Nordic immunohistochemical Quality Control

NordiQC

Workshop in Diagnostic Immunohistochemistry
Aalborg University Hospital, 2-4 October 2019

Optimization of antibodies, protocols and controls
Hematolymphoid markers

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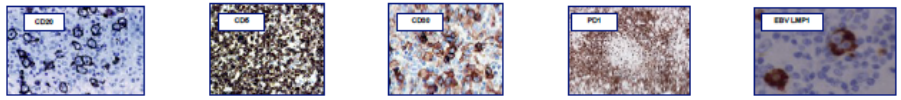
Courtesy: Steve Hamilton-Dutoit

Useful antigens in haematopathology

- CD45
- B-cell 'specific'
  - CD19
  - CD20
  - CD79α
  - Pax-5
  - OCT-2 / BOB1
  - Ig
- T-cell 'specific'
  - CD3
  - CD5
  - CD2
  - CD7
  - CD1a
  - CD4
  - CD8
  - PD-1/CXCL-13 (TFH)

- Other
  - CD30
  - CD10
  - Bcl-2
  - Bcl-6
  - ALK
  - c-myc
  - CD21
  - CD23
  - CD15
  - TdT
  - Cyclin-D1
  - SOX-11
  - CD56
  - TIA-1, granzyme, perforin

- Other
  - EBV
    - LMP1
    - EBNA2
    - (EBER)
  - CD56
  - CD57
  - EMA
  - S100
  - CD68
  - CD163



Mission impossible

The challenge

Basic IHC panel for lymphoma diagnosis

- CD45
- CD20
- CD79α
- (PAX-5)
- kappa/lambda
- CD3
- CD5
- CD30
- CD43
- Bcl-2
- Bcl-6
- CD23 (CD21)
- Cyclin-D1
- Ki-67

Courtesy: Steve Hamilton-Dutoit

Relative frequency of lymphoid malignancies

10	3	1
B-Cell	Hodgkin	T-Cell

Antigen	NQC assessments	Latest Run	Pass rate (%)	Optimal (%)
CD20	√	Run 35	95	77
CyclinD1	√	Run 47	94	54
CD3	√	Run 37	92	66
Ki67	√	Run B13	89	72
Pax5	√	Run 53	86	40
CD45	√	Run 37	82	56
BCL2	√	Run 28	82	44
CD79a	√	Run 45	79	51
CD5	√	Run 34	79	46
BCL6	√	Run 55	77	45
CD23	√	Run 34	73	38
CD30	√	Run 43	71	34
Kappa	√	Run 18	41	14
Lambda	√	Run 15	34	15
CD43	-	-	-	-

23%

86%

Focus on the basic lymphoid markers/panel

+ Update on additional markers assessed by NordiQC during the period 2017-2018

B-Cell lymphoma markers – "lineage specific" (1):

Marker (localization)	Control	High exp. (HE)	Low exp. (LE)	Non exp. (NE)
CD19 (membr.) LE-CD19, BT51E	Tonsil/Appendix	Mantle zone-, germinal centre- & interfollicular B-cells	Plasma cells	No staining of other cell types including T-cells and epithelial cells of the appendix.
CD20 (membr.). L26, 7D1, EP7	Tonsil/Appendix	Mantle zone-, germinal centre- & interfollicular B-cells	None	No staining of other cell types including T-cells and epithelial cells of the appendix.
CD79a (membr. + cytopl) JCB117, SP18	Tonsil/Appendix	Mantle zone B-cells and plasma cells	Germinal centre B-cells	No staining of other cell types including T-cells and epithelial cells of the appendix.
BSAP (PAX5) (nuclear) 1EW, 24, DAK-PAX5, MX017, SP34, EP156, BSR59, BV6	Tonsil/Appendix	Mantle zone-, germinal centre- & interfollicular B-cells*	None	No staining of other cell types including T-cells and epithelial cells of the appendix.
IgK (membr. + cytopl). pAb A0191	Tonsil	Plasma cells (App. 50%)	Mantle zone B-cells (App. 50 %)	No staining of other cell types including T-cells (weak background staining my be seen)
IgL (membr. + cytopl) pAb A0193	Tonsil	Plasma cells (App. 50%)	Mantle zone B-cells (App. 50 %)	No staining of other cell types including T-cells (weak background staining may be seen)
IgM (membr. + cytopl) pAb A0425, 760-2654	Tonsil	Plasma cells (app. 35%)	Virtually all mantle zone B-cells	No staining of other cell types including T-cells (weak background staining may be seen)
OCT-2 & BOB.1	See Hodgkin Lymphoma panel			

* A weak cytoplasmic staining reaction in B-cells must be accepted. In the technical calibration phase, it is recommended to verify the protocol on Hodgkin lymphoma, classical subtype.

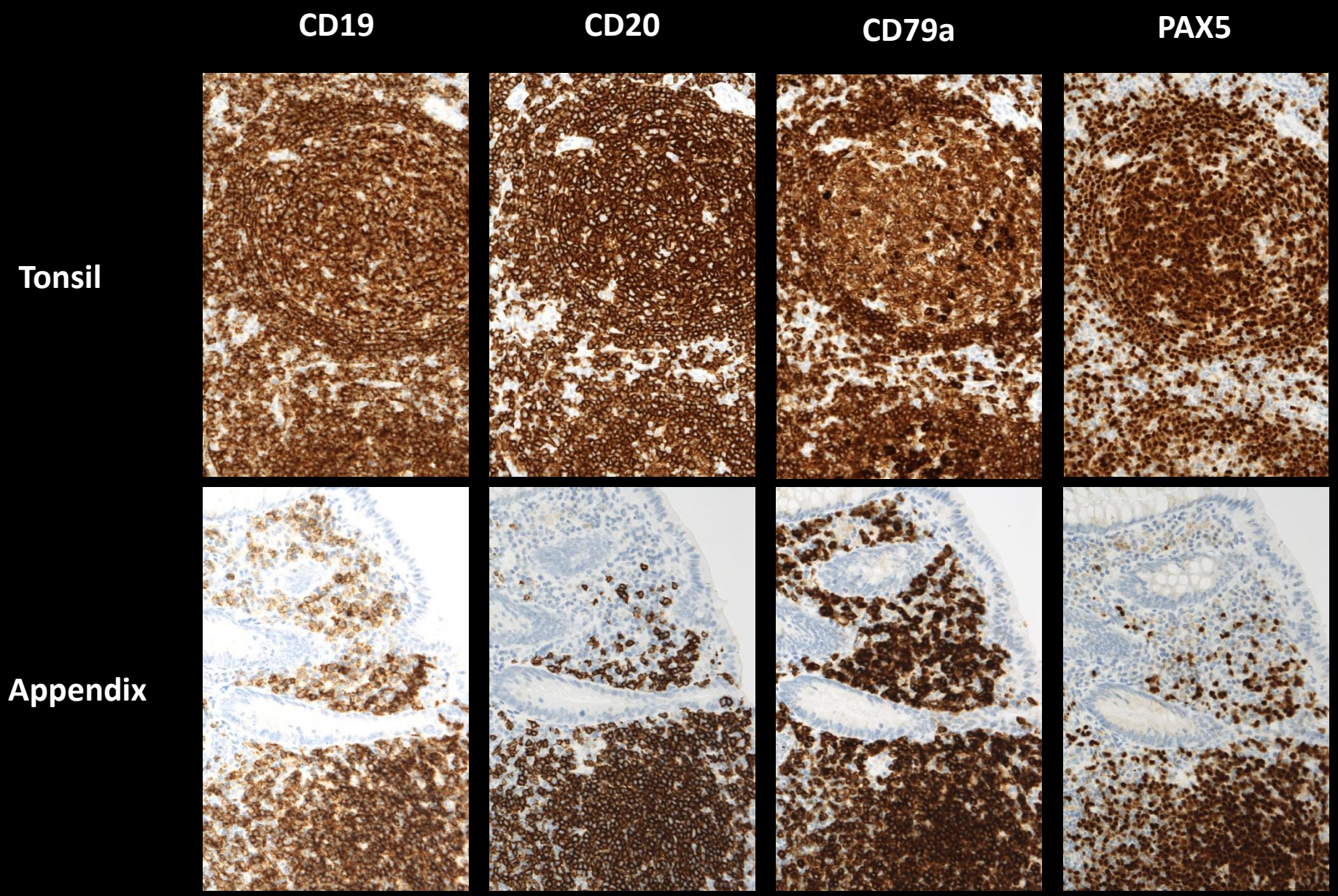
Clones (mAbs, rmAbs & pAbs) providing optimal results (NordiQC assessments)

HE: Strong staining intensity/reactions should be expected

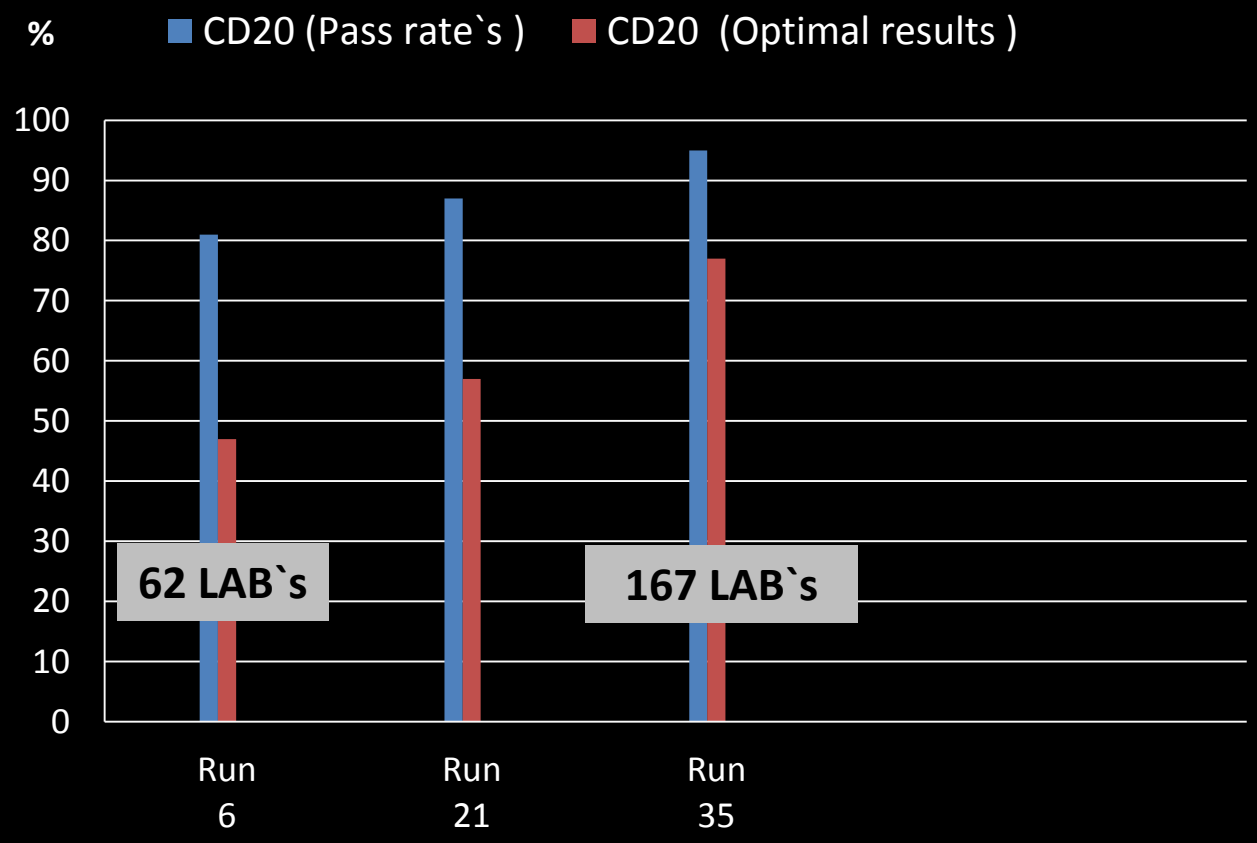
LE: An at least weak to moderate staining intensity/reactions should be expected

NE: No staining/reactions should be expected

B-Cell lymphoma markers - lineage “specific” (1):



CD20



CD20/ Run 31 (2012):

Sufficient: 95%

Optimal: 77%

Success due to very robust Abs

Clone L26 used by 97% of the LAB`s

J Clin Pathol 1998;51:364–369

Levels of expression of CD19 and CD20 in chronic B cell leukaemias

Lia Ginaldi, Massimo De Martinis, Estella Matutes, Nahla Farahat, Ricardo Morilla, Daniel Catovsky

Table 1 Mean ABC (antibody binding capacity) values × 10^3 in normal peripheral blood B lymphocytes and B lineage leukaemias

Antigen	Normal B cells	CLL	PLL	MCL	SLVL	HCL
CD19	22 (7)	13 (7)	16 (9)	10 (7)	15 (11)	38 (16)
(p value)*		<0.001	<0.05	<0.001	<0.05	<0.001
CD20	94 (16)	65 (11)	129 (47)	123 (51)	167 (72)	312 (110)
(p value)*		<0.001	<0.01	<0.05	<0.001	<0.001

Values are mean (SD); *comparison with normal peripheral blood B lymphocytes. CLL, chronic lymphatic leukaemia; HCL, hairy cell leukaemia; MCL, mantle cell lymphoma; PLL, prolymphocytic leukaemia; SLVL, splenic lymphoma with villous lymphocytes.

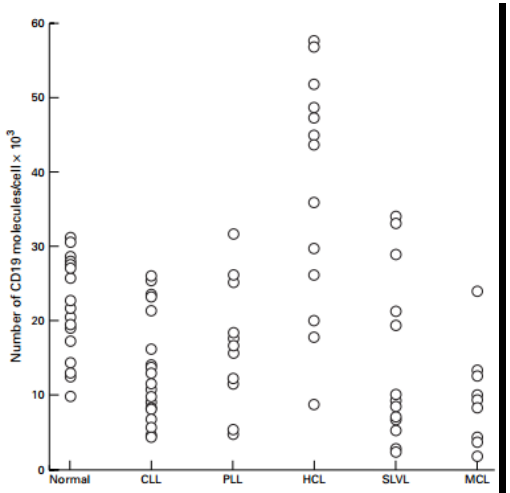


Figure 2 Distribution of individual ABC values for CD19 in normal peripheral blood B lymphocytes and B cell leukaemias.

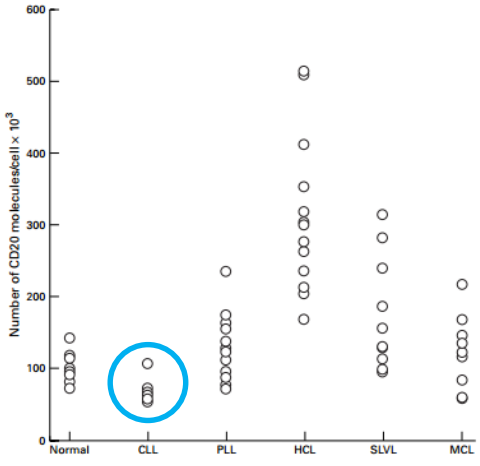


Figure 4 Individual ABC values for CD20 in normal peripheral blood B cells and B lineage leukaemias.

Prevodnik et al. Diagnostic Pathology 2011, 6:33
http://www.diagnosticpathology.org/content/6/1/33

DIAGNOSTIC PATHOLOGY

RESEARCH Open Access

The predictive significance of CD20 expression in B-cell lymphomas

Veronika Kloboves Prevodnik[1*], Jaka Lavrenčak[1], Mateja Horvat[2] and Barbara Jezeršek Novaković[3]

Abstract

Background: In our recent study, we determined the cut-off value of CD20 expression at the level of 25 000 molecules of equivalent soluble fluorochrome (MESF) to be the predictor of response to rituximab containing treatment in patients with B-cell lymphomas. In 17.5% of patients, who had the level of CD20 expression below the cut-off value, the response to rituximab containing treatment was significantly worse than in the rest of the patients with the level of CD20 expression above the cut-off value. The proportion of patients with low CD20 expression who might not benefit from rituximab containing treatment was not necessarily representative. Therefore the aim of this study was to quantify the CD20 expression in a larger series of patients with B-cell lymphomas which might allow us to determine more reliably the proportion of patients with the CD20 expression below the cut-off.

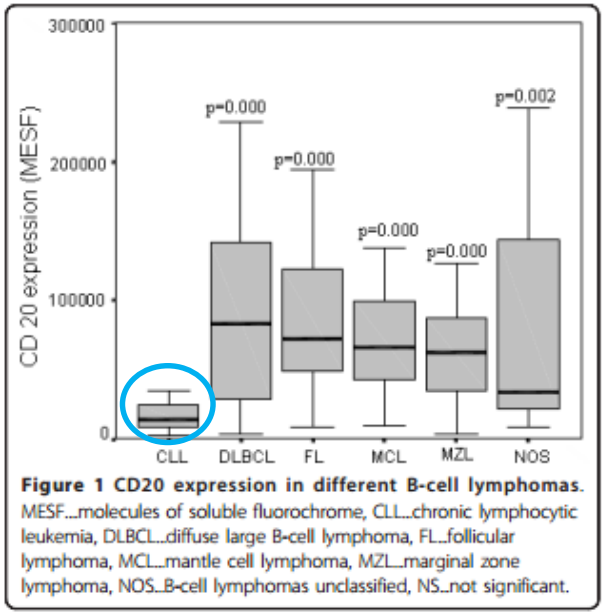
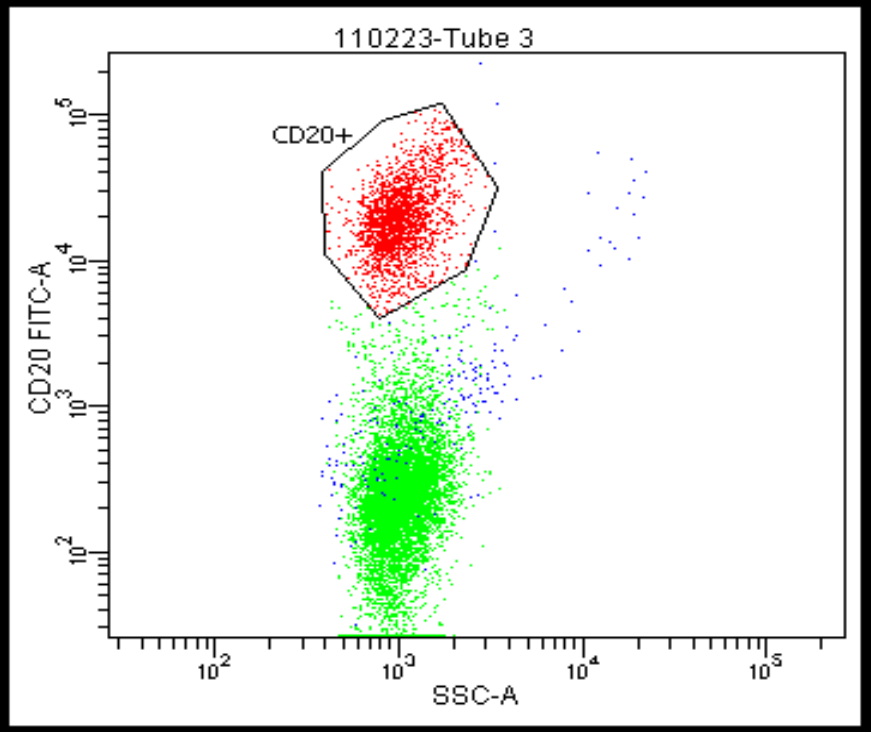


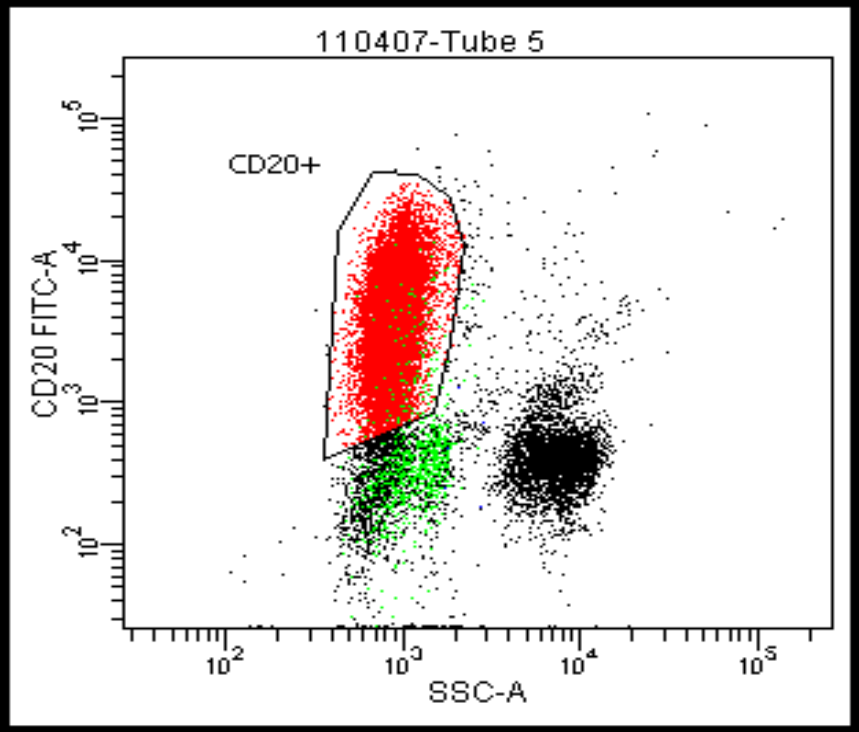
Figure 1 CD20 expression in different B-cell lymphomas. MESF...molecules of soluble fluorochrome, CLL...chronic lymphocytic leukemia, DLBCL...diffuse large B-cell lymphoma, FL...follicular lymphoma, MCL...mantle cell lymphoma, MZL...marginal zone lymphoma, NOS...B-cell lymphomas unclassified, NS...not significant.

In the calibration phase of CD20 – test on tissue material diagnosed with CLL (10-20 cases) as most of these cases express CD20 at lower level compared to normal lymphoid tissue or other lymphoid malignancies



Normal Lymph node

CD20 strong positive

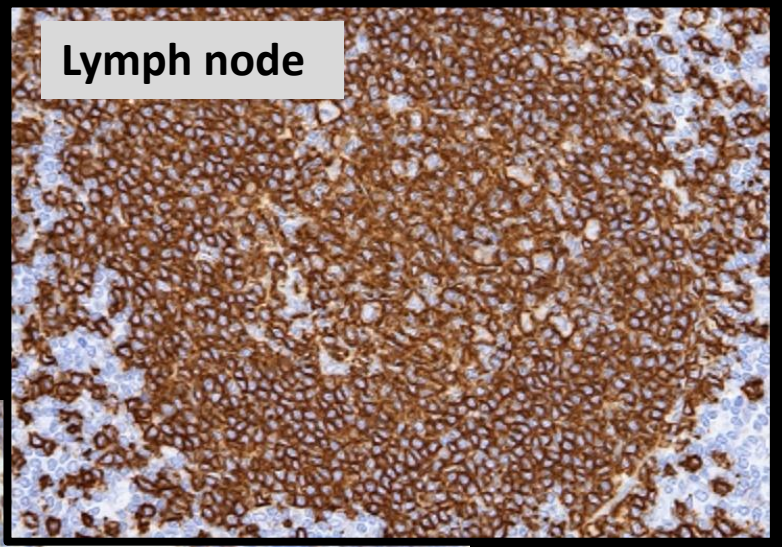


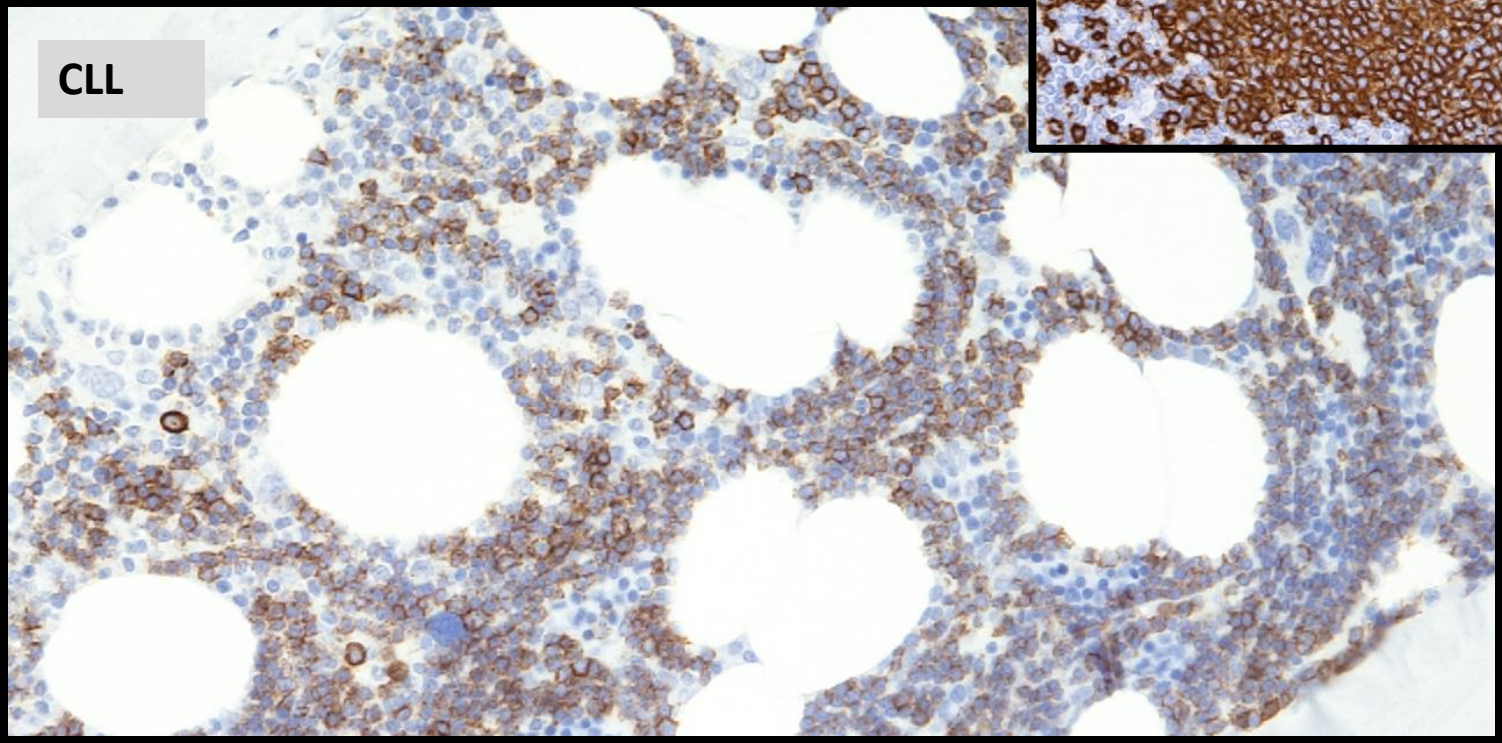
Bone Marrow Aspirate / CLL patient

Marker profile: CD19+, CD5+, CD10-neg, CD20-dim, CD38-neg, CD23+, Kappa+

CD20-dim reaction in the vast majority of the neoplastic B-cells (CLL)

CD20





B-CLL`s in bone marrow specimens often display weak/dim reaction (flowcytometry). A weak to moderate, predominantly membranous staining of the majority of the neoplastic B-cells should be seen.

CD20

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

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **L26**	104	Biocare Cell Marque Dako Master Diagnóstica Leica/Novocastra Scytek Thermo/NeoMarkers Zymed Zytomed Systems	73	25	5	1	94 %	94 %
mAb clone **7D1**	1	Leica/Novocastra	1	0	0	0	-	-
rmAb clone **EP7**	1	Epitomics	1	0	0	0	-	-
pAb **RB-9013-P**	1	Thermo/NeoMarkers	0	0	1	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Ready-To-Use Abs								
mAb clone **L26, 760-4380**	38	Ventana	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	Leica/Novocastra	1	0	0	0	-	-
mAb clone **MJ1, PA0906**	2	Leica/Novocastra	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.

