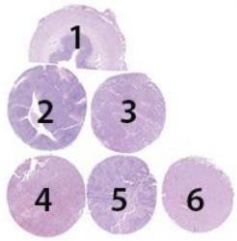
Assessment Run 57 2019
Lung Anaplastic Lymphoma Kinase (lu-ALK)

Purpose
Evaluation of the technical performance and level of analytical sensitivity and specificity of IHC tests among NordiQC participants for EML4-ALK translocations in lung adenocarcinomas. The translocation occurs through a paracentric inversion between EML4 and ALK genes located in the short arm of chromosome 2 and induces an EML4-ALK fusion protein being expressed in 2–4% of lung adenocarcinomas and is a target for ALK tyrosine inhibitors as crizotinib, ceritinib, and alectinib.

Material
The slide to be stained for lu-ALK comprised:



1. Appendix, 2. Tonsil, 3. Merkel cell carcinoma, 4. Anaplastic large cell lymphoma (ALCL) with ALK translocation, 5. Lung adenocarcinoma without EML4-ALK translocation. 6. Lung adenocarcinoma with EML4-ALK translocation.

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing lu-ALK staining as optimal included:

- A distinct, moderate to strong nuclear and cytoplasmic staining reaction of virtually all neoplastic cells in the anaplastic large cell lymphoma (ALCL).
- An at least weak to moderate granular cytoplasmic staining reaction of virtually all neoplastic cells in the lung adenocarcinoma with EML-ALK translocation.
- An at least weak to moderate granular cytoplasmic staining reaction of virtually all neoplastic cells in the Merkel cell carcinoma.
- An at least weak to moderate granular cytoplasmic staining reaction of ganglion cells and dispersed axons in the appendix.
- No staining of neoplastic cells in the lung adenocarcinoma without ALK rearrangement.
- No staining of epithelial cells in the appendix and tonsil.

Participation

Number of laboratories registered for lu-ALK, run 57	218
Number of laboratories returning slides	201 (92%)

Results
201 laboratories participated in this assessment. 168 (84%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies used and assessment marks given (see page 2).

The most frequent causes of insufficient staining reactions were:
- Less successful primary antibodies (mAb clone ALK1)
- Too low concentration of the primary antibody
- Use of detection systems with low sensitivity

Performance history
This was the fourth NordiQC assessment of lu-ALK. A significant increase of the pass rate was seen compared to run 51 in 2017 (see Table 2).

Table 2. **Proportion of sufficient results for lu-ALK in the four NordiQC runs performed**

	Run 39 2013	Run 45 2015	Run 51 2017	Run 57 2019
Participants, n=	146	176	189	201
Sufficient results	49%	67%	61%	84%

Conclusion
The mAb clone **OTI1A4** and the rmAb clone **D5F3** are both highly recommendable Abs for demonstration of EML4-ALK translocation in lung adenocarcinoma. Irrespective of selected clone, Heat Induced Epitope Retrieval (HIER) at high pH, use of a sensitive 3-step polymer/multimer based detection system and appropriate calibration of the titer of the primary antibody were crucial for an optimal performance.

Optimal staining results were also seen with the mAb clone **5A4,** but the analytical sensitivity was lower compared to mAb clone OTI1A4 and rmAb clone D5F3.
The Ventana Ready-To-Use system based on the rmAb clone D5F3 and using the recommended protocol settings was the most successful assay with an impressive overall pass rate of 97%.
Appendix is recommendable as positive and negative external tissue control, in which ganglion cells of the myenteric plexus and dispersed axons must show an at least weak to moderate staining reaction and no staining should be seen in smooth muscle cells and epithelium.
Lung adenocarcinomas with and without ALK translocation can be applied as supplemental external positive and negative tissue control and are crucial at the validation/verification phase of the IHC methods. ALCLs will typically express a too high level of antigen and cannot be recommended as the only positive tissue control for lu-ALK.

