Template for Reporting Results of Biomarker Testing of Specimens from Patients with Carcinoma of the Breast

Version: 1.4.1.1
Protocol Posting Date: November 2021
This biomarker template is not required for accreditation purposes but may be used to facilitate compliance with CAP Accreditation Program Requirements

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With guidance from the CAP Cancer and CAP Pathology Electronic Reporting Committees.
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Accreditation Requirements
Completion of the template is the responsibility of the laboratory performing the biomarker testing and/or providing the interpretation. When both testing and interpretation are performed elsewhere (eg, a reference laboratory), synoptic reporting of the results by the laboratory submitting the tissue for testing is also encouraged to ensure that all information is included in the patient's medical record and thus readily available to the treating clinical team.

Summary of Changes

v 1.4.1.1
- Repositioned Note under HER2 by in situ Hybridization

Reporting Template

Protocol Posting Date: November 2021
Select a single response unless otherwise indicated.

CASE SUMMARY: (Breast Biomarker Reporting Template)
Includes interpretative content from the CAP/ASCO HER2 Guidelines (2018)
Completion of the template is the responsibility of the laboratory performing the biomarker testing and / or providing the interpretation. When both testing and interpretation are performed elsewhere (e.g., a reference laboratory), synoptic reporting of the results by the laboratory submitting the tissue for testing is also encouraged to ensure that all information is included in the patient's medical record and thus readily available to the treating clinical team.
Core data elements in this template comply with the CAP Accreditation requirements for HER2 and hormone receptor testing. Core data elements should be reported only for tests performed. If some studies were performed on different specimen(s), the specimen number(s) should be provided.

TEST(S) PERFORMED

Test(s) Performed (Note A) (select all that apply)
___ Estrogen Receptor (ER) Status (Note B)
Estrogen Receptor (ER) Status
___ Positive (greater than 10% of cells demonstrate nuclear positivity)#
Percentage of Cells with Nuclear Positivity#
___ Specify %: _____ %
--OR--
Select range below:
___ 11-20%
___ 21-30%
___ 31-40%
___ 41-50%
___ 51-60%
___ 61-70%
___ 71-80%
___ 81-90%
___ 91-100%
Average Intensity of Staining
___ Weak
___ Moderate
___ Strong
___ Low Positive (1-10% of cells with nuclear positivity)##
+Specify Percentage of Cells with Nuclear Positivity: _____ %
Average Intensity of Staining
___ Weak
___ Moderate
___ Strong
Status of Internal Controls
___ Internal control cells present and stain as expected
___ Internal control cells absent###
___ Other (specify): _____
___ Negative (less than 1%)
___ Internal control cells present and stain as expected

___ Internal control cells absent###
___ Other (specify): _____
___ Cannot be determined (indeterminate)####
___ Internal control cells present; no immunoreactivity of either tumor cells or internal controls
___ Other (specify): _____

 # Percentage of cells with nuclear positivity for ER may be reported as a specific number or a range if more than 10%.
Invasive carcinoma cases with 1 to 10% of cells staining for ER (not PgR) are reported as "Low Positive" and the following report comment is recommended: "The cancer in this sample has a low level (1-10%) of ER expression by IHC. There are limited data on the overall benefit of endocrine therapies for patients with low level (1-10%) ER expression but they currently suggest possible benefit, so patients are considered eligible for endocrine treatment. There are data that suggest invasive cancers with these results are heterogeneous in both behavior and biology and often have gene expression profiles more similar to ER negative cancers." The Low Positive designation applies only to invasive carcinoma, and is not used for Progesterone receptor or DCIS.
For cases in which no internal controls are present and the ER result is either negative or Low Positive, the following report comment is recommended: "No internal controls are present, but external controls are appropriately positive. If needed, testing another specimen that contains internal controls may be warranted for confirmation of ER status." When a tumor is negative but no internal control cells are present, the pathologist must exercise judgment as to whether the assay can be interpreted as a true negative. This should include consideration of histologic type and grade, cold ischemia and fixation times, and the status of external controls. If the pathologist decides that hormone receptor status cannot be determined, the test should be reported as such and repeated on another block or specimen.
Technical issues prevent the test from being reported as positive, negative, or equivocal. This may occur if specimen handling was inadequate, if artifacts (crush or edge artifacts) make interpretation difficult, or if the analytic testing failed.

