StressMarq Biosciences INC.

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MOLECULAR SIGNATURE®

Dibromo-tyrosine ELISA Kit

Catalog no. SKT-140-96 (96-Well Kit)

Catalog no. SKT-140-480 (5 x 96-Well Kit)

Colorimetric detection of dibromo-tyrosine

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GENERAL INFORMATION

Materials Supplied

Catalog No.	Item	Quantity/Size
SKC-140A	Dibromo-tyrosine: BSA Coated Plate	1 Plate
SKC-140C	Dibromo-tyrosine Standard	1 vial/75µl
SKC-140F	Dibromo-tyrosine: Biotin Conjugated Monoclonal Antibody	1 vial/75µl
SKC-140G	Streptavidin Poly HRP Concentrate	1 vial/150µl
SKC-140H	Streptavidin Poly HRP Diluent	1 vial/13 mL
SKC-0001	Sample and Standard Diluent (Red)	1 vial/50mL
SKC-0002	Dibromo-tyrosine Antibody Diluent (Blue)	1 vial/13mL
SKC-0003	Wash Buffer Concentrate (10X)	1 vial/ 50mL
SKC-0004	TMB Substrate	1 vial/13mL
SKC-0005	Stop Solution	1 vial/13mL
SKC-0009	Plate Covers	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 MOLECULAR SIGNATURE® 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, except the **Dibromo-tyrosine Standard,** which should be stored at **-20°C**. For optimum storage, the Dibromo-tyrosine Standard should be aliquoted into smaller portions and then stored appropriately. Avoid repeated freeze/thaw cycles (10 µL of standard can prepare a triplicate standard curve.

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 9-11).

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 room temperature before use in the assay.

INTRODUCTION

Background

Dibromo-tyrosine is produced by the oxidative bromination of tyrosine residues. This reaction occurs via eosinophil peroxidase (EPO), an enzyme released by activated eosinophils. Upon activation of eosinophils, a respiratory burst occurs releasing elevated levels of O2 and H202. In the oxidation of tyrosine, EPO utilizes H202 to catalyze the peroxidation of physiological levels of bromine found within plasma to generate the brominating reagent hypobromous acid (HOBr) (Figure 1). [1-5]

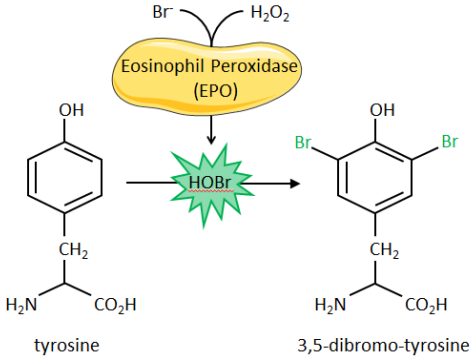

Figure 1. Bromination of tyrosine

Eosinophils play an immunomodulatory role through their recruitment to host sites of parasitic invasion. EPO levels also contribute to diseases such as asthma, cancers and allergic disorders where cellular activation is found to occur at pathological sites [6-10].

Brominated products such as 3,5-dibromo-tyrosine serve as biological markers for *in vivo* eosinophil-mediated tissue damage which allows for understanding the overall roll oxidative stress has on pathways implicated in diseased states within organisms [4]

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About This Assay

StressMarq's Dibromo-tyrosine ELISA is a competitive assay that can be used for the quantification of 3,5-dibromo-tyrosine in urine, plasma, and other sample matrices. The ELISA utilizes an dibromo-tyrosine-coated plate and an biotin-conjugated antibody for detection which allows for an assay range of 0.078 - 5 µg/mL, with a sensitivity of 0.04 µg/mL. Additional kit highlights are quick incubation times, stable reagents, and an easy to use protocol.

It is important to note that the dibromo-tyrosine antibody used in this assay recognizes both free dibromo-tyrosine and brominated residues within a protein. Since complex samples such as plasma, are comprised of mixtures of protein fragments and free 3,5-dibromo-tyrosine, concentrations of 3,5-dibromo-tyrosine reported by ELISA methodology may not coincide with literature values where the free residue is typically measured. This should be kept in mind when analyzing and interpreting experimental results.

