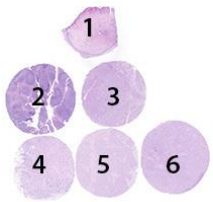
Assessment Run B26 2018

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	1-5%	Weak to moderate
3.	Breast carcinoma	0%	Negative
4.	Breast carcinoma	90- 100%	Moderate to strong
5.	Breast carcinoma	40-50%	Weak to strong
6.	Breast carcinoma	60-80%	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), and an at least weak to moderate nuclear staining reaction in the majority of both the columnar and basal squamous epithelial cells in the uterine cervix.

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, B26	373
Number of laboratories returning slides	355 (95%)

One laboratory returned a PR slide with an ER Ab. This laboratory was not included in the results below.

Results

354 laboratories participated in this assessment. 245 of 354 (70%) 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.
- Use of detection systems with low sensitivity.

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. However, the rmAb clone SP1 seemed to be a more robust Ab and performed overall better than rmAb clone EP1 and mAb clone 6F11. The corresponding Ready-To-Use (RTU) system from Ventana/Roche provided the highest proportion of sufficient and optimal results. In this assessment, false negative staining reaction was the prominent feature of insufficient 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, B26**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **6F11**	16	Leica/Novocastra	2	5	2	7	44%	100%
rmAb clone **EP1**	12	Dako/Agilent	2	4	4	2	50%	60%
rmAb clone **SP1**	20 6 2 1 1	Thermo Scientific Cell Marque Spring Bioscience Abcam Diagnostic Biosystems	14	7	4	5	70%	95%
rmAb clone **S21-V**	1	DB Biotech	-	-	-	1	-	-
mAb clone **1D5**	1	Biocare Medical	-	-	-	1	-	-
Ready-To-Use antibodies								
mAb clone **1D5 IR/IS657**	1	Dako/Agilent	-	-	-	1	-	-
mAb clones **1D5 + ER-2-123 SK310**	1	Dako/Agilent	-	-	-	1	-	-
mAb clone **6F11 PA0009/PA0151**	11	Leica	1	1	2	7	18%	-
rmAb **EP1 IR/IS084**	32	Dako/Agilent	2	7	13	10	28%	-
rmAb **EP1 IR/IS084**[3]	9	Dako/Agilent	2	-	5	2	22%	-
rmAb **EP1 GA084**	33	Dako/Agilent	4	18	6	5	67%	69%
rmAb **EP1 GA084**[3]	2	Dako/Agilent	1	1	-	-	-	-
rmAb clone **SP1 790-4324/5**	193	Ventana/Roche	75	91	21	6	86%	85%
rmAb clone **SP1 790-4324/5**[3]	1	Ventana/Roche	-	1	-	-	-	-
rmAb clone **SP1 249R-1**	4	Cell Marque	2	1	-	1	-	-
rmAb clone **SP1 KIT-0012**	2	Maixin	1	-	-	1	-	-
rmAb **SP1 M3011**	1	Spring Biosystems	-	-	-	1	-	-
rmAb clone **SP1 MAD-000306QD**	1	Master Diagnostica	-	-	1	-	-	-
rmAb clone **SP1 RM-9101-R7**	3	Thermo Scientific	2	1	-	-	-	-
Total	354		108	137	58	51	-	
Proportion			31%	39%	16%	14%	70%	

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, B26
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 Cell Conditioning 1 (CC1, Ventana) (1/3)* or Novocastra Epitope Retrieval Solutions pH 6 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:50 using a 3-layer detection system. Using these protocol settings, 6 of 6 (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 Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (1/7) or TRS High pH (Dako) (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:40-1:50 using a 3-layer detection system. Using these protocol settings, 3 of 5 (60%) laboratories produced a sufficient staining result.

rmAb clone **SP1**: Protocols with optimal results were all based on HIER using TRS pH 9 (Dako) (3/4), CC1 (Ventana) (6/10), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (4/7) or Tris-EDTA pH 9 (1/4) as retrieval buffer. The rmAb was typically diluted in the range of 1:25-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 18 of 19 (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 pH 9.0 (3-in-1)	TRS pH 6.1 (3-in-1)	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**	-	-	0/1**	-	1/3	-	0/7	0/2
rmAb clone **EP1**	1/7 (14%)	-	1/1	-	0/1	-	0/1	-
rmAb clone **SP1**	0/1	-	3/4	-	6/10 (60%)	-	4/7 (57%)	-

