*Oxis*Research™

A Division of OXIS Health Products, Inc.

BIOXYTECH® GST-340™

Spectrophotometric Assay for Glutathione-S-Transferase
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Catalog Number 21046

INTRODUCTION

The Analyte

Glutathione S-transferase (GST) catalyzes the conjugation of the thiol group of glutathione to electrophilic xenobiotics (1). It thus utilizes glutathione to scavenge potentially toxic compounds, including those produced as a result of oxidative stress (2), and is part of the defense mechanism against the mutagenic, carcinogenic and toxic effects of such compounds. Erythrocyte GST has also been proposed to function as a hemin-transport protein in developing erythroid cells (3). GST activity is present in virtually all human tissues but is especially prevalent in the liver, which plays a major role in detoxification of xenobiotics. GST is actually a family of enzymes, which differ in the nature of the xenobiotic they will utilize as substrate. There are at least nine major classes of human GST isozymes (4). Within each major class there are multiple isozymes. Expression of these different isozymes is organ specific. Human genetic polymorphisms in GST contribute to predisposition to disease as well as responsiveness to treatment (5,6). GST activity is also inducible (6), and GST activity is present throughout the phylogenetic scale (7). All GST isozymes catalyze the same reaction, shown schematically below, where X is a xenobiotic and GSH is glutathione.

$$X + GSH \xrightarrow{GST} G\text{-}S\text{-}X + H^+$$

Principle of the Assay

The isozymes have widely varying K_m and V_{max} values towards different xenobiotics and GSH. Thus, in a tissue extract containing a mixture of isozymes, the contribution of each to the overall GST activity will depend on the xenobiotic used and the reaction conditions.

The great variation in enzymatic activity of GST isozymes towards different xenobiotics makes the design of a single assay for all GST isozymes impractical. In practice, a variety of different substrates have been employed. These differ not only in reactivity towards the different isozymes but also in the ease with which the reaction can be monitored. The substrate with perhaps the broadest range of isozyme detectablility, coupled with ease of monitoring the reaction, is 1-chloro-2,4-dinitrobenzene (CDNB) (8). The conjugation of this compound with GSH proceeds by nucleophilic aromatic substitution of chlorine by the thiol group of GSH, producing a dinitrophenyl thioether and chloride ion (Figure 1).

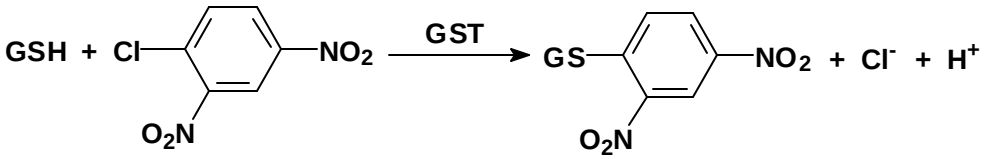


Figure 1. The reaction of CDNB with GSH.

Product formation is accompanied by the appearance of an absorption band at 340 nm, which makes possible a spectrophotometric assay for GST activity. As shown in Figure 2, addition of GST results in the gradual increase of the dinitrophenyl thioether chromophore. The non-enzymatic reaction of CDNB and GSH at pH 6.5 is slow relative to the enzyme-catalyzed reaction.

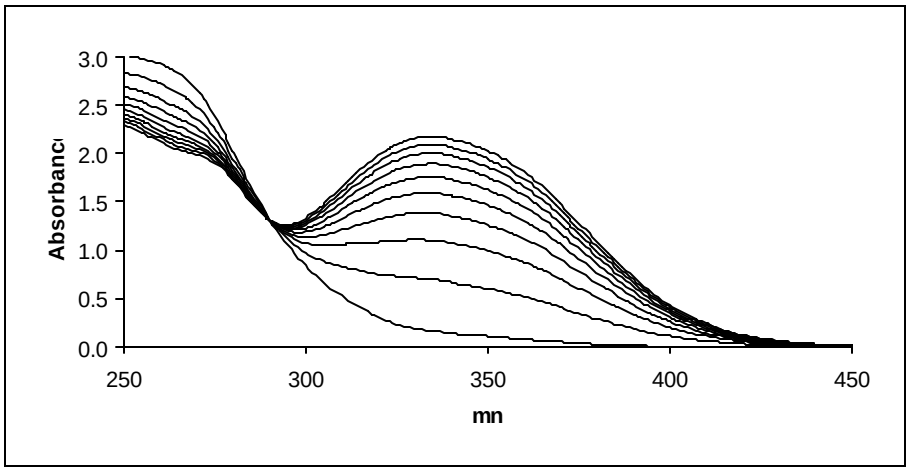


Figure 2. The reaction of CDNB and GSH in the presence of GST. The lowest scan at 340 nm is the CDNB/GSH mixture before addition of GST. Subsequent scans were taken at two minute intervals after enzyme addition and show increasing absorbance at 340 nm and decreasing absorbance at 250 nm, with an isosbestic point at about 290 nm.

