Mitochondrial Superoxide Assay Kit with MitoNeoD

Cat #: KTA9012 Size:200 T/1000 T

	Mitochondrial Superoxide Assay Kit with MitoNeoD		
REF	**Cat #:** KTA9012	LOT	**Lot #:** Refer to product label
	Applicable samples: Adherent cells, suspension cells		
	Storage: Stored at -20℃ for 6 month, protected from light		

Assay Principle

Superoxide anion (O_2•$^-$) is a free radical anion formed by the reduction of an oxygen molecule with one electron. It is the primary form of reactive oxygen species (ROS). Mitochondrial O_2•$^-$ mainly originates from the leakage of electrons in the electron transport chain, with key sites including complex I and complex III. The inhibition of complex I, reverse electron transfer, or accumulation of ubiquinone/ubiquinol can increase its generation. The direct reactivity of O_2•$^-$ is limited, and its harm is mainly mediated by secondary reactions: Superoxide dismutase (SOD) catalyzes its conversion into H_2O_2, which, with the participation of Fe^{2+}, undergoes the Fenton reaction to generate hydroxyl radicals (·OH); ·OH is extremely reactive and can cause lipid peroxidation, protein oxidation/carbonylation, and mitochondrial DNA oxidative damage (such as the formation of 8-oxodG). The imbalance between the generation and clearance of mitochondrial O_2•$^-$ can lead to oxidative stress. Current studies suggest that this oxidative stress is closely related to pathological processes such as neurodegenerative diseases (such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease), cardiovascular diseases, and metabolic disorders.

MitoNeoD (also known as MND, Mito-NeoD, Mito Neo D, Mito Neo-D) is a fluorescent probe that can rapidly and specifically target mitochondria and selectively detect mitochondrial O_2•$^-$. Its structure consists of three functional units: the reduced phthalazine core serves as an O_2•$^-$ responsive group; when not oxidized, the fluorescence is extremely weak, and upon oxidation, a red fluorescent product is generated; the new octyl substitution can spatially prevent the product from embedding into DNA; the 6-carbon-deuterium bond undergoes kinetic isotope effect (KIE) to reduce the non-specific oxidation rate and improve the selectivity for O_2•$^-$; the triphenylphosphonium lipophilic cation, driven by membrane potential, crosses the cell membrane and accumulates in the mitochondrial matrix to achieve mitochondrial targeting.

Compared with the mitochondrial superoxide detection kit based on MitoSOX Red, the fluorescence product of MitoNeoD can emit fluorescence without binding to DNA, and the signal is located in the mitochondria with low interference to mitochondrial function. Therefore, it outperforms MitoSOX Red in terms of positioning accuracy, signal specificity, and low perturbation to the physiological state of mitochondria. This kit is suitable for fluorescence detection systems such as fluorescence microscopes, laser confocal microscopes, fluorescence microplate readers, and flow cytometers. The fluorescence detection channel is the red channel (Ex/Em = 545 nm/610 nm).

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Materials Supplied and Storage Conditions

Kit components	Size		Storage conditions
	200 T	1000 T	
MitoNeoD（5 mM）	20 μL	100 μL	-20℃, protected from light
Assay Buffer	40 mL	100 mL×2	4℃
mSox UP	40 μL	200 μL	-20℃, protected from light

Note: This kit is available in 200 T/1000 T specifications for use with fluorescence microscopes, fluorescence microplate readers, and laser confocal microscopes. Taking the 96-well plate as an example, each well contains 100 μL of the system, and can be used for 200 and 1000 detections respectively; taking the 24-well plate as an example, each well contains 200 μL of the system, and can be used for 100 and 500 detections respectively, and so on. When used with a flow cytometer, each sample is in a 200 μL system, and can be used for 100 and 500 detections respectively.

Materials Required but Not Supplied

·Cryogenic centrifuge, fluorescence microscope, flow cytometer, fluorescence microplate reader, laser confocal microscope, MitoNeoD detection by fluorescence microscopy: Ex/Em = 545/610 nm, detection by flow cytometry: PE channel

·Precision pipettes, disposable pipette tips

·Deionized water

·Phosphate buffer solution (PBS, pH 7.4)

Reagent Preparation

mSox UP Working Reagent: Dilute mSox UP with the cell culture medium at a ratio of 1:1000. The mSox UP working solution should be prepared immediately before use and the entire operation process should avoid exposure to light; the diluted working solution should not be stored for a long time.

