I. Testing tumor markers on every breast carcinoma (whether primary or metastatic):

The determination of estrogen receptor (ER), progesterone receptor (PR) and HER2 status is a crucial step in the pathologic evaluation of breast cancers. Indeed, the current American Society of Clinical Oncology (ASCO) guidelines on the use of tumor markers recommend that *ER, PR, and HER2 be tested on every primary breast carcinoma, as well as metastatic foci* if the results could influence treatment.

**Hormone receptors (ER/PR):**

ER is a nuclear transcription factor, activated by estrogen. It controls the development and differentiation of normal, hyperplastic, and neoplastic breast epithelial cells. PR is an estrogen-related gene, the expression of which is indicative of a functional ER pathway.

Approximately 75% of invasive breast carcinomas are positive for hormone receptors, with slightly more tumors being ER-positive than they are PR-positive. Both ER and PR are relatively weak prognostic factors, but both are strong predictive factors for response to endocrine therapy (selective ER modulators such as tamoxifen, antagonists that degrade the receptor such as fluvastatin, and estrogen receptor deprivation methods such as ovarian ablation or aromatase inhibitors).

Currently, the gold standard method for ER and PR assessment is *immunohistochemical staining* (Advantages include: lower cost, nuclear staining pattern, rapid turn around time and ability to assay small tissue samples such as needle core biopsies)

**Human Epidermal Growth Factor Receptor 2 (HER2):**

Carcinoma cells depend on changes in molecular genetics (inherited or acquired) for their growth and survival. Thus, these alterations constitute potential targets for therapeutic agents. *Amplification of HER2 gene (also known as ERB-B2) is the ultimate example of an acquired molecular alteration that promotes the maintenance and growth of carcinoma.*

*HER2* gene (proto-oncogene on chromosome 17q12) is a member of the Human Epidermal Growth Factor Receptor (HER) family of receptors including HER1 (EGFR), HER3, and HER4; all of which regulate normal cell proliferation and cell survival through intracellular signaling pathways. Thought to be an early event in the pathogenesis of HER2-positive Breast cancers, *HER2 amplification* (or *HER2 overexpression*) plays a role in sustaining multiple cancer
pathways by: 1) Promoting formation of new blood vessels (angiogenesis), 2) Improving growth signals and thereby driving tumor proliferation, 3) facilitating mitotic activity, and 4) enhancing invasive capabilities.

Approximately 20% of all invasive breast carcinomas are positive for HER2, conferring a more aggressive clinical course. Indeed, HER2 amplification is a prognostic marker of poor outcome in the absence of adjuvant therapy, independent of lymph node status, tumor size, grade, and hormone receptor (ER/PR) status. It is associated with:

- Significantly decreased disease-free and overall survival (OS)
- Increased rate of metastases (including lymph node metastasis)
- Decreased time to recurrence (compared to HER2-negative tumors)

The importance of accurate HER2 testing:

Progress made in understanding HER2 molecular biology led to a breakthrough in therapy for HER2-positive breast cancer, which continues to be the model of successful targeted therapy.

Located on the cell surface, HER2 receptors represent an ideal target for agents such as monoclonal antibodies (e.g., trastuzumab) and other small-molecule inhibitors of the HER2 tyrosine kinases (e.g., lapatinib). Studies involving metastatic breast carcinoma as well as early stage breast cancer demonstrated a significant improvement in prognosis with anti-HER2 therapy. The emergence of resistance to anti-HER2 therapy gave rise to novel agents including pertuzumab (monoclonal antibody), neratinib (irreversible kinase inhibitor), and DM1 (a conjugate that helps deliver trastuzumab specifically to HER2-overexpressing tumors and improve its potency).

The main clinical reason to assess HER2 status is to select patients who would benefit from treatment. With 1) the predictive importance of HER2, 2) the advances in anti-HER2 therapy and 3) its established and remarkable efficacy against HER2-positive Breast cancers (BC), the latest ASCO/CAP guidelines recommend routine testing of HER2 (along with ER and PR, as aforementioned) in women with primary invasive BC as well as metastatic foci. Thus, assessment of HER2 status in an accurate, precise and reproducible manner is of utmost importance.

