STRESSMARQ
BIOSCIENCES INC.
www.stressmarq.com

8-hydroxy-2-deoxy Guanosine EIA Kit

Catalog# SKT-120-96 (96 well kit)

Catalog# SKT-120-480 (480 well kit)

StressXpress®

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Materials Supplied

Catalog Number	Item	96 wells Quantity/Size	480 wells Quantity/Size
SKC-120A	8-hydroxy-2-deoxy Guanosine Monoclonal Antibody	1 vial/100 dtn	1 vial/500 dtn
SKC-120B	8-hydroxy-2-deoxy Guanosine AChE Tracer	1 vial/100 dtn	1 vial/500 dtn
SKC-120C	8-hydroxy-2-deoxy Guanosine EIA Standard	1 vial	1 vial
SKC-120D	EIA Buffer Concentrate (10X)	2 vials/10 ml	4 vials/10 ml
SKC-120E	Wash Buffer Concentrate (400X)	1 vial/5 ml	1 vial/12.5 ml
SKC-120F	Tween 20	1 vial/3 ml	1 vial/3 ml
SKC-120G	Goat Anti-Mouse IgG Coated Plate	1 plate	5 plates
SKC-120H	Plate Cover	1 cover	5 covers
SKC-120I	Ellman's Reagent	3 vials/100 dtn	6 vials/250 dtn
SKC-120J	EIA Tracer Dye	1 vial	1 vial
SKC-120K	EIA Antiserum Dye	1 vial	1 vial

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® EIA 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

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeat pipettor.
3. A source of 'UltraPure' water. Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for EIA.
4. Materials used for **Sample Preparation** (see page 12).

INTRODUCTION

Background

8-hydroxy-2-deoxy Guanosine (8-OH-dG) is produced by the oxidative damage of DNA (see Figure 1, on page 6) by reactive oxygen and nitrogen species and serves as an established marker of oxidative stress.[1-4] Hydroxylation of guanosine occurs in response to both normal metabolic processes and a variety of environmental factors (*i.e.*, anything that increases reactive oxygen and nitrogen species). Increased levels of 8-OH-dG are associated with the aging process as well as with a number of pathological conditions including cancer, diabetes, and hypertension.[5-9]

In complex samples such as plasma, cell lysates, and tissues, 8-OH-dG can exist as either the free nucleoside or incorporated in DNA. Once the blood enters the kidney, free 8-OH-dG is readily filtered into the urine, while larger DNA fragments remain in the bloodstream. Because of the complexity of plasma samples, urine is a more suitable matrix for the measurement of free 8-OH-dG than plasma. Urinary levels of 8-OH-dG range between 2.7-13 ng/mg creatine, while plasma levels of free 8-OH-dG have been reported to be between 4-21 pg/ml as determined by LC-MS.[10-11]

About This Assay

StressMarq's 8-OH-dG EIA is a competitive assay that can be used for the quantification of 8-OH-dG in urine, cell culture, plasma, and other sample matrices. The EIA utilizes an anti-mouse IgG-coated plate and a tracer consisting of an 8-OH-dG-enzyme conjugate. This format has the advantage of providing low variability and increased sensitivity compared to assays that utilize an antigen-coated plate. Our EIA typically displays IC_{50} (50% B/B_0) and IC_{80} (80% B/B_0) values of approximately 100 and 30 pg/ml, respectively.

It is important to note that the 8-OH-dG antibody used in this assay recognizes both free 8-OH-dG and DNA-incorporated 8-OH-dG. Since complex samples such as plasma, cell lysates, and tissues are comprised of mixtures of DNA fragments and free 8-OH-dG, concentrations of 8-OH-dG reported by EIA methodology will not coincide with those reported by LC-MS where the single nucleoside is typically measured. This should be kept in mind when analyzing and interpreting experimental results.

