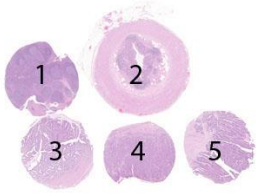
Assessment Run 49 2017
MLH1

Material
The slide to be stained for MLH1 comprised:



1. Tonsil, 2. Appendix, 3-4. Colon adenocarcinoma with loss of MLH1 expression, 5. Colon adenocarcinoma with normal MLH1 expression.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing MLH1 staining as optimal were:

- An at least weak to moderate distinct nuclear staining reaction of virtually all cells in the appendix.
- An at least weak to moderate distinct nuclear staining reaction of virtually all mantle zone B-cells and a moderate to strong nuclear staining reaction of the germinal centre B-cells.
- A moderate to strong nuclear staining in virtually all neoplastic cells of the colon adenocarcinoma no. 5.
- No nuclear staining reaction of neoplastic cells of the colon adenocarcinomas no. 3 and 4, but a distinct nuclear staining reaction in the majority of other cells (stromal cells, lymphocytes etc.).
- A weak cytoplasmic staining reaction was accepted.

Participation

Number of laboratories registered for MLH1, run 49	245
Number of laboratories returning slides	224 (91%)

Results
224 laboratories participated in this assessment, 59% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining were:
- Use of less successful primary antibodies – including mAb clone M1 showing aberrant nuclear staining
- Too low concentration of the primary antibody
- Use of less sensitive detection systems

Performance history
This was the fourth NordiQC assessment of MLH1. A significant decrease in pass rate to 59% was observed (see table 2).

Table 2. **Proportion of sufficient results for MLH1 in four NordiQC runs**

	Run 13 2005	Run 30 2010	Run 40 2014	Run 49 2017
Participants, n=	25	85	142	224
Sufficient results	72%	57%	73%	59%

This decrease may be explained by the high number of false positive staining reactions seen with the widely used mAb clone M1. 55 of 80 (69%) of the laboratories using mAb clone M1 experienced false positive nuclear staining reaction in neoplastic cells in at least one of the colon adenocarcinoma with known loss of MLH1.

Conclusion
The mAb clones **ES05**, **G168-15** and **GM011** could all be used to obtain an optimal staining for MLH1. In this assessment, the mAb clone ES05 was most successful, both as concentrate and as RTU format (Dako/Agilent and Leica/Novocastra). Irrespective of which of the three clones applied, HIER in an alkaline buffer and use of a sensitive and specific 3-step polymer/multimer based detection system gave the highest proportion of optimal results. The concentration of the primary antibody must be carefully calibrated.
In contrast to previous assessments, the mAb clone M1 could not be used to obtain optimal staining for MHL1. All the slides stained with mAb clone M1 showed – to some degree – aberrant nuclear staining in

the neoplastic cells in one of the colon adenocarcinomas known to lack MLH1 expression. This is the second report of aberrant nuclear staining with mAb clone M1[(1)] which should encourage not only laboratories and NordiQC but also Ventana/Roche to explore the nature of this aberrant nuclear staining. Tonsil is a recommendable positive tissue control for MLH1: Mantle zone B-cells must show an at least weak to moderate nuclear staining reaction, while a moderate to strong nuclear staining reaction must be seen in proliferating germinal centre B-cells. Tumour tissue, e.g. colon adenocarcinoma with loss of MLH1 expression must be used as negative tissue control, in which no nuclear staining reaction in the neoplastic cells should be seen.

