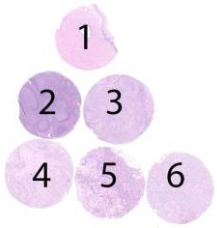
Assessment Run B21 2016

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 strong
3.	Breast carcinoma	0%	Negative
4.	Breast carcinoma	40-60%	Weak to moderate
5.	Breast carcinoma	60-80%	Weak to strong
6.	Breast carcinoma	80-100%	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 lymphocytes 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 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 was seen in any of the breast carcinomas no. 4, 5 and 6 or false positive staining reaction was seen in the breast carcinoma no. 3.

Participation

Number of laboratories registered for ER, run B21	364
Number of laboratories returning slides	343 (94%)

Results
343 laboratories participated in this assessment. 306 (89%) 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:
- Insufficient HIER - too short efficient HIER time and/or use of a non-alkaline buffer
- Too low concentration of the primary Ab.
- Less successful primary Ab.

Conclusion
The mAb clones **1D5**, **6F11** and rmAb clones **EP1** and **SP1** could all be used to provide an optimal result for ER. The rmAb clone **SP1** both as concentrate in a laboratory developed assay and as Ready-To-Use (RTU) format (Ventana) 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 and tonsil are appropriate positive and negative tissue controls for ER. In the uterine cervix,

virtually all stromal, columnar epithelial and squamous epithelial cells must show a moderate to strong and distinct nuclear staining reaction. In tonsil dispersed germinal centre lymphocytes must show a distinct, weak to moderate nuclear staining reaction. No staining should be seen in endothelial cells and the vast majority of peripheral lymphocytes.

Table 1. **Antibodies and assessment marks for ER, run B21**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1D5**	3 1	Dako/Agilent Zytomed	0	1	1	2	-	-
mAb clone **6F11**	27	Leica/Novocastra	9	14	4	0	85%	91%
rmAb clone **EP1**	18 1	Dako/Agilent Cell Marque	8	7	4	0	79%	77%
rmAb clone **SP1**	28 4 3 2	Thermo/Neomarkers Spring Bioscience Immunologic Cell Marque	28	8	1	0	97%	97%
Ready-To-Use antibodies								
mAb clone **1D5 IR/IS657**	7	Dako/Agilent	3	2	1	1	71%	100%
mAb clones **1D5 + ER-2-123 K4071/SK310**	2	Dako/Agilent	0	1	1	0	-	-
mAb clone **6F11 PA0151**	7	Leica/Novocastra	1	4	2	0	71%	100%
rmAb **EP1 IR/IS084**	61	Dako/Agilent	28	17	8	8	74%	87%
rmAb clone **SP1 790-4324/5**	173	Ventana/Roche	129	40	4	0	98%	98%
rmAb clone **SP1 MAD-000306QD**	2	Master Diagnostica	0	2	0	0	-	-
rmAb clone **SP1 ILM30142-R25**	1	Immunologic	1	0	0	0	-	-
rmAb clone **SP1 KIT-0012**	1	Maixin	1	0	0	0	-	-
rmAb clone **SP1 RM-9101-R7**	1	Thermo/Neomarkers	1	0	0	0	-	-
rmAb + mAb clone cocktail **EP1+6F11 IPI3150**	1	Biocare	1	0	0	0	-	-
Total	343		210	96	26	11	-	
Proportion			61%	28%	8%	3%	89%	

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 B21
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 (TRS) pH 6.1 (Dako) (1/2)*, Bond Epitope Retrieval Solution 1 (BERS1; Leica) (1/2), BERS2 (Leica) (3/11), Cell Conditioning 1 (CC1; Ventana) (1/5), PT Module buffer 1, pH 6 (1/1) or Tris-EDTA/EGTA pH 9 (2/2) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:300 depending on the total sensitivity of the protocol employed. Using these protocol settings, 21 of 23 (91%) 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) (5/9), TRS pH 9 (Dako) (2/4) 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, 10 of 13 (77%) 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) (3/4), TRS 9 (Dako) (3/3), CC1 (Ventana) (11/11) BERS2 (Leica) (6/7), Tris-EDTA/EGTA pH 9 (3/7) or Citrate pH 6 (2/5) 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, 35 of 36 (97%) laboratories produced a sufficient staining result.

