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FRONTIERS IN LIFE SCIENCE

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CellFluor™ GST <Cell-based GST Activity Assay Reagent>

Catalog NO. FDV-0030

Product Background

Glutathione *S*-Transferases (GSTs) are widely conserved in nature from bacteria to plants and animals. In human, over 20 members are identified and classified into three categories: cytosolic, mitochondrial, and membrane-bound microsomal members. Cytosolic GSTs consist of 6 subfamilies including α (GSTA), μ (GSTM), π (GSTP), θ (GSTT), ο (GSTO) and ζ (GSTZ). Mitochondrial member is κ (GSTK) and microsomal members are MGSTs and membrane associated proteins in eicosanoid and glutathione metabolism (MAPEGs). GSTs are phase-II detoxification enzymes and commonly play an important role in detoxification of hydrophobic and electrophilic compounds including endogenous toxic metabolites or xenobiotics by conjugating with glutathione (GSH) to produce glutathione-conjugate (GS-conjugates) (Figure 1). Generally GSTs have two types of substrate-binding site, called G-site and H-site, for GSH and hydrophobic substrate (xenobiotics), respectively. When GSTs bind to GSH as the first substrate, GSTs catalyze and stabilize thiol group of GSH as a thiolate anion. Once hydrophobic and electrophilic xenobiotics bind to GSTs as the second substrate, GSTs transfer them to GSH to form GS-conjugates. GS-conjugates are released from GSTs and quickly exported to extracellular space by multidrug resistance-associated protein (MRP) transporters. Through the above processes, GSTs detoxify toxic compounds.

As many studies suggested expression level of GSTs are significantly increased in cancer cells, GSTs are considered as anticancer drug-resistant enzymes in malignant cancer cells through the neutralization of drugs. To understand biological functions of GSTs, research tools for monitoring GST activity are very important. Although several reagents including classical GST substrate CDNB (1-chrolo-di-nitrobenzen) for this purpose have been developed, the probes which can be applied into measurement of intracellular GST activity are highly limited. To physiological function of GSTs, the tool for live cell-based GST activity assay is desired.