Table 1. **Antibodies and assessment marks for MLH1, run 49**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **BS29**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **ES05**	28 20 1	Leica/Novocastra Dako/Agilent BD Pharmingen	20	22	6	1	86%	92%
mAb clone **G168-15**	9 4 2 1	BD Pharmingen Biocare Zytomed Maixin	7	4	3	2	67%	92%
mAb clone **G168-728**	5	Cell Marque	0	1	2	2	-	-
mAb clone **GM011**	4	Genemed Biotechnologies	3	1	0	0	-	-
mAb **M1**	1	Cell Marque	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **BS29 MAD-00726QD**	1	Master Diagnostica	0	1	0	0	-	-
mAb clone **ES05 IR079/IS079**	56	Dako/Agilent	32	14	9	1	82%	97%
mAb clone **ES05 PA0610**	6	Leica/Novocastra	3	1	1	1	67%	60%
mAb clone **ES05 AM703-5M/AM703-10M**	1	BioGenex	1	0	0	0	-	-
mAb clone **G168-15 PM220**	1	Biocare	0	1	0	0	-	-
mAb clone **G168-15 PDM 148**	1	Zytomed	0	0	1	0	-	-
mAb clone **G168-728 285M-17/285M-18/285M**	2	Cell Marque	0	1	0	1	-	-
mAb clone **G168-728 MAD-000372QD**	1	Master Diagnostica	0	1	0	0	-	-
mAb clone **M1 790-4535**	79	Ventana/Roche	0	17	61	1	22%	-
Total	224		67	65	83	9	-	
Proportion			30%	29%	37%	4%	59%	

1) Proportion of sufficient stains (optimal or good)
2) Proportion of sufficient stains with optimal protocol settings only, see below.

Detailed analysis of MLH1, Run 49
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **BS29**: One protocol with an optimal result was based on 20 min. HIER using Tris-EDTA / EGTA pH 9, 30 min. incubation of the primary Ab diluted 1:100 and BioSite Histo Plus HRP Polymer anti-Mouse (Nordic Biosite) (KDB-10007) as detection system.

mAb clone **ES05**: Protocols with optimal results were all based on HIER using Cell Conditioning 1 (CC1, Ventana) (4/19)*, Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (7/9), TRS pH 9 (Dako) (1/1), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (3/9), Tris-EDTA/EGTA pH 9 (1/3), TRS pH 6.1 (Dako) (1/2), Tris-HCl pH 9 (1/1), Bond Epitope Retrieval Solution 1 (BERS1, Leica) (1/3) or Citrate pH 6 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:10-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 44 of 48 (92%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **G168-15**: Protocols with optimal results were all based on HIER using CC1 (Ventana) (4/8) or BERS2 (Leica) (3/5), as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings, 11 of 12 (92%) laboratories produced a sufficient staining result.

mAb clone **GM011**: Protocols with optimal results were all based on HIER using TRS High pH (Dako) (3/4). The mAb was diluted 1:200 in Renoir Red and a 3-layer EnVision Flex (Dako GV800/GV823) detection system was employed. Using this protocol setting, 4 of 4 (100%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for MLH1 for the most commonly used antibodies as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako OMNIS**		**Ventana BenchMark GX / 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 **ES05**	6/8** (75%)	0/1	1/1	-	4/19 (21%)	-	3/9 (33%)	1/3
mAb clone **G168-15**	-	-	-	-	4/8 (50%)	-	3/5 (60%)	-

* 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 **ES05,** product no. **IR079/IS079,** Dako/Agilent, Autostainer Link / Classic:
Protocols with optimal results were typically based on 10-20 min. HIER using TRS High pH (3-1) (Dako), 20-30 min. incubation of the primary Ab and a 3-layer EnVision FLEX+ (Dako K8004/DM828) as detection system. Using these protocol settings, 30 of 31 (97%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **ES05,** product no. **PA0610,** Leica/Novocastra, BOND Max / BONDIII:
Protocols with optimal results were typically based on 10-30 min. HIER using Bond Epitope Retrieval Solution 2 (Leica) or Novocastra Epitope Retrieval Solutions pH 6 (Leica), 15-40 min. incubation of the primary Ab and a 3-layer Bond Refine (Leica DS9800) as detection system. Using these protocol settings, 3 of 5 (60%) laboratories produced a sufficient staining result.

