DetectX® Glutathione Reductase

Fluorescent Activity Kit

1 Plate Kit – Catalog No. K009-F1

Species Independent

Sample Types Tested:

Serum, Plasma, Red Blood Cells, 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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SUPPLIED COMPONENTS & STORAGE

		K009-F1	Description
Black Half Area 96-well Plate	Quantity	1	Non-treated 96-well half area plate
	Catalog No.	X023-1EA	
Glutathione Reductase Standard	Volume	40 µL	Glutathione Reductase at 200 mU/mL in stabilizing solution
	Catalog No.	C023-40UL	
ThioStar® Detection Reagent	Quantity	1 vial	Store in sealed pouch with desiccant. Must be reconstituted with dry DMSO.
	Catalog No.	C025-1EA	
Dry DMSO (Anhydrous)	Volume	2 mL	Dimethyl sulfoxide, dried over molecular sieves. **May be stored at room temperature*.**
	Catalog No.	X022-2ML	
Assay Buffer Concentrate 2X	Volume	60 mL	2X concentrate that must be diluted
	Catalog No.	X021-60ML	
NADPH	Quantity	1 vial	Reduced β-nicotinamide adenine dinucleotide 2'-phosphate. Must be reconstituted with NADPH diluent and stored with desiccant.
	Catalog No.	X032-1EA	
NADPH Diluent	Volume	5 mL	A phosphate buffer for diluting NADPH
	Catalog No.	X034-5ML	
Oxidized Glutathione	Volume	3 mL	Oxidized Glutathione (GSSG) in stabilizing solution
	Catalog No.	C024-3ML	

*When stored at 4ºC, DMSO will freeze. It can be stored tightly capped at room temperature.

This kit should be stored at 4ºC until the expiration date on the kit.

OTHER MATERIALS REQUIRED

- Distilled or deionized water
- Adjustable pipettes with disposable tips. Repeater pipettes or multichannel pipettes with corresponding tips are also recommended.
- High-quality polypropylene test tubes for Standard and Sample preparation. **Do not use glass tubes.**
- Microcentrifuge
- A fluorescence 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 performing linear regression analysis. Contact your plate reader manufacturer for details.
- Optional: Isotonic saline, 0.9% (for Red Blood Cell Samples)
- Optional: 1X Phosphate Buffered Saline (PBS, for Cell Lysates)

PRECAUTIONS

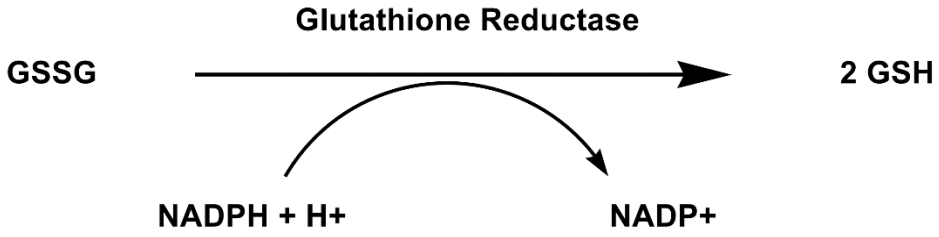
- Read this insert completely prior to using the product.
- This kit may not perform as described if any reagent or procedure is replaced or modified. Do not interchange reagents from different kit lots.
- Take appropriate safety precautions, such as: avoid breathing fumes, wear personal protective equipment (gloves, clothing, eye, and face protection), and familiarize yourself with SDS documents.
    - https://www.arborassays.com/documentation/msds/K009-F1_MSDS.pdf
- Ensure all buffers used for samples are azide free. ThioStar® will react with strong nucleophiles. Buffers containing the preservatives sodium azide, Proclin™ and Kathon™ will react with the substrate.

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, with the enzyme glutathione peroxidase (GP), is the acting reductant responsible for minimizing harmful hydrogen peroxide cellular levels. The regeneration of GSH is catalyzed by GR[2]. GR is a 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 like NADPH act as hydrogen donors in various 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:



ASSAY PRINCIPLE

The DetectX® Glutathione Reductase (GR) Fluorescent Activity Kit is designed to quantitatively measure glutathione reductase activity in a variety of samples. Please read the complete kit insert before performing this assay.

