ABEL® ANTIOXIDANT TEST KIT
for SINGLET OXGYEN with SPECIAL PHOLASIN®

ABEL®-81M
Microplate Luminometer Test Kit

A chemiluminescent antioxidant test for measuring the capacity of samples to scavenge singlet oxygen.

CONTENTS

Kit components sufficient for 100x 200µL tests

A. 1x Special Antioxidant Pholasin® (reconstituted to 5ml)
B. 1x Singlet Oxygen Primer (reconstituted to 5ml)
C. 1x Singlet Oxygen Generator (reconstituted to 5ml)
D. 1x Reconstitution Buffer for Singlet Oxygen Generator (25ml)
E. 1x Reconstitution and Assay Buffer (25ml)
F. 1x L-Histidine standard (reconstituted to 5mM)
G. 1x 96 well microplate

STORAGE CONDITIONS AND SHELF LIFE

Reagent	Format	Temperature	Shelf Life
Antioxidant Pholasin®	Freeze Dried	-20°C or lower	12 months
	Reconstituted	DISCARD UNUSED PRODUCT	
Singlet Oxygen Primer	Powder	-20°C or lower	12 months
	Reconstituted	2-8°C	1 month
Singlet Oxygen Generator	Powder	-20°C or lower	12 months
	Reconstituted	DISCARD UNUSED PRODUCT	
Reconstitution Buffer for Singlet Oxygen Generator	Frozen	-20°C or lower	12 months
	Liquid	2-8°C	1 month
Reconstitution and Assay Buffer	Frozen	-20°C or lower	12 months
	Liquid	2-8°C	1 month
L-Histidine Standard	Freeze Dried	-20°C or lower	12 months
	Reconstituted	DISCARD UNUSED PRODUCT	

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. Pholasin® and Histidine are supplied in a vial that has been sealed under vacuum. It is important that you **do not remove the rubber insert before reconstituting**. Please read the instructions carefully.
Do not use any damaged items; contact your supplier for advice.

INTRODUCTION

ABEL® is an acronym for 'analysis by emitted light.' The ABEL® antioxidant assays, of Knight Scientific Limited, are designed to generate light from a reaction between the photo-protein Pholasin® and a variety of reactive oxygen species (ROS). The sample, once introduced into the assay, will compete with Pholasin® for the ROS; the result of this competition is a reduction in the amount of light emitted as compared to the assay in which no sample is present, which is sometimes referred to as 'quenching.' An ABEL®-RAC (relative antioxidant capacity) mg score can then be calculated and used in various quality control measures.

ANTIOXIDANT TESTING USING SINGLET OXYGEN

The ABEL®-80M antioxidant assay provides a method for measuring the capacity of a sample to quench singlet oxygen generated within the assay. It is an excellent means to determine the suitability of materials as antioxidants with the potential to prevent lipid oxidation resulting from singlet oxygen attack.

Edible oils and lipids in general are readily oxidized during processing and storage via photosensitized oxidation, in which singlet oxygen initiates an attack on double bonds. The conjugated and non-conjugated lipid hydroperoxides formed by singlet oxygen attack lead to the production of off-flavor compounds with the reduction in quality of the oil.

Other factors that affect the oxidative stability of edible oils are temperature, light, processing and fatty acid composition of the oils. The fatty acid composition of the oils is especially relevant to their oxidative stability. Fish oils, for example, are especially prone to oxidative attack by singlet oxygen because they contain significant amounts of polyunsaturated omega-3 fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

While peroxyl radicals also play a significant role in the oxidation of lipids, the initial attack is usually initiated by singlet oxygen, leading then to the production of peroxyl radicals.

Introducing antioxidants with high affinities for singlet oxygen into processed foods with high lipid content can protect the food from attack by singlet oxygen, and thus extend its shelf life.

The ABEL®-80 singlet oxygen test is used to assess the antioxidant capacity of materials against attack by singlet oxygen.

WHAT IS SINGLET OXYGEN?

Molecular oxygen, though a free radical, is chemically inert due to its two unpaired electrons in its outermost orbit having the same quantum number which imposes a spin restriction as electrons spinning in the same direction are unreactive. This restriction can be removed by moving one of the unpaired electrons in a way that alleviates this restriction. This mechanism requires input of energy and generates the singlet states of oxygen: Delta singlet oxygen (^1D_g O_2) and Sigma singlet oxygen ($^1S_g O_2$) with Delta singlet oxygen the most common in biological systems and usually decays to the Sigma state.

PROTOCOL

All reagents must be at ambient temperature before use.

Reconstitution of Pholasin®

The Pholasin® has been specially formulated to be reconstituted with 5mL of Reconstitution and Assay Buffer.

1. Load a syringe with 5mL Reconstitution and 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.
4. Then carefully remove and dispose of the needle (using good laboratory practice).
5. Replace the screw cap and invert and roll the bottle at least 5 times to dissolve the contents.
6. Reconstituted antioxidant Pholasin® must not be frozen and should be stored at 2-8oC for up to 1 day. Any leftover reconstituted Pholasin® must be discarded

Reconstitution of the Histidine Standard

- Follow the same procedure for the reconstitution of Pholasin®, however, use 2ml of the Reconstitution and Assay Buffer for Singlet Oxygen in place of 5ml.

Reconstitution of Single Oxygen Primer

- Pipette 5ml of the Reconstitution and Assay Buffer for Singlet Oxygen into the bottle with powdered the Single Oxygen Primer.
- Invert and roll the bottle at least 5 times to dissolve the contents.

Reconstitution of Single Oxygen Generator

- Pipette 5ml of the Reconstitution and Assay Buffer for Single Oxygen Generator into the bottle with the powdered Single Oxygen Generator.

SAMPLE PREPARATION

1. **Identify the correct solvent for your sample**
   - Place a small amount of sample into a microcentrifuge tube, and add the solvent you wish to identify.
   - Repeat this with multiple solvents to ensure correct identification. Note the solvent that best dissolves the sample.

   If testing oil, check the sample in 100% and 50% acetone.

2. **Dissolve your sample in the appropriate solvent at a concentration of 10mg/mL.**
   - For solid and oil samples, weigh out a mass of the sample, and dilute to the above concentration with the appropriate solvent (e.g. 50mg of sample in 5mL of solvent).
   - For liquid samples with a mass similar to water, make the dilution with 10uL/mL (e.g. 50uL of sample in 5mL of solvent)

   Ensure it has fully dissolved before use.

3. **Create a set of 1:10 dilutions.**
   - You will need 5 dilutions to test the sample at different concentrations.
   - If your solvent is water/buffer, follow the 'Water-soluble samples' diagram on the following page.

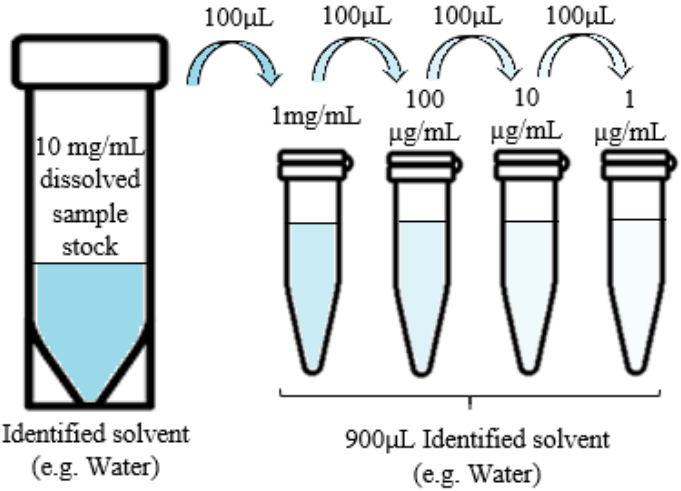
     Then test the following concentrations:
     10mg/mL, 1mg/mL, 100µg/mL, 10µg/mL, 1µg/mL.
   - If your solvent is ethanol or acetone, follow the 'Oil soluble samples' diagram on the following page.

     Then test the following concentrations:
     1mg/mL, 100µg/mL, 10µg/mL, 1µg/mL, 100ng/mL.

Water-soluble samples:

For samples dissolvable in water or buffer

1:10 Dilution: Example for sample in water

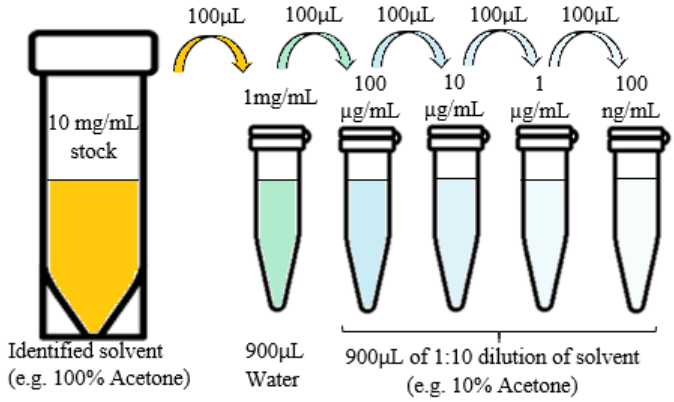


Oil soluble samples:

For oils, dissolve in 100% or 50% acetone.

For other samples try 100% or 50% Ethanol.

1:10 Dilution: Example for Oils in Acetone



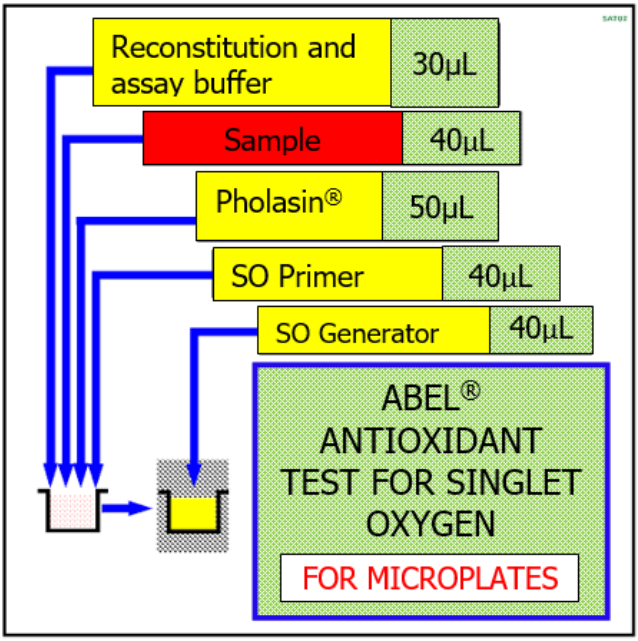
TEST PROCEDURE

1. Prime the luminometer injector with the Singlet Oxygen (SO) Generator.

2. Pipette the following reagents, in the following order, into each well of the microplate:
   - 40μl diluted sample or solvent control
   - 30μl Reconstitution and Assay Buffer for Singlet Oxygen
   - 40μl Single Oxygen Primer
   - 50μl special antioxidant Pholasin®

3. Place the microplate into the luminometer and run as follows: 40μl Singlet Oxygen Generator injected per well after 10 seconds of baseline analysis, and wait for a further 30 seconds.

