Total Phenolic Content Assay Kit

Cat# AOX-17

INSTRUCTION MANUAL ZBM0126.00

STORAGE CONDITIONS

All orders are delivered via Federal Express Priority courier.
All orders must be processed immediately upon arrival.

Gallic Acid Standard

Remove from box and store at -20°C

All Other Reagents and Assay Plates

Store at room temperature

Long-term storage:

Reagents are good for at least 3 months after arrival if stored properly.

For *in vitro* Use Only

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

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INTRODUCTION

Free radicals and reactive oxygen species (ROS) are highly reactive molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS react with cellular components, damaging DNA, carbohydrates, proteins, and lipids causing cellular and tissue injury. Excess production of reactive oxygen species can also lead to inflammation, premature aging disorders, and several disease states, including cancer, diabetes, and atherosclerosis. Organisms have developed complex antioxidant systems to protect themselves from oxidative stress, however, excess ROS can overwhelm the systems and cause severe damage.

Phenolic compounds in plants and other food provide antioxidant capacity by scavenging oxygen radicals, thereby reducing the effects of ROS. Common classes of phenolic compounds, flavonoids, tannins and coumarins, have been widely studied for their antioxidant capacity and are commonly found in fruits, tea and wine. The ZenBio Total Phenolic Content Assay can assess the amount of phenolic compounds in a test sample through their reaction with the Folin-Ciocalteu reagent. Gallic Acid [3,4,5-Trihydroxybenzoic acid], a simple phenolic compound, serves as a positive control comparator. This is an endpoint assay measuring the change in absorbance at 765nm caused by phenolic compounds reacting with the Folin-Ciocalteu. The phenolic content of a sample can be normalized to equivalent Gallic Acid units to quantify the total of the phenolic compounds present.

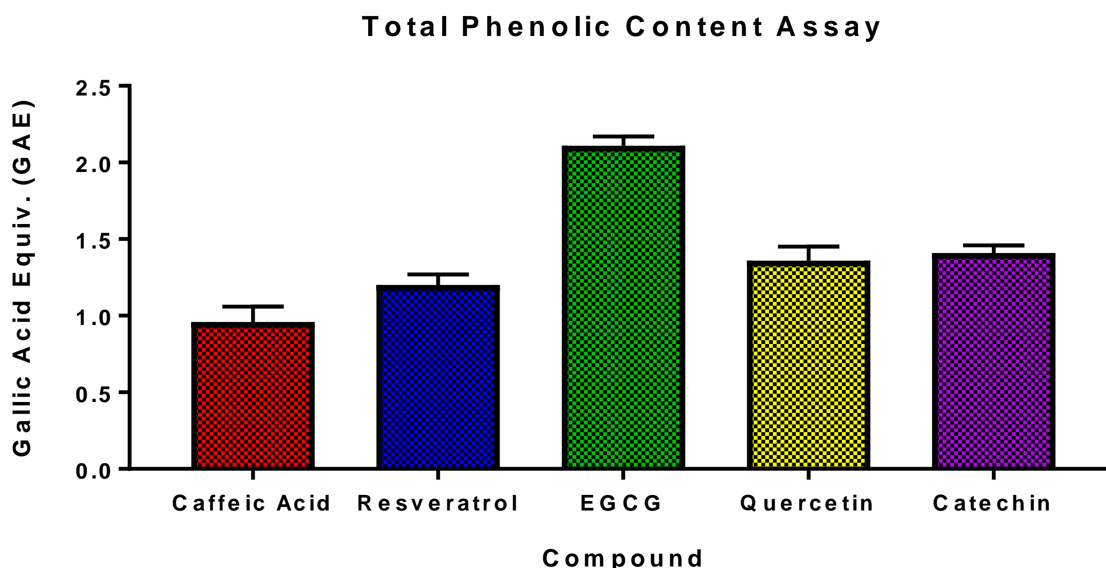
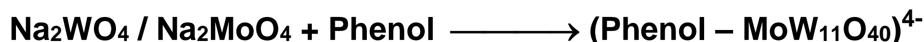


Figure 1. Gallic Acid Equivalence of Common Antioxidants

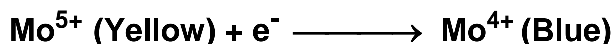
Caffeic acid, resveratrol, Epigallocatechin gallate (EGCG), quercetin and catechin were tested for their total phenolic content relative to gallic acid.

