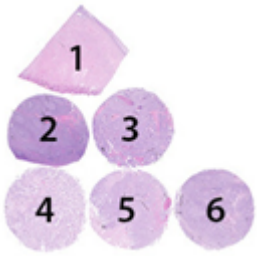
Assessment Run B29 2020

Progesterone receptor (PR)

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 PgR1294, 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.	Uterine cervix	80-90%	Moderate to strong
2.	Tonsil	0%	Negative
3.	Breast carcinoma	100%	Moderate to strong
4.	Breast carcinoma	30-80%**	Weak to strong
5.	Breast carcinoma	20-70%**	Weak to strong
6.	Breast carcinoma	0%***	Negative



*PR-status and staining pattern as characterized by NordiQC reference laboratories using the mAb clones 16 and PgR1294.
** PR expression heterogenous
*** Focal PR expression was observed in one of the TMAs used. Participant's slides were compared to nearest NordiQC reference slide.

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. 3, 4 and 5.
- No nuclear staining reaction in the neoplastic cells in the breast carcinoma no. 6 – In one of the TMAs used in the assessment, focal positivity was observed in the reference slides. In these instances, participant's results were compared to the nearest NordiQC reference slide.
- No nuclear staining reaction of any cells in the tonsil.

A PR IHC result was 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 reaction but less than the reference range.

A PR IHC result was classified as **borderline** if 1) ≥1% and <10% of the neoplastic cells showed a nuclear staining reaction in one or more of the breast carcinomas no. 3, 4 and 5. 2) If a distinct nuclear staining reaction was seen in ≥10% of germinal centre B-cells in the tonsil.

The staining reactions were classified as **poor** if a false negative (<1% positivity in the breast carcinomas no. 3-5) or a false positive staining reaction (≥1% in the breast carcinoma no. 6) was seen.

Participation

Number of laboratories registered for PR, run B29	377
Number of laboratories returning slides	365 (97%)

Results
365 laboratories participated in this assessment. 90% achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Less successful protocol modifications for the Ventana RTU system based on rmAb clone 1E2
- Too low concentration of the primary antibody
- Use of detection systems with low sensitivity
- Technical issues

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.
75% of the participants used Ready-To-Use (RTU) systems from Ventana, Dako and Leica and in total obtained a pass rate of 98% when applying these assays as "plug-and-play".
In this assessment, a false positive staining reaction was the predominant feature of the insufficient results, but false negative 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 B29**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **16**	34 1 1 1	Leica/Novocastra Biocare Monosan Thermo Fisher	19	12	6	-	84%	51%
mAb clone cocktail **16 + SAN27**	4	Leica/Novocastra	3	1	-	-	-	-
mAb clone **PgR 636**	17 1 1	Dako Agilent Biocare Enquire	11	6	-	2	89%	58%
mAb clone **PgR 1294**	9	Dako Agilent	5	3	-	1	89%	56%
mAb clone **C4D10**	1	Celnovte	1	-	-	-		
rmAb clone **SP2**	1	Diagnostic BioSystems	1	-	-	-	-	-
rmAb clone **SP42**	2 1	Zytomed Cell Marque	-	2	1	-	-	-
rmAb clone **Y85**	1	Cell Marque	1	-	-	-	-	-
rmAb clone **ZR4**	1	Zeta Corporation	-	1	-	-	-	-
Ready-To-Use antibodies							Suff.[1]	OR[2]
mAb clone **16 PA0312 (VRPS[3])**	8	Leica/Novocastra	7	1	-	-	100%	88%
mAb clone **16 PA0312 (LMPS[4])**	13	Leica/Novocastra	6	7	-	-	100%	46%
mAb clone **16 MAD-000670QD**	2	Master Diagnostica	-	2	-	-	-	-
mAb **PgR 636 IR/IS068 (VRPS[3])**	5	Dako Agilent	5	-	-	-	100%	100%
mAb **PgR 636 IR/IS068 (LMPS[4])**	25	Dako Agilent	13	10	2	-	92%	52%
mAb **PgR 1294 GA090 (VRPS[3])**	25	Dako Agilent	20	5	-	-	100%	80%
mAb **PgR 1294 GA090 (LMPS[4])**	16	Dako Agilent	11	5	-	-	100%	69%
rmAb clone **1E2 790-2223/4296 (VRPS[3])**	51	Ventana	25	24	2	-	96%	49%
rmAb clone **1E2 790-2223/4296 (LMPS[4])**	140	Ventana	64	54	19	3	84%	46%
rmAb clone **EP2 AN711-5M**	1	BioGenex	1	-	-	-	-	-
rmAb clone **SP2**	2	Maixin	-	2	-	-	-	-

