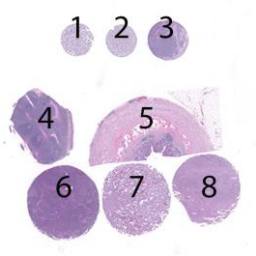
Assessment Run 45 2015
Lung Anaplastic Lymphoma Kinase (lu-ALK)

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



1. Cell line without EML4-ALK translocation*, 2. Cell line with EML4-ALK translocation*, 3. Merkel cell carcinoma, 4. Tonsil, 5. Appendix, 6. Anaplastic large cell lymphoma with ALK translocation, 7. Lung adenocarcinoma with EML4-ALK translocation 8. 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.

* The two cell lines (Horizon, UK) were excluded from the final evaluation due to an aberrant and unexpected intracytoplasmic dot-like staining reaction of the cell line without EML4-ALK translocation. The reaction was mainly seen for the Ventana Ready-To-Use system for lu-ALK and most likely caused by the tyramide based amplification kit interacting with an unknown epitope sequence in the cells. Despite the aberrant staining reaction of the cell line, a sufficient result in all the histological specimens was seen.

Participation

Number of laboratories registered for ALK-lu, run 45	197
Number of laboratories returning slides	177(90%)

Results
177 laboratories participated in this assessment. One used an inappropriate antibody (Ab). Of the remaining 176 laboratories, 67% 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 second NordiQC assessment of lu-ALK. A significant increase in the pass rate was seen compared to run 39 in 2013 (see table 2).

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

	Run 39 2013	Run 45 2015
Participants, n=	146	176
Sufficient results	49%	67%

Conclusion
The mAb clones **5A4**, **OTI1A4** and the rmAb clone **D5F3** are all recommendable Abs for demonstration of EML4-ALK translocation in lung adenocarcinoma. Irrespective of selected clone and HIER settings, appropriate calibration of the titre of the primary antibody was crucial for an optimal performance.
The Ventana Ready-To-Use systems based on the rmAb clone D5F3 were the most successful assays with an overall pass rate of 94%.
Lung adenocarcinomas with and without ALK translocation must be applied as positive and negative tissue

controls when the assay is used for lung adenocarcinoma. ALCLs will typically have a too high 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 45**

Concentrated antibodies	n	Vendor	Optimal	Good	Borderline	Poor	Suff.[1]	Suff. OPS[2]
mAb clone **5A4**	46 3 2 1 1 1	Leica/Novocastra Thermo/NeoMarkers Monosan Abcam Biocare Zytomed	24	16	13	1	74%	81%
mAb clone **ALK1**	8	Dako	0	0	3	5	0%	-
mAb clone **OTI1A4**	5	ORIGENE	4	1	0	0	100%	100%
rmAb clone **D5F3**	21 1	Cell Signaling PrimeBioMed	18	2	1	1	91%	95%
rmAb clone **SP8**	2	Thermo/NeoMarkers	0	0	1	1	-	-
Ready-To-Use antibodies								
mAb clone **5A4 PA0306**	3	Leica/Novocatra	0	1	2	0	-	-
mAb clone **5A4 API3041**	1	Biocare	1	0	0	0	-	-
mAb clone **5A4 MAB-0281**	1	Maixin	1	0	0	0	-	-
mAb **5A4 MAD-001720QD**	1	Master Diagnostica	0	0	0	1	-	-
mAb **ALK1 IR641**	15	Dako	0	0	4	11	0%	-
mAb clone **ALK1 790/800-2918**	10	Ventana	0	1	6	3	10%	-
mAb clone **ALK1 204M-18**	1	Cell Marque	0	0	0	1	-	-
mAb clone **ALK1 GA641**	1	Dako	0	0	0	1	-	-
rmAb clone **D5F3 790-4794**	47	Ventana	41	4	2	0	96%	96%
rmAb clone **D5F3 790-4843** (CDx assay)	4	Ventana	3	0	1	0	-	-
Unknown	1	Unknown	1	0	0	0	-	-
Total	176		93	25	33	25	-	
Proportion			53%	14%	19%	14%	67%	

