



Mitochondrial Membrane Potential Detection Kit

Instruction Manual

Catalog #280002 Mitochondrial Membrane Potential Detection Kit

Revision B

Research Use Only. Not for Use in Diagnostic Procedures.

280002-12



Agilent Technologies

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

United States and Canada

Agilent Technologies

Stratagene Products Division

11011 North Torrey Pines Road

La Jolla, CA 92037

Telephone (858) 373-6300

Order Toll Free (800) 424-5444

Technical Services (800) 894-1304

Email techservices@agilent.com

World Wide Web www.genomics.agilent.com

Europe

Location	Telephone
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292

All Other Countries

Please contact your local distributor. A complete list of distributors is available at www.genomics.agilent.com.

Mitochondrial Membrane Potential Detection Kit

CONTENTS

Materials Provided	1
Storage Conditions	1
Additional Materials Required	1
Introduction	2
Preprotocol Considerations	3
Preparing the JC-1 Reagent	3
Preparing the 10× Assay Buffer	3
Staining Protocol for Flow Cytometry	4
Inducing the Cells.....	4
Staining the Cells.....	4
Analyzing the Cells	5
Suspension Cell Staining Protocol for Fluorescence Microscopy	6
Inducing the Cells.....	6
Staining the Cells.....	6
Analyzing the Cells	7
Staining Protocol for Fluorescence Ratio Detection^{7,8}	7
Monolayer Cell Staining Protocol for Fluorescence Microscopy	8
Inducing the Cells.....	8
Staining the Cells.....	8
Analyzing the Cells	8
Troubleshooting	9
Preparation of Reagents	9
References	9
Endnotes	9
MSDS Information	9
Staining Quick-Reference Protocol for Flow Cytometry	10

Mitochondrial Membrane Potential Detection Kit

MATERIALS PROVIDED

Materials provided ^a	Quantity
JC-1 reagent (lyophilized)	1 tube
10× assay buffer	60 ml

^a The Mitochondrial membrane potential detection kit provides sufficient reagents for 100 reactions.

STORAGE CONDITIONS

JC-1 reagent: Store the JC-1 reagent at –20°C, protected from light and moisture (preferably in a desiccator). Once reconstituted, full activity is guaranteed for 6 months. Avoid subjecting the reconstituted reagent to multiple freeze-thaw cycles by dispensing into small working aliquots.

10× Assay Buffer: Store the 10× Assay buffer at 4°C upon receipt.

ADDITIONAL MATERIALS REQUIRED

Phosphate-buffered saline (PBS; see *Preparation of Reagents*)
Dimethyl sulfoxide (DMSO)
Flow cytometer, fluorescence microscope, or fluorescence plate reader

Revision B

© Agilent Technologies, Inc. 2010.

INTRODUCTION

The mitochondrial permeability transition is an important step in the induction of cellular apoptosis. During this process, the electrochemical gradient (referred to as $\Delta\Psi$) across the mitochondrial membrane collapses. The collapse is thought to occur through the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm, which promotes the activation of caspases, which are directly responsible for apoptosis.¹⁻⁴

The Mitochondrial membrane potential detection kit uses a unique fluorescent cationic dye, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide), to signal the loss of mitochondrial membrane potential.⁵ In healthy non-apoptotic cells, the dye stains the mitochondria bright red.⁶ The negative charge established by the intact mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates. When the critical concentration is exceeded, J-aggregates form which become fluorescent red. Whereas, in apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria. In these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells which show red and green fluorescence (see Figure 1). The aggregate red form has absorption/emission maxima of 585/590 nm.⁵ The green monomeric form has absorption/emission maxima of 510/527 nm. The JC-1 monomers and aggregates give strong positive signals, capable of yielding both qualitative and quantitative results. Detection methods include flow cytometry, fluorescence microscopy, and a fluorescent 96-well plate reader format.

