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OXIDATIVE STRESS KITS

ABEL® ANTIOXIDANT TEST KIT
with **PHOLASIN®**
for **PEROXYNITRITE**

Microplate Test Kit
ABEL-40M2

A chemiluminescent test for assessing antioxidant capacity of a sample to scavenge peroxynitrite formed by the reaction between superoxide and nitric oxide released from SIN-1. This assay is especially good for clinical studies aimed at assessing oxidative stress arising from peroxyl radical-mediated lipid peroxidation. It can also be used for assessing antioxidants in food, cosmetics and other media.

CONTENTS

Kit components sufficient for 100 x 200µL tests

A. 1 x bottles Pholasin® (50µg: reconstitute to 5mL)
B. 1 x 50mL Reconstitution & Assay Buffer for Peroxynitrite assay
C. 2 x 12mg SIN-1 (mixed with an inert material)
D. 1 x 25mL Water for dissolving SIN-1
E. 1 x Vit E Analogue Standard: VEA Standard (reconstitute to 2mL)
F. 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: PEROXYNITRITE KIT	Liquid	-20°C or lower	12 months
		2-8°C	1 month
VEA STANDARD	Freeze Dried	-20°C or lower	12 months
	Reconstituted	2-8°C in the dark	48 hours
SIN-1	Powder	-20°C or lower & dry	12 months
	Solution	Discard any remaining	DISCARD
WATER	Liquid	-20°C or lower	12 months
		2-8°C	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.

All reagents must be kept at room temperature or if possible at the temperature at which the assay is run.

Pholasin® and Vitamin E Analogue (VEA) Standard are supplied in vials that have been sealed under vacuum. It is important that you do not remove the rubber insert until the bottles have been reconstituted with buffer that has been injected through the septum.

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

INTRODUCTION

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, $C_6H_{10}N_4O_2 \cdot HCl$).

$O_2^{\bullet -}$ (superoxide) + $NO^{\bullet}$ (nitric oxide) $\rightarrow$ $ONOO^-$ (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 gradually be consumed, delaying the time at which the maximum peak of light occurs, which because of competition with Pholasin® is of lower intensity.

The time at which peak luminescence occurs (peak time) after adding SIN-1, is expressed in Vit 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.

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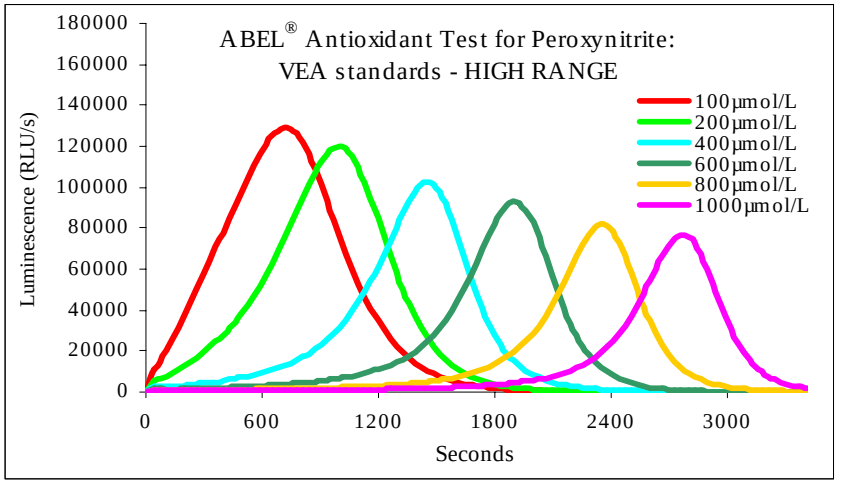
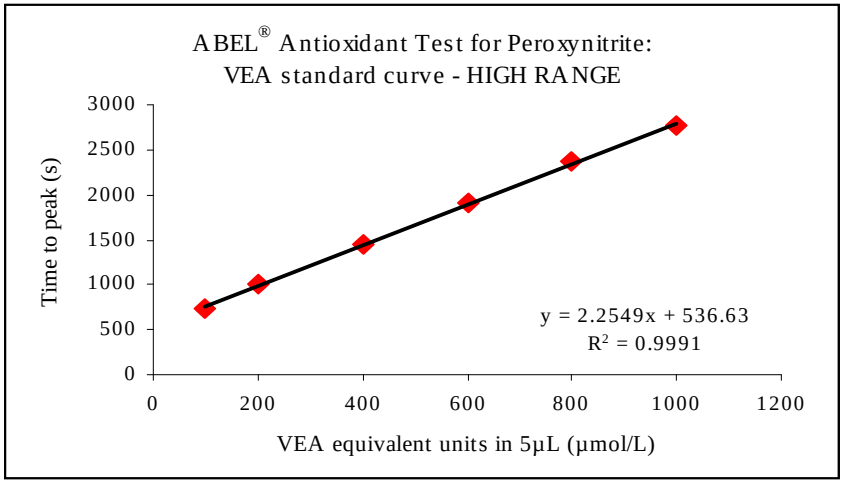




The antioxidant capacity of a sample can be expressed in VEA equivalent units (µmol L^{-1}) from a standard curve of concentration of VEA in 5µL sample against time to peak luminescence (after addition of SIN-1). Sample equivalent concentrations are best obtained from the linear regression of the times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

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.

