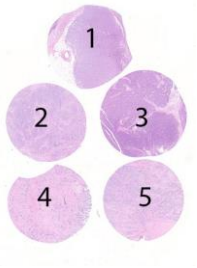
Assessment Run H8 2015

HER-2 ISH (BRISH or FISH)

Material

Table 1: **Content of the multi-block used for the NordiQC HER-2 ISH assessment, run H8 (same as for H7)**

	HER-2 IHC*	Dual - SISH**	FISH***	FISH***
	IHC score	**HER2/chr17 ratio¤**	**HER2/chr17 ratio¤**	**HER2 copies**
1. Breast carcinoma	2+	NA	1.1 – 1.3	< 4, ≥ 4 < 6
2. Breast carcinoma	3+	>6, clusters	>6, clusters	≥ 6
3. Breast carcinoma	0	0.9	1.0 – 1.1	< 4
4. Breast carcinoma	2+	1.5	1.2 – 1.5	< 4
5. Breast carcinoma	2+	1.6	1.5 – 1.9	< 4, ≥ 4 < 6



* PATHWAY® (Ventana), data from two reference labs.
** Inform HER-2 Dual ISH kit (Ventana), range of data from one reference lab.
*** HER2 FISH pharmDX™ Kit (Dako) and HER2 FISH (Zytovision), range of data from two NordiQC labs.
¤HER2/chr17: HER-2 gene/chromosome 17 ratio

All tissues were fixed for 24 - 48 hours in 10% neutral buffered formalin according to the ASCO/CAP 2013 guidelines for tissue preparation of breast tissue for HER-2 ISH analysis.

HER-2 BRISH, Technical assessment

The main criteria for assessing a BRISH HER-2 analysis as technically **optimal** was the ability to correctly interpret the signals and thus evaluate the HER2/chr17 ratios in all five tissues.

Staining was assessed as **good**, if the HER2/chr17 ratios could be evaluated in all five tissues, but the interpretation was slightly compromised e.g. due to excessive retrieval, weak or excessive counterstaining or focal negative areas.

Staining was assessed as **borderline** if one of the tissues could not be evaluated properly e.g. due to weak signals, large negative areas with no signals (> 25% of the core) or a low signal-to-noise ratio due to excessive background staining.

Staining was assessed as **poor** if two or more of the tissue cores could not be evaluated properly e.g. due to weak signals, large negative areas with no signals (> 25% of the core) or a low signal-to-noise ratio due to excessive background staining.

HER-2 BRISH and FISH interpretation

For both BRISH and FISH, participating laboratories were asked to submit a scoring sheet with their interpretation of the HER2/chr17 ratio. Results were compared to NordiQC FISH data from reference laboratories to analyze scoring consensus.
Consensus scores from the NordiQC FISH reference laboratories

- Breast ductal carcinomas, no. 3 and 4: non-amplified
- Breast ductal carcinoma no. 2: (highly) amplified
- Breast ductal carcinoma no. 1 and 5: non-amplified or equivocal

The most recent ASCO/CAP guidelines (2013) were applied for the interpretation of the HER-2 status:
Unamplified: HER2/chr17 ratio < 2.0 using a dual probe assay or an average < 4 HER-2 gene copies per cell/nucleus (both dual and single probe assay)
Equivocal: HER2/chr17 ratio of < 2.0 using a dual probe assay with an average of ≥ 4 and < 6 HER-2 gene copies per cell/nucleus (both dual and single probe assay)
Amplified: Using a dual probe assay a HER2/chr17 ratio of ≥ 2.0 and an average ≥ 4 HER-2 copies per cell/nucleus. Using a single probe assay an average of ≥ 6 HER-2 copies per cell/nucleus.

Participation

Number of laboratories registered for HER-2 BRISH	122
Number of laboratories returning slides	103 (84%)
Number of laboratories returning scoring sheet	95 (92%)
Number of laboratories registered for HER-2 FISH	53
Number of laboratories returning scoring sheet	49 (93%)

Results BRISH, technical assessment

In total, 103 laboratories participated in this assessment. 73 laboratories (71%) achieved a sufficient mark (optimal or good). Results are summarized in Table 2.

