OxyIHC™ Oxidative Stress Detection Kit

50 reactions

Catalog No. S7450

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

The **OxyIHC™ Oxidative Stress Detection Kit (S7450)** contains the chemical and immunological reagents necessary to detect protein oxidation in various tissues from a variety of organs and animal species. The OxyIHC test method involves chemical derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH). This chemical reaction results in proteins being covalently coupled to DNP at their carbonyl sites. The DNP-derivatized proteins are then detected using an antibody that specifically binds to the DNP moiety. Subsequent incubation with biotin conjugated secondary antibody, streptavidin conjugated HRP, and development using a 3,3' diaminobenzidine (DAB) staining allows immunohistochemical detection of protein oxidation.

Background

Reactive oxygen species (ROS) have been implicated in pathological processes including cancer, apoptosis, aging, neurodegenerative diseases, chronic inflammatory diseases, pulmonary diseases, and cardiovascular diseases (for reviews, see ref. 1-4). Living organisms are continually exposed to potentially harmful oxygen free radicals that are generated thru normal cellular functions as well as from environmental factors (1, 5-7). Yet, these reactive molecules are mitigated by the presence of antioxidants and therefore organisms exist with a delicate balance between oxidants and antioxidants (7-9). During times of oxidative stress there is an imbalance in favor of the oxidants. The end result of which is that free radicals are able to attack and modify sub-cellular components including nucleic acids, lipids and proteins (7, 10-12).

Proteins are one of the major targets of reactive species. Oxidation of proteins modifies the side chains of methionine, histidine, and tyrosine and forms cysteine disulfide bonds (16-19). Metal catalyzed oxidation of proteins introduces carbonyl groups (aldehydes and ketones) at lysine, arginine, proline or threonine residues in a site-specific manner (16, 20-22). The oxidative modification of proteins can modulate biochemical characteristics of proteins such as enzymatic activity (21-23), DNA binding activities of transcription factors (24-26), and the susceptibility to proteolytic degradation (12, 25-28). While a relationship between protein oxidation and aging has been suggested (29-31), little is known about the importance of oxidative modification of individual proteins in the pathophysiology of free radical mediated processes. Not surprisingly, carbonyl modification of proteins has become a key biomarker for the identification of oxidative stress.

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Kit Components: (Store at 2-8ºC)

Part #	Component	Amount
CS205119	10X Wash Buffer	One 25 mL bottle.
CS205110	10X Antigen Retrieval Buffer	One 1.25 mL bottle
CS205111	5X Blocking Buffer	One 2.5 mL bottle
CS205112	Primary Antibody	One 125 μL vial
CS205113	DAB-A	One 500 μL vial
CS205114	DAB-B	One 12 mL bottle
CS205115	Antibody Diluent	One ready to use 12.5 mL bottle
CS205116	Biotinylated Secondary Antibody	One ready to use 12.5 mL bottle
CS205109	Streptavidin conjugated HRP	One ready to use 12.5 mL bottle
CS205118	Hematoxylin	One ready to use 12.5 mL bottle
CS205128	DNPH Solution	One ready to use 12.5 mL bottle
CS204989	Derivatization Control Solution	One ready to use 12.5 mL bottle

Materials Required But Not Provided

- General lab supplies such as microcentrifuge tubes, pipets and pipet tips.
- Methanol
- Chloroform
- Acetic Acid
- Glass slides and coverslips
- Plastic coverslips
- Drying Oven
- Mounting media
- Xylene/Histoclear™
- EtOH
- 2N HCl
- Microscope
- 3% H_2O_2
- PAP Pen
- Humidified chamber
- Xylene resistant gloves

Warnings & Safety Precautions

- Hazardous Material: The DNPH Solution and the Derivatization Control Solution contain hydrochloric acid and are harmful if swallowed or inhaled. DAB is a suspected carcinogen and must be handled with caution.

  Caution: Eye, hand, face, and clothing protection should be worn when handling this material. If direct contact occurs, wash areas of contact immediately with water.

- All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

Storage & Stability

The OxyIHC kit components should be stored at 2-8ºC. Kit components are stable for 6 months from the date of receipt if stored and handled properly.

Preparation of Reagents

Follow the directions below to prepare reagents and solutions necessary for detecting oxidative stress using the OxyIHC Kit. The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and a failure to produce accurate data. The reagent volume provided: sufficient for 50 reactions

Methacarn Fixative*

Methacarn is a non-crosslinking protein-precipitating fixative. To make 50 mL of Methacarn, mix 30 mL of 100% methanol, 15 mL of Chloroform, and 5 mL of Glacial Acetic Acid. Methacarn can be stored at room temperature for 1 month.

