TaqMan Advanced miRNA Assays—superior performance for miRNA detection and quantification

In this white paper, we show that Applied Biosystems™ TaqMan™ Advanced miRNA Assays offer:

- A simplified workflow for miRNA analysis
- Single-base discrimination of related miRNAs
- Superior sensitivity for detecting low-abundance miRNAs
- Greater accuracy in measuring small fold-changes in serum

Cells utilize diverse mechanisms for regulating the function of their genomes. MicroRNAs (miRNAs), although discovered relatively recently, are now recognized as components of gene expression regulation. miRNAs are an abundant and ubiquitous class of endogenous, noncoding, small RNA involved in posttranscriptional gene regulation for a range of cellular functions [1,2]. Expression patterns of miRNAs have been linked to many important biological processes including cellular metabolism and intracellular signaling, as well as cell division and cell death [1]. Consequently, altered miRNA expression has been associated with several pathological processes, including cancer [3], Alzheimer's disease [4], immune-related diseases, and many others [1].

Because miRNAs are associated with human health issues, the last decade has seen a growing interest in miRNA-related research along with a need for sensitive and specific tools to detect and characterize the growing number of sequences found. The sensitivity and specificity of RT-qPCR makes it the method of choice for quantifying miRNA expression [5,6]. However, miRNAs can be challenging targets when using this method—their short length (~22 nt) and often very high sequence homology can lead to difficulties when attempting to accurately detect and differentiate closely related miRNAs [5,6]. In addition, the growing interest in using circulating miRNAs as biomarkers in fluids such as plasma, serum, or cerebrospinal fluid (CSF) raises other issues related to sensitivity and limit of detection [7].

The most popular RT-qPCR methods for reliably measuring miRNAs include Applied Biosystems™ TaqMan™ MicroRNA Assays that employ target-specific stem-loop primers for cDNA synthesis, and SYBR™ Green dye–based chemistries with universal cDNA synthesis. Although a variety of qPCR-based analysis solutions are currently available, they may lack either sensitivity or specificity, have cumbersome workflows, or have limited instrument and software compatibility. To address these challenges, we have developed a workflow that includes both a universal RT step and the superior sensitivity and specificity of TaqMan™ probe-based chemistry (Figure 1). In this workflow, the Applied Biosystems™ TaqMan™ Advanced miRNA cDNA Synthesis Kit is used to incorporate a 3′ poly(A) tail and 5′ adapter sequence to exclusively target and universally amplify all mature miRNAs present within a sample. Prior to detection with specific TaqMan Advanced miRNA Assays, the cDNA is further amplified using universal miR-Amp primers in order to maximize detection of low-expressing miRNA targets while maintaining the relative expression levels of the total miRNA population. This key step improves the sensitivity of the assay significantly, without affecting sequence specificity. Furthermore, TaqMan Advanced miRNA Assays can be run using standard protocols on a range of qPCR instruments, and data can be analyzed using preexisting parameters on a variety of software analysis programs [8].

