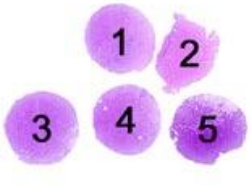
Assessment Run B6 2008

Progesterone Receptor (PR)

The slide to be stained for Progesterone Receptor (PR) comprised:

No.	Tissue	PR-positivity*	PR-intensity*
1.	Breast ductal carcinoma	Negative	-
2.	Uterine cervix	80-90%	Moderate to strong
3.	Breast ductal carcinoma	40-60%	Moderate to strong
4.	Breast ductal carcinoma	60-80%	Moderate to strong
5.	Breast ductal carcinoma	90-100%	Strong



*PR-status and staining pattern was characterized by reference laboratories using the mAb clone PgR 636 and the rmAb clone 1E2.

Criteria for assessing a PR staining as optimal included:

- A moderate to strong, distinct nuclear staining of the columnar epithelial cells, the basal squamous epithelial cells and the stromal cells in the uterine cervix.
- A moderate to strong, distinct nuclear staining of the ductal breast carcinomas no. 3, 4 & 5 in accordance with the PR status.
- No nuclear staining of the PR negative ductal breast carcinoma no. 1 – only epithelial cells in remnants of normal glands should show a positive reaction.

111 laboratories submitted stains. At the assessment 55 achieved optimal marks (50%), 36 good (32%), 17 borderline (15 %) and 3 poor marks (3 %).

The following Abs were used:
mAb clone **PgR 636** (Dako, n=48; NeoMarkers/Thermo, n=1)
mAb clone **16** (Novocastra/Leica, n=15; Monosan, n=1)
mAb clone **1A6** (Novocastra/Leica, n=4; BioCare, n=1; Zhongshan Golden Bridge Biotechnology, n=1)
mAb clone **PR-1** (ImmunoVision, n=2; Zytomed, n=1)
mAb clone **PgR 1294** (Dako, n=2)
mAb clone **PR 88** (BioGenex, n=1)
mAb clone **hPRa 2 + hPRa 3** (NeoMarkers, n=1)
mAb clone cocktail **16/SAN 27** (Novocastra/Leica, n=1)
rmAb clone **1E2** (Ventana, n=29)
rmAb clone **SP2** (NeoMarkers/ Thermo, n=4; DCS, n=1)

Optimal staining for PR in this assessment was only obtained with the mAb clones **PgR 636** (27 out of 49), **PgR 1294** (1 out of 2), **16** (14 out of 16), the mAb cocktail **16/SAN 27** (1 out of 1) and the rmAb **1E2** (12 out of 27).

All optimal protocols were based on heat induced epitope retrieval (HIER) and the following main protocol settings:

PgR 636: The HIER buffers used were Tris-EDTA/EGTA pH 9.0 (11/20)*; Target Retrieval Buffer pH 9, (Dako) (6/9), EDTA/EGTA pH 8 (1/2), Target Retrieval Solution pH 6.1 (Dako) (2/3), Bond Epitope Retrieval Solution 2 (Bond, Leica) (2/4), or Citrate pH 6.0 (5/10). The mAb was typically diluted in the range of 1:50 – 1:600 depending on the total sensitivity of the protocol employed or as a Ready-To-Use Ab. With these settings 40 out of 43 (93 %) laboratories produced a sufficient staining (optimal or good).

PgR 1294: The HIER buffer used was a Citrate buffer pH 6.0 (1/2). The Ab was used as a Ready-To-Use Ab. With these settings 2 out of 2 (100 %) laboratories produced a sufficient staining (optimal or good).

16: The HIER buffers used were Tris-EDTA/EGTA pH 9.0 (8/8); Target Retrieval Buffer pH 9, (Dako) (2/2)*, Citrate pH 6.0 (2/2) or Cell Conditioning1 (BenchMark, Ventana) (2/3). The mAb was typically diluted in the

range of 1:50 – 1:500 depending on the total sensitivity of the protocol employed. With these settings 14 out of 15 (93 %) laboratories produced a sufficient staining (optimal or good).