* 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:
One protocol with an optimal result was based on HIER using BERS2 20 min., 60 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Only one laboratory used these protocol settings (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 20-30 min. at 97-98°C), 20 min. incubation of the primary Ab and EnVision FLEX (K8000/K8002) with rabbit linker (K8009/K8019) as detection system. Using these protocol settings, 3 of 3 laboratories produced a sufficient staining result.
9 laboratories used product no IR084/IS084 on other platforms. These were not included in the description above.

mAb clone **EP1**, product no. **GA084**, Dako, Dako Omnis:
Protocols with optimal results were typically based on HIER using TRS High pH (efficient heating time 30 min. at 97°C), 10-25 min. incubation of the primary Ab and Envision FLEX (GV800) as detection system. Using these protocol settings, 22 of 32 (69%) laboratories produced a sufficient staining result.
2 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.), 16-40 min. incubation of the primary Ab and IView (760-091), UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings, 153 of 179 (85%) 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**	1/4	0/4	8/16 (50%)	2/16 (13%)
Dako Omnis rmAb EP1 **GA084**	18/27 (67%)	3/27 (11%)	4/6 (67%)	1/6 (17%)
Leica Bond mAb 6F11 **PA009/PA0151**	0/4	0/4	2/7 (29%)	1/7 (14%)
VMS Ultra/XT/GX rmAb SP1 **790-4324/4325**	33/37 (89%)	12/37 (32%)	133/156 (85%)	63/156 (40%)

* 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 a significant decrease in pass rate was seen (see Graph 1 below). The circulated material challenged the overall diagnostic sensitivity of the protocols on a higher level than in previous runs. 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. 6, 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.
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 all of the insufficient results (109 of 109). No laboratories obtained a false positive staining reaction.

The three most commonly used Abs – both as concentrated formats and RTU systems – were rmAb clones SP1, used by 66% (235 of 354), EP1, used by 25% (88 of 354) and mAb clone 6F11, used by 7% (27 of 354). The rmAb clone SP1 performed significantly better than both rmAb clone EP1 and mAb clone 6F11 with a total pass rate of 83% (195 of 235), 40% optimal. rmAb clone EP1 obtained a total pass rate of 46% (41 of 88), 12% optimal, and mAb clone 6F11 gave an overall pass rate of 33%, 11% optimal.

17% (60 of 354) of the participants used Abs as concentrated formats within laboratory developed (LD) assays. The three most commonly 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 platforms (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 22% (2 of 9) was seen, one of which was optimal. In comparison, HIER in an alkaline buffer provided a pass rate of 63% (32 of 51), 33% optimal. Using a 2-layer detection system a pass rate of 16% (3 of 19) was seen, 11% optimal. When using a 3-layer detection system, a pass rate of 76% (31 of 41) was seen, 39% optimal. Grouped together, the LD assays in this run provided a pass rate of 57 % (34 of 60). 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.

Ready-To-Use (RTU) Abs were used by 83% (294 of 354) of the participants. 96% (282 of 294) 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 86%. 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.
Use of OptiView or UltraView with amplification as detection system was the most successful modification observed, used by 35 laboratories. 33 laboratories (94%) obtained a sufficient result. The most common modification observed was a shortened HIER time. 85 laboratories used HIER for <40 min and 81% (69 of 85) obtained a sufficient result.

The Dako/Agilent RTU system IR084/IS084 for Autostainer based on the rmAb EP1 provided an overall pass rate of 28%. Four laboratories used the RTU as recommended by Dako, with one sufficient staining result. 16 laboratories modified the protocol with a pass rate of 50% (8 of 16). The most common modification observed was use of FLEX+ and rabbit linker, used by 14 laboratories. 8 laboratories (57%) obtained a sufficient result.

