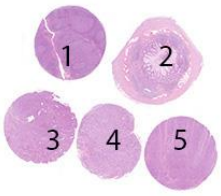
Assessment Run 61 2021

Mismatch repair protein MSH6 (MSH6)

Purpose
Evaluation of the technical performance and level of analytical sensitivity and specificity of IHC tests among NordiQC participants for MSH6 status in colon adenocarcinomas. Loss of MSH6 function due to gene mutation or epigenetic changes is characterized by absence of nuclear expression in neoplastic cells, whereas intact nuclear MSH6 expression indicates normal MSH6 function and no gene mutations.

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 in cells with coexisting nuclear staining reaction was accepted.

*Focal areas with preserved MSH6 expression in the neoplastic cells was seen in few slides.

Participation

Number of laboratories registered for MSH6, run 61	323
Number of laboratories returning slides	278 (86%)

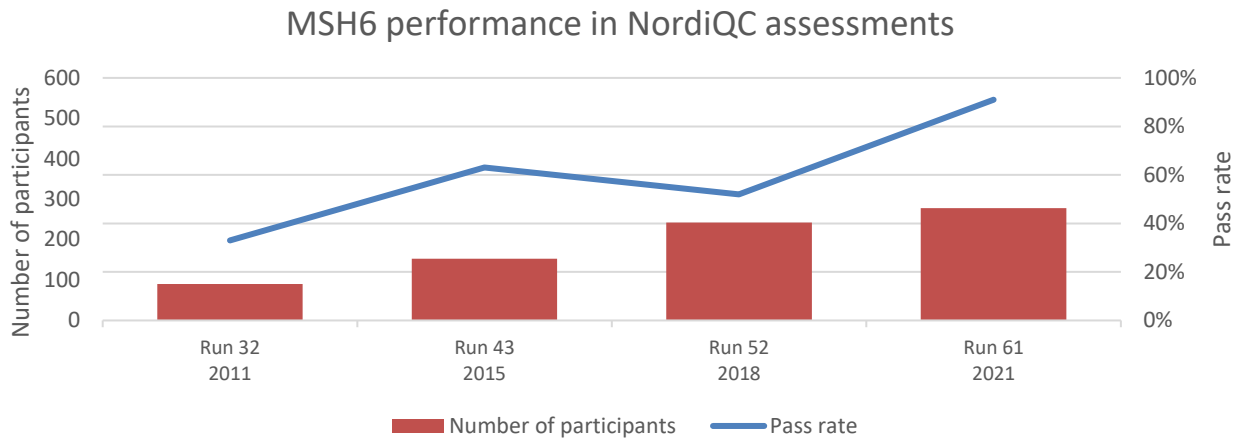
The number of laboratories returning slides has decreased in this run 61 compared to previous assessments, due to the COVID-19 pandemic and associated postal delays. All slides returned after the assessment were assessed and received advice if the result being insufficient but were not be included in this report.

Results
278 laboratories participated in this assessment. 252 (92%) 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 less successful clones
- Too low concentration of the primary antibody

Performance history
This was the fourth NordiQC assessment of MSH6. A significant increase in the pass rate was seen compared to the previous runs (see Figure 1).

Figure 1. **Proportion of sufficient results for MSH6 in the four NordiQC runs performed**



Conclusion

Optimal staining results could be obtained with many Abs as the rabbit monoclonal antibodies (rmAb) clones **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/Agilent), Autostainer (Dako/Agilent), Bond (Leica) and BenchMark (Ventana/Roche).

The access to several high quality RTU systems for MSH6 was instrumental for the improved pass rate in this run. Especially the recently introduced RTU system of rmAb clone SP93 (Ventana/Roche) showed a significantly superior performance compared to the terminated system based on clone 44, being widely used in run 52.