Suff. (clone L26)

HIER (preferable in alkaline buffer`s)

1:75-1:2000

All detection systems

Insuff. (clone L26)

Omission of HIER

Too low conc. of primary Ab

Provided optimal results on the 3 main platforms (Ventana Benchmark, Dako Autostainer and Leica BOND)

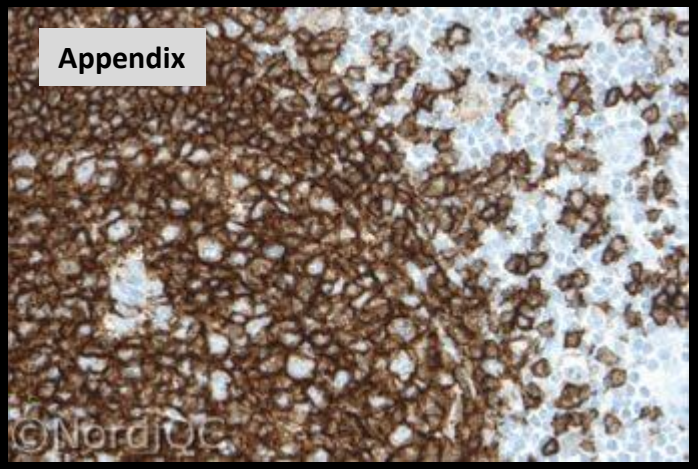


Fig. 1a. Lymphatic tissue in the appendix showing an optimal staining reaction for CD20 using the mAb clone L26 in a RTU format on the BenchMark platform. **HIER was performed** using Cell Conditioning 1. A very strong membranous staining reaction is seen in virtually all the B-cells.

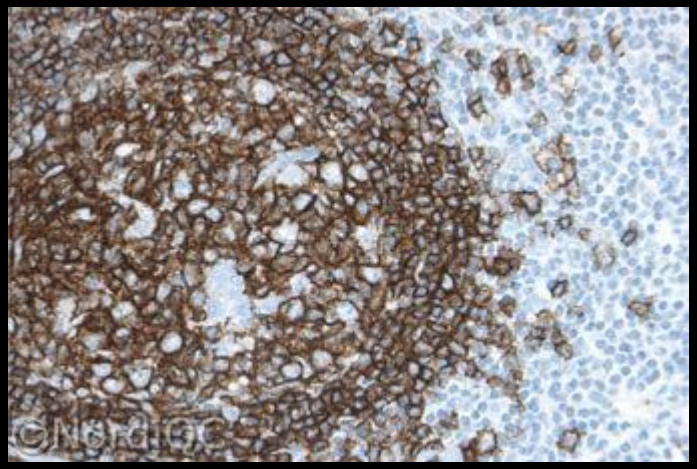


Fig. 1b. Lymphatic tissue in the appendix. Same field as in Fig. 1a. Insufficient staining for CD20 using the mAb clone L26 in a RTU format at the BenchMark platform. **No HIER was performed**. A moderate to strong staining reaction is seen in virtually all the B-cells. The normal B-cells are high expressors 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).

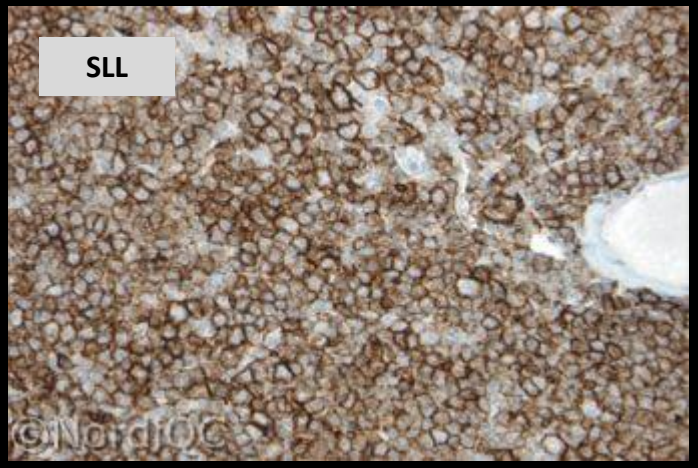


Fig. 2a. B-CLL. Optimal staining reaction for CD20. Same protocol as in Fig. 1a. A moderate to strong membranous staining is seen in virtually all the neoplastic cells.

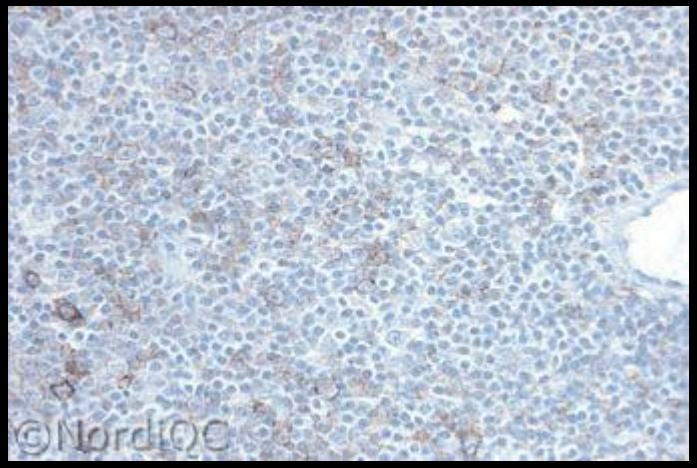


Fig. 2b. B-CLL. Insufficient staining for CD20 using the same protocol as in Fig. 1b. Omitting HIER, only scattered cells are positive. The majority of the neoplastic cells are negative. Compare with the optimal result in Fig. 2a, same field.

Lymphoma panel: CD20

Optimal protocol settings (NQC)

CD20	Retrieval buffers	Titer	Detection systems	RTU	Detection
mmAb **L26**	**HIER High pH or Low pH buffer**	**1:75-1:2000**	**2 & 3-step**	**Dako (IR604)**	**Flex** **Flex+**
	CC1	**-**	**-**	**Ventana (760-2531)**	**iView** **UltraView** **OptiView**
mmAb **7D1**	**HIER Low pH buffer (BERS1)**	**1:200**	**3-step**		**BOND Refine**
rmAb **EP7**	**HIER Low pH buffer (Citrate buffer pH6)**	**1:100**	**-**	**-**	**-**

Control material / Tonsil:

An strong, distinct membranous staining reaction of all B-cells in the tonsil.

No staining of other cellular structures

CD79a

Table 1. **Antibodies and assessment marks for CD79a, run 45**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **11D10**	1	Leica/Novocastra	0	0	0	1	-	-
mAb clone **11E3**	3	Leica/Novocastra	0	0	0	3	-	-
mAb clone **HM57**	2	Dako	0	0	0	2	-	-
mAb clone **JCB117**	94 3	Dako Thermo/NeoMarkers	37	35	19	6	74%	74%
rmAb clone **SP18**	12 3 2 1 1	Thermo/NeoMarkers Spring Bioscience Cell Marque Nordic Biosite Zytomed	21% 4	14	0	1	95%	83%
Ready-To-Use antibodies								
mAb clone **11E3 PA0192**	6	Leica/Novocastra	0	0	3	3	-	-
mAb clone **HM46/A9 PM067**	1	Biocarea	0	0	0	1	-	-
mAb clone **JCB117 IR/IS621**	40	Dako	23	11	5	1	85%	89%
mAb **JCB117 GA621**	11	Dako	9	2	0	0	100%	100%
mAb **JCB117 760-2639***	2	Ventana/Cell Marque	0	1	1	0	-	-
mAb clone **JCB117 PA0599**	1	Leica/Novocastra	0	0	0	1	-	-
rmAb clone **SP18 790-4432**	58	Ventana	86% 50	6	0	2	97%	96%
rmAb clone **SP18 MAD-00032QD**	2	Master Diagnostica	0	0	2	0	-	-
rmAb clone **SP18 179R-18**	1	Cell Marque	0	1	0	0	-	-
rmAb clone **SP18 RMA-0552**	1	Maixin	1	0	0	0	-	-
Total	245		124	70	30	21	-	
Proportion			51%	28%	12%	9%	79%	

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

Optimal (clone JCB117)

HIER (preferable alkaline buffer)

1:25-1:600

2 & 3 step detection systems

Optimal (clone SP18)

HIER (CC1)

1:300-1:500

OptiView (Ventana Benchmark)

Insufficient results

Too short inefficient HIER

Too low conc. of primary Ab

Less successful primary Abs

Table 3: **Proportion of optimal results for CD79a 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 **JCB117**	9/16** (56%)	0/1	11/31 (36%)	-	6/8 (75%)	2/2
rmAb clone **SP18**	0/2	-	4/6 (67%)	-	0/2	-

* 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).

mAb clone JCB117 provided optimal results on the 3 main platforms but……

The proportion of optimal results were lower on the Ventana Benchmark instruments compared to other platforms

<u>In concordance with Run 29, 2010 (mAb JCB117):</u>

Dako Autostainer /BOND platforms, 36 out of 39 of the protocols (92%) gave a sufficient result (77% optimal)

Ventana BenchMark instruments, 17 out of 25 protocols (68%) gave a sufficient staining (12 % optimal)

High Ab concentration (1:25 – 1:100) gave optimal results.

Use rmAb SP18 on the Ventana Benchmark platforms

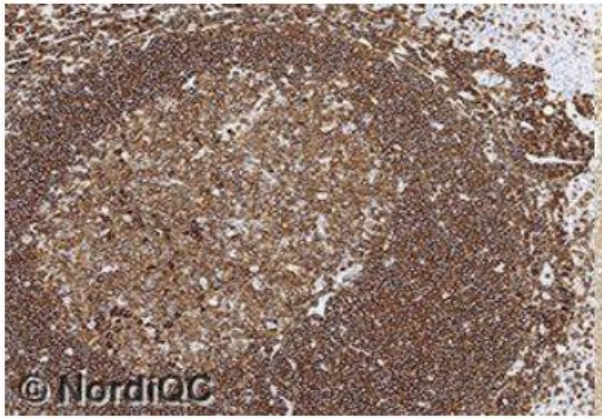


Fig. 1a
Optimal CD79a staining of the tonsil using the mAb clone JCB117 as Ready-To-Use format (GA621, Dako), with HIER in TRS High pH 9 for 30 min., a 3-step polymer based detection kit and performed on Omnis, Dako.
Mantle zone B-cells show an intense membranous staining reaction, while the germinal centre B-cells show a moderate staining reaction. Plasma cells and late stage germinal centre B-cells show a strong cytoplasmic staining reaction.
Also compare with Figs. 2a – 5a, same protocol.

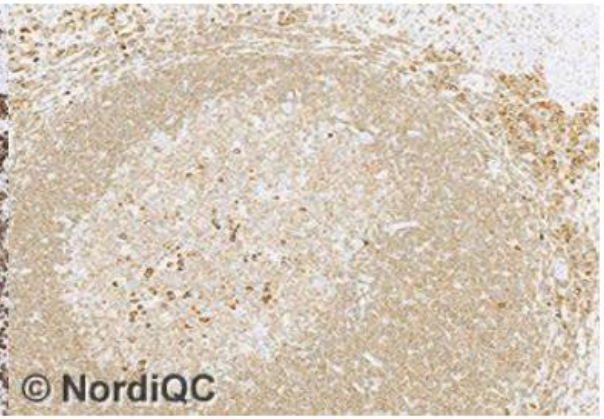


Fig. 1b
CD79a staining of the tonsil using the mAb clone JCB117 with an insufficient protocol – same field as in Fig. 1a.
The primary Ab was used at a titre of 1:500 and a 2-step multimer based detection system providing a too low sensitivity.
The mantle zone B-cells and the late stage germinal centre B-cells are demonstrated, while the germinal centre B-cells only show a weak and diffuse staining reaction.
Also compare with Figs. 2b & 3b – same protocol.

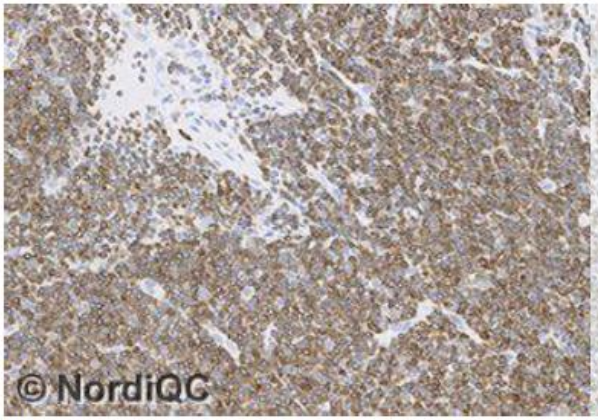


Fig. 2a
Optimal CD79a staining of the B-CLL using same protocol as in Fig. 1a.
Virtually all the neoplastic cells show a moderate and distinct membranous staining reaction.
No background reaction is seen.

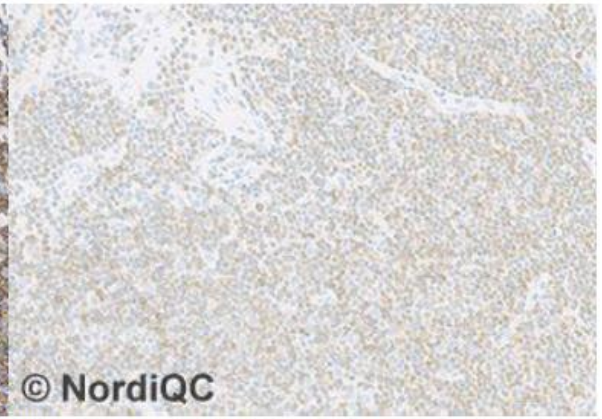


Fig. 2b
Insufficient CD79a staining of the B-CLL using same protocol as in Fig. 1b - same field as in Fig. 2a.
The neoplastic cells only show a weak and equivocal staining reaction.
Also compare with Fig. 3b – same protocol.

Problem:

Protocol with too low sensitivity

- Low concentration of primary

- Low sensitive detection system

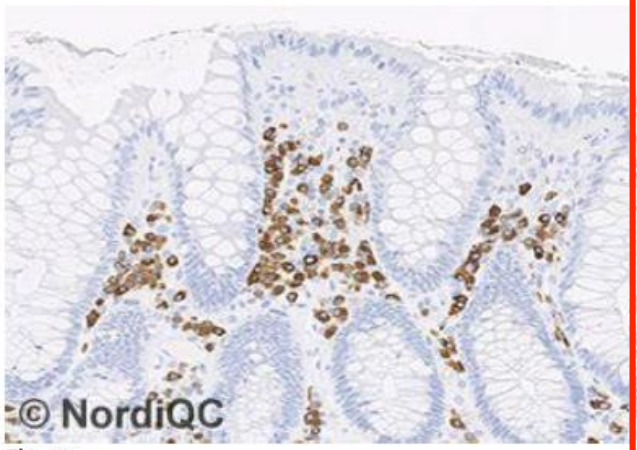


Fig. 4a
Optimal CD79a staining of colon using same protocol as in Figs. 1a - 3a.
Plasma cells show a moderate to strong cytoplasmic staining reaction.
No background reaction is seen.

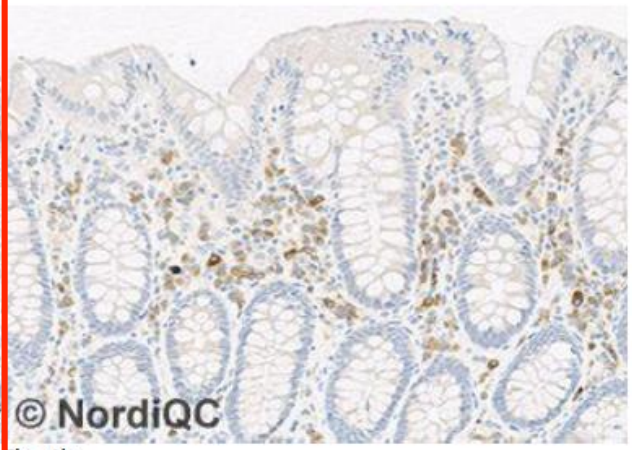


Fig. 4b
CD79a staining of the colon using an insufficient protocol based on the mAb clone 11E3.
The intensity and proportion of plasma cells demonstrated is reduced compared to the level expected.
However also compare with Fig. 5b – same protocol

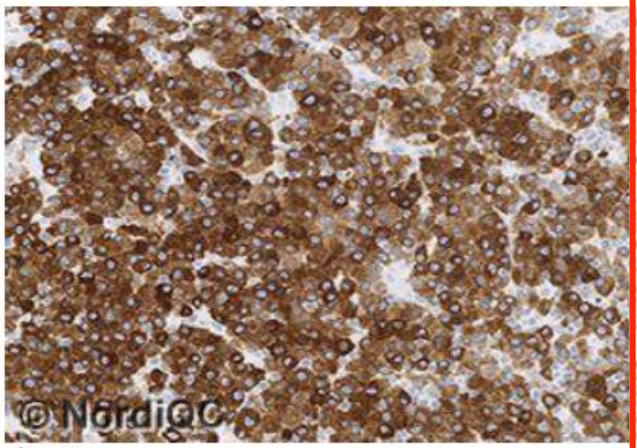


Fig. 5a
Optimal CD79a staining of the plasmacytoma using same protocol as in Figs. 1a - 4a.
Virtually all neoplastic cells show a moderate cytoplasmic staining reaction.

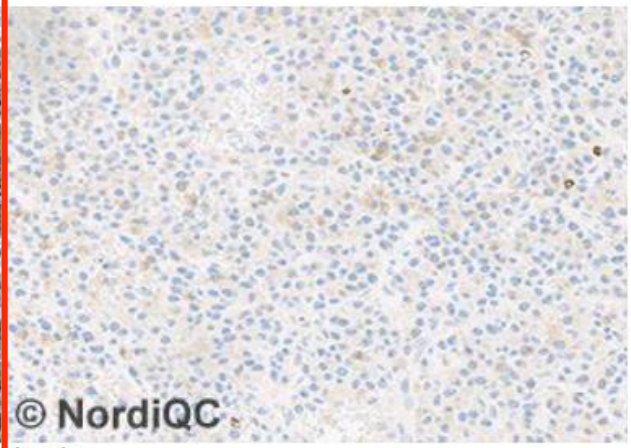


Fig. 5b
Insufficient CD79a staining of the plasmacytoma using same protocol as in Fig. 4b.
Only scattered normal B-cells are demonstrated, while the neoplastic cells are negative.
9 of 9 protocols based on mAb clone 11E3 provided an insufficient result due to a too weak or completely false negative staining reaction in both the plasmacytoma and the precursor B-ALL.

Problem:

Less successful primary Ab

mAb clone 11E3

Lymphoma panel: CD79a

Optimal protocol settings (NQC)

CD79a	Retrieval buffers	Titer	Detection systems	RTU	Detection
mmAb **JCB117**	**HIER High pH or Low pH buffer**	**1:25-1:600**	**2&3-step**	**Dako/Agilent (IR621)** **Dako/Agilent (GA621)**	**Flex** **Flex+**
rmAb **SP18**	**CC1**	**1:300-1:500**	**2&3-step**	**Ventana (790-4432)**	**UltraView** **OptiView**

Tonsil and Appendix/Colon is recommended as positive and negative control:

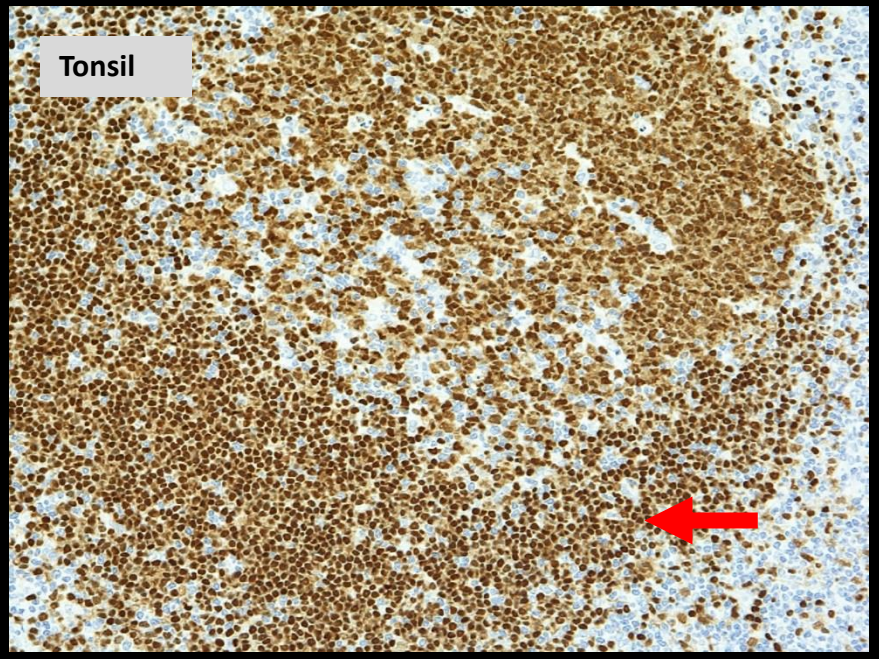
A strong, distinct membranous staining reaction of B-cells in the mantle zone in the tonsil

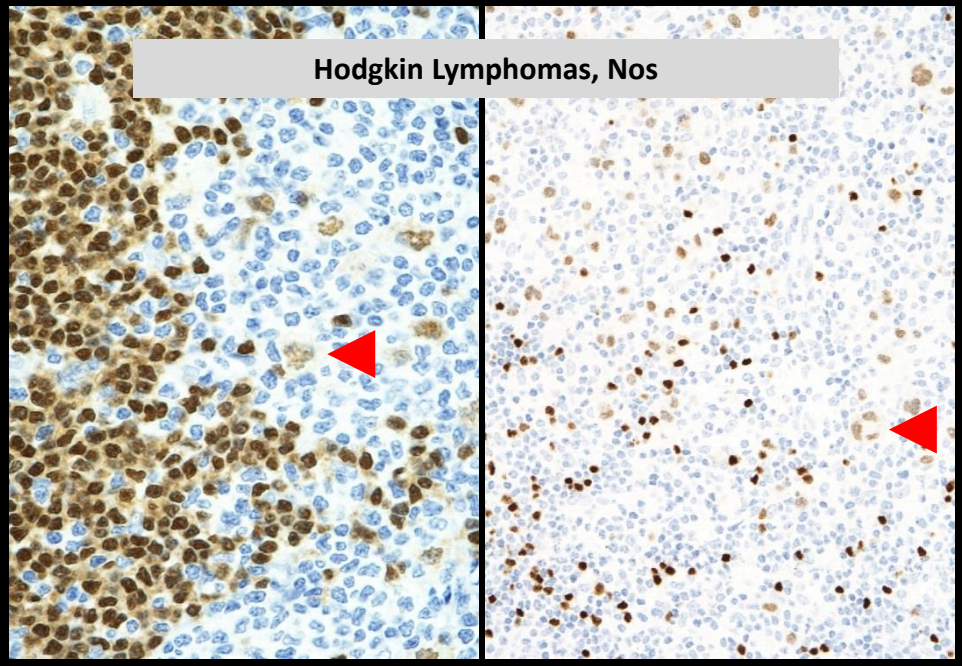
A moderate staining reaction of germinal centre B-cells

Plasma cells should show a strong cytoplasmic staining reaction

Epithelial cells in the appendix/colon should be negative

PAX-5





A moderate to strong, nuclear staining of virtually all the mantle zone B-cells, the germinal centre B-cells and the interfollicular B-cells in the tonsil.

<u>In addition:</u>

The majority of the Hodgkin and Reed-Sternberg cells in Hodgkin lymphomas often displays a weak nuclear reaction in the neoplastic cells.

PAX5 (Run 53)

Table 1. **Antibodies and assessment marks for BSAP, run 53**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1EW**	9	Leica/Novocastra	7	2	0	0	-	-
mAb clone **24**	6 2	BD Biosciences Immunologic	3	2	1	2	-	-
mAb clone **BC/24**	2	Biocare Medical	0	2	0	0	-	-
mAb clone **MX017**	1	Immunologic	1	0	0	0	-	-
~~mAb clone **ZR007**~~	~~1~~	~~Biogenex~~	~~0~~	~~1~~	~~0~~	~~0~~		
mAb clone **DAK-Pax5**	23	Agilent/Dako	15	7	0	1	96%	100%
rmAb clone **BSR59**	1	Nordic Biosite	1	0	0	0	-	-
rmAb clone **BV6**	1	Diagnostic Biosystems	1	0	0	0	-	-
rmAb clone **EP156**	1	Cell marque	1	0	0	0	-	-
rmAb clone **SP34**	12 3 2	Cell Marque Thermo Scientific Spring Biosciences	4	11	2	0	88%	100%
pAb **RB-9406**	3	Thermo Scientific	0	0	1	2	-	-
Ready-To-Use antibodies								
mAb clone **1EW PA0552**	5	Leica/Novocastra	2	2	1	0	-	-
mAb clone **BC/24 PM207**	1	Biocare Medical	1	0	0	0	-	-
mAb clone **24 312M-18**	1	Cell marque	0	1	0	0	-	-
mAb clone **MX017 MAB-0706**	1	Maixin	1	0	0	0	-	-
mAb clone **MX017 MAD-000694QD**	1	Master Diagnostica	1	0	0	0	-	-
mAb clone **DAK-Pax5 IS/IR650**	23	Agilent/Dako	19	3	1	0	96%	100%
mAb clone **DAK-Pax5 IS/IR650**[3]	3	Agilent/Dako	3	0	0	0	-	-
mAb clone **DAK-Pax5 GA650**	24	Agilent/Dako	24	0	0	0	100%	100%
mAb clone **DAK-Pax5 GA650**[3]	1	Agilent/Dako	1	0	0	0	-	-
rmAb clone **EP156 8500-C010**	2	Sakura Finetek	2	0	0	0	-	-
rmAb clone **RBT-PAX5 BSB 5862**	1	BioSB	0	0	0	1	-	-
rmAb clone **SP34 790-4420**	33	Ventana	3	23	7	0	79%	75%
rmAb clone **SP34 312R-18**	35	Cell Marque	2	25	8	0	77%	100%
Total	198		92	79	21	6	-	
Proportion			46%	40%	11%	3%	86%	

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.

Most common primary Abs
mAb DAK-Pax5 and rmAb SP34

rmAb SP34 as LD or RTU assays:

Low proportion of optimal results
- **Poor signal to noise ratio**

AS or Omnis (optimal results):
HIER in TRS pH9 or TRS pH 6.1 (10-20 ` at 95-99C), primary Ab Inc (15-30`), Flex/Flex+

Benchmark Ultra/XT/GX (optimal results):
HIER in CC1 (32-90`), primary Ab Inc (16-44`), UV+ amp or OV

Optimal (DAK-Pax5)

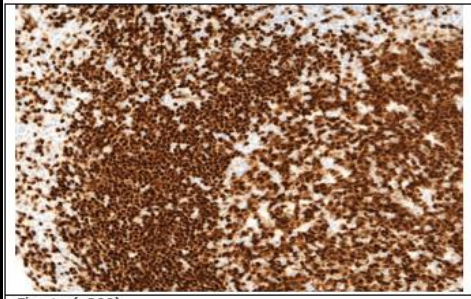
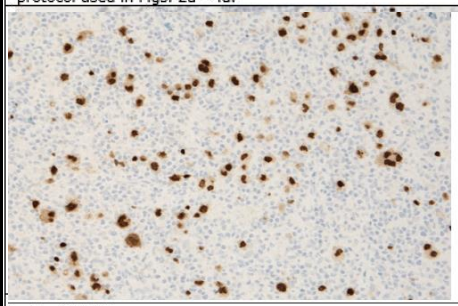
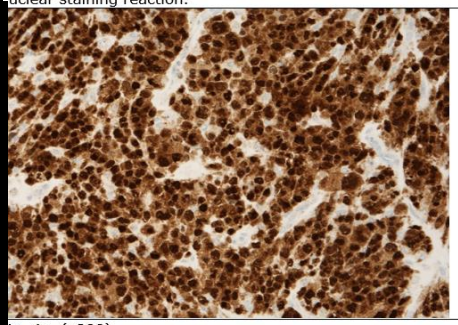


Fig. 1a.(x200)
Optimal BSAP staining reaction of the tonsil using the mAb clone DAK-Pax5, optimally calibrated, HIER in TRS (3-1) pH 9 (Dako) and a 3-step polymer based detection system (Flex+/Dako).
All mantle zone and germinal centre B-cells show a strong and distinct nuclear staining reaction. Cytoplasmic staining reaction in positive B-cells must be accepted. No staining reaction is observed in other cellular structures including T-cells. Same protocol used in Figs. 2a - 4a.



ig. 3a. (x200)
)ptimal BSAP staining of the Hodgkin Lymphoma (classical ype) using same protocol as in Figs. 1a and 2a. The vast najority of Hodgkin and Reed-Sternberg cells, intermingling etween B- and T-cells, show a moderate to strong but distinct uclear staining reaction.



ig. 4a. (x200)
)ptimal BSAP staining of the DLBCL using same protocol as in igs. 1a – 3a. All the neoplastic cells display a strong and istinct nuclear staining reaction. Cytoplasmic staining reaction f the neoplastic cells must be accepted.

