assay designs
SIMPLIFY YOUR SCIENCE®

Stressgen

> Hydrogen Peroxide Fluorometric Detection Kit

Catalog # 907-028

Sufficient Reagents for 500 Tests

For use with cells, culture supernates, and other biological matrices

Store at 4°C

Check our website for additional protocols, technical notes and FAQs

For proper performance, use the insert provided with each individual kit received

Table of Contents

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

Assay Designs
734.668.6113 or 800.833.8651
www.assaydesigns.com

Introduction

Assay Designs' Hydrogen Peroxide Fluorometric Detection Kit is a sensitive cell-based assay to detect hydrogen peroxide or peroxidase activity from culture supernates, cells, and other biological matrices. The kit may also be used to measure peroxidase activity. This kit provides a fast one-step homogenous assay that is adaptable to kinetic and High Throughput applications.

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 NFκB and other factors, hydrogen peroxide mediated pathways have been linked to asthma, inflammatory arthritis, atherosclerosis, diabetes, 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.

Principle

The Hydrogen Peroxide Fluorometric Detection Kit utilizes a non-fluorescent substrate, 10-Acetyl-3, 7- dihydroxyphenoxazine (ADHP), to detect H_2O_2. H_2O_2 oxidizes ADHP in a one to one ratio to produce a fluorescent product, Resorufin. This oxidation is catalyzed by Peroxidase in a homogeneous no wash assay system.

ADHP can be utilized to detect H_2O_2 release from cells or enzyme coupled reactions[14-20].

Reaction:

H_2O_2 + Detection reagent (non-fluorescent) $\xrightarrow{\text{Peroxidase}}$ Resorufin (fluorescent)

Excitation 530-571 nm

Emission 590-600 nm

Materials Supplied

1. **Reaction Buffer Concentrate**
   20 mL, Product No. 80-1702
   A 5X Buffer Concentrate, pH 7.4

2. **Detection Reagent (ADHP)**
   1 vial, Product No. 80-1703
   One vial is sufficient for 500 tests

3. **Horseradish Peroxidase**
   1 vial, Product No. 80-1705
   18.9 units of enzyme

4. **3% Hydrogen Peroxide**
   200 µL, Product No. 80-1704
   A 3% stabilized solution of hydrogen peroxide

Storage

Store all undiluted reagents at 4°C.

Materials Needed but Not Supplied

1. Dimethyl sulfoxide (DMS0)
2. Black 96 well plates
3. Fluorescence plate reader
4. Deionized water
5. Superoxide dismutase (optional, See Reagent Preparation #4)

Assay Designs
734.668.6113 or 800.833.8651
www.assaydesigns.com

Reagent Preparation

1. **1X Reaction Buffer**
   Prepare the a 1X solution of the Reaction Buffer by diluing 4 mL of Reaction Buffer Concentrate with 16 mL of deionized water. This should be sufficient for performing 100 tests at 100µL each and for preparing the solutions that follow. This can be stored at 4ºC until the kit expiration date, or for 3 months, whichever is earlier.

2. **20 mM Hydrogen Peroxide**
   The 3% Hydrogen Peroxide solution is 0.88 M. Prepare a 20 mM solution by adding 22.7 µL of the provided H_2O_2 to 977 µL of 1X Reaction Buffer. Once diluted, the H_2O_2 should be used promptly, as it degrades rapidly.

3. **10 U/mL HRP (Horseradich Peroxidase)**
   Add 0.5 mL of 1X Reaction buffer to the vial of Horseradish Peroxidase provided. Transfer this 0.5 mL to a larger tube. Add an additional 1.39 mL of the 1X Reaction Buffer to the tube and mix gently to combine. Once diluted, the unused HRP should be stored at -20ºC as single use aliquots.

4. **10mM Detection Reagent**
   Dissolve the contents of the vial in 500 µL of DMSO. Allow the contents to sit at room temperature for 10 minutes. Gently pipet up and down several times to dissolve any clumps. Once dissolved the detection reagent should be used promptly and any remaining reagent should be aliquotted in single use vials and stored at -70ºC. Avoid repeated freeze thaw cycles.

   At NADH levels above 10 µM and glutathione concentrations above 300 µM, the Detection Reagent (ADHP) oxidizes; this could result in aberrant readings. To minimize this interference it is recommended to add superoxide dismutase (SOD) at a final concentration of 40 U/mL to the reaction[21].