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 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.

A GR standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Samples are diluted in 1x Assay Buffer and added to the wells of the plate.

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.

REAGENT PREPARATION

Except for the reagents listed below, all kit components are ready for use.

Reagent	Preparation	Stability
1X Assay Buffer	Warm 2X Assay Buffer to room temperature and mix thoroughly by inversion. Mix 1 volume 2X Assay Buffer Concentrate with 1 volume deionized water.	1X Assay Buffer is stable for 3 months at 4°C
Reconstituted ThioStar® Detection Reagent	Warm ThioStar® Detection Reagent to room temperature and pulse spin in a microcentrifuge. Add 1.8 mL of the provided DMSO and nutate for 5 minutes to mix thoroughly.	Reconstituted ThioStar® is stable for 2 months at 4ºC.
Reconstituted NADPH	Warm NADPH to room temperature. Add 3 mL NADPH Diluent and nutate for 5 minutes to mix thoroughly.	Reconstituted NADPH is stable for 2 weeks at 4ºC.

SAMPLE PREPARATION

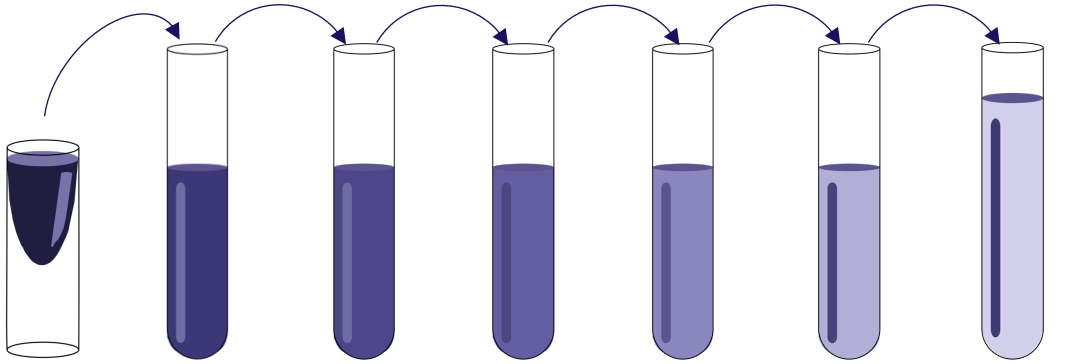
For samples containing particulates, centrifuge prior to use. Upon collection, all samples should be frozen rapidly and stored at -80˚C until testing.

Sample Type	Procedure
Serum and Plasma	• Prepare a ≥ 40-fold dilution of sample by diluting each sample in 1X Assay Buffer. • Samples may require further dilution with 1X Assay Buffer to fall within the standard curve range.
Red Blood Cells (RBCs)	1. Collect blood in the presence of heparin or EDTA. 2. Centrifuge the sample at 600 x *g* for 15 minutes at 4ºC. Aspirate and remove plasma and white cell layer from the RBC layer. 3. Gently wash RBCs twice with three volumes of isotonic saline (0.9%). 4. Centrifuge the sample at 600 x *g* for 10 minutes and discard the saline after each wash. 5. Lyse the RBCs by adding four volumes of cold, deionized water. Vortex cells and incubate for 10 minutes at 4˚C. 6. Centrifuge at 14,000 rpm for 10 minutes at 4˚C. 7. Collect the supernatant and store on ice until assaying or freeze at -80ºC in aliquots for later use. • Ensure sample hemoglobin concentration is ≤ 0.625 mg/mL before running in this assay. • Hemoglobin levels in RBCs can be measured using the DetectX® Hemoglobin Detection kit, K013-H1.
Cell Lysates	1. Wash cell pellets in cold PBS. Resuspend at 1-40 x 10^6 cells/mL in cold PBS. 2. Lyse by vigorous vortexing, freeze/thaw cycling or other suitable disruption method. 3. Centrifuge samples at 14,000 rpm for 10 minutes at 4°C. Collect supernatant. 4. Store on ice until assaying or freeze at -80ºC in aliquots for later use. • During validation, 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.**

