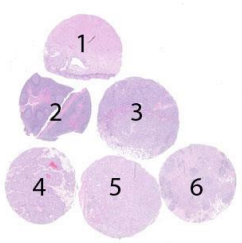
Assessment Run B23 2017

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	90 - 100%	Moderate to strong
5.	Breast carcinoma	50 - 70%	Weak to moderate
6.	Breast carcinoma	60 - 80%	Weak to moderate



*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 results 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 lymphocytes 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 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 and 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 B23	400
Number of laboratories returning slides	394 (99%)

Results

394 laboratories participated in this assessment. 362 (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 reactions were prominent features 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 supplement 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 B23**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1D5**	1	Dako/Agilent	0	0	0	1	-	-
mAb clone **6F11**	22 1	Leica/Novocastra Celnovte	10	11	1	1	91%	86%
rmAb clone **EP1**	15 1	Dako/Agilent Cell Marque	6	5	5	0	69%	89%
rmAb clone **SP1**	30 3 3 1	Thermo/Neomarkers Cell Marque Spring Bioscience Immunologic	27	7	2	1	92%	97%
Ready-To-Use antibodies								
mAb clone **1D5 IR/IS657**	4	Dako/Agilent	0	2	2	0	-	-
mAb clone **1D5 BMS008**	1	Zytomed	0	0	1	0	-	-
mAb clones **1D5 + ER-2-123 SK310**	2	Dako/Agilent	0	1	0	1	-	-
mAb clone **6F11 PA0009/PA0151**	14	Leica	7	6	1	0	93%	100%
rmAb **EP1 IR/IS084**	59	Dako/Agilent	34	21	3	1	93%	95%
rmAb **EP1 GA084**	16	Dako/Agilent	12	3	1	0	94%	92%
rmAb **EP1 AN710-5M**	1	Biogenex	0	1	0	0	-	-
rmAb **EP1 249R-2**	1	Cell Marque	0	0	1	0	-	-
rmAb clone **SP1 790-4324/5**	209	Ventana/Roche	122	79	7	1	96%	95%
rmAb clone **SP1 249R-1**	3	Cell Marque	0	2	1	0	-	-
rmAb clone **SP1 KIT-0012**	2	Maixin	1	1	0	0	-	-
rmAb clone **SP1 RMPD001**	1	Diagnostic Biosystems	0	0	0	1	-	-
rmAb clone **SP1 ILM30142-R25**	1	Immunologic	1	0	0	0	-	-
rmAb clone **SP1 MAD-000306QD**	1	Master Diagnostica	0	1	0	0	-	-
rmAb clone **SP1 M3011**	1	Spring Bioscience	1	0	0	0	-	-
rmAb clone **SP1 RM-9101-R7**	1	Thermo/Neomarkers	1	0	0	0	-	-
Total	394		222	140	25	7	-	
Proportion			56%	36%	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 B23
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **6F11**: Protocols with optimal results were based on HIER using Target Retrieval Solution High pH (TRS; Dako Omnis) (1/1)*, Bond Epitope Retrieval Solution 2 (BERS2; Leica) (5/8), Cell Conditioning 1 (CC1; Ventana) (2/3), Citrate pH 6 (1/3) or Unknown (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, 12 of 14 (86%) 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 TRS pH 9 (3-in-1) (Dako) (5/10) or CC1 (Ventana) (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:25-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 8 of 9 (89%) 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) (3/3), TRS High pH (Dako Omnis) (3/3), CC1 (Ventana) (9/11), BERS2 (Bond,Leica) (6/9), Tris-EDTA/EGTA pH 9 (3/5) or Citrate pH 6 (2/4) as retrieval buffer. The rmAb was typically diluted in the range of 1:25-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings, 33 of 34 (97%) 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	ER2 pH 9.0	ER1 pH 6.0
mAb clone **6F11**	1/1	0/1	2/2	-	2/4	0/3
rmAb clone **EP1**	5/9 (56%)	-	1/1	-	-	-
rmAb clone **SP1**	6/6 (100%)	0/1	9/10 (90%)	-	6/9 (67%)	-