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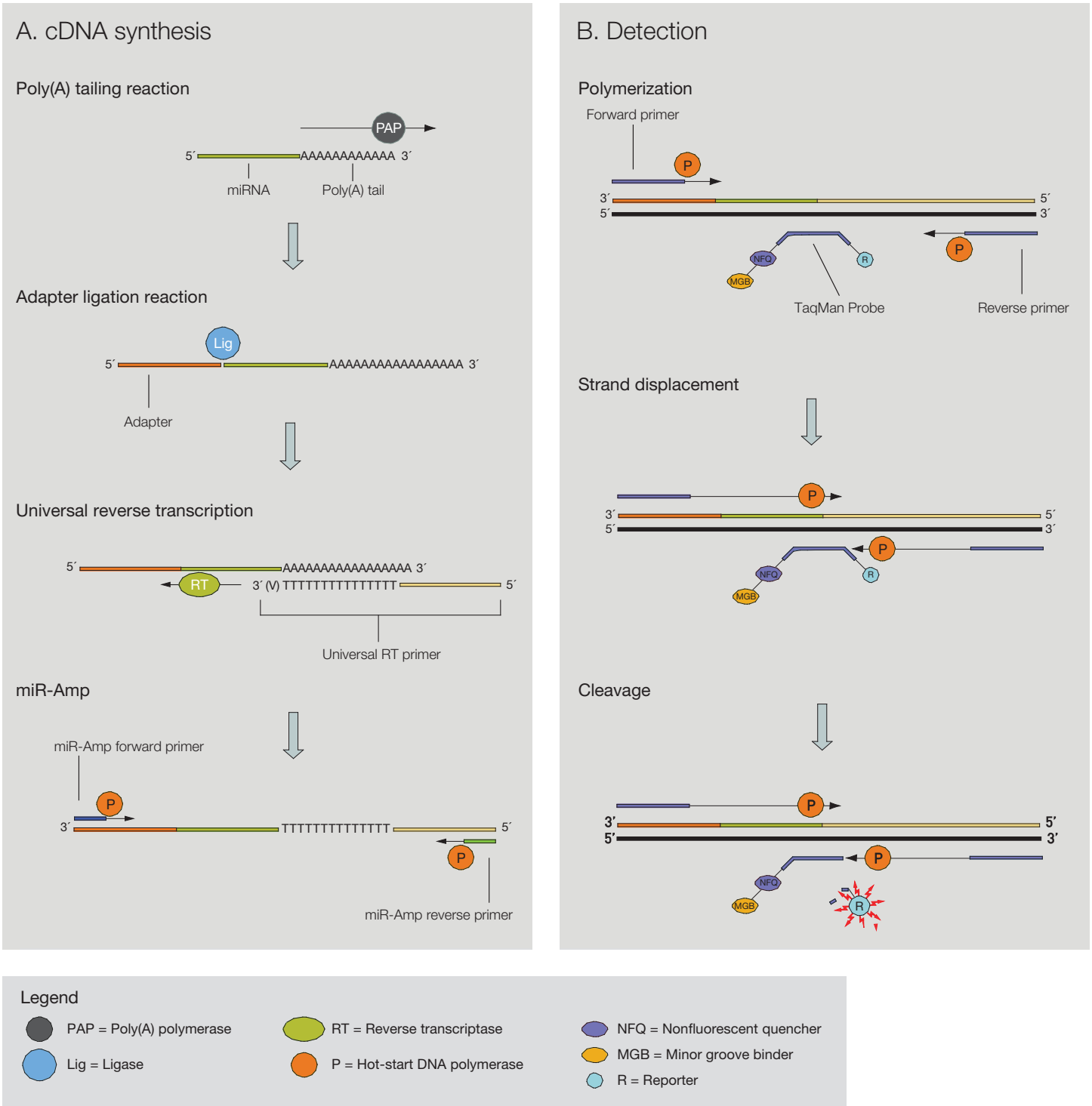


Figure 1. The TaqMan Advanced miRNA Assay workflow. (A) The cDNA synthesis workflow incorporates a 3′ poly(A) addition and 5′ adapter ligation step, followed by reverse transcription with a universal RT primer. An optional miR-Amp reaction is then performed to uniformly increase miRNA cDNA prior to detection by qPCR. **(B)** Each TaqMan Advanced miRNA Assay employs a TaqMan minor groove binder (MGB) probe that specifically anneals to a complementary sequence between the forward and reverse primer sites. The MGB modification increases melting temperature without increasing probe length, allowing for design of shorter probes. When the probe is intact, the proximity of the reporter dye to the nonfluorescent quencher (NFQ) results in suppression of reporter fluorescence. Probe cleavage by DNA polymerase during primer extension separates the reporter dye from the NFQ, resulting in increased fluorescence of the reporter. Note: Primer and probe placement is designed to maximize specificity and will vary for each assay.

The performance of TaqMan Advanced miRNA Assays was evaluated in this study by performing a similar set of standardized tests as described in Mestdagh et al. [9]. In particular, we examined specificity by measuring cross-reactivity between the highly conserved let-7 miRNA family, limit of detection by performing serial dilution experiments, and accuracy by measuring small differences of miRNA concentrations in serum.

