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The Relevance of External Quality Assessment for Molecular Testing for *ALK* Positive Non-Small Cell Lung Cancer: Results from Two Pilot Rounds Show Room for Optimization

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Abstract

Background and Purpose: Molecular profiling should be performed on all advanced non-small cell lung cancer with non-squamous histology to allow treatment selection. Currently, this should include *EGFR* mutation testing and testing for *ALK* rearrangements. *ROS1* is another emerging target. *ALK* rearrangement status is a critical biomarker to predict response to tyrosine kinase inhibitors such as crizotinib. To promote high quality testing in non-small cell lung cancer, the European Society of Pathology has introduced an external quality assessment scheme. This article summarizes the results of the first two pilot rounds organized in 2012–2013.

Materials and Methods: Tissue microarray slides consisting of cell-lines and resection specimens were distributed with the request for routine *ALK* testing using IHC or FISH. Participation in *ALK* FISH testing included the interpretation of four digital FISH images.

Results: Data from 173 different laboratories was obtained. Results demonstrate decreased error rates in the second round for both *ALK* FISH and *ALK* IHC, although the error rates were still high and the need for external quality assessment in laboratories performing *ALK* testing is evident. Error rates obtained by FISH were lower than by IHC. The lowest error rates were observed for the interpretation of digital FISH images.

Conclusion: There was a large variety in FISH enumeration practices. Based on the results from this study, recommendations for the methodology, analysis, interpretation and result reporting were issued. External quality assessment is a crucial element to improve the quality of molecular testing.

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Introduction

Lung cancer is amongst the leading causes of cancer related mortality worldwide [1]. Approximately 85% of lung cancers are non-small cell lung cancers (NSCLC), traditionally divided into three major cell types: adenocarcinoma, squamous cell carcinoma and large cell carcinoma [2]. Over the past decade, the availability of molecular targeted therapies has increased the progression-free

survival for patients with NSCLC, adenocarcinoma in particular [3–6].

The approach of using biomarkers to select treatments that are tailored to individual patient profiles is referred to as precision medicine. In advanced NSCLC, *EGFR* gene mutations and *ALK* rearrangements are currently critical biomarkers to predict treatment response. The fusion protein from *ROS1* rearrangement is an emerging target.

In 2007, it was first reported that an inversion on chromosome 2p resulted in the creation of an *EML4-ALK* fusion gene in lung cancer [7]. Multiple *EML4-ALK* variants, represented by different *EML4* breakpoints, have been identified, as well as other fusion partners for *ALK*, such as *KIF5B* and *TFG* [8–10]. *ALK* rearrangements result in oncogenic fusions which lead to constitutive activity of the *ALK* tyrosine kinase with subsequent effects on proliferation, migration and survival [11]. Lung cancers harboring *ALK* rearrangements represent a unique subpopulation of lung cancer patients. The frequency of the *EML4-ALK* rearrangement ranges from 2% to 7% in unselected NSCLC patients [3,12]. The frequency is higher in NSCLC patients with adenocarcinoma histology, non or light cigarette smoking history, and younger age, regardless of ethnicity [3,12,13]. However, these clinical characteristics are not shared by all carriers and molecular characterization is necessary to determine treatment eligibility [3,14,15].

ALK rearrangements are pharmacologically targetable with the small molecule tyrosine kinase inhibitor (TKI) crizotinib. In 2011, the FDA granted accelerated approval of crizotinib in response to the manifested clinical benefit.

Routine molecular diagnostics need to include evaluations for both *EGFR* mutations and *ALK* rearrangements [13,15,16]. It is expected that testing for *ROS1* rearrangements will be included soon. *ROS1* is another receptor tyrosine kinase that forms fusions in NSCLC and has shown responsiveness to crizotinib [17]. Diagnostic testing laboratories have been expected to rapidly introduce and perform molecular testing for NSCLC. For successful patient treatment, it is of great importance that molecular test results are accurate, highly reliable, and presented in a timely fashion. In 2012, the European Society of Pathology (ESP) proposed an external quality assessment (EQA) scheme to promote high quality biomarker testing in NSCLC for *EGFR* mutation analysis and *ALK* rearrangement detection. From 2014 on, *ROS1* testing is also included. The scheme aims to assess and improve the current status of molecular testing in NSCLC, to provide education and remedial measures, to permit inter-laboratory comparison and to allow validation of test methods by distributing validated material harboring well-defined aberrations. For *EGFR*, EQA results have been reported [18]. This article summarizes the results of the two *ALK* testing pilot rounds of the ESP Lung EQA scheme, organized in 2012–2013 with the purpose to reflect the current status of *ALK* rearrangement testing practices and to issue recommendations for the improvement of testing quality.

