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Research

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An Unbalanced Exon-Expression qPCR-based Assay for Detection of *ALK* Translocation (Fusion) in Lung Cancer

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ABSTRACT

Non-Small Cell Lung Cancer (NSCLC) constitutes 85-90% of all lung cancer. Accurate diagnosis and selection of targeted therapies in lung cancer depends on robust detection of the molecular events that underlie its pathogenesis. Since patients having a rearrangement in the Anaplastic Lymphoma Kinase (*ALK*) gene respond well to treatment with crizotinib, identification of such *ALK* mutations is necessary for the successful treatment of NSCLC. The most common rearrangement of the *ALK* gene in NSCLC involves fusion with echinoderm microtubule-associated protein-like 4 (EML 4) as the upstream partner. Current testing methods for this rearrangement (IHC and/or FISH) can be very subjective due to high operator variability. They require expert interpretation by a pathologist and have a long turnaround time. The FDA-approved Fluorescence *In Situ* Hybridization (FISH) test has been shown to lack sensitivity and is generally acknowledged to fail to detect rearrangements in up to 60% of patients. Here, we have adapted an approach described earlier and optimized it for use with degraded RNA obtained from Formalin-Fixed Paraffin-Embedded (FFPE) sections. This method is based on the unbalanced expression of 5'- and 3'-regions (exons) of the *ALK* gene. It is also applicable to the detection of other cancer-relevant gene rearrangements e.g. ROS 1 or RET that result in increased expression of the 3'-kinase domain. Patients with these rearrangements have been shown to respond to crizotinib and cabozantinib, respectively. Using NSCLC cell lines we demonstrate that our method is cost-effective, reproducible, sensitive, objective, and easy to use. Unlike FISH, it does not require interpretation by several scorers and it can be performed in any clinical laboratory with access to a qPCR instrument. Here we present the protocol for the method and validation with 197 clinical samples.

KEYWORDS: Non-small cell lung cancer; Targeted therapy; Unbalanced exon-expression; *EML4-ALK* rearrangement.

ABBREVIATIONS: NSCLC: Non-Small Cell Lung Cancer; PCR: Polymerase Chain Reaction; qRT-PCR: quantitative Reverse-Transcriptase-PCR; FFPE: Formalin-Fixed Paraffin Embedded.

INTRODUCTION

Lung cancer is the most common cancer and leading cause of death from cancer in Canada. Lung cancer represented 14% (26,600) of all new cancers diagnosed in Canada and accounted for 27% (20,900) deaths in 2015. This results in the death of 57 Canadians every day and the numbers will continue to rise with the aging population. Non-Small Cell Lung Cancer (NSCLC) constitutes 85-90% of all lung cancers.[1]

Therapeutic options for many cancers, including advanced lung cancer, are generally

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limited to chemotherapies, for which response rates are typically 20-30% with only modest increases (3-5 months) in Progression-Free Survival (PFS). In contrast, personalized therapy based on patients genotypes offer much higher response rates (approximately 75%), longer PFS (9-13 months) and fewer side effects. Between 3-11% of patients have activating mutations caused by various rearrangements in the Anaplastic Lymphoma Kinase (*ALK*) gene, which are targetable by the tyrosine kinase inhibitor, crizotinib (Pfizer, PF-02341066).[2] Additional second and third-generation ALK inhibitors are approved or in development such as ceritinib (Novartis), alectinib (Roche-GenenTech), lorlatinib (Pfizer), brigatinib (Ariad), entrectinib (Ignyta). Identifying patients with *ALK* rearrangements is currently expensive, time-consuming and subject to misinterpretation.[3]

Accurate diagnosis and selection of targeted therapies in lung cancer depends on robust detection of the molecular events that underlie its pathogenesis. It is known that patients having an activating mutation (rearrangement) in the *ALK* gene respond better to treatment with crizotinib (a tyrosine kinase inhibitor) than lung cancer patients with other etiologies of the disease. Therefore, identifying patients with *ALK* mutations becomes a prerequisite to successful and efficient treatment of lung cancer. The most common rearrangement of the *ALK* gene in NSCLC involves fusion with echinoderm microtubule-associated protein-like 4 (EML4) as the upstream partner.[4] There are many known variants of *EML4-ALK* fusions depending on which EML4-exon is fused with *ALK* exon 20.[4-6] For example, in the variant 1 EML4 exon 13 is fused with the ALK exon 20 (E13:A20) and in the variant 7 EML4 exon 14 is fused with *ALK* exon 20 (E14:A20). Other known upstream partner genes that are fused with the *ALK* gene in NSCLC are *TFG*,[7] *KIF5B*[8,9] and *KLC1*.[10] However, the existing methods that detect only specific, e.g. *EML4-ALK* translocations or those with known upstream fusion genes, are of limited scope.

The current testing methods for the *EML4-ALK* rearrangement are immunohistochemistry (IHC)[11-13] and/or Fluorescence *In Situ* Hybridization (FISH) using the Vysis Break Apart Probe.[14] These tests use a solid tissue biopsy and can be very subjective due to high operator variability. They require expert interpretation by a pathologist and have a long turnaround time (TAT). The ALK IHC test, which measures expression of protein, is only semi-quantitative. The staining intensity score (1-4) that is used to determine *ALK*-positive or negative samples,varies depending on the fusion points (*EML-ALK*) and the promotor strength of the upstream partner. An upstream partner of similar promotor strength as *ALK* would give a negative result by IHC but positive result by FISH. In contrast, epigenetic changes in the promotor region may also cause higher expression of an unrearranged *ALK* gene resulting in positive IHC but negative FISH. The FDA-approved FISH test has been shown to lack sensitivity and is generally acknowledged to fail to detect rearrangements in up to 60% of patients.[15,16] The FISH test also fails to identify cases where epigenetic change may cause upregulation of an unrearranged *ALK* gene.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) methods allow the detection of gene rearrangements using RNA extracted from a patient's tumor.[4-6] They have been shown to be more accurate in determining rearranged *ALK* compared to FISH,[17] enable rapid processing and are highly specific for particular rearrangements depending on the primers used, but will not detect unknown rearrangements. Here, we have adapted a method described by Wang et al[18] and optimized it for use with degraded RNA obtained from Formalin-Fixed Paraffin-Embedded (FFPE) sections. This method is based on the unbalanced expression of 5'- and 3'-regions (exons) of the *ALK* gene. In the normal unrearranged *ALK* gene, the expression levels of both 5'- and 3'- exons are equal, whereas in the rearranged-*ALK* gene, expression of the 3'-region (kinase domain) is higher than that of the 5'-region. It is also notable that *ALK*-positive patients identified by RT-PCR and Next Generation Sequencing show similar responses to crizotinib as those identified by FISH.[18,19]

The robust and quantitative RT-PCR (qRT-PCR) method we have developed can detect unbalanced 5'-and 3'-exon expression indicative of *ALK* rearrangement. Since the primers used to amplify the exons are not based on specific fusion-points, the test detects all known and unknown variants and all upstream fusion partners of *ALK* can be determined by sequencing amplicons if necessary. In addition, the test can also identify high *ALK*-expressers that may be caused by epigenetic changes or mutations in the promoter region. This unbalanced 5'-and 3'-exon expression approach is also applicable for detection of other cancer-relevant gene rearrangements that result in increased expression of the 3'-kinase domain, e.g. ROS1 or RET. This is particularly relevant to patients with these rearrangements as they have been shown to respond to crizotinib[20] and cabozantinib,[21] respectively. This method is reproducible, quantitative, sensitive (to 1% positive cells), requires only 50-100 ng RNA, does not require highly trained personnel and can be performed in any clinical laboratory with access to a qPCR instrument. Here we present the protocol for the method and validation with 197 clinical samples.

MATERIAL AND METHODS

Clinical Samples and Cell Lines

Fifteen FFPE slides containing 2×10 µm sections from Canadian *ALK* (CALK) centers were provided by Dr. Ming Tsao, Department of Pathology, University Health Network, Princess Margaret Cancer Center and University of Toronto, ON, Canada. A second set of 182 specimens of 20 µm curls from FFPE sections were obtained from our tissue bank at the Nova Scotia Health Authority and Dalhousie University, Halifax, NS, Canada.

The lung adenocarcinoma cell line A 549 harboring the normal unrearranged *ALK* gene was obtained from the American

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Type Culture Collection and propagated as recommended. The lung adenocarcinoma cell line H3122 harboring a rearranged *ALK* gene (EML4 exon13: *ALK* exon20; *ALK* Variant 1) was a kind gift from Dr. Jeffrey Engelmann, Massachusetts General Hospital, Boston, MA, USA. The lung adenocarcinoma cell line HCC78 carrying the SLC34A2 exon4: ROS1 exon 32 fusion was obtained from Dr. John D. Minna, The University of Texas Southwestern Medical Center, Dallas, TX, USA. This study was approved by the Capital Health Research Ethics Board (CDHA-RS/2013-090) and all participating individuals signed inform consent.

Primers and Stock Preparation

Primers were designed to span exon-exon boundaries to minimize amplification from contaminating genomic DNA (Table 1, Suppl. Table 1). Primers were obtained from IDT (Coralville, IA, USA) and resuspended in RNase-free water to a working stock concentration of 10 µM.

Total RNA Isolation

Tumor-containing tissue was scraped from FFPE slides (1×20 µm or 2×10 µm sections) into a 1.5 mL Eppendorf tube using a fresh scalpel blade treated with RNA-Zap (Ambion Inc., Austin, TX, USA). Deparaffinization was achieved by the addition of 1 mL xylene, vigorous vortexing for 10 s, and centrifugation at 14000 rpm for 2 min. The supernatant was carefully removed by pipetting without disturbing the pellet and the pellet washed with 1 mL 100% ethanol, vortexed, and centrifuged at 14000 rpm for 2-5 min. The supernatant was again removed by pipetting without disturbing the pellet and any residual ethanol was removed using a fine pipet tip. The tube was then incubated at room temperature (15-25 °C) or at 37 °C for 10 min or until all residual ethanol had evaporated. Care was taken that deparaffinization was complete and the tissue pellet was not over-dried preceding lysis.

Total RNA was extracted from FFPE sections using the RNeasy® FFPE Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's instruction. Total RNA was eluted into 50 µL of RNase-free water. Total RNA from fresh cells was purified using the RNeasy® kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Human reference RNA was obtained from Life Technologies (Waltham, MA, USA). RNA concentration was determined using a NanoDrop-1000 (Nano Drop Technologies, Wilmington, DE, USA) and stored at -80 °C.

Reverse Transcription

Total RNA (500 ng), unless indicated otherwise, was reverse-transcribed using the Superscript III kit (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instruction. cDNA was used immediately for qPCR or stored frozen at -20°C.

RT-qPCR: qPCR reactions (10 µL) contained 5 µL 2X Kapa CybrFast qPCR mix (Kapa Biosystems, Boston, MA, USA), 10 pmol each of forward and reverse primers and 1 µL of undiluted cDNA. All reactions were performed in triplicate. The control cDNA was from the A549 cell line. The positive controls were

Primer	For/Rev	Sequence (5' > 3')	Amplicon Size (bp)
Assay optimization with cell lines			
ALK-Ex-1	For	GAGGCGATCTTGGAGGGTTG	154
ALK-Ex-1	Rev	CCACTTCCGACGCCTTCTTC	
ALK-Ex-29	For	AAAGAAGGAGCCACACGACAG	185
ALK-Ex-29	Rev	CGTAATTGACATTCCCACAAGG	
GAPDH 102-F	For	CAAGATCATCAGCAATGCCT	193
GAPDH 192-R	Rev	CTCTCCAGAACATCATCCCT	
Assay validation with clinical samples			
ALK14-15-FP	For	GTGAACAGAAGCGTGCATGAG	91
ALK14-15-RP	Rev	GCACCGGCACTCCATCCTTC	
ALK17-18-FP	For	CCACTGGGCATCCTGTACACC	112
ALK17-18-RP	Rev	CCATGTGACATTCGTCTACCTCAC	
ALK22-23-FP	For	CCTGAAGTGTGCTCTGAACAGG	87
ALK22-23-RP	Rev	GCGAACAATGTTCTGGTGGTTG	
GAP4-5-FP	For	GCCAAGGTCATCCATGACAAC	92
GAP4-5-RP	Rev	GGGCCATCCACAGTCTTCTG	

Table 1. List of primers used for *ALK* exon-expression assay.

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cDNA from the *ALK*-positive H3122 cell line or the ROS1-positive HCC78 cell line. The negative control was RNase-free water.

qPCR was performed in a Light Cycler® 480 Real-Time PCR System (Roche Applied Science, Laval, QC, Canada) in 384-well plates according to the Kapa CybrFast qPCR kit cycling conditions (once at 95 °C for 3 min; 45 times at 95 °C for 20 sec, 60 °C for 20 sec, 72 °C for 20 sec) followed by melt-curve analysis.

DATA ANALYSIS

The raw data was exported from the Light Cycler-480 software and an average Ct was calculated for each triplicate reaction. Fold-change was calculated using ΔΔCt method[22] as below:

ΔCt = Ct (Target A-Treated) – Ct (Ref B-Treated),
ΔCt = Ct (Target A-Control) – Ct (Ref B-Control),

Therefore, the fold-change for *ALK* exon14-15 using *GAPDH* as reference and A549 as control will be:

$\Delta\Delta Ct = \Delta Ct\ (ALK\ exon14\text{-}15^{FFPE} - GAPDH^{FFPE}) - \Delta Ct\ (ALK\ exon14\text{-}15^{A549} - GAPDH^{A549})$

The fold change = $2^{(-\Delta\Delta Ct)}$

The *ALK* 14-15 exon served as the normalizer. Fold-change for the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) exon 4-5 was used to assess the level of degradation of the FFPE-derived RNAs compared to the cell line controls. For validation set 1, fold-change of the *ALK* exon 22-23 was determined after normalization with the *ALK* exon 14-15 to identify *ALK* rearrangement-positive or -negative samples. For highly degraded FFPE samples (validation set 2), normalization with *ALK* exon 14-15 and/or 17-18 gave inconsistent results. This was mainly because of RNA degradation. In an unrearranged ALK gene, normalized expression values decrease from the 5'- to the 3'-end. However, in a rearranged *ALK* gene, expression of the 5'-exon will be higher than that of the middle exon, but expression of the 3'-exon will be higher than the middle despite the effect of RNA degradation. Therefore, GAPDH exon 4-5-normalized expression of *ALK* exon 14-15, 17-18 and 22-23 was used to determine *ALK*-positives. A sample was called positive when *ALK* exon 22-23 expression was higher than the *ALK* exon 17-18, which is at the 5'-position to the fusion point (*ALK* exon 20). The identity of the upstream fusion partner is not known; only the presence of a rearrangement resulting in up-regulation of the 3' kinase domain of *ALK* is determined.

The utility of the exon-expression assay for the detection of the *ROS1* gene fusion was demonstrated using HCC78 cell line containing the SLC34A2: *ROS1* rearrangement. Primers designed for the *ROS1* 5'-end (exon 7 and 12) and *ROS1* 3'-end (exon 35 and 39) and GAPDH were used (Supplementary Table 1). Fold-change was derived as described above after normalization with *GAPDH*.

RESULTS AND DISCUSSION

As shown in Figure 1, expression of both 5'-exon 1 and 3'-exon 29 of intact *ALK* is similar and lower (higher Ct value; blue circle and arrow) compared to the 3'-exon 29 in a rearranged *ALK* (red arrows). The two groups of amplification curves in both groups are due to undiluted and 10-fold diluted starting template cDNA

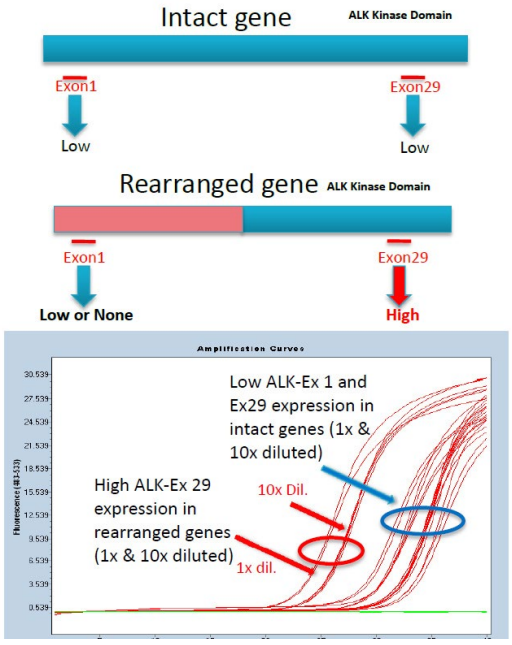

Figure 1: *ALK* exon-expression assay for rearrangements. Amplification curves are shown for RNA isolated from cell lines containing unarranged and rearranged *ALK* genes as described in Material and Methods.

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used in the qPCR reactions. Unbalanced expression is observed when the *ALK* gene is rearranged irrespective of the rearrangement partner or fusion points in the case of *EML4-ALK* variants. Therefore, the greatest advantage of this method is the ability to detect not only known but also unknown *EML4-ALK* variants as well as fusion with other known and unknown upstream partners (e.g. KIF5B, TFG, KLC1), which upon further study may prove sensitive to crizotinib. Identification of fusion points and upstream partner can be determined, if desired, by qPCR of each *ALK*-exon and sequencing of the amplicon containing the fusion partner.

To determine the sensitivity of detection of the test, a titration was performed with the known *EML4-ALK* rearranged cell line H3122 into the A549 cell line containing the unrearranged *ALK* (Figure 2). *ALK* unbalanced 3'-exon29 expression can be detected with high confidence (*p*-value 0.0025) with as few as 1% H3122 cells in A549 background. In contrast, since tumors are heterogeneous, FISH-positive cell count variations are inherent.[23] For example, 5-95% FISH-positive cells were scored for the sample CALK-FFPE-11 by 2 individual operators at twelve centers. The accepted cutoff value is 15% FISH positive cells. This sample was confirmed negative for *ALK* rear-

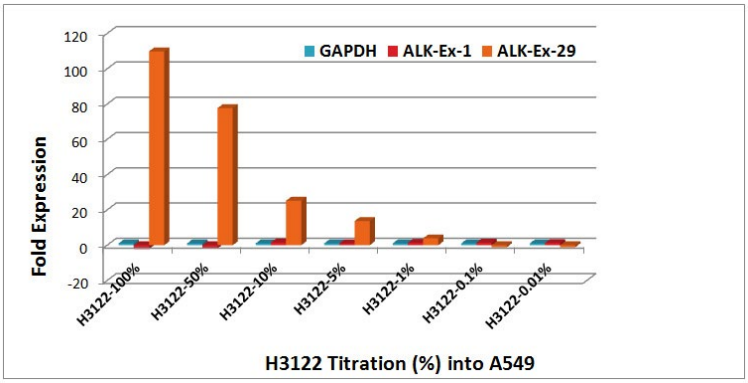

Figure 2: Determination of sensitivity of detection of rearranged ALK gene by dilution. H3122 RNA (EML13:*ALK*20) was diluted into A549 (intact ALK). All p-values for assays down to 1% H3122 RNA were 0-0.0025.

Sample Name	ALK Exon-Expression qRT-PCR					FISH*	
	GAPDH		*ALK*- 3'-exon		Positive / Negative	FISH- % Positive cells[a]	Positive / Negative
	Fold Change	*p* value	Fold Change	*p* value			
H3122 (Pos. Ctr.)	-1.32	0.009	219.79	0.0000	Positive	N/A	N/A
CALK-FFPE-1	-49.52	0.00029	14.12	0.015	Positive	50-99	Positive
CALK-FFPE-4	-134.67	0.00002	4.42	0.000328	Positive	25-70	Positive
CALK-FFPE-7	-29.31	0.000028	4.84	0.00007	Positive	15-80	Positive
CALK-FFPE-8[b]	-105.17	0.000025	2.25	0.0381	Positive	24-70	Positive
CALK-FFPE-9	-27.22	0.00002	2.54	0.236	Negative	0-5	Negative
CALK-FFPE-10	-171.65	0.000025	2.8	0.0053	Positive	5-75	Positive
CALK-FFPE-11	-15.17	0.000047	1.92	0.1427	Negative	5-95	Negative
CALK-FFPE-12	-2.68	0.00165	240.51	0.000004	Positive	25-90	Positive
CALK-FFPE-13	-130.99	0.000025	1.45	0.19844	Negative	5-12	Negative
CALK-FFPE-14	-30.69	0.000028	1.47	0.126358	Negative	5-10	Negative
CALK-FFPE-15[c]	-434.54	0.000024	-1.25	0.22542	Negative	10-60	Positive
CALK-FFPE-17	-88.85	0.000025	14.16	0.000051	Positive	20-90	Positive
CALK-FFPE-19	-33.2051	0.000304	58.35	0.000005	Positive	30-90	Positive
CALK-FFPE-23	-168.89	0.000025	2.32	0.077846	Negative	5-10	Negative
CALK-FFPE-27	-123.06	0.000025	5.72	0.000759	Positive	15-60	Positive
CDHA-A4[d]	-23.16	0.00032	16.45	0.000212	Positive	N/A	N/A
CDHA-C-1[d]	-6.96	0.000491	43.31	0.0023	Positive	N/A	N/A

Note: A549 Negative control and reference sample; *H3122-ALK* Positive control sample
a: Distribution of % abnormal *ALK* signals from 200 nuclei counted by 2 technologist from 12 Centers
b: only 160ng RNA available for RT
c: FFPE section was very bloody; GAPDH >400-fold degraded
d: Known Positive FFPE from Dr Zhaolin Xu
*: From Reference[17]

Table 2: Validation of *ALK* exon expression assay using clinical FFPE samples from *CALK* centers. Fold change of GAPDH represents the amount of RNA degradation compared to H3122 cell line RNA, which is relatively intact (-1.32). Fold change of *ALK* represents the expression of the 3'-exon22-23 relative to the 5'-exon17-18 after normalization with *ALK* exon14-15 as described in the Materials and Methods. *ALK* positive tumors have a fold change of greater than 2 with a *p*-value <0.05. N/A, FISH test was not performed.

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rangement using RT-PCR method (Table 2). However, in RNA extracted from a 10-20 µm FFPE section, all RNA species are available for detection by qPCR, and RNA contributed by as little as 1% *ALK*-positive cells is detected.

Due to the limited availability of patients' FFPE samples together with limited amounts of total RNA available after purification, a titration experiment was performed to determine the minimal amounts of RNA required for cDNA conversion prior to qPCR. Most reverse transcription kit protocols suggest using 500-1000 ng total RNA for cDNA synthesis. We converted 10-500 ng total RNA into cDNA and evaluated unbalanced exon-expression using H3122 and HCC78 cell lines harboring rearranged *ALK* and *ROS1*, respectively (Figure 3). Total RNA amounts from 10-500 ng showed >50-fold higher 3'-end expression compared to the 5'-end with very high confidence (*p*-value 0.000001). However, total amounts of 20-100 ng RNA converted into cDNA provided optimal performance. This demonstrates an additional advantage of this method where a single 10 µm FFPE section or a very small biopsy sample may be sufficient to determine rearranged *ALK* and/or *ROS1* gene.

The ROS1 5'-end exons (7 and 9) have a higher Ct value (lower expression) compared to ROS1 3'-end exons (35 and 39) in HCC78 cell line (Supplementary Figure 1). As expected, expression of the housekeeping gene *GAPDH* is similar in the A549 cell line containing intact *ROS1* gene and *HCC78*. The sensitivity of the *ROS1* exon-expression assay was determined by titration of *HCC78* RNA into A549 RNA (Supplementary Figure 2). As little as 1% *HCC78* RNA in A549 RNA was detectable (*p*-value 0.000001). Validation of the *ROS1* exon-expression assay was not performed due to unavailability of clinical samples.

The *ALK* exon-expression assay was validated in a blinded manner using 15 FFPE samples from NSCLC patients from *CALK* centers (Validation set 1). Two known positive FFPE samples were also included. We were unable to obtain amplification using *ALK* exon1 and *ALK* exon29 and *GAPDH* 102F and 192 R primers (Table 1), indicating that RNA from FFPE samples was degraded to sizes smaller than these amplicons (154-193 bp). To address this, new exon-exon spanning primers for *ALK* exons 14-15, 17-18 and 22-23 were designed that generated smaller amplicons (87-112 bp; Table 1). The *ALK* exon 14-15 was used to normalize and determine relative expression of *ALK* exon 17-18 and *ALK* exon 22-23 and also to determine the amount of RNA degradation in FFPE samples compared to cell line RNA. H3122 RNA exhibited no degradation (fold-change of -1) whereas FFPE RNAs showed varying amounts of degradation (Table 2). Samples that had >2-fold higher expression of 3'-exon 22-23 and a *p*-value 0.05 or lower were scored as *ALK*-positive. Of the 15 CALK samples, 14 were concordant with FISH results (Table 2). *CALK*-FFPE-11 was *ALK*-negative using the exon-expression assay. Interestingly, this sample showed very high variability by FISH (5-95% cells FISH-positive as scored by two technologists at 12 centers) but was called negative when subsequently tested by RT-PCR[17] in agreement with our results (Table 2). Similar heterogeneity in FISH results has been reported previously.[23] *CALK*-FFPE-15 exon-expression assay results were not concordant with the FISH results; this is probably because the FFPE section for this sample was very bloody and also showed a very high level of RNA degradation (*GAPDH* fold-change; -434-fold), making it impossible to assess with certainty.

A second set of 182 validation samples were single 20 µM curls (Validation set 2). Good concordance was seen between the exon-expression assay and FISH. Similar to Validation set 1, A549 and H3122 cells were used as negative and positive controls and relative *GAPDH* expression was used to assess RNA degradation in the FFPE samples. Table 3 shows the results of six samples that had equivocal results. These samples showed varying and high levels of RNA degradation when compared with cell line RNA (Table 3). When RNA is highly degraded, the accuracy to assess fold-changes between 5'-end and 3'-end expression by ΔΔCt method is compromised. However, the test still yields a result and can be confirmed with IHC and/or FISH. Degradation of any transcript starts from the 3'-end and proceeds towards the 5'-end which is capped to prevent degradation. Sample RE-12-04 T RNA showed RNA degrada-

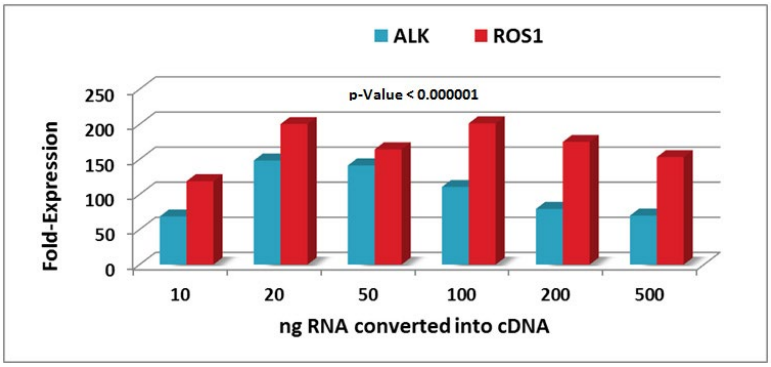

Figure 3: Determination of sensitivity of detection of rearranged *ALK* and ROS1 genes. Decreasing amounts of total RNA were used in the assay as described in the Materials and Methods.

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Sample Name	FISH						*ALK* Exon-Expression by qRT-PCR (*ALK* exon14-15 and 17-18 normalizer)						*ALK* Exon-Expression by qRT-PCR (*GAPDH* exon4-5 normalizer)							
	% Abnormal cells					FISH results -NSHA	*GAPDH* Exon 4-5		*ALK* Exon 22-23		Result qPCR method	Concordance with FISH	*ALK* Exon 14-15		*ALK* Exon 17-18		*ALK* Exon 22-23		Result qPCR method	Concordance with FISH
	Read 1	Read 2	Read 3	Read 4	Read 5		Fold-Change	*p* value	Fold-Change	*p* value			Fold-Change	*p* value	Fold-Change	*p* value	Fold-Change	*p* value		
RE-12-04 T	48%	74%	NA	NA	NA	Pos.	-513.00	0.0008	-1.01	0.8963	Neg.	NO	299.55	0.0003	156.50	0.0170	284.05	0.0154	Pos.	YES
RE-12-04 AB	56%	80%	NA	NA	NA	Pos.	-185.00	0.0013	2.29	0.0900	Neg.	NO	185.25	0.0000	99.96	0.0000	312.27	0.0169	Pos.	YES
RE-12-04 BL	9%	14%	1%	34%	NA	Neg.	-2.50	0.0311	3.54	0.0178	Pos.	NO	2.50	0.0124	-2.85	0.0003	3.33	0.0064	Pos.	NO
RE-12-04 W	8%	4%	9%	16%	NA	Neg.	-316.00	0.0008	2.68	0.0130	Pos.	NO	135.92	0.0310	41.45	0.2410	54.06	0.0012	Pos.	NO
RE-12-04 BV	41%	36%	4%	6%	26%	Neg.[a]	-3.56	0.0035	2.02	0.0195	Pos.	NO	9.36	0.0017	1.33	0.0074	7.11	0.0005	Pos.	NO
RE-12-05 AU	15%	18%	8%	8%	12%	Neg.[a]	-72.58	0.0010	1.56	0.4207	Neg.	NO	74.88	0.0461	70.36	0.0080	113.50	0.0042	Pos.	NO

[a]Additional confirmatory FISH test performed at *CALK* center Toronto. This was also scored as Negative.

Table 3: Results of *ALK* exon-expression assay for Validation set 2 samples. Fold change of GAPDH exon4-5 represents the amount of RNA degradation compared to H3122 cell line RNA, which is relatively intact (-1.32). *ALK*-positive tumors have a fold change >2 with a *p*-value <0.05 (shown in red). For GAPDH exon4-5 normalized data, *ALK* positive tumors have higher fold change for *ALK* 3'-exon22-23 relative to *ALK* 5'-exon17-18 with a *p*-value <0.05 (shown in red). qPCR results are scored positive (Pos.) or Negative (Neg.) and concordance with FISH result is scored YES or NO.

tion of >500-fold and did not clearly show >2-fold change with high confidence when normalized with *ALK* exon 14-15/ 17-18. However, *GAPDH*-normalized expression values (284 for exon 22-23 compared to 156 for exon 17-18) indicated that this sample is *ALK*-positive (*p*-value<0.05; Table 3). Similarly, sample RE-12-04 AB showed ~185-fold degradation of RNA and only 2.3-fold higher expression of 3'-end ALK with *p*-value 0.09 when normalized with *ALK* exon14-15/17-18. Again, GAPDH-normalized expression (312 for exon 22-23 compared to 100 for exon 17-18) suggests this sample is *ALK*-positive (*p*-value <0.01; Table 3). Both RE-12-04 T and RE-12-04 AB samples were *ALK*-positive by the FISH test with 48-74% and 56-80% abnormal cells, respectively (Table 3).

Sample RE-12-04-BL was positive by exon-expression assay using both normalization methods (Table 3). This sample was negative by FISH even though average of the 4 reads is ~15% abnormal cells. Two other samples, RE-12-04-W and RE-12-04-BV were also positive with both normalization methods. However, these samples were negative by the FISH with positive cell counts of 9±5% and 23±16% (average±standard deviation), respectively (Table 3). Sample RE-12-05-AU was found positive by using *GAPDH* as normalizer (113.5 for exon 22-23 compared to 70.36 for exon 17-18) but not when exon 14-15/17-18 was used. This sample was negative by the FISH test with 12±5% counted as FISH-positive (Table 3). It is possible that an epigenetic modification of the *ALK* gene or a rearrangement that alters FISH probe binding would not be detected by FISH and caused relative up-regulation of exon 22-23 in this tumor.

Clearly, the sensitivity of the assay depends on the quality of the RNA. As seen for the validation samples, high levels of RNA degradation can lead to a decrease in the ability to determine *ALK*-positivity by the exon-expression assay. Therefore, we compared the RNA quality from ten representative FFPE samples and RNA isolated from a fresh-frozen lung tumor sample and a commercially available human reference RNA. We used a qPCR method measuring 93bp and 193bp amplicons of the highly expressed housekeeping gene, *GAPDH*. Average Ct values of three replicates for both amplicons are shown in Suppl. Table 2. The Ct values for both amplicons from fresh-frozen lung tumor and reference human RNA are similar and lower, indicating little or no degradation of RNA, whereas RNA from all FFPE samples showed 10-15 Ct value higher or not even detectable (Ct>35). This translates into 1000-100,000-fold degradation of RNA obtained from FFPE samples compared to fresh-frozen samples. Such samples may necessitate confirmation with another method.

CONCLUSION

The qPCR-based exon-expression assay we have developed is demonstrably objective, robust and sensitive for the detection of rearranged *ALK* genes when compared to FISH and IHC. As shown by our preliminary results with *ROS1*, this method also shows great promise as generalized test for rearranged genes implicated in various kinds of cancer. This is particularly relevant since *ROS1*-positive patients also respond to crizotinib. Since detection of rearranged genes in patient samples uses RNA, the sensitivity and robustness of this test is compromised when RNA is highly degraded, and can result in false-negative calls. We have observed this phenomenon with two sets of valida-

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tion FFPE samples showing high degrees of RNA degradation. However, fresh patient samples may have less RNA degradation, which would minimize false-negative calls; therefore, we highly recommend using fresh-frozen or RNA Later-preserved biopsies for this assay.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Canadian Cancer Society. Web site. http://www.cancer.ca/en/cancer-information/cancer-type/lung/lung-cancer/non-small-cell-lung-cancer/?region=ns. Accessed September 6, 2016.

2. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. 2010; 363: 1693-703. doi: 10.1056/NEJMoa1006448

3. Bang YJ. The potential for crizotinib in non-small cell lung cancer: a perspective review. *Ther Adv Med Oncol*. 2011; 3(6): 279-291. doi: 10.1177/1758834011419002

4. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature*. 2007; 448(7153): 561-566. doi: 10.1038/nature05945

5. Choi YL, Takeuchi K, Soda M, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. *Cancer Res*. 2008; 68(13): 4971-4976. doi: 10.1158/0008-5472.CAN-07-6158

6. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. *Clin Cancer Res*. 2008; 14(20): 6618-6624. doi: 10.1158/1078-0432.CCR-08-1018

7. Rikova K, Guo A, Zeng Q, et al. Global survey of phosphotyrosine signaling identifies oncogenic kinases in lung cancer. *Cell*. 2007; 131(6): 1190-1203. doi: 10.1016/j.cell.2007.11.025

8. Takeuchi K, Choi YL, Togashi Y, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. *Clin Cancer Res*. 2009; 15(9): 3143-3149. doi: 10.1158/1078-0432.CCR-08-3248

9. Suehara Y, Arcila M, Wang L, et al. Identification of KIF5B-RET and GOPC-ROS1 fusions in lung adenocarcinomas through a comprehensive mRNA-based screen for tyrosine kinase fusions. *Clin Cancer Res*. 2012; 18(24): 6599-6608. doi: 10.1158/1078-0432.CCR-12-0838

10. Togashi Y, Soda M, Sakata S, et al. KLC1-ALK: A novel fusion in lung cancer identified using a formalin-fixed paraffin-embedded tissue only. *PLoS One*. 2012; 7(2): e31323. doi: 10.1371/journal.pone.0031323

11. Yi ES, Boland JM, Maleszewski JJ, et al. Correlation of IHC and FISH for ALK gene rearrangement in non-small cell lung carcinoma: IHC score algorithm for FISH. *J Thorac Oncol*. 2011; 6(3): 459-465. doi: 10.1097/JTO.0b013e318209edb9

12. Mino-Kenudson M, Chirieac LR, Law K, et al. A novel, highly sensitive antibody allows for the routine detection of ALK-rearranged lung adenocarcinomas by standard immunohistochemistry. *Clin Cancer Res*. 2010; 16(5): 1561-1571. doi: 10.1158/1078-0432.CCR-09-2845

13. Paik PK, Arcila ME, Fara M, et al. Clinical characteristics of patients with lung adenocarcinomas harboring BRAF mutations. *J Clin Oncol*. 2011; 29(15): 2046-2051. doi: 10.1200/JCO.2010.33.1280

14. Shaw AT, Solomon B, Kenudson MM. Crizotinib and testing for ALK. *J Natl Compr Canc Netw*. 2011; 9: 1335-1341. Web site. http://www.jnccn.org/content/9/12/1335.short. Accessed September 6, 2016.

15. Pekar-Zlotin M, Hirsch FR, Soussan-Gutman L, et al. Fluorescence in situ hybridization, immunohistochemistry, and next-generation sequencing for detection of EML4-ALK rearrangement in lung cancer. *Oncologist*. 2015; 20(3): 316-322. doi: 10.1634/theoncologist.2014-0389

16. Peled N, Palmer G, Hirsch FR, et al. Next-generation sequencing identifies and immunohistochemistry confirms a novel crizotinib-sensitive ALK rearrangement in a patient with metastatic non-small-cell lung cancer. *J Thorac Oncol*. 2012; 7(9): e14-e16. doi: 10.1097/JTO.0b013e3182614ab5

17. Cutz JC, Craddock KJ, Torlakovic E, et al. Canadian anaplastic lymphoma kinase study: a model for multicenter standardization and optimization of ALK testing in lung cancer. *J Thorac Oncol*. 2014; 9(9): 1255-1263. doi: 10.1097/JTO.0000000000000239

18. Wang R, Pan Y, Li C, et al. The use of quantitative real-time reverse-transcriptase PCR for 5' and 3' portions of ALK transcripts to detect ALK rearrangements in lung cancers. *Clin*

ISSN 2377-1658 http://dx.doi.org/10.17140/PRRMOJ-4-132

Cancer Res. 2012; 18(17): 4725-4732. doi: 10.1158/1078-0432.CCR-12-0677

19. Wang Y, Zhang J, Gao G, et al. EML4-ALK fusion detected by RT-PCR confers similar response to crizotinib as detected by FISH in patients with advanced non-small-cell lung cancer. *J Thorac Oncol*. 2015; 10(11): 1546-1552. doi: 10.1097/JTO.0000000000000668

20. Bergethon K, Shaw AT, Ignatius Ou SH, et al. ROS1 rearrangements define a unique molecular class of lung cancers. *J Clin Oncol*. 2012; 30(8): 863-870. doi: 10.1200/JCO.2011.35.6345

21. Drilon A, Wang L, Hasanovic A, et al. Response to cabozantinib in patients with RET fusion-positive lung adenocarcinomas. *Cancer Discov*. 2013; 3(6): 630-635. doi: 10.1158/2159-8290.CD-13-0035

22. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29(9): e45. doi: 10.1093/nar/29.9.e45

23. Camidge DR, Kono SA, Flacco A, et al. Optimizing the detection of lung cancer patients harboring anaplastic lymphoma kinase (ALK) gene rearrangements potentially suitable for ALK inhibitor treatment. *Clin Cancer Res*. 2010; 16(22): 5581-5590. doi: 10.1158/1078-0432.CCR-10-0851

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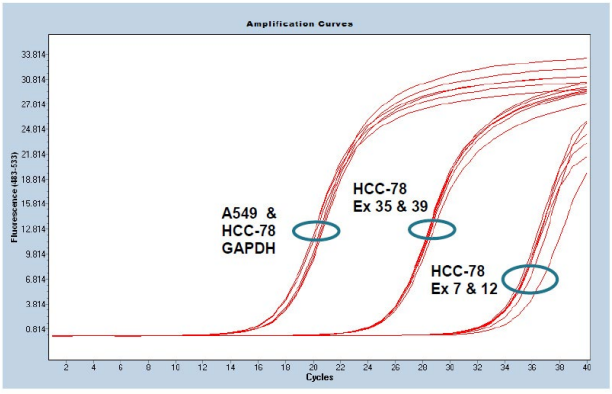
Supplementary Data

Primer	For/Rev	Sequence (5' > 3')	Amplicon Size
GAPDH 102-F	For	CAAGATCATCAGCAATGCCT	193bp
GAPDH 192-R	Rev	CTCTCCAGAACATCATCCCT	
ROS1-Ex-7	For	TGAGAGCTCAAGTCCCGACAC	130bp
ROS1-Ex-7	Rev	GGTTCTCTGTGTCCCTGCATC	
ROS1-Ex-12	For	CTGTGCGTATTGTGGAGAGTTG	137bp
ROS1-Ex-12	Rev	TAGGATGAGATGGGAAGCAGAG	
ROS1-Ex-35	For	ATTGAAAATCTTCCTGCCTTCC	127bp
ROS1-Ex-35	Rev	TGATTTCTCCACTTCCAACTCC	
ROS1-Ex-39	For	CAGCTAGAAATTGCCTTGTTTCC	125bp
ROS1-Ex-39	Rev	CAGGCCTTCCCCTCTCTTTC	

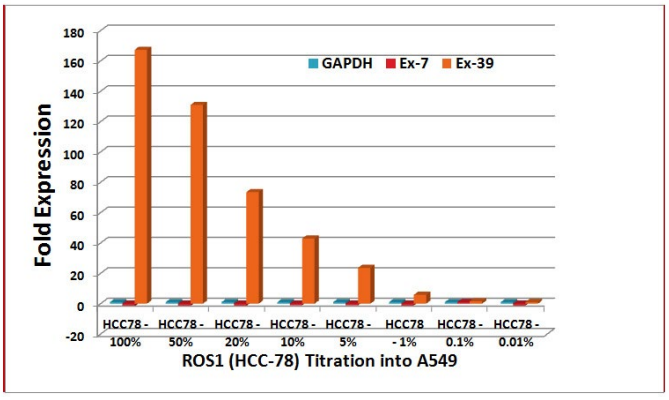
Supplementary Table 1: List of primers used for *ROS1* exon expression assay.

Sample Name	**GAPDH: Average Ct Value** (3 replicates)	
	93 bp amplicon	**193 bp** amplicon
Lung-FFPE 1	28.88	33.37
Lung-FFPE 2	27.17	29.93
Lung-FFPE 3	30.02	>35.00
Lung-FFPE 4	29.46	>35.00
Lung-FFPE 5	28.60	32.49
Lung-FFPE 6	25.65	27.91
Lung-FFPE 7	27.55	31.15
Lung-FFPE 8	26.51	28.52
Lung-FFPE 9	27.35	29.79
Lung-FFPE 10	27.55	30.34
Fresh-Frozen Lung Tumor	16.89	15.98
Human Reference RNA	16.30	14.74

Supplementary Table 2: Comparison of RNA quality between RNA isolated from FFPE samples and a fresh-frozen sample by qRT-PCR. RNA was extracted from ten FFPE samples and one fresh-frozen sample and compared with Human Reference RNA by qPCR as described in the Materials and Methods.



Supplementary Figure 1: Detection of *ROS1* fusion by exon-expression assay. RNA was extracted from the HCC-78 cell line carrying SLC34A2-ROS1 fusion (SLC34A2 Ex4:*ROS1* Ex 32). A549 ROS1 exon 7, 12, 35 and 39 amplify similar as HCC-78 ROS1 exon 7 and 12 (not shown).



Supplementary Figure 2: Determination of sensitivity of detection of translocated *ROS1* by dilution of HCC-78 RNA (SLC34A2 Ex4:*ROS1* Ex 32) into A549 RNA (intact *ROS1*). All *p*-Values for assays down to 1% HCC-78 RNA were 0-0.000001.