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Glutaredoxin Fluorometric Activity Assay

Item No. 500239

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening kit, store individual components as stated below.

Item Number	Item	Quantity/ Size	Storage
32589	Potassium Phosphate Assay Buffer (10X)	1 vial/10 ml	-20°C
32592	Glutathione Assay Reagent	1 vial	-20°C
32587	NADPH Assay Reagent	1 vial	-20°C
32590	Glutathione Reductase	1 vial	-20°C
32591	Assay Stabilizing Reagent	1 vial	-20°C
32593	Glutaredoxin Substrate	1 vial/1.2 ml	-20°C
32588	Eosin Standard	3 vials	-20°C
32594	Glutaredoxin Positive Control	1 vial/10 µl	-80°C
400091	Half-Volume 96-Well Solid Plate (black)	1 plate	RT
400023	Foil Plate Cover	1 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring fluorescence with excitation and emission wavelengths of 520 nm and 560 nm, respectively
2. An orbital microplate shaker (optional)
3. Adjustable pipettes; multichannel or repeating pipettor recommended
4. A source of pure water; glass-distilled water or pure water is acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
5. Protease inhibitor; recommended for sample preparation and dilution. *NOTE: Nuclear Extraction Protease Inhibitor Cocktail (100X) is available for purchase from Cayman (Item No. 10009303).*

INTRODUCTION

Background

Glutaredoxins (Grxs) are thiol-disulfide oxidoreductases with a role in the maintenance of cellular thiol redox homeostasis.[1,2] Grx1, the isoform encoded by *GLRX1*, is ubiquitously expressed and localizes to the cytosol, nucleus, and mitochondrial intermembrane space.[1] It contains two active site cysteine residues, which catalyze the deglutathionylation of glutathionylated proteins and the reduction of proteins disulfides to regulate redox signal transduction and repair oxidized proteins.[1,3,4] The oxidized active site of Grx1 is reduced by GSH, forming oxidized GSSG which is then reduced to GSH by glutathione reductase and NADPH. Grx2a, Grx2b, and Grx2c isoforms are produced by alternative splicing of *GLRX2*, the gene encoding Grx2, with Grx2a localized to the mitochondria and Grx2b and Grx2c in the cytoplasm and nucleus. Grx2 also contains two active site cysteine residues and catalyzes the glutathione-dependent reduction of disulfides, acting as an electron donor for ribonucleotide reductase or sulfate reduction, and regulating protein levels of glutathione mixed disulfides.[2] In its inactive state, Grx2 is a homodimer linked by a single [2Fe-2S] cluster, which acts as a redox sensor that drives monomerization and activation of Grx2 under conditions of oxidative stress.[2,5,6] The oxidized active site in Grx2 is reduced by GSH and, unlike other eukaryotic Grxs, is also a substrate for thioredoxin reductase (TrxR). Grx2 catalyzes the reversible glutathionylation of mitochondrial complex I to regulate superoxide production and facilitates the reduction of protein disulfides, as well as other glutathionylated proteins and low molecular weight substrates, such as Coenzyme A disulfide (CoA-disulfide), under conditions of oxidative stress.[7,8] Knockdown of *GLRX1* or *GLXR2* enhances oxidative stress-induced apoptosis *in vitro*.[7,9] Grx deficiencies are associated with increased insulin resistance, inflammation, and mitochondrial dysfunction in animal models.[10,11]

About This Assay

Cayman's Glutaredoxin Fluorometric Activity Assay Kit provides a convenient method of measuring Grx activity in cell lysates and tissue samples. In this reaction, the substrate eosin-GS-BSA (self-quenched) is reduced by Grx, giving the fluorescent product eosin-GSH that can be detected at an emission wavelength of 560 nm. Grx becomes oxidized during the reaction and then reduced by glutathione to produce GSSG, which is recycled by a coupled reaction *via* glutathione reductase and NADPH (see Figure 1). The increase in signal is directly proportional to the Grx activity in the sample.

