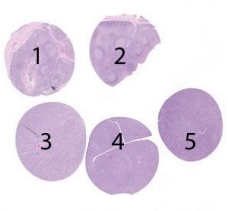
Assessment Run 49 2017
CD5

Material
The slide to be stained for CD5 comprised:



1. Tonsil fixed 24h, 2. Tonsil fixed 48h, 3-4. Mantle cell lymphomas (MCL), 5. B-cell chronic lymphatic leukemia (B-CLL).

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CD5 staining as optimal included:

- A strong and distinct, predominantly membranous staining reaction of virtually all T-cells in both T-zones and in germinal centres in the tonsils.
- An at least weak to moderate, distinct membranous staining reaction of dispersed B-cells in the mantle zones of the tonsil (core 1) and of the majority of B-cells in the mantle zones of the tonsil (core 2).
- A strong, distinct membranous staining reaction of all neoplastic cells in the MCL (core 3) and virtually all neoplastic cells of the B-CLL.
- An at least weak to moderate, distinct membranous staining reaction of all the neoplastic cells in the MCL (core 4).
- A strong, distinct membranous staining reaction of all T-cells intermingling with the neoplastic cells of the MCLs and of the B-CLL.
- No staining reaction of germinal centre B-cells.

Staining reaction of squamous epithelial cells lining tonsillar crypts was accepted, as this was seen with all the antibodies (Abs) used in this assessment.

Participation

Number of laboratories registered for CD5, run 49	297
Number of laboratories returning slides	278 (94%)

Results
278 laboratories participated in this assessment. 256 (92%) achieved a sufficient mark (optimal or good). Table 1 summarizes the Abs used and the assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Insufficient HIER (too short heating time)
- Unexplained technical issues

Performance history
This was the fifth NordiQC assessment of CD5. The overall pass rate was high and has increased significantly compared to the result obtained in run 34, 2012 (see table 2).

Table 2. **Proportion of sufficient results for CD5 in the five NordiQC runs performed**

	Run 8 2003	Run 17 2006	Run 24 2008	Run 34 2012	Run 49 2017
Participants, n=	65	88	119	187	278
Sufficient results	65%	66%	68%	79%	92%

Conclusion
The mAb clone **4C7** and rmAb clone **SP19** could both be used to obtain an optimal staining for CD5. In this assessment, the Ready-To-Use (RTU) systems from the main IHC providers obtained superior pass rates and higher proportion of optimal results compared to laboratory developed (LD) tests. Within LD

tests, optimal results required efficient HIER, preferable in alkaline buffer, and careful calibration of the primary Ab titre.
Tonsil is recommendable as positive and negative tissue control for CD5. Virtually all T-cells must show a strong membranous staining reaction, while an at least weak to moderate and distinct staining reaction must be seen in dispersed mantle zone B-cells in secondary follicles. No staining reaction must be seen in the germinal center B-cells.

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

Concentrated antibodies	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 antibodies								
mAb clone **4C7 IR/IS082**	39	Dako/Agilent	27	10	1	1	95%	97 %
mAb clone **4C7 IR/IS082**[3]	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.

Detailed analysis of CD5, run 49
The following protocol parameters were central to obtain optimal staining.

Concentrated antibodies
mAb **4C7**: Protocols with optimal results were all based on heat induced epitope retrieval (HIER) using alkaline buffer as Cell Conditioning 1 (CC1) (BenchMark, Ventana) (18/31)*, TRS High pH (3-1) (Dako/Agilent) (13/19), TRS High pH (1/1), Bond Epitope Retrieval Solution 2 (Bond, Leica) (8/17), Epitope Retrieval Solution pH 9 (RE7119, Leica/Novocastra) (1/1) or TRIS-EDTA pH 9 (2/5) as retrieval buffer. The mAb was typically diluted in the range of 1:20 – 1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings, 55 of 59 (93%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

rmAb **SP19**: Protocols with optimal results were based on HIER using alkaline buffer as CC1 (BenchMark, Ventana) (9/12), TRS High pH (3-1) (Dako/Agilent) (2/7), or TRIS-EDTA pH 9 (2/2) as retrieval buffer. One protocol with an optimal result was based on HIER in standard citric based buffer pH 6 (1/1) and one laboratory obtained an optimal mark without performing any pre-treatment at all (1/1). The mAb was typically diluted in the range of 1:25 – 1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings, 19 of 23 (83%) laboratories produced a sufficient staining. (optimal or good)

