Assessment Run B30 2020

Estrogen receptor (ER)

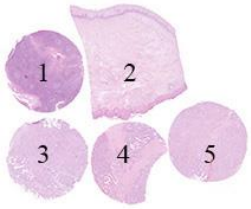
Purpose

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	70-90%**	Weak to strong
4.	Breast carcinoma	90-100%	Moderate to strong
5.	Breast carcinoma	Negative	-



* ER-status and staining pattern as characterized by NordiQC reference laboratories using the rmAb clones EP1 and SP1.
**ER expression slightly heterogenous.

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 the columnar epithelial cells, 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.
- An at least weak to moderate distinct nuclear staining in the appropriate proportion of the neoplastic cells in the breast carcinomas no. 3 and 4.
- No nuclear staining in the neoplastic cells in the breast carcinoma 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 no. 3 and 4 showed an at least weak nuclear staining reaction but less than 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, excessive counterstaining or impaired morphology.

- An ER IHC result was assessed as **borderline** if ≥ 1% and < 10% of the neoplastic cells in one of the breast carcinomas no. 3 and 4 showed a nuclear staining reaction. A negative staining reaction of the cells expected to be demonstrated in tonsil/cervix can also be marked as **borderline.**

- An IHC result was assessed as **poor** if a false negative staining (< 1%) was seen in one of the breast carcinomas no. 3 and 4 or false positive staining (≥ 1%) was seen in the breast carcinoma no. 5.

An IHC result can also be assessed as **borderline/poor** related to technical artefacts, e.g. poor signal-to-noise ratio, excessive counterstaining, impaired morphology and/or excessive staining reaction hampering the interpretation.

Participation

Number of laboratories registered for ER, B30	386
Number of laboratories returning slides	363 (94%)

Results
363 laboratories participated in this assessment. 334 of 363 (92%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks given (see page 3).

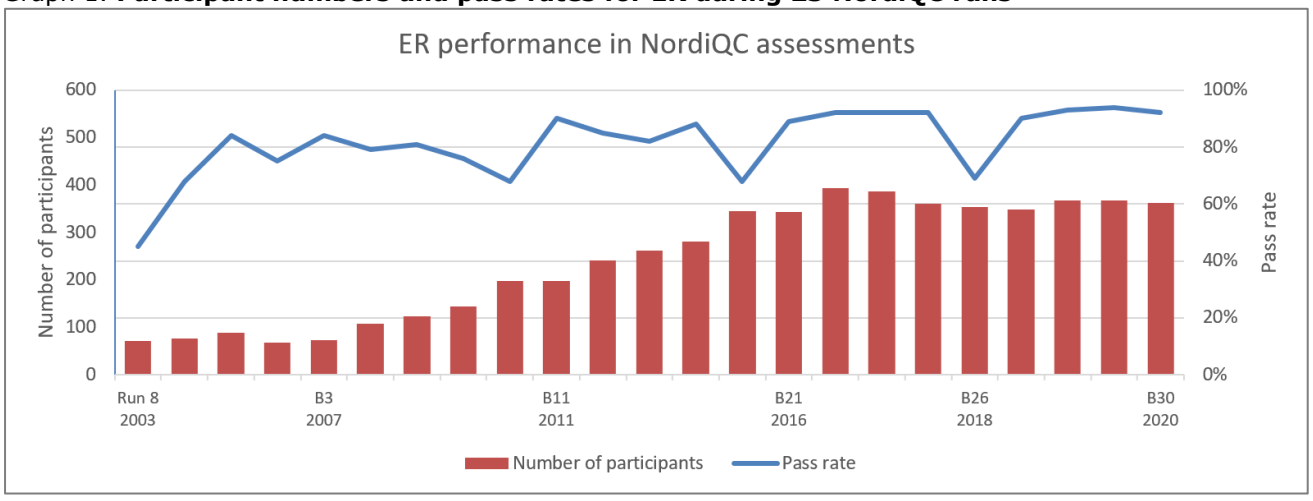
The most frequent causes of insufficient staining reactions were:
- Insufficient Heat Induced Epitope Retrieval (HIER) in an acidic buffer
- Use of detection systems with low sensitivity
- Technical issues

Conclusion
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 ER. 86% of the participants used Ready-To-Use (RTU) systems for the demonstration of ER. The RTU systems from Ventana and Dako used as "plug-and-play" assays provided a pass rate of 94%. In this assessment, low analytical sensitivity giving a too weak or false negative staining reaction was the predominant feature of insufficient results.

