

Purpose

Evaluation of the technical performance and level of analytical sensitivity and specificity of IHC tests performed by the NordiQC participants for demonstration of Progesterone receptor (PR) expression in breast carcinomas. IHC, based on the mAb clones 16 and PgR 1294, performed in a NordiQC reference laboratory served as reference standard methods and were used to identify breast carcinomas with the dynamic, diagnostic and critical relevant expression levels of PR. The obtained score in NordiQC is indicative of the performance of the IHC tests, but due to the limited number and composition of samples internal validation and extended quality control (e.g. regularly measurement of PR results) is needed.

Material

The slide to be stained for PR comprised the following tissues#:

No.	Tissue	PR-positivity*	PR-intensity*
1.	Tonsil	0%	Negative
2.	Uterine cervix	80-90%	Moderate to strong
3.	Breast carcinoma	0%	Negative
4.	Breast carcinoma	30-70%**	Weak to strong
5.	Breast carcinoma	90-100%**	Moderate to strong



* PR-status and staining pattern as characterized by NordiQC reference laboratories using the mAb clones 16 and PgR 1294.

** PR expression heterogenous.

Same donor materials as in run B31

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

Criteria for assessing a PR IHC result as **optimal** included:

- A moderate to strong, distinct nuclear staining reaction of most columnar epithelial and stromal cells (with the exception of endothelial cells and lymphoid cells) and an at least weak but distinct nuclear staining reaction in most basal squamous epithelial cells in the uterine cervix.
- An at least weak to moderate distinct nuclear staining reaction in the appropriate proportion of the neoplastic cells in the breast carcinomas no. 4 and 5.
- No nuclear staining reaction in the neoplastic cells in the breast carcinoma no. 3 and no more than a weak cytoplasmic staining reaction in cells with a strong nuclear staining reaction.
- No staining reaction in squamous epithelial cells or germinal centre B-cells in the tonsil.

A PR IHC result was classified as **good** if $\geq 10\%$ of the neoplastic cells in the breast carcinomas no. 4 and 5 showed an at least weak nuclear staining reaction but significantly reduced proportion compared to the reference range.

An at least weak to moderate nuclear staining reaction in the majority of the stromal, columnar and basal squamous epithelial cells in the uterine cervix.

A PR IHC result was assessed as **borderline** if $\geq 1\%$ and $< 10\%$ of the neoplastic cells in one of the breast carcinomas no. 4 and 5 showed a nuclear staining reaction. A significantly reduced number of neoplastic cells demonstrated in combination with a negative staining reaction in the uterine cervix can also be marked as **borderline**.

A PR IHC result can also be assessed as **borderline**, if the signal-to-noise ratio was low, e.g., because of cytoplasmic reaction, excessive counterstaining, impaired morphology hampering the interpretation and/or a distinct nuclear staining reaction was seen in $\geq 10\%$ of germinal centre B-cells in the tonsil.

A PR IHC result was assessed as **poor** if a false negative staining ($< 1\%$) was seen in one of the breast carcinomas no. 4 and 5. or false positive staining ($\geq 1\%$) was seen in the breast carcinoma no. 3.

A PR IHC result can also be assessed as **poor** in case of extreme poor signal-to-noise ratio, impaired morphology etc hampering the interpretation.

Participation

Number of laboratories registered for PR, run B33	432
Number of laboratories returning slides	405 (94%)

At the date of assessment, 94% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

One laboratory performed PR on the HER2 slide and was thus excluded from the assessment.

Results

404 laboratories participated in this assessment. 91% achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 3).

