Version 29-05/24

User's Manual

DNA Damage ELISA kit

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

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

General Description

The DNA Damage ELISA (enzyme-linked immunosorbent assay) is a fast and sensitive competitive immunoassay for the detection and quantitation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine, serum, and saliva samples. 8-OHdG has become a frequently used biomarker of oxidative DNA damage and oxidative stress. Measurement of urinary 8-OHdG may be useful as an indicator of oxidative damage.

The DNA Damage ELISA uses an 8-OHdG monoclonal antibody to bind, in a competitive manner, 8-OHdG in the sample, standard or pre-bound to the wells of the 96-well immunoassay plate. Anti-8-OHdG bound to 8-OHdG in the sample or standard are washed away while those captured by the immobilized 8- OHdG are detected with a secondary antibody: HRP conjugate. The assay is developed with tetramethylbenzidine substrate and the absorbance is measured in a microplate reader at 450nm. The intensity of the yellow color is inversely proportional to the concentration of 8-OHdG.

Intracellular and extracellular free radical species can be potentially damaging to the living cell. Intracellular free radical species are produced as a result of normal metabolism and extracellular forms are produced as a result of ultraviolet radiation or ionizing radiation. Reactive oxygen species (ROS) are of particular interest in the research of oxidative damage and disease. The various ROS include the highly reactive hydroxy radical ($^{\bullet}OH$), superoxide radical ($O_2^{\bullet-}$), hypochlorite ion ($OCl^{\bullet-}$) and non-radical hydrogen peroxide (H_2O_2). 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 oxidized1 . Cells have acquired a number of defense mechanisms to cope with oxidative damage by ROS and other free radicals. The simplest defense mechanisms involve Vitamin C and E intercepting free radicals, becoming radicals themselves and protecting cellular biomolecules from damage. Complex defense mechanisms involve enzymes such as superoxide dismutase, catalase and glutathione peroxidase that have evolved to reduce ROS levels. Low background levels of damage occur even in normal cells because ROS have a tendency to escape the defense mechanisms. However, when the defense mechanisms cannot prevent the accumulation of ROS, then there is an increase in cellular damage.

Modified lipids and proteins are removed via normal lipid-, protein- turnover mechanisms. However, modified DNA cannot be replaced and has to be repaired. Numerous DNA repair mechanisms have evolved in the cell and have become the focus of research in many disease states. Removal of DNA damage and restoration of the continuity of the DNA duplex response, activation of the DNA damage checkpoint, which stops 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. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is a modified nucleoside base, which is the most commonly studied and detected by-product of DNA damage that is excreted in the urine upon DNA repair. Urinary 8-OHdG and its analogs, 8- hydroxyguanosine and 8-hydroxyguanine, are linked to many degenerative diseases. The association of ROS and the use of 8-OHdG as a biomarker of oxidative stress have been investigated in many diseases, including bladder and prostate cancer cystic fibrosis, atopic dermatitis and rheumatoid arthritis. 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. 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.

CRITICAL ASSAY PARAMETERS AND NOTES

1. The DNA Damage ELISA kit contains a pre-coated microtiter plate (8-OHdG Immunoassay Plate) with removable wells to allow assaying on separate occasions. Run both standards and samples in duplicate.
2. Include a standard curve each time the assay is performed.
3. The following kit components should be brought to room temperature prior to use: 8-OHdG Immunoassay Plate, Sample Diluent, Wash Buffer, Antibody Diluent, HRP Conjugate Diluent, TMB Substrate, Stop Solution 2.
4. 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.
5. 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.

6. 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.
7. This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettors are calibrated.
8. 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.
9. Exercise appropriate laboratory safety precautions when performing this assay.
10. In this protocol, room temperature refers to 20-28°C. The room temperature should remain within this range throughout the assay.

Reagents And Materials Provided

The DNA Damage 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.

1. 8-OHdG Immunoassay Plat, 96 well plate 12 x 8 removable strips and plate frame. Pre-coated plate with 8-OHdG: BSA conjugate
2. 8-OHdG Standard, 25 µL, 10 µg/mL stock solution of 8-hydroxy-2'deoxyguanosine
3. Sample Diluent, 50 mL Buffer to dilute standards and samples
4. 20× Wash Buffer, 100 mL Concentrated solution of buffer and surfactant
5. Anti-8-OHdG, 25 µL Monoclonal antibody specific for 8-OHdG
6. Antibody Diluent, 6 mL Buffer for dilution of Anti-8-OHdG
7. Anti-Mouse IgG: HRP Conjugate, 25 µL Anti-Mouse IgG conjugated to horseradish peroxidase
8. HRP Conjugate Diluent, 11 mL Buffer for dilution of Anti-Mouse IgG: HRP Conjugate
9. TMB Substrate, 10 mL Stabilized tetramethylbenzidine substrate

10. Stop Solution 2, 10 mL Acid stop solution to stop color reaction
11. Plate Sealer Two adhesive plate sealers

Materials Required But Not Supplied

1. Deionized or distilled water
2. Precision pipettors capable of accurately delivering 1 to 1000 µL
3. Disposable pipette tips
4. 5, 10, 25 mL pipettes for reagent preparation
5. 1 L graduated cylinder
6. Squirt bottle, manifold dispenser, or automated microtiter plate washer
7. Disposable polypropylene tubes
8. Microtiter plate reader capable of measuring absorbance at 450 nm

Storage

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

Unused wells of the 8-OHdG Immunoassay Plate should be resealed with desiccant in the foil pouch provided and stored at 4°C until the kit's expiry date.

