## Superoxide measurements by HPLC

The Redox Molecular Signaling Core utilizes a Shimadzu Prominence 20 series HPLC to quantitate superoxide level in either cell or tissue samples. To initiate superoxide measurements, contact the Redox Molecular Signaling core facility at **RedoxMolSignalCore@lsuhsc.edu** and schedule a meeting with Core Leaders to discuss the project timeline and deliverables.

To be provided by investigator:

- A completed Work Order Form brought to the meeting with Core Leaders.
- The test samples prepared according the following SOPs (performed in the dark):
  For cell samples → Procedure A
  For plasma samples → Procedure B
  For tissue samples → Procedure C

  Note 1: Enrichment experiment of 2-OH-$e^+$ can be requested in the core if the concentration of 2-OH-E+ in the sample is expected to be below 60 nmol/L.

  Note 2: For mitochondrial superoxide measurements, the mitochondria-targeted hydroethidine (MitoSOX™ Red) can be used (stock solution: 5 mM MitoSOX in DMSO, diluted 5 μM MitoSOX in medium for cell treatment).

To be generated by the core:

- Superoxide HPLC analysis report

Timeline: Timeline may depend on sample number and core schedule. Typical timelines range around 2 weeks.

## Procedure A: Preparing superoxide samples from cells

Equipment and materials

- HE or DHE—5-ethyl-5, 6-dihydro-6-phenyl-3, 8-diaminophenanthridine (Fluka, #37291)
- DMSO, ACS spectrophotometric grade (Sigma-Aldrich, cat. no. 15,493-8)
- Dulbecco's PBS (DPBS; Sigma, cat. no. D8537)
- Triton X-100 (Sigma-Aldrich, cat. no. T9284)
- 1 M phosphate buffer pH 2.6
- 0.2 M $HClO_4$ in MeOH: Add 85.5 ml of 70%$HClO_4$ per 4.915 ml of ice-cold MeOH. Keep the resulting solution in the 4$^{O}$C refrigerator.
- Centrifuge.

Procedure:

- Typically, one confluent well of a 6-well dish provides suitable material for superoxide measurements.
- Incubate the cells with media containing 10 μM HE (5 μl of 20 mM HE dissolved in DMSO per 10 ml of medium) for 30 min. For treatments longer than 30 minutes, only include HE during the final 30 min. of treatment. Include a blank control with cells that did not receive HE treatment.
- Remove the media and wash cells twice with ice-cold PBS buffer.
- Scrape the cells immediately in 1 ml of ice-cold DPBS, transfer the cell suspension into a 1.5 ml EP tube, and place the tube on ice.
- Pellet the cells by centrifugation: 5min ×1000g at 4℃.
- Aspirate the excess DPBS. (Note: cells can be stored in -80℃ for several weeks at this step)
- Lyse the cells with DPBS containing 0.1% Triton X-100 (~200 μl for each well of 6 well plate) by passing the mixture through a p200 pipette tip ten times or by sonication (2 X 10 sec.) at 20 KHz. (Note: Add 5 mM KCN or 5 U/ml of catalase into the lysis buffer if the samples are expected to contain high levels of peroxidases)
- Centrifuge at 10000 g at 4℃ for 5 min.
- Transfer > 5 μl of lysate supernatant for determining protein concentration.
- Transfer 100 μl of the lysate supernatant into a fresh tube containing 100 μl of the MeOH/$HClO_4$ solution, vortex the tube for 10s, and place it back on ice for 1~2 h to stimulate protein precipitation.
- Pellet the protein precipitate by centrifugation: 10 min ×12,000g at 4℃.
- Transfer 100 μl of the supernatant to a fresh tube containing 100 μl of 1 M phosphate buffer (PH 2.6, to induce $KClO_4$ salt production), vortex for 5s.
- Pellet the excess buffer and $KClO_4$ precipitate by centrifugation: 5 min ×12,000g at 4℃.
- Transfer the supernatant into the new microcentrifuge tubes and place them at 4$^{o}$C for short term (< 2 weeks) storage. For long term storage, place tubes at -20 $^{o}$C.