Table 2: **HER-2 BRISH systems and assessment marks for BRISH HER-2.**

Two colour HER-2 systems	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
INFORM™ HER-2 Dual ISH **800-4422, 780-4422**	74	Ventana	34	14	19	7	65%	69%
INFORM™ HER-2 Dual ISH + IHC **800-4422 + HER2 IHC**	4	Ventana	3	0	1	0	-	-
DuoCISH pharmDx™ **SK109**	8	Dako	4	3	1	0	88%	100%
Zyto*Dot*® 2C **C-3022 / C-3032**	5	ZytoVision	4	0	1	0	80%	80%
One colour HER-2 systems								
INFORM™ HER-2 SISH **780-4332**	4	Ventana	4	0	0	0	100%	100%
Zyto*Dot*® **C-3003**	7	ZytoVision	5	1	0	1	86%	86%
HER-2 CISH **In-house**	1	In-house	0	1	0	0	-	-
Total	103		54	19	22	8	-	-
Proportion			52%	18%	21%	8%	71%	-

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

Comments

In this assessment, optimal demonstration and evaluation of the HER-2 gene amplification status in all cores of the multitissue block could be obtained both by single and dual-colour systems as shown in table 2. Minor focal staining artefacts were accepted if they did not compromise the overall interpretation of each of the 5 individual tissue cores. Artefacts were silver precipitates, excessive background staining or negative areas most likely caused by technical issues as slides drying out during the staining process or inadequate washing etc. In this run, ISH rejection criteria defined in the 2013 ASCO/CAP HER2 guidelines were applied. In brief, repeated test must be performed if more than 25% of the signals/cells cannot be interpreted due to artefacts. In these cases, the staining result was rated as insufficient (poor or borderline). For the most commonly used HER-2 BRISH assay, the INFORM™ HER-2 Dual ISH (Ventana), technical adequate result was thus provided in 69% using appropriate protocol settings being identified as essential for a technical optimal staining result.

Optimal protocol settings: Two-colour HER-2 systems

For the **INFORM™ Dual ISH systems 800-4422** and **780-4422** (Ventana), optimal demonstration of HER-2 BRISH typically was based on HIER in Cell Conditioning 2 (CC2) for 24-32 min. at 86-90˚C and subsequent proteolysis in P3 for 8 - 16 min. at 36-37˚C. The HER-2 SISH probe was typically applied for 6 hours at 50-52˚C, while the chr17 probe was applied for 2 hours at 42-44˚C.

Using these protocol settings, sufficient results (optimal or good) were seen in 69% of the submitted protocols (38 of 55). 17 laboratories used a protocol with optimal settings, but for unexplained reasons, completely false negative staining or excessive background staining (e.g. due to silver precipitates) in the entire slide or in large areas of one or more of the tissue cores were seen. No reason for these insufficient results could be related to the applied protocols, reagents, platform (BenchMark XT, GX or Ultra) or any other protocol parameter. This observation has been seen in the latest runs and might indicate a less robust and reproducible performance of the protocols on the used instruments.

The remaining insufficient results were characterized by impaired morphology hampering interpretation. This pattern was typically caused by excessive retrieval and as a consequence the nuclei were almost totally digested and virtually no counterstaining could be seen.

Four laboratories used the **INFORM™ Dual ISH systems 800-4422** (Ventana) in combination with an immunohistochemical demonstration for **HER-2 PATHWAY®** (Ventana). An optimal demonstration of HER-2 BRISH using this assay was based on HIER in Cell Conditioning 2 (CC2) for 32 min. at 75-90°C and subsequent proteolysis in P3 for 8-20 min. at 36-37°C. The HER-2 SISH probe was typically applied for 6 hours at 50-52°C, while the chr17 probe was applied for 2 hours at 42-44°C. HER-2 PATHWAY® was performed with iVIEW as detection system. Both BenchMark ULTRA and XT could be used as stainer platform.
Using these protocol settings, sufficient results were seen in 75% of the submitted protocols (3 of 4).

For the **DuoCISH™ system SK109** (Dako), protocol settings with optimal results typically were based on HIER in pre-treatment buffer in a waterbath or microwave oven for 10 min. at 95 - 98°C and subsequent proteolysis in pepsin for 2-3 min. at 37°C (both reagents included in the HER2 DuoCISH pharmDX kit SK109). The HER-2 and chr17 probes were applied for 14–20 hours at 45°C and visualized by the detection reagents provided in the DuoCISH™ kit SK109.
Using these protocol settings, sufficient results were seen in 100% of the submitted protocols (6 of 6).

