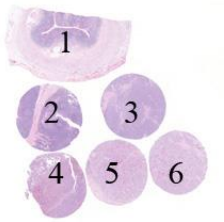
## Assessment Run 51 2017

# Lung Anaplastic Lymphoma Kinase (lu-ALK)




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

1. Appendix, 2. Tonsil, 3. Merkel cell carcinoma, 4. Anaplastic large cell lymphoma with ALK translocation, 5. Lung adenocarcinoma with EML4-ALK translocation
6. Lung adenocarcinoma without 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 dispersed neoplastic cells in the Merkel cell carcinoma.
- An at least weak to moderate granular cytoplasmic staining reaction of ganglion cells 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 51 | 209 |
| Number of laboratories returning slides | 189 (90%) |

**Results**
189 laboratories participated in this assessment. 115 (61%) achieved a sufficient mark (optimal or good). Table 1 summarizes the antibodies used and assessment marks (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 third NordiQC assessment of lu-ALK. A slight decrease of the pass rate was seen compared to run 45 in 2015 (see Table 2).

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

| | Run 39 2013 | Run 45 2015 | Run 51 2017 |
|---|---|---|---|
| Participants, n= | 146 | 176 | 189 |
| Sufficient results | 49% | 67% | 61% |

**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, 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. Sufficient staining results were also seen with the mAb clone **5A4** but the analytical sensitivity was significantly lower compared to mAb clone OTI1A4 and rmAb D5F3.
The Ventana Ready-To-Use system based on the rmAb clone D5F3 and using the recommended protocol settings were the most successful assay with an impressive overall pass rate of 100%.
Lung adenocarcinomas with and without ALK translocation must be applied as positive and negative tissue controls when the assay is used for lung carcinoma. ALCLs will typically express a too high level of antigen

expression and cannot be recommended as the only positive tissue control for ALK. Appendix is an excellent supplemental positive tissue control, in which ganglion cells of the myenteric plexus must show an at least weak to moderate staining reaction.

Table 1. **Antibodies and assessment marks for lu-ALK, run 51**

| Concentrated antibodies | n | Vendor | Optimal | Good | Borderline | Poor | Suff.[1] | Suff. OPS[2] |
|---|---|---|---|---|---|---|---|---|
| mAb clone **5A4** | 43<br>1<br>1<br>1<br>1 | Leica/Novocastra<br>Abcam<br>Biocare<br>Monosan<br>ThermoFisher | 1 | 15 | 24 | 7 | 34% | 22% |
| mAb clone **ALK1** | 2<br>1 | Dako<br>Cell Marque | 0 | 0 | 0 | 3 | - | - |
| rmAb clone **D5F3** | 23 | Cell Signaling | 6 | 12 | 3 | 2 | 78% | 94% |
| mAb clone **OTI1A4** | 13 | ORIGENE | 10 | 3 | 0 | 0 | 100% | 100% |
| Ready-To-Use antibodies | | | | | | | | |
| mAb clone **5A4**<br>**PA0306** | 6 | Leica/Novocatra | 0 | 0 | 6 | 0 | - | - |
| mAb clone **5A4**<br>**MAB-0281** | 1 | Maixin | 0 | 0 | 1 | 0 | - | - |
| mAb **5A4**<br>**MAD-001720QD** | 1 | Master Diagnostica | 0 | 0 | 1 | 0 | - | - |
| mAb clone **5A4**<br>**MS-1104-R7** | 1 | ThermoFisher | 0 | 1 | 0 | 0 | - | - |
| mAb **ALK1**<br>**IR641** | 9 | Dako | 0 | 0 | 1 | 8 | - | - |
| mAb clone **ALK1**<br>**GA641** | 4 | Dako | 0 | 0 | 0 | 4 | - | - |
| mAb clone **ALK1**<br>**790/800-2918** | 7 | Ventana | 0 | 0 | 2 | 5 | - | - |
| rmAb clone **SP8**<br>**AN770** | 1 | BioGenex | 0 | 0 | 0 | 1 | - | - |
| rmAb clone **D5F3**<br>**790-4796** | 70 | Ventana | 53 | 12 | 4 | 1 | 93% | 100% |
| rmAb clone **D5F3**<br>**790-4796**[3] | 2 | Ventana | 1 | 0 | 1 | 0 | - | - |
| mAb clone **OTI1A4**<br>**8344-C010** | 1 | Sakura Finetek | 1 | 0 | 0 | 0 | - | - |
| Total | 189 | | 72 | 43 | 43 | 31 | - | |
| Proportion | | | 38% | 23% | 23% | 16% | 61% | |