Tonsil is recommendable as positive tissue control for MSH6. Virtually all mantle zone B-cells must show an at least weak to moderate, distinct nuclear staining reaction, while a 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 61**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR
mAb clone **44**	2 2 2 2 1	Bicare BD Biosciences Cell Marque Diagnostic BioSystems BioGenex	0	0	7	2	0%	0%
mAb clone **PU29**	1	Leica Biosystems	0	0	1	0	-	-
rmAb clone **BSR100**	2	Nordic Biosite	2	0	0	0	-	-
rmAb clone **EP49**	23 22 1 1 1	Dako/Agilent Epitomics Abcam Cell Marque BioSB	39	7	2	0	96%	81%
rmAb clone **EPR3945**	4 1	Abcam Thermo Scientific	4	0	1	0	80%	80%
rmAb clone **SP93**	7	Cell Marque	6	0	1	0	86%	86%
mAb clone **BC19**	1	Biocare Medical	1	0	0	0	-	-
mAb clone **ZM99**	2	Zeta Corporation	0	0	1	1	-	-
Ready-To-Use antibodies								
rmAb **SP93 760-5092**[3]	42	Ventana/Roche	38	2	1	1	95%	91%

rmAb **SP93 760-5092**[4]	62	Ventana/Roche	54	6	2	0	97%	87%
rmAb clone **EP49 GA086**[3]	13	Dako/Agilent	10	3	0	0	100%	77%
rmAb clone **EP49 GA086**[4]	5	Dako/Agilent	5	0	0	0	100%	100%
rmAb clone **EP49 IR086**[3]	13	Dako/Agilent	11	2	0	0	100%	85%
rmAb clone **EP49 IR086**[4]	49	Dako/Agilent	39	10	0	0	100%	80%
rmAb clone **EP49 8326-C010**	3	Sakura Finetek	3	0	0	0	-	-
mAb clone **BC19 API3115H**	1	Biocare Medical	0	0	1	0	-	-
mAb clone **C5D11 CMM-0202**	1	Celnovte	1	0	0	0	-	-
rmAb clone **IHC026 IHC026**	1	GenomeMe	1	0	0	0	-	-
rmAb clone **EP49 PA0990**	3	Leica Biosystems	1	1	1	0	-	-
mAb clone **44 PDM 147**	2	Diagnostic Biosystems	0	0	2	0	**-**	**-**
rmAb clone **EP49 MAD-000635QD**	2	Master Diagnostica	1	0	0	1	-	-
mAb clone **MX056 MAB-0831**	2	Maixin	2	0	0	0	-	-
rmAb clone **SP93 287R**	3	Cell Marque	2	1	0	0	-	-
rmAb clone **44 287M-10/17/18**	1	Cell Marque	0	0	1	0	-	-
Total	278		220	32	21	5		
Proportion			79%	12%	7%	2%	91%	

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of optimal results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).

Detailed analysis of MSH6, Run 61
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
rmAb clone **BSR100**: Protocols with optimal results were all based on Heat Induced Epitope Retrieval (HIER) using Cell Conditioning 1 (CC1, Ventana/Roche) (1/1)* or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:100-1:300 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) (7/7), CC1 (Ventana/Roche) (18/21), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (8/11), Bond Epitope Retrieval Solution 1 (BERS1, Leica) (1/1), TRS pH 6.1 (3-in-1) (Dako/Agilent) (1/1) or unknown (2/3) as retrieval buffer. The rmAb was typically diluted in the range of 1:20-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings, 44 of 45 (98%) 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) (3/3). 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 4 (100%) laboratories produced an optimal staining result.

rmAb clone **SP93**: Protocols with optimal results were all based on HIER using CC1 (Ventana/Roche) (6/7) as retrieval buffer. The rmAb was diluted in the range of 1:50-1:200 depending on the total sensitivity of

the protocol employed. Using these protocol settings, 6 of 6 (100%) laboratories produced an optimal staining result.
mAb clone **BC19**: One protocol with an optimal result was based on HIER using BERS2 (Leica) (1/1) as retrieval buffer. The mAb was diluted 1:50.

Table 2. **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/Roche 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 **BSR100**	-	-	-	-	1/1**	-	-	-
rmAb clone **EP49**	2/3	1/1	7/7 (100%)	-	18/20 (90%)	-	8/10 (80%)	1/1
rmAb clone **EPR3945**	-	-	-	-	4/4	-	-	-
rmAb clone **SP93**	-	-	-	-	6/6 (100%)	-	-	-
mAb clone **BC19**	-	-	-	-	-	-	1/1	-

