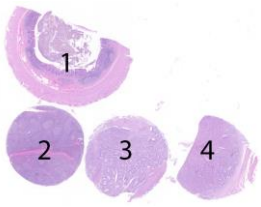
Assessment Run 43 2015

Mismatch repair protein MSH6 (MSH6)

Material
The slide to be stained for MSH6 comprised:



1. Appendix 2. Tonsil fixed for 24 hours, 3. Colon adenocarcinoma with normal MSH6 expression, 4. Colon adenocarcinoma 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, but a distinct nuclear staining reaction in the vast majority of other cells (stromal cells, lymphocytes etc).
- A generally weak cytoplasmic staining reaction was accepted.

Participation

Number of laboratories registered for MSH6, run 43	173
Number of laboratories returning slides	153 (88%)

Results
153 laboratories participated in this assessment. 96 (63%) 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 primary antibodies
- Use of less sensitive detection systems
- Too low concentration of the primary antibody

Performance history
This was the second NordiQC assessment of MSH6. A significant increase of the pass rate was seen compared to run 32, 2011.

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

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

Conclusion
The rmAb clones **EP49, EPR3945** and **SP93** were the most successful antibodies and could all be used to obtain an optimal staining result for MSH6.The concentrated formats of the rmAb clones **EP49** and **EPR3945** provided optimal staining results on the three main platforms from Dako, Leica and Ventana. Irrespective of the clone applied, efficient HIER 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.
mAb clone 44 gave a less successful result and cannot be recommended for diagnostic use.
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.

Table 1. **Antibodies and assessment marks for MSH6, run 43**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **44**	12 2 1 1	BD Biosciences Cell Marque Diagnostic Biosystems Zeta	0	1	14	1	6%	-
mAb clone **BC/44**	6	Biocare	1	3	2	0	67%	67%
mAb clone **PU29**	6	Leica/Novocastra	0	1	4	1	17%	-
mAb clone **SPM525**	1	Zytomed Systems	0	0	0	1	-	-
rmAb clone **EP49**	20 12	Epitomics Dako	22	7	3	0	91%	91%
rmAb clone **EPR3945**	4 2 1 1	Abcam Epitomics Gene Tex Nordic Biosite	5	3	0	0	100%	100%
rmAb clone **SP93**	2 1	Cell Marque Spring Bioscience	3	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **44** **790-4455**	33	Ventana	1	9	22	1	30%	40%
mAb clone **44** **287M**	2	Cell Marque	0	0	2	0	-	-
mAb clone **44** **PDM 147**	1	Diagnostic Biosystems	0	0	1	0	**-**	**-**
mAb clone **44** **081374**	1	Invitrogen/Life Technologies	0	0	1	0	-	-
mAb clone **44** **MAB-0643**	1	Maixin	1	0	0	0	-	-
mAb **BC/44** **M265**	2	Biocare	0	1	1	0	-	-
rmAb clone **EP49** **IR086**	35	Dako	24	8	3	0	91%	96%
rmAb clone **EP49** **MAD-000635QD**	2	Master Diagnostica	1	1	0	0	-	-
rmAb clone **EP49** **AN780-5M**	1	Biogenex	1	0	0	0	-	-
rmAb clone **SP93** **287R**	2	Cell Marque	1	1	0	0	-	-
rmAb **SP93** **M3931**	1	Spring Bioscience	1	0	0	0	-	-
Total	153		61	35	53	4	-	
Proportion			40%	23%	35%	2%	63%	

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

Detailed analysis of MSH6, Run 43
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **BC/44**: One protocol with an optimal result was based on HIER using Cell Conditioning 1 (CC1; Ventana) (1/4)* as retrieval buffer). The mAb was diluted 1:100. Using this protocol setting 2 of 3 (67%) laboratories produced a sufficient staining result (optimal or good).
* (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) (2/4), TRS pH 9 (Dako) (1/3), TRS pH low 6.1 (3-in-1) (2/2), CC1 (Ventana) (10/13), Bond Epitope Retrieval Solution 2 (BERS2; Leica) (3/5), Borg Decloaker pH 9.5 (Biocare) (1/1) or Tris-EDTA/EGTA pH 9 (3/4) as retrieval buffer. 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 29 of 32 (91%) laboratories produced a sufficient staining result.

rmAb clone **EPR3945**: Protocols with optimal results were all based on HIER using TRS pH 9 (3-in-1) (Dako) (1/2), CC1 (Ventana) (2/3), BERS2, (Leica) (1/2) or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:50-1:500 depending on the total sensitivity of the protocol employed. Using these protocol settings 8 of 8 (100%) laboratories produced a sufficient staining result.

rmAb clone **SP93**: Protocols with optimal results were all based on HIER using CC1 (Ventana) (2/2) or Tris-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:15-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 3 of 3 (100%) laboratories produced an optimal staining result.

Table 3. **Proportion of optimal results for MSH6 for the most commonly used antibody as concentrate on the 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	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**	3/7** (43%)	2/2	9/12 (75%)	-	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 **44**, product no. **790-4455**, Ventana, BenchMark XT:
One protocol with an optimal result was based on HIER using Cell Conditioning 1 (efficient heating time 80 min.), 20 min. incubation of the primary Ab and OptiView (760-700) + amplification kit as detection system. Using these protocol settings 2 of 5 laboratories produced a sufficient staining result (optimal or good).

mAb clone **44**, product no. **MAB-0643**, 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 KIT-5230 (Maixin) as detection system.

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) or TRS pH 9 (efficient heating time 10-30 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 28 (96%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP49**, product no. **AN780-5M**, Biogenex, BioGenex i6000:
One protocol with optimal result was based on HIER using Citrate pH 6 (MWO) for 10 min., 60 min. incubation of the primary Ab and Super Sensitive (QD400-60KE) as detection system.

Comments
In this assessment and in concordance with the observations in the previous assessment of MSH6, run 32 2011, 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 61% of the insufficient results (35 of 57.) 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, wherefore it is of decisive importance that the normal cells within and around the neoplastic cells show a distinct positive nuclear staining reaction, serving as internal positive control. In the remaining 39% of the insufficient results both a too weak specific staining reaction and an excessive background staining compromising the interpretation was seen. This staining pattern was in particular observed for the mAb clone 44.

Using a concentrated Ab, optimal result could be obtained by many clones as listed in table 1. However, the rmAb clones EP49, EPR3945 and SP93 were most successful. Irrespective of the clone applied, careful calibration of the titre, efficient HIER and use of a non-biotin based detection system 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, providing the titre of the primary Ab was adjusted to the level of sensitivity for the system.
The rmAb clone EP49 was the most widely used Ab for demonstration of MSH6 and provided a high proportion of sufficient staining results and optimal results could be obtained on all three main IHC systems from Dako, Leica and Ventana (see table 3).

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).

The widely used mAb clone 44 gave a low proportion of sufficient and optimal results (see table 1) both as concentrated format within a laboratory developed (LD) assay and as RTU system (Ventana). The calibration of the protocols based on mAb clone 44 was very challenging as the clone seemed to have a relative low affinity for the specific nuclear MSH6 antigen combined with a cross-reaction with a cytoplasmic protein. If the laboratories used a low titre of the clone in order to eliminate the unspecific cytoplasmic staining reaction, the specific nuclear staining was significantly reduced giving a too weak nuclear staining reaction. On the other hand, if the mAb clone 44 was applied within a highly sensitive IHC system e.g. based on HIER in an alkaline buffer and a 3-step labelled polymer system, excessive background and cytoplasmic staining reaction was seen compromising the interpretation of the specific nuclear staining reaction.
Using the mAb clone 44 as a concentrate in a LD assay a pass rate of 6% (1 of 16) was seen. No optimal result was obtained.
For the Ventana RTU system for MSH6 based on the mAb clone 44, a pass rate of 30% (10 of 33) was seen (1 protocol was optimal). Sufficient results were not obtained using the official protocol settings provided by Ventana.

