**Standard Operating Procedure:   
Biochemical Analyses of Superoxide Dismutase (with parallel BCA protein determination)**

CM, CYW  
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**Kit:** Invitrogen™ Superoxide Dismutase (SOD) Colorimetric Activity Kit, 2 x 96 Tests, Catalog number [EIASODC](https://www.thermofisher.com/order/catalog/product/EIASODC?SID=srch-srp-EIASODC)   
  
\*UPON RECEIPT, STORE KIT IN FREEZER (-20°C) UNTIL USE. ONCE OPEN, STORE AT 4°C AND USE WITHIN 2 WEEKS.

**Additional materials needed but not supplied:**

* Plate reader (450 nm SOD, 562 nm Protein)
* Precision pipettes (8 channel, 10-200 uL; single channel, 10-200uL, single channel, 5mL)
* 1.5 mL tubes for creating serial dilution of standards and diluting samples
* 10 mL tubes for preparing reagents (1x XO, 1x Substrate)
* Vortex for mixing solutions
* Optional: incubator set at room temp (20°C) for stable temperature conditions
* Supernatant for all samples (frozen): 125-150uL

1. Remove kit from freezer and allow to reach room temperature (this can take several hours).   
   Place samples from freezer into fridge to thaw.
2. Prepare reagents

Note: Reagent volumes are per plate as we carried at one plate per day. 2 plates total needed for all samples. We carried out plates over 2 consecutive days.

1X Xanthine Oxidase (25 uL per well)

1. Prepare and label 10mL tube (**Label: 1x XO**)
2. Add 2.4 mL of Xanthine Oxidase Buffer to tube.
3. Vortex Xanthine Oxidase (XO) Concentrate (25X) prior to pipetting.
4. Add 100 uL of Xanthine Oxidase Concentrate (25X) to tube. Pipette from bottom of tube.
5. Vortex to ensure good mixing.

1X Substrate (50 uL per well)

1. Prepare and label 10 mL tube (**Label: 1X Substrate**)
2. Add 4.5 mL of Substrate Diluent to tube.
3. Vortex Substrate Concentrate prior to pipetting.
4. Add 500 uL of Substrate Concentrate to tube.
5. Vortex to ensure good mixing.
6. Blank plate (450 nm) prior to preparation of samples and standards.

Note: not sure this is necessary but did just in case.

1. Prepare samples:
2. Remove samples from fridge and leave on benchtop to warm to room temperature.
3. Dilute samples in Assay Buffer as per pre-determined dilution factor

Note: We trialed dilution factors of 1:4, 1:24 (Plate 1), and 1:1, 1:3 (Plate 2). We found that 1:4 and 1:24 were in the very low range. We propose NOAA try 0 (no dilution) and 1:1 (for direct comparison).

1. Vortex samples to ensure mixing.
2. Use samples within **2 hours of preparation**.
3. Prepare standards:

Note: We ran 1 set of standards per plate and made new standards for each of two plates (as ran over more than one day).

1. Prepare and label 8 x 1.5 mL tubes: 4, 2, 1.0, 0.50, 0.25, 0.125, 0.0625, and 0 U/mL SOD (Stds 1-8).
2. Prepare SOD standard: Reconstitute by adding 250 uL Assay Buffer. Vortex and incubate for 5 min at room temperature. Label as: 4 U/mL SOD.
3. Transfer 120 uL of the 4 U/mL SOD into a Std 1 tube ONLY.
4. Add 75 uL Assay Buffer to remaining 7 tubes (Std 2-8 tubes) ONLY.
5. Carry out serial dilution as shown on P.2 of information sheet. Add 75 uL of SOD standard (Std1) to Std 2 tube and mix thoroughly. Repeat for Stds 3-7 but NOT Std 8 (0 U/mL). Mix thoroughly between steps.
6. Use standards within **2 hours of preparation.**
7. Store remaining reconstituted SOD standard in frozen aliquots.
8. Prepare plate:

Note: You will require standards, diluted samples, 1x XO and 1x Substrate. All reagents, standards, and samples should be at room temperature prior to use. Mix well prior to use.

1. Add 10uL of standards and samples in duplicate to plate as per plate layout below.
2. Add 50uL of 1x Substrate to every well.
3. Add 25 uL of 1x XO into each well.
4. Incubate plate for 20 minutes at room temperature.
5. Read absorbance at 450 nm.

SOD Plate Layout:

8 standards; 3 treatments (Ctrl, OA, HW (plus baseline (BL)); 3 timepoints (T1=20°C, T2=24°C, T3=24 hr recovery) per treatment (e.g. T1OA, T2OA T3OA); 6 samples per treatment per timepoint (1-6), 2 replicates per samples (1,1).

