Analysis By ABEL Emitted Light

ABEL® ANTIOXIDANT TEST KITS WITH PHOLASIN®

from

KSL Knight Scientific Ltd

Knight Scientific Limited

ABEL® ANTIOXIDANT TEST KITS WITH PHOLASIN®

for measuring the capacity of a sample to scavenge free radicals and other oxidants

The ABEL®[1] (Analysis By Emitted Light) antioxidant test kits, of Knight Scientific Limited, measure the capacity of a sample to scavenge free radicals and other oxidants. If the sample has already been exposed to free radicals and/or oxidants then its complement of antioxidants will be very much reduced, leaving the sample with a diminished capacity to deal with the free radicals and oxidants that are generated in the assay.

These chemiluminescent test kits contain the unique photoprotein Pholasin® which emits light in the presence of free radicals and certain oxidants. The antioxidant capacity of the sample under test is expressed as the percentage reduction of peak luminescence of Pholasin® observed during the course of the test in the presence and absence of the sample. The antioxidant capacity of the sample under test is expressed as the percentage reduction of peak luminescence of Pholasin® (and/or the delay in time in which the peak luminescence is observed) during the course of the test in the presence and absence of the sample. The antioxidant capacity, that is the ability to scavenge free radicals and oxidants, is determined separately in the different antioxidant tests.

ABEL® Antioxidant Test for superoxide and other free radicals is excellent at screening for antioxidants such as ascorbic acid

ABEL® Antioxidant Test for halogenated oxidants such as hypochlorous acid assesses antioxidants such as albumin

ABEL® Antioxidant Test using peroxynitrite is a total antioxidant capacity (TAC) test and can screen for lipophilic antioxidants such as Vitamin E as well as the hydrophilic antioxidant glutathione (GSH).

ABEL® Antioxidant Test for hydroxyl radical is an excellent assay for assessing antioxidants in a variety of materials including plants.

ABEL® Enzyme-generated Superoxide Antioxidant & Superoxide Dismutase Quantification Test, excellent for quantifying levels of superoxide and measuring the activity of superoxide dismutase (SOD) and SOD mimetics.

The superoxide and other free radicals, halogenated oxidant and hydroxyl radical tests are individual flash assays which can be completed in 10 to 30 seconds. The peroxynitrite assay is best suited for running with large number of samples a 96 well microplate can be assayed in 20 to 60 minutes, depending upon the antioxidant capacity of the sample. The superoxide quantification assay is suited for 10 to 12 samples in a microplate and can be assayed in 10 to 20 minutes. All the assays require between 5µL and 50µL of sample volume, depending upon the type of assay and whether the format is for a tube luminometer or a microplate luminometer. Typical samples that can be assayed include: serum, synovial fluid, cerebrospinal fluid, seminal plasma, fluid surrounding the alveoli of the lungs, water, or indeed any other fluid. The kits contain Pholasin® that has been formulated specifically for use in antioxidant testing together with all other necessary reagents.

[1] ABEL and Pholasin are the registered trademarks of Knight Scientific Limited (KSL)

The ABEL® Antioxidant kits are specific for particular groups of oxidants. whereas the assay using peroxynitrite is the best for measuring total antioxidant capacity, especially of people and animals and for use in testing ingredients and finished products. The ABEL® TAC test with peroxynitrite is now The ABEL® Superoxide and Other Free Radicals Test kit contains proprietary solutions to generate superoxide anion. The ABEL® Superoxide Quantification Test kit contains xanthine and xanthine oxidase which generate known quantities of the superoxide anion. In the ABEL® Halogenated Antioxidant Test kit, chloramine-T, which releases hypochlorous acid, is used as a model for hypochlorous and hypobromous acids, as well as, for the longer-lived derived chloramines and bromamines. In the peroxynitrite kit SIN-1 (3-morpholinosydnonimine hydrochloride) releases superoxide and nitric oxide simultaneously with the consequent formation of peroxynitrite.

WHAT IS PHOLASIN® AND WHAT DOES IT DO?

Pholasin® is the photoprotein of the marine, rock-boring bioluminescent mollusc, *Pholas dactylus*, the Common Piddock. Pholasin® does not glow on its own,[2] but needs to be switched on. Free radicals and other reactive oxygen species produced by activated leucocytes (and other types of cell) turn on the light. Pholasin® is an ultrasensitive detector of activated leucocytes.

Pholasin® emits light in the presence of:

- free radicals: superoxide anion, singlet oxygen, nitric oxide and possibly hydroxyl radical and/or ferryl radical;
- oxidants such as: hypochlorous and hypobromous acids, chloramines, bromamines and peroxynitrite
- enzymes: peroxidases and certain oxidases

Pholasin® is isolated and purified by Knight Scientific Limited (KSL) from piddocks cultivated in land-based systems.

FREE RADICALS

Free radicals are molecules that contain atoms with unpaired electrons. Molecules are most stable if all their electrons are paired; free radicals are very unstable, that is they are very reactive. When reacting with another molecule the second molecule loses an electron to the free radical that now, with a full complement of paired electrons, is more stable. However, in the process, the second molecule itself becomes a reactive free radical and a chain reaction is thus initiated. The chain is broken when two free radicals combine. If the second molecule should be important to the biochemistry of the organism then the whole organism may suffer as a result of the initial free radical attack.

As an example, consider the fate of the all important polyunsaturated lipids in a cell membrane. Attack by a free radical on one of the carbon to carbon double bonds can lead to the formation of the corresponding lipid peroxyl radical and the initiation of a chain reaction in which the final products, lipid peroxides, no longer maintain the integrity of the cell wall. This process of lipid peroxidation may be initiated by the hydroxyl radical $OH^{\bullet}$ in a reaction in which iron, as Fe^{++}, is involved. Hence the use of chelation therapy in the iron overload disease such as that which ensues when children have eaten their parent's iron tablets.

[2] In solution Pholasin® emits low-level light referred to as the resting glow

REACTIVE OXYGEN SPECIES

SUPEROXIDE, HYDROXYL RADICAL AND HYDROGEN PEROXIDE

In biological systems much attention is focussed on the two free radicals, superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$), formed by the reduction of oxygen, and on hydrogen peroxide (which is not a free radical). There is a specific enzyme complex, the NADPH oxidase system, usually associated with phagocytic cells, which when activated releases large quantities of superoxide. Superoxide is also produced by leakage of electrons onto oxygen during the electron transport chain, and as a by-product of the activity of enzymes such as xanthine oxidase and aldehyde oxidase as well as by the auto-oxidation of hydroquinones, catecholamines and thiols. Hydrogen peroxide is formed from superoxide (usually catalysed by superoxide dismutase), as well as, by the enzymes D-amino acid oxidase and amine oxidase. Hydroxyl radicals can be formed by either superoxide anion or from hydrogen peroxide especially in the presence of the transition metals, iron and copper.

HALOGENATED OXIDANTS

The reaction of the neutrophil and monocyte granule enzyme myeloperoxidase with hydrogen peroxide (derived from superoxide) and chlorine leads to the production of hypochlorous acid, whereas eosinophil peroxidase reacts with hydrogen peroxide and bromine to produce hypobromous acids. Both oxidants are highly reactive and participate in the killing of micro-organisms. At sites of inflammation, however, leucocytes may accumulate in vast numbers, and there the release of large amounts of these granule enzymes can cause much damage.

Halogenated oxidants are capable of oxidising many biological molecules and especially those containing –SH groups. Hypochlorous acid can damage cartilage but it also rapidly inactivates α_1-antiprotease, the enzyme that prevents proteolytic enzymes such as elastase from destroying tissue.

The extent to which inactivation of the halogenated oxidants occurs in vivo depends largely upon the environment of the oxidant. In plasma HOCl is relatively unimportant because it reacts preferentially with albumin (concentration 50-60mg mL^{-1}) rather than with α_1-antiprotease (1-3mg mL^{-1}). However, in many other biological fluids such as cerebrospinal fluid, synovial fluid, and fluid surrounding the alveoli of the lungs, the concentrations of albumin are very much lower. In such environments the inactivation of α_1-antiprotease can be significant. Ascorbic acid is also oxidised by HOCl and its presence as an antioxidant in biological fluids can prevent the inactivation of α_1-antiprotease and thus protect tissue from damage.

It should be noted that HOCl can activate collagenase (which digests native collagen) and gelatinase (which attacks denatured collagen) and so presents a very great threat of damage.

PEROXYNITRITE

Peroxynitrite anion ($ONOO^-$) is produced by the reaction between nitric oxide and superoxide anion. This reaction is important to the organism when it occurs in inflammatory cells, such as neutrophils and macrophages, as the resultant peroxynitrite kills bacteria and other microorganisms. Excessive production of peroxynitrite, however, can damage normal tissue by oxidation and nitration. For example, its reaction with DNA, can lead to breaks in the chain, modification of the bases and mutations. Peroxynitrite can give rise to radical chain reactions, including lipid peroxidation. Nitration occurs mainly

on

phenols, as in the nitration of tyrosine residues in proteins. It is known to initiate lipid peroxidation of phosphatidylcholine liposomes and low density lipoprotein.

While the damage by peroxinitrite to some molecules is harmful to the organism, the function of others molecules, such as vitamin C and E, includes the harmless removal of excess peroxynitrite.

CLINICAL INTEREST AND APPLICATIONS OF THE ABEL® ANTIOXIDANT TESTS WITH PHOLASIN®

When free radicals attack, damage occurs. Free radicals can injure and even kill cells, they can damage DNA resulting in mutations, and they can attack enzymes and other proteins. They are continually produced in the body and are also continually destroyed by *antioxidants*. These include specialised *enzymes*, such as superoxide dismutase, which converts the free radical superoxide anion to the oxidant hydrogen peroxide, and catalase and glutathione peroxidase, which act on peroxides. Other antioxidants present are *scavengers*, such as vitamin C, vitamin E and any *molecule* such as albumin that will itself be attacked and thus protect from attack more precious molecules.

FREE RADICALS HAVE BEEN IMPLICATED IN A WIDE RANGE OF CONDITIONS INCLUDING:

- ageing
- alcoholism
- asthma
- atherosclerosis
- cancer
- cardiovascular disease
- cataracts
- diabetes
- exposure to excess oxygen
- hepatitis
- infectious diseases
- infertility
- inflammation
- inflammatory diseases
- ischaemia - reperfusion injury
- liver disease
- neurological conditions
- oxidative stress induced by toxins, asbestos and other fibres, air pollutants, Paraquat
- radiation-induced tissue injury
- renal failure and dialysis
- respiratory diseases
- respiratory distress syndrome
- septic shock
- smoking
- stroke
- tissue injury in iron and copper overload
- ultraviolet induced skin injury
- vascular diseases

The antioxidant test kits have applications to food testing, where free radicals for example are produced during irradiation.

