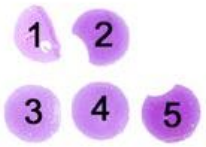
Assessment Run B5 2008

Estrogen Receptor alpha (ER)

The slide to be stained for estrogen receptor alpha (ER) comprised:

No.	Tissue	ER-positivity*	ER-intensity*
1.	Uterine cervix	80-90%	Moderate to strong
2.	Breast ductal carcinoma, basal-like subtype	Negative**	Negative to weak
3.	Breast ductal carcinoma, luminal A subtype	60-80%	Weak to moderate
4.	Breast ductal carcinoma, luminal A subtype	80-90%	Moderate to strong
5.	Breast ductal carcinoma, luminal A subtype	90-100%	Strong



All tissues were fixed in 10% neutral buffered formalin.
*ER-status and staining pattern as characterized by four reference laboratories using the mAb clones 1D5, 6F11 and the rmAb clone SP1.
** Using a cut-off level of 10% positive nuclei (current standard used in Denmark, Sweden and Finland).

Criteria for assessing an ER staining as optimal included:

- A strong, distinct nuclear staining of both the columnar and squamous epithelial cells and most of the stromal cells (with the exception of endothelial cells and lymphoid cells) in the uterine cervix.
- A strong, distinct nuclear staining of the appropriate proportion of the neoplastic cells in the breast ductal carcinomas no. 2- 5.
- No more than a weak cytoplasmic reaction in cells with strong nuclear staining.

107 laboratories submitted stains. At the assessment 43 achieved optimal marks (40 %), 42 good (39 %), 20 borderline (19 %) and 2 poor marks (2 %).

The following Abs were used:
rmAb clone **SP1** (NeoMarkers/Thermo Scientific, n=23; Ventana, n=22; Diagnostic Biosystems, n=1).
mAb clone **1D5** (Dako, n=29; Biocare, n=1; Zytomed System, n=1).
mAb clone **6F11** (Novocastra, n=23; Biocare, n=2; Ventana, n=1).
mAb clones **1D5+6F11** (NeoMarkers/Thermo Scientific, n=1).
mAb clones **1D5+ER-2-123** (Dako ER/PR pharmDx, n=4).

Optimal staining for ER in this assessment was obtained with the rmAb **SP1** (31 out of 46), the mAb **6F11** (10 out of 26), the mAb **1D5** (1 out of 31) and the mAb clones **1D5+ER-2-123** (1 out of 4).

All optimal protocols, independent of the Ab, were based on heat induced epitope retrieval (HIER) in buffers as follows:

rmAb **SP1**: Tris-EDTA/EGTA pH 9 (8/11)*, Cell Conditioning1 (Benchmark, Ventana) (18/24), Target Retrieval Solution pH 9.0 (Dako) (3/3), EDTA/EGTA pH 8 (1/1) or Bond Epitope Retrieval Solution 2 (Bond, Leica Microsystems) (1/1).
The Ab was typically used in the range of 1:25 - 1:400 or applied as a Ready-To-Use (RTU) Ab. Using these settings 42 out of 43 (98 %) obtained a sufficient staining.
* (number of optimal results/number of laboratories using this buffer)

mAb **6F11**: Tris-EDTA/EGTA pH 9 (4/10) or Bond Epitope Retrieval Solution 2 (Bond, Leica Microsystems) (4/5).
The Ab was typically used in the range of 1:10 – 1:500 depending on the total sensitivity of the protocol employed. Using these settings 13 out of 13 (100 %) obtained a sufficient staining.

mAb **1D5**: Tris-EDTA/EGTA pH 9 (1/20) and the Ab was diluted 1:50. Using these settings 7 out of 13 (54 %) obtained a sufficient staining.

mAb **1D5+ER-2-123**: Target Retrieval Solution pH 6.1 (Dako TRS, S1699/1700). The Ab was used as a Ready-

To-Use Ab. Using these settings 3 out of 4 (75 %) obtained a sufficient staining.

Table 1 shows the cumulated data from all 6 NordiQC ER assessments. The mAb clone 6F11 and the rmAb SP1 seem to be the most robust Abs for ER resulting in the largest proportion of optimal results.

Table 1. **Cumulated data from 6 runs showing the pass rates for the 5 Abs used.**

	Run 8 - B5 All protocol settings			**Run 8 - B5 Optimal protocol settings***		
	Protocols	**Sufficient**	**Optimal**	**Protocols**	**Sufficient**	**Optimal**
mAb clone 1D5	193	120 (62%)	39 (20%)	109	77 (71%)	39 (36%)
mAb clones 1D5 + 6F11	3	3 (100%)	0 (0%)	-	-	-
mAb clones 1D5 + ER-2-123	8	7 (88%)	2 (25%)	8	7 (88%)	2 (25%)
mAb 6F11	190	142 (75%)	71 (37%)	150	128 (85%)	71 (47%)
rmAb SP1	91	81 (89%)	57 (63%)	84	81 (96%)	57 (68%)

* HIER and dilution range of the Ab in the 6 individual assessments giving an optimal result.

