StressMarq Biosciences INC.

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StressXpress®

GRP78 ELISA Kit (Rat)

Catalog# SKT-137-96 (96-Well Kit)

Catalog# SKT-137-480 (5 x 96-Well Kit)

Colorimetric detection of Rat GRP78

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GENERAL INFORMATION

Materials Supplied

Catalog No.	Item	Quantity/Size
SKC-137A	Anti-Rat GRP78 Immunoassay Plate	1 Plate
SKC-137B	Recombinant Rat GRP78 Standard	2 vials/56.7ng
SKC-0001	Standard and Sample Diluent	1 vial/50mL
SKC-0002	Anti-Rat GRP78 Biotinylated Antibody Concentrate	1 vial/150µL
SKC-0003	10X Wash Buffer Concentrate	1vial/100mL
SKC-137F	Anti-Rat GRP78 Biotinylated Antibody Diluent	1 vial/13mL
SKC-137G	Streptavidin Poly HRP Concentrate	1 vial/150µL
SKC-137H	Streptavidin Poly HRP Diluent	1 vial/13mL
SKC-0004	TMB Substrate	1 vial/13mL
SKC-0005	Stop Solution	1 vial/13mL
SKC-0009	Plate Cover	2

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

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with StressMarq Biosciences Inc.'s StressXpress® ELISA Kit. This kit may not perform as described if any reagent or procedure is replaced or modified.

StressMarq suggests running all ELISA kits in triplicate, but replication must be defined by the user.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service 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).

Storage and Stability

This kit will perform as specified if the components are stored as directed and used before the expiration date indicated on the outside of the box.

All reagents are stable as supplied at 4°C.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and a repeat pipettor.
3. Deionized or distilled water
4. Materials used for **Sample Preparation** (see page 9-10).

Assay Precautions

- All ELISA reagents must be at room temperature before use.
- Vigorous plate washing is essential.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Substrate. Do not expose the TMB solution to glass, foil or metal. If the solution is blue before use, do NOT use it.
- Individual components may contain preservatives. Wear gloves while preforming the assay. Please follow proper disposal procedures.
- Include a standard curve each time the assay is performed.
- Run both standards and samples in triplicate.
- Buffers may crystallize over time. Warm crystallized buffer until the salt crystals return to solution. Ensure that your components return to RT before use in the assay.

INTRODUCTION

Background

GRP78 is a ubiquitously expressed, 78-kDa glucose-regulated protein, and is commonly referred to as an immunoglobin chain binding protein (BiP). The BiP proteins are categorized as stress response proteins because they play an important role in the proper folding and assembly of nascent protein and in the scavenging of misfolded proteins in the endoplasmic reticulum lumen. Translation of BiP is directed by an internal ribosomal entry site (IRES) in the 5' nontranslated region of the BiP mRNA. BiP IRES activity increases when cells are heat stressed (1).

GRP78 is also critical for maintenance of cell homeostasis and the prevention of apoptosis (2). Luo et al. have provided findings that suggest GRP78 is essential for embryonic cell growth and pluripotent cell survival (3).

In terms of diseases, GRP78 has been shown to be a reliable biomarker of hypoglycemia, to serve a neuroprotective function in neurons exposed to glutamate and oxidative stress (4), and its protein levels are reduced in the brains of Alzheimer's patients (5). Also, the induction of the GRP78 protein that results in severe glucose and oxygen deprivation could possible lead to drug resistance to antitumor drugs (6, 7).

About This Assay

StressMarq's GRP78 ELISA (Rat) is a sandwich ELISA that can be used for the quantification of Rat GRP78 in Tissue Lysates. The ELISA utilizes an Anti-Rat GRP78 monoclonal antibody-coated plate and a Biotinylated antibody for detection which allows for an assay range of 0 to 50 ng/mL, with a sensitivity of 0.15 ng/mL. The other highlights of this kit are a quick incubation time, stable reagents, and an easy to use protocol.

