Assessment Run B24 2017

Estrogen receptor (ER)

Material

The slide to be stained for ER comprised:

No.	Tissue	ER-positivity*	ER-intensity*
1.	Uterine cervix	80 - 90%	Moderate to strong
2.	Tonsil	< 2 - 5%	Weak to strong
3.	Breast carcinoma	0%	Negative
4.	Breast carcinoma	60 - 80%	Weak to moderate
5.	Breast carcinoma	60 - 80%	Weak to moderate
6.	Breast carcinoma	90 - 100%	Moderate to strong

*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 Yaziji et al. (1).

Criteria for assessing ER staining result as **optimal** were:

- Moderate to strong, distinct nuclear staining reaction of virtually all columnar epithelial cells, basal squamous epithelial cells and most stromal cells (except endothelial and lymphoid cells) in the uterine cervix.
- An at least weak to moderate nuclear staining reaction of dispersed germinal centre macrophages and squamous epithelial cells of the tonsil.
- At least weak to moderate distinct nuclear staining reaction in the appropriate proportion of the neoplastic cells in the breast carcinomas no. 4, 5 and 6.
- No nuclear staining reaction of neoplastic cells in the breast carcinoma no. 3.
- No more than a weak cytoplasmic staining reaction in cells with strong nuclear staining reaction.

The staining reactions were classified as **good** if ≥ 10 % of the neoplastic cells in the breast carcinomas no. 4, 5 and 6 showed an at least weak nuclear staining reaction (but significantly less than the range of the reference laboratories).

The staining reactions were classified as **borderline** if ≥ 1 % but < 10 % of the neoplastic cells showed a nuclear staining reaction in one or more of the breast carcinomas no. 4, 5 & 6.

The staining reactions were classified as **poor** if a false negative or false positive staining reaction was seen in one of the breast carcinomas.

Participation

Number of laboratories registered for ER, run B24	400
Number of laboratories returning slides	386 (97%)

Results

386 laboratories participated in this assessment. 357 (92%) of these achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining results were:
- Too low concentration of the primary Ab.
- Insufficient HIER - too short efficient HIER time and/or use of a non-alkaline buffer.
- Less successful primary Ab.

Conclusion

The mAb clone **6F11** and rmAb clones **EP1** and **SP1** could all be used to provide an optimal result for ER. The corresponding Ready-To-Use (RTU) systems from Dako/Agilent, Leica and Ventana/Roche provided the highest proportion of sufficient and optimal results. In this assessment, false negative staining reaction was the prominent feature of insufficient staining results. Uterine cervix is an appropriate positive tissue control for ER. Virtually all stromal, columnar epithelial and squamous epithelial cells must show a moderate to strong and distinct nuclear staining reaction. Lymphocytes and endothelial cells must be negative. As a supplement control to monitor the technical sensitivity of the assay, tonsil seems to be very valuable. In tonsil, an at least weak to moderate nuclear staining reaction of dispersed germinal centre macrophages and squamous epithelial cells must be seen.

Table 1. **Antibodies and assessment marks for ER, run B24**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **6F11**	21 1	Leica/Novocastra Celnovte	11	7	3	1	82%	85%
rmAb clone **EP1**	18 1	Dako/Agilent Cell Marque	9	8	2	0	89%	75%
rmAb clone **SP1**	24 3 3 1 1 1 1	Thermo/Neomarkers Cell Marque Spring Bioscience Immunologic BioCare Zytomed Diagnostic BioSystems	23	3	6	2	76%	74%
Ready-To-Use antibodies								
mAb clone **1D5 IR/IS657**	2	Dako/Agilent	0	2	0	0	-	-
mAb clones **1D5 + ER-2-123 SK310**	2	Dako/Agilent	0	1	1	0	-	-
mAb clone **6F11 PA0009/PA0151**	14	Leica	6	6	2	0	86%	100%
rmAb **EP1 IR/IS084**	55	Dako/Agilent	36	15	3	1	93%	95%
rmAb **EP1 GA084**	21	Dako/Agilent	16	5	0	0	100%	100%
rmAb **EP1 AN710-5M**	1	Biogenex	0	0	0	1	-	-
rmAb clone **SP1 790-4324/5**	205	Ventana/Roche	166	33	4	2	97%	97%
rmAb clone **SP1 249R-1**	4	Cell Marque	2	1	1	0	-	-
rmAb clone **SP1 KIT-0012**	2	Maixin	2	0	0	0	-	-
rmAb clone **SP1 RMPD001**	1	Diagnostic Biosystems	1	0	0	0	-	-
rmAb clone **SP1 ILM30142-R25**	1	Immunologic	1	0	0	0	-	-
rmAb clone **SP1 MAD-000306QD**	1	Master Diagnostica	1	0	0	0	-	-
rmAb clone **SP1 RM-9101-R7**	2	Thermo/Neomarkers	2	0	0	0	-	-
Total	386		276	81	22	7		
Proportion			71%	21%	6%	2%	92%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.