* 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:
Protocols with optimal results were typically based on HIER using BERS1 or BERS2 (Leica) 20-30 min., 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings, 10 of 10 (100%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP1**, product no. **IR084/IS084**, Dako, 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.

rmAb clone **EP1**, product no. **GA084**, Dako, Dako Omnis:
Protocols with optimal results were typically based on HIER using TRS High (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, 12 of 13 (92%) laboratories produced a sufficient staining result.

rmAb clone **SP1**, product no. **790-4324/4325**, Ventana, BenchMark XT, GX, ULTRA:
Protocols with optimal result typically based on HIER using CC1 (efficient heating time 12-64 min.), 8-64 min. incubation of the primary Ab and Iview (760-091), UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings, 120 of 126 (95%) 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 (Pressure Cooker) using Citrate pH 6 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 4. **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**	10/12 (83%)	4/12 (33%)	24/25 (96%)	17/25 (68%)
Dako Omnis rmAb EP1 **GA084**	9/9 (100%)	8/9 (89%)	4/5 (80%)	4/5 (80%)
Leica Bond mAb 6F11 **PA009/PA0151**	0/3	0/3	7/8 (88%)	5/8 (63%)
VMS Ultra/XT/GX rmAb SP1 **790-4324/4325**	35/35 (100%)	21/35 (60%)	165/173 (95%)	100/173 (58%)

* 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 81% of the insufficient results (26 of 32). A 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 4), in which 90-100% of the neoplastic cells were expected to be demonstrated. Demonstration of ER in the breast carcinomas no. 5 and 6, in which an at least weak nuclear staining reaction of 50% of the neoplastic cells was expected, was much more challenging and required a carefully calibrated protocol.

20% (77 of 394) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays. The three most widely used antibodies, 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, Leica and Ventana), see tables 1 and 2. The rmAb clone SP1 was most successful and provided the highest proportion of sufficient and optimal results. Irrespective of the clone applied, efficient HIER, preferable in an alkaline buffer, was a central parameter for optimal results. When using HIER in a non-alkaline buffer, such as citrate pH 6, a pass rate of 82% (14 of 17 protocols) was seen, 24% optimal. HIER in an alkaline buffer provided a pass rate of 90% (52 of 58 protocols), 67% optimal. In addition, a main prerequisite for optimal performance seemed to be careful calibration of the primary Ab and thus 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.

Ready-To-Use (RTU) antibodies were used by 80% (317 of 394) of the laboratories. 96% of the laboratories used a complete RTU system including the pre-diluted primary Ab, specified ancillary reagents and an IHC stainer platform from the three main IHC system providers, Dako, Leica and Ventana.

The Ventana 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 96%. 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 typically adjusting incubation time of the primary Ab, HIER time and/or detection system as shown in table 4.
No significant difference in the proportion of sufficient and optimal results was seen comparing vendor recommended protocol settings and off-label use.
In this assessment use of OptiView as detection system was the most successful modification observed. 23 laboratories used OptiView and all obtained an optimal result. The insufficient results were typically caused by reduced HIER and/or incubation time of the primary Ab in combination with UltraView as detection system.

The Dako 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 4. If protocols were performed according to the recommendations provided by Dako, a pass rate of 83% (10 of 12) was obtained of which 33% were optimal. Laboratory modified protocol settings provided a higher pass rate and most noticeable a significantly increased proportion of optimal