Materials and Methods

A pilot EQA scheme consisting of two rounds was set up. Tissue microarray (TMA) slides that consisted of NSCLC cell-lines and resection specimens were distributed. Three expert laboratories (University of Groningen, the Netherlands, UK NEQAS ICC & ISH, United Kingdom and VU University Medical Center, Amsterdam) provided material for this EQA program. All patient samples were leftover tissues that were obtained as part of routine care and testing from the three laboratories mentioned above and then handed over to the researchers anonymously. These laboratories signed a statement that the patient material was obtained according to the national legal requirements for the use of patient samples. Informed consent is not a mandatory prerequisite for the use of patient derived material, since samples for test validation are exempt from research regulations requiring informed consent. The treating physician was responsible to obtain informed consent from the patients to use their tissues and

data for research purposes and this consent is kept in the patient's medical file. The authors had no contact with the patients or received any patient identifying information. The samples were to be analyzed for the presence of *ALK* rearrangements using IHC or FISH. In addition to the TMA slides, participation in *ALK* FISH included the interpretation of four digital FISH images which were accessible online. The *ALK* FISH digital cases were provided in close collaboration with UK NEQAS ICC & ISH.

In both rounds, mock clinical information was provided for several cases, for which the delivery of a full written report was requested. Report content was assessed in agreement with established standards/guidelines on reporting [19–21].

A central database was used for the submission of the results, accessible through the ESP Lung EQA Scheme website. Through their personal account, participants could access their results, scheme documentation and assessor feedback. Upon registration, each laboratory was assigned a unique EQA identity number to guarantee anonymity. A team of medical and technical experts supported the validation of the samples and the evaluation of the scheme results. Results were discussed during an assessment meeting to obtain final consensus scores. Participants received individual feedback and a general report with aggregated scheme results.

The set-up of both rounds slightly differed and the scheme was a pilot for the development and standardization of homogeneous testing material. Eight samples (four resection specimens and four cell lines) and twelve samples (six resection specimens and six cell lines) were prepared and sent to the participants for respectively the first and second round. Different cell lines either with or without *ALK* break were routinely fixed with neutral-buffered formalin, mixed with agar and embedded in paraffin (reflecting routine pathology tissue block) were included. Results from samples for which less than 75% of the participants were able to obtain a result were not taken into account to assess performance [22]. Consequently, for *ALK* FISH, 3/8 and 7/12 samples were regarded as educational samples for respectively the first and second round. For the assessment, the accepted cases were two resection specimens and three cell lines for the first round of *ALK* FISH, and for the second round, five resection specimens were included. For *ALK* IHC, all samples were approved in both rounds.

For *ALK* FISH, it was requested for each case to report the number of neoplastic cell nuclei without hybridization signals, the number of neoplastic nuclei with fused signal, with split signal and with a single red signal. An algorithm automatically generated the number of neoplastic nuclei with FISH signal, the number of neoplastic nuclei with split or single red, and the fraction of FISH positive and negative nuclei. Participants were asked to then determine the outcome of the *ALK* FISH test (positive/negative). Samples for which a laboratory did not obtain results due to sample quality or technical failures were not assessed. For *ALK* FISH TMA and *ALK* FISH Digital, error rates were calculated based on the samples for which a minimum of 50 nuclei were enumerated, in order to exclude uninformative cases. This paper does not emphasize the marking criteria and assigned scores, as the scoring criteria slightly differed for the pilot rounds, but the study aims to reflect the current status of *ALK* rearrangement testing practices in molecular pathology laboratories.

