amsbio

MitoShift™ Kit

for the Assessment of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Catalog # 6305-100-K

100 Tests

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UK & Rest of World
184 Milton Park, Abingdon
OX14 4SE, Oxon, UK
Tel: +44 (0) 1235 828 200
Fax: +44 (0) 1235 820 482

Switzerland
Centro Nord-Sud 2E
CH-6934 Bioggio-Lugano
Tel: +41 (0) 91 604 55 22
Fax: +41 (0) 91 605 17 85

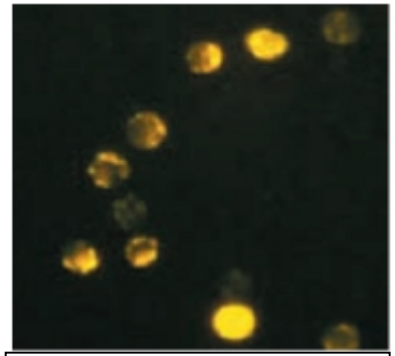
Deutschland
Bockenheimer Landstr. 17/19
60325 Frankfurt/Main
Tel: +49 (0) 69 779099
Fax: +49 (0) 69 13376880

United States
23591 El Toro Rd, Suite #167
Lake Forest, CA 92630
Tel: + 1 800 987 0985
Fax: + 1 949 265 7703

amsbio
info@amsbio.com

I. Background

In non-apoptotic, healthy cells, cellular energy produced during mitochondrial respiration, is maintained as an electrochemical gradient that constitutes a high mitochondrial transmembrane potential ($\Delta\Psi_m^{high}$). This membrane potential enables the cell to drive the synthesis of ATP, and its disruption is associated with uncoupling of oxidative phosphorylation, generation of superoxide free radicals and release of mitochondrial matrix-associated Ca^{2+} into the cytosol. Additionally, leakage of key apoptotic mitochondrial proteins such as cytochrome C, HtrA2/Omi, SMAC/Diablo and apoptosis inducing factor (AIF) have been associated with loss of $\Delta\Psi_m$. Thus, decreases in membrane potential have been used as a characteristic apoptotic marker.



Healthy WEHI 7.1 cells appear yellow. When observed by fluorescence confocal microscopy or by flow cytometry (ex: 488; em: 565) a loss in yellow fluorescence corresponds to an efflux of the MitoShift™ dye from mitochondria thereby indicating early apoptotic cells.

Trevigen's **MitoShift™ Kit** uses a unique cationic dye (tetramethylrhodamine ethylester) that can be used to visualize mitochondria in cells. Although the maximum absorption/emission of this dye is 549 nm and 575 nm respectively, an excitation of 488 nm and emission at 565 nm was used successfully to monitor mitochondrial function in intact cells. In healthy cells, the mitochondria appear as punctuate, perinuclear, yellow-red staining as observed by fluorescence confocal microscopy or by flow cytometry. Upon disruption of the mitochondrial potential, the dye shifts out of the mitochondria into the cytoplasm, where it produces diffuse orange fluorescence.

Mitochondrial events can be monitored at the single mitochondrial level, and the distribution of the dye across the mitochondrial membrane can be analyzed to quantitate $\Delta\Psi_m$ using a ratiometric approach based on the Nernst equation. Trevigen's **MitoShift™ Kit** has been used to evaluate the influence of drugs or cytotoxins on cellular mitochondria or to quickly estimate their effects on a cell population or detect apoptosis in known models.

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the products contained within the MitoShift Kit may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS sheets are available.

III. Materials Supplied

Component	Amount Provided	Storage	Catalog #
*MitoShift™	100 µl	4 °C	6305-100-01
Valinomycin (1 mM)	100 µl	4 °C	6305-100-02
10X Reaction Buffer	30 ml	4 °C	6300-100-02
Stabilizer Solution	5 ml	4 °C	6300-100-03

(*Trevigen recommends storing with desiccant.)

IV. Materials/Equipment Required But Not Supplied

Equipment
1. Micropipettors
2. 37 °C, 5% CO_2 incubator
3. Fluorescence microscope equipped with rhodamine long pass filter
4. FACS machine
5. Humidity chamber (please see the appendix)

Reagents
1. 1X PBS (10X PBS available from Trevigen)
2. Cell culture material
3. Deionized H_2O

Disposables
1. Serological pipettes
2. Microscope slides and coverslips
3. Micropipettor tips

V. Assay Protocol

NOTE: Mitochondrial membrane potential is pH sensitive. For optimal results, ensure that all reagents used are buffered between pH 7.0 and 8.0 and analyze rapidly. Cell culture media may be used in place of the reaction buffer provided.