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

Detailed analysis of lu-ALK, Run 45
The following protocol parameters were central to obtain optimal staining:

Concentrated antibodies
mAb clone **5A4**: Protocols with optimal results were typically based on HIER using Target Retrieval Solution (TRS) pH 9 (3-in-1) (Dako) (6/10)*, TRS pH 9 (Dako) (2/7), Cell Conditioning 1 (CC1, Ventana) (5/20), Bond Epitope Retrieval Solution 2 (BERS2, Leica) (6/9), BERS1 (Leica) (1/1) or Tris-EDTA pH 9 (4/5) as retrieval buffer. The mAb was typically diluted in the range of 1:10-1:50. Using these protocol settings, 34 of 42 (81%) laboratories produced a sufficient staining result (optimal or good).
* (number of optimal results/number of laboratories using this HIER buffer)

mAb clone **OTI1A4**: Protocols with optimal results were based on HIER using TRS pH 9 (3-in-1) (Dako) (1/1), TRS pH 9 (Dako) (2/2) or CC1 (Ventana) (1/1) as retrieval buffer. The mAb was diluted in the range of 1:50-1:1000. Using these protocol settings, 4 of 4 (100%) laboratories produced a sufficient staining result.

rmAb clone **D5F3**: Protocols with optimal results were based on HIER using TRS pH 9 (3-in-1) (Dako) (5/6), TRS pH 9 (Dako) (2/2), CC1 (Ventana) (1/2), BERS2 (Leica) (4/4), Tris-EDTA pH 9 (4/4), EDTA/EGTA pH 8 (1/2) or Citrate pH 6.7 (1/1) as retrieval buffer. The mAb was diluted in the range of 1:50-1:250. Using these protocol settings, 20 of 21 (95%) 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 3 main IHC systems***

Concentrated antibodies	**Dako Autostainer Link / Classic**		**Ventana BenchMark XT / Ultra**		**Leica Bond III / Max**	
	TRS pH 9.0	TRS pH 6.1	CC1 pH 8.5	CC2 pH 6.0	ER2 pH 9.0	ER1 pH 6.0
mAb clone **5A4**	6/9** (67%)	-	4/17 (24%)	-	6/6 (100%)	1/1
rmAb clone **D5F3**	7/8 (88%)	0/1	1/2	-	4/4	-

* 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. **API3041**, Biocare, IntelliPATH: One protocol with an optimal result was based on HIER using Borg Decloaker pH 9.5 (Biocare) in a pressure cooker (efficient heating time 15 min. at 110°C), 30 min. incubation of the primary Ab and MACH 4 Universal HRP-Polymer (M4U534) as detection system.

mAb clone **5A4**, product no. **MAB-0281**, Maixin, manual staining:
One protocol with an optimal result was based on HIER using Citrate buffer pH 6 (efficient heating time 2 min. at 120°C), 60 min. incubation of the primary Ab. and KIT-5230 (Maixin) as detection system.

rmAb clone **D5F3** product no. **790-4794**, Ventana, BenchMark GX, XT and Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 32-92 min.), 16-44 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 45 of 47 (96%) laboratories produced a sufficient staining result.

rmAb clone **D5F3** product no. **790-4843**, CDx Assay, Ventana, BenchMark XT and Ultra:
Protocols with optimal results were typically based on HIER using Cell Conditioning 1 (efficient heating time 72-92min.), 16-36 min. incubation of the primary Ab. and OptiView (760-700) + amplification kit (760-099) as detection system. Using these protocol settings, 3 of 3 (100%) laboratories produced a sufficient staining result.

