INSTRUCTIONS

Pierce™ Quantitative Peroxide Assay Kits

23280 23285

0526.2

Number	Description
23280	**Pierce Quantitative Peroxide Assay Kit, aqueous-compatible formulation**
	Kit contents:
23281	**Reagent A**, 1mL, Composition: 25mM ammonium ferrous (II) sulfate, 2.5M H_2SO_4
23282	**Reagent B**, 2 × 50mL, Composition: 100mM sorbitol, 125µM xylenol orange in water
23285	**Pierce Quantitative Peroxide Assay Kit, lipid-compatible formulation**
	Kit contents:
23281	**Reagent A**, 1mL, Composition: 25mM ammonium ferrous (II) sulfate, 2.5M H_2SO_4
23283	**Reagent C**, 2 × 50mL, Composition: 4mM BHT, 125µM xylenol orange in methanol

Xylenol orange is (*o*-cresolsulfonephthalein-3´-3´-bis-[methyliminodiacetic acid sodium salt]).

CAS numbers for chemical components of formulations:

methyl alcohol: #67-56-1	sorbitol: #50-70-4	xylenol orange: #1611-35-4
BHT: #28-37-0	sulfuric acid: #7664-93-9	ferrous sulfate: #7783-85-9

Storage: Upon receipt store kit at 4° C. Product shipped at ambient temperature.

Introduction

The Thermo Scientific Pierce Quantitative Peroxide Assay Kits detect peroxide based on oxidation of ferrous to ferric ion in the presence of xylenol orange. One assay kit has an aqueous-compatible formulation that includes sorbitol, which provides sensitivity enhancement. The other assay kit has a lipid-compatible formulation that may be used without extraction; however, because it does not contain sorbitol, the assay may be less sensitive. Most proteins do not interfere with these assays, although some metal chelators may require use of a blank (i.e., control). Pierce Quantitative Peroxide Assays are suitable for H_2O_2 measurement in the following applications: quantitating detergent peroxide, monitoring cellular activity, determining protein glycation and measuring peroxide accumulation in lipid.

In these assays, hydroperoxides convert the Fe^{2+} to Fe^{3+} at acidic pH. With the aqueous-compatible formulation, peroxide first reacts with sorbitol, converting it to a peroxyl radical, which in turn initiates Fe^{2+} oxidation to Fe^{3+}. In the lipid-compatible formulation, the peroxide converts the Fe^{2+} to Fe^{3+} directly. In a sulfuric acid solution, the Fe^{3+} complexes with the xylenol orange dye to yield a purple product with maximum absorbance at 560nm. The molar extinction coefficient of the xylenol orange-Fe^{3+} complex is 1.5×10^4 $M^{-1}cm^{-1}$ in 25mM H_2SO_4 at room temperature. The complex in both assays absorbs strongest between 540 and 580nm but absorbs measurably up to 620nm. When using microplates, the best wavelength for measurement is 595nm (best signal-to-noise). The maximum absorbance of the assay components before addition of peroxide is at 430nm.

Important Product Information

- Excessive H_2O_2 (above 1mM) can result in low absorbance measurements caused by a bleaching effect on the dye. Ensure that results are accurate by preparing a 1:100 dilution of the sample and perform the assay in the same manner as the undiluted sample. If the absorbance of the diluted sample is higher than or similar to the original reading, then there is excessive peroxide in the sample.
- The protocols use a 1:10 (sample:WR) ratio. This ratio can be changed to accommodate samples with relatively high levels of peroxide [e.g., use 1:100 (sample:WR)] as long as the dilution is accounted for when comparing to the assay standards.
- The working range of the assay is from 1µM to 1mM peroxide in a sample assayed using the Standard Procedure [1:10 (sample:WR) ratio]. Above 1mM, a bleaching effect occurs, causing a decrease in absorbance and loss of linearity in the assay.
- The standard curve is not linear over the entire assay range (see Figure 1). Use a quadratic or best-fit curve to the standard points. Alternatively, use a linear fit for individual 100µM increments (e.g., from 1-100µM, 101-200µM, etc.); each incremental standard curve will be more horizontal in slope than the previous one until a plateau is reached at about 1mM.

Reagent Preparation for Aqueous-Compatible Procedure

Working Reagent (WR)

Mix 1 volume of Reagent A with 100 volumes of Reagent B.

Notes:

- Prepare at least 1mL of WR for each sample and standard replicate to be assayed in cuvettes. Prepare at least 200µL of WR for each sample and standard replicate to be assayed in a microplate.
- The WR is stable for at least 12 hours.