Table 4 summarises 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 strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the specific IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for MLH1 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako Autostainer Classic/Link mAb ES05 **IR079/IS079**	94% 17/18	89% 16/18	89% 17/19	58% 11/19
VMS Ultra/XT mAb M1 **790-4535**	75% (3/4)	0% (0/4)	19% (14/74)	0% (0/74)

* 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 >25%, detection kit – only protocols performed on the specified vendor IHC stainer integrated.

Comments
In this assessment for MLH1 the prevalent features of insufficient results were false positive reactions or a too weak specific staining reaction. 67% of the insufficient results (60 of 90 laboratories) were due to false positive nuclear staining in one or both of the colon carcinomas with known loss of MLH1 or a general poor signal to noise ratio. This staining pattern was typically characterized by a diffuse and granular/dot like nuclear staining reaction of the neoplastic cells in parts of the two colon adenocarcinomas (especially tissue core no. 4) with loss of MLH1 expression and/or excessive cytoplasmic and background staining compromising interpretation. These staining patterns were seen for the mAb clones M1 (see Fig. 5a and Fig. 5b) and G168-728 (see Fig. 6b). The remaining 33% of insufficient results (30 of 90 laboratories) were characterized by weak or false negative staining reaction of the cells expected to be demonstrated. Most laboratories could demonstrate MLH1 in cells with high-level antigen expression as proliferating germinal centre B-cells in the tonsil, basal epithelial cells of the appendix and neoplastic cells in the colon adenocarcinoma with normal MLH1 expression. Demonstration of MLH1 in cells with low-level antigen expression as resting mantle zone B-cells, smooth muscle cells and stromal cells was more challenging and required an optimally calibrated protocol. Identification of loss of MLH1 expression in tumours is characterized by a negative nuclear staining reaction of the neoplastic cells. Consequently, it is of decisive importance that normal cells within and around the neoplastic cells show a distinct positive nuclear staining reaction, serving as reliable internal positive tissue control.

34% (76 of 224) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for MLH1. The mAb clone ES05 was the most widely used Ab for demonstration of MLH1 and provided a high proportion of sufficient staining results (see table 1). Optimal results could be obtained on all three main IHC systems from Dako/Agilent, Leica/Novocastra and Ventana/Roche using the clone as concentrate (see table 3). The highest proportion of optimal results was seen on the Dako Autostainer with 75%, compared to Leica Bond and Ventana Benchmark with 33% and 21% respectively, indicating that mAb clone ES05 could be easier to optimize on the Dako Autostainer. On all three platforms, efficient HIER in an alkaline buffer in combination with a sensitive non-biotin based detection system and a titre in the range of 1:10-1:100 were the main protocol prerequisites for optimal results. Especially use of 3-step polymer/multimer based detection systems seemed to provide higher proportions of optimal results compared to 2-step polymer/multimer based systems.
The mAb clone G168-15 as concentrated format could be used to obtain optimal results on both the Leica and Ventana systems, whereas no laboratories used the clone as concentrate on the Dako system. On both platforms, optimal protocols were all based on efficient HIER at high pH, antibody titre in the range of 1:25 to 1:100 and use of a 3-layer polymer/multimer-based system. Using these settings, the proportions of optimal were 60% (3 of 5) on the Leica Bond platform and 50% (4 of 8) on the Ventana BenchMark platform. The mAb clone G168-728 used as concentrate was found to be less successful, as a consistent aberrant cytoplasmic staining reaction was seen in nerves and endothelial cells often together with a too weak specific nuclear staining. In addition, an aberrant dot-like/granular nuclear staining reaction was seen in neoplastic cells in parts of the colon adenocarcinoma in tissue core no. 4 without MLH1 expression (see Fig. 6b).
The recently introduced mAb clone GM011 showed promising results. All four laboratories using this clone achieved sufficient staining results with 3 assessed as optimal.

66% (148 of 224) of the laboratories used Abs in Ready-To-Use formats. The most widely used RTU systems for MLH1 were the Ventana 790-4535 system based on the mAb clone M1, and the Dako IR079/IS079 system, based on mAb clone ES05 and tailored for the Dako Autostainer.
In this assessment and in concordance with previous assessments, the Dako IR079/IS079 system provided the highest pass rate and proportion of optimal results (see table 1). Used according to the recommended protocol settings, the IR079/IS079 system had a pass rate of 94%, 89% optimal (see table 4). Lacking a RTU alternative tailored for the Dako Omnis platform, 16 laboratories used the IR079/IS079 system on the Dako Omnis. Despite using similar protocol settings as recommended for the Dako Autostainer, the pass rate dropped to 63%, with an optimal proportion of only 25%. These data suggest that the use of the IR079/IS079 system on the Dako Omnis platform within a laboratory developed (LD) assay, requires profound adjustment of the protocol settings and a "direct" transfer of the original Autostainer protocol cannot be applied (see Fig 1 – Fig 4).
50% of the laboratories using RTU systems, used the Ventana 790-4535 RTU system based on the mAb clone M1, but contrary to previous assessments the pass rate was very low. Only 22% of the laboratories obtained sufficient result and none an optimal score. The main reason was an unexpected aberrant nuclear staining reaction of neoplastic cells in the colon adenocarcinoma tissue core no. 4, a tumour known not to express MLH1. The aberrant nuclear staining was primary seen in certain areas of the tumour. These areas generally accounted for more than 10% of the neoplastic cells. Occasionally the aberrant staining reaction was both diffuse and granular/dot-like in appearance (see Fig. 5a), whereas other cases only showed a dot-like nuclear staining reaction (see Fig. 5b). In previous NordiQC assessments aberrant nuclear staining

have not been observed with mAb clone M1, but the Canadian Immunohistochemistry Quality Control program (cIQc) reported in their MLH1 assessment in 2014: *"Four different clones were used. Clone M1 did appear to give false positive staining of core 4, for some labs"* [1]. No explanation was given for this reported false positive staining. Likewise, no explanation can be given for the aberrant nuclear staining in the neoplastic cells in colon adenocarcinoma no. 4 seen in the recent NordiQC assessment. More than 10 different lot. no. were used in this assessment and to some degree they all showed aberrant nuclear staining in core no 4. In the previous NordiQC MLH1 assessment in 2014, optimal results were typically obtained by modified and laboratory validated protocol settings using UltraView +amplification or OptiView +/-amplification as detection system compared to the recommendations given in the package inserts for the RTU format. In contrast, the present assessment shows a very poor pass rate in general, but especially for the modified protocols using more sensitive protocol settings than the recommendations given for the RTU format. Typically, sufficient staining results could only be achieved using the recommended protocol setting or with minor modifications (see table 4). The sufficient protocols were typically based on HIER in CC1 for 40-64 min., incubation in RTU Ab for 16-32 min. and UltraView +/-amplification or OptiView as detection system. With these settings, it was possible to reduce the problem with aberrant nuclear staining in the neoplastic cells to an acceptable level (dot like and weak) and number (less than 10%), but at the expense of a reduced staining intensity of stromal cells and lymphocytes compared to optimal protocols with one of the other mAb e.g. clone ES05, GM011 or G168-15 (see Fig. 4a, Fig. 5b and Fig. 6a). More data must be generated to elucidate on this aberrant nuclear staining reaction.

Controls

Tonsil was found to be a recommendable positive tissue control for MLH1. Virtually all mantle zone B-cells must show at an at least weak to moderate nuclear staining reaction, while a moderate to strong nuclear staining reaction must be seen in the proliferating germinal centre B-cells.

Colon adenocarcinoma with loss of MLH1 expression is recommended as negative tissue control for MLH1. No nuclear staining reaction should be seen in the neoplastic cells, whereas a nuclear staining reaction must be seen in stromal cells.

