ABEL®-RAC™

Test Kit to be used with a variety of ABEL® Chemiluminescent Antioxidant Test Kits with Pholasin®

ABEL®-RAC™ TEST KITS

TO BE USED WITH A VARIETY OF

ABEL® ANTIOXIDANT CHEMILUMINESCENT TEST KITS with PHOLASIN®

CONTENTS

Kit components sufficient for sample dilutions to produce four ABEL®-RAC mg determinations

A. 65 x 1.5mL microcentrifuge tubes
B. 5 x 15ml tubes
C. ABEL®-RAC™ Analysis Template specific for each Reactive oxygen species (ROS)

WHAT IS ABEL®-RAC?

ABEL®-RAC™ [1] is a method to quantify the antioxidant activity or power of a mass (usually mg) of a finished product or the individual ingredients in the product against different reactive oxygen (ROS) challenges. The ABEL®-RAC™ score is usually expressed as a unit of antioxidant activity per mg[2] of test material. It is a highly reproducible score, which is especially important when trying to re-produce batches of products made from natural ingredients.

Just recreating the formula is unlikely to lead to a new batch of product with the exact same or better efficacy even when using material from the same supplier in which the concentration of the active components are quoted as the same or higher. This is because the antioxidant **activity** of a material, that is how good the material is at neutralising different reactive oxygen species (ROS), is the result of a multitude of complex factors.

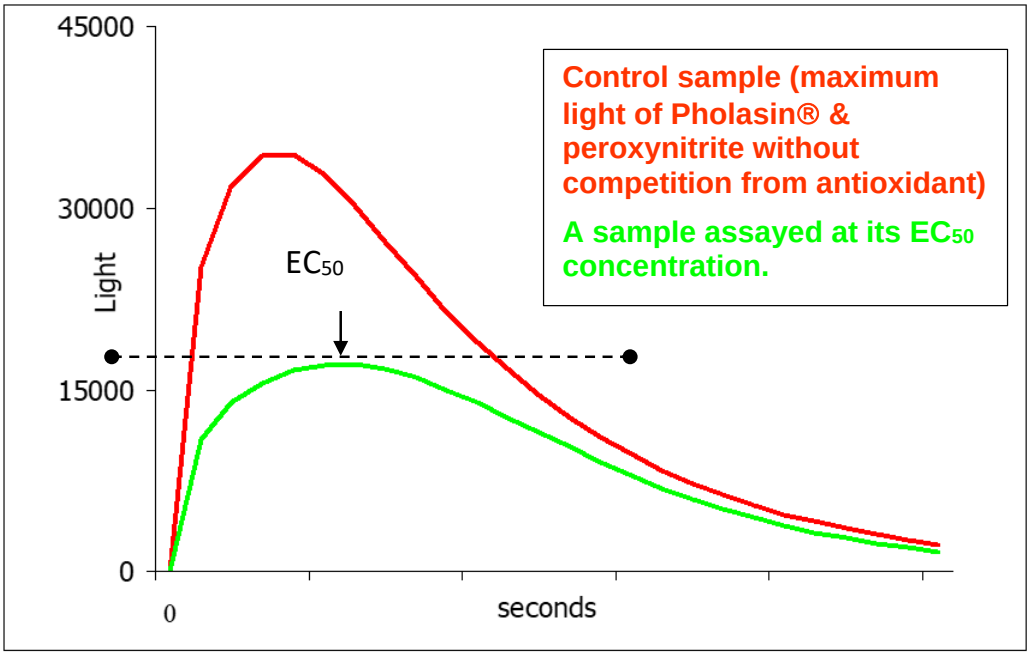
It is sometimes beneficial to obtain ABEL®-RAC™ scores using a range of solvents in order to determine the activity of the water or oil soluble

[1] Analysis By Emitted Light—Relative Antioxidant Capacity
[2] Other mg score can be converted into other ABEL-RAC units such as g or kg for example.

components in a natural material.

ABEL®-RAC and EC_{50}

ABEL®-RAC™ is intimately linked to the value of the EC_{50}. The figure below shows a simplistic diagram illustrating the relationship between the control sample where 100% light is emitted from Pholasin® when challenged (in this case) with and the EC_{50}.