Comments
In concordance with the previous NordiQC assessment for lu-ALK (run 39, 2013), the prominent feature of an insufficient staining result was a too weak or completely false negative staining reaction of the structures expected to be demonstrated. Virtually all the participating laboratories were able to demonstrate EML4-ALK in the neoplastic cells of the ALCL, whereas the Merkel cell carcinoma and of special interest the lung adenocarcinoma with EML-4 ALK translocation was more challenging and required an optimally calibrated IHC system.
The overall pass rate was in particular influenced by the choice of the primary Ab. An optimal result could only be obtained by the use of the mAb clones 5A4, OTI1A4 and the rmAb clone D5F3, whereas the mAb clone ALK1 gave a high proportion of insufficient results irrespective of being applied within protocol settings identical to clones giving successful and optimal IHC results.
Protocols based on mAb clone ALK1, applied either as a concentrate within a laboratory developed (LD) assay (n=8) or as Ready-To-Use (RTU) format (n=27), gave an insufficient result in 97% (34 of 35) of the protocols. No optimal staining results were obtained using this clone. In general, 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.
In contrast, the widely used mAb clone 5A4, rmAb clone D5F3 and the recently introduced mAb clone OTI1A4, all applied as concentrate within a LD assay, provided a high proportion of sufficient and optimal results. For all three clones efficient HIER preferable in an alkaline buffer, careful calibration of the titre of

the primary Ab and especially the use of a sensitive 3-step polymer/multimer based detection system were the main prerequisites for a sufficient and optimal staining result.
Optimal staining results could be obtained for 5A5 and D5F3 on all the three main IHC platforms (Dako, Ventana and Leica), see table 2. A comparison could not be made for the OTI1A4, since protocols for this clone were not submitted for all platforms.
As seen in the previous NordiQC assessment, the Ventana RTU systems based on the rmAb clone D5F3 prod. no. 790-4794 and 790-4843 were the most successful and robust assays for lu-ALK giving an overall pass rate of 94% (48 of 51 laboratories) out of which 86% was 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 XT/GX as stainer platform. Using these settings, an overall pass rate of 96% (27 of 28 laboratories) was seen and 89% received an optimal score. However, also slightly modified protocol settings such as reduced HIER time and/or adjustment of the incubation time of the primary Ab could be used to obtain sufficient and optimal staining results.

This was the second NordiQC assessment of lu-ALK and regardless of many new participants, a significantly increase of the pass rate compared to run 39 in 2013 (see table 2) was seen. The pass rate was improved from 49% in 2013 to 67% in the current run. The primary reason for this improvement seems to be closely related to reduced use of mAb clone ALK1, increased use of superior clones for LD assays and the extended use of optimally calibrated RTU systems for lu-ALK.
In Run 39, the less successful mAb clone ALK1 was used by 43% (62 of 146) compared to 20% (35 of 176) in this run. The most successful assay for lu-ALK, Ventana RTU systems 790-4794 and 790-4843 were used by 29% in this run compared to 14% in run 39.

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 EML4-ALK rearrangement both in lung adenocarcinoma and lymphomas, both materials must be included as positive tissue controls (both for the initial calibration/validation process as well as daily performance controls). Typically, ALCLs will display an intense staining reaction due to a high level of ALK protein, whereas lung adenocarcinomas will shows a weak to moderate staining reaction due to a reduced level of ALK protein. 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 lu-ALK a weak to strong granular cytoplasmic staining reaction was seen in the ganglion cells in appendix. If these cells were negative, a too weak or false negative staining reaction was seen in the lung adenocarcinoma with EML4-ALK translocation. 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.