The Bioxytech® GST-340™ assay is based upon the GST-catalyzed reaction between CDNB and GSH. One unit of GST activity is defined as the amount of enzyme producing 1 µmol of CDNB-GSH Conjugate/min under the conditions of the assay.

REAGENTS

Materials Provided (Sufficient for 100 Tests)

- Buffer — Potassium phosphate, pH 6.5, 100 mL
- GSH — Lyophilized, Glutathione, 4 vials
- CDNB — 1-Chloro-2,4-dinitrobenzene in ethanol, 5 mL
- Sample Diluent — Lyophilized, Potassium phosphate, GSH, BSA, 5 vials

Materials Required But NOT Provided

- Spectrophotometer
- Cuvettes with 1 cm optical path
- Water bath, 25°C
- Pipettes, preferably adjustable, capable of accurately pipetting 5-1000 µL

Warnings and Precautions

- The Sample Diluent contains bovine serum albumin (BSA).
- CDNB (1-chloro-2,4-dinitrobenzene) is toxic by inhalation, swallowing or contact with the skin. In case of exposure to the eyes, rinse immediately with water and seek medical attention. In case of contact with the skin, wash with soapy water. Wear protective gloves and use eye protection.
- The CDNB solution contains denatured ethanol. Denatured ethanol contains methanol and isopropyl alcohol. Flammable liquid. Vapor harmful. May be fatal or cause blindness if swallowed. Avoid contact with skin. Wear protective gloves and eye protection.

Reagent Storage and Handling

Do not allow the reagent bottles to sit at room temperature for long periods of time. When not in use, place the bottles at 2-8°C. Subsequent to reconstitution, unused portions of GSH and Sample Diluent should be kept frozen at –20°C. The CDNB solution should be protected from light. Unopened reagents are stable until the indicated expiration date.

PROCEDURE

Reagent Preparation

- GSH: Just prior to use, reconstitute 1 vial with 2.70 mL of deionized water. One vial is sufficient for 25 GST assays. If fewer assays are to be run on a given day, incubate the required volume at 25°C and freeze the remainder at –20°C. Unused GSH solution may be stored at –20°C for up to one week.
- Sample Diluent: Just prior to use reconstitute one vial with 20 mL deionized water. Keep on wet ice. Unused buffer may be kept frozen at –20°C for up to one week.
- Prepare Buffer/CDNB Mixture according to Table 1 for the number of assays to be run. This solution should be prepared on the day it is to be used and incubated at 25°C. Discard any unused solution at the end of the day.

Table 1
Preparation of Working Solutions

	Number of Assays to be Run				
Component	1	10	15	20	25
Buffer	0.880 mL	9.680 mL	14.200 mL	8.480 mL	22.880 mL
CDNB	0.020 mL	0.220 mL	0.320 mL	0.420 mL	0.520 mL

Sample Preparation Guidelines

These procedures are intended as a starting point for sample preparation. The researcher will have to adjust them as necessary for their particular sample types.

Erythrocytes: Collect blood using heparin or EDTA as anticoagulant. Collect and wash erythrocytes in cold 0.85% NaCl or phosphate buffered saline. Lyse cells by adding 4 volumes of cold deionized water to 1 volume of packed cells (1/5). Suspend cells and rapidly freeze the sample. Thaw in a water bath at 20-25°C and dilute an aliquot of lysate 1/4 with Sample Diluent to give a net 1/20 dilution of the packed red cells. Store on wet ice and assay as described below. Diluted aliquots may be frozen at –70°C for later analysis. Results may be normalized to hemoglobin for inter-sample comparison.

Solid Tissue: Homogenize ~200 mg of tissue in 1.0 mL of cold Sample Diluent. Dilute an aliquot of the homogenate with Sample Diluent and store on wet ice. Assay as described below. Homogenate may be frozen at –70°C for later analysis. Results may be normalized to total protein for inter-sample comparison.

Enzyme concentration: The appropriate degree of sample dilution must be determined experimentally. Regardless of sample type, samples must be diluted with Sample Diluent to give 20-140 mU/mL and kept on wet ice.

Assay (in cuvettes)

Use of a thermostatted cell holder maintained at 25°C is recommended.

1. Pipet 900 µL of Buffer/CDNB Mixture into a test tube incubated in a 25°C water bath.
2. Add 50 µL of GSH solution.
3. Immediately add 50 µL of diluted sample or deionized water (blank). Vortex to mix.
4. Transfer to a cuvette and record A_{340} vs. time for five minutes.