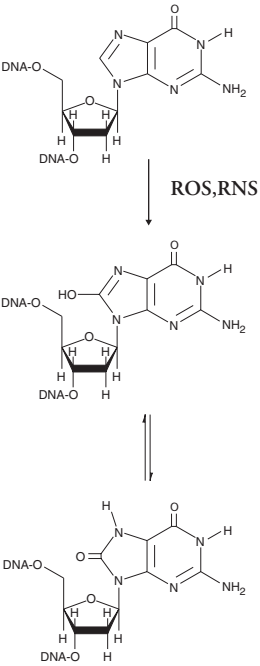


Figure 1. Oxidation of Guanosine

Description of StressXpress® Series Competitive EIAs[12,13]

This assay is based on the competition between 8-hydroxy-2-deoxy guanosine (8-OH-dG) and a 8-OH-dG-acetylcholinesterase (AChE) conjugate (8-OH-dG Tracer) for a limited amount of 8-OH-dG Monoclonal Antibody. Because the concentration of the 8-OH-dG Tracer is held constant while the concentration of 8-OH-dG varies, the amount of 8-OH-dG Tracer that is able to bind to the 8-OH-dG Monoclonal Antibody will be inversely proportional to the concentration of 8-OH-dG in the well. This antibody-8-OH-dG complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of 8-OH-dG Tracer bound to the well, which is inversely proportional to the amount of free 8-OH-dG present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound 8-OH-dG Tracer}] \propto 1/[\text{8-OH-dG}]$$

A schematic of this process is shown in Figure 2, below.

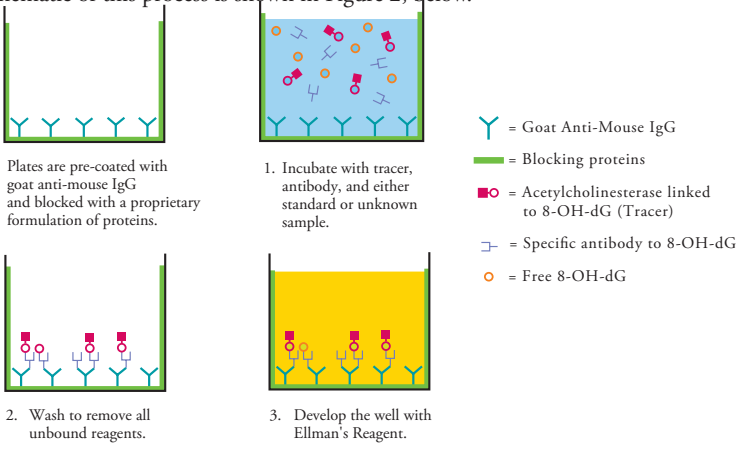


Figure 2. Schematic of the Molecular Signature™ Series EIA

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *Electrophorus electricus*, contains an avid acetylcholinesterase (AChE) capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s^{-1}) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in Molecular Signature™ Series enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman's Reagent. This reagent consists of acetylthiocholine and 5,5'-dithio-*bis*-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 3, on page 9). The non-enzymatic reaction of thiocholine with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a 'stop' reagent, and the plate may be read whenever it is convenient.

