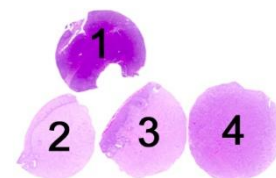


The slide to be stained for p16 comprised:

1. Tonsil, 2. Uterine cervix, 3. Uterine cervical high grade squamous intraepithelial lesion (HSIL), 4. Uterine cervical squamous cell carcinoma.

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 of scattered squamous epithelial cells in tonsillar lymphocyte rich areas of the reticulated crypt epithelium and in scattered follicular dendritic cells in the germinal centres.
- A moderate to strong nuclear and cytoplasmic staining in virtually all the neoplastic cells throughout the entire cell layer of the HSIL.
- A moderate to strong nuclear and cytoplasmic staining in the majority of the neoplastic cells of the uterine cervix squamous cell carcinoma.
- No staining in the normal cervical squamous epithelial cells.

A weak staining in scattered fibroblasts and columnar epithelial cells was accepted.

96 laboratories participated in this assessment. 90 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and scores for p16, run 26**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff. <sup>1</sup>	Suff. OPS <sup>2</sup>
mAb clone <b>JC8</b>	13 6 4 2 1 1	Santa Cruz Biocare Immunologic Diagnostic Biosystem ID Labs NeoMarkers	23	4	0	0	100 %	100 %
mAb clone <b>6H12</b>	7	Novocastra	0	4	3	0	57 %	100 %
mAb clone <b>G175-405</b>	6	Becton Dickinson	1	3	2	0	67 %	100 %
mAb clone <b>16P07</b>	2	NeoMarkers	1	0	0	1	-	-
mAb clone <b>16P04</b>	1	NeoMarkers	1	0	0	0	-	-
mAb clone <b>E6H4</b>	1	Dako	0	1	0	0	-	-
rmAb clone <b>EP4353Y</b>	2	Epitomics	0	0	1	1	-	-
pAb <b>abcam 7962</b>	1	Abcam	0	0	1	0	-	-
<b>Ready-To-Use Abs</b>								
mAb clone <b>E6H4</b> , (9511, 9517, 9512, 9518, 9521)	46	mtm Laboratories AG	16	30	0	0	100 %	100 %
mAb clone <b>E6H4</b> , OA315	1	Dako	0	1	0	0	-	-
mAb clone <b>16P04</b> , 760-4267	1	Ventana	0	1	0	0	-	-
mAb clone <b>6H12</b> , ZM0205	1	Zymed	0	0	1	0	-	-
<b>Total</b>	96		42	44	8	2	-	-
<b>Proportion</b>			44 %	46 %	8 %	2 %	70 %	100 %

1) Proportion of sufficient stains (optimal or good)

2) Proportion of sufficient stains with optimal protocol settings only, see below.

The following central protocol parameters were used to obtain an optimal staining:

### Concentrated Abs

mAb clone **JC8**: All protocols giving an optimal result were based on heat induced epitope retrieval (HIER) using Cell Conditioning 1 (BenchMark, Ventana) (9/9)\*, Tris-EDTA/EGTA pH 9 (6/7), Target Retrieval Solution pH 9 (FLEX TRS high pH, Dako, (6/6), Bond Epitope Retrieval Solution 2 (Bond, Leica) (1/1), or EDTA/EGTA pH8 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:25– 1:800 depending on the total sensitivity of the protocol employed. Using these protocol settings all of 23 (100 %) laboratories produced a sufficient staining (optimal or good).

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

mAb clone **G175-405**: The protocol giving an optimal result was based on HIER using Cell Conditioning 1 (BenchMark, Ventana) (1/1) as retrieval buffer. The mAb was diluted 1:25.

mAb clone **16P07**: The protocol giving an optimal result was based on HIER using Bond Epitope Retrieval Solution 2 (Bond, Leica) (1/1) as retrieval buffer. The mAb was diluted 1:640.

mAb clone **16P04**: The protocol giving an optimal result was based on heat induced epitope retrieval (HIER) using Cell Conditioning 1 (BenchMark, Ventana) (1/1)\* as retrieval buffer. The mAb was diluted of 1:600.

### Ready-To-Use Abs

mAb clone **E6H4**, prod. no 9511, 9517, 9512, 9518, 9521, MTM Laboratories: The protocols giving an optimal result were all based on HIER using Cell Conditioning 1 (BenchMark, Ventana) (6/15), Tris-EDTA/EGTA pH 9 (6/13), Bond Epitope Retrieval Solution 2 (Bond, Leica) (1/2), Bond Epitope Retrieval Solution 1 (Bond, Leica) (1/1), Citrate pH 6 (1/4) or "unknown" retrieval buffer (KIT) (1/6) as retrieval buffer. 12 out of the 16 labs obtaining an optimal result used the mAb as a RTU Ab, 4 labs used the mAb in the range 1:2- 1:10. Using these protocol settings all of 44 (100 %) laboratories produced a sufficient staining.