Table 1. **Antibodies and assessment marks for lu-ALK, run 57**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5A4**	27 2 1 1 1	Leica/Novocastra Monosan Abcam DBS Unknown	8	18	4	2	81%	89%
mAb clone **OTI1A4 / 1A4**	24 1	Origene Nordic Biosite	20	3	1	1	92%	96%
rmAb clone **D5F3**	28	Cell Signaling	11	14	3	0	89%	91%
rmAb clone **RBT-ALK1**	1	BioSB	0	0	0	1	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**	6	Leica/Novocatra	2	0	3	1	-	
mAb clone **5A4 PA0831**	1	Leica/Novocatra	1	0	0	0	-	
mAb clone **5A4 API3041**	1	BioCare	0	1	0	0	-	
mAb **ALK1 GA641**	1	Dako	0	0	0	1	-	
mAb clone **ALK1 IR641**	2	Dako	0	0	1	1	-	
mAb clone **ALK1 790/800-2918**	5	Ventana	0	0	1	4	-	
mAb clone **MX064 MAB-0848**	1	Maixin	0	1	0	0	-	
mAb clone **OTI1A4 / 1A4 8344-C010**	1	Sakura Finetek	0	1	0	0	-	
rmAb clone **D5F3 790-4794 / 790-4796**	95	Ventana	76	12	4	3	93%	
rmAb clone **D5F3 790-4794**[3]	1	Ventana	0	0	1	0	-	
rmAb clone **SP144 AN874-5M(10M)**	1	BioGenex	0	0	0	1	-	
Total	201		118	50	18	15	-	
Proportion			59%	25%	9%	7%	84%	

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 Ventana BenchMark systems (Ultra/XT) but used by laboratories on different platforms (e.g. Dako Autostainer)

Detailed analysis of lu-ALK, Run 57
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **5A4**: Protocols with optimal results were all based on HIER using either Cell Conditioning solution 1 (CC1, Ventana) (4/13) *, Target Retrieval Solution (TRS) High pH (3-in-1) (Dako) (2/8) or Bond Epitope Retrieval Solution 2 (BERS2, Leica) (2/5) as retrieval buffer. The mAb was diluted in the range of 1:10-1:40. Using these protocol settings, 17 of 19 (89%) laboratories produced a sufficient staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **OTI1A4 / 1A4**: Protocols with optimal results were all based on HIER using either TRS, High pH (Dako) (14/15), TRS High pH (3-in-1) (Dako) (3/3), CC1 (Ventana) (1/2), BERS2 (Leica) (1/2) or Bond Epitope Retrieval Solution 1 (BERS1, Leica) (1/1) as retrieval buffer. The mAb was diluted in the range of 1:100-1:3,000. Using these protocol settings, 22 of 23 (96%) laboratories produced a sufficient staining result.

rmAb clone **D5F3**: Protocols with optimal results were all based on HIER using either CC1 (Ventana) (5/10), BERS2 (Leica) (2/8) or TRS High pH (3-in-1) (Dako) (4/5) as retrieval buffer. The rmAb was diluted in the range of 1:50-1:250. Using these protocol settings, 21 of 23 (91%) laboratories produced a sufficient staining result.

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

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Dako Omnis**		**Ventana BenchMark XT / Ultra**		**Leica 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	ER2 pH 9.0	ER1 pH 6.0
mAb clone **5A4**	2/8** (25%)	-	0/3	-	4/13 (31%)	-	2/5 (40%)	-
mAb clone **OTI1A4 / 1A4**	3/3	-	14/15 (93%)	-	1/2	-	1/2	1/1
rmAb clone **D5F3**	3/4	-	0/5 (0%)	-	5/10 (50%)	-	2/8 (25%)	-

* 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 **5A4,** product no. **PA0306**, Leica/Novocastra, Bond III / Max:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 20-30 min. at 99-100°C), 15-20 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 2 of 4 (50%) produced a sufficient staining result (optimal or good).

mAb clone **5A4,** product no. **PA0831**, Leica/Novocastra, Bond III / Max:
One protocol with an optimal result was based on HIER using BERS2 (efficient heating time 30 min. at 100°C), 15 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system.

rmAb clone **D5F3** product no. **790-4794 or 790-4796**, Ventana, BenchMark GX, XT and Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 64-92 min.), 16-24 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 70 of 73 (96%) laboratories produced a sufficient staining result.

Table 4 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 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.

Table 4. **Proportion of sufficient and optimal results for lu-ALK 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 D5F3 **790-4794 or 790-4796**	97% (61/63)	87% (55/63)	84% (27/32)	66% (21/32)
Leica BOND mAb 5A4 **PA0306**	1/2	1/2	1/4	1/4

* 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 integrated.

Comments

In this assessment and in concordance with the previous NordiQC lu-ALK assessments, the prevalent feature of an insufficient result was a too weak or false negative staining reaction of cells expected to be demonstrated. This pattern was seen in 88% of the insufficient results (29 of 33 laboratories). The remaining 12% insufficient results were characterized by a poor signal-to-noise ratio and false positive staining reaction compromising interpretation (see Fig. 8). Virtually all the participating laboratories were able to demonstrate ALK in the neoplastic cells of the ALCL, whereas the Merkel cell carcinoma and the lung adenocarcinoma with EML-4 ALK translocation were more challenging and required an optimally calibrated IHC system (see Figs. 1 – 4).

