oxidized DNA

(8-hydroxy-2’-deoxyguanosine, 8-OHdG)
ELISA

EK-ODG 96 tests

For Research Use Only (RUO)

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ENGLISH

INTENDED USE

The BÜHLMANN oxidized DNA ELISA kit is intended for the direct and quantitative determination of 8-hydroxy-2'-deoxyguanosine present in human urine. A serum application is available on the website: www.buhlmannlabs.ch

PRINCIPLE OF THE ASSAY

The BÜHLMANN oxidized DNA ELISA is a competitive immunoassay. A polyclonal antibody (Ab) specific for mouse antibody has been coated onto the wells of the microtiter plate. During the first incubation, oxidized DNA (8-OHdG) present in the prediluted urine samples and the calibrators, respectively, competes with biotinylated 8-OHdG for the binding sites of the specific mouse anti-8-OHdG antibody. Simultaneously, the complexes bind to the anti-mouse-antibody coated to the microtiter plate. After washing, streptavidin conjugated to horseradish peroxidase (HRP) is added, which binds during a second incubation step to the Biotin-Ab complexes. Unbound enzyme label is removed by a second washing step and tetramethylbenzidin (TMB) Substrate Solution is added to the wells. During the following incubation step, a colored product is formed in inverse proportion to the amount of 8-OHdG present in the sample. Upon addition of acidic Stop Solution the color changes from blue to yellow and can be measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Microtiter Plate precoated with Goat–anti-mouse polyclonal Ab.	12 x 8 wells	B-ODG-MP	Ready to use
Plate Sealer	3 pieces		
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 ml	B-ODG-WB	Dilute with 900 ml of deionised water
Incubation Buffer with preservatives	1 bottle 50 ml	B-ODG-IB	Ready to use
Calibrator [1] lyophilized 8OHdG in a buffer matrix with preservatives	1 vial	B-ODG-CA	Add 1 ml of Incubation Buffer
Control Low / High [2] lyophilized 8OHdG within human urine	2 vials	B-ODG-CONSET	Add 1 ml of Incubation Buffer
Antiserum Monoclonal anti-8OHdG Antibody in a buffer matrix with preservatives	1 vial 5.5 ml	B-ODG-AS	Ready to use
Biotin Conjugate lyophilized 8OHdG conjugated to biotin in a buffer matrix with preservatives	1 vial	B-ODG-BC	Add 5.5 ml of Incubation Buffer
Enzyme Label streptavidin conjugated HRP in a buffer with preservatives	1 vial 11 ml	B-ODG-EL	Ready to use
TMB Substrate TMB in citrate buffer with H_2O_2	1 vial 11 ml	B-TMB	Ready to use
Stop Solution 0.25 M sulfuric acid	1 vial 11 ml	B-STS	Ready to use **Corrosive agent**

Table 1

[1] After reconstitution the actual 8-OHdG concentration of the Calibrator is 1 µg/ml. The urine samples are to be diluted 1:10 for the measurement in the ELISA. To take the dilution into account for the final calculations the Calibrator is labelled with 10 µg/ml 8-OHdG.

[2] The Controls contain lot-specific amounts of 8-OHdG. Refer to the QC Data Sheet for actual concentrations.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened Reagents	
The unopened kit components are stable at 2-8°C. Do not use the kit after the expiration date.	
Opened / Reconstituted Reagents	
Microtiter Plate	Return unused strips immediately to the aluminium pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store for up to 2 months at 2-8°C.
Diluted Wash Buffer	Store for up to 4 months at 2-8°C.
Controls	Stable at 2-8 °C for at least 4 months
Calibrators	
Biotin Conjugate	Stable at 2-8 °C for at least 1 month
Antiserum	Stable at 2-8°C until expiration date
Incubation Buffer	
Enzyme Label	
Substrate Solution	Store in darkness at 2-8°C until expiration date.
Stop Solution	Store at 18-28°C until expiration date

Table 2

WARNINGS AND PRECAUTIONS

The controls of this kit contain components of human origin. All products containing human source material should be handled in accordance with good laboratory practice using appropriate precautions.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes with disposable tips for 50 µl, 100 µl and 1000 µl.
- Multishot pipettes with disposable tips for 50 µl and 100 µl.
- Disposable polystyrene or polypropylene tubes for the preparation of calibrators and sample dilutions.
- 1000 ml cylinder for the dilution of the Wash Buffer concentrate.
- Blotting Paper
- Microtiter Plate washer or squeeze bottle for the Wash Buffer.
- Microtiter Plate rotator.
- Microtiter Plate reader for measurement of absorbance at 450 nm.

