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ARBOR ASSAYS™
Interactive Assay Solutions™

MULTI SPECIES
MOST SENSITIVE

DetectX®

Glutathione Reductase
Fluorescent Activity Kit

1 Plate Kit Catalog Number K009-F1

Sample Types Validated:

Serum, Plasma, RBCs and Cell Lysates

Please read this insert completely prior to using the product.
For research use only. Not for use in diagnostic procedures.

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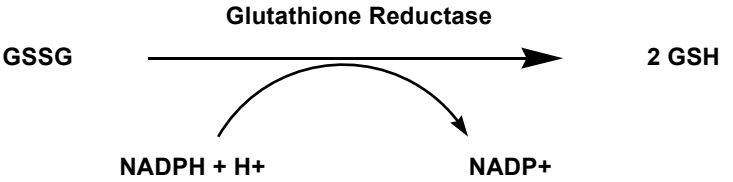
BACKGROUND

Glutathione reductase (GR) plays an indirect but essential role in the prevention of oxidative damage within the cell by helping to maintain appropriate levels of intracellular glutathione (GSH). GSH, in conjuction with the enzyme glutathione peroxidase (GP), is the acting reductant responsible for minimizing harmful hydrogen peroxide cellular levels[1]. The regeneration of GSH is catalyzed by GR[2]. GR is an ubiquitous 100-120 kDa dimeric flavoprotein that catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione, using β-nicotinamide dinucleotide phosphate (NADPH) as the hydrogen donor[3]. Molecules such as NADPH act as hydride donors in a variety of enzymatic processes. NADPH has been suggested to also act as an indirectly operating antioxidant, given its role in the re-reduction of GSSG to GSH and thus maintaining the antioxidative power of glutathione.

The general GR reaction is shown below:



The most widely used procedure to measure GR is to monitor the oxidation of NADPH as a decrease in absorbance at 340 nm[4]. However this method suffers from the absorbance of many biological molecules at 340 nm.

This DetectX® assay determines GR activity by directly measuring the amount of GSH generated from the reduction of GSSG by reacting the GSH with a non-fluorescent molecule, ThioStar®, to covalently bind the free thiol group on GSH and yield a highly fluorescent product.

1. Meister, A. “The Glutathione-Ascorbic Acid Antioxidant Systems in Animal” J. Biol. Chem., 1994 269:9397-9400.
2. Andersen, Helle Raun, et al. “Antioxidative Enzyme Activities in Human Erythrocytes” Clin. Chem. 1997 43(4):562-568.
3. Massey, V. and Willams, C.H. “On the Reaction Mechanism of Yeast Glutathione Reductase”. J.Biol.Chem. 1965 240(11):4470-4480.
4. Carlberg, I. and Mannervik, B. “Glutathione reductase” Methods Enzymol. 1985 113:484-490.

ASSAY PRINCIPLE

The DetectX® Glutathione Reductase (GR) Fluorescent Activity Kit is designed to quantitatively measure glutathione reductase (GR) activity in a variety of samples. Please read the complete kit insert before performing this assay. A GR standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. The kit utilizes a proprietary non-fluorescent molecule, ThioStar®, that will covalently bind to the free thiol group on GSH generated in the reduction of oxidized glutathione (GSSG) to yield a highly fluorescent product. After mixing the sample or standard with ThioStar® and incubating at room temperature, the fluorescent product is read at 510 nm in a fluorescent plate reader with excitation at 390 nm.

Background thiol content is read first after 5 minutes, followed by addition of GSSG and NADPH which uses the standard or sample GR to convert the oxidized glutathione, GSSG, into free GSH, which then reacts with the ThioStar® to yield the signal related to GR activity The activity of GR in the sample is calculated from the generated signal. We have provided a 96 well plate for measurement but this assay is adaptable for higher density plate formats. The end user should ensure that their HTS black plate is suitable for use with these reagents prior to running samples.

RELATED PRODUCTS

Kits	Catalog No.
Glutathione Fluorescent Detection Kit	K006-F1/F5
Glutathione Colorimetric Detection Kit	K006-H1
Glutathione S-Transferase Fluorescent Activity Kit	K009-F1
Hemoglobin Colorimetric Detection Kit	K013-H1
Hemoglobin High Sensitivity Colorimetric Detection Kits	K013-HX1/HX5

Reagents	Catalog No.
Glutathione Mouse Monoclonal Antibody, 50 µg Mouse IgG2a, Clone L4H raised to glutathione conjugated to KLH Applications: Western blotting, Immunoassay and Immunoprecipitation	A001-50UG

SUPPLIED COMPONENTS

Black Half Area 96 Well Plate
See: www.ArborAssays.com/resources/#general-info for plate dimensions.