SUMMARY



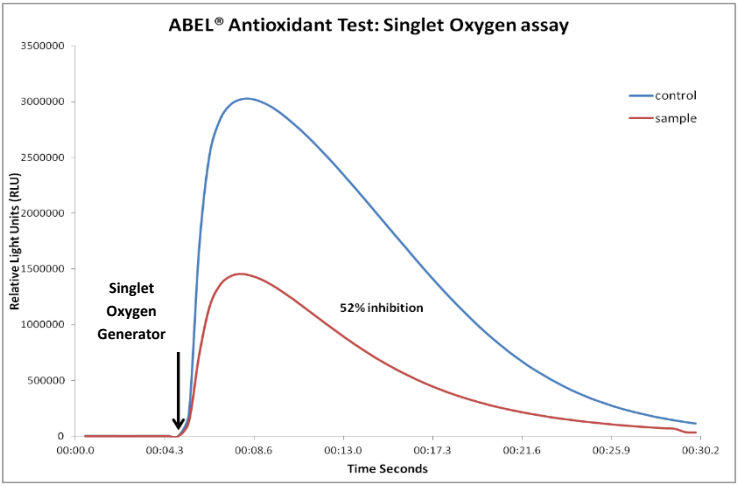
RESULTS

After the assay is finished, obtain the data results and compare the various dilution concentrations to the control.
Identify the most suitable concentration to focus on in further tests. This will be the concentration that gives a relative light unit (RLU) score that is 50% of the solvent control.
Carry out repeats on this concentration to find the average RLU. From this average RLU and the solvent control RLU, you can calculate the percentage inhibition for the sample at a fixed concentration.

For example, a sample with a 50% inhibition at 1µg/mL can quenched more singlet oxygen that a sample with 50% inhibition at 100µg/mL

For further analysis, refer to the ABEL-RAC test kit available from KSL.

TYPICAL ANALYSIS



STANDARD CURVE FOR L-HISTIDINE STANDARD

1. Reconstitute the histidine standard according to the protocol.

2. Prepare the Histidine concentrations required.

Follow the table below to make up 6 Histidine concentrations to be used to create the standard curve.

For example, pipetting 10µL of 5mM Histidine into 190µL of assay buffer gives a Histidine solution with a concentration of 0.25mM, which will be diluted when added to the assay to a final concentration of 0.05mM.

5mM Histidine required (µl)	Reconstitution and assay buffer for singlet oxygen (µl)	Histidine equivalent units in 200µl dilution (*mM*)	Histidine concentration in 200µl assay final volume (*mM*)
10	190	0.25	0.05
20	180	0.5	0.1
40	160	1	0.2
60	140	1.5	0.3
80	120	2	0.4
100	100	2.5	0.5

3. Pipette into the well: 40µl histidine dilution sample (prepared according to the table), 30µl Reconstitution and Assay Buffer, 40µl Urea Peroxide, and 50µl Pholasin®.

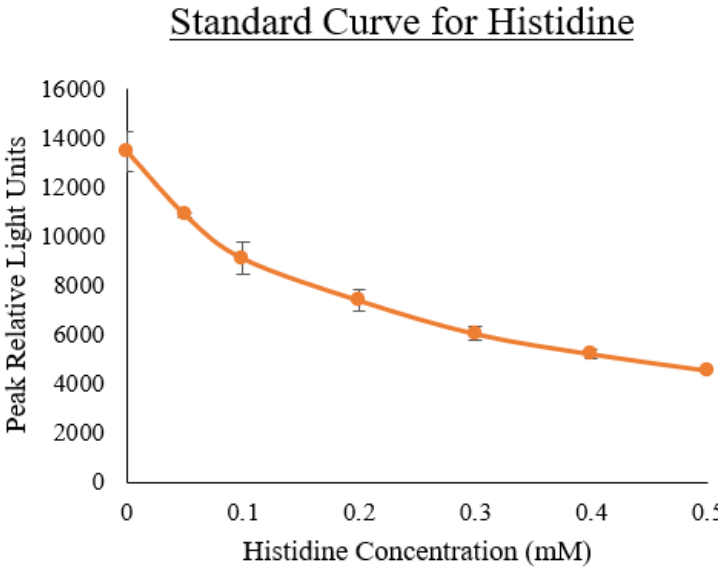
4. Repeat with each concentration, including a buffer control.

5. Place the microplate into the luminometer and run as follows:

40µl Single Oxygen Generator injected per well after 10 seconds of baseline analysis, and wait for a further 30 seconds.

6. Plot the maximum relative light units for each concentration of histidine onto a standard curve, as seen below.

The antioxidant capacity of a sample can be expressed as equivalent to *mM Histidine* from this standard curve of differing concentration of Histidine plotted against time of peak luminescence (after the injection of Singlet Oxygen Generator). Sample equivalent concentrations are best obtained from the linear regression of the times to peak.



In such linear regressions, the *x* axis represents time to peak and the *y* axis represents *mM* of Histidine standard. By multiplying the *x* value of the sample by the dilution factor (5), the Histidine equivalent units will be obtained.

ANTIOXIDANT TEST KIT

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