MitoNeoD Working Reagent: Prepare before use. Before using the Assay Buffer, let it equilibrate to room temperature. Dilute MitoNeoD with the Assay Buffer at a ratio of 1:1000. The staining working solution for MitoNeoD should be prepared immediately and used up promptly. Avoid light during the operation; the diluted working solution should not be stored for a long time.

Assay Procedure

A. Fluorescence microscopy analysis

1. For adherent cells

(1) Taking adherent cells cultured in 96-well plates as an example, an appropriate amount of cells is inoculated into the wells, and the cells are processed after they reach the desired density.

(2) Optional step: Positive control preparation. Rinse the cells in the wells with PBS twice, add 100 μL mSox UP Working Regent to the wells, induce at 37℃ in a CO_2 cell incubator for 2-4 h. During this period, observe the cell morphology. After the cell morphology changes, remove the mSox UP Working Regent and rinse the cells in the wells with PBS twice.

Note: After most cells were induced by mSox UP for 2-4 h, the level of superoxide significantly increased. After staining with MitoNeoD, a distinct red fluorescence signal could be observed, while normal cells that were not induced showed only a weak red fluorescence signal. The optimal concentration and incubation time of mSox UP for different cells vary, and it is recommended to optimize the conditions yourself. Moreover, some cells may even be insensitive to this inducer.

(3) Remove the PBS, then add 100 μL of MitoNeoD Working Regent. Incubate at 37℃ in a CO_2 cell incubator for 20-30 min.

Note: The working concentration of MitoNeoD varies depending on the type of cells and experimental conditions. It should be determined through preliminary experiments. It is recommended to start with a concentration of 5 μM, and then screen for an appropriate concentration within the range of 2.5-10 μM. If the positive control signal is weak, increase the concentration; if the background signal is high, decrease the concentration. When conducting tests on specific cells, it is advisable to conduct a preliminary experiment first.

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(4) After incubation is complete, discard MitoNeoD Working Regent and rinse the cells twice with PBS.

(5) If mitochondrial staining is required, use MitoGreen (TraKine™ Mitochondrial Staining Kit (Green Fluorescence), KTC4003) to stain for 10 min. Unless otherwise specified, discard the PBS, add 100 μL Assay Buffer, and observe under a fluorescence microscope or laser confocal microscope.

2. For non-adherent cells

(1) Prepare the samples according to the flow cytometry analysis procedure.

(2) Optional step: Preparation of positive control. Centrifuge 300 g for 5 min to collect cells (cell density approximately 1×10^6). Wash twice with PBS, then add 1 mL of mSox UP Working Regent. Induce at 37℃ in a CO_2 cell incubator for 2-4 h. During this period, observe the cell morphology. After the cell morphology changes, centrifuge again at 300 g for 5 min to collect the cells. Discard the mSox UP Working Regent and immerse the cells in PBS for 2 times.

Note: After most cells were incubated with mSox UP for 2-4 h, the level of superoxide significantly increased. After staining with MitoNeoD, a distinct red fluorescence signal could be observed, while normal cells without induction showed only a weak red fluorescence signal. The optimal concentration and incubation time of mSox UP for different cells vary, and it is recommended to optimize the conditions yourself. Moreover, some cells may even be insensitive to this inducer.

(3) Centrifuge at 300 g for 5 min to collect the cells. Discard the supernatant and add 200 μL of MitoNeoD Working Regent. Incubate at 37℃ in a CO_2 cell incubator for 20-30 min.

Note: The working concentration of MitoNeoD varies depending on the type of cells and experimental conditions. It should be determined through preliminary experiments. It is recommended to start with a concentration of 5 μM, and then screen for an appropriate concentration within the range of 2.5-10 μM. If the positive control signal is weak, increase the concentration; if the background signal is high, decrease the concentration. When conducting tests on specific cells, it is advisable to conduct a preliminary experiment first.

(4) Centrifuge at 300 g for 5 min to collect the cells, then wash them twice with PBS.