***Important Note**: Because this assay detects carbonyl derivatives, tissue fixed with aldehyde containing fixatives such as formaldehyde, paraformaldehyde or glutaraldehyde should not be used. Tissue should be fixed with Methacarn for optimal results.*

10X Wash Buffer:

The Wash Buffer provided is supplied as a 10X concentrate. Prior to use, dilute the concentrate to 1X strength using Milli-Q™ water. For example, to make 250 mL of working dilution wash buffer, add 225 mL of Milli-Q™ water to 25 mL of 10X wash buffer.

10X Antigen Retrieval Buffer:

The Antigen Retrieval Buffer (ARB) provided is supplied as a 10X concentrate. Prior to use, dilute the concentrate to 1X strength using Milli-Q™ water. For example, to make 12.5 mL of working dilution ARB, add 11.25 mL of Milli-Q™ water to 1.25 mL of 10X Antigen Retrieval Buffer.

***Note:** the 10X Antigen Retrieval Buffer contains 0.1% Kathon 300 as a preservative.*

5X Blocking Buffer:

The Blocking Buffer provided is supplied as a 5X concentrate. Prior to use, dilute the concentrate to 1X strength using Milli-Q™ water. For example, to make 12.5 mL of working dilution Blocking Buffer add 10 mL of Milli-Q™ water to 2.5 mL of 5X Blocking buffer.

Primary Antibody: Dilute the primary antibody 1:100 with Antibody Diluent immediately before use.

DAB-A/B mix:

Dilute 1 part DAB-A into 24 parts DAB-B immediately before use. For example, add 10 μL of DAB-A into 240 μL of DAB-B.

Specimen Handling and Methacarn Fixation:

Researchers should optimize the tissue extraction procedure for their own applications. The following protocol has been successfully employed for several different tissue types.

1. Isolate non-fixed fresh tissue as desired. Transfer into a 50 mL tube and wash twice with PBS.

   ***Note**: Specimens should be handled carefully and promptly to avoid compromising specimen integrity. For proper fixative penetration small tissue blocks (less than 1 cm thick) are recommended. If the tissue size is large, the animal can be perfused with Methacarn.*

2. Incubate tissue in Methacarn fixative for at least 4 hours at room temperature (RT) or overnight at 2-8^{o}C.

   ***Note**: Depending on the size of the tissue, longer fixation may be required. Typically, a whole mouse brain requires at least a 4 hour fixation. Researchers should optimize the fixation procedure for their own applications.*

3. Following fixation transfer tissue into a 50 mL tube and wash 3 times with PBS; at least 5 minutes per wash.

4. Paraffin-embed specimens using standard laboratory protocol. Proper in-house quality control and verification measures should be taken to determine suitability of specimen types for immunohistochemical methodologies.

5. Section the tissues as desired. For optimum resolution by immunohistochemistry, 4 to 8 micron (μm) thick sections are recommended.

6. Place tissue sections on glass slides then dry in oven. Deparaffinize and rehydrate tissue sections using standard methods (typically Xylene treatment followed by a series of ethanol washes).

   ***Note:** Tissue sections should be used the same day they are deparaffinized.*

Assay Protocol:

For best results, each experiment should include several control experiments to verify any results obtained using the OxyIHC kit. Reactions with DNPH solution and Derivatization Control Solution as well as plus and minus oxidative stress treatment (for example treating live tissue with a known oxidative stress inducer such as H_2O_2) should be included in every test that is performed. Additional tests that may also be run include omitting antibody and/or streptavidin to verify specificity.

1. Obtain slides with deparaffinized and rehydrated tissue (see above for suggested protocol).

2. Perform antigen unmasking of tissue by adding 250 µL of 1X Antigen Retrieval Buffer per slide.

3. Place a glass coverslip on top of the slide and incubate in a steamer for 20 minutes.

4. Allow the slide to cool down for 15 minutes at room temperature.

5. Wash the tissue by incubating it in 250 µL of 1X Wash Buffer for 5 minutes. Repeat the wash 2 additional times.

   ***Note:** Following every wash and incubation step in this protocol, the liquid is removed from the sample with a gentle "flick" of the slide over the sink. After removal of liquid, gently tap the edge of the slide on a paper towel to remove excess wash buffer but be careful so that you do not touch the tissue. It is also important to note that the sample tissue should not be allowed to dry out at any time during this protocol prior to the dehydration step (Step 18).*

6. Blot-dry the area around the tissue and then use a PAP Pen® to create a hydrophobic barrier to contain the staining reagents. Allow the PAP Pen® barrier to dry for at least 2 minutes at room temperature but ensure that the tissue remains hydrated.