Insufficient (DAK-Pax5)

Fig. 1b.(x200)
Insufficient staining of BSAP in the tonsil using the mAb clone DAK-Pax5, too diluted, HIER in TRS (3-1) pH 6 (Dako) and the less sensitive detection system Flex (Dako) – same field as in Fig. 1a.
B-cells of the mantle zone and germinal centres only display weak to moderate staining intensity of the nuclei(compare with Fig. 1a). Same protocol used in Figs. 2b - 4b.

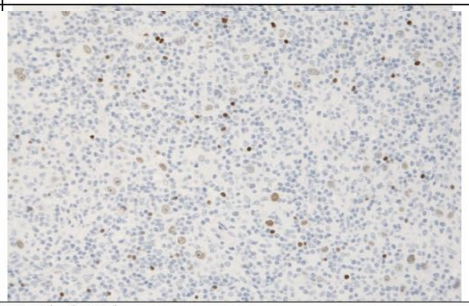


Fig. 3b. (x200)
Insufficient BSAP staining of the Hodgkin Lymphoma (classical type) using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The neoplastic cells are only faintly demonstrated and a proportion of Hodgkin and Reed-Sternberg cells are false negative.

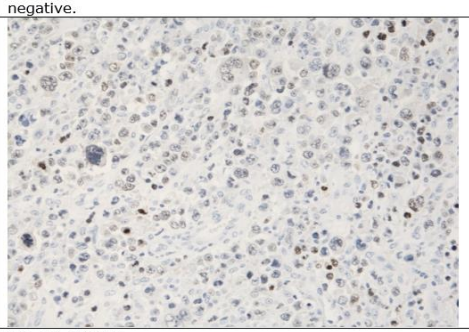


Fig. 4b. (x200)
Insufficient BSAP staining of the DLBCL using same protocol as in Figs. 1b and 3b - same field as in Fig. 4a. The staining intensity of the nuclei´s are barely visible and a significant proportion of the neoplastic cells are false negative.

Frequent causes of insufficient staining reactions were:

- **Too low concentration of the primary antibody**
- **Use of low sensitivity detection systems**

Insufficient (rmAbSP34)

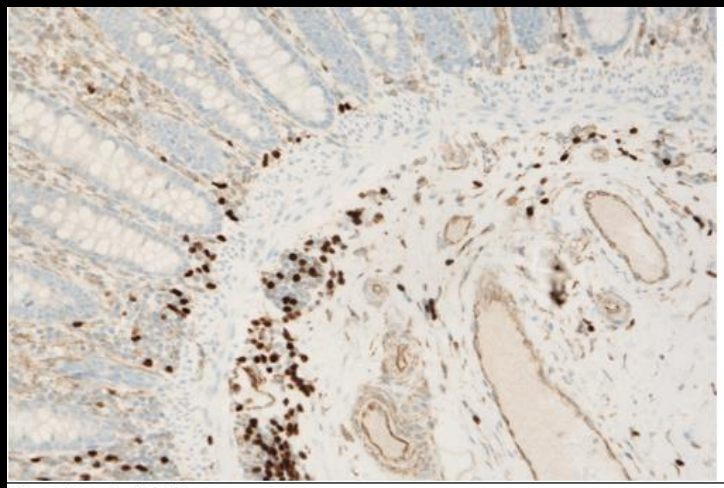


Fig. 5a. (x200)
Insufficient BSAP staining of the colon. The protocol was based on the rmAb clone SP34 as RTU format (790-4420, lot. no. Y18596, Ventana), HIER in CC1 and OptiView (Ventana) as the detection – same protocol used in Fig. 5b, but with a different lot. no. (both slides stained in a NQC reference lab.). Typical reaction pattern seen with the rmAb 34. The B-ce expected nuclear staining reaction, but vast majo cells (e.g. endothelia cells) displays an unaccepta cytoplasmic staining reaction providing a poor sig ratio.

Sufficient (rmAbSP34)

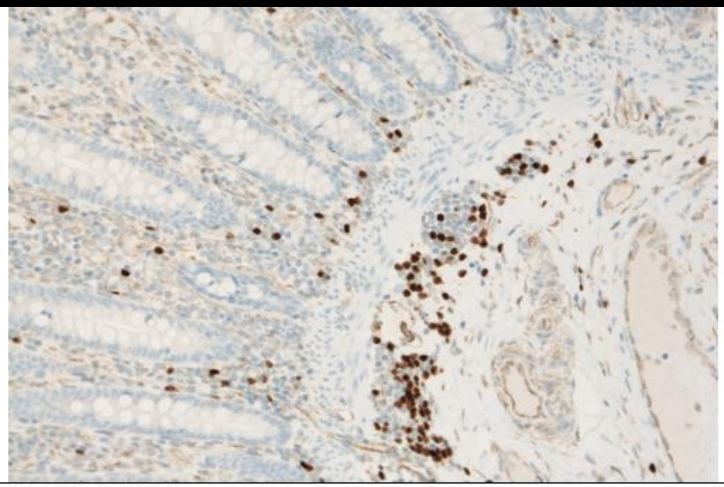


Fig. 5b.(x200)
Sufficient BSAP staining (good) of the colon using the same protocol as in Fig. 5a, but with lot.no. Y05958 (primary Ab). It has been observe from NQC reference labs, but also seen in this assessment, that there are lot-to-lot variation of concentrates/RTU's based on the rmAb SP34. Only a faint to

NordiQC ref. Lab:

Lot to lot variations ?

The most frequent causes of insufficient staining reactions were:

- **Too low concentration of the primary antibody**
- **Use of low sensitivity detection systems**
- **False positive staining reaction or poor signal-to-noise ratio of assays based on the rmAb SP34**
- **Previous run: Cross reactivity or contamination (CK20) ?**

Lymphoma panel: PAX5 (most common markers)
Optimal protocol settings (NQC)

PAX5	Retrieval buffers	Titre	Detection	RTU	Detection
mmAb **DAK-PAX5**	**HIER High pH, mod. & standard low pH**	**1:20-1:100**	**2 & 3-step**	**Dako (IS/IR/GA650)**	**Flex/ Flex+**
rmAb **SP34**	**HIER High pH**	**1:50-1:100**	**2 & 3-step**	**Ventana (790-4420)**	**UltraView + Amp OptiView**
mmAb **1EW**	**HIER High pH & standard low pH**	**1:25-1:50**	**2 & 3-step**	**Leica (PA0552)**	**BOND Refine**
mmAb **24**	**HIER High pH**	**1:20-1:50**	**2 & 3-step**	**-**	**-**

Control material / Tonsil or Appendix:

A distinct moderate to strong nuclear staining reaction of virtually all mantle zone B-cells, germinal centre B-cells and interfollicular peripheral B-cells in the tonsils and appendix.

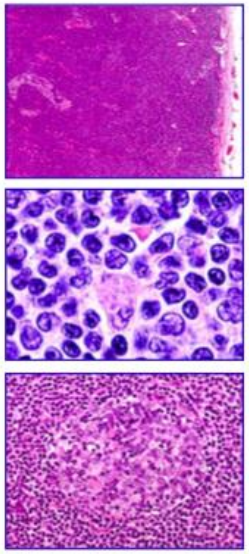
No staining reaction of other cells, including T-cells, squamous epithelial cells of the tonsils and columnar epithelial cells of the appendix.

Tech tip: Use Hodgkin Lymphoma`s in the calibration phase

Immunophenotype: Small B-Cell Lymphomas

	CD20	CD79A	CD10	CD23	CD5	CD43	bcl-2	CyclinD1	TdT
CLL	+	+	-	+	+	+	+	-	-
FL	+	+	+	-	-	-	+	-	-
MCL	+	+	-	-	+	+	+	+	-
LPL	+	+	-	-	-	-/+	+	-	-
MZL	+	+	-	-	-	-/+	+	-	-
SMZ	+	+	-	-	-	-/+	+	-	-
MALT	+	+	-	-	-	-/+	+	-	-
HCL	+	+	-	-	-	-	+	-	-
BLB	-/+	+	+/-	+/-	-	-	+	-	+

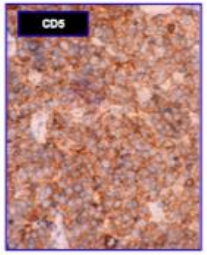
Mantle Cell Lymphoma

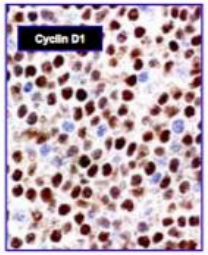


Morphology
- small-medium lymphocytes
- cleaved / irregular
- blastoid variant
- nodular / mantle / diffuse

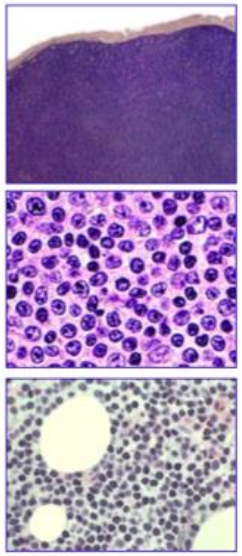
Immunology

surface Ig	+
CD19, 20, 22, 79a	+
CD5	+
CD23	-
Cyclin D1	+
CD10	-





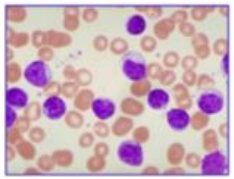
B-cell Small Lymphocytic Lymphoma (CLL)

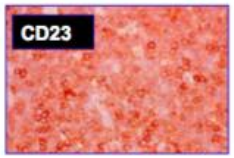


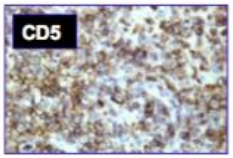
Morphology
- small lymphocytes
- proliferation centres

Immunology

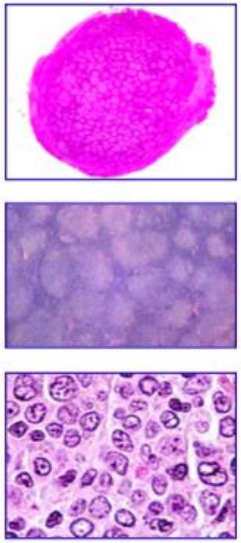
surface IgMD weak	
CD19, 20, 79a	+
CD5	+
CD23	+
CD10, CycD1	-







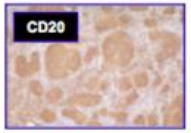
Follicular Lymphoma

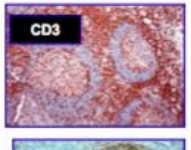


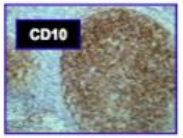
Morphology
- germinal centre cells
- CBs & CCs
- follicular

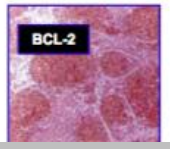
Immunology

surface Ig	+
CD19, 20, 22, 79a	+
BCL-2	+
CD10	+/-
Bcl-6	+
CD5	-









Courtesy: Steve Hamilton-Dutoit

B-Cell lymphoma markers (2)

Marker (localization)	Control	High exp. (HE)	Low exp. (LE)	Non exp. (NE)
BCL2 (cytopl. + nuclear) 124, 100/D5, BCL/100/D5, 100	Tonsil/Appendix	Mantle zone B-cells & T-cells (including intra germinal centre T-cells)	Basal cells (squamous epithelium) in surface epithelium of the tonsil & columnar cells lining basal compartment of the crypts (appendix)	Germinal centre B-cells (tonsil)
CD10 (cytopl. + membr.) 56C6, GI191E/A8	Tonsil/Kidney	Germinal centre B-cells (Tonsil, moderate to strong intensity). Proximale tubuli (Kidney)	Scattered neutrophil granulocytes	Mantle zone B-cells and squamous epithelial cells (tonsil)
CD23 (membr.) 1B12, DAK-CD23, BS20, SP23	Tonsil	Follicular dendritic cells in the germinal centres	Mantle zone B-cells and scattered interfollicular B-cells	No staining of T-cells
CyclinD1 (nuclear) SP4, EP12	Tonsil	Suprabasal squamous epithelial cells, scattered lymphocytes and endothelial cells	Germinal centre macrophages	Mantle zone B-cells and germinal centre B-cells
SOX11 (nuclear) SOX11-C1, MRQ-58	MCL`s /Tonsil	MCL	MCL	Tonsil (all cells)
CD43 (membr.) DF-T1	Tonsil/Appendix	T-cells in the T-zone (tonsil)	Intra germinal centre T-cells (an at least moderate expression) , macrophages (tonsil, germinal centres) and activated B-cells (Ig pos)	Mantle zone B-cells of germinal centres (tonsil) and epithelium (app.)
CD5 (see T-cells) & TdT (see blasts/bonus material)				

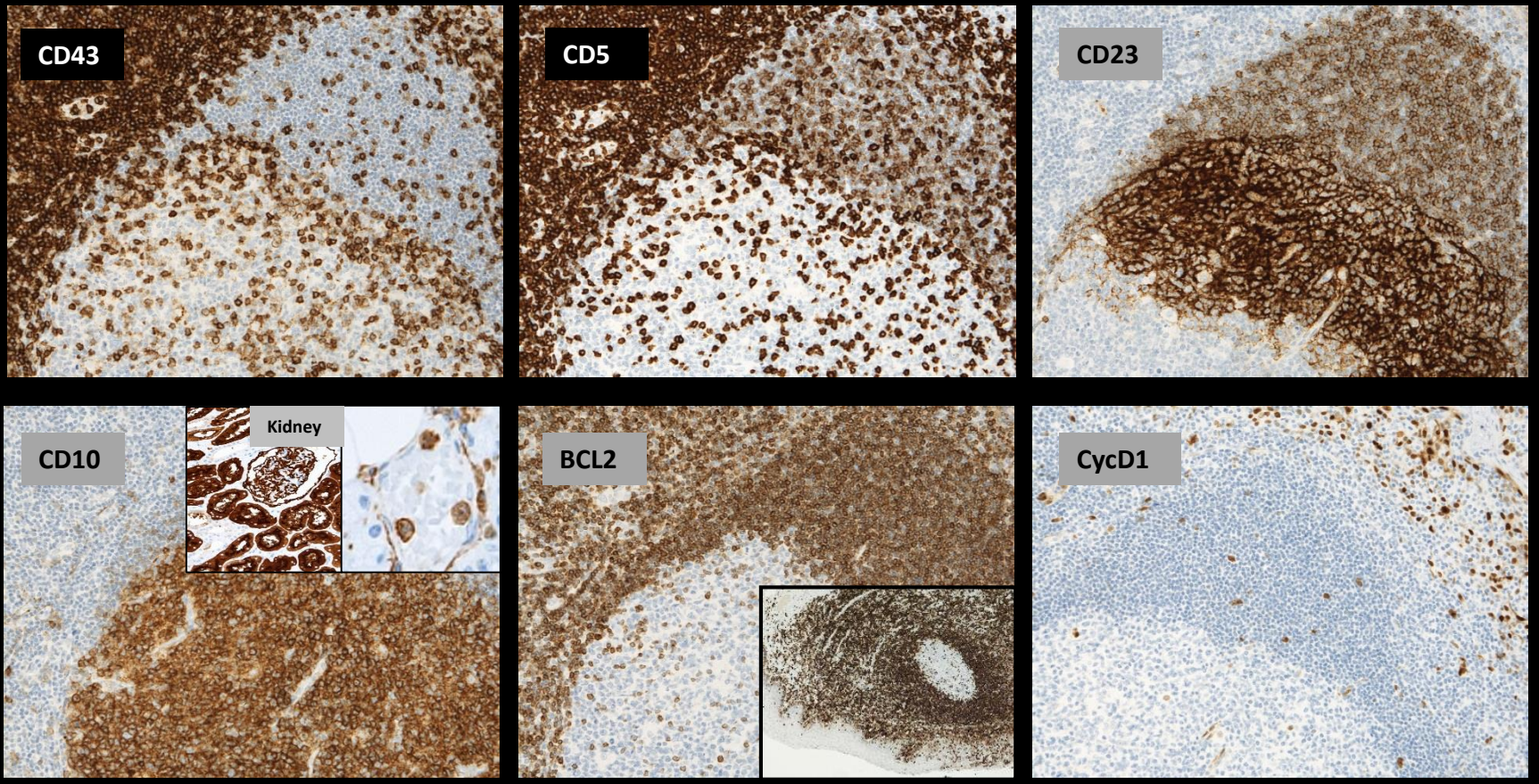
Clones (mAbs, rmAbs & pAbs) providing optimal results (NordiQC assessments)

HE: Strong staining intensity/reactions should be expected

LE: An at least weak to moderate staining intensity/reactions should be expected

NE: No staining/reactions should be expected

B-Cell lymphoma markers (2)



Tonsil

CD5 and CD43 are in principal T-cell markers, but very helpful in classification of small B-cell lymphomas (low grade)

BCL2

Table 1. Abs and assessment marks for Bcl-2, run 28

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **124**	98 1	Dako Cell Marque	49	35	15	0	85 %	86 %
mAb clone **100/D5**	5 1 1 1	NeoMarkers Biocare Immunologic Master Diagnostica	2	5	1	0	89 %	100 %
mAb clone **bcl-2/100/D5**	5	Novocastra	3	1	0	1	80 %	-
mAb clone **100**	2	BioGenex	2	0	0	0	-	-
mAb clone **3.1**	2	Novocastra	0	2	0	0	-	-
mAb clone **Bcl-2-100**	1	Zymed	0	0	1	0	-	-
mAb clone **8C8**	1	NeoMarkers	0	1	0	0	-	-
Ready-To-Use Abs								
mAb clone **124, IR614**	14	Dako	10	4	0	0	100 %	100 %
mAb clone **124, 760-4240**	18	Ventana/Cell Marque	0	8	9	1	44 %	-
mAb clone **124, MON-RTU1011**	1	Monosan	0	0	1	0	-	-
mAb clone **bcl-2/100/D5, PA0117**	2	Leica	2	0	0	0	-	-
mAb clone **100/D5, PM003**	1	Biocare	0	1	0	0	-	-
mAb clone **100/D5, 760-2693**	1	Ventana	0	1	0	0	-	-
Total	155		68	58	27	2	-	-
Proportion			44 %	38 %	17 %	1 %	82 %	-

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

Optimal Protocols

HIER preferable in alkaline buffer (high pH)

Careful calibration of primary Ab

3-step detection systems

Insufficient results

Low concentration of the primary Ab

Platform dependent mAb clone 124

BCL-2

mAb clone 124: The staining result was influenced by the platform used for the staining.

LD assay (mAb clone 124)	Pass Rate`s (%)
Ventana Benchmark	50% (21 of 42)
Dako Autostainer	97% (59 of 61)

Only 10% (4 of 42) were assessed as optimal on the Ventana Benchmark platform and optimal protocols were based on high concentration of the Ab (1:10 – 1:20), efficient HIER by Standard CC1, and UltraView + amplification as the detection system.

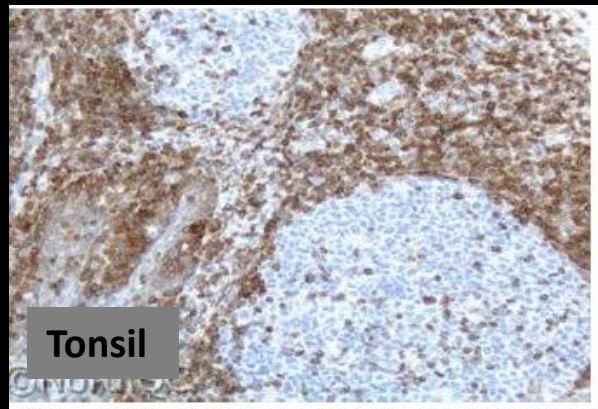
RTU assay (mAb clone 124)	Pass Rate`s (%)	Optimal (%)
Ventana Benchmark (760-4240)	44% (8 of 18)	0% (0 of 18)
Dako Autostainer (IR614)	100% (14of 14)	71% (10 of 14)

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 (K8000) or Flex+ (K8002) as the detection system.

RTU format (Ventana/Cell Marque) - No optimal results

Vendor protocol recommendations: HIER in CC1 (Standard), 16 min inc. in primary Ab and UltraView as the detection system.

BCL-2



ig. 2a. High magnification of the optimal Bcl-2 staining of the onsil shown in Fig. 1a. The scattered T-cells within the erminal centre show a distinct staining and also the basal quamous epithelial cells (left) show a weak to moderate taining. Same protocol as in Fig. 1a.

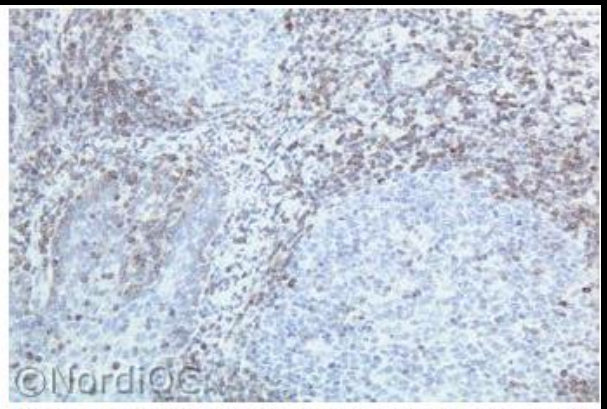
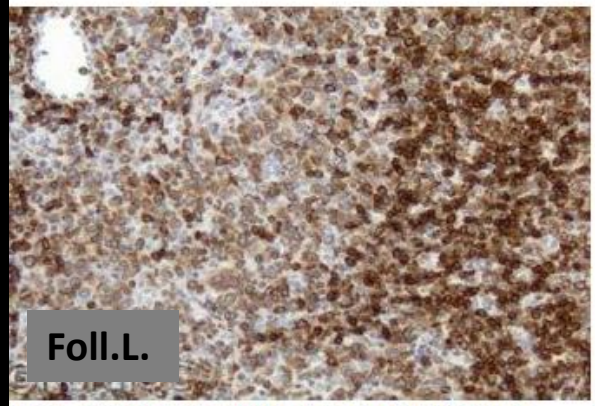


Fig. 2b. High magnification of the insufficient Bcl-2 staining of the tonsil shown in Fig. 1b – same field as in Fig. 2a. Only the grouped peripheral lymphocytes show a distinct staining, while the germinal centre T-cells and the basal squamous epithelial cells virtually are negative. Same protocol as in Fig. 1b.



ig. 3a. Optimal Bcl-2 staining of the follicular lymphoma rade III using same protocol as in Figs. 1a & 2a. Virtually all he neoplastic show a moderate staining, while the remnants f the normal lymphocytes (right) show a strong staining.

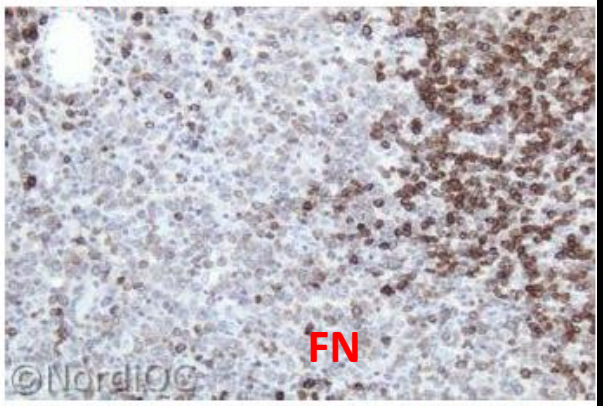


Fig. 3b. Insufficient Bcl-2 staining of the follicular lymphoma grade III using same protocol as in Figs. 1b & 2b. – same field as in Fig. 3a. The normal lymphocytes show a moderate staining, while the neoplastic cells only show a weak, equivoca staining.

Problem:

Protocol with too low sensitivity

mAb clone 124

Too low conc of the primary Ab

Lymphoma panel: BCL-2

Optimal protocol settings (NQC)

BCL-2	Retrieval buffers	Titre	Detection	RTU	Detection
mmAb **124**	**HIER High pH & mod. Low pH**	**1:10-1:400**	**2 & 3-step**	**Dako (IS503/IR503)**	**Flex/ Flex+**
mmAb **100/D5**	**HIER High pH**	**1:20-1:40**	**3-step**	**Leica (PA0117)**	**BOND Refine**
mmAb **BCL2/100/D5**	**HIER High pH & mod. Low pH**	**1:50-1:140**	**2 & 3-step**	**-**	**-**
mmAb **100**	**HIER High pH**	**1:200-1:1200**	**2 & 3-step**	**-**	**-**

Control material / Tonsil:

A moderate to strong predominantly cytoplasmic staining of virtually all the peripheral B- and T-cells in the tonsils.

An at least weak cytoplasmic staining of the basal squamous epithelial cells of the tonsil.

No staining reaction in the germinal centre B-cells.

CD23

Table 1. **Antibodies and assessment marks for CD23, run 50**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **1B12**	51 3 2 2 2	Leica/Novocastra Cell Marque Biocare Thermo F. Scientific Monosan	22	27	8	3	82%	87%
mAb clone **DAK-CD23**	12	Agilent/Dako	5	4	2	1	75%	100%
mAb clone **BS20**	1	Nordic Biosite	1	0	0	0	-	-
mAb clone **MRQ-57**	1		0	0	1	0	-	-
mAb clone **MHM6***	1	Agilent/Dako	1	0	0	0	-	-
rmAb clone **SP23**	25 3 3 1 1	Thermo S./ Neomarkers Spring Bioscience Cell Marque Immunologic Diagnostic Biosystems	20	9	4	0	88%	90%
Ready-To-Use antibodies								
mAb clone **1B12 PA0169**	9	Leica/Novocastra	8	0	1	0	89%	100%
mAb clone **1B12* PA0169**	3	Leica/Novocastra	0	2	1	0	-	-
mAb clone **1B12 123M-18**	1	Cell Marque	0	0	1	0	-	-
mAb clone **1B12 PM100**	1	Biocare	0	1	0	0	-	-
mAb clone **1B12 PDM143**	1	Diagnostic Biosystems	0	0	1	0	-	-
mAb clone **DAK-CD23 IR781**	31	Agilent/Dako	24	5	1	1	94%	92%
mAb clone **DAK-CD23 IR781***	7	Agilent/Dako	3	4	0	0	100%	-
mAb clone **DAK-CD23 GA781**	15	Agilent/Dako	14	1	0	0	100%	100%
mAb clone **DAK-CD23 GA781***	1	Agilent/Dako	0	1	0	0	-	-
rmAb clone **SP23 790-4408**	78	Roche/Ventana	43	34	1	0	99%	99%
rmAb clone **SP23 123R-17/18**	5	Cell Marque	3	1	1	0	80%	100%
rmAb clone **SP23 MAD-00333QD**	3	Master Diagnostica	2	0	0	1	-	-
rmAb clone **SP23 M3231**	2	Spring Bioscience	0	2	0	0	-	-
rmAb clone **SP23 RMA-0504**	1	Maixin	0	1	0	0	-	-
rmAb clone **SP23 IR800***	1	Agilent/Dako	1	0	0	0	-	-
rmAb clone **EP75 123R-27/28**	1	Cell Marque	1	0	0	0	-	-
pAb **AR460-5/10R**	1	Biogenex	0	0	0	1	-	-
Total	269		148	92	22	7	-	
Proportion			55%	34%	8%	3%	89%	

1) Proportion of sufficient stains (optimal or good), 2) Proportion of sufficient stains with optimal protocol settings only, see below. 3) RTU system developed for the Leica/Novocastra full-automatic system (BOND III/MAX) but used by laboratories on e.g. a Ventana Benchmark Ultra (Roche/Ventana), 4) RTU system developed for the Agilent/Dako semi-automated systems (Autostainer) but used by laboratories on the Omnis (Agilent/Dako). 5) RTU used in a manual assay.
*Product has been discontinued by the vendor.

Optimal protocols:

HIER in alkaline buffer

HIER in mod. Low pH buffer (Dako) /DAK-CD23

3-step pol./mul. Detection systems.

Careful calibration of the primary Ab.

HIER in BERS2/1 (10-20 min/95-100℃), BOND refine

HIER in TRS pH 6.1 (30 min/97℃), Flex/Flex+

HIER in CC1 (24- 98 min/95-100 ℃), Ultra/OptiView with or without amp.

