

## Assessment Run B31 2021 Estrogen receptor (ER)

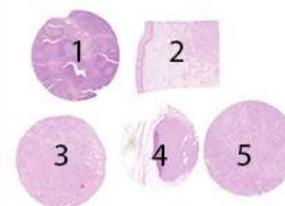
### Purpose

Evaluation of the technical performance and level of analytical sensitivity and specificity of IHC tests performed by the NordiQC participants for demonstration of estrogen receptor (ER) expression in breast carcinomas. IHC, based on the rmAb clones SP1 and EP1, 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 ER. 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 ER results) is needed.

### Material

The slide to be stained for ER comprised:

No.	Tissue	ER-positivity*	ER-intensity*
1.	Tonsil	1-5%	Weak to moderate
2.	Uterine cervix	80-90%	Moderate to strong
3.	Breast carcinoma	Negative	-
4.	Breast carcinoma	60-80%**	Weak to moderate
5.	Breast carcinoma	90-100%	Moderate to strong



\* ER-status and staining pattern as characterized by NordiQC reference laboratories using the rmAb clones EP1 and SP1.

\*\*ER expression slightly heterogenous.

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

Criteria for assessing an ER staining as **optimal** included:

- A moderate to strong, distinct nuclear staining of virtually all the columnar epithelial cells, most squamous epithelial and stromal cells (with the exception of endothelial cells and lymphoid cells) in the uterine cervix.
  - An at least weak to moderate nuclear staining reaction in scattered follicular dendritic cells/T-cells and squamous epithelial cells in the tonsil.
  - An at least weak to moderate distinct nuclear staining in the appropriate proportion of the neoplastic cells in the breast carcinomas no. 4 and 5.
  - No nuclear staining in the neoplastic cells in the breast carcinoma no. 3.
  - No more than a weak cytoplasmic reaction in cells with a strong nuclear staining reaction.
- An ER 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 less than the reference range. An at least weak to moderate nuclear staining reaction in the majority of the stromal, columnar and squamous epithelial cells in the uterine cervix and in the dispersed cells expected to be positive in the tonsil.

An IHC result was also assessed as **good**, if the signal-to-noise ratio was low, e.g., because of moderate cytoplasmic reaction, excessive counterstaining or impaired morphology.

- An ER 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 negative staining reaction of the cells expected to be demonstrated in tonsil/uterine cervix can also be marked as **borderline**.
- An 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.

An IHC result can also be assessed as **borderline/poor** related to technical artefacts, e.g. poor signal-to-noise ratio, excessive counterstaining, impaired morphology and/or excessive staining reaction hampering the interpretation.

### Participation

Number of laboratories registered for ER, B31	424
Number of laboratories returning slides	380 (90%)

The number of laboratories returning slides has decreased in this run B31 compared to previous assessments, due to the COVID-19 pandemic and associated postal delays. All slides returned after the assessment were assessed and received advice if the result being insufficient but the results were not included in this report.

Two laboratories were excluded from the assessment. One laboratory used PR on the ER slide and one laboratory experienced issues with the circulated NordiQC slides, providing a partial or entire aberrant/false negative staining result.

## Results

378 laboratories participated in this assessment. 321 of 378 (85%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks given (see page 3).

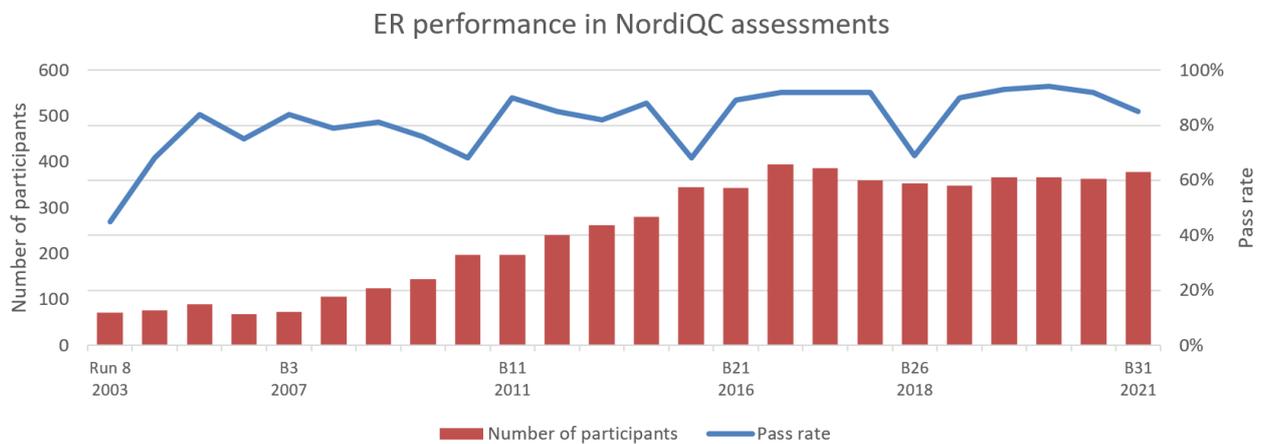
The most frequent causes of insufficient staining reactions were:

- Insufficient Heat Induced Epitope Retrieval (HIER) in an acidic buffer
- Use of detection systems with low sensitivity

## Performance history

This was the 24<sup>th</sup> NordiQC assessment of ER. The proportion of sufficient results has been relatively stable in the recent runs from 2016-2021 (except for run B26) at a high and satisfactory level (see Graph 1).

Graph 1. **Participant numbers and pass rates for ER during 24 NordiQC runs**



Fluctuations in pass rates, as seen in run B26 and at lesser degree this run B31, is likely caused by more challenging material circulated compared to other runs. In order to secure the consistency of the material circulated, NordiQC evaluates the material with two reference standard methods and monitor the ER expression levels throughout all TMAs used in the assessment. Fluctuation in pass rates can also be influenced by many new participants and in this run 10% (n=40) more participants were registered compared to run B30.

## Conclusion

The rabbit monoclonal antibodies (rmAb) clones **SP1** and **EP1** and the mouse monoclonal Ab (mAb) clone **6F11** could all be used to provide an optimal result for ER. 86% of the participants used Ready-To-Use (RTU) systems for the demonstration of ER. The RTU systems from Ventana and Dako used as "plug-and-play" assays provided a pass rate of 91%. In this assessment, low analytical sensitivity giving a too weak or false negative staining reaction was the predominant feature of insufficient results.

Uterine cervix and tonsil can be recommended as positive tissue controls for ER. In uterine cervix, virtually all squamous and columnar epithelial cells must show a moderate to strong and distinct nuclear staining reaction. Endothelial cells and lymphocytes must be negative in this tissue.

Tonsil was especially found recommendable as a tool to monitor the level of analytical sensitivity for the demonstration of ER. Dispersed follicular dendritic cells<sup>2</sup> in germinal centers and squamous epithelial cells must show an at least weak but distinct nuclear staining reaction. In addition, tonsil can be used as negative tissue control, as B-cells in mantle zones and within germinal centers must be negative.

Table 1. **Antibodies and assessment marks for ER, B31**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff. <sup>1</sup>	OR <sup>2</sup>
mAb clone <b>6F11</b>	14	Leica Biosystems	6	5	3	1	73%	40%
	1	Thermo Scientific						
rmAb clone <b>EP1</b>	12	Dako/Agilent	2	7	2	3	64%	14%
	1	Cell Marque						
	1	BioGenex						
rmAb clone <b>SP1</b>	10	Thermo Scientific	12	5	2	1	85%	60%
	7	Cell Marque						
	1	Zytomed Systems						
	1	Abcam						
	1	Diagnostic Biosystems						
rmAb clone <b>BP6026</b>	1	Bailing Biotechnology Co., Ltd	0	0	1	0	-	-
rmAb clone <b>IHC423</b>	1	GenomeMe	0	1	0	0	-	-
rmAb clone <b>ZR2</b>	1	Zeta Corporation	0	0	0	1	-	-
Ready-To-Use antibodies							Suff. <sup>1</sup>	OR <sup>2</sup>
mAb clone <b>6F11 PA0009/PA0151 (VRPS<sup>3</sup>)</b>	1	Leica Biosystems	0	0	0	1	-	-
mAb clone <b>6F11 PA0009/PA0151 (LMPS<sup>4</sup>)</b>	14	Leica Biosystems	5	4	4	1	64%	36%
mAb clone <b>6F11 PDM048-10MM</b>	1	Diagnostic BioSystems	0	0	1	0	-	-
rmAb <b>EP1 IR/IS084 (VRPS<sup>3</sup>)</b>	8	Dako/Agilent	2	3	0	3	63%	25%
rmAb <b>EP1 IR/IS084 (LMPS<sup>4</sup>)</b>	28	Dako/Agilent	9	14	3	2	82%	32%
rmAb <b>EP1 GA084 (VRPS<sup>3</sup>)</b>	27	Dako/Agilent	14	12	1	0	96%	52%
rmAb <b>EP1 GA084 (LMPS<sup>4</sup>)</b>	26	Dako/Agilent	16	7	3	0	88%	62%
rmAb <b>EP1 8361-C010</b>	4	Sakura Finetek	1	3	0	0	-	-
rmAb clone <b>SP1 790-4324/5 (VRPS<sup>3</sup>)</b>	44	Ventana/Roche	24	17	2	1	93%	55%
rmAb clone <b>SP1 790-4324/5 (LMPS<sup>4</sup>)</b>	160	Ventana/Roche	86	56	15	3	89%	54%
mAb clone <b>1D5 IR/IS657</b>	1	Dako/Agilent	0	1	0	0	-	-
rmAb clone <b>SP1 249R-17/18</b>	4	Cell Marque	2	1	1	0	-	-
rmAb clone <b>SP1 BRB053</b>	1	Zytomed Systems	0	0	1	0	-	-
rmAb clone <b>SP1 MAD-000306QD/V</b>	2	Master Diagnostica Vitro SA	0	1	0	1	-	-
rmAb clone <b>SP1 KIT-0012</b>	2	Maixin	2	0	0	0	-	-
rmAb clone <b>SP1 M3011</b>	1	Spring Biosystems	1	0	0	0	-	-
rmAb+mAb clones <b>SP1+6F11 PM308</b>	1	Biocare Medical	0	1	0	0	-	-
rmAb clone <b>SP1 RMPD001</b>	1	Diagnostic BioSystems	1	0	0	0	-	-
Total	378		183	138	39	18		
Proportion			48%	37%	10%	5%	85%	

1) Proportion of sufficient results (optimal or good) ( $\geq 5$  assessed protocols).

2) Proportion of optimal results ( $\geq 5$  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 ER, B31

The following protocol parameters were central to obtain optimal staining:

### Concentrated antibodies

mAb clone **6F11**: Protocols with optimal results were based on HIER using Cell Conditioning 1 (CC1, Ventana/Roche) (3/4)\*, Bond Epitope Retrieval Solution 2 (BERS2, Leica) (2/5) or Target Retrieval Solution (TRS) pH 9 (Dako/Agilent) (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:200 and combined with a 3-layer detection system. Using these protocol settings, 10 of 10 (100%) laboratories produced a sufficient staining result (optimal or good).

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

rmAb clone **EP1**: Protocols with optimal results were based on HIER using TRS pH 9 (3-in-1) (Dako/Agilent) (2/7) as retrieval buffer. The rmAb was typically diluted in the range of 1:60-1:100 and combined with either a 2- or 3-layer detection system. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

rmAb clone **SP1**: Protocols with optimal results were typically based on HIER using CC1 (Ventana/Roche) (8/11), TRS pH 9 (Dako/Agilent) (2/2) or Tris-EDTA pH 9 (2/2) as retrieval buffer. The rmAb was typically diluted in the range of 1:25-1:300 and combined with either a 2- or 3-layer detection system. Using these protocol settings, 14 of 16 (88%) laboratories produced a sufficient staining result.

Table 2 summarizes the overall proportion of optimal staining results when using the three most frequently used concentrated Abs on the most commonly used IHC staining platforms.

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

Concentrated antibodies	Dako/Agilent Autostainer		Dako/Agilent Omnis		Ventana BenchMark XT/Ultra/GX		Leica Bond III / Max	
	TRS High pH 9.0	TRS Low pH 6.1	TRS High pH 9.0	TRS Low pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone <b>6F11</b>	-	-	1/1**	-	3/4	-	2/5 (20%)	-
rmAb clone <b>EP1</b>	2/3	-	-	-	-	-	-	-
rmAb clone <b>SP1</b>	-	-	2/2	-	8/11 (73%)	-	-	-

\* 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 **6F11**, product no. **PA0009/PA0151**, Leica Biosystems, Bond III/Bond Max:

Protocols with optimal results were based on HIER using BERS2 for 20-30 min., 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings, 6 of 6 (100%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP1**, product no. **IR084/IS084**, Dako/Agilent, Dako Autostainer+/Autostainer Link:

Protocols with optimal results were based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-30 min. at 97-98°C), 20-40 min. incubation of the primary Ab and EnVision FLEX (K8000/K8002) or EnVision FLEX+ with rabbit linker (K8009/K8019) as detection system. Using these protocol settings, 18 of 24 (75%) laboratories produced a sufficient staining result.

10 laboratories used product no IR084/IS084 on other platforms. These were not included in the description above.

mAb clone **EP1**, product no. **GA084**, Dako/Agilent, Dako Omnis:

Protocols with optimal results were typically based on HIER using TRS High pH (efficient heating time 20-30 min. at 97°C), 10-30 min. incubation of the primary Ab and EnVision FLEX (GV800) or EnVision FLEX+ with rabbit linker (GV800+GV809) as detection system. Using these protocol settings, 48 of 50 (96%) laboratories produced a sufficient staining result.

3 laboratories used product no. GA084 on other platforms. These were not included in the description above.

rmAb clone **SP1**, product no. **790-4324/4325**, Ventana/Roche, BenchMark GX, XT, ULTRA:

Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 16-76 min.), 8-44 min. incubation of the primary Ab and UltraView (760-500) with or without UltraView Amplification kit (760-080), iView (760-091) or OptiView (760-700) as detection system. Using these protocol settings, 175 of 194 (90%) laboratories produced a sufficient staining result.

2 laboratories used product no 790-4324/4325 on other platforms. These were not included in the description above.

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 systems changing basal protocol settings. Only protocol assays performed on the specific 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
Dako AS48 rmAb EP1 <b>IR084/IS084</b>	5/8 (63%)	2/8 (25%)	15/18 (83%)	8/18 (44%)
Dako Omnis rmAb EP1 <b>GA084</b>	26/27 (96%)	14/27 (52%)	22/23 (96%)	15/23 (65%)
Leica Bond mAb 6F11 <b>PA009/PA0151</b>	0/1	0/1	9/13 (69%)	5/13 (38%)
VMS Ultra/XT/GX rmAb SP1 <b>790-4324/4325</b>	41/44 (93%)	24/44 (55%)	140/158 (89%)	84/158 (53%)

\* 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

The assessment criteria were in the latest run B30 and this run B31, and in concordance to the ASCO/CAP 2020 recommendation on ER IHC testing, increasingly anchored on the focus on the tissue controls, tonsil and uterine cervix.

According to both previous NordiQC results and the recommendations from ASCO/CAP 2020, especially tonsil is recommended to ensure an appropriate low limit of demonstration of ER. If both breast carcinomas (tissue cores no. 4 and 5) showed the expected staining reaction both regarding intensity and proportion of cells demonstrated, but the tissue controls were too weak or a reduced proportion of positive cells were seen in these controls, the overall result was assessed as “good” or “borderline” depending on the overall performance.

In this assessment and as seen in previous NordiQC runs for ER, the most common feature of an insufficient staining result was inadequate protocol sensitivity, resulting in a weak or false negative staining reaction, with reduced detection of the ER epitope. This pattern was seen in 91% of the insufficient results (52 of 57). In 5% (3 of 57) a false positive nuclear staining reaction was observed in lymphocytes in tonsil. In the remaining 4% (2 of 57), poor signal-to-noise ratio was seen.

Virtually all laboratories were able to demonstrate ER in the high-level ER-expressing breast carcinoma (tissue core no. 5), in which 90-100% of the neoplastic cells were expected to be demonstrated and by the NordiQC reference standard methods, the cells showed a moderate to strong intensity. Demonstration of ER in the heterogeneous mid-level ER-expressing breast carcinoma (tissue core no. 4), in which an at least weak nuclear staining reaction of 60-80% of the neoplastic cells was expected, was much more challenging.

**Ready-To-Use (RTU) Abs** were used by 86% (326 of 378) of the participants. 87% (283 of 326) of these laboratories obtained a sufficient staining result, 50% optimal (163 of 326).

