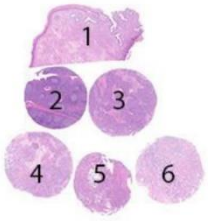
Assessment B25 2018

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



*ER-status and staining pattern as characterized by the 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:

- A 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.
- An 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 and 6.

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

Participation

Number of laboratories registered for ER, B25	377
Number of laboratories returning slides	362 (96%)

Results

362 laboratories participated in this assessment. One laboratory could not be assessed because of missing cores on the returned slide. This laboratory will not be in included in the results below.
332 of 361 (92%) 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.

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 supplemental 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, B25**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **6F11**	22 1	Leica/Novocastra Celnovte	10	8	4	1	78%	87%
rmAb clone **EP1**	12 2 1	Dako/Agilent Cell Marque BioGenex	7	6	2	0	87%	91%
rmAb clone **SP1**	22 4 3 1 1 1	Thermo Scientific Cell Marque Spring Bioscience Immunologic BioCare Zytomed	22	6	2	2	88%	93%
rmAb clone **S21-V**	1	DB Biotech	0	0	0	1	-	-
mAb clone **1D5**	1	Dako/Agilent	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **1D5 IR/IS657**	1	Dako/Agilent	0	0	1	0	-	-
mAb clones **1D5 + ER-2-123 SK310**	2	Dako/Agilent	0	1	1	0	-	-
mAb clones **1D5 + ER-2-123 K4071**	1	Dako/Agilent	0	1	0	0	-	-
mAb clone **6F11 PA0009/PA0151**	10	Leica	5	3	2	0	80%	100%
rmAb **EP1 8361-C010**	1	Sakura Finetek	1	0	0	0	-	-
rmAb **EP1 IR/IS084**	45	Dako/Agilent	17	24	3	1	91%	94%
rmAb **EP1 GA084**	24	Dako/Agilent	14	8	2	0	92%	94%
rmAb clone **SP1 790-4324/5**	196	Ventana/Roche	123	66	7	0	96%	96%
rmAb clone **SP1 249R-1**	4	Cell Marque	2	2	0	0	-	-
rmAb clone **SP1 KIT-0012**	1	Maixin	1	0	0	0	-	-
rmAb clone **SP1 RMPD001**	1	Diagnostic Biosystems	0	0	1	0	-	-
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 RM-9101-R7**	1	Thermo Scientific	1	0	0	0	-	-
Total	361		204	127	25	5	-	
Proportion			57%	35%	7%	1%	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, B25
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) (1/1)*, Cell Conditioning 1 (CC1, Ventana) (2/4), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (5/9), or Novocastra 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, 13 of 15 (87%) 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) (6/10) or unknown (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:20-1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings, 10 of 11 (91%) 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) (4/5), CC1 (Ventana) (7/9), BERS2 (Leica) (6/8), Tris-EDTA/EGTA pH 9 (3/5) or Citrate pH 6 (2/5) 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, 28 of 30 (93%) 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 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	0/2	2/4	-	5/9 (56%)	0/3
rmAb clone **EP1**	6/10 (60%)	-	0/2	-	-	-
rmAb clone **SP1**	4/5 (80%)	-	7/9 (78%)	-	6/8 (75%)	-

* 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 15-30 min., 15-60 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) or Bond Polymer Refine Red (DS9390) 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, 29 of 31 (94%) 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(3-in-1) (efficient heating time 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, 17 of 18 (94%) laboratories produced a sufficient staining result.
3 laboratories used product no. GA084 on AutoStainer48. These were not 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 8-90 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, 189 of 196 (96%) laboratories produced a sufficient staining result.
One laboratory used product no 790-4324/4325 on Leica Bond. 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 strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the specific IHC stainer device 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**	14/15 (93%)	7/15 (47%)	19/20 (95%)	7/20 (35%)
Dako Omnis rmAb EP1 **GA084**	12/13 (92%)	8/13 (62%)	7/8 (88%)	5/7 (63%)
Leica Bond mAb 6F11 **PA009/PA0151**	1/3	0/3	7/7 (100%)	5/7 (71%)
VMS Ultra/XT/GX rmAb SP1 **790-4324/4325**	35/36 (97%)	23/36 (64%)	154/160 (96%)	100/160 (62%)

* 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 97% of the insufficient results (29 of 30). 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. Demonstration of ER in the breast carcinoma no. 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.

