Production of Superoxide Ion in the Mitochondria

MitoSOX Red Mitochondrial Superoxide Indicator (Molecular Probes M36008) 10x50 µg
Storage: -20°C in darkness, aliquot required.
Shelf life: 6 months.
MitoSOX is supplied in packaging with an oxygen-deprived pouch, extending its shelf life. Vials should be returned to the pouch after use. Vials should be brought to room temperature before opening.
MitoSOX is a derivative of ethidium bromide.

HBSS with Calcium and Magnesium

MitoSOX Red is oxidized by superoxide ions but not by other ROS or reactive nitrogen species. Oxidation of the probe is prevented by superoxide dismutase. Upon oxidation, the probe becomes highly fluorescent due to its binding to nucleic acids. The positively charged probe accumulates in the mitochondria.

MitoSOX is used here at **2.5 µM** but can be increased up to **3.5 µM** for a greater delta.

MitoSOX also accumulates in dead cells, necessitating a double staining with **Sytox Blue**.

Sytox Blue Storage: -20°C, stable for 1 year (can be aliquoted).

APC Annexin V: 25 tests, 5 µL/test (store at 4°C in darkness).
Identifies apoptotic cells (early-stage).
Calcium-dependent.

Culture Medium: DMEM/high glucose 10% FBS 1% penicillin/streptomycin.

Caution: Protect from light.

MitoSOX Excitation & Emission:

- Excited by **488 nm blue laser**
- **Ex:** 510 nm
- **Em:** 580 nm (similar to PE)

Annexin V Excitation & Emission:

- Excited by **650 nm red laser**
- **Emission Peak:** 660 nm (similar to APC)

Sytox Blue Excitation & Emission:

- Excited by **405 nm violet laser**

- **Ex:** 444 nm
- **Em:** 480 nm (similar to Pacific Blue)

EXPERIMENTAL PROTOCOLS

Cells should not exceed **100% confluence** in the last **two passages**.
Cells should reach **80% confluence** before the experiment, and fresh culture medium should be added beforehand.

PROTOCOL

1. **Cell Seeding (Day -3):**
   - **Petri dish (35 mm):** 100,000 cells per dish.
2. **Solution Preparation:**
   - **MitoSOX Reagent Solution (2.5 mM):**
     - Dissolve **50 µg MitoSOX** in **26.4 µL DMSO**.
     - Aliquot and store at -20°C.
     - Prepare fresh (< 15 min before use).
     - Can be reused once.
   - **Culture Medium and HBSS:** Pre-warmed to **37°C**.
3. **Wash Cells with PBS.**
4. **Add MitoSOX Red at Final Concentration of 2.5 µM:**
   - Dilute **1:1000** in **1 mL medium**.
   - (1 µL per 1 mL medium).
5. **Incubate Cells for 30 min at 37°C, Covered with Aluminum Foil.**
6. **Wash Cells with 1 mL PBS.**
7. **Trypsinize Cells:**
   - **500 µL trypsin per well**.
   - Ensure a single-cell suspension (microscopic check).
   - Add **1 mL medium**, transfer to flow cytometry tubes.
8. **Wash Wells with 1 mL PBS to Recover All Cells.**
9. **Wash Cells 2x with HBSS+ (Centrifuge 5 min, 1200 rpm, RT).**
10. Discard the supernatant and gently mix the cell pellet.
11. Resuspend the cells in **100 µL of 1X Binding Buffer per 500,000 cells**.
    - If proceeding directly to flow cytometry, resuspend in **500 µL of PBS or HBSS**.
    - Ensure **500,000 cells per tube** for optimal analysis.
12. Add **5 µL of Annexin V** and incubate for **20 minutes at RT in the dark**.
13. Add **400 µL of 1X Binding Buffer per tube**.

14. Add **5 µL Sytox Blue**.
15. Incubate **5 minutes at RT, protected from light**.
16. **Keep Tubes on Ice and Analyze by Flow Cytometry Within 30 Minutes.**
    - Exclude dead cells, analyze only main cell population.

Manual Compensation Calculation:
Include **unstained cells** in a separate control tube.

Results Calculation:

Mean (treated population) - Mean (untreated population) = delta mean

Troubleshooting:

- **MitoSOX Red strongly stains dead cells**.
- Use additional markers:
  - **Early apoptosis:** Annexin V
  - **Dead cells:** Sytox Blue

Stain Index Calculation:
Use **Antimycin-treated cells (max peak)** and **Sytox Blue** to exclude dead cells. For single-peak distribution:

(Median Treated Pop PE+ - Median Untreated Pop PE-)/ rSDpop-

DATA ANALYSIS

Statistical Analysis:

1. **Verify sample independence** using **Kruskal-Wallis test**.
2. **Mann-Whitney test (GraphPad):**
   - **Column → Create → Paste data**.
   - **Column Analyze → t-test (non-parametric)**.
   - **Select groups to compare, choose Unpaired, and use Mann-Whitney test.**
   - **Results include p-value.**

GraphPad Graphing:

- **Box and Violin Plots**: Data → Box and Violin
- **Dose-Response Curves**: XY Analysis → Non-Linear Regression → Sigmoidal Dose-Response.