**The Ventana/Roche RTU system, 790-4324/4325 for BenchMark based on the rmAb clone SP1** was in this assessment the most widely applied assay being used by 53% of the participants and gave an overall pass rate of 90%. 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, vendor recommended protocol settings were used by only 22% of the laboratories. Use of OptiView detection was observed to be a successful protocol modification, resulting in an optimal staining result for 90% (26 of 29) of users, compared to 55% for the manufacturer’s protocol (see Table 3). Protocols based on OptiView detection gave a pass rate of 100% (29 of 29 users).

**The Dako/Agilent RTU system GA084 for Omnis, based on rmAb clone EP1** was used by 13% of the participants and gave an overall pass rate of 96%. The proportion of sufficient and optimal results obtained by the vendor recommended protocol settings was slightly decreased compared to laboratory

modified protocols as shown in Table 3. The modified protocols either increased incubation time of the primary Ab and/or added a rabbit linker for the detection system.

**The Dako/Agilent RTU system IR084/IS084 for Autostainer, also based on the rmAb EP1** was used by 7% of the participants and provided an overall pass rate of 77%. As shown in Table 3, 70% (18 of 26) of the laboratories modified the protocol settings and obtained a relatively significant higher pass rate and increased proportion of optimal results compared to laboratories using the RTU system according to the Dako recommended protocol settings. The most common and successful modification observed was use of FLEX+ with rabbit linker as detection system. A modification including rabbit linker was used by 16 laboratories: 81% obtained a sufficient mark and 50% being optimal staining results.

**The Leica RTU system PA009/PA0151 for BOND based on mAb 6F11**, was used by 4% of the laboratories and gave an overall pass rate of 64%. In this assessment, vendor recommended protocol settings based on HIER in BERS1 for 20 min., 15 min. incubation of the primary Ab and Bond Refine as detection system was used by one participant, with an insufficient result. Laboratories using a protocol modification enhancing analytical sensitivity by using HIER in BERS2 obtained a pass rate of 88% (7 of 8), 63% optimal. However high pH retrieval should be used with caution with this clone due to the concerning number of false positive staining reactions noted by this modification in run B28. Protocol modifications increasing incubation time of the primary antibody alone did not yield any optimal results and resulted in an overall weak staining.

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 and previous assessments, modifications can be very successful but may also generate sub-optimal or aberrant results and therefore must be carefully monitored.

**Concentrated antibody formats** with laboratory-developed (LD) assays were applied by 14% (52 of 378) of the participants. The three Abs, mAb clone 6F11 and rmAb clones EP1 and SP1 used in a LD assay all could provide sufficient and optimal results on the main IHC platforms (Dako/Agilent, Leica Biosystems and Ventana/Roche), see Tables 1 and 2. Irrespective of the clone applied, careful calibration of the primary Ab concentration in combination with efficient HIER, preferably in an alkaline buffer (except for mAb clone 6F11 as discussed in run B28 and B15), and use of a sensitive 3-layer detection system were found to be the common core elements for an optimal performance. 75% (39 of 52) of the laboratories used a 3-layer detection system and obtained a pass rate of 80%, 44% optimal, compared to 2-layer detection system with a significant lower pass rate at 54%, 23% optimal.

### Controls

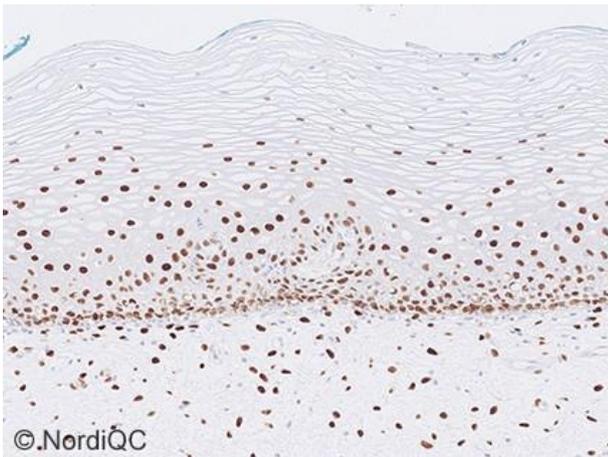
In concordance with previous NordiQC runs, uterine cervix and tonsil was found to be valuable positive and negative tissue controls for ER staining: In the uterine cervix, optimal results were characterized by virtually all epithelial cells throughout the squamous epithelium and in the glands showing 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.

