

Material

The slide to be stained for HER-2 comprised the following 5 tissues:

	IHC	FISH
	HER-2 score* (0, 1+, 2+, 3+)	HER-2/chr17 ratio**
1. Breast ductal carcinoma	0-1+	1.2 – 1.4
2. Breast ductal carcinoma	0-1+	1.2 – 1.5
3. Breast ductal carcinoma	1-2+	1.4 – 1.7
4. Breast ductal carcinoma	2-3+	2.5 – 2.8
5. Breast ductal carcinoma	3+	> 6.0



* HER-2 immunohistochemical score (see table below) as achieved by using the two FDA approved kits and antibodies, HercepTest™, Dako, and PATHWAY®, Ventana, in NordiQC reference laboratories.

** HER-2 gene/chromosome 17 Ratio achieved by using HER-2 FISH pharmDX™ Kit, Dako, and Inform HER-2 Dual colour ISH, Ventana.

All carcinomas were fixed for 24 - 48 h in 10 % neutral buffered formalin.

IHC scoring system according to the guidelines given by ASCO/CAP:

Score 0	No staining is observed or cell membrane staining is observed in less than 10% of the tumour cells.
Score 1+	A faint perceptible membrane staining can be detected in more than 10% of the tumour cells. The cells are only stained in part of their membrane.
Score 2+	A weak to moderate complete membrane staining is observed in more than 10% of the tumour cells.
Score 3+	A strong complete membrane staining is observed in more than 30% of the tumour cells.

Criteria for assessing a HER-2 staining as optimal included:

- A clear and unequivocal staining marked as score 0 or 1+ in the breast ductal carcinomas no. 1 & 2.
- A clear and unequivocal staining marked as score 1+ or 2+ in the breast ductal carcinoma no. 3.
- A clear and unequivocal staining marked as score 2+ or 3+ in the breast ductal carcinoma no. 4.
- A clear and unequivocal staining marked as score 3+ in the breast ductal carcinoma no. 5.
- No or only a weak cytoplasmic reaction that did not affect the interpretation of the true membranous HER-2 reaction.

A staining was assessed as good, if the HER-2 gene amplified tumour no. 5 showed a 2+ reaction (an equivocal 2+ IHC staining should always be analyzed by ISH according to the ASCO/CAP guidelines and the national guidelines in Scandinavia) and the other breast carcinomas showed a reaction pattern as described above.

A staining was assessed as borderline if the signal-to-noise ratio was low, e.g., because of moderate cytoplasmic reaction, excessive counterstaining or excessive retrieval hampering the interpretation.

A staining was assessed as poor in case of a false negative staining (e.g. the 3+ tumour and the 2+ tumour with gene amplification showing a 0 or 1+ reaction) or a false positive staining (e.g. the 0, 1+ and 2+ tumours without gene amplification showing a 3+ reaction).

Results

253 laboratories participated in this assessment. 83 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. The IHC systems/Abs used and the assessment marks given:

FDA-/CE-IVD approved HER-2 systems	N	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
PATHWAY® rmAb clone 4B5, 790-2991	72	Ventana	62	9	1	0	99 %	100 %
CONFIRM™, rmAb clone 4B5, 800-2996	38	Ventana	33	3	0	2	95 %	97 %
rmAb clone 4B5, 790-4493	9	Ventana	9	0	0	0	100 %	100 %
HercepTest™ SK001	28	Dako	19	4	0	5	82 %	91 %
HercepTest™ K5204	7	Dako	2	1	0	4	43 %	67 %
HercepTest™ K5207	12	Dako	6	2	0	4	67 %	78 %
Oracle™ mAb clone CB11, TA9145	7	Leica	1	3	0	3	57 %	67 %
Abs for in-house HER-2 systems, conc. Ab	N	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone CB11	5	Leica/Novocastra	4	1	1	0	83 %	100 %
	1	BioGenex						
mAb clone e-2-4001+3B5	1	Thermo/NeoMarkers	0	1	0	0	-	-
rmAb clone EP1045Y	1	Biocare	2	0	0	0	-	-
	1	Epitomics						
rmAb clone EP3	1	Epitomics	1	0	0	0	-	-
rmAb clone SP3	16	Thermo/NeoMarkers	9	5	0	5	74 %	85 %
	2	Spring						
	1	Master Diagnostica						
pAb clone A0485	49	Dako	22	9	2	16	63 %	63 %
Abs for in-house HER-2 systems, RTU Ab								
mAb clone CB11, RTU-CB11	2	Leica/Novocastra	1	0	1	0	-	-
Total	253		171	38	5	39	-	-
Proportion			68 %	15 %	2 %	15 %	83 %	-