The most frequent causes of insufficient staining were:

- Less successful antibodies
- Insufficient HIER
- Too low concentration of the primary Ab.

In this assessment the prevalent feature of an insufficient staining was either a generally too weak staining or a staining with a poor signal-to-noise ratio. The former was typically characterized by only a faint cytoplasmic staining of the neoplastic cells of the HSIL and a significant reduction in the proportion and intensity of the cells expected to be demonstrated in the squamous cell carcinoma. This pattern was seen either when an otherwise successful clone (such as G175-405) was used too diluted, or a less successful clone (in this test) as the mAb clone EP4353Y. The insufficient result due to a poor signal-to-noise ratio was characterized by a positive reaction in the structures supposed to be identified, but at the same time accompanied by a general background reaction a diffuse staining in virtually all the normal cervical squamous and columnar epithelial cells.

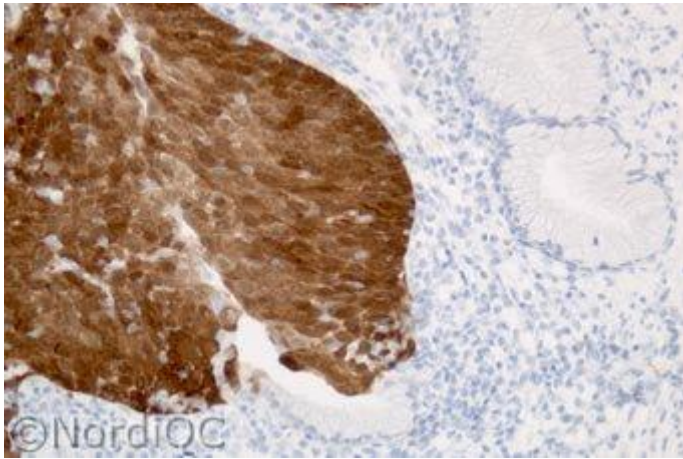
As positive and negative control for p16<sup>ink4a</sup> the HSIL and the normal cervix uteri was valuable providing the reaction pattern was obtained as described above in the "Criteria for assessing a p16<sup>ink4a</sup> staining". However it was also observed that the germinal centre follicular dendritic cells constantly in all the optimal protocols irrespective of the clone applied displayed a weak to moderate but distinct nuclear and cytoplasmic staining, which might indicate that these normal cells can be useful as critical quality staining indicator for p16<sup>ink4a</sup>. It was seen that the two most widely used clones JC8 from e.g. Santa Cruz and others and the clone E6H4 gave same high proportion of sufficient results (100 %). However it was unexpected, that the proportion of optimal results was higher, when the clone JC8 (85 %) was used and calibrated to an in-house assay compared to the number (35 %) obtained with the clone E6H4 and Ready-To-Use kit assay of this. In this context it has to be stressed, that only 23 laboratories (50 %) used the p16<sup>ink4a</sup> kit according to the instructions from the vendor. The p16<sup>ink4a</sup> kit from mtm laboratories AG was the only CE IVD labelled marker for p16<sup>ink4a</sup>, whereas the other markers were labelled as Research Use Only. It was only possible to find information about the clones and regulatory status of the individual markers at a few homepages of the companies. E.g. the clone JC8 could only be identified at the homepage from Santa Cruz.

### Conclusion

The mAb clones JC8, E6H4, G175-405, 16P04 and 16P07 are all recommendable markers for p16<sup>ink4a</sup>. The mAb clone E6H4 from mtm laboratories AG is the only CE IVD labelled marker for p16.

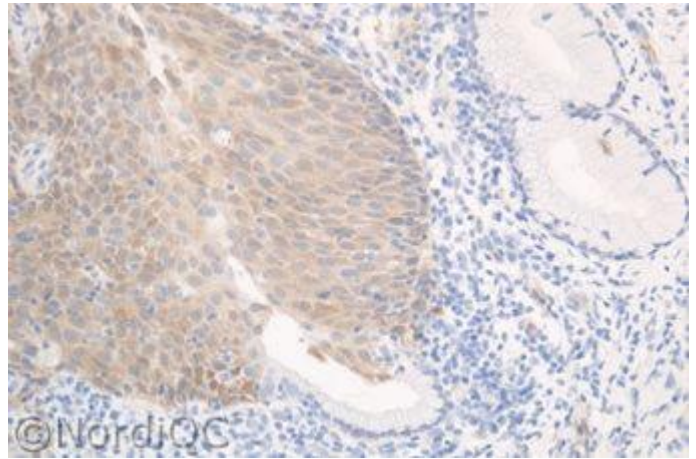
HIER is mandatory to obtain an optimal result.

