Laboratory Testing for Her2 Status in Breast Cancer

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Overview

- Clinical relevance of Her2 status for treatment of breast cancer
- Standard approaches for determining Her2 status in breast cancer
- Current concepts and controversies in Her2 testing

Who gets breast cancer?

- Breast cancer is one of the most common malignancies to affect women
- About 1 in 8 women will be diagnosed with breast cancer at some point in her lifetime
- Most cases of breast cancer are sporadic, but a small percentage (5-10%) are related to a heritable gene mutation, most commonly *BRCA1* or *BRCA2*
- Having a first degree relative with breast cancer increases a woman's chance of developing breast cancer
- Screening mammography is recommended for older women
  - US Preventive Services Task Force: Every 2 years starting at age 50
  - American Cancer Society, others: Every 2 years starting at age 40

How is breast cancer treated?

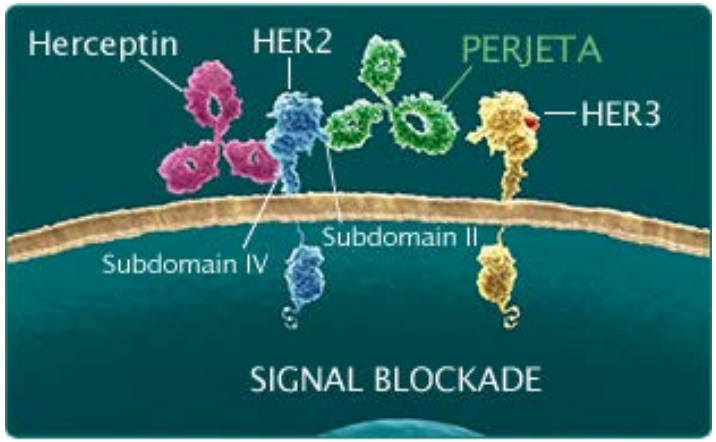
- Surgery: excision with or without sentinel lymph node biopsy
  - Breast conserving: lumpectomy, partial mastectomy
  - Mastectomy
- Chemotherapy: before and/or after surgery
- Radiation

- Targeted therapies
  - Hormone therapy: Tamoxifen, aromatase inhibitors
  - Her2 targeted therapy for cancers with overexpression of the gene *ERBB2*, commonly called Her2 or Her2/neu

- Treatment is based on testing for ER, PR, and Her2 status, as well as cancer grade and stage.

Her2 targeted therapy

- Herceptin (trastuzumab)
- Others: pertuzumab (Perjeta), T-DM1 (Kadcyla), and lapatinib (Tykerb)
- Recent data shows that a combination of pertuzumab, trastuzumab, and docetaxel (PTD) improved progression free survival compared to patients who had only trastuzumab and docetaxel (TD)[1,2]



source:
http://www.perjeta.com/hcp/moa

1. CLEOPATRA trial. Most recent: Swain et al, *NEJM* 2015 Feb 19;372(8):724-34.
2. NeoSphere trial. Gianni et al, *Lancet Oncol* 2012 Jan;13(1):25-32.

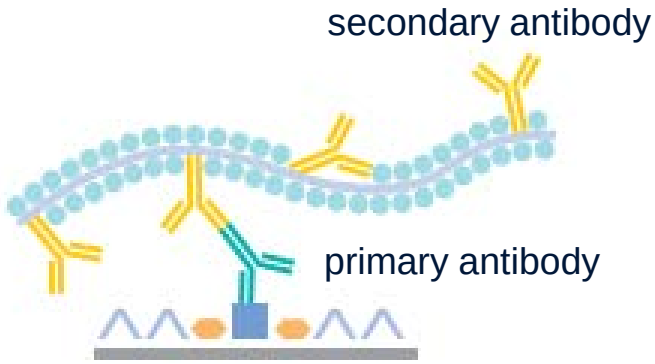
ER, PR, and Her2

- Proteins made by some breast cancers
- ER and PR: Hormone receptors
  - ER: estrogen receptor
  - PR (PgR): progesterone receptor
  - Tested by immunohistochemistry; immunoreactivity in 1% or more cancer cells is considered positive[1]
- Her2: Growth factor receptor
  - Encoded by gene *ERBB2*, also known as Her2/neu, V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog, *etc*.
  - Tested by immunohistochemistry and/or *in situ* hybridization

[1]Hammond MEH, Hayes DF, Dowsett M, et al. American Society of Clinical Oncology/ College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. *Arch Pathol Lab Med*. 2010;134:907–922.

Methods for assessing Her2 status in breast cancer: Immunohistochemistry

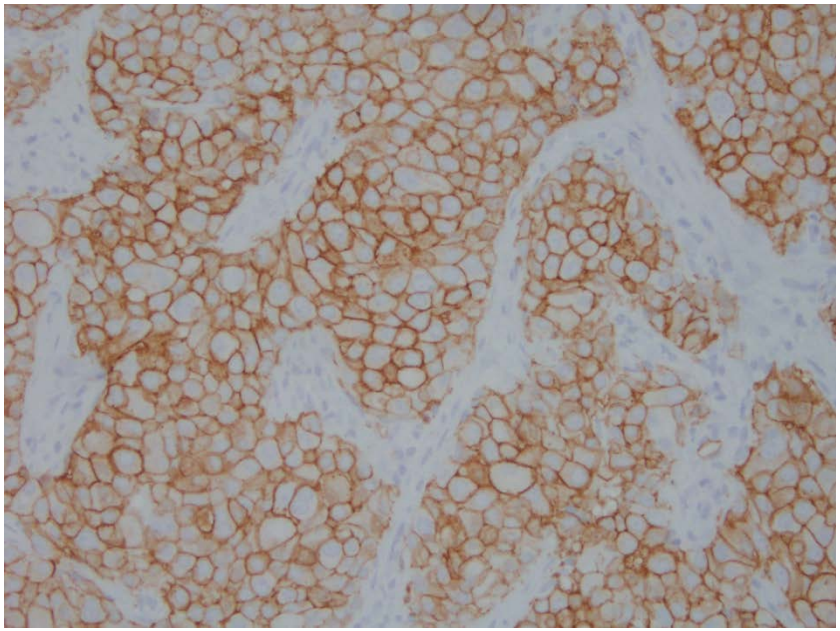
- Antibody directed to Her2 protein, detected with a secondary antibody conjugated to a substrate (horseradish peroxidase)
- Chromogen (DAB) is used to generate stain where Her2 protein binds primary + secondary antibody



Adapted from HercepTest™ Interpretation Manual (Dako)

Methods for assessing Her2 status in breast cancer: Immunohistochemistry

- Staining intensity is correlated to the number of Her2 protein molecules per cell
- Scored according to the intensity and completeness of staining of the cell membrane, where Her2 protein resides
  - Negative (0 or 1+)
  - Equivocal (2+)
  - Positive (3+)



