Cell Meter™ Fluorimetric Mitochondrial Superoxide Activity Assay Kit

Green Fluorescence

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 16060 (200 assays)	Keep in freezer Avoid exposure to light	Fluorescence Microscope Flow Cytometer

Introduction

Mitochondria are major producers of cellular superoxide. The production of low to moderate levels of superoxide is critical for the proper regulation of many essential cellular processes including gene expression, signal transduction, and muscle adaptation to endurance exercise training. Uncontrolled mitochondrial superoxide production can trigger cellular oxidative damage that contributes to the pathogenesis of a wide variety of disorders including cancer, cardiovascular diseases, neurodegenerative diseases and aging. The detection of intracellular mitochondrial superoxide is of central importance to understanding proper cellular redox regulation and the impact of its dysregulation on various pathologies. Cell Meter™ Fluorimetric Mitochondria Superoxide Activity Assay Kit uses our unique superoxide indicator to quantify superoxide level in live cells. MitoROS™ 520 is live-cell permeant and can rapidly and selectively target superoxide in mitochondria. It generates red fluorescence when it reacts with superoxide, and can be easily read at Ex/Em = 490/530 nm. The Cell Meter™ Fluorimetric Mitochondria Superoxide Activity Assay Kit provides a sensitive, one-step fluorimetric assay to detect mitochondrial superoxide in live cells with one hour incubation. This kit can be used for flow cytometry and fluorescence microscopy applications.

Kit Components

Components	**Amount**
Component A: MitoROS™ 520	1 vial
Component B: Assay Buffer	1 bottle (20 mL)
Component C: DMSO	100 µL

Assay Protocol for One 96-well Plate

Brief Summary

Prepare cells in growth medium → Treat the cells with test compounds to induce superoxide → Add MitoROS™ 520 working solution → Stain the cells at 37 °C for 60 minutes → Monitor the fluorescence increase at Ex/Em= 490/530 nm

Note: Thaw all the components at room temperature before use.

1. Prepare cells:

1.1 For adherent cells: Plate cells overnight in growth medium at 10,000 to 40,000 cells/well/90 µL for a 96-well plate or 2,500 to 10,000 cells/well/20 µL for a 384-well plate.

1.2 For non-adherent cells: Centrifuge the cells from the culture medium and suspend the cell pellets in culture medium at 100,000-200,000 cells/well/90 µL for a 96-well poly-D lysine plate or 25,000-50,000 cells/well/20 µL for a 384-well poly-D lysine plate. Centrifuge the plate at 800 rpm for 2 minutes with brake off prior to your experiment. *Note: Each cell line should be evaluated on an individual basis to determine the optimal cell density.*

2. Prepare MitoROS™ 520 working solution:

2.1 Prepare MitoROS™ 520 stock solution (500X): Add 50 µL of DMSO (Component C) into the vial of MitoROS™ 520 (Component A), and mix them well.
Note: 25 µL of reconstituted MitoROS™ 520 stock solution is enough for 1 plate. Unused portion can be aliquoted and stored at < -20 °C for more than one month if the tubes are sealed tightly and kept from light. Avoid repeated freeze-thaw cycles.

2.2 Prepare MitoROS™ 520 working solution: Add 5 µL of 500X DMSO reconstituted MitoROS™ 520 stock solution (from Step 2.1) into 2 mL of Assay Buffer (Component B), and mix them well. This working solution needs to be prepared freshly before use. *Note: The working solution is not stable; prepare it as needed before use.*

3. Run superoxide assay:

3.1 Treat cells with 10 µL of 10X test compounds (96-well plate) or 5 µL of 5X test compounds (384-well plate) in medium or your desired buffer (such as PBS or HHBS). For control wells (untreated cells), add the corresponding amount of compound buffer.

3.2 To induce superoxide, incubate the cell at 37 °C for a desired period of time, protected from light.
Note: We treated RAW 264.7 macrophage cells with 5 µM Antimycin A (AMA) at 37 ºC for 2 hours to induce superoxide. See Figure 1 for details.

3.3 Add 100 µL/well (96-well plate) or 25 µL/well (384-well plate) of MitoROS™ 520 working solution (from Step 2.2) into the cell plate.

