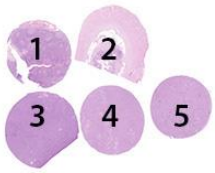
Assessment Run 59 2020
CD10

Purpose
Evaluation of the technical performance and, in particular, the level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for CD10, discriminating Diffuse Large B-Cell Lymphoma (DLBCL) of Germinal centre B-cell like (GCB) from non-GCB subtype. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for CD10 (see below). Cases diagnosed with DLBCL were classified according to Hans[1] algorithm in which neoplastic B-cells of the GCB phenotype is characterized being CD10 positive or present with the phenotype $CD10^{-}BCL6^{+}MUM1^{-}$. A cut-off value of ≥30% positive neoplastic B-cells was applied for each individual marker.
[1]Hans CP, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 2004;103:275-82.

Material
The slide to be stained for CD10 comprised:



1. Tonsil, 2. Appendix, 3. Burkitt Lymphoma (BL), 4. DLBCL (GCB subtype), 5. DLBCL (non-GCB subtype).

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CD10 staining as optimal included:

- A moderate to strong, distinct membranous staining reaction of virtually all germinal centre B-cells in tonsil and of scattered stromal cells in the appendix.
- An at least moderate, distinct membranous staining reaction of virtually all neoplastic B-cells in the Burkitt lymphoma.
- A moderate to strong, distinct membranous staining reaction of virtually all the neoplastic B-cells in the DLBCL (GCB subtype).
- An at least weak to moderate, distinct staining reaction of neutrophil granulocytes in all the specimens.
- No staining reaction of the neoplastic B-cells in the DLBCL (non-GCB subtype), mantle zone B-cells and squamous epithelial cells of the tonsil.

Participation

Number of laboratories registered for CD10, run 59	361
Number of laboratories returning slides	295 (82%)

The number of laboratories returning slides decreased in this run 59 compared to previous assessments, due to the COVID-19 pandemic. All slides returned after the assessment will be assessed, and receive advice if the result is insufficient, but will not be included in this report.

Results
295 laboratories participated in this assessment. 79% achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and the assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration or too short incubation time of the primary antibody.
- Omission of HIER.
- Insufficient HIER (use of a citric based buffer or too short efficient HIER time).
- Less sensitive detection systems.
- Less successful performance of the RTU system 790-4506 (Ventana) based on the rmAb clone SP67.
- Unexplained technical issues.

Performance history
This was the fifth NordiQC assessment of CD10. The overall pass rate decreased significantly compared to the result obtained in run 39, 2013 (see Table 2).

Table 2. **Proportion of sufficient results for CD10 in the five NordiQC runs performed**

	Run 6 2006	Run 16 2006	Run 27 2009	Run 39 2013	Run 59 2020
Participants, n=	43	89	137	229	295
Sufficient results	63 %	72 %	74 %	91 %	79%

Conclusion

The mAb clones **56C6**, **MX002** and the rmAb clone **SP67** could all produce optimal results for demonstration of CD10. HIER in alkaline buffer, precise calibration of the primary Ab and use of a 3-step polymer or multimer based detection system were the main prerequisites for an optimal result. The RTU systems GA648 (Dako) and PA0270 (Leica), based on the mAb clone 56C6, were most successful and in particular when applied in compliance with protocol settings as recommended by vendors.
Tonsil is recommended as positive and negative tissue control for CD10. Virtually all the germinal centre B-cells must show an at least moderate but distinct membranous staining reaction, whereas mantle zone B-cells and squamous epithelial cells must be negative. Scattered neutrophil granulocytes must display an at least weak membranous/cytoplasmic staining reaction.

Table 1. **Antibodies and assessment marks for CD10, run 59**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **56C6**	67 10 7 5 4 2 1 1 1	Leica/Novocastra Cell marque Biocare Medical Agilent/Dako Monosan/Sanbio Thermo Scientific Diagnostic Biosystem Immunologic Zytomed Systems	57	21	13	7	80%	58 %
mAb clone **MX002**	1	Fuzhiou Maixin Biotech	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **56C6 GA648 (VRPS)**[3]	33	Agilent/Dako	31	2	0	0	100%	94 %
mAb clone **56C6 GA648 (LMPS)**[4]	23	Agilent/Dako	21	1	1	0	97%	91%
mAb clone **56C6 IR/IS648 (VRPS)**[3]	3	Agilent/Dako	0	1	2	0	-	-
mAb clone **56C6 IR/IS648 (LMPS)**[4]	17	Agilent/Dako	15	2	0	0	100%	88%
mAb clone **56C6 PA0270/0131 (VRPS)**[3]	11	Leica Biosystems	10	1	0	0	100%	91%
mAb clone **56C6 PA0270/0131 (LMPS)**[4]	13	Leica Biosystems	10	2	1	0	92%	77%
mAb clone **56C6 MAD-002022QD**	3	Master Diagnostica	2	1	0	0	-	-
mAb clone **56C6 110M-10/17/18**	2	Cell Marque	1	0	1	0	-	-
mAb clone **56C6 PM129**	1	Biocare Medical	0	1	0	0	-	-
rmAb clone **56C6 AM451**	1	BioGenex	0	0	0	1	-	-
rmAb clone **56C6 PDM107**	1	Diagnostic Biosystems	0	0	1	0	-	-
mAb clone **GM003 8253-C010**	1	Sakura Finetek	0	0	0	1	-	-
rmAb clone **SP67 790-4506 (VRPS)**[3]	4	Ventana/Roche	0	2	1	1	-	-
rmAb clone **SP67 790-4506 (LMPS)**[4]	83	Ventana/Roche	19	30	28	6	59%	23%

Total	295		167	64	48	16	-	
Proportion			57%	22%	16%	5%	79%	

1) Proportion of sufficient results (optimal or good) (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 asessed protocols).

Detailed analysis of CD10, Run 59
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb **56C6**: Protocols with optimal results were all based on Heat Induced Epitope Retrieval (HIER) using an alkaline buffer as Cell Conditioning 1 (CC1, Ventana) (37/59)*, Target Retrieval Solution (TRS) High pH (3-in-1) (Dako) (7/13), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (11/17), Novocastra Epitope Retrieval Solution pH 9 (1/1) or TRIS-EDTA pH 9 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:10– 1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings, 63 of 74 (85%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb **MX002**: One protocol with an optimal result was based on HIER using CC1 (Ventana) as retrieval buffer. The mAb was diluted 1:300 and OptiView (Ventana) was used as detection system.

Table 3. **Proportion of optimal results for CD10 for the most commonly used antibody as concentrate on the 4 main IHC systems***

Concentrated antibody	**Dako Autostainer Link/Classic**		**Dako Omnis**		**Ventana BenchMark GX /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 **56C6**	2/3**	-	4/6 (67%)	-	35/50 (70%)	-	9/13 (69%)	0/1

* 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 **56C6,** product no. **GA648,** Dako, Omnis:
Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (efficient heating time 30min. at 97°C), 12-20 min. incubation of the primary Ab and EnVision FLEX+ (GV800/GV823 + GV821) as detection system. Using these protocol settings, 34 of 34 (100%) laboratories produced a sufficient result. Applying vendor recommended protocol settings (VRPS), the proportion of sufficient results was 100% (33/33) and 94% (31/33) were optimal (see Tables 1 and 4). Two laboratories used the RTU format off-label (deviant platforms).

mAb clone **56C6,** product no. **IR/IS648,** Dako, Autostainer+ /Autostainer Link:
Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (efficient heating time 10-20 min. at 95-97°C), 20-30 min. incubation of the primary Ab and EnVision Flex+ (K8000/K8002) as detection system. Using these protocol settings, 9 of 9 (100%) laboratories produced an optimal result. 19% (3/16) of the laboratories applied VRPS of which none provided an optimal result (see Tables 1 and 4). Four laboratories used the RTU format off-label (deviant platforms).