The most frequent causes of insufficient staining in run B5 were:
- Insufficient HIER (citrate pH 6.0 for the clone 1D5 and/or too short heating time)
- Too low concentration of the primary antibody.

In this assessment the prevalent feature of an insufficient staining was a general too weak reaction and especially too low proportion of positive cells in the ductal carcinoma no. 3 with 60-80% positivity. As found in the previous runs the uterine cervix could be used as an appropriate control and critical stain quality indicator for the ER staining. In the optimal protocols almost all epithelial cells throughout the layers showed a distinct nuclear reaction compared to the protocols giving insufficient results in which only few epithelial cells were demonstrated. For the first time in a NordiQC ER assessment, a basal-like subtype of breast ductal carcinoma was included. The tumour was classified as triple negative, p63 & CK5 positive with a proliferation index of >50% based on Ki67 staining. In the reference laboratories the tumour was found to give a weak nuclear reaction in 1-5% of the cells, i.e. deemed ER negative using a cut-off level of 10%. However the ER status of this tumour varied surprisingly much in the different protocols used by the participants as illustrated in table 2.

Table 2. **Variation in ER status in the basal-like subtype breast ductal carcinoma.**

ER status	0	< 1%	1 - 10%	> 10%
No. of laboratories	22	17	50	18

A positive ER staining reaction (cut-off level of 10% positive nuclei) was seen with the mAb clone 6F11 and the rmAb clone SP1 typically when used relatively concentrated, combined with an efficient HIER and a highly sensitive detection system. The positive nuclei was generally weakly stained, the proportion of ER positive nuclei typically about 20-30%. Depending on the cut-off level used by the individual laboratory, the result would have an impact on the choice of treatment for this tumour. Using the 10% cut-off level, 83% of the laboratories would have classified the tumour as negative, whereas 17% would have classified it as positive. As NordiQC at the moment do not have other data on this case (such as mRNA analysis of the ER expression or treatment response), both positive and negative staining reactions was accepted. However, from this observation a new focus era for the immunohistochemical demonstration of ER may have come.
Previously the false negative ER reactions in breast carcinomas was a major problem, as demonstrated in both UK NEQAS and NordiQC, where up to 50% of the results have shown to be false negative, but there are virtually no data on "false positive" ER demonstration in breast carcinomas. If the low level of ER positivity in the basal-like carcinoma is considered as truly positive and suited for endocrine therapy, only 17% of the laboratories delivered a sufficient result for ER using a cut off level at 10%.

This was the 6th assessment of ER in NordiQC with following proportions of sufficient results. Table 3 shows the development in number of participants and pass rates. The slight drop in pass rate from run B3 to run B5 is probably due to the many new laboratories participating for the first time.

Table 3. **Number of participants and pass rates for ER during 6 runs.**

	Run 8 2003	Run 10 2004	Run 13 2005	Run B1 2006	Run B3 2007	Run B5 2008
Participants, n	71	77	89	68	73	107
Sufficient results%	45%	67%	84%	75%	84%	79%

Conclusion
The mAb clones 6F11 and the rmAb SP1 seem to be the most robust Abs for ER. Efficient HIER is mandatory. For optimal demonstration of ER an alkaline buffer is preferable. The concentration of the Ab must be carefully calibrated using an appropriate control such as the uterine cervix in which both the epithelial cells and most stromal cells must show a strong distinct nuclear reaction with minimal cytoplasmic reaction.

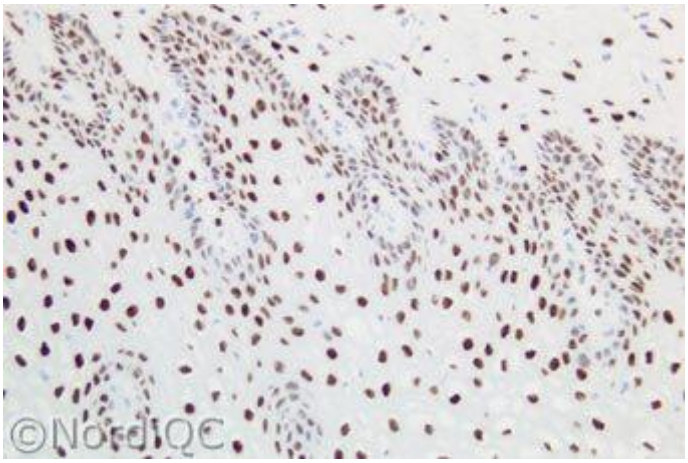


Fig. 1a
Optimal ER staining of the uterine cervix using the rmAb clone SP1. Virtually all the squamous epithelial cells show a distinct nuclear staining. The majority of the stromal cells are demonstrated while all endothelial and lymphoid cells are negative.