PRINCIPLE OF THE ASSAY

The Folin-Ciocalteu reagent contains phosphomolybdic/phosphotungstic acid complexes which can oxidize phenolic compounds in a basic buffer resulting in the formation of $O_2^{\bullet -}$. This radical anion further reacts to generate MoO^{4+}, which absorbs strongly at 765nm. The increase in absorbance at 765nm is proportional to the concentration of phenolic compounds in the test sample.



$$Na_2WO_4 / Na_2MoO_4 + Phenol \longrightarrow (Phenol - MoW_{11}O_{40})^{4-}$$



$$Mo^{5+} (Yellow) + e^{-} \longrightarrow Mo^{4+} (Blue)$$

ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Color	UNIT	QTY	STORAGE
Blank Assay Plates	96-well assay plates, blank	---	PLATE	2	-----
AOX Assay Buffer	20 ml	CLEAR	BOTTLE	1	RT
Folin-Ciocalteu Reagent	550 µl	AMBER	VIAL	1	RT
Gallic Acid Standard	250 µl, 1mM	AMBER	VIAL	1	-20°C
Tray	For multi-channel pipetters, clear polyvinyl	---	EACH	2	RT

Other equipment/reagents required but not provided with the kit:

- Multi-channel Pipet , single channel pipet and pipet tips
- Tubes for preparing dilutions.
- Distilled water
- Plate reader with a filter of ~765 nm

Reagents that might interfere with the assay results:

The F-C reagent is not specific for phenolic compounds, reducing sugars and ascorbic acid can interfere with the reagent. Other interfering substances include proteins, tyrosine, formic acid, acetic acid and L-cysteine.

ASSAY PROCEDURE

1. Prepare 10% Folin-Ciocalteu (F-C) reagent by adding 500 µL stock to a tube containing 4.5 mL of distilled water. Mix by vortexing. 2 mL is needed per plate.
2. Prepare Gallic Acid standard curve using water according to the following table:

Standard	Final Conc. (µM)	Water (µL)	Volume (µL)	Gallic Acid Solution
Std1000	1000	0	100	1000 µM solution
Std500	500	100	100	Std1000
Std250	250	100	100	Std500
Std125	125	100	100	Std250
Std62.5	62.5	100	100	Std125
Std31.25	31.25	100	100	Std62.5
Std15.6	15.6	100	100	Std31.25
Std0	0	100	0	0

Mix each new dilution thoroughly before proceeding to the next step.

3. Prepare test sample dilutions in water as necessary.
4. Add 10 µL of samples and Gallic Acid Standards to triplicate wells.
5. Add 20 µL of 10% F-C reagent (from step 1) to each well.
6. Add 80 µL of Assay Buffer to all wells.
7. Incubate at RT, shaking for 120 minutes.
8. Determine absorbance using plate reader at a wavelength of 765 nm.

DATA ANALYSIS

Generate standard curve: see example below (Collected using a SpectraMax iD3)
[DO NOT use this standard curve to generate your data. This is an example.]

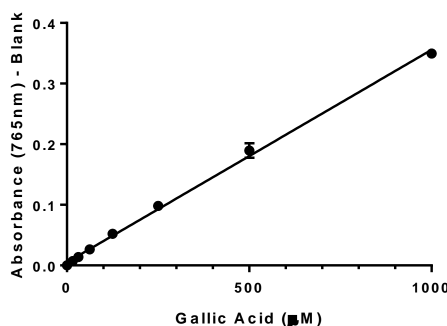
Raw Absorbance Values

Gallic Acid µM	Abs1	Abs2	Abs3
1000	0.3848	0.3854	0.3808
500	0.2175	0.2368	0.2159
250	0.1308	0.1324	0.1337
125	0.0855	0.0867	0.0867
62.5	0.06	0.0603	0.0608
31.25	0.047	0.0486	0.0477
15.625	0.0402	0.0416	0.0418
0	0.0329	0.0328	0.0364