Kit-0013								
rmAb clone **Y85 8360-C010**	1	Sakura Finetek	-	1	-	-	-	-
Total	365		193	136	30	6	-	
Proportion			53%	37%	8%	2%	90%	

1) Proportion of sufficient results (optimal or good) (≥5 asessed protocols).
2) Proportion of optimal results (≥5 asessed 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 B29
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) pH 9 (Dako) (5/7)*, Cell Conditioning 1 (CC1, Ventana) (6/13), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/6), Bond Epitope Retrieval Solution 1 (BERS1, Leica) (4/6), PT module 1 buffer pH 6 (Thermo Fisher) (1/1) or Tris-EDTA pH 9 (TE) (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:400, depending on the total sensitivity of the protocol employed.
Using these protocol settings, 29/32 (91%) 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 pH 9 (Dako) (7/9), BERS2 (Leica) (1/3), TRS pH 6.1 (Dako) (1/2), Diva Decloaker Solution pH6 (Biocare) (1/1) or Citrate pH 6 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:500, depending on the total sensitivity of the protocol employed.
Using these protocol settings, 15/15 (100%) laboratories produced a sufficient staining result.

mAb clone **PgR 1294**: Protocols with optimal results were based on HIER using CC1 (Ventana) (4/5) or TRS High pH (Dako) (1/3) as retrieval buffer. The mAb was diluted in the range of 1:50-1:525, depending on the total sensitivity of the protocol employed.
Using these protocol settings, 8/8 (100%) 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**		**Leica 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**	2/3	-	2/3	-	6/13 (46%)	-	1/6 (17%)	4/6 (67%)
rmAb clone **PgR 636**	7/8 (88%)	1/2	-	-	0/1	-	1/3	-
mAb clone **PgR1294**	0/1	-	1/2	-	4/5 (80%)	-	0/1	-

* 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, Bond Max, Bond III: Protocols with optimal results were typically based on HIER using BERS1 or BERS2 (efficient heating time 20-40 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, 17 of 17 (100%) laboratories produced a sufficient staining result (optimal or good).
2 laboratories used product no. PA0312 on other platforms than Bond (Leica). Data was not included in the description above.

mAb clone **PgR 636**, product no. **IS068/IR068**, Dako, 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), 15-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems.

Using these protocol settings, 20/20 (100%) laboratories produced a sufficient staining result.
6 laboratories used product no. ISO068/IR068 on other platforms than Autostainer+/Link (Dako). Data was not included in the description above.

mAb clone **PgR 1294** product no. **GA090**, Dako, Omnis: Protocols with optimal results were typically based on HIER using TRS High pH (efficient heating time 30 min.), 10-27 min. incubation of the primary Ab and EnVision Flex/Flex+ (GV800/GV021) as detection system.
Using these protocol settings, 38/38 (100%) laboratories produced a sufficient staining result.
One laboratory used product no. GA090 on another platform than Omnis (Dako). Data was not included in the description above.

