Glutathione Assay Kit (GSH, GSSG and Total) (Catalog #K264-100; 100 assays; Store kit at -20oC)

I. Introduction:

Glutathione is the major intracellular low-molecular-weight thiol that plays a critical role in cellular defense against oxidative stress in tissues and cells. Commercially available glutathione detection kits, such as the DTNB-enzyme cycling glutathione assay kit or the Monochlorobimane based assay kit hardly distinguish between reduced glutathione (GSH; FW: 307) and oxidized glutathione (GSSG; FW: 612). BioVision's Glutathione Detection Kit provides a unique, convenient tool for detecting GSH, GSSG, and total glutathione individually. In the assay, OPA, reacts with GSH (not GSSG), generating fluorescence, so GSH can be specifically quantified. Adding a reducing agent converts GSSG to GSH, so (GSH + GSSG) can be determined. To measure GSSG specifically, a GSH Quencher is added to remove GSH, preventing reaction with OPA (while GSSG is unaffected). Reducing agent is then added to destroy excess quencher and to convert GSSG to GSH. Thus, GSSG can be specifically quantified. The kit provides a unique procedure and buffer formula to eliminate protein thiol interference and to stabilize GSH and GSSG in solution. The assay is easy to perform and detects 2-400 ng/µl of GSH, GSSG or total glutathione.

II. Kit Contents:

Kit Component	100 Assays	Cap Code	Part Number
Glutathione Assay Buffer	30 ml	WM	K264-100-1
PCA (Perchloric Acid, 6 N)	2 ml	Red	K264-100-2
KOH (6 N)	2 ml	Blue	K264-100-3
OPA Probe (o-phthalaldehyde)	0.2 ml	Brown	K264-100-4
Reducing Agent Mix	1 vial	Green	K264-100-5
GSH Quencher	20 µl	Purple	K264-100-6
GSH Standard (FW: 307)	1 mg	Yellow	K264-100-7

III. Reagent Reconstitution and Storage:

OPA Probe, Reducing Agent Mix, GSH Quencher: Add 0.85 ml H2O to the OPA probe, mix. Dissolve both the Reducing Agent and the GSH Quencher in 1.05 ml of dH2O separately. Store at –20oC. Use within two months.

GSH Standard: Accurately dissolve in 45 l dH2O and add 5 µl PCA to stabilize the standard GSH stock solution (20 µg/µl). Store at –20oC. Use within two months.

IV. Sample Collection and Storage*:

Tissue and Cell Samples:

GSH is labile and cell preparations will oxidize it rapidly. Keep all samples and reagents ice cold and work as rapidly as possible. Prepare centrifuge tubes with 20 µl PCA on ice to receive samples. Homogenize 2-4 x106 cells or 40 mg tissue on ice with 100 µl of ice cold Glutathione Assay Buffer. Take 60 µl of each homogenate to a prechilled tube containing PCA and vortex several seconds to achieve a uniform emulsion. Keep on ice for 5 min. Spin 2 min at 13,000 G at 4oC, collect supernatant (containing glutathione) and discard the protein pellet. The sample can then be stored at –80oC, stable for a month.

Serum or Other Liquid Samples:
Freeze samples immediately upon acquisition and keep frozen until ready for processing. Take 60 □l thawed sample to centrifuge tube containing 20 µl ice cold PCA, vortex and keep on ice for 5 min. Spin for 2 min at 13,000 G at 4oC. Collect the supernatant. The sample can then be stored at –80oC, stable for a month.

V. Assay Protocol:
1. Standard Curve: Add 10 µl of the 20 µg/µl standard GSH stock to 990 µl of Assay Buffer to generate a 0.2 µg/µl working standard solution. Add 0, 2, 4, 6, 8, 10 µl to a 96-well plate to generate 0, 0.4, 0.8, 1.2, 1.6, 2.0 µg/well GSH. Bring the volume to 90 µl with Assay Buffer.
Note: If the concentration of your assay samples is lower than the above standard range, the standard can be further diluted 10-fold to generate 0, 40, 80, 120, 160, 200 ng/well GSH by following the same procedure.

2. Preparation of Samples for Assays: Add 20 µl of ice cold 3N KOH to 40 µl of PCA preserved samples (as prepared in Section IV) to precipitate PCA and neutralize the samples (pH should be 5-10). Keep on ice for 5 min then spin 2 min at 13,000 G at 4oC. Transfer 10 µl of the neutralized samples to a 96-well plate. You may choose to add several dilutions (e.g., 1-10 µl) of your samples to ensure the readings are within the standard curve range.

A. To Detect GSH: Bring the sample volume to 90 µl with Assay Buffer.

B. To Detect Total Glutathione: Bring the sample well to 80 µl with Assay Buffer. Do a background control without sample. Add 10 µl of Reducing Agent Mix to the wells, mix well, incubate at room temperature for 10 min to convert GSSG to GSH.

C. To Detect GSSG: Bring the sample well volume to 70 µl with Assay Buffer. Do a background control without sample. Add 10 µl of GSH Quencher, mix well, and incubate at room temperature for 10 min to quench GSH. Then add 10 µl of Reducing Agent Mix to destroy the excess GSH Quencher and convert GSSG to GSH.
3. Assay: Add 10 µl of OPA Probe into the standard and sample wells, mix well, incubate at room temperature for 40 min. Read samples and standards on a fluorescence plate reader equipped with Ex/Em = 340/420 nm. We suggest adjusting the plate reader settings so that the background reading without glutathione is at about 50-150 RFU.
4. Calculations: Subtract background reading from sample readings. Plot RFU vs GSH standard. Apply the sample readings to the standard curve to get glutathione amount in each sample.
Glutathione Concentration = Ga/Sv
Where Ga: Glutathione amount from standard curve.
Sv: Sample volume added to the sample wells (Note: The original samples have been diluted 2-fold by performing the procedures above).

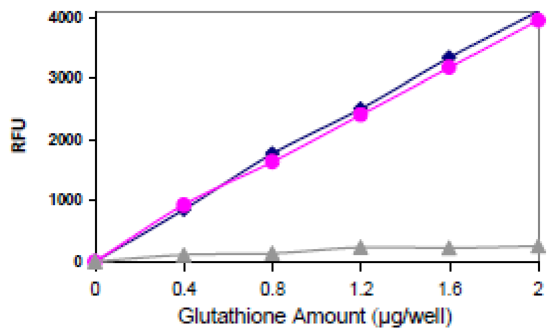

Fig 1. Glutathione assays were prepared using standard GSH and GSSG following kit instructions. Diamond (blue) represents GSH standard curve. Circle (pink) represents standard curve produced by adding GSSG + GSH Quencher + Reducing Agent Mix. Triangle (gray) represents GSSG standard without adding Reducing Agent mix.

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer • Omission of a step in the protocol • Plate read at incorrect wavelength • Use of a different 96-well plate	• Assay buffer must be at room temperature • Refer and follow the data sheet precisely • Check the wavelength in the data sheet and the filter settings of the instrument • Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type • Samples prepared in a different buffer • Samples were not deproteinized (if indicated in datasheet) • Cell/ tissue samples were not completely homogenized • Samples used after multiple free-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples	• Refer data sheet for details about incompatible samples • Use the assay buffer provided in the kit or refer data sheet for instructions • Use the 10 kDa spin cut-off filter or PCA precipitation as indicated • Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope • Aliquot and freeze samples if needed to use multiple times • Troubleshoot if needed, deproteinize samples • Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used	• Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer datasheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components • Pipetting errors in the standard • Pipetting errors in the reaction mix • Air bubbles formed in well • Standard stock is at an incorrect concentration • Calculation errors • Substituting reagents from older kits/ lots	• Thaw and resuspend all components before preparing the reaction mix • Avoid pipetting small volumes • Prepare a master reaction mix whenever possible • Pipette gently against the wall of the tubes • Always refer the dilutions in the data sheet • Recheck calculations after referring the data sheet • Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength • Samples contain interfering substances • Use of incompatible sample type • Sample readings above/below the linear range	• Check the equipment and the filter setting • Troubleshoot if it interferes with the kit • Refer data sheet to check if sample is compatible with the kit or optimization is needed • 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.		