Light microscopy

[Immunoperoxidase - using immunon sequenza coverplate technique](http://stehm.uvic.ca/docs/prep/microwave/protocols.php#immunoperoxidase)

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| **Immunoperoxidase - using immunon sequenza coverplate technique** |

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| Sample type | Paraffin sections on glass slides |
| Application | Immunohistochemistry |
| Specimen(s) | Routine formalin or "prefer" fixed paraffin embedded tissue sections mounted on poly-1-lysine coated (positively charged) slides and dried at 55°C for 1 hour.  |
| Submitted by | Rick Giberson, [Ted Pella, Inc.](http://www.tedpella.com) and Bruce Caukins, formerly with Pathology Sciences Medical Group, Chico, CA  |
| Instrument used | BioWave™, Ted Pella, Inc. Microwave system with PELCO ColdSpot®, vacuum chamber, and variable wattage. |
| Sample size | 3-5 µm sections on slides |
| Sample container | Microwave-safe plastic coplin jars (Thermo-Shandon #1001361) and coverplate system or other microwave safe staining containers should be used. Place sample container on PELCO ColdSpot®, and place temperature probe in right rear port of ColdSpot. |
| Benefits | Compared to routine bench methods or an automated immunostainer the submitted microwave protocol reduces turnaround times by about 75% over either bench or automated methods. Also, background seems to be reduced by the use of the microwave when compared to the other two methods.  |
| Cautions | The primary antibody dilution used in microwave staining may need to be more highly concentrated that what is used for bench protocols employing an overnight step or longer in primary antibody. We found that the dilution (1:800) used for an overnight labeling step for a primary antibody against vimentin required a 1:100 dilution to deliver the same results from a 6 minute microwave labeling step as the overnight step at 4°C. A 1:200 dilution used in the microwave produced a clearly negative result. All other antibody dilutions (secondary and tertiary) can be left alone and have always worked. We have had no problems with the primary antibody dilutions having incubation times of less than a few hours.  |
| Note | Cut 1 slide for most antibodies and 2 slides if digestion is required, plus 1 additional slide for normal serum control. Frozen sections of fresh tissue and cytologic cyto-spin preparations may be used if air dried for 48 hours followed by fixation in cold (4°C) acetone for 10 min. |
| Control | Known positive tissue (see data sheets supplied with specific primary antibodies for recommended controls), fixed like specimen and cut at 4 microns. Run a control slide with each stain batch. In addition stain an extra section of test tissue with normal serum (as a negative control) and stain positive control tissue with specific serum and normal serum. If negative, repeat procedure (check expiration dates of reagents). Protease or trypsin predigestion or Antigen Retrieval technique may be required. |

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| **Step** | **Reagent** | **Power** | **Time** | **TR** |
| 1 | 50 ml Coplin Jar, xylene | 250 W | 4 min | none |
| 2 | 50 ml Coplin Jar, 95-100% ETOH | 250 W | 1 min | none |
| 3 | 50 ml Coplin Jar, Wash in DI Water |  | Bench step, 30 sec |  |
| 4 | Antigen Retrieval (if required) |  | No specific method is recommended |  |
| 5 | Transfer slides to coverplate system (See Figure 1) |  |  |  |
| 6 | Block Endogenous Peroxidase - 3 drops (˜ 100 µl) | 250 W | 1 min | none |
| 7 | Buffer rinse approx. 1 ml (1 to 3 x 1 min) | 250 W | 1 min | none |
| 8 | Blocking step - 3 drops to approx. 1 ml | 250 W | 1 min on, 1 min off, 1 min on | none |
| 9 | Primary antibody - 3 drops (approx. 100 µl) | 250 W | 2 min on, 2 min off, 2 min on | none |
| 10 | Buffer Rinse approx. 1 ml (1 to 3 x 1 min) | 250 W | 1 min | none |
| 11 | Secondary Antibody - 3 drops (approx. 100 µl) | 250 W | 2 min on, 2 min off, 2 min on | none |
| 12 | Buffer Rinse approx. 1 ml (1 to 3 x 1 min) | 250 W | 1 min | none |
| 13 | Tertiary Attachment - 3 drops (approx. 100 µl) | 250 W | 2 min on, 2 min off, 2 min on | none |
| 14 | Buffer Rinse approx. 1 ml (1 to 3 x 1 min) | 250 W | 1 min | none |
| 15 | Chromagen - 100 approx. 500 µl | 250 W | 1-6 min | none |
| 16 | Rinse in DI Water approx. 1 ml (1 to 3 x 1 min) | 250 W | 1 min | none |
| 17 | Counter Stain - 100 approx. 500 µl | 250 W | 1 min | none |
| 18 | Rinse in DI Water approx. 1 ml (1 to 3 x 1 min) | 250 W | 1 min | none |
| 19 | Remove Slides From Coverplate System | 250 W | 1 min | none |
| 20 | Mount Coverslip and View | 250 W | 1 min | none |

TR = temperature restriction

Place temperature probe in ColdSpot® port when temperature restriction (TR) is "none" and set TR to > 50°C. This modification was developed with a variable wattage, continuous power microwave oven fitted with a Cold Spot® to eliminate hot/cold spots. This procedure uses Shandon Immunon Sequenza Coverplate system (See attached illustrations on loading slides in clips).



Figure 1a: Front view of coverplate that is placed on the glass slide to form a capillary gap for the staining reagents to flow through. The arrow indicates the notch in the front clip of the coverplate that connects with the tab in Fig 1c as shown by the arrow.



Figure 1b: Rear view of coverplate showing the tabs (marked with << marks) that postion and hold the slide (tissue side down) in position prior to placing the complex into the cassette base.



Figure 1c: Cassette base - microwave holder for coverplate and slide. The cassette base holds the coverplate and slide complex together to maintain the capillary gap during microwave. The lid is not needed for microwave protocols.

##### Detailed reagent prep information

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| Detection system  | Universal Streptavidin/Biotin Peroxidase. Bring all reagents to room temperature before use.  |
| Enzyme digestion solution  | Optional  |
| Counterstain  | Hematoxylin (Gill's #1)  |
| Mounting medium  | "Advantage", Innovex/Biosciences #NB300  |

##### Comments

Paraffin-embedded sections are hydrated to water and treated with an endogenous peroxidase block and a protein blocking agent to reduce non-specific binding of antibodies. The tissues are then sequentially incubated with 1) primary antibody, which binds to specific tissue antigens; 2) secondary (link) antibody, which binds to the primary antibody; and 3) streptavidin-enzyme reagent (label), which links to the secondary antibody. The secondary antibody is polyvalent and universal and will bind to primary antibodies derived from rabbit, goat, mouse, guinea pig, sheep or rat. The secondary antibody is also conjugated with biotin. The third step uses streptavidin conjugated with horseradish peroxidase. The peroxidase then serves as the label. Addition of enzyme substrate and a chromogen (AEC) results in a colored precipitate at the sites of tissue antigen. Visualization is aided by counterstaining with hematoxylin. Microwaves cause most reactions and staining to proceed more quickly.

##### Results

A positive reaction is indicated by a colored precipitate at sites of specific cellular antigen localization. AEC will appear pink to reddish brown. Results should be interpreted in light of the appropriate staining of all positive and negative controls.

##### References

Lipshaw/Immunon, OmniTags Universal Streptavidin/Biotin Affinity Immunostaining Systems Instruction Manual, 11/92

DAKO, LSAB2 System, Peroxidase, Universal, Instructions, 1993

Carson,F.L., Histotechnology: A Self-Instructional Text, ASCP Press, 1990, pg. 236