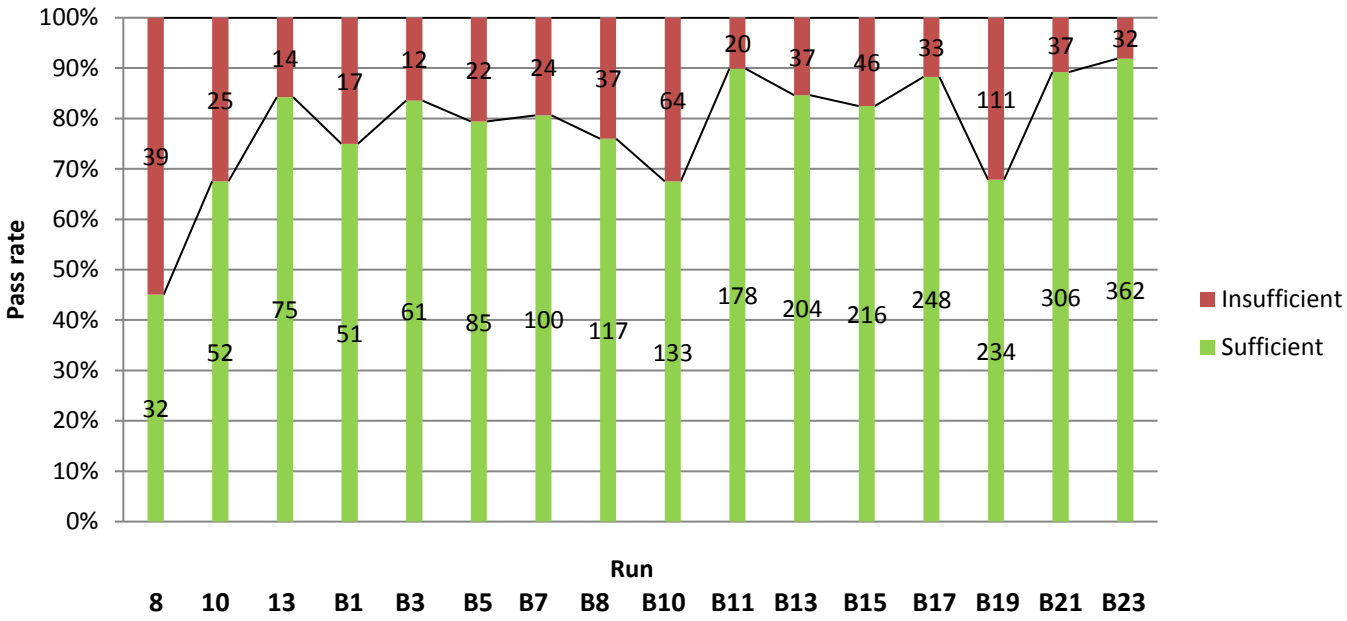
results. Especially, use of FLEX+ and rabbit linker was successful as 93% (14 of 15 protocols) based on this detection system gave an optimal result.

The Dako 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 (using HIER in TRS High for 30 min., 10 min. incubation of the primary Ab with FLEX as detection system and staining performed on Omnis), a pass rate of 100% (9 of 9) was obtained, 89% optimal.

The Leica RTU system PA0009/PA0151 for BOND gave an overall pass rate of 96%. Optimal results were only obtained by laboratory modified protocols using prolonged HIER time for 30 min. and/or HIER in BERS2 and not using HIER in BERS1 for 20 min. as recommended by Leica.

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

Fig. 1. **Participant numbers and pass rates for ER during 16 runs**



The consistent and high proportion of sufficient results can be explained by many factors: A 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 few laboratories. HIER was mainly performed by alkaline buffers.
Focusing on RTU systems from the main IHC system providers (Dako, Leica and Ventana) and grouped together in this run provided a pass rate of 95% (283 of 297 laboratories).

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 tool to monitor the level of analytical sensitivity for the IHC demonstration of ER and was in fact superior to uterine cervix. It was observed, that dispersed germinal centre lymphocytes (most likely T-cells) and squamous epithelial cells were distinctively demonstrated in virtually all protocols providing an optimal result in the other tissues included. If the germinal centre lymphocytes were negative, a reduced proportion of ER positive cells in the other tissues were seen and a too weak or even false negative staining was seen in the breast carcinomas no. 5 and 6. Simultaneously, tonsil can be used as supplementary negative tissue control, as B-cells in mantle zones and within germinal centres must be negative.
In order to validate the specificity of the IHC protocol further, a 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 high sensitive protocol is being applied, whereas the sensitivity cannot be evaluated in the normal epithelial cells 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.

