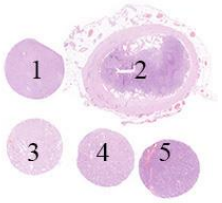
Assessment Run 65 2022

Lung Anaplastic Lymphoma Kinase (ALK (lung))

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 EML4-ALK 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 ALK (lung) comprised:



1. Anaplastic large cell lymphoma with ALK translocation, 2, Appendix, 3. Lung adenocarcinoma with EML4-ALK translocation*, 4. Lung adenocarcinoma without EML4-ALK translocation*, 5. Merkel cell tumor.
*confirmed by FISH

All tissues were fixed in 10% neutral buffered formalin.

Criteria for assessing ALK (lung) 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 ALK (lung), run 65	273
Number of laboratories returning slides	256 (94%)

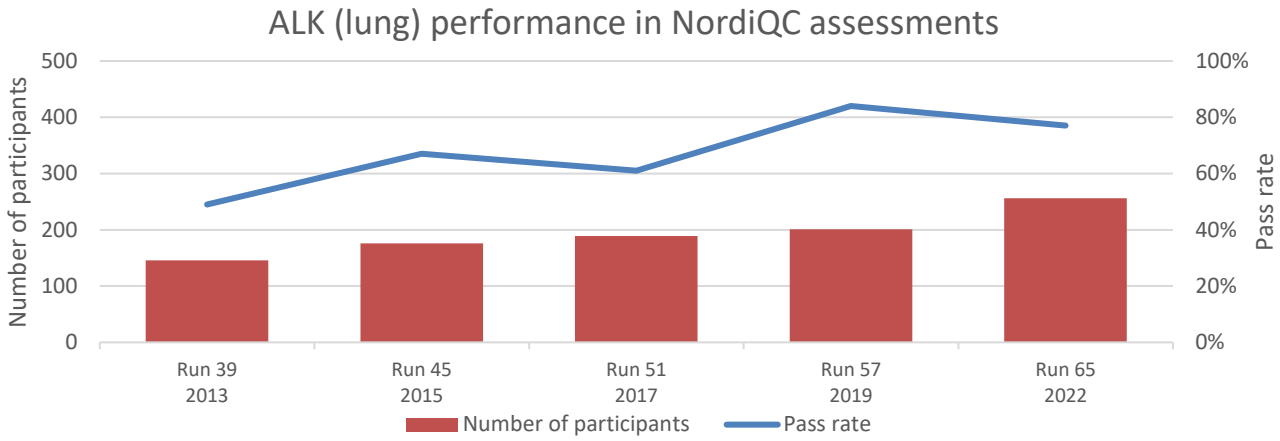
Results
At the date of assessment, 94% of the participants had returned the circulated NordiQC slides. All slides returned after the assessment were assessed and laboratories received advice if the result was insufficient, but the data were not included in this report.

256 laboratories participated in this assessment. 198 (77%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies used and assessment marks given (see page 3).

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 fifth NordiQC assessment of ALK (lung). A slightly decrease of the pass rate was seen compared to run 57 in 2019 (see Graph 1).

Graph 1. **Proportion of sufficient results for ALK (lung) in the five NordiQC runs performed**



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 Dako/Agilent Ready-To-Use (RTU) system based on the mAb clone OTI1A4 was the most successful assay with a pass rate of 100%, all optimal. The Ventana/Roche RTU system based on the rmAb clone D5F3 was the most used assay and using the recommended protocol settings providing an overall pass rate of 95%.
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 ALK (lung).

