

ab109912 – Monoamine Oxidase B Enzyme Specific Activity Microplate Assay Kit

Instructions for Use

For the quantitative measurement of Monoamine Oxidase B activity from Human Samples

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

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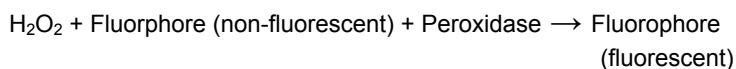
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1. Introduction

Monoamine oxidase B (MAOB, P27338) is a 59 kDa enzyme in the mitochondrial outer membrane that catalyzes the oxidative deamination of biotic and xenobiotic monoamines (EC 1.4.3.4). MAOB has a role in the metabolism of neuroactive and vasoactive amines in neurons and astroglia. MAOB is also found in heart, liver, GI, placenta and blood. Two monoamine oxidase isoforms exist, MAOA and MAOB. In vivo MAOA metabolizes norepinephrine, serotonin and dopamine while MAOB metabolizes dopamine preferentially. MAO inhibitors are very powerful antidepressant drugs but are rarely used to treat this condition. Several MAOB selective inhibitors exist including lasebemide, pargyline, rasagiline and selegiline. Selegiline is also used to treat early stage Parkinson's disease and senile dementia in combination with L-DOPA treatment. MAOs perform the following reaction:



The generated hydrogen peroxide can then be detected by an H_2O_2 sensitive probe:



ab109912 (MS747) improves upon other MAO assays in two ways. First, this assay immunocaptures only MAOB in each well. This removes all other peroxidases and oxidases (including MAOA) and thus eliminating background signal. Second, after the activity measurement, the quantity of enzyme is measured in the same well/s by adding a MAOB specific antibody which is detected by a colorimetric label (HRP). This reaction takes place in a time dependant manner proportional to the amount of enzyme captured in each well. By combining activity and quantity measurements, the relative specific activity can be determined. Specific activity is a useful for measuring up or down regulation of activity by site-specific modification or damage.

This assay works using human tissue samples and many different human cultured cell lines. As noted above not all cells contain monoamine oxidases, for example no significant amounts of MAOB have been detected in HL60 or fibroblast cells. Typical ranges for several human sample types are described below. It is highly recommended to prepare multiple dilutions for each sample to ensure that each is in the working range of the assay (see Data Analysis section).

The table below shows the typical working ranges for different sample types:

Cultured cell extracts:

HeLa 10 μ g - 1 mg/ml

HepG2 10 μ g - 1 mg/ml

Tissue extracts (e.g. heart) 1 μ g - 0.3 mg/ml

Tissue mitochondria (e.g. liver) 1 μ g - 0.3 mg/ml

2. Assay Summary

Prepare Sample (approximately 1 hour)

- Extract the sample pellet
- Incubate on ice for 20 minutes.
- Centrifuge at 12,000 x g for 20 minutes and then collect supernatant.
- Determine protein concentration and dilute to within recommendation range



Load plate (2 hours)

- Load sample(s) on the plate being sure to include a dilution series of a reference or normal sample and a buffer control as null reference.
- Incubate 2 hrs at room temperature.



Activity measurement (1 hour)

- Empty wells and wash each well twice with 300 μ l 1X Wash Buffer.
- Prepare activity solution
- Measure activity in fluorescence microplate reader 60 minutes. Ex 495, Em 529nm.



Detector antibody incubation (30 minutes)

- Empty wells and wash each well twice with 300 μ l 1X Wash Buffer.
- Add 100 μ l of 1X Detector antibody to every well used.
- Incubate for 30 minutes at room temperature.



HRP label incubation (30 minutes)

- Empty wells and wash each well twice with 300 μ l 1X Wash Buffer.
- Add 100 μ l of 1X HRP Label to every well used.
- Incubate for 30 minutes at room temperature.



