Fluoro: SSAO™

Semicarbazide-Sensitive Amine Oxidase Detection Kit

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Notes
Revised 06/06
Updated – 1/07

CELL TECHNOLOGY

I. Assay Principle:

Semicarbazide-sensitive amine oxidase (**SSAO**) is a common name for a widely distributed enzyme in nature. In man this enzyme is present in the vascular system and circulates in plasma. SSAO differ from the monoamine oxidases A and B in substrate and inhibitor patterns. These enzymes have been widely studied and their tissue distribution, molecular properties, substrate specificities and inhibitor sensitivities are extensively reviewed (2,3).

SSAO exists in two forms: tissue bound and soluble (plasma SSAO). Tissue bound SSAO acitvity is associated with blood vessels, mainly in smooth muscle layers, however it is also associated with spleen, placenta, bone marrow, kidney, sclera, retina, endothelial cells, adipocytes, chondrocytes and fibroblasts. (4,5). It is expected and evidence suggests that Plasma SSAO originates from the cleavage of membrane-bound form. The possible sources of plasma SSAO are still unclear, but it has been suggested that it may be derived from liver, retina, placenta and bone tissue (6,7,8).

SSAO's functional role has been suggested to be involved in: apoptosis, atherogenesis, cell adhesion, leucocyte trafficking, glucose transport and local production of hydrogen peroxide. Elevated levels of SSAO have been reported in congestive heart failure, diabetes mellitus, alzheimer's disease and various other inflammatory diseases. Furthermore, by products of SSAO deamination, such as formaldehyde and methylglyoxal, have been proposed to be involved in pathogenesis of cancer, aging and atherosclerosis (1 review).

The Fluoro SSAO detection kit utilizes a non- fluorescent detection reagent to measure H_2O_2 released from the conversion of Benzylamine to Benzaldehyde via SSAO. Furthermore H_2O_2 oxidizes the detection reagent in a 1:1 stoichiometry to produce a fluorescent product resorufin. This oxidation is catalyzed by Peroxidase.

Reaction:

Benzylamine + O2+ H2O + SSAO ⟶ Benzaldehyde + NH3 + H2O2

H_2O_2 + Detection reagent (non-fluorescent) + Peroxidase ⟶ Resorufin(fluorescent)
Excitation 530-571nm
Emission 590-600nm

II. Storage:

1. The kit contains multiple storage temperature components. Please see labels of individual components for storage instructions.
2. Once a vial of the Detection reagent is opened, it should be used promptly and frozen since it is subject to oxidation by air.
3.

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 10µM. If you are using your own buffer, keep the 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 and Storage (for 500 assays).
Unopened kit can be stored at 4-8^{0}C for two weeks except for Part # 6006 and 7004 which must be stored at –20^{0}C immediately upon arrival. For long term storage see table below.

Description	Part#	Long term Storage Kit or Reconstitution of Reagents.
1 Bottle: 5X Reaction Buffer pH 7.4	3019	4-8^{0}C
1 vial: Detection reagent	4008	Aliquot in single use vials: Below –20^{0}C
1 vial: Horseradish Peroxidase	6005	4-8^{0}C
1 vial: SSAO substrate Benzylamine	7001	Aliquot in vials: Below –20^{0}C
1 vial: SSAO Enzyme	6006	Aliquot in single use vials: Below –20^{0}C
1 vial Pargyline: Monoamine Oxidase B inhibitor [9-11]	7003	Aliquot in vials: Below –20^{0}C
1 vial Semicarbazide: Semicarbazide-sensitive amine oxidase inhibitor.	7004	Aliquot in single use vials: Below –20^{0}C

Materials required but not supplied:

1. Dimethyl sulfoxide (DMSO)
2. Black 96-well plates
3. Fluorescence plate reader
4. Deionized water
5. Resorufin (optional Sigma Cat# 424455 or R3257.Consult Sigma on how to dissolve the dye).