mAb clone **56C6,** product no. **PA0270/0131,** Leica, BOND III/BOND MAX:
Protocols with optimal results were typically based on HIER using BERS2 (efficient heating time 20-30 min. at 100°C), 15-20 min. incubation of the primary Ab and BOND Refine (DS9800) as the detection system. Using these protocol settings, 16 of 16 (100%) laboratories produced a sufficient result. Applying VRPS, the proportion of sufficient results was 100% (11/11) and 91% (10/11) of were optimal (see Tables 1 and 4). Three laboratories used the RTU format off-label (deviant platforms).

rmAb clone **SP67**, product no. **790-4506**, Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER in CC1 (efficient heating time 40-64 min. at 94-100°C), 16-40 min. incubation of the primary Ab and UltraView with amplification (760-500/760-080) or OptiView with or without amplification (760-700/760-099) as detection systems. Using these protocol

settings, 26 of 37 (70%) laboratories produced a sufficient result. 5% (4/87) of the laboratories applied VRPS of which none provided an optimal result (see Table 1 and 4).

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems (≥10 asessed 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 (in Table 1 LMPS also includes off label use on deviant IHC stainers).

Table 4. **Proportion of sufficient and optimal results for CD10 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
Dako Omnis mAb 56C6 **GA648**	100% (33/33)	94% (31/33)	100% (21/21)	95% (20/21)
Dako AS mAb 56C6 **IR648**	1/3	0/3	100% (13/13)	85% (11/13)
Leica Bond III/Max mAb 56C6 **PA270/0131**	100% (11/11)	91% (10/11)	90% (9/10)	70% (7/10)
VMS Ultra/XT/GX rmAb SP67 **790-4506**	2/4	0/4	59% (49/83)	23% (19/83)

* Protocol settings recommended by vendor – retrieval method and duration, Ab incubation times, detection kit, IHC stainer/equipment.
** Modifications included: retrieval method, retrieval duration, retrieval reagents, Ab incubation time and detection kit. Only protocols performed on the specified vendor IHC stainer were included.

Comments

In this fifth NordiQC assessment of CD10, the prevalent feature of an insufficient result was either a too weak or completely false negative staining reaction of cells expected to be demonstrated and was seen in 92% (59/64) of the insufficient results. The majority of these laboratories were unable to demonstrate CD10 in the germinal centre B-cells of the tonsil, providing the obligatory staining pattern and intensity as outlined for optimal staining criteria (see above), but also challenged by demonstration of CD10 in the neutrophil granulocytes (all specimens), the neoplastic B-cells of the DLBCL (GCB subtype) and the neoplastic cells of the BL, all requiring a carefully calibrated protocol for optimal performance. In 33% (21 of 64) of the insufficient results, a general too weak and indistinct membranous staining reaction was seen, and in particular, observed with the RTU product 790-4506 (Ventana) based on the rmAb SP67, typically using tyramide based amplification in combination with OptiView as the detection system (see Figs. 5a-6a).

mAb clone 56C6 and the rmAb clone SP67 were the most widely used antibodies for the demonstration of CD10 and in total, applied by 99% (293/295) of the participants.
Used as a concentrate in a laboratory developed (LD) assay, mAb clone 56C6 gave an overall pass rate of 80% (78/98). As shown in Table 3, a relative high proportion of optimal results could be obtained on the three fully automated IHC platforms from Dako, Leica and Ventana. The most common cause of an insufficient staining result was use of a protocol with too low analytical sensitivity, typically using no pre-treatment, inefficient HIER (citric based buffer or too short HIER time), too short incubation time in the primary Ab, and in particular, too low concentration of the primary Ab affecting the overall performance of the assays. For protocols assessed as insufficient, and e.g. performed on a Benchmark platform using efficient HIER in CC1 at 95-100°C (average HIER time of 48 min., range 24-64 min.), incubation time in primary Ab (average incubation time of 35 min., range 16-60 min.) and a 3-step multimer based detection system (UltraView+Amp./OptiView) was used, the average dilution factor of the primary Ab was 1:103 (range 1:20-500). In comparison, and for protocols assessed as optimal using similar protocols settings based on HIER in CC1 at 95-100°C (average HIER time of 52 min., range 8-92min.), incubation time in primary Ab (average incubation time of 38 min., range 30-60 min) in combination with a 3-step multimer based detection system (UltraView+Amp./OptiView), the average dilution factor of the primary Ab was 1:34 (range 1:10-200), indicating that the titer of the primary Ab could significantly influence the overall performance of the assays.
In this assessment and applying the mAb clone 56C6 within a LD assay, only 6% (6/98) of the protocols were based on a 2-step polymer/multimer detection system. Although data is inconclusive, the protocols providing an optimal result (3/3) used efficient HIER in an alkaline buffer, optimal dilution range (1:10-1:50) and importantly, with prolonged incubation time in the primary Ab (50-60 min.). Two protocols

applied similar protocol settings, but with reduced incubation time of the primary Ab (20-30 min.) and both were assessed as insufficient.
Thus, it must be emphasized that all parameters must be optimized and carefully calibrated to provide an IHC protocol that is able to demonstrate CD10 in cellular structures with both low- and high-level CD10 expression, accomplishing purpose of the test, and, in this assessment, to be used for classification and subtyping of DLBCL providing both prognostic and predictive information.

Overall, Ready-To-Use (RTU) systems or RTU formats were used by 66% (196/295) of the participants. In this assessment, the RTU systems GA648 (Agilent/ Dako) and PA270/0131 (Leica) based on the mAb clone 56C6 provided superior results (see Table 1 and 4). Grouped together, all protocols (44/44) following Vendor Recommended Protocol Settings (VRPS) were assessed as sufficient of which 93% (41/44) were optimal. A high proportion of sufficient and optimal results could also be obtained using Laboratory Modified Protocol settings (LMPS), typically adjusting HIER time and/or incubation time in the primary Ab. The RTU system IR/IS648 (Agilent/ Dako), based on the mAb clone 56C6, also provided a high proportion of sufficient and optimal results but only by applying LMPS, typically using Flex+ instead of Flex as the detection system (see Table 4). Three laboratories followed instructions strictly as given by the vendor and only one provided a sufficient result assessed as good. The VRPS for IR/IS648 gave a generally too weak staining reaction and a reduced level of analytical sensitivity which most likely was caused by application of the recommended 2-step detection system Flex. Thus, use of a 3-step polymer detection system (Flex+) seemed to be the main parameter for the improved performance of these assays by LMPS. This observation is also supported by the high proportion of optimal results obtained with RTU systems GA648 (Omnis, Dako) or PA0270/0131 (Bond, Leica) in which VRPS are based on 3-step polymer detection systems, Flex+ (Dako) and Bond Refine (Leica), respectively (see Table 4).

In this assessment, the RTU system from Ventana (790-4506) based on the rmAb SP67 was used by 29% (87/295) of the participants providing an overall pass rate of 59% (51/87) of which 22% (19/87) were optimal. As shown in Table 4, only 5% (4/87) of the protocols were based on VRPS and 50% (2/4) were assessed as sufficient of which none were optimal. The protocols assessed as optimal were based on HIER in CC1 in combination with a 3-step multimer based detection system, most frequently being OptiView with or without amplification (84%, 16 of 19). However, in a significant proportion of protocols 31% (22 of 70) these settings were unsuccessful and provided an insufficient result. This result deviated from the previous run 39 (2013) in which 96% of the laboratories produced a sufficient result (optimal or good) applying similar protocol settings. No single technical parameters could be identified discriminating optimal from insufficient results e.g. the average HIER time in CC1 was 61 min. (range 40-100 min.) at 95-100° for the group obtaining an optimal result, whereas protocols with an insufficient result were based on comparable protocol settings with an average HIER time of 64 min. (range 16-264 min.). The same pattern was seen for the incubation time of the primary Ab in which an average incubation time of 24 min. (range 8-40 min.) and 26 min. (range 4-60 min.) provided optimal and insufficient results, respectively. Thus, it is difficult to precisely elucidate upon the discrepancy in the performance, but the RTU format seems demanding from a technical point of view, which might also be reflected by the high number of laboratories trying to optimize an assay that should work as a "true plug and play system" (see Table 4). Finally, 14% (11 of 81) of the participants applied the RTU system in combination with UltraView (2-step multimer) as the detection system, providing inferior performance with an overall pass rate of 9% (1 of 11).