Table 1. **Antibodies and assessment marks for ALK (lung), run 65**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	OR[2]
mAb clone **5A4**	26 2 1 1 2 2 1	Leica Biosystems Monosan Abcam DBS Biocare Medical Zytomed Systems Invitrogen	8	9	14	4	49%	23%
mAb clone **OTI1A4***	19 1 1 1	Origene Nordic Biosite Cell Signaling Zeta Corporation	16	6	0	0	100%	73%
mAb clone **IHC509**	1	GenomeMe	0	0	1	0	-	-
rmAb clone **D5F3**	19	Cell Signaling	7	9	3	0	84%	36%
rmAb clone **ALK1**	3 1	Dako/Agilent Cell Marque	0	0	0	4	-	-
rmAb clone **QR017**	1	Quartett	0	1	0	0	-	-
rmAb clone **SP8**	1	BioGenex	0	0	0	1	-	-
rmAb clone **ZR305**	1	Zeta Corporation	0	0	1	0	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**/PA0831 (VRPS)[3]**	2	Leica Biosystems	1	1	0	0	-	-
mAb clone **5A4 PA0306*/PA0831 (LMPS)[4]**	10	Leica Biosystems	4	3	2	1	70%	40%
mAb clone **5A4 API3041**	1	BioCare	0	0	1	0	-	-
mAb clone **5A4 CAM-0170**	1	Celnovte	0	1	0	0	-	-
mAb clone **5A4 MAD-001720QD**	1	Master Diagnostica	0	0	1	0	-	-
mAb clone **ALK1 GA641**	3	Dako/Agilent	0	0	0	3	-	-
mAb clone **ALK1 IR641**	4	Dako/Agilent	0	0	0	4	-	-
mAb clone **ALK1 790/800-2918 (LMPS)[4]**	10	Ventana/Roche	1	0	1	8	10%	10%
mAb clone **137E9E8 PA132**	1	Abcarta	0	0	0	1	-	-
mAb clone **OTI1A4 / 1A4 8344-C010**	1	Sakura Finetek	1	0	0	0	-	-
mAb clone **OTI1A4 / 1A4 GA785 (VRPS)[3]**	12	Dako/Agilent	12	0	0	0	100%	100%
mAb clone **OTI1A4 / 1A4 GA785 (LMPS)[4]**	4	Dako/Agilent	4	0	0	0	-	-
rmAb clone **D5F3 790-4794 (VRPS)[3]**	73	Ventana/Roche	62	7	1	3	95%	85%
rmAb clone **D5F3 790-4794 (LMPS)[4]**	48	Ventana/Roche	36	9	3	0	94%	75%
rmAb clone **SP8 RMPD007**	1	Diagnostic BioSystems	0	0	0	1	-	-
Total	256		152	46	28	30		
Proportion			59%	18%	11%	12%	77%	

1) Proportion of sufficient stains (optimal or good) (≥5 assessed protocols).
2) Proportion of Optimal Results (≥5 assessed protocols).
3) Vendor Recommended Protocol Settings (VRPS) to a specific RTU product applied on the vendor recommended platform(s) (≥5 assessed protocols).
4) Laboratory Modified Protocol Settings (LMPS) to a specific RTU product (≥5 assessed protocols).
*) OTI1A4 is called 1A4 by some vendors
**) Product no. PA0306 has been terminated and replaced by PA0831.

Detailed analysis of ALK (lung), Run 65
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/Roche) (2/15)*, Target Retrieval Solution (TRS) High pH (3-in-1) (Dako/Agilent) (4/7) or Bond Epitope Retrieval Solution 2 (BERS2, Leica Biosystems) (2/8) as retrieval buffer. The mAb was typically diluted in the range of 1:10-1:50. Using these protocol settings, 17 of 29 (59%) laboratories produced a sufficient staining result (optimal or good).
* (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/Agilent) (10/13), TRS High pH (3-in-1) (Dako/Agilent) (2/2), CC1 (Ventana/Roche) (2/3), BERS2 (Leica Biosystems) (1/2) or Bond Epitope Retrieval Solution 1 (BERS1, Leica Biosystems) (1/1) as retrieval buffer. The mAb was diluted in the range of 1:50-1:2,000. Using these protocol settings, 20 of 20 (100%) laboratories produced a sufficient staining result.

rmAb clone **D5F3**: Protocols with optimal results were all based on HIER using either CC1 (Ventana/Roche) (5/11), BERS2 (Leica Biosystems) (1/4) or TRS High pH (3-in-1) (Dako/Agilent) (1/1) as retrieval buffer. The rmAb was diluted in the range of 1:50-1:250. Using these protocol settings, 13 of 16 (81%) laboratories produced a sufficient staining result.

