ANALYSIS OF FUNDAMENTAL RNA QUALITY FACTORS IN ARCHIVAL FORMALIN FIXED AND PARAFFIN EMBEDDED TISSUE

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RESEACH BACKGROUND

The widespread adoption of transcriptional profiling of tumors has been limited by the inability to isolate high quality RNA from routine surgical specimens. Most research studies have relied on frozen tissue, which is not practical for most specimens in a routine clinical care environment. RNA can be isolated from formalin fixed, paraffin embedded (FFPE) tissue and utilized in RT-PCR based assays, however RNA quantity and quality are significant issues in assay development. FFPE tissue is anticipated to remain the specimen preservation means of choice for diagnostic histopathology, having been built on over a century fund of knowledge. Other approaches including candidate fixatives have been investigated, however none meet the demands of clinical practice as a economic universal fixative, nor do they provide the *reproducible artifact* of formalin fixation. Individual groups are working on expanding these applications, unfortunately little data exist comparing different fixation methodologies. Therefore, any data on the impact of tissue handling and processing on RNA quantity and quality is of interest to those developing clinical assays based on RNA analysis.

The isolation of RNA from FFPE tissue was first reported in 1988 by Rupp *et al*, but quality metrics to quantify the quality of RNA isolated from FFPE tissue are limited. The quality of RNA obtained from FFPE tissue is widely variable. Methods of isolation no doubt impact quantity of RNA recovered, however quality within FFPE specimens dependent on pre-analytical conditions including warm ischemia time, and fixation conditions. Manuscripts routinely were described fixation and processing "per standard protocol" when in fact there is a lack of standardization of fixation and tissue processing protocols. The process of tissue handling and processing from patient to paraffin block is too frequently hidden behind a curtain from researchers, further limiting the appreciation of the importance of standardization of tissue handling. Namely, there are three major steps (warm ischemia, fixation and tissue processing) in transition from patient to paraffin block. (i) Warm ischemia, referred to the time of transfer from an operation room to pathology laboratory, may vary from minutes to hours. In most instances specimens are held at room temperature, however in some cases, or if long delays are anticipated, they may be refrigerated. Some specimens, more commonly small biopsies, are directly placed in fixative and transported to pathology. (ii) Within the pathology laboratory, optimally the specimen is dissected and sectioned into appropriate size for fixation and processing with minimal delay. However, the length of fixation can vary greatly, largely depended on specimen size and time of day of receipt of the specimen. (iii) Finally, the specimen is placed on a tissue processor, in which the specimen is serially dehydrated in graded alcohols. The length of this process routinely varies from 4 to 12 hours, however protocols that are either shorter or longer are regularly encountered. Finally, the tissue is surrounded in a mold with hot paraffin, to form a paraffin block, which can be cut with a microtome. At this point, the specimen is a FFPE platform.

A few studies demonstrated that the over extended times of the fixation and embedding processing and storages of the embedded specimens are resulted in poor quality of RNA. However, the pre-analytical steps of tissue handling and processing have not been systematically studied for impact on the quality of RNA isolated. In this study, we dissect the warm ischemia time and its impact to RNA quality and quantity. In addition, we sought to define the points in the process of formalin fixation, and paraffin embedding that impacted the RNA quality.

Design of Study

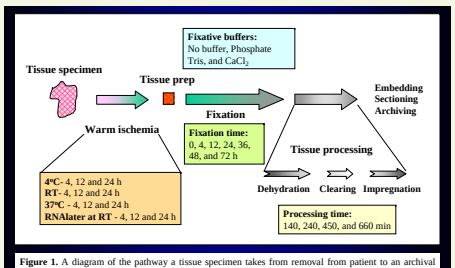

Figure 1. A diagram of the pathway a tissue specimen takes from removal from patient to an archival tissue block. After devitalization of the specimen, there is a variable length of time before the specimen is placed in fixative. During this period, called "warm ischemia" the tissue lacks oxygen and anoxic metabolic pathways take over. The specimen undergoes some form of preparation before fixation, depending on the size and complexity of the specimen. The specimen fixes, optimally in 10 volumes of fixative for a duration suitable for complete penetration of the fixative (1 mm/hr). After fixation, the specimen, the specimen undergoes "processing" on an automated instrument that serially replaces the fluids within a retort to accomplish the replacement of water with paraffin. This three step processes (dehydration, clearing and impregnation) is accomplished with a variable number of steps (12-14) with more steps devoted to dehydration than clearing or impregnation. Some steps may contain the same reagent, in an effort to complete the exchange of solvents. Most tissue processors operate under vacuum. After tissue processing, the specimen is "embedded" surrounded with paraffin that acts as a sold support for microtomy. Colored boxes were presented the conditions examined in this study.

Warm ischemia

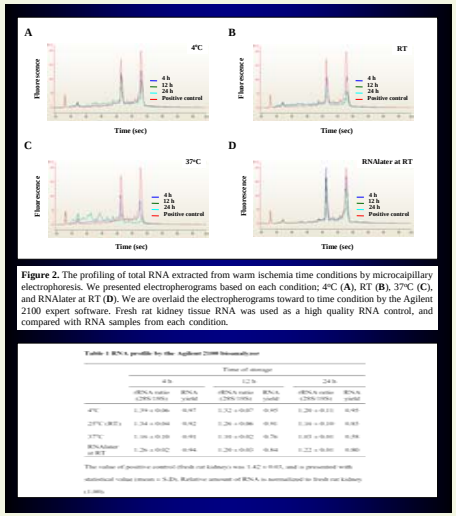

Figure 2. The profiling of total RNA extracted from warm ischemia time conditions by microcapillary electrophoresis. We presented electropherograms based on each condition; 4°C (**A**), RT (**B**), 37°C (**C**), and RNAlater at RT (**D**). We are overlaid the electropherograms toward to time condition by the Agilent 2100 expert software. Fresh rat kidney tissue RNA was used as a high quality RNA control, and compared with RNA samples from each condition.

Fixative buffers

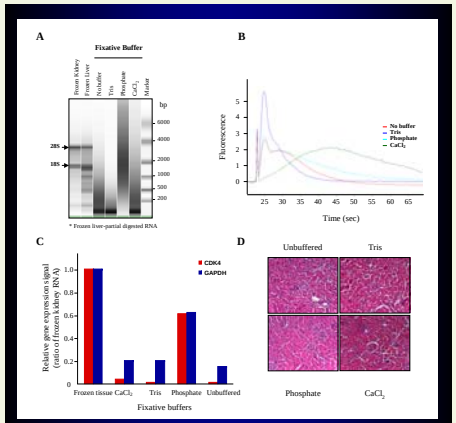

Figure 3. Evaluation of RNA quality and H & E staining depend on fixative buffers. Total RNA was extracted from mouse kidney. Tris-, phosphate- and $CaCl_2$-buffered formalin as well as formalin only fixed tissues coupled with paraffin-embedded processing. The total RNA samples (200 ng) were analyzed on an RNA LabChip using the Agilent 2100 bioanalyzer, and present a gel-like image (**A**) and an electropherogram (**B**). *CDK4* and *GAPDH* gene expressional profiles were measured with 500 and 200 ng of total RNA extracted from those samples, respectively. Relative expressional signal of both genes are normalized to that of frozen kidney tissue (**C**). Data are representative of three independent experiments, and are expressed as mean. Representative histology data were presented as a H & E image (**D**).

Fixation time

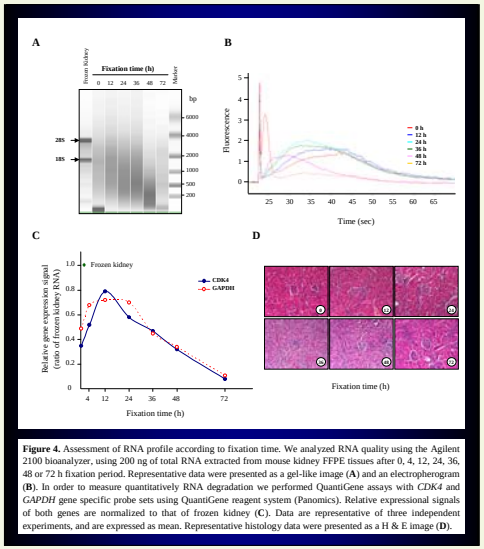

Figure 4. Assessment of RNA profile according to fixation time. We analyzed RNA quality using the Agilent 2100 bioanalyzer, using 200 ng of total RNA extracted from mouse kidney FFPE tissues after 0, 4, 12, 24, 36, 48 or 72 h fixation period. Representative data were presented as a gel-like image (**A**) and an electropherogram (**B**). In order to measure quantitatively RNA degradation we performed QuantiGene assays with *CDK4* and *GAPDH* gene specific probe sets using QuantiGene reagent system (Panomics). Relative expressional signals of both genes are normalized to that of frozen kidney (**C**). Data are representative of three independent experiments, and are expressed as mean. Representative histology data were presented as a H & E image (**D**).