Use of tonsil as a control tissue is especially recommended as a tool to monitor the analytical sensitivity for the IHC demonstration of ER and was in fact superior to uterine cervix. It was observed, that dispersed cells (most likely follicular dendritic cells<sup>2</sup>) in germinal centers and squamous epithelial cells were distinctively demonstrated in virtually all protocols providing an optimal result. If the follicular dendritic cells were negative, a reduced proportion of ER positive cells were seen in the other tissues and most critically a too weak or even false negative staining was seen in breast carcinomas no. 3 and 4. In addition, tonsil can be used as supplementary negative tissue control, as B-cells in mantle zones and within germinal centers must be negative.

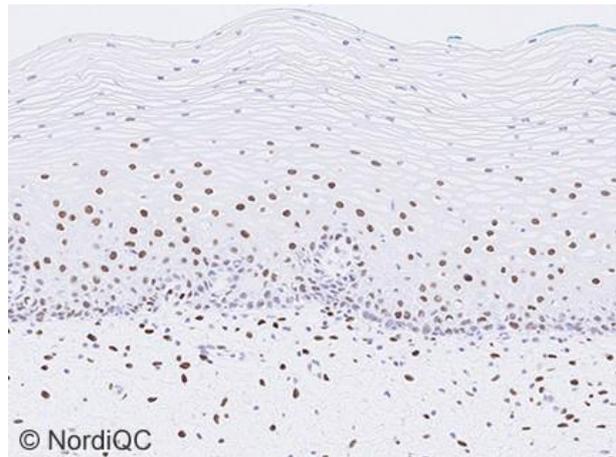
To validate the specificity of the IHC protocol further, an ER negative breast carcinoma must be included as primary negative tissue control, in which only remnants of normal epithelial and stromal cells should be ER positive, serving as internal positive tissue control. Positive staining reaction of the stromal cells in breast tissue indicates that the IHC protocol provides a high analytical sensitivity for ER, whereas the analytical sensitivity cannot reliably be evaluated in normal epithelial cells in breast as they typically express moderate to high levels of ER.

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 Varela, 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

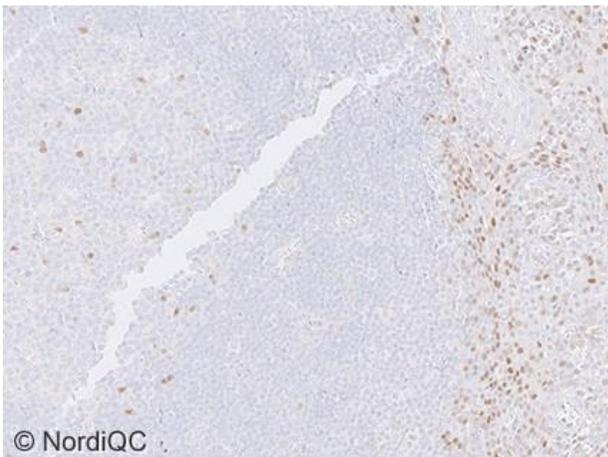
2. Sapino A, Cassoni P, Ferrero E, Bongiovanni M, Righi L, Fortunati N, Crafa P, Chiarle R, Bussolati G. Estrogen receptor alpha is a novel marker expressed by follicular dendritic cells in lymph nodes and tumor-associated lymphoid infiltrates. *Am J Pathol.* 2003 Oct;163(4):1313-20. PubMed PMID: 14507640



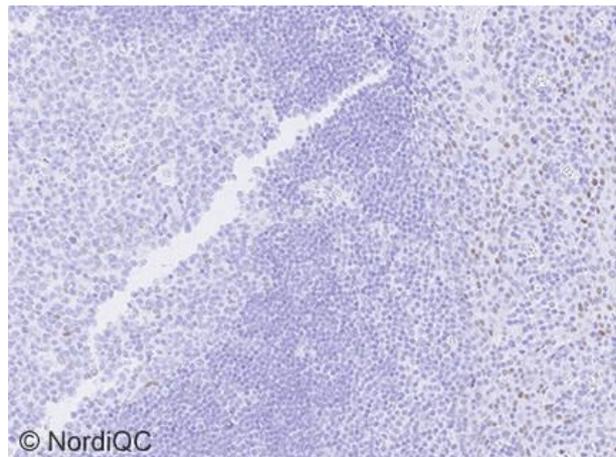
**Fig. 1a**  
Optimal ER staining of the uterine cervix using the rmAb clone SP1 based RTU format from Ventana/Roche, using HIER in an alkaline buffer and a 3-step polymer-based detection system.  
Virtually all squamous epithelial and stromal cells show a moderate to strong nuclear staining reaction. Endothelial and lymphoid cells are negative. Also compare with Figs. 2a-5a, same protocol.



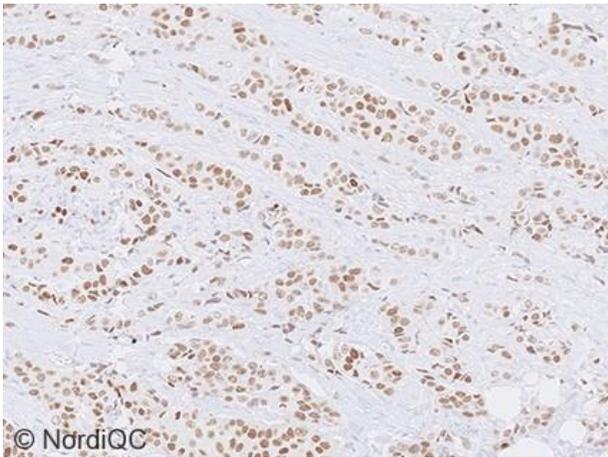
**Fig. 1b**  
ER staining of the uterine cervix using the rmAb clone EP1 based RTU format for Dako Autostainer, using HIER in an alkaline buffer and 2-step detection system – same field as in Fig. 1a.  
The intensity and proportion of squamous epithelial and stromal cells demonstrated is significantly reduced compared to the level expected and seen in Fig. 1a. However, also compare with Figs. 2b- 4b, same protocol.



**Fig. 2a**  
Optimal ER staining of the tonsil using same protocol settings as in Fig. 1a.  
A moderate, distinct nuclear staining reaction is seen in dispersed follicular dendritic cells in the germinal center and squamous epithelial cells.  
No nuclear staining reaction is seen in the mantle zone B-cells and in general a high signal-to-noise ratio is seen.



**Fig. 2b**  
Insufficient ER staining of the tonsil using same protocol settings as in Fig. 1b.  
Only a faint equivocal staining reaction is observed in few follicular dendritic cells in the germinal center and squamous epithelial cells.  
Compare with Fig. 2a – same field.

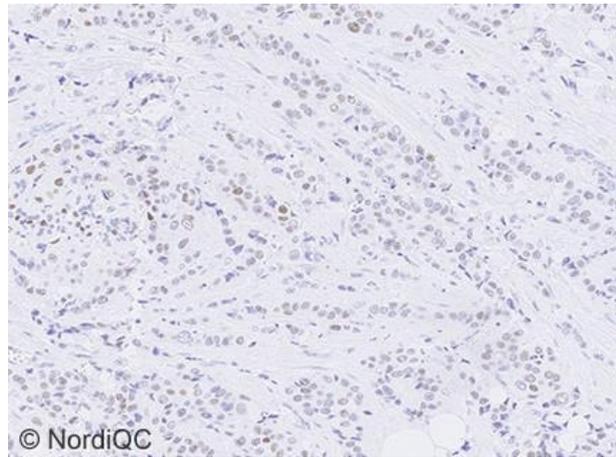


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Fig. 3a

Optimal ER staining of the breast carcinoma no. 5 with 90-100% cells being positive using same protocol as in Figs. 1a-2a.

The neoplastic cells display a moderate and distinct nuclear staining reaction.

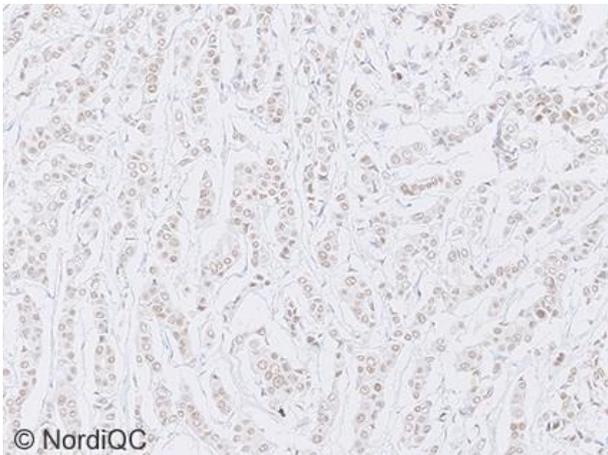


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Fig. 3b

ER staining of the breast carcinoma no. 5 with expected 90-100% cells being positive using same protocol as in Figs. 1b-2b.

The staining intensity and proportion of positive cells seen being reduced compared to the optimal result in Fig. 3a.

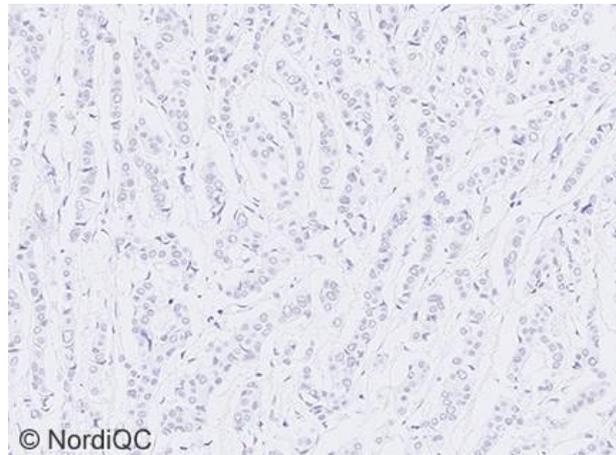


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Fig. 4a

Optimal ER staining of the breast carcinoma no. 4 with minimum 60-80% of the neoplastic cells expected to be positive using same protocol as in Figs. 1a-3a.

The majority of the neoplastic cells display a weak and distinct nuclear staining reaction.



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Fig. 4b

Insufficient and false negative ER staining of the breast carcinoma no. 4 with expected 60-80% cells being positive using same protocol as in Figs. 1b-3b.

<1% of the neoplastic cells are positive.

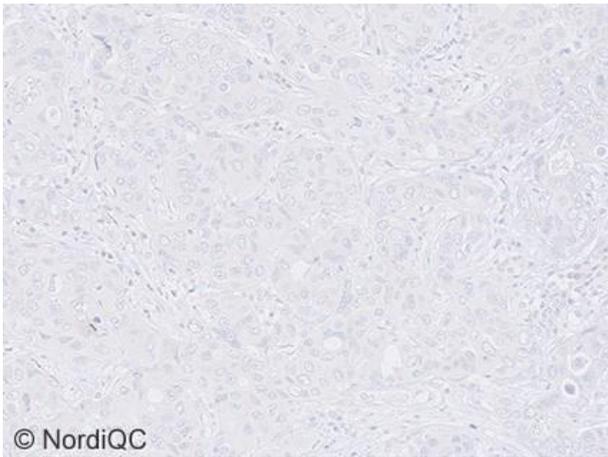


Fig. 5a  
Optimal ER staining of the breast carcinoma no. 3 expected to be negative using same protocol as in Figs. 1a-4a.  
No nuclear staining reaction is seen and a high signal-to-noise ratio is observed facilitating the read-out.

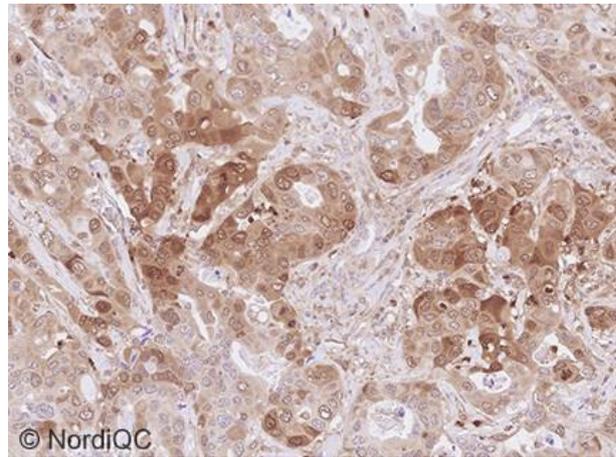


Fig. 5b  
Insufficient and false positive ER staining of the breast carcinoma no. 3 expected to be negative. The protocol based on rmAb clone ZR2 in a concentrated format, using HIER in an alkaline buffer and a 3-step polymer-based detection system.  
An aberrant cytoplasmic staining reaction is seen in virtually all cells compromising the read-out, and in addition of most concern a false positive nuclear staining reaction is seen in the majority of the neoplastic cells.

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