# FRAP Antioxidant Assay Kit
## (100 point kit)
Cat# AOX-11

**INSTRUCTION MANUAL ZBM0119.00**

## STORAGE CONDITIONS

All orders are delivered via Federal Express Priority courier at 4°C.
All orders must be processed immediately upon arrival. Any adverse conditions upon arrival must be reported within 7 days.

**Ferrous Standard, FRAP Probe and $FeCl_3$ solution**
Remove from box and store at -20°C
**FRAP Acetate Buffer**
Store at 4°C
**Assay plate and Feeding Tray**
Store at Room Temperature
**Long-term Storage**
Reagents are good for at least 3 months upon 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 these systems and cause severe damage.

The Zen-Bio FRAP (Ferric Reducing Antioxidant Power) Assay Kit can be used to determine the antioxidant capacity of biological fluids, cells, and tissue. It can also be used to assay the antioxidant activity of naturally occurring or synthetic compounds for use as dietary supplements, topical protection, and therapeutics. The assay measures the increase in absorbance due to the reduction of Fe(III) to Fe(II) at low pH in the presence of a chelating probe, tripyridyltriazine (TPTZ). An Fe(II) solution serves as the positive control comparator to determine the sample's reducing capacity. The FRAP assay is an endpoint assay measuring the increase in blue absorbance at 540-600nm. The antioxidant activity in biological fluids, cells, tissues, and natural extracts can be normalized to equivalent Fe(II) units to quantify the composite antioxidant activity present. This assay measures antioxidant activity by electron donation and when combined with Zen-Bio's other antioxidant assay kits, provides a comprehensive analysis of a test sample's antioxidant activity.

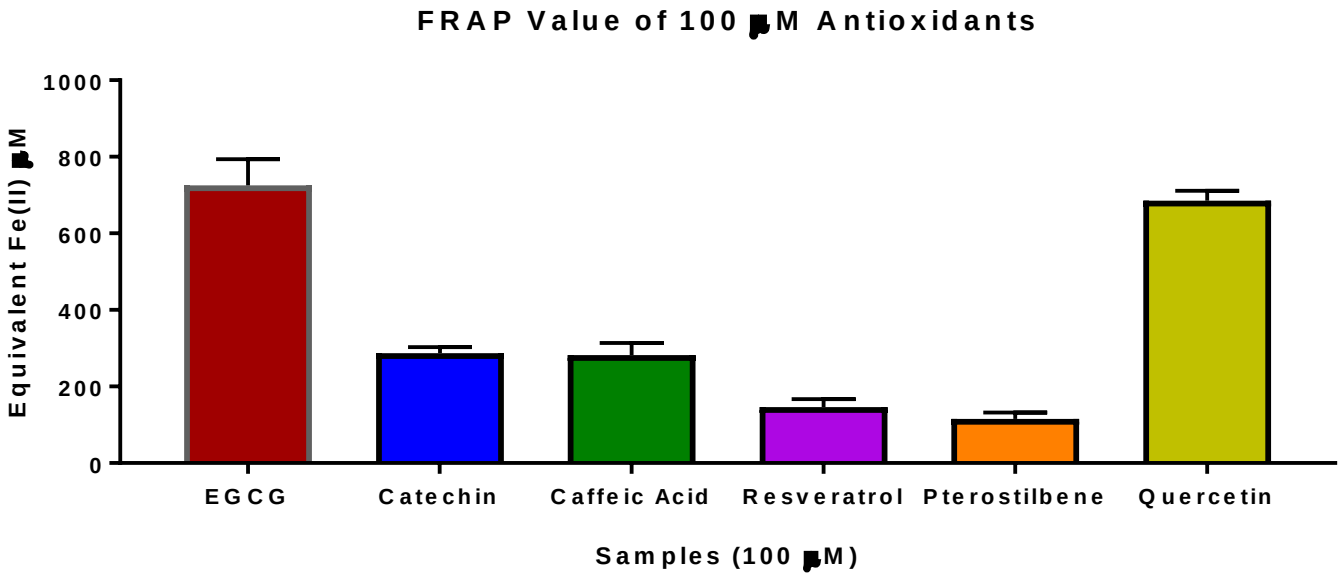
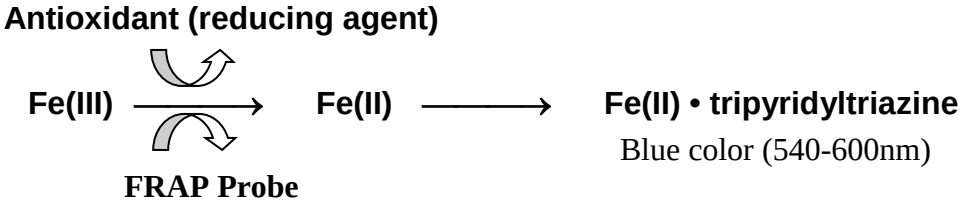



