The OncoNetwork Consortium: A global collaborative research study on the development and verification of an Ion AmpliSeq™ RNA Lung Fusion Panel

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ABSTRACT

Chromosomal translocations and corresponding gene fusions play an important role in the initiation of tumorigenesis and these processes have been strongly associated with distinct tumor subtypes. The recent association of ALK, ROS and RET fusion transcripts and its potential as lung tumor predictive biomarkers has increased the need of a technology that could detect these biomarkers starting from limited amount of material. Life Technologies and OncoNetwork consortium collaborated for the development of a lung fusion panel based on Ion AmpliSeq™ RNA chemistry. The OncoNetwork consortium is comprised of twelve - translational cancer research institutes with many years of experience in adopting the latest molecular techniques like next generation sequencing (NGS)- for lung research. Life technologies and OncoNetwork consortium agreed to work together for the development of a lung fusion panel based on Ion AmpliSeq™ RNA chemistry. Material and Methods: Consortia's requirements for the panel development : 1) detect all variants of ALK, ROS1, or RET fusion transcripts described in COSMIC in a single reaction using 10 ng of total RNA 2) Include 5' and 3' ALK, ROS1, RET gene expression assays as an indicator of a translocation at this gene. 3) Include endogenous RNA assay controls to determine if the quality of the results could be affected by RNA quality. 4) Provide similar results on archived FFPE samples tested by FISH. Human lung adenocarcinoma cell lines H2228 (EML4-ALK positive), HCC78 (SLC4A2-ROS1 positive) ,LC-2/ad (CCDC6-RET positive) and Ambion® FirstChoice® Human Brain Reference (HBR) RNA was used as positive and negative control for the study. Formalin fixed paraffin embedded (FFPE) tissue was isolated using different extraction methods. After amplification using Ion AmpliSeq™ RNA chemistry samples were sequenced on the Ion Torrent PGM™ sequencer using the Ion PGM™ 200 Sequencing Kit. The presence of the fusions was confirmed by custom TaqMan® gene expression assays when possible. Preliminary results of the panel on ALK or ROS1 or RET positive cell lines and FFPE archived cancer research samples gave good concordance with FISH results. Expected negative samples were confirmed negative. We found a KIF5B-RET fusion positive sample in a sample not previously tested for RET fusions. Two of the expected positive samples by FISH were found negative due to limited amount to tumor cells present in the sample. Cell line RNA dilutions were performed to determine the panel's limit of detection. We demonstrate a limit of detection of 1 % tumor RNA in the presence of 99 % normal RNA using the panel with 10 ng of RNA extracted by cell lines. Gene expression controls work well as an indicator of the RNA quality and of the translocation presence. The Ion AmpliSeq™ RNA lung fusion panel research workflow is easier and faster to perform in comparison to the FISH method. The results obtained to date are highly encouraging for panel to be used in the clinical research setting. More data needs to be analyzed before a final conclusion is made.

INTRODUCTION

Life Technologies is collaborating with an international consortium for the development of lung fusion panel. The OncoNetwork (see figure below) is comprised of twelve-translational cancer research institutes with many years of experience in adopting the latest molecular techniques like next generation sequencing (NGS)- for lung research. Life technologies and OncoNetwork consortium agreed to work together for the development of a lung fusion panel based on Ion AmpliSeq™ RNA chemistry.

MATERIALS AND METHODS

Human lung adenocarcinoma cell lines H2228, HCC78 and LC-2/ad RNA was isolated using Invitrogen™ TRIzol® reagent. Ambion® FirstChoice® Human Brain Reference (HBR) RNA was used as a negative control for the study. Formalin fixed paraffin embedded (FFPE) tissue was isolated using Ambion® RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE . 10 ng of RNA for each samples were then processed using the Ion AmpliSeq™ RNA Library Kit and the Ion AmpliSeq™ RNA Lung Fusion Panel . Libraries were quantified using the Agilent® Bioanalyzer® instrument on a High Sensitivity DNA chip and prepared for sequencing on the Ion OneTouch™ System with 200 Template Kit v2. Molecular barcodes were added during library preparation and 6-8 samples per Ion 318™ chip were sequenced on the Ion PGM™ using the Ion PGM™ 200 Sequencing Kit. After sequencing, data was analyzed to give the number of counts per amplicon in the Ion AmpliSeq™ RNA Lung Fusion Panel

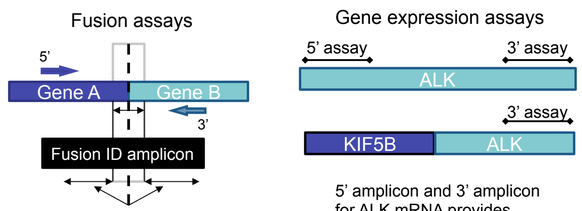
RESULTS

Table 1. Variants for ALK, ROS1, RET on Ion AmpliSeq™ RNA Lung Fusion Panel

FUSION TRANSCRIPT	COSMIC ID	5' GENE	3' GENE
EML4-ALK fusion 1	*	EML4	ALK
EML4-ALK fusion 2	COSF409	EML4	ALK
EML4-ALK fusion 3	*	EML4	ALK
EML4-ALK fusion 4	*	EML4	ALK
EML4-ALK fusion 5	COSF477	EML4	ALK
EML4-ALK fusion 6	COSF478	EML4	ALK
EML4-ALK fusion 7	COSF479	EML4	ALK
EML4-ALK fusion 8	COSF1062	EML4	ALK
EML4-ALK fusion 9	COSF1064	EML4	ALK
EML4-ALK fusion 10	COSF413	EML4	ALK
EML4-ALK fusion 11	COSF487	EML4	ALK
EML4-ALK fusion 12	COSF1296	EML4	ALK
EML4-ALK fusion 13	COSF1366	EML4	ALK
Kif5b-ALK fusion 1	COSF1257	KIF5B	ALK
Kif5b-ALK fusion 2	COSF1058	KIF5B	ALK
Kif5b-ALK fusion 3	COSF1060	KIF5B	ALK
KCL1-ALK fusion 1	COSF1276	KCL1	ALK
EZR-ROS1 fusion 1	COSF1267	EZR	ROS1
GOPC-ROS1 fusion 1	COSF1139	GOPC	ROS1
GOPC-ROS1 fusion 2	COSF1188	GOPC	ROS1
LRIG3-ROS1 fusion 1	COSF1269	LRIG3	ROS1
SCD4-ROS1 fusion 1	COSF1265	SDC4	ROS1
SCD4-ROS1 fusion 2	COSF1280	SDC4	ROS1
SCD4-ROS1 fusion 3	COSF1278	SDC4	ROS1
SCD4-ROS1 fusion 4	*	SDC4	ROS1
SCL34A2-ROS1 fusion 1	COSF1198	SLC34A2	ROS1
SCL34A2-ROS1 fusion 2	COSF1261	SLC34A2	ROS1
TPM3-ROS1 fusion 1	COSF1273	TPM3	ROS1
CD74-ROS1 fusion 1	COSF1202	CD74	ROS1
CD74-ROS1 fusion 2	COSF1200	CD74	ROS1
KIF5B-RET fusion 1	COSF1232	KIF5B	RET
KIF5B-RET fusion 2	COSF1253	KIF5B	RET
KIF5B-RET fusion 3	COSF1234	KIF5B	RET
KIF5B-RET fusion 4	COSF1255	KIF5B	RET
KIF5B-RET fusion 5	COSF1231	KIF5B	RET
KIF5B-RET fusion 6	COSF1262	KIF5B	RET
KIF5B-RET fusion 7	COSF1236	KIF5B	RET
CCDC6-RET fusion 1	COSF1271	CCDC6	RET

All fusion transcripts on this chart were included in the V2 design panel. Nucleotide sequence data was obtained from the COSMIC database, hosted by Sanger Institute, NCBI GenBank®k entries and public literature.

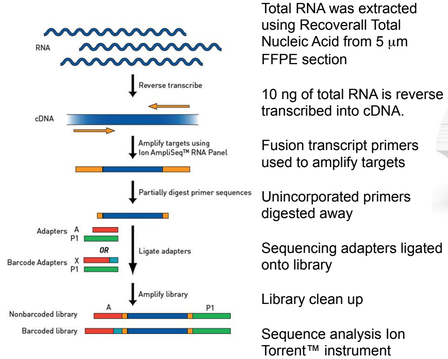
Figure 1. Ion AmpliSeq™ RNA lung fusion panel primer design strategy



Fusion specific amplicon provides nucleotide sequence through the junction of the two fusion genes

5' amplicon and 3' amplicon for ALK mRNA provides additional information on expression levels. Equivalent levels will be observed in normal samples, 3' bias will be present in samples with ALK translocation

Figure 2. Ion AmpliSeq™ RNA workflow for detection of fusion transcripts



Total RNA was extracted using Recoverall Total Nucleic Acid from 5 µm FFPE section

10 ng of total RNA is reverse transcribed into cDNA.

Fusion transcript primers used to amplify targets

Unincorporated primers digested away

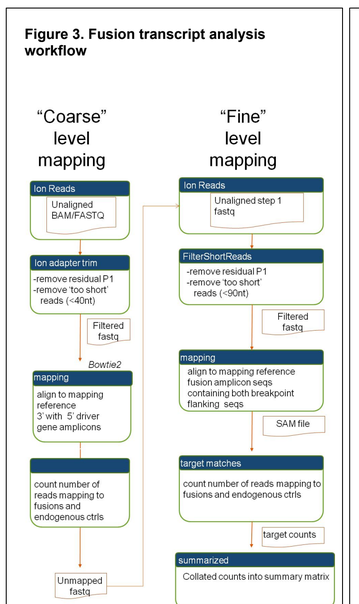
Sequencing adapters ligated onto library

Library clean up

Sequence analysis Ion Torrent™ instrument

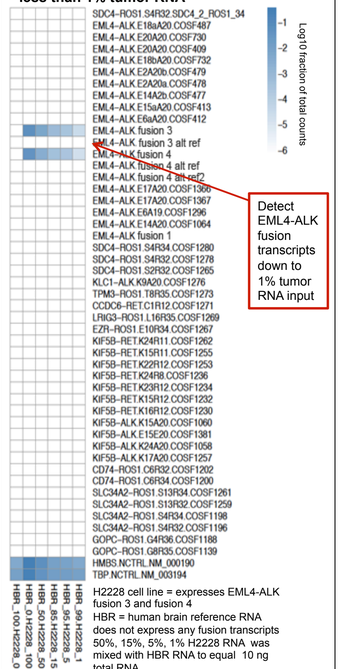
Fusion transcript AmpliSeq™ RNA libraries were sequenced on Ion PGM™ system and Ion 318™ chip

Figure 3. Fusion transcript analysis workflow



Sequence analysis pipeline that was used to map sequencing information from AmpliSeq RNA fusion panel. R statistical software package was used to translate the log10 fraction of total counts into a heat map as shown in Figure 4.

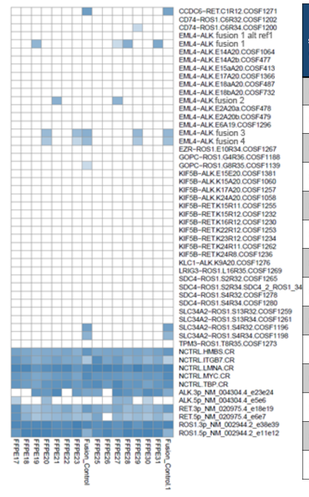
Figure 4. Detection of fusion transcript in less than 1% tumor RNA



Detect EML4-ALK fusion transcripts down to 1% tumor RNA input

H2228 cell line = expresses EML4-ALK fusion 3 and fusion 4
HBR = human brain reference RNA does not express any fusion transcripts 50%, 15%, 5%, 1% H2228 RNA was mixed with HBR RNA to equal 10 ng total RNA

Figure 4. Onconetwork verification of an Ion AmpliSeq™ RNA Lung Fusion Panel



Lung sample (FFPE)	FISH Results % positive	Ion AmpliSeq™ RNA OncoNetwork Panel results	3':5' ALK assays ratio	RT-PCR Results
FFPE17	Negative	Negative	ALK<10	Negative
FFPE18	Negative	Negative	ALK<10	Negative
FFPE19	Positive 36%	E13A20, AB462411	> 36000 fold ALK	V1 (E13A20)
FFPE20	Positive >15%	E6bA20, E6aA20	> 25 fold ALK	V3 (E6aA20 and E6bA20
FFPE21	Positive 30%	E20A20	> 7200 fold ALK	V2 (E20A20)
FFPE22	Negative	Negative	ALK<10	Negative
FFPE23	Positive 80%	E6bA20, E6aA20	> 13300 fold ALK	Negative
FFPE25	Negative 5%	Negative	ALK<10	Negative
FFPE26	Negative 3%	Negative	ALK<10	Negative
FPE27	Positive >15%	E20A20; E13A20	> 45 fold ALK	V (E20A20)
FFPE28	Positive >15%	E13A20	> 17 fold ALK	V1 (E13A20)
FFPE29	Positive >15%	E6bA20; E6aA20	> 3228 fold ALK	V3 (E6aA20 and E6bA20
FFPE30	Positive >15%	Negative	> 860 fold ALK	Negative
FFPE31	Positive >15%	E13A20	> 393 fold ALK	V1 (E13A20)

Table 2. Onconetwork verification of Ion AmpliSeq™ RNA Lung Fusion Panel

Ion AmpliSeq™ RNA Lung Fusion Panel (V2) verification						
	Concordance with FISH			Concordance with all methods		
Test Site	# FFPE samples correctly called by the Ion AmpliSeq™ RNA Lung Fusion Panel	# of samples tested by FISH	% Concordance with FISH	# FFPE samples correctly called by the Ion AmpliSeq™ RNA Lung Fusion Panel	# of samples tested with FISH,IHC,RT,NGS	% Concordance with all methods
IGR	5	6	80,00%	11	12	91,67%
INSERM	14	14	100,00%	14	14	100,00%
Kinki	4	4	100,00%	4	4	100,00%
ARUP	5	5	100,00%	8	8	100,00%
TOTAL	28	29	96,55%	37	38	97,37%

Samples procurement: FISH, IHC, qRT-PCR or NGS were performed by the indicated Onconetwork consortium site. A total of 38 samples were externally verified using the Ion AmpliSeq™ RNA Lung Fusion Panel . We observed 96,55% concordance with FISH and 97,37 considering all the methods and the panel including the 5'3' ALK gene expression assay ratio assays . There were no false positives observed in this dataset. The samples that were called negative by Ion AmpliSeq™ RNA Lung Fusion Panel were from samples estimated to be ~15% + by FISH. Raw data supplied to Thermo Fisher Scientific for analysis as outlined in Fig. 3.

CONCLUSIONS

FISH is time and cost consuming and not suitable for multiple translocation analysis. We developed a research test that enables simultaneous detection of multiple variants of ALK, RET or ROS1 fusion transcripts in single panel: Ion AmpliSeq™ RNA lung fusion panel

The Ion AmpliSeq™ RNA lung fusion panel shows:
- Robust performance from all 5 RNA quality control assays from low total RNA input (10 ng) and from degraded RNA isolated from formalin fixed paraffin embedded tissue (RIN < 3; data not shown)
- High sensitivity: fusion transcripts can be detected in less than 1% tumor RNA in the presence of 99% normal RNA
- Good concordance with other methods: >97,37 % concordance.

These preliminary data demonstrate that the panel may be used in the clinical research setting to better classify lung and colon tumors at the molecular level. An updated version of the panel including more variants and NTRK1 translocation is under development

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