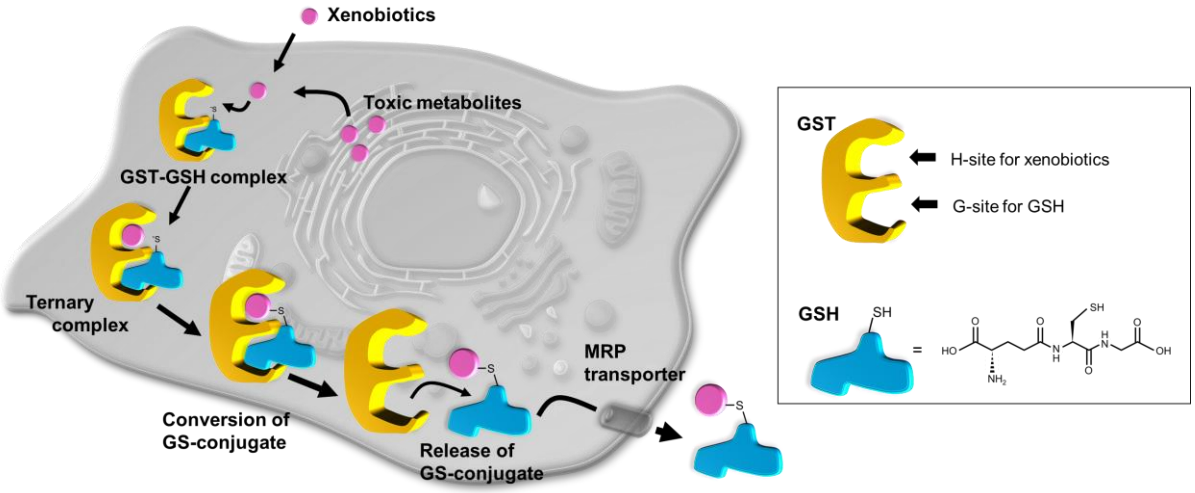


Figure 1. Overview of the detoxification process of GSTs

CellFluor™ GST, another name DNs-Rh or bis-DNs-Rh (ref.1-4), is a Rhodamine 110 derivative which protected by DNBs (**d**i**n**itro**b**enzene**s**ulfonamide). This probe shows very low fluorescence (quantum yield = 0.0007). After deprotected by GSTs via coupling of DNB-glutathione conjugates, Rhodamine 110 was released and emits strong green fluorescence (quantum yield = 0.645, S/N ratio ~900). An important advantage of **CellFluor™ GST** is high cell-permeability and this probe can measure intracellular GST activity by green fluorescent intensity under live cell condition. As the DNB group is a well-characterized substrate for various types of GST members, **CellFluor™ GST** is able to monitor pan-GST activity at least including 6 cytosolic GST subfamilies and MGST family. As only addition of **CellFluor™ GST** to cell culture medium is required to monitor intracellular GST activity, flexible applications are available. **CellFluor™ GST** is a powerful tool not only to investigate physiological GST activities in live cell upon any biological stimulation, but also to develop GST inhibitors under live cell condition.

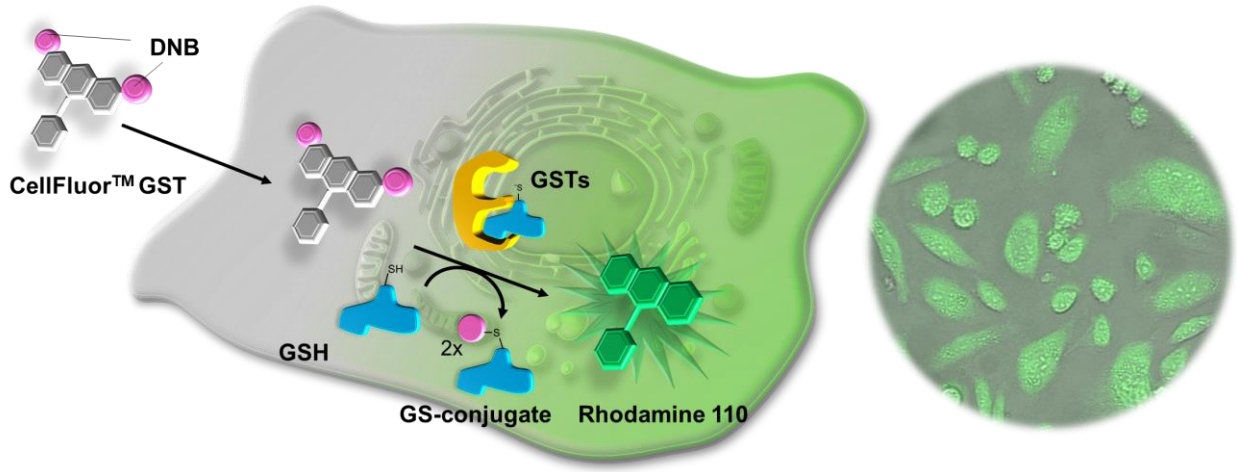


Figure 2 Principle of cell-based GST sensing of CellFluor™ GST

Description

Catalog Number: FDV-0030
Size : 0.1 µmol
Formulation : $C_{32}H_{18}N_6O_{15}S_2$
Molecular weight : 790.6 g/mol
Solubility : Soluble in DMSO
Ex/Em: 496/520 nm (After conversion to Rhodamine 110)
*Commercial FITC filter sets are available.

