

# Highly sensitive and specific LNA™-enhanced detection technologies for microRNA expression analysis

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## Introduction

MicroRNAs (miRNAs) comprise a family of highly conserved small non-coding RNAs (~22 nt). As regulators of post-transcriptional gene expression, miRNAs play an essential role in many parts of development, differentiation, and physiological processes. The accurate and specific expression analysis of miRNAs is complicated by their short length and sequence similarities between miRNAs in the same family. The specific quantification of a 22 nt sequences with single nucleotide mismatches is a significant challenge. We have developed a highly sensitive real-time PCR method for quantification of miRNAs. One of the advantages of the Locked Nucleic Acid (LNA™) technology is that very short high-affinity miR-specific

primers can be designed, thus working under general PCR conditions. The LNA™ primer design enables a simple and robust two-step method employing two different miRNA-specific primers: A miRNA-specific RT primer is employed in the first-strand cDNA synthesis, and for the following SYBR® Green-based quantitative PCR detection, a LNA™-enhanced primer targets the miRNA sequence at the opposite end. Hence, the method offers accurate quantification of specific miRNAs directly from total RNA.

## Conclusions

The miRCURY LNA™ microRNA PCR System offers highly sensitive and specific detection of mature microRNAs. The

LNA™-enhanced primers enable detection of as few as 10 RNA copies and microRNA detection is typically possible from 10pg of total RNA starting material. In addition, the microRNA primer sets offer a wide dynamic range with a linear readout of miRNA concentrations spanning up to 8 orders of magnitude. The LNA™ enhanced primers allow superior discrimination between microRNA sequences with single nucleotide mismatches. The miRCURY LNA™ microRNA PCR System also allows specific discrimination between the mature and precursor forms of microRNAs.

Figure 1: A simple two-step procedure for microRNA real-time PCR