The EC_{50} is the concentration of a material that has sufficient antioxidant capacity to compete with Pholasin® for a particular ROS enough to reduce the light of the Solvent Control by 50%. The EC_{50} value (mg/mL) of a sample is calculated from the equation of a linear regression obtained from plotting the sample concentration (mg/mL) against peak light values taken from the light response curves. ABEL®-RAC™ scores are calculated by multiplying the reciprocal of the EC_{50} value by 100.

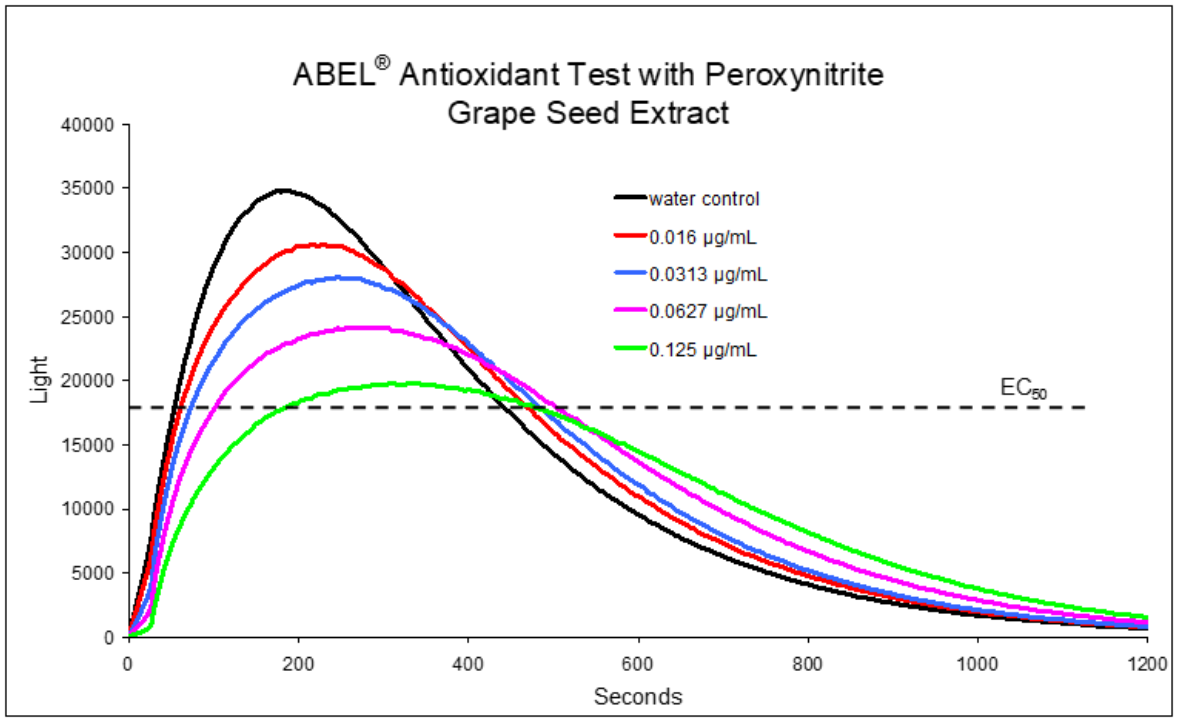
Generating the Data to Produce an ABEL-RAC™ Score

To obtain an accurate measure of the EC_{50} a sample to be tested must first be diluted, sometimes by a number of orders of magnitude. The object of these dilutions is to obtain a range of concentrations that when measured in the assay result in maximum light values proportional to their concentration.

Access to a template, specific for each ABEL Antioxidant ROS assay, will be provided by a link to an Excel workbook with a pass word to open the Excel file specific for a particular ABEL® Antioxidant test. When peak light values for each concentration are put into a table in the template an instant calculation of the EC_{50} and derived ABEL-RAC™ mg score is obtained. You can then save and print the template.

The template uses the linear regression equation.

A series of concentrations are measured, and the concentration leading to an approximate 50% reduction in emitted light is determined. This is used to create a further narrower range of concentrations, of which the maximum peak light (measured as relative light units (RLU)) for each concentration is used to create a linear regression of 3 - 4 points. We suggest 3 to 4 points as we aim to obtain at least 3 points that lie above the theoretical EC_{50} value. By analysing this regression, the ABEL®-RAC score can be calculated.



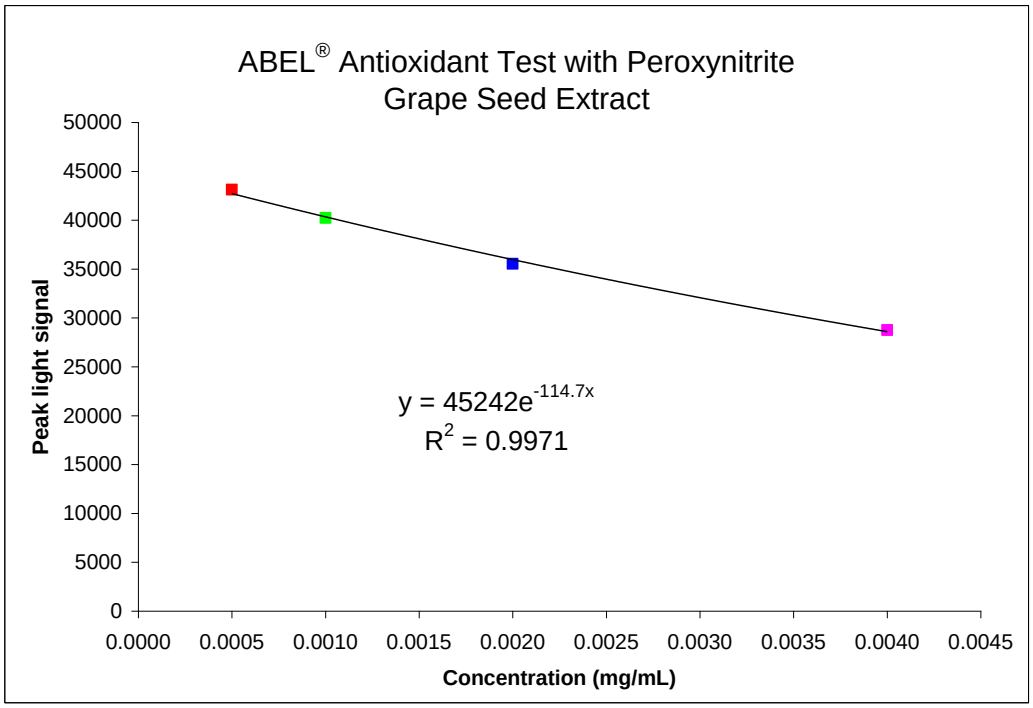
Antioxidant Capacity Scores: EC_{50} Values and ABEL-RAC™ Scores

Separate light response curves are produced for a range of sample concentrations as well as a solvent control (see example above). The peak light values of each concentration are used in the calculation.

The EC_{50} of a material is related to its antioxidant capacity. The greater the amount of material required to reduce the light by half, the weaker the antioxidant capacity. Therefore, high EC_{50} values indicate low antioxidant capacity.

Calculating the EC_{50} using the KSL template

The EC_{50} value (mg/mL or µL/mL) of a sample is calculated from the equation of an exponential regression analysis obtained from plotting the sample concentration (mg/mL or µL/mL) against peak light values taken from the light response curves (see below).



To obtain a reliable EC_{50} value, at least 3 sample concentrations are needed, which should produce a peak light signal of between 0% and 50% of the solvent control peak. At least one concentration's peak should be close to the no solvent control and another just above 50% of the solvent control peak. It is essential that you have at least 3 points producing a curve of nearly perfect linearity. This is necessary because the EC_{50} is a derived value of the concentration on the graph at which the regression curve crosses the actual 50% light value of the solvent control.

The following equation is generated from such an exponential regression:

$$y = Ae^{Bx}$$

This equation can be rearranged so that the EC_{50} in mg/mL or µL/mL (x) may be calculated when y = 50% of the maximum light value of the solvent control:

$$x = (\ln(y/A))/B$$

(A & B are values returned from modelling the exponential curve)

Converting the EC_{50} value of a sample to an ABEL-RAC score

To make the test results more readily understandable, the EC_{50} values of samples are converted to a positive ABEL-RAC™ score for each free radical or oxidant used to challenge the test material. ABEL-RAC™ scores are calculated by multiplying the reciprocal of the EC_{50} value by 100. Therefore, the higher the ABEL-RAC™ score per mg or µL, the higher the antioxidant capacity of the sample. This becomes a much easier value to understand. The higher the value the greater the antioxidant capacity.

ABEL-RAC™ scores are adjusted to take into account any dilutions made of sample working concentrations when adding the sample to the assay mixture in cuvettes or microplate wells.