rmAb clone **1E2** product no. **790-2223/4296,** Ventana, BenchMark GX, XT/Ultra:
Protocols with optimal result were typically based on HIER using CC1 (efficient heating time 32-64 min.), 12-32 min. incubation of the primary Ab and iView (760-091), UltraView (760-500) or OptiView (760-700) as detection system.
Using these protocol settings, 125/141 (89%) 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**	8/8 (100%)	7/8 (88%)	11/11 (100%)	5/11 (45%)
Dako Autotstainer+/ Autostainer Link mAb PgR 636 **IS068/IR068**	5/5 (100%)	5/5 (100%)	19/19 (100%)	10/19 (53%)
Dako Omnis mAb PgR 1294 **GA090**	25/25 (100%)	20/25 (80%)	15/15 (100%)	10/15 (67%)
Ventana BenchMark GX/XT/Ultra rmAb 1E2 **790-2223/790-4296**	49/51 (96%)	25/51 (49%)	118/140 (84%)	64/140 (46%)

* 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 B29 for PR, an overall pass rate of 90% was observed similar to most of the previous assessments except B24. The features of insufficient staining results were either characterized by false negative/too weak staining reaction, false positive staining reactions or a general poor signal-to-noise ratio.
A false negative or too weak staining reaction was seen in 28% of the insufficient results (10 of 36 laboratories). Virtually all laboratories were able to demonstrate PR in the breast carcinoma no. 3 with a high PR expression level in 100% of the neoplastic cells, whereas the demonstration of PR in the breast carcinomas no. 4 and 5, in which at least a weak nuclear staining reaction of >20-30% of the neoplastic cells was expected, was more challenging and required a carefully calibrated protocol.
In 56% of the insufficient results (20 of 36 laboratories), a false positive staining result was observed and mainly characterized by both weak to moderate but distinct nuclear staining reaction of germinal centre B-cells in the tonsil and a diffuse nuclear staining reaction in many neoplastic cells of the breast carcinoma no 6, expected to be PR negative as defined by the NordiQC reference standard methods based on the mAb clones 16 and PgR 1294. In this context it has to be specified, that the breast carcinoma no. 6 in one of the TMAs used for the assessment did show focal PR positivity by the NordiQC reference methods and this was taken into account during the evaluation and assessment meeting. A false positive result was thus only reported in case of an aberrant nuclear staining reaction in tonsil and/or positive reaction in the breast carcinoma no. 6 clearly defined as PR negative in the nearest reference slides.
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 17% of the insufficient results were caused by a general poor signal-to-noise ratio compromising the interpretation.

Ready-To-Use (RTU) Abs were used by 79% (289 of 365) of the participants. 95% (274 of 289) 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.
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 52% of the participants and it gave an overall pass rate of 87%. 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 27% of the laboratories provided a superior overall pass rate of 96% compared to laboratory modified protocol settings giving a pass rate of 84%. The insufficient results for the Ventana RTU system based on rmAb clone 1E2 were mainly characterized by false positive staining reactions similar to the observations in runs B20 and B26. No single protocol parameter causing this aberrant result was identified, but typically a combination of more modifications was found to be less successful. Typically, the protocols giving false positive results were based on a reduced HIER time (e.g. 20-32 min. in CC1) in combination with a prolonged incubation time of the primary antibody compared to the official recommendation given in the package insert.

The Dako/Agilent RTU system GA090 for Omnis, based on mAb clone PgR 1294 was used by 11% of the participants and gave an overall pass rate of 100%. The proportion of optimal results obtained by the vendor recommended protocol settings and by laboratory modified protocols were relatively similar as shown in Table 3 and both settings provided an overall high proportion of optimal results.

The Dako/Agilent RTU system IR068/IS084 for Autostainer, based on the mAb clone PgR 636, also provided an overall pass rate of 100%. As shown in Table 3, 79% (19 of 24) of the laboratories modified the protocol settings and for this group a reduced proportion of optimal results compared to laboratories using the RTU system accordingly to the vendor recommended protocol settings was seen. The "less" successful modifications observed were omission of FLEX+ linker and/or reduced HIER time.

