Creative Diagnostics®

Version 18-04/24

User's Manual

DNA/RNA Oxidative Damage (High Sensitivity) ELISA Kit

REF **DEIA4986**

Σ **96T, 480T**

RUO

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.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

CD has developed this immunoassay for the measurement of DNA/RNA oxidative damage that detects all three oxidized guanine species; 8-hydroxy-2'-deoxyguanosine from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA.

General Description

DNA and RNA are damaged by oxidation during aging and in a variety of disease states including cancer.[1-3] Guanine is the base is most prone to oxidation, and the repair processes initiated to correct the damage release multiple oxidized guanine species into the urine, including 8-hydroxyguanosine (8-OHG), 8-hydroxy-2'- deoxyguanosine (8-OHdG), and 8-hydroxyguanine. Some commercial vendors offer immunoassays that detect only 8-OHdG. CD's DNA/RNA Oxidative Damage (High Sensitivity) ELISA Kit is a competitive assay that can be used to measure 8-OHdG, 8-OHG, and 8-hydroxyguanine. Since the antibody used in the assay recognizes damaged nucleic acid species other than 8-OHdG, the value obtained with our assay will be higher than that obtained by competitor ELISAs or by LC/MS analysis that measure a single species. As such, caution is recommended when comparing results obtained from this kit to those obtained by other methods.

Principles of Testing

This assay is based on the competition between oxidatively damaged guanine species and an 8-OH-dG-acetylcholinesterase conjugate (DNA/RNA Oxidative Damage Tracer) for a limited about of DNA/RNA Oxidative Damage Monoclonal antibody. Because the amount of tracer is held constant while the concentration of oxidatively damaged guanine varies, the amount of tracer that is able to bind to the monoclonal antibody will be inversely proportional to the concentration of oxidatively damaged guanine in the well. This antibody-oxidatively damaged guanine complex binds to the goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of DNA/RNA Oxidative Damage Tracer bound to the well, which is inversely proportional to the amount of free 8-OH-dG present in the well during the incubation; or Absorbance $\propto$ [Bound DNA/RNA Oxidative Damage Tracer] $\propto$ 1/[8-OH-dG]

Reagents And Materials Provided

1. DNA/RNA Oxidative Damage ELISA Monoclonal Antibody, 1 vial/100 dtn
2. DNA/RNA Oxidative Damage AChE Tracer, 1 vial/100 dtn
3. DNA/RNA Oxidative Damage ELISA Standard, 1 vial
4. ELISA Buffer Concentrate (10X), 2 vials/10 ml
5. Wash Buffer Concentrate (400X), 1 vial/5 ml
6. Polysorbate 20, 1 vial/3 ml

7. Goat Anti-Mouse IgG Coated Plate, 1 plate
8. 96-Well Cover Sheet, 1 cover
9. Ellman's Reagent 3 vials/100 dtn
10. ELISA Tracer Dye, 1 vial
11. ELISA Antiserum Dye, 1 vial

Materials Required But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeating pipettor.
3. A source of 'UltraPure' water. Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA.
4. Materials used for Sample Preparation

Storage

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Specimen Collection And Preparation

Proper sample storage and preparation are essential for consistent and accurate results. Please read this section thoroughly before beginning the assay.

General Precautions

1. All samples must be free of organic solvents prior to assay.
2. Samples that cannot be assayed immediately should be stored as indicated below.
3. Samples of mouse or rat origin may contain antibodies which interfere with the assay by binding to the goat anti-mouse plate. We recommend that all mouse and rat samples be purified prior to use in this assay.

Urine

Urine samples should be stored at -20°C immediately after collection. Samples should be diluted a minimum of 1:250 and up to greater than 1:1,000 to fall within the range of the standard curve. Interference in urine is infrequent; dilutions appropriate for this assay (i.e., dilutions falling between 20-80% B0) show a direct linear correlation between immunoreactivity and oxidized guanine concentration. Urinary concentrations of oxidized guanine vary considerably and, as with any urinary marker, we recommend standardizing the values obtained by ELISA to creatinine levels.

