Assessment Run 55 2019

Bcl-6 protein (Bcl-6)

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

1. Tonsil, 24h fixation, 2. Tonsil, 48h fixation, 3. Mantle cell lymphoma (MCL), 4-5. Follicular lymphomas (FL), 6. Diffuse large B-cell lymphoma (DLBCL).

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 tonsils.
- An at least weak to moderate, distinct nuclear staining reaction of the majority of the squamous epithelial cells in the tonsils.
- A moderate to strong, distinct nuclear staining reaction of the neoplastic cells in the two follicular lymphomas.
- An at least moderate, distinct nuclear staining reaction of the majority of the neoplastic cells in the DLBCL.
- No staining reaction of the neoplastic cells in the MCL (dispersed remnants of normal B-cells show moderate to strong nuclear staining reactions).

Participation

Number of laboratories registered for Bcl-6, run 55	289
Number of laboratories returning slides	279 (97%)

Results
279 laboratories participated in this assessment. 216 (77%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Insufficient Heat Induced Epitope Retrieval (HIER) (to short HIER time or use of low pH antigen retrieval buffer)
- too low concentration of the primary antibody
- less successful performance of the Ready-to-use (RTU) system 760-4241 (Ventana) based on the mAb GI191E/A8
- unexplained technical issues

Performance history
This was the fourth NordiQC assessment of Bcl-6. The pass rate increased marginally compared to the previous runs 42, 2014 (see Table 2).

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

	Run 17 2006	Run 28 2010	Run 42 2014	Run 55 2019
Participants, n=	69	132	228	279
Sufficient results	42%	48%	74%	77%

Conclusion
The mAbs clones **GI191E/A8**, **LN22**, **PG-B6p** and the rmAb clone **ER278** 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 highly sensitive detection system and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result. The mAb clones **PG-B6p** and **LN22** provided the highest proportion of sufficient and optimal results applied as an RTU format (Dako and Leica, respectively). The RTU system based on the mAb clone GI191E/A8 (Ventana) provided a lower pass rate and was challenged by poor signal-to-noise ratio and false positive results. Tonsil is recommended as tissue control: Virtually all germinal centre B-cells must show a moderate to strong nuclear staining reaction and the majority of squamous epithelial cells must display an at least weak nuclear intensity. No staining reaction should be seen in the vast majority of mantle zone B-cells and interfollicular

lymphocytes.

Table 1. **Antibodies and assessment marks for Bcl-6, run 55**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **GI191E/A8**	18 1 1	Cell Marque Immunologic Zytomed Systems	11	4	4	1	75%	92%
mAb clone **LN22**	50 2	Leica/Novocastra Diagnostic Biosystems	29	14	8	1	83%	90%
mAb clone **PG-B6p**	29	Agilent/Dako	16	6	3	4	76%	88%
Ready-To-Use antibodies								
mAb clone **GI191E/A8 760-4241**	88	Roche/Ventana	22	29	28	9	58%	76%
mAb clone **GI191E/A8 227M-9x**	4	Cell Marque	1	1	1	1	-	-
mAb clone **LN22 PA0204**	18	Leica/Novocastra	14	4	0	0	100%	100%
mAb clone **LN22 PM410**	1	Biocare	1	0	0	0	-	-
mAb clone **LN22 MAD-00638QD**	2	Master Diagnostica	2	0	0	0	-	-
mAb clone **PG-B6p IR/IS625**	23	Agilent/Dako	8	14	1	0	96%	100%
mAb clone **PG-B6p IR/IS625**[3]	4	Agilent/Dako	1	2	1	0	-	-
mAb clone **PG-B6p GA625**	34	Agilent/Dako	18	15	1	0	97%	96%
mAb **MX042 MAB-0746**	1	Maixin	1	0	0	0	-	-
rmAb **EP278 8461-C010**	2	Sakura Finetek	2	0	0	0	-	-
rmAb **EP278 277R-2x**	1	Cell Marque	0	1	0	0	-	-
Total	279		126	90	47	16	-	
Proportion			45%	32%	17%	6%	77%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Ready-to-use product developed for a specific semi/fully automated platform by a given manufacturer but inappropriately applied by laboratories on other non-validated semi/fully automatic systems or used manually.

