TREVIGEN® Instructions

For Research Use Only. Not for Use In Diagnostic Procedures.

Anti-8-hydroxyguanine antibody (Anti-8-oxo-dG)

Monoclonal Antibody for Detection of 8-oxo-dG

Cat# 4359-MC-100

コスモ・バイオ株式会社

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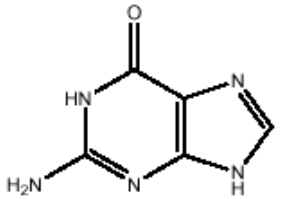
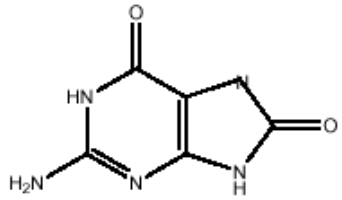
I. Introduction

Mutagenic reactive oxygen species are implicated in cancer, neurodegenerative disorders such as Alzheimer's disease (1), and in apoptosis. Oxidative damage can cause formation of 8-hydroxyguanine (i.e. 7,8-dihydro-8-oxo-guanine, 7-oxo-7, 8-dihydroguanine), often termed 8-oxo-dG. The production of 8-hydroxyguanine is almost exclusively elicited by oxidative stress with the main attack site by oxidative radicals at the N7-C8 bond. DNA polymerases preferentially insert adenine opposite 8-hydroxyguanine. Therefore, without repair these oxidative damage adducts can lead to G to T transitions. The 8-hydroxyguanine lesion causes mutational frequencies of 1 - 5% (mainly G:C to T:A transitions) and is one of the most abundant oxidative lesions.



guanine



8-hydroxyguanine

In Escherichia coli, repair of 8-hydroxyguanine may occur prior to replication via the base excision pathway. During replication formamidopyrimidine (FaPy) DNA glycosylase can excise the damaged base. Without repair 8-hydroxyguanine is commonly matched with adenine by DNA polymerases. Mut Y may initiate base excision of adenine on the undamaged strand, with correction to an 8-hydroxyguanine:cytosine pairing followed by repair by FaPy glycosylase. Mutation due to 8-hydroxyguanine typically occurs through the G to T transition when the 8-hydroxyguanine is mispaired with adenine, which then pairs with thymine during replication.

Trevigen's anti-8-oxo-dG antibody allows detection and quantitation of 8-hydroxyguanine in DNA, cell and tissue samples. The antibody was raised against 8-hydroxyguanine coupled to BSA, and is provided purified in phosphate buffered saline (PBS), 0.01% sodium azide. The antibody is recommended for use in immunofluorescence and immunohistochemistry (1-4). The antibody has similar binding affinity for 8-hydroxydeoxyguanosine and for 8-hydroxyguanine. Cross-reaction

with guanine and guanosine occurs at concentrations 800 to 20,000-fold higher than for 8-hydroxyguanine. The antibody will cross react with structurally related 8-hydroxyguanine derivatives such as 5-hydroxymethyluridine. This protocol is provided as a guide and incubation times, temperatures and antibody concentrations may need optimization.

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. DAB is a known carcinogen and should be disposed of according to local and state laws.
3. Trevigen assumes no liability for damage resulting from handling or contact with Trevigen products. Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. MSDS are available on request.

III. Materials Supplied

Catalog Number	Component	Amount Provided	Storage Temperature
4359-MC-100	**Monoclonal Anti-8-oxodG antibody**	**100µl**	**+4°C**

For long term storage, aliquot and store at -20°C up to one year.

IV. Materials/Equipment Required But Not Supplied

Reagents:
Proteinase K*
70% ethanol
eosin or 1% Methyl Green*
methanol
StrepABC complex or Streptavidin-peroxidase* 1 mg/ml
DNase-free RNase
30% hydrogen peroxide
DAB*
Tris base
1 M Tris-HCl, pH 7.5
HCl
0.4 M NaCl
100 mM EDTA, pH 8
Fetal bovine serum
10X PBS*

Equipment:
Microscope

* Available from Trevigen, see Section VIII, page 11.

