Product Manual

Xanthine/Hypoxanthine Assay Kit

Catalog Number

MET-5150 **100 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Xanthine and hypoxanthine are naturally occurring purine derivatives. Xanthine is created from guanine by guanine deaminase, from hypoxanthine by xanthine oxidoreductase, and from xanthosine by purine nucleoside phosphorylase. Xanthine is used as a building block for human and animal drug medications, and is an ingredient in pesticides. In vitro, xanthine and related derivatives act as competitive nonselective phosphodiesterase inhibitors, raising intracellular cAMP, activating Protein Kinase A (PKA), inhibiting tumor necrosis factor alpha (TNF-α) as well as and leukotriene synthesis. Furthermore, xanthines can reduce levels of inflammation and act as nonselective adenosine receptor antagonists.

Hypoxanthine is sometimes found in nucleic acids such as in the anticodon of tRNA in the form of its nucleoside inosine. Hypoxanthine is a necessary part of certain cell, bacteria, and parasitic cultures as a substrate and source of nitrogen. For example, hypoxanthine is often a necessary component in malaria parasite cultures, since Plasmodium falciparum needs hypoxanthine to make nucleic acids as well as to support energy metabolism. Recently NASA studies with meteorites found on Earth supported the idea that hypoxanthine and related organic molecules can be formed extraterrestrially. Hypoxanthine can form as a spontaneous deamination product of adenine. Because of its similar structure to guanine, the resulting hypoxanthine base can lead to an error in DNA transcription/replication, as it base pairs with cytosine. Hypoxanthine is typically removed from DNA by base excision repair and is initiated by N-methylpurine glycosylase (MPG).

Cell Biolabs' Xanthine/Hypoxanthine Assay Kit is a simple fluorometric assay that measures the amount of total xanthine and hypoxanthine present in biological samples in a 96-well microtiter plate format. Each kit provides sufficient reagents to perform up to 100 assays*, including blanks, xanthine standards, and unknown samples. Sample xanthine/hypoxanthine concentrations are determined by comparison with a known xanthine standard. The kit has a detection sensitivity limit of 6.25 µM xanthine/hypoxanthine.

Note: Each sample replicate requires 2 assays, one treated with xanthine oxidase (+XO) and one without (-XO). Xanthine/hypoxanthine is calculated from the difference in RFU readings from the 2 wells.

Assay Principle

Cell Biolabs' Xanthine/Hypoxanthine Assay Kit measures total xanthine/hypoxanthine within biological samples. Hypoxanthine is converted into xanthine by xanthine oxidase (XO) and then xanthine is converted into uric acid and hydrogen peroxide by XO. The resulting hydrogen peroxide is then detected with a highly specific fluorometric probe. Horseradish peroxidase catalyzes the reaction between the probe and hydrogen peroxide, which bind in a 1:1 ratio. Samples are compared to a known concentration of xanthine standard within the 96-well microtiter plate format. Samples and standards are incubated for 15 minutes and then read with a standard 96-well fluorometric plate reader (Figure 1).

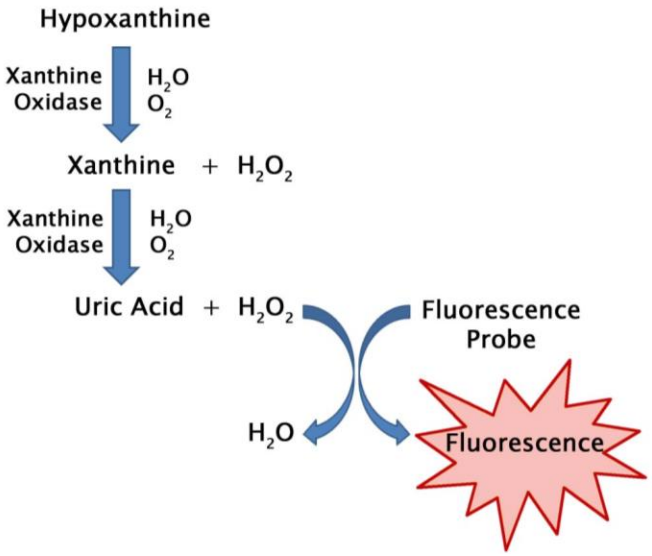

Figure 1. Xanthine/Hypoxanthine Assay Principle.

Related Products

1. MET-5147: Guanine Assay Kit (Colorimetric)
2. MET-5148: Guanine Assay Kit (Fluorometric)
3. MET-5149: Guanosine Assay Kit
4. MET-5090: Adenosine Assay Kit
5. MET-5092: Inosine Assay Kit

Kit Components

1. Xanthine Standard (Part No. 51501C): One 100 µL tube at 20 mM.
2. 10X Assay Buffer (Part No. 268002): One 25 mL bottle of 500 mM sodium phosphate pH 7.4.
3. Fluorometric Probe (Part No. 50231C): One 50 µL tube in DMSO.
4. HRP (Part No. 234402-T): One 10 µL tube of a 100 U/mL solution in glycerol.
5. Xanthine Oxidase (Part No. 50904D): one 100 µL tube at 2.5 U/mL.