This was the fifth assessment of CD10 in NordiQC. The pass rate was 79% which is a significant decrease compared to the result obtained in run 39, 2013 (see Table 2). The most important parameters influencing the final result in negative direction were:
1) Use of the mAb clone 56C6 within a LD-assay utilizing the primary Ab with too low concentration, providing too weak or false negative staining results.
2) Use of no-pretreatment or less efficient HIER in a non-alkaline buffer. Ten laboratories applied these settings, and 40% (4/10) produced a sufficient result of which none were assessed as optimal.
3) Use of a less sensitive 2-step multimer/polymer-based detection systems e.g. UltraView (Ventana) or Flex (Dako). Data analysis based on integrating all protocol settings (e.g. all formats of primary Abs and incubation times applied, all HIER buffers and HIER times used, etc.) demonstrated that protocols based on a 2-step multimer/polymer based detection system gave a pass rate of 37% (10/27) (optimal or good), whereas protocols based on a 3-step multimer/polymer based detection system e.g. Bond Refine (Leica), Flex+ (Dako) or OptiView with or without amplification (Ventana), gave a pass rate of 82% (220/267).
4) Use of the less successful RTU system 790-4506 (Ventana) based on the rmAb SP67.
Importantly, laboratories should use a robust Ab, calibrate the protocols correctly and verify the results accordingly to the expected antigen level of the recommended tissue control materials (see below).

Controls

Tonsil is recommended as positive and negative tissue control for CD10. Virtually all the germinal centre B-cells must show an at least moderate but distinct membranous staining reaction, which should be identified even at low power magnification (4x). It must be emphasized that the individual germinal center B-cells are clearly outlined showing the contours of the membranes and not showing a diffuse, indistinct staining reaction of the B-cells – see Figs. 1a and 1b. The mantle zone B-cells and squamous epithelial cells must be negative. Scattered neutrophil granulocytes must display an at least weak staining reaction.

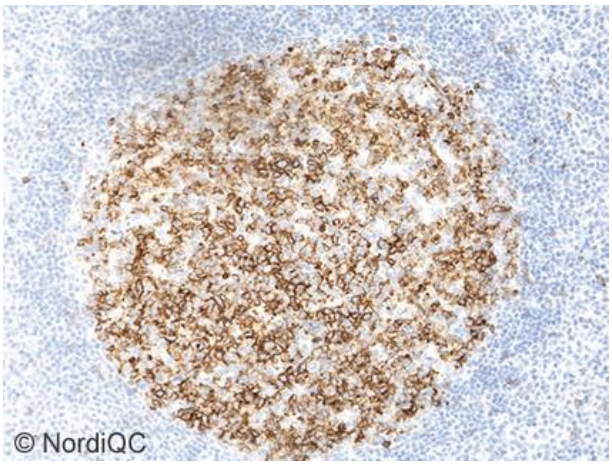


Fig. 1a (x200)
Optimal staining for CD10 of the tonsil using the mAb clone 56C6 as a concentrate (1:30), efficient HIER in an alkaline buffer (BERS2, Leica) and a 3-step polymer based detection system (Bond Refine, Leica) - same protocol used in Figs. 2a-4a. Virtually all germinal centre B-cells show a distinct, moderate to strong membranous staining reaction, whereas mantle zone B-cells display the expected negative staining reaction.

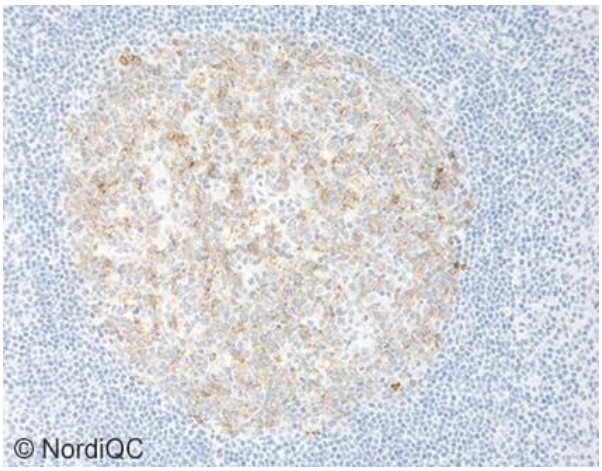


Fig. 1b (x200)
Insufficient staining for CD10 of the tonsil using the mAb clone 56C6 as concentrate (too diluted), efficient HIER in BERS2 and Bond Refine as the detection system - same protocol used in Figs. 2b–4b. The intensity of the staining reaction is significantly reduced and the intra germinal centre B-cells display an inaccurate membranous staining reaction, with the individual B-cells not being distinctively outlined. Too low concentration of the primary Ab was one of the most prevalent parameters causing an insufficient staining result using the mAb clone 56C6 within a LD assay - compare with Figs. 1a-4b.

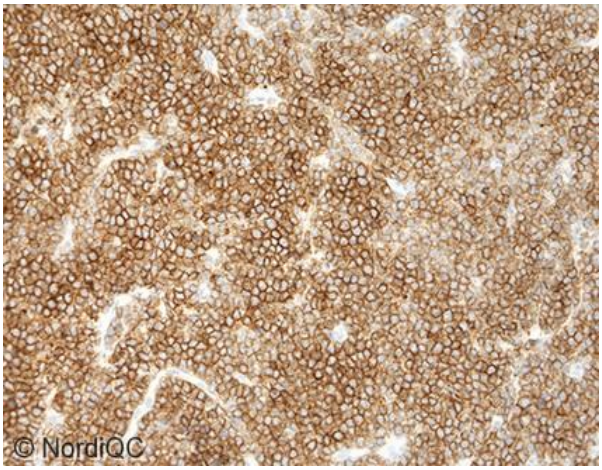


Fig. 2a (x200)
Optimal staining for CD10 in the BL using same protocol as in Fig. 1a. All the neoplastic B-cells show a moderate to strong, distinct membranous staining reaction.

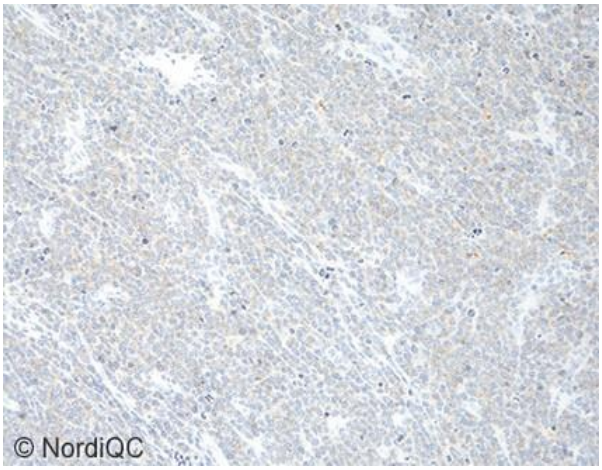


Fig. 2b (x200)
Insufficient staining for CD10 in the BL using same protocol as in Fig. 1b. Virtually all neoplastic B-cells display an indistinct faint or completely false negative staining reaction - compare with Fig. 2a.

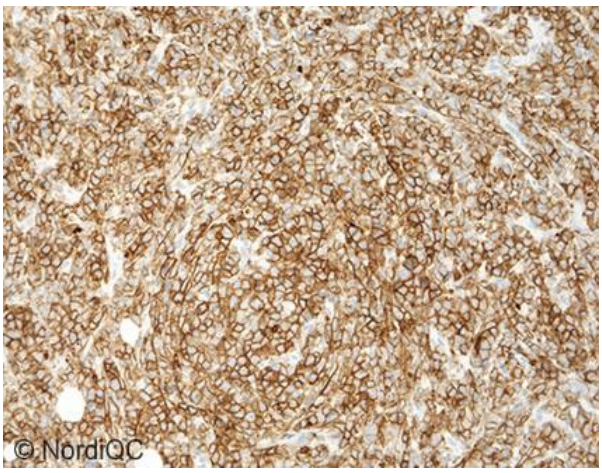


Fig. 3a (x200)
Optimal staining for CD10 of the DLBCL (GCB subtype) using same protocol as in Figs. 1a and 2a. All the neoplastic B-cells show a strong, distinct membranous staining reaction.

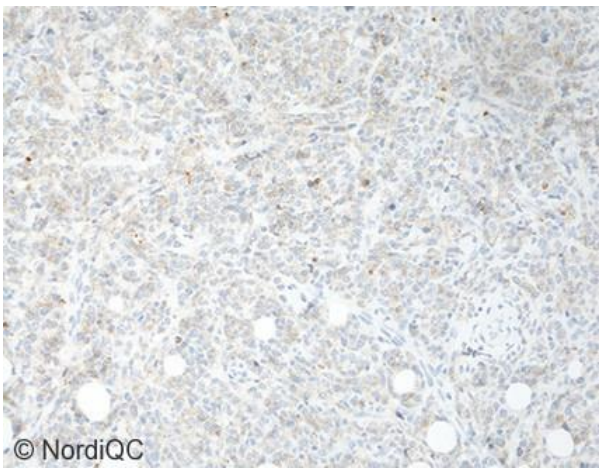


Fig. 3b (x200)
False negative staining for CD10 of the DLBCL (GCB subtype) using same protocol as in Figs. 1b and 2b. The neoplastic B-cells only display a faint indistinct staining reaction. Also, the proportion of positive neoplastic cells are below the cut-off of 30%, and consequently, could be misclassified according to Hans algorithm - compare with Fig. 3a.

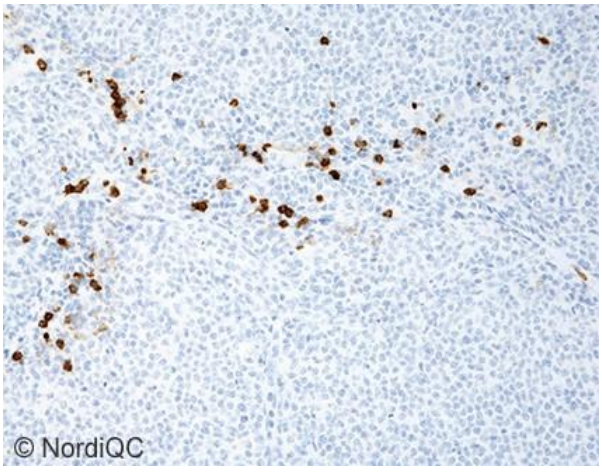


Fig. 4a (x200)
Optimal staining for CD10 of the DLBCL (non-GCB subtype) using same protocol as in Figs. 1a-3a. All the neoplastic B-cells are negative. Scattered neutrophil granulocytes display a weak to moderate staining reaction.

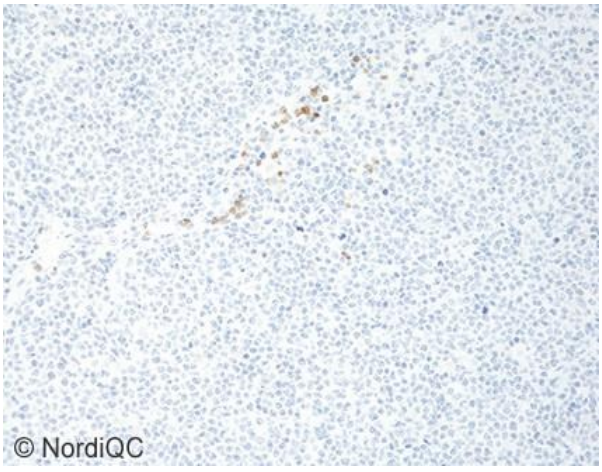


Fig. 4b (x200)
Insufficient staining for CD10 of the DLBCL (non-GCB subtype) using same protocol as in Figs. 1b-3b. Although the neoplastic B-cells show the expected negative reaction pattern, the protocol provided an overall too low analytical sensitivity - compare with Figs. 1a-3b. In addition, the neutrophil granulocytes display a too weak staining intensity or are false negative - compare with Fig. 4a.