43% (86 of 201) of the laboratories used Abs as concentrated formats within laboratory developed (LD) assays for ALK. The mAb clones 5A4, OTI1A4 and the rmAb clone D5F3 were the most widely used antibodies (see Table 1). Within LD assays for ALK, all three clones had a high proportion of sufficient and optimal staining results. In concordance with previous assessments, clone OTI1A4 was especially successful with a general pass rate of 92% (23 of 25) with 80% optimal. Optimal staining results were obtained on all 4 main IHC systems. With rmAb clone D5F3, optimal staining results were obtained on 3 of the main IHC systems, as no optimal staining results were recorded on the Dako Omnis system. The general pass rate for rmAb clone D5F3 reached 89% (25 of 28) with 39% optimal. For both clones, efficient HIER in an alkaline buffer, careful calibration of the titer of the primary Ab (see Figs. 5 – 7) and especially use of a sensitive 3-step polymer/multimer based detection system were the main prerequisites for a sufficient and optimal staining result. Compared to the last ALK assessment in 2017 (Run 51), the mAb clone 5A4 showed an improved performance, displaying a pass rate of 81% (26 of 32) with 25% optimal. In 2017, the pass rate was only 34% with 2% optimal. The reason for these differences is unclear but could be related to a more challenging material circulated in the 2017 ALK assessment. In that run, especially a low-level ALK expressing Merkel cell carcinoma was a challenge for laboratories using the mAb clone 5A4. Optimal staining results were obtained on 3 of the main IHC systems, as no optimal staining results were recorded on the Dako Omnis system. For the mAb clone 5A4, efficient HIER in an alkaline buffer, careful calibration of the titer of the primary Ab and especially use of a sensitive 3-step polymer/multimer based detection system were the main prerequisites for a sufficient and optimal staining result similar to the observations for D5F3.

57% (115 of 201) of the laboratories used Abs in Ready-To-Use (RTU) formats. This was a minor increase compared to the previous ALK assessment in 2017, where 54% of the laboratories used a RTU format. Only RTU systems based on rmAb clone D5F3 and mAb clone 5A4 provided optimal staining results. The Ventana RTU systems based on the rmAb clone D5F3 (prod. no. 790-4794 and 790-4796) were the most successful and robust assays for ALK giving an overall pass rate of 93% (88 of 95 laboratories) with 80% optimal. Optimal results were typically obtained using the officially recommended protocol based on extended HIER in CC1 (92 min.), 16 min. incubation of the primary Ab, OptiView + amplification kit as detection system and BenchMark Ultra/XT/GX as stainer platform. Using these settings, an overall pass rate of 97% (61 of 63 laboratories) was seen and 87% received an optimal score. Slightly modified protocol settings such as reduced HIER time and/or adjustment of the incubation time of the primary Ab could also be used to obtain sufficient and optimal staining results, but the general pass rate was lower, with 84% being sufficient and 66% optimal.
The Leica Bond RTU system based on mAb clone 5A4 (prod. no. PA0306) was used by 6 laboratories. Despite the use of the officially recommended protocol or protocol setups with similar levels of analytical sensitivity the pass rate was only 33% (2 of 6). The recently introduced PA0831 RTU system (Leica) was used by one laboratory and using the vendor recommended protocol settings, optimal staining result was obtained. Both the PA0831 and the PA0306 RTU systems are based on mAb 5A4, but the datasheets suggest higher concentration of antibody in the PA0831 RTU compared to PA0306, This, and the vendor

recommended protocol settings based on prolonged HIER in BERS2, could indicate improvements in analytical sensitivity. Future ALK assessments might reveal if that is the case.

In concordance with previous assessments, RTU systems based on mAb clone ALK1 gave an insufficient result in 100% (8 of 8) of the protocols. In most cases, the mAb clone ALK1 gave the expected staining reaction in the ALCL, but an insufficient (too weak or false negative) result in the lung adenocarcinoma with EML4-ALK translocation and Merkel cell carcinoma (see Fig. 1 – 4). This indicates that mAb clone ALK1 is not fit for purpose demonstrating ALK fusion protein in EML-ALK translocated lung adenocarcinomas.

