

ApoTrack™ Cytochrome c Apoptosis ICC Antibody Kit

MSA07

Rev.1

DESCRIPTION

ApoTrack™ Cytochrome c Apoptosis ICC Antibody Kit: 2 color immunocytochemistry of cytochrome c and mitochondria.

Kit Contents:

Item	MSA07
Anti-Cytochrome c monoclonal antibody (clone 37BA11)	50 µg
Anti-Complex V α monoclonal antibody (clone 15H4C4)	50 µg
Goat anti-mouse IgG _{2a} - FITC secondary antibody	50 µg
Goat anti-mouse IgG _{2b} - TXRD secondary antibody	50 µg
Buffer (10X PBS, pH 7.4)	100 mL
Detergent (0.1% (v/v) Triton® X-100 in PBS)	30 mL
100% normal goat serum	20 mL

Storage:

Store Goat Serum at -20°C. Store all other components at 4°C.

INTRODUCTION

Mitochondria have an important role in apoptosis. One of the crucial players in apoptosis is cytochrome *c*, which, under apoptotic conditions, is released from the intermembrane space of the mitochondria to the cytosol. Once in the cytosol, cytochrome *c* combines with an adaptor subunit APAF-1 in the presence of dATP, leading to dimerization of APAF-1 and activation of a cysteine protease, caspase 9. Caspase 9 triggers activation of other caspases, which, in turn, selectively destroy certain proteins such as DNA replicating proteins and cytoskeletal proteins resulting in cell death. Release of cytochrome *c* from mitochondria is an early marker of apoptosis.

The ApoTrack™ Cytochrome *c* Apoptosis ICC Antibody Kit, MSA07, contains an anti-cytochrome *c* monoclonal antibody (IgG_{2a} isotype) and a goat anti-mouse IgG_{2a}-FITC secondary antibody enabling the researchers to observe the location of cytochrome *c* in cells by fluorescence microscopy. This kit also contains an anti-Complex V subunit α (CV α) monoclonal antibody (IgG_{2b} isotype) and a goat anti-mouse IgG_{2b}-TXRD secondary antibody. Complex V is an inner mitochondrial membrane protein and serves as a mitochondrial marker since it is not released into the cytoplasm during apoptosis unlike cytochrome *c*. Both primary antibodies are reactive in human, mouse and rat cells.

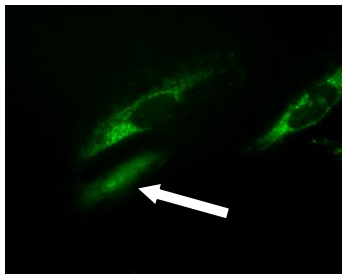


Figure 1. Staurosporine-treated HeLa cells analyzed with anti-cytochrome *c* antibody. The white arrow indicates cytochrome *c* release from mitochondria.

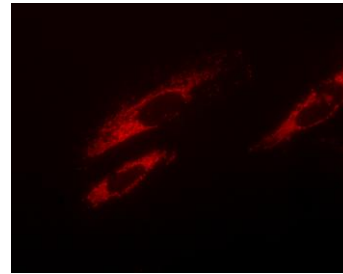


Figure 2. Staurosporine-treated HeLa cells analyzed with anti-Complex V α antibody.

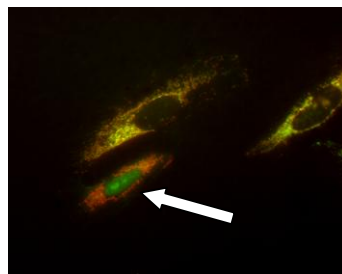


Figure 3. Figure 1 and 2 merged.

ADDITIONAL MATERIALS REQUIRED

- Cells of interest grown on coverslips
- Apoptosis inducer of interest
- 6-well tissue culture plates or 30-mm tissue culture dishes
- 4% (v/v) paraformaldehyde in PBS, for fixing cells
 - paraformaldehyde can be purchased as a 20% (v/v) solution (Electron Microscopy Services, www.emsdiasum.com, cat# 15713)
- Antigen retrieval buffer (100 mM Tris, 5% (w/v) urea, adjust pH to 9.5 with HCl)
- Broad-tipped forceps, 4½" long (Fisher Scientific www.fishersci.com, cat# 10-300)
- Coverglass staining jar (Electron Microscopy Services www.emsdiasum.com, cat# 72242-01)
- Waterbath heated to 95°C
- Nucleic acid stain for staining nuclei, if desired, e.g. DAPI
- Microscope glass slides
- Mounting medium
 - Commercially available brands include ProLong Antifade Kit (Life Technologies-Invitrogen, www.invitrogen.com, cat# P7481).
- Fluorescence microscope fitted with filters for:
 - Texas Red (TXRD) dye (Excitation λ 590nm; Emission λ 620nm).
 - Oregon Green 488 or Fluorescein isothiocyanate (FITC) dye (Excitation λ 488 nm; Emission λ 520 nm).
 - DAPI dye (Excitation λ 360 nm; Emission λ 460 nm), if staining the nuclei with DAPI.

These filters can be purchased from suppliers such as Chroma Technology Corp (www.chroma.com) or Omega Optical Inc (www.omegafilters.com).