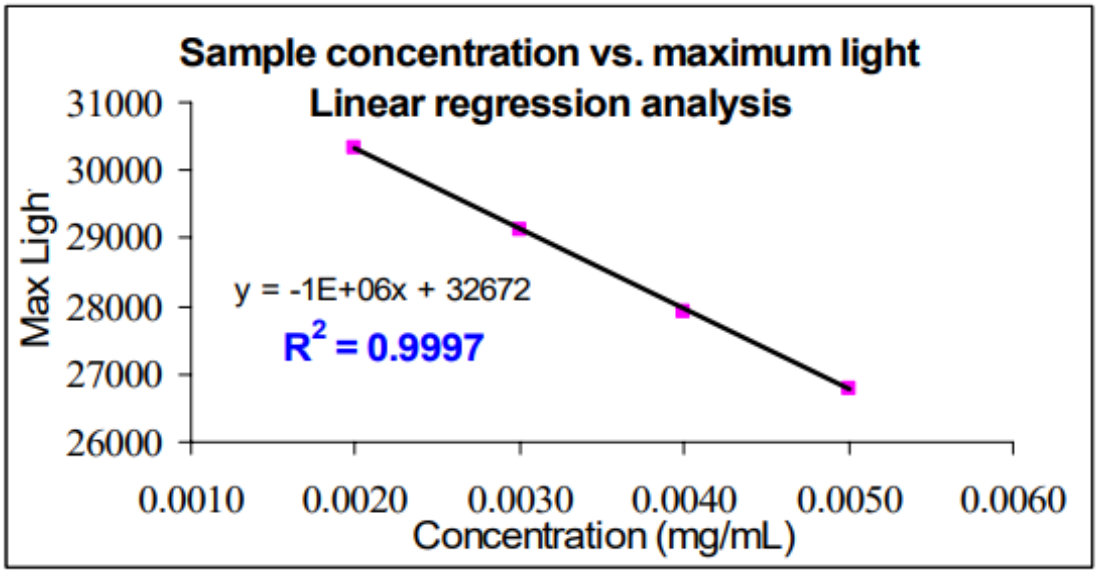
The above is performed in the template with all steps are shown on the template.

ABEL-RAC mg scores

ABEL®-RAC mg scores are the reciprocal of the EC_{50} multiplied by 100 ($1/EC_{50}$ x 100). The higher the ABEL®-RAC mg score the higher the antioxidant capacity of the sample.

ABEL®-RAC scores are expressed per mg of dried material or µL of a liquid for each of the ROS used to challenge the material. There will be different ABEL®-RAC scores for each ROS challenge used as the material under test is likely to contain molecules of greater or lesser affinity for particular ROS.

In order to calculate the theoretical EC_{50} of a sample a linear regression analysis is performed (automatically in the template) on the relationship between sample concentration (mg or µL) and maximum light values, obtained from light response curves, of a range of sample concentrations (see below).



Some materials will be better antioxidants against some ROS than others. These scores can be used directly in formulations and can also be used to identify positive and negative synergies between more than one material. Also, carrying out antioxidant assays with the material to be tested dissolved in different solvents such as water, 100% alcohol and 50% alcohol will lead to the calculation of different ABEL®-RAC scores which will give an insight into the nature of the material being tested.

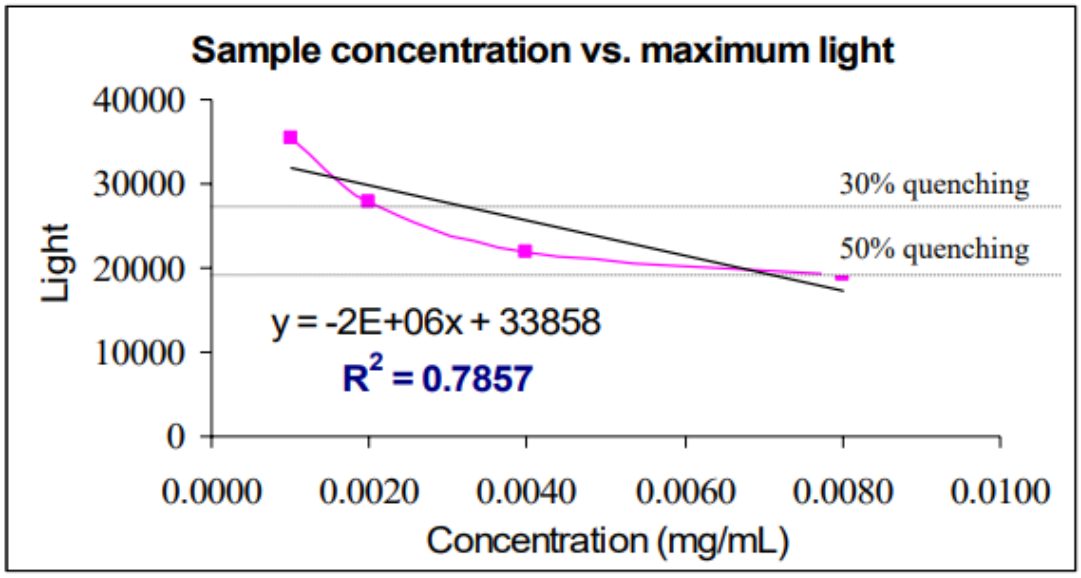
There are several important factors to be considered when using the **ABEL-RAC™ linear template:**

