



HT 8-oxo-dG ELISA Kit

**For the detection and quantitation of
8-hydroxy-2'-deoxyguanosine in urine,
serum and saliva samples**



Catalog # 4370-096-K



I. Introduction

Oxidized lipids and proteins are removed during lipid and protein turnover mechanisms.¹ However, modified DNA cannot be replaced and has to be repaired.¹ Numerous DNA repair mechanisms have evolved in the cell and have become the focus of research in many disease states.² Removal of DNA damage and restoration of the continuity of the DNA duplex, activation of the DNA damage checkpoint, which stops the cell cycle and prevents the transmission of damaged chromosomes, changes in the transcriptional response of the cell and apoptosis are some of the important DNA damage response reactions.²

8-hydroxy-2'-deoxyguanosine (8-oxo-dG) is a modified nucleoside base, which is the most commonly studied and detected by-product of DNA damage that is excreted upon DNA repair.³ The presence of 8-oxo-dG and its analogs, 8-hydroxyguanosine and 8-hydroxyguanine in the urine, is associated with many degenerative disease states.³ The association of reactive oxygen species (ROS) and the use of 8-oxo-dG as a biomarker of oxidative stress have been investigated in many diseases, including bladder and prostate cancer,^{3,4} cystic fibrosis,⁵ atopic dermatitis⁶ and rheumatoid arthritis.⁷ Parkinson's disease, Alzheimer's disease and Huntington's disease are neurodegenerative diseases that are thought to be caused by exposure to neurotoxins in people with a genetic predisposition for these diseases.^{8,9} Oxidative stress is associated with the pathogenesis of these diseases and elevated levels of DNA damage have been measured in a wide range of neurological conditions.^{8,9}

A. Assay Design

Trevigen's **HT 8-oxo-dG ELISA** (enzyme-linked immunosorbent assay) is a fast and sensitive competitive immunoassay for the detection and quantitation of 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) in urine, serum and saliva samples. 8-oxo-dG has become a frequently used biomarker of oxidative DNA damage and oxidative stress. Measurement of urinary 8-oxo-dG may be useful as an indicator of oxidative damage. The kit features a 96 strip well plate pre-bound with 8-oxo-dG, an 8-oxo-dG monoclonal antibody, an enzyme-labeled secondary antibody, detection substrates and buffer to provide a complete, robust assay flexible for your experimental design.

The 8-oxo-dG monoclonal antibody binds, in a competitive manner, to 8-oxo-dG in the sample, standard or pre-bound wells of the immunoassay plate. Anti-8-oxo-dG bound to 8-oxo-dG in the sample or standard are washed away while those captured by the immobilized 8-oxo-dG are detected with a secondary antibody: HRP conjugate. The assay is developed with tetramethylbenzidine substrate (TMB) and the absorbance is measured in a microplate reader at 450 nm. The intensity of the yellow color is inversely proportional to the concentration of 8-oxo-dG.

B. Scientific Background

Intracellular and extracellular free radical species can be potentially damaging to the living cell.¹ Intracellular free radical species are produced as a result of normal metabolism, and inflammation whereas extracellular forms are produced as a result of ultraviolet or ionizing radiation.¹ The various ROS include the highly

reactive hydroxyl radical (OH^{\bullet}), superoxide radical ($\text{O}_2^{\bullet-}$), hypochlorite ion ($\text{OCl}^{\bullet-}$) and non-radical hydrogen peroxide (H_2O_2).¹ DNA, lipids, and proteins are cellular targets for oxidative damage by ROS and the order of preference for modification depends on location of ROS production, availability of metal ions, and the relative ability for the target to be oxidized.¹ Cells have acquired a number of defense mechanisms to cope with oxidative damage by ROS and other free radicals.¹ The simplest defense mechanisms involve vitamin C and E intercepting free radicals, becoming radicals themselves and protecting cellular biomolecules from damage.¹ Complex defense mechanisms involve enzymes such as superoxide dismutase, catalase and glutathione peroxidase that have evolved to reduce ROS levels.¹ Low background levels of damage occur even in normal cells because ROS have a tendency to escape these defense mechanisms.¹ Indeed, when defense mechanisms cannot prevent the accumulation of ROS, there is an increase in cellular damage.¹