Test Type
___ Food and Drug Administration (FDA) cleared (specify test / vendor): _____
___ Laboratory-developed test
___ +Non-U.S.-based health systems
___ Health Canada Approved (specify test / vendor): _____
___ Other (specify): _____

Primary Antibody
___ SP1
___ 6F11
___ 1D5
___ Other (specify): _____

+Scoring System
___ No separate scoring system used
___ Allred
+Proportion Score: _____
+Intensity Score: _____
+Total Allred Score: _____
___ Other scoring system
+Specify System: _____
+Specify Score Result: _____

___ Progesterone Receptor (PgR) Status (Note B)

Progesterone Receptor (PgR) Status
___ Positive#

Percentage of Cells with Nuclear Positivity
___ Specify %: _____ %
--OR--
Select range below:
___ 1-10% (specify)#: _____ %
___ 11-20%
___ 21-30%
___ 31-40%

___ 41-50%
___ 51-60%
___ 61-70%
___ 71-80%
___ 81-90%
___ 91-100%

Average Intensity of Staining

___ Weak
___ Moderate
___ Strong

___ Negative (less than 1%)
___ Internal control cells present and stain as expected
___ Internal control cells absent##
___ Other (specify): _____

___ Cannot be determined (indeterminate)###
___ Internal control cells present; no immunoreactivity of either tumor cells or internal controls
___ Other (specify): _____

 # Percentage of cells with nuclear positivity may be reported as a specific number or a range if more than 10%.
When a tumor is negative but no internal control cells are present, the pathologist must exercise judgment as to whether the assay can be interpreted as a true negative. This should include consideration of histologic type and grade, cold ischemia and fixation times, and the status of external controls. If the pathologist decides that hormone receptor status cannot be determined, the test should be reported as such and repeated on another block or specimen.
Technical issues prevent the test from being reported as positive, negative, or equivocal. This may occur if specimen handling was inadequate, if artifacts (crush or edge artifacts) make interpretation difficult, or if the analytic testing failed.

Test Type

___ Food and Drug Administration (FDA) cleared (specify test / vendor): _____
___ Laboratory-developed test
___ +Non-U.S.-based health systems
___ Health Canada Approved (specify test / vendor): _____
___ Other (specify): _____

Primary Antibody

___ 1E2
___ 636
___ 16
___ SP2
___ 1A6
___ 1294
___ 312
___ Other (specify): _____

+Scoring System

___ No separate scoring system used
___ Allred
+Proportion Score: _____
+Intensity Score: _____
+Total Allred Score: _____
___ Other scoring system
+Specify System: _____
+Specify Score Result: _____

___ HER2 by Immunohistochemistry (Note C)

HER2 by Immunohistochemistry

___ Negative (Score 0)
___ Negative (Score 1+)
___ Equivocal (Score 2+)
Percentage of Cells with Uniform Intense Complete Membrane Staining
___ Specify percentage: _____ %
___ Other (specify): _____
___ Cannot be determined
___ Positive (Score 3+)
Percentage of Cells with Uniform Intense Complete Membrane Staining
___ Specify percentage: _____ %
___ Other (specify): _____
___ Cannot be determined
___ Cannot be determined (indeterminate) (explain)
Test Type
___ Food and Drug Administration (FDA) cleared (specify test / vendor): _____
___ Laboratory-developed test
___ +Non-U.S.-based health systems
___ Health Canada Approved (specify test / vendor): _____
___ Other (specify): _____
Primary Antibody
___ 4B5
___ HercepTest
___ A0485
___ SP3
___ CB11
___ Other (specify): _____
___ HER2 by in situ Hybridization (Note C)
HER2 by in situ Hybridization
"Number of Observers" and "Number of Invasive Tumor Cells Counted" are required only when Negative or Positive is selected.
___ Negative (not amplified)
___ Positive (amplified)
___ Cannot be determined (indeterminate) (explain)
___ Not performed
___ Pending
Number of Observers: _____
Number of Invasive Tumor Cells Counted: _____ cells
Method (select all that apply)
___ Dual probe assay
Average Number of HER2 Signals per Cell: _____
Average Number of CEP17 Signals per Cell: _____
HER2 / CEP17 Ratio: _____
___ Single probe assay
Average Number of HER2 Signals per Cell: _____
+Aneusomy (as defined by vendor kit used)
___ Not identified
___ Present (explain): _____
+Heterogeneous Signals
___ Not identified
___ Present

+Percentage of Cells with Amplified HER2 Signals
___ Specify percentage: _____ %
___ Other (specify): _____
___ Cannot be determined

Test Type
___ Food and Drug Administration (FDA) cleared (specify test / vendor): _____
___ Laboratory-developed test
___ +Non-U.S.-based health systems
___ Health Canada Approved (specify test / vendor): _____
___ Other (specify): _____

___ +Ki-67 (Note D)
+Specify Percentage of Cells with Nuclear Positivity: _____ %
+Primary Antibody
___ MIB1
___ SP6
___ MM1
___ 30-9
___ IR / IS626
___ Other (specify): _____

