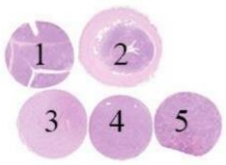
Assessment Run 63 2021

CD3

Table 1 updated 16.12.2021

Purpose
Evaluation of the technical performance, level of analytical sensitivity and specificity of IHC tests among the NordiQC participants for CD3, typically used in the classification of malignant lymphomas and leukemias. Relevant clinical tissues, both normal and neoplastic, were selected to display a broad spectrum of antigen densities for CD3 (see below).

Material
The slide to be stained for CD3 comprised:



1. Tonsil, 2. Appendix, 3. Diffuse large B-cell lymphoma (DLBCL), 4. Angioimmunoblastic T-cell lymphoma (AITL), 5. Peripheral T-cell lymphoma (PTCL-NOS)

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing a CD3 staining as optimal included:

- A moderate to strong and distinct predominantly membranous staining reaction of all T-cells both in the interfollicular T-zones and in the germinal centers of the tonsil.
- A moderate to strong and distinct predominantly membranous staining reaction of the intra-epithelial T-cells in the appendix.
- An at least weak to moderate and distinct predominantly membranous staining reaction of the majority of the neoplastic T-cells in the two T-cell lymphomas.
- No staining of other cells. Especially the normal B-cells in the tonsil and neoplastic cells of the DLBCL should be negative.

Participation

Number of laboratories registered for CD3, run 63	382
Number of laboratories returning slides	355 (93%)

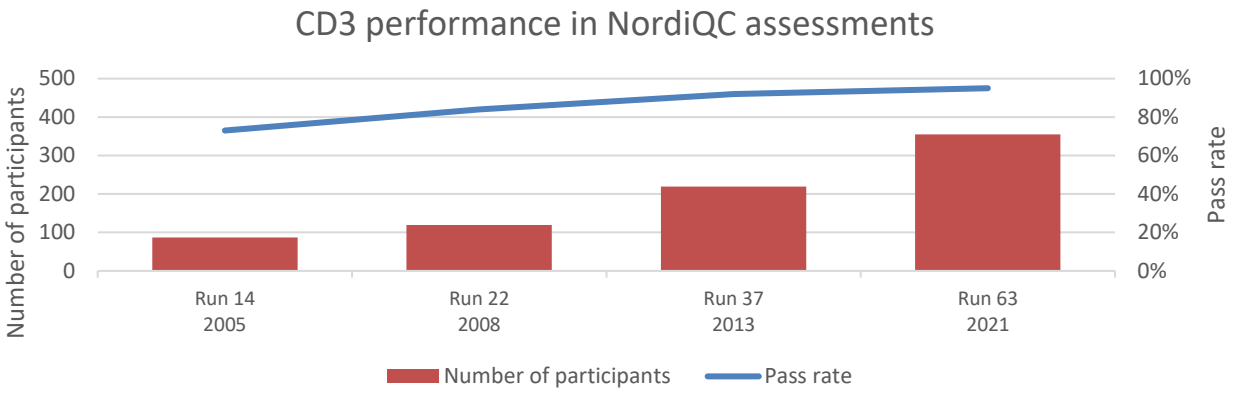
Results
At the date of assessment, 93% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

355 laboratories participated in this assessment and 95% achieved a sufficient mark (optimal or good). Table 1 summarizes antibodies (Abs) used and assessment marks (see page 3).

The most frequent causes of insufficient staining were:
- Inefficient Heat Induced Epitope Retrieval (HIER) – too short time or use of acidic buffer.
- Too low concentration of the primary antibody or too short incubation time.
- Less sensitive detection systems used in combination with other low sensitivity protocol parameters.

Performance history
This was the fourth NordiQC assessment of CD3. An increase in pass rate was observed compared to previous runs (see Graph 1), which primarily is due to the use of robust primary antibodies and well calibrated Ready-To-Use (RTU) systems (see Table 1).

