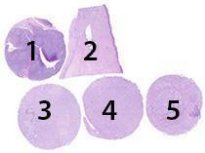
Assessment Run B28 2019

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	40-60%	Weak to moderate
4.	Breast carcinoma	90-100%	Moderate to strong
5.	Breast carcinoma	Negative	-



*ER-status and staining pattern as characterized by the 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 Yaziji 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 germinal centre macrophages/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 of neoplastic cells in the breast carcinoma no. 5 and no more than a weak cytoplasmic reaction in cells with a strong nuclear staining.

A staining was classified as **good** if ≥10 % of the neoplastic cells in the breast ductal carcinomas no. 3 and 4 showed an at least weak nuclear staining reaction, but less than the reference range.
An at least week to moderate nuclear staining in the majority of the uterine columnar and squamous epithelial cells and in the dispersed cells expected to be positive in the tonsil.

A staining was assessed as **borderline** if ≥ 1 % but <10 % of the neoplastic cells in one of the breast carcinomas no. 3 and 4 showed a nuclear staining reaction.

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

Participation

Number of laboratories registered for ER, B28	374
Number of laboratories returning slides	367 (98%)

Results
367 laboratories participated in this assessment. 342 of 367 (93%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks given (see page 2).

Conclusion
The rabbit monoclonal antibody (rmAb) clones **SP1** and **EP1** and the mouse monoclonal Ab (mAb) clone **6F11** could all be used to provide an optimal result for ER. 80% of the participants used Ready-To-Use (RTU) systems for the demonstration of ER. The RTU systems from Ventana, Dako and Leica used as "plug-and-play" assays provided a pass rate of 99%. In this assessment, too weak or false negative staining reaction was the predominant feature of insufficient results, but also false positive results were observed. 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. Lymphocytes and endothelial cells must be negative.
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.

Table 1. **Antibodies and assessment marks for ER, B28**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1D5**	1	Biocare Medical	-	-	1	-	-	-
mAb clone **6F11**	15	Leica/Novocastra	5	9	1	-	93%	100%
mAb clone **ER88**	1	BioGenex	-	-	-	1	-	-
rmAb clone **EP1**	12 1	Dako/Agilent Cell Marque	6	5	2	-	85%	89%
rmAb clone **SP1**	17 8 1 1	Thermo Scientific Cell Marque Abcam Diagnostic Biosystems	14	11	1	1	93%	95%
Ready-To-Use antibodies								
mAb clone **1D5 IR657**	1	Dako/Agilent	1	-	-	-	-	
mAb clones **1D5 + ER-2-123 SK310**	2	Dako/Agilent	-	2	-	-	-	
mAb clone **6F11 PA0009/PA0151**	17	Leica	5	9	-	3	82%	
rmAb **EP1 IR/IS084**	27	Dako/Agilent	15	10	1	1	93%	
rmAb **EP1 IR/IS084**[3]	7	Dako/Agilent	3	2	2	-		
rmAb **EP1 GA084**	38	Dako/Agilent	28	10	-	-	100%	
rmAb **EP1 GA084**[3]	3	Dako/Agilent	2	-	-	1	-	
rmAb clone **SP1 790-4324/5**	204	Ventana/Roche	103	91	7	3	95%	
rmAb clone **SP1 790-4324/5**[3]	1	Ventana/Roche	1	-	-	-	-	
rmAb clone **SP1 249R-17/18**	3	Cell Marque	1	2	-	-	-	
rmAb clone **SP1 KIT-0012**	1	Maixin	1	-	-	-	-	
rmAb clones **SP1 OAA301T60**	1	Biocare Medical	-	1	-	-	-	
rmAb **SP1 M3011**	1	Spring Biosystems	-	1	-	-	-	
rmAb clone **SP1 MAD-000306QD**	1	Master Diagnostica	-	1	-	-	-	
rmAb clone **EP1 8361-C010**	1	Sakura Finetek	-	1	-	-	-	
rmAb clone **SP1 RMPD001**	1	Diagnostics Biosystem	1	-	-	-	-	
r/mAb clones **6F11 + SP1 PM308**	1	Biocare Medical	1	-	-	-	-	
Total	367		187	155	15	10	-	
Proportion			51%	42%	4%	3%	93%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) RTU system used on a different platform than it was developed for.

