RNA quality assessment of long-term storage biobank samples from colorectal cancer patients for sequencing and profiling

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Abstract

Samples from patients with colorectal cancer (CRC) are valuable tools for understanding the development, progression, and treatment of the disease. However, to date, the integrity of long-term preserved human specimens in biobanks has not been well understood. In this study, we investigated the RNA quality of 12-year-stored specimens, including frozen and formalin-fixed, paraffin-embedded (FFPE) tumor tissues from CRC patients at the Siriraj Biobank. We assessed the RNA quality of 12-year and 2-year storage samples using three technologies: next-generation sequencing (NGS), Nanostring nCounter® platform, and GeoMx® digital spatial profiling (DSP). We found that the RNA quality of 12-year storage frozen tissues was consistent with the criteria for RNA sequencing. Although RNA in long-term storage FFPE tissues was degraded, the normalized counts of RNA from the 12-year-stored FFPE samples were comparable to those from the 2-year-stored FFPE samples in the Nanostring nCounter® gene expression assay. For histological staining, clear tissue microanatomy was observed in the FFPE blocks stored for 12 years. In GeoMx® DSP, there was no statistically significant difference in the normalized counts of RNA from the 12- and 2-year stored FFPE samples. Our results suggest the potential utilization of long-term storage biobank specimens from patients with CRC for NGS, Nanostring nCounter® gene expression analysis, and GeoMx® DSP.

Introduction

Molecular investigations of patient samples are extremely useful for determining the origin, prognosis, and therapeutic strategy for diseases [1]. Next-generation sequencing (NGS) of DNA and RNA is becoming increasingly important for exploring and understanding complex diseases, including cancer, via variants and gene expression [2–5]. Alternatively, the direct detection of RNA extracted from patient samples has become more attractive for basic, pharmaceutical, and clinical research because of the lack of need for reverse transcription, amplification, and enzymes [6]. NanoString nCounter® is a digital readout technology developed for multiplexed direct detection of nucleic acids using fluorescent color-coded barcode probes [7]. Compared to any other current RNA analysis technique, Nanostring nCounter® technology is superior to formalin-fixed paraffin-embedded (FFPE) materials [7]. Recently, approaches for investigating the spatial distribution of RNA have promoted transcriptomic studies [8]. GeoMx® digital spatial profiling (DSP) is a commercialized approach that integrates Nanostring nCounter® technology and enables high-level multiplex spatial profiling of RNA in FFPE tissue samples from patients [9]. For molecular studies of patient-derived specimens using these advanced technologies, a sufficient quantity and quality of target molecules are necessary.

Hospital-based biobanking intends to collect patient samples of bodily fluids or tissues for research to understand health and disease [10–12]. At most hospitals, including Siriraj Hospital, fresh tumor tissues from patients were stored at -80°C, along with FFPE tumor tissue samples in the biobank. Biobanking now faces several challenges, such as collecting standard protocols and storage time, which influence sample quality to variable degrees [10]. In particular, RNA is a highly degradable molecule whose quality declines with storage conditions and duration [11, 12]. Thus, protecting reagents, such as RNAlater, have
been widely utilized to protect patient-derived RNA from denaturation in frozen tissues [13–17]. Nevertheless, patient-derived FFPE materials prepared by the pathology department are still susceptible, possibly limiting downstream RNA analyses [18, 19]. To date, the integrity of nucleic acids, especially RNA, in long-term storage biobank specimens from patients is not fully understood for subsequent sequencing and profiling [20–23].

In this study, we focused on colorectal cancer (CRC) which is the third most common cancer worldwide with highly heterogeneous transcriptome levels; therefore, studies on human CRC are necessarily dependent on the quantity and quality of RNA obtained from patient tissues in the biobank [24, 25]. This study aimed to investigate RNA quality in 12-year-stored specimens from CRC patients at Siriraj Hospital for downstream transcriptome sequencing, direct detection, and spatial profiling.