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 51**
The following protocol parameters were central to obtain optimal staining:

**Concentrated antibodies**
mAb clone **5A4**: One protocol with an optimal result was based on heat induced epitope retrieval (HIER) using Cell Conditioning 1 (CC1, Ventana) in 64 min. at 100°C. The mAb was diluted 1:20 and incubated for 32 min. at 36°C using OptiView with tyramide amplification as detection system. Using similar protocol settings 2 of 9 (22%) laboratories produced a sufficient staining result.

mAb clone **OTI1A4**: Protocols with optimal results were all based on HIER using either Target Retrieval Solution (TRS), High pH (Dako) (5/5) *, TRS High pH (3-in-1) (Dako) (2/3), CC1 (Ventana) (1/2) Bond Epitope Retrieval Solution 2 (BERS2, Leica) (1/1) or Tris-EDTA pH 9 (1/1), as retrieval buffer. The mAb

was diluted in the range of 1:100-1:1,500. Using these protocol settings, 13 of 13 (100%) laboratories produced a sufficient staining result.
* (number of optimal results/number of laboratories using this HIER buffer)

rmAb clone **D5F3**: Protocols with optimal results were all based on HIER using either BERS2 (Leica) (2/7), CC1 (Ventana) (2/6) or TRS High pH (3-in-1) (Dako) (2/3), as retrieval buffer. The rmAb was diluted in the range of 1:50-1:200. Using these protocol settings, 15 of 16 (94%) 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** | 0/9** (0%) | | 0/3 | - | 1/22 (5%) | - | 0/9 (0%) | 0/1 |
| mAb clone **OTI1A4** | 2/2 | - | 5/5 (100%) | - | 1/2 | - | 1/1 | - |
| rmAb clone **D5F3** | 2/3 | 0/1 | 0/3 | - | 2/6 (33%) | - | 2/7 (29%) | 0/1 |

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

**Ready-To-Use antibodies and corresponding systems**
mAb clone **OTI1A4,** product no. **8344-C010**, Sakura Finetek, Genie:
One protocol with an optimal result was based on 45 min. HIER using Sakura Finetek Tissue-Tek Genie High pH Antigen Retrieval Buffer, 30 min. incubation of the primary Ab and Tissue-Tek PRO DAB Detection Kit (8826-K250) 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 Cell Conditioning 1 (efficient heating time 88-104 min.), 16-20 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 63 of 63 (100%) 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** | 100% (56/56) | 82% (46/56) | 64% (9/14) | 50% (7/14) |

* 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 97% of the insufficient results (72 of 74 laboratories). The remaining 3% insufficient results were characterized by a poor signal-to-noise ratio and false positive staining reaction compromising interpretation (see Fig. 9). 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 Fig. 1 – 4).

46% (87 of 189) 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). As seen in previous NordiQC assessment, the overall pass rate was in particular influenced by the choice of the primary Ab, with the rmAb clone D5F3 and mAb clone OTI1A4 giving the highest proportion of sufficient and optimal staining results. Within LD assays for ALK, mAb clone OTI1A4 had an impressive general pass rate of 100% (13 of 13) with 77% 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 78% (18 of 23) with 26% 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.
51% (44 of 87) of LD assays for ALK were based on the mAb clone 5A4. Surprisingly, mAb clone 5A4 had a relative low pass rate in this assessment compared to the previous assessment in 2015. In the present assessment, mAb clone 5A4 had a pass rate of 34% compared to 74% in 2015. The difference in the proportion of optimal staining result was even more notable, with only 2% being optimal in the present assessment compared to 44% in the 2015 assessment. The reason for these differences is unclear but could be related to a more challenging material circulated in the present ALK assessment. In the present run, especially the low-level ALK expressing Merkel cell carcinoma was a challenge for laboratories using the mAb clone 5A4 (see Fig. 6b). The highest pass rate for mAb clone 5A4 was recorded on the Leica Bond platform where 78% of the laboratories (7 of 9) achieved sufficient staining results. In comparison, only 44% (4 of 9) and 14% (3 of 22) achieved sufficient staining results on the Dako Autostainer and Ventana Benchmark platforms.
In concordance with previous assessments no sufficient staining results were seen when mAb clone ALK1 was used as a concentrate within LD assay for ALK.