A. Microscopic/Confocal Observation

a. Staining of Monolayer Cells

1. Dilute the 10X Reaction Buffer to 1X with distilled water and prewarm to room temperature before use. To increase the stability of the dye for viewing under the microscope, add 20 µl of Stabilizer Solution for every 1 ml of 1X Reaction Buffer prepared.
2. To dilute the MitoShift™ dye, add 1 µl of dye to 10 µl of 1X Reaction Buffer prepared in the previous step (with or without the Stabilizer solution). Vortex to homogenize. Again, add 1 µl from this solution to 1000 µl of 1X reaction buffer and vortex again. A starting concentration of 100 nM (1:10,000 dilution) is suggested, although optimization may be required depending upon the cell type under investigation.
3. Remove media and wash cells in 1X reaction buffer.
4. Cover the cells with diluted MitoShift™ solution prepared in step 2.

5. Incubate at room temperature, in the dark for 30-90 minutes to let the dye equilibrate. A humidity chamber (please see Appendix) may be used to avoid evaporation and sample drying.

6. Wash the cells with 1 ml of 1X Reaction Buffer with Stabilizer Solution.

7. Observe. Depending on the settings, yellow or red-orange punctuate staining will be apparent in healthy cells under the microscope. Diffuse orange fluorescence will appear in the cytoplasm of cells containing damaged mitochondria.

8. Other treatments can also be applied to the cells at this point to evaluate their effects on mitochondrial $\Delta\Psi_m$. Note that mitochondrial reactions occur quickly after insult therefore constant observation or recording may be required to observe the shift.

b. Staining of Suspension Cells

1. Harvest about 1 x 10^6 cells per sample by centrifugation at 250 x g for 5 minutes at room temperature.

2. Dilute the 10X Reaction Buffer to 1X with distilled water. To increase the stability of the dye for analysis, add 20 µl of Stabilizer Solution for every 1 ml of 1X Reaction Buffer prepared.

3. To dilute the MitoShift™ dye, add 1 µl of dye to 10 µl of 1X Reaction Buffer prepared in step 1 on page 2 (with or without the Stabilizer Solution). Vortex to homogenize. Again, add 1 µl from this dilution to 1000 µl of 1X reaction buffer. A general starting concentration of 100 nM (1:10,000 dilution) is suggested, keeping in mind that optimization may be required according to cell type.

4. Resuspend cells in 1 ml of diluted MitoShift™ solution, prepared in step 3.

5. Incubate samples at room temperature, in the dark for 30-90 minutes to let the dye equilibrate.

6. Centrifuge cells at 250 x g and discard supernatant.

7. Resuspend cells in 1 ml of 1X Reaction Buffer with Stabilizer solution at room temperature.

8. Observe. Depending on the settings, yellow or red-orange punctuate staining will be apparent in healthy cells under the microscope. Diffuse orange fluorescence will appear in the cytoplasm of cells containing damaged mitochondria.

9. Other treatments can also be applied to the cells at this point to evaluate their effects on mitochondrial $\Delta\Psi_m$. Note that mitochondrial reactions occur quickly after insult therefore constant observation or recording may be required to observe the shift.

B. Analysis by Flow Cytometry

The use of MitoShift™ in flow cytometry will be appropriate when an endpoint assay can give information about the status of a cell population. Absence of fluorescence in this assay will indicate cells with depolarized mitochondria or dead cells.

1. Harvest about 1 x 10^6 cells per sample by centrifugation at 250 x g for 5 minutes at room temperature.

2. Dilute the 10X Reaction Buffer to 1X with distilled water.

3. To dilute the MitoShift™ dye, add 1 µl of dye to 10 µl of 1X Reaction Buffer prepared in step 1 (page 2). Vortex to homogenize solution. Again, add 1 µl from this dilution to 1,000 µl of 1X reaction buffer and vortex again. A general starting concentration of 100 nM (1:10,000 dilution) is suggested, keeping in mind that optimization may be required according to cell type.

4. Resuspend cells in 1 ml of diluted MitoShift™ solution, prepared in the previous step.

5. Incubate samples at room temperature, in the dark for 30-90 minutes to let the dye equilibrate.

5. Wash samples in 1 ml of PBS with centrifugation at 250 x g.
   Note: Keep samples shielded from light until analysis. Stained cells can be kept at room temperature until analyzed.

6. Resuspend cells in 1 ml of PBS and analyze promptly by flow cytometry (use a 488 nm argon laser for excitation and FL2 for detection).

