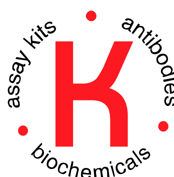

KAMIYA BIOMEDICAL COMPANY

Hydrogen Peroxide Assay

For the quantitative chemiluminescent determination of Hydrogen Peroxide in colorless tissue culture media and buffers

Cat. No. KT-130

For research use only, not for use in diagnostic procedures.

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DESCRIPTION

The **K-ASSAY**® Chemiluminescent Hydrogen Peroxide Assay is a complete kit for the quantitative determination of Hydrogen Peroxide in colorless tissue culture media and buffers. Please read the complete package insert before performing this assay.

INTRODUCTION

Hydrogen Peroxide (H_20_2) is a reactive oxygen metabolic byproduct that serves as a key regulator for a number of oxidative stress-related states. Functioning through NF-kappaB 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. Refer to Sample Handling, page 2.

COMPONENTS

1. **White Microtiter Plate, 1 each**
   Break apart microtiter plate. Store at room temperature in the plastic ziploc bag provided to prevent contamination.
2. **Hydrogen Peroxide Standard, 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 Hemoglobin containing preservatives.
5. **Hydrogen Peroxide Assay Layout Sheet, 1 each**

STORAGE

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

MATERIALS NEEDED BUT NOT SUPPLIED

1. Precision pipets for volumes between 25 µL and 1,000 µL.
2. Repeater pipet for dispensing 50 µL.
3. Plate Luminometer with one injector capable of flash luminescence.
4. Graph paper for plotting the standard curve.

SAMPLE HANDLING

The **K-ASSAY**® Hydrogen Peroxide Assay is compatible with samples in tissue culture media and buffer. Samples in these matrices can be read directly from the standard curve, provided the standards have been diluted in the same diluent as the samples. A tissue culture medium **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 standard 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 standards 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.

PROCEDURAL NOTES

1. Do not mix components from different lot numbers or use reagents beyond the expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with the reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards 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

1. Hydrogen Peroxide Standard(s)

In buffer:

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

Pipet 975 µL of buffer into tube #1. Pipet 750 µL of buffer into tubes #2 - #6.

Add 25 µL of the 100,000 ng/mL standard 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. See Hydrogen Peroxide Assay Layout Sheet for dilution details.

In Tissue Culture Media:

Allow the 100,000 ng/mL Hydrogen Peroxide Standard Solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5.

Pipet 900 µL of tissue culture media into tube #1. Pipet 750 µL of tissue culture media into tubes #2 -#5.

Add 100 µL of the 100,000 ng/mL standard 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 be10,000, 2,500, 625, 156.25 and 39.06 ng/mL respectively. See Hydrogen Peroxide Assay Layout Sheet for dilution details.

ASSAY PROCEDURE

All samples should be run in duplicate.

All samples should be allowed to warm to room temperature for at least 30 minutes prior to use.

1. Determine the number of wells to be used and put any remaining wells back into plastic ziploc bag. Store unused wells at room temperature.
2. Pipet 50 µL of standard diluent (buffer or tissue culture media) into duplicate Blank (Zero Standard) wells.
3. Pipet 50 µL of Standards #1 through #6 for buffer, or #1 through #5 for tissue culture media, into duplicate wells.

4. Pipet 50 µL of Samples into duplicate wells.
5. Pipet 50 µL of Substrate into the Blank, Standards 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 standard and sample by subtracting the average Blank RLU from the average RLU for each standard and sample:

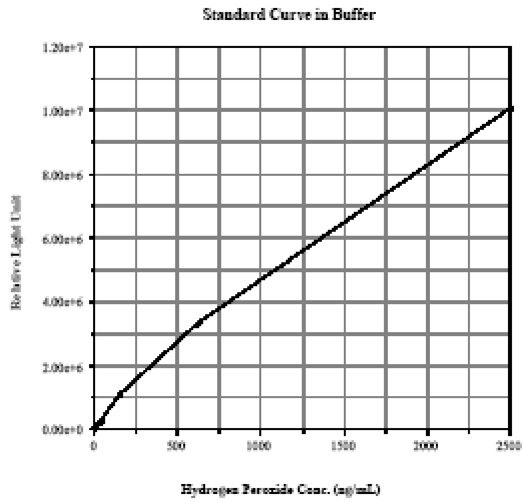
   Average Net RLU = Average RLU - Average Blank RLU

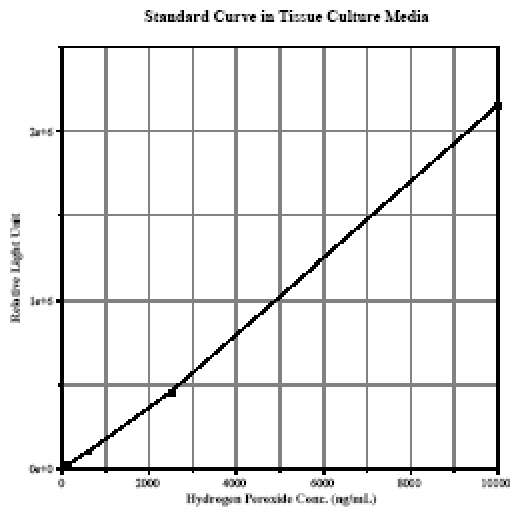
2. Using linear graph paper, plot the Average Net RLU for each Standard versus Hydrogen Peroxide Concentration. Approximate a straight line through the points. The concentration of Hydrogen Peroxide in the unknowns can be determined by interpolation.

TYPICAL RESULTS

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

Typical standard curves are shown below. The curves must not be used to calculate Hydrogen Peroxide concentrations; each user must run standard curve for each assay.





Sample	BUFFER Average RLU	BUFFER Net RLU	BUFFER **Hydrogen Peroxide ng/mL**	TISSUE CULTURE MEDIA Average RLU	TISSUE CULTURE MEDIA Net RLU	TISSUE CULTURE MEDIA **Hydrogen Peroxide ng/mL**
Blank	(129,892)			(146,118)		
S1	10,160,143	10,030,251	**2,500**	2,291,888	2,145,770	**10,000**
S2	3,462,480	3,332,588	**625**	598,190	452,072	**2,500**
S3	1,228,303	1,098,411	**156.25**	247,691	101,573	**625**
S4	396,837	266,945	**39.06**	165,902	19,784	**156.25**
S5	201,917	72,025	**9.77**	150,207	4,089	**39.06**
S6	157,039	27,147	**2.44**			

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

Hydrogen Peroxide sensitivity was calculated by determining the average RLU bound for sixteen (16) wells run as the zero standard, and comparing to the average RLU for sixteen (16) wells run with Standard #6 for Buffer or Standard #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 standard curve.

Buffer Sensitivity

RLU for Zero Standard = 129,168 ± 9,374 (7.3%)

RLU for Standard #6 = 148,271 ± 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 = $\frac{18,748}{19,102}$ x 2.44 ng/mL = **2.39 ng/mL**

Tissue Culture Media

RLU for Zero Standard =	143,039 ± 2,949 (2.1%)
RLU for Standard #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 = $\frac{5,898}{19,370}$ 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			TISSUE CULTURE MEDIA		
	Hydrogen Peroxide (ng/mL)	Inter-assay (%CV)	Intra-assay (%CV)	Hydrogen Peroxide (ng/mL)	Inter-assay (%CV)	Intra-assay (%CV)
Low	27.33	7.8		433.1	8.9	
Medium	55.25	6.1				
High	1,135.85	8.4		880.4	3.9	
Low	26.49		9.6	483.04		3.4
Medium	51.57		6.7			
High	1,167.56		5.8	911.85		4.0

FOR RESEARCH USE ONLY

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