For *ALK* IHC, it was requested to use the H-score procedure as described by Ruschoff et al. [23]. This modified H-score procedure is educational as it gives a better understanding of the *ALK* IHC sensitivity and reliability [14]. In the first round, a cut-off IHC score of 32 was determined by the mean score plus standard deviation from the laboratories on the IHC negative

Table 1. Laboratory characteristics.

Laboratory characteristics		Round 1		Round 2	
		N	% (n = 67)	N	% (n = 149)
Laboratory settings	Community Hospital	30	48%	80	54%
	University Hospital	24	36%	30	20%
	University	6	9%	17	11%
	Private	3	4%	16	11%
	Industry	2	3%	3	2%
	Private Hospital	0	0%	2	1%
	Other	2	3%	1	1%
Clinical tests offered on lung biopsy samples in routine diagnostics	ALK rearrangement detection	56	84%	110	74%
	EGFR mutation detection	/	/	128	86%
	KRAS mutation detection	/	/	112	75%
Analysis performed under the authority of the department of pathology	Yes	61	91%	116	78%
	No	6	9%	33	22%
Tests most frequently ordered on lung biopsies	EGFR	/	/	132	89%
	ALK	/	/	49	33%
	KRAS	/	/	44	30%
	None of the above	/	/	7	5%

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specimens (except outliers with H-score >100). The same threshold for positivity/negativity was applied in the second round.

For the statistical analysis, scheme error rates from both rounds for FISH digital and FISH TMA were compared using the Mann-Whitney U test. Scheme error rates for IHC were compared using an unpaired t-test. The level of significance was set at $\alpha = 0,05$.

Results

In total, 173 different laboratories (primarily from European Union countries) participated in the pilot rounds. In the first round, 29 laboratories submitted results for ALK IHC, 55 for ALK FISH TMA, and 67 laboratories performed the interpretation of the digital ALK FISH images. In the second round, 58 laboratories submitted results for ALK IHC, 104 for ALK FISH TMA, and 106 for the ALK FISH digital cases. For the data-analysis, missing values were ignored and only valid answers to questions were included, which explains why sample sizes slightly differ. Laboratory characteristics are listed in Table 1. The total number of laboratories that provided information was used as the denominator to calculate percentages. Because it was sometimes possible to indicate more than one answer, percentages may not add up to 100%.

The majority of the participants were set in a community hospital or university hospital environment. The analysis was mostly performed under the authority of the department of pathology. Regarding the interpretation of ALK FISH, a pathologist was most frequently involved (23% and 27% of laboratories in the first and second round, respectively), in some cases assisted by a scientist (18% and 27%) or a technician (20%

and 13%). A scientist alone performed the FISH reading in 15% of the laboratories in both the first and second round. The final reading conclusion was the responsibility of a pathologist alone in more than half of the laboratories (52% and 59% in the first and second round). A pathologist in cooperation with a scientist was responsible in 13% and 14% of the laboratories. A scientist alone was responsible for the final reading conclusion in 16% and 12% of the laboratories in the first and second round.

ALK FISH digital results

Results for both rounds of the ALK FISH digital subscheme are summarized in Table 2. There were no clear differences in the error rates depending on the number of nuclei enumerated. Enumeration practices were evaluated on sample level and on laboratory level. In both rounds, the bulk of the participants enumerated 50–100 nuclei for each case. At laboratory level, in the first round, 34/67 laboratories (51%) counted ≥ 50 cells for each sample. In the second round, an increase was observed to 77/106 (73%). Table 3 illustrates the performance of the labs that participated in both rounds for each subscheme. Improvement in enumeration practices was defined as enumeration of ≥ 50 cells in a larger number of samples in the second round compared to the first round.

A decrease was observed in the error rates between both rounds (error rates were calculated taking only the samples for which ≥ 50 nuclei were enumerated into account). In the first round, 7 out of 195 scored samples were incorrectly assigned (3,6%), while in the second round, 4 errors out of 366 scored samples (1,1%) occurred. The comparison of the number of errors made for laboratories

Table 2. Results for the interpretation of digital ALK FISH images.

Round	Sample ID	ALK rearrangement status	Number of labs that counted <50 nuclei	Number of labs that counted 50–100 nuclei	Number of labs that counted >100 nuclei	Number of FP/FN from labs that counted <50 nuclei	Number of FP/FN from labs that counted 50–100 nuclei	Number of FP/FN from labs that counted >100 nuclei
Round 1	LUNG12.109	negative	12 (18%)	38 (58%)	16 (24%)	1 (8%)	0 (0%)	1 (6%)
	LUNG12.110	negative	21 (32%)	43 (65%)	2 (3%)	0 (0%)	0 (0%)	0 (0%)
	LUNG12.111	positive	15 (23%)	45 (70%)	4 (6%)	0 (0%)	0 (0%)	0 (0%)
	LUNG12.112	positive	13 (22%)	37 (62%)	10 (17%)	3 (23%)	6 (16%)	0 (0%)
	Average		15.3 (23.8%)	40.8 (63.8%)	8.0 (12.5%)	1.0 (7.75%)	1.5 (4.0%)	0.3 (1.5%)
Round 2	LUNG12.213	negative	19 (18%)	74 (70%)	11 (10%)	0 (0%)	0 (0%)	0 (0%)
	LUNG12.214	positive	11 (11%)	69 (65%)	24 (23%)	0 (0%)	0 (0%)	0 (0%)
	LUNG12.215	negative	10 (10%)	66 (62%)	27 (25%)	0 (0%)	1 (2%)	2 (7%)
	LUNG12.216	positive	9 (9%)	68 (64%)	27 (25%)	0 (0%)	1 (1%)	0 (0%)
	Average		12.3 (12.0%)	69.3 (65.3%)	22.3 (20.75%)	0 (0%)	0.5 (0.8%)	0.5 (1.8%)

FP, false positives; FN, false negatives.
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that participated in both rounds and that counted ≥ 50 nuclei for each case can be found in Table 3.

ALK FISH TMA results

Table 4 summarizes the ALK FISH TMA results for both rounds. In both rounds, the majority of the participants enumerated 50–100 nuclei for each case. Again, there were only small differences in the error rates depending on the number of nuclei enumerated. In the first round, 30/55 laboratories (55%) counted ≥ 50 cells for each sample; in the second round there was an increase to 81/104 (78%).

For the TMA cases there was also a decrease in the error rates between the two rounds. In the first round, 14 out of 193 scored samples were incorrectly assigned (7,3%), while in the second round, 22 errors out of 423 scored samples (5,2%) occurred. Comparison of the enumeration performance and number of errors made for laboratories participating in both rounds can be found in Table 3.

ALK IHC results

Results for both ALK IHC rounds are provided in Table 5. In the first round, 30/230 scored cases (13,0%) were incorrectly called (false positive or false negative). In the second round, a decrease to 44/540 (8,2%) was observed. Table 3 illustrates the performance for the labs that participated in both IHC rounds.

Summary scheme error rates

The Mann-Whitney U test revealed no significant differences between both rounds for Digital FISH ($U = 7, z = -0.308, p = 0.758$) or FISH TMA ($U = 9, z = -0.731, p = 0.465$). For IHC, an unpaired t-test showed no significant difference between the first ($M = 0.13, SD = 0.06$) and second round ($M = 0.08, SD = 0.05$); $t(18) = 1.845, p = 0.082$. Although not statistically significant, comparing the error rates in both rounds suggests a learning effect (Table 6). The smallest error rates were observed for the digital cases, assessing only the post-analytical interpretation phase. In both rounds, the error rate for ALK FISH TMA was lower than the error rate for ALK IHC TMA.

Methods used

The most frequently used method for FISH analysis was the Vysis ALK break apart FISH probe kit (Abbott Molecular, Illinois, USA), used by over 70% of the participants. For IHC, the most frequently used antibodies were clone 5A4 and clone D5F3 for the first and second round, respectively. An overview of the used methods and the error rate per method is provided in Tables 7 and 8. For the percentage of laboratories that used a certain method, the total number of laboratories that provided information was used as the denominator. Because it was possible to indicate more than one used method, percentages may not add up to 100%.