The Leica RTU system PA0312 for Bond, based on the mAb clone 16, provided an overall pass rate of 100%. As shown in Table 3, 58% (11 of 19) of the laboratories modified the protocol settings and for this group a reduced proportion of optimal results was seen compared to laboratories using the RTU system accordingly to the vendor recommended protocol settings. The "less" successful modification observed was related to the use of a low pH buffer for HIER substituting BERS2 pH 9 as retrieval buffer.

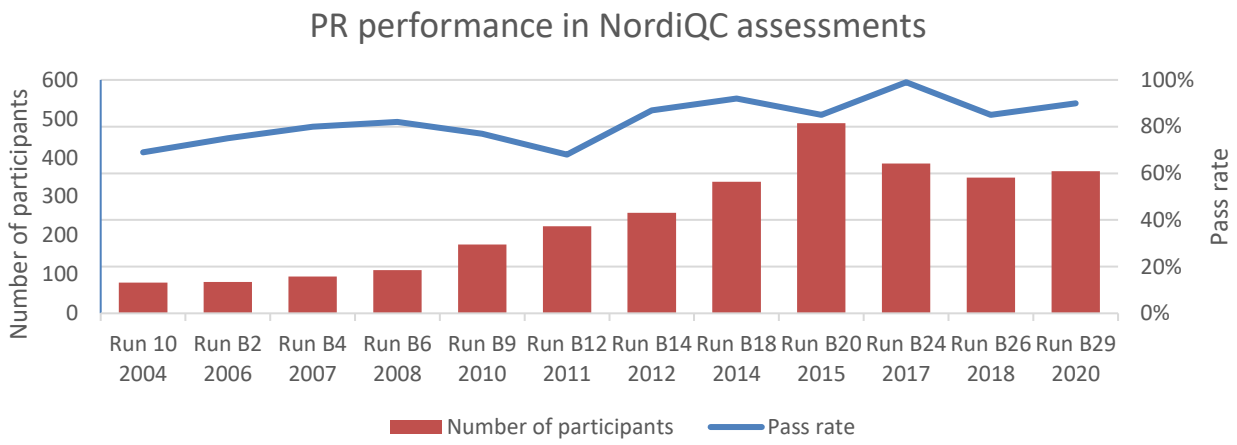
Overall, the RTU systems from the above mentioned three main vendors being applied in full compliance with the recommended protocol settings gave a pass rate of 98% and 64% optimal.
In general, it must be emphasized that modifications of vendor recommended protocol settings for the RTU systems including migration of the RTU Abs to another platform than the intended, require a meticulous validation process for the end-users. As seen in this assessment, modifications can be successful but potentially also generate aberrant results and therefore must be carefully monitored.

21% (76 of 335) 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), 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, 54% 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 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 is reduced in the uterine cervix of post-menopausal women and thus especially demonstration of PR in squamous epithelial cells can be compromised.
Tonsil is recommendable as negative tissue control, in which no nuclear staining should be seen.

Performance history
This was the twelfth 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**



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 Jan 13. doi: 10.5858/arpa.2019-0904-SA

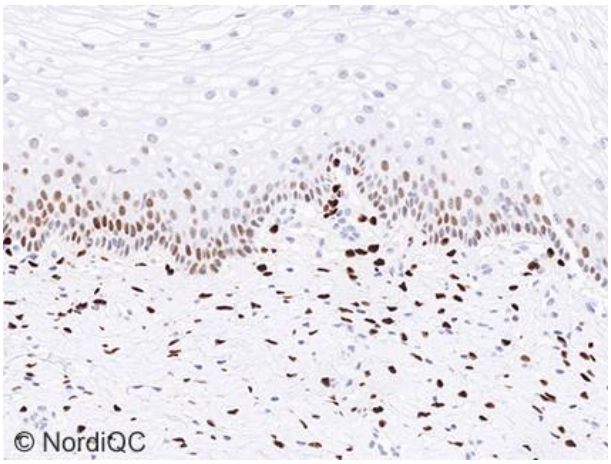


Fig. 1a
Optimal staining result for PR of the uterine cervix using the Ventana RTU system based on the rmAb clone 1E2. The protocol was performed in compliance to the protocol settings recommend by Ventana using HIER in CC1 for 64 min., 16 min. in primary Ab and UltraView as detection system and applied 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.