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

Ready-To-Use antibodies and corresponding systems
rmAb clone **SP93**, product no. **760-5092**, Ventana/Roche, Ventana Benchmark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER in CC1 (efficient heating time for 32-64 min. at 100°C), 8-32 min. incubation at 36°C of the primary Ab and UltraView (760-500) or OptiView (760-700) with or without amplification (760-080/760-099) as detection system. Using these protocol settings 99 of 102 (97%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP49**, product no. **IR086**, Dako/Agilent, 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 10-25 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 27 of 27 (100%) laboratories produced a sufficient staining result (optimal or good).
35 laboratories used product no IR084/IS084 on other platforms. These were not included in the description above.

rmAb clone **EP49**, product no. **GA086**, Dako/Agilent, Omnis:
Protocols with optimal results were typically based on HIER in TRS pH 9 (efficient heating time 20-30 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+/FLEX++ (GV8000/GV823/GV809) as detection system. Using these protocol settings 16 of 16 (100%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP49**,product no. **8326-C010**, Sakura Finetek, Tissue-Tek Genie:
Protocols with optimal results were all based on HIER in Tissue-Tek Genie High pH Antigen Retrieval (efficient heating time 45 min. at 98°C), 30 min. incubation of the primary Ab and Tissue-Tek Genie Pro Detection Kit, DAB (8826-K250) as detection system. Using these protocol settings 3 of 3 laboratories produced an optimal staining result.

rmAb clone **EP49**, product no. **PA0990**, Leica Biosystems, Bond III:
One protocol with an optimal result was based on HIER using BERS2 for 20 min., 30 min. incubation of the primary Ab and Bond Refine (DS9800) as detection system. Only one laboratory used these protocol settings.

Table 3 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 3. **Proportion of sufficient and optimal results for MSH6 for the most commonly used RTU IHC systems**

RTU systems	**Vendor recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS BenchMark rmAb clone **SP93, 760-5092**	95% (40/42)	91% (38/42)	97% (59/61)	87% (53/61)
Dako AS rmAb clone **EP49, IR086**	100% (13/13)	85% (11/13)	100% (14/14)	79% (11/14)
Dako Omnis rmAb clone **EP49, GA086**	100% (13/13)	77% (10/13)	(3/3)	(3/3)

* Protocol settings recommended by vendor – retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Modifications included: retrieval method, retrieval duration, retrieval reagents, Ab incubation time and detection kit. Only protocols performed on the specified vendor IHC stainer were included.

Comments

The pass rate has in this assessment increased significantly from previously assessments (see Table 2, page 4) and the pass rate of 52% in the latest run 52 was improved to 91% in this run 61. The access to several high quality RTU systems for MSH6 was instrumental for the improved pass rate.
Ventana/Roche has e.g. terminated the sale of the previously widely used RTU product based on the less successful mAb clone 44 and have introduced the FDA-approved RTU system based on rmAb clone SP93. This affected both the use of the different clones and especially the pass rate in the two NordiQC assessments. In run 52, 35% (85 of 242) of the participants used the mAb clone 44 and in run 61, only 4% (12 of 278) used the mAb clone 44. The use of rmAb clone SP93 increased from 5% (12 of 242) in run 52, to 41% (114 of 278) in this run 61. In run 52 the overall pass-pate for participants using mAb clone 44 was 5% and in run 61, the pass rate for participants using rmAb clone SP93 was 96%.

In this assessment and in concordance with the observations in the previous assessments of MSH6, the prevalent feature of an insufficient staining reaction was a too weak or false negative nuclear staining reaction of the cells expected to be demonstrated. Too weak or false negative staining reaction was seen in 77% of the insufficient results (20 of 26). The majority of the laboratories were 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. 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 tissue control. 8% (2 of 26) of the insufficient staining reactions were caused by a false positive nuclear staining reaction in the neoplastic cells in a tumour with loss of MSH6. In the remaining 15% of the insufficient results a poor signal-to-noise ratio or a granular staining reaction (compromising interpretation) was seen.

27% (75 of 278) 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 and mAb clone BC19 (see Table 1). Irrespective of the clone applied, careful calibration of the titre, efficient HIER, preferably at high pH and 3-step polymer/multimer based detection system were the main protocol prerequisites for optimal results.
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/Agilent, Leica and Ventana/Roche (see Table 2).
In contrast, no sufficient staining result was registered with the mAbs clones 44, PU29 and ZM99.

Ready-To-Use (RTU) antibodies was used by 73% (203 of 278) of the laboratories. The use of RTU products has increased from 53% in 2015, 61% in 2018 till 73% in this run 61.