Best performance:

RTU clone 1B12 (PA0169, Leica)

RTU clone DAK-CD23 (IR/GA781, Dako)

RTU format SP23 (790-4408, Ventana)

Table 3. **Proportion of optimal results for CD23 for the most commonly used antibodies as concentrate on the 3 main IHC systems***

Concentrated antibodies	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 **1B12**	4/6** (67%)	-	2/4	-	7/19 (37%)	-	8/10 (80%)	0/2
mAb clone **DAK-CD23**	0/3	3/3	-	-	0/1	-	2/3	-
rmAb clone **SP23**	1/1	-	0/1	1/1	10/17 (59%)	0/1	3/3	1/1

* 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)

Table 4. **Proportion of sufficient and optimal results for CD23 for the most commonly used RTU IHC systems**

RTU systems	Recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS mAb **IR781**	100% (7/7)	100% (7/7)	92% (22/24)	71% (17/24)
Dako Omnis mAb **GA781**	100% (7/7)	100% (7/7)	100% (4/4)	75% (3/4)
Leica BOND MAX/III mAb **PA0169**	100% (4/4)	100% (4/4)	80% (4/5)	80% (4/5)
VMS Ultra/XT rmAb **790-4408**	100% (3/3)	0% (0/3)	99% (71/72)	59% (43/72)

* 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 integrated.

UltraView

mAb clone 1B12 challenging on the Ventana Benckmark

Optimal results:

Efficient HIER in CC1 , high conc. of the primary Ab (1:10-20), 3-step mul. detection system

Alternative: Use SP23

Optimal results:

Efficient HIER in CC1 and the use of a 3-step mul. detection system (UltraView with amp. or OptiView)

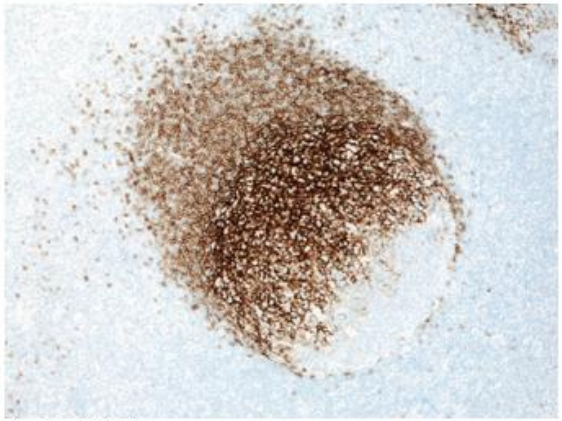


Fig. 1a (x100)
Optimal staining reaction for CD23 of the tonsil using the mAb clone 1B12 as concentrate, careful calibrated (1:10), HIER in an alkaline buffer (CC1, Ventana) and a 3-step multimer based detection system (OptiView, Ventana) - same protocol used in Figs. 2a - 3a. The majority of B-cells in the mantle zone show a moderate but distinct membranous staining reaction. The follicular dendritic cells of the germinal centres display a strong staining reaction - compare with Fig.1b.

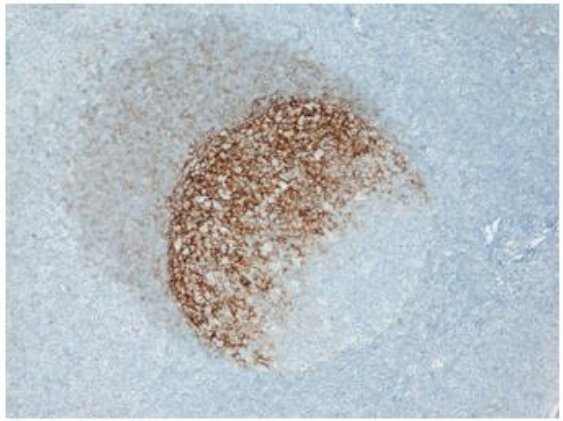


Fig. 1b (x100)
Insufficient staining reaction for CD23 of the tonsil using the mAb clone 1B12 as concentrate (too diluted, 1:50), HIER in CC1 and with a too low sensitive detection system (UltraView, Ventana) - same protocol used in Figs. 2b - 3b. The intensity of the staining reaction is significantly reduced and the majority of B-cells in the mantle zone show an equivocal staining reaction - compared with Fig. 1a (same field).

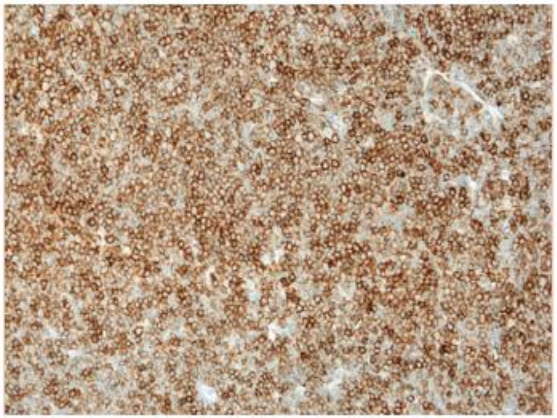


Fig. 3a (x200)
Optimal staining reaction for CD23 of the B-CLL, tissue core no. 4, using same protocol as in Figs. 1a and 2a. The vast majority of the neoplastic cells show a strong membranous staining reaction – compare with Fig. 3b.

Fig. 3b (x200)
Insufficient staining reaction for CD23 of the B-CLL, tissue core no. 4, using same protocol as in Figs. 1b and 2b – same field as in Fig. 3a.
The majority of the neoplastic cells displays reduced staining intensity and a significant proportion of neoplastic cells are false negative.

mAb clone 1B12 (Ventana Benchmark Ultra)

Problem

Too diluted

Too low sensitive detection system

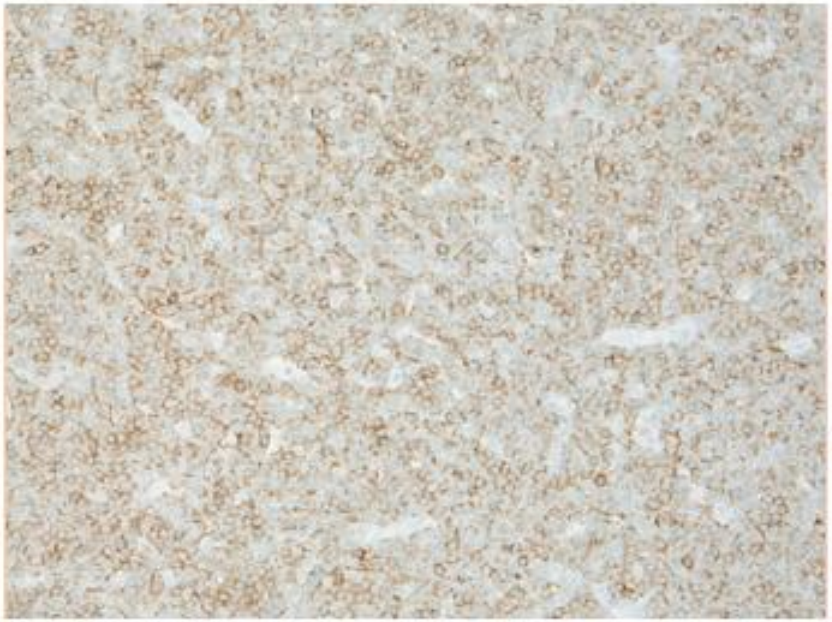


Fig. 4a (x200)
Good staining reaction for CD23 of the B-CLL, tissue core no. 5, using the rmAb clone SP23 in a RTU format (790-4408, Benchmark, Ventana), HIER in CC1 and with a 2-step multimer detection system (UltraView). Although the majority of the neoplastic cells show a weak to moderate distinct membranous staining reaction, the system can be optimized – see Fig. 4b.

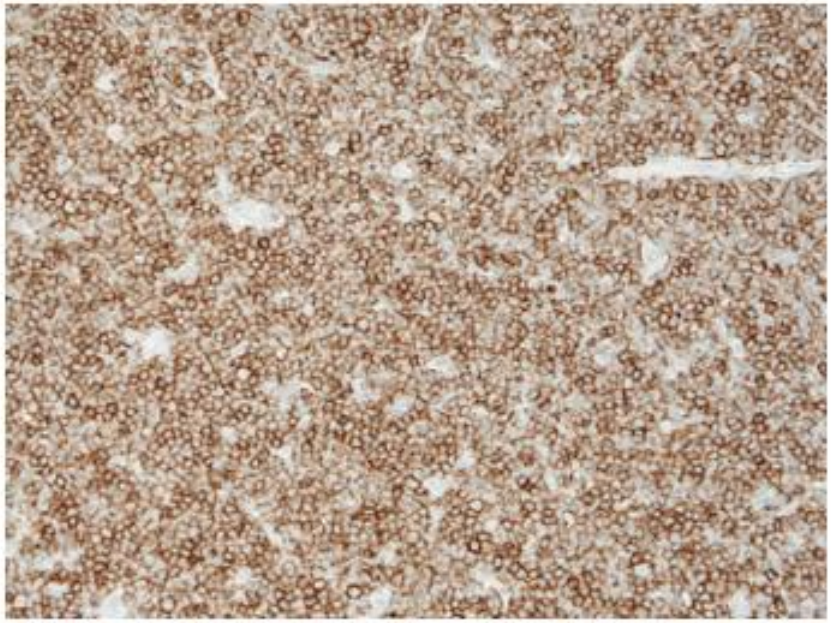


Fig. 4b (x200)
Optimal staining reaction for CD23 of the B-CLL, tissue core no. 5, using the same system as in Fig. 4a but with a 3-step multimer detection system (OptiView) – same field as in Fig. 4a.
Virtually all neoplastic cells show a strong membranous staining reaction. For this RTU system, the use of OptiView or UltraView with amplification significantly increased the proportion of optimal results.

UltraView versus OptiView

Lymphoma panel: CD23

Optimal protocol settings (NQC)

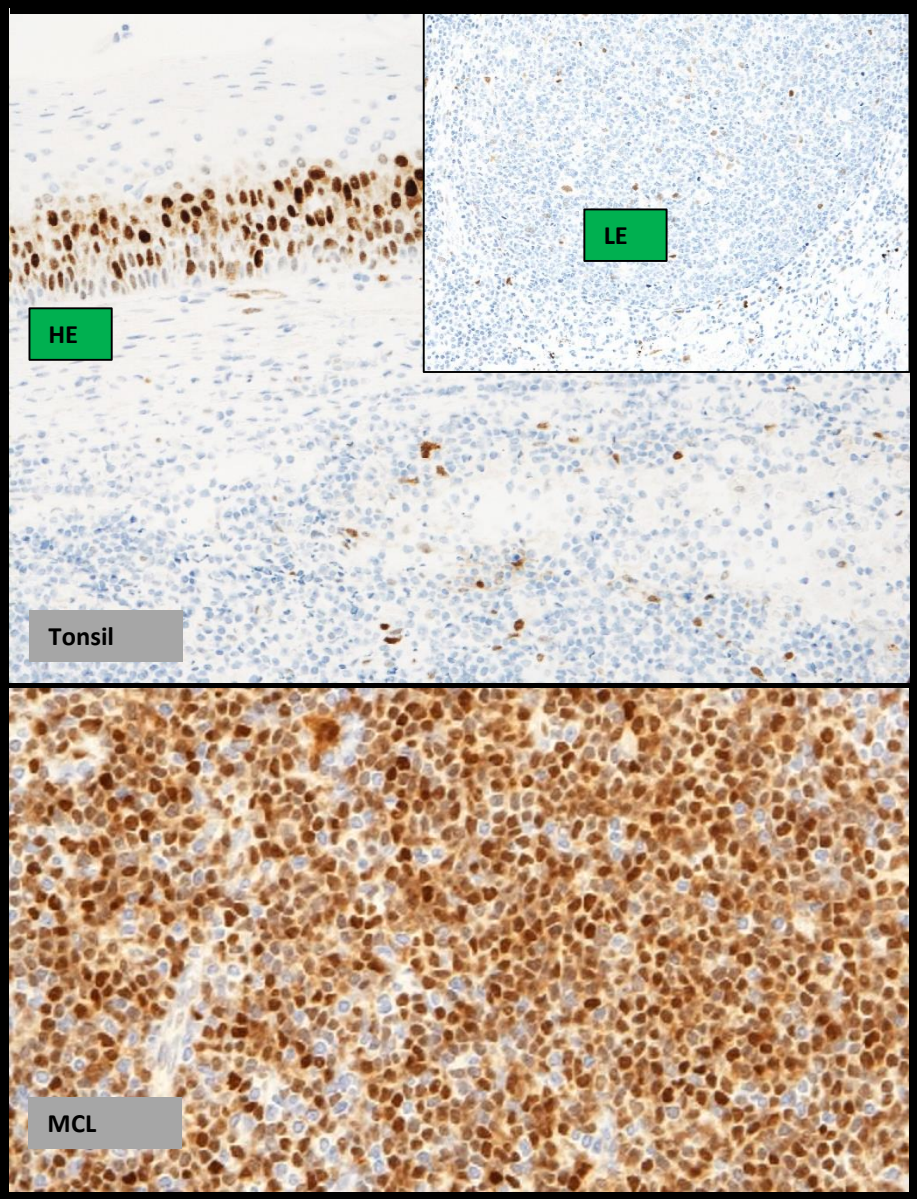
CD23	Retrieval buffers	Titre	Detection	RTU	Detection
mmAb 1B12	HIER High pH or Low pH	1:10-1:50	3-step	Leica (PA0169)	BOND refine
rmAb SP23	HIER High pH or Low pH	1:20-1:100	3-step	Ventana (790-4408)	UltraView + Amp* OptiView
DAK-CD23	HIER mod. Low pH or High pH	1:25-1:100	3-step	Dako (IR/GA781)	Flex/Flex+

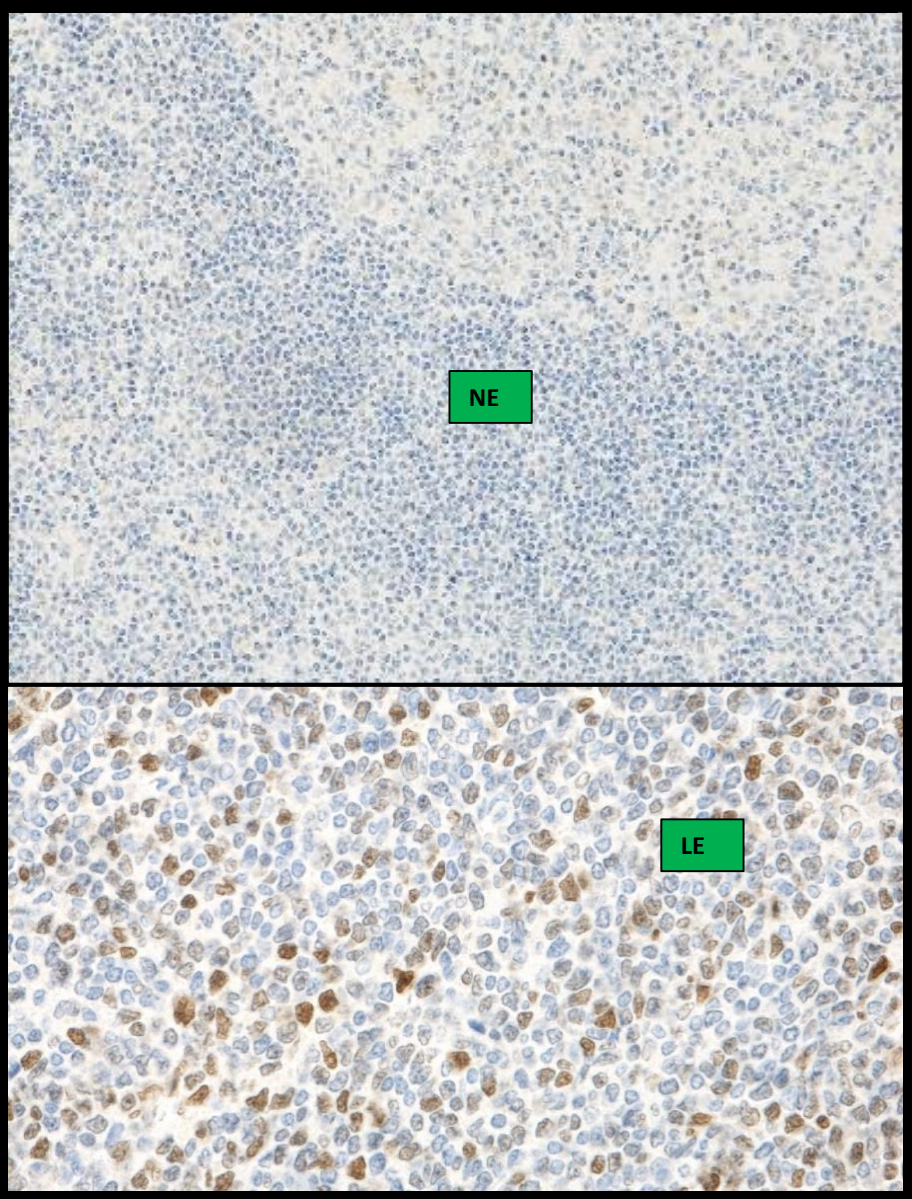
* Optimal results could also be obtained with the detection system UltraView without amplification but at overall lower frequency compared to laboratories using UltraView with amplification

Control material / Tonsil:

An at least weak to moderate, distinct membranous staining of the activated B-cells in the mantle zone of the germinal centres in the tonsils.

CyclinD1 & SOX11





Cyclin D1

Performance history

This was the fifth NordiQC assessment of CyD1. The pass rate was comparable to the previous run and maintained at a high and satisfactory level, as shown in table 2.

Table 2. **Proportion of sufficient results for CyD1 in the five NordiQC runs performed**

	Run 9 2003	Run 17 2006	Run 19 2007	Run 33 2011	Run 47 2016
Participants, n=	57	87	92	179	257
Sufficient results	53%	59%	75%	90%	94%

Increased pass rate & optimal performance

Poor clones

mAb DCS6
mAb P2D11F11
pAbs

Robust rabbit monoclonal Abs

rmAb EP12
rmAb SP4

CycD1

Table 1. **Antibodies and assessment marks for CyD1, run 47**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **P2D11F11**	4	Leica/Novocastra	0	2	2	0	-	-
rmAb clone **EP12**	13 1 1	Dako/Agilent Cell Marque Epitomics	8	6	1	0	93%	98%
	69 6 5	Thermo/Neomarkers Cell Marque Biocare						
rmAb clone **SP4**	4 2 1	Spring Bioscience Zytomed Immunologic	36	45	6	3	90%	92%
	1 1 1	Maixin Nordic Biosite Thermo/Pierce						
Unknown	1	Eptitomics	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **P2D11F11 RTU-CYCLIN D1-GM**	1	Leica/Novocastra	0	1	0	0	-	-
rmAb clone **EP12 IR/IS083**	57	Dako/Agilent	33	23	1	0	98%	100%
rmAb clone **EP12 MAD-000630QD**	3	Master Diagnostica	1	2	0	0	-	-
rmAb **EP12 PME432**	1	Biocare	1	0	0	0	-	-
rmAb **EP12 PA0046**	1	Leica/Novocastra	0	1	0	0	-	-
rmAb clone **EPR2241(IHC)-32 AN474**	1	Biogenex	0	1	0	0	-	-
rmAb clone **SP4 790-4508**	72	Ventana/Roche	54	17	1	0	99%	100%
rmAb clone **SP4 760-4282***	5	Cell Marque/Ventana	5	0	0	0	-	-
rmAb clone **SP4 IR152***	2	Dako	0	2	0	0	-	-
mAb clone **SP4 RM-9104-R7**	2	Thermo/Neomarkers	0	1	1	0	-	-
rmAb clone **SP4 241R-18**	1	Cell Marque	1	0	0	0	-	-
rmAb clone **SP4 RMA-0541**	1	Maixin	1	0	0	0	-	-
Total	257		140	102	12	3	-	
Proportion			54%	40%	5%	1%	94%	

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

Optimal (rmAb EP12 & SP4)

Efficient HIER in alkaline buffer (20 min)

1:20-1:200 (EP12)

1:20-1:150 (SP4)

2 & 3 step detection systems

Insufficient results

Too low concentration of the primary antibody

Less successful primary antibody

Unexplained technical issues

Table 3. **Proportion of optimal results for CyD1 for the most commonly used antibodies as concentrate on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer / Omnis		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
rmAb clone **EP12**	4/5** (80%)	-	3/5 (60%)	-	1/2	-
rmAb clone **SP4**	20/41** (64%)	0/1	11/31 (49%)	-	2/15 (13%)	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)

Bond™ Polymer Refine Detection
Catalog No: DS9800

Intended Use

This detection system is for *in vitro* diagnostic use.

Bond Polymer Refine Detection is a biotin-free, polymeric horseradish peroxidase (HRP)-linker antibody conjugate system for the detection of tissue-bound mouse and rabbit IgG and some mouse IgM primary antibodies. It is intended for staining sections of formalin-fixed, paraffin-embedded tissue on the Bond™ automated system.

The clinical interpretation of any staining or its absence should be complemented by morphological studies and proper controls. They should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

The Bond Polymer Refine Detection Kit must be used with laboratory best practice in the use of tissue controls. For assurance, laboratories should stain each patient sample in conjunction with positive, negative, and other tissue specific controls as needed.

Summary and Explanation

Immunohistochemical techniques can be used to demonstrate the presence of antigens in tissue and cells (see "Using Bond Reagents" in your Bond user documentation).

Bond Polymer Refine Detection utilizes a novel controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates. The detection system avoids the use of streptavidin and biotin, and therefore eliminates non-specific staining as a result of endogenous biotin.

Bond Polymer Refine Detection works as follows:

- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity.
- A user-supplied specific primary antibody is applied.
- Post Primary IgG linker reagent localizes mouse antibodies.
- Poly-HRP IgG reagent localizes rabbit antibodies.
- The substrate chromogen, 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB), visualizes the complex via a brown precipitate.
- Hematoxylin (blue) counterstaining allows the visualization of cell nuclei.

Using Bond Polymer Refine Detection in combination with the Bond automated system reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting and reagent application.

The detection system Bond Refine acts by nature as a 2 step polymer system for detection of rabbit polyclonal or rabbit monoclonal primary antibodies

Only enhances reactions with mouse primary antibodies due to the Post Primary IgG linker (Rabbit antibody)

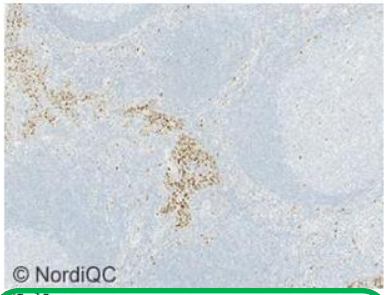


Fig. 1a
Optimal staining for Cyclin D1 of the tonsil, tissue core no. 1, using the rmAb clone SP4-R as Ready-To-Use format (Ventana prod. no. 790-4508) using HIER in CC1 for 64 min. and UltraView as detection system.
Even at low power field squamous epithelial cells, dispersed endothelial cells and germinal centre macrophages can be identified.
Also compare with Figs. 2a - 4a, same protocol.

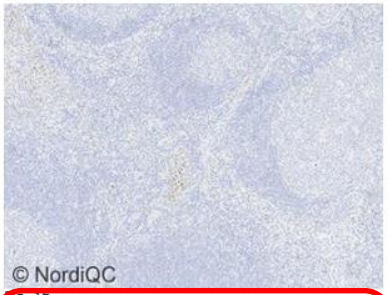


Fig. 1b
Insufficient staining for Cyclin D1 of the tonsil, tissue no. 1, using the rmAb clone SP4 by a laboratory developed assay giving a too low sensitivity (too low. conc. of the primary Ab) - same field as in Fig. 1a.
The proportion of positive cells and the intensity of the staining reaction are significantly reduced compared to the result obtained in Fig. 1a.
Also compare with Figs. 2b - 4b, same protocol.

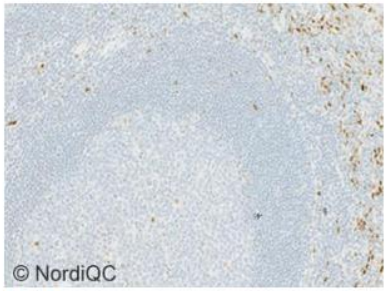


Fig. 2a
Optimal staining for Cyclin D1 of the tonsil, tissue core no. 1, using same protocol as in Fig. 1a. High power field x200.
Virtually all squamous epithelial cells, dispersed endothelial cells and germinal centre macrophages show a moderate to strong nuclear staining reaction. The vast majority of lymphocytes are negative and no background staining is seen.

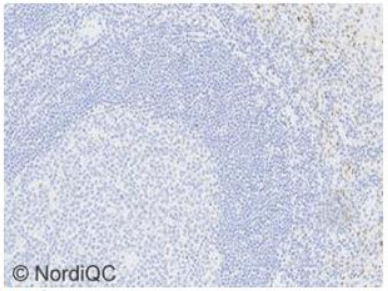


Fig. 2b
Insufficient staining for Cyclin D1 of the tonsil, tissue core no. 1, using same protocol as in Fig. 1b - same field as in Fig. 2a.
Only scattered squamous epithelial cells show a weak and equivocal staining reaction, while endothelial cells and germinal centre macrophages are negative.
Also compare with Fig. 3b, same protocol.

Fig. 3a
Optimal staining for Cyclin D1 of the mantle cell lymphoma, tissue core no. 4, using same protocol as in Figs. 1a & 2a.
Virtually all the neoplastic cells show a distinct, moderate to strong nuclear staining reaction.

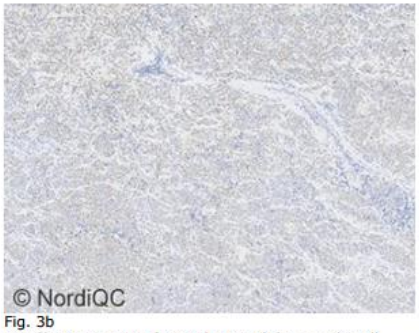


Fig. 3b
Insufficient staining for Cyclin D1 of the mantle cell lymphoma, tissue core no. 4, using same protocol as in Figs. 1b & 2b – same field as in Fig. 3a.
The proportion of positive cells and the intensity of the staining reaction are significantly reduced compared to the result expected and obtained in Fig. 3a.

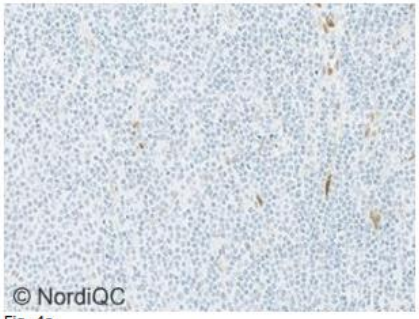


Fig. 4a
Optimal staining for Cyclin D1 of the B-CLL using same protocol as in Figs. 1a - 3a.
The neoplastic cells are negative, while scattered endothelial cells show a moderate nuclear staining reaction serving as internal positive tissue control.

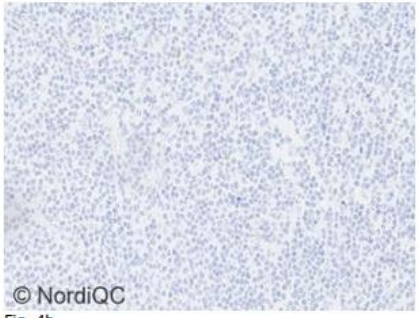


Fig. 4b
Staining for Cyclin D1 of the B-CLL using same insufficient protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
No staining is seen.

