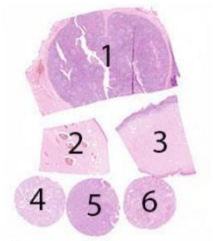
Assessment Run 72 2024
$p16^{ink4a}$ (p16)

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 lesions in histological uterine cervical samples and head and neck squamous cell carcinomas. Relevant clinical tissues, both normal and neoplastic disorders, were selected to display a broad spectrum of p16 antigen expression (see below).

Material
The slide to be stained for p16 comprised:



1. Tonsil, 2. Uterine cervix, 3. Cervical carcinoma in situ (CIN3), 4. Cervical squamous cell carcinoma, 5-6. Head and neck squamous cell carcinoma (HNSCC)

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 to moderate, 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 both the cervical and head and neck squamous cell carcinoma, tissue core no. 5.
- A moderate to strong nuclear and cytoplasmic staining in the majority of the neoplastic cells throughout the entire cell layers of the CIN3 lesion.
- No staining reaction in virtually all normal cervical squamous epithelial cells.
- No staining reaction in virtually all neoplastic cells of the head and neck squamous cell carcinoma, tissue core no. 6.

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

KEY POINTS FOR p16 IMMUNOASSAYS
- A high overall pass rate of 88% was observed in this 1' assessment focusing on p16 in both cervical lesions and HNSCCs.
- The mAb clone **E6H4** was used by 64% of all participants.
- The p16 **RTU** systems from Ventana/Roche and Dako/Agilent applied by vendor were most successful giving pass rates of 97% and 100%, respectively.
- The performance of the Ventana/Roche RTU system based on mAb clone E6H4, was impaired by prolonging the Ab incubation time for ≥ 28 min. giving an excessive background reaction and/or false positive result.
- The mAb **MX007** produced the highest pass-rate among the concentrated formats.

Participation

Number of laboratories registered for p16, run 72	453
Number of laboratories returning slides	431 (95%)

All slides returned after the assessment were assessed and participants received advice if the result was insufficient - data were not included in this report.

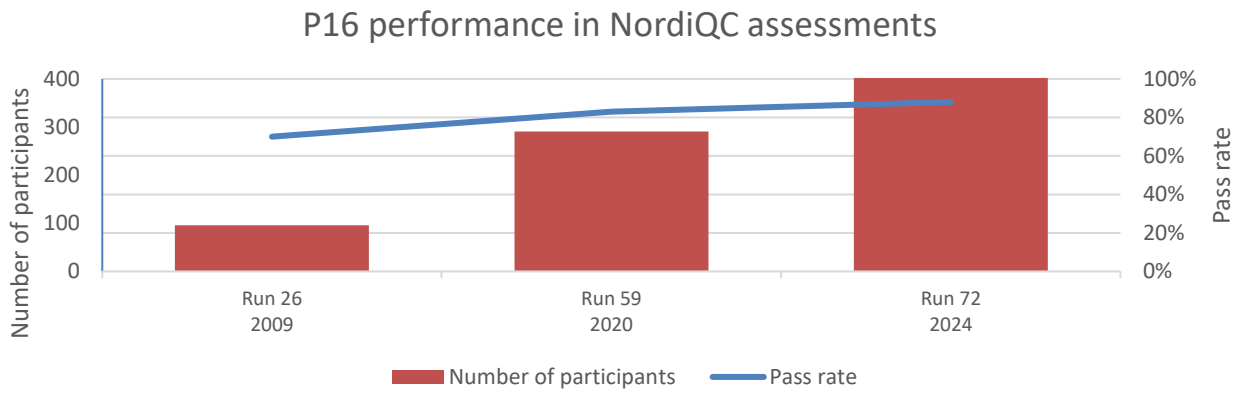
Results
431 laboratories participated in this assessment. 379achieved a sufficient mark (optimal or good). One laboratory used an inappropriate antibody and data will not be included in the report. Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 3 and 4).