**Figure 1. Effects of antioxidants in FRAP assay**

EGCG, Catechin, Caffeic Acid, Resveratrol, Pterostilbene and Quercetin were tested for their antioxidant activity in the FRAP antioxidant assay at 100 µM.

## PRINCIPLE OF THE ASSAY

The Ferric Reducing Antioxidant Power (FRAP) assay measures the reducing capacity of antioxidants by their ability to reduce Fe(III) to Fe(II) in an acidic environment. The tripyridyltriazine FRAP Probe forms a colorless complex with Fe(III), however, it forms a blue colored complex with Fe(II). This reduction reaction occurs by electron donation from a reducing agent to the Fe(III) complex generating the Fe(II) complex. Antioxidants stimulate this reaction, increasing the Fe(II) product and the concomitant increase in absorbance at wavelengths between 540-600nm. The maximum absorbance generated over the reaction time (up to 60 minutes) is proportional to the reducing power of the test sample. This antioxidant reducing power is assessed by comparing it to an Fe(II) standard curve.




**[Antioxidants induce the reduction of Fe(III) by electron donation]**

# ITEMS INCLUDED IN THE KIT

| ITEM | DESCRIPTION | Cap Color | UNIT | QTY | STORAGE |
|---|---|---|---|---|---|
| Clear Assay Plate | 96-well assay plate, clear | --- | PLATE | 1 | ----- |
| FRAP Assay Buffer | 35 ml | --- | CLEAR BOTTLE | 1 | 4°C |
| Ferrous Standard | 50 µl $FeSO_4$ solution | --- | 50 µl / VIAL | 1 | -20°C |
| FRAP Probe | 2 ml solution | --- | AMBER BOTTLE | 1 | -20°C |
| $FeCl_3$ Solution | 2 ml solution | | AMBER BOTTLE | 1 | -20°C |
| Multichannel Pipette Reservoirs | Clear polyvinyl reservoir | --- | EACH | 1 | ----- |

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

- Multi-channel pipette, single channel pipette and pipette tips
- 96-well microplate or tubes for preparing standards.
- Tubes for preparing working solutions
- Microplate reader able to perform absorbance measurement at 593nm (or wavelength between 540-600nm)

# SAMPLE PREPARATION

## Cell Lysate Preparation

1. Scrape ~1 x$10^6$ cells and centrifuge at 1,000xg to prepare a cell pellet. DO NOT use proteolytic enzymes such as trypsin but scrape using a rubber policeman or cell scraper tool.
2. Homogenize or sonicate the cell pellet on ice in 1ml ice cold AOX Assay buffer
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and keep on ice until ready to use in the assay. [Alternatively, pass supernatant through a 10 kDa molecular weight cutoff filter to reduce protein content.]
5. If not using the same day, store the samples at -80°C.
6. Data is expressed as Ferrous equivalents per cell number (i.e. µmole Fe(II)/$10^6$ cells)

