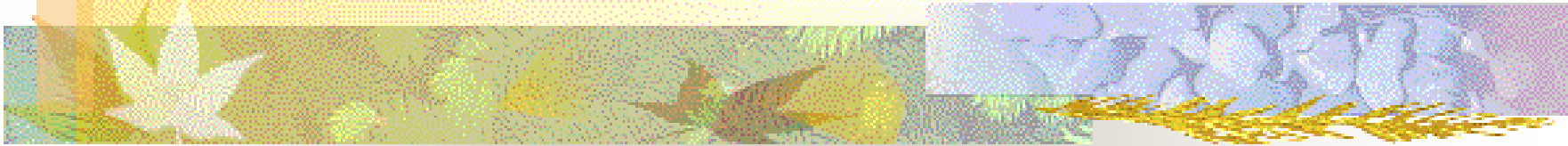


# Determination of prognostic markers in breast cancer:

*quality control and standardisation*



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# Prognostic parameters in breast cancer

- Morphologic:
  - Tumour size.
  - Lymph node status.
  - Histologic grade.
  - Histologic subtype.
  - Lymphovascular invasion.
- Biologic:
  - Oestrogen receptor.
  - Progesterone receptor.
  - c-erbB2 (HER2).
  - MIB1 (Ki67) proliferation index.



# Prognostic parameters in breast cancer - Morphologic

- Synoptic reports and minimal datasets.
- Pathologist training & experience.
- Team based specialty reporting.
- Multidisciplinary conferences:
  - Radiology-pathology conferences.
  - Breast tumour boards.
- Continual medical education in breast pathology.

SIZE OF INVASIVE TUMOUR: \_\_\_\_\_ mm in maximum dimension  
WHOLE SIZE OF TUMOUR (INVASIVE + DCIS): \_\_\_\_\_ mm in maximum dimension  
EXTENT OF TUMOUR: Localised / Multiple foci

HISTOLOGICAL TUMOUR TYPE: Ductal NOS, others \_\_\_\_\_  
GRADE: 1 2 3 Not assessable (Tubule score-\_\_\_\_; nuclear pleomorphism-\_\_\_\_; mitotic score-\_\_\_\_)  
[mitotic count: \_\_\_\_ per 10 high power fields; field diameter: \_\_\_\_ mm]

ASSOCIATED DCIS: None/Minimal (less than or equal to 25%)/Extensive (more than 25%)  
Grade of DCIS: High/ Intermediate/Low  
Subtype of DCIS: Mixed/Comedo/Solid/Micropapillary/Cribriform

ASSOCIATED LCIS: Present/Absent

PERITUMORAL LYMPHOVASCULAR INVASION: Yes/Not Seen /Possible/Not assessable

PAGET'S DISEASE: Present/Absent

**[FOR WIDE EXCISION-Remove this heading in final report]**

**RESECTION MARGINS**

Deep margin : \_\_\_\_\_ mm away from invasive/in situ carcinoma  
Superior margin : \_\_\_\_\_ mm away from invasive/in situ carcinoma  
Inferior margin : \_\_\_\_\_ mm away from invasive/in situ carcinoma  
Lateral margin : \_\_\_\_\_ mm away from invasive/in situ carcinoma  
Medial margin : \_\_\_\_\_ mm away from invasive/in situ carcinoma  
Anterior margin : \_\_\_\_\_ mm away from invasive/in situ carcinoma

