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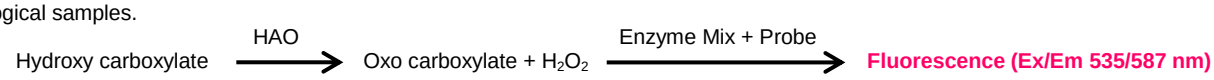
Hydroxy Acid Oxidase Activity Assay Kit (Fluorometric)

07/19

(Catalog # K2010-100; 100 assays; Store at -20ºC)

I. Introduction:

Hydroxy Acid Oxidase (HAO) (EC 1.1.3.15), also known as glycolate oxidase is a flavin mononucleotide dependent enzyme that oxidizes alpha hydroxy acid to oxo acid. Glycolate is the primary substrate for HAO, which gets oxidized to glyoxalate. HAO contributes to the general pathway of fatty acid α-oxidation and plays an important role in ethylene glycol poisoning. It is expressed in liver, kidney and pancreas. The most well studied enzyme of this family is the plant glycolate oxidase that has an essential role in photorespiration. BioVision's Hydroxy Acid Oxidase Activity Kit is a simple plate-based fluorometric assay for measuring HAO activity in biological samples. HAO oxidizes hydroxy carboxylate, which is accompanied by the release of H_2O_2. H_2O_2 then oxidizes a non-fluorescent probe to a fluorescent product in the presence of enzyme mix via an enzymatic reaction. The assay can detect as low as 1 µU of HAO activity in biological samples.



II. Applications:

Measurement of HAO activity

III. Sample Type:

- Animal tissue lysate (Liver tissue)
- Plant tissue lysate
- Cell lysate
- Recombinant enzyme
- Purified protein

IV. Kit Contents:

Components	K2010-100	Cap Code	Part Number
HAO Assay Buffer	25 ml	WM	K2010-100-1
HAO Dilution Buffer	200 µl	Amber	K2010-100-2
HAO Substrate	1 vial	White	K2010-100-3
HAO Enzyme Mix	1 vial	Green	K2010-100-4
HAO Probe	200 µl	Red	K2010-100-5
H_2O_2 Standard	100 µl	Yellow	K2010-100-6
HAO Positive Control	10 µl	Purple	K2010-100-7

V. User Supplied Reagents and Equipment:

- 96-well white plate with flat bottom
- Multi-well spectrophotometer
- Distilled water
- 10 kDa Spin Column (BV# 1997)

VI. Storage Conditions and Reagent Preparation:

Upon arrival, store the kit at -20°C, protected from light. Briefly centrifuge the small vials before opening. Read the entire protocol before performing the assay. Components are stable for at least three months.

- **HAO Assay Buffer:** Warm to room temperature (RT) before use.
- **HAO Dilution Buffer:** Keep on ice when in use.
- **HAO Substrate** and **HAO Enzyme Mix:** Reconstitute HAO Substrate with 220 µl H_2O and enzyme mix with 220 µl HAO Assay Buffer. Aliquot and store at -20ºC in the dark. Thaw on ice before use.
- **HAO Probe and H_2O_2 Standard (0.88 M):** Thaw at RT.
- **HAO Positive Control:** Aliquot and store at -20°C. Always keep on ice when in use.

VII. HAO Activity Assay Protocol:

1. **Sample preparation:** Homogenize cells (4 x 10^5 cells) or tissue (10 mg) with 100 µl HAO Assay Buffer to perform lysis. Tissues such as plant leaf, should be ground mechanically in HAO Assay Buffer to break the cell wall. Keep on ice for 10 min followed by centrifugation at 10,000 x *g* and 4ºC for 15 min. Collect the supernatant and estimate the protein concentration using any preferred method. We recommend using BCA protein assay kit (BV# K813-2500). Protein concentration should range between 0.025-0.15 µg/µl. Dilute the lysate if needed using HAO Assay Buffer. Prepare two wells for each Sample labeled as Sample Background Control (**SBC**), and Sample. Add the same 2-4 µl Sample (up to 0.3 µg protein) into each of these wells. For Positive Control, dilute the provided HAO Positive Control in HAO Dilution Buffer at 1:4 and add 4 µl of the diluted HAO Positive Control into the desired well(s). Adjust the volume to 50 µl/well with HAO Assay Buffer. For Substrate Control (**SC**) wells, add 50 µl of HAO Assay Buffer.

Notes:

a) For Sample(s) with high background such as liver tissue lysate, dilute the lysate with HAO assay buffer 5-10 times and filter through 10 kDa Spin Columns (BV# 1997). Small molecules will be removed in the ultrafiltrate and the ultraconcentrate is used for the HAO activity assay.
b) We recommend using the Samples for activity analysis immediately. Otherwise, store the Sample(s) at -80ºC for 3-4 days.
c) For Unknown Samples, we suggest testing several concentrations to ensure that the readings are within the Standard Curve range.
d) Do not dilute Positive Control in HAO Assay Buffer. Do not dilute the entire vial at one time. Dilute enough for the number of Positive Control being run at a time.