Tissue processing time

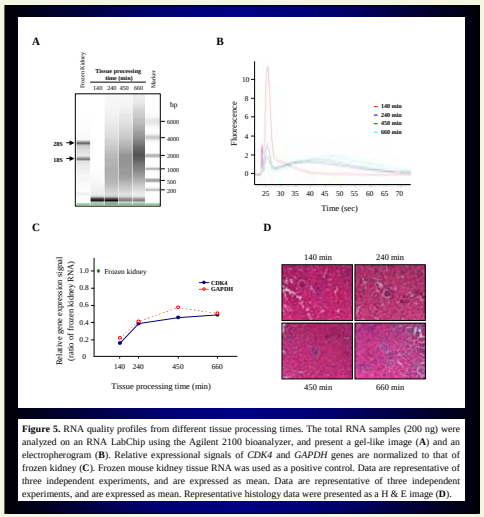

Figure 5. RNA quality profiles from different tissue processing times. The total RNA samples (200 ng) were analyzed on an RNA LabChip using the Agilent 2100 bioanalyzer, and present a gel-like image (**A**) and an electropherogram (**B**). Relative expressional signals of *CDK4* and *GAPDH* genes are normalized to that of frozen kidney (**C**). Frozen mouse kidney tissue RNA was used as a positive control. Data are representative of three independent experiments, and are expressed as mean. Data are representative of three independent experiments, and are expressed as mean. Representative histology data were presented as a H & E image (**D**).

Optimization of mRNA targeting

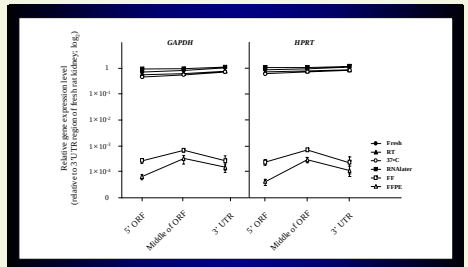

Figure 6. Gene-target optimization for amplification of *GAPDH* and *HPRT* mRNA from fresh, FF and FFPE tissue RNA isolates. Real-time quantitative RT-PCR is performed in three different target regions (5'- and middle- region of ORF aw well as 3' untranslational area of target gene) with gene-specific probe sets (see supplementary Table 1), designed by Primer Express software (Applied Biosystems). The *x* axis shows the region which is amplified for the target gene. The *y* axis shows expression values for targeted RNA regions (log scale). Fresh rat kidney tissue RNA was used as a high quality RNA control, and relative expression levels of each sample are normalized to the fresh tissue RNA. Data are representative of three independent experiments, and are expressed as mean ± SD.

A hypothetical basic model of FFPE

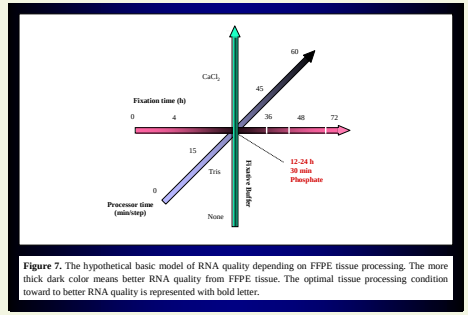

Figure 7. The hypothetical basic model of RNA quality depending on FFPE tissue processing. The more thick dark color means better RNA quality from FFPE tissue. The optimal tissue processing condition toward to better RNA quality is represented with bold letter.

CONCLUSIONS

- There are significant impacts on RNA recovery quantity and quality depend on warm ischemia condition. At 4ºC, the ratio of 28S and 18S rRNA was relatively stable for up to 12 h, whereas progressive degradation of these two rRNA species was observed after 12 h of incubation. When comparing the finding of observed at 4ºC, the 28S/18S ratio decreased early (after 4 h incubation) at 25ºC. In contrast to results of tissue held at 4 and 25ºC, representative rRNA pattern was almost completely disappeared after 12 h incubation at 37ºC. Alternatively, RNAlater sustained the stability of RNA up to after 24 h incubation at 25ºC but the 28S/18S ratio was marginally decreased when compared to untreated condition.
- Fixation time and buffer-type do affect the quality of the morphology and RNA quality. The optimal time for better results is believed to be a 12-24 h formalin fixation period.
- High quality RNA was associated with longer tissue processing programs. The condition of 660 minutes (30 min/ stage) produced the highest quality RNA, and H & E staining showed also better histology.
- Studying the RNA profiling from FFPE tissue, we demonstrate that a middle region of target gene could be a promising target for RNA analysis using real-time quantitative RT-PCR. This data suggests that RNA within FFPE could be processed random degradation mode, not 5' to 3' directional.