3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

PeroxiDetect™ Kit
For the Determination of Aqueous and Lipid Hydroperoxides

Catalog Number **PD1**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

The measurement of peroxides in biological systems is an important factor in determining the degree of free radicals present in specific tissues.

Lipid peroxidation has been proposed to contribute to various pathophysiological cell and tissue abnormalities. For example, increased levels of lipid peroxidation products in red blood cells were found to correlate well with the onset of diabetes mellitus in pregnant women, being strongly elevated during the third trimester of pregnancy.[1] Cholesterol hydroperoxides analyzed from normal and alcoholic patients show an increased level of accumulation in the patients having excessive blood alcohol.[2,3]

Measurement of hydrogen peroxide in tissues has been used to study several aspects of free radical damage such as skin aging as induced by UV light,[4] and the effect of hydrogen peroxide (H_2O_2) as an inducer of elevated tyrosinase levels in melanoma cells.[5] Hydrogen peroxide has been shown to be a potent mitogen for growth arrested cultured human aortic smooth muscle cells.[6]

Assessment of oxidative damage in tissue exposed to oxidative stress has been difficult to achieve by direct measurement and the methods used for the direct determination of hydroperoxides (*e.g.*, iodine oxidation or coupled oxidation of NADPH) can be insensitive to the amounts of peroxide formed.[7] The use of thiobarbituric acid (TBA) as a reagent for the measurement of lipid peroxidation has been called into question.[8]

The method in this kit has a detection range for H_2O_2 in aqueous solutions of 1–7 nmoles per reaction volume or for lipid hydroperoxides in organic solvents of 1–16 nmoles per reaction volume.

The procedure is based on the fact peroxides oxidize Fe^{2+} to Fe^{3+} ions at acidic pH.[9] The Fe^{3+} ion will form a colored adduct with xylenol orange (XO, 3,3′-bis[*N,N*-bis(carboxymethyl)aminomethyl]-*o*-cresolsulfonephthalein, sodium salt), which is observed at 560 nm. The ε^M at 560 nm of the XO-Fe^{3+} colored adduct in aqueous solution is 15,000.

In aqueous solutions containing hydrogen peroxide, the colored product is enhanced by the addition of sorbitol to the reaction mixture. First, sorbitol is converted by the hydrogen peroxide to a peroxyl radical. This radical initiates the oxidation of Fe^{2+} ion to Fe^{3+} ion and the formation of the colored adduct with xylenol orange.[7]

For lipid hydroperoxides the aqueous reagent is unsuitable, since sorbitol causes extensive peroxidation of the lipid itself leading to a false signal. Lipid hydroperoxides are measured using a methanolic reagent containing xylenol orange and butylated hydroxytoluene (BHT). BHT is an antioxidant that prevents the effect of excess peroxidation.[7]

Components

Sufficient reagents are supplied for 100 tests of aqueous peroxide and 100 tests of organic peroxide.

Aqueous Peroxide Color Reagent 100 ml
Catalog Number A9344
Aqueous solution containing 100 mM sorbitol and 125 μM xylenol orange.

Organic Peroxide Color Reagent 1 bottle
Catalog Number O8262
Vial containing 480 μmoles of butylated hydroxytoluene (BHT) and 15 μmoles of xylenol orange dried *in situ.* After reconstitution using 90% methanol, the solution will contain 4 mM BHT and 125 μM xylenol orange.

Ferrous Ammonium Sulfate Reagent 2 ml
Catalog Number F6675
25 mM ferrous ammonium sulfate in 2.5 M sulfuric acid

tert-Butyl Hydroperoxide (t-BuOOH) 1 ml
Catalog Number 458139
70% aqueous solution

Hydrogen Peroxide Color Reagent (H_2O_2) 1 ml
Catalog Number H9533
30% aqueous solution

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 Fe^{2+} to Fe^{3+} ions under acidic conditions. Fe^{3+} ions will then form a colored adduct with xylenol orange, which is observed at 560 nm. The reaction can be described as:

$$Fe^{2+} + R\text{-}OOH \rightarrow Fe^{3+} + RO^{\bullet} + OH^{-}$$

$$Fe^{3+} + XO \rightarrow Fe^{3+}\text{-}XO \text{ (Colored adduct)}$$

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).

<u>Determination for Aqueous Peroxide Solutions</u>
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 H_2O_2 per reaction volume.

2. 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 µl of Ferrous Ammonium Sulfate Reagent).

Procedure for Determination of Aqueous Peroxides

1. Follow the reaction scheme (see Table 1) for preparation of the H_2O_2 standard curve by placing 0, 10, 20, 40, 60, and 80 µl of the 100 µM standard hydrogen peroxide solution in labeled tubes. Bring the final volume in each tube to 100 µl with deionized water.

   Table 1.
   Reaction Scheme for H_2O_2 Standard Curve

100 µM H_2O_2 Solution (µl)	nmoles per reaction volume
0	0
10	1.0
20	2.0
40	4.0
60	6.0
80	8.0

2. Place up to 100 µl of the test sample in a tube. Bring the final volume to 100 µl with deionized water.

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 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.

5. Plot a standard curve of nmoles of H_2O_2 against A_{560} 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

1. 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.

2. 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 µl of Ferrous Ammonium Sulfate Reagent).

3. 200 µM *tert*-Butyl hydroperoxide (t-BuOOH) Standard Solution - To prepare a 200 µM 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 µM 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 H_2O_2 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 $CHCl_3$/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

1. Carone, D. *et al.,* Eur. J. Obstet. Gyn. Rep. Biol., **51**, 103-109 (1993).
2. Adachi, J. *et al.,* Alcohol. Clin. Exp. Res., **22**, 103S-107S (3rd Suppl.) (1998).
3. Adachi, J. *et al.,* Alcohol. Clin. Exp. Res., **23**, 96S-100S (4th Suppl.) (1999).
4. Masaki, H. *et al.,* Biochem. Biophys. Res. Comm., **206**, 474-479 (1995).
5. Karg, E. *et al.,* J. Invest. Dermatol., (2nd Suppl.), **100**, 209S-213S (1993).
6. Herbert, J-M. *et al.,* FEBS Lett., **395**, 43-47 (1996).
7. Jiang, Z-Y. *et al.,* Lipids, **26**, 853-856 (1991).
8. Gutteridge, J.M.C., and Halliwell, B., Trends in Biochem. Sci., **15**, 129-135 (1990).
9. Gupta, B.L., Microchem. J., **18**, 363-374 (1970).
10. Yagi, K. *et al.,* Biochem. Int., **12**, 367-371 (1986).
11. Jiang, Z-Y. *et al.*, FEBS Lett., **268**, 69-71 (1990).
12. Nourooz-Zadeh, J. *et al.,* Anal. Biochem., **220**, 403-409 (1994).

EM,NDH,MAM 06/13-1