V. Preparation of reagent working solutions:

1. **1 X Reaction Buffer part# 3019:** To prepare a 1X solution of Reaction buffer, dilute the 5X buffer 1:5 with Di water. For example to prepare 20 ml of 1X Reaction buffer add 4ml of 5X Reaction buffer to 16ml of deionized water.

2. **Horseradish Peroxidase part# 6005.** Make a 100X stock solution of HRP by diluting it 1:18.9 with 1X Reaction Buffer. For example take out 5.29uL µL of the HRP reagent and add 94.71 µL 1X Reaction Buffer. Make enough HRP for 1 days worth of experiments.

3. **Detection Reagent Part# 4008:** Dissolve the contents of one vial in 500µL of DMSO. Allow the contents to sit at Room Temperature for 15 minutes. Next gently pipette up and down several times to dissolve any clumps. Once dissolved the detection reagent should be used promptly and any remaining reagent can be aliquated and frozen at --70^{o}C. Avoid repeated freeze thaw cycles.
Note: Protect the Detection Reagent from light.

4. **Semicarbazide:** Semicarbazide-sensitive amine oxidase SSAO vial Part#6006 is at

10 units/mL.

5. **Substrate: Benzylamine Hydrocholride Part# 7001.** Make a .5M solution by adding 0.6 mL of Di water to the vial. Allow the vial to sit at room temperature for 15 minutes. Gently vortex the vial form time to time.

6. **Pargyline Part# 7003.** Make a 100mM solution by reconstituting the Vial with 0.5mL of Di water. Gently vortex the vial and allow the contents to dissolve at room temperature for 15 minutes.
7. **Semicarbazide part# 7004:** is at a concentration of 0.5M. Dilute in 1X Reaction Buffer as needed.

VI. Assay Protocol: Detection of SSAO in Samples.

1. Prepare 10ml **reaction cocktail** for 100 assays:
   100µL Detection Reagent .
   100µLl HRP of 100X HRP.
   100µL of SSAO Substrate.
   9.7ml of 1X Reaction buffer
   The volume of the **reaction cocktail** can be scaled down or up (as needed) provided that the **ratios** of the ingredients are kept constant.
   Note: *See Technical note #1.*

2. Positive control: Make a 2X solution of the SSAO enzyme by diluting the enzyme (part# 6006) 1:50 in 1X reaction buffer. The resulting SSAO solution will have an activity of approximately .2units/mL. One unit results in the oxidation of one micromole of benzylamine per minute at 25^0C. The SSAO enzyme can be used as a positive control.
   Note: *See Technical note #2.*

3. Preparation of samples.
   Samples (microsomes, cell membrane preparations or serum) can be diluted in 1X Reaction Buffer. Since Benzylamine is a substrate for both SSAO and Monoamine oxidase B, pargyline, a Monoamine oxidase B inhibitor can be added to samples 30 minutes prior to the assay. Pargyline can be added to a final concentration of 0.5mM to each sample and incubated for 30 minutes at 37^0C.
   Note: *See Technical note #2 & #3 and 4.*

4. Assay.
   1. To a black 96 well plate add 100 µL of sample or positive control to each individual wells.
   2. Add 100 µL of Reaction Cocktail prepared in Section VI step 1 above.
   3. Incubate the sample at 37^0C for 1-3 hours.
      Note: Each investigator should optimize the incubation time for their particular application.

   4. Optional: A standard curve can be prepared from resorufin (fluorescent end product of DETECTION REAGENT) to determine moles of product produced from DETECTION REAGENT. Standard curve can be constructed in the 1X Reaction Buffer, from 0 – 50 µM. Pipette 200uL of this standard curve to individual wells, prior to reading your samples.

