Product Manual

Hydrogen peroxide colorimetric detection kit

Catalog #: ADI-907-015

96 Determination Kit

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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DESCRIPTION

The Hydrogen peroxide colorimetric detection kit is a complete kit for the quantitative determination of Hydrogen Peroxide in biological fluids and tissue culture media. Please read the complete kit insert before performing this assay. The kit is designed to measure low concentrations of H_20_2 in biological matrices. The kit has a color reagent that contains a dye, xylenol orange, in an acidic solution with sorbitol and ammonium iron sulfate that reacts to produce a purple color proportional to the concentration of H_20_2 in the sample. The exact mechanism of the color reaction is not known, but probably involves coordinated iron reacting with H_20_2 and the dye molecule.

INTRODUCTION

Hydrogen Peroxide (H_20_2) is a reactive oxygen metabolic by-product that serves as a key regulator for a number of oxidative stress-related states.[1,2] Functioning through NF kappa-B and other factors, hydroperoxide-mediated pathways have been linked to asthma, inflammatory arthritis, atherosclerosis, diabetic vasculopathy, osteoporosis, a number of neurodegenerative diseases and Down's Syndrome.[3-11] Perhaps the most intriguing aspect of H_20_2 biology is the recent report that antibodies have the capacity to convert molecular oxygen into hydrogen peroxide to contribute to the normal recognition and destruction processes of the immune system.[12,13] Measurement of this reactive species will help to determine how oxidative stress modulates varied intracellular pathways.

PRECAUTIONS

Handle with care

Protect from light

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

1. Dispose of the contents of the plate with care. Attention should be taken in handling because of unknown effects of the contents.
2. We test this kit's performance in a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results. Refer to Sample Handling, page 4.
3. The standard is light-sensitive and should be protected from direct light for prolonged periods of time.

MATERIALS SUPPLIED

1. **Half-Area Microtiter Plate, 1 each, Catalog No. 80-2404:**

   The plate is ready to use.

2. **Hydrogen Peroxide Standard, 0.5ml Catalog No. 80-0941:**

   A solution of Hydrogen Peroxide at 100,000ng/mL in water with preservatives.

3. **Hydrogen Peroxide Color Reagent, 11ml, Catalog No. 80-0968**

   A solution of colorimetric substrate in dilute acid.

4. **Hydrogen Peroxide Assay Layout Sheet, 1 each, Catalog No. 30-0170**

5. **Plate Sealer, 2 each, Catalog No. 30-0012**

Reagents require separate storage conditions.

STORAGE

All components of this kit, except the Hydrogen Peroxide Color Reagent, are stable at 4°C until the kit's expiration date. The Hydrogen Peroxide Color Reagent must be stored at -20˚C.

OTHER MATERIALS NEEDED

1. Recommended sample diluent, 50mM Phosphate, pH 6.0.
2. Precision pipets for volumes between 34µl and 1,000µl.
3. Repeater pipet for dispensing 100µl.
4. Microplate reader capable of reading between 540 and 570nm, ideally 550nm.
5. Graph paper for plotting the standard curve.

SAMPLE HANDLING

The Hydrogen peroxide colorimetric detection kit is compatible with samples in a wide range of matrices. Samples diluted sufficiently into the recommended Sample Diluent of 50mM Phosphate, pH 6.0 can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. **If the end user chooses to use a different buffer as the Sample Diluent, it is up to the end user to determine if the buffer works in the assay, the appropriate dilution of samples, and assay validation.**

Samples in Tissue Culture Media can also be read in the assay provided the standards have been diluted into the Tissue Culture Media. There will be a small change in the standard curve associated with running the standards and samples in media. Media containing ferrous salts should be avoided as they will interfere with sensitive detections. The presence of pH indicator will not affect relative assay detection as long as standards and samples are in the same media.

PROCEDURAL NOTES

1. Do not mix components from different lot numbers or use reagents beyond the expiration date.
2. Allow all reagents, with the exception of the Hydrogen Peroxide Color Reagent, to warm to room temperature for at least 30 minutes before opening.
3. The Hydrogen Peroxide Color Reagent must be kept at 4˚C during use.
4. Standards can be made up in either glass or plastic tubes.
5. Pre-rinse the pipet tip with the reagent, use fresh pipet tips for each sample, standard and reagent.
6. Pipet standards and samples to the bottom of the wells.
7. Add the reagent to the side of the well to avoid contamination.

REAGENT PREPARATION

Hydrogen Peroxide Standard

Allow the 100,000ng/mL Hydrogen Peroxide standard solution to warm to room temperature. Label six 12 x 75 mm tubes #1 through #6.

Pipet 966µl of sample diluent (buffer or Tissue Culture Media) into tube #1. Pipet 500µl of Diluent into tubes #2 - #6.

Add 34µl of the 100,000ng/mL standard to tube #1. Vortex thoroughly. Add 500µl of tube #1 to tube #2 and vortex thoroughly. Add 500µl of tube #2 to tube #3 and vortex. Continue this for tubes #4 through #6.

The concentration of Hydrogen Peroxide in tubes #1 through #6 will be 3,400, 1,700, 850, 425, 212.5 and 106.25ng/mL respectively. This converts to 100µM, 50µM, 25µM, 12.5µM, 6.25µM and 3.125µM respectively. See Hydrogen Peroxide Assay Layout Sheet for dilution details.

ASSAY PROCEDURE

Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.

1. Determine the number of wells to be used. Cover unused wells tightly with a plate sealer. **DO NOT REUSE WELLS**!
2. Pipet 50µl of sample diluent (buffer or Tissue Culture Media) into duplicate Blank (Zero Standard) wells.
3. Pipet 50µl of Standards #1 through #6 into duplicate wells.
4. Pipet 50µl of Samples into duplicate wells.
5. Pipet 100µl of Color Reagent into the Blank, Standards and Sample wells.
6. Mix well by shaking or tapping the side of the plate for 10 seconds.
7. Incubate for 30 minutes at room temperature.
8. Blank the plate reader against the blank wells, read the optical density between 540 and 570nm, preferably at 550nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

CALCULATION OF RESULTS

Several options are available for the calculation of the concentration of Hydrogen Peroxide in the samples. We recommend that the data be handled by an immunoassay software package utilizing a four parameter logistic curve fitting program (such as AssayBlaster™, catalog number ADI-28-0002). Such software is often supplied by plate reader manufacturers. If this type of data reduction software is not readily available, the concentration of Hydrogen Peroxide can be calculated as follows:

1. Calculate the average net Optical Density (OD) for each standard and sample by subtracting the average Blank OD from the average OD for each standard and sample:

   Average Net OD = Average OD - Average Blank OD

2. Plot the Average Net OD for each Standard versus Hydrogen Peroxide Concentration. Approximate a straight line through the points. The concentration of Hydrogen Peroxide in the unknowns can be determined by interpolation.