Peroxide Standards

Serially dilute a 30% (8.8M) hydrogen peroxide stock solution to achieve 8-10 standards in the concentration range of 1-1000µM (Figure 1).

Notes:

- A 30% hydrogen peroxide solution can be standardized using $43.6 M^{-1} cm^{-1}$ as the molar extinction coefficient for H_2O_2 at 240nm. Then, prepare the high standard (1000µM) by adding 100µL of 30% hydrogen peroxide to 880mL of ultrapure water or buffer.

Aqueous-Compatible Procedure

1. Add 10 volumes of WR to 1 volume of sample. For example, in a microplate, add 20µL of sample (or standard) and 200µL of WR to each well.
2. Mix and incubate assay reactions for 15-20 minutes at room temperature. This incubation step is necessary for the reaction to reach an endpoint. Once formed, the complex is relatively stable, but absorbance measurements are optimal if taken on the same day the reaction is performed.
3. Measure absorbance at 560-620nm (560nm is optimal in a spectrophotometer; 595nm is optimal if using a plate reader).
4. Calculate the concentration of peroxide in the sample by reference to its assay absorbance compared to the standard curve (see the Important Product Information Section for correct curve-fitting procedures for the standards).

Reagent Preparation for Lipid-Compatible Procedure

Working Reagent (WR)	Mix 1 volume of Reagent A with 100 volumes of Reagent C. **Notes:** • Prepare at least 1mL of WR for each sample and standard replicate to be assayed in cuvettes. Prepare at least 200µL of WR for each sample and standard replicate to be assayed in a microplate. • The WR is stable for at least 12 hours.
Peroxide Standards	Serially dilute a 30% (8.8M) hydrogen peroxide stock solution in methanol to make 8-10 standards in the concentration range of 1-1000µM (Figure 1). Refer to the Aqueous-Compatible Procedure for more information on diluting the peroxide standard. For the "TCEP Reference," dilute Thermo Scientific Bond-Breaker TCEP Solution, Neutral pH (Product No. 77720; 0.5M) (see Related Thermo Scientific Products) to make a 10mM stock solution by diluting 1 part of the TCEP Solution with 49 parts of methanol.

Lipid-Compatible Procedure

- If samples may contain metals, then produce "test" or methanol-only replicates, as well as "TCEP Reference" replicates. Subtract the absorbance values of the "TCEP Reference" from that of the "test" replicates to eliminate the metal effects. Create both types of replicates for each peroxide concentration standard. Use the absorbance difference at each standard concentration as the value for creating the standard curve. If the absorbance of the "test" and "TCEP Reference" are equal, then metals are not present and the "TCEP Reference" steps do not need to be performed.
- Plasma hydroperoxide levels may be determined using the lipid-compatible formulation of the Pierce Quantitative Peroxide Assay Kit (Prod. No. 23285). Since plasma is quite low in peroxide content, the assay absorbance measurement often will be less than about 0.100. Because of endogenous plasma iron, a "TCEP Reference" must be assayed as described. This protocol is based on the work by Nourooz-Zadeh, J., *et.al.*[1]
- If using the assay with lipid samples that are not extracted, prepare a blank that omits Reagent A. Subtracting the absorbance for this blank from the sample tested in WR controls endogenous iron interferences. A blank may also be necessary if the sample contains other transition metals or a protein having chelating properties or strong absorbance characteristics at the wavelengths used for measurement.

1. For each sample to be tested, pipette 90µL of sample into microcentrifuge tubes.

   Note: For each plasma sample or other metal-containing sample to be tested, pipette 90µL of sample into microcentrifuge tubes labeled "test" and "TCEP Reference." Use a minimum of two replicates for each "test" and "TCEP Reference." In this case, each peroxide standard must also be created as "test" and "TCEP Reference" replicates.
2. Add 10µL of methanol to each "test" tube and 10µL of 10mM TCEP in methanol to each "TCEP Reference" tube.
3. Vortex tubes and incubate for 20-30 minutes at room temperature.
4. To each tube, add 900µL of WR.
5. Vortex tubes and incubate for 20-30 minutes.
6. Centrifuge at 12,000 × *g* for 5-10 minutes.
7. Transfer supernatant to a cuvette (900µL) or microwell plate (300µL).
8. Measure absorbance at 560-620nm (560nm is optimal in a spectrophotometer or 595nm if using a plate reader) as described in Aqueous Compatible Procedure.
9. For samples containing metals, subtract the "TCEP Reference" absorbance from the "test" absorbance and generate the hydrogen peroxide standard curve using the difference at each peroxide concentration. Calculate the concentration of the samples against this standard curve.