C. Assay Procedure Summary

1. Bring to room temperature: 8-oxo-dG Immunoassay Plate, 20X Wash Buffer, Sample Diluent, Antibody Diluent, HRP Conjugate Diluent, TMB Substrate and Stop Solution 2.
2. Prepare 8-oxo-dG Standard and samples in Sample Diluent.
3. Add 50 μL prepared standards and samples in duplicate to the appropriate wells (please see template on page 14) of 8-oxo-dG Immunoassay Plate.
4. Add 50 μL diluted Anti-8-oxo-dG to each well, except the blank. Cover immunoassay plate.
5. Incubate plate at room temperature for 1 hour.
6. Wash wells 6 times using 300 μL /well of 1X Wash Buffer.
7. Add 100 μL diluted Anti-Mouse IgG: HRP Conjugate to each well, except the blank. Cover immunoassay plate.
8. Incubate plate at room temperature for 1 hour.
9. Wash wells 6 times using 300 μL /well of 1X Wash Buffer.
10. Add 100 μL TMB Substrate to each well.
11. Incubate at room temperature for 15 minutes (preferably in the dark).
12. Add 100 μL Stop Solution 2 to each well.
13. Measure absorbance at 450 nm.
14. Plot the 8-oxo-dG standard curve and calculate 8-oxo-dG sample concentrations. (Use our calculations worksheet: <http://www.trevigen.com/8-oxo-dGELISA.php>).

II. Materials

A. Precautions

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

1. The activity of the Anti-Mouse IgG horseradish peroxidase conjugate (cat# 4370-096-07) is affected by nucleophiles such as azide, cyanide and hydroxylamine.
2. The Stop Solution 2 (cat# 4370-096-10) is a 1 Normal (1N) solution of hydrochloric acid. This solution is corrosive; please use caution when handling.

Please read the complete Instructions for Use before performing this assay.

B. Materials Included

Trevigen's 8-oxo-dG ELISA kit contains the following components in sufficient quantities for 96 wells. These reagents are sufficient to assay one standard curve and 39 samples in duplicate or two standard curves and 30 samples in duplicate.

| Catalog # | Component | Size | Description |
|-------------|-------------------------------|---------------|--|
| 4370-096-01 | 8-oxo-dG Immunoassay Plate | 96 well plate | 12 × 8 removable strips and plate frame. Pre-coated plate with 8-oxo-dG: BSA conjugate |
| 4370-096-02 | 8-oxo-dG Standard | 25 µL | 10 µg/mL stock solution of 8-hydroxy-2'-deoxyguanosine |
| 4370-096-03 | Sample Diluent | 50 mL | Buffer to dilute standards and samples |
| 4370-096-04 | 20X Wash Buffer | 100 mL | Concentrated solution of buffer and surfactant |
| 4370-096-05 | Anti-8-oxo-dG | 25 µL | Monoclonal antibody specific for 8-oxo-dG |
| 4370-096-06 | Antibody Diluent | 6 mL | Buffer for dilution of Anti-8-oxo-dG |
| 4370-096-07 | Anti-Mouse IgG: HRP Conjugate | 25 µL | Anti-Mouse IgG conjugated to horseradish peroxidase |
| 4370-096-08 | HRP Conjugate Diluent | 12 mL | Buffer for dilution of Anti-Mouse IgG: HRP Conjugate |
| 4370-096-09 | TMB Substrate | 12 mL | Stabilized tetramethylbenzidine substrate |
| 4370-096-10 | Stop Solution 2 | 11 mL | Acid stop solution to stop color reaction |

C. Storage of Materials

All reagents are stable as supplied at 4 °C, except the 8-oxo-dG Standard, which should be stored at -20 °C. For optimum storage, the 8-oxo-dG Standard should be aliquotted into smaller portions and stored at -20 °C to avoid repeated freeze/thaw cycles.

Unused wells of the 8-oxo-dG Immunoassay Plate should be resealed with desiccant in the foil pouch provided and stored at 4 °C until the kit expiration date.