(5) Place the prepared cell suspension sample on a slide, cover the cells with a cover glass, and observe under a fluorescence microscope or a confocal laser microscope. Alternatively, drop the cell suspension into a 96-well plate, let it stand for 5 min, and then observe under a fluorescence microscope or a confocal laser microscope.

Note: For smaller cells (such as Jurkat), the fluorescence signal after MitoNeoD staining is relatively weak, and it is difficult to observe clearly with a regular fluorescence microscope. It is recommended to use a laser confocal microscope with higher detection sensitivity for observation.

B. Flow cytometry detection

1. For adherent cells

(1) Taking adherent cells cultured in 96-well plates as an example, a black 96-well enzyme-linked plate is selected. Appropriate amounts of cells are inoculated into the wells, and the cells are allowed to grow to the desired density before being processed.

(2) Optional step: Positive control preparation. Rinse the cells in the wells with PBS twice, add 100 μL mSox UP Working Regent to the wells, induce at 37℃ in a CO_2 cell incubator for 2-4 h. During this period, observe the cell morphology. After the cell morphology changes, remove the mSox UP Working Regent and rinse the cells in the wells with PBS twice.

Note: After most cells were induced by mSox UP for 2-4 h, the level of superoxide significantly increased. After staining with MitoNeoD, a distinct red fluorescence signal could be observed, while normal cells that were not induced showed only a weak red fluorescence signal. The optimal concentration and incubation time of mSox UP for different cells vary, and it is recommended to optimize the conditions yourself. Moreover, some cells may even be insensitive to this inducer.

(3) Remove the PBS, then add 100 μL of MitoNeoD Working Regent. Incubate at 37℃ in a CO_2 cell incubator for 20-30 min.

Note: The working concentration of MitoNeoD varies depending on the type of cells and experimental conditions. It should be determined through preliminary experiments. It is recommended to start with a concentration of 5 μM, and then screen for an appropriate concentration within the range of 2.5-10 μM. If the positive control signal is weak, increase the concentration; if the background signal is high, decrease the concentration. When conducting tests on specific cells, it is advisable to conduct a preliminary experiment first.

(4) After the incubation process is completed, discard the MitoNeoD Working Regent and rinse the cells twice with PBS.

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(5) The prepared cell samples were digested using a mild digestive solution, and then centrifuged at 300 g for 5 min to collect the cells. The cells were then washed twice with PBS.

(6) Discard the PBS, add an appropriate amount of Assay Buffer to resuspend the cells, and then analyze them using a flow cytometer.

2. For non-adherent cells

(1) Cultivate the cells to the optimal density. After trypan blue staining, the cell viability rate is above 95%.

(2) Optional step: Preparation of positive control. Centrifuge 300 g for 5 min to collect cells (cell density approximately 1×10^6). Wash twice with PBS, then add 1 mL of mSox UP Working Regent. Induce at 37℃ in a CO_2 cell incubator for 2-4 h. During this period, observe the cell morphology. After the cell morphology changes, centrifuge again at 300 g for 5 min to collect the cells. Discard the mSox UP Working Regent and immerse the cells in PBS for 2 times.

Note: After most cells were incubated with mSox UP for 2-4 h, the level of superoxide significantly increased. After staining with MitoNeoD, a distinct red fluorescence signal could be observed, while normal cells without induction only showed a weak red fluorescence signal. The appropriate concentration and incubation time of mSox UP for different cells vary, and it is recommended to optimize the best conditions by yourself. Moreover, some cells may even be insensitive to this inducer.

(3) Centrifuge at 300 g for 5 min to collect the cells. Discard the supernatant and add 200 μL of MitoNeoD Working Regent. Incubate at 37℃ in a CO_2 cell incubator for 20-30 min.

Note: The working concentration of MitoNeoD varies depending on the type of cells and experimental conditions. It should be determined through preliminary experiments. It is recommended to start with a concentration of 5 μM, and then screen for an appropriate concentration within the range of 2.5-10 μM. If the positive control signal is weak, increase the concentration; if the background signal is high, decrease the concentration. When conducting tests on specific cells, it is advisable to conduct a preliminary experiment first.

(4) Centrifuge at 300 g for 5 min to collect the cells. Wash them twice with PBS. Add an appropriate amount of Assay Buffer to resuspend the cells, and then analyze them using a flow cytometer.