Quantity measurement (15 minutes)

- Empty wells, wash each four times with 300 μ l 1X Wash Buffer.
- Add 100 μ l of Development Solution.
- Measure OD₆₀₀ at 20-second intervals for 15 minutes at room temperature.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

Part Number	Item	Quantity
8209506	100X Benzylamine Substrate	0.25 ml
8201093	Extraction Buffer	15 ml
8209204	20X Buffer	25 ml
8209507	500X Fluorophore	50 µl
8209508	500X Peroxidase	50 µl
8209203	10X Blocking Solution	10 ml
8209803	1X Development Solution	12 ml
8209509	100X Detector Antibody	0.125 ml
8209510	100X HRP Label	0.125 ml
8209511	96-well Microplate (12 strips)	1
5206003	12-channel Reagent Reservoir	1

4. Storage and Handling

All components are shipped cold. For long term storage keep 500X Fluorophore and 100X Benzylamine substrate at -80°C. Store all other components store at 4°C.

5. Additional Materials Required

- Fluorescence plate reader – Activity (Ex 495, Em 529 nm), Quantity (Absorbance 450,600, or 650 nm)
- Multichannel pipette (50 - 300 µl) and tips
- 1.5-ml microtubes
- A fine needle
- Optional 1N HCl

6. Preparation of Buffers and Samples

Note: This protocol contains detailed steps for measuring MAOB activity in human samples. Be completely familiar with the protocol before beginning the assay. Do not deviate from the specified protocol steps or optimal results may not be obtained.

A. Buffer Preparation

1. Prepare 1X Wash Buffer by adding 25 ml 20X Buffer to 475 ml deionized water.
2. Prepare 1X Incubation Buffer by adding 10 ml 10X Blocking Solution to 90 ml 1X Wash Buffer.
3. Before use (in Assay Method Step A4) prepare 1X Activity Solution by adding 50 μ l 500X Fluorophore, 50 μ l 500X Peroxidase, and 0.25 ml 100X Benzylamine to 24.65 ml 1X Wash Buffer. A 12 channel reagent reservoir is provided for serial dilution of inhibitors or drugs if desired.
4. Before use (in Assay Method Step B2) prepare 1X Detector Antibody by adding 0.125 ml 100X Detector Antibody to 12.375 ml 1X Incubation Buffer.

5. Before use (in Assay Method Step B4) prepare 1X HRP Label by adding 0.125 ml 100X HRP Label to 12.375 ml 1X Incubation Buffer.

B. Sample Preparation

Note: Samples must be detergent extracted by the provided Extraction buffer. To do this tissue homogenates, mitochondria preparations or cell pellets must be prepared.

1. For cultured cells, mitochondrial preparations or tissue homogenates: Pellet the sample by centrifugation and resuspend the pellet in 9 volumes of Sample Extraction Buffer.
2. Incubate extracts on ice for 20 minutes. Centrifuge at 12,000 x g at 4°C for 20 minutes. Remove the supernatant and discard the pellet.
3. Determine the protein concentration of the supernatant extract (a protein assay method unaffected by the presence of detergent is critical e.g. BCA assay recommended).

4. The sample should be diluted to within the working range of the assay (in Assay Method Step A1). Undiluted extract can be frozen at -80°C.

7. Assay Method

A. Activity Measurement

1. Sample should be diluted in 1X Incubation Buffer. A dilution series of samples is recommended to ensure that samples are within the working range of the activity and the quantity assays. Also, include a buffer control (1X Incubation Buffer only) as a null or background reference. Add 100 µl of each diluted sample into individual wells.
2. Cover/seal the plate and incubate for 2 hours at room temperature with shaking
3. Wash the plate:

Empty the wells by turning the plate over a receptacle and firmly shaking out the well contents in one rapid downward motion. Dispose of biological samples appropriately.

Rapidly add 300 µl 1X Wash Buffer to each well. The wells must not become dry during any step.

Repeat this wash once more for a total of two washes. After the last wash strike the microplate surface onto paper towels to remove excess liquid.