**[FOR MASTECTOMY-Remove this heading in final report]**

**RESECTION MARGINS:**

DEEP MARGIN: Involved/Uninvolved by DCIS/invasive tumour

Clearance: \_\_\_\_\_ mm away

RADIAL MARGINS: Involved/Uninvolved by DCIS/invasive tumour

Closest radial margin (not anterior or posterior) is \_\_\_\_\_

Clearance: \_\_\_\_\_ mm away

Extent of margin involvement: Focal/Extensive

AXILLARY LYMPH NODES: \_\_\_ out of \_\_\_ lymph nodes show metastatic tumour

Size of largest nodal metastatic focus: \_\_\_\_\_ mm

SENTINEL LYMPH NODE: Not sampled/Sampled - metastatic tumour present/absent





# Prognostic parameters in breast cancer - Biologic

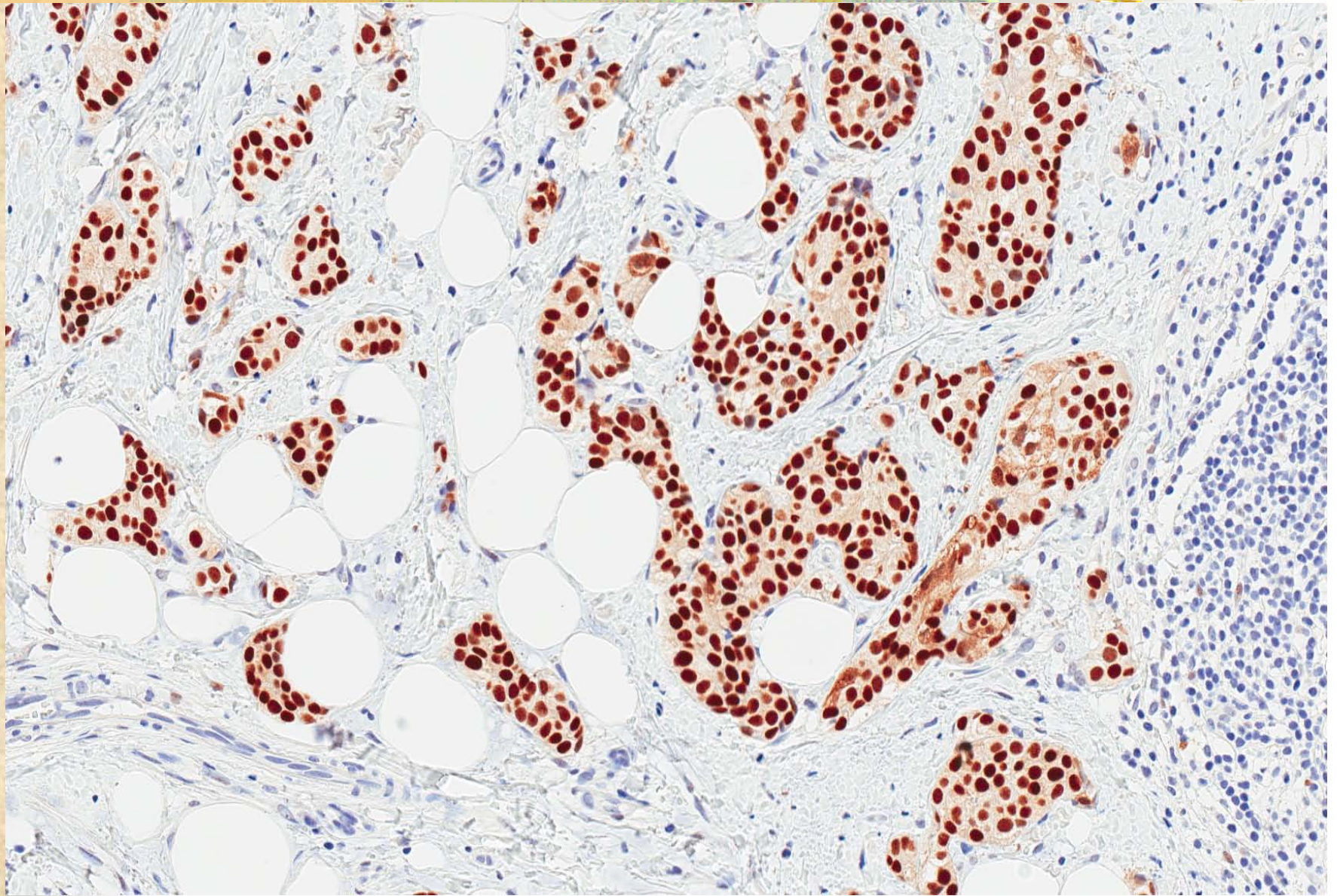
## ■ Scope:

- ER, PR, c-erbB2.
- Factors contributing to variability in reporting and results.
- Approach to standardisation and quality control.



# Oestrogen receptor

- Nuclear hormone receptor activated by oestrogen (17 $\beta$ -oestradiol).
- DNA transcription factor regulating gene expression.
- 2 different forms of ER (ER $\alpha$ , ER $\beta$ ).
- ER $\alpha$  in breast, endometrium, ovary, hypothalamus.