Table 3. **Proportion of optimal results for CD5 for the most commonly used antibodies as concentrate on the 3 main fully-automated IHC systems***

Concentrated antibodies	**Dako/Agilent Omnis**		**Ventana BenchMark XT / Ultra/GX**		**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 **4C7**	3/5 ** (60%)	-	15/26 (58%)	-	5/11 (45%)	-
rmAb clone **SP19**	2/3	-	8/11 (73%)	-	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)

Table 4. **Proportion of optimal results for CD5 for the most commonly used antibodies as concentrate on the 3 main semi-automated IHC systems ***

Concentrated antibodies	**Dako/Agilent Autostainer Link / Classic**		**Thermo/LabVision Autostainer 36/48/72**		**Biocare Medical IntelliPATH**	
	TRS pH 9.0	TRS pH 6.1	HIER buffer H pH 9.0	HIER Buffer L pH 6.0	Borg pH 9.0	Diva pH 6.2
mAb clone **4C7**	8/11 ** (73%)	-	-	-	2/2	-
rmAb clone **SP19**	0/4	-	-	-	-	-

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

Ready-To-Use antibodies and corresponding systems
mAb clone **4C7,** product no. **IR/IS082,** Dako/Agilent Autostainer+ /Autostainer Link:
Protocols with optimal results were based on HIER using TRS pH 9 (3-1) (efficient heating time 10-20 min. at 95-99°C), 20-30 min. incubation of the primary Ab and EnVision Flex (K8000/K8002/K8010/K8012) as detection system. Using these protocol settings, 32 of 33 (97%) laboratories produced a sufficient result. One laboratory obtained an optimal result using same protocol settings as above but without performing any pre-treatment at all.

mAb clone **4C7,** product no. **PA0168,** Leica/Novocastra BOND III/BOND MAX:
Protocols with optimal results were based on HIER using BERS2 or BERS1 (efficient heating time 20-30 min. at 99-100°C), 15-30 min. incubation of the primary Ab and BOND Refine (DS9800) as detection system. Using these protocol settings, 9 of 10 (90%) laboratories produced a sufficient result.

rmAb clone **SP19,** product no. **790-4451,** Ventana Benchmark Ultra/XT:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 4-90 min.) and 12-60 min. incubation of the primary Ab. UltraView (760-500) or OptiView (760-700) with or without amplification (760-080 or 860-099, respectively) were used as detection systems. Two protocols with optimal marks were based on no pre-treatment at all. Using these protocol settings, 87 of 88 (99%) laboratories produced a sufficient staining result.

Table 5 summarises 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 accordingly to the vendor recommendations and by laboratory modified systems changing basal protocol settings. Only protocols performed on the specific IHC stainer device are included, whereas e.g. Dako RTU Ab formats applied on a Ventana stainer device were excluded.

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.

Comments

In this fifth NordiQC assessment of CD5, the prevalent feature of an insufficient result was a generally too weak staining reaction of cells expected to be demonstrated and/or a false negative staining result. This pattern was seen in all the insufficient results (n=22). The majority of the laboratories were able to stain CD5 in T-cells (all specimens) and neoplastic cells of the MCL (core 3), whereas demonstration of CD5 in mantle zone B-cells in the tonsils and neoplastic B-cells of the MCL (core 4) was more challenging and required a carefully calibrated protocol. In 14% (3 of 22) of the insufficient results, a poor signal-to-noise ratio (1 protocol) or weak to false negative staining reaction (2 protocols) due to unexplained technical issues was seen.