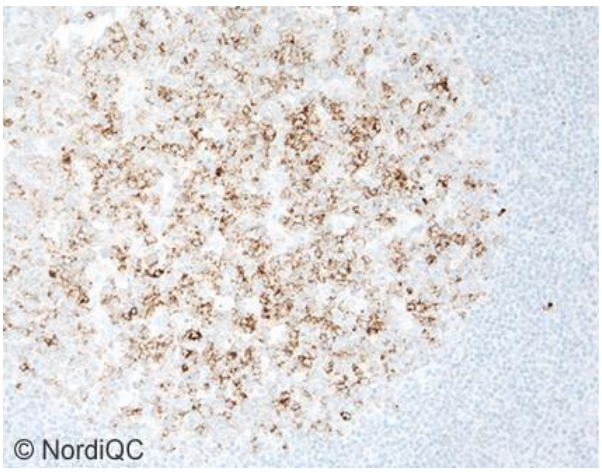


Fig. 5a (x200)
Insufficient staining for CD10 of the tonsil using the RTU system 790-4506 (Ventana) based on the rmAb clone SP67, HIER in CC1 and OptiView with amplification as the detection system - same protocol used in Figs. 5b-6a. The intensity and proportion of positive intra germinal centre B-cells is reduced. The reaction product is indistinct, displaying a granular appearance without a characteristic membranous accentuation. The RTU system seems challenging from a technical point of view as similar protocol settings could provide either a sufficient or insufficient result (see description in comments) – compare with optimal result in Fig. 1a.

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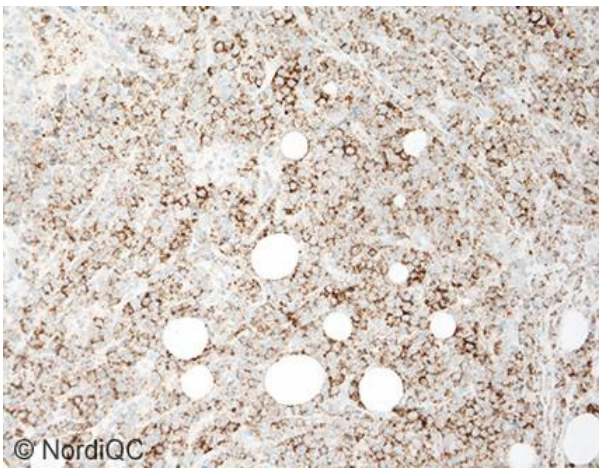

Fig. 5b (x200)
Insufficient staining for CD10 of the DLBCL (GCB subtype) using the same protocol as in Fig. 5a. Although this DLBCL might be classified correctly according to Hans algorithm, the protocol provides an overall too low analytical sensitivity (see Fig. 6a). Furthermore, the neoplastic B-cells display the same aberrant staining pattern as described in Fig. 5a, compromising interpretation due to imprecise staining reactions and a reduced proportion of positive neoplastic cells - compare with the optimal result in Fig. 3a.

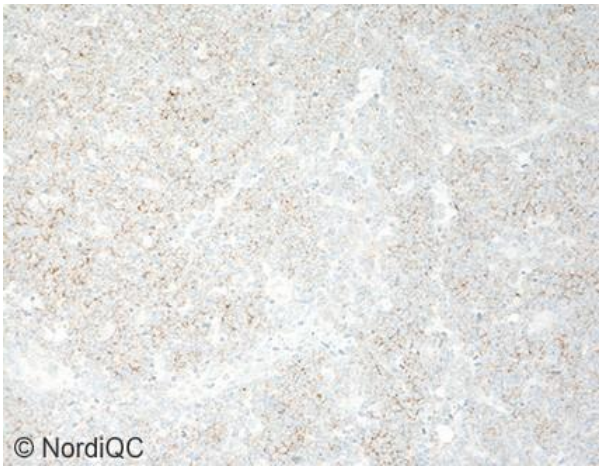

Fig. 6a (x200)
Insufficient staining for CD10 of the BL using the same protocol as in Figs. 5a-5b. The vast majority of the neoplastic B-cells display an aberrant punctuated staining reaction, obscuring the interpretation. This can be a challenge applying tyramide based detection systems such as OptiView with amplification in contrast to "standard" non-tyramide based IHC assays - compare with optimal protocol in Fig. 2a.

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