**Materials And Methods**

**Patients’ sample collection and storage.**

CRC tissues from all patients were surgically collected at Siriraj Hospital, Faculty of Medicine Siriraj Hospital, Mahidol University. Similar to an earlier report [26], after operative processing, the tissues were cut into 5–10 mm pieces, immediately placed in chilled RNealater (Invitrogen, MA, USA), and stored at -80°C in the Siriraj Biobank until RNA extraction. To prepare FFPE samples, tissues were fixed in 10% formalin, dehydrated using ascending grades of ethyl alcohol, embedded in hot paraffin wax, partially sectioned and mounted on a slide for histopathological analysis, and stored at room temperature in the pathology department until further use.

**RNA isolation from frozen tumor tissues.**

A lysis buffer together with lysing matrix Z (MP Biomedicals, Santa Ana, CA, USA) was applied to all frozen tissues (30 mg) before homogenization using a FastPrep-24 5G instrument (MP Biomedicals, CA, USA) at a speed of 6.0 m/s for 40-s ON and 3-min OFF cycles. During the OFF phase, sample tubes were placed on ice. The homogenization step was continued until a clear lysate was obtained. An RNeasy mini kit (Qiagen, Hilden, Germany) was used to extract total RNA, according to the manufacturer’s instructions. Approximately 30 µL RNA samples were obtained.

**RNA isolation from FFPE tumor tissues.**

Total RNA was extracted from two sectioned FFPE tissues (5 µm thickness) using a high-purity FFPE RNA isolation kit (Roche Diagnostics, Indianapolis, IN, USA), strictly according to the manufacturer’s instructions. Approximately 30 µL RNA samples were obtained.

**RNA quantity assessment.**

The RNA concentration, ratio of absorbance at 260 nm and 280 nm (A260/A280), and ratio of absorbance at 260 nm and 230 nm (A260/A230) were analyzed using a Nanodrop 8000
spectrophotometer (Thermo Fisher Scientific, MA, USA). The RNA integrity number (RIN) was assessed using an Agilent RNA 6000 Nano LabChip kit and an Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA). To calculate the RIN, the ratio of 28s and 18s ribosomal RNA bands was analyzed from 1 µL of the extracted RNA sample.

**RNA sequencing.**

RNA library preparation and transcriptome sequencing were conducted by Novogene Co. Ltd. (Singapore) and Vishuo Biomedical Ltd. (Thailand). Briefly, the qualified RNA samples (1 µg total RNA, RIN > 7) were enriched using oligo (dT) beads before mRNA was randomly fragmented using a fragmenting buffer. Next, the mRNA template and random hexamers were used to synthesize cDNA. Next, second-strand synthesis was initiated by adding DNA polymerase I, RNase H, dNTPs, and a custom second-strand synthesis buffer. Both ends were repaired and ligated with adaptors, followed by size selection of adaptor-ligated DNA and PCR amplification. After the PCR products were cleaned and validated, libraries were loaded on an Illumina HiSeq platform (Illumina, San Diego, CA, USA). Sequencing was performed using a 2 × 150 bp paired-end (PE) configuration with 8.0 Gb raw data per sample. Raw data, error rate, quality score of 20 (Q20), quality score of 30 (Q30), and GC content were calculated simultaneously to ensure the quality of the sequencing data.

**NanoString nCounter® gene expression assay.**

RNA profiling was performed using a NanoString nCounter® analysis system (NanoString Technologies, WA, USA), according to the manufacturer’s instructions. The PanCancer progression panel kit was used to measure the expression of 770 target genes in RNA samples (300 ng) extracted from FFPE tumor tissues. Raw counts were normalized to the geometric mean counts of 11 housekeeping genes (HRNP1, RPL27, RPL9, RPL6, RPL30, OAZ1, PTMA, RPS29, UBC, RPS12, and RPS16) and spiked-in controls.

**Hematoxylin and eosin staining.**

For morphological analysis, a slide of a FFPE tumor section (5 µm thickness) was prepared, stained with hematoxylin and eosin (H&E), and observed under an Olympus Bx43 microscope with a DP73 digital camera attached to CellSens Standard software (Olympus Optical, Co. Ltd, Tokyo, Japan).