One Plate — Catalog Number X023-1EA

Glutathione Reductase Standard
Glutathione Reductase at 200 mU/mL in a special stabilizing solution.

40 µL — Catalog Number C023-40UL

ThioStar® Detection Reagent
ThioStar thiol detection substrate stored in a ziploc pouch with desiccant. Reconstitute with dry DMSO.

1 vial — Catalog Number C025-1EA

Dry DMSO
Dry Dimethyl sulfoxide solvent over molecular sieves. **May be stored at room temperature.**

2 mL — Catalog Number X022-2ML

Assay Buffer Concentrate
A 2x concentrated phosphate buffer containing detergents and stabilizers.

60 mL — Catalog Number X021-60ML

NADPH
Reduced β-nicotinamide adenine dinucleotide 2'-phosphate freeze dried with stabilizers stored in a desiccator.

1 vial — Catalog Number X032-1EA

NADPH Diluent
A phosphate buffer containing detergents and stabilizers.

5 mL — Catalog Number X034-5ML

Oxidized Glutathione
Oxidized Glutathione (GSSG) in a special stabilizing solution.

3 mL — Catalog Number C024-3ML

STORAGE INSTRUCTIONS

All components of this kit should be stored at 4°C until the expiration date of the kit. DMSO, when stored at 4°C, will freeze. DMSO can be stored tightly capped at room temperature.

OTHER MATERIALS REQUIRED

Repeater pipet with disposable tips capable of dispensing 15 µL and 25 µL.

Fluorescence 96 well plate reader capable of reading fluorescent emission at 510 nm, with excitation at 390 nm.

Software for converting raw relative fluorescent unit (FLU) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details. Set plate parameters for a 96-well Corning Costar 3686 plate. See: www.ArborAssays.com/resources/#general-info for plate dimension data.

Arbor Assays has available for free download on our website an Excel spreadsheet useful in subtracting out sample thiol background at: www.ArborAssays.com/resources/#general-info

PRECAUTIONS

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

Dimethyl sulfoxide is a powerful aprotic organic solvent that has been shown to enhance the rate of skin absorption of skin-permeable substances. Wear protective gloves when using the solvent especially when it contains dissolved chemicals.

ThioStar® Detection Reagent should be stored at 4°C in the desiccated pouch. Allow desiccated pouch to warm to room temperature prior to opening. ThioStar will react with strong nucleophiles. Buffers containing the preservatives sodium azide, Proclin™ and Kathon™ will react with the substrate.

Reconstituted ThioStar in DMSO stored at 4°C in the desiccated pouch can be used up to 2 months later. The background on the reconstituted ThioStar will increase slowly over time but the increase will not affect the assay results obtained.

ACTIVITY STANDARDIZATION

The Glutathione Reductase standard used in this kit has been calibrated using an enzymatic method adapted from reference 4.

SAMPLE TYPES

This assay has been validated for human serum, EDTA and heparin plasma, and isolated erythrocytes. Most cell lysates should also be compatible. Samples containing visible particulate should be centrifuged prior to using. GR activity varies across tissues and species, however we expect this kit to measure GR activity from sources other than human. The end user should evaluate recoveries of GR activity in samples from other species being tested.

SAMPLE PREPARATION

Any samples requiring larger dilutions or with GR activities outside the standard curve range should be diluted further with Assay Buffer to obtain readings within the standard curve.

Serum and Plasma Samples

Store separated serum or plasma on ice until assaying or freeze in aliquots for later use. Samples must be diluted ≥ 1:40 in Assay Buffer prior to running in the kit.

Erythrocytes (RBCs)

Blood is collected in the presence of heparin or EDTA. The sample is then centrifuged and the plasma and white cell layer are removed from the RBC layer. The RBCs are suspended and gently washed twice with three volumes of isotonic saline (0.9%), separating the cells by centrifugation at 600 x g for 10 minutes and discarding the saline after each step. To lyse the RBCs, four volumes of cold deionized water are added to the RBCs. The cells are then vortexed and incubated for 10 minutes at 4°C, or allowed to undergo a freeze/thaw. Samples are centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant collected. Store on ice until assaying or freeze in aliquots for later use. For erythrocyte lysates, hemoglobin content should be ≤ 0.625 mg/mL in the dilution ran in the assay.

For normalization of results, hemoglobin levels in RBCs can be easily measured using the DetectX® Hemoglobin Detection kit, K013-H1.