54% (102 of 189) of the laboratories used Abs in Ready-To-Use (RTU) formats. This was a minor increase compared to the previous ALK assessment in 2015, where 48% of the laboratories used the RTU format. Only RTU systems based on rmAb clone D5F3 and mAb OTI1A4 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% (65 of 70 laboratories) with 76% 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 100% (56 of 56 laboratories) was seen and 82% 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 significantly lower, with 64% being sufficient and 50% optimal.
In concordance with the findings with the LD assays, RTU systems based on mAb clone ALK1 gave an insufficient result in 100% (20 of 20) 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. This indicates that mAb clone ALK1 is not fit for purpose when it comes to detecting ALK protein in EML-ALK translocated lung adenocarcinomas.
6 laboratories used the Leica Bond RTU system based on mAb clone 5A4 (prod. no. PA0306) - all with insufficient staining results. In general, PA0306 gave the expected strong staining reaction in the ALCL and positive, but weak, reaction in the lung adenocarcinoma with EML4-ALK translocation (see Fig. 5b), but in all cases a false negative reaction was seen in the Merkel cell carcinoma (see Fig. 6b).

**Controls**
In order to evaluate the 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 the assessment, appendix was found to be a valuable supplemental positive tissue control, useful for evaluating the sensitivity of the assay: In virtually all optimal protocols for 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. 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 assessment in 2015, the Merkel cell carcinoma proved to be very 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.

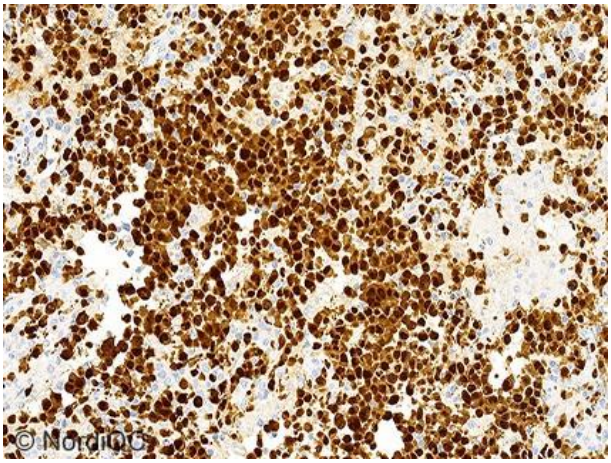



Fig. 1a
Optimal ALK staining of the ALCL with ALK rearrangement using the mAb clone OTI1A4 optimally calibrated, using HIER at High pH and a 3-step polymer based detection system performed on Bond, Leica. The neoplastic cells show an intense nuclear and cytoplasmic staining reaction. Despite the intense staining reaction, a high signal-to-noise ratio is provided and no background staining is seen. Also compare with Figs. 2a - 4a, same protocol.

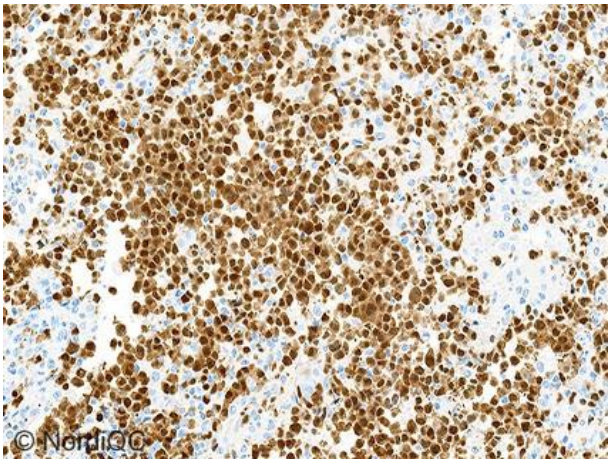



Fig. 1b
ALK staining of the ALCL with ALK rearrangement using an insufficient protocol providing a too low sensitivity for the demonstration of ALK rearrangement in lung adenocarcinoma - same field as in Fig. 1a. The protocol was based on the mAb clone ALK1, using similar settings as in Fig. 1a. The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