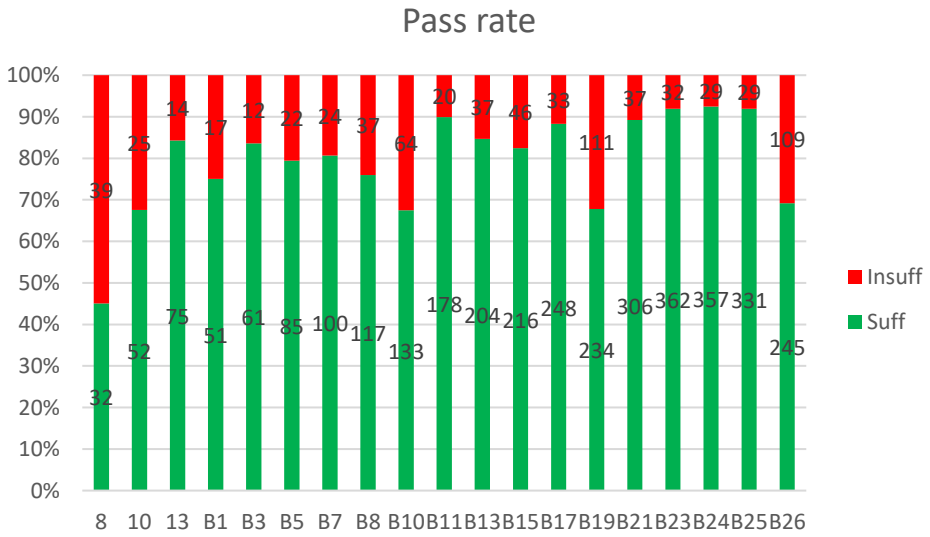
The Dako/Agilent RTU system GA084 for Omnis, also based on rmAb clone EP1, gave an overall pass rate of 67%. Sufficient results could be obtained both by the vendor recommended protocol and by laboratory modified protocols as shown in Table 3. Using protocols according to the recommendations provided 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 67% (18 of 27) was obtained. Laboratory modified protocol settings provided the same pass rate of 67%. The six modified protocols all increased incubation time of the primary Ab.

The Leica RTU system PA009/PA0151 for BOND gave an overall pass rate of 18%. Sufficient results were only obtained by laboratory modified protocols settings using HIER in BERS2 for 20 min. as opposed to performing HIER in BERS1 for 20 min. as recommended by Leica.

12 laboratories used RTU assays on other platforms than the intended. 5 of 12 (42%) obtained a sufficient result (25% optimal). However, it must be emphasized, that 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 nineteenth NordiQC assessment of ER. The proportion of sufficient results was significantly reduced compared to the latest run (see Graph 1).

Graph 1. **Participant numbers and pass rates for ER during 19 runs**



The decrease of sufficient results can be caused by many factors. The circulated material challenged the diagnostic sensitivity of the Abs and protocols on a higher level than in the previous assessments, and might be the main reason for the low pass rate.

No obvious other cause to the decreased pass rate could be identified in the submitted data. A daily variation of the laboratory performance – due to e.g. period since last maintenance, fading Abs or reagents, could be the reason for the different scores for similar protocol settings.

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 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 are 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 breast 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.

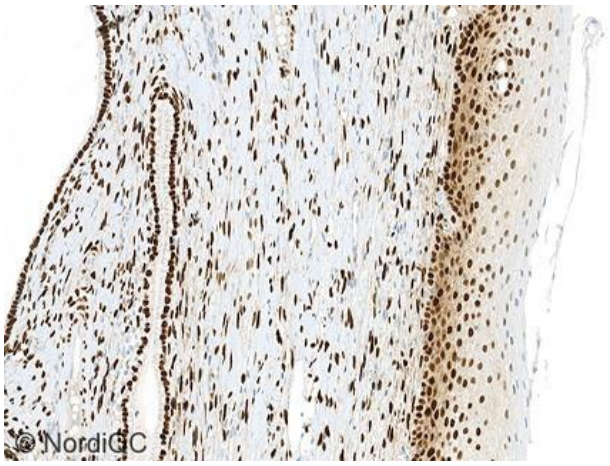


Fig. 1a
Optimal ER staining of the uterine cervix using the rmAb clone SP1 in an RTU format with optimal protocol settings.
Virtually all squamous and columnar epithelial cells show a moderate to strong nuclear staining reaction. The majority of the stromal cells are demonstrated and only endothelial and lymphoid cells are negative. Also compare with Figs. 2a – 4a, same protocol.