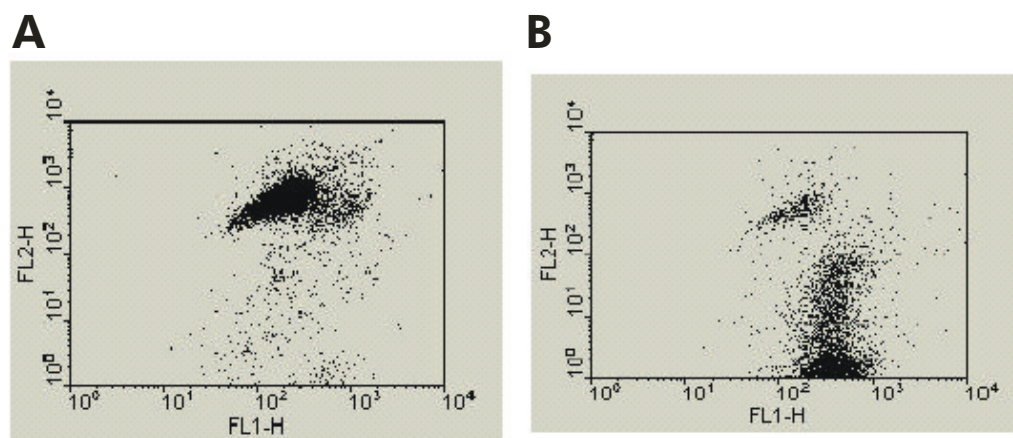


FIGURE 1 Jurkat cells were stimulated with (A) DMSO or (B) 1.5 μ M staurosporine for 3 hours. The cells were then labeled with the JC-1 reagent for 15 minutes. After washing, cell fluorescence was measured on a flow cytometer using FL1 and FL2 channels.

PREPROTOCOL CONSIDERATIONS

Preparing the JC-1 Reagent

1. Reconstitute the lyophilized JC-1 reagent with 500 μ l of DMSO to obtain a 100 $\times$ stock solution. Mix by inverting the vial several times at room temperature until the lyophilized contents are completely dissolved.
2. Aliquot the reconstituted JC-1 reagent in small amounts sufficient for one day of experimental work and store the remainder at -20°C in amber vials, away from moisture and light.
3. Immediately before use, dilute the 100 $\times$ JC-1 reagent 1:100 in 1 $\times$ assay buffer, or the desired warmed media. Vortex the solution. To remove any undissolved particulate matter, centrifuge the dye-buffer solution for 1 minute at 13,000 $\times g$ and carefully transfer the supernatant, without disturbing the pelleted debris, into a fresh tube. Protect the reagent from light at all times.

Preparing the 10 $\times$ Assay Buffer

Warm the 10 $\times$ assay buffer until any salt crystals are completely dissolved, if necessary. Prepare a 1 $\times$ working concentration of the 10 $\times$ assay buffer by diluting the buffer 1:10 in dH₂O.

STAINING PROTOCOL FOR FLOW CYTOMETRY

Inducing the Cells

Cells should be cultured to a density of 1×10^6 cells/ml or less.

Note *The optimal cell density should be determined specifically for each cell type and apoptosis induction method.*

Induce apoptosis in cells using methods, conditions, and time points according to the desired protocol. Set up a duplicate experiment to perform an uninduced control reaction.

Staining the Cells

Note *Protect the JC-1 reagent from the light at all times.*

1. Following induction for the required activation time, transfer 500 μ l, containing 1×10^6 induced cells, into a sterile centrifuge tube. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant.
2. Resuspend the cells in 500 μ l of $1\times$ JC-1 reagent solution (see *Preparing the JC-1 Reagent* under *Preprotocol Considerations*). Incubate the cells in standard growth conditions (i.e., 37°C and 5% CO_2 in a humidified incubator) for 15 minutes.
3. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant.
4. Resuspend the cell pellet in 2 ml of $1\times$ assay buffer or cell culture medium. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant. Repeat this wash step once.
5. Resuspend the cell pellet in 500 μ l of $1\times$ assay buffer or fresh cell culture medium. The cells are now ready for analysis by flow cytometry and must be analyzed immediately.

Analyzing the Cells

In live non-apoptotic cells, JC-1 exists as a monomer in the cytosol which stains green, and also accumulates as aggregates in the mitochondria which stain red. In apoptotic and dead cells, JC-1 exists in the monomeric form only, staining the cytosol green.

Mitochondria containing red JC-1 aggregates in healthy cells are detectable in the FL2 channel. Green JC-1 monomers in apoptotic cells are detectable in the FL1 channel (FITC).