For ALK FISH, the Repeat-Free Poseidon ALK/EML4 t(2;2) inv(2) Fusion Probe (Kreatech Diagnostics, Amsterdam, the Netherlands) revealed a high error rate of 50% in the second round (Table 7). For IHC, the smallest error rates were observed for clones 5A4 and D5F3 (Table 8).

Clinical result reporting

Evaluation of the written reports for the second round ($n = 102$) showed that a case-specific clinical interpretation was missing in 74% and 79% of the reports for an ALK positive and ALK negative case, respectively. Patient name and date of birth were correctly present in the majority of the reports, as well as a

Table 3. Performance of laboratories that participated in both rounds.

Subscheme	Nr of labs with improved enumeration performance	Nr of labs with equal enumeration performance	Nr of labs with worse enumeration performance	Nr of labs with a decrease in nr of errors	Nr of labs with equal nr of errors	Nr of labs with higher nr of errors
ALK FISH Digital	14/39 (36%)	22/39 (56%)	3/39 (8%)	1/37 (3%)	36/37 (97%)	0/37 (0%)
ALK FISH TMA	13/35 (37%)	17/35 (49%)	5/35 (14%)	7/29 (24%)	20/29 (69%)	2/29 (7%)
ALK IHC	/	/	/	3/14 (21%)	9/14 (64%)	2/14 (14%)

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specification of the methods used (FISH kit information or IHC antibody). However, a specification of the aberrations tested and the threshold of the method were not mentioned in 46% and 47% of the FISH reports. The total number of neoplastic cells analyzed and the number of cells with split and/or single signal were missing in 23% of the FISH reports. For IHC, the threshold for positivity/negativity was not defined in 81% of the reports, and the staining intensity was missing in 39% of the reports.

Discussion

Major advances have been made in the management of patients with NSCLC, with improved treatment response and survival following the introduction of molecular targeted TKI therapies focusing on *EGFR* mutations and *ALK* rearrangements. The increasing importance of morphology-based studies such as IHC or FISH has made the pathologist's involvement a key element in precision medicine for NSCLC [2,24].

In reply to the growing demands, laboratories have introduced molecular testing for NSCLC in routine diagnostics. Regular participation in quality assurance programs is crucial to ensure a high quality of testing service and to warrant patient safety [15,18,19].

Our results show that in the majority of the participating laboratories, *ALK* testing is performed under the authority of the pathology department. This is a necessity as FISH and IHC are both histological tests. Pathology review and assessment of section quality is essential considering the diversity and heterogeneity of tumor tissue [19], as false negatives may be due to poor fixation or insufficient neoplastic cell content [14,18].

Three methods are generally used in routine diagnostics for *ALK* rearrangement detection: FISH, RT-PCR, and immunohistochemistry for aberrant expression of *ALK* protein [12,14,25]. Importantly, every assay should undergo validation in the laboratory before clinical interpretation and should be subject to regular internal and external quality controls [14,19,24]. The FDA approved test to determine *ALK* status is the Vysis LSI *ALK* dual color, break apart rearrangement probe (Abbott Molecular, Illinois, USA) [12,14]. Although other IVD-CE labeled kits are available in Europe, this kit was by far the most frequently used method in both pilot rounds. *ALK* break apart (or split-signal) probes detect disruption of the *ALK* 2p23 locus but do not identify the partner fusion gene [3,25]. Surprisingly, the *ALK/EML4* fusion probe is still occasionally used, although these probes miss the translocation of *ALK* with partners other than *EML4*. In the second round, the fusion probe revealed a high error rate of 50%. The cut-off values used during the clinical trials to prove the efficacy of crizotinib can be transferred from the Vysis probe to other break apart probes since the design (size + location) is highly similar [26]. The ZytoLight TriCheck (ZytoVision, Bremerhaven, Germany), used by approximately 7% of the participants in both

rounds, can identify the presence of an *ALK* rearrangement and if the rearrangement partner is *EML4*. Today it is still under discussion whether it is important to know the fusion partner of *ALK* in relation to expected response to *ALK* TKIs [27,28].