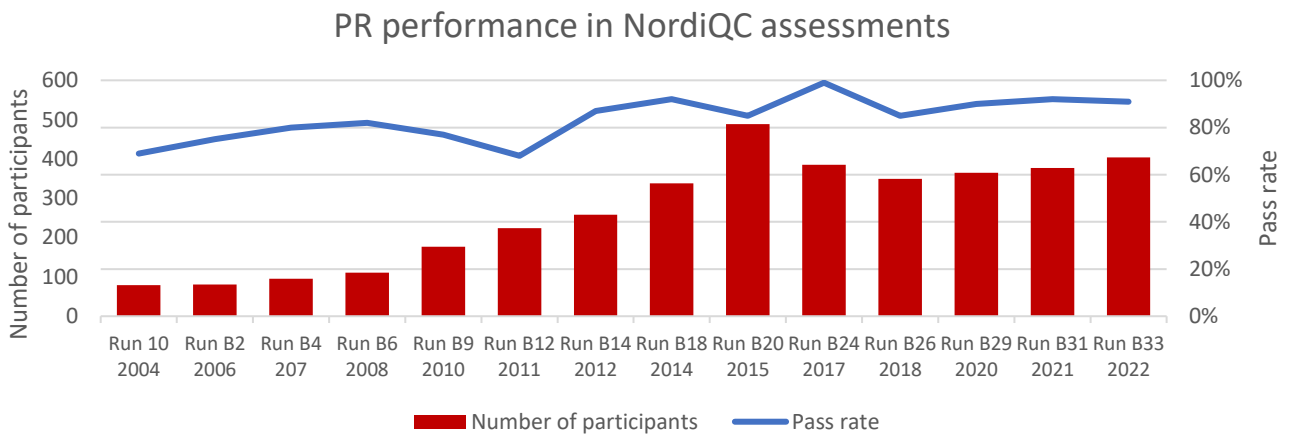
The most frequent causes of insufficient staining reactions were:

- Use of detection systems with low sensitivity
- Use of less successful primary Ab
- Inefficient HIER

Performance history

This was the 14th NordiQC assessment of PR. The pass rate was in concordance with previous assessments, except run B24 which was exceptionally high, as shown in Graph 1:

Graph 1. **Pass rate in the NordiQC assessments for PR**



Conclusion

The widely used mouse monoclonal antibodies (mAb) clones **16**, **PgR 636**, **PgR 1294** and the rabbit monoclonal Ab (rmAb) clone **1E2** could all be used to provide an optimal result for PR.

78% of the participants used Ready-To-Use (RTU) systems from Ventana/Roche, Dako/Agilent and Leica Biosystems and in total obtained a pass rate of 93% when applying these assays as "plug-and-play".

In this assessment, a false negative staining reaction was the predominant feature of the insufficient results, but false positive results were also observed.

Uterine cervix and tonsil in combination can be recommended as positive and negative tissue controls for PR. In uterine cervix, virtually all stromal cells and columnar epithelial cells must show a moderate to strong nuclear staining reaction. Most critically, the majority of basal squamous epithelial cells must show an at least weak but distinct nuclear staining reaction. Tonsil is an appropriate negative tissue control – no nuclear staining reaction should be seen.

Table 1. **Antibodies and assessment marks for PR, run B33**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	OR ²
mAb clone 16	37	Leica Biosystems						
	1	Monosan	27	10	2	0	95%	69%
	1	DCS						
mAb clone cocktail 16 + SAN27	3	Leica Biosystems	1	1	1	0	-	-
rmAb clone BP6081	1	Biolynx	0	0	1	0	-	-
mAb clone PgR 636	11	Dako/Agilent	5	6	0	0	100%	45%
mAb clone PgR 1294	15	Dako/Agilent	9	4	2	0	87%	60%
rmAb clone SP2	1	Diagnostic BioSystems						
	1	Thermo Fisher Scientific	2	0	0	0	-	-
rmAb clone SP42	3	Zytomed	0	0	3	0	-	-
rmAb clone YR85	1	Thermo Fisher Scientific	0	0	1	0	-	-
rmAb clone ZR290	1	Zeta Corporation	0	1	0	0	-	-
rmAb clone QR014	1	Quartett	1	0	0	0	-	-
Ready-To-Use antibodies								
Ab clone 16 PA0312 (VRPS³)	9	Leica Biosystems	9	0	0	0	100%	100%
mAb clone 16 PA0312 (LMPS⁴)	13	Leica Biosystems	7	5	1	0	92%	54%
mAb clone 16 MAD-000670QD	3	Master Diagnostica	0	1	0	2	-	-
mAb PgR 636 IR/IS068 (VRPS³)	8	Dako/Agilent	6	2	0	0	100%	75%
mAb PgR 636 IR/IS068 (LMPS⁴)	19	Dako/Agilent	16	3	0	0	100%	84%
mAb PgR 1294 GA090 (VRPS³)	41	Dako/Agilent	18	15	7	1	80%	44%
mAb PgR 1294 GA090 (LMPS⁴)	23	Dako/Agilent	17	5	1	0	96%	74%
rmAb clone 1E2 790-2223/4296 (VRPS³)	63	Ventana/Roche	38	24	1	0	98%	60%
rmAb clone 1E2 790-2223/4296 (LMPS⁴)	142	Ventana/Roche	87	41	13	1	90%	61%
mAb clone IHC751 IHC751	1	GenomeMe	1	0	0	0	-	-
rmAb clone MXR008 RMA-0895	2	Fuzhou Maixin	2	0	0	0	-	-
rmAb clone EP2 AN711-5M	1	BioGenex	0	0	1	0	-	-
rmAb clone Y85 8360-C010	2	Sakura Finetek	2	0	0	0	-	-
Total	404		248	118	34	4		
Proportion			61%	29%	8%	1%	91%	

1) Proportion of sufficient results (optimal or good) (≥ 5 assessed protocols).

