The slide to be stained for MLH1 comprised:


All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a MLH1 staining as optimal included:

- An at least weak to moderate nuclear staining reaction of almost all cells in the appendix.
- An at least weak to moderate nuclear staining reaction in the mantle zone B-cells and a moderate to strong nuclear staining reaction in the germinal centre B-cells.
- A moderate to strong nuclear staining in the neoplastic cells of the colon adenocarcinoma no. 4.
- A negative staining reaction in the neoplastic cells of the colon adenocarcinoma no. 3, and a distinct nuclear reaction in all other cells.
- A weak cytoplasmic reaction was accepted.

85 laboratories participated in this assessment. 57 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. Abs and assessment marks for MLH1, run 30

<table>
<thead>
<tr>
<th>Concentrated Abs</th>
<th>N</th>
<th>Vendor</th>
<th>Optimal</th>
<th>Good</th>
<th>Borderl.</th>
<th>Poor</th>
<th>Suff.¹</th>
<th>Suff. OPS²</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb clone G168-15</td>
<td>33</td>
<td>BD Pharmingen</td>
<td>10</td>
<td>11</td>
<td>15</td>
<td>6</td>
<td>50 %</td>
<td>70 %</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Biocare</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Zytomed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mAb clone ES05</td>
<td>16</td>
<td>Novocastra/Leica Dako</td>
<td>11</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>84 %</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Dako</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mAb clone G168-728</td>
<td>1</td>
<td>BD Pharmingen</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ready-To-Use Abs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mAb clone ES05, IR079</td>
<td>7</td>
<td>Dako</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>mAb clone ES05, PA0610</td>
<td>1</td>
<td>Leica</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mAb, clone G168-728, 760-4264</td>
<td>11</td>
<td>Ventana</td>
<td>0</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>18 %</td>
<td>-</td>
</tr>
<tr>
<td>mAb, clone G168-728, 285M &amp; CMA869</td>
<td>3</td>
<td>Cell Marque</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mAb, clone G168-728, 15 PM220</td>
<td>1</td>
<td>Biocare</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td></td>
<td>30</td>
<td>19</td>
<td>26</td>
<td>10</td>
<td>12 %</td>
<td>57 %</td>
</tr>
</tbody>
</table>

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

Following central protocol parameters were used to obtain an optimal staining:

**Concentrated Abs**

mAb clone G168-15: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Tris-EDTA/EGTA pH 9 (3/13)*, TRS pH 9 (Dako) (2/8), TRS pH 9 (3-in-1, Dako) (1/3), Bond Epitope Retrieval Solution 2 (Bond, Leica) (3/8) or EDTA/EGTA pH 8 (1/1) as the 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 16 out of 23 (70 %) laboratories produced a sufficient staining (optimal or good).

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

mAb clone **ES05**: The protocols giving an optimal result were all based on HIER using either Tris-EDTA/EGTA pH 9 (3/3), TRS pH 9 (Dako) (2/2), TRS pH 9 (3-in-1, Dako) (3/3), Bond Epitope Retrieval Solution 1 (Bond, Leica) (1/1) or Bond Epitope Retrieval Solution 2 (Bond, Leica) (2/2) as the retrieval buffer. The mAb was typically diluted in the range of 1:25-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 11 out of 11 (100 %) laboratories produced an optimal staining.

**Ready-To-Use Abs**

mAb clone **ES05** (prod. no. IR079, Dako): The protocols giving an optimal result were all based on HIER in PT-Link using TRS pH 9 or TRS pH 9 (3-in-1) and an incubation time of 20 min in the primary Ab and EnVision Flex (K8000) or Flex+ (K8002) as the detection system. Using these protocol settings all of 7 (100 %) laboratories produced an optimal staining.

mAb clone **ES05** (prod. no. PA0610, Leica): The protocol giving an optimal result was based on HIER using Bond Epitope Retrieval Solution 2 (Bond, Leica) and an incubation time of 15 min in the primary Ab and Bond Polymer Refine Detection (DS9800) as the detection system.

mAb clone **G168-728** (prod. no. CMA869, Cell Marque): The protocol giving an optimal result was based on HIER using Bond Epitope Retrieval Solution 2 (Bond, Leica) and an incubation time of 16 min in the primary Ab and Bond Polymer Refine Detection (DS9800) as the detection system.

The most frequent causes of insufficient staining reactions were:

- Less successful Ready-To-Use (RTU) mAb clone G168-728 (Ventana, 11/15 insufficient)
- Use of low sensitive detection systems
- Too low concentration of the primary Ab
- HIER in a non-alkaline buffer

In this assessment and in concordance to the previous NordiQC assessment for MLH1, run 13, 2005, the prevalent feature of an insufficient staining was a too weak or false negative staining of the majority of the cells expected to be demonstrated. The majority of the laboratories were able to demonstrate MLH1 in the cells with a high antigen expression as the proliferating germinal centre B-cells and the basal epithelial cells of the appendix, whereas the demonstration of MLH1 in cells with a low antigen expression as 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 the identification of loss of MLH1 in tumours is characterized by a negative immunoreaction of the neoplastic cells wherefore it is of decisive importance that the nonneoplastic cells within and around the tumour are stained, serving as internal positive control.