Table 3. **Proportion of optimal results for ALK (lung) 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**	4/7** (57%)	-	0/4	-	2/15 (13%)	-	2/7 (29%)	-
mAb clone **OTI1A4 / 1A4**	2/2	-	10/12 (83%)	-	2/3	-	1/2	1/1
rmAb clone **D5F3**	1/1	-	0/3	-	5/11 (45%)	-	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
mAb clone **5A4,** product no. **PA0306/PA0831**, Leica Biosystems, Bond III / Max:
Protocols with optimal results were based on HIER using BERS2 (efficient heating time 20-40 min. at 99-100°C), 15-30 min. incubation of the primary Ab and Bond Polymer Refine (DS9800) as detection system. Using these protocol settings, 7 of 8 (88%) produced a sufficient staining result (optimal or good).

mAb clone **OTI1A4,** product no. **GA785**, Dako/Agilent, Omnis:
Protocols with optimal results were based on HIER using TRS High pH (efficient heating time 30 min. at 97-99°C), 20-30 min. incubation of the primary Ab and EnVision Flex (GV800/GV823+GV821) as detection system. Using these protocol settings, 16 of 16 (100%) produced a sufficient staining result.

rmAb clone **D5F3** product no. **790-4794**, Ventana/Roche, BenchMark GX, XT and Ultra:
Protocols with optimal results were typically based on HIER using CC1 (efficient heating time 92 min.), 16 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 110 of 117 (94%) 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 ALK (lung) 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**	95% (69/73)	85% (62/73)	93% (41/44)	80% (35/44)
Dako Omnis mAb OTI1A4 **GA785**	100% (12/12)	100% (12/12)	(4/4)	(4/4)
Leica BOND mAb 5A4 **PA0306/PA0831**	(2/2)	(1/2)	75% (6/8)	50% (4/8)

* 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 ALK (lung) 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 95% of the insufficient results (55 of 58). The remaining 5% insufficient results were characterized by a poor signal-to-noise ratio and/or false positive staining reaction compromising the interpretation. 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.

33% (84 of 256) 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 could provide sufficient and optimal staining results. In concordance with previous assessments, clone OTI1A4 was especially successful with a general pass rate of 100% (22 of 22) with 73% optimal. Optimal staining results were obtained on all 4 main IHC systems (see Table 3). 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 84% (16 of 19) with 36% optimal. For both clones, 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. Compared to the last ALK assessment in 2019 (Run 57) the mAb clone 5A4 showed a significantly reduced pass rate of 49% (17 of 35), 23% optimal. In 2019 a pass rate of 81% (26 of 32) with 25% optimal. No obvious reason for the reduced pass rate has been identified.
The mAb clone 5A4 gave an optimal staining result 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.

67% (172 of 256) of the laboratories used Abs as Ready-To-Use (RTU) formats. The Ventana RTU system based on the rmAb clone D5F3 (prod. no. 790-4794) was the most used assay for ALK giving an overall pass rate of 94% (114 of 121 laboratories) with 81% 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.
The Leica Bond RTU system based on mAb clone 5A4 (prod. no. PA0306/PA0831) was used by 10 laboratories. Only two laboratories used the vendor recommended protocol, both obtaining sufficient results. Minor adjustments in HIER time and/or incubation time of primary Ab were seen for the modified protocols, giving a pass rate of 75% (6 of 8) (see Table 4).
The new Dako/Agilent RTU system for Omnis (prod. no. GA785) based on mAb clone OTI1A4 was the most successful assay, used by 16 laboratories, all with optimal results. All laboratories used EnVision Flex+ as detection system, as recommended by vendor.

In concordance with previous assessments, concentrated Abs and RTU systems based on mAb clone ALK1 gave a low pass rate of only 6% (1 of 17). 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. This indicates that mAb clone ALK1 is not fit for purpose demonstrating ALK fusion protein in EML-ALK translocated lung adenocarcinomas.

Controls
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 ALK (lung), 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. 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 ALK (lung) 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.

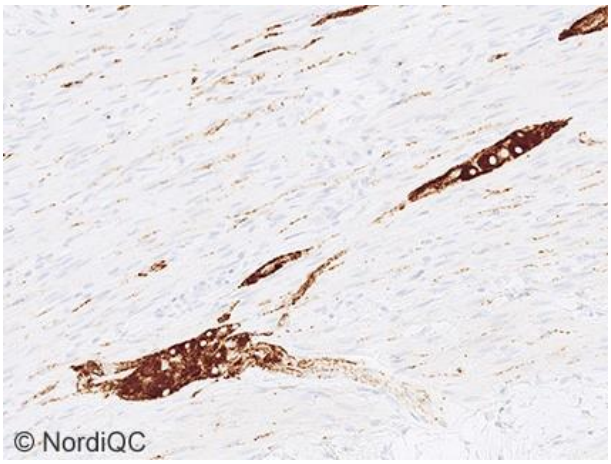


Fig. 1a
Optimal ALK staining of axons and ganglion cells in appendix using the rmAb clone D5F3 RTU (Ventana/Roche, 790-4794) by vendor recommended protocol settings. The ganglion cells of the myenteric plexus display a moderate to strong, distinct cytoplasmic staining reaction, while the axons display a weak to moderate staining reaction.
Also compare with Figs. 2a - 6a, same protocol.