___ +Multiparameter Gene Expression / Protein Expression Assay (Note E)
+Name of Assay: _____
+Results
___ Low risk
___ Moderate risk
___ High risk
+Recurrence Score: _____
+Other Result (explain): _____

Cold Ischemia and Fixation Times
___ Meet requirements specified in latest version of the ASCO / CAP Guidelines
___ Do not meet requirements specified in latest version of the ASCO / CAP Guidelines
___ Cannot be determined (explain): _____

+Cold Ischemia Time (minutes): _____ min

+Fixation Time (hours): _____ hours

+Testing Performed on Block Number(s) (specify): _____

METHODS

+Fixative
___ Formalin
___ Other (specify): _____

+Image Analysis
___ Not performed
___ Performed
+Specify Method: _____

+Biomarkers Scored by Image Analysis (select all that apply)

___ ER
___ PgR
___ HER2 by IHC
___ HER2 by ISH
___ Ki-67
___ Other (specify): _____

COMMENTS

Comment(s): _____

Explanatory Notes

A. Results

It is recommended that hormone receptor and HER2 testing be done on all primary invasive breast carcinomas and on recurrent or metastatic tumors.[1,2,3,4] If hormone receptors and HER2 are both negative on a core biopsy, repeat testing on a subsequent specimen should be considered, particularly when the results are discordant with the histopathologic findings. When multiple invasive foci are present, the largest invasive focus should be tested. Testing smaller invasive carcinomas is also recommended if they are of different histologic type or higher grade. Other biomarker tests (eg, Ki-67 or multigene expression assays) are optional and are not currently recommended for all carcinomas. Fresh tissue should not be used for special studies (eg, RNA expression profiling or investigational studies) unless the invasive carcinoma is of sufficient size that histologic evaluation and ER, PgR, and HER2 assessment will not be compromised.

Guidelines published by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) require recording specific pre-analytic and analytic variables that can affect test results.[5,6] Such variables include:

Cold ischemia time (time between tissue removal and initiation of fixation) *and* time of fixation. Alternatively, laboratories may record the time the specimen was removed from the patient and the time the specimen was placed in formalin. Both the time the tissue is removed from the patient and the time it is placed in fixative must be communicated to the processing laboratory. These times are used to determine if the specimen meets requirements specified in latest version of the ASCO/CAP guidelines for cold ischemia time and fixation time. Reporting these times in the pathology report is optional.

- Type of fixative, if other than buffered formalin
- Treatment of the tissue that could potentially alter immunoreactivity (eg, decalcification)[7]
- Status of controls:
    - Internal – normal epithelial cells positive or negative for ER and PgR
    - External – type and expected level of expression
- Adequacy of sample for evaluation
- Primary antibody clone
- Regulatory status (FDA cleared versus laboratory-developed test)

Information regarding assay validation or verification should be available in the laboratory. Any deviation(s) from the laboratory's validated methods should be recorded. Appropriate positive and negative controls should be used and evaluated.

References

1. National Comprehensive Cancer Network (NCCN) Clinical Practice Guideline in Oncology, Version 3.2017. www.nccn.org/professionals/physician_gls/PDF/breast.pdf. Accessed December 18, 2017.
2. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol.* 2007;25(33):1-26.
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4. Arslan C, Sari E, Aksoy S, Altundag K. Variation in hormone receptor and HER-2 status between primary and metastatic breast cancer: review of the literature. *Expert Opin Ther Targets*. 2011;15(1):21-30.
5. Allison KH, Hammond MEH, Dowsett M, et al. Estrogen and progesterone receptor testing in breast cancer: ASCO/CAP guideline update. *Arch Pathol Lab Med* doi: 10.5858/arpa.2019-0904-SA.

6. Wolff AC, Hammond MEH, Allison KH, et al. HER2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline focused update. *Arch Pathol Lab Med*. 2018;142(11):1364-1382.
7. Arber JM, Arber DA, Jenkins KA, Battifora H. Effect of decalcification and fixation in paraffin-section immunohistochemistry. *Appl Immunohistochem.* 1996;4:241-248.

B. Estrogen Receptor and Progesterone Receptor Testing

Scientific rationale: Normal breast epithelial cells have receptors for estrogen and progesterone and proliferate under their influence. Most breast carcinomas also express these receptors and may be stimulated to grow by these hormones. Removal of endogenous hormones by oophorectomy or blocking hormonal action pharmaceutically (eg, with tamoxifen or aromatase inhibitors) can slow or prevent tumor growth and prolong survival.

Clinical rationale: Hormone receptor status is determined primarily to identify patients who may benefit from hormonal therapy.[1] About 75% to 80% of invasive breast cancers are positive for ER and PgR, including almost all well-differentiated cancers and most moderately differentiated cancers, and studies have shown a substantial survival benefit from endocrine therapy among patients with ER-positive tumors.[2] True ER-negative, PgR-positive carcinomas are extremely rare, but patients with such tumors are also considered eligible for hormonal therapy. Receptor status is only a weak prognostic factor.