## Procedure B: Preparing superoxide samples from mouse plasma

Equipment and materials

- HE or DHE—5-ethyl-5, 6-dihydro-6-phenyl-3, 8-diaminophenanthridine (Fluka, #37291)
- DMSO, ACS spectrophotometric grade (Sigma-Aldrich, cat. no. 15,493-8)
- Microtainer plasma separator tubes (BD Biosciences, Cat. No. 365958).
- Tissue homogenizer
- Mouse Surgery tools
- 1 M phosphate buffer pH 2.6
- 0.2 M HClO4 in MeOH: Add 85.5 ml of 70% HClO4 per 4.915 ml of ice-cold MeOH.

Procedure:

- Administer 0.3 mg of HE (0.12 ml DMSO, 0.18 ml dd$H_2O$) / 30 mg mouse intra-peritoneally.
- After 1h, mouse should be euthanized.
- Collect blood and isolate plasma by plasma separator tubes (WHAT VOLUME IS REQUIRED?).
- Transfer 100 μl of the lysate supernatant into a fresh tube containing 100 μl of the MeOH/$HClO_4$ solution, vortex the tube for 10s, and place it back on ice for 1~2 h to stimulate protein precipitation.
- Pellet the protein precipitate by centrifugation: 10 min ×12,000g at 4℃.
- Transfer 100 μl of the supernatant to a fresh tube containing 100 μl of 1 M phosphate buffer (PH 2.6, to induce $KClO_4$ salt production), vortex for 5s.
- Pellet the excess buffer and $KClO_4$ precipitate by centrifugation: 5 min ×12,000g at 4℃.
- Transfer the supernatant into the new microcentrifuge tubes and place them at 4°C for short term (< 2 weeks) storage. For long term storage, place tubes at -20 °C.

**Procedure C: Preparing superoxide samples from mouse tissue**

Equipment and materials

- HE or DHE—5-ethyl-5, 6-dihydro-6-phenyl-3, 8-diaminophenanthridine (Fluka, #37291)
- DMSO, ACS spectrophotometric grade (Sigma-Aldrich, cat. no. 15,493-8)
- Microtainer plasma separator tubes (BD Biosciences, Cat. No. 365958).
- Tissue homogenizer
- Mouse Surgery tools
- 1 M phosphate buffer pH 2.6
- 0.2 M HClO4 in MeOH: Add 85.5 ml of 70% HClO4 per 4.915 ml of ice-cold MeOH.

Procedure:

- Administer 0.3 mg of HE (0.12 ml DMSO, 0.18 ml ddH2O) /30 mg mouse intra-peritoneally.
- After 1h, mouse should be euthanized, and blood should be removed from tissue by excising the portal vein and flushing the left ventricle with PBS. Collect the desired tissues.
- Homogenize the collected tissues with 50 mM phosphate buffer (pH 7.4) (10 μl per mg of tissue). Note: Add 5 mM KCN or 5 U/ml of catalase into homogenize buffer if samples with high levels of peroxidases.
- Transfer 100 μl of the lysate supernatant into a fresh tube containing 100 μl of the MeOH/$HClO_4$ solution, vortex the tube for 10s, and place it back on ice for 1~2 h to stimulate protein precipitation.
- Pellet the protein precipitate by centrifugation: 10 min ×12,000g at 4℃.
- Transfer 100 μl of the supernatant to a fresh tube containing 100 μl of 1 M phosphate buffer (PH 2.6, to induce $KClO_4$ salt production), vortex for 5s.
- Pellet the excess buffer and $KClO_4$ precipitate by centrifugation: 5 min ×12,000g at 4℃.
- Transfer the supernatant into the new microcentrifuge tubes and place them at 4°C for short term (< 2 weeks) storage. For long term storage, place tubes at -20 °C.