   Note: *Leave space between the tissue and the PAP Pen® barrier as the PAP Pen® solution may adversely affect staining of the tissue, causing false negative results.*

7. Add 250 μL of the DNPH solution. For control slides add 250 μL of Derivatization Control Solution. Incubate slides in a humidified chamber for 30 minutes at room temperature (RT) in the dark.

8. Wash with 250 μL of 1X Wash Buffer for 5 minutes at RT. Repeat the wash 2 additional times.

9. Add 250 μL of 1X Blocking Buffer and incubate in a humidified chamber for 30 minutes at RT.

10. Add 250 μL of freshly diluted primary antibody and cover with a plastic cover slip. Incubate in a humidified chamber for 1-2hr at RT or overnight at 4°C.

11. Wash with 250 μL of 1X Wash Buffer for 5 minutes at RT. Repeat the wash 2 additional times.

12. Add 250 μL of biotinylated secondary antibody and incubate in a humidified chamber for 30 minutes at RT.

13. Wash with 250 μL of 1X Wash Buffer for 5 minutes at RT. Repeat the wash 2 additional times.

14. Add 250 μL of 3% H_2O_2 and incubate in a humidified chamber for 10 minutes at RT. This should quench peroxidases endogenous to your tissue

15. Wash with 250 μL of 1X Wash Buffer for 5 minutes at RT. Repeat the wash 2 additional times.

16. Add 250 μL of streptavidin conjugated HRP and incubate in a humidified chamber for 30 minutes at RT.

17. Wash with 250 μL of 1X Wash Buffer for 5 minutes at RT. Repeat the wash 2 additional times.

18. Add 250 μL of freshly prepared DAB-A/B mixture and incubate at RT for 2 to 5 minutes. To prevent overstaining, monitor color development. When sections are developed to the desired amount, immerse the slides in 1X wash buffer.

19. For counter staining, add 250 μL of Hematoxylin and incubate at RT for 2 to 5 minutes. To prevent overstaining monitor the color development.

20. When sections are developed to the desired amount, immerse the slides in MilliQ H_2O.

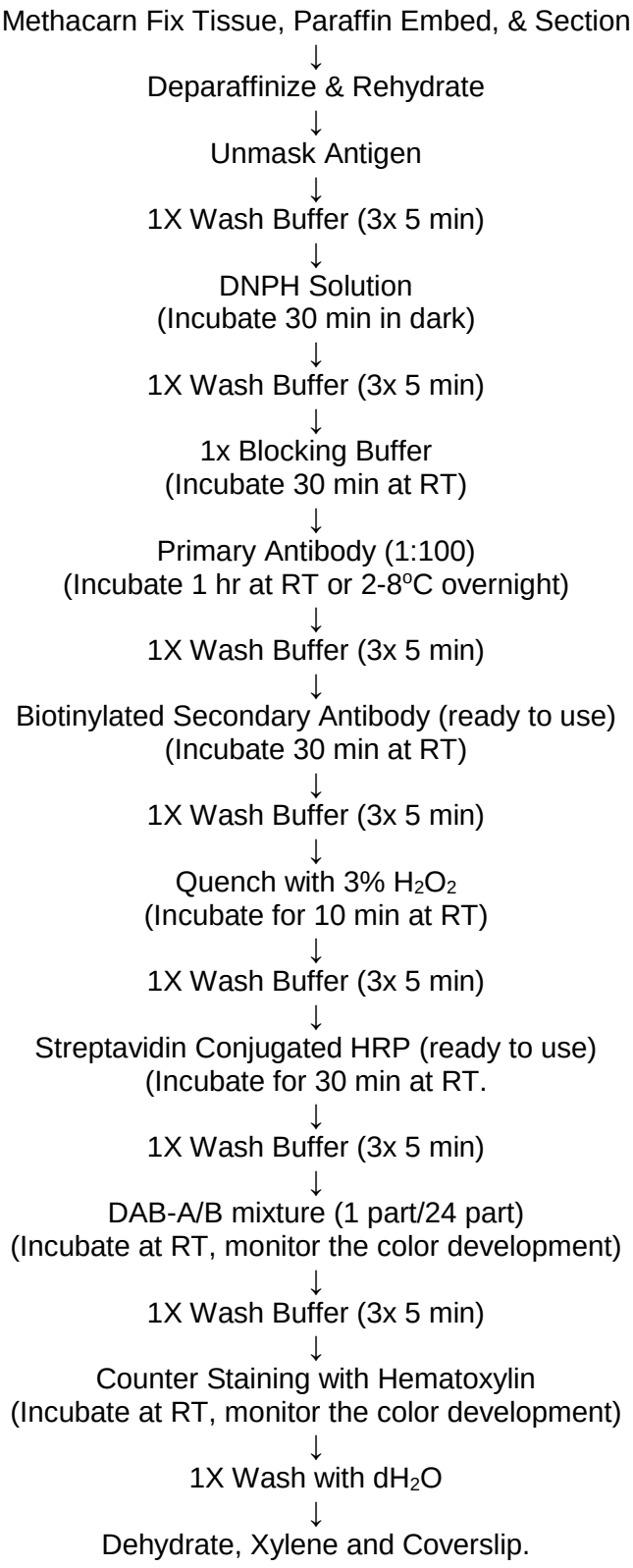
21. Dehydrate slides with 250 μL of 100% EtOH. Complete two rounds of incubations (5 minutes each, for a total of 10 minutes) at RT.

