StressMarq Biosciences INC.

Discovery through partnership | Excellence through quality

**StressXpress®**

# Glutathione Reductase Activity Kit

Catalog# SKT-204 (96-Well Kit)

Fluorometric detection of glutathione reductase activity by the amount of GSH generated from the reduction of GSSG

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# GENERAL INFORMATION

## Materials Supplied

| Catalog Number | Reagent | Quantity | Description |
|---|---|---|---|
| SKC-204A | Black Half Area 96 Well Plate | One plate | - |
| SKC-204B | Glutathione Reductase Standard | 40 µL | Glutathione Reductase at 200 mU/mL in a special stabilizing solution. |
| SKC-204C | StressXpress® Detection Reagent | 1 vial | StressXpress® thiol detection substrate stored in a desiccator. Reconstitute with dry DMSO. |
| SKC-204D | Dry DMSO | 2 mL | Dry Dimethyl sulfoxide solvent over molecular sieves. May be stored at room temperature. |
| SKC-204E | Assay Buffer Concentrate | 60 mL | A 2x concentrated phosphate buffer containing detergents and stabilizers. |
| SKC-204F | NADPH | 1 vial | Reduced ß-nicotinamide adenine dinucleotide 2'-phosphate freeze dried with stabilizers stored in a desiccator. |
| SKC-204G | NADPH Diluent | 5 mL | A phosphate buffer containing detergents and stabilizers. |
| SKC-204H | Oxidized Glutathione | 3 mL | Oxidized Glutathione (GSSG) in a special stabilizing solution. |

If any of the items listed above are damaged or missing, please contact our Customer Service department at (250) 294-9065. We cannot accept any returns without prior authorization.

**WARNING: Not for human or animal disease diagnosis or therapeutic drug use.**

## Precautions

As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete booklet 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.

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

## Storage

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. Can be stored tightly capped at room temperature.

## Materials Needed But Not Supplied

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. Plate Dimensions (in mm): Well Depth: 10.54; Plate Length: 127.8; Plate Width: 85.5; A1 Row Offset: 11.2; A1 Column Offset: 14.3.

You can find an Excel spreadsheet, useful in subtracting out sample thiol background, availible on this product's website page.

## Activity Standardization

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

**Please read this booklet completely prior to using the product.**

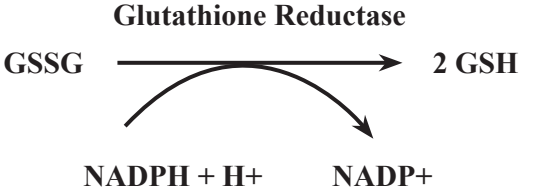
FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

# INTRODUCTION

## 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 ß-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 340nm[4]. However this method suffers from the absorbance of many biological molecules at 340nm.

This StressXpress® Glutathione Reductase Fluorescent Activity 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 to covalently bind the free thiol group on GSH and yield a highly fluorescent product.

## Assay Overview

The Glutathione Reductase *StressXpress®* Fluorescent Activity kit is designed to quantitatively measure glutathione reductase (GR) activity in a variety of samples. Please read the complete kit booklet 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 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 StressXpress® Detection Reagent 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 StressXpress® Detection Reagent 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.

# PRE-ASSAY PREPARATION

## Sample Types

**Sample Types Validated:**
Serum, Plasma, RBCs and Cell Lysates

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.

### Cell Lysates

Washed cell pellets are resuspended at 1-40x$10^6$ 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 4x$10^7$ 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. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine GR activity. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

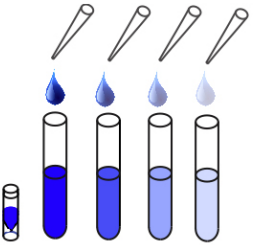
### Assay Buffer Preparation

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 six 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.



| | Standard 1 | Standard 2 | Standard 3 | Standard 4 | Standard 5 | Standard 6 |
|---|---|---|---|---|---|---|
| Buffer Volume (µL) | 390 | 200 | 200 | 200 | 200 | 200 |
| Addition | Stock | Standard 1 | Standard 2 | Standard 3 | Standard 4 | Standard 5 |
| Volume of Addition (µL) | 10 | 200 | 200 | 200 | 200 | 200 |
| Final Concentration (mU/mL) | 5 | 2.5 | 1.25 | 0.625 | 0.3125 | 0.156 |

### StressXpress® Detection Reagent

Remove the vial of StressXpress® Reagent from the desiccator and add 1.8 mL of the provided DMSO to the vial. Vortex thoroughly. Store any unused reconstituted Detection Reagent at 4°C in the desiccator and use within 2 weeks.

### 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

1. Use the plate layout sheet on page 23 of the booklet to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3686 plate. Plate Dimensions (in mm): Well Depth: 10.54; Plate Length: 127.8; Plate Width: 85.5; A1 Row Offset: 11.2; A1 Column Offset: 14.3.
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 StressXpress® 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

You can find an Excel spreadsheet, useful in subtracting out sample thiol background, availible on this product's website page.