II. Established HER2 Testing Methods:

With HER2 gene amplification in direct correlation with HER2 expression levels at the mRNA and protein levels, determination of HER2 status can be made at either of these levels. The two most commonly used methods are: 1) Detection of HER2 protein overexpression by using immunohistochemistry and 2) Evaluation of HER2 gene amplification by fluorescence in situ hybridization (FISH). Both methods can be applied on formalin-fixed paraffin-embedded (FFPE) tumor samples.
**Immunohistochemical staining:**

HER2 protein expression can be evaluated by an immunohistochemical (IHC) staining pattern of tumor cells and is interpreted through a specially devised scoring system (See Figure 1). Two assays are available for this purpose including Dako Herceptest and Ventana Pathway.

![Diagram of IHC scoring system]

**Figure 1.** HER2 protein expression, interpreted using the 2013 ASCO/CAP HER2 testing guidelines.

The immunohistochemical test is relatively quick and inexpensive. Results are used using a conventional bright-field microscope. Stained slides do not degrade and, thus, can be stored for long periods (See Table 1). This widely used test allows morphologic features of breast tumors to be viewed in parallel.

However, it is only about 90% accurate, dependent on multiple preanalytical factors, and subject to interobserver variation.

**Fluorescence in situ hybridization (FISH) testing:**

This morphology-based, dual probe method identifies HER2 gene copy numbers in conjunction with the number of chromosome 17 centromere (CEP 17) copy numbers. It can be applied to cell blocks or cytologic specimens. When compared to immunohistochemistry, the FISH technique is relatively insensitive to pre-analytical factors (DNA is more stable than
protein), and results in a more objective and quantitative score (See Figure 2 and Table 1).

However, it is a relatively advanced and expensive technique that requires specialized equipment and expertise, and results in an impermanent signal. Reproducibility of this technique is dependent on the thickness of the tissue sections, recognition of the invasive component and the interpretation of the signals.

Figure 2. HER2 gene status assessment by in situ hybridization (ISH), using the ASCO/CAP HER2 testing guidelines

In most laboratories, FISH test is performed if the HER2 test result by IHC is reported as “equivocal”. Three FISH assays have been approved by the FDA: Abott Pathvysion, Ventana Inform, and Dako PharmDX).

The ASCO/CAP guidelines require that the duration of specimen fixation be between 6 to 72 hours for both IHC and ISH methods.

It is important to mention that tumor heterogeneity can lead in discordant HER2 results by FISH and IHC techniques. Tumor heterogeneity is defined as more than 1 population of tumor cells in terms of HER2 amplification (with >5% but <50% of the tumor cells showing amplification). In addition, chromosome 17 polysomy (multiple copies of the entire chromosome) can account for some breast carcinoma cases that show 3+ results by IHC, but are not HER2 amplified when the HER2/chromosome 17 ratio is evaluated.
III. Additional Relatively Recent HER2 Testing Methods:

CISH and SISH:

Chromogenic in situ hybridization or CISH constitutes a modification of the FISH method for the detection of HER2 gene amplification. The assay can be used as a primary test to detect HER2 status or to retest equivocal immunohistochemical results. The most widely used type of CISH assay is the dual-color CISH (dc-CISH): Inclusion of the chromosome 17 probe (CEP17) in dc-CISH allows calculation of the HER2/CEP17 ratio; thus, excluding chromosome 17 polysomy (multiple copies of the entire chromosome).

dc-CISH is based on the same principle as 2-color FISH and uses a similar scoring system with a HER2/CEP17 ratio of 2 or more being indicative of HER2 gene amplification. In addition, dc-CISH allows the assessment of HER2 status in conjunction with histopathologic examination and the ease of bright-field microscopy.

SISH is a fully automated technique to detect chromogenic signals. Scored similarly to CISH, SISH is faster to perform than FISH and requires only a conventional light microscope.

A comparison of immunohistochemistry, FISH, CISH and SISH is summarized in Table 1.

