Best Practices For Hormone Receptor Testing by Immunohistochemistry

David J. Dabbs, M.D.
Professor of Pathology
University of Pittsburgh School of Medicine
Chief of Pathology, Magee-Women's Hospital
Pittsburgh,Pennsylvania, USA

- An ad-hoc group of pathologists, laboratory scientists and technical experts, representing academia, community hospitals, industry and reference laboratories, conducted a full day consensus meeting (Santa Barbara, CA; January 27, 2008) to discuss these critically important issues in an effort to develop rational evidence-based guidelines for best practices in the assessment of ER by IHC

Consensus Recommendations on Estrogen Receptor Testing in Breast Cancer By Immunohistochemistry

Appl Immunohistochem Molec Morph

In press, 2008

- What steps led up to this meeting?
- What does history tell us?

- Schinzinger (1889) suggested “endocrine abalation” in treatment of breast cancer.
- Beatson (1896) performed the first operation to remove ovaries in a patient with inoperable breast cancer. “8 months after the operation the disease had disappeared”.
- Boyd (1900) 54 patients, 35% complete remission of disease.

Jensen and Jacobsen 1960

- Radioisotpic (“radioactive”) estrogen accumulates in target tissues-pituitary gland, vagina, uterus.
- Radioisotopes were found in the cytoplasm and nucleus of target cells.
- Suggest that ablation of the pituitary or adrenal gland may be a treatment to eliminate sources of estrogen.

…next several decades

- Should removal of ovaries in patients with breast cancer be prophylactic, or therapeutic, based on advanced stage?

“Prelude to ER Testing”

- Lewison EF. “Castration in the treatment of advanced breast cancer” Cancer 1965;18:1558-62.
- Sander S. The in vitro uptake of estradiol in biopsies from 25 breast cancer patients. Acta Pathol et Microbiol Scand 1968;74: 301-302.
- Korenman et al J Clin Endocrinol Metab Specific Estrogen Binding of the Cytoplasm of Human Breast Carcinoma 1970;30:639-45

Dextran-Coated Charcoal/Ligand Binding Method

- Principle: measurement of available cytoplasmic estrogen receptor binding proteins (ERBP), measured as a fraction of the total sample protein content.

DCC/LB..the steps

- Homegenate of tissue-centrifuge and isolate “cytosol”
- Cytosol total protein measured
- Sucrose density gradient fractionates the cytosol
- exposed to tritiated (radioisotopic) estrogen, binds to ER.
- DCC removes unbound estrogen
- Scintillation counting.
- Exposure to estrogen to determine “nonspecific binding”
- Final result expressed in “femtomoles/mg cytosol protein”
- Femto= ten to the minus 15. (.000000000000001)

Ferherty PG et al Br J Cancer 1971;25:697-710

- DCC step in the ligand-binding method aids in reducing non-specific ER binding receptors.
- More receptors found in postmenopausal women than premenopausal.
- May have prognostic value for treatment regimens.

Dextran-Coated Charcoal Method/Ligand Binding

- Requires large amount of fresh tissue.
- Immediate freezing of fresh tissue when removed from patient.
- Radioactive reagents.
- Carcinogenic reagents
- Expensive laboratory equipment not usually found in hospitals.

- "Blind sampling". Samples for assay are largely independent of what is examined histologically.
- Tumor-poor cellularity may lead to false negative assay result.
- Non-tumor areas sampled, necrotic areas yield false negative results.
- No direct visualization of assay sample*.

Pertschuck et al Cancer 1978; 41: 907-11
Immunofluorescent Detection of Estrogen Receptors in Breast Cancer

- Using estrogen polymer, labeled with fluorescein.
- Principle: the polymer binds to the estrogen receptor and is localized with a fluoresence microscope.
- Receptors were found in the cytoplasm and nucleus.
- 90% correlation with DCC/LB method.

- Transport expense (on dry ice to reference labs)
- Scatchard plot analysis (binding coefficients)
- QA issues were the same: quality control, test results with "standardized" test specimens.