**GeoMx® DSP.**

In DSP, 5-µm thick FFPE sections were deparaffinized, heated in ER2 solution (Leica Biosystems, Baden-Wurttemberg, Germany) at 100°C for 20 min, and treated with 1 µg/mL proteinase K at 37°C for 15 min. Hybridization using the GeoMx® immune pathway panel for 84 target mRNAs was performed overnight at 37°C in a hybridization oven. To remove unbound probes, the slides were washed twice with 50% formamide/2X SSC buffer at 37°C for 25 min. The prepared slides were incubated with a tri-color fluorescence morphology marker panel targeting pan-cytokeratin or pan-CK (epithelial and tumoral regions), CD45 (immune cells), and SYTO13 (nuclear). The stained slides were loaded onto a GeoMx® instrument (NanoString Technologies) and scanned before regions of interest (ROI) (215–625 µm in
diameter, n ≥ 4) were selected. UV illumination was performed twice, once for the PanCK segment and once for the CD45 segment. Hybridization and purification of heat-dried oligos to unique NanoString barcodes were performed using the nCounter® Prep Station (NanoString Technologies). The barcodes were counted on the nCounter® analysis system using standard procedures (NanoString Technologies). The normalized counts were calculated using five housekeeping genes: RAB7A, OAZ1, UBB, POLR2A, and SDHA.

**Statistical analysis.**

Prism 9 software (GraphPad Software, Inc., CA, USA) was used for all the statistical analyses. The Mann-Whitney U test or T test was used to compare whether there was a difference in the dependent variable between the two independent groups. Statistical significance was set at p-value < 0.05.

**Results**

**Long-term storage frozen tumor tissues for RNA sequencing.**

We first determined the RNA integrity of CRC tissues stored for 12 and 2 years at -80°C in the Siriraj biobank for NGS. In Table 1, the quality characteristics of RNA extracted from the 12-year-stored tissues are assessed in comparison to those from the 2-year-stored tissues. The means of A260/280 (> 1.8) and A260/230 (> 1.7) indicated that RNA samples extracted from 12- and 2-year-stored tissues were acceptably pure (no or relatively low protein and salt contamination) (Table 1) [26–28]. A minimum of 1 µg of total RNA with an RIN value above 7 is required for subsequent library preparation in RNA sequencing [29, 30]. As the RNA concentration shown in Table 1, 1 µg of total RNA was obtained from all prepared stocks (≈ 30 µL). For RIN, among all the samples, only one sample extracted from a 12-year-stored tissue was below the cut-off of < 7 and possibly risk in the sequencing (Fig. 1(a)) [30]. Regarding the quality summary of the sequence read data, there was no significant difference in the raw data of RNA samples from the 12- and 2-year-stored tissues (Fig. 1(b)). In Fig. 1(c-f), the error rate, Q20, Q30, and GC content, which are generally calculated to reflect the quality of sequencing data, of all RNA samples are shown. The sequencing error rate in RNA samples from both groups was < 0.05% (Fig. 1(c)). For RNA extracted from 12-year-stored tissues, the mean Phred Q20 and Q30 scores were 97.92% and 94.08%, respectively (Fig. 1(d)). Similarly, the Q20 and Q30 means were 97.51% and 92.93%, respectively, for RNA extracted from the 2-year-stored tissues (Fig. 1(e)). The mean GC content was nearly 50% in both groups (Fig. 1(f)). Statistically, there was a significant difference between the two independent groups for most quality characteristics, as shown in Table 1 and Fig. 1; however, the difference was small, except for RNA concentration. According to Novogene’s Q30 standard of ≥ 85% for high throughput and high accuracy, all RNA samples prepared from 12-year-stored CRC tissues were suitable for NGS.
Table 1
Quality metrics for RNA extracted from frozen CRC tissues stored for 12 and 2 years.

