

Chapter 2

Combination of TUNEL Assay with Immunohistochemistry for Simultaneous Detection of DNA Fragmentation and Oxidative Cell Damage

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Abstract

Oxidative cell damage causes disruption of DNA via formation of 8-hydroxy-2'-deoxyguanosine and can trigger apoptotic cell death. The cells damaged by oxidative stress can either become apoptotic, or recover. Therefore, it is helpful to employ a parallel assay that would confirm whether cells experiencing oxidative damage undergo apoptosis. Our paper describes the technique that combines immunohistochemical detection of 8-hydroxy-2'-deoxyguanosine with the TUNEL assay. This permits simultaneous detection of oxidative damage and apoptosis at a single-cell level. We have developed simple and reliable protocols which can be used with cultured cells and slide-mounted tissue sections. These techniques can be employed in research dealing with high-throughput drug screening, toxicology, and cancer.

Key words: Oxidative stress, 8-hydroxy-2'-deoxyguanosine, Immunohistochemistry, TUNEL assay, Apoptosis, Cultured cells, Tissue sections, Double-labeling

1. Introduction

The TUNEL assay is a very powerful technique for the detection of DNA fragmentation in cells. Based on terminal deoxynucleotidyl transferase (TdT)-mediated nick end-labeling, TUNEL allows for *in situ* detection of DNA breaks in both apoptotic and necrotic cells (1, 2). That's why the TUNEL assay is widely used to study cellular mechanisms underlying embryonic development and morphogenic redistribution of cells (3), aging (2, 4), tumorigenesis (5, 6), and neurodegenerative processes (7). Although TUNEL detects DNA fragmentation, which appears to represent the end-point of apoptotic DNA degradation, it cannot detect the causes of such degradation which can be, for example, an

oxidative stress induced by reactive oxygen species (ROS) and oxygen radicals. Oxidative damage has been implicated in many neurodegenerative disorders including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, and Parkinson's disease (8–11). Oxidative stress leads to the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) which, in turn, causes disruptive DNA modifications. Immunohistochemical detection of 8-OHdG appears to be a valuable technique, allowing for identification of possible oxidative DNA damage induced by ROS. However, measuring the extent of oxidative damage alone has a limited value because it does not answer the question whether it triggers apoptotic DNA damage or not.

Since apoptotic DNA fragmentation is preceded by oxidative DNA damage, the sequential application of each technique allows quantification of cells during the early phase (8-OHdG-positive) and late phase (TUNEL-positive) of apoptosis. Because oxidative damage may not result in cell death, measuring the number of TUNEL- and 8-OHdG-positive cells helps to determine a threshold of cellular sensitivity to oxidative stimuli at which DNA oxidation results in DNA damage.

Protocols that combine the TUNEL assay and immunohistochemical labeling for 8-OHdG can be used with cultured cells and with sections. Briefly, after doing immunohistochemistry for 8-OHdG labeling, the same specimens are subjected to the TUNEL assay. This technique is easy to perform and it can be customized for a large variety of cells and tissues.

2. Materials

1. Dissociated (primary culture) rat dorsal root ganglia (DRG) neurons from 3-day-old Sprague-Dawley rat pups.
2. DRG dissociation solution: 10 mg/mL of collagenase/dispase (Roche Molecular Biochemicals, Indianapolis, IN) in Hank's Balanced Salt Solution (HBSS; Gibco BRL, Grand Island, NY).
3. Culture media for DRG neurons (all reagents except NGF are from Sigma-Aldrich, St. Louis, MO): Ham's medium (F-12; Gibco BRL, Grand Island, NY) supplemented with 5% heat-inactivated horse serum and 5% fetal bovine serum, 50 ng/mL nerve growth factor (R&D Systems, Minneapolis, MN), 4.4 mM glucose, 2 mM l-glutamine, penicillin (50 µg/mL) and streptomycin (50 µg/mL).
4. Chemical fixation of cultured DRG neurons: 4% formaldehyde fixative.