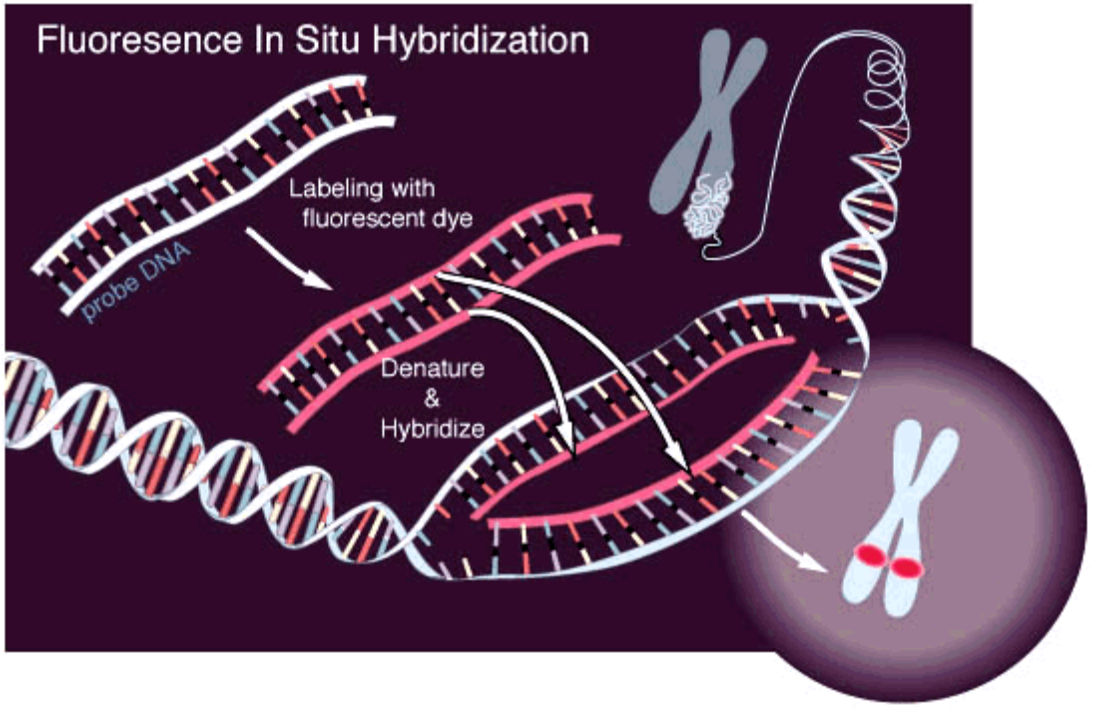
Her2 positive
3+ staining intensity
HercepTest (Dako)

Pros and Cons of Her2 Immunohistochemistry

- Pros
  - Inexpensive
  - Detects Her2 overexpression regardless of mechanism
  - Can visualize with brightfield microscopy under low power, allowing rapid assessment of entire tissue sample tested
- Cons
  - False negatives will be undetected due to lack of internal control
  - Subjective, semi-quantitative interpretation

Methods for assessing Her2 status in breast cancer: *In situ* hybridization

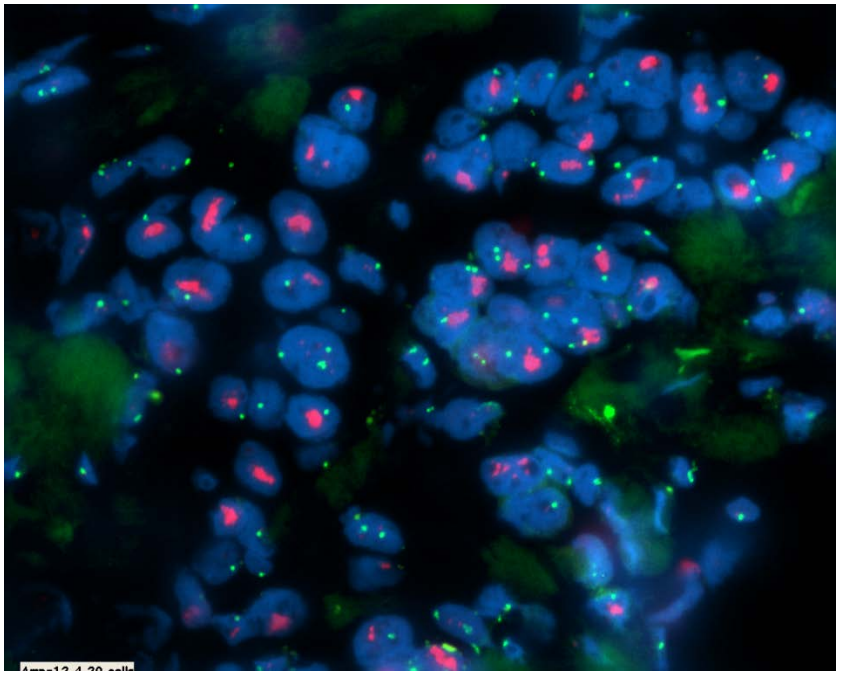
- FISH: fluorescent labeled probe
- Brightfield in situ hybridization is similar but uses non-fluorescent labeling to allow visualization by brightfield microscopy



source: http://en.wikipedia.org/wiki/Fluorescence_in_situ_hybridization

Methods for assessing Her2 status in breast cancer: *In situ* hybridization (FISH)

- FISH slide is scored by enumerating signals for the target (Her2) and the control (CEP17) (chromosome 17 centromere)
- Her2/CEP17 ratio and average Her2 signal count per cell are both used to determine Her2 status
  - Amplified
  - Non-amplified
  - Equivocal
  - Indeterminate



Her2 positive
Her2/CEN-17 ratio ≥2
HER2 IQFISH pharmDx™

Pros and Cons of Her2 FISH

- Pros
  - Less subjective than immunohistochemistry; an absolute quantitative score is generated
  - Her2 amplification by FISH correlates well with overexpression by IHC in breast cancer
  - Direct genetic evaluation of individual tumor cells *in situ* on a slide, allows for evaluation of cell to cell variability, sub-clonal populations
- Cons
  - More expensive than immunohistochemistry
  - More time consuming interpretation
  - Analytic difficulties related to control locus, which can also be abnormal in cancer

ASCO CAP Guidelines (2007)

- Standardization of immunohistochemistry and FISH assays
- Specified tissue handling and formalin fixation times
- Mandated external proficiency testing
- Defined 3 categories of results:

Method	Negative	Equivocal	Positive
IHC	No staining or weak, complete membrane staining <10%	Weak, non-uniform staining ≥10% or Uniform intense membrane staining ≤30%	Uniform intense membrane staining >30%
FISH	Single probe: <4/cell Dual probe: Ratio < 1.8	Single probe: 4-5.9/cell Dual probe: Ratio 1.8-2.2	Single probe: ≥6.0/cell Dual probe: Ratio >2.2

ASCO CAP Updated Guidelines (2013)

– Re-defined 3 categories of results:

Method	Negative	Equivocal	Positive
IHC	No staining or faint, barely perceptible staining	Incomplete and/or weak to moderate membrane staining >10%, or uniform intense membrane staining **≤10%**	Uniform intense membrane staining **>10%**
FISH	Single probe: <4/cell Dual probe: **<4/cell, and ratio <2.0**	Single probe: 4-5.9/cell Dual probe: **4-5.9/cell, and ratio <2.0**	Single probe: ≥6.0/cell Dual probe: Ratio **≥2.0 or ≥6.0/cell**

– Created new category: Indeterminate (technical issues preventing interpretation of test)

What Changed in the 2013 Guidelines?

- Negative:
  - Immunohistochemistry: Re-defined 0 and 1+
  - FISH: **Ratio <2.0 *and* <4 average Her2 copies per cell** (was ratio <1.8)
- Equivocal:
  - Immunohistochemistry: **≤10% intense membrane staining** (was <30%)
  - FISH: **4 to 5.9 average Her2 copies per cell** (was ratio 1.8-2.2)
- Positive:
  - Immunohistochemistry: **>10% intense membrane staining** (was ≥30%)
  - FISH: **Ratio ≥2.0 *or* ≥6 average Her2 copies per cell** (was ratio >2.2)

Additional Changes in the 2013 Guidelines

- Included guidance on new technologies
  - Brightfield in situ hybridization: guidelines same as FISH
  - DNA microarray and mRNA expression assays: insufficient evidence to support clinical use for Her2 status
- Resolved discordance between different existing methodologies
  - Single vs. dual probe FISH assays
- Minimized false negatives by lowering thresholds for equivocal and amplified/positive
- Broadened recommendations: Her2 testing on all primary and recurrent/metastatic breast cancers
- Promoted early testing of all breast cancers (diagnostic biopsy instead of excision)
- Provided route for resolving discrepancies between Her2 testing and histology
- Updated definition of genetic heterogeneity

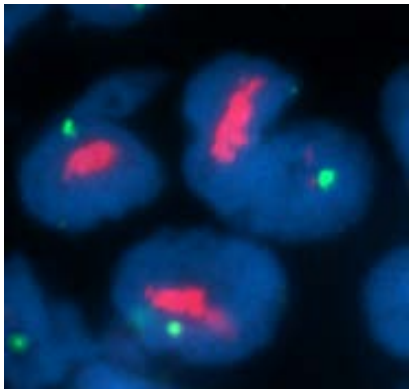
Clinical Impact of Changed Guidelines

HER2 Amplification Status of IHC Equivocal (2+) Cases by Percent Membrane Staining

