A Survey of Tissue Specimen Collection Techniques: Impact on Biomarker Data Quality

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ABSTRACT

Prognostic and diagnostic biomarker development is currently hindered by access to high quality, clinically annotated biospecimens. To address this limiting factor for translational research, we established a state-of-the-art biorepository facility to procure clinically annotated biospecimens from remote network locations, and isolate high quality nucleic acids for long-term storage and molecular analysis. We describe here a long-term project comparing methods of sample collection and storage, including stabilization media, on the quality of RNA for genomics research. Tissues known for the presence of high and low RNase (kidney/liver and colon/lung, respectively) were collected as FFPE, fresh, frozen in Liquid Nitrogen (LN_2), frozen with stabilization media (RNA*later*®), and LN_2 frozen tissues post-treated with stabilization media (RNA*later*®-ICE). Total RNA isolated from all tissues was assessed for quality, and analyzed using expression arrays. At zero time point, no major differences in RNA quantity were observed due to collection and storage treatments. Further analysis of RNA quality with QC arrays revealed intact RNA free of inhibitors of reverse transcription and PCR amplification, genomic and general DNA contamination. Hierarchical clustering of Oncogene and Tumor Suppressor genes revealed three distinct groups; 1) Fresh, LN_2 and RNA*later*®-ICE, 2) RNA*later*® and 3) FFPE suggesting that RNA stabilization treatments may influence gene expression patterns. Currently, experiments are underway to assess the RNA quality on global gene expression patterns and establish a baseline, to be compared to the RNA quality and gene expression patterns at 6, 12 and 24 month post-collection.

INTRODUCTION

Catholic Health Initiatives (CHI), a non-profit, faith-based organization, which operates in 19 states and includes 73 community-hospitals, is committed to bringing cutting-edge research and most-effective treatment options to all its patients. To facilitate the advancement and integration of personalized medicine, CHI's Institute for Research & Innovation (CIRI) has established the Center for Translational Research (CTR).

Current biomarker (both prognostic and diagnostic) development strategies are hindered by access to high quality, clinically annotated biospecimens. To address this limiting factor, the CTR's state-of-the-art biorepository facility is tasked to procure clinically annotated biospecimens from remote network locations, and isolate high quality nucleic acids for long-term storage and/or downstream molecular analysis. Since CHI hospitals are spread across the United States of America (USA), many in remote or rural areas, implementing technologies to standardize sample collection and shipment, while maintaining their quality for downstream molecular analysis is crucial.

While a number of stabilization methods are currently available to preserve human tissues (Table 1), to date, there are no comprehensive studies comparing how the different stabilization media and different tissues may affect molecular profiles, particularly those of gene expression patterns[2-5]. With increased emphasis on discovering novel genes (biomarkers) for therapeutic decision choices, selecting technologies with zero to minimal effect on whole genome transcriptome is crucial.

Here, we describe a long-term project aimed at evaluating the impact of different sample collection and storage procedures on the quality of RNA and gene expression patterns.

Table 1: Current Collection and Stabilization Methodologies

Method	Pros	Cons
Fresh	High RNA quality and yields	Requires immediate extraction of tissue
Liquid Nitrogen (LN_2)	High RNA quality and yields	Requires pulverization of tissue and rapid homogenization to minimize RNA degradation
FFPE	Preserves tissue morphology, widely available with clinically annotated data	Crosslinking and chemical modification degrades and fragments nucleic acids, resulting in compromised quality of molecular data
RNA*later*®	Suitable for both short term and long term storage at different temperatures	Passive diffusion of aqueous sulfate salts requires small sample size. Yields denatured proteins only
RNA*later*®-ICE	Stabilizes RNA in tissues during the transition of tissue from frozen to a non-frozen state	Alcohol based solution requires overnight storage at -20˚ C prior to RNA isolation. Yields denatured proteins only
AllProtect Tissue Reagent	Stabilizes DNA, RNA, and protein for both short term and long term storage at different temperatures	Proteins are denatured thus, requiring separate tissue for the isolation of native proteins
PAXgene Tissue System	Stabilizes DNA , RNA and histomorphology for both short term and long term storage at different temperatures	Does not yield protein products

METHODS

SAMPLE TYPE AND STORAGE CONDITIONS

All tissues were collected following an institutional review board (IRB) approved collection protocol. Eighteen (18) single aliquots were dissected from each of the three different types of tissues (kidney, liver, and colon representative of high and low RNase activity) and processed as described in Figure 1.