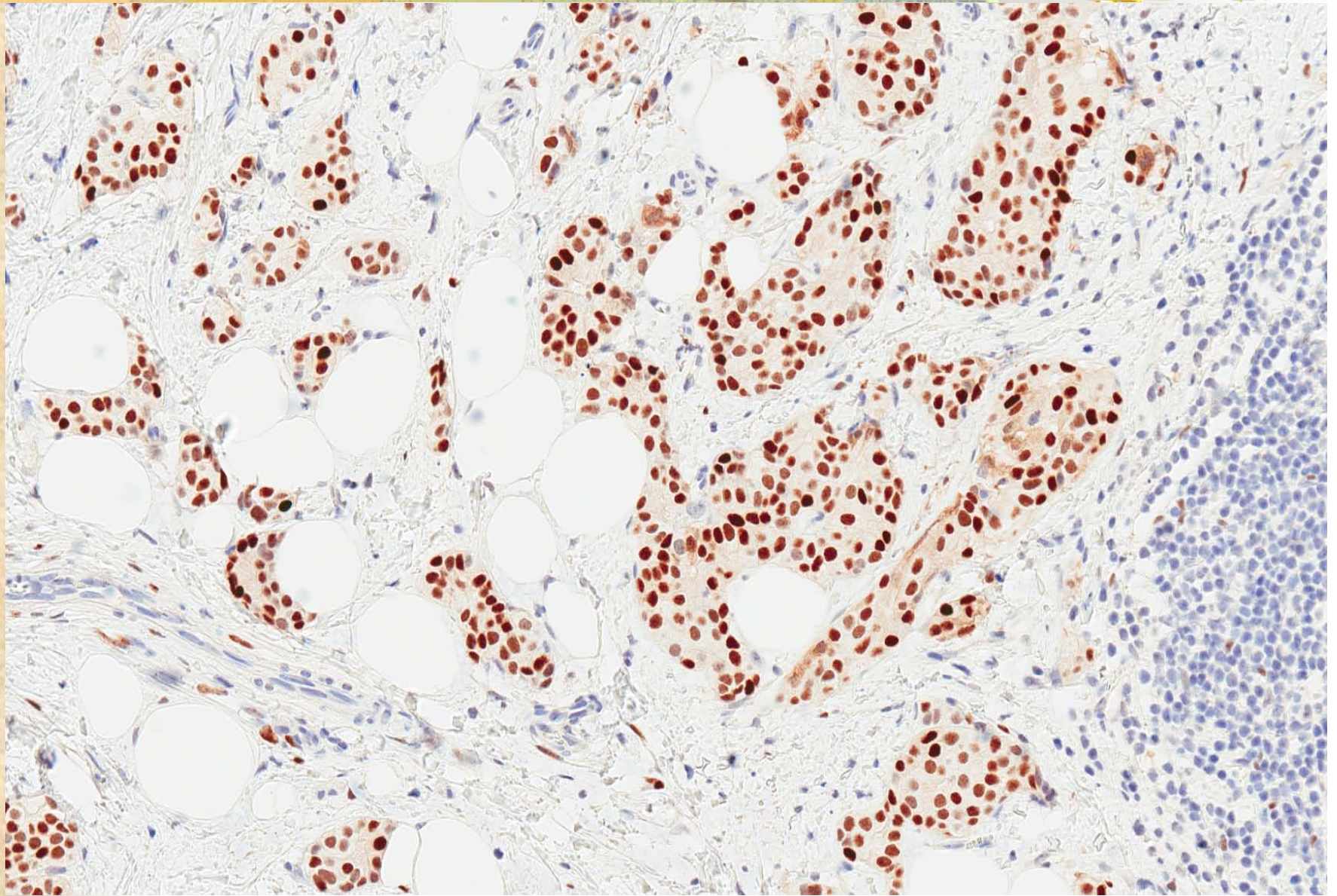


Oestrogen receptor



# Progesterone receptor

- Nuclear hormone receptor, also known as NR3C3 (nuclear receptor subfamily 3, group C, member 3).
- Progesterone binds to PR, triggering downstream transcription effects.
- Oestrogen is needed to induce PR.



Progesterone receptor



# Hormone receptor status (ER, PR)

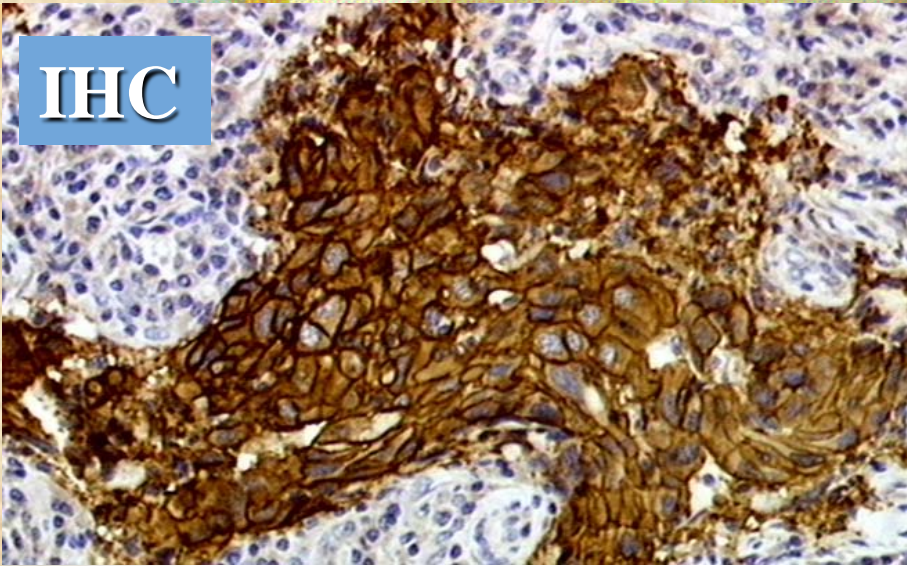
- ER and PR status predicts response to therapeutic and adjuvant hormonal treatment.
- Nuclear staining.
- Evaluated only in the invasive component.
- Proportion of invasive cancer cells, and intensity of staining, scored.



## c-erbB2 (HER2)

- Human epidermal growth factor receptor 2.
- Member of the EGFR family (4 EGFR receptors).
- Cell membrane surface bound receptor tyrosine kinase involved in signal transduction pathways, implicated in cell growth and differentiation.
- Orphan receptor, dimerises with other HER2 receptors.
- Proto-oncogene located on long arm of chromosome 17 (17q21-22).

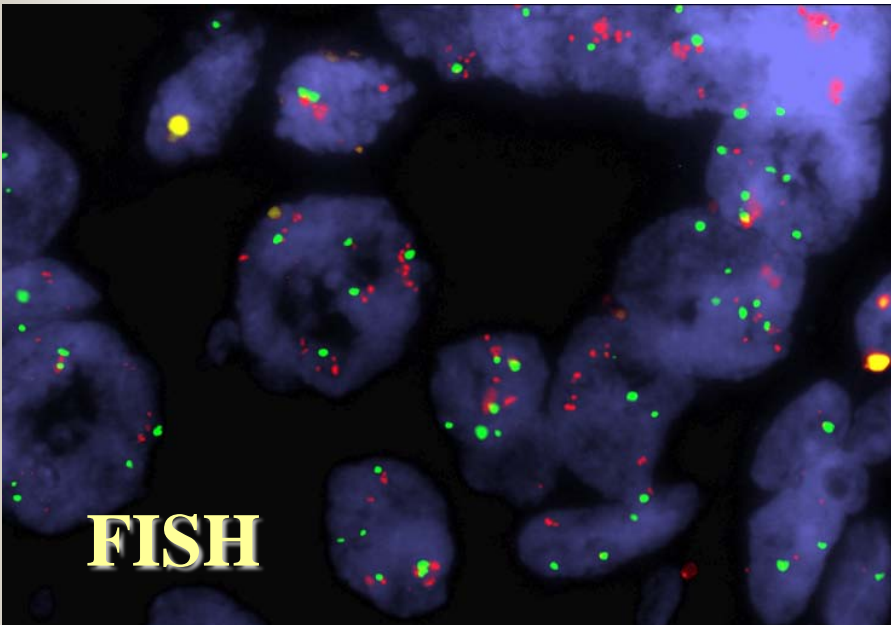
**IHC**



## Her2/neu (cerbB2)

- erbB2 protein overexpression or gene amplification (located on 17q21) occurs in about 10% to 20% of breast cancers.
- Associated with tumours of high histologic grade & reduced survival.
- Herceptin (trastuzumab).

