

Product Information

Superoxide Anion Assay Kit

Catalog Number **CS1000**

Storage Temperature 2–8 °C

TECHNICAL BULLETIN

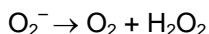
Product Description

The superoxide anion (O_2^-) is a short-lived radical generated by the addition of an electron to oxygen. It is formed in response to environmental factors such as UV light, cigarette smoke, environmental pollutants, and γ -radiation, or by oxidases like xanthine oxidase or NADPH oxidase. Once formed, O_2^- attacks cellular components causing damage to lipids, proteins, and DNA. This can initiate numerous diseases, including cancer, atherosclerosis, rheumatoid arthritis, diabetes, liver damage, and central nervous system disorders.

Superoxide anions play a key role in the immune system, protecting the animal from infectious organisms. The highly reactive O_2^- anion is released by stimulated leukocytes including monocytes, macrophages, and polymorphonuclear leukocytes. In immune cells, the superoxide anion is produced by the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.^{1,2}



The enzyme superoxide dismutase (SOD) suppresses the activity of the superoxide anion by converting O_2^- to hydrogen peroxide.



The Superoxide Anion Assay Kit provides a useful qualitative cell-based assay for the measurement of O_2^- anion status in cells. The kit can be used to test changes in superoxide anion levels following oxidative stress directly in whole cells. In addition, the kit can be also used for the detection of superoxide dismutase (SOD) activity. This activity was tested on erythrocytes, leucocytes, and plasma.

The kit method is based on the oxidation of luminol by superoxide anions resulting in the formation of chemiluminescence light.³⁻⁵ This method utilizes a specific, non-toxic enhancer that amplifies the chemiluminescent signal.

The kit includes a superoxide anion producing system (xanthine/xanthine oxidase) for a positive control and the superoxide dismutase enzyme for the repression of the system (a negative control).

The kit was tested on U937 cells and human leucocytes.

Components

The kit is sufficient for 100 assays in 96 well plates.

Assay Buffer Catalog Number A5980	25 ml
Assay Medium Catalog Number A6105	20 ml
Enhancer Solution 40 mM solution Catalog Number E4281	500 μ l
Luminol Solution Catalog Number L5043	500 μ l
Xanthine Solution Catalog Number X0254	500 μ l
Xanthine Oxidase from bovine milk Catalog Number X4878	0.5 units
Superoxide Dismutase from bovine liver (4 units/ μ l) Catalog Number S6696	400 units

Reagents and Equipment Required but Not Provided

- Phorbol 12-myristate 13-acetate (PMA, Catalog Number P8139) or any other suitable stimulator for the tested cells
- Nunc-Immuno™ MicroWell™ 96 well polystyrene plates, white (Catalog Number P8616)
- Human Interferon- γ (Catalog Number I3265) for the activation of U937 cells
- Dimethyl sulfoxide (DMSO, Catalog Number D8418 or equivalent)
- Sodium chloride (Catalog Number S3014 or equivalent)
- Supplement growth medium
- Luminometer
- Multichannel pipette

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

PMA Solutions – PMA (Catalog Number P8139, not provided) is required in order to stimulate U937 cells to produce superoxide anions. Stimulating other cells (e.g., polymorphonuclear neutrophils or mononuclear cells) or cell lines to produce superoxide anions may require the use of other inducers (e.g., fMLP, zymozan opsonized particles, or target cells⁶) and a specific protocol according to the literature.

- Prepare a 1 mg/ml PMA Stock Solution in DMSO. Vortex vigorously until the PMA completely dissolves. Store at -20°C .
- Prepare a 20 $\mu\text{g}/\text{ml}$ PMA Working Solution by diluting the PMA Stock Solution (1 mg/ml) 50-fold in 150 mM NaCl solution. Vortex the diluted PMA solution. Store at room temperature and use within 1 hour.

Xanthine Oxidase Working Solution – If needed, dilute an aliquot of the Xanthine Oxidase (Catalog Number X4878) with Assay Buffer (Catalog Number A5980) to obtain a 2 unit/ml solution. 2 μl of the Xanthine Oxidase Working Solution are required for each Xanthine/Xanthine Oxidase activity assay. Mix well by vortex. Keep on ice.

Xanthine Working Solution – Dilute an aliquot of the Xanthine Solution (Catalog Number X0254) 20-fold with Assay Buffer (Catalog Number A5980). 100 μl of the Xanthine Working Solution are required for each Xanthine/Xanthine Oxidase activity assay. Mix well by vortex. Keep on ice.

Storage/Stability

The kit is shipped on wet ice and storage at $2-8^{\circ}\text{C}$ is recommended.

Procedure

A. Preparation of U937 Cells

The following procedure is for U937 cells. The cells are first activated by human interferon- γ and then stimulated to produce superoxide anion with PMA.

1. Seed $\sim 1.0 \times 10^7$ cells (i.e., 20 ml of $\sim 5 \times 10^5$ cells/ml) in growth medium (RPMI-1640 supplemented with 10% FCS).
2. Add 40 units/ml of human interferon- γ to the flask.
3. Grow the cells for 3–5 days. After 2 days replace the growth medium with a growth medium containing human interferon- γ (40 units/ml).
4. Collect the cells in a conical tube and centrifuge at $3,000 \times g$ for 5 minutes. Remove the supernatant.
5. Resuspend the cells in 1 ml of fresh growth medium. Count the number of cells in the cell suspension.
6. Incubate the cells for 30 minutes at 37°C . Centrifuge at $3,000 \times g$ for 5 minutes. Remove the supernatant.
7. Resuspend the cells in the Assay Medium (Catalog Number A6105) to a concentration of 5.0×10^5 to 1.0×10^6 cells/100 μl . Store on ice until use (no longer than 1 hour).

B. Assay for Superoxide Anions in U937 Cells

Work in duplicates. Store all solutions provided on ice while preparing the reaction mixtures in the plate according to Table 1. The reaction is initiated by the addition of the activated cells.

Table 1.

Reaction Scheme for Superoxide Anion Detection in U937 cells

Component	Assay Buffer (A5980) μl	Luminol Solution (L5034) μl	Enhancer Solution (E4281) μl *	PMA Working Solution μl **	SOD (S6696) μl
Test (U937 Cells)	89	5	5	1	—
Control No PMA	90	5	5	—	—
Control No Enhancer	94	5	—	1	—
Control + SOD	88	5	5	1	1

* The effective final concentration of the enhancer in the well can vary from 0.1–2.5 mM depending on the cells and luminometer used. The enhancer concentration may require a titration within this range, to optimize the chemiluminescence measurement.

** This stimulating agent is suitable for stimulation of U937 cells. *Other cell lines may require a different superoxide anion production stimulator.*

1. Set the luminometer for the appropriate sensitivity according to the luminometer used.
2. Add the reaction components to the 96 well plate according to Table 1 and mix using a horizontal shaker or by pipetting. Avoid introducing bubbles into the sample during pipetting.
3. Start the reaction by adding to each well 100 μl of cell suspension in Assay Medium (5.0×10^5 to 1.0×10^6 cells/well).
4. Measure the luminescence intensity every 5–10 minutes during a 4-hour period.

C. Assay of Superoxide Anion Production by the Xanthine/Xanthine Oxidase System

This system serves as a positive control for the superoxide anion measurement using a superoxide anion producing system, Xanthine/Xanthine Oxidase. This assay system can also be used for superoxide dismutase (SOD) activity measurement in biological samples.

Table 2.

Reaction Scheme for the Xanthine/Xanthine Oxidase System

Sample*	Component	Assay Buffer (A5980) μl	Luminol Solution (L5034) μl	Enhancer Solution (E4281) μl	Xanthine Oxidase Working Solution μl	SOD 4 units/μl (S6696) Or SOD containing supernatant μl **
1	Positive control Xanthine/Xanthine Oxidase system	88	5	5	2	—
2	Negative Control – No Enhancer	93	5	—	2	—
3	Negative Control – No enzyme	90	5	5	—	—
4	SOD	87	5	5	2	1 (SOD)
5	SOD test - SOD containing supernatant	88-X	5	5	2	X (1-10 μl) SOD containing supernatant

* Samples 1–4 in Table 2 are controls for the superoxide anion measurement. For the detection of SOD activity in biological samples, the Xanthine/Xanthine Oxidase system serves as the superoxide producing system. The superoxide anion produced is the substrate for the SOD tested. In this case the experiment set includes a fifth sample, which is the SOD containing supernatant.

** For assays for the detection of SOD activity in biological samples it is recommended to add a sample in which the SOD positive control (Catalog Number S6696) is diluted 10 to 20-fold in assay buffer. In this sample the superoxide anion will not be totally converted to H_2O_2 and thus be measurable.

1. Set the luminometer for the appropriate sensitivity according to the luminometer used.
2. Add the reaction components to the 96 well plate according to Table 2 and mix using a horizontal shaker or by pipetting. Avoid introducing bubbles into the sample during pipetting.

- Start the reaction by adding 100 μ l of the Xanthine Working Solution to each well to initiate the reaction. It is recommended to use a multichannel pipette in order to have a simultaneous initiation of the reaction in all wells, since the superoxide anion production is immediate.
- Immediately** measure the luminescence intensity for 3–5 minutes at 30 seconds intervals.

Results

Figure 1.

Superoxide Anion Production by U937 cells

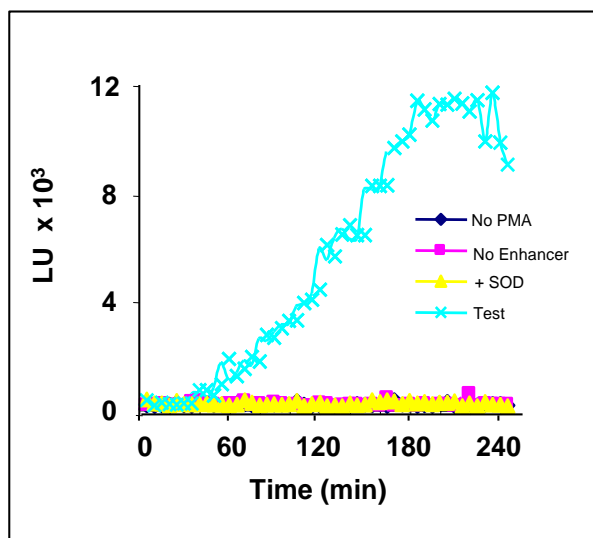
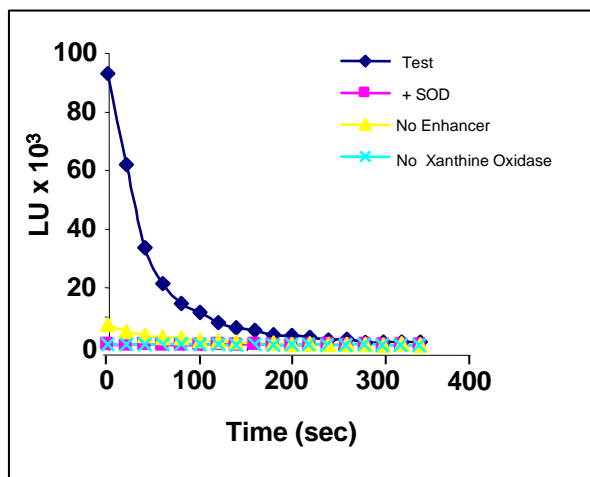


Figure 2.

Superoxide Anion Production by the Xanthine/Xanthine Oxidase System



In the presence of SOD the superoxide anion produced is immediately converted to hydrogen peroxide and therefore, there is no enhancement in the luminescence intensity.

The units of SOD provided with this kit are determined according to Sigma's unit definition: One unit will inhibit reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 at 25 °C in a 3.0 ml reaction volume. Xanthine oxidase concentration should produce an initial ΔA_{550} of 0.025 ± 0.005 per minute.

References

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