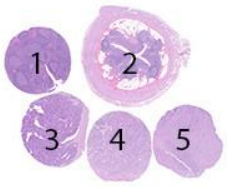
Assessment Run 52 2018

Mismatch repair protein MSH6 (MSH6)

Material
The slide to be stained for MSH6 comprised:



1. Tonsil 2. Appendix 3. Colon adenocarcinoma with normal MSH6 expression, 4-5. Colon adenocarcinomas with loss of MSH6 expression.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing MSH6 staining as optimal included:

- 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, distinct nuclear staining reaction of the germinal centre B-cells in the tonsil
- A moderate to strong, distinct nuclear staining reaction of virtually all neoplastic cells in the colon adenocarcinoma no. 3
- No nuclear staining reaction of the neoplastic cells in the colon adenocarcinomas no. 4 and 5, but a distinct nuclear staining reaction in the vast majority of other cells (stromal cells, lymphocytes etc).

A general weak cytoplasmic staining reaction was accepted.

Participation

Number of laboratories registered for MSH6, run 52	253
Number of laboratories returning slides	242 (96%)

Results
242 laboratories participated in this assessment. 127 (52%) of these 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 the less successful mAb clone 44
- Use of less sensitive detection systems
- Too low concentration of the primary antibody
- Insufficient Heat Induced Epitope Retrieval (HIER)

Performance history
This was the third NordiQC assessment of MSH6. A significant decrease of the pass rate was seen compared to run 43, 2015.

Table 2. **Proportion of sufficient results for MSH6 in the two NordiQC runs performed**

	Run 32 2011	Run 43 2015	Run 52 2018
Participants, n=	90	153	242
Sufficient results	33%	63%	52%

Conclusion
Optimal staining results could be obtained with the rmAb clones **BSR100, EP49, EPR3945** and **SP93.** Irrespective of the clone applied, efficient 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. The concentrated format of the rmAb clone **EP49** provided optimal staining results on the four main stainer platforms - Omnis (Dako), Autostainer (Dako), Bond (Leica) and BenchMark (Ventana).
Extremely low proportion of sufficient staining results was seen with the mAb clone 44, both as concentrate in laboratory developed (LD) assays and as Ready-To-Use (RTU) systems. In total, only 5% (4 of 85) achieved sufficient staining results with none being optimal, emphasizing the need for many laboratories to consider a change of clone in their MSH6 assays for diagnostic use.
The rmAb clone EP49 based Dako Autostainer RTU system provided a high proportion of sufficient results. Using the recommended protocol settings, the RTU system was the most successful assay with an overall pass rate of 100% with 65% being optimal.
The recently introduced rmAb clone SP93 based Ventana Benchmark RTU system was used by 2

laboratories. Using the recommended protocol settings, both achieved optimal staining results, indicating this RTU system being the obvious alternative to the "old" mAb clone 44 based RTU system on the Ventana BenchMark system.

Tonsil is recommendable as positive tissue control for MSH6. Mantle zone B-cells must show an at least weak to moderate, distinct nuclear staining reaction, while moderate to strong, distinct nuclear staining reaction must be seen in proliferating germinal centre B-cells. Tumour tissue, e.g. colon adenocarcinoma with loss of MSH6 expression must be used as negative tissue control, in which no nuclear staining reaction of the neoplastic cells must be seen. Stromal cells within the tumour tissue must exhibit distinct nuclear staining reaction serving as internal positive tissue control.