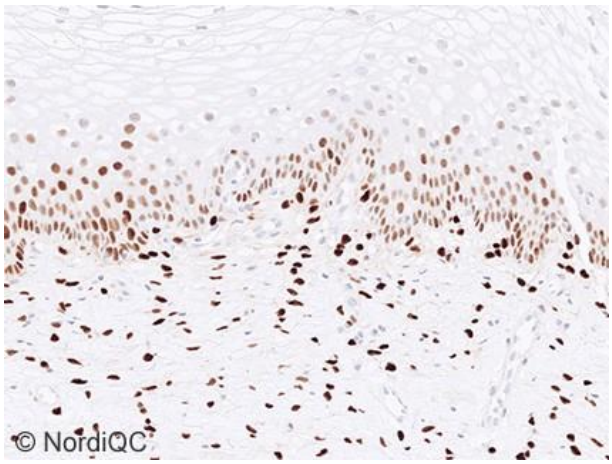


Fig. 1b
Staining result for PR of the uterine cervix, using the Ventana RTU system based on the rmAb clone 1E2 with laboratory modified protocol settings reducing the HIER time in CC1 to 32 min. and prolonging the incubation time in primary Ab to 36 min. UltraView was used as detection system and applied on BenchMark Ultra.
The result is as expected but also compare with Figs. 2b and 4b, same protocol.

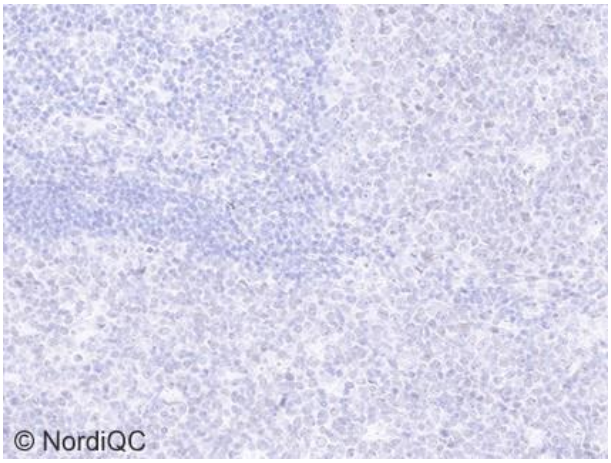


Fig. 2a
Optimal staining result for PR of the tonsil using same protocol as in Fig. 1a.
No nuclear staining reaction is seen.

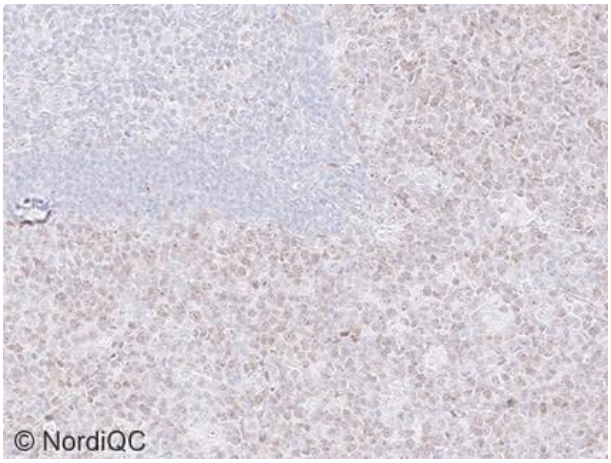


Fig. 2b
Insufficient staining result for PR of the tonsil – same field as in Fig. 4a.
The vast majority of germinal center B-cells show a weak to moderate and aberrant false positive nuclear staining reaction.
This aberrant staining reaction was only seen for the rmAb clone 1E2 with laboratory modified protocol settings as outlined above typically by reducing the efficient HIER time and at the same time prolonging the incubation time in primary Ab.