The mAb clone 4C7 was the most widely used antibody for the demonstration of CD5. Used as a concentrate in a LD assay, mAb clone 4C7 gave an overall pass rate of 89% (71 of 80). As shown in table 3, optimal results could be obtained on all three main fully-automated IHC systems from Dako/Agilent, Leica/Novocastra and Ventana/Roche. Optimal performance could also be accomplished on the semi-automated IHC systems from Dako/Agilent (Autostainer Link/Classic) and Biocare Medical (IntelliPATH) (see table 4). Efficient HIER in an alkaline buffer, careful calibration of the primary Ab and use of a sensitive detection system were the central parameters for optimal results. Correct titre of the primary Ab was crucial as this significantly influenced the technical and analytical sensitivity of the assay. For protocols based on the concentrate of mAb clone 4C7, use of HIER in alkaline buffer applied in combination with a standard 2- or 3-step multimer/polymer detection system, the Average Dilution Value (ADV) for optimal results was 1:142 (range 1:10-1:1,200), whereas a ADV of 1:282 (range 1:20-1:1,150) was seen in protocols with insufficient results. Consequently, the titer must be carefully calibrated to provide an IHC protocol that is able to demonstrate CD5 in structures with both low-level and high-level CD5 expression, which is the range seen in different haematological neoplasias (e.g. subtypes of B-CLL). In this assessment, the pass rate for protocols using the mAb 4C7 as concentrated format was not influenced by the choice of detection system (2- versus 3-step multimer/polymer detection systems).

The rmAb clone SP19 used as concentrated format within a LD assay also provided a relatively high pass rate of 91% (20 of 22). Optimal results could be obtained on both the fully-automated IHC systems from Roche/Ventana (Benchmark XT/Ultra/GX) or Dako/Agilent (Omnis) (Table 3) and the semi-automated IHC system from Dako/Agilent (Autostainer Link/Classic) (see table 4). As for the mAb clone 4C7, the rmAb SP19 also required efficient HIER (especially efficient heating time ≥ 20 min. at 97-100°C in alkaline buffer) or high temperature at 110°C for 3 min. (TRIS/EDTA based buffer pH9) or 10 min. (Citrate based buffer pH6), careful calibration of the primary Ab and use of a sensitive detection system.

Ready-To-Use (RTU) formats were used by 62% (171 of 278) of the laboratories. The Ventana RTU system based on rmAb clone SP19 (790-4451) was the most widely used RTU system applied by 88 laboratories. An overall pass rate of 99% was seen, 86% optimal. Optimal results could both be obtained by the use of vendor recommended or laboratory modified protocol settings (see table 5). Although there was no significant difference in the pass rate using either UltraView or OptiView as the detection system, the proportion of optimal results was lower using UltraView (all protocol settings). 76% (26 of 34) were optimal using UltraView, whereas 97% (31 of 32) produced an optimal result if OptiView was used.

In this assessment, the RTU systems PA0168 and IR/IS082 based on the mAb clone 4C7 from Leica/Novocastra and Agilent/ Dako, respectively, also provided a high number of sufficient and optimal results (see table 1). These systems were used by 18% (51 of 278) of the laboratories.

For laboratories using the Dako/Agilent RTU system IR/IS082 (Autostainer Link/Classic), an overall pass rate of 95% was obtained of which 69% (27 of 39) were optimal. Optimal results could be obtained by using vendor protocol recommendations or by using laboratory modified protocol settings (mostly adjusting HIER time). Three laboratories used a Flex+ protocol – none of these was assessed as optimal (all marked as good). The Dako/Agilent RTU system IR/IS082 was used by 13 laboratories on the Omnis platform. The proportion of sufficient and optimal results was comparable to that obtained on the Autostainer Link/Classic. The basic protocol parameters on the Omnis were HIER in TRS High pH for 30 min., incubation in primary Ab for 20 min. and Flex as the detection system (20 min. incubation). In that context, the ideal RTU format of a primary Ab is used within a system with precise information on vendor recommended protocol settings, equipment, reagents and results expected. Therefore, it is not advisable to use a RTU format of the primary Ab on a system/platform for which it has not been developed and validated, although it might produce optimal results (see table 1).

For the Leica RTU system PA0168 (BOND III/MAX), an overall pass rate of 92% was obtained of which 75% (9 of 12) were optimal. Both vendor protocol recommendations and laboratory modified protocol settings (typically adjusting HIER buffer, HIER time and incubation time in primary Ab) could produce optimal results.

This was the fifth NordiQC assessment of CD5 and a pass rate of 92% was obtained, which is a significant improvement compared to the result obtained in run 34, 2012 (see table 2). Especially the extended use of robust and successful Ready-To-Use (RTU) systems/formats used by 62% of the participants (171 of 278) in this assessment compared to 38% (72 of 187) in run 34, 2012 had a positive impact on the pass rate. For LD assays, most laboratories used the corresponding concentrated formats of mAb clone 4C7 and rmAb SP19, whereas none used the less successful mAb clone CD5/54/F6. Essentially, efficient HIER preferable in alkaline buffer, carefully calibration of the primary Ab and the use of a sensitive detection system are still prerequisites for optimal results.

Controls
Tonsil is recommendable as positive and negative tissue control, in which dispersed B-cells in the mantle zone of the secondary follicles must display a weak to moderate and distinct membranous staining reaction. If these cells were negative or only faintly demonstrated, the proportion of positive neoplastic cells especially in the mantle cell lymphoma, core no. 4, was reduced or false negative. T-cells will be strongly stained. No staining must be seen in the germinal center B-cells.

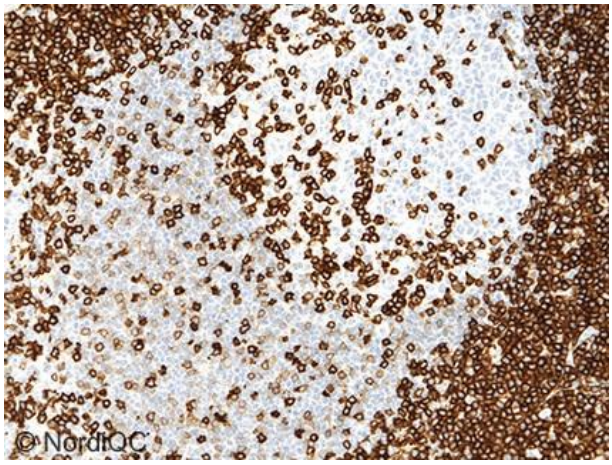


Fig. 1a (x200)
Optimal staining for CD5 of the tonsil, core 1, using the mAb 4C7 as a concentrate, HIER in an alkaline buffer (BERS2) and a polymer based detection system (BOND Refine, Leica) - same protocol used in Figs. 2a - 5a. The T-cells in the interfollicular T-zone and within the germinal centre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

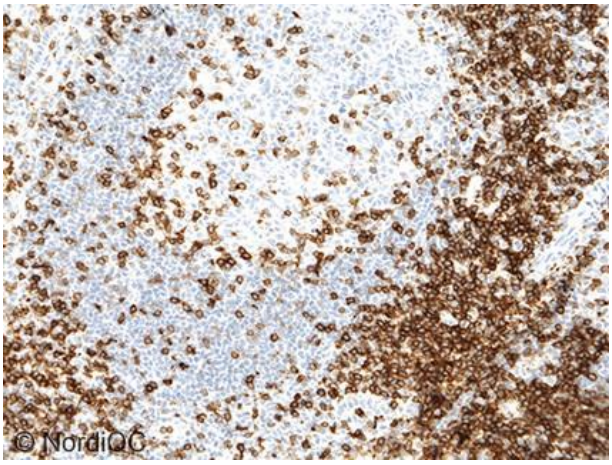


Fig. 1b (x200)
Insufficient staining for CD5 of the tonsil, core 1, using the mAb clone 4C7 as concentrate (too diluted), HIER in an alkaline buffer (BERS2, too short time) and BOND Refine (Leica) as the detection system - same protocol used in Figs. 2b – 5b. The intensity of the staining reaction, both of germinal centre T-cells and mantle zone B-cells, is reduced - compare with Fig. 1a (same field).

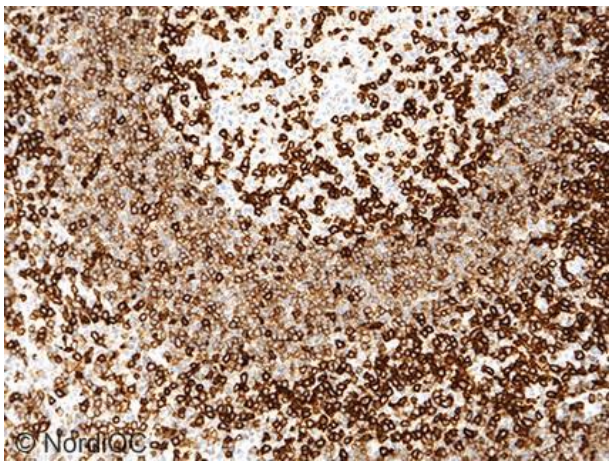


Fig. 2a (x200)
Optimal staining for CD5 in the tonsil, core 2, using same protocol as in Fig. 1a. T-cells are strongly stained and the majority of mantle zone B-cells show a weak to moderate staining reaction.

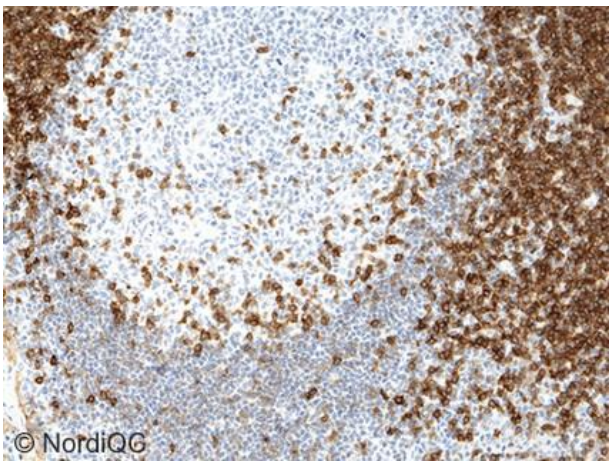


Fig. 2b (x200)
Insufficient staining for CD5 in the tonsil, core 2, using same protocol as in Fig. 1b. The intensity of the staining reaction in T-cells is reduced compared to the result obtained in Fig. 2a. However most important and critical; no staining reaction in the mantle zone B-cells is observed – compare with Fig. 2a (same field).

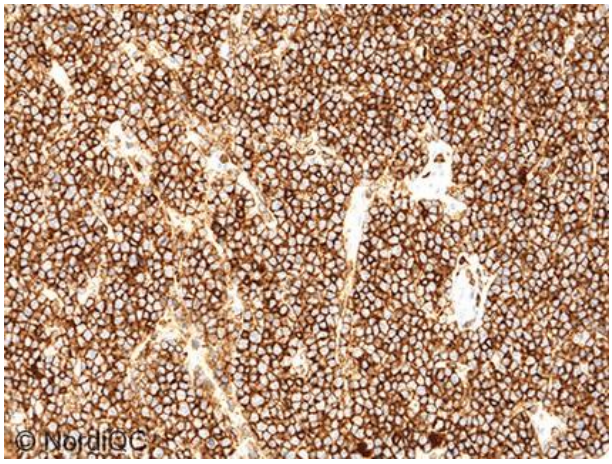


Fig. 3a (x200)
Optimal staining for CD5 of the MCL, core 3, using same protocol as in Figs. 1a and 2a.
All the neoplastic shows a moderate to strong but distinct membranous staining reaction.

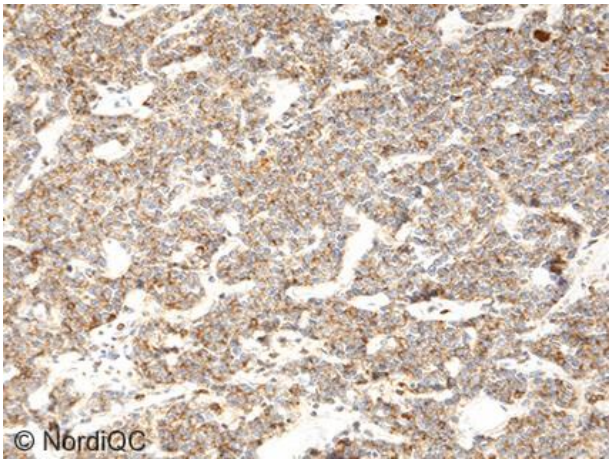


Fig. 3b (x200)
Staining for CD5 of the MCL, core 3, using same insufficient protocol as in Figs. 1b and 2b.
The intensity of the neoplastic cells is significantly reduced and displays an indistinct membranous staining reaction pattern - compare with Fig. 3a (same field).

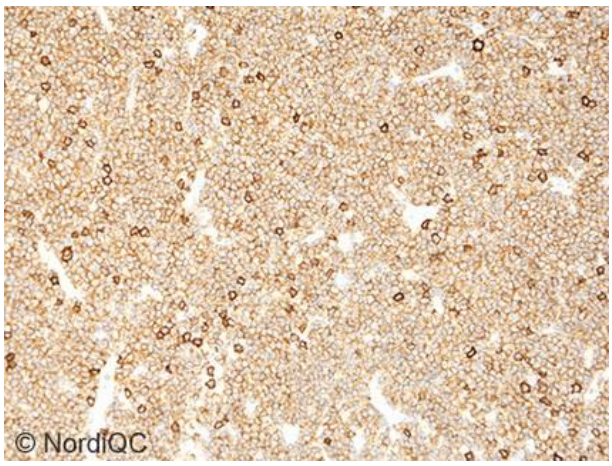


Fig. 4a (x200)
Optimal staining for CD5 of the MCL, core 4, using same protocol as in Figs. 1a - 3a. Virtual all the neoplastic cells show a weak to moderate, distinct membranous staining reaction. T-cells intermingling with the neoplastic cells show a strong membranous staining reaction.

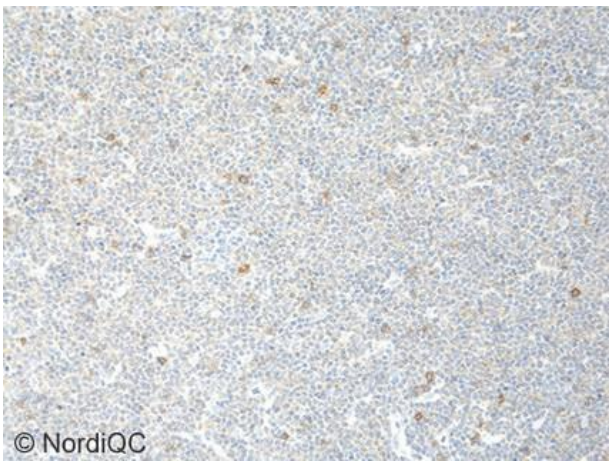


Fig. 4b (x200)
Insufficient staining for CD5 of the MCL, core 4, using same protocol as in Figs. 1b -3b. The neoplastic cells are false negative and only T-cells with reduced intensity are demonstrated - compare with Fig. 4a (same field).

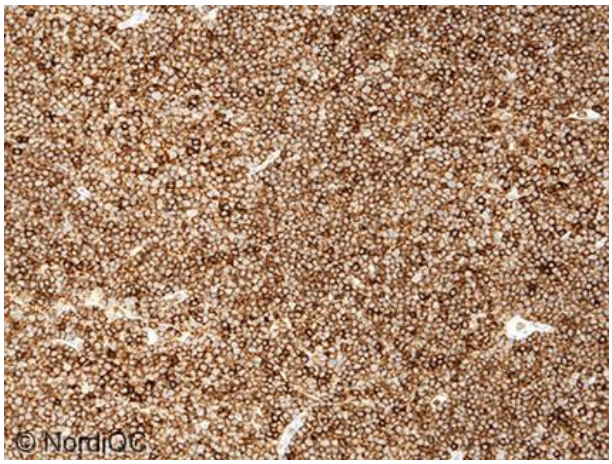


Fig. 5a (x200)
Optimal staining for CD5 of the B-CLL using same protocol as in Figs. 1a - 4a. All the neoplastic cells show a strong and distinct membranous staining reaction.

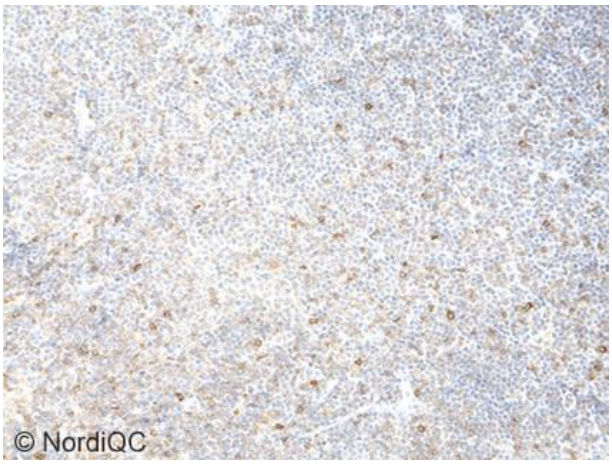


Fig. 5b (x200)
Insufficient staining for CD5 of B-CLL using same protocol as in Figs. 1b - 4b. The vast majority of neoplastic cells are false negative or shows reduced intensity. T-cells display a moderate staining intensity - compare with Fig. 5a (same field).

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