CycD1 clone SP4

Too low concentration of the primary Ab

Lymphoma panel: CyD1

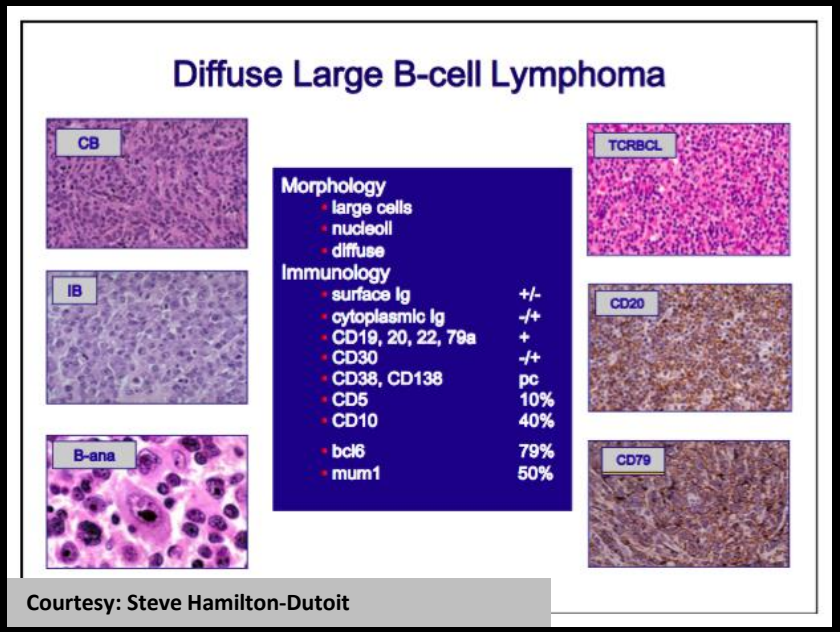
Optimal protocol settings (NQC)

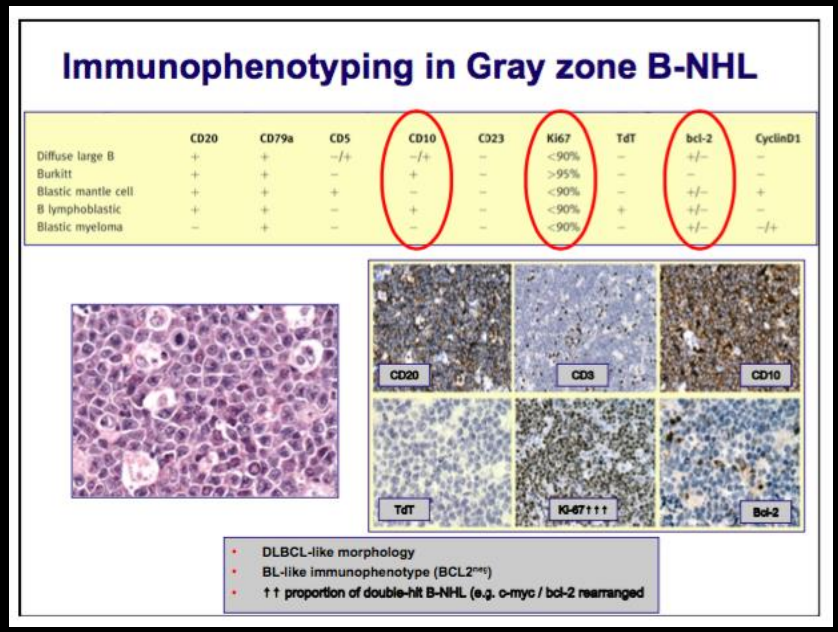
CyD1	Retrieval buffers	Titre	Detection	RTU	Detection
rmAb EP12	HIER High pH	1:20-1:200	2 & 3-step	Dako (IS/IR083)	Flex/Flex+
				Biocare (PME432)	MACH4
rmAb SP4	HIER High pH	1:20-1:150	2 & 3-step	Ventana (790-4508)	UltraView +/- Amp OptiView

Control material / Tonsil:

A moderate to strong, distinct nuclear staining reaction of virtually all suprabasal squamous epithelial cells, scattered lymphocytes and endothelial cells

An at least weak, distinct nuclear staining reaction of germinal centre macrophages





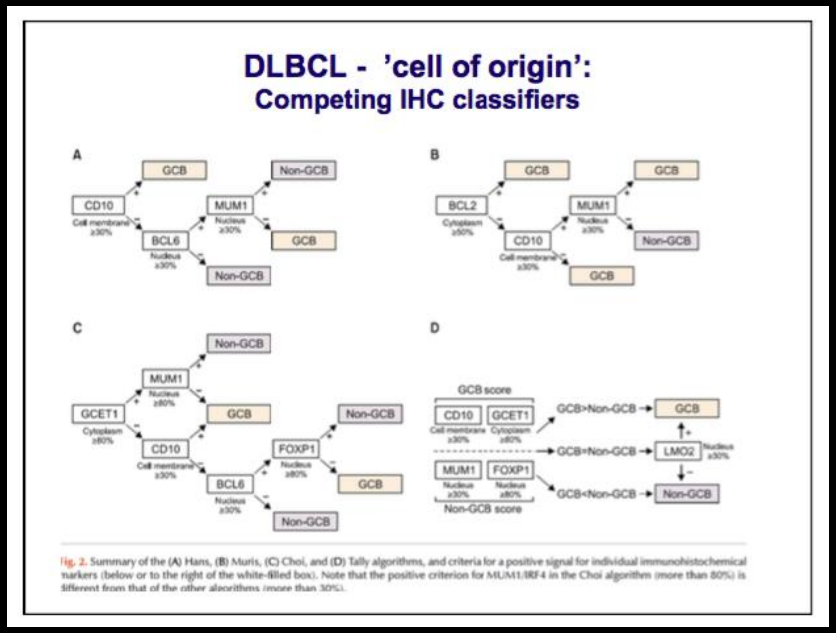


Fig. 2. Summary of the (A) Hans, (B) Muris, (C) Choi, and (D) Tally algorithms, and criteria for a positive signal for individual immunohistochemical markers (below or to the right of the white-filled box). Note that the positive criterion for MUM1/IRF4 in the Choi algorithm (more than 80%) is different from that of the other algorithms (more than 30%).

Diffuse Large B-cell Lymphoma (DLBCL)

- Differential diagnosis / Gray zone B-NHL

- IHC classification (subtypes/GC versus non-GC) and prognosis

BCL6
MUM1
CD138
Ki67

CMYC
FOXP1
GCET1

B-Cell lymphoma markers (3) - Diffuse Large B-Cell Lymphoma

Marker (localization)	Control	High exp. (HE)	Low exp. (LE)	Non exp. (NE)
BCL6 (nuclear) LN22, PG-B6p, SP18	Tonsil	Germinal centre B-cells	Squamous epithelial cells	The vast majority of cells in the mantle zones and interfollicular areas
MUM1 (nuclear). MUM1p, EAU32, EP190	Tonsil/Colon	Late stage germinal centre B-cells (tonsil) Plasma cells (tonsil & colon)	"Mantle zone B-lymphocytes (tonsil) "	Epithelia cells and smooth muscle cells (lamina muscularis propria) in the colon.
CD138 (membr.) B-A38, B-B4, MI15	Tonsil	Plasma cells and squamous epithelial cells	Activated germinal centre B-cells	Mantle zone B-cells and T-cells
Ki67 (nuclear) MIB-1, BS4, GM001, K2, UMAB107, 30-9, SP6	Tonsil/lLiver	All germinal centre B-cells (dark zone) in the tonsil	Most germinal centre B-cells (light zone) in the tonsil	99% of "normal" hepatocytes should be negative
FOXP1 (nuclear) EP137	Tonsil/Liver	Virtually all mantle zone B-cells T-cells are positive	App. 50% of germinal centre B-cells in the tonsil (moderate intensity) T-cells are positive	The vast majority of hepatocytes are negative
GCET1 (cytopl) RAM341	Tonsil	Intra germinal centre B-cells (centroblast) – moderate to strong intensity	None	All other cells including T-cells
CMYC (nuclear) EP121, Y69	Tonsil/appendix	Activated intragerminal centre B-lymphocytes and scattered lymphocytes in interfollicular zones	App. 10-20 % of the mantle zone B-cells. Suprabasal squamous epithelial cells in the tonsil often displays moderate intensity.	Luminal epithelia cells of the appendix. The basal crypt epithelia cells displays moderate intensity.
CD10, see B-cell lymphoma markers (2) & TdT, see blast`s/bonus material				

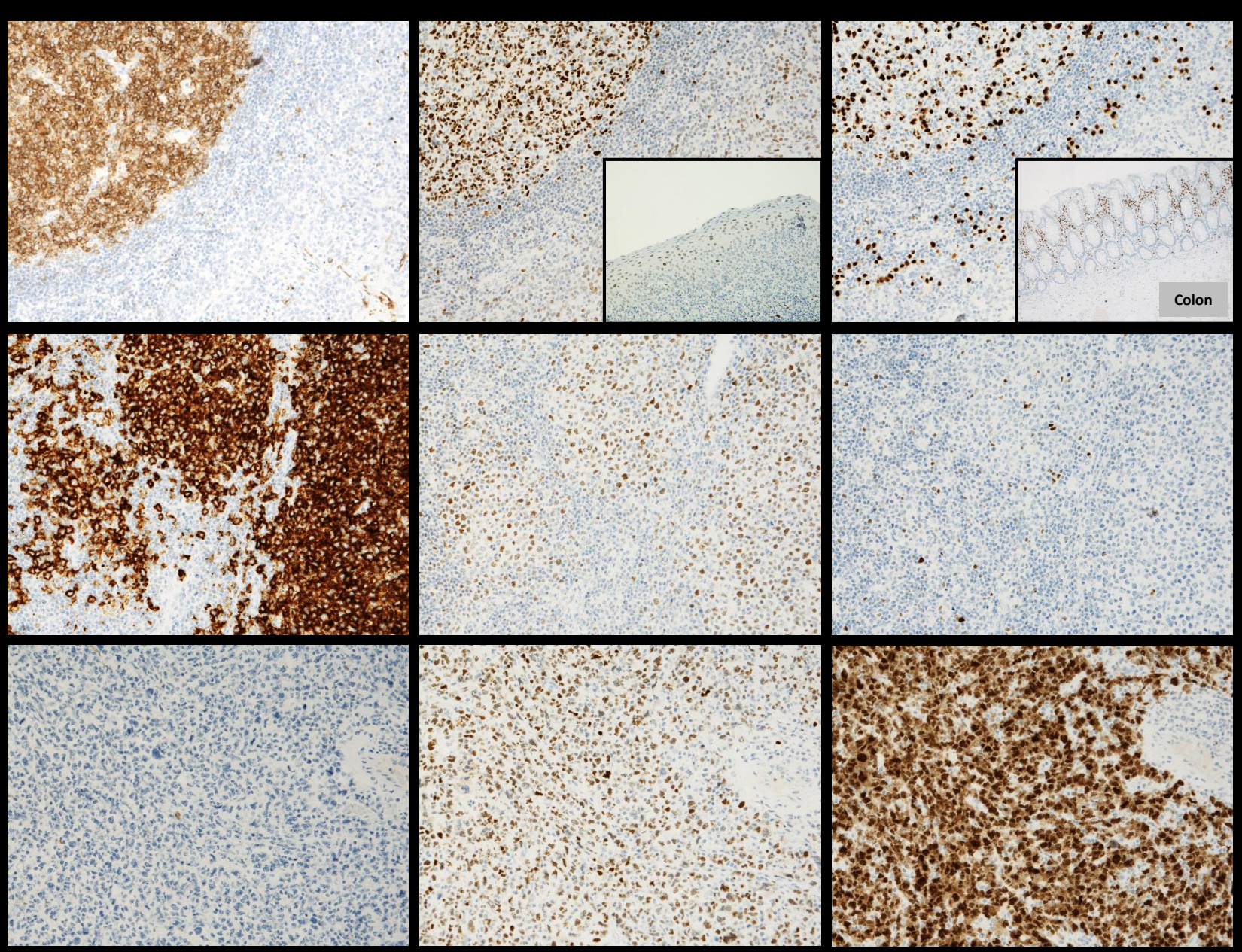
Clones (mAbs, rmAbs & pAbs) providing optimal results (NordiQC assessments)

HE: Strong staining intensity/reactions should be expected

LE: An at least weak to moderate staining intensity/reactions should be expected

NE: No staining/reactions should be expected

DLBCL: HANS classification



BCL6

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.

Optimal results
1) HIER in alkaline buffers (pH 8-9)
2) 3-step pol./mul. Detec. systems

<u>Insufficient results</u>

Too short HIER time/use of low pH buffer

Too low concentration of the primary antibody

Less successful performance of the RTU system 760-4241 (GI191E/A8, Ventana) – FP/PSN

Unexplained technical issues

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.

BCL6

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).

RTU system 760-4241
Applied by 32% of all LABS

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%

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)

If protocols were based on a titer of the concentrated format in the "optimal dilution range", 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.

BCL6

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.

RTU systems IS/IR/GA625 (mAb PG-B6p, Dako): 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.

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).

RTU system 760-4241 (mAb GI191E/A8, Ventana): The pass rate was only 58% (51 of 88) of which 25% (22 of 88) of the protocols were assessed as optimal. Insufficient staining reaction - poor signal-to-noise ratio or false positive staining reaction.

Of 22 protocols giving optimal results, 21 (95%) were based on OptiView with or without amplification.

BCL6

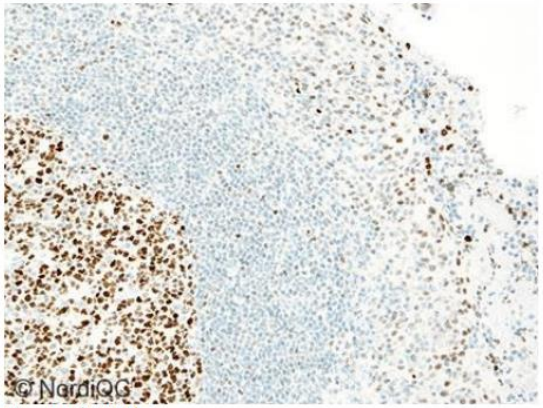


Fig. 1a (X200)
Optimal Bcl-6 staining of the tonsil using the mAb clone GI191E/A8 optimally calibrated, HIER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview, Benchmark Ultra, Ventana). Virtually all germinal centre B-cells show a moderate to strong nuclear staining reaction and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

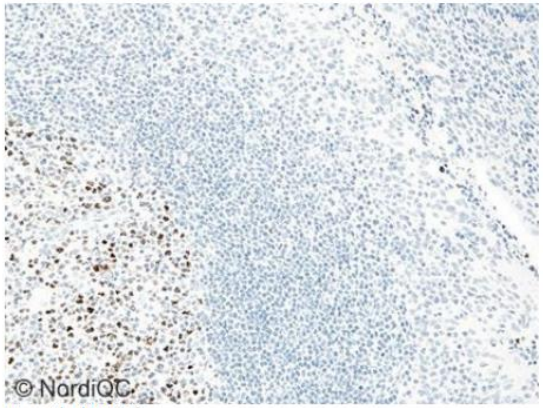


Fig. 1b (X200)
Insufficient Bcl-6 staining of the tonsil using the mAb clone GI191E/A8 HIER in CC1 (32 min.) and a 3-step multimer based detection system (Optiview + amplification, Benchmark Ultra, Ventana). The protocol provided too low sensitivity due to too low titre of the primary Ab (1:800) - same 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.

Too diluted primary Ab

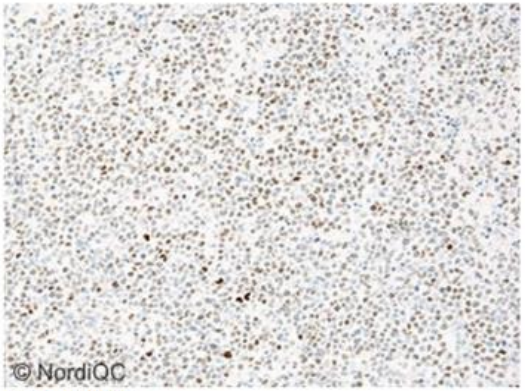


Fig.4a (X200)
Optimal Bcl-6 staining of the DLBCL using same protocol as in Figs. 1a - 3a. The vast majority of neoplastic cells show an at least moderate but distinct nuclear staining reaction. No background staining is observed.

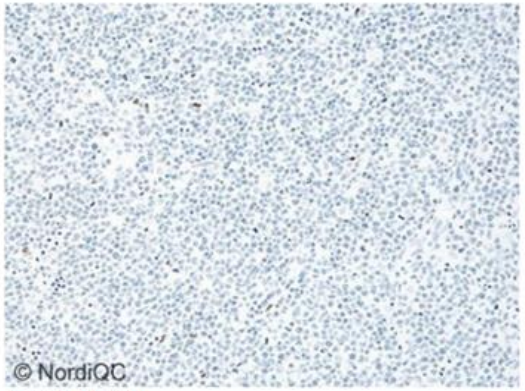


Fig. 4b (X200)
Insufficient Bcl-6 staining result of the DLBCL using same protocol as in Figs. 1b - 3b. The neoplastic cells are false negative. Only few scattered normal B-cells are demonstrated - same field as in Fig. 4a.

BCL6

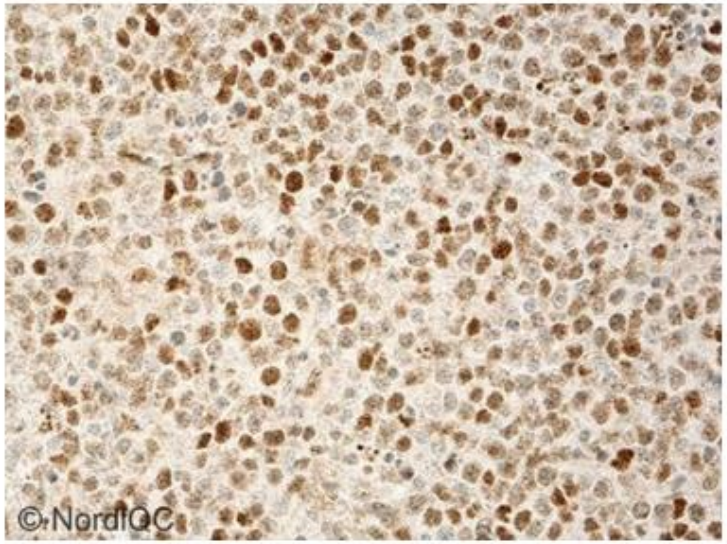


Fig.5a (X400)
Insufficient Bcl-6 staining result of the DLBCL using the RTU product 760-4241 (Ventana) based on the mAb clone GI191E/A8, HIER in CC1 (64 min.) and a 3-step multimer based detection system (UltraView with amplification, Benchmark Ultra, Ventana).
Although the protocol provided the expected 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.

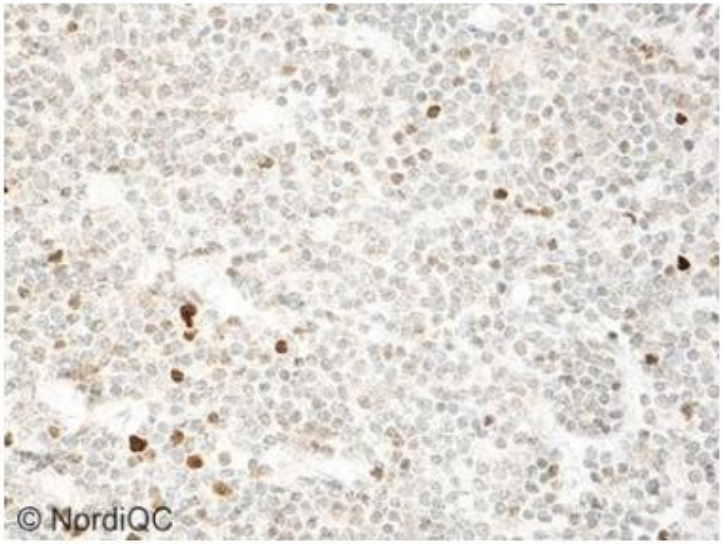


Fig. 5b (X400)
Insufficient Bcl-6 staining of the MCL using same protocol as in Fig. 5a. A significant proportion of the neoplastic cells display a weak to moderate, and distinct nuclear staining reaction (false positive) - compare with optimal result in Fig. 2a.

FP

Lymphoma panel: BCL6

Optimal protocol settings (NQC)

BCL6	Retrieval buffers	Titre	Detection	RTU	Detection
mmAb GI191E/A8	HIER High pH	1:50-1:200	3-step	Ventana 760-4241	UltraView +/-Amp.* OptiView +/- Amp.
mmAb LN22	HIER High pH	1:25-200	3-step	Leica PA0204	BOND Refine
mmAb PG-B6p	HIER High pH	1:10-1:50	3-step	Dako IR/IS/GA 625	Flex+

* Optimal results could also be obtained with the detection system UltraView with or without amplification but at overall lower frequency compared to laboratories using OptiView (only 5% (2/40) of the protocols were assessed as optimal result).

Control material / Tonsil:

An at least weak to moderate distinct nuclear staining reaction of the majority of the squamous epithelial cells in the tonsil.

Strong nuclear staining of germinal centre B-cells

CMYC

Table 1. **Antibodies and assessment marks for C-MYC, run 56**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **9E10**	2	Diagnostic BioSystems	0	0	0	2	-	-
rmAb clone **EP121**	26 15 12 1 1	Cell Marque Epitomics Biocare Medical Abcam Sakura Finetek	17	20	13	5	67%	72%
rmAb clone **Y69**	39 1	Abcam Eurobio	12	14	5	9	65%	79%
Ready-To-Use antibodies								
rmAb clone **EP121 PME 415**	2	Biocare Medical	1	0	0	1	-	-
rmAb clone **EP121 PME 415** [3]	2	Biocare Medical	0	1	0	1	-	-
rmAb clone **EP121 395R-18** [4]	5	Cell Marque	3	1	1	0	-	-
rmAb clone **EP121 RMA-0803**	1	Maixin	0	1	0	0	-	-
rmAb clone **Y69 MAD-000487QD-7/N**	2	Master Diagnostica	0	1	1	0	-	-
rmAb clone **Y69 790-4628**	61	Ventana/Roche	8	19	16	18	44%	60%
rmAb clone **Y69 790-4628** [5]	3	Ventana/Roche	0	1	1	1	-	-
Total	173		41	58	37	37	-	
Proportion			24%	33%	21,5%	21,5%	57%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) RTU system developed for the BioCare IntelliPath system, but used on other systems.
4) RTU system developed for a manual Cell Marque protocol, but used on various automated systems.
5) RTU system developed for the Ventana BenchMark system, but used on other systems.

Optimal results
1) HIER in alkaline buffers (pH 8-9)
2) 3-step pol./mul. systems

<u>Insufficient results</u>

Too low concentration of the primary antibody

Poor signal-to-noise ratio of assays based on the UltraView amplification kit

Use of less sensitive detection systems

Too short efficient Heat Induced Epitope Retrieval (HIER) time

CMYC

Table 4 summarizes the proportion of sufficient and optimal marks for the most commonly used RTU systems. The performance was evaluated both as "true" plug-and-play systems performed strictly according 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 C-MYC for the most commonly used RTU IHC systems**

RTU-systems	Recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
VMS Ultra/XT/GX rmAb Y69 **790-4628**	41% (7/17)	6% (1/17)	45% (20/44)	16% (7/44)

* 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.

84% of all RTU systems were based on rmAb clone Y69 from Ventana (790-4628)

Laboratory modified protocol settings - Two different sub-groups

Sub-group A: Applied reduced sensitivity protocol settings compared to the official recommendations from the vendor, typically using too short HIER time and the less sensitive detection system UltraView: Suff 11% (none optimal)

Sub-group B: Applied enhanced sensitive protocol settings compared to the official recommendations from the vendor (CC1 64`, 16 min primary Ab, Ultra/Optiview), typically prolonging incubation time in primary Ab and using and/or the use of OptiView with tyramide amplification: Suff 69% (27% optimal)

CMYC

Optimal

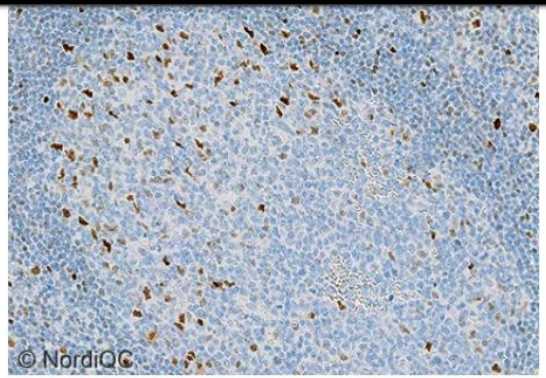


Fig. 1a
Optimal C-MYC staining reaction of the tonsil using the rmAb clone EP121, optimally calibrated, HIER in CC1 (Ventana) and a 3-step multimer based detection system (OptiView/Ventana). A moderate to strong, distinct nuclear staining reaction is displayed in approximately 10% of lymphocytes scattered both in the interfollicular zones and in the reactive germinal centers of the tonsil. A weak, distinct nuclear staining reaction is displayed in 10-20% of mantle zone B-cells. No background staining is seen. Same protocol used in Figs. 2a - 4a.

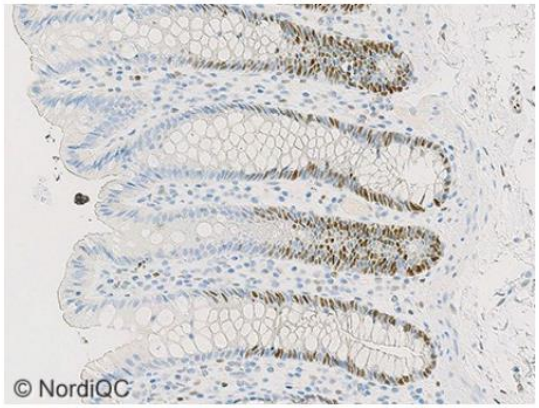


Fig. 2a
Optimal staining of C-MYC in the colon using same protocol as in Fig. 1a. Scattered epithelial cells in the basal crypts of the colon display a weak to moderate, distinct nuclear staining reaction. No background staining is seen.

Insufficient

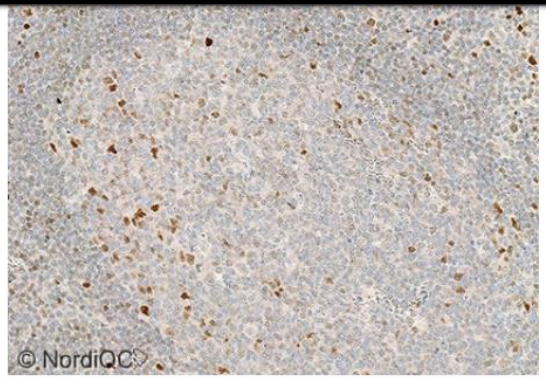


Fig. 1b
Insufficient C-MYC staining reaction of the tonsil using the rmAb clone Y69 based RTU system 790-4628 (Ventana) according to the recommended protocol settings (UltraView amplification based) – same field as in Fig. 1a. Excessive background staining – most likely related to the use of the UltraView amplification kit - combined with weak specific nuclear staining reaction results in poor signal-to-noise. Compare with Fig. 1a. Same protocol used in Figs. 2b - 4b.

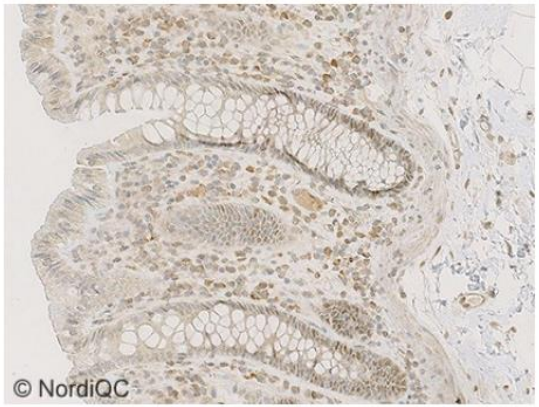


Fig. 2b
Insufficient staining of C-MYC in the colon using same protocol as in Fig. 1b – same field as in Fig. 2a. The staining intensity in epithelial cells in basal crypts cells is significantly reduced while general background staining is notable. Compare with Fig. 2a.

Problem:

Poor Signal to Noise ratio

Weak

Related to the UltraView amp. Kit (batch problems)

CMYC

Optimal

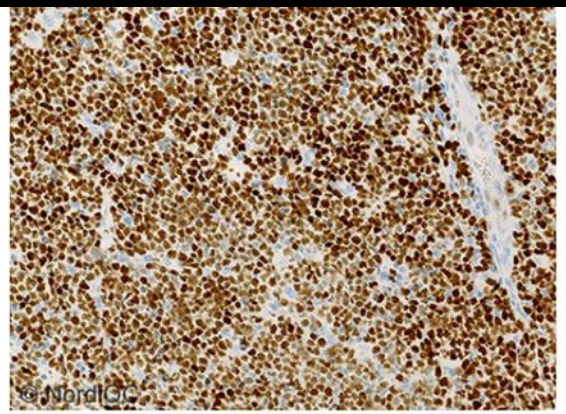


Fig. 3a
Optimal C-MYC staining of the Burkitt lymphoma using same protocol as in Figs. 1a and 2a. Virtually all neoplastic cells display a strong, distinct nuclear staining reaction. No background staining is seen.