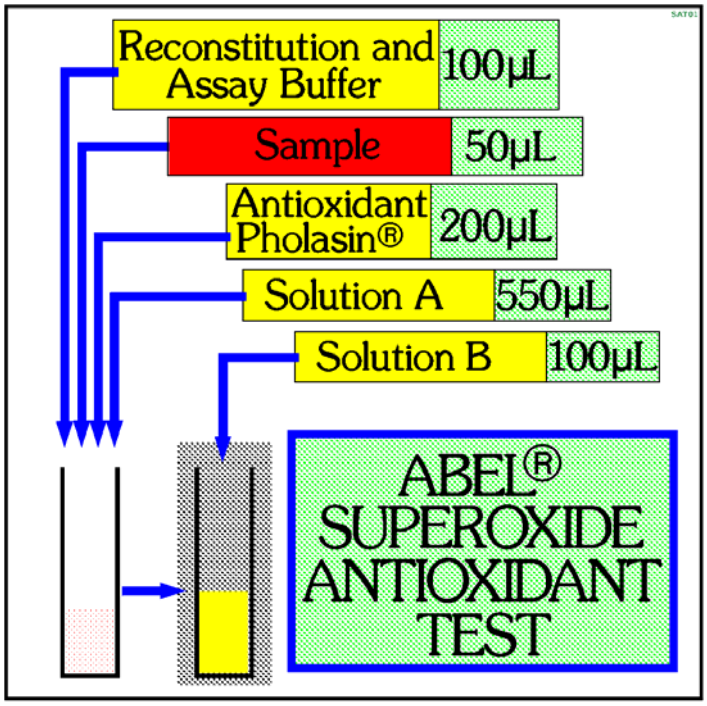
ANTIOXIDANT TEST FOR SUPEROXIDE AND OTHER FREE RADICALS

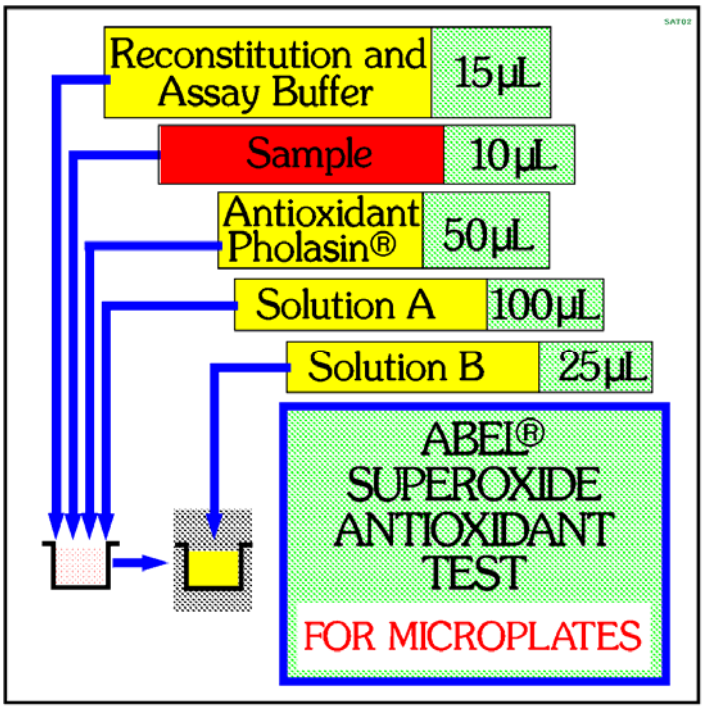
BACKGROUND

Superoxide is generated instantaneously when Solution B is added to Solution A. Solution B is injected into a cuvette/well containing Solution A and Pholasin®, with and without a 50µL sample (tube luminometer) or 5-10µL sample (microplate luminometer) of fluid of unknown antioxidant capacity. (The fluid can be heparin- or citrate-plasma (not EDTA), serum, synovial fluid, cell supernatant, process water, etc.). If Pholasin® is present when the superoxide is generated, light will be emitted. If there are antioxidants in the sample capable of scavenging superoxide, then these antioxidants will compete with Pholasin® for the superoxide and less light will be detected. Controls containing no sample are run with each assay. The antioxidant capacity can be expressed as the percentage reduction of peak luminescence or in equivalent ascorbate units. To obtain ascorbate equivalent units, an ascorbate standard curve is run as part of the assay (for details see section Standard curve for ascorbate).

SAMPLE PREPARATION AND CHEMILUMINESCENT ASSAY

The antioxidant Pholasin® is provided freeze-dried and under vacuum. It is reconstituted by injecting 5 or 10mL of the Antioxidant Reconstitution and Assay Buffer (provided in the kit) through the rubber insert. A syringe and needle (not provided in the kit) must be used to reconstitute the contents of the bottle. **On no account should the rubber insert be removed while the contents of the bottle are under vacuum,** as this is likely to lead to the loss of some of the contents. The test can be performed on either a tube luminometer or a microplate luminometer.





Into a luminometer cuvette add the following:

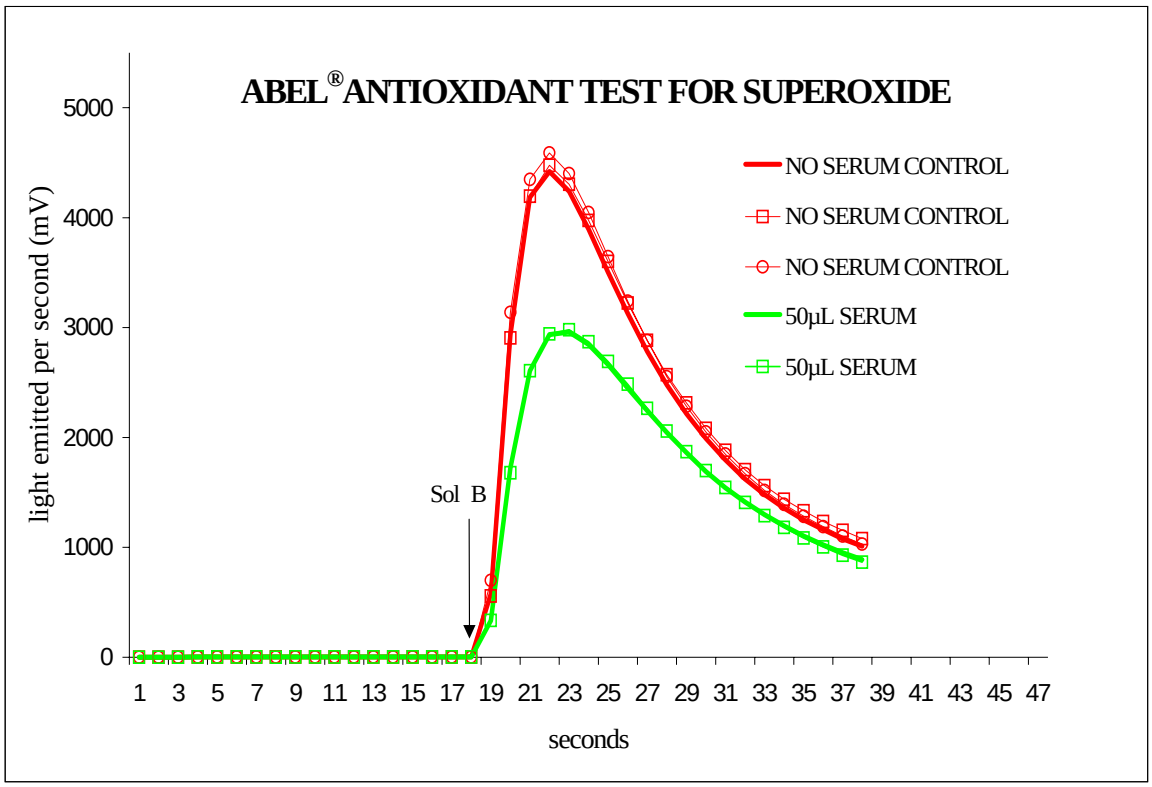
- 150 µL Assay Buffer (for controls) or 100 µL Assay Buffer + 50 µL sample
- 200 µL Antioxidant Pholasin®
- 550 µL Solution A or

Into a microplate well add the following:

- 25 µL Assay Buffer (for controls) or 15 µL Assay Buffer + 10 µL sample
- 50 µL Antioxidant Pholasin®
- 100 µL Solution A

Mix the contents of the cuvettes, ideally in a vortex mixer, or shake the microplate, before putting them into the luminometer and equilibrating to the selected temperature (ambient or 25°C). The assay is initiated when Solution B (100µL for the tube luminometer and 25µL for the microplate luminometer) is injected into the cuvette or microplate well, which must be in the light measuring position. Continue the measurement of light for a further 20 seconds if you wish to record the complete reaction (although the peak of luminescence, which is the crucial measurement, is reached within about 5 seconds after the addition of Solution B).

TYPICAL ANALYSIS



STANDARD CURVE FOR ASCORBATE

1. Reconstitute the L-ascorbic acid (sodium salt) with 5mL Reconstitution & Assay Buffer, giving a final concentration of 1mmol L^{-1}. Important: do not remove rubber insert until after reconstituting the ascorbate.
2. Pipette into a cuvette:
   200µL Pholasin
   50µL Reconstitution and Assay Buffer
   550µL Solution A
   100µL of 1mmol L^{-1} ascorbate + R&A Buffer, as shown in the table below.
3. Pipette into a microplate well:
   50µL Pholasin
   5µL Reconstitution and Assay Buffer
   100µL Solution A
   20µL of 1mmol L^{-1} ascorbate + R&A Buffer

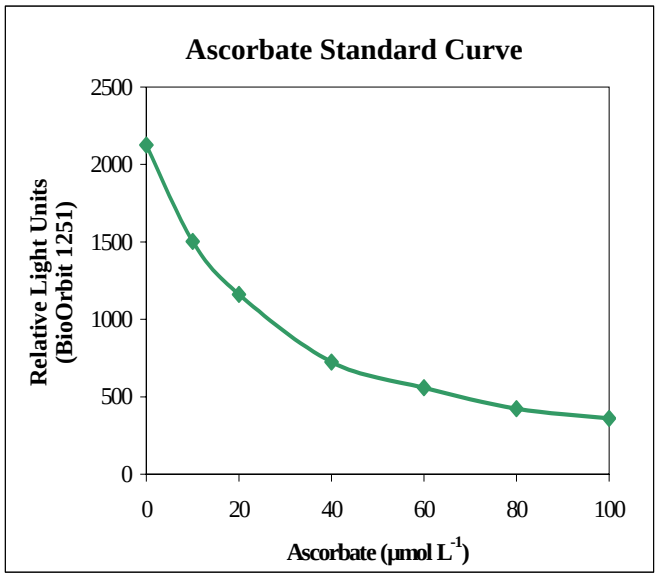
as shown in the table below.