TaqMan Advanced miRNA Assays allow single-base discrimination of closely related miRNAs

miRNAs can share a large degree of sequence homology, and are grouped in highly conserved families of nearly identical sequence. miRNAs regulate their target mRNAs by binding to a 7 nt complementary region generally located in the 3′ UTR. Complementarity to nucleotides 2–7 at the 5′ end of the miRNA (the seed region) is a major determinant in target recognition. It is now clear that single-base changes in this region of miRNAs can correspond to a >50% shift in the spectrum of mRNA targets [10]. Thus, is it important to have confidence that the tools employed to study closely related miRNAs have the necessary discriminatory power to rule out cross-reactivity, which could lead to misleading results.

Cross-reactivity of TaqMan Advanced miRNA Assays was tested between four hsa-let-7 miRNAs (Table 1) by using synthetically generated targets spiked into bacteriophage MS2 RNA at 5 x 10^6 copies/µg RNA following the protocol outlined in Mestdagh et al. [8]. qPCR experiments were run on the Applied Biosystems™ ViiA™ 7 Real-Time PCR System. Detection cutoff was set at a C_t of 35. Replicate results for all assay-target combinations were compared and cross-reactivity was calculated relative to the exact match for each miRNA. All TaqMan Advanced miRNA Assay designs draw on the Applied Biosystems™ TaqMan™ Assay design pipeline with consistent performance and high specificity across close homologs.

Table 1. Sequences of closely related hsa-let-7 targets and corresponding assays used to demonstrate specificity of TaqMan Advanced miRNA Assays.

hsa-let7 family member	miRNA sequence	Cat. No.
hsa-let-7a-5p	UGA GGU AGU AGG UUG UAU AGU U	478575_mir
hsa-let-7b-5p	UGA GGU AGU AGG UUG UGU GGU U	478576_mir
hsa-let-7c-5p	UGA GGU AGU AGG UUG UAU GGU U	478577_mir
hsa-let-7d-5p	AGA GGU AGU AGG UUG CAU AGU U	478439_mir

Table 2. Percent reactivity of TaqMan Advanced miRNA Assays tested against each hsa-let-7 family member and calculated based on exact-match results.

	Target			
Assay	hsa-let-7a-5p	hsa-let-7b-5p	hsa-let-7c-5p	hsa-let-7d-5p
hsa-let-7a-5p	**100**	0	0	0
hsa-let-7b-5p	0	**100**	0	0
hsa-let-7c-5p	1.5	0.1	**100**	0
hsa-let-7d-5p	0	0	0	**100**

Table 3. TaqMan Advanced miRNA Assays used for limit of detection assessments with serially diluted total RNA from human liver.

miRNA name	Relative expression in liver	miRNA sequence	Cat. No.
hsa-miR-16-5p	High	UAGCAGCACGUAAAUAUUGGCG	477860_mir
hsa-miR-21-5p	High	UAGCUUAUCAGACUGAUGUUGA	477975_mir
hsa-miR-141-3p	Medium	UAACACUGUCUGGUAAAGAUGG	478501_mir
hsa-miR-130b-5p	Low	ACUCUUUCCCUGUUGCACUAC	477899_mir

TaqMan Advanced miRNA Assays exhibited zero cross-reactivity for the majority of target combinations (Table 2). For hsa-let-7c that differs from hsa-let-7a by a single base (G/A), results show less than 2% reactivity. For comparison, a different qPCR-based system produced cross-reactivity up to 17% [11]. Thus, TaqMan Advanced miRNA Assays are capable of discriminating closely related miRNA sequences, differing by as little as a single nucleotide.

TaqMan Advanced miRNA Assays demonstrate superior sensitivity for detecting low-abundance miRNAs

While RT-qPCR can be regarded as a gold standard in sensitivity for miRNA quantification, reliable detection of low-abundance miRNAs can be challenging due their short length and high sequence homology [5,6]. The TaqMan Advanced miRNA Assay workflow incorporates a unique miR-Amp universal amplification step, combined with proven TaqMan Assay design and chemistry, to enable consistent and reliable results down to as little as 1 pg of RNA. This level of sensitivity is required for challenging investigations such as the study of noninvasive biomarkers where circulating miRNA levels are limited [7].