Detailed analysis of ER, B28
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **6F11**: Protocols with optimal results were based on Heat Induces Epitope Retrieval (HIER) using Cell Conditioning 1 (CC1, Ventana) (1/3)*, Target Retrieval Solution (TRS) High pH (Dako) (1/1) or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (3/5) 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 9 (100%) 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 CC1 (Ventana) 1/2, TRS High pH (Dako) (4/8) or BERS2 (Leica) (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:25-1:100 and combined with a 3-layer detection system. Using these protocol settings, 8 of 9 (89%) laboratories produced a sufficient staining result.

rmAb clone **SP1**: Protocols with optimal results were based on HIER using CC1 (Ventana) (4/8), TRS pH 9 (Dako) (3/5), BERS2 (Leica) (5/7), Tris-EDTA pH 9 (1/1) or unknown (1/1) as retrieval buffer. The rmAb was typically diluted in the range of 1:30-1:300 and combined with either a 2- or 3-layer detection system. Using these protocol settings, 19 of 20 (95%) 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 stainer 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**		**Leica Bond III / Max**	
	TRS High pH	TRS Low pH	TRS High pH	TRS Low pH	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **6F11**	-	-	1/1**	-	1/3	-	3/5	0/3
rmAb clone **EP1**	3/4	-	1/2	-	1/2	-	1/1	-
rmAb clone **SP1**	-	-	3/4	-	4/8	-	5/6	-

* 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 either Bond Epitope Retrieval 1 (BERS1) or BERS2 20-30 min., 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings, 9 of 10 (90%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP1**, product no. **IR084/IS084**, Dako Agilent, Autostainer+/Autostainer Link:
Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-25 min. at 97-98°C), 15-40 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, 25 of 27 (93%) laboratories produced a sufficient staining result.
7 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 based on HIER using TRS High pH (efficient heating time 30 min. at 97°C), 10-30 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, 38 of 38 (100%) 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 XT, GX, ULTRA:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 32-64 min.), 12-40 min. incubation of the primary Ab and UltraView (760-500) with or without UltraView/iView Amplification kit (760-080) or OptiView (760-700) as detection system. Using these protocol settings, 175 of 184 (95%) laboratories produced a sufficient staining result.
One laboratory used product no 790-4324/4325 on Dako Autostainer. This was 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 protocols 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**	8/9 (89%)	4/9 (44%)	17/18 (94%)	11/18 (61%)
Dako Omnis rmAb EP1 **GA084**	23/23 (100%)	16/23 (70%)	15/15 (100%)	12/15 (75%)
Leica Bond mAb 6F11 **PA009/PA0151**	5/5 (100%)	3/5 (60%)	9/12 (75%)	3/12 (25%)
VMS Ultra/XT/GX rmAb SP1 **790-4324/4325**	28/28 (100%)	18/28 (64%)	166/176 (94%)	85/176 (48%)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered >25%, detection kit. Only protocols performed on the specified vendor IHC stainer are included.

Comments

In this assessment and in concordance with previous NordiQC runs for ER, the prominent feature of an insufficient staining result was a too weak or false negative staining reaction. This pattern was seen in 52% of the insufficient results (13 of 25).

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 reference standard methods showed a moderate to strong intensity. Demonstration of ER in the breast carcinoma no. 3, in which an at least weak nuclear staining reaction of 40-60% of the neoplastic cells was expected, was much more challenging and required a carefully calibrated protocol.

28% (7 of 25) of the insufficient results were characterized by a poor signal-to-noise ratio e.g. excessive cytoplasmic staining reaction and/or diffuse background reaction compromising the interpretation. In the remaining 20% (5 of 25), a false positive staining reaction of the breast carcinoma tissue core no. 5 was seen.

Ready-To-Use (RTU) Abs were used by 84% (310 of 367) of the participants. 94% (292 of 310) of the laboratories used a complete RTU system including the pre-diluted primary Ab, specified ancillary reagents and IHC stainer platform.

The Ventana/Roche RTU system, based on the rmAb clone SP1 (790-4324/4325) to be performed on the BenchMark platform, was in this assessment the most widely used assay being used by 56% of the participants and gave an overall pass rate of 95%. 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 14% of the laboratories and provided a slightly improved overall pass rate and proportion of optimal results compared to laboratory modified protocol settings. Especially the use of UltraView with amplification kit was found to be less successful providing an inferior pass rate of 65% (11 of 17) and typically providing a reduced signal-to-noise ratio. Use of OptiView was observed to be a more successful modification of the protocol providing a pass rate of 97% (29 of 30).