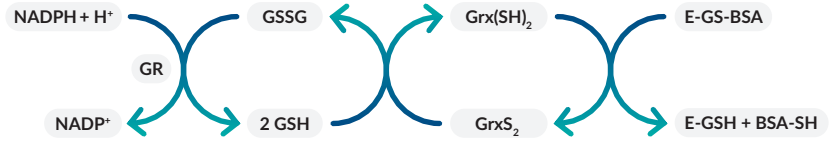

Figure 1. Reaction Schematic

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Potassium Phosphate Assay Buffer (1X) – (Item No. 32589)**

   This vial contains 10 ml of Potassium Phosphate Assay Buffer (10X). Mix 2 ml of Potassium Phosphate Assay Buffer (10X) with 18 ml of pure water. This Potassium Phosphate Assay Buffer (1X), should be used for reconstituting the kit components and diluting the Eosin Standard. This diluted buffer will be stable for one week when stored at 4°C.

2. **Glutathione Assay Reagent – (Item No. 32592)**

   This vial contains lyophilized reduced glutathione. Immediately prior to assaying, reconstitute the entire contents of the vial with 2 ml of Potassium Phosphate Assay Buffer (1X). This will make a sufficient volume of Glutathione Assay Reagent to assay an entire 96-well plate. The reconstituted Glutathione Assay Reagent will be stable for one week when stored at -20°C.

3. **NADPH Assay Reagent – (Item No. 32587)**

   This vial contains lyophilized NADPH. Immediately prior to assaying, reconstitute the entire contents of the vial with 2 ml of Potassium Phosphate Assay Buffer (1X). This will make a sufficient volume of NADPH Assay Reagent to assay an entire 96-well plate. The reconstituted NADPH Assay Reagent will be stable for one week when stored at -20°C.

4. **Glutathione Reductase – (Item No. 32590)**

   This vial contains lyophilized glutathione reductase. Immediately prior to assaying, reconstitute the entire contents of the vial with 2 ml of Potassium Phosphate Assay Buffer (1X). This will make a sufficient volume of Glutathione Reductase to assay an entire 96-well plate. The reconstituted Glutathione Reductase will be stable for one week when stored at -20°C.

5. **Assay Stabilizing Reagent – (Item No. 32591)**

   This vial contains lyophilized Assay Stabilizing Reagent. Immediately prior to assaying, reconstitute the entire contents of the vial with 2 ml of Potassium Phosphate Assay Buffer (1X). This will make a sufficient volume of Assay Stabilizing Reagent to assay an entire 96-well plate. The reconstituted Assay Stabilizing Reagent will be stable for one month when stored at -20°C.

6. **Glutaredoxin Substrate – (Item No. 32593)**

   This vial contains 1.2 ml of a Glutaredoxin substrate solution. The reagent is ready to use as supplied. This is a sufficient volume of substrate to assay an entire 96-well plate.

7. **Eosin Standard – (Item No. 32588)**

   Each vial contains lyophilized Eosin Standard. Immediately prior to performing the assay, reconstitute the entire contents of one vial with 2 ml of Potassium Phosphate Assay Buffer (1X) to make a 10 µM Eosin Standard solution. This will make a sufficient volume of Eosin Standard to assay an entire 96-well plate. This will be used for making the eosin standard curve dilutions. Discard any unused reconstituted standard.

8. **Glutaredoxin Positive Control – (Item No. 32594)**

   This vial contains 10 µL of human recombinant Grx1. Make Grx diluent by mixing 900 µl of Potassium Phosphate Assay Buffer (1X) with 100 µL of reconstituted Assay Stabilizing Reagent. Mix 2 µL of the Glutaredoxin Positive Control with 398 µL of Grx diluent and keep the vial on ice. This diluted Glutaredoxin Positive Control is ready to use in the assay. Store any undiluted Glutaredoxin Positive Control at -80°C. The diluted Glutaredoxin Positive Control is stable for 2 hours when kept on ice. Discard unused diluted Glutaredoxin Positive Control.

Sample Preparation

Tissue Homogenate

1. Prior to dissection, rinse the tissue with PBS, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 100 mM Tris-HCl, pH 7.8) supplemented with protease inhibitor per 250-500 mg of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, store the sample at -80°C.
5. Determine protein concentration. It is recommended to use Cayman's Protein Determination (BCA) Kit (Item No. 701780), Micro BCA Protein Assay Kit (Item No. 760200), Protein Determination Kit (Item No. 704002) or a similar protein determination assay to measure the total protein concentration.
6. Dilute tissue homogenate to 3-10 mg/ml protein concentration in Assay Buffer (1X) supplemented with protease inhibitor before assaying.