This was the fourth NordiQC assessment of ALK and a pass rate of 84% was obtained, which was a significant improvement compared to the result obtained in run 51, 2017 (see Table 2). The extended use of the robust and successful RTU system 790-4794 based on the rmAb clone D5F3 could explain some of the improvement. In 2017 64% (70 of 110) of the Ventana users used the 790-4794 RTU system. In the present assessment, this number increased to 75% (94 of 125). Likewise, for LD assays an extended use of the successful mAb clone OTI1A4 also contribute to the improved pass rate.

Controls

In order to evaluate the analytical sensitivity and specificity of the IHC assay for EML4-ALK translocation, the selection of control material must reflect the diagnostic use of the assay. If the assay is to be used for the demonstration of ALK rearrangements both in lung adenocarcinoma (EML4-ALK) and lymphomas, these two materials must be included as positive tissue controls (both for the initial calibration/validation process but also as daily performance controls). Typically, ALCLs will display an intense staining reaction due to a high expression level of ALK protein, whereas lung adenocarcinomas (EML4-ALK) will show a weak to moderate staining reaction due to lower levels of ALK protein expression. Negative tissue controls, as tonsil and lung non-small cell carcinoma without ALK rearrangement, should also be included. The ALK status of all the included positive and negative tissue controls must be confirmed by FISH in the validation process.

In this assessment and in concordance with previous assessments, appendix was found to be a valuable and recommendable external positive tissue control, especially useful to evaluate the level of the analytical sensitivity of the assay: In virtually all optimal protocols for lu-ALK, a weak to strong granular cytoplasmic staining reaction was seen in the ganglion cells and a weak to moderate reaction in the axons. If these cells/structures were negative, a too weak or even completely false negative staining reaction was seen in the lung adenocarcinoma with EML4-ALK translocation and Merkel cell carcinoma (see Figs. 1 – 7). In general, the mAb clone OTI1A4 and rmAb clone D5F3 gave a stronger and more extensive staining reaction of ganglion cells compared to mAb clone 5A4. This could reflect a higher analytical sensitivity of these two clones.

In this assessment and in concordance with the previous assessments, the Merkel cell carcinoma proved to be challenging. Merkel cell carcinomas do not harbour ALK translocations/inversions, but more than 90% show aberrant/overexpression of ALK protein (1,2). The amount of ALK protein is generally much lower than in ALCL, most often on par with low level ALK expressing lung adenocarcinoma with EML4-ALK translocation. This makes Merkel cell carcinomas an important addition to the positive tissue controls needed for lu-ALK assays, at least for the initial calibration/validation process.

1. Filtenborg-Barnkob BE, Bzorek M. Expression of anaplastic lymphoma kinase in Merkel cell carcinomas. Hum Pathol. 2013 Jul 31;44(8):1656–64.

2. Veija T, Koljonen V, Bohling T, Kero M, Knuutila S, Sarhadi VK. Aberrant expression of ALK and EZH2 in Merkel cell carcinoma. BMC Cancer. 2017 Mar 31;17(1):236.

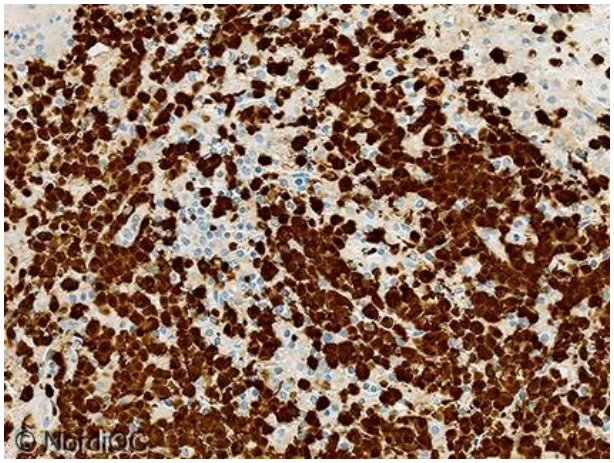


Fig. 1a
Optimal ALK staining of the ALCL with ALK rearrangement using the rmAb clone D5F3 RTU (Ventana, 790-4794) by vendor recommended protocol settings. The neoplastic cells show an intense nuclear and cytoplasmic staining reaction. Despite the intense staining reaction, a high signal-to-noise ratio is provided, and no background staining is seen. Also compare with Figs. 2a - 4a, same protocol.