C. Fluorescence microplate reader detection

1. For adherent cells

(1) Taking adherent cells cultured in 96-well plates as an example, an appropriate amount of cells is inoculated into the wells, and the cells are processed after they reach the desired density.

(2) Optional step: Positive control preparation. Rinse the cells in the wells with PBS twice, add 100 μL mSox UP Working Regent to the wells, induce at 37℃ in a CO_2 cell incubator for 2-4 h. During this period, observe the cell morphology. After the cell morphology changes, remove the mSox UP Working Regent and rinse the cells in the wells with PBS twice.

Note: After most cells were induced by mSox UP for 2-4 h, the level of superoxide significantly increased. After staining with MitoNeoD, a distinct red fluorescence signal could be observed, while normal cells that were not induced showed only a weak red fluorescence signal. The optimal concentration and incubation time of mSox UP for different cells vary, and it is recommended to optimize the conditions yourself. Moreover, some cells may even be insensitive to this inducer.

(3) Remove the PBS, then add 100 μL of MitoNeoD Working Regent. Incubate at 37℃ in a CO_2 cell incubator for 20-30 min..

Note: The working concentration of MitoNeoD varies depending on the type of cells and experimental conditions. It should be determined through preliminary experiments. It is recommended to start with a concentration of 5 μM, and then screen for an appropriate concentration within the range of 2.5-10 μM. If the positive control signal is weak, increase the concentration; if the background signal is high, decrease the concentration. When conducting tests on specific cells, it is advisable to conduct a preliminary experiment first.

(4) After the incubation process is completed, discard the MitoNeoD Working Regent and rinse the cells twice with PBS.

(5) Remove PBS and add 100 μL of Assay Buffer. Then, perform the detection using a fluorescence microplate reader with an excitation wavelength of 545 nm and an emission wavelength of 610 nm.

2. For non-adherent cells

(1) Cultivate the cells to the optimal density. After trypan blue staining, the cell viability rate is above 95%.

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(2) Optional step: Preparation of positive control. Centrifuge 300 g for 5 min to collect cells (cell density approximately 1×10^6). Wash twice with PBS, then add 1 mL of mSox UP Working Regent. Induce at 37℃ in a CO_2 cell incubator for 2-4 h. During this period, observe the cell morphology. After the cell morphology changes, centrifuge again at 300 g for 5 min to collect the cells. Discard the mSox UP Working Regent and immerse the cells in PBS for 2 times.

Note: After most cells were incubated with mSox UP for 2-4 h, the level of superoxide significantly increased. After staining with MitoNeoD, a distinct red fluorescence signal could be observed, while normal cells without induction only showed a weak red fluorescence signal. The appropriate concentration and incubation time of mSox UP for different cells vary, and it is recommended to optimize the best conditions by yourself. Moreover, some cells may even be insensitive to this inducer.

(3) Centrifuge at 300 g for 5 min to collect the cells. Discard the supernatant and add 200 μL of MitoNeoD Working Regent. Incubate at 37℃ in a CO_2 cell incubator for 20-30 min.

Note: The working concentration of MitoNeoD varies depending on the type of cells and experimental conditions. It should be determined through preliminary experiments. It is recommended to start with a concentration of 5 μM, and then screen for an appropriate concentration within the range of 2.5-10 μM. If the positive control signal is weak, increase the concentration; if the background signal is high, decrease the concentration. When conducting tests on specific cells, it is advisable to conduct a preliminary experiment first.

(4) Centrifuge for 5 min to collect the cells. Wash twice with PBS. Resuspend the cells with an appropriate amount of Assay Buffer. Add them to a black 96-well microplate. Detect using a fluorescence microplate reader. Excitation wavelength: 545 nm; Emission wavelength: 610 nm.

