Assessment Run B40 2025

Estrogen receptor (ER)

Purpose
Evaluation of the technical performance and level of analytical sensitivity and specificity of IHC tests performed by the NordiQC participants for demonstration of estrogen receptor (ER) expression in breast carcinomas. IHC, based on the rmAb clones SP1 and EP1, performed in a NordiQC reference laboratory served as reference standard methods and were used to identify breast carcinomas with the dynamic, diagnostic and critical relevant expression levels of ER. The obtained score in NordiQC is indicative of the performance of the IHC tests, but due to the limited number and composition of samples, internal validation and extended quality control (e.g. regularly measurement of ER results) is needed.

Material
The slide to be stained for ER comprised:

No.	Tissue	ER-positivity*	ER-intensity*
1.	Breast carcinoma	50-80%	Weak to moderate
2.	Uterine cervix	80-90%	Moderate to strong
3.	Breast carcinoma	90-100%	Moderate to strong
4.	Tonsil	1-5%	Weak to moderate
5.	Breast carcinoma	0%	Negative

* ER-status and staining pattern as characterized by NordiQC reference laboratory using the rmAb clones EP1 and SP1.

All tissues were fixed in 10% neutral buffered formalin for 24-48 hours and processed according to Allison et al.[1]

Criteria for assessing an ER staining as **optimal** included:

- A moderate to strong, distinct nuclear staining of virtually all columnar epithelial cells (if present), and most squamous epithelial and stromal cells (with the exception of endothelial cells and lymphoid cells) in the uterine cervix.
- An at least weak to moderate nuclear staining reaction in scattered follicular dendritic cells/T-cells and squamous epithelial cells in the tonsil, easily identified at low magnification (5x).
- An at least weak to moderate distinct nuclear staining in the appropriate proportion of the neoplastic cells in the ER positive breast carcinomas, tissue cores no. 1 and 3.
- No nuclear staining in the neoplastic cells in the ER negative breast carcinoma, tissue core no. 5.
- No more than a weak cytoplasmic reaction in cells with a strong nuclear staining reaction.

- An ER IHC result was classified as **good** if ≥ 10% of the neoplastic cells in the breast carcinomas, tissue cores no. 1 and 3, showed an at least weak nuclear staining reaction but in a significantly reduced proportion compared to the reference range. Alternatively or additionally, if a reduced but still weak to moderate nuclear staining reaction in the uterine columnar and squamous epithelial cells and/or in the dispersed cells expected to be positive in the tonsil was seen.

- An IHC result was also assessed as **good**, if the signal-to-noise ratio was low, e.g., because of moderate cytoplasmic reaction, background staining, excessive or inselective counterstaining or impaired morphology. The ER IHC result was still acceptable in all tissues.

- An ER IHC result was assessed as **borderline** if ≥ 1% and < 10% of the neoplastic cells in one or both of the breast carcinomas, tissue cores no. 1 and 3, showed a nuclear staining reaction. A negative staining reaction of the cells expected to be demonstrated in tonsil/uterine cervix can also be assessed as **borderline.**

  An IHC result could also be assessed as **borderline**, if the signal-to-noise ratio was low, e.g., because of moderate cytoplasmic reaction, excessive/inselective counterstaining or impaired morphology, to the extent where interpretation of the ER IHC result was compromised.

- An ER IHC result was assessed as **poor** if a false negative staining (< 1%) was seen in one of the breast carcinomas, tissue cores no. 1 and 3, or false positive staining (≥ 1%) was seen in the breast carcinoma, tissue core no. 5. Poor signal-to-noise ratio or poor morphology as described above could also result in a grade of **poor** where interpretation was severely hampered.

KEY POINTS FOR ER IHC ASSAYS

- Tonsil and uterine cervix are highly recommendable tissue controls to monitor analytical sensitivity and specificity.
- RTU assays were used by 94% of all participants.
- The rmAb clone EP1 was most successful, with high pass rates both applied as RTU or LD assay.
- The Dako/Agilent RTU system for Dako Omnis based on rmAb clone EP1 gave a pass rate of 87%. Close to the mean level of 92% seen in runs B36-B40 using VRPS.
- The Ventana/Roche RTU system based on rmAb clone SP1 showed significantly inferior performance and linked to faulty batches of primary antibody with reduced analytical sensitivity, as noted in the recent Roche recall notice.

Participation

Number of laboratories registered for ER, B40	457
Number of laboratories returning slides	427 (93%)

At the date of assessment, 427 (93%) of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

Results

427 laboratories participated in this assessment run and of these 303 (71%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks given (see page 4 and 5).

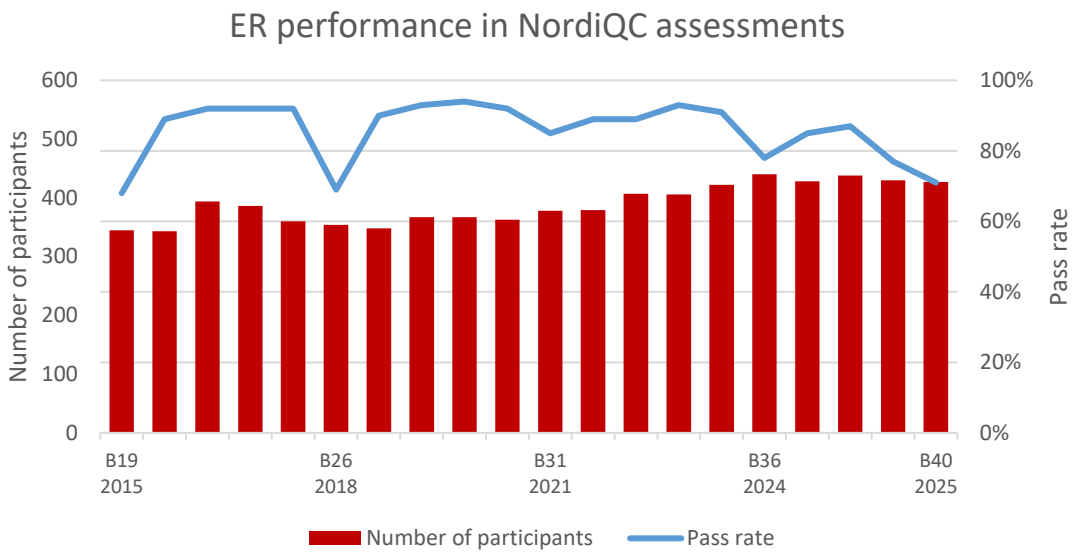
The commonest failing, accounting for 91% (114/125) of the insufficient results in this assessment, was low analytical sensitivity giving a too weak (84%; 105/125) or false negative staining reaction (7%; 9/125). This generally manifested as a severely reduced number of cells showing positive staining compared to the reference slide. Low analytical sensitivity and weak demonstration of ER was occasionally complicated by excessive or "inselective" counterstaining (where nuclei were difficult to distinguish from cytoplasm), or poor signal-to-noise ratios, leading to difficulties in scoring. No instances of a false positive result of the tumour expected to be negative for ER were observed in this run.