**FISH**

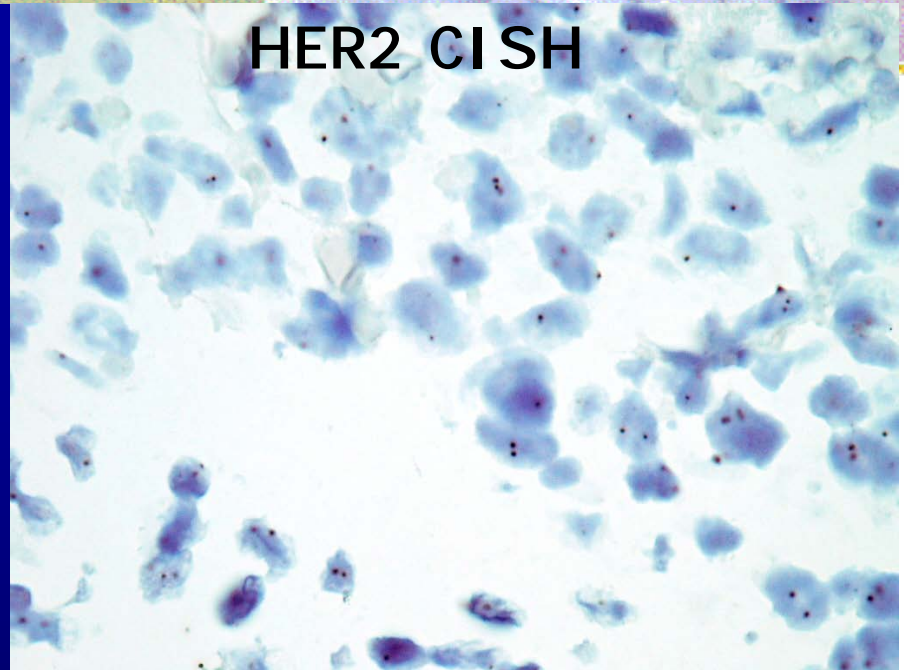


HER2 immunostaining

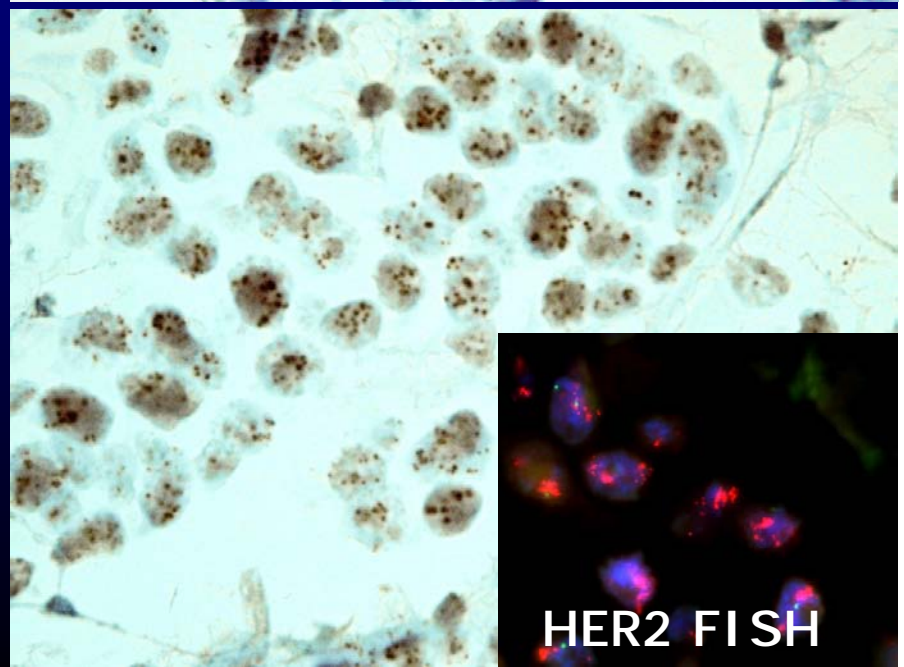
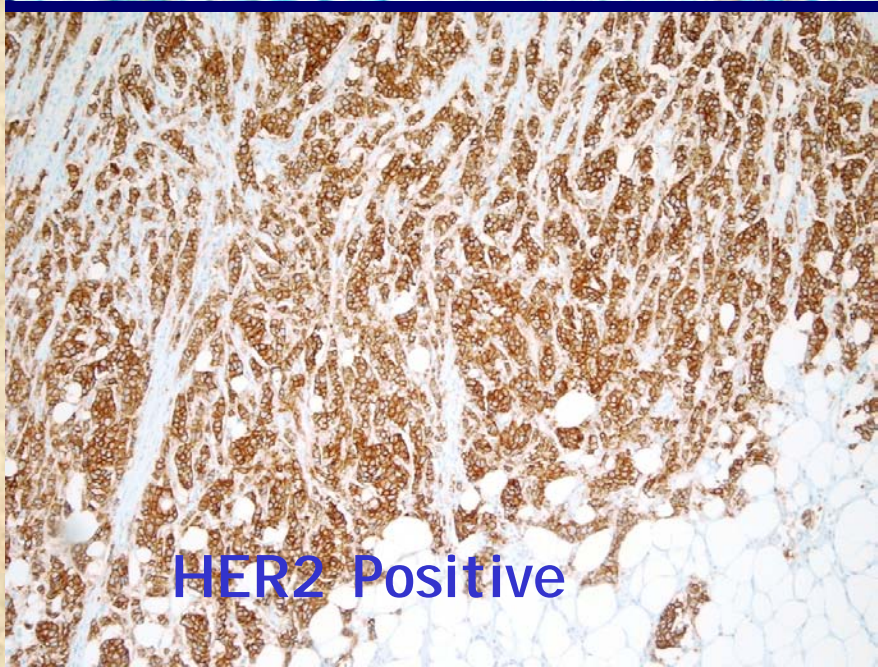


