Assessment Run B15 2013
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.	Breast carcinoma	0 %	Negative
3.	Breast carcinoma	40 - 60 %	Weak to moderate
4.	Breast carcinoma	60 - 80 %	Weak to moderate
5.	Breast carcinoma	80 - 100 %	Moderate to strong

*ER-status and staining pattern as characterized by NordiQC reference laboratories using the mAb clone SP1.

All tissues were fixed in 10% neutral buffered formalin for 24-48 hours and processed according to Yaziji et al. (1).

Criteria for an optimal ER staining included:

- Moderate to strong, distinct nuclear staining of both the columnar and basal squamous epithelial cells and most of the stromal cells (except endothelial and lymphoid cells) in the uterine cervix.
- At least weak to moderate distinct nuclear staining in the appropriate proportion of the neoplastic cells in the breast carcinomas no. 3, 4 and 5.
- No nuclear staining of neoplastic cells in breast carcinoma no. 2.
- No more than a weak cytoplasmic reaction in cells with a strong nuclear staining.

The stainings reactions were classified as good if ≥ 10 % of the neoplastic cells in the breast carcinomas no. 3, 4 and 5 showed an at least weak nuclear staining (less than the range of the reference laboratories).

The staining reactions were classified as borderline if ≥ 1 % and < 10 % of the neoplastic cells showed a nuclear staining in one of the breast carcinomas no. 3, 4 & 5.

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

262 laboratories participated in this assessment. 77 % achieved a sufficient mark. Table 1 summarizes antibodies (Abs) used and marks.

Table 1. **Antibodies and assessment marks for ER run B15**

Concentrated Antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1D5**	8 2 2	Dako Immunologic Zytomed	4	4	1	3	67 %	100 %
mAb clone **6F11**	24 4 2	Leica/Novocastra Vector Monosan	2	12	5	11	47 %	50 %
mAb clones **1D5+6F11**	1	Thermo/Neomarkers	0	0	0	1	-	-
rmAb **EP1**	10 1	Dako Epitomics	7	3	0	1	91 %	100 %
rmAb **SP1**	25 3 2 2 1	Thermo/Neomarkers Immunologic Cell Marque Spring Dako	20	6	7	1	76 %	79 %

	1	Diagnostic BioSystems						
Ready-To-Use Antibodies								
mAb clone **1D5** **IR657**	8	Dako	2	1	2	3	38 %	100 %
mAb clone **6F11** **PA0151**	5	Leica/Novocastra	0	1	0	4	20 %	0 %
mAb clone **6F11** **RTU-ER-6F11**	1	Leica/Novocastra	1	0	0	0	-	-
mAb clones **1D5 + ER-2-123** **K4071/SK310**	4	Dako	0	3	1	0	-	-
mAb clone **6F11 +** rmab clone **SP1** **PM308**	1	Biocare	1	0	0	0	-	-
rmAb clone **EP1** **IR084**	35	Dako	12	9	10	4	60 %	81 %
rmAb clone **EP1** **ZA-0102**	1	Zhongshan	0	0	1	0	-	-
rmAb clone **SP1** **790-4324/25**	114	Ventana	99	13	2	0	98 %	99 %
rmAb clone **SP1** **249R**	2	Cell Marque	1	0	0	1	-	-
rmAb clone **SP1** **IR151**	1	Dako	0	0	0	1	-	-
rmAb clone **SP1** **MAD-000306QD**	1	Master Diagnostica	1	0	0	0	-	-
rmAb clone **SP1** **RM-9101-R7**	1	Thermo/Neomarkers	0	1	0	0	-	-
Total	262		150	53	29	30	-	
Proportion			57 %	20 %	11 %	11 %	77 %	

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 B15
The following protocol parameters were central to obtain an optimal staining:

Concentrated antibodies
mAb clone **1D5**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using either Target Retrieval Solution pH 9 (TRS pH 9;Dako) (1/3)*, PT Module Buffer 1, pH 6 (PM1X, Thermo) (1/1), Tris-EDTA/EGTA pH 9 (1/2) or EDTA/EDTA pH 8 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:60-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 5 of 5 (100 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone **6F11**: Protocols with optimal results were all based on HIER using either TRS pH 9, 3-in-1 (Dako) (1/5) or Tris-EDTA/EGTA pH 9 (1/5) as retrieval buffer. The mAb was diluted in the range of 1:75-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 3 of 6 (50 %) laboratories produced a sufficient staining (optimal or good).

rmAb clone **EP1**: Protocols with an optimal result were all based on HIER using either TRS pH 9, 3-in-1 (Dako) (3/5), TRS pH 9 (Dako) (2/3), Cell Conditioning 1 (CC1; Ventana) (1/1) or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:40-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 9 of 9 (100 %) laboratories produced a sufficient staining (optimal or good).

rmAb clone **SP1**: Protocols with optimal results were all based on HIER using either TRS pH 9, 3-in-1 (Dako) (3/4), TRS pH 9 (Dako) (1/5), CC1 (BenchMark, Ventana) (6/10), Bond Epitope Retrieval Solution 2 (BERS2; Leica) (3/3), BERS 1 (Leica) (1/1), Tris-EDTA/EGTA pH 9 (3/7), EDTA/EDTA pH 8 (1/1) or

Citrate pH 6 (2/3) 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 26 of 33 (79 %) laboratories produced a sufficient staining (optimal or good).

Ready-To-Use (RTU) antibodies
mAb clone **1D5** (product.no. IR657, Dako): Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (heating time 20-30 min at 99°C), 30 min incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 2 of 2 (100 %) laboratories produced an optimal staining.

mAb clone **6F11** (product. no. RTU-ER-6F11, Leica/Novocastra): The protocol with an optimal result was based on HIER using BERS 1 (Leica), 17 min incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system.

mAb clone **6F11** + rmAb clone **SP1** (product. no. PM308, Biocare): The 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.

rmAb clone **EP1** (product.no. IR084, Dako): Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (heating time 20 min at 95-99°C), 20 min incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 13 of 16 (81 %) laboratories produced a sufficient staining (optimal or good).

rmAb clone **SP1** (prod. no. 790-4324/25, Ventana): Protocols with optimal results were all based on HIER using short, mild or standard Cell Conditioning 1, 8-60 min incubation of the primary Ab and iView (760-091), UltraView (760-500) or OptiView (760-700) as detection system. Using these protocol settings 112 of 113 (99 %) laboratories produced a sufficient staining (optimal or good).

The most frequent causes of insufficient staining reactions were:

- Insufficient HIER (too short efficient HIER time)
- Too low concentration of the primary Ab.
- False positive staining reaction when using the mAb clone 6F11 (no single cause identified)

In this assessment the prominent feature of an insufficient staining result was a too weak or false negative staining reaction, especially of the breast carcinomas no. 3 & 4, in which at least a weak nuclear staining of 40-80 % of the neoplastic cells was expected. This pattern was seen in 42 of the insufficient 59 results (71 %) and was typically caused by insufficient HIER and/or too low concentration of the primary Ab, irrespectively of the clone applied. In the remaining insufficient cases, a false positive staining reaction (n=15) or a poor signal-to-noise ratio (n=2) was observed. The false positive staining reaction was characterized by a weak to moderate but distinct nuclear staining reaction in > 10 % of the neoplastic cells in the breast carcinoma no. 2, which was classified ER negative when assessed by the reference laboratories (protocol based on the rmAb clone SP1) and the vast majority of the participating laboratories. The aberrant false positive staining was only seen for the mAb clone 6F11 (Leica & Vector), whereas as all other Abs and RTU systems consistently gave a negative staining result in this tumour. It was not possible to identify any single parameter, e.g. HIER conditions (pH of the buffer and HIER time), concentration and/or incubation time of the primary Ab, lot-to-lot variations of the primary Ab or use of high sensitive detection systems that could account for this pattern. Although efficient HIER in an alkaline buffer, primary Ab titre of the mAb clone 6F11 in the range of 1:50-100 and a 3-step polymer based detection system typically was applied by the laboratories producing a false positive staining reaction, identical protocol settings gave optimal staining reactions in other laboratories.
In this context, it must be considered if the breast carcinoma no. 2 expressed low levels of ER that only was revealed by the use of a very sensitive protocol and the mAb clone 6F11. However, based on the observation that all other Abs (with equal or superior sensitivity in the remaining cores), gave a completely negative staining of the breast carcinoma no. 2, the positive staining reaction of the mAb clone 6F11 was considered to be false positive. NordiQC is in contact with Leica to further investigate the observation and staining pattern for the mAb clone 6F11.