STANDARD PREPARATION

1. Label tubes Standard 1 through Standard 6. Do not use glass tubes.
2. Pulse spin the Glutathione Reductase Standard in a microcentrifuge.
3. Add 390 µL 1X Assay Buffer to Standard 1 tube.
4. Add 200 µL 1X Assay Buffer to Standard 2 – 6 tubes.
5. Add 10 µL of the GR Standard stock solution to Standard 1 tube. Vortex thoroughly.
6. Transfer 200 µL of Standard 1 into Standard 2 tube to make a 2-fold dilution. Vortex thoroughly.
7. Transfer 200 µL of the mixed solution from Standard 2 into Standard 3 tube to make a 2-fold dilution. Vortex thoroughly.
8. Continue serially diluting into the remaining tubes. This process and the final concentrations are summarized in the table below.



	Standard 1	Standard 2	Standard 3	Standard 4	Standard 5	Standard 6
1X Assay Buffer (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 Concentration (mU/mL)	5.0	2.5	1.25	0.63	0.31	0.16

⚠ **Use all Standards within 2 hours of dilution.**

ASSAY PROTOCOL

Before You Begin:

- **Room Temperature for this assay is defined as 22ºC – 24ºC.**
- **Ensure all reagents have been warmed to room temperature.**
- **Dilute Samples as described in Sample Preparation.**
- **Run all Standards and Samples in duplicate.**
- Use the blank plate template on the back page of this booklet to design your plate layout and aid in proper sample and standard identification.
- Set plate parameters on the plate reader for a 96-well Corning Costar 3686 plate. See ArborAssays.com for plate dimension data.

1. Add 25 µL Samples or Standards into duplicate wells in the plate. Add 25 µL of 1X Assay Buffer into duplicate wells as the Zero standard.
2. Add 15 µL of reconstituted ThioStar® Detection Reagent to each well.
3. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
4. Incubate at room temperature for 5 minutes.
5. Read the plate immediately using a fluorescent emission at 510 nm with excitation at 370 - 410 nm. Proceed immediately to the next step.
6. Add 25 µL Oxidized Glutathione to each well.
7. Add 25 µL reconstituted NADPH to each well.
8. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
9. Incubate at room temperature for 15 minutes.
10. Read the plate immediately using a fluorescent emission at 510 nm with excitation at 370 - 410 nm.

CALCULATION OF RESULTS

Follow the instructions below or use the online tool: https://www.myassays.com/assay.aspx?id=938

1. Gather all raw data FLU readings from each Sample and Standard, including the Zero Standard.
2. Subtract the background thiol signal present in the samples by subtracting the FLU values from the first plate reading (Protocol Step 5) from the FLU values from the second plate reading (Protocol Step 10) (Corrected FLU).

EXAMPLE:

Sample	Replicate 1 FLU (Background, First Reading)	Replicate 1 FLU (Total, Second Reading)	Replicate 1 Corrected FLU
Zero Standard	600	1,753	1,153
Standard 1	629	50,294	49,665
Sample 1	945	6,258	5,313

3. Average the duplicate Corrected FLU readings for each Sample, Standard, and Zero Standard (Mean FLU).

EXAMPLE:

Sample	Replicate 1 Corrected FLU	Replicate 2 Corrected FLU	Mean FLU
Zero Standard	1,153	1,186	1,170
Standard 1	49,665	51,234	50,450
Sample 1	5,313	5,093	5,203

4. Subtract the Zero Standard Mean FLU from the Mean FLU for each Sample and Standard (Net FLU).

EXAMPLE:

Sample	Mean FLU	Zero Standard Mean FLU	Net FLU
Standard 1	50,450	1,170	49,280
Sample 1	5,203	1,170	4,033

5. Plot the standard curve with Net FLU for the Standards on the y-axis and GR activity (mU/mL) on the x-axis. Perform a linear regression.

   Use the slope and Y-intercept of the regression line, together with the Net FLU to calculate the GR activity in the diluted Samples using the equation below. If the diluted GR activity is outside of the range of the Standards, the Sample should be prepared again at a more appropriate dilution.

$$\text{Sample GR Activity (mU/mL)} = \frac{\text{(Net FLU)} - \text{(Y-intercept)}}{\text{Slope}}$$

EXAMPLE:

Sample	Net FLU	Sample GR Activity (mU/mL)
Sample 1	4,033	0.36

6. If the original sample was diluted, multiply the GR activity by the Sample Dilution Factor to determine the activity of GR in the original sample.