An efficient HIER in an alkaline buffer such as Tris-EDTA/EGTA pH 9, BERS2 (Leica) or TRS pH 9 (Dako) - irrespective of the mAb clone applied - seemed mandatory to obtain an optimal staining. If combined with the use of a 3-step polymer based system (e.g., EnVision Flex+, Dako, or Refine, Leica) a very robust protocol was achieved. With HIER in an alkaline buffer and a 3-step polymer system, 33 out of 37 laboratories (89 %) obtained a sufficient result, of which 25 (67 %) were optimal. Using HIER in an alkaline buffer and a less sensitive 2-step polymer or multimer based system (e.g. EnVision Flex, Dako or UltraView, Ventana) only 12 out of 38 (32 %) obtained a sufficient result, of which 5 (13 %) were optimal.

In this assessment the newly launched mAb clone ES05 from Novocastra/Leica and Dako obtained a very high pass rate both as a concentrate (84 %) and in the RTU format (100 %). The mAb clone G168-728 was less successful especially in the RTU format from Ventana and Cell Marque, where only 4 out of 15 laboratories (27 %) using this product obtained a sufficient result, of which only 1 was assessed as optimal. The optimal result was obtained using the Bond III, Leica and HIER in BER2 and a 3-step polymer based system, Refine Leica.

A significant difference in the overall performance for MLH1 was also related to the IHC platform applied. Only 6 out of 25 (24 %) protocols performed on the fully automated platform BenchMark XT or Ultra, Ventana were assessed as sufficient, none were optimal. In contrast, 10 out of 13 (77 %) protocols performed on a similar fully automated platform Bond-max or Bond III were assessed as sufficient, out of which 8 (62 %) were optimal. Tonsil is a recommendable positive control for MLH1: The mantle zone B-cells must show at least a weak to moderate nuclear staining, while a moderate to strong staining must be seen in the proliferating germinal centre B-cells.

This was the 2nd assessment of MLH1 in NordiQC. The proportion of sufficient results decreased from 72 % in run 13, 2005, to 57 % in the current run (Table 2). The lower pass rate may be due to several factors (new tissue material circulated, many new participants).
Table 2. Proportion of sufficient results for MLH1 in the two NordiQC runs performed

<table>
<thead>
<tr>
<th></th>
<th>Run 13 2005</th>
<th>Run 30 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants, n=</td>
<td>25</td>
<td>85</td>
</tr>
<tr>
<td>Sufficient results</td>
<td>72 %</td>
<td>57 %</td>
</tr>
</tbody>
</table>

**Conclusion**

The mAb clones G168-15 and ES05 can both be used to obtain an optimal staining for MLH1. In this assessment the mAb clone ES05 was most successful, both as concentrate and in an RTU format. HIER in alkaline buffer and the use of a 3-step polymer based detection system gave the most robust protocol. Tonsil is a recommendable positive control for MLH1: The mantle zone B-cells must show at least a weak to moderate nuclear staining, while a moderate to strong staining must be seen in the proliferating germinal centre B-cells.

Fig. 1a
Optimal MLH1 staining of the tonsil using the mAb clone ES05 optimally calibrated, using HIER in an alkaline buffer and a 3-step polymer based detection system. Virtually all the mantle zone B-cells show a distinct, moderate to strong nuclear staining, while the germinal centre B-cells show a strong nuclear staining.

Fig. 1b
Insufficient MLH1 staining of the tonsil using the mAb clone ES05 with an insensitive protocol (2-step polymer and too low concentration of the primary Ab) - same field as in Fig. 1a. Only the germinal centre B-cells are demonstrated, while the mantle zone B-cells expressing limited MLH1 are virtually unstained. Also compare with Figs. 2b & 3b, same protocol.

Fig. 2a
Optimal staining for MLH1 of the appendix using same protocol as in Fig. 1a. The majority of the epithelial and the stromal cells show a moderate to strong nuclear staining. Only a weak cytoplasmic staining is seen.

Fig. 2b
Insufficient staining for MLH1 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 significantly reduced compared to the result in Fig. 2a. Also compare with Fig. 3b, same protocol.
Fig. 3a
Optimal MLH1 staining of the colon adenocarcinoma no. 3 (with loss of MLH1 protein) using same protocol as in Figs. 1a & 2a. The neoplastic cells are negative, while the entrapped normal crypts, the lymphocytes and stromal cells show a distinct nuclear staining, serving as internal positive control.

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

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