2) Proportion of optimal results (≥ 5 assessed protocols).

3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s).

4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s) or other platforms.

Detailed analysis of PR, run B33

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

mAb clone **16**: Protocols with optimal results were based on Heat Induced Epitope Retrieval (HIER) using Target Retrieval Solution (TRS) High pH (3-in-1) (Dako/Agilent) (1/2)*, TRS pH 9 (Dako/Agilent) (4/4), Cell Conditioning 1 (CC1, Ventana/Roche) (6/15), Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (9/11), Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (6/6) or Citrate buffer (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:800, depending on the total sensitivity of the protocol employed.

Using these protocol settings, 37/39 (95%) laboratories produced a sufficient staining result (optimal or good).

* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **PgR 636**: Protocols with optimal results were based on HIER using TRS High pH (3-in-1) (Dako/Agilent) (3/7) TRS pH 9 (Dako/Agilent) (1/1) or Tris-EDTA buffer (1/2). The mAb was typically diluted in the range of 1:100-1:500, depending on the total sensitivity of the protocol employed.

Using these protocol settings, 10/10 (100%) laboratories produced a sufficient staining result.

mAb clone **PgR 1294**: Protocols with optimal results were based on HIER using CC1 (Ventana/Roche) (5/8) or TRS High pH (Dako/Agilent) (4/5) as retrieval buffer. The mAb was diluted in the range of 1:25-1:100, depending on the total sensitivity of the protocol employed.

Using these protocol settings, 10/11 (91%) laboratories produced a sufficient staining result.

Table 2. **Optimal results for PR using concentrated antibodies on the main IHC systems***

Concentrated antibodies	Dako/Agilent Autostainer		Dako/Agilent Omnis		Ventana/Roche BenchMark XT / Ultra / GX		Leica Biosystems 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 16	1/2**	-	4/4	-	6/15 (40%)	-	9/11 (82%)	6/6 (100%)
rmAb clone PgR 636	3/7 (43%)	-	1/1	-	-	-	0/1	-
mAb clone PgR1294	0/1	-	4/5 (80%)	-	5/8 (63%)	-	-	-

* 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 **16**, product no. **PA0312**, Leica Biosystems, Bond Max/Bond III:

Protocols with optimal results were typically based on HIER using BERS1 or BERS2 (efficient heating time 10-30 min. at 100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system.

Using these protocol settings, 20 of 20 (100%) laboratories produced a sufficient staining result (optimal or good).

2 laboratories used product no. PA0312 on other platform than Bond (Leica Biosystems). Data was not included in the description above.

mAb clone **PgR 636**, product no. **IS068/IR068**, Dako/Agilent, Autostainer+/Autostainer Link:

Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-30 min. at 95-99°C), 10-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems.

Using these protocol settings, 25/25 (100%) laboratories produced a sufficient staining result.

2 laboratories used product no. ISO068/IR068 on another platform than Autostainer+/Link (Dako/Agilent). Data was not included in the description above.

mAb clone **PgR 1294** product no. **GA090**, Dako/Agilent, Omnis:

Protocols with optimal results were typically based on HIER using TRS High pH (efficient heating time 20-30 min.), 10-30 min. incubation of the primary Ab and EnVision Flex/Flex+ (GV800/GV021) as detection system.

Using these protocol settings, 55/64 (86%) laboratories produced a sufficient staining result.

rmAb clone **1E2** product no. **790-2223/4296**, Ventana/Roche, BenchMark GX/XT/Ultra: Protocols with optimal result were typically based on HIER using CC1 (efficient heating time 24-64 min.), 8-64 min. incubation of the primary Ab and iView (760-091), UltraView (760-500) or OptiView (760-700) as detection system.

Using these protocol settings, 190/205 (93%) laboratories produced a sufficient staining result.

