StressMarq Biosciences INC.

Discovery through partnership | Excellence through quality

StressXpress®

HSP70 ELISA Kit

Catalog# SKT-105-96 (96-Well Kit)

Catalog# SKT-105-480 (5 x 96-Well Kit)

Colorimetric detection of HSP70

TABLE OF CONTENTS

GENERAL INFORMATION

Materials Supplied

Catalog No.	Item	Quantity/Size
SKC-105A	Anti-Hsp70 Immunoassay Plate	1 Plate
SKC-105B	5X Hsp70 Extraction Reagent	1 vial/10mL
SKC-105C	Recombinant Hsp70 Standard	2 vials
SKC-105D	Standard and Sample Diluent	1 vial/50mL
SKC-105E	10X Wash Buffer Concentrate	1vial/100mL
SKC-105F	Anti-Hsp70 Biotinylated Antibody Concentrate	1 vial/150µL
SKC-105G	Anti-Hsp70 Biotinylated Antibody Diluent	1 vial/13mL
SKC-105H	Streptavidin: HRP Concentrate	1 vial/150µL
SKC-105I	Streptavidin: HRP Diluent	1 vial/13mL
SKC-105J	TMB Substrate	1 vial/13mL
SKC-105K	Stop Solution	1 vial/13mL
SKC-105L	Pre-treatment Buffer	1 vial/ 13mL

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

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

All reagents are stable as supplied at 4°C. Unused wells should be resealed with desiccant in the foil pouch provided, and stored at 4°C until the kits expiry date.

Materials Needed But Not Supplied

- Ultra pure water
- Additional reagents and materials for cell lysate and tissue extract preparation, including protease inhibitors
- Precision pipettors, with disposable plastic tips
- Polypropylene or polyethylene tubes to prepare samples − do not use polystyrene, polycarbonate or glass tubes
- A container to prepare 1X Wash Buffer
- A wash bottle or an automated 96-well plate washer

- Disposable reagent reservoirs
- A standard microtiter plate reader for measuring absorbance at 450 nm
- Adhesive plate sealers

Assay Precautions

- All ELISA reagents must be at room temperature (20-25°C) 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 TMB Substrate 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 performing the assay. Please follow proper disposal procedures.

INTRODUCTION

Background

Hsp70 genes encode abundant heat-inducible 70-kDa hsps (hsp70s). In most eukaryotes hsp70 genes exist as part of a multigene family. They are found in most cellular compartments of eukaryotes including nuclei, mitochondria, chloroplasts, the endoplasmic reticulum and the cytosol, as well as in bacteria. The genes show a high degree of conservation, having at least 5O% identity (2). The N-terminal two thirds of hsp70s are more conserved than the C-terminal third. Hsp70 binds ATP with high affinity and possesses a weak ATPase activity which can be stimulated by binding to unfolded proteins and synthetic peptides (3). When hsc70 (constitutively expressed) present in mammalian cells was truncated, ATP binding activity was found to reside in an N-terminal fragment of 44 kDa which lacked peptide binding capacity. Polypeptide binding ability therefore resided within the C-terminal half (4). The structure of this ATP binding domain displays multiple features of nucleotide binding proteins (5). All hsp70s, regardless of location, bind proteins, particularly unfolded ones. The molecular chaperones of the hsp70 family recognize and bind to nascent polypeptide chains as well as partially folded intermediates of proteins preventing their aggregation and misfolding. The binding of ATP triggers a critical conformational change leading to the release of the bound substrate protein (6). The universal ability of hsp70s to undergo cycles of binding to and release from hydrophobic stretches of partially unfolded proteins determines their role in a great variety of vital intracellular functions such as protein synthesis, protein folding and oligomerization and protein transport.

About This Assay

StressMarq Biosciences Inc.'s StressXpress® ELISA Kit is for the detection of human Hsp70 in cell lysates, and tissue extracts. Each kit contains sufficient components to quantitate the Hsp70 concentration in up to 40 samples, tested in duplicate.

