ABEL® ANTIOXIDANT TEST KIT
for HYDROXYL RADICALS
with PHOLASIN®

Microplate Luminometer Kit

ABEL-51M

A chemiluminescent test for measuring the capacity of a sample of fluid such as water, food extract, plasma, serum, etc to scavenge free radicals such as the hydroxyl radical

CONTENTS

Kit components sufficient for 100 x 200mL tests

A. 1 x bottle antioxidant Pholasin® for 100 tests

B. 1 x 25mL Reconstitution and Assay Buffer for Hydroxyl radical microplate assay

C. 1 x 25mL Solution A

D. 1 x 10mL Solution B

E. 1 x D-mannitol standard (reconstitute 1mL: 8mmol L^{-1})

F. 1 x D-mannitol standard (reconstitute 2mL: 80mmol L^{-1})

G. 1x 96 well white luminometer microplate

REAGENT	FORMAT	TEMPERATURE	SHELF LIFE
PHOLASIN (Antioxidant)	Freeze Dried	-20ºC	up to 12 months
	Reconstituted	DISCARD UNUSED PRODUCT	
RECONSTITUTION & ASSAY BUFFER FOR PHOLASIN	Frozen	-20ºC or lower	up to 1 month
	Liquid	2-8ºC	up to 12 months
SOLUTION A	Liquid	Room Temp.	up to 12 months
SOLUTION B	Liquid	2-8ºC	up to 12 months
D-MANNITOL STANDARDS	Freeze Dried	Room Temp or lower	up to 12 months
	Reconstituted	2-8ºC	up to 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 at room temperature (or above) at the start of the assay. Crystals may form in Solution A during storage at 2-8^{o}C but they will dissolve easily with gentle warming.

Pholasin® and D-mannitol 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.**

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

INTRODUCTION

The hydroxyl radical is the most reactive free radical known and will attack virtually anything in its path. It should, however, be noted that many organic solvents used to dissolve ingredients used in cosmetic preparations, such as ethanol, methanol and DMSO, are very strong quenchers of hydroxyl radical. While it is possible to control for the antioxidant properties of such solvents, the halogenated oxidant and peroxynitrite assays are recommended as the most appropriate assays for materials dissolved in such solvents.

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.

This kit can also be used to generate an ABEL-RAC mg hydroxyl radical score. Contact Knight Scientific for more information.

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®

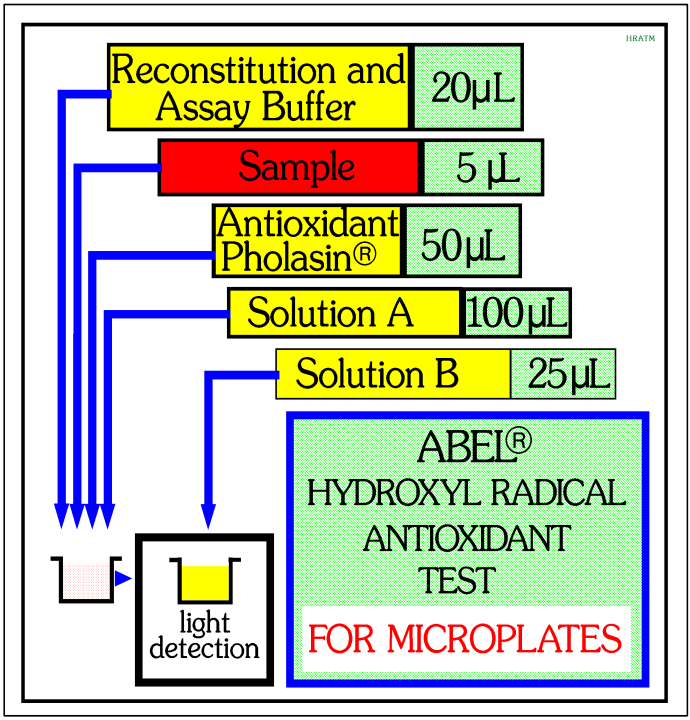
100µL Solution A

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.