HER2 Negative

HER2 CISH



HER2 Positive



HER2 FISH

## Table 2. HER2 Testing Results

	IHC HER2 Protein Expression	Testing Method FISH HER2 Gene Amplification
Positive	3+*	<i>HER2</i> /CEP 17 ratio >2.2 or Average <i>HER2</i> gene copy number >6†
Equivocal	2+	<i>HER2</i> /CEP 17 ratio of 1.8-2.2 or Average <i>HER2</i> gene copy number 4-6†
Negative	0-1+	<i>HER2</i> /CEP 17 ratio <1.8 or Average <i>HER2</i> gene copy number <4†

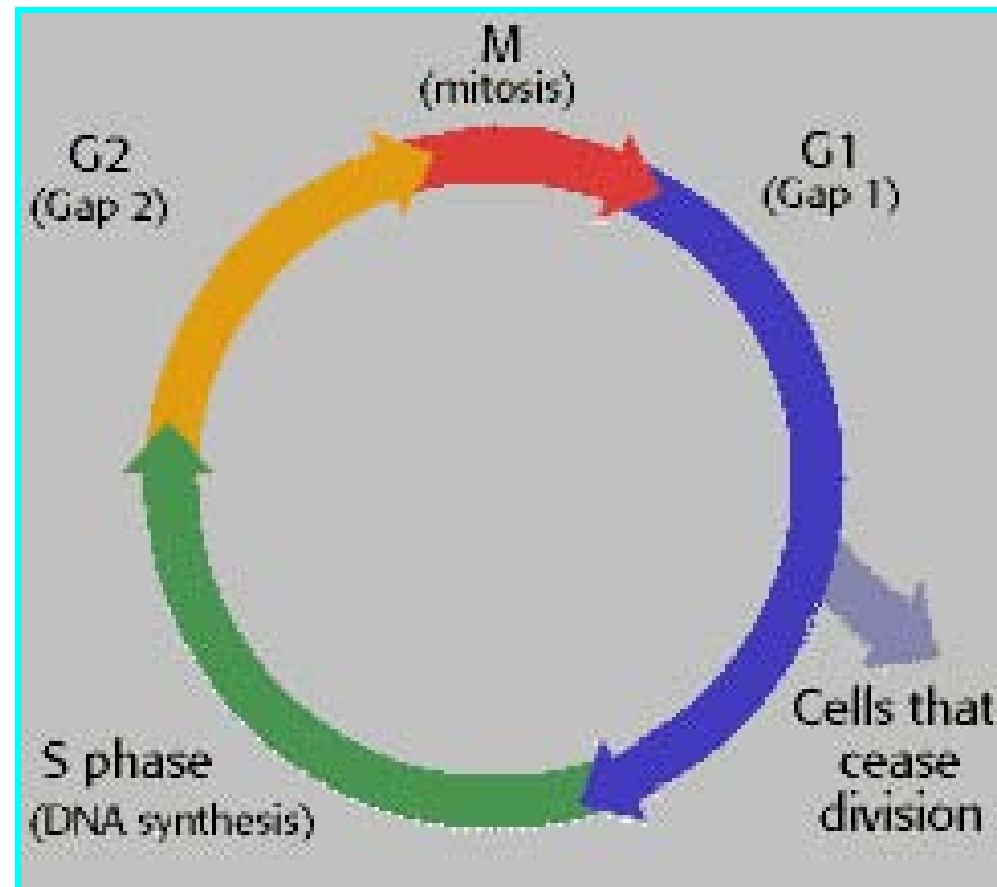
JCO 2007

- Validation of tests
- Internal quality control
- External proficiency tests
- Accredited laboratory

# Cell proliferation in breast cancer: Ki67

- Ki67 is a labile non-histone nuclear protein that is tightly linked to the cell cycle.
- Expressed in proliferating cells in mid G<sub>1</sub> phase, increasing in level through S and G<sub>2</sub>, peaking in M phase.
- Rapidly catabolised at the end of M phase.
- Undetectable in resting (G<sub>0</sub> and early G<sub>1</sub>) cells.