Table 2. **Optimal results for ER of the three most commonly used 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**	0/1	1/2	1/5 (20%)	-	3/11 (27%)	1/2
rmAb clone **EP1**	6/11 (55%)	0/1	1/1	-	-	-
rmAb clone **SP1**	6/7 (86%)	-	10/10 (100%)	-	4/6 (86%)	-

* 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 **1D5**, product no. **IR/IS657**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min. at 97-98°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings, 5 of 5 (100%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **6F11**, product. no. **PA0151**, Leica/Novocastra:
One protocol with an optimal result was based on HIER using BERS 1 (Leica) 30 min., 36 min incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system.

rmAb clone **EP1**, product no. **IR/IS084**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min. at 96-98°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings, 41 of 47 (87%) laboratories produced a sufficient staining result.

rmAb clone **SP1**, product no. **790-4324/5**, Ventana, BenchMark XT, GX, ULTRA:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 20-64 min.), 8-64 min. incubation of the primary Ab and UltraView (760-500) +/- amplification kit or OptiView (760-700) as detection system. Using these protocol settings, 125 of 128 (98%) 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-5230 as detection system.

mAb clone **6F11** + rmAb clone **EP1** product. no. **IPI3150**, Biocare, IntelliPath:
One protocol with an optimal result was based on HIER using Diva Decloaker pH 6.2 (Biocare) in a Pressure Cooker, 45 min incubation of the primary Ab and MACH4 (4U534) as detection system.

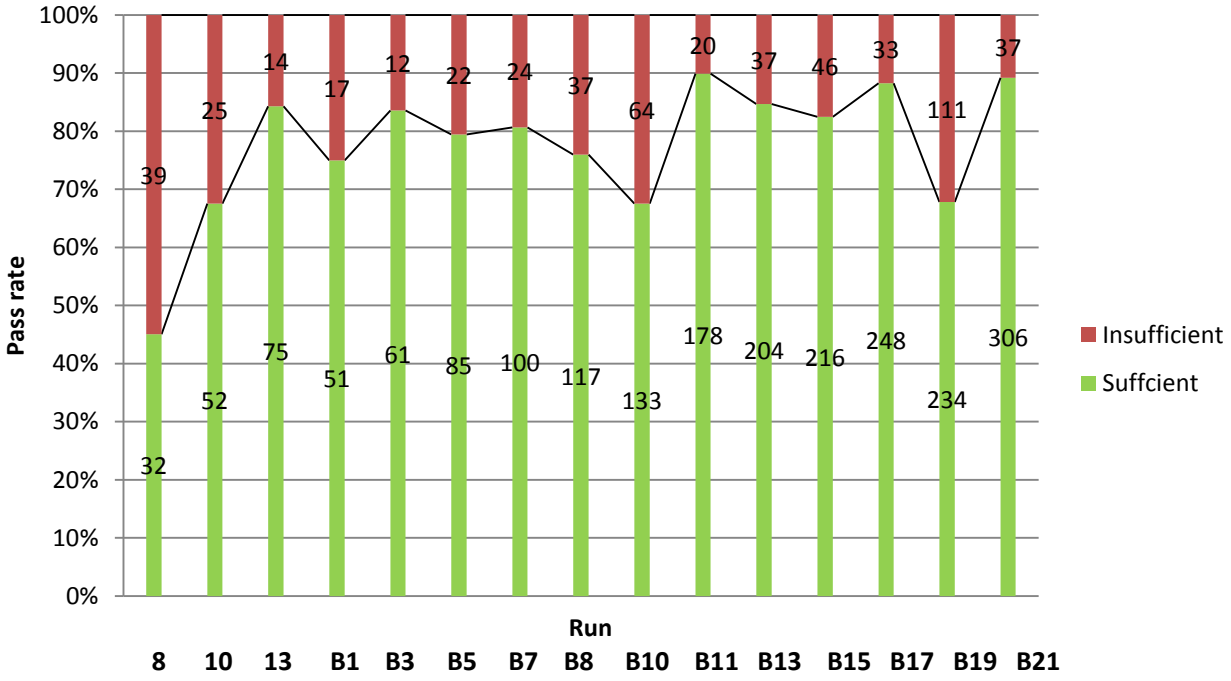
Comments
In this assessment and in concordance with the previous run B19, the prominent feature of an insufficient staining result was a too weak or false negative staining reaction. This pattern was seen in 92% of the insufficient results (34 of 37). A poor signal-to-noise ratio and/or inadequate counterstaining compromising the interpretation characterized the remaining insufficient results.
Virtually all laboratories were able to demonstrate ER in the high level ER expressing breast carcinoma, tissue core no. 6, in which 80-100% 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 40% of the neoplastic cells was expected, was much more challenging and required a carefully calibrated protocol.

