TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

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Superoxide Dismutase Assay Kit

Catalog# 7500-100-K

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Reagent kit for the analysis of Superoxide Dismutase in cell extracts

Sufficient reagents for 100 Reactions

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I. Background

The production of superoxide radicals, via immune responses and normal metabolism, is a substantial contributor, if not the primary cause, of pathology associated with neurodegenerative diseases, ischemia reperfusion injury, atherosclerosis and aging.[1-3] Superoxide Dismutases (SODs) catalyze the dismutation of the superoxide radical ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2) which diffuses into the intermembrane space or mitochondrial matrix (Fig. 1), and thus, SODs provide an important defense against the toxicity of superoxide radicals.[4]

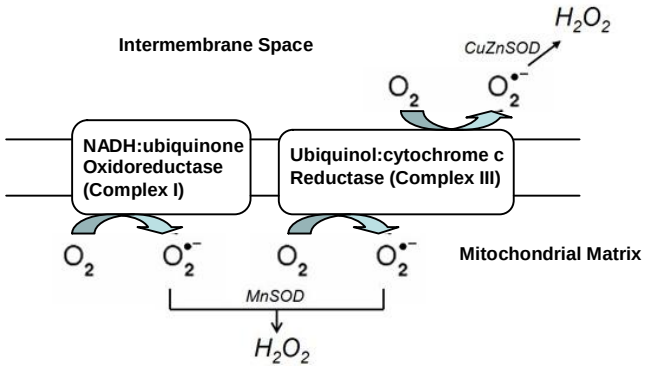


Figure 1. Hydrogen peroxide production by SODs.

In Trevigen's **Superoxide Dismutase Assay** ions, generated from the conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxidase (XOD), converts NBT to NBT-diformazan. NBT-diformazan absorbs light at 550 nm. SODs reduce superoxide ion concentrations and thereby lower the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBT-diforma-zan is a measure of SOD activity present in your experimental sample[5] (Fig. 2).

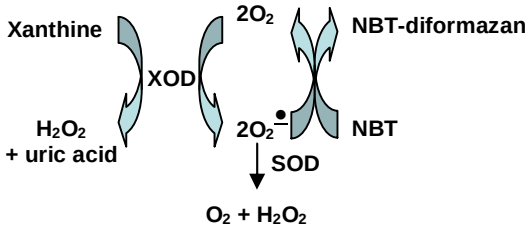


Figure 2. XOD and SOD cooperation in the inhibition of NBT-diformazan formation.

Trevigen's SOD assay is free of interference by other catalytic activities, and is ideal for assaying SOD in mammalian cell lysates. The kit contains the proper lysis buffer and the reagents needed for 100 experimental tests, 50 positive controls, and 50 negative controls. Unlike some other assay kits for SOD, this system is not greatly disturbed by trace metals. The assay is performed in as little as 6 minutes and relative SOD activity of the experimental sample is determined from percent inhibition of the rate of formation of NBT-diformazan. Furthermore, the assay is suitable for the assay of isozymes SOD1 (cytosolic Cu/Zn-SOD), SOD2 (mitochondrial Mn-SOD), and SOD3 (extracellular Cu/Zn-SOD).

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical and toxicological properties of the provided products may not yet have been fully investigated, therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.

III. Materials Supplied

Component	Quantity	Storage	Catalog #
SOD (0.5-1unit/µl)	200 µl	4 °C	7500-100-01
25X SOD Reaction Buffer	12.0 ml	4 °C	7500-100-02
Xanthine Solution	1.5 ml	4 °C	7500-100-03
NBT Solution	6.0 ml	4 °C	7500-100-04
XOD Solution	2.0 ml	4 °C	7500-100-05
20X Cell Lysis Solution	12.0 ml	4 °C	7500-100-06

IV. Reagents/Equipment Required But Not Supplied

Equipment

1. Visible spectrophotometer to read absorbance at 550 nm
2. Cuvettes (disposable or quartz, with at least a 1.5 ml volume)
3. Pipettor
4. Pipette tips
5. Pipette aid
6. Pasteur pipette and bulb
7. Centrifuge (for cell lysis)
8. Timer

Reagents

1. SOD Inhibitors or cells/tissue to be tested
2. 10X PBS (cat# 4870-500-6)
3. Distilled water
4. Protease inhibitors (optional, e.g. phenylmethylsulfonylfluoride (PMSF))
5. Reagents to determine protein concentration
6. Ficoll-Paque™ (erythrocyte, lymphocyte and monocyte preparations) (GE Healthcare cat# 17-1440-03).
7. Ethanol and Chloroform (erythrocyte preparation)

Disposables

1. 15 ml conical tubes (adherent and suspension cell preparation)
2. 50 ml conical tubes (tissue preparation)

V. Reagent Preparation

Prior to each experiment, prepare the necessary amount of 1X Cell Lysis Solution by diluting the 20X Cell Lysis Solution with dH_2O. All other reagents are ready for use. Store all components at 4 ^oC until needed and avoid contamination.

VI. Preparation of Cell and Tissue Extracts

Choose the appropriate protocol in Section A to Process Sample before proceeding to Section B: Please note that samples should be kept on ice to maintain enzyme activity.

Section A. Processing Samples

Suspension cells:

1. Centrifuge 2 to 6 x 10^6 suspension cells at 250 x g for 10 minutes at 4ºC. Discard the supernatant.
2. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to a 1.5 ml microtube on ice. Centrifuge, discard supernatant, and place on ice.
3. Proceed to Section B**.** Preparation of Cytosolic Extracts

Adherent cells:

1. Wash 2 to 6 x 10^6 adherent cells with 1X PBS. Adherent cells may be harvested by gentle trypsinization.
2. Transfer to a 15 ml tube on ice. Centrifuge at 250 x g for 10 minutes at 4ºC and discard the supernatant.
3. Suspend the cell pellet in 1 ml of ice-cold 1X PBS and transfer to 1.5 ml microtube on ice. Centrifuge, discard supernatant, and place on ice.
4. Proceed to Section B. Preparation of Cytosolic Extracts

Erythrocytes (Note: Mn- and Fe-SODs are inactivated by the recommended chloroform/EtOH extraction, however, hemoglobin or albumin can inhibit the generation of NBT-diformazan.[6]) For measurement of Cu/Zn-SOD:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Paque™ or similar reagent and centrifuge at 800 X g for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Remove the remaining liquid from above the red blood cell/leukocyte pellet. Wash the pellet with 10 cell volumes of PBS.
4. Determine the packed cell volume and add 10 cell volumes of cold dH_2O. Mix well and incubate on ice for 10-15 minutes to lyse the red blood cells. Lysis occurs when the opaque solution changes to a brilliant clear red solution, indicating the release of hemoglobin.
5. Precipitate the hemoglobin by adding 0.25 volumes of ethanol and 0.15 volumes of chloroform. Shake for 1 min and centrifuge at 10,000 x g for 10 minutes at 4˚C.
6. Recover the clear top layer and dialyze, using 6-8 kDa cut-off tubing, overnight at 4°C against 1X PBS or 50 mM Potassium Phosphate, pH 7.8.
7. Centrifuge the dialyzed erythrocyte extract to remove any precipitate that formed during the dialysis and place on ice.
8. Proceed to Section B. Preparation of Cytosolic Extracts

Lymphocytes and Monocytes:

1. Dilute anticoagulated blood with an equal volume of PBS. Layer over Ficoll-Paque™ or similar reagent and centrifuge at 800 X g for 25 min at 12°C with the BRAKE OFF in a swinging bucket rotor.
2. Collect the mononuclear cells (lymphocytes and monocytes) at the interphase and transfer to another tube.
3. Dilute the blood mononuclear cells with 5 volumes of PBS and centrifuge at 400 x g for 10 minutes at 4ºC. Discard the supernatant
4. Suspend the cell pellet in 1 ml of ice-cold **1X** PBS and transfer to a prechilled 1.5 ml microtube. Centrifuge, discard supernatant, and place on ice.
5. Proceed to Section B. Preparation of Cytosolic Extracts.

Tissue:

1. Remove tissue and place in cold PBS in a 50 ml conical tube. Repeatedly wash the tissue with PBS to remove blood clots and other debris.
2. Transfer the tissue to a Petri dish on ice and mince the tissue to small pieces with surgical scissors.
3. Transfer the tissue pieces to a clean stainless steel sieve. Place the sieve with the tissue pieces in a Petri dish which contains about 20 ml of cold 1X PBS.
4. Create a single cell suspension of the tissue as follows: Using a pestle or a round bottom tube, grind the tissue pieces thoroughly until the bulk of the tissue passes through the sieve.
5. Transfer the PBS containing the single cell suspension to a 50 ml conical tube. Fill with cold PBS and mix by inverting the tube several times. Let the tube stand on ice for 1 minute to allow large aggregates of tissue to settle out of solution.
6. Carefully transfer the supernatant containing the single cell suspension to a clean 50 ml conical centrifuge tube. Centrifuge at 400 x g for 10 minutes at 4ºC. Discard the supernatant. Suspend the cell pellet in 1 ml of ice-cold **1X** PBS and transfer to a pre-chilled 1.5 ml microtube on ice. Centrifuge, discard the supernatant, and place on ice.
7. Proceed to Section B: Preparation of Cytosolic Extracts