In this run a pass rate of 63% was seen, which was a significant improvement compared to 33% in run 32, 2011. It is difficult to conclude on the specific causes for the improvement of the pass rate obtained in this run, as many laboratories participated for the first time and many laboratories have changed their IHC systems compared to the previous run in 2011. However, the availability and extended use of high quality antibodies for MSH6 seems to be one of the central elements. In run 32, 2011 11% of the participants (10 of 90) used the rmAb clones EP49 and EPR3945. In this run 49% (75 of 153) of the participants used one of these clones either within a LD assay or a RTU system. Grouped together, these clones obtained an overall pass rate of 92% (69 of 75).

Controls
Tonsil was found to be a recommendable positive tissue control for MSH6. 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 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.

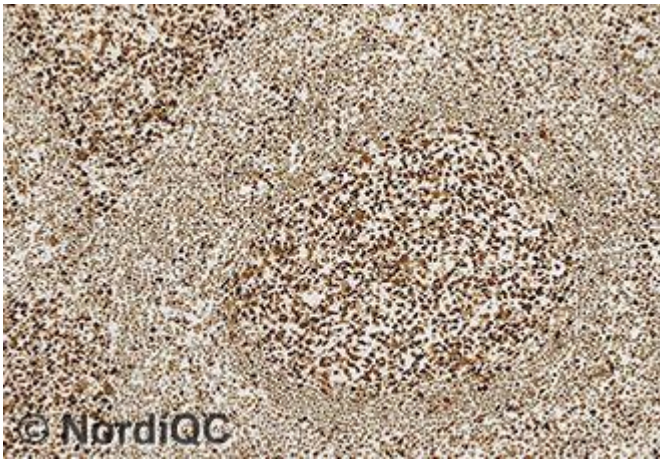


Fig. 1a (X100)
Optimal MSH6 staining reaction of the tonsil using the rmAb clone EP49, optimally calibrated, HIER in an alkaline buffer and a 3-step multimer based detection system (OptiView, Ventana). Virtually all mantle zone B-cells show a moderate and distinct nuclear staining reaction, while the germinal centre B-cells show a strong nuclear staining reaction. Also compare with Figs. 2a - 4a, same protocol.

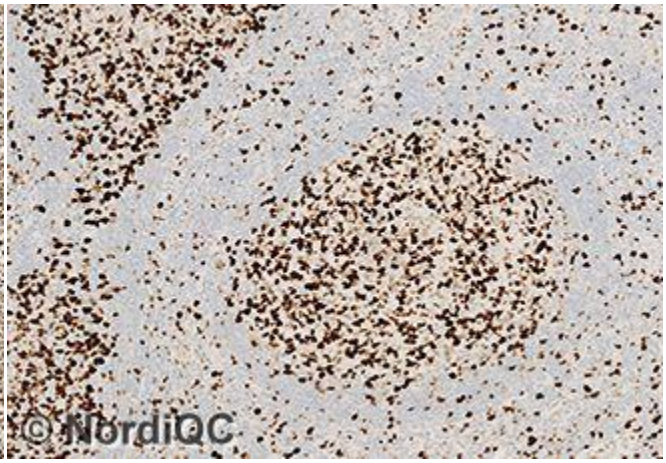


Fig. 1b (X200)
Insufficient MSH6 staining reaction of the tonsil using the rmAb clone EP49 with a protocol providing a too low sensitivity- same field as in Fig. 1a.
Only the germinal centre B-cells are distinctively demonstrated, while mantle zone B-cells expressing low level MSH6 virtually are unstained. The protocol was based on OptiView + amplification (Ventana) and the combination of a too low titre of the primary Ab and the use of tyramide based amplification provided an inadequate balance of the staining reaction.
Also compare with Figs. 2b - 4b, same protocol.