According to Abbott Molecular scoring criteria, a nucleus is considered positive if it contains at least one split signal or one isolated red signal. A first enumerator should count 50 nuclei. Cases with >50% and <10% positive nuclei are considered positive and negative respectively. If a sample shows between 10–50% positive nuclei, a second enumerator should also count 50 nuclei. If the average of the two readings contains at least 15% positive cells, the sample is considered positive. The kit specifies uninformative specimens as those in which fewer than 50 nuclei within the scribed area can be enumerated. In our evaluation these cases were therefore not included to calculate and compare the scheme error rates. It has been shown that the sensitivity and specificity of the kit increase as the number of tumor areas and number of nuclei scored increase [29,30]. Our results showed that *ALK* rearrangement status was often determined on the evaluation of less than 50 nuclei by many participants. The percentage of false positive and false negative results upon enumeration of <50 nuclei did not reveal a clear difference compared to the percentage upon enumeration of ≥50 tumor nuclei. These findings correlate with the fact that the *ALK* rearrangement appears to be a homogenous event in the tumor population [26,29]. Enumeration of <50 nuclei is not advisable because this number is based on the minimal number that is statistically needed to be able to reliably define a sample without FISH break signals (<15% of nuclei) as a case without *ALK* rearrangement. In addition, the predictive value of phase III trial is based on this. Remarkably, some participants enumerated a large number of nuclei (e.g. >600 evaluated nuclei for case 12.215), which is not a requirement for daily practice. FISH interpretation should be performed in areas of the slide with clear signals, which are clearly distinct from the nuclear fluorescent 'noise' as well as from the background [15]. Importantly, selection of neoplastic nuclei is essential, and to this end sufficient morphological knowledge in FISH stained slides is obligatory, which stresses involvement of a pathologist.

It is not a surprise that the TMA FISH error rates were substantially higher than those of the digital FISH images. The FISH digital subscheme specifically assesses the interpretation of identical digital images whereas the TMA FISH error rate also incorporates variation in serial TMA sections, technical execution, and reading. Suboptimal *ALK* FISH procedure may lead to a low signal versus background ratio, increasing the chance for interpretation errors.

Although FISH is used as a standard test, it demonstrates considerable inter-observer variability. Therefore, experienced (> 100 cases/year) and well-trained FISH reviewers/enumerators are necessary. If the clinical scientist is well trained and experienced in histo- and cytomorphology with specialized training in solid tumor

Table 4. ALK FISH TMA results.

Round	Sample ID	ALK rearrangement status	Number of labs that counted <50 nuclei	Number of labs that counted 50–100 nuclei	Number of labs that counted >100	Number of FP/FN from labs that counted <50 nuclei	Number of FP/FN from labs that counted 50–100 nuclei	Number of FP/FN from labs that counted >100 nuclei
Round 1	LUNG12.101	positive	11 (22%)	37 (74%)	2 (4%)	2 (18%)	4 (11%)	0 (0%)
	LUNG12.105	positive	11 (26%)	26 (62%)	5 (12%)	2 (18%)	6 (23%)	1 (20%)
	LUNG12.106	negative	10 (19%)	34 (64%)	9 (17%)	1 (10%)	1 (3%)	0 (0%)
	LUNG12.107	positive	11 (21%)	34 (64%)	8 (15%)	0 (0%)	2 (6%)	0 (0%)
	LUNG12.108	negative	10 (20%)	34 (68%)	6 (12%)	0 (0%)	0 (0%)	0 (0%)
Average		10.6 (21.6%)	33.0 (66.4%)	6.0 (12.0%)	1 (9.2%)	2.6 (8.6%)	0.2 (4.0%)	
Round 2	LUNG12.202	positive	10 (11%)	46 (51%)	34 (38%)	1 (10%)	14 (30%)	1 (3%)
	LUNG12.204	negative	8 (9%)	48 (52%)	37 (40%)	0 (0%)	1 (2%)	2 (5%)
	LUNG12.206	negative	7 (8%)	56 (60%)	30 (32%)	0 (0%)	0 (0%)	1 (3%)
	LUNG12.208	positive	6 (6%)	55 (57%)	36 (37%)	0 (0%)	1 (2%)	1 (3%)
	LUNG12.210	negative	6 (7%)	48 (53%)	36 (40%)	0 (0%)	0 (0%)	1 (3%)
Average		7.4% (8.2%)	50.6 (54.6%)	34.6 (37.4%)	0.2 (2.0%)	3.2 (6.8%)	1.2 (3.4%)	

FP, false positives; FN, false negatives.
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Table 5. ALK IHC results.

Round	Sample ID	ALK rearrangement status	Average IHC score	Standard deviation IHC score	Number of FP/FN
Round 1	LUNG 12.101	positive	154	53	2 (7%)
	LUNG 12.102	positive	113	73	6 (22%)
	LUNG 12.103	negative	15	51	2 (7%)
	LUNG 12.104	negative	24	49	5 (17%)
	LUNG 12.105	positive	118	61	3 (10%)
	LUNG 12.106	negative	25	53	6 (21%)
	LUNG 12.107	positive	154	62	2 (7%)
	LUNG 12.108	negative	13	32	4 (14%)
	Average	/	/	/	3,8 (13,1%)
Round 2	LUNG12.201	negative	18	52	5 (12%)
	LUNG12.202	negative	16	51	5 (11%)
	LUNG12.203	negative	5	22	2 (5%)
	LUNG12.204	negative	6	23	2 (4%)
	LUNG12.205	negative	18	47	7 (16%)
	LUNG12.206	negative	1	4	0 (0%)
	LUNG12.207	negative	9	34	4 (9%)
	LUNG12.208	positive	163	58	4 (9%)
	LUNG12.209	positive	137	69	7 (16%)
	LUNG12.210	negative	2	11	1 (2%)
	LUNG12.211	positive	143	68	6 (13%)
	LUNG12.212	negative	1	6	1 (2%)
	Average	/	/	/	3,7 (8,3%)

FP, false positives; FN, false negatives.
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FISH analysis, he/she can be responsible for the technical performance and molecular interpretation. A pathologist should at least be responsible for the selection of the right cells, the review of the interpretation and the authorization of the pathology report [14,15]. Our data demonstrate that a pathologist was responsible for the final conclusion in the majority of the laboratories. Participating laboratories indicated that scientists and technicians were often involved in FISH enumeration. In this set-up it is important that the clinical scientist can consult a pathologist at any time in case of doubt concerning the location of the tumor cell area.

ALK IHC, if carefully clinically validated according to ISO 15189, may be considered as a screening method to select

specimens for ALK FISH testing [15]. It is a cost-effective screening tool which correlated significantly with ALK FISH, using a number of antibodies including the 5A4 and D5F3 [25,31–33]. However, discrepancies are reported also and need to be elucidated [34]. The 5A4 and D5F3 antibodies were the most frequently used clones in our study and revealed the smallest error rates, which is in accordance with literature [32,33] and the findings of a recent NordiQC assessment [35]. Not surprisingly however, the error rates for IHC were greater than for FISH. Recently different validation projects for ALK IHC tests were done in collaboration with a lot of laboratories [36]. Moreover, on the website of NORDIQC (<http://www.nordiqc.org/>), advice on IHC staining protocols is given for several antibody clones.

Table 6. Scheme error rates per subscheme for both pilot rounds.

Subscheme	Error rate round 1*		Error rate round 2*	
Digital ALK FISH	3,6%	0,5% due to FP 3,1% due to FN	1,1%	0,8% due to FP 0,3% due to FN
ALK FISH TMA	7,3%	0,5% due to FP 6,7% due to FN	5,2%	4,0% due to FP 1,2% due to FN
ALK IHC	13,0%	7,4% due to FP 5,6% due to FN	8,2%	5,0% due to FP 3,2% due to FN

*Error rate=(number of FP + number of FN)/total number of informative results FP, false positives; FN, false negatives.
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Table 7. ALK FISH methods used in the scheme and error rate per probe.