V. Immunoperoxidase Assay

i. Sample Preparation

Preparation of Suspension Cells

Cells grown in suspension can be fixed in solution and then spotted onto pretreated glass microscope slides for processing. This method is quick and easy and requires no special equipment.

Method:

1. Harvest cell suspension by centrifugation at 500 x g for 5 minutes at room temperature.
2. Discard media and resuspend cells in 5 ml 70% ethanol at -20°C. Let stand for 10 minutes at 4ºC.
3. Spot 1 x 10^5 cells onto a clean glass microscope slide. Air dry for 10 minutes at room temperature.
   Note: Glass slides pretreated for electrostatic adherence are recommended. Other pretreatments (e.g. gelatin) can cause increased background staining.
4. Immerse in 70% ethanol at room temperature for 10 minutes and then air dry overnight at room temperature or dry at 45^0C for 2 hours.
5. Immerse in 1X PBS and proceed to Immunoperoxidase detection on page 7.

Preparation of Monolayer Cells

Sterile Chamber Slides
For optimal outcomes, cells should be grown on a surface that allows for both fixation and direct labeling such as sterile chamber slides. Remove the chamber walls and gasket after fixation.

Sterile Slides
Other cell culture methods include culturing directly on microscope slides. The slides must be sterile and, if necessary, pretreated to ensure cell adhesion. Sterilize microscope slides by autoclaving in a large glass Petri dish. If needed, coat slides with sterile poly-L-lysine or collagen. Place sterile microscope slides in culture vessel directly before plating cells.

Sterile Glass Cover Slips
Sterilize cover slips by autoclaving in a large glass Petri dish. If needed, coat cover slips with sterile poly-L-lysine or collagen. Place sterile glass cover slips in wells of tissue culture dishes (12 mm cover slips fit into 24 well tissue culture plates) using fine tipped sterile forceps. Handle only at edges prior to cell plating.

Method:

1. Remove media from cells and rinse once with 1X PBS at room temperature.
2. Fix cells for 10 minutes on ice with 70% ethanol at -20°C.
3. Rinse cells with 1X PBS, for 2 minutes each wash.
4. Proceed to Immunoperoxidase detection on page 7.

Preparation of Fresh Frozen Tissue

Rapid-freeze tissue or biopsy immediately after removal by immersing in liquid nitrogen or on dry ice. Store frozen tissue at –70°C or lower. Samples may be embedded in a cutting matrix prior to freezing. Position the sample within cutting matrix in a suitable container. Immerse embedded tissue in isopentane chilled on dry ice. Using the cutting matrix, attach the sample to cutting block. Equilibrate the temperature of the cryostat before sectioning. Collect sections between 10-20 μm on glass slides pretreated for electrostatic adherence.

1. **Dry overnight at room temperature or for at least 2 hours at 45°C on a slide warmer.**
2. **Immerse for 10 minutes in 70% ethanol at -20°C.**
3. **Wash two times in 1X PBS, 2 minutes each wash.**
4. **Proceed to Immunoperoxidase detection on page 7.**

Preparation of Paraffin Embedded Tissue

1. Deparaffinize five-micron tissue sections twice in Xylene for 10 min each.
2. Rehydrate for 1 minute each in the following ethanol solutions: A, 100% Ethanol; B, 100%, Ethanol; C, 90% Ethanol; D, 90% Ethanol; E, 70% Ethanol.
3. Wash twice in PBS (2 minutes each).
4. Antigen retrieval if needed (this step is needed only if you want to stain the tissues for other antigens too, if only 8-oxodG to detected go direct to step 10).
5. Antigen retrieval solution of 10mM citrate acid, pH6 is added into a container containing slides.
6. Place a container in a microwave on high (700 watt oven) to cook for 20 minutes.
7. Check level of retrieval solution to avoid slides dry.
8. Cool for **20 minutes**
9. Wash slides
10. Cover tissue sections with 100ul of 10ug/ml proteinase K, in PBS and incubate for **15-30 minutes at 37°C**.
11. Proceed to Immunoperoxidase detection.

ii. Immunoperoxidase Detection

Note: RNase treatment is optional but recommended in studies of mitochondrial DNA oxidation to increase the sensitivity of the detection method. For RNA oxidation studies, omit the RNase step. The antibody recognizes 8-oxoG in RNA as well as in DNA.