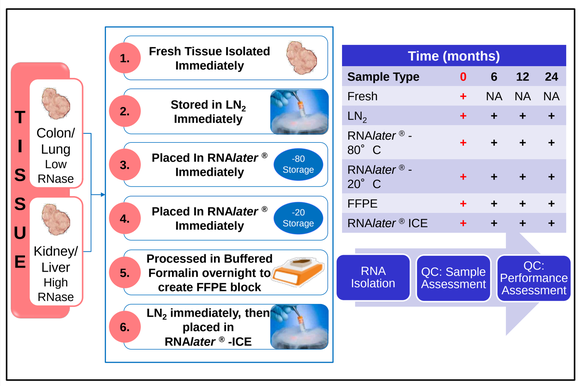

Figure 1: Schematic diagram of tissue collection and storage methods
For each sample, 4 sections each were placed into Liquid Nitrogen (LN_2), RNAlater® frozen at-20˚ C & at -80˚ C, and RNAlater®-ICE post LN_2 storage; one section each was immediately processed to total RNA isolation (fresh tumor) or processed to FFPE tissue.

METHODS (cont.)

RNA EXTRACTION AND QUALITY ASSESSMENT

Total RNA from all three tissue types was extracted with a Qiagen RNeasy® Mini Kit , while the total RNA from 10µ FFPE tissue was extracted with the Qiagen RNeasy® FFPE Kit following manufacturer's protocol. Total RNA yield and QC was assessed with Nanodrop 2000 (ThermoScientific, Wilmington, DE) and the Experion Bioanalyzer (BioRad, Hercules, CA), respectively. The presence of PCR inhibitors and the contamination of total RNA with non-transcribed genomic DNA and general DNA was assessed with SABiosciences RT² RNA QC PCR Arrays following manufacturers protocols. In addition, the RNA QC PCR arrays assess the integrity of total RNA by monitoring the expression levels of two housekeeping genes, ACTB (high expresser) and HPRT1 (low expresser).

GENE EXPRESSION ANALYSIS

The impact of stabilization media and storage conditions on gene expression profiles was assessed by processing total RNA with the Human Oncogenes & Tumor Suppressor Genes RT² Profiler™ PCR Array. In addition to the qPCR Array, whole genome transcriptome analysis will also be performed by using the Affymetrix U133 Plus2.0 chips at 0, 6, 12, and 24 month time points.

DATA ANALYSIS

Raw ct values were processed following manufacturer instructions (www.sabiosciences.com/pcrarraydataanalysis.php and pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) prior to normalizing data to house keeping genes by using the geNorm VBA applet (genomebiology.com.2002/3/7/research/0034). Hierarchical clustering and principle component analysis (PCA) were performed using Pearson correlation distance (Bioconductor package HTqPCR, version 1.4.0).

RESULTS

Our results, to date, indicated that RNA yields were much higher in fresh than all other treatments, with exception of colon. Reduced yield in Colon may be due to increased warm ischemic time associated with the surgical procedure (Figure 2A). No major differences were observed in the quality of total RNA due to different stabilization media, with the exception of FFPE samples (Figure 2B). We consider a RQI of 0-3 as Poor quality, 4-6 as Moderate quality, and 7-10 as Good quality.

Data obtained from RNA QC arrays revealed no detectable PCR inhibitors and DNA contamination suggesting that the RNA isolated is of high quality for downstream analysis as revealed by the differential expression patterns of low and high expresser genes (Figure 3).