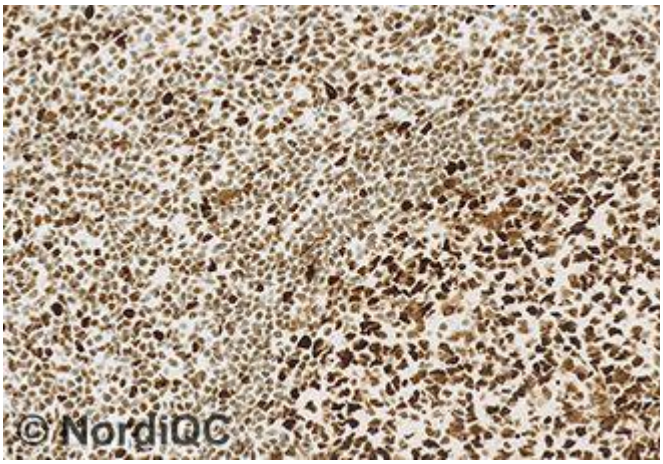


Fig. 2a (x200)
High magnification of the optimal MSH6 staining reaction of the tonsil shown in Fig. 1a. The staining reaction expected is obtained and virtually all cells show a distinct nuclear staining reaction. Proliferating germinal centre B-cells display a strong nuclear staining intensity, while dormant mantle zone B-cells and T-cells show a weak to moderate nuclear staining reaction. Also compare with Figs. 3a and 4a, same protocol.

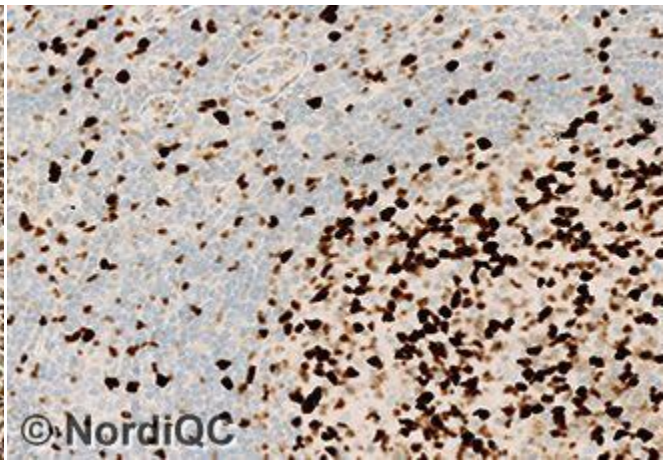


Fig. 2b (x200)
High magnification of the insufficient MSH6 staining reaction of the tonsil shown in Fig. 1b. The majority of proliferating germinal centre B-cells and dispersed interfollicular cells show an intense nuclear staining reaction, whereas dormant mantle zone B-cells and T-cells virtually are unstained and false negative.
Inappropriate calibration of the tyramide based protocol provided an aberrant range of the MSH6 expression and cells thus being either strongly positive or negative.