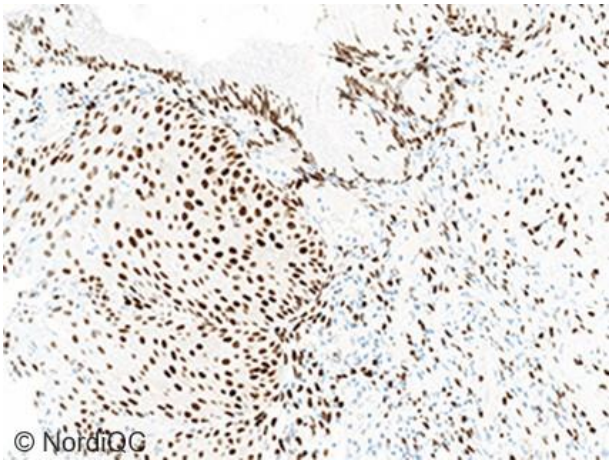


Fig. 1a
Optimal ER staining of the uterine cervix using the rmAb clone SP1 as Ready-To-Use format, Ventana 790-4325 with HIER in CC1 and UltraView as detection system.
Virtually all squamous and columnar epithelial cells show a moderate to strong, distinct nuclear staining reaction.
The majority of the stromal cells are demonstrated and only endothelial and lymphoid cells are negative. Also compare with Figs. 2a – 6a, same protocol.

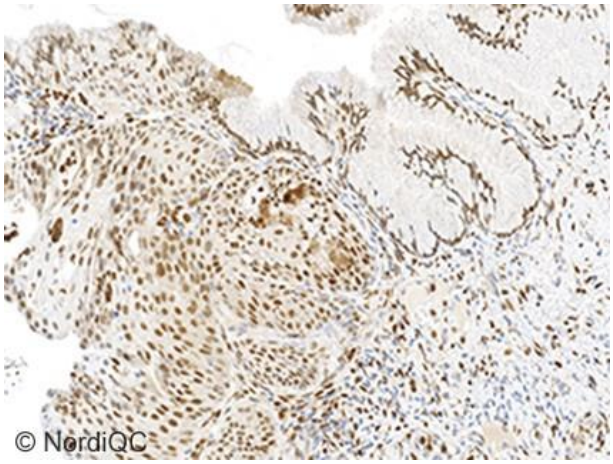


Fig. 1b
Insufficient ER staining of the uterine cervix - same field as in Fig. 1a.
The intensity of the staining reaction in the squamous and especially in columnar epithelial cells is reduced and at the same time a reduced signal-to-noise ratio is observed.
Also compare with Figs. 2b - 4b, same protocol. The protocol was based on the mAb clone 1D5 with HIER in alkaline buffer and a 3-step polymer based detection system.

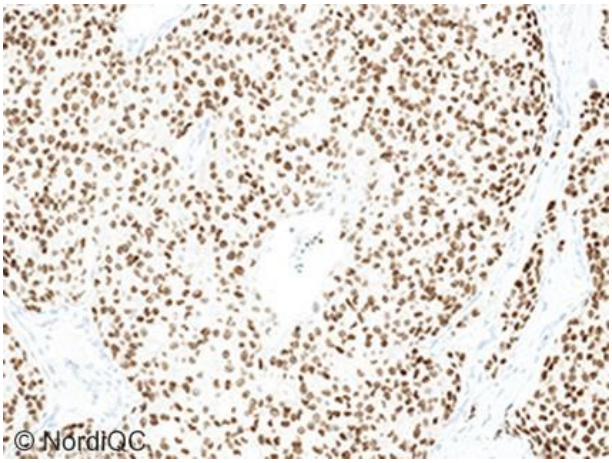


Fig. 2a
Optimal ER staining of the breast ductal carcinoma no. 4 with 90 – 100% cells positive using same protocol as in Fig. 1a.
Virtually all neoplastic cells show a strong, distinct nuclear staining reaction with only a weak cytoplasmic staining reaction.
No background staining is seen.