5. Read samples using excitation: 530-570nm (570nm is optimal) and measure fluorescence at 590-600nm.

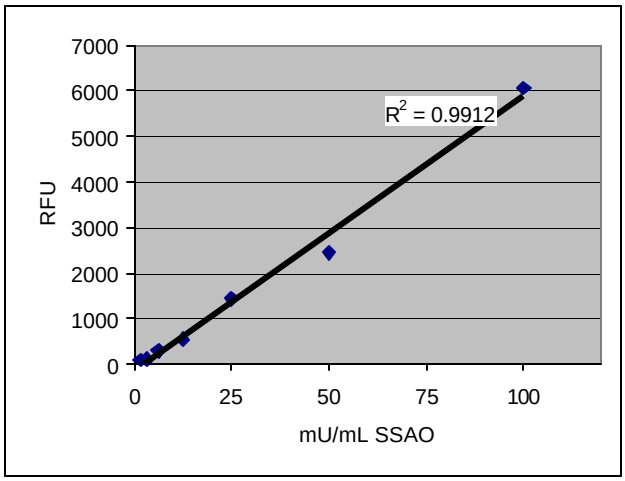

Figure 1. Bovine serum Semicarbazide-sensitive amine oxidase was serially diluted in 1X Reaction buffer. The serially diluted samples were run as described in the protocol. The samples were read after a 3 hours incubation period. Excitation: 530nm and emission: 590nm.

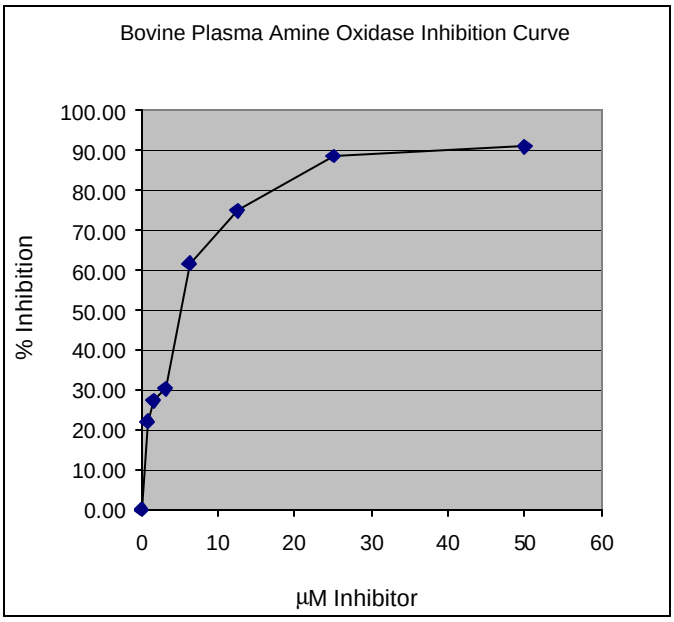

Figure 2. Bovine Plasma Amine Oxidase (.5U/mL) was incubated with various concentrations of semicarbazide. The activity of Bovine Plasma Amine Oxidase was then measured with the Fluoro SSAO kit as described. Data point reading taken after 2 hours at 37^0C.

VIII. Technical Notes:

1. Final concentration of SSAO substrate Benzylamine = 2.5mM. Each investigator should titrate benzylamine to optimize the kinetic reaction for their particular enzyme.

2. SSAO Enzyme= Bovine Plasma Amine Oxidase I.U.B.: 1.4.3.6.
   Caution for serum samples: Serum may contain catalase activity that will interfere with the assay. A catalase inhibitor should be used in such cases to suppress catalase activity (Do not use Azide as it will also suppress HRP activity).

3. For detailed information on inhibitor concentrations see reference 10.

4. The fluorescent product of the detection reagent is no stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 5µM.
   If you are using your own buffer, keep the reaction between pH 7-8 (optimal pH 7.4).
5. At NADH levels above 10µM, detection reagent 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 [12].
   At glutathione (reduced form GSH) above 300µM, detection reagent 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 [12].

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