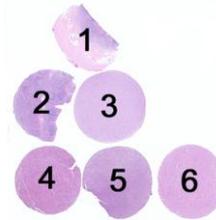


Material

The slide to be stained for CD20 comprised:

1. Appendix, 2. Tonsil, 3. Diffuse large B-cell lymphoma (DLBCL), 4. Precursor-B-Acute lymphatic leukaemia (Pre-B-ALL), 5. B-Chronic lymphatic leukaemia (B-CLL), 6. Plasmacytoma.



All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CD20 staining as optimal included:

- A strong, predominantly membranous staining of the mantle zone B-cells, the germinal centre B-cells and the interfollicular B-cells in the tonsil and the appendix.
- A strong membranous staining of virtually all the neoplastic cells of the DLBCL.
- A moderate to strong membranous staining of virtually all the neoplastic cells of the B-CLL.
- A negative staining of the Pre-B-ALL (only scattered matured neoplastic cells and entrapped normal B-cells may be demonstrated).
- A negative staining of the plasmacytoma (only the remnants of normal B-cells should be demonstrated).
- A negative staining of all other cell types.

167 laboratories participated in this assessment. 95 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. Abs and assessment marks for CD20, run 35.

Concentrated Abs:	N	Vendor	Optimal	Good	Borderline	Poor	Suff. ¹	Suff. OPS ²
mAb clone L26	104	BioCare Cell Marque Dako Master Diagnóstica Novocastra/Leica Scytek Thermo Scientific Zymed Zytomed Systems	73	25	5	1	94 %	94 %
mAb clone 7D1	1	Novocastra/Leica	1	0	0	0	-	-
rmAb clone EP7	1	Epitomics	1	0	0	0	-	-
pAb RB-9013-P	1	Neomarker	0	0	1	0	-	-
Not specified	1		1	0	0	0	-	-
Ready-To-Use Abs:								
mAb clone L26, 760-4380	38	Ventana/Cell Marque	35	1	2	0	95 %	100 %
mAb clone L26, IR604/N1502	17	Dako	15	2	0	0	100 %	100 %
mAb clone L26, PM004	1	BioCare	1	0	0	0	-	-
mAb clone L26, CD20-L26-R-7-CE	1	Novocastra/Leica	1	0	0	0	-	-
mAb clone MJ1, PA0906	2	Novocastra/Leica	0	2	0	0	-	-
Total	167		128	30	8	1	-	-
Proportion			77 %	18 %	4 %	<1 %	95 %	-

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 **L26**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Cell Conditioning 1 (20/34)* TRS pH 9 (3-in-1, Dako) (13/14), Bond Epitope Retrieval Solution 2 (11/14), TRS pH 9 (10/13), Tris-EDTA/EGTA pH 9 (8/11), Bond Epitope Retrieval Solution 1 (3/5), Citrate pH 6 (3/4), EDTA/EGTA pH 8 (3/3), Cell Conditioning 2 (1/3) or TRS pH 6.1 (1/1) (3-in-1, Dako). The mAb was diluted in the range of 1:75–1:2000 depending on the total sensitivity of the protocol employed. Using these protocol settings 96 out of 102 (94 %) laboratories produced a sufficient staining (optimal or good).

* (number of optimal results/number of laboratories using this buffer)

mAb clone **7D1**: The protocol giving an optimal result was based on heat induced epitope retrieval (HIER) using Bond Epitope Retrieval Solution 1 (1/1). The mAb was diluted 1:200.

rmAb clone **EP7**: The protocol giving an optimal result was based on heat induced epitope retrieval (HIER) using Citrate pH 6 (1/1). The rmAb was diluted 1:100.

Ready-To-Use Abs

mAb clone **L26 (760-2531, Ventana/Cell Marque)**: The protocols giving an optimal result were all based on HIER using mild or standard Cell Conditioning 1, an incubation time of 8 to 44 min of the primary Ab at 36°C and UltraView (Ventana, 760-500), OptiView (Ventana 760-700) or IView (Ventana 760-091) as the detection system. Using these protocol settings 36 out of 36 (100 %) laboratories produced a sufficient staining (optimal or good).

mAb clone **L26 (IR604, Dako)**: The protocols giving an optimal result were all based on HIER using TRS pH 9, TRS pH 9 (3-in-1), TRS pH 6.1 or Bond Epitope Retrieval Solution 1, and an incubation time of 15 to 25 min in the primary Ab and a 2 or 3-step polymer system, EnVision (Dako K8000/K8002/K5007) or Bond Polymer Refine (Novocastra/Leica) as the detection system. Using these protocol settings 17 out of 17 (100 %) laboratories produced a sufficient staining (optimal or good).