1. Wash fixed samples twice in 1X PBS, 5 minutes each.
2. *__**Optional step**__: Incubate sample in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.4 mM NaCl, 100 μg/ml RNase for 1 hour at 37°C.
3. Wash twice with 1X PBS (2 minutes each).
4. Denature the DNA by soaking the slides in 4 N HCl for 5 minutes at room temperature.
5. Neutralize the sample by soaking the slides in 50 mM Tris-base for 5 minutes at room temperature.
6. Wash twice in PBS (2 minutes each).
7. Block non-specific binding sites by incubating the tissue sections in 10mM Tris-HCl, pH 7.5, 10% fetal bovine serum, for 30 minutes at room temperature.
8. Incubate the tissue sections with 50ul per slide of Mouse Monoclonal Anti-8-Hydroxyguanine Antibody (4359-MC-100, clone 4E9) at a concentration of **1:300** to **1:500** diluted in PBS, containing 10% bovine serum or 1% BloxAll™, for **1 hour at RT or overnight at 4^{0}C**. *Titration of the antibody is advised to obtain optimal results*.
9. Wash twice in PBS (2 minutes each).
10. Add secondary antibody Biotinylated Anti-Mouse Immuno-globulins at a concentration of **1:100** dilution (for optimal dilution refer to the suppliers recommendations) in PBS for **30 minutes at room temperature**.
11. Wash twice in PBS (2 minutes each).
12. Add StrepABC complex and incubate for **30 minutes at room temperature** (*StrepABC can be replaced with Strep-HRP, but with lower sensitivity for detection of 8-oxodG)*
13. Wash twice in PBS (2 minutes each).
14. Incubate in 0.5μg/ml diaminobenzamide tetrahydrochloride (DAB), 0.001% hydrogen peroxide in 1X PBS for 10 minutes at room temperature. When using Trevigen's DAB (Cat# 4800-30-07) thaw DAB reagent at 37°C until completely thawed (about 10 minutes) and just before use combine:
    a. 250 μl of DAB
    b. 50 μl of 30% hydrogen peroxide
    c. 50 ml of 1X PBS
15. Rinse in water.

iii. Counter Staining

Staining with eosin

1) Counter stain with eosin for 1 minute.
2) Dehydrate in 100% ethanol followed by Xylene for 3 times, 4 minutes each, and then cover slides.

Staining with Methyl Green

1) Counterstain with 1% Methyl Green (Cat# 4800-30-18) for 30 seconds to 5 minutes.
2) Wash and dehydrate the samples using either method (a) for cells, or (b) for tissues:

(a) Ethanol method**:**

1) Wash away excess counterstain in deionized water until water is colorless.
2) Dehydrate sample by dipping 10 times in 95% ethanol and 10 times in 100% ethanol.
3) Clarify sample by dipping 10 times in 2 changes of xylenes.
4) Mount with appropriate mounting media e.g. Trevigen mounting media (Cat# 4865-25) or Permount®.

(b) Butanol method:

1) Dip in 1-butanol until sample turns from blue to green (5-10 sec).
2) Dip in fresh 1-butanol until excess counterstain is washed away (5-10 sec).
3) Clarify sample by dipping 10 times in 2 changes of xylenes.

Mount with appropriate mounting media e.g. Trevigen mounting media (Cat# 4865-25) or Permount®.

VI. Controls and Data Interpretation

If desired a positive sample can be created by treating an unfixed specimen of cells or tissues with 10-200 μm H_2 O_2 in complete media for 15 minutes at 37°C.

Stop the reaction by washing twice with cold PBS. Process this positive control in the same manner as the experimental samples. For positive tissue control we recommend the use of Hepatocellular Carcinoma tissue, which has been reported to contain 8-oxodG positive cells (Reference 4.)