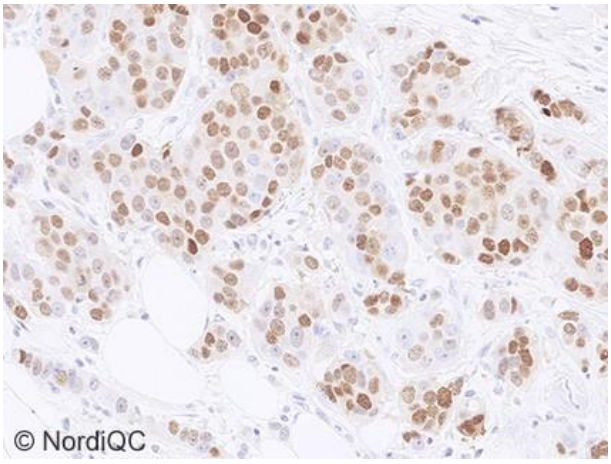


Fig. 3a
Optimal staining for PR of the breast carcinoma no. 5 with 30-80% cells positive using same protocol as in Figs. 1a-2a.
The PR positive cells are easily recognized, and the appropriate proportion of cells is demonstrated.

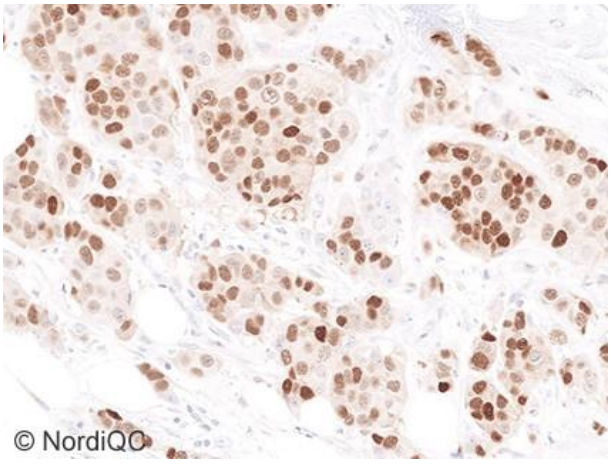


Fig. 3b
Staining for PR of the breast carcinoma no. 5 with 30-80% cells positive using same protocol as in Figs. 1b-2b – same field as in Fig. 3a.
The expected proportion of cells being positive is demonstrated – a slightly increased cytoplasmic staining is seen, but the result is fully sufficient. However, also compare with Fig. 4b, same protocol.

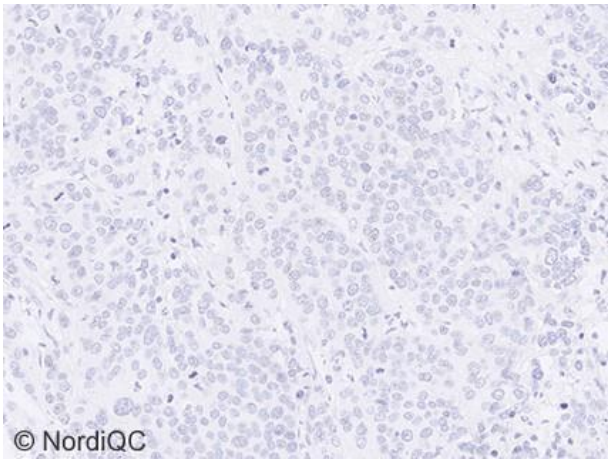


Fig. 4a
Optimal staining for PR of the breast carcinoma no. 6 expected to be negative using same protocol as in Figs. 1a-3a.
No nuclear staining reaction in the neoplastic cells is seen. The PR status was tested and confirmed by two different Abs and protocol settings in the NordiQC reference laboratory.