Application

- *in cellulo* GST activity assay in live cells (Fluorescent plate leader, fluorescent imaging or flow cytometry)
- *in vitro* GST activity assay

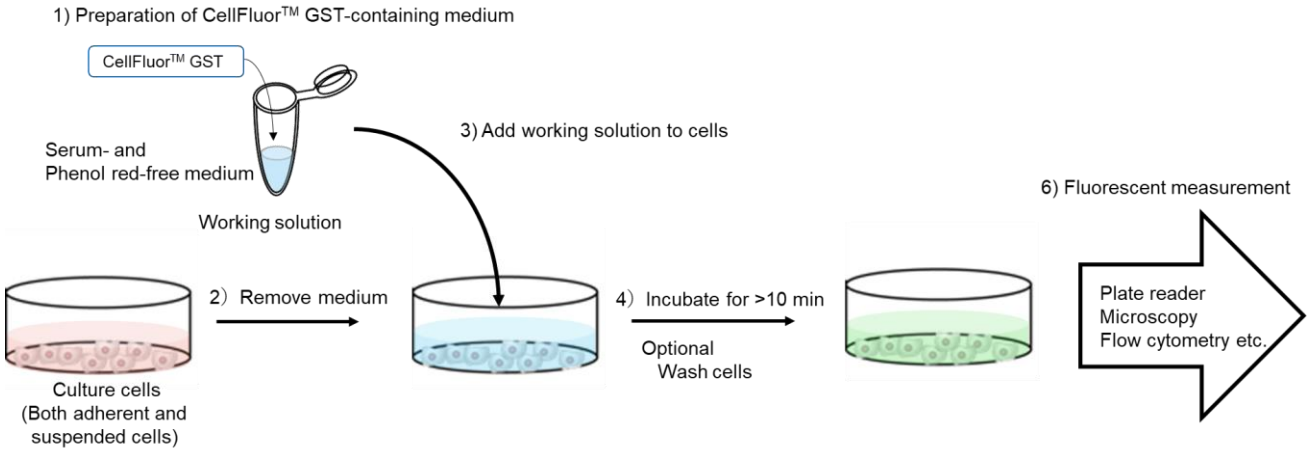
Reconstitution and Storage

Reconstitution : Add 100 µL of 100% DMSO into vial to prepare 1 mM stock solution.
Storage (solution) :
Store powder at -20°C.
After reconstitution in DMSO, aliquot and store at -20 °C. Avoid repeated freeze-thaw cycles.

How to use

General procedure for detection of intracellular GST activities

1. Prepare 1-30 μM **CellFluor™ GST** in serum- and phenol red-free medium or adequate buffers such as HEPES-buffered saline (HBS; *e.g.* 20 mM HEPES (pH 7.4), 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$).
   NOTE: Please optimize concentration of reagent and reaction medium for your experiments.
2. Remove culture medium
3. Add **CellFluor™ GST**-containing working solution prepared in step-1 to cells
4. Incubate cells for >10 min
   NOTE: Empirically optimize reaction time for your experiments.
5. Measure fluorescent intensity by the fluorescent plate reader or flow cytometry without any wash step.
   In the case of fluorescent imaging, cells are wash by PBS etc and quickly observed by fluorescent microscopies.