Using a laboratory developed (LD) assay, the three most widely used antibodies, mAb clone 6F11, rmAb clones EP1 and SP1 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 66% (8 of 12 protocols) was seen, of which 33% were optimal. HIER in an alkaline buffer provided a pass rate of 91% (64 of 70) and 57% 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 the choice of detection system being either a 2- or 3-step system was of less importance.

The Ventana RTU format of the rmAb clone SP1 was in this assessment the most successful assay and gave a pass rate of 98%. An optimal result 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 as detection kit) and by laboratory defined modifications of the protocol typically adjusting the incubation time of the primary Ab and/or a reduced HIER time. Protocols based on the recommended settings applying HIER in CC1 for 64 min. provided a higher proportion of optimal results compared to a reduced HIER time to e.g. 24-32 min. Protocols based on HIER in CC1 for 64 min. provided a higher proportion of optimal results of 83% (50 of 60) compared to 63% (53 of 84) if HIER was performed for 24-32 min. in CC1 – all other protocol settings were not adressed.
Similar observations were made for the Dako RTU format of the rmAb clone EP1. An optimal result could both by obtained by the protocol settings given by Dako and by laboratory defined protocol settings. A pass rate of 85% of which 58% were optimal were obtained if the basic protocol settings for HIER (20 min.), incubation times in primary Ab and detection kit were performed as recommended, compared to a pass rate of 65% and 35% optimal using modified protocol settings for these central parameters. Especially reduced efficient HIER time to 10 min. in combination with a reduced incubation time of the polymer caused an increased number of insufficient results.

Performance history
This was the 15th NordiQC assessment of ER. The proportion of sufficient results was increased compared to the last run and now back at a comparable level as seen from run B11 and onward. (Figure 1).

Fig. 1. **Participant numbers and pass rates for ER during 15 runs**



The increased proportion of sufficient results can be caused by many factors. A confident 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. In this assessment run B21 it was thus observed that both the use of the

less successful mAb clone 1D5 was reduced and HIER was mainly performed by alkaline buffers. Focusing on the RTU systems from the main IHC system providers (Dako, Leica and Ventana), grouped together in this run provided a pass rate of 91% (219 of 243 laboratories). In this context it has to be mentioned, that the Ventana RTU system based on the rmAb clone SP1 provided a pass rate of 98% and was used by more than 50% of the laboratories in this assessment for ER.
The pass rates in the individual assessments for ER can also be related to the choice of material circulated and level of ER expression in the tumours included. ER status for all tissues are characterized by NordiQC using rmAb clones SP1 and EP1, as listed under material circulated.

Controls
In concordance with previous NordiQC runs, uterine cervix was found to be an appropriate and 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 included and found to be highly recommendable as tool to monitor the level of analytical sensitivity for the IHC demonstration of ER and 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. If the germinal centre lymphocytes 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. 3 and 4.
In order to validate the specificity of the IHC protocol, ER negative breast carcinoma must be included 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 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.

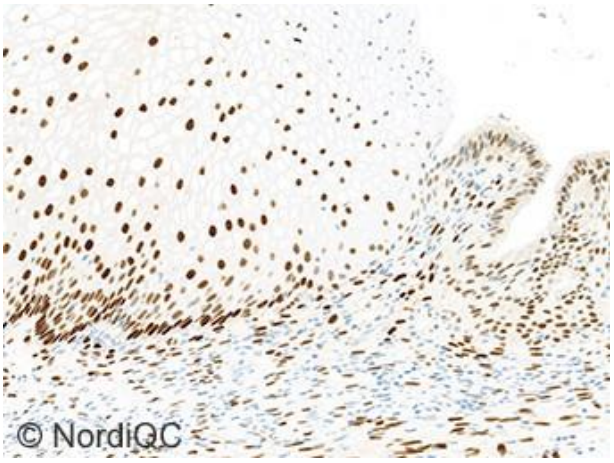


Fig. 1a
Optimal ER staining result of the uterine cervix using the rmAb clone SP1as Ready-To-Use format, Ventana 790-4324 with HIER in CC1 and UltraView as detection system. Virtually all the squamous and columnar epithelial cells show a moderate to strong, distinct nuclear staining reaction. The majority of the stromal cells are demonstrated and only endothelial and lymphoid cells are negative. Also compare with Figs. 2a – 4a, same protocol.

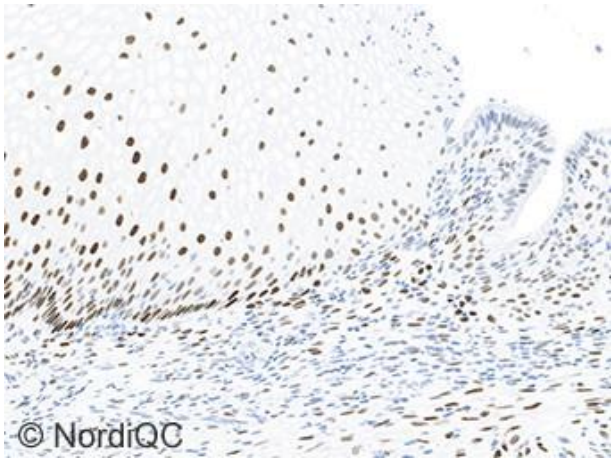


Fig. 1b
Insufficient ER staining result of the uterine cervix - same field as in Fig. 1a.
The proportion and intensity of the staining reaction in the squamous and especially in columnar epithelial cells is reduced. Also compare with Figs. 2b - 4b, same protocol.
The protocol was based on the rmAb clone EP1 applied with protocol settings giving a too low sensitivity – most likely due to a too dilute titre of the primary Ab and insufficient HIER.

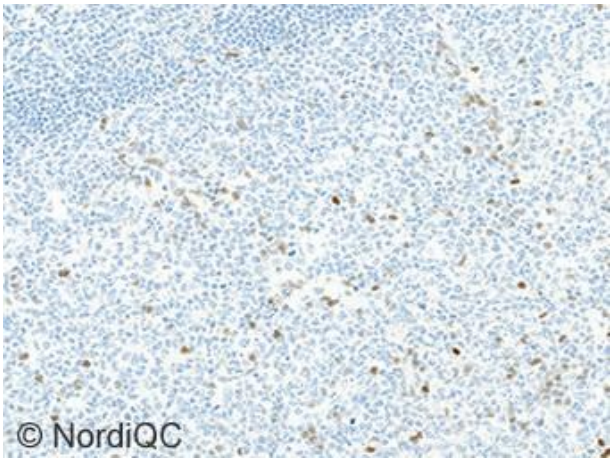


Fig. 2a
Optimal ER staining result of the tonsil using same protocol as in Fig. 1a
A weak to moderate nuclear staining reaction of dispersed germinal centre lymphocytes is seen.
The nuclear staining reaction can be seen at low magnification, x100.
However note that the vast majority of lymphocytes are negative.

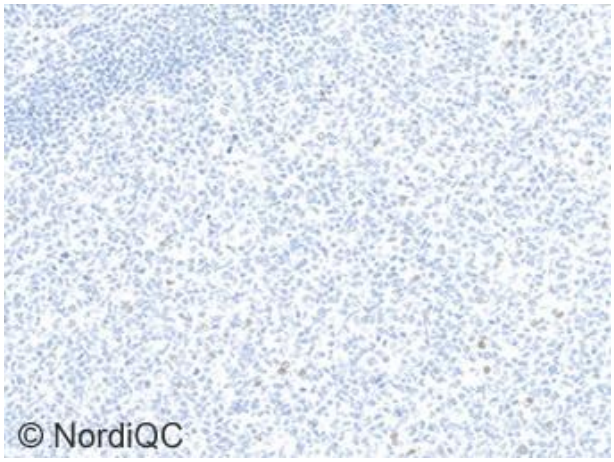


Fig. 2b
Insufficient ER staining result of the tonsil using same protocol as in Fig. 1b – same field as in Fig. 2a.
Compared to the result obtained in Fig. 2a, only a faint nuclear staining reaction in a significantly reduced proportion of germinal centre lymphocytes is seen.

