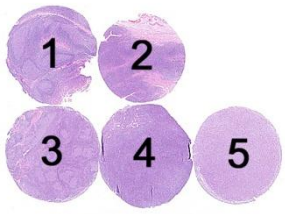
Assessment Run 28 2010

Bcl-6 protein (Bcl-6)

The slide to be stained for Bcl-6 comprised:
1. Tonsil, 24 h., 2. Tonsil, 48 h., 3. Follicular lymphoma, grade I, 4. Follicular lymphoma, grade II, 5. Diffuse large B-cell lymphoma.
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**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **PG-B6p**	57 2 1 1	Dako Diagnostic BioSystems Master Diagnostica NeoMarkers	10	15	27	9	40 %	60 %
mAb clone **LN22**	13	Novocastra	6	1	6	0	54 %	100 %
mAb clone **GI191E/A8**	8 2 1	Cell Marque Immunologic CNIO	0	2	6	3	18 %	-
mAb clone **P1F6+PG-B6p**	4	NeoMarkers	1	1	1	1	-	-
mAb clone **P1F6**	1	Biocare	0	0	0	1	-	-
Ready-To-Use Abs								
mAb clone **PG-B6p, IR625**	14	Dako	2	5	6	1	50 %	71 %
mAb clone **GI191E/A8, 760-4241**	23	Ventana/Cell Marque	7	11	5	0	78 %	81 %
mAb clone **GI191E/A8, ZM0011**	1	Zymed	0	0	0	1	-	-
mAb clone **LN22, PA0204**	3	Leica	0	2	1	0	-	-
mAb clone **P1F6, PM223**	1	Biocare	0	1	0	0	-	-
Total	132		26	38	52	16	-	-
Proportion			20 %	29 %	39 %	12 %	49 %	-

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

diluted in the range of 1:10– 1:40 depending on the total sensitivity of the protocol employed. Using these protocol settings 21 out of 35 (60 %) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

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.

mAb clones **P1F6+PG-B6p**: The protocol giving an optimal result was based on HIER using Bond Epitope Retrieval Solution 2 (Bond, Leica) as the retrieval buffer. The mAb was diluted 1:10. Using these protocol settings both of 2 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% H_2O_2)

In this assessment and in concordance to the previous assessment run 17, 2007, the prevalent feature of an insufficient staining was a generally too weak or completely false negative staining reaction of the cells expected to stain. Virtually all laboratories could demonstrate Bcl-6 in the normal germinal centre B-cells in the two tonsils, whereas the demonstration of Bcl-6 in the follicular lymphoma grade I and in particular the diffuse large B-cell lymphoma was more challenging and required a correctly calibrated protocol. Some of the insufficient stains were characterized by a poor signal-to-noise ratio and/or by an excessive counterstaining compromising the interpretation.
The immunohistochemical demonstration of Bcl-6 seems to require a highly sensitive protocol primarily based on 1) Efficient HIER in an alkaline buffer, 2) A high concentration of the Ab and 3) A robust (preferably a 3-step polymer/multimer based) detection system.
It was observed that apparently identical protocols in some cases gave completely different staining patterns with different levels of sensitivity, which may indicate that other methodological parameters than those submitted to NordiQC could affect the staining result. From internal studies in NordiQC reference laboratories it appears that the Bcl-6 epitope detected by the most commonly used mAb clone PG-B6p is impaired by 3 % H_2O_2 used for blocking of endogenous peroxidase – see photos Figs. 4a & 4b. Thus, it is recommended either to use a lower concentration of H_2O_2 or completely omit the endogenous peroxidase blocking step when clone PG-B6p is used. Omission of peroxidase blocking was actually done by some laboratories obtaining optimal staining results.

In concordance with the previous assessment, Run 17, 2006, tonsil is found to be a recommendable control for Bcl-6 provided that a strong distinct nuclear reaction is seen in the majority of the germinal centre B-cells and - even more important - an at least weak to moderate nuclear reaction is seen in the majority of the squamous epithelial cells. Virtually all labs obtaining this reaction pattern were assessed as sufficient.

This was the second assessment of Bcl-6 in NordiQC. 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.

Table 2. **Proportion of sufficient results for Bcl-6 in the two NordiQC runs performed**

	Run 17 2006	Run 28 2010
Participants, n=	69	132
Sufficient results	87 %	48 %

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.

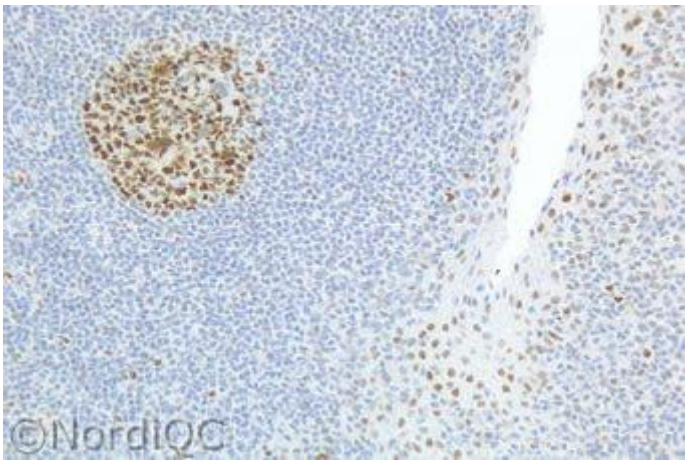


Fig. 1a
Optimal Bcl-6 staining of the tonsil using the mAb clone PG-B6p optimally calibrated and with HIER in an alkaline buffer. Virtually all the germinal centre B-cells show a strong and distinct nuclear staining, while the majority of the squamous epithelial cells show a weak to moderate nuclear staining.

