ABEL® SUPEROXIDE and SUPEROXIDE DISMUTASE QUANTIFICATION KIT with PHOLASIN® XANTHINE and XANTHINE OXIDASE

Microplate Test Kit

ABEL-60M

The ABEL 61 series are chemiluminescent test kits for quantifying low levels of superoxide produced as a by product of the production of uric acid from the enzyme catalysed oxidation of xanthine with xanthine oxidase. The assay can be used to quantify the superoxide produced by cells as well as for assessing the antioxidant capacity of therapeutic reagents and ingredients in foods, nutraceuticals and cosmetics. The activity of superoxide dismutases and mimetics of this enzyme can be quantified as well as inhibitors of xanthine oxidase

CONTENTS

Kit components sufficient for 100 x 200 mL tests

1 x bottles 50 µg Pholasin® (reconstitute to 5 mL)

1 x 50 mL Reconstitution & Assay Buffer for xanthine oxidase.

1 x 10 mL Reconstitution Buffer for xanthine

1 x bottles of xanthine (reconstitute with 2.5mL buffer to obtain 16 mM)

1 bottles xanthine oxidase: 51.25 mU

1 bottles superoxide dismutase: 125 U

1 x 96 well white microplates

STORAGE CONDITIONS AND SHELF LIFE

REAGENT	FORMAT	TEMPERATURE	SHELF LIFE
PHOLASIN®	Freeze Dried	-20°C or lower	12 months
	Reconstituted	-20°C or lower	1 month
RECONSTITUTION & ASSAY BUFFER: XANTHINE OXIDASE	Liquid	-20°C or lower	12 months
		2-8°C	1 month
RECONSTITUTION BUFFER: XANTHINE	Freeze Dried	-20°C or lower	12 months
	Reconstituted	2-8°C	1 month
XANTHINE OXIDASE	Freeze Dried	-20°C or lower & dry	12 months
	Reconstituted	Discard any remaining	DISCARD
XANTHINE	Freeze Dried	-20°C or lower	12 months
	Reconstituted	2-8°C	7 days
SUPEROXIDE DISMUTASE	Freeze Dried	-20°C or lower	12 months
	Reconstituted	-20°C or lower	1 month

If any packs are damaged or bottles appear to have leaked, do not use the items, but contact your supplier for advice.

This kit is supplied for research use only. Responsibility will not be accepted for misuse of the kit components. This kit contains glass items, and the use of sharps is recommended; these should be handled with due care and disposed of correctly according to good laboratory practice.

This kit contains sufficient reagents for 100 tests of 200 mL each.

INTRODUCTION

The ABEL 61 series are chemiluminescent test kits for quantifying superoxide (anion radical O2·-) produced as a by product of the production of uric acid from the enzyme reaction of xanthine with xanthine oxidase and for use as an antioxidant test and for measuring the activity of superoxide dismutase (SOD) The assay can be used to quantify the superoxide produced by cells as well as assessing the antioxidant capacity of therapeutic reagents and ingredients in foods, nutraceuticals and cosmetics.

One unit of xanthine oxidase catalyses the oxidation of 1µmol xanthine to uric acid with the concomitant production of 2µmol superoxide per minute at 250C with the rate of reaction doubling for approximately every 100C increase in temperature

Pholasin®, an ultrasensitive chemiluminescent detector of superoxide can detect concentrations in this assay as low as 50 fmol per minute, which is in the order produced by small numbers of cells. Higher amounts of superoxide can also be generated for use in antioxidant and other assays.

This assay has many advantages over the non-specific indirect cytochrome C method.

The activity of superoxide dismutases and mimetics of this enzyme can be quantified very easily. As SOD will compete with Pholasin® for any superoxide produced in x/xo system less light will be emitted in the presence of SOD. From a set of SOD standards the amount of SOD or a mimetic of SOD in a sample to be tested can be determined by the amount of light emitted in the presence of Pholasin®

Assay with Superoxide Dismutase Standards.

1. Pipette into each well of a microplate:
   - 10 µL assay buffer + SOD*
   - 50µL reconstituted Pholasin®
   - 20µL of reconstituted xanthine

* Adjust SOD volume for desired concentration (see tables).
For example 20 µL SOD + 90 mL of assay buffer = 100 mL total

2. Inject into each well 20µL of xanthine oxidase

Table High Range. These concentrations of superoxide dismutase should be used with 2 nmol/minute of Superoxide.

25 U/mL Superoxide Dismutase (µL added)	R&A XO Buffer (µL added)	Superoxide Dismutase in 200µL (mU)
2	108	50
4	106	100
10	100	250
20	90	500
40	70	1000

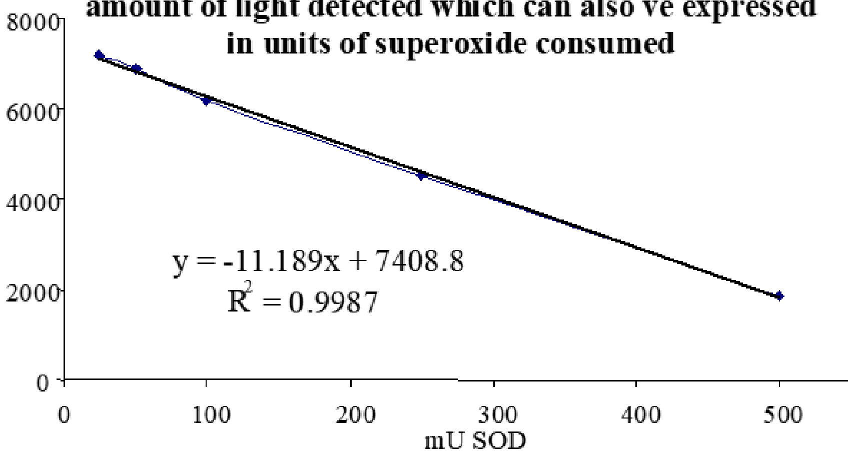
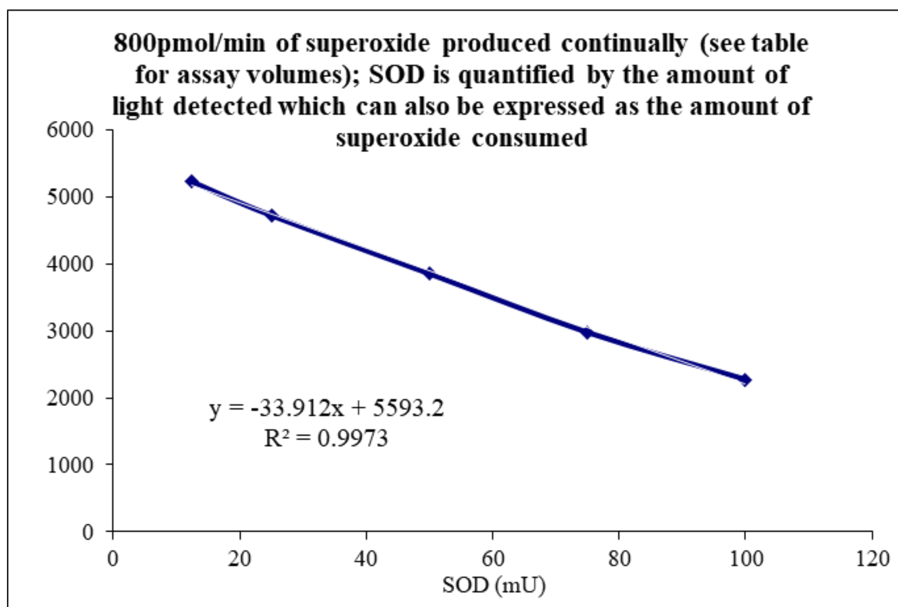
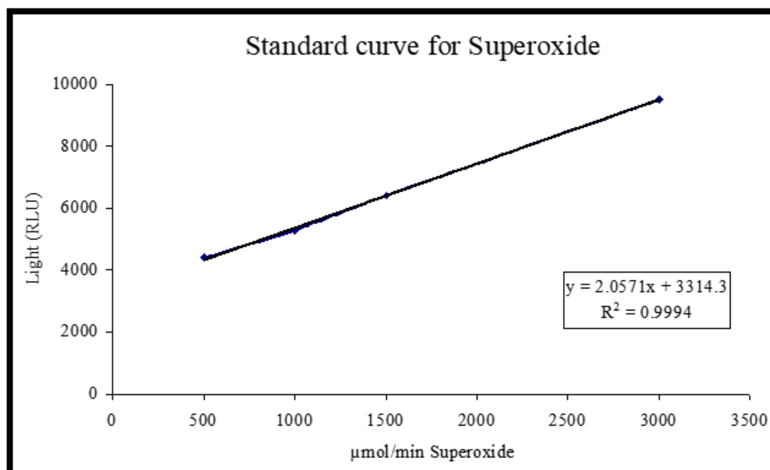


Table Mid Range SOD. These concentrations of superoxide dismutase should be used with 800 pmol/minute of superoxide

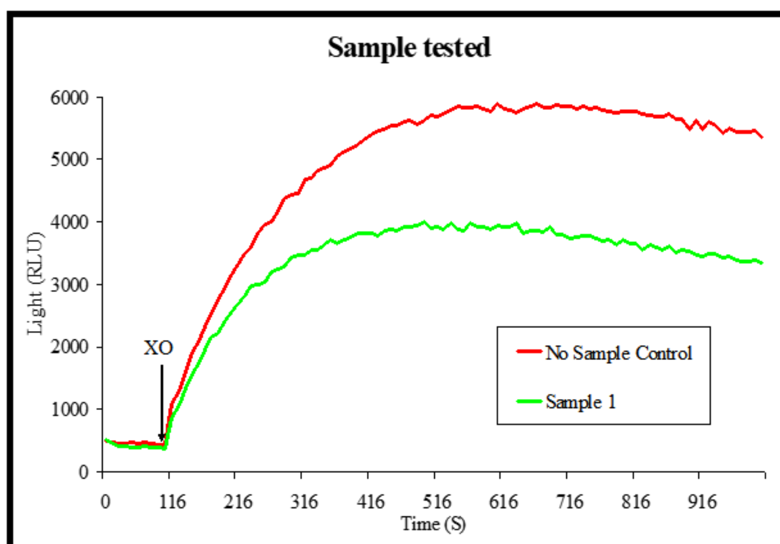
2.5U/mL Superoxide Dismutase (µL added)	R&A XO Buffer (µL added)	Concentration of Superoxide Dismutase (mU/mL)	Amount of Superoxide Dismutase in 200µL microplate well (mU)
5	105	62.5	12.5
10	100	125	25
20	90	250	50
30	80	375	75
40	70	500	100





APPLICATIONS OF THE ASSAY

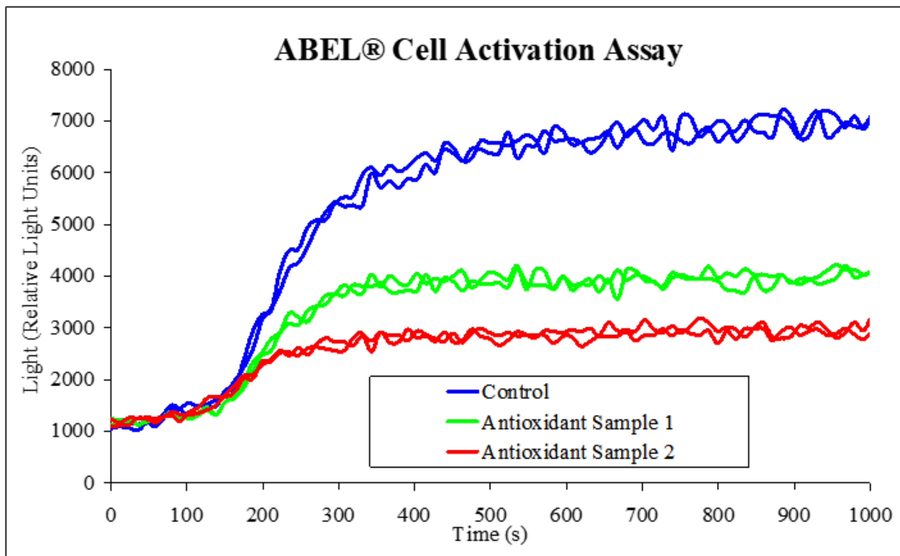
The antioxidant capacity of a sample can be expressed by the amount of superoxide it quenches.



Samples	Maximum light Relative Light Units (RLU)	Superoxide measured µmol/min	Superoxide quenched µmol/ min
No Sample Con-	6000	1310	------
Antioxidant sam-	4000	330	980

Quantifying Superoxide Produced by Living Cells

Superoxide is produced in cells containing the NADPH oxidase system after activation of this system with substances such as phorbol-12-myristate-13-acetate (PMA). See **ABEL® Cell Activation Assay** which can be used to measure the respiratory burst produced of leucocytes from as little as 0.2µL of blood. The system can be used as an antioxidant test in which superoxide is produced by living cells instead of xanthine oxidase and xanthine.



Samples	Max Light (RLU)	Superoxide µmol/min	Intercept	Gradient
Control	7200	**2200**	2747.5	2021.2
Antioxidant sample 1	4200	**720**	2747.5	2021.2
Antioxidant sample 2	3400	**320**	2747.5	2021.2

1. Pipette into each well of a microplate:
   - 100µL assay buffer + 10µL sample
   - 50µL reconstituted Pholasin®
   - 20µL of reconstituted xanthine
2. Inject into each well 20 µL of xanthine oxidase into each well, preferably with an automatic dispenser. Alternatively, add xanthine oxidase very quickly before putting the plate in the luminometer. [Tip: a delay in injection of xanthine oxidase allows the basal chemiluminescence to be determined.] .
3. Measure light for 0.5 to 1.0 second in each well; measure each well in turn (plate mode). It is recommended that a maximum of 24 wells be measured at one assay.
4. Run assay for a total of 30 minutes in the first instance; this time may be reduced (or extended) once the time to reach maximum velocity is identified. Cycle times should be kept to a minimum.
5. When the assay is run at 37°C instead of 25°C the total assay time can be reduced.

PRODUCTION OF SUPEROXIDE

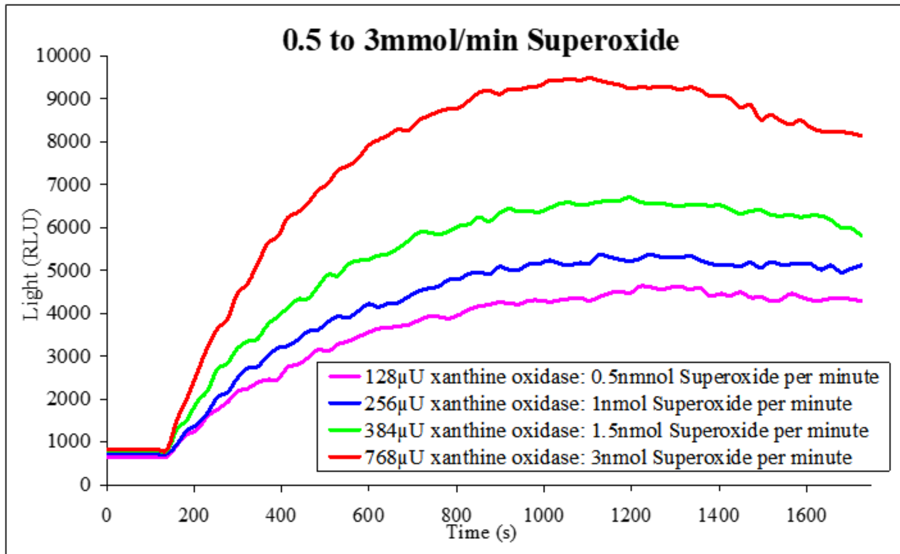
When Pholasin® and xanthine are in excess the amount of superoxide generated per minute can be determined by the amount of xanthine oxidase used in the assay. See chart below

Volumes of xanthine oxidase and superoxide produced

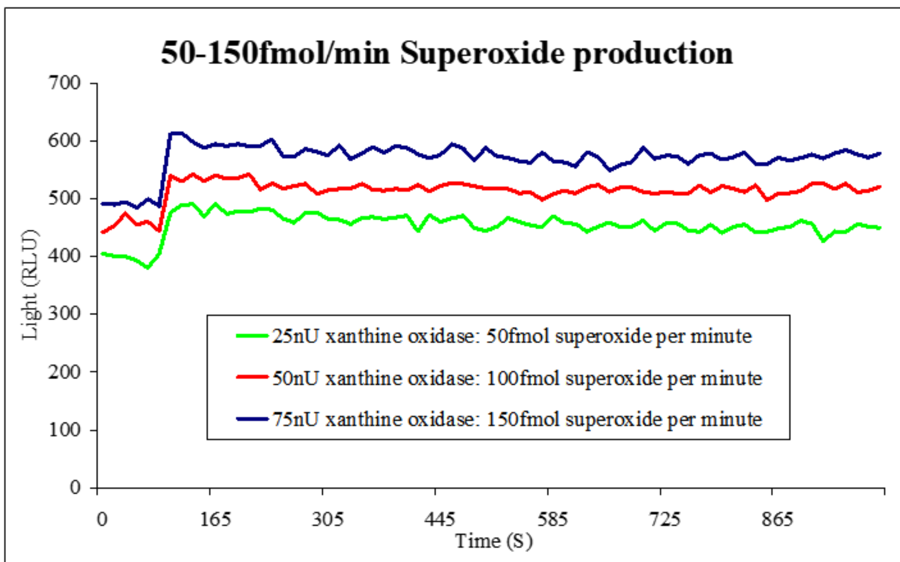
Volume 10.25 mU/mL xanthine oxidase (µL added)	Equivalent Units XO µU of xanthine oxidase (200µL well)	Superoxide (nmol/min)
50	512.5	2
40	410.0	1.6
30	307.5	1.2
20	205.0	0.8
10	102.5	0.4

SUPEROXIDE QUANTIFICATION

The concentration of xanthine oxidase used in the assay can be used to quantify the rate of superoxide production.



The assay is extremely sensitive to superoxide production and levels as low as 50fmol per minute have been measured



PROTOCOL

Reconstitution of Pholasin®

The Pholasin® (50µg) must be reconstituted with 5mL of Reconstitution & Assay Buffer supplied for the Xanthine Oxidase Assay (R&A XO buffer).

1. Load a syringe with 5mL R&A XO buffer
2. Fit a needle (1 inch, 21 gauge; 0.8 x 25mm) to the syringe.
3. Remove the protective screw cap from the vial of Pholasin® making sure to leave the rubber insert in place. Carefully push the needle through the rubber septum. There is no need to push the plunger because as soon as the septum is pierced, the syringe contents will be drawn automatically into the vial. However, be sure that the syringe is emptied completely.
4. Replace the screw cap and invert and roll the bottle at least 5 times to dissolve the contents.
5. Reconstituted Pholasin® with xanthine oxidase reconstitution and assay buffer can be stored frozen at –20°C. After reconstitution remove sufficient for use on a particular day and freeze remaining volume.

Reconstitution of Xanthine

Xanthine reconstituted with 2.5mL Buffer for Dissolving Xanthine produces a 16mM solution; reconstitution with 1mL produces 40mM

1. Load a syringe with 2.5mL (or less) of Buffer for Dissolving Xanthine1
2. Fit a needle (1 inch, 21 gauge; 0.8 x 25mm) to the syringe.
3. Remove the protective screw cap from the vial of xanthine making sure to leave the rubber insert in place. Carefully push the needle through the rubber septum. There is no need to push the plunger because as soon as the septum is pierced, the syringe contents will be drawn automatically into the vial. However, be sure that the syringe is emptied completely.
4. Replace the screw cap and invert and roll the bottle at least 5 times to dissolve the contents.
5. Reconstituted xanthine can only be used for a maximum of 7 days.

Reconstitution of Xanthine Oxidase

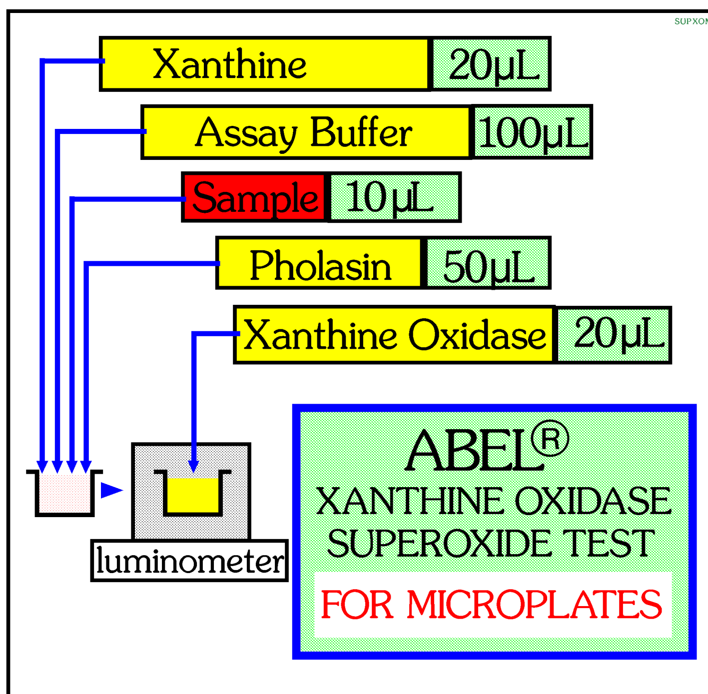
The vial contains 51.25 mU of Xanthine Oxidase. When reconstituted with 5 mL R&A XO buffer 10.25 mU/mL solution is obtained. For reconstitution of XO, use the procedure as described for Pholasin® above. Discard any unused product.

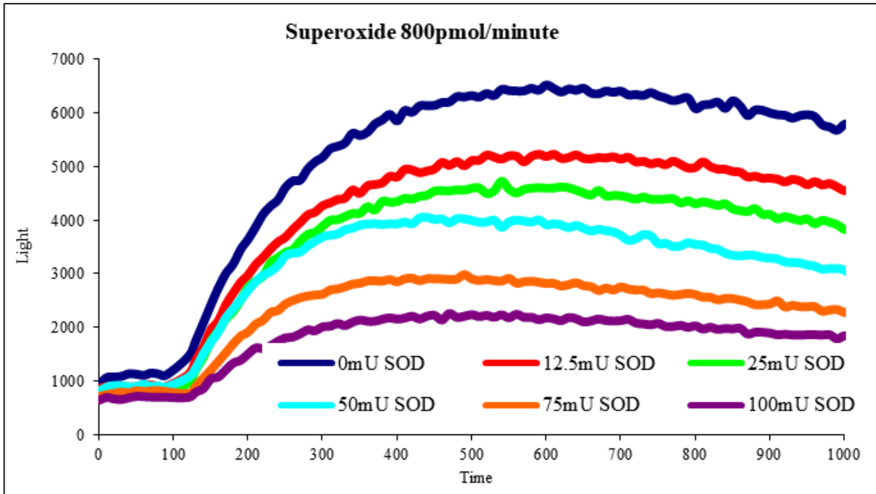
Reconstitution of Superoxide Dismutase (SOD)

One unit of activity is defined as the amount of SOD that inhibits cytochrome C reduction by 50% under specified assay conditions

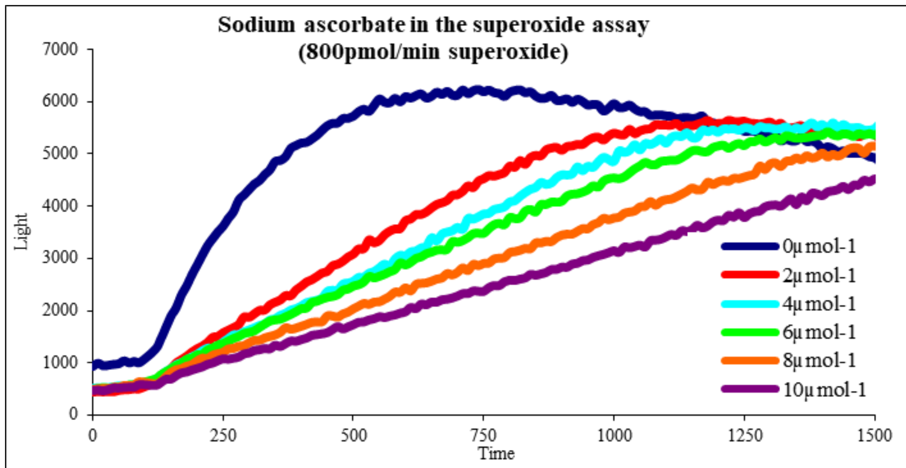
1. Each bottle of freeze dried SOD contains 125 units.
2. Reconstitute the freeze dried SOD (125 units) with 5 mL Reconstitution and Assay buffer for xanthine oxidase by injecting 5 mL buffer through the rubber septum using the same procedure as described for the other reagents.
3. When reconstituted with 5 mL of R&A xanthine oxidase buffer a solution containing 25 unit/mL is obtained.

TEST PROCEDURE





Light measured in the luminometer with the xanthine/xanthine oxidase system, when SOD of different concentrations (12.5mU-62.5mU) had been added to the microplate well before the assay.



Sodium ascorbate is a non enzyme antioxidant; it contrasts enzyme antioxidants such as SOD and SOD mimetics by being consumed in the assay. A feature of consumed vs enzyme activity is a linear increase in light compared to a steady light emission in the enzyme reaction.

Analysis By
ABEL
Emitted Light

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