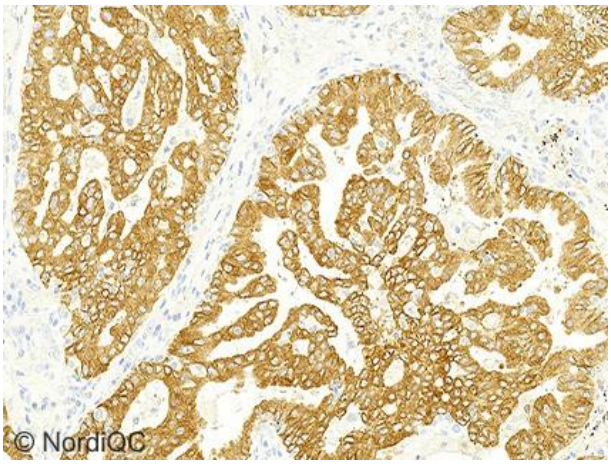



Fig. 2a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1a. Most of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction. No background staining is seen.

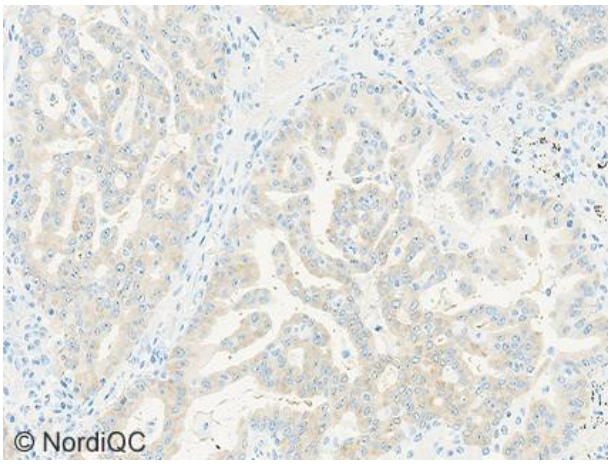



Fig. 2b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1b - same field as in Fig. 2a. The neoplastic cells only display a very faint cytoplasmic staining reaction.

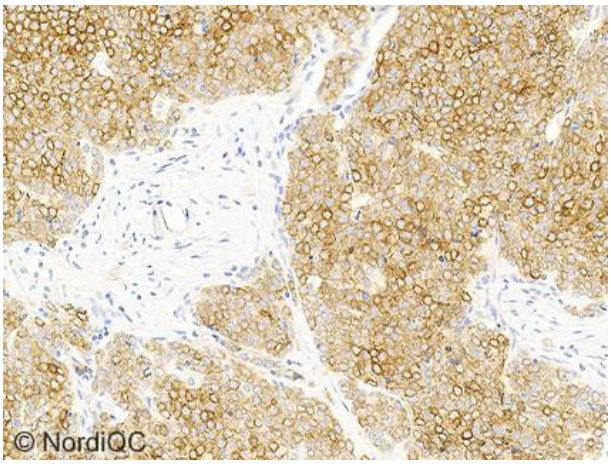



Fig. 3a
Optimal ALK staining of the Merkel cell carcinoma using same protocol as in Figs. 1a - 2a. Most of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction. No background staining is seen.

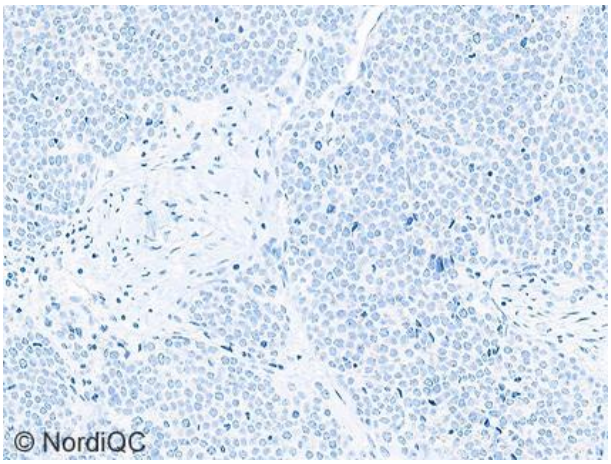



Fig. 3b
Insufficient ALK staining of the Merkel cell carcinoma using same protocol as in Figs. 1b - 2b - same field as in Fig. 3a. The neoplastic cells are all false negative.

