Assessment Run 28 2010
Bcl-6 protein (Bcl-6)

The slide to be stained for Bcl-6 comprised:
All tissues were fixed in 10 % neutral buffered formalin.

Criteria for assessing a Bcl-6 staining as optimal included:

- A moderate to strong distinct nuclear staining of the majority of the normal germinal centre B-cells in the two tonsils.
- An at least weak to moderate distinct nuclear reaction of the majority of the squamous epithelial cells in the tonsils.
- A moderate to strong distinct nuclear staining of the neoplastic cells of the two follicular lymphomas.
- An at least weak to moderate nuclear staining of the majority of the neoplastic cells of the diffuse large B-cell lymphoma.

132 laboratories participated in this assessment. 48 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. Abs and assessment marks for Bcl-6, run 28

<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 PG-B6p</td>
<td>57</td>
<td>Dako Diagnostic BioSystems Master Diagnostica NeoMarkers</td>
<td>10</td>
<td>15</td>
<td>27</td>
<td>9</td>
<td>40 %</td>
<td>60 %</td>
</tr>
<tr>
<td>mAb clone LN22</td>
<td>13</td>
<td>Novocastra</td>
<td>6</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>54 %</td>
<td>100 %</td>
</tr>
<tr>
<td>mAb clone G1191E/A8</td>
<td>8</td>
<td>Cell Marque Immunologic CNIO</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>18 %</td>
<td>-</td>
</tr>
<tr>
<td>mAb clone P1F6+PG-B6p</td>
<td>4</td>
<td>NeoMarkers</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mAb clone P1F6</td>
<td>1</td>
<td>Biocare</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Ready-To-Use Abs

<table>
<thead>
<tr>
<th>mAb clone PG-B6p, IR625</th>
<th>14</th>
<th>Dako</th>
<th>2</th>
<th>5</th>
<th>6</th>
<th>1</th>
<th>50 %</th>
<th>71 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb clone G1191E/A8, 760-4241</td>
<td>23</td>
<td>Ventana/Cell Marque</td>
<td>7</td>
<td>11</td>
<td>5</td>
<td>0</td>
<td>78 %</td>
<td>81 %</td>
</tr>
<tr>
<td>mAb clone G1191E/A8, ZM0011</td>
<td>1</td>
<td>Zymed</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mAb clone LN22, PA0204</td>
<td>3</td>
<td>Leica</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mAb clone P1F6, PM223</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>132</td>
<td></td>
<td>26</td>
<td>38</td>
<td>52</td>
<td>16</td>
<td>20 %</td>
<td>29 %</td>
</tr>
</tbody>
</table>

Proportion

<p>| | | | | | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>20 %</td>
<td>29 %</td>
<td>39 %</td>
<td>12 %</td>
<td>49 %</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 PG-B6p: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) in one of the following buffers: Tris-EDTA/EGTA pH 9 (2/17)*, Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH, Dako)(6/18), or Bond Epitope Retrieval Solution 2 (Bond, Leica) (2/4). The mAb was typically
including new, more challenging tissue material circulated and many laboratories participating for the first time. This was the second assessment of Bcl-6 epitope detected by the most commonly used mAb clone PG-B6p. The proportion of sufficient results declined from 87 % in run 17, 2006 to 48 % in the current run – see table 2. The lower pass rate may be due to several factors including new, more challenging tissue material circulated and many laboratories participating for the first time.

**mAb clone LN22:** The protocols giving an optimal result were all based on HIER using one of the following buffers: Tris-EDTA/EGTA pH 9 (3/6), Cell Conditioning 1 (BenchMark, Ventana) (2/2), or Bond Epitope Retrieval Solution 2 (Bond, Leica) (1/4). The mAb was typically diluted in the range of 1:40–1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 7 out of 7 (100 %) laboratories produced a sufficient staining.

**Ready-To-Use Abs**

- **mAb clone PG-B6p** (prod. no IR625, Dako): The protocols giving an optimal result were all based on HIER in PT-Link using Target Retrieval Solution pH 9 (EnVision FLEX TRS high pH), an incubation time of 20 min in the primary Ab and EnVision Flex+ (K8002) as the detection system. Using these protocol settings 5 out of 7 (71 %) laboratories produced a sufficient staining.

- **mAb clone GI191E/A8** (prod. no. 760-4241, Ventana/Cell Marque): The protocols giving an optimal result were based on HIER using mild, standard or extended HIER in Cell Conditioning 1 (BenchMark, Ventana), an incubation time of 32 – 60 min in the primary Ab and iView (760-091) or Ultra View (760-500) as the detection system. 3 labs used amplification kit. Using these protocol settings 17 out of 21 (81 %) laboratories produced a sufficient staining.

The most frequent causes of insufficient staining results were:
- Insufficient HIER (in particular with citrate pH 6 as the heating buffer)
- Too low concentration of the primary antibody
- Use of low sensitive detection systems (in particular for mAb clone PG-B6p)
- Excessive counter staining compromising the interpretation
- Possibly inappropriate blocking of endogenous peroxidase (3% H₂O₂)

In this assessment and in concordance with the previous assessment, Run 17, 2006, tonsil is found to be a recommendable control for Bcl-6 in NordiQC. The proportion of sufficient results has declined from 61 % in the previous run to 48 % in the current run – see table 2. The lower pass rate may be due to several factors including new, more challenging tissue material circulated and many laboratories participating for the first time.
Table 2. **Proportion of sufficient results for Bcl-6 in the two NordiQC runs performed**

<table>
<thead>
<tr>
<th></th>
<th>Run 17 2006</th>
<th>Run 28 2010</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants, n=</td>
<td>69</td>
<td>132</td>
</tr>
<tr>
<td>Sufficient results</td>
<td>87 %</td>
<td>48 %</td>
</tr>
</tbody>
</table>

**Conclusion**

The mAb clones GI 191E/A8, LN22, PG-B6p and P1F6+PG-B6p and are useful antibodies for Bcl-6. HIER in an alkaline buffer is mandatory for optimal performance. Normal tonsil is a recommendable positive control: Both the germinal centre B-cells and the squamous epithelial cells must show a distinct nuclear staining.

*Fig. 1a*

Optimal Bcl-6 staining of the tonsil using the mAb clone PG-B6p optimally calibrated and with HIER in an alkaline buffer. Virtually all the germinal centre B-cells show a strong and distinct nuclear staining, while the majority of the squamous epithelial cells show a weak to moderate nuclear staining.

*Fig. 1b*

Insufficient Bcl-6 staining of the tonsil using the mAb clone BG-B6p - same field as in Fig. 1a. The germinal centre B-cells only show a weak to moderate nuclear staining, while the squamous epithelial cells are virtually negative – also compare with Figs. 2b – 3b, same protocol.

*Fig. 2a*

Optimal Bcl-6 staining of the follicular lymphoma grade I using same protocol as in Fig. 1a. Virtually all the neoplastic cells show a moderate, distinct nuclear staining.

*Fig. 2b*

Insufficient Bcl-6 staining of the follicular lymphoma grade I using same protocol as in Fig. 1b - same field as in Fig 2a. The neoplastic cells only show a weak and equivocal staining.
Fig. 3a
Optimal Bcl-6 staining of the diffuse large B-cell lymphoma using same protocol as in Figs. 1a & 2a. The majority of the neoplastic cells show a moderate, distinct nuclear staining.

Fig. 3b
Insufficient Bcl-6 staining of the diffuse large B-cell lymphoma using same protocol as in Figs. 1b & 2b – same field as in Fig. 3a. The neoplastic cells only show a weak and equivocal staining.

Fig. 4a
Optimal Bcl-6 staining using the mAb clone PG-B6p diluted 1:20, incubated for 30 min. at room temp. and visualized with EnVision+ K4007, Dako. Endogenous peroxidase blocking was performed with a 0.03% H₂O₂ solution (K4007 provided in the kit) for 10 min after HIER in an alkaline buffer. The majority of the germinal centre B-cells shows a distinct staining. Compare with Fig. 4b using same protocol, except for peroxidase blocking performed with 3 % H₂O₂.

Fig. 4b
Bcl-6 staining using the same protocol as in Fig. 4a (same field), apart from the peroxidase blocking, which was performed with 3 % H₂O₂. This seems to deteriorate the epitope detected by the clone PG-B6p. This effect was most marked when the blocking in 3 % H₂O₂ was performed after HIER, but also seen if performed before HIER. 3 % H₂O₂ is used as routine in many laboratories and also as a blocking reagent provided in commercially distributed visualization kits from various companies.

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