Tonsil appears to be a recommendable control: The follicular dendritic cells must show an at least moderate nuclear and cytoplasmic staining, while no reaction should be seen in the germinal centre B-cells.



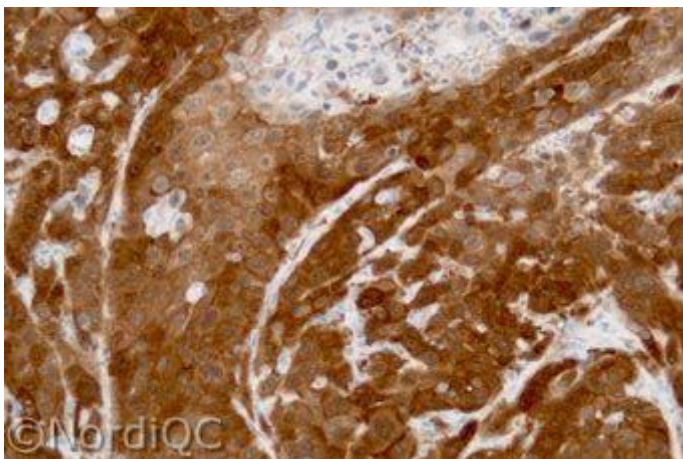
**Fig. 1a**  
Optimal staining for p16<sup>ink4a</sup> of the HSIL using the mAb clone E6H4 and CINtec™ kit according to the instructions from mtr laboratories AG.

Virtually all the neoplastic cells show a strong nuclear and cytoplasmic staining in a diffuse distribution. No background reaction is seen. Also compare with Figs. 2a – 4a – same protocol.



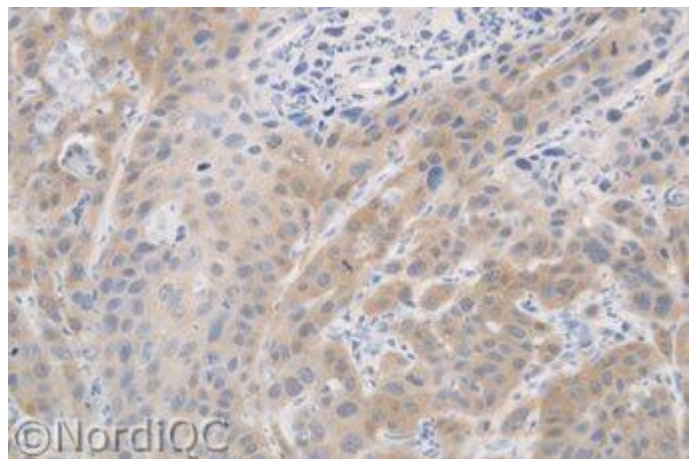
**Fig. 1b**  
Insufficient staining for p16<sup>ink4a</sup> of the HSIL using the rmAb clone EP4353Y in a less successful protocol - same field as in Fig. 1a.

The neoplastic cells only show a weak staining and at the same time a weak background reaction is seen. Also compare with Figs. 2b – 4b – same protocol.

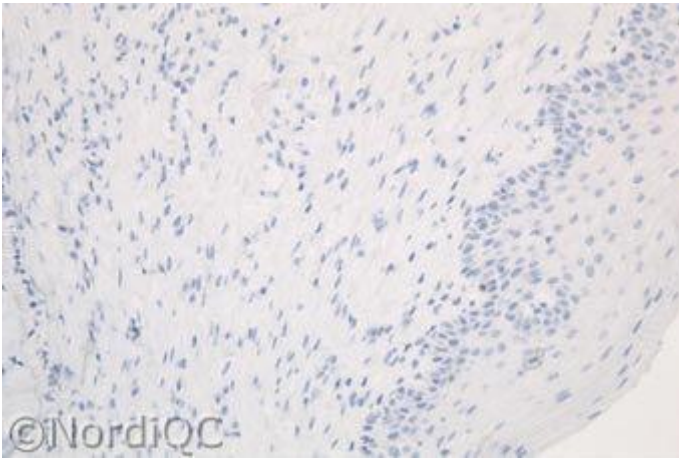


**Fig. 2a**  
Optimal staining for p16<sup>ink4a</sup> of the uterine cervical squamous cell carcinoma using same protocol as in Fig. 1a.

