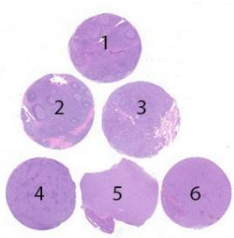
Assessment Run 42 2014

Bcl-6 protein (Bcl-6)

Material
The slide to be stained for Bcl-6 comprised:

1. Tonsil, 24h fixation, 2. Tonsil, 48h fixation*, 3. Follicular lymphoma grade I, 4. Follicular lymphoma grade II, 5. Diffuse large B-cell lymphoma, non-Germinal Centre B-cell type (DLBCL non-GCB), 6. DLBCL, GCB.



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 reaction of virtually all normal germinal centre B-cells in the tonsil fixed 24h.
- An at least weak to moderate distinct nuclear staining reaction of the majority of the squamous epithelial cells in the tonsil fixed 24h.
- A moderate to strong distinct nuclear staining reaction of the neoplastic cells in the two follicular lymphomas.
- An at least weak to moderate nuclear staining reaction of the majority of the neoplastic cells in the DLBCL, GCB subtype, tissue core no. 6.
- No or only a nuclear staining reaction in dispersed neoplastic cells of the DLBCL, non-GCB subtype, tissue core no. 5

* The tonsil fixed for 48h (tissue core no 2) was excluded from the assessment due to an aberrant inconsistent staining reaction in the circulated material.

Participation

Number of laboratories registered for Bcl-6, run 42	244
Number of laboratories returning slides	228 (93%)

Results
228 laboratories participated in this assessment. Of these, 168 (74%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- too low concentration of the primary antibody
- less successful performance of the mAb clone PG-B6p
- use of low sensitivity detection systems

Performance history
This was the third NordiQC assessment of Bcl-6. An increased pass rate was seen compared to the two previous runs 17, 2006 and 28, 2010 (see table 2).

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

	Run 17 2006	Run 28 2010	Run 42 2014
Participants, n=	69	132	228
Sufficient results	42%	48%	74%

Conclusion
The mAbs clones **GI191E/A8**, **LN22** and **PG-B6p** could all be used to produce optimal staining results for Bcl-6. Irrespective of the clone applied, efficient HIER in alkaline buffer, use of a high sensitive detection system and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result. The mAb clones **GI191E/A8** and **LN22** provided the highest proportion of sufficient and optimal results both as concentrated format and as Ready-To-Use format (Ventana and Leica, respectively). Tonsil is recommended as positive and negative tissue control: Virtually all germinal centre B-cells must show a moderate to strong nuclear staining reaction, while the majority of squamous epithelial cells must display an at least weak nuclear staining reaction. No staining reaction must be seen in the vast majority of mantle zone B-cells and interfollicular T- and B-cells.

Table 1. **Antibodies and assessment marks for Bcl-6, run 42**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **GI191E/A8**	13 1 1	Cell Marque Immunologic Zytomed	6	8	0	1	93%	100%
mAb clone **LN22**	38 2 1 1 1	Leica/Novocastra DBS Biocare BioGenex Zeta Corporation	20	16	4	3	84%	100%
mAb clone **PG-B6p**	43 1 1	Dako DBS Thermo/Neomarkers	9	22	11	3	69%	86%
Ready-To-Use antibodies								
mAb clone **GI191E/A8 760-4241**	59	Ventana/Cell Marque	24	25	9	1	83%	84%
mAb clone **GI191E/A8 227M-9x**	1	Cell Marque	0	0	1	0	-	-
mAb clone **LN22 PA0204**	10	Leica/Novocastra	3	7	0	0	100%	100%
mAb clone **LN22 PM410**	1	Biocare	1	0	0	0	-	-
mAb clone **LN22 MAD-00638QD**	1	Master Diagnostica	0	1	0	0	-	-
mAb clone **PG-B6p IR/IS625**	44	Dako	4	17	21	2	48%	75%
mAb clone **PG-B6p GA625**	7	Dako	2	2	3	0	57%	75%
mAb **PG-B6p MAD-004023QD**	2	Master Diagnostica	0	1	1	0	-	-
Total	228		69	99	50	10	-	
Proportion			30%	44%	22%	4%	74%	

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

Detailed analysis of Bcl-6, Run 42

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies

mAb clone **GI191E/A8**: Protocols with optimal results were all based on HIER using Cell Conditioning 1 (CC1; Ventana) (6/14)* as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 9 of 9 (100%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **LN22**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (2/2), CC1 (Ventana) (9/18) or Epitope Retrieval Solution 2 (BERS2; Leica) (9/11) as retrieval buffer. The mAb was typically diluted in the range of 1:25-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 27 of 27 (100%) laboratories produced a sufficient staining result.