Detailed analysis of ER, run B24

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

mAb clone **6F11**: Protocols with optimal results were based on heat induced epitope retrieval (HIER) using Target Retrieval Solution High pH (TRS, Dako Omnis) (1/1)*, Bond Epitope Retrieval Solution 2 (BERS2, Leica) (8/11), Cell Conditioning 1 (CC1, Ventana) (1/1), or Novacastra Epitope Retrieval Solutions pH 6 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 11 of 13 (85%) 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) (6/13), Bond Epitope Retrieval Solutions 1 (BERS1, Leica) (1/1) or unknown (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:40-1:100 depending on the total sensitivity of the

protocol employed. Using these protocol settings, 9 of 12 (75%) laboratories produced a sufficient staining result.

rmAb clone **SP1**: Protocols with optimal results were all based on HIER using TRS pH 9 (3-in-1) (Dako) (1/5), CC1 (Ventana) (10/10), BERS2 (Leica) (7/11), Tris-EDTA/EGTA pH 9 (2/4), Citrate pH 6 (2/5) or DSB Montage EDTA Antigen Retrieval Solution (Diagnostic BioSystems) (1/1) as retrieval buffer. The rmAb was typically diluted in the range of 1:10-1:250 depending on the total sensitivity of the protocol employed. Using these protocol settings, 23 of 31 (74%) laboratories produced a sufficient staining result.

Table 2. **Optimal results for ER using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer / Omnis**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS 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	1/2	-	8/11 (73%)	0/3
rmAb clone **EP1**	6/13 (46%)	-	-	-	-	1/1
rmAb clone **SP1**	1/5 (20%)	-	10/10 (100%)	-	7/11 (64%)	-

* 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 typically based on HIER using BERS2 (Leica) 20-30 min., 15-60 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings, 5 of 5 (100%) 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 typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-20 min. at 97-98°C), 20-40 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings, 36 of 38 (95%) laboratories produced a sufficient staining result.
13 laboratories used product no IR084/IS084 on Omnis. 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(3-in-1) (efficient heating time 20-30 min. at 97°C), 10-30 min. incubation of the primary Ab and Envision FLEX (GV800) or Envision FLEX+ (GV800++GV821) as detection system. Using these protocol settings, 20 of 20 (100%) laboratories produced a sufficient staining result.
1 laboratory used product no GA084 on AutoStainer48 and the data was not used 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 8-90 min.), 8-60 min. incubation of the primary Ab and Iview (760-091), UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings, 199 of 205 (97%) laboratories produced a sufficient staining result.

rmAb clone **SP1**, product no. **KIT-0012**, Maixin, Manual staining:
One protocol with an optimal result was based on HIER (Water bath) using TrisEDTA/EGTA for 20 min. and 60 min. incubation of the primary Ab and KIT-0038 as detection system. Using these protocol settings, 2 of 2 100%) laboratories produced a sufficient staining result.