3. Inject Solution B when the cuvette or microplate well is in the light measuring position (100µL for the tube luminometer and 25µL for the microplate luminometer).
4. The total volume is 1mL in the cuvette and 200µL in the microplate well.

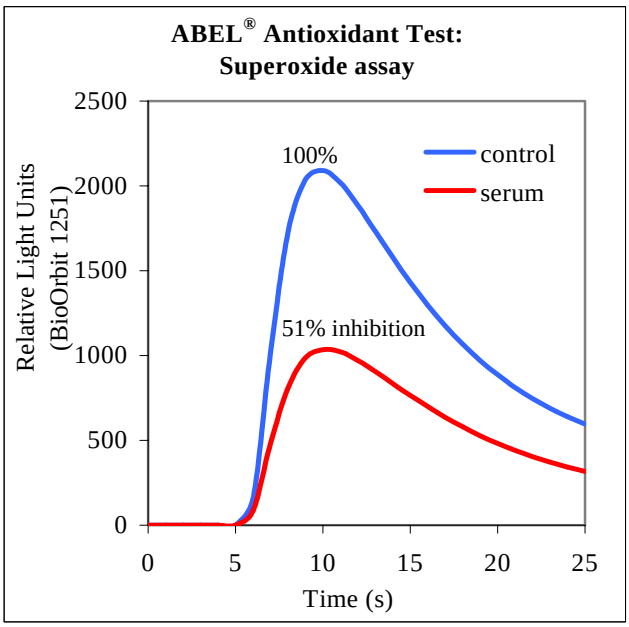
Table. Volumes of ascorbate and R&A Buffer added to cuvette or microplate well as the sample.

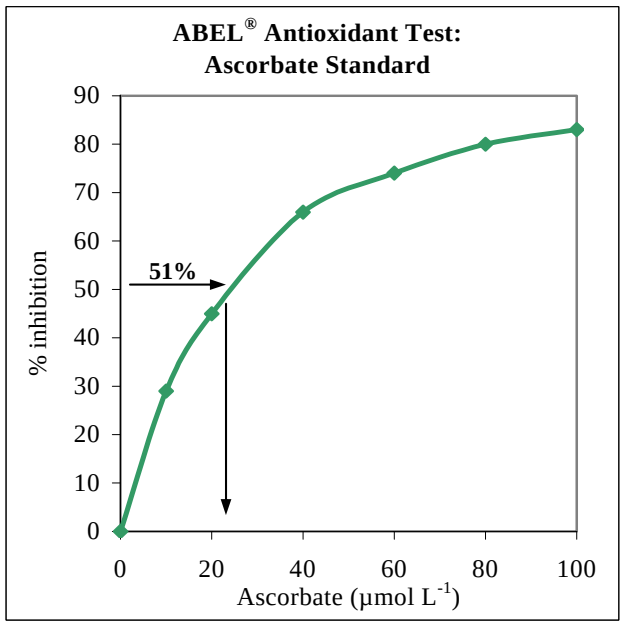
1mmol L^{-1} ascorbate (µL added)		R&A Buffer (µL added)		Ascorbate in cuvette/well (µmol L^{-1})	Ascorbate in sample (µmol L^{-1})
cuvette	*microplate*	*cuvette*	*microplate*		
0	0	100	20	0	0
10	2	90	18	10	200
20	4	80	16	20	400
40	8	60	12	40	800
60	12	40	8	60	1200
80	16	20	4	80	1600
100	20	0	0	100	2000

Example of an ascorbate standard curve obtained from a tube luminometer



TYPICAL ANALYSIS





Results can be expressed as percent inhibition compared to a control, or as Ascorbate equivalent antioxidant units.

Ascorbate Equivalent Antioxidant Units:

51% inhibition of 50µL (cuvette) or 10µL (microplate) sample in a 1:20 dilution
≡ 25µmol L^{-1} asc units
→ 500 µmol L^{-1} ascorbate equivalent units in pure sample

ANTIOXIDANT TEST FOR HALOGENATED OXIDANTS

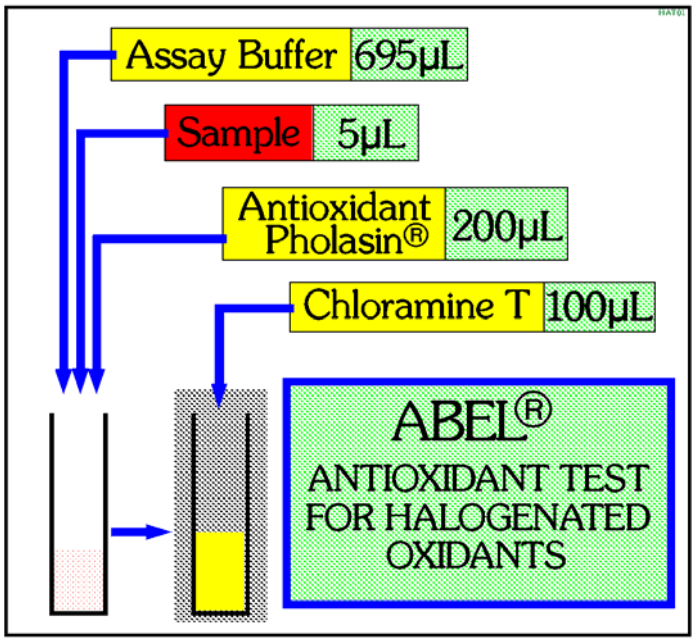
BACKGROUND

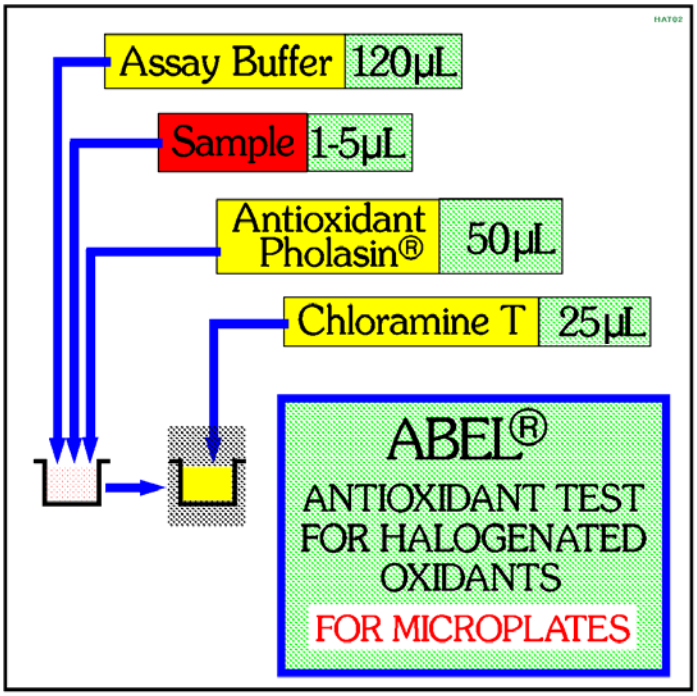
Chloramine-T (4.4mmol L^{-1} final concentration) is added to cuvettes/wells containing: Pholasin® with and without a 5-50µL sample of fluid of unknown antioxidant capacity. (The fluid can be heparin, citrate or EDTA plasma, serum, synovial fluid, cell supernatant, process water, etc.) If Pholasin® is present when the chloramine-T is injected, light will be emitted; if the sample contains antioxidants capable of scavenging chloramine-T, then these antioxidants will compete with Pholasin® for the Chloramine-T and less light will be detected. Controls containing no sample are run with each assay. The antioxidant capacity can be expressed as the percentage reduction of peak luminescence or in equivalent albumin units (albumin is a well-known scavenger of halogenated oxidants). To obtain albumin equivalent units, an albumin standard curve is run as part of the assay (for details see section Standard curve for albumin).

SAMPLE PREPARATION AND CHEMILUMINESCENT ASSAY

The antioxidant Pholasin® is provided freeze-dried and under vacuum. It is reconstituted by injecting 5 or 10mL of the Pholasin® Reconstitution and Assay Buffer (provided in the kit) through the rubber insert. A syringe and needle (not provided in the kit) must be used to reconstitute the contents of the bottle. On no account should the rubber insert be removed while the contents of the bottle are under vacuum as this is likely to lead to the loss of some of the contents.

Chloramine-T is provided pre-weighed (50mg) so that when reconstituted with 5mL of Chloramine-T Reconstitution Buffer it yields a 44mmol L^{-1} solution. It is important not to use the Pholasin® Reconstitution and Assay Buffer for reconstituting Chloramine-T, use the special buffer provided in the kit. The test can be performed on either a tube luminometer or a microplate luminometer.





Into a tube luminometer cuvette add the following:

- 700µL Pholasin® Reconstitution and Assay Buffer (for controls) or 695µL buffer + 5µL sample
- 200µL reconstituted antioxidant Pholasin®