mAb clone **PG-B6p**: Protocols with optimal results were all based on HIER using TRS pH 9 (3-in-1) (Dako) (2/8), TRS pH 9 (Dako) (2/6), CC1 (Ventana) (1/13), Tris-EDTA/EGTA pH 9 (3/6) or EGTA/EDTA pH 8 (1/2) as retrieval buffer. The mAb was diluted in the range of 1:10-1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings 18 of 21 (86%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for Bcl-6 for the two most commonly used antibodies 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
mAb clone **PG-B6p**	4/12** (33%)	-	1/11 (9%)	-	0/4	-
mAb clone **LN22**	2/2	-	9/16 (56%)	-	8/8 (100%)	-

* 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 **GI191E/A8**, product no. **760-4241**, Ventana/Cell Marque, BenchMark XT/Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 30-90 min.), 12-52 min. incubation of the primary Ab. and UltraView (760-500) +/- amplification kit or OptiView (760-700) +/- amplification kit as detection systems. Using these protocol settings 41 of 49 (84%) laboratories produced a sufficient staining result (optimal or good).

mAb clone **LN22**, product no. **PA0204**, Leica, Bond-max/Bond-III:
Protocols with optimal results were all based on HIER using BERS 2 pH 9 (Bond, Leica) (efficient heating time 20-30 min. at 99-100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings 8 of 8 (100%) laboratories produced a sufficient staining result.

mAb clone **LN22**, product no. **PM410**, Biocare, intelliPATH:
One protocol with an optimal result was based on HIER using Diva pH 6.2 (Biocare) in a pressure cooker, 60 min. incubation of the primary Ab and MACH4 (M4U534) as detection system.

mAb clone **PG-B6p** product no. **IS625/IR625** 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 min. at 97-99°C),20-30 min. incubation of the primary Ab and EnVision FLEX+ (K8000/K8002) as detection system. Using these protocol settings 6 of 8 (75%) laboratories produced a sufficient staining result.

mAb clone **PG-B6p**, product no. **GV625**, Dako, OMNIS:
Protocols with optimal results were based on HIER using TRS pH 9 (efficient heating time 30 min. at 97°C),12½ min. incubation of the primary Ab and EnVision FLEX+ (GV800+GV821) as detection system. Using these protocol settings 3 of 4 (75%) laboratories produced a sufficient staining result.

Comments
In this NordiQC assessment for Bcl-6 and in concordance to the previous runs 17 and 28, the prevalent features of an insufficient staining result was a generally too weak or completely false negative staining reaction of the cells expected to be demonstrated. This was observed in 95% of the insufficient results (57 of 60). Virtually all laboratories were able to demonstrate Bcl-6 in high-level antigen expressing cells, such as normal germinal centre B-cells in the tonsil fixed for 24h in NBF and the neoplastic cells of the follicular lymphoma grade II, tissue core no 4. In contrast, Bcl-6 in low-level antigen expressing cells as squamous epithelial cells of the tonsil, the neoplastic cells of the follicular lymphoma grade I, tissue core 3 and the DLBCL GCB subtype, tissue core no 6 could only be demonstrated with an optimally calibrated protocol. The remaining insufficient results were caused by impaired morphology or a poor signal-to-noise ratio.

Optimal staining results could be obtained with all three mAbs used for Bcl-6; clones GI191E/A8, LN22 and PG-B6p. Used as a concentrate within a laboratory developed (LD) assay, mAb clones GI191E/A8 and LN22 gave an overall higher pass rate and proportion of optimal results compared to the mAb clone PG-B6p (see table 1). Especially the mAb clone LN22 provided a high proportion of optimal results. 47% (20 of 43 protocols) of LD assays based on the mAb clone LN22 were assessed as optimal compared to 20% (9 of 45 protocols) for protocols based on the mAb clone PG-B6p. It was shown in previous assessments for Bcl-6 that the mAb clone PG-B6p provided an inferior result if endogenous peroxidase blocking was performed by treatment with 3% H_2O_2 prior to the incubation of the primary antibody. This is a standard method on many automated IHC platforms such as the Ventana BenchMark and Leica BOND. The mAb clone LN22 seems to be more robust in this matter and, as shown in table 3, optimal results could be obtained on all three main IHC platforms using standard operating procedures.
For all three clones, careful calibration of the titer of the primary Ab, efficient HIER in an alkaline buffer and use of a highly sensitive detection system were the main protocol prerequisites for optimal results.