Graph 1. **Proportion of sufficient results for CD3 in the four NordiQC runs performed**



Conclusion
The mAb clones **F7.2.38**, **LN10**, **PS1**, **ZM45** the rmAb clones **2GV6**, **SP7, MRQ-39, EP41, EP177, BP6039 DMC-39** and the pAb **A0452** and **103A-74** can all be recommended for demonstration of CD3. Both concentrated formats and especially the Ready-To-Use systems from Dako, Leica and Ventana gave a high proportion of sufficient and optimal results. HIER is mandatory for a sufficient result. The Ready-to-Use product GA503 for Dako Omnis is from vendor recommended used with TRS low, but data from this assessment indicates TRS high is to be preferred due to aberrant cytoplasmic staining in the epithelial compartment of the appendix and plasma cells when used with low pH buffer.

Tonsil and appendix are appropriate tissue controls for CD3. Both aggregated T-cells in the interfollicular areas and dispersed T-cells in the mantle zone and within the germinal centers must show a moderate to strong distinct membranous staining reaction, while B-cells must be negative. In the appendix a moderate to strong and distinct predominantly membranous staining reaction of the intra-epithelial T-cells should be seen without cytoplasmic staining of the columnar epithelial cells and plasma cells.

Table 1. **Antibodies and assessment marks for CD3, Run 63**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **LN10**	30	Leica Biosystems	24	4	2	-	93%	80%
mAb clone **F7.2.38**	16 1	Dako/Agilent Invitrogen	8	4	5	-	71%	47%
rpAb **A0452**	27	Dako/Agilent	20	4	3	-	89%	74%
rmAb clone **SP7**	6 1	Thermo Scientific Zytomed systems	2	4	1	-	86%	29%
rmAb clone **MRQ-39**	4	Cell Marque	2	-	2	-	-	-
mAb clone **PS1**	2 1	Leica Biosystems Biocare	3	-	-	-	-	-
mAb clone **BS103**	1	Nordic Biosite	-	-	1	-	-	-
mAb clone **ZM45**	1	Zeta Corporation	1	-	-	-	-	-
rmAb clone **EP41**	1	Epitomics	1	-	-	-	-	-
rmAb clone **BP6039**	1	Biolynx Biotechnology	1	-	-	-	-	-
rmAb clone **DCM-39**	1	DCS	1	-	-	-	-	-
rmAB clone **RBT-CD3**	1	Bio SB	-	1	-	-	-	-
rpAb **103A-74**	3	Cell Marque	1	1	-	1	-	-
rpAb **CP215C**	1	Biocare Medical	-	-	-	1	-	-
Conc total	98		64	18	14	2	84%	65%
Ready-To-Use antibodies							Suff.[1]	OR.[2]
rmAb clone **2GV6 790-4341**[3]	13	Ventana/Roche	13	-	-	-	100%	100%
rmAb clone **2GV6 790-4341**[4]	124	Ventana/Roche	106	17	1	-	99%	86%
pAb clone **IR/IS503**[3]	15	Dako/Agilent	14	1	-	-	100%	93%
pAb clone **IR/IS503**[4]	7	Dako/Agilent	7	-	-	-	100%	100%
pAb clone **GA503**[3]	37	Dako/Agilent	-	37	-	-	100%	-
pAb clone **GA503**[4]	31	Dako/Agilent	20	10	1	-	97%	65%
mAb clone **LN10 PA0553**[3]	12	Leica Biosystems	12	-	-	-	100%	100%
mAb clone **LN10 PA0553**[4]	9	Leica Biosystems	9	-	-	-	100%	100%
rmAb clone **EP177**[3]	2	Sakura	2	-	-	-	-	-
rmAb **MRQ-39 103R/97**	1	Cell Marque	1	-	-	-	-	-
mAb clone **PS1 AM322-5M**	1	BioGenex	1	-	-	-	-	-
mAb clone **C3E7 CCM-0332**	1	Celnovte	1	-	-	-	-	-
rmAb clone **EP41 MAD-000621QD**	4	Master Diagnostica Vitro SA	3	-	1	-	-	-
RTU total	257		189	65	3	-	99%	74%
Total	355		253	83	17	2	95%	
Proportion			71%	23%	5%	1%		

1) Proportion of sufficient results (optimal or good). (≥5 asessed protocols).
2) Proportion of Optimal Results (OR).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 asessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product applied either on the vendor recommended platform(s), non-validated semi/fully automatic systems or used manually (≥5 asessed protocols).

