Technical Bulletin

Peroxidase Activity Assay Kit

Catalog Number MAK092

Product Description

Peroxidase is an enzyme found broadly in biological systems that utilizes hydrogen peroxide in the oxidation of various substrates.

The Peroxidase Activity Assay Kit provides a simple and direct procedure for measuring peroxidase activity in a variety of biological samples. Peroxidase catalyzes the reaction between H_2O_2 and the probe, resulting in a colorimetric (570 nm) or fluorometric (λ_{Ex} = 535/λ_{Em} = 587 nm) product proportional to the peroxidase activity present. One unit of peroxidase is defined as the amount of enzyme that reduces 1.0 µmole of H_2O_2 per minute at 37 °C.

The kit is suitable for the determination of peroxidase activity in cell culture supernatant, serum, plasma, urine, and other biological fluids.

Components

The kit is sufficient for 100 colorimetric or fluorometric assays in 96-well plates.

- Assay Buffer — 25 mL
  Catalog Number MAK092A
- Fluorescent Peroxidase Substrate, in DMSO — 0.2 mL
  Catalog Number MAK092B
- H_2O_2 Substrate, 0.88 M — 0.1 mL
  Catalog Number MAK092C
- HRP Positive Control — 1 vial
  Catalog Number MAK092D

Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescence or spectrophotometric multiwell plate reader
- 96-well plates. It is recommended to use black plates with clear bottoms for fluorescence assays and clear plates for colorimetric assays. Cell culture or tissue culture treated plates are **not** recommended.
- Dounce tissue grinder set
  (Catalog Number D9063 or equivalent)
- Refrigerated microcentrifuge capable of RCF ≥ 1,000 × *g*

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C, protected from light.

Preparation Instructions

Briefly centrifuge small vials prior to opening.

Assay Buffer: Bring to room temperature prior to use.

Fluorescent Peroxidase Substrate: Prior to use, briefly warm at 37 °C for 1-2 minutes to completely melt DMSO solution, mix well. Store at –20 °C.

H_2O_2 Substrate: See Standard Curve Preparation Procedure.

HRP Positive Control: Reconstitute the vial with 1 mL of Assay Buffer. The reconstituted HRP Positive Control solution is stable for one day at 2-8 °C and one month at -20 °C. Keep the solution on ice while in use.

Procedure

Sample Preparation

Both the colorimetric and fluorometric assays require 50 µL of sample for each reaction (well).

1. Collect cell culture supernatant, serum, plasma, urine, and/or other biological fluids.
2. Centrifuge test samples for 15 minutes at 1,000 × *g* within 30 minutes of collection to remove insoluble materials. Retain supernatant for assay. Assay immediately or aliquot and store the samples at -80 °C. Avoid repeated freeze-thaw cycles.
3. Add 2-50 µL of Sample (S) into desired well(s) in 96-well plate. For unknown samples, test different amounts of Sample to ensure the readings are within the Standard Curve range.
4. Adjust the total volume of each Sample (S) well to 50 µL with Assay Buffer.

HRP Positive Control Preparation

1. Dilute HRP Positive Control 1:199 in Assay Buffer.
2. Add 1 µL of diluted Positive Control into desired wells in 96-well plate.
3. Adjust the total volume of each Positive Control well to 50 µL by adding 49 µL of Assay Buffer.

Colorimetric Standard Curve Preparation

1. Prepare a 12.5 mM H_2O_2 Substrate solution by mixing 5 µL of the 0.88 M H_2O_2 Substrate with 347 µL of Assay Buffer. Mix well by pipetting (**do not** vortex), then aliquot and store at -20 °C. The diluted H_2O_2 Substrate is stable for one day at 2-8 °C and one month at -20 °C.
2. Prepare a 0.1 mM H_2O_2 Substrate Solution by mixing 10 µL of the 12.5 mM H_2O_2 Substrate solution with 1240 µL of Assay Buffer.
3. Prepare H_2O_2 Standards in separate wells of the 96-well plate according to Table 1.

Table 1.
Preparation of Colorimetric H_2O_2 Standards

Well	0.1 mM H_2O_2 Substrate Solution	Assay Buffer	H_2O_2 (nmol/well)
1	-	50 µL	0
2	10 µL	40 µL	1
3	20 µL	30 µL	2
4	30 µL	20 µL	3
5	40 µL	10 µL	4
6	50 µL	-	5

Fluorometric Standard Curve Preparation

1. Prepare a 0.1 mM H_2O_2 Substrate solution as directed in Steps 1 and 2 of the Colorimetric Standard Curve Preparation Procedure.
2. Prepare a 0.01 mM H_2O_2 Substrate solution by mixing 100 µL of the 0.1 mM H_2O_2 Substrate solution with 900 µL of Assay Buffer.
3. Prepare H_2O_2 Standards in separate wells of the 96-well plate according to Table 2.

Table 2.
Preparation of Fluorometric H_2O_2 Standards

Well	0.01 mM H_2O_2 Substrate Solution	Assay Buffer	H_2O_2 (pmol/well)
1	-	50 µL	0
2	10 µL	40 µL	100
3	20 µL	30 µL	200
4	30 µL	20 µL	300
5	40 µL	10 µL	400
6	50 µL	-	500

Standard Curve Reaction Mix

1. Dilute HRP Positive Control 1:199 in Assay Buffer.
2. Mix enough reagents for the number of assays to be performed. For each Standard well, prepare 50 µL of Standard Curve Reaction Mix according to Table 3.