Table 1. **Antibodies and assessment marks for MSH6, run 52**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **44**	5 4 2 1	Bicare BD Biosciences Cell Marque Im Path	0	1	4	7	8%	-
mAb clone **PU29**	5	Leica/Novocastra	0	0	4	1	0%	-
rmAb clone **BSR100**	2	Nordic Biosite	2	0	0	0	-	-
rmAb clone **EP49**	30 27 1	Dako/Agilent Epitomics Immunologic	31	13	7	7	76%	78%
rmAb clone **EPR3945**	6 1 1 1	Abcam Epitomics Thermo Scientific Unknown	4	1	4	0	56%	57%
rmAb clone **SP93**	5 3	Cell Marque Spring Bioscience	4	1	2	1	63%	100%
Ready-To-Use antibodies								
mAb clone **2D4B5 AM454-10M**	1	Biogenex	0	0	1	0	**-**	**-**
mAb clone **44 790-4455**	67	Ventana/Roche	0	3	38	26	4%	-
mAb clone **44 287M**	2	Cell Marque	0	0	0	2	-	-
mAb clone **44 PDM 147**	3	Diagnostic Biosystems	0	0	3	0	**-**	**-**
mAb clone **44 BSB6143**	1	BIOSB	0	0	0	1	-	-
rmAb clone **EP49 IR086**	43	Dako/Agilent	28	13	2	0	95%	95%
rmAb clone **EP49 IR086**[3]	18	Dako/Agilent	2	13	3	0	83%	-
rmAb clone **EP49 IR086**[4]	6	Dako/Agilent	4	1	1	0	83%	-
rmAb clone **EP49 MAD-000635QD**	2	Master Diagnostica	2	0	0	0	-	-
rmAb clone **EP49 RAM-0770**	1	Maixin	1	0	0	0	-	-
rmAb clone **SP93 287R**	2	Cell Marque	1	0	0	1	-	-
rmAb **SP93 790-5092**	2	Ventana/Roche	2	0	0	0	-	-
Total	242		81	46	69	46	-	
Proportion			33%	19%	29%	19%	52%	

1) Proportion of sufficient stains (optimal or good),
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) RTU system developed for the Dako/Agilent semi-automatic system (Dako Autostainer) but used by laboratories on the full-automatic Dako Omnis system.
4) RTU system developed for the Dako/Agilent semi-automatic system (Dako Autostainer) but used by laboratories on different platforms (e.g. Ventana Benchmark and Biocare Intellipath).

Detailed analysis of MSH6, Run 52
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
rmAb clone **BSR100**: Protocols with optimal results were all based on HIER using Bond Epitope Retrieval Solution 2 (BERS2; Leica) (1/1)* or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:100-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 2 of 2 (100%) laboratories produced an optimal staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **EP49**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako/Agilent) (2/3), TRS pH 9 (Dako/Agilent) (5/5), Cell Conditioning Solution 1 (CC1, Ventana/Roche) (16/33), BERS2 (Leica) (6/13), EDTA pH 7.8 (1/1) or Tris-EDTA/EGTA pH 9 (1/2) as retrieval buffer. The rmAb was typically diluted in the range of 1:20-1:250 depending on the total sensitivity of the protocol employed. Using these protocol settings, 43 of 55 (78%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EPR3945**: Protocols with optimal results were all based on HIER using CC1 (Ventana/Roche) (3/6) or BERS2, (Leica) (1/1). The rmAb was diluted in the range of 1:25-1:1,000 depending on the total sensitivity of the protocol employed. Using these protocol settings, 4 of 7 (57%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **SP93**: Protocols with optimal results were all based on HIER using CC1 (Ventana/Roche) (4/4) as retrieval buffer. The rmAb was diluted in the range of 1:20-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 4 of 4 (100%) laboratories produced an optimal staining result.

Table 3. **Proportion of optimal results for MSH6 for the most commonly used antibodies as concentrates on the 4 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
rmAb clone **EP49**	2/3 **	0/1	5/5 (100%)	-	16/33 (48%)	-	6/13 (46%)	-
rmAb clone **EPR3945**	-	-	0/1	-	3/6 (50%)	-	1/1	-
rmAb clone **SP93**	-	-	-	-	4/4 (100%)	-	0/2	-

* 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)

Ready-To-Use antibodies and corresponding systems
rmAb clone **EP49**, product no. **IR086**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (efficient heating time 10-30 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (Dako/Agilent, K8000/K8002) as detection system. Using these protocol settings 40 of 42 (95%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP49**, product no. **RMA-0770**, Maixin, manual:
One protocol with an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 (Waterbath) for 20 min., 60 min. incubation of the primary Ab and MaxVision III DAB (Maixin) as detection system.

