



Results That Rock!

Ultra Amplifying IHC Kit

Cellular Stress

Catalogue Number: AAK7, AAK8

About This Assay:

This assay utilizes novel licensed technology aimed at removing limitations associated with the antibody staining of frozen and paraffin embedded tissue sections. The technology includes specific buffer formulations for antigen exposure and optimized antibody formulations. It also includes a unique device called the Antibody Amplifier™. Using the Antibody Amplifier™, microscope slides are submerged in a greatly diluted antibody solution (1 in 10,000 as described in the Table on page 3). Incubating slides in antibody solution while rocking on a standard laboratory rocker guarantees even staining, and reproducible results. Please note that the antibody formulations used in this kit are specifically optimized for use in the Ultra Amplifying IHC kit and may not work as a stand-alone reagent.

Technical Service Contact Information

Phone: (803) 407-0506

Fax: (803) 407-0260

E-Mail: prohisto@yahoo.com

*Best to contact by email, and please leave a phone number you can be reached at. In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

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GENERAL INFORMATION

Introduction/Background:

Cellular stress is associated with many diseases, including chronic inflammation, autoimmunity, cancer, diabetes, neurodegenerative disorders, hypertension, and many more (1-7). Associated with cellular stress is DNA damage, post-translational modification of proteins, such as nitration of tyrosine residues, and elevated levels of p53. Phosphorylation of serine 15 causes p53 stabilization. The Ultra-Amplifying IHC kits is a highly sensitive kit that accurately, and reproducibly detects DNA damage, nitrotyrosine, p53 and phosphorylation of p53.

Kit Contents:

Catalog Number	Item	Quantity/Size
AA1	Antibody Amplifier™	1
AA3	Amplifying Antibody Dilution Buffer (1x)	500 ml
AA4	Amplifying Wash Buffer (20x)	500 ml
AA6	Epitope Unmasking Solution (10x)	250 ml
N/A	Anti-DNA Damage (8-OHdG) (mouse monoclonal) (suggested dilution: 1 in 10,000)	50 µl Reacts with 8-OHdG on DNA/RNA of all species
N/A	Anti-Nitrotyrosine (mouse monoclonal) (suggested dilution: 1 in 10,000)	50 µl Reacts with human, mouse, rat, dog
N/A	Anti-p53 (mouse monoclonal) (suggested dilution: 1 in 10,000)	50 µl Reacts with human, mouse, rat, money, hamster
N/A	Anti-p53-Phospho-Serine 15 (mouse monoclonal) (suggested dilution: 1 in 10,000)	50 µl Reacts with human, mouse

If any of the items listed above are damaged or not in compliance with the kit ordered, please contact our Customer Service department at (803) 407-0506.

This kit is not for human or animal disease diagnosis or therapeutic drug use.

Precautions:

Please read these instructions carefully before beginning this assay.

The reagents in this kit have been tested and formulated to work exclusively with ProHisto's Ultra Amplifying IHC Series. This kit may not perform as described if any reagent or procedure is replaced or modified. **For research use only.**

Storage and Stability:

This kit will perform as specified if the antibodies are stored as directed at -20°C and the indicated buffers are stored at 4°C (AA3, Amplifying Antibody Dilution Buffer; and AA6, Epitope unmasking solution) or room temperature (AA4, Amplifying Wash Buffer) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied for ABC Protocol:

1. Biotinylated Secondary Antibody
2. Avidin-Biotin Complex Kit (e.g. Vectastain Kits from Vector Labs).
3. DAB reagents
4. Xylene
5. Ethanol
5. 1x PBS
7. Peroxidase Blocking Solution (3% H₂O₂ in PBS)
30% H₂O₂.....1 part (e.g. 10 ml)
1XPBS.....9 parts (e.g. 90 ml)
8. Coverslips
9. Methyl Green (1% in water) or Hematoxylin (Gill-1)
10. Slide racks and holders
11. Permount
12. ddH₂O
13. A standard laboratory rocker
14. Pipettors and tips

Materials Needed But Not Supplied for DAKO Protocol:

1. Kits (these are expensive, but in our opinion well worth the money, as they give very good staining):
 - If your primary antibody is a mouse monoclonal, use: DAKO Envision + System, HRP (DAB), cat#K4006 – Try to use same LOT of antibody for all slides in project
 - If your primary antibody is a rabbit polyclonal, use: DAKO Envision + System, HRP (DAB), cat#K4011– Try to use same LOT of antibody for all slides in project
2. Xylene
3. Ethanol
4. 1x PBS
5. Coverslips
6. Methyl Green (1% in water) or Hematoxylin (Gill-1)
7. Slide racks and holders
8. Permount
9. ddH₂O
10. A standard laboratory rocker
11. Pipettors and tips

Materials Needed But Not Supplied for IF Protocol:

1. Fluorescent-tagged Secondary Antibody
2. Serum from secondary antibody's host (only if background is an issue).
3. Xylene
4. Ethanol
5. Coverslips
6. Slide racks and holders
7. ddH₂O
8. Anti-Fade Solution (e.g. Prolong Gold from Invitrogen Catalog # S36936, or Prolong Gold Anti-Fade Solution plus DAPI from Invitrogen Catalog # P36931)
9. A standard laboratory rocker
10. Pipettors and tips

PROTOCOLS

Sample Types:

Note: If cell lines or frozen sections are being used as opposed to paraffin-embedded ones, please use the following guidelines before moving onto the general protocols in the following section ('Performing the Assay').

Preparation of Slides (skip if paraffin-embedded tissue):

A. Cell line:

1. Grow cultured cells on sterile glass cover slips.
2. Wash briefly with 1X PBS.
3. Fix 10 minutes in formalin (3.7% formaldehyde- dilute 37 formaldehyde 10X in 1X PBS). Keep wet.
4. Wash in 1X Amplifying Wash Buffer (Catalog# AA4) for 5 minutes.

B. Frozen Sections:

1. Snap frozen fresh tissues in liquid nitrogen or isopentane pre-cooled in liquid nitrogen, embedded in OCT compound in cryomolds. Store frozen blocks at -80°C .
2. Cut 4-8 μm thick cryostat sections and mount on superfrost plus slides or gelatin coated slides. Store slides at -80°C until needed.
3. Fix in formalin (3.7% formaldehyde- dilute 37% formaldehyde 10X in 1X PBS)
4. Wash in 1X Amplifying Wash Buffer (Catalog # AA4).

Once you have completed these, please move to Section 8 in the Assay Protocol on Page 7.

Performing the Assay

IMPORTANT NOTE

For the following protocol(s), use each antibody (Anti-DNA Damage, Anti-Nitrotyrosine, Anti-p53, and Anti-p53-Phospho-Serine 15) **separately**. You cannot co-incubate the antibodies, or you will not know what the end signal is. Carry out the following protocol(s) for Anti-DNA Damage, then Anti-Nitrotyrosine, then Anti-p53, then Anti-p53-Phospho-Serine 15, such that you will carry out 4 separate assays.

IHC PROTOCOL USING THE ABC METHOD

1. Place slides in a glass slide holder and place in the following solutions serially (at room temperature):
 - a. Xylene: 5 minutes swishing every 2 minutes (in fume hood).
 - b. Xylene: 5 minutes, swishing every 2 minutes (in fume hood).
 - c. 100% Ethanol: 2 minutes, swishing every 30 seconds.
 - d. 95% Ethanol: 2 minutes, rocking
 - e. 80% Ethanol, 2 minutes rocking.
 - f. 70% Ethanol, 2 minutes rocking.
 - g. 50% Ethanol, 2 minutes rocking.
 - h. ddH₂O: 3 minutes, rocking. Repeat one time.
2. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.
3. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) another 2 times, but for three minutes each this time.
4. Transfer slide holder to plastic, autoclavable container and submerge in 1X Epitope Unmasking Solution (Catalog # AA6).
5. Cover in foil and autoclave for 20-30 minutes.
6. Retrieve sample from autoclave and place on counter to let cool at room temperature for 20 minutes (Continuing submersion in Epitope Unmasking Solution (Catalog # AA6).

NOTE: For fatty tissue, such as brain or breast, use these directions for antigen retrieval:

Place slides in slide rack. Lower slide rack into 600 mL gently boiling 1X Epitope Unmasking Solution (Catalog # AA6). Boil gently for exactly 8 minutes, and then remove from the heat source (a large beaker filled with 1x Epitope Unmasking Solution (Catalog # AA6), with boiling chips on the bottom and sitting on a heat block works well). Slowly add 500 mLs of ddH₂O to bring buffer temperature down. Quickly put slide rack in glass slide holder and wash twice with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes each time.

7. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.

8. *Note: Use of the Antibody Amplifier™ is optional at this point. However, ensure that the slides are completely covered by the peroxidase blocking solution.*

Wipe off back and sides of slide with a kim-wipe, lay flat on bench top and cover tissue with 1-3 drops of peroxidase block (3% H₂O₂ in 1X PBS). Incubate at room temperature for 30 minutes, checking every 5-10 minutes to ensure the samples do NOT dry out (If they are starting to dry out, add more peroxidase block).

9. Put slides back into slide carrier and wash for 3 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat two times.

10. Dilute primary antibody in Amplifying Antibody Dilution Buffer (Catalog # AA3). Use a minimum of 3 mLs per slide to completely submerge in the Antibody Amplifier™. The suggested dilution for Anti-DNA Damage, Anti-Nitrotyrosine, and Anti-p53 is 1 in 10,000. The suggested dilution for Anti-p53-Phospho-Serine 15 is 1 in 10,000. Place lid onto the Antibody Amplifier™ and place on a standard laboratory rocker. Rock overnight at 4°C. After use, store primary antibodies at -20°C. The antibodies can generally be re-used up to 4X without compromising sensitivity.

11. Put slides back into glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.

12. Incubate slides in biotinylated anti-mouse secondary antibody. Secondary antibody is usually provided as part of avidin-biotin complex staining kits (e.g. Vectastain kit by Vector Labs is recommended). Secondary antibody dilution is 1:2000 in Amplifying Antibody Dilution Buffer (Catalog # AA3), but optimal dilution is at the discrepancy of the end-user (the use of the Antibody Amplifier™ rocking on a standard laboratory rocker often allows the user to significantly titrate out antibodies from that which is recommended by the supplier). Incubate slides rocking at room temperature for one hour in Antibody Amplifier™. Remember that each slide needs 3 mLs for full submersion.

13. As soon as the slides are incubating, mix up an Avidin-Biotin Complex (ABC) solution (e.g. found in Vectastain kits from Vector Labs). The ABC solution is diluted 1:1000 (e.g. 5 µl A + 5 µl B in 10 mLs 1X PBS) but optimal dilution is at the discrepancy of the end-user (the use of the Antibody Amplifier™ rocking on a standard laboratory rocker often allows the user to significantly titrate out ABC solutions from that which is recommended by the supplier). Remember if using the Antibody Amplifier™, to make 3 mLs per slide. Let the mixed solution sit at room temperature for 30-60 minutes before using.

- 14.** Put slides in glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.
- 15.** Incubate slides in pre-made ABC solution, rocking at room temperature for 1 hour in the Antibody Amplifier™.
- 16.** Make up DAB solution according to the manufacturer's instructions. 100-200 µL per slide is needed.
- 17.** Wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.
- 18.** Wash for 3 minutes in ddH₂O.
- 19.** Wipe slides around tissue with kim-wipes, keeping tissue moist, and add DAB solution. Check the staining of one slide first by testing a positive control: Incubate with 100-200 µL of DAB solution, starting at 30 seconds. Monitor using the microscope at 40X. Keep note of DAB times. Wash slides as soon as staining is optimal with ddH₂O.
NOTE: All slides that you are comparing must be incubated for the same time period with DAB once this time is established with the positive control.
- 20.** Wash slides with ddH₂O for 3 minutes.
- 21.** Counter-stain using Methyl Green: Filter 3-10 mLs of methyl green using a syringe filter into a 15 mL tube. Add 200 µL of methyl green onto tissue and incubate at room temperature for 2 minutes (or whatever time has been established). Rinse in containers of ddH₂O until slides are clean and let air dry, with slides standing vertically on their ends.
- 22.** After tissues are completely dry, add one drop of Permount, put on coverslip, and smooth out bubbles by pushing down on the coverslip using the big/blunt end of the pipettor tip. Label slides with: date, antibody, dilution and timing. After 4-5 hours, you can clean off extra Permount with Xylene (The Xylene is optional-only if too much Permount was added, and it is covering tissue.)

IHC PROTOCOL USING KITS MANUFACTURED BY DAKO

For customers who prefer to use DAKO kit (DAKO Envision + System, Catalog # K4006, or DAKO Envision + System Catalog # K4011)

1. Place slides in a glass slide holder and place in the following solutions serially (at room temperature):
 - a. Xylene: 5 minutes swishing every 2 minutes (in fume hood).
 - b. Xylene: 5 minutes, swishing every 2 minutes (in fume hood).
 - c. 100% Ethanol: 2 minutes, swishing every 30 seconds.
 - d. 95% Ethanol: 2 minutes, rocking
 - e. 80% Ethanol, 2 minutes rocking.
 - f. 70% Ethanol, 2 minutes rocking.
 - g. 50% Ethanol, 2 minutes rocking.
 - h. ddH₂O: 3 minutes, rocking. Repeat one time.
2. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.
3. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) another 2 times, but for three minutes each this time.
4. Transfer slide holder to plastic, autoclavable container and submerge in 1X Epitope Unmasking Solution (Catalog # AA6).
5. Cover in foil and autoclave for 20-30 minutes.
6. Retrieve sample from autoclave and place on counter to let cool at room temperature for 20 minutes (Continuing submersion in Epitope Unmasking Solution (Catalog # AA6)).

NOTE: For fatty tissue, such as brain or breast, use these directions for antigen retrieval:

Place slides in slide rack. Lower slide rack into 600 mL gently boiling 1X Epitope Unmasking Solution (Catalog # AA6). Boil gently for exactly 8 minutes, and then remove from the heat source (a large beaker filled with 1x Epitope Unmasking Solution (Catalog # AA6), with boiling chips on the bottom and sitting on a heat block works well). Slowly add 500 mLs of ddH₂O to bring buffer temperature down. Quickly put slide rack in glass slide holder and wash twice with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes each time.
7. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.
8. Take each individual slide, and with a kimwipe, wipe off the back of the slide, and around the tissue. Be sure not to touch the tissue, and that it keeps wet. Cover tissue with 1-3 drops of Peroxidase Block (comes with the DAKO kit).

Incubate at room temperature 30 minutes, checking every 5-10 minutes to make sure samples don't dry out. If you observe some drying, place more Peroxidase Block onto the tissue.

9. Put slides back into slide carrier and wash for 3 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat two times.

10. Dilute primary antibody in Amplifying Antibody Dilution Buffer (Catalog # AA3). Use a minimum of 3 mLs per slide to completely submerge in the Antibody Amplifier™. The suggested dilution for Anti-DNA Damage, Anti-Nitrotyrosine, and Anti-p53 is 1 in 10,000. The suggested dilution for Anti-p53-Phospho-Serine 15 is 1 in 10,000. Place lid onto the Antibody Amplifier™ and place on a standard laboratory rocker. Rock overnight at 4°C. After use, store primary antibodies at -20°C. The antibodies can generally be re-used up to 4X without compromising sensitivity.

11. Put slides back into glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.

12. Wipe off the back of slides and around tissues as above with a kim wipe. Add 1-3 drops of Labeled Polymer (comes with DAKO kit) straight from the bottle, enough to cover tissue. Incubate at room temperature for 30 minutes. Add polymer as needed so that the tissue does not dry out.

13. As soon as slides are incubating, mix up the DAB solution. The ratio of the solution is 1 ml solution 3a (Buffered Substrate in DAKO kit): 1 drop 3b (liquid DAB and chromagen in DAKO kit). Mix well. Make enough so there is 200 ul per slide. Let the mixed solution sit at room temperature 30-60 minutes before using.

14. After incubation with Labeled Polymer, put slides in glass slide rack, and wash 2 x 5 minutes in 1x Amplifying Wash Buffer (Cat# AA4).

15. Wash slides in ddH₂O 1 x 3 minutes.

16. Wipe slides around tissue with kim-wipes, keeping tissue moist, and add DAB solution. Check the staining of one slide first by testing a positive control: Incubate with 100-200 µL of DAB solution, starting at 30 seconds. Monitor using the microscope at 40X. Keep note of DAB times. Wash slides as soon as staining is optimal with ddH₂O.

NOTE: All slides that you are comparing must be incubated for the same time period with DAB once this time is established with the positive control.

17. Wash slides with ddH₂O for 3 minutes.

18. Counter-stain using Methyl Green: Filter 3-10 mLs of methyl green using a syringe filter into a 15 mL tube. Add 200 µL of methyl green onto tissue and incubate at room temperature for 2 minutes (or whatever time has been established). Rinse in containers of ddH₂O until slides are clean and let air dry, with slides standing vertically on their ends.

19. After tissues are completely dry, add one drop of Permount, put on coverslip, and smooth out bubbles by pushing down on the coverslip using the big/blunt end of the pipettor tip. Label slides with: date, antibody, dilution and timing. After 4-5 hours, you can clean off extra Permount with Xylene (The Xylene is optional-only if too much Permount was added, and it is covering tissue.)

IMMUNOFLUORESCENCE PROTOCOL

The following outlines an ImmunoFluorescence (IF) protocol, using Alexa Fluor conjugated secondary antibodies on paraffin-embedded tissues, to allow optimal fluorescent detection markers for IHC.

- 1.** Place slides in a glass slide holder and place in the following solutions serially (at room temperature):
 - a. Xylene: 5 minutes swishing every 2 minutes (in fume hood).
 - b. Xylene: 5 minutes, swishing every 2 minutes (in fume hood).
 - c. 100% Ethanol: 2 minutes, swishing every 30 seconds.
 - d. 95% Ethanol: 2 minutes, rocking
 - e. 80% Ethanol, 2 minutes rocking.
 - f. 70% Ethanol, 2 minutes rocking.
 - g. 50% Ethanol, 2 minutes rocking.
 - h. ddH₂O: 3 minutes, rocking. Repeat one time.
- 2.** Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.
- 3.** Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) another 2 times, but for three minutes each this time.
- 4.** Transfer slide holder to plastic, autoclavable container and submerge in 1X Epitope Unmasking Solution (Catalog # AA6).
- 5.** Cover in foil and autoclave for 20-30 minutes.
- 6.** Retrieve sample from autoclave and place on counter to let cool at room temperature for 20 minutes (Continuing submersion in Epitope Unmasking Solution (Catalog # AA6).

NOTE: For fatty tissue, such as brain or breast, use these directions for antigen retrieval:

Place slides in slide rack. Lower slide rack into 600 mL gently boiling 1X Epitope Unmasking Solution (Catalog # AA6). Boil gently for exactly 8 minutes, and then remove from the heat source (a large beaker filled with 1x Epitope Unmasking Solution (Catalog # AA6), with boiling chips on the bottom and sitting on a heat block works well). Slowly add 500 mLs of ddH₂O to bring buffer temperature down. Quickly put slide rack in glass slide holder and wash twice with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes each times.

7. Wash with 1X Amplifying IHC Wash Buffer (Catalog # AA4) for 5 minutes, rocking at room temperature. Repeat once.

8. *Optional to reduce background fluorescence:* Block for 30 minutes at room temperature using serum from secondary antibody's host.

9. Put slides back into slide carrier and wash in 1X Amplifying IHC Wash Buffer (Catalog # AA4) for three minutes. Repeat twice.

10. Dilute primary antibody in Amplifying Antibody Dilution Buffer (Catalog # AA3). Use a minimum of 3 mLs per slide to completely submerge in the Antibody Amplifier Eclipse™. The suggested dilution for Anti-DNA Damage, Anti-Nitrotyrosine, and Anti-p53 is 1 in 10,000. The suggested dilution for Anti-p53-Phospho-Serine 15 is 1 in 10,000. Place lid onto the Antibody Amplifier Eclipse™ and place on a standard laboratory rocker. Rock overnight at 4°C. After use, store primary antibody at -20°C. The antibody can generally be re-used up to 4X without compromising sensitivity.

11. Put slides back into glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Repeat once.

12. Prepare dilution of fluorescent anti-mouse secondary antibody. Secondary antibody dilution is 1:5000 in 1X PBS MADE IN THE DARK, but optimal dilution is at the discrepancy of the end-user (the use of the Antibody Amplifier Eclipse™ rocking on a standard laboratory rocker often allows the user to significantly titrate out antibodies from that which is recommended by the supplier). Incubate slides rocking at room temperature for one hour in Antibody Amplifier Eclipse™. Remember that each slide needs 3 mLs for full submersion. If you wish to store and re-use the secondary antibody, make the dilution buffer as follows: 0.01 M PBS pH 7.2, 0.05% thimerosal. This solution can be stored at 4°C wrapped in tinfoil for 6 months, do not expose secondary antibody to light.

13. Incubate slides rocking at room temperature for 1-2 hours in the Antibody Amplifier Eclipse™. Remember that each slide needs 3 mLs.

- 14.** Prepare Prolong Gold Anti-fade Solution. Take out of freezer and let thaw at room temperature for 1 hour. Do not artificially speed up warming.
- 15.** Put slides in tinfoil wrapped glass slide carrier and wash for 5 minutes in 1X Amplifying IHC Wash Buffer (Catalog # AA4). Keep slides in the dark- do not expose to light. Repeat once.
- 16.** Wash slides in ddH₂O for 3 minutes.
- 17.** Prepare fluorescent microscope according to manufacturer's instructions.
- 18.** Under dim lighting, use a kim-wipe to dry most of the slide, keeping the mounted tissue moist. Add 1 drop of room temperature Prolong Gold Anti-Fade Solution to the center of tissue. Cover with a coverslip, gently removing air bubbles.
- 19.** To view immediately, tack down the corners of the coverslip with clear nail polish. After viewing, to permanently seal the slide, paint the edges of the coverslip with the clear nail polish and let dry. Put the slides into the Antibody Amplifier Eclipse™, and allow the slides to cure in total darkness for 2-24 hours depending on the thickness of the tissue and the relative humidity of the environment.
- 20.** To keep slides in optimal condition indefinitely, store them upright in a covered slide box containing a dessicant at -20°C.

Notes on the Use of the Antibody Amplifier™/ Antibody Amplifier Eclipse™

1. Each Antibody Amplifier™/ Antibody Amplifier Eclipse™ has 12 wells (1 slide per well), and therefore holds 12 slides.
2. Each well in the Antibody Amplifier™/ Antibody Amplifier Eclipse™ requires approximately 3 mLs of solution. Because of the characteristics of the amplification technology and chemistries used in this kit, it is important to use the correct amount of antibody. Please follow the instructions carefully for the relevant antibodies in each kit.
3. The Antibody Amplifier™/ Antibody Amplifier Eclipse™ is designed to be stackable, and therefore, an experiment can be carried out with multiple Antibody Amplifiers™/ Antibody Amplifier Eclipses™. We suggest, however, that you secure them together with laboratory tape.
4. Following placement of slides into the Antibody Amplifier™, the chamber with the lid on is placed on a standard laboratory rocker during incubation.
5. The Antibody Amplifier™/ Antibody Amplifier Eclipse™ is chemical resistant (e.g. to xylenes).
6. The scientist can decide either to use the Antibody Amplifier™/ Antibody Amplifier Eclipse™ during the entire procedure, or alternatively only during specific parts of the assay. In all cases, however, the scientist should use the Antibody Amplifier™/ Antibody Amplifier Eclipse™ during antibody incubation.
7. Slides can be taken out of chambers with any pipette tip or forceps.
8. For more information, or trouble-shooting, please see our website (www.prohisto.com), call 803-407-0506, or (preferable) email us at prohisto@yahoo.com (we monitor the email site every 30 minutes).

PHOTOS

Anti-DNA Damage (8-OHdG) (mouse monoclonal):

1. Here is an example of the anti-DNA/RNA damage antibody diluted at 1 in 10,000 in Amplifying Antibody Dilution Buffer (see Figure 1 below), submerging each slide in this solution, and incubating while rocking overnight at 4°C in the Antibody Amplifier™. However, the optimum titration should be determined by the end-user, and may depend on such issues as the tissue type, and method of fixation.

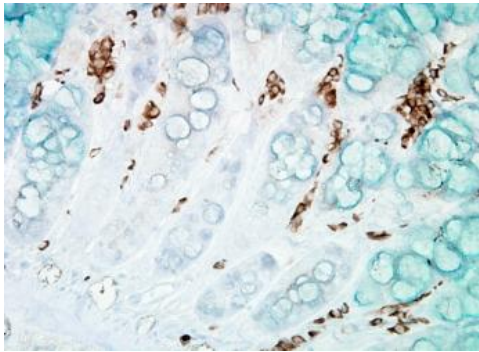


Figure 1. Detection of DNA Damage (8-OHdG) in formalin-fixed, paraffin-embedded colon tissue from a mouse with colitis.

Anti-Nitrotyrosine (mouse monoclonal):

1. Here is an example of the anti-nitrotyrosine antibody diluted at 1 in 10,000 in Amplifying Antibody Dilution Buffer (see Figure 2 below), submerging each slide in this solution, and incubating while rocking overnight at 4°C in the Antibody Amplifier™. However, the optimum titration should be determined by the end-user, and may depend on such issues as the tissue type, and method of fixation.

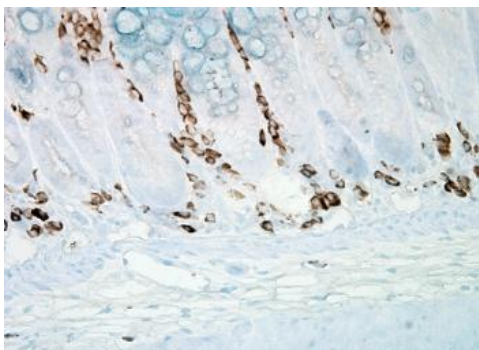


Figure 2. Detection of nitrotyrosine in formalin-fixed, paraffin-embedded colon tissue from a mouse with colitis.

Anti-p53 (mouse monoclonal):

1. Here is an example of the anti-p53 antibody diluted at 1 in 10,000 in Amplifying Antibody Dilution Buffer (see Figure 3 below), submerging each slide in this solution, and incubating while rocking overnight at 4°C in the Antibody Amplifier™. However, the optimum titration should be determined by the end-user, and may depend on such issues as the tissue type, and method of fixation.

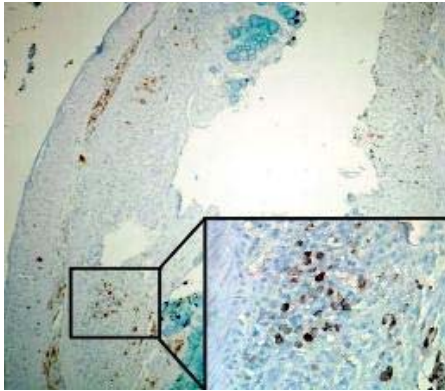


Figure 3. Detection of p53 in formalin-fixed, paraffin-embedded colon tissue from a mouse with colitis.

Anti-p53-Phospho-Serine 15 (mouse monoclonal):

1. Here is an example of the anti- p53-Phospho-Serine 15 antibody diluted at 1 in 10,000 in Amplifying Antibody Dilution Buffer (see Figure 4 below), submerging each slide in this solution, and incubating while rocking overnight at 4°C in the Antibody Amplifier™. However, the optimum titration should be determined by the end-user, and may depend on such issues as the tissue type, and method of fixation.

Figure 4A. Detection of p53-Phospho-Serine 15 in formalin-fixed, paraffin-embedded colon tissue from a mouse with colitis.

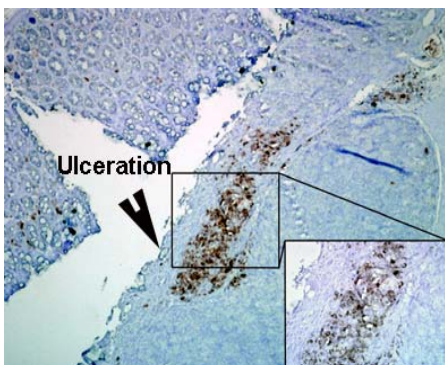
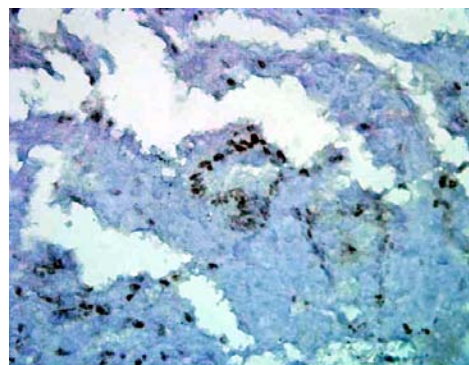


Figure 4B. Detection of p53-Phospho-Serine 15 in formalin-fixed, paraffin-embedded colon tissue in human colon cancer.



TROUBLESHOOTING

Problem	Possible Cause	Solution
Weak or no staining	Inadequate deparaffinization	Deparaffinize sections longer, or change fresh xylenes and ethanol
	Inactive primary antibodies –improper storage?	Replace with a new batch of antibodies. Aliquot antibodies into smaller volumes and avoid repeated freeze and thaw cycles.
	Antibody concentration was too low	Increase the concentration; or run a serial dilution test to determine the optimal dilution that gives the best signal to noise ratio
	Inadequate antibody incubation time	Increase antibody incubation time
	Inadequate or improper tissue fixation	Increase duration of post-fixation or try different fixatives.
	Tissue overfixation	Reduce the duration of post-fixation. If the tissue has already been over-fixed, perform the appropriate or recommended antigen retrieval procedure.
	Incompatible secondary and primary antibodies	Use secondary antibody that will interact with the primary antibody.
	Inactive secondary antibody	Replace with a new batch of antibodies.
	Defective or incompatible enzyme substrate system	Replace with a new batch of reagents.
	Inadequate substrate incubation time	Increase the substrate incubation time.
	Incorrect mounting medium	Choose a correct mounting medium.
	Reagents applied in wrong order or steps omitted	Check notes or procedure used.
Over-staining	The concentration of primary and/or secondary antibodies was too high	Reduce antibody concentration or perform a titration to determine the optimal dilution for primary and secondary antibodies.
	Incubation time was too long	Reduce incubation time.
	Sections dried out	Avoid sections drying out, by adding the appropriate amount of solution
High background	Inadequate washing	Wash at least three times between steps
	Non-specific binding of primary antibodies to tissue	Non-specific binding may be reduced by using a higher dilution of primary antibodies.
	Diffusion of tissue antigen due to inadequate fixation	Increase duration of post-fixation.
	Sections dried out	Avoid sections drying out by adding appropriate volumes of solution.
	DAB step too long	Reduce DAB time.
Tissue comes off slide	Too harsh treatment for antigen retrieval	Use a soft-boiling method.
	Washing too much	Decrease wash steps to 1x3 min. between incubations.

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