The most frequent causes of insufficient staining reactions were:

- Too high or low concentration of the primary Ab
- Use of less successful primary Ab
- Less successful laboratory modified protocol settings for the Ventana/Roche RTU system based on E6H4 (e.g. pronged incubation and/or mitigation to other platforms)

Performance history
This was the third NordiQC assessment of p16. An increased pass rate of 88% was seen in this run 72, compared to 83% in the previous run 59 (see Table 2).

Graph 1. **Proportion of sufficient results for p16 in the three NordiQC runs performed**



Controls
Tonsil appears to be a recommendable positive and negative tissue control. The germinal centre macrophages/dendritic cells must show an at least weak to moderate 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.

Conclusion
In this 1'assessment of p16 IHC focusing on both cervical lesions and HNSCCs the mAb clones **JC2**, **JC8, MX007**, **6H12** and **E6H4** were all found to be recommendable markers for $p16^{ink4a}$.
Efficient HIER in an alkaline buffer, careful calibration of the primary Ab and preferrable use of a 3-step polymer/multimer detection system were the main prerequisites for optimal performance. It was noted that for the JC2 clone the titer range for optimal performance was highly different depending on vendor.
The mAb clone E6H4 as RTU formats (Ventana/Roche) was used by 64% (274 of 430) of the laboratories. When using the RTU systems as recommended by Ventana/Roche, a pass rate of 96% was obtained, 63% being optimal. The majority of insufficient staining results were characterized by a false positive staining reaction and/or excessive background.
The newly introduced JC8 RTU product for Dako Omnis provided the highest pass-rate with a 100% being optimal using the vendor recommended protocol setting.

Table 1a. **Overall results for p16, run 72**

	n	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
Concentrated antibodies	73	37	20	12	4	78%	51%
Ready-To-Use antibodies	357	204	118	33	2	90%	57%
Total	430	241	138	45	6		
Proportion		56%	32%	11%	1%	88%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of Optimal Results.

Table 1b. **Concentrated antibodies and assessment marks for p16, run 72**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **JC2**	21 9 1 1	Cell Marque Diagnostic Biosystems Gennova Zeta Corporation	19	9	2	2	88%	59%
mAb clone **MX007**	1 9 4 5	Fuzhou Maixin Biotech Immunologic Master Diagnostica Zeta Corporation	12	5	2	-	90%	63%
mAb clone **BC42**	4	Biocare Medical	-	1	3	-	-	-
mAb clone **G175-405**	2 1	BD Pharmingen Zeta Corporation	1	-	1	1	-	-
mAb clone **IHC116**	2	GenomeMe	-	1	1	-	-	-
mAb clone **JC8**	4	Santa Cruz	1	3	-	-	-	-
mAb clone **R15-A**	4	DB Biotech	2	-	2	-	-	-
mAb clone **IHC216**	1	GenomeMe	1	-	-	-	-	-
rmAb clone **RBT-p16**	1	Bio SB	1	-	-	-	-	-
rmAb clone **EP435Y-129R**	1	Abcam	-	-	-	1	-	-
rmAb clone **QR019**	1	Quartett	-	-	1	-	-	-
rmAb clone **ZR407**	1	Zeta Corporation	-	1	-	-	-	-
Total	73		37	20	12	4		
Proportion			51%	27%	16%	6%	78%	

1) Proportion of sufficient stains (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results.