1. A narrow range of **3 - 4 sample concentrations** must be used in order for an accurate curve to be plotted automatically in the template.
2. **The R^2 value for the linear regression curve must be ≥ 0.9900.** In order that the test concentrations fall within a linear range we recommend that the strongest sample concentration used should result in no more than 25-30% light quenching. (Although as long as the relationship is still linear this is not mandatory). This is why we suggest 3-4 samples as sometimes only 3 samples above this cut-off point are available.

We have found that when the concentration of an antioxidant sample is increased beyond the level at which ~30% light quenching is achieved a saturation effect begins to take place in which increasing the antioxidant concentration appears to have diminishing returns with respect to antioxidant activity (i.e. reduction in the light signal).

Eventually a point is reached when no matter how much you increase the concentration of a sample you will not get any further quenching of light (i.e. when quenching = 100%). When this saturation effect occurs, the relationship between sample concentration and maximum light strays away from a linear model (see below) and the linear template cannot be applied (R^2 values will fall below 0.99).

The theoretical EC_{50} value used in the calculation is the point at which the linear regression curve crossed the 50% maximum light obtained from the No Sample Solvent Control.



INSTRUCTIONS FOR USING THE ABEL-RAC™ LINEAR TEMPLATE

1. **For a new sample with unknown antioxidant capacity, test a wide range of concentrations in the first assay by following the instructions bellow. When carrying out the assay, refer to your specific antioxidant kit instructions.**

SAMPLE PREPARATION

A. Identify the correct solvent for your sample

1. Place a small amount of sample into a microcentrifuge tube, and add the solvent you wish to identify.
2. 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.

B. Dissolve your sample in the appropriate solvent at a concentration of 10mg/mL.

1. 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).
2. 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.

C. Create a set of 1:10 dilutions.

1.You will need 5 dilutions to test the sample at different concentrations.

2.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.

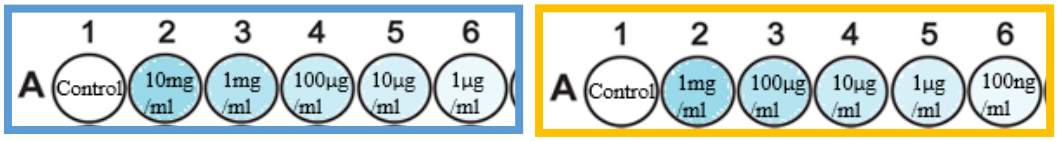
3. If your solvent is ethanol or acetone, follow the 'Oil soluble

samples' diagram on the following page.

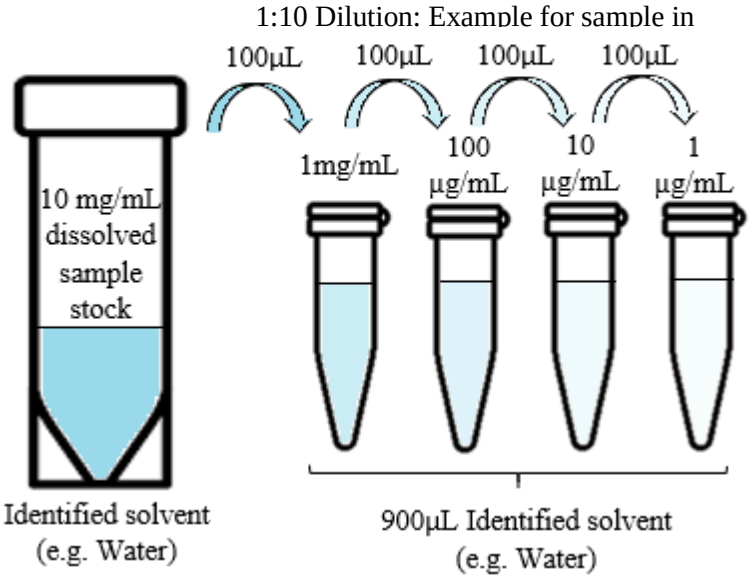
Then test the following concentrations:
1mg/mL, 100µg/mL, 10µg/mL, 1µg/mL, 100ng/mL.