1) Proportion of sufficient stains (optimal or good), 2) Proportion of sufficient stains with optimal protocol settings only, see below.

3) mAb: mouse monoclonal antibody, rmAb: rabbit monoclonal antibody, pAb: polyclonal antibody.

FDA/CE IVD approved systems

PATHWAY® rmAb clone **4B5** (Ventana): 62 out of 72 (86 %) protocols were assessed as optimal. The protocols giving an optimal result were typically based on Heat Induced Epitope Retrieval (HIER) in Cell Conditioning 1 (CC1), mild or standard, in the BenchMark XT or Ultra, an incubation time in the primary Ab in the range of 12 – 36 min. and either iView or UltraView being used as the detection kit. Using these protocol settings 69 out of 69 (100 %) laboratories produced a sufficient staining (optimal or good).

CONFIRM™ rmAb clone **4B5** (Ventana): 33 out of 38 (87 %) protocols were assessed as optimal. The protocols giving an optimal result were typically based on HIER in CC1, mild or standard, in the BenchMark XT or Ultra, an incubation time in the primary Ab in the range of 8 – 32 min. and either iView, UltraView or OptiView being used as the detection kit. Using these protocol settings 35 out of 36 (97 %) laboratories produced a sufficient staining.

790-4493 rmAb clone **4B5** (Ventana): 9 out of 9 (100 %) protocols were assessed as optimal. The protocols giving an optimal result were typically based on HIER in CC1, mild or standard, in the BenchMark XT or Ultra, an incubation time in the primary Ab in the range of 8 – 32 min. and either iView or UltraView being used as the detection kit.

HercepTest™ SK001 (Dako): 19 out of 28 (68 %) protocols were assessed as optimal. The protocols giving an optimal result were typically based on HIER in HercepTest epitope retrieval solution at 97 - 99°C for 40 min in a water bath or PT Link and an incubation time of 30 min in the primary Ab. Using these protocol settings 21 out of 23 (91 %) laboratories produced a sufficient staining.

HercepTest™ K5204 (Dako): 2 out of 7 (29 %) protocols were assessed as optimal. One protocol giving an optimal result were based on HIER in HercepTest epitope retrieval solution at 99°C for 40 min in a water bath and an incubation time of 30 min in the primary Ab. The other protocol used a Pressure Cooker for HIER and an incubation time of 30 min in the primary Ab. Using these protocol settings 2 out of 3 (67 %) laboratories produced a sufficient staining.

HercepTest™ K5207 (Dako): 6 out of 12 (50 %) protocols were assessed as optimal. The protocols giving an optimal result were based on HIER in HercepTest epitope retrieval solution at 97 - 99°C for 20-40 min in a water bath or PT Link and an incubation time of 30 min in the primary Ab. Using these protocol settings 7 out of 9 (78 %) laboratories produced a sufficient staining.

Oracle™ (Leica) mAb clone CB11: 1 out of 7 (14 %) protocols was assessed as optimal. The protocol giving an optimal result was based on HIER in Bond Epitope Retrieval Solution (BERS1) for 25 min. and an incubation time for 30 min. in the primary Ab. Using similar protocol settings 4 out of 6 (67 %) laboratories produced a sufficient staining.

Abs for in-house systems (conc. Ab)

mAb **CB11**: 4 out of 6 protocols (67 %) were assessed as optimal. The protocols giving an optimal staining were based on HIER using CC1 (BenchMark, Ventana) (2/2)*, Bond Epitope Retrieval Solution 2 (BERS 2; Bond, Leica) (1/1) or Tris-EDTA/EGTA pH 9 (1/2). The mAb CB11 was diluted in the range of 1:70-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings 4 out of 4 (100 %) laboratories produced a sufficient staining.

* (number of optimal results/number of laboratories using this buffer)

rmAb **EP1045Y**: 2 out of 2 protocols (100 %) were assessed as optimal. The protocols giving an optimal staining were based on HIER using Diva Decloaker pH 6.2 (Biocare) (1/1) or Tris-EDTA/EGTA pH 9 (1/1). The rmAb EP1045Y was diluted in the range of 1:50-1:150 depending on the total sensitivity of the protocol employed.

rmAb **EP3**: 1 out of 1 protocols (100 %) were assessed as optimal. The protocol giving an optimal staining was based on HIER in a pressure cooker using Citrate pH 6. The rmAb EP3 was diluted 1:100 using a streptavidin-biotin based detection system.

rmAb **SP3**: 9 out of 19 (47 %) protocols were assessed as optimal. The optimal protocols were based on HIER using either TRS pH 9 (Dako) (1/1), CC1 (BenchMark, Ventana) (1/3), BERS 2 (Bond, Leica) (3/4), Tris-EDTA/EGTA pH 9 (2/3) or Citrate pH 6 (2/5). The rmAb clone SP3 was typically diluted in the range of 1:50-150 depending on the total sensitivity of the protocol employed. Using these protocol settings 11 out of 13 (85 %) laboratories produced a sufficient staining.

pAb **A0485**: 22 out of 49 (45 %) protocols were assessed as optimal. All protocols giving an optimal staining were based on HIER using either Target Retrieval Solution (TRS) low pH 6.1 (Dako) (10/25), TRS pH 9 (Dako) (4/6), TRS pH 9 (3-in-1) (Dako) (1/1), CC1 (BenchMark, Ventana) (2/3), BERS 1 (Bond, Leica) (1/1), Tris-EDTA/EGTA pH 9 (2/3) or Citrate pH 6 (2/10). The pAb A0485 was typically diluted in the range of 1:200-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings 26 out of 41 (63 %) laboratories produced a sufficient staining.

Abs for in-house systems (RTU Ab)

mAb **CB11, RTU-CB11**: 1 out of 2 (50 %) protocols was assessed as optimal. The optimal protocol was based on HIER using Citrate pH 6 in a water bath for 20 min. at 98°C, an incubation time of 30 min. in the primary Ab and NOVOLINK (RE7140) as the detection system.

Comments

In this assessment and in concordance with the previous NordiQC assessments for HER-2 IHC, the prevalent feature of an insufficient HER-2 staining was a too weak or false negative staining reaction, which particularly and most critically was observed as a 0/1+ IHC reaction in the HER-2 gene amplified breast carcinoma no. 4. This tumour was shown to be IHC 2+ in the NordiQC reference laboratories using both HercepTest™ (Dako) and PATHWAY® (Ventana), and showed a low level of HER-2 gene amplification (ratio 2.5 – 2.8) by ISH. The weak or false negative staining reactions were seen in 71 % of the

insufficient results (31 out of 44 laboratories), whereas 27 % (12 out of 44 laboratories) were characterized by a poor signal-to-noise ratio caused by an excessive cytoplasmic staining reaction hampering the interpretation and in 2 % (1 out of 44 laboratories) a false positive staining was seen in one or more of the 3 HER-2 non-amplified tumours, no. 1, 2 & 3.

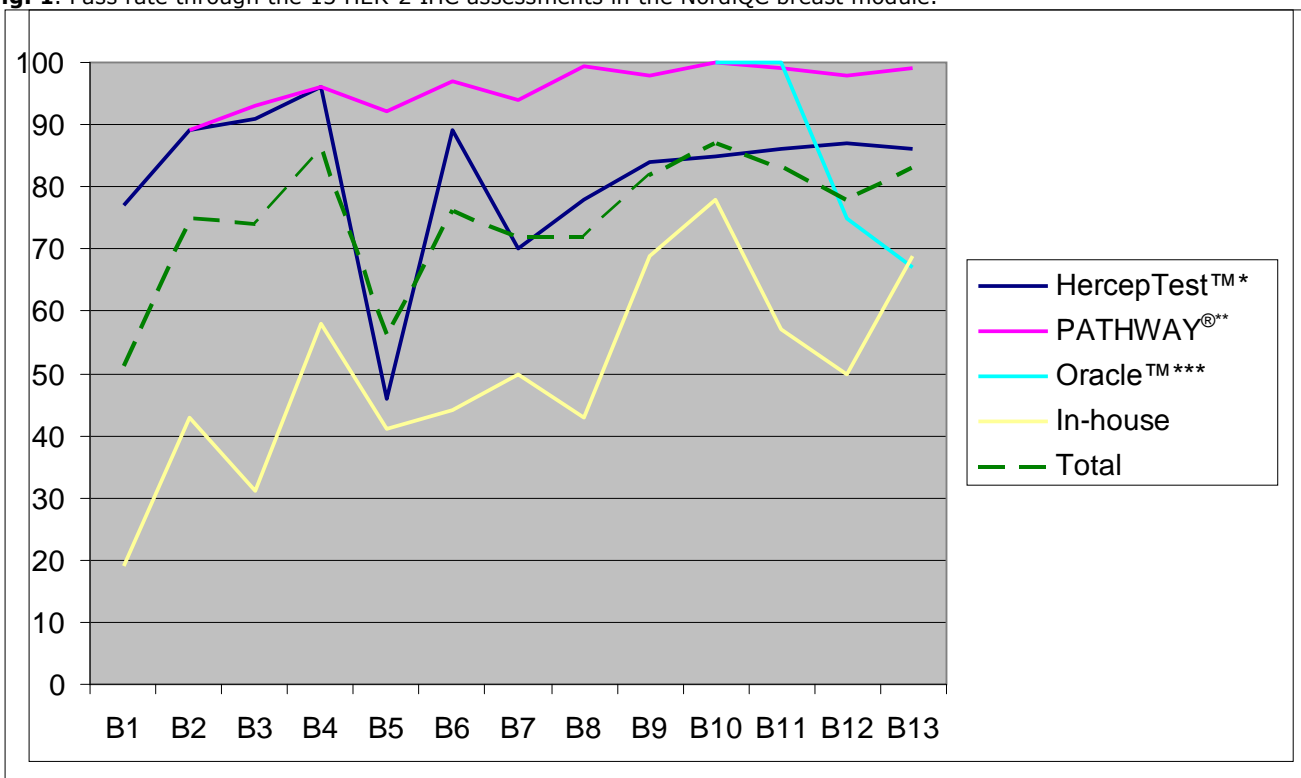
The false negative results and results with a poor signal-to-noise ratio were seen for both the in-house validated assays and the FDA-/CE-IVD approved systems, while the false positive result was seen by an in-house validated assay. The weak and false negative results were for the in-house systems typically caused by a too low sensitivity of the protocol e.g. a too low concentration of the primary Ab, or a protocol based on a RTU Ab not applied within a system for which this product was calibrated.

For the FDA-/CE-IVD approved systems the false negative reactions could in part be related to the use of other protocol settings than recommended by the producers, e.g., too short incubation time in the primary Ab and detection system and/or insufficient HIER, but in a few cases no reason for the insufficient result could be identified. This was seen for both the HercepTest™ (Dako) and Oracle™, (Leica).

This was the 13th NordiQC HER-2 IHC assessment in the breast cancer module. As illustrated in Fig. 1, the FDA-/CE-IVD approved systems such as PATHWAY®/CONFIRM™ (Ventana, rmAb clone 4B5) and HercepTest™ (Dako) have constantly given superior pass rates compared to the in-house HER-2 assays. Thus, the average pass rate in the 13 consecutive runs has been 96 % for PATHWAY®/CONFIRM™ (Ventana, rmAb clone 4B5), and 82 % for HercepTest™ (Dako), while only 50 % for the in-house assays. Grouped together, the FDA-/CE-IVD approved IHC systems gave in the current run a pass rate of 89 % (154 out of 173 laboratories), which was comparable to the pass rates and performances seen in the previous runs. The pass rate for the in-house validated assays as a group was 69 % (55 out of 80 laboratories), which was an increase compared to the pass rates for this group in the previous runs. For unexplained reasons the FDA-/CE-IVD approved system Oracle™, Leica showed a decline in the pass rates and proportion of sufficient results in this and the previous run B12. At present no single parameter or plausible cause for the decline can be identified and as only a relatively small number of participants have used the Oracle™ system, no conclusions can be made.

The use of in-house validated HER-2 assays is still used by a considerable proportion of laboratories, despite the pass-rates for this group consistently have provided an inferior pass rate and shown to be less accurate as an immunohistochemical assay for the semi-quantitative demonstration of HER2: In this run 32 % of the laboratories (n=80) used an in-house HER-2 validated assay, compared to 33 % and 31 % in runs B12 and B11, respectively.

Fig. 1. Pass rate through the 13 HER-2 IHC assessments in the NordiQC breast module.



* HercepTest™ code no. K5204, K5206, K5207 & SK001, Dako

** PATHWAY® & CONFIRM™, rmAb clone 4B5, Ventana

*** Oracle™, Leica

Scoring consensus

The laboratories were requested to submit their own scores (0, 1+, 2+, 3+) on the stained sections. For 149 out of the 219 laboratories (68 %) responding, the scores on all the tissues in the multi-tissue sections given by the laboratories were in concordance with the scores given by the NordiQC assessor group. A sufficient staining combined with an interpretation in concordance with the NordiQC assessors was seen in 79 % (142 out of 179), which was a decrease from 89 % obtained in the previous run B12. An insufficient staining combined with an interpretation in concordance with the NordiQC assessor group was seen in 18 % (7 out of 40) of the laboratories.

In this context it was observed that some laboratories more or less were biased in the interpretation by the previously published assessment criteria and HER2 expression profiles in the tissues submitted. E.g. the tumour no 4, expected to be 2+ to 3+ was by many laboratories interpreted as 2+, despite the submitted staining result of this core clearly and indisputably only could be interpreted as 0-1+ by the NordiQC assessor team.

Conclusion

The FDA-/CE-IVD approved HER-2 IHC systems PATHWAY®/CONFIRM™ rmAb clone 4B5 (Ventana), and HercepTest™ (Dako) were in this assessment the most reliable methods for the semi-quantitative IHC determination of the HER-2 protein expression. In-house validated assays are still used by many laboratories and as observed in the previous runs gave a high proportion of insufficient results. The inclusion of the 2+ tumours (from run B5 onwards) with and without HER-2 gene amplification is essential to evaluate the IHC HER-2 performance and the robustness of the protocols used by the participants. The scoring consensus for laboratories with insufficient staining result was very low and focus must be addressed to improve and to secure both the technical performance but also the interpretation of the immunohistochemical HER2 analysis.

Figs 1a and 1b – optimal staining results, same protocol

Figs 2a and 2b – insufficient staining results - false negative, same protocol

Figs 3a and 3b – insufficient staining results – false positive and excessive retrieval, same protocol

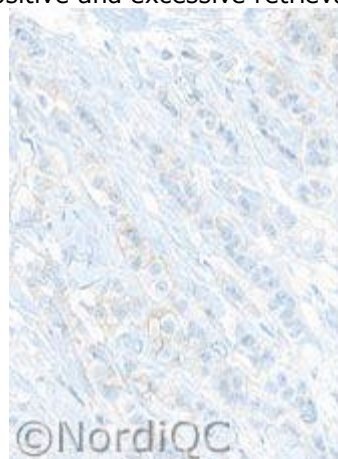
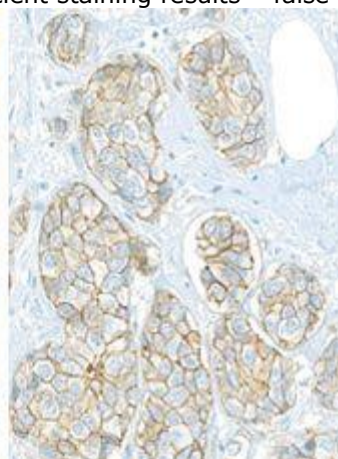
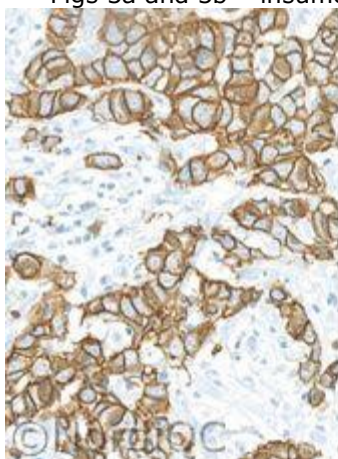


Fig 1a

Left: Optimal staining for HER-2 of the breast ductal carcinoma no. 5 with a ratio of HER-2 / Chromosome 17 of > 6.0.

> 30 % of the neoplastic cells show a strong and complete membranous staining reaction corresponding to 3+.

Right: Optimal staining for HER-2 of the breast ductal carcinoma no. 4 with a ratio of HER-2 / Chromosome 17 of 2.5 – 2.9.

> 10 % of the neoplastic cells show a moderate complete membranous staining reaction corresponding to 2+.

Fig 1b

Left: Optimal staining for HER-2 of the breast ductal carcinoma no. 3 with a ratio of HER-2 / Chromosome 17 of 1.4 – 1.7.

> 10 % of the neoplastic cells show a weak incomplete membranous staining reaction corresponding to 1+.

Right: Optimal staining for HER-2 of the breast ductal carcinoma no. 2 with a HER-2 / Chromosome 17 ratio of 1.2 – 1.5.

A faint membranous staining is seen in < 10 of the neoplastic cells corresponding to 0.

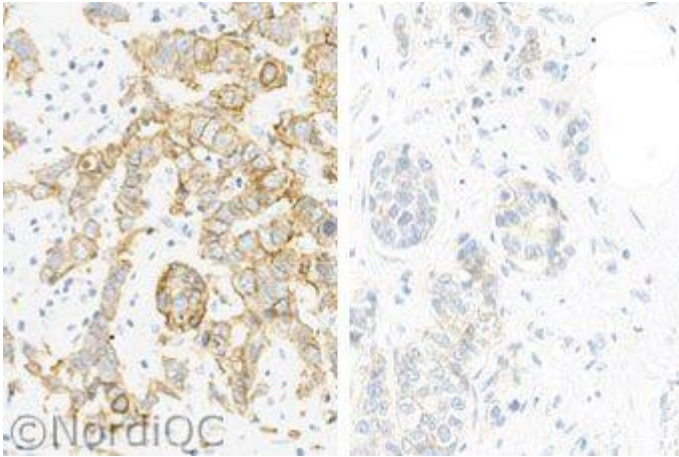


Fig 2a

Left: Staining for HER-2 of the breast ductal carcinoma no. 5 with a ratio of HER-2 / Chromosome 17 of > 6.0 . $> 30\%$ of the neoplastic cells show a strong and complete membranous staining corresponding to 3+.

Right: Insufficient staining for HER-2 of the breast ductal carcinoma no. 4 with a ratio of HER-2 / Chromosome 17 of 2.5 - 2.9. $> 10\%$ of the neoplastic cells show a faint perceptible membrane staining corresponding to 1+, but does not meet the criteria to be classified as 2+ and will not be referred to ISH.

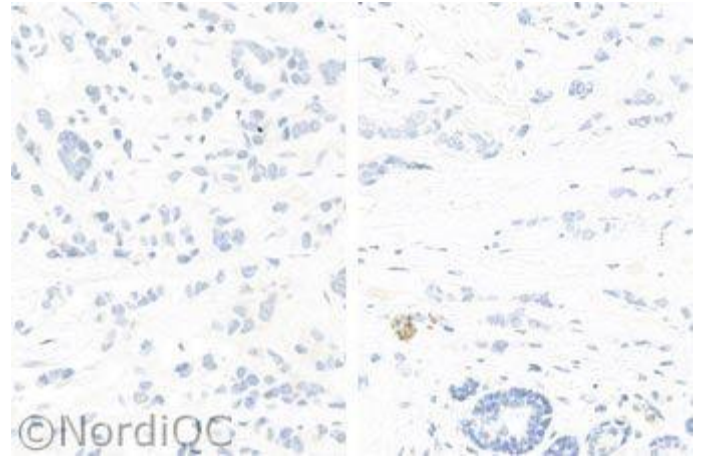


Fig 2b

Left: Staining for HER-2 of the breast ductal carcinoma no. 3 with a ratio of HER-2 / Chromosome 17 of 1.4 - 1.7. A faint membranous staining is seen in $< 10\%$ of the neoplastic cells corresponding to 0.