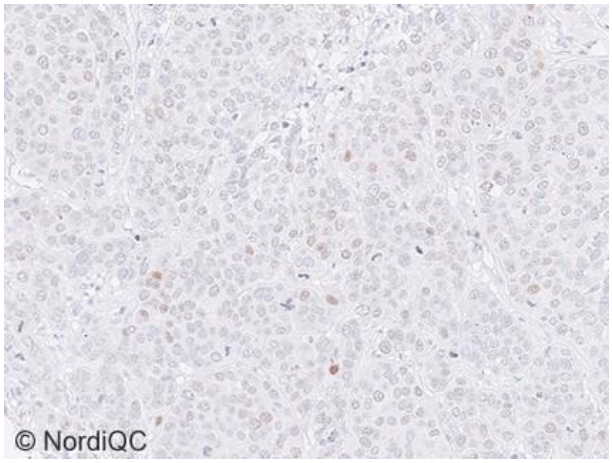


Fig. 4b
Insufficient staining for PR of the breast carcinoma no. 6 expected to be negative – same field as in Fig. 4a.
Most neoplastic cells show a weak and aberrant false positive nuclear staining reaction.
Same protocol as used in Figs. 1b-3b – of central importance also see Fig. 2b showing same aberrant and false positive result in the tonsil.

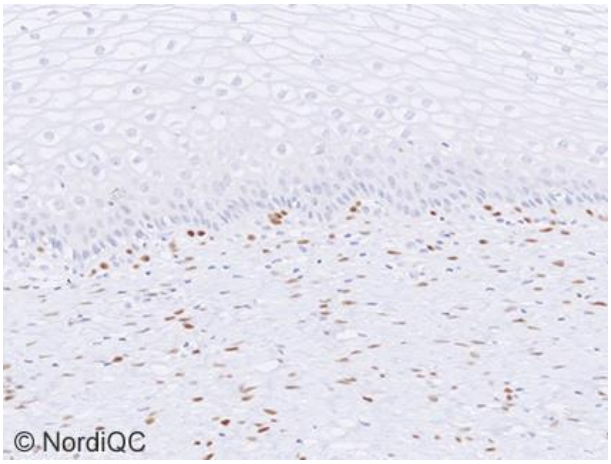


Fig. 5a
Insufficient staining for PR of the uterine cervix with a protocol giving a too low analytical sensitivity – same field as in Fig. 1a.
The stromal cells are demonstrated, but the basal squamous epithelial cells are virtually negative. Also compare with Figs. 5b left and right – same protocol.

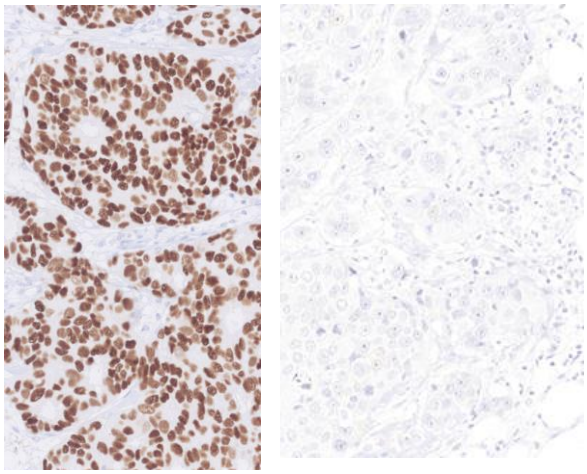


Fig. 5b
Left: Staining for PR of the breast carcinoma no. 3 with a result as expected. Virtually all neoplastic cells show a strong nuclear staining reaction.
Right: An insufficient and false negative staining of the breast carcinoma no 5.
The protocol provided too low analytical sensitivity only giving the expected result in tissues/cells with high level PR expression, but not calibrated to demonstrate PR in structures with reduced PR expression levels.

SN/LE/RR 06.04.2020