Validation of Capture and Detection Antibodies

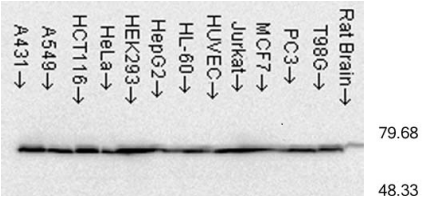


Figure 1: Western blot analysis of Hsp70 using the capture antibody

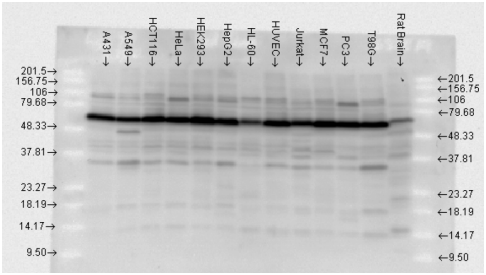


Figure 2: Western blot analysis of Hsp70 using the detection antibody

ASSAY OVERVIEW

1. Prepare Standard and samples in Standard and Sample Diluent.
2. Add 100 µL of Standard to appropriate wells.

   Note. Run each standard in duplicate.
3. Add 50 µL of Pre-Treatment Buffer to all sample wells.
4. Add 50 µL of sample to appropriate wells.

   Note: Run each sample in duplicate.
5. Cover plate with Plate Sealer and incubate at 37°C for 2 hours.
6. Wash plate four times with 1X Wash Buffer.

   Note: Gently squeeze the sides of the plate frame before washing to ensure all strips remain securely in the frame. Empty plate contents. Use a squirt 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.
7. Add 100 µL of Detection Antibody Working Solution to each well.
8. Cover plate with Plate Sealer and incubate at 37°C for 2 hours.
9. Wash plate four times with 1X Wash Buffer as described in step 6.
10. Add 100 µL of Streptavidin-HRP Working Solution to each well.
11. Cover plate with Plate Sealer and incubate at room temperature for 30 minutes.
12. Wash plate four times with 1X Wash Buffer as described in step 6.
13. Add 100 µL of TMB Substrate to each well.
14. Develop the plate in the dark at room temperature for 30 minutes.

    Note: Do not cover plate with Plate Sealer.
15. Stop reaction by adding 100 µL of Stop Solution to each well.
16. Measure absorbance on a plate reader at 450 nm.

Sample Preparation

Cell Lysate Preparation

1. Prepare and treat cells as desired.
2. For adherent cells, remove media and rinse cells with ice-cold PBS. Harvest cells with trypsin-EDTA or by using a cell scraper. Centrifuge at 500 x g for 5 minutes. For suspension cells, harvest by centrifugation at 500 x g for 5 minutes.
3. Wash cells by re-suspending the cell pellet in ice-cold PBS. Pellet cells by centrifugation at 500 x g for 5 minutes. Repeat wash for a total of three (3) washes with ice-cold PBS.
4. Use a pipette to carefully remove and discard the supernatant, leaving the cell pellet as dry as possible. The cell pellet may be frozen at -70ºC and lysed at a later time, if desired.
5. Calculate the amount of 1X Extraction Reagent required. For every 1 X 10^6 to 1X 10^7 cells, use 1 mL of 1X Extraction Reagent.
6. Prepare 1X Extraction Reagent by diluting 1 part 5X Extraction Reagent with 4 parts ice-cold ultra pure water. For example, if 5 mL of 1X Extraction Buffer is required, dilute 1 mL of 5X Extraction Reagent with 4 mL of ultra pure water.

Note: Use of alternative extraction buffers may contain components which could interfere and compromise the performance of the assay, producing inaccurate results. For best results, use the 1X Extraction Reagent included in this kit.

7. Add protease inhibitors to the 1X 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.
8. Add appropriate amount of ice-cold 1X Extraction Reagent including protease inhibitors to the cell pellet.

Note: If excess buffer is used for lysed cells, the protein concentration will be low.

9. Pipet up and down to break up the cell pellet until the cell suspension is homogeneous and no clumps are visible.
10. Incubate on ice for 30 minutes with occasional mixing or sonification.

Note: To increase protein yields and decrease sample viscosity, aspirate the cell pellet 5-10 times through a 21 ½ gauge needle or sonicate the cell pellet for 30 seconds with 50% pulse during the incubation.