Uterine cervix and tonsil can 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. Endothelial cells and lymphocytes must be negative in this tissue.
Tonsil was especially found recommendable 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 but 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.

Performance history
This was the twenty-third NordiQC assessment of ER. The proportion of sufficient results has been relatively stable in the recent runs from 2016-2020 (except for run B26) at a high and satisfactory level (see Graph 1).

Graph 1. **Participant numbers and pass rates for ER during 23 NordiQC runs**



Fluctuations in pass rates, as seen in run B26, is likely caused by more challenging materiel circulated compared to other runs. In order to secure the consistency of the material circulated, NordiQC evaluates the material with two reference standard methods and monitor the ER expression levels throughout all TMAs used in the assessment.

Table 1. **Antibodies and assessment marks for ER, B30**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **6F11**	15	Leica/Novocastra	9	2	4	-	73%	60%
mAb clone **C6H7**	1	Cenovte	-	1	-	-	-	-
rmAb clone **EP1**	9 1 1	Dako/Agilent Cell Marque BioGenex	4	4	3	-	73%	36%
rmAb clone **SP1**	14 4 3 1 1	Thermo Scientific Cell Marque Zytomed Systems Abcam Diagnostic Biosystems	18	2	3	-	87%	78%
rmAb clone **BP6026**	1	Bailing Biotechnology Co., Ltd	1	-	-	-	-	-
Ready-To-Use antibodies							Suff.[1]	OR[2]
mAb clone **6F11 PA0009/PA0151 (VRPS[3])**	2	Leica Biosystems	-	1	1	-	-	-
mAb clone **6F11 PA0009/PA0151 (LMPS[4])**	13	Leica Biosystems	6	2	5	-	62%	46%
mAb clone **6F11 PDM048-10MM**	1	Diagnostic BioSystems	1	-	-	-	-	-
rmAb **EP1 IR/IS084 (VRPS[3])**	7	Dako/Agilent	1	5	1	-	86%	14%
rmAb **EP1 IR/IS084 (LMPS[4])**	31	Dako/Agilent	19	8	4	-	87%	61%
rmAb **EP1 GA084 (VRPS[3])**	24	Dako/Agilent	14	10	-	-	100%	58%
rmAb **EP1 GA084 (LMPS[4])**	20	Dako/Agilent	11	8	1	-	95%	55%
rmAb **EP1 8361**	2	Sakura Finetek	2	-	-	-	-	-
rmAb clone **SP1 790-4324/5 (VRPS[3])**	38	Ventana/Roche	21	16	1	-	97%	55%
rmAb clone **SP1 790-4324/5 (LMPS[4])**	166	Ventana/Roche	100	61	5	-	97%	60%
rmAb clone **SP1 249R-17/18**	2	Cell Marque	2	-	-	-	-	-
rmAb clone **SP1 MAD-000306QD/V**	1	Master Diagnostica Vitro SA	-	1	-	-	-	-
rmAb clone **SP1 KIT-0012**	4	Maixin	1	2	1	-	-	-
rmAb clone **SP1 M3011**	1	Spring Biosystems	1	-	-	-	-	-
Total	363		211	123	29	0		
Proportion			58%	34%	8%	0%	92%	

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, B30
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **6F11**: Protocols with optimal results were based on HIER using Cell Conditioning 1 (CC1, Ventana) (3/4)* or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (6/7) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:200 and combined with a 3-layer detection system. Using these protocol settings, 9 of 11 (82%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **EP1**: Protocols with optimal results were based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (2/4) or TRS High pH (Dako) (2/3) as retrieval buffer. The rmAb was typically diluted in the range of 1:50-1:100 and combined with a 3-layer detection system. Using these protocol settings, 5 of 5 (100) laboratories produced a sufficient staining result.