<table>
<thead>
<tr>
<th></th>
<th>12-year-stored</th>
<th>2-year-stored</th>
<th>p-value $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>100</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>A260/280 ratio $^1$</td>
<td>1.95 ± 0.21</td>
<td>2.08 ± 0.01</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>A260/230 ratio $^1$</td>
<td>1.86 ± 0.25</td>
<td>1.92 ± 0.08</td>
<td>0.6800</td>
</tr>
<tr>
<td>RNA concentration$^1$ (ng/µL)</td>
<td>229.75 ± 101.24</td>
<td>352.02 ± 79.03</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

$^1$ Data was shown as the mean ± standard deviation (SD) and determined using a Nanodrop 8000 spectrophotometer. $^2$ Mann-Whitney U test.

Long-term storage FFPE tumor tissue samples for amplification-free RNA expression analysis.

Next, we studied RNA in CRC patient-derived FFPE tumor tissues stored at room temperature for 12 and 2 years in the Siriraj biobank for RNA expression analysis using the NanoString nCounter® technology. The number, purity, and concentration of RNA samples extracted from the 12- and 2-year-stored FFPE specimens are illustrated in Table 2, indicating that all samples had sufficient quantity with acceptable purity. After analyzing the RNA expression of 770 target genes included in the human PanCancer progression panel, the obtained raw counts were normalized to the 11 housekeeping genes to counteract technical and intra-sample variabilities. Figures 2(a) and 2(b) show the total raw counts and total normalized counts of all target genes in the RNA samples extracted from the 12- and 2-year-stored FFPE samples. Although the total raw counts of the 2-year-stored FFPE samples were higher than those of the 12-year-stored FFPE samples, no significant difference was observed in the total normalized counts (Figs. 2(a) and 2(b)). Thus, this result demonstrates the potency of 12-year-stored FFPE samples from CRC patients for amplification-free RNA expression analysis.

Table 2
Quality metrics for RNA extracted from FFPE tumor tissue samples stored for 12 and 2 years.

<table>
<thead>
<tr>
<th></th>
<th>12-year-stored</th>
<th>2-year-stored</th>
<th>p-value $^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>36</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>A260/280 ratio $^1$</td>
<td>1.89 ± 0.05</td>
<td>1.96 ± 0.07</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>A260/230 ratio $^1$</td>
<td>1.67 ± 0.26</td>
<td>1.76 ± 0.30</td>
<td>0.0381</td>
</tr>
<tr>
<td>RNA concentration$^1$ (ng/µL)</td>
<td>544.15 ± 440.30</td>
<td>479.96 ± 227.43</td>
<td>0.7736</td>
</tr>
</tbody>
</table>
Data was shown as the mean ± SD and determined using a Nanodrop 8000 spectrophotometer. \(^2\) Mann-Whitney U test.

**Long-term storage frozen tumor tissues for RNA sequencing.**

To check the morphological correlation between 12- and 2-year-stored FFPE tumor tissue samples from CRC patients, we performed H&E staining. The results for both sample groups clearly showed the histological structures of hematoxylin and eosin (Fig. 3). As shown in Fig. 3, the overall H&E staining of FFPE tumor tissue samples stored for 12 and 2 years was adequate. Based on the histopathological features, morphological changes in the cell nuclei were clearly observed in the cancer cells in terms of nuclear size and chromatin compaction in the two sample groups (Fig. 3). Cancer cells contained large nuclei with pink cytoplasm (Fig. 3). Their chromatin varied from an irregular or broad chromatin distribution to dense and homogenous purple chromatin (Fig. 3). Furthermore, infiltration of inflammatory cells, mainly eosinophils, was evident in both the 12- and 2-year-stored FFPE samples (Fig. 3).

In the GeoMx® DSP study, we clearly observed the spatial profile of three morphological markers, Pan-CK, CD45, and SYTO13, in the 12- and 2-year-stored FFPE samples; thus, the tumor region could be distinguished from the tumor microenvironment (Figs. 4(a) and 4(b)). At least four similar ROIs (either the tumor or tumor microenvironment) of each specimen were selected for 84 RNA targets in the GeoMx® immune pathway panel. As a result of normalization to the five housekeeping genes, there was no statistically significant difference in the normalized barcode counts of 12- and 2-year-stored samples (Fig. 4(c)). These findings indicate the potential of 12-year-stored FFPE samples from CRC patients for H&E staining as well as RNA spatial profiling.