## Tissue Lysate Preparation

1. Homogenize tissue samples on ice in cold buffer at ~20mg tissue per ml cold buffer.
2. Centrifuge at 10,000 x g for 15 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay. [Alternatively, pass supernatant through a 10 kDa molecular weight cutoff filter to reduce protein content.]
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as Ferrous equivalents per gram of starting sample (i.e. µmole Fe(II)/g)

## Plasma Preparation

1. Collect the blood in a tube containing heparin as anticoagulant.
2. Centrifuge at 1,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Ferrous equivalents per volume sample (i.e. µmole Fe(II)/L)

## Serum Preparation

1. Collect the blood in a tube WITHOUT any anticoagulant. Allow the blood to clot.
2. Centrifuge at 2,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Ferrous equivalents per volume sample (i.e. µmole Fe(II)/L)

## Saliva Collection

1. Collect whole saliva for a defined period of time (i.e. 1-5 minutes) into polypropylene tubes.
2. Immediately place on ice or store at -80°C for later analysis.
3. Data is expressed as micromole Ferrous equivalents per volume sample (i.e. µmole Fe(II)/L)

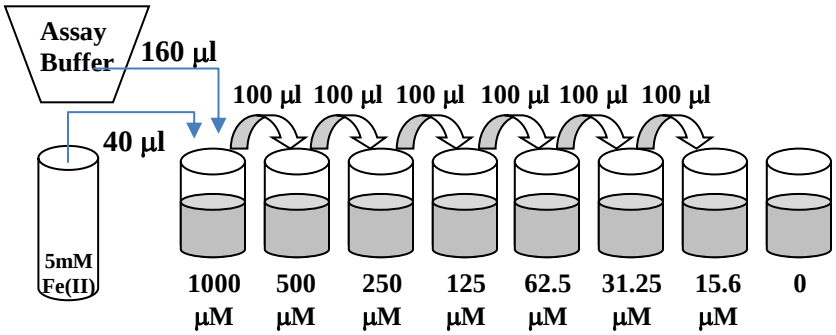
## Food Extract Preparation

1. Weigh the starting material and homogenize in a small volume ice cold buffer or water. [ex. 5g of material in 100mL water].
2. Filter or centrifuge homogenates to obtain supernatant.
3. Store small aliquots at -80°C for analysis.
4. When ready to assay, keep thawed samples on ice.
5. Data is expressed as Ferrous equivalents per gram of starting sample (i.e. µmole Fe(II)/g)

# ASSAY PROCEDURE

**THIS KIT PROVIDES SUFFICIENT REAGENTS TO ASSAY 100 WELLS. AT LEAST 8 OF THESE WELLS ARE REQUIRED FOR FE(II) STANDARDS**

1. Warm FRAP Assay Buffer to room temperature before running the assay. Thaw FRAP Probe, Ferrous Standard and $FeCl_3$ solutions and place on ice.
2. Set-up plate reader to read absorbance at 593nm (or convenient wavelength between 540-600nm).
3. Prepare Ferrous standards as follows in a separate plate (not provided) or tubes (not provided): Briefly spin down the contents of the 5 mM Ferrous Standard tube after thawing. Pipette **40 µl** of the 5 mM Ferrous Standard solution into a tube/well containing **160 µl** Assay Buffer and mix well. This produces a diluted Ferrous standard of **1000 µM**. Pipette **100 µl** of AOX Assay Buffer into 7 tubes/wells. Using the table and diagram below, prepare Ferrous standards 500, 250, 125, 62.5, 31.25, 15.6 and 0 µM. Mix each new dilution thoroughly before proceeding to the next. The **1000 µM** standard dilution serves as the highest standard, and assay buffer serves as the zero standard (or blank).