Western Blot Validation of Capture Antibody

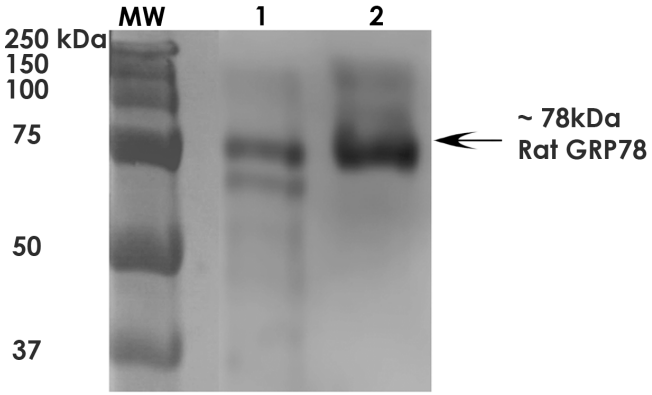

Figure 1. Western blot analysis of rat lysates showing the detection of ~78 kDa GRP78 protein using Anti-GRP78 Antibody, Clone 6G3-1B9, 1:1000 Dilution. MW ladder. Lane 1: Rat Liver Lysate. Lane 2: Rat Kidney Lysate; Load: 10 µg per lane. Block: 5% milk + TBST, 60min at RT. Primary antibody: Mouse Anti- GRP78 monoclonal antibody, Clone 6G3-1B9, incubated for 60 min at RT. Secondary antibody: Goat Anti-Mouse HRP antibody, 1:200 dilution, 60min at RT. Development: ECL solution for 6min at RT. Predicted/Observed size: ~ 78 kDa. Other band(s):~70kDa.

Assay Overview

1. Prepare Standard and samples in Standard and Sample Diluent.
2. Add 100 µL of Standard or sample to appropriate wells.
3. Cover plate with Plate Sealer and incubate at RT for 1 hour.
4. Wash plate four times with 1X Wash Buffer.
5. Add 100 µL of Biotinylated Antibody Working Solution to each well.
6. Cover plate with Plate Sealer and incubate at at RT for 1 hour.
7. Wash plate four times with 1X Wash Buffer.
8. Add 100 µL of Streptavidin Poly HRP Working Solution to each well.
9. Cover plate with Plate Sealer and incubate at RT for 30 minutes.
10. Wash plate four times with 1X Wash Buffer.
11. Add 100 µL of TMB Substrate to each well.
12. Develop the plate in the dark at room temperature for 30 minutes.
13. Stop reaction by adding 100 µL of Stop Solution to each well.
14. Measure absorbance on a plate reader at 450 nm.

PRE-ASSAY PREPARATION

Sample Preparation

Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay.

NOTE: Prepare at least 350µL of your diluted sample to permit assay in triplicate (approximately 100µL/ well).

General Precautions

- All samples must be free of organic solvents prior to assay.
- Samples that cannot be assayed immediately should be stored as indicated below.
- Please be advised that all suggested dilutions below are simply recommended as a starting point, and it may be necessary to adjust the dilution based on experimental results.

Tissue Extract Preparation

1. Harvest tissue to be analyzed. Tissues my be flash frozen, stored at -70°C and prepared at a later time, if desired.
2. Calculate the amount of Extraction Reagent required (Not Supplied). An appropriate reagent could be 1X Wash Buffer with the protease inhibitor. For each 0.5 cm^3 piece of tissue, use 1 mL of Extraction Reagent.
3. Add protease inhibitors to the Extraction Reagent. Examples of an appropriate protease inhibitor cocktail includes 0.1 mM PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin, and 1 µg/mL pepstatin. Alternatively, a commercially available protease cocktail, obtainable from a variety of scientific reagent vendors, may also be used.
4. Place the tissue in a mortar and add sufficient volume of liquid nitrogen to cover the tissue.
5. Allow the liquid nitrogen to evaporate. The tissue should be thoroughly frozen.
6. Grind the frozen tissue to a powder with a pestle.

7. Add appropriate amount of ice-cold Extraction Reagent including protease inhibitors to the processed tissue.
8. Continue to homogenize the tissue with the pestle until the tissue suspension is homogeneous.
9. Transfer the extract to a fresh micro centrifuge tube and centrifuge at ~21,000 x g for 10 minutes at 4°C.
10. Transfer the supernatant (tissue extract) to a fresh tube for analysis. Avoid disturbing the cell pellet. Discard the cell pellet once the supernatant is harvested. Filter using a 0.2micron PES membrane (ie. Spin column). The tissue extract is now ready for analysis in the assay.
11. Alternatively, store the tissue extract in single-use aliquots at -70°C. It is recommended that a protein determination assay be performed and the extracts aliquoted into convenient amounts prior to storing at -70°C to avoid multiple freeze-thaw cycles.