Assay Overview

1. Prepare standard and samples in the Sample and Standard Diluent.
2. Add 50 µL of prepared standards and samples in triplicate to appropriate wells.
3. Add 50 µL of the diluted Dibromo-tyrosine-Biotin antibody to the appropriate wells.
4. Cover plate with Plate Cover and incubate at 37 °C for 1 hour.
5. Wash plate 4 times with 1X Wash Buffer.
6. Add 100 µL of Streptavidin-HRP Working Solution to each well.
7. Cover plate with Plate Sealer and incubate at room temperature for 30 minutes.
8. Wash plate 4 times with 1X Wash Buffer.
9. Add 100 µL of TMB Substrate to each well.
10. Develop the plate in the dark at room temperature (20-25°C) for 30 minutes.
11. Stop reaction by adding 100 µL of Stop Solution to each well.
12. Measure absorbance on plate reader at 450 nm.

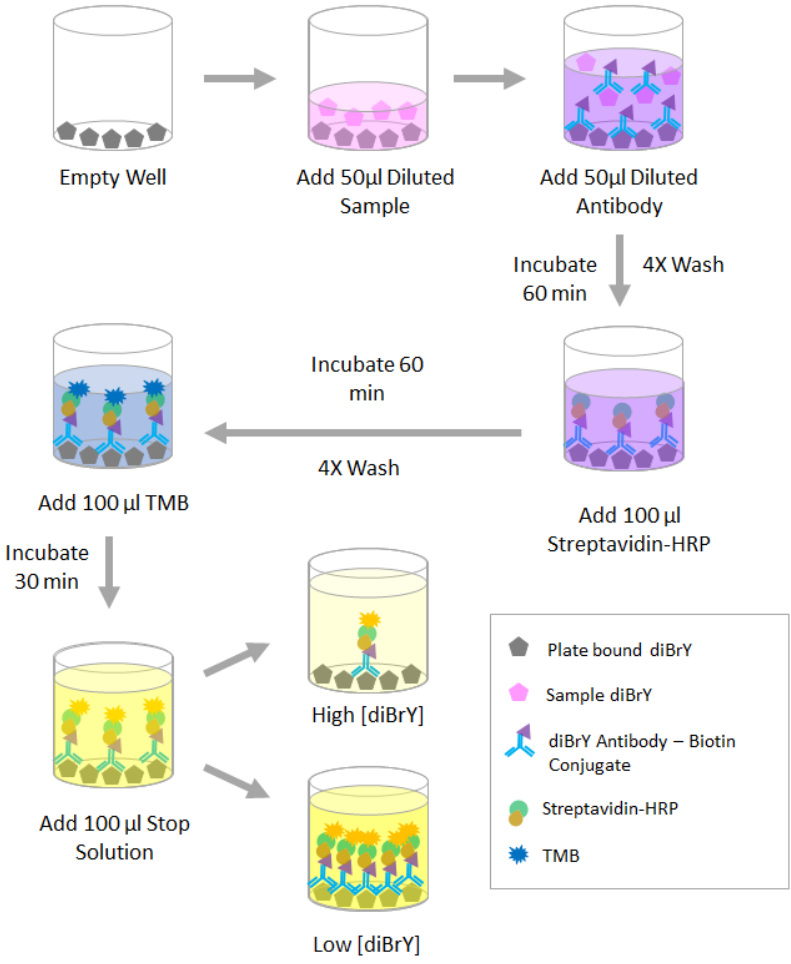

Figure 2. Schematic of the dibromo-tyrosine MOLECULAR SIGNATURE® competitive ELISA

PRE-ASSAY PREPARATION

Proper sample storage and preparation are essential for consistent and accurate results. Caution should be taken during sample work up, to avoid inadvertent oxidation of undamaged DNA. Please read this section thoroughly before beginning the assay.

NOTE: Prepare at least 180 µL of your diluted sample to permit assay in triplicate.

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.

Urine

Interference in urine is infrequent; dilutions appropriate for this assay show a direct linear correlation between dibromo-tyrosine immunoreactivity and dibromo-tyrosine concentration (see figure 3). Urinary concentrations of dibromo-tyrosine can vary considerably and can be standardized against creatinine levels if required.

Storage: Fresh urine samples should be centrifuged at 2,000 x g for 10 minutes or filtered with a 0.2 µm filter before this assay, and stored at -20°C immediately after collection.

Dilution: Dilute urine samples 1:1 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing. For example: 50 µL of sample into 50 µL of Sample and Standard Diluent.

Plasma/Serum

The concentration of free dibromo-tyrosine in plasma is very low relative to the level of Protein-incorporated dibromo-tyrosine. Glomerular filtration results in excretion of dibromo-tyrosine into the urine, while the Protein-incorporated dibromo-tyrosine remains in the blood. The differing fates of free versus Protein-incorporated dibromo-tyrosine should be considered in experimental design. If you choose to measure Protein-incorporated dibromo-tyrosine in plasma, it is possible to purify Protein using a commercially available kit and treat the Protein with a combination of proteases to liberate the individual amino acids. Due to the complexities of measuring dibromo-tyrosine in plasma, urine is often a more appropriate matrix.

Storage: Collect plasma using established methods and store at -80°C.

Dilution: Serum samples may be diluted 1:1 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

Culture Media Samples

Storage: Collect culture media samples and store at -80°C.

Dilution: Fetal bovine serum contains dibromo-tyrosine, therefore assays should either be performed in serum-free medium or PBS; these samples may be assayed directly. If the dibromo-tyrosine concentration is high enough to dilute the sample 10-fold with Sample and Standard Diluent, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted 1:10 with Sample and Standard Diluent), dilute the standards in the same culture medium as that used for the experiment. This will ensure that the matrix for the standards is comparable to the samples. We recommend that a standard curve be run first to ensure that the assay will perform in a particular culture medium.

Tissue Samples

Storage: Snap-freeze tissue samples in liquid nitrogen immediately after collection. Store at -80°C until use.

Usage: When ready to use the samples, thaw and add 5 ml of homogenization buffer (0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA) per gram of tissue. Homogenize the sample using either a Polytron-type homogenizer or a sonicator. Centrifuge at 1,000 x g for 10 minutes and purify the supernatant

using a commercially available protein extraction kit. Digest protein using X or equivalent) following the manufacturer's instructions. Adjust the pH to 7.5-8.5 using 1 M Tris. Add 1 unit of Y per 100 µg of protein and incubate at 37°C for 30 minutes. Boil for 10 minutes and place on ice until use.

Reagent Handling Preparation

Standard Preparation (S1-S8)

NOTE: The Standard should be aliquoted into smaller portions before use to ensure product integrity. Avoid freeze/thaw cycles. (10 µL of Standard can prepare a triplicate standard curve).

1. Centrifuge the Dibromo-tyrosine Standard vial before removing the cap. This process will assure that all of the standard is collected and available for use.
2. Label seven (7) polypropylene tubes, each with one of the following standard values: 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.625 µg/mL, 0.312 µg/mL, 0.156 µg/mL and 0.0781 µg/mL.
3. Add 500 µL of Sample and Standard Diluent to Tube #1.
4. Add 250 µL of Sample and Standard Diluent to Tube #2, 3, 4, 5, 6 and 7.
5. Add 10 µL of the Dibromo-tyrosine Standard (255 µg/mL) to Tube #1 for a concentration of 5 µg/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 Sample and Standard Diluent to another 1.5 mL polypropylene tube (Tube #8), which is the zero standard (0 µg/mL).

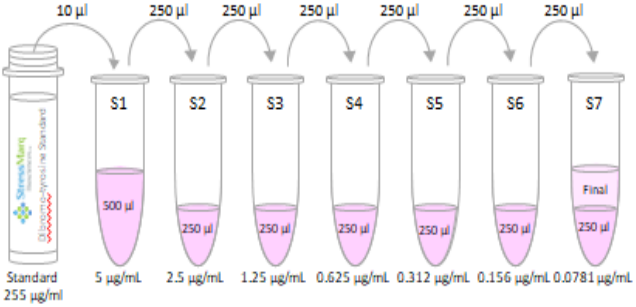

Figure 3. Preparation of dibromo-tyrosine standards

Dibromo-tyrosine-Biotin Conjugate Monoclonal Antibody Preparation

1. Determine the amount of Dibromo-tyrosine Antibody Working Solution required. For every strip-well used (8-wells), prepare 0.5 mL of working antibody solution.