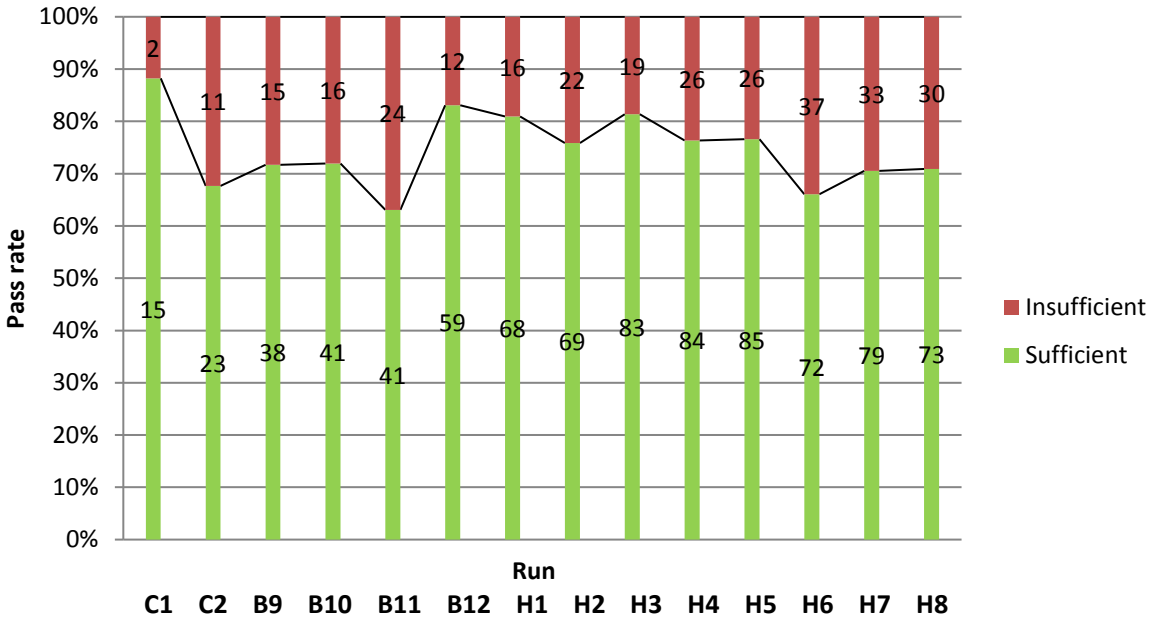
For the **Zyto*Dot*® 2C system C-3022** (ZytoVision), protocol settings with optimal results typically were based on HIER in EDTA pH 8 in a waterbath for 15 min. at 95-98°C and subsequent proteolysis in pepsin for 2-5 min at room temperature, hybridization at 37°C for 16-18 hours and visualization with the ZytoVision detection kit C-3022. Using these settings sufficient results were seen in 80% of the submitted protocols (4 of 5).

One-colour HER-2 systems
For the **INFORM™ SISH systems 780-4332** (Ventana), protocol settings with optimal results typically were based on HIER in CC2 for 28 min. at 93°C and subsequent proteolysis in P3 for 4-8 min. at 36°C. The HER-2 SISH probe was applied for 6 hours at 50°C.
Using these or similar protocol settings optimal results were seen in 100% of the submitted protocols (4 of 4).

For the **Zyto*Dot*® CISH system C-3003**, ZytoVision, protocol settings with optimal results typically were based on HIER in EDTA pH 8 in a waterbath for 15 min. at 95-98°C and subsequent proteolysis in pepsin for 2-5 min. at room temperature, hybridization at 37°C 16-18 hours and visualization with the ZytoVision detection kit C-3003. Using these or similar protocol settings, sufficient results were seen in 86% of the submitted protocols (6 of 7).

Performance history
This was the 14th assessment of HER-2 BRISH in NordiQC and a relatively consistent pass rate has been seen observed in the latest runs. Data is shown in table 3.