Pertschuck et al Cancer 1978; 41: 907-11
Immunofluorescent Detection of Estrogen Receptors in Breast Cancer

- "The technique can be performed by the average surgical pathology laboratory"
- "in general, tumors with less than 10% positive cells were negative by DCC/LB, and those with 11-20% positive were borderline by DCC/LB".

Antoniades et al. Am J Clin Pathol 1979;71: 497-503.
Correlation of Estrogen Receptor Levels with Histology and Cytomorphology in Human Mammary Cancer.

- Histology/cytologic criteria from NSABP.
- Strong correlation with better differentiated tumors, especially tubular and lobular cancers.

Hasson et al Cancer 1981;47: 138-39.
Comparison of Estrogen Receptor Levels in Breast Cancer Samples from Mastectomy and Frozen Tissue Samples.

- Devitalized tissue may yield false negative results:
- Comparison of fresh frozen tissue and the subsequent mastectomy specimen.
- Markedly lower DCC/LB results in mastectomy sample.
- A low expressor may become falsely negative.

Eusebi et al Tumori 1981;67:315-23
A Two Stage Method for Estrogen Receptor Analysis:
Correlation with Morphologic Parameters of Breast Carcinoma

- Enhanced sensitivity over direct methods.
- Nuclear expression dominates.
- Correlates well with morphology; better-differentiated tumors are “estrogen rich”.

Shimada et el. Proc Natl Acad Sci 1985; 82:483-7.

- Immunocytochemical staining of estrogen receptor in paraffin sections of breast cancer by use of monoclonal antibodies: Comparison with frozen sections.
- Frozen, paraffin (IP and ABC methods)
- All correlate well with DCC/LB

McCarty et al Estrogen Receptor Analysis:Correlation of Immunohistochemical and Biochemical Methods 1985;Arch Pathol Lab Med 109:716-21

- Use of the “H Score” (Histochemical).
- The sum of proportion of cells with nuclear staining times the intensity of staining (graded 0-4).
- Frozen tissue with antibody H22 (Abbot).
- Quantitative comparison with biochemical method, sensitivity 93%, specificity 89% based on clinical outcome.

Berger et al. Comparison of ICA for PR with Biochemical Method. 1989; 49: 5176-9.

- First introduction of antibody to PR.
- Allowed for routine analysis of PR.
- PR was not routinely done with the DCC method because it required substantial tissue.

Quality Issues with DCC/Ligand Binding Method: Thorpe SM Breast Cancer Res Treat 1987;9: 175-89

- Biopsy Composition/inability to distinguish normal from tumor
- Homegenization
- Incubation time
- Adsorption of ligand-free surfaces
- Adsorption of free steroid by DCC
- Adsorption of cytosol protein by DCC
- Scintillation counting

PR is an independent prognostic factor and ER+/PR+ patients respond better as a group (70% responders) to endocrine therapy

- Thorpe S, et al. Breast Cancer Res Treat 1986;7:91-8.
- McGuire W et al. Semin Oncol 1985;12:12-6.
- McGuire W et al. Cancer 1977;39:2934-47
- Pertschuk L et al. Cancer 1990;66:1663-70.

Shousha T et al. J Clin Pathol 1990;43:239-42

- 60 cases negative for ER by DCC were reassessed by IHC.
- 6 weakly positive (10%)
- 3 moderately positive (5%)-these were tubular & lobular cancers and SHOULD have been DCC positive.

Pertschuk et al ER1D5 in paraffin predicts endocrine response better than ICA or cytosol methods. Cancer 1996;77:2514-9

- Percent of cells staining (10%), exclusive of intensity.

The Call to Standardize HR by IHC

- Esteban et al. J Cell Biochem Suppl 1994 19:138-42
- Pertschuk et al J Cell Biochem Suppl 1994;19:134-7.