Section B. Preparation of Cytosolic Extracts from Cells and Tissue

1. Measure the approximate volume of the cell pellets prepared above (except for erythrocytes) and suspend the cells in 5-10 volumes of cold 1X Cell Lysis Solution. Incubate the cell suspensions on ice, with periodic vortexing, for 30 minutes.
2. Microcentrifuge the disrupted cell suspension at 10,000 x g for 10 minutes at 4ºC to remove insoluble material. Recover the supernatant to a fresh tube prechilled on ice. Occasionally, the pellet may float and can easily be removed with a pipet tip.
3. Determine the protein concentration of the cleared cell lysate.
4. If not assaying for SOD immediately, snap-freeze the cleared cell extract in 100 µl aliquots by immersing in liquid nitrogen and store at -80ºC. Avoid repeated freezing and thawing of the extract.
5. The detection of SOD in subcellular fractions is detailed in reference [7].

Section C. Differentiation between Mn- Fe- and Cu/Zn-SOD activity

1. Mn- and Fe-SODs can be inactivated by adding 400 µl or 800 µl of ice-cold chloroform/ethanol (37.5/62.5 (v/v)) to 250 µl of erythrocyte lysate or 500 µl of cell/tissue lysate, respectively, shaking for 30 sec, and then centrifuging at 2,500 x g for 10 min. Assay the upper aqueous phase for Cu/Zn-SOD immediately or freeze in aliquots at -80°C.

2. The addition of cyanide ion to a final concentration of 2 mM inhibits more than 90% of SOD1 activity. SOD2 is unaffected by cyanide.

3. SOD3 (extracellular Cu/Zn-SOD) is isolated from the extracellular matrix of tissue. SOD3 has been found in serum and in cerebrospinal, ascitic, and synovial fluids. Ensure that all cells are removed from the extracellular fluid by centrifuging at 250 x g for 10 minutes at 4ºC. Assay the supernatant for SOD3 activity.

VII. Assay Protocol

1. The assay is performed at room temperature. All components, except cell lysates, should be brought to room temperature before use. The total reaction volume is 1.5 ml. The volume of the reagent components is 107.5 µl. Therefore, the volume of deionized water required is: 1500 µl – 107.5 µl – vol. sample. Briefly vortex each reagent immediately before use.

2. To a disposable cuvette add the following components in order:

dH_20	From Step 1
25 x Reaction Buffer	60 µl
Xanthine Solution	7.5 µl

3. Mix thoroughly by repeated pipetting with a clean Pasteur pipette.

4. Add 30 µl of NBT Solution and repeat Step 3.

5. Add your cell lysate and repeat Step 3.

6. Place the cuvette into a spectrophotometer, read absorbance at 550 nm or set the absorbance reading to zero.

7. Just before use, briefly vortex the Xanthine Oxidase (XOD) Solution and add 10 µl to the cuvette. Quickly repeat Step 3.

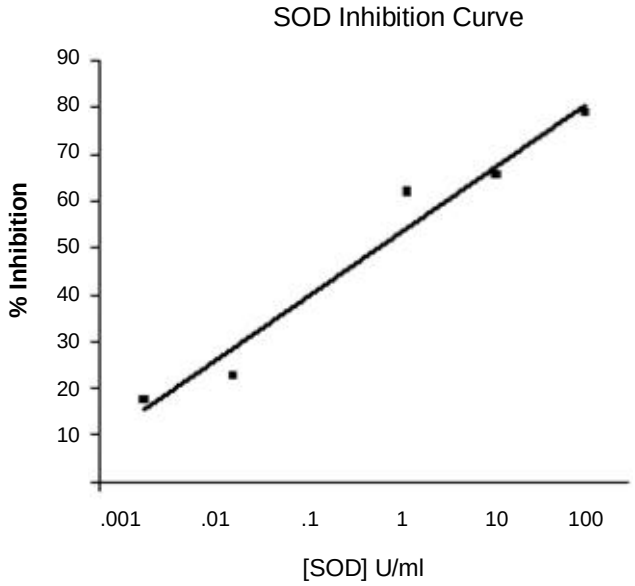
8. Immediately place the cuvette in the spectrophotometer, start a timer or stopwatch and record the absorbance reading every 30 seconds for 5 minutes. The first time point will be at 30 seconds and the final time point will be at 5 minutes 30 seconds.

A. Controls

1. Negative Control
   A negative control must be performed. It includes all components except SOD or cell lysate. In this case, the increase in absorbance due to generation of superoxide radical proceeds maximally.

2. Positive Control
   The kit contains sufficient SOD for generating 50 individual positive controls. The SOD has an activity of 0.5-1 unit/µl (1 unit is the amount of SOD which inhibits the rate of increase in absorbance due to NBT-diformazan formation by 50%). A typical standard curve would include the following SOD concentrations: 0.01 unit, 0.1 unit, 1 unit, and 10 units. For the 0.01 and 0.1 unit points, dilute ~1 µl of SOD to 100 µl and 10 µl, respectively, with 1 x Reaction Buffer and use 1 µl of each. Add ~1 µl and ~10 µl of undiluted SOD for the 1 unit and 10 unit activity points, respectively. Briefly vortex the SOD immediately before use.

Figure 3: Plot of SOD concentration vs. % inhibition of the rate of increase of absorbance at 550nm due to the reduction of NBT to NBT-diformazan by the superoxide radical ($O_2^{\bullet -}$).



VIII. Data Interpretation and SOD Activity Determination

1. Determine the rate of increase in absorbance units (A) per minute for the negative control and for the test sample(s).

$$\frac{A_{550}\ @\ 5min.30\ sec. - A_{550}\ @\ 30\ sec.}{5\ min} = \Delta\ A_{550n}/minute$$

2. Determine the % inhibition for the test sample(s):

$$\frac{[(\Delta\ A_{550n}/minute)_{negative\ control} - (\Delta\ A_{550n}/minute)_{test}]}{(\Delta\ A_{550n}/minute)_{negative\ control}} \times 100 = \%\ Inhibition$$

IX. References

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X. Related Products Available From Trevigen

Contact Trevigen for details of our unique product line for studying DNA damage and repair. All of Trevigen's kits include highly qualified enzymes, substrates, buffers, full instructions for use, and a synopsis specific for your kit.

PARP Assay Kits:

Catalog #	Description	Size
4520-096-K	**HT PARP in vivo Pharmacodynamic Assay II**	96 samples
4684-096-K	**HT Colorimetric PARP/Apoptosis Assay**	96 samples
4685-096-K	**HT Chemiluminescent PARP/Apoptosis Assay**	96 samples
4690-096-K	**HT F Homogeneous PARP Inhibition Assay Kit**	96 tests
4667-250-01	**Recombinant Human PARP Enzyme**	250 µl
4668-100-1	**Recombinant Human PARP (High Specific Activity)**	1000 Units

DNA Damage Antibodies:

Catalog #	Description	Size
4410-PC-100	**Fen-1**	100 µl
4411-PC-100	**γ-H2AX**	100 µl
4350-MC-100	**UVssDNA**	100 µg
4354-MC-050	**anti-8-oxo-dG**	50 µl

CometAssay®:

Catalog #	Description	Size
4250-050-K	**CometAssay® Kit**	**50 samples**
4251-050-K	**CometAssay® Silver Kit**	**50 samples**
4252-040-K	**CometAssay® Higher Throughput Kit**	**40 samples**
4253-096-K	**CometAssay® Kit 96 Wells**	**96 samples**
4254-200-K	**CometAssay® Silver Staining Kit**	**200 samples**
4256-010-CC	**CometAssay® Control Cells**	**10 assays**
4250-050-ES	**CometAssay® Electrophoresis System (ES)**	**each**

FLARE™ Assay Kits:

Catalog #	Description	Damage Recognized	Size
4040-100-FK **4040-100-FM**	Fpg Kit	**8-oxoguanine, DNA containing formamidopyrimidine moieties**	75 samples 100 samples
4045-01K-FK **4045-01K-FM**	Endonuclease III Kit	**Thymine Glycol, 5,6-dihydrothymine, urea, 5-hydroxy-6-hydrothymine, 5,6-dihydrouracil, alloxan, 5-hydroxy-6-hydrouracil, uracil glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxycytosine,5-hydroxyuracil, methyltartonylurea**	75 samples 100 samples
4130-100-FK **4130-100-FM**	hOGG1 Kit	**8-oxoguanine, DNA containing formamidopyrimidine moieties**	75 samples 100 samples
4055-100-FK **4055-100-FM**	T4-PDG Kit	**Cis-syn isomers of cyclobutane pyrimidine dimers**	75 samples 100 samples
4065-100-FK **4065-100-FM**	cv-PDG Kit	**Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers**	75 samples 100 samples
4100-100-FK **4100-100-FM**	UVDE Kit	**Cyclobutane pyrimidine dimers, (6-4) photoproducts**	75 samples 100 samples

Oxidative Damage Kits

Catalog #	Description	Size
7510-100-K	**Glutathione Reductase Assay**	100 Reactions
7513-500-K	**HT Glutathione Reductase Assay Kit**	500 tests
7511-100-K	**HT Glutathione Assay Kit**	384 tests
7512-100-K	**HT Glutathione Peroxidase Assay Kit**	480 tests
7501-500-K	**HT Superoxide Dismutase Assay Kit**	500 tests
4370-096-K	**HT 8-oxo-dG ELISA Kit**	96 Samples