Table 3: **Proportion of sufficient results for HER-2 BRISH in the NordiQC assessments**



HER-2 ISH interpretation and scoring consensus:

Table 4: **NordiQC FISH amplification data**

	NordiQC FISH HER2/chr17 ratio	**NordiQC FISH HER2 copies**	**NordiQC HER-2 amplification status**
1. Breast ductal carcinoma	1.1 – 1.3	<4, ≥ 4 and < 6*	Non-amplified / Equivocal
2. Breast ductal carcinoma	> 6.0, clusters	> 6	Amplified
3. Breast ductal carcinoma	1.0 – 1.1	< 4	Non-amplified
4. Breast ductal carcinoma	1.2 – 1.5	< 4	Non-amplified
5. Breast ductal carcinoma	1.5 – 1.9	<4, ≥ 4 and < 6*	Non-amplified / Equivocal

* data from 2 different NordiQC reference laboratories.

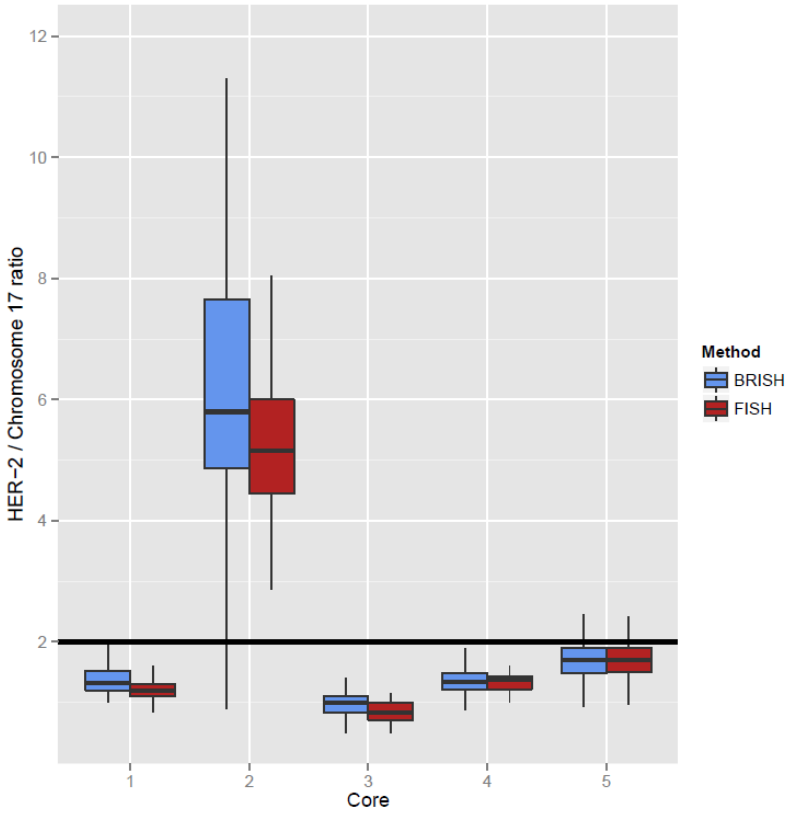
Scoring sheets were completed by 144 of the 156 participating laboratories. These evaluations were compared to the HER-2 FISH amplification status obtained by the NordiQC reference laboratories, summarized in Figs. 1 and 2. For laboratories performing FISH, the consensus rate was 82% (40 of 49 laboratories) compared to only 62% for the laboratories using BRISH (59 of 95 laboratories).

In general, for both BRISH and FISH, high consensus rates were observed between participants and NordiQC regarding the HER-2 amplification status in the breast carcinomas tissue cores no. 2, 3 and 4. The interpretation of HER-2 amplification status was more challenging for tissue core no. 1 and 5. Tumour no. 1 was by the NordiQC reference laboratories classified as non-amplified with a HER-2 ratio of 1.1 – 1.3, but simultaneously showed polysomia and in areas a level of ≥ 4 but less < 6 HER-2 gene copies was identified. Consequently it was accepted to classify the tumour both as non-amplified and equivocal using the ASCO/CAP 2013 guidelines. This tumour was in particular by laboratories using BRISH frequently classified as amplified.
Tumour no. 5 was by the NordiQC laboratories characterized to be non-amplified or equivocal. The tumour showed HER-2 ratio in the range of 1.5 – 1.9 and in areas a level of ≥ 4 but less < 6 HER-2 gene copies was identified.
For both BRISH and FISH, disagreement of the interpretation of the HER-2 amplification status between the participants and NordiQC data were related to "overrating" of the HER-2 status and thus classification of a false positive result. This was, as mentioned above, more frequently observed for laboratories performing BRISH compared to FISH. The inferior precision of the interpretation of the HER-2 status using BRISH was in part shown to be related to the technical quality of the protocol applied. For the results assessed as technically optimal, a consensus rate for the interpretation of 74% was seen, whereas a

