www.rockland.com
tech@rockland.com
+1 484.791.3823

Protocol

AccuSignal™ DNA Damage (8-OHdG) 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.

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.

Parameter	Specification
Range	0.94 pg/ml – 60 pg/ml
Sensitivity	<0.59ng/mL
Specificity	8-hydroxy-2-deoxy guanosine
Cross-reactivity	8-hydroxy Guanosine (23%); 8- hydroxy Guanine (23%); Guanosine (<0.01%)

Kit Components

Product	Storage	Size	Item No.
8-Hydroxy-2-Deoxy Guanosine: BSA Coated Plate	-20°C	1 plate	KOA0887A
Target Protein Standard	-20°C	1 vial (100 µL)	KOA0887B
Conjugated Monoclonal Antibody	-20°C	1 vial (75 µL)	KOA0887D
Sample and Standard Diluent (Red)	4°C	1 vial (50 ml)	KOD0102
Antibody Diluent (Blue)	4°C	1 vial (13 ml)	KOD0104
Wash Buffer Concentrate	4°C	1 vial (50 ml)	KOD0105
TMB Substrate	4°C	1 vial (13 ml)	KOC0102
Stop Solution	4°C	1 vial (13 ml)	KOC0103
Plate Cover		2	KOC0104

Note:

This kit will perform as specified if the components are stored as directed and used before the expiration date.

All reagents are stable as supplied at 4°C, except after receipt, store the protein standard, detection antibody, and plate at -20°C. 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).

Additional Materials Required but not Provided

- Microplate reader in standard size at 450nm
- Automated plate washer
- Adjustable pipettes and pipette tips
- Clean tubes and Eppendorf tubes
- Distilled or deionized water

General Considerations

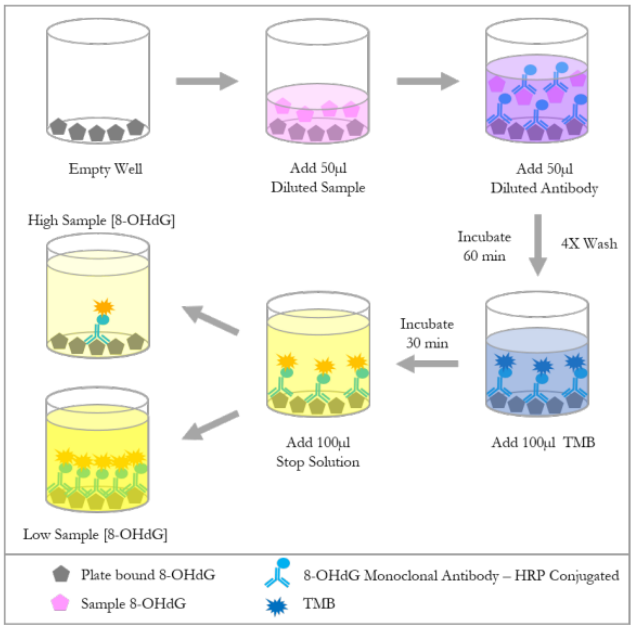
Please read the following instructions before starting the experiment.

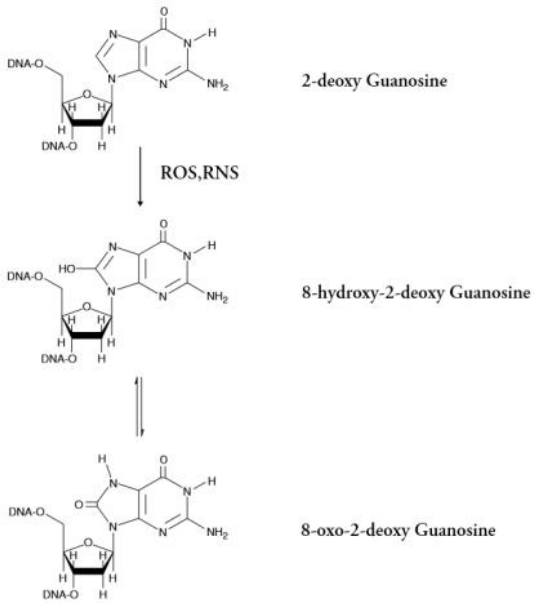
- To inspect the validity of experimental operation and the appropriateness of sample dilution proportions, pilot experiment using standards and a small number of samples is recommended.
- The TMB Color Developing agent is colorless and transparent before using, contact us if it is not the case.
- Before using the kit, briefly spin down the vials.
- For statistical reasons, we recommend both standard and samples should be assayed with a minimum of two replicates (duplicates).
- 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.
- Do not mix or substitute reagents or materials from other kit lots or vendors.
- In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution be prewarmed in 37°C for 30 min before using.

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.





Reagent Preparation

Sample Preparation and Sample Dilution

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).

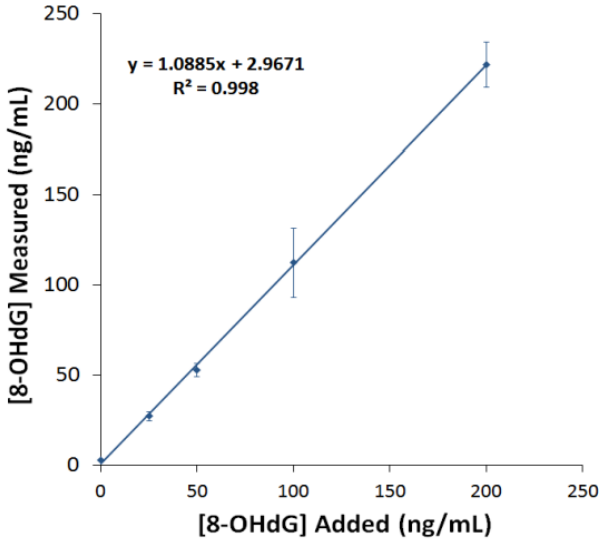
- All samples must be free of organic solvents prior to assay.
- Samples that cannot be assayed immediately should be stored as indicated below.
- Please be advised that all suggested dilutions below are simply recommended as a starting point, and it may be necessary to adjust the dilution based on experimental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

Note: Levels of the target protein may vary between different samples. Estimation of the concentration of a target protein in the sample and the optimal dilution factors for each sample must be determined by the investigator. The diluted target protein concentration should fall near the middle of the linear regime in the standard curve. Sample diluent buffer should be used for dilution of samples.

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 the standard is collected and available for use.

2. Label tubes:

Tube #	Label
1	60ng/mL
2	30ng/mL
3	15ng/mL
4	7.5ng/mL

5	3.75ng/mL
6	1.875ng/mL
7	0.94ng/mL
8	0pg/mL (Blank sample diluent buffer)

3. Add 490 µL of Sample and Standard Diluent to Tube #1, 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.

4. Add 250 µL of Sample and Standard Diluent to Tube #2, 3, 4, 5, 6 and 7.

5. Transfer 250 µL from Tube #1 to Tube #2. Mix well.

6. 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.

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

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.

Note: The solution should be prepared no more than 2 hours prior to the experiment.

- The total volume should be 0.1 mL/well x (the number of wells). Prepare 100–200 µL more of the solution than the total volume required to compensate for pipetting errors.
- Biotinylated anti-Human TSP2 antibody should be diluted 1:100 with the antibody diluent buffer and mixed thoroughly. For example, add 1 µL Biotinylated Anti-Human TSP2 antibody to 99µL antibody diluent buffer.

Assay Procedure

Note 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.

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.

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℃).
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

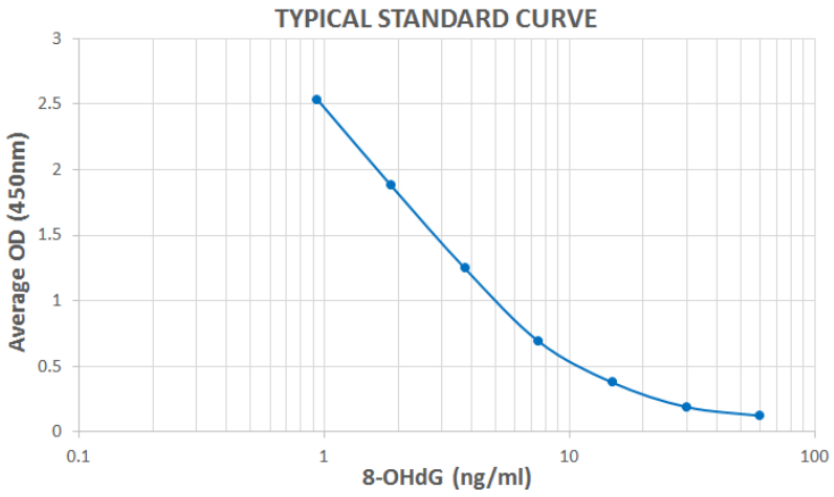
The following procedure is recommended for preparation of the data prior to graphical analysis.

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.

Kit Specifications



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 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.

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%.

Troubleshooting

Visit www.rockland.com/ELISA-kit-troubleshooting