Cytochrome c Releasing Apoptosis Assay Kit

(Catalog #BN00525; 100 assays; Store kit at –20° C)

I. Introduction:

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. Assay Genie'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.

II. Kit Contents:

Component	BN00525	Cap Code	Part No.
Mitochondria Extraction Buffer	10 ml	NM	BN00525-1
5X Cytosol Extraction Buffer	20 ml	WM	BN00525-2
DTT (1 M)	110 µl	Blue	BN00525-3
500X Protease Inhibitor Cocktail	1 vial	Red	BN00525-4
Anti-Cytochrome c mouse mAb	0.1 ml	Green	BN00525-5

III. Mitochondria/Cytosol Fractionation Protocol:

A. General Consideration and Reagent Preparation:

- Read the entire protocol before beginning the procedure.
- After opening the kit, store buffers at 4° C. Store antibody, Protease Inhibitor Cocktail, and DTT at –20° C.
- Add 250 µl DMSO to dissolve the 500X Protease Inhibitor Cocktail before use.
- 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.
- 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.
- Be sure to keep all buffers on ice at all times during the experiment.

B. 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. Resuspend cells with 1.0 ml of 1X Cytosol Extraction Buffer Mix containing DTT and Protease Inhibitors (as prepared in Section A). Incubate on ice for 10 minutes.
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.

Note: 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. 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. Resuspend the pellet in 0.1-ml Mitochondrial Extraction Buffer Mix containing DTT and protease inhibitors (as prepared in section A), vertex for 10 seconds and save as Mitochondrial Fraction.
9. Load 10 µg each of the cytosolic and mitochondrial fractions isolated from uninduced 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.

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