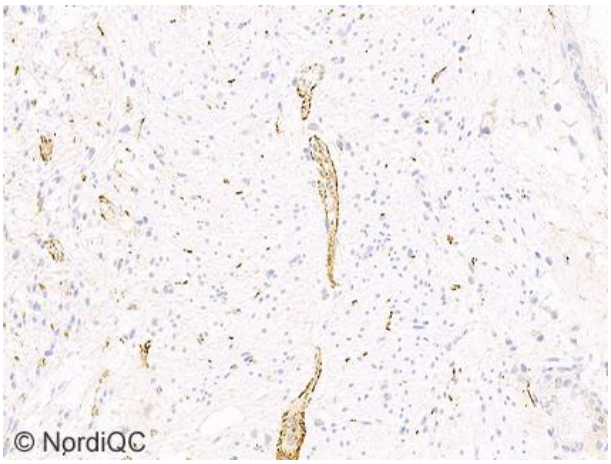



Fig. 4a
Optimal ALK staining of the appendix using same protocol as in Figs. 1a - 3a. The ganglion cells of the myenteric plexus display a moderate, distinct cytoplasmic staining reaction, while the axons display a weak to moderate staining reaction.

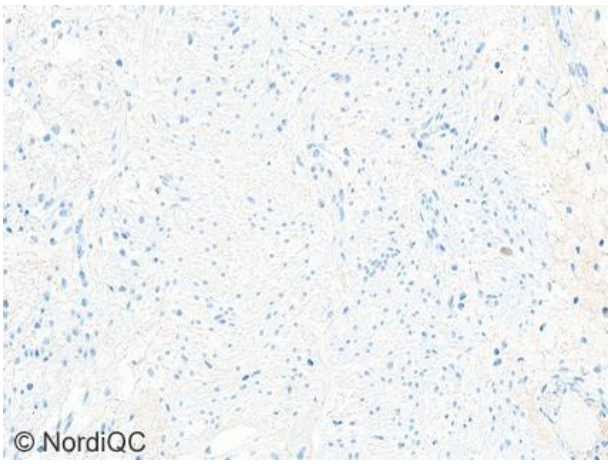



Fig. 4b
Insufficient ALK staining of the appendix using same protocol as in Figs. 1b - 3b - same field as in Fig. 4a. Both ganglion cells and axons are unstained. This seems to predict false negative reaction in both the Merkel cell carcinoma and lung adenocarcinoma with ALK rearrangement, see Fig. 2b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

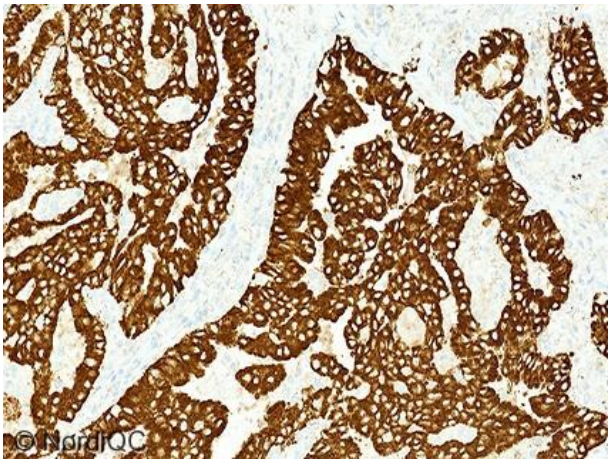



Fig.5a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using the Ventana RTU system based on rmAb D5F3 following the recommended Ventana protocol settings. Virtually all neoplastic cells display a very strong granular cytoplasmic staining reaction. No background staining is seen. Compare with Fig. 5b.

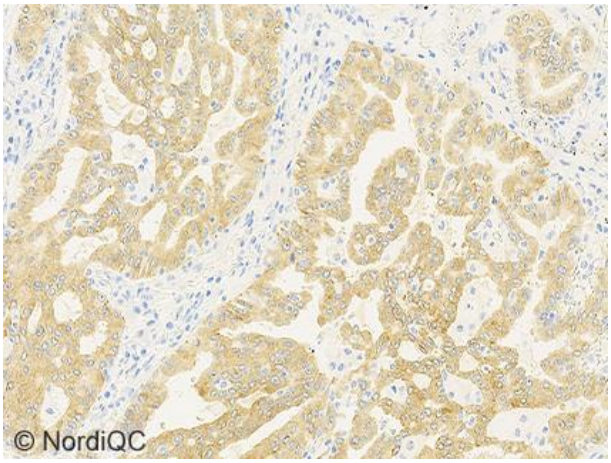



Fig. 5b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using the Leica/Novocastra RTU system based on mAb 5A4 following the recommended Leica/Novocastra protocol settings. The majority of neoplastic cells display a weak to moderate granular cytoplasmic staining reaction. Compare with Fig. 5a (same field).