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<th>HER2 Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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| Immunohistochemistry (IHC) | • Performed in most pathology laboratories  
• Relatively quick, cheap, and easy to perform  
• IHC-stained slides can be stored for long periods of time (years)  
• Morphologic features of cells can still be determined in the IHC-assayed section | • Susceptible to variations in testing protocol  
• Semi-quantitative and subjective score interpretation |
| FISH | • Less affected by pre-analytic factors than IHC (DNA more stable than protein)  
• More objective and quantitative score interpretation than IHC (Numeric result)  
• Identifies HER2+ tumors within IHC 2+ cases | • Costly (Requires fluorescence microscope and digital photography)  
• Signal decays over time  
• Areas of invasive carcinoma can be challenging to identify under fluorescence microscope |
| CISH | • Lower costs when compared to FISH  
• Requires only standard light microscope  
• Morphologic and histopathologic features of specimen can be assessed simultaneously  
• Relatively fast interpretation of staining result  
• Staining remains stable for long periods of time (like IHC) | • Not as widespread as FISH (Little experience in interpreting the results)  
• If not performed and interpreted adequately, can give false-positive results |
| SISH | • Requires only standard light microscope  
• Technique fully automated and rapidly performed  
• HER2 and CEP17 assays can be performed on contiguous slides  
• Staining remains stable for long periods of time  
• Relatively easy to interpret | • New technology; therefore, little experience |
Quantitative RT-PCR:

Oncotype DX (Genomic Health; Redwood City, CA) assay detects HER2 mRNA overexpression using quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). This technology is not currently used to make treatment decisions. It requires tissue microdissection if DCIS shows stronger HER2 positivity or if little carcinoma in relation to stroma is present. In addition, this technique will not detect heterogeneity of HER2 overexpression.

IV. Emerging Potential HER2 Testing Methods:

Multiple studies have proven concordant results among the different ISH techniques in accurately determining HER2 gene status, although costs are somewhat variable. Still, ISH remains a semi-quantitative test since it requires an experienced individual to scan an entire slide and select a representative area for scoring, which is done by counting the signals in at least 20 representative tumor cells using a fluorescent or a bright-field microscope.

Working with FFPE samples to come up with a fully quantitative method for HER2 assessment represents a great challenge. The extensive DNA damage and degradation upon exposure to formaldehyde (fixative solution) leads to short DNA fragments, not conducive to producing high-quality copy number (CN) data. Furthermore, the chemically induced DNA alterations render the FFPE samples unsuitable for enzyme-driven approaches, including polymerase chain reaction (PCR). Another major hurdle is the relatively small quantity of DNA present in FFPE samples; most CN platforms require large amounts of DNA.

Recently, molecular inversion probe (MIP) technology has proven to be successful in overcoming the challenges of FFPE tissue samples, generating high-quality CN data from archived FFPE tissue with minimal DNA requirements (60 ng).

Despite some limitations (including inability to assess tumor heterogeneity or differentiate between in situ and invasive carcinoma), the MIP platform, with its ability to generate high-quality CN data from highly degraded FFPE-derived DNA, can potentially be used as a sole clinical test for HER2 amplification status, especially when the designated field of tumor on the selected slide is highly cellular and composed predominantly of invasive carcinoma.

MIP analysis can also be considered as an adjunct tool when HER2 status is equivocal by both FISH and IHC methods. One benefit of MIP arrays is the minimal or absent operator bias, leading to more objective results (See Table 2). Genome-wide CN analysis and detection of polysomy and loss of heterozygosity remain the most promising aspects of this technology, not only in breast cancer but also in other solid tumors.
Table 2. Comparison of FISH and MIP methodologies

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<tr>
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<th>FISH</th>
<th>MIP Array</th>
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<tr>
<td>Sample type</td>
<td>FFPE, FNA, and frozen tissue</td>
<td>FFPE, FNA, and frozen tissue</td>
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</table>
| Required amount of tumor (for high-quality copy number data) | Quantity: ~ 100 cells
Tumor fraction: Any | Quantity: 60-80 ng of DNA
Tumor fraction: 30% of viable tumor |
| Detection              | Semi-automated by fluorescent microscopy | Automated by microarray based detection    |
| Genome-wide CN profiling | No                                      | Yes                                        |
| Polysomy and LOH       | No                                        | Yes                                        |
| Operator dependent     | Yes                                       | No                                         |
| Intra-tumoral heterogeneity | Identifiable by careful observer     | Indistinguishable                            |

References: