Assessment Run H23 2023
HER2 (BRISH or FISH)

Purpose
The primary focus of this assessment is evaluation of the technical performance of HER2 Brightfield in-situ hybridization (BRISH) tests performed by the NordiQC participants for demonstration and establishment of the HER2 gene amplification level in breast carcinomas. In addition, the participants are asked to interpret and score the amplification status in the breast carcinomas and submit these to NordiQC in order to evaluate the inter-observer variability. The evaluation of inter-observer concordance is applicable for participants using either BRISH based tests or Fluorescent in-situ hybridization (FISH) based tests. The obtained assessment marks in NordiQC is indicative of the performance of the tests but due to the limited number and composition of samples, internal validation and extended quality control, e.g. regularly measuring the HER2 results, is necessary.

Material

Table 1. **Content of the multi-block used for the NordiQC HER2 ISH assessment, run H23**

	**HER2 IHC***	**Dual - BRISH****	**FISH*****	**FISH*****
	IHC score	**HER2/chr17 ratio¤**	**HER2/chr17 ratio¤**	**HER2 copies**
1. Breast carcinoma	0	0.6 - 0.9	0.6 - 1.0	1.3 - 1.7
2. Breast carcinoma	3+	3.4	5.8	9.6
3. Breast carcinoma	1+	1.5 - 1.7	1.3 - 1.4	2.5 - 2.6
4. Breast carcinoma	3+	2.5 - 3.3	2.3 - 3.4	4.3 - 6.3
5. Breast carcinoma	2+	1.1 - 1.2	1.0 - 1.1	1.9 - 2.6

* PATHWAY® (Ventana/Roche), data from two reference labs.
** Ventana HER2 Dual ISH DNA Probe Cocktail, data from one reference lab.
*** HER2 FISH (Zytovision), range of data from two tests from one reference lab.
¤HER2/chr17: HER2 gene/chromosome 17 ratio.

All tissues were fixed for 24-72 hours in 10% neutral buffered formalin according to the ASCO/CAP 2013/2018 guidelines for tissue preparation of breast tissue for HER2 ISH analysis.

HER2 BRISH, Technical assessment
The NordiQC assessors evaluate the technical quality of the BRISH tests and at this point do not conduct a precise estimation of the HER2 amplification status. The main criteria for the technical evaluation are as listed below.

The main criteria for assessing a BRISH HER2 analysis as technically **optimal** were the ability to 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.

HER2 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 and BRISH data from reference laboratories to analyze scoring consensus.

Consensus scores from the NordiQC BRISH/FISH reference laboratories

- Breast carcinoma, no. 1, 3 and 5: non-amplified
- Breast carcinoma, no. 2 and 4: amplified

The ASCO/CAP 2018 guidelines were applied for the interpretation of the HER2 status:

Amplified: HER2/chr17 ratio ≥ 2.0 using a dual probe assay with an average ≥ 4 HER2 copies per cell/nucleus. Using a single probe assay an average of ≥ 6 HER2 copies per cell/nucleus. (Group 1)

Equivocal (Additional work-up required):

HER2/chr17 ratio of ≥ 2.0 using a dual probe assay with an average of < 4 HER2 gene copies per cell/nucleus (Group 2)

HER2/chr17 ratio of < 2.0 using a dual probe assay with an average of ≥ 6 HER2 gene copies per cell/nucleus (Group 3)

HER2/chr17 ratio of < 2.0 using a dual probe assay with an average of ≥ 4 and < 6 HER2 gene copies per cell/nucleus (both dual and single probe assay) (Group 4)

Unamplified: HER2/chr17 ratio < 2.0 using a dual probe assay with an average < 4 HER2 gene copies per cell/nucleus (both dual and single probe assay) (Group 5)

