Xanthine Oxidase Fluorometric Assay Kit

Item No. 10010895

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
10010972	XO Assay Buffer (10X)	1 vial
10010973	XO Sample Buffer (10X)	1 vial
10010974	Xanthine Oxidase Standard	2 vials
10010975	XO Detector	3 vials
10010976	XO Horseradish Peroxidase	2 vials
700001	DMSO Assay Reagent	1 vial
400017	96-Well Solid Plate (black)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring fluoescence using excitation wavelength of 520-550 nm and emission wavelength of 585-595 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Xanthine oxidase (XO), or xanthine oxidoreductase, is a complex molybdoflavoenzyme which, in humans, is recognized as the terminal enzyme of purine catabolism, catalyzing the hydroxylation of hypoxanthine to xanthine and then to uric acid. When acting as an NADH oxidase, XO is a generator of superoxide, a powerful reactive oxygen species (ROS). XO has also been noted to produce hydrogen peroxide (H_2O_2) and superoxide during ischemia-reperfusion injury.[1,2] Due to their highly reactive nature, these ROS affect various molecular components of the cell, with excess amounts leading to cell degeneration and death.

XO is present in nearly all species. In mammalian tissues, XO is found predominantly in the liver and intestine. Human XO activity is almost exclusively limited to these tissues, with only trace levels found elsewhere in the body. However, in several disease states, levels of circulating XO have been seen to increase dramatically. This is especially true of liver disease, during which circulating levels of XO may be 1,000-fold greater.[2]

About This Assay

Cayman's Xanthine Oxidase Fluorometric Assay Kit provides a simple and accurate method for quantifying xanthine oxidase activity. The assay is based on a multistep enzymatic reaction (see Figure 1) in which xanthine oxidase first produces H_2O_2 during oxidation of hypoxanthine. In the presence of horseradish peroxidase, the H_2O_2 reacts with ADHP (10-acetyl-3,7-dihydroxyphenoxazine) in a 1:1 stoichiometry to produce the highly fluorescent compound resorufin.[3] Resorufin fluorescence can be easily analyzed with an excitation wavelength of 520-550 nm and an emission wavelength of 585-595 nm.

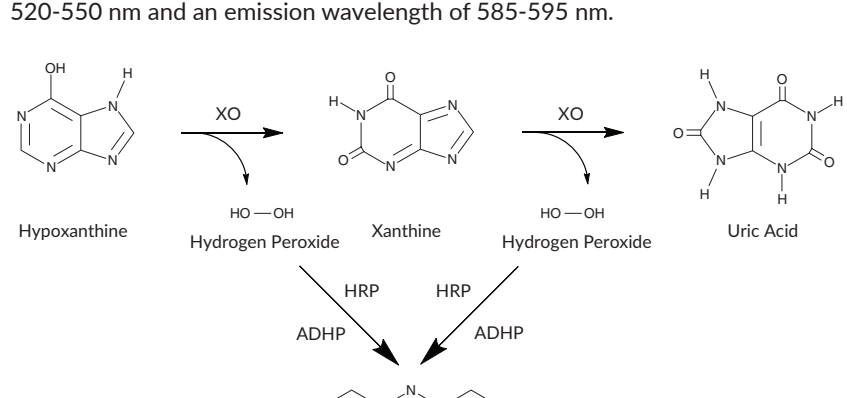


Figure 1. Xanthine oxidase assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **XO Assay Buffer (10X) - (Item No. 10010972)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. The diluted Assay Buffer is used in the preparation of the Assay Cocktail. The diluted Assay Buffer is stable for at least one week if stored at 4°C.

2. **XO Sample Buffer (10X) - (Item No. 10010973)**

   Dilute 3 ml of Sample Buffer concentrate with 27 ml of HPLC-grade water. The diluted Sample Buffer, 100 mM Tris-HCl, pH 7.5, should be used for preparation of standards and dilution of samples. The diluted Sample Buffer is stable for six months when stored at 4°C.

3. **Xanthine Oxidase Standard - (Item No. 10010974)**

   The vial contains 35 µl of 2 U/ml xanthine oxidase solution. Thaw vial on ice. Vortex gently to mix contents and spin briefly to ensure contents are in the bottom of the vial. In a separate tube, add 20 µl of Xanthine Oxidase Standard to 380 µl of diluted Sample Buffer. Store on ice until use. The diluted enzyme standard is stable for one day when stored at 4°C.

4. **XO Detector - (Item No. 10010975)**

   The vial contains a lyophilized powder of ADHP (10-acetyl-3,7-dihydroxyphen-oxazine). Immediately prior to adding to the Assay Cocktail (See under step 5 of **Performing the Assay**, on page 15), reconstitute the Detector with 200 µl of Dimethylsulfoxide (Item No. 700001). The reconstituted Detector is stable for 15 minutes. After 15 minutes, increased background fluorescence may occur.