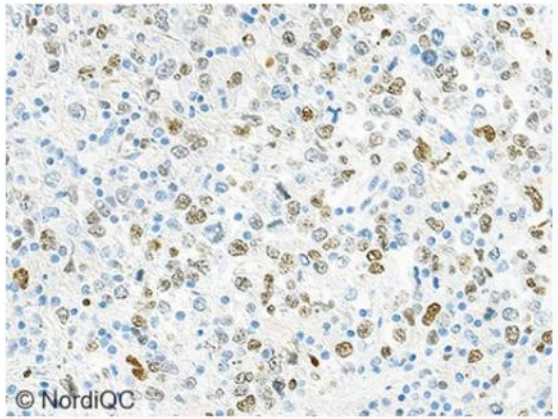


Fig. 4a
Optimal C-MYC staining of the DLBCL, tissue core no. 4, using same protocol as in Figs. 1a – 3a. Approximately 40% of the neoplastic cells display a moderate and distinct nuclear staining reaction. No background staining is seen.

Insufficient

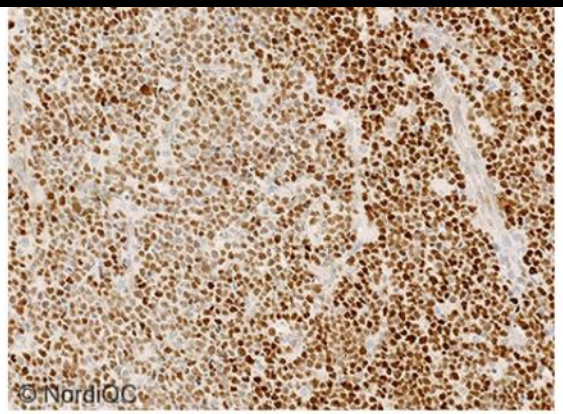


Fig. 3b
Insufficient C-MYC staining of the Burkitt lymphoma using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. Virtually all neoplastic cells display a distinct nuclear staining reaction, but staining intensity is reduced compared to Fig. 3a. In this solid tumour tissue background staining is not evident but compare to Figs. 1b, 2b and 4b.

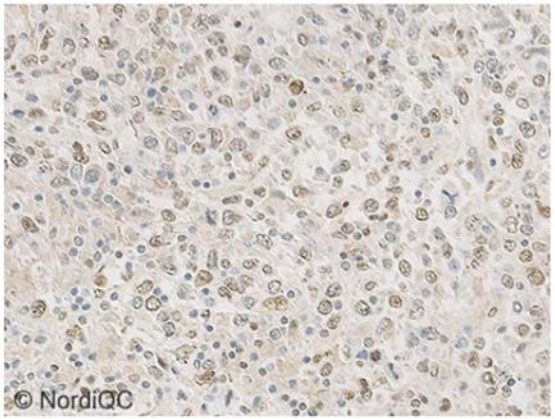


Fig. 4b
Insufficient C-MYC staining of the DLBCL, tissue core no. 4, using same protocol as in Figs. 1b – 3b - same field as in Fig. 4a. Weak specific staining reaction combined with background staining makes diagnostic interpretation complicated. Compare with Fig. 4a.

Problem:

Poor Signal to Noise ratio

Weak

Related to the UltraView amp. Kit (batch problems)

Lymphoma panel: CMYC

Optimal protocol settings (NQC)

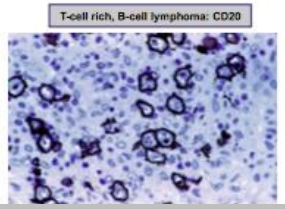
CMYC	Retrieval buffers	Titre	Detection	RTU	Detection
rmAb Y69	HIER High pH	1:40-1:100	3-step	Ventana 790-4628	UltraView +Amp.* OptiView +/- Amp.
rmAb EP121	HIER High pH	1:10-100	3-step		
* Batch problems with the UltraView amplification KIT					

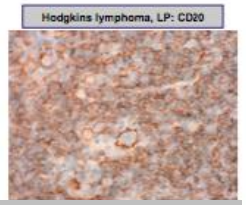
Tonsil and colon are recommended as positive and negative tissue controls for C-MYC, respectively. In tonsil, protocols must be calibrated to provide a moderate to strong, distinct nuclear staining reaction in approximately 10% of lymphocytes scattered both in the interfollicular zones and in the reactive germinal centers of the tonsil. A weak, distinct nuclear staining reaction of mantle zone B-cells (app. 10-20%) should also be seen. In colon, a weak to moderate nuclear staining reaction should be displayed in scattered epithelial cells in the basal crypts, whereas the luminal epithelial cells and smooth muscle cells of the tunica muscularis should display no nuclear staining reaction. As a supplement to tonsil and colon, especially in the technical calibration phase, it is recommended to verify the protocol on Burkitt lymphomas and DLBCLs with C-MYC rearrangements.

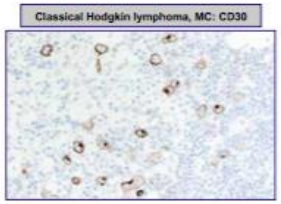
Hodgkins lymphoma: differential diagnosis

	CD20	CD79a	T-cell antigen	CD4 CD8	CD30	CD15	EMA
Nodular lymphocyte predominant HL	+	+	–	–	–/+	–	+
Classical HL	–/+	–/+	–	–	+	+	+
T-cell rich large B-cell lymphoma	+	+	–	–	–	–	–
Anaplastic large cell lymphoma	–	–	+/–	CD8>CD4> CD4&8 -ve	+	–	+

Key
+/– The lymphoma cells are commonly but not always positive
–/+ The lymphoma cells are usually but not always negative







Courtesy: Steve Hamilton-Dutoit

HL *vs* ALCL: Immunophenotype

	HL	ALK - pos T/null - ALC	ALK - neg T/null - ALC
ALK	-	+	-
EBV	> 40 %	-	-
CD30	+	+	+
CD15	ca. 90 %	< 5 %	- / +
EMA	-	ca. 50 %	ca. 50 %
PAX5	> 80 %	-	-
CD20	ca. 25 %	-	-
CD3	ca. 2 %	+ / -	+ / -
CD45	-	ca. 50 %	ca. 50 %
CD43	-	most +	most +
Granzyme/ perforin	10 – 20 %	ca. 90 %	ca. 70 %
TCR genes	G	R	R
Ig genes	R (single cell)	G	G

Marker / Neoplasm	Classical Hodgkin Lymphoma Hodgkin/Reed-Sternberg cells	Nodular lymphocytic predominantly Hodgkin lymphoma L & H (popcorn cells)
CD30	+	-/+
CD15	+/-	-
PAX5	+ (weak)	+ (strong)
BCL6	-/+	+
OCT2/BOB.1	- (both or one)	+ (both)
CD57	- (no rosettes)	+ (rosettes surrounding L & H)
EBV-EBER	+/-	-
EBV-LMP1	-/+	-

+ > 90% positive; +/- 50-90% positive; -/+ 10-50% positive; - < 10% positive.

Hodgkin Lymphoma

- Differential diagnosis
- IHC classification (subtypes) / classical HL vs N-LPHL

CD30
CD15

OCT2
BOB.1
CD57

EBV-EBER/EBV-LMP1/EBV-EBNA-2
ALK

Hodgkin lymphoma markers

Marker (localization)	Control	High exp. (HE)	Low exp. (LE)	Non exp. (NE)
CD30 (membr. + Golgi) Ber-H2, CON6D/5, 1G12, JCM182, rmAb EP154	Tonsil	None	Interfollicular activated B- and T-cells and perifollicular germinal centre B-cells (moderate intensity)	All other cells
CD15 (membr. + cytopl.) Carb-3, MMA and HI98	Tonsil/Kidney	Epithelial cells of the renal proximal tubules (predominantly membr.) Neutrophils	Follicular dendritic cells in the germinal centres (Tonsil)	All other cells
BOB.1 (nuclear + cytopl.) SP92	Tonsil	Germinal centre B-cells & plasma cells	Mantle zone B-cells	T-cells
OCT2 (nuclear) EP284	Tonsil	Germinal centre B-cells & plasma cells	Mantle zone B-cells ("moderate intensity")	"T-cells"
CD57 (membr.) TB01	Tonsil/Appendix	Intragerminal centre activated T-cells and NK-cells in the T-zone (Tonsil)	Schwann cells of peripheral nerves (ganglionic neurons) in the appendix	Epithelia cells of the Appendix. Neuroendocrine cells displays a distinct staining reaction
EBV-EBER/EBV-LMP1/EBV-EBNA2 **ALK (See markers for the Lung panel / Ole Nielsen)**				

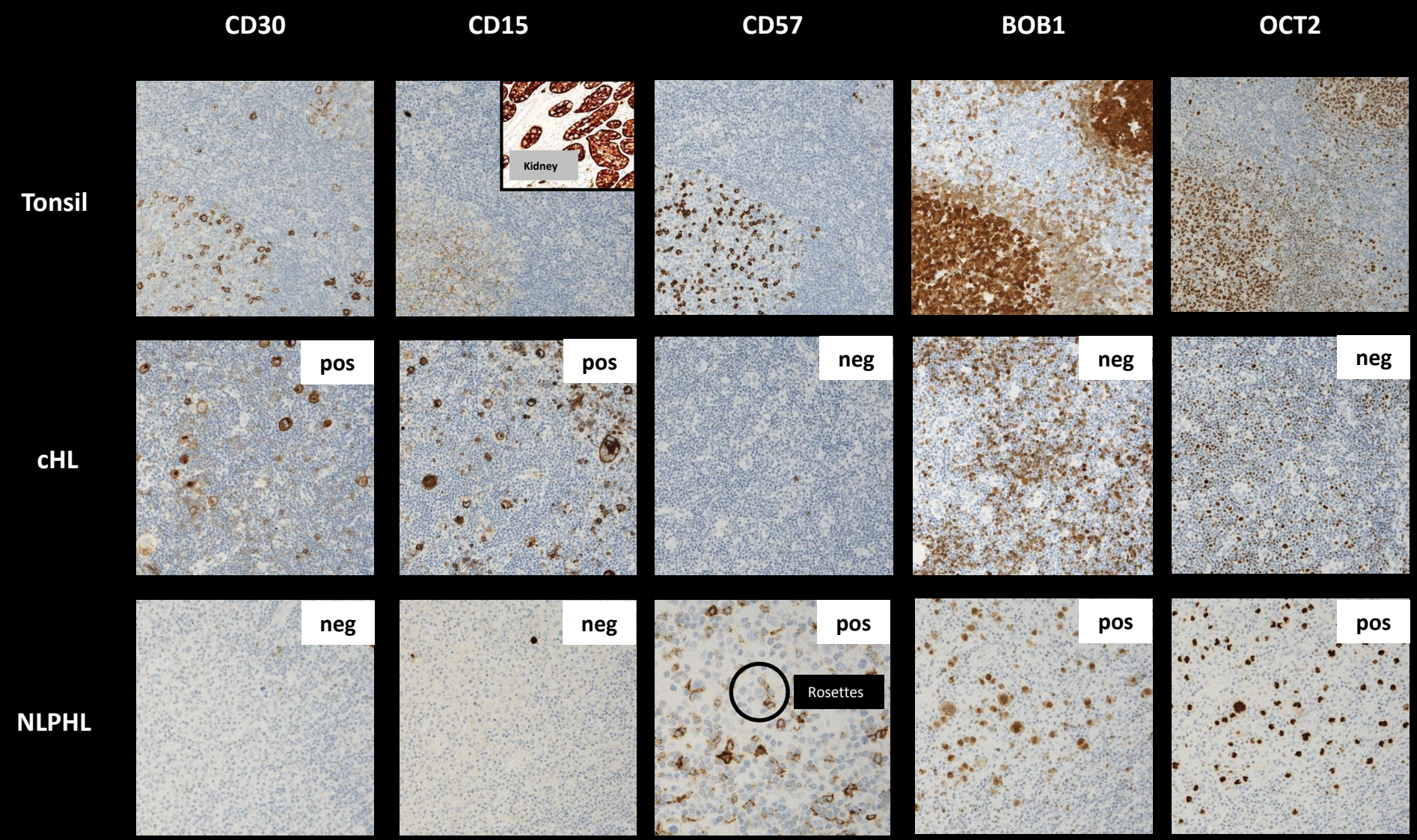
Clones (mAbs, rmAbs & pAbs) providing optimal results (NordiQC assessments)

HE: Strong staining intensity/reactions should be expected

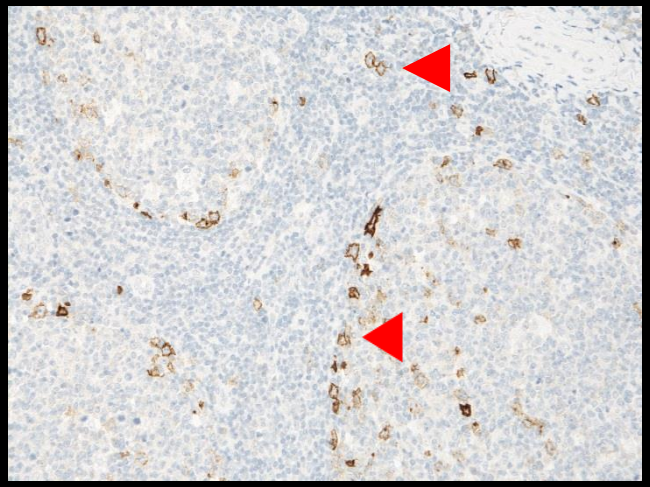
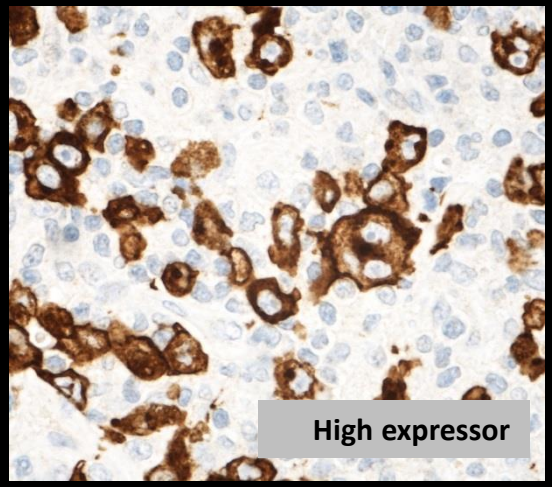
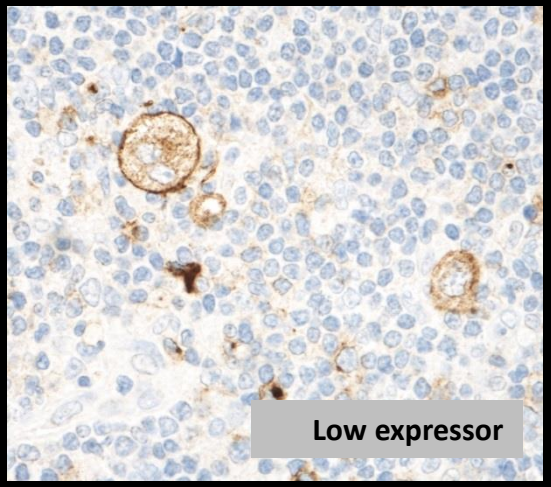
LE: An at least weak to moderate staining intensity/reactions should be expected

NE: No staining/reactions should be expected

Hodgkin lymphoma markers



CD30

Tonsil	Hodgkin Lymphomas, Nos	
	 High expressor	 Low expressor

An at least weak to moderate and distinct membranous staining reaction of interfollicular activated B- and T-cells and perifollicular germinal centre B-cells in the tonsil.

<u>In addition:</u>

Calibrate the assay using classical Hodgkin Lymphomas with “known” weak expression for CD30 (membranous or Golgi reaction) of the neoplastic cells.

CD30

Performance history
This was the fifth NordiQC assessment of CD30. The overall pass rate increased compared to run 43, 2015 (see Table 2).

Table 2. **Proportion of sufficient results for CD30 in the five NordiQC runs performed**

	Run 11 2004	Run 25 2009	Run 31 2011	Run 43 2015	Run 51 2017
Participants, n=	74	126	172	252	282
Sufficient results	92%	78%	77%	71%	83%

Table 1. **Antibodies and assessment marks for CD30, run 51**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **Ber-H2**	94 10 2 2 2 2 1	Agilent/Dako Cell Marque Thermo S./Neomarkers Diagnostic Biosystems Immunologic Zytomed Systems Nordic Biosite	53	41	13	6	83%	84%
mAb clone **JCM182**	10	Leica/Novocastra	6	2	1	1	80%	100%
mAb clone **1G12**	6	Leica/Novocastra	0	4	1	1	67%	-
mAb clone **CON6D/5**	5	Biocare Medical	4	0	1	0	80%	100%
mAb clone **HRS4**	1	Thermo Scientific	0	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **Ber-H2 IS/IR602**	30	Agilent/Dako	18	11	1	0	97%	96%
mAb clone **Ber-H2 IS/IR602**[3]	21	Agilent/Dako	15	4	1	1	90%	-
mAb clone **Ber-H2 790-4858**	75	Roche/Ventana	34	27	8	6	81%	87%
mAb **Ber-H2 MAD-002045QD**	2	Master Diagnostica	2	0	0	0	-	-
mAb **Ber-H2 130M-XX**	2	Cell Marque	0	0	0	2	-	-
mAb clone **Ber-H2 MS-361-R7**	1	Thermo S. /Neomarkers	1	0	0	0	-	-
mAb clone **Ber-H2 MAB-0023**	1	Maxin	0	1	0	0	-	-
mAb clone **JCM182 PA0790**	10	Leica/Novocastra	7	2	1	0	90%	90%
mAb clone **1G12 PA0153**	3	Leica/Novocastra	0	1	2	0	-	-
mAb clone **HRS4 AM351-5/10**	1	BioGenex	0	1	0	0	-	-
mAb clone **unknown 8265-C010**	1	Sakura Finetek USA	0	0	1	0	-	-
Total	282		140	94	31	17	-	
Proportion			50%	33%	11%	6%	83%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) RTU system developed for the Agilent/Dako semi-automated systems (Autostainer) but used by laboratories on the Omnis (Agilent/Dako), Ventana Benchmark XT/Ultra or manually.

Robust primary Abs:

mAb clone BER-H2
mAb clone JCM182
mAb clone CON6D/5

Optimal protocol settings

HIER in alkaline buffer
HIER in mod. Low pH buffers (TRS or Diva)

mAb clone BER-H2 (conc, dil. 1:20-1:100):

6/8 opt. (75%)~ Mod. Low pH buffers

47/94opt. (50%)~ Alkaline pH buffers

Detection System: 3-step mul./pol.

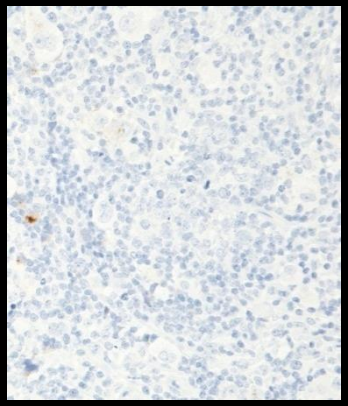
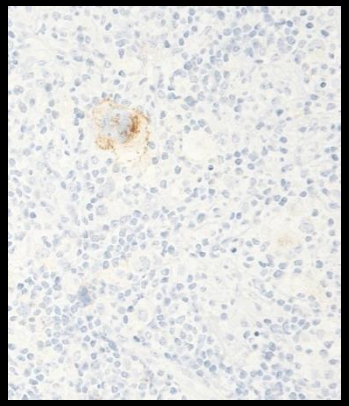
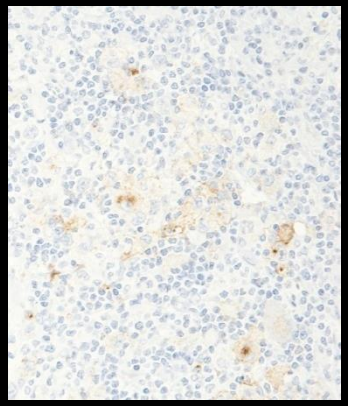
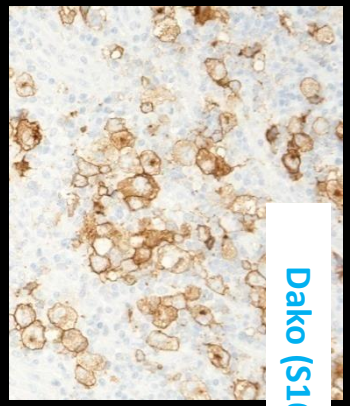
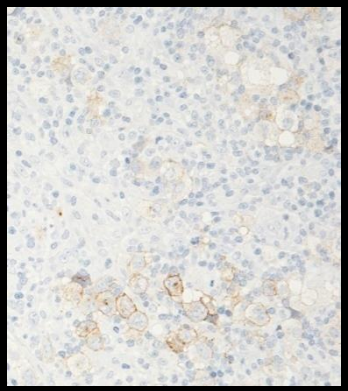
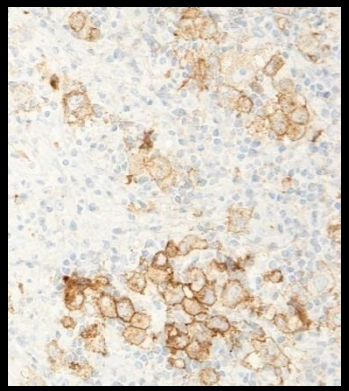
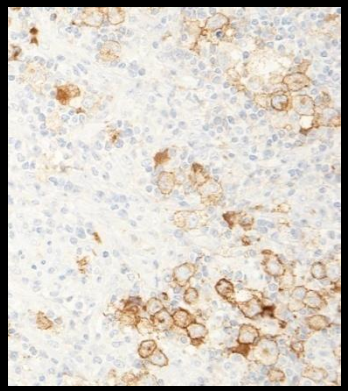
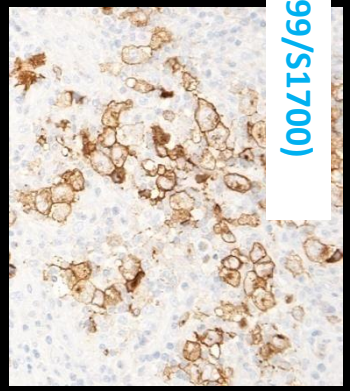
CD30 – Detection systems

mAb BER-H2 (LD-assay):

The overall pass rate for participants using a 3-step polymer/multimer based detection system (e.g. Bond Refine (Leica), Envision Flex+ (Dako) and OptiView (Ventana)) was 87% (78 of 88) of which 53% (47 of 88) were assessed as optimal.

In comparison and for laboratories using a 2-step polymer/multimer based detection system (e.g. Envision Flex (Dako) and UltraView (Ventana)), the overall pass rate was only 59% (13 of 22) of which 18% (4 of 22) were assessed as optimal.

Antibody - Antigen retrieval - Platform ?

	Ci pH 6 MWO / 20 min	EDTA pH 8 MWO / 20 min	TE pH 9 MWO / 20 min	TRS pH 6.1 MWO / 20 min
CD30 Clone ConD6/B5				
CD30 Clone Ber-H2				

Dako (S1699/S1700)

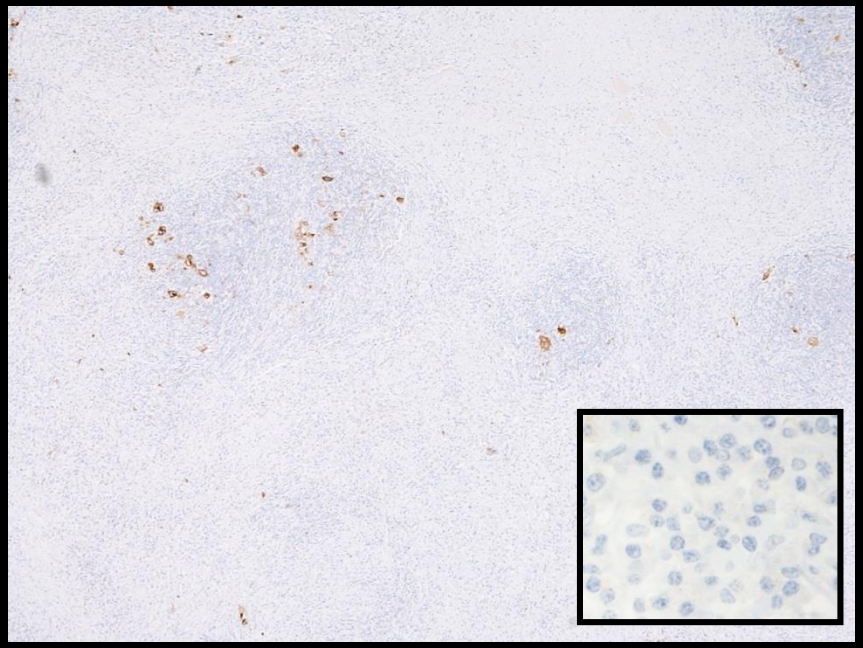
Hodgkin Lymphoma

HIER (modified low pH buffer)

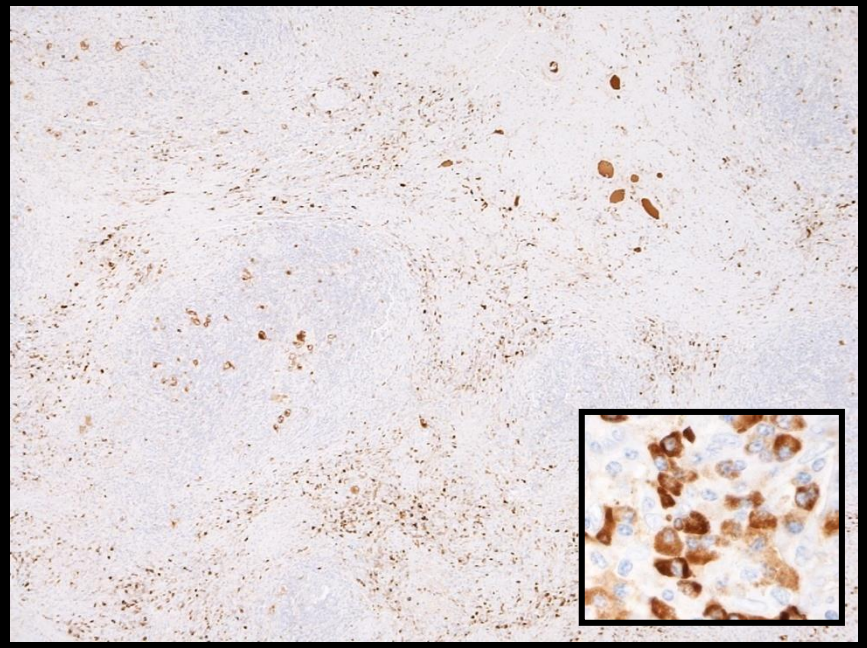
CD30

Hodgkin Lymphoma

Clone ConD6/B5



Clone Ber-H2



Note: No un-specific staining of plasma cells using the clone ConD6/B5

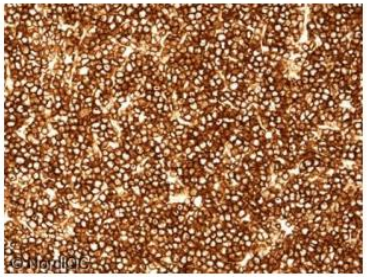


Fig. 1a (x200)
Optimal CD30 staining of the ALCL using the mAb clone CON6D/5 as concentrate, HIER in an modified low pH buffer (TRS pH 6.1, Dako) and a 3-step polymer based detection system (Flex+, Dako Omnis). Same protocol used in Figs. 2a - 5a. All neoplastic cells show a strong predominantly membranous staining reaction - compare with Fig. 1b.

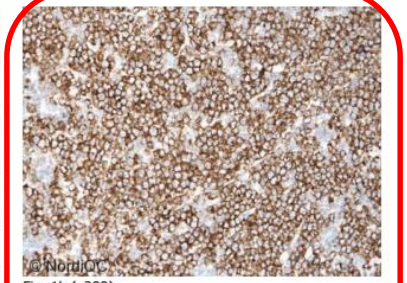


Fig. 1b (x200)
Insufficient staining for CD30 of the ALCL using the mAb clone CON6D/5 as concentrate (too diluted), HIER in Diva Decloaker solution pH 6.2 (excessive) and MACH1 (Biocare) as detection system – same protocol used in Figs. 2b – 6b. Staining intensity of the neoplastic cells are reduced - compare with Fig. 1a (same field), but also with Fig. 2a-5b.