20% (72 of 361) of the participants used Abs as concentrated formats within laboratory developed (LD) assays. The three most common Abs, mAb clone 6F11 and 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 57% (8 of 14) was seen, 21% optimal. HIER in an alkaline buffer provided a pass rate of 90% (52 of 58), 62% optimal. In addition, an important prerequisite for optimal performance seemed to be careful calibration of the primary Ab i.e., adjustment of the titer 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 83 % (60 of 72)

Ready-To-Use (RTU) antibodies were used by 80% (289 of 361) of the participants. 94% (272 of 289) of the laboratories used a complete RTU system including the pre-diluted primary Ab, specified ancillary reagents and the specific IHC stainer platform.

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 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 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 laboratory modified protocol settings.
Use of OptiView as detection system was the most successful modification observed. 24 laboratories used OptiView and all obtained a sufficient result. The most common modification observed was a shortened HIER time. 96 laboratories used HIER for ≤40 min and 96% (92 of 96) obtained a sufficient result.

The Dako/Agilent RTU system IR084/IS084 for Autostainer based on the rmAb EP1 provided an overall pass rate of 91%. 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 93% (14 of 15) was obtained of which 47% were optimal. Laboratory modified protocol settings provided an equal pass rate of 95% but a decreased proportion of optimal results of 35%. However, among these, use of FLEX+ and rabbit linker was successful, as 63% (5 of 8 protocols) based on this detection system gave an optimal result.

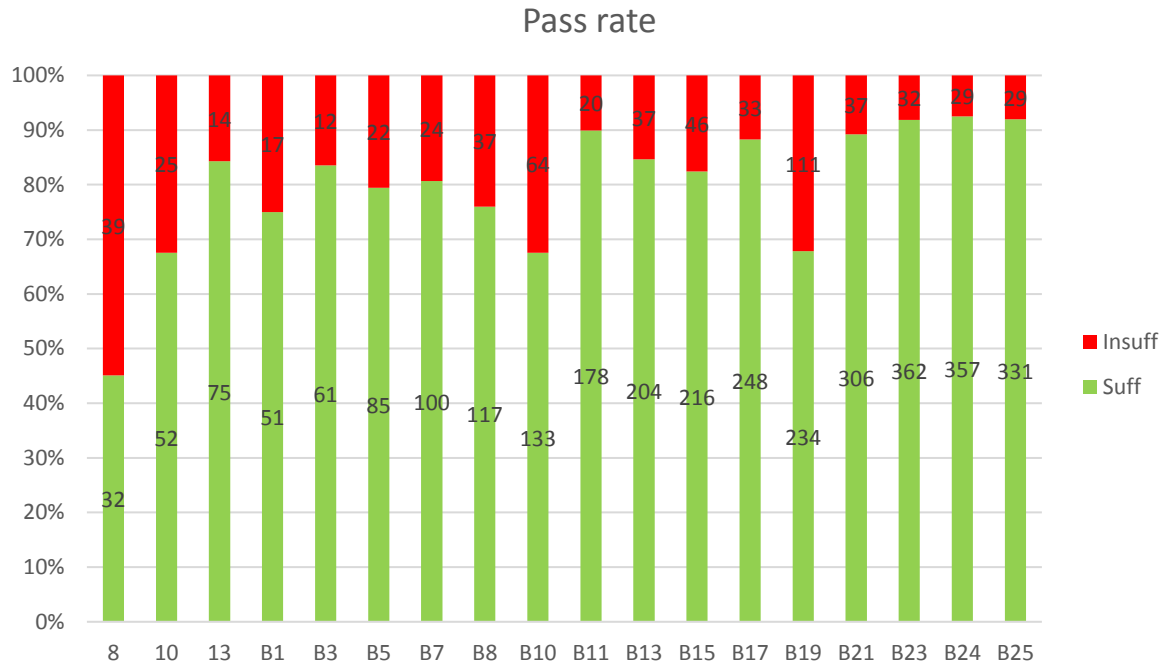
The Dako/Agilent RTU system GA084 for Omnis, also based on rmAb clone EP1, gave an overall pass rate of 92%. Optimal results could be obtained both by the vendor recommended protocol and by laboratory modified protocols as shown in Table 3. Using protocols according to the recommendations provided by Dako (HIER in TRS High for 30 min., 10 min. incubation of the primary Ab with FLEX as detection system), a pass rate of 92% (12 of 13) was obtained. Laboratory modified protocol settings provided a pass rate of 88%.