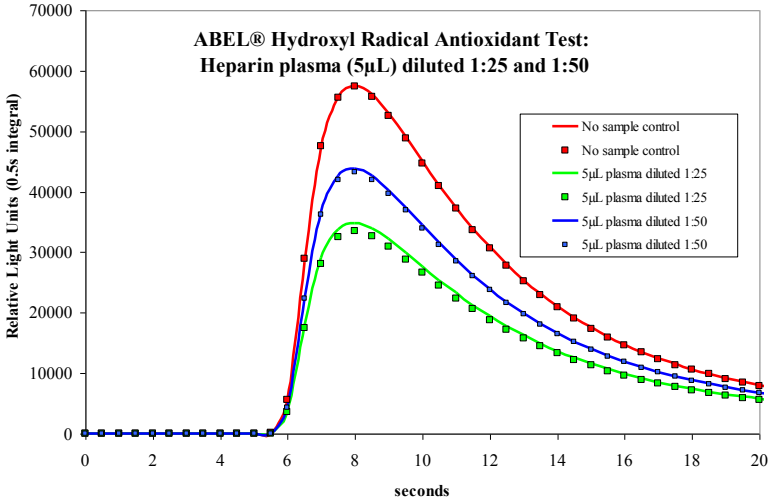
The antioxidant capacity of the sample can be expressed as the percentage reduction of peak luminescence as follows:

[(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).



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**]

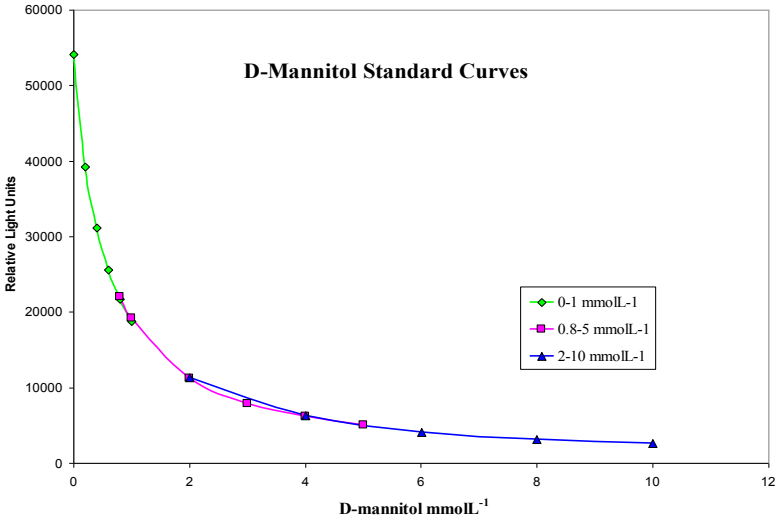
- The total volume in the well is now 200µL.

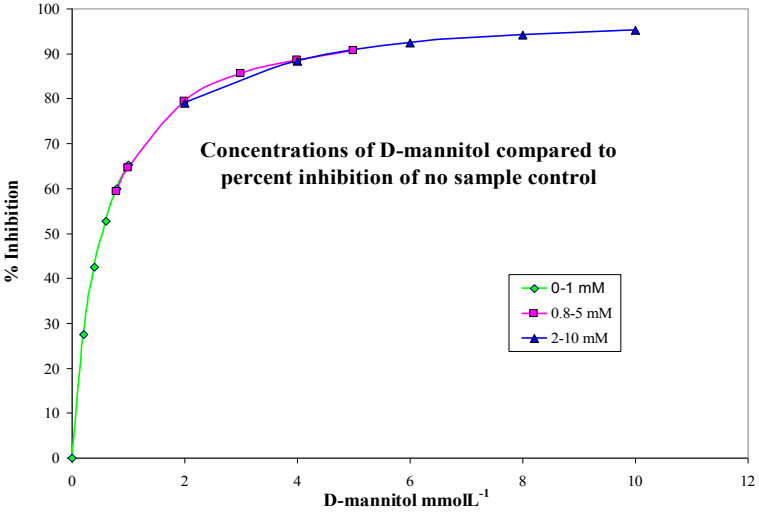
Table Low Range. Volumes of D-mannitol and R&A Buffer added to the microplate well. Low range: 0-1 mmol L^{-1} equivalent to 0-40 mmol L^{-1} D-mannitol in 5µL sample

8 moll L^{-1} D-mannitol (µL)	**Recon & Assay Buffer (µL)**	**D-mannitol (mmol L^{-1}) in 200µL**	**D-mannitol equivalent units (mmol L^{-1}) in 5µL sample**
0	25	0	0
5	20	0.2	8
10	15	0.4	16
15	10	0.6	24
20	5	0.8	32
25	0	1	40

Table High Range. Volumes of D-mannitol and R&A Buffer added to the microplate well. High range: 2-10 mmol L^{-1} equivalent to 0-400 mmol L^{-1} D-mannitol in 5µL sample.

80 mmol L^{-1} D-mannitol (µL)	**Recon & Assay Buffer (µL)**	**D-mannitol (mmol L^{-1}) in 200µL**	**D-mannitol equivalent units (mmol L^{-1}) in 5µL sample**
0	25	0	0
5	20	2	80
10	15	4	160
15	10	6	240
20	5	8	320
25	0	10	400





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

Analysis By ABEL Emitted Light

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