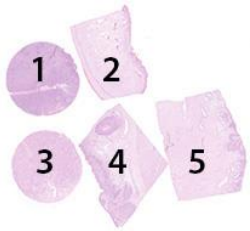
Assessment Run 59 2020
$p16^{ink4a}$ (p16)

Purpose
Evaluation of the technical performance and level of analytical sensitivity and specificity of the IHC assays for p16 performed by the NordiQC participants, identifying HPV associated cervical lesions in histological uterine cervical samples. Relevant clinical tissues, both normal and neoplastic disorders, were selected to display a broad spectrum of p16 antigen expression (see below).

The slide to be stained for p16 comprised:

1. Tonsil, 2. Uterine cervix, 3. Cervical squamous cell carcinoma,
4-5. High-grade squamous intraepithelial (HSIL) lesions



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a p16 staining as optimal included:

- A moderate to strong nuclear and cytoplasmic staining reaction in scattered reticulated crypt epithelial cells in the tonsil.
- An at least weak, but distinct nuclear and cytoplasmic staining reaction in dispersed germinal centre macrophages/dendritic cells in the tonsil.
- A moderate to strong nuclear and cytoplasmic staining reaction in virtually all the neoplastic cells in the cervical squamous cell carcinoma.
- A moderate to strong nuclear and cytoplasmic staining in the majority of the neoplastic cells throughout the entire cell layers of both the HSIL lesions.
- No staining in virtually all normal cervical squamous epithelial cells.

A weak staining reaction in scattered fibroblasts, endothelial cells and columnar epithelial cells was expected and accepted.

Participation

Number of laboratories registered for p16, run 59	352
Number of laboratories returning slides	291 (83%)

The number of laboratories returning slides has decreased in this run 59 compared to previous assessments, due to the COVID-19 pandemic. All slides returned after the assessment will be assessed, and receive advice if the result is insufficient, but will not be included in this report.

Results
291 laboratories participated in this assessment. 241 achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary Ab
- Use of less successful primary Ab
- Poor performance of the mAb clone E6H4 on the Dako Omnis platform

Performance history
This was the second NordiQC assessment of p16. An increased pass rate of 83% was seen this run 59, compared to 70% in the previous run 26 (see Table 2).

Table 2. **Proportion of sufficient results for p16 in the two NordiQC runs performed**

	Run 26, 2009	**Run 59, 2020**
Participants, n=	96	291
Sufficient results	70%	83%

Conclusion
The mAb clones **JC2**, **MX007**, **6H12** and **E6H4** are all recommendable markers for p16^{ink4a}. The mAb clone E6H4 (Ventana, RTU) was used by 70% (228 of 291) of the laboratories. When using the RTU system as recommended by Ventana, a pass rate of 100% was obtained. The majority of insufficient staining results were characterized by a too weak or false negative staining reaction, which was mostly seen when using too low concentration of the primary Ab or when applying the Ventana RTU on the Dako Omnis platform.
HIER, preferably in an alkaline buffer, and careful calibration of the primary Ab were the main prerequisites for optimal results.

Tonsil appears to be a recommendable positive and negative tissue control. The germinal centre macrophages/dendritic cells must show an at least weak but distinct nuclear and cytoplasmic staining reaction. Scattered reticulated crypt epithelial cells must show a moderate to strong nuclear and cytoplasmic staining reaction, while no reaction should be seen in the vast majority of lymphocytes and normal superficial squamous epithelial cells.

Table 1. **Antibodies and assessements for p16, run 59**

Concentrated Abs	**N**	**Vendor**	**Optimal**	**Good**	**Borderl.**	**Poor**	**Suff.[1]**	**OR[2]**
mAb clone **JC8**	1	Immunologic	0	1	0	0	-	-
mAb clone **JC2**	4 3 4 3 1	Cell Marque Gennova Diagnostic Biosystems Zytomed Systems Zeta Corporation	7	4	4	0	73%	47%
mAb clone **BC42**	1	Biocare Medical	0	0	1	0	-	-
mAb clone **G175-405**	7 1 2	BD Pharmingen Biogenex Zeta Corporation	0	1	8	1	10%	0%
mAb clone **GM501**	1	Gene Tech	0	1	0	0	-	-
mAb clone **IHC116**	2 1	GeneAb DCS	0	1	2	0	-	-
mAb clone **MX007**	5 9 1	Fuzhou Maixin Biotech Immunologic Nordic Biosite	11	3	1	0	93%	73%
mAb clone **R15-A**	1	DB Biotech	1	0	0	0	-	-
rmAb clone **R19-D**	2	DB Biotech	0	0	1	1	-	-
rmAb clone **RBT-p16**	1	Bio SB	1	0	0	0	-	-
Ready-To-Use Abs								
mAb clone **6H12, PA0016 (VRPS)[3]**	2	Leica Biosystems	1	1	0	0	-	-
mAb clone **6H12, PA0016 (LMPS)[4]**	1	Leica Biosystems	1	0	0	0	-	-
mAb clone **BC42, API3231**	1	Biocare Medical	0	0	1	0	-	-
mAb clone **BC42, VLTM3231**	1	Biocare Medical	1	0	0	0	-	-
mAb clone **E6H4, 9511 (VRPS)[3]**	1	Ventana	0	1	0	0	-	-
mAb clone **E6H4, 9511 (LMPS)[4]**	61	Ventana	29	23	8	1	85%	48%
mAb clone **E6H4, 805/825-4713 (VRPS)[3]**	36	Ventana	25	11	0	0	100%	69%
mAb clone **E6H4, 805/825-4713 (LMPS)[4]**	130	Ventana	64	47	18	1	85%	49%

mAb clone **G175-405, AM540**	1	Biogenex	0	0	0	1	-	-
mAb clone **JC2, PDM575**	2	Diagnostic Biosystems	1	0	1	0	-	-
mAb clone **MX007, 8313-C010**	2	Sakura Finetek	0	2	0	0	-	-
mAb clone **MX007, MAD-00690QD**	3	Master Diagnostica	3	0	0	0	-	-
Total	291		145	96	45	5		
Proportion			50%	33%	15%	2%	83%	

1) Proportion of sufficient stains (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) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 asessed protocols).

Detailed analysis of p16, Run 59
The following central protocol parameters were used to obtain an optimal staining:

Concentrated antibodies
mAb clone **JC2**: Protocols with optimal results were based on Heat Induced Epitope Retrieval (HIER) using Cell Conditioning 1 (CC1, Ventana) (3/4)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (1/2), TRS, High pH (Dako) (2/4) or TRS pH 6.1 (3-in-1) (Dako) (1/1). The mAb was diluted in the range of 1:100–200 and a 3-step polymer detection system was applied. Using these protocol settings, 9 of 10 (90%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this buffer).

mAb clone **MX007**: Protocols with optimal results were based on HIER using CC1 (Ventana) (5/6), TRS pH 9 (3-in-1) (Dako) (2/2), TRS, High pH (Dako) (3/6) or Bond Epitope Retrieval Solution 1 (BERS1, Leica) (1/1) as retrieval buffer. The mAb was diluted in the range of 1:100-2,000 depending on the total sensitivity of the protocol applied. Using these protocol settings, 14 of 15 (93%) laboratories produced a sufficient staining result.

mAb clone **R15-A**: One protocol with an optimal result was based on HIER using TRS pH 9 (3-in-1) (Dako) as retrieval buffer. The mAb was diluted 1:100. Only one laboratory used this clone.

rmAb clone **RBT-p16**: One protocol with an optimal result was based on HIER using CC1 (Ventana) as retrieval buffer. The rmAb was diluted of 1:400. Only one laboratory used this clone.

Table 3. **Proportion of optimal results for p16 for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **JC2**	1/1**	1/1	2/4	-	3/4	-	-	-
mAb clone **MX007**	2/2	-	3/6	-	5/6	-	-	1/1

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective systems.
** (number of optimal results/number of laboratories using this buffer).

Ready-To-Use antibodies and corresponding systems
mAb clone **6H12**, product no **PA0016**, Leica Biosystems, Bond III/Max:
Protocols with optimal results were based on HIER using BERS1 or Bond Epitope Retrieval 2 (BERS2) (efficient heating time 15-20 min. at 100°C), 15 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system.
Using these protocol settings, 3 of 3 laboratories produced a sufficient staining result.

mAb clone **E6H4**, product no. **805-4713/825-4713**, Ventana/Roche, BenchMark Ultra/GX/XT:
Protocols with optimal results were based on HIER using CC1 (efficient heating time typically 16-64 min. at 95-100°C), 12-48 min. incubation of the primary Ab and UltraView (760-500) with or without amplification (760-080) or OptiView (760-700) as detection system.
Using these protocol settings, 137 of 141 (97%) laboratories produced a sufficient staining result.

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems (≥10 asessed protocols). 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 protocols performed on the intended IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for p16 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Ventana BenchMark Ultra/GX/XT mAb **E6H4**	36/36 (100%)	25/36 (69%)	94/106 (89%)	61/106 (58%)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered, detection kit – only protocols performed on the specified vendor IHC stainer were included.

Comments
In this assessment, the prevalent feature of an insufficient staining was either a generally too weak or false negative staining reaction, seen in 72% (36 of 50). 24% (12 of 50) of the insufficient results were characterized by a poor signal-to-noise ratio. In the remaining two insufficient results (4%), a false positive staining reaction was observed.

The weak or false negative staining reactions were seen in tonsil, the cervical squamous cell carcinoma and HSIL (tissue core no. 5). In tonsil, the majority of all laboratories were able to stain p16 in the reticulated crypt epithelial cells, whereas demonstration of p16 in germinal centre macrophages/dendritic cells was much more challenging and required a carefully calibrated protocol. When a too weak or completely false negative staining reaction in the germinal centre macrophages/dendritic cells was observed, the neoplastic cells in the carcinoma displayed a significantly reduced intensity. Of more critical impact, the "block-positivity" expected in the HSILs (especially tissue core no. 5) was reduced and only the basal part of the HSIL lesion being positive.

In 24% (12 of 50) of the insufficient results, a poor signal-to-noise ratio was seen, characterized by a positive staining reaction in structures expected to be demonstrated, but at the same time accompanied by a general background reaction and a diffuse staining reaction in virtually all the normal cervical squamous and columnar epithelial cells.

17% (50 of 291) of the laboratories used a concentrated format within a laboratory developed (LD) assay for the demonstration of p16.

The mAb clone MX007 was the most successful Ab within a LD assay, with a pass rate of 93% (14 of 15), 73% optimal (see Table 1). The clone could be applied with various protocol settings, and on both fully automated and semi-automated platforms. All protocols used HIER (preferably in an alkaline buffer, see Table 3) as pre-treatment, and the majority of participants used a 3-step polymer-based detection system (14 of 15).

The mAb clone JC2 obtained a pass rate of 73% (11 of 15), 47% optimal (see Table 1). All optimal protocols were based on HIER and a 3-step polymer-based detection system. Laboratories applying a 2-step detection system increased the concentration of the primary Ab in order to enhance the technical and analytical sensitivity, however, the overall result was inferior to 3-steps system as a general too weak staining reaction was obtained.

The mAb clone G175-405 was less successful and obtained a pass rate of only 10% (1 of 10), none optimal (see Table 1). The laboratories applied protocol settings similar to the mAb clones MX007 and JC2, with a high concentration of the primary Ab, HIER in an alkaline buffer, and 2- or 3-layer detection system. However, the majority of the laboratories obtained a too weak or false negative staining reaction.

241 of 291 (83%) of the laboratories used an RTU format for the demonstration of p16. Ideally, an RTU format of a primary Ab should be used within a system that has been thoroughly validated, providing

precise information on vendor recommended protocol settings, equipment, reagents and performance characteristic (expected reaction patterns).

mAb clone E6H4 (Ventana) were the most widely used clone. 95% (228 of 241) of the RTU assays were based on the Ventana clone.

Using the Ventana RTU formats (805-4713/825-4713) for BenchMark platforms, 36 laboratories applied protocol settings as recommended by Ventana, with a pass rate of 100%, 69% optimal (see Table 4). The majority (n=106) of the laboratories modified the protocols, obtaining a pass rate of 89% (94 of 106), 58% optimal (see Table 4). The most common modification was prolonged incubation of the primary Ab. For laboratories using UltraView as detection system, the majority reduced HIER time to e.g. 36 min. from 64 min. as recommended. Both modifications could provide sufficient and optimal results. 24 laboratories used the mAb E6H4 on different platforms than BenchMark. A significantly decreased pass rate of 38% (9 of 24), 13% optimal, was seen when using the Ventana RTU off-label on other IHC platform.

Using the Ventana kit (9511) developed for manual staining and Autostainer platforms, only one laboratory used the product as recommended by Ventana, obtaining a result assessed as "Good" (see Table 1). The remaining 61 laboratories used the Ventana primary Ab "off-label" with either other reagents than provided in the kit and/or on other platforms, with a pass rate of 85% (52 of 61), 48% optimal.

In total, 17 laboratories used either the Ventana RTU formats 805-4713/825-4713 or 9511 on the Dako Omnis platform, with a pass rate of 24% (4 of 17), only one optimal (6%) clearly indicating an inferior performance of the Ventana RTU format based on mAb clone E6H4 on Omnis compared to BenchMark. Off-label use cannot be recommended and requires meticulous validation by the laboratories.

The newly launched Leica RTU (PA0016) based on mAb clone 6H12 was used by three laboratories, all with sufficient results (see Table 1).

Controls
Tonsil appears to be a recommendable positive and negative tissue control. Scattered reticulated crypt epithelial cells must show a moderate to strong nuclear and cytoplasmic staining reaction. Dispersed germinal centre macrophages/dendritic cells must show an at least weak but distinct nuclear and cytoplasmic staining reaction.
No staining reaction should be seen in the vast majority of lymphocytes and normal superficial squamous epithelial cells.

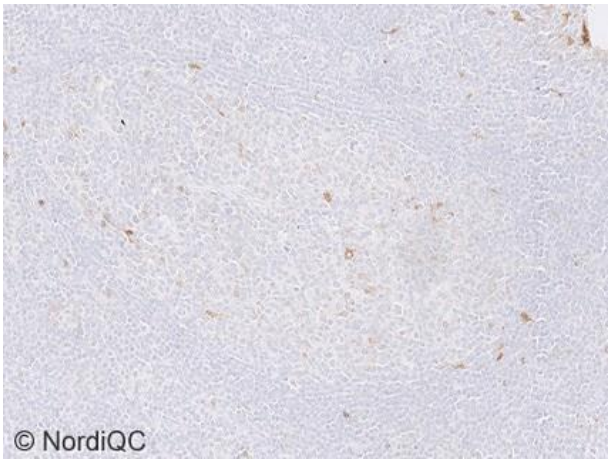


Fig. 1a
Optimal p16 staining of the tonsil using the Ventana RTU format 805-4713/825-4713 based on mAb clone E6H4 using recommended protocol settings. Dispersed germinal centre macrophages/dendritic cells show a weak, distinct nuclear and cytoplasmic staining reaction. Same protocol used in Figs. 2a-4a.

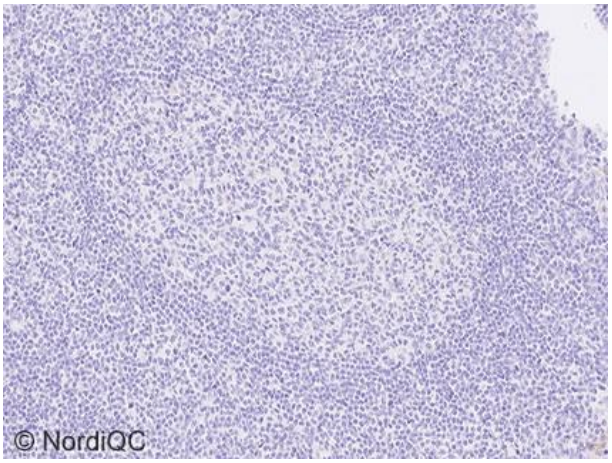


Fig. 2a
Insufficient p16 staining of the tonsil using the less successful mAb clone G175-405, providing a too low level of technical and analytical sensitivity. Same protocol used in Figs. 2b-4b. Virtually all germinal centre macrophages are negative. Same area as in Fig. 1a.

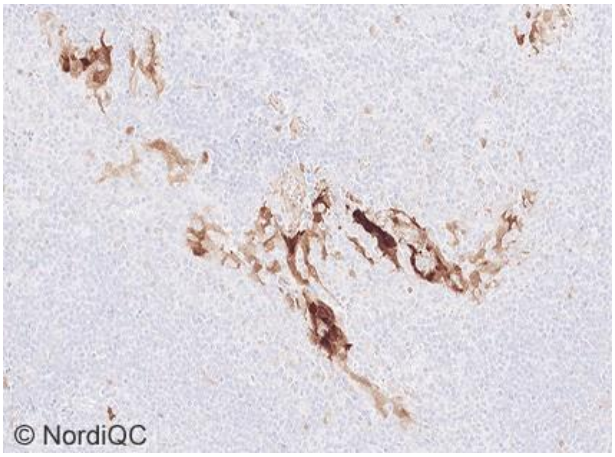


Fig. 2a
Optimal p16 staining of the tonsil using same protocol as in Fig. 1a. Scattered reticular epithelial cells display a moderate to strong nuclear and cytoplasmic staining reaction.

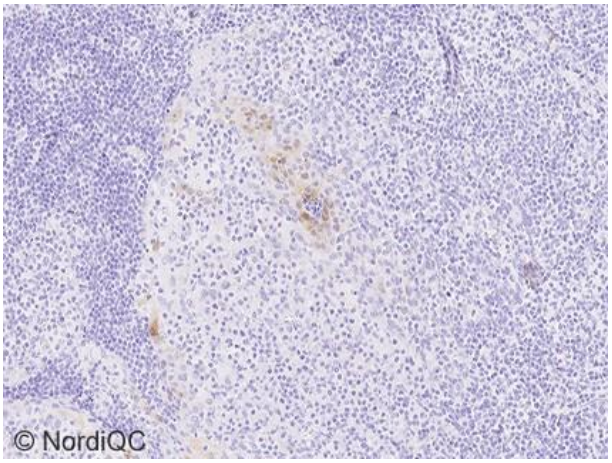


Fig. 2b
Insufficient p16 staining of the tonsil using same protocol as in Fig. 1b. Only few reticular epithelial cells show a faint staining reaction. Same area as in Fig. 2a.

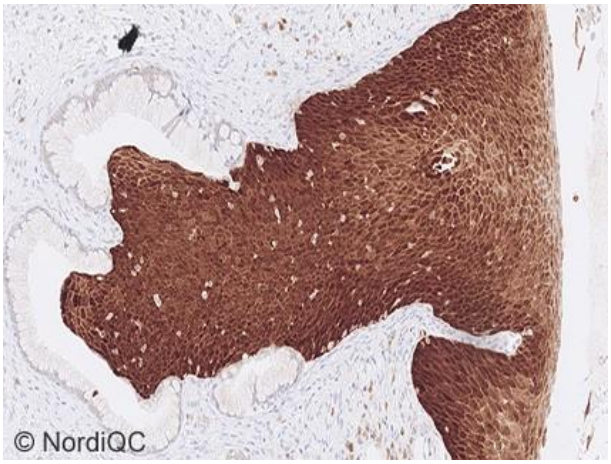


Fig. 3a
Optimal p16 staining of HSIL, tissue core no. 4, using same protocol as in Figs. 1a and 2a. A moderate to strong nuclear and cytoplasmic staining reaction is seen in all neoplastic cells throughout the cell layers – "block-positivity".