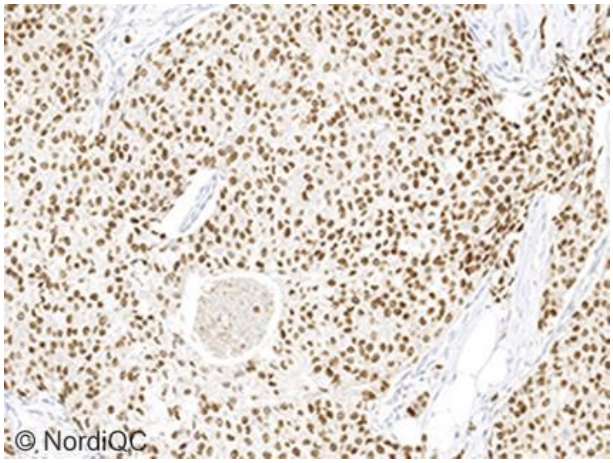


Fig. 2b
ER staining of the breast ductal carcinoma no. 4 with 90 – 100% cells positive using same protocol as in Fig. 1b – same field as in Fig. 2a.
The majority of neoplastic cells are demonstrated. However also compare with Figs. 3b and 4b – same protocol.

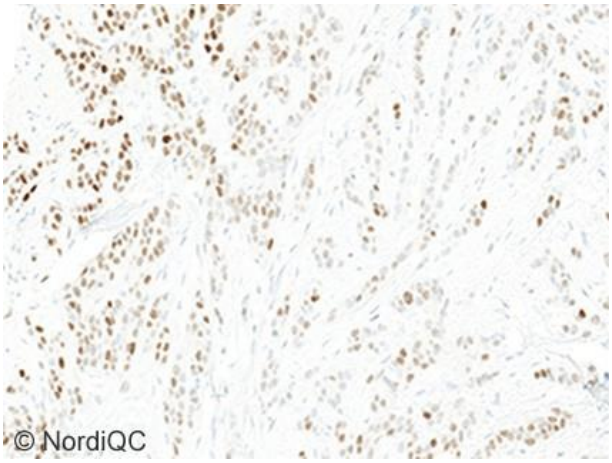


Fig. 3a
Optimal ER staining of the breast ductal carcinoma no. 6 with 60 – 80% cells positive using same protocol as in Figs. 1a and 2a.
The majority of neoplastic cells show a weak to moderate and distinct nuclear staining reaction.
No background staining is seen.

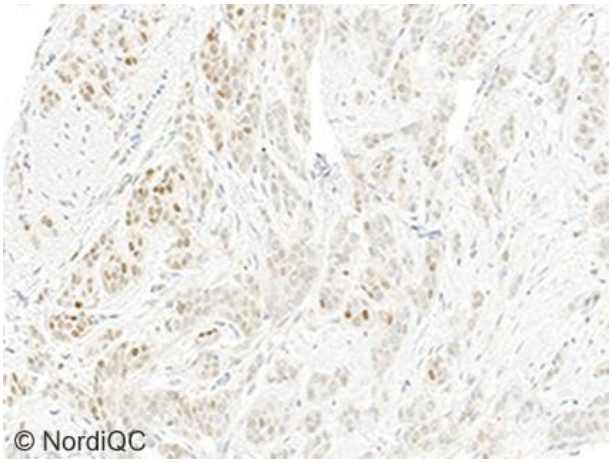


Fig. 3b
Insuffcient ER staining of the breast ductal carcinoma no. 6 with 60 – 80% cells positive using same protocol as in Figs. 1b and 2b.
The intensity and proportion of cells demonstrated is significantly reduced compared to the level expected. In addition an excessive cytoplasmic staining reaction compromises the interpretation.