rmAb clone **EP49**, product no. **MAD-000635QD**, Master Diagnostica, MD-Stainer:
One protocol with an optimal result was based on HIER using Tris-EDTA/EGTA pH 9 for 20 min., 45 min. incubation of the primary Ab and Master Polymer Plus (Master Diagnostica, MAD-000230QP) as detection system.

rmAb clone **SP93**, product no. **760-5092**, Ventana/Roche, Ventana Benchmark GX/XT/Ultra:
Two protocols with optimal results were using identical protocol settings based on HIER in CC1 (Ventana/Roche) (efficient heating time for 64 min. at 100°C), 12 min. incubation at 36°C of the primary Ab and OptiView (Ventana/Roche, 760-700) as detection system.

Table 4 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 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 MSH6 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS mAb clone **44, 790-4455**	0% (0/2)	0% (0/2)	5% (3/65)	0% (0/65)
Dako AS rmAb clone **EP49, IR086**	100% (20/20)	65% (13/20)	91% (20/22)	69% (15/22)
VMS rmAb clone **SP93, 760-5092**	100% (2/2)	100% (2/2)	-	-

* 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 and in concordance with the observations in the previous assessment of MSH6, run 43 2015, the prevalent feature of an insufficient staining reaction was a too weak or false negative nuclear staining reaction of the majority of the cells expected to be demonstrated. Too weak or false negative staining reaction was seen in 89% of the insufficient results (102 of 115) The majority of the laboratories was able to demonstrate MSH6 in cells with high-level antigen expression as proliferating germinal centre B-cells and basal epithelial cells of the appendix, whereas demonstration of MSH6 in cells with low antigen expression as resting mantle zone B-cells, smooth muscle cells and stromal cells could only be obtained by an optimally calibrated protocol (see Fig. 1-7). In this context, it has to be emphasized that identification of loss of MSH6 in tumours is characterized by a negative staining reaction of the neoplastic cells, consequently it is of decisive importance that the normal cells among and around the neoplastic cells show a distinct positive nuclear staining reaction, serving as internal positive control. In the remaining 11% of the insufficient results both a too weak specific staining reaction and a poor signal to noise ratio or an excessive background staining (compromising interpretation) was seen. This staining pattern was in particular observed for the mAb clone 44 (see Fig. 4b).

39% (94 of 242) of the laboratories used Abs as concentrated format within laboratory developed (LD) assays for MSH6. Optimal staining result could be obtained with the rmAbs clones BSR100, EP49, EPR3945 and SP93 (see Table 1). Irrespective of the clone applied, careful calibration of the titre, efficient HIER at high pH. were the main protocol prerequisites for optimal results. Both 2- and 3-step polymer/multimer based detection systems could be used to provide an optimal result, but 3-step polymer/multimer based detection systems provided the highest proportion of optimal staining result at 61% (31 of 51) compared to 38% (10 of 26) for the 2-step polymer/multimer detection systems. The rmAb clone EP49 was the most widely used Ab for demonstration of MSH6 and provided a high proportion of sufficient staining results. Optimal results could be obtained on all four main IHC systems from Dako, Leica and Ventana (see Table 3). In contrast, no optimal staining result was registered with the mAbs clones 44, PU29 and 2D4B5.