The most frequent causes of insufficient staining were:

- Too low concentration of the primary antibody.
- Omission of HIER.
- pAb showing cross reactivity.

The prevalent feature of the insufficient results was a general too weak staining of both the normal and neoplastic cells supposed to be demonstrated, resulting in a negative or weak staining of the membranes especially in the neoplastic cells the B-CLL (Fig. 2b). The insufficient stainings were mainly related to the omission of HIER or to the use of low concentration of the primary Ab. The polyclonal antibody **RB-9013-P** showed an unacceptable cross reactivity resulting in false positive staining in the plasmacytoma and in peripheral nerves (Fig. 3b and Fig. 4b). Tonsil and appendix are appropriate for control tissue: the staining of the B-cells should be as strong as possible with no reaction in other cells (epithelial cells, muscle cells, nerve cells etc.). HIER is mandatory to obtain an optimal reaction. In concordance with previous observations (Run 21 in 2007) HIER in high pH buffers gives the most efficient retrieval of the CD20 epitopes, but optimal staining could also be achieved with mild citrate based HIER, provided the concentration of the antibody was increased.

This was the 3rd assessment of CD20 in NordiQC, as CD20 also was assessed in run 6, 2002 and run 21, 2007 (table 2). The proportion of sufficient results have once again increased. Even though the number of participants has almost tripled since 2002, the proportion of sufficient results has increased from initially 81% to an encouraging 95%.

Table 2. **Proportion of sufficient results for CD20 in the three NordiQC runs performed**

	Run 6, 2002	Run 21, 2007	Run 35, 2012
Participants, n=	62	115	167
Sufficient results	81 %	87 %	95 %

Conclusion

The mAb clone L26 and 7D1 and the mAb EP7 are useful for the demonstration of CD20. HIER is mandatory to obtain an optimal result. Concentration of the primary Ab should be carefully calibrated. Tonsil and appendix are appropriate controls: The mantle zone B-cells and the germinal centre B-cells must show a very strong reaction. No other cells should stain.

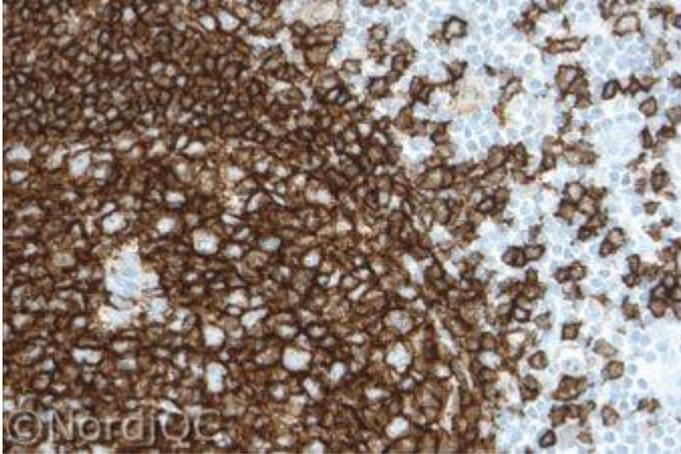


Fig. 1a
Lymphatic tissue in the appendix showing an optimal staining reaction for CD20 using the mAb clone L26 in a RTU format on the BenchMark platform. HIER was performed using Cell Conditioning 1. A very strong membranous staining reaction is seen in virtually all the B-cells.

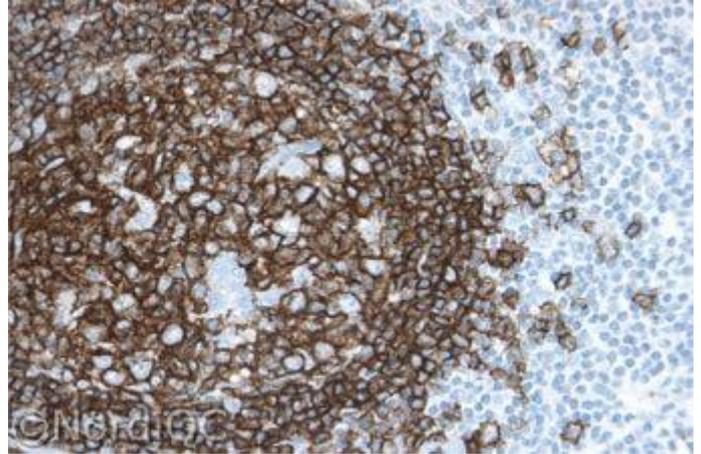


Fig. 1b
Lymphatic tissue in the appendix. Same field as in Fig. 1a. Insufficient staining for CD20 using the mAb clone L26 in a RTU format at the BenchMark platform. No HIER was performed. A moderate to strong staining reaction is seen in virtually all the B-cells. The normal B-cells are high expressors of CD20, hence the relatively strong reaction. Even so, the staining intensity should be improved in order to detect low expressors of CD20 (e.g. B-CLL in Fig. 2a and 2b).

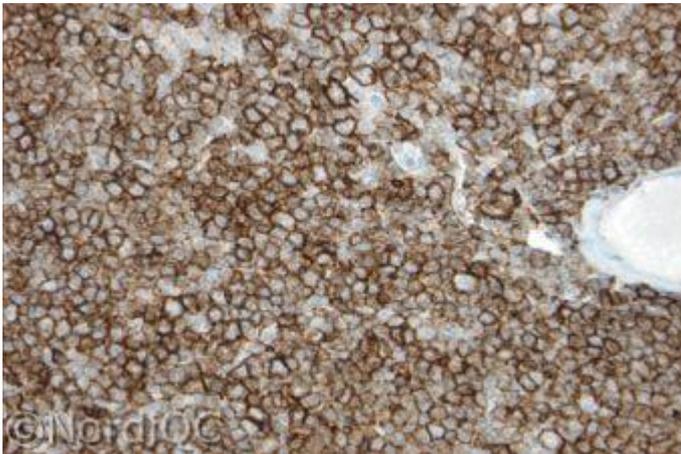


Fig. 2a
B-CLL. Optimal staining reaction for CD20. Same protocol as in Fig. 1a. A moderate to strong membranous staining is seen in virtually all the neoplastic cells.

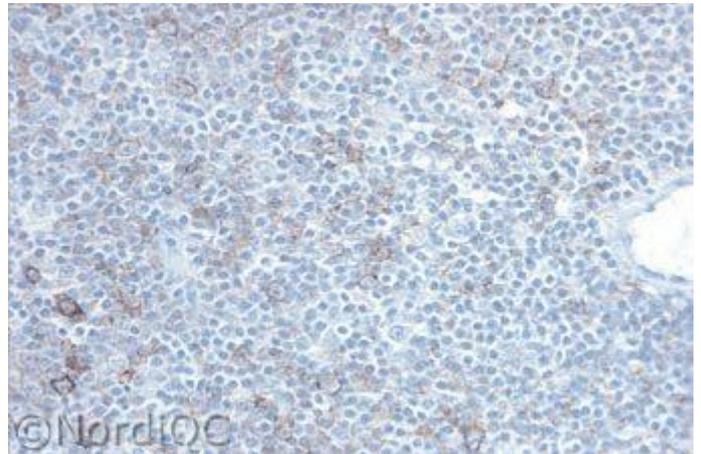


Fig. 2b
B-CLL. Insufficient staining for CD20 using the same protocol as in Fig. 1b. Omitting HIER, only scattered cells are positive. The majority of the neoplastic cells are negative. Compare with the optimal result in Fig. 2a, same field.

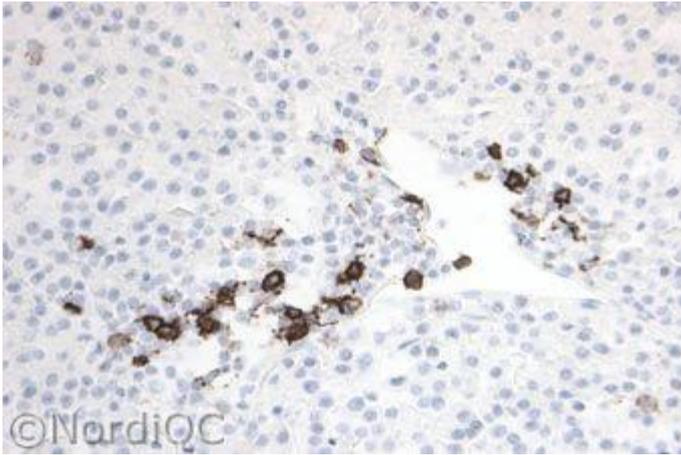


Fig. 3a
Plasmacytoma. Optimal staining reaction for CD20. Same protocol as in Figs. 1a & 2a. The neoplastic cells are all negative. Scattered normal B-cells are strongly positive.

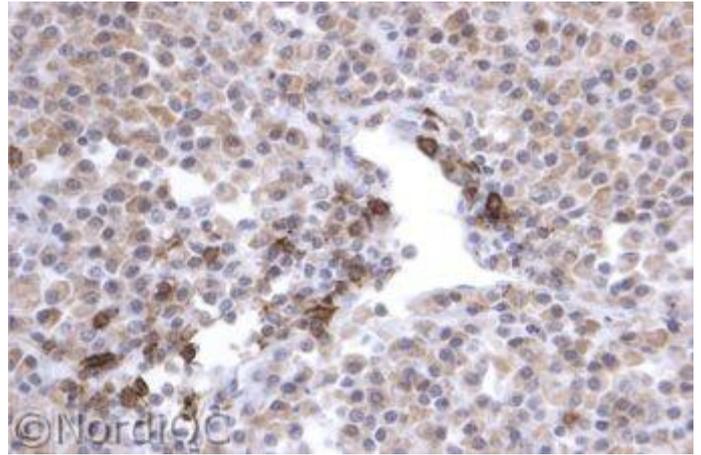


Fig. 3b. Plasmacytoma. Same field as in Fig. 3a. An insufficient staining reaction for CD20 is seen. The protocol is based on the pAb RB-9013-P giving a false positive staining reaction in the neoplastic plasma cells. Virtually all the neoplastic cells show a moderate staining reaction.

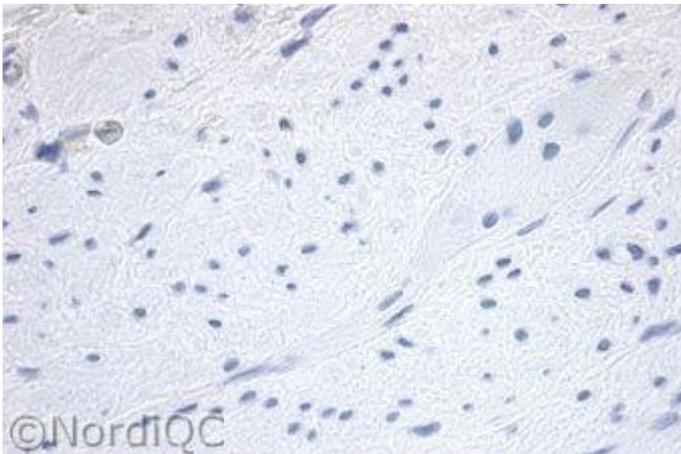


Fig. 4a
Normal appendix. Optimal staining reaction for CD20. Same protocol as in Figs. 1a - 3a. No staining is seen in non-B-cells.

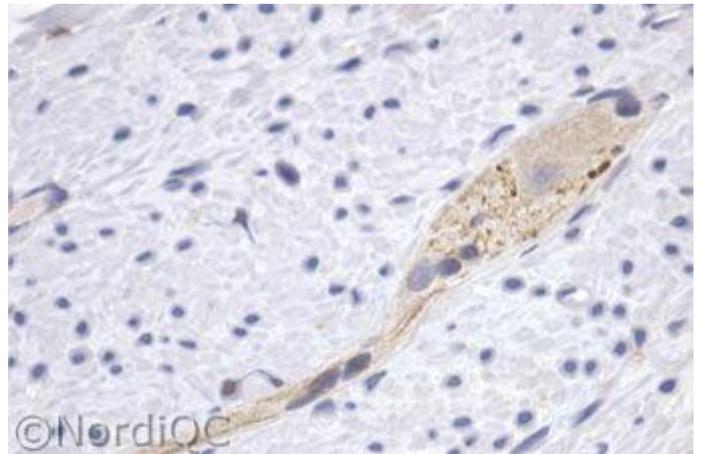


Fig. 4b
Normal appendix. Same field as in Fig. 4a. Insufficient staining reaction for CD20 using the same protocol as in Fig. 3b based on the pAb RB-9013-P giving a false positive staining reaction in the peripheral nerves.

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