5. **XO Horseradish Peroxidase (HRP) - (Item No. 10010976)**

   The vial contains a lyophilized powder of horseradish peroxidase (HRP). Reconstitute the reagent with 200 µl of HPLC-grade water. The reconstituted HRP is stable for one week when stored at -20°C.

6. **DMSO Assay Reagent - (Item No. 700001)**

   The vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.

Sample Preparation

Plasma

Typically, human plasma has xanthine oxidase levels which fall below the detection level of this kit. However, in some disease states the XO concentration may increase to detectable levels.[2]

1. Collect blood using an anticoagulant such as heparin or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month while stored at -80°C.

Serum

Typically, human serum has xanthine oxidase levels which fall below the detection level of this kit. However, in some disease states the XO concentration may increase to detectable levels.[2]

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The serum sample will be stable for one month while stored at -80°C.

Tissue Homogenate

1. Prior to dissection, rinse tissue with PBS (phosphate buffered saline solution, pH 7.4) to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 100 mM Tris- HCl, pH 7.5, containing protease inhibitors of choice; see **Interferences** on page 19) per gram weight of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of xanthine oxidase standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
B	B	B	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
C	C	C	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
D	D	D	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
E	E	E	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
F	F	F	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
G	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
H	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42

A-F = Standards
S1-S42 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples and standards be assayed at least in duplicate.
- 42 samples can be assayed in duplicate or 28 in triplicate.
- Monitor the fluorescence using an excitation wavelength of 520-550 nm and an emission wavelength of 585-595 nm.

Standard Preparation

Further dilute the Xanthine Oxidase Standard by transferring 10 µl of the previously diluted Standard to 990 µl of Sample Buffer. This will produce the 1 mU/ml stock used to prepare the standards. Take six clean glass test tubes and mark them A-F. Add the amount of Xanthine Oxidase Standard and Sample Buffer to each tube as described below in Table 1. Keep the standards on ice until aliquotted into the 96-well plate.

Tube	XO Standard (µl) (1 mU/ml)	Sample Buffer (µl)	Final Concentration (µU/ml)
A	0	1,000	0
B	20	980	20
C	40	960	40
D	60	940	60
E	80	920	80
F	100	900	100

Table 1. Preparation of XO standards

Performing the Assay

1. **Xanthine Oxidase Standard Wells** - add 50 µl of Xanthine Oxidase Standard (tubes A-F) per well in the designated wells on the plate (see **Sample plate format**, Figure 2, page 12).
2. **Sample Wells** - add 50 µl of sample to two wells. To obtain reproducible results, xanthine oxidase levels in the sample should fall within the range of the standard curve.
3. Cover the plate with the plate cover provided.
4. Prepare the Assay Cocktail by mixing the following reagents in a test tube: Diluted Assay Buffer (4.9 ml), Detector (50 µl), and HRP (50 µl). *NOTE: This volume provides enough cocktail to run the entire 96-well plate. Use the cocktail within 10 minutes of preparation for best results.*
5. Remove the plate cover and initiate the reactions by adding 50 µl of freshly prepared Assay Cocktail to all the wells being used.
6. Cover the plate with the plate cover and incubate for 45 minutes at 37°C.
7. Remove the plate cover and read the fluorescence using an excitation wavelength of 520-550 nm and an emission wavelength of 585-595 nm.

ANALYSIS

Calculations

1. Calculate the average fluorescence of each standard and sample.
2. Subtract the fluorescence value of the standard A from itself and all other values (both standards and samples). This is the corrected fluorescence.
3. Plot the corrected fluorescence values (from step 2 above) of each standard as a function of the final concentration of xanthine oxidase from Table 1. See Figure 3, on page 18, for a typical standard curve.
4. Calculate the xanthine oxidase activity of the samples using the equation obtained from the linear regression of the standard curve substituting adjusted fluorescence values for each sample. One unit is defined as the amount of enzyme that will catalyze the conversion of one µmol of hypoxanthine to uric acid and generates one µmol of hydrogen peroxide per minute at 37°C.

Xanthine Oxidase (µU/ml) =

$$\left[\frac{\text{(Adjusted sample fluorescence) - (y-intercept)}}{\text{Slope}}\right] \text{ x Sample dilution}$$

Performance Characteristics

Precision:

When a series of seventy-seven rat liver samples were assayed on the same day, the intra-assay coefficient of variation was 1.9%. When a series of fifteen rat liver samples were assayed on fifteen different days under the same experimental conditions, the inter-assay coefficient of variation was 3.9%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0.01-0.10 mU/ml Xanthine Oxidase.

Representative Xanthine Oxidase Standard Curve

The standard curve presented here is an example of the data typically provided with this kit; however, your results will not be identical to these. You must run a new standard curve - do not use the one below to determine the values of your samples.