Table 3.
Preparation of Standard Curve Reaction Mix

Reagent	Volume
Fluorescent Peroxidase Substrate	2 µL
Diluted HRP Positive Control	48 µL

3. Mix well and add 50 µL of Standard Curve Reaction Mix to each Standard well.
4. Mix well and incubate at room temperature for 5 minutes.
5. For colorimetric assay, measure the absorbance at 570 nm (A). For fluorometric assay, measure fluorescence intensity (RFU) at λ_{Ex} = 535/λ_{Em} = 587 nm.

Sample and HRP Positive Control Reaction Mix

1. Mix enough reagents for the number of assays to be performed. For each Sample and HRP Positive Control well, prepare 50 µL of Sample and HRP Positive Control Reaction Mix according to Table 4.

Table 4.
Preparation of Sample and HRP Positive Control Reaction Mix

Reagent	Volume
Assay Buffer	46 µL
Fluorescent Peroxidase Substrate	2 µL
12.5 mM H_2O_2 Substrate	2 µL

2. Mix well and add 50 µL of Sample and HRP Positive Control Reaction Mix to each Sample and HRP Positive Control well.
3. Mix well.
4. Incubate the plate at 37 °C. After 3 minutes, take the initial measurement. For colorimetric assays, measure the absorbance at 570 nm ($A_{Initial}$). For fluorometric assays, measure fluorescence intensity ($RFU_{Initial}$) at λ_{Ex} = 535/λ_{Em} = 587 nm).
5. Incubate the plate at 37 °C for another 30 minutes to 2 hours. Incubation time will depend on the peroxidase activity in the samples. Protect the plate from light during the incubation.
6. For colorimetric assays, measure the absorbance (A_{Final}) after incubation is complete. For fluorometric assays, measure RFU_{Final} after incubation is complete.
7. Alternatively, take measurements every 3-5 minutes in kinetic method and choose the period of linear range which falls within the H_2O_2 Standard Curve to calculate the peroxidase activity of the Samples. It is essential that the final measurement falls within the linear range of the standard curve.

Results

1. Use optical density (A) or fluorescence intensity (RFU) measured in Step 5 of the Standard Curve Reaction Mix Procedure.
2. Determine ΔOD or ΔF by subtracting the Blank value (Standard #1) from the remaining standard values.
3. Plot the ΔOD or ΔF against standard concentrations and determine the slope of the H_2O_2 Standard Curve by linear regression.
4. Calculate the change in absorbance (ΔA) or fluorescence intensity (ΔRFU) for each sample:

$$\Delta A = A_{Final} - A_{Initial}$$

or

$$\Delta RFU = RFU_{Final} - RFU_{Initial}$$

5. Apply the ΔA or ΔRFU to the H_2O_2 Standard Curve to get B nmol of H_2O_2 generated by peroxidase in the given reaction time.

Peroxidase Activity (nmol/min/mL or mU/mL) =

$$\frac{B}{T \, x \, V} \, x \, DF$$

where

B = H_2O_2 amount from standard Curve (in nmol)

T = Incubation time (minutes)

V = The sample volume added into the reaction well (mL)

DF = Sample dilution factor (DF = 1 for undiluted Samples)

Unit Definition: One unit of Peroxidase is the amount of enzyme that will oxidize 1.0 µmol of H_2O_2 per minute at 37 °C.

Figure 1.
Typical Colorimetric Standard Curve

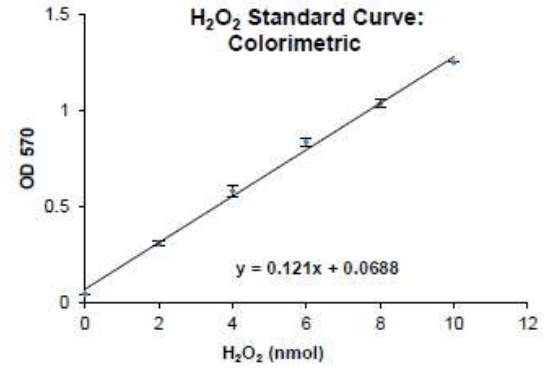
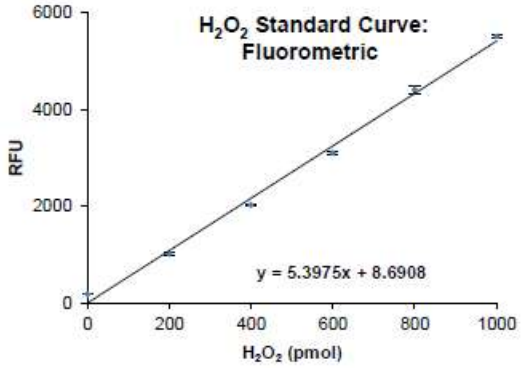


Figure 2.
Typical Fluorometric Standard Curve



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Document MAK092 Rev 09/22