Fluoro H_2O_2™

Hydrogen Peroxide/Peroxidase Detection Kit

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Revised protocol 5/06

Notes
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I. Assay Principle:

The Fluoro H_2O_2 detection kit utilizes a non-fluorescent detection reagent to measure H_2O_2. H_2O_2 oxidizes the detection reagent in a 1:1 stoichiometry to produce a fluorescent product resorufin. This oxidation is catalyzed by Peroxidase in a homogeneous no wash assay system.
The detection reagent can be utilized to measure H_2O_2 release from cells or enzyme coupled reactions (1-7)
Reaction:

H_2O_2 + Detection reagent (non-fluorescent) $\xrightarrow{\text{Peroxidase}}$ Resorufin(fluorescent)
Excitation 530-571nm
Emission 590-600nm

II. Storage:

1. Short term (several weeks): at 2-4°C and away from light.
2. Long term: see individual components.
3. Once a vial of the Detection reagent is opened, it should be used promptly since it is subject to oxidation by air.

III. Warnings and Precautions:

1. For Research use only. Not for use in diagnostic procedures.
2. Practice safe laboratory procedures by wearing protective clothing and eyewear.
3. The fluorescent product of the detection reagent is no stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 10 ☐M. If you are using reaction between pH 7-8 (optimal pH 7.4).
4. NADH and glutathione (reduced form: GSH) may interfere with the assay. See Technical note #5.

IV. Part # 5016. Kit contents (for 500 assays):

1. **Part # 3011. 5X Reaction Buffer:** 25 ml pH 7.4.
2. **Part # 4007. Detection reagent:** One vial for 500 assays.
3. **Part # 3012. Hydrogen Peroxide:** 200µL of a stabilized 3% solution.
4. **Part # 6004. Horseradish Peroxidase:** 18.9 Units of enzyme

Materials required but not supplied:

1. Dimethyl sulfoxide (DMSO)
2. Black 96-well plates
3. Fluorescence plate reader
4. Deionized water

V. Preparation of reagent working solutions:

1. **20 ml of 1X Reaction buffer Part# 3011:** 4ml of 5X Reaction buffer is added to 16ml of deionized water to make 1X reaction buffer. This should be sufficient for performing 100 assays of 100µL each and for preparing stock solutions of the enzyme and H_2O_2.

Note: **It is important to equilibrate the buffer to room temperature before use as crystals may form on storage. This can be done by warming in a 37ºC water bath or incubator for a few minutes.**

2. **20mM Hydrogen Peroxide Part # 3012:** To 977µL of 1X Reaction buffer, add 22.7µL of the 3% H_2O_2 (0.88M) to make a 20mM solution. Once diluted, the H_2O_2 should be used promptly as it degrades rapidly.
3. **10U/ml Horseradish Peroxidase Part# 6004 :** Quickly spin down the contents of the vial before opening. To the contents of the vial, add 1.89ml of 1X Reaction buffer.
   Once diluted, the unused HRP should be stored at -20°C as single use aliquots.
4. **10mM Detection Reagent Part# 4007:** Dissolve the contents of the vial in 500µL of DMSO.
   Once opened, should be used promptly and any remaining reagent can be aliquoted and frozen at -70°C. **Avoid repeated freeze thaw cycles.**

VI. Assay Protocol: Detection of Hydrogen Peroxide in Supernatants

1. Prepare 5ml **reaction cocktail** (for 100 assays) as follows:
   100µL of 10mM Detection Reagent (50 µM final)
   200µL of 10U/ml HRP (0.1 U/ml)
   4.7ml of 1X Reaction buffer

2. To prepare an H_2O_2 standard curve, dilute the appropriate amount of 20mM H_2O_2 in 1X Reaction buffer to make concentrations ranging from 0 to 10µM, each in a volume of 100µL (for duplicate runs).
   Note: *See Technical note #4.*

3. Be sure to include a positive and negative (no H_2O_2) control in the assay if a standard curve is not used.

4. Pipette 50µL of the H_2O_2 dilutions, controls and samples into the wells of a 96- well plate.

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

6. Incubate for 10 minutes, at RT, away from light.

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

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

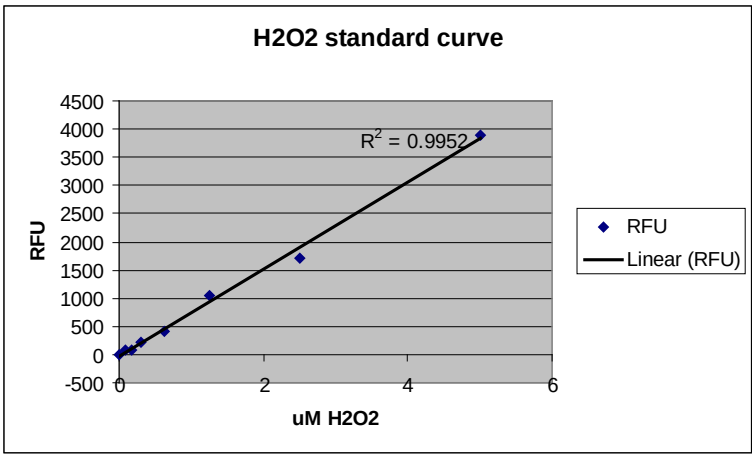

Figure 1: Example of standard curve in 1X reaction Buffer.