Plasma/Serum

This assay has not been thoroughly validated for measurement of DNA/RNA oxidized species in plasma or serum. Our recommendation is to make 2-3 dilutions of the sample within the range of 1:25 - 1:100 directly into ELISA Buffer and then follow the assay protocol as instructed.

Culture Medium Samples

Collect culture medium samples and store at -80°C. Fetal bovine serum contains oxidized guanine species, therefore assays should either be performed in serumfree medium or PBS; these samples may be assayed directly. If the concentration of damaged guanine species is high enough to dilute the sample 10-fold with ELISA Buffer, the assay can be performed without any modifications. When assaying less concentrated samples (where samples cannot be diluted 1:10 with ELISA Buffer), dilute the standard curve 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

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

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

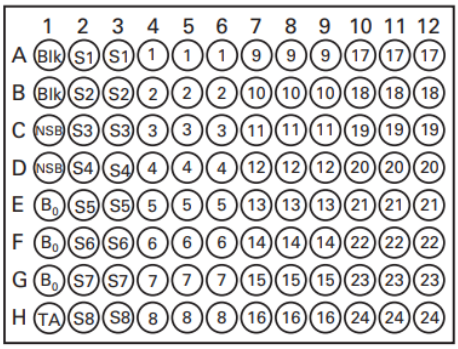
Saliva samples should be stored at -80°C immediately after collection. Samples may be assayed directly after appropriate dilution.

Plate Preparation

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 4°C. Be sure the packet is sealed with the desiccant inside.

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B0), and an eight point standard curve run in duplicate. **NOTE:** Each assay must contain this minimum configuration in order to ensure accurate and reproducible results. Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, 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 using a convenient spreadsheet offered by CD. We suggest you record the contents of each well on the template sheet provided.



Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8
1-24 - Samples

Reagent Preparation

NOTE: Water used to prepare all ELISA reagents and buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for ELISA.

Store all diluted buffers at 4°C; they will be stable for about two months.

1. **ELISA Buffer Preparation**

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. **NOTE:** It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.

2. **Wash Buffer Preparation**

   5 ml vial Wash Buffer Concentrate (400X): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20.

   Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer).

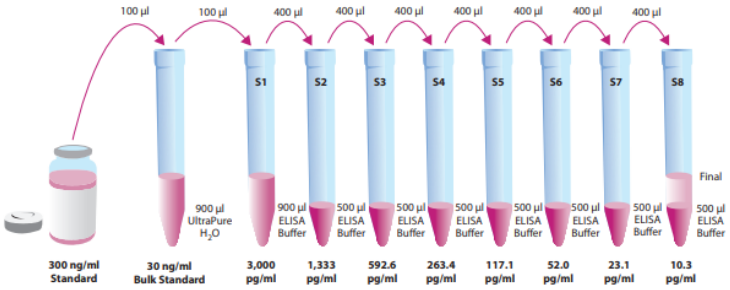
   NOTE: Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

3. **DNA/RNA Oxidative Damage ELISA Standard**

   Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the DNA/RNA Oxidative Damage ELISA Standard into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 30 ng/ml. Store this solution at 4°C; it will be stable for approximately six weeks.

   NOTE: If assaying culture medium samples that have not been diluted with ELISA Buffer, culture medium should be used in place of ELISA Buffer for dilution of the standard curve.

   To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 500 µl ELISA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (30 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 400 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove400 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. The diluted standards may be stored at 4°C for no more than 24 hours.



4. **DNA/RNA Oxidative Damage AChE Tracer**

   Reconstitute the DNA/RNA Oxidative Damage AChE Tracer as follows: 100 dtn DNA/RNA Oxidative Damage AChE Tracer: Reconstitute with 6 ml ELISA Buffer.

   Store the reconstituted DNA/RNA Oxidative Damage AChE Tracer at 4°C (do not freeze!) and use within four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

   Tracer Dye Instructions (optional)

   This dye may be added to the tracer, if desired, to aid in visualization of tracercontaining wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

5. **DNA/RNA Oxidative Damage ELISA Monoclonal Antibody**

   Reconstitute the DNA/RNA Oxidative Damage ELISA Monoclonal Antibody as follows: 100 dtn DNA/RNA Oxidative Damage ELISA Monoclonal Antibody: Reconstitute with 6 ml ELISA Buffer.

   Store the reconstituted DNA/RNA Oxidative Damage ELISA Monoclonal Antibody at 4°C. It will be stable for at least four weeks. A 20% surplus of antibody has been included to account for any incidental losses.

   Antiserum Dye Instructions (optional)

   This dye may be added to the antibody, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody or add 300 µl of dye to 30 ml of antibody).

Assay Procedure

Pipetting Hints

1. Use different tips to pipette each reagent.
2. 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).
3. Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. ELISA Buffer

   Add 100 µl ELISA Buffer to NSB wells. Add 50 µl ELISA Buffer to B0 wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer in the NSB and B0 wells (i.e., add 50 µl culture medium to NSB and B0 wells and 50 µl ELISA Buffer to NSB wells).

2. DNA/RNA Oxidative Damage ELISA Standard

   Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. Samples

   Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. DNA/RNA Oxidative Damage AChE Tracer

   Add 50 µl to each well except the TA and the Blk wells.

5. DNA/RNA Oxidative Damage ELISA Monoclonal Antibody

   Add 50 µl to each well except the TA, the NSB, and the Blk wells.

Incubation of the Plate

Cover each plate with plastic film and incubate 18 hours at 4°C.

Development of the Plate

1. Reconstitute Ellman's Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells): 100 dtn vial Ellman's Reagent: Reconstitute with 20 ml of UltraPure water.

   NOTE: Reconstituted Ellman's Reagent is unstable and should be used the same day it is prepared; protect the Ellman's Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman's Reagent to each well.
4. Add 5 µl of tracer to the TA wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (i.e., B0 wells ≥0.3 A.U. (blank subtracted)) in 90-120 minutes.

Reading the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman's Reagent from splashing on the cover. **NOTE:** Any loss of Ellman's Reagent will affect the absorbance readings. If Ellman's Reagent is present on the cover, use a pipette to transfer the Ellman's Reagent into the well. If too much Ellman's Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman's Reagent.
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B0 wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B0 wells are in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 2.0, wash the plate, add fresh Ellman's Reagent and let it develop again.

Calculation

Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as either %B/B0 versus log concentration using a four-parameter logistic fit or as logit B/B0 versus log concentration using a linear fit.

Preparation of the Data

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

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B0 wells.
3. Subtract the NSB average from the B0 average. This is the corrected B0 or corrected maximum binding.
4. Calculate the B/B0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/B0 for a logistic four-parameter fit, multiply these values by 100.)

   NOTE: The TA values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B0 divided by the actual TA (10X measured absorbance) will give the %Bound. This value should closely approximate the %Bound that can be calculated from the Sample Data. Erratic absorbance values and a low (or no) %Bound could indicate the presence of organic solvents in the buffer or other technical problems.

Plot the Standard Curve

Plot %B/B0 for standards S1-S8 versus 8-hydroxy-2'-deoxyguanosine concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be lineraized using a logit transformation. The equation for this conversion is shown below. **NOTE:** Do not use %B/B0 in this calculation.

logit (B/B0) = ln [B/B0/(1 - B/B0)]

Plot the data as logit (B/B0) versus log concentrations and perform a linear regression fit.

Determine the Sample Concentration

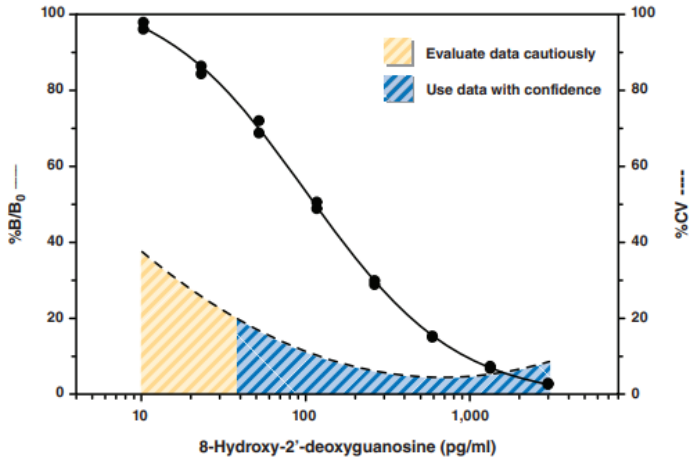
Calculate the B/B0 (or %B/B0) value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. **NOTE:** Remember to account for any concentration or dilution of the sample prior to the addition to the well. Samples with %B/B0 values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference which could be eliminated by purification.