D. Materials Required but not Provided

- Deionized or distilled water
- Precision pipettors capable of accurately delivering 1 to 1000 µL
- Disposable pipette tips
- 5, 10, 25 mL pipettes for reagent preparation
- 1 L graduated cylinder
- Squirt bottle, manifold dispenser, or automated microtiter plate washer

- Disposable polypropylene tubes
- Microtiter plate reader capable of measuring absorbance at 450 nm
- Adhesive plate sealers or plastic wrap

III. Performing the Assay

A. Critical Assay Parameters and Notes

- Trevigen's **HT 8-oxo-dG ELISA kit** contains a pre-coated microtiter plate (8-oxo-dG Immunoassay Plate) with removable wells to allow assaying on separate occasions.
- Run both standards and samples in duplicate.
- Include a standard curve each time the assay is performed.
- The following kit components should be brought to room temperature prior to use: 8-oxo-dG Immunoassay Plate, Sample Diluent, Wash Buffer, Antibody Diluent, HRP Conjugate Diluent, TMB Substrate, and Stop Solution 2.
- Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents are ready to use and all required strip-wells are secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.
- For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 15 minutes.
- Mix all reagents and samples gently, yet thoroughly, prior to use. Avoid foaming of reagents.
- To avoid cross contamination, change disposable pipette tips between the addition of each standard, sample, and reagent. Use separate reagent troughs/reservoirs for each reagent.
- This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated.
- Consistent, thorough washing of each well is critical. If using an automatic washer, check washing head before use. If washing manually, ensure all wells are completely filled at each wash. Air bubbles should be avoided.
- Exercise appropriate laboratory safety precautions when performing this assay.
- In this protocol, room temperature refers to 20–28 °C. The room temperature should remain within this range throughout the assay.

NOTE: The components in each kit lot # have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot #s.

B. Sample Preparation

Extraction of Samples

Urine Samples

Fresh clear urine samples can be diluted in Sample Diluent and used directly in the assay. Samples containing precipitate should be centrifuged at 2,000 × g for 10 minutes, or filtered using a 0.45 µm filter, prior to use in the assay.

Urine samples can be frozen at $-70\text{ }^{\circ}\text{C}$ and assayed at a later date. Filter samples using a $0.45\text{ }\mu\text{m}$ filter and add suitable antibiotics, such as gentamicin at $30\text{ }\mu\text{g/mL}$, or 0.05% sodium azide, prior to storing at $-70\text{ }^{\circ}\text{C}$.

Serum Samples

- 1) Collect whole blood using established methods.
- 2) Allow samples to clot at room temperature for 30 minutes.
- 3) Centrifuge at $2,700 \times g$ for 10 minutes, taking precautions to avoid hemolysis.
- 4) Remove serum. Transfer the serum to a labeled polypropylene tube. The serum collected is now ready for analysis using the HT 8-oxo-dG ELISA kit.
- 5) Alternatively, the serum sample can be frozen at $\leq -20\text{ }^{\circ}\text{C}$ and assayed at a later date. It is recommended that the serum be aliquotted to convenient volumes prior to storing at $\leq -20\text{ }^{\circ}\text{C}$ to avoid multiple freeze thaw cycles.

Saliva Samples

Collect sample in centrifuge tube. To clarify, freeze sample at $-70\text{ }^{\circ}\text{C}$ for 1 hour. Thaw sample on ice, and centrifuge at $2,000 \times g$ for 10 minutes. Transfer clarified supernatant to clean tube for use in the assay. Alternatively, the clarified saliva samples can be frozen at $\leq -20\text{ }^{\circ}\text{C}$ and assayed at a later date. It is recommended that the saliva sample be aliquotted to convenient volumes prior to storing at $\leq -20\text{ }^{\circ}\text{C}$ to avoid multiple freeze thaw cycles.

Dilution of Samples

Samples should be prepared as described above. Dilute prepared urine samples 1:20 (v/v) as a suggested starting dilution in Sample Diluent. Serum samples may be diluted 1:20 (v/v) appropriately in Sample Diluent as a suggested starting dilution. Dilute saliva samples 1:8 (v/v) as a suggested starting dilution in Sample Diluent. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

- 1) Dilute prepared samples (i.e. urine, serum, saliva) in Sample Diluent. Prepare at least $150\text{ }\mu\text{L}$ of diluted sample to permit assaying in duplicate.
- 2) Mix thoroughly.
- 3) Samples are now ready to be used in the Assay Procedure (see page 7, Section IIID). Samples may be left at room temperature while Reagents are being prepared (see Section IIIC).

C. Reagent Preparation

NOTES: 1) All reagents should be freshly prepared prior to use. Once prepared, reagents should be kept at room temperature for the duration of the assay.

2) The preparation of the reagents is based on using the complete 1×96 well assay, unless otherwise noted. If only a portion of the immunoassay plate is to be used, please store all components as described above.

