IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

FkGSH-01

Patent pending

FLUORESCENT GLUTATHIONE ASSAY KIT 96 well

Glutathione (GSH) is a tripeptide (γ-Glu-Cys-Gly) present at very high concentrations throughout living organisms (from 1 to 10 mM), and exists in three major forms: reduced sulphydryl (GSH), glutathione disulfide (GSSG) or bound to Cys residues in proteins (PSSG).

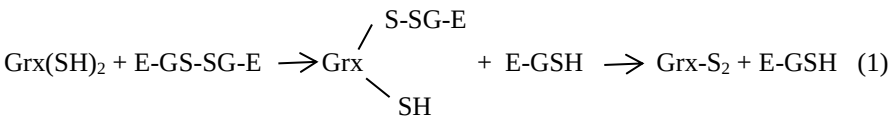
From its first described role in heavy metals and xenobiotics detoxification, it is now known that GSH plays a key role in cell redox homeostasis, gene expression, cell proliferation and apoptosis mainly by binding to Cys residues in protein, known as protein glutathionylation (PSSG). GSH is used by glutaredoxin (Grx) to reduce disulfides. This reaction is usually coupled to the NADPH driven reduction og GSSG by glutathione reductase (GR).

The ratio GSH/GSSG has been used as an indicator of the redox level in cells but this parameter can also be estimated by the quantification of PSSG. In fact, PSSG has the advantage of being more stable than GSSG.

Within mammalian cells, glutathione exists mainly (>98%) in the thiol-reduced form (GSH). In fact the oxidized form, GSSG, is toxic in the cytosol and it is either reduced by glutathione reductase or exported from cells when its cytosolic concentration increases. Vice versa in plasma glutathione is present mainly as the oxidized form both as GSSG and mixed disulfides (Cysteinyl-glutathione and PSSG).

This kit is designed to measure the amount of total glutathione (GSH+GSSG) in cells and tissue homogenates and the total amount (including GSSC and protein mixed disulfides with glutathione) in plasma [1]. The assay is simple and sensitive based on eosinGSH that emits a strong fluorescent signal detectable at 545nm after excitation at 520nm.

The fluorescent substrate, Di-E-GSSG, is cleaved by Grx. This generates two molecules of E-GSH increasing the fluorescence emission coupled to oxidation of the active site in Grx (reaction 1-3).



Please read this manual thoroughly before using the kit. The reagents will suffice to measure 100 wells. Some of the reagents needed to prepare the samples are not included in this kit.

IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

Table of Contents

KIT CONTENT

Required for the assay (but not supplied)

1. Fluorescence micro plate reader with 520 nm excitation / 545 nm emission like Perkin Elmer EnSpire.
2. 96 black micro titer plates.
3. Sulfosalicylic acid, Triton-X-100, 2-vinylpyridine, triethanolamine (for sample preparation).
4. Distilled water.

Supplied components:

Assay buffer (KE buffer) x2 - white cap

Potassium phosphate, pH 7.5 and EDTA.

Preparation procedure:

Dissolve the content of each tube with 1 ml distilled water, yielding 1 M potassium phosphate pH 7.5 and 10 mM EDTA (1 M KE buffer).

Store at -20°C.

Reduced glutathione (GSH) – yellow cap

Lyophilized GSH.

Preparation procedure:

Dissolve the content with 50 µl distilled water yielding 0.1 M GSH.

Store at -20°C.

Make a 10 µM GSH aliquot from the 0.1 M GSH stock using distilled water.

Not stabile in low concentrations, prepare fresh before each assay

IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

Assay stabilizing reagent – transparent cap

Lyophilized stabilizing reagent.

Preparation procedure:

Dissolve the content with 100 µl distilled water to make 10 mg/ml

Store at -20°C.

β-NADPH – purple cap

Lyophilized reduced β-NADPH containing stabilizing reagents.

Preparation procedure:

Dissolve the content with 50 µl distilled water, yielding 40 mg/ml reduced β-NADPH.

Store at -20°C. Not stabile for long durations once diluted.

Baker yeast glutathione reductase (GR) – brown cap

20 µl of 50 µM glutathione reductase in 50 percent glycerol, 75 mM Tris-Cl, 1 mM EDTA, pH 7.5.

Preparation procedure:

Add 30 µl of 10 times diluted Assay buffer to make 50 µl of 20 µM glutathione reductase.

Cap and shake the tube. For long term use make aliquots before dilution.

Store at -20°C.

Human Glutaredoxin 1 (hGrx-1) – blue cap

156 µg recombinant hGrx-1 lyophilized from 10 µl of 50 mM Tris-HCl, 1 mM EDTA, pH 7.5 (TE).

Preparation procedure:

Dissolve the content with 100 µl distilled water yielding 1.56 mg/ml (130 µM) of hGrx-1.