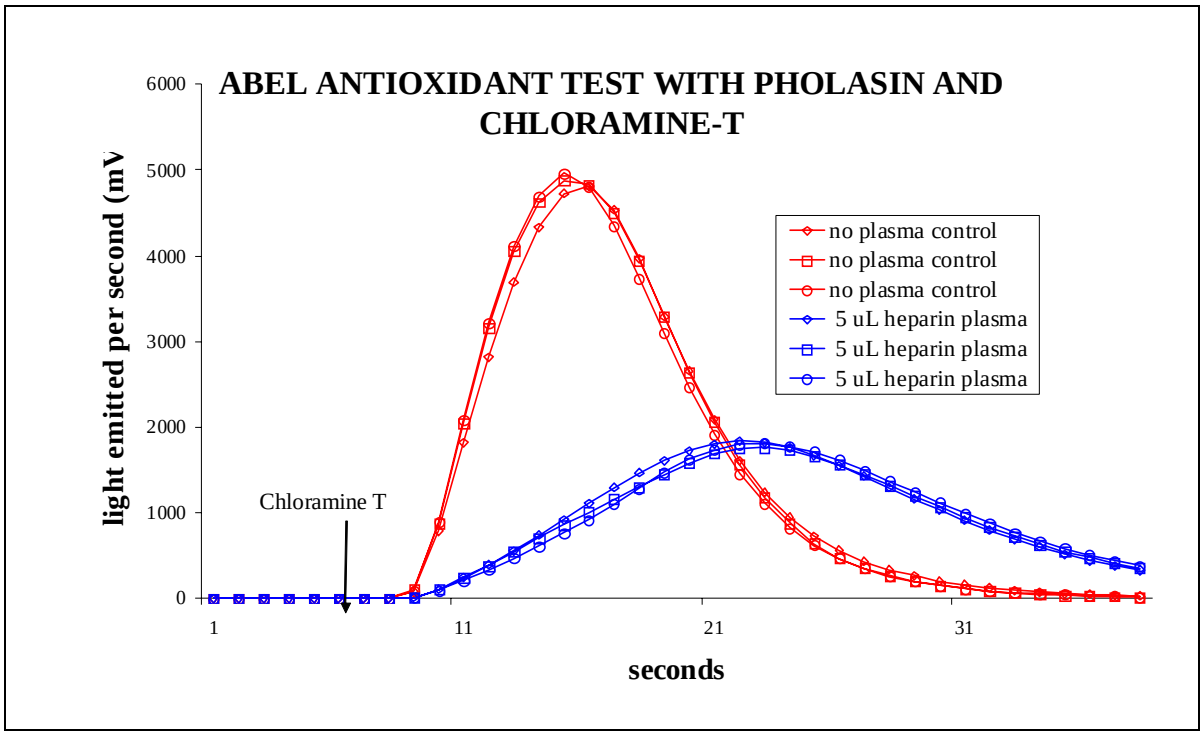
or

Into a microplate well add the following:

- 125µL Reconstitution and Assay Buffer (for controls) or 120µL buffer + 1-5µL sample (adjust the volume of the buffer so that the total volume of buffer + sample is 125µL)
- 50µL reconstituted antioxidant Pholasin®

Mix the contents of the cuvettes, ideally using a vortex mixer, or shake the plate before putting them into the luminometer and equilibrating to the selected temperature (ambient or 25°C). The assay is initiated when Chloramine-T (100µL for the tube luminometer and 25µL for the microplate luminometer) is injected into the cuvette or the microplate well, which must be in the light measuring position. Continue the measurement of light for a further 30 seconds if you wish to record the complete reaction (although the peak of luminescence, which is the crucial measurement, is reached within about 5 seconds after the addition of Chloramine-T).

TYPICAL ANALYSIS



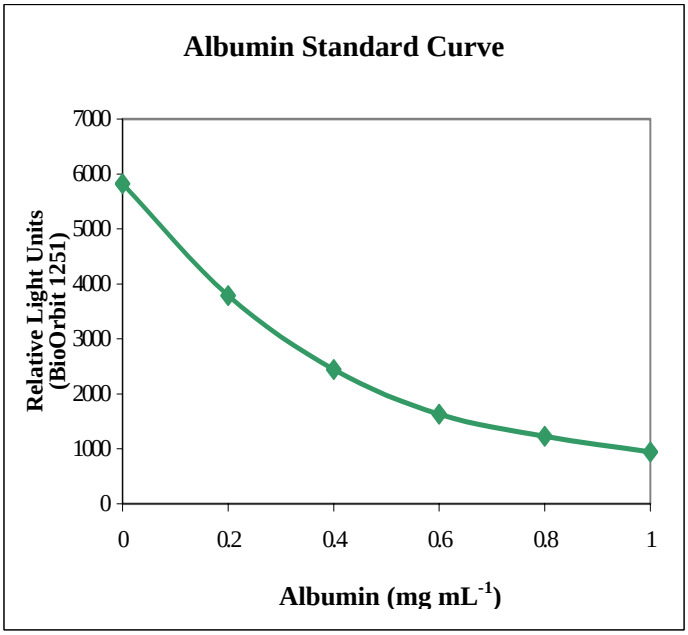
STANDARD CURVE FOR ALBUMIN

1. Make up a 1% solution of albumin (albumin, bovine, fraction V) in Reconstitution & Assay Buffer. Always use the same source of albumin and always make up fresh solution as it can become a pro-oxidant when it is stored in solution.
2. Pipette into a cuvette:
   200µL Pholasin
   600µL Reconstitution and Assay Buffer
   100µL of 1% albumin in R&A Buffer + R&A Buffer, as shown in the table below.
3. Inject 100µL Chloramine-T when the is in the light measuring position.
4. The total volume in the cuvette is 1mL.

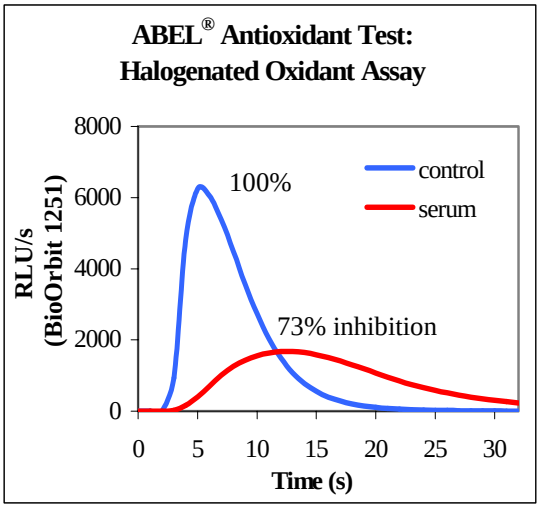
Table. Volumes of albumin and R&A Buffer added to cuvette as the sample.

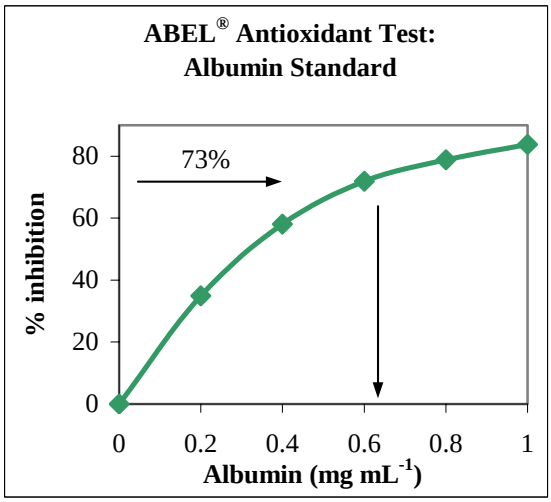
1% Albumin (µL)	R&A Buffer (µL)	Albumin in cuvette (mg mL^{-1})	Albumin equivalent units in 5µL sample (mg mL^{-1})
0	100	0.0	0
20	80	0.2	40
40	60	0.4	80
60	40	0.6	120
80	20	0.8	160
100	0	1.0	200

Example of an albumin standard curve obtained from a tube luminometer



TYPICAL ANALYSIS





Results can be expressed as percent inhibition of emitted light compared to a control, or as albumin equivalent antioxidant units.

Albumin Equivalent Antioxidant Units:

73% inhibition of 5µL sample in a 1:200 dilution ≡ 0.62mg mL^{-1}
→ 124 mg mL^{-1} albumin equivalent units in pure sample

ANTIOXIDANT TEST FOR PEROXYNITRITE

BACKGROUND

Peroxynitrite is formed in the assay by the reaction between superoxide and nitric oxide, released simultaneously and continually from a 2.5mmol L^{-1} solution of SIN-1 (3-morpholino-sydnonimine HCl; $C_6H_{10}N_4O_2$. HCl).

$$O_2^{\bullet -}\ \text{(superoxide)} + NO^{\bullet}\ \text{(nitric oxide)} \rightarrow ONOO^{-}\ \text{(peroxynitrite)}$$

In the assay, when 50µL of 10mmol L^{-1} SIN-1 is injected into a microplate well containing Pholasin®, light of gradually increasing intensity is detected, reaching a peak after a few minutes. If there are antioxidants in the sample capable of scavenging peroxynitrite, such as Vitamin E, then these will compete with the Pholasin® for the peroxynitrite. Any antioxidants in the sample will be gradually consumed, delaying the time at which the maximum peak of light is emitted, as well as, competing for the Pholasin®, resulting in a peak of lower intensity.

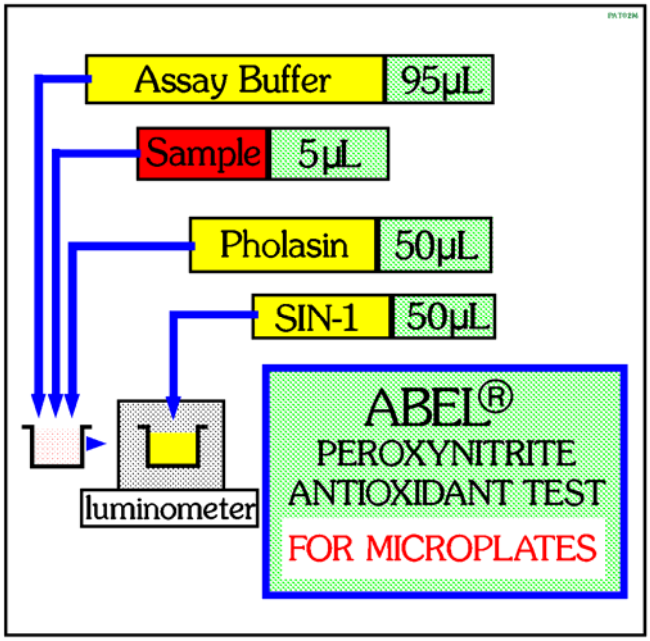
The time at which peak luminescence occurs (peak time) after adding SIN-1, is expressed in Vitamin E analogue equivalent units, VEA units, using 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid. A standard curve is run as part of the assay; the formula for the linear regression can then be used to convert times-to-peak into VEA equivalent units.

SAMPLE PREPARATION AND CHEMILUMINESCENT ASSAY

The Pholasin® is provided freeze-dried and under vacuum. It is reconstituted by injecting 10mL of the Peroxynitrite Reconstitution and Assay Buffer (provided in the kit) through the rubber insert. A syringe and needle (not provided in the kit) must be used to reconstitute the contents of the bottle. **On no account should the rubber insert be removed while the contents of the bottle are under vacuum** as this is likely to lead to the loss of some of the contents.

SIN-1 is provided pre-weighed (12mg) together with some inert material. Pipette 6mL of the water (provided in the kit) to make a 2mg mL^{-1} solution.

The test is best performed on a microplate luminometer. The assay will work at ambient temperature or above; the total assay time can be reduced by running the assay (and standards) at a temperature higher than 25°C (such as 30 or 37°C).