11. Transfer the mixture to a fresh micro centrifuge tube and centrifuge at ~21,000 x g for 10 minutes at 4ºC.
12. Transfer the supernatant (cell lysate) to a fresh tube for analysis. Avoid disturbing the cell pellet. Discard the cell pellet once the supernatant is harvested. The cell lysate is now ready for analysis in the assay.
13. Alternatively, store the cell lysate in single-use aliquots at -70ºC. It is recommended that a protein determination assay be performed and the extracts aliquoted into convenient amounts prior to storing at -70ºC to avoid multiple freeze-thaw cycles.

Tissue Extract Preparation

1. Harvest tissue to be analyzed. Tissues my be flash frozen, stored at -70ºC and prepared at a later time, if desired.
2. Calculate the amount of 1X Extraction Reagent required. For each 0.5 cm^3 piece of tissue, use 1 mL of 1X Extraction Reagent.
3. Prepare 1X Extraction Reagent by diluting 1 part 5X Extraction Reagent with 4 parts ice-cold ultra pure water. For example, if 5 mL of 1X Extraction Buffer is required, dilute 1 mL of 5X Extraction Reagent with 4 mL of ultra pure water.

Note: For best results, use the 1X Extraction Reagent included in this kit.Use of alternative extraction buffers may contain interfering components and compromise assay performance.

4. Add protease inhibitors to the 1X 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.

5. Place the tissue in a mortar and add sufficient volume of liquid nitrogen to cover the tissue.
6. Allow the liquid nitrogen to evaporate. The tissue should be thoroughly frozen.
7. Grind the frozen tissue to a powder with a pestle.
8. Add appropriate amount of ice-cold 1X Extraction Reagent including protease inhibitors to the processed tissue.
9. Continue to homogenize the tissue with the pestle until the tissue suspension is homogeneous.
10. Transfer the extract to a fresh micro centrifuge tube and centrifuge at ~21,000 x g for 10 minutes at 4ºC.
11. 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. The tissue extract is now ready for analysis in the assay.
12. Alternatively, store the tissue extract in single-use aliquots at -70ºC. It is recommended that a protein determination assay be performed and the extracts aliquoted into convenient amounts prior to storing at -70ºC to avoid multiple freeze-thaw cycles.

Sample Handling

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions when handling and disposing of infectious agents.
- 100 µl of diluted sample is required per well.
- Samples must be assayed in duplicate each time the assay is performed.
- Samples should be frozen if not analyzed shortly after harvest. For long-term storage, aliquot and freeze samples. Avoid repeated freeze-thaw cycles when storing samples.
- If particulate is present in samples, centrifuge prior to analysis.
- If the integrity of the sample is of concern, make a note on the Plate Template and interpret results with caution.

Sample Dilution

- Samples must first be diluted prior to testing.
- Suggested starting dilutions for samples:

  For cell and tissue lysates, dilute samples 1:4 in Standard and Sample Diluent. For example, dilute 35 µL of sample in 105 µL Standard and Sample Diluent. Mix well.

Note: If values for samples are not within the range of the standard curve, optimal sample dilutions need to be determined.

- Prepare at least 125 µL of sample in Standard and Sample Diluent. Mix samples well prior to analysis.

Note: Serum or plasma samples are not recommended.

Other Reagent Handling/ Preparation

Standard Preparation

1. Label seven (7) tubes, one for each of the standard curve points: 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.563 ng/mL and 0.781 ng/mL.
2. Reconstitute the Recombinant Hsp70 Standard vial with 1 mL of Standard and Sample Diluent for a concentration of 83.3 ng/mL. Mix well.
3. Add 400 µL of Standard and Sample Diluent to Tube #1
4. Add 250 µL of Standard and Sample Diluent to Tubes #2, 3, 4, 5, 6 and 7.
5. Add 600 µL of the 83.3 ng/mL reconstituted standard to Tube #1 for a concentration of 50 ng/mL. Mix well.
6. Transfer 250 µL from Tube #1 to Tube #2. Mix well.
7. Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, mix well, etc.) up to and including Tube #7.
8. Finally, add 250 µL of Sample and Standard Diluent to another tube (Tube #8), which is the zero standard (0 ng/mL).