Paraformaldehyde powder is toxic and it is required to wear mask and gloves and use the chemical hood when working with this chemical.

- (a) Prepare solution A (phosphate buffered saline, PBS): Fill 1 L beaker with 900 mL of distilled water and dissolve 0.23 g of NaH_2PO_4 (anhydrous), 1.15 g Na_2HPO_4 (anhydrous), and 9 g NaCl. Adjust pH to 7.4 using 1 M NaOH and/or 1 M HCl;
 - (b) Prepare solution B (8% formaldehyde): Slowly dissolve 8 g of paraformaldehyde powder in 100 mL of deionized water using heating stir plate. After temperature reaches 58°C turn the heat off and add one to two drops of 1 M NaOH to clear formaldehyde solution. Continue stirring for another 20–30 min, cool it down and filter it using filter paper such as Whatman #1;
 - (c) Monitor the temperature of formaldehyde solution using a thermometer to avoid heating this solution above 58°C . Formaldehyde solution heated above 58°C is not useful as a fixative: it should be discarded and new batch has to be made;
 - (d) Four percent formaldehyde fixative: is prepared by mixing one part of Solution A with one part of Solution B. For solution stored at 4°C its expiration date is about 4 weeks.
5. DRG culture dishes and other accessories: Multiwell (4 or 8 wells) chamber slides and sterile 35 mm Petri dishes, 70% alcohol, Pasteur pipettes, scissors, forceps, dissecting microscope, and sterile hood for dissection and dissociation of DRG neurons. $37^\circ\text{C}/\text{CO}_2$ humidified incubator to culture dissociated DRG neurons.
 6. Reagent to induce oxidative stress: prepare 0.5 mM solution of H_2O_2 by adding 0.5 μL of 30% H_2O_2 to 8.8 mL of Hank's Balanced Salt Solution (HBSS; Gibco BRL, Grand Island, NY). Prepare 5 μM solution of H_2O_2 by adding 100 μL of 1 mM H_2O_2 solution to 9.9 mL of HBSS. Store at 4°C . Calculate the volume of each reagent needed for stimulation cells in two multiwell (4-well or 8-well) chamber slides.
 7. Dilution buffer: PBS containing 1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton X-100 (v/v), and 0.01% sodium azide.
 8. Anti-8-OHdG (primary) antibodies for immunocytochemistry: Prepare working solution of 5 $\mu\text{g}/\text{mL}$ of mouse monoclonal anti-8-OHdG antibodies (Cat # 12501, clone 15A3; QED Bioscience Inc., San Diego, CA) in a dilution buffer. This primary antibody solution will be used to incubate both dissociated DRG neurons and tissue sections mounted onto

histological slides. This solution can be stored at 4°C for up to 1 month and for longer storage it is recommended to make small volume aliquots and store them at -20°C for up to 1 year and avoid their repeated freeze-thaw cycles.