Flow Cytometer Instrument Set Up

Typical Flow Cytometer Settings

Typical settings for the analysis of JC-1 staining on a BD FACSCalibur System (BD Biosciences, San Jose, CA) flow cytometer are as follows:

FL1 PMT voltage 511

FL2 PMT voltage 389

Compensation: FL1 – 10.5% FL2

FL2 – 25.9% FL1

Two-Parameter Analysis

1. Run the uninduced control sample first. Generate a log FL1 (X-axis) versus log FL2 (Y-axis) dot plot. Add regions R2 and R3 to the dot plot.

Note *On instruments where it is not possible to add regions to the dot plot, add quadrants to the dot plot instead.*

2. Adjust the FL1 and FL2 PMT voltages to register a dual positive population in region 2 (R2). The peak of the dual positive population should fall within the second and third log decade scale of FL1 and FL2.
3. The region 2 (R2) gate should be adjusted to include >95% of events. This number will vary depending on the condition of the cells.
4. Run the induced sample, using the PMT settings established above for the uninduced control sample. A population of cells should appear in region 3 (R3). This reflects a loss of red emission on the FL2 axis, which corresponds to the loss of mitochondrial membrane potential in induced cells.

Note *If the induced sample exhibits only a minimal decrease in red emission as compared to the uninduced sample, increase the FL2-%FL1 compensation.*

SUSPENSION CELL STAINING PROTOCOL FOR FLUORESCENCE MICROSCOPY

Inducing the Cells

Cells should be cultured to a density of 1×10^6 cells/ml or less.

Note *The optimal cell density should be determined specifically for each cell type and apoptosis induction method.*

Induce apoptosis in cells using methods, conditions, and time points according to the desired protocol. Set up a duplicate experiment to perform an uninduced control reaction.

Staining the Cells

Note *Protect the JC-1 reagent from the light at all times.*

1. Following induction for the required activation time, transfer 500 μ l of the induced cell suspension into a sterile centrifuge tube. The cell density should be 1×10^6 cells/ml. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant.
2. Resuspend the cells in 500 μ l of $1\times$ JC-1 reagent solution (see *Preparing the JC-1 Reagent* under *Preprotocol Considerations*). Incubate the cells in standard growth conditions (i.e., 37°C and 5% CO_2 in a humidified incubator) for 15 minutes.
3. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant.
4. Resuspend the cell pellet in 2 ml of $1\times$ assay buffer. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant. Repeat this wash step.
5. Resuspend the cell pellet in 300 μ l of $1\times$ assay buffer. The cells are now ready for analysis by fluorescence microscopy and must be analyzed immediately.

Analyzing the Cells

Observe the samples immediately with a fluorescence microscope using a dual-bandpass filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red® dyes. JC-1 aggregates are detected with a bandpass filter designed to detect rhodamine (excitation/emission = 540/570 nm) or Texas Red dye (excitation/emission = 590/610 nm). JC-1 monomers are detected with a bandpass filter designed to detect fluorescein (excitation/emission = 490/520 nm).

In live non-apoptotic cells, JC-1 exists as a monomer in the cytosol which stains green, and also accumulates as aggregates in the mitochondria which stain red. In apoptotic and dead cells, JC-1 exists in the monomeric form only, staining the cytosol green.

STAINING PROTOCOL FOR FLUORESCENCE RATIO DETECTION^{7,8}

Note *Protect the JC-1 reagent from the light at all times.*

Transfer 100 µl of the stained cell suspension (from step 5 in *Staining the Cells* in *Suspension Cell Staining Protocol for Fluorescence Microscopy*) into each of three wells of a black 96-well plate. Measure red (585/590 nm) and green (510/527 nm) fluorescence using a fluorescence plate reader. Determine the ratio of red-to-green fluorescence. The ratio of red-to-green fluorescence will be decreased in dead cells and cells undergoing apoptosis, compared to healthy non-apoptotic cells.

MONOLAYER CELL STAINING PROTOCOL FOR FLUORESCENCE MICROSCOPY

Inducing the Cells

Grow the cells on a glass cover slip in a Petri dish or in a chamber slide. Induce apoptosis in cells using methods, conditions, and time points according to the desired protocol. Set up a duplicate experiment to perform an uninduced control reaction.