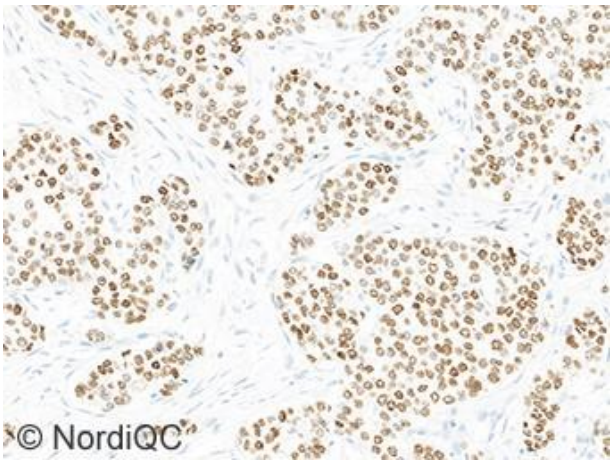


Fig. 3a
Optimal ER staining result of the breast ductal carcinoma no. 6 with 80 – 100% cells positive using same protocol as in Figs. 1a and 2a.
The vast majority of the neoplastic cells show a strong, distinct nuclear staining reaction with only a weak cytoplasmic staining reaction.
No background staining is seen.

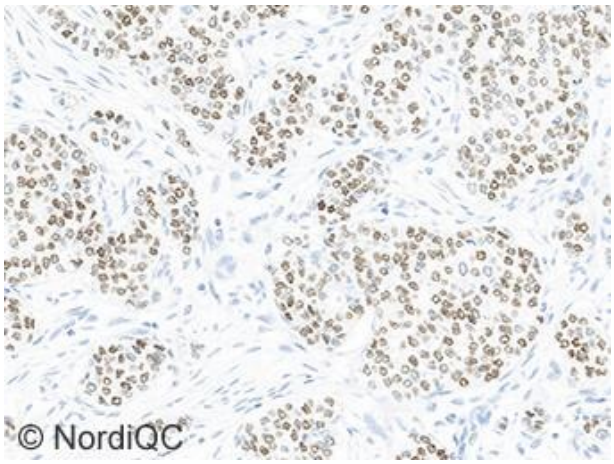


Fig. 3b
ER staining result of the breast ductal carcinoma no. 6 with 80 – 100% cells positive using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
The majority of neoplastic cells are demonstrated.
However also compare with Fig. 4b – same protocol.

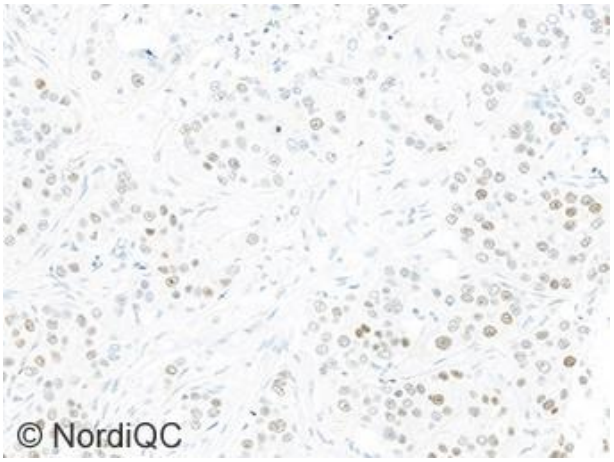


Fig 4a
Optimal ER staining result of the breast ductal carcinoma no. 4 with 40 – 60% cells positive using same protocol as in Figs. 1a – 3a.
A weak but distinct nuclear staining reaction in the appropriate proportion of the neoplastic cells is seen.

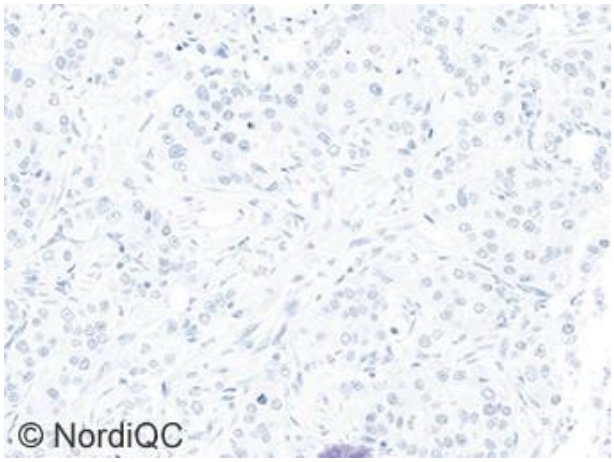


Fig 4b
Insufficient ER staining result of the breast ductal carcinoma no. 4 with 40 – 60% cells positive using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
A false negative staining reaction is seen.

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