SPECIMEN COLLECTION AND STORAGE

- Urine: Less than 100 µl of urine are needed.
- Collect patient urine and keep the specimen refrigerated. Aliquots may be stored at 2-8°C for up to 7 days, keep at –20 °C for up to 4 months.
- Serum: Collect at least 1.0 ml of blood or for 0.5 ml of. blood into plain tubes, avoid hemolysis, leave to clot for 45 min at room temperature (18-28°C). Centrifuge at 1800 x g for 15 min at room temperature and collect the serum. Interferences of lipemic, hemolytic and icteric samples have not been investigated.
- A serum application is available on the website: www.buhlmannlabs.ch.

PROCEDURAL NOTES

- The enzyme (HRP) used as the label is inactivated by oxygen and is highly sensitive to sodium azide, thimerosal, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Therefore, use only deionized or distilled high quality water.
- If the initial concentration of an unknown sample reads greater than the highest calibrator (312.5 ng/ml), the sample should be further diluted with incubation buffer and re-assayed according to the assay procedure. The resulting dilution factor must be accounted for final calculations.
- **Calibrator dilution:** Reconstitute the calibrator. Prepare a fresh standard curve each time a new assay is performed. In order to obtain the standard curve, serial dilutions of the Calibrator are prepared as follows:
  - Label five tubes S1 through S5 and pipet 930 µl of Incubation buffer (IB) into tube S1 and 300 µl IB into tubes S2 through S5.
  - Pipet 30 µl of reconstituted Calibrator (10 µg/ml) into tube S1 and vortex.
  - Transfer 200 µl from S1 to S2, vortex. Transfer 200 µl from S2 to S3, vortex. Continue to transfer 200 µl from each tube until dilution series is completed. The corresponding concentrations of 8-OHdG will be:
    S1 312.5 ng/ml S4 20 ng/ml
    S2 125 ng/ml S5 8 ng/ml
    S3 50 ng/ml

The diluted Calibrators are stable for 8 hours at 2-8°C. Prepare new dilutions each time a new assay is performed.

ASSAY PROCEDURE

1. Centrifuge urine samples for 5 min. at 10'000 g and dilute samples 1:10 with Incubation Buffer (e.g. 50 µl of urine + 450 µl of Incubation Buffer).
2. Dilute Calibrator according to the scheme mentioned above
3. Prepare a plate with sufficient strips to test the desired number of samples. Remove excess strips from the holder and immediately reseal them in the foil pouch together with the desiccant packs. Store refrigerated.

   Important: Equilibrate the reagents to 18-25 °C before performing the assay.
4. Wash the coated wells twice using at least 300 µl of Wash Buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

5a. Pipet 50 µl of Calibrator 10 µg/ml (Blank) in duplicate into wells A1+A2.
Pipet 50 µl of Incubation Buffer (B0) in duplicate into wells B1+B2.
Pipet 50 µl of Calibrator S5 (8.0 ng/ml) in duplicate into wells C1+C2.
Pipet 50 µl of Calibrator S4 (20 ng/ml) in duplicate into wells D1+D2.
Pipet 50 µl of Calibrator S3 (50 ng/ml) in duplicate into wells E1+E2.
Pipet 50 µl of Calibrator S2 (125 ng/ml) in duplicate into wells F1+F2.
Pipet 50 µl of Calibrator S1 (312.5 ng/ml) in duplicate into wells G1+G2.

5b. Pipet 50 µl of the Low Control into wells H1+H2.
Pipet 50 µl of the High Control into wells A3+A4.

5c. Pipet 50 µl of each diluted sample (1:10) in duplicate into the subsequent wells.

6. Pipet 50 µl Biotin Conjugate into all wells.
7. Pipet 50 µl Antiserum into all wells.
8. Cover the plate with a Plate Sealer, place the plate on a plate rotator set at **400-600 rpm** and incubate for **5 minutes at 18-25 °C.** Then incubate the plate **at 2-8°C for 2 hours ± 5 min.**
9. Remove and discard the Plate Sealer. Empty the wells and wash three times using at least 300 µl of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
10. Pipet 100 µl of Enzyme Label to all wells.
11. Cover the plate with a Plate Sealer, place the plate on a plate rotator set at **400-600 rpm** and incubate for 30 ± 1 minutes at 18-25 °C.
12. Remove and discard the Plate Sealer. Empty the wells and wash three times using at least 300 µl of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.
13. Pipet 100 µl of the TMB Substrate Solution to all wells.
14. Cover the plate with a Plate Sealer, place the plate on a plate rotator set at **400-600 rpm**, protect the plate from direct light and incubate for 15 ± 1 minutes at 18-25 °C.
15. Pipet 100 µl of Stop Solution to all wells. Remove air bubbles with a pipette tip. Proceed to step 15 within 30 minutes.
15. Read the absorbance at 450 nm in a microtiter plate reader within 30 minutes.

RESULTS

Standard Curve

Record the absorbance at 450 nm for each calibrator and the blanks. Calculate the average of the duplicates, subtract the Blank and record the blank-corrected average absorbance). Calculate the binding (B) of each duplicate of calibrators as the percentage of B0 (B/B0 %), with the Blank-corrected absorbance of B0 as 100 %.

$$B / B_0\ (\%) = \frac{\text{net absorbance of Calibrator}}{\text{net absorbance of B0 (Incubation Buffer)}} \times 100$$

Plot the percent bound (vertical axis) versus the concentration of 8-OHdG in ng / ml (horizontal axis) using a lin/log graph paper. Draw the best fitting curve or calculate the standard curve using a four parameter logistic. See Figure 1 for typical data (results and standard curve*). These results and standard curve are provided for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.*

Samples and Controls

Record the absorbance at 450 nm for each sample well. Average the duplicate values, subtract the mean of the blank wells and record the corrected average absorbance. Calculate, as described above, the binding of each sample duplicate as a percentage of B_0, with the Blank-corrected absorbance of the B0 (100%). Locate the B/B_0 value of the samples on the vertical axis, draw a horizontal line intersecting the standard curve and read the 8-OHdG concentration (ng/ml) from the horizontal axis.

QUALITY CONTROL

Reliable results will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following this package insert.

The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the controls are lot-specific and printed on the QC data sheet delivered with the kit.

If the precision of the assay is not within the established limits and repetition excludes errors in technique, check the following issues: i) pipetting, temperature and timers ii) ELISA reader settings iii) expiration dates of reagents iv) storage and incubation conditions v) TMB Substrate Solution should be colorless vi) purity of water.

LIMITATIONS

- The reagents supplied with this kit are optimized to measure 8-OHdG in human urine.
- Optimal Incubation temperature is 20-25 °C for Enzyme Label and TMB.
- Cross reactivity: the kit measure also 8-hydroxyguanine and 8-hydroxyguanosine (see performance characteristics)

PERFORMANCE CHARACTERISTICS

Intra-Assay Precision: 6.8 %. The intra-assay precision was calculated from 20 pairs of values of 6 urine samples assayed in a single run according to the assay procedure. The values are presented in Table 4.

Inter-Assay Precision: 11.7 %. The inter-assay precision of the ELISA was calculated from 4 urine samples (2 spiked) and 20 aliquots of the low and high control. The samples and controls were assayed in duplicates in 20 different runs according to the assay procedure. The values are presented in Table 5 .

Analytical Sensitivity of the ELISA: < 5 ng/ml. The minimal detectable dose of 8-OHdG was calculated to be 5 ng/ml by subtracting two standard deviations from the mean absorbance of the blank (Incubation Buffer) and intersecting this value with the standard curve obtained in the same run.

Functional Sensitivity: < 13.5 ng/ml. Six native urine samples and three spiked urines with values between 4.9 and 363 ng/ml 8-OHdG were assayed 20 times in duplicates in one assay. The % CV and the mean values were calculated for each sample. The functional sensitivity was observed at 15 % CV. The resulting precision profile in Figure 2 allows the precise measurement between 13.5 and 312.5 ng/ml.

Dilution Linearity: 96.5 %. Two urine samples and three spiked urine were diluted with Incubation Buffer and subsequently assayed according to the assay procedure. The expected values were calculated from the observed value found with the first dilution. The results are presented in Table 6.

Spiking Recovery: 102.5 %. Three urine samples were spiked with different amounts of 8-OHdG. The samples were measured before and after spiking according to the assay procedure. The results are presented in Table 7.

Specificity: Cross-reaction of the monoclonal anti-8OHdG antibody with 19 analogues has been determined at 50 % binding. For the following substances a significant cross-reaction has to be reported.

8-Hydroxyguanosine: 20 %
8-Hydroxyguanine: 17 %

The results are presented in Table 8.

Table 3: **Example of Results**

	Absorb. (OD)	CV (%)	B/B_0 (%)	Conc. (ng/ml)
Blank	**0.054**			
B 0	2.499		101.7	
B 0	2.417		98.3	
B 0 Avg.	**2.458**	**2.3**	**100.0**	**0.0**
Cal S1	2.312		94.1	
Cal S1	2.258		91.8	
Cal A Avg.	**2.285**	**1.7**	**93.0**	**8.0**
Cal S2	1.875		76.3	
Cal S2	1.775		72.2	
Cal B Avg.	**1.825**	**3.9**	**74.3**	**20.0**
Cal S3	1.057		43.0	
Cal S3	1.015		41.3	
Cal C Avg.	**1.036**	**2.9**	**42.1**	**50.0**
Cal S4	0.450		18.3	
Cal S4	0.430		17.5	
Cal D Avg.	**0.440**	**3.1**	**17.9**	**125.0**
Cal S5	0.145		5.9	
Cal S5	0.138		5.6	
Cal E Avg.	**0.142**	**3.3**	**5.8**	**312.5**
Control low	1.495			
Control low	1.320			
Control low Avg.	**1.408**	**8.8**		**33.6**
Control high	0.300			
Control high	0.275			
Control high Avg.	**0.288**	**6.0**		**173.9**

ED-20 = 107.5 ng/ml ED-50 = 40.8 ng/ml ED-80 = 16.2 ng/ml

Figure 1: **Example of Standard Curve**

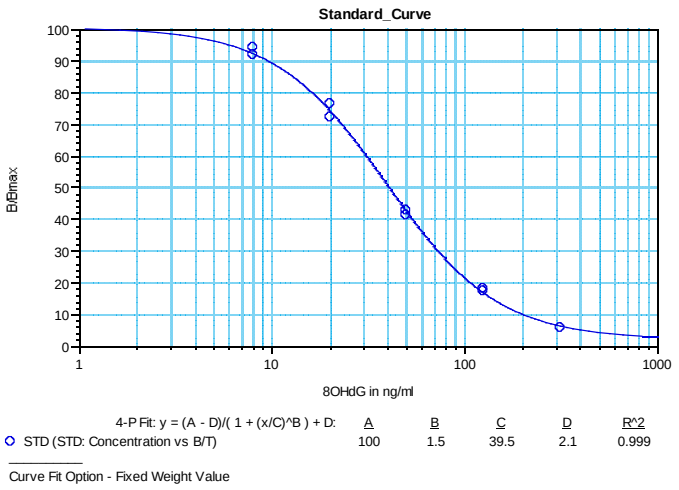

Figure 2: **Precision Profil**

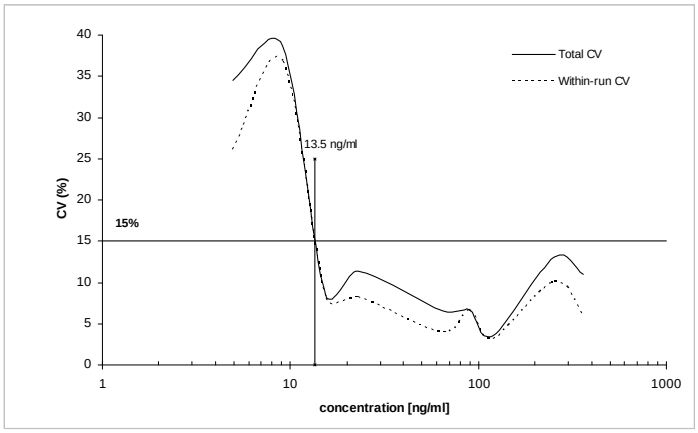

Table 4: **Intra-Assay Precision**

Sample	Mean [ng/ml]	SD [ng/ml]	CV [%]
1	15.3	1.1	7.2
2	23.0	2.2	9.7
3	64.7	3.8	5.9
4	88.7	4.2	4.8
5	119.4	3.4	2.8
6	253.0	27.0	10.6
Mean			**6.8**

Table 5: **Inter-Assay Precision**

Sample	Mean [ng/ml]	SD [ng/ml]	CV [%]
7	15.4	2.6	17
8	34.5	4.4	12.7
9	52.9	5.3	10.0
10	115.3	11.7	10.1
Low Ctrl	35.4	4.8	13.6
High Ctrl	180.0	12.1	6.7
Mean			**11.7**

Table 6: **Dilution Linearity/Parallelism**

Sample	Dilution	Observed [ng/ml]	Expected [ng/ml]	O/E [%]
S1	1:10	76.9	-	-
	1:20	36.1	38.5	93.9
	1:40	18.1	19.2	94.1
	Mean			**94.0**
S2	1:10	156.1	-	-
	1:20	72.5	78.1	92.9
	1:40	42.1	39.0	107.9
	1:80	20.4	19.5	104.5
	Mean			**101.7**
S3	1:10	356.5	-	-
	1:20	222.0	-	-
	1:40	125.7	111.0	113.2
	1:80	52.0	55.5	93.7
	1:160	23.3	27.8	84.0
	1:320	12.4	13.9	89.4
	Mean			**95.1**
S4	Mean			**98.4**
S5	Mean			**93.4**
Mean				**96.5**

Table 7: **Spiking Recovery**

Sample	ng/ml	Spiked with [ng/ml]	Observed [ng/ml]	Expected [ng/ml]	O/E [%]
S1	19.6	10	26.8	29.6	90.5
		20	31.4	39.6	79.3
		50	62.8	69.6	90.2
		100	110.6	119.6	92.5
		200	206.4	219.6	94.0
	Mean				**89.3**
S2	37.8	10	48.3	47.8	101.0
		20	53.8	57.8	93.1
		50	90.1	87.8	102.6
		100	141.4	137.8	102.6
		200	252.7	237.8	106.3
	Mean				**101.1**
S3	32.8	10	46	42.8	107.5
		20	59.5	52.8	112.7
		50	99.3	82.8	119.9
		100	165.9	132.8	124.9
		200	280.0	232.8	120.3
	Mean				**117.1**
Mean					**102.5**

Table description: cf. “Results” (page 3), “Performance Characteristics”.

Table 8: **Cross Reactivity**

Molecule	**% Inhibition at IC_{50}**
8-hydroxydeoxyguanosine	**100**
8-hydroxyguanosine	20
8-hydroxyguanine	17
2’-Deoxyguanosine monohydrate	<0.01
0^6-Methyl-2’-deoxyguanosine	<0.01
8-Hydroxy-2’-deoxyadenosine	<0.01
2’-deoxyinosine	<0.01
2’-deoxycytidine	<0.01
Thymidine	<0.01
2’-deoxyuridine	<0.01
Guanosine	<0.01
8-mercaptoguanosine	<0.01
8-Bromoguanosine	<0.01
7-methylguanosine	<0.01
Guanine	<0.01
Creatine	<0.01
Creatinine	<0.01
Uric acid	<0.01
Urea	<0.01

APPENDIX II
REFERENCES

1. Marcus S. Cooke et al.: *Urinary 8-Oxo-2’Deoxyguanosine – Source, Significance and Supplements.* Free Rad. Res. **32**, 381-397 (2000).

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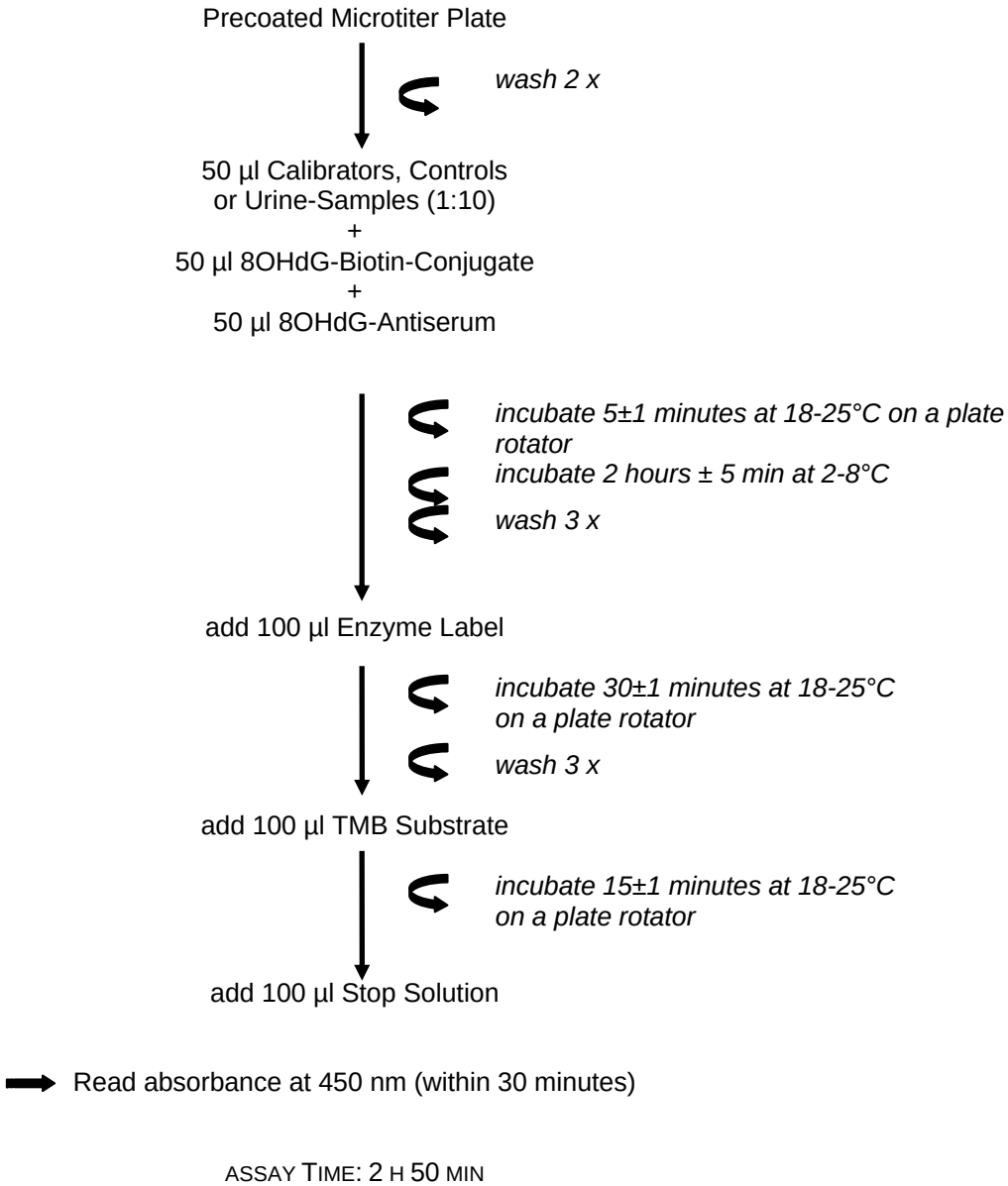
Oxidised DNA ELISA



ASSAY TIME: 2 H 50 MIN

Symbol	Explanation
	Use By
REF	Catalogue number
LOT	Batch code
IVD	*In Vitro* Diagnostic Medical Device
	Contains sufficient for <n> tests
	Consult Instructions for Use
	Temperature limitation
	Upper limit of temperature
MP	Microtiterplate
BUF WASH 10X	Wash Buffer Concentrate (10x)

Symbol	Explanation
BUF INC	Incubation Buffer
CAL	Calibrator
CONTROL L	Control Low
CONTROL H	Control High
Ab	Antiserum
BC	Biotin Conjugate
EL	Enzyme Label
SUBS TMB	TMB Substrate
SOLN STOP	Stop Solution

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