## Assessment Run B36 2023

# 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. | Tonsil | 1-5% | Weak to moderate |
| 2. | Uterine cervix | 80-90% | Moderate to strong |
| 3. | Breast carcinoma | 50-90% | Weak to moderate |
| 4. | Breast carcinoma | 90-100% | Moderate to strong |
| 5. | Breast carcinoma | 0% | Negative |


* ER-status and staining pattern as characterized by NordiQC reference laboratories 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 squamous epithelial, columnar epithelial cells (if present) 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 breast carcinomas, tissue cores no. 3 and 4.
- No nuclear staining in the neoplastic cells in 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. 3 and 4 showed an at least weak nuclear staining reaction but in a significantly reduced proportion compared to the reference range. An at least weak to moderate nuclear staining reaction in the majority of the uterine columnar and squamous epithelial cells and in the dispersed cells expected to be positive in the tonsil.

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

- 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. 3 and 4, showed a nuclear staining reaction. A negative staining reaction of the cells expected to be demonstrated in tonsil/uterine cervix can also be marked 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 was compromised.

- An IHC result was assessed as **poor** if a false negative staining (< 1%) was seen in one of the breast carcinomas, tissue cores no. 3 and 4, 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.

**Participation**

| | |
|---|---|
| Number of laboratories registered for ER, B36 | 466 |
| Number of laboratories returning slides | 440 (94%) |

At the date of assessment, 94% 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**

440 laboratories participated in this assessment. 342 of 440 (78%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks given (see page 4).

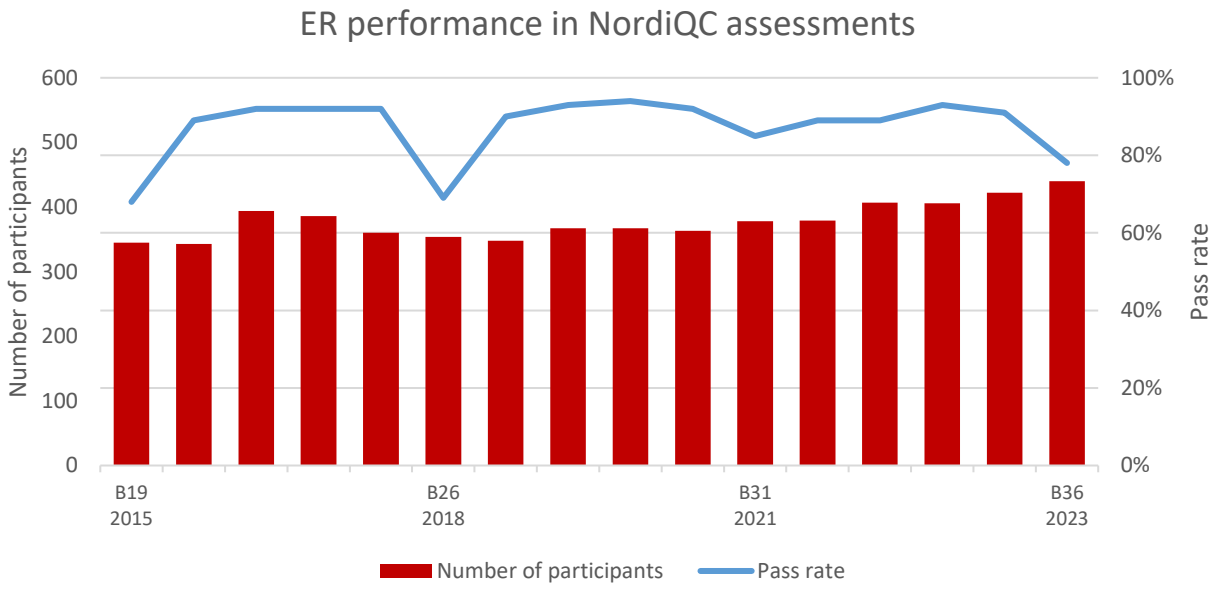
The most common staining faults reported were weak staining and excessive counterstaining hindering interpretation, and the most frequent causes of insufficient staining reactions were:

- Insufficient Heat Induced Epitope Retrieval (HIER) time or HIER in acidic buffer (weak staining)
- Excessive HIER or primary Ab incubation time (scoring hindered by excessive background or poor signal/noise ratio)
- Use of detection systems with low sensitivity (weak staining)
- Unexplained inferior performance of the Ventana/Roche IHC RTU system based on rmAb clone SP1

**Performance history**

In this run the pass rate of 78% (proportion of sufficient results) was a significant reduction compared to run B35 (91%) although previously the pass rate had been stable at a high and satisfactory level in runs between 2015-2023, with the exception of runs B19 and B26 (see Graph 1).

Graph 1. **Participant numbers and pass rates for ER from 2015 - 2023**




Fluctuations in pass rates (e.g. as seen in runs B19 and B26), can be caused by the circulation of more challenging material. In order to ensure the consistency of the material circulated, NordiQC evaluates the material with two reference standard methods and monitors the ER expression levels throughout all TMAs used in the assessment. Fluctuation in pass rates may also be influenced by new participants and in average 5% more participants have been registered for each of the last three runs, but similar pass rates being observed for both existing and new registered participants. In this run, the decline in pass rate was in particular attributed to an inferior performance using the most common combination of primary antibody and staining platform, RTU format of rmAb clone SP1 (790-4324/790-4325) on the Ventana BenchMark platform group. As for Run B35, over half of the participants used this combination (B35 = 61%; B36 = 58%). However, the pass rate for this group fell from 95% in run B35 to 78% in this run. The insufficient results were for the Ventana/Roche RTU system mainly characterized by reduced analytical sensitivity. At present no single parameter causing the low pass rate e.g. certain lot no of SP1 790-4324/4325 has been identified.

**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 (91%, 400 of 440) used Ready-To-Use (RTU) systems, with the majority of these (258 of 400, 65%) using the Ventana/Roche platform. Both RTU and concentrated primary antibody formats could be used successfully: the pass rate for participants using RTU antibodies was 80%, versus 60% for concentrated formats. "Plug and play" RTU assays (where a RTU format was used on its intended fully automated platform) gave an overall pass rate of 84% across the two major manufacturers platforms (Ventana/Roche BenchMark: 79% and Dako/Agilent Omnis: 91%).

The commonest failing, accounting for 86% (84/98) of insufficient results in this assessment, was low analytical sensitivity giving a weak or false negative staining reaction. Low analytical sensitivity and weak demonstration of ER was often further complicated by excessive or "inselective" counterstaining (where nuclei were difficult to distinguish from cytoplasm), or sections showing poor signal-to-noise ratios, leading to difficulties in scoring.

Uterine cervix and tonsil continue to be recommended as positive tissue controls for ER. In uterine cervix, virtually all squamous and columnar 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 1. **Antibodies and assessment marks for ER, B36**

| Concentrated antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | OR[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **6F11** | 12 | Leica Biosystems | 3 | 3 | 5 | 1 | 50% | 25% |
| rmAb clone **EP1** | 10 | Dako/Agilent | 1 | 7 | 1 | 1 | 80% | 10% |
| rmAb clone **SP1** | 9<br>3<br>2<br>1<br>1<br>1 | Thermo Sci./ePredia<br>Cell Marque<br>Zytomed<br>AbCam<br>BioCare<br>DCS | 3 | 7 | 5 | 2 | 59% | 18% |
| rmAb clone **QR013** | 1 | Quartett | 0 | 0 | 1 | 0 | - | - |
| Ready-To-Use antibodies | | | | | | | Suff.[1] | OR[2] |
| mAb clone **6F11**<br>**PA0009/PA0151 (VRPS[3])** | 4 | Leica Biosystems | 0 | 0 | 2 | 2 | - | - |
| mAb clone **6F11**<br>**PA0009/PA0151 (LMPS[4])** | 16 | Leica Biosystems | 1 | 9 | 5 | 1 | 63% | 6% |
| rmAb **EP1**<br>**IR/IS084 (VRPS[3])** | 4 | Dako/Agilent | 1 | 1 | 2 | 0 | - | - |
| rmAb **EP1**<br>**IR/IS084 (LMPS[4])** | 31 | Dako/Agilent | 6 | 21 | 2 | 2 | 87% | 19% |
| rmAb **EP1**<br>**GA084 (VRPS[3])** | 43 | Dako/Agilent | 13 | 26 | 4 | 0 | 91% | 30% |
| rmAb **EP1**<br>**GA084 (LMPS[4])** | 25 | Dako/Agilent | 4 | 19 | 2 | 0 | 92% | 16% |
| rmAb **EP1**<br>**AY710-50G** | 1 | BioGenex | 0 | 0 | 0 | 1 | - | - |
| rmAb **EP1**<br>**8361-C010** | 3 | Sakura Finetek | 1 | 2 | 0 | 0 | - | - |
| rmAb clone **SP1**<br>**790-4324/4325 (VRPS[3])*** | 59 | Ventana/Roche | 8 | 43 | 8 | 0 | 86% | 14% |
| rmAb clone **SP1**<br>**790-4324/4325 (LMPS[4])*** | 197 | Ventana/Roche | 49 | 104 | 40 | 4 | 78% | 25% |
| rmAb clone **SP1**<br>**249R-17/18** | 6 | Cell Marque | 2 | 3 | 0 | 1 | 83% | 33% |
| rmAb clone **SP1**<br>**MAD-000306QD/V**<br>**MAD-000306QD-7/N** | 3 | Master Diagnostica Vitro SA | 0 | 0 | 2 | 1 | - | - |
| rmAb clone **SP1**<br>**RMPD001** | 1 | Diagnostic BioSystems | 0 | 0 | 0 | 1 | - | - |
| rmAb clone **SP1**<br>**GT205602** | 1 | Gene Tech | 1 | 0 | 0 | 0 | - | - |
| rmAb clone **SP1**<br>**BRB056** | 1 | Zytomed Systems | 0 | 0 | 1 | 0 | - | - |
| rAb clone **BP6139**<br>**I12192e-05** | 1 | BioLynx | 0 | 0 | 1 | 0 | - | - |
| Ab clone **658G3A2**<br>**PA212** | 1 | Abcarta | 1 | 0 | 0 | 0 | - | - |
| Ab clone **C6H7**<br>**CEM-0081** | 1 | Celnovte | 1 | 0 | 0 | 0 | - | - |
| Ab clone **DA155**<br>**DMRD0191** | 1 | Shenzhen Dartmon Biotech. | 0 | 1 | 0 | 0 | - | - |
| Ab clone **MXR030**<br>**RMA-1065** | 1 | Fuxhou Maixin | 1 | 0 | 0 | 0 | - | - |
| Total | 440 | | 96 | 246 | 81 | 17 | 342 | |
| Proportion | | | 22% | 56% | 18% | 4% | 78% | |

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) to a specific RTU product applied on the vendor recommended platform(s).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s) or other platforms.

**Detailed analysis of ER: Run B36**
The following protocol parameters were central to obtain optimal staining:

**Concentrated antibodies**
mAb clone **6F11**: Three of 12 laboratories obtained optimal results: all were based on high pH HIER, using Cell Conditioning 1 (CC1, Ventana/Roche) (2/2)* or Bond Epitope Retrieval Solution 2 (BERS2) pH 9.0 (Leica Biosystems) (1/9) as retrieval buffer. The mAb was diluted in the range of 1:25-1:50 and combined with a 2- or 3-layer detection system. Using these protocol settings, 5 of 5 (100%) of laboratories produced a sufficient staining result (optimal or good), and 3 of 5 of laboratories produced an optimal result. Low pH HIER (Leica Biosystems Bond Epitope Retrieval Solution 1, pH 6.0) was employed by one laboratory, without a sufficient result.
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **EP1**: One of 10 laboratories obtained an optimal result, using a protocol based on high pH HIER, using Target Retreival Solution (TRS) pH 9 (3-in-1) (Dako/Agilent) (1/4) as retrieval buffer. The rmAb was diluted 1:80 and combined with a 2-layer detection system.

rmAb clone **SP1**: Three of 17 laboratories obtained optimal results. Protocols with optimal results were typically based on high pH HIER, using CC1 (Ventana/Roche) (2/7) or TRS pH 9 (Dako/Agilent) (1/3) as retrieval buffer. The rmAb was typically diluted in the range of 1:30-1:200 and combined with either a 2- or 3-layer detection system. Using these protocol settings, 10 of 15 (67%) laboratories produced a sufficient staining result.

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** | | **Dako/Agilent Omnis** | | **Ventana BenchMark XT/Ultra/GX** | | **Leica Bond III / Max** | |
|---|---|---|---|---|---|---|---|---|
| | 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** | - | - | - | - | 2/2** | - | 1/9 (11%) | 0/1 |
| rmAb clone **EP1** | 1/4 | - | 0/3 | - | 0/2 | - | - | - |
| rmAb clone **SP1** | - | - | 1/3 | - | 2/7 (29%) | - | 0/4 | 0/1 |

* 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).

**Ready-To-Use antibodies and corresponding systems**

mAb clone **6F11,** product. no. **PA0009/PA0151**, Leica Biosystems, Bond Prime/Bond III/Bond Max:
Only one optimal result was obtained, using the following protocol: HIER in BERS2 (high pH) for 30 min., 15 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Of the 20 laboratories using this antibody, 8/10 (80%) were able to achieve sufficient results using a protocol based on HIER using BERS2 (20-40 min.), 15-60 min. incubation of primary Ab and Bond Polymer Refine Detection. Eight laboratories used a protocol using HIER with BERS1 (low pH), but none achieved a sufficient result.

rmAb clone **EP1**, product no. **IR084/IS084**, 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 10-20 min. at 97-98°C; mode = 20 min.), 20-40 min. incubation of the primary Ab (mode = 20 min.) and EnVision FLEX (K8000/SM802, K8010/DM822) or EnVision FLEX+ (K8002/SM802) as detection system, with or without Rabbit Linker (K8009, K8019). Of the laboratories using these protocol settings, 18 of 21 (86%) produced a sufficient staining result.
*10 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 20-30 min. at 97°C, mode = 30 min.), 10-27 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, 58 of 62 (94%) produced a sufficient staining result.
*1 laboratory used product no. GA084 on another platform and is not included in the description above.*

rmAb clone **SP1**, product no. **790-4324/4325**, Ventana/Roche, BenchMark GX, XT, ULTRA, ULTRA Plus: Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 8-92 min. (mode = 64 min.), 16-40 min. incubation of the primary Ab (modes = 16, 32 min.) and UltraView (760-500) with or without UltraView Amplification kit (760-080), or OptiView (760-700) with or without optiView Amplification kit (760-099/860-099) as detection system. Using these protocol settings, 193 of 239 (81%) laboratories produced a sufficient staining result.
*3 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 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<br>rmAb EP1<br>**IR084/IS084** | 2/4 | 1/4 | 19/21 (90%) | 5/21 (24%) |
| Dako Omnis<br>rmAb EP1<br>**GA084** | 39/43 (91%) | 13/43 (30%) | 22/24 (92%) | 4/24 (17%) |
| Leica Bond<br>mAb 6F11<br>**PA009/PA0151** | 0/4 | 0/4 | 10/16 (63%) | 1/16 (6%) |
| VMS Ultra/XT/GX<br>rmAb SP1<br>**790-4324/4325** | 51/59 (86%) | 8/59 (14%) | 150/194 (77%) | 47/194 (24%) |

* 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 on 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 86% of the insufficient results (84 of 98). A weak or false negative staining reaction was further complicated by excessive or "inselective" counterstain in 14% (14 of 98) of the insufficient results. In one case, the counterstain was so excessive as to make resolution of the ER signal impossible, and in one case excessive background staining rendered the ER staining uninterpretable. Clinically relevant false positive staining reaction, where >1% of cells in the negative breast tumour (tissue core no. 5) stained unequivocally, was seen in one of the insufficient results. Poor signal-to-noise ratio and excessive background were seen in 11% and 3% of insufficient staining results respectively.

Virtually all laboratories were able to demonstrate ER in the high-level ER-expressing breast carcinoma (tissue core no. 4), in which 90-100% of the neoplastic cells were expected to be demonstrated and by the NordiQC reference standard methods, the cells showed a moderate to strong intensity. Demonstration of ER in the heterogeneous mid-level ER-expressing breast carcinoma (tissue core no. 3), in which an at least weak nuclear staining reaction of 50-90% of the neoplastic cells was expected, was much more challenging.

In this run an increased number of sufficient results assessed as good (56% of all results) was observed in comparison to Run B35 and previous runs, and the majority (76%) of these were characterized by reduced analytical sensitivity manifesting as significantly fewer cells staining positive for ER than expected. Other features included a poor-signal-to noise ratio, excessive background reactions and/or excessive or "inselective" nuclear counterstaining. The observation on both excessive background and excessive nuclear counterstaining was again primarily related to staining on the Omnis platform (Dako/Agilent), as discussed in Run B35. "Inselective" counterstaining, where nuclei were difficult to resolve, was primarily related to staining on the Ventana BenchMark platform group.

**Ready-To-Use (RTU) Abs** were used by 91% (400 of 440) of the participants. 80% (318 of 400) of these laboratories obtained a sufficient staining result, 22% optimal (89 of 400).

**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% of all the participants and gave an overall pass rate of 80%. Laboratory modified protocols (LMPS) were used by the majority (77%) 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 were used by only 23% of the laboratories and provided a slightly higher overall pass rate compared to LMPS as shown in Tables 1 and 3, although the optimal rate was somewhat higher with LMPS. Use of OptiView detection was a successful protocol modification, resulting in a slightly reduced pass rate of 82% (32/39 users) versus 86% for the manufacturer's protocol, but with an optimal score rate of 44% (17 of 39) compared to 14% using VRPS (see Table 3). Use of UltraView amplification in addition to the base detection system gave a pass rate of 100% (16 of 16) and an optimal rate of 63% (10 of 16).

**The Dako/Agilent RTU system GA084 for Omnis, based on rmAb clone EP1** was used by 15% of the participants and gave an overall pass rate of 91%. The proportion of sufficient results of 91% and 92% obtained by the VRPS and LMPS, respectively, were almost identical, whereas the VRPS provided 30% optimal results compared to 16% for laboratories applying LPMS. Seven laboratories used a modified protocol including rabbit linker, with a pass rate of 100% and 13 laboratories increased the primary antibody incubation time to 15-30 minutes, with a pass rate of 92%.

**The Dako/Agilent RTU system IR084/IS084 for Autostainer, also based on the rmAb EP1** was used by 6% of the participants and provided an overall pass rate of 84%. As shown in Table 3, 84% (21 of 25) of the laboratories modified the protocol settings and obtained a higher pass rate compared to laboratories using the RTU system according to the Dako/Agilent recommendations.
The commonest and most successful modification included use of a rabbit linker and was used by 10 laboratories: 90% of these obtained a sufficient mark (9/10) with 40% (4/10) optimal.

**The Leica RTU system PA0009/PA0151 for BOND based on mAb 6F11,** was used by 5% (20 of 440) of the participants and gave an overall pass rate of 50%. In this assessment, VRPS based on HIER in BERS1 (low pH) for 20 min., 15 min. incubation of the primary Ab and Bond Refine as detection system was used by four participants, with none achieving sufficient results. Laboratories using a protocol modification increasing analytical sensitivity by using HIER in BERS2 (high pH) for 20 min. obtained a pass rate of 83% (5 of 6), although no optimal results were obtained. Extending the HIER time in BERS2 to 30 minutes without increasing the primary Ab incubation time gave an optimal result (1/1), whilst protocols extending the primary incubation time alone, or in combination with extended HIER in BERS2 gave a pass rate of 66% (2/3), with no optimal results. In this run, false positive staining of the negative tumour (breast carcinoma no. 5) was not seen with high pH retrieval, as previously noted sporadically from run to run, even with extended retrieval times of up to 40 mins.

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 9% (40 of 440) of the participants, a reduction 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. Overall, the rmAb clone SP1 was the most commonly used concentrated antibody, with 18% of laboratories attaining an optimal result across all platforms. Sufficient results were obtained with all three Abs on the main IHC platforms (Dako/Agilent, Leica Biosystems and Ventana/Roche), see Tables 1 and 2. The most robust antibody clone in this run was EP1, with an overall pass rate of 81%. The overall pass rate for all laboratories using concentrated antibody formats in this run was 60% (24 of 40), with 18% (7of 40) obtaining optimal results.

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

In this run, 2-layer detection systems performed slightly more successfully than 3-layer detection systems: the majority of the laboratories using concentrated antibody formats used a 3-layer detection system (63%, 25/40), with 47% (15 of 40) using a 2-layer system. Laboratories using a 3-layer system obtained a pass rate of 52%, with 16% optimal, compared to 2-layer detection system, with a pass rate of 73%, 20% 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 distinctively 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. 3 and 4. 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

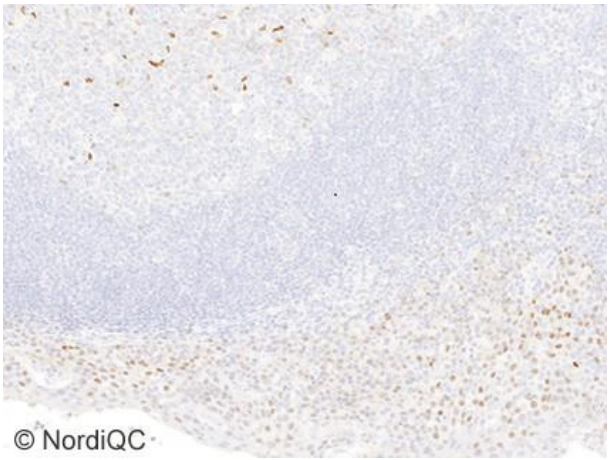



Fig. 1a
Optimal ER staining of the tonsil using the rmAb clone SP1 as RTU format (790-4324) from Ventana/Roche by vendor recommended protocol settings using HIER in CC1 (pH 8.5) for 64 min., incubation time 16 min. in primary Ab and visualized by UltraView and performed on BenchMark Ultra.
A distinct nuclear staining reaction in both dispersed follicular dendritic cells in the germinal center and many squamous epithelial cells can be identified at even low magnification (10x).
Also compare with Figs. 2a - 4a, same protocol.

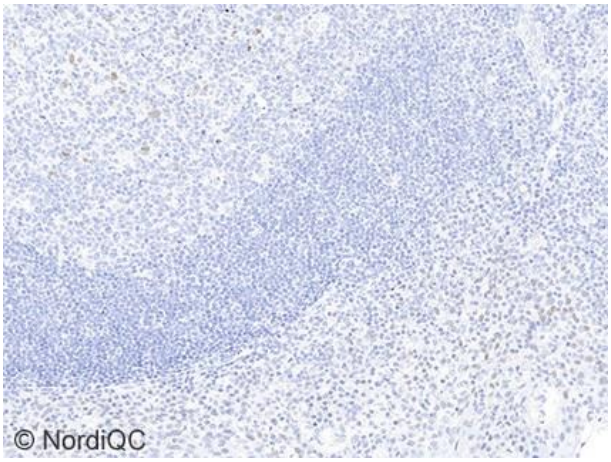



Fig. 1b
Insufficient ER staining of the tonsil using the rmAb clone SP1 as RTU format (790-4324) from Ventana/Roche by reduced HIER time and primary Ab incubation compared to the vendor recommended protocol – same field as in Fig. 1a.
Only a faint equivocal staining reaction in few follicular dendritic cells in the germinal center and squamous epithelial cells is observed.
Also compare with Figs. 2b - 4b, same protocol.

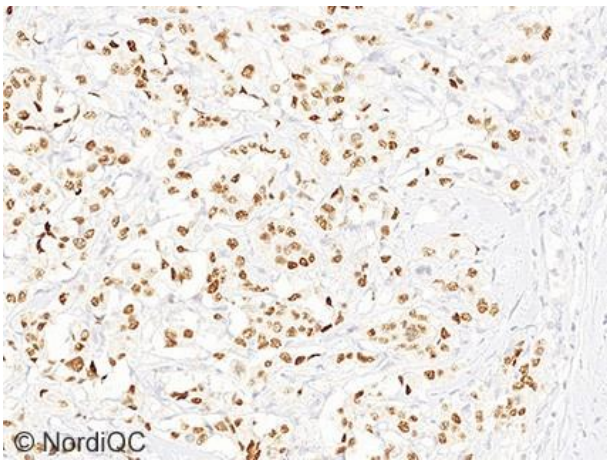



Fig. 2a
Optimal ER staining of the breast carcinoma, tissue core no. 4, with 90-100% cells being positive (moderate to strong) using same protocol as in Figs. 1a – 4a.
The neoplastic cells display a moderate to strong and distinct nuclear staining reaction.

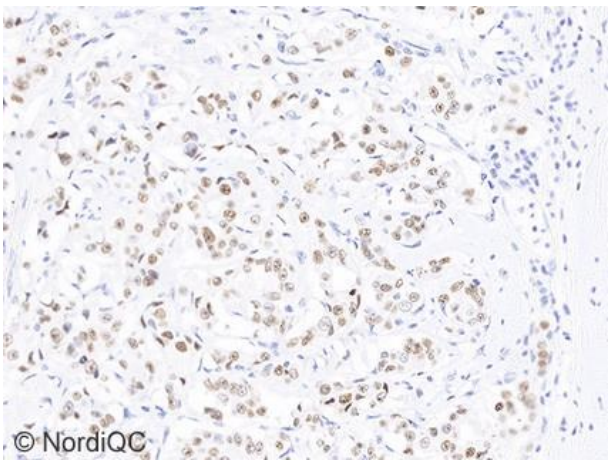



Fig. 2b.
ER staining of the breast carcinoma, tissue core no. 4, with 90-100% cells being positive using the same protocol as in Figs. 1b - 4b.
The neoplastic cells display a clear positive staining reaction for ER.
However also compare with Fig. 3b, same protocol.

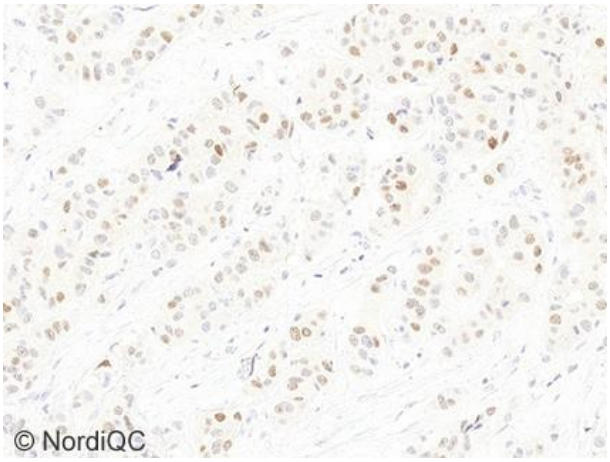



Fig. 3a
Optimal ER staining of the breast carcinoma, tissue core no. 3, with 50-90% of the neoplastic cells expected to be positive (weak to moderate) using same protocol as in Figs. 1a – 4a.
The majority of the neoplastic cells display a weak but distinct nuclear staining reaction.

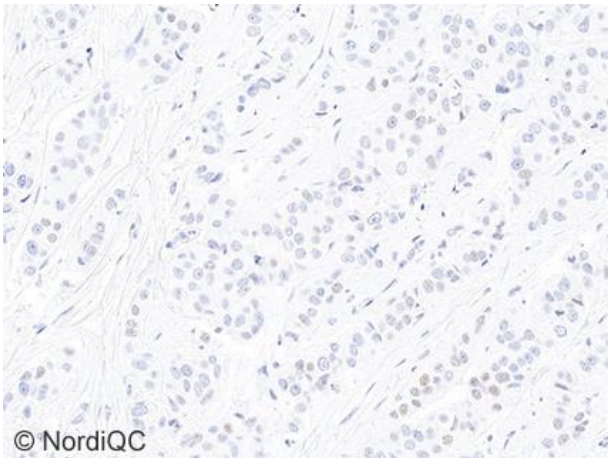



Fig. 3b
Insufficient (borderline) ER staining of the breast carcinoma, tissue core no. 3, with 50-90% of the neoplastic cells expected to be positive (weak to moderate) using same protocol as in Figs. 1b - 4b.
Only dispersed neoplastic cells show a weak and equivocal nuclear staining reaction for ER.

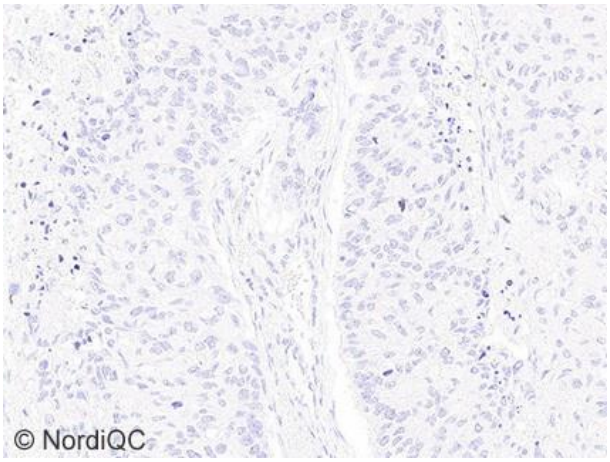



Fig. 4a
Optimal ER staining of the breast carcinoma, tissue core no. 5, expected to be negative using same protocol as in Figs. 1a – 3a. No nuclear staining reaction is seen and a high signal-to-noise ratio is observed.

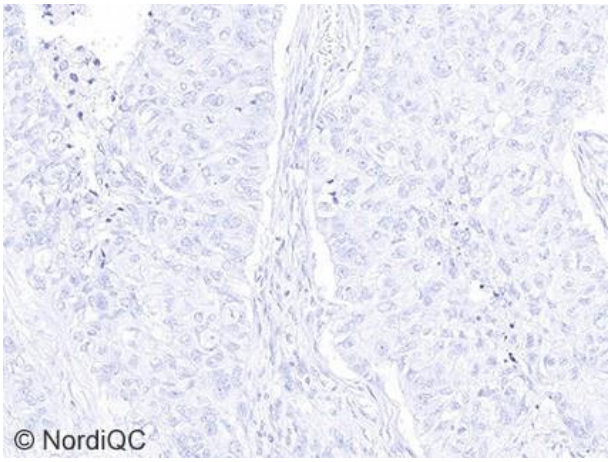



Fig. 4b
ER staining of the breast carcinoma, tissue core no. 5, expected to be negative using same protocol as in Fig. 1b - 3b.
No nuclear staining reaction is observed.

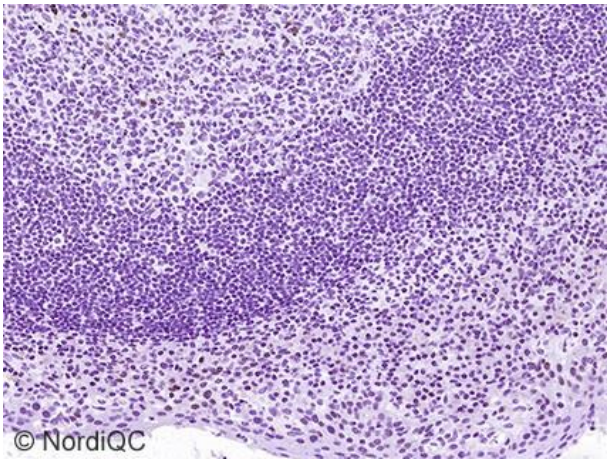



Fig. 5a
Insufficient ER staining of the tonsil using the rmAb SP1 as RTU format by a protocol providing a combination of reduced analytical sensitivity and excessive counterstaining compromising the evaluation of the ER IHC staining quality.
The intense counterstaining makes it virtually impossible to evaluate if the immunohistochemical critical assay performance controls (squamous epithelial cells and follicular dendritic cells in germinal centres) are positive or negative. Also compare with Fig. 5b, same protocol.

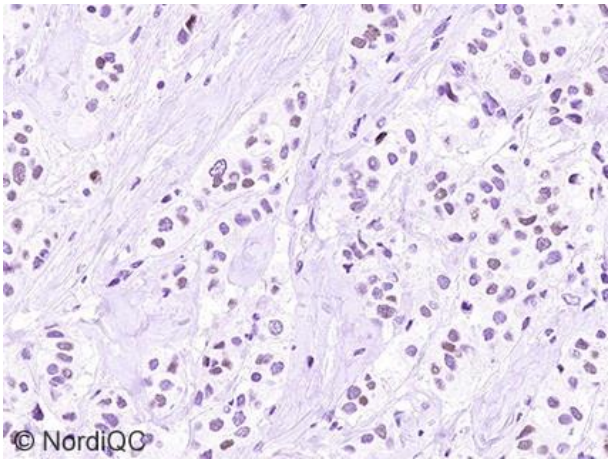



Fig. 5b
Insufficient ER staining of the breast carcinoma, tissue core no. 3, with 50-90% of the neoplastic cells expected to be positive (weak to moderate) using same protocol as in Fig. 5a.
The read-out and determination of ER positivity is significantly hampered due to the intense counterstaining.

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