Reagent Handling/ Preparation

Standard Preparation

NOTE: Standards are meant to be used within the time frame of the kit. Do NOT reconstitute until you are ready to proceed with the rest of the test. If you have extra at the end, it can be stored at -80˚C for a few days at maximum.

1. Reconstitute standard vial with 567 µL of Standard and Sample Diluent for a concentration of 100 ng/mL. Mix well.
2. Label seven (7) tubes, one for each additional standard curve point: 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.56 ng/mL, and 0.78 ng/mL.
3. Pipet 250 µl of Standard and Sample Diluent into each tube.
4. Serial dilute the 100 ng/mL standard 1:1 with Standard and Sample Diluent. Perform dilution by mixing 250 µL of the previous standard with 250 µL of Standard and Sample Diluent. Continue until reach the standard value of 0.78 ng/mL.
5. Use Standard and Sample Diluent only as the zero standard value.

1X Wash Buffer Preparation

- Prepare 1X Wash buffer by diluting 10X Wash Buffer in ultra pure water. For example, if preparing 1 L of 1X Wash Buffer, dilute 100 mL of 10X Wash Buffer into 900 mL of ultra pure water. Mix well. Store reconstituted 1X Wash Buffer at 2-8°C for up to one (1) month. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.

Biotinylated Antibody Working Solution Preparation

- Determine the amount of Biotinylated Antibody Working Solution required. For every strip-well used (8-wells), prepare 1 mL of Biotinylated Antibody Working Solution.
- Prepare Biotinylated Antibody Working Solution by diluting Biotinylated Antibody Concentrate 1:100 with Biotinylated Antibody Diluent. For example, if 12 mL of Biotinylated Antibody Working Solution is required (one whole plate), dilute 120 µL of Biotinylated Antibody Concentrate in 12 mL Biotinylated Antibody Diluent. Mix well prior to use.

Streptavidin Poly HRP Working Solution Preparation

- Determine the amount of Streptavidin Poly HRP Working Solution required. For every strip-well used (8-wells), prepare 1 mL of Streptavidin Poly HRP Working Solution.
- Prepare Streptavidin Poly HRP Working Solution by diluting Streptavidin Poly HRP Concentrate 1:100 with Streptavidin Poly HRP Diluent. For example, if 12 mL of Streptavidin Poly HRP Working Solution is required (one whole plate), dilute 120 µL of Streptavidin Poly HRP Concentrate in 12 mL Streptavidin Poly HRP Diluent. Mix well prior to use.

Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. *NOTE: If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 2-4°C. Be sure the packet is sealed with the desiccant inside.*

For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 2, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis. We suggest you record the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	Blk	Blk	Blk	8	8	8	16	16	16
B	S2	S2	S2	1	1	1	9	9	9	17	17	17
C	S3	S3	S3	2	2	2	10	10	10	18	18	18
D	S4	S4	S4	3	3	3	11	11	11	19	19	19
E	S5	S5	S5	4	4	4	12	12	12	20	20	20
F	S6	S6	S6	5	5	5	13	13	13	21	21	21
G	S7	S7	S7	6	6	6	14	14	14	22	22	22
H	S8	S8	S8	7	7	7	15	15	15	23	23	23

S1 – S7: 50 - 0.78 ng/ml Standards
S8: Zero Standard
Blk: Blank
1 – 23: Samples

Figure 2. Sample plate format

Performing the Assay

Assay Hints

- Use different tips to pipette the buffer, standard, sample, and antibody.
- 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.
- Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.

Well	Standard OR Sample Preparation	Standard and Sample Diluent	Antibody Preparation	Antibody Diluent	Total Volume Per Well
Standard (S1-S7)	100 µL	Included in Standard Prep	100 µL	Included in Ab. Prep	200 µL
Zero Standard (S8)	-	100 µL	100 µL	Included in Ab. Prep	200 µL
Blank	-	100 µL	-	100 µL	200 µL
Samples (1-23)	100 µL	Included in Sample Prep	100 µL	Included in Ab. Prep	200 µL

Table 1: Pipetting Summary

Sample Incubation

- Determine the number of strips required. Leave these strips in the plate frame. Place unused strips in the foil pouch with desiccant and seal tightly. Store unused strips at 2-8°C. After completing assay, keep the plate frame for additional assays.
- Use a Plate Template to record the locations of the standards and unknown samples within the wells.