consensus rate of 50% was seen for results assessed as non-optimal (good, borderline and poor). Excessive background staining, inadequate counterstaining, chromogene or silver precipitates most likely compromised the interpretation.

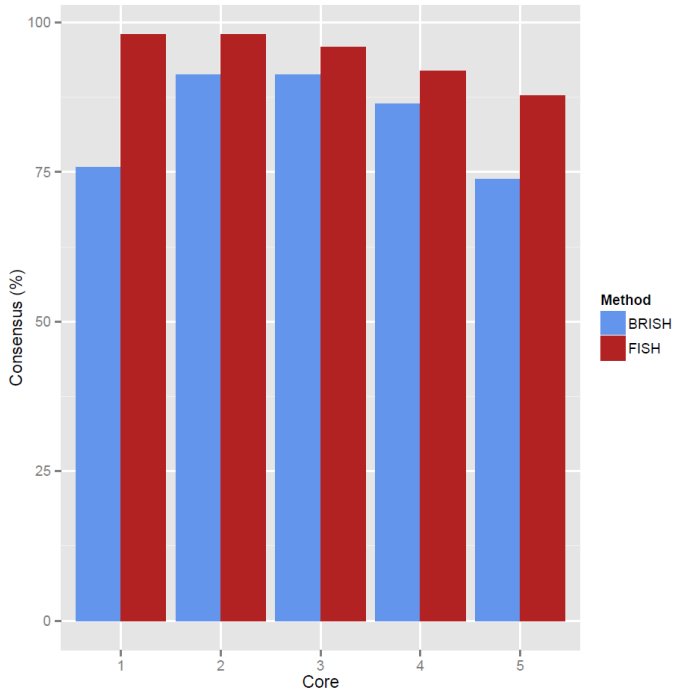
The overall interpretation of amplification ratios and consensus rates of the participants are shown in Figs. 1 and 2.

Fig. 1



NordiQC HER-2 ISH run H8: participants interpretation of amplification status

Fig. 2



NordiQC HER-2 ISH run H8: consensus between participants and NordiQC

No technical evaluation of FISH protocols was performed. Table 5 shows the FISH assay used by the participants and concordance level to the NordiQC data observed. In this matter it has to be emphasized that it was not possible to identify the cause of an aberrant interpretation of the HER-2 status whether this was related to the technical performance of the FISH assay or the interpretation by the observer(s).

Table 5: **FISH assays used and level of consensus HER-2 status to NordiQC reference data**

	Number	Consensus rate
Dako K5331	4	75%
Dako K5731	9	89%
Dako GM333	3	67%
Leica TA9217	3	100%
Kreatech	3	33%
Pathvysion / Abbot	16	94%
ZytoVision, Zytolight	7	86%
Other	4	75%

Conclusion

In this assessment and in concordance with previous NordiQC HER-2 ISH runs, technical optimal demonstration of HER-2 BRISH could be obtained by the commercially available two-colour HER-2 systems INFORM™ HER-2 Dual ISH (Ventana), DuoCISH™(Dako) and ZytoDot® 2C (ZytoVision).

The single-colour HER-2 systems INFORM™ HER-2 ISH (Ventana) and ZytoDot® (ZytoVision) could also be used to produce a technical optimal HER-2 demonstration.

For all systems, retrieval settings – HIER and proteolysis - must be carefully balanced to provide sufficient demonstration of HER-2 (and chr17 signals) and preserved morphology.

Despite optimal protocol settings being applied a high proportion of technical insufficient results were seen, indicating other issues are influencing the quality of the BRISH assays. Especially the capability of present instrumentation technique to provide a reproducible performance of the protocols might be a central factor. Attention must be paid to interpretation in order to obtain correct HER-2 amplification status.

