Assessment Run 47 2016

Cyclin D1 (CyD1)

Material
The slide to be stained for CyD1 comprised:

1. Tonsil fixed 24h, 2. Tonsil fixed 48h, 3. B-CLL, 4-5. Mantle cell lymphomas

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing CyD1 staining as optimal included:

- A moderate to strong, distinct nuclear staining reaction of virtually all suprabasal squamous epithelial cells, scattered lymphocytes and endothelial cells in the two tonsils.
- An at least weak, distinct nuclear staining reaction of germinal centre macrophages in tonsils.
- A moderate to strong and distinct nuclear staining reaction of virtually all neoplastic cells in the two mantle cell lymphomas.
- No nuclear staining reaction of neoplastic cells in the B-CLL (whereas a moderate nuclear staining reaction should be seen in scattered endothelial cells).
- No staining of germinal centre lymphocytes and the vast majority of mantle zone B-cells and interfollicular lymphocytes in the tonsils.

A weak cytoplasmic staining reaction was accepted provided that this did not complicate interpretation.

Participation

Number of laboratories registered for CyD1, run 47	278
Number of laboratories returning slides	257 (92%)

Results
257 laboratories participated in this assessment. 242 (94%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies (Abs) used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Less successful primary antibody
- Unexplained technical issues

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%

Conclusion
The rmAb clones **EP12** and **SP4** were the most widely used clones for CyD1. Both can be recommended both within a laboratory developed (LD) assay and in a Ready-To-Use (RTU) system.
In LD assays, HIER in an alkaline buffer and careful calibration of the primary antibody were the main requirements for optimal performance. An optimal result could be obtained for both antibodies on the most commonly used IHC platforms.
In this assessment, the RTU systems from Dako and Ventana based on EP12 and SP4, respectively, provided the highest proportion of sufficient and optimal results.
Tonsil is recommendable as positive and negative tissue control: Virtually all suprabasal squamous epithelial cells, scattered lymphocytes and endothelial cells must show a moderate to strong distinct nuclear staining reaction, whereas an at least weak but distinct staining reaction of germinal centre macrophages should be seen. Mantle zone B-cells and germinal centre B-cells should be negative. The positive staining of endothelial cells is a valuable internal positive tissue control for CyD1.

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%
rmAb clone **SP4**	69 6 5 4 2 1 1 1 1	Thermo/Neomarkers Cell Marque Biocare Spring Bioscience Zytomed Immunologic Maixin Nordic Biosite Thermo/Pierce	36	45	6	3	90%	92%
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

Detailed analysis of CyD1, Run 47

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
rmAb clone **EP12**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (4/5)*, Cell Conditioning 1 (CC1, Ventana) (3/5) or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/2) as retrieval buffer. The mAb was diluted in the range of 1:20-1:200. Using these protocol settings, 47 of 48 (98%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **SP4**: Protocols with optimal results were typically based on HIER using TRS pH 9 (3-in-1) (Dako) (6/9), TRS pH 9 (Dako) (3/5), CC1 (Ventana) (20/42), BERS2 (Leica) (3/16), BORG Decloaker pH 9.5 (Biocare) (1/3) or Tris-EDTA pH 9 (2/8) as retrieval buffer. The mAb was typically diluted in the range of 1:20-1:150. Using these protocol settings, 77 of 84 (92%) laboratories produced a sufficient staining result.

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)

Ready-To-Use antibodies and corresponding systems
rmAb clone **EP12**, product no. **IS083/IR083**, Dako, Autostainer+/Autostainer Link:
Protocols with optimal results were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) or TRS pH 9 (efficient heating time 10-20 min. at 95-99°C), 15-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection systems. Using these protocol settings, 46 of 46 (100%) laboratories produced a sufficient staining result (optimal or good).

rmAb clone **EP12**, product no. **PME432**, Biocare, IntelliPATH
One protocol with an optimal result was based on HIER using BORG decloaker pH 9.5 in a Pressure Cooker, 30 min. incubation of the primary Ab. MACH 4 Universal HRP-Polymer (MRH534) was used as detection system.

rmAb clone **SP4**, product no. **790-4508**, Ventana, BenchMark GX/XT/Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 16-64 min.) and 8-60 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) were used as detection system. Using these protocol settings, 68 of 68 (100%) laboratories produced a sufficient staining result.

rmAb clone **SP4**, product no. **RMA-0541**, Maixin, manual staining:
One protocol with an optimal result was based on HIER using Tris-EDTA pH 9 in a waterbath, 60 min. incubation of the primary Ab. and KIT-5230 (Maixin) as detection system.