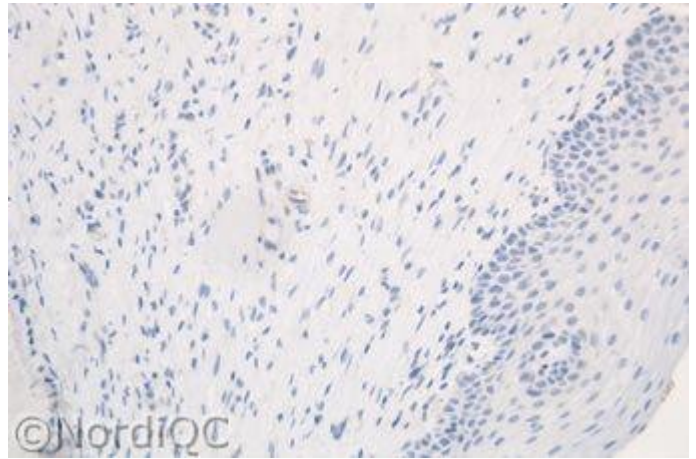
Virtually all the neoplastic cells show a strong nuclear and cytoplasmic staining.



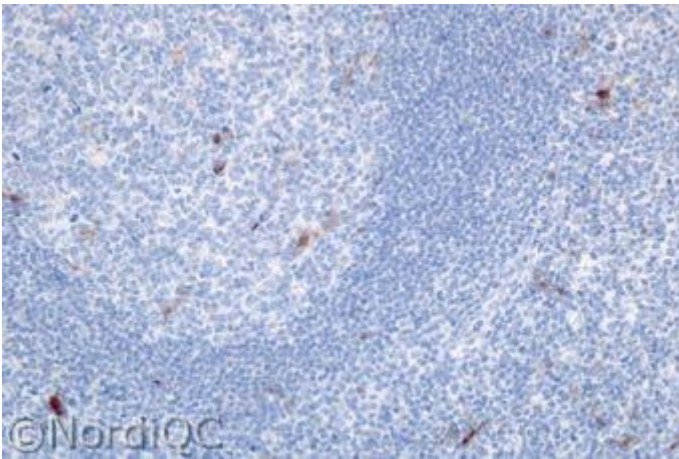
**Fig. 2b**  
Insufficient staining p16<sup>ink4a</sup> of the uterine cervical squamous cell carcinoma using same protocol as in Fig. 1b. - same field as in Fig. 2a. The majority of the neoplastic cells only show a weak staining.



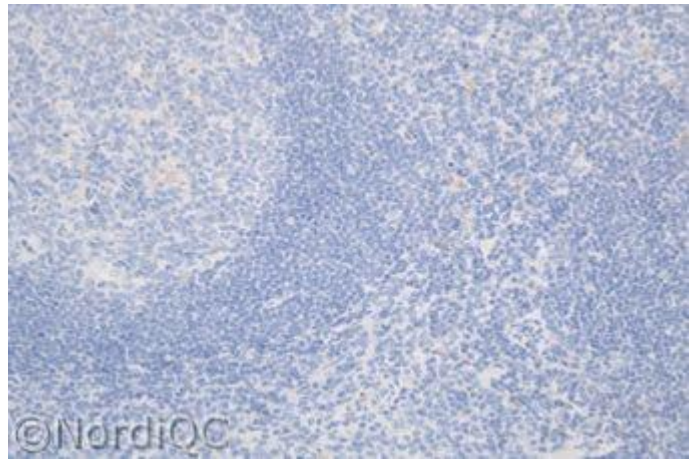
**Fig. 3a**  
Optimal staining for p16<sup>ink4a</sup> of the normal uterine cervix using same protocol as in Figs. 1a & 2a. Despite a strong staining in the HSIL and the uterine cervical squamous cell carcinoma, no reaction is seen in the normal squamous epithelial cells.



**Fig. 3b**  
Staining for p16<sup>ink4a</sup> of the normal uterine cervix using same insufficient protocol as in Figs. 1b & 2b - same field as in Fig. 3a. No staining is seen, however also compare with Figs. 1b & 2b.



**Fig. 4a**  
Optimal staining for p16<sup>ink4a</sup> of the tonsil using same protocol as in Figs. 1a - 3a. Scattered germinal centre macrophages show a distinct nuclear and cytoplasmic staining.



**Fig. 4b**  
Insufficient staining for p16<sup>ink4a</sup> of the tonsil using same protocol as in Figs. 1b - 3b, same field as in Fig. 4a. None or only a dubious reaction is seen in the germinal centre macrophages.

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