| Final Conc. (µM) | Buffer (µL) | Volume (µL) | Iron(II) Solution |
|---|---|---|---|
| 1000 | 160 | 40 | 5mM Stock |
| 500 | 100 | 100 | 1000 µM Std |
| 250 | 100 | 100 | 500 µM Std |
| 125 | 100 | 100 | 250 µM Std |
| 62.5 | 100 | 100 | 125 µM Std |
| 31.25 | 100 | 100 | 62.5 µM Std |
| 15.6 | 100 | 100 | 31.25 µM Std |
| 0 | 100 | 0 | 0 |

4. Add 20 µL of samples or Ferrous standards (with zero standard) to individual wells of the assay plate provided. [**IF THE AOX ACTIVITY OF THE TEST SAMPLES IS UNKNOWN, WE RECOMMEND PREPARING SEVERAL DILUTIONS IN AOX ASSAY BUFFER.]**
5. Prepare the Working FRAP Reagent by combining 14.4 mL Assay Buffer with 1.8 mL $FeCl_3$ Solution and 1.8 mL FRAP Probe solution in a tube (not supplied). Mix well.
6. Add 180 µL of the Working FRAP Reagent to each well. Mix well with pipette or on horizontal shaker. Incubate the reaction for 15 minutes at room temperature. [Some reactions may take up to 60 minutes to complete. Multiple absorbance readings may be taken over the course of the reaction to verify completion. Longer reaction times can increase background readings.]
7. Place assay plate in microplate reader and determine absorbance at 593nm (or wavelength between 540-600nm).

# FERROUS STANDARD CURVE _____

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

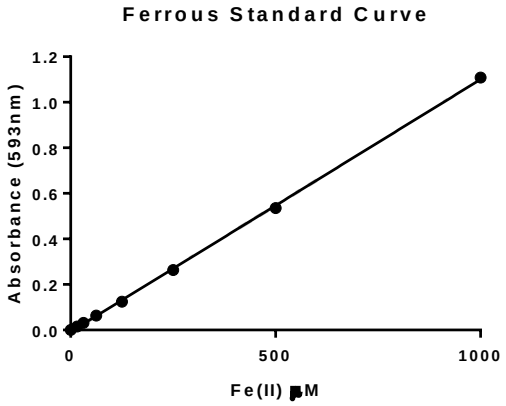
| Fe(II) µM | Abs1 | Abs2 | Abs3 |
|---|---|---|---|
| 1000 | 1.3177 | 1.3254 | 1.3138 |
| 500 | 0.7459 | 0.7259 | 0.7644 |
| 250 | 0.4691 | 0.4792 | 0.4736 |
| 125 | 0.3321 | 0.3324 | 0.3389 |
| 62.5 | 0.2726 | 0.2754 | 0.2741 |
| 31.25 | 0.2427 | 0.2414 | 0.2428 |
| 15.625 | 0.2256 | 0.2264 | 0.2258 |
| 0 | 0.2102 | 0.2106 | 0.2102 |

Absorbance minus No Fe(II) control

| Fe(II) µM | Abs1 | Abs2 | Abs3 | Avg | Stdev |
|---|---|---|---|---|---|
| 1000 | 1.107367 | 1.115067 | 1.103467 | 1.108633 | 0.005903 |
| 500 | 0.535567 | 0.515567 | 0.554067 | 0.535067 | 0.019255 |
| 250 | 0.258767 | 0.268867 | 0.263267 | 0.263633 | 0.00506 |
| 125 | 0.121767 | 0.122067 | 0.128567 | 0.124133 | 0.003842 |
| 62.5 | 0.062267 | 0.065067 | 0.063767 | 0.0637 | 0.001401 |
| 31.25 | 0.032367 | 0.031067 | 0.032467 | 0.031967 | 0.000781 |
| 15.625 | 0.015267 | 0.016067 | 0.015467 | 0.0156 | 0.000416 |
| 0 | -0.00013 | 0.000267 | -0.00013 | -9.3E-18 | 0.000231 |

y=0.0011x – 0.0068

$r^2$=0.9996




- Subtract the No Fe(II) control absorbance value from all absorbance values for each test sample.
- Use the Fe(II) standard curve to calculate the µM Fe(II) equivalence for each test sample.
- If test samples were diluted, use the dilution factor to determine Fe(II) equivalence.