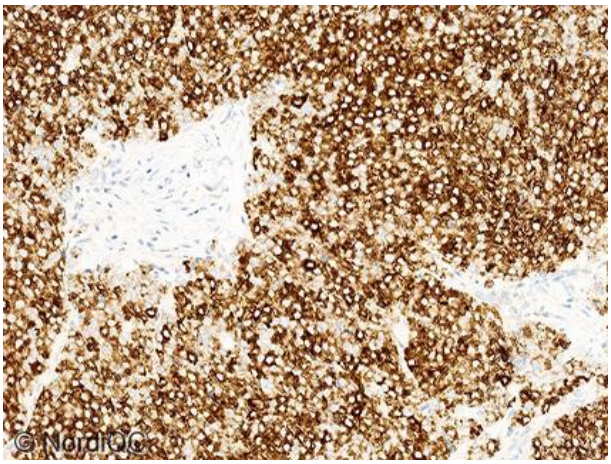



Fig. 6a
Optimal ALK staining of the Merkel cell carcinoma using the same protocol as in Fig. 5a. Virtually all neoplastic cells display a strong granular cytoplasmic staining reaction. No background staining is seen. Compare with Fig. 6b.

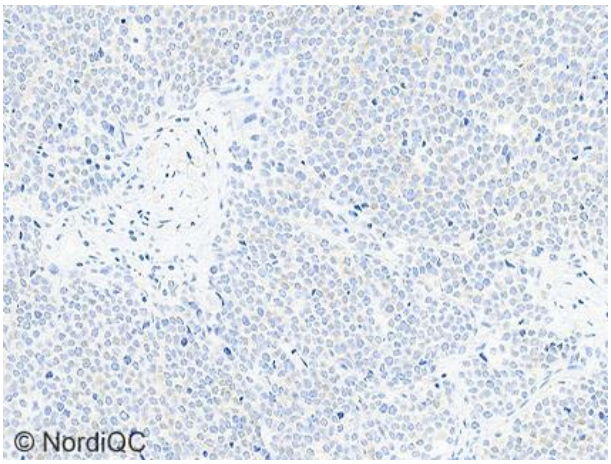



Fig. 6b
Insufficient ALK staining of the Merkel cell carcinoma using the same protocol as in Fig. 5b. Only a few scattered neoplastic cells display a faint cytoplasmic staining reaction, while the vast majority is negative. Compare with Fig. 6a (same field).

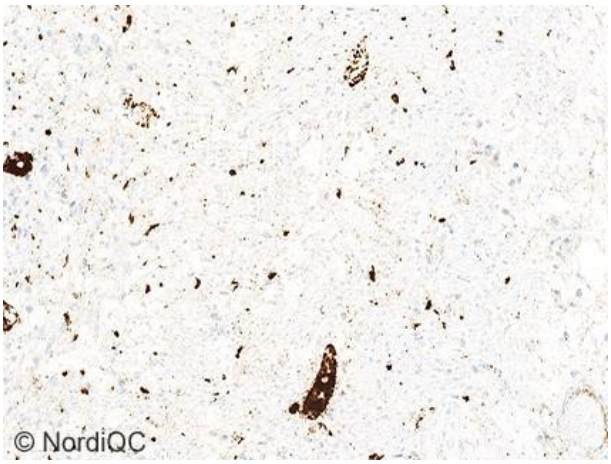



Fig. 7a
Optimal ALK staining of the appendix using same protocol as in Figs. 5a - 6a. The ganglion cells of the myenteric plexus display a very strong, distinct cytoplasmic staining reaction, while the axons display a moderate to strong staining reaction.

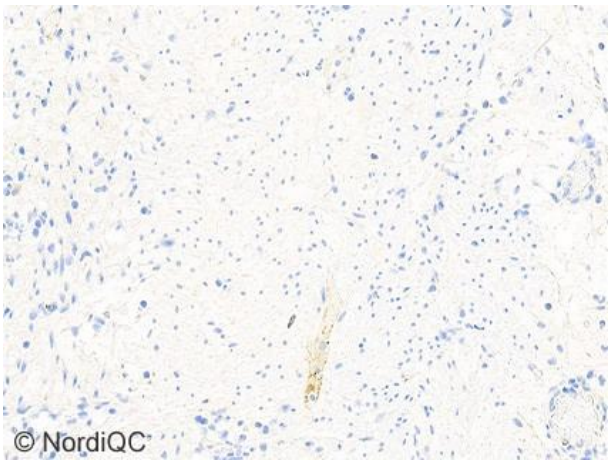



Fig. 7b
Insufficient ALK staining of the appendix using same protocol as in Figs. 5b - 6b - same field as in Fig. 7a. Some ganglion cells are faintly positive but the axons are unstained. The unstained axons seem to predict a false negative staining reaction in Merkel cell carcinoma – see Fig. 6b.

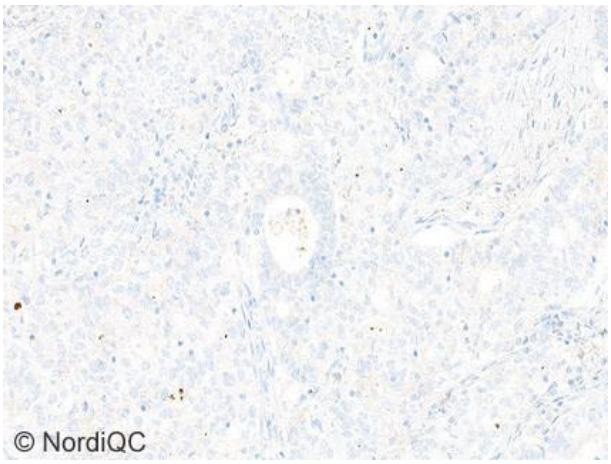



Fig. 8a
Optimal ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Figs. 5a - 7a. The neoplastic cells are all negative.

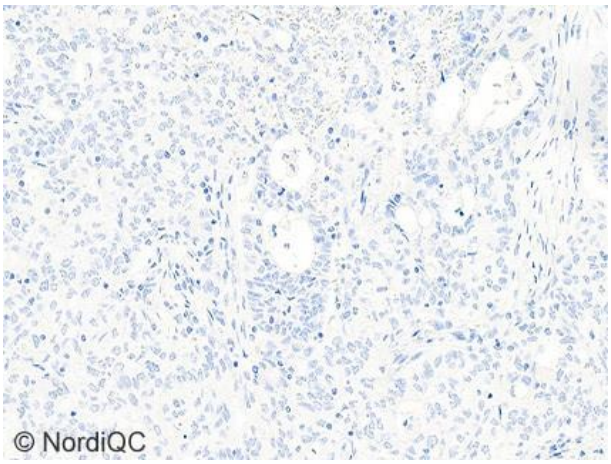



Fig. 8b
Optimal ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Figs. 5b - 7b. The neoplastic cells are all negative. Compare with Fig. 8a (same field).

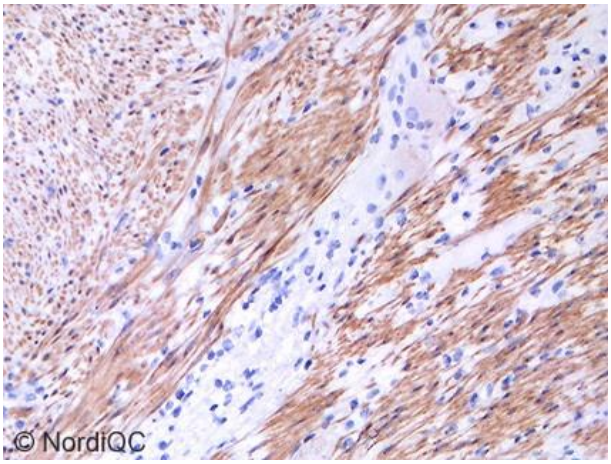



Fig. 9
Insufficient ALK staining of the appendix using the rmAb clone SP8. Both false negative and false positive staining reaction was recorded. Ganglion cells are false negative, and smooth muscle cells are false positive.

ON/LE/MV/RR 04.12.17