To determine the overall similarity and differences within groups of data both features and samples (normalized) were subjected to principle components analysis and hierarchical clustering using Pearson correlation distance (Bioconductor package HTqPCR, version 1.4.0). Results of the gene and sample cluster are shown in Figure 4. Samples by and large clustered according to the tissue type and within tissue type into three groups: : 1) FFPE alone, 2) RNAlater® (-80˚ C and -20˚ C) and 3) fresh, LN_2, and RNAlater® – ICE, although FFPE samples branch within a given cluster. Results of PCA are shown in Figure 5. Similar to hierarchical clustering samples by and large group according to tissue type. Though preliminary, the results presented above indicate that tissue, not collection method is the largest source of variance in the data.

To identify the differentially expressed transcripts (DETs) relative to fresh tissue, Venn diagrams were created where the intersections of collection methods are compared across samples (Figure 6).

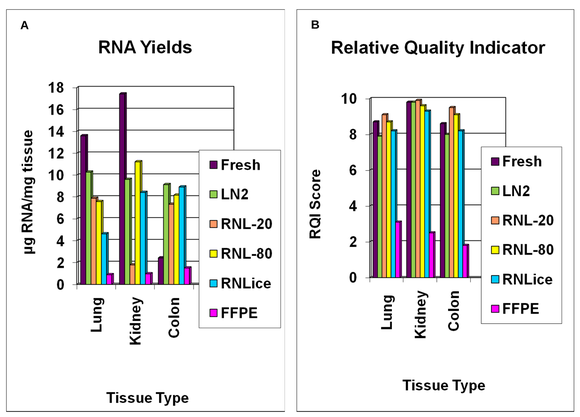

Figure 2: Total RNA isolation and quality for Lung, Kidney, and Colon samples

RESULTS (cont.)

