DNA Damage (8-OHdG) AccuSignal™ ELISA Kit

AccuSignal competitive ELISA (Enzyme-Linked Immunosorbent Assay) Kit is *an in vitro* enzyme-linked immunosorbent assay designed for the quantitative detection of 8-hydroxy-2-deoxy Guanosine (8-OHdG) in cell lysates, plasma, sample matrices and urine. The ELISA utilizes an 8-hydroxy-2-deoxy Guanosine-coated plate and an HRP-conjugated antibody for detection which allows for an assay range of 0.94 - 60 ng/ml, with a sensitivity of 0.59 ng/ml. The other highlights of this kit are a quick incubation time of 60 minutes, stable reagents, and an easy to use protocol.

I. KIT COMPONENTS

DNA Damage (8-OHdG) AccuSignal™ ELISA Kit			
Component	Catalog Number	Description	Size
8-Hydroxy-2-Deoxy Guanosine: BSA Coated Plate	KOA0887A	96-well plate pre-coated with 8-Hydroxy-2-Deoxy Guanosine: BSA	1
Target Protein Standard	KOA0887B	8-Hydroxy-2-Deoxy Guanosine standard	1 vial (100 µL)
Conjugated Monoclonal Antibody	KOA0887D	8-Hydroxy-2-Deoxy Guanosine HRP-conjugated monoclonal antibody	1 vial (75 µL)
Sample and Standard Diluent (Red)	KOD0102	Sample and standard diluent	1 vial (50 ml)
Antibody Diluent (Blue)	KOD0104	8-Hydroxy-2-Deoxy Guanosine antibody diluent	1 vial (13 ml)
Wash Buffer Concentrate	KOD0105	Wash buffer concentrate (10X)	1 vial (50 ml)
TMB Substrate	KOC0102	TMB color developing reagent	1 vial (13 ml)
Stop Solution	KOC0103	Stop solution	1 vial (13 ml)
Plate Cover	KOC0104	Adhesive cover	2

II. STORAGE AND STABILITY CONDITIONS

All reagents are stable as supplied at 4°C, except the protein **standard,** which should be stored at -20°C after receipt. For optimum storage, the 8-OHdG Standard should be aliquoted into smaller portions and then stored appropriately. Avoid repeated freeze/thaw cycles (10µL of Standard can prepare a triplicate standard curve).

III. INTRODUCTION

The 8-OHdG AccuSignal ELISA is a competitive assay that can be used for the quantification of 8-OHdG in urine, cell culture, plasma, and other sample matrices. The ELISA utilizes an 8-hydroxy-2-deoxy Guanosine-coated plate and an HRP-conjugated antibody for detection which allows for an assay range of 0.94 - 60 ng/ml, with a sensitivity of 0.59 ng/ml. The other highlights of this kit are a quick incubation time of 60 minutes, stable reagents, and an easy to use protocol.

It is important to note that the 8-OHdG antibody used in this assay recognizes both free 8-OHdG and DNA-incorporated 8-OHdG. Since complex samples such as plasma, cell lysates, and tissues are comprised of mixtures of DNA fragments and free 8-OHdG, concentrations of 8-OHdG reported by ELISA 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.

IV. REQUIRED EQUIPMENT AND REAGENTS NOT INCLUDED

- Microplate reader capable of measuring absorbance at 450 nm
- Automated plate washer
- Adjustable pipettes and pipette tips
- Clean tubes and Eppendorf tubes
- Distilled or deionized water

V. GENERAL CONSIDERATIONS

Please read the following instructions before starting the experiment.

- All ELISA reagents must be at room temperature before use.
- Vigorous plate washing is essential.
- Before using the Kit, briefly spin down the vials.
- Do not let 96-well plate to dry, this will inactivate active components on plate.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Use new adhesive plate cover for each incubation step.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Do not mix or substitute reagents or materials from other kit lots or vendors.
- Avoid microbial contamination of reagents and equipment. Automated plate washers can easily become contaminated thereby causing assay variability.
- Take care not to contaminate the TMB Substrate. Do not expose the TMB solution to glass, foil or metal. If the solution is blue before use, do NOT use it.
- Individual components may contain preservatives. Wear gloves while preforming the assay. Please follow proper disposal procedures.
- Include a standard curve each time the assay is performed.
- Run both standards and samples in triplicate.
- Buffers may crystallize over time. Warm crystallized buffer until the salt crystals return to solution.

VI. ELISA PROTOCOL

A. Sample Preparation

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

NOTE: Prepare at least 180 µL of your diluted sample to permit assay in triplicate (approximately 50 µL/ well).

