

Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

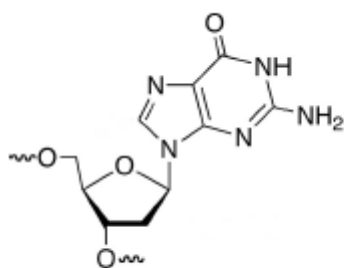
HT 8-oxo-dG ELISA Kit II

96 Tests Cat# 4380-096-K

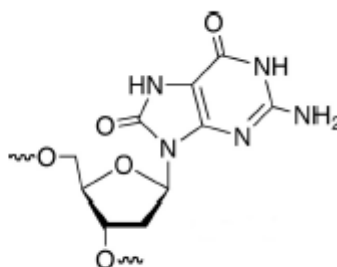
High throughput ELISA to quantify 8-OHdG in DNA, plasma, urine and saliva samples

I. Introduction

Mutagenic reactive oxygen species (ROS) are implicated in cancer, neurodegenerative disorders such as Alzheimer's disease (1), and in apoptosis (2). When exposed to oxidative radicals, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is formed and can serve as a sensitive indicator of physiological and environmental damage to DNA. The production of 8-hydroxyguanine is almost exclusively elicited by oxidative stress with the main attack site by oxidative radicals at the N7-C8 bond. 8-hydroxyguanine is labile resulting in an abasic lesion whereby DNA polymerase preferentially insert adenine opposite abasic site. Therefore, without repair these oxidative damage adducts can lead to G to T transitions (3). The 8-hydroxyguanine lesion causes mutational frequencies of 1 - 5% (mainly G:C to T:A transitions) and is one of the most abundant oxidative lesions (4).



deoxyguanosine



8-hydroxy-2'-deoxyguanosine

8-OHdG is a frequently used biomarker of oxidative DNA damage and oxidative stress. To address this need Trevigen offers a validated HT 8-oxo-dG ELISA kit II, a fast and sensitive immunoassay for the detection and quantitation of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

in DNA, plasma, urine and saliva samples. This assay employs a 96 strip well pre-coated with 8-OHdG, an anti-8-OHdG monoclonal mouse antibody, an HRP conjugated secondary antibody, and colorimetric detection substrate to construct a high throughput assay flexible for your experimental design. The 8-OHdG monoclonal antibody binds competitively to 8-OHdG immobilized on pre-coated wells and in solution. Antibody bound to 8-OHdG in the sample is washed away while antibody bound to 8-OHdG attached to the well is retained. Detection is performed with HRP conjugate and colorimetric substrate. Product formation is inversely proportional to amount of 8-OHdG present in sample.

Important features of the assay include:

- 1) colorimetric, non-radioactive format;
- 2) high throughput 96 strip wells;
- 3) dynamic range from 3.13 nM to 200 nM (0.89 ng/ml to 56.7 ng/ml); and,
- 4) sensitivity at 2 nM (0.57 ng/ml) 8-OHdG.

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the chemicals and reagents contained in the HT 8-oxo-dG ELISA Kit II may not yet have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products. MSDS are available on request.
3. Although this assay has been validated for use with DNA, plasma, urine, and saliva. Some samples may contain higher levels of interfering factors that can compromise the performance of the assay, or produce inaccurate results.
4. The components in each kit lot number have been quality assured and validated in this specific combination only; please do not mix them with components from other kit lot numbers.

III. Materials Supplied

Catalog Number	Component	Amount Provided (Cat# 4380-096-K)	Amount Provided (Cat# 4380-192-K)	Storage Temperature
4380-096-01	8-OHdG Standard, 20 µM	20 µl	2X 20 µl	-20°C
4380-096-02	Assay Diluent	50 ml	2X 50 ml	4°C
4380-096-03	8-OHdG monoclonal antibody	20 µl	2X 20 µl	-20°C
4380-096-04	Goat anti-Mouse IgG-HRP	20 µl	2X 20 µl	-20°C
4380-096-P	Pre-coated 96-stripwell plate	1 plate	2 plates	4°C
4380-096-05	100X Cations	500 µl	2X 500 µl	4°C
4380-096-06	DNase I (5 Units/µl)	40 µl	2X 40 µl	-20°C
4380-096-07	Alkaline Phosphatase (1 Unit/µl)	40 µl	2X 40 µl	-20°C
4822-96-08	TACS-Sapphire	10 ml	2X 10 ml	4°C