General procedure for *in vitro* GST activity assay

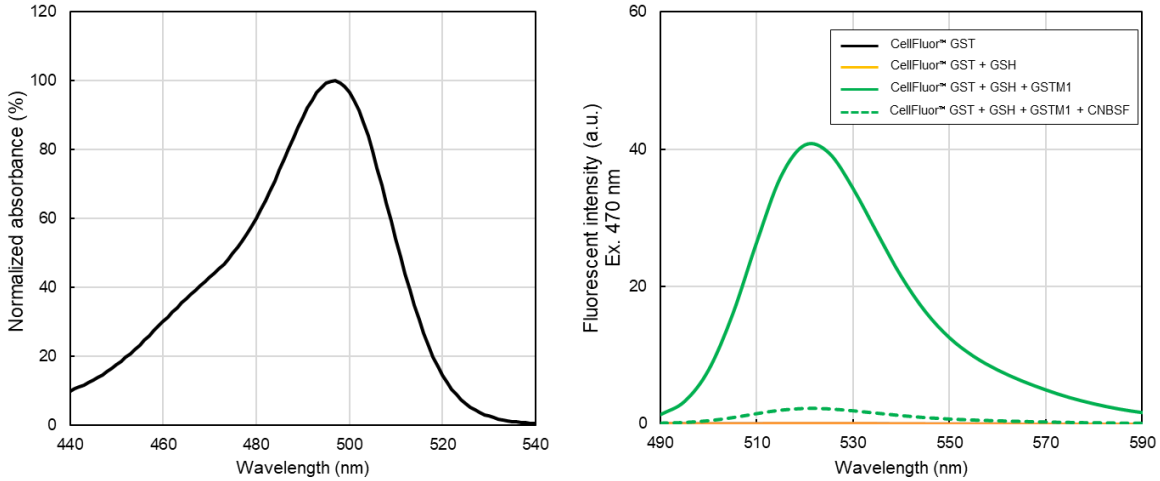
1. Prepare assay solution containing **CellFluor™ GST** (1-10 μM), GSH (10-100 μM) and any GST-samples such as cell/tissue lysates, purified enzymes etc.
   NOTE: Assay buffer depends on experiments. Buffer condition should be optimized for your experiments.
2. Incubate for 30-60 min and measure fluorescent intensity (maximum Ex/Em 496/520 nm).
   NOTE: Over 60 min incubation is not recommended. **CellFluor™ GST** slightly reacts with free-thiol group including GSH but reaction efficiency to free-thiol is much lower than that to GSTs. As *in vitro* GST activity assay requires addition of exogenous GSH, fluorescent background by exogenous GSH may be detected under long-term incubation. The measurement of a simple mixture of **CellFluor™ GST** and GSH without GST-containing samples is highly recommended as a negative control.

Reference data

Absorbance spectrum of Rhodamine 110 and GST-dependent fluorescent spectrum of CellFluor™ GST

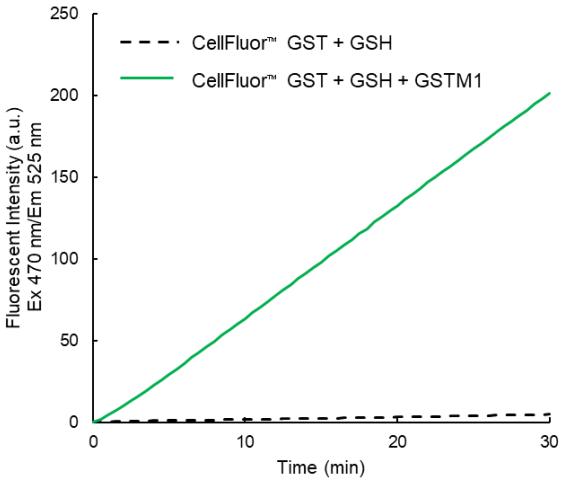
Left: Normalized absorbance spectrum of Rhodamine 110. Maximum absorbance ~ 495 nm

Right: Fluorescent spectrum of **CellFluor™ GST** with or without GSH and recombinant GSTM1 enzyme. Fluorescent spectrum of **CellFluor™ GST** (1 µM) with or without GSH, recombinant GSTM1, and CNBSF, a GST inhibitor, excited at 470 nm in phosphate buffer (50 mM sodium phosphate (pH 7.4), 150 mM NaCl) was measured. While **CellFluor™ GST** only and in the presence of 10 µM GSH showed little fluorescent intensity, GSTM1 clearly increased fluorescent intensity (maximum ~525 nm). When GSTM1 was pre-incubated with GSH and CNBSF (a GST irreversible inhibitor), fluorescent intensity was dramatically suppressed.