[1] B Gilks, J Garratt and E Torlakovic. Assessors' report for cIQc Run 38: MMR immunostaining (May 2014). Canadian Immunohistochemistry Quality Control Program

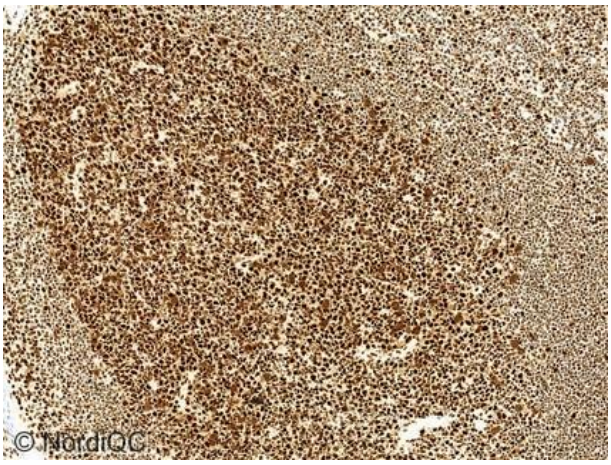


Fig. 1a (x100)
Optimal staining reaction for MLH1 of the tonsil using the mAb clone ES05 in a RTU format (IR079/IS079) on the Dako Omnis instrument. With careful calibration of the "Autostainer-RTU"-system (IR079/IS079) e.g. prolonged incubation times in HIER and primary Ab the system can produce optimal result on the Dako Omnis. Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the germinal centre B-cells show a strong nuclear staining reaction.
Also compare with Figs. 2a, 3a and 4a, same protocol.

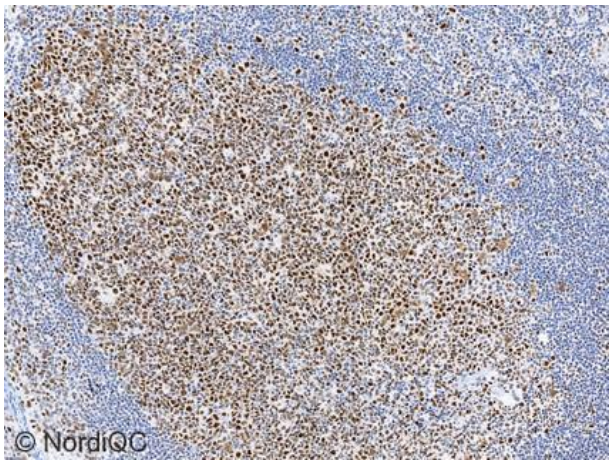


Fig. 1b (x100)
Insufficient staining reaction for MLH1 of the tonsil using the mAb clone ES05 in a RTU format (IR079/IS079) as "plug-and-play" on the Dako Omnis. A simple transfer of the Dako Autostainer protocol to the Dako Omnis, results in a protocol with too low sensitivity.
Compare with Fig. 1a - same field. Only the germinal centre B-cells are demonstrated, while the mantle zone B-cells expressing a low level of MLH1 are virtually unstained.
Also compare with Figs. 2b, 3b and 4b, same protocol.

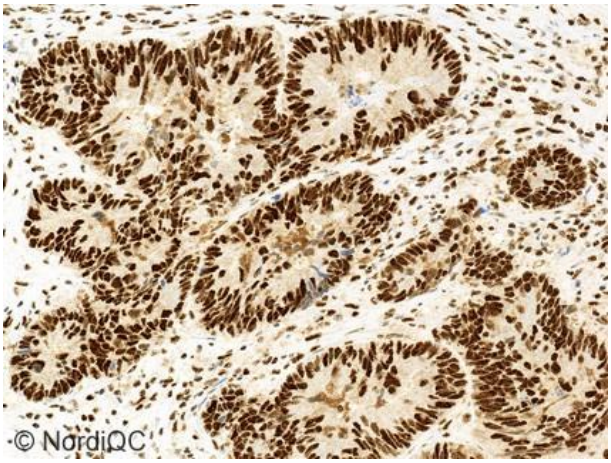


Fig. 2a (x200)
Optimal staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 5, with normal MLH1 expression using same protocol as in Fig. 1a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction. A high signal-to-noise ratio is obtained. No background staining is seen and a distinct nuclear staining reaction in the stromal cells is seen.