2. Prepare the working antibody solution. by diluting the Dibromo-tyrosine-Biotin Conjugate Antibody Concentrate 1:100 with Dibromo-tyrosine Antibody Diluent. For example, if 6 mL of Antibody Preparation is required (one whole plate), dilute 60 µL of Antibody in 6 mL of Dibromo-tyrosine Antibody Diluent. Mix well prior to use.

Streptavidin Poly HRP Working Solution Preparation

1. Determine the amount of Streptavidin Poly HRP Working Solution required. For every strip-used (8-wells), prepare 1 mL of Streptavidin Poly HRP Working Solution

2. Prepare the 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.

1X Wash Buffer Preparation

1. Prepare 1X Wash buffer by diluting 10X Wash Buffer in distilled or deionized water. For example, if preparing 500 mL of 1X Wash Buffer, dilute 50 mL of 10X Wash Buffer into 450 mL of distilled water. Mix well.

Store reconstituted 1X Wash Buffer at 2-8 ºC for up to one (1) month. Do not use 1X Wash Buffer if it becomes visibly contaminated during storage.

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.*

A suggested plate format is shown in Figure 5, 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 (page 28)

	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: 5 - 0.078 µg/mL Standards
S8: Zero Standard
Blk: Blank
1 – 23: Samples

Figure 4. 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).
- Always add the Antibody Working Solution after the rest of the reagents, as this is a competitive assay.
- Tape the well strips together with lab tape 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)	50 µL	Included in Standard Prep	50 µL	Included in Ab. Prep	100 µL
Zero Standard (S8)	-	50 µL	50 µL	Included in Ab. Prep	100 µL
Blank	-	50 µL	-	50 µL	100 µL
Samples (1-23)	50 µL	Included in Sample Prep	50 µL	Included in Ab. Prep	100 µL

Table 1. Pipetting summary

Addition of the Reagents

1. Add 50 µl (in triplicate) of each of the following to appropriate wells:

- Prepared Dibromo-tyrosine Standard (Tube #1 through Tube #7) into wells labelled S1-S7.
- Zero Standard (Tube #8- Sample and Standard Diluent, which represents 0 ug/mL) into wells labelled S8.
- Samples (previously prepared - see **Sample Preparation**, pages 10-12) into wells labelled 1-23.

2. Add 50 µl of the previously diluted Dibromo-tyrosine Antibody Working Solution to each well, except the blank.

3. Add 50 µl of Standard and Sample Diluent and 50 µl of Antibody Diluent into wells labelled as the blank.

Incubate the Plate

- Cover each plate with the plate cover and incubate 1 hour at 37˚C.

Plate Washing

1. Carefully remove adhesive plate cover. Gently squeeze the long sides of the plate frame before washing to ensure all strips remain securely in the frame.
2. Empty plate contents. Use a multi-channel pipette to fill each well completely (300 µl) 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: Follow the same procedure when using an automated plate washer as well. Take care to avoid microbial contamination of equipment. Automated plated washers can easily become contaminated thereby causing assay variability.

Addition of the HRP Conjugate

- Add 100 µl of the Streptavidin-HRP working solution to each well containing standard, sample or blank.

Incubate the Plate

- Cover each plate with the plate cover and incubate for 30 minutes at room temperature (20-25˚C).

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 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. Cover carefully with the second provided plate cover.
3. Allow the enzymatic color reaction to develop at room temperature (20-25ºC) in the dark for 30 minutes. The substrate reaction yields a blue solution.
4. After 30 minutes, carefully remove the plate cover, and 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.

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. StressMarq Biosciences Inc. has a computer spreadsheet available for data analysis. Please visit our website (http://www.stressmarq.com/wp-content/uploads/SKT-140-Dibromo-Tyrosine-ELISA-Kit-Calculations-Worksheet.xlsx) to obtain a free copy of this convenient data analysis tool. Instructions on how to use this analysis tool are detailed on the excel file.

