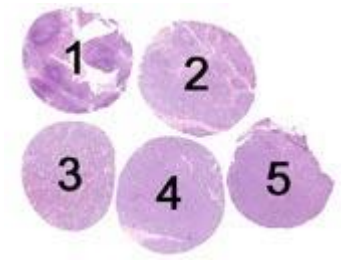
Assessment Run 33 2011

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 a CyD1 staining as optimal included:

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

A weak cytoplasmic staining reaction was accepted, providing this did not complicate the interpretation.

179 laboratories participated in this assessment. 90 % achieved a sufficient mark. In table 1 the antibodies (Abs) used and marks are summarized.

Table 1. **Abs and assessment marks for CyD1, run 33**

Concentrated Abs	N	Vendor	Optimal	Good	Borderl.	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **DCS-6**	2	Dako	0	0	1	1	-	-
mAb clone **P2D11F11**	5	Leica/Novocastra	0	5	0	0	100 %	-
rmAb clone **SP4**	79 15 4 1 1 1 1 1	Thermo/NeoMarkers Dako Biocare Cell Marque Immunologic Maxin Master Diagnostica Spring Zytomed	56	36	8	4	88 %	95 %
Ready-To-Use Abs								
mAb clone **P2D11F11**	2	Leica/Novocastra	0	2	0	0	-	-
rmAb clone **EP12 PME432**	1	Biocare	1	0	0	0	-	-
rmAb clone **EP12 IR083**	1	Dako	1	0	0	0	-	-
rmAb clone **SP4 760-4282**	34	Ventana/Cell Marque	23	10	0	1	97 %	100 %
rmAb clone **SP4 IS/IR152**	23	Dako	18	4	0	1	96 %	100 %
rmAb clone **SP4 RM-9104-R7**	2	Thermo/NeoMarkers	1	0	0	1	-	-
rmAb clone **SP4 241R-17**	1	Cell Marque	0	1	0	0	-	-
rmAb clone **SP4-R 790-4508**	4	Ventana	1	2	1	0	-	-
Total	179		101	60	10	8	-	-
Proportion			56 %	34 %	6 %	4 %	90 %	-

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

Following central protocol parameters were used to obtain an optimal staining:

Concentrated Abs
rmAb clone **SP4**: The protocols giving an optimal result were all based on heat induced epitope retrieval (HIER) using either Cell Conditioning 1 (CC1; BenchMark, Ventana) (15/22)*, Bond Epitope Retrieval Solution 2 (BERS 2; Bond, Leica) (6/17), Target Retrieval Solution pH 9 (3-in-1) (TRS pH 9; Dako) (13/18), TRS pH 9 (Dako) (6/15), Borg Decloaker pH 9.5 (Biocare) (2/2), Tris-EDTA/EGTA pH 9 (8/16), EDTA/EGTA pH 8 (1/4) or Citrate pH 6 (3/7) as the retrieval buffer.
The mAb was typically diluted in the range of 1:20-1:75 depending on the total sensitivity of the protocol employed. Using these protocol settings 79 out of 83 (95 %) laboratories produced a sufficient staining (optimal or good).
2 laboratories with an optimal result used a combined pre-treatment by using HIER in CC1 (BenchMark, Ventana) followed by enzymatic pre-treatment in Protease 3 (Ventana). Using these protocol settings 3 out of 3 laboratories produced a sufficient staining.
*(number of optimal results/number of laboratories using this buffer)

Ready-To-Use Abs
rmAb clone **EP12** (prod. no. PME432, Biocare): The protocol giving an optimal result was based on HIER in a Pressure Cooker (30 sec. at 125°C) using Borg Decloaker pH 9.5 as HIER buffer, an incubation time of 30 min in the primary Ab and MACH4 Universal HRP polymer (MRH534) as the detection system.

rmAb clone **EP12** (prod. no. IR083, Dako): The protocol giving an optimal result was based on HIER in PT-Link (heating for 20 min at 97°C) using TRS pH 9 (3-in-1) as HIER buffer and an incubation time of 20 min in the primary Ab and EnVision Flex (K8000) as the detection system.

rmAb clone **SP4** (prod. no. 760-4282, Ventana/Cell Marque): The protocols giving an optimal result were based on HIER using mild, standard or extended CC1, an incubation time of 24-60 min in the primary Ab and iView (760-091) or UltraView (760-500) with or without amplification kit as the detection system.
Using these protocol settings 31 out of 31 (100 %) laboratories produced a sufficient staining.

rmAb clone **SP4** (prod. no. IS/IR152, Dako): The protocols giving an optimal result were all based on HIER in PT-Link using either TRS pH 9 (3-in-1) or TRS pH 9 as HIER buffer, a heating time for 10 - 30 min at 95-97°C, an incubation time of 20-30 min in the primary Ab and EnVision Flex (K8000) or Flex+ (K8002) as the detection system. Using these protocol settings 22 out of 22 (100 %) laboratories produced a sufficient staining (optimal or good).

rmAb clone **SP4-R** (prod. no. 790-4508, Ventana): The protocol giving an optimal result was based on HIER using standard CC1, an incubation time of 64 min in the primary Ab and UltraView (760-500) with amplification kit as the detection system.

The most frequent causes of insufficient stains were:

- Too low concentration of the primary antibody
- Insufficient HIER (too short efficient heating time)
- Less successful primary Ab.

In this assessment and in concordance with the previous NordiQC assessments for CyD1 the prevalent feature of an insufficient staining was a too weak or false negative reaction of the cells expected to be demonstrated. A too weak or false negative staining was seen in 67 % of the insufficient results (12 out of 18). In the remaining 33 % both a too weak and a false positive staining was seen.
The too weak staining was in virtually all cases characterized by a weak or equivocal nuclear staining in both the neoplastic cells of the mantle cell lymphomas, the suprabasal squamous epithelial cells of the tonsils and scattered endothelial cells. The identification of endothelial cells was found to be a reliable internal positive control in all the 5 tissues stained and in particular valuable in the B-CLL, in which the neoplastic cells were negative for CyD1.
A too low concentration of the primary Ab (clone SP4) and/or a too short HIER time were the main reasons for the insufficient stains. The false positive staining reactions and poor signal-to-noise ratios were mainly caused by the use of mAb clone DCS-6, but could also seen with rmAb clone SP4 e.g., giving a moderate nuclear staining in germinal centre B-cells and a weak but widespread nuclear staining in lymphocytes. No obvious reason for this aberrant pattern for the rmAb clone SP4 could be identified. In a couple of cases contamination with another primary Ab may be the reason for false positivity.

The tonsil was found to be a reliable control for CyD1: The majority of the suprabasal squamous epithelial cells, scattered lymphocytes and endothelial cells must show a moderate to strong distinct nuclear staining reaction. If these cells were negative or only weakly demonstrated, the neoplastic cells in the two mantle cell lymphomas were negative or only showed an equivocal reaction. Virtually all mantle zone B-cells and germinal centre B-cells should be negative.

The newly launched rmAb clone EP12 gave a staining reaction virtually identical with that of rmAb clone SP4, when the clone was used as a Ready-To-Use format from Dako and optimized for the Dako Autostainer Link system. A slightly different reaction pattern was seen when the clone was applied as a Ready-To-Use format from Biocare and optimized for the intelliPATH FLX system. With the latter, a distinct staining of the proliferation centres of the B-CLL was observed that was not seen with clone SP4. The staining of the proliferation centres was in this assessment accepted as a true positive reaction but has to be evaluated further (as a cross-reaction with e.g., Cyclin D2 might be considered).
This was the 4th assessment of CyD1 in NordiQC (Table 2). A major increase in the pass rate has been achieved during all 4 runs, despite that the number of participants nearly is doubled in this run compared with the previous run in 2007.

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

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

The marked improvement of the pass rate for CyD1 is highly influenced by the expanding use of the rmAb clone SP4 (table 3), which has shown to be a highly robust marker for CyD1 that can be applied in practically all IHC stainer systems. In all the four NordiQC assessments for CyD1 the rmAb clone SP4 has shown a superior performance compared to the other, older Abs used.

Table 3. **Proportion of sufficient results related to the most commonly Abs used**

	Run 9 2003		**Run 17 2006**		**Run 19 2007**		**Run 33 2011**		**Total**	
	Num.	Suff.	Num.	Suff.	Num.	Suff.	Num.	Suff.	Num.	Suff.
mAb clone **DCS-6**	25	6	11	0	5	0	2	0	38	6 (16%)
mAb clone **P2D11F11**	24	18	16	4	3	2	7	7	50	31 (62%)
rmAb clone **SP4**	3	3	55	44	80	64	165	150	303	261 (86%)

Conclusion

The rmAb clone SP4 is the most widely used clone for CyD1 and was found to be very robust giving an overall pass rate of 95 - 100 %. HIER is mandatory for an optimal performance, preferably in an alkaline buffer. The clone can be applied with the commonly used IHC platforms.
Tonsil is a recommendable control: The majority of suprabasal squamous epithelial cells, scattered lymphocytes and endothelial cells must show a moderate to strong distinct nuclear staining reaction. Virtually all mantle zone B-cells and germinal centre B-cells should be negative. The positive staining of endothelial cells is a valuable internal positive control for CyD1 in e.g., B-CLL.

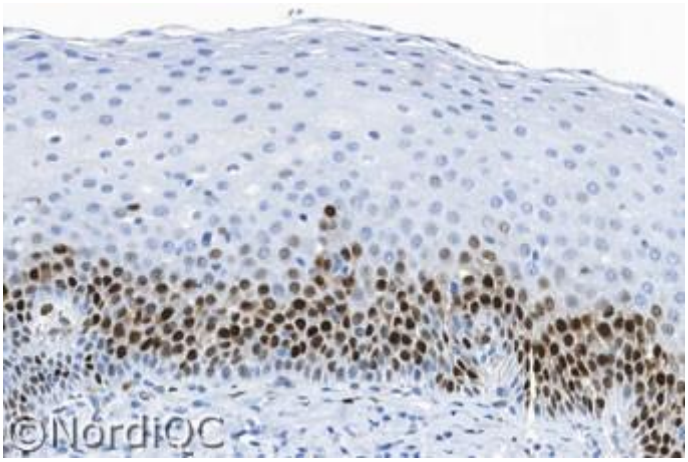


Fig. 1a
Optimal staining for Cyclin D1 of the tonsil no. 1 using the rmAb clone SP4 optimally calibrated and with HIER in an alkaline buffer.
The suprabasal squamous epithelial cells show a moderate to strong nuclear staining reaction and only a faint intra-cytoplasmic staining.

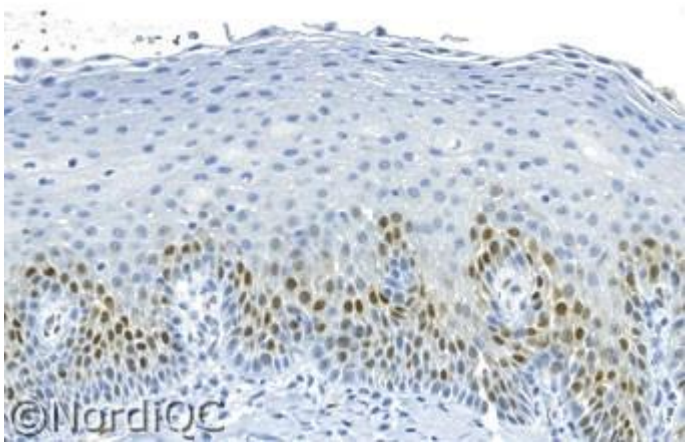


Fig. 1b
Insufficient staining for Cyclin D1 of the tonsil no. 1 using the rmAb clone SP4 by a protocol with a too low sensitivity (too low. conc. of the primary Ab) - same field as in Fig. 1a.
The proportion of positive cells and the intensity of the staining reaction are significantly reduced compared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

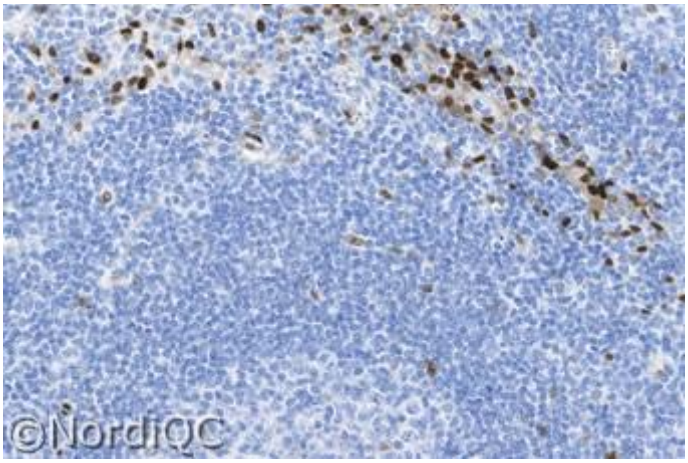


Fig. 2a
Optimal staining for Cyclin D1 of the tonsil no. 2 using same protocol as in Fig. 1a.
Both the squamous epithelial cells and scattered endothelial cells show a moderate to strong nuclear staining reaction and only a faint intra-cytoplasmic staining. No background staining is seen.

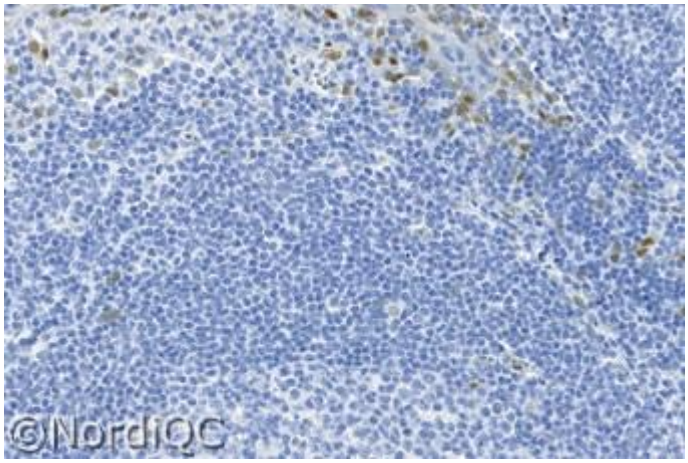


Fig. 2b
Insufficient staining for Cyclin D1 of the tonsil no. 2 using same protocol as in Fig. 1b. - same field as in Fig. 2a.
Only scattered squamous epithelial cells show a weak and equivocal staining reaction, while the endothelial cells are negative.
Also compare with Fig. 3b., same protocol.

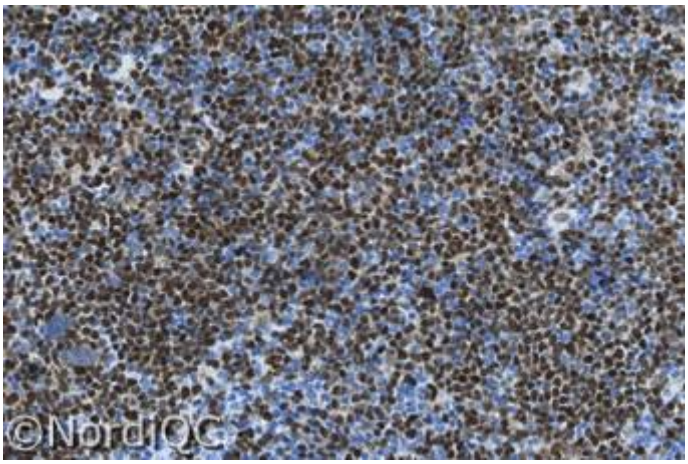


Fig. 3a
Optimal staining for Cyclin D1 of the mantle cell lymphoma no. 4 using same protocol as in Figs. 1a & 2a.
Virtually all the neoplastic cells show a distrinct, moderate to strong nuclear staining reaction.

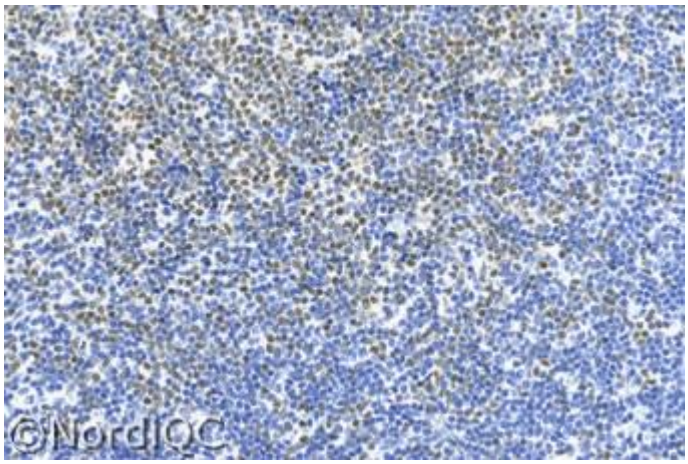


Fig. 3b
Insufficient staining for Cyclin D1 of the mantle cell lymphoma no. 4 using same protocol as in Figs. 1b. & 2b. – same field as in Fig. 3a.
The proportion of positive cells and the intensity of the staining reaction are significantly reduced compared to the result obtained in Fig. 3a.

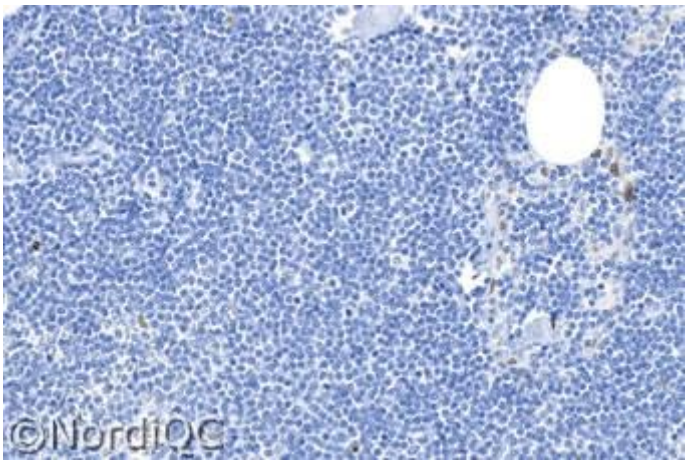


Fig. 4a
Optimal staining for Cyclin D1 of the B-CLL using same protocol as in Figs. 1a. - 3a.
The neoplastic cells are negative, while scattered endothelial cells show a moderate nuclear staining reaction serving as internal positive control.

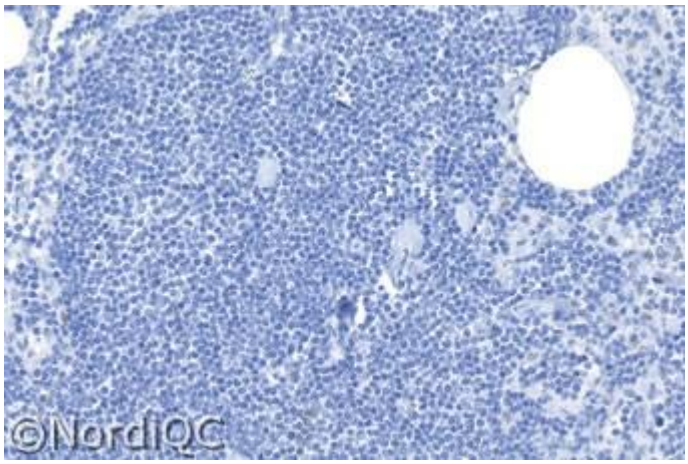


Fig. 4b
Insufficient staining for Cyclin D1 of the B-CLL using same protocol as in Figs. 1b. - 3b. – same field as in Fig. 4a.
No staining is seen in any cells.

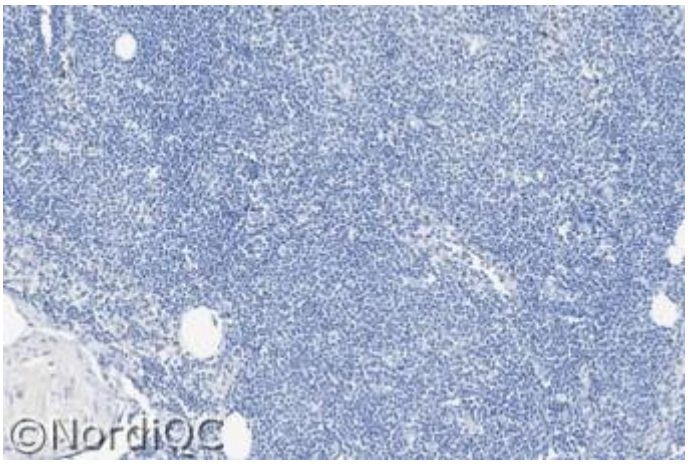


Fig. 5a
Optimal staining for Cyclin D1 of the B-CLL using a protocol based on the rmAb clone SP4.
At low magnification only scattered endothelial cells are demonstrated, while the neoplastic cells are negative.
This staining pattern was seen in all optimal protocols based on the rmAb clone SP4.
Also compare with Fig. 5b – same field, but a protocol based on the newly launched rmAb clone EP12.

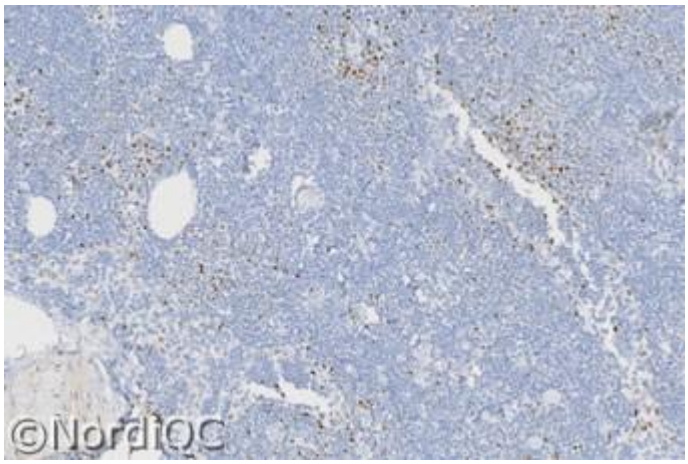


Fig. 5b
Optimal staining for Cyclin D1 of the B-CLL using a protocol based on the rmAb clone EP12.
At low magnification the majority of the neoplastic cells are negative, but a positive staining reaction is seen in the proliferation centres of the B-CLL.
This staining pattern was seen in both protocols based on the rmAb clone EP12.
Otherwise the staining pattern was identical to the pattern obtained for the rmAb clone SP4, as shown in Figs. 1a – 3a.

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