Kinetic measurement of GST activity *in vitro*

Fluorescent intensity (Ex 470 nm/Em 525 nm) in the presence and absence of human recombinant GSTM1 (0.2 mg/ml) in assay buffer (50 mM sodium phosphate (pH 7.4), 150 mM NaCl) containing 5 µM **CellFluor™ GST** and 10 µM GSH was measured over time for 30 min. Whereas slow fluorescence increased was observed under the condition without GSTM1, in the presence of GSTM1 fluorescent intensity was remarkably increased and a linear increase in signal was observed in the 30 min range.

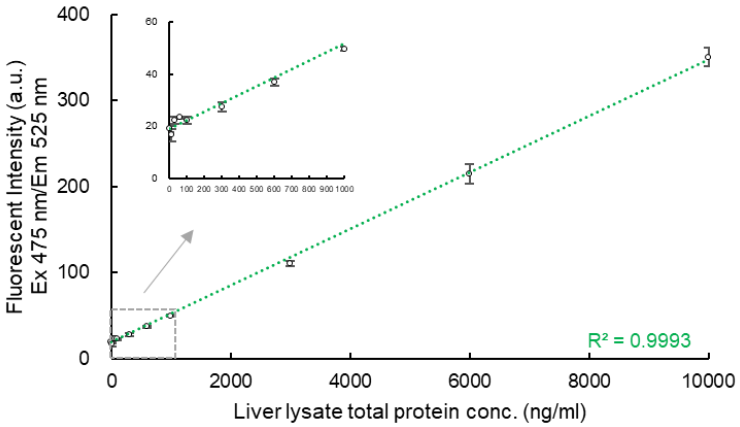


NOTE-1: The other cytosolic GST submembers including GSTA1, GSTP1, GSTO1, GSTT1, GSTZ1 showed similar results.

NOTE-2: As **CellFluor™ GST** could react with thiol group weakly. In *in vitro* assay, **CellFluor™ GST** assay needs reduced GSH as a cofactor of GST. High concentration of reduced GSH may be background signal of **CellFluor™ GST** probe. Please empirically optimize concentration of GSH and setting of adequate negative control experiments is highly recommended.

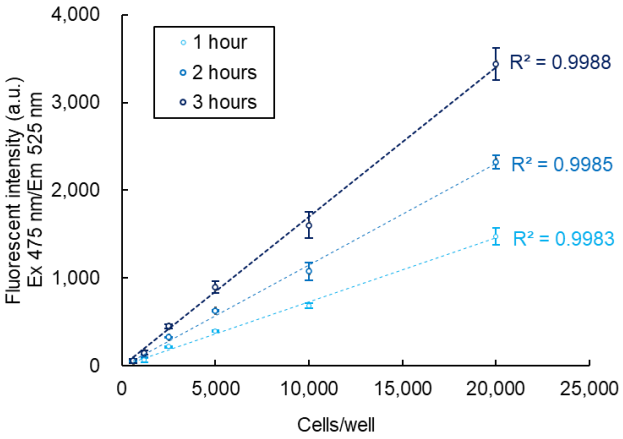
Dose-dependent fluorescent response of CellFluor™ GST using liver lysate

Mouse liver lysate was added to assay buffer (50 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% NP-40, 100 µM GSH) to be a total protein concentration of 10-10,000 ng/ml. **CellFluor™ GST** (final conc. 1 µM) was added to them and incubated for 30 min at RT. Fluorescent intensity (Ex 475±5 nm/Em 525±10 nm) was measured by a fluorescent plate reader. A total protein dose-dependent fluorescence response was confirmed with high correlation coefficient (R^2=0.9996). The detection limit in this experiment was estimated to be around 100 ng/ml liver total protein. When a conventional CDNB assay (GSH 1 mM, CDNB 1 mM) was performed using the same liver samples. Then, the detection limit was around 1000 ng/ml.