Store at -20°C.

For long term use make aliquots.

Fluorescent substrate – green cap

Lyophilized fluorescent substrate.

Preparation procedure:

Dissolve the content in 1.0 ml distilled water yielding 200µM of fluorescent substrate.

Dilute to 40µM before use in assay.

Store at -20°C.

IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

SUGGESTED PREPARATION OF SAMPLES

Plasma/Serum

To obtain plasma, collect blood using anticoagulant, centrifuge 1000xg for 10 min at 4°C and take the supernatant.

To obtain serum, collect blood *without* anticoagulant, leave the sample at room temperature for 30 min to allow the blood to clot, centrifuge 2000xg 15 min at 4°C and take the supernatant.

Plasma and serum can be used directly; we recommend to use 5-30 µl plasma in the assay and adjust the volume to fit your sample to the standard curve.

The assay can be optimized for 384 well plates using 3-5µl of plasma or serum samples.

Tissues and Cells

Cell preparation:

1. Collect cells (1-10 x 10^6) and centrifuge at 1000xg for 10 min. Scrape the cells if adherent rather than use trypsin.
2. Remove supernatant; wash the cell pellet with 1 ml PBS save 10µl to perform protein determination and centrifuge at 1000xg for 5 min.
3. Remove the supernatant. From now on the samples should be kept in ice. Dissolve the pellet in 0.3ml 0.1% Triton-X-100 and 2% sulfosalicylic acid (fresh prepared) in 0.1 M KE pH 7.5 to lyse the cells and sonicate with 10 burst for 10 sec.
4. Centrifuge 10000xg for 10 min at 4°C and collect the supernatant.
5. The acidified cell lysate supernatants can be divided in two and used directly for total-GSH or derivatized for GSSG assay following the Rahman *et al* sample preparation [2]. Importantly for GSSG measurement the samples must be fast mixed with vinylpyridine (4µl of 1:10 diluted vinylpyridine in 0.1 KE for 100µl of supernatant). After vortexing for 15s, samples are incubated for 2h at RT in a fume hood. To inactivate non-reacted vinylpyridine add 6 µl of triethanolamine (1:6 diluted in KE buffer) and incubate 10 min.

Tissue preparation:

1. Homogenize the tissue sample in 0.1% Triton-X in 0.1 M KE buffer pH 7.5 ice cold.
2. Centrifuge at 1000xg for 5 min in 4°C to remove the debris. Collect supernatant and save 10µl to perform protein determination.
3. Add 2% sulfosalicylic acid (fresh prepared) and centrifuge at 1000xg for 10 min in 4°C. Collect the supernatant.
4. The acidified supernatants can be divided in two and used directly for total-GSH or derivatized for GSSG assay following the Rahman *et al* sample preparation [2]. Importantly for GSSG measurement the samples must be fast mixed with vinylpyridine (4µl of 1:10 diluted vinylpyridine in 0.1 KE for 100µl of supernatant). After vortexing for 15s, samples are incubated for 2h at RT in a fume hood. To inactivate non-reacted vinylpyridine add 6 µl of triethanolamine (1:6 diluted in KE buffer) and incubate 10 min.

We recommend to measure the protein concentrations of all your non-plasma samples and make diluted aliquots of supernatant corresponding to 2µg/µl protein to use in the assay. This makes it easier to fit the samples in the standard curve and to calculate [GSH] / [protein].

IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

GLUTATHIONE ASSAY

General information

- The final volume of the assay is 100 µl per well and performed at room temperature.
- The final assay should be carried out in triplicate to increase accuracy but it is the user's choice.
- Record the emission at 545 nm after excitation at 520 nm for 20-60 minutes

We recommend as first step to check that samples fit within your standard curve.

a) For cell samples we recommend about 1-5 µg of total protein.
b) For plasma samples we recommend using 10-30 µl pure plasma.

If a sample is not within the standard curve, dilute the samples accordingly.

Assay procedure

1. Prepare MASTER MIX (for 9+1wells).

	10 wells	**Master Mix concentration**	**Final concentration in well**
Assay buffer	181 µl	0.9 M KE	0.18 M KE
Stabilizing reagent	5 µl	0.25 mg/ml	0.05 mg/ml
NADPH 40mg/ml	5 µl	1 mg/ml	0.2 mg/ml
GR 20µM	5 µl	0.5 µM	0.1 µM
hGrx-1 130µM	4 µl	2.6 µM	0.52 µM

IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

2. To execute the GSH assay, follow the protocol suggested in table 1.

Well	1	2	3	4	5	6	Sample	Sample Background
Final [GSH]	**Blank**	**0.2 µM**	**0.4 µM**	**0.8 µM**	**1.6 µM**	**3.2 µM**	**-**	**-**
MASTER MIX	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	20 µl	-
Distilled water	55 µl	53 µl	51 µl	47 µl	39 µl	23 µl	55 - x µl	55 - x µl
GSH 10µM	-	2 µl	4 µl	8 µl	16 µl	32 µl	-	-
Assay Buffer	-	-	-	-	-	-	-	20 µl
Sample	-	-	-	-	-	-	x µl	x µl

1. Incubate for 10 min to ensure all GSSG is reduced to GSH.
2. Add 25 µl of 40 µM fluorescent substrate to each well.
3. Make sure to shake the plate properly (for ca 30 seconds) - If the substrate is not fully mixed it will make the first 5-10 minutes unusable.
4. Record the emission at 545 nm after excitation at 520 nm for maximum 60 minutes.

IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

DATA TREATMENT AND CALCULATION OF RESULTS

1. Select the linear range of the standard curve (for example 10-30 minutes) and the same range for the samples to determine rate of reaction (Δfluorescence per minute).
2. Calculate the rate of reaction as ΔFluorescence/ΔTime, meaning the slope of the curve.
3. Subtract all samples' slopes with their corresponding background's slopes
4. If you have duplicates or triplicates, calculate the mean values.
5. Using the standard curve equation extrapolate the amount of GSH in samples.
6. Normalize for amount of protein considering every dilution done to the sample.

TIPS:

Plasma and serum samples can have a very different background activity and can be influenced by the healthy condition of the blood donor and other variables. To obtain maximum accuracy when calculating the concentration of your samples we recommend to measure the same sample with three different concentrations, like using 5-10-20 µl of plasma to get a linear correlation (if all of them are within the standard curve) that you can use when you are calculating your results. Plasma and serum samples with visible sign of hemolysis should be discarded and are unreliable.

To calculate the concentration of GSH:
- Run 5-10-20µl sample
- Plot sample slope against the volume used for each sample and calculate the **intercept** of the trend line. The three different measures should be aligned.
- ***GSH* c*oncentration in well = (Sample slope –intercept) / Standard curve slope***

Normalize for amount of protein and consider every dilution done in the process.

IMCO Corporation Ltd AB
PO Box 21195
SE -100 31 Stockholm
Sweden
www.imcocorp.se

Figure 1. Typical standard curve

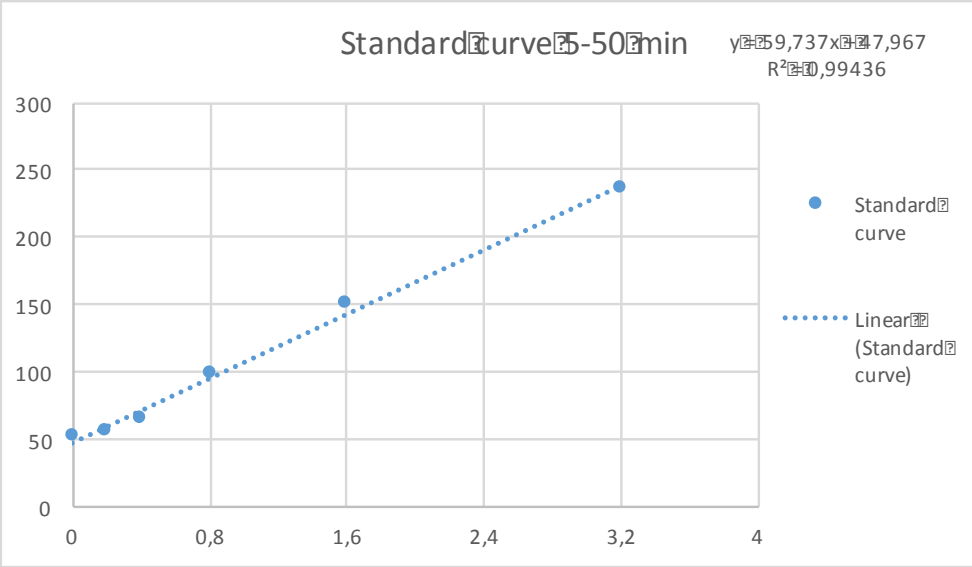


Fig 1. Typical glutathione standard curve recorded at 545 nm emission after 520 nm excitation.

Slope (ΔFluorescence/minute) between 5 and 50 minutes plotted against GSH content for the corresponding well.

Note: The y-axis values may vary depending on the fluorescent plate reader.

REFERENCES

1. Coppo, L., Ogata, F. T., Santhosh, S. M., Sventelius, T., & Holmgren, A. (2019). Enzymatic glutaredoxin-dependent method to determine glutathione and protein S-glutathionylation using fluorescent eosin-glutathione. *Analytical biochemistry*, *568*, 24-30.
2. Rahman, I., Kode, A., & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature protocols*, *1*(6), 3159.