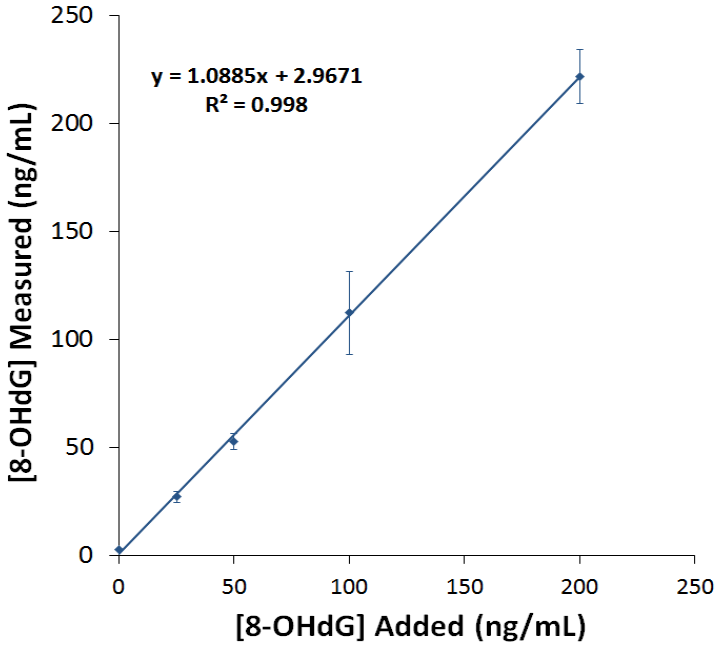
- All samples must be free of organic solvents prior to assay.
- Samples that cannot be assayed immediately should be stored as indicated below.
- Please be advised that all suggested dilutions below are simply recommended as a starting point, and it may be necessary to adjust the dilution based on experimental results.

Urine

Interference in urine is infrequent; dilutions appropriate for this assay show a direct linear correlation between 8-OHdG immunoreactivity and 8-OHdG concentration (see figure below). Urinary concentrations of 8-OH-dG can vary considerably and can be standardized against creatinine levels if required.

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

Dilution: Dilute urine samples 1:20 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing. For example: 9 µL of sample into 171 µL of Sample and Standard Diluent.



Recovery of 8-hydroxy-2-deoxy Guanosine from urine:
Urine samples were spiked with 8-OHdG, diluted as described in the Sample Preparation and analyzed using the 8-OHdG ELISA Kit. The y-intercept corresponds to the amount of 8-OHdG in un-spiked urine. Error bars represent standard deviations obtained from multiple dilutions of each sample.

Plasma/Serum

The concentration of free 8-OHdG in plasma is very low relative to the level of DNA-incorporated 8-OHdG. Glomerular filtration results in excretion of 8-OHdG into the urine, while the DNA-incorporated 8-OHdG remains in the blood. The differing fates of free *versus* DNA-incorporated 8-OHdG should be considered in experimental design. If you choose to measure DNA-incorporated 8-OHdG 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-OHdG in plasma, urine is often a more appropriate matrix.

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

Dilution: Serum samples may be diluted 1:20 (v:v) in Sample and Standard Diluent as the starting dilution prior to testing.

Culture Media Samples

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

Dilution: Fetal bovine serum contains 8-OHdG, therefore assays should either be performed in serum-free medium or PBS; these samples may be assayed directly. If the 8-OHdG concentration is high enough to dilute the sample 10-fold with Sample and Standard Diluent, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted 1:10 with Sample and Standard Diluent), dilute the standards in the same culture medium as that used for the experiment. This will ensure that the matrix for the standards is comparable to the samples. We recommend that a standard curve be run first to ensure that the assay will perform in a particular culture medium.

Cell Lysates

Storage: Collect lysates using established methods and store at -80°C until use.

Usage: 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

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

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

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

Dilution: Saliva samples can be prepared 1:8 (v:v) in Sample and Standard Diluent as a suggested starting dilution.

B. Standard Preparation

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

1. Centrifuge the 8-hydroxy-2-deoxy Guanosine Standard vial before removing the cap. This process with assure that all of the standard is collected and available for use.
2. Label seven (7) polypropylene tubes, each with one of the following standard values: 60 ng/ml, 30 ng/ml, 15 ng/ml, 7.5 ng/ml, 3.75 ng/ml, 1.875 ng/ml and 0.94 ng/ml.
3. Add 490 µL of Sample and Standard Diluent to Tube #1.
4. Add 250 µL of Sample and Standard Diluent to Tube #2, 3, 4, 5, 6 and 7.
5. Add 10 µL of the 3.06 µg/ml 8-hydroxy-2-deoxy Guanosine Standard to Tube #1 for a concentration of 60 ng/ml. Mix well.
6. Transfer 250 µL from Tube #1 to Tube #2. Mix well.

