ab155895

Peroxidase Activity Assay Kit

Instructions for Use

For the sensitive and accurate measurement of peroxide activity in biological samples.

View kit datasheet: www.abcam.com/ab155895
(use www.abcam.cn/ab155895 for China, or www.abcam.co.jp/ab155895 for Japan)

This product is for research use only and is not intended for diagnostic use.
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1. Overview

Peroxidases (EC number 1.11.1.x) are a large family of enzymes that typically catalyze a reaction of the form: ROOR' + electron donor (2 e-) + 2H+ → ROH + R'OH. For many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. Peroxidases can contain a heme cofactor in their active sites, or alternately redox-active cysteine or selenocysteine residues.

Abcam’s Peroxidase Assay Kit provides a convenient colorimetric and fluorometric means to measure the peroxidase activity in biological samples. In the presence of Peroxidase, the OxiRed Probe reacts with H₂O₂ in a 1:1 stoichiometry to produce the redfluorescent oxidation product, resorufin. The resorufin is quantified by colorimetric (λmax = 570nm) or fluorometric methods (Ex/Em = 535/587 nm). The assay is simple, direct, highly sensitive and high throughput-ready. The detection limit is 0.1 mU per well via colorimetric or 0.01 mU per well via fluorometric method, based on our unit definition.
2. Protocol Summary

Reagent Preparation
\[\downarrow\]
Sample Preparation
\[\downarrow\]
Standard Curve Preparation
\[\downarrow\]
Positive Control
\[\downarrow\]
Reaction Mix
\[\downarrow\]
Measurement and Calculation
3. Components and Storage

A. Kit Components

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Buffer</td>
<td>25 mL</td>
</tr>
<tr>
<td>OxiRed™ Probe (in DMSO)</td>
<td>0.2 mL</td>
</tr>
<tr>
<td>(\text{H}_2\text{O}_2) Substrate</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>(0.88 M)</td>
<td></td>
</tr>
<tr>
<td>HRP positive control</td>
<td>1 vial</td>
</tr>
</tbody>
</table>

* Store the kit at -20°C and protect from light. Please read the entire protocol before performing the assay. Avoid repeated freeze/thaw cycles.

Warm assay buffer to room temperature before use. Briefly centrifuge all small vials prior to opening.

B. Additional Materials Required

- 96-well clear plate with flat bottoms
- Multi-well spectrophotometer (ELISA reader)
4. Assay Protocol

A. Reagent Preparation

1. H$_2$O$_2$ Substrate:
   Dilute H$_2$O$_2$ Substrate to 12.5 mM by adding 5 µl of H$_2$O$_2$ substrate (0.88 M) to 347 µl Assay Buffer. The diluted H$_2$O$_2$ Substrate is stable for one day at 4°C and one month at -20°C.

2. HRP Positive Control:
   Add 1 ml assay buffer into lyophilized HRP to prepare HRP solution. The HRP solution is stable for one day at 4°C and one month at -20°C.

3. OxiRed™ Probe:
   Before use, briefly warm at 37°C for 1-2 min to completely melt DMSO solution, mix well. Store at –20°C.

B. Hydrogen Peroxide Assay Protocol

1. Sample Preparation:
   Collect cell culture supernatant, serum, plasma, urine, and other biological fluids. Centrifuge test samples for 15 minutes at 1000 x g within 30 min of collection to remove particulate pellet. Assay immediately or aliquot and store the samples at -80°C. Avoid repeated freeze-thaw cycles. Add 2-50 µl samples into each well and adjust the final volume to 50 µl with Assay Buffer.
2. **Standard Curve Preparations:**

   **For colorimetric assay:**
   Dilute H₂O₂ substrate solution to 0.1 mM by adding 10 µl of H₂O₂ substrate solution (12.5 mM) to 1240 µl Assay Buffer, mix well. Add 0, 10, 20, 30, 40, 50 µl into a series of wells in duplicate and adjust the final volume to 50 µl with Assay Buffer to generate 0, 1, 2, 3, 4, 5 nmol/well of H₂O₂ standard.