Comments
In this fifth NordiQC run for CyD1, a pass rate of 94% was observed. Only 15 of 257 (6%) laboratories obtained an insufficient mark due to a too weak staining intensity or false negative reaction in cellular structures expected to be demonstrated.

The majority of laboratories used the rmAb clone SP4. In total, 173 of 257 (67%) laboratories used this clone either as a concentrate within a laboratory developed (LD) assay or as RTU format. Within LD assays, rmAb clone SP4 provided optimal staining results on all three main IHC systems, see table 3. However, for unexplained reasons, a noticeable lower proportion of optimal results were obtained on the BOND IHC system, compared to BenchMark (Ventana) and Autostainer (Dako) as shown in table 3. Despite applying comparable protocol settings for clone rmAb SP4 on the three systems, only 13% of the results (2 of 15) on the BOND system were assessed as optimal compared to 64% and 49% on Autostainer and BenchMark, respectively. As mentioned, the reasons for this difference are unclear and no single parameter causing this, was identified.
Efficient HIER in alkaline buffer and careful calibration of the primary Ab were the two most central parametres for optimal results. Choice of detection system was of less importance. Both 2-step and 3-step

multimer / polymer based detection systems could be used to obtain optimal staining results, provided that the titre of the primary Ab was adjusted. Five protocols were based on HIER using citrate at pH 6 none of which resulted in an optimal mark.
The recently introduced rmAb clone EP12 also provided optimal staining results on all three main IHC systems within LD assays.
Overall, using one of these clones within a LD assay a pass rate of 91% (95 of 105) was obtained of which 42% were optimal.

Corresponding RTU systems based on the rmAb clones SP4 and EP12 were used by 57% (146 of 257) of all laboratories.
For the Ventana RTU system based on the rmAb clone SP4 (790-4508), a pass rate of 99% was seen. Optimal results could be obtained both by use of the vendor recommended protocol settings (HIER in CC1 for 64 min. 16-24 min. incubation time of the primay Ab and UltraView as detection system) and laboratory modified protocols, typically reducing HIER time and/or change of detection system. In a few cases, a weak unexpected nuclear staining reaction was seen in mantle zone B-cells of the tonsils. This pattern was seen by protocols using a reduced HIER time (range of 32 – 36 min.). This staining pattern was evaluated as sufficient and marked as "Good", as an otherwise optimal and expected result was achieved.

The Dako RTU system IR083/IS083 based on the rmAb EP12 provided a pass rate of 98% and both vendor recommended and laboratory modified protocol settings could produce optimal results. A reduced efficient HIER time from the recommended 20 min. to 10 min. was found less successful as 4 of 4 protocols all were assessed as "Good" and thus none evaluated as optimal. It was observed that the product IR083 intended for Autostainer Link 48 was used by 9 laboratories on the Omnis system (Dako) and by 3 laboratories on the BOND system (Leica) and an optimal result could be generated on both IHC platforms. On Omnis, an optimal result was obtained by HIER in TRS High for 30 min., 25 min. incubation time of the primary Ab and EnVision FLEX as detection system. On BOND, an optimal result was based on HIER in BERS2 for 20 min., 15 min. incubation time of the primary Ab and Refine DAB as detection system.