## Data Analysis Example

| µM sample | Raw Absorbance 593nm | | | Remove 0 Fe(II) value | | |
|---|---|---|---|---|---|---|
| | Abs 1 | Abs 2 | Abs3 | Abs 1 | Abs 2 | Abs3 |
| 100 | 0.94 | 0.9303 | 0.916 | 0.730 | 0.720 | 0.706 |
| 33.33 | 0.4691 | 0.4785 | 0.4737 | 0.259 | 0.268 | 0.263 |
| 11.11 | 0.2874 | 0.3064 | 0.303 | 0.077 | 0.096 | 0.093 |

Use the standard curve above to calculate the µM Fe(II) equivalence and determine µmole Fe(II) per µmole of sample.

| µM sample | µM Fe(II) equivalence | | | µmoles Fe(II) / µmole Sample | | | µmoles Fe(II) / µmole | |
|---|---|---|---|---|---|---|---|---|
| | Value 1 | Value 2 | Value 3 | Value 1 | Value 2 | Value 3 | Avg | Std. Dev. |
| 100 | 669.5 | 660.7 | 647.7 | 6.70 | 6.61 | 6.48 | 7.26 | 0.68 |
| 33.33 | 241.4 | 250.0 | 245.6 | 7.24 | 7.50 | 7.37 | | |
| 11.11 | 76.2 | 93.5 | 90.4 | 6.86 | 8.42 | 8.14 | | |

# APPENDIX A: Plate layout_____

| H | G | F | E | D | C | B | A | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | 1 |
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| | | | | | | | | 11 |
| | | | | | | | | 12 |

# APPENDIX B: Protocol Flowchart

**FRAP ASSAY**

Prior to assay, thaw reagents and bring assay buffer to room temperature. Keep Fe(II) standard on ice.

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Make necessary Standard and test sample dilutions in Assay Buffer.

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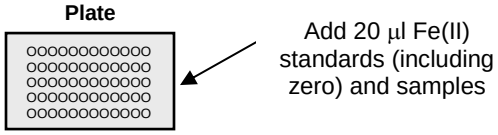
Add 20 µl/well Fe(II) standards (with zero) and test samples to the assay plate.

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Prepare FRAP Working Reagent.

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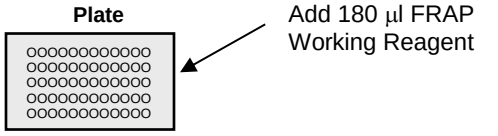
Add 180 µl FRAP Working Reagent per well and incubate at Room Temperature for 10-60 minutes.

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Read Absorbance at 593nm in a microplate reader. Alternatively read at a wavelength between 540-600nm.

## REFERENCES


1. *Analytical Chemistry* 239: 70-76, 1996.
2. *Methods Enzymol.* 299:15-27, 1999.
3. *J.* Agric. Food Chem. 48: 3396-3402, 2000

# FREQUENTLY ASKED QUESTIONS

1. **Is it alright that my absorbance values are different than those in the sample data but still generate a good Ferrous Standard curve?** 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 Ferrous standards still generate a robust standard curve, the assay is functioning appropriately.

2. **Should I dilute my sample for testing its FRAP activity?** In order to accurately determine the FRAP activity of your sample, the absorbance value must fall on the Ferrous standard curve. We recommend preparing several serial dilutions of your test sample using the assay buffer to ensure that you generate usable absorbance values.

3. **How do I know that the reaction is complete?** When the absorbance value no longer significantly increases and is stable, the reaction is complete. Most reactions are complete within 5-10 minutes, however, some samples react much more slowly and require longer incubation times.