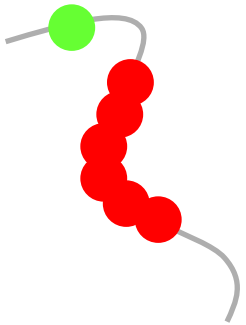
IHC 2+	FISH Amplified (2013 Guidelines) (%)
2+ Cases (All)	58/707 (8.2%)
2+ Cases, 10-30% Membrane Staining	38/507 (7.5%)
Strong	1/8 (12.5%)
Moderate	17/136 (12.5%)
Weak	20/363 (5.5%)

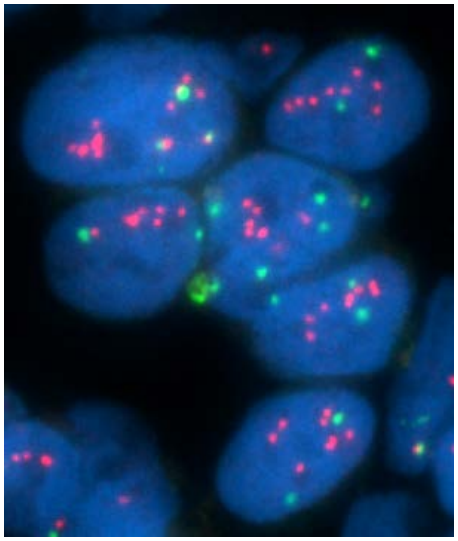
Poster presentation at San Antonio Breast Cancer Symposium, December 2014
Gulbahce et al, Effect of the New 2013 ASCO / CAP Guidelines on HER2 Reporting.

Patterns of Her2 amplification

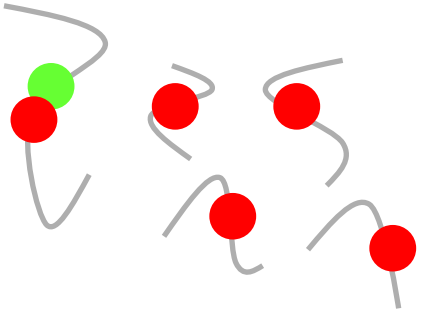


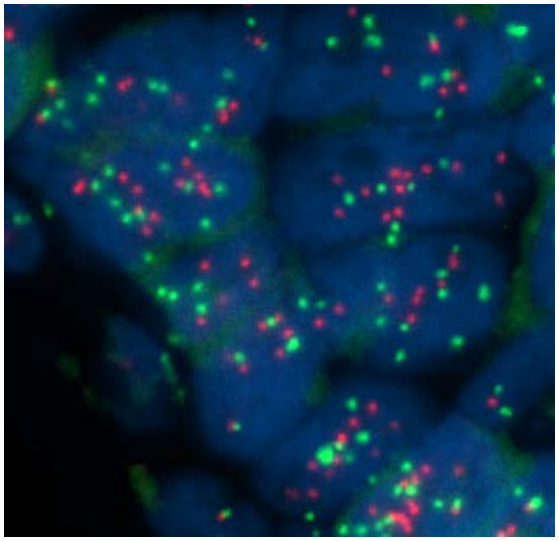
intrachromosomal
(“stacked” signals)