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**	12/13 (92%)	6/13 (46%)	25/26 (96%)	21/26 (81%)
Dako Omnis rmAb EP1 **GA084**	11/11 (100%)	8/11 (73%)	9/9 (100%)	8/9 (89%)
Leica Bond mAb 6F11 **PA009/PA0151**	3/3	0/3	9/11 (82%)	5/11 (45%)
VMS Ultra/XT/GX rmAb SP1 **790-4324/4325**	44/47 (94%)	36/47 (77%)	154/157 (98%)	129/157 (82%)

* 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 the 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 90% of the insufficient results (26 of 29). Poor signal-to-noise ratio, false positive staining reaction and/or inadequate counterstaining compromising the interpretation characterized the remaining insufficient results. Virtually all laboratories were able to demonstrate ER in the high-level ER expressing breast carcinoma (core 6), in which 90-100% of the neoplastic cells were expected to be demonstrated. Demonstration of ER in the breast carcinomas no. 4 and 5, in which an at least weak nuclear staining reaction of 60% of the neoplastic cells was expected, was much more challenging and required a carefully calibrated protocol.

19% (75 of 386) of the participants used Abs as concentrated formats within laboratory developed (LD) assays. The three Abs, mAb clone 6F11, rmAb clones EP1 and SP1 used in a LD assay could provide sufficient and optimal results on the main IHC systems (Dako/Agilent, Leica and Ventana/Roche), see Tables 1 and 2. Irrespective of the clone applied, efficient HIER, preferable in an alkaline buffer, was a central protocol parameter for optimal results. When using HIER in a non-alkaline buffer, such as citrate pH 6, a pass rate of 53% (9 of 17) was seen, 35% optimal. HIER in an alkaline buffer provided a pass rate of 90% (52 of 58), 64% optimal. In addition, an important prerequisite for optimal performance seemed to be careful calibration of the primary Ab i.e. adjustment of the titre to the overall level of sensitivity of the IHC system, whereas choice of detection system, being either a 2- or 3-step system, was of less importance. Grouped together, the LD assays in this run provided a pass rate of 81 % (61 of 75)

Ready-To-Use (RTU) antibodies were used by 81% (311 of 386) of the participants. 89% (278 of 311) of the laboratories used a complete RTU system including the pre-diluted primary Ab, specified ancillary reagents and an IHC stainer platform from on of the three main IHC system providers (Dako/Agilent, Leica and Ventana/Roche).

The Ventana/Roche RTU system, based on the rmAb clone SP1 (790-4324/4325), was in this assessment the most widely used assay 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.
No significant difference in the proportion of sufficient and optimal results was seen comparing vendor recommended protocol settings and off-label use.
Use of OptiView as detection system was the most successful modification observed. 26 laboratories used OptiView and all obtained a sufficient result.

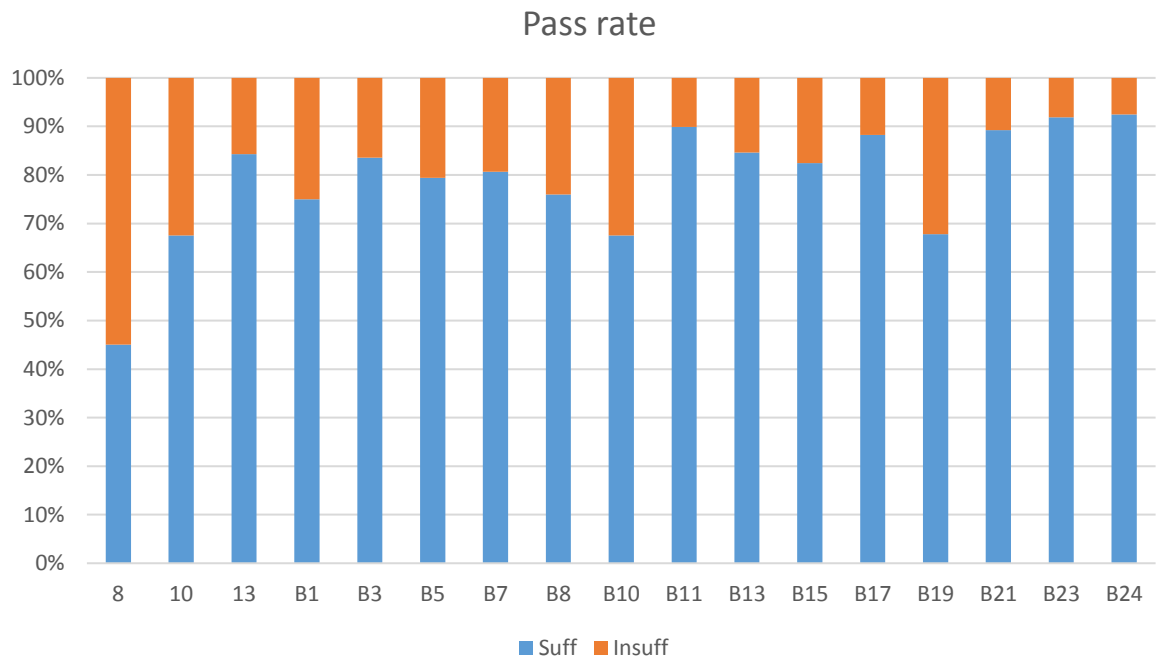
The Dako/Agilent RTU system IR084/IS084 for Autostainer based on the rmAb EP1 provided an overall pass rate of 93%. Both vendor recommended (20 min. incubation of the primary Ab, HIER in TRS High for 20 min. and EnVision FLEX as detection kit) and laboratory modified protocol settings could produce optimal results as shown in Table 3. If protocols were performed according to the recommendations provided by Dako, a pass rate of 92% (12 of 13) was obtained of which 46% were optimal. Laboratory modified protocol settings provided an equal pass rate of 96% and most noticeable an increased proportion of optimal results of 81%. Especially, use of FLEX+ and rabbit linker was successful, as 88% (15 of 17 protocols) based on this detection system gave an optimal result.

The Dako/Agilent RTU system GA084 for Omnis, also based on rmAb clone EP1, was more successful than the Autostainer RTU system. Using protocols according to the recommendations provided by Dako (HIER in TRS High for 30 min., 10 min. incubation of the primary Ab with FLEX as detection system), a pass rate of 100% (11 of 11) was obtained, 73% optimal. Laboratory modified protocol settings also provided a pass rate of 100% and most noticeable an increased proportion of optimal results (89%).

The Leica RTU system PA009/PA0151 for BOND gave an overall pass rate of 86%. Optimal results were only obtained by laboratory modified protocols settings using HIER in BERS2 for 20-40 min. as opposed to performing HIER in BERS1 for 20 min. as recommended by Leica.

Performance history
This was the 17th NordiQC assessment of ER. The proportion of sufficient results was similar compared to the latest run (see Graph 1).

Graph 1. **Participant numbers and pass rates for ER during 17 runs**



The consistent high proportion of sufficient results in the last runs can be caused by many factors: Harmonization and use of optimized protocol settings for LD assays and extended use of properly calibrated RTU systems for ER seem to have an impact. Less successful Abs, as mAb clone 1D5, is now only used by a few laboratories. HIER was mainly performed by alkaline buffers.
Focusing on RTU systems from the main IHC system providers (Dako/Agilent, Leica and Ventana/Roche), grouped together in this run provided a pass rate of 96% (283 of 295 laboratories) compared to the LD assays with a pass rate of 81 % (61 of 75)

Controls
In concordance with previous NordiQC runs, uterine cervix was found to be an appropriate positive tissue control for ER staining: In optimal protocols, virtually all epithelial cells throughout the layers of the squamous epithelium and in the glands showed 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.
In this assessment tonsil was again included and found to be highly 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 germinal centre cells (most likely macrophages) and squamous epithelial cells were distinctively demonstrated in virtually all protocols providing an optimal result. If the germinal centre macrophages were negative, a reduced proportion of ER positive cells were seen in the other tissues and a too weak or even false negative staining was seen in the breast carcinomas no. 4 and 5. Simultaneously, tonsil can be used as supplementary negative tissue control, as B-cells in mantle zones and within germinal centres 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 must be ER positive, serving as internal positive tissue control. Positive staining reaction of the stromal cells in

breast tissue indicates that a highly sensitive protocol is being applied, whereas the sensitivity cannot be evaluated in normal epithelial cells in mamma as they express 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.

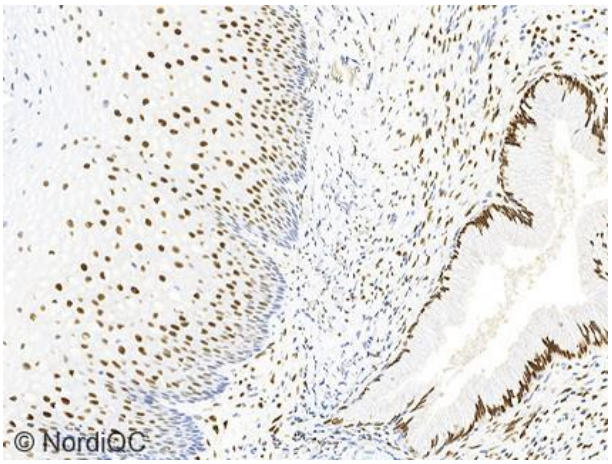


Fig. 1a
Optimal ER staining of the uterine cervix using the rmAb clone EP1 as a concentrate carefully calibrated, with efficient HIER and a 3-step polymer based detection system.
Virtually all squamous and columnar epithelial cells show a moderate to strong, distinct nuclear staining reaction. The majority of the stromal cells are demonstrated and only endothelial and lymphoid cells are negative. Also compare with Figs. 2a – 5a, same protocol.

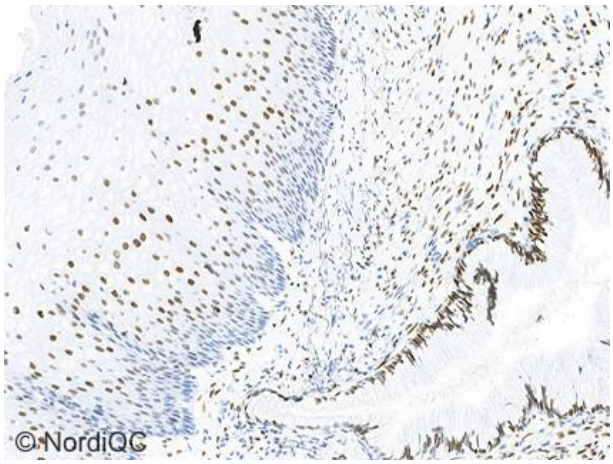


Fig. 1b
ER staining of the uterine cervix using an insufficient protocol - same field as in Fig. 1a.
The proportion of positive stained squamous epithelial cells is reduced.
Also compare with Figs. 2b - 4b, same protocol. The protocol was based on the rmAb clone EP1 as a concentrate too diluted, with HIER in alkaline buffer and a 3-step polymer based detection system.

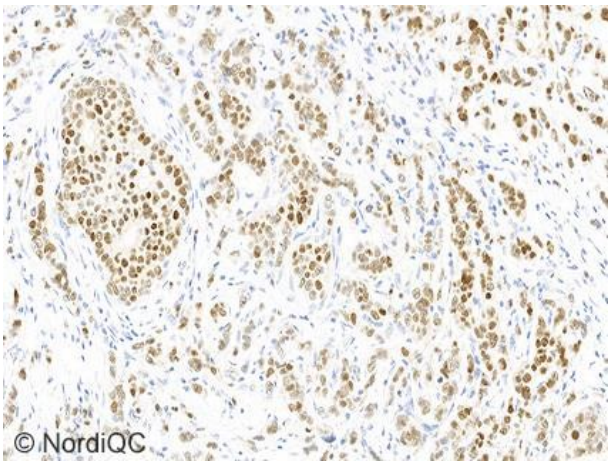


Fig. 2a
Optimal ER staining of the breast ductal carcinoma no. 6 with 90 – 100% cells positive, using same protocol as in Fig. 1a.
Virtually all neoplastic cells show a moderate, distinct nuclear staining reaction with only a weak cytoplasmic staining reaction.
No background staining is seen.

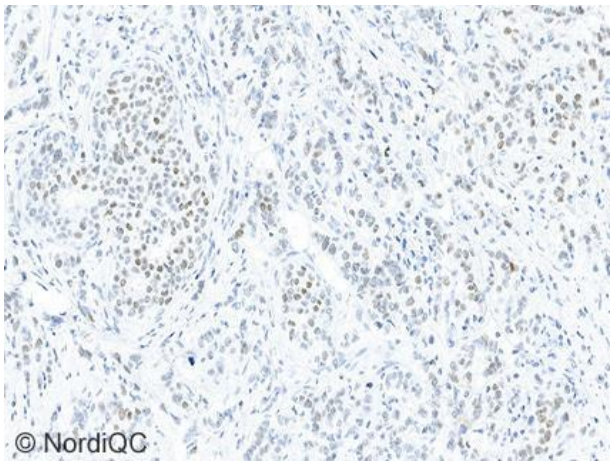


Fig. 2b
ER staining of the breast ductal carcinoma no. 6 with 90 – 100% cells positive, using same protocol as in Fig. 1b – same field as in Fig. 2a.
The intensity and proportion of cells demonstrated is significantly reduced compared to the level expected.
Also compare with Figs. 3b and 4b – same protocol.

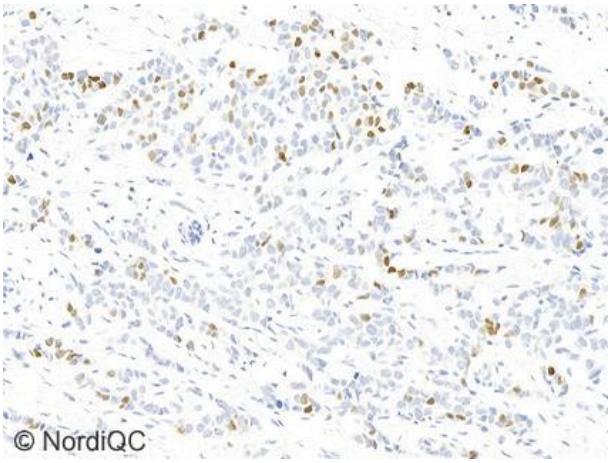


Fig. 3a
Optimal ER staining of the breast ductal carcinoma no. 5 with 60 – 80% cells positive, using same protocol as in Figs. 1a and 2a.
The majority of neoplastic cells show a weak to moderate and distinct nuclear staining reaction.
No background staining is seen.

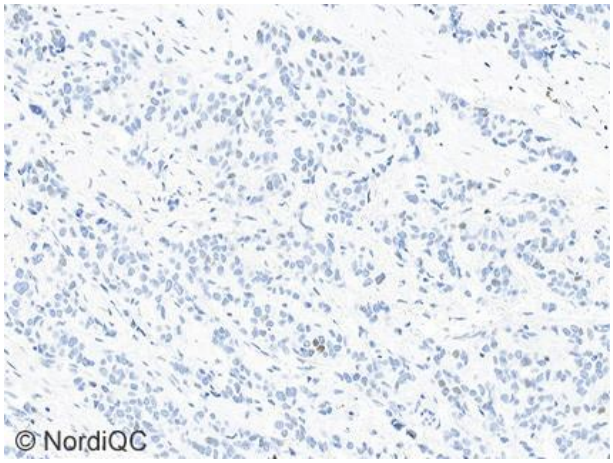


Fig. 3b
Insufficient ER staining of the breast ductal carcinoma no. 5 with 60 – 80% cells positive, using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
The carcinoma is virtually negative. The intensity and proportion of cells demonstrated is significantly reduced compared to the level expected.

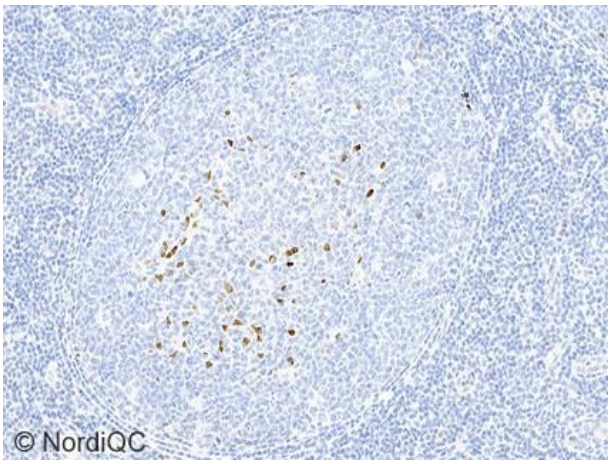


Fig. 4a
Optimal ER staining of the tonsil using same protocol as in Figs. 1a – 3a.
Dispersed germinal centre cells show a weak to moderate nuclear staining reaction.
Note that the vast majority of lymphocytes are negative.

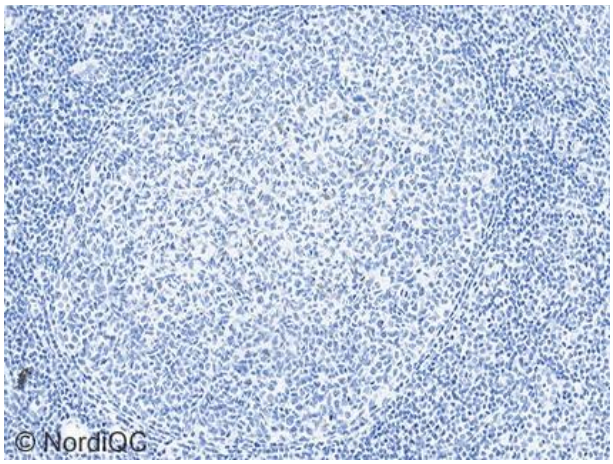


Fig. 4b
Insufficient ER staining of the tonsil using same protocol as in Figs. 1b – 3b
The germinal centre cells are virtually negative.
Tonsil seems to be the preferred positive tissue control for ER and provides a more reliable evaluation of the level of analytical sensitivity compared to uterine cervix.

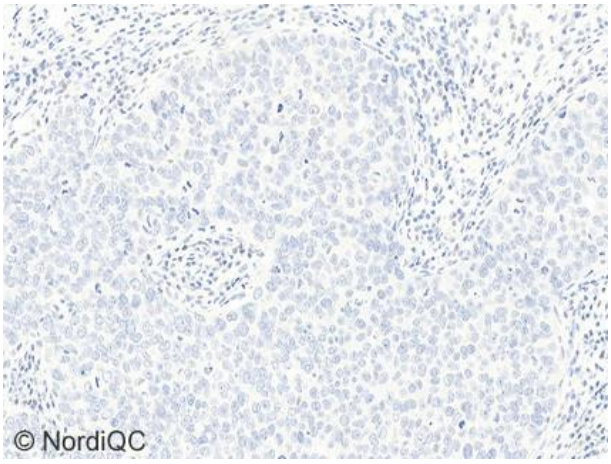


Fig. 5a
Optimal ER staining result of the breast carcinoma no. 3 with no ER expression.
Only dispersed stromal cells show a weak nuclear staining reaction, while all neoplastic cells are unstained.
Same protocol as in Figs. 1a – 4a.

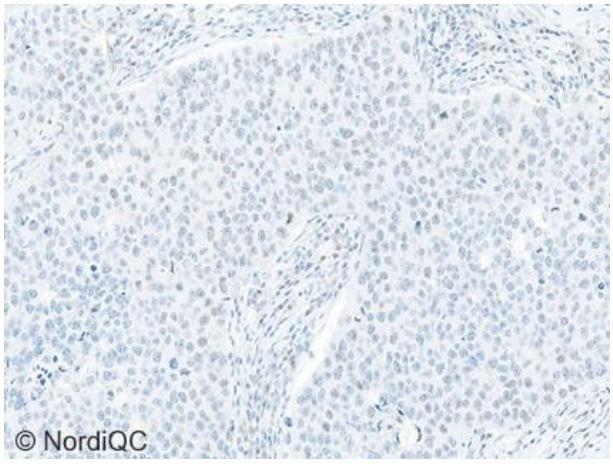


Fig. 5b
Insufficient ER staining of the breast ductal carcinoma no. 3 with no ER expression using the rmAb clone SP1 as Ready-To-Use format (Ventana, 790-4325) with HIER in CC1 and UltraView as detection system.
A weak nuclear staining reaction is seen in the vast majority of the neoplastic cells. The insufficient result most likely was caused by a prolonged incubation time of the primary Ab and/or inadequate buffer washing.

HLK/LE/RR 07.12.17