Cell Lysates

Washed cell pellets are resuspended at 1-40 x 10^6 cells/mL in cold PBS and are lysed by vigorous vortexing, freeze/thaw cycling or other suitable disruption method. Samples are centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant collected. Store on ice until assaying or freeze in aliquots for later use. A sample of approximately 200,000 cells/mL (1:200 dilution of 4 x 10^7 cells/mL Jurkats) resulted in a GR reading of 3.66 mU/mL. The protocol might require adjustment for other cell types.

Use all samples within 2 hours of dilution.

REAGENT PREPARATION

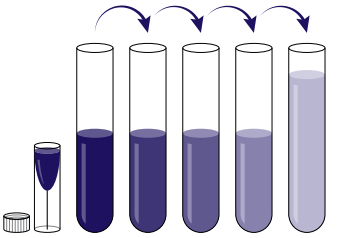
Allow the kit reagents to come to room temperature for 30 minutes. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Assay Buffer Preparation

Prepare the Assay Buffer by diluting one part of the 2x Assay Buffer Concentrate 1:2 with one part deionized water. It is stable for up to 3 months when stored at 4°C.

Standard Preparation

GR Standards are prepared by labeling test tubes as #1 through #6. Briefly spin vial of standard in a microcentrifuge to ensure contents are at bottom of vial. Pipet 390 µL of Assay Buffer into tube #1 and 200 µL into tubes #2 to #6. Carefully add 10 µL of the Glutathione Reductase Standard to tube #1 and vortex completely. Take 200 µL of the GR solution in tube #1 and add it to tube #2 and vortex completely. Repeat these serial dilutions for tubes #3 through #6. The concentration of GR in tubes 1 through 6 will be 5, 2.5, 1.25, 0.625, 0.3125, and 0.156 mU/mL.



Use all Standards within 2 hour of preparation.

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
Buffer Volume (µL)	**390**	200	200	200	200	200
Addition	Stock	Std 1	Std 2	Std 3	Std 4	Std 5
Volume of Addition (µL)	**10**	200	200	200	200	200
Final Conc (mU/mL)	5	2.5	1.25	0.625	0.3125	0.156

ThioStar® Detection Reagent

Allow the ziploc bag to warm **completely** to room temperature prior to opening and remove the vial of ThioStar Reagent. Add 1.8 mL of the provided DMSO to the vial. Vortex thoroughly. Store any unused reconstituted Detection Reagent at 4°C in the ziploc pouch with desiccant and use within 2 months.

NADPH

Allow the ziploc bag to warm **completely** to room temperature prior to opening and remove the vial of NADPH. Add 3 mL of the NADPH Diluent to the NADPH vial and vortex thoroughly. Store any unused reconstituted NADPH at 4°C for no more than 2 weeks.

ASSAY PROTOCOL

We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine GR activity.

1. Use the plate layout sheet on the back page of the insert to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3686 plate.
   See: www.ArborAssays.com/resources/#general-info for plate dimension data.
2. Pipet 25 µL of samples or standards into duplicate wells in the plate.
3. Pipet 25 µL of Assay Buffer into duplicate wells as the Zero standard.
4. Add 15 µL of the ThioStar® Detection Reagent to each well using a repeater pipet.
5. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
6. Incubate at room temperature for 5 minutes.
7. Read the fluorescent signal from each well in a plate reader capable of reading the fluorescent emission at 510 nm with excitation at 370-410 nm. Please contact your plate reader manufacturer for suitable filter sets. This data will be used to subtract any background thiol signal in samples.
8. Add 25 µL of the Oxidized Glutathione to each of the wells using a repeater pipet.
9. Add 25 µL of the NADPH to each of the wells using a repeater pipet.
10. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
11. Incubate at room temperature for 15 minutes.
12. Read the fluorescent emission at 510 nm with excitation at 370-410 nm. Please contact your plate reader manufacturer for suitable filters.

CALCULATION OF RESULTS

Arbor Assays has available for free download on our website an Excel spreadsheet useful in subtracting out sample thiol background at: www.ArborAssays.com/resources/#general-info

After subtracting the background thiol FLU readings for each well from step 7, average the duplicate FLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean FLUs for the zero standard. The activities obtained should be multiplied by the dilution factor to obtain neat sample values.

Or use the online tool from MyAssays to calculate the data:
www.myassays.com/arbor-assays-glutathione-reductase-fluorescent-kit.assay

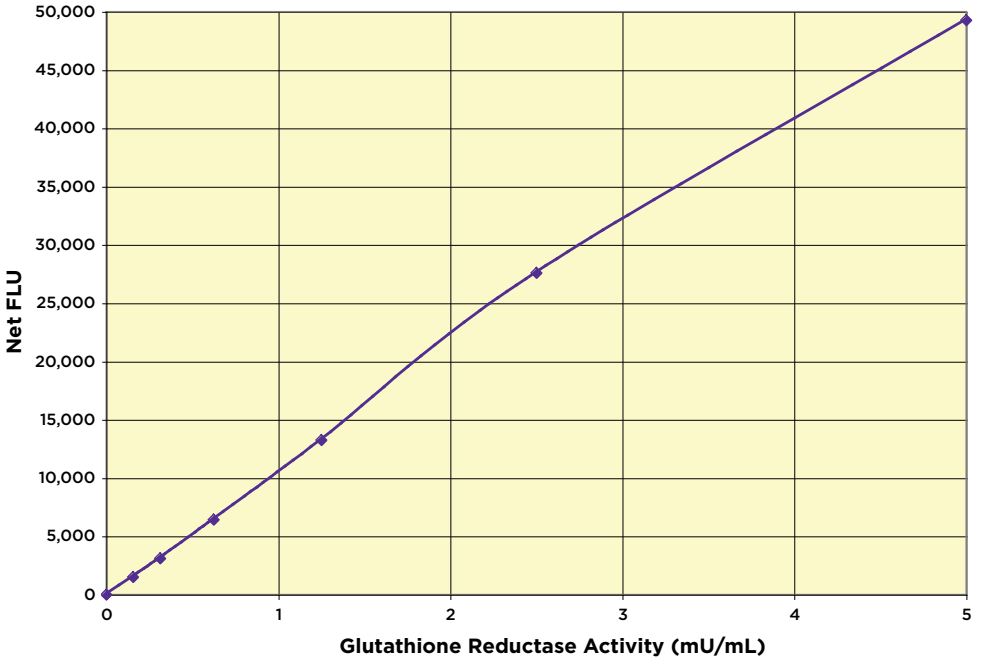
*The MyAssays logo is a registered trademark of MyAssays Ltd.

TYPICAL DATA

Sample	Mean FLU	Net FLU	GR Activity (mU/mL)
Zero	1,170	0	0
Standard 1	50,450	49,280	5
Standard 2	28,768	27,599	2.5
Standard 3	14,429	13,260	1.25
Standard 4	7,609	6,440	0.625
Standard 5	4,279	3,110	0.3125
Standard 6	2,680	1,510	0.156
Sample 1	5,203	4,034	0.415
Sample 2	7,951	6,782	0.671

Always run your own standard curve for calculation of results. Do not use this data.

Typical Standard Curve



Always run your own standard curve for calculation of results. Do not use this data.

VALIDATION DATA

Sensitivity and Limit of Detection

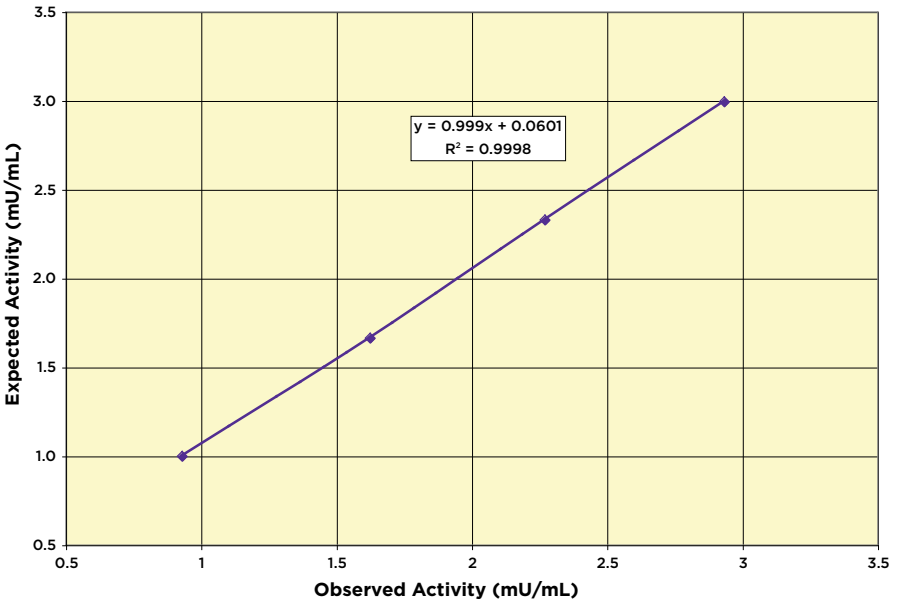
Sensitivity was calculated by comparing the FLUs for twenty wells run for each of the zero and standard #6. The detection limit was determined at two (2) standard deviations from the zero along the standard curve. **Sensitivity was determined as 0.009 mU/mL.**