16/SAN 27: The HIER buffer used was Tris-EDTA/EGTA pH 9.0 (1/1). The mAb was diluted 1:300.

1E2: The HIER buffer used were Cell Conditioning1 (BenchMark, Ventana) (12/29). The mAb was used as a Ready-To-Use Ab (Ventana). With these settings 26 out of 29 (90 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

The most frequent causes of insufficient staining were:
- Too low concentration of the primary antibody
- Less successful primary antibody
- Insufficient epitope retrieval – too short efficient HIER time
- "Too sensitive" protocol settings (for the rmAbs SP2 and 1E2).

In this assessment (and in concordance with the previous PR assessments run B2 & B4), the insufficient results were caused by a false negative, a false positive staining or a staining with a poor signal-to-noise ratio hampering the interpretation. A too weak or false negative staining was seen in 9 out of 20 (55 %) of the insufficient results, while in 7 out of 20 (35 %) a too strong staining and false positive reaction was seen.

Virtually all laboratories could demonstrate PR in the ductal breast carcinoma no. 5 (high expressor, 90-100 % positivity), whereas the prevalent feature of the insufficient staining was a too weak or entirely false negative staining of the ductal breast carcinoma no. 3 (low expressor, 40-60 % positivity).

Unexpectedly, 35 % of the insufficient results were due to a false positive nuclear staining in the ductal breast carcinoma no. 1. The false positive reaction was mainly seen by protocols based on the two rabbit monoclonal antibodies clone 1E2 (3 out of 27) and SP2 (2 out 5) but also with the mAb clone 1A6 (2 out 6). When a false positive reaction was observed in the PR negative tumour also a false positive nuclear reaction was seen in scattered non-epithelial cells as lymphocytes and endothelial cells, and for the clone 1A6 also a diffuse cytoplasmic reaction was seen. No single parameter causing the false positive reaction could by identified, but the combination of efficient HIER, e.g., in pressure cooker, and usage of a high Ab concentration seemed to be the main causes.

This was the fourth assessment of PR. Throughout, a constant increase of the pass rate has been seen (table 1).

Table 1. **Pass rate for PR in four runs**

	Run 10 2004	Run B2 2006	Run B4 2007	Run B6 2008
Participants, n=	79	81	95	111
Sufficient results	69%	75%	78%	82%

Multiple factors may contribute to the improvement, but especially the focus on the choice of Ab and an appropriate control such as normal uterine cervix seem to be central parameters for an improved demonstration of PR. Table 2 shows the cumulated pass rates for the most widely used Abs in the 3 recent runs for PR, B2, B4 and B6.

Table 2. **Cumulated pass rate for PR in three runs**

	Total B2, B4 & B6	
	Protocols analyzed	**Sufficient**
mAb clone 1A6	17	8 (47%)
mAb clone 16	51	40 (78%)
mAb clone PgR 636	129	104 (81%)
rmAb 1E3	57	52 (91%)
rmAb SP2	16	7 (44%)

From the table 2 it is clear that the 3 most widely used clones also show the most successful performance in the three runs, which statistically of course can be related to the difference in the number of protocols analyzed for each Ab and does not implicit show the exact performance of each of the Abs, when the protocol parameters

have been optimized for each individual Ab. However, the data is in line with the results observed by UK NEQAS (Ibrahim M et al. Am J Clin Pathol 2008;129:398-409), where the pass rates for the clones PgR 636, 1A6 and SP2 were 85%, 40 % and 20%, respectively.