ALK FISH rearrangement detection method	Round 1			Round 2			
	Percentage of labs that used the probe (n = 60)	Number of FP/FN obtained with the probe	Total number of samples analysed with the probe	Percentage of samples resulting labs that used the probe (n = 103)	Number of FP/FN obtained with the probe	Total number of samples analysed with the probe	Percentage of samples resulting in a FP/FN
ALK FISH DNA Probe, Split Signal (Dako, Glostrup, Denmark)	12%	3	29	10,3%	0	38	0,0%
ZytoVision (Bremerhaven, Germany), not further specified	/	/	/	2%	1	9	11,1%
Master Diagnostica (Granada, Spain)	/	/	/	1%	/	/	/
Repeat-Free Poseidon ALK (Kreatech Diagnostics, Amsterdam, the Netherlands)	2%	1	5	20,0%	0	10	0,0%
Repeat-Free Poseidon ALK/EML4 t(2;2) inv(2) Fusion Probe (Kreatech Diagnostics, Amsterdam, the Netherlands)	7%	0	16	0,0%	2	4	50,0%
Vysis ALK break apart FISH probe kit (Abbott Molecular, Illinois, USA)	82%	11	157	7,0%	16	311	5,1%
ZytoLight SPEC ALK Dual Color Break Apart Probe (ZytoVision, Bremerhaven, Germany)	/	/	/	/	0	4	0,0%
ZytoLight SPEC ALK/EML4 TriChec Probe (ZytoVision, Bremerhaven, Germany)	7%	0	14	0,0%	2	30	6,7%

FP, false positives; FN, false negatives.
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Table 8. Antibodies used for ALK IHC in the scheme and error rate per antibody.

ALK IHC antibody	Round 1					Round 2				
	Percentage of labs that used the antibody (n = 22)	Number of FP/FN obtained with the antibody	Total number of samples analysed with the antibody	Percentage of samples resulting in a FP/FN	Percentage of labs that used the antibody (n = 62)	Number of FP/FN obtained with the antibody	Total number of samples analysed with the antibody	Percentage of samples resulting in a FP/FN	Percentage of samples resulting in a FP/FN	
5A4	64%	6	104	5.8%	40%	5	185	2.7%		
D5F3	14%	1	16	6.3%	44%	30	291	10.3%		
ALK1	18%	9	32	28.1%	15%	7	35	20.0%		
SP8	9%	8	16	50.0%	2%	1	11	9.1%		
4C5B8	/	/	/	/	2%	1	6	16.7%		

FP, false positives; FN, false negatives.
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Our study demonstrates improvement of *ALK* testing after only two EQA rounds. This suggests that laboratories constructively use the assessors' feedback from the previous round to enhance their performance. Participation in EQA facilitates rapid exposure of errors and the timely implementation of corrective and preventive actions. However, other factors such as increased expertise and experience may play a part. It is expected that larger datasets, spanning a larger number of EQA participations will demonstrate a statistically significant improvement. On scheme level, the error rates for both *ALK* FISH and *ALK* IHC were lower in the second round and the *ALK* FISH digital scheme demonstrated an error rate of only 1.1%. Error rates for *ALK* FISH TMA and *ALK* IHC were still high (>5%), which stresses the need for continued education through EQA. Progress was also seen on individual laboratory level. For FISH analysis, improvements were observed both in the number of errors made and in enumeration practices.

Reporting of test results should take into account sample adequacy relative to the assay performance characteristics and limitations, and clinical reports should be readily interpretable by non-expert clinicians [19,21]. Previous EQA schemes have exposed existing deficiencies in clinical reporting [18,37]. Our results show that the content of reports for *ALK* rearrangement detection should be improved. Especially, a case-specific clinical interpretation, predicting the effect of the rearrangement status on therapy response, should be integrated in each report since a clear and concise assessment of the clinical implications of the result is crucial to fully inform treatment options.

Maintenance of quality assurance measures, including stringent internal quality controls and continued education by repeated EQA participations is essential to ensure high testing quality and rapid exposure of errors in order to warrant appropriate treatment choices. This article has demonstrated improvement in the performance of *ALK* FISH and *ALK* IHC in two consecutive EQA rounds. Several recommendations were made to improve the quality of *ALK* testing.

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Author Contributions

Conceived and designed the experiments: LT KM ES ET ED. Performed the experiments: LT KM ES ET ED. Analyzed the data: LT VT KZ PP KK KM LB ES ET ED. Contributed reagents/materials/analysis tools: LT KM ES ET ED. Contributed to the writing of the manuscript: LT VT PP KZ ED.

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