WHOLE TRANSCRIPTOME RNA-SEQ WITH ION TORRENT PLATFORM: FFPE, FRESH LCM AND FFPE LCM SAMPLES. INCREASINGLY DIFFICULT

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INTRODUCTION

Next generation sequencing (NGS) technologies revolutionized cancer research by making it possible to study the complexity of cancer using high throughput deep sequencing methodologies. Formalin-fixed, paraffin-embedded (FFPE) tissue samples stored in diagnostic pathology archives represent an invaluable biobank for retrospective clinical research. Laser capture microdissection (LCM) allows to capture a population of homogeneous target cells from a heterogeneous tissue section. The method thus significantly improves the accuracy of the cell-specific molecular profiling, as it eliminates noise derived from cell contamination.

The development of reliable NGS-based methods for use with low-quality FFPE tissue-derived nucleic acids (such as those derived from LCM FFPE tissues) would open the diagnostic pathology archives to high-throughput profiling, facilitating extensive retrospective clinical studies. In particular, RNA-seq analisys allows to evaluate the whole transcriptome sequence for each gene; this is extremely important in FFPE tissue gene expression analysis which could lead to a lack of information using conventional techniques (e.g. Real-Time PCR) due to the non uniformity of the sequence.

In our laboratory, we developed some different methods that would allow us to work optimally with very low amount of material, based on the technique of the SMARTer technology and on the Ion Torrent protocols that we modified carefully. We analyzed the whole transcriptome of different samples: FFPE, fresh and FFPE LCM samples on the Ion Proton Sequencer System (Life Technologies, Grand Island, NY).

MATERIALS AND METHODS

Samples: 12 FFPE tissue (brain tumor); 20 LCM FFPE samples of breast cancer (from a maximum of 700 cells to a minimum of 100 cells) and 13 LCM fresh frozen samples from mouse brain cells (up to 20 cells).

Laser capture microdissection: Sections 2 μm thick. The PALM RoboMover automatic laser microdissector (Carl Zeiss) was used.

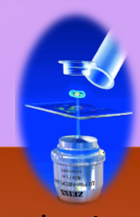
RNA extraction: We used the automated system Maxwell 16 (Promega).

LIBRARY PREPARATION

FFPE SAMPLES

- Ribosomal Rna removal with RiboGone Mammalian kit (Clontech Laboratories)
- cDNA Synthesis and amplification with SMARTer Universal Low Input RNA kit (Clontech Laboratories)
- No fragmentation step because they are FFPE so degraded
- Adapters ligation with Ion TargetSeq Exome Enrichment kit (Life Technologies)
- Size selection with E-Gel SizeSelect 2% Agarose Gel (Life Technologies)
- Amplification with Platinum PCR SuperMix High Fidelity (Life Technologies) **22 cycles**

NO LCM



FRESH and FFPE LCM SAMPLES

- No ribosomal RNA removal for the insufficient amount material
- cDNA Synthesis and amplification with SMARTer Universal Low Input RNA kit (Clontech Laboratories)
- Fragmentation step (only for fresh tissue) with Ion Shear Plus Reagents (Life Technologies)
- Adapters ligation with Ion TargetSeq Exome Enrichment kit (Life Technologies)
- Size selection with E-Gel SizeSelect 2% Agarose Gel (Life Technologies)
- Amplification with Platinum PCR SuperMix High Fidelity (Life Technologies) **22 cycles**

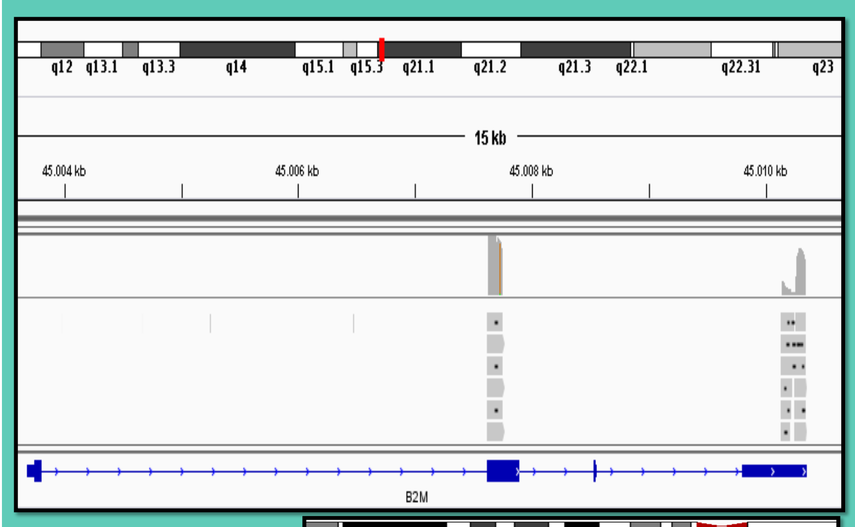
LCM

Template Preparation and Sequencing: **Ion OneTouch 2 and ES Instruments were used to prepare enriched Ion PI Ion Sphere Particles (ISPs) and loaded in the chip and placed on the Ion Torrent Sequencer; *Bioinformatics analysis*: we used several command line software included in Bio-Linux a custom version of Ubuntu 12.04 LTS.**

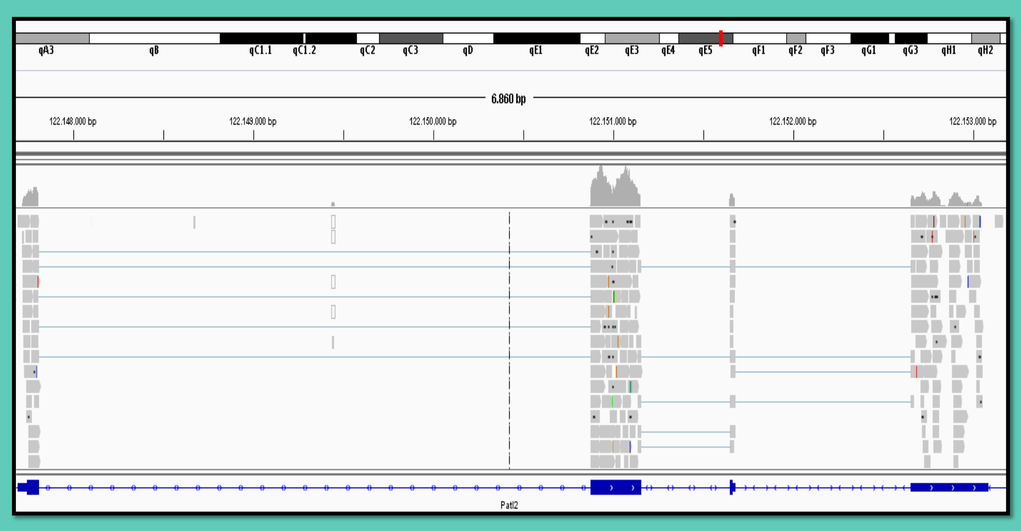
RESULTS AND DISCUSSION

We developed high performance method to analyze the whole transcriptome of our FFPE samples, obtaining a very high number of reads (78,186,377 usable reads) perfectly comparable with samples with a large amount of RNA such as samples obtained from cells or fresh tissues. Unfortunately for FFPE tissues the results that we obtained with commercial kit (Ion Total RNA-Seq, Life Technologies) following exactly their protocol, weren't optimal like those obtained with other types of samples (fresh, frozen…). In fact we achieved a lot of reads but we had a non-uniformity of the sequence coverage. Figures A and B show the sequence coverage of the reads in RNA-seq experiments comparing FFPE and fresh frozen tissue respectively. Exons (in blue) are partially covered by the reads in FFPE tissue (with old protocol). Instead with our modified protocol, the coverage become exactly comparable to samples of good quality (figure C). In figure D is shown the mapping quality comparing old and new method.

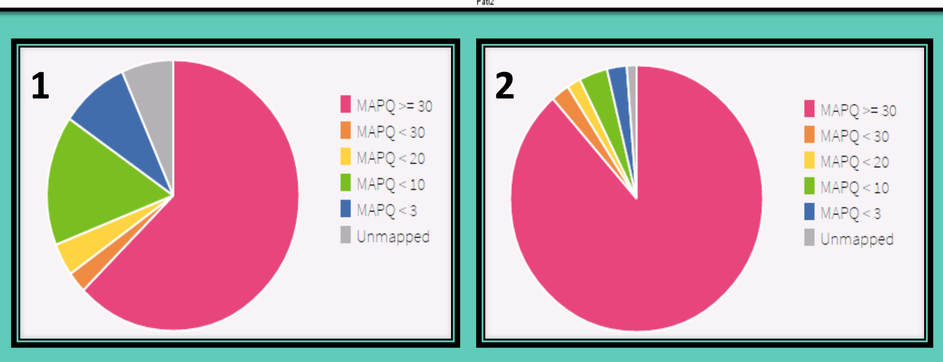
A. FFPE tissue with old method



B. Fresh frozen tissue



C. FFPE LCM tissue with our protocol



D. Mapping quality: 1) FFPE tissue old method;2) FFPE tissue with our protocol

FUTURE PERSPECTIVES

The combination of SMARTer Universal Low Input RNA kit and Ion TargetSeq Exome Enrichment kit, in addition to some improvements to their conventional protocols, provides to obtain excellent results and so analyze the whole transcriptome of different types of challenging samples (FFPE, LMC..). Eventually, but not less important, we stress that this application has never been done with the Ion Torrent platform.