Ensure you carry out a solvent control, as many solvents (ethanol, acetone) have antioxidant properties.

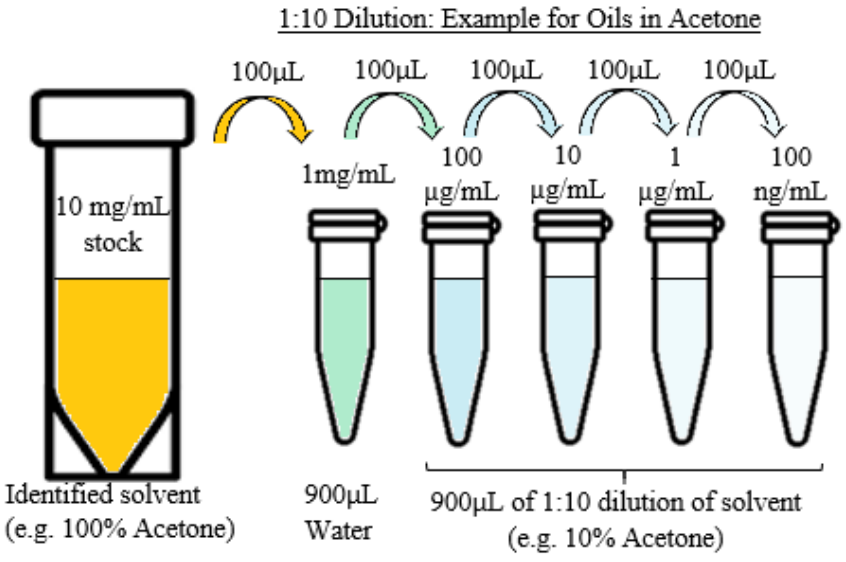
Below is the recommended plate layout for both variations of dilutions:



Water-soluble samples:
For samples dissolvable in water or buffer



Oil soluble samples:
For oils, dissolve in 100% or 50% acetone.
For other samples try 100% or 50% Ethanol.

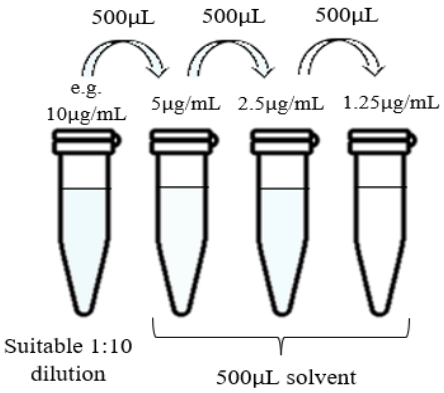


2. Based on the results of the first test, estimate the approximate concentration which produces a 50% decrease in the light signal, compared to the solvent control. If required, re-test a couple of extra concentrations to check this.

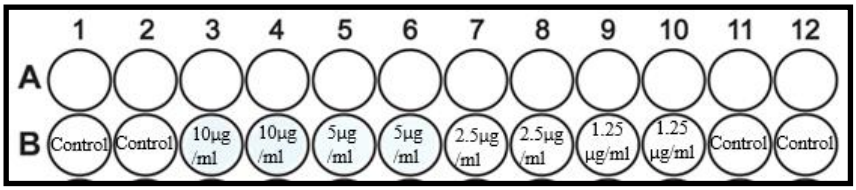
3. From the estimated concentration that causes ~50% reduction in light, make a narrow range of dilutions to be tested in the next assay. These concentrations should be tested using adjacent duplicates to obtain reliable results.

NARROW RANGE 1:2 DILUTIONS

Using the concentration identified as suitable by the previous 1:10 dilutions, create a series of further 1:2 dilations by diluting 500µL of the concentration in 500µL of solvent. Follow the figure on the right, using the example of 10µg/mL as the starting concentration.