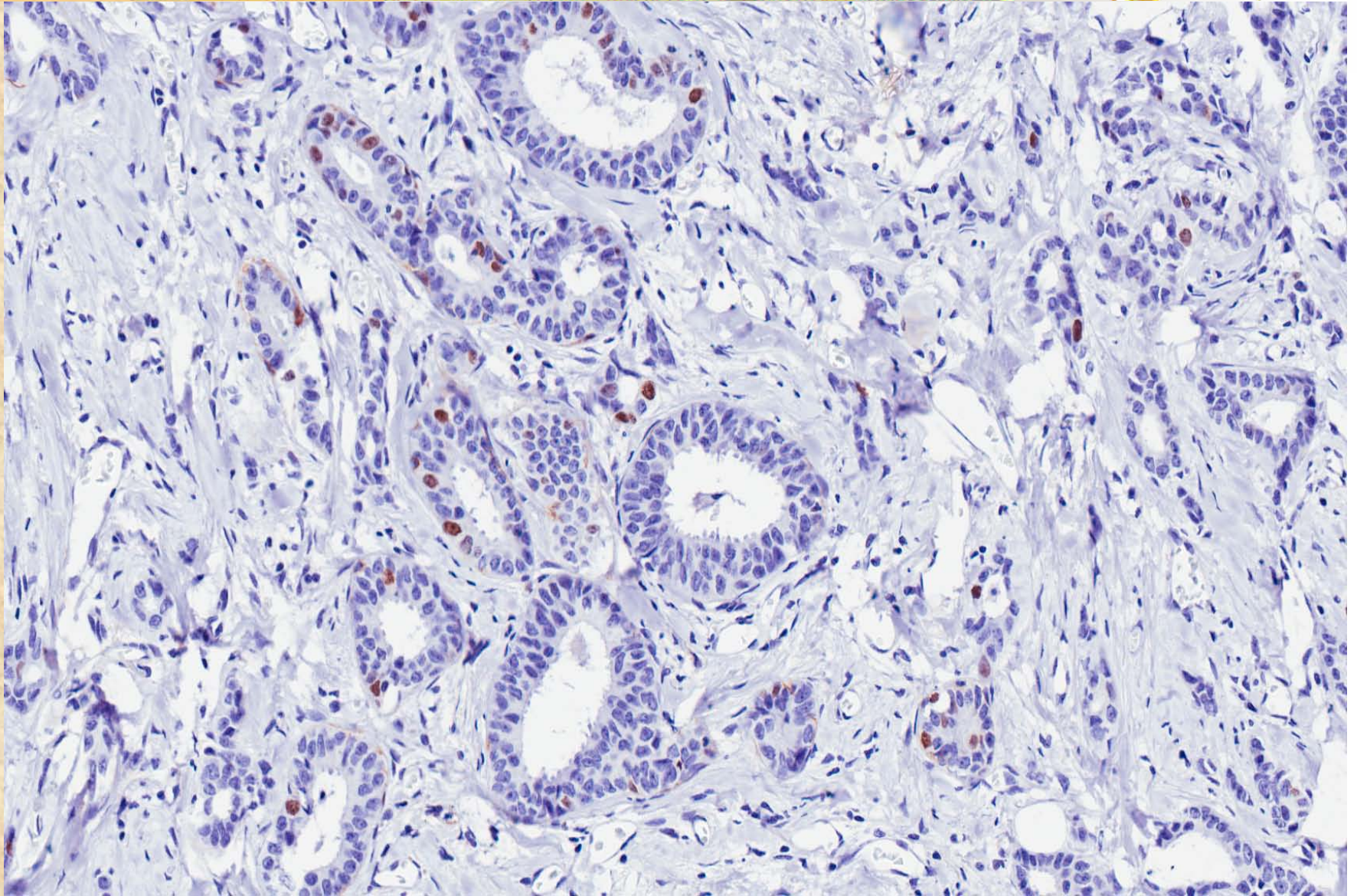
## Cell cycle





# Cell proliferation in breast cancer: Ki67

- Marker of cell proliferation.
- % Ki67 positive tumour cells can stratify patients with breast cancer into good and poor prognostic categories.
- Predictor of response to chemotherapy.



Ki67



# Evaluation of biologic prognostic markers

- Accuracy.
- Consistency.
- Reliability.