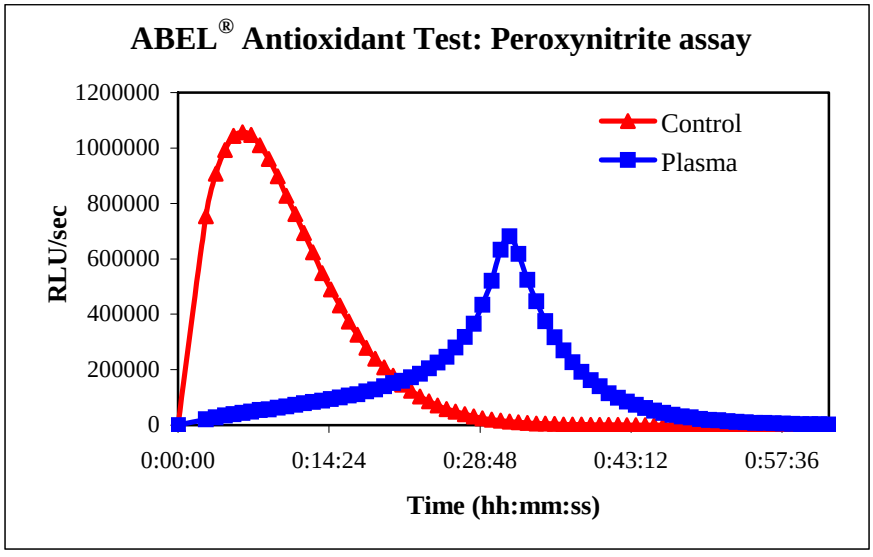
Into a microplate well add the following:

- 100µL Peroxynitrite Reconstitution and Assay Buffer (for controls) or 95µL Buffer + 5µL sample
- 50µL reconstituted Pholasin®

Shake the plate before putting it into the luminometer and equilibrate to the selected temperature. 50µL of 2mg mL^{-1} solution of SIN-1 is injected into each well using an automatic dispenser, after the assay has commenced. Alternatively, add the SIN-1 very quickly before putting the plate in the luminometer.

Measure light for 0.5 to 1.0 second in each well; measure each well in turn. Repeat the cycle of measurements for a total of 60 minutes in the first instance; this time may be reduced (or extended) when the approximate time for the sample to reach its peak is determined. Please ensure that the intervals between repeat measurements are minimal.

TYPICAL ANALYSIS



STANDARD CURVE FOR VITAMIN E ANALOGUE (VEA) STANDARD

1. Reconstitution of the VEA standard

The VEA standard (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) has been specially formulated to be reconstituted with 2mL of R&A PN buffer to obtain a 0.5mmol L^{-1} solution. Keep reconstituted bottle in the dark as the product is light sensitive and store at 2-8^{o}C.

- Load a syringe with 2mL R&A PN buffer and fit a needle (1 inch, 21 gauge, 0.8 x 25 mm) to the syringe.
- Remove the protective screw cap from the vial of VEA standard and push the needle through the 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. Replace the screw cap and invert and roll the bottle until the contents are fully dissolved.
- Make a 1:10 dilution of the 0.5mmol L^{-1} VEA stock to obtain a 50µmol L^{-1} VEA stock that is used in the High range VEA std curve. (Take 1mL of 0.5mmol L^{-1} VEA standard and add to the bottle labeled 'HIGH RANGE VEA standard', which contains 9mL of R&A Buffer).
- Make a further 1:10 dilution of the 50µmol L^{-1} to obtain a 5µmol L^{-1} VEA std stock that is used in the Low range VEA std curve. (Take 1mL of 0.05mmol L^{-1} VEA stock and add to the bottle labeled 'LOW RANGE VEA standard', which contains 9mL of R&A Buffer).

2. To decide which range of VEA standards to include in the testrun, run about three concentrations from the high range (HR) and three from the low range (LR) of VEA standards (see tables below) together with 2-3 of your samples to get an idea where the samples will come out. Express the time to peaks of the samples in VEA equivalent units (in 5µL of sample) and then determine which standard concentration range that needs to be included in each run.
3. Pipette into a well: 50µL Pholasin
   100 µL of VEA standard + R&A PN Buffer,
   as shown in the tables below.
4. Inject 50µL SIN-1 when the well is in the light measuring position.
5. The total volume in the well is 200µL.

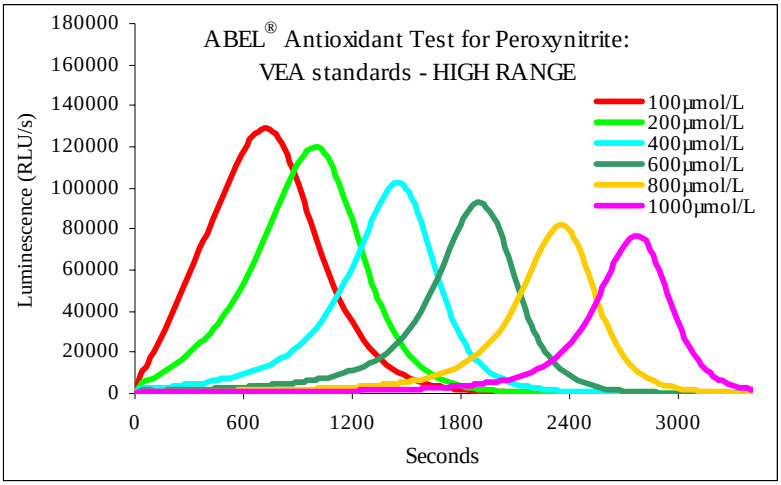
Any unused reconstituted VEA standard can be stored in the dark at 2-8^{o}C for up to 2 days.

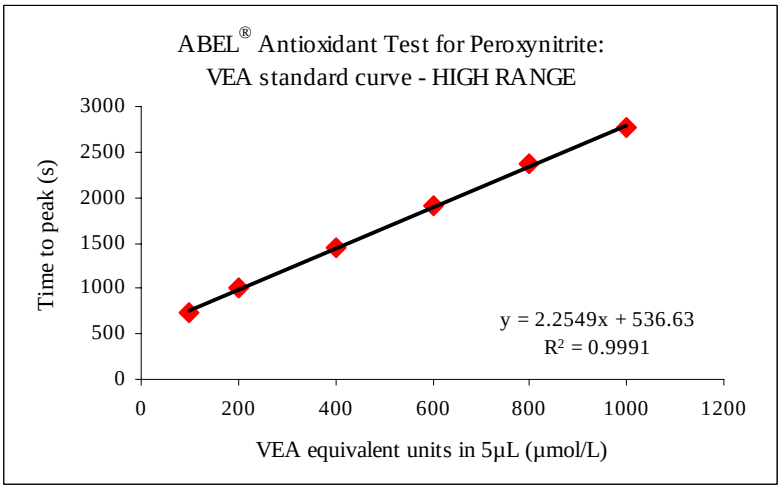
Table High Range. Volumes of VEA standard and R&A PN Buffer added to the microplate well. High range:100-1000µmol L^{-1} VEA equiv units.

50µmol L^{-1} Vit E Analogue Standard (µL added)	R&A PN Buffer (µL added)	Vit E Analogue VEA in 200µL (µmol L^{-1})	Vit E Analogue VEA equivalent units in 5µL sample (µmol L^{-1})
10	90	2.5	100
20	80	5	200
40	60	10	400
60	40	15	600
80	20	20	800
100	0	25	1000

Table Low Range. **.** Volumes of VEA standard and R&A PN Buffer added to the microplate well. Low range:2-40µmol L^{-1} VEA equiv units.

5µmol L^{-1} Vit E Analogue Standard (µL added)	R&A PN Buffer (µL added)	Vit E Analogue VEA in 200µL (µmol L^{-1})	Vit E Analogue VEA equivalent units in 5µL sample (µmol L^{-1})
2	98	0.050	2
5	95	0.125	5
10	90	0.250	10
20	80	0.500	20
30	70	0.750	30
40	60	1.000	40





The antioxidant capacity of a sample can be expressed in VEA equivalent units (µmol L^{-1}) from a standard curve of concentration of VEA in 5µL sample against time to peak luminescence (after addition of SIN-1). Sample equivalent concentrations are best obtained from the linear regression of the times to peak:

y = time to peak of sample, x = the unknown VEA equiv units (µmol L^{-1}). For example, using the above equation, the VEA equiv units of a specific sample are: x=(y-536.63)/2.2549.

ANTIOXIDANT TEST FOR HYDROXYL RADICALS

BACKGROUND

Hydroxyl radicals are generated in the assay instantaneously when Solution B comes into contact with Solution A. If Pholasin® (a photoprotein which reacts with hydroxyl radicals to emit light) is present when the hydroxyl radicals are generated light will be emitted. If there are substances (other than Pholasin®) in the sample capable of reacting with hydroxyl radicals (antioxidants), these will compete with Pholasin® for hydroxyl radicals and less light will be emitted. The antioxidant capacity of a sample can thus be expressed as the percent inhibition of light.of Pholasin® compared to the no-sample control or in D-mannitol equivalent units (a well characterized scavenger of hydroxyl radicals) derived from a standard curve. .

A wide variety of samples can be used including: heparin or citrate plasma, but not EDTA plasma, serum, synovial and other body fluids, cell supernatants, environmental samples, process water, food extracts & ingredients, personal care products, nutraceuticals and many more.

EQUIPMENT REQUIRED

A microplate luminometer with at least one injector which must be capable of injecting into a well at the same time light is measured. Temperature control and mixing is recommended but not essential.

PROTOCOL

Reconstitution of Pholasin®

The antioxidant Pholasin® has been specially formulated to be reconstituted with 5mL of Hydroxyl Radical Reconstitution and Assay Buffer supplied in the kit.

1. Load a syringe with 5mL the Reconstitution & Assay 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: remove syringe and leave needle in place; then carefully remove needle.
4. Replace the screw cap and invert and roll the bottle at least 5 times to dissolve the contents.
5. Reconstituted antioxidant Pholasin® **MUST NOT BE FROZEN** and should be stored at 2-8°C.

Samples

Any liquid sample (5µL) of unknown antioxidant capacity can be tested for its capacity to quench hydroxyl radicals. It is strongly recommended that the sample be diluted in buffer and re-assayed if the antioxidant capacity of the undiluted 5µL sample is greater than 90%. You should dilute the sample to obtain approximately 50% inhibition of light and then multiply the antioxidant capacity result by the dilution factor. If acetone extracts are used these must be diluted at least 50% with water or buffer. Always use a sample of the extraction medium as a control.

TEST PROCEDURE

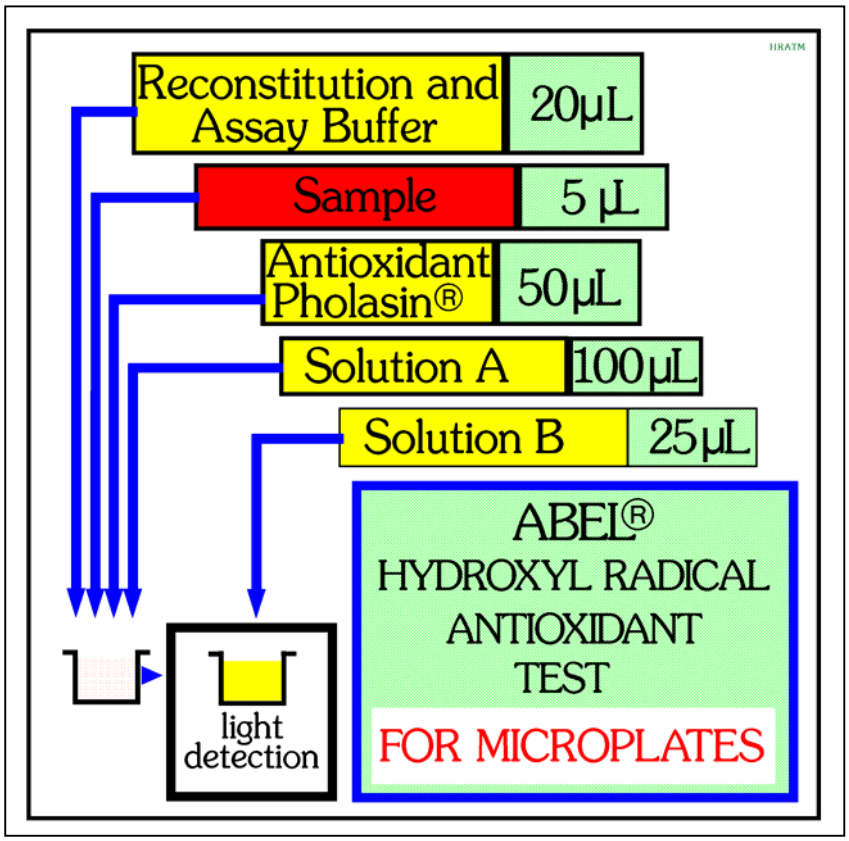
Pipette into a microplate well:

- 25µL assay buffer or 20µL assay buffer + 5µL sample
- 50µL Pholasin®
- 100µL Solution A

While the microplate well is in the light measuring position 25µL Solution B is injected. The luminescent peak is reached within 5 seconds. If Solution B is added to Solution A before the plate is put into the luminometer then no light will be detected because the hydroxyl radicals will have reacted OUTSIDE the luminometer. A control, without sample, but with a volume of buffer equal to the volume of the sample is run with each assay. The antioxidant capacity of the sample can be expressed as the percentage reduction of peak luminescence as follows

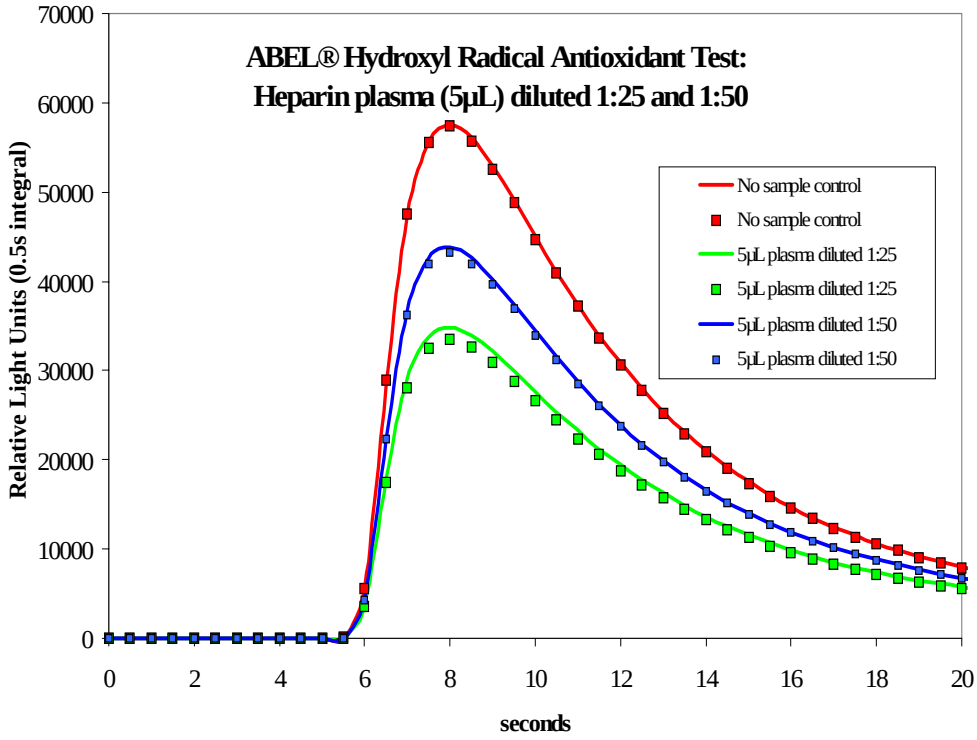
[(peak, control) – (peak, sample)] x 100/(peak, control)

The reduction of peak luminescence can also be expressed in equivalent D-mannitol units. To obtain D-mannitol equivalent units, a D-mannitol standard curve is run as part of the assay (see section Standard curve for D-mannitol).



SUMMARY OF TEST PROCEDURE

TYPICAL ANALYSIS

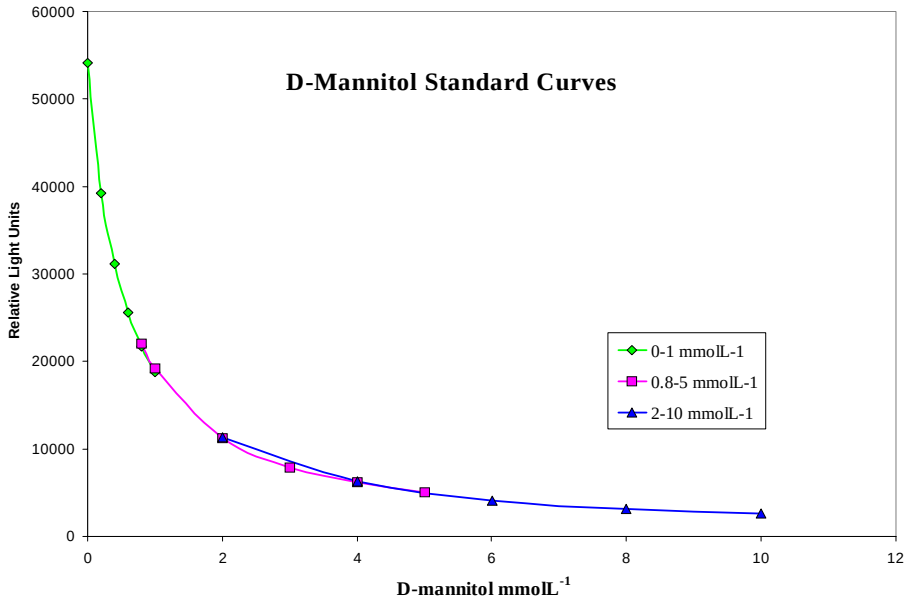


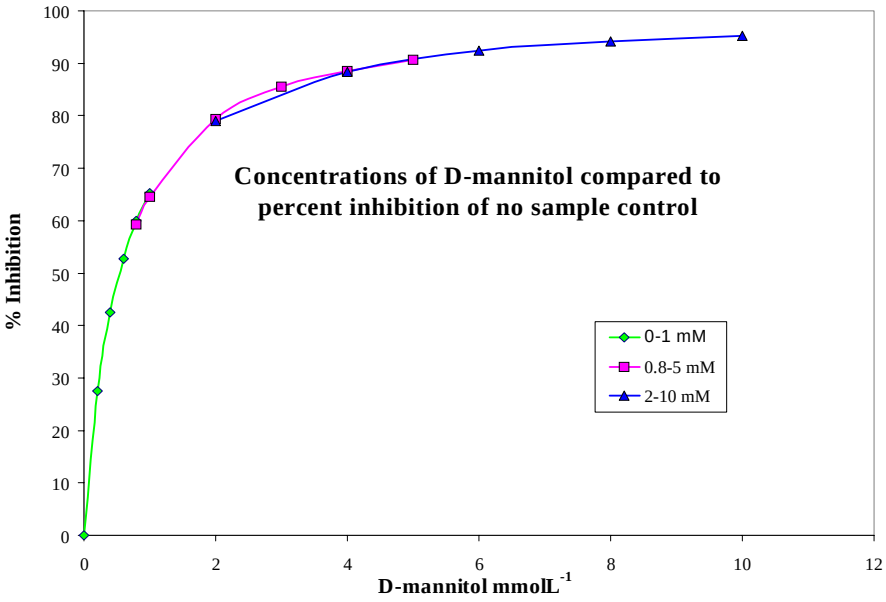
STANDARD CURVE FOR D-MANNITOL

- To decide which range of D-mannitol standards to include in the test run, run three standard curves: 0-1 mmol L^{-1}, 0.8-5 mmol L^{-1} and 2-10 mmol L^{-1}. All three standards curves overlap (see graph 'D-mannitol Standard Curves'.)
- Reconstitute each bottle of D-mannitol standard with the volume of reconstitution and assay buffer as instructed.
- Remove the protective screw cap from the vial of D-mannitol and 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.
- Replace the screw cap and invert and roll the bottle at least 5 times to dissolve the contents.
- Any unused D-mannitol should be stored at 2-8°C.
- Pipette into a well: 50µL Pholasin, 100µL Solution A, 25µL of D-mannitol + R&A buffer, as shown in the tables below.

NOTE: For best results, inject Pholasin and Solution A. If not possible, then pipette the Pholasin into the wells just prior to measuring.

- Inject 25µL Solution B when the well is in the light measuring position. [**It is essential that Solution B is injected**]
- The total volume in the well is now 200µL.





Results can be expressed as percent inhibition compared to no sample control (=100%) or as D-mannitol equivalent units.

ANTIOXIDANT TEST WITH ENZYME GENERATED SUPEROXIDE

BACKGROUND

Superoxide (anion radical $0_2^{\cdot-}$) is produced as a by product of the production of uric acid, from the enzyme reaction of xanthine with xanthine oxidase. This is used 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.

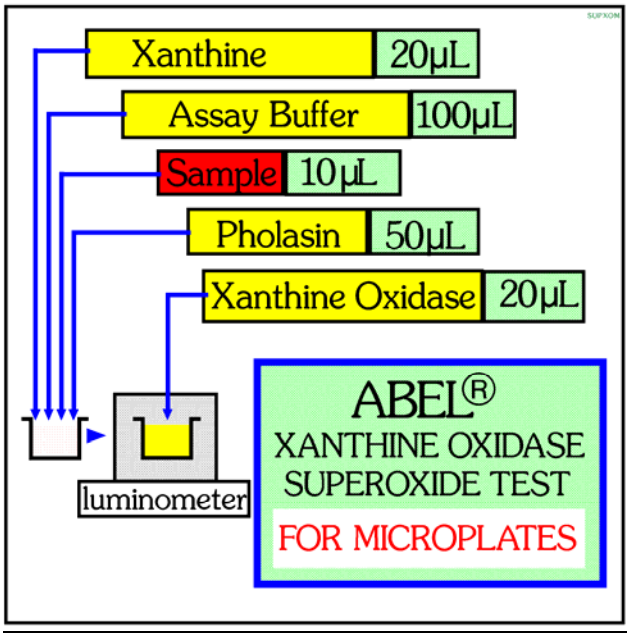
Pholasin® is an ultrasensitve chemiluminescent detector of superoxide can detect concentrations in this assay as low as 50fmol per minute, which is in the order produced by small numbers of cells. Higher amounts of superoxide can also be generated for use in antioxidant and other assays. This assay has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

SAMPLE PREPARATION AND CHEMILUMINESCENT ASSAY

The Pholasin® and xanthine oxidase provided is freeze-dried and under vacuum. It is reconstituted by injecting 5mL of the Xanthine Oxidase Reconstitution and Assay Buffer (provided in the kit) through the rubber insert. A syringe and needle (not provided in the kit) must be used to reconstitute the contents of the bottle. On no account should the rubber insert be removed while the contents of the bottle are under vacuum**,** as this is likely to lead to the loss of some of the contents.

Xanthine is provided and should be reconstituted with 2.5mL of Buffer for Dissolving Xanthine, this yields a 16mmol L^{-1} solution. It is important not to use the Xanthine Oxidase Reconstitution and Assay Buffer for reconstituting xanthine, use the special buffer provided in the kit. This test should be performed on a microplate luminometer.



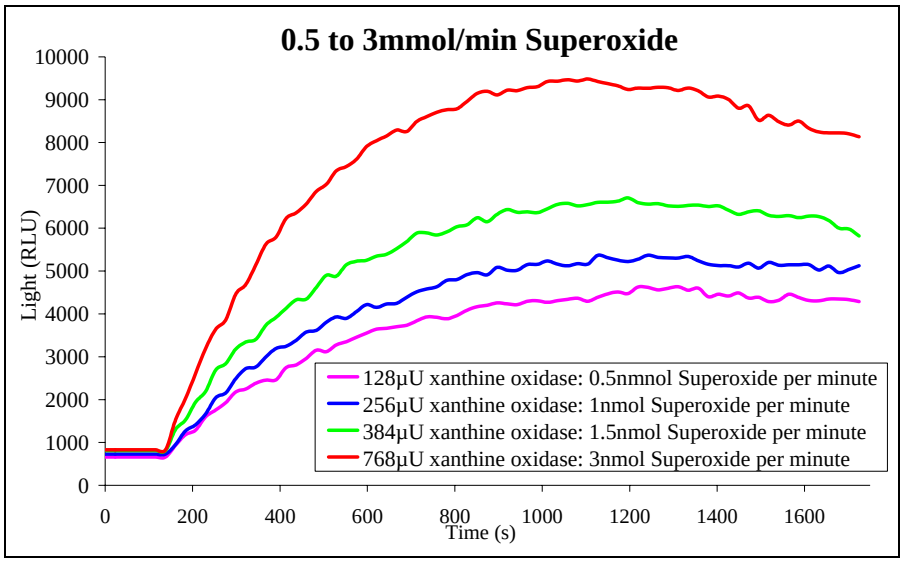
Into a microplate well add the following:

- 110µL Xanthine Oxidase Reconstitution and Assay Buffer (for controls) or 100µL Buffer + 10µL sample
- 50µL reconstituted Pholasin®
- 20µL reconstituted xanthine

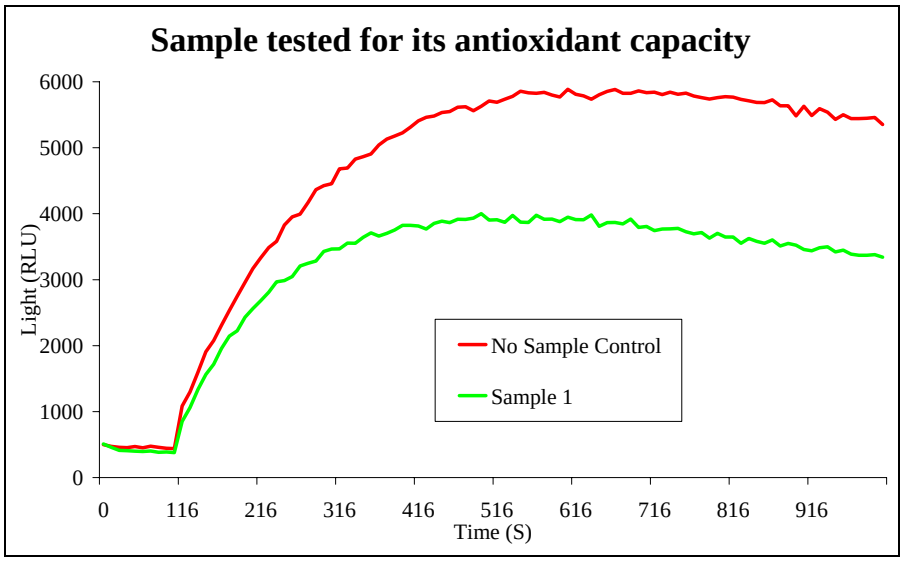
Shake the plate before putting it into the luminometer and equilibrate to the selected temperature. 20µL of xanthine oxidase is injected into each well using an automatic dispenser, after the assay has commenced (after 10 cycles for example). Alternatively, add the xanthine very quickly before putting the plate in the luminometer.

The optimum amount of samples to be assayed is 10 to 12, measureing light for 0.5 to 1.0 second in each well; this will give a short cycle time so that the kinetics of the reaction can be observed. Repeat the cycle of measurements for a total of 30 minutes in the first instance; this time may be reduced (or extended) when the approximate time for the sample to reach its peak is determined.

TYPICAL ANALYSIS



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.



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

SUPEROXIDE DISMUTASE

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

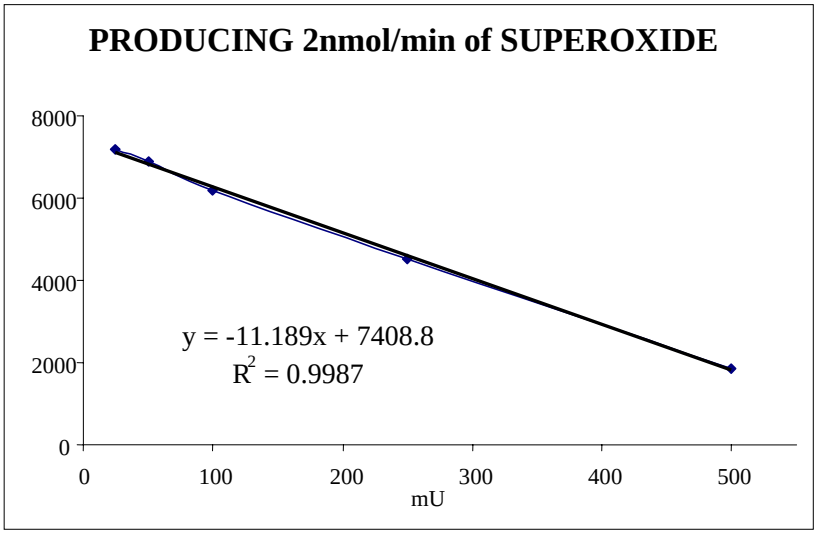
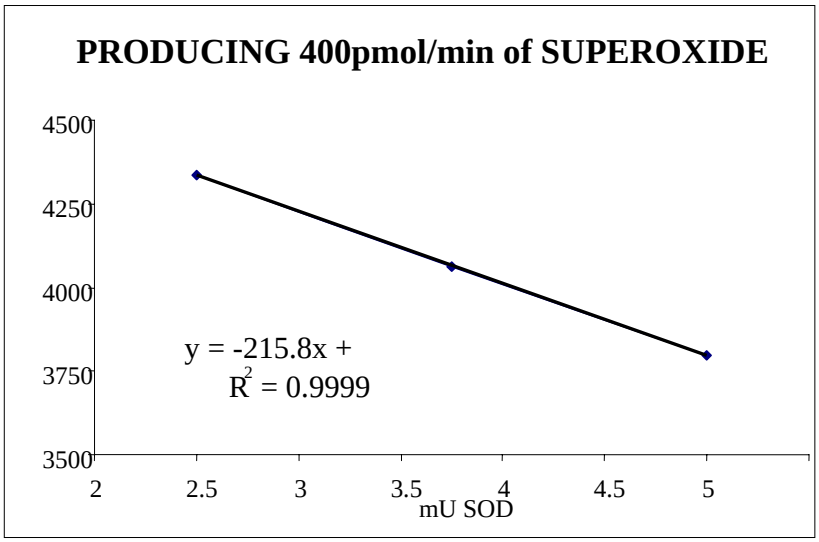
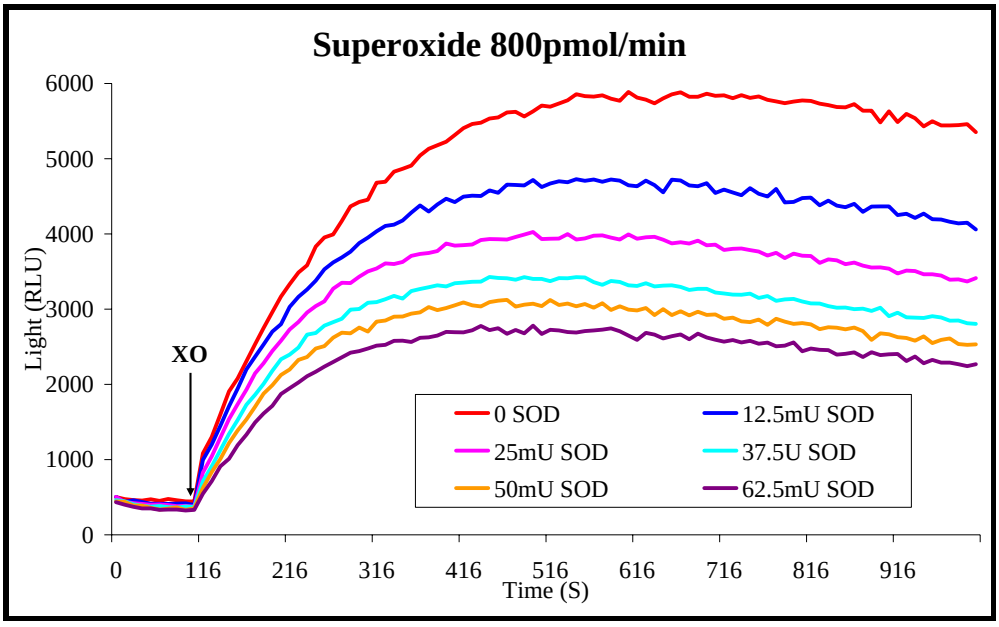


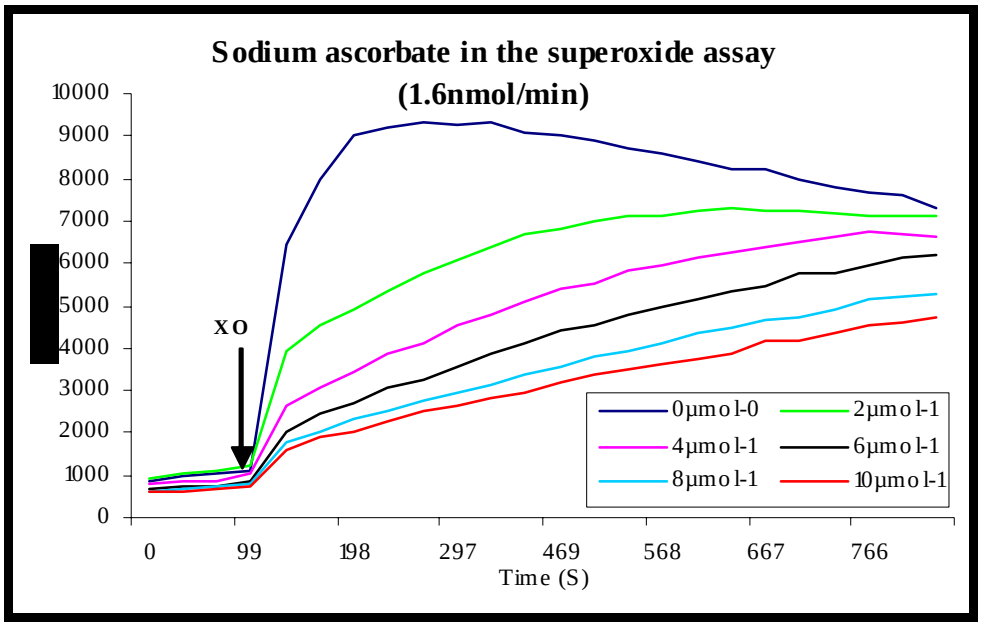
Table Low Range SOD. This concentration of Superoxide Dismutase should be used with 400-800pmol/minute of Superoxide

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





SOD of different concentrations (12.5mU-62.5mU) is in the microplate wells together with xanthine, Pholasin and buffer at the start of the assay; xanthine oxidase is injected to initiate the reaction with xanthine in which superoxide is produced.



Sodium ascorbate is a non enzymatic antioxidant, it contrasts antioxidants such as SOD and SOD mimetcs by being consumed in the assay. A feature of consumed verses enzyme activity is a linear increase in light compared to a steady light emision in the enzyme reaction.

FEATURES AND BENEFITS

FEATURE	BENEFIT
• easy-to-use kits	• require little operator training • reduce technician time
• short assay time; the crucial measurement namely the maximum luminescence is reached in about 5 seconds	• high throughput of samples • ability to carry out large number of tests
• specificity	• allows a sample to be assessed for its antioxidant capacity towards a particular type of free radical or oxidant.
• excellent reproducibility	• confidence in results
• good value	• ability to assay all samples and not have to select a limited number in order to keep within budget
• easily automated	• saves technician time • high throughput • best reproducibility
• non-hazardous reagents	• require no special handling or disposal procedures

FREQUENTLY ASKED QUESTIONS

QUESTION	ANSWER
1. What kind of samples can be analysed?	Serum, plasma, synovial fluid or any other fluid from which the cells have been removed.
2. Which anticoagulant should be used if plasma is to be used?	Heparin, or citrate may be used in all the antioxidant tests; EDTA must **NOT** be used in the test for superoxide.
3. Can the samples be stored frozen and analysed at a later date?	Yes. Samples are preferably stored at -70^{o}C and should not be refrozen once thawed.
4. At what temperature should the tests be carried out?	The tests will work at most temperatures but the rate of the reaction and the peak of luminescence will increase with increasing temperature. It is therefore essential that for any comparative work the tests are performed at the same controlled temperature.
5. What volume of sample should I use?	This will depend upon the nature of the sample and the test. For the superoxide test 50µL plasma or serum has been found to be optimal whereas 5µL plasma or serum was best for the halogenated antioxidant test. Larger volumes should be used in the halogenated test if the fluid is one in which albumin is not present in high concentrations.
6. Do I need to use a luminometer that has automatic injectors?	No. But the cuvette must be in position in front of the light detector when you inject Solution B for the superoxide test, or Chloramine T for the halogenated antioxidant test because the reactions are so rapid.

7. Do I need to mix the sample during the measurement of light?	No. The tests take between 15 and 30 seconds and the force of injecting Solution B or Chloramine-T is sufficient to mix the contents of the cuvette. However, be sure to mix the contents of the cuvette (a vortex mixer is ideal) before placing it in the luminometer.
8. Are there any special tubes and cuvettes that should be used?	No.
9. Can I use the antioxidant Pholasin® in the cell activation tests?	No. The Pholasin® is formulated differently.
10. What are Solutions A and B?	These are proprietary reagents that have been formulated in such a way that superoxide is produced instantaneously when Solution B is added to Solution A.
11. Can Solutions A and B be mixed together and then added to the sample?	No. Superoxide will be produced and consumed before you start the assay.
12. Can I use the Pholasin® in the cell activation kits for measuring antioxidant status?	No. The formulation is different.
13. How do these test compare to tests that measure total antioxidant status?	The ABEL® tests from KSL are designed to assess the capacity of a sample to scavenge particular groups of free radicals and/or oxidants: either superoxide-like free radicals or halogenated oxidants. As no antioxidant can scavenge every type of free radical or oxidant the present assays are more specific.

RECOMMENDED EQUIPMENT

Luminometer: We recommend a tube or microplate luminometer with automatic mixing/shaking, automatic injection and temperature control.

Syringes and needles: Antioxidant Pholasin® is supplied sealed under vacuum and is reconstituted to working strength by injecting Reconstitution and Assay Buffer through the rubber insert with a syringe and needle. Do not remove the rubber insert prior to inserting the needle because some powder might escape when the vacuum is released. Needles and syringes are not included in the kits. We recommend using 1 inch, 21 gauge needles and standard 5mL and 10mL syringes.

REAGENTS FOR SUPEROXIDE, HALOGENATED OXIDANT AND HYDROXYL RADICAL TESTS:

STORAGE CONDITIONS AND SHELF LIFE

REAGENT	FORMAT	TEMPERATURE	SHELF LIFE
PHOLASIN® (SUPEROXIDE, HYDROXYL RADICAL & HALOGENATED TEST)	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted	2-8°C; **DO NOT FREEZE**	up to 1 month
RECONSTITUTION & ASSAY BUFFERS FOR PHOLASIN®	Liquid	-20°C or lower	up to 12 months
		2-8°C	up to 1 month
RECONSTITUTION BUFFER FOR CHLORAMINE –T	Liquid	-20°C or lower	up to 12 months
		2-8°C	up to 1 month
SOLUTION A*	Liquid	Room temperature	up to 12 months
SOLUTION B*	Liquid		up to 12 months
D-MANNITOL STANDARD	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted	DISCARD UNUSED PRODUCT	
L-ASCORBIC ACID	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted	DISCARD UNUSED PRODUCT	
CHLORAMINE-T*	Powder	2-8°C	up to 12 months
	Reconstituted	DISCARD UNUSED PRODUCT	

** Can be stored frozen but Solution A may need gentle warming to dissolved crystals that may form.*

REAGENTS FOR PEROXYNITRITE TEST:

STORAGE CONDITIONS AND SHELF LIFE

REAGENT	FORMAT	TEMPERATURE	SHELF LIFE
PHOLASIN® (PEROXYNITRITE TEST)	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted	-20°C or lower	up to 1 month
PN RECONSTITUTION & ASSAY BUFFER	Liquid	-20°C or lower	up to 12 months
		2-8°C	up to 1 month
VIT E ANALOGUE VEA STANDARD	Freeze dried	-20°C or lower	up to 12 months
	Reconstituted	2-8°C in the dark	48 hours
SIN-1	Powder	-20°C or lower & dry	up to 12 months
	Solution	Discard any remaining	DISCARD
WATER FOR DISSOLVING SIN-1	Liquid	-20°C or lower	up to 12 months
		2-8°C	up to 1 months

REAGENTS FOR SUPEROXIDE QUANTIFICATION TEST:

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

ORDERING INFORMATION

	Catalogue number
Superoxide & other free radicals:	KSL-ABEL-21 (25 x 1mL tests) KSL-ABEL-22 (50 x 1mL tests) KSL-ABEL-23 (100 x 1mL tests) KSL-ABEL-24 (500 x 1mL tests) KSL-ABEL-21M2 (100 x 200µL tests, microplate)
Halogenated Oxidants:	KSL-ABEL-31 (25 x 1mL tests) KSL-ABEL-32 (50 x 1mL tests) KSL-ABEL-33 (100 x 1mL tests) KSL-ABEL-34 (500 x 1mL tests) KSL-ABEL-31M (100 x 200µL tests, microplate)
Peroxynitrite:	KSL-ABEL-41M2 (200 x 200µL tests, microplate) KSL-ABEL-41M3 (200 x 200µL tests, microplate)
Hydroxyl Radical	KSL-ABEL-51M (100 x 200 µL tests, microplate) KSL- ABEL-53 (100 x 1mL tests)
Enzyme generated Superoxide	KSL-ABEL-60M (100 x 200µL tests, microplate) KSL-ABEL-61M (200 x 200µL tests, microplate)

CONTRACT TESTING

Knight Scientific Limited offers contract testing and research services contact Dr Jan Knight for further information.

ABEL® products are developed and manufactured by:

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