61% (148 of 242) of the laboratories used Abs in Ready-To-Use (RTU) formats. This was a minor increase compared to the previous MSH6 assessment in 2015, where 53% of the laboratories used the RTU format. The most widely used RTU systems for MSH6 were the mAb clone 44 based **790-4455** from Ventana/Roche intended for use on the Ventana BenchMark System and the rmAb EP49 based **IR086** from Dako/Agilent intended for use on the Dako Autostainer System. In concordance with previous assessments, the proportion of sufficient staining results was extremely low for the 790-4455 system (see Figs. 5-7). 67 laboratories used the 790-4455 RTU system and only 4% (3 of 67) achieved sufficient staining results (see Table 1). The good news is that the newly introduced rmAb clone SP93 based **790-5092** system intended for use on the BenchMark system seems to exhibit higher analytic sensitivity than the old RTU system. Two laboratories used the new 790-5092 RTU system and using the recommended protocol settings, both achieved optimal staining results (see Figs. 5-7).
The Ready-To-Use (RTU) system from Dako based on the rmAb clone EP49 gave a high proportion of sufficient and optimal results. Optimal results for the Dako RTU system could both be obtained by using the official protocol recommendations given by Dako but also by laboratory modified protocol settings (typically adjusting incubation time of the primary Ab and/or choice of detection system). Using the recommended protocol settings, the rmAb clone EP49 based Dako Autostainer RTU system was the most successful assay with an overall pass rate of 100% with 65% being optimal. The **IR086** RTU system was developed for the Dako Autostainer system but frequently used on other systems. In the current assessment, 18 laboratories used IR086 on the Dako Omnis system. The proportion of sufficient results was acceptable at 83% (15 of 18) compared to 95% (41 of 43) on the Dako Autostainer system, but the

proportion of optimal staining results was only 11% (2 of 18) compared to 65% (28 of 43) on the Dako Autostainer system. Data collected from the Dako Omnis users suggest that the protocol settings must be modified if optimal staining results should be achieved using the IR086 on the Dako Omnis system. HIER at high pH should be prolonged to 30-45 min. and a 3-step polymer based (EnVision Flex+) detection system must be used instead of the recommended 2-step polymer based (EnVision Flex) detection system. These observations underlines that used on other systems than developed for, RTU Abs must be considered as a LD assay, thus requiring a thorough technical calibration and diagnostic validation before used in the daily diagnostic practice.

This was the third assessment of MSH6 in NordiQC (see Table 2). The pass rate dropped to 52% compared to 63% in run 43 in 2015. The reason for this decrease in pass rate is unclear, but many new laboratories participated in the current MSH6 assessment and for laboratories participating for the first time (n=107), the pass rate was only 44%. In concordance with previous MSH6 assessments the primary reason for the generally poor pass rate was the large number of laboratories still using less successful primary antibodies as mAbs clone 44 and clone PU29. Grouped together, these clones obtained an overall pass rate of 4% (4 of 90) with none being optimal.

Controls
Tonsil was found to be a recommendable positive tissue control for MSH6. Virtually all 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 the proliferating germinal centre B-cells.
Colon adenocarcinoma with loss of MSH6 expression is recommended as negative tissue control. No nuclear staining reaction should be seen in the neoplastic cells, whereas a nuclear staining reaction must be seen in stromal cells serving as internal positive tissue control.

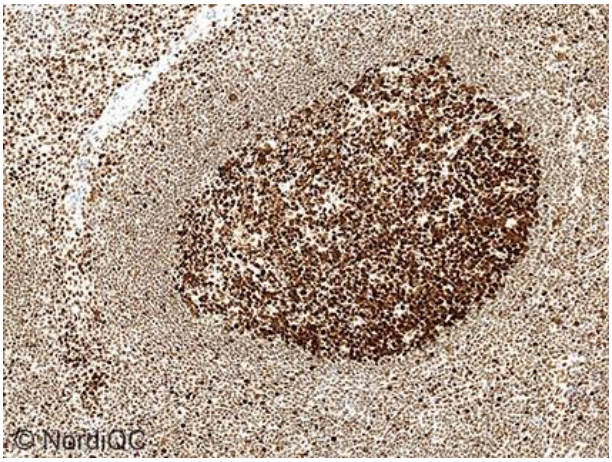


Fig. 1a
Optimal MSH6 staining reaction of the tonsil using the rmAb clone EP49, optimally calibrated, HIER in an alkaline buffer (BERS2, Leica) and a 3-step polymer-based detection system (Refine, Leica). Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the germinal centre B-cells show a strong nuclear staining reaction. Also compare with Figs. 2a - 4a, same protocol.