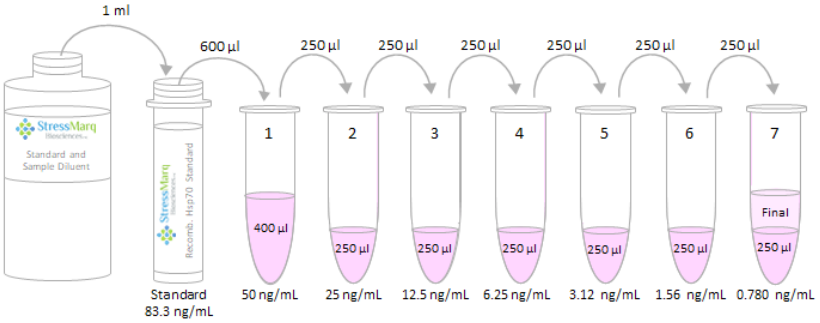


Figure 3. Preparation of the Hsp70 Standard

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 2-8ºC 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-HRP Working Solution Preparation

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

Performing the Assay

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 standard to each appropriate well. Run each standard or blank in duplicate.
2. Add 50 µL of Pre-Treatment Buffer and add 50 µL of appropriately diluted sample to all sample wells.
3. Carefully cover wells with a new adhesive plate cover. Incubate for 37°C for 2 hours.
4. 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.

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, sample or blank. Mix well by gently tapping the plate several times.
2. Carefully attach a new adhesive plate cover. Incubate plate for two (2) hours at 37°C.
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-HRP Incubation

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

1. Add 100 µL of Streptavidin-HRP Working Solution to each well containing standard, sample or blank.
2. Carefully attach a new adhesive plate cover. Incubate plate for 30 minutes at room temperature, 20-25°C.
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. Do NOT cover plate with a plate sealer. 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

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used.

Calculations

- Duplicate absorbance values should be within 10% of each other. Care should be taken when interpreting data with differences in absorbance values greater than 10%.

1. Prepare a standard curve to determine the amount of Hsp70 in an unknown sample. Plot the average absorbance obtained for each standard concentration on the vertical (Y) axis versus the corresponding Hsp70 concentration on the horizontal (X) axis using graph paper or curve-fitting software.
2. Calculate the Hsp70 concentration in unknown samples using the prepared standard curve. Determine the amount of Hsp70 in each unknown sample by noting the Hsp70 concentration (X axis) that correlates with the absorbance value (Y axis) obtained for the unknown sample.
3. Multiply the Hsp70 concentration obtained by the dilution factor to determine the amount of Hsp70 in the undiluted sample.

Performance Characteristics

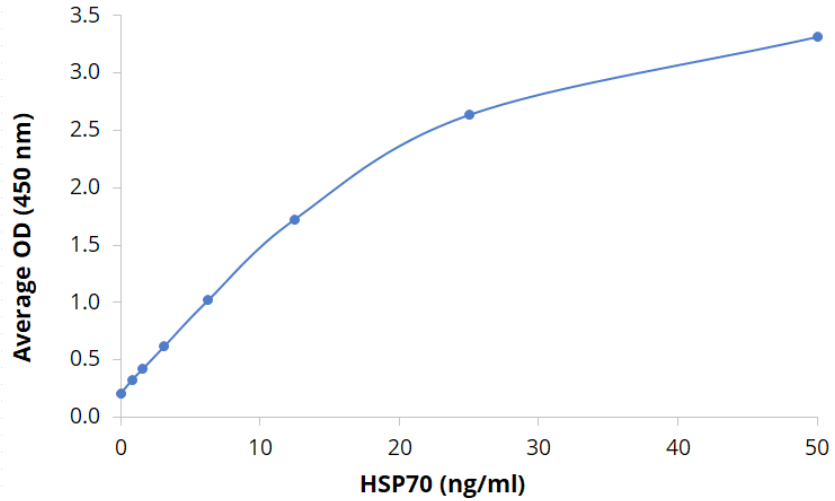


Figure 4. Typical standard curve

Note: This typical standard curve was generated using the Hsp70 ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.

Assay Range: 0.781-50 ng/mL

- Suggested standard curve points are 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.563 ng/mL, 0.781 ng/mL, and 0 ng/mL.