Temperature of Reagents

Bring the following reagents to room temperature prior to use:

- 8-oxo-dG Immunoassay Plate (cat# 4370-096-01)
- Sample Diluent (cat# 4370-096-03)
- Wash Buffer (cat# 4370-096-04)
- Antibody Diluent (cat# 4370-096-06)
- HRP Conjugate Diluent (cat# 4370-096-08)

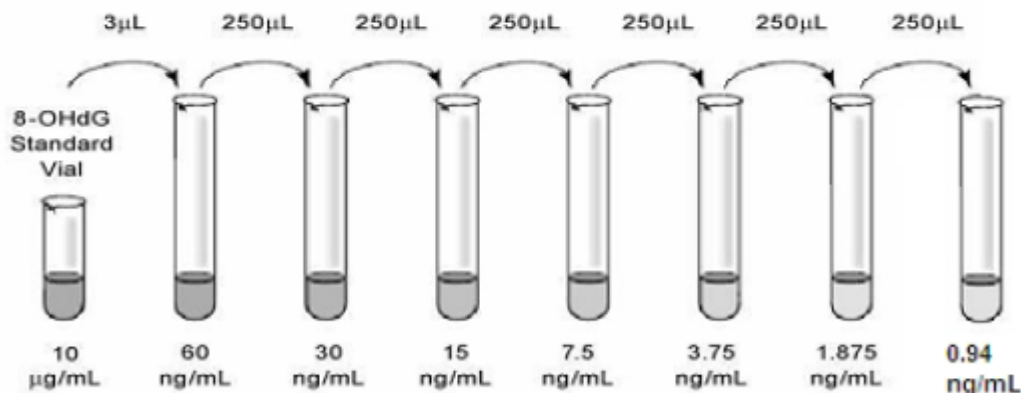
- TMB Substrate (cat# 4370-096-09)
- Stop Solution 2 (cat# 4370-096-10)

8-oxo-dG Standard (cat# 4370-096-02)

NOTE: The 8-oxo-dG Standard will withstand two freeze/thaw cycles to allow preparation of a second standard curve. However, to ensure product integrity, it is suggested that the 8-oxo-dG Standard be aliquotted into smaller portions and any remaining 8-oxo-dG Standard be discarded after the second use. Avoid repeated freeze/thaw cycles.

The 8-oxo-dG Standard is used to generate a standard curve with 7 points, ranging from 0.94–60 ng/mL.

- 1) Centrifuge the 8-oxo-dG Standard vial before removing the cap. This process will assure that the entire standard is collected and available for use.
- 2) Label seven (7) polypropylene tubes, each with one of the following standard values: 60 ng/mL, 30 ng/mL, 15 ng/mL, 7.5 ng/mL, 3.75 ng/mL, 1.875 ng/mL, 0.94 ng/mL.
- 3) Add 500 μ L of Sample Diluent to Tube #1.
- 4) Add 250 μ L of Sample Diluent to Tube #2, 3, 4, 5, 6, and 7.
- 5) Add 3 μ L of the 8-oxo-dG Standard stock solution (10 μ g/mL) to Tube #1.
- 6) Mix thoroughly.
- 7) Transfer 250 μ L from Tube #1 to Tube #2.
- 8) Mix thoroughly.
- 9) Similarly, complete the dilution series to generate the remaining standards (250 μ L from Tube #2 to Tube #3, mix thoroughly, etc) up to and including Tube #7.
- 10) Finally, add 250 μ L Sample Diluent to another 1.5 mL polypropylene tube (Tube # 8), which is the zero standard (0 ng/mL).



Wash Buffer (cat# 4370-096-04)

- 1) Bring the 20X Wash Buffer to room temperature and swirl gently to dissolve any crystals that may have formed from storage.
- 2) Dilute 100 mL of the 20X Wash Buffer with 1,900 mL of deionized or distilled water. 1X Wash Buffer is stable at room temperature for up to 4 weeks. For longer-term storage, 1X Wash Buffer should be stored at 4 °C.

NOTE: 100mL of 20X Wash Buffer has been provided in this kit, which is sufficient for the preparation of 2L of 1X Wash Buffer. The minimum required volume of 1X Wash Buffer is 350 mL (if the complete plate is used at once). The additional 1X Wash Buffer is supplied to allow for multiple assays or alternative washing techniques.