   **For fluorometric assay:**
   Dilute H₂O₂ substrate solution to 0.01 mM by adding 100 µl of H₂O₂ substrate solution (0.1 mM) to 900 µl Assay Buffer, mix well. Add 0, 10, 20, 30, 40, 50 µl into a series of wells in duplicate and adjust the final volume to 50 µl with Assay Buffer to generate 0, 100, 200, 300, 400, 500 pmol/well of H₂O₂ standard.

3. **Standard Curve Measurement:**
   Dilute HRP positive control solution 1:199 in Assay Buffer. For each well, prepare a total 50 µl Reaction Mix containing 2 µl OxiRed Probe and 48 µl diluted HRP positive control solution, mix well. Incubate for 5 min and measure the OD at 570 nm or RFU at Ex/Em = 535/587 nm in a micro plate reader.

4. **Positive Control Preparation:**
   Use 1 µl of diluted positive control solution into the desired well(s) and adjust the final volume to 50 µl with Assay Buffer.
5. **Reaction Mix:**

Mix enough reagents for the number of assays to be performed. For each well, prepare a total 50 µl Reaction Mix:

- Assay Buffer 46 µl
- OxiRed Probe solution 2 µl
- H₂O₂ Substrate solution 2 µl

*The fluorometric assay is ~10 times more sensitive than the colorimetric assay. Use 0.4 µl of the probe per reaction to decrease the background reading/increase detection sensitivity significantly.*

Add 50 µl of the Reaction Mix to each test samples and HRP positive control. Mix well; incubate the mix for 3 min at 37°C.

6. **Measurement**

Measure OD 570 nm (A0) for colorimetric assay or Ex/Em = 535/587 nm (R0) for fluorometric assay. Incubate for another 30 min to 2 hr at 37°C to measure OD at 570 nm (A1) or fluorescence at Ex/Em = 535/587 nm (R1) again, incubation times will depend on the peroxidase activity in the samples. We recommend measuring the OD or fluorescence in a kinetic method (preferably every 3 – 5 min) and choose the period of linear range, which falls within H₂O₂ Standard Curve to calculate the peroxidase activity of the samples.
5. Data Analysis

**Calculation:** Plot the H$_2$O$_2$ Standard Curve. Calculate the Peroxidase activity of the test samples: $\Delta A = A_1 - A_0$ or $\Delta RFU = R_1 - R_0$, apply the $\Delta A$ or $\Delta RFU$ to the H$_2$O$_2$ Standard Curve to get $B$ nmol of H$_2$O$_2$ generated by peroxidase in the given time.

\[
\text{Peroxidase Activity} = \frac{B}{(T \times V)} \times \frac{\text{Sample Dilution Factor}}{\text{nmol/min/ml}} = \text{mU/ml}
\]

Where:

- $B$ is the amount of H$_2$O$_2$ from standard curve (nmol).
- $\Delta T$ is the time incubated (min).
- $V$ is the sample volume added into the reaction well (ml).