rmAb clone **SP1**: Protocols with optimal results were typically based on HIER using CC1 (Ventana) (9/9), TRS pH 9 (Dako) (2/2), BERS2 (Leica) (4/4) or Tris-EDTA pH 9 (3/3) as retrieval buffer. The rmAb was typically diluted in the range of 1:25-1:200 and combined with either a 2- or 3-layer detection system. Using these protocol settings, 19 of 19 (100%) 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**	-	-	-	-	3/4**	-	6/7	-
rmAb clone **EP1**	2/3	-	2/2	-	-	-	-	-
rmAb clone **SP1**	0/1	-	2/2	-	9/9	-	4/4	-

* 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/Novocastra, Bond III/Bond Max:
Protocols with optimal results were based on HIER using BERS2 for 20-30 min., 15-60 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings, 7 of 7 (100%) laboratories produced a sufficient staining result (optimal or good).

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 10-30 min. at 97-98°C), 20-40 min. incubation of the primary Ab and EnVision FLEX (K8000/K8002) or EnVision FLEX+ with rabbit linker (K8009/K8019) as detection system. Using these protocol settings, 23 of 25 (92%) laboratories produced a sufficient staining result.
10 laboratories used product no IR084/IS084 on other platforms. These were not included in the description above.

mAb clone **EP1**, product no. **GA084**, Dako, Dako Omnis:
Protocols with optimal results were typically based on HIER using TRS High pH (efficient heating time 30 min. at 97°C), 10-27 min. incubation of the primary Ab and EnVision FLEX (GV800) or EnVision FLEX+ with rabbit linker (GV800+GV809) as detection system. Using these protocol settings, 37 of 38 (97%) laboratories produced a sufficient staining result.
3 laboratories used product no. GA084 on other platforms. These were not included in the description above.

rmAb clone **SP1**, product no. **790-4324/4325**, Ventana, BenchMark GX, XT, ULTRA:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 8-76 min.), 8-64 min. incubation of the primary Ab and UltraView (760-500) with or without UltraView Amplification kit (760-080), iView (760-091) or OptiView (760-700) as detection system. Using these protocol settings, 181 of 187 (97%) laboratories produced a sufficient staining result.
10 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 rmAb EP1 **IR084/IS084**	7/8 (88%)	1/8 (13%)	18/20 (90%)	14/20 (70%)
Dako Omnis rmAb EP1 **GA084**	24/24 (100%)	14/24 (58%)	16/17 (94%)	8/17 (47%)
Leica Bond mAb 6F11 **PA009/PA0151**	1/2	0/2	8/13 (62%)	6/13 (46%)
VMS Ultra/XT/GX rmAb SP1 **790-4324/4325**	37/38 (97%)	21/38 (55%)	151/156 (97%)	93/156 (60%)

* 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 and detection kit. Only protocols performed on the specified vendor IHC stainer are included.

Comments
The assessment criteria were in this run and in concordance to the ASCO/CAP 2020 recommendation on ER IHC testing increasingly anchored on the focus on the tissue controls, tonsil and cervix.
According to both previous NordiQC results and the recommendations from ASCO/CAP 2020, especially tonsil is recommended to ensure an appropriate low limit of demonstration of ER. If both breast carcinomas (tissue cores no. 3 and 4) showed the expected staining reaction both regarding intensity and proportion of cells demonstrated, but the tissue controls were too weak or a reduced proportion of positive cells were seen in these controls, the overall result was assessed as "good" or "borderline" depending on the overall performance. The modification did not affect a significant change in the pass-rate or proportion of good vs. optimal scores in this assessment compared to the previous assessments.

In this assessment and as seen in previous NordiQC runs for ER, the most common feature of an insufficient staining result was inadequate protocol sensitivity, resulting in a weak or false negative staining reaction, with reduced detection of the ER epitope. This pattern was seen in 86% of the insufficient results (25 of 29). In 7% (2 of 29) a false positive nuclear staining reaction was observed in lymphocytes in tonsil. In the remaining 7% (2 of 29), poor signal-to-noise ratio, excessive cytoplasmic staining reaction or faulty counterstaining was seen.

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 no. 3, in which an at least weak nuclear staining reaction of 50-90% of the neoplastic cells was expected, was more challenging.