1. Add 100 µL of appropriately diluted standards or samples to each well. Run each standard, sample, or blank in duplicate.
2. Carefully cover wells with a new adhesive plate cover. Incubate for one (1) hour at Room Temperature.
3. Carefully remove adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

Plate Washing

1. Gently squeeze the long sides of plate frame before washing to ensure all strips remain securely in the frame.
2. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with 1X Wash Buffer, then empty plate contents. Repeat procedure three additional times for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate plate contents from all wells and flood wells with 1X Wash Buffer. Repeat procedure three additional times for a total of FOUR washes. Additional washes may be necessary. Blot plate onto paper towels or other absorbent material.

Take care to avoid microbial contamination of equipment. Automated plate washers can easily become contaminated thereby causing assay variability.

Biotinylated Antibody Incubation

- Prepare only the required amount of Biotinylated Antibody Working Solution for the number of strips being used.

1. Add 100 µL of Biotinylated Antibody Working Solution to each well containing standard, or sample. Mix well by gently tapping the plate several times.
2. Carefully attach a new adhesive plate cover. Incubate plate for one (1) hour at Room Temperature.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

Streptavidin Poly HRP Incubation

- Prepare only the required amount of Streptavidin Poly HRP Working Solution for the number of strips being used.

1. Add 100 µL of Streptavidin Poly HRP Working Solution to each well containing standard, or sample.
2. Carefully attach a new adhesive plate cover. Incubate plate for 30 minutes at Room Temperature.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FOUR times with 1X Wash Buffer as described in the Plate Washing section.

TMB Substrate Incubation and Reaction Stop

- Only remove the required amount of TMB Substrate and Stop Solution for the number of strips being used.
- Do NOT use a glass pipette to measure the TMB Substrate solution. Do NOT cover the plate with aluminum foil or metalized mylar. Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate. If the solution is blue before use, DO NOT USE IT!

1. Add 100 µL of TMB Substrate into each well.
2. Allow the enzymatic color reaction to develop at room temperature (20-25°C) in the dark for 30 minutes. The substrate reaction yields a blue solution.
3. After 30 minutes, stop the reaction by adding 100 µL of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.

Absorbance Measurement

Note: Evaluate the plate within 30 minutes of stopping the reaction.

1. Wipe underside of wells with a lint-free tissue.
2. Measure the absorbance on an ELISA plate reader set at 450 nm.

ANALYSIS

Data Analysis Methods

This kit can be analyzed using any of the following methods:

A. Many plate readers come with data reduction software that plot data automatically.

B. The following procedure is recommended for preparation of the data prior to graphical analysis.

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD bound.
2. Plot Net OD versus Concentration of Rat GRP78 for the standards. Sample concentrations may be calculated off of Net OD values using the desired curve fitting.
3. Samples that read at concentrations outside of the standard curve range will need to be re-analyzed using a different dilution. Make sure to multiply sample concentrations calculated off the curve by the dilution factor used during sample preparation to get starting sample concentration.

Performance Characteristics

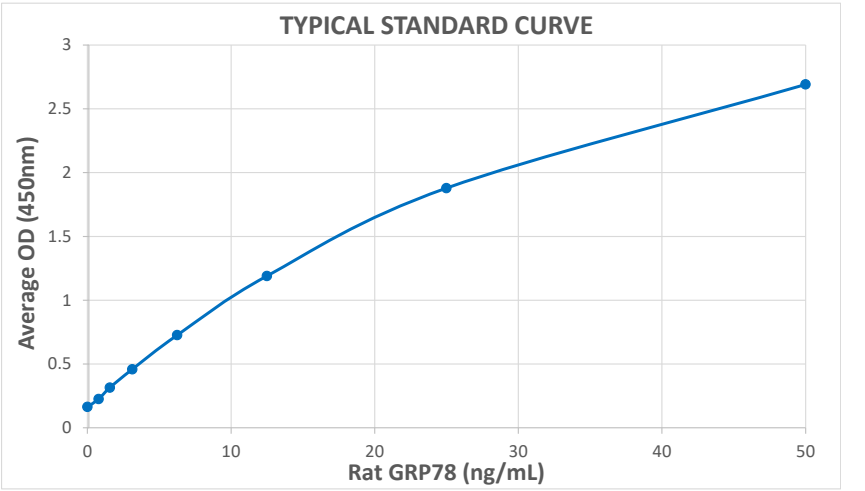

Figure 4. Typical standard curve
NOTE: This typical standard curve was generated using the Rat GRP78 ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.