Note: One unit is defined as the amount of enzyme that will convert 1.0 µmole of xanthine to uric acid per min at pH 7.5 at 25°C. About 50% of the activity is obtained with hypoxanthine as substrate.

Materials Not Supplied

1. Phosphate Buffered Saline (PBS)
2. Standard 96-well fluorescence black microtiter plate and/or black cell culture microplate

Storage

Upon receipt, store the 10X Assay Buffer at room temperature and store the rest of the kit at -20ºC. The Fluorometric Probe is light sensitive and must be stored accordingly. Avoid multiple freeze/thaw cycles.

Note: After thawing Xanthine Oxidase for the first time, make smaller aliquots and store at -20°C.

Preparation of Reagents

- 1X Assay Buffer: Dilute the stock 10X Assay Buffer 1:10 with deionized water for a 1X solution. Stir or vortex to homogeneity. Store at room temperature.
- Reaction Mix: Prepare a Reaction Mix by diluting the Fluorometric Probe 1:100, HRP 1:500, and Xanthine Oxidase 1:50 into 1X Assay Buffer. For example, add 10 µL Fluorometric Probe stock solution, 2 µL HRP stock solution, and 20 µL of Xanthine Oxidase to 968 µL of 1X Assay Buffer for a total of 1 mL. This Reaction Mix volume is enough for 20 assays. The Reaction Mix is stable for 1 day at 4ºC.

Note: Prepare only enough for immediate use by scaling the above example proportionally.

- Control Mix: Prepare a Reaction Mix (without xanthine oxidase) by diluting the Fluorometric Probe 1:100, and HRP 1:500 in 1X Assay Buffer. For example, add 10 µL Fluorometric Probe stock solution, and 2 µL HRP stock solution to 988 µL of 1X Assay Buffer for a total of 1 mL. This Control Mix volume is enough for 20 assays. The Control Mix is stable for 1 day at 4ºC.

Note: Prepare only enough for immediate use by scaling the above example proportionally.

Preparation of Samples

- Cell culture supernatants: Cell culture media containing xanthine and hypoxanthine should be avoided. To remove insoluble particles, centrifuge at 10,000 rpm for 5 min. The cell conditioned media may be assayed directly or diluted as necessary in PBS.

Note: Maintain pH between 7 and 8 for optimal working conditions as the Fluorometric Probe is unstable at high pH (>8.5).

- Tissue lysates: Sonicate or homogenize tissue sample in PBS and centrifuge at 10,000 x g for 10 minutes at 4°C. The supernatant may be assayed directly or diluted as necessary in PBS.
- Cell lysates: Resuspend cells at 1-2 x 10^6 cells/mL in PBS. Homogenize or sonicate the cells on ice. Centrifuge to remove debris. Cell lysates may be assayed undiluted or diluted as necessary in PBS.
- Serum, plasma or urine: To remove insoluble particles, centrifuge at 10,000 rpm for 5 min. The supernatant may be assayed directly or diluted as necessary in PBS.

Notes:

- *All samples should be assayed immediately or stored at -80°C for up to 1-2 months. Run proper controls as necessary. Optimal experimental conditions for samples must be determined by the investigator. Always run a standard curve with samples.*
- *Samples with NADH concentrations above 10 µM and glutathione concentrations above 50 µM will oxidize the Fluorometric Probe and could result in erroneous readings. To minimize this interference, it is recommended that superoxide dismutase (SOD) be added to the reaction at a final concentration of 40 U/mL (Votyakova and Reynolds, Ref. 2).*
- *Avoid samples containing DTT or β-mercaptoethanol since the Fluorometric Probe is not stable in the presence of thiols (above 10 µM).*

Preparation of Standard Curve

Prepare fresh Xanthine standards according to Table 1.

Standard Tubes	20 mM Xanthine Standard (µL)	PBS (µL)	Xanthine (µM)
1	10	490	400
2	250 of Tube #1	250	200
3	250 of Tube #2	250	100
4	250 of Tube #3	250	50
5	250 of Tube #4	250	25
6	250 of Tube #5	250	12.5
7	250 of Tube #6	250	6.25
8	0	250	0

Table 1. Preparation of Xanthine Standards.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each sample, including unknowns and standards, should be assayed in duplicate or triplicate.