As observed in previous ER assessments, all of the 4 most widely used Abs for ER, (mAb clones 1D5 and 6F11 and the rmAb clones EP1 and SP1) could be used to produce optimal staining results.
The RTU format of the rmAb SP1 (Ventana) were used by 114 laboratories, and provided the highest proportion of sufficient and optimal results (98 % and 87 %, respectively).

In table 2 the overall performance of the four most widely used Abs for ER in the latest 10 NordiQC assessments is listed.

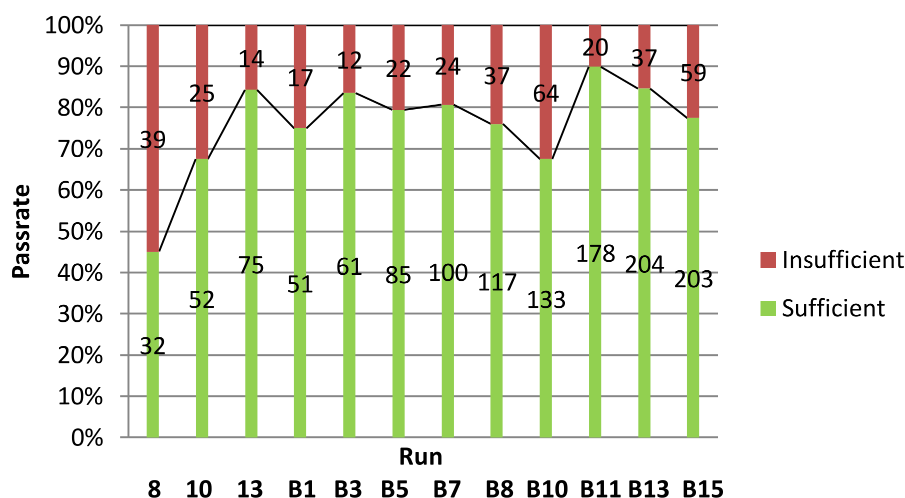
Table 2. **Results for the four most widely used Abs in the latest 10 NordiQC ER assessments**

	All ER assessments* All protocol settings			All ER assessments* Optimal protocol settings**		
	Protocols	**Sufficient**	**Optimal**	**Protocols**	**Sufficient**	**Optimal**
mAb clone 1D5	348	205 (59 %)	68 (20 %)	188	131 (70 %)	68 (37 %)
mAb clone 6F11	374	269 (72 %)	139 (37 %)	263	223 (85 %)	139 (53 %)
rmAb clone EP1	73	54 (74 %)	39 (53 %)	48	44 (92 %)	39 (81 %)
rmAb clone SP1	797	704 (88 %)	566 (71 %)	761	701(92 %)	566 (74 %)

*Runs 8, 10, 13, B1, B3, B5, B7, B8, B10, B11, B13 & B15.
** HIER settings and dilution range of the Ab in all assessments giving an optimal result.

Effect of external quality assessment
This was the 12th NordiQC assessment of ER. A decrease in proportion of sufficient results was seen compared to the latest runs (Figure 1).