The newly introduced RTU system from Ventana/Roche based on rmAb clone SP93 (790-5093) was the most widely used system and obtained high pass-rates both following the vendor recommended protocol settings or modifying the protocol of 95% and 97% respectively (see Table 3). The most common modifications were increased incubation time of primary Ab and/or a decreased time for HIER. Some laboratories applied OptiView Amplification kit, which in few cases gave a granular staining reaction, compromising the interpretation.

The Dako/Agilent RTU systems based on rmAb clone EP49 IR086 and GA086 for Autostainer link 48 and Omnis, respectively, provided pass-rates at 100% using both recommended and laboratory modified protocol settings. The recommended protocol for the Omnis RTU is based on a 4-layer detection system, while the Autostainer RTU is based on a 2-layer detection system. In this assessment a successful performance could be obtained when applying the Autostainer format IR086 on Omnis, whereas this approach was found inferior in run 56 providing a reduced level of analytical sensitivity compromising the identification of internal positive stroma cells. Despite the encouraging pass rate for interchangeability of RTU formats for the two systems in this run, an off-label use cannot be recommended. The different observations in the two runs can be related to extended use of the 4-layer detection system on Omnis and/or less challenging material circulated.

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 most stromal cells serving as internal positive tissue control.

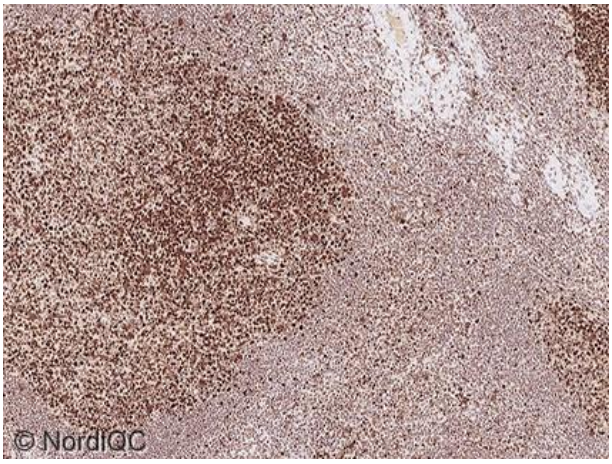


Fig. 1a
Optimal MSH6 staining reaction of the tonsil using the Dako RTU GA086 format based on rmAb clone EP49, using the recommended protocol settings with HIER in an alkaline buffer (TRS pH 9, Dako) and a 4-step polymer-based detection system (EnVision Flex with both Rabbit and Mouse Linker, Dako). 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 - 5a, same protocol.

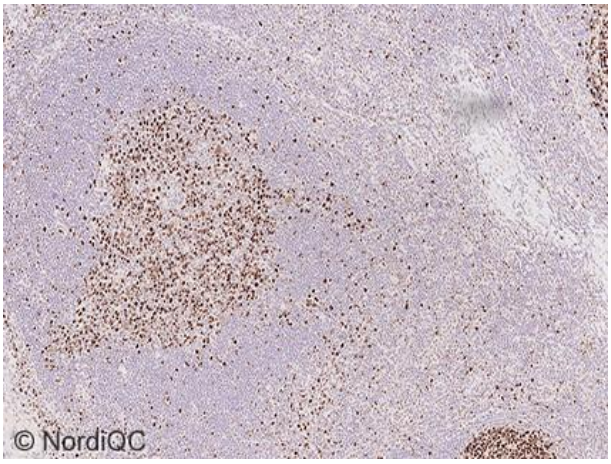


Fig. 1b
Insufficient MSH6 staining reaction of the tonsil using the mAb clone 44 with a protocol providing too low analytic sensitivity - same field as in Fig. 1a. Only the germinal centre B-cells are distinctively demonstrated, while mantle zone B-cells low level MSH6 expression virtually are unstained. 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 the insufficient staining result. Also compare with Figs. 2b - 4b, same protocol.