Detailed analysis of Bcl-6, Run 55
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) (11/17)* 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, 11 of 12 (92%) 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) (4/8), CC1 (Ventana) (12/21), Epitope Retrieval Solution 2 (BERS2; Leica) (12/18) or Tris-EDTA / EGTA pH 9 (1/2) 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, 28 of 31 (90%) 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) (7/13), CC1 (Ventana) (6/9) or BERS2 (Leica) (3/3) as retrieval buffer. The mAb was typically diluted in

the range of 1:10-1:25 depending on the total sensitivity of the protocol employed. Using these protocol settings, 15 of 17 (88%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for Bcl-6 for the three most commonly used antibodies as concentrate on the four main IHC systems***

Concentrated antibodies	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	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 **GI191E/A8**	0/0**	0/0	0/0	0/0	10/12 (83%)	0/0	0/0	0/0
mAb clone **LN22**	1/1	-	3/3	-	8/12 (67%)	-	10/15 (67%)	0/1
mAb clone **PG-B6p**	3/7	-	2/2	-	5/6	-	2/2	-

* 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 CC1 (efficient heating time 32-64 min.), 16-32 min. incubation of the primary Ab. and OptiView (760-700) +/- amplification kit as detection systems. Using these protocol settings, 25 of 33 (76%) 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 typically based on HIER using BERS2 pH 9 (Bond, Leica) (efficient heating time 20-30 min. at 99-100°C), 15-20 min. incubation of the primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings, 13 of 13 (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 (efficient heating time 15 min. at 110°C), 30 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) (efficient heating time 10-20 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, 15 of 15 (100%) laboratories produced a sufficient staining result.

mAb clone **PG-B6p**, product no. **GA625**, Dako, OMNIS:
Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (efficient heating time 30 min. at 97°C), 12½ min. incubation of the primary Ab and EnVision FLEX+ (GV800/823+GV821) as detection system. Using these protocol settings, 24 of 25 (96%) laboratories produced a sufficient staining result.

rmAb clone **EP278**, product no. **8461-C010**, Sakura Finetek, Tissue-Tek Genie:
Protocols with optimal results were based on HIER using Tissue-Tek Genie High pH Antigen Retrieval Solution (Sakura Finetek) (efficient heating time 30-45 min. at 98°C), 30 min. incubation of the primary Ab and Tissue-Tek Genie Pro DAB kit (8826-K250) as detection system.

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems (≥10 assessed protocols). The performance was evaluated both as "true" plug-and-play systems performed strictly accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the intended IHC stainer device are included.

Table 4. **Proportion of sufficient and optimal results for Bcl-6 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako Autostainer mAb PG-B6p **IR625**	(6/6)	(1/6)	94% (15/16)	44% (7/16)
Dako Omnis mAb PG-B6p **GA625**	96% (22/23)	43% (10/23)	100% (11/11)	72% (8/11)
VMS Ultra/XT/GX mAb GI191E/A8 **760-4241**	(4/5)	(1/5)	56% (44/78)	27% (21/78)
Leica Bond III mAb LN22 **PA0204**	100% (10/10)	80% (8/10)	(7/7)	(5/7)

* Protocol settings recommended by vendor – Retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Significant modifications: retrieval method, retrieval duration and Ab incubation time altered >25%, detection kit – only protocols performed on the specified vendor IHC stainer were included.

Comments

In this assessment for Bcl-6, the prevalent feature of an insufficient staining reaction was either poor signal-to-noise ratio or false positive staining reaction of cells expected to be negative e.g., the nuclei of the neoplastic cells in the MCL. This was observed in 68% (43 of 63) of the insufficient results and primarily related to use of the mAb GI191E/A8 RTU system on the Ventana Benchmark platform (79%, 34 of 43). Too weak or false negative staining reaction was seen in 29% of the insufficient results (18 of 63). Virtually all laboratories were able to demonstrate Bcl-6 in high-level antigen expressing cells, such as normal germinal centre B-cells in the tonsils and the neoplastic cells of the follicular lymphoma, tissue core 5. However, demonstration of Bcl-6 in low-level antigen expressing cells as squamous epithelial cells of the tonsil, the neoplastic cells of the follicular lymphoma, tissue core 4, and the DLBCL was more challenging and required an optimally calibrated protocol. Unexplained technical issues caused the remaining insufficient results.

Used within a laboratory developed (LD) assay, the mAb clones GI191E/A8, LN22 and PG-B6p could all be used to obtain an optimal staining result for Bcl-6 (see Table 1). As shown in Table 3, the mAb clones LN22 and PG-B6p could provide optimal results on the four main IHC-platforms from the most common vendors. The mAb clone GI191E/A8 provided the highest proportion of optimal results (83%) but only on the Ventana Benchmark platform (no staining was performed on other platforms applying optimal protocol settings). 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. If protocols were based on a titer of the concentrated format in the "optimal dilution range" as listed above, HIER was performed in an alkaline buffer, and a 3-step polymer/multimer based detection system as EnVision FLEX+ (Dako), Optiview (Ventana) or Bond Refine (Leica) was applied, a pass rate of 92% (57 of 62) was seen, out of which 69% (43 of 62) were optimal.

In this assessment, the RTU systems from Leica and Dako (based on the mAb clone LN22 and PG-B6p, respectively) were the most successful assays for demonstration of Bcl-6. Best performance was obtained with the RTU system PA0204 (mAb LN22, Leica), providing a pass rate of 100% (18 of 18) of which 78% (14 of 18) were assessed as optimal. The pass rate for the RTU systems IS/IR/GA625 (mAb PG-B6p, Dako) was 96% (55 of 57) of which 46% (26 of 57) were optimal. However, proportion of optimal results were significant higher when the RTU system was applied on the Omnis (GA625) compared to the Autostainer (IS/IR625), 53% (18 of 34) and 35% (8 of 23), respectively, and may partly be explained by the fact (as noted in the previous run 44, 2015) that the mAb clone PG-B6p is sensitive to peroxidase blocking. On the Omnis, this step is performed after application of the primary Ab. If participants are not aware of this problem, the peroxidase blocking step may be performed after HIER and before incubation in primary Ab, thus impacting antigenicity of the recovered protein (Bcl-6).
As shown in Table 4 for the systems described above, both vendor and laboratory modified protocol setting could be used to obtain a sufficient or optimal result for Bcl-6. For the RTU system GA625 based on the mAb clone PG-B6p (Omnis, Dako), the proportion of optimal results was significant higher applying laboratory modified protocol settings (typically prolonging incubation time of primary Ab) compared to the recommendation (12½ min incubation of the primary Ab) given by the vendor (72% and 43%, respectively).
The Ventana RTU system 760-4241 based on the mAb clone GI191E/A8, was inferior in performance compared to the RTU systems from the other major vendors. The pass rate was only 58% (51 of 88) of

which 25% (22 of 88) of the protocols were assessed as optimal. As described above, the major problems with insufficient staining reaction was either a poor signal-to-noise ratio or false positive staining reaction. As shown in Table 4, the vast majority of laboratories modified the vendor recommended protocol settings, typically substituting the UltraView detection system with either UltraView with amplification or OptiView (with or without amplification). The increased sensitivity gained by this modification may account for some of the problems related to excessive background staining or false positive reactions. However, of the 22 protocols giving optimal results, 21 used OptiView with or without amplification and only one laboratory applied the recommended UltraView as detection system in compliance with the package insert from Ventana. Contrary for protocols assessed as insufficient, the majority of laboratories (66%, 24 of 36) applied a 2-step multimer detection system (UltraView with or without amplification) and only 4 of the insufficient results were caused by too weak staining reactions. This indicates that the RTU product needs re-calibration as the pass rate was lower compared to LD assays based on the same clone GI191E/A8. The antibody seems robust and can provide high proportion of sufficient and optimal results as long as optimal protocol settings are applied (see Table 1 and 3).

This was the fourth assessment of Bcl-6 in NordiQC (see Table 2). The pass rate in this run 55 increased slightly compared to the latest run 42, 2014. In this assessment, the major problem causing insufficient results was related to the use of the mAb clone GI191E/A8 and especially applied as RTU format from Ventana. If assays were not modified, which in principle should not be done with a plug-and-play RTU system, poor signal-to-noise ratio or false positive staining reaction were seen. On the other hand, it was uplifting that the number of too weak or false negative staining reactions has decreased significantly compared to previous runs for Bcl-6. In total, only 6% (18 of 279) of the slides were assessed as insufficient due to too weak or false negative staining result - typically using too short HIER time, too diluted primary Ab or a less sensitive detection system.

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 should display a positive nuclear staining reaction.

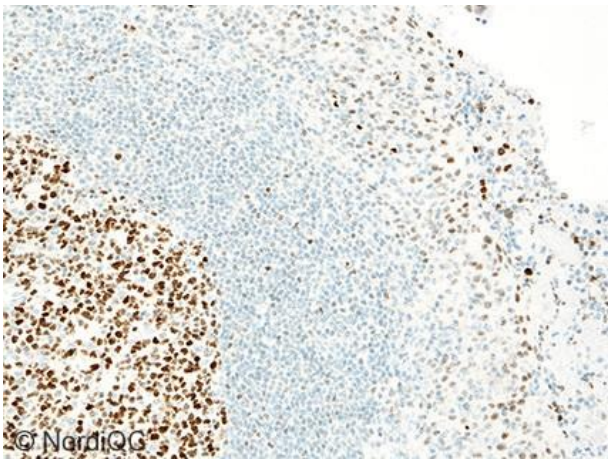

Fig. 1a (X200)
Optimal Bcl-6 staining of the tonsil using the mAb clone GI191E/A8 optimally calibrated, HIER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview, Benchmark Ultra, Ventana). Virtually all germinal centre B-cells show a moderate to strong nuclear staining reaction and the nuclei of squamous epithelium display weak to moderate intensity – same protocol used in Figs. 2a-4a.

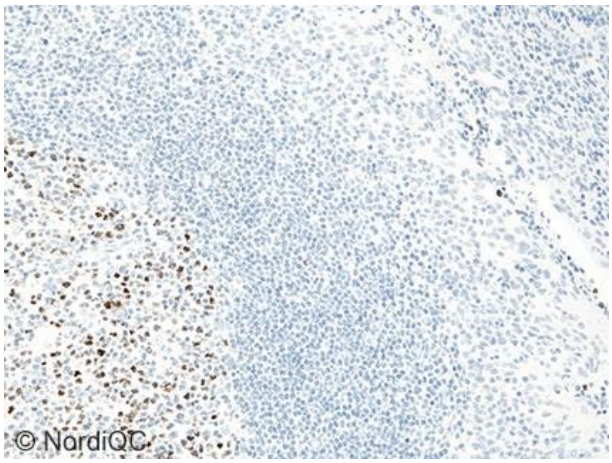

Fig. 1b (X200)
Insufficient Bcl-6 staining of the tonsil using the mAb clone GI191E/A8 HIER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview + amplification, Benchmark Ultra, Ventana). The protocol provided too low sensitivity due to too low titre of the primary Ab (1:800) - same field as in Fig. 1a. The proportion of positive germinal centre B-cells is significantly reduced and virtually all nuclei of squamous epithelium are false negative – same protocol used in Figs. 2b-4b.

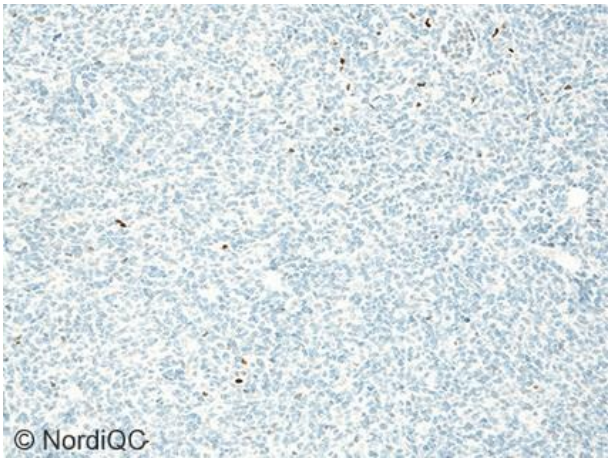

Fig. 2a (X200)
Optimal Bcl-6 staining result of the MCL using same protocol as in Fig. 1a. All nuclei of neoplastic cells display no staining reaction. Only dispersed normal B-cells display a weak to strong nuclear staining reaction.

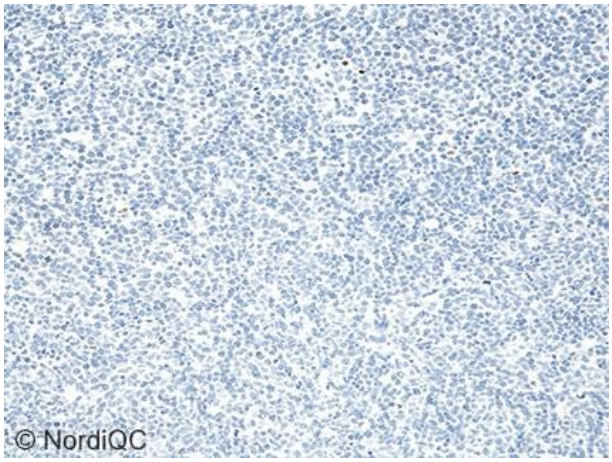

Fig. 2b (X200)
Insufficient Bcl-6 staining result of the MCL using same protocol as in Fig. 1b. Although the staining pattern is similar to the optimal protocol seen in Fig. 2a, the assay provided too weak staining reaction in critical tissue specimens – compare fig. 3a-3b and 4a-4b. Also, the proportion of normal dispersed B-cells intermingling with the neoplastic cells of the MCL is significantly reduced - same field as in Fig. 2a.

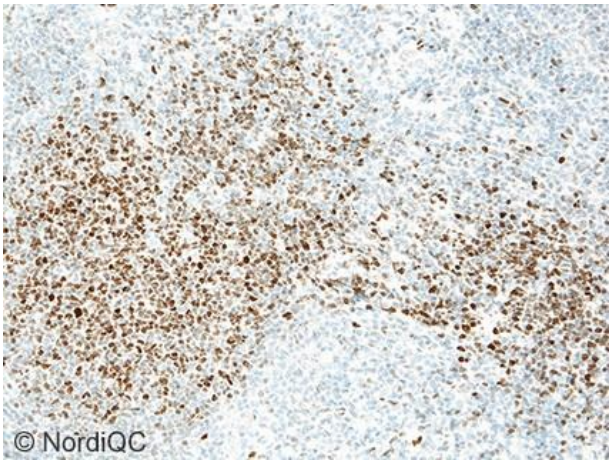

Fig. 3a (X200)
Optimal Bcl-6 staining of the FL (tissue core 4) using same protocol as in Figs. 1a and 2a. The majority of the neoplastic cells show a moderate to strong nuclear staining reaction.

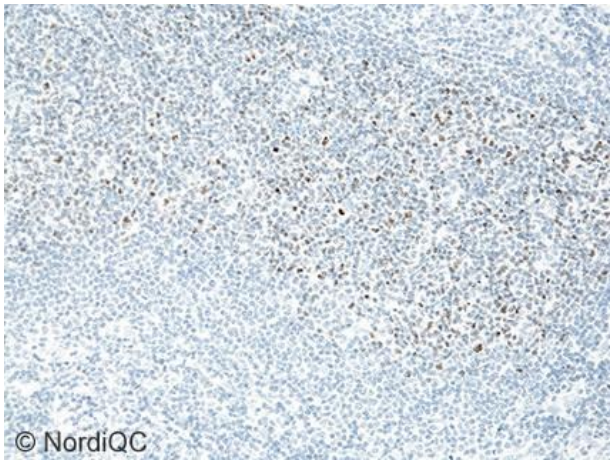

Fig. 3b (X200)
Insufficient Bcl-6 staining of the FL (tissue core 4) using same protocol as in Figs. 1b and 2b. The proportion and staining intensity of the neoplastic cells is significantly reduced compared to the level in Fig. 3a

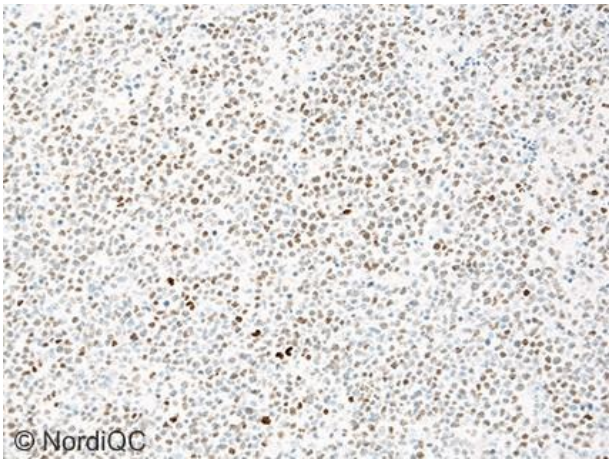

Fig.4a (X200)
Optimal Bcl-6 staining of the DLBCL using same protocol as in Figs. 1a - 3a. The vast majority of neoplastic cells show an at least moderate but distinct nuclear staining reaction. No background staining is observed.