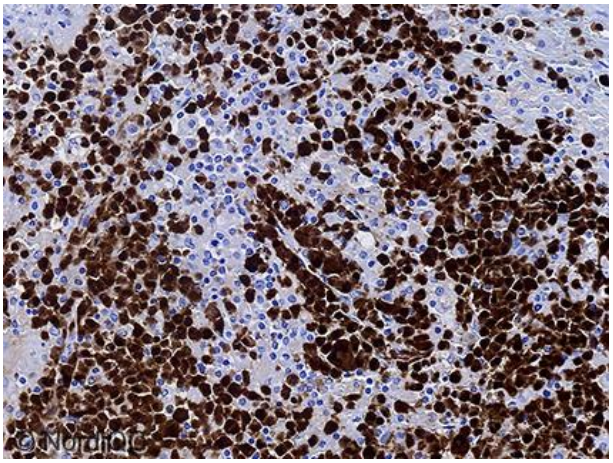


Fig. 1b
ALK staining of the ALCL with ALK rearrangement using an insufficient protocol providing a too low sensitivity for the demonstration of ALK rearrangement in lung adenocarcinoma - same field as in Fig. 1a. The protocol was based on the mAb clone ALK1 RTU (Ventana, 790-2918), using vendor recommended protocol settings. The neoplastic cells of the ALCL are demonstrated, however also compare with Figs. 2b – 4b, same protocol.

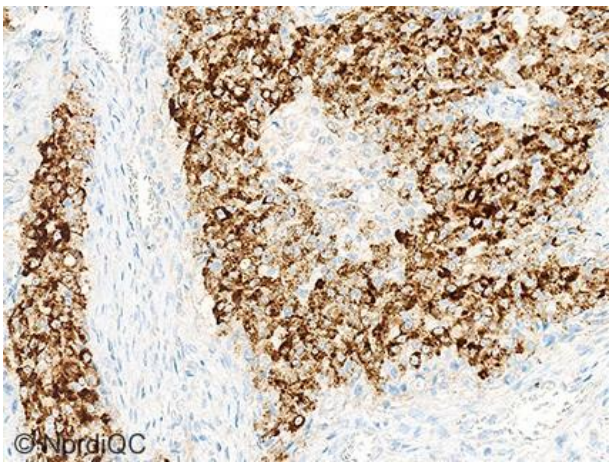


Fig. 2a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1a. Most of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction. No background staining is seen.

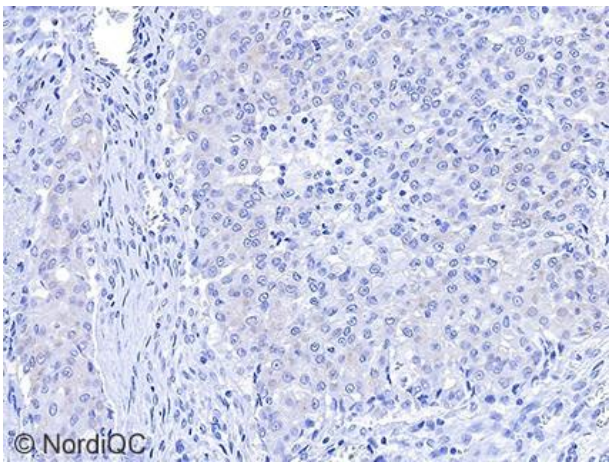


Fig. 2b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1b - same field as in Fig. 2a. The neoplastic cells are false negative.

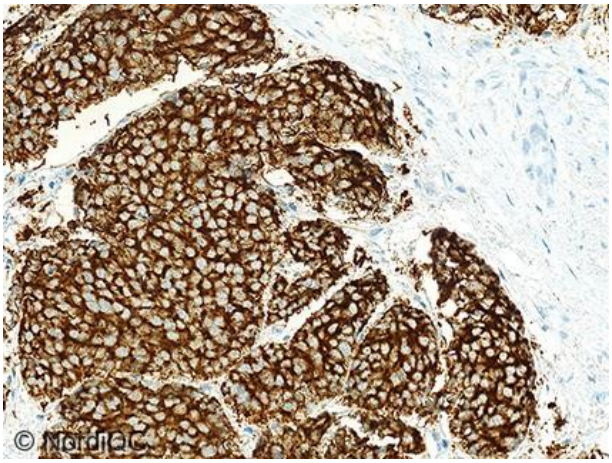


Fig. 3a
Optimal ALK staining of the Merkel cell carcinoma using same protocol as in Figs. 1a - 2a. Virtually all the neoplastic cells show a strong granular cytoplasmic staining reaction. No background staining is seen.