Figure 1. **Proportion of sufficient ER staining reaction in the NordiQC assessments performed**



There has been a slight decrease in the proportion of sufficient result in the last two NordiQC assessments of ER. A small difference regarding the pass rates was observed for the laboratories participating in the ER assessment for the first time compared to the laboratories also participating in the latest assessment run B13, 2012: Pass rate for new participants were 67 % (36 of 54 laboratories), whereas the pass rate was 80 % (167 of 208 laboratories) for the laboratories participating in both runs.

Controls
In concordance with previous runs, uterine cervix was found to be an appropriate and recommendable positive control for the ER staining: In optimal protocols virtually all the 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. If the staining intensity in the epithelial cells of the uterine cervix was significantly reduced, a too weak or even false negative staining was seen in the breast carcinomas no. 3 and in particular no. 4.
In order to validate the specificity of the IHC protocol, ER negative breast carcinoma must be included. Only remnants of normal epithelial and stromal cells must be ER positive in this tissue serving as internal positive control. Positive staining reaction of the stromal cells indicates that a high sensitive protocol is being applied, whereas the sensitivity cannot be evaluated in the normal epithelial cells as they express high concentrations of ER (http://www.nordiqc.org/Run-35-B13-H1/Assessment/assessment-B13-ER.htm).

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.

Conclusion

The mAb clone **1D5** and the rmAb clones **EP1** and **SP1** were in this assessment the most robust Abs for demonstration of ER. The Ready-To-Use format of the rmAb clone **SP1** (Ventana) provided the highest proportion of sufficient and optimal results.
In this assessment, false negative or false positive staining reactions were prominent features of insufficient staining results. Uterine cervix is an appropriate positive control. Virtually all the 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.

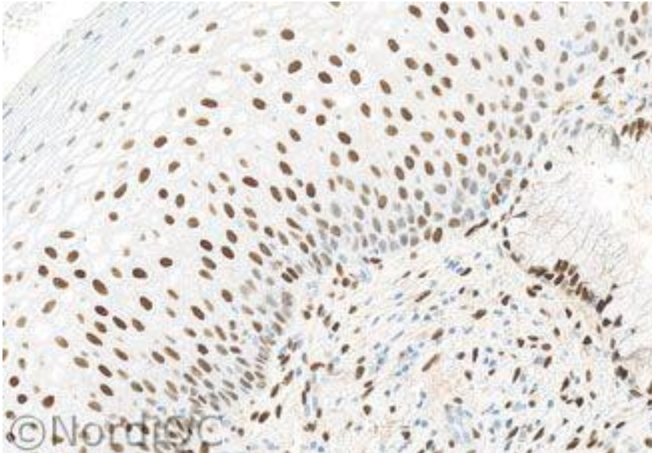


Fig 1a
Optimal ER staining of the uterine cervix using the rmAb clone SP1 optimally calibrated and with HIER in an alkaline buffer. Virtually all the squamous and columnar epithelial cells show a moderate to strong, distinct nuclear staining reaction. The majority of the stromal cells are demonstrated and only endothelial and lymphoid cells are negative.