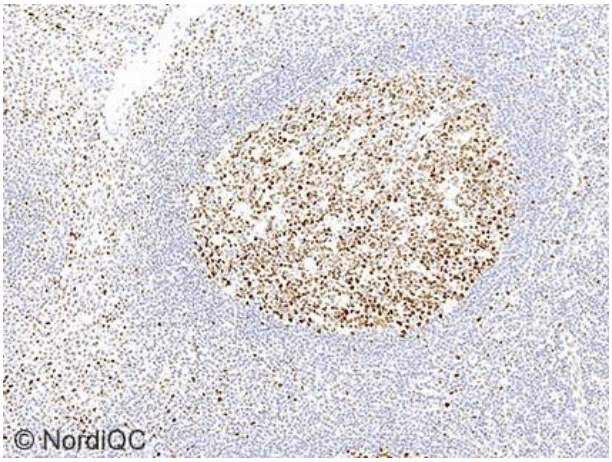


Fig. 1b
Insufficient MSH6 staining reaction of the tonsil using the rmAb clone EP49 with a protocol providing too low analytic sensitivity - same field as in Fig. 1a. Only the germinal centre B-cells are distinctively demonstrated, while mantle zone B-cells expressing low level MSH6 virtually are unstained. This protocol was also based on HIER in an alkaline buffer (BERS2, Leica) and a 3-step polymer-based detection system (Refine, Leica). Too low concentration of the primary antibody in combination with too short HIER time resulted in insufficient staining result. Also compare with Figs. 2b - 3b, same protocol.

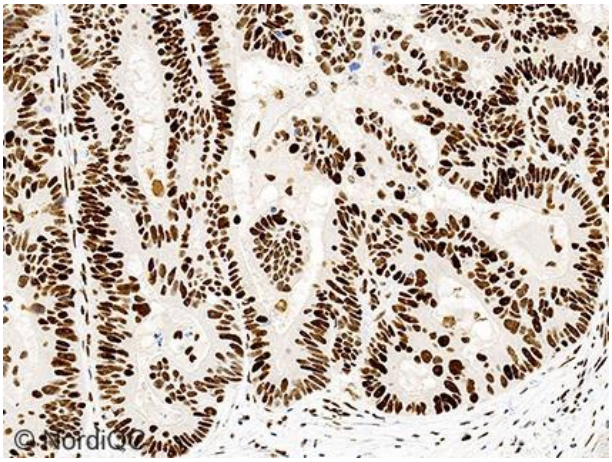


Fig. 2a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 3, with normal MSH6 expression using same protocol as in Fig. 1a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction. A high signal-to-noise ratio is obtained with only faint cytoplasmic staining. No background staining is seen and a distinct nuclear staining reaction in the stromal cells is seen.

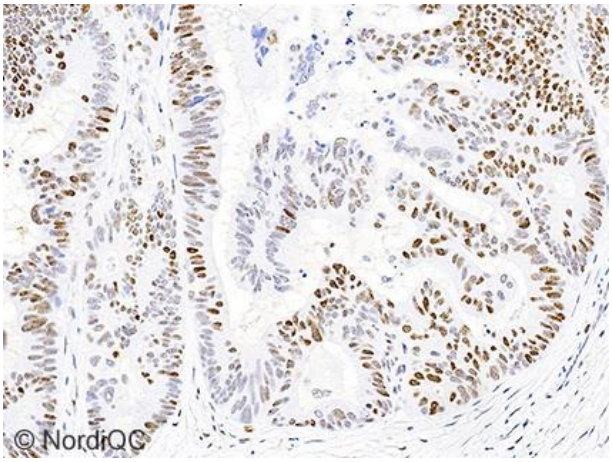


Fig. 2b
MSH6 staining reaction for of the colon adenocarcinoma, tissue core no. 3, using same protocol as in Fig. 1b - same field as in Fig. 2a. The proportion and the intensity of cells demonstrated is reduced compared to the result expected and shown in Fig. 2a. Also note that stromal cells are virtually negative. Also compare with Fig. 3b, same protocol.