Assay Specificity and Species Reactivity

- This assay is specific for Hsp70.
- The Hsp70 ELISA has been validated for the detection of human Hsp70

Sensitivity

- The calculated sensitivity of the Hsp70 ELISA is 0.18 ng/mL.

Assay Limitations

- This assay has been validated for use with cell lysates, and tissue extracts. Other sample types or matrices (e.g. urine, cerebrospinal fluid, cell culture supernatant, etc.) may contain interfering factors that can compromise the performance of the assay or produce inaccurate results.
- Some samples may contain higher levels of interfering factors that can produce abnormal results.
- If values for samples are not within the range of the standard curve, optimal sample dilutions need to be determined.
- The use of assay reagents not provided in this kit can compromise the performance of this assay.
- Do not mix components with reagents from other kits with different lot numbers.

References

1. Zho, J. (1998) *Cell.* 94: 471-480.
2. Boorstein W. R., Ziegelhoffer T. & Craig E. A. (1993) *J. Mol. Evol*. 38 (1) 1-17.
3. Rothman J. (1989) *Cell* 59: 591 -601.
4. DeLuca-Flaherty *et al.* (1990) *Cell*. 62: 875-887.
5. Bork P., Sander C. & Valencia A. (1992) *Proc. Nut1 Acad. Sci. USA* 89: 7290-7294.
6. Fink A.L. (1999) *Physiol. Rev.* 79: 425-449.
7. Smith D.F., et al., (1993) *Mol. Cell. Biol.* 13(2):869-876.
8. Prapapanich V., et al., (1996) *Mol. Cell. Biol.* 16(11):6200-6207.
9. Fernandez-Funez et al., (2000) *Nature.* 408(6808):101-106.

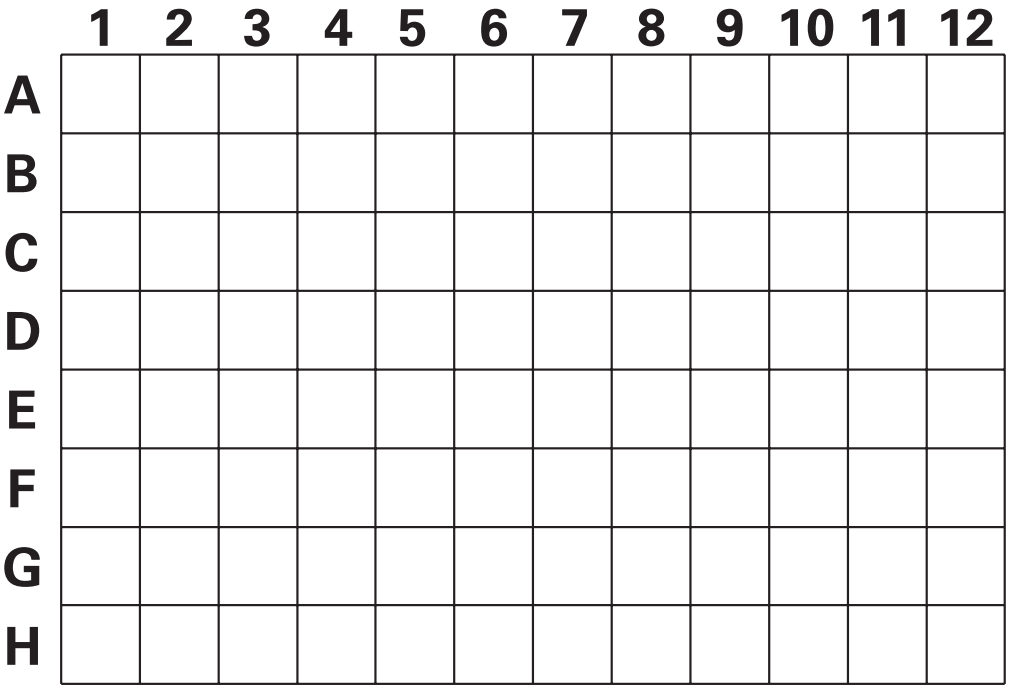
Warranty and Limitation of Remedy

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.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

NOTES