SOLiD™ System Approaches for Small RNA Expression Profiling of Formalin-fixed, Paraffin-embedded (FFPE) Tissue Samples

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ABSTRACT

Archived formalin-fixed paraffin-embedded (FFPE) specimens represent excellent resources for biomarker discovery, but it has been a major challenge to study gene expression in these samples due to mRNA degradation and modification during fixation and processing. MicroRNAs (miRNAs) regulate gene expression at the post-transcriptional level and are considered important regulators of cancer progression. Next generation sequencing technologies such as the SOLiD™ System provide an ideal method for measuring the abundance of miRNA molecules in different cancer stages and provide insightful information on tumorigenesis. However, there is currently no available method to systematically study miRNA expression in FFPE samples on next generation sequencing platforms.

We have designed and developed a ligation-based miRNA detection method to capture small RNA sequences in FFPE samples and convert them into templates suitable for sequencing on the SOLiD™ System. Total RNA was isolated from matched cancer or normal adjacent FFPE and fresh frozen tissues using the Ambion RecoverAll™ kit. Enriched small RNA from these samples was used for library preparation, followed by sequencing on the SOLiD™ system. Our results show that small RNA extracted from FFPE samples was successfully converted to small RNA libraries. The expression profiles from FFPE and fresh frozen samples were in good correlation, suggesting that miRNA molecules are less affected by sample degradation and RNA-protein crosslinking. This study provides a foundation for miRNA expression analysis on the SOLiD™ system using FFPE samples in cancer and other diseases.

INTRODUCTION

FFPE samples, when associated with clinical records, provide invaluable information on clinically useful biomarkers. Despite significant efforts for developing techniques to identify biomarkers from FFPE samples, little progress has been made due to poor quality of extracted RNA from this type of sample. RNA from FFPE tissue is normally degraded to fewer than 300-nt in length, and fixing tissues with formalin leads to RNA-RNA and RNA-protein crosslinking. Although it is challenging to study mRNA expression in FFPE samples, a few studies have shown that processes of miRNA extraction, labeling, and microarray hybridization from FFPE samples are highly reproducible and add little variation to the results [1,2,3]. These results shed light on studying miRNA expression in FFPE samples using next generation sequencing systems (NGS) which are now increasingly being used for quantitative transcriptomics and identification of novel transcripts, and are considered as an alternative to microarrays.

MATERIALS AND METHODS

FFPE and fresh frozen tissue preparation- a lung tumor sample, identified as a moderately differentiated adenocarcinoma, a lung tumor normal adjacent sample from the same patient, and a breast cancer normal adjacent sample, were (a) fresh frozen and (b) formalin fixed and paraffin embedded into a block. For formalin fixation, samples were cut into 2mm thick pieces, fixed in 10% Neutral Buffered Formalin for 4 hours, processed on a Tissue-Tek® V.I.P tissue processor, and subsequently paraffin embedded.

RNA extraction- RNA was extracted from the fresh frozen samples using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) and from the FFPE tissue using RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion, Austin, TX) following the manufacturer's protocols. For FFPE samples, two 40 µm sections were used for extraction.

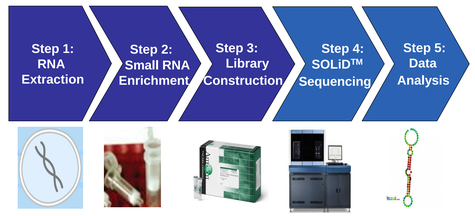
Small RNA enrichment- The Purelink miRNA purification kit (Invitrogen, Carlsbad, CA) was used to enrich small RNA from total RNA isolated from either fresh-frozen or FFPE tissue following a modified protocol in the SOLiD™ Total RNA-Seq Kit manual (Appendix B, Small RNA Enrichment). Enriched small RNA was checked on Agilent® Small RNA chip for size distribution and Nanodrop for quantitation.

Small RNA library preparation- 100-200 ng of enriched small RNA from FFPE samples and enriched small RNA from fresh-frozen samples containing ~25 ng miRNA were used in the SOLiD™ total RNA-Seq kit following manufacturer's instructions with the following exceptions: (a) 60-70 nt range was selected from 10% Novex® TBU gel instead of 60-80 nt in the standard protocol. (b) 18 cycles of PCR were used as default. Each sample type was done in duplicates and each library was barcoded differently. Final libraries were eluted in 10 µL Elution buffer and 1 µL was run on an Agilent DNA 1000 chip to evaluate library quality.

SOLiD™ sequencing and data analysis- SOLiD™ small RNA libraries were pooled and the standard SOLiD™ emPCR workflow was used to generate template-containing beads. Samples were run on SOLiD™ V3.5 and 6-18 million 35 bp reads were generated for each sample. Sequencing data was mapped using the SOLiD™ Small RNA analysis pipeline. miRBase (V13) coverage and Spearman Correlation on miRBase mapping were calculated and compared.