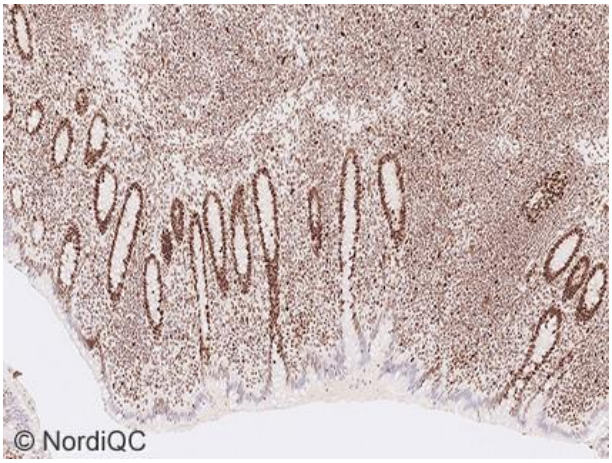


Fig. 2a
Optimal MSH6 staining reaction of the appendix using same protocol as in Fig. 1a. Virtually all cells show an at least weak to strong nuclear staining reaction. Also compare with Figs. 3a - 5a, same protocol.

Fig. 2b
Insufficient MSH6 staining reaction of the appendix, using same protocol as in Fig. 1b - same field as in Fig. 2a. Only crypt epithelial cells and dispersed lymphocytes are positive. Also compare with Fig. 3b - 5b, same protocol.

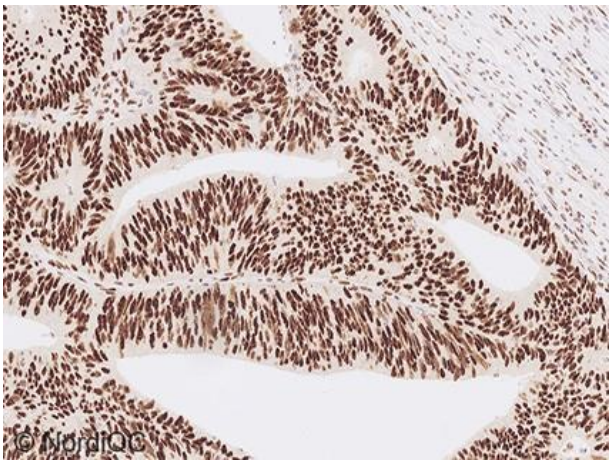


Fig. 3a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 3, with preserved MSH6 expression using same protocol as in Figs. 1a - 2a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction. Stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control. Also compare with Figs. 4a - 5a, same protocol.

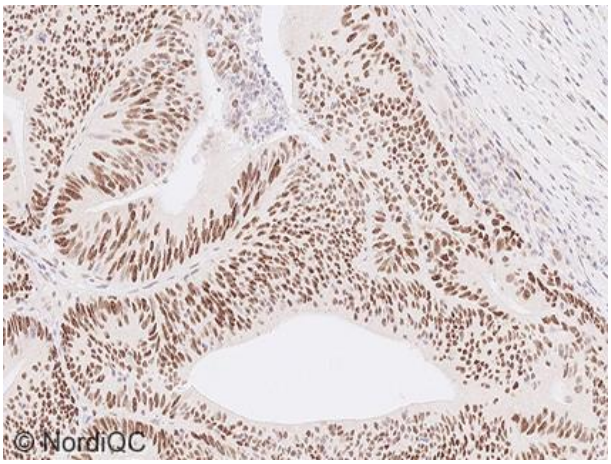


Fig. 3b
MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 3, with preserved MSH6 expression using same protocol as in Figs. 1b - 2b – same field as in Fig. 3a. A weak to moderate staining reaction was seen in the neoplastic cells. The proportion and the intensity of stromal cells demonstrated is reduced compared to the optimal result in Fig. 3a (same area).

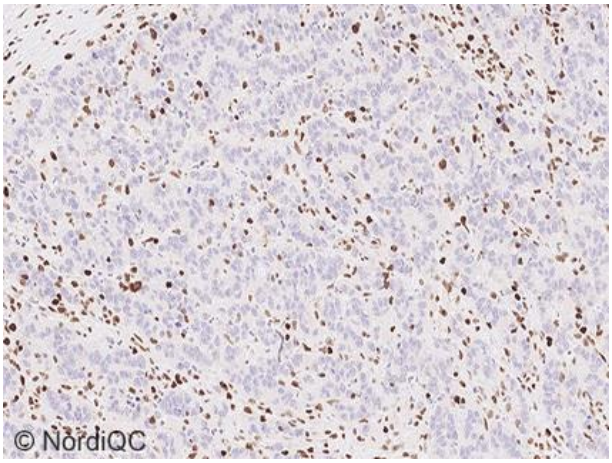


Fig. 4a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using same protocol as in Figs. 1a - 3a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control. No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

