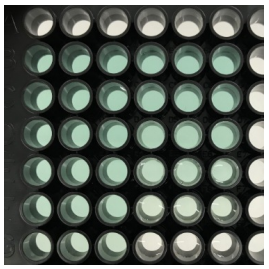
Jul 26, 2019

ABTS decolorization assay – in vitro antioxidant capacity



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We use this protocol in our group and it is working.

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Abstract

This protocol describes how to perform the ABTS decolorization assay to assess potential in vitro antioxidant capacity of molecules and extracts using microtiter plates. Procedures are based on the method described in R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radic. Biol. Med. 26 (1999) 1231–1237.

Materials

MATERIALS

- Ultrapure water (Type 1)
- Corning® 96 well NBS™ Microplate **Sigma Aldrich Catalog #**CLS3651
- 22′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) **Sigma Aldrich Catalog #**A1888
- Ammonium persulfate (APS) **Sigma Aldrich Catalog #**A3678
- (±)-6-Hydroxy-2578-tetramethylchromane-2-carboxylic acid (Trolox) **Sigma Aldrich Catalog #**238813
- Phosphate buffered saline (PBS)

Equipment

SpectraMax M3 Multi-Mode Microplate Reader	NAME
Microplate Reader	TYPE
Molecular Devices	BRAND
8002482	SKU
https://www.moleculardevices.com/	LINK
Multi-mode microplate readers (Absorbance, Fluorescence (top/bottom read), and Luminescence (top/bottom read)). Ranges: Abs, 200–1000 nm; FL, 250–850 nm; Lumi, 250–850 nm. Light source: Xenon flashlamp. Detector: Silicon photodiode, Photomutiplier tube. Readtypes: Endpoint, Kinetic, Spectrum scan, and Well scan.	SPECIFICATIONS

ABTS radical preparation

1 **Prepare a 7 mM ABTS (e.g., A1888, Sigma-Aldrich) stock solution in ultrapure water.**

Example: Dilute 3.8408 mg in a final volume of 1,000 µL[1].

[1]Note that you will need 570 µL (190 µL for each replicate) of the final ABTS• stock solution per sample; 400 µL of the ABTS solution produces approximatelly 10,000 µL of ABTS• stock solution (see step 6).

2 **Prepare a 245 mM APS (e.g., A3678, Sigma-Aldrich) solution in ultrapure water.**

Example: Dilute 5.5909 mg in a final volume of 100 µL.

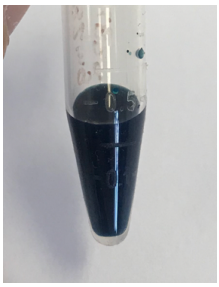
3 **Add APS to the ABTS solution so that the final APS concentration is 2.45 mM. This step is necessary to generate the ABTS radical (ABTS•).**

Example: Add 5.05 µL of APS to 500 µL of ABTS; final volume 505.05 µL.

ABTS solution before APS addition.

ABTS solution right after APS addition.

4 **Incubate it overnight (12–16 h) at room temperature in the dark.**



ABTS$^{\bullet}$ solution after overnight incubation.

5 **Check the concentration of the ABTS radical (ABTS$^{\bullet}$) stock solution at 734 nm.**

Example: Dilute 10 uL of ABTS$^{\bullet}$ solution in a final volume of 1,000 µL using ultrapure water; read it at 734 nm.

6 **Prepare an ABTS$^{\bullet}$ solution that absorbs ~0.700 at 734 nm.**

Example: If the solution prepared in the previous step absorbed 0.689, dilute 100 µL of the ABTS$^{\bullet}$ stock solution in a final volume of 9,842.9 µL[2].

[2]Remember that you will need 190 µL per well in the assay; each sample/control/standard is analyzed in triplicate (3 wells).

Example: If you are going to assess two samples at four concentrations each, build a standard curve with six concentrations, and read a blank reaction control (all in triplicate), you will need 8,550 µL of the ABTS$^{\bullet}$ stock solution (absorbing ~0.700).

7 **Check the absorbance at 734 nm, adjust if necessary, and store until use.**

Example: If the solution prepared in the previous step absorbed 0.805, diluted it 1.15-fold (9,000 µL in a final volume of 10,350 µL) to achieve an absorbance of ~0.700.

This is what an ABTS$^{\bullet}$ solution that absorbs ~0.700 at 734 nm looks like.

Trolox standard solutions preparation

8 **Prepare a 2 mM Trolox (e.g., 238813, Sigma-Aldrich) stock solution in PBS[3].**

Example: Dilute 1.0012 mg in a final volume of 2,000 µL.

[3]If compatible, use the same solvent/buffer in which the sample to be analyzed is prepared to make the Trolox standard solutions.

9 **Prepare several Trolox solutions at concentrations from 12.5 to 400 µM[4] using PBS in Eppendorf tubes.**

Example: First, dilute the 4 mM stock by 5-fold to 400 µM, and dilute it serially to 200, 100, 50, 25, and 12.5 µL (e.g., 100 µL of the previous concentration + 100 µL PBS).

[4]Under the conditions described in this protocol, the change in absorbance at 734 nm is linear within the 12.5–400 µM Trolox range (see *Calculation section*).