To assess limits of detection, Invitrogen™ Human Liver Total RNA was diluted and 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg were tested with four assays chosen to represent a range of expression levels in liver (Table 3). TaqMan Advanced miRNA Assays and Exiqon miRCURY LNA™ microRNA assays were compared in parallel, following all manufacturer protocols [12].

Across each assay and every dilution tested in this study, TaqMan Advanced miRNA Assays exhibited lower C_t values allowing for overall greater sensitivity compared with the equivalent miRCURY LNA microRNA assay (Figure 2). Furthermore, for several dilutions (100 pg, 10 pg, and 1 pg) the miRCURY LNA hsa-miR-141-3p assay failed to amplify, and the 1 pg sample failed for the miRCURY LNA hsa-miR-16 assay. TaqMan Advanced miRNA Assays also demonstrated superior reproducibility over 3 technical replicates, exhibiting consistently tight standard deviation values, most notably at the lower dilution points and for the low-expression target hsa-miR-130b-5p (Table 4). Greater sensitivity and lower variability from TaqMan Advanced miRNA Assays indicates that low-expressing genes are identified more reliably across various dilutions and targets. Conversely, lower sensitivity of miRCURY LNA microRNA assays may result in false-negative data, possibly leading to misinterpretation of biological processes.

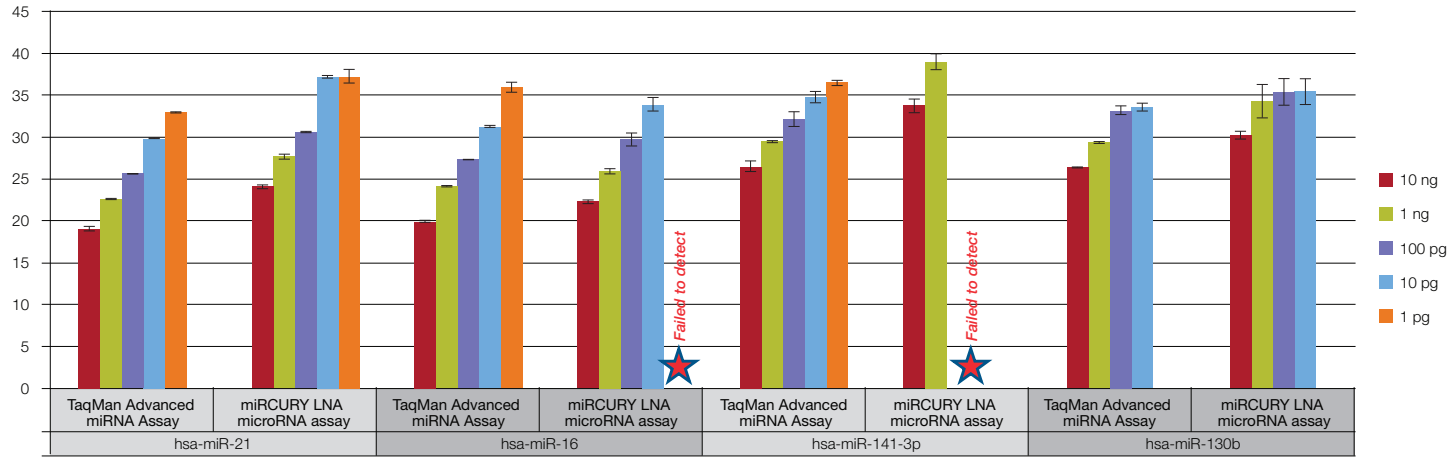


Figure 2. Amplification of serially diluted total RNA from human liver. Across all four assays chosen to represent high, medium, and low relative target abundance, higher sensitivity was obtained with TaqMan Advanced miRNA Assays compared with miRCURY LNA microRNA assays. Variability is also limited across all TaqMan Advanced miRNA Assays, with error bars representing standard deviations calculated from triplicate reactions.