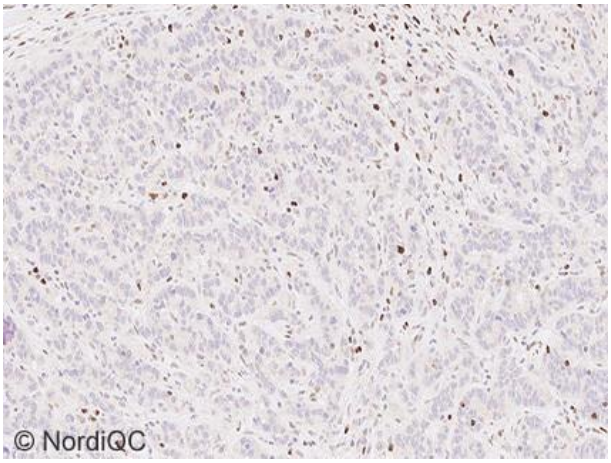


Fig. 4b
MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using same protocol as in Figs. 1b – 3b. The neoplastic cells are negative. The proportion and the intensity of stromal cells demonstrated is reduced compared to the optimal result in Fig. 4a (same area).

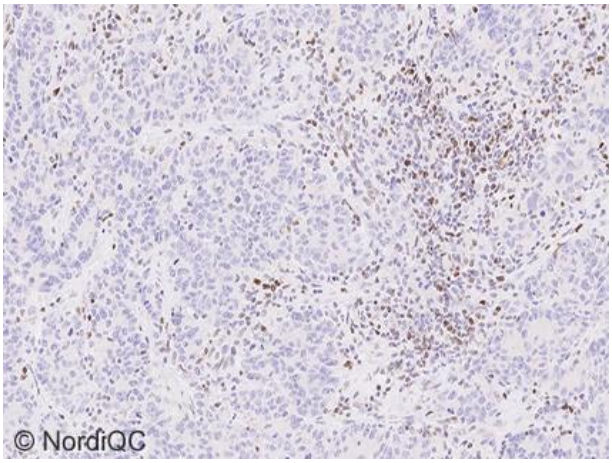


Fig. 5a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 5, with loss of MSH6 expression using same protocol as in Figs. 1a – 4a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control. No background staining is seen.

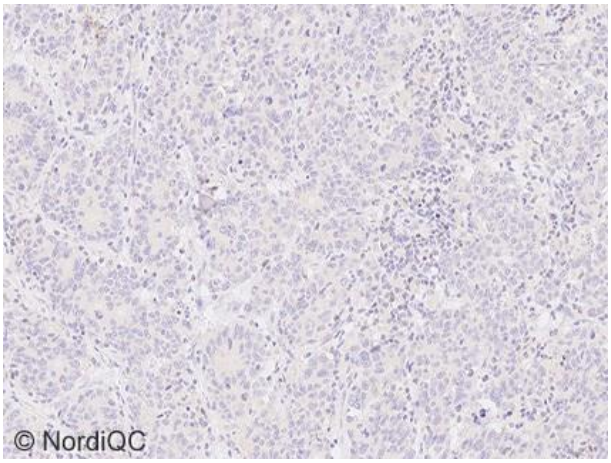


Fig. 5b
Insufficient MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using same protocol as in Figs. 1b – 4b. The neoplastic cells are negative as expected, but as only scattered stromal cells display a faint nuclear staining reaction, the staining pattern cannot reliably be interpreted. Compare with the optimal result in Fig. 5a (same area).

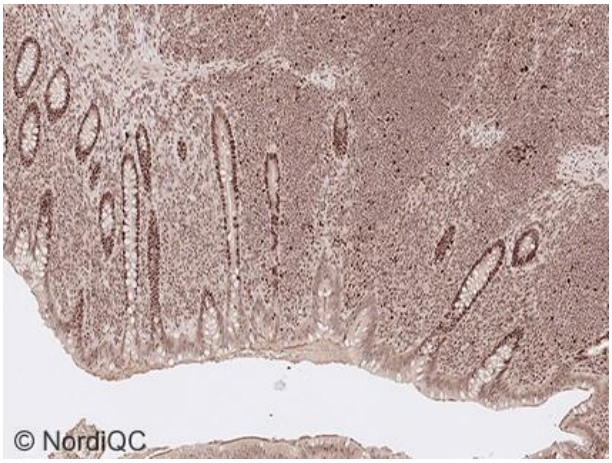


Fig. 6a
MSH6 staining reaction of the appendix, using the mAb clone ZM99, using HIER in an alkaline buffer and a 3-layer detection system. Virtually all cells show an at least moderate nuclear staining reaction. However, also a cytoplasmic staining reaction is observed, giving a poor signal-to-noise ratio. Compare with optimal result in Fig. 2a. Also compare with insufficient result in Fig. 6b – same protocol.