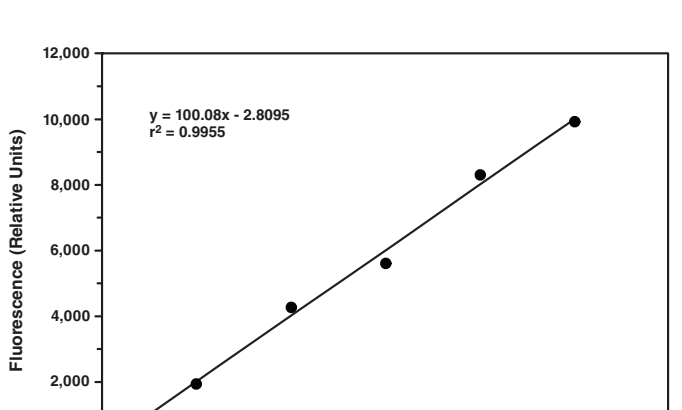


Figure 3. Xanthine Oxidase standard curve

RESOURCES

Interferences

The following reagents were tested in the assay for interference in the assay:

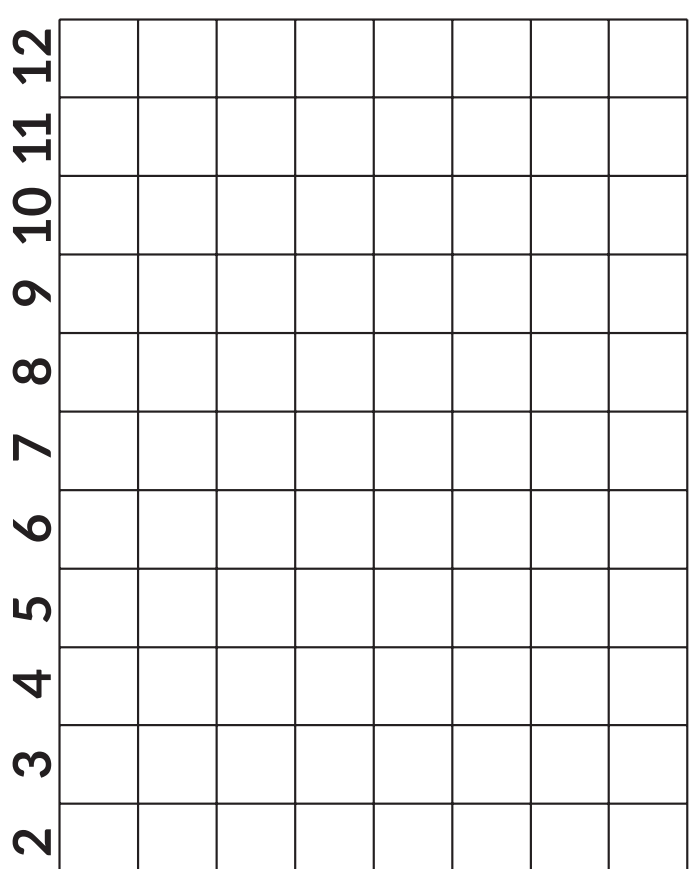
	Reagent	Will Interfere (Yes or No)
Detergents	1% Polysorbate 20	Yes
	≤0.5% Polysorbate 20	No
	1% Triton X-100	Yes
	≤0.5% Triton X-100	No
	10 mM Chaps	No
Buffers	Phosphate	No
	HEPES	No
	MES	Yes
	Tris	No
Protease/Chelators	200 µM PMSF	Yes
	1 mM EDTA	No
	1 mM EGTA	Yes
	10 µg/ml Antipain	No
Others	5% Glycerol	Yes
	≤1% Glycerol	No
	0.1% BSA	Yes
Solvents	Ethanol	No
	Methanol	Yes
	DMSO	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Poor fluorescence of both standards and samples	Plate was not incubated at 37°C	Make sure to incubate plate at 37°C
Xanthine Oxidase was not detected in the sample	Sample was too dilute	Re-assay the sample using a lower dilution
Fluorescence of sample fell above standard curve	The sample is too concentrated	Dilute your sample with Sample Buffer and re-assay
The Xanthine Oxidase standard curve did not work	A. Standards were not diluted properly B. Standard has degraded	A. Set-up the standards again according to Table 1 and re-assay B. Be sure to keep the standards on ice until aliquotting into plate

References

1. Brown, James. *et al.* Xanthine oxidase produces hydrogen peroxide which contributes to reperfusion injury of ischemic, isolated, perfused rat hearts. *J. Clin. Invest.* **81**, 1297-1301 (1988).
2. Harrison, Roger. Structure and function of xanthine oxidoreductase: Where are we now? *Free Radical Biology & Medicine* **33(6)**, 774-797 (2002).
3. Amundson, D.M. and Zhou, M. Fluorometric method for the enzymatic determination of cholesterol. *J. Biochem. Biophys. Meth.* **38**, 43-52 (1999).



NOTES

Warranty and Limitation of Remedy

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