The most frequent causes of insufficient staining reactions were:
- Low reproducibility of the most commonly applied RTU system from Ventana/Roche based on rmAb clone SP1 with intended use on the BenchMark platforms
- Extended use of lot numbers of RTU formats of clone SP1 on the Benchmark platform recalled by Roche for inadequate sensitivity.
- Use of detection systems with low analytical sensitivity
- Insufficient HIER time or HIER in acidic buffer (weak staining)

Performance history

In this run the pass rate dropped to 71% and continued the decline observed in the previous run B39 with a pass rate of 77%. These two runs have shown a quite significant anomalous reduced pass rate compared to a stable and satisfactory level of 85-94% in runs between 2015-2024, with the exception of runs B19 and B26 (see Graph 1).

Graph 1. **Participant numbers and pass rates for ER from 2015 - 2025**



Fluctuations in pass rates in between assessment runs can be caused by many factors, related both to the protocols applied by the participants, circulation of more challenging material, variations in assessment criteria or similar factors. To ensure the consistency of the material circulated, NordiQC evaluates the selected tissue materials with two reference standard methods and in addition monitors the ER expression levels throughout all TMAs used in the assessment. NordiQC also strives to include testing material that show comparable and diagnostically-relevant levels of antigen expression in between each run. However, as in previous runs, similar pass rates continue to be observed for both existing and newly registered participants. As for run B36 (and more recently, B39), a reduced pass rate in this run was largely attributed to an inferior performance of the Ventana/Roche RTU system based on rmAb clone SP1 (790-4324/790-4325) on the BenchMark platform group. Over the last five runs, this RTU system was employed by an average of 57% of participants (range = 55% to 58%). The overall pass rate for the system fell from 95% in run B35 to 67% in the current run. The insufficient results across this period have overwhelmingly been characterized by reduced analytical sensitivity. For this run B40, the unsatisfactory performance of the Ventana/Roche RTU system was related to the extended use of lots of the RTU primary Ab being recalled by Roche in the period of this assessment run.

Conclusion

In this assessment, the rabbit monoclonal antibodies (rmAb) clones **SP1** and **EP1** and the mouse monoclonal Ab (mAb) clone **6F11** could all be used to provide an optimal result for demonstration of ER. The majority of participants (94%, 402 of 427) used RTU systems, with the majority of these (251 of 402, 62%) using the Ventana/Roche SP1 RTU product on the BenchMark platforms. Both RTU and concentrated primary antibody formats could be used successfully: however, the pass rate for participants using RTU antibodies was 71% (287 of 402) and slightly superior to 64% (16 of 25) for concentrated formats (Table 1a). In this run, the most robust performance was seen using the Dako/Agilent RTU system based on rmAb clone EP1 for Omnis used either as a true "plug and play" (vendor-recommended) assay, with a pass rate of 87% (22% optimal), or with modifications (90% pass, 37% optimal) (Tables 1a, 1c and 3). This clone also showed a similar performance as a true "plug and play" on the Link Autostainer platform with a pass rate 83%. In contrast the widely used RTU IHC system from Ventana/Roche based on rmAb clone SP1 provided significanly lower pass rates of 69% and 66% when applied with vendor recommended and laboratory modified protocol settings, respectively.

Uterine cervix and tonsil continue to be recommended as positive tissue controls for ER. In uterine cervix, virtually all squamous epithelial cells must show a moderate to strong and distinct nuclear staining reaction, whereas endothelial cells and lymphocytes must be negative. Tonsil is particularly recommended as a tool to monitor the level of analytical sensitivity for the demonstration of ER. Dispersed follicular dendritic cells[2] in germinal centers and squamous epithelial cells must show an at least weak, distinct nuclear staining reaction. In addition, tonsil can be used as negative tissue control, as B-cells in mantle zones and within germinal centers must be negative.

Table 1a. **Overall results for ER, run B40**

	n	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
Concentrated antibodies	25	6	10	7	2	64%	24%
Ready-To-Use antibodies	402	98	189	108	7	71%	24%
Total	427	104	199	115	9		
Proportion		24%	47%	27%	2%	71%	

1) Proportion of sufficient results (optimal or good).
2) Proportion of optimal results.

Table 1b. **Concentrated antibodies and assessment marks for ER, run B40**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **6F11**	10	Leica Biosystems	2	5	2	1	70%	20%
rmAb clone **EP1**	3 2	Dako/Agilent CellMarque	2	2	1	0	80%	40%
rmAb clone **SP1**	4 3 1 1	Thermo Sci./ePredia Cell Marque AbCam Diagnostic Biosystems	1	3	4	1	44%	11%
rmAb clone **QR013**	1	Quartett	1	-	-	-	-	-
Total	25		6	10	7	2		
Proportion			24%	40%	28%	8%	64%	

1) Proportion of sufficient results (optimal or good). (≥5 assessed protocols).
2) Proportion of optimal results.