VII. Assay Protocol: Detection of Hydrogen Peroxide from Cells.

The Fluoro H_2O_2™ detection kit can be used to measure the release of H_2O_2 from cells. The following is a suggested protocol and can be modified to suit your particular research needs.

1. The reaction cocktail should be prepared in media that is used in your cell culture system. Serum should be reduced or avoided, as it will interfere with the reaction cocktail and may contain catalase activity, consequently compromising sensitivity. Suggested media to use:
   A. Hanks Balanced Salt Solution
   B. Krebs Ringers Phosphate Buffer
   C. Serum Free Media (use with caution).

2. Just prior to use, prepare 5ml **reaction cocktail** (for 50-100 assays) as follows:
   100µL of 10mM Detection Reagent (50 µM final)
   200µL of 10U/ml HRP (0.1 U/ml)
   4.7ml of Media (media as suggested above or used in your experiment).

3. Harvest cells and wash in fresh media (see media step V 1). Plate cells in a 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.

   Construct a standard H_2O_2 curve (see above step IV 2) in the same media in which the cells are plated in. Keep the volumes in the cell sample and standard H_2O_2 curve constant.
   Note: *Each investigator should optimize the cell concentration and volume for their particular protocol. See Technical note 4.*

5. Activate your cells according to your experimental protocol.

6. After activation add 50-100 µl of reaction cocktail to your cells and standard curve.

7. Incubate for 10 minutes, at RT, away from light.

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

VIII. Assay Protocol: Detection of Peroxidase Activity:

The Fluoro H_2O_2 ™ kit can also be used to assay peroxidase activity.

1. Prepare 5ml of **reaction cocktail**(for 100 assays) as follows:
   500 µL of 20mM H_2O_2 solution(2 mM final concentration)
   50 µL of 10mM Detection Reagent (100µM final concentration)
   4.45 ml of 1X Reaction buffer

2. To prepare a Peroxidase standard curve, make dilutions of the supplied Horse radish peroxidase in 1X Reaction buffer to make concentrations ranging from 0 to 25 mU/ml, each in a volume of 100 µL for duplicate runs.

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

3. Be sure to include a positive and negative (no H_2O_2) control in the assay if a standard curve is not used.

4. Pipette 50µL of the H_2O_2 dilutions, controls and samples into the wells of a 96- well plate.

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

6. Incubate, away from light, if necessary.
   Note: 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: 530-570nm and emission at 590-600nm in a fluorescent plate reader.
8. Subtract the background fluorescence (mean RFU value without H_2O_2) from each reading.

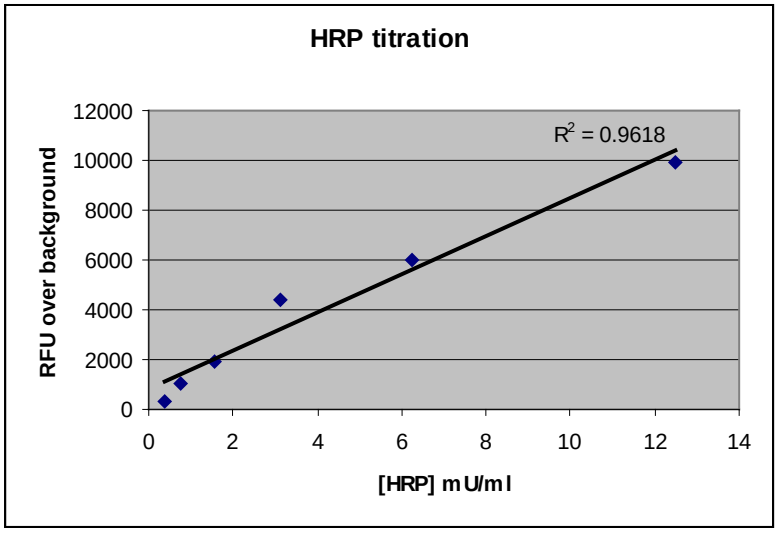

Figure 2: Standard curve of Peroxidase in 1X Reaction buffer

IX. Technical Notes:

1. The final concentrations of H_2O_2 will be 2X lower than used since the final reaction volume is 100µL. (0 to 5µM). The final concentration of the detection reagent is 100µM and that of HRP is 0.2U/ml in a 100µl reaction.
2. 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. The reaction cocktail once prepared, cannot be stored.
4. Preparation of H_2O_2 standard curve: 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.
5. At NADH levels above 10 µM, false oxidation results from side chain reaction between NADH and HRP. This could result in aberrant readings. To minimize this interference it is recommended to add superoxide dismutase (SOD) at 40U/mL to the reaction [8].
   At glutathione (reduced form GSH) above 300 µM, false oxidation results from side chain reaction between GSH and HRP. This could result in aberrant readings. To minimize this interference it is recommended to add superoxide dismutase (SOD) at 40U/mL to the reaction [8].
6. The 5X reaction buffer should be equilibrated to room temperature before use as crystals may form upon storage.

References:

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