Absorbance minus no Gallic Acid Control

Gallic Acid µM	Abs1	Abs2	Abs3	Avg	Stdev
1000	0.3508	0.3514	0.3468	0.3496	0.003
500	0.1835	0.2028	0.1819	0.1894	0.012
250	0.0968	0.0984	0.0997	0.0983	0.001
125	0.0515	0.0527	0.0527	0.0523	0.001
62.5	0.0260	0.0263	0.0268	0.0263	0.000
31.25	0.0130	0.0146	0.0137	0.0137	0.001
15.625	0.0062	0.0076	0.0078	0.0072	0.001
0	-0.0011	-0.0012	0.0024	0.0000	0.002

$y = 0.0003x + 0.0048$
$r^2 = 0.9988$



1. Determine the background absorbance value by averaging the absorbance values of the three no Gallic Acid wells.
2. Subtract the average background absorbance from all of the absorbance values.
3. Plot Gallic Acid concentration vs. average background corrected absorbance.
4. Use the standard curve to calculate the µM Gallic Acid Equivalents (GAE) for each sample.
5. If the samples were diluted, use the dilution factor to determine the GAE for the stock solution.

Data Analysis Example

µM Sample	Raw Absorbance 765nm			Remove no Gallic Acid Abs.		
	Abs1	Abs2	Abs3	Abs1	Abs2	Abs3
200	0.1282	0.1293	0.1286	0.0942	0.0953	0.0946
66.7	0.0716	0.0721	0.0697	0.0376	0.0381	0.0357
22.2	0.0486	0.0492	0.0477	0.0146	0.0152	0.0137

Use the standard curve above to calculate the µM GA equivalence and determine the µmole GA per µmole of sample.

µM Sample	µM Gallic Acid Equivalents			µmole GA / µmole sample			µmole GA / µmole sample	
	Value1	Value2	Value3	Value1	Value2	Value3	Avg.	Std Dev
200	297.9	301.6	299.2	1.49	1.51	1.50	1.52	0.098
66.7	109.2	110.9	102.9	1.64	1.66	1.54		
22.2	32.6	34.6	29.6	1.47	1.56	1.33		

APPENDIX A: Plate layout ________________________________

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

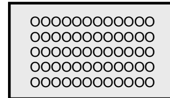
APPENDIX B: Protocol Flowchart

TOTAL PHENOLIC CONTENT ASSAY

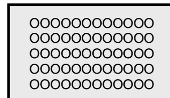
Prior to assay, prepare 10% F-C Reagent Solution. Prepare Gallic Acid Standard Curve.

Make necessary test compound dilutions in water.

Add 10 µl/well samples or standards to assay plate.



Add 20 µl/well 10% F-C Reagent to all the wells of the plate.



Add 80 µl Assay Buffer to each well. Incubate at RT for 120 minutes.

Measure the optical density of each well at 765 nm using a spectrophotometer plate reader.

REFERENCES

1. Gülçin, *Arch Toxicol*, **86**:345-391, 2012
2. Apak *et al.*, J. Agric. Food Chem., **64**:997-1027, 2016

FREQUENTLY ASKED QUESTIONS

1. **Is it alright that my absorbance values are lower than those in the sample data?** Yes, the absorbance values detected by the spectrophotometer are based on the sensitivity of the instrument used. Our data was collected using a SpectrMax iD3 multi-mode microplate reader, other instruments vary in sensitivity and can give lower values. If the Gallic Acid standards still generate a robust standard curve, the assay is functioning appropriately.

2. **Should I dilute my sample for testing its total phenolic content**? In order to accurately determine the total phenolic content of your sample, the absorbance value must fall on the Gallic Acid standard curve. We recommend preparing several serial dilutions of your test sample to ensure that you generate usable absorbance values.