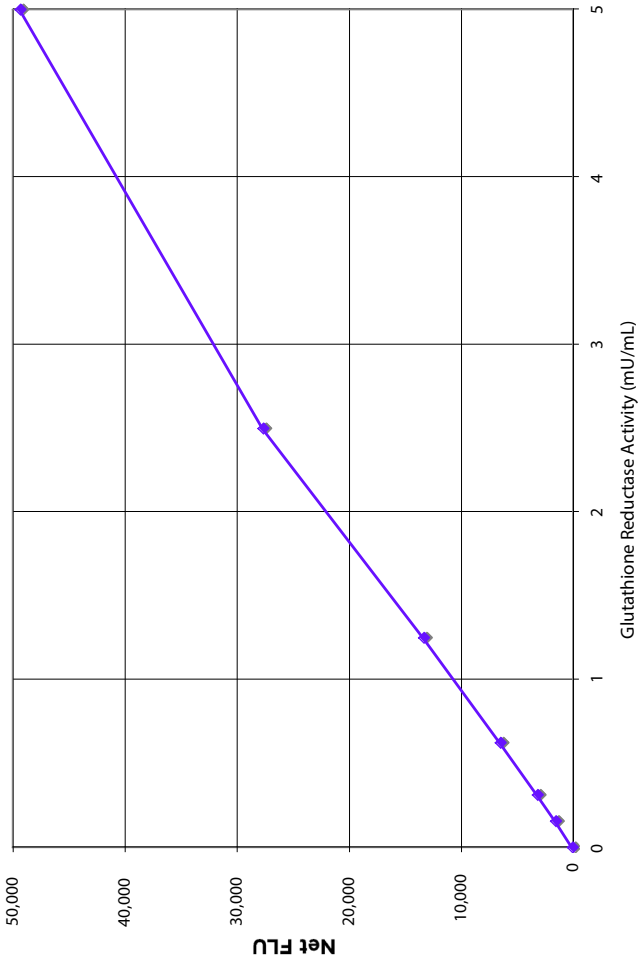
After subtracting the background thiol FLU readings for each well from step 6, average the duplicate FLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean FLUs for the zero standard. The activities obtained should be multiplied by the dilution factor to obtain neat sample values.

## 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



## 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.

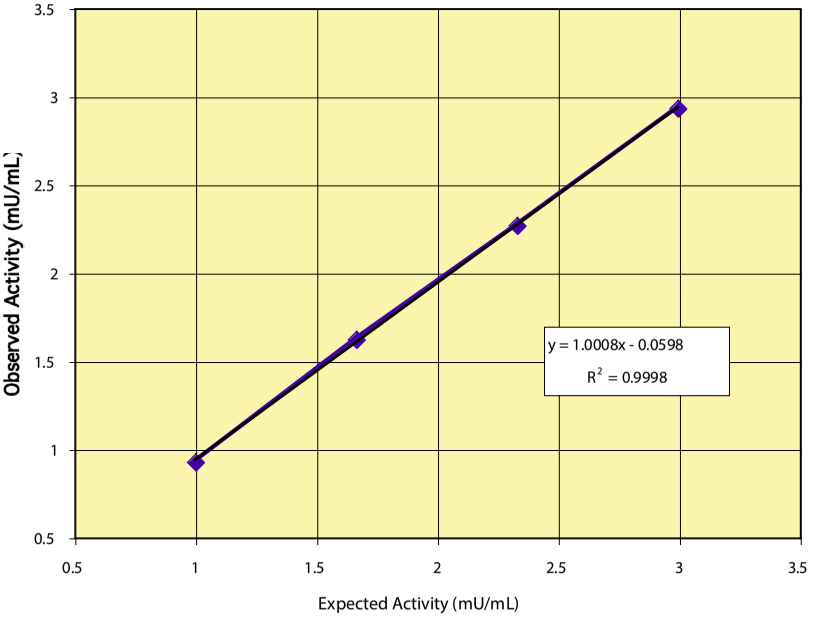
Sensitivity was determined as 0.009 mU/mL.