Anti-8-oxo-dG (cat# 4370-096-05)

- 1) Centrifuge the vial before removing the cap to ensure maximum product recovery.
- 2) Dilute 20 μ L of Anti-8-oxo-dG in 5 mL of Antibody Diluent in a polypropylene tube. If using only a portion of the plate, dilute only what is needed for number of wells used.
- 3) Mix gently by inversion.
- 4) Reagent is now ready to be used in the Assay Procedure (see Section III.D, below.).
- 5) Do not re-use or store any remaining diluted Anti-8-oxo-dG.

Anti-Mouse IgG: HRP Conjugate (cat# 4370-096-07)

- 1) Centrifuge the vial before removing the cap to ensure maximum product recovery.
- 2) Dilute 22 μ L of the Anti-Mouse: HRP Conjugate in 11 mL of the HRP Conjugate Diluent in a polypropylene tube. If only using a portion of the plate, dilute only what is needed for the number of wells used.
- 3) Mix gently by inversion.
- 4) Reagent is now ready to be used in the Assay Procedure (see below).
- 5) Do not re-use or store any remaining diluted Anti-Mouse IgG: HRP Conjugate.

D. Assay Procedure

Determine the Required Number of Wells

- 1) Refer to the 8-oxo-dG Plate Template on page 14 to determine the number of wells to be used.
- 2) Remove the 8-oxo-dG Immunoassay Plate from the packaging and note the color of the desiccant pack. Silica beads should be blue. Pink beads indicate that moisture is present and the performance of the plate may be compromised.
- 3) If less than 96 pre-coated microtiter wells are needed, remove the excess wells from the frame and return them to the foil pouch.
- 4) Reseal the pouch containing the unused wells and store at 4 °C.

Addition of Standards, Samples, and Anti-8-oxo-dG Antibody

- 1) Add 50 μ L (in duplicate) of each of the following to appropriate wells:
 - Prepared 8-oxo-dG Standard (Tube #1 through Tube #7)
 - Zero Standard (Sample Diluent, which represents 0 ng/mL)
 - Samples (previously prepared - see Sample Preparation, page 4)
- 2) Add 50 μ L of the previously diluted Anti-8-oxo-dG to each well, except the blank.
- 3) Cover wells with an adhesive plate sealer or plastic wrap and incubate at room temperature for 1 hour.

NOTE: For each step in the procedure, total dispensing time for the addition of the reagents and samples to the assay plate should not exceed 15 minutes.

Washing

- 1) Aspirate liquid from all wells.
- 2) Add 300 μ L of 1X Wash Buffer to all wells, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle.
- 3) Repeat the aspirating and washing 5 more times, for a total of 6 washes.
- 4) After the 6th addition of 1X Wash Buffer, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels (10 seconds).

Addition of Anti-Mouse IgG:HRP Conjugate

(previously diluted, see previous page)

- 1) Add 100 μ L of the previously diluted Anti-Mouse IgG:HRP Conjugate to each well, except the blank.
- 2) Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 1 hour.
- 3) Wash plate 6 times as previously described with 1X Wash Buffer and pat dry on paper towel (10 seconds).

Addition of TMB Substrate and Stop Solution

- 1) Add 100 μ L of the TMB Substrate to the wells. Color development should be visible within 1 minute of addition to the plate.
- 2) Incubate the plate at room temperature for 15 minutes (preferably in the dark).
- 3) Add 100 μ L of the Stop Solution 2 to the wells in the same order that the TMB Substrate was added.

Measuring Absorbance

- 1) Set up the microplate reader according to the manufacturer's instructions.
- 2) Set wavelength at 450 nm.
- 3) Measure the absorbance. If the plate cannot be read immediately, it should be covered and kept at room temperature. The absorbance should be read within 30 minutes of adding the Stop Solution 2.

E. Calculation of Results

Determination of 8-oxo-dG Concentrations on the Web

- 1) Use the Calculations Worksheet provided on the website:
<http://www.trevigen.com/8-oxo-dGELISA.php>. (MS Excel is required).
- 2) Follow the steps on the instructions page for using the Calculations Worksheet.