IV. Materials/Equipment Required But Not Supplied

Reagents/Disposables:

1. Biological specimens to be tested
2. 1XPBS containing 0.1% Tween 20 (PBST)
3. 0.2M HCl or 5% Phosphoric acid
4. DNA extraction kit, i.e. Qiagen Flexigen Kit for cultured cells (recommendation) and Qiagen PAXgene kit for tissues (recommendation)
5. pipette and tips
6. microcentrifuge tubes and conical tubes

Equipment:

1. Pipette-aid, pipettor and multichannel pipettor
2. Wash bottle or microstrip wells plate washer (optional)
3. 96-well plate reader with 450 nm filter
4. Vortexer and microcentrifuge
5. Heat block
6. Incubator set at 25 °C
7. -20 °C and 4 °C storage

V. Reagent Preparation

1. PBS + 0.1% Tween 20 Wash Solution (PBST)

Prepare 500 ml of 1X PBST containing 1X PBS and 0.1% Tween 20 in a wash bottle for washing strip wells.

2. 8-OHdG Standard

The kit contains 20 µl of 8-OHdG standard (Cat# 4380-096-01) at a concentration of 20 µM. Centrifuge the standard vial before opening cap. Aliquot and avoid repeated freeze/thaw cycles. Serially dilute the standard with Assay Diluent (Cat# 4380-096-02) just before use. The volume of each dilution should be 150 µl or greater. The recommended final concentrations are 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM and 3.13 nM. The standard curve requires 25 µl/well of each 8-OHdG dilution performed in triplicate. Table 1 describes a serial dilution protocol for 8-OHdG standards.

Diluted 8-OHdG standard should be used immediately and any remainder discarded.

Table 1: Dilution of 8-OHdG Standard

8-OHdG Concentr.	200 nM (56.7 ng/ml)	100 nM (28.3 ng/ml)	50 nM (14.2 ng/ml)	25 nM (7.1 ng/ml)	12.5 nM (3.54 ng/ml)	6.25 nM (1.77 ng/ml)	3.13 nM (0.89 ng/ml)
8-OHdG Standard 20 µM	3 µl	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl
Assay Diluent	297 µl	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl

3. Anti-8-OHdG Monoclonal Antibody

Just before use, dilute anti-8-OHdG monoclonal antibody (Cat# 4380-096-03) 250-fold with Assay Diluent (Cat# 4380-096-02). A total of 25 µl/well of diluted anti-8-OHdG monoclonal antibody is required in the assay. For 96 wells, dilute 12 µl of anti-8-OHdG monoclonal antibody into 3 ml of Assay Diluent and add 25 µl/well with a multichannel pipettor.

4. Goat Anti-Mouse-IgG-HRP Conjugate

Just before use, dilute Goat anti-Mouse IgG-HRP conjugate (Cat# 4380-096-04) 500-fold with Assay Diluent (Cat# 4380-096-02). A total of 50 µl/well of diluted goat anti-mouse IgG-HRP conjugate is required in the assay. For 96 wells, dilute 12 µl of Goat anti-Mouse-HRP conjugate into 6 ml of Assay Diluent and add 50 µl/well with a multichannel pipettor.

5. TACS-Sapphire™

Pre-warm TACS-Sapphire™ (Cat# 4822-96-08) to room temperature before use. TACS-Sapphire is a colorimetric substrate that turns blue in the presence of Horseradish Peroxidase (HRP). The addition of an equal volume of 0.2 M HCl or 5% phosphoric acid stops the reaction to generate a yellow color that can be read at 450 nm. A total of 50 µl is required per well. For 96 wells, distribute 6 ml of substrate with a multichannel pipettor.

VI. Preparation of Sample

DNA samples:

Cultured cells

For suspension cells:

1. Grow $1-5 \times 10^6$ suspension cells in complete medium in a suitable tissue culture plate or flask.
2. Count the cells. Harvest cells by centrifugation and remove growth medium.
3. Wash one time with 1XPBS. Suspend cell pellets at 1×10^6 cells/ml in icecold 1X PBS. For example, add 5 ml 1XPBS to 5×10^6 cells. Aliquot 1 ml into 1.5 ml microtubes.
4. Centrifuge at $10,000 \times g$ for 10 sec at 4°C. Discard supernatant.

Go to **DNA Extraction**. (Cell pellets can be flash frozen in liquid nitrogen and stored at -80 °C for later use).

For adherent cells:

1. Grow $1-5 \times 10^6$ adherent cells in complete medium in a suitable tissue culture dish or flask until 75% confluent.
2. Remove the growth medium and harvest cells by trypsinization or a method of choice. Count the cells.

3. Wash one time with 1XPBS. Suspend the cell pellets at 1×10^6 cells/ml in icecold 1X PBS. For example, add 5 ml 1XPBS to 5×10^6 cells. Aliquot 1 ml into 1.5 ml microtubes.

4. Centrifuge at $10,000 \times g$ for 10 sec at 4°C . Discard supernatant.

Go to **DNA Extraction** (Cell pellets can also be flash frozen in liquid nitrogen and stored at -80°C for later use).

Tissue samples

1. The tissue specimen should be cut into a 2 mm cube and weigh approximately 8–12 mg in mass.

Go to **DNA Extraction**.

DNA Extraction

1. Extract DNA by a desired method or commercial Extraction kit and dissolve DNA in pH 7.0-8.0 buffer.

2. Quantitate DNA spectrophotometrically ($1\text{OD}_{260} = 50 \mu\text{g/ml}$).

3. Add 100X Cations (Cat# 4380-096-05) to DNA solution for final 1X concentration.

4. Add 2 μl DNase I (Cat# 4380-096-06) per 50 μg DNA and incubate for 1 hour at 37°C .

5. Add 2 μl Alkaline Phosphatase (Cat# 4380-096-07) per 50 μg DNA and incubate 1 hour at 37°C .

6. Assay immediately or aliquot and store at -20°C .

Plasma samples:

1. Withdraw blood according to standard procedures using Sodium Heparin or EDTA as anticoagulant. 2. Collect plasma by centrifugation at $2-8^\circ\text{C}$ in a horizontal rotor (swinging bucket) in a proper adaptor for 15 minutes at $1,500 \times g$ within 30 minutes of blood collection.

3. Carefully remove the plasma and assay immediately or store at $\leq -20^\circ\text{C}$ in aliquots for later use. Avoid repeated freeze-thaw cycles.

Urine samples:

1. Collect urine according to standard procedure into a sterile container.

2. To clarify centrifuge $2,000 \times g$ for 15 minutes, or filter using a $0.45 \mu\text{m}$ filter to remove precipitate.

3. Assay immediately or store at $\leq -20^\circ\text{C}$ in aliquots for later use. Avoid repeated freeze-thaw cycles.

Saliva samples:

1. Collect saliva according to standard procedure in centrifuge tube.

2. To clarify centrifuge at $2,000 \times g$ for 15 minutes.

3. Carefully remove supernatant and assay immediately or store at $\leq -20^\circ\text{C}$ in aliquots for later use. Avoid repeated freeze-thaw cycles.

VII. Assay Protocol

1. Remove pre-coated strip wells from foil pouch and bring to room temperature. See Section XII Appendix for sample plate layout.

Note: If less than 96 pre-coated wells are needed, remove excess strips frame and return to foil pouch and store at 4 °C with desiccant. (Data performance will be compromised if desiccant color changes from blue to pink).

2. Prepare serial dilutions of the 8-OHdG Standard (Section V item 2) and samples (Section VI). 150 µl final volumes are recommended to assay in triplicate.

NOTES:

DNA samples diluted 1:5 (v/v) in Assay Diluent is a recommended starting dilution. Clarified plasma, urine and saliva samples diluted 1:10 in Assay Diluent is a recommended starting dilution. If samples generate values greater than the 200 nM standard, assay at a higher sample dilution. If samples generate values lower than 3.13 nM standard, assay at a lower sample dilution.

3. Add 25 µl of 8-OHdG standards and clarified samples to appropriate wells. Add 25 µl of Assay Diluent to 0 nm 8-OHdG and blank wells (background control). See Section XII Appendix for sample plate layout.

4. Add 25 µl of anti 8-OHdG monoclonal solution (Section V item 3) to all wells except blank wells. Add 25 µl of Assay Diluent to blank wells instead. Mix thoroughly without causing air bubbles. Cover wells with film sealer and incubate at 25 °C for 1 hour.

5. Gently remove film sealer and wash wells 4 times with PBST (300 µl/well). Ensure liquid is removed by tapping plate onto paper towels.

6. Add 50 µl of diluted Goat anti-Mouse IgG-HRP conjugate (Section V item 4) to all wells except blank wells. Add 50 µl of Assay Diluent to blank wells. Cover wells with film sealer and incubate at 25°C for 1 hour. Place TACSSapphire™ at 25 °C to pre-warm.

7. Gently remove the film sealer and wash wells 4 times with PBST (300 µl/well). Ensure liquid is removed by tapping plate onto paper towels.

8. Add 50 µl of pre-warmed TACS-Sapphire™ colorimetric substrate to all wells and incubate **in the dark**, for 15 minutes at 25°C. Stop the reactions by adding 50 µl 0.2M HCl or 5% Phosphoric Acid to all wells mixing well. Immediately read the absorbance at 450 nm.

VIII. Data Interpretation

Determination of 8-OHdG Concentrations on the Web

- 1) Use Calculations Worksheet provided on Trevigen website: <http://www.trevigen.com/8-OHdGELISA.php>. (MS Excel is required).
- 2) Follow instruction steps on Calculations Worksheet.

Alternative Method to Determine 8-OHdG Concentrations

- 1) Calculate average absorbance measurement for each 8-OHdG Standard, sample and blank.

2) Subtract the blank average from 8-OHdG Standard and sample averages to determine relative absorbance.

3) Plot the log of 8-OHdG Standard concentrations (nM) on the X-axis versus relative absorbance on the Y-axis.

4) The standard curve is a 2nd order polynomial function represented by the equation: $y = a + bx + cx^2$, where y is the relative absorbance, x is the log of 8-OHdG concentration in nM and a, b and c are coefficients. Calculate the 8-OHdG sample concentrations using the polynomial equation or interpolation from the standard curve.

5) Multiply by the dilution factor for the final sample 8-OHdG concentration. For example, if the sample was diluted 1:5, the value generated from the polynomial equation or the standard curve must be multiplied by 5 to calculate the final sample 8-OHdG concentration.

Example data

The following figures are examples of 8-oxodG ELISA Kit II results. The data below is for reference only and should not be used to interpret actual results.

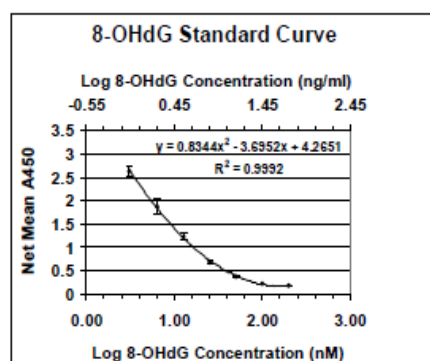


Figure 1. Typical 8-OHdG Standard Curve. 8-OHdG Standards were prepared in Section V and assayed according to the protocol in Section VII.

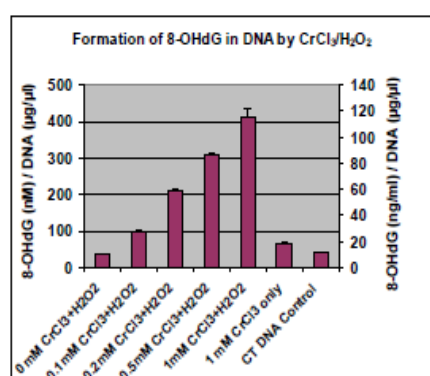


Figure 2. Formation of 8-OHdG in Calf Thymus DNA by $\text{CrCl}_3/\text{H}_2\text{O}_2$. Calf thymus DNA was treated with 1–1000 μM CrCl_3 plus 0.5 mM H_2O_2 as described by (5). The reaction was terminated by ethanol precipitation and DNA digested by DNase I (Cat# 4380-096-06) and Alkaline Phosphatase (Cat# 4380-096-07) as described in Section VI. 8-OHdG concentration was measured according to assay protocol.

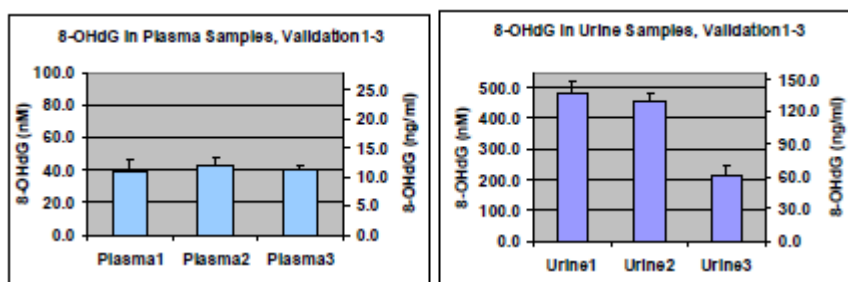


Figure 3. Assay Validations of the 8-OHdG levels in plasma and urine samples from healthy donors. Validation of assay reproducibility was determined by obtaining biofluid samples from three healthy donors. Sample extracts were prepared as described in Section VI, aliquoted, and frozen. Test samples were assayed on three successive days according to assay protocol. Variation is minimal indicating the assay is well suited to obtain baseline 8-OHdG levels as shown in Figure 3.

IX. Performance Characteristics

Intra-Assay Precision: The intra-assay precision (within-run precision): three samples of known concentration were tested nine times in a single run. The overall intra-assay coefficient of variation was calculated to be <10%.

Inter-Assay Precision: The inter-assay precision (between-run precision): three samples of known concentration were tested nine times in three separate runs. The overall inter-assay coefficient of variation was calculated to be <15%.

Sensitivity of the ELISA: The LLD (low limit of detection) of 8-OHdG was calculated to be 2 nM (0.57 ng/ml).

Spike and Recovery: Test samples were spiked with three different levels of 8-OHdG and analyzed for recovery before and after spiking. The calculated overall mean is between 80%-120%.

Dilution Linearity: Test samples were serially diluted in the Assay Diluent and subsequently measured by the assay. Dilution recovery is assessed by comparing observed vs. expected values based on undiluted samples. The calculated overall mean is between 80%-120%.

Specificity: Cross-reactivity of the anti-8-OHdG monoclonal with eight analogues of 8-OHdG was tested at 50% binding. 8-hydroxyguanosine and 8-hydroxyguanine have a significant cross-reaction. The cross-reactivity reports are as follows:

Table 2: Specificity

Cross-Reactant	% Inhibition at IC ₅₀
8-Hydroxydeoxyguanosine	100
8-Hydroxyguanosine	~30
8-Hydroxyguanine	~20
8-Mercatoguanosine	~4
8-Bromoguanosine CMP	<0.01
Guanosine	<0.01
Guanine	<0.01
2'-Deoxyinosine	<0.01
N2-Methylguanosine	<0.01

X. References

1. Nunomura, A., Perry, G., Pappolla, M.A., Wade, R., Hirai, K., Chiba, S., and Smith, M.A. (1999) *Journal of Neuroscience* **19**:1959-1964.
2. Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004) *Annu. Rev. Biochem.* **73**: 39-85.
3. Wood, M. L., Dizdaroglu, M., Gajewski, E. and Essigmann, J. M. (1990) *Biochemistry*, **29**, 7024-7032.
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5. Tsou, T., Chen, C., Liu, T., and Yang, J. (1996) *Carcinogenesis* **17**, 103- 108.