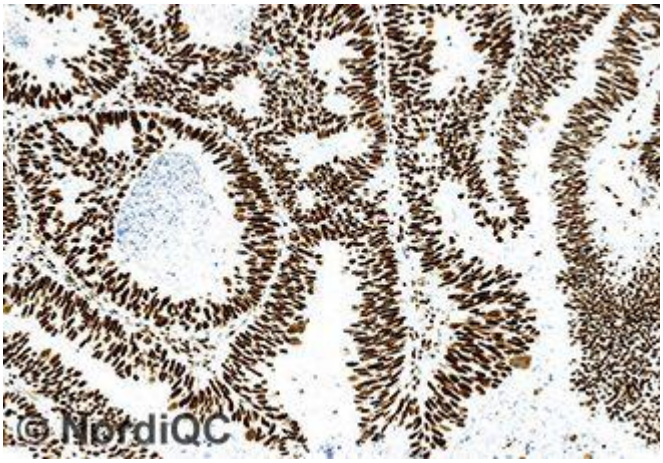


Fig. 3a (X100)
Optimal MSH6 staining reaction of the colon adenocarcinoma tissue core no. 3 with normal MSH6 expression using same protocol as in Figs. 1a and 2a. Virtually all neoplastic cells show a moderate to strong nuclear staining reaction. A high signal-to-noise ratio is obtained. No background staining is seen and a distinct nuclear staining reaction in the stromal cells is seen.

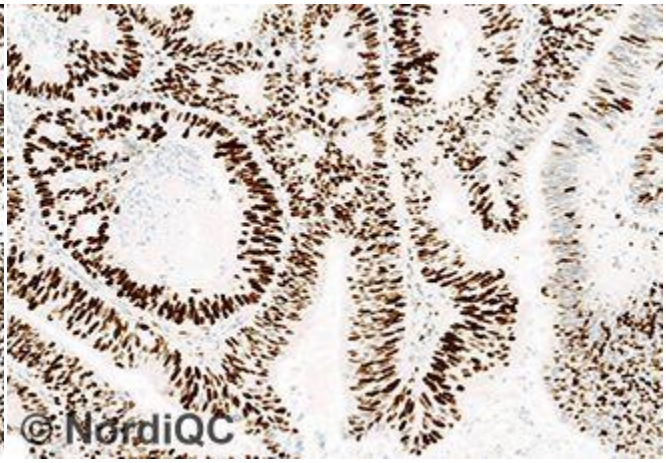


Fig. 3b (X100)
MSH6 staining reaction for of the colon adenocarcinoma tissue core no. 3 using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The proportion and the intensity of cells demonstrated is slightly reduced compared to the result expected and shown in Fig. 3a. Also note that stromal cells are virtually negative. Also compare with Fig. 4b, same protocol.

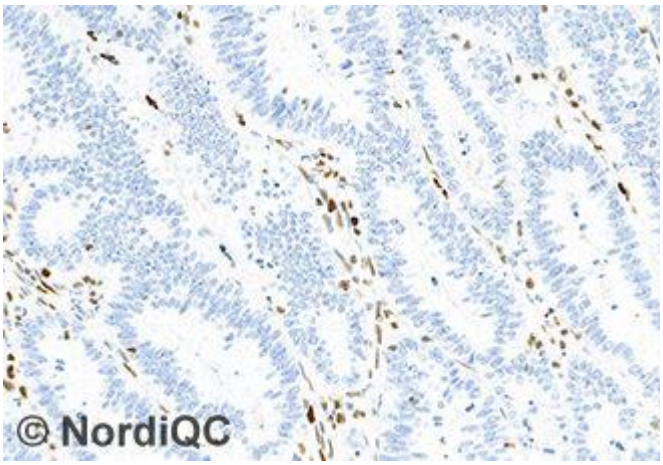


Fig. 4a (X200)
Optimal MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1a - 3a. The neoplastic cells are negative, while stromal cells show a distinct nuclear staining reaction serving as internal positive tissue control.