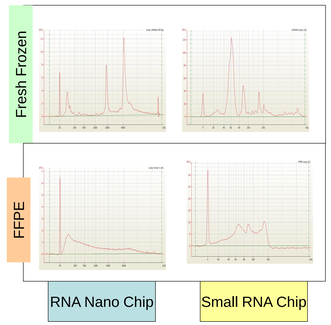
RESULTS

Figure 1. Workflow for FFPE miRNA analysis on SOLiD™



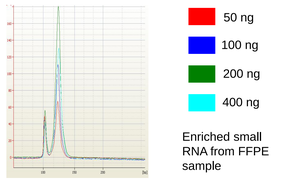
Basic workflow for FFPE miRNA analysis on SOLiD™
Step 1: Isolate FFPE total RNA using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion, Austin, TX).
Step 2: Enrich small RNA (<150 nts) using PureLink® miRNA isolation kit (Invitrogen, Carlsbad, CA), starting from 1-50 µg total RNA.
Step 3: Quantify enriched small RNA on Qubit® fluorometer (Invitrogen, Carlsbad, CA) and use 100-200 ng enriched RNA as input for SOLiD™ Total RNA-Seq Kit (Ambion, Austin, TX). Follow small RNA protocol using a gel size-select 60-70 nts cDNA and PCR amplify using 18 cycles.
Step 4: emPCR and SOLiD™ sequencing following standard workflow
Step 5: Data analysis

Figure 2. Bioanalyzer traces for FFPE and Fresh Frozen lung tumor samples



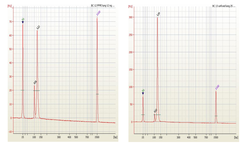
Fresh frozen and FFPE total RNA from lung tumor samples were diluted to ~100 ng/µL and analyzed using an Agilent® 2100 Bioanalyzer™ with both RNA nano and small RNA chips. RNA nano chip data showed that the RNA Integrity Number (RIN) was 9.6 for the fresh frozen lung and 1.6 for the paired FFPE sample, suggesting very poor RNA quality of the FFPE sample. The median size for FFPE sample is ~350 nts. Small RNA chip data confirmed that several small RNA species, such as tRNA and 5S rRNA, were degraded in FFPE sample.

Figure 3. Input titration for SOLiD™ Total RNA-Seq Kit



Bioanalyzer traces for SOLiD™ small RNA libraries starting from different input amounts are shown above. PureLink™ miRNA isolation kit-enriched small FFPE RNA was used for SOLiD™ total RNA-Seq ligation following a modified protocol. The data shows that highest yield is achieved with 200 ng input, indicating a saturation point between 200-400 ng input. Thus, enriched small RNA sample with100-200 ng input amount is recommended.

Figure 4. SOLiD™ small RNA Libraries

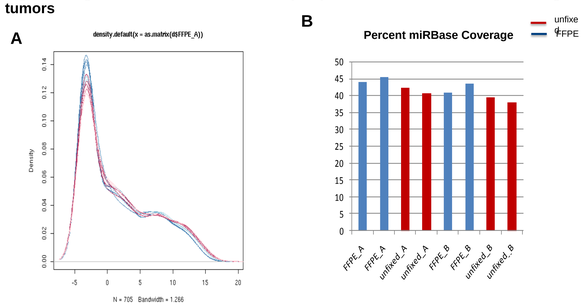


Bioanalyzer traces for SOLiD™ small RNA libraries from FFPE (left) and fresh frozen lung (right) samples. Peaks around 123-124 bp are considered as miRNA ligation products. Peaks around 101-103 bp are from adaptor self-ligation by-products. Libraries generated from FFPE samples show no differences compared to those from fresh frozen RNA.

Table 1. Sequencing reads

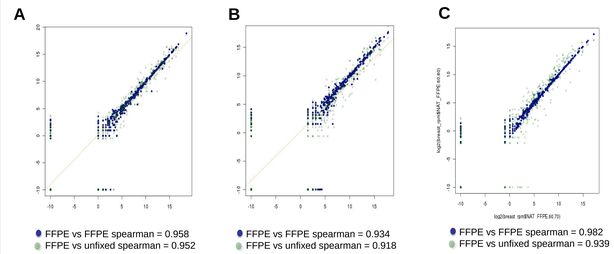
Sample	Total Reads	Map to miRBase	Map to Genome (exclude miRBase Mapped Reads)
Lung tumor FFPE	14,521,963	3,100,866	2,112,326
Fresh-Frozen Lung tumor	17,549,554	5,436,550	2,508,831
Lung tumor NAT FFPE	6,337,377	662,576	1,528,317
Fresh-Frozen Lung tumor NAT	9,538,451	2,805,842	2,209,212
Breast Cancer NAT FFPE	6,494,230	1,394,704	1,455,849
Fresh-Frozen Breast Cancer NAT	7,565,899	3,253,150	1,429,489

Figure 5. log2 RPM normalized miRBase (V13) distribution and detection in lung tumors



5A. Log(2) RPM normalized miRBase distribution in lung tumor samples. The density plot reveals that log2(RPM) distributions are largely the same between FFPE and fresh-frozen (unfixed) lung samples. 5B. Log(2) RPM normalized miRBase detection in lung tumor samples (RPM>0.3). There were no significant differences in detection of miRBase with coverage ranging from ~37%-46%.

Figure 7. log2 normalized pair-wise comparison between FFPE replicates and matched FFPE and fresh-frozen libraries



Pair-wise Spearman correlation comparison indicates that replicates show slightly less variability than FFPE vs. Fresh-frozen samples. A. lung adenocarcinoma tumor. B. lung adenocarcinoma normal adjacent. C. breast cancer normal adjacent.

CONCLUSIONS

- A SOLiD™ small RNA library preparation workflow from FFPE samples has been established. Libraries from FFPE tumor/normal adjacent tissues and their matched fresh-frozen samples have been successfully constructed and sequenced on SOLiD™ system.
- Although percentage miRBase mapping is less in FFPE sample, very similar miRBase distribution and detection coverage are observed in paired FFPE and fresh frozen samples. A good correlation of miRNA expression levels between FFPE and fresh frozen samples with Spearman Correlation > 0.9 is also observed.
- Although the RNA extracted from FFPE samples is often compromised, it is demonstrated that the robustness of miRNA profiling in FFPE material which could provide a source of study material for large scale or retrospective studies. This study has confirmed that miRNA species can be successfully extracted and analyzed from archived sources.

REFERENCES

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2. Peltier HJ, Latham GJ, RNA 2008, 14:844
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