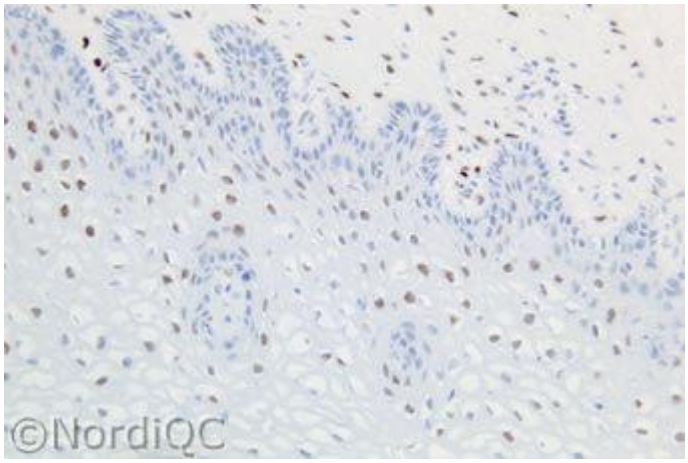


Fig. 1b
Insufficient staining for ER staining of the uterine cervix – same field as in Fig. 1a. Only scattered epithelial and stromal cells show a weak to moderate nuclear staining. Also compare with Fig. 2b and 3b – same protocol.

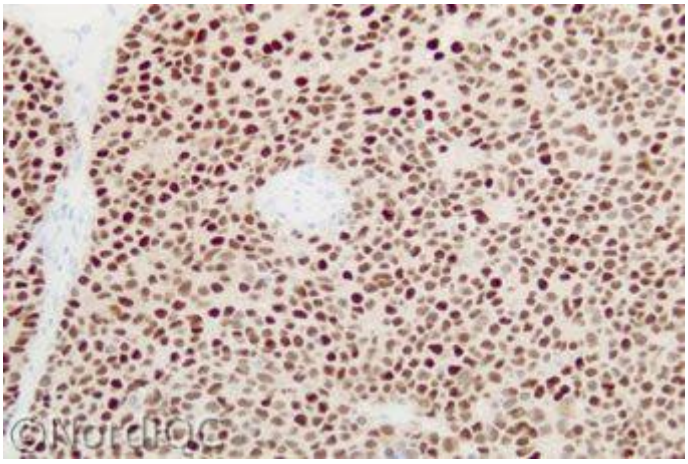


Fig. 2a
Optimal ER staining of the breast ductal carcinoma with 90 – 100 % cells positive. All the nuclei of the neoplastic cells are strongly stained with a weak cytoplasmic reaction – note the cytoplasmic reaction is only seen in the neoplastic cells, while the background is negative. Same protocol as in Fig. 1a.

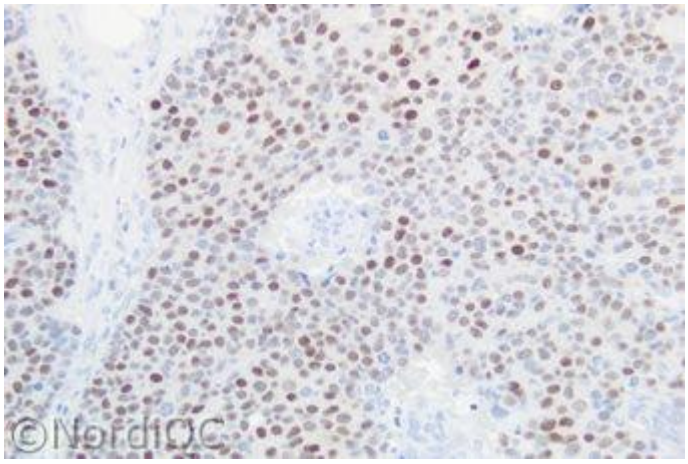


Fig. 2b
ER staining of the ductal breast carcinoma with 90 – 100 % cells positive using an insufficient protocol – same field as in Fig. 2a. The majority of the nuclei of the neoplastic cells are stained, but weaker than seen in Fig. 2a. However, compare with Fig. 3b – same protocol.