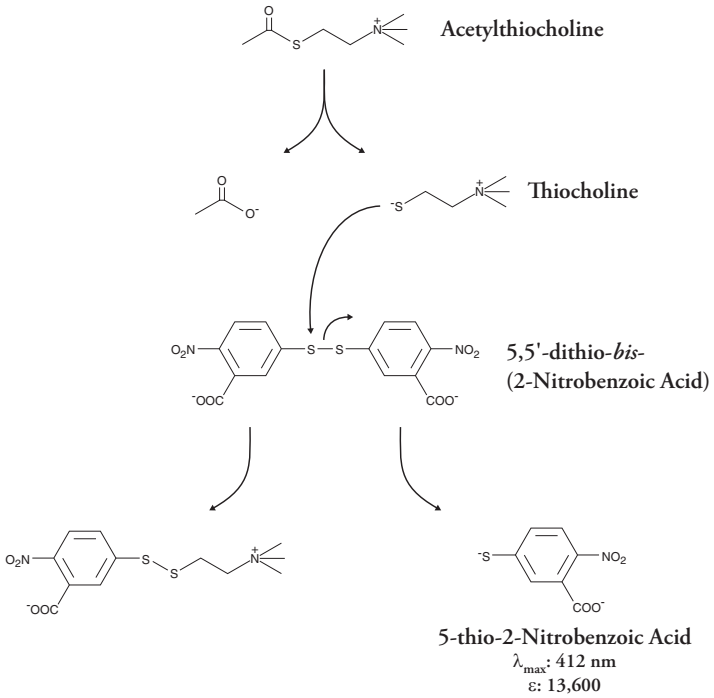


Figure 3. Reaction catalyzed by acetylcholinesterase

Definition of Key Terms

Blank: background absorbance caused by Ellman's Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

B_0 (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

%B/B_0 (%Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding (B_0) well.

Standard Curve: a plot of the %B/B_0 values *versus* concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for EIA.

Buffer Preparation

Store all buffers at 4°C; they will be stable for about two months.

1. **EIA Buffer Preparation**

   Dilute the contents of one vial of EIA Buffer Concentrate (**Catalog# SKC-120D**) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. *NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.*

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer (96-well kit; Catalog# SKC-120E): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Tween 20 (**Catalog# SKC-120F**).

   OR

   12.5 ml vial Wash Buffer (480-well kit; Catalog# SKC-120E): Dilute to a total volume of 5 liters with UltraPure water and add 2.5 ml of Tween 20 (**Catalog# SKC-120F**).

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Tween 20 (0.5 ml/liter of Wash Buffer).

NOTE: Tween 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

Sample Preparation

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

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.

Urine

Urine samples should be stored at -20°C immediately after collection. Interference in urine is infrequent; dilutions appropriate for this assay (*i.e.*, dilutions falling between 20-80% B_0) show a direct linear correlation between 8-OH-dG immunoreactivity and 8-OH-dG concentration (see figure 4, on page 14). Urinary concentrations of 8-OH-dG can vary considerably and can be standardized against creatinine levels if required.

Plasma/Serum

Collect plasma using established methods and store at -80°C. The concentration of free 8-OH-dG in plasma is very low relative to the level of DNA-incorporated 8-OH-dG. Glomerular filtration results in excretion of 8-OH-dG into the urine, while the DNA-incorporated 8-OH-dG remains in the blood. The differing fates of free *versus* DNA-incorporated 8-OH-dG should be considered in experimental design. If you choose to measure DNA-incorporated 8-OH-dG in plasma, it is possible to purify DNA using a commercially available kit and treat the DNA with a combination of nuclease and alkaline phosphatase to liberate the individual bases. Due to the complexities of measuring 8-OH-dG in plasma, urine is often a more appropriate matrix.

Culture Media Samples

Collect culture media samples and store at -80°C. Fetal bovine serum contains 8-OH-dG, therefore assays should either be performed in serum-free medium or PBS; these samples may be assayed directly. If the 8-OH-dG concentration is high enough to dilute the sample 10-fold with EIA Buffer, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted 1:10 with EIA Buffer), dilute the standard curve 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.

Cell Lysates

Collect lysates using established methods and store at -80°C until use. Purify DNA using a commercially available extraction kit. Digest DNA using nuclease P1 (Sigma N8630 or equivalent) following the manufacturer's instructions. Adjust pH to 7.5-8.5 using 1M Tris. Add 1 unit of alkaline phosphatase per 100 µg of DNA and incubate at 37°C for 30 minutes. Boil for 10 minutes and place on ice until use.

Tissue Samples

Snap-freeze tissue samples in liquid nitrogen immediately after collection. Store at -80°C until use. 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 DNA extraction kit. Digest DNA using nuclease P1 (Sigma N8630 or equivalent) following the manufacturer's instructions. Adjust the pH to 7.5-8.5 using 1 M Tris. Add 1 unit of alkaline phosphatase per 100 µg of DNA and incubate at 37°C for 30 minutes. Boil for 10 minutes and place on ice until use.