CELL PREPARATION FOR ANALYZING APOPTOSIS

It is recommended that the cells of interest are grown at least 24 hours before inducing apoptosis. The following method has been used by us for inducing apoptosis in HeLa cells:

1. Grow the cells in the appropriate tissue culture medium under sterile conditions, on glass coverslips in 6-well tissue culture plates or 30-mm culture dishes, for at least 24 hours at 37°C in a CO₂ incubator before inducing apoptosis.
2. When the cells are approximately 60% confluent, induce apoptosis (e.g. with staurosporine or Actinomycin D) for the desired time. Remember to have control samples of cells that are not treated with the apoptosis inducer.
3. Aspirate the culture medium and gently rinse the cells twice in PBS at room temperature. Do not let the cells dry out.
4. Fix the cells by incubating them in 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature.

5. Rinse the cells three times with PBS.
6. The cells can be stored in 0.02% (w/v) sodium azide in PBS at 4°C overnight or be analyzed immediately using the Apoptosis Detection Kit, MSA07.

IMMUNOCYTOCHEMISTRY METHOD

1. Dilute the 10X PBS, provided in the kit, 10-fold by adding the contents (100 mL) to 900 mL of deionized water.
2. Depending on cell type and fixation method, antigen retrieval may improve signal. Preheat the Antigen Retrieval Buffer (100 mM Tris, 5% (w/v) urea, pH 9.5) to 95°C. This can be done by heating the buffer in a coverglass staining jar which is placed in a waterbath at 95°C. Using a small pair of broad-tipped forceps, carefully place the coverslips in the Antigen Retrieval Buffer in the coverglass staining jar, making note of which side of the coverslips the cells are on. Heat the coverslips at 95°C for 10 minutes. Remove the coverslips from the Antigen Retrieval Buffer and immerse them, with the side containing the cells facing up, in PBS, in the tissue culture plates.
3. Rinse the coverslips thoroughly 5-6 times in PBS.
4. Add sufficient (approximately 0.5 mL) 0.1% Triton[®] X-100 to each coverslip and incubate the cells for 10 min at room temperature. This process permeabilizes the cells.
5. Rinse the coverslips 5 times in PBS.
6. Dilute an aliquot of the 100% normal goat serum 10-fold by adding 9 volumes of PBS to one volume of 100% normal goat serum. Freeze the remaining 100% goat serum at -20°C.
7. Block the cells by adding sufficient (approximately 0.5 mL) 10% normal goat serum made in the above step to each coverslip.
8. Incubate the cells for one hour at room temperature in this blocking solution.
9. Incubate the cells in anti-cytochrome *c* monoclonal antibody and anti-Complex Va monoclonal antibody, at 4°C overnight, or at room temperature for 2 hours. MitoSciences recommends using a final concentration of 2 µg/mL anti-Cytochrome *c* antibody and 2 µg/mL anti-Complex Va antibody, using 10% goat serum as the diluent.
10. Dilute an aliquot of 10% goat serum 10 fold with PBS (i.e. 1 volume 10% goat serum and 9 volumes PBS).
11. Rinse the coverslips in 1% goat serum, 5-6 times, to remove unbound primary antibodies.
12. Incubate the cells in goat anti-mouse IgG_{2a}-FITC secondary antibody and goat anti-mouse IgG_{2b}-TXRD secondary antibody, diluted in 10% goat serum, for 1 hour at room temperature, **away from light**. A final concentration of 2 µg/mL of each of the secondary antibodies is recommended.

Note: The goat anti-mouse IgG_{2a}-FITC antibody binds to the anti-Cytochrome *c* monoclonal antibody since the anti-Cytochrome *c* mAb is of IgG_{2a} isotype. The goat anti-mouse IgG_{2b}-TXRD antibody binds to the anti-Complex Va monoclonal antibody since the anti-Complex Va mAb is of IgG_{2b} isotype.

13. Rinse the coverslips, in 1% goat serum, *away from light*, 5-6 times, to remove unbound secondary antibodies.
14. The nuclei may be stained at this stage using a DNA-binding stain such as DAPI (300 ng/mL in 1% goat serum for 10 minutes).
15. Mount the coverslips on microscope slides with a drop of mounting medium, such as ProLong Antifade (Invitrogen).
16. When the mounting medium has dried, visualize the cells using a fluorescence microscope equipped with a Texas Red filter, a Fluorescein isothiocyanate filter, and a DAPI filter if the nuclei were stained with DAPI.

INTREPERTATION OF RESULTS

Healthy cells will exhibit co-localization of cytochrome *c* and Complex V α in the mitochondria.

Cells which have undergone apoptosis will exhibit spreading of cytochrome *c* immunoreactivity from mitochondria to the cytosol whereas Complex V α remains localized in the mitochondria.

REFERENCES

Gottlieb R.A and Granville D.J. (2002) Analyzing mitochondrial changes during apoptosis. *Methods* 26, 341-347.

Bossy-Wetzel E and Green D.R. (2000) Assays for Cytochrome *c* Release from Mitochondria during Apoptosis. *Methods in Enzymology* 322, 235-242.

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This guide is for quick reference only. Be completely familiar with the previous details of this document before performing the assay.