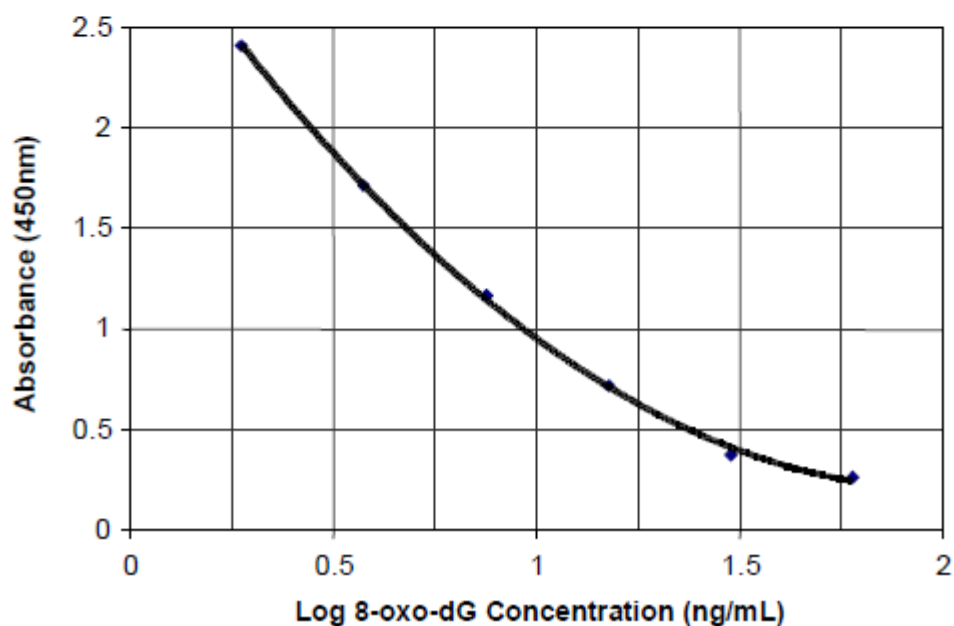


Alternative Method to Determine 8-oxo-dG Concentrations

- 1) Calculate the average of the duplicate absorbance measurements for each standard and sample.
- 2) Calculate the average of the duplicate absorbance measurements for the blank.
- 3) Subtract the average value obtained in Step #2 (blank) from the values obtained in Step #1 (standards and samples).
- 4) To generate the standard curve, plot the log of the 8-oxo-dG Standard concentrations (ng/mL) on the X-axis, and the absorbance measurements for the corresponding 8-oxo-dG standards on the Y-axis.
- 5) The standard curve is a 2nd order polynomial function represented by the equation: $y = a + bx + cx^2$, where y is the average absorbance measurement of the sample, x is the log of 8-oxo-dG concentration in ng/mL and a, b and c are coefficients. Calculate the 8-oxo-dG sample concentrations either by using the polynomial equation above or by interpolating the sample concentrations from the standard curve.
- 6) Multiply by the dilution factor for the final sample 8-oxo-dG concentration. For example, if the sample was diluted 1:20 prior to assaying, the value generated from the polynomial equation or the standard curve must be multiplied by 20 to calculate the final sample 8-oxo-dG concentration.

IV. Performance Characteristics

A. Typical 8-oxo-dG Standard Curve



B. Sensitivity

The sensitivity of the 8-oxo-dG ELISA has been determined to be 0.59 ng/mL. The standard curve has a range of 0.94-60 ng/mL.

C. Precision

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 Trevigen DNA Damage ELISA has been determined to be < 10%.

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 Trevigen DNA Damage ELISA has been determined to be < 10%.

D. Specificity

Trevigen's HT 8-oxo-dG ELISA also detects 8-hydroxyguanosine (product of oxidative RNA damage) and 8-hydroxyguanine (product of oxidative DNA damage by hydroxyl radicals).

Other related compounds were also identified, and these cross-reactants were diluted in sample diluent and assayed at concentrations of 100x, 10x, 1x, 0.1x and 0.01x of the high standard concentration. Cross-reactivity was calculated from the ED50 of the cross-reactant divided by the ED50 of the standard curve. Where ED50's could not be obtained for a cross-reactant, it was assumed that cross-reactivity was less than 0.016% (lowest 8-oxo-dG standard divided by the highest cross-reactant concentration).

| <u>Cross Reactant</u> | <u>Cross-reactivity</u> |
|---------------------------------|-------------------------|
| Guanosine | <0.016% |
| 8-Bromoguanosine | <0.016% |
| 2'-Deoxyinosine | <0.016% |
| 8-Mercaptoguanosine | 3.5% |
| N ² -Methylguanosine | <0.016% |



E. Limitations of the Assay

- This assay has been validated for use with urine, serum and saliva. 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.
- Although this assay has been validated for use with urine, serum and saliva samples, some samples may contain higher levels of interfering factors that can produce anomalous 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.

V. References

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