In this assessment two cell lines (origin Horizon Discovery, UK) with and without EML4-ALK translocation were included in the material circulated to the participants.
The cell line with EML4-ALK translocation was found to be very informative regarding the level of sensitivity needed for a sufficient lu-ALK IHC assay and a high concordance of the IHC assessment marks was seen between the cell line and the histological specimens. In 46 of 49 results assessed as insufficient due to a too weak or false negative result of the lung adenocarcinoma with EML4-ALK translocation the same pattern was seen for the staining reaction of the cell line with translocation.
However, it was observed that the cell line without translocation frequently displayed an aberrant and unexpected distinct intracytoplasmic dot-like staining reaction. This staining reaction was only seen with protocols performed on the Ventana BenchMark platform typically based on OptiView + amplification kit as detection system. In 31 of 51 protocols, evaluated as optimal in all the histological specimens, an extensive aberrant cytoplasmic staining reaction was seen in the EML-ALK negative cell line. Subsequent tests performed in the NordiQC reference laboratories have indicated that the aberrant staining reaction in the cell lines is proportional to the level of sensitivity of the Ventana detection system and in particular related to the use of tyramide based amplification step (required for the Ventana RTU system).
In negative reagent controls, omitting the primary Ab, the same aberrant level of intracytoplasmic staining reaction was observed thus eliminating the primary Ab as a cause for the staining reaction. In addition, it was revealed that in the negative reagent control, both cell lines displayed an aberrant intracytoplasmic dot-like staining reaction.
Due to this observation, the two cell lines could not reliably be used as positive and negative controls to evaluate the level of sensitivity and specificity of the assays applied by the participants and consequently the cell lines were not used by NordiQC for the final assessment marks given.

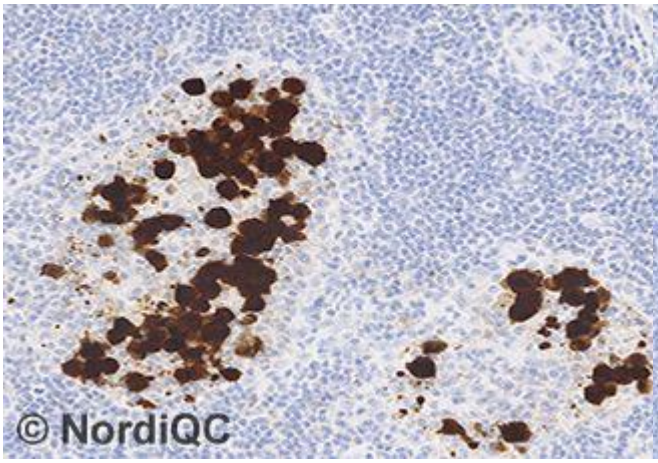


Fig. 1a
Optimal ALK staining of the ALCL with ALK rearrangement using the mAb clone OTI1A4 optimally calibrated, HIER in TRS High pH 9 (Dako), a 3-step polymer based detection system and performed on Omnis, Dako.
The neoplastic cells show an intense nuclear and cytoplasmic staining reaction. Despite the intense staining reaction, a high signal-to-noise ratio is provided and no background staining is seen.
Also compare with Figs. 2a - 6a, same protocol.

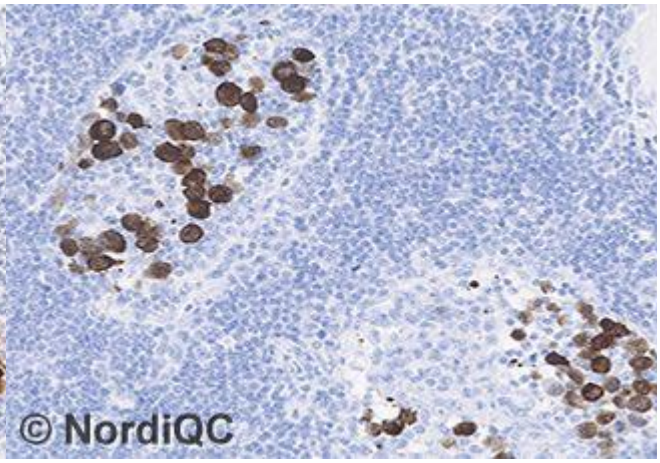


Fig. 1b
ALK staining of the ALCL with ALK rearrangement using an insufficient protocol providing a too low sensitivity for the demonstration of ALK rearrangement in lung adenocarcinoma - same field as in Fig. 1a.
The protocol was based on the mAb clone ALK1, HIER in an alkaline buffer, a 3-step polymer based detection system and performed on the Autostainer Link 48, Dako.
The neoplastic cells of the ALCL are demonstrated, however also compare with Figs. 2b – 5b, same protocol.

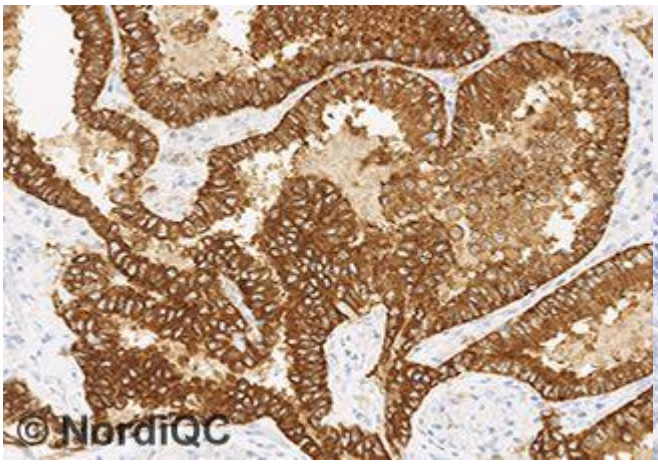


Fig. 2a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1a.
The majority of the neoplastic cells show a moderate to strong granular cytoplasmic staining reaction.
No background staining is seen.

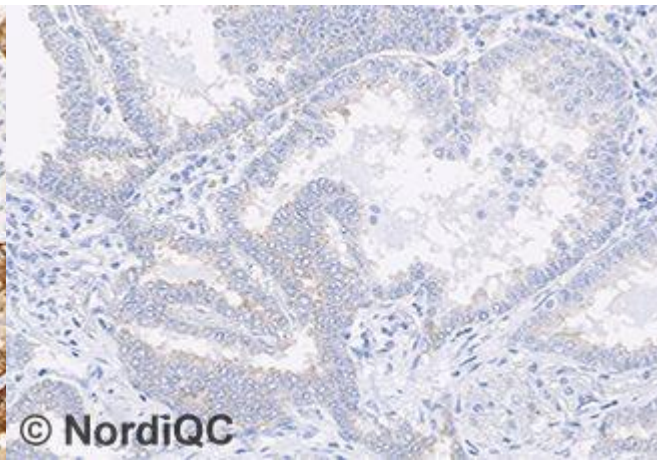


Fig. 2b
Insufficient ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 1b - same field as in Fig. 2a.
Only scattered neoplastic cells show a faint cytoplasmic staining reaction, while the vast majority is negative.

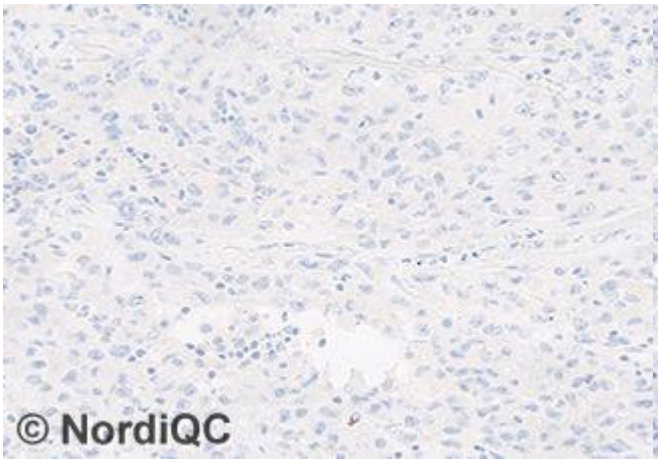


Fig. 3a
Optimal ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Figs. 1a and 2a.
The neoplastic cells are all negative.

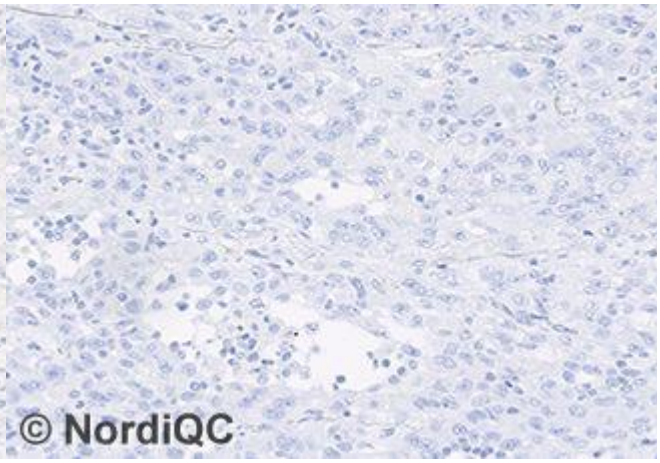


Fig. 3b
ALK staining of the lung adenocarcinoma without ALK rearrangement using same insufficient protocol as in Figs. 1b and 2b - same field as in Fig. 3a.
The neoplastic cells are all negative.

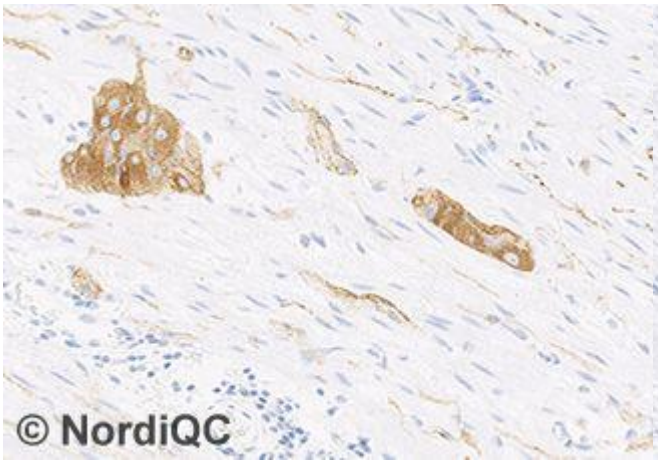


Fig. 4a
Optimal ALK staining of the appendix using same protocol as in Figs. 1a - 3a. The ganglion cells of the myenteric plexus show a moderate, distinct cytoplasmic staining reaction, while the axons show a weak to moderate staining reaction.

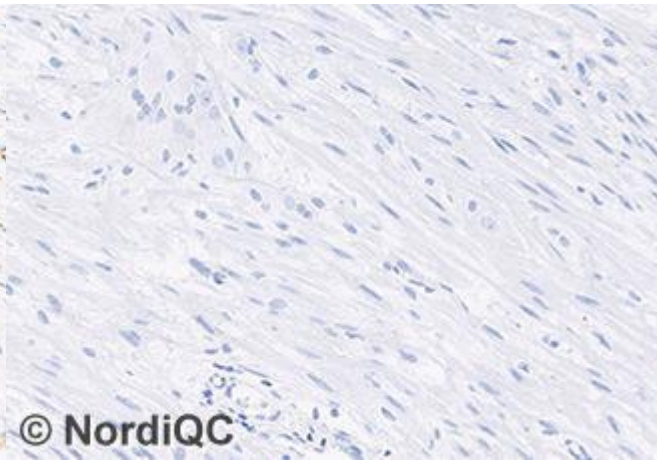


Fig. 4b
Insufficient ALK staining of the appendix using same protocol as in Figs. 1b - 3b. - same field as in Fig. 4a.
The ganglion cells and axons are unstained.

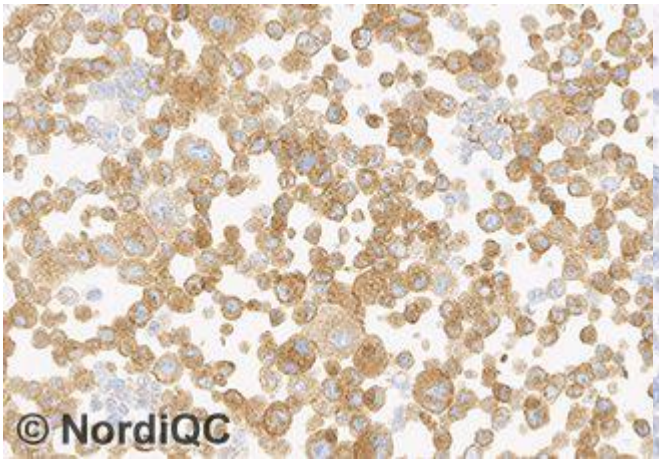


Fig. 5a
Optimal ALK staining of the cell line with ALK rearrangement using same protocol as in Figs. 1a - 4a. The wast majority of cells show moderate granular cytoplasmic staining reaction.

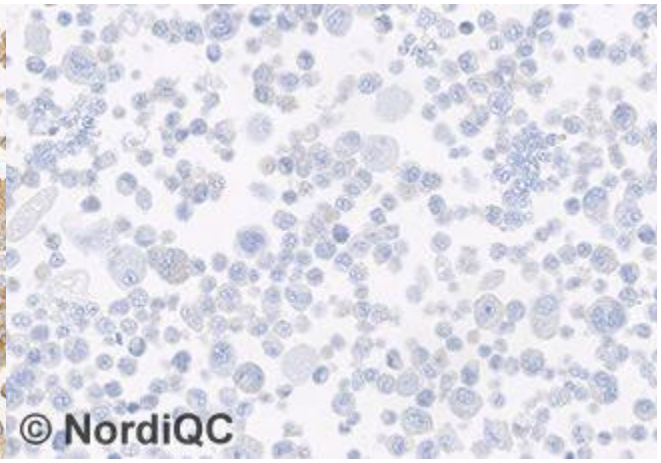


Fig. 5b
Insufficient ALK staining of the cell line with ALK rearrangement using same protocol as in Figs. 1b - 4b. Only scattered neoplastic cells show a faint and equivocal staining reaction.
The cell line was in this assessment evaluated as adequate to monitor the level of sensitivity needed for a sufficient result to demonstrate ALK in lung adenocarcinoma.

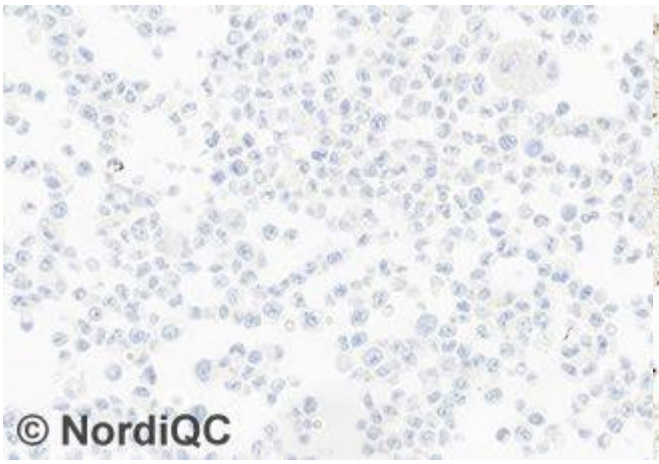


Fig. 6a
Optimal ALK staining of the cell line without ALK rearrangement using same protocol as in Figs. 1a – 5a. All cells are unstained.

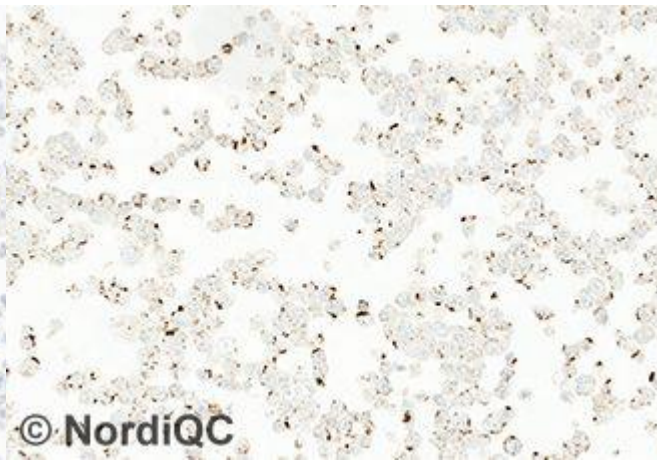


Fig. 6b
Aberrant ALK staining of the cell line without ALK rearrangement using the Ventana Ready-To-Use system based on the rmAb clone D5F3, prod. No. 790-4794. The vast majority of cells show an intracytoplasmic dot-like staining reaction.
This aberrant result was seen in a high number of protocols based on this system (31 of 51). As the system otherwise provided the results expected in all the histological specimens tested, the unexpected result in the cell line was not encountered in the final assessment score.
The positive staining reaction most likely was due to the tyramide based amplification step interacting with an unknown sequence in the cell lines. As such negative reagent controls omitting the primary antibody revealed same reaction in both cell lines included.
Also compare with Figs. 7a and 7b using same protocol/system in the two lung adenocarcinomas, where the result expected is obtained.

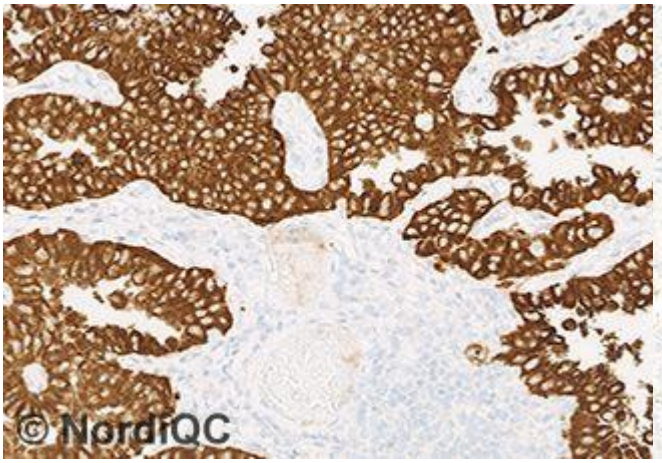


Fig. 7a
Optimal ALK staining of the lung adenocarcinoma with ALK rearrangement using same protocol as in Fig. 6b based on the Ventana Ready-To-Use system, prod. No 790-4794.
All neoplastic cells show an intense cytoplasmic staining reaction. No background reaction is seen.

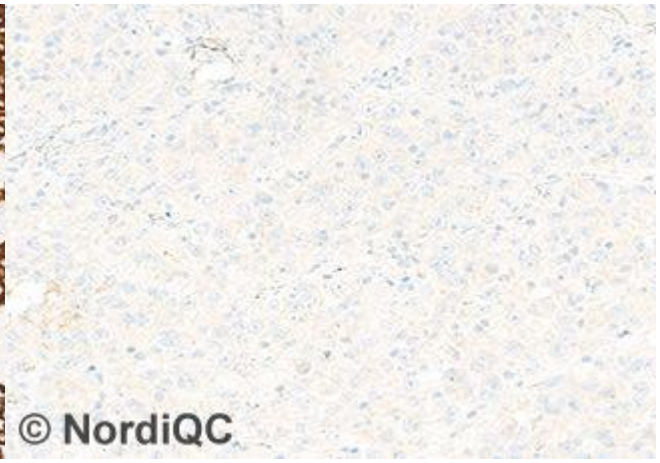


Fig. 7b
Optimal ALK staining of the lung adenocarcinoma without ALK rearrangement using same protocol as in Fig. 6b and 7a based on the Ventana Ready-To-Use system, prod. No 790-4794.
No staining reaction is seen.

SN/LE/MV/RR 06.12.2015