Right: Staining for HER-2 of the breast ductal carcinoma no. 2 with a HER-2 / Chromosome 17 ratio of 1.2 - 1.5. No staining reaction is seen corresponding to 0.

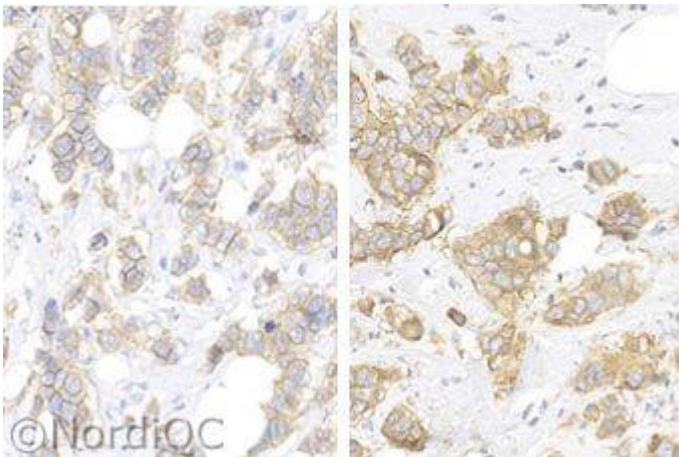


Fig 3a

Left: Staining for HER-2 of the breast ductal carcinoma no. 5 with a ratio of HER-2 / Chromosome 17 of > 6.0 . $> 10\%$ of the neoplastic cells show a weak to moderate and complete membranous staining reaction corresponding to 2+.

Right: Insufficient staining for HER-2 of the breast ductal carcinoma no. 4 with a ratio of HER-2 / Chromosome 17 of 2.5 - 2.9.

An excessive cytoplasmic staining reaction complicates the interpretation of the membranous staining reaction. Also compare with Figs. 3b, left & right, same protocol.

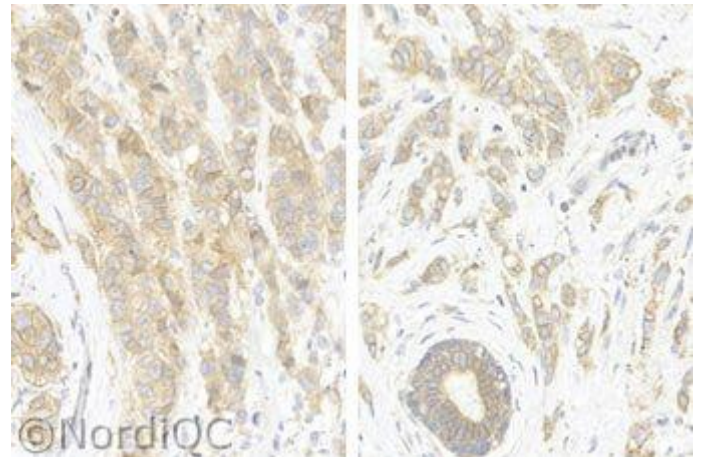


Fig 3b

Left: Insufficient staining for HER-2 of the breast ductal carcinoma no. 3 with a ratio of HER-2 / Chromosome 17 of 1.4 - 1.7. An excessive cytoplasmic staining reaction complicates the interpretation of the membranous staining reaction.

Right: Insufficient staining for HER-2 of the breast ductal carcinoma no. 2 with a HER-2 / Chromosome 17 ratio of 1.2 - 1.5.

An excessive cytoplasmic staining reaction complicates the interpretation of the membranous staining reaction. Also note the strong staining reaction in the normal epithelial cells.

SN/MV/LE 11-7-2012