22. Immerse into 250 μL of Xylene and incubate for 5 minutes at RT. Repeat this step 2 additional times, for a total of 3 immersions (15 minutes)

23. Add mounting media to the sample, and cover the slide with a plastic slip. Allow to dry overnight and visualize slides on a light microscope.

Figure 3: Quick Reference for OxylHC™ Procedure

*(**NOTE**: 250 µL are needed for each incubation and wash)*



Example Data

An experiment was carried out to demonstrate the effectiveness of the OxyIHC kit. Briefly, brain tissue from 12 month old Alzheimer's disease transgenic mice (B6;SJL-Tg(APPSWE)2576Kha) was isolated and methacarn fixed as described above. These mice carry a transgene coding for the 695-amino acid isoform of human Alzheimer ß-amyloid (Aß) precursor protein and show elevated levels of amyloid plaque formation. Furthermore, amyloid plaques have been linked to oxidative stress. The OxyIHC protocol was used to determine oxidative stress in wild type versus transgenic mouse brain (Figure 1 and 2). This protocol was also used to examine a human brain of an Alzheimer patient (Figure 3).

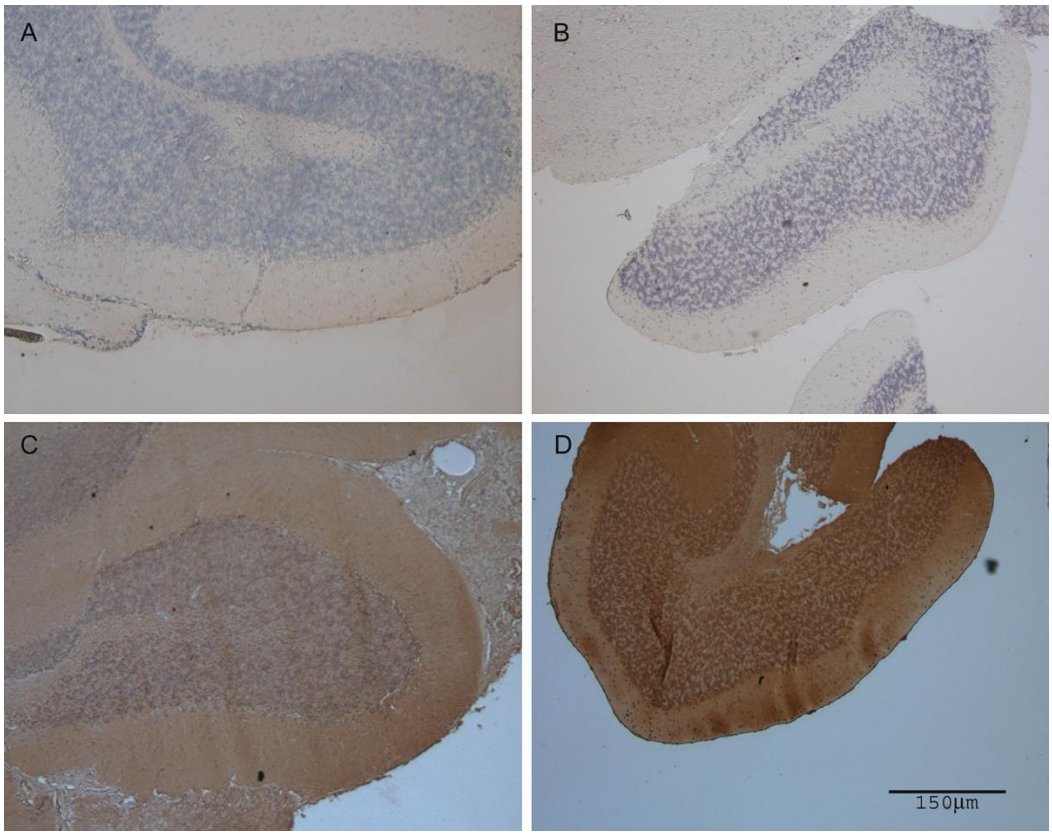


Figure 1: OxyIHC identified oxidative stress in the cerebellar cortex of the Alzheimer's disease transgenic mouse model. Following Methacarn fixation, the brain tissues were paraffin embedded and sectioned. They were then deparaffinized and antigen retrieval was performed according to standard laboratory protocol. Panels A and C are sections from wild type while panels B and D are sections from the transgenic mice. Negative control reactions were performed with the Derivatization Control Solution (panel A and B) and showed minimal DAB reactivity with only Hematoxylin staining. Whereas staining with DNPH resulted in immunoreactivity (panel C and D). Panel C shows basal levels of staining in wild type brain tissue. Panel D shows the Alzheimer's disease transgenic mice is under increased oxidative stress.