*Standardisation*

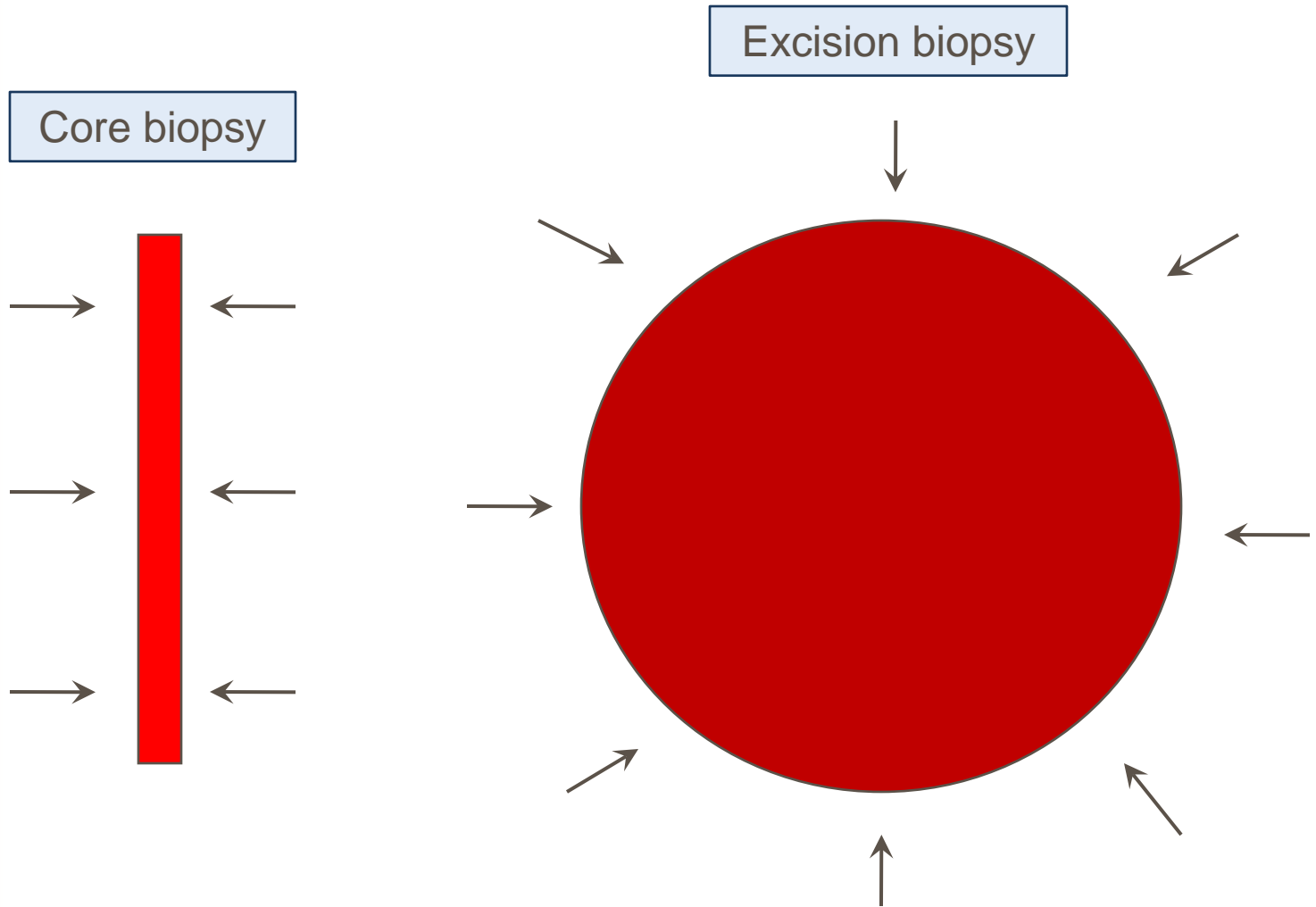
*Quality assurance*



# Evaluation of biologic prognostic markers

- Fixation duration and type of fixative.
- Nature of specimen tested:
  - Core biopsy vs excision specimen.
  - Cell block prepared from needle aspirates.
- Testing platform:
  - Immunohistochemistry (protein).
  - In-situ hybridisation (gene).
  - Expression profiling (mRNA).

# Formalin fixation rate $\sim 1$ mm/h





# Effect of fixation period on HER2 FISH

**SUMMARY** This study investigated if formalin fixation duration affects HER2/neu gene amplification detection by fluorescence in situ hybridization (FISH) in breast cancer. Tumor tissues from 35 cases were divided into three groups and subjected to two formalin fixation protocols per group (12 hr, 27 hr in the first; 2 hr, 17.5 hr in the second; 28.5 hr, 541 hr in the third) before FISH analysis. There was no significant difference in FISH signal detection between the two different fixation protocols in the first two groups. In the third, no signal was detected in 4/6 cases fixed for an extended duration.

(J Histochem Cytochem 50:1693–1696, 2002)

Reliable results for HER2/neu amplification are not compromised by the usual range of routine fixation periods of surgical breast specimens before processing and FISH analysis. Breast specimens fixed for a shortened period are also suitable for FISH analysis. However, a fixation period of more than a week appears to compromise the results obtained by FISH.



# Cell block

- Derived from fine needle aspirates.
- Cell blocks initially fixed in 50% ethanol (4-12 hrs), followed by formalin fixation (minimum 6 hrs).
- Tissue blocks formalin-fixed within 4-8 hrs for 6-48 hrs.
- ER and PR by IHC show good agreement between cell and tissue block samples (weighted Kappa of 0.773 and 0.785, respectively).
- HER2 shows only moderate agreement with a weighted kappa of 0.571.
- Increased discrepant results may be due to ethanol fixation causing false positive increased HER2 expression.

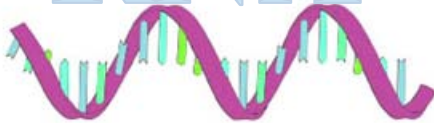
## Methods of interrogation



Genome



RNA



Transcriptome



**PROTEIN**

Proteome

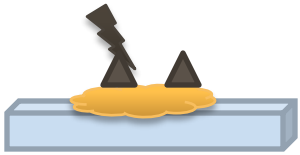
DNA *In situ* hybridisation (ISH)  
[ Fluorescence (FISH)/  
Chromogenic (CISH)/  
Silver (SISH)]

Expression profiling

Immunohistochemistry

# Immunohistochemistry (IHC)

Antigen Retrieval



Blocking



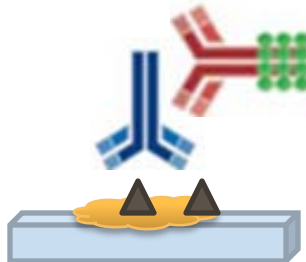
Primary antibody



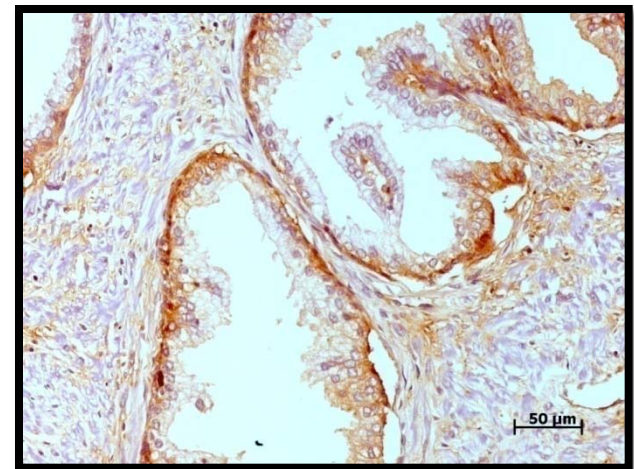
Substrate-Chromogen addition



Labelled Secondary antibody



Counterstain,  
dehydrate, mount  
and visualize





# Evaluation of biologic prognostic markers

- Immunohistochemistry:
  - Type of antibody.
  - Antigen retrieval method.
  - Scoring and cutoff thresholds.

**Table 2**  
**Main Technical Variables for Immunohistochemical Receptor Assays\***

Variable	Proportional Use by Laboratories (%)				Mann-Whitney <i>U</i>	<i>P</i> (2-Tailed)
	With Reproducible Assays		Others			
	Mean	95% CI	Mean	95% CI		
<b>Antigen retrieval</b>						
Sodium citrate buffer <sup>†</sup>	81	73-91	84	80-88	30.000	.833
Microwave oven	34	28-40	60	54-66	0.000	.001
Pressure cooker	54	50-58	26	23-30	0.000	.001
<b>Main primary antibodies</b>						
<b>Estrogen receptor</b>						
Clone 1D5, Dako, Glostrup, Denmark	81	78-83	67	62-73	0.000	.008
Clone 6F11, Novocastra, Newcastle-upon-Tyne, England	17	14-20	19	12-26	7.000	.242
<b>Progesterone receptor</b>						
Clone 1A6, Novocastra	64	60-68	46	40-52	0.000	.021
Clone 1A6, other suppliers <sup>‡</sup>	12	0-26	20	6-34	6.000	.564
<b>Detection systems</b>						
<b>Avidin-biotin systems</b>						
Dako	59	54-66	53	48-58	15.000	.072
Vector	32	26-36	12	10-14	0.000	.001
Other detection systems <sup>§</sup>	9	6-12	35	30-40	0.000	.001
<b>Automation</b>						
None	65	62-69	63	59-68	20.500	.221
Full <sup>  </sup>	18	14-24	18	14-22	28.000	.672
Partial <sup>¶</sup>	14	10-20	16	14-18	31.000	.913

CI, confidence interval.

\* Comparison between laboratories with sensitive and reproducible assays and all other laboratories participating during the same 2-year period.

<sup>†</sup> Sodium citrate buffer, pH 6.0-6.2; 0.01- to 0.2-mol/L concentration.

<sup>‡</sup> Use of other progesterone receptor clones ranged from only 4% (CI, 0%-8%) to 12% (CI, 4%-18%).

<sup>§</sup> Includes avidin-biotin systems from various suppliers.

<sup>||</sup> Includes Optimax, Biogenex (San Ramon, CA); Horizon, Dako; Techmate 500, Dako; Immunostainer, Lab Vision (Newmarket, England); Cadenza, Life Sciences International (Basingstoke, England); Ventana ES, Ventana Medical Systems (Strasbourg, France); and NEXES, Ventana.

<sup>¶</sup> Sequenza, Life Sciences.



# Immunohistochemical scoring method for ER, PR

- Harvey et al. *J Clin Oncol* 1999;17:1474–1481.
  - 9-point, semiquantitative ‘Allred’ score (ranging from 0–8) was performed on a series of almost 2000 patients.
  - Results correlated with response to adjuvant endocrine therapy.
  - Strong direct association between level of ER expression (Allred score), and response to hormonal therapy.
  - Allred scores greater than 2 (corresponds to as few as 1% of cells showing weak immunostaining signal) predicted the largest number of patients who benefited from adjuvant endocrine therapy.
  - Similar cutoff for PR.



## IMMUNOHISTOCHEMICAL EXPRESSION OF HORMONE RECEPTORS IN INVASIVE BREAST CARCINOMA: CORRELATION OF RESULTS OF H-SCORE WITH PATHOLOGICAL PARAMETERS

AYE AYE THIKE, MEI JUAN CHNG, STEPHANIE FOOK-CHONG\* AND PUAY HOON TAN

**H-score:** (3+ % strong nuclear staining) + (2+ % moderate nuclear staining) + (1+ % weak nuclear staining), with the score ranging from 0 to 300.

ER and PR positivity was defined using a H-score of 50 and above. Tumours with H-scores of 50–99 were considered weakly receptor positive.

**Current method:** percentage of at least moderately stained tumour cell nuclei was assessed and ER and PR positivity defined using a cut-off of 10% and above.

*Very good agreement for ER ( $k = 0.879$ ) and good agreement for PR ( $k = 0.662$ ).*



# Evaluation of ER and PR

- Discordance rates:
  - SGH data based on archival triple negative cases from 1994-2007 subjected to repeat immunohistochemical assessment – 26 of 679 (4%) showed presence of one or more markers.  
*(longitudinal retrospective study with changes in fixation and immunohistochemical methods)*


IMMUNOHISTOCHEMISTRY: The invasive carcinoma cells show the following staining characteristics:

Intensity of staining (1+ to 3+)	Percentage of cells stained	Conclusion	Comments
ER			
PR			
c-erb-B2			

*Note to pathologists: Please select one of the indexes below for c-erb-B2.*

- (1) The specimen was fixed in formalin for 6-48 hours. A negative result for c-erb-B2 by immunohistochemical methods may not be valid if the specimen has been fixed for <6 hours or >48 hours.
- (2) The specimen was fixed in formalin for <6 hours / >48 hours / indeterminate number of hours. A negative result for c-erb-B2 by immunohistochemical methods may not be valid if the specimen has been fixed for <6 hours or >48 hours. Consideration should be given to performing confirmatory analysis by FISH.

Note : Paraffin sections of the formalin fixed tissue are stained for estrogen receptor using Neomarker RM9101-S, progesterone receptor using Neomarker RM9102-S and c-erb-B2 using Neomarker RM9103-S. The detection system used is the Ventana multimer detection kit. For estrogen and progesterone receptor study, a result is considered positive if at least 10% of the lesional cells display a minimal 2+ nuclear staining pattern. For c-erb-B2, a test is considered positive if at least 30% of the lesional cells exhibit 3+ cell membrane staining and a borderline/equivocal result is given when at least 10% of the lesional cells show 2+ cytoplasmic membrane staining. Results which fail to fulfil the above criteria are considered negative.



# Immunohistochemical evaluation of ER, PR, c-erbB2

- Preanalytical variables:
  - Nature of specimen.
  - Fixative and duration of fixation.
- Analytical variables:
  - Immunohistochemical methods.
- Interpretive variables:
  - Scoring thresholds.
  - Invasive vs in situ component.



# Clues to questionable ER and PR results

- Internal and external controls are not working.
- Grade 1 tumours reported as ER, PR negative.
- Special subtypes eg tubular, cribriform, mucinous carcinoma reported as ER/PR negative.
- Medullary carcinoma reported as ER/PR/HER2 positive.



# Quality assurance programs

- College of American Pathologists.
- Royal College of Pathologists of Australasia.
- UK National External Quality Assessment Scheme (NEQAS).



# Approach

- Synoptic reports.
- Antibody, probe & result validation.
- Guidelines and recommendations.
- External quality assurance programs.
- Laboratories with high volume load, reliable internal quality controls.
- Regular reviews/discussion at breast multidisciplinary conferences and tumour boards.