Cell number-dependent fluorescent response of CellFluor™ GST in cell-based assay

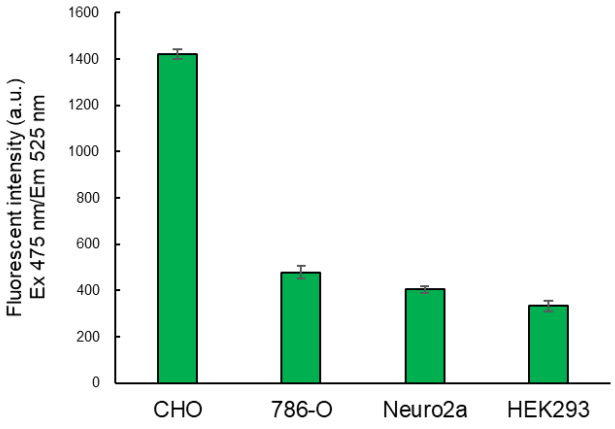
CHO cells were seeded in 96 well plate in 2x dilution series from 2 x 10^5 cell/well and cultured in DMEM containing 10% FBS for 4 hours. After cell adhesion was confirmed, the culture media were replaced with serum- and phenol red-free DMEM containing 2 µM **CellFluor™ GST**. Fluorescent intensities were measured every hour with a fluorescent plate reader (Ex 475±5 nm/ Em 525±10 nm). Time-dependency of fluorescent response was observed, and fluorescent intensity was highly correlated with cell number at all reaction times. The detection limit was estimated to be about 300 cell/well in this experiment.



Application data

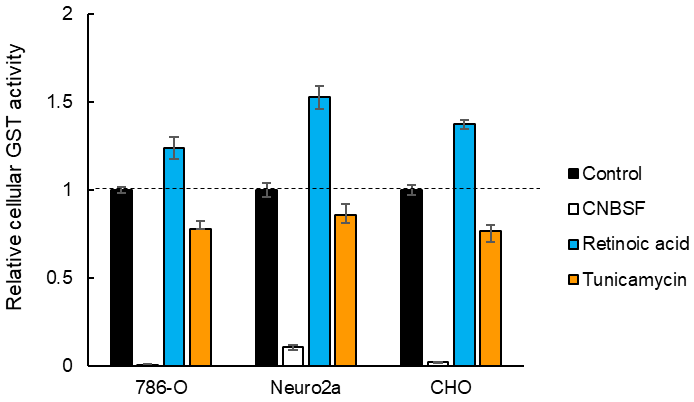
Cell-based comparison of GST activities

Four cell line (Chinese hamster ovarian cancer cell line CHO, human renal cancer cell line 786-O, mouse neuroblastoma cell line Neuro2a, and human fetal kidney cell line HEK293) were seeded in 96 well plate at 1 x 10^4 cell/well and cultured in DMEM containing 10% FBS for 20 hours. Then, the medium was replaced with serum-free and phenol red-free DMEM containing 2 µM **CellFluor™ GST** and cells were incubated for 1 hour. Fluorescent intensities (Ex 475±5 nm/ Em 525±10 nm) was measured with a fluorescence plate reader without medium exchange. Among the four cell lines tested here, CHO cells showed the highest GST activities.



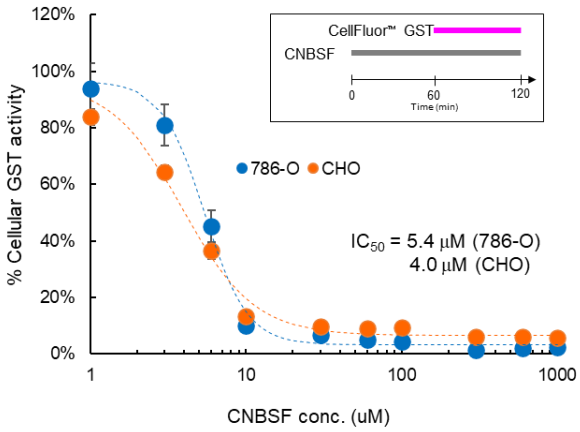
Cell-based assay for monitoring drug-induced activity change of GST