The Leica RTU system PA009/PA0151 for BOND gave an overall pass rate of 80%. 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.

14 laboratories used RTU assays on other platforms than the intended. 12 of 14 (86%) obtained a sufficient result (36% optimal). However, despite the encouraging results, the protocol must be meticulously validated by the end-users, when changing the systems to another platform than it was developed for.

Performance history
This was the eighteenth 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 18 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.
The circulated material for this assessment contained tumours with a high proportion of positive neoplastic cells. Protocols with low sensitivity would have been challenged if a tumour with only 30% positive neoplastic cells were included in the circulated material.
Focusing on RTU systems, grouped together in this run provided a pass rate of 94% (271 of 289 laboratories) compared to the LD assays with a pass rate of 83 % (60 of 72)

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.
Tonsil was 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 carcinoma no. 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.

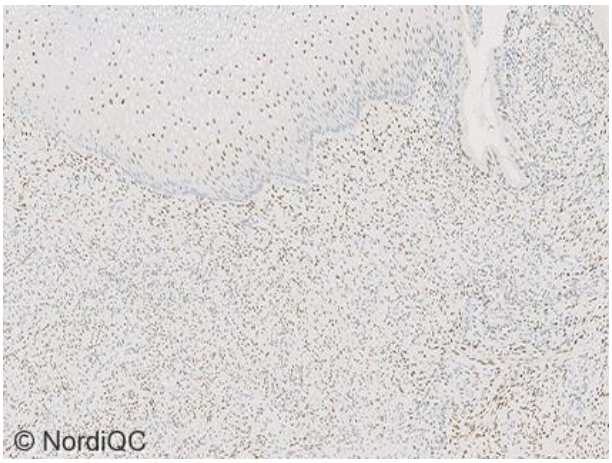


Fig. 1a
Optimal ER staining of the uterine cervix using the rmAb clone SP1 in an RTU format, with optimal protocol settings.
Virtually all squamous and columnar epithelial cells show a moderate to strong, distinct nuclear staining reaction. The majority of the stromal cells are demonstrated and only endothelial and lymphoid cells are negative. Also compare with Figs. 2a – 3a, same protocol.

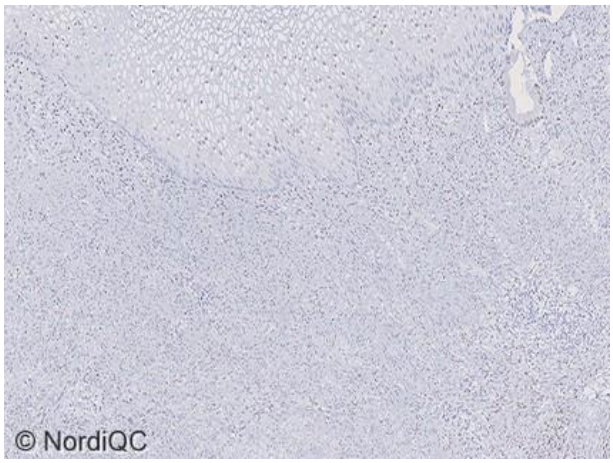


Fig. 1b
ER staining of the uterine cervix using an insufficient protocol - same field as in Fig. 1a.
The proportion of positive stained squamous epithelial cells is significantly reduced.
Also compare with Figs. 2b - 3b, same protocol. The protocol was based on the rmAb clone SP1 as a concentrate with insufficient HIER in citrate buffer and a 3-step polymer based detection system.

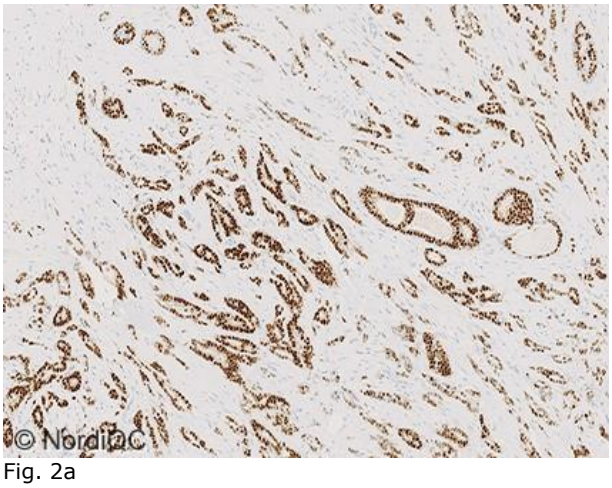


Fig. 2a
Optimal ER staining of the breast ductal carcinoma no. 4 with 90 – 100% cells positive using same protocol as in Fig. 1a.
Virtually all neoplastic cells show a moderate to strong, distinct nuclear staining reaction with only a weak cytoplasmic staining reaction.
No background staining is seen.

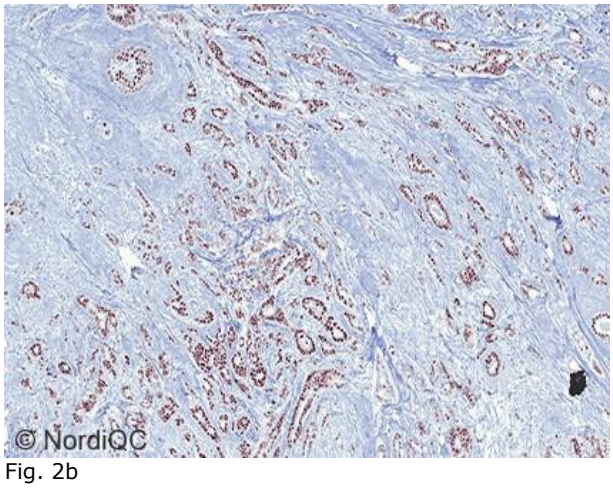


Fig. 2b
ER staining of the breast ductal carcinoma no. 4 with expected 90 – 100% cells positive using same protocol as in Fig. 1b.
Only cells showing a strong nuclear staining reaction are seen. Cells showing a weaker staining intensity are difficult to see due to an excessive counterstain.
Also compare with Figs. 3b – same protocol.

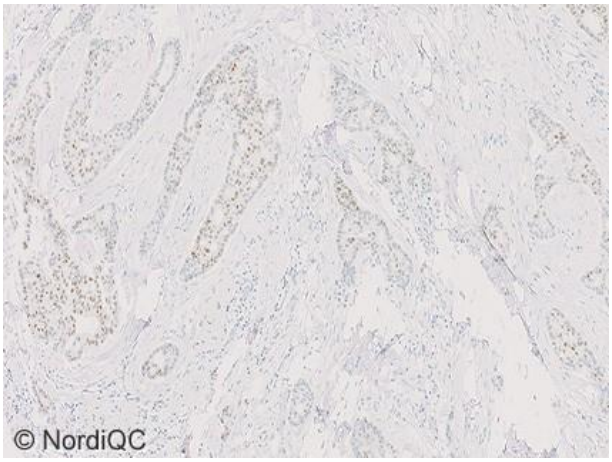


Fig. 3a
Optimal ER staining of the breast ductal carcinoma no. 6 with 90 – 100% cells positive using same protocol as in Figs. 1a and 2a.
The majority of neoplastic cells show a weak and distinct nuclear staining reaction.
No background staining is seen.

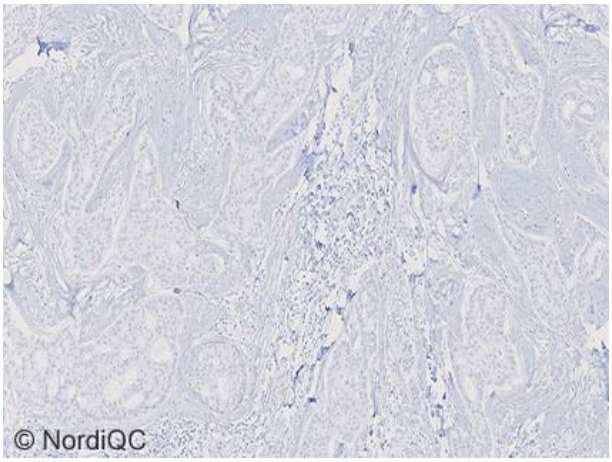


Fig. 3b
Insufficient ER staining of the breast ductal carcinoma no. 6 with expected 90 – 100% cells positive using same protocol as in Figs. 1b and 2b.
The carcinoma is virtually negative. The intensity and proportion of cells demonstrated is significantly reduced compared to the level expected.

HLK/LE/RR 13.04.18