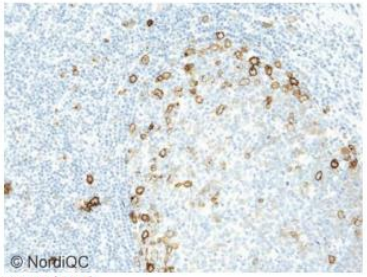


Fig. 2a (x200)
Optimal staining for CD30 in the tonsil, tissue core no 2, using same protocol as in Fig. 1a. The activated B- and T-cells, particularly B-cells located at the rim of the germinal centres, show a moderate to strong predominantly membranous staining reaction.

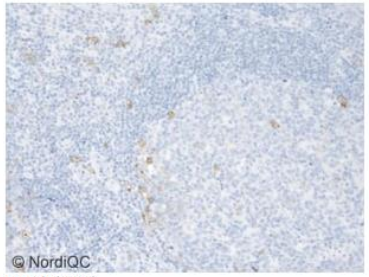


Fig. 2b (x200)
Insufficient staining for CD30 in the tonsil, tissue core no 2, using same protocol as in Fig 1b. The proportion of activated B- and T-cells is significantly reduced and staining intensity is too weak - compare with Fig. 2a (same field).

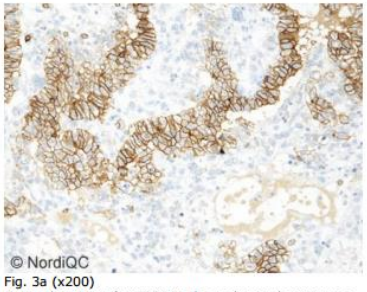


Fig. 3a (x200)
Optimal staining for CD30 in the embryonal carcinoma using same protocol as in Fig. 1a. All the neoplastic cells displays a strong continuous membranous staining reaction.

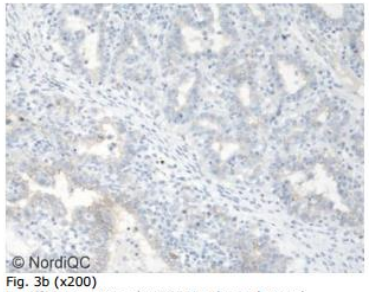


Fig. 3b (x200)
Insufficient staining for CD30 in the embryonal carcinoma using same protocol as in Fig. 1b. The neoplastic cells are false negative or only display a faint inconsistent membranous staining reaction - compare with Fig. 3a (same field).

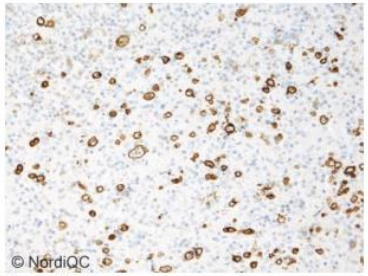


Fig. 4a (x200)
Optimal staining for CD30 of the Hodgkin lymphoma, tissue core no 5, using same protocol as in Fig. 1a - 3a. Virtually all the neoplastic cells show a strong predominately membranous staining reaction.

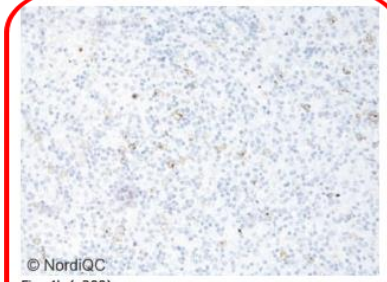


Fig. 4b (x200)
Insufficient staining for CD30 of the Hodgkin lymphoma, tissue core no 5, using same protocol as in Fig. 1b -3b. Staining intensity of the neoplastic cells is too weak or false negative - compare with Fig. 4a (same field).

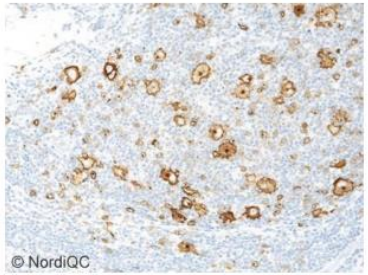


Fig. 5a (x200)
Optimal staining for CD30 of the Hodgkin lymphoma, tissue core no 6, using same protocol as in Fig. 1a - 4a. Virtually all Reed-Sternberg and Hodgkin cells show a moderate to strong, distinct membranous and cytoplasmic dot-like staining pattern.

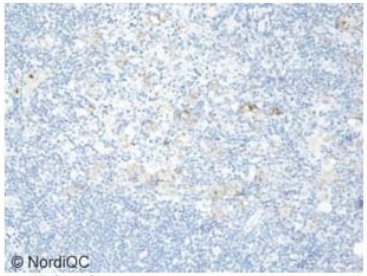


Fig. 5b (x200)
Insufficient staining for CD30 of the Hodgkin lymphoma, tissue core no 6, using same protocol as in Fig. 1b - 4b. The Reed-Sternberg and Hodgkin cells only display weak, inconsistent membranous staining reaction. In addition, the cytoplasmic dot-like staining reaction of the Reed-Sternberg and Hodgkin cells is weak and proportion of positive cells is significantly reduced - compare with Fig. 5a (same field).

CD30 clone CON6D/5 (HIER mod. Low pH buffers)

Protocol providing too low sensitivity (red frame) - Too diluted and the use of a low sensitive detection system)

Lymphoma panel: CD30

Optimal protocol settings (NQC)

CD30	Retrieval buffers	Titre	Detection	RTU	Detection
mAb **BER-H2**	**HIER High pH & mod. Low pH**	**1:20-1:100**	**3-step**	**Dako (IS602/IR602)**	**Flex/ Flex+**
				Ventana (790-4858)	**UltraView + Amp OptiView .**
mAb clone **JCM182**	**HIER High pH & Low pH**	**1:25-1:100**	**3-step**	**Leica (PA0790)**	**BOND Refine**
mAb **CON6D/5**	**HIER mod. Low pH**	**1:25-1:100**	**3-step (Flex+)**		

Control material / Tonsil:

An at least weak to moderate and distinct membranous staining reaction of interfollicular activated B- and T-cells and perifollicular germinal centre B-cells in the tonsil.

T-Cell lymphoma markers (1)

Marker (localization)	Control	High exp. (HE)	Low exp. (LE)	Non exp. (NE)
CD3 (membr.) F7.2.38, LN10, PS1, JCM182, EP449E, SP7, 2GV6, pAb A0542	Tonsil / Appendix	T-cells in the T-zone	T-cells in the mantle zones and within the germinal centres (moderate to strong intensity)	All other cells including B-cells and epithelia cells of the appendix
CD5 (membr.) 4C7, SP19	Tonsil / Appendix	T-cells	Dispersed mantle zone B-cells	All other cells including B-cells and epithelia cells of the appendix
CD4 (membr.) 4B12, 1F6, SP34, EP204, EPR6855	Tonsil / Appendix	Helper/inducer T-cells	Germinal centre macrophages	All other cells including B-cells and epithelia cells of the appendix
CD8 (membr.) C8/144B, 4B11, 1A5	Tonsil / Appendix	T-cytotoxic/suppressor cells & NK cells	None	All other cells including B-cells and epithelia cells of the appendix
CD1a (membr.) O10, EP3622	Tonsil/Skin/Thymus	The Langerhans' cells in the squamous epithelium (tonsil & skin) and cortical thymocytes (Thymus)	None	All other cells including epitheliums
CD2 (membr) AB75, SP304, BS60	Tonsil / Appendix	See CD3	See CD3	See CD3
CD7 (membr.) CBC.37, BSR9, BS8	Tonsil / Appendix	See CD3	See CD3	See CD3
In addition to the previous panels EBV-EBER/EBV-LMP1				

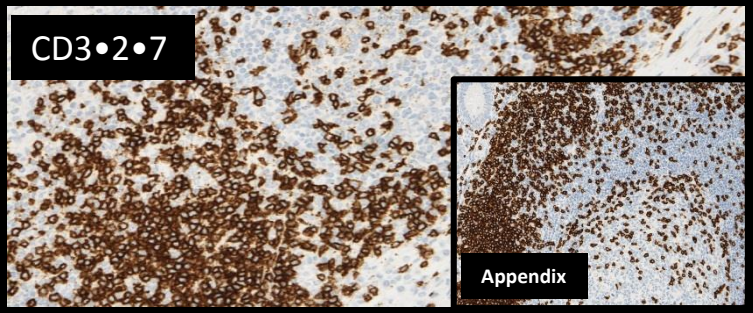
Clones (mAbs, rmAbs & pAbs) providing optimal results (NordiQC assessments)

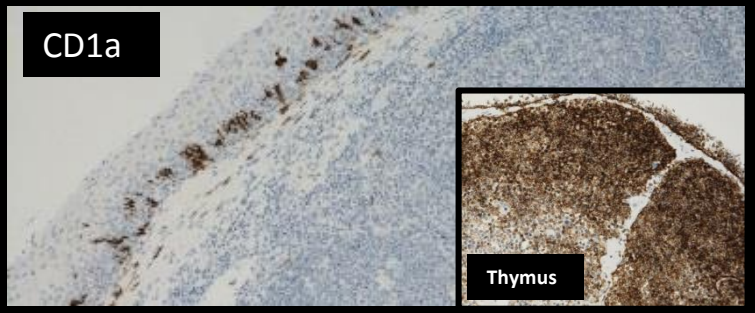
HE: Strong staining intensity/reactions should be expected

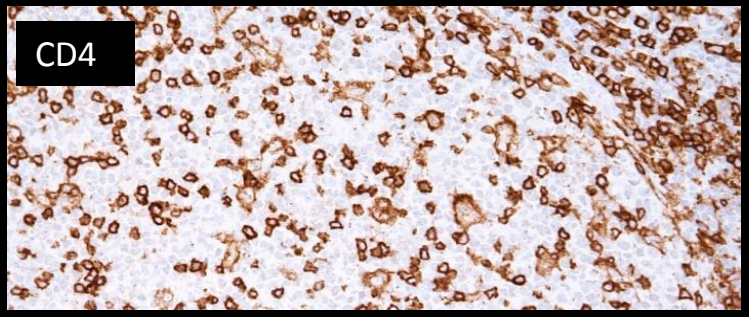
LE: An at least weak to moderate staining intensity/reactions should be expected

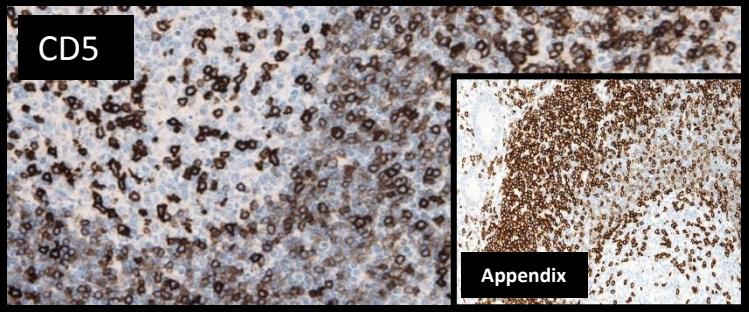
NE: No staining/reactions should be expected

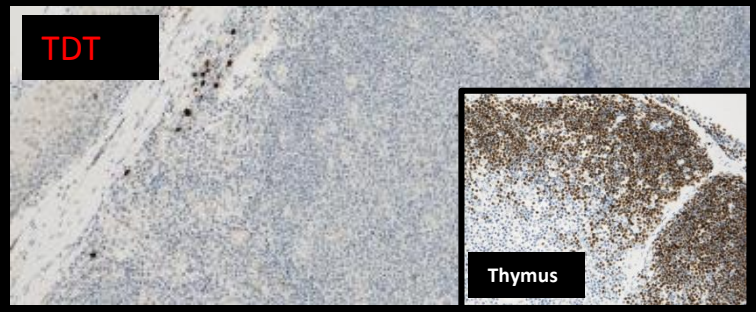
T-Cell lymphoma markers (1):





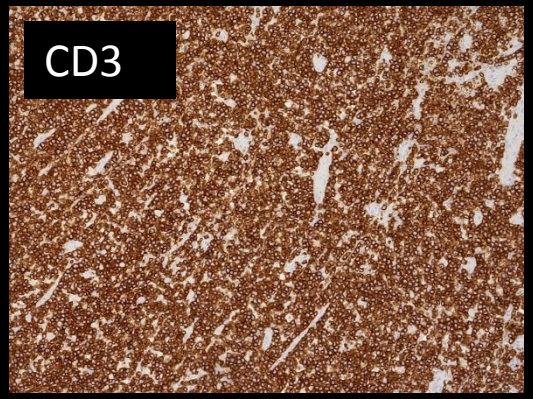


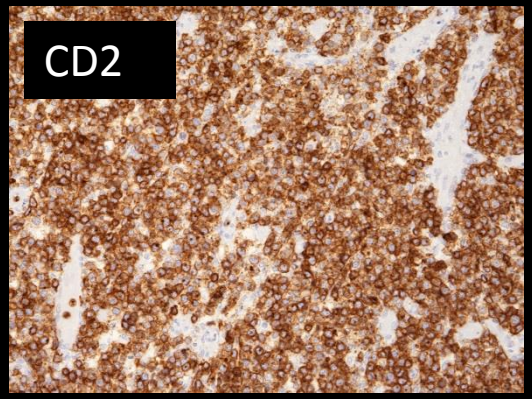


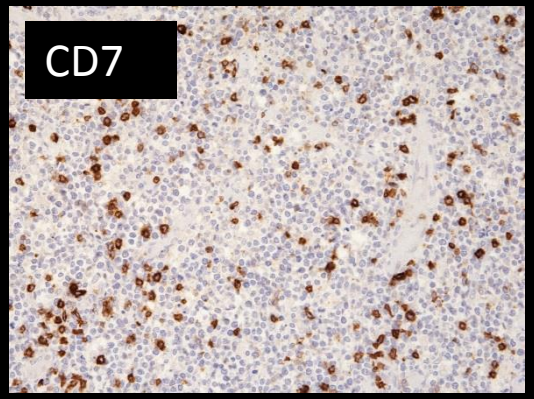


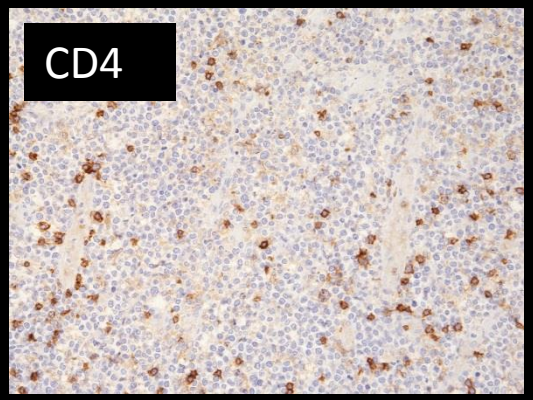
Tonsil

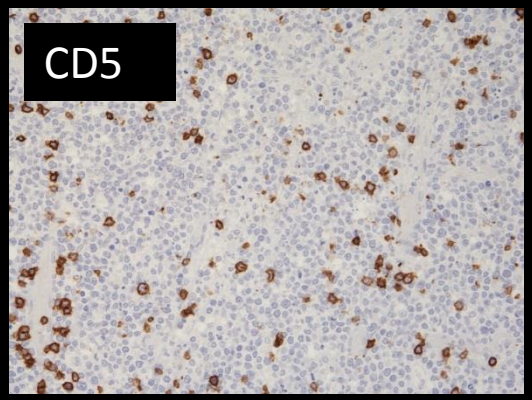
T-cell Lymphoma`s immunophenotype: Complex

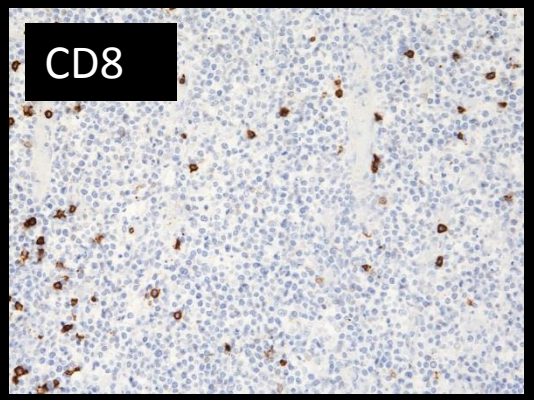












Note: Loss of T cell markers (CD7, CD4 and CD5)

CD3

Table 1. Abs and assessment marks for CD3, run 37

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **F7.2.38**	24	Dako	16	2	6	0	75 %	95 %
mAb clone **LN10**	12	Leica/Novocastra	5	5	2	0	83 %	100 %
mAb **PS1**	25 3 2 1 1	Leica/Novocastra Monosan Biocare Gene Tech Vector	18	10	4	0	88 %	92 %
rmAb **EP41**	1	Epitomics	0	1	0	0	-	-
rmAb **EP449E**	1	Epitomics	1	0	0	0	-	-
rmAb **SP7**	18 1 1	Thermo/NeoMarkers Cell Marque Zytomed	6	11	3	0	85 %	89 %
pAb **A0542**	29	Dako	14	13	2	0	93 %	96 %
Ready-To-Use Abs								
mAb clone **LN10 PA0553**	10	Leica/Novocastra	10	0	0	0	100 %	100 %
mAb clone **PS1 CD3-PS1-R-7**	1	Leica/Novocastra	0	1	0	0	-	-
mAb clone **PS1 PM110**	1	Biocare	1	0	0	0	-	-
rmAb clone **2GV6 790-4341**	54	Ventana	51	3	0	0	100 %	100 %
rmAb clone **EP272 MAD-000325QD**	1	Master Diagnostica	1	0	0	0	-	-
rmAb clone **MRQ-39 103R**	1	Cell Marque	1	0	0	0	-	-
pAb **IR503/IS503**	31	Dako	20	10	1	0	97 %	97 %
pAb clone **N1580**	1	Dako	0	1	0	0	-	-
Total	219		144	57	18	0	-	
Proportion			66 %	26 %	8 %	0 %	92 %	

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

Optimal Protocols

HIER preferable in alkaline buffer

Careful calibration of primary Ab

2 and 3-step detection systems

Insufficient results

Inefficient HIER (too low temp. or too short time)

Low concentration of the primary Ab

Platform dependent mAb F7.2.38

CD3

Table 2. Optimal results for CD3 using concentrated Abs on the 3 main IHC systems*

Table 2. **Optimal results for CD3 using concentrated antibodies on the 3 main IHC systems***

Concentrated antibodies	Dako Autostainer Link / Classic		Ventana BenchMark XT / Ultra		Leica Bond III / Max	
Buffer	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 **F7.2.38**	92 % 11/12**	-	0 % 0/4	0 % 0/1	-	-
mAb clone **PS1**	63 % 5/8	-	50 % 5/10	-	50 % 4/8	100 % 2/2
pAb **A0542**	64 % 9/14	-	18 % 2/11	-	100 % 1/1	-

* Antibody concentration applied as listed above, HIER buffers and detection kits used as provided by the vendors of the respective platforms.
** (number of optimal results/number of laboratories using this buffer)

mAb F7.2.38 performed less successful on the Ventana Benchmark platform compared to protocols with similar settings applied on Dako Autostainers

<u>Alternative : Use Ventana`s RTU system (790-4341) based on the rmAb 2GV2</u>

54 protocols (100% sufficient/94% optimal), HIER in CC1 and iView, UltraView or OptiView

CD3

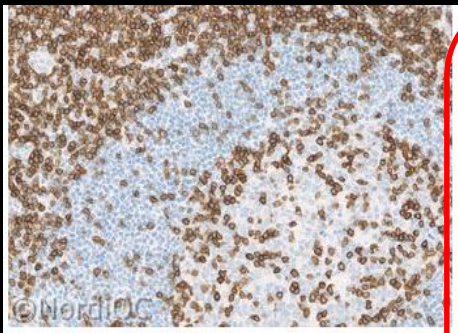


Fig. 1a. Optimal CD3 staining of the tonsil using the rmAb clone 2GV6, Ready-To-Use, Ventana. Virtually all the T-lymphocytes in the T-zone and within the germinal centre show a strong and distinct membranous staining reaction. No background staining or staining of the B-cells is seen. Also compare with Figs. 2a – 3a, same protocol.

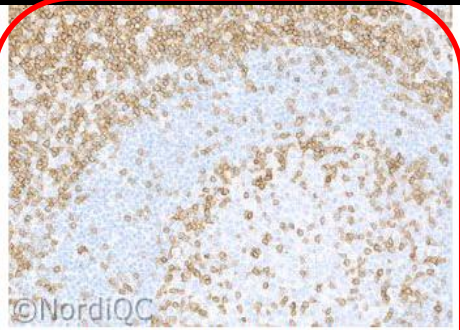


Fig. 1b. CD3 staining of the tonsil using the mAb clone F7.2.38 by protocol settings giving a too low sensitivity - same field as in Fig. 1a. The vast majority of the T-lymphocytes are demonstrated. A slightly weaker and less intense staining reaction is seen. However also compare with Figs. 2b – 3b, same protocol.

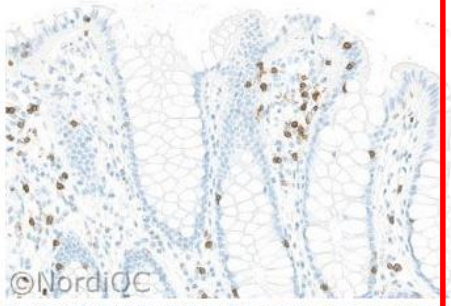


Fig. 2a. Optimal CD3 staining of the colon using same protocol as in Fig. 1a. The dispersed intraepithelial T-lymphocytes show a distinct staining reaction. The columnar epithelial cells are negative and no background staining is seen.

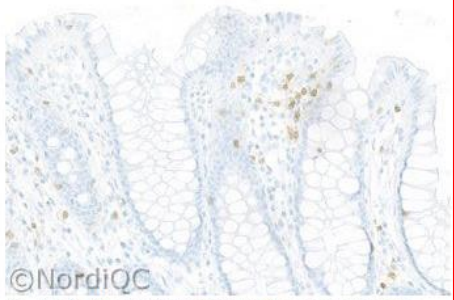


Fig. 2b. Insufficient CD3 staining of the colon using same protocol as in Fig. 1b – same field as in Fig. 2a. The intraepithelial T-lymphocytes are virtually negative. Also compare with Fig. 3b, same protocol.

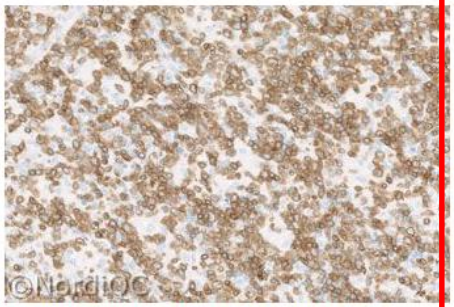


Fig. 3a. Optimal CD3 staining of the peripheral T-cell lymphoma, NOS, using same protocol as in Figs. 1a & 2a. Virtually all the neoplastic cells show a moderate to strong and distinct predominantly membranous staining reaction. No background staining is seen.

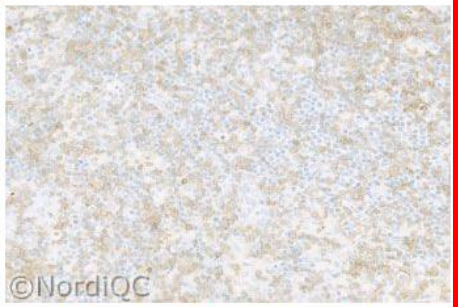


Fig. 3b. Insufficient CD3 staining of the peripheral T-cell lymphoma, NOS, using same protocol as in Figs. 1b & 2b – same field as in Fig. 3a.
The proportion and intensity of the neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained in Fig. 3a.

Problem:

Low sensitive protocols

Too low HIER temperature

Too short HIER time

Too low concentration of the primary Ab

Too low sensitivity of the detection system

All these parameters should be carefully calibrated to give optimal results = focus on critical staining indicators

Lymphoma panel: CD3

Optimal protocol settings (NQC)

CD3	Retrieval buffers	Titre	Detection	RTU	Detection
mmAb **F7.2.38**	**HIER High pH**	**1:50-1:200**	**2 & 3-step**	**-**	**-**
pAb **A0452**	**HIER High pH**	**1:50-1:300**	**2 & 3-step**	**Dako (IS503/IR503)**	**Flex/ Flex+**
mmAb **LN10**	**HIER High pH & Low pH**	**1:50-1:140**	**2 & 3-step**	**Leica (PA0553)**	**BOND Refine**
mAb clone **PS1**	**HIER High pH & Low pH**	**1:40-1:100**	**2 & 3-step**	**Biocare (PM110)**	**MACH4**
rmAb **2GV2**	**HIER High pH (CC1)**	**-**	**-**	**Ventana (790-4341)**	**iView UltraView OptiView**
rmAb **SP7**	**HIER High pH**	**1:100-1:200**	**2 & 3-step**	**-**	**-**

Control material / Tonsil:

A moderate to strong, distinct predominantly membranous staining reaction of all T-cells.

No staining of other cellular structures

CD5

Table 1. **Antibodies and assessment marks for CD5, run 49**

Concentrated	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **4C7**	55 11 6 4 2 1 1	Leica/Novocastra Dako/Agilent Thermo S./LabVision Biocare Medical Cell Marque BioGenex Monosan	43	28	9	0	89%	93%
rmAb clone **SP19**	9 7 6 2	Thermo S./LabVision Cell Marque Spring Bioscience Zytomed Systems	15	5	2	2	83%	83%
rmAb clone **EP77**	1 1	Cell Marque Zeta	0	0	2	0	-	-
pAb **E2474**	1	Spring Bioscience	0	1	0	0	-	-
Ready-To-Use								
mAb clone **4C7 IR/IS082**	39	Dako/Agilent	27	10	1	1	95%	97 %
mAb clone **4C7 IR/IS082**	13	Dako/Agilent	7	5	1	0	92%	-
mAb clone **4C7 PA0168**	12	Leica Biosystems	9	2	1	0	92%	90%
mAb clone **4C7 PA0168**[4]	7	Leica Biosystems	3	3	0	1	86%	-
mAb clone **4C7 205M-17/18**	1	Cell Marque	1	0	0	0	-	-
mAb clone **4C7 MS-393-R7**	1	Thermo S./LabVision	1	0	0	0	-	-
mAb clone **4C7 AM430-5/10**	1	BioGenex	1	0	0	0	-	-
mAb clone **4C7 PDM095**	1	Diagnostic BioSystems	1	0	0	0	-	-
mAb clone **4C7 PM099**	1	Biocare medical	0	1	0	0	-	-
rmAb clone **SP19 790-4451**	88	Ventana/Roche	76	11	1	0	99%	99%
rmAb clone **SP19 205R-17/18**	4	Cell Marque	4	0	0	0	-	-
rmAb clone **SP19 KIT-0033**	1	Maixin	1	0	0	0		
rmAb clone **EP77 MAD-000602QD**	2	Master Diagnostica	0	1	0	1	-	-
Total	278		189	67	17	5	-	
Proportion			68%	24%	6%	2%	92%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only (see below).
3) RTU system developed for the Dako/Agilent semi-automatic system (Autostainer) but used by laboratories on the Omnis platform (Dako/Agilent).
4) RTU system developed for the Leica Biosystem full-automated systems (BOND III/MAX) but used by laboratories on different platforms (e.g. Ventana Benchmark) or manually.

Efficient HIER, preferable in alkaline buffer and careful calibration of the primary Ab titre

Insufficient protocols

Too low primary Ab concentration

mAb 4C7: ADF 1:142 (range 1:10-1:1200) / Opt. result

mAb 4C7: ADF 1:282 (range 1:20-1:1500) / Insuff. result

RTU systems gave higher pass rate compared to Laboratory developed assays

Best performance: rmAb clone SP19, 790-4451 (Ventana)

CD5, Run 49

Table 5. **Comparison of pass rates for vendor recommended and laboratory modified RTU protocols**

RTU systems	Vendor recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
Dako AS48 mAb 4C7 **IR/IS082**	94% (16/17)	71% (12/17)	95% (21/22)	68% (15/22)
Leica BOND mAb 4C7 **PA0168**	100% (3/3)	100% (3/3)	89% (8/9)	67% (6/9)
VMS Ultra/XT rmAb SP19 **790-4451**	100% (6/6)	33% (2/6)	90% (70/78)	68% (53/78)

* 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 are included.

Optimal results could be obtained by using vendor recommended or laboratory modified protocol settings – all vendors (see table 5).