786-O, Neuro2a and CHO cells were seeded in 96 well plates at 1 x 10^4 cell/well, and cultured in DMEM containing 10% FBS for 4 hours. Then media were replaced to serum-free DMEM, and cells were cultured in the presence of retinoic acid (5 µM) or Tunicamycin (1 µg/ml) for 20 hours. Only CNBSF (100 µM), a potent irreversible GST inhibitor (Funakoshi Catalog No. #FDV-0031), was incubated in HBS for 1 hour. The medium was replaced with HBS, **CellFluor™ GST** (2 µM) was added, and cells were incubated for 1 hour. Then fluorescence intensity was measured with a fluorescent plate reader (Ex 475±5 nm/ Em 525±10 nm) without medium change. In all cell lines, cellular GST activity was markedly suppressed by the addition of CNBSF. Retinoic acid increased GST activity and tunicamycin decreased GST activity.



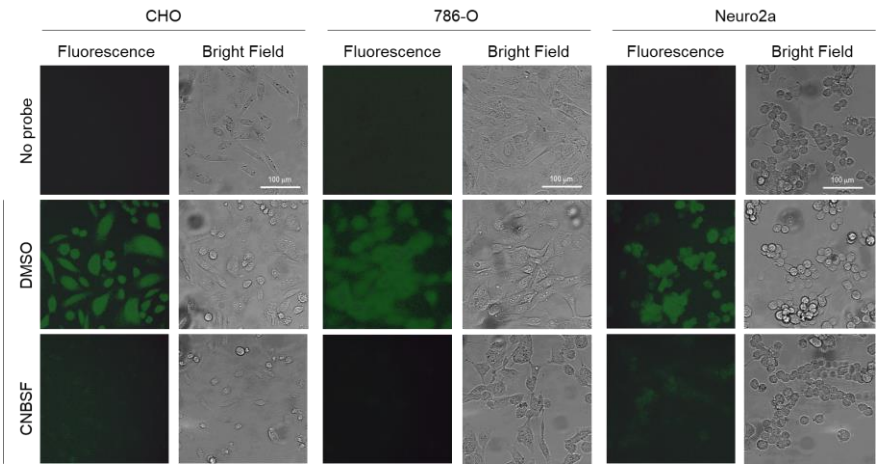
Cell-based assay for estimation of IC_{50} value of a GST inhibitor, CNBSF

786-O and CHO cells were seeded in 96 well plate at 1 x 10^4 cell/well and cultured in DMEM containing 10% FBS for 20 hours. Then culture media were replaced with HBS, treated with 1-1000 µM CNBSF for 1 hour, and further treated with 2 µM **CellFluor™ GST** for 1 hour. The fluorescent intensities were measured with a fluorescence plate reader (Ex 475±5 nm/ Em 525±10 nm). Both 786-O and CHO cell lines showed similar IC_{50} values of CNBSF around 4-5 µM in cell-based assay.