Method: Hormone receptor status is most often determined in formalin-fixed, paraffin-embedded tissue sections by immunohistochemistry (IHC). Only nuclear staining is considered positive. Use of single-gene expression assays are not recommended for routine use.

Quality assurance: There are many tissue and technical variables that can affect test results,[2,3,4,5] and the assays must be validated to ensure their accuracy.[6] External proficiency testing surveys for ER and PgR are invaluable tools to help ensure that assays perform as expected, and they are available from the CAP and other organizations.

False-negative results: Failure to detect ER or PgR is the greatest problem with this assay because patients may not receive effective therapy. This may occur if specimen handling was inadequate, if artifacts (crush or edge artifacts) make interpretation difficult, or if the analytic testing failed. To avoid false-negative results, appropriate internal and external controls should be positive. *When a tumor is negative (non-immunoreactive), it is essential that the internal control cells be assessed to ensure that they show positive staining (as expected). If the internal controls are also negative, the test should not be reported as negative but should be considered indeterminate ("Cannot be determined").[2] The test should be repeated on another block or specimen.*

When a tumor is negative but no internal control cells are present in the test section, the pathologist must exercise judgment as to whether the assay can be interpreted as a true negative. This should include consideration of histologic type and grade, cold ischemia and fixation times, and the status of external controls. If the pathologist decides that hormone receptor status cannot be determined, the test should be reported as such and repeated or performed on another block or specimen.

Reasons for false-negative results include the following:

- Exposure of tumor cells to heat (eg, carcinomas transected by using cautery during surgery)
- Prolonged cold ischemic time, which may result in antigenic degradation. One hour or less is preferable[7,8]
- Under or overfixation; fixation for at least 6 hours in buffered formalin is recommended,[2] and prolonged fixation can also diminish immunoreactivity[5,9]

- Type of fixative: ER is degraded in acidic fixatives such as Bouin's and B-5; formalin should be buffered to ensure pH range between 7.0 and 7.4
- Decalcification, which may result in loss of immunoreactivity[10]
- Nonoptimized antigen retrieval
- Type of antibody
- Dark hematoxylin counterstain obscuring faintly positive diaminobenzidine (DAB) staining

False-positive results: False-positive results occur less frequently.[11] Rare reasons would be the use of an impure antibody that cross-reacts with another antigen or misinterpretation of entrapped normal cells or an in situ component as invasive carcinoma. False-positive tests can also be generated by image analysis devices that mistakenly count overstained nuclei. It has been suggested that highly sensitive assays may detect very low levels of ER in cancers that will not respond to hormonal therapy, but that has not been proven by a clinical trial.

False-negative and false-positive results can be reduced by paying attention to the following:

- Staining of normal breast epithelial cells. Normal epithelial cells serve as a positive internal control and should always be assessed. If the normal cells are negative, repeat studies on the same specimen or on a different specimen should be considered. If normal cells are not present (eg, core biopsy) and the test results are negative, testing should be repeated on another block or subsequent specimen.
- External controls (must stain as expected). These controls help ensure that the reagents have been appropriately dispensed onto the slide with the clinical sample.
- Correlation with histologic type and grade of the cancer. The study should be repeated if the results are discordant (eg, ER-negative low-grade carcinoma).

Reporting guidelines: ASCO and the CAP have issued recommendations for reporting the results of immunohistochemical assays for ER and PgR (Table 1).[2] Studies using both IHC and the ligand binding assay suggest that patients with higher hormone receptor levels have a higher probability of response to hormonal therapy, but expression as low as 1% positive staining has been associated with clinical response. As a result, the guidelines recommend classifying all cases with at least 1% positive cells as receptor positive.[2] For patients with low ER expression (1% to 10% weakly positive cells), the decision on endocrine therapy should be based on an analysis of its risks and potential benefits.