This was the fifth NordiQC assessment of CyD1. Consistent improvement of the pass rate has been obtained since the first assessment in run 9, 2003. The pass rate improved from 53% in 2003 to 94% in the current run. The primary reason for this improvement seems closely related to the extended use of rmAb clones SP4 and EP12 at the expense of "old" clones as DCS6 and P2D11F11. In the current run, 98% (251 of 257) used rmAb clones SP4 and EP12 while only 2% (5 of 257) used P2D11F11. In run 9, 5% (3 of 57) used rmAb clone SP4, while 86% (49 of 57) used mAb clones DCS6 or P2D11F11.
The access to and use of optimally calibrated RTU systems for CyD1 also has impacted the increase in pass rate.

Controls
Tonsil can be recommended as positive and negative tissue control for CyD1. Virtually all suprabasal squamous epithelial cells, scattered lymphocytes and endothelial cells must show a moderate to strong distinct nuclear staining reaction, whereas a weak nuclear staining reaction of germinal centre macrophages should be seen. A weak cytoplasmic staining reaction together with the specific nuclear reaction must be accepted. Mantle zone B-cells and germinal centre B-cells should be negative.

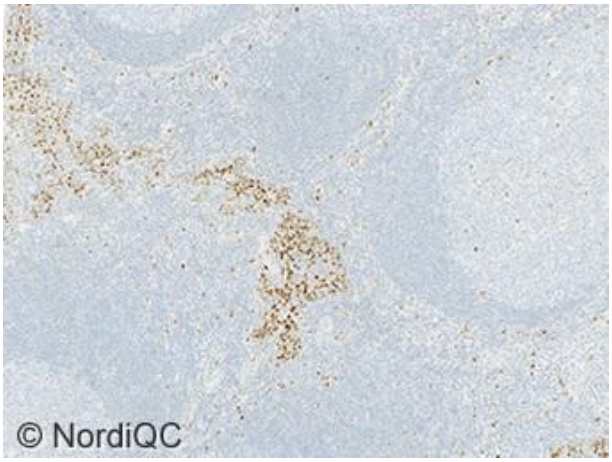

Fig. 1a
Optimal staining for Cyclin D1 of the tonsil, tissue core no. 1, using the rmAb clone SP4-R as Ready-To-Use format (Ventana prod. no. 790-4508) using HIER in CC1 for 64 min. and UltraView as detection system.
Even at low power field squamous epithelial cells, dispersed endothelial cells and germinal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

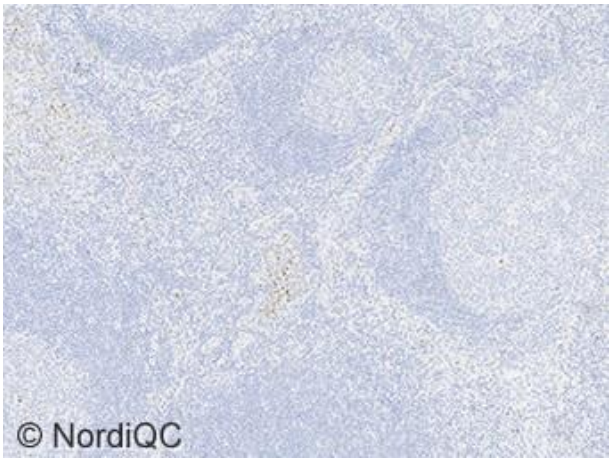

Fig. 1b
Insufficient staining for Cyclin D1 of the tonsil, tissue no. 1, using the rmAb clone SP4 by a laboratory developed assay giving a too low sensitivity (too low. conc. of the primary Ab) - same field as in Fig. 1a.
The proportion of positive cells and the intensity of the staining reaction are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

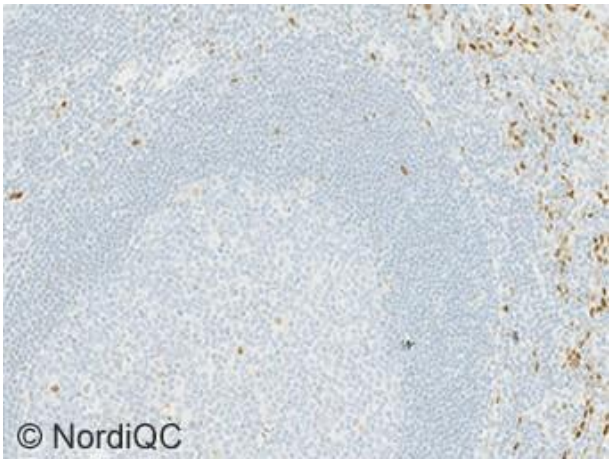

Fig. 2a
Optimal staining for Cyclin D1 of the tonsil, tissue core no. 1, using same protocol as in Fig. 1a. High power field x200.
Virtually all squamous epithelial cells, dispersed endothelial cells and germinal centre macrophages show a moderate to strong nuclear staining reaction. The vast majority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

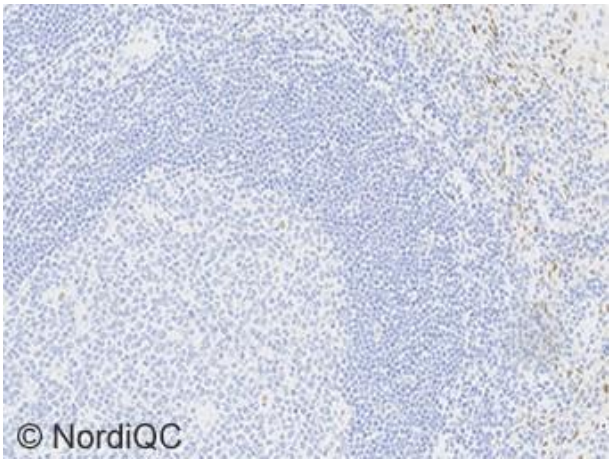

Fig. 2b
Insufficient staining for Cyclin D1 of the tonsil, tissue core no. 1, using same protocol as in Fig. 1b - same field as in Fig. 2a.
Only scattered squamous epithelial cells show a weak and equivocal staining reaction, while endothelial cells and germinal centre macrophages are negative.
Also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

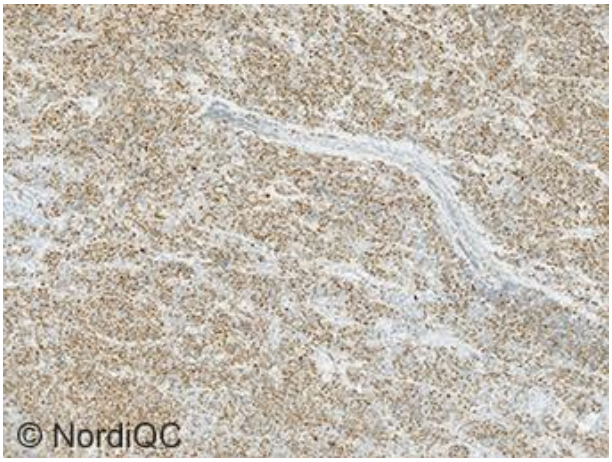

Fig. 3a
Optimal staining for Cyclin D1 of the mantle cell lymphoma, tissue core no. 4, using same protocol as in Figs. 1a & 2a.
Virtually all the neoplastic cells show a distinct, moderate to strong nuclear staining reaction.

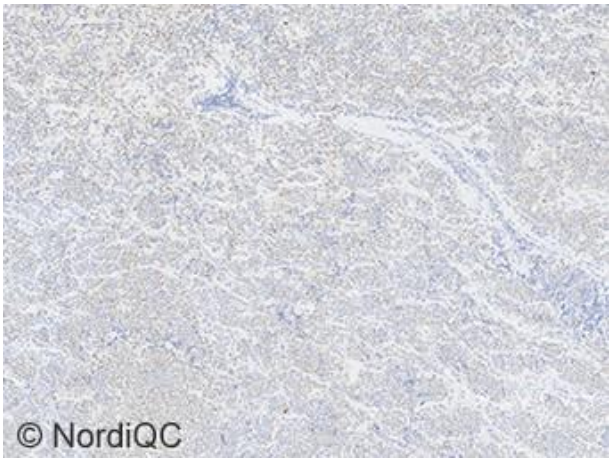

Fig. 3b
Insufficient staining for Cyclin D1 of the mantle cell lymphoma, tissue core no. 4, using same protocol as in Figs. 1b & 2b – same field as in Fig. 3a.
The proportion of positive cells and the intensity of the staining reaction are significantly reduced compared to the result expected and obtained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

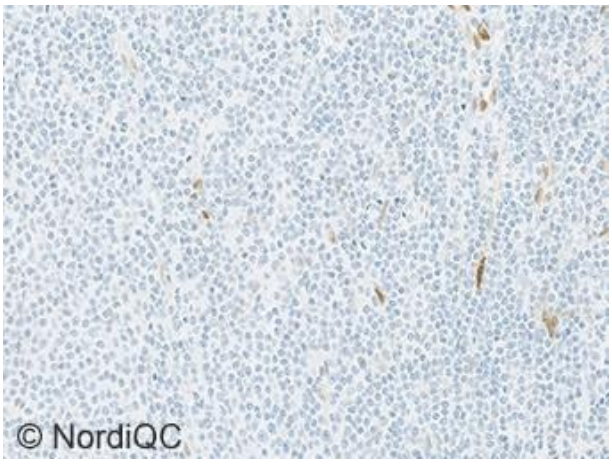

Fig. 4a
Optimal staining for Cyclin D1 of the B-CLL using same protocol as in Figs. 1a - 3a.
The neoplastic cells are negative, while scattered endothelial cells show a moderate nuclear staining reaction serving as internal positive tissue control.

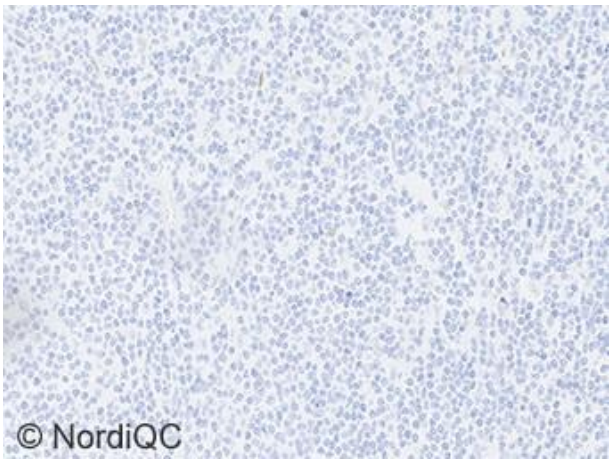

Fig. 4b
Staining for Cyclin D1 of the B-CLL using same insufficient protocol as in Figs. 1b - 3b – same field as in Fig. 4a.
No staining is seen.

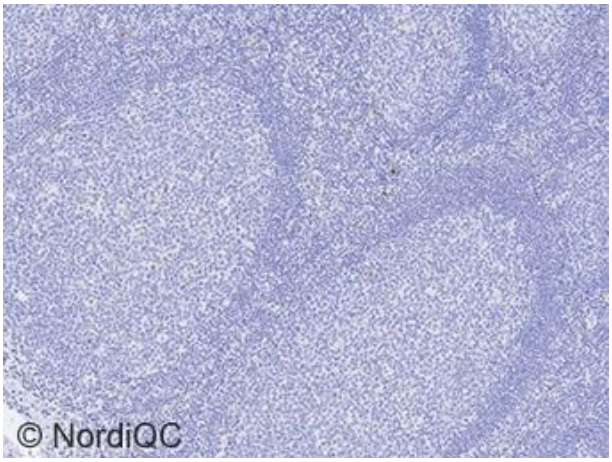

Fig. 5a
Insufficient staining for Cyclin D1 of the tonsil, tissue core no. 1. A combination of a protocol giving a too low sensitivity and excessive counterstaining compromises the interpretation and a reduced number of positive cells cells is demonstrated
Also compare with Fig. 5b, same protocol.

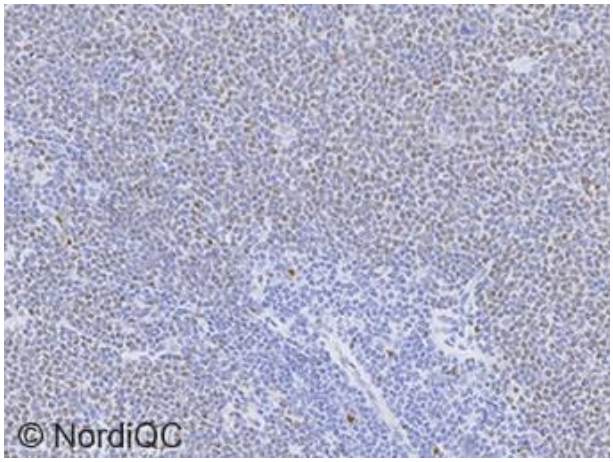

Fig. 5b
Insufficient staining for Cyclin D1 of the mantle cell lymphoma, tissue core no. 4. The combination of a protocol giving a low sensitivity and excessive counterstaining compromises the interpretation of the signal in the neoplastic cells.

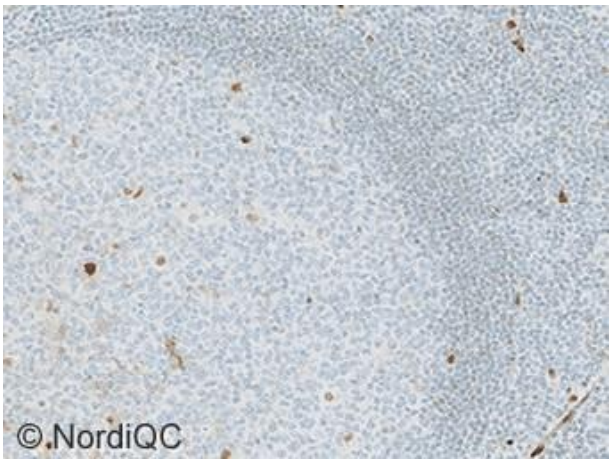

Fig. 6a
Staining for Cyclin D1 of the tonsil, tissue core no. 1, using the rmAb clone SP4-R as Ready-To-Use format (Ventana prod. no. 790-4508) using HIER in CC1 for 32 min. and OptiView as detection system.
A weak unexpected distinct nuclear staining reaction is seen in virtually all mantle zone B-cells. This staining pattern was marked as "Good", as an otherwise optimal and expected result was achieved in both the 2 mantle cell lymphomas and of particular interest in the B-CLL expected to be negative.
Also see Fig. 6b, B-CLL, same protocol.

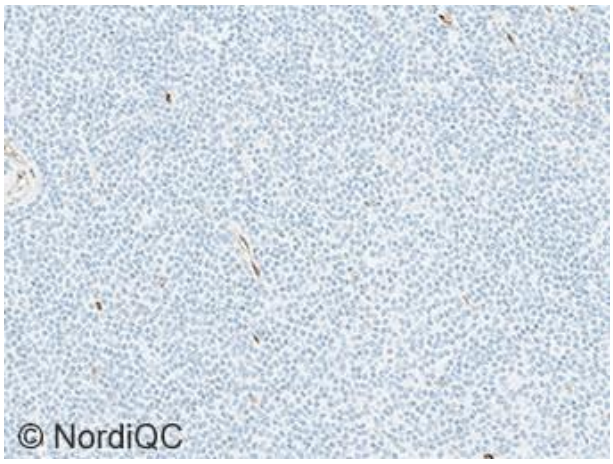

Fig. 6b
Staining for Cyclin D1 of the B-CLL using same protocol as in Fig. 6a.
The neoplastic cells are negative, while scattered endothelial cells show a moderate nuclear staining reaction.
Despite the aberrant staining pattern in the tonsil, the protocol provided the overall result expected.
The combination of reduced HIER time and a more sensitive detection system might have impacted the staining pattern.
Compare Figs. 1a and 6a.

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