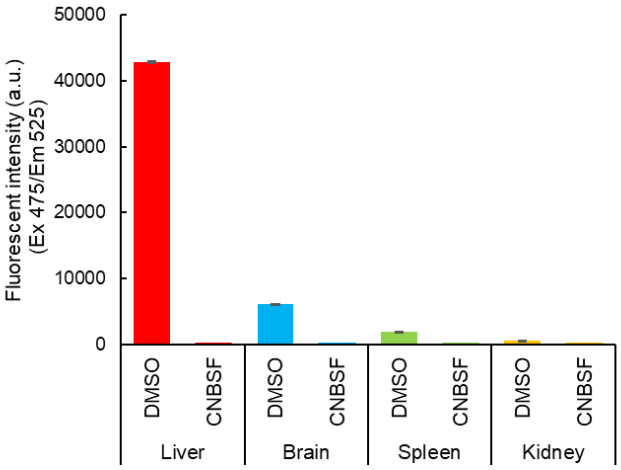
Fluorescent imaging

Three cell lines (CHO, 786-O, and Neuro2a) were seeded into glass bottom dishes and culture for 20 hours in 10% FBS-containing DMEM. Culture medium was replaced to serum- and phenol red-free DMEM and **CellFluor™ GST** (final 30 µM) was incubated for 10 min. Just before observation, the culture media were replaced to fresh serum- and phenol red-free DMEM and quickly observed by epi-fluorescent microscopy (Ex 435-475 nm/Em 530-543 nm). In all cell lines, green fluorescent signals were observed from inside of the cells. On the other hand, when 100 µM CNBSF, a potent irreversible GST inhibitor, was pretreated to cells for 30 min, the fluorescent signals were dramatically suppressed.



In vitro assay for comparison of tissue GST activities

Four mouse tissues (liver, brain, spleen, and kidney) were cut into small pieces, lysed in lysis buffer (50 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% NP-40), and centrifuged to obtain soluble fraction. The total protein concentration of the soluble fraction was determined by the BCA assay and prepared to 1 mg/ml for each tissue. Each tissue lysate was added to assay buffer (50 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% NP-40, 100 µM GSH) to a final concentration of 0.1 mg/ml of total protein. After 30 min reaction at RT, fluorescence intensity was measured with a fluorescence plate reader (Ex 475±5 nm/ Em 525±10 nm). The samples were also pretreated with CNBSF (100 µM final concentration) prior to the addition of **CellFluor™ GST**. In the four tissues tested, significantly stronger GST activity was observed in the liver. In all tissues, the activity was inhibited by CNBSF.



Reference

1. Shibata *et al., Bioorg. Med. Chem. Lett.*, **18**, 2246-2249 (2008) Rhodamine-based fluorogenic probe for imaging biological thiol.
2. Alander *et al., Anal. Biochem.*, **390**, 52-56 (2009) Characterization of a new fluorogenic substrate for microsomal glutathione transferase 1.
3. Zhang *et al., J. Am. Chem. Soc.*, **133**, 14109-14119 (2011) Synthesis and characterization of a series of highly fluorogenic substrates for glutathione transferases, a general strategy.
4. Shishido *et al.*, *Chembiochem.*, 20, 900-905 (2019) A covalent inhibitor for Glutathione S-Transferase Pi (GSTP1-1) in human cells.

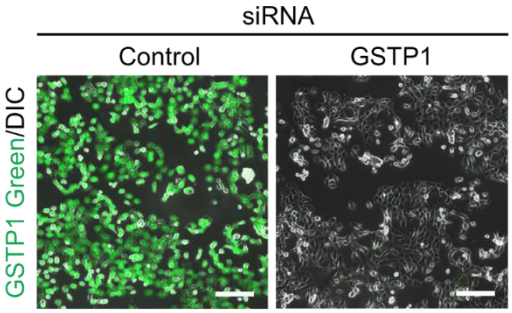
Related products

CellFluor™ GSTP1

CellFluor™ GSTP1 is an activity assay probe for pi-class GST (GSTP1). Since GSTP1 is highly expressed in various cancer cells among GST family, GSTP1 is considered as one of the anti-cancer drug-resistant and LPO-derived aldehydes neutralizing enzymes. CellFluor™ GSTP1, a world's first reagent specific for GSTP1, is only visualized GSTP1 activity among the other GST family members in live cells.

Catalog No. FDV-0034
Size 1 set
- 0.1 mg CellFluor™ GSTP1
- 0.5 mg supplemental reagent MK571)

Features
- Highly specific for GSTP1 among cytosolic GST family members
- Optimized in live cell experiments (not compatible with *in vitro* assay)



CNBSF <Irreversible GST Inhibitor >

CNBSF is a novel GST inhibitor which irreversibly blocks GST enzymes. CNBSF has membrane-permeability and can be applied into live cell experiments.

Catalog No. FDV-0031
Size 10 mg
Features
- Membrane-permeable and irreversible inhibitor
- Broad specificity for various GST family members

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