Typical Data

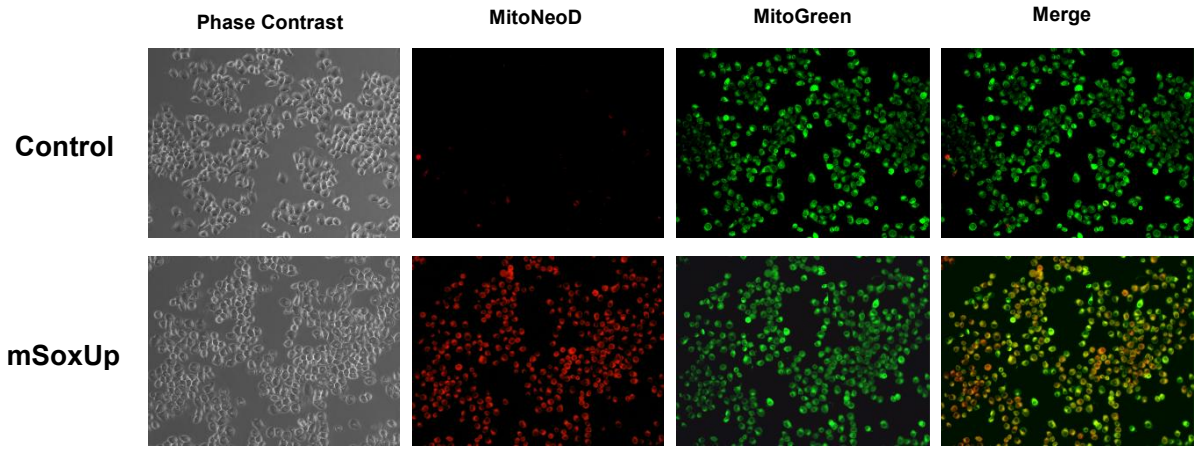


Figure 1. Fluorescence microscopic results of Hela cells stained with the mitochondrial superoxide detection kit. After Hela cells were not treated or treated with the positive control reagent mSox UP, staining was performed using this kit and MitoGreen. The green fluorescence represents the mitochondria of all cells labeled with MitoGreen. This kit does not provide MitoGreen; if needed, you can order the TraKine™ mitochondrial staining kit (green fluorescence) (KTC4003) from Abbkine.

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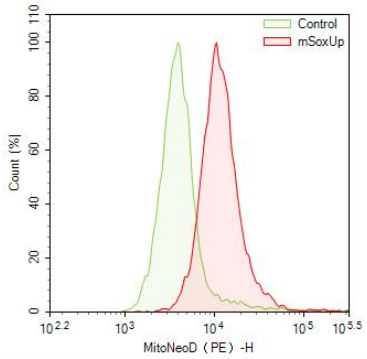


Figure 2. Flow cytometry result diagram of Jurkat cells stained with mitochondrial superoxide detection kit.

Precautions

1. To avoid cross-contamination, tip changes when adding different samples and different reagents.
2. Make sure all components and equipment are at the right temperature before the experiment starts.
3. Fluorescent dyes have quenching problems, please pay attention to avoid light as much as possible to slow down fluorescence quenching.
4. For your safety and health, please wear a lab coat and wear disposable gloves.

FAQ

1. After staining with the kit, there is no significant difference in the signals between the PC group and the NC group. How should this be handled?

A: For certain sensitive cell types, such as HEK293, the manipulation process may cause cellular damage, leading to higher positive signals in the NC group. Therefore, it is recommended to modify the procedure by first loading the probe and then inducing. After staining with MitoNeoD for 30 min, wash the cells twice with PBS. Add mSox UP Working Reagent to the PC group and incubate for 2–4 hours, while adding regular culture medium to the NC group. Place both groups into a CO_2 incubator.

2. When diluting mSox UP, should we use the basal medium or the complete medium?

A: All are acceptable. If the mSox UP Working Regent is prepared using the complete medium, the induction effect is not good. In this case, it is advisable to replace it with the induction agent prepared using the basic medium. Also, during the induction period, the NC group should also be switched to the basic medium.

Recommended Products

Catalog No.	Product Name
KTA4001	Mitochondrial Membrane Potential Assay Kit (JC-1)
KTA4002	Mitochondrial Permeability Transition Pore Assay
KTA9010	Oxygen Consumption Rate (OCR) Assay Kit
KTB1910	CheKine™ Reactive Oxygen Species (ROS) Detection Fluorometric Assay Kit (Green Fluorescence)

Disclaimer

The reagent is only used in the field of scientific research, not suitable for clinical diagnosis or other purposes.

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