Detailed analysis of CD3, Run 63

The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **LN10**: Protocols with optimal results were all based on HIER using either Bond Epitope Retrieval Solution 2 (BERS2; Leica Biosystems) (14/16)*, Bond Epitope Retrieval Solution 1 (BERS1; Leica Biosystems) (1/1), Cell Conditioning 1 (CC1; Ventana/Roche) (4/6), Target Retrieval Solution (TRS, Dako/Agilent) pH 9 (3-in-1) (3/5) or TRIS-EDTA/EGTA pH 9 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:400 depending on the total sensitivity of the protocol employed. Using these protocol settings 28 of 30 (93%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone **F7.2.38**: Protocols with optimal results were all based on HIER using either TRS pH 9 (3-in-1) (2/8)*, TRS pH 6 (Dako/Agilent) (1/1), CC1 (3/6), BERS2 (1/1) or Diagnostic Biosystems EDTA Buffer pH 8,0 (1/1) as retrieval buffer. The mAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 9 of 14 (64%) laboratories produced a sufficient staining.

pAb **A0542**: Protocols with optimal results were all based on HIER using either TRS pH 9, 3-in-1 (2/5), TRS pH 9 (3/3), BERS2 (1/2), CC1 (14/15) or Tris-EDTA/EGTA pH 9 (2/3) as retrieval buffer. The pAb was typically diluted in the range of 1:50-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 20 of 20 (100%) laboratories produced a sufficient staining.

rmAb clone **SP7**: Protocols with optimal results were all based on HIER using either TRS pH 9 (3-in-1) (1/1) or CC1 (1/4) as retrieval buffer. The rmAb was typically diluted in the range of 1:150-1:200 depending on the total sensitivity of the protocol employed. Using these protocol settings 2 of 5 (40%) laboratories produced a sufficient staining.

Table 2. **Proportion of optimal results for CD3 for the most commonly used antibody as concentrate on the four main IHC systems***

Concentrated antibody	**Dako/Agilent Autostainer**		**Dako/Agilent Omnis**		**Ventana/Roche BenchMark XT / Ultra**		**Leica Biosystems 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	BERS2 pH 9.0	BERS 1 pH 6.0
mAb clone **LN10**	1/2**	-	2/3	-	4/6 (67%)	-	14/16 (88%)	1/1
mAb clone **F7.2.38**	0/3	-	2/5 (40%)	1/1	3/6 (50%)	-	1/1	-
rpAb **A0452**	2/5 (40%)	-	3/3	-	14/15 (93%)	-	1/2	-
rmAb clone **SP7**	1/1	-	-	-	1/4	-	-	-

* 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
rmAb clone **2GV6** (prod. no. 790-4341, Ventana/Roche): Protocols with an optimal result were all based on HIER using CC1 for 24-64 min., 8-44 min. incubation of the primary Ab and UltraView with or without amplification (760-500/760-080) or OptiView with or without amplification (760-700/760-099) as detection systems. Using these protocol settings 129 of 129 (100%) laboratories produced a sufficient staining (optimal or good).

pAb **IR503** (product.no. IR503, Dako/Agilent): Protocols with an optimal result were typically based on HIER in PT-Link using TRS pH 9 (3-in-1) (heating time 10-20 min. at 95-98°C), 10-30 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (K8000/K8002) as detection system. Using these protocol settings 22 out of 22 (100%) laboratories produced a sufficient staining.

pAb **GA503** (product.no. GA503, Dako/Agilent): Protocols with an optimal result were typically based on HIER using TRS pH 9 (17/19)* or TRS Low pH 6.1 (3/48) (heating time 20-30 min. at 97°C), 10-20 min. incubation of the primary Ab and EnVision FLEX/FLEX+ (GV800/GV823) as detection system. Using these protocol settings 61 out of 61 (100%) laboratories produced a sufficient staining (optimal or good).
* (number of optimal results/number of laboratories using this buffer)

mAb clone **LN10** (product. no. PA0553, Leica Biosystems): Protocols with optimal results were all based on HIER using BERS2 or BERS1 (heating time 10-30 min. at 98-100°C), 15-30 min. incubation of the

primary Ab and Bond Polymer Refine Detection (DS9800) as detection system. Using these protocol settings 21 out of 21 (100%) laboratories produced a sufficient staining.

