

A Highly Sensitive Fluorimetric Assay for Thiol Quantification

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Introduction

Thiols play important roles in biochemistry, both as components of protein structures and as metabolic intermediates. The most abundant cellular thiol, reduced glutathione (GSH), is a natural reservoir of the reductive capacity of a cell. It functions as a component of the intracellular and extracellular redox buffer combating oxidative stress. Here we report the development of a novel fluorimetric assay for thiol detection using fluorescence resonance energy transfer (FRET) techniques. We designed and developed a new thiol quantitation assay kit using a detection reagent labeled with a quencher, QXL™ 520, and a fluorophore, 5-carboxyfluorescein (5-FAM). Upon reaction of the detection reagent with thiol, fluorescence is released and can be monitored at excitation/emission=490/520 nm. Increase in fluorescence is proportional to the thiol concentration. The assay features a simple "add-mix-measure" protocol and is sensitive with a linear range of 0.04-160 μM for GSH. Common contaminants, such as DMSO and Triton X-100, are well tolerated in the assay. The thiol detection reagent was also validated with cell lysates for determination of total GSH.

Assay Principle

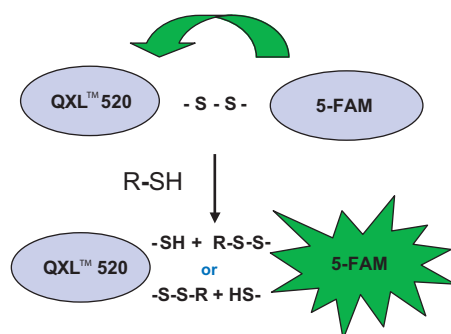


Figure 1. FRET-based principle of SensoLyte® 520 Thiol Quantitation Kit. Thiol Detection Reagent provided in the kit carries 5-FAM/QXL™ 520 FRET pair. Fluorescence of 5-FAM is quenched by QXL™ 520. Upon the reaction with thiols fluorescence of 5-FAM is released and can be monitored at Ex/Em=490/520 nm.

Materials and Methods

- SensoLyte® 520 Thiol Quantitation Assay Kit (Cat# 72138)
 - ✓ Thiol Detection Reagent - synthesized using 5-FAM and QXL™ 520 acid purified by HPLC and characterized by UV-Vis, MALDI-MS and LC-MS
 - ✓ Assay Buffer
 - ✓ Reduced GSH
- DTT, Homocysteine (Hcy), DMSO, Triton-X-100, NADPH, Glutathione Reductase (GR) and DTNB (Sigma, St. Louis, MO)

SensoLyte® 520 Thiol Quantitation Assay Kit was used as recommended by the protocol. The reaction volumes were 10 μl of thiol containing sample and 90 μl of Thiol Detection Reagent. Assays were done in 96-well black opaque plates. Fluorescence was measured using FlexStation 384II (Molecular Devices, Sunnyvale, CA)

Results

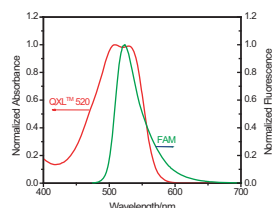


Figure 2. 5-FAM and QXL™ 520 is a new donor - acceptor pair for Thiol FRET reagents. The absorption spectrum of QXL™ 520 overlaps with the emission spectrum of 5-FAM. Hydrophilicity of QXL™ 520 results in better solubility of the peptide substrate.

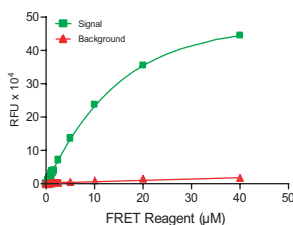


Figure 3. Dose response of Thiol Detection Reagent. Fluorescence was measured at 30 min after incubation of GSH with a range of concentrations of Thiol Detection Reagent.

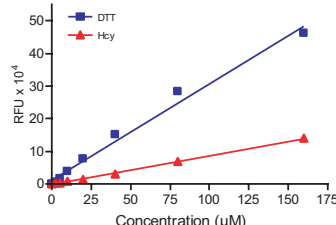


Figure 4. Validation of Thiol Detection Reagent. Fluorescence was measured at 30 min after incubation of Thiol Detection Reagent with serial dilution of DTT, and Hcy.

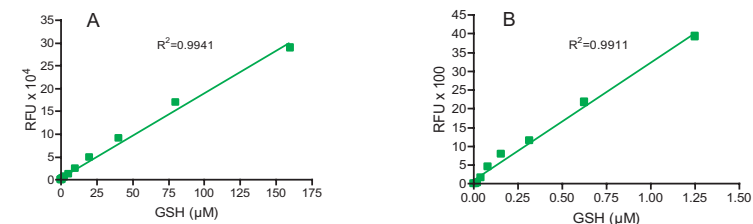


Figure 5. GSH titration with Thiol Detection Reagent. Sensitivity of assay at 30 min incubation was 39 nM. A. Linearity and sensitivity of SensoLyte® 520 Thiol Quantitation Assay Kit; B. Detection of lower thiol concentrations.

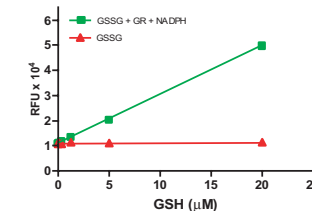


Figure 6. Measurement of converted GSH. Oxidized GSH (GSSG) is first converted to reduced GSH by GR and NADPH. Reduced GSH is then determined at 30 min after incubation with Thiol Detection Reagent.

Table 1. Contaminants Tolerance

Contaminant	Concentration in assay
DMSO	2.5%
Triton X-100	0.5%

*Tolerance was defined as less than 10% perturbation

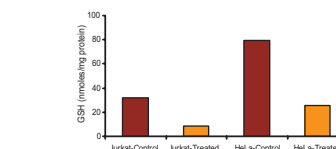


Figure 7. GSH quantification in apoptotic vs non-apoptotic cells. Cells were treated with staurosporine for 8-24 hrs. Cell lysates were deproteinized and then measured for total GSH in the presence of GR and NADPH.

Table 2. Comparison with DTNB*

Reagent	Read-out	Linear Range (μM)
Thiol FRET	Ex/Em=490/520 nm	0.04 - 160
DTNB	OD=405 nm	1.5 - 100

*DTNB, Ellman's Reagent [5, 5'-dithiobis (2-nitrobenzoic acid)]

Conclusions

- We have developed the novel SensoLyte® 520 Thiol Quantitation Assay Kit, based on FRET principle.
- The SensoLyte® 520 Thiol Quantitation Assay Kit detects picomole amounts of thiols using convenient homogenous format.
- Thiol FRET Detection Reagent provided in the kit was validated for use with different thiols and biological samples.