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Technical Datasheet

EZAssay™ GST Activity Estimation Kit

Product Code: CCK028

1. Introduction:

The Glutathione *S*-transferases (GSTs) comprise of a family of water-soluble enzymes found in prokaryotic and eukaryotic cells. They are located in the cytosol, mitochondria and microsomes. These enzymes contribute to cell survival by detoxification of harmful endogenous compounds and breakdown of xenobiotics. They are often referred to as phase II enzymes as they are active in the second phase of xenobiotic metabolism. The family of mammalian GSTs consists of eight classes of cytosolic isoenzymes namely, Alpha, Mu, Pi, Sigma, Theta, Zeta, Kappa and Omega.

GSTs can act on a wide variety of substrates of endogenous and xenobiotic origin. Endogenously derived substrates include by-products of normal metabolism, products of lipid peroxidation such as 4-hydroxyalkenals as well as aldehydes, epoxides, hydroperoxides and alkenes generated from tissue damage. Xenobiotic substrates include environmental pollutants, carcinogens, and also, in some cases, anti-cancer drugs. One of the reasons for acquired drug resistance to cancer therapeutic agents in cancer cells is the elevated activity of GST.

The mechanism of detoxification involves neutralization of the electrophilic, reactive sites of toxic compounds by attaching them to the tripeptide glutathione. This reaction is catalyzed by GSTs. The compounds are rendered water-soluble and are eliminated.

2. About the kit:

HiMedia's EZAssay™ GST Activity Estimation kit is simple and convenient tool for sensitive and reproducible detection and quantitation of GST activity in an array of samples like serum, cell lysates and tissue homogenates. This assay is based on the GST-catalyzed reaction between reduced glutathione and the GST substrate 1-chloro-2,4-dinitrobeneze (CDNB). CDNB can react with the broadest range of GST isoenzymes.

The conjugation of glutathione to the CDNB substrate results in an increase in absorbance at 340nm.

The components of one kit are sufficient to perform 100 tests including controls, standards and samples.

3. Kit Contents:

Contents		Quantity	Storage
Code	**Description**		
CCK028 (Part A)	Assay buffer	100ml	2-8°C
CCK028 (Part B)	Sample buffer	25ml	2-8°C
CCK028 (Part C)	GST standard	400µl	-30 to -10°C
CCK028 (Part D)	Glutathione, reduced	100mg	2-8°C
CCK028 (Part E)	Substrate solution	1.5ml	-30 to -10°C

4. Materials required but not provided in the kit:

- Test sample (serum/plasma/cells/tissue)
- Adjustable pipettes and pipette aid
- Flat-bottom 96-well microtiter plates or Quartz cuvettes
- 96-well microplates reader capable of measuring absorbance between 340nm or Spectrophotometer capable of measuring absorbance between 340nm
- Cell Culture Grade Water

5. General Guidelines:

Accuracy

- To obtain statistically significant data, perform the assay in triplicates or more.
- Accuracy of the assay depends on pipetting skills of the personnel. Inappropriate addition and mixing practices may result in erroneous and false-positive or false-negative results.

- Use of a repeating pipettor is recommended to pipette reagents. This saves time and helps maintain more precise incubation times.
- Pipette tip should be equilibrated with the reagent before use. This is carried out by slowly filling up the tip with reagent and gently expelling the contents several times.
- Care should be taken so that no bubbles are introduced into the wells during pipetting or mixing of the reagents.

Procedural precautions

- Do not leave the reagent bottles and sample bottles open for prolonged duration. Replace the caps immediately after use.

6. Directions for use:

Users are advised to review entire procedure before starting the assay

1. Preparation of reagents:

Assay buffer

Dilute the Assay buffer 1:2 with cell-culture grade water. Use the diluted buffer for the assay. The diluted buffer can be stored at 4°C for about one month. Ensure that the pH of the buffer is 6.5. If required, adjust using 1N NaOH or 1N HCl. Equilibrate the buffer to room temperature before use.

Sample buffer

Dilute the Sample buffer 1:2 with cell-culture grade water. Use the diluted Sample buffer for diluting GST control or samples, if required. The diluted buffer can be stored at 4°C for about one month. Ensure that the pH of the buffer is 6.5. If required, adjust using 1N NaOH or 1N HCl.

GST Standard

The control consists of a solution of Glutathione S-transferase enzyme from equine liver. The enzyme should be aliquoted in small quantities into vials to avoid repeated freeze-thaw cycles.

Glutathione, reduced (GSH)

Prepare 200mM solution of reduced glutathione in cell culture-grade water. The solution is stable for 24 hours when stored on ice. It is recommended that solutions be made freshly, as per the required quantity. For long term storage, aliquot the solution and store at –20 °C.

Note: Aqueous solutions of GSH get readily oxidized when exposed to air to form oxidized glutathione. Keep the solution container capped between use.

Substrate solution

The substrate is a solution of 1-Chloro-2,4-dinitrobenzene (CDNB). The solution is ready to be used as supplied.

2. Preparation of Samples:

Plasma

1. Collect blood using anticoagulant.
2. Centrifuge at 1900 - 2300rpm for 10 minutes at 4°C.
3. Collect the top yellow plasma layer without disturbing the lower layers.
4. Store plasma on ice. For long term storage (one month), freeze at -80°C.

Cell lysate

1. Collect 2 x 10^6 cells/ml in PBS.
2. Sonicate 3X for 5 second intervals at 40V setting over ice.
3. Centrifuge at about 2000rpm for 15 minutes at 4°C.
4. Use supernatant for the assay.
5. Store at -80°C if not assaying immediately.

Tissue homogenate

1. Rinse the tissue thoroughly in 1X PBS or saline to remove all traces of blood.
2. Homogenize the tissue in 5-10ml of cold diluted Sample buffer.
3. Centrifuge the tissue homogenate at 5000rpm for 5 minutes. Use the supernatant for the assay.
4. Store at -80°C if not assaying on the same day.

3. Assay Procedure:

1. The assay buffer and the substrate solution should be at room temperature before use.
2. Add 930µl assay buffer to a 1ml quartz cuvette. Using this as a blank solution, set the spectrophotometer to zero.
   Note: Wipe the external surface of cuvettes with lint-free tissue paper to minimize the handling error.
3. Add 10µl GSH and 10µl substrate solution to the cuvette. Mix by covering the cuvette with parafilm and inverting several times.
4. Read absorbance at 340nm every one minute to obtain 5-6 readings. These readings will be the Blank readings.
5. For positive control, add 4-6µl of GST standard to the above substrate mixture and mix thoroughly.

6. For test sample, add 50µl of test sample to the substrate mixture and mix thoroughly.
7. Read absorbance at 340nm every one minute to obtain 5-6 readings. These readings will be the Standard/Sample readings.
8. The procedure is summarized in Table 1.

Table 1: Assay procedure

	Positive Control	Test
Assay buffer	930µl	930µl
Set spectrophotometer reading to zero at 340nm.		
GSH	10µl	10µl
Substrate solution	10µl	10µl
Read absorbance at 340nm every 30sec to obtain 5-6 readings		
GST Standard	4-6µl	--
Sample	--	50µl
Read absorbance at 340nm every 30sec to obtain 5-6 readings		

Note: You can measure the absorbance using a microplate reader. Divide each volume quantity by five to obtain a total volume of 200µl. Follow the same procedure as given above.

7. Result Analysis:

1. Calculate change in absorbance (ΔA_{340}) per minute for Blank, Standard and Sample.

$$\Delta A_{340} / \min (\_) = \frac{A_{340}(\text{time } 2) - A_{340}(\text{time } 1)}{\text{time2(min)} - \text{time1(min)}}$$

2. Subtract ΔA_{340}/min of Standard and Sample from ΔA_{340}/min of Blank.

$$\Delta A_{340} / \min = \Delta A_{340} / \min (\text{standard/sample}) - \Delta A_{340} / \min (\text{blank})$$

3. Calculate GST activity

GST activity (µ*Mole/ml/min*) =

$$\frac{\Delta A_{340}/\min \times \text{reaction volume (ml)} \times \text{sample dilution factor}}{\text{ext coeff of GSH - DNB adduct} \times \text{sample volm added to well (ml)}}$$

Extinction coefficient for test in cuvette (path length = 1cm**): $9.6 mM^{-1}cm^{-1}$

Reaction volume = 1ml

Sample volume = 50µl

**For cuvette with a different path length, coefficient should be calculated as: 9.6 × path length in cm

GST activity

$$= \frac{\Delta A_{340}/\min \times 1}{9.6 \times 0.05 ml} \times \text{sample dilution factor}$$

8. Storage and Shelf life:

- On receipt, store the kit components at temperatures indicated on individual labels. (Refer section 3).
- Use before the expiry date given on the product label.

9. Advantages:

- **Easy reagent preparation**: Reagents of required concentration provided, requiring only simple dilutions for reagent preparation
- **Flexibility**: Different types of samples (serum, cells and tissue) can be analyzed.
- **Sensitivity and accuracy:** As the assay is based on an enzyme-substrate reaction, it is very sensitive. The GSH-CDNB reaction can be directly correlated to GST activity in the sample.
- **Compatibility with multiple instruments**: The absorbance can be read using a spectrophotometer or a microplate reader

10. Troubleshooting Points:

Problems	Possible Causes	Recommended Solutions
Erratic values; Dispersion of duplicates/triplicates	Pipetting errors	Do not splash contents of the well; equilibrate the pipette tips before pipetting each reagent
	Air bubbles formed in the tube/cuvette	Pipette gently against the wall of the tubes; remove the bubbles by gently tapping the side of the tube/cuvette
	Use of chilled assay buffer	Assay buffer must be at room temperature before use

	Samples prepared in different buffer	Use only the buffer provided in the kit for sample preparation
	Samples/Standard used after multiple freeze-thaw cycles	Aliquot samples/standard before freezing
	Use of old or inappropriately stored samples	Use fresh samples or store at correct temperatures
	Use of reagents diluted previously and stored for long durations	Prepare fresh dilutions of all reagents; refer to datasheet for storage of diluted reagents
Readings do not follow a linear pattern for Standard	Use of partially thawed components	Thaw all components to room temperature and mix gently before use
	Air bubbles formed in the tube/cuvette	Pipette gently against the wall of the tubes; remove the bubbles by gently tapping the side of the tube/cuvette
	Dilutions of standard stock not prepared correctly	Refer to the datasheet for dilutions of standards; equilibrate the pipette tips while pipetting out standard of each dilution
	Calculation errors	Recheck calculations after referring to the datasheet
	Substituting reagents from older kits/lots	Only use the components given in the kit
Reaction rate was too fast or too slow	Too much or too little enzyme was added to the tube/cuvette	Dilute or concentrate the sample with sample buffer and reassay

11. References:

1. Habig WH, Pabst MJ, Jakoby WB. Glutathione-S-Transferases. The First Enzymatic Step in Mercapturic Acid Formation. J. Biol. Chem. 249, 7130-7139 (1974).
2. Jakoby WB. The Glutathione-S-Transferases: A group of multifunctional detoxification proteins. Adv. Enzymol. Relat. Areas Mol. Biol. 46, 383-414 (1978).