Unit Definition: One unit of Peroxidase is the amount of enzyme that will oxidize 1.0 μmol of H$_2$O$_2$ per min at 37°C.
Figure 1: (a) H$_2$O$_2$ standard curve - colorimetric (b) H$_2$O$_2$ standard curve - fluorometric. Assays were performed following kit protocol.
## 6. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Assay buffer at wrong temperature</td>
<td>Assay buffer must not be chilled - needs to be at RT</td>
</tr>
<tr>
<td>Protocol step missed</td>
<td></td>
<td>Re-read and follow the protocol exactly</td>
</tr>
<tr>
<td>Plate read at incorrect wavelength</td>
<td></td>
<td>Ensure you are using appropriate reader and filter settings (refer to datasheet)</td>
</tr>
<tr>
<td>Unsuitable microtiter plate for assay</td>
<td></td>
<td>Fluorescence: Black plates (clear bottoms); Luminescence: White plates; Colorimetry: Clear plates. If critical, datasheet will indicate whether to use flat- or U-shaped wells</td>
</tr>
<tr>
<td>Unexpected results</td>
<td>Measured at wrong wavelength</td>
<td>Use appropriate reader and filter settings described in datasheet</td>
</tr>
<tr>
<td>Samples contain impeding substances</td>
<td></td>
<td>Troubleshoot and also consider deproteinizing samples</td>
</tr>
<tr>
<td>Unsuitable sample type</td>
<td></td>
<td>Use recommended samples types as listed on the datasheet</td>
</tr>
<tr>
<td>Sample readings are outside linear range</td>
<td></td>
<td>Concentrate/ dilute samples to be in linear range</td>
</tr>
<tr>
<td>Samples with inconsistent readings</td>
<td>Unsuitable sample type</td>
<td>Refer to datasheet for details about incompatible samples</td>
</tr>
<tr>
<td>Samples prepared in the wrong buffer</td>
<td></td>
<td>Use the assay buffer provided (or refer to datasheet for instructions)</td>
</tr>
<tr>
<td>Problem</td>
<td>Reason</td>
<td>Solution</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>Cell/ tissue samples not sufficiently homogenized</td>
<td>Increase sonication time/ number of strokes with the Dounce homogenizer</td>
<td></td>
</tr>
<tr>
<td>Too many freeze-thaw cycles</td>
<td>Aliquot samples to reduce the number of freeze-thaw cycles</td>
<td></td>
</tr>
<tr>
<td>Samples contain impeding substances</td>
<td>Troubleshoot and also consider deproteinizing samples</td>
<td></td>
</tr>
<tr>
<td>Samples are too old or incorrectly stored</td>
<td>Use freshly made samples and store at recommended temperature until use</td>
<td></td>
</tr>
<tr>
<td>Lower/ Higher readings in samples and standards</td>
<td>Not fully thawed kit components</td>
<td>Wait for components to thaw completely and gently mix prior to use</td>
</tr>
<tr>
<td></td>
<td>Out-of-date kit or incorrectly stored reagents</td>
<td>Always check expiry date and store kit components as recommended on the datasheet</td>
</tr>
<tr>
<td></td>
<td>Reagents sitting for extended periods on ice</td>
<td>Try to prepare a fresh reaction mix prior to each use</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation time/ temperature</td>
<td>Refer to datasheet for recommended incubation time and/ or temperature</td>
</tr>
<tr>
<td></td>
<td>Incorrect amounts used</td>
<td>Check pipette is calibrated correctly (always use smallest volume pipette that can pipette entire volume)</td>
</tr>
<tr>
<td>Standard curve is not linear</td>
<td>Not fully thawed kit components</td>
<td>Wait for components to thaw completely and gently mix prior use</td>
</tr>
<tr>
<td></td>
<td>Pipetting errors when setting up the standard curve</td>
<td>Try not to pipette too small volumes</td>
</tr>
<tr>
<td></td>
<td>Incorrect pipetting when preparing the reaction mix</td>
<td>Always prepare a master mix</td>
</tr>
<tr>
<td>Problem</td>
<td>Reason</td>
<td>Solution</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Air bubbles in wells</td>
<td>Air bubbles will interfere with readings; try to avoid producing air bubbles and always remove bubbles prior to reading plates</td>
<td></td>
</tr>
<tr>
<td>Concentration of standard stock incorrect</td>
<td>Recheck datasheet for recommended concentrations of standard stocks</td>
<td></td>
</tr>
<tr>
<td>Errors in standard curve calculations</td>
<td>Refer to datasheet and re-check the calculations</td>
<td></td>
</tr>
<tr>
<td>Use of other reagents than those provided with the kit</td>
<td>Use fresh components from the same kit</td>
<td></td>
</tr>
</tbody>
</table>
UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp

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