8. Optimize setting of apparatus and incubation times as necessary.

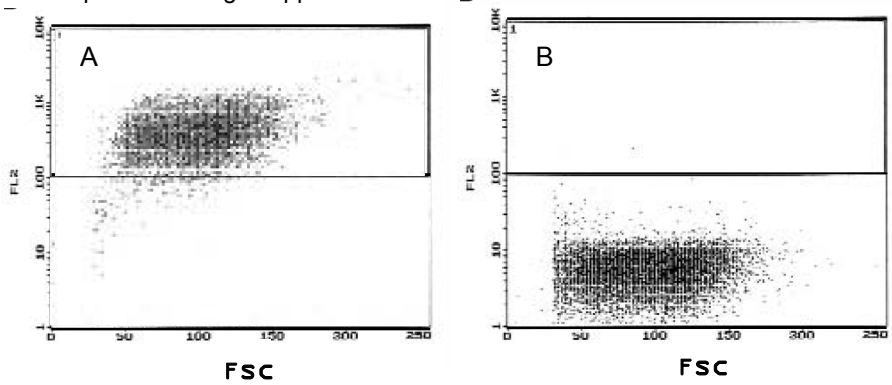


Fig.1. Healthy WEHI 7.1 mouse lymphocytes A) and cells treated with etoposide for 2 hours with overnight recovery B) were analyzed by flow cytometry using the PE channel (FL2). A distinctive shift in the fluorescence occurs when the mitochondrial potential is disturbed in apoptotic cells.

VI. Controls

A. Healthy Cells

Controls are necessary to evaluate the level of staining in healthy cells. Some cell lines may be too sensitive to pH variations to allow the use of the MitoShift™ assay. Viable counts of a healthy sample stained with trypan blue will also be helpful to assess general viability. Use of Fetal Bovine Serum (FBS) supplemented media to incubate and wash the cells may improve the results for sensitive cells lines or if extended periods of time are necessary to observe results. For flow cytometry and confocal experiments, unstained samples are also necessary to establish the level of autofluorescence of the cells.

B. Disrupted $\Delta\Psi_m$ Control

Treatment of cells with certain drugs that disturb the mitochondrial membrane potential provide an appropriate positive control. The valinomycin included in the kit is a potent potassium ionophore and a known apoptosis inducer. Treatment of a cell sample with 100 nM valinomycin diluted in media or reaction buffer, will immediately disrupt the mitochondrial membrane potential and the fluorescence will be releseased from the mitochondria into the cytoplasm. Valinomycin and as an alternative, carbonyl cyanide p-(trifluoro-methoxy) phenylhydrazone (FCCP, 250 nM) are also used to depolarize the mitochondrial potential before addition of mitochondrial activators.

VII. References

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5. Furlong, I.J. *et al.* 1998. Induction of apoptosis by valinomycin: mitochondrial permeability transition causes intracellular acidification. *Cell Death Differ* **5**:214-221.

6. Harvey, K., J. D. Ludovic, D.S. Ucker. 2000. Caspase-dependent Cdk activity is a requisite effector of apoptotic death events. *J. Cell Biol.* **148**:59-72**.**

7. Barnhart, B.C., *et al*. 2005. CD95 apoptosis resistance in certain cells can be overcome by noncanonical activation of caspase-8. *Cell Death Differ*. **12**:25-37.

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9. Xu, G., *et al.* 2006. Activin receptor-like kinase 7 induces apoptosis through up-regualtion of Bax and down-regulation of xiap in normal and malignant ovarian epithelial cell lines. *Mol. Cancer Res*. **4**: 235-246.

VIII. Related products available from Trevigen.

Catalog #	Description	Size
6300-100-K	DePsipher™ Mitochondrial Potential Assay Kit	100 tests
4817-60-K	FlowTACS™ Apoptosis Detection Kit	60 samples
4822-96-K	HT TiterTACS™ Assay Kit	96 tests
4830-01-K	TACS® Annexin V FITC Kit	100 samples
4835-01-K	TACS® Annexin V Biotin Kit	100 samples
4815-30-K	TumorTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4823-30-K	NeuroTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4827-30-K	CardioTACS ™ *In Situ* Apoptosis Detection Kit	30 samples
4829-30-K	DermaTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4826-30-K	VasoTACS™ *In Situ* Apoptosis Detection Kit	30 samples
4828-30-DK	TACS•XL® DAB *In Situ* Apoptosis Detection Kit	30 samples
4828-30-BK	TACS•XL® Blue Label *In Situ* Apoptosis Detection Kit	30 samples
4810-30-K	TACS® 2 TdT DAB *In Situ* Apoptosis Detection Kit	30 samples
4811-30-K	TACS® 2 TdT Blue Label *In Situ* Apoptosis Detection Kit	30 samples
4812-30-K	TACS® 2 TdT Fluorescein Apoptosis Detection Kit	30 samples
4850-20-ET	TACS® Apoptotic DNA Laddering Kit EtBr	20 samples
4892-010-K	Cultrex® Calcein-AM Cell Viability Kit	1000 tests
4684-096-K	HT Colorimetric PARP/Apoptosis Assay	96 tests
4685-096-K	HT Chemiluminescent PARP/Apoptosis Assay	96 tests

Antibodies

Catalog #	Description	Size
4411-PC-100	Anti-Phosphorylated Histone-γ-H2AX polyclonal	100 µl
2290-MC-100	Anti-murine-Bcl-2 mAb (clone YTH-10C4)	100 µg
2291-MC-100	Anti-human-Bcl-2 mAb (clone YTH-8C8)	100 µg
2300-MC-100	Anti-human-Bcl-XL mAb (clone YTH-2H12)	100 µg
2280-MC-100	Anti-mouse-Bax mAb (clone YTH-5B7)	100 µg
2281-MC-100	Anti-human-Bax mAb (clone YTH-6A7)	100 µg
2282-MC-100	Anti-human-Bax mAb (clone YTH-2D2)	100 µg
2305-PC-100	Anti-cleaved human/mouse-Caspase 3 polyclonal	40 µg
6360-PC-100	Anti-human/mouse-PBR polyclonal	100 µl
4335-MC-100	Anti-PAR polymer mAb (10HA)	100 µl
4336-BPC-100	Anti- PAR polymer polyclonal	100 µl
4338-MC-50	Anti-human/murine-PARP mAb (clone C2-10)	50 µg

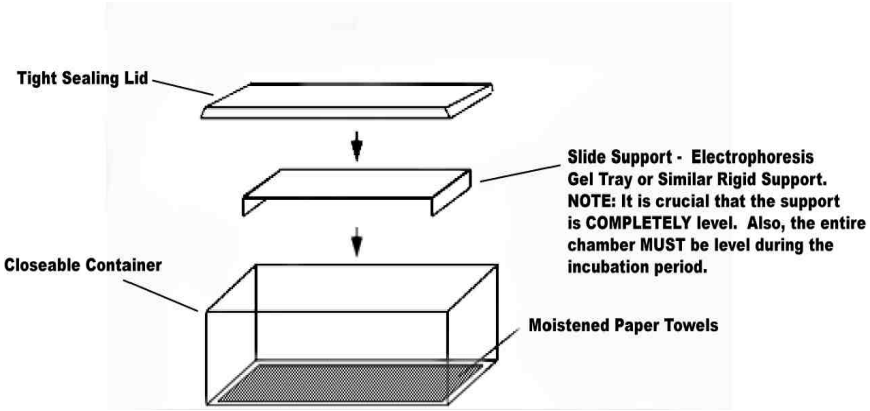
Accessories

Catalog #	Description	Size
4800-30-40	Tissue Control Slides	2 ea.
4800-30-20	Cell Culture Control Slides	2 ea.
4864-100	Treated Glass Microscope Slides w/3 sample Hydrophobic Barrier	100 ea.
4867-100	Hydrophobic Coverslips	100 ea.
4862-10	Coverslips 24 x 60 mm, No. 1.5	10 x 1 oz.
4865-25	Mounting Medium	25 ml
4800-30-14	Strep-Fluorescein	30 µl
4830-100-03	Propidium Iodide	30 µl
4870-500-6	10X PBS	6 x 500 ml
4869-500-6	Apoptosis Grade™ H_2O	6 x 500 ml
4820-30-13	Blue Counterstain	50 ml
4825-30-RL	Red Label	30 samples
4878-05-02	Cytonin™ IHC	2 x 5 ml

IX. Appendix

Appendix A. Humidity Chamber

To prevent evaporation it is recommended that 30-90 minute incubations are carried out in a humidity chamber. A humidity chamber can be made using a plastic box with a tight fitting lid and two glass rods or other support. Place paper towel on the bottom of the box and wet thoroughly with water. Lay the glass rods parallel to each other and less than one slide length apart on the wet tissue. Position the slides on the glass rods and seal the plastic box with the lid. Ensure that the slides are horizontal.



UK & Rest of World
184 Milton Park, Abingdon
OX14 4SE, Oxon, UK
Tel: +44 (0) 1235 828 200
Fax: +44 (0) 1235 820 482

Switzerland
Centro Nord-Sud 2E
CH-6934 Bioggio-Lugano
Tel: +41 (0) 91 604 55 22
Fax: +41 (0) 91 605 17 85

Deutschland
Bockenheimer Landstr. 17/19
60325 Frankfurt/Main
Tel: +49 (0) 69 779099
Fax: +49 (0) 69 13376880

United States
23591 El Toro Rd, Suite #167
Lake Forest, CA 92630
Tel: + 1 800 987 0985
Fax: + 1 949 265 7703

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