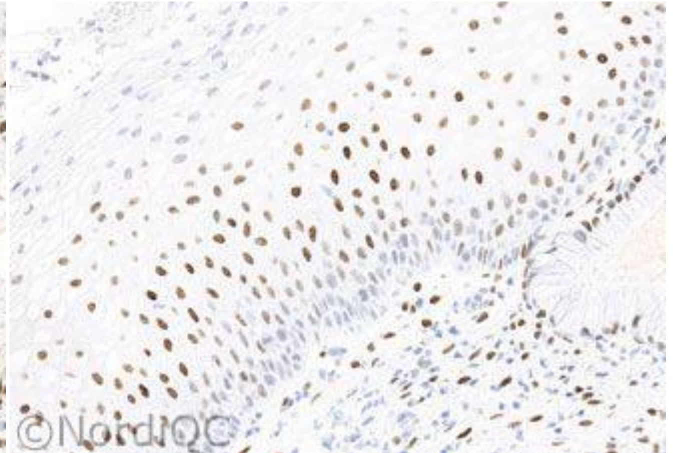


Fig 1b
Insufficient ER staining of the uterine cervix, same field as in Fig. 1a. The proportion and intensity of the staining reaction in the squamous and columnar epithelial cells is reduced. Also compare with Figs. 2b - 4b – same protocol. The protocol was based on the rmAb clone SP1 applied with protocol settings giving a too low sensitivity – most likely a combination of insufficient HIER and a too dilute primary Ab.

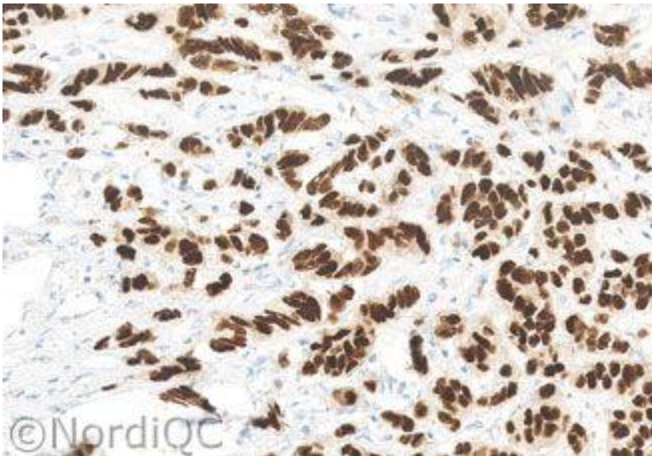


Fig 2a
Optimal ER staining of the breast ductal carcinoma no. 5 with 80 – 100 % cells positive. Virtually all the nuclei of the neoplastic cells show a strong, distinct nuclear staining reaction with only a weak cytoplasmic staining reaction. No background staining is seen.
Same protocol as in Fig. 1a.

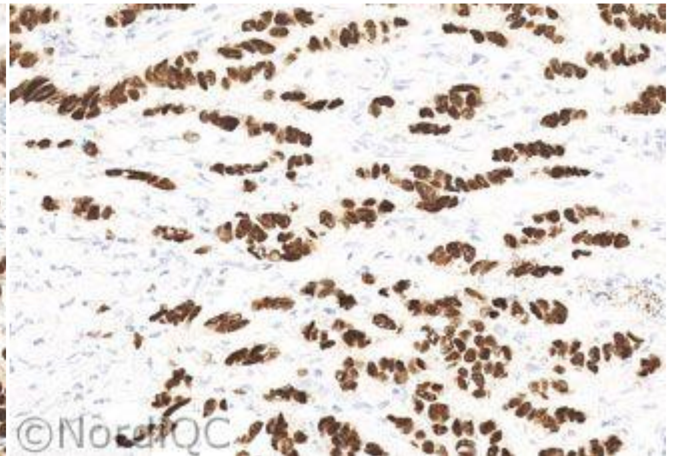


Fig 2b
ER staining of the breast ductal carcinoma no. 5 with 80 – 100 % cells positive using the same insufficient protocol as in Fig. 1b – same field as in Fig. 2a. Virtually all the neoplastic cells are demonstrated, but also compare with Figs. 2b - 4b – same protocol.