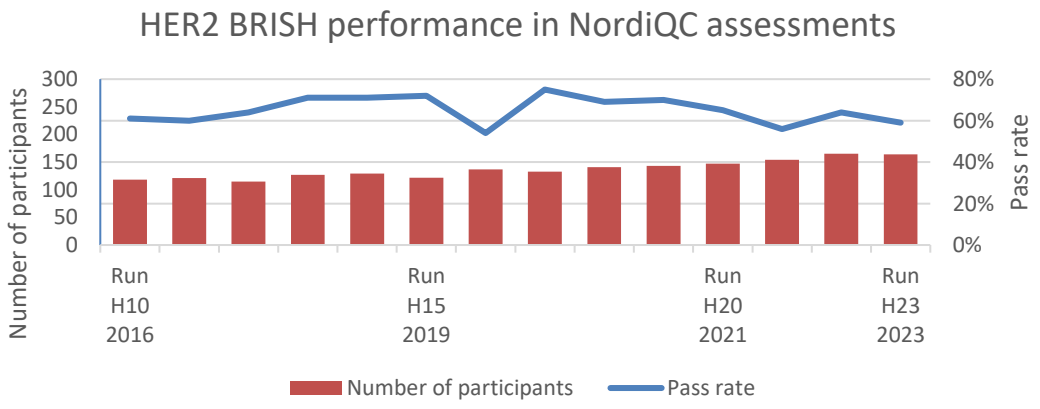
Participation

Number of laboratories registered for HER2 BRISH	175
Number of laboratories returning slides	163 (93%)
Number of laboratories returning scoring sheet	142
Number of laboratories registered for HER2 FISH	68
Number of laboratories returning scoring sheet	65

At the date of technical assessment meeting, 93% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment meeting were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

Performance history
In this assessment run H23 the overall pass rate was virtually on par compared to the levels obtained since run H10 2016 as illustrated in Graph 1, and still at a low level. Concordant to the previous NordiQC runs, the ISH rejection criteria defined in the 2013/2018 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 technical artefacts as large negative areas, excessive background and similar technical issues.

Graph 1. **Proportion of sufficient results for HER2 BRISH in NordiQC assessments, 2016 – 2023**



Results BRISH, technical assessment
In total, 163 laboratories participated in this assessment. 96 laboratories (59%) achieved a sufficient mark (optimal or good). Results are summarized in Table 2.

Table 2. **HER2 BRISH systems and assessment marks for BRISH HER2 run H23.**

Two colour HER2 systems	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
INFORM™ HER2 Dual ISH **780-4422/ 800-4422**	10	Ventana/Roche	4	1	4	1	50%	40%
VENTANA HER2 Dual ISH **800-6043**	127	Ventana/Roche	55	29	35	8	66%	43%
VENTANA HER2 Dual ISH + IHC **800-6043 + HER2 IHC (GPA*)**	19	Ventana/Roche	1	5	6	7	32%	5%
Zyto*Dot*® 2C **C-3022 / C-3032**	5	ZytoVision	1	0	3	1	20%	20%
One colour HER2 systems								
Zyto*Dot*® **C-3003**	2	ZytoVision	0	0	2	0	-	-
Total	163		61	35	50	17		
Proportion			37%	21%	31%	10%	59%	

1) Proportion of Sufficient Results (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
* GPA; Gene Protein Assay (HER2 BRISH + PATHWAY HER2 IHC).

Comments
In this run and in concordance with the latest assessments, the vast majority of participants (96%) used BRISH HER2 systems from Ventana/Roche. 90% (146 of 163 participants) used the VENTANA HER2 Dual ISH DNA Probe Cocktail (800-6043) and 6% (10 of 163) the INFORM™ HER2 Dual ISH assay (780-4422/800-4422).
12% of participants (19 of 163) used the VENTANA HER2 Dual ISH DNA Probe Cocktail (800-6043) in combination with HER2 IHC providing a Gene Protein Assay (GPA). In the evaluation of the technical assessment, only the HER2 BRISH results were addressed.

As shown in Table 2, a technically optimal performance for the demonstration of HER2/Chr17 signals permitting an adequate evaluation of the HER2 gene amplification status in the five breast carcinomas included in the multi-tissue block was obtained both by the two Ventana/Roche dual-colour BRISH systems and the ZytoVision ZytoDot® 2C system.
The insufficient results were most frequently characterized by large negative areas in one or more of the breast carcinoma samples, but also caused by impaired morphology, generally weak or missing signals for either HER2 and/or chr17.
In line with the previous NordiQC runs, the ISH rejection criteria defined in the 2013/2018 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 as listed above. In these cases, the staining results were thus rated as insufficient (poor or borderline). 64% (43 of 67) of the insufficient results were characterized by large negative areas covering more than 25% of one or more of the breast carcinomas. In the remaining 36% of the insufficient results these were caused by different artefacts as impaired morphology, excessive counterstaining, weak signals, silver precipitates and in more cases also negative areas were seen at the same time. Minor focal staining artefacts were accepted if they did not compromise the overall interpretation in each of the five individual tissue cores. In this context it has to be emphasized that focal negative areas <25% was accepted and did not impact the assessment mark and consequently also observed for sufficient results evaluated as optimal or good.