Saliva

Saliva samples should be stored at -80°C immediately after collection. Samples may be assayed directly after appropriate dilution.

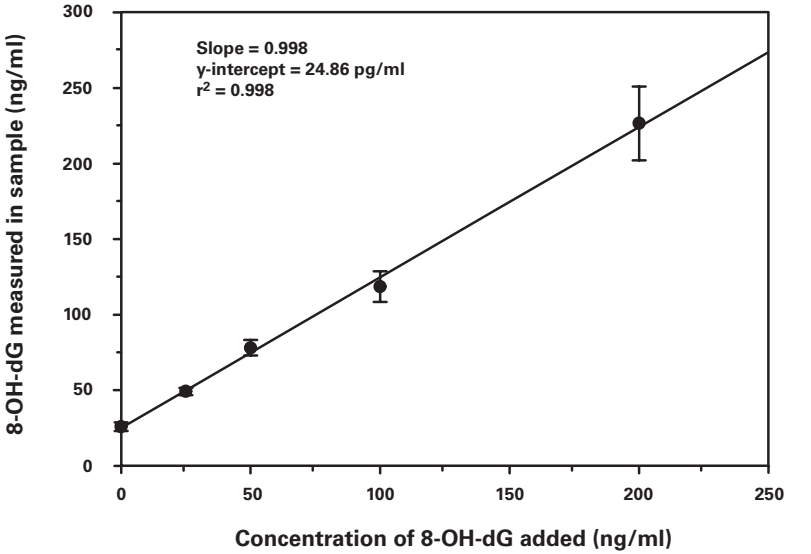


Figure 4. Recovery of 8-hydroxy-2-deoxy Guanosine from urine
Urine samples were spiked with 8-OH-dG, diluted as described in the **Sample Preparation** section and analyzed using the 8-OH-dG EIA Kit. The y-intercept corresponds to the amount of 8-OH-dG in unspiked urine. Error bars represent standard deviations obtained from multiple dilutions of each sample.

Preparation of Assay-Specific Reagents

8-hydroxy-2-deoxy Guanosine EIA Standard

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the 8-OH-dG standard (**Catalog# SKC-120C**) into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 30 ng/ml. Store this solution at 4°C; it will be stable for approximately six weeks.

NOTE: If assaying culture medium samples that have not been diluted with EIA Buffer, culture medium should be used in place of EIA Buffer for dilution of the standard curve.

To prepare the standard for use in EIA: Obtain 8 clean test tubes and number them #1 through #8. Aliquot 900 µl EIA Buffer to tube #1 and 500 µl EIA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (30 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 400 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. The diluted standards may be stored at 4°C for no more than 24 hours.

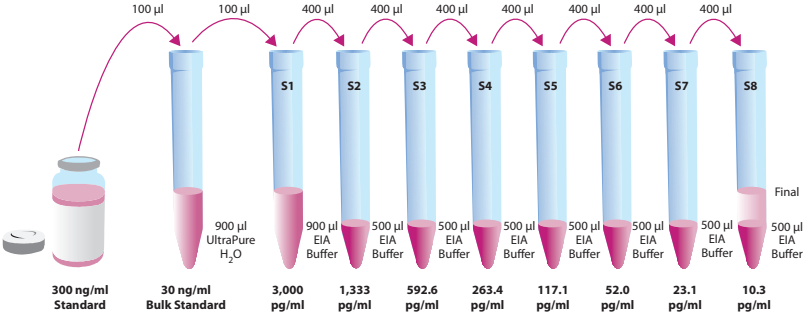


Figure 5. Preparation of the 8-hydroxy-2-deoxy guanosine standards

8-hydroxy-2-deoxy Guanosine AChE Tracer (Catalog# SKC-120B)

Reconstitute the 8-hydroxy-2-deoxy Guanosine Tracer as follows:

100 dtn 8-hydroxy-2-deoxy Guanosine AChE Tracer (96-well kit: Catalog#SKT-120-96)): Reconstitute with 6 ml EIA Buffer.

OR

500 dtn 8-hydroxy-2-deoxy Guanosine AChE Tracer (480-well kit: Catalog#SKT-120-480)): Reconstitute with 30 ml EIA Buffer.

Store the reconstituted 8-hydroxy-2-deoxy Guanosine Tracer at 4°C *(do not freeze!)* and use within four weeks. A 20% surplus of 8-hydroxy-2-deoxy Guanosine Tracer has been included to account for any incidental losses.

Tracer Dye Instructions (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

8-hydroxy-2-deoxy Guanosine Monoclonal Antibody (Catalog# SKC-120A)

Reconstitute the 8-hydroxy-2-deoxy Guanosine Monoclonal Antibody as follows:

100 dtn 8-hydroxy-2-deoxy Guanosine Monoclonal Antibody (96-well kit: Catalog#SKT-120-96)): Reconstitute with 6 ml EIA Buffer.

OR

500 dtn 8-hydroxy-2-deoxy Guanosine Monoclonal Antibody (480-well kit: Catalog#SKT-120-480): Reconstitute with 30 ml EIA Buffer.

Store the reconstituted 8-hydroxy-2-deoxy Guanosine Antibody at 4°C. It will be stable for at least four weeks. A 20% surplus of 8-hydroxy-2-deoxy Guanosine Antibody has been included to account for any incidental losses.

Antiserum Dye Instructions (optional)

This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum or add 300 µl of dye to 30 ml of antiserum).

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.*

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B_0), and an eight point standard curve run in duplicate. *NOTE: Each assay must contain this minimum configuration in order to ensure accurate and reproducible results.* Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 6, 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 31).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	S1	S1	1	1	1	9	9	9	17	17	17
B	Blk	S2	S2	2	2	2	10	10	10	18	18	18
C	NSB	S3	S3	3	3	3	11	11	11	19	19	19
D	NSB	S4	S4	4	4	4	12	12	12	20	20	20
E	B_0	S5	S5	5	5	5	13	13	13	21	21	21
F	B_0	S6	S6	6	6	6	14	14	14	22	22	22
G	B_0	S7	S7	7	7	7	15	15	15	23	23	23
H	TA	S8	S8	8	8	8	16	16	16	24	24	24

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Figure 6. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette the buffer, standard, sample, tracer, 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.

Addition of the Reagents

1. **EIA Buffer**

   Add 100 µl EIA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl EIA Buffer to Maximum Binding (B_0) wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for EIA Buffer in the NSB and B_0 wells (*i.e.*, add 50 µl culture medium to NSB and B_0 wells and 50 µl EIA Buffer to NSB wells).

2. **8-hydroxy-2-deoxy Guanosine Standard**

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **8-hydroxy-2-deoxy Guanosine AChE Tracer**

   Add 50 µl to each well *except* the Total Activity (TA) and the Blank (Blk) wells.

5. **8-hydroxy-2-deoxy Guanosine Monoclonal Antibody**

   Add 50 µl to each well *except* the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

Well	EIA Buffer	Standard/ Sample	Tracer	Antibody
Blk	-	-	-	-
TA	-	-	5 µl (at devl. step)	-
NSB	100 µl	-	50 µl	-
B_0	50 µl	-	50 µl	50 µl
Std/Sample	-	50 µl	50 µl	50 µl

Table 1. Pipetting Summary

Incubate the Plate

Cover each plate with plastic film (**Catalog# SKC-120H**) and incubate 18 hours at 4°C.

Develop the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   100 dtn vial Ellman's Reagent (96-well kit; Catalog# SKC-120I): Reconstitute with 20 ml of UltraPure water.

   OR

   250 dtn vial Ellman's Reagent (480-well kit; Catalog# SKC-120I): Reconstitute with 50 ml of UltraPure water.

NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of tracer to the Total Activity wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (*i.e.*, B_0 wells ≥0.3 A.U. (blank subtracted)) in 90-120 minutes.

Read the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. *NOTE: Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.*
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B_0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B_0 wells are in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 2.0, wash the plate, add fresh Ellman's Reagent and let it develop again.

ANALYSIS

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as $\%B/B_0$ *versus* log concentration using either a 4-parameter logistic or log-logit curve fit.

NOTE: StressMarq Biosciences Inc. has a computer spreadsheet available for data anaylsis. Please visit our website (http://www.stressmarq.com/kit_data/SKT-120/analysis.html) to obtain a free copy of this convenient data analysis tool.

Calculations

Preparation of the Data

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

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B_0 wells.
3. Subtract the NSB average from the B_0 average. This is the corrected B_0 or corrected maximum binding.
4. Calculate the $\%B/B_0$ (% Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B_0 (from Step 3). Multiply by 100 to obtain $\%B/B_0$. Repeat for S2-S8 and all sample wells.

NOTE: The total activity (TA) values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B_0 divided by the actual TA (10X measured absorbance) will give the % Bound. This value should closely approximate the % Bound that can be calculated from the ***Sample Data*** *(see page 24). Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 27 for* ***Troubleshooting****).*

Plot the Standard Curve

Plot $\%B/B_0$ for standards S1-S8 *versus* 8-hydroxy-2-deoxy guanosine concentration using linear (y) and log (x) axis and fit the data to a 4-parameter logistic equation.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is as follows, *NOTE: Do not use $\%B/B_0$ in this calculation:*

$$\textbf{logit } (B/B_0) = \ln [B/B_0/(1 - B/B_0)]$$

Plot the data as logit (B/B_0) *versus* log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the $\%B/B_0$ value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. *NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well.* Samples with $\%B/B_0$ values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample would indicate interference which could be eliminated by purification.

Performance Characteristics

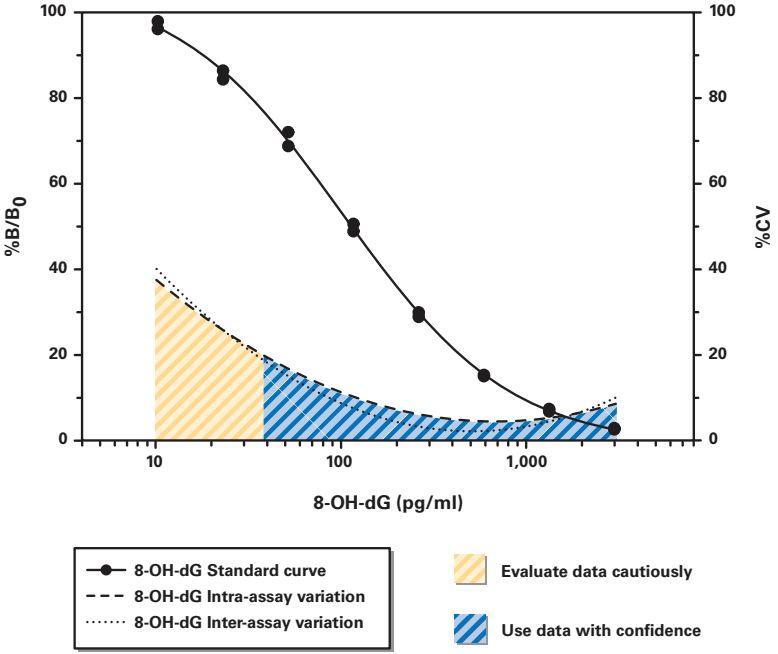
Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You **must** run a new standard curve - do not use this one to determine the values of your samples. Depending on the development conditions and the purity of the water used, your results could differ substantially from the data presented below.