Table 3 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 3. **Proportion of sufficient and optimal results for CD3 for the most commonly used RTU IHC systems**

RTU systems	**Recommended protocol settings***		**Laboratory modified protocol settings****	
	Sufficient	Optimal	Sufficient	Optimal
VMS Ultra/XT rmAb 2GV6 **790-4341**	100% (13/13)	100% (13/13)	99% (123/124)	86% (106/124)
Dako AS pAb **IR503**	100% (15/15)	93% (14/15)	100% (7/7)	100% (7/7)
Dako Omnis pAb **GA503**	100% (37/37)	0% (0/37)	97% (30/31)	65% (20/31)
Leica Bond III/MAX mAb LN10 **PA0553**	100% (12/12)	100% (12/12)	100% (9/9)	100% (9/9)

* 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, detection kit – only protocols performed on the specified vendor IHC stainer integrated.

Comments
In this assessment and in concordance with the previous NordiQC assessments of CD3, the predominant reason for an insufficient result was a too weak staining reaction of the cells expected to be demonstrated. This pattern was seen in 95% (18/19) of the insufficient results.
Virtually all laboratories could demonstrate CD3 in the aggregated T-cells in the interfollicular T-zones in the tonsils, whereas detection of CD3 in dispersed T-cells (e.g. in the mantle zones of germinal centres of the tonsil and in mucosa of the colon) was much more challenging and required an optimally calibrated protocol. Protocol settings with a too low technical sensitivity typically resulted in a weak diffuse membranous staining reaction of the neoplastic T-cells in the T-cell lymphomas as shown in Fig. 3b.

98 laboratories used a concentrated antibody format of CD3 providing a general pass-rate of 84% and 65% optimal results. Both mouse monoclonal, rabbit monoclonal and rabbit polyclonal antibodies were used in this assessment all with optimal results.

The most widely used concentrate was the mAb clone LN10 from Leica Biosystems which was able to produce optimal results on all 4 main IHC platforms (see Table 2). The LN10 clone had the highest pass rate in general and applied as a concentrate 93% of all protocols passed (28/30) with 80% being optimal. The antibody was typically diluted in the range of 1:50-400 depending on the total sensitivity of the protocol. Optimal results were only seen with 3-layer detection systems. The 2 insufficient protocols were also based on 3-layer detection system however both with a short HIER time in combination with a low concentration of the primary antibody.

27 laboratories used the rabbit polyclonal antibody A0452. The antibody provided a pass rate of 89%, 74% being optimal. This antibody was frequently applied on the Ventana BenchMark platform with an optimal level of 93% (see Table 2). For optimal results, the antibody could be diluted in the range of 1:50-200. Similar to the mAb clone LN10, insufficient results were caused by a too low concentration of the primary antibody and/or too short efficient HIER.

The mouse monoclonal antibody F7.2.38 17 was used be 17 laboratories. All optimal protocols were based on a 3-layer detection system compared to the five insufficient results all using a 2-layer detection system.

72% (257/355) of the laboratories were using RTU products with an overall pass rate of 99%, 74% being optimal.
The Ready-To-Use system 790-4341 from Ventana/Roche based on the rabbit monoclonal antibody clone 2GV6 was used by 137 participants. Only 13 of these used the vendor recommended protocol settings based on HIER in CC1 for 40 min. at 100°C, primary Ab incubation of 16 min. and OptiView as detection system. All 13 results were assessed as optimal. The remaining 124 laboratories modified the protocol, typically adjusting HIER time and/or primary Ab incubation time. Overall, the obtained pass rate of 99% was encouraging, but the proportion of optimal results reduced to 86%. Especially reduced HIER time to

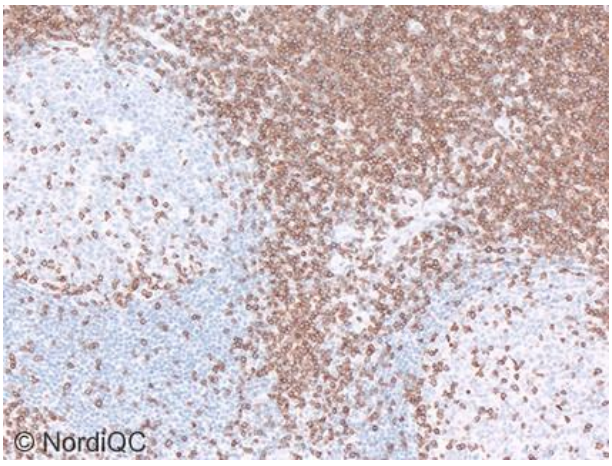
less than 40 min. seemed to have a negative effect, whereas the change to UltraView was less critical, despite the intensity of the staining reaction was reduced in the cells demonstrated (see Figs. 5a and 5b).