EXAMPLE:

Sample	Sample GR Activity (mU/mL)	Sample Dilution Factor	Original Sample GR Activity (mU/mL)
Sample 1	0.36	40x dilution	14.4

TYPICAL DATA

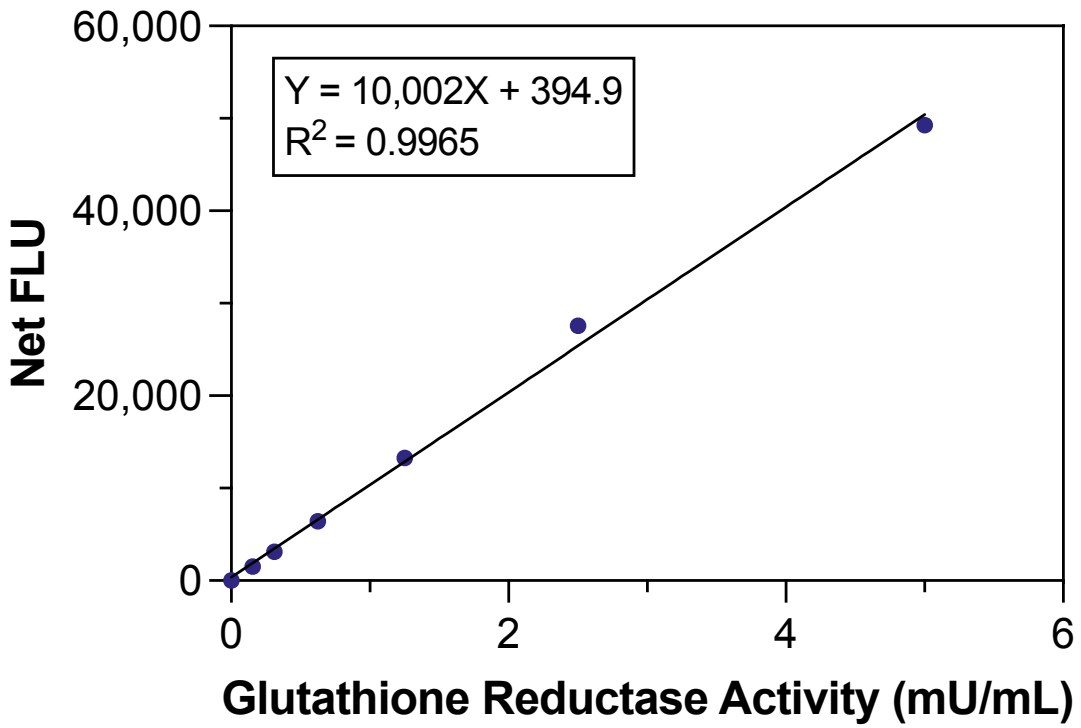
⚠ **Always run your own standard curve. This data should NOT be used to interpret results.**

Sample	Mean FLU	Net FLU	GR Activity (mU/mL)
Standard 1	50,450	49,280	5
Standard 2	28,768	27,598	2.5
Standard 3	14,429	13,259	1.25
Standard 4	7,609	6,439	0.63
Standard 5	4,279	3,109	0.31
Standard 6	2,680	1,510	0.16
Zero	1,170	0	0
Sample 1	5,203	4,033	0.36
Sample 2	7,951	6,781	0.64

Glutathione Reductase Unit Definition

The Glutathione Reductase is calibrated using an enzymatic method adapted from reference 4.

Typical Standard Curve



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 standard deviations from the zero along the standard curve.

Sensitivity was determined as 0.009 mU/mL.

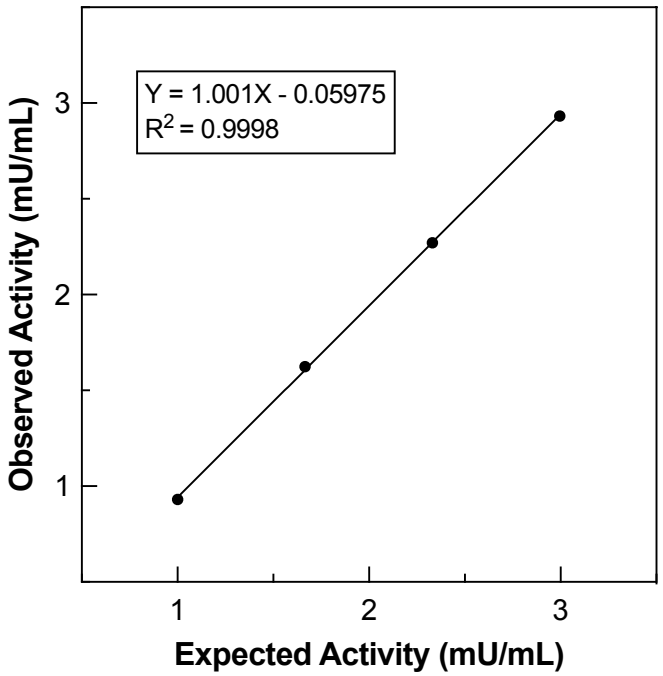
The Limit of Detection was determined in a similar manner by comparing the FLUs for twenty wells run for each of the Zero Standard and a low activity sample.

The Limit of Detection was determined as 0.011 mU/mL.

Linearity

Linearity was determined in human cell lysates by diluting two lysate preparations with known GR activity with 1X Assay Buffer. One sample had a GR activity of 0.335 mU/mL (Low Sample), and a second had a GR activity of 3.66 mU/mL (High Sample). The two samples were mixed in the ratios given below and the measured activities were compared to the expected values for each given ratio.

Low Sample	High Sample	Expected Activity (mU/mL)	Observed Activity (mU/mL)	% Recovery
80%	20%	1.00	0.93	93.0
60%	40%	1.67	1.62	97.5
40%	60%	2.33	2.27	97.4
20%	80%	3.00	2.93	97.9
			Mean Recovery	**96.5%**



Intra Assay and Inter Assay Precision

For intra assay precision, five samples were diluted in 1X Assay Buffer, and 16 replicates were run in one assay. For inter assay precision, five samples were diluted in 1X Assay Buffer and duplicates of each sample were run in 22 assays over multiple days by 4 operators. The %CV represents the variation in activity (not fluorescence level) as determined using a standard curve.

	Intra Assay Precision		Inter Assay Precision	
Sample	GR Activity (mU/mL)	% CV	GR Activity (mU/mL)	% CV
1	3.35	2.7	3.35	5.0
2	2.38	5.6	2.36	12.6
3	1.75	3.9	1.64	6.8
4	0.56	3.7	0.62	10.6
5	0.27	3.6	0.27	10.5

SAMPLE VALUES

Five human serum and 5 human EDTA plasma samples were diluted in 1X Assay Buffer and tested in the assay. The adjusted average activity and sample range are shown below.

Sample Type	Adjusted Average Activity (mU/mL)	Adjusted Activity Range (mU/mL)
Serum	28.3	24.1 – 33.6
Plasma	28.4	25.3 – 31.7

INTERFERENCE

Potentially interfering substances were evaluated in the assay and the change in signal was calculated.

Interferent	Effect
DMSO (5.0%)	7.4% increase
DMF (5.0%)	6.2% increase
Methanol (5.0%)	1.7% decrease
Triton X-100 (1%)	4.1% increase
Tween-20 (1%)	11.5% increase
SDS (0.01%)	3.1% decrease
Hemoglobin (0.625 mg/mL)	9.8% decrease

TROUBLESHOOTING

Issue	Possible Cause & Solution
Reagent Shortage	• Check under the cap for additional reagent. Pulse spin reagent containers to collect contents prior to opening when possible. • When using a multichannel pipette, return unused reagent to container for later use.
Erratic Values	• Prerinse pipet tips with desired reagent prior to aspirating the required volume. • Deliver volume with care to prevent splashing into adjacent wells.
Low Signal	• Confirm tools, equipment, reagents, and containers used do not contain any trace of sodium azide. • Verify the plate reader is set to fluorescent emission at 510 nm with excitation at 370-410 nm. • Confirm reagents are at room temperature prior to use.