Adjuvant Therapy for Breast Cancer National Institutes of Health Consensus Development Conference Statement November 1-3, 2000.

- The decision whether to recommend adjuvant hormonal therapy should be based on the presence of hormone receptors, as assessed by immunohistochemical staining of breast cancer tissue.

Adjuvant Therapy for Breast Cancer National Institutes of Health Consensus Development Conference Statement November 1-3, 2000.

- Adjuvant hormonal therapy should be recommended to women whose breast tumors contain hormone receptor protein, regardless of age, menopausal status, involvement of axillary lymph nodes, or tumor size. While the likelihood of benefit correlates with the amount of hormone receptor protein in tumor cells, patients with any extent of hormone receptor in their tumor cells may still benefit from hormonal therapy.
- Hormonal adjuvant therapy should not be recommended to women whose breast cancers do not express hormone receptor protein.

Recommendations for Improved Standardization in Immunohistochemistry

Appl Immunohistchem Molec Morph

2007; 15:124-33

Fisher ER et al Cancer 2005;103:164-73
Solving the Dilemma of the Immunohistochemical and Other Methods Used for Scoring ER and PR in Patients with Invasive Breast Cancer

- Compared ER/PR results of DCC/IHC, NSABP B-09, 402 patients.
- Any or none; intensity/percent; computer assisted image analysis.
- The presence of any nuclear ER expression is “related significantly to overall survival at 5 and 10 years, regardless of scoring method.

BEST PRACTICES
DIAGNOSTIC IMMUNOHISTOCHEMISTRY

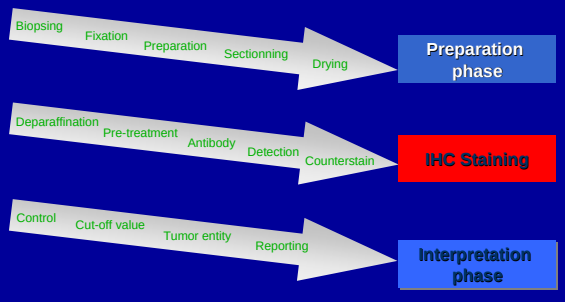
Reference/Regulatory Agencies

- Clinical and Laboratory Standards Institute
- College of American Pathologists
- CLIA 88 (Clinical Laboratory and Improvement Act of 1988)

The Total Test-Immunohistochemistry
Taylor C. Arch Pathol Lab Med 2000; 124:945.

Elements of testing	QA issues	Responsibility
Clinical question;test selection	Indications;stain selection; specimen collection fixation etc	Pathologist;clinician; tecnologists.
Technology/methodology	Reagents;protocols;sensitivity, specificity, qual, prof testing.	Pathologist/technologist
Results:validation/reporting	Criteria (+, -);report content; tat.	Pathologist/tech
Interpretation	Qualifications;prof testing integration of report	Pathologist/clinician

Different procedures intra- and intra laboratory compromise standardization



3^{14} = 4.8 mio procedures (3 choices in 14 steps)

Best Practice IHC: Pre-Analytic

- Tissue Acquisition: Time.
- Tissue Gross Sectioning.
- Tissue Fixative: 10% NBF.
- Tissue Fixation: Minimum/Maximum.
- Tissue Gross Sections for Microtomy 4-5mm thick.

Best Practice IHC; Pre-Analytic

- Tissue Processor: Formalin.
- Change solutions weekly/daily.
- No solutions >37C
- Paraffin 60C
- Remove blocks from paraffin.
- Embedding.

Best Practices IHC: Pre-Analytic Antibody Optimization

- Antibody package insert!
- Dilutions-one above & below insert rec.
- AR low and high pH
- Two detection systems
- For categorical (+/-) results-25 samples, 10 high, 10 intermediate, 5 negative for expression

Best Practices IHC: Pre-Analytic

- Tissues microtomy-4-5 microns
- Tissue adherence to slide (baking) removes water
- Tissue de-waxing in xylene
- Technician competence in IHC

Test Battery Suggested for Screening an Optimal AR Protocol

Buffer	pH 1-2	pH 7-8	pH 10-11
120 C	Slide #1	Slide #4	Slide #7
100 C	Slide # 2	Slide #5	Slide #8
90 C	Slide #3	Slide #6	Slide #9

Best Practices IHC: Pre-Analytic

- New Antibody Lots:
- 3 samples: one high, intermediate/negative.

IHC Procedure

- Primary antibody application to slide
- Secondary antibody/ with detection agent
- Localization (color reaction) developed
- Counterstain and coverslip

Best Practices IHC: Pre-Analytic

- Endogenous peroxidase block step
- Antigen retrieval

Best Practices IHC: Analytic

- Document immunostaining
- What is positive and negative
- Semi-quantitate immunostaining
- Comment on appropriate positive and negative internal controls
- Comment on appropriate positive and negative external controls

Best Practices IHC: Post-Analytic

- Document Fixative and fixation.
- Document antibody source, clone, targeted antigen.
- Metrics-for predictive/prognostic markers.
- Metrics-for comparison to other testing methods-outside labortory reports; Her2 FISH.

Tissue Fixation

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- Communication/coordination with operating room personnel and clinics is essential for proper specimen acquisition.

Recommendation #1

- Breast resection specimens must be sectioned fresh as thin as possible ~0.5cm, placed in fixative as quickly as possible (<1 hour) and the “time in formalin” recorded.
- Tissue sections must be immersed in an adequate volume of fixative (ratio of tissue/fixative = 1:20) within a maximum of one hour from removal.

Recommendation #2

- Breast core biopsies should be fixed and processed in an identical manner to excision specimens.
- Specimen acquisition/time into formalin should be recorded.

- Inclusion of normal tissue with tumor in the same cassette is desirable.

Recommendation #3

- Only 10% aqueous phosphate buffered formaldehyde* pH 7.0 -7.4 (10% phosphate buffered formalin) should be used as the fixative for breast tissue samples.
- *Accrued data on HRT and clinical outcomes have been performed on FFPE tissues.

Alternative Fixatives

- A formal cross validation study requires a minimum of 100 samples that are fixed in both the alternative fixative and 10% neutral phosphate-buffered formalin.

Recommendation # 5

- Alcohol-fixed fine needle aspirations (FNA): If there is a clinical suspicion of breast cancer that may need ER analysis and an FNA is performed, then all efforts should be made to collect a portion of the cytology specimens in formalin.
- Validation required with appropriate alcohol fixed cytology specimens.

Recommendation #4

- The time that the samples spend in 10% phosphate-buffered formalin should be standardized for all breast specimens to help ensure adequate and uniform fixation. Minimum fixation times of at least 8* hours, not to exceed to 72 hours, unless validated by the Medical Director.
- *Avoids alcohol fixation and promotes AR standardization. **Goldstein et al. Am J Clin Pathol 2003;120: 86-92**

Tissue Processing

Recommendation # 6

- Breast cancer specimens should be processed in conventional processors.
- Alternative processors (microwave enhanced) need to be validated by the Medical Director on 100 samples.

Recommendation # 8

- It is strongly recommended that none of the tissue processor solutions, excluding paraffins, should exceed 37C if the processor contains breast tissue for potential ER and other biomarker testing.
- Revalidation is required if significant changes in processor solutions or paraffin types are changed.

Recommendation # 7

- The first formalin container(s) in the tissue processor should always be newly replenished.
- Time in formalin includes the processor exposure to formalin.

Recommendation #9

- Paraffin in tissue processors or embedding centers should not be warmed over 60C, and the tissue should not be kept in heated paraffin for extended periods of time.

Documentation

- Communication/coordination with surgical sites that supply tissues and the rationale for doing this are essential.

Recommendation #10

- It is recommended to include a designated field on the requisition sheet for recording time into formalin, and time out.
- "Time in formalin" can be dictated into a gross description. A surrogate marker of "time out of formalin" is when the processor begins.
- Time of collection recording is encouraged at clinic sites where biopsies are performed. A surrogate marker could be the date of collection.

STANDARDIZING ANALYTICAL VARIABLES IN ER TESTING

- For any IHC reaction to take place, all of its components should be properly functioning, namely the primary antibody, the detection system and the chromogen. A drop of 'sensitivity' of any of these components will lead to an inadequate assay, with potentially false-negative results

Recommendation #11

- IHC estrogen receptor assays should be performed with one of three antibody clones; 1D5, 6F11 and SP1.
- ER assays should be performed by standardized methods; preferably using FDA approved test kits.

- The original biochemical assay and appropriately optimized immunohistochemical assays will show a spectrum of ER or PR content in individual cells.
- For IHC, this means that some cells will be negative, 3+, 2+ and 1+.

Recommendation #12

- Positive and negative test controls should be included with every estrogen receptor IHC batch run.

- Internal positive controls: In this context an internal control consists of tissue (cells) in the same section (or a separate section from the same patient specimen) as the 'test' section.
- External positive control tissues: normal tissue from the same patient or from a different patient.

- Metrics: It is highly desirable to maintain laboratory metrics for each prognostic/predictive test results in order to monitor for potential analytical drift. For example, published literature indicates that 70 to 80 percent of breast cancers are ER positive. This should be a benchmark for each laboratory to monitor.

- Other external positive controls: Other sources of ER positive control tissue are benign gynecologic tissues, such as endomyometrium, cervica/endocervix and ovarian tissue.

STANDARDIZING INTERPRETATION OF ER ASSAYS

- the NIH Consensus Statement, published in 2001, recommended “any nuclear ER” as allowing a patient to be eligible for endocrine therapy.

- Cheang et al using SP1 clone and compared it to clone 1D5: 1% positive cells cutoff for a positive result. (4105 pts)

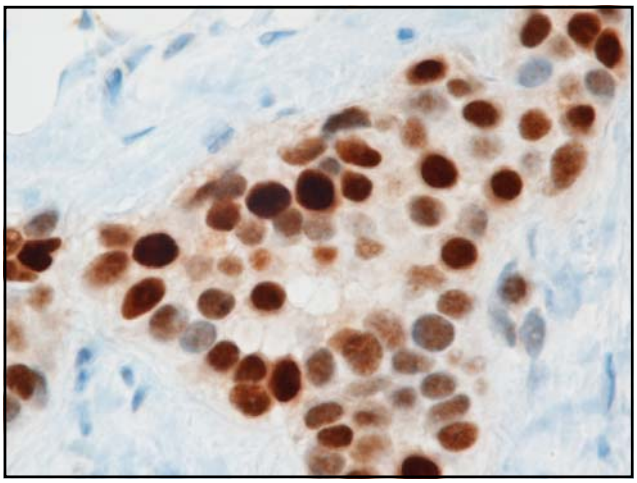
- Harvey et al (1999), these investigators, using the 6-F11 clone for ER IHC recorded the proportion of positive cells and the intensity of staining (Allred score) and correlated the results with clinical outcomes in a large cohort of breast cancer patients treated with adjuvant tamoxifen. (1982 pts)

Recommendation #13

- A commonly employed threshold for positive results for ER IHC assays in term of the potential benefit from adjuvant endocrine therapy is one percent positive tumor cells with a 1+ or greater signal.

Recommendation #14

- The interpretation of ER assays should include an evaluation of both the percentage of positive tumor cell nuclei and the intensity of the staining reaction.



Recommendation #15

signal (__%), 1+ (__%), 2+ (__%), 3+ (__%)

- The IHC assay for ER should be optimized so that the staining can capture this dynamic range in terms of the distribution and intensity of staining, and the level of expression should therefore be a part of the interpretative results of these tests

- negative ER results on needle core biopsies should be repeated on the surgical excision.