Table 4. C_t and standard deviation (SD) values obtained following qPCR of serially diluted total RNA from human liver.

	hsa-miR-21				hsa-miR-16				hsa-miR-141-3p				hsa-miR-130b-5p			
	TaqMan Advanced miRNA Assay		miRCURY LNA microRNA assay		TaqMan Advanced miRNA Assay		miRCURY LNA microRNA assay		TaqMan Advanced miRNA Assay		miRCURY LNA microRNA assay		TaqMan Advanced miRNA Assay		miRCURY LNA microRNA assay	
RNA input	C_t	SD	C_t	SD	C_t	SD	C_t	SD	C_t	SD	C_t	SD	C_t	SD	C_t	SD
10 ng	19.06	0.28	24.09	0.23	19.91	0.11	22.27	0.25	26.50	0.58	33.71	0.78	26.36	0.08	30.19	0.47
1 ng	22.60	0.04	27.65	0.29	24.11	0.07	25.95	0.30	29.46	0.06	39.00	NA*	29.37	0.11	34.26	1.97
100 pg	25.63	0.01	30.59	0.07	27.34	0.00	29.70	0.77	32.12	0.87	ND	NA	33.18	0.51	35.40	1.55
10 pg	29.79	0.08	37.24	0.15	31.27	0.09	33.88	NA*	34.78	0.64	ND	NA	33.58	0.45	35.45	NA*
1 pg	32.91	0.05	37.24	0.79	35.95	0.64	ND	NA	36.40	0.28	ND	NA	ND	NA	ND	NA

ND	Not detected, no amplification
	Amplification with C_t >35

*SD could not be calculated since only one replicate produced a C_t value.

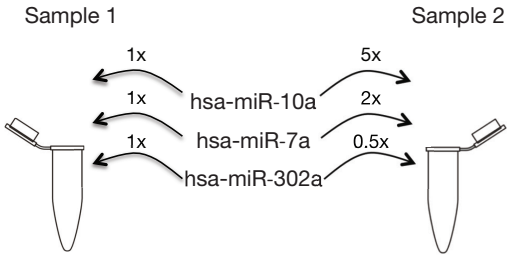
TaqMan Advanced miRNA Assays show greater accuracy in measuring small fold-changes in serum

To determine accuracy of expression, serum samples 1 and 2 were generated by spiking 3 different synthetic miRNA targets, hsa-miR-10a, hsa-miR-7a, and hsa-miR-302a, into the samples. The 3 miRNAs were spiked at equimolar concentrations in serum sample 1 (6 x 10^4 copies/µg of RNA). In serum sample 2, hsa-miR-10a was spiked at 5x the concentration of serum sample 1, hsa-miR-7a was spiked at 2x the concentration of serum sample 1, and hsa-miR-302a was spiked at 0.5x the concentration of serum sample 1 [9]. Accuracy was determined by comparing expected and observed concentration differences between serum sample 1 and 2 for all 3 miRNAs studied. As seen in Figure 3, TaqMan Advanced miRNA Assays more accurately quantified small differences in miRNA concentrations when compared to miRCURY LNA microRNA assays.

TaqMan Advanced miRNA Assays have a unique sensitivity advantage facilitated by miR-Amp universal amplification

To determine the performance equivalence in the universal miR-Amp step, an equimolar pool of 426 synthetic targets (6 x 10^5 copies of each miRNA) was reverse-transcribed using the TaqMan Advanced cDNA Synthesis Kit as per standard protocol, and a modified protocol without the miR-Amp step. The resulting cDNA was analyzed using 64 randomly selected targets and the corresponding TaqMan Advanced miRNA Assays. The difference in C_t values between the pools with and without miR-Amp universal amplification was calculated (Figure 4). A consistent shift of 7 to 9 in C_t was observed across the range of randomly selected TaqMan Advanced miRNA Assays with no amplification bias and increased sensitivity for low-abundance miRNAs.

A.



B.