Additional Information

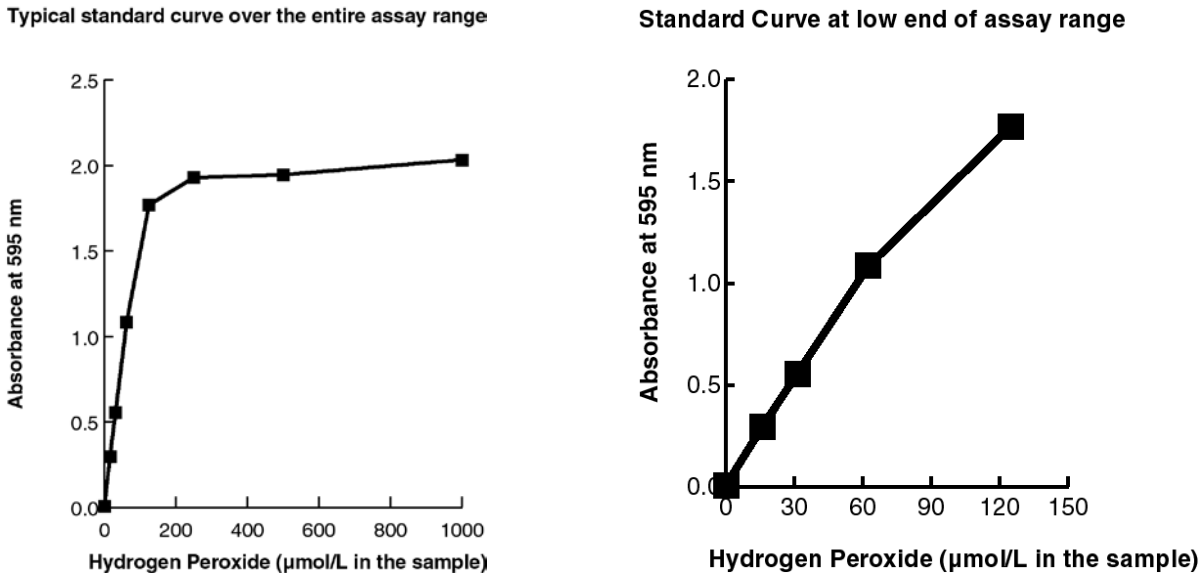

Figure 1. Thermo Scientific Pierce Quantitative Peroxide Assay absorbance response to hydrogen peroxide concentration. A 1mM (1000µM) solution of hydrogen peroxide was initially made by diluting a 30% H_2O_2 stock 1:9000 (11µL of 30% H_2O_2 into 100mL of ultrapure water). This sample was then serially diluted with ultrapure water 1:2 (100µL of ultrapure water + 100µL of the previous dilution) for a total of seven samples. Ultrapure water was used as the blank. In microplate wells, 200µL of the Working Reagent was added to 20µL of the diluted H_2O_2 standards. Samples were mixed and incubated for 15 minutes at room temperature. Absorbances were measured at 595nm using a plate reader. The blank value was subtracted from all sample measurements.

Related Thermo Scientific Products

77720	**Bond-Breaker™ TCEP Solution, Neutral pH**
20490	**TCEP•HCl**, Tris[2-carboxyethyl]phosphine hydrochloride, 1g
15041	**96-Well EIA Plates**, corner-notched, clear, 100 per package
15031	**8-Well EIA Strip Plates**, corner-notched, clear, 100 per package
15082	**MicroTube Racked System**

General References

Jiang, Z.-Y., Hunt, J.V. and Wolff, S.P. (1992). Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low-density lipoprotein. *Anal Biochem* **202:**384-9.

Jiang, Z.-Y., Woollard, A.C.S. and Wolff, S.P. (1990). Hydrogen peroxide production during experimental protein glycation. *FEBS* **268(1)**:69-71.

Jiang, A.-Y., Woollard, A.C.S. and Wolff, S.P. (1991). Lipid hydroperoxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. *Lipids* **26:**853-6.

Cited Reference

1. Nourooz-Zadeh, J., Tajaddini-Sarmadi, J. and Wolff, S.P. (1994). Measurement of plasma hydroperoxide concentrations by the ferrous oxidation - Xylenol Orange assay. *Anal Biochem* **220:**403-9.

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