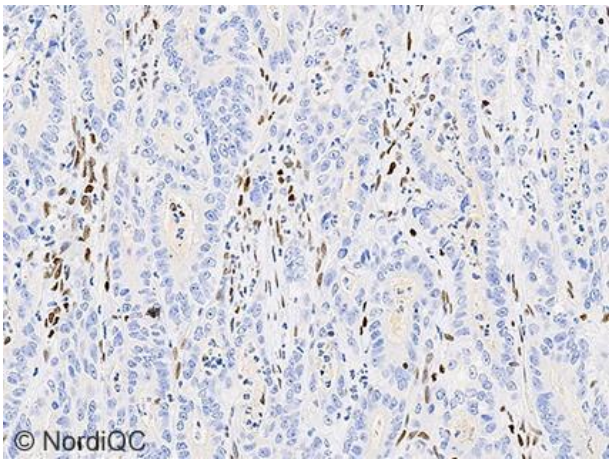


Fig. 3a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 5, with loss of MSH6 expression using same protocol as in Figs. 1a - 2a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

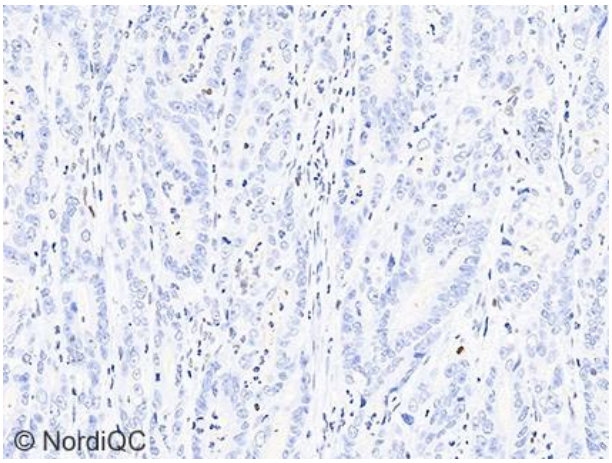


Fig. 3b
Insufficient MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 5, with loss of MSH6 expression using same protocol as in Figs. 1b - 2b – same field as in Fig. 3a. No nuclear staining reaction in the neoplastic cells is seen, but as only an equivocal nuclear staining reaction in the normal stromal cells is present, the staining pattern cannot reliably be interpreted.

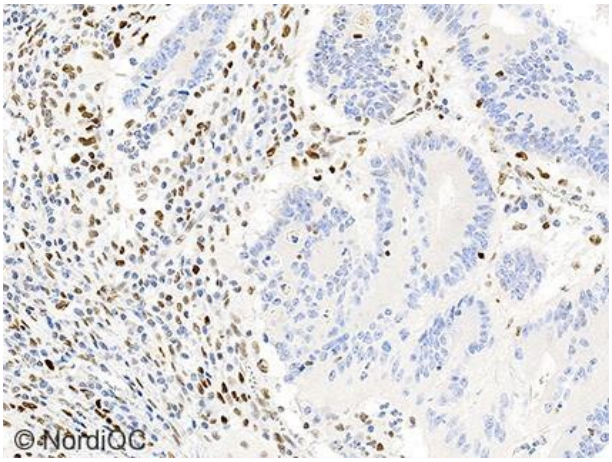


Fig. 4a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using same protocol as in Figs. 1a - 3a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control. No background staining is seen.

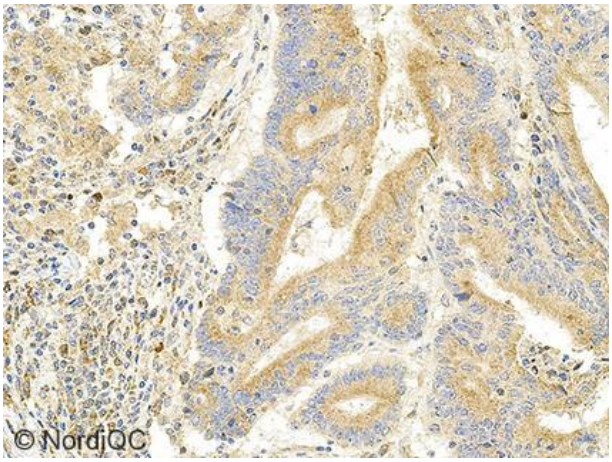


Fig. 4b
Insufficient MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression. Using the mAb clone 44 with similar protocol settings as in Fig 1a – 4a. Poor signal-to-noise reaction is seen. Excessive cytoplasmic staining in both the neoplastic cells and in the stromal cells obscures the interpretation of the nuclear staining. This staining pattern was typically seen when the mAb clone 44 was applied with a highly sensitive protocol. Compare with Fig. 4a – same field.