3.4 Incubate the cells at 37 °C for 1 hour, and take images using fluorescence microscope with a FITC filter.

Data Analysis

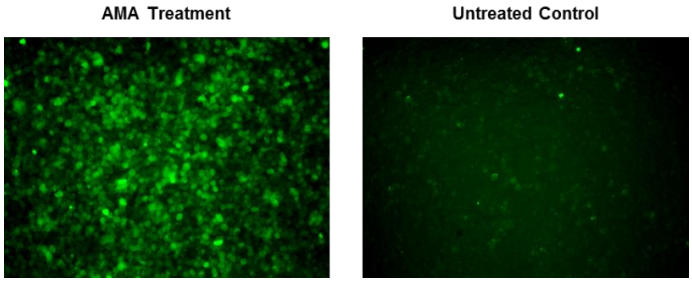

Figure 1. Fluorescence images of superoxide measurement in macrophage cells using cat#16060. RAW 264.7 cells at 100,000 cells/well/100 µL were seeded overnight in a 96-well black wall/clear bottom plate. AMA Treatment: Cells were treated with 5 µM Antimycin A (AMA) at 37 ºC for 2 hours, then incubated with MitoROS™ 520 for 1 hour. Untreated Control: RAW 264.7 cells were incubated with MitoROS™ 520 at 37 ºC for 1 hour without AMA treatment. The fluorescence signal was measured using fluorescence microscope with a FITC filter.

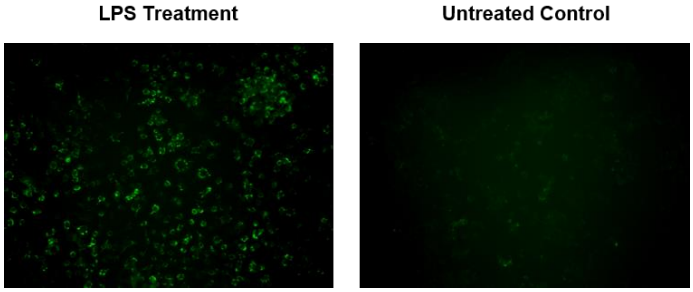

Figure 2. Fluorescence images of endogenous superoxide measurement in macrophage cells using Cat#16060. RAW 264.7 cells at 100,000 cells/well/100 µL were seeded overnight in a 96-well black wall/clear bottom plate. LPS Treatment: Cells were incubated with MitoROS™ 520 for 1 hour, then treated with 200 µg/mL of lipopolysaccharide (LPS) at 37 ºC for 16 hours. Untreated Control: RAW 264.7 cells were incubated with MitoROS™ 520 at 37 ºC for 1 hour without LPS treatment. The fluorescence signal was measured using fluorescence microscope

Assay Protocol for Flow Cytometry Analysis

Brief Summary

Prepare cells in growth medium → Treat the cells with test compounds to induce superoxide → Stain the cells at 37 °C for 60 minutes→ Monitor the fluorescence intensity with a flow cytometer

1. Prepare cells:

Prepare cells at the density from 5×10^5 to 1×10^6 cells/mL.
Note: Each cell line should be evaluated on the individual basis to determine the optimal cell density for apoptosis induction.

2. Prepare 500X MitoROS™ 520 stock solution: Add 100 µL of DMSO (Component C) into the vial of MitoROS™ 520 (Component A), and mix them well.

3. Run ROS Assay:

3.1 Treat cells as desired.

3.2 To induce superoxide, incubate the cells at 37 °C for a desired period of time, protected from light.
Note: We treated Jurkat cells with 50 µM Antimycin A (AMA) at 37 ºC for 2 hours to induce superoxide.

3.3 Add 1 µL/0.5 mL cells of MitoROS™ 520 stock solution (500X) (from Step 2) into the cells.

3.4 Incubate the cells in a 5% CO_2, 37 °C incubator for 1 hour, and monitor the fluorescence intensity using a flow cytometry in FL1 channel (Ex/Em= 488/520 nm).

References

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2. Brand, M.D., C. Affourtit, T.C. Esteves, K. Green, A.J. Lambert, S. Miwa, J.L. Pakay, N. Parker. 2004. Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. Free Radic. Biol. Med. 37:755.
3. Mukhopadhyay P., Rajesh M, Hasko G, Hawkins BJ, Madesh M, Pacher P. 2007. Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. Nat. Protoc. 2:2295.
4. Fang, H., M. Chen, Y. Ding, W. Shang, J. Xu, X. Zhang, W. Zhang, K. Li, Y. Xiao, F. Gao, et al. 2011. Imaging superoxide flash and metabolism-coupled mitochondrial permeability transition in living animals. Cell Res. 21:1295.