4. Immediately add the prepared Activity Solution minimizing the production of bubbles: Add 200 µl 1X Activity Solution with or without desired inhibitor to wells.
5. Pop bubbles immediately and record fluorescence in the fluorescence microplate reader prepared as follows:

Mode:	Kinetic
Wavelength:	Ex 495, Em 529 nm
Time:	Minimum 15 min to 1 hour (or longer as desired)
Interval	1 to 2 min
Shaking:	Shake between readings

Alternative – In place of a kinetic reading, record the fluorescence in all wells at a user defined time/s.

6. Record the data for analysis and proceed to section 7.2 – Quantity Measurement.

B. Quantity Measurement

1. Repeat the wash procedure in step A3 Collect and dispose of solutions and inhibitors appropriately.
2. Add 100 µl of 1X Detector Antibody to each well used. Cover/seal the plate and incubate for 30 minutes at room temperature with shaking.
3. Repeat the wash procedure in step A3.
4. Add 100 µl of 1X HRP Label to each well used. Cover/seal the plate and incubate for 30 minutes at room temperature with shaking. Meanwhile, prepare the microplate spectrophotometer using the parameters described below (Step B6).
5. Repeat the wash procedure in step A3 however performing a total of **four** washes.
6. Add 100 µl HRP Development Solution to each empty well, rapidly pop bubbles, and immediately record the blue color development in the microplate reader prepared as follows:

Mode:	Kinetic
Wavelength:	600 or 650 nm
Time:	Up to 15 min
Interval	20 sec to 1 min
Shaking:	Shake between readings

*Alternative – In place of a kinetic reading, record the endpoint OD data at a **user defined** time at (i) 600/650 nm or (ii) stop the reaction by adding 50 µl stop solution (1N HCl) to each well and record the OD at 450 nm (note that an intense, sub-saturated, blue color can become saturated at 450 nm after stopping).*

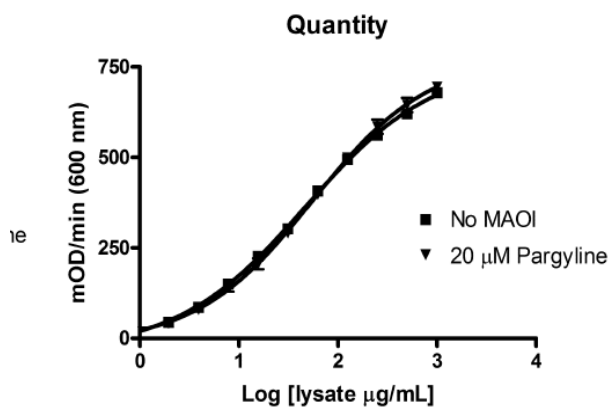
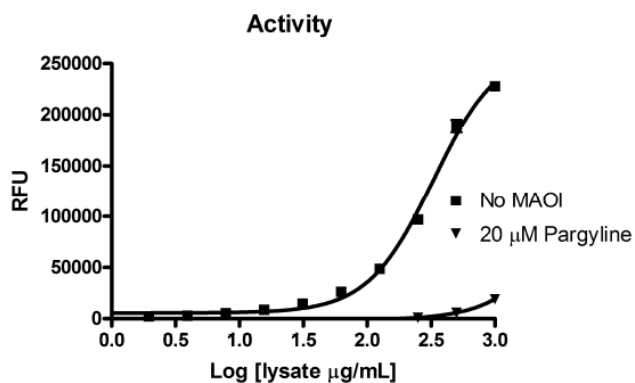
7. Record the data for analysis and proceed to Data Analysis.

8. Data Analysis

Example data sets are shown below illustrating data manipulation and analysis of MAOB activity/quantity measurements in the human cell line HepG2 treated with the MAOB selective inhibitors pargyline (CAS 555-57-5), selegiline (CAS 14611-51-9) and an MAOA selective inhibitor clorgyline (CAS 17780-72-2).