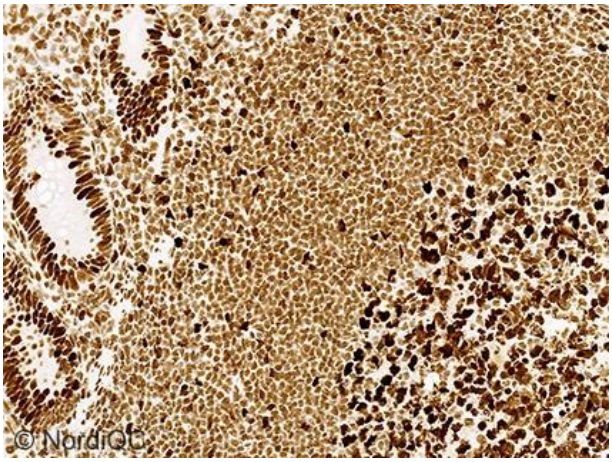


Fig. 5a
Optimal MSH6 staining reaction of the appendix using the **rmAb clone SP93 based 790-5092** RTU system for the Ventana BenchMark platform following the recommended protocol settings. Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the germinal centre B-cells and the columnar epithelia cells show a strong nuclear staining reaction. Also compare with Figs. 6a and 7a, same protocol.

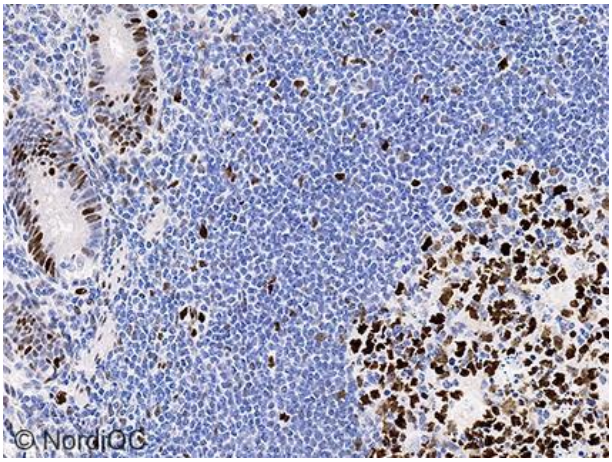


Fig. 5b
Insufficient MSH6 staining reaction of the appendix using the **mAb clone 44 based 790-4455** RTU system for the Ventana BenchMark platform using a laboratory modified protocol setting. Prolonged incubation time in the RTU Ab and an amplification step was used compared to the recommended protocol. In spite of these "sensitivity-boosting" steps the protocol showed too low analytical sensitivity. Only the germinal centre B-cells and most of the columnar epithelia cells are distinctively demonstrated, while mantle zone B-cells expressing low level MSH6 are virtually unstained. Also compare with Figs. 6b and 7b, same protocol.

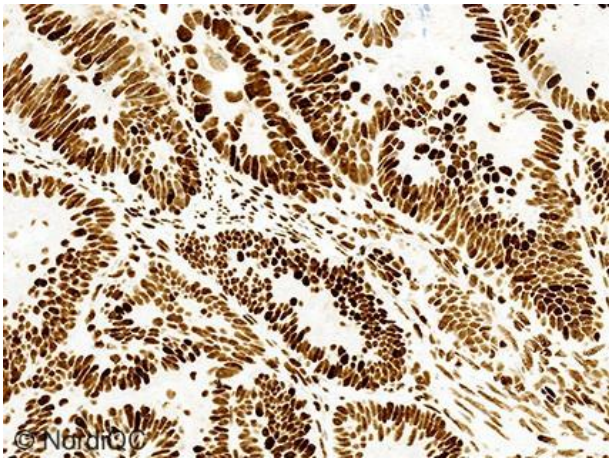


Fig. 6a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 3, with normal MSH6 expression using same protocol as in Fig. 5a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction. A high signal-to-noise ratio is obtained. No background staining but a distinct nuclear staining reaction in the stromal cells is seen.