Ready-To-Use (RTU) Abs were used by 86% (312 of 363) of the participants. 94% (293 of 312) of these laboratories obtained a sufficient staining result, 57% optimal (179 of 312).

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 56% of the participants and gave an overall pass rate of 97%. Optimal results could be obtained both by the vendor recommended protocol settings (16 min. incubation of the primary Ab, HIER in CC1 for 64 min. and UltraView or iView as detection kit) and by laboratory modified protocols adjusting incubation time of the primary Ab, HIER time and detection system as shown in Table 3. In this assessment, vendor recommended protocol settings were used by only 19% of the laboratories. Use of OptiView detection was observed to be a successful protocol modification, resulting in an optimal staining result for 86% (24 of 28) of users, compared to 55% for the manufacturer's protocol (see Table 3). OptiView detection gave a pass rate of 96% (27 of 28 users).

The Dako/Agilent RTU system GA084 for Omnis, based on rmAb clone EP1 was used by 12% of the participants and gave an overall pass rate of 98%. The proportion of sufficient and optimal results obtained by the vendor recommended protocol settings was slightly increased compared to laboratory

modified protocols as shown in Table 3. The modified protocols either increased incubation time of the primary Ab and/or added a rabbit linker for the detection system.

The Dako/Agilent RTU system IR084/IS084 for Autostainer, also based on the rmAb EP1 was used by 11% of the participants and provided an overall pass rate of 87%. As shown in Table 3, 71% (20 of 28) of the laboratories modified the protocol settings and obtained a significant higher proportion of optimal results compared to laboratories using the RTU system according to the Dako recommended protocol settings. The most common and successful modification observed was use of FLEX+ and rabbit linker as detection system. A modification including rabbit linker was used by 17 laboratories: 100% obtained sufficient and 82% optimal results.

The Leica RTU system PA009/PA0151 for BOND based on mAb 6F11, was used by 4% of the laboratories and gave an overall pass rate of 60%. In this assessment, vendor recommended protocol settings based on HIER in BERS1 for 20 min., 15 min. incubation of the primary Ab and Bond Refine as detection was used by two participants, one with a sufficient result. Laboratories using a protocol modification enhancing analytical sensitivity by using HIER in BERS2 obtained a pass rate of 100% (7 of 7), 86% optimal. However high pH retrieval should be used with caution with this clone due to the concerning number of false positive staining reactions noted using this modification in run B28. Protocol modifications increasing incubation time of the primary antibody alone did not yield any optimal results and resulted in weak staining.

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 applied by 14% (51 of 363) of the participants. The three Abs, mAb clone 6F11 and rmAb clones EP1 and SP1 used in a LD assay all could provide sufficient and optimal results on the main IHC platforms (Dako/Agilent, Leica and Ventana/Roche), see Tables 1 and 2. Irrespective of the clone applied, careful calibration of the primary Ab concentration in combination with efficient HIER, preferably in an alkaline buffer, and use of a sensitive 3-layer detection system were found to be the common core elements for an optimal performance.

Controls

In concordance with previous NordiQC runs, uterine cervix and tonsil was found to be valuable positive and negative tissue controls for ER staining: In the uterine cervix, optimal results were characterized by virtually all epithelial cells throughout the squamous epithelium and in the glands showing a moderate to strong and distinct nuclear staining reaction. 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 especially recommended as a tool to monitor the analytical sensitivity for the IHC demonstration of ER and was in fact superior to uterine cervix. 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, a reduced proportion of ER positive cells were seen in the other tissues and most critically a too weak or even false negative staining was seen in breast carcinomas nos. 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 uterine cervix using the rmAb clone EP1 diluted 1:100, HIER in an alkaline buffer and a 3-step polymer-based detection system.
Virtually all squamous epithelial and stromal cells show a moderate to strong nuclear staining reaction.
Endothelial and lymphoid cells are negative.
Also compare with Figs. 2a-4a, same protocol.