When using a microplate reader, we suggest that the following layout is used:

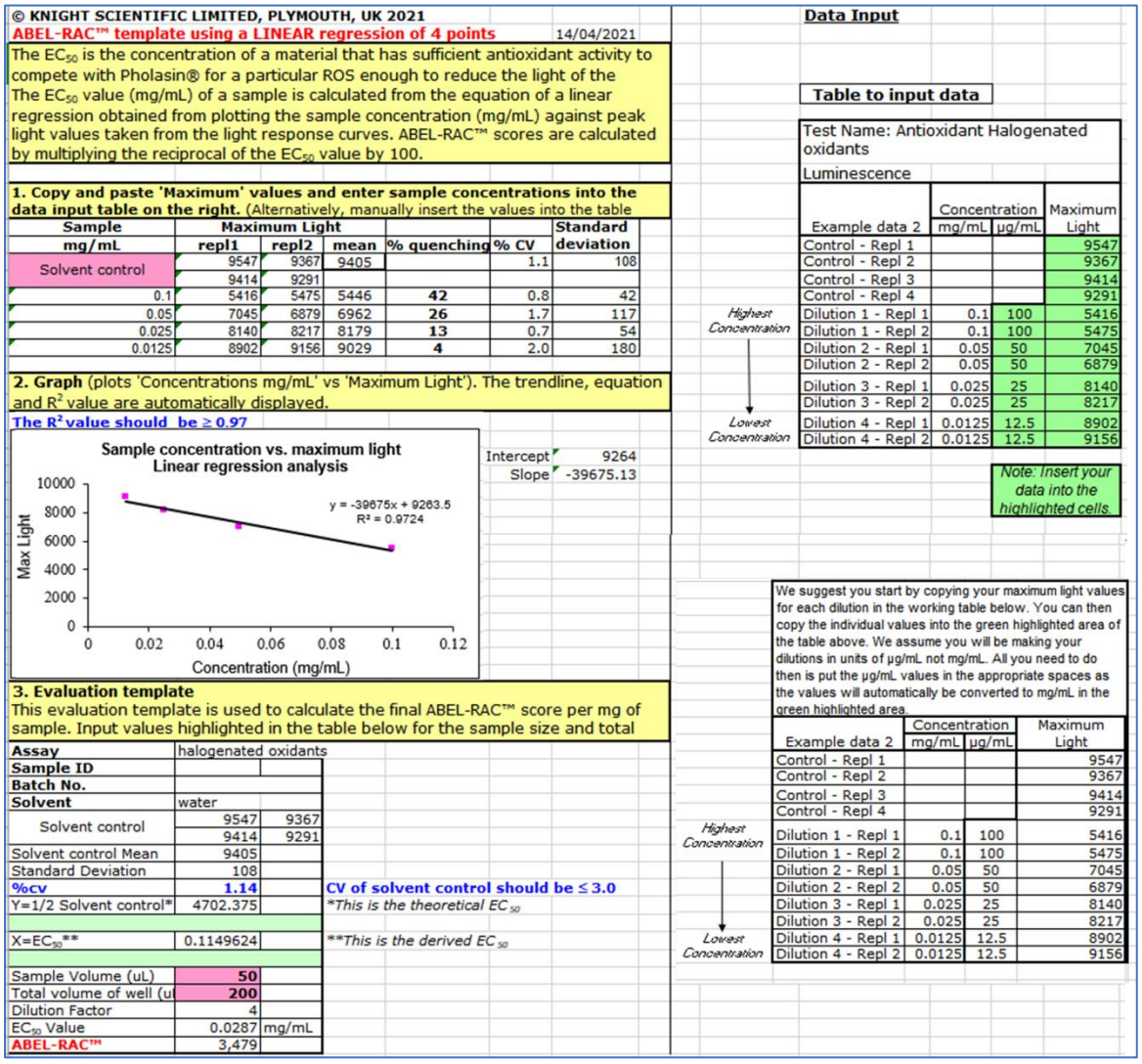


We recommended that each sample occupies one row with NSC or solvent controls at the beginning and the end.

Note: In some cases 'edge effects' on the microplate can cause a slight increase in the light signal in the outside wells (columns 1 & 12). When this occurs use the NSC maximum values in the wells of columns 2 & 11, corresponding to their respective rows.