A. Calculation of drug effect upon enzyme specific activity.

Using the supplied 12-channel reagent reservoir the following two-fold dilution series of a HepG2 cell extract was prepared in incubation buffer: 0, 1, 2, 4, 8, 16, 32, 64, 125, 250, 500, 1000 µg/ml of HepG2 extract. This sample series was applied to the microplate and incubated as specified in the protocol. Activity buffer was prepared \pm 20 µM pargyline. Activity was measured over a 35 minute period and then followed by the ELISA quantity measurement. As shown in Figure 1 the enzyme specific activity was affected significantly by 20 µM pargyline (90%).



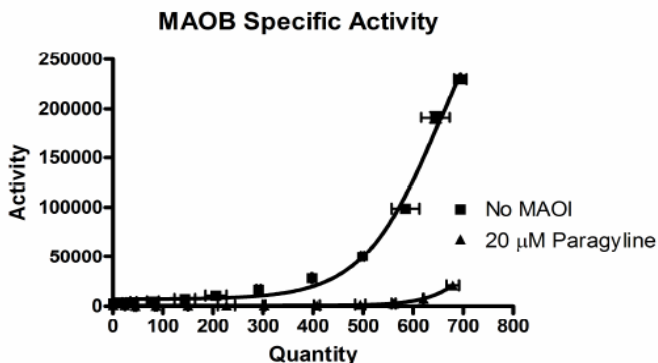


Figure 1: Data was collected using a microplate reader and software, capable of 4-parameter analysis of data. The raw data was exported for 4-parameter fit analysis. Activity was clearly measurable in the 16-1000 $\mu\text{g}/\text{ml}$ range and quantity in the range 1-1000 $\mu\text{g}/\text{ml}$ when such a fit was applied. The MAOB specific inhibitor pargyline inhibited activity 90% while not affecting quantity.

B. Determination of IC_{50} of Inhibitors.

1. The IC_{50} of selegiline, pargyline and clorgyline against MAOB was determined using the ab109912 (MS747) kit. Pargyline and selegiline are selective for MAOB while clorgyline, a selective inhibitor of MAOA, is much less effective against MAOB.

2. A normal HepG2 lysate was applied to each microplate well at 125 µg/ml. Meanwhile using the supplied 12-channel reagent reservoir the following ten-fold dilution series of each inhibitor was prepared in Activity solution: 0, 10^{-4} , 10^{-3} , 0.01, 0.1, 1, 10, 100 µM
3. After two hour sample incubation, wells were washed according to the protocol and the inhibitor series in activity solution was added to the plate using a multi-channel pipette. Activity rate was measured over 35 minutes and followed by the ELISA quantity measurement. In Figure 2 the MAOB activity was significantly affected by pargyline with an IC₅₀ 0.26 µM, and selegiline with an IC₅₀ of 80 nM while clorgyline inhibited the MAOB isoform with an IC₅₀ of more than 100 µM. The amount of bound enzyme was not significantly affected by the treatments.

Activity and Quantity - effect of MAO inhibitors

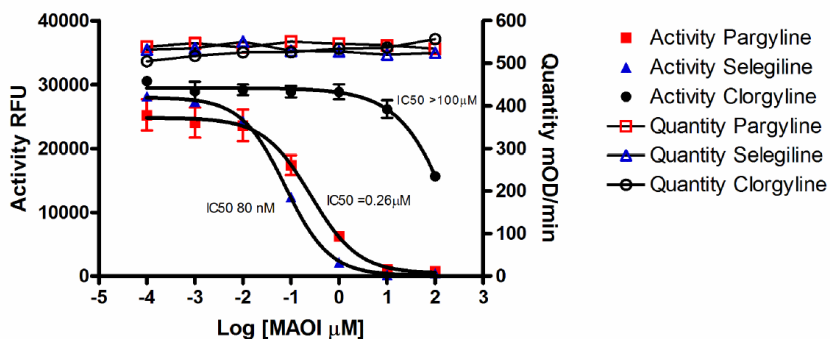


Figure 2: Data was collected using a microplate reader and software, capable of 4-parameter analysis of data. Raw data was exported for 4-parameter fit analysis and IC₅₀ determination.

C. Reproducibility

Intra-Assay: CV < 7 %

Inter-Assay: CV < 10 %

9. Specificity

Species Reactivity: Human

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