Assessment Run 44 2015

CD4

Recommended CD4 protocols Recommended CD4 control tissue

Material

The slide to be stained for CD4 comprised:



1. Tonsil, 2. Colon, 3. Liver, 4. Hodgkin lymphoma (classical type), 5. Peripheral T-cell Lymphoma, NOS.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CD4 staining as optimal included:

- A strong, distinct, predominantly membranous staining reaction of virtually all helper/inducer T-cells in the T-zones and within the germinal centres in the tonsil.
- A moderate to strong, distinct, predominantly membranous staining reaction of intraepithelial T-cells in the colon mucosa
- An at least moderate, distinct, predominantly membranous staining reaction of the majority of macrophages as germinal centre macrophages in the tonsil, macrophages in lamina propria of the colon mucosa and Kupffer cells in the liver
- An at least weak to moderate staining reaction of endothelial cells in the liver sinusoids
- An at least weak to moderate, distinct, predominantly membranous reaction of the majority of neoplastic cells in the T-cell lymphoma
- No staining of other cells. Especially all B-cells, Hodgkin/Reed-Sternberg cells, squamous epithelial cells in the tonsil and columnar epithelial cells in the colon should be negative

Participation

Number of laboratories registered for CD4, run 44	254
Number of laboratories returning slides	234 (92%)

Results

234 laboratories participated in this assessment. 184 (79%) achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 2).

The most frequent causes of insufficient staining reactions were:
- Too low concentration of the primary antibody
- Insufficient HIER (too low temperature and/or too short heating time)
- Less successful performance of mAb clone 4B12 on the Ventana BenchMark and Leica BOND platforms
- Unexplained technical issues

Performance history

This was the 3rd NordiQC assessment of CD4. An increased pass rate was seen compared to run 29, 2010 (see table 2).

Table 2. **Proportion of sufficient results for CD4 in the three NordiQC runs performed**

	Run 14 2005	Run 29 2010	Run 44 2015
Participants, n=	59	129	234
Sufficient results	72%	63%	79%

Conclusion

The mAbs clones **4B12**, **1F6** and the rmAb clones **SP34, EP204, EPR6855** could all be used to obtain optimal staining results for CD4. Irrespective of the clone applied, efficient HIER, use of appropriate primary Ab tailored to the choice of IHC system and careful calibration of the primary antibody were the most important prerequisites for an optimal staining result. The Ready-To-Use systems for CD4 from Ventana and Dako provided the highest proportion of sufficient and optimal results.
Tonsil is recommended as positive and negative tissue controls: All helper/inducer T-cells must show a distinct and strong membranous staining reaction, while germinal centre macrophages must at least display a moderate staining reaction. No staining reaction should be seen in other cells including B-cells and squamous epithelial cells of the tonsil.

Table 1. **Antibodies and assessment marks for CD4, run 44**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **4B12**	23 13 8 1 1	Leica/Novocastra Dako Thermo/NeoMarkers Monosan Immunologic	5	22	10	9	59%	82%
mAb clone **1F6**	10	Leica/Novocastra	4	3	2	1	70%	75%
mAb clone **BC/1F6**	1	Biocare	0	1	0	0	-	-
rmAb clone **SP35**	17 7 2	Cell Marque Spring Biosciences Immunlogic	11	11	3	1	85%	86%
rmAb clone **EP204**	3	Nordic Biosite Zeta	2	1	0	0	-	-
rmAb clone **EPR6855**	1	Epitomics/Abcam	1	0	0	0	-	-
Ready-To-Use antibodies								
mAb clone **4B12 IS/IR649**	51	Dako	13	24	8	6	73%	81%
mAb clone **4B12 PA0368**	7	Leica/Novocastra	0	1	2	4	14%	-
mAb clone **4B12 PA0427**	1	Leica/Novocastra	0	1	0	0	-	-
mAb clone **4B12 MS-1528-R7**	1	Thermo/NeoMarkers	0	0	0	1	-	-
mAb clone **1F6 MONX10330**	1	Monosan	0	1	0	0	-	-
rmAb clone **BC/1F6 PM153**	1	BioCare	0	1	0	0	-	-
rmAb clone **SP35 790-4423**	74	Ventana	63	10	0	1	99%	100%
rmAb clone **SP35 104R-17/104R-18**	4	Cell Marque	1	2	1	0	-	-
rmAb clone **SP35 RMA-0620**	2	Maixin	1	1	0	0	-	-
rmAb clone **EP204 MAD-000600QD**	3	Master Diagnostica	-	2	1	-	-	-
rmAb clone **EP204 AN722-5M**	1	BioGenex	1	-	-	-	-	-
rmAb clone **EP204 104R-28**	1	Cell Marque	-	1	-	-	-	-
Total	234		102	82	27	23	-	
Proportion			44%	35%	12%	9%	79%	

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

Detailed analysis of CD4, Run 44
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **4B12**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (3/9)*, Tris-EDTA/EGTA pH 9 (1/7) or Borg Decloaker pH 9.5 (Biocare) (1/2) as retrieval buffer. The mAb was typically diluted in the range of 1:40-1:150 depending on the total sensitivity of the protocol employed. Using these protocol settings 9 of 11 (82%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **1F6**: Protocols with optimal results were all based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (1/1), BERS2 (Leica) (2/4) or CC1 (Ventana) (1/4) as retrieval buffer. The

mAb was typically diluted in the range of 1:20-1:50 depending on the total sensitivity of the protocol employed. Using these protocol settings 6 of 8 (75%) laboratories produced a sufficient staining result.

rmAb clone **SP35**: Protocols with optimal results were all based on HIER using CC1 (Ventana) (9/19), CC2 (Ventana) (1/1) and TRS pH 9 (Dako) (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:10-1:100 depending on the total sensitivity of the protocol employed. Using these protocol settings 18 of 21 (86%) laboratories produced a sufficient staining result.

rmAb clone **EP204**: Protocols with optimal results were based on HIER using TRS pH 9 (Dako) (2/2) as retrieval buffer. The rmAb was diluted 1:25 using a 3-step polymer based detection kit.

rmAb clone **EPR6855**: One protocol with an optimal result was based on HIER using TRS pH 9 (3-in-1) (Dako) as retrieval buffer. The rmAb was diluted 1:100 using a 3-step polymer based detection kit.

Table 3. **Proportion of optimal results for CD4 for the three most commonly used antibodies as concentrate on the three 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 **4B12**	3/8** (38%)	-	0/5 (0%)	-	0/9 (0%)	-
mAb clone **1F6**	1/1	-	1/3	-	2/4	-
rmAb clone **SP35**	1/3	-	9/19 (47%)	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)

Ready-To-Use antibodies and corresponding systems
mAb clone **4B12**, product no. **IS649/IR649**, 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 97-98°C), 20-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 26 of 32 (81%) laboratories produced a sufficient staining result.

rmAb clone **SP35**, product no. **790-4423**, Ventana, BenchMark XT/Ultra:
Protocols with optimal result were typically based on HIER using Cell Conditioning 1 (efficient heating time 32-64 min.) and 16-72 min. incubation of the primary Ab. UltraView (760-500) +/- amplification kit or OptiView (760-700) were used as detection systems. Using these protocol settings 50 of 50 (100%) laboratories produced a sufficient staining result.

rmAb clone **SP35**, product no. **RMA-0620**, Maxin:
One protocol with an optimal result was based on HIER using Citrate buffer pH 6 (efficient heating time 2 min. at 120°C), 60 min. incubation of the primary Ab. and KIT-5230 (Maixin) as detection system.

rmAb clone **EP204**, product no. **AN722-5M**, BioGenex:
One protocol with an optimal result was based on HIER using AR-10 solution (efficient heating time 10 min. at 100°C), 60 min. incubation of the primary Ab. and Super Sensitive Polymer-HRP (QD400-60K, BioGenex) as detection system.

Comments
In this third NordiQC assessment for CD4, the prevalent features of an insufficient staining result was a generally too weak or completely false negative staining reaction of the cells expected to be demonstrated. This was observed in 94% of the insufficient results (47 of 50). The majority of laboratories were able to demonstrate CD4 in high-level antigen expressing cells, as normal helper/inducer T-cells in the tonsil and the Hodgkin lymphoma, whereas demonstration of CD4 in low-level antigen expressing cells as the neoplastic cells in the T-lymphoma, the germinal centre macrophages in the tonsil, Kupffer and endothelial cells in the liver sinusoids were more challenging and could only be achieved using appropriate protocol settings. Typically, too low concentration of the primary Ab, insufficient HIER (too low temperature/too short time) and/or less successful performance of the mAb clone 4B12 on the Ventana BenchMark and Leica BOND platform were the main parameters causing a too weak and insufficient staining reaction for CD4.

Optimal demonstration of CD4 could be achieved with the mAbs clones 4B12, 1F6 and the rmAbs SP35, EP204 and EPR6855 (table 1).
The rmAb clone SP35 was the most robust and successful antibody and provided the highest overall pass rate both as a concentrate within a laboratory developed assay or as RTU format e.g. Ventana, 790-4423. Applied as a concentrate, optimal result could be obtained on both a Dako Autostainer and Ventana BenchMark platform. A pass rate of 83% (5/6) and 85% (17/20), respectively for rmAb clone SP35 on these two platforms were observed. There was no significant difference in performance using either a 2-step multimer or polymer based detection system (e.g. UltraView) or a 3-step detection system (e.g. Ultraview with amplification), indicating very high robustness of the rmAb SP35. No explanation for an insufficient result in 3 of 4 laboratories could be identified as protocol settings applied were similar to protocols with optimal results. In the remaining protocol excessive HIER was used compromising the morphology.

11 laboratories used the mAb clone 1F6 as a concentrate and 72% (8/11) produced a sufficient result out of which 36% (4/11) were assessed as optimal. Although the number of optimal results obtained with this primary Ab was low, the mAb 1F6 provided optimal results on all three main platforms (table 3).The 4 protocols with an optimal mark were based on efficient HIER in an alkaline buffer (e.g. BERS2, Leica), the use of a high concentration of the primary Ab (1:20-1:50) and the use of a sensitive 3-step multimer/polymer system (e.g Bond Refine, Leica). As mentioned in the previous assessments for CD4, the antigen detected by the mAb clone 1F6 is deteriorated by blocking of endogenous peroxidase in > 1 % H_2O_2 after HIER. Therefore, both the concentration of H_2O_2 and when to apply the blocking step (before or after HIER) must be taken into consideration. Optimally, the peroxidase blocking step should be performed after incubation of the primary Ab.

Compared to the other concentrated Abs, the mAb clone 4B12 gave the overall lowest pass rate of 59% (27/46) of which only 11% (5/46) were assessed as optimal. Careful calibration of all parameters involved in the IHC staining process seems to be a prerequisite for this challenging antibody. Mandatory for an optimal performance of the mAb clone 4B12 is use of high sensitive detection systems, efficient HIER and precise calibration of the titre of the Ab. In addition, the choice of the clone must be tailored to the IHC system used, as this had a high impact on the final result: In this run and in concordance with the previous NordiQC assessment of CD4 (Run 29, 2010), all 6 protocols based on the mAb clone 4B12 as concentrate on the BenchMark IHC system (Ventana) gave an insufficient result. The constantly inferior performance of the mAb clone 4B12 on the Ventana BenchMark platform should prompt laboratories to substitute the mAb 4B12 with a more robust clone, e.g. rmAb SP35.
For participants using the mAb 4B12 as concentrate on the Leica Bond-III/MAX platform, the pass rate was only 54% (7/13) and none were assessed as optimal (table 3). The reason for insufficient results obtained on the Leica Bond-III/MAX platform is currently not known as the sensitivity of the protocol settings were similar to other IHC systems providing optimal results.
The best performance of the concentrated format of mAb clone 4B12, was obtained on the Autostainer Link / Classic IHC system (Dako) giving an overall pass rate of 85% (11/13) of which 23% (3/13) were assessed as optimal.

Although data is preliminary and that no firm conclusion at this point can be drawn on the performance of the concentrated and RTU formats of the rmAbs EP204 or EPR6855 (probably the same rmAb produced by the company Epitomics), the performance seems promising (see Fig. 6a – Fig. 6b). Four protocols based on the rmAbs EP204/EPR6955 as concentrates were all assessed as sufficient and 75% (3/4) were assessed an optimal. These primary rmAbs may be a good alternative especially to the more demanding mAb clone 4B12.

In this assessment, the Ready-To-Use (RTU) systems from Ventana (790-4423) and Dako (IR/IS649) based on the rmAb clone SP35 and mAb 4B12, respectively, provided a higher pass rate and proportion of optimal results compared to laboratory developed (LD) assays using same clones as concentrate (see table 1).
Optimal results for these two RTU products were typically obtained using the official protocol recommendations given by the companies. Laboratory modified protocol settings (typically adjusting HIER, incubation time of the primary Ab and/or choice of detection system) could also provide sufficient and optimal result.
The RTU system based on the rmAb clone SP35 from Ventana (790-4423) gave a superior performance compared to all other RTU systems applied. All 73 protocols (100%) provided a sufficient result and 86% (63/73) were assessed as optimal. One protocol assessed as poor (see table 1) was based on the RTU format 790-4423 (Ventana) within a LD assay performing HIER in Tris-EDTA buffer (pressure cooker), and performing the staining on a Dako Autostainer platform. It should be emphasized that RTU formats of the individual clones are developed for a specific concept, typically optimized to the vendor's platform

including reagents needed for optimal performance. Therefore, laboratories should only use a RTU format committed to a specific automatic platform and the RTU format should as a minimum be used with the basic protocol settings as recommended by the supplier/company as illustrated in Fig. 5a - 5b.
The Dako RTU system (IR/IS649) based on the mAb clone 4B12 gave a pass rate of 73% (37/51) of which 25% (13/51) were assessed as optimal, which was significantly inferior to the Ventana RTU system.
The RTU format PA0368 based on the mAb 4B12 by Leica/Novocastra has been reformulated and replaced by PA0427. This product was only used by one laboratory and thus no conclusions can be generated on the performance of this reformulated format. However, of participants using the product PA0368 (now discontinued by Leica /NovoCastra) only 14% (1/7) produced a sufficient result, none of which was assessed as optimal

In this run a pass rate of 79% was obtained, which is an improvement compared to 63% in run 29, 2010. The availability and extended use of high quality and robust RTU systems for CD4, especially the RTU system based on the rmAb SP35 from Ventana (790-4463), seems to be one of the central elements. In run 29, 2010 22% of the participants (28 of 129) used one of the above mentioned RTU systems from either Dako or Ventana. In this run, 53% (124 of 234) of the participants used a RTU system from one of these two vendors and grouped together a pass rate of 89% was obtained.

Controls
Tonsil is recommended as positive and negative tissue control for CD4. In the tonsil, protocol must be calibrated to provide a distinct and strong membranous staining reaction in all helper/inducer T-cells. Germinal centre macrophages should at least display a moderate and distinct staining reaction. No staining reaction must be seen in other cells, including B-cells and epithelial cells of the tonsil.
As a supplement to tonsil, especially in the technical calibration phase, it is recommended to verify the protocol on liver tissue. The Kupffer cells and endothelial cells in the liver sinusoids must at least display a moderate, distinct staining reaction.

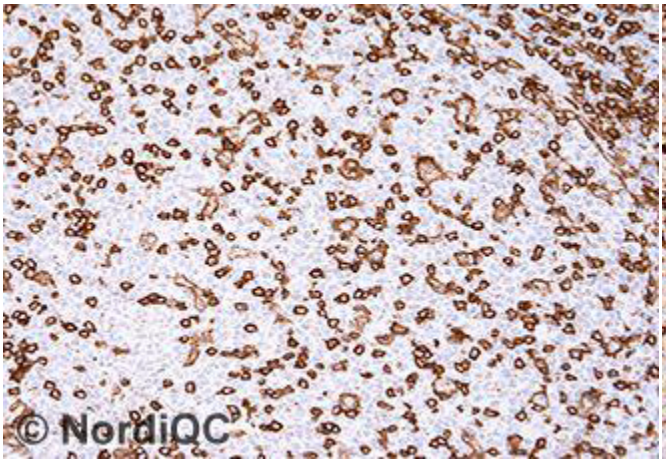


Fig. 1a (x200)
Optimal CD4 staining of the tonsil using the rmAb clone SP35 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a 3-step multimer based detection system (OptiView, Ventana) - same protocol used in Figs. 2a - 4a. The inducer/helper T-cells show a strong staining reaction, while the germinal centre macrophages show a moderate and distinct membranous staining reaction - compare with Fig.1b.

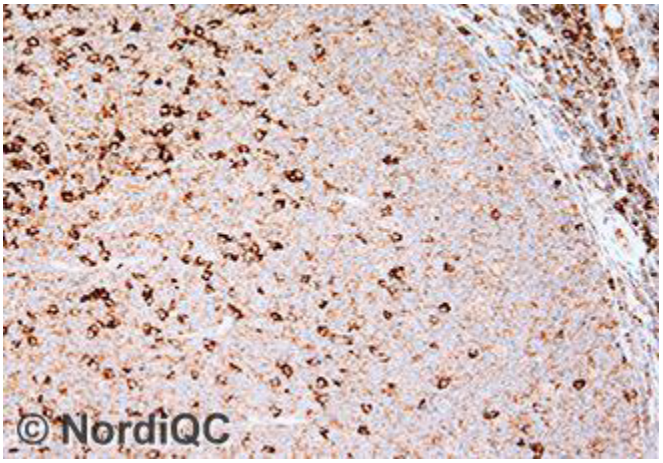


Fig. 1b (x200)
Insufficient CD4 staining of the tonsil using the mAb 4B12 as a concentrate, HIER in an alkaline buffer (CC1 pH 8.5) and a multimer based detection system (OptiView with Tyramide amplification, Ventana) -same protocol used in Figs. 2b-4b. The protocol provided a too low sensitivity, but also poor signal-to-noise ratio and false positive staining. No staining of germinal centre macrophages is seen and simultaneously B-cells are labelled. The pattern of too weak staining reaction was observed with all protocols based on the mAb 4B12 performed on the Ventana BenchMark platform - compare with Fig. 1a (same field).

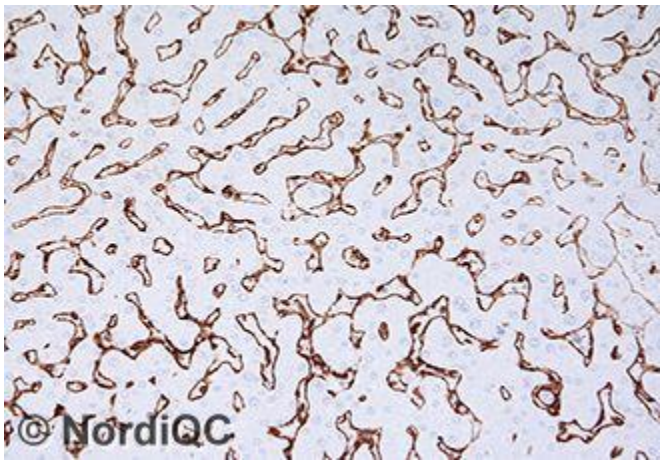


Fig. 2a (x200)
Optimal CD4 staining of the liver using same protocol as in Fig. 1a. The endothelial cells lining the sinusoids and the Kupffer cells show a moderate to strong predominantly membranous staining reaction, while the liver cells are unstained. - compare with Fig.2b.

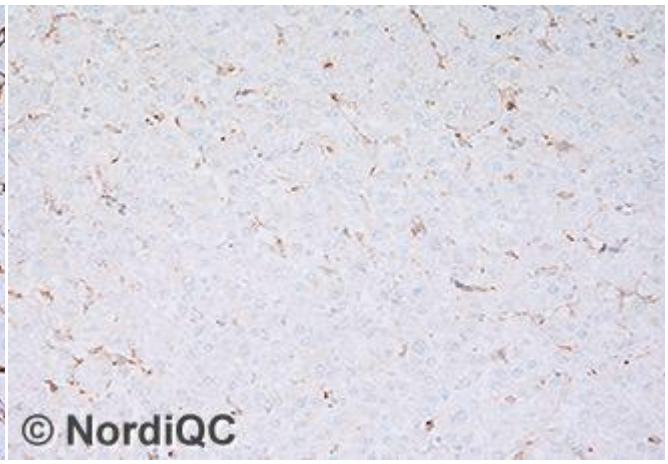


Fig. 2b (x200)
Insufficient CD4 staining of the liver using same protocol as in Fig. 1b. The endothelial cells and the Kupffer cells show only a weak and equivocal staining reaction - compare with Fig. 2a (same field).

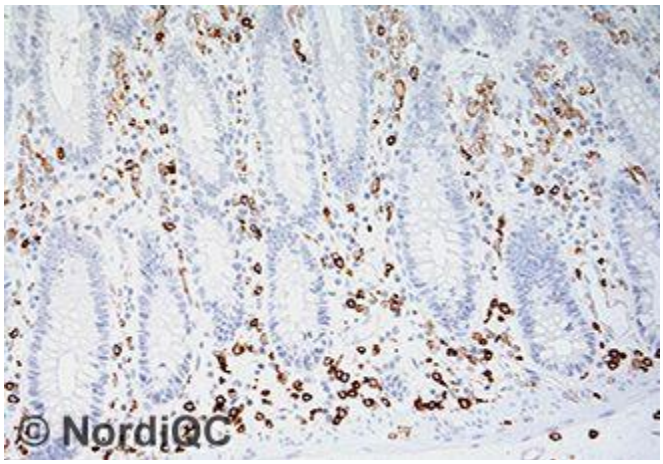


Fig. 3a (x200)
Optimal CD4 staining of the colon using same protocol as in Figs. 1a & 2a. The T-cells in lamina propria show a strong membranous staining reaction, while scattered macrophages in lamina propria display a weak to moderate membranous staining reaction - compare with Fig.3 b.

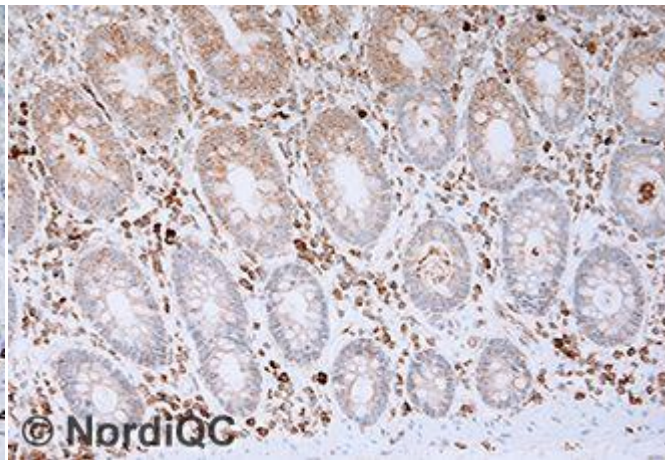


Fig. 3b (x200)
Insufficient CD4 staining of the colon using same protocol as in Figs. 1b & 2b. The vast majority of T-cells show a strong staining reaction, but an aberrant cytoplasmic staining of epithelial cells and general background staining compromises the interpretation - compare with Fig. 3a (same field).

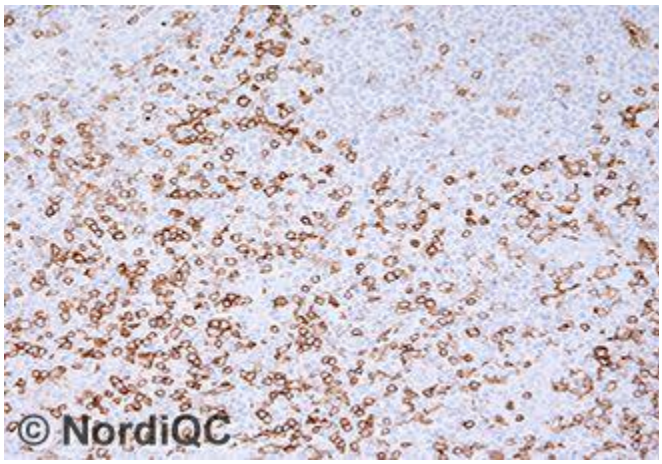


Fig. 4a (x200)
Optimal CD4 staining of the T-cell lymphoma using same protocol as in Figs. 1a - 3a. The neoplastic T-cells show a weak to moderate membranous staining intensity while intermingling normal inducer/helper T-cells show a strong staining reaction - compare with Fig.4 b.

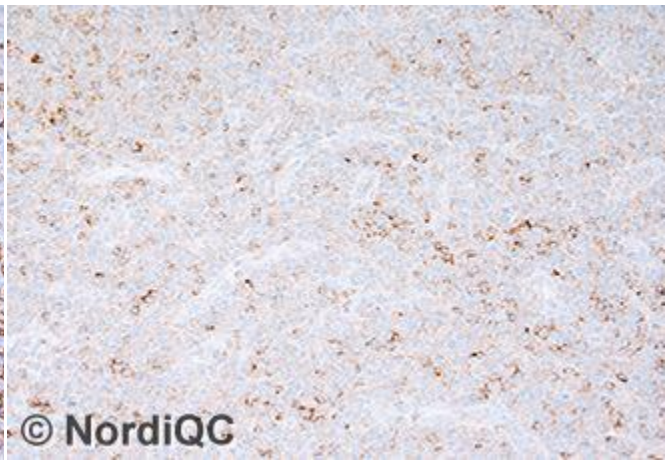


Fig. 4b (x200)
Insufficient CD4 staining of the T-cell lymphoma using same protocol as in Fig. 1b-3b. Both the neoplastic T-cells and the normal inducer/helper T-cells show a too weak and faint staining intensity - compare with Fig.4a (same field).

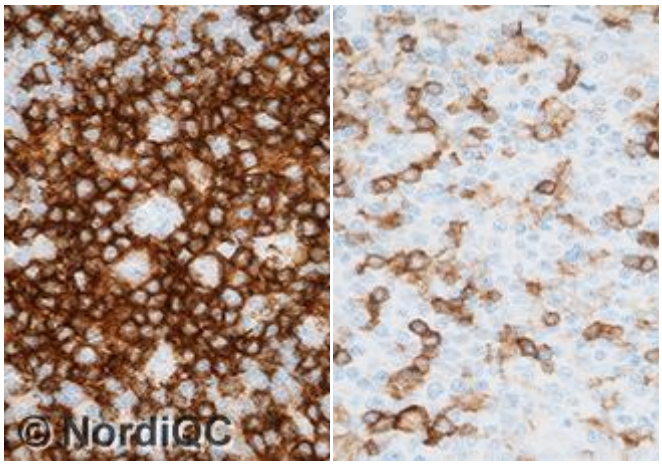


Fig. 5a (x400)
Optimal CD4 staining using the mAb clone 4B12 as RTU format prod. Id. IR649 with protocol settings as recommended by the vendor (Dako); HIER in an alkaline buffer (TRS pH 9) and a 3-step polymer based detection system (Flex+, Dako).
Left: A strong membranous staining reaction of the inducer/helper T-cells in the Hodgkin Lymphoma is seen, while Hodgkin/Reed-Sternberg cells are negative.
Right: A weak to moderate membranous staining reaction of the neoplastic T-cells in the T-cell lymphoma is seen, while normal T-cells show a strong staining reaction.
Compare with an insufficient protocol based on the same RTU format but within a laboratory developed assay, see Fig. 5b.

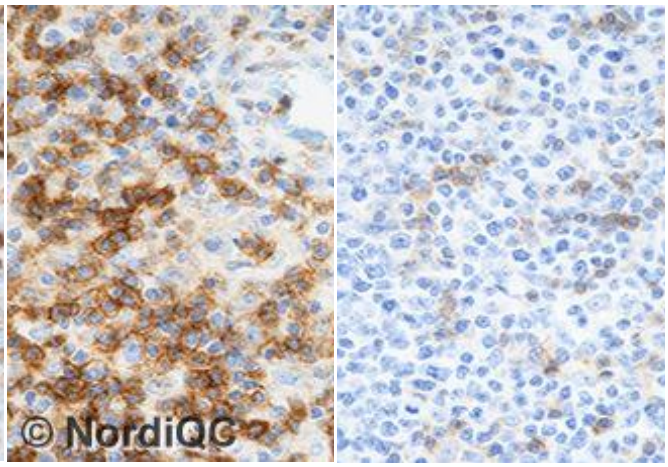


Fig. 5b (x400)
Insufficient CD4 staining based on the RTU format IR649 (Dako) within a laboratory develop assay, using a moderate sensitive 2-step polymer based detection system (Flex, Dako). The proportion and intensity of cells demonstrated in both the Hodgkin lymphoma (left) and the T-cell lymphoma (right), is significantly reduced – compare with Fig. 5a (same fields).

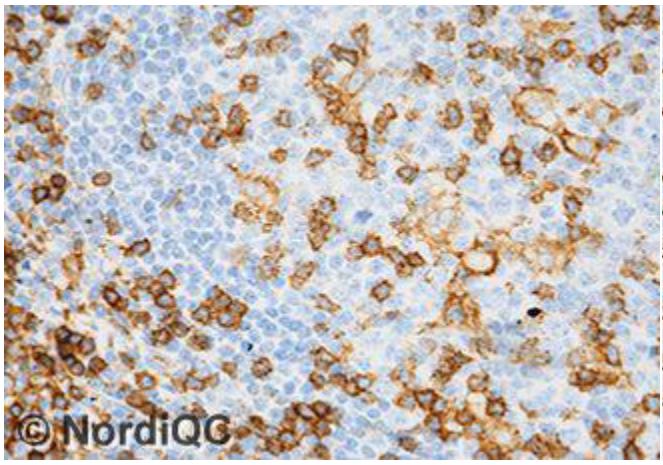


Fig. 6a (x400)
Optimal CD4 staining of the tonsil based on the rmAb EP204 as a concentrate, HIER in an alkaline buffer (TRS pH 9) and a 3-step polymer based detection system (Flex+, Dako) - same protocol in Fig. 6b.
The inducer/helper T-cells are strongly stained, while the germinal centre macrophages show a moderate but distinct membranous staining reaction.

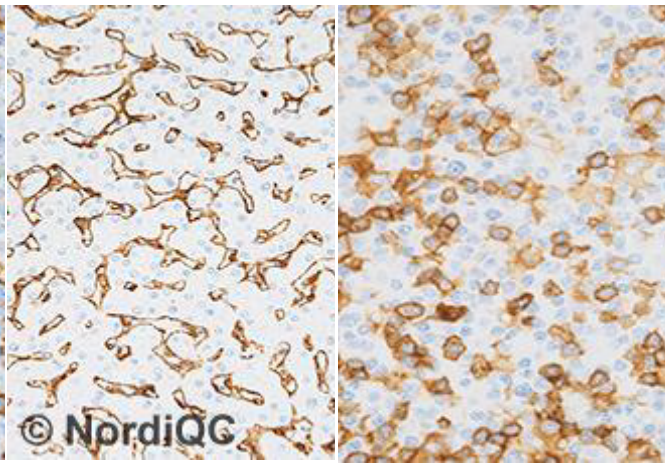


Fig. 6b
Optimal CD4 staining using the rmAb clone EP 204 with the same protocol settings as in Fig. 6a.
Left (x200): The endothelial cells lining the sinusoids and the Kupffer cells in the liver show a moderate to strong membranous staining reaction.
Right (x400): The neoplastic T-cells in the T-cell lymphoma show a weak to moderate membranous staining intensity.

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