Especially choice of detection system seemed to have a high impact on the proportion of sufficient and optimal results. If the LD assay was based on a titer of the concentrated format in the "optimal dilution range" as listed above and applied with HIER in an alkaline buffer and a 2-step polymer/multimer based detection system as EnVision FLEX (Dako) or UltraView (Ventana), a pass rate of 68% (27 of 40 protocols) was seen, out of which 15% (6 of 40 protocols) were optimal. If the same protocol settings were applied with a 3-step polymer/multimer based detection system as EnVision FLEX+ (Dako), Optiview (Ventana) or Bond Refine (Leica), a pass rate of 93% (39 of 42 protocols) was seen and 62% (26 of 42 protocols) were optimal. Only protocols submitted with unambiguous information regarding the use of 2-step versus 3-step systems were incorporated in this comparison.

In this assessment, the two RTU systems from Leica and Ventana (based on the mAb clone LN22 and GI191E/A8, respectively) provided comparable pass rates and proportion of optimal results to LD assays using the same clones (see table 1). The Dako RTU systems based on the mAb clone PG-B6p showed a slightly lower pass rate.
For the Ventana RTU system, optimal results were typically obtained by modified and laboratory validated protocol settings using prolonged HIER and UltraView + amplification or OptiView +/- amplification as detection system instead of the recommendations given in the package inserts.
Optimal results for the Dako and Leica RTU systems could be obtained by both the official recommendations and by modified protocol settings of e.g. HIER time and incubation time of the primary antibody.

It is difficult to conclude on the specific causes for the significant improvement of the pass rate in this run, as many laboratories participated for the first time and many laboratories have changed their IHC systems completely compared to the previous run in 2010. However, the extended use of the robust clones GI191E/A8 and LN22 providing successful results on all 3 main IHC systems within a LD assay seems to be one of the central elements. In run 28 (2010) 32% of the participants (24 of 90) used one of the two clones within a LD assay compared to 56% (58 of 103) in this run. As shown in table 1 and 3 the clones GI191E/A8 and LN22 provided a higher proportion of sufficient results compared to clone PG-B6. The availability and increased use of highly sensitive 3-step polymer/multimer based detection systems both for LD assays and RTU systems also seemed to contribute to the improved pass rate.

Controls
Normal tonsil is recommended as positive and negative tissue control. Virtually all the germinal centre B-cells must show a moderate to strong nuclear staining reaction, while an at least weak to moderate nuclear staining reaction must be seen in the majority of squamous epithelial cells. In the mantle zones and interfollicular areas only dispersed cells must show a nuclear staining reaction.

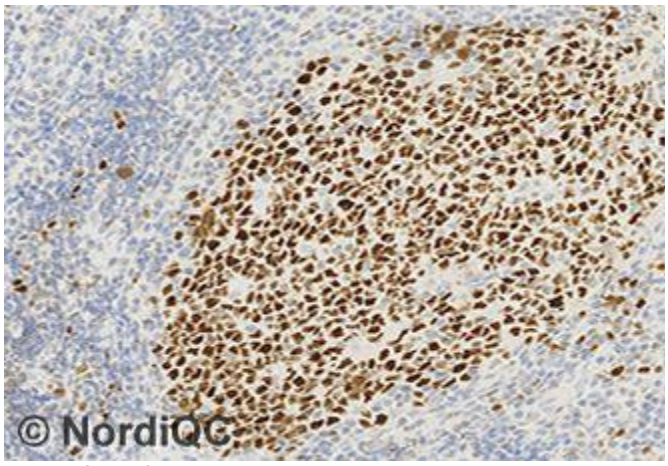


Fig. 1a (X200)
Optimal staining result for Bcl-6 of the tonsil using the mAb clone LN22 optimally calibrated, using HIER in an alkaline buffer (ER2 pH 9.0 Leica) and a 3-step polymer based detection system (Refine, Leica). Virtually all germinal centre B-cells show a moderate to strong nuclear staining reaction. No background staining is observed – also compare with Figs. 2a - 4a, same protocol.

