RT-qPCR

Reliable cDNA synthesis from FFPE-derived RNA for RT-qPCR and targeted sequencing

Introduction

Formalin-fixed, paraffin-embedded (FFPE) tissue is one of the most valuable resources for molecular research and translational medicine, providing access to vast collections of archived clinical specimens. However, formalin fixation and long-term storage introduce RNA fragmentation and chemical modifications that may complicate downstream molecular analyses.

Although improvements in RNA extraction have increased nucleic acid recovery from FFPE tissues, RNA integrity remains highly variable between specimens and even between adjacent tissue sections from the same FFPE block. Consequently, successful downstream applications rely heavily on reverse transcription reagents capable of efficiently converting fragmented RNA into representative cDNA.

Invitrogen™ SuperScript™ V VILO™ Master Mix was developed to improve cDNA synthesis efficiency from challenging RNA samples while maintaining sensitivity across a broad range of RNA quality.

In this application note, SuperScript V VILO Master Mix performance was evaluated using a diverse panel of archived human FFPE tissues differing in tissue origin, storage duration, and RNA integrity. The resulting cDNA was assessed by qPCR and compared with another commercially available reverse transcription master mix. Compatibility with targeted RNA sequencing was also evaluated to assess the suitability of the generated cDNA for multiple downstream applications.

Highlights

- Sensitive RT-qPCR performance across highly variable and degraded FFPE RNA
- Reproducible cDNA synthesis across independent RNA extractions from heterogeneous FFPE tissue sections
- Earlier target detection than with another commercially available reverse transcription master mix
- Broad recovery of cancer-associated targets in downstream targeted RNA sequencing

Recommendations for working with FFPE RNA

1. Use a specialized kit for good FFPE RNA recovery, such as the Applied Biosystems™ MagMAX™ FFPE DNA/RNA Ultra Kit.
2. Use a trusted cDNA synthesis solution, such as Invitrogen™ SuperScript™ V Reverse Transcriptase or SuperScript™ V VILO™ Master Mix, Primer-Free.
3. Avoid oligo(dT) for highly degraded RNA; use gene-specific primers or random primers instead, with random primers up to 5 µM if needed for better recovery of short transcripts.
4. Use short PCR/qPCR amplicons, ideally ~60–100 bp, especially for samples with low DV200.
5. Ensure efficient genomic DNA removal or, where possible, design PCR primers that discriminate against gDNA, for example, by targeting exon-exon junctions or spanning introns.

Table 1. Overview of tissue samples used in this study.

Sample ID	FFPE block age at analysis, years	Tissue origin	Tumor type (histology)	Tumor content (% surface area)
A1	1	Colon	Colorectal adenocarcinoma	60
A2	1	Kidney	Renal cell carcinoma, clear cell type	75
A3	1	Prostate	Prostate adenocarcinoma	55
B1	6	Colon	Colorectal adenocarcinoma	75
B2	6	Kidney	Renal cell carcinoma, clear cell type	60
B3	6	Prostate	Prostate adenocarcinoma	85
C1	11	Colon	Colorectal adenocarcinoma	70
C2	11	Kidney	Renal cell carcinoma, clear cell type	95
C3	11	Prostate	Prostate adenocarcinoma	90

Materials and methods

FFPE sample panel

Nine human FFPE tissue samples representing colon, kidney, and prostate tumors were included in the study (Table 1). For each tissue type, three independent FFPE blocks, approximately 1, 6, and 11 years old at the time of analysis, were selected to represent recently archived, intermediate-age, and older specimens. The panel therefore encompassed variation in tissue origin, tumor type, and archival duration, enabling evaluation of cDNA synthesis performance across FFPE samples with different expected levels of RNA preservation.

RNA extraction

FFPE tissue blocks were sectioned into 20 µm–thick slices using a Thermo Scientific™ Shandon™ Finesse™ 325 Microtome. Tissue sections were deparaffinized with xylene, and total RNA was purified using the Applied Biosystems™ MagMAX™ FFPE DNA/RNA Ultra Kit. Four independent RNA extractions were performed for each FFPE block using separate tissue sections, generating 36 RNA extracts in total. RNA concentration was measured using a Thermo Scientific™ NanoDrop™ spectrophotometer. RNA quality was assessed using the Agilent™ 2100 Bioanalyzer™ system and Agilent™ RNA 6000 Pico Kit, and reported as DV200, defined as the percentage of RNA fragments longer than 200 nucleotides.

Reverse transcription

Purified RNA was reverse transcribed using SuperScript V VILO Master Mix, Primer-Free or with another commercially available primer-free RT master mix from an alternative supplier. Each reverse transcription reaction contained 40 ng of total RNA, and random primers were added to a final concentration of 5 µM. Reactions were performed according to the manufacturers' recommended protocols.

RT-qPCR assays

Quantitative PCR was performed using Applied Biosystems™ TaqMan™ Fast Advanced Master Mix on an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System. Gene expression was evaluated using Applied Biosystems™ TaqMan™ Gene Expression Assays targeting *MYC, VEGFA, AMACR,* and *ACTB*. The selected targets represent genes known to be overexpressed in the corresponding cancer tissue types included in the study, while *ACTB* was used as a reference gene. For all qPCR reactions, cDNA represented 10% of the total reaction volume. Amplification was performed using the recommended fast cycling protocol.

Targeted RNA sequencing

RNA concentration was quantified using the Invitrogen™ Qubit™ RNA HS Assay Kit on an Invitrogen™ Qubit™ 4 Fluorometer.

First-strand cDNA was generated using SuperScript V VILO Master Mix, Primer-Free, supplemented with 5 µM random hexamers. Each reaction contained 20 ng of sample RNA and was performed according to the recommended protocol. Target-specific amplification was performed using Invitrogen™ Platinum™ Multiplex PCR Master Mix with a primer panel targeting fifty oncogenes/tumor suppressor genes. The primer panel consisted of 100 individual primers (50 primer pairs), targeting 50 amplicons. The average amplicon length was 150 bp. Each PCR reaction contained 20% cDNA input. PCR amplification was performed according to the manufacturer's recommendations.

Amplicons were purified using magnetic bead–based cleanup. Sequencing libraries were prepared using the Invitrogen™ Collibri™ ES DNA Library Prep Kit according to the recommended protocol. Final libraries were evaluated using a 2100 Bioanalyzer system and sequenced on the Illumina MiSeq™ platform. Sequencing performance was assessed based on target recovery, coverage, and read depth.

Table 2. RNA yield and integrity across the FFPE sample panel.

Sample ID	FFPE block age at analysis, years	Tissue	Mean RNA yield (µg per extraction)	DV200 (%)	
				Mean ± SD	Range*
A1	1	Colon	3.0	70 ± 9	64–83
A2	1	Kidney	2.7	61 ± 12	48–77
A3	1	Prostate	1.2	54 ± 20	28–74
B1	6	Colon	1.5	26 ± 17	14–51
B2	6	Kidney	0.7	24 ± 17	10–48
B3	6	Prostate	2.1	19 ± 17	7–45
C1	11	Colon	0.8	17 ± 14	4–37
C2	11	Kidney	2.3	31 ± 21	15–61
C3	11	Prostate	2.0	28 ± 16	15–51

* The DV200 range represents the minimum and maximum values observed across the four extractions.

Results

FFPE samples span a broad range of RNA quality

RNA was successfully purified from all nine FFPE tissue blocks. For each block, four independent extractions were performed using separate tissue sections. RNA integrity varied both between blocks and among extractions from the same block (Table 2).

RNA integrity was evaluated using the DV200 value, which represents the percentage of RNA fragments longer than 200 nucleotides. Differences between blocks may be related to tissue type, archival duration, and fixation history. Variation among extractions from the same block most likely reflects within-block heterogeneity, as adjacent sections can differ in cellular composition, fixation-related damage, and RNA preservation despite having the same thickness and being processed under identical extraction conditions.

A clear association was observed between archival duration and RNA integrity. Samples approximately 1 year old generally showed the highest DV200 values, whereas 6- and 11-year-old samples showed greater RNA fragmentation. Across the sample panel, mean DV200 values ranged from approximately 17% to 70%, providing a broad range of RNA quality for evaluation of downstream reverse transcription performance. Despite this variability, RNA suitable for RT-qPCR analysis was recovered from the samples.

SuperScript V VILO Master Mix consistently generates amplifiable cDNA

SuperScript V VILO Master Mix enabled detection of all tested gene targets across the FFPE sample panel, despite substantial differences in RNA integrity. Consistent results were observed across the four independent RNA extractions from each FFPE block (Figure 1A). Although RNA integrity varied among sections from the same block, the resulting cDNA supported reproducible target detection across the assays tested.

C_q values generally decreased as DV200 increased, reflecting the increased availability of longer, amplifiable RNA fragments in samples with higher DV200 values (Figure 1B). These results demonstrate reliable cDNA synthesis from FFPE-derived RNA spanning a broad range of tissue types, archival ages, and RNA quality.

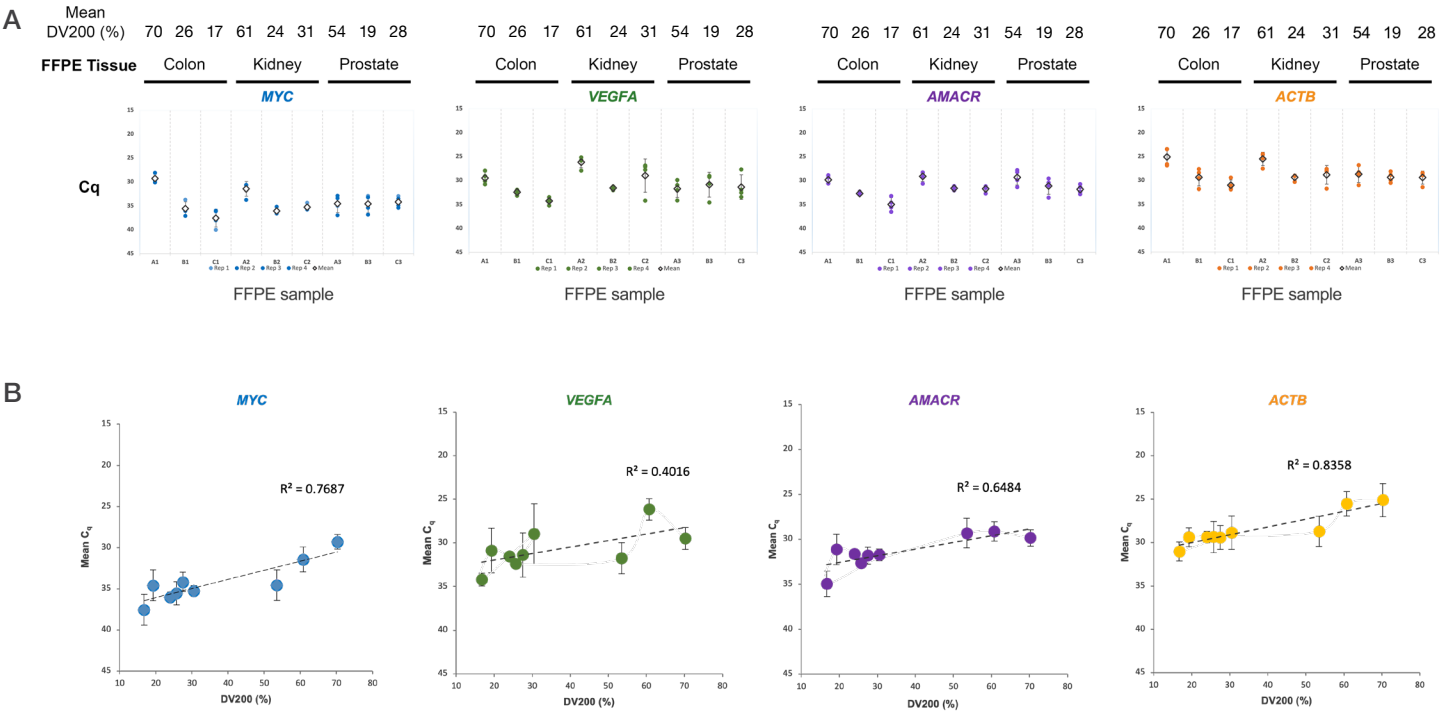

Figure 1. C_q values across FFPE samples and independent RNA extractions. C_q values for *MYC, VEGFA, AMACR,* and *ACTB* were determined using cDNA generated with SuperScript V VILO Master Mix. Four independent RNA extractions were evaluated for each FFPE block. **(A)** Consistent amplification was maintained across independent extractions. Diamonds indicate mean C_q, and error bars indicate standard deviation. Each dot represents one extraction (n = 4). **(B)** Earlier target detection was generally observed in samples with higher DV200 values. Dots indicate mean C_q, and error bars indicate standard deviation.

SuperScript V VILO Master Mix enables earlier target detection by RT-qPCR

SuperScript V VILO Master Mix generally enabled earlier target detection than another commercially available reverse transcription master mix across the tested assays. Earlier C_q values were observed with SuperScript V VILO Master Mix in 91% of paired comparisons, with improvements of up to approximately 5 cycles (Figure 2).

Mean ΔC_q values were positive for the four assays, indicating an overall performance advantage for SuperScript V VILO Master Mix. The mean improvement ranged from approximately one to more than two cycles, depending on the target. Together, these results demonstrate greater recovery of amplifiable target cDNA from FFPE-derived RNA under the conditions tested.

Compatibility with targeted RNA sequencing

SuperScript V VILO Master Mix generated cDNA that supported targeted RNA sequencing across the nine FFPE specimens, despite differences in tissue type, archival duration, and RNA integrity. Across the sample panel, the resulting cDNA supported recovery of a broad range of cancer-associated target intervals, with 42–49 of 50 evaluated targets detected per specimen (Figure 3A). Cancer-relevant target recovery was high, with a broad range of cancer-associated targets detected at high sequencing depth (Figure 3B).

Consistent recovery was observed across targets associated with major oncogenic and tumor-suppressor pathways, including RAS–MAPK, PI3K–AKT, cell-cycle regulation, DNA-damage response, and receptor tyrosine kinase signaling (Figure 4). Forty-two targets were detected in at least eight of nine samples, and 21 targets reached a mean depth of at least 10x. Together, these results show that FFPE-derived cDNA generated with SuperScript V VILO Master Mix was suitable for downstream targeted sequencing across biologically diverse FFPE samples.

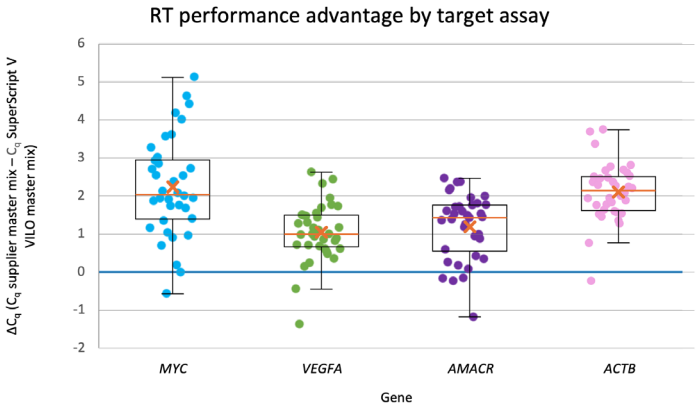

Figure 2. Earlier RT-qPCR target detection with SuperScript V VILO Master Mix. FFPE-derived RNA was reverse transcribed using SuperScript V VILO Master Mix or a commercially available primer-free RT master mix, followed by qPCR analysis of *MYC, VEGFA, AMACR,* and *ACTB*. ΔC_q was calculated as C_q obtained with the supplier RT master mix minus C_q obtained with SuperScript V VILO Master Mix. Each dot represents an individual FFPE RNA extraction replicate analyzed by RT-qPCR. Boxes represent the interquartile range (25th–75th percentile), the horizontal line indicates the median, whiskers show the data spread, and diamonds indicate the mean ΔC_q. Positive ΔC_q values correspond to earlier target detection with SuperScript V VILO Master Mix.

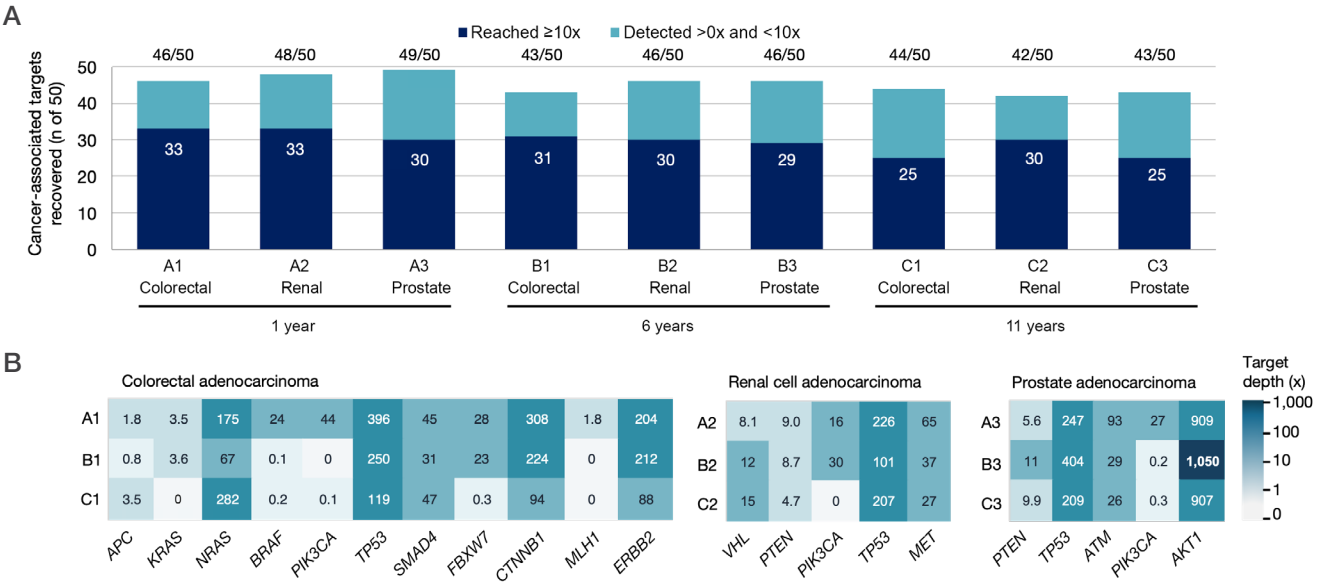

Figure 3. Broad cancer-relevant target recovery across FFPE samples. Targeted sequencing was performed using targets associated with a 50-plex–targeted amplicon panel. Libraries were normalized to 27,000 read pairs per sample. **(A)** Across the sample panel, 42–49 of the 50 evaluated targets were detected per specimen, corresponding to 407 recovered sample–target observations out of 450. In addition, 25–33 of the detected targets across the sample panel reached ≥10x sequencing depth. **(B)** Sequencing depth of cancer-relevant targets is shown for each sample. Most targets were detected at ≥10x sequencing depth.

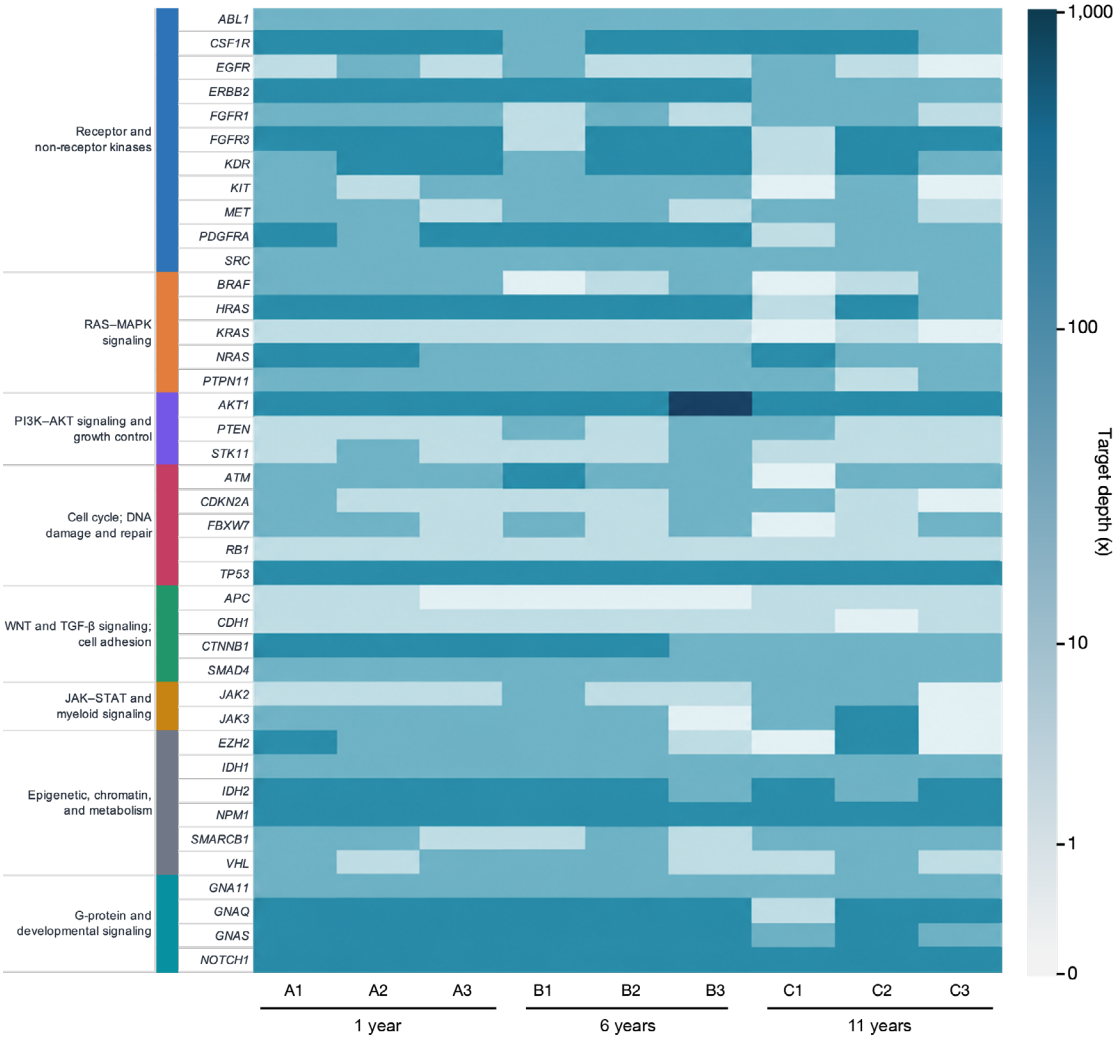

Figure 4. Sequencing depth of cancer-associated targets across biological pathways. The heatmap summarizes sequencing depth for 40 cancer-associated targets detected across the nine FFPE samples after normalization to 27,000 read pairs per sample. Targets are grouped by major cancer-related biological pathways, including receptor and non-receptor kinase signaling, RAS–MAPK signaling, PI3K–AKT signaling, cell-cycle and DNA-damage regulation, WNT and TGF-beta signaling, JAK–STAT signaling, epigenetic regulation, and G-protein signaling. Color intensity represents the mean per-base sequencing depth across each target interval, displayed on a logarithmic scale.

Conclusions

FFPE-derived RNA remains one of the most challenging sample types for gene expression analysis because RNA integrity can vary substantially with tissue type, archival duration, and heterogeneity within individual tissue blocks. In this study, SuperScript V VILO Master Mix consistently generated cDNA from FFPE RNA spanning a tested range of DV200 values. The workflow supported reproducible RT-qPCR detection across independent RNA extractions and generally enabled earlier target detection than another commercially available primer-free RT master mix. SuperScript V VILO Master Mix also generated cDNA compatible with targeted RNA sequencing, supporting broad recovery of cancer-associated target intervals across a panel of nine FFPE tissue specimens. Together, these results demonstrate that SuperScript V VILO Master Mix offers a versatile reverse transcription solution for RT-qPCR and targeted RNA sequencing workflows using challenging archived FFPE samples.

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