1. Pipette into a well: 50µL Pholasin
   100 µL of VEA standard + R&A PN Buffer,
   as shown in the tables below
2. Inject 50µL SIN-1 when the well is in the light measuring position.
3. The total volume in the well is 200µL

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 assay will work at ambient temperatures or above; the total assay time can be reduced significantly by running the assay (and standards) at a temperature higher than 25°C (such as 30°C).

EQUIPMENT REQUIRED

A microplate luminometer with temperature control and automatic injectors is ideal. However, if an injector is not available then SIN-1 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®

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

1. Load a syringe with 5mL R&A PN 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® for the Peroxynitrite Antioxidant Assay can be stored frozen at –20°C. After reconstitution remove sufficient for use on a particular day and freeze remaining volume.

Dissolving SIN-1

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

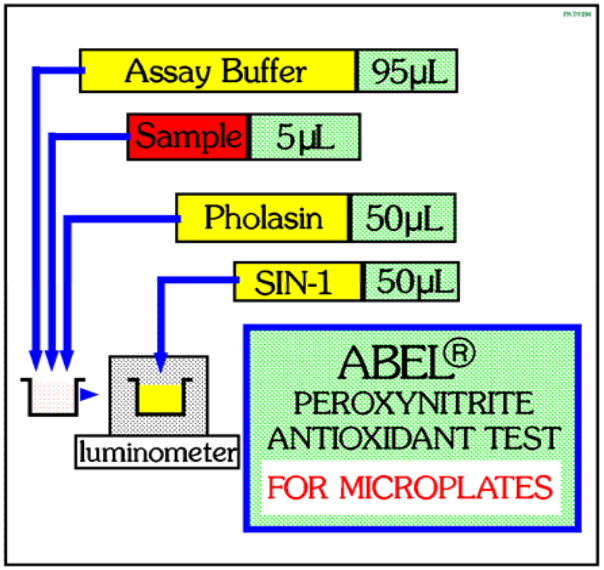
Reconstitution of the VEA standard

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°C.

1. Load a syringe with 2mL R&A PN buffer.
2. Fit a needle (1 inch, 21 gauge) to the syringe.

3. 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.
4. Replace the screw cap and invert and roll the bottle until the contents are fully dissolved.
5. 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
6. 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.
7. Any unused reconstituted VEA standard can be stored in the dark at 2-8°C for up to 2 days.

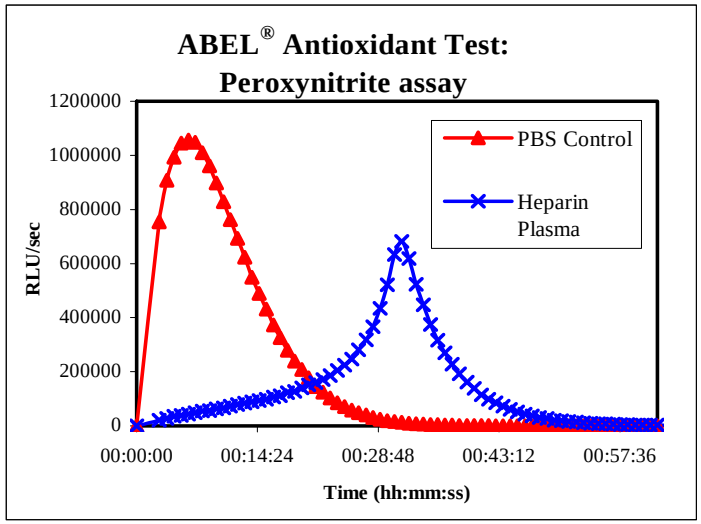
TEST PROCEDURE



1. Pipette into each well of a microplate:
   - 100µL assay buffer (control) or 95µL assay buffer + 5µL sample
   - 50µL reconstituted Pholasin®
2. Always include at least four different concentrations of the VEA standard with each experimental run to be able to express the results in VEA equivalent units. To choose the right concentrations see 'Standard curves for VEA standard'.

3. Inject 50µL of 2mg mL^{-1} solution of SIN-1 into each well, preferably with an automatic dispenser. Alternatively, add SIN-1 very quickly before putting the plate in the luminometer.
4. Measure light for 0.5 to 1.0 second in each well; measure each well in turn.
5. Repeat cycle of measurements for a total of 60 minutes in the first instance; this time may be reduced (or extended) in order to obtain peak time of sample. Keep intervals between repeat measurements to a minimum.
6. The total assay time can be reduced significantly when the assay is run at 30°C instead of ambient.

TYPICAL ANALYSIS



STANDARD CURVE FOR VITAMIN E ANALOGUE (VEA) STANDARD

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 table on p6) together with 2-3 of your