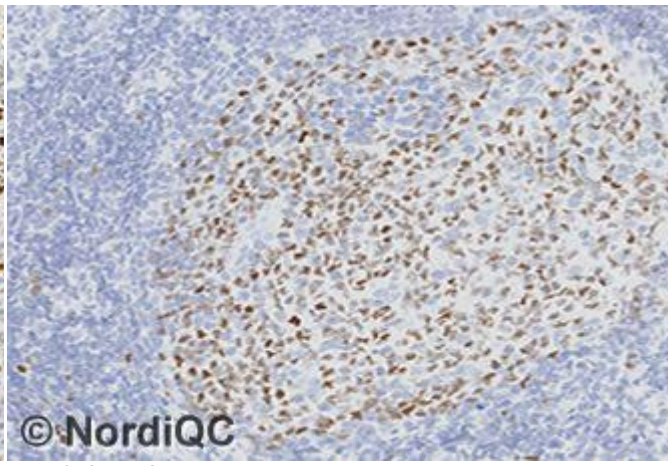


Fig. 1b (X200)
Staining result for Bcl-6 of the tonsil using an insufficient protocol based on the mAb clone LN22. The protocol provided a too low sensitivity most likely due to a too low titre of the primary Ab and use of a 2-step polymer based detection system (EnVision FLEX, Dako) - same field as in Fig. 1a. The majority of germinal centre B-cells are demonstrated, but the intensity is significantly reduced – also compare with Figs. 2b - 4b, same protocol.

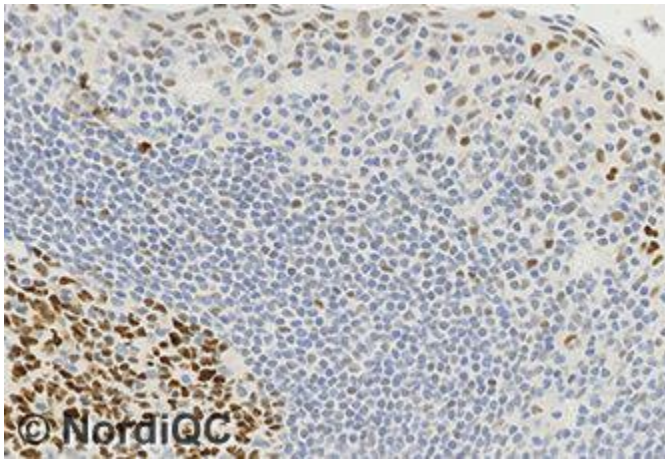


Fig. 2a (X200)
Optimal staining result for Bcl-6 of the tonsil using same protocol as in Fig. 1a. The majority of squamous epithelial cells with low-level Bcl-6 expression show a weak to moderate nuclear staining reaction, while the germinal centre B-cells with high-level expression show a moderate to strong nuclear staining reaction.

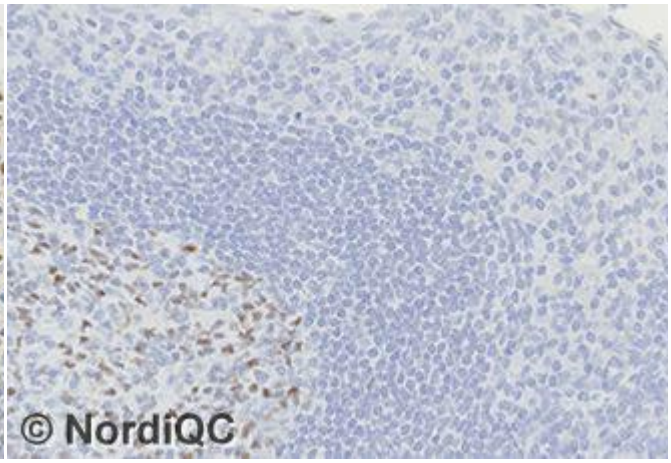


Fig. 2b (X200)
Insufficient staining result for Bcl-6 of the tonsil using same protocol as in Fig. 1b - same field as in Fig. 2a. Only germinal centre B-cells are demonstrated, whereas squamous epithelial cells with low-level Bcl-6 expression are negative - also compare with Fig. 3b, same protocol.

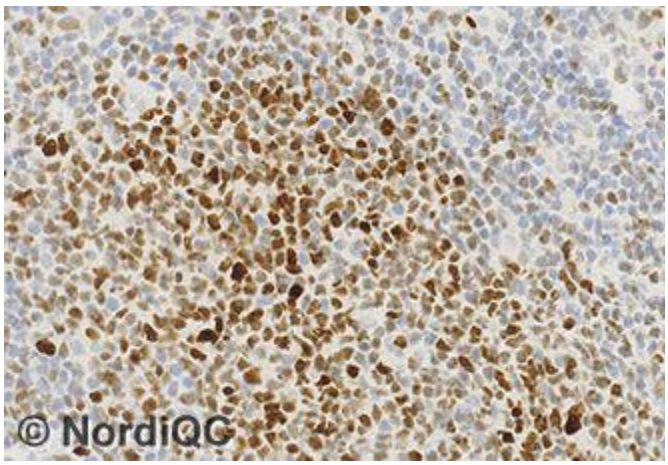


Fig. 3a (X200)
Optimal staining result for Bcl-6 of the follicular lymphoma grade I using same protocol as in Figs. 1a & 2a. The majority of the neoplastic cells show a moderate to strong nuclear staining reaction.

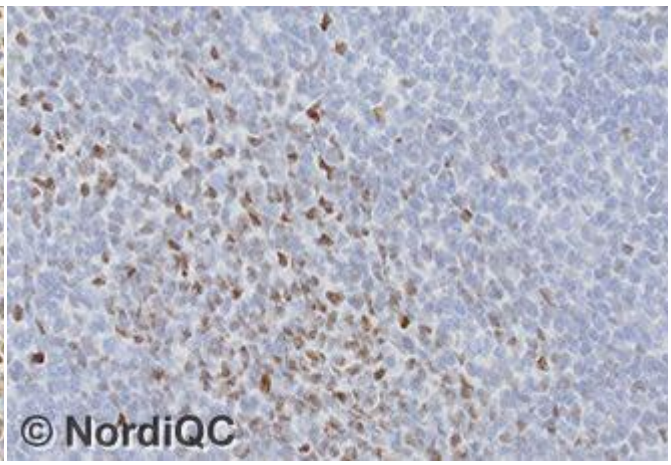


Fig. 3b (X200)
Insufficient staining result for Bcl-6 of the follicular lymphoma grade I using same protocol as in Figs. 1b & 2b. – same field as in Fig. 3a. The proportion and staining intensity of the neoplastic cells is significantly reduced compared to the level expected and obtained in Fig. 3a.

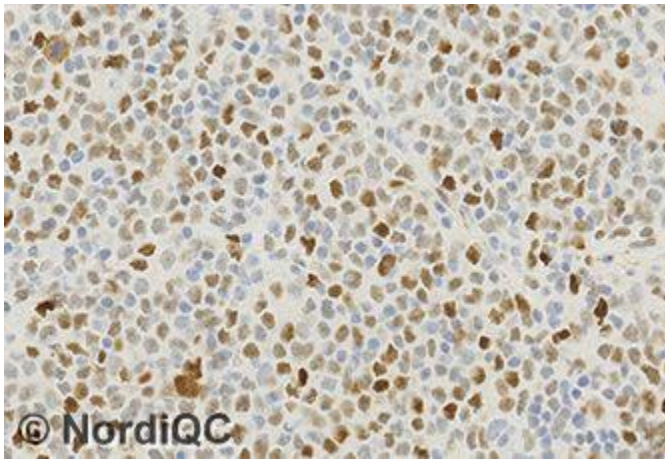


Fig.4a (X200)
Optimal staining result for Bcl-6 of the DLBCL, GCB subtype using same protocol as in Figs. 1a - 3a. The vast majority of neoplastic cells show a weak to strong and distinct nuclear staining reaction. No background staining is observed.

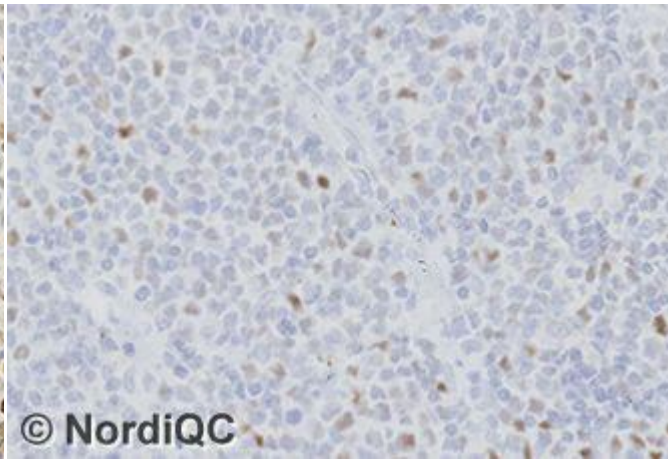


Fig. 4b (X200)
Insufficient staining result for Bcl-6 of the DLBCL, GCB subtype using same protocol as in Figs. 1b - 3b. – same field as in Fig. 4a. Only dispersed neoplastic cells are demonstrated.

SN/RR/LE/MV 11-11-2014