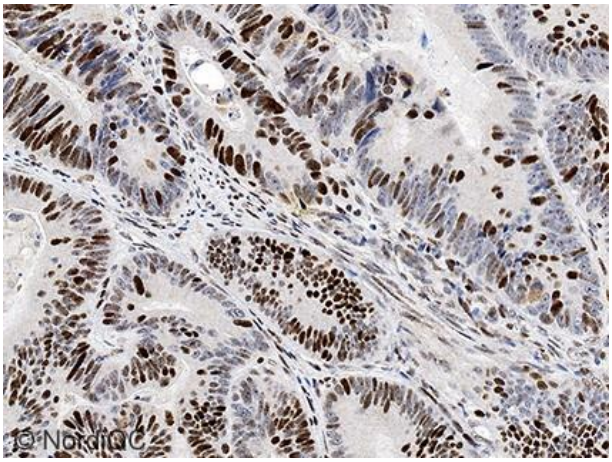


Fig. 6b
MSH6 staining reaction for of the colon adenocarcinoma, tissue core no. 3, using same protocol as in Fig. 5b - same field as in Fig. 6a. The proportion and the intensity of tumour cells and stromal cells demonstrated is clearly reduced compared to the result expected and shown in Fig. 6a. Also compare with Fig. 7b, same protocol.

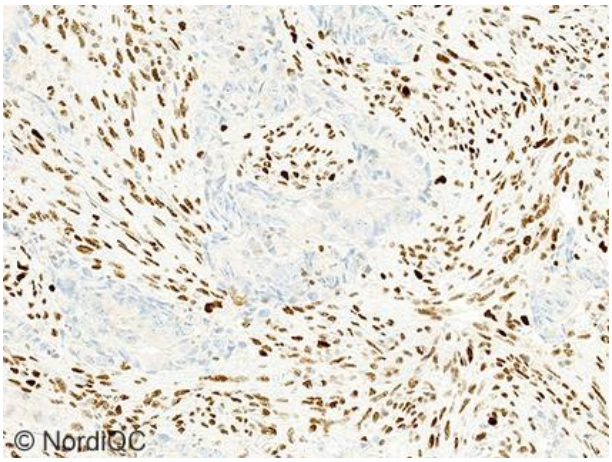


Fig. 7a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using same protocol as in Figs. 5a and 6a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

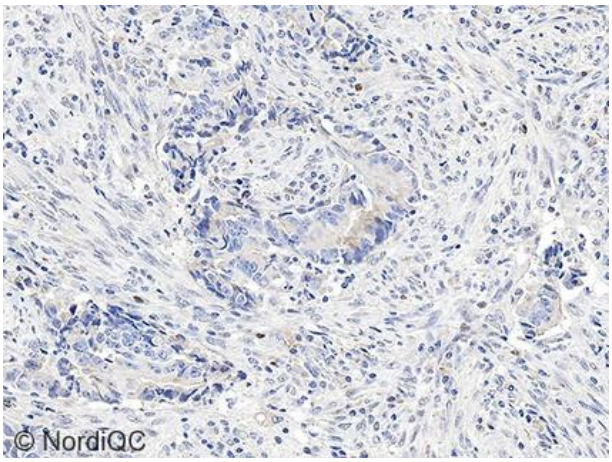


Fig. 7b
Insufficient MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using same protocol as in Figs. 5b and 6b – same field as in Fig. 7a. No nuclear staining reaction in the neoplastic cells is seen, but as virtually no nuclear staining reaction in the normal stromal cells is present, the staining pattern cannot reliably be interpreted.

ON/LE/MV/RR 10.04.18