In this assessment the overall pass rate was slightly reduced compared to the level seen in the latest run H22 as illustrated in Graph 1, and also to the cumulated average level of 64% obtained in the NordiQC assessment runs performed for HER2 BRISH from 2016-2023 and no significant improvement in pass rates has been obtained in this period. In this assessment run H23, the combined GPA assay (VENTANA HER2 Dual ISH 800-6043 + HER2 IHC) was found less successful giving a pass rate of 32%, 5 % optimal. The insufficient results were typically characterized by a successful IHC test for HER2 and as such showing a distinct and strong 3+ IHC membranous reaction of the neoplastic cells in the tumour tissue core no. 2, but only scattered cells displaying HER2 gene signals despite being highly amplified (Her2/Chr17 ratio of 3.4-5.8 and > 6 HER2 signals pr cell) and likewise also in the IHC 2+ tissue core no. 5 without gene amplification being identified as 2+ IHC but only few cells having HER2/Chr 17 signals. The central protocol settings e.g. HIER time/temp., HIER buffers and proteolysis reported for the GPA assay were similar to the settings reported for the single use of the VENTANA HER2 Dual ISH 800-6043 assay and thus not possible to identify any parameters causing the very low pass rate in this run.

As indicated from Table 2, a consolidation and harmonization of methods used for HER2 BRISH has been effectuated, but despite this consolidation it has not been possible to progress the pass rates being more or less stable at the same level for 6-7 years. It is difficult to identify the exact root cause for the relatively low and disappointing pass rate and it can be impacted by many parameters and at different sites as both the laboratories, NordiQC and BRISH system vendors. From the laboratory perspective, the consolidation of both the choice of BRISH systems and also a certain harmonization of the protocols applied for these systems have been implemented. From NordiQC, the same assessment criteria have been applied, same requirements for tissue processing procedures for the samples used for the assessments (ASCO/CAP guidelines) but the samples originating from different donors. From the BRISH vendor site, precise and validated guidelines concerning protocol set-up for the recently launched VENTANA HER2 Dual ISH DNA Probe Cocktail (800-6043) are provided. However, these fundamentals have not been successful to improve and maintain a stable pass rate at a satisfactory level in the NordiQC HER2 BRISH assessment runs. An assessment evaluation by external quality programs gives an input to the performance of a specific analysis but cannot be used isolated to judge the quality (precision and accuracy) of this analysis and internal quality measurement must always be conducted in combination with the external quality assessment. In this aspect, for the laboratories receiving an insufficient mark as borderline or poor and caused by e.g. large negative areas >25% in the samples, they are encouraged to perform an internal analysis of retests needed on daily basis, general quality observed and if needed take contact to the vendor of the BRISH system to make a plan how to improve the reproducibility of the analysis.

Optimal protocol settings: Two-colour HER2 systems
127 laboratories used the **VENTANA Dual ISH system 800-6043** (Ventana/Roche).
Optimal demonstration of HER2 BRISH using this assay was typically based on the vendor recommended protocol settings based on a 2-step Heat Induced Epitope Retrieval (HIER) procedure using Cell Conditioning 1 (CC1) at 84°C followed by Cell Conditioning 2 (CC2) at 82°C and subsequent proteolysis in ISH Protease 3 or Protease 3 for 12-20 min. at 36-37°C. The HER2 and chr17 probe cocktail being applied for 60 min. at 44°C following a denaturation step at 80°C for 8 min. – both steps and parameters are fixed by the vendor.
Among the laboratories reporting these protocol settings a pass rate of 70% (60 of 86) was obtained, 48% being optimal.

19 laboratories used the **VENTANA Dual ISH system 800-6043** (Ventana/Roche) in combination with immunohistochemical demonstration for **HER2 PATHWAY®** (Ventana/Roche). The optimal result using this GPA assay, was based on HIER in CC2 for 24 min. and a subsequent proteolysis in ISH Protease 3 for 20 min. at 36°C. Among the laboratories using the GPA assay a pass rate of 32% was obtained, 5% optimal.

5 laboratories used the **ZytoDot® 2C system C-3022 / C-3032** (ZytoVision)
One protocol provided an optimal demonstration of HER2 BRISH and was based on HIER in EDTA, PT-0002-500 (ZytoVision) for 15 min. at 95°C, proteolysis in pepsin for 12 min. at 37°C, hybridization at 37°C for 16 hours following a denaturation at 75°C for 9 min. and visualization with the ZytoVision detection kit C-3022.