Table 1. Reporting Results of Estrogen Receptor (ER) and Progesterone Receptor (PgR) Testing

Result	Criteria	Comments
Positive	Immunoreactive tumor cells present (≥1%)	Invasive carcinomas with 1 to 10% of cells staining for ER (not PgR) are reported as "Low Positive" and the following report comment is recommended: "The cancer in this sample has a low level (1-10%) of ER expression by IHC. There are limited data on the overall benefit of endocrine therapies for patients with low level (1-10%) ER expression but they currently suggest possible benefit, so patients are considered eligible for endocrine treatment. There are data that suggest invasive cancers with these results are heterogeneous in both behavior and biology and often have gene expression profiles more similar to ER negative cancers." The Low Positive designation applies only to invasive carcinoma, and is not used for Progesterone receptor or DCIS.
Negative	<1% immunoreactive tumor cells present	

Definition of a negative result: The ASCO/CAP guidelines recommend that carcinomas with <1% positive cells be considered negative for ER and PgR.[2] In the Allred system (see Table 2), the survival of patients whose carcinomas had a score of 2 (corresponding to <1% weakly positive cells) was similar to that of patients whose carcinomas were completely negative for ER.[9] Therefore, a score of 2 was considered to be a negative result. Carcinomas with <1% positive cells and intensity scores of 2 or 3 would have a total score of 3 or 4 and be considered positive. These are rare carcinomas, and their response to hormonal therapy has not been specifically studied.

Quantification of ER and PgR: There is a wide range of receptor levels in cancers as shown by the biochemical ligand binding assay and as observed with IHC. Patients whose carcinomas have higher levels have improved survival when treated with hormonal therapy.[9,11] Quantification systems may use only the proportion of positive cells or may include the intensity of immunoreactivity:

- Number of positive cells: The number of positive cells can be reported as a percentage or within discrete categories (figure 1 below).
- Intensity: Refers to degree of nuclear positivity (ie, pale to dark). The intensity can be affected by the amount of protein present, as well as the antibody used and the antigen retrieval system. In most cancers, there is heterogeneous immunoreactivity with pale to darkly positive cells present.

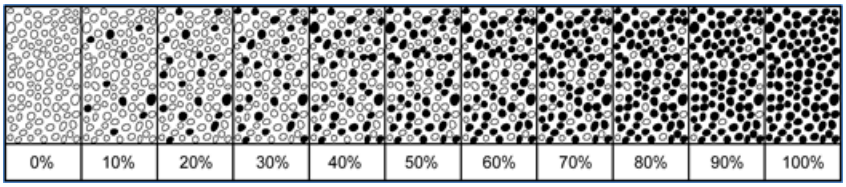

Figure 1. Quantification of Immunohistochemical Findings. The percentage of positive cells can be visually estimated.

Two methods of quantifying ER by using both intensity and percentage of positive cells are the Allred score[9] (Table 2) and the H score[12] (Table 3). The 2 systems classify carcinomas into similar, but not identical, groups.[13] If high-affinity antibodies are used with sensitive detection systems, most carcinomas will fall into clearly positive (score 7 or 8) or clearly negative (score 0) categories by Allred score.[14,15] A small group of carcinomas (<1% of total) show intermediate levels of immunoreactivity.

Quantitation can also be performed by using the proportion of positive cells. In one study, carcinomas were scored as 0 (<1% positive), 1 (1% to 25% positive), 2 (>25% to 75% positive), and 3 (>75% positive).[16] The same results were obtained when scored by visual analysis or by image analysis. The proportion of positive cells correlated with the results of the biochemical assay and with prognosis. In another study, carcinomas with small numbers of positive cells (between 1% and 10%) had a prognosis between cancers with no or rare positive cells (<1%) and cancers with >10% positive cells.[11]

Table 2. Allred Score* for Estrogen and Progesterone Receptor Evaluation

Proportion Score	Positive Cells, %	Intensity	Intensity Score
0	0	None	0
1	<1	Weak	1
2	1 to 10	Intermediate	2
3	11 to 33	Strong	3
4	34 to 66		
5	≥67		

* The Allred score combines the percentage of positive cells and the intensity of the reaction product in most of the carcinoma.[9] The 2 scores are added together for a final score with 8 possible values. Scores of 0 and 2 are considered negative. Scores of 3 to 8 are considered positive.

Table 3. H Score* for Estrogen and Progesterone Receptor Evaluation

		Calculation of H Score
Cell Signal	**Percentage of Cells**	**Value Multiplied**
Cells with no signal		% x 0 = 0
Cells with weak signal		% x 1 =
Cells with moderate signal		% x 2 =
Cells with strong signal		% x 3 =
Total score =		

* The H score is determined by multiplying the percentage of cells demonstrating each intensity (scored from 0 to 3) and adding the results.[12] There are 300 possible values. In this system, <1% positive cells is considered to be a negative result.

References

1. Harris L, Fritsche H, Mennel R, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol.* 2007;25(33):1-26.
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3. Yaziji H, Taylor CR, Goldstein NS, et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. *Appl Immunohistochem Mol Morphol.* 2008;16(6):513-520.
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6. Fitzgibbons PL, Murphy DA, Hammond EH, Allred DC, Valenstein PN. Recommendations for validating estrogen and progesterone receptor Immunohistochemistry assays. *Arch Pathol Lab Med.* 2010;134(6):930-935.