Table 1c. **Ready-To-Use antibodies and assessment marks for p16, run 72**

Ready-To-Use antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **E6H4, 805/825-4713 (VRPS)[3]**	94	Ventana/Roche	59	32	3	-	97%	63%
mAb clone **E6H4, 805/825-4713 (LMPS)[4]**	123	Ventana/Roche	49	54	20	-	80%	40%
mAb clone **E6H4, 9511 (VRPS)[3]**	3	Ventana/Roche	1	1	1	-	-	-
mAb clone **E6H4, 9511 (LMPS)[4]**	54	Ventana/Roche	34	18	2	-	96%	64%
mAb clone **JC8, GA783 (VRPS)[3]**	15	Dako/Agilent	15	-	-	-	100%	100%
mAb clone **JC8, GA783 (VRPS)[4]**	12	Dako/Agilent	11	1			100%	92%
mAb clone **6H12, PA0016 (VRPS)[3]**	22	Leica Biosystems	14	6	2	-	91%	64%
mAb clone **6H12, PA0016 (LMPS)[4]**	5	Leica Biosystems	2	2	-	1	80%	40%
mAb clone **BC42, API3231**	6	Biocare Medical	5	-	1	-	83%	83%
mAb clone **MX007, 8313-C010**	3	Sakura Finetek	2	1	-	-	-	-
mAb clone **JC2, 416M-10/17/18**	3	Cell Marque	2	-	-	1	-	-
mAb clone **JC2, MSG123**	1	Zytomed systems	-	1	-	-	-	-
mAb clone **JC2, Z256/MP**	1	Zeta Corporation	-	-	1	-	-	-
mAb clone **JC2, PDM575**	6	Diagnostic Biosystems	5	-	1	-	83%	83%
mAb clone **MX007, MAD-00690QD**	3	Master Diagnostica	1	1	1	-	-	-
mAb clone **MX007, MAB-0673**	1	Fuzhou Maixin	1	-	-	-	-	-
mAb clone **IHC116 IHC116-7**	1	GenAb	1	-	-	-	-	-
rmAb clone **GM501 GT233002**	1	Gene Tech	1	-	-	-	-	-
Ab clone **598A6G3**	1	Abcarta	-	-	1	-	-	-
Ab clone **BPM6147**	1	Biolynx Biotechnology	-	1	-	-	-	-
Ab clone **BY001**	1	BioIn Biotechnology	1	-	-	-	-	-
Total	357		204	118	33	2		
Proportion			57%	33%	9%	1%	90%	

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 assessed protocols)

Detailed analysis of p16, Run 72
The following protocol parameters were central to obtain 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/Roche) (3/4)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako/Agilent) (2/2), TRS, High pH (Dako/Agilent) (11/18) or Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (3/8) as retrieval buffer. The mAb was diluted in the range of 1:100–1.000 depending on the total sensitivity of the protocol applied. Using these protocol settings, 27 of 30 (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/Roche) (3/6), TRS pH 9 (3-in-1) (Dako/Agilent) (1/2), Citrate pH 6 (1/1), TRS High pH (Dako/Agilent) (1/4), TRS Low pH 6.1 (Dako/Agilent) (1/1), Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (2/2) or BERS2 (Leica Biosystems) (2/2) as retrieval buffer. The mAb was diluted in the range of 1:100-800 depending on the total sensitivity of the protocol applied. Using these protocol settings, 15 of 17 (88%) laboratories produced a sufficient staining result.

Table 2. **Proportion of optimal results for p16 for the most commonly used antibody concentrates on the 4 main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer[1]**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark[2]**		**Leica Biosystems Bond[3]**	
	TRS pH 9.0	TRS pH 6.1	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	BERS2 pH 9.0	BERS1 pH 6.0
mAb clone **JC2**	2/2**	-	11/18 (61%)	-	3/4	-	3/8 (38%)	-
mAb clone **MX007**	1/2	1/1	1/4	1/1	3/6 (50%)	-	2/2	2/2

* 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)
1) Autostainer Classical, Link 48.
2) BenchMark GX, XT, Ultra, Ultra Plus
3) Bond III, Prime, Max

Ready-To-Use antibodies and corresponding systems
mAb clone **E6H4**, product no. **805-4713/825-4713**, Ventana/Roche, BenchMark Ultra/Ultra plus/GX/XT:
Protocols with optimal results were based on HIER using CC1 (efficient heating time typically 20-64 min. at 95-100°C), 8-28 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, 145 of 154 (94%) laboratories produced a sufficient staining result.

mAb clone **JC8**, product no. **GA783**, Dako/Agilent, Dako Omnis:
Protocols with optimal results were typically based on HIER using TRS pH 9 (efficient heating time 30 min. at 97°C) and 8-30 min. incubation of the primary Ab and EnVision Flex+ (GV800/GV823/GV821) as detection system. Using these protocol settings, 26 of 26 (100%) laboratories produced a sufficient staining result.

