Xanthine Oxidase Activity Colorimetric/Fluorometric Assay Kit (#BN00930)

(Catalog #BN00930; 100 assays; Store kit at –20° C)

I. Introduction:

Xanthine oxidase (XO, EC 1.17.3.2) is present in appreciable amounts in the liver and jejunum in healthy individuals. However, in various liver disorders, XO is released into circulation. Therefore, determination of serum XO level serves as a sensitive indicator of acute liver damage such as jaundice. Assay Genie has developed an easy and sensitive assay to determine XO in variety of samples. In the assay, XO oxidizes xanthine to hydrogen peroxide (H_2O_2) which reacts stoichiometrically with GenieRed Probe to generate color (at λ = 570 nm) and fluorescence (at Ex/Em = 535/587 nm). Since the color or fluorescence intensity is proportional to XO content, the XO activity can be accurately measured. The kit detects 1-100 mU xanthine oxidase in 100 µl reaction volume.

II. Kit Contents:

Components	BN00930	Cap Code	Part Number
XO Assay Buffer	25 ml	WM	BN00930-1
GenieRed Probe (in DMSO)	200 µl	Red	BN00930-2A
XO Enzyme Mix	lyophilized	Green	BN00930-4
XO Substrate Mix	lyophilized	Purple	BN00930-5
XO Positive Control	8 µl	Blue	BN00930-6
H_2O_2 Standard (0.88 M)	0.1 ml	Yellow	BN00930-7

III. Reagent Preparation and Storage Conditions:

GenieRed Probe: Ready to use as supplied. (Need to warm > 20°C to melt frozen DMSO).Store at -20°C, use within two months.

XO Enzyme Mix: Dissolve with 220 µl dH_2O. Pipette up and down to dissolve completely.

XO Substrate Mix: Dissolve with 220 µl dH_2O. Pipette up and down to dissolve completely.

XO Positive Control: Dilute with 92 µl dH_2O. Pipette up and down to dissolve completely. All components in kit should store at -20° C and use within two months.

IV. Xanthine Oxidase Assay Protocol:

1. Standard Curve Preparations:

Dilute 4 µl of 0.88 M H_2O_2 Standard into 348 µl dH_2O to generate 10 mM H_2O_2 Standard, then dilute 20 µl of 10 mM H_2O_2 Standard into 980 µl dH_2O to generate 0.2 mM H_2O_2 Standard.

Colorimetric assay: Add 0, 10, 20, 30, 40, 50 µl of the 0.2 mM H_2O_2 Standard into 96-well plate in duplicates, bring the total volume to 50 µl each well with dH_2O to generate 0, 2, 4, 6, 8, 10 nmol/well H_2O_2 Standard.

Fluorometric assay: Dilute 50 µl fresh 0.2 mM H_2O_2 into 950 µl dH_2O to generate 10 µM H_2O_2 Standard. Add 0, 10, 20, 30, 40, 50 µl of the 10 µM H_2O_2 into 96-well plate in duplicates, bring volume to 50 µl with dH_2O to generate 0, 0.1, 0.2, 0.3, 0.4, 0.5 nmol/well H_2O_2 Standard.

2. Sample and Positive Control Preparations: Prepare test samples in 50 µl/well with assay buffer in a 96-well plate. Serum can be directly added into sample wells, and adjust volume to 50 µl/well with dH_2O. Tissues or cells can be extracted with 4 volumes of the Assay Buffer, centrifuge (16,000 x g, 10 min) to get clear XO extract. For the positive control, add 5 µl positive control solution to wells, adjust volume to 50 µl/well with dH_2O. H_2O_2 in the sample will generate background. It is important to set up a background control. We suggest using several doses of your sample to ensure the readings are within the linear range.

3. Reaction Mix Preparation: Mix enough reagents for the number of assays and standard to be performed. For each well, prepare a total 50 µl Reaction Mix containing:

Xanthine Oxidase Measurement	Background Control
44 µl Assay Buffer	46 µl Assay Buffer
2 µl Substrate Mix	----------
2 µl Enzyme Mix	2 µl Enzyme Mix
2 µl GenieRed Probe**	2 µl GenieRed Probe

** For the fluorescent assay, dilute GenieRed probe 10X to reduce background readings.

4. Add 50 µl of the reaction mix to each well containing the H_2O_2 Standard, Positive Control, and test samples, mix well.

5. Measure the plate immediately (OD = 570 nm for colorimetric assay or at Ex/Em = 535/587 nm for fluorometric assay) at T_1 to read A_1, measure again at T_2 after incubating the reaction at 25° C for 10 - 20 min (or incubate longer time if the sample XO activity is low) to read A_2, protect from light. The signal generated by XO is $\Delta A = A_2 - A_1$

Notes:

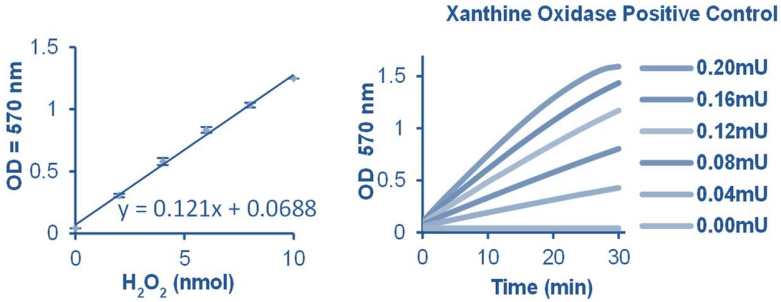
1) It is essential to read A_1 and A_2 in the reaction linear range. It will be more accurate if you read the reaction kinetics. Then choose A_1 and A_2 in the reaction linear range.
2) Read H_2O_2 standard after 20 min incubation without subtract A1. The standard is stable for a few hours.

6. Calculation: Subtract background from all readings. Plot the H_2O_2 standard Curve. Apply sample ΔA to the H_2O_2 standard curve to get B nmol of H_2O_2 (H_2O_2 generated between T_1 and T_2 in the reaction by XO).

$$\text{XO Activity} = \frac{B}{(T2-T1)\times V} \text{ x Sample Dilution Factor = nmol/min/ml = mU/ml.}$$

Where: **B** is the amount of H_2O_2 generated by XO from standard curve (in nmol).
T_1 is the time of the first reading (A1) (in min).
T_2 is the time of the second reading (A2) (in min).
V is the pretreated sample volume added into the reaction well (in ml).

Unit Definition: One unit xanthine oxidase is defined as the amount of enzyme catalyzes the oxidation of xanthine, yielding 1.0 µmol of uric acid and H_2O_2 per minute at 25° C.



FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures until use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		