KAMIYA BIOMEDICAL COMPANY

Hydrogen Peroxide Assay

For the quantitative determination of hydrogen peroxide in colorless tissue culture media and buffers

Cat. No. KT-130

For research use only.

PRODUCT INFORMATION

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INTENDED USE
The Hydrogen Peroxide Assay is for the quantitative determination of hydrogen peroxide in colorless tissue culture media and buffers. For research use only.

DESCRIPTION
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. 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. 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. Measurement of this reactive species will help to determine how oxidative stress modulates varied intracellular pathways.

PRECAUTIONS
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. The microtiter plate supplied in the kit has been selected for its low luminescent background and excellent reproducibility.
3. 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.

COMPONENTS
1. White Microtiter Plate
   Break apart microtiter plate. Store at room temperature in the plastic ziploc bag provided to prevent contamination.
2. Hydrogen Peroxide Calibrator, 0.5 mL
   A solution of Hydrogen Peroxide at 100,000 ng/mL in water with preservatives.
3. Hydrogen Peroxide Substrate, 6 mL
   A solution of chemiluminescent substrate in aqueous buffers. Substrate is sensitive to light. Store in the dark. Protect from ambient room light.
4. Hydrogen Peroxide Trigger Solution, 15 mL
   A solution of Bovine Hemoglobin containing preservatives.
5. Assay Layout Sheet

STORAGE
All components of this kit are stable at 4°C until the kit's expiration date.

MATERIALS REQUIRED BUT NOT PROVIDED
- Precision pipettes for volumes between 25 µL and 1,000 µL.
- Repeater pipette for dispensing 50 µL.
- Plate Luminometer with one injector capable of flash luminescence.
- Graph paper for plotting the calibration curve.

SAMPLE HANDLING
The Hydrogen Peroxide Assay is compatible with samples in tissue culture media and buffer. Samples in these matrices can be read directly from the calibration curve, provided the calibrators have been diluted in the same diluent as the samples. A tissue culture media **without** phenol red or other color indicators is recommended for use in this assay.

Phenol red has been shown to interfere.

This assay is not designed for samples which could potentially contain Hemoglobin. The hydrogen peroxide in the calibrator or sample reacts with Hemoglobin, which in this system is used as the trigger. The product of this reaction then reacts with the provided substrate, yielding light as a final product. The problem with biological samples containing Hemoglobin is it will trigger the Substrate before the plate is placed in the plate reader and the reaction is measured.

An alternative method for biological samples would be to directly pipet the calibrators or samples and Hemoglobin into the wells and use the Substrate as the trigger in the luminometer. However, after using this method, the trigger lines used will have to be designated for this Substrate only and will have to be replaced for other triggers. This alternative method is offered only as a suggestion.

PRECAUTIONS

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to RT for at least 30 minutes before opening.
3. Calibrators can be made up in either glass or plastic tubes.
4. Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, calibrator and reagent.
5. Pipette calibrators and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells can be kept at room temperature in the plastic ziploc bag provided. The wells should be used in the frame provided.
8. **This assay uses a luminescent measurement of Hydrogen Peroxide concentration. The luminescent signal is typically represented as Relative Light Units (RLU). Different luminometers will display different RLU readings. Please see the luminometer instruction manual for details.**

REAGENT PREPARATION

Hydrogen Peroxide Calibrators

In Buffer

Allow the 100,000 ng/mL calibrator solution to warm to RT. Label six 12 x 75 mm glass tubes #1 through #6. Pipette 975 µL of buffer into tube #1. Pipette 750 µL of buffer into tubes #2 through #6. Add 25 µL of the 100,000 ng/mL calibrator to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL 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 2,500, 625, 156.25, 39.06, 9.77 and 2.44 ng/mL respectively.

Tissue Culture Media

Allow the 100,000 ng/mL calibrator solution to warm to RT. Label five 12 x 75 mm glass tubes #1 through #5. Pipette 900 µL of tissue culture media into tube #1. Pipette 750 µL of tissue culture media into tubes #2 through #5. Add 100 µL of the 100,000 ng/mL calibrator to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5.

The concentration of Hydrogen Peroxide in tubes #1 through #5 will be 10,000, 2,500, 625, 156.25 and 39.06 ng/mL respectively.

ASSAY PROCEDURE

All samples should be run in duplicate.
All samples should be allowed to warm to RT for at least 30 minutes prior to use.