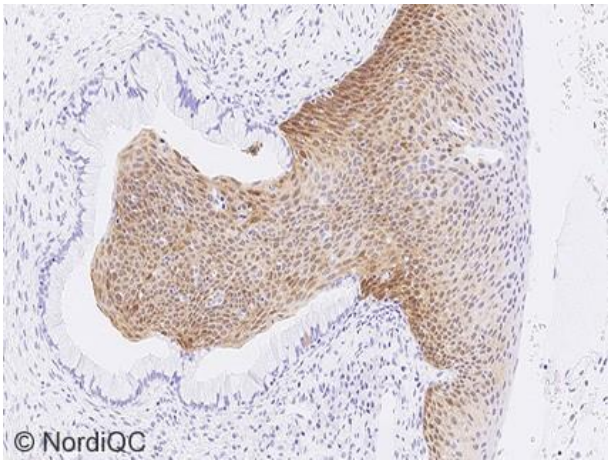


Fig. 3b
Insufficient staining of HSIL, tissue core no. 4, using same protocol as in Figs. 1b and 2b. A moderate staining reaction is only seen in the basal layer The "block-positivity" is reduced and only the basal part of the lesion distinctively demonstrated. Same area as in Fig. 3a.

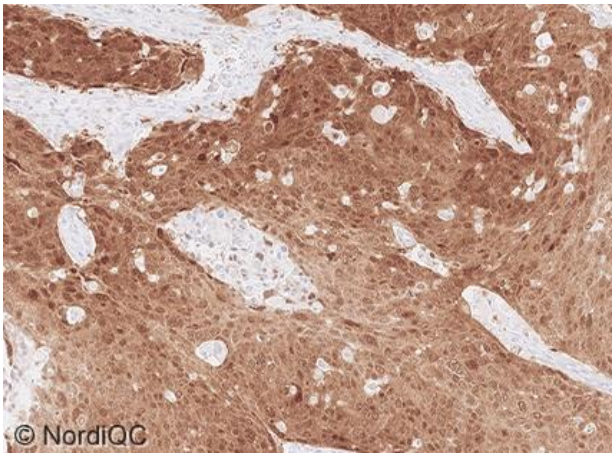


Fig. 4a
Optimal p16 staining of the cervical squamous cell carcinoma, using same protocol as in Figs. 1a - 3a. A moderate to strong nuclear and cytoplasmic staining reaction is seen in all neoplastic cells.

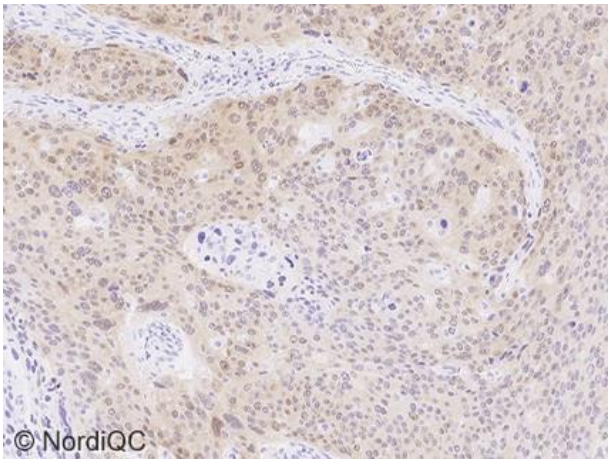


Fig. 4b
Insufficient staining of the cervical squamous cell carcinoma, using same protocol as in Figs. 1b – 3b. The neoplastic cells display a weak cytoplasmic staining reaction and only scattered neoplastic cells display a faint nuclear staining reaction. Same area as in Fig. 4a.

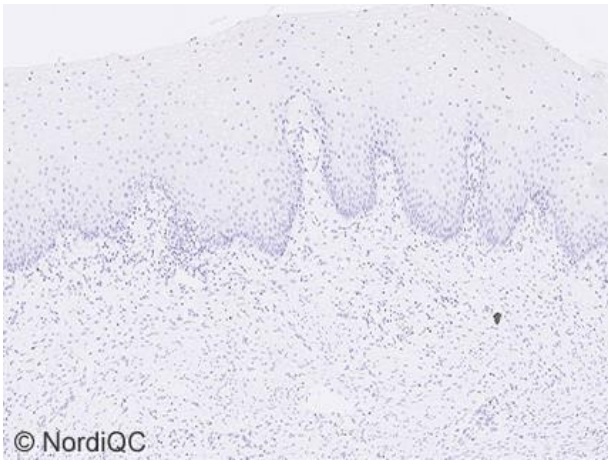


Fig. 5a
Optimal p16 staining of the uterine cervix, using the mAb clone JC2 as a concentrated format with HIER in an alkaline buffer and a 3-step polymer detection system. No staining reaction is seen in the squamous epithelial cells. Same protocol used in Fig. 6a.

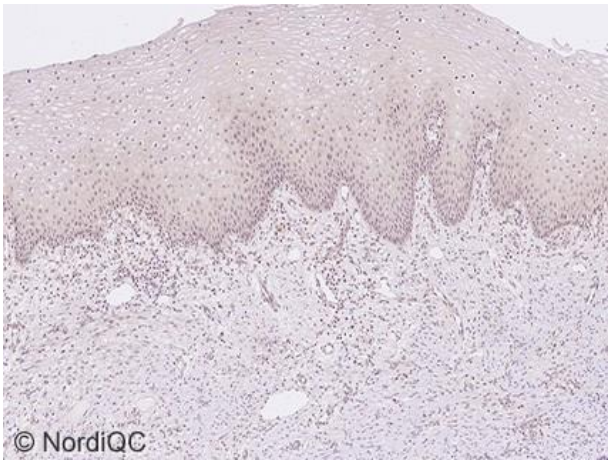


Fig. 5b
Insufficient staining of the uterine cervix, using the mAb clone G175-405 as a concentrated format with HIER in an alkaline buffer and a 3-step polymer detection system. Same protocol used in Fig. 6b. An aberrant false positive nuclear staining reaction is observed in virtually all cell types. Same area as in Fig. 5a.

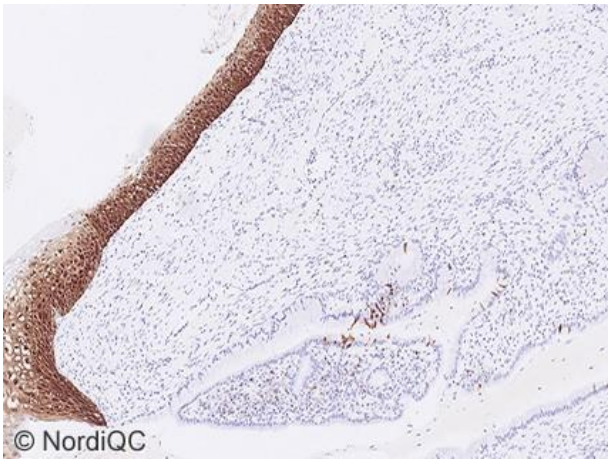


Fig. 6a
Optimal staining of HSIL, tissue core no. 5, using same protocol as in Fig. 5a. The neoplastic cells display a moderate to strong nuclear and cytoplasmic staining reaction, and only few scattered columnar epithelial cells are demonstrated. A high signal-to-noise ratio is observed facilitating the interpretation.

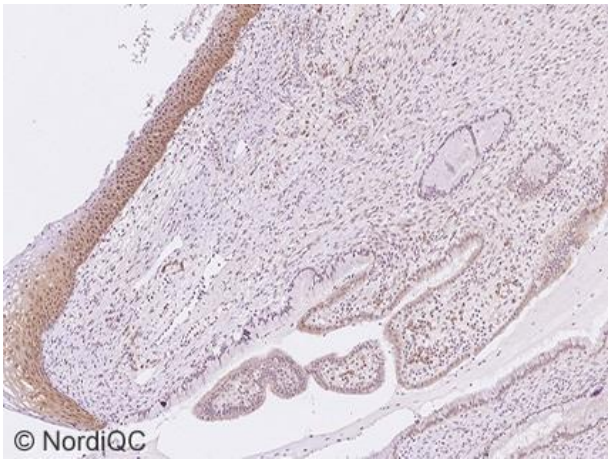


Fig. 6b
Insufficient staining of HSIL, tissue core no. 5, using same protocol as in Fig. 5b. A weak to moderate nuclear and cytoplasmic staining reaction is seen in the neoplastic cells. However also a weak but distinct aberrant false positive, nuclear staining reaction is observed in the stromal cells, and the majority of columnar epithelial cells compromising the interpretation.

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