	Raw Data		Average	Corrected
Total Activity	2.105	2.101	2.103	
NSB	0.000	0.000	0.000	
B_0	1.551	1.492		
	1.480	1.397	1.480	1.480

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
3,000	0.041	0.038	0.041	0.038	2.8	2.6
1,333	0.108	0.100	0.108	0.100	7.3	6.7
592.6	0.226	0.221	0.226	0.221	15.3	15.0
263.4	0.442	0.426	0.442	0.426	29.9	28.8
117.1	0.722	0.748	0.722	0.748	48.8	50.5
52.0	1.017	1.064	1.017	1.064	68.7	71.9
23.1	1.278	1.248	1.278	1.248	86.3	84.3
10.3	1.449	1.421	1.449	1.421	97.9	96.0

Table 2. Typical results



50% B/B_0 - 115 pg/ml
Detection Limit (80% B/B_0) - 33 pg/ml

Figure 8. Typical standard curve

Precision:

The intra- and inter-assay CV's have been determined at multiple points on the standard curve. These data are summarized in the graph on page 25.

Dose (pg/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
3,000	6.2	8.4
1,333	6.1	4.6
592.6	9.6	4.8
263.4	4.7	5.5
117.1	9.3	4.5
52.0	11.6	10.7
23.1	†	†
10.3	†	†

Table 3. Intra- and inter-assay variation
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.
†Outside of the recommended usable range of the assay.

Specificity:

Compound	Cross-reactivity
8-hydroxy-2-deoxy Guanosine	100%
8-hydroxy Guanosine	23%
8-hydroxy Guanine	23%
Guanosine	<0.01%

Table 4. Specificity of the 8-hydroxy-2-deoxy Guanosine Monoclonal Antibody

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
High NSB (>0.035)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Rewash plate and redevelop
Very low B_0	A. Contamination of water with organic solvents B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of UltraPure water B. Return plate to shaker and reread later
Low sensitivity (shift in dose response curve)	Standard is degraded	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by EIA[14]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

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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.

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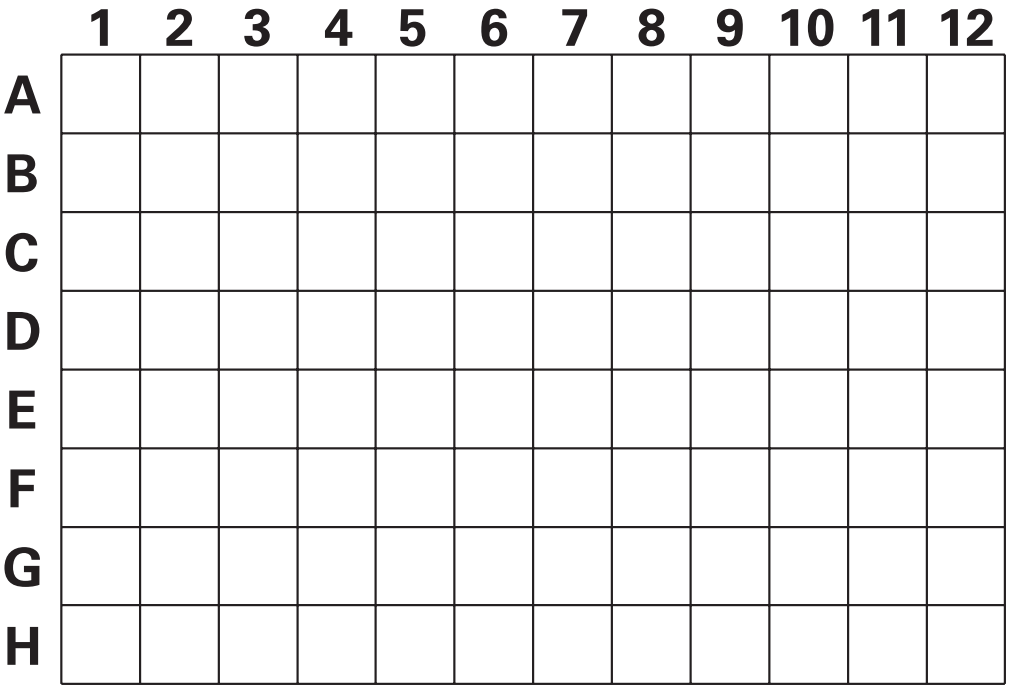
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