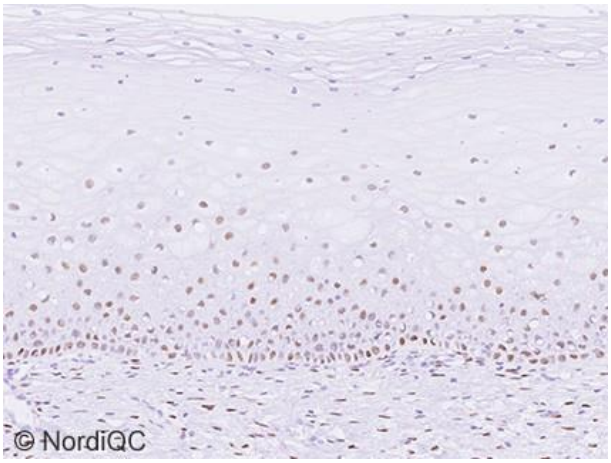


Fig. 1b
ER staining of the uterine cervix using the rmAb clone EP1 by an insufficient protocol – same field as in Fig. 1a.
A dilution of 1:20, HIER in an alkaline buffer and a 2-step detection systems was used.
The intensity and proportion of squamous epithelial and stromal cells demonstrated is reduced.
However, also compare with Figs. 2b- 4b, same protocol.

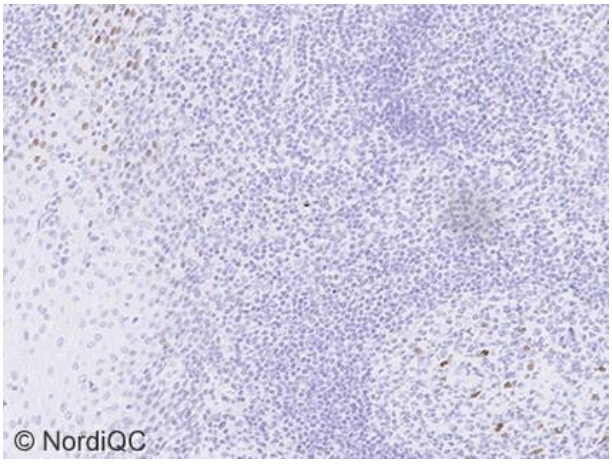


Fig. 2a
Optimal ER staining of the tonsil using same protocol settings as in Fig. 1a.
A moderate to strong, distinct nuclear staining reaction is seen in dispersed follicular dendritic cells in the germinal center and squamous epithelial cells.
No nuclear staining reaction is seen in the mantle zone B-cells and in general a high signal-to-noise ratio is seen.

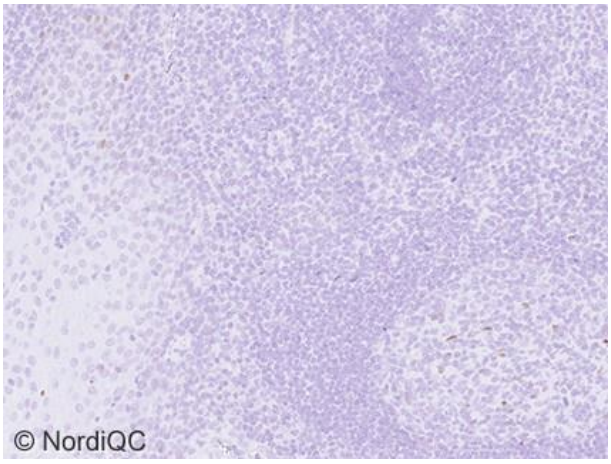


Fig. 2b
Insufficient ER staining of the tonsil using same protocol settings as in Fig. 1b.
Only a faint equivocal staining reaction is observed in few follicular dendritic cells in the germinal center and squamous epithelial cells.
Compare with Fig. 2a – same field.

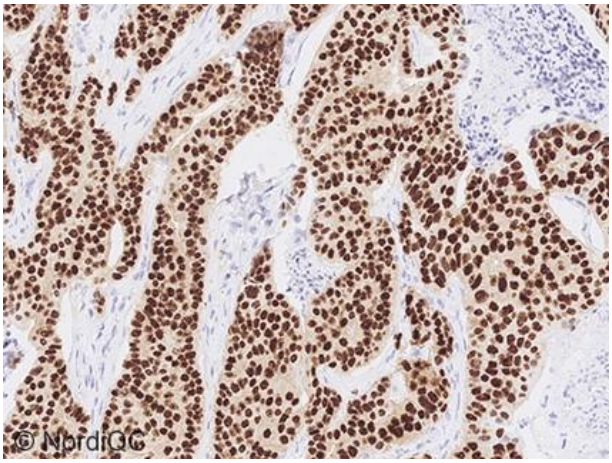


Fig. 3a
Optimal ER staining of the breast carcinoma no. 4 with 90-100% cells being positive using same protocol as in Figs. 1a-2a.
The neoplastic cells display a moderate and distinct nuclear staining reaction.