7. Yildiz-Aktas IZ, Dabbs DJ, Bhargava R. The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma. *Mod Pathol*. 2012;25(8):1098-1105.
8. Neumeister VM, Anagnostou V, Siddiqui S, et al. Quantitative assessment of effect of preanalytic cold ischemic time on protein expression in breast cancer tissues. *J Natl Cancer Inst.* 2012;104(23)1815-1824.
9. Harvey JM, Clark GM, Osborne CK, et al. Estrogen receptor status by immunohistochemistry is superior to the ligand binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol.* 1999;17(5):1474-1481.
10. Arber JM, Arber DA, Jenkins KA, Battifora H. Effect of decalcification and fixation in paraffin-section immunohistochemistry. *Appl Immunohistochem.* 1996;4:241-248.
11. Viale G, Regan MM, Maiorano E, et al. Prognostic and predictive value of centrally reviewed expression of estrogen and progesterone receptors in a randomized trial comparing letrozole and tamoxifen adjuvant therapy for postmenopausal early breast cancer: BIG 1-98. *J Clin Oncol.* 2007:25(25):3846-3852.
12. McCarty KS Jr, Miller LS, Cox EB, et al. Estrogen receptor analyses: correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch Pathol Lab Med.* 1985;109(8):716-721.
13. Shousha S. Oestrogen receptor status of breast carcinoma: Allred/H score conversion table. *Histopathology.* 2008;53(3):346-347.
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15. Nadji M, Gomez-Fernandez C, Ganjei-Azar P, Morales AR. Immunohistochemistry of estrogen and progesterone receptors reconsidered: experience with 5,993 breast cancers. *Am J Clin Pathol.* 2005:123(1);21-27.
16. Turbin DA, Leung S, Cheang MC, et al. Automated quantitative analysis of estrogen receptor expression in breast carcinoma does not differ from expert pathologist scoring: a tissue microarray study of 3,484 cases. *Breast Cancer Res Treat.* 2008;110(3):417-426.

C. HER2 (ERBB2) Testing

Scientific rationale: A subset of breast carcinomas (approximately 15% to 20%) overexpress human epidermal growth factor receptor 2 (HER2; HUGO nomenclature *ERBB2*). Protein overexpression is usually due to gene amplification. Assays for gene copy number, mRNA quantity, and protein generally give similar results; gene amplification correlates with protein overexpression in about 95% of cases. In a small subset of carcinomas (probably <5%), protein overexpression may occur by different mechanisms. Overexpression is both a prognostic and predictive factor.

Clinical rationale: HER2 status is primarily evaluated to determine patient eligibility for anti-HER2 therapy. It may identify patients who have a greater benefit from anthracycline-based adjuvant therapy.

Methods: HER2 status can be determined in formalin-fixed paraffin-embedded tissue by assessing protein expression on the membrane of tumor cells using IHC or by assessing the number of HER2 gene copies using in situ hybridization (ISH). When both IHC and ISH are performed on the same tumor, the results should be correlated. The most likely reason for a discrepancy is that 1 of the assays is incorrect, but in a small number of cases there may be protein overexpression without amplification, amplification without protein overexpression, or marked intratumoral heterogeneity.

HER2 (ERBB2) Testing by Immunohistochemistry

Factors altering the detection of HER2 (ERBB2) by IHC have not been studied as well as for ER and PgR. It is recommended that tissue be fixed in buffered 10% formalin for at least 6 hours unless another fixative has been validated. External proficiency testing surveys for HER2 are available from the CAP and other organizations. These surveys are invaluable tools to ensure that the laboratory assays are working as expected.

False-positive IHC results for HER2 may be due to:

- Edge artifact. This is usually seen in core biopsies, where cells near the edges of the tissue stain stronger than in the center, possibly because antibody pools at the sides. Specimens with stronger staining at the edge of the tissue should be interpreted with caution.
- Cytoplasmic positivity, which can obscure membrane staining and make interpretation difficult.
- Overstaining (strong membrane staining of normal cells). May be due to improper antibody titration (concentration too high).
- Misinterpretation of ductal carcinoma in situ (DCIS). High-grade DCIS is often HER2 positive. In cases with extensive DCIS relative to invasive carcinoma (particularly microinvasive carcinoma), HER2 scoring may mistakenly be done on the DCIS component. Care must be taken to score only the invasive component.

False-negative IHC results for HER2 may be due to:

- Prolonged cold ischemia time.
- Tumor heterogeneity. When a negative result is found, but only a small biopsy sample was tested, repeat testing on a subsequent specimen with a larger area of carcinoma should be considered, particularly if the tumor has characteristics associated with HER2 positivity (ie, tumor grade 2 or 3, weak or negative PgR expression, increased proliferation index).
- Improper antibody titration (concentration too low)

False-negative and false-positive results can be reduced by paying attention to the following:

- Tissue controls. External controls must stain as expected. There are no normal internal controls for HER2 protein assessment by IHC.
- Correlation with histologic and other biomarker results. If the HER2 test is negative by IHC, but the tumor has characteristics associated with HER2 positivity (see above), repeating the test by ISH should be considered.

Reporting guidelines: ASCO and CAP have issued recommendations for reporting the results of HER2 testing by IHC (Table 4).[1]

Table 4. Reporting Results of HER2 Testing by Immunohistochemistry (IHC)

Result	Criteria
Negative (Score 0)	No staining observed *or* Membrane stating that is incomplete and is faint/barely perceptible and within ≤10% of tumor cells
Negative (Score 1+)	Incomplete membrane staining that is faint/barely perceptible and within >10% of tumor cells*
Equivocal (Score 2+)†	Weak to moderate complete membrane staining in >10% of tumor cells *or* Complete membrane staining that is intense but within ≤10% of tumor cells*
Positive (Score 3+)	Complete membrane staining that is intense and >10% of tumor cells*

* Readily appreciated using a low-power objective and observed within a homogeneous and contiguous population of invasive tumor cells.

† Must order reflex test (same specimen using ISH) or order a new test (new specimen if available, using IHC or ISH).

HER2 Testing by In Situ Hybridization

Fluorescence in situ hybridization (FISH), chromogenic in situ hybridization (CISH), and silver-enhanced in situ hybridization (SISH) studies for *HER2* determine the presence or absence of gene amplification. Some assays use a single probe to determine the number of HER2 gene copies present, but most assays include a chromosome enumeration probe (CEP17) to determine the ratio of HER2 signals to copies of chromosome 17. Although 10% to 50% of breast carcinomas have more than 2 CEP17 copies, only 1% to 2% of carcinomas show true polysomy (ie, duplication of the entire chromosome).

Failure to obtain results with ISH may be due to the following:

- Prolonged fixation in formalin (>1 week)[2]
- Fixation in non-formalin fixatives[3]
- Procedures or fixation involving acid (eg, decalcification) may degrade DNA[4]
- Insufficient protease treatment of tissue

External proficiency testing surveys for HER2 by ISH are available from CAP and other organizations. These surveys are invaluable tools to ensure that the laboratory assays are working as expected.

Reporting guidelines: ASCO and CAP have issued recommendations for reporting the results of HER2 testing by ISH (Tables 5 and 6).[1]

Dual Probe ISH Group Definitions:

Group 1 = HER2/CEP17 ratio ≥2.0; ≥4.0 HER2 signals/cell
Group 2 = HER2/CEP17 ratio ≥2.0; <4.0 HER2 signals/cell
Group 3 = HER2/CEP17 ratio <2.0; ≥6.0 HER2 signals/cell
Group 4 = HER2/CEP17 ratio <2.0; ≥4.0 and <6.0 HER2 signals/cell
Group 5 = HER2/CEP17 ratio <2.0; <4.0 HER2 signals/cell

Table 5. Reporting Results of *HER2* Testing by In Situ Hybridization (single-probe assay)

Result	Criteria (single-probe assay)
Negative	· Average HER2 copy number <4.0 signals/cell · Average HER2 copy number ≥4.0 and <6.0 signals/cell and concurrent IHC 0, 1+ or 2+ · Average HER2 copy number ≥4.0 and <6.0 signals/cell and concurrent dual probe ISH Group 5
Positive	· Average HER2 copy number ≥6.0 signals/cell · Average HER2 copy number ≥4.0 and <6.0 signals/cell and concurrent IHC 3+ · Average HER2 copy number ≥4.0 and <6.0 signals/cell and concurrent dual probe ISH Group 1

Table 6. Reporting Results of *HER2* Testing by In Situ Hybridization (dual-probe assay)

Result	Criteria (dual-probe assay)
Negative	· Group 5
Negative* (see comment)	· Group 2 and concurrent IHC 0-1+ or 2+ · Group 3 and concurrent IHC 0-1+ · Group 4 and concurrent IHC 0-1+ or 2+
Positive*	· Group 2 and concurrent IHC 3+ · Group 3 and concurrent IHC 2+ or 3+ · Group 4 and concurrent IHC 3+
Positive	· Group 1

*For Groups 2-4 final ISH results are based on concurrent review of IHC, with recounting of the ISH test by a second reviewer if IHC is 2+ (per 2018 CAP/ASCO Update recommendations).

Comment for Group 2 Negative result: *Evidence is limited on the efficacy of HER2-targeted therapy in the small subset of cases with HER2/CEP17 ratio ≥2.0 and an average HER2 copy number <4.0/cell. In the first generation of adjuvant trastuzumab trials, patients in this subgroup who were randomized to the trastuzumab arm did not appear to derive an improvement in disease free or overall survival, but there were too few such cases to draw definitive conclusions. IHC expression for HER2 should be used to complement ISH and define HER2 status. If IHC result is not 3+ positive, it is recommended that the specimen be considered HER2 negative because of the low HER2 copy number by ISH and lack of protein overexpression.*

Comment for Group 3 Negative result: *There are insufficient data on the efficacy of HER2-targeted therapy in cases with HER2 ratio <2.0 in the absence of protein overexpression because such patients were not eligible for the first generation of adjuvant trastuzumab clinical trials. When concurrent IHC results are negative (0-1+), it is recommended that the specimen be considered HER2 negative.*

Comment for Group 4 Negative result: *It is uncertain whether patients with ≥4.0 and <6.0 average HER2 signals/cell and HER2/CEP17 ratio <2.0 benefit from HER2 targeted therapy in the absence of protein overexpression (IHC 3+). If the specimen test result is close to the ISH ratio threshold for positive, there is a high likelihood that repeat testing will result in different results by chance alone. Therefore, when IHC results are not 3+ positive, it is recommended that the sample be considered HER2 negative without additional testing on the same specimen.*

Important issues in interpreting ISH are the following:

- Identification of invasive carcinoma: A pathologist should identify on the hematoxylin and eosin (H&E) or HER2 IHC slide the area of invasive carcinoma to be evaluated by ISH.
- Identification of associated DCIS: In some cases, DCIS will show gene amplification, whereas the associated invasive carcinoma will not. ISH analysis must be performed on the invasive carcinoma.

Some cancers have a low level of HER2 expression as determined by equivocal results by both IHC and ISH analysis. Repeat testing may be helpful to exclude possible technical problems with the assays but often does not result in definitive positive or negative results.

Either the number of *HER2* genes or the ratio of *HER2* to *CEP17* can be used to determine the presence of amplification. In the majority of carcinomas, both methods give the same result. In unusual cases, the 2 methods give different results, usually due to variation in the number of *CEP17* signals. Some studies have shown that chromosome 17 abnormalities can lead to alterations of the *HER2/CEP17* ratio, potentially leading to equivocal or incorrect ISH results.[5] In such cases, gene copy number may be a more accurate reflection of HER2 status. If there is a second contiguous population of cells with increased HER2 signals/cell, and this cell population consists of more than 10% of tumor cells on the slide (defined by image analysis or by visual estimation of the ISH or IHC slide), a separate counting of at least 20 non-overlapping cells must also be done within this cell population and also reported. An overall random count is not appropriate in this situation.

References

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D. Ki-67 Testing

Ki-67 is a nuclear protein found in all phases of the cell cycle and is a marker of cell proliferation. The monoclonal antibody MIB-1 is the most commonly used antibody for assessing Ki-67 in formalin-fixed paraffin-embedded tissue sections. The percentage of Ki-67 positive tumor cells determined by IHC is often used to stratify patients into good and poor prognostic groups, but there is a lack of consensus on scoring, definition of low versus high expression, an appropriate cut point for positivity, or which part of the tumor should be scored (eg, leading edge, hot spots, overall average).[1] There is also a paucity of data on the effects of pre-analytic variables (eg, ischemic time, length of fixation, antigen retrieval) on Ki-67 staining. For these reasons, routine testing of breast cancers for Ki-67 expression is not currently recommended by either ASCO or the National Comprehensive Cancer Network (NCCN).

References
1. Dowsett M, Nielsen TO, A'Hern R, et al. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in breast cancer working group. *J Natl Cancer Inst.* 2011;103(22):1656-1664.

E. Multigene Expression Assays

Scientific rationale: Breast cancers vary greatly in histologic appearance, expression of biomarkers, response to treatment, and prognosis. Assays that detect variations in gene expression by mRNA levels have confirmed the diversity of gene expression patterns underlying these observations.

Clinical rationale: It may be possible to use multigene expression assays to identify specific tumor subtypes and improve our ability to assess prognosis and likelihood of response to specific treatments.[1]

Methods: A variety of multigene and multiprotein expression assays are available, most of which are proprietary assays developed and performed by a single laboratory. Multigene assays detect gene expression patterns by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) or by hybridization of labeled nucleic acids derived from the tumor to a number of small, immobilized, synthetic DNA strands (microarrays). Using these methods, numerous gene products can be examined simultaneously in the same sample. Some of the assays have been optimized for use on formalin-fixed tissue, while others require frozen tissue.

Reporting guidelines: Pathologists may choose to incorporate results of proprietary assays into their own reports if this would make the information more accessible for patient care.

References
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