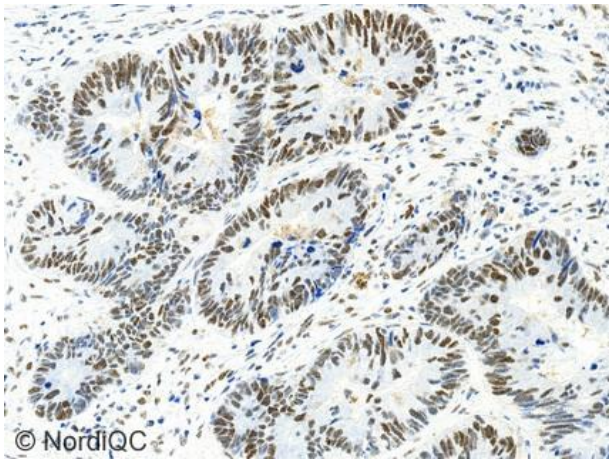


Fig. 2b (x200)
Insufficient staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 5, using same protocol as in Fig. 1b - same field as in Fig. 2a. The proportion of positive cells and the intensity of the staining reaction is reduced compared to the result in Fig. 2.
Also compare with Fig. 3b and 4b, same protocol.

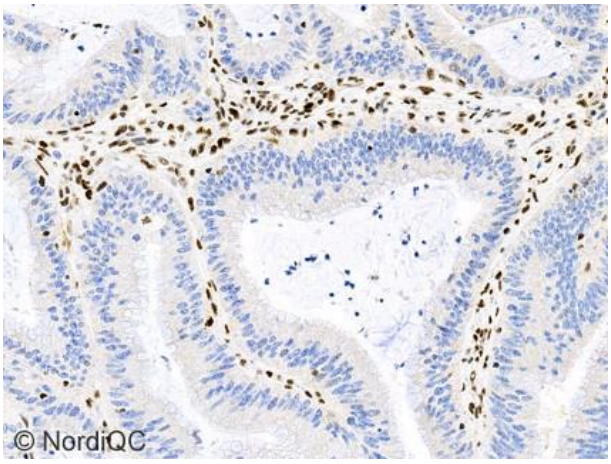


Fig. 3a (x200)
Optimal staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 3, with loss of MLH1 using same protocol as in Figs. 1a and 2a. The neoplastic cells are negative, while lymphocytes and stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

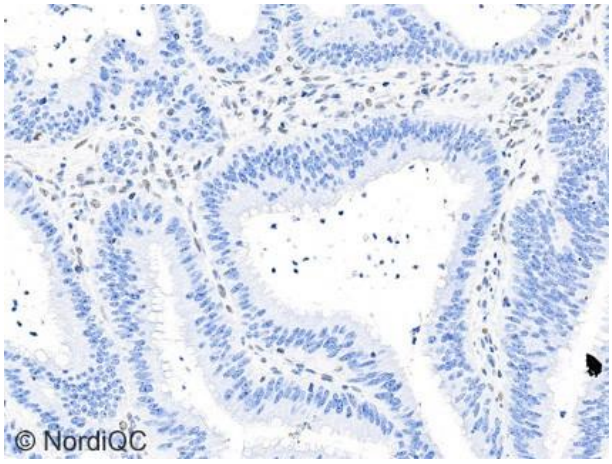


Fig. 3b (x200)
Insufficient staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 3, with loss of MLH1 using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
No staining reaction in the neoplastic cells is seen, but as virtually no nuclear staining reaction is seen in the normal stromal cells, the staining pattern can't reliably be interpreted.

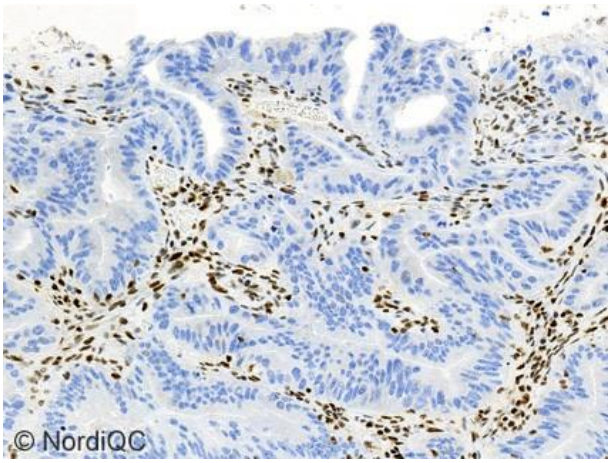


Fig. 4a (x200)
Optimal staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 4, with loss of MLH1 using same protocol as in Figs. 1a - 3a.
The neoplastic cells are negative, while lymphocytes and stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

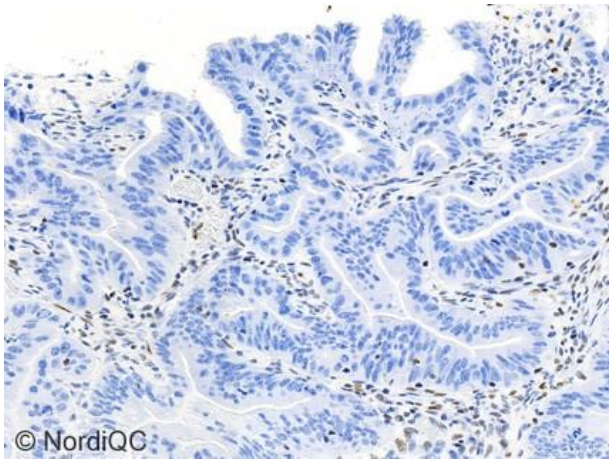


Fig. 4b (x200)
Insufficient staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 4, with loss of MLH1 using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
No staining reaction in the neoplastic cells is seen, but as also virtually no nuclear staining reaction is seen in the normal stromal cells, the staining pattern can't reliably be interpreted.

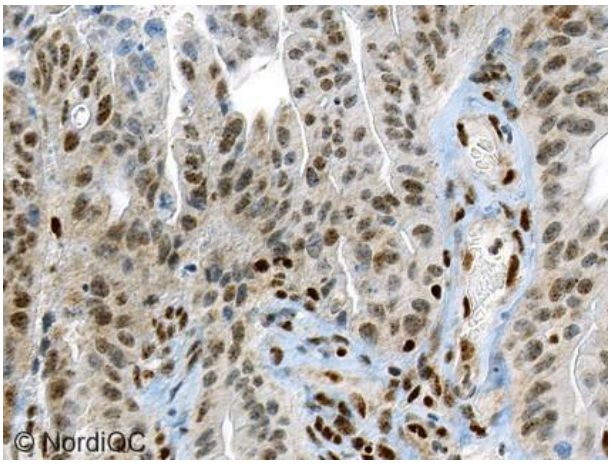


Fig. 5a (x400)
Insufficient MLH1 staining of the colon adenocarcinoma no. 4 with loss of MLH1 using the mAb clone M1 in a RTU format (790-4535) on the Ventana BenchMark platform in **a sensitive 3-layer detection system** with tyramide amplification (OptiView with Amp). Besides the expected nuclear staining in lymphocytes and stromal cells, a strong aberrant nuclear staining (both diffuse and granular/dot like) is seen in the neoplastic cells in larger areas of this tumour.