4. When the assay has finished copy and paste the maximum light values for the concentrations tested, and the solvent controls, from the evaluation software into the ABEL-RAC™ linear template spreadsheet and follow the instructions on the template to obtain your ABEL-RAC™ score.

The image below shows an example of the results of a completed ABEL-RAC™ template. You will be provided with a template specific to the antioxidant kit purchased.



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ABEL-RAC™ template using a LINEAR regression of 4 points 14/04/2021

The EC_{50} is the concentration of a material that has sufficient antioxidant activity to compete with Pholasin® for a particular ROS enough to reduce the light of the The EC_{50} value (mg/mL) of a sample is calculated from the equation of a linear regression obtained from plotting the sample concentration (mg/mL) against peak light values taken from the light response curves. ABEL-RAC™ scores are calculated by multiplying the reciprocal of the EC_{50} value by 100.

1. Copy and paste 'Maximum' values and enter sample concentrations into the data input table on the right. (Alternatively, manually insert the values into the table

Sample mg/mL	Maximum Light repl1	repl2	mean	% quenching	% CV	Standard deviation
Solvent control	9547	9367	9405		1.1	108
	9414	9291				
0.1	5416	5475	5446	**42**	0.8	42
0.05	7045	6879	6962	**26**	1.7	117
0.025	8140	8217	8179	**13**	0.7	54
0.0125	8902	9156	9029	**4**	2.0	180

2. Graph (plots 'Concentrations mg/mL' vs 'Maximum Light'). The trendline, equation and R^2 value are automatically displayed.

The R^2 value should be ≥ 0.97

Intercept 9264
Slope -39675.13

3. Evaluation template

This evaluation template is used to calculate the final ABEL-RAC™ score per mg of sample. Input values highlighted in the table below for the sample size and total

Assay	halogenated oxidants		
Sample ID			
Batch No.			
Solvent	water		
Solvent control	9547	9367	
	9414	9291	
Solvent control Mean	9405		
Standard Deviation	108		
%cv	**1.14**		**CV of solvent control should be ≤ 3.0**
Y=1/2 Solvent control*	4702.375		*This is the theoretical EC_{50}
X=EC_{50}**	0.1149624		**This is the derived EC_{50}
Sample Volume (uL)	**50**		
Total volume of well (u	**200**		
Dilution Factor	4		
EC_{50} Value	0.0287	mg/mL	
ABEL-RAC™	3,479		

Data Input

Table to input data

Test Name: Antioxidant Halogenated oxidants

Luminescence

	Example data 2	Concentration mg/mL	Concentration µg/mL	Maximum Light
	Control - Repl 1			9547
	Control - Repl 2			9367
	Control - Repl 3			9414
	Control - Repl 4			9291
Highest Concentration	Dilution 1 - Repl 1	0.1	100	5416
	Dilution 1 - Repl 2	0.1	100	5475
	Dilution 2 - Repl 1	0.05	50	7045
	Dilution 2 - Repl 2	0.05	50	6879
	Dilution 3 - Repl 1	0.025	25	8140
	Dilution 3 - Repl 2	0.025	25	8217
Lowest Concentration	Dilution 4 - Repl 1	0.0125	12.5	8902
	Dilution 4 - Repl 2	0.0125	12.5	9156

Note: Insert your data into the highlighted cells.

We suggest you start by copying your maximum light values for each dilution in the working table below. You can then copy the individual values into the green highlighted area of the table above. We assume you will be making your dilutions in units of µg/mL not mg/mL. All you need to do then is put the µg/mL values in the appropriate spaces as the values will automatically be converted to mg/mL in the green highlighted area.

	Example data 2	Concentration mg/mL	Concentration µg/mL	Maximum Light
	Control - Repl 1			9547
	Control - Repl 2			9367
	Control - Repl 3			9414
	Control - Repl 4			9291
Highest Concentration	Dilution 1 - Repl 1	0.1	100	5416
	Dilution 1 - Repl 2	0.1	100	5475
	Dilution 2 - Repl 1	0.05	50	7045
	Dilution 2 - Repl 2	0.05	50	6879
	Dilution 3 - Repl 1	0.025	25	8140
	Dilution 3 - Repl 2	0.025	25	8217
Lowest Concentration	Dilution 4 - Repl 1	0.0125	12.5	8902
	Dilution 4 - Repl 2	0.0125	12.5	9156

For further help and advice, please contact telephone or e-mail:

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