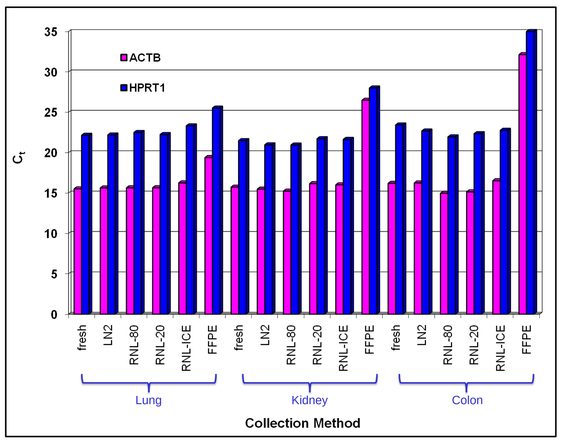

Figure 3: Housekeeping genes as an indicator of qPCR quality

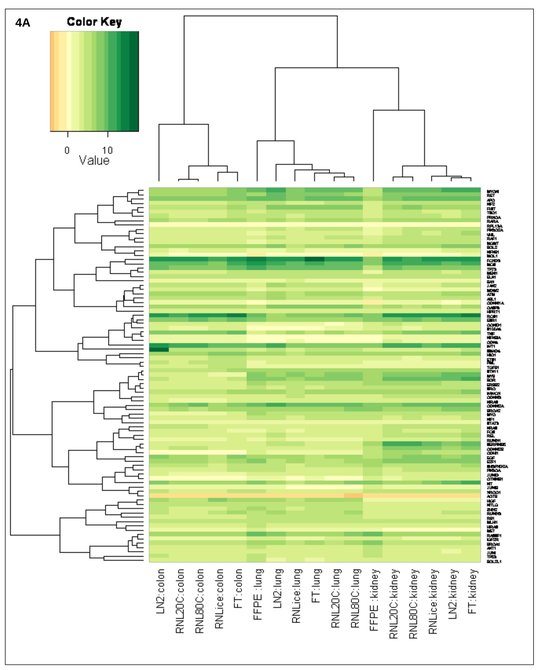

Figure 4A: Hierarchical clustering of genes and samples

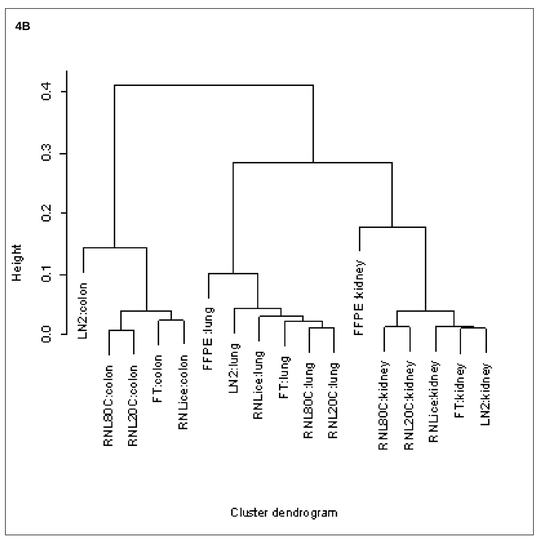

Figure 4B: Hierarchical clustering of Collection Methods and Samples

RESULTS (cont.)

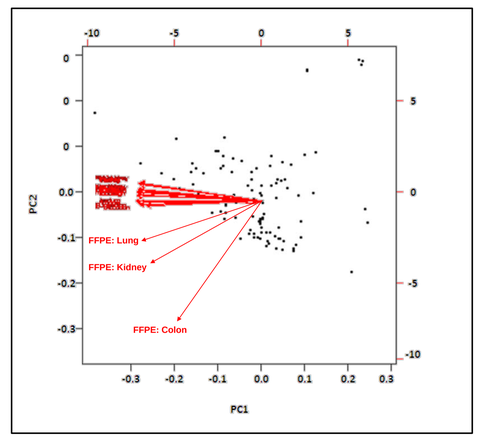

Figure 5: Principle Components Analysis (PCA)

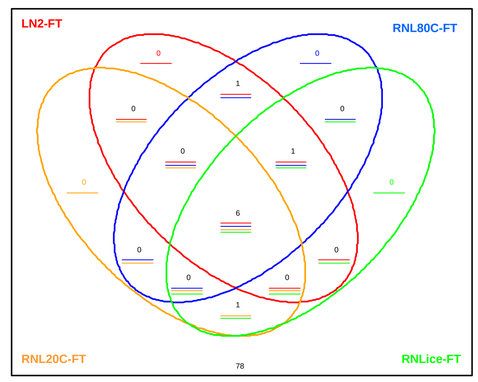

Figure 6: Venn diagram of DETs

ONGOING STUDIES

Our ongoing studies include the collection of additional samples to perform both targeted and whole genome transcriptome analysis to identify changes in gene expression profiles included by tissue stabilization media over a period of two (2) years. Other approaches include the evaluation of new tissue and nucleic acid stabilization media such as AllProtect and DNAGard on downstream molecular analysis. Completion of our studies is expected to illustrate the influence of stabilization media on gene expression patterns, and identify the most effective stabilization media thus, enabling the standardization of biorepository specimen collection and handling.

REFERENCES

1. National Cancer Institute's *Best Practices for Biospecimen Resources,* June 2007.
2. Mutter GL, Zahrieh D, Liu C, Neuberg D, Finkelstein D, Baker HE, Warrington JA. (2004). Comparison of frozen and RNALater solid tissue storage methods for use in RNA expression microarrays, BMC Genomics. 5(1):88.
3. Chowdary D, Lathrop J, Skelton J, Curtin K, Briggs T, Zhang Y, Yu J, Wang Y, Mazumder A. (2006). Prognostic gene expression signatures can be measured in tissues collected in RNAlater preservative, *Journal of Molecular Diagnostics*. 8(1):31-9.
4. Abdueva D, Wing M, Schaub B, Triche T, Davicioni E. (2010) Quantitative expression profiling in formalin-fixed paraffin-embedded samples by Affymetrix microarrays. *Journal of Molecular Diagnostics 12(4):409-17.*
5. Gaby Hoffmann, Jooske Ijzer, Bas Brinkhof, Baukje A Schotanus, Ted SGAM van den Ingh, Louis C Penning, and Jan Rothuizen. (2009). Comparison of different methods to obtain and store liver biopsies for molecular and histological research. *Comparative Hepatology* 8:3.
6. Qiagen protocols

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