HER2 ISH interpretation and scoring consensus

Table 3. **NordiQC FISH amplification data***

	NordiQC FISH HER2/chr17 ratio	**NordiQC FISH HER2 copies**	**NordiQC HER2 amplification status**
1. Breast carcinoma	0.6 - 1.0	<4	Non-amplified
2. Breast carcinoma	5.8	>6	Amplified
3. Breast carcinoma	1.3 - 1.4	<4	Non-amplified
4. Breast carcinoma	2.3 - 3.4	>4	Amplified
5. Breast carcinoma	1.0 - 1.1	<4	Non-amplified

* data from one NordiQC reference laboratory.

No technical evaluation of FISH protocols was performed. Table 4 shows the ISH assays used by the participants and concordance level to the NordiQC data observed. It has to be emphasized that it was not possible to identify the cause of an aberrant interpretation of the HER2 status whether this was related to the technical performance of the FISH assay or the interpretation by the observer(s).

Table 4. **ISH assays used and level of consensus HER2 status to NordiQC reference data, H23**

BRISH	n*	Vendor	Consensus	No consensus	Consensus rate
INFORM™ HER2 Dual ISH **780-4422/ 800-4422**	8	Ventana/Roche	7	1	88%
VENTANA HER2 Dual ISH **800-6043**	111	Ventana/Roche	85	26	77%
VENTANA HER2 Dual ISH + IHC **800-6043 + HER2 IHC (GPA)**	16	Ventana/Roche	16	0	100%
Zyto*Dot*® 2C **C-3022 / C-3032**	5	ZytoVision	4	1	80%
Zyto*Dot*® **C-3003**	2	ZytoVision	2	0	-
FISH					
PathVysion HER-2 DNA **6N4630 / 30-161060**	12	Abbott	12	0	100%
HER2 IQFISH **GM333**	4	Dako/Agilent	4	0	-
HER2 IQFISH **K5731**	13	Dako/Agilent	12	1	92%
SureFISH **G110144G-8**	1	Dako/Agilent	1	0	-
BOND HER2 FISH system **TA9217**	7	Leica Biosystems	7	0	100%
HER2/CEN17 FISH probe **MF2001**	1	Maixin	1	0	-
FISH Kit **MAD-FISH-MDS**	2	Master Diagnostica	2	0	-
FISH ERB2 probe **KBI-10701**	1	Kreatech	1	0	
Rembrandt Her-2-C17 probe **C801K.5206**	2	PanPath	2	0	-
ZytoLight **Z-2015 / Z-2020/ Z-2077**	18	ZytoVision	18	0	100%
ZytoMation ERBB2/CEN17 Dual Color FISH Probe **Z-2292**	4	ZytoVision	4	0	-
Total	207		178	29	
Proportion			86%	14%	

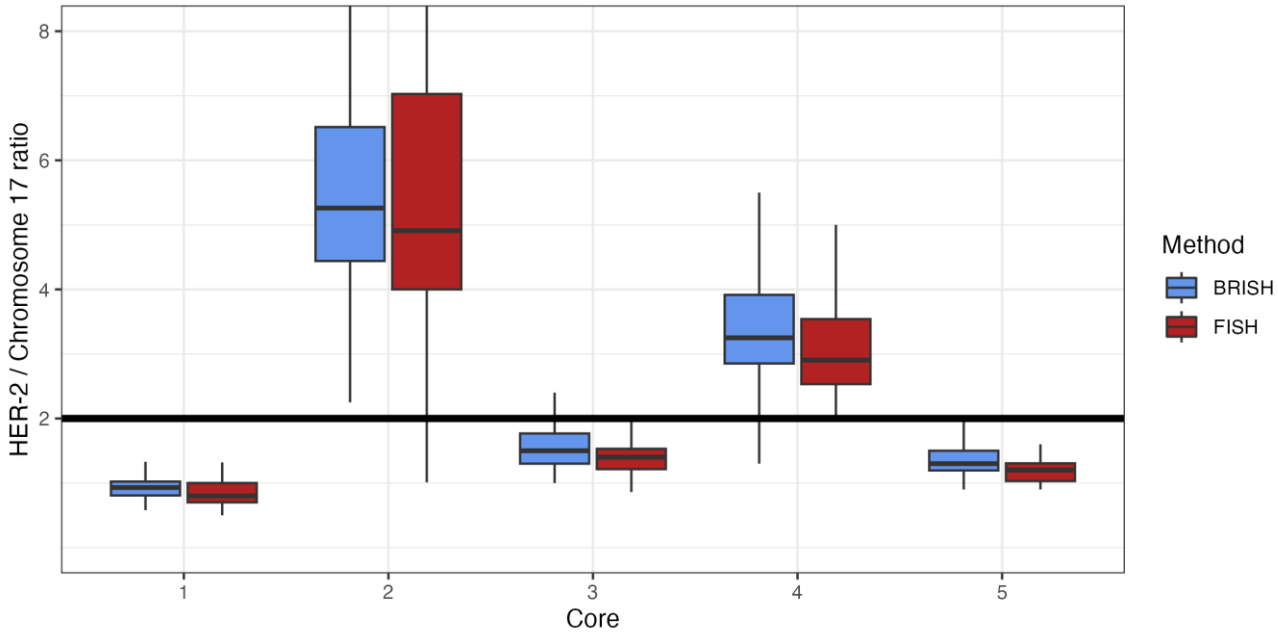
*The number varies from Table 2. Not all participants have submitted a scoring sheet.

207 of the 231 (90%) participating laboratories completed scoring sheets on the NordiQC homepage. These evaluations were compared to the HER2 ISH amplification status obtained by the NordiQC reference laboratories, summarized in Graph 2 and 3 (see page 6). For the laboratories performing FISH, the consensus rate was 98%, and 80% for laboratories using BRISH.

For BRISH, it was observed that the consensus rates for interpretation of the individual cores were virtually identical for laboratories that produced a staining reaction assessed as technically sufficient (Optimal and Good) and for laboratories with an insufficient mark (Borderline and Poor) being 81% and 80%, respectively. Despite a result evaluated as insufficient by the NordiQC assessor group, laboratories typically still were able to correctly evaluate the slide and settle the HER2 gene status. The ISH rejection criteria as outlined by the 2013/2018 ASCO/CAP HER2 guidelines and being applied by NordiQC indicate retest is required if more than 25% of the signals/cells cannot be interpreted due to artefacts such as silver precipitate, excessive background or negative areas without gene signals. The material in the assessment consisted of breast tumours with relatively homogenous HER2 expression, which permitted correct evaluation even in slides with large negative areas. This is not always the case in diagnostic settings with heterogeneous HER2 expression, biopsy material with limited tumor mass or HER2 evaluation in specific "hot-spot areas" identified by HER2 IHC.
The discrepancies for read-out among participants were mostly related to tissue core no. 3. This tissue was by NordiQC and 63/65 (98%) of the participants performing FISH scored as non-amplified, but by 20/142 (14%) of the participants performing BRISH classified as HER2 amplified or equivocal. The breast

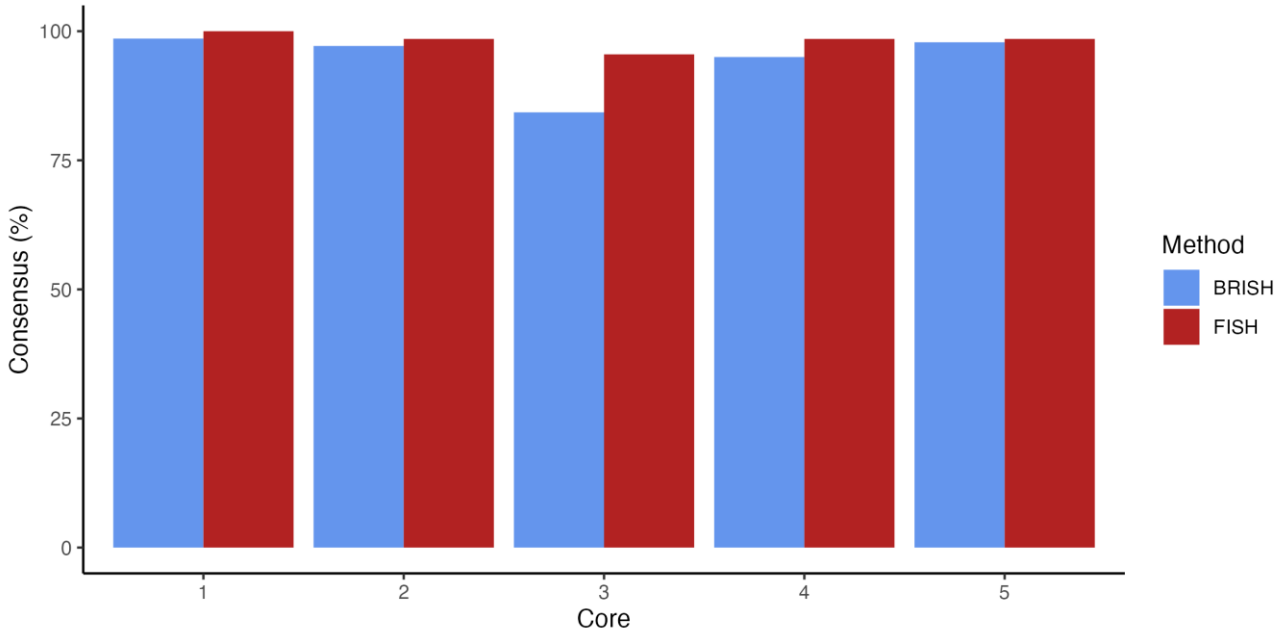
carcinoma, tissue core no. 3 was by the NordiQC reference ISH methods characterized as HER2 negative (IHC 1+) with a HER2/Chr17 ratio of 1.3-1.7 and HER2 copy number of 2.5-2.6.

Participants overall interpretation of amplification ratios and consensus rates are shown in Graph 2 and 3.



Graph 2

NordiQC HER2 ISH run H23: Participant interpretation of amplification status



Graph 3

NordiQC HER2 ISH run H23: Consensus depending on method

Conclusion

In this assessment a technical optimal demonstration of HER2 BRISH could be obtained by both the two Ventana/Roche two-colour HER2 systems **VENTANA HER2 Dual ISH** and **INFORM™ HER2 Dual ISH** and also by the **ZytoVision ZytoDot® 2C** system .

Overall focusing on the technical quality of the HER2 BRISH assays a relatively low pass rate of only 59% was obtained. This level has almost been consistent for the last runs.

For the most commonly used assay, the **VENTANA HER2 Dual ISH 800-6043** assay, being used by 127 participants the overall pass rate was 66% and 43% optimal and comparable to the level in run H22.

The insufficient results were mainly caused by large negative areas in one or more of the included tissue cores. In addition, also impaired morphology, excessive background and more artefacts in combination characterized the insufficient results.
Despite an assay harmonization and application of best practice protocols have been accomplished in the latest runs for HER2 BRISH, the overall pass rate is still only at a moderate level.

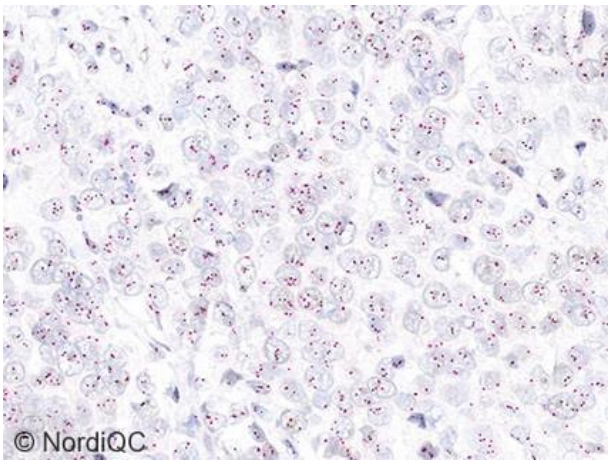


Fig. 1a
Optimal demonstration of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, of the breast carcinoma no. 1 without HER2 gene amplification:
HER2/chr17 ratio 0.6 - 1.0, <4 HER2 copies*.
The HER2 genes are stained black and chr17 red.
The morphology is well preserved, and signals distinctively demonstrated.
NordiQC and virtually all participants interpreted this tumour as non-amplified.

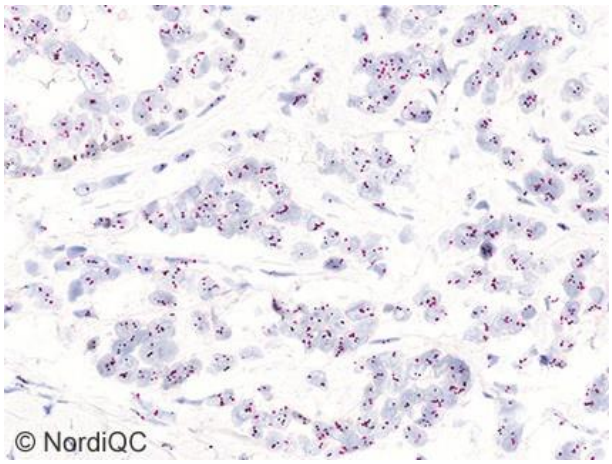


Fig. 1b
Optimal demonstration of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, of the breast carcinoma no. 5 without HER2 gene amplification:
HER2/chr17 ratio 1.0 – 1.2, <4 HER2 copies*.
The HER2 genes are stained black and chr17 red.
NordiQC and virtually all participants interpreted this tumour as non-amplified.

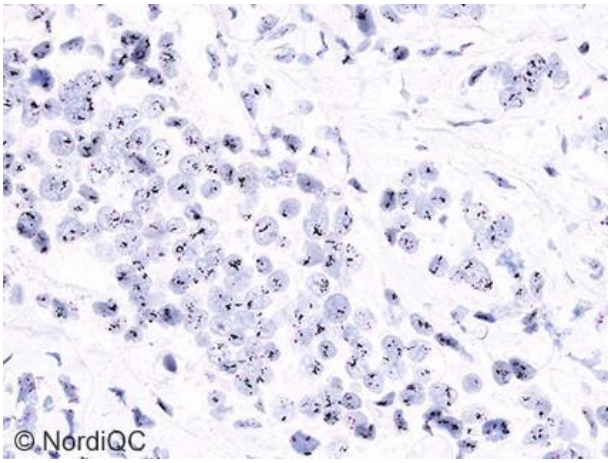


Fig. 2a
Optimal demonstration of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, of the breast carcinoma no. 2 with HER2 gene amplification:
HER2/chr17 ratio 3.4 – 5.8, >6 HER2 copies *.
The HER2 genes are stained black and chr17 red.
NordiQC and virtually all participants interpreted this tumour as amplified.

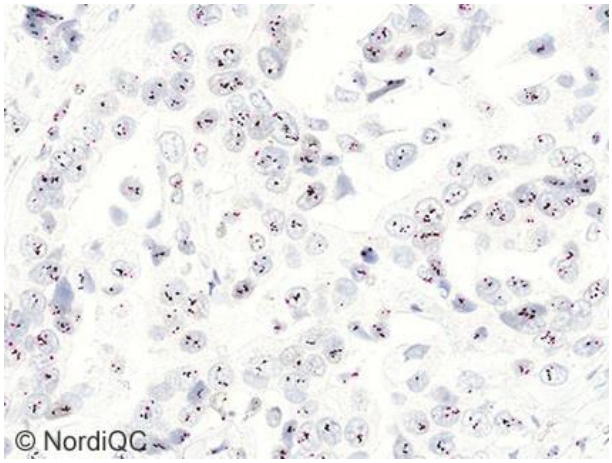


Fig. 2b
Optimal demonstration of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, of the breast carcinoma no. 4 with HER2 gene amplification:
HER2/chr17 ratio 2.3-3.4, ≥4 HER2 copies*.
The HER2 genes are stained black and chr17 red.
The signals are distinctively demonstrated in all the neoplastic cells.
NordiQC and most participants interpreted this tumour as amplified.

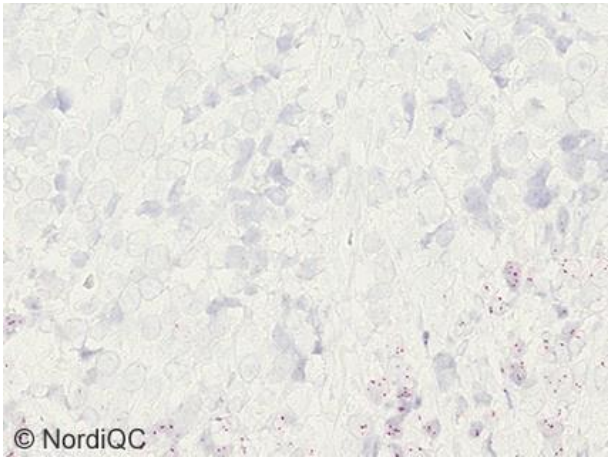


Fig. 3a
Insufficient staining of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, of the breast carcinoma no. 1 without HER2 gene amplification:
HER2/chr17 ratio 0.6 - 1.0, <4 HER2 copies*.
The HER2 genes are stained black and chr17 red.
The vast majority of cells and large areas (>25% of areas with neoplastic cells) are totally negative. This aberrant staining reaction / "negative spot artefact" was most likely caused by a technical issue during the staining process in the BenchMark instrument.

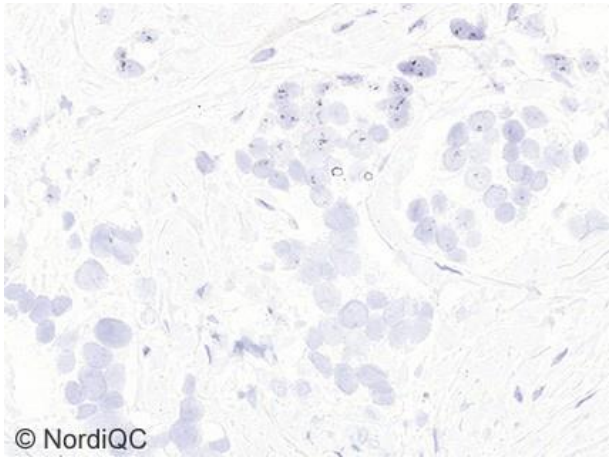


Fig. 3b
Insufficient staining of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, of the breast carcinoma no. 2 with HER2 gene amplification:
HER2/chr17 ratio 3.4 – 5.8, >6 HER2 copies*.
The HER2 genes are stained black and chr17 red.
The vast majority of cells and large areas (>25% of areas with neoplastic cells) are totally negative – in this field few large neoplastic cells show indication of amplification, but read-out compromised as Chr17 signals are missing and overall about 75% cells are without any signals. This aberrant staining reaction / "negative spot artefact" was most likely caused by a technical issue during the staining process in the BenchMark instrument.
Compare with Fig. 2a – same tumour.

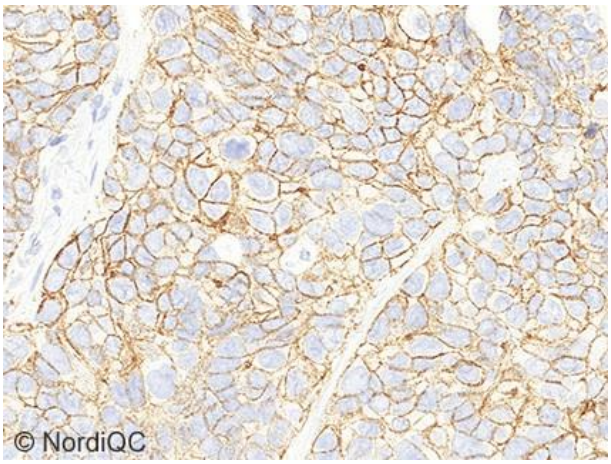


Fig. 4a
Insufficient staining of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, in combination with HER2 IHC using PATHWAY, Ventana/Roche, of the breast carcinoma no. 5 without HER2 gene amplification:
HER2/chr17 ratio 1.0 – 1.2, <4 HER2 copies*.
The gene protein assay (GPA) labels the HER2 genes black, chr17 red and HER2 protein brown.
The IHC level is interpreted as 2+ but the vast majority of neoplastic cells are totally negative concerning HER2 and Chr 17 signals and thus cannot reliably be scored.
This aberrant staining reaction / "negative spot artefact" was most likely caused by a technical issue during the staining process in the BenchMark instrument.

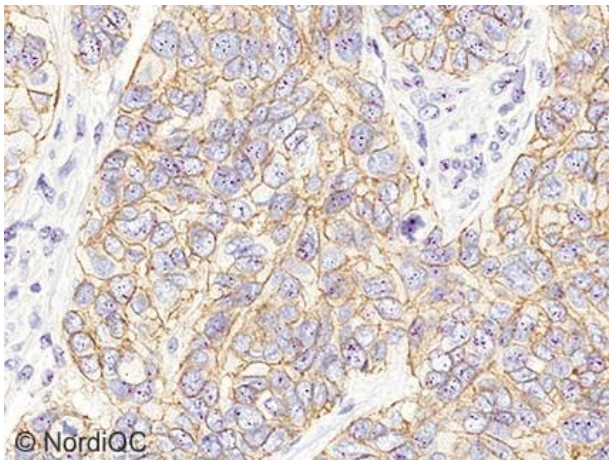


Fig. 4b
Optimal staining of the HER2 gene status using the VENTANA HER2 Dual ISH kit cat. no. 800-6043, Ventana/Roche, in combination with HER2 IHC using PATHWAY, Ventana/Roche, of the breast carcinoma no. 5 without HER2 gene amplification:
HER2/chr17 ratio 1.0 – 1.2, <4 HER2 copies*.
The gene protein assay (GPA) labels the HER2 genes black, chr17 red and HER2 protein brown.
The IHC level is interpreted as 2+ and the GPA assay visualizes the HER2 protein expression and the HER2/chr17 gene status simultaneously.
The participant interpreted this tumour as non-amplified.
NordiQC and virtually all participants also interpreted this tumour as non-amplified.
Compare with Fig. 1b. – same tumour.

* Range of data from FISH and BRISH performed in two NordiQC reference laboratories.

SN/LE 13.04.2023