Typical Standard Curve

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. Your results could differ substantially.

	Raw Data		Average	Corrected
Total Activity	2.105	2.101	2.103	
NSB	0.000	0.000	0.000	
B_0	1.551	1.492		
	1.480	1.397	1.480	1.480

Dose (pg/ml)	Raw Data		Corrected		%B/B_0	
3,000	0.041	0.038	0.041	0.038	2.8	2.6
1,333	0.108	0.100	0.108	0.100	7.3	6.7
592.6	0.226	0.221	0.226	0.221	15.3	15.0
263.4	0.442	0.426	0.442	0.426	29.9	28.8
117.1	0.722	0.748	0.722	0.748	48.8	50.5
52.0	1.017	1.064	1.017	1.064	68.7	71.9
23.1	1.278	1.248	1.278	1.248	86.3	84.3
10.3	1.449	1.421	1.449	1.421	97.9	96.0



Assay Range = 10.3-3,000 pg/ml
Sensitivity (defined as 80% B/B_0) = 30 pg/ml
Mid-point (defined as 50% B/B_0) = 90-140 pg/ml

The sensitivity and mid-point were derived from the standard curve shown above. The standard was diluted with ELISA Buffer.

Precision

The intra- and inter-assay CVs have been determined at multiple points on the standard curve.

Dose (pg/ml)	%CV* Intra-assay variation	%CV* Inter-assay variation
3,000	6.2	8.4
1,333	6.1	4.6
592.6	9.6	4.8
263.4	4.7	5.5
117.1	9.3	4.5
52.0	11.6	10.7
23.1	†	†
10.3	†	†

*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.

†Outside of the recommended usable range of the assay.

Specificity

Compound	Cross Reactivity
8-hydroxy-2'-deoxyguanosine	100%
8-hydroxyguanosine	38%
8-hydroxyguanine	23%
Guanosine	<0.01%

Precautions

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with CD's AChE ELISA Kits. This kit may not perform as described if any reagent or procedure is replaced or modified. When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Trace organic contaminants in the water source B. Poor pipetting/technique	A. Replace activated carbon filter or change source of UltraPure water
High NSB (>10% of B_0)	A. Poor washing B. Exposure of NSB wells to specific antibody	A. Rewash plate and redevelop
Very low B_0	A. Trace organic contaminants in the water source B. Plate requires additional development time C. Dilution error in preparing reagents	A. Replace activated carbon filter or change source of UltraPure water B. Return plate to shaker and re-read later
Low sensitivity (shift in dose response curve)	Standard is degraded	Replace standard
Analyses of two dilutions of a biological sample do not agree (*i.e.*, more than 20% difference)	Interfering substances are present	Purify sample prior to analysis by ELISA[7]
Only Total Activity (TA) wells develop	Trace organic contaminants in the water source	Replace activated carbon filter or change source of UltraPure water

References

1. Gan, W., Nie, B., Shi, F., et al. Age-dependent increases in the oxidative damage of DNA, RNA, and their metabolites in normal and senescenceaccelerated mice analyzed by LC-MS/MS: Urinary 8-oxoguanosine as a novel biomarker of aging. Free Radic. Biol. Med. 52, 1700-1707 (2012).
2. Shi, F., Nie, B., Gan, W., et al. Oxidative damage of DNA, RNA and their metabolites in leukocytes, plasma and urine of Macaca mulatta: 8-oxoguanosine in urine is a useful marker for aging. Free Radic. Res. 46(9), 1093-1098 (2012).
3. Roszkowski, K. and Olinski, R. Urinary 8-oxoguanine as a predictor of survival in patients undergoing radiotherapy. Cancer Epidemiol. Biomarkers Prev. 21, 629-634 (2012).