The Ready-To-Use systems IS/IR503 and GA503 from Dako/Agilent for Autostainer 48 and Omnis, respectively, based on a rabbit polyclonal antibody provided a pass rate of 100% on both platforms. The recommended protocol settings for the Autostainer platform was however superior to protocol settings recommended for Omnis, as the proportion of optimal results was 93% on Autostainer compared to 0% for Omnis.
On the Autostainer platform the TRS pH 9 (3-in-1) was recommended as HIER buffer, whereas TRS low pH 6.1 was recommended for the Dako Omnis. Using the low pH buffer on Omnis an aberrant cytoplasmic staining reaction was seen in many plasma cells and columnal epithelial cells of the appendix which is not to be expected and not found present using the TRS high pH buffer (see Figs. 6a and 6b). 19 participants modified the protocol for Omnis and substituted the TRS low pH buffer to TRS High pH and 95% (18/19) obtained an optimal result. These data clearly indicate that the vendor protocol recommendations for the Omnis GA503 CD3 RTU system should be changed.

The Ready-To-Use system PA0553 from Leica Biosystem based on the mAb clone LN10 provided 100% optimal results both using the vendor recommended and laboratory modified protocol settings. Changes were typically related to prolonged primary Ab incubation time to 20 min. instead of 15 min. as recommended.

In the last assessment for CD3, run 37 2013, 54% (119/219) of the participants used concentrated formats with an overall pass rate of 86% and 50% being optimal. In this run the pass rate of 84% for this group was very similar, however the number of optimal results has increased resulting in an optimal proportion of 65%. Especially the extended use of the robust LN10 clone and general change to 3-layer detection systems seems to be the main reason for the positive impact.
In run 37, 46% of the laboratories used a Ready-to-Use format of CD3 compared to 72% in this assessment. The widely used Ready-To-Use systems from Ventana/Roche, Leica Biosystems and Dako/Agilent based on 2GV6, LN10 and a polyclonal antibody, respectively, were all very successful giving a pass rate of 100% for this group with the vendor recommended protocol settings. In conclusion the overall pass rate has increased since the last assessment and the access to both concentrated formats of CD3 antibodies providing optimal results on all 4 of the main IHC platforms and corresponding optimally calibrated Ready-to-Use systems seem to be the main pillars for the improvement.

Controls
Tonsil and appendix are recommendable tissue controls for CD3. Both aggregated T-cells in the interfollicular areas and dispersed T-cells in the mantle zone and within the germinal centers must show a moderate to strong distinct membranous staining reaction, while B-cells must be negative. In the appendix a moderate to strong and distinct predominantly membranous staining reaction of the intra-epithelial T-cells should be seen without cytoplasmic staining of the columnar epithelial cells or plasma cells.



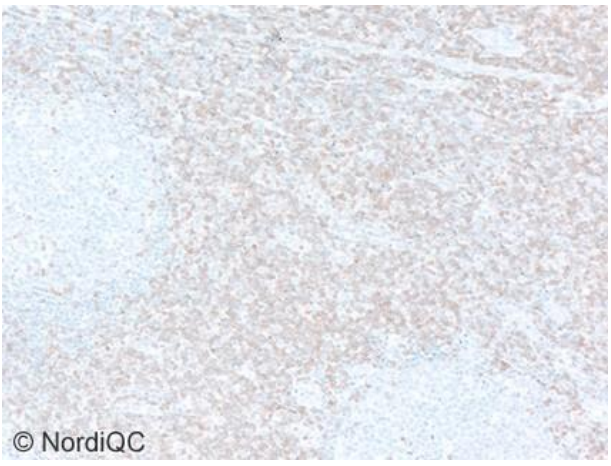


Fig. 1a (x100)
Optimal staining for CD3 of the tonsil using the pAb A0452 on the Ventana BenchMark with HIER in an alkaline buffer (CC1 for 48 min.), primary Ab dilution of 1:100, incubated for 32 min. and OptiView as detection system.
Virtually all the T-lymphocytes in the T-zone and within the germinal 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 – 4a, same protocol.