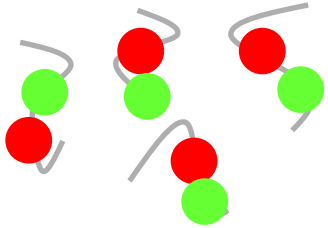


extrachromosomal



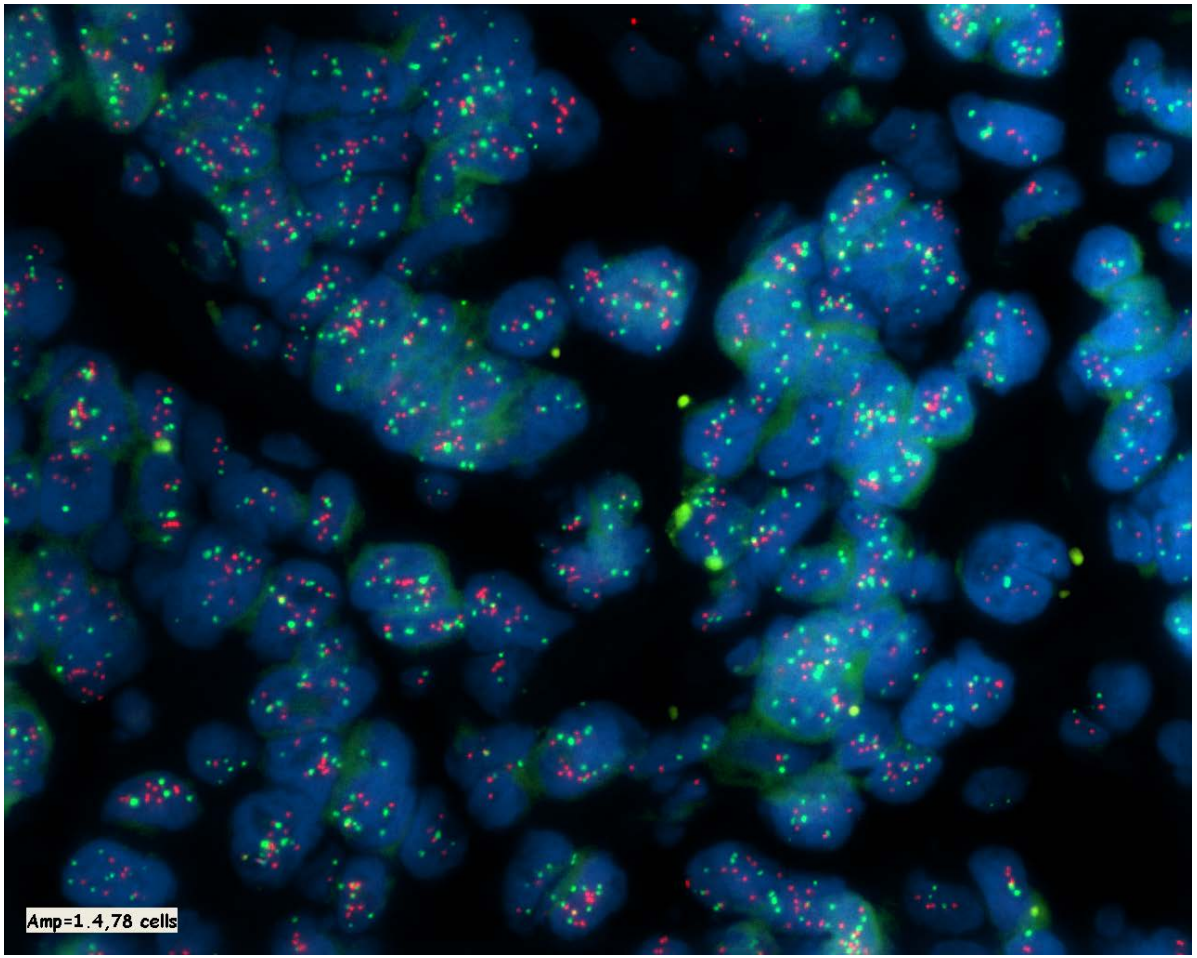


co-amplification



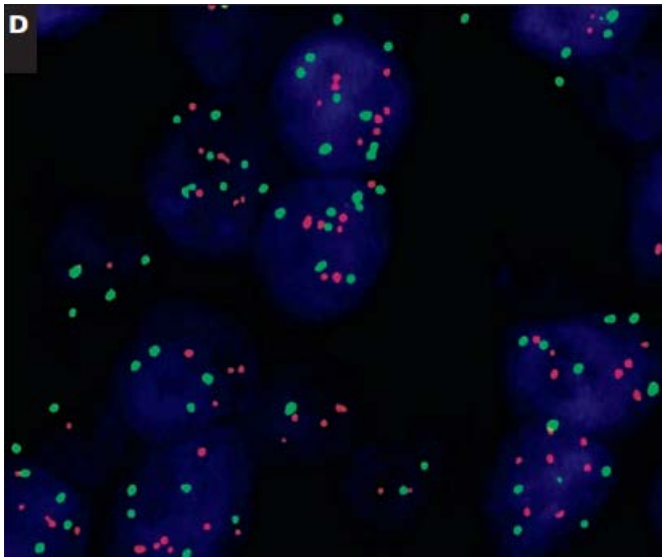
Challenges in Her2 FISH Testing

1. Polysomy / Co-amplification

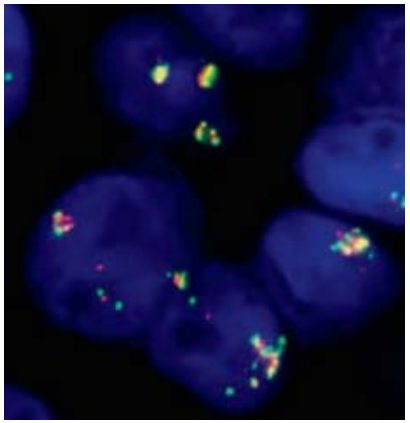


Chromosome 17 “polysomy” in Her2 FISH testing

- Extra copies of chromosome 17 centromere
- 3 or more copies of CEP17 (avg/cell): ~8% of cases, mostly those with 4-6 Her2 copies per cell (equivocal range)[1]



common



rare

Colocalization (“Coamplification”)[2]

1. Wolff AC et al. ASCO/CAP guideline for Her2 testing (2007)
2. Starczynski et al. Am J Clin Pathol. 137**,** 595-605 (2012)

What is “polysomy”?

- Extra whole copies of a chromosome
- Normal diploid state is 2 copies
- 3 or more copies is polysomy
- Polysomy is harder to define on FFPE sections due to signal truncation
  - Average signal count for diploid state is < 2 in FFPE
  - Polysomy has been defined in the medical literature as average signal counts as low as 1.86[1] and ranging up to >3
  - Most commonly adopted threshold is mean of ≥3 CEP17 signals per nucleus[2]

1. Watters et al. Breast cancer research and treatment. 2003 77(2):109-14, 2003.
2. Hanna et al. Modern Pathology 27:4-18, 2014.

Does chromosome 17 “polysomy” affect Her2 expression?

- Most cases not associated with Her2 protein or mRNA overexpression[1,2]

TABLE 2. Breakdown of CEP17 Polysomy by IHC, Mean *HER2* Copy Number, Mean CEP17 Copy Number, Mean *HER2*/CEP17 Ratio, and ISH for HER2 mRNA

IHC	CEP17+	Mean *HER2* Copy	Mean CEP17	Mean *HER2*/CEP17 Ratio	ISH for HER2 mRNA
0–1+	39/56 (69%)	4.2	3.1	1.4	16/16 without *HER2* gene overexpression
2+	15/56 (27%)	4.6	3.0	1.5	9/9 without *HER2* gene overexpression
3+	2/56 (3%)	4.5	3.1	1.5	1/1 without *HER2* gene overexpression

- Absolute Her2 signal number per cell of 6.0 or greater is correlated with overexpression of Her2, regardless of the Her2/CEP17 ratio[2]

1. Downs-Kelly et al, AJSP 2005 Sep;29(9):1221-7. (data shown above: Table 2)
2. Dal Lago et al. Molecular cancer therapeutics. 2006 Oct;5(10):2572-9.

Where are the extra copies of chromosome 17 centromere?

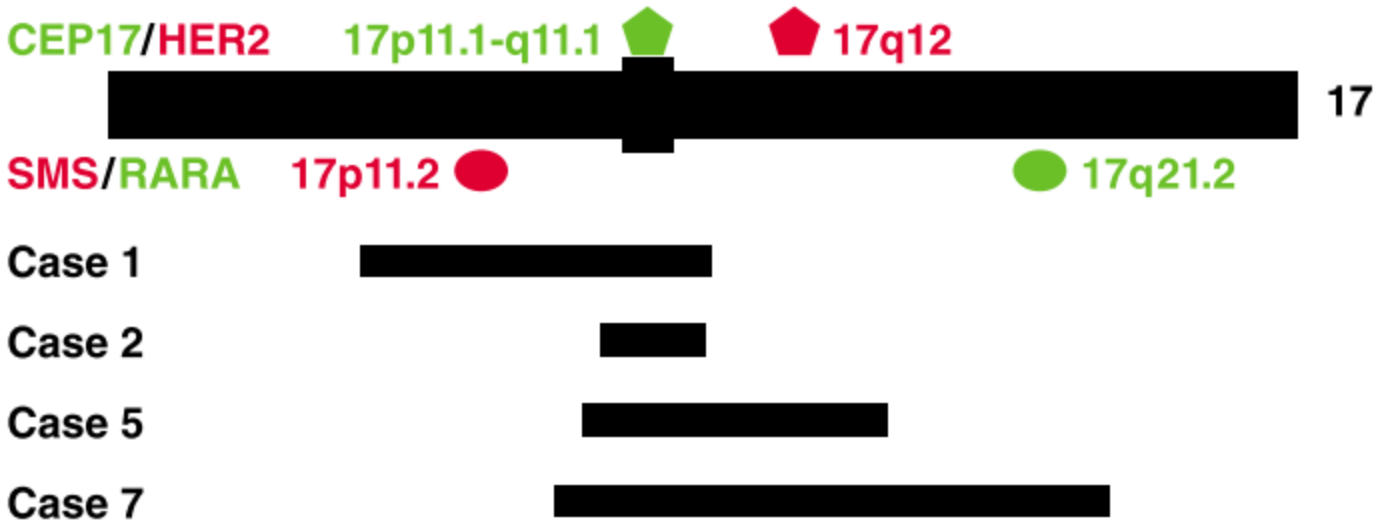
- In extra whole copies of chromosome 17 (“true polysomy”)
- In extra structurally abnormal (deleted, duplicated, rearranged) DNA molecules:
  - Extra structurally abnormal copies of chr17 (centromere 17 present)