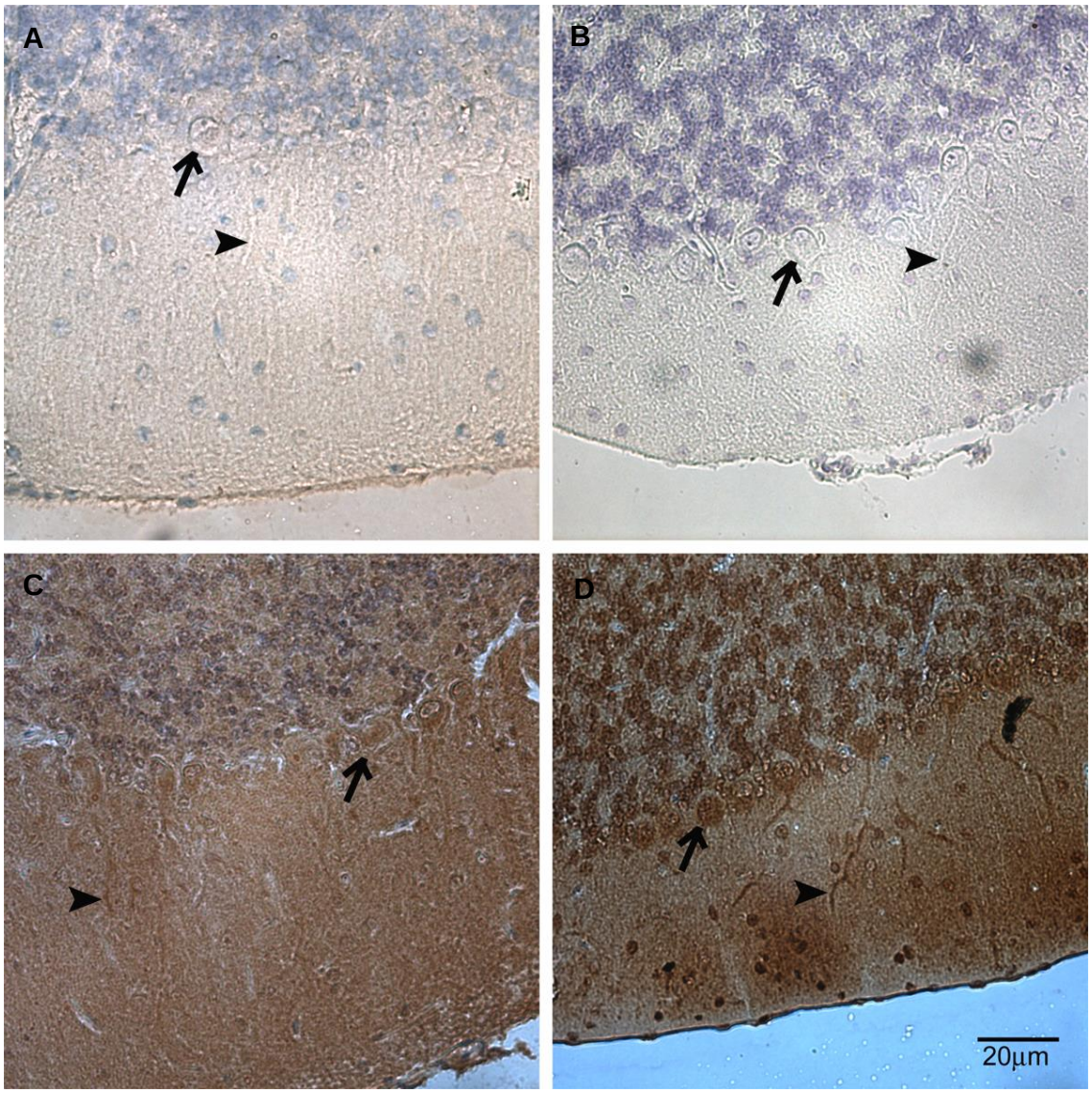
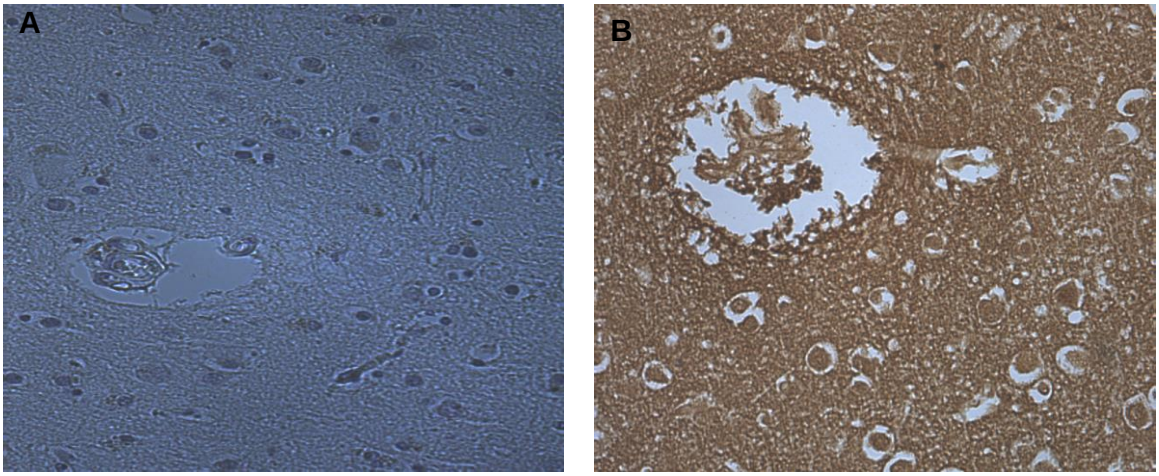


Figure 2: Higher power magnification of tissue from figure 1 showing the row of large Purkinje cells lying between the outer and inner cortical layers. Arrows delineate cell bodies of Purkinje neurons and the arrow head their dendritic branching. Negative control reactions (panel A and B) show minimal DAB reactivity with only Hematoxylin staining primarily in the granular layer. Staining with DNPH resulted in strong immunoreactivity with Purkinje neurons in the transgenic mouse model (arrows in panel D) but minimal staining in the wild type control (arrows in panel C).



-DNPH **+DNPH**

Figure 3: OxyIHC identified oxidative stress in a human brain of an Alzheimer patient. The brain tissue was fixed and stained according to the OxyIHC protocol. Negative control reactions were performed with the Derivatization Control Solution and showed minimal DAB reactivity with only Hematoxylin staining (panel A). Staining with DNPH resulted in strong immunoreactivity (panel B).

Troubleshooting

Poor positive staining or no positive staining with little if any background staining:

- Anti-DNP antibody was omitted, used at the wrong concentration, or used in the wrong order.
- Streptavidin-HRP reagent was omitted, used at the wrong concentration, or used in the wrong order.
- Use a longer incubation time for the Anti-DNP Antibody.
- Do not let slides dry out! Keep wet at all times during the testing procedure.
- Protocol procedure was not followed correctly or steps were omitted.

High background staining:

- Anti-DNP antibody should be diluted further.
- Increase the volume, time, and/or the number of washes between steps.

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