NOTE: This assay has been validated in mouse liver extracts with protein concentrations of 10, 7, 5, and 3 mg/ml.

Cell Lysate

1. Collect cells (~5 x 10^6) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, use a rubber policeman instead of proteolytic enzymes.
2. Homogenize the cell pellet in 0.5-1 ml cold buffer (*i.e.*, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA) supplemented with protease inhibitor.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying on the same day, store the sample at -80°C.
5. Determine protein concentration. It is recommended to use Cayman's Protein Determination (BCA) Kit (Item No. 701780), Micro BCA Protein Assay Kit (Item No. 760200), Protein Determination Kit (Item No. 704002) or a similar protein determination assay to measure the total protein concentration.
6. Dilute cell lysate to 0.25-1.5 mg/ml protein concentration in Assay Buffer (1X) supplemented with protease inhibitor before assaying.

NOTE: This assay has been validated in Daudi cell lysates with protein concentrations of 1.4, 0.7, and 0.35 mg/ml.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, the Eosin Standard, blank, and Glutaredoxin Positive Control must be assayed with the samples. It is suggested that each sample, positive control, sample background, and positive control background be assayed in at least duplicate and that the contents of each well are recorded on the template sheet provided on page 26. A typical layout of samples to be measured in duplicate is provided below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	PC	PC	4	4	8	8	12	12	16	16
B	S2	S2	PCB	PCB	B4	B4	B8	B8	B12	B12	B16	B16
C	S3	S3	1	1	5	5	9	9	13	13	17	17
D	S4	S4	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
E	S5	S5	2	2	6	6	10	10	14	14	18	18
F	S6	S6	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
G	S7	S7	3	3	7	7	11	11	15	15	19	19
H	Blk	Blk	B3	B3	B7	B7	B11	B11	B15	B15	B19	B19

S1-S7 = Standard Wells
Blk = Blank Wells
PC = Positive Control Wells
PCB = Positive Control Background Wells
1-19 = Sample Wells
B1-B19 = Sample Background Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that an multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- All reagents except Glutaredoxin Positive Control must be equilibrated to room temperature before beginning the assay. The assay is performed at room temperature.
- The final volume of the assay is 100 µl in all of the wells.
- It is not necessary to use all the wells on the plate at one time. However, a standard curve must be run simultaneously with each set of samples.
- Prepare the Assay Cocktail just before its addition to the wells.
- It is recommended to mix by pipetting or placing on a plate shaker after the addition of samples and Glutaredoxin Positive Control.
- The addition of the Glutaredoxin Substrate must be done as quickly as possible.
- Protect the plate from light after the addition of Glutaredoxin Substrate.
- Mixing by pipetting and/or placing on a plate shaker for 1 minute must be done prior to assaying in a plate reader.
- Monitor the fluorescence with an excitation wavelength of 520 nm and an emission wavelength of 560 nm.

Standard Preparation

Take eight clean test tubes and mark them A-H. Aliquot the Eosin Standard and diluent to each tube as described in Table 1, below.

Tube	Eosin Standard (µl)	Assay Buffer (1X) (µl)	Final Eosin Concentration (µM)
1		400	0
2	50	450	1
3	100	400	2
4	200	300	4
5	300	200	6
6	400	100	8
7	400	0	10

Table 1. Preparation of the eosin standards

Performing the Assay

1. Add 100 µl of the standards (tubes A-F) per well in the designated wells on the plate (see **Sample Plate** Format, Figure 2, page 12). Protect the plate from light.
2. Prepare the **Assay Cocktail** by mixing the following reagents: Assay Buffer (1X) (30 µl), reconstituted Glutathione Assay Reagent (10 µl), reconstituted NADPH Assay Reagent (10 µl), reconstituted Glutathione Reductase Assay Reagent (10 µl), reconstituted Assay Stabilizing Reagent (10 µl). *NOTE: The volumes of reagents given are for the use of exactly 1 well. Adjust the volumes of the reagents accordingly.*
3. Prepare the **Background Cocktail** by mixing the following reagents: Assay Buffer (1X) (60 µl), reconstituted Assay Stabilizing Reagent (10 µl). *NOTE: The volumes of reagents given are for the use of exactly 1 well. Adjust the volumes of the reagents accordingly.*
4. **Positive Control Wells**

   Add 70 µl of **Assay Cocktail** and 20 µl of diluted Glutaredoxin Positive Control to designated wells on the plate (see **Sample Plate** format, Figure 2, page 12).
5. **Blank Wells**

   Add 70 µl of **Assay Cocktail** and 20 µl of Assay Buffer (1X) to the designated wells on the plate (see **Sample Plate** format, Figure 2, page 12).
6. **Sample Wells**

   Add 70 µl of **Assay Cocktail** and 20 µl of sample to the designated wells on the plate (see **Sample Plate** format, Figure 2, page 12).
7. **Sample/Positive Control Background**

   Add 70 µl of **Background Cocktail** and 20 µl of sample or positive control to the designated wells on the plate (see **Sample Plate** format, Figure 2, page 12).

Well	Assay Cocktail	Background Cocktail	Positive Control	Sample	Assay Buffer (1X)
Glutaredoxin Positive Control	70 µl	-	20 µl	-	-
Sample	70 µl	-	-	20 µl	-
Sample Background	-	70 µl	-	20 µl	-
Glutaredoxin Positive Control Background	-	70 µl	20 µl	-	
Blank	70 µl	-	-	-	20 µl

Table 2. Pipetting summary

8. Initiate the reaction by adding 10 µl of **Glutaredoxin Substrate** to all the sample, background, positive control, and blank wells. Protect plate from light. **DO NOT** add substrate to standard wells.
9. **Immediately** mix thoroughly by slowly pipetting several times; or, by placing on an orbital plate shaker for one minute while protecting the plate from light. *NOTE: This step is crucial for minimizing noise in the first 5 minutes of reading.*
10. **Immediately** measure the fluorescence with excitation at 520 nm and emission at 560 nm once every minute for 30 minutes at room temperature.

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard at time 0.
2. Plot the average relative fluorescence unit (RFU) of each standard as a function of the final concentration of eosin from Table 1. The slope of this curve is called f-slope. See Figure 3, for a typical standard curve.

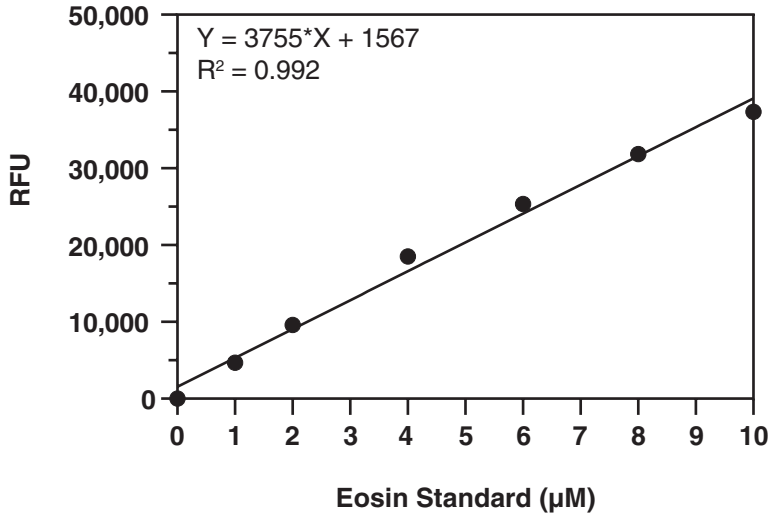

Figure 3. Eosin standard curve

3. Determine the change in RFU per minute for each positive control, positive control background, sample, sample background, and blank by either::

   Plotting the average fluorescence values as a function of time to obtain the slope (rate) of the linear portion of the curve. The 5-15 minute range is generally recommended.

   OR

   Select two points on the linear portion of the curve and determine the change in fluorescence during that time using the following equation.

$$\text{RFU/min} = \frac{\text{RFU (Time 2)} - \text{RFU (Time 1)}}{\text{Time 2 (min)} - \text{Time 1 (min)}}$$

See Figure 4 for typical activity of the glutaredoxin positive control and blank.

4. Subtract rate RFU/min of the blank well from the RFU/min for the sample and positive control wells to calculate the c-slope or each individual sample and control.
5. To calculate GRX activity, subtract the rate RFU/min of the background wells from c-slope of the corresponding sample wells, then divide this value by f-slope and multiply by sample dilution (see formula below).

$$\text{Grx Activity} \left(\frac{\mu M}{\text{min.}}\right) = \frac{\text{c-slope - background} \left(\frac{\text{RFU}}{\text{min.}}\right)}{\text{f-slope}} \text{ x sample dilution}$$

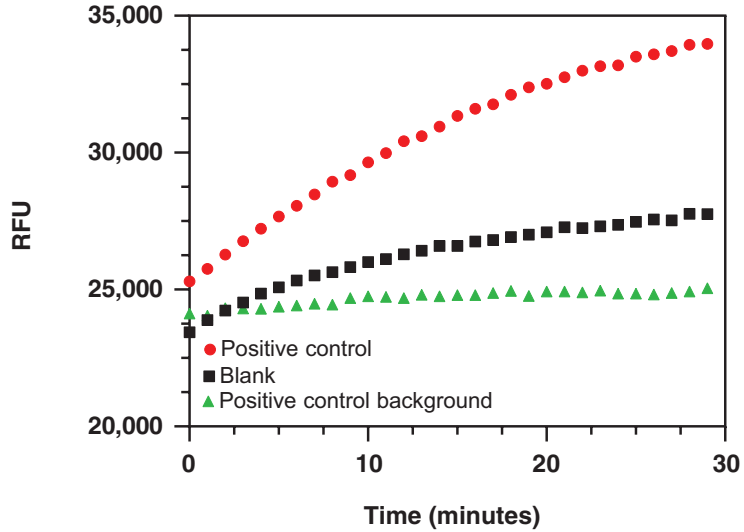

Figure 4. Activity of the Glutaredoxin Positive Control and blank

Performance Characteristics

Sensitivity:

The limit of detection for this assay is 0.066 ± 0.013 µM/min.

Precision:

When a series of 24 blank and Glutaredoxin Positive Control measurements were performed on the same day under the same experimental conditions, the intra-assay coefficients of variation were 6 and 4%, respectively.

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[12]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

Where σ: Standard deviation
µ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z´ factor is 1.0. A robust assay has a Z´ factor >0.5. The Z´ factor for Cayman's Glutaredoxin Fluorometric Activity Assay Kit was determined to be 0.71.

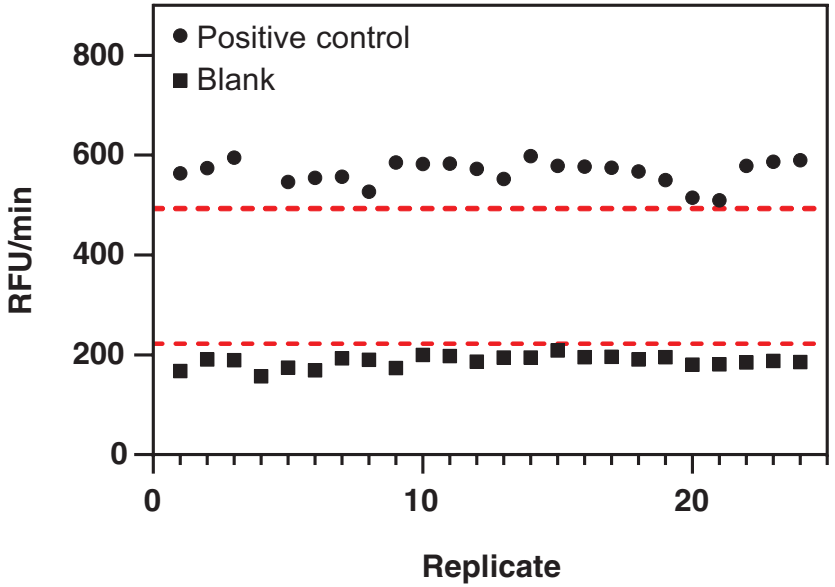

Figure 5. Typical Z′ data for the Glutaredoxin Fluorometric Activity Assay Kit. Data are shown from 24 replicates each for Glutaredoxin Positive Control and a blank sample prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.71. The red lines correspond to three standard deviations from the mean for each control value.

Interferences

The following reagents were tested for interference in the assay.

Reagent		Will Interfere (Yes or No)
Buffers	HEPES	No
	Tris (pH 8.0)	Yes
Detergents	Polysorbate 20 (1%)	Yes
	Polysorbate 20 (0.1%)	Yes
	Triton X-100 (1%)	Yes
	Triton X-100 (0.1%)	Yes
	SDS (1%)	Yes
	SDS (0.1%)	Yes
	CHAPS (1%)	Yes
	CHAPS (0.1%)	Yes
Chelators	EDTA (1 mM)	No
	EGTA (1 mM)	No
Protease Inhibitors/ Enzymes	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
	Leupeptin (10 µg/ml)	No
	Trypsin (10 µg/ml)	No
Solvents	Dimethylsufoxide (5%)	Yes
	Ethanol (5%)	No
	Methanol (5%)	No
Others	BSA (0.1%)	Yes
	Dithiotreitol (1 mM)	Yes
	Glycerol (10%)	No
	Sodium Chloride (150 mM)	Yes

Table 2. Interferences

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in the sample wells	A. Grx activity was too low to detect B. The sample does not contain Grx, or the sample contains an interfering reagent	A. Repeat the assay using a more concentrated sample B. Check for possible interference (see page 21)
The fluorometer exhibited 'MAX' values for the wells	The *gain* setting was too high	A. Reduce the *gain* and repeat the assay B. Establish the *gain* with the Eosin Standard prior to assaying samples

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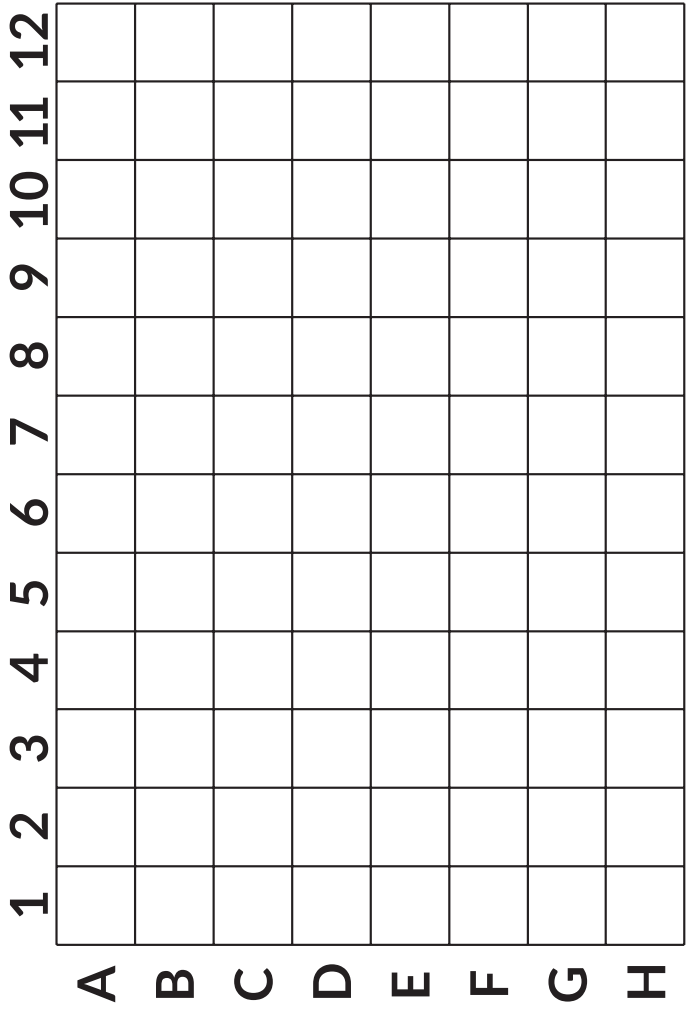

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.