As described previously the uterine cervix seems to be an appropriate control for the evaluation of the sensitivity of the PR staining. In an optimal protocol almost all the columnar epithelial cells, the basal squamous epithelial cells and the stromal cells must show a strong and distinct nuclear staining with only a minimal cytoplasmic reaction. However, differences are seen depending on the Ab selected. When using the mAb clone 1A6, the basal squamous epithelial cells are negative and a cytoplasmic reaction is seen in the intermediate and superficial squamous epithelial cells, while the clone PgR 636 gives an intense cytoplasmic reaction in the columnar epithelial cells.

Conclusion
The mAb clones **PgR 636**, **16** and the rmAb clone **1E3** are all well performing and robust Abs for PR. HIER is mandatory. The concentration of the Ab must be carefully calibrated on an appropriate control such as the uterine cervix.

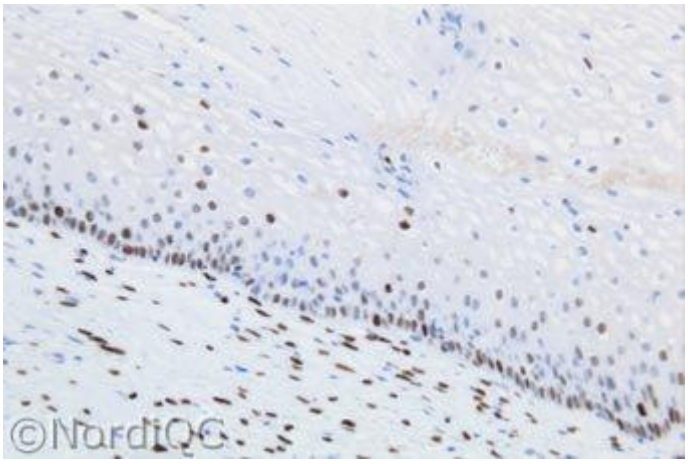


Fig. 1a
Optimal staining for PR of the cervix using the rmAb clone 1E2. The stromal cells show a strong nuclear staining and the basal squamous epithelial cells show a moderate staining.

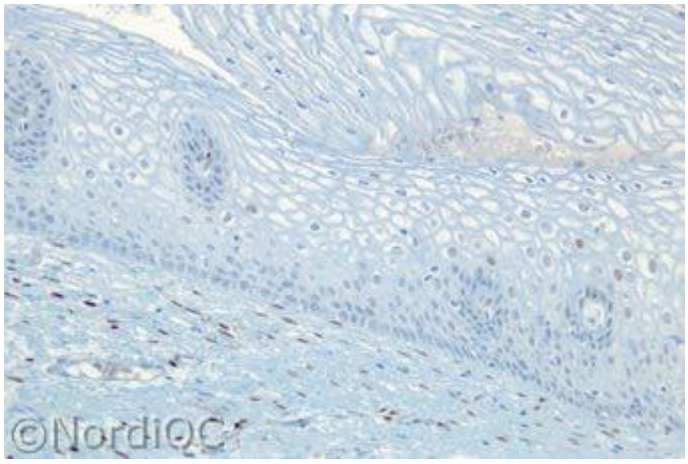


Fig. 1b
Insufficient staining for PR of the cervix, same field as in Fig. 1a using the rmAb clone SP2 with protocol settings giving a too low sensitivity. The stromal cells show a weak to moderate nuclear staining, but the basal squamous epithelial cells are virtually negative. Compare with Fig. 2b – same protocol.

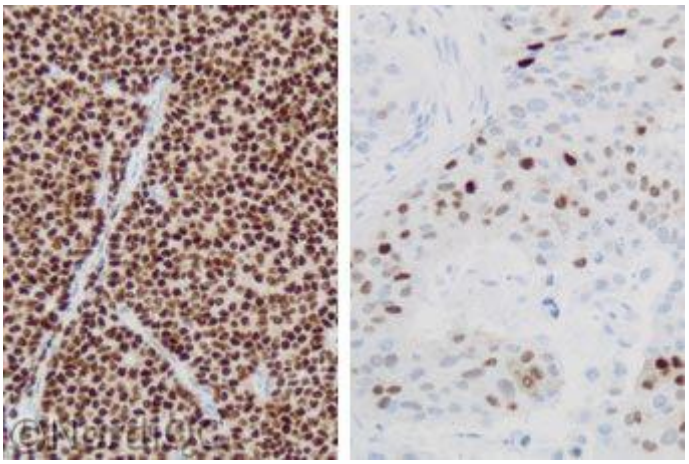


Fig. 2a
Optimal staining for PR using same protocol as in Fig. 1a.
Left: Ductal breast carcinoma no. 5 in which almost 100% of the neoplastic cells show a strong nuclear reaction.
Right: Ductal breast carcinoma no. 3 in which approximately 50% of the neoplastic cells show a moderate to strong staining.

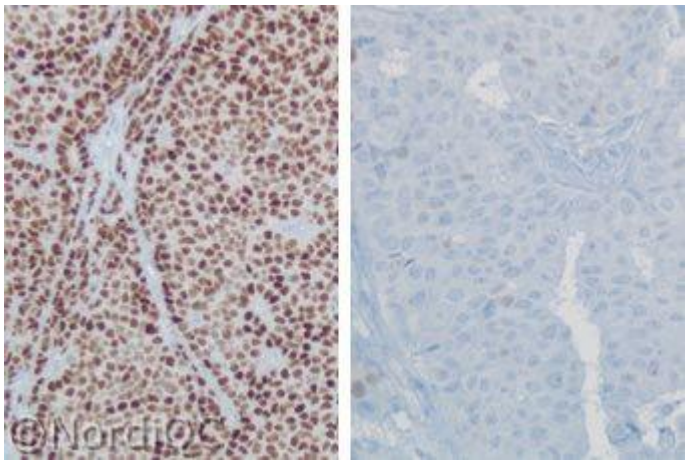


Fig. 2b
Staining for PR using same protocol as in Fig. 1b.
Left: Ductal breast carcinoma no. 5 in which almost 100% of the neoplastic cells show a moderate to strong nuclear reaction.
Right: Insufficient staining for PR of the ductal breast carcinoma no. 3. The proportion and intensity of the positive cells is significantly reduced compared to Fig. 2a left.

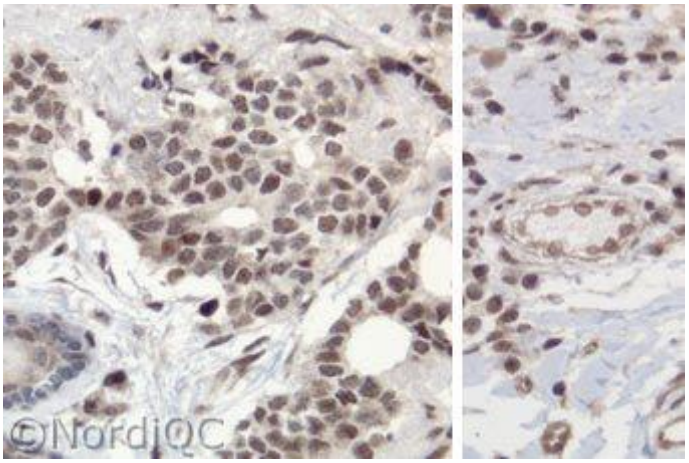


Fig. 3a
Insufficient staining for PR of the PR negative ductal breast carcinoma no. 1 using the mAb clone 1A6 too concentrated. The majority of the neoplastic cells show a false positive nuclear reaction.
Insert: Also note the false positive staining in e.g. lymphocytes, endothelial and muscle cells.

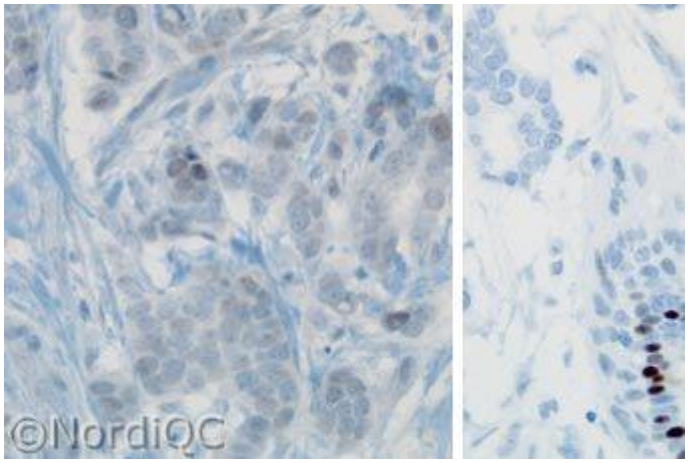


Fig. 3b
Insufficient staining for PR of the PR negative ductal breast carcinoma no. 1 using the rmAb 1E2. A weak but positive staining is seen in > 10% of the neoplastic cells.
Insert: Optimal staining of the same tumour using the mAb clone PgR 636. Only the normal epithelial cells show a positive staining.

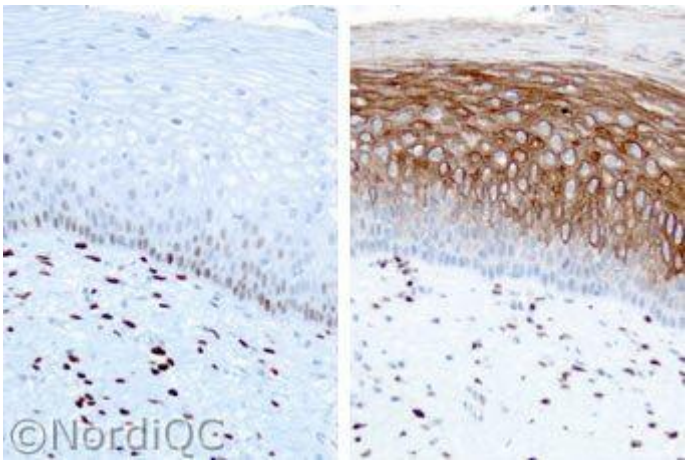


Fig. 4a
Staining pattern for PR in the cervix.
Left: Using the clones 1E2, 16, 16+SAN 27, PgR 636 & 1294 all gave a distinct nuclear staining in the stromal cells and the basal squamous epithelial cells.
Right: Using the clone 1A6, the basal squamous epithelial cells were negative, while a cytoplasmic staining was seen in the intermediate squamous epithelial cells.

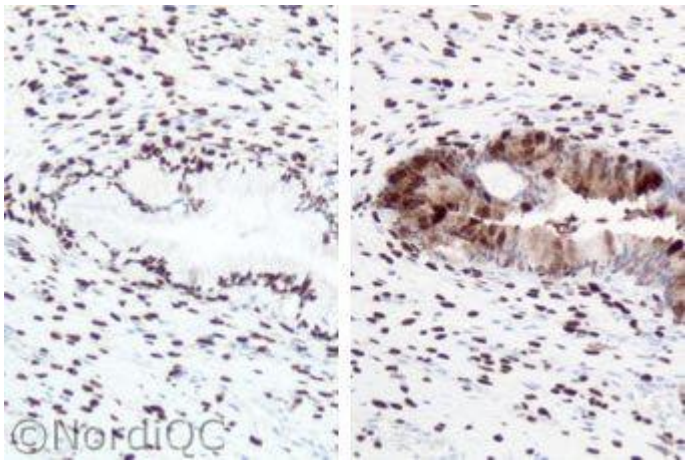


Fig. 4b
Staining pattern for PR in the cervix.
Left: Using the clones 1A6, 1E2, 16, 16+SAN 27 & 1294 all gave a distinct nuclear staining in the columnar epithelial cells and the stromal cells.
Right: Using the clone PgR 636 also a moderate to strong cytoplasmic staining was seen in the columnar epithelial cells.

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