Assay Range: 0 - 50 ng/mL

Sensitivity:

- The sensitivity of the Rat GRP78 ELISA kit has been determined to be 0.15 ng/mL.

Precision:

1. Intra-Assay Precision (Within Run Precision)

- To determine Intra-Assay Precision, three samples of known concentration were assayed thirty times on one plate. The intra-assay coefficient of variation of the Rat GRP78 ELISA has been determined to be <10%.

2. Inter-Assay Precision (Between Run Precision)

- To determine Inter-Assay Precision, three samples of known concentration were assayed thirty times in three individual assays. The inter-assay coefficient of variation of the Rat GRP78 ELISA has been determined to be <15%.

Assay Limitations:

- If samples generate greater values than the highest standard, the samples should be re-assayed at a higher sample dilution. Similarly, if samples generate lower values than the lowest standard, the samples should be re- assayed at a lower sample dilution.
- The use of assay reagents not provided in this kit or amendments to the protocol can compromise the performance of this assay.
- The components in each kit lot number have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Poor Standard Curve	A. Improper standard solution B. Standard degraded C. Curve doesn't fit scale D. Pipetting Error	A. Confirm dilutions are made correctly. B. Store and handle standard as recommended. C. Try plotting using different scales D. Use calibrated pipettes and proper pipetting technique.
No Signal	A. Plate washings too vigorous B. Wells dried out	A. Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually. B. Do not allow wells to dry out. Cover the plate for incubations.
High Background	A. Wells are insufficiently washed B. Contaminated wash buffer C. Waiting too long to read the plate after adding stop solution	A. Wash wells as per protocol B. Prepare fresh wash buffer C. Read plate immediately
Low sensitivity	A. Standard is degraded B. Mixing or substituting reagents from other kits	A. Replace standard B. Avoid mixing components

References

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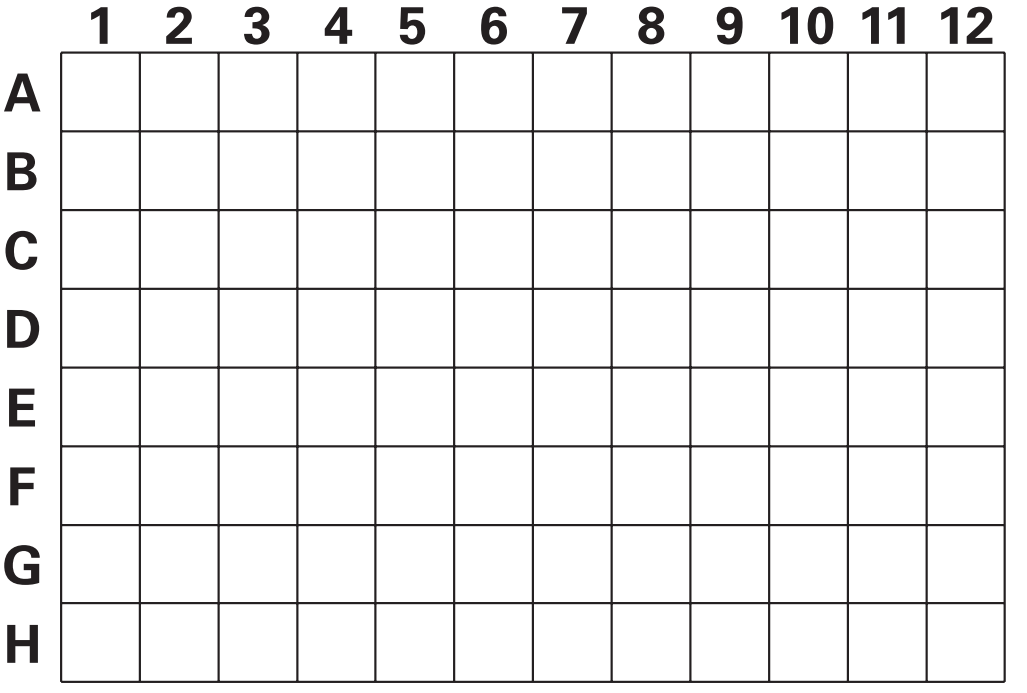
Warranty and Limitation of Remedy

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.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NOTES