

Ferrous Ammonium Sulfate Reagent Catalog Number F6675 25 mM ferrous ammonium sulfate in 2.5 M sulfuric acid	2 ml
<i>tert</i> -Butyl Hydroperoxide (t-BuOOH) Catalog Number 458139 70% aqueous solution	1 ml
Hydrogen Peroxide Color Reagent (H ₂ O ₂) Catalog Number H9533 30% aqueous solution	1 ml

Reagents and Equipment Required but Not Provided.

(Catalog Numbers are given where appropriate)

- UV/visible spectrophotometer with appropriate cuvettes
- 2.5 M sulfuric acid (to be used to prepare a Working Reagent Blank, if necessary; see Procedure section)
- Methanol, spectrophotometric grade
- 90% Methanol, prepared by mixing 108 ml of methanol with 12 ml of deionized water (120 ml total volume)

Precautions and Disclaimer

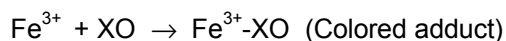
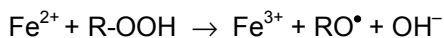
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

Store the kit at 2–8 °C. The components of this kit, as supplied, are stable for at least 24 months.

Procedures

Peroxides will convert Fe²⁺ to Fe³⁺ ions under acidic conditions. Fe³⁺ ions will then form a colored adduct with xylenol orange, which is observed at 560 nm. The reaction can be described as:



Where:

XO = xylenol orange

R = H or a lipidic group

The final product is stable for several hours after the initial color development of 30 minutes.

If endogenous iron or other transition metals are suspected to be present in the sample, they may cause an increase in the signal. Compensate for the high signal by preparing a Working Reagent Blank (100 volumes of the Aqueous or Organic Peroxide Color Reagent with 1 volume of 2.5 M sulfuric acid solution, instead of the Ferrous Ammonium Sulfate Reagent). The result will then be obtained by subtracting the sample value obtained with the Working Color Reagent from the sample value obtained with the Working Reagent Blank.

Non-ionic detergents such as TWEEN® 20, TWEEN 80, and Triton™ X-100 may contain high levels of endogenous peroxides and will give false results. If these detergents are vital for the extraction of the specific proteins needed, use a suitable grade of detergent with low peroxide content such as Catalog Numbers P6585 (TWEEN 20), P8192 (TWEEN 80), or X100PC (Triton X-100).

Determination for Aqueous Peroxide Solutions

High concentrations of fructose, sorbitol, sucrose, glucose, and formic acid will cause an increase in the color yield when using the Working Color Reagent for aqueous peroxides,¹¹ and samples containing these chemicals will be difficult to assay using the reagent.

This procedure is optimized for a final reaction volume of 1.1 ml. If a smaller reaction volume is desired, the assay can be performed in a multiwell plate using 20 µl of sample and 200 µl of Working Color Reagent. The small scale reaction is **not** recommended for determination for organic or lipid hydroperoxides, due to evaporation of the methanol based solutions.

Preparation Instructions

1. 100 µM Hydrogen Peroxide Standard - To prepare a standard solution of 100 µM hydrogen peroxide for comparison with aqueous peroxides, dilute the 30% (~9.8 M) solution of hydrogen peroxide (Catalog Number H9533) with deionized water 9.8-fold to prepare a 1 M solution. Dilute the 1 M hydrogen peroxide with two serial 10-fold dilutions to prepare a 10 mM solution. Determine the exact concentration of the 10 mM hydrogen peroxide solution by measuring the absorbance at 240 nm, using A_{240} of 10 mM solution = 0.436 to calculate the concentration. Dilute to exactly 1 mM using the calculated concentration and then dilute 10-fold to obtain a 100 µM solution. The reaction is linear from 1–7 nmoles of H₂O₂ per reaction volume.

- Working Color Reagent for aqueous peroxides - Prepare the Working Color Reagent by mixing 100 volumes of Aqueous Peroxide Color Reagent with 1 volume of Ferrous Ammonium Sulfate Reagent (*i.e.*, 10 ml of Aqueous Peroxide Color Reagent with 100 μ l of Ferrous Ammonium Sulfate Reagent).

Procedure for Determination of Aqueous Peroxides

- Follow the reaction scheme (see Table 1) for preparation of the H₂O₂ standard curve by placing 0, 10, 20, 40, 60, and 80 μ l of the 100 μ M standard hydrogen peroxide solution in labeled tubes. Bring the final volume in each tube to 100 μ l with deionized water.

Table 1.