Sample preparation

10 **Dilute[5] the samples at the desired concentrations[6].**

[5]If a solvent other than PBS is necessary to dilute the sample, prepare adequate solvent controls in the next step.

Example: If you need 50% (v/v) DMSO to dilute your sample, also assess 10% DMSO (without sample) solution in the assay to check for any interference.

[6]For peptides, we usually use 2.0, 1.0, 0.5, and 0.25 mg/mL concentrations. Note that for peptides with strong activity (i.e., 100% ABTS$^{\bullet}$ scavenging), we further dilute them to 0.125–0.031 mg/mL.

ABTS decolorization assay

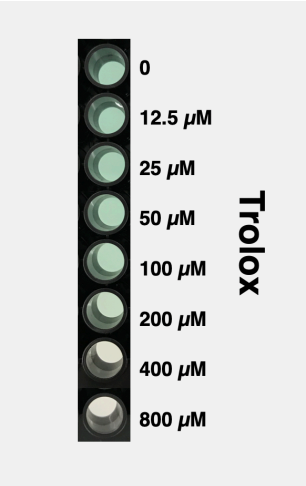
11 **Pipet 10 µL of PBS (or other solvent used) plus 190 µL of ultrapure water into separate microtiter plate wells in triplicate.** These will be used as reference/zero for the readings.

12 **Pipet 10 µL of each trolox standard solution (including the control[7], solvent only) and each sample dilution into separate microtiter plate wells in triplicate.**

[7]These are the reference wells of maximum ABTS$^{\bullet}$ concentration, in which no sample or standard were added.

13 **Add 190 µL of the ABTS$^{\bullet}$ solution prepared in Step 7 into each well.**

14 **Mix the wells content using the 'shake' function of the microtiter plate reader, incubate it for 5 minutes in the dark and read it at 734 nm.**



Calculation

15 **Using the average values of the triplicates for each standard concentration, calculate the decolorization effect caused by each samples/standard relative to the absorbance of the control (ABTS$^{\bullet}$ + solvent) at 734 nm.**

$$Decolorization\ (\%) = \left(\frac{Control\ Abs - Sample\ Abs}{Control\ Abs}\right) \times 100$$

Example:

Trolox		Absorbance at 734 nm				Decolorization (%)
μM	mg/mL	Read 1	Read 2	Read 3	**Average**	
0	0	0.399	0.402	0.402	0.401	0.00
12.5	0.0031	0.383	0.392	0.387	0.387	3.41
25	0.0063	0.379	0.381	0.380	0.380	5.24
50	0.0125	0.355	0.357	0.357	0.356	11.14
100	0.0250	0.312	0.314	0.310	0.312	22.19
200	0.0501	0.229	0.232	0.227	0.229	42.81
400	0.1001	0.064	0.062	0.059	0.062	84.62

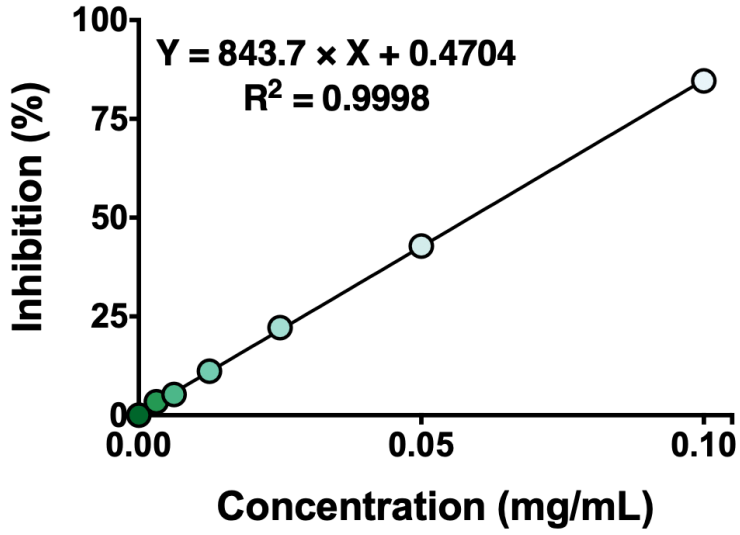
Trolox standard solutions.

Sample	Absorbance at 734 nm				Decolorization (%)
mg/mL	Read 1	Read 2	Read 3	**Average**	
0.25	0.203	0.199	0.205	0.202	49.54

Sample.

16 **Build a standard curve: plot trolox concentrations in mg/mL on the X-axis and decolorization (%) on the Y-axis. Calculate a linear regression (Y = a × X + b; e.g., 'add a linear trendline' in Microsoft Excel).**

Example:



17 **Calculate sample's antioxidant capacity relative to that of trolox using the equation generated in the previous step.**

$$Trolox - eq\ (mg/mg)\ = \left(\frac{Sample\ decolorization\ (\%) - b}{a}\right) \div Sample\ concentration\ (mg/mL)$$

Example:

$$Trolox - eq\ (mg/mg)\ = \left(\frac{49.54 - 0.4704}{843.7}\right) \div 0.250$$

$$Trolox - eq\ (mg/mg)\ = 0.233$$