1. Determine the number of wells to be used and put any remaining wells into plastic ziploc bag. Store unused wells at room temperature.
2. Pipette 50 µL of calibrator diluent (Buffer or Tissue Culture Media) into duplicate blank (Zero Calibrator) wells.
3. Pipette 50 µL of Calibrators #1 through #6 for Buffer or #1 through #5 for Tissue Culture Media into duplicate wells.
4. Pipette 50 µL of Samples into duplicate wells.
5. Pipette 50 µL of Substrate into the blank, calibrator and sample wells.
6. Mix well by shaking or tapping the side of the plate for 10 seconds.
7. Place microtiter plate in luminometer for chemiluminescent measurement.
8. Inject 50 µL of Hydrogen Peroxide Trigger into each well. Immediately read in luminometer for 5 seconds.
9. Determine integrated light output for the 5 second read time in Relative Light Units (RLU).

For Step 8: Program your luminometer to inject with zero delay between injection and light detection. Read time should be set at 5 seconds.

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 a software package utilizing a 4 parameter logistic curve-fitting program. 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 Relative Light Units (RLU) for each calibrator and sample by subtracting the average Blank RLU from the average RLU for each calibrator and sample.
   Average Net RLU = Average RLU - Average Blank RLU
2. Using linear graph paper, plot the Average Net RLU for each calibrator 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 from another assay.

Buffer:

Sample	Average RLU	Net RLU	Hydrogen Peroxide (ng/mL)
BLANK	(129,892)		
S1	10,160,143	10,030,251	2,500
S2	3,462,480	3,332,588	625
S3	1,228,303	1,098,411	156.25
S4	396,837	266,945	39.06
S5	201,917	72,025	9.77
S6	157,039	27,147	2.44

Tissue Culture Media:

Sample	Average RLU	Net RLU	Hydrogen Peroxide (ng/mL)
BLANK	(146,118)		
S1	2,291,888	2,145,770	10,000
S2	598,190	452,072	2,500
S3	247,691	101,573	625
S4	165,902	19,784	156.25
S5	150,207	4,089	39.06

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.

Sensitivity

Sensitivity was calculated by determining the average RLU bound for sixteen (16) wells run as the zero Calibrator, and comparing to the average RLU for sixteen (16) wells run with Calibrator #6 for Buffer or Calibrator #5 for Tissue Culture Media. The detection limit was determined as the concentration of hydrogen peroxide measured at two (2) standard deviations from the zero along the calibration curve.

Buffer:
RLU for Zero Calibrator = 129,168 $\pm$ 9,374 (7.3%)
RLU for Calibrator #6 = 148,271 $\pm$ 5,254 (3.5%)
Delta RLU (2.44-0 ng/mL) = 148,271-129,168 = 19,102
2 SD's of S0 = 18,748
Sensitivity = 18,748/ 19,102 x 2.44 ng/mL = 2.39 ng/mL

Tissue Culture Media:
RLU for Zero Calibrator = 143,039 ± 2,949 (2.1%)
RLU for Calibrator #6 = 162,409 ± 3,513 (2.2%)
Delta RLU (39.1-0 ng/mL) = 162,409-143,039 = 19,370
2 SD's of S0 = 5,898
Sensitivity = 5,898/ 19,370 x 39.1 ng/mL = 11.91 ng/mL

Linearity

Buffer:
A sample containing 1,146 ng/mL hydrogen peroxide was serially diluted 7 times 1:2 in Buffer 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 0.9841 with a correlation coefficient of 0.9998.

Tissue Culture Media:
A sample containing 8,184 ng/mL hydrogen peroxide was serially diluted 6 times 1:2 in Tissue Culture Media 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 0.9441 with a correlation coefficient of 0.9997.

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.

Buffer:

	Hydrogen Peroxide (ng/mL)	Intra-assay % CV	Inter-assay % CV
Low	26.49	9.6	
Medium	51.57	6.7	
High	1,167.56	5.8	
Low	27.33		7.8
Medium	55.25		6.1
High	1,135.85		8.4

Tissue Culture Media:

	Hydrogen Peroxide (ng/mL)	Intra-assay % CV	Inter-assay % CV
Low	483.04	3.4	
Medium			
High	911.85	4.0	
Low	33.1		8.9
Medium			
High	880.4		3.9

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KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com