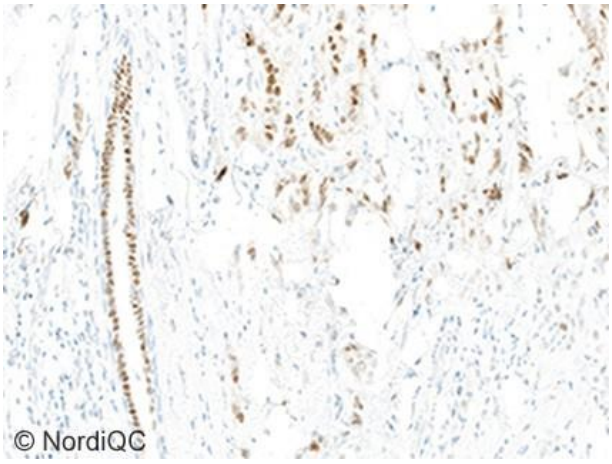


Fig. 4a
Optimal ER staining of the breast ductal carcinoma no. 5 with 50 – 70% cells positive using same protocol as in Figs. 1a - 3a.
The majority of neoplastic cells show a moderate and distinct nuclear staining reaction.
Virtually all normal epithelial cells lining the breast ductal gland (left) are demonstrated.
No background staining is seen.

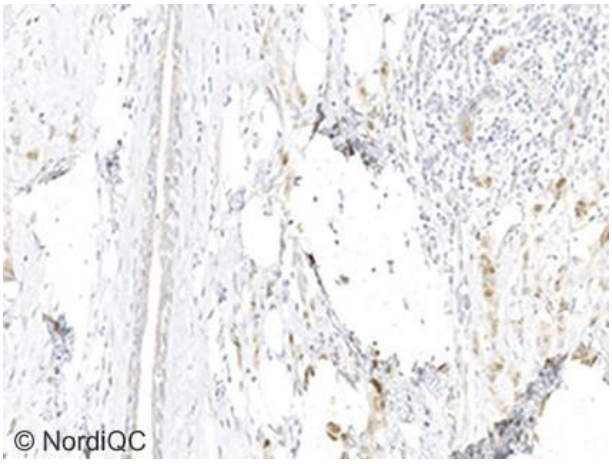


Fig. 4b
Insuffcient ER staining of the breast ductal carcinoma no. 5 with 50 – 70% cells positive using same protocol as in Figs. 1b – 3b.
The intensity and proportion of cells demonstrated is significantly reduced compared to the level expected.
This is seen in both the neoplastic cells and the normal epithelial cells.

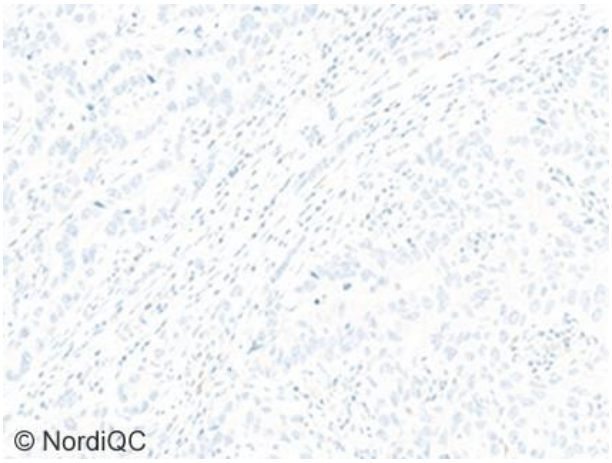


Fig. 5a
Optimal ER staining result of the breast carcinoma no. 3 with no ER expression.
Only dispersed stromal cells show a weak nuclear staining reaction, while all neoplastic cells are unstained.
Same protocol as in Figs. 1a – 4a.

