

“ Highly Sensitive 8-OHdG Check ” Instructions

The 8-OHdG ELISA kit is a competitive *in vitro* enzyme-linked immunosorbent assay for quantitative measurement of the oxidative DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine, tissue, serum, plasma and so on. (Japanese Patent No.3091974)

1. Kit Contents

(1) 8-OHdG Microtiter plate	: coated with 8-OHdG (8×12wells, split type)	1 plate
(2) Primary antibody	: monoclonal antibody specific for 8-OHdG	1 vial
(3) Primary antibody solution	: phosphate buffered saline	1 vial (ca.6ml)
(4) Secondary antibody	: HRP-conjugated antibody	1 vial
(5) Secondary antibody solution	: phosphate buffered saline	1 vial (ca.12ml)
(6) Chromatic solution	: 3,3',5,5'-tetramethylbenzidine	1 vial (ca.0.25ml)
(7) Diluting solution	: hydrogen peroxide/citrate-phosphate buffered saline	1 vial (ca.12ml)
(8) Washing solution(×5)	: 5 times concentrated phosphate buffered saline*	2 vials (ca.26ml×2)
(9) Reaction terminating solution	: 1M phosphoric acid	1 vial (ca.12ml)
(10) Standard 8-OHdG solution	: 0.125, 0.25, 0.5, 1, 4, 10 ng/ml	1vial each total 6 vials (ca.1ml/vial)
(11) Plate seal		1 sheet

* Dilute ⑧Washing solution by 5 times (v/v) with distilled water for use.

2. Storage

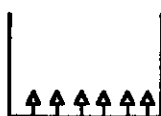
Store all reagents at 2-8°C.

The components should be used before the expiration date indicated on the outside of the box.

3. Materials and Equipments Required But Not Provided

- (1) Pipettes and Pipette Tips: 10µl - 200µl
- (2) 8-channel Pipette and Pipette Tips: 50µl - 200µl or 50µl - 300µl
- (3) Buffer and Reagent Reservoirs for 8-channel Pipettes
- (4) Absorbent Towels
- (5) Refrigerator
- (6) Microtiter Plate Reader of Capable of Reading Absorbency at 450nm

4. Principles of Procedure

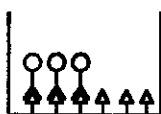


- (1) The 8-OHdG monoclonal antibody and the sample or standard are added to the microtiter plate which has been coated with 8-OHdG. The 8-OHdG monoclonal antibody reacts competitively with the 8-OHdG bound on the plate and the 8-OHdG in samples solution. Therefore higher concentrations of 8-OHdG in the sample solution lead to a reduced binding of the antibody to the 8-OHdG on the plate.



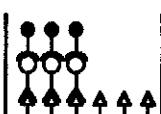
- (2) The antibodies which are bound to the 8-OHdG in the sample are washed away from the antibodies that have bound to the 8-OHdG coated on the plate.

- (3) An enzyme-labeled secondary antibody, which is added to the plate, binds to the monoclonal antibody which is bound to the 8-OHdG coated on the plate.



- (4) Unbound enzyme-labeled secondary antibody is removed by a wash step.

- (5) Addition of a chromatic substrate results in the development of color in proportion to the amount of antibody bound to the plate.



- (6) The color reaction is terminated and the absorbance is measured.

5. Assay Procedure

Allow the reagents shall be used to reach room temperature before use, and proceed through the following 10 steps.