NADH and glutathione may interfere with the assay.

The fluorescent product of the detection reagent is not stable in the presence of thiols (DTT or 2-mercap-toethanol). Keep these reactants below 10 µM. If using your own buffer, keep the reaction between pH 7-8 (optimal pH 7.40).

Sample Handling

The optimal dilution for a specific sample must be determined by the investigator. Due to the unstable nature of hydrogen peroxide, immediate sample analysis is recommended. Several dilutions should be assayed to ensure the samples fall within the range of the assay. Extremely high concentrations of analyte will result in lower fluorescence due to the oxidation of the fluorescent reaction product.

Assay Procedure: H_2O_2 Activity in Culture Supernates

1. Prepare 5 mL Reaction Cocktail (sufficient for 100 tests) as follows:
   100 µL of 10 mM Detection Reagent
   200 µL of 10 U/mL HRP
   4.7 mL of 1X Reaction buffer
   Use immediately; do not store the Reaction Cocktail for later use.

2. To prepare a H_2O_2 standard curve, dilute the appropriate amount of 20 mM H_20_2 in 1X Reaction buffer to make concentrations ranging from 0 to 10 µM, each in a volume of at least 100 µL (for duplicate runs, for improved precision preparing more than 100 µL is recommended).

   It is important to prepare the standard curve in the same matrix or media as your sample. If your samples are in PBS, your can use our **1X Reaction Buffer** to construct your standard curve. If your samples are in media, prepare your standard curve in the same media. Serum based media tends to suppress the fluorescent signal.

   If a standard curve is not used, two wells with and two wells without H_2O_2 are recommended as positive and negative controls for the assay.

   Note: High concentrations of H_2O_2 (>100µM) will result in lower fluorescence due to oxidation of the fluorescent reaction product, resorufin to non-fluorescent resazurin.

3. Pipet 50 µL of the standard curve, controls, and samples to the bottom of a black 96 well plate.

4. Pipet 50 µL of the Reaction Cocktail to each well to begin the reaction.

5. Incubate the plate for 10 minutes at room temperature in the dark.

6. Measure fluorescence at excitation 530-570 nm (570 nm is optimal) and emission 590-600 nm in a fluorescent plate reader.

7. Subtract background fluorescence (mean RFU value without H_2O_2) from each reading.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

All standards, controls, and samples should be run in duplicate.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Assay Designs
734.668.6113 or 800.833.8651
www.assaydesigns.com

Assay Procedure: H_2O_2 Activity from Cells

1. The Reaction Cocktail should be prepared in the same non-conditioned media that is used in your cell culture system. Serum should be reduced or avoided, as it will interfere with the reaction and may contain catalase which can compromise sensitivity.

   Suggested media to use:
   - Hanks Balanced Salt Solution
   - Krebs Ringers Phosphate Buffer
   - Serum Free Media (use with caution)

2. Just prior to use, prepare 5 mL Reaction Cocktail (for 100 tests) as follows:
   - 100 µL of 10 mM Detection Reagent
   - 200 µL of 10 U/mL HRP
   - 4.7 mL of Media (as suggested above).

   Use immediately; do not store the Reaction Cocktail for later use.

All standards, controls, and samples should be run in duplicate.

Pipet the reagents to the sides of the wells to avoid possible contamination.

3. Harvest cells and wash in fresh media. Plate cells in a black 96 well plate at a concentration of 10,000 to 50,000 cells per well in a volume of 50 to 100 µL. Include the appropriate negative controls to measure background fluorescence.
   1. Media alone
   2. Non-activated cells

   Note: High concentrations of H_2O_2 (>100µM) will result in lower fluorescence due to oxidation of the fluorescent reaction product, resorufin to non-fluorescent resazurin. Each investigator must optimize the cell concentration and volume for their particular protocol.

4. Prepare a H_2O_2 Standard Curve by diluting the appropriate amount of 20 mM H_2O_2 in the same non-conditioned media in which the cells are plated in. The concentrations should range from 0 to 10 µM, each in a volume of at least 100 µL. For duplicate wells and improved precision, preparing more than 100 µL is recommended. Keep the volumes in the sample and H_2O_2 Standard Curve constant.