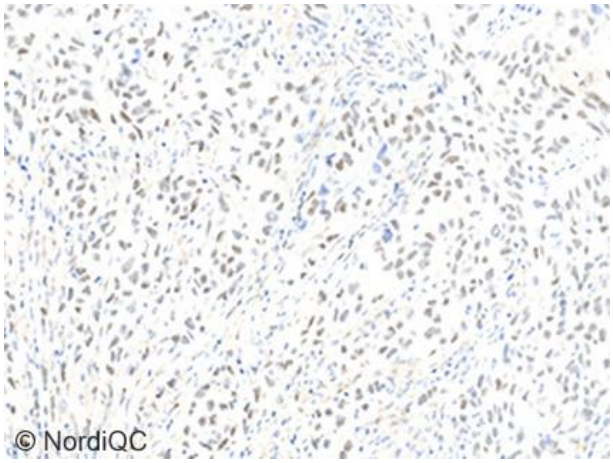


Fig. 5b
Insufficient ER staining of the breast ductal carcinoma no 3 with no ER expression.
A weak to moderate nuclear staining reaction is seen in the vast majority of the neoplastic cells.. The insufficient result most likely was caused by a combination of a too concentrated format of the mAb clone 6F11 and a protocol with a very high sensitivity (HIER in an alkaline buffer and a 3-step polymer based detection system).

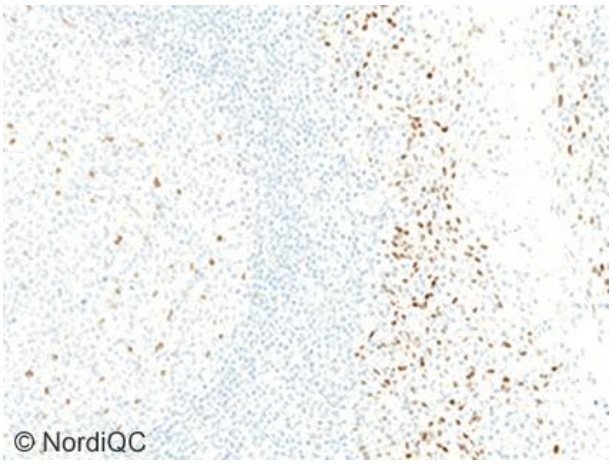


Fig. 6a
Optimal ER staining result of the tonsil using same protocol as in Figs. 1a – 5a.
Dispersed germinal centre lymphocytes and squamous epithelial cells show a weak to moderate nuclear staining reaction.
The nuclear staining reaction can be seen at low magnification, x100. However note that the vast majority of lymphocytes are negative.

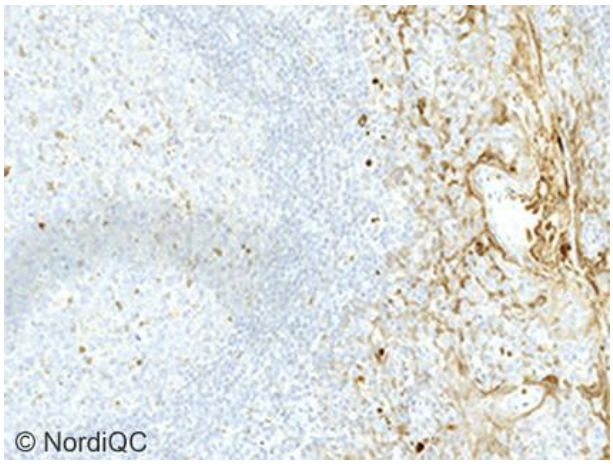


Fig. 6b
Insuffcient ER staining result of the tonsil using same protocol as in Figs. 1b – 4a.
Dispersed germinal centre lymphocytes show a weak to moderate nuclear staining reaction, but a general background staining and excessive cytoplasmic staining reaction in squamous epithlial cells compromises the interpretation.
Tonsil is recommendable as positive and negative tissue control, in which the optimal result must be as shown in Fig. 6a.

SN/LE/MV/RR 03.04.2017