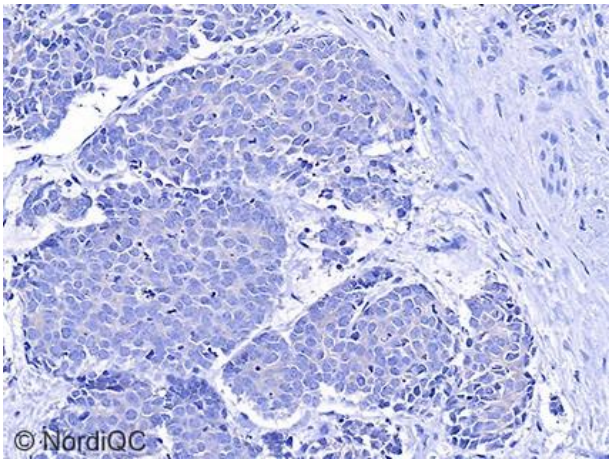


Fig. 3b
Insufficient ALK staining of the Merkel cell carcinoma using same protocol as in Figs. 1b - 2b - same field as in Fig. 3a. The neoplastic cells are false negative.

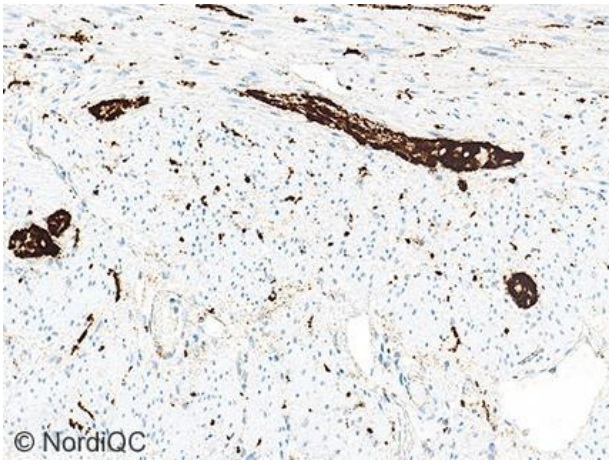


Fig. 4a
Optimal ALK staining of the appendix using same protocol as in Figs. 1a - 3a. The ganglion cells of the myenteric plexus display a moderate to strong, distinct cytoplasmic staining reaction, while the axons display a weak to moderate staining reaction.

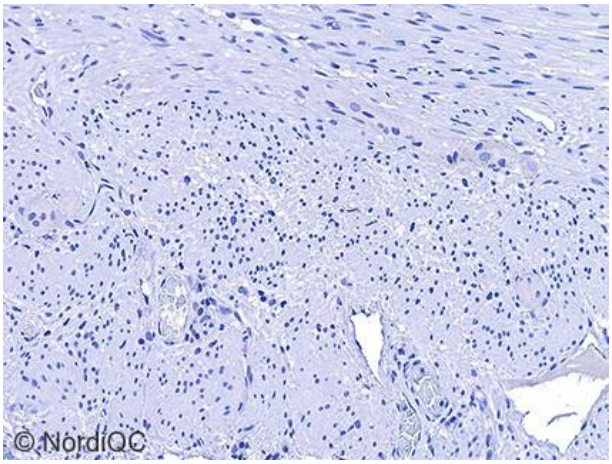


Fig. 4b
Insufficient ALK staining of the appendix using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a. Both ganglion cells and axons are unstained. This seems to predict false negative reaction in both the Merkel cell carcinoma and lung adenocarcinoma with ALK rearrangement, see Fig. 2b and 3b.

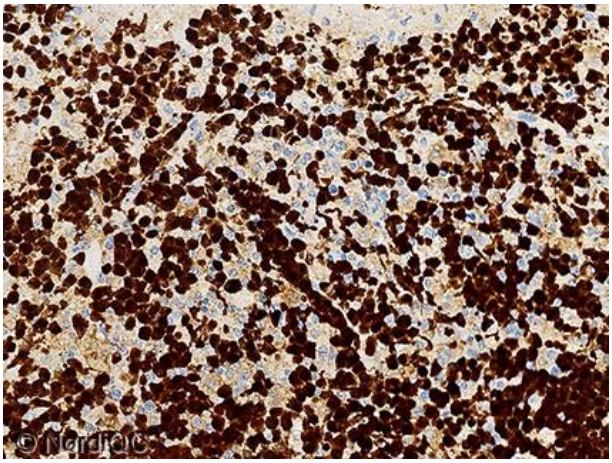


Fig.5a
Optimal ALK staining of the ALCL with ALK rearrangement using the mAb clone OTI1A4 in an optimally calibrated LD assay on the Dako OMNIS instrument. The neoplastic cells show an intense nuclear and cytoplasmic staining reaction. Despite the intense staining reaction, a high signal-to-noise ratio is provided, with minimal background staining. Also compare with Figs. 6a and 7a, same protocol. Compare with Fig. 5b.