Staining the Cells

Note *Protect the JC-1 reagent from the light at all times.*

1. Remove the cell culture media from the cells. Add sufficient 1× JC-1 reagent solution (see *Preparing the JC-1 Reagent* under *Preprotocol Considerations*) to cover the cells. Incubate the cells in standard growth conditions (i.e., 37°C and 5% CO₂ in a humidified incubator) for 15 minutes.
2. Remove the JC-1 reagent and wash the cells once with 1× assay buffer.
3. After removing the assay buffer, add a drop of PBS to the cells and add a coverslip. The cells are now ready for analysis by fluorescence microscopy and must be analyzed immediately.

Analyzing the Cells

Observe the samples immediately with a fluorescence microscope using a dual-bandpass filter designed to simultaneously detect fluorescein and rhodamine or fluorescein and Texas Red dyes. JC-1 aggregates are detected with a bandpass filter designed to detect rhodamine (excitation/emission = 540/570 nm) or Texas Red dye (excitation/emission = 590/610 nm). JC-1 monomers are detected with a bandpass filter designed to detect fluorescein (excitation/emission = 490/520 nm).

In live non-apoptotic cells, JC-1 exists as a monomer in the cytosol which stains green, and also accumulates as aggregates in the mitochondria which stain red. In apoptotic and dead cells, JC-1 exists in the monomeric form only, staining the cytosol green.

TROUBLESHOOTING

Observation	Suggestion
Low signal observed	Analyze the sample immediately following the wash steps. Over time, the dye will leach out of the cells and into the assay buffer, resulting in low signal.
	Avoid photobleaching by minimizing the exposure of dye-containing samples to light.
Non-apoptotic samples contain green-stained cells only	Ensure that the cells are healthy prior to the induction of apoptosis.

PREPARATION OF REAGENTS

Phosphate-Buffered Saline (PBS)

150 mM NaCl
20 mM Na₂HPO₄
Adjust the pH to 7.4 with HCl

REFERENCES

1. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S. *et al.* (1999) *J Cell Biol* 144(5):891–901.
2. Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J. *et al.* (1998) *Proc Natl Acad Sci U S A* 95:14681–14686.
3. Basanez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E. *et al.* (1999) *Proc Natl Acad Sci U S A* 96:5492–5497.
4. Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) *Cell* 94:481–490.
5. Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A. *et al.* (1991) *Proc Natl Acad Sci U S A* 88:3671–3675.
6. Cossarizza, A., Baccarani-Contri, M., Kalashnikova, G. and Franceschi, C. (1993) *Biochem Biophys Res Commun* 197(1):40–45.
7. Reers, M., Smith, T. W. and Chen, L. B. (1991) *Biochemistry* 30:4480–4486.
8. White, R. J. and Reynolds, I. J. (1996) *J Neuroscience* 16:5688–5697.

ENDNOTES

Texas Red® is a registered trademark of Molecular Probes, Inc.

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at <http://www.genomics.agilent.com>. MSDS documents are not included with product shipments

STRATAGENE

An Agilent Technologies Division

Mitochondrial Membrane Potential Detection Kit

Catalog #280002

STAINING QUICK-REFERENCE PROTOCOL FOR FLOW CYTOMETRY

- ◆ Cells should be cultured to a density of 1×10^6 cells/ml or less.

Note *The optimal cell density should be determined specifically for each cell type and apoptosis induction method.*

- ◆ Induce apoptosis in cells using methods, conditions, and time points according to the desired protocol.
- ◆ Transfer 500 μ l, containing 1×10^6 induced cells, into a sterile centrifuge tube. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant.

Note *Protect the JC-1 reagent from the light at all times.*

- ◆ Resuspend the cells in 500 μ l of $1 \times$ JC-1 reagent solution. Incubate the cells in standard growth conditions (i.e., 37°C and 5% CO₂ in a humidified incubator) for 15 minutes.
- ◆ Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant.
- ◆ Resuspend the cell pellet in 2 ml of $1 \times$ assay buffer or cell culture medium. Centrifuge the cell suspension for 5 minutes at $400 \times g$ at room temperature. Discard the supernatant. Repeat this wash step once.
- ◆ Resuspend the cell pellet in 500 μ l of $1 \times$ assay buffer or fresh cell culture medium. The cells are now ready for analysis by flow cytometry and must be analyzed immediately.
- ◆ Mitochondria containing red JC-1 aggregates in healthy cells are detectable in the FL2 channel. Green JC-1 monomers in apoptotic cells are detectable in the FL1 channel (FITC).