Target name	hsa-miR-10a	hsa-miR-7a	hsa-miR-302a
Expected	2.3	1	–1
TaqMan Advanced miRNA Assays	2.4	0.8	–1.2
miRCURY LNA microRNA assays	2.9	0	–0.5

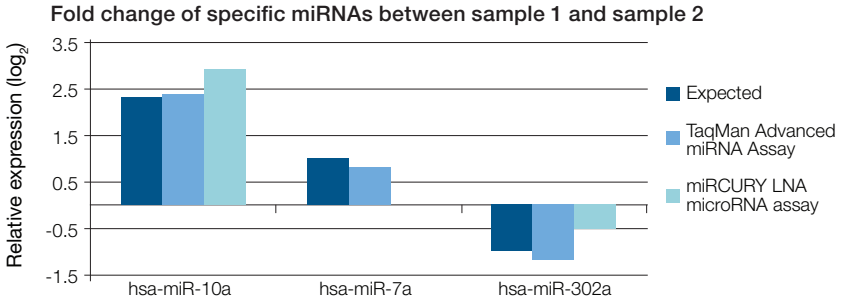
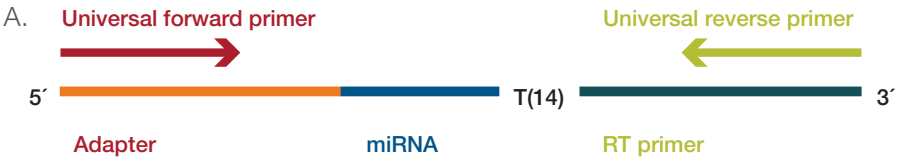


Figure 3. Relative mean ΔC_t values for synthetic miRNAs spiked into serum. (A) Serum sample 2 was spiked with varying concentrations of 3 synthetic miRNAs, while serum sample 1 was spiked with fixed concentrations of the same miRNAs. **(B)** The miRNAs were quantified by RT-qPCR and concentration differences expressed as relative expression (log_2) values.

A.



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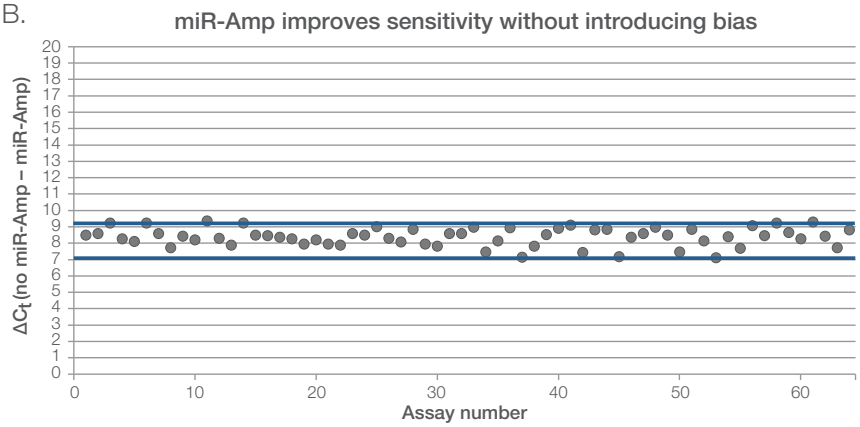

Figure 4. Increased sensitivity following miR-Amp universal amplification. (A) Diagrammatic representation of miR-Amp universal primers that facilitates uniform amplification of all miRNAs present in a sample. **(B)** ΔC_t values are consistent across 64 TaqMan Advanced miRNA Assays performed on cDNA pools generated with or without miR-Amp universal amplification, demonstrating an increase in sensitivity of 7 to 9 cycles, with no amplification bias.

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Setting a new standard in miRNA quantification

In the past twenty years, research of miRNAs has grown rapidly, resulting in a better understanding of the critical importance of these non-translated elements that globally regulate gene expression in so many important biological processes [11]. Further, the presence of circulating miRNAs in biofluids, coupled with clear correlations of their expression linked to pathological conditions, creates the possibility for use as biomarkers and future diagnostic tools [7]. Here, we have demonstrated that TaqMan Advanced miRNA Assays have the potential to support these current and future research applications with a miRNA RT-qPCR workflow that is sensitive, robust, and streamlined. Exhibiting excellent specificity down to a single-base difference and superior sensitivity without amplification bias, the TaqMan Advanced miRNA Assay workflow was specifically developed to create a new standard for miRNA RT-qPCR.

References

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Ordering information

Product	Quantity	Cat. No.
TaqMan Advanced miRNA cDNA Synthesis Kit	50 reactions	A28007
TaqMan Advanced miRNA Assays	250 qPCR reactions (20 µL)	A25576

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