Negative controls that may be included when optimizing labeling conditions are:

a) omission of primary antibody
b) omission of secondary antibody
c) preadsorption of primary antibody with free 8-oxodG

Each of these controls should be negative for brown nuclear staining. If brown DAB staining is observed, conditions to improve the signal to noise ratio should be incorporated e.g. alter dilutions and/or incubation times and temperatures, select alternative blocking agents, increase number and length of washes, increase quenching time. Brown nuclear staining (DAB) is indicative of the presence of 8-oxo-dG. The relative staining of treated cells should be several folds higher compared to untreated cells. Low levels of DAB staining may be expected in untreated control samples due to the presence of endogenous 8-oxodG in the nucleoside precursor pool and steady state levels in DNA.

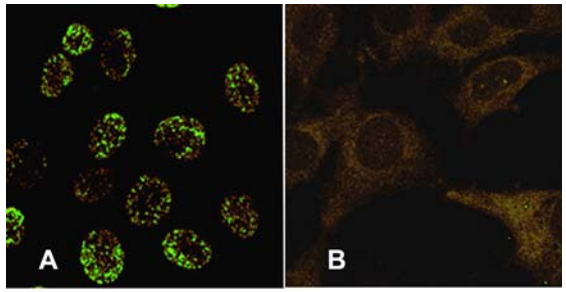


Figure 1. H_2O_2 treated (A) and untreated (B) cells stained with 4E9 antibody according to the protocol using anti-mouse FITC antibody.

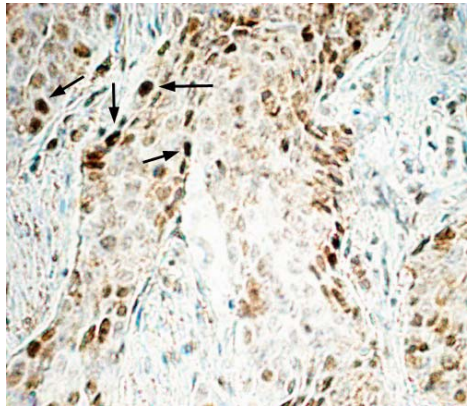


Figure 2. Breast carcinoma tissue stained for 8-oxodG using the 4E9 antibody and anti-mouse IgG-HRP. Developed using enhanced DAB method. Arrows indicates the cells strongly reacting with the antibody.

VII. References

1) Nunomura, A., G. Perry, M.A. Pappolla, R. Wade, K. Hirai, S. Chiba, and M.A. Smith. 1999.RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. Journal of Neuroscience **19:**1959-1964.
2) Yarborouhg, A., Y.-J. Zhang, T.-M. Hsu, and R. Santella. 1996. Immunoperoxidase detection of 8-hydroxydeoxyguanosine in aflatoxin B1-treated rat liver and human oral mucosal cells. Cancer Research **56:**683-688.
3) Yin, B., R.M. Whyatt, F.P. Perera, M.C. Randall, T.B. Cooper, and R. Santella. 1995. Determination of 8-hydroxydeoxyguanosine by an immunoaffinity chromatography-monoclonal-antibody based ELISA. Free Radical Biology & Medicine **18:**1023-1032.
4) Ichiba M, Maeta Y, Mukoyama T, Saeki T, Yasui S, Kanbe T, Okano J-i, Tanabe Y, Hirooka Y, Yamada S, Kurimasa A, Murawaki Y, Shiota G. Expression of 8-hydroxy-20-deoxyguanosine in chronic liver disease and hepatocellular carcinoma. Liver International 2003: **23:**338–345.

VIII. Related Products Available From Trevigen

4800-30-01	Proteinase K	30 µl
4800-30-06	Strep-HRP	30 µl
4800-30-07	DAB and DAB Enhancer	3.75 ml
4800-30-18	1% Methyl Green	50 ml
4800-30-14	Strep-FITC	30 µl
4861-100	Treated Glass Microscope Slides	100 slides
4864-100	Treated Glass Microscope Slides (3 ring)	100 slides
4865-25	Mounting medium	25 ml
4866-20	Fluorescence Mounting Medium	20 ml
4870-500-6	10X PBS	6 X 500 ml

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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