7. Similarly, complete the dilution series to generate the remaining standards (250 µL from Tube #2 to Tube #3, mix well, etc.) up to and including Tube #7.
8. Finally, add 250 µL Sample and Standard Diluent to another 1.5ml polypropylene tube (Tube #8), which is the zero standard (0 ng/ml).

Standard Dilution Preparation					
Standard #	Volume to Dilute (µL)	Diluent (µL)	Total Volume (µL)	Starting Concentration (µg/ml)	Final Concentration (ng/ml)
1	10 (Standard 3.06 µg/ml)	490	500 µL	3.06	60.0
2	250	250	500 µL	0.06	30.0
3	250	250	500 µL	0.03	15.0
4	250	250	500 µL	0.015	7.5
5	250	250	500 µL	0.0075	3.75
6	250	250	500 µL	0.00375	1.875
7	250	250	500 µL	0.00187	0.937
8	0	250	250 µL	0	0

C. Reagent Preparation

1X Wash Buffer Preparation

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

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

8-hydroxy-2-deoxy Guanosine: HRP Conjugate Monoclonal Antibody Preparation

Determine the amount of Antibody Preparation required. For every strip-well used (8-wells), prepare 0.5 ml of Antibody Preparation.

Prepare Antibody Preparation by diluting the 8-hydroxy-2-deoxy Guanosine: DNA Damage Peroxidase Monoclonal Antibody 1:100 with 8-hydroxy-2-deoxy Guanosine: DNA Damage Monoclonal Antibody Diluent. For example, if 6 ml of Antibody Preparation is required (one whole plate), dilute 60 µL of Antibody in 6 ml of 8-hydroxy-2-deoxy Guanosine Antibody Diluent. Mix well prior to use.

D. Assay Procedure

The 96-well plate included with this kit is supplied ready to use. It is not necessary to rinse the plate 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.*

We recommend assaying samples in triplicate.

A suggested plate format is shown 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.

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

Assay Hints

- Use different tips to pipette the buffer, standard, sample, and antibody.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.
- Always add the Antibody Preparation after the rest of the reagents, as this is a competitive assay.
- Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.

1. Add 50 µL (in triplicate) of each of the following to appropriate wells:
    - Prepared 8-hydroxy-2-deoxy Guanosine Standard (Tube #1 through Tube #7) into wells labelled S1-S7
    - Zero Standard (Tube #8- Sample and Standard Diluent, which represents 0 ng/mL) into wells labelled S8
    - Samples (previously prepared) into wells labelled 1-23
2. Add 50 µL of the previously diluted 8-hydroxy-2-deoxy Guanosine Antibody Preparation to each well, except the blank.

3. Add 50 µL of Standard and Sample Diluent and 50 µL of Antibody Diluent into wells labelled as the blank.
4. Cover each plate with the plate cover and incubate 1 hour at room temperature (20- 25˚C).
5. Carefully remove adhesive plate cover. Gently squeeze the long sides of the plate frame before washing to ensure all strips remain securely in the frame.
6. Empty plate contents. Use a multi-channel pipette to fill each well completely (300 µL) with 1X Wash buffer, then empty plate contents. Repeat procedure three additional times, for a total of FOUR washes. Blot plate onto paper towels or other absorbent material.

   NOTE: Follow the same procedure when using an automated plate washer as well. Take care to avoid microbial contamination of equipment. Automated plated washers can easily become contaminated thereby causing assay variability.
7. Add 100 µL of TMB Substrate into each well.

   Note: Only remove the required amount of TMB Substrate and Stop Solution for the number of strips being used. Do NOT use a glass pipette to measure the TMB Substrate solution.

   Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate. If the solution is blue before use, DO NOT USE IT.
8. Cover carefully with the second provided plate cover.
9. Allow the enzymatic color reaction to develop at room temperature (20-25ºC) in the dark for 30 minutes. The substrate reaction yields a blue solution.
10. After 30 minutes, carefully remove the plate cover, and stop the reaction by adding 100 µL of Stop Solution to each well. Tap plate gently to mix. The solution in the wells should change from blue to yellow.
11. Wipe underside of wells with a lint-free tissue.
12. Measure the absorbance on an ELISA plate reader set at 450 nm.

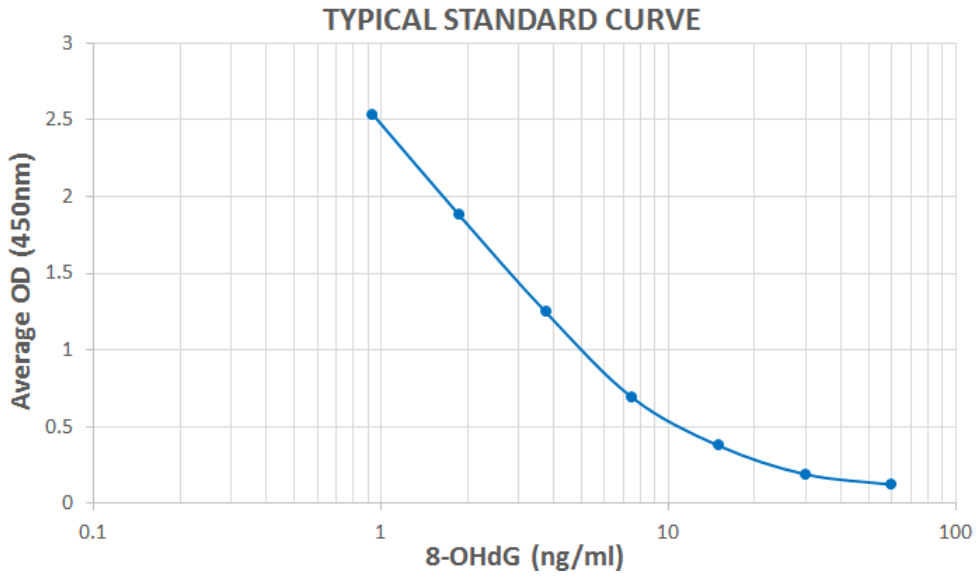
    Note: Evaluate the plate within 30 minutes of stopping the reaction.

Calculations

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

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average Blank OD from the average OD bound.
2. Plot Net OD versus Concentration of 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.

VII. 8-OHdG ACCUSIGNAL™ ELISA KIT STANDARD CURVE



This typical standard curve was generated using the 8-OHdG ELISA Kit Protocol. This standard curve is for demonstration only. A standard curve must be generated for each assay.

Assay Range	0.94 ng/ml-60 ng/ml
Sensitivity	0.59 ng/ml
Specificity/Cross-reactivity	8-hydroxy-2-deoxy guanosine (100%); 8-hydroxy Guanosine (23%); 8-hydroxy Guanine (23%); Guanosine (<0.01%)

Assay Limitations:

- 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.
- 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.

VIII. INTRA AND INTER ASSAY PRECISION

Intra-Assay 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 <5%.

Inter-Assay 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 <5%.

VIII. ASSAY SUMMARY

- Prepare standard and samples in the Sample and Standard Diluent.
- Add 50 µL of prepared standards and samples in triplicate to appropriate wells.
- Add 50 µL of the diluted antibody preparation to the appropriate wells.
- Cover plate with Plate Cover and incubate at room temperature (20-25°C) for 1 hour.
- Wash plate 4 times with 1X Wash Buffer.
- Add 100 µL of TMB Substrate to each well.
- Cover plate and develop the plate in the dark at room temperature for 30 minutes.
- Add 100 µL of Stop Solution to each well.
- Measure absorbance on a plate reader at 450 nm.
- Plot the standard curve and calculate sample concentrations.

IX. TROUBLESHOOTING

Problem	Possible Causes	Recommended Solutions
Poor Standard Curve	A. Improper standard solution B. Standard degraded C. Curve doesn't fit scale D. Pipetting Error	A. Confirm dilutions are made correctly. B. Store and handle standard as recommended. C. Try plotting using different scales D. Use calibrated pipettes and proper pipetting technique.
No Signal	A. Plate washings too vigorous B. Wells dried out C. Target present below detection levels of kit	A. Check and ensure correct pressure in automatic wash system. Pipette wash buffer gently if washes are done manually. B. Do not allow wells to dry out. Cover the plate for incubations.

		C. The basic range of DNA to use, if the damage is low, 100 µg/ml - if maximally damaged, 1ng/ml and dilute from there.
High Background	A. Wells are insufficiently washed B. Contaminated wash buffer C. Waiting too long to read the plate after adding stop solution	A. Wash wells as per protocol B. Prepare fresh wash buffer C. Read plate immediately
Low sensitivity	A. Standard is degraded B. Mixing or substituting reagents from other kits	A. Replace standard B. Avoid mixing components

X. RELATED PRODUCTS

Component	Catalog #	Size
10x TBS pH 7.5	MB-012	1000 ml
10x TTBS pH 7.5	MB-013	1000 ml
10X PBS pH 7.2	MB-008	1000 ml
10x PBST pH 7.2	MB-075-1000	1000 ml