Xanthine/Hypoxanthine Fluorometric/Colorimetric Assay Kit

Item No. 702340

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
10010973	Xanthine Oxidase Sample Buffer (10X)	1 vial/5 ml	-20°C
400582	Xanthine Standard	1 vial/35 µl	-20°C
400583	Xanthine/Hypoxanthine Developer Enzyme	1 vial	-20°C
400584	Xanthine Oxidase Enzyme	1 vial	-20°C
400610	MaxiProbe	1 vial/250 µl	-20°C
400115	Half-Area 96-Well Solid Plate (black, clear bottom)	1 plate	RT

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.

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical's Xanthine/Hypoxanthine Fluorometric/Colorimetric Assay Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

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 as directed in the **Materials Supplied** section (see page 3) 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 fluorescence with excitation and emission wavelengths of 535 and 587 nm, respectively, or a plate reader capable of measuring absorbance at 570 nm
2. An orbital microplate shaker
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of pure water; glass-distilled water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Aluminum foil
6. Materials used for **Sample Preparation** (see page 10)

INTRODUCTION

Background

Xanthine and hypoxanthine are products of purine catabolism.[1,2] Hypoxanthine is formed from the ATP metabolic intermediate inosine by nucleoside phosphorylase. Xanthine is formed from hypoxanthine by xanthine oxidase (XO), a dual-function enzyme that also converts xanthine to uric acid. It is also generated from guanine by guanine deaminase. Serum levels of xanthine are increased and associated with the formation of xanthine renal stones and cognitive disability in several inborn errors of purine metabolism such as Lesch-Nyhan syndrome and hereditary xanthinuria, diseases characterized by a deficiency in hypoxanthine-guanine phosphoribosyltransferase (HPRT) or very low levels of XO, respectively.[1,3,4] Increased serum levels of hypoxanthine are associated with hypoxia, pneumonia, leukemia, perinatal asphyxia, and cerebral ischemia.[2,5] Plasma levels of hypoxanthine are increased in patients experiencing non-traumatic chest pain and is a potential biomarker for the early detection of acute cardiac ischemia.[2,6]

About This Assay

Cayman's Xanthine/Hypoxanthine Assay Kit provides fluorometric and colorimetric methods for measuring xanthine and hypoxanthine in tissue homogenates, cell lysates, cell culture supernatants, and biological fluids, such as serum, plasma, and urine. In the assay, XO catalyzes the conversion of xanthine and hypoxanthine to uric acid and hydrogen peroxide (H_2O_2). In the presence of a developer enzyme, H_2O_2 reacts stoichiometrically with MaxiProbe to produce a fluorogenic/chromogenic compound (Figure 1). The fluorescence of this compound can be quantified at excitation and emission wavelengths of 535 and 587 nm, respectively. Alternatively, the absorbance can be measured at 570 nm. Background signal generated from H_2O_2 and other interferences found inherently in samples that are not produced by XO in the assay can be corrected by omitting the enzyme in sample background reactions.

It is at the user's discretion to choose the mode of measurement (and corresponding standard curve preparation) that best fits their needs. When read fluorometrically, this assay has a range of 0.04-5 µM and a limit of detection (LOD) of 0.04 µM. When read colorimetrically, the range is 0.18-100 µM with a LOD of 0.18 µM.

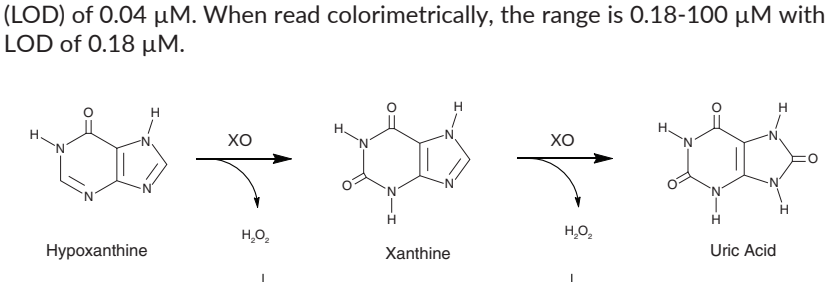


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Xanthine Oxidase Sample Buffer (10X) - (Item No. 10010973)**

   This vial contains 5 ml of concentrated xanthine oxidase sample buffer. Thaw at room temperature and dilute the contents with 45 ml pure water. Be certain to rinse the vial to remove any salts that may have precipitated. If not using all of the Xanthine Oxidase Sample Buffer (10X) at one time, save the remaining buffer at -20°C. The Xanthine Oxidase Sample Buffer (1X) will be stable for 6 months when stored at 4°C.

   NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with pure water.

2. **Xanthine Standard - (Item No. 400582)**

   This vial contains 35 µl of 25 mM xanthine, which is ready to use as supplied. Thaw at room temperature and vortex vigorously to ensure that the solid is completely dissolved. If not using all at once, aliquot and store at -20°C. Avoid multiple freeze-thaw cycles.

3. **Xanthine Oxidase Enzyme - (Item No. 400584)**

   This vial contains a lyophilized powder of xanthine oxidase. Reconstitute the contents of the vial with 150 µl of pure water and place on ice. One vial provides a sufficient volume to assay 100 wells. The reconstituted Xanthine Oxidase Enzyme will be stable for at least 1 week when stored at 4°C. Do not freeze.

4. **Xanthine/Hypoxanthine Developer Enzyme - (Item No. 400583)**

   This vial contains lyophilized Xanthine/Hypoxanthine Developer Enzyme. Reconstitute the contents of the vial with 150 µl of pure water and store on ice. One vial provides a sufficient volume to assay 100 wells. The reconstituted enzyme will be stable for at least 1 week when stored at 4°C. Do not freeze.

5. **MaxiProbe - (Item No. 400610)**

   The vial contains 250 µl of MaxiProbe in DMSO and is ready to use as supplied. One vial contains a sufficient amount to evaluate approximately 80 wells if using the colorimetric method or 100 wells if using the fluorometric method. The solution will be stable for one hour at room temperature if protected from light with the cap closed. If not planning to use MaxiProbe all at once, prepare aliquots and store at -20°C. Avoid multiple freeze-thaw cycles.

Sample Preparation

Enzymes in the samples may interfere with the assay. Therefore, it is recommended to deproteinate all the test samples using ultrafiltration with 10 kDa cut-off spin filters following the manufacturer's protocol prior to the assay. Generally, it is recommended to assay each sample in several dilutions.

Plasma

Collect blood in vacutainers containing heparin or EDTA for plasma samples. To obtain plasma, centrifuge blood at 1,000 x g for 15 minutes at 4°C. Pipette off the top plasma layer without disturbing the white buffy layer. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. Filter plasma samples through 10 kDa cut-off spin filters following the manufacturer's protocol and dilute with Xanthine Oxidase Sample Buffer (1X) to test in different dilutions or if high concentrations of xanthine are expected.

Serum

Collect blood in vacutainers without a coagulant for serum samples. Allow samples to clot undisturbed for 30-60 minutes at room temperature. To obtain serum, centrifuge at 1,000-2,000 x g for 15-30 minutes at 4°C. Pipette off the top serum layer. Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C. Filter serum samples through 10 kDa cut-off spin filters following the manufacturer's protocols and dilute with Xanthine Oxidase Sample Buffer (1X) if testing in several dilutions or if high concentrations of xanthine are expected.

Urine

Urine samples should be assayed immediately or stored at -20°C immediately after collection. Filter samples through 10 kDa cut-off spin filters following the manufacturer's protocols and dilute with the Xanthine Oxidase Sample Buffer (1X) at least 1:4 to fall within the range of the standard curve. It is recommended that the values obtained from urine samples be standardized to creatinine levels using Cayman's Creatinine ELISA Kit (Item No. 502330), Cayman's Creatinine (urinary) Colorimetric Assay Kit (Item No. 500701), or a similar assay.

Tissue Homogenate

Collect tissue and process immediately or store at -80°C. Mince tissue into small pieces. Homogenize 100 mg of minced tissue in 1 ml of Xanthine Oxidase Sample Buffer (1X) then centrifuge at 10,000 x g for 15 minutes at 4°C. Transfer the supernatant to another tube and store on ice or at -80°C for long-term storage. Filter the samples through 10 kDa cut-off spin filters following the manufacturer's protocol. To fall within the range of the standard curve, it may be necessary to dilute samples with Xanthine Oxidase Sample Buffer (1X) prior to the assay.

Cell Lysate

Collect cells (~10-20 x 10^6 cells) by centrifugation (*i.e.* 1,000-2,000 x g for 10 minutes at 4°C). Lyse cells in 1-2 ml of Xanthine Oxidase Sample Buffer (1X) OR a suitable lysis buffer, such as RIPA (Item No. 10010263), following the manufacturer's protocol. Centrifuge at 15,000 x g for 10 minutes at 4°C and use the supernatant for analysis. If not assaying the same day, store at -80°C. Filter the samples through 10 kDa cut-off spin filters following the manufacturer's protocol. To fall within the range of the standard curve, it may be necessary to dilute samples with Xanthine Oxidase Sample Buffer (1X) prior to the assay.

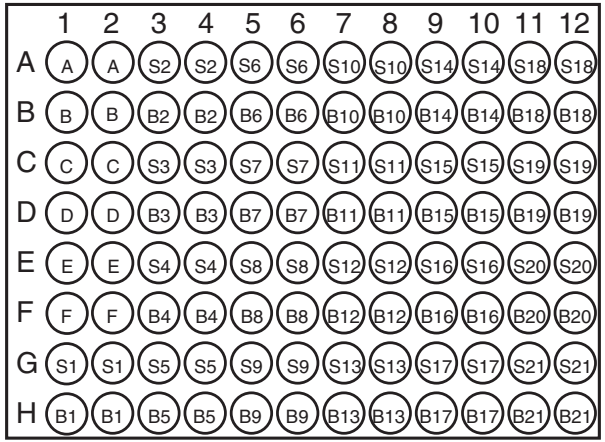
Cell Culture Supernatant

Remove cells from the medium by centrifugation. Filter the samples through 10 kDa cut-off spin filters following the manufacturer's protocol. To fall within the range of the standard curve, it may be necessary to dilute samples with Xanthine Oxidase Sample Buffer (1X) prior to the assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. It is suggested that each sample and standard be assayed at least in duplicate (triplicate is preferred). A typical layout of standards and samples to be measured in duplicate is shown in Figure 2 below. It is suggested that the contents of each well are recorded on the template sheet provided (see page 29).



A-F = Standards
S1-S21 = Sample Wells
B1-B21 = Sample Background Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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 in the assay is 100 µl in all of the wells.
- The enzymes should be kept on ice and all other reagents should be kept at 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 be assayed at least in duplicate (triplicate is preferred), but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with excitation and emission wavelengths of 535 and 587 nm, respectively, or monitor the absorbance at 570 nm.

Standard Curve Preparation

NOTE: This assay can be read using fluorescence or absorbance. Choose the standard curve preparation that matches the format needed. Both standard curves do not need to be prepared.

Fluorometric Standard Curve Preparation

Dilute 10 µl of the 25 mM Xanthine Standard with 990 µl of Xanthine Oxidase Sample Buffer (1X) to yield a 0.25 mM bulk standard. Label six clean glass or polystyrene test tubes A-F. Pipette 490 µl of Xanthine Oxidase Sample Buffer (1X) to tube A. Pipette 200 µl of Xanthine Oxidase Sample Buffer (1X) to tubes B-F. Transfer 10 µl of the bulk standard (0.25 mM) to tube A. Mix gently. Serially dilute the standard by removing 200 µl from tube A and placing it into tube B. Mix gently. Next, remove 200 µl from tube B and place it into tube C. Mix gently. Repeat the process for tubes D-E. Do not add any standard to tube F. This tube is the zero point of the standard curve.

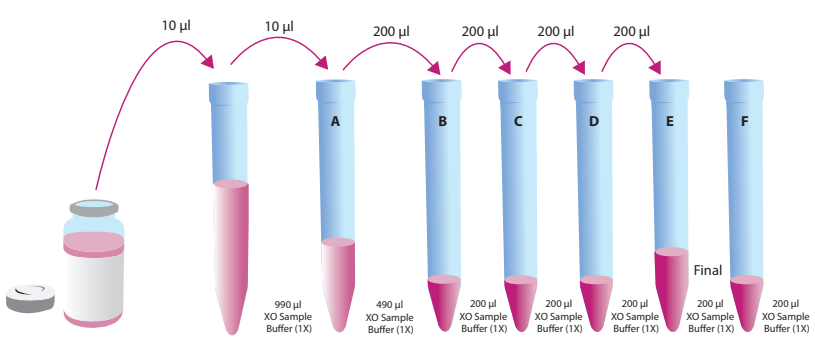


Figure 3. Preparation of the xanthine standards for the fluorometric assay format

Colorimetric Standard Curve Preparation

Dilute 10 µl of the 25 mM Xanthine Standard with 990 µl of Xanthine Oxidase Sample Buffer (1X) to yield a 0.25 mM bulk standard. Label six clean glass or polystyrene test tubes A-F. Pipette 240 µl of Xanthine Oxidase Sample Buffer (1X) to tube A. Pipette 200 µl of Xanthine Oxidase Sample Buffer (1X) to tubes B-F. Transfer 160 µl of the bulk standard (0.25 mM) to tube A. Mix gently. Serially dilute the standard by removing 200 µl from tube A and placing it into tube B. Mix gently. Next, remove 200 µl from tube B and place it into tube C. Mix gently. Repeat the process for tubes D-E. Do not add any standard to tube F. This tube is the zero point of the standard curve.

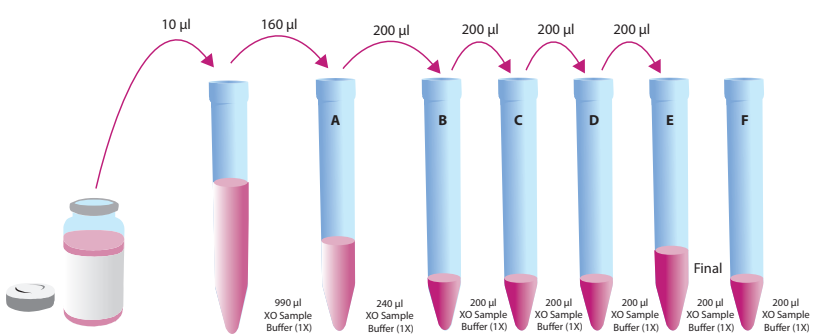


Figure 4. Preparation of the xanthine standards for the colorimetric assay format

Performing the Assay

NOTE: Use the Half-Area 96-Well Solid Plate (Black, Clear Bottom) (Item No. 400115) for both fluorometric and colorimetric assays.

1. **Reaction Mix and Background Mix:** prepare sufficient volumes of the Reaction and Background mixes according to Table 1 and use within 10 minutes of preparation. Scale the volumes up or down as needed. Each well requires 50 µl of either mixes. Each of the volumes calculated below include a 20% surplus.

Component	Fluorometric Assay		Colorimetric Assay	
	Volume in the Reaction Mix (µl)	Volume in the Background Mix (µl)	Volume in the Reaction Mix (µl)	Volume in the Background Mix (µl)
Reconstituted Xanthine/Hypoxanthine Developer Enzyme	60	60	60	60
Reconstituted Xanthine Oxidase Enzyme	60	--	60	--
MaxiProbe	15	15	60	60
Xanthine Oxidase Sample Buffer (1X)	2,865	2,925	2,820	2,880
Final Volume (# of Wells)	3,000 (50)	3,000 (50)	3,000 (50)	3,000 (50)

Table 1. Preparation of reaction and background mixes for fluorometric and colorimetric assays (50 wells)

2. **Standard Wells:** add 50 µl of standard per well in the designated wells on the plate (see Sample plate format, Figure 2, page 12).
3. **Sample Wells**: add 50 µl of sample to at least two wells.
4. **Sample Background Wells**: add 50 µl of sample to at least two wells. If several dilutions of the same sample are being tested, separate background wells should be included for each dilution.
5. Initiate the reactions by adding 50 µl of the Reaction mix to all standard and sample wells and 50 µl of Background mix to all sample background wells.
6. Cover the plate with aluminum foil to protect from light, shake for 20 seconds on a plate shaker, and incubate for 30 minutes at room temperature.
7. Remove the foil and read fluorescence at excitation and emission wavelengths of 535 and 587 nm, respectively. If the colorimetric method is used, read absorbance at 570 nm.

ANALYSIS

Calculations

1. Determine the average signal of each standard, sample, and sample background well.
2. Subtract the average signal value of standard F (zero standard) from itself and all other standards to obtain corrected signal values.
3. Plot the corrected signal values of each standard as a function of the concentration of xanthine. See Figures 5 and 6, on pages 21 and 22, for typical standard curves.
4. Subtract the average signal values of the sample background wells from the average signal values of the corresponding sample wells to yield the corrected sample measurement (CSM).
5. Calculate xanthine/hypoxanthine concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the CSM for each sample.

$$\text{[xanthine/hypoxanthine] (µM)} = \left[\frac{\text{CSM - (y-intercept)}}{\text{slope}}\right] \text{x sample dilution}$$

Performance Characteristics

The LOD for the fluorometric assay is 0.04 µM. The LOD for the colorimetric assay is 0.18 µM. The Lower Limit of Quantification (LLOQ) for the fluorometric assay is 0.08 µM and the LLOQ for the colorimetric assay is 1.6 µM.

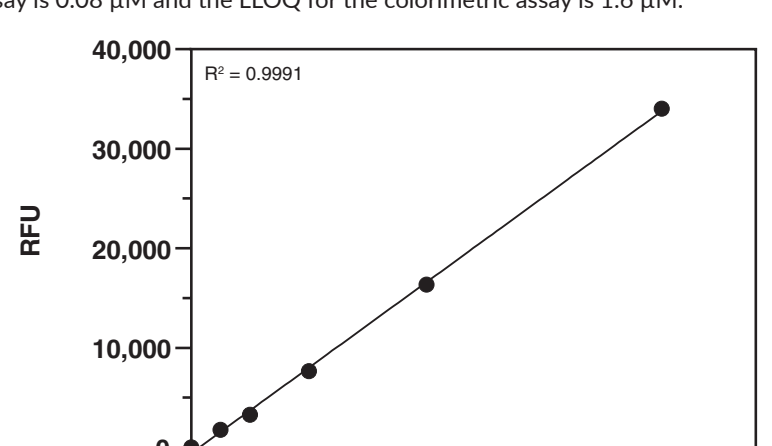


Figure 5. Xanthine standard curve for the fluorometric assay

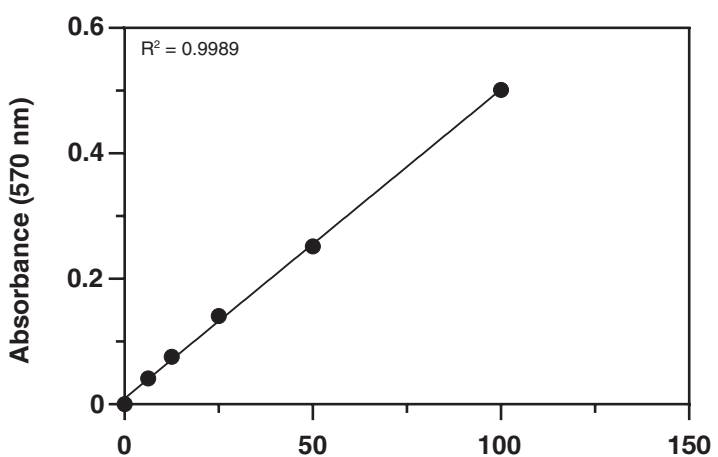


Figure 6. Xanthine standard curve for the colorimetric assay

Parallelism

To assess parallelism, human plasma, serum, rat liver homogenate, cell lysates, urine, and cell culture supernatant were processed as described in the Sample Preparation section, serially diluted with Xanthine Oxidase Sample Buffer (1X), and evaluated using the Xanthine/Hypoxanthine Fluorometric/Colorimetric Assay Kit. Measured xanthine concentrations were plotted against the dilution factor. The results are shown below.

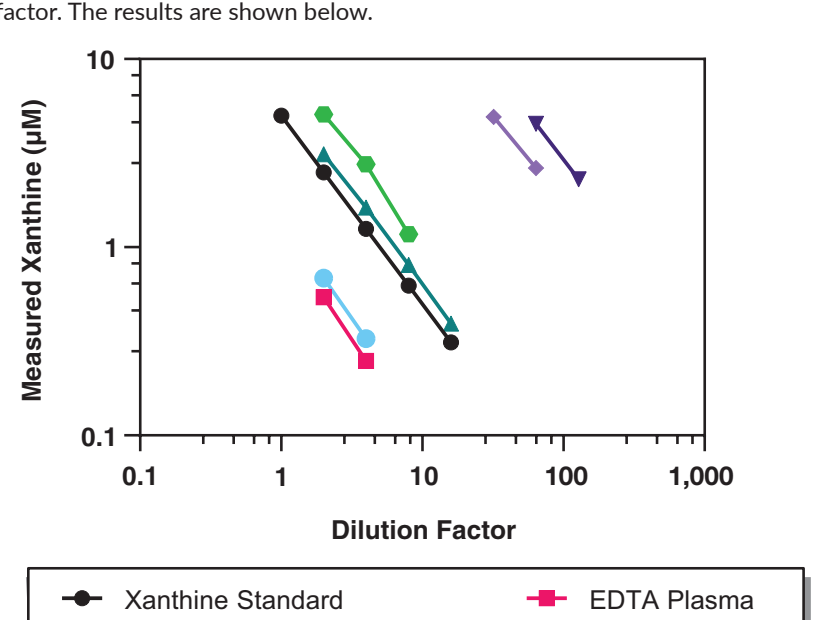


Figure 7. Parallelism of various matrices in the Xanthine/Hypoxanthine Fluorometric/Colorimetric Assay

Spike and Recovery

Serum and HepG2 cell culture supernatant were spiked with different amounts of xanthine, processed as described in the Sample Preparation section, serially diluted with Xanthine Oxidase Sample Buffer (1X), and evaluated using the Xanthine/Hypoxanthine Fluorometric/Colorimetric Assay Kit. The results are shown below. The error bars represent standard deviations obtained from multiple dilutions of each sample.

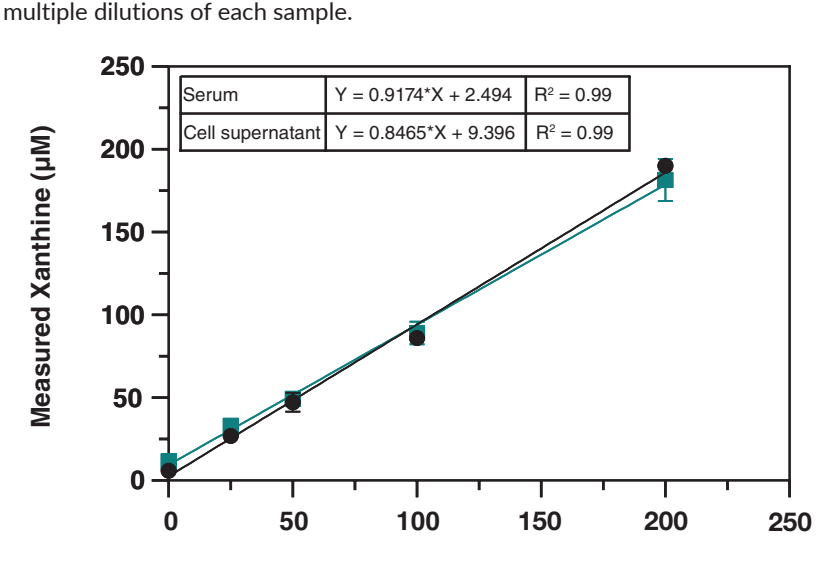


Figure 8. Spike and recovery of xanthine in serum and cell culture supernatant

Precision

When a series of 24 replicates of serum and urine measurements were performed on the same day, the intra-assay coefficient of variation was 2% and 11%, respectively for the fluorometric assay and 10% and 9%, respectively for the colorimetric assay. When a series of five serum and urine measurements were performed on different days under the same experimental conditions, the inter-assay coefficient of variation was 3% and 6%, respectively in the fluorometric assay and 3.1% and 4%, respectively in the colormetric assay.

Interferences

The following reagents were tested for interference in the assay.

Reagent		Will Interfere (Yes or No)
Buffers	25 mM MES	No
	25 mM HEPES	No
	10 mM PBS	No
	100 mM TRIS	No
	RIPA	No
Detergents	Polysorbate 20 (1%)	Yes
	Triton X-100 (1%)	Yes
Chelators	1 mM EDTA	No
Protease Inhibitors	Antipain (50 µg/ml)	No
	Bestatin (40 µg/ml)	No
	Chymostatin (60 µg/ml)	No
	E-64 (10 µg/ml)	No
	Leupeptin (0.5 µg/ml)	No
	Pepstatin (0.7 µg/ml)	No
	Phosphoramidon (330 µg/ml)	No
	Pefabloc SC (1 mg/ml)	No
	Aprotinin (2 µg/ml)	No

Table 2. Interferences

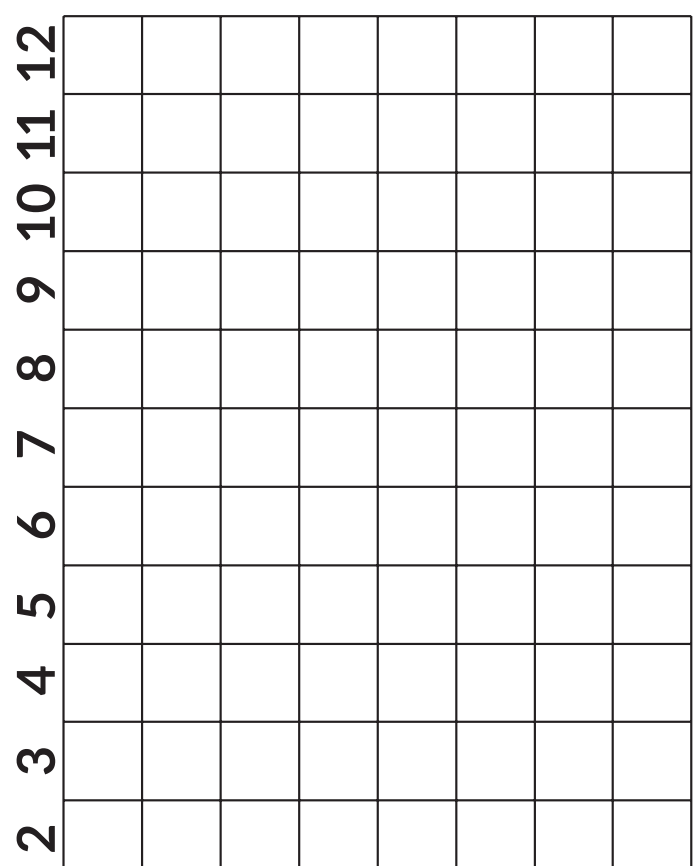
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s) C. Poor mixing of reaction mix and background mix D. Interferences from sample matrix from unfiltered sample	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 C. Mix the reaction mix and backround mix by inverting D. Filter the sample before assay
No fluorescence or absorbance was detected above background in the sample wells	Sample was too dilute	Re-assay the sample using a lower dilution
Measured xanthine/ hypoxanthine concentration was above the highest point in the standard curve	The xanthine/hypoxanthine concentration was too high in the sample	Dilute samples with Xanthine Oxidase Sample Buffer (1X) and re-assay *NOTE: Remember to account for the dilution factor when calculating the xanthine/ hypoxanthine concentration*
The fluorometer exhibited 'MAX' value for the wells	The *gain* setting is too high	Reduce the *gain* and re-read

	Standard Wells (µl)	Sample Wells (µl)	Sample Background Wells (µl)
Prepare Reaction and Background mixes according to Table 1.			
Standard	50	--	--
Sample	--	50	50
Reaction Mix	50	50	--
Background Mix	--	--	50
Cover plate with a foil, shake for 20 seconds, and incubate for 30 minutes at room temperature. Measure fluorescence at ex/em 535/587 nm or absorbance at 570 nm.			

Table 3. Assay summary



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NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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