**Discussion**

Collectively, our results suggest the integrity of RNA in long-term storage biobank samples from patients with CRC for downstream sequencing and profiling analyses. Here, the RNA integrity of the patient-derived materials in each analysis was focused on and compared between RNA from 12- and 2-year-stored samples. Since RNA quality and stability were retained in the samples stored for up to six years at -80°C \(^3\), the 2-year-stored sample group was utilized to ensure the integrity of RNA derived from the 12-year-stored sample group in this study.

First, transcriptome sequencing of RNA samples extracted from the tumor tissues of CRC patients stored for 12 and 2 years was performed. All the extracted RNA samples possessed good purity (i.e., low contaminants from proteins and salts). Our quality control data showed that all RNA samples extracted from the 12-year-stored specimens reached a Q30 of greater than 85%, an illumina high-quality standard in NGS, implying no errors and ambiguities for all sequencing read information. Although one RNA sample from a 12-year-stored tumor tissue had an RIN lower than 7, there was no problem with the quality summary of this sample. This finding is consistent with previous studies, which indicated no adverse effects of long-term storage at -80°C on the quality of RNA extracted from the stored tissues and
the potential of the degraded RNA from tumor samples with a RIN > 5 for RNA sequencing [10, 11, 32].
The statistical difference in most quality characteristics between the 12- and 2-year-stored groups was
very small and did not affect the quality of the RNA samples in RNA sequencing. Long-term storage has
been suggested to have a negative effect on RNA quantity. To our knowledge, this study is the first to
validate the integrity of RNA from tumor tissues stored for more than ten years for RNA sequencing.

Next, gene expression analysis of RNA samples extracted from 12- and 2-year-stored FFPE specimens
was conducted. We employed NanoString nCounter® technology because this technique allows single-
molecule direct detection without amplification using unique fluorescent barcodes to digitally detect
different RNA targets (up to 800 genes) in a single run [33]. RNA with acceptable purity was extracted
from both sample groups. Our gene analysis results indicated that long-term storage adversely affected
the total raw counts of RNA targets. Interestingly, the barcode count difference for 12- and 2-year-stored
groups could be counteracted by housekeeping gene normalization. This finding suggests that long-term
storage of FFPE tumor specimens (≤ 12 years) is promising for RNA expression analysis using
Nanostring nCounter® technology.

Finally, H&E staining and DSP of the 12- and 2-year-stored FFPE specimens were studied. Histological
staining demonstrated that the tumor and normal cell contents could be clearly determined in both
sample groups. This is in accordance with a previous study showing that the histomorphology of a 20-
year-stored FFPE tumor sample was adequately retained [11]. In GeoMx® DSP, it was possible to clearly
identify the tumor and tumor microenvironment regions in the two sample groups after
immunomorphological staining. In addition, the results of RNA spatial profiling indicated no adverse
impact from the 12-year storage on the normalized count analysis for RNA targets, supported by an
earlier study of an 8-year-stored FFPE tumor block using GeoMx® DSP [34]. Taken together, these
findings provide evidence for the promise of FFPE tumor specimens stored for up to 12 years for DSP.

To the best of our knowledge, the present study is the first attempt to report a proof of concept that RNA
in CRC patient tumor specimens stored for over ten years is suitable for RNA sequencing, expression
analysis, and DSP in the specified techniques. Since acceptable RNA integrity varies with downstream
applications, RNA integrity for other analytical techniques should be verified next. Tumor biobanks have
made important contributions to translational studies [35]. Omics studies of patient-derived tumor
materials have been widely performed to explore and understand diseases [36]. In particular, patient-
derived tumor tissues and FFPE specimens are valuable resources for medical research because they are
commonly collected and stored in hospital biobanks [11, 34]. Therefore, our findings showing the
potential utilization of long-term storage biobank samples from CRC patients would promote research
aimed at overcoming this complex disease.

