High correlation of miRNA quantitation data from matched FFPE and snap-frozen tissues using TaqMan® MicroRNA Assays

Abstract
This application note describes the successful isolation and detection of miRNAs from formalin- or paraformalin-fixed, paraffin-embedded (FFPE) samples and demonstrates the accurate and robust measurement of miRNA expression from comparable snap-frozen samples using TaqMan® MicroRNA Assays. The data indicate that miRNAs are robustly and reproducibly amplified from FFPE samples using real-time PCR. Comparison to matched snap-frozen samples demonstrates high concordance between the data generated from the two sample sources, confirming that the FFPE fixation process does not adversely affect miRNA quantitation.

Introduction

MicroRNAs (miRNAs), a class of small non-coding RNAs, are responsible for significant post-transcriptional regulation of gene expression. As publications describing miRNA function and targets accumulate, it is clear that miRNAs are involved in regulating diverse biological processes, including cell development, differentiation, apoptosis, and proliferation [1].

There have been many inquiries into the possible role of miRNAs in cancer. Several publications document the differential expression of miRNAs between malignant and normal tissues, indicating that miRNAs play a role in tumor formation and that their expression patterns may prove useful for tumor classification and treatment [2–4].

One potential source of samples for the study of human cancer are FFPE tissues, since they are generally retrieved with extensively documented clinicopathological histories. The ability to isolate nucleic acids from archived samples is an invaluable tool for the study of human cancer on a molecular level. While standard methods of preservation using formalin and paraformalin are ideal for maintaining tissue structure and preventing putrefaction, they pose challenges for the molecular analyses of these samples. Nucleic acids become trapped and modified through protein–nucleic acid and nucleic acid–nucleic acid crosslinks. RNA isolated from FFPE samples is often fragmented to a random range of sizes and chemically modified to a degree that is incompatible with many molecular analysis techniques. To address this challenge, an optimized extraction and quantification protocol was described by Life Technologies scientists [5].

Comparison of miRNA profiles from FFPE and snap-frozen human tissues

Researchers from the Department of Pathology at the Erasmus MC-University Medical Center Rotterdam, The Netherlands, led by Prof. Leendert H.J. Looijenga, analyzed miRNA expression in archived human germ cell tumors using high throughput real-time PCR [6]. Their results support observations that miRNAs are involved in regulating stem cell differentiation [7]. To confirm that results were not affected by the FFPE fixation process, the researchers performed a comparative study using matched testis and seminoma samples either preserved by FFPE fixation or snap-frozen immediately upon collection. Total RNA was extracted from the snap-frozen samples using the

RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE
This kit has been optimized for isolation of total nucleic acids, including microRNAs, from FFPE tissues. The RNA protocol does not require overnight Proteinase K digestion, and it is possible to deparaffinize samples and perform downstream analysis in a single day. Yields are typically >50% of those from unfixed tissue from the same sample source. The recovered nucleic acids are suitable for real-time PCR, mutation screening, and microarray analysis.

***mir*Vana™ miRNA Isolation Kit**
This kit allows the efficient isolation of small RNA–containing total RNA and also the enrichment of small RNAs (<200 nt) to increase sensitivity in downstream analyses. The quick and easy procedure is ideal for miRNA, siRNA, shRNA, and snRNA analyses. The kit is compatible with virtually all cell and tissue types.

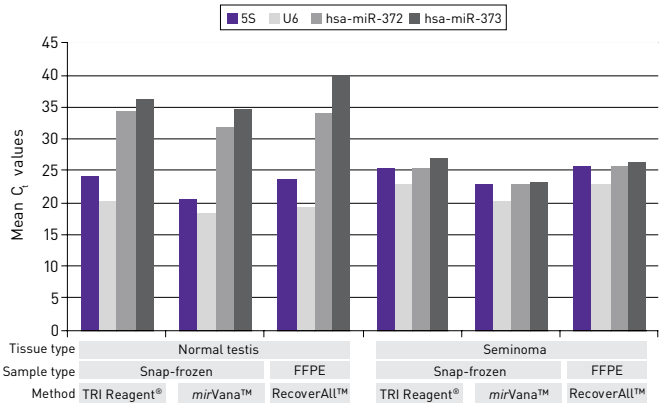


Figure 1. Highly comparable results of miRNA quantitation in FFPE tissues and snap-frozen samples. Total RNA was extracted using TRI Reagent® Solution or the *mir*Vana™ miRNA Isolation Kit for the snap-frozen samples, and the RecoverAll™ kit for the FFPE tissues. Individual small RNAs were detected by real-time PCR using TaqMan® MicroRNA Assays. C_t values are the means of triplicates. Data courtesy of Leendert Looijenga and Ad Gillis, Erasmus MC-University Medical Center Rotterdam, The Netherlands.

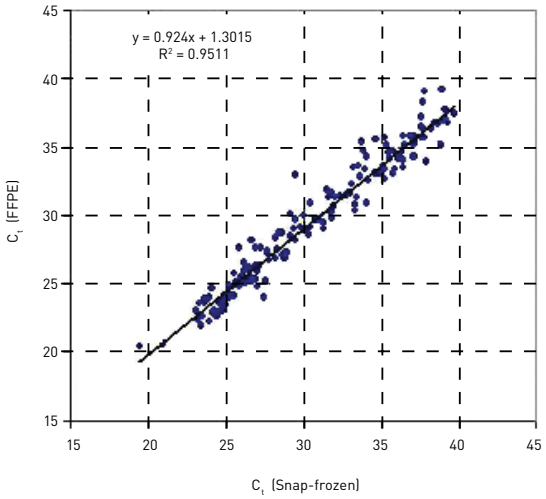


Figure 2. High correlation of mean C_t values of 154 miRNA assays from paired FFPE and snap-frozen cells. RNA was extracted from snap-frozen cells using the *mir*Vana™ miRNA Isolation Kit, and from FFPE cells using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE. Identical amounts of total RNA were used in TaqMan® MicroRNA Assays. Real-time PCRs were run in triplicate, and the points represent the means of the replicates. Correlation coefficient (R^2) = 0.9511 with p = 0.000. Data courtesy of Orla Sheils, University of Dublin, Ireland.

*mir*Vana™ miRNA Isolation Kit or TRI Reagent® Solution. Total RNA was extracted from FFPE samples using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE.

Reverse transcription was performed using identical amounts of input total RNA and the small RNA-specific stem-loop RT primer provided in the TaqMan® MicroRNA Assay Kit. After reverse transcription, an aliquot of the reaction was used for TaqMan® probe–based real-time PCR. In this experiment, four different small RNAs (5S, U6, hsa-miR-372, and hsa-miR-373) were detected in the samples (Figure 1).

In a similar but larger study, Orla Sheils and colleagues from the Department of Histopathology at the University of Dublin, Ireland, compared the expression profiles of 160 miRNAs in matched samples of snap-frozen and FFPE cells (cells isolated from FFPE tissues by laser capture microdissection) [8]. Total RNA was isolated from ~2 x 10^6 FFPE-preserved thyroid cancer cells and from ~1.7 x 10^5 snap-frozen cells using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE and the *mir*Vana™ miRNA Isolation Kit, respectively. Life Technologies TaqMan® MicroRNA Assays were used for miRNA expression profiling. The real-time PCRs were run in triplicate for each miRNA quantitated (Figure 2).

The results presented in the two studies described demonstrate a high degree of correlation of miRNA detection irrespective of the approach for preserving the tissue, confirming that miRNA expression levels are well preserved in FFPE samples.

Reproducibility of miRNA detection in FFPE samples

Prof. Leendert Looijenga in Rotterdam performed a follow-up study to confirm the reproducibility of the detected miRNA expression level in FFPE tissues across a small population by comparing the expression levels of 157 miRNAs in five biological replicates of yolk sac tumor FFPE samples, each resected from a different individual. Total RNA was extracted from the FFPE samples with the RecoverAll™ kit, and identical amounts were run with each TaqMan® MicroRNA Assay. The results show that miRNAs are reproducibly quantitated across biological replicates from FFPE tissues (Figure 3).

The data presented in this document clearly demonstrate a high correlation of miRNA expression patterns between FFPE and matched snap-frozen cells as shown in Figures

TRI Reagent® Solution
This simple and versatile reagent allows the isolation of DNA-free RNA, RNA-free DNA, and protein. TRI Reagent® Solution gives higher yields than traditional guanidine thiocyanate or cesium chloride methods, and it can be used with human, plant, yeast, bacterial, and viral samples. Reactions are easily scalable depending on starting material and desired yield.

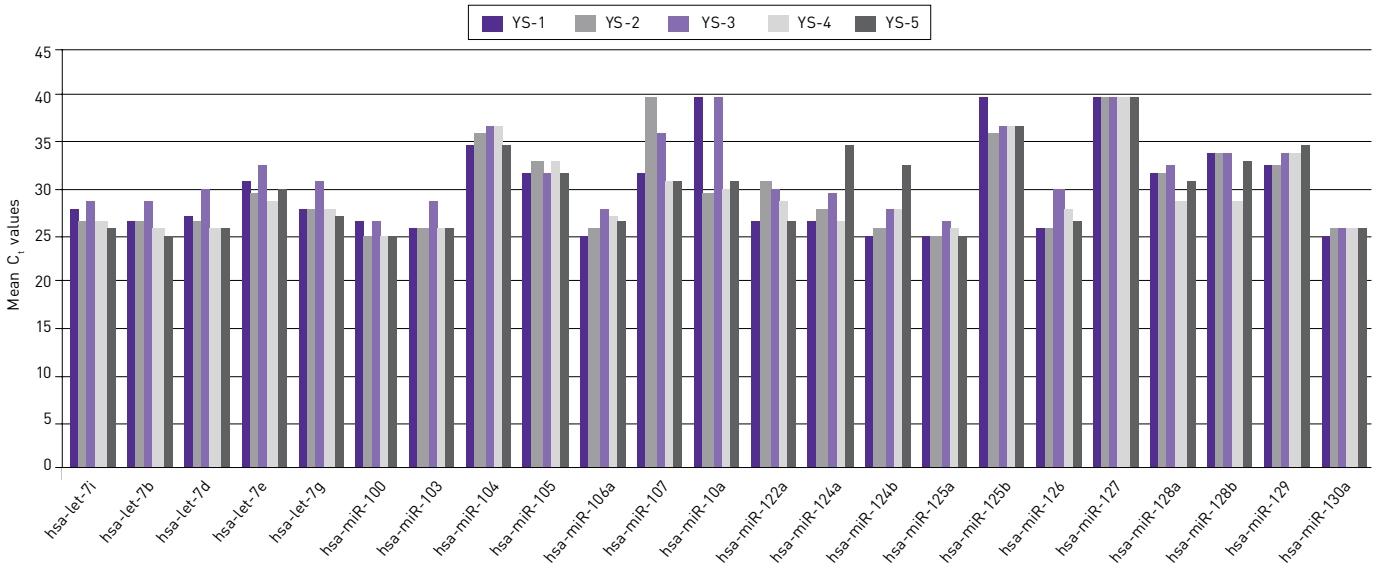


Figure 3. Expression levels of 23 representative miRNAs in five biological replicates of yolk sac tumor FFPE samples (YS-1 to YS-5). FFPE tumor samples were between 8 and 20 years old. RNA was isolated with the RecoverAll™ kit for FFPE according to the manufacturer's protocol. A total of 157 miRNAs were analyzed per sample. Mean C_t values from triplicate PCR reactions are shown for some representative miRNAs. Data courtesy of Leendert Looijenga and Ad Gillis, Erasmus MC-University Medical Center Rotterdam, The Netherlands.

1 and 2. There is also very high reproducibility of miRNA detection from FFPE samples as shown in the analysis of biological replicates (Figure 3). A general workflow to extract and amplify miRNA from FFPE tissues is shown in Figure 4.

Benefits of miRNA analysis from FFPE tissues

The detection of mRNAs from archival material has been challenging due to its labile nature and the deleterious effects of enzymatic fragmentation and/or RNA modification induced by formalin fixation. As a potential solution, it has been suggested that small PCR amplicons (shorter than 130 nucleotides) could have utility as robust markers in gene expression studies using FFPE tissues [5].

In contrast, mature miRNAs have the advantage of already being small (20–22 nucleotides) and potentially represent a difficult target for enzymatic or chemical cleavage. In addition, it is hypothesized that miRNAs are further protected from the effects of formalin fixation by their tight association with proteins of the RNA-induced silencing complex (RISC). This hypothesis was supported by data indicating that miRNAs are tightly associated with RISC *in vivo* [9]. Tang and co-workers used real-time PCR to find the proportion of miRNAs that were associated stably with RISCs *in vivo* under physiological conditions. The authors concluded that only 1–3% of mature miRNAs were free in cells.

Irrespective of the mechanisms involved, it would appear that, compared to mRNA, miRNAs are not as susceptible to the deleterious fixation effects associated with FFPE samples and, as a consequence, represent a set of biomarkers that can be used to unlock the wealth of information embodied by FFPE sample collections.

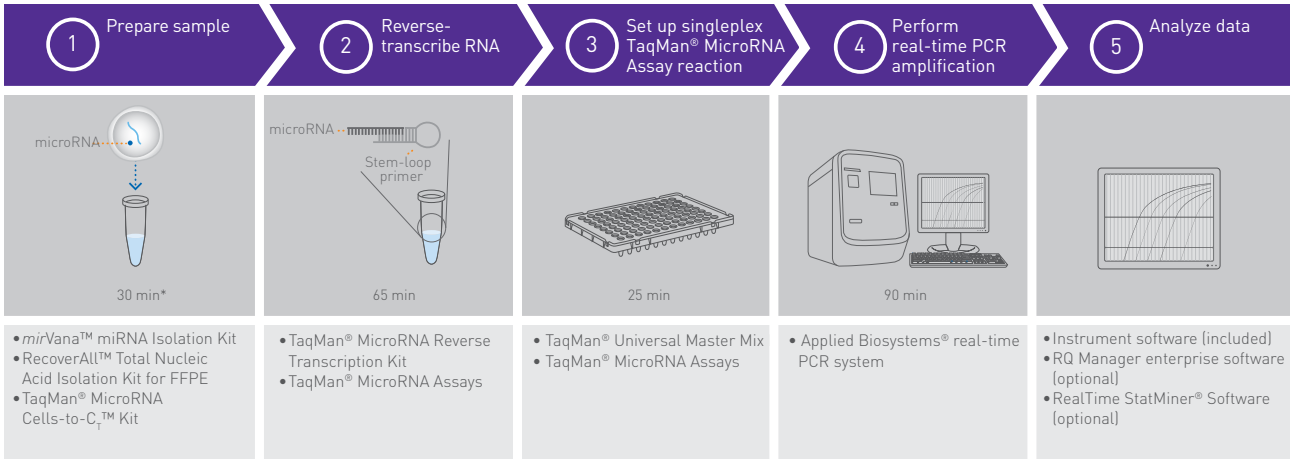
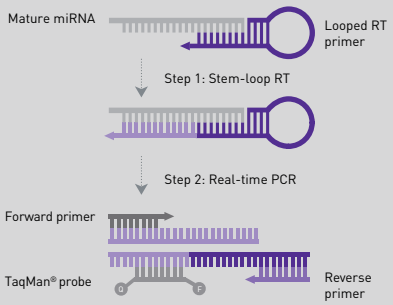


Figure 4. Life Technologies workflow for microRNA extraction and quantification from FFPE tissues.

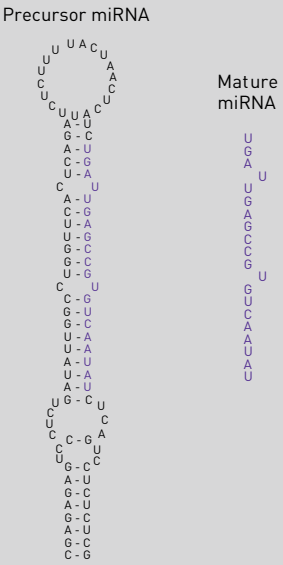
*Total workflow time using the *mir*Vana™ miRNA Isolation Kit = 3 hr 30 min. Total time may vary depending on number of samples and pipetting method used.

TaqMan® MicroRNA Assays

TaqMan® MicroRNA Assays have a simple, two-step protocol that requires only reverse transcription with a miRNA-specific primer, followed by real-time PCR with TaqMan® probes. The assays are highly specific and target only mature miRNAs, not their precursors, ensuring biologically relevant results. The assays require only 1–10 ng of total RNA. The fast and scalable, two-step quantitative RT-PCR assay provides high-quality results in under 3 hours.



TaqMan® MicroRNA Assay



Precursor and mature miRNA

MicroRNA functional analysis workflow

Select miRNA inhibitor or mimics and controls	Optimize and transfect cells	Assess target message levels (optional)	Assess target protein levels or other biological effect
*mir*Vana™ miRNA Inhibitors	Lipofectamine® RNAiMAX Transfection Agent	TaqMan® Gene Expression Cells-to-C_T™ Kit	Western-*SuperStar*™ Immunodetection System
*mir*Vana™ miRNA Mimics	*mir*Vana™ miRNA Isolation Kit	TaqMan® Gene Expression Assays	pMIR-REPORT™ miRNA Expression Reporter Vector System
		Applied Biosystems® real-time PCR systems	Dual-Light® Combined Reporter Gene Assay System

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Ordering information

Description	Quantity	Cat. No.
miRNA isolation		
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	40 purifications	AM1975
*mir*Vana™ miRNA Isolation Kit	Up to 40 purifications	AM1560
TRI Reagent® Solution	100 mL	AM1975
Real-time PCR		
TaqMan® MicroRNA Reverse Transcription Kit	200 rxns	4366596
	1,000 rxns	4366597
TaqMan® Universal PCR Master Mix	200–2,000 rxns	Varies
TaqMan® MicroRNA Assays (Inventoried; small-scale)*	50 RT (15 µL) and 150 PCR rxns (20 µL)	4427975
TaqMan® MicroRNA Assays (Made-to-Order; extra small-scale)	25 RT (15 µL) and 75 PCR rxns (20 µL)	4440885
TaqMan® MicroRNA Assays (Made-to-Order; small-scale)	50 RT (15 µL) and 150 PCR rxns (20 µL)	4440886
TaqMan® MicroRNA Assays (Made-to-Order; medium-scale)	750 RT (15 µL) and 750 PCR rxns (20 µL)	4440887
TaqMan® MicroRNA Assays (Made-to-Order; large-scale)	2,900 RT (15 µL) and 2,900 PCR rxns (20 µL)	4440888

*Assays are available individually, or in species sets. For a current list of available TaqMan® MicroRNA Assays and ordering information, go to lifetechnologies.com/taqmanmirna

Functional analysis		
*mir*Vana™ miRNA Inhibitors	5 nmol	4464084
- Negative Control	5 nmol	4464076
*mir*Vana™ miRNA Mimics	5 nmol	4464066
- Negative Control	5 nmol	4464058
*mir*Vana™ miRNA Isolation Kit	40 purifications	AM1560
Lipofectamine® RNAiMAX Transfection Reagent	1.5 mL	13778-150
TaqMan® Gene Expression Cells-to-C_T™ Kit	100 rxns	AM1728
TaqMan® Gene Expression Assays	250 20 µL rxns	4331182