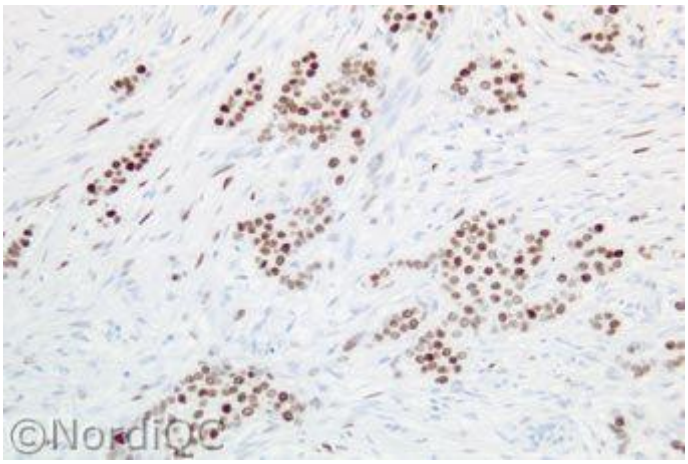


Fig. 3a
Optimal ER staining of the breast ductal carcinoma with 80 – 90 % cells positive. The majority of the nuclei show a moderate to strong staining. Same protocol as in Fig. 1a and 2a.

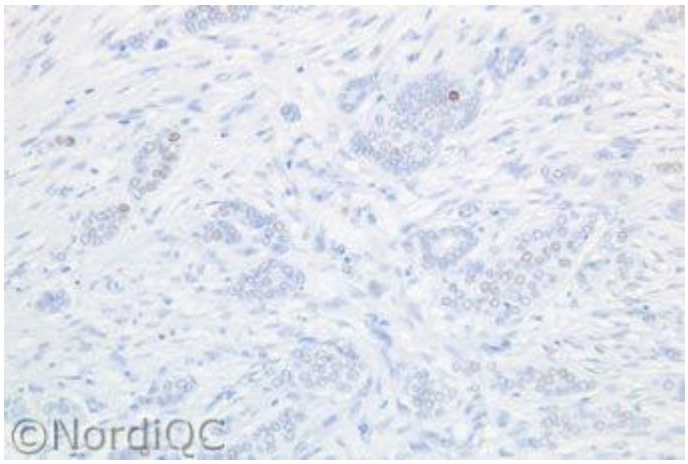


Fig. 3b
ER staining of the breast ductal carcinoma with 80 – 90 % cells positive using same protocol as in Fig. 1b and 2b. < 10 % of the neoplastic cells show a nuclear staining.

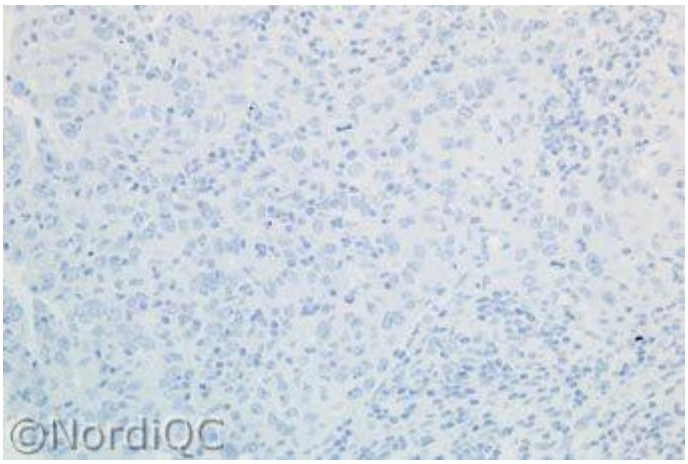


Fig. 4a
Optimal ER staining of the breast ductal carcinoma, basal-like subtype using same protocol as in Figs. 1a – 3a. All cells are unstained.

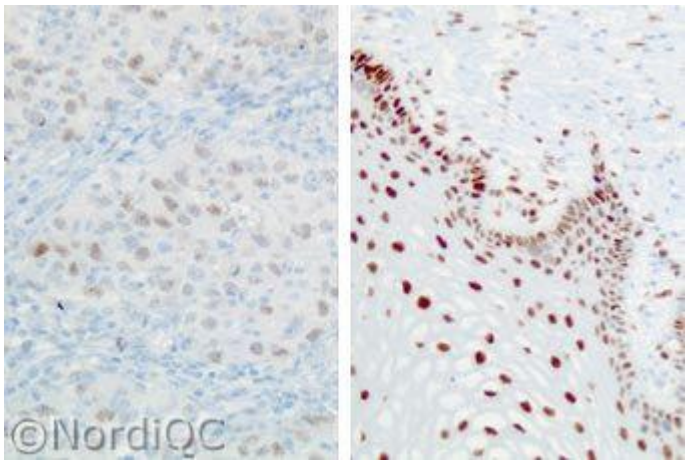


Fig. 4b
Staining for ER using the rmAb clone SP1 with a highly sensitive protocol.
Left: > 40-60 % of the nuclei of the breast ductal carcinoma, basal-like subtype show a weak to moderate staining.
Right: The uterine cervix shows a reaction similar to the expected result and the result obtained with the protocol in Figs. 1a.

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