- (1) Reconstitute primary antibody with primary antibody solution. Allow complete dissolving.
- (2) Add 50 μ l sample or standard solution to well, as shown in plate diagram. To ensure accuracy, do not use outer most wells.
- (3) Add 50 μ l reconstituted primary antibody to each well except blank well. Shake plate by hand to ensure complete mixing of solution in all wells. Cover plate with adhesive strip, making sure it is sealed tightly. Incubate at 4°C for over night.
- (4) Throw away the solution of wells in the sink. Remove the residual solutions from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Wash assay plate 3 times using this procedure with washing solution, 250 μ l per well per wash. **Do not let wells dry before proceeding to the next step.**
- (5) Reconstitute secondary antibody with secondary antibody solution. Allow complete dissolving.
- (6) Add 100 μ l reconstituted secondary antibody to each well. Shake plate by hand to ensure complete mixing of solution in all wells. Cover plate with adhesive strip, making sure it is sealed tightly. Incubate at room temperature for 1 hour.
- (7) Repeat step (4).
- (8) Reconstitute chromatic solution (enzyme substrate solution) with 100 times volume of diluting solution. Add 100 μ l reconstituted enzyme substrate to each well and shake plate by hand to ensure complete mixing of solution in all wells. Incubate at room temperature for 15 minutes. The incubation should be done in the dark, i.e. shield the plate with aluminum foil. Blue color should be formed in wells of 8-OHdG standards with intensity proportional to decreasing concentrations of 8-OHdG.
- (9) Add 100 μ l reaction terminating solution and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification.
- (10) After terminating the reaction, read absorbance at 450 nm in a plate reader.
Use a standard curve to determine the amount of 8-OHdG present in samples. Generate the standard curve by plotting absorbance vs. log (concentration of sample). Then use the absorbance values obtained for the samples to determine the concentrations.

6. Guides

(1) Sample Pretreatment:

To assay properly, please pre-treat samples desired bellow. In addition, please avoid repeated freeze thaw samples. In order to confirm the aptitude of assay methods on a new sample, implementing recovery test of standard 8-OHdG added into new sample is recommended.

- ① Urine: If it's clear, pretreatment is not needed.

Centrifuging at 2,000 ~ 5,000g for 10 ~ 15 minutes is recommended for opaque samples only.

- ② Serum: Blood samples must be separated to serum immediately. To separate interfering substances, filtration of serum using an ultra filter (cut off molecular weight 10,000) is necessary. Pre-treat ultra filter following manufacture's protocols. In order to reduce deviation, diluting samples by more than twice, while paying attention to concentration range is suggested.

- ③ DNA in tissue: It's necessary to extract samples and digest DNA before using this kit.

(2) Measurement:

- ① Please pay attention to the reaction conditions in order to avoid the affection of reaction conditions.
- ② Thoroughly Washing of Microtiter Plate: It's recommendable to invert the plate and tap it smartly onto absorbent towels in order to remove the residual solutions from all wells, because solutions remained in well influence measurement value.
- ③ Cleansing of Instruments and Vessels: Please use clean instruments and vessels (such as tips, reservoirs for 8-channel pipette etc.). If such tools are used repeatedly, please boil or steep them into alkaline cleanser, then wash thoroughly and dry them before use.
- ④ Be careful not to shake it violently when chromatic solution is diluted.
- ⑤ Adjustment of PH for Samples: It is necessary to maintain PH of a sample mixed with primary solution between 6.0 and 8.0. It's recommendable for abnormal urine samples to be diluted with PBS by 3 times.

(3) Split Usage:

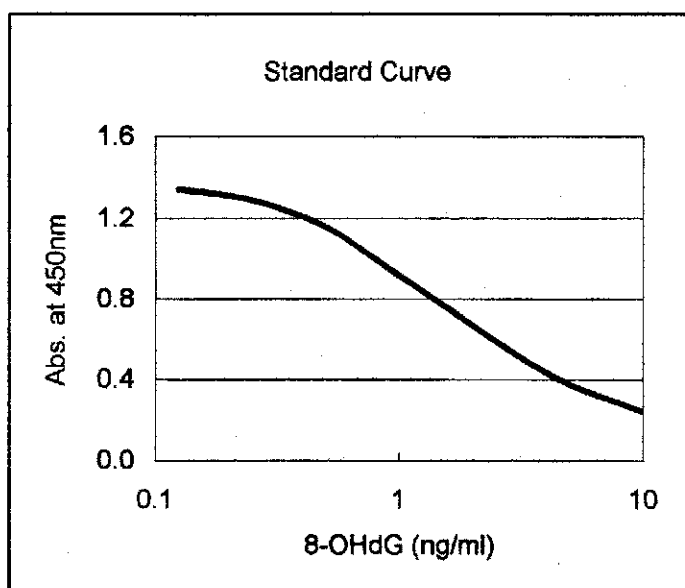
- ① Remained parts of kit (plate and reagents) must be kept at 2-8°C and used within two weeks after open.
- ② Plate and reagents except chromatic solution shall be used to reach room temperature before use.
- ③ Necessary volume of chromatic solution may be added to adequate volume only of diluting solution just before the reaction. Keep chromatic solution in the dark.

(4) Wells Usage:

- ① To avoid edge effects, the use of outer most wells is not recommended. To maintain the uniform temperature within the wells, please add same volume (50µl) PBS or DW to the unused wells.
- ② Blank well: At the operation of step (3), those wells which are not added the reconstituted primary antibody, will serve as blank wells. **High absorbance in blank well could be due to a)cross well contamination by standard solution or sample b)inadequate washing of wells with washing solution c)overexposure to light after substrate has been added.**
- ③ The figure below shows a typical arrangement for sample loading in triplicates for each sample. Wells indicated with a cross-mark (x) in A and H lines are not used. With this arrangement, a maximum of 18 samples can be assayed in a plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	x	x	x	x	x	x	x	x	x	x	Blank
B	STD 0.125ng/ml (x3)				1	(x3)		7	(x3)		13	(x3)
C	0.25ng/ml (")				2	(")		8	(")		14	(")
D	0.5ng/ml (")				3	(")		9	(")		15	(")
E	1ng/ml (")				4	(")		10	(")		16	(")
F	4ng/ml (")				5	(")		11	(")		17	(")
G	10ng/ml (")				6	(")		12	(")		18	(")
H	Blank	x	x	x	x	x	x	x	x	x	x	Blank

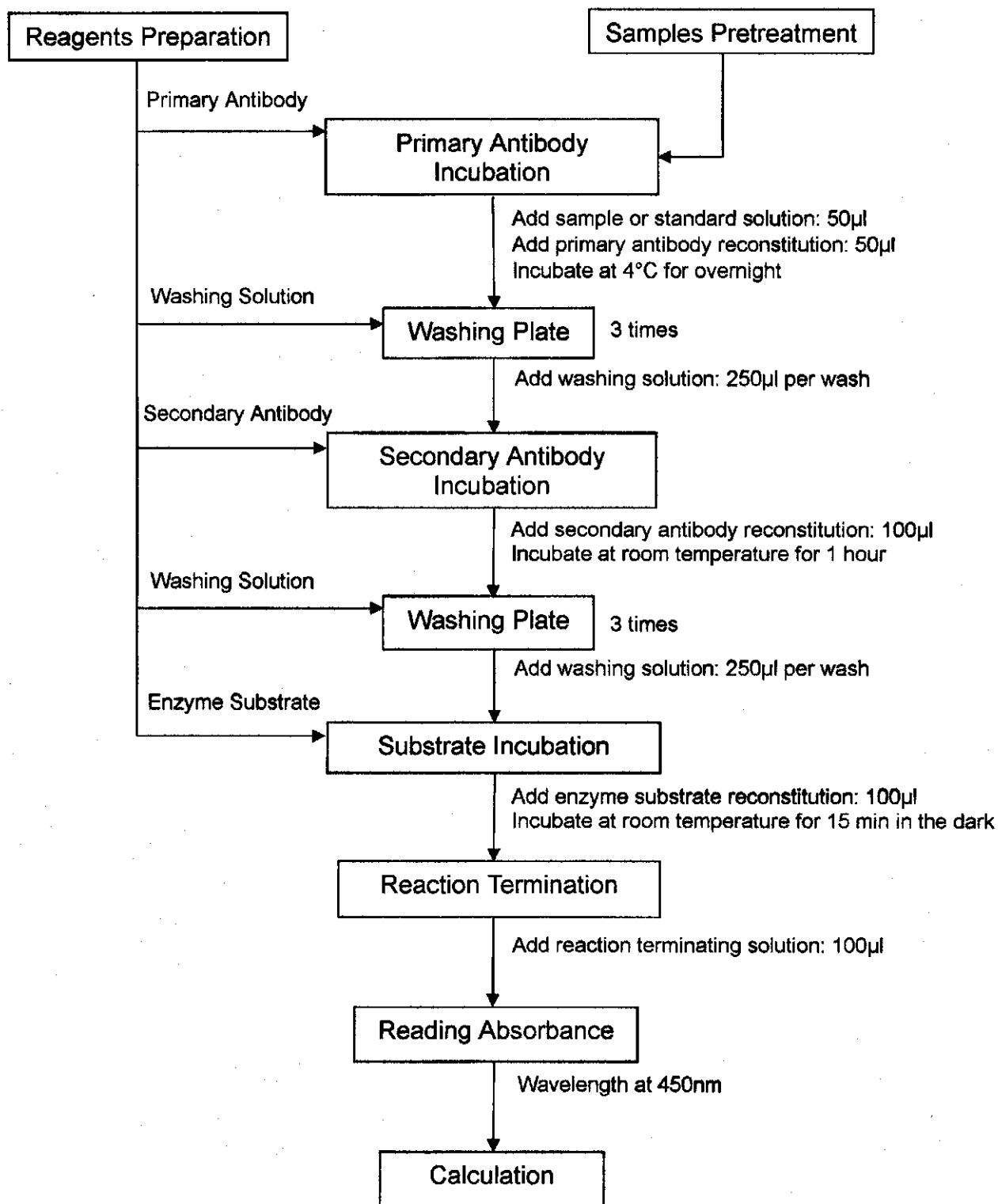
7. Standard Curve



8. References

- (1) S.Toyokuni, T.Tanaka, Y.Hattori, Y.Nishiyama, A.Yoshida, K.Uchida, H.Hiai, H.Ochi, and T.Osawa. Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: Its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab.Invest.* **76**, pp365-374 (1997)
- (2) T.Shimoike, T.Inoguchi, F.Umeda, H.Nawata, K.Kawano and H.Ochi. The meaning of serum levels of advanced glycosylation end products in diabetic nephropathy. *Metabolism* **49(8)** pp1030-1035 (2000)
- (3) T.Kuragano, T.Kuno, C.Yamamoto, Y.Nagura, S.Takahashi, K.Kanmatsuse Oxidative Stress on DNA in chronic renal failure: the influence of different hemodialysis membranes. *J Artif. Organs* **4** pp320-325 (2001)
- (4) Y.Ibuki, T.Warashina, T.Noro, R.Goto Coexposure to benzo[a]pyrene plus ultraviolet A induces 8-oxo-7,8-dihydro-2'-deoxyguanosine formation in human skin fibroblasts: preventive effects of anti-oxidant agents. *Environmental Toxicology and Pharmacology* **12** pp37-42 (2002)
- (5) M.Kakimoto, T.Inoguchi, T.Sonta, H.Y.Yu, M.Imamura, T.Etoh, T.Hashimoto and H.Nawata Accumulation of 8-hydroxy-2'-deoxyguanosine and mitochondrial DNA deletion in kidney of diabetic rats. *Diabetes* **51** pp1588-1595 (2002.5)
- (6) M.Takane, N.Sugano, H.Iwasaki, Y.Iwano, N.Shimizu and K. Ito New biomarker evidence of oxidative DNA damage in whole saliva from clinically healthy and periodontally diseased individuals. *J.Periodontol* **73(5)** pp551-554 (2002.5)
- (7) M.S.Cooke, M.D.Evans and J.Lunec DNA repair: insights from urinary lesion analysis. *Free Radical Research* **36(9)** pp929-932 (2002)
- (8) H.W.Kim, A.Murakami, M.V.Williams and H.Ohigashi Mutagenicity of reactive oxygen and nitrogen species as detected by co-culture of activated inflammatory leukocytes and AS52 cells. *Carcinogenesis* **24(2)** pp235-241 (2003)

9. Assay Flowchart



Japan Institute for the Control of Aging
710-1 Haruoka, Fukuroi City, Shizuoka Pref., Japan, 437-0122
E-mail: biotech@jaica.com URL: <http://www.jaica.com/biotech/e>
Tel: +81-538-49-0125 Fax: +81-538-49-1267