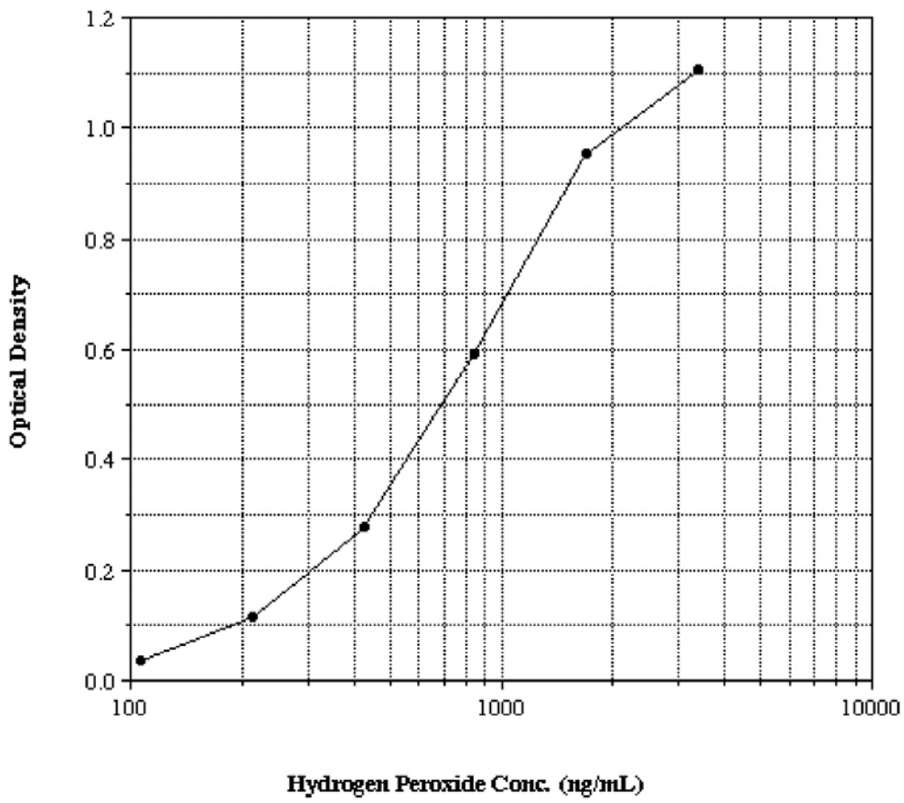
TYPICAL RESULTS

The results shown below are for illustration only and should not be used to calculate results.

Sample	Average OD	Net OD	Hydrogen Peroxide	
			(ng/mL)	µM
Blank	0.306			
S1	1.412	1.106	**3,400**	**100**
S2	1.262	0.956	**1,700**	**50**
S3	0.899	0.593	**850**	**25**
S4	0.585	0.279	**425**	**12.5**
S5	0.421	0.115	**212.5**	**6.25**
S6	0.341	0.035	**106.25**	**3.125**
Unknown1	0.838	0.532	**767.6**	**22.58**
Unknown2	1.200	0.894	**1,555.1**	**45.74**

TYPICAL STANDARD CURVES

Typical standard curves are shown below. These curves must not be used to calculate pERK concentrations; each user must run a standard curve for each assay.



PERFORMANCE CHARACTERISTICS

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[14].

Sensitivity

Hydrogen Peroxide sensitivity in 50mM Phosphate, pH 6.0 was calculated by determining the average OD bound for sixteen (16) wells run as the zero standard, and comparing to the average OD for sixteen (16) wells run with Standard #6. The detection limit was determined as the concentration of Hydrogen Peroxide measured at two (2) standard deviations from the zero along the standard curve.

OD for Zero Standard =	0.277 ± 0.020 (7.3%)
OD for Standard #6 =	0.362 ± 0.051 (14.0%)
Delta OD (106.25-0ng/mL) =0.362-0.277 =	0.085
2 SD's of Zero Standard =	0.041
Sensitivity = $\frac{0.041}{0.085}$ x 106.25ng/mL =	51.25ng/mL

Linearity

A sample containing 2,880ng/mL Hydrogen Peroxide was serially diluted 5 times 1:2 in the recommended sample diluent and measured in the assay. The data was plotted graphically as actual Hydrogen Peroxide concentration versus measured Hydrogen Peroxide concentration. The line obtained had a slope of 0.9342 with a correlation coefficient of 0.9983.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Hydrogen Peroxide and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of Hydrogen Peroxide in multiple assays (n=8). The precision numbers listed below represent the percent coefficient of variation for the concentrations of Hydrogen Peroxide determined in these assays as calculated by a curve fitting program.

	Hydrogen Peroxide (ng/mL)	Intra-assay %CV	Inter-assay %CV
Low	314.2		10.0
Medium	772.1		3.0
High	1,606.2		4.0
Low	326.46	2.2	
Medium	768.21	1.7	
High	1,732.93	5.7	

SAMPLE RECOVERIES

Please refer to pages **4-5** for Sample Handling recommendations and Standard preparation.

Hydrogen Peroxide concentrations were measured in horse heparinized plasma, human serum, human urine and tissue culture media. Hydrogen Peroxide was spiked into the undiluted samples of these matrices which were then diluted with the appropriate diluent and assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Horse heparin Plasma 94.5 >1:64	Horse heparin Plasma 94.5 >1:64	Horse heparin Plasma 94.5 >1:64
Human Serum 94.7 >1:64	Human Serum 94.7 >1:64	Human Serum 94.7 >1:64
Human Urine 90.6 >1:64	Human Urine 90.6 >1:64	Human Urine 90.6 >1:64
Tissue Culture Media 105.6 None	Tissue Culture Media 105.6 None	Tissue Culture Media 105.6 None

* See Sample Handling instructions on page 4 for details.

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