PLATE 1

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Std 1 | 1 | T1Ctrl1 | 1 | T2Ctrl1 | 1 | T3Ctrl1 | 1 | T1OA 1 | 1 | T2OA 1 | 1 |
| B | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| C | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| D | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| F | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| G | 7 | 7 |  |  |  |  |  |  |  |  |  |  |
| H | 8 | 8 |  |  |  |  |  |  |  |  |  |  |

PLATE 2

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Std 1 | 1 | T3OA 1 | 1 | T1HW1 | 1 | T2HW1 | 1 | T3HW1 | 1 | BL 1 | 1 |
| B | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| C | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| D | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| F | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| G | 7 | 7 |  |  |  |  |  |  |  |  |  |  |
| H | 8 | 8 |  |  |  |  |  |  |  |  |  |  |

**BCA Protein Determination for SOD samples**

**Kit:** BCA Protein Pierce™ BCA Protein Assay Kit, 500mL, 10 x 1 mL glass ampules, 250 tubes or 2,500 microplate assays, Catalog number [23227](https://www.thermofisher.com/order/catalog/product/23227)

\*UPON RECEIPT, STORE KIT AT ROOM TEMPERATURE.

**Additional materials needed but not supplied:**

* Assay Buffer (from SOD kit) for diluent
* 96 micro-well plates
* Plate reader (562 nm)
* Plate shaker
* Precision pipettes (8 channel, 10-200 uL; single channel, 10-200uL, single channel, 5mL)
* 1.5 mL tubes for creating serial dilution of standards and diluting samples
* 50 mL tubes for BCA working reagent and SOD Assay Buffer (standard diluent)
* Incubator (set to 37°C).

USE Microplate procedure (sample to WR ratio = 1:8)

1. Turn on incubator (set to 37°C).
2. Move samples from freezer into fridge to thaw.
3. Prepare Standards
4. Prepare Standards according to Table 1 of User Guide using 9 x 1.5 mL tubes. Label A-I. Keep at room temperature.

Note: For SOD protein determination, use SOD kit Assay Buffer as diluent.

1. Prepare samples:
2. Remove samples from fridge and leave on benchtop to warm to room temperature.
3. If necessary, dilute samples in Assay Buffer as per per-determined dilution factor

Note: We did not dilute our samples but propose NOAA use 1:1 or 1:2 dilutions.

1. Vortex samples to ensure mixing.
2. Use samples within **2 hours of dilution**.
3. Prepare BCA working reagent (WR)

Note: Reagent volumes are for 2 plates as we carried at both plates (covering all samples) in one day.

1. Determine volume of BCA working reagent (WR): (#standards + #samples) x (#replicates) x (200uL) (See User Guide). Therefore, (9+60) x (2) x (200) x 2 plates = ~55 mL (Note: we made 65 mL)
2. Make up WR at a ratio of 50:1 (Reagent A : Reagent B). For 65 mL, mix 65 mL of Reagent A with 1.3 mL Reagent B

NOTE: WR is stable for several days in closed container at room temperature.

1. Prepare Plate
2. Pipette 25 µL of each standard or sample replicate into a microplate well (working range = 20–2000 µg/mL) according to plate layout below.
3. Add 200 µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
4. Cover the plate and incubate at 37°C for 30 minutes.
5. Equilibrate the plate to room temperature prior to reading (5-10 minutes).
6. Measure plate absorbance (562 nm).

Protein Plate Layout:

9 standards, 3 treatments (Ctrl, OA, HW (plus baseline (BL)); 3 timepoints (T1=20°C, T2=24°C, T3=24 hr recovery) per treatment (e.g. T1OA, T2OA T3OA); 6 samples per treatment per timepoint (1-6), 2 replicates per samples (1,1).

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Std A | SA | T1Ctrl1 | 1 | T2Ctrl1 | 1 | T3Ctrl1 | 1 | T1OA 1 | 1 | T2OA 1 | 1 |
| B | B | B | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| C | C | C | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| D | D | D | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| E | E | E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| F | F | F | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| G | G | G | H | H | I | I |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | Std A | SA | T3OA 1 | 1 | T1HW1 | 1 | T2HW1 | 1 | T3HW1 | 1 | BL 1 | 1 |
| B | B | B | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| C | C | C | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| D | D | D | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| E | E | E | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| F | F | F | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| G | G | G | H | H | I | I |  |  |  |  |  |  |
| H |  |  |  |  |  |  |  |  |  |  |  |  |