   It is important to prepare the Standard Curve in the same matrix or media as your sample. If your samples are in PBS, your can use our 1X Reaction Buffer to construct your standard curve. If your samples are in media, prepare your standard curve in the same media. Serum based media tends to suppress the fluorescent signal.

   If a Standard Curve is not used, two wells with and two wells without H_2O_2 are recommended as positive and negative controls for the assay.

5. Pipet 50-100 µL Standard Curve to duplicate wells of the plate. Keep the volumes of the sample Standard Curve constant.

6. Activate cells according to your experimental protocol.

7. Add 50-100 µL of Reaction Cocktail to your cells and Standard Curve.

8. Incubate the plate for 10 minutes at room temperature in the dark.

9. Measure fluorescence at excitation 530-570 nm and emission 590-600 nm in a fluorescent plate reader.

Assay Procedure: Peroxidase Activity

1. Prepare 5 mL of Reaction Cocktail (for 100 tests) as follows:
   500 µL of 20 mM H_2O_2 solution
   50 µL of 10 mM Detection Reagent
   4.45 mL of 1X Reaction Buffer
   Use immediately; do not store the Reaction Cocktail for later use.

2. Prepare a Peroxidase Standard Curve by diluting the supplied horseradish peroxidase in 1X Reaction Buffer to make concentrations ranging from 0 to 25 mU/mL, each in a volume of at least 100 µL for duplicate runs.

   Note: High levels of HRP (~100 mU/mL) will produce lower fluorescence than 1 mU/mL, because the excess HRP oxidizes the fluorescent reaction product, resorufin to non-fluorescent resazurin.

   If a Standard Curve is not used, two wells with and two wells without HRP are recommended as positive and negative controls for the assay.

3. Pipette 50 µL of the Standard Curve, controls, and samples in the wells of a 96- well plate.

4. Add 50 µL of the reaction cocktail to each well to begin the reaction.

5. Incubate, away from light, if necessary. This step may need to be optimized as incubation times could vary from a few minutes to several minutes.

7. Measure the fluorescence at excitation 530-570 nm and emission 590-600 nm in a fluorescent plate reader.

8. Subtract the background fluorescence (mean RFU value without HRP) from each reading.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

All standards, controls, and samples should be run in duplicate.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Assay Designs
734.668.6113 or 800.833.8651
www.assaydesigns.com

Calculation of Results

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

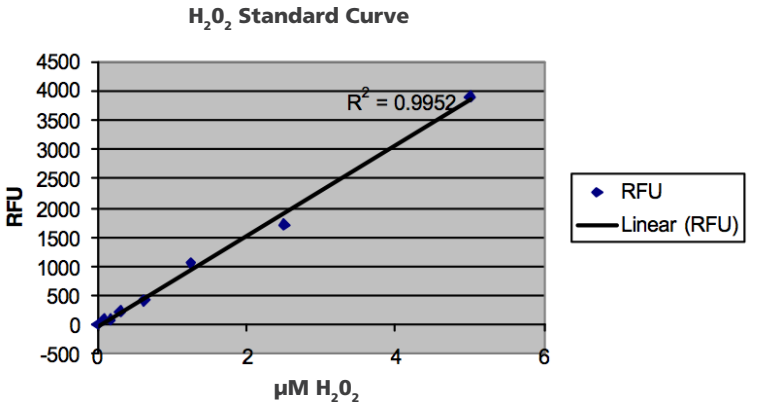
Several options are available for the calculation of hydrogen peroxide and horseradish peroxidase activity in samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the activity can be calculated as follows:

1. Calculate the average net RFU for each standard and sample by subtracting the average 0 U/mL RFU from the average RFU for each standard and sample.
2. Using linear graph paper, plot the Average Net RFU for each standard versus hydrogen peroxide or horseradish peroxidase activity (U/mL) in each standard. Appropximate a line through the points. The concentration of the unknowns can be determined by interpolation.

