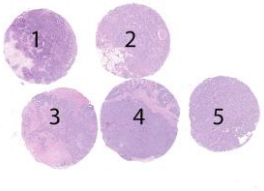
Assessment Run B20 2015
HER-2 IHC

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

	IHC: HER-2 Score* (0, 1+, 2+, 3+)	FISH: HER-2 gene/chr 17 ratio**
1.Breast carcinoma	3+	> 6.0 (clusters) (a)
2.Breast carcinoma	0-1+	0.9 – 1.2 (u)
3.Breast carcinoma	1-2+	1.2 – 1.6 (u)
4.Breast carcinoma	2-3+	2.3 – 2.8 (a)
5.Breast carcinoma	0-1+***	1.2 – 1.5 (u)



* HER-2 immunohistochemical score (see table below) as achieved by using the three FDA approved kits and antibodies, HercepTest™ Dako, Oracle™ Leica and PATHWAY® Ventana, in NordiQC reference laboratories.
** HER-2 gene/chromosome 17 ratios achieved using Zyto*Light* ® SPEC HER2/CEN 17 Dual Color FISH (Zytovision)
*** scattered cells < 1% focally showed a moderate to strong complete membranous staining reaction.

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

IHC scoring system according to the 2013 ASCO/CAP guidelines

Score 0	No staining is observed or incomplete membrane staining is observed in ≤ 10% of the tumour cells.
Score 1+	A faint perceptible and incomplete membrane staining is observed in more than 10% of the tumour cells.
Score 2+	A weak to moderate circumferential incomplete membrane staining is observed in more than 10% of the tumour cells or an intense circumferential complete membranous staining in ≤ 10% of the tumour cells.
Score 3+	An intense circumferential complete membrane staining is observed in more than 10% of the tumour cells.

Criteria for assessing a HER-2 staining as **optimal** were:

- Staining corresponding to score 0 or 1+ in carcinomas no. 2 and 5.
- Staining corresponding to score 1+ or 2+ in carcinoma no. 3.
- Staining corresponding to score 2+ or 3+ in carcinoma no. 4.
- Staining corresponding to score 3+ in carcinoma no. 1.
- No or only weak cytoplasmic reaction that did not interfere with the interpretation.

Staining was assessed as **good**, if (1) the HER-2 gene amplified tumour no. 1 showed a 2+ reaction and the other breast carcinomas showed reaction pattern as described above (equivocal 2+ IHC staining should always be analyzed by ISH according to the ASCO/CAP guidelines) ***or*** (2) the HER-2 gene non-amplified tumour no. 2 and/or 5 showed a 2+ reaction and the other breast carcinomas showed the expected reaction pattern.

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.

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

Participation

Number of laboratories registered for HER2, run B19	513
Number of laboratories returning slides	480 (94%)

Results
480 laboratories participated in this assessment. 2 laboratories only submitted the scoring sheet and 1 laboratory submitted a damaged slide. Of the remaining 477 laboratories, 90% achieved a sufficient mark. Assessment marks for IHC HER-2 assays and HER-2 antibodies are summarized in table 1.