Fig. 1b (x100)
Insufficient staining for CD3 of the tonsil using the pAb A0452 on the Ventana BenchMark with HIER in an alkaline buffer (CC1 for 32 min.), primary Ab dilution of 1:400, incubated for 20 min. and OptiView as detection system – same field as Fig. 1.a.
The vast majority of the T-lymphocytes are demonstrated. A weaker, less intense and more diffuse staining reaction is seen. Same protocol as Figs. 2b-4b.

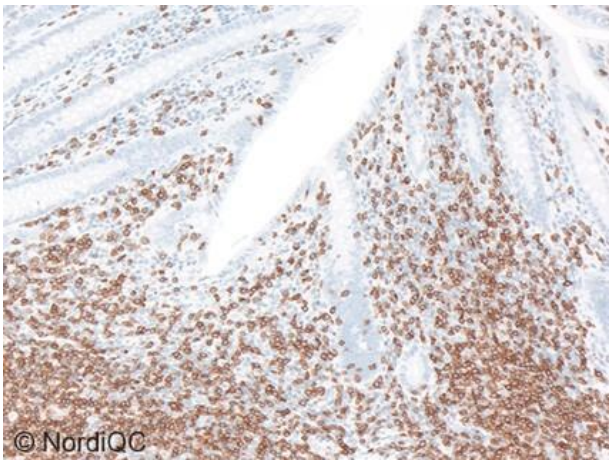


Fig. 2a (x100)
Optimal staining of the appendix using same protocol settings as Fig. 1a.
The dispersed intraepithelial T-lymphocytes in the mucosa show a distinct staining reaction. The columnar epithelial cells are negative and no background staining is seen.

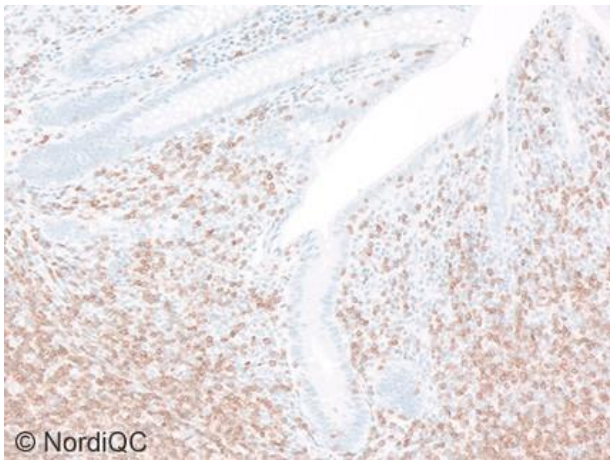


Fig. 2b (x100)
Insufficient staining of the appendix using same protocol settings as Fig. 1b - same field as in Fig. 2a.
The intraepithelial T-lymphocytes are only weakly stained and proportion being reduced compared to Fig. 2a.

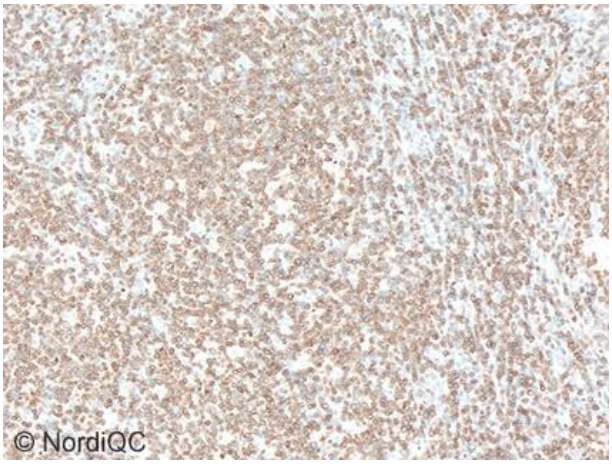


Fig. 3a (x100)
Optimal CD3 staining of the Angioimmunoblastic T-cell lymphoma using same protocol as in Figs. 1a-2a.
Virtually all the neoplastic cells show a moderate to strong and distinct predominantly membranous staining reaction. No background staining is seen.

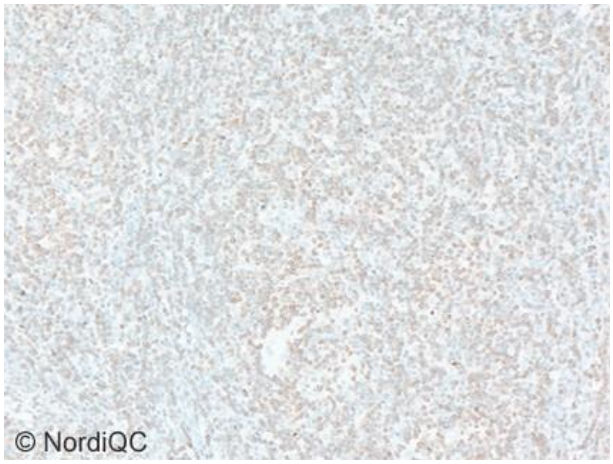


Fig. 3b (x100)
Insufficient CD3 staining of the Angioimmunoblastic T-cell lymphoma using same protocol as in Figs. 1b-2b – same field as in Fig. 3a. The proportion and intensity of the neoplastic cells demonstrated is significantly reduced compared to the level expected and obtained in Fig. 3a.

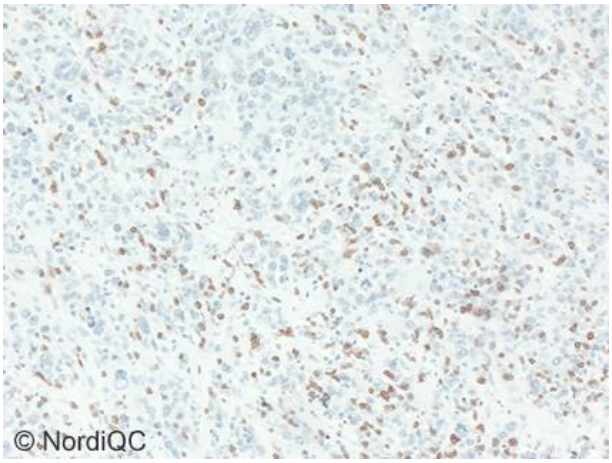


Fig. 4a (x100)
Optimal CD3 staining of the Diffuse Large B-cell lymphoma using same protocol as in Figs. 1a-3a. All the neoplastic cells are negative, whereas the residual T-cells are positive with a moderate to strong and distinct predominantly membranous staining reaction.

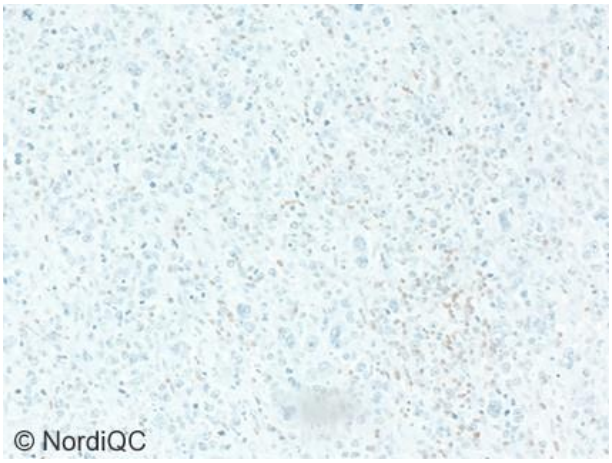


Fig. 4b (x100)
Insufficient CD3 staining of the Diffuse Large B-cell lymphoma using same protocol as in Figs. 1b-3b. All the neoplastic cells are negative, and no background staining is seen. The residual T-cells are only weakly positive with a diffuse membranous staining reaction.

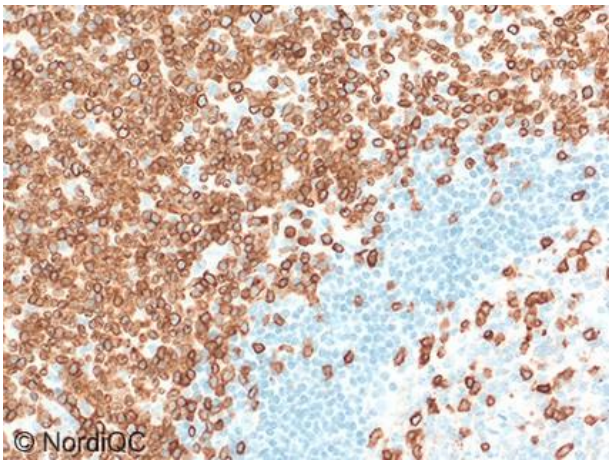


Fig. 5a (x200)
Optimal staining of the Peripheral T-Cell lymphoma using the Ventana/Roche RTU system (790-4341) based on the rmAb clone 2GV6 by the vendor recommended protocol settings with OptiView as detection system. All neoplastic cells (left) and normal T-cells in entrapped germinal center show a moderate to strong and distinct predominantly membranous staining reaction.

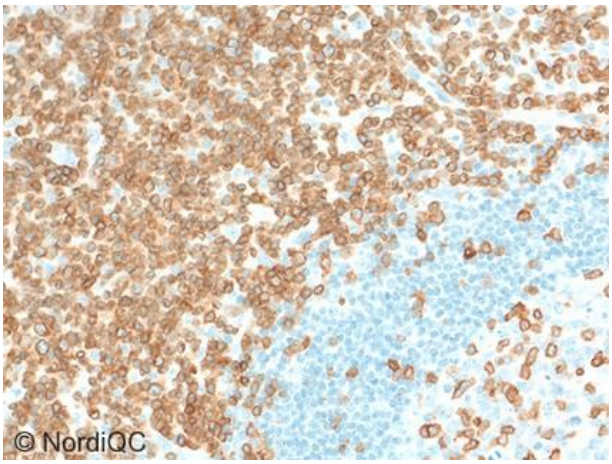


Fig. 5b (x200)
Optimal staining of the Peripheral T-Cell Lymphoma using Ventana/Roche RTU system (790-4341) based on the rmAb clone 2GV6 with UltraView as detection system – otherwise same protocol settings as in Fig. 5a.
Virtually all neoplastic cells show a moderate and distinct predominantly membranous staining reaction, but with a slightly less intensity than with OptiView as detection system.

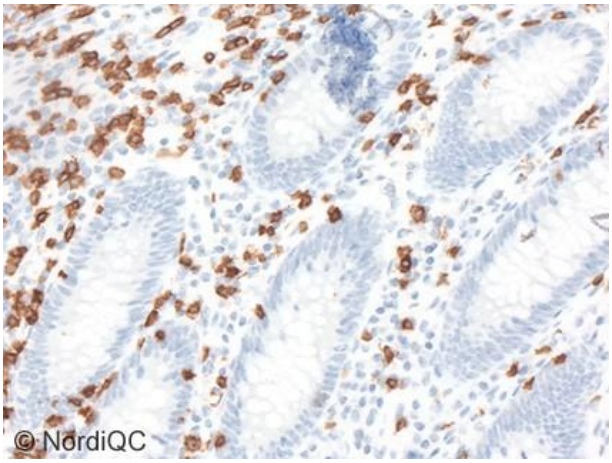


Fig. 6a (x200)
Optimal staining of the appendix using the Dako/Agilent RTU system GA503 based on a pAb on the Omnis with HIER in an alkaline buffer (TRS High).
The columnar epithelial cells and plasma cells are negative and no background staining is seen.

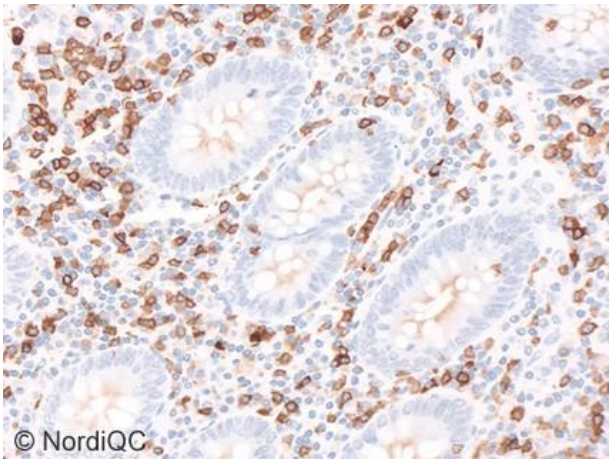


Fig. 6b (x200)
Staining of the appendix using the Dako/Agilent RTU system GA503 based on a pAb on the Omnis with HIER in an acidic buffer (TRS Low) – otherwise same protocol settings as in Fig. 6b.
The columnar epithelial cells show an aberrant cytoplasmic staining reaction and some of the plasma cells show a weak but distinct cytoplasmic staining reaction.

TJ/LE/SN 27.11.2021
Updated HLK/SN 16.12.2021