9. Fluorescent detection (secondary) antibodies for 8-OHdG visualization: Donkey anti-mouse conjugated with NL-493 (Cat # NL009; R&D Systems, Inc., Minneapolis, MN). Dilute secondary antibodies 1:100 with dilution buffer and use it as a working solution (can be stored at 4°C for up to 1 month).
10. Fluorescent detection of TUNEL reaction: Prepare 1:100 working solution of Streptavidin conjugated with NL-557 (Cat # NL999; R&D Systems, inc., Minneapolis, MN) with dilution buffer (can be stored at 4°C for up to 1 month).
11. Mounting medium for fluorescent labels: i-BRITE Plus mounting medium (Cat # SF40000-1; Neuromics, Edina, MN). This medium minimizes loss of fluorescence by fluorescent probes due to photobleaching during examination under the fluorescence microscope.
12. TUNEL assay: TACS 2 TdT DAB kit (Cat # 4810-30-K; Trevigen, Gaithersburg, MD; <http://www.trevigen.com>). This kit is also available for ordering through VWR International, LLC (<http://www.vwr.com>).
13. Chromogenic system for 8-OHdG detection: HRP-AEC Cell and Tissue Staining kit (CTS 003; R&D Systems, Inc., Minneapolis, MN);
14. Chromogenic system for TUNEL detection: HRP-DAB Cell and Tissue Staining kit (CTS 002; CTS 005 or CTS 008; R&D Systems, Inc., Minneapolis, MN) combined with DAB enhancer (CTS010; R&D Systems, Inc., Minneapolis, MN);
15. Mounting medium for chromogenic labels: Aqueous mounting medium (CTS011; R&D Systems, Inc., Minneapolis, MN).
16. Waterproof pen to label slides: SHUR/MARK pen
17. PAP pen: to draw a hydrophobic line surrounding tissue section mounted onto histological slide (Cat # Z377821, Sigma-Aldrich, St Louis, MO). This hydrophobic barrier prevents leakage of incubation reagents from the slide.
18. Slide incubation chamber: "SlideShow" tray with transparent cover (Cat # 6844-30CL; Newcomer Supply of Middleton WI, <http://www.newcomersupply.com>)
19. Microscopy: Bright field/fluorescence microscope (Provis; Olympus, Hauppauge, NY) equipped with microscope digital camera (DP71; Olympus Hauppauge, NY) and fluorescence filter sets (460–490 nm excitation/510–550 nm emission and 541–551 nm excitation/572–607 nm emission).

3. Methods

All procedures were performed at room temperature unless stated otherwise. If protocol calls for incubation at room temperature, reagents stored at 4°C should be adjusted to room temperature before they added to cell and tissue samples. Since 3-amino-9-ethylcarbazole (AEC) and 3,3'Diaminobenzidine (DAB) are potential carcinogens wear gloves to avoid contact of these reagents with skin. It is recommended that each staining experiment be performed in at least duplicate, in case some samples dry out during the incubation and cannot be used to complete the experiment.

3.1. Double-Labeling of Cultured DRG Neurons for 8-OHdG and TUNEL Reaction

1. Prepare suspension of rat neonatal DRG neurons in a sterile hood (see Note 1). Culture DRG neurons in multiwell chamber slides in the 37°C/CO₂ humidified incubator for 2–3 days and after that cells can be used in double-labeling experiments.
2. Transfer chamber slides with cells from the 37°C/CO₂ humidified incubator into the sterile hood. Wait for approximately 20 min to allow temperature of the culture medium in the chamber slide to decrease from 37°C to ambient.
3. Using a sterile pipette gently remove the culture medium from each well: position the tip of the pipette into the corner of the well to avoid disturbing cells that can cause their detachment. Save collected culture medium in a sterile tube for further use.
4. Add reagents to designated wells to induce oxidative stress. In one or two wells add plain HBSS: cells in these wells will serve as control groups (see Note 2). Place the chamber slide back into the 37°C/CO₂ humidified incubator and incubate for 30 min.
5. Transfer the chamber slide from the incubator into a sterile hood. Discard oxidative stress reagents from designated wells and rinse them three times with sterile HBSS. Discard HBSS and add culture medium that has been collected in step 3. Return chamber slides with cells into the 37°C/CO₂ humidified incubator and incubate for additional 18–24 h (see Note 3).
6. Transfer the chamber slide from the incubator to a lab bench, discard the culture medium and add 4% formaldehyde into each well. Fix cells for 10 min and then wash them 3 × 15 min with PBS.
7. Remove the upper chamber from the slide using tools provided by the slide chamber vendor.
8. Place slides horizontally into humidity chamber, add primary anti-8-OHdG antibodies and incubate 3 h. Alternatively, cells

- may be incubated overnight at 4°C. Adding too much primary antibodies may cause their leakage from the slide causing samples to dry.
9. Wash slide 3 × 15 min in PBS, place it horizontally into a humid chamber, add secondary anti-mouse NL-493 conjugated antibodies and incubate for 1 h.
 10. Wash slide 3 × 15 min in PBS and start TUNEL assay.
 11. Rinse slide with DNase-free water and permeabilize cells for 30 min with CytoPore reagent provided with TUNEL kit.
 12. Rinse slide with DNase-free water and then transfer it into a Coplin jar containing TdT labeling buffer: follow dilution recommendations provided with TUNEL kit. Incubate for 5 min.
 13. Prepare TdT labeling mixture. Calculate the volume of TdT mixture: 150 µl is required to cover the entire area of the slide with cells. Combine TdT labeling components (TdT dNTP mix, 50× Mn²⁺, TdT enzyme and TdT labeling buffer) as recommended in the TUNEL kit insert (see Note 4). Apply TdT labeling mixture and cover it with 22 × 60 mm coverslip. Do not press the coverslip too hard to avoid the leakage of the labeling mixture. Place slide horizontally into a humid chamber and incubate at 37°C for 1 h.
 14. Transfer slide with coverslip into a Coplin jar containing TdT Stop Buffer: follow dilution recommendations provided in TUNEL kit protocol. Keep slide in stop buffer for 2 min and then pull it slowly out of the buffer to remove coverslip by washing it off. If coverslip remains sticking to the slide, lift it gently with fine forceps. Transfer uncovered slides with tissue section into another Coplin jar filled with Stop Buffer and incubate for 5 min.
 15. Wash slide in PBS for 5 min.
 16. Place slide horizontally into a humid chamber, apply three to five drops of Streptavidin-NL-557 solution and incubate for 1 h.
 17. Stop the reaction by rinsing slides with PBS and then wash them 3 × 10 min with PBS in a Coplin jar.
 18. Mount under coverslips using mounting medium for fluorescent labels and examine the staining under the fluorescence microscope (see Note 5).

3.2. Double-Labeling for 8-OHdG and TUNEL Reaction on Paraffin- Embedded Tissue Sections

Double-labeling immunofluorescence appears to be more convenient than the procedure utilizing chromogenic labels: it is faster and results in a clear distinction between color labels (see Note 6). However, the presence of autofluorescent pigment, lipofuscin, in human tissues can obscure fluorescent labels used for labeling tissue targets. For example, high density of lipofuscin in

Alzheimer's brain leaves no other option but to employ chromogenic rather than fluorescent labels for immunohistochemical experiments. We utilized Alzheimer's brain tissue sections to develop a two-color chromogenic detection protocol that can be used on the same tissue section. This technique allows obtaining a good contrast and accurate separation of color labels and can be used for simultaneous immunohistochemical detection of DNA fragmentation (see Note 7) and oxidative damage to RNA and DNA on the same tissue section.

1. Mark slides with tissue sections using waterproof SHUR/MARK pen.
2. Outline the tissue section with Pep-pen making at least a 2 mm gap between the outer edge of the tissue section and the inner edge of the Pep-pen line. Allow Pep-pen drawing to dry for at least 5 min.
3. Place slide into a Coplin jar and deparaffinize paraffin-embedded tissue sections by treating them sequentially with the following reagents:
 - (a) Xylene – two baths, 10 min each;
 - (b) 100% ethanol – two baths, 10 min each;
 - (c) 95% ethanol – two baths, 10 min each;
 - (d) 70% ethanol – two baths, 10 min each.
4. Wash slides 2 × 5 min in PBS.
5. Wash slides in DNase-free water for 5 min.
6. Treat tissue section with Proteinase K solution (see Notes 8 and 9). It will be necessary to calculate the working volume of Proteinase K solution assuming that 50 is required to cover 2 × 1 cm² tissue area. Dilute Proteinase K concentrate with DNase-free water using the ratio recommended in the TUNEL kit data sheet. Apply Proteinase K solution onto tissue section and cover them gently with the coverslip of appropriate size. Do not press coverslip too hard to avoid the leakage of Proteinase K solution. Place this slide horizontally into a humid chamber and incubate for 30 min.
7. Transfer the slide with coverslips into a Coplin jar containing DNase-free water and keep it there for 2 min. Pull the slide slowly from water to facilitate removal of the coverslip. Transfer uncovered slides with tissues into another Coplin jar with fresh portion of DNase-free water.
8. Remove slide from the Coplin jar, shake excess water, place it horizontally and add three to five drops of H₂O₂ blocking reagent from Cell and Tissue Staining kit. Incubate 10 min in a humid chamber.
9. Rinse slide in DNase-free water for 5 min.

10. Transfer slide into a Coplin jar containing TdT labeling buffer prepared according to dilution recommendations specified in the TUNEL kit protocol. Incubate for 5 min.
11. Prepare TdT labeling mixture. Calculate the volume of TdT mixture assuming that 50 μL is required to cover $2 \times 1 \text{ cm}^2$ of tissue area. Combine TdT labeling components (TdT dNTP mix, $50\times \text{Mn}^{2+}$, TdT enzyme and TdT labeling buffer) as recommended in the TUNEL kit protocol. Apply 50 μL of TdT labeling mixture onto tissue section and cover it gently with coverslip of appropriate size. Do not press the coverslip too hard to avoid the leakage of the labeling mixture. Place slide horizontally into a humid chamber and incubate it for 1 h at 37°C .
12. Transfer slide with coverslip into a Coplin jar containing TdT stop buffer: follow up dilution recommendations provided in TUNEL kit protocol. Keep slide in a stop buffer for 2 min and then pull it slowly from the buffer to facilitate the removal of the coverslip. Transfer uncovered slide into another Coplin jar with fresh portion of stop buffer and incubate for 5 min.
13. Wash slide in PBS for 5 min.
14. Place slide horizontally into a humid chamber and apply three to five drops of Streptavidin-HRP solution from Cell and Tissue staining kit. Incubate 30 min.
15. Wash slide 2×5 min in PBS.
16. Place slide horizontally onto a microscope stage and apply mixture of DAB (from Cell and Tissue Staining kit, CTS002; R&D Systems, Inc., Minneapolis, MN) with DAB enhancer to cover entire tissue section. Monitor the development of dark-blue color under the microscope using $4\times$ or $10\times$ power lens. Three to ten minutes is needed for developing strong blue-black labeling of nuclei in apoptotic and/or necrotic cells.
17. Discard DAB mixture from the slide.
18. Wash slide in PBS for 20 min.
19. Place slide horizontally and add three to five drops of H_2O_2 blocking reagent from Cell and Tissue Staining kit. Incubate 10 min in a humid chamber.
20. Rinse slides in PBS and perform avidin-biotin blocking procedure using reagents from Cell and Tissue Staining kit. Place slide horizontally into a humid chamber and add three to five drops of avidin blocking solution and incubate for 15 min. Rinse slide in PBS, place it horizontally and add three to five drops of biotin blocking solution and incubate 15 min.
21. Wash slide in PBS for 20 min.
22. Place slide into a humid chamber and apply primary anti-8-OHdG antibodies. 80–150 μL of antibody solution is needed to cover the tissue section on the slide (see Notes 10 and 11).

23. Incubate with primary anti-8-OHdG antibodies 16–24 h at 4°C.
24. Wash slide 3 × 15 min in PBS.
25. Repeat step 14.
26. Repeat step 15.
27. Place slide horizontally onto the microscope stage and apply AEC solution from Cell and Tissue Staining kit (CTS003; R&D Systems, Inc., Minneapolis, MN) to cover entire tissue section. Monitor the development of red color under the microscope using 4× or 10× lens. Strong red color usually develops in 1–3 min.
28. Repeat step 18.
29. Cover tissue sections with aqueous mounting medium and let them dry.
30. Use bright-field microscope equipped with digital color camera to collect images of tissue section double-labeled for TUNEL and 8-OHdG.

4. Notes

1. Isolation of DRGs, their cell dissociation, and culturing described in this chapter includes the following steps. Decapitate 3-day-old Sprague-Dawley rats (use 70% alcohol to sterilize surgical instruments (scissors and forceps) and decapitated bodies). Under dissection microscope dissect DRGs from the thoracic and lumbar segments of the spinal cord and incubate them in collagenase/dispase digestion solution for 30 min in the 37°C/CO₂ incubator. Dissociation of cells can be accomplished by triturating DRGs through large, medium, and small diameter flame constricted pipettes: 50–60 times/per pipette. Digestion is terminated by adding 10 mL of sterile HBSS into cell suspension and centrifuging it at 500 × *g* for 10 min. Supernatant is discarded and the pellet is resuspended in the same volume of HBSS followed by centrifugation. Supernatant is discarded and the resulting pellet is resuspended in 5 mL of F-12 culture medium. This cell suspension is added into wells of sterile multiwell chamber slides for further manipulations.
2. It is necessary to include different types of controls to be sure that TUNEL signal is confined to true apoptotic or/and/ necrotic cells rather than was generated nonspecifically in normal cells. There are three types of controls addressing the

specificity issue: (a) *reagent-specific*, (b) *target-specific* and (c) *positive tissue control*.

- (a) *Reagent-specific* control answers the question whether components of the TdT mixture interact with each other in a specific manner. This type of control includes incubating cell and tissue specimens with TdT mixture that does not contain terminal deoxynucleotidyl transferase (TdT enzyme). It is expected that that cell nuclei will not be labeled. Some postmortem tissues stored for a long time in formalin may show staining caused by nonspecific tissue binding of either streptavidin-HRP or DAB chromogen. However, the cytoplasmic pattern of such tissue staining can be easily recognized from specific TUNEL reaction which is always confined to cell nuclei.
 - (b) *Target-specific* control helps to determine whether TUNEL assay detects only apoptotic/necrotic cells. This is done by comparing labeling in normal tissues versus tissues undergoing apoptosis or necrosis. To employ a target-specific control, it is recommended to include normal cells and tissues with specimens undergoing apoptosis/necrosis. Although normal specimens may contain cells with TUNEL-positive nuclei (due to naturally occurring apoptosis), their number is expected to be much lower in comparison with apoptotic/necrotic specimens.
 - (c) *Positive tissue* control is done to prove that TUNEL assay is both reagent- and target-specific. This type of control includes inducing apoptosis/necrosis in normal cells and tissues by treating them with nuclease. Nuclease treatment causes DNA breaks and results in increased number of cells with TUNEL-positive nuclei.
3. Cells and tissue sections should not dry during the incubation and washing steps: specimens found dry should be excluded from experiment because this may result in nonspecific background. Because partial drying may be overlooked by an operator fatigued by processing a large number of specimens, hence it is recommended to interpret staining of tissue margins (which is more prone to partial drying) with caution.
 4. The TUNEL kit manufactured by Trevigen includes Streptavidin-HRP and DAB chromogen reagents which can be used to obtain strong single-color brown-labeled nuclei. It appears that using similar reagents from R&D Systems' Cell and Tissue Staining kit and DAB chromogen, combined with R&D Systems' DAB enhancer producing dark-blue color, gives a better color separation when combined with AEC (red color) chromogen. Alternatively, similar reagents can be purchased separately from other vendors. However, using

staining kits saves a lot of time because they don't require any time-consuming optimization.

