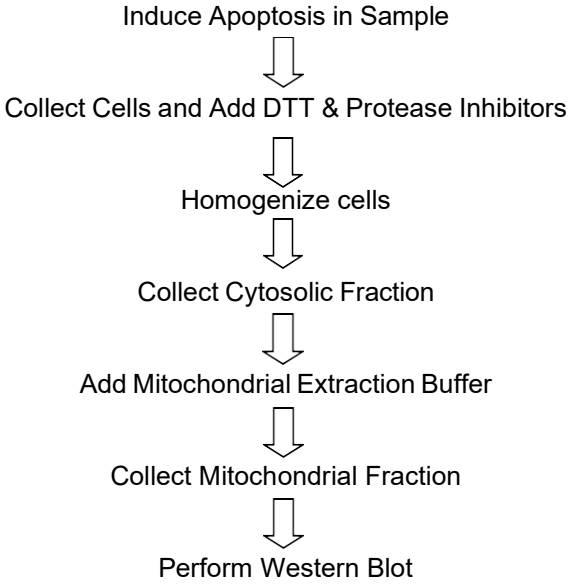
1. Overview

Cytochrome c plays an important role in apoptosis. The protein is located in the space between the inner and outer mitochondrial membranes. An apoptotic stimulus triggers the release of cytochrome c from the mitochondria into cytosol where it binds to Apaf-1. The cytochrome c/Apaf-1 complex activates caspase-9, which then activates caspase-3 and other downstream caspases.

Novus Biologicals's Cytochrome c Releasing Apoptosis Assay Kit provides an effective means for detecting cytochrome c translocation from mitochondria into cytosol during apoptosis. The kit provides unique formulations of reagents to isolate a highly enriched mitochondria fraction from cytosol. The procedure is so simple and easy to perform; no ultracentrifugation is required and no toxic chemicals are involved. Cytochrome c releasing from mitochondria into cytosol is then determined by Western blotting using the cytochrome c antibody provided in the kit.

2. Protocol Summary



3. Components and Storage

A. Kit Components

Item	Quantity
Mitochondria Extraction Buffer	10 mL
5X Cytosol Extraction Buffer	20 mL
DTT (1 M)	110 µL
500X Protease Inhibitor Cocktail (Lyophilized)	1 vial
Anti-Cytochrome c Mouse mAb	100 µL

* Store kit at -20°C.

- Be sure to keep all buffers on ice at all times during the experiment.
- Read the entire protocol before beginning the procedure.
- After opening the kit, store buffers at +4°C. Store the antibody, Protease Inhibitor Cocktail, and DTT at -20°C.

PROTEASE INHIBITOR COCKTAIL: Add 250 µL DMSO before use.

MITOCHONDRIA EXTRACTION BUFFER MIX: Before use, prepare just enough Mitochondria Extraction Buffer Mix for your experiment: Add 2 µL Protease Inhibitor cocktail and 1 µL DTT to 1 mL of Mitochondria Extraction Buffer.

CYTOSOL EXTRACTION BUFFER MIX: Dilute the 5X Cytosol Extraction Buffer to 1X buffer with ddH_2O. Before use, prepare just enough Cytosol Extraction Buffer Mix for your experiment: Add 2 µL Protease Inhibitor cocktail and 1 µL DTT to 1 mL of 1X Cytosol Extraction Buffer.

B. Additional Materials Required

- Microcentrifuge
- Pipettes and pipette tips
- Dounce tissue grinder
- Orbital shaker

4. Assay Protocol

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture *without* induction.

2. Collect cells (5 x 10^7) by centrifugation at 600 x g for 5 minutes at 4°C.

3. Wash cells with 10 mL of ice-cold PBS. Centrifuge at 600 x g for 5 minutes at 4°C. Remove supernatant.

4. a) Cells: Re-suspend cells with 1ml of 1X Cytosol Extraction Buffer Mix containing DTT and Protease Inhibitors. Incubate on ice for 10 minutes.

b) Frozen tissue may be suitable (although not tested) but would recommend fresh. If you must use frozen: washing the tissue with ice cold PBS and then resuspend each 10 mg of tissue in 1ml of cytosol extraction buffer.

5. Homogenize cells in an ice-cold Dounce tissue grinder. Perform the task with the grinder on ice. We recommend 30-50 passes with the grinder; however, efficient homogenization may depend on the cell type.

Notes:

a) To check the efficiency of homogenization, pipette 2-3 µL of the homogenized suspension onto a coverslip and observe under a microscope. A shiny ring around the nuclei indicates

that cells are still intact. If 70-80% of the nuclei do not have the shiny ring, proceed to step 7. Otherwise, perform 10-20 additional passes using the Dounce tissue grinder.

b) Excessive homogenization should also be avoided, as it can cause damage to the mitochondrial membrane which triggers release of mitochondrial components.

6. Transfer homogenate to a 1.5 mL microcentrifuge tube, and centrifuge at 700 x g for 10 minutes at 4°C.

7. Collect supernatant into a fresh 1.5 mL tube, and centrifuge at 10,000 x g for 30 minutes at 4°C. Collect supernatant as Cytosolic Fraction.

8. Re-suspend the pellet in 100 µL Mitochondrial Extraction Buffer Mix containing DTT and protease inhibitors (as prepared in section A), vortex for 10 seconds and save as Mitochondrial Fraction.

9. Load 10 µg each of the cytosolic and mitochondrial fractions isolated from un-induced and induced cells on a 12% SDS-PAGE. Then proceed with standard Western blot procedure and probe with cytochrome c antibody (1:200 dilution is recommended).

Note:

The anti-Cytochrome c antibody is a mouse monoclonal antibody that reacts with denatured human, mouse, and rat cytochrome c.