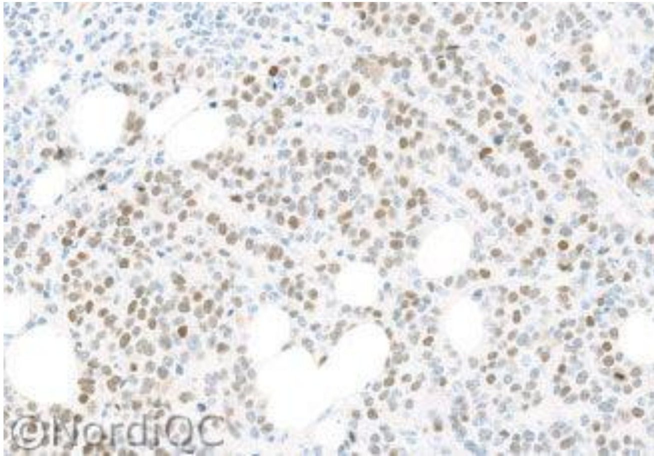


Fig 3a
Optimal ER staining of the breast ductal carcinoma no. 4 with 60 – 80 % cells positive. A weak to moderate and distinct nuclear staining is seen in the appropriate proportion of the neoplastic cells. Same protocol as in Figs. 1a and 2a.

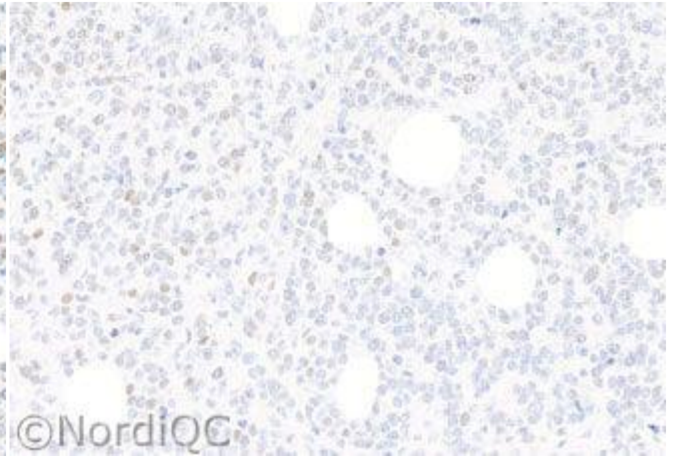


Fig 3b
Insufficient ER staining of the breast ductal carcinoma no. 4 with 60 – 80 % cells positive using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a. Only dispersed neoplastic cells show a weak nuclear staining reaction.

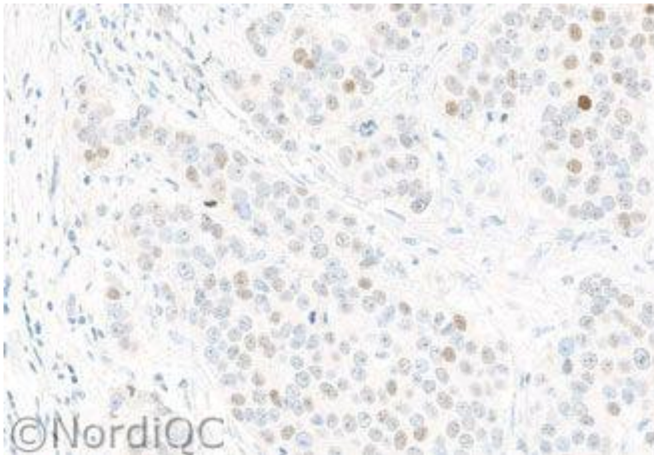


Fig 4a
Optimal ER staining of the breast ductal carcinoma no. 3 with 40 – 60 % cells positive. A weak to moderate nuclear staining reaction is seen in the appropriate proportion of the neoplastic cells and no background staining is seen. Same protocol as in Figs. 1a – 3a.