Note: Each sample replicate requires two paired wells, one to be treated with Xanthine Oxidase (+XO) and one without the enzyme (-XO) to measure endogenous background.

2. Add 50 µL of each standard into wells of a black microtiter plate suitable for a fluorescence plate reader.
3. Add 50 µL of each unknown sample to each of two separate wells.
4. Add 50 µL of Reaction Mix to all standard wells and one half of the paired sample wells.
5. Add 50 µL of Control Mix to the remaining paired sample wells.
6. Mix the well contents thoroughly and incubate for 15 minutes at room temperature protected from light.

Note: This assay is continuous (not terminated) and therefore may be measured at multiple time points to follow the reaction kinetics.

7. Read the plate with a fluorescence microplate reader equipped for excitation in the 530-570 nm range and for emission in the 590-600 nm range.

Example of Results

The following figure demonstrates typical Xanthine/Hypoxanthine Assay Kit (Fluorometric) results. One should use the data below for reference only. This data should not be used to interpret or calculate actual sample results.

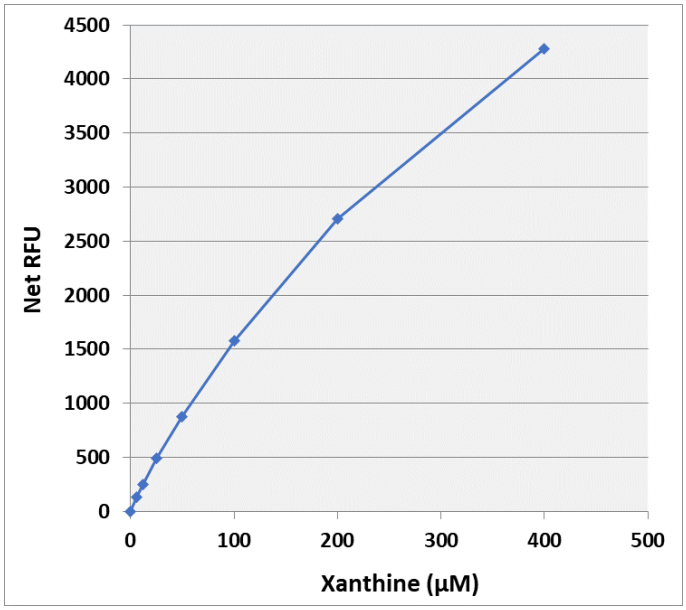

Figure 2: Xanthine Standard Curve.

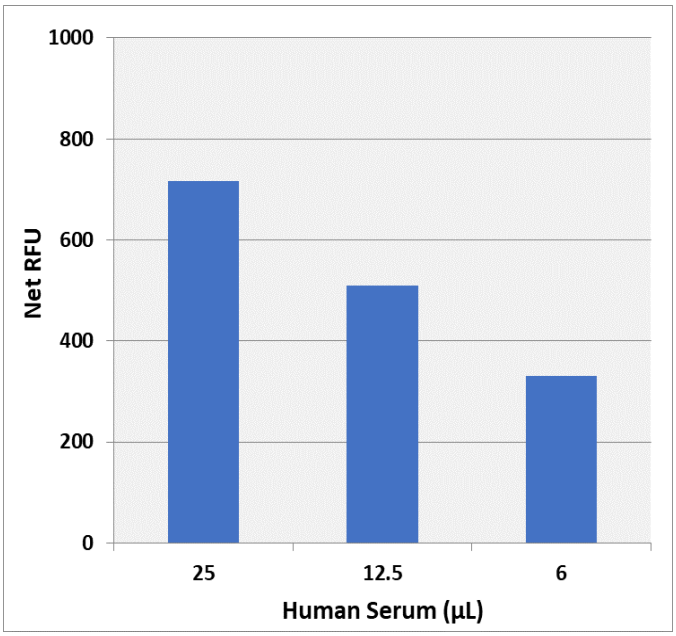

Figure 3: Xanthine/Hypoxanthine detection in human serum using the Xanthine/Hypoxanthine Assay Kit.

Calculation of Results

1. Determine the average Relative Fluorescence Unit (RFU) values for each sample, control, and standard.
2. Subtract the average zero standard value from itself and all standard values.
3. Graph the standard curve (see Figure 2).
4. Subtract the sample well values without Xanthine Oxidase (-XO) from the sample well values containing Xanthine Oxidase (+XO) to obtain the difference. The fluorescence difference is due to the Xanthine Oxidase activity.

$$\textbf{Net RFU} = (\textbf{RFU}_{+XO}) - (\textbf{RFU}_{-XO})$$

5. Compare the net RFU of each sample to the standard curve to determine and extrapolate the quantity of Xanthine/Hypoxanthine present in the sample. Only use values within the range of the standard curve.

References

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