Table 1: **Assessment marks for IHC assays and antibodies run B20, HER-2 IHC**

FDA approved HER-2 assays	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
PATHWAY® rmAb clone **4B5, 790-2991**	185	Ventana	167	12	0	6	97%	98%
CONFIRM™, rmAb clone **4B5, 790-4493**	75	Ventana	73	1	0	1	99%	100%
CONFIRM™, rmAb clone **4B5, 800-2996**	2	Ventana	2	0	0	0	-	-
HercepTest™ **SK001**	59	Dako	33	21	0	5	92%	93%
HercepTest™ **K5207**	15	Dako	2	9	1	3	73%	90%
HercepTest™ **K5204**	17	Dako	2	10	2	3	71%	86%
Oracle™ mAb clone **CB11, TA9145**	13	Leica	0	5	0	8	39%	-
Antibodies[3] for laboratory developed HER-2 assays, conc. antibody	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **10A7**	1	Leica/Novocastra	0	0	0	1	-	-
mAb clone **CB11**	11 5 1 1	Leica/Novocastra Cell Marque Biocare Klinipath	3	9	2	4	67%	75%
mAb clone **UMAB36**	1	ORIGENE	0	1	0	0	-	-
rmAb clone **EP3**	1	Biocare	0	1	0	0	-	-
rmAb clone **SP3**	14 1 1 1 1 1	Thermo/NeoMarkers Cell Marque Immunologic Spring Bioscience Thermo/Pierce Zytomed	11	3	0	5	74%	87%
pAb clone **A0485**	63	Dako	32	24	0	7	89%	89%
Antibodies for laboratory developed HER-2 assays, RTU	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **CB11, RTU-CB11**	2	Leica/Novocastra	1	1	0	0	-	-
mAb clone **CB11, 237M-18**	1	Cell Marque	0	0	0	1	-	-
rmAb clone **EP3, PRO47-6ml**	1	PathnSito	1	0	0	0	-	-
rmAb clone **SP3, MAD-000308QD**	1	Master Diagnostics	1	0	0	0	-	-
rmAb clone **SP3, M3031**	1	Spring Bioscience	0	1	0	0	-	-
Ab clone **MXR001, RMA-0701**	1	Maixin	1	0	0	0	-	-
pAb **E2441**	1	Spring Bioscience	0	0	0	1	-	-
Total	477		329	98	5	45	-	-
Proportion			69%	21%	1%	9%	90%	-

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

Detailed Analysis
FDA/CE IVD approved assays

PATHWAY® rmAb clone **4B5** (790-2991, Ventana): 167 of 185 (90%) protocols were assessed as optimal. Protocols with optimal results were typically based on heat inducd epitope retrieval (HIER) in Cell Conditioning 1 (CC1) (efficient heating time 20-64 min.) in BenchMark XT, GX or Ultra, 12 – 44 min. incubation of the primary Ab and Iview, UltraView or OptiView as detection kit. Using these protocol settings 178 of 182 (98%) laboratories produced a sufficient staining result (optimal or good).

CONFIRM™ rmAb clone **4B5** (790-4493, Ventana): 73 of 75 (97%) protocols were assessed as optimal. Protocols with optimal results were typically based on HIER in CC1 (efficient heating time 20-64 min.) in BenchMark XT, GX or Ultra, 12 – 36 min. incubation of the primary Ab and Iview, UltraView or OptiView as

detection kit. Using these protocol settings 74 of 74 (100%) laboratories produced a sufficient staining result.

CONFIRM™ rmAb clone **4B5** (800-2996, Ventana): 2 of 2 protocols were assessed as optimal. Both protocols were based on HIER in CC1 mild or standard in the BenchMark XT and Ultra, 16 – 32 min. incubation of the primary Ab and UltraView as detection kit.

HercepTest™ pAb (SK001, Dako): 33 of 59 (60%) protocols were assessed as optimal. Protocols with optimal results 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 30 min. incubation of the primary Ab. Using these protocol settings 51 of 55 (93%) laboratories produced a sufficient staining result.

HercepTest™ pAb (K5207, Dako): 2 of 15 (13%) protocols were assessed as optimal. One protocol was performed on a BOND III (Leica). The other protocol was based on HIER in HercepTest™ epitope retrieval solution at 97°C for 40 min. in a water bath or PT link and 30 min. incubation of the primary Ab. Using these or comparable protocol settings 9 of 10 (90%) laboratories produced a sufficient staining result.

HercepTest™ pAb (K5204, Dako): 2 of 17 (12%) protocols were assessed as optimal. One protocol with optimal results was based on HIER in TRS pH9 at 95°C in a water bath for 30 min. The other protocol was based on HIER in HercepTest™ epitope retrieval solution at 99°C for 40 min in a water bath and 30 min. incubation of the primary Ab. Using these or comparable protocol setting 6 of 7 (86%) laboratories produced a sufficient staining result.

Concentrated antibodies for laboratory developed (LD) assays
mAb **CB11**: 3 of 18 (17%) protocols were assessed as optimal. Optimal protocols were based on HIER using Target Retrieval Solution (TRS) pH 9 (Dako) (1/3)*, PT Module Buffer 1, pH 6 (Thermo) (1/1) or EDTA/EGTA pH 8 (1/1). The mAb clone CB11 was diluted in the range of 1:100-400 depending on the total sensitivity of the protocol employed. Using these protocol settings 3 of 4 (75%) laboratories produced a sufficient staining result (optimal or good).

rmAb **SP3**: 11 of 19 (60%) protocols were assessed as optimal. Optimal protocols were based on HIER using either Cell Conditioning 1 (CC1) (BenchMark, Ventana) (3/5), Bond Epitope Retrieval Solution 2 pH 9 (BERS2, Leica) (4/4), BERS1 pH 6 (Leica) (1/1), Tris-EDTA/EGTA pH 9 (1/3) or Citrate pH 6 (2/3). The rmAb clone SP3 was typically diluted in the range of 1:40-100 depending on the total sensitivity of the protocol employed. Using these protocol settings 13 of 15 (87%) laboratories produced a sufficient staining result.

pAb **A0485**: 32 of 63 (51%) protocols were assessed as optimal. Optimal protocols were based on HIER using either TRS low pH 6.1 (Dako) (18/34), TRS pH 9 (3-in-1) (Dako) (1/3), TRS pH 9 (Dako) (7/7), CC1 (BenchMark, Ventana) (2/4), BERS1 pH 6 (Bond, Leica) (2/8) or Citrate pH 6 (2/6). The pAb A0485 was typically diluted in the range of 1:200-1,000 depending on the total sensitivity of the protocol employed. Using these protocol settings 54 of 61 (89%) laboratories produced a sufficient staining result.

Comments
In this assessment and in concordance with the previous NordiQC assessments of HER-2 IHC, insufficient HER-2 staining result was characterized by a too weak or false negative staining reaction. This was particularly and most critically observed as 0/1+ IHC reaction in the low level HER-2 gene amplified breast carcinoma core no. 4. This tumour was established as a IHC 2+ in the NordiQC reference laboratories using the three FDA/CE-IVD HER-2 IHC assays; PATHWAY® (Ventana), HercepTest™ (Dako) and Oracle™ (Leica) and showed a low level of HER-2 gene amplification (ratio 2.3 – 2.8) by ISH. False negative staining reaction of the breast carcinoma no. 4 was seen in 88% of the insufficient results (44 of 50).
The remaining insufficient results were typically characterized by a poor signal-to-noise ratio, impaired morphology or excessive counterstaining complicating the interpretation.
In contrast to previous NordiQC assessments, no false positive 3+ IHC staining in the HER-2 non-amplified tumours were observed.
False negative results were seen both by laboratory developed (LD) and FDA-/CE-IVD approved HER-2 assays.
False negative results were for the LD assays typically caused by a too low sensitivity of the protocol applied (e.g. too low concentration of the primary Ab, too short incubation time of the primary Ab and/or insufficient HIER). For the FDA-/CE-IVD approved systems no single cause for insufficient and false negative staining reactions could be identified from the protocols submitted.

In this assessment, the FDA-/CE-IVD approved HER-2 IHC assays from Ventana and Dako, PATHWAY® /CONFIRM™ or HercepTest™, respectively, provided a higher pass-rate superior to LD assays as illustrated in Fig. 1. PATHWAY®/CONFIRM™ have provided a consistently high pass rate throughout all HER-2 IHC runs in NordiQC. The FDA/CE-IVD approved system Oracle™, Leica has shown a noticeable decline in the proportion of sufficient results in the latest three NordiQC HER-2 IHC assessments. At present, no single cause for the decline can be identified, and, as only a relatively small number of participants used the

Oracle™ system, no conclusions can be drawn. In this context it has to be emphasized that the material circulated has been processed according to the recommendations given by ASCO/CAP e.g. concerning time to and time in 10% NBF and the level of HER-2 protein expression is monitored by NordiQC throughout the material used for the assessment and in addition verified by FISH. To monitor the range of HER-2 protein expression in the slides circulated approximately every 50th slide is stained by NordiQC to serve as reference for the expected level of HER-2 IHC expression.

The proportion of laboratories using LD assays is relatively consistent. In this run, 25% of the participants (n=121) used LD assays compared to 26 - 31% in the last 9 assessments. Despite an overall improvement of the pass rate for LD HER-2 assays from run B1 to B20 has been achieved, the pass rate and proportion of optimal results still is inferior to the FDA/CE-IVD approved systems as PATHWAY® /CONFIRM™ and HercepTest™. In general, the two FDA-/CE-IVD approved HER-2 assays provided a proportion of optimal results of 81% (279 of 343), whereas only 41% of LD HER-2 assays were assessed as optimal (50 of 121). As shown in Fig. 2, LD HER-2 assays both provided a reduced proportion of sufficient results but also a shift from optimal to good, typically caused by 2+ staining reaction in one or both of the HER-2 non-amplified tumours (no. 2 and 5) expected to show a 0/1+ staining reaction. The staining reaction of 2+ in these tumours would not directly lead to a wrong diagnosis but require an additional ISH test due to the less precise IHC result.

The overall pass rate of 90% obtained in this assessment was largely comparable to the pass rates seen in the last 5 runs. The material composed for the last runs have been virtually identical, whereas the number of new participants in this run increased significantly compared to the previous runs.
A slight difference regarding the pass rates was observed for the laboratories participating in the HER-2 IHC assessment for the first time compared to the laboratories also participating in the latest assessments.
For the laboratories participating for the first time the pass rate was 80% (100 of 125 laboratories), whereas the pass rate was 93% (330 of 355 laboratories) for the laboratories participating in previous runs.

Figure 1. **Pass rates of 20 HER-2 IHC assessments in the NordiQC breast cancer module**

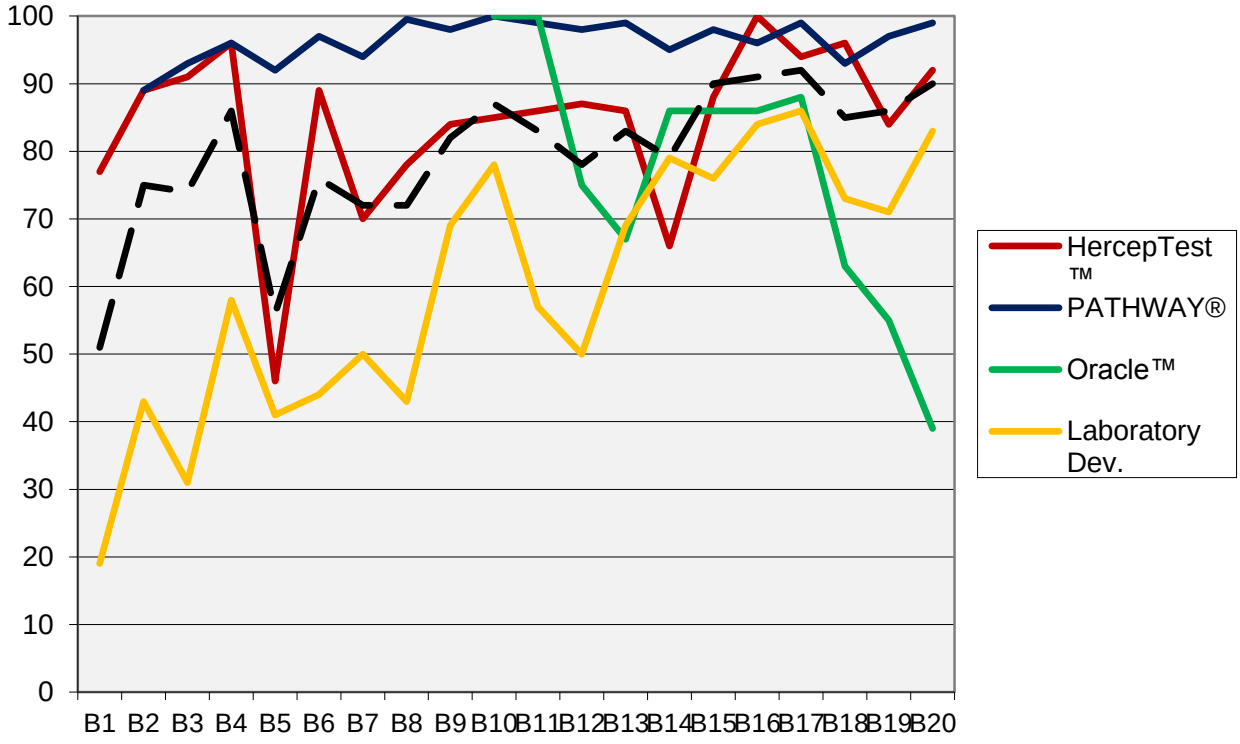
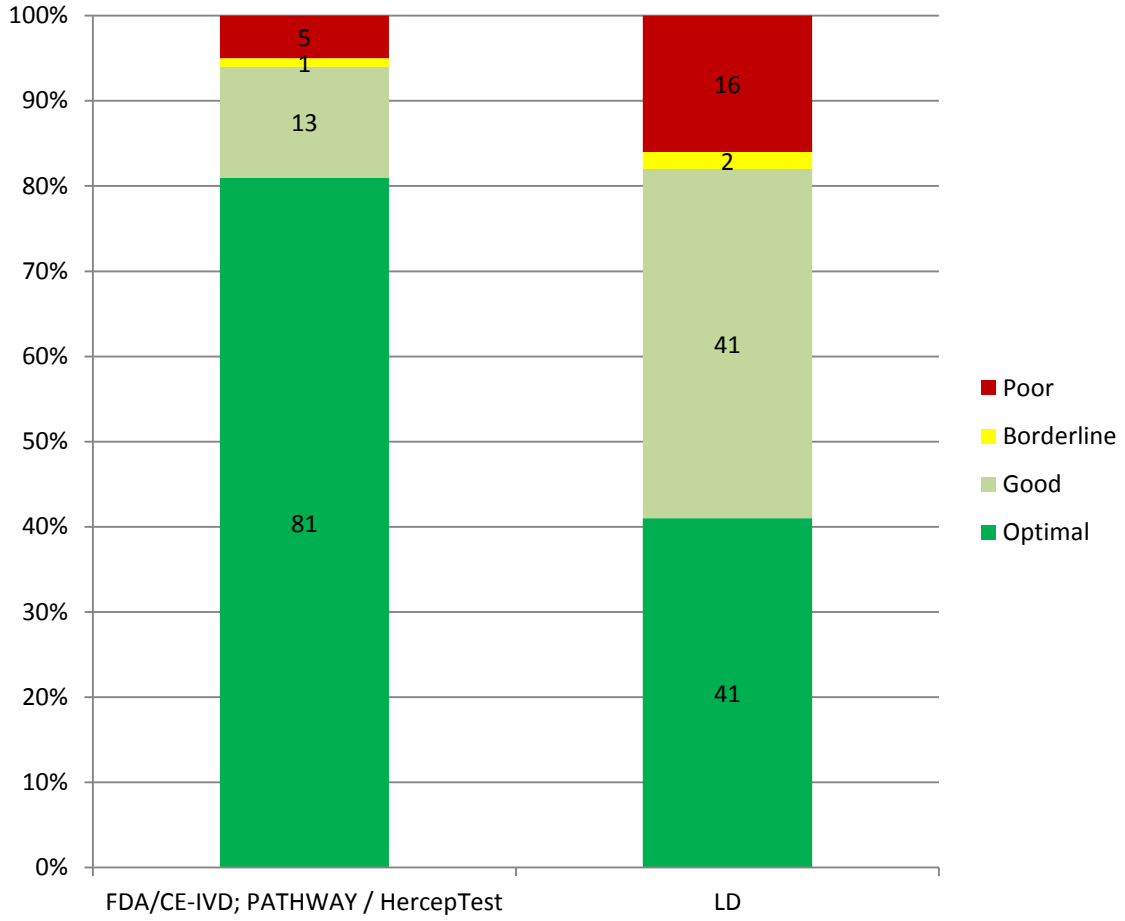


Figure 2. **Proportion of assessment marks using FDA-/CE-IVD and LD assays**



Scoring consensus

The laboratories were requested to submit their own scores (0, 1+, 2+, 3+) of their stained sections, which was effectuated by 88% (421 of 477) of participants. For 341 of the 421 participants (81%) responding, scores for all the tissues in the multi-tissue sections were in concordance with the NordiQC assessor group using the ASCO/CAP 2013 interpretation guidelines. This was a slight decrease compared to the level of 89% observed in run B19. Sufficient staining and interpretation in agreement with the NordiQC assessors were seen in 84% (312 of 373), a decrease from 94% in run B19. Insufficient staining and interpretation in concordance with the NordiQC assessor group was seen in 62% (29 of 47) of the participants. Typically the tumour no. 1 was interpreted as 2+ by the laboratory, but 0-1+ by the NordiQC assessor group. This was to some extent extraordinary as the ASCO/CAP 2013 HER-2 IHC guidelines have lowered the staining threshold for 2+ tumours and thus a higher level of consensus was expected.

Conclusion

The FDA-/CE-IVD approved HER-2 IHC assays **PATHWAY®** & **CONFIRM™** rmAb clone 4B5 (Ventana), and **HercepTest™** (Dako) were in this assessment the most precise assays for the semi-quantitative IHC determination of HER-2 protein expression. Laboratory developed assays produced a lower pass-rate and were less precise for the HER-2 status, requiring an additional ISH test for final evaluation.
Inclusion of 2+ tumours with and without HER-2 gene amplification in control material is essential to evaluate precision and performance stability of the IHC HER-2 assays used by laboratories.
Use of insufficiently calibrated laboratory assays may have severe implications for the patients as well as se health care system.[1]

[1] Vyberg M, Nielsen S, Røge R, Sheppard B, Ranger-Moore J, Walk E, Gartemann J, Rohr UP, Teichgräber V. Immunohistochemical expression of HER2 in breast cancer: socioeconomic impact of inaccurate tests. BMC Health Serv Res. 2015 Aug 29;15:352. doi: 10.1186/s12913-015-1018-6. PubMed PMID: 26318869; PubMed Central PMCID: PMC4553016.

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, same protocol

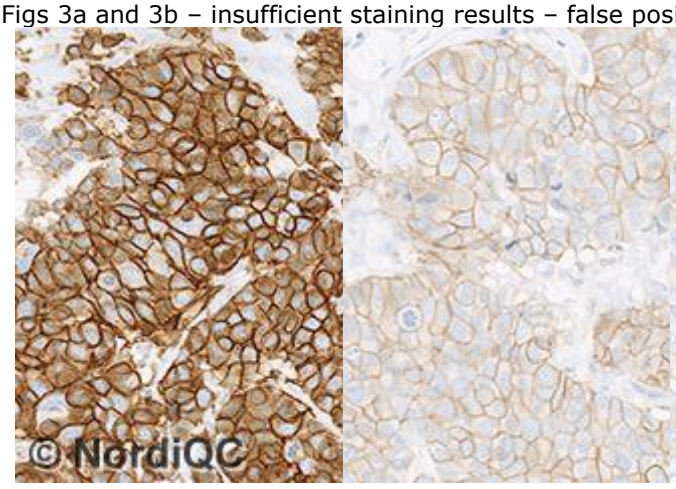


Fig. 1a
Left: Optimal staining result for HER-2 of the breast ductal carcinoma no. 1 with a ratio of HER-2 / Chr17 of > 6.0.
> 10 % of the neoplastic cells show an intense and complete membranous staining reaction corresponding to 3+.
Right: Optimal staining result for HER-2 of the breast ductal carcinoma no. 4 with a ratio of HER-2 / Chr17 of 2.3 – 2.8.
> 10 % of the neoplastic cells show a weak to moderate and complete membranous staining reaction corresponding to 2+.

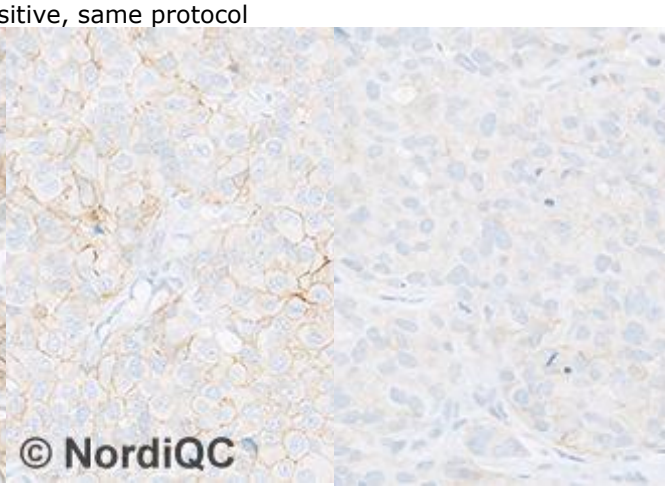


Fig. 1b
Left: Optimal staining result for HER-2 of the breast ductal carcinoma no. 3 with a ratio of HER-2 / Chr17 of 1.2 – 1.6.
> 10 % of the neoplastic cells show a weak to moderate membranous staining reaction corresponding to 2+.
Right: Optimal staining result for HER-2 of the breast ductal carcinoma no. 5 with a HER-2 / Chr17 ratio of 1.2–1.5.
> 10 % of the neoplastic cells show a faint membranous staining reaction corresponding to 1+.

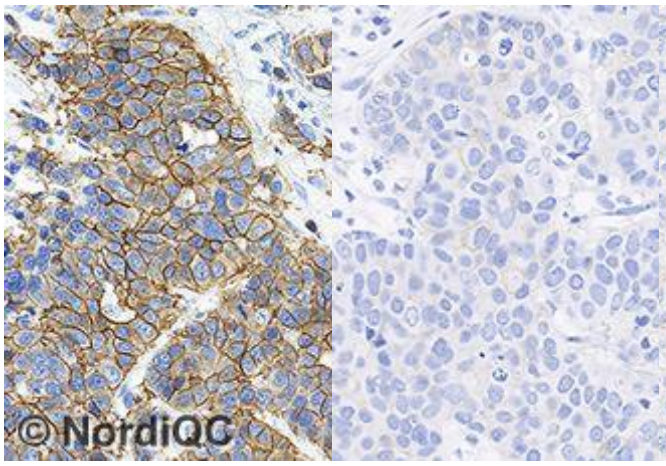


Fig. 2a
Left: Staining result for HER-2 of the breast ductal carcinoma no. 1 with a ratio of HER-2 / Chr17 of > 6.0.
> 10 % of the neoplastic cells show a strong and complete membranous staining reaction corresponding to 3+.
Right: Insufficient and false negative staining result for HER-2 of the breast ductal carcinoma no. 4 with a ratio of HER-2 / Chr17 of 2.3 – 2.8.
> 10 % of the neoplastic cells show a faint perceptible membranous staining reaction corresponding to 1+, but does not meet the criteria to be classified as 2+ and will not be referred to ISH.

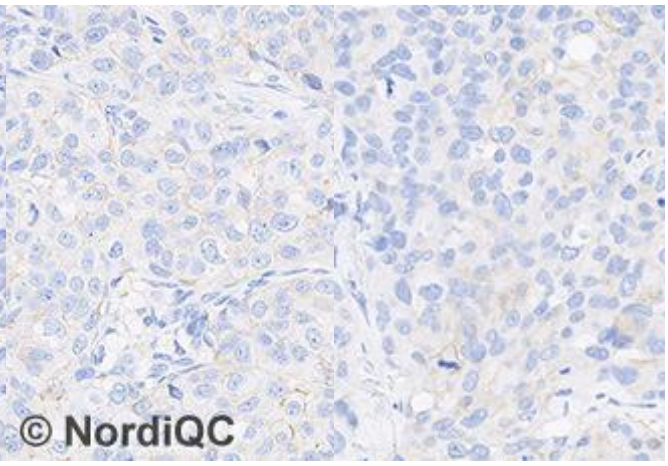


Fig. 2b
Left: Staining result for HER-2 of the breast ductal carcinoma no. 3 with a ratio of HER-2 / Chr17 of 1.2 – 1.6.
> 10 % of the neoplastic cells show a faint perceptible membranous staining reaction corresponding to 1+.
Right: Staining result for HER-2 of the breast ductal carcinoma no. 5 with a HER-2 / Chr17 ratio of 1.2 – 1.5.
> 10 % of the neoplastic cells show a faint membranous staining reaction corresponding to 1+.

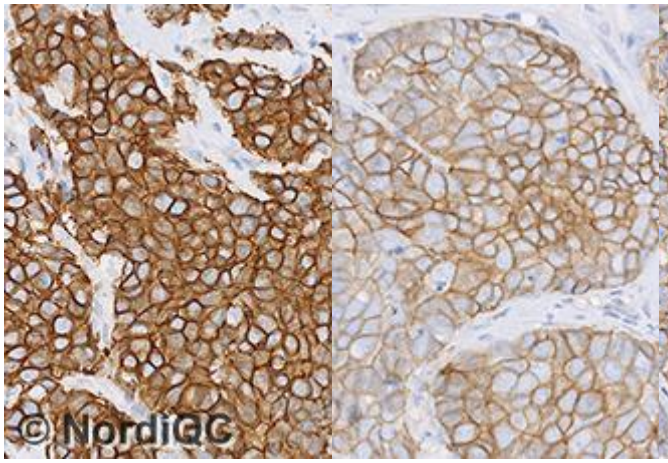


Fig. 3a
Left: Staining result for HER-2 of the breast ductal carcinoma no. 1 with a ratio of HER-2 / Chr17 of > 6.0.
> 10 % of the neoplastic cells show an intense and complete membranous staining reaction corresponding to 3+.
Right: Staining result for HER-2 of the breast ductal carcinoma no. 4 with a ratio of HER-2 / Chr17 of 2.3 – 2.8.
> 10 % of the neoplastic cells show a strong and complete membranous staining reaction corresponding to 3+.

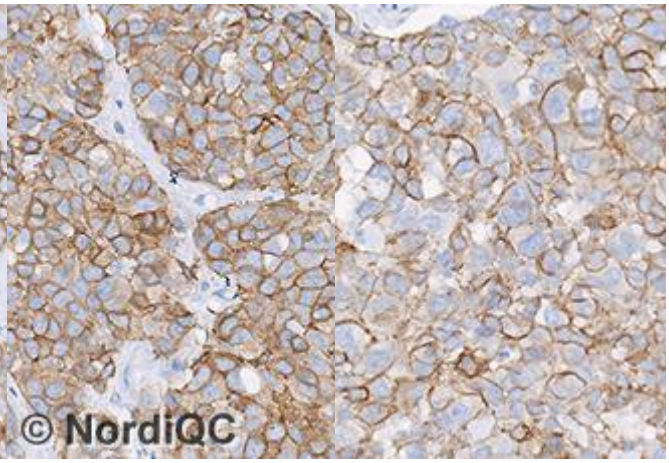


Fig. 3b
Left: Insufficient and false positive staining result for HER-2 of the breast ductal carcinoma no. 3 with a ratio of HER-2 / Chr17 of 1.2 – 1.6.
> 10 % of the neoplastic cells show an intense and complete membranous staining reaction corresponding to 3+.
Right: Insuffient and false positive staining result for HER-2 of the breast ductal carcinoma no. 5 with a HER-2 / Chr17 ratio of 1.2 – 1.5.
> 10 % of the neoplastic cells show a strong complete membranous staining reaction corresponding to 3+.

SN/LE/RR 11.12.2015