Specimen Collection And Preparation

*Caution should be taken during sample work up, to avoid inadvertent oxidation of undamaged DNA.

1. **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 xg for 10 minutes, or filtered using a 0.45 µm filter, prior to use in the assay.

Urine samples can be frozen at -70°C and assayed at a later date. Filter samples using a 0.45 µm filter and add suitable antibiotics, such as gentamicin at 30 µg/mL, or 0.05% sodium azide, prior to storing at -70°C.

Serum Samples

a) Collect whole blood using established methods.

b) Allow samples to clot at room temperature for 30 minutes.

c) Centrifuge at 2700 xg for 10 minutes, taking precautions to avoid hemolysis.

d) Remove serum. Transfer the serum to a labeled polypropylene tube. The serum collected is now ready for analysis using the DNA Damage ELISA kit.

e) Alternatively, the serum sample can be frozen at ≤ -20°C and assayed at a later date. It is recommended that the serum be aliquotted to convenient volumes prior to storing at ≤-

20°C to avoid multiple freeze thaw cycles.

Saliva Samples

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

2. **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 prepared 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.

a) Dilute prepared samples (i.e. urine, serum, saliva) in Sample Diluent. Prepare at least 150 µL of diluted sample to permit assaying in duplicate.

b) Mix thoroughly.

c) Samples are now ready to be used in the Assay Procedure. Samples may be left at room temperature while Reagents are being prepared.

Reagent Preparation

NOTE: 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 previously described.

1. Temperature of reagents Bring the following reagents to room temperature prior to use:

8-OHdG Immunoassay Plate

Sample Diluent

Wash Buffer

Antibody Diluent

HRP Conjugate Diluent

TMB Substrate

Stop Solution 2

2. 8-OHdG Standard

NOTE: The 8-OHdG 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-OHdG Standard be aliquotted into smaller portions and any remaining 8-OHdG Standard be discarded after the second use. Avoid repeated freeze/thaw cycles.

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

a) Centrifuge the 8-OHdG Standard vial before removing the cap. This process will assure that all of the

standard is collected and available for use.

b) 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.

c) Add 500 µL of Sample Diluent to Tube #1. Add 250 µL of Sample Diluent to Tube #2, 3, 4, 5, 6, and 7.

d) Add 3 µL of the 8-OHdG Standard stock solution (10 µg/mL) to Tube #1.

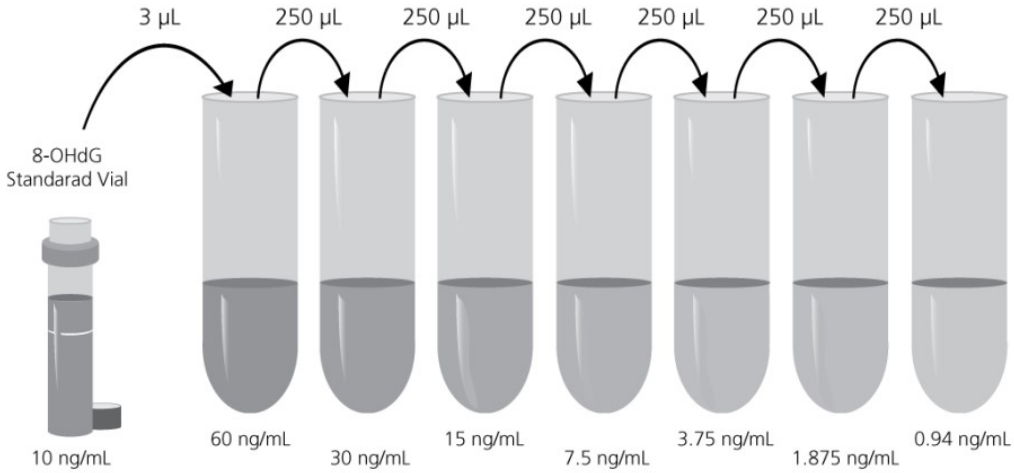
e) Mix thoroughly.

f) Transfer 250 µL from Tube #1 to Tube #2.

g) Mix thoroughly.

h) Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, mix thoroughly, etc.) up to and including Tube #7.

i) Finally, add 250 µL Sample Diluent to another 1.5 mL polypropylene tube (Tube #8), which is the zero standard (0 ng/mL).



3. Wash Buffer

a) Bring the 20× Wash Buffer to room temperature and swirl gently to dissolve any crystals that may have formed from storage.

b) Dilute the 100 mL of 20× Wash Buffer with 1900 mL of deionized or distilled water. Once diluted, the 1× Wash Buffer is stable at room temperature for up to 4 weeks. For longer-term storage, the Wash Buffer should be stored at 4°C.

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

4. Anti-8-OHdG

a) Centrifuge the vial before removing the cap to ensure maximum product recovery.

b) Dilute 20 µL of Anti-8-OHdG in 5 mL of Antibody Diluent in a polypropylene tube. If only using a

portion of the plate, dilute only what is needed for number of wells used.

c) Mix gently by inversion.

d) Reagent is now ready to be used in the Assay Procedure.

e) Do not re-use or store any remaining diluted Anti-8-OHdG.

5. ANTI-MOUSE IgG: HRP CONJUGATE

a) Centrifuge the vial before removing the cap to ensure maximum product recovery.

b) Dilute 22µL of the Anti-Mouse: HRP Conjugate in 11mL 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.

c) Mix gently by inversion.

d) Reagent is now ready to be used in the Assay Procedure.

e) Do not re-use or store any remaining diluted Anti-Mouse IgG: HRP Conjugate.

Assay Procedure

1. **Determine the required number of wells**

a) Refer to the 8-OHdG Plate Immunoassay Template to determine the number of wells to be used.

b) Remove the 8-OHdG 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.

c) If less than 96 pre-coated microtiter wells are needed, remove the excess wells from the frame and return them to the foil pouch.

d) Reseal the pouch containing the unused wells and store at 4°C.

2. **Addition of standards, samples and Anti-8-OHdG antibody**

a) Add 50 µL (in duplicate) of each of the following to appropriate wells:

- Prepared 8-OHdG Standard (Tube#1 through Tube #7)
- Zero Standard (Sample Diluent, which represents 0 ng/mL)
- Samples (previously prepared)

b) Add 50 µL of the previously diluted Anti-8-OHdG to each well, except the blank.

c) 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.

3. **Washing**

a) Aspirate liquid from all wells.

b) Add 300 µL of 1× Wash Buffer to all wells, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle.

c) Repeat the aspirating and washing 5 more times, for a total of 6 washes.

d) After the 6th addition of 1× Wash Buffer, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.

4. **Addition of Anti-mouse IgG: HRP conjugate (previously diluted)**

   a) Add 100 µL of the previously diluted Anti-Mouse IgG: HRP Conjugate to each well, except the blank.

   b) Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 1 hour.

   c) Wash plate as described in Step #3.

5. **Addition of TMB substrate and stop solution**

   a) Add 100 µL of the TMB Substrate to the wells. Color development should be visible within 1 minute of addition to the plate.

   b) Incubate the plate at room temperature for 15 minutes (preferably in the dark).

   c) Add 100 µL of the Stop Solution 2 to the wells in the same order that the TMB Substrate was added.

6. **Measuring absorbance**

   a) Set up the microplate reader according to the manufacturer's instructions.

   b) Set wavelength at 450 nm.

   c) 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.

Summary

1. Bring to room temperature: 8-OHdG Immunoassay Plate, 20× Wash Buffer, Sample Diluent, Antibody Diluent, HRP Conjugate Diluent, TMB Substrate and Stop Solution 2.
2. Prepare 8-OHdG Standard and samples in Sample Diluent.
3. Add 50 µL prepared standards and samples in duplicate to wells of 8-OHdG Immunoassay Plate.
4. Add 50 µL diluted Anti-8-OHdG to each well, except the blank. Cover immunoassay plate.
5. Incubate plate at room temperature for 1 hour.
6. Wash wells 6× using 300 µL/well of 1× 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× using 300 µL/well of 1× 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-OHdG standard curve and calculate 8-OHdG sample concentrations.

Calculation

Several options are available for the calculation of 8-OHdG in the samples. We recommend that the data be handled by an immunoassay software package utilizing a weighted 4 parameter logistic (4PL) curve fitting program.

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:

Average Net OD = Average Sample OD - Average Blank OD

2. Plot Net OD versus Concentration of 8-OHdG 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.

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

2. **Inter-Assay Precision (Between Run Precision)**

To determine Inter-Assay Precision, three samples of known concentration were assayed thirty times in three individual assays. The Inter-Assay Coefficient of variation of the DNA Damage ELISA has been determined to be < 10%.

Sensitivity

The sensitivity of the DNA Damage ELISA kit has been determined to be 0.59 ng/mL.

The standard curve has a range of 0.94 - 60ng/mL.

Specificity

The DNA Damage 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 identified, and these crossreactants were diluted in sample diluent and assayed at concentrations of 100×, 10×, 1×, 0.1×, and 0.01× of high standard concentration. Cross-reactivity was calculated from 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-OHdG standard divided by the highest cross-reactant concentration).

Guanosine < 0.016%

8-Bromoguanosine < 0.016%

2'-Deoxyinosine < 0.016%

8-Mercaptoguanosine 3.5%

N_2-Methylguanosine < 0.016%

Limitations

1. This assay has been validated for use with urine. 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.
2. Although this assay has been validated for use with urine and serum samples, some samples may contain higher levels of interfering factors that can produce anomalous results.
3. 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 reassayed at a lower sample dilution.
4. The use of assay reagents not provided in this kit or amendments to the protocol can compromise the performance of this assay.
5. 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.