The Limit of Detection was determined in a similar manner by comparing the FLUs for twenty wells run for each of the zero and a low activity serum sample. **The Limit of Detection was determined as 0.011 mU/mL.**

Linearity

Linearity was determined by taking Jurkat cell lysates at 40 x 10^6 cells/mL diluted to 200,000 and 20,000 cells/mL and mixing in the ratios given below. The measured activities were compared to the expected values based on the ratios used.

Low Cell #	High Cell #	Expected Activity (mU/mL)	Observed Activity (mU/mL)	% Recovery
80%	20%	1.000	0.930	93.0
60%	40%	1.665	1.624	97.5
40%	60%	2.330	2.270	97.4
20%	80%	2.995	2.933	97.9
			Mean Recovery	**96.5%**



Intra Assay Precision

Five native samples were diluted in Assay Buffer and run in replicates of 16 in an assay. The mean and precision of the calculated GR activities were:

Sample	Glutathione Reductase Activity (mU/mL)	%CV
1	3.35	2.7
2	2.38	5.6
3	1.75	3.9
4	0.56	3.7
5	0.27	3.6

Inter Assay Precision

Five native samples were diluted in Assay Buffer and run in duplicates in twenty-two assays run over multiple days by four operators. The mean and precision of the calculated GR activities were:

Sample	Glutathione Reductase Activity (mU/mL)	%CV
1	3.35	5.0
2	2.36	12.6
3	1.64	6.8
4	0.62	10.6
5	0.27	10.5

SAMPLE VALUES

Ten random human serum and EDTA plasma samples were tested in the assay. Values ranged from 24.1 to 33.6 mU/mL with an average of 28.4 mU/mL.

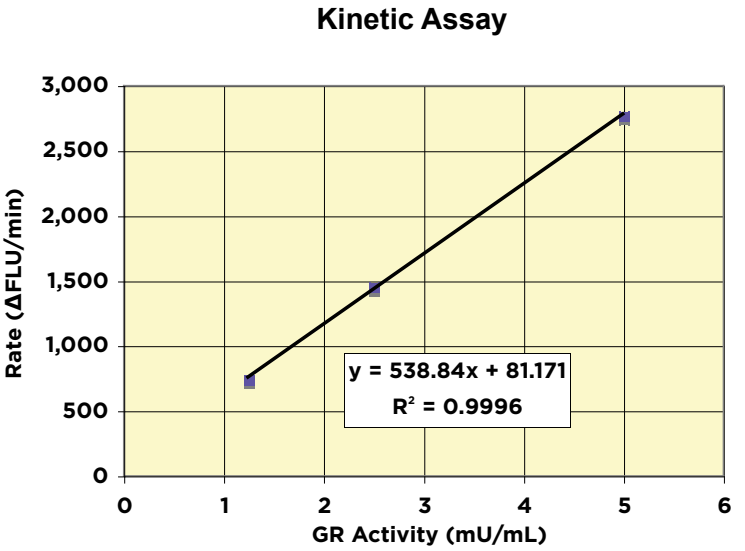
INTERFERENCES

A variety of solvents and detergents were tested as possible interfering substances in the assay. Less than 10% change was seen in the GR activity in the presence of 5% methanol, DMSO or DMF in the sample. Three detergents were also tested: Triton X-100, Tween 20 and SDS. At 1% concentration in the sample, both Triton and Tween showed modest increases in activity, whereas SDS showed < 3.1% decrease at 0.01%.

Hemoglobin levels of 0.0625% (0.625 mg/mL) in the sample showed < 10% decrease in GR activity.

END POINT VERSUS KINETIC ACTIVITY

The assay can also be run as a kinetic assay. A Jurkat cell lysate was read in both an end point and in a kinetic assay. In the end point measurement, it had an activity of 3.66 mU/mL and in the kinetic assay, an activity of 3.58 mU/mL. A typical standard curve for the kinetic assay is shown below.



LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

For details concerning this kit or to order any of our products please contact us:

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OFFICIAL SUPPLIER TO ISWE

Arbor Assays and the International Society of Wildlife Endocrinology (ISWE) signed an exclusive agreement for Arbor Assays to supply ISWE members with EIA kits for wildlife conservation research.

ASSAYS™
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