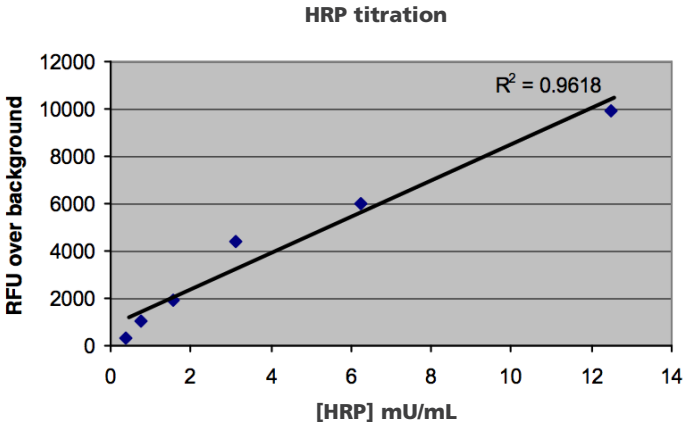
Samples with concentrations outside of the standard curve range will need to be reanalyzed using a higher dilution.

Typical Results

Example of standard curve in 1X Reaction Buffer. The results shown below are for illustration only and should not be used to calculate results from another assay.



Standard curve of Peroxidase in 1X Reaction buffer. The results shown below are for illustration only and should not be used to calculate results from another assay.



Assay Designs
734.668.6113 or 800.833.8651
www.assaydesigns.com

References

1. Davies, K. J. (1999) IUBMB Life. **48**, 41-7.
2. Zhang, J., ***et al***. (2001) Antioxid. Redox Signal. **3**, 493-504.
3. Li, N., Karin, M. (1999) FASEB J. **13**, 1137-43.
4. Kim, D.K., ***et al***. (2001) J. Cell Sci. **114**, 4329-34.
5. Emelyanov, A., **et al**. (2001) Chest. **120**, 1136-9.
6. Uesuge, M., ***et al***. (2000) J. Immunol. **165**, 6532-7.
7. Okuda, M., ***et al***. (2001) Artherioscler. Thromb. Vasc. Biol. **21**, 1483-7.
8. Peiro, C., ***et al***. (2001) Br. J. Pharmacol. **133**, 967-74.
9. Mody, N., **et al**. (2001) Free Radic. Biol. Med. **165**, 509-19.
10. Halliwell, B. (2001) Drugs Aging. **18**, 685-716.
11. Sanji, E., ***et al***. (2001) Biochem. Biophys. Res. Commun. **287**, 1003-8.
12. Wentworth Jr, P., ***et al***. (2001) Science. **293**, 1806-11.
13. Wentworth, AD., ***et al***. (2000) PNAS. **97**, 10930-5.
14. Zhou, M., ***et al***. (1997) Anal Biochem. **253**, 162.
15. Mohanty, J.G., ***et al***. (1997) J. Immunol Methods. **202**, 133.
16. Tatyana, V., ***et al***. (2001) J. Neurochem. **79**, 266.
17. Song, C., ***et al***. (2001) Am J. Physiol Lung Cell Mol Physiol. **281**, L993.
18. Richer, S., Ford, W.C.L. (2001) Mol Hum Reprod. **7**, 237.
19. Gutheil, W., ***et al***. (2000) Anal Biochem. **287**, 196.
20. Peus, D.,***et al***. (1999) J Invest Dermatol. **112**, 751.
21. Votyakova, V., Reynolds, I. (2004) Archives of Biochemistry and Biophysics. **431**, 138-144.

Notes

Assay Designs
734.668.6113 or 800.833.8651
www.assaydesigns.com

Limited Warranty

Assay Designs, Inc. warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

MSDS (Material Safety Data Sheet) available online

Assay Designs must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if Assay Designs is not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

Assay Designs, Inc.
5777 Hines Drive
Ann Arbor, Michigan
USA

tel: 734.668.6113 or 800.833.8651
fax: 734.668.2793
info@assaydesigns.com
www.assaydesigns.com

technical support: technical@assaydesigns.com

Kits for Charity™

Assay Designs has always been an active contributor to a number of local, national and international charities. We have broadened our charitable contributions by implementing a program called Kits for Charity™.

Each quarter, Assay Designs will feature a different non-religious and non-political charitable organization on our website. For each kit sold during this time period, we will make a monetary contribution to the featured charity. Please check our website for the current quarter's charity to see what organization your purchases are helping to support.

If you have any suggestions for future Kits for Charity™ recipients, please contact us at 800.833.8651 or 734.668.6113.

Contact Us

For more details concerning the information within this kit insert, or to order any of the Assay Designs' products, please call (734) 668-6113 between 8:30 a.m. and 5:30 p.m. EST. Orders or technical questions can also be transmitted by fax or e-mail 24 hours a day.

Catalog No. 25-0608

Last Revised: 28 June 2007