CITATIONS

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.

RELATED PRODUCTS

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

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.

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CONTACT INFORMATION

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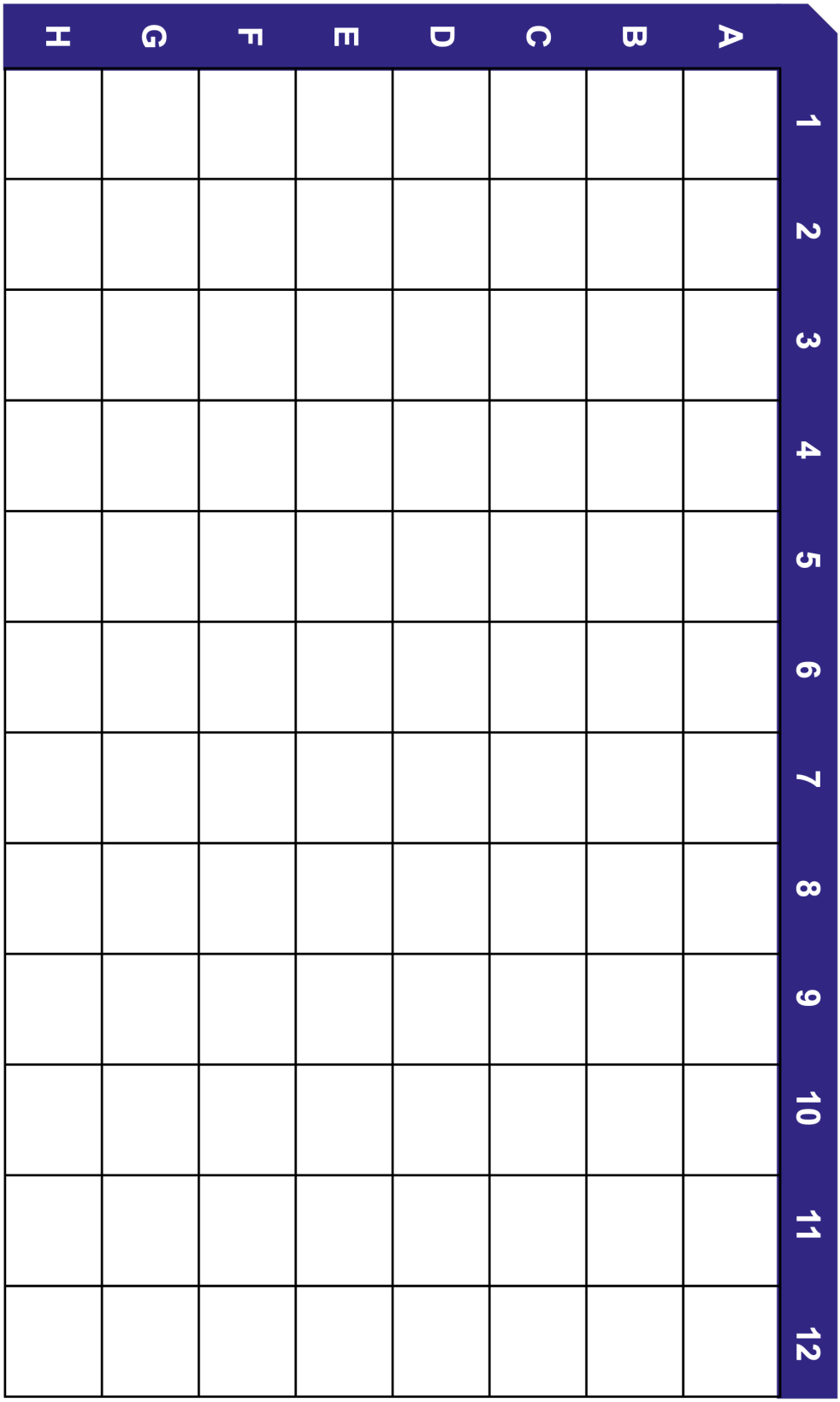
OFFICIAL SUPPLIER TO ISWE

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

PLATE LAYOUT



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