Multiplexed Mass Spectrometry-based Assay to Quantify Translocation Markers from NSCLC FFPE Tissue

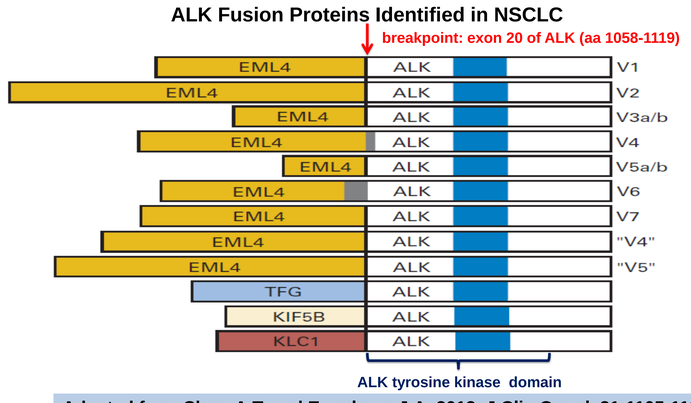
Wei-Li Liao[1], Sheeno Thyparambil[1], Eunkyung An[1], Christopher P. Hartley[2], Patrick Ma[3], Jaime Rodriguez[4], Ignacio Wistuba[4], Jon Burrows[1], Todd Hembrough[1], and Laura J. Tafe[2]

[1]OncoPlex Diagnostics Inc., Rockville, MD, [2]Dartmouth-Hitchcock Medical Center, Lebanon, NH,
[3]Cleveland Clinic Cancer Institute, Cleveland, OH, [4]MD Anderson Cancer Center, Houston, TX.

Overview

- While FISH is the standard diagnostic test to detect *ALK*, *ROS1* or *RET* translocation, it is low-throughput and performing FISH on multiple targets is tissue-consuming. Therefore, a higher-throughput multiplex method is necessary especially for the detection of oncogenic drivers of low frequencies (*ALK* rearrangement incidence rate: 2-5%; *ROS1* and *RET*: 1-2%).
- Quantitation of protein may provide a more relevant measure of the ALK pathway. Therefore, a specific, objective, sensitive, and accurate proteomics-based quantitative assay would be ideal.
- In this report, we developed a clinically-validated multiplex MS assay to quantify ALK, ROS1, and RET protein levels from formalin-fixed paraffin-embedded (FFPE) NSCLC tissues.
- We are running the assay in a CLIA-certified-CAP-accredited laboratory to concurrently assess protein expression levels for translocation markers and several diagnostic and potentially targetable biomarkers, e.g. TTF1, K7, p63, K5, EGFR, HER2, HER3, MET, KRAS and IGF1R, from NSCLC biopsies.

ALK Fusion Proteins Identified in NSCLC



Adapted from Shaw A.T. and Engelman J.A. 2013. J Clin Oncol. 31:1105-1111.

Methods

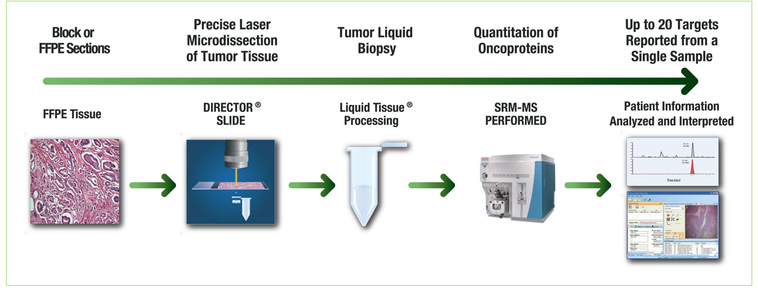


Figure 1: Liquid Tissue®-SRM workflow for analysis of proteins from FFPE tissue.

Results

Translocation Markers- SRM Assay Development

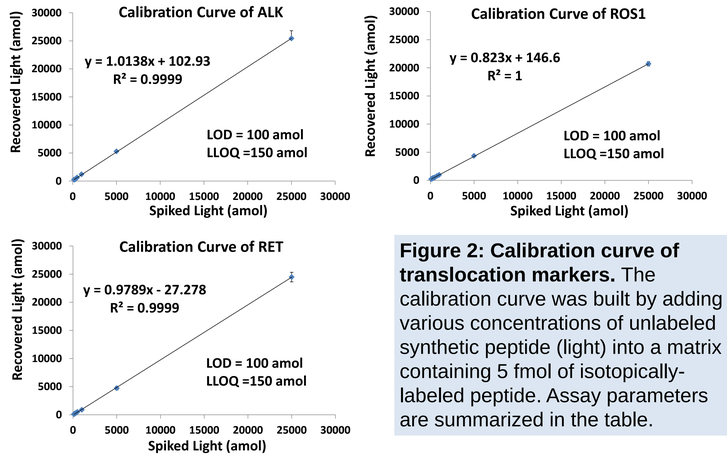


Figure 2: Calibration curve of translocation markers. The calibration curve was built by adding various concentrations of unlabeled synthetic peptide (light) into a matrix containing 5 fmol of isotopically-labeled peptide. Assay parameters are summarized in the table.

Protein	Sensitivity*	Assay Range* (amol)		Linearity*	Precision**	Specificity
		Low	High			
ALK	LLOD: 100	150	25,000	Standard Curve R^2=0.9999	<25% CV	Peptide is unique for and specific to ALK
ROS1	LLOD: 100	150	25,000	Standard Curve R^2=1	<25% CV	Peptide is unique for and specific to ROS1
RET	LLOD: 100	150	25,000	Standard Curve R^2=0.9999	-----	Peptide is unique for and specific to RET

***** Based on 5 replicates of each point on a standard curve in *Pyrococcus* lysate background.
******Precision is based on six replicates of clinical sample runs.

Quantitation of ALK and ROS1 in Rearrangement Positive and Negative Tissues

Sample Type	Sample ID	*ALK* or *ROS1* Translocation Status	SRM (amol/µg)	
			ALK	ROS1
NSCLC	C0060-P1R-A	Unknown	ND	696.00
NSCLC	C0383-T1LR-A	ROS1 translocation +	ND	377.08
Cell Line	H3122	ALK FISH +	396.3	ND
NSCLC	DH4	ALK FISH +	437.65	ND
NSCLC	C0556-T1LR-A	ALK FISH +	336.55	ND
NSCLC	DH2	ALK FISH +	242.6	ND
NSCLC	DH3	ALK FISH +	223.25	ND
NSCLC	DH5	ALK FISH +	216.4	ND
NSCLC	DH1	ALK FISH +	175.25	ND
NSCLC	C0558-T1LR-A	ALK FISH +	153.90	ND
NSCLC	C0557-T1LR-A	ALK FISH +	138.85	ND
NSCLC	C0560-T1LR-A	ALK FISH +	136.32	ND
NSCLC	DH6	ALK FISH +	117.65	ND
NSCLC	C0559-T1LR-A	ALK FISH +	106.45	ND
NSCLC	DH9	ALK FISH +	ND	ND
NSCLC	DH7	unknown	ND	ND
Brain/met	DH8	ALK FISH negative	ND	ND
NSCLC	DH10	ALK FISH negative	ND	ND
NSCLC	DH11	ALK FISH negative	ND	ND

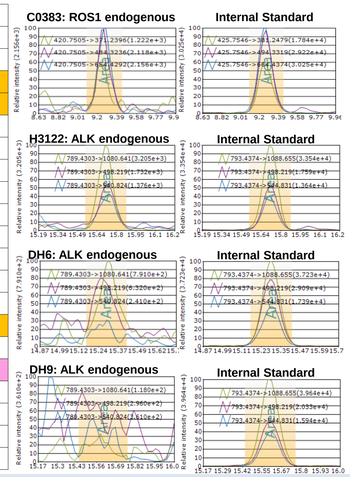
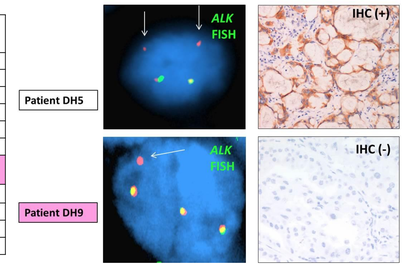


Figure 3: Summary of the expression of translocation markers in eighteen FFPE NSCLC tissues and H3122 cells. *ALK* or *ROS1* translocation status is listed and samples were analyzed by mass spectrometry to quantitate the expression of ALK and ROS1 protein. Analytes were quantitated in triplicate 1 µg injections. Pinpoint spectra on the right represent highlighted rows in the table.

Comparison of SRM with *ALK* DNA Sequence, FISH, and IHC in DH9

DNA sequence analysis of the ALK SRM peptide:

Sample ID	SRM (amol/µg)	DNA Sequencing Result	*ALK* FISH	ALK IHC (cell signaling D5F3)
DH1	175.25	WT	positive	positive
DH2	242.6	Not performed	positive	positive
DH3	223.25	Not performed	positive	positive
DH4	437.65	WT	positive	positive
DH5	216.4	Not performed	positive	positive
DH6	117.65	WT	positive	positive
DH9	ND	Heterozygous point mutation	positive	Negative
DH7	ND	WT	Not performed	Negative
DH8	ND	WT	Negative	Negative
DH10	ND	WT	Negative	Negative
DH11	ND	WT	Negative	Negative



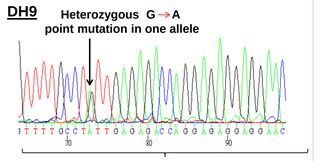


Figure 4: Comparison of SRM with *ALK* FISH, IHC and DNA sequencing in 11 samples. The table summarizes SRM data, sequencing results for ALK peptide-encoding region, and FISH/IHC status. The upper figures represent FISH and paired IHC for DH5 and DH9. In both cases, *ALK* FISH testing shows deletion of the 5' (green) signal with retained 3' (orange) signal consistent with *ALK* rearrangement. Arrows indicate the re-arranged red signal. ALK IHC, however, is negative in DH9.

Analysis of Lung OncoPlex in FFPE NSCLC Tissues

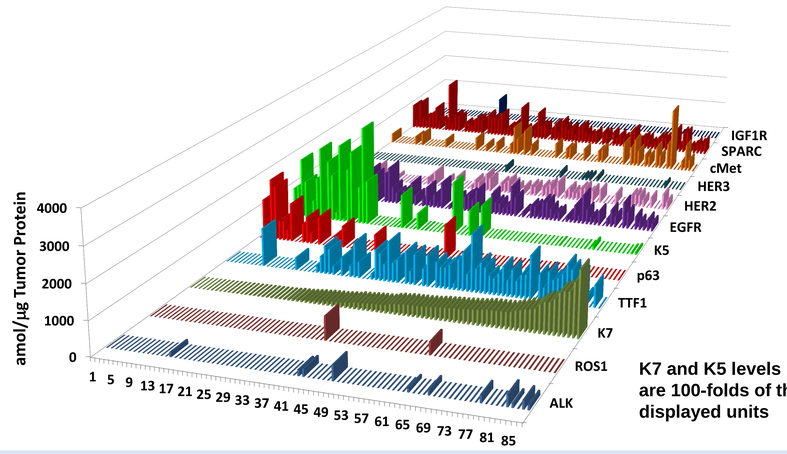


Figure 5: NSCLC tissue expression for each of the targets within the Lung OncoPlex as a multiplex analysis, sorted by K7 expression from low to high, left to right. The 87 samples represent a mixture of 12 *ALK* rearrangement positive controls and a cohort of 75 ALK negative NSCLC. The Lung OncoPlex assay not only confirmed pathologist's subtyping but also quantified the other potentially targetable biomarkers.

Conclusions

- We have developed a quantitative mass spectrometry-based assay for ALK and ROS1 to evaluate protein expression level in FFPE samples.
- Including these markers within the lung OncoPlex assay allows simultaneous assessment of multiple clinically actionable gene rearrangements and biomarker targets.
- The multiplexed proteomic screening of patient tissue could be performed at the time of initial biopsy to maximize information in limited tissue. Clinicians could use the information to strategically order appropriate tests, leading to the best patient care.