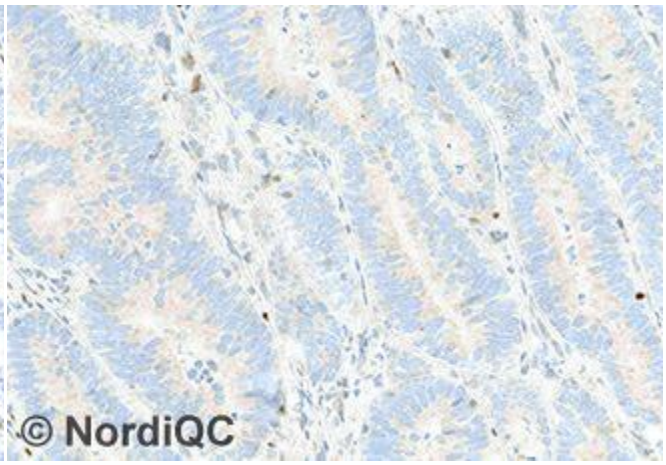


Fig. 4b (x200)
Insufficient MSH6 staining reaction of the colon adenocarcinoma no. 4 with loss of MSH6 expression using same protocol as in Figs. 1b - 3b – same field as in Fig. 4a. No nuclear staining reaction in the neoplastic cells is seen, but as only an equivocal nuclear staining reaction in the normal stromal cells is present, the staining pattern cannot reliably be interpreted. In addition a weak aberrant cytoplasmic staining reaction is seen.

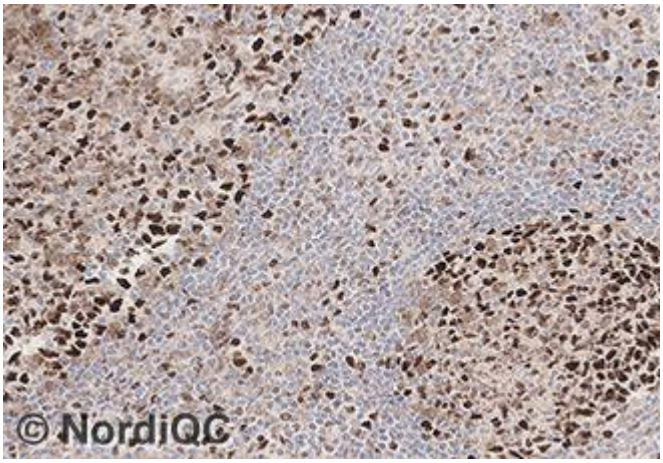


Fig. 5a (x100)
Insufficient MSH6 staining reaction of the tonsil using the mAb clone 44 with HIER in an alkaline buffer and a 3-step polymer based detection system. Mantle zone B-cells only show a faint and equivocal nuclear staining reaction, whereas germinal centre B-cells show a moderate to strong nuclear staining reaction. Simultanously a cytoplasmic staining reaction is seen complicating the interpretation. The mAb clone 44 was shown to be challenging to calibrate and the vast majority of protocols based on the clone either gave a false negative result and/or a poor signal-to-noise ratio.

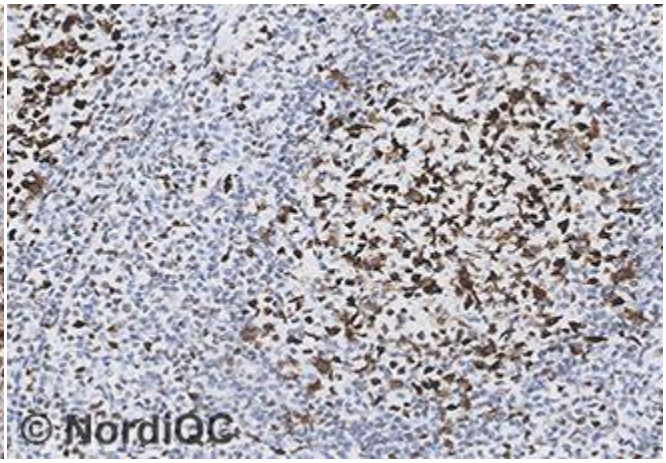


Fig. 5b (x200)
Insufficient MSH6 staining reaction of the tonsil using the rmAb clone EP49. Excessive HIER (too long efficient HIER time) and/or extensively drying of the sections after the IHC assay was completed, caused impairment of the morphology and consequently the interpretation was compromised. Virtually all nuclei display a wrinkled appearance.

SN/RR/LE/MV 15-3-2015