mAb clone **6H12**, product no **PA0016**, Leica Biosystems, Bond III/Prime/Max:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 10-20 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, 24 of 26 (92%) 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 3. **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 mAb E6H4 **805-4713/825-4713** **OptiView**	97% (38/39)	61% (24/39)	75% (39/52)	35% (18/52)
Ventana BenchMark mAb E6H4 **805-4713/825-4713** **UltraView**	96% (53/55)	64% (35/55)	95% (55/57)	45% (26/57)
Dako Omnis mAb clone JC8 **GA783**	100% (15/15)	100% (15/15)	100% (11/11)	91% (10/11)
Leica Bond mAb clone 6H12 **PA0016**	91% (20/22)	64% (14/22)	80% (4/5)	40% (2/5)

* 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 are integrated.

Comments
In this assessment, the prevalent feature of an insufficient staining result was characterized by an excessive background reaction in combination with either a too weak or false positive staining reaction and in total observed in 82% (42 of 51) of all the insufficient results.
The remaining insufficient results were related to a false negative result or a granular staining reaction interfering with the interpretation.

The weak or false negative staining reactions were most frequently seen in tonsil and the cervical intraepithelial neoplasia (CIN3) but also observed in the cervical squamous cell carcinoma and HNSCC, 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 center 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 center macrophages/dendritic cells was observed, the neoplastic cells in the two HPV associated carcinomas also displayed a significantly reduced intensity. Of more critical impact, the strong "block-positivity" expected in the CIN3 was reduced and primarily the intermediate and basal part of the lesion remained strongly positive.

In 53% (27 of 51) of the insufficient results, an aberrant and false positive nuclear staining reaction was seen and typically observed in the benign squamous epithelial cells of the uterine cervix, tissue core no. 2. The pattern was in most cases also accompanied by a general background reaction or cytoplasmic staining reaction in the normal columnar epithelial structures of the CIN3 (tissue core no. 3).

17% (73 of 430) of the laboratories used a concentrated format within a laboratory developed (LD) assay for the demonstration of p16.

The mAb clone **JC2** was the most used clone with a pass rate of 88%, 59% being optimal (see Table 1b). 3 of the 4 insufficient protocols were based on a too high concentration of the primary antibody on the Dako Omnis platform causing an excessive background or false positive staining reaction. Using the concentrated format from Cell Marque the titre ranges obtaining optimal results were 1:200-800 dependent on the total sensitivity of the protocol whereas the JC2 clone purchased from Diagnostic Biosystems required a titre range of 1:50-200 using similar protocol settings for the two products compared.
All protocols were based on an alkaline HIER buffer and a 3-step detection system and optimal results were produced on all of the main IHC platforms.

The mAb clone **MX007** was the most successful Ab within a LD assay, with a pass rate of 90% (17 of 19), 63% optimal (see Table 1b). The clone could be applied with various protocol settings, and on both fully automated and semi-automated platforms. All protocols used HIER as pre-treatment, and a 3-step detection system. The 2 insufficient protocols were due to either a too high or too low analytical sensitivity of the protocol applied.

Of the remaining 22 protocols the results were very mixed but optimal results were seen by various protocols and clones of which especially the mAb clone **JC8** from Santa Cruz displayed promising results.

83% (357 of 430) of all the participants used a Ready-to-use (RTU) system. The Ventana/Roche RTU systems based on mAb clone E6H4 were most widely used and in total applied by 77% (274 of 357) of participants using p16 IHC RTU systems.

Using the Ventana/Roche RTU formats of clone **E6H4** (805-4713/825-4713) for BenchMark platforms, 94 laboratories applied protocol settings as recommended by Ventana/Roche. The recommendation for OptiView as detection system using CC1 for 48 min. and an Ab incubation for 8-12 min. displayed a pass rate of 97%, 61% being optimal. Slides downgraded to "good" displayed an excessive background reaction and was seen in 13 of 14 cases. Using UltraView as detection system within recommended protocol settings as CC1 for 64 min. and 16-20 min. Ab incubation the pass rate was 96%, 64% being optimal. Also using UltraView the excessive background was observed in 12 of 18 cases marked as good.

120 laboratories applied the RTU formats of clone **E4H6** (805-4713/825-4713) within a laboratory modified protocol with a pass rate of 95% for UltraView and only 75% for OptiView with a level of optimal of 45% and 35%, respectively. The main cause for the insufficient results were false positive results combined with excessive background reaction. For protocols with an Ab incubation above 28 min. 54% (30 of 56) displayed either a false positive result or excessive background, but for protocols with an Ab incubation less than 28 min. only 32% (49 of 153) displayed the same problems, indicating that a short Ab incubation is preferred.

Using the Ventana/Roche RTU kit (9511, clone E4H6) developed for manual staining and Autostainer platforms, only 3 laboratories used the product as recommended by Ventana/Roche, obtaining mixed results (see Table 1c). The remaining 54 laboratories used the Ventana/Roche primary Ab "off-label" with either other reagents than provided in the kit and/or on other platforms, with a pass rate of 96% (52 of 54), 64% optimal.

In total, 23 laboratories used either the Ventana/Roche RTU formats 805-4713/825-4713 or 9511 on other platforms than Ventana, with a pass rate of 78% (18 of 23), 48% (11 of 23) being optimal indicating an inferior performance of the Ventana/Roche RTU formats based on mAb clone E6H4 on other platforms compared to BenchMark. In general and in this specific instance, off-label use cannot be recommended and requires meticulous validation by the laboratories.

In this assessment, the newly launched Dako/Agilent RTU system for p16 based on clone **JC8** (GA783) with intended use on Dako Omnis was most successful. In total 15 laboratories used the product with the vendor recommended protocol settings with 100% being optimal. 12 laboratories applied the product as a laboratory modified protocol only adjusting the Ab incubation time with a pass-rate of 100%, 92% being optimal. The one protocol not receiving an optimal mark did not use mouse linker as recommended from vendor.

The Leica RTU system (PA0016) based on mAb clone **6H12** was used by 27 laboratories, 22 applying it by the vendor recommended protocol with a pass-rate of 91%, 64% being optimal. 5 laboratories applied the product as a laboratory modified protocol with a pass-rate 80%, 40% optimal. The product was only applied on the Bond platforms. Overall the results were as expected but in 41% of the results evaluated (11 of 27) a weak staining or excessive background reaction was observed.

This was the third assessment of p16 in NordiQC (see Graph 1), but the first when both cervical and head and neck samples were included. The proportion of sufficient results was 88% which is an improvement from 83% achieved in the previous run 59 (2020) and overall the highest pass rate for p16 to date. Similar to the previous run, the main parameters contributing to the positive development were related to the access to newly introduced robust concentrated Abs as clone MX007 and extensive use of well performing RTU systems from the main IHC system providers. In this context both the newly launched p16 RTU system from Dako/Agilent and the established RTU system from Ventana/Roche both gave a high pass rate of 100% and 97% when applied by recommended protocol settings.
Although some clones seem to be relatively more robust, it is important to conclude that sufficient HIER and careful calibration of the primary antibody concentration together with a sensitive 3-step detection system is an important prerequisite for an optimal p16 staining reaction.

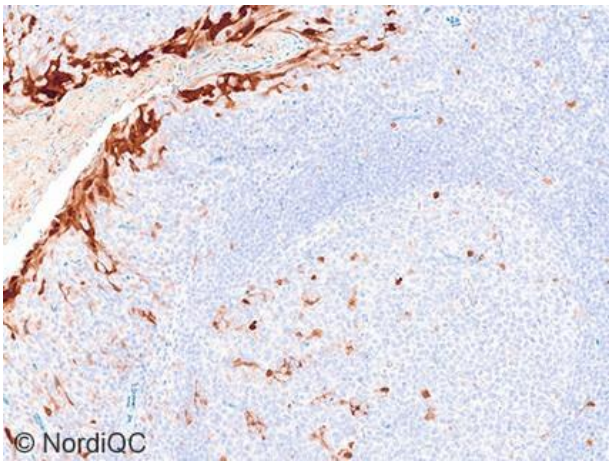


Fig. 1a
Optimal p16 staining reaction of the tonsil using the Dako/Agilent RTU format GA783 based on mAb clone JC8 using recommended protocol settings. Dispersed germinal center macrophages/dendritic cells show a weak, distinct nuclear and cytoplasmic staining reaction. Same protocol used in Figs. 2a-4a.

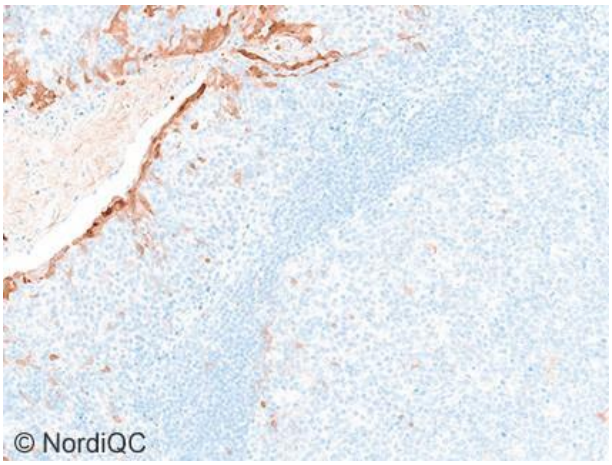


Fig. 1b
Insufficient p16 staining reaction of the tonsil using the Ventana/Roche RTU format 805-4713/825-4713 based on the mAb clone E6H4 as a laboratory modified protocol providing a too low level of technical and analytical sensitivity. Same protocol used in Figs. 2b-3b. Virtually all germinal center macrophages are negative and the squamous crypt epithelial cells being reduced in intensity. Same area as in Fig. 1a.

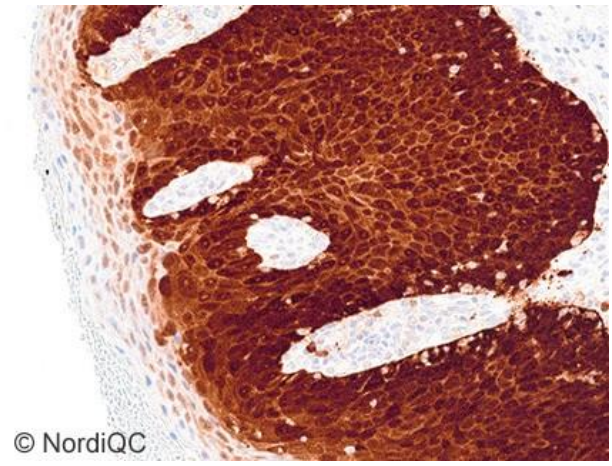


Fig. 2a
Optimal p16 staining reaction of CIN3, tissue core no. 3, using same protocol as in Fig. 1a. A moderate to strong nuclear and cytoplasmic staining reaction is seen in all neoplastic cells throughout the cell layers – "block-positivity".

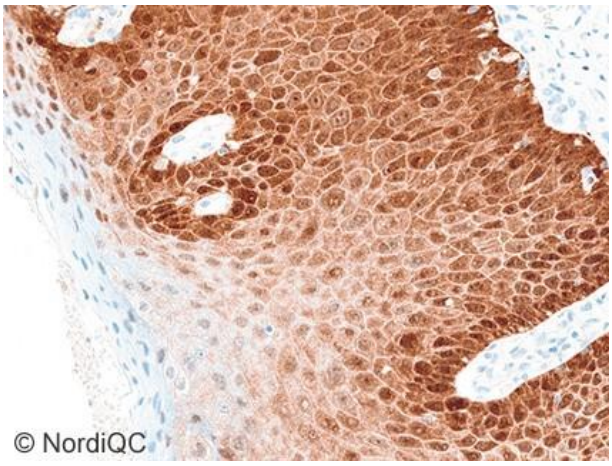


Fig. 2b
Staining reaction of CIN3, tissue core no. 3, using same protocol as in Fig. 1b. A weak to moderate staining reaction is seen throughout the cell layers. The "block-positivity" is reduced compared to Fig 2a. Same area.

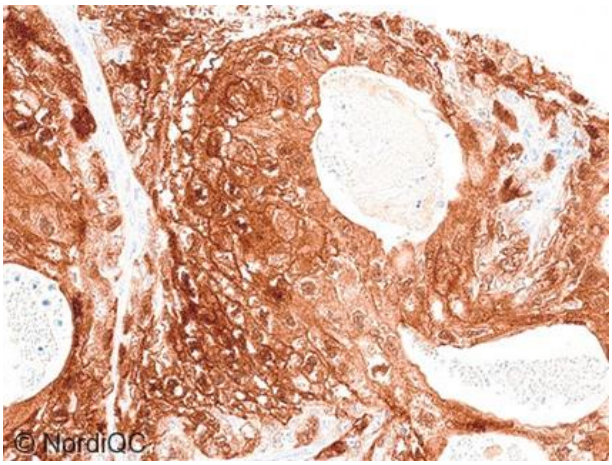


Fig. 3a
Optimal p16 staining reaction of the cervical squamous cell carcinoma, using same protocol as in Figs. 1a - 2a. A moderate to strong nuclear and cytoplasmic staining reaction is seen in all neoplastic cells.

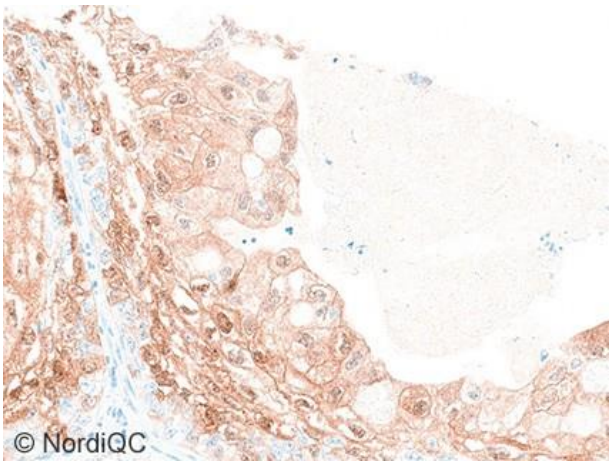


Fig. 3b
Insufficient staining reaction of the cervical squamous cell carcinoma, using same protocol as in Figs. 1b – 2b. The neoplastic cells display a weak to moderate cytoplasmic staining reaction and only scattered neoplastic cells display a nuclear staining reaction.

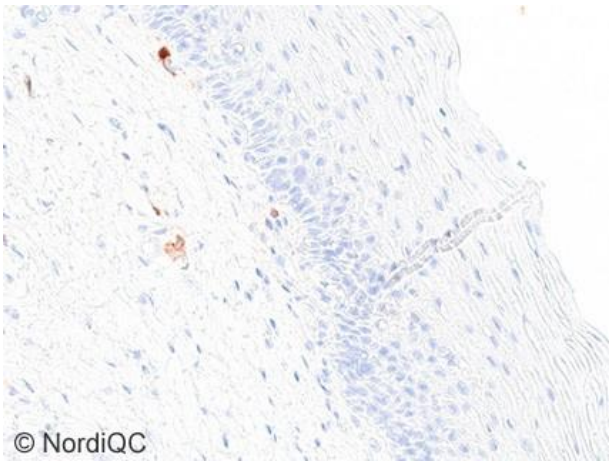


Fig. 4a
Optimal p16 staining reaction of the uterine cervix using same protocol as in Figs. 1a - 3a. No staining reaction is seen in the squamous epithelial or vast majority of stromal cells. As expected only scattered stromal fibroblasts/macrophages are demonstrated.

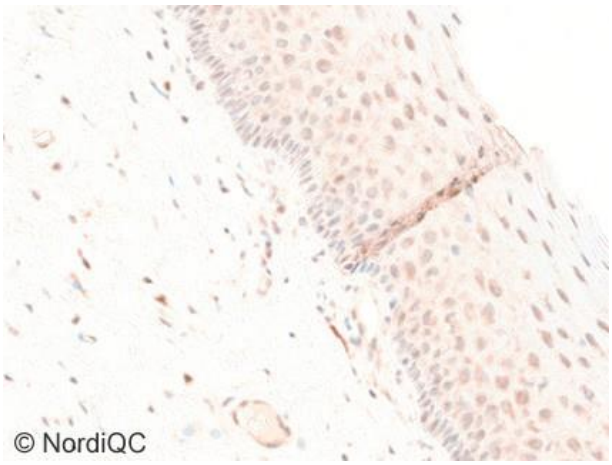


Fig. 4b
Insufficient staining reaction of the uterine cervix, using the Ventana RTU format 805-4713/825-4713 based on the mAb clone E6H4 as a laboratory modified protocol with a too long primary Ab incubation time (32 min.). Same protocol used in Figs. 5a-b. An aberrant false positive nuclear staining reaction is observed in virtually all cell types in both the epithelial layer and stromal compartment. Same area as in Fig. 4a.

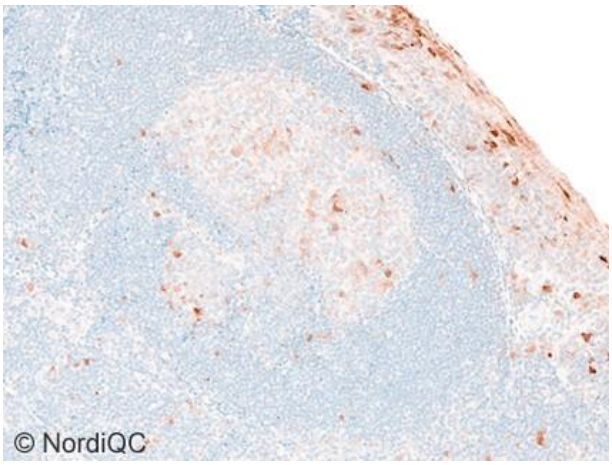


Fig. 5a
Staining reaction of tonsil, using same insufficient protocol as in Figs. 4b and 5b.
Dispersed germinal center macrophages/dendritic cells show a weak, nuclear and cytoplasmic staining reaction but a general and excessive background staining is seen in both the germinal centers and the squamous epithelial cells.

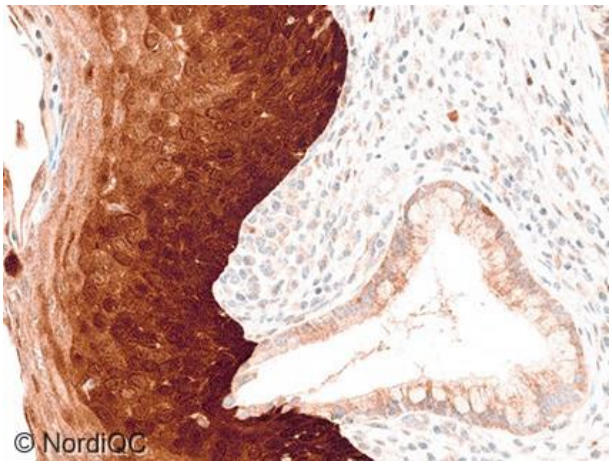


Fig. 5b
Insufficient p16 staining reaction of CIN3, tissue core no. 3, using same protocol as in Figs. 4b and 5a. A moderate to strong nuclear and cytoplasmic staining reaction is seen in all neoplastic cells throughout the cell layers – "block-positivity" but an excessive background reaction is displayed throughout the entire core with a positive nuclear staining reaction of the stromal cells and a positive cytoplasmic staining of the columnar epithelial cells.

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