The Dako/Agilent RTU system GA084 for Omnis, based on rmAb clone EP1 was used by 10% of the participants and gave an overall pass rate of 100%. The proportion of sufficient and optimal results obtained by the vendor recommended protocol settings and by laboratory modified protocols were similar 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, provided an overall pass rate of 93%. As shown in Table 3, 67% (18 of 27) of the laboratories modified the protocol settings and obtained a slightly higher pass rate and proportion of optimal results compared to laboratories using the RTU system accordingly to the Dako recommended protocol settings. The most common and

successful modification observed was use of FLEX+ and rabbit linker as detection system. This was used by 16 laboratories and all obtained a sufficient result of which 75% being optimal.

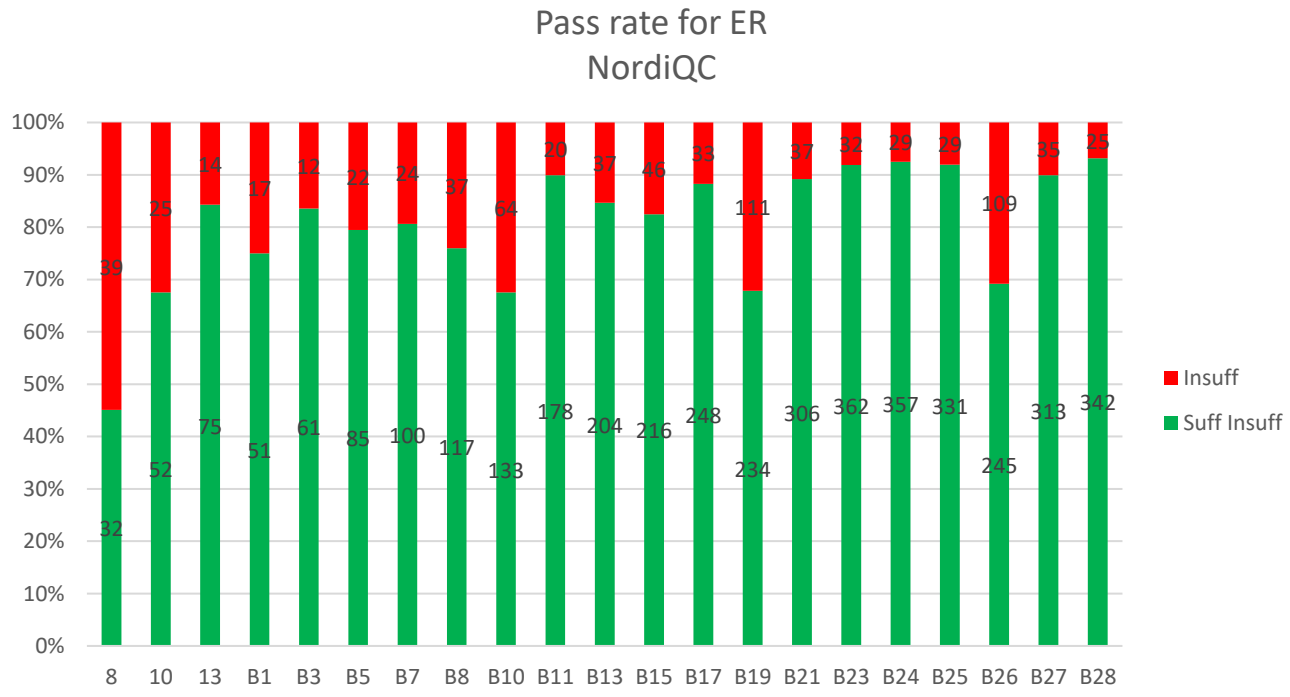
The Leica RTU system PA009/PA0151 for BOND gave an overall pass rate of 82%. In this assessment, vendor recommended protocol settings based on HIER in BERS1 for 20 min., 15 min. incubation of the primary Ab and Bond Refine as detection system provided an improved pass rate and proportion of optimal results compared to laboratory modified protocol settings (see Table 3). For unexplained reasons and in contrast to recent runs for ER, protocol modifications increasing the level of analytical sensitivity e.g. by performing HIER in BERS2 and/or prolonging the time in primary Ab was found less successful as these changes occasionally induced a poor signal-to-noise ratio and of particular concern induced an aberrant diffuse nuclear staining reaction of the neoplastic cells of the breast carcinoma tissue core no. 5 that was characterized as ER negative by the NordiQC reference laboratory.

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

16% (57 of 367) of the participants used Abs as concentrated formats within laboratory developed (LD) assays. Similar to the data generated for the RTU systems, 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, a careful calibration of the primary Ab in combination with efficient HIER, preferable in an alkaline buffer, and use of a sensitive 3-layer detection system were found to be the core elements for an optimal performance

Performance history
This was the twenty-first NordiQC assessment of ER. The proportion of sufficient results has been relatively stable in the recent runs from 2016-2019 except for run B26 (see Graph 1) at a high and satisfactory level.

Graph 1. **Participant numbers and pass rates for ER during 21 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.

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.

Especially tonsil was found recommendable 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 the breast carcinoma no. 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. Yaziji H, Taylor CR, Goldstein NS, Dabbs DJ, Hammond EH, Hewlett B, Floyd AD, Barry TS, Martin AW, Badve S, Baehner F, Cartun RW, Eisen RN, Swanson PE, Hewitt SM, Vyberg M, Hicks DG; Members of the Standardization Ad-Hoc Consensus Committee.
Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry.
Appl Immunohistochem Mol Morphol. 2008 Dec;16(6):513-20. PubMed PMID: 18931614.

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

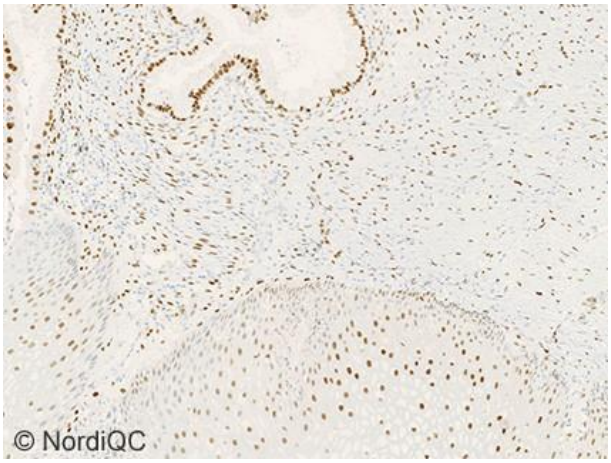


Fig. 1a
Optimal ER staining of the uterine cervix using the rmAb clone SP1 in a RTU format with optimal protocol settings. Virtually all squamous and columnar epithelial cells show a moderate to strong nuclear staining reaction. The majority of the stromal cells are demonstrated and only endothelial and lymphoid cells are negative. Also compare with Figs. 2a-5a, same protocol.

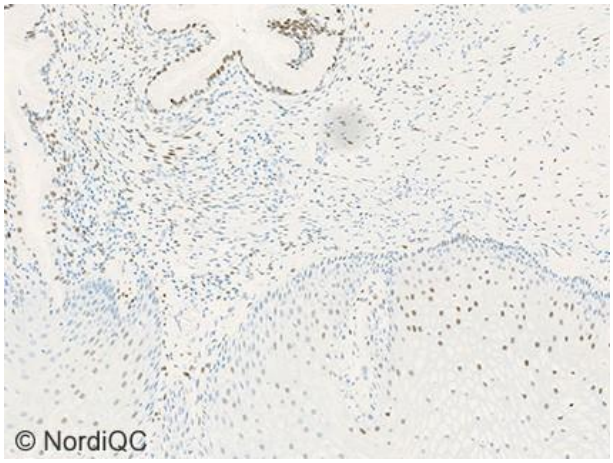


Fig. 1b
ER staining of the uterine cervix using an insufficient protocol – same field as in Fig. 1a.
The intensity and proportion of stromal, squamous and columnar epithelial cells demonstrated are significantly reduced.
Also compare with Figs. 2b- 4b, same protocol. The protocol was based on the rmAb clone SP1 as an RTU with too short efficient HIER time and incubation time in primary antibody.

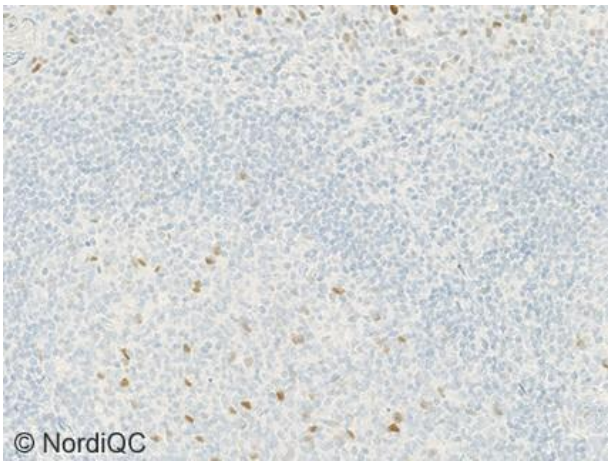


Fig. 2a
Optimal ER staining of the tonsil using same protocol settings as in Fig. 1a.
A moderate to strong, distinct nuclear staining reaction is seen in the squamous epithelial cells and in dispersed follicular dendritic cells in the germinal center.

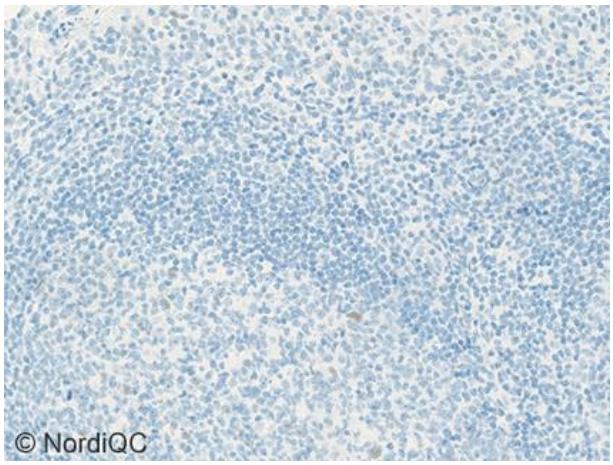


Fig. 2b
Insufficient ER staining of the tonsil using same protocol settings as in Fig. 1b.
Only a faint staining reaction is observed in the squamous epithelial cells and dispersed follicular dendritic cells in the germinal center. Compare with Fig. 2a – same field.

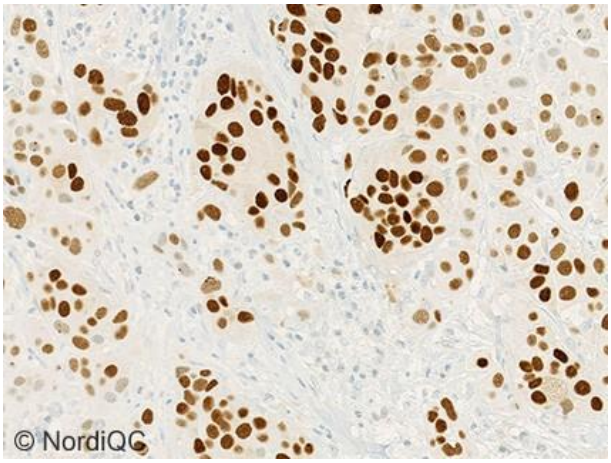


Fig. 3a
Optimal ER staining of the breast carcinoma no. 4 with 90-100% cells being positive using same protocol as in Figs. 1a-2a.
Virtually all neoplastic cells show a strong, distinct nuclear staining reaction with only a weak cytoplasmic staining reaction.

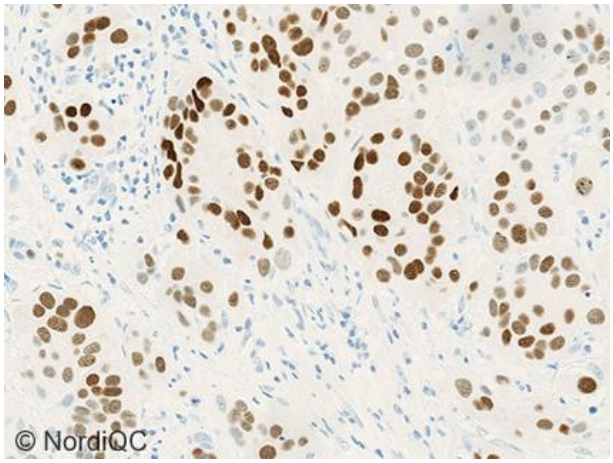


Fig. 3b
ER staining of the breast carcinoma no. 4 with 90- 100% cells positive using same protocol as in Figs. 1b-2b.
The proportion and intensity of neoplastic cells demonstrated is as expected. However also compare with Fig. 4b.

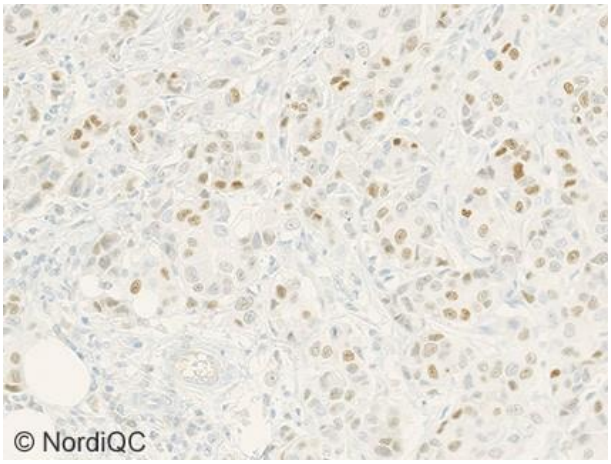


Fig. 4a
Optimal ER staining of the breast carcinoma no. 3 with 40-60% cells being positive using same protocol as in Figs. 1a-3a.
The neoplastic cells display a weak to moderate and distinct nuclear staining reaction.
No background staining is seen.

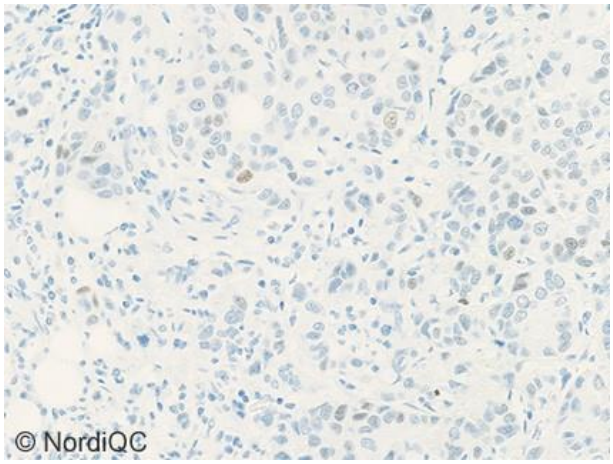


Fig. 4b
Insufficient ER staining of the breast carcinoma no. 3 with expected 40-60% cells being positive using same protocol as in Figs. 1b-3b.
Only scattered neoplastic cells are demonstrated showing an equivocal positivity.

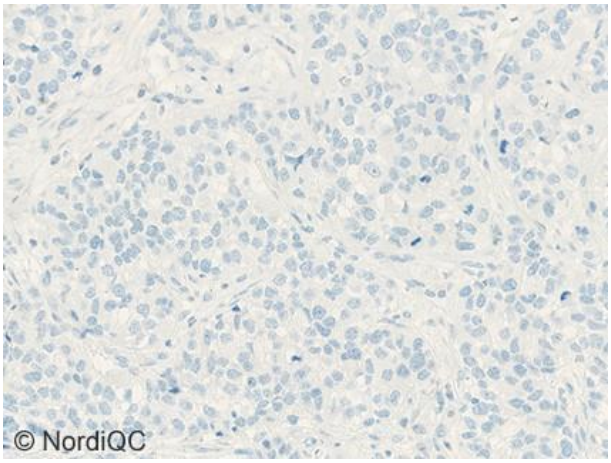


Fig. 5a
Optimal ER staining of the breast carcinoma no. 5 expected to be ER negative using the same protocol as in Figs. 1a-4a.
No staining reaction is seen.
The negative reaction was characterized by the NordiQC reference laboratory using rmAb clones SP1 and EP1 and same result was obtained by 99% of all participants.

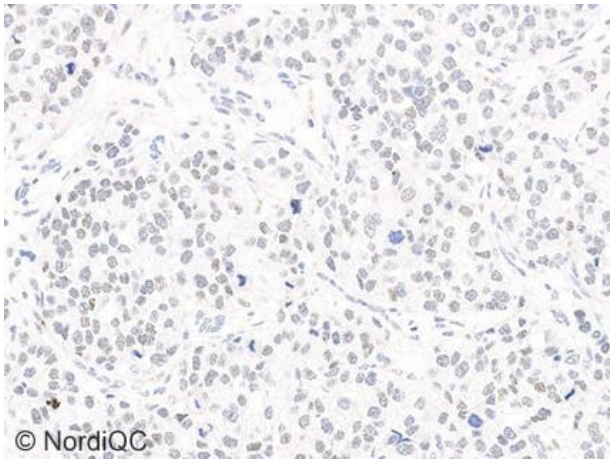


Fig. 5b
Insufficient ER staining of the breast carcinoma no. 5 expected to be ER negative. The majority of neoplastic cells show a weak positive nuclear staining reaction.
The protocol was based on the mAb 6F11 as a RTU format using modified protocol settings compared to vendor recommendations. HIER was performed in an alkaline buffer and the time in primary Ab was prolonged significantly.

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