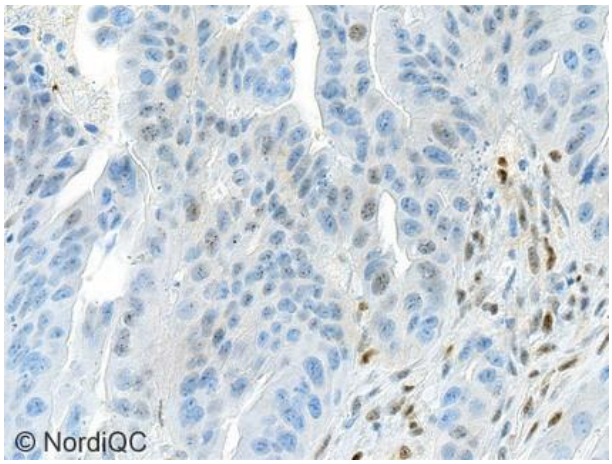


Fig. 5b (x400)
Sufficient (Good) MLH1 staining of the colon adenocarcinoma no. 4 with loss of MLH1 using the mAb clone M1 in a RTU format (790-4535) on the Ventana BenchMark platform in a **2-layer detection system** (UltraView). The use of a less sensitive detection system reduces the problem with aberrant nuclear staining in the neoplastic cells to an acceptable level (weak and dot like) and number, but at the same time reduces the staining intensity of stromal cells and lymphocytes compared to an optimal protocol (see Fig. 6a). Compare also with Fig. 5a (same field)

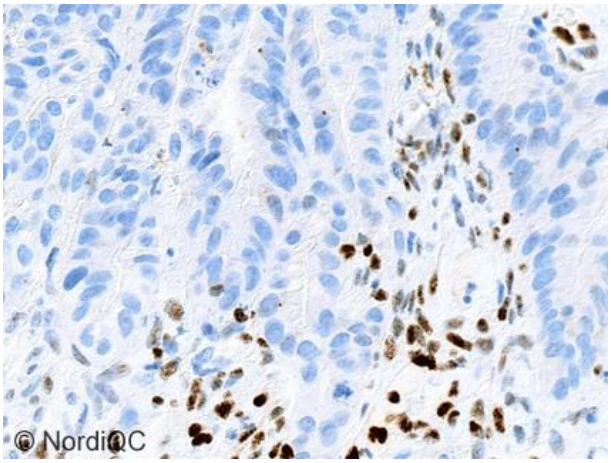


Fig. 6a (x400)
Optimal staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 4, with loss of MLH1 using mAb G168-15 in an optimally calibrated protocol on the Ventana BenchMark platform. The protocol was similar to the protocol used in Fig. 5a; **a sensitive 3-layer detection system** with tyramide amplification (OptiView with Amp).
No aberrant nuclear staining is seen in the neoplastic cells, only lymphocytes and stromal cells show nuclear staining reaction.
Compare with Fig. 5a and Fig. 5b (same field).

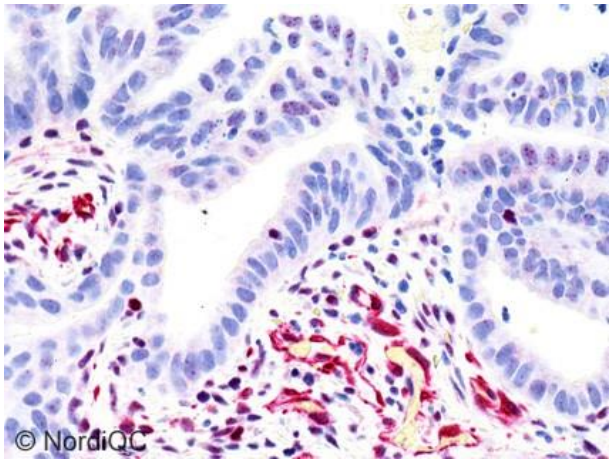


Fig. 6b (x400)
Insufficient staining reaction for MLH1 of the colon adenocarcinoma, tissue core no. 4, with loss of MLH1 using the mAb clone G168-728. An aberrant cytoplasmic staining reaction in e.g. macrophages and endothelial cells generally complicates the interpretation, as the nuclear staining reaction in the stromal cells cannot be identified with certainty. This staining pattern was frequently seen for the mAb clone G168-728. In addition the interpretation is compromised by an aberrant dot like nuclear staining reaction (in this case weak) in the neoplastic cells in larger areas of the adenocarcinoma, tissue core no. 4, very similar to the observations with mAb clone M1. Compare with Fig. 5a, 5b and 6a.

ON/SN/LE/RR 14.04.2017