    or

  - Separate “marker” (structurally abnormal, unidentifiable) chromosomes

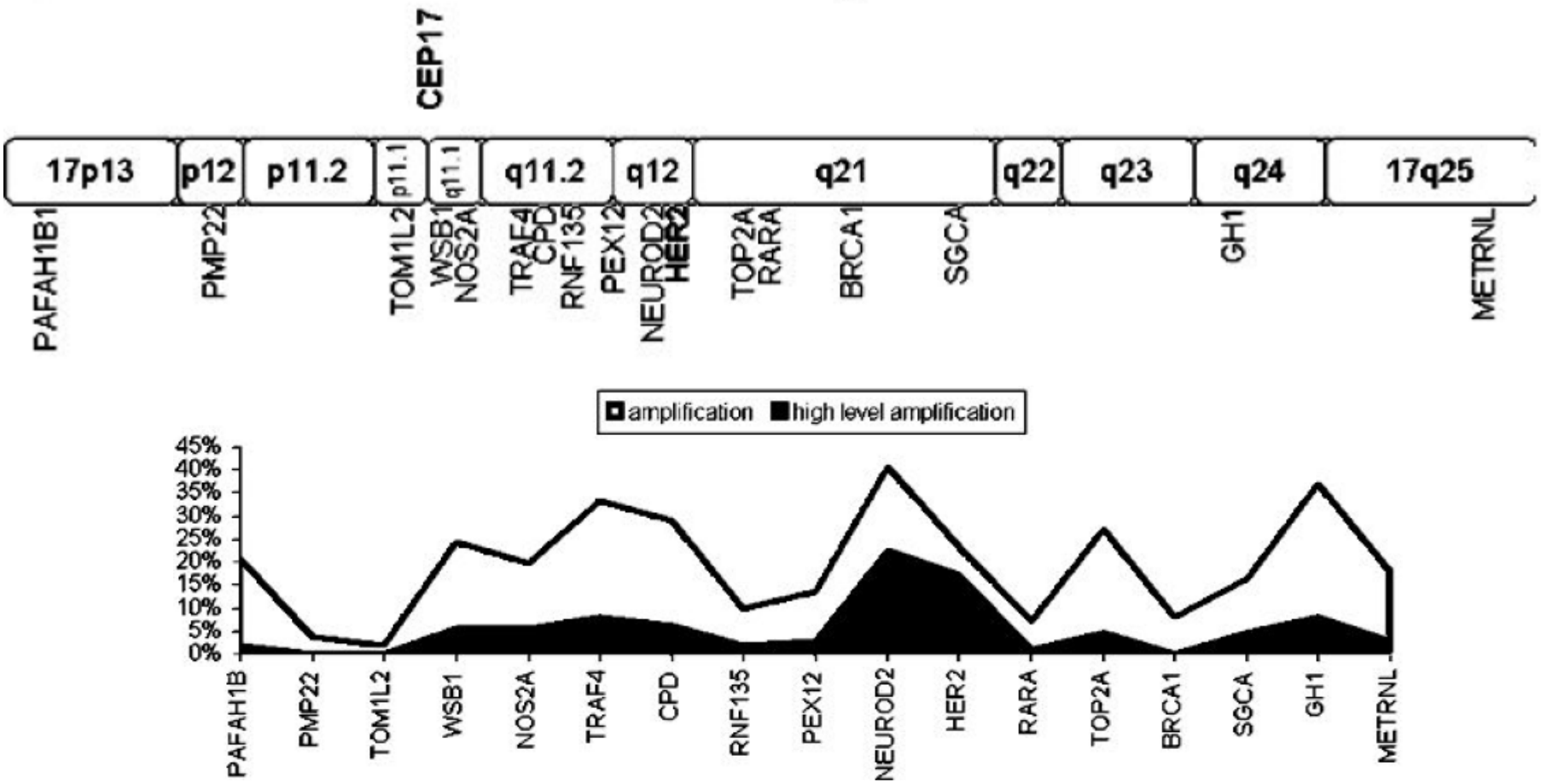
  - **Co-amplification: discrete segments of the genome are amplified together, often in tandem on the same chromosome or on separate “marker” (structurally abnormal, unidentifiable) chromosomes**

FISH with SMS/RARA probes to resolve Her2 status



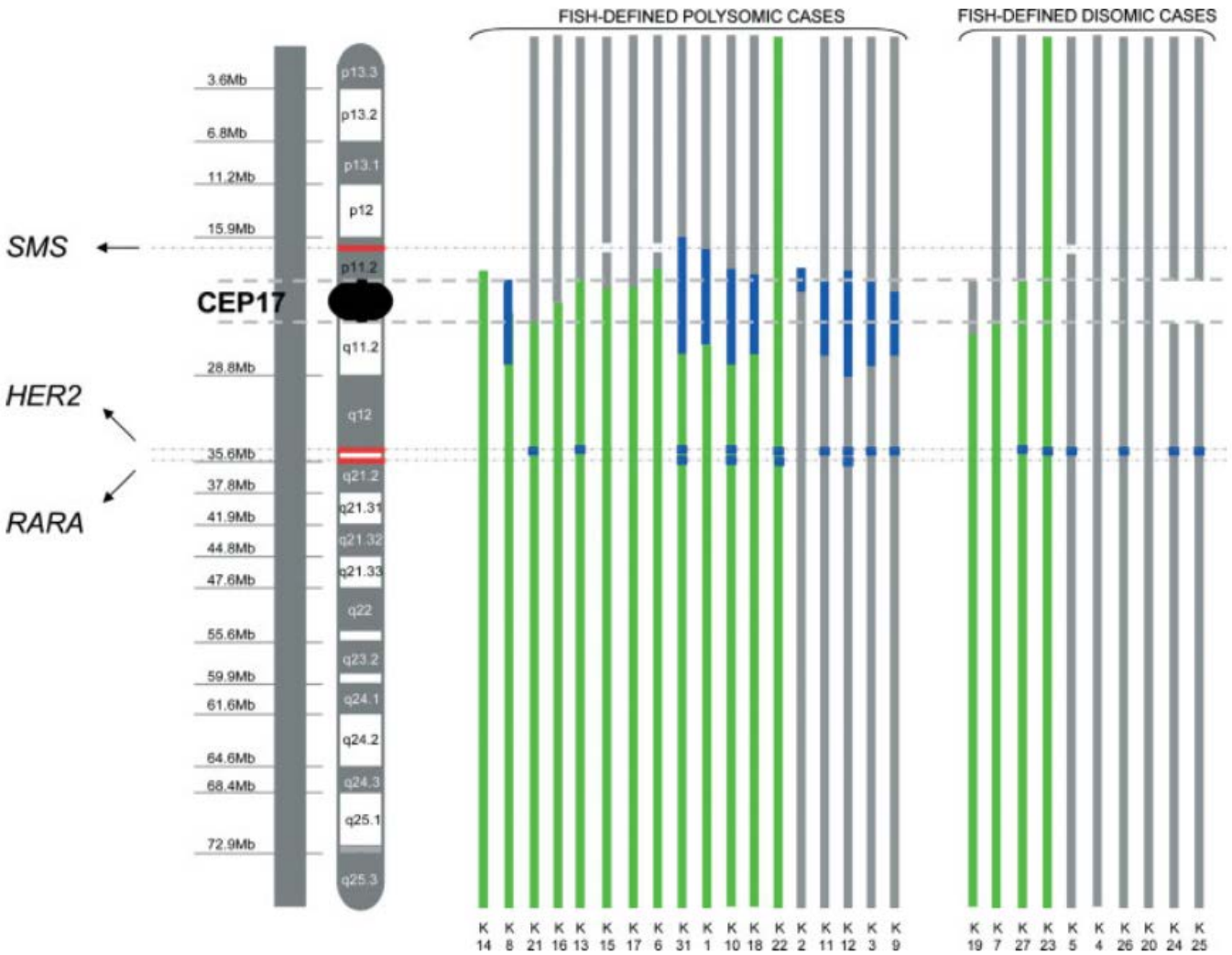
Troxell ML et al. *Am J Clin Pathol*. 126**:** 709-16, 2006

Multiplex ligation-dependent probe amplification (MLPA) – chromosome 17



Moelans, C. B., et al. Breast Cancer Res Treat. 120**:** 1-7, 2010

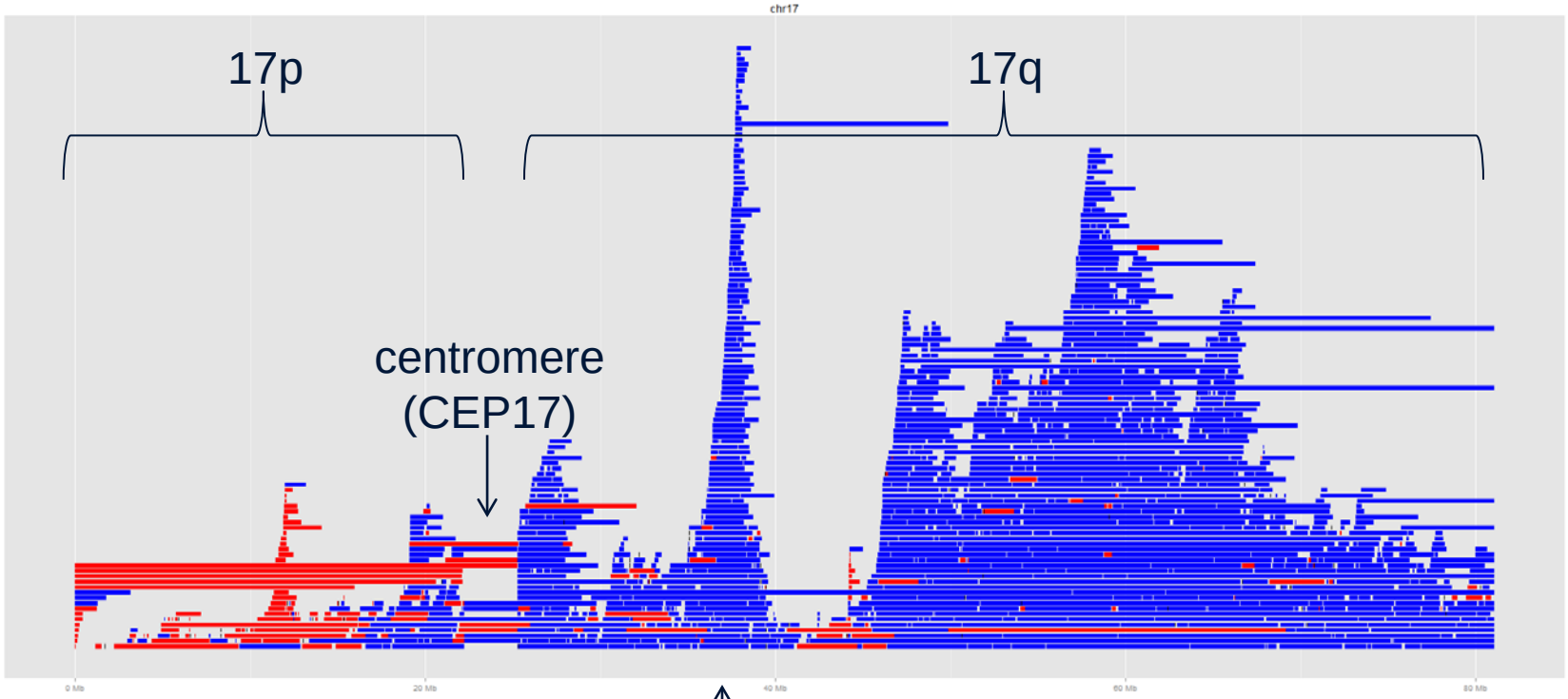
Microarray CGH: chromosome 17



Green: gain
Blue: amplification
Gray: no change
(White): deletion

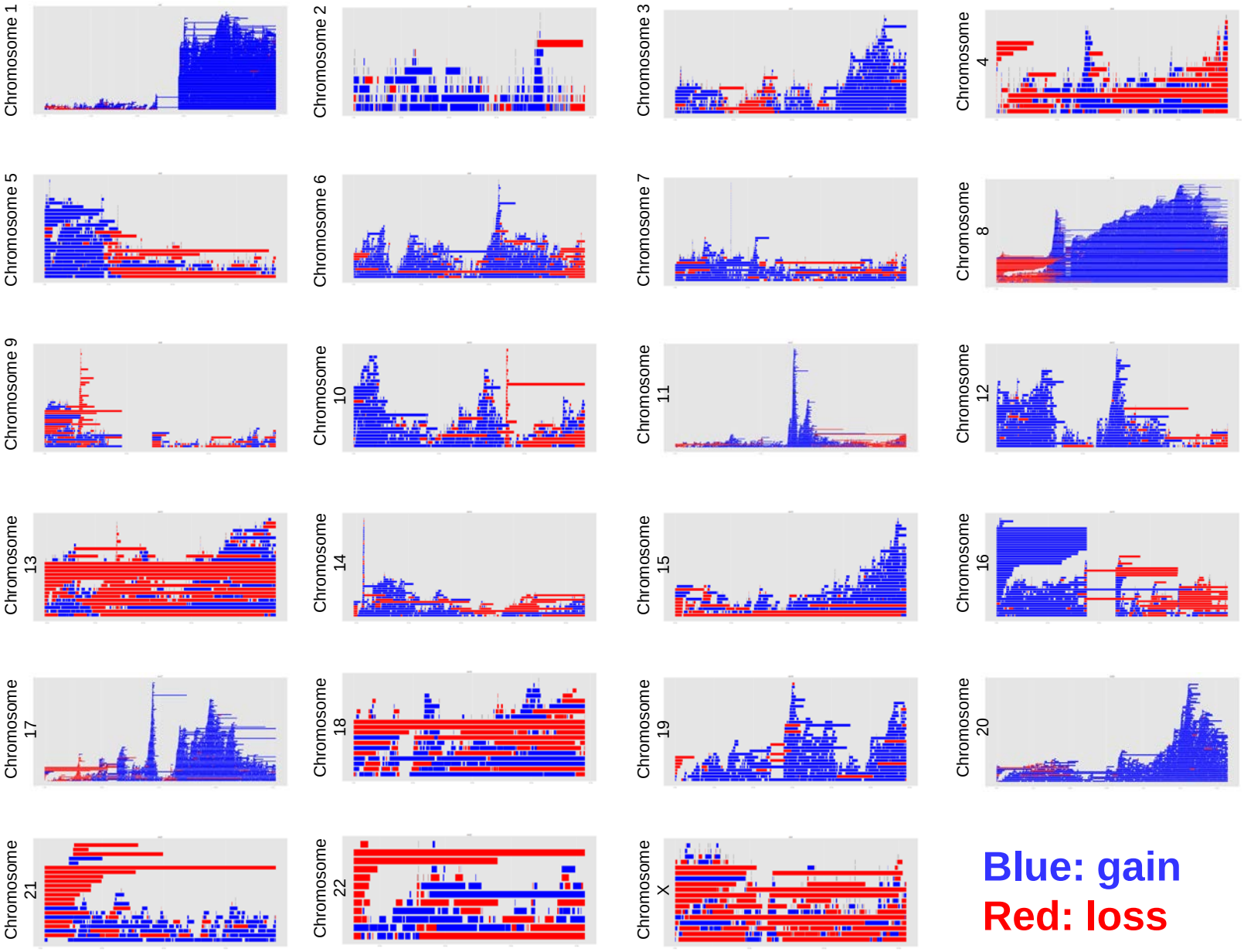
Marchio et al, J Pathol. 219**:** 16-24, 2009

TCGA: DNA copy number on 773 breast tumors (SNP microarray) *Nature* 490: 61-70, 2012



Graphic courtesy of Wei Shen

Blue: gain
Red: loss



Blue: gain

Red: loss

Graphic courtesy of Wei Shen & Lisa Collins

Copy number gains in the context of the cancer genome

- Entire genome may be present in 3 or more copies (on average), i.e. “polyploidy,” confounding the definition of “normal” or “control” for the genome
  - Polyploidy may not be detected on microarray analysis, depending on the software tools and bioinformatic approach used for analysis
- Adult solid tumors are known to have complex genomes, characterized by gains, losses, allelic imbalances encompassing large portions of the genome
- Absolute copy number per cell can be estimated by some techniques, but not others
  - FISH, flow cytometry, cytogenetics: individual cell analysis
- Reference/ “control” region(s) may also be abnormal

(The Search for a Perfect Control)

- CEP17 is co-amplified in a fraction of cases
- Another gene region on chromosome 17 may be used as a control

But…..

- No region of the genome is immune to copy number changes in cancer

And…

- Chromosome 17 is especially prone to copy number changes in breast cancer

Resolution of Equivocal Her2 FISH

- ASCO-CAP 2013 Guidelines recommend using an alternate control probe for a gene on chromosome 17

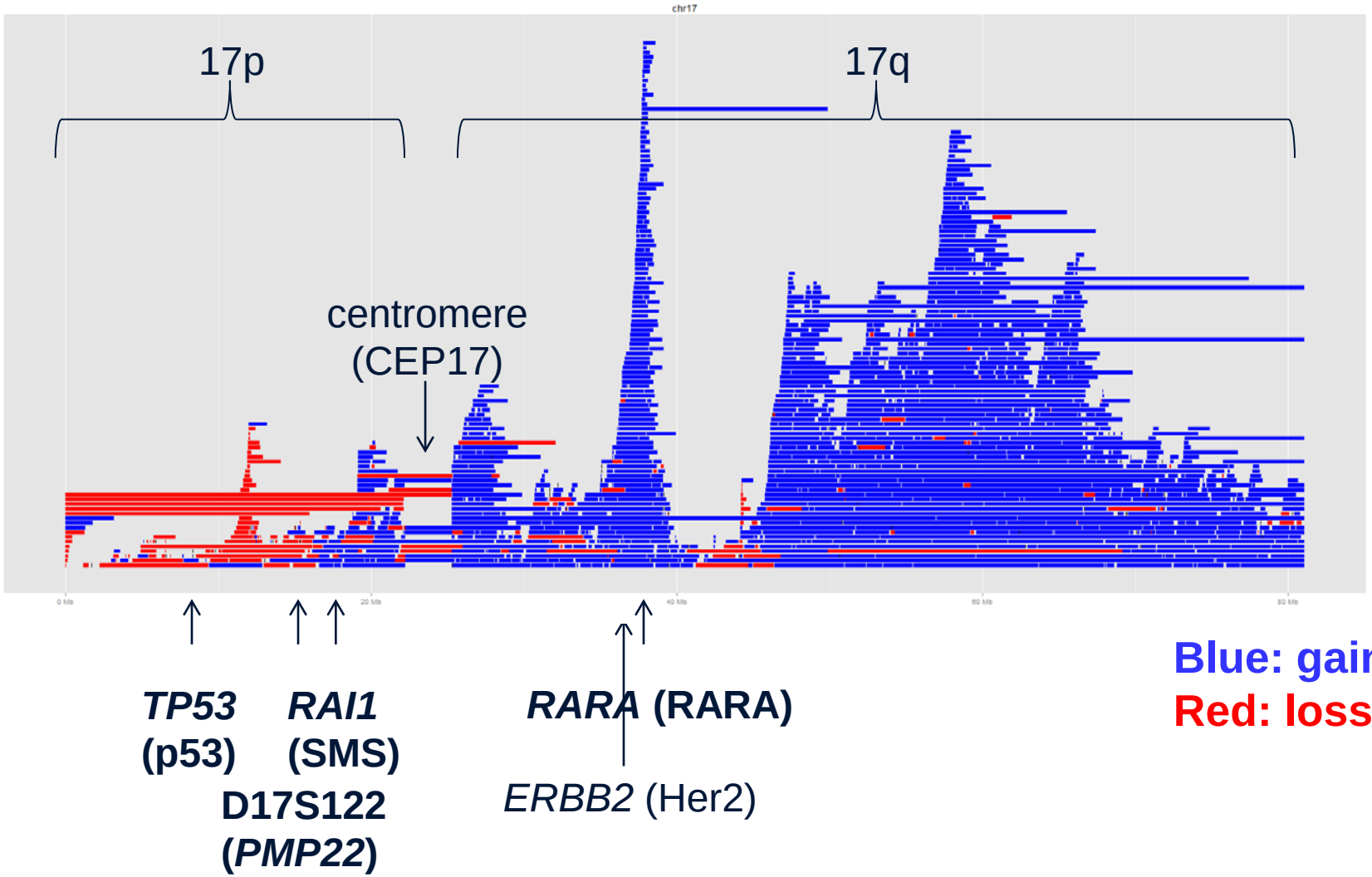
- What if the alternate control probe is also abnormal (deleted or amplified)?
  - No guidelines on interpretation or further reflex testing

Cut-off values for alternate control probe

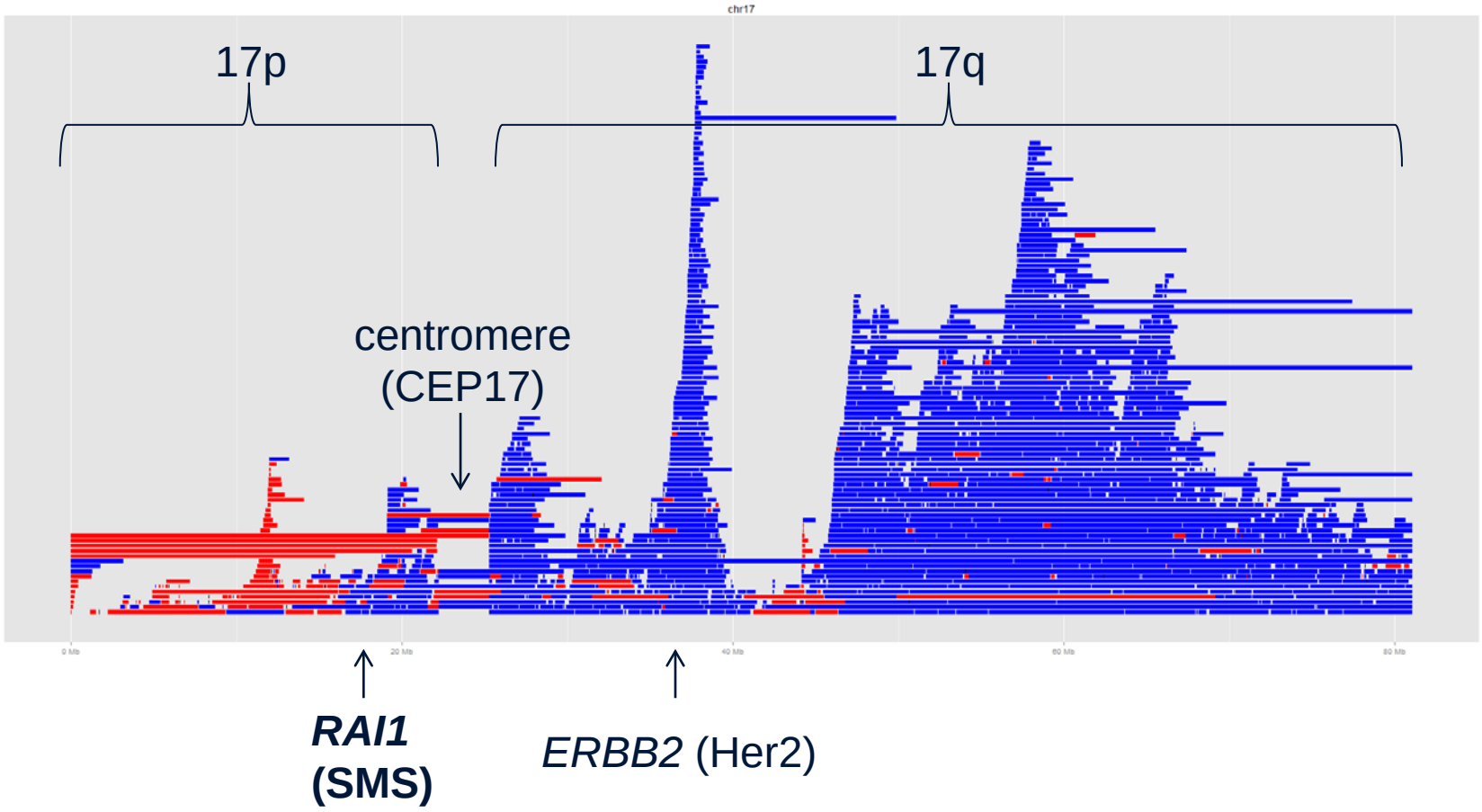
Study	Probe(s) used	Cutoff: Deleted	Cutoff: Amplified
Troxell (2006)	SMS (*RAI1*), RARA	none	Not specified
Tse (2011)	SMS (*RAI1*), RARA, TP53	none, highest of 3 probes <2.6 used as new control to calculate Her2 ratio	≥2.6
Mansfield (2013)	D17S122 (*PMP22*)	none	Not specified*

* CEP17 ≥6.0 was defined as co-amplification of chromosome 17 centromere

Alternate control probes used to resolve Her2 double equivocals



Reflex FISH Testing for Double Equivocals



12/773 (1.6%) in TCGA study copy number alterations at *RAI1*

Challenges in Her2 FISH Testing

2. Genetic Heterogeneity

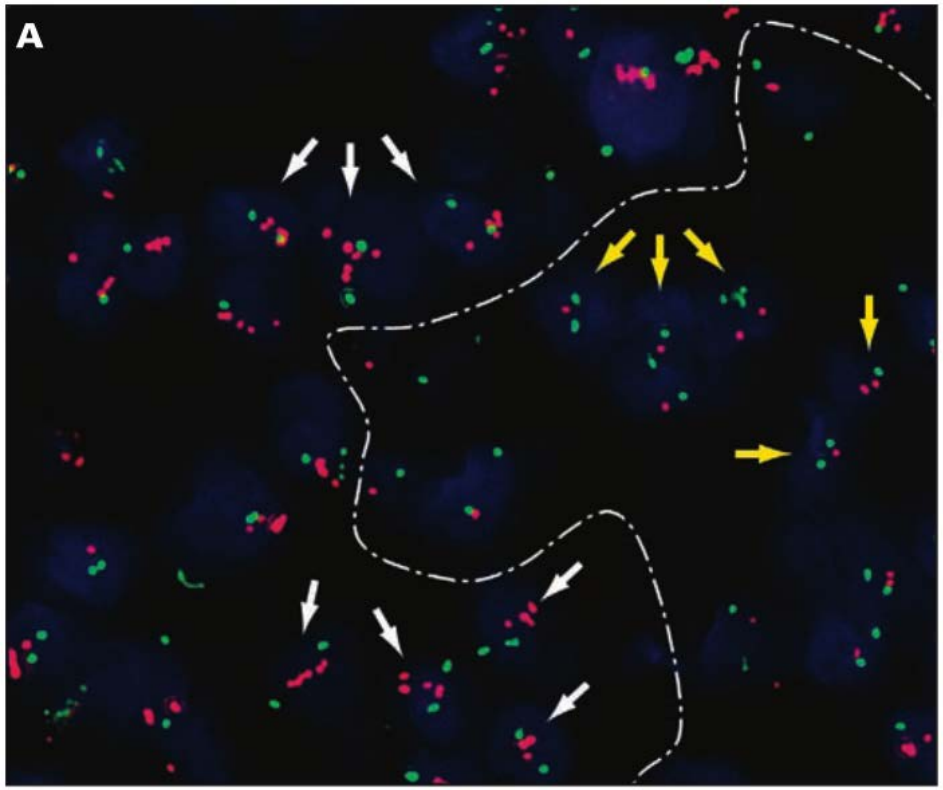


Fig. 1A from Starczynski et al. HER2 gene amplification in breast cancer: a rogues' gallery of challenging diagnostic cases: UKNEQAS interpretation guidelines and research recommendations. *Am J Clin Pathol*. 137**,** 595-605, 2012.

Genetic Heterogeneity

- A subpopulation of tumor cells shows amplification, while the rest of the tumor is non-amplified
- 2009 guideline: More than 5% but less than 50% infiltrating tumor cells with a ratio higher than 2.2
  - Must report % amplified, pattern (scattered or discrete population) and whether cells are histologically distinctive
  - Problems with spurious “amplified” cells defined only by ratio of individual cells (e.g. 1 green and 3 red signals)
- 2013 update: More than 10% infiltrating tumor cells with increased Her2 signals/cell
  - Only reported if there is a discrete subpopulation of amplified cells, and score the amplified and non-amplified cell populations separately

1. Vance, G. H., et al., 2009. Genetic heterogeneity in HER2 testing in breast cancer: panel summary and guidelines. Arch Pathol Lab Med. 133, 611-2.

Cell	CEP17	H2N	Ratio
1	2	4	2.000
2	1	4	4.000
3	2	4	2.000
4	3	4	1.333
5	2	5	2.500
6	2	3	1.500
7	3	4	1.333
8	3	6	2.000
9	3	4	1.333
10	2	5	2.500
11	3	4	1.333
12	3	4	1.333
13	3	4	1.333
14	3	5	1.667
15	3	5	1.667
16	3	5	1.667
17	3	5	1.667
18	3	4	1.333
19	1	3	3.000
20	3	5	1.667
n	60	60	60.000
Total	124	233	126.500
Mean	2.0667	3.8833	1.879
d dev	0.8206	1.0591	0.823
SEM	0.1059	0.1367	0.106

CAP-EP classification: Heterogeneous based on 20% of individual cells with ratio >2.2

Original classification: Equivocal based on ratio of mean HER2 to CEP17 signals being 1.8-2.2

Allison et al. Frequency of HER2 heterogeneity by fluorescence in situ hybridization according to CAP expert panel recommendations: time for a new look at how to report heterogeneity. *Am J Clin Pathol*. 136**,** 864-71, 2011.

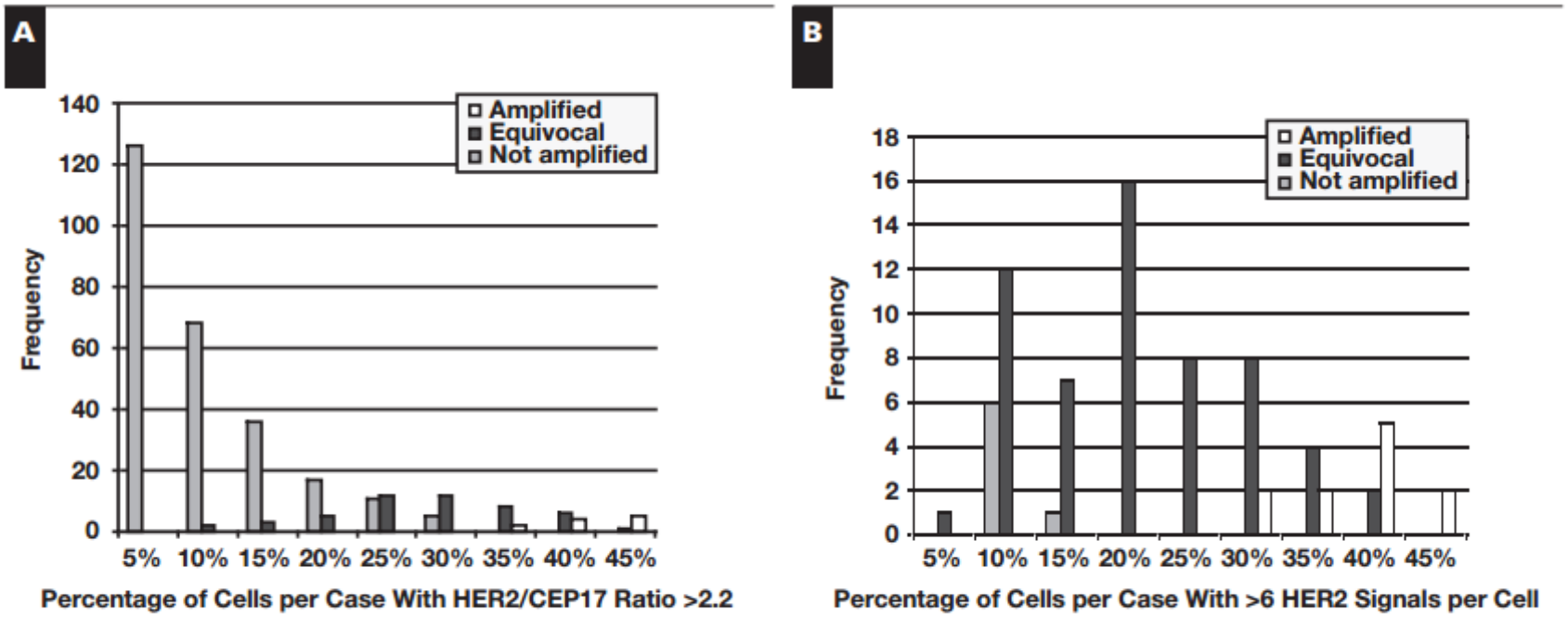


Figure 3 Standard College of American Pathologists/American Society of Clinical Oncology classification by HER2/centromere 17 (CEP17) ratio (**A**) and number of signals per cell (**B**).

Allison et al. Frequency of HER2 heterogeneity by fluorescence in situ hybridization according to CAP expert panel recommendations: time for a new look at how to report heterogeneity. *Am J Clin Pathol*. 136**,** 864-71, 2011.

Summary

- Immunohistochemistry and in situ hybridization (ISH, FISH) are the recommended methods for determining Her2 status for treatment with Her2-targeted therapy
- Neither method is 100% sensitive or specific
- Updated ASCO-CAP (2013) guidelines have resulted in increased proportion of patients being eligible for Her2-targeted therapy
- Her2-positive cases are not a homogeneous group
  - Borderline positive cases may not be as responsive to Her2-targeted therapy
- Challenges in Her2 laboratory testing include polysomy / co-amplification, and genetic heterogeneity

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