Calculations

1. Determine the slopes (Δ A_{340}/min) of the sample and blank runs from the A_{340} vs. time plots.
2. Determine the net rate by subtracting the blank slope from the sample slope.

$$\text{Net Rate } (\Delta A_{340}/\text{min}) = \text{Slope}_{\text{sample}} - \text{Slope}_{\text{blank}}$$

3. Calculate the GST activity in the original, undiluted sample from the equation below:

$$\text{GST Activity (mU/mL)} = \frac{\text{Net Rate}}{0.0096} \times \text{Dilution Factor}$$

Where: 0.0096 = the extinction coefficient of the CDNB-GSH adduct
Dilution Factor = total amount by which the sample was diluted

Example

A red blood cell lysate was diluted with Sample Diluent and assayed in triplicate, along with a blank, according to the above procedure (Table 2). The hemoglobin concentration of the diluted sample was 3.45 mg/mL.

Table 2
Enzyme Activity in Erythrocyte Lysate

Slope (Δ A_{340}/min)					
Assay	Run 1	Run 2	Run 3	Mean	Net Rate
Blank	0.0035	0.0033	0.0034	0.0034	---
Sample	0.0144	0.0146	0.0144	0.0145	0.0110

1. Calculate the mean of the three runs of blank and sample.
2. Subtract the mean blank slope from the mean sample slope to get the Net Rate.
3. Calculate the GST activity in the sample (Note: since 50µL of sample was used in a final volume of 1000 µL, the dilution factor is 20).

GST (mU/mL) = (0.0110/0.0096) x (20) = 22.9 mU/mL

4. Express activity per mg hemoglobin

GST (mU/mg Hb) = 22.9 mU/mL/3.45 mg Hb/mL = 6.64 mU/mg Hb

Alternative Microplate Assay Procedure

Reagent and sample preparation are identical to the cuvette method. The results will differ from the cuvette method for three reasons: 1) the pathlength will not be 1 cm, 2) the bandpass of the filter will be different from the monochromator of the spectrophotometer, and, 3) the temperature will likely not be exactly 25°C.

These conditions will not prevent comparison of results from a series of samples to determine relative GST activities. By running a given sample in both the cuvette and microplate methods, the researcher can determine a correction factor to convert microplate rates to activities in mU/mL in the standard cuvette assay. A suggested protocol for the microplate assay follows.

1. Pipet 180 µL of Buffer/CDNB Mixture into a well.
2. Add 10 µL of GSH solution.
3. Immediately add 10 µL of sample or deionized water (Blank).
4. Mix and record A_{340} for 5 minutes.

Calculations

Calculations are identical to those for the cuvette method. If the researcher has determined a conversion factor as described above, the activity may be expressed as mU/mL. Alternatively, the Net Rates may be compared to determine relative GST activities of samples.

PERFORMANCE CHARACTERISTICS

Precision

The precision of the assay was determined by assaying GST controls, at three different concentrations, stored at –70°C. Each sample was assayed in duplicate on 10 different occasions over a one-week period (Table 3).

Table 3
Precision

mU/mL Enzyme in Cuvette	1.42	4.20	8.20
Mean (ΔA_{340}/min)	0.0136	0.0403	0.0787
Intra-assay Precision (%CV)	0.89%	0.80%	1.95%
Total Precision (%CV)	1.05%	0.89%	2.13%

Sensitivity – Lower Limit of Detection (LLD)

The Lower Limit of Detection (LLD) is a measure of the lowest activity distinguishable from zero with confidence. Using Sample Diluent, the ΔA_{340}/min for a blank was determined and the mean and standard deviation of the slope was calculated. The LLD (~99.5% confidence) is the amount of enzyme giving a rate 3.29 times the standard deviation of the blank (Table 4).

Table 4
Lower limit of Detection

Number of Data Points	11
Mean ΔA_{340}/min	0.00339
Standard Deviation	0.000176
LLD (mU enzyme/cuvette)	0.060

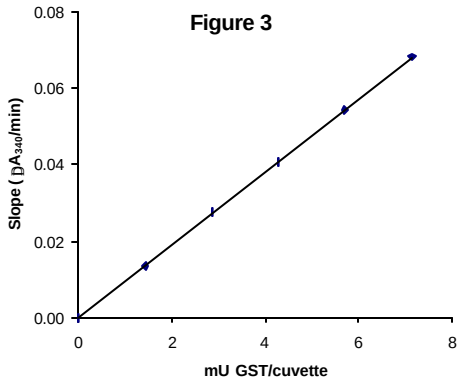
Assay Range

The theoretical range of the assay is from the LLD to the highest rate for which a linear slope is obtained. As a practical matter, the effective LLD is determined by the sensitivity of the spectrophotometer. A conservative estimate of the working range is 1-7 mU of enzyme/cuvette. This will provide an adequate slope at the lower end of the range to determine an accurate rate and a sufficiently long linear region of the A_{340} vs. time plot at the upper end of the range to give an accurate rate.

Linearity on Dilution

Purified GST was diluted and assayed under standard conditions. As shown in the graph (Figure 3), ΔA_{340}/min vs. mU of enzyme per cuvette is linear on dilution between 0 – 7 mU/cuvette.

Figure 3



Recovery

Pure GST was added to Sample Diluent and a red blood cell lysate. The recovery of added enzyme was then calculated by dividing the Net lysate activity by the Spiked Diluent activity as seen in Table 5.

Table 5
Recovery of Enzyme Added to Erythrocyte Lysate

Sample	Measured	Added	% Recovery
Lysate	70	---	---
Spiked Diluent	112	112	---
Spiked Lysate	178	108	96

Specificity

The activity of purified equine liver GST was measured in the presence of various substances commonly used in enzyme isolation (Table 6). The percent activity was determined relative to a control with no additions. The concentration of each addition is given in the assay mixture.

Table 6
Interference by Selected Substances

Substance	Concentration in Reaction Mixture	GST Activity ΔA_{340}/min	% of Control
None	---	0.0527	100
Urea	600 mM	0.0456	86
Triton X-100	0.10%	0.0354	67
Sucrose	6.0%	0.0524	99
Na_2SO_4	100 mM	0.0520	99
$(NH_4)_2SO_4$	100 mM	0.0471	89
BSA	1 mg/mL	0.0527	100

Abbreviations: BSA, bovine serum albumin.

Sucrose, Na_2SO_4 and BSA do not interfere in the assay. Urea and $(NH_4)_2SO_4$ cause a slight reduction in GST activity. Triton X-100 has a significant effect.

Effect of Thiols on GST Activity

Thiols react non-enzymatically with CDNB, increasing the blank reaction rate. They also can serve as substrates for GST, as shown below in Table 7.

Table 7
Selected Thiols as GST Substrates

Compound	Conc. in Rxn Mixture	Slope Enzyme Rxn	Slope Blank Rxn	Net Slope	% of Control
GSH	1.00 mM	0.0801	0.0034	0.0767	100
DTT	64.9 mM	0.0563	0.0210	0.0353	46
Mercaptoethanol	14.3 mM	0.0499	0.0128	0.0371	48

The data compare the rate of the enzymatic reaction using GSH, the natural substrate, with dithiothreitol (DTT) and 2mercaptoethanol (2ME). The concentrations of the latter two thiols in the reaction mixture correspond to 0.10% solutions. For each thiol, the blank and enzymatic rates were determined and the net enzymatic rate calculated. The concentrations of DTT and mercaptoethanol are much higher than GSH, indicating they are much less effective substrates for GST. However, at the concentrations used, their activity as GST substrates is significant compared to GSH itself (last column). If either thiol is used in the isolation of GST, it could be present in the reaction mixture at concentrations in the range of 0.1%, as used in the above study. The effect of such a concentration of either DTT or 2ME on the standard GST assay was examined and the data are presented in Table 8.

Table 8
Effect of 0.1% DTT or 2ME on GST Activity

Thiol Added	Conc. in Rxn Mixture	Slope Enzyme Rxn	Slope Blank Rxn	Net Slope	% of Control
None	---	0.0801	0.0034	0.0767	100
DTT	64.9 mM	0.0908	0.0230	0.0678	88
Mercaptoethanol	14.3 mM	0.0850	0.0169	0.0681	89

With no added thiol, the reaction rate reflects GST conjugation of GSH to CDNB under standard assay conditions, and is defined as 100% activity. When the reaction is run with the addition of either DTT or 2ME, both the blank and enzymatic rates are affected. The net slope is the difference between the two and, as can be seen, there is an apparent reduction in measured GST activity to 88-89% of the control.

It may seem paradoxical that these thiols, which the previous experiment showed to be substrates for GST, should reduce rather than elevate the net enzymatic rate. The explanation, however, is straightforward. Both DTT and 2ME are much poorer substrates for the enzyme than GSH, producing product at a much slower rate. Therefore, to the extent that either thiol is bound to the active site of GST the ability of the enzyme to produce product is reduced relative to the reaction where only GSH is present. Thus, either thiol, when present with the natural substrate, GSH, behaves as an inhibitor since it slows the net formation of product**.**

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