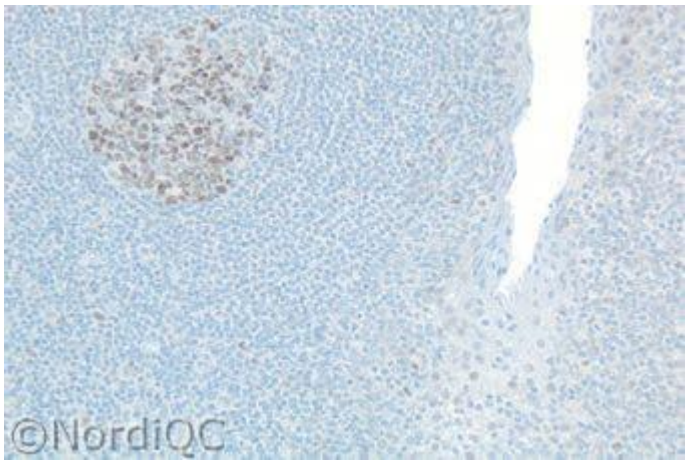


Fig. 1b
Insufficient Bcl-6 staining of the tonsil using the mAb clone BG-B6p - same field as in Fig. 1a. The germinal centre B-cells only show a weak to moderate nuclear staining, while the squamous epithelial cells are virtually negative – also compare with Figs. 2b – 3b, same protocol.

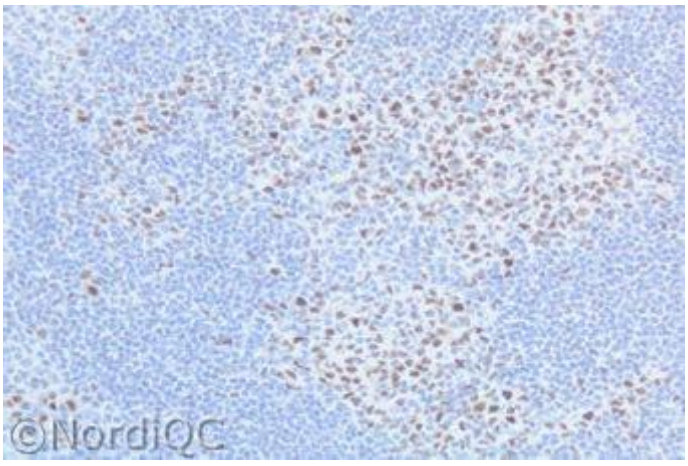


Fig. 2a
Optimal Bcl-6 staining of the follicular lymphoma grade I using same protocol as in Fig. 1a. Virtually all the neoplastic cells show a moderate, distinct nuclear staining.

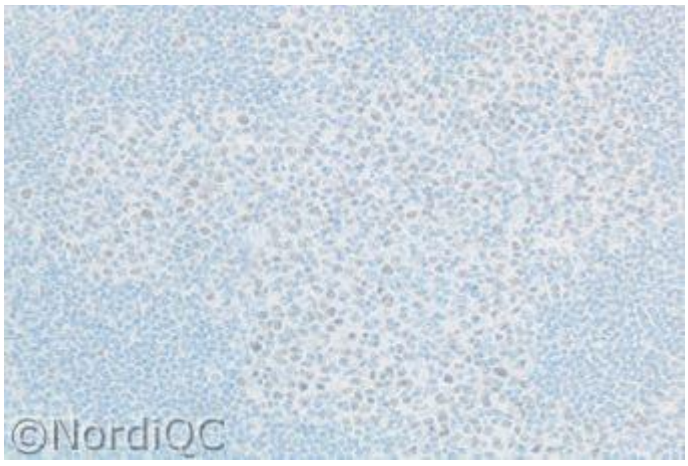


Fig. 2b
Insufficient Bcl-6 staining of the follicular lymphoma grade I using same protocol as in Fig. 1b - same field as in Fig 2a. The neoplastic cells only show a weak and equivocal staining.

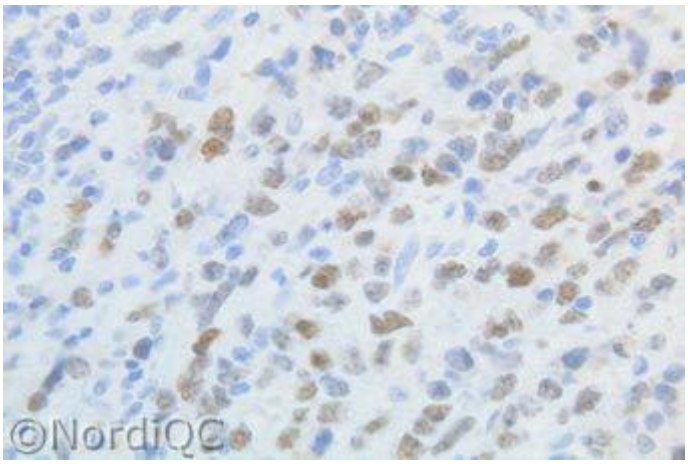


Fig. 3a
Optimal Bcl-6 staining of the diffuse large B-cell lymphoma using same protocol as in Figs. 1a & 2a. The majority of the neoplastic cells show a moderate, distinct nuclear staining.

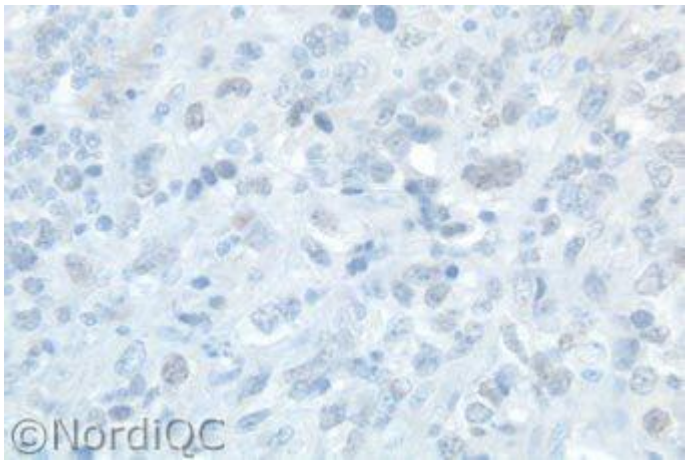


Fig. 3b
Insufficient Bcl-6 staining of the diffuse large B-cell lymphoma using same protocol as in Figs. 1b & 2b – same field as in Fig. 3a. The neoplastic cells only show a weak and equivocal staining.

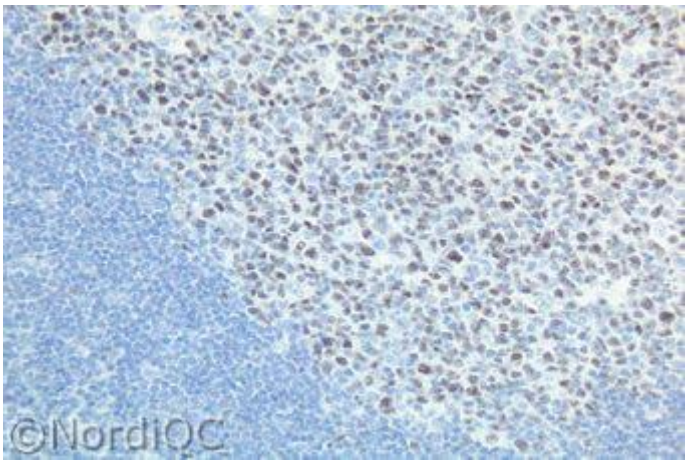


Fig. 4a
Optimal Bcl-6 staining using the mAb clone PG-B6p diluted 1:20, incubated for 30 min. at room temp. and visualized with EnVision+ K4007, Dako. Endogenous peroxidase blocking was performed with a 0.03% H_2O_2 solution (K4007 provided in the kit) for 10 min after HIER in an alkaline buffer.
The 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 % H_2O_2.

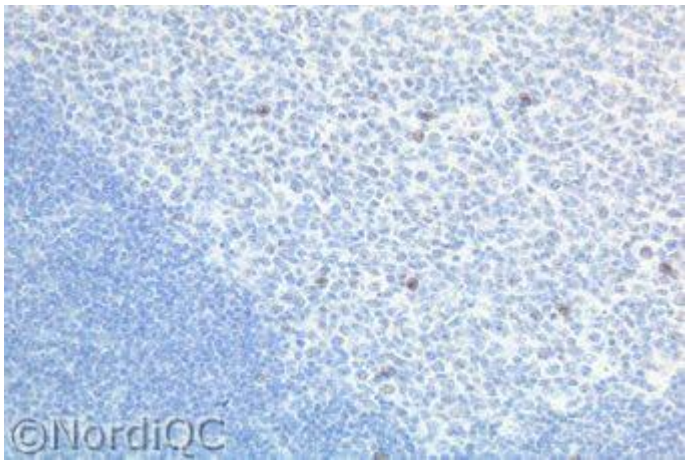


Fig. 4b
Bcl-6 staining using the same protocol as in Fig. 4a (same field), apart from the peroxidase blocking, which was performed with 3 % H_2O_2. This seems to deteriorate the epitope detected by the clone PG-B6p. This effect was most marked when the blocking in 3% H_2O_2 was performed after HIER, but also seen if performed before HIER. 3 % H_2O_2 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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