Conclusions

We demonstrated that the RNA integrity of long-term storage biobank samples from patients with CRC is
applicable for sequencing and profiling. RNA extracted from 12-year-stored specimens reached the gold
standard for NGS. In the Nanostring nCounter® gene expression assay, the normalized expression of RNA targets extracted from the 12-year-stored FFPE tumor tissues was not statistically different from that of the 2-year-stored group. Moreover, the microanatomy of the 12-year-stored FFPE tumor tissues could be possibly analyzed by H&E staining. Finally, the 12-year-stored FFPE tumor tissues were available for immuno-morphological staining and RNA profiling in GeoMx® DSP. Our results suggest the potential utilization of long-term storage biobank samples from patients toward translational research.

Declarations

Author contributions

V.T. and P.T. contributed to the conceptualization. O.A., K.T. and P.T. contributed to the methodology. T.S., A.C. and P.T. contributed to the validation. T.S., O.A., K.T., T.S., A.C., A.N. and P.T. contributed to the formal analysis. T.S., O.A., K.T., A.N. and P.T. contributed to the investigation. M.P., V.T. and P.T. contributed to the resource. T.S., O.A., K.T. and P.T. contributed to the data curation. T.S. and K.T. contributed to the writing—original draft preparation, T.S., M.P., V.T. and P.T. contributed to the writing—review and editing. T.S. and K.T. contributed to the visualization. V.T. and P.T. contributed to the supervision. V.T. and P.T. contributed to the project administration. M.P., V.T. and P.T. contributed to the funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Ethics approval

This study was approved by the Siriraj Institutional Review Board (COA No. Si105/2021 and Si348/2019, SIRB Protocol No. 864/2563(IRB1 and 895/2561(EC1). This study was conducted in accordance with the International Guidelines for Human Research Protection, as well as the Declaration of Helsinki.

Consent to participate

Written informed consent was obtained from all patients with CRC.

Data availability statement

All of the relevant data for RNA sequencing (GSE220148) and Nanostring nCounter (GSE220149) shown in this study are publicly available in the Gene Expression Omnibus (GEO) under the SuperSeries with accession number GSE220150. Gene expression data (normalized counts) for DSP is available in the supplementary file. The rest of the data are also available from the corresponding authors upon request.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

References


Figures
Figure 1

Quality characteristics of RNA extracted from 12- and 2-year-stored tumor tissues of CRC patients for RNA sequencing. Box plots of (a) RIN value, (b) Raw data, (c) Error rate, (d) Q20, (e) Q30 and (f) GC content of the RNA samples. RIN, RNA integrity number, was examined on an Agilent 2100 bioanalyzer. Raw data, raw reads multiplied by sequence length. Error rate, percentage of incorrectly-called bases. Q20 and Q30 indicate the percentage of bases whose Qphred > 20 and 30 or the percentage of bases whose
correct base recognition rate over 99% and 99.9% in total bases, respectively. GC content, G base and C base accounted for the overall base. A p-value was calculated by Mann-Whitney U test.

Figure 2

Violin plots depicting (a) total raw counts and (b) total normalized counts for the 770 target genes in nanoString nCounter® gene expression profiling of RNA samples extracted from 12- and 2-year-stored FFPE tumor tissue samples of CRC patients. A p-value was determined by Mann-Whitney U test.
Figure 3

H&E staining of representative (a) 12- and (b) 2-year-stored FFPE tumor tissue samples from CRC patients. Blue, hematoxylin (cell nuclei). Pink, eosin (extracellular matrix and cytoplasm). Higher magnification of the square area is shown in the corresponding bottom photograph. Scale bars for the top photographs in each panel are 50 µm and the bottom photographs are 20 µm.
Digital spatial profiling (DSP) of 12- and 2-year-stored FFPE tumor tissue samples from CRC patients in GeoMx® DSP system. Representative ROI tumor images of (a) 12- and (b) 2-year-stored samples. Green, Pan-CK (epithelial and tumoral regions). Pink, CD45 (immune cells). Blue, SYTO13 (nuclear). Scale bar, 250 μm. (c) Normalized barcode counts for 84 target genes in the GeoMx® immune pathways panel. A p-value was calculated by T test.
Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryfileofnormalizedcountsforDSP.xlsx