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ABEL® SUPEROXIDE and SUPEROXIDE DISMUTASE Quantification Kit with PHOLASIN® XANTHINE and XANTHINE OXIDASE

Microplate Test Kit
ABEL-60M

The ABEL 60 series are chemiluminescent test kits for quantifying low levels of superoxide produced as a by product in 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 200µL tests

A. 1 x bottles 50µg Pholasin® (reconstitute to 5 mL)
B. 1 x 50mL Reconstitution & Assay Buffer for xanthine oxidase.
C. 1 x 10mL Reconstitution Buffer for xanthine
D. 1 x bottles of xanthine
(reconstitute with 2.5mL buffer to obtain 16mM)
A. 1 x bottles xanthine oxidase: 51.25mU
B. 1 x bottles superoxide dismutase: 125 U
C. 1 x 96 well white microplates

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 µL each.

INTRODUCTION

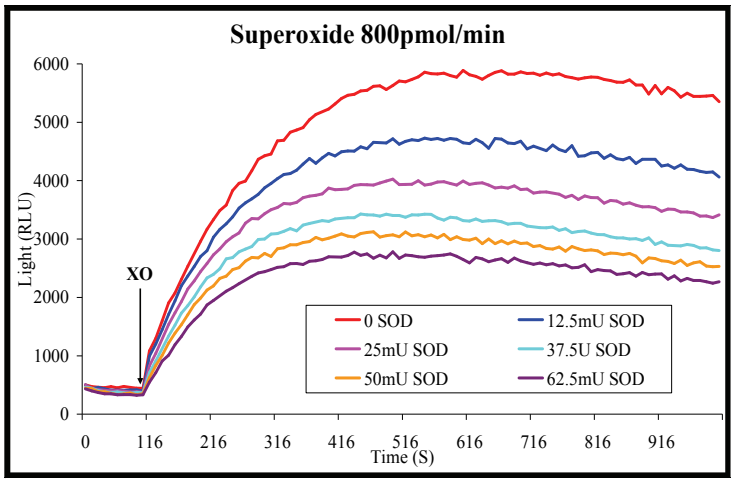
The ABEL 61 series are chemiluminescent test kits for quantifying superoxide (anion radical $0_2^{\cdot-}$) produced as a by product of the production of uric acid from the enzyme reaction of xanthine with xanthine oxidase and for use as an antioxidant test and for measuring the activity of superoxide 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 25^0C with the rate of reaction doubling for approximately every 10^0C increase in temperature

Pholasin®, an ultrasensitve chemiluminescent detector of superoxide can detect concentrations in this assay as low as 50fmol 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®



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.

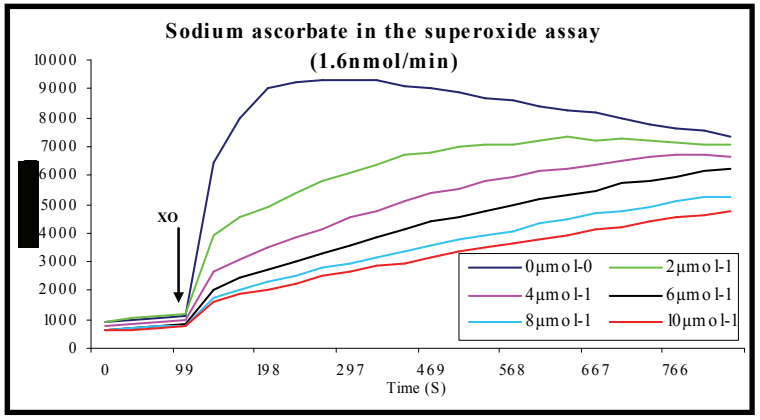
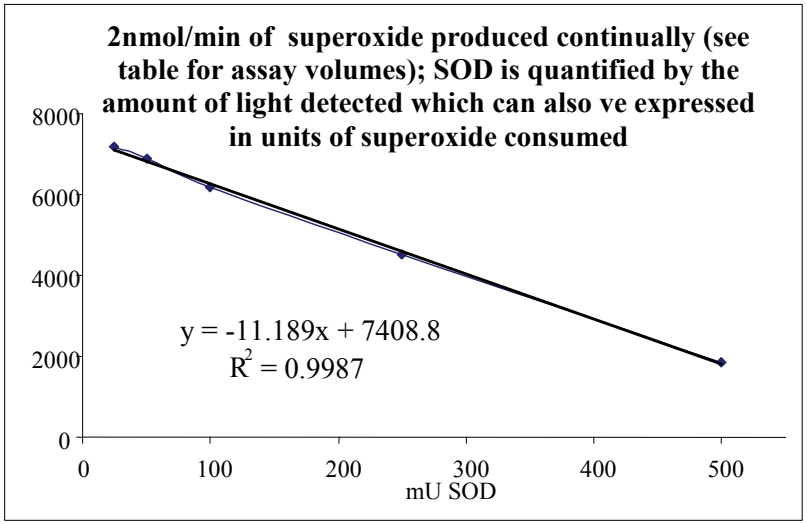
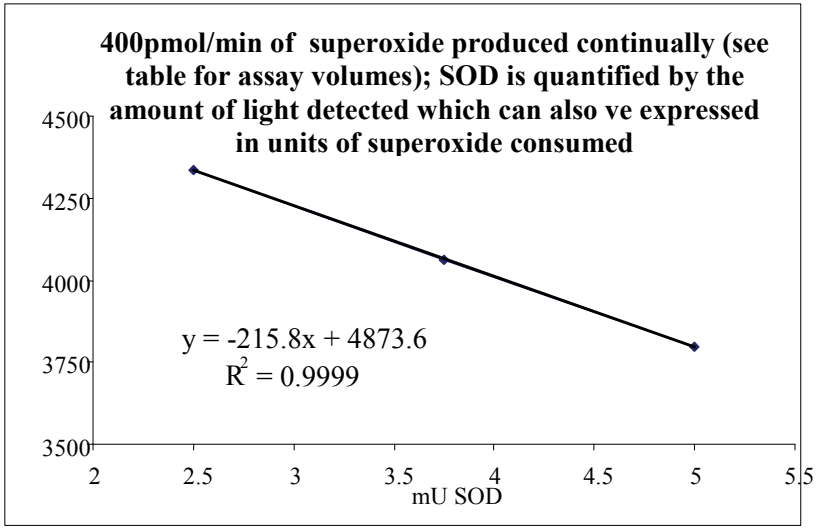


Figure 13. Sodium ascorbate is a non enzyme antioxidant; it contrasts enzyme antioxidants such as SOD and SOD mimetcs by being consumed in the assay. A feature of consumed vs enzyme activity is a linear increase in light compared to a steady light emission in the enzyme reaction

High range SOD standards



Low Range SOD standards



EQUIPMENT REQUIRED

A microplate luminometer with temperature control and automatic injectors is ideal. However, if an injector is not available then xanthine oxidase can be pipetted into each well before putting the plate into the luminometer. The assay can be adapted for tube luminometers (seek guidance from supplier).

PROTOCOL

Reconstitution of Pholasin®

Reconstitute the Pholasin® (50µg) with 5mL of Reconstitution & Assay Buffer for 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. Check 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. Use the procedure for reconstitution as described for reconstituting Pholasin® above.
2. Reconstituted xanthine can be used for a maximum of 7 days if stored between 0 and 7^0C.

Reconstitution of Xanthine Oxidase

The vial contains 51.25mU of Xanthine Oxidase. When reconstituted with 5mL R&A XO buffer 10.25mU/mL solution is obtained.

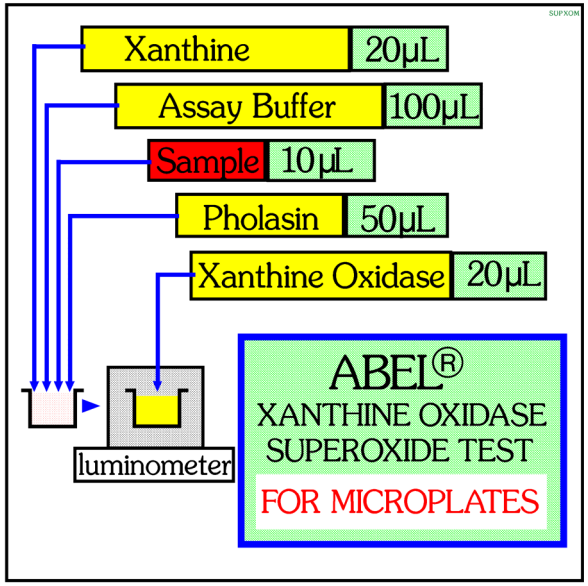
1. Use the procedure for reconstitution as described for reconstituting Pholasin® above.
2. Discard any unused product.

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 5mL Reconstitution and Assay buffer for xanthine oxidase by injecting 5mL buffer through the rubber septum using the same procedure as described for the other reagents.
3. When reconstituted with 5mL of R&A xanthine oxidase buffer a solution containing 25unit/mL is obtained.

TEST PROCEDURE



Assay with Superoxide Dismutase.

1. Pipette into each well of a microplate:
   - 100µL assay buffer + 10µL sample
   - 50µL reconstituted Pholasin®
   - 20µL of reconstituted xanthine
2. Add SOD; adjust the amount of assay buffer added to a total volume of 100µL.
   For example 25µL SOD +75µL of assay buffer = 100µL total
3. Inject into each well 20µL of xanthine oxidase

Table High Range SOD This concentration of Superoxide Dismutase should be used with 2nmol/minute of Superoxide.

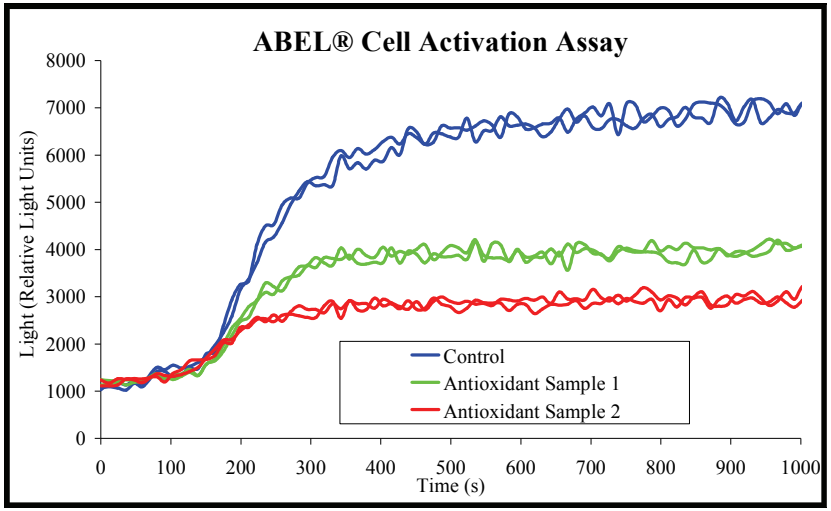
25U/mL Superoxide Dismutase (µL added)	R&A XO Buffer (µL added)	Superoxide Dismutase in 200µL (mU)
2	98	50
4	96	100
10	90	250
20	80	500
40	60	1000

Table Low Range SOD. This concentration of Superoxide Dismutase should be used with 400-800pmol/minute of Superoxide

250mU/mL Superoxide Dismutase (µL added)	R&A XO Buffer (µL added)	Superoxide Dismutase in 200µL (mU)
10	90	2.5
15	85	3.75
20	80	5
25	75	6.25
50	50	12.5

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	Maximum light relative light unites (RLU)	Superoxide measured pmol/min	Superoxide quenched µmol/min
Control	7200	1890	------
Antioxidant sample 1	4200	430	1460
Antioxidant sample 2	3400	40	1850

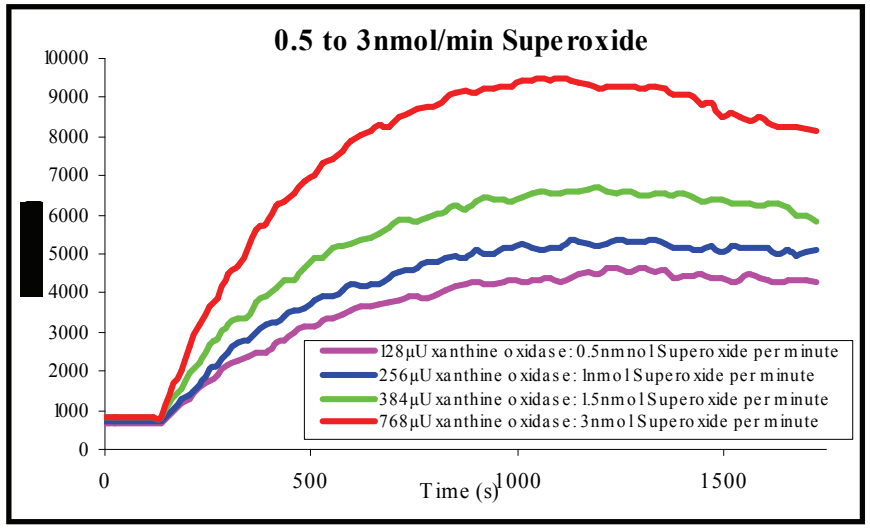
1. Pipette into each well of a microplate:
   - 100µL assay buffer + 10µL sample
   - 50µL reconstituted Pholasin®
   - 20µL of reconstituted xanthine
2. Inject 20µL of 10.25mU/mL xanthine oxidase into each well, preferably with an automatic dispenser. Alternatively, add xanthine oxidase very quickly 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

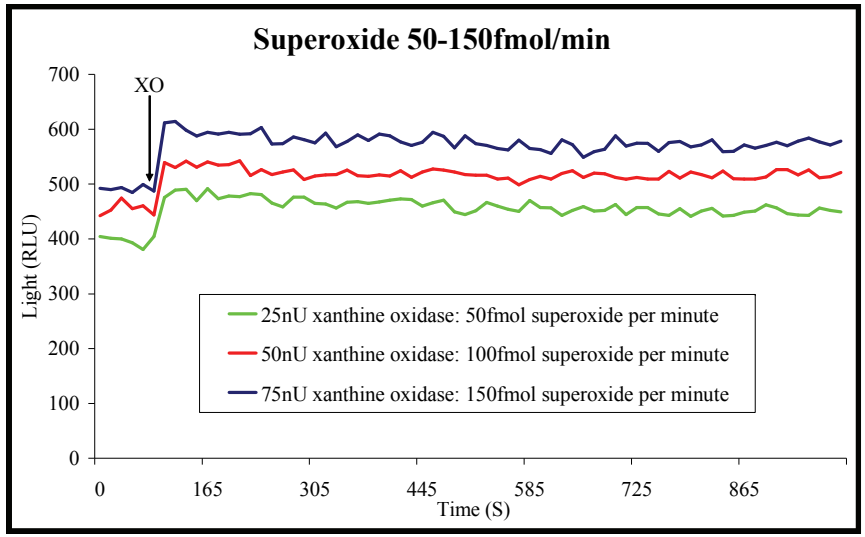
When Pholasin® and xanthine are in excess the amount of superoxide generated per minute can be determined by the amount of xanthine oxidase used in the assay. See chart below.

Volumes of xanthine oxidase and superoxide produced

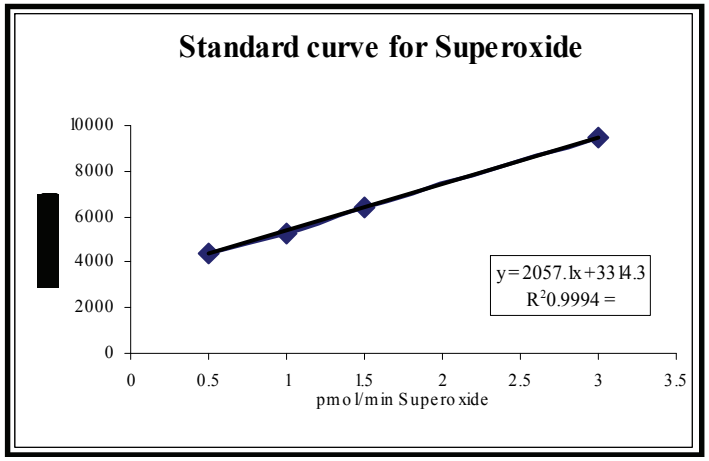
Volume 10.25mU/mL xanthine oxidase (µL added)	Equivalent Units XO µU of xanthine oxidase (200µL well)	Superoxide (nmol/min)
50	512.5	2
40	410.0	1.6
30	307.5	1.2
20	205.0	0.8
10	102.5	0.4



As Pholasin is an ultrasensitive detector of superoxide the assay can be used to measure superoxide as low as 50fmol per minute.

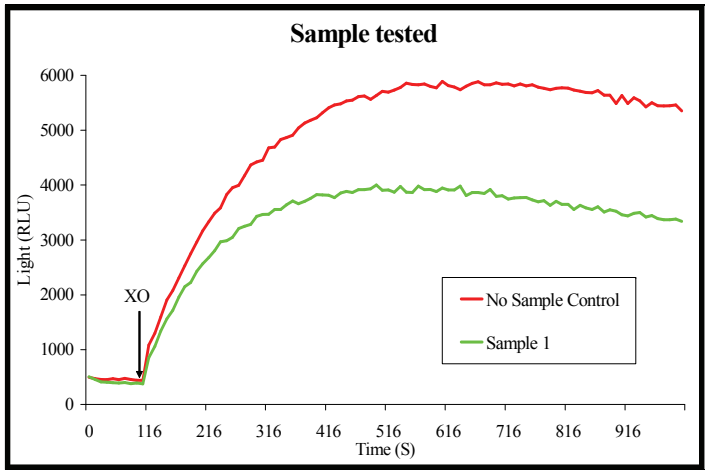


To convert luminescent readings into equivalent moles of superoxide a standard curve of light detected against superoxide generated (determined by the amount of xanthine oxidase used in the assay) can be produced. An example is presented below



APPLICATIONS OF THE ASSAY
Antioxidant Testing

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 Control	6000	1310	------
Antioxidant sample	4000	330	980

Using the standard curve above the data was obtained from the formula: (Max Light – Intercept)/Gradient