Reaction Scheme for H₂O₂ Standard Curve

100 μ M H ₂ O ₂ Solution (μ l)	nmoles per reaction volume
0	0
10	1.0
20	2.0
40	4.0
60	6.0
80	8.0

- Place up to 100 μ l of the test sample in a tube. Bring the final volume to 100 μ l with deionized water.
- Add 1 ml of the Working Color Reagent to each tube, mix, and incubate at room temperature (25 °C) for ~30 minutes until color formation is complete.
- Read each standard and test sample at 560 nm in a spectrophotometer using water as a reference. If the signal is too high, it is possible to extend the range by reading the absorbance of the solutions at 590 nm.
- Plot a standard curve of nmoles of H₂O₂ against A₅₆₀ and calculate the nmoles of peroxide in the test sample (see Results section, calculations).

Determination for Organic or Lipid Hydroperoxide Solutions

Performing a small scale reaction in a multiwell plate is **not** recommended for determination for organic or lipid hydroperoxides, due to evaporation of the methanol based solutions.

Preparation Instructions

- Organic Peroxide Color Reagent - Reconstitute the contents of the vial (Catalog Number O8262) with 120 ml of a 90% methanol solution. After reconstitution this solution can be stored at 2–8 °C for at least 6 months. However, since methanol is a volatile solvent the concentration of this product may increase with time due to evaporation. Note: Using 100% methanol will result in lower absorbance readings.
- Working Color Reagent for organic or lipid hydroperoxides - Prepare the Working Color Reagent by mixing 100 volumes of the reconstituted Organic Peroxide Color Reagent with 1 volume of Ferrous Ammonium Sulfate Reagent (*i.e.*, 10 ml of Organic Peroxide Color Reagent with 100 μ l of Ferrous Ammonium Sulfate Reagent).
- 200 μ M *tert*-Butyl hydroperoxide (t-BuOOH) Standard Solution - To prepare a 200 μ M standard solution, dilute the 70% (~7 M) solution of *tert*-Butyl hydroperoxide (Catalog Number 458139) with methanol 7-fold to prepare a 1 M solution. Dilute the 1 M *tert*-butyl hydroperoxide with three serial 10-fold dilutions in methanol to prepare a 1 mM solution. Dilute the 1 mM solution 5-fold in methanol to prepare a 200 μ M solution. The reaction is linear from 1–16 nmoles of t-BuOOH per reaction volume.

Procedure for Determination of Organic or Lipid Hydroperoxides

If the amounts of H₂O₂ and lipid hydroperoxide in the sample are desired, this can be determined by using the Working Color Reagent for organic hydroperoxides. Assay the sample "as is" for total peroxide content and also after reduction with addition of tris(2-carboxyethyl) phosphine (TCEP, Catalog Number C4706) at a final concentration of 1 mM.¹² A reagent blank containing TCEP at a final concentration of 1 mM is recommended. This procedure will reduce virtually all the lipid hydroperoxide present and the result will be the inorganic peroxide content. The difference between the two readings is the true lipid hydroperoxide content.

When assaying for lipid hydroperoxides, it may be necessary to extract the lipid soluble components from the tissue. This can be performed by a $\text{CHCl}_3/\text{MeOH}$ extraction procedure.¹⁰

1. Follow the reaction scheme (see Table 2) for preparation of the *tert*-butyl hydroperoxide standard curve by placing 0, 5, 10, 20, 40, 60, and 80 μl of the 200 μM *tert*-butyl hydroperoxide standard solution in labeled tubes. Bring the final volume in each tube to 100 μl with 90% or 100% methanol.

Table 2.
Reaction Scheme for *tert*-BuOOH Standard Curve

200 μM t-BuOOH Solution (μl)	nmoles per reaction volume
0	0
5	1
10	2
20	4
40	8
60	12
80	16

2. Place up to 100 μl of test sample in a tube. Bring the final volume to 100 μl with 90% or 100% methanol.
3. Add 1 ml of the Working Color Reagent to each tube, mix, and incubate at room temperature (25 °C) for ~30 minutes until color formation is complete.
4. Read each standard and sample at 560 nm in a spectrophotometer using 90% or 100% methanol as a reference. If the signal is too high, it is possible to extend the range by reading the absorbance of the solutions at 590 nm.
5. Plot a standard curve of nmoles of t-BuOOH against A_{560} and calculate the nmoles of peroxide in the test sample (see Results section, calculations).

Results

Calculation

Calculate the nmole peroxide/ml in the test sample using the standard curve:

nmole peroxide/ml =

$$\frac{[A_{560}(\text{sample}) - A_{560}(\text{blank})] \times \text{dilution factor}}{[A_{560}(1 \text{ nmole peroxide})] \times \text{sample volume}}$$

Where:

dilution factor – factor for dilution of original sample
 $[A_{560}(1 \text{ nmole peroxide})]$ – calculated from H_2O_2 or t-BuOOH standard curve
 sample volume – volume of sample (ml)

References

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