2. **H_2O_2 Standard Curve Generation:** Dilute 10 µl 0.88M H_2O_2 Standard into 870 µl dH_2O to generate 10 mM H_2O_2 Standard. Dilute 10 µl of the 10 mM H_2O_2 Standard into 990 µl dH_2O to generate a 0.1 mM H_2O_2 Standard. Further dilute 100 µl of the 0.1 mM H_2O_2 Standard into 900 µl dH_2O to generate 10 µM H_2O_2 Standard.

   Add 0, 2, 4, 6, 8, 10 µl of the 10 µM H_2O_2 Standard into a 96-well white plate in duplicates to generate 0, 20, 40, 60, 80, 100 pmol/well H_2O_2 Standard. Adjust the volume of each well to 50 µl with HAO Assay Buffer.
3. **Reaction Mix:** Mix enough reagents for the number of assays to be performed. Make sufficient amount of each type of the mix to add 50 µl to all assay wells of that type.

	H_2O_2 Standard Curve/SBC Mix	**Reaction Mix**
HAO Assay Buffer	46 µl	44 µl
HAO Substrate	-	2 µl
HAO Enzyme	2 µl	2 µl
HAO Probe	2 µl	2 µl

   Mix well. Add 50 µl of the H_2O_2 Standard Curve/SBC Mix to "H_2O_2 Standard Curve" and "**SBC**" wells and 50 µl of the Reaction Mix to **SC**, Sample and Positive Control wells.

   Notes:

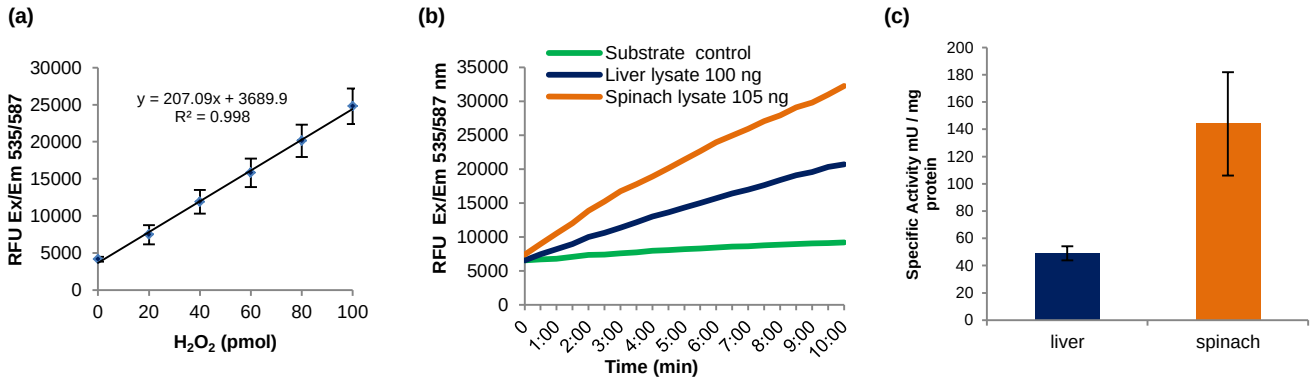
   a). Have the plate reader ready at Ex/Em 535/587 nm in a kinetic mode at RT set to record fluorescence every 30 sec.

   b). Prepare reaction mix immediately before adding to wells.
4. **Measurement:** Immediately start recording fluorescence in kinetic mode at 30 sec intervals for 35 -400 min at RT. Standard Curve may be read in either kinetic or end point mode.
5. **Calculation:** Subtract the 0 Standard readings from all Standard readings and **SBC** readings from Sample readings respectively. If the Substrate Control (**SC**) reading is higher than the **SBC** reading, subtract the Substrate Control readings from the Sample readings instead. Plot the H_2O_2 Standard Curve. Choose any two time points within the linear portion of the curve (t_1 & t_2) for each Sample. Apply the corrected Sample readings to the H_2O_2 Standard Curve to get **ΔM** pmol of H_2O_2 formed during the reaction time ($\Delta t = t_2 - t_1$).

Sample HAO Specific Activity = ΔM / (Δt x P) (pmol / (min x µg)) = µUnits / µg or mUnits / mg

Where: **ΔM =** linear change in H_2O_2 concentration during Δt (pmol)
Δt = $t_2 - t_1$ (min)
P = Sample protein amount added to well (µg)

Unit Definition: One unit of HAO is the amount of enzyme that produces1 µmol of H_2O_2 per minute at pH 7.5 at RT.



Figures. a). H_2O_2 Standard Curve. **b).** Enzyme kinetics of Rat Liver lysate (100 ng protein) and Spinach Leaf lysate (105 ng protein). **c).** HAO specific activity in Rat liver and Spinach lysates. Experiments were conducted according to kit protocol.

VIII. Related Products:

Oxalate Oxidase Activity Assay Kit (Fluorometric (K509)
Oxalate (Oxalic Acid) Colorimetric Assay Kit (K663)
Oxalate Decarboxylase Activity Colorimetric Assay Kit (K664)

FOR RESEARCH USE ONLY! Not to be used on humans.