<u>RTU 790-4451 (rmAb SP19) / Ventana Benchmark (all protocol settings):</u>

Proportion of optimal results was influenced by the choice of detection system:

- **76% (26 of 34) produced an optimal result using UltraView as the detection system**
- **97% (31 of 32) produced an optimal result using OptiView as the detection system.**

CD5, Run 49

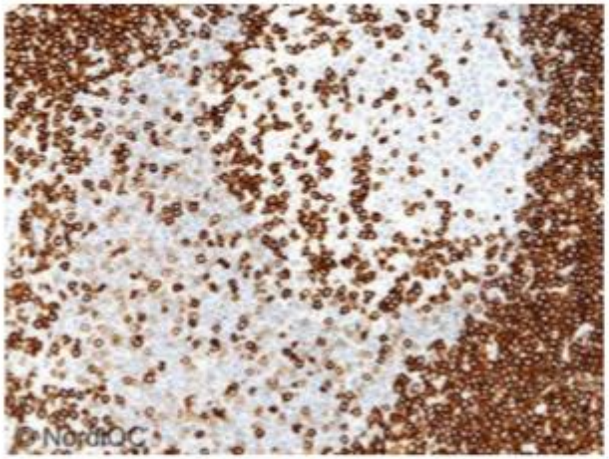


Fig. 1a (x200)
Optimal staining for CD5 of the tonsil, core 1, using the mAb 4C7 as a concentrate, HIER in an alkaline buffer (BERS2) and a polymer based detection system (BOND Refine, Leica) - same protocol used in Figs. 2a - 5a. The T-cells in the interfollicular T-zone and within the germinal centre show a strong distinct membranous staining reaction. Dispersed B-cells in the mantle zone show a weak to moderate but distinct membranous staining reaction.

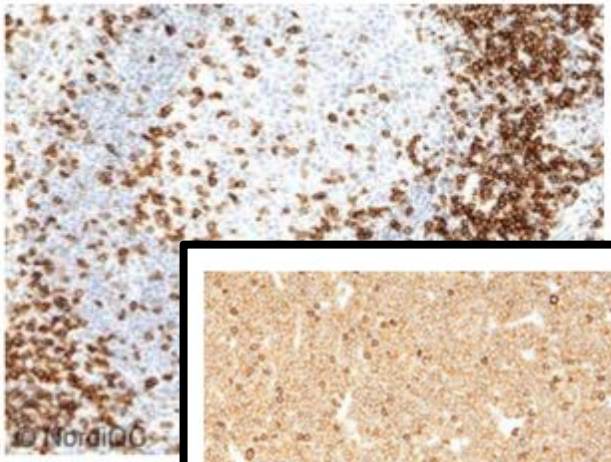


Fig. 1b (x200)
Insufficient stain
mAb clone 4C7 a
alkaline buffer (
(Leica) as the de
Figs. 2b – 5b. Th
germinal centre
- compare with F

Fig. 4a (x200)
Optimal staining for CD5 of the MCL, core 4, using same protocol as in Figs. 1a - 3a. Virtual all the neoplastic cells show a weak to moderate, distinct membranous staining reaction. T-cells intermingling with the neoplastic cells show a strong membranous staining reaction.

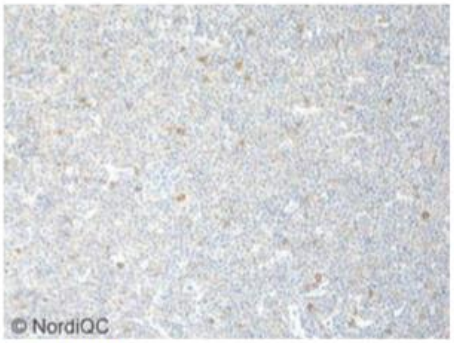


Fig. 4b (x200)
Insufficient staining for CD5 of the MCL, core 4, using same protocol as in Figs. 1b -3b. The neoplastic cells are false negative and only T-cells with reduced intensity are demonstrated - compare with Fig. 4a (same field).

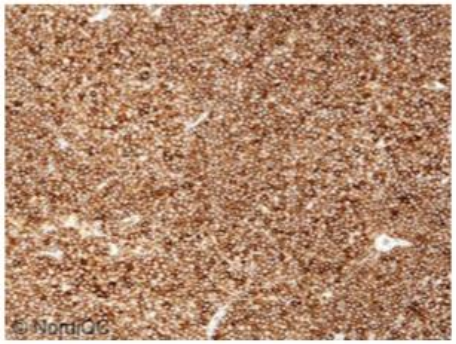


Fig. 5a (x200)
Optimal staining for CD5 of the B-CLL using same protocol as in Figs. 1a - 4a. All the neoplastic cells show a strong and distinct membranous staining reaction.

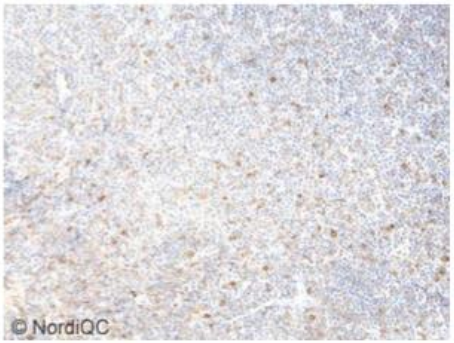


Fig. 5b (x200)
Insufficient staining for CD5 of B-CLL using same protocol as in Figs. 1b - 4b. The vast majority of neoplastic cells are false negative or shows reduced intensity. T-cells display a moderate staining intensity - compare with Fig. 5a (same field).

Problem: Too diluted primary Ab and inefficient HIER (too short time)

Lymphoma panel: CD5

Optimal protocol settings (NQC)

CD5	Retrieval buffers	Titre	Detection	RTU	Detection
mmAb 4C7	HIER High pH or mod. Low pH	1:20-1:200	2 & 3-step	Leica (PA0168)	BOND refine
				Dako (IS/IR082)	Flex
rmAb SP19	HIER High pH	1:25-1:100	2 & 3-step	Ventana (790-4451)	iView UltraView +/- Amp* OptiView

* Optimal results could also be obtained with the detection system UltraView without amplification but at overall lower frequency compared to laboratories using UltraView with amplification

Control material / Tonsil:

An at least weak to moderate and distinct membranous staining reaction of dispersed B-cells in the mantle zone of the secondary follicles in the tonsils.

Strong membranous staining of T-cells

T-Cell lymphoma markers (2)

Marker (localization)	Control	High exp. (HE)	Low exp. (LE)	Non exp. (NE)
PD-1 (membr.) **NAT105**	Tonsil/	Follicular centre T-cells (helper T-cells)	Scattered extrafollicular and mantle zone lymphocytes	All other cells
CXCL-13 (cytopl.) **53610**	Tonsil	Follicular centre T-cells (helper T-cells), scattered T-cells in the mantle zone and interfollicular areas	None	All other cells
Granzyme B (cytopl.) **GrB-7**	Tonsil	Activated cytotoxic T-cells & NK cells	None	All other cells including B-cells
TIA-1 (cytopl.) **TIA-1**	Tonsil	Cytotoxic T-cells & NK cells	Dispersed unstimulated T-cells, NK-cells and some myeloid cells	All other cells including B-cells

Blast marker(s)

Marker (localization)	Control	High exp. (HE)	Low exp. (LE)	Non exp. (NE)
TdT (nuclear) **SEN28, EP266**	Thymus/Tonsil	Dispersed immature T-cells in the interfollicular zones of tonsils.	Cortical thymocytes (moderate intensity)	Mantle zone and germinal centre B-cells.

Clones (mAbs, rmAbs & pAbs) providing optimal results (NordiQC assessments)

HE: Strong staining intensity/reactions should be expected

LE: An at least weak to moderate staining intensity/reactions should be expected

NE: No staining/reactions should be expected

TdT

Table 1. **Antibodies and assessment marks for TdT, run 52**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **SEN28**	66 3 2 1 1	Leica/Novocastra Diagnostic Biosystems Thermo/NeoMarkers Monosan Immunologic	20	29	19	5	67%	70%
rmAb clone **EP266**	13 2 1 1	Agilent/Dako Cell Marque Diagnostic Biosystems Epitomics	11	3	3	0	82%	87%
pAb **A3524**[3]	2	Agilent/Dako	0	1	1	0	-	-
pAb **ILP 0049**	3	Immunologic	0	1	2	0	-	-
pAb **338A-76**	2	Cell Marque	0	0	1	1	-	-
pAb **CP134**	1	Biocare Medical	0	1	0	0	-	-
pAb **44811**	1	Menarini Diagnostics	0	1	0	0	-	-
Ready-To-Use antibodies								
mAb clone **SEN28 PA0339**	11	Leica/Novocastra	6	5	0	0	100%	100%
mAb clone **SEN28 PA0339**[4]	5	Leica/Novocastra	2	1	1	1	-	-
mAb clone **SEN28 8243-C010**	1	Sakura FineTek	1	0	0	0	-	-
mAb clone **SEN28 MAB-0197**	1	Maixin	1	0	0	0	-	-
mAb clone **SEN28 MS-1105-R7**	1	Thermo/Neomarkers	0	1	0	0		
rmAb clone **EP266 IR093**	36	Agilent/Dako	26	8	2	0	94%	95%
rmAb clone **EP266 IR093**[4]	17	Agilent/Dako	17	0	0	0	100%	100%
rmAb clone **EP266 MAD-000659QD**	2	Master Diagnostica	1	1	0	0	-	-
rmAb clone **EP266 338R-28**	1	Cell Marque	1	0	0	0	-	-
rmAb clone **EP266**	1	Unknown	0	1	0	0		
pAb **760-2670**	45	Ventana/Cell Marque	1	39	4	1	89%	100%
pAb **338A-78**	4	Cell Marque	0	4	0	0	-	-
pAb **IR001**[3]	1	Agilent/Dako	0	1	0	0	-	-
Total	225		87	97	33	8	-	
Proportion			39%	43%	15%	3%	82%	

1) Proportion of sufficient stains (optimal or good).
2) Proportion of sufficient stains with optimal protocol settings only, see below.
3) Product discontinued.
4) 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.

Robust antibodies

mAb clone SEN28
rmAb clone EP266

HIER in alkaline buffer

mAb clone SEN28
2- or 3-step mul./pol detection sys

rmAb clone EP266
3-step mul./pol detection sys.

Inappropriate platforms ?
88% (15 of 17) on the Omnis?

Proportion of optimal results ?

RTU better than LD assays

TdT

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

Concentrated antibodies	Dako Autostainer Link / Classic		Dako Omnis		Ventana 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 **SEN28**	3/3**	-	2/4	-	8/30 (27%)	-	2/5 (40%)	0/2
rmAb clone **EP266**	1/3	-	2/2	-	5/8 (63%)	-	1/1	-

* 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)

Low proportion of optimal results ?

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

RTU systems	Recommended protocol settings*		Laboratory modified protocol settings**	
	Sufficient	Optimal	Sufficient	Optimal
Leica BOND MAX/III mAb SEN28 **PA0339**	100% (3/3)	0% (0/3)	100% (8/8)	75% (6/8)
Dako AS mAb EP266 **IR093**	92% (11/12)	50% (6/12)	100% (20/20)	90% (18/20)
VMS Ultra/XT/GX pAb **760-2670**	0% (0/1)	0%(0/1)	89% (34/38)	3% (1/38)

* 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.

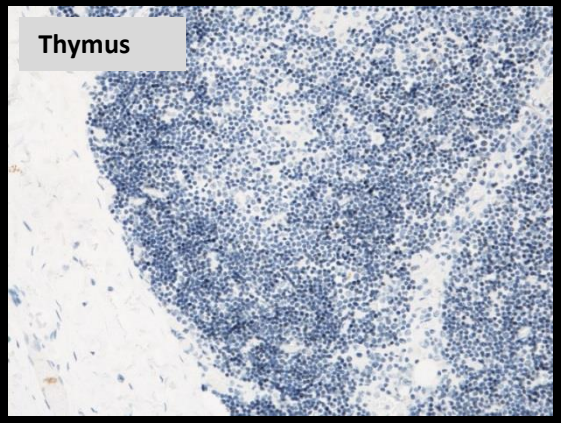
Prolonging inc. time

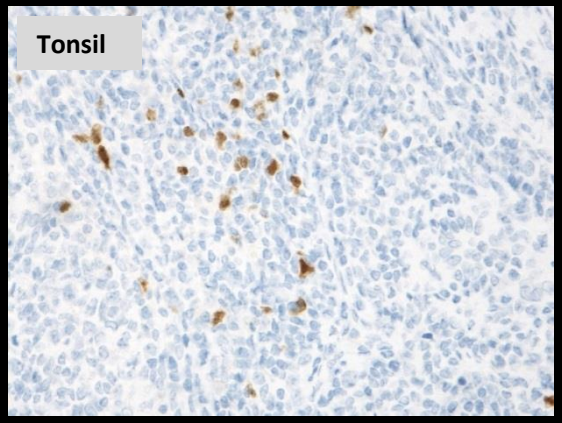
Substituting Flex with Flex+

Poor signal-to-noise ratio

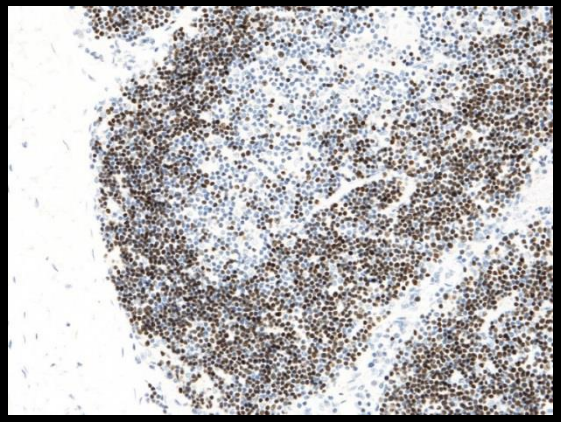
TdT: A marker sensitive to the choice of antibody diluent ?

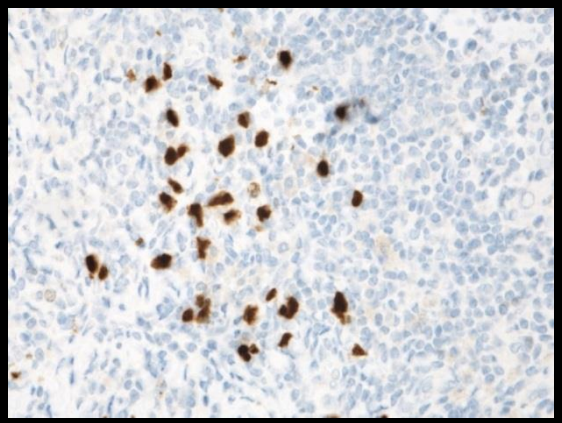
TdT, SEN28 1:50
Dako dil. pH 7.3





TdT, SEN28 1:50
Renoir Red pH 6.2

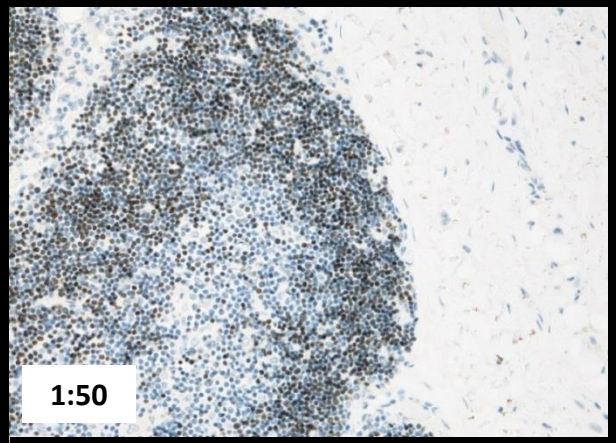


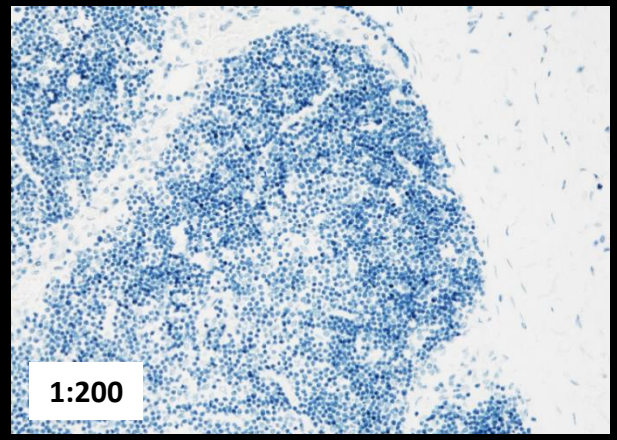


Omnis: HIER/HIGH pH 24`, Flex+ Mouse (10+20`)

TdT: A marker sensitive to the choice of antibody diluent ?

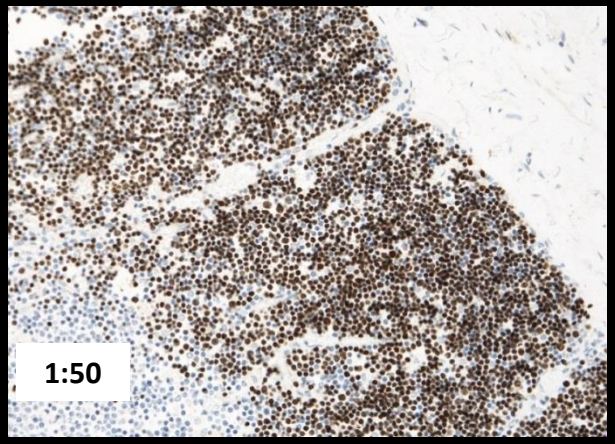
TdT, EP266
Dako dil. pH 7.3

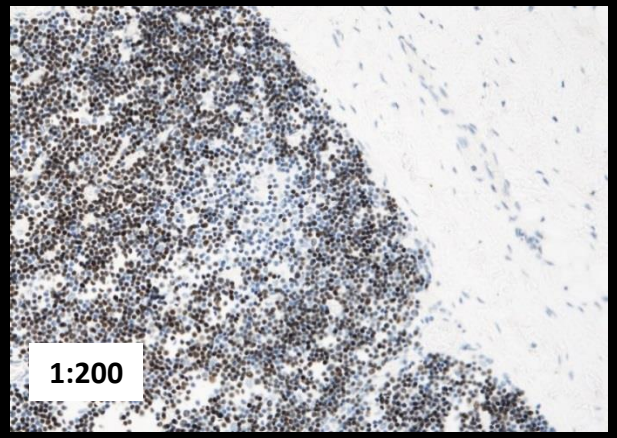




Omnis: HIER/HIGH pH 24`, Flex+ Rabbit (10+20`)

TdT, EP266
Renoir Red pH 6.2





Thymus

TdT

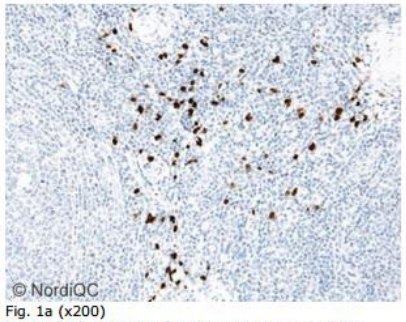


Fig. 1a (x200)
Optimal TdT staining of tonsil using the mAb clone SEN28, optimally calibrated, HIER in TRS (3-1) pH 9 (Dako) and a 3-step polymer based detection system (Flex+/Dako).
Dispersed pre-mature T-cells of the interfollicular zones show a strong and distinct nuclear staining reaction. Same protocol used in Figs. 2a - 4a.

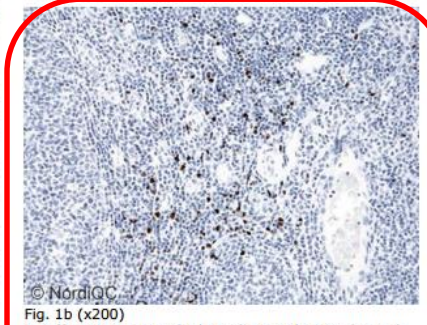


Fig. 1b (x200)
Insufficient staining of TdT in the tonsil using the mAb clone SEN28, too diluted and applying the less sensitive detection system Flex (Dako) – same field as in Fig. 1a. Although the pre-mature T-cells of the interfollicular zones display a relative strong nuclear staining intensity, the protocol provided too low sensitivity (compare Figs. 1a - 4b). Same protocol used in Figs. 2b - 4b.

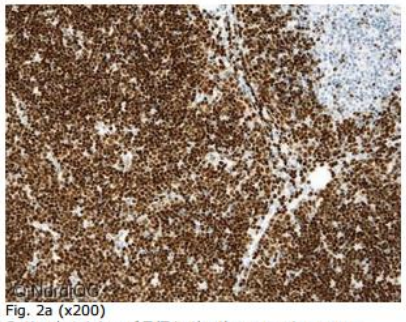


Fig. 2a (x200)
Optimal staining of TdT in the thymus using same protocol as in Fig. 1a. Immature cortical thymocytes and scattered pre-mature T-cells of medulla show a strong and distinct nuclear staining reaction.

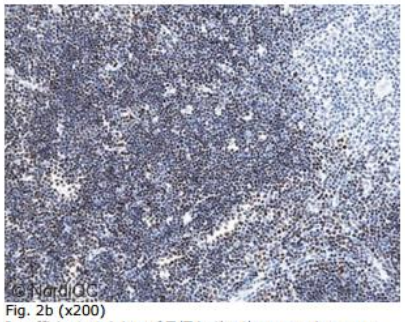


Fig. 2b (x200)
Insufficient staining of TdT in the thymus using same protocol as in Fig. 1b – same field as in Fig. 2a.
The staining intensity and proportion of positive cortical thymocytes is significantly reduced.

Optimal result

HIER (TRS pH9)/ Optimal calibrated primary Ab and use of Flex+ as the detection system

Insufficient result:

HIER (TRS pH9)/ Too diluted primary Ab and use of the less sensitive detection system Flex

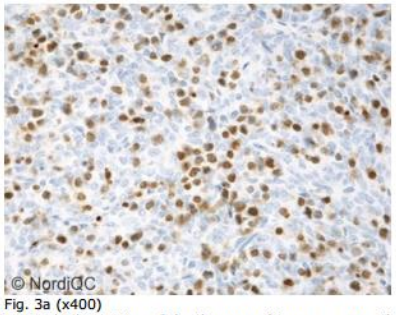


Fig. 3a (x400)
Optimal TdT staining of the thymoma (tissue core no. 4) using same protocol as in Figs. 1a and 2a. The vast majority of immature T-cells intermingling between the neoplastic cells show a weak to moderate but distinct nuclear staining reaction.

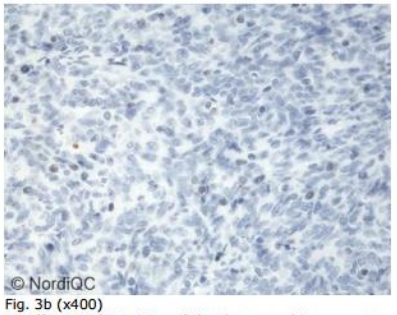


Fig. 3b (x400)
Insufficient TdT staining of the thymoma (tissue core no. 4) using same protocol as in Figs. 1b and 2b - same field as in Fig. 3a. The immature T-cells intermingling between the neoplastic cells are false negative or only faintly demonstrated in a small fraction of the total population of T-cells.

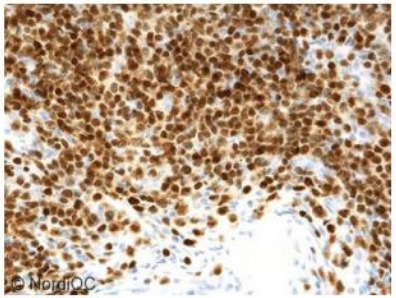


Fig. 4a (x400)
Optimal TdT staining of the thymoma (tissue core no. 5) using same protocol as in Figs. 1a – 3a. Virtually all the immature T-cells show a strong and distinct nuclear staining reaction.

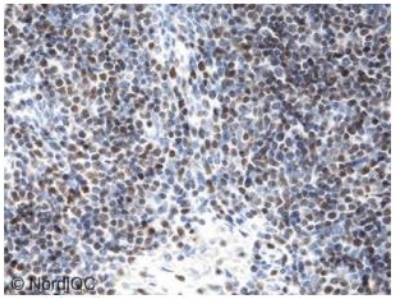


Fig. 4b (x400)
Insufficient TdT staining of the thymoma (tissue core no. 5) using same protocol as in Figs. 1b and 3b - same field as in Fig. 4a. The staining intensity of the immature T-cells is significantly reduced.

Blast panel: TdT

Optimal protocol settings (NQC)

TdT	Retrieval buffers	Titre	Detection	RTU	Detection
mAb SEN28	HIER High pH	1:20-1:50	2 & 3-step	Leica (PA0339)	BOND refine
rmAb EP266	HIER High pH	1:25-1:100	2 & 3-step	Dako (IR093)	Flex/Flex+

Control material / Thymus:

An at least moderate and distinct nuclear staining reaction of cortical thymocytes.

Lymphoma´s (Basic panel): Antibodies

Based on the result`s in NordiQC (> 5 protocols pr. clone assessed in the latest run)

Target	High scoring clones	Low scoring clones
CD20	mmAb: L26	-
Pax5 (BSAP)	mmAb: DAK-PAX5 & 24 & 1EW, rmAb: BV6 & BSR59 & EP156	pAb: RB-9406 , mmAb: 24[#] & 1EW (PO blocking)* & SP34[◊]
BCL2	mmAb: 124 & 100/D5 & BCL2/100/D5	mmAb: 124[#]
CD5	mmAb: 4C7, rmAb: SP19	mmAb: CD5/54/F6
BCL6	mmAb: GI181E/A8 & LN22 & PG-B6p	mmAb: PG-B6p (PO blocking) *
CD23	mmAb: 1B12 & DAK-CD23 & BS20 , rmAb: SP23	mmAb: 1B12[#]
CD30	mmAb: BER-H2 & JCM182 & "CON6D/5"	-
Kappa	pAb: A0191	All other pAbs and mmAbs
Lambda	pAb: A0193	All other pAbs and mmAbs
CD79a	mmAb: JCB118, rmAb: SP18	mmAb: 11E3 & "HM57" & JCB118[#] , rmAb: SP18[‡]
CD3	mmAb: F7.2.38 & LN10 & PS1, rmAb: SP7 & 2GV6, pAb: A0542	-
CyD1	rmAb : EP12 & SP4	mmAb: P2D11F11
CD45	mmAb: 2B11+PD7/26 & X16/99 & "RP2/18 (RTU, Ventana)"	-
Ki67	mmAb: MIB-1 & K2 & UMAB107, rmAb: SP6 & "30-9 (RTU, Ventana)"	-
CD43	mmAb: DF-T1 ?	?

[#]Platform issues (Ventana)
[‡]Platform issues (Autostainer / BOND)
*PO blocking before appl. of the primary Ab
[◊] Lot variations

Hematolymphoid markers

Go for primary Abs providing high proportion of optimal results and carefully calibrated the titre

NordiQC website ~ Recommended controls / iCAPCs

Use efficient HIER in app. buffer`s (20-40 min at 97°C-100°C)

For CD30 clone CON6D/5A, HIER in mod. low pH buffer`s is mandatory

Use a sensitive 3-step polymer/multimer detection system

In addition, consider other parameters that may influence the quality of the IHC-staining

Platform dependent primary Abs
Epitopes sensitive to H_2O_2 blocking
Lot - to - lot variations
Too much counterstain
…….

Thank you

CMYC

Table 3. **Proportion of optimal results for C-MYC for the two most commonly used antibody concentrates on the four main IHC systems***

Concentrated antibodies	Dako Autostainer Link / Classic		Dako Omnis		Ventana 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
rmAb clone **EP121**	0/8** (0%)	-	5/15 (33%)	-	7/21 (33%)	-	2/6 (33%)	-
rmAb clone **Y69**	3/9 (33%)	0/2	0/4	-	7/16*** (44%)	-	2/5 (20%)	-

* 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)
***(One laboratory used a combined HIER and protease protocol)

Too low conc. of the primary Ab

Although the proportion of optimal results on the three fully automated platforms was an identical 33%, a significant difference was observed comparing the pass rates.

Omnis (Dako): Pass rate of 80% (all based 3-step polymer detection system)

Bond (Leica): Pass rate of 83% (all based 3-step polymer detection system)

Benchmark (Ventana): Pass rate of 62% (based on 2 and 3-step multimer detection system)
Pass rate of 76% (3-step multimer detection system)