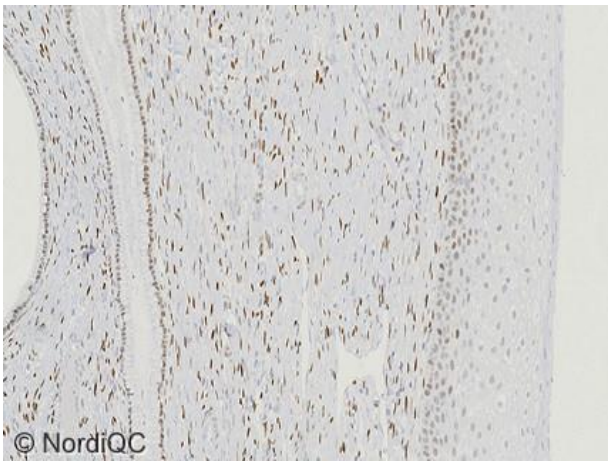


Fig. 1b
ER staining of the uterine cervix using an insufficient protocol - same field as in Fig. 1a.
The proportion of positive stained squamous epithelial cells is significantly reduced.
Also compare with Figs. 2b - 4b, same protocol. The protocol was based on the rmAb clone SP1 as an RTU with too short efficient HIER time and a 2-step polymer based detection system.

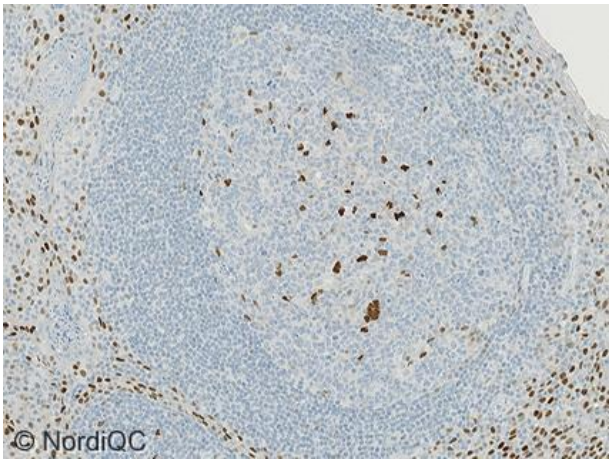


Fig. 2a
Optimal ER staining of tonsil using same protocol settings as in Fig. 1a.
A moderate to strong, distinct nuclear staining reaction is seen in the squamous epithelial cells and in dispersed germinal centre macrophages.

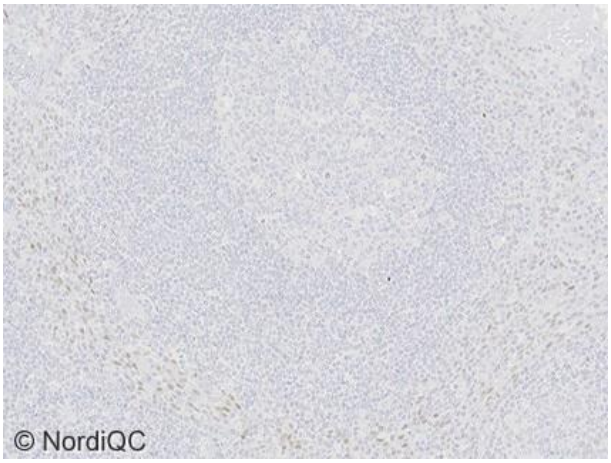


Fig. 2b
ER staining of tonsil using same protocol settings as in Fig. 1b.
Only squamous epithelial cells demonstrate a faint staining reaction. All germinal centre cells are virtually negative.

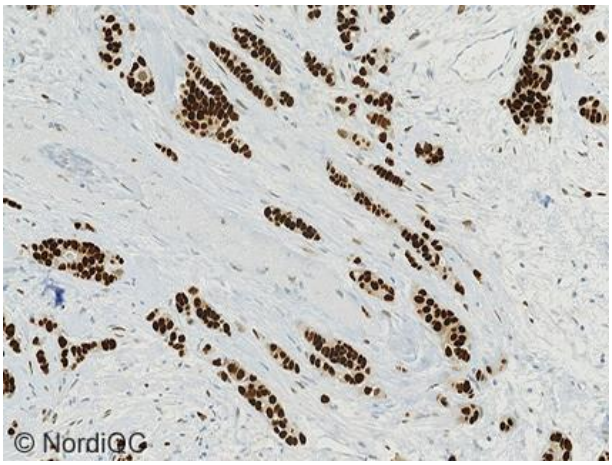


Fig. 3a
Optimal ER staining of the breast ductal carcinoma no. 4 with 90-100% cells positive using same protocol as in Fig. 1a.
Virtually all neoplastic cells show a strong, distinct nuclear staining reaction with only a weak cytoplasmic staining reaction. No background staining is seen.

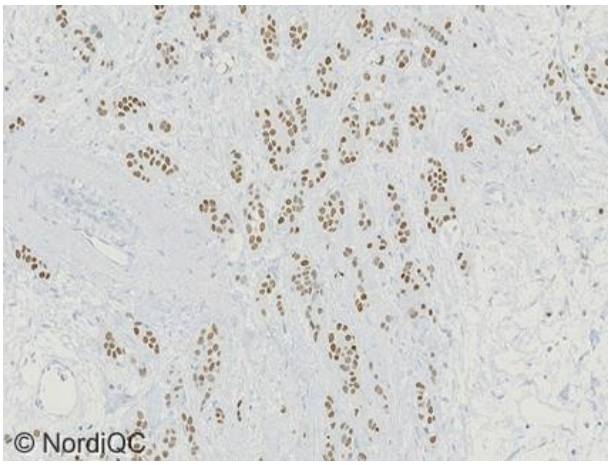


Fig. 3b
ER staining of the breast ductal carcinoma no. 4 with expected 90-100% cells positive using same protocol as in Fig. 1b.
The proportion of positive stained neoplastic cells are as expected, but the intensity is much weaker compared to Fig. 3b.

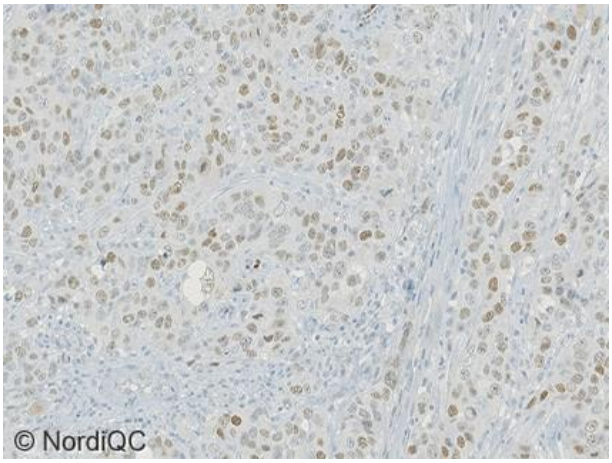


Fig. 4a
Optimal ER staining of the breast ductal carcinoma no. 6 with 60-80% cells positive using same protocol as in Figs. 1a - 3a.
The majority of neoplastic cells show a weak to moderate distinct nuclear staining reaction.
No background staining is seen.

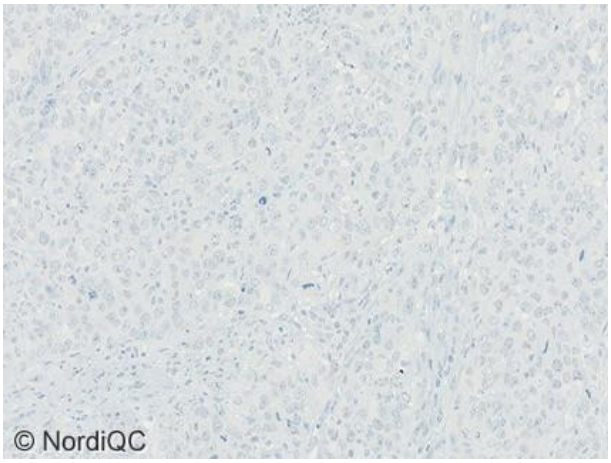


Fig. 4b
Insufficient ER staining of the breast ductal carcinoma no. 6 with expected 60-80% cells positive using same protocol as in Figs. 1b - 3b.
The carcinoma is virtually negative. The intensity and proportion of cells demonstrated is significantly reduced compared to the level expected.

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