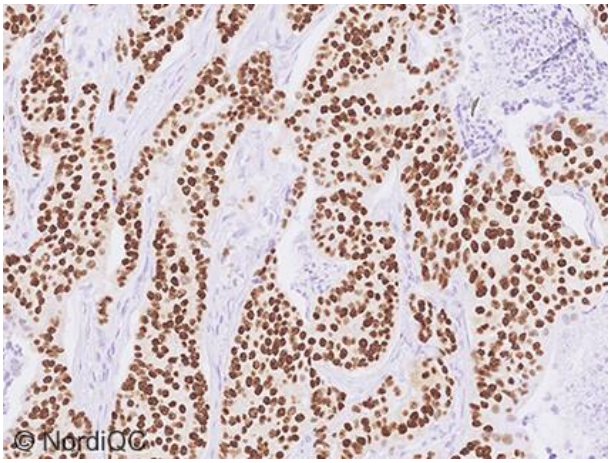


Fig. 3b
ER staining of the breast carcinoma no. 4 with expected 90-100% cells being positive using same protocol as in Figs. 1b-2b.
A reduced staining intensity is seen compared to optimal result in Fig. 3a.

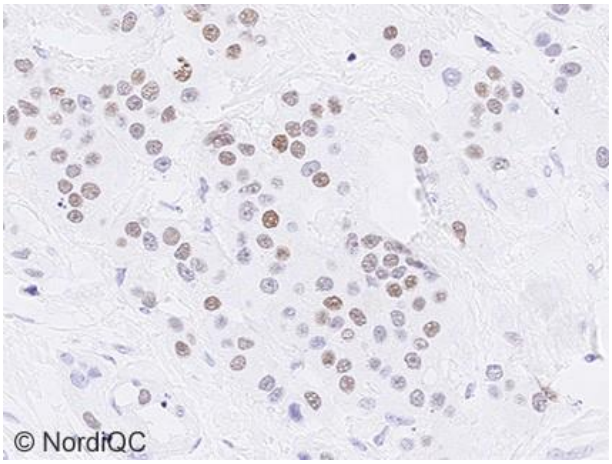


Fig. 4a
Optimal ER staining of the breast carcinoma no. 3 with minimum 60% of the neoplastic cells expected to be positive using same protocol as in Figs. 1a-3a.
The majority of the neoplastic cells display a weak to moderate and distinct nuclear staining reaction.

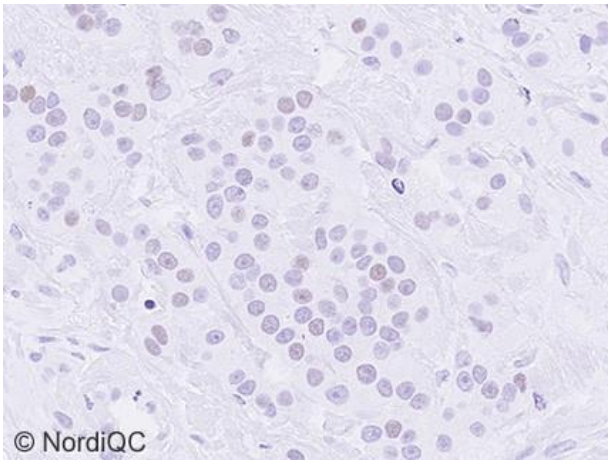


Fig. 4b
Insufficient and false negative ER staining of the breast carcinoma no. 3 with expected 60-90% cells being positive using same protocol as in Figs. 1b-3b.
<10% of the neoplastic cells are positive.

HLK/LE/SN 07.12.2020