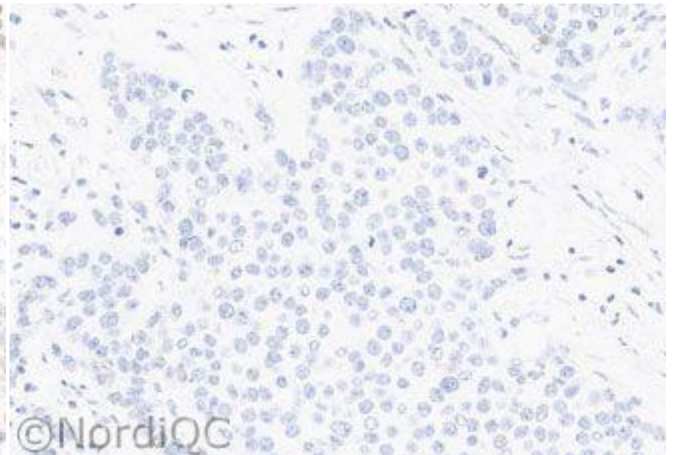


Fig 4b
Insufficient ER staining of the breast ductal carcinoma no. 3 with 40 – 60 % cells positive using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a. A false negative staining reaction is seen.

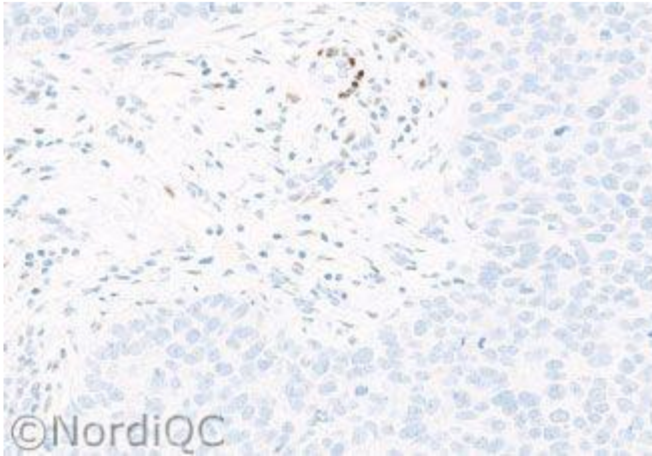


Fig 5a
ER staining of the breast ductal carcinoma no. 2. No nuclear staining reaction is seen in the neoplastic cells and only scattered stromal cells show a distinct nuclear staining reaction.
Same protocol as in Figs. 1a – 4a.
This staining pattern was seen in all protocols (n=225) based on the mAb clone 1D5 and the rmAb clones EP1 and SP1.
15 of 37 protocols based on the mAb clone 6F11 gave a weak nuclear staining reaction as seen in Fig. 5b.

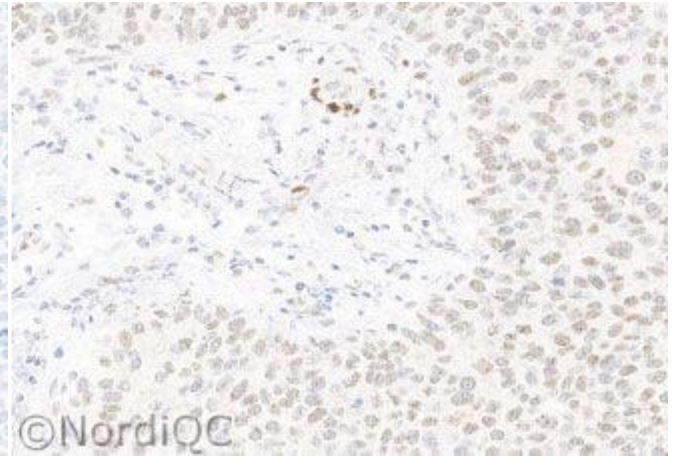


Fig 5b
ER staining of the breast ductal carcinoma no 2. based on the mAb clone 6F11, HIER in an alkaline buffer and a 3-step polymer based detection system. A weak nuclear staining reaction is seen in the vast majority of the neoplastic cells.
The positive staining reaction in this tumour was only seen for the mAb clone 6F11 and typically seen by using efficient HIER in an alkaline buffer and a 3-step polymer based detection system.
At present no conclusive data is available to determine if this tumour is true ER positive or the mAb clone 6F11 provided a false positive staining. The tumour was thus discarded from the final evaluation in this assessment.

SN/RR/LE 15-3-2013