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 # | Observed Activity (mU/mL) | Expected Activity (mU/mL) | % Recovery |
|---|---|---|---|---|
| 100% | 0% | 0.335 | -- | -- |
| 80% | 20% | 0.930 | 1.000 | 93.0 |
| 60% | 40% | 1.624 | 1.665 | 97.5 |
| 40% | 60% | 2.270 | 2.330 | 97.4 |
| 20% | 80% | 2.933 | 2.995 | 97.9 |
| 0% | 100% | 3.660 | -- | -- |
| | | | Mean Recovery | 96.5% |


3.5
3
2.5
2
1.5
1
0.5
Observed Activity (mU/mL)
y = 1.0008x - 0.0598
R2 = 0.9998
0.5
1
1.5
2
2.5
3
3.5
Expected Activity (mU/mL)

**Intra Assay Precision**

Five native samples 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

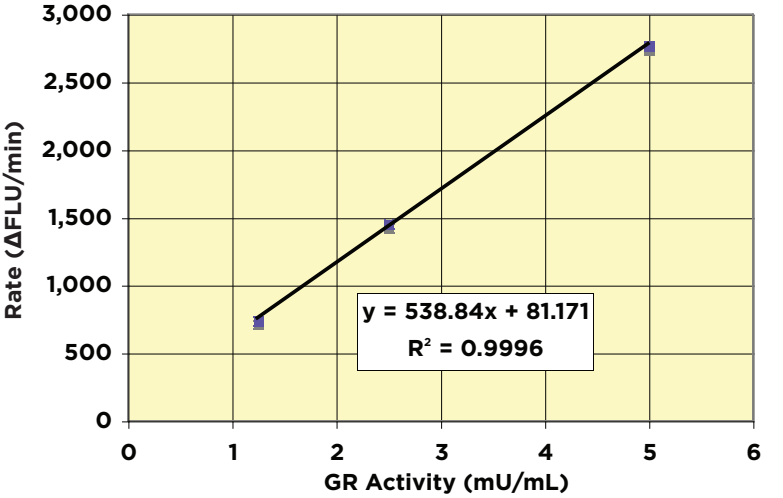
A variety of solvents and detergents were tested as possible interfering substances in the assay. Less than 10% change was seen in the GR activity in the presence of 5% methanol, DMSO or DMF in the sample. Three detergents were also tested, Triton X-100, Tween 20 and SDS. At 1% concentration in the sample,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

## Kinetic Assay



## References


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.


## Warranty and Limitation of Remedy

StressMarq Biosciences Inc. makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. StressMarq **warrants only** to the original customer that the material will meet our specifications at the time of delivery. StressMarq will carry out its delivery obligations with due care and skill. Thus, in no event will StressMarq have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if StressMarq is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of StressMarq, its directors or its employees.

Buyer's **exclusive remedy** and StressMarq's sole liability hereunder shall be limited to a refund of the purchase price, or at StressMarq's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to StressMarq within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

**For further details, please refer to our Warranty and Refund Policy located on our website and in our catalog**.

## Contact Information

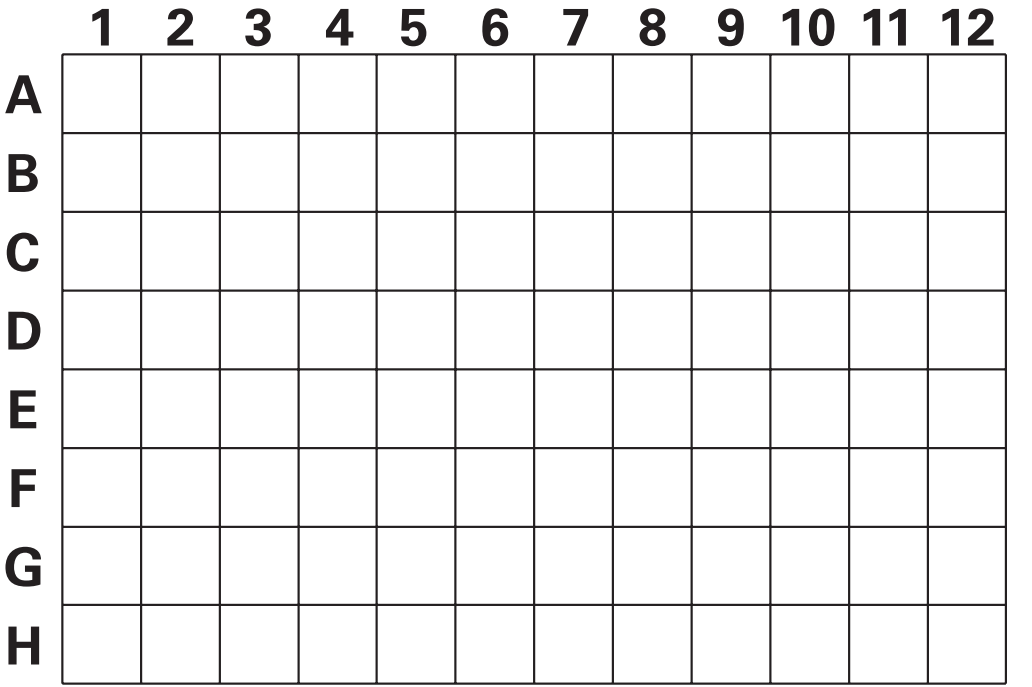
**Phone:** 250-294-9065

**Fax:** 250-294-9025

**E-Mail:** techsupport@stressmarq.com

**Hours:** M-F 9:00 AM to 5:00 PM PST

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).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## NOTES