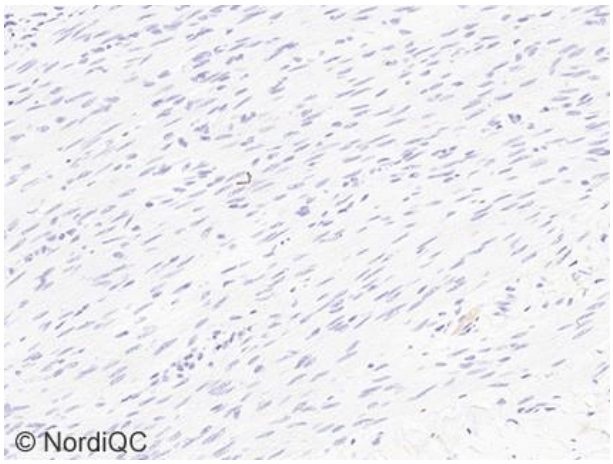


Fig. 1b
Insufficient ALK staining of the appendix using same protocol as in Figs. 1b - 4b - same field as in Fig. 1a. Both ganglion cells and axons are unstained. The protocol was based on the mAb clone ALK1 RTU (Dako/Agilent, GA641), using vendor recommended protocol settings.

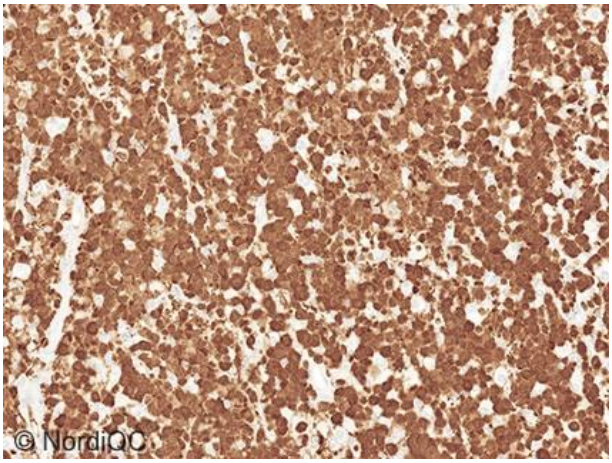


Fig. 2a
Optimal ALK staining of the ALCL with ALK rearrangement using same protocol as in Fig. 1a. The neoplastic cells show an intense nuclear and cytoplasmic staining reaction.

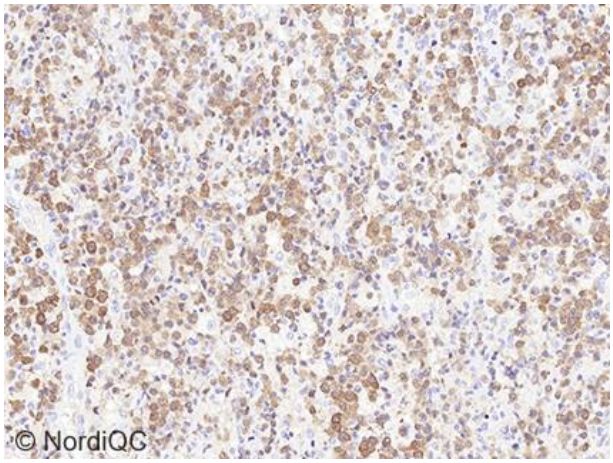


Fig. 2b
ALK staining of the ALCL with ALK rearrangement using same protocol as in Fig. 1b. The neoplastic cells are demonstrated, however, the intensity and proportion of positive cells are significantly reduced – compare with optimal result in Fig. 2a, same area.

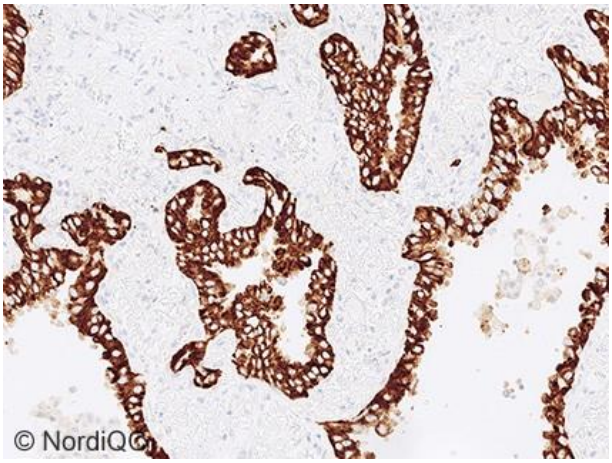


Fig. 3a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Figs. 1a – 2a. Most of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction. No background staining is seen.

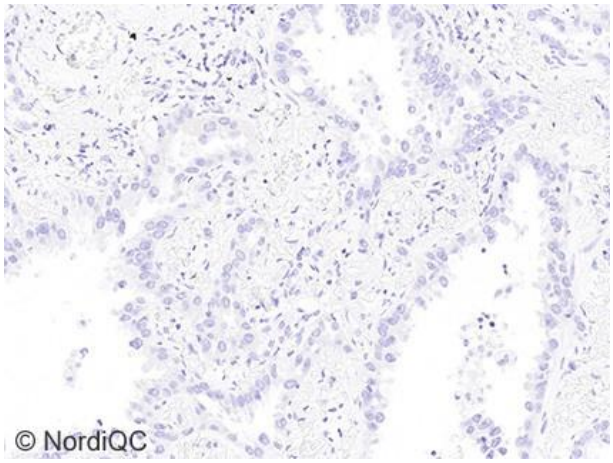


Fig. 3b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Figs. 1b – 2b - same field as in Fig. 3a. The neoplastic cells are false negative.

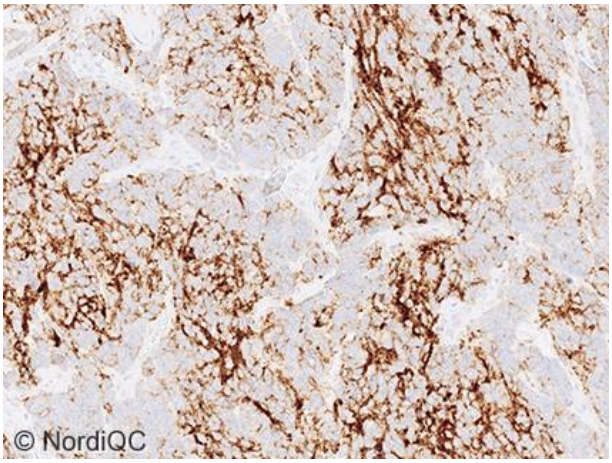


Fig. 4a
Optimal ALK staining of the Merkel cell carcinoma using same protocol as in Figs. 1a - 3a. Virtually all the neoplastic cells show a strong granular cytoplasmic staining reaction. No background staining is seen.

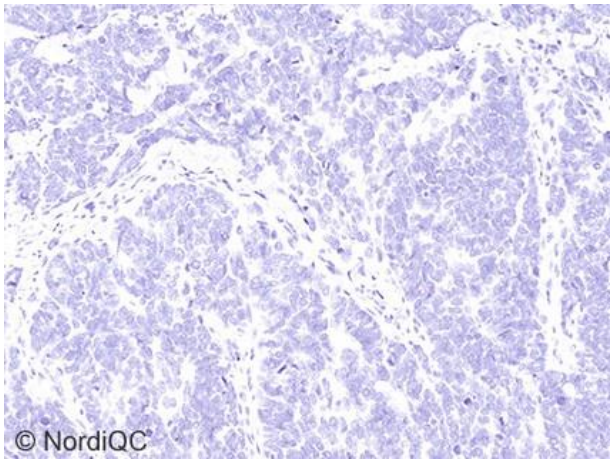


Fig. 4b
Insufficient ALK staining of the Merkel cell carcinoma using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a. The neoplastic cells are false negative.

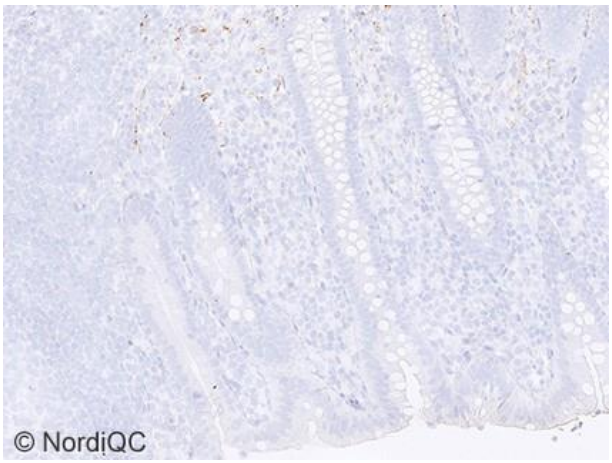


Fig. 5a
Optimal ALK staining of lamina propria in appendix using the same protocol as in Fig. 1a – 4a. Deprived nerves show a weak staining reaction. The epithelial cells are negative.

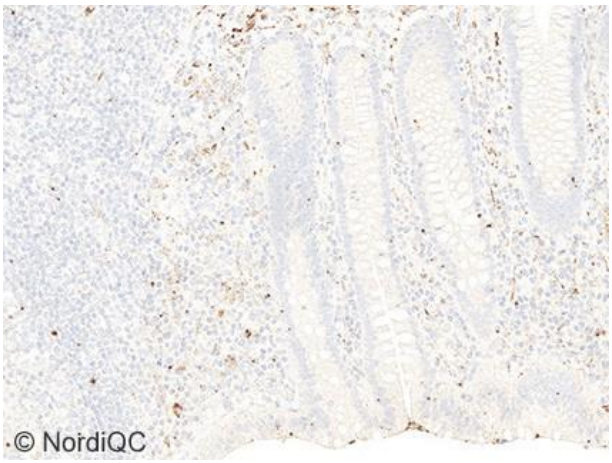


Fig. 5b
ALK staining of lamina propria in appendix using an insufficient protocol based on the mAb clone D5F3 diluted 1:50 and OptiView with amplification as detection system. The use of thyramide amplification combined with a high concentration of the primary Ab causes an aberrant granular staining reaction in "non-nerve" derived structures/cells. Compare with Fig. 5a (same field).

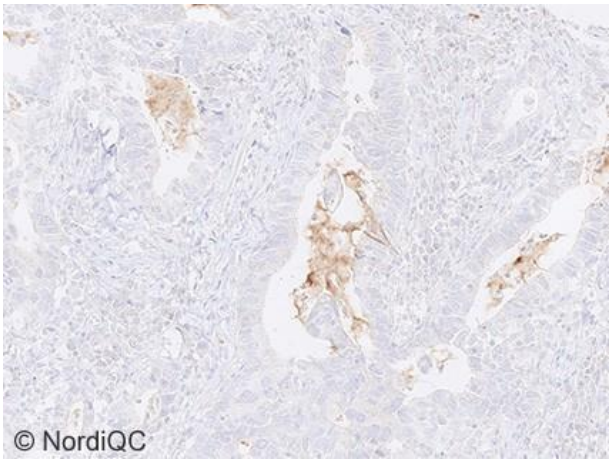


Fig. 6a
Optimal ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Figs. 1a - 5a. No staining reaction is seen.

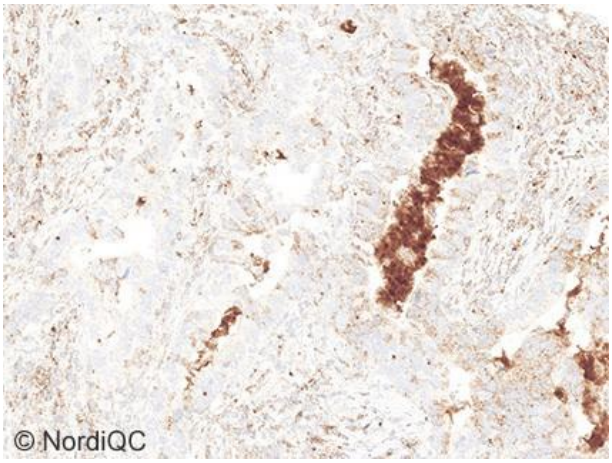


Fig. 6b
Insufficient ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Fig. 5b. The use of tyramide amplification combined with a high concentration of the primary Ab causes an aberrant false positive granular staining reaction in the neoplastic cells.

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