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 protocol settings (LPMS) changing basal protocol settings. Only protocols performed on the intended 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
Leica BOND MAX/ BOND III mAb 16 PA0312	9/9 (100%)	9/9 (100%)	11/11 (100%)	7/11 (64%)
Dako Autostainer+/ Autostainer Link mAb PgR 636 IS068/IR068	8/8 (100%)	6/8 (75%)	17/17 (100%)	15/17 (88%)
Dako Omnis mAb PgR 1294 GA090	33/41 (80%)	18/41 (44%)	22/23 (96%)	17/23 (74%)
Ventana BenchMark GX/XT/Ultra rmAb 1E2 790-2223/790-4296	62/63 (98%)	38/63 (60%)	128/142 (90%)	87/142 (61%)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.

** Modifications included: retrieval method, retrieval duration, retrieval reagents, Ab incubation time and detection kit. Only protocols performed on the specified vendor IHC stainer are included.

Comments

In this NordiQC assessment B33 for PR, an overall pass rate of 91% was observed similar to most of the previous assessments except run B24. The features of insufficient staining results were either characterized by false negative/too weak staining reactions, false positive staining reactions or a general poor signal-to-noise ratio.

A false negative or too weak staining reaction was seen in 82% of the insufficient results (31 of 38).

Virtually all laboratories were able to demonstrate PR in the breast carcinoma no. 5 with a high PR expression level in 90-100% of the neoplastic cells, whereas the demonstration of PR in the breast carcinoma no. 4, in which at least a weak nuclear staining reaction of >30% of the neoplastic cells was expected, was more challenging and required a carefully calibrated protocol.

In 10% of the insufficient results (4 of 38), a false positive staining result was observed and characterized by a weak to moderate but distinct nuclear staining reaction of germinal centre B-cells and/or squamous epithelial cells in the tonsil and/or a diffuse nuclear staining reaction in $\geq 1\%$ of the neoplastic cells of the breast carcinoma no. 3, expected to be PR negative as defined by the NordiQC reference standard methods based on the mAb clones 16 and PgR 1294. In order to account for heterogeneity and monitor the target analyte (PR) expression levels in the individual tumour cores included in NordiQC TMA blocks, reference slides are always made throughout the blocks. Every 50th slide throughout the blocks were thus stained for PR by the two reference standard methods and used during the assessment meeting as reference points.

The remaining 8% (3 of 38) of the insufficient results were caused by a general poor signal-to-noise ratio or an excessive background staining compromising the interpretation.

Ready-To-Use (RTU) Abs were used by 81% (327 of 404) of the participants. 97% (316 of 327) of the laboratories used a complete RTU system including the pre-diluted primary Ab, specified ancillary reagents and IHC stainer platform from either Ventana/Roche, Dako/Agilent or Leica Biosystems (see Table 3). The proportion of participants using RTU systems seems consolidated as same level seen in the last two assessments.

The Ventana/Roche RTU system, based on the rmAb clone 1E2 (790-2223/4296) to be performed on the BenchMark platform, was in this assessment the most widely used assay being used by 51% (205 of 377) of the participants and it gave an overall pass rate of 93%. 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. In this assessment, the vendor recommended protocol settings, being used by 31% (63 of 205) of the laboratories, provided a superior

overall pass rate of 98% compared to laboratory modified protocol settings giving a reduced pass rate of 90%. The insufficient results for the Ventana RTU system based on mAb clone 1E2 were mainly characterized by a too weak or false negative staining reaction.

The Dako/Agilent RTU system GA090 for Omnis, based on mAb clone PgR 1294 was used by 16% of the participants (64 of 404) and gave an overall pass rate of 86%. The pass rate was 80%, when the protocol was based on the vendor recommended protocol settings and inferior to the pass rate of 96% obtained by modified protocols, as shown in Table 3. Also, the proportion of optimal results were higher for modified protocols compared to the recommended protocols giving a proportion of 74% and 44%, respectively. The "positive" protocol modification was typically related to the use of a more sensitive detection system EnVision FLEX+ instead of EnVision FLEX giving a pass rate of 100%, 76% optimal.

The Dako/Agilent RTU system IR068/IS068 for Autostainer, based on the mAb clone PgR 636, provided an overall pass rate of 100%. As shown in Table 3, 68% (17 of 25) of the laboratories modified the protocol settings obtaining a pass rate of 100%, 88% optimal. Only minor adjustments in the protocol settings were seen.

The Leica RTU system PA0312 for Bond, based on the mAb clone 16, provided an overall pass rate of 100% and 100% being optimal. As shown in Table 3, 55% (11 of 20) of the laboratories modified the protocol settings obtaining a pass rate of 100% and 64% being optimal. The reduced proportion of optimal results was typically characterized by excessive background reaction and associated with adjustment of HIER and/or primary Ab incubation time. Only minor adjustments in the protocol settings were seen.

Overall, the RTU systems from the above mentioned three main vendors being applied in full compliance with the recommended protocol settings gave a pass rate of 93% and 59% optimal. In general, it must be emphasized that modifications of vendor recommended protocol settings for RTU systems including migration of the RTU Abs to another platform than the intended, require a meticulous validation process by the end-users. As seen in this assessment, modifications can be successful but potentially also generate aberrant results and therefore must be carefully monitored.

In addition to the three established vendors mentioned above, new RTU systems are introduced. In this run for PR both the Sakura Finetek and Maixin, Fuzhou RTU systems were found successful, as shown in Table 1.

19% (77 of 404) of the participants used Abs as concentrated formats within laboratory developed (LD) assays. Similar to the data generated for the RTU systems, the Abs, mAb clones 16, PgR 636 and PgR 1294 were widely used and could all provide sufficient and optimal results on the main IHC platforms (Ventana/Roche, Dako/Agilent and Leica Biosystems), see Tables 1 and 2. Irrespective of the clone applied, a careful calibration of the primary Ab in combination with efficient HIER, preferable in an alkaline buffer, and use of a sensitive 3-layer detection system were found to be the core elements for an optimal performance.

For LD assays based on concentrated formats an overall pass rate of 87% was obtained, 58% optimal.

Controls

As observed in previous NordiQC assessments of PR, uterine cervix is an appropriate positive tissue control to monitor the level of analytical sensitivity for the PR assay: With an optimal protocol, virtually all columnar epithelial cells and stromal cells should show a moderate to strong nuclear staining reaction with only a minimal cytoplasmic reaction, whereas the majority of basal squamous epithelial cells must show an at least weak and distinct nuclear staining reaction. No staining must be seen in endothelial cells and lymphocytes. However, it must be taken into consideration that the PR expression level can be reduced in the uterine cervix of e.g. post-menopausal women and thus especially demonstration of PR in squamous epithelial cells hereby can be compromised. From in-house NordiQC data, the usage of uterine cervix as positive tissue control will require a screening of the samples with a validated PR IHC protocol for appropriate selection of a sample with the described expression pattern.

Tonsil is recommendable as negative tissue control, in which no nuclear staining should be seen.

1. Kimberly H. Allison, M. Elizabeth H. Hammond, Mitchell Dowsett, Shannon E. McKernin, Lisa A. Carey, Patrick L. Fitzgibbons, Daniel F. Hayes, Sunil R. Lakhani, Mariana Chavez-MacGregor, Jane Perlmutter, Charles M. Perou, Meredith M. Regan, David L. Rimm, W. Fraser Symmans, Emina E. Torlakovic, Leticia Varella, Giuseppe Viale, Tracey F. Weisberg, Lisa M. McShane, and Antonio C. Wolff. Estrogen and Progesterone Receptor Testing in Breast Cancer: American Society of Clinical Oncology/College of American Pathologists Guideline Update. Arch Pathol Lab Med. 2020 May;144(5):545-563

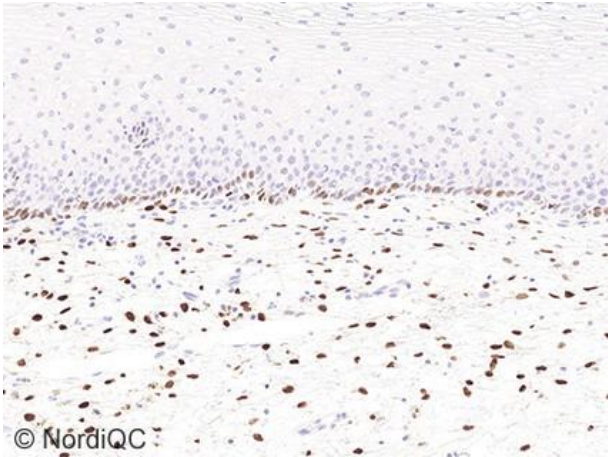


Fig. 1a
 Optimal staining result for PR of the uterine cervix using the Ventana/Roche RTU system based on the rmAb clone 1E2. The protocol was performed in compliance with the protocol settings recommend by Ventana using HIER in CC1 for 64 min., 16 min. incubation in primary Ab and UltraView as detection system and applied on BenchMark Ultra. The vast majority of basal squamous epithelial cells show a moderate nuclear staining reaction, whereas the stromal cells show a moderate to strong nuclear staining reaction.

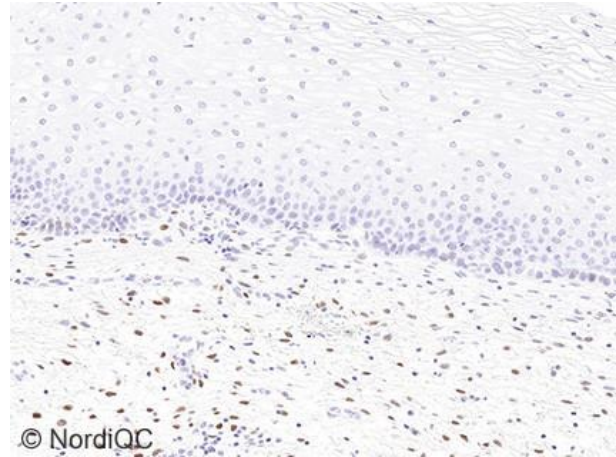


Fig. 1b
 Insufficient staining result for PR of the uterine cervix, using the Dako/Agilent RTU system for Omnis based on the rmAb clone PgR 1294. The protocol provided a too low analytical sensitivity primarily caused by use of a 2-step polymer based detection system. The majority of stromal cells are demonstrated whereas only cattered basal squamous epithelial cells show a weak nuclear staining reaction – same field as in Fig. 1a.

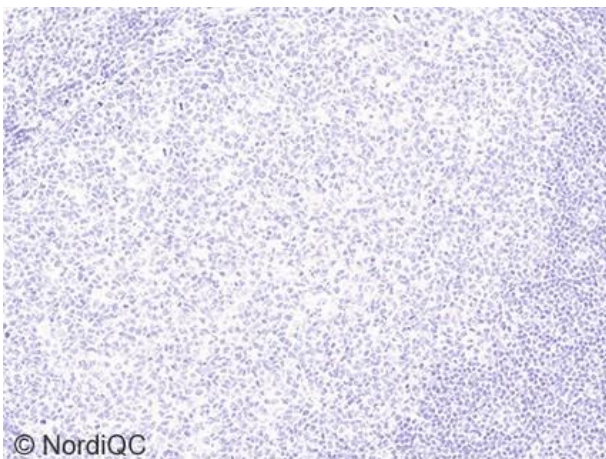


Fig. 2a
 Optimal staining result for PR of the tonsil using same protocol as in Fig. 1a. No nuclear staining reaction is seen.

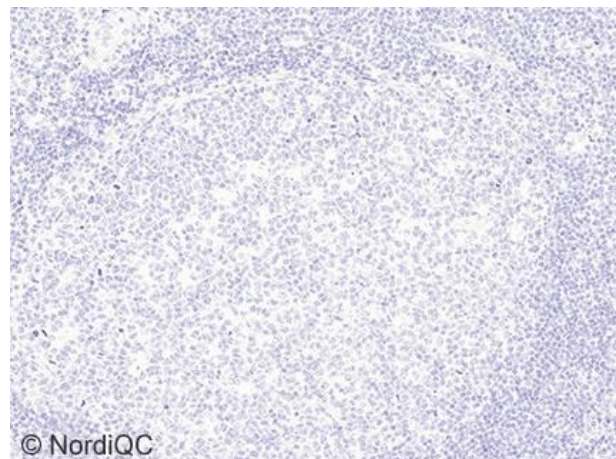


Fig. 2b
 Staining result for PR of the tonsil using same protocol as in Fig. 1b. No nuclear staining reaction is seen.

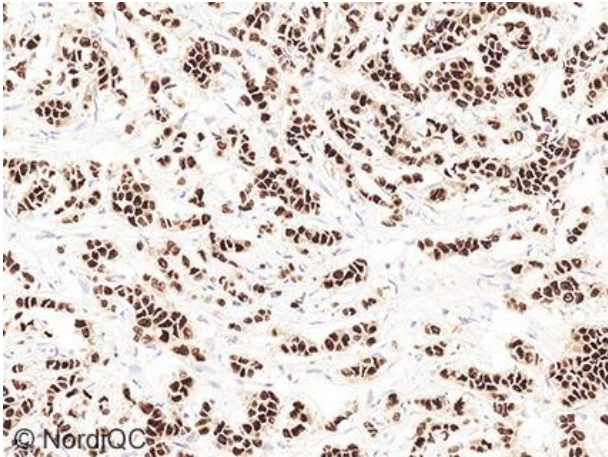


Fig. 3a
Optimal staining for PR of the breast carcinoma no. 5 with 90-100% cells positive using same protocol as in Figs. 1a-2a. Virtually all neoplastic cells show a strong nuclear staining reaction.

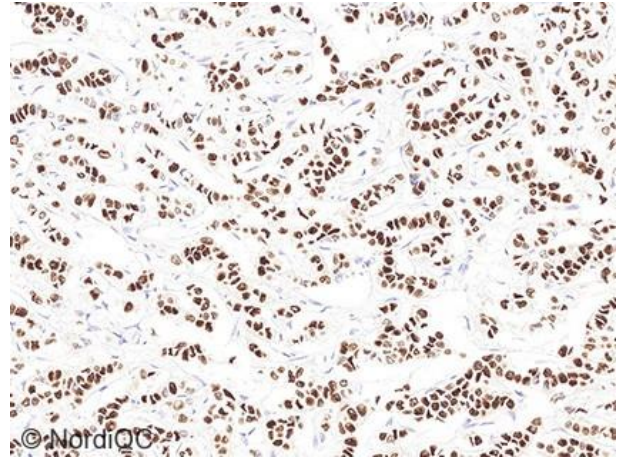


Fig. 3b
Staining for PR of the breast carcinoma no. 5 with 90-100% cells positive using same protocol as in Figs. 1b-2b – same field as in Fig. 3a. The expected proportion of cells being positive is demonstrated.

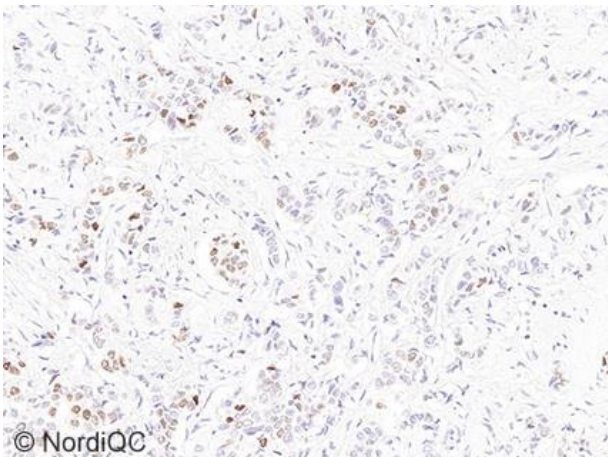


Fig. 4a
Optimal staining for PR of the breast carcinoma no. 4, with at least 30% of the neoplastic cells showing a weak but distinct nuclear staining reaction - using same protocol as in Figs. 1a-3a.

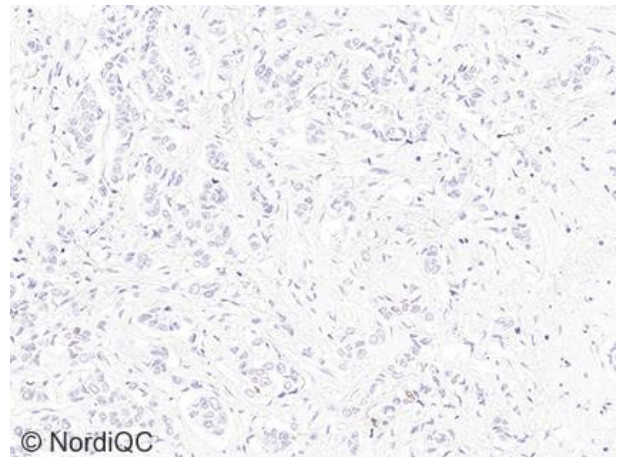


Fig. 4b
Insufficient staining for PR of the breast carcinoma no. 4 expected to be positive in minimum 30% of the neoplastic cells – same field as in Fig. 4a. <1% of the neoplastic cells are positive, giving a false negative result for PR. Same protocol as used in Figs. 1b-3b.

HLK/LE/SN 08.04.2022