Laboratories performing FISH achieved a higher consensus rate for the interpretation of HER-2 amplification status compared to laboratories performing BRISH.

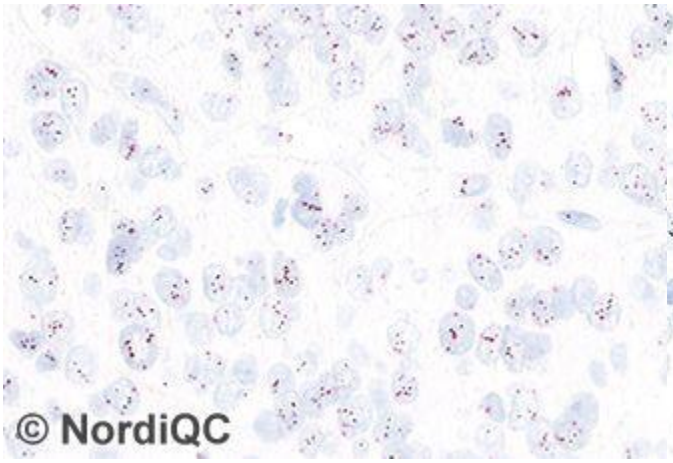


Fig. 1a
Optimal demonstration of the HER-2 gene status using the INFORM™ Dual ISH kit cat. no. 800-4422/780-4422, Ventana, of the breast carcinoma no. 1 without gene amplification: HER-2/chr. 17 ratio 1.1 – 1.3*.
The HER-2 genes are stained black and chr. 17 red.
The signals are distinctively demonstrated.
Many cells show polysomia and in areas a level of ≥ 4 but less < 6 HER-2 gene copies is identified.

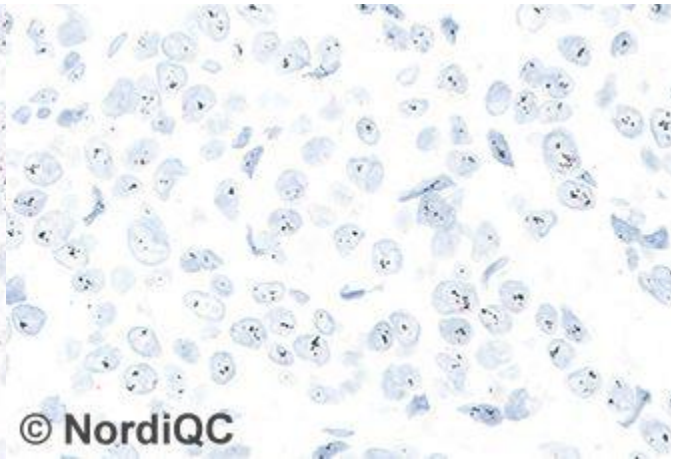


Fig. 1b
Optimal demonstration of the HER-2 gene status using the INFORM™ Dual ISH kit cat. no. 800-4422/780-4422, Ventana, of the breast carcinoma no. 2 with gene amplification: HER-2/chr. 17 ratio >6.
The HER-2 genes are stained black and chr. 17 red.
The HER-2 signals are mostly located in clusters and distinctively demonstrated.

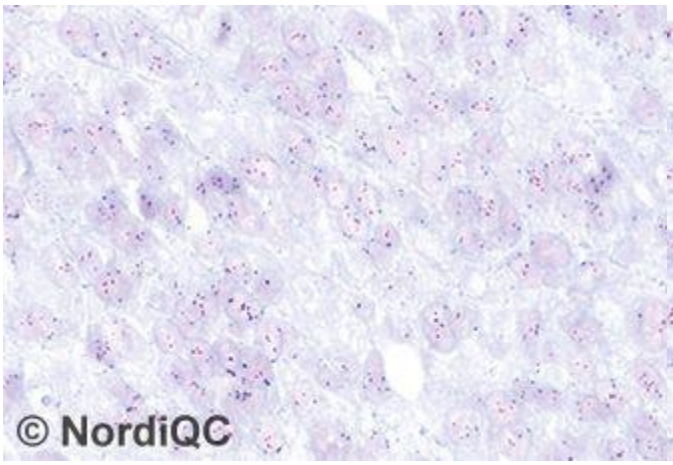


Fig. 2a
Optimal demonstration of the HER-2 gene status using the DuoCISH pharmDx™ cat. no. SK109, Dako, of the breast carcinoma no. 1 without gene amplification:
HER-2/chr. 17 ratio 1.1 – 1.3.
The HER-2 genes are stained red and chr. 17 blue.
Many cells show polysomia and in areas a level of ≥ 4 but less < 6 HER-2 gene copies is identified.

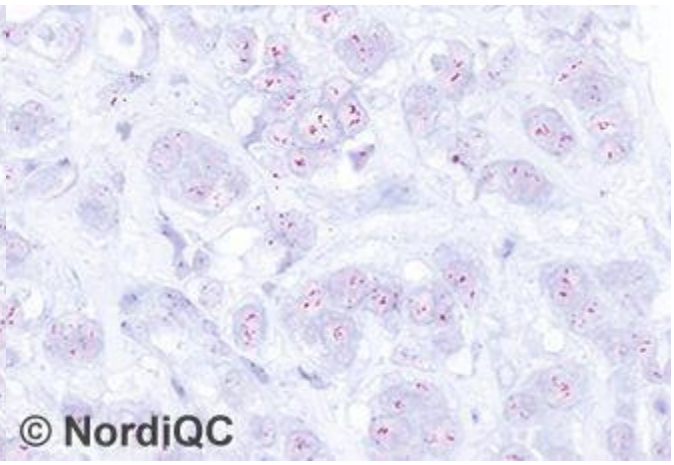


Fig. 2b
Optimal demonstration of the HER-2 gene status using the DuoCISH pharmDx™ cat. no. SK109, Dako, of the breast carcinoma no. 2 with gene amplification:
HER-2/chr. 17 ratio >6.
The HER-2 genes are stained red and chr. 17 blue.
The HER-2 signals are mostly located in clusters and distinctively demonstrated.

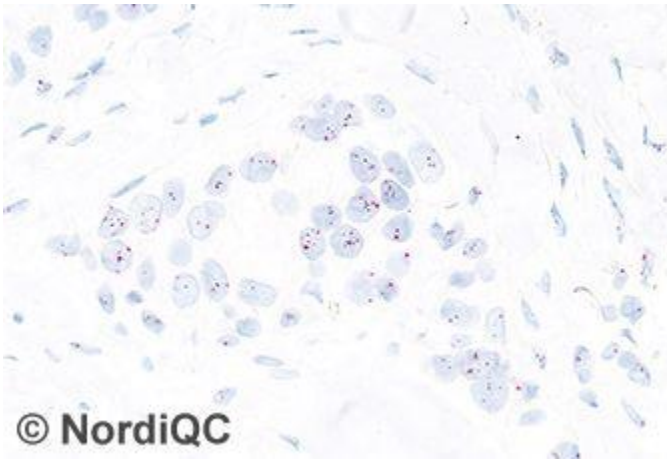


Fig. 3a
Optimal demonstration of the HER-2 gene status using the INFORM™ Dual ISH kit cat. no. 800-4422/780-4422, Ventana, of the breast carcinoma no. 4 showing a HER-2/chr. 17 ratio of 1.2 – 1.5.
The HER-2 genes are stained black, chr. 17 red.
Also compare with Fig. 3b using same assay but in combination with IHC for HER-2 providing a gene protein assay (GPA) and thus information on both HER-2 gene and HER-2 protein level simultaneously.

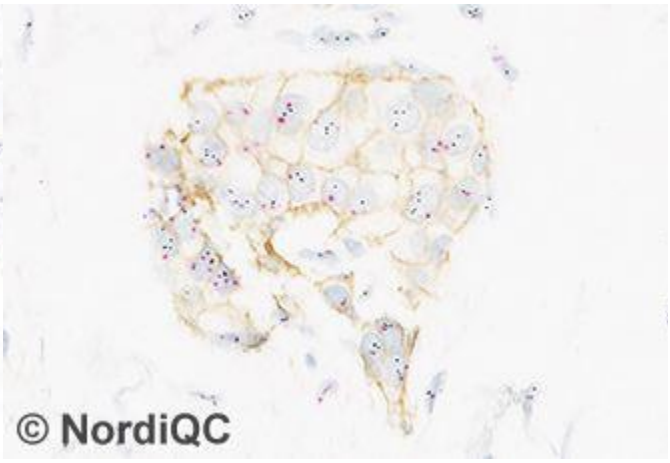


Fig. 3b
Optimal demonstration of the HER-2 gene status using the INFORM™ Dual ISH kit cat. no. 800-4422/780-4422, Ventana, of the breast carcinoma no. 4 showing a HER-2/chr. 17 ratio of 1.2 – 1.5.
In addition HER-2 protein is demonstrated by IHC using PATHWAY, Ventana.
The HER-2 genes are stained black, chr. 17 red and HER-2 protein brown.
The IHC level is interpreted as 2+ and the GPA visualizes hot-spots where to evaluate the HER-2 gene status precisely.
The tumour was by the NordiQC reference laboratories and by the vast majority of participants scored as non-amplified.

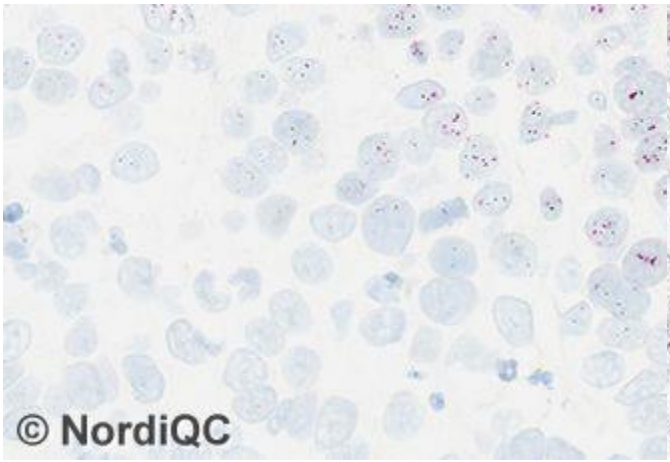


Fig. 4a
Insufficient staining for the HER-2 gene using the INFORM™ Dual ISH kit cat. No. 800-4422/780-4422, Ventana, of the breast carcinoma no. 1 without gene amplification: HER-2/chr. 17 ratio 1.1 – 1.3*.
The vast majority of the neoplastic cells are negative and only in scattered cells HER-2 and chr. 17 signals can be identified. This aberrant reaction most likely was caused by a technical problem during the staining process in the BenchMark instrument. Same protocol settings were applied as used in Figs. 1 and 2.
Negative areas of < 25% in each of the tissue cores were accepted.
The laboratory reported the result as technically insufficient and new test required.

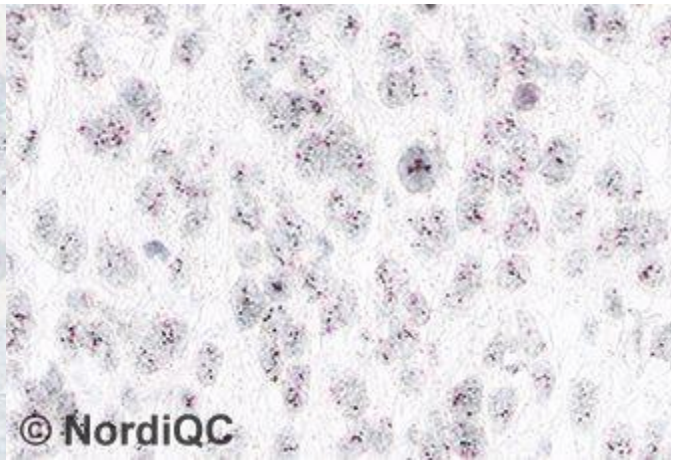


Fig. 4b
Insufficient staining for the HER-2 gene using the INFORM™ Dual ISH kit cat. No. 800-4422/780-4422, Ventana, of the breast carcinoma no. 3 without HER-2 gene amplification: HER-2/chr. 17 ratio 1.0 – 1.1*.
Due to extensive silver precipitates the HER-2 gene status cannot reliably be interpreted. This aberrant reaction most likely was caused by a technical problem during the staining process in the BenchMark instrument. Same protocol settings were applied as used in Figs. 1 and 2.
The laboratory reported the result as technically insufficient and new test required.

SN/LE/MV/RR 11.12.2015