Hotspot mutation and fusion transcript detection from the same non-small lung adenocarcinoma sample

Angie Cheng[1], Varun Bagai[1], Joey Cienfuegos[1], Natalie Hernandez[1], Mu Li[1], Jeff Schageman[1], Richard Fekete[1], Rosella Petraroli[2], Alexander Vlassov[1], and Susan Magdaleno[1]

[1]Life Technologies, 2130 Woodward St. Ste 200 Austin, TX, USA, 78739 [2]Life Technologies, Italy

ABSTRACT

The presence of certain chromosomal rearrangements and the subsequent fusion gene derived from translocations has been implicated in a number of cancers. Hundreds of translocations have been described in the literature recently but the need to efficiently detect and further characterize these chromosomal translocations is growing exponentially. The two main methods to identify and monitor translocations, fluorescent *in situ* hybridization (FISH) and comparative genomic hybridization (CGH) are challenging, labor intensive, the information obtained is limited, and sensitivity is rather low. Common sample types for these analyses are biopsies or small tumors, which are very limited in material making the downstream measurement of more than one analyte rather difficult; obtaining another biopsy, using a different section or splitting the sample can raise issues of tumor heterogeneity. The ability to study mutation status as well as measuring fusion transcript expression from the same sample is powerful because you're maximizing the information obtained from a single precious sample and eliminating any sample to sample variation. Here we describe the efficient isolation of two valuable analytes, RNA and DNA, from the same starting sample without splitting, followed by versatile and informative downstream analysis. This methodology has been applied to FFPE and degraded samples as well as fresh tissues, cells and blood. DNA and RNA were recovered from the same non-small lung adenocarcinoma sample and both mutation analysis, as well as fusion transcript detection was performed using the Ion Torrent PGM™ platform on the same Ion 318™ chip. Using 10ng of DNA and 10ng of RNA input, we applied the Ion AmpliSeq™ Colon and Lung Cancer panel to analyze over 500 COSMIC mutations in 22 genes and the Ion AmpliSeq™ RNA Lung Fusion panel to detect 40 different fusion transcripts.

MATERIALS AND METHODS

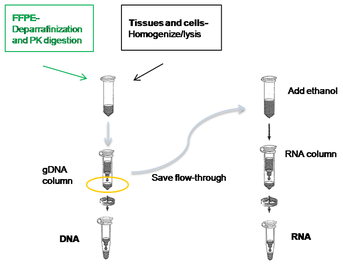
FFPE samples were purchased from Asterand. One 10um section for each sample was deparrafinized with xylene and protease digested. DNA and RNA were then isolated from the same sample. The obtained nucleic acid were quantified with the NanoDrop 8000. In addition to the nanodrop, RNA was also assessed using the Agilent 2100 Bioanalyzer instrument. A pico chip was ran to obtain RNA integrity information. As expected, samples were degraded with RIN values of <2.3. Yield varied between the different samples but for DNA it ranged between 800ng to 5ug from one 10um section and for RNA it ranged between 300ng to 2ug from one 10um section.

Real-time PCR was performed using the isolated DNA and RNA with plus and minus RT (reverse transcriptase) controls to assess cross nucleic acid contamination in each fraction by calculating a dCt (minus RT-plus RT). For DNA, a low dCt is expected and a high dCt for RNA is expected. 30ng of total RNA was reverse transcribed in 20ul using the SuperScript® VILO™cDNA synthesis kit for gene expression and the TaqMan® MicroRNA Reverse Transcription kit was used for miRNA expression. 2ul of the cDNA was used in a 10ul qPCR reaction using the TaqMan® Universal Master Mix II, with no UNG. For gene expression, the ACTB TaqMan® Gene Expression assay ID Hs03023880_g1 was used. Both primers and probe map within a single exon producing a 139bp amplicon. For miRNA expression, TaqMan® MicroRNA assays for let7e and mir24 were used. Reactions were run on the 7900HT real time PCR machine under standard cycling conditions.

DNA and RNA Ampliseq libraries were made with 10ng starting input according to the protocol for library prep. DNA libraries used the Ion AmpliSeq™ Colon and Lung Cancer panel which contain 90 amplicons so targets were amplified with 22 cycles. RNA libraries were made with the Ion AmpliSeq™ RNA Lung Fusion panel and targets were amplified with 24 cycles. Libraries were barcoded using the Ion Xpress™ Barcode Adapters. Final libraries were quantified by qPCR using the Ion Library Quantitation kit. Samples were templated using the Ion Torrent's One Touch™ System with the Ion One Touch™ 200 Template Kit v2. Standard input amounts were used for templating as recommended for Ampliseq libraries. Samples were then sequenced on the Ion PGM™ using the Ion PGM™ 200 Sequencing Kit on a 318 chip. DNA and RNA libraries were individually sequenced or pooled together to be sequenced on the same 318 chip.

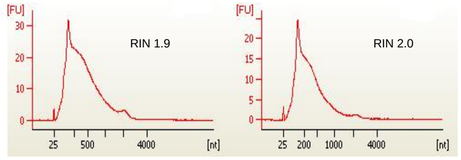
RESULTS

Figure 1a. DNA and RNA Isolation Workflow



DNA and RNA were isolated from the same FFPE lung patient samples-single 10uM section. The respective nucleic acid was quantified with the NanoDrop 8000 UV-Vis spectrophotometer. Yield varied depending on the section but on average for DNA 800ng-5ug and for RNA 300ng-2ug.

Figure 1b. Agilent 2100 Bioanalyzer Traces

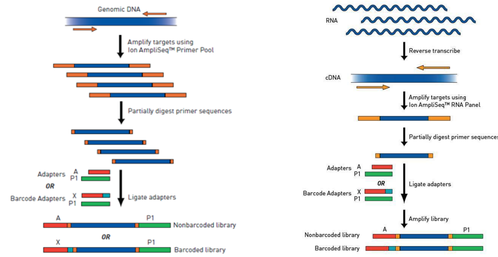


In addition to measuring RNA concentration with the nanodrop, RNA integrity was assessed with the Agilent 2100 Bioanalyzer using a pico chip.

Figure 2. qPCR-Gene Expression and miRNA Expression

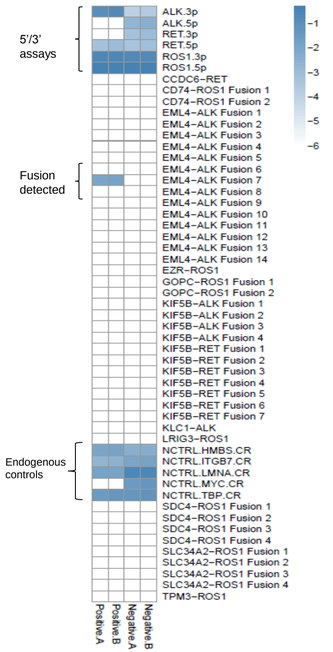
qPCR was performed on the isolated DNA and RNA with plus and minus RT controls to look at cross nucleic acid contamination. For gene expression, we looked at beta-actin using assay ID Hs03023880_g1. Both primers and probe map within a single exon which gives an amplicon size of 139bp. A dCt was calculated to assess the amount of cross contamination. The DNA fraction had a small dCt, close to zero, indicating it had very little RNA contamination whereas the RNA fraction had a big dCt, 13-16, indicating it had very little DNA contamination as well. miRNA expression was also evaluated for let7e and mir24.

Figure 3. Ion AmpliSeq™ DNA and RNA for targeted sequencing



RNA was reverse transcribed into cDNA. After reverse transcription, the DNA and RNA protocols were unified. The DNA and cDNA were amplified with the Colon and Lung cancer primer panel or the OncoNetwork Lung RNA Fusion Transcript panel (respectively). Primers were partially digested and adapters ligated onto the amplicons. After libraries were purified, they were sequenced on the PGM.

Figure 4. Detection of a fusion transcript in a FFPE patient sample



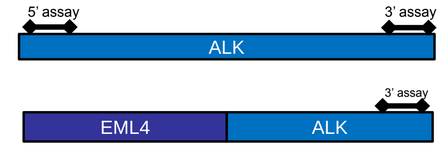
DNA and RNA AmpliSeq libraries were sequenced on Ion 318 chips. (Heat map here was condensed to only show a subset of the FFPE samples we screened). We set a detection threshold of at least 10 counts.

Heat map shows one fusion negative patient and one fusion positive patient either:
A-sequenced together (DNA and RNA) on the same chip or **B**-RNA libraries sequenced individually on a separate chip.

From 10ng of total RNA, we detected an EML4-ALK fusion in one of the patient samples. Approximately 1% of the reads were allocated to the fusion in both positive sample A and B.

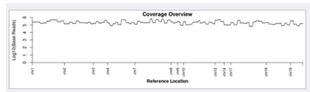
The positive sample contains a high 5'/3' ALK ratio which corresponds with the EML4-ALK fusion detected (see next figure).

Figure 5. Differential expression of 5' and 3' ends of ALK, RET and ROS1



Included in the fusion panel are primers to detect a differential expression between 5' and 3' ends of ALK, RET or ROS1. These assays could potentially be used as additional controls to detect a translocation presence. We have data demonstrating at least for ALK that this is feasible. Equivalent levels were observed in normal samples but a 3' bias were present in samples with ALK translocations such as the fusion positive sample highlighted in the heat map.

Figure 6. DNA Coverage Analysis and Variant Caller



Number of mapped reads	1,399,451
Percent reads on target	95.95%
Average base coverage depth	13,907
Uniformity of base coverage	99.97%

Amplicon Read Coverage		Target Base Coverage	
Number of amplicons	90	Bases in target regions	10,031
Percent assigned amplicon reads	95.95%	Percent base reads on target	90.59%
Average reads per amplicon	14,920	Average base coverage depth	13,907
Uniformity of amplicon coverage	100.00%	Uniformity of base coverage	99.97%
Amplicons with at least 1 read	100.00%	Target base coverage at 1x	100.00%
Amplicons with at least 20 reads	100.00%	Target base coverage at 20x	100.00%
Amplicons with at least 100 reads	100.00%	Target base coverage at 100x	100.00%
Amplicons with at least 500 reads	100.00%	Target base coverage at 500x	100.00%
Amplicons with no strand bias	100.00%	Target bases with no strand bias	97.86%
Amplicons reading end-to-end	94.44%		

Here is an example of one of the sample's results-DNA sequencing results were analyzed using the Coverage Analysis plug in. We achieved over 99% uniformity. In addition to the plug in, we also ran the Variant Caller using customized parameters to detect low frequency variants. The algorithm identified 10 unique variants in the fusion negative patient versus 40 unique variants in the fusion positive patient.

CONCLUSIONS

We have developed a protocol for the isolation of DNA and RNA from the same sample in both fixed FFPE degraded samples and unfixed samples such as tissues, cells and blood. We have been able to maximize the information we obtained from a single precious sample. We achieve high yield and purity for the nucleic acids recovered which are robust in various assays such as qPCR and sequencing without sacrificing one analyte over another. Here we demonstrated with one application the utility of having DNA and RNA from the same sample; one can analyze the DNA for mutation detection and from RNA fusion transcript detection from a single precious sample. We utilized two panels in collaboration with the OncoNetwork Consortia. The fusion panel enables the detection of multiple variants of ALK, RET or ROS1 fusion transcripts in a single tube starting from 10ng RNA extracted from FFPE samples and the DNA panel contain primers to analyze hotspot and targeted regions of key genes implicated in lung and colon cancers.