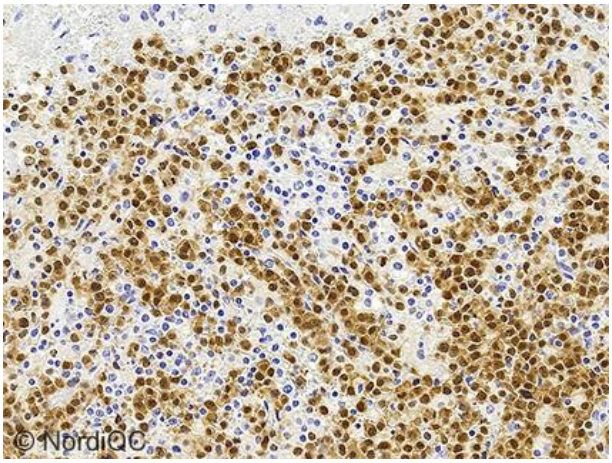


Fig. 5b
ALK staining of the ALCL with ALK rearrangement using an insufficient protocol providing a too low sensitivity for the demonstration of ALK rearrangement in lung adenocarcinoma - same field as in Fig. 5a. The protocol was based on the mAb clone OTI1A4 used in a very low concentration on the BioCare IntelliPath instrument. The vast majority of neoplastic cells of the ALCL are weakly to moderately demonstrated, however also compare with Figs. 6b and 7b, same protocol. Compare with Fig. 5a (same field).

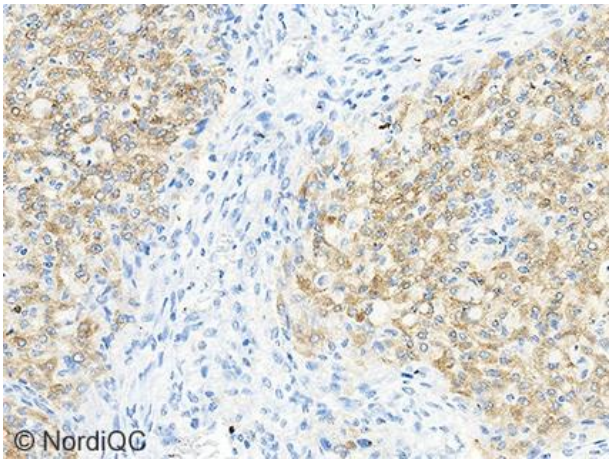


Fig. 6a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 5a. Most of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction. No background staining is seen. Compare with Fig. 6b.

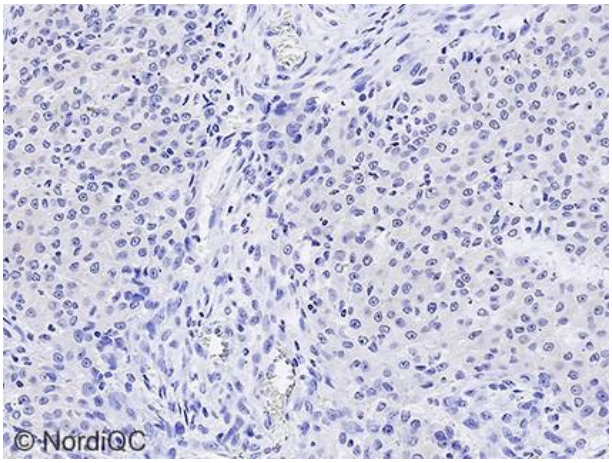


Fig. 6b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 5b. The neoplastic cells are all false negative. Compare with Fig. 6a (same field).

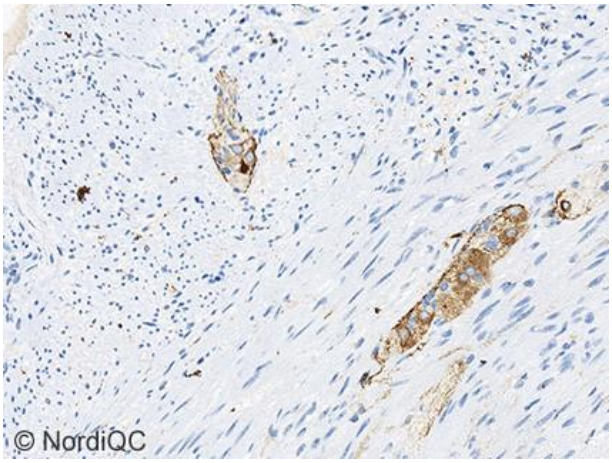


Fig. 7a
Optimal ALK staining of the appendix using same protocol as in Figs. 5a - 6a. The ganglion cells of the myenteric plexus display a moderate to strong, distinct cytoplasmic staining reaction, while the axons display a weak to moderate staining reaction.

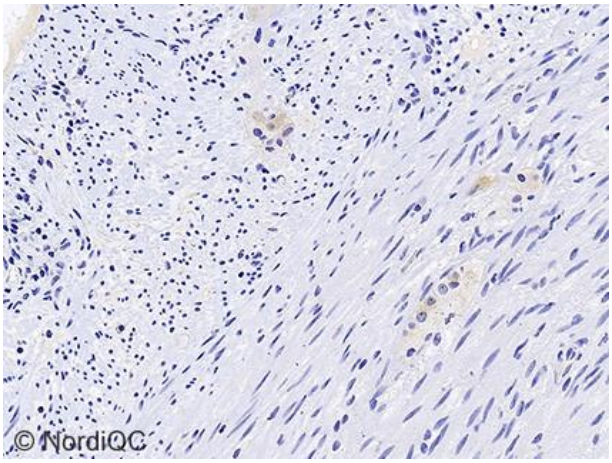


Fig. 7b
Insufficient ALK staining of the appendix using same protocol as in Figs. 5b - 6b - same field as in Fig. 7a. A few ganglion cells are faintly positive, but the axons are unstained. The faintly stained ganglion cells and unstained axons seem to predict a false negative staining reaction in Merkel cell carcinoma and lung adenocarcinoma with ALK rearrangement – see Fig. 6b.

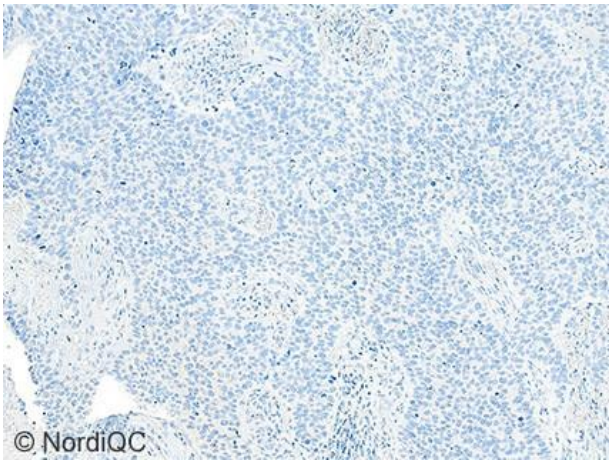


Fig. 8a
Optimal ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Figs. 1a - 4a. The neoplastic cells are all negative.

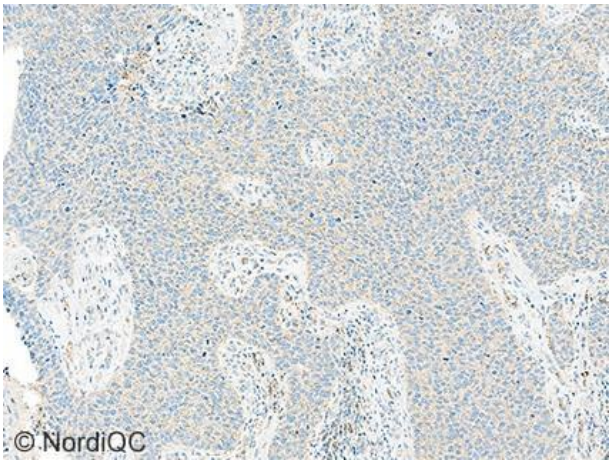


Fig. 8b
Insufficient ALK staining of the lung adenocarcinoma without ALK rearrangement using a LD assay based on rmAb clone D5F3 as a concentrate in a protocol with similar protocol settings as the Ventana recommended ALK protocol (Figs. 1a – 4a). But the use of a too high concentration of the primary Ab within highly sensitive protocol settings results in false positive staining reaction in both neoplastic cells and normal stromal cells. Compare with Fig. 8a (same field).

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