C. 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 dibromo-tyrosine 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

Standard Curve

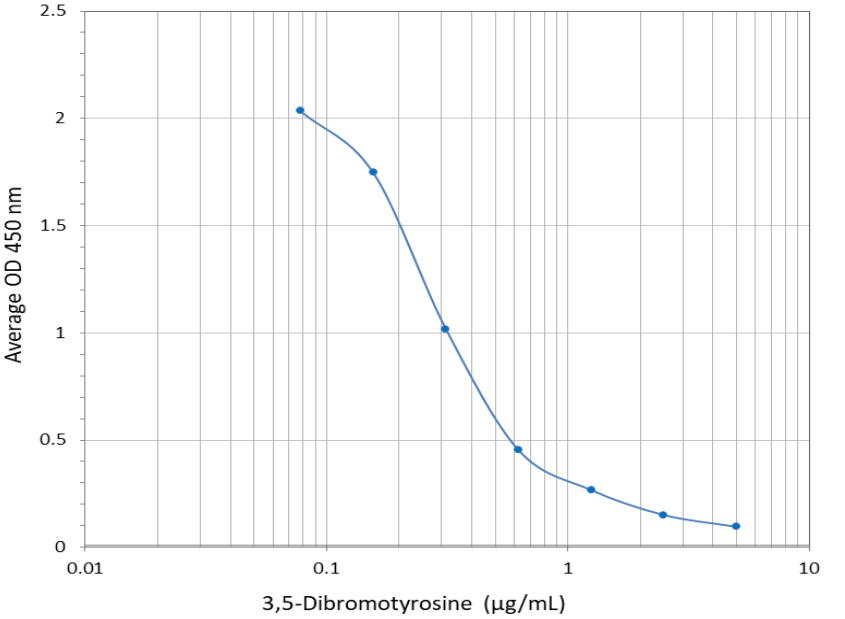

Figure 5. Typical standard curve

NOTE: This typical standard curve was generated using the Dibromo-tyrosine ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.

Assay range: 0.078 µg/mL - 5 µg/mL

Sensitivity

- The sensitivity of the Dibromo-tyrosine ELISA kit has been determined to be 0.04 µg/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 dibromo-tyrosine 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 dibromo-tyrosine has been determined to be <10%.

Specificity

- No detectable cross-reactivity was observed at assay range 0.078 µg/mL to 5 µg/mL for tyrosine, di-tyrosine, 3-nitro-tyrosine or 3-bromo-tyrosine.

Matrix Impact

- Spike recovery and linearity of dilution studies yielded 10% precision across dilutions

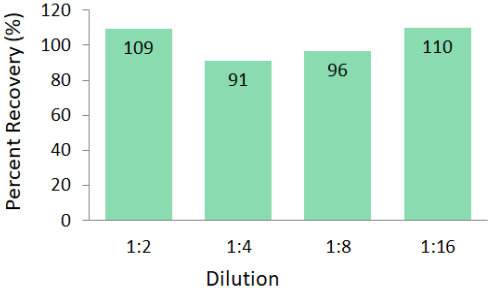

Figure 6. Dibromo-tyrosine spike recovery in human plasma

Urine Analysis

- Urine samples were spiked with dibromo-tyrosine, diluted as described in the Sample Preparation (page 10) section and analyzed using the dibromo-tyrosine ELISA Kit. The y-intercept corresponds to the amount of dibromo-tyrosine in un-spiked urine. Error bars represent standard deviations obtained from multiple dilutions of each sample.

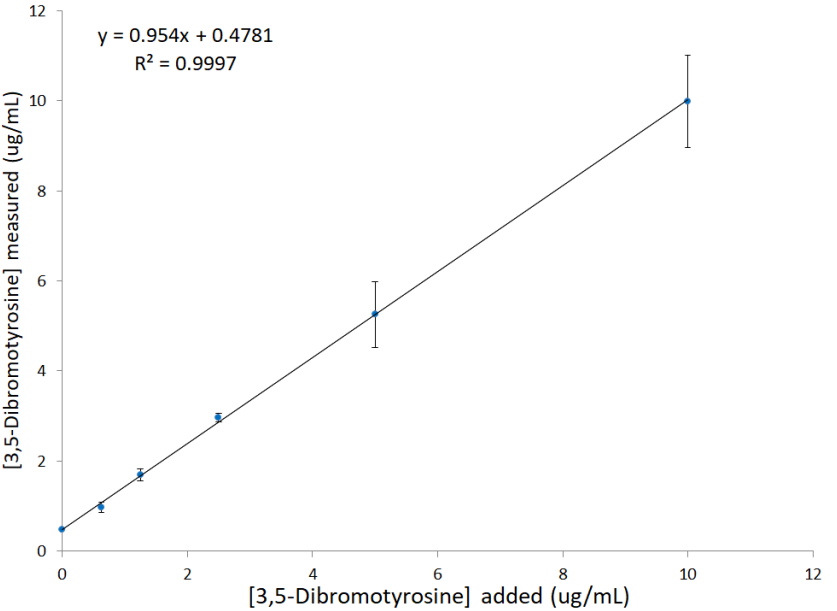

Figure 7. Recovery of dibromo-tyrosine from urine

Example: Sample Comparison

- Comparison of the 3,5-dibromo-tyrosine concentration between a diabetic smoker and a non-diabetic non-smoking individual was carried out using the Dibromo-tyrosine ELISA kit.

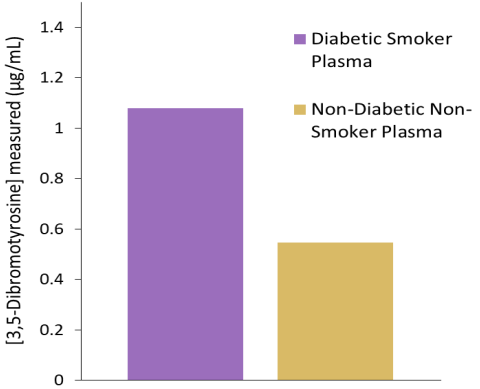

Figure 8. Comparison of dibromo-tyrosine in healthy versus diabetic smokers

NOTE: The above data is for illustrative purposes and should not be taken as a statistically relevant result. Each researcher must consider their sample population and type individually and draw their own conclusions to be statistically relevant or otherwise.

Assay Limitations

- This assay has been validated for use with urine and plasma. Other sample types or matrices (e.g. tissue and cell extracts, cerebrospinal fluid, cell culture supernatant, etc.) may contain interfering factors that can compromise the performance of the assay, or produce inaccurate results.
- 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

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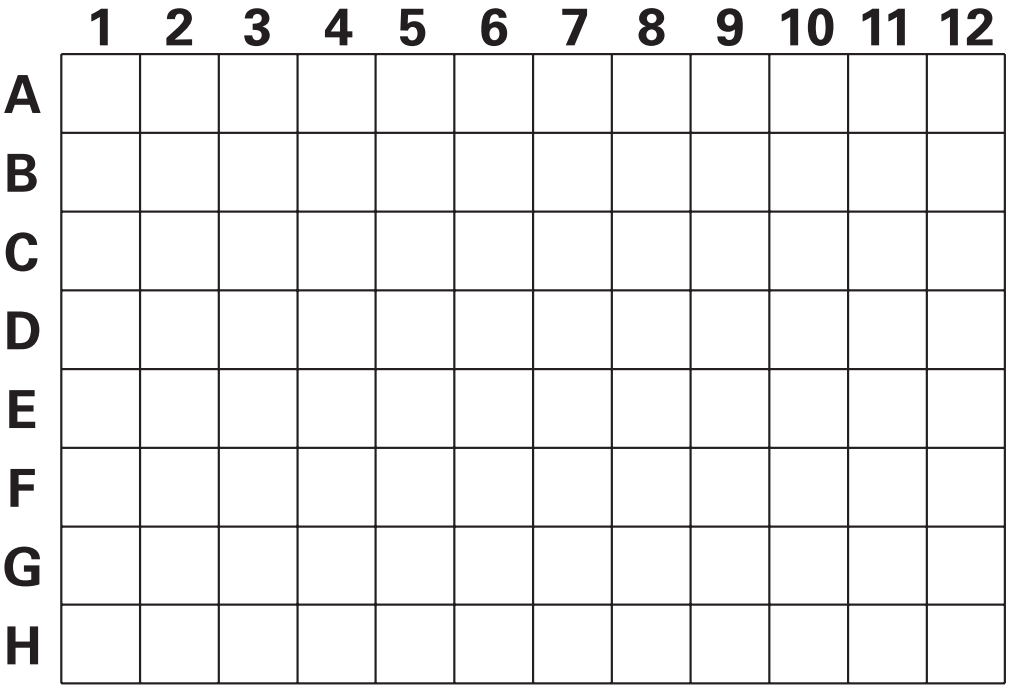
Warranty and Limitation of Remedy

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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