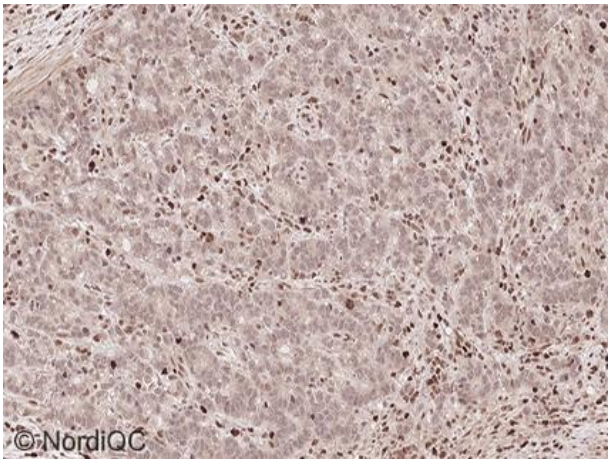


Fig. 6b
Insufficient MSH6 staining reaction for of the colon adenocarcinoma, tissue core no. 4, using same protocol as in Fig. 6a - same field as in Fig. 4a. The neoplastic cells, expected to be negative, shows a weak nuclear staining reaction, giving a false positive staining result.

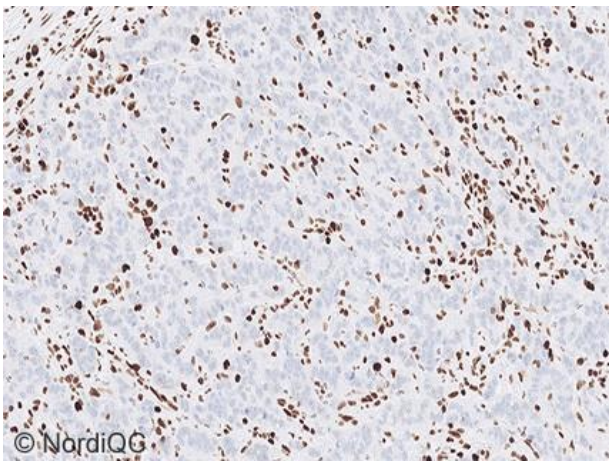


Fig. 7a
Optimal MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using rmAb clone SP93 based 790-5092 RTU system (Ventana/Roche), using the recommended protocol settings. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

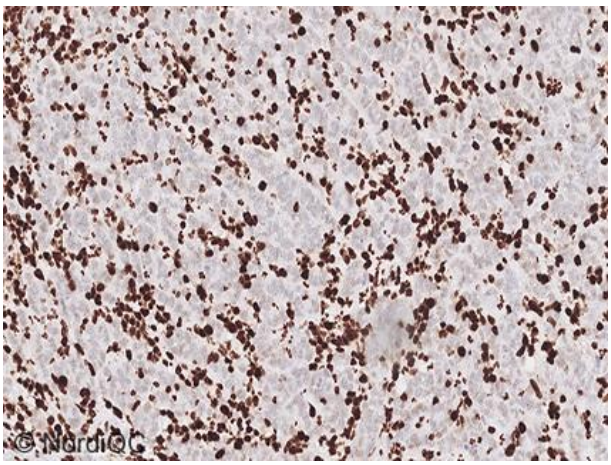


Fig. 7b
Insufficient MSH6 staining reaction of the colon adenocarcinoma, tissue core no. 4, with loss of MSH6 expression using rmAb clone SP93 based 790-5092 RTU system (Ventana/Roche), modifying the protocol settings by applying OptiView Amplification. An aberrant diffuse granular staining reaction is observed in the nuclei of the neoplastic cells, compromising the interpretation. Compare to optimal result in Fig. 7a (same area).

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