

Brigham and Women's Hospital
Pathology Department
Standardized Immunohistochemistry Protocol
March 17, 2009

I. Reagents

A. Antibodies: dilute in PBS pH 7.4 immediately before use

- **Bcl-2** Oncoprotein (murine clone bcl-2/100/D5), Novocastra, used at 1:100
- **Beta-Catenin** murine monoclonal (Clone 14, BD Transduction Laboratories, Cat#610154). Shipped in about 600ul. Aliquot in 100ul tubes and store at -20'C-0'C. Use at 1:300.
- **Cyclin B1** (murine Clone 7A9, Novocastra Cat# NCL-Cyclin B1)
- **Cyclin E** (murine monoclonal HE12, Santa Cruz cat#sc-247) 1:400
- **CD31, endothelial cell.** Clone JC70A, monoclonal mouse antibody (Dako catalog #M0823), use at 1:100 dilution at room temperature for 1 hour. Cross reacts with some white blood cells (nk cells, myeloid, t-cells, B-cell precursors).
- **Cytokeratin (Pan-K).** Anti-human cytokeratin, Mouse monoclonal IgG clone MNF116. Dako Cat number M0821, 1ml liquid. Use at 1:100 with antigen retrieval. 1' AB incubation either overnight 4'C or room temperature 2 hours.
- **Cytokeratin (PanK).** Anti-human pankeratin cocktail of AE1 and AE3 murine anti-human IgG. (Dako #M3515, Ascites 0.2 mL/1 mL). use at 1:100 1'Ab incubation 2 hours at RT. This works for destained H&E slides as well.
- **E2F6 Transcription factor:** (GenWayBio Cat#10-003-42161) supplied as 100ug lyophilized RABBIT IgG. Reconstitute in 100ul Water to give antibody concentration of 1000ug/ml. Use at 1:200 dilution.
- **Estrogen Receptor:** Anti-estrogen receptor alpha, murine antibody ER-ID5 (Dako, cat M7047) used at 1:300 dilution.
- **gamma-H2AX**, phosphorylated form of the core histone H2AX that localizes to double stranded DNA breaks Millipore Corp. Item: Anti-phospho-Histone H2A.X (Ser139), clone JBW301, Mouse monoclonal antibody Item #: 05-636, use at 1:3000. Vendor: Millipore Corp.
- **HIF1**, stain for oxidative damage. AbCam HIF1 alpha antibody (H1alpha67 murine monoclonal) (ab1): Use at 1:500.
- **Hox A10:** rabbit polyclonal against human Homeobox A10 gene (Polyclonal A10/1135 from Honami Naora). use at 1:200 dilution.
- **Hox A11:** rabbit polyclonal against human Homeobox A11 gene (Polyclonal A11/1131 from Honami Naora). use at 1:200 dilution.
- **MIB-1** (murine monoclonal clone MIB-1) DAKO, Cat.M7240, use at 1:100
- **MLH1** murine (Clone G168-728, BD PharMingen Cat.#554073, formerly 13291A) at 1:200
- **p53** murine (Murine Clone PAb 1801, Novocastra distributed under Novocastra Cat# NCL-p53-1801 by Vision Biosystems in the USA, 1-781-616-1190) Shipped

lyophilized to be reconstituted in 1ml of ice cold STERILE water. Aliquot in 100ul tubes and store at -20°C. Use this reconstituted antibody at 1:300

- **p63** murine monoclonal 4AF (from C.Crum) used at 1:100
- **Phospho-HistoneH3** (for mitotic prophase to telophase) rabbit polyclonal phospho H3 from Upstate Biotechnology (Cat# 06-570), 200ul supplied at 1ug/1ul. Use at 0.1 ug/ml (1:10,000 dilution).
- **Phospho-PTEN** (Ser380/Thr382/383) rabbit polyclonal, Cell Signalling Cat#9554, use at 1:50 dilution
- **Progesterone Receptor:** anti-progesterone receptor murine antibody IA6 (Vector: Cat VP-P975) used at 1:1000 dilution.
- **PTEN;** Murine anti-human PTEN clone 6H2.1. Cascade Bioscience (cat.# ABM2051), use at **1:100 (best for old blocks)** to 1:300 dilution. Other suppliers we have tested and which work very well at same dilutions are: DAKO 6h2.1 and Millipore 6h2.1
- **WT1** mouse monoclonal Dako Cat#M3561, antibody Clone 6F-H2 @ 1:200 dil.
- **ZEB1** Rabbit anti-Zfhep {Douglas Darling #1642 D88}, supplied via Richer. Use stock at 1:5000.

Intermediate working solution: Prepare intermediate 50x dilution by diluting 1ul of stock in 50ul of diluent (Diluent PBS 10x @pH7.4 with 1mg/mg BSA).

To use, dilute intermediate working solution 100x.

PTEN is a somewhat tricky protein to stain in paraffin sections. The below are some of the key parameters essential for success.

Hints for PTEN Immunohistochemistry:

- Only one antibody works on paraffin sections, and that is 6h2.1. Others stain everything permissively, and yield no nulls. We have successfully used 6h2.1 preparations from Cascade, Dako, and Millipore. All can be used at about the same dilution.
- Antigenicity of slides drops with time after sections are cut. We cut and use slides within 1 week, maximum 2. If you use slides that have been stored for a long time, signal is reduced and background goes up, much like you see with ER staining.
- Antigen retrieval is critical.
- Primary antibody incubation overnight greatly improves reliability. We have tried short (few hours) primary incubations and it sometimes works, sometimes doesn't.
- The “prettiness” of our sections showing protein deficient glands (nulls) is often mentioned. A very big part of achieving an optimal contrast between stained and unstained areas is having good matching of the detection system (DAB, brown) with counterstain (methyl green). I have seen many H&E counterstained preps where the null glands are darkly stained by H&E and thus fail to show. We tried Toluidine blue once, and failed, because it somehow washed out much of the signal. Try methyl green.
- Rapid tissue fixation is important, and probably the reason that biopsies work much better than hysterectomies that sit around for a few hours before fixation.

B. Buffers

- Citrate buffer, pH 6.4. Measure 2.94g of sodium citrate (Sigma S-4641) into 1 liter flask. Dissolve in 970 mL of di water. Adjust pH to 6.4 with HCl
- PBS, pH 7.4 without Mg or Ca (GibcoBRL cat.# 70011-044) dilute 10X PBS stock 1 part : 9 parts di water
Gibco PBS is as follows:

| GIBCO PBS pH7.4, 10x | MW | Concentration (mg/L) | Molarity (mM) |
|--|-----------|-----------------------------|----------------------|
| Potassium Phosphate monobasic (KH ₂ PO ₄) | 136 | 1440 | 10.59 |
| Sodium Chloride (NaCl) | 58 | 90000 | 1551.72 |
| Sodium Phosphate dibasic (Na ₂ HPO ₄ -7H ₂ O) | 268 | 7950 | 29.66 |

- primary Antibody Diluent (See table below):
PBS, pH7.4, with 0.88% NaCl and 0.10 % BSA mixed as below. May be stored at 4°C up to one week.

| 1' Ab Diluent | Stock | 1x | vol stock for 10ml 1x | vol stock for 50ml 1x |
|---------------|-------------|-----------|-----------------------|-----------------------|
| PBS, pH 7.4 | 10x (above) | 1x | 1000 ul | 5ml |
| BSA, pentax V | 10% | 0.10% | 100ul | 500ul |
| Water | -- | to volume | 8.9ml | 44.5 |
| Total | | | 10ml | 50ml |

- C. Xylene
- D. 100% Ethanol
- E. 95% Ethanol
- F. 70% Ethanol
- G. 3% hydrogen peroxide in methanol (20 mL H₂O₂ + 180 mL methanol)
- H. Protein Block (DAKO cat.# X0909)
- I. ABC Elite Kit (mouse IgG) (Vector Labs cat.# pk6102)
- J. Superfrost Plus Slides, (Fisher cat.# 12-550-10)
- K. Copper (II) Sulfate, hydrate 98% (Aldrich, cat.#209201-1kg)
- L. Liquid DAB-Plus Substrate Kit (Zymed Labs; cat.# 00-2020)
- M. Methyl Green; ready to use (DAKO cat.# S1962)
- N. PermOUNT (Fisher cat.# SP15-100)

II. Procedure

- A.** Cut tissue section 4-5 μm thick, transfer section into a room temp water bath, and transfer section into a 48° C waterbath and adhere to slide (Superfrost Plus)
- Bake slides at 37° C overnight to dry sections
 - Bake tissue sections in oven at 60° C for 2 hours
 - Slides must be used within 4 days
 - Most cases require: H&E stained section, PTEN IMPOX, negative control.
 - Label slides appropriately (i.e. PTEN 1:300, or H&E)
 - Place slides in plastic (25-slide capacity) vertical carrier. Next five steps occur in a 250mL vertical bin in hood
- B.** Deparaffinize and rehydrate tissue sections using a xylene and ethanol series:
1. Xylene: x 3 at 5 minutes each
 2. 100% EtOH: x 2 at 5 minutes each
 3. 95% EtOH: x 1 at 5 minutes
 4. 70% EtOH: x1 at 5 minutes
 5. dH₂O: x 2 at 5 minutes each
- *****note: H&E slides are either left in water or pulled for staining at this point
- C.** Antigen retrieval (microwave): slides are to be evenly spaced in the vertical carrier (green bin) for microwaving session
- Microwave antigen retrieval:
1. Place slides in 1000mL citrate buffer, pH 6.4 at room temp.
 2. Place 1L beaker with suspended rack in microwave with probe inserted through the lid
 3. Set microwave at 199°F (~ 93° C), holding temperature
 4. Microwave slides at 199°F for 30 minutes, without opening door, agitating or replacing buffer.
- D.** Remove bin from microwave, and cool in a water bath for 15 minutes. Place rack into dH₂O for 5 minutes, then in 1 x PBS for 5 minutes.
- E.** Quenching of endogenous peroxides (25mL of 30% H₂O₂ +225mL MeOH)
1. Treat sections with 3% H₂O₂ in methanol for 10 minutes
 2. Place in tap water bath for 5 minutes to rinse
- F.** Remove slides from rack, dry around tissue sections with a kimwipe, and ring tissue with a pap pen smear (Zymed labs). Tissue must not dry out. Place each slide in a humidity chamber as it is prepared.

G. Blocking:

1. Cover tissue sections with Protein Block (DAKO X0909) (~3-4 drops)
2. Incubate slides for at least 10 minutes in humid chamber, room temp.

*Alternative Protocol to use of Protein Block in this step G:
Precoat Slides for 20 minutes with normal serum of species of secondary Ab.*

Option 1: In Vectastain ABC kit: add 3 drops of blocking serum (normal serum) to 10 ml of I'Ab buffer missing I'Ab.

Option 2: Dilute serum 1:100 in I'Ab buffer missing I'Ab.

H. Primary antibody incubation:

Overnight at 4° C with slides in a humid chamber

****Note:** primary antibody is applied individually to each slide after decanting and tapping edge of slide to remove excess blocking reagent.

- 1) Place stock Ab on ice
- 2) Mix and dilute Ab using antibody Diluent as indicated depending on the Ab
- 3) Decant excess liquid on slide and add 250ul of Ab (negative controls with 250ul of 1 x PBS pH 7.4
- 3) Place slides in humid chamber
- 4) Incubate overnight at 4° C (alternative incubation is 1hr at R.T. in humid chamber)

I. Wash of primary antibody

- It is crucial to separate the groups (- Cont, Ab1, Ab2, etc.) during the initial work to avoid cross contamination. For this reason, prepare separate 250mL bins with 1 x PBS pH 7.4 for each slide group.
- Thoroughly wash each slide by dipping 6 x in a 50ml tube of 1 x PBS, pH 7.4 (use a separate tube for each slide group)
- Place all specific Ab slides in separate 250mL PBS filled bins. Repeat for (-) controls.

J. Detection

1. Drain off excess buffer, wipe the back of each slide.
2. Add 250ul of diluted biotinylated IgG secondary antibody (Vector Laboratories cat.# PK6102): 1 drop of concentrated biotinylated IgG in 10 mL 1 x PBS, pH 7.4.
3. Place in humid chamber and incubate for 60 minutes at room temperature.
3. Prepare Vectastain ABC reagent (2 drops of reagent A to 5mL PBS pH 7.4, mix

immediately, then add 2 drops of reagent B to the solution. Mix and allow reagent to sit for at least 30 minutes before use.

4. Wash slides for 5 minutes in 1 x PBS buffer
5. Drain off excess buffer, wipe the back of the slide and apply 250ul of the Vectastain ABC reagent. Incubate for 60 min @ RT in a humid chamber.
6. Remove secondary antibody by placing all slides into two successive bins of PBS for 5 minutes each min.

K. DAB Substrate Chromogen: Liquid DAB-PLUS Substrate Kit (Zymed Laboratories; 00-2020) in HOOD w/ gloves
***Trojanowski, J.Q. et al. J Histochem.Cytochem. 31:1217, 1983.

1. Follow Zymed kit instructions including modified steps below

*Kit preparation:

Add 1 drop solution #1 into 1ml of dH₂O. Invert and vortex.

Add 1 drop of solution #2 and 1 drop solution #3. Invert and vortex. (store away from light until use, prepare within 30 minutes of use)

2. Decant excess buffer from slides and lay out each slide onto tray.
3. Add 250ul DAB Substrate solution to each slide.
4. Incubate for 3 minutes in dark.
5. Drain DAB on slide into beaker, rinse in 3 changes of dH₂O (6 dips each, all slides in rack)

*Treat all DAB containers with bleach before disposal

L. Stain enhance 0.5% CuSO₄ in dH₂O (DAB Enhancer reagent, prepare within 30 minutes of use)

1. Stain slides Copper Sulfate solution (green bin) for 2 minutes.
2. Rinse slides in 3 changes of dH₂O (6 dips per bin)
* CuSO₄ solution must be disposed of with hazardous waste

M. Counterstain (DAKO cat.# S1962, Methyl Green)

Important Note: Methyl Green is applied in an aqueous solution, but is quickly washed out by water and ethanol. Once it gets to Xylene, stain is stable and slides can sit in xylene for longer periods. This requires that all transfers post staining in 100% ethanol are as rapid as possible, with good mixing.

Staining Protocol:

1. Blot excess water from slides, place in Methyl Green to stain for 2 minutes.
2. Dehydration steps: 100% Ethanol x 3 (3 very quick vigorous dips each, with rapid blotting of rack and quick transfer to next step), Xylenes x 3 (5 minutes each)

3. Mount with coverslip while specimens are still wet with xylene using permanent aqueous medium (Fisher; PermOUNT cat.# SP15-100)
4. Slides dry in open overnight.

Alternative Protocol/III: Alternative Protocol: Double staining in One slide

Slides were double stained for endothelial cells (red chromagen) and stromal breakdown (brown chromagen) as follows. Dewaxed rehydrated 5µm paraffin sections underwent microwave antigen retrieval before adding primary anti-HIF1 antibody (AbCam HIF1 alpha antibody) at 1:300 dilution. Primary antibody was incubated overnight at 4°C, washed, incubated with appropriate secondary biotinylated immunoglobulin (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) and signal detected by sequential addition of avidin peroxidase and 3,3'-diaminobenzidine. The second primary antibody (CD31, endothelial cell. Clone JC70A, monoclonal mouse antibody Dako catalog #M0823) was incubated at room temperature for 2 hours, washed, incubated with appropriate secondary biotinylated immunoglobulin and signal detected using an ABC-AP Kit (Cat#AK-5000; Vector Labs, Burlingame, CA) and sequential addition of Liquid Permanent Red Chromagen: (DAKO, Burlingame, CA, Cat K0640). Slides were counterstains with methyl green, airdried, and mounted with PermOUNT.

Example of sequence and reagent list:

Step 1: HIF1. Overnight 4°C incubation. DAB

HIF1, stain for oxidative damage. AbCam HIF1 alpha antibody [H1alpha67 murine monoclonal] (ab1): Use at 1:300.

Detection by DAB:

Dewaxed rehydrated 4µm paraffin sections underwent microwave antigen retrieval before adding primary anti-HIF1 antibody (AbCam HIF1 alpha antibody) at 1:300 dilution. Primary antibody was incubated overnight at 4°C, washed, incubated with appropriate secondary biotinylated immunoglobulin (Vectastain ABC kit, Vector Laboratories, Inc., Burlingame, CA) and signal detected by sequential addition of avidin peroxidase and 3,3'-diaminobenzidine.

Step 2: CD31.

CD31, endothelial cell. Clone JC70A, monoclonal mouse antibody (Dako catalog #M0823), use at 1:50 dilution at room temperature for 2 hour. Cross reacts with some white blood cells (nk cells, myeloid, t-cells, B-cell precursors).

Vectastain ABC-AP Kit (Cat#AK-5000; Vector Labs, Burlingame, CA)

Liquid Permanent Red Chromagen: DAKO (Cat K0640)

Detection by Red Chromagen:

The second primary antibody (CD31, endothelial cell. Clone JC70A, monoclonal mouse antibody Dako catalog #M0823) was incubated at room temperature for 2 hours, washed, incubated with appropriate secondary biotinylated immunoglobulin and signal detected using an ABC-AP Kit (Cat#AK-5000; Vector Labs, Burlingame, CA) and sequential addition of Liquid Permanent Red Chromagen: (DAKO, Burlingame, CA, Cat K0640).

Slides were counterstains with methyl green, airdried, and mounted with PermOUNT.

Notebook recording for IMPOX experiments:

Following is an example of how an experiment might be recorded. Include all essential elements for each experiment.

Slide labeling should be permanent and allow matching of each slide to detailed notebook notes keyed by date. Minimal slide labeling requirements includes: 1)Block ID, 2)experiment date; 3)primary antibody used; 4)level number.

EXAMPLE:

PTEN training IMPOX run: 2blocks, 3 antibodies 7/7/2004

GOALS:

Run multiple blocks in duplicate for 3 ab's with neg control

TISSUES: paraffin blocks - cut @5um 7/1/2004, baked 24hr 37°C, 2hr 60°C

| | | |
|--------|-------|-----|
| Case | Block | Dx |
| 94-398 | a | PE |
| 99-023 | 3c | EIN |

ANTIBODIES:

| | | |
|--------|-------|------------------|
| Target | Ab | Working Dilution |
| PTEN | 6h2.1 | 1:100 |
| ER | ERID5 | 1:300 |
| PR | IA6 | 1:100 |

DESIGN: Slide levels as noted

| | 6h2.1 | ERID5 | IA6 | none (-) | Total # |
|---------------------|--------------|--------------|--------------|-------------|---------|
| 94-398 | slide 3/7 | 4/8 | 5/9 | 6 | 7 |
| 99-023 | 1/5 | 2/6 | 3/7 | 4 | 7 |
| Totals | 4 | 4 | 4 | 2 | 14 |
| Vol 1'Ab mix needed | 5x250-1250ul | 5x250-1250ul | 5x250-1250ul | 3x250=750ul | |
| 1'ab buffer | 1250 ul | 1250ul | 1250ul | 750ul | |
| vol 1'Ab(vol/dil) | 12.5ul | 4.2ul | 12.5ul | 0ul | |

PROTOCOL:

Slides hydrated
 Ag. retrieval in MW 93°C, 30 minutes
 Pre-incubated with powerblock.
 1' Ab incubation O/N 4°C
 IN:2pm 7/7/04
 OUT: 10am 7/8/04
 Washed, 2' detection with anti-mouse Vectastain Kit
 Methyl Green counter, then dehydrated and coverslipped

RESULTS:

Reviewed 7/12/04 with GM
 Controls worked. Replicates OK.