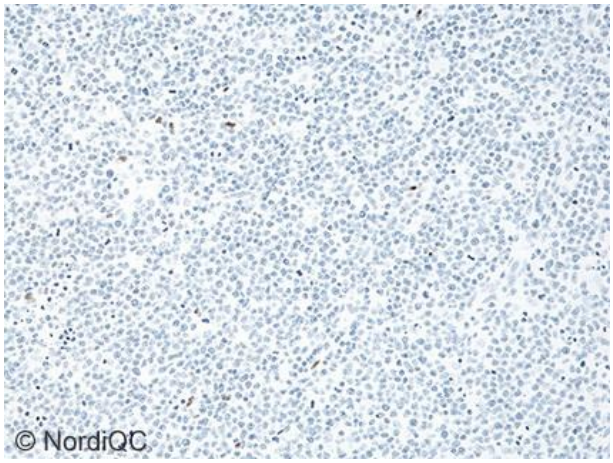

Fig. 4b (X200)
Insufficient Bcl-6 staining result of the DLBCL using same protocol as in Figs. 1b - 3b. The neoplastic cells are false negative. Only few scattered normal B-cells are demonstrated - same field as in Fig. 4a.

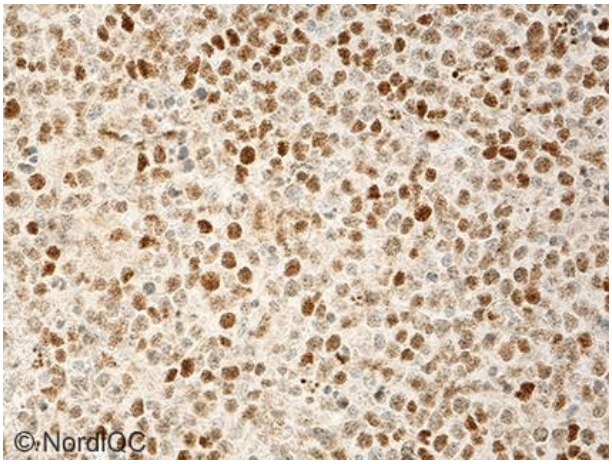

Fig.5a (X400)
Insufficient Bcl-6 staining result of the DLBCL using the RTU product 760-4241 (Ventana) based on the mAb clone GI191E/A8, HIER in CC1 (64 min.) and a 3-step multimer based detection system (UltraView with amplification, Benchmark Ultra, Ventana).
Although the protocol provided the expected reaction pattern of the neoplastic cell in the DLBCL, the typical problem of an insufficient result applying this assay was too much background and/or a false positive staining result, compromising interpretation of the specific signals - see Fig. 5b.

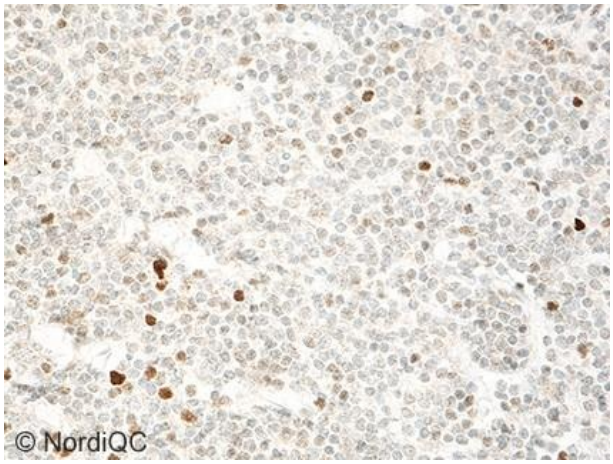

Fig. 5b (X400)
Insufficient Bcl-6 staining of the MCL using same protocol as in Fig. 5a. A significant proportion of the neoplastic cells display a weak to moderate, and distinct nuclear staining reaction (false positive) - compare with optimal result in Fig. 2a.

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