Table 1c. **Ready-To-Use antibodies and assessment marks for ER, run B40**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **6F11** **PA0009/PA0151 (VRPS[3])**	2	Leica Biosystems	0	0	1	1	-	-
mAb clone **6F11** **PA0009/PA0151 (LMPS[4])**	15	Leica Biosystems	4	6	5	0	67%	27%
rmAb **EP1** **IR084 (VRPS[3])**	6	Dako/Agilent	1	4	1	0	83%	17%
rmAb **EP1** **IR084 (LMPS[4])**	18	Dako/Agilent	5	9	4	0	78%	28%
rmAb **EP1** **GA084 (VRPS[3])**	46	Dako/Agilent	10	30	6	0	87%	22%
rmAb **EP1** **GA084 (LMPS[4])**	33	Dako/Agilent	12	16	4	1	85%	36%
rmAb **EP1** **8361-C010**	3	Sakura Finetek	1	1	1	0	-	-
rmAb clone **SP1** **790-4324/4325 (VRPS[3])***	71	Ventana/Roche	12	37	21	1	69%	17%
rmAb clone **SP1** **790-4324/4325 (LMPS[4])***	185	Ventana/Roche	49	77	55	4	68%	26%
rmAb clone **SP1** **249R-17/18**	8	Cell Marque	1	3	4	0	50%	13%
rmAb clone **SP1** **MAD-0003060-DS-2/ MAD-000306QD**	2	Master Diagnostica Vitro SA	0	1	1	0	-	-
rmAb clone **SP1** **RMPD001**	1	Diagnostic BioSystems	0	1	0	0	-	-
rmAb clone **SP1** **GT205602**	1	Gene Tech	1	0	0	0	-	-
rmAb clone **SP1** **BRB053** **NOA-RB053**	5	Zytomed Systems	0	2	3	0	40%	0%
rmAb clone **SP1** **ALR 301 G7**	1	BioCare Medical	0	1	0	0	-	-
rmAb clone **SP1** **M3011**	1	Spring Biosystems	0	0	1	0	-	-
rmAb clones **SP1+6F11** **PM308**	1	BioCare Medical	1	0	0	0	-	-
Ab clone **DY49837** **4911432**	1	Dakewe/BioSci	0	1	0	0	-	-
rmAb clone **QR013** **P-E001-30**	1	Quartett	0	0	1	0	-	-
Ab clone **MXR030** **RMA-1065**	1	Fuzhou Maixin	1	0	0	0	-	-
Total	402		98	189	108	7		
Proportion			24%	47%	27%	2%	71%	

1) Proportion of sufficient results (optimal or good) (≥5 assessed protocols).
2) Proportion of optimal results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) refer to a specific RTU product applied on the vendor recommended platform(s) according to the manufacturer's recommended protocol settings.
4) Laboratory Modified Protocol Settings (LMPS) refer to a specific RTU product applied either on the vendor recommended platform(s) or other platforms .

Detailed analysis of ER: Run B40
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **6F11**: Two of 10 laboratories obtained optimal results: based on Heat Induced Epitope Retrieval (HIER) using Bond Epitope Retrieval Solution 2 (BERS2) pH 9.0 (Leica Biosystems) or Target Retrieval Solution (TRS) pH 9 (Dako/Agilent) as retrieval buffer. The mAb was diluted in the range of 1:25-1:30 and combined with a 2- or 3-layer detection system (Leica Polymer Refine, or Dako EnVision FLEX plus mouse linker).

rmAb clone **EP1**: Two of 5 laboratories obtained an optimal result, using a protocol based on HIER using BERS2 pH 9.0 (Leica Biosystems) or Cell Conditioning 1 (CC1, Ventana/Roche) as retrieval buffer. The rmAb was diluted 1:10-1:40 and combined with a 2-layer detection system. A sufficient result was obtained by 2 of 2 labs using these parameters.

rmAb clone **SP1**: One of 9 laboratories obtained an optimal result. The protocol with optimal result was based on HIER using TRS pH 9 (Dako/Agilent) (1/1) as retrieval buffer. The rmAb was diluted in 1:100 and combined with a 3-layer detection system (Dako EnVision FLEX plus rabbit linker).

Table 2 summarizes the overall proportion of optimal staining results when using the three most frequently used concentrated Abs on the most commonly used IHC staining platforms.

Table 2. **Optimal results for ER using concentrated antibodies on the main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer[1]**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark[2]**		**Leica Biosystems Bond[3]**	
	TRS High pH 9.0	TRS Low pH 6.1	TRS High pH 9.0	TRS Low pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **6F11**	-	-	1/1**	-	0/1	-	1/7 (10%)	0/1
rmAb clone **EP1**	-	-	0/1	-	1/2	-	1/2	-
rmAb clone **SP1**	-	-	1/1	-	0/4	-	0/2	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms.
** (number of optimal results/number of laboratories using this buffer).
1) Autostainer Classical, Link 48.
2) BenchMark GX, XT, Ultra, Ultra Plus
3) Bond III, Prime

Ready-To-Use antibodies and corresponding systems
mAb clone **6F11,** product. no. **PA0009/PA0151**, Leica Biosystems Bond III/Bond Max/Bond PRIME:
Four optimal results were obtained, using the following protocol: HIER in BERS2 (high pH) for 20-30 min., 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) or Bond-PRIME Polymer DAB Detection (DS9284) as detection system. Using these protocol settings, 7 of 9 (78%) laboratories were able to achieve sufficient results, 44% optimal. Three laboratories used a protocol using HIER with Bond Epitope Retrieval Solution 1 (BERS1, low pH), but none achieved a sufficient result.

rmAb clone **EP1**, product no. **IR084**, Dako/Agilent, Dako Autostainer+/Autostainer Link:
Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 20 min. at 97-98°C), 20 min. incubation of the primary Ab and EnVision FLEX (K8000/SM802, K8024/SM802) or EnVision+ Rabbit (K4003) as detection system, most commonly with Rabbit Linker (K8009, K8019)(4 of 5). Of the laboratories using these protocol settings, 10 of 11 (91%) produced a sufficient staining result, 45% optimal.
6 laboratories used product no IR084/IS084 on other platforms. These were not included in the description above.

rmAb clone **EP1**, product no. **GA084**, Dako/Agilent, Dako Omnis:
Protocols with optimal results were typically based on HIER using TRS High pH (efficient heating time 30 min. at 97°C), 10-28 min. incubation of the primary Ab (mode = 10 min.) and EnVision FLEX (GV800/GV823) with or without rabbit linker (GV809) as detection system. Of the laboratories using these protocol settings, 64 of 71 (90%) produced a sufficient staining result, 21 of 71 (30%) optimal.
3 laboratories used product no. GA084 on another platform and are not included in the description above.

rmAb clone **SP1**, product no. **790-4324/4325**, Ventana/Roche, BenchMark XT, ULTRA, ULTRA Plus:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 30-92 min. (mode = 64 min.), 16-60 min. incubation of the primary Ab (modes = 16 and 32 min.) and UltraView (760-500) with or without UltraView Amplification kit (760-080), or OptiView (760-700) without

amplification as detection system. Using these protocol settings, 158 of 232 (68%) laboratories produced a sufficient staining result, 57 of 232 (25%) optimal.
9 laboratories used product no 790-4324/4325 on other platforms. These were not included in the description above.

Table 3 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocol assays performed on the specific IHC platform(s) indicated on the datasheet are included.

Table 3. **Comparison of pass rates for vendor recommended and laboratory modified RTU protocols**

RTU systems	**Vendor recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS48 rmAb EP1 **IR084**	5/6 (83%)	1/6 (17%)	9/12 (75%)	4/12 (33%)
Dako Omnis rmAb EP1 **GA084**	40/46 (87%)	10/46 (22%)	27/30 (90%)	11/30 (37%)
Leica Bond III/Max mAb 6F11 **PA0009/PA0151**	0/2	0/2	6/10 (60%)	2/10 (20%)
VMS Ultra/XT/Ultra Plus rmAb SP1 **790-4324/4325**	49/71 (69%)	12/71 (17%)	117/176 (66%)	45/176 (26%)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Modifications included: retrieval method, retrieval duration, retrieval reagents, Ab incubation time, detection kit and use of amplification. Only protocols performed on the specified vendor IHC stainer are included.

Comments
Since Run B30, the assessment criteria continue to be centered on the tissue controls, tonsil and uterine cervix, in concordance to the ASCO/CAP 2020 recommendation on ER IHC testing. The results of previous NordiQC ER assessments, along with the ASCO/CAP guidelines (2020) strongly suggest that the use of tonsil as positive control material is essential to ensure an appropriate lower limit of sensitivity for demonstration of ER.

As in previous NordiQC runs for ER, the most common feature of an insufficient staining result in this assessment was inadequate analytical sensitivity, resulting in a weak or false negative staining reaction, with reduced detection of the ER epitope. This was seen in 92% of the insufficient results (114 of 124), with 10% of these weak staining reactions further complicated by sub-optimal counterstaining. Clinically relevant false positive staining reaction, where >1% of cells in the negative breast tumour showed positive signal was not observed in this run.

Demonstration of ER was successfully achieved by the majority of laboratories in the high-level ER-expressing breast carcinoma (tissue core no. 3), in which 90-100% of the neoplastic cells showed a moderate to strong intensity with the NordiQC reference standard assays. However, adequate detection of ER in the heterogeneous mid-level ER-expressing breast carcinoma (tissue core no. 1), in which an at least weak nuclear staining reaction of 50-80% of the neoplastic cells was expected (ER positivity level depending on TMA), proved more challenging.

Similarly to runs B36, B37 and B39, where an average of 52% of all results were assessed as good, the proportion of sufficient results assessed as good in this run was 47% (199 of 427 results). This is in contrast to runs B32-B35, which showed an average of 32% of all results in this category. The results assessed as good were again overwhelmingly characterized by reduced analytical sensitivity manifesting as significantly fewer cells staining positive for ER than expected.

Ready-To-Use (RTU) Abs were used by 94% (402 of 427) of the participants. 71% (287 of 402) of the laboratories using RTU formats obtained a sufficient staining result, 24% optimal (98 of 402).

The Ventana/Roche RTU system, 790-4324/4325 for BenchMark based on the rmAb clone SP1 was in this assessment the most widely applied assay being used by 58% (247 of 427) of all the participants and gave an overall pass rate of 67% (166 of 247), 23% optimal. Laboratory modified protocols (LMPS) were used by the majority (71%, 176 of 247) of participants using this system. Optimal results could be obtained both by the vendor recommended protocol settings (VRPS) (16 min. incubation of the primary Ab, HIER in CC1 for 64 min. and UltraView or iView as detection kit) and by laboratory modified protocol settings (LMPS) adjusting incubation time of the primary Ab, HIER time, detection systems and use of amplification as shown in Table 3. In this assessment, VRPS, used by only 29% (71 of

247) of the laboratories, provided a near identical pass rate compared to LMPS as shown in Tables 1c and 3, although the optimal rate was higher with LMPS.

Increasing the incubation time in primary antibody to 32 min. was the most commonly used single modification to the VRPS. This modification gave a slightly improved pass rate (73%) to the VRPS (69%) for laboratories using this protocol (22 of 30), although a reduced optimal rate of 10% versus 17% for VRPS was seen. Increasing the primary antibody incubation time to 40 min. further improved the pass rate to 100% (7 of 7), 43% optimal.

Use of OptiView as a substitute for UltraView detection as the sole modification to the VRPS was the second most commonly used (and most successful) single modification, with a pass rate of 88% (14 of 16), 50% optimal. Protocol modifications using OptiView detection (with or without alteration of primary incubation and HIER time) were in general highly successful, resulting in a pass rate of 80% (39 of 49 users), and a significantly improved optimal score rate of 57% (28 of 49) compared to 17% obtained using VRPS (see Table 3). Addition of UltraView Amplification as the sole modification to the VRPS was also highly successful, with a pass rate of 86% (6 of 7), 27% optimal. Together, as in runs B38 and B39, these observations suggest that use of the recommended UltraView detection system is associated with relatively reduced analytical sensitivity compared to the OptiView system, although analytical sensitivity for UltraView also may be increased by the addition of amplification kit.

As noted in B39, the Ventana/Roche RTU system for ER has in the 5 latest assessment runs exhibited a consistent, relatively significant decline from the mean pass rate of 95% (range 90%-97%) obtained in the 5 previous runs B30-B35, to an average pass rate of 82% (range = 67-95%) in runs B36-B40.
This declined pass rate and inferior performance of the Ventana/Roche RTU system, which especially has been observed in runs B39 and this run B40, is without question related to a reduced reproducibility of the CONFIRM anti-Estrogen Receptor (ER) (SP1) Rabbit Monoclonal Primary Antibody prod. Id. **790-4324/4325**. This was also observed and notified by Roche/Ventana as a recall of 11 different lot numbers of this product was communicated in a warning letter to all users of this early September 2025. In this assessment a pass rate of 63% was seen for protocols based on VRPS and using one of the 11 affected lots compared to 93% using an unaffected lot no. (accordingly to the warning letter).
In this context, NordiQC again encourages the participants to update the registered protocols on the NordiQC website and secure that the actual data including lot no. for primary Ab is updated to the protocol applied for the result submitted for assessment. If not updated, this compromise the validity of data analysis and tailored recommendations in case of an insufficient assessment mark.

The Dako/Agilent RTU system GA084 for Omnis, based on rmAb clone EP1 was used by 18% of the participants (76 of 427) and gave an overall pass rate of 88%, 28% optimal. The proportion of sufficient results was very similar for laboratories using the VRPS or LMPS, although LPMS provided 37% optimal results compared to 22% for laboratories applying VRPS.
The Dako/Agilent RTU system GA084 for Dako Omnis has shown a stable performance throughout the latest 5 assessment runs for ER. In runs B36-B40, the average pass rate has been 92% (range 87-95%) when applied by VRPS.

Six laboratories used the VRPS with the addition of rabbit linker and this was found to be a highly successful modification resulting in a pass rate of 100%, 67% optimal. Overall, modified protocols including rabbit linker obtained a pass rate of 100% (17 of 17), 53% optimal. Ten laboratories increased the primary antibody incubation time to 15-30 minutes as the sole modification from VRPS, obtaining a pass rate of 90%, 20% optimal.

The Dako/Agilent RTU system IR084 for Autostainer, also based on the rmAb EP1 was used by 4% (18 of 427) of the participants and provided an overall pass rate of 78%, 28% optimal. As shown in Table 3, 67% (12 of 18) of the laboratories modified the protocol settings and obtained a pass rate of 75% (33% optimal), compared to 83% pass and 17% optimal for laboratories using the VRPS.
The commonest and most successful modification (used by 8 laboratories) included addition of a rabbit linker with a pass rate of 88%, 50% optimal. However, the low number of laboratories using this format precludes a definitive analysis of protocol efficacy.

The Leica RTU system PA0009/PA0151 for BOND III and Max based on mAb 6F11, was in total used by 3% (12 of 427) of the participants and gave an overall pass rate of 63%, 25% optimal. In this assessment, VRPS based on HIER in BERS1 (low pH) for 20 min., 15 min. incubation of the primary Ab and Bond Refine DAB as detection system was used by two participants, with neither achieving sufficient results. One laboratory extended the HIER time in BERS1 to 40 minutes but did not achieve a sufficient result. Laboratories using a protocol modification increasing analytical sensitivity by using HIER in BERS2 (high pH) for 20 min. without extending the incubation time in primary antibody obtained a pass rate of 50% (1 of 2), with no optimal results. Further extending use of BERS2 to 30-40 minutes without increasing the primary Ab incubation time was a successful modification, giving 100% sufficient (4 of 4)

but no optimal results. Epitope retrieval using BERS2 for 20 min. with an extended primary antibody incubation time of 30 min. was employed by two laboratories: 1 of 2 obtained a sufficient result, with 1 optimal. As noted in previous runs, increasing analytical sensitivity by using BERS2 and/or extended incubation times must be balanced against the risk of inducing false positivity in the negative tumour, or a poor signal to noise ratio.
In this analysis only protocols performed on Bond II and Max was incorporated. At present the RTU is not validated by Leica Biosystems for Bond Prime.

In general, it must be emphasized that modifications of vendor recommended protocol settings for the RTU systems including migration of the RTU Abs to another platform than the intended, require a meticulous validation process for the end-users. As seen in this and previous assessments, modifications can be very successful but may also generate sub-optimal or aberrant results and therefore must be carefully monitored.

Concentrated antibody formats with laboratory-developed (LD) assays were used by 6% (25 of 427) of the participants, continuing the downward trend from 11% in run B35. The three most commonly applied Abs mAb clone 6F11, rmAb clones EP1 and SP1 used in a LD assay could all provide an optimal result on the tissues supplied in this run, and sufficient results were obtained with all three Abs on the main IHC platforms (Dako/Agilent, Leica Biosystems and Ventana/Roche), see Tables 1b and 2. The overall pass rate for all laboratories using concentrated antibody formats in this run was 64% (16/25), with 24% (6/25) obtaining optimal results. Whilst the pass rate is lower than that seen for laboratories using RTU formats (64% vs 71%, respectively), the optimal rate of 24% was identical across both formats, indicating that concentrated formats may still be employed effectively.

The rmAb clone 6F11 continues to be the most commonly used concentrated antibody: however, poor-signal-to-noise ratio and excessive background with a borderline false positive staining reaction in the negative tumour was associated with prolonged HIER in an alkaline buffer, and this was the leading cause of insufficient results with this clone. In previous runs, these conditions and/or too high a concentration of the primary antibody were seen to cause similar issues, with false positive staining of the negative tumour in the most extreme cases.

Although used by fewer participants, the most robust antibody clone used in a concentrated format in this run was rmAb clone EP1, with an overall pass rate of 80%, 14% optimal.

Irrespective of the clone applied, careful calibration of the primary Ab concentration in combination with efficient HIER, preferably in an alkaline buffer (with caveats for mAb clone 6F11 as discussed here, and in runs B39, B35, B28 and B15) were found to be the common core elements for an optimal performance.

In this run, 2-layer and 3-layer detection systems showed similar performance:. The majority of the laboratories using concentrated antibody formats used a 3-layer detection system (72%, 18 of 25) and 28% (7 of 25) used a 2-layer system. Laboratories using a 2-layer detection system with concentrated antibody formats obtained a pass rate of 71% (43% optimal), compared to a pass rate of 61% with 3-layer systems, 17% optimal. The opposite trend was seen with laboratories using RTU antibody formats, where the majority (79%, 316 of 402) used 2-layer detection systems. For laboratories using RTU formats, 2-layer systems gave a pass rate of 71% (19% optimal) with 3-layer systems giving a pass rate of 73% (44% optimal).

Controls
In concordance with previous NordiQC runs, uterine cervix and tonsil were found to be valuable positive and negative tissue controls for ER staining: In the uterine cervix, optimal results were characterized by a moderate to strong, distinct nuclear staining reaction in virtually all epithelial cells throughout the squamous epithelium and in the glands. In the stromal compartment, moderate to strong nuclear staining reaction was seen in most cells except endothelial and lymphatic cells.

Use of tonsil as a control tissue is particularly recommended as a tool to monitor analytical sensitivity for the demonstration of ER, and this tissue is superior to uterine cervix in this context. It was observed, that dispersed cells (most likely follicular dendritic cells[2]) in germinal centers and squamous epithelial cells were prominently demonstrated in virtually all protocols providing an optimal result. If the follicular dendritic cells were negative or weakly demonstrated, a reduced proportion of ER positive cells were seen in the other tissues and, most critically, an unsatisfactory weak or even false negative staining pattern was seen in breast carcinomas, tissue cores no. 1 and 3. In addition, tonsil can be used as supplementary negative tissue control, as B-cells in mantle zones and within germinal centers must be negative.

To validate the specificity of the IHC protocol further, an ER negative breast carcinoma must be included as primary negative tissue control, in which only remnants of normal epithelial and stromal cells should be ER positive, serving as internal positive tissue control. Positive staining reaction of the stromal cells in

breast tissue indicates that the IHC protocol provides a high analytical sensitivity for ER, whereas the analytical sensitivity cannot reliably be evaluated in normal epithelial cells in breast as they typically express moderate to high levels of ER.

1. Kimberly H. Allison, M. Elizabeth H. Hammond, Mitchell Dowsett, Shannon E. McKernin, Lisa A. Carey, Patrick L. Fitzgibbons, Daniel F. Hayes, Sunil R. Lakhani, Mariana Chavez-MacGregor, Jane Perlmutter, Charles M. Perou, Meredith M. Regan, David L. Rimm, W. Fraser Symmans, Emina E. Torlakovic, Leticia Varella, Giuseppe Viale, Tracey F. Weisberg, Lisa M. McShane, and Antonio C. Wolff. Estrogen and Progesterone Receptor Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Guideline Update. Arch Pathol Lab Med. 2020 May;144(5):545-563

2. Sapino A, Cassoni P, Ferrero E, Bongiovanni M, Righi L, Fortunati N, Crafa P, Chiarle R, Bussolati G. Estrogen receptor alpha is a novel marker expressed by follicular dendritic cells in lymph nodes and tumor-associated lymphoid infiltrates. Am J Pathol. 2003 Oct;163(4):1313-20. PubMed PMID: 14507640

Fig. 1a
Optimal ER staining reaction of the uterine cervix using the rmAb clone EP1 as RTU format (790-4324) from Ventana/Roche, using HIER in CC1 (pH 8.5) for 64 min., incubation time 16 min. in primary Ab and visualized by OptiView and performed on BenchMark Ultra.
The lot no. of the RTU primary Ab was not on the Roche list with problematic lots with reduced analytical sensitivity.
Virtually all squamous epithelial and stromal cells show a moderate to strong nuclear staining reaction.
Also compare with Figs. 2a – 5a, same protocol.

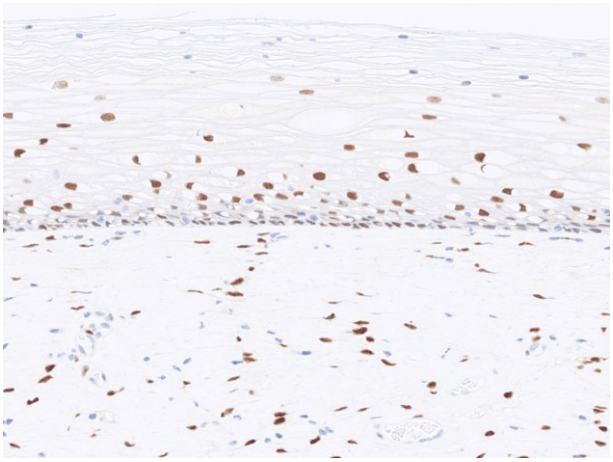


Fig 1b
ER staining reaction of the uterine cervix using the rmAb clone P1 as RTU format (790-4324) from Ventana/Roche, using HIER in CC1 (pH 8.5) for 64 min., incubation time 16 min. in primary Ab and visualized by UltraView and performed on BenchMark Ultra.
The protocol was applied in concordance to vendor recommendations but providing a general too low level of analytical sensitivity. The unexpected inferior performance was most likely related to the lot no. of the RTU format being listed in the Ventana/Roche customer bulletin TP-02561 indicating "Unacceptable light staining with specific lots.
A reduced intensity and proportion of squamous epithelial cells demonstrated is seen.
Also compare with Figs. 2b – 5b, same protocol.

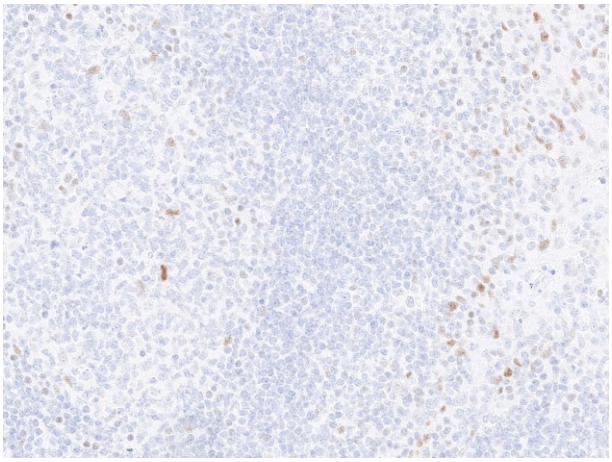


Fig. 2a
Optimal ER staining reaction of the tonsil using the same protocol as in Fig. 1a.
A distinct nuclear staining reaction in both dispersed follicular dendritic cells/T-cells in the germinal center and many squamous epithelial cells can be identified at even low magnification (10x).

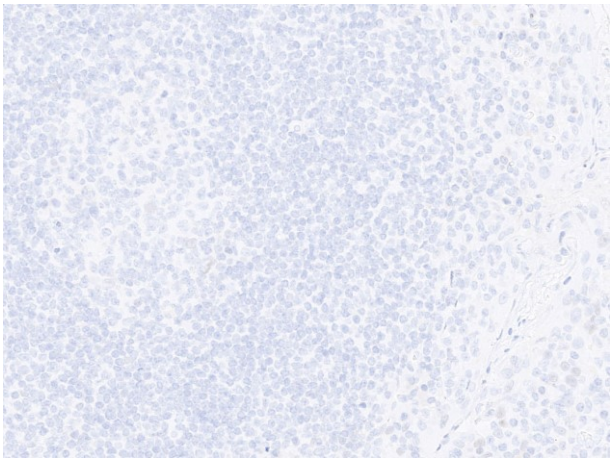


Fig. 2b.
Insufficient ER staining reaction of the tonsil using the same protocol as in Fig. 1b.
Only a faint equivocal staining reaction in few squamous epithelial cells is observed, whereas all follicular dendritic cells/T-cells are completely false negative.

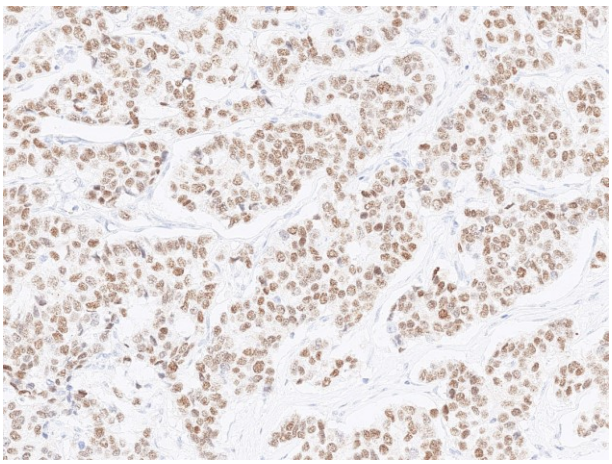


Fig. 3a
Optimal ER staining reaction of the breast carcinoma, tissue core no. 3, with 90-100% cells being positive (moderate to strong) using same protocol as in Figs. 1a – 2a.
The neoplastic cells display a moderate to strong and distinct nuclear staining reaction.

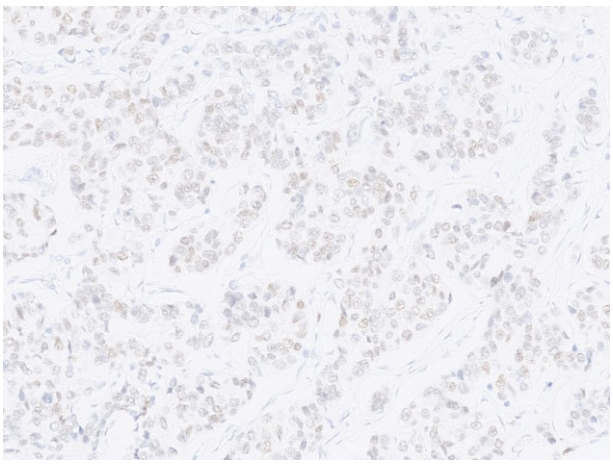


Fig. 3b.
ER staining reaction of the breast carcinoma, tissue core no. 3, with 90-100% cells being positive using the same protocol as in Figs. 1b – 2b.
More than 10% of the neoplastic cells display a positive staining reaction for ER, but the intensisty and proportion of cells demonstrated is significantly reduced compared to the level expected and obtained in both Fig. 3a (Ventana/Roche RTU 790-4324 using Optiview) and Fig. 6b. (Dako/Agilent RTU GA084).
However also compare with Fig. 4b, same protocol.

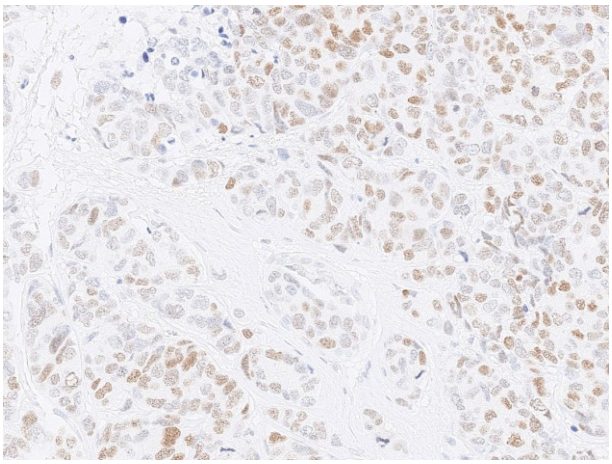


Fig. 4a
Optimal ER staining reaction of the breast carcinoma, tissue core no. 1, with 50-80% of the neoplastic cells expected to be positive (weak to moderate) using same protocol as in Figs. 1a – 3a.
About 60-70% of the neoplastic cells display a weak to moderate but distinct nuclear staining reaction.

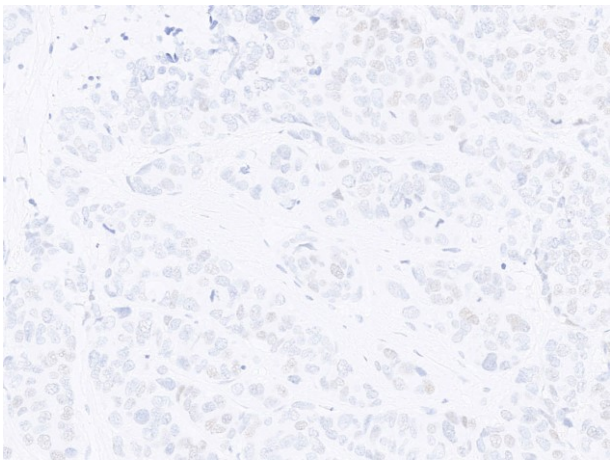


Fig. 4b
Insufficient ER staining reaction of the breast carcinoma, tissue core no. 1, with 50-80% of the neoplastic cells expected to be positive (weak to moderate) using same protocol as in Figs. 1b – 3b.
Only scattered (<1% overall) neoplastic cells show a weak and equivocal nuclear staining reaction for ER.

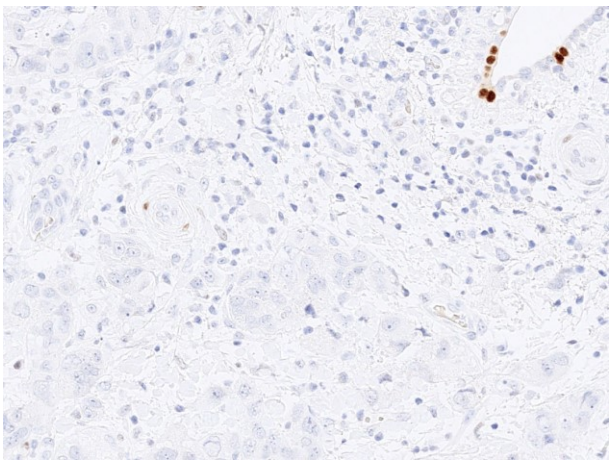


Fig. 5a
Optimal ER staining of the breast carcinoma expected to be ER negative, tissue core no. 5, using the same protocol as in Figs. 1a - 4a. No staining reaction in the neoplastic cells is seen, whereas a moderate to strong nuclear staining reaction is seen in dispered epithelial cells lining the normal gland (top right) and serving as internal positive tissue control and supplemental to the external tissues controls (tonsil and uterine cervix).

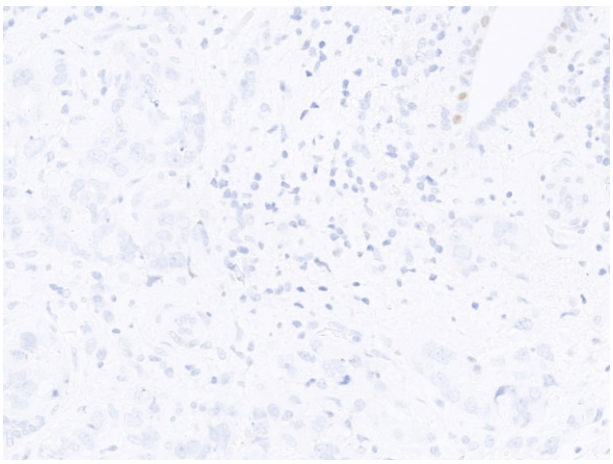


Fig. 5b
ER staining of the breast carcinoma expected to be ER negative, tissue core no. 5, using the same protocol as in Figs. 1b – 4b. As expected, no staining reaction in the neoplastic cells is seen, whereas a weak nuclear staining reaction is seen in dispered epithelial cells lining the normal gland (top right).
The use of normal epithelial cells to serve as internal positive tissue control can be less reliable, as expression level is not consistent and in this case normal cells with a higher expression level as seen in the breast carcinoma, tissue core no. 1.

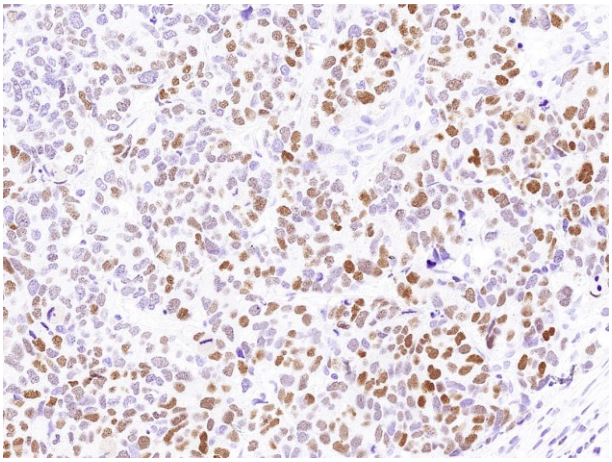


Fig. 6a
Optimal ER staining reaction of the breast carcinoma, tissue core no. 1, with 50-80% of the neoplastic cells expected to be positive (weak to moderate) using the Dako/Agilent ER RTU system for Dako Omnis based on the rmAb clone EP1. The protocol was applied in concordance to vendor protocol recommendations based on HIER in TRS High pH and EnVision FLEX as detection system. .
About 50-60% of the neoplastic cells display a weak to moderate but distinct nuclear staining reaction.

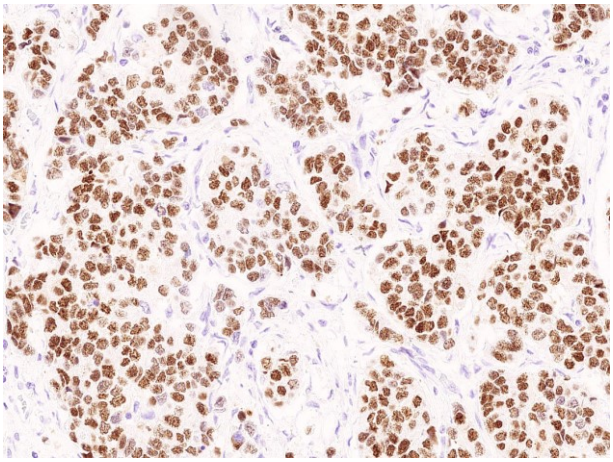


Fig. 6b
Optimal ER staining reaction of the breast carcinoma, tissue core no. 3, with 90-100% cells being positive (moderate to strong) using same protocol as in Fig. 6a. Virtually all neoplastic cells display a moderate to strong and distinct nuclear staining reaction.

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