5. When manipulating digital images, brightness and contrast should be adjusted on "control" and "experimental" samples using identical parameters to avoid misrepresentation of staining results.
6. The double-staining protocol presented in this chapter can be used for other tasks including embryonic development, cancer research, aging and neurodegenerative disorders, and toxicological studies. For example, this technique can be employed for qualitative and quantitative pollution monitoring using cultured cells as a pollution probe.
7. Apoptosis and/necrosis determined by TUNEL needs to be also confirmed by morphological criteria such as cell membrane blebbing, shrinkage of cells, and the formation apoptotic bodies.
8. Proteinase K treatment may cause tissue damage recognized as detached (floating) tissue fragments and/or holes of irregular size. In this case, it is recommended to reduce the duration of enzymatic treatment from 30 min to 10–20 min. Cultured cells are more sensitive to the damaging effects of Proteinase K and therefore enzymatic treatment on cytological samples is not recommended. Alternatively, cells may be permeabilized by incubating them in 0.1–0.3% Triton X-100/PBS solution for 20 min.
9. To enhance the signal intensity of 8-OHdG labeling, cells and tissues may be treated with Proteinase K (10 µg/mL in PBS) from 10 to 30 min in a humid chamber at 37°C, but see Note 8.
10. The specificity of labeling for 8-OHdG is determined by two factors: (a) specificity of 8-OHdG antibodies and (b) specificity of immunohistochemical reagents. In addition, the specificity of staining may be evaluated using (c) negative control.
 - (a) Evaluating the specificity of anti-8-OHdG antibodies is needed to confirm that immunohistochemical labeling resulted from the interaction of antibodies with their cognate (8-OHdG) target, rather than caused by their cross-reactivity with irrelevant antigens. This can be accomplished using a so-called absorption control. Briefly, mix anti- 8-OHdG antibodies (taken in working dilution, i.e. 5 µg/mL) with 10 µg/mL purified 8-OHdG antigen (Sigma-Aldrich, St Louis, MO). Mix well and incubate either 5 h at room temperature or overnight at 4°C. Since these antibodies recognize RNA-derived 8-OHdG as well, it is also helpful to do absorption control using 8-OHdG reactant. It is expected that intensity of labeling will decrease when using absorption control

mixtures since soluble antigens occupy binding sites on anti-8-OHdG antibodies and thus reduce capacity to interact with intracellular 8-OHdG targets.

- (b) Nonspecific signal can sometimes be present due to sticking of immunohistochemical reagents to fixed cells and sections. The simplest way to test this would be the incubation of specimens by omitting anti-8-OHdG antibody from the working solution: lack of labeling proves specificity of immunohistochemical reagents. However, if non-specific labeling is observed, additional steps are required to block it. For example, nonspecific background staining may be caused by free aldehyde groups present in tissues that are fixed with formaldehyde or glutaraldehyde solutions: free aldehyde groups are capable of reacting with secondary antibodies “cross-linking” them to the tissue. To neutralize free aldehyde groups, specimens can be treated with 0.5 mg/mL of sodium borohydrate (NaBH_4) for 10–20 min at room temperature. Alternatively, free aldehyde groups can be blocked by incubating samples in 10% normal horse or donkey serum for 5–30 min at room temperature. The blocking of free aldehyde groups is done before applying primary antibodies.
 - (c) A negative control can be a specimen that is known to lack the antigen of interest. For example, the antigen can be intentionally destroyed. Since anti-8-OHdG antibodies target DNA and/or RNA, their degradation is expected to result in reduced or abolished cell and tissue labeling. To do such a control, DNA and RNA may be degraded by pretreatment of specimen with either 10 U/ μL of DNase I or 10 U/ μL S1 DNase or 5 $\mu\text{g}/\mu\text{L}$ of RNase from 30 min to 1 h in a humid chamber at 37°C. Alternatively, specimens can be treated with all three enzymes combined together which appear to degrade DNA and RNA targets more efficiently than using individual enzymes.
11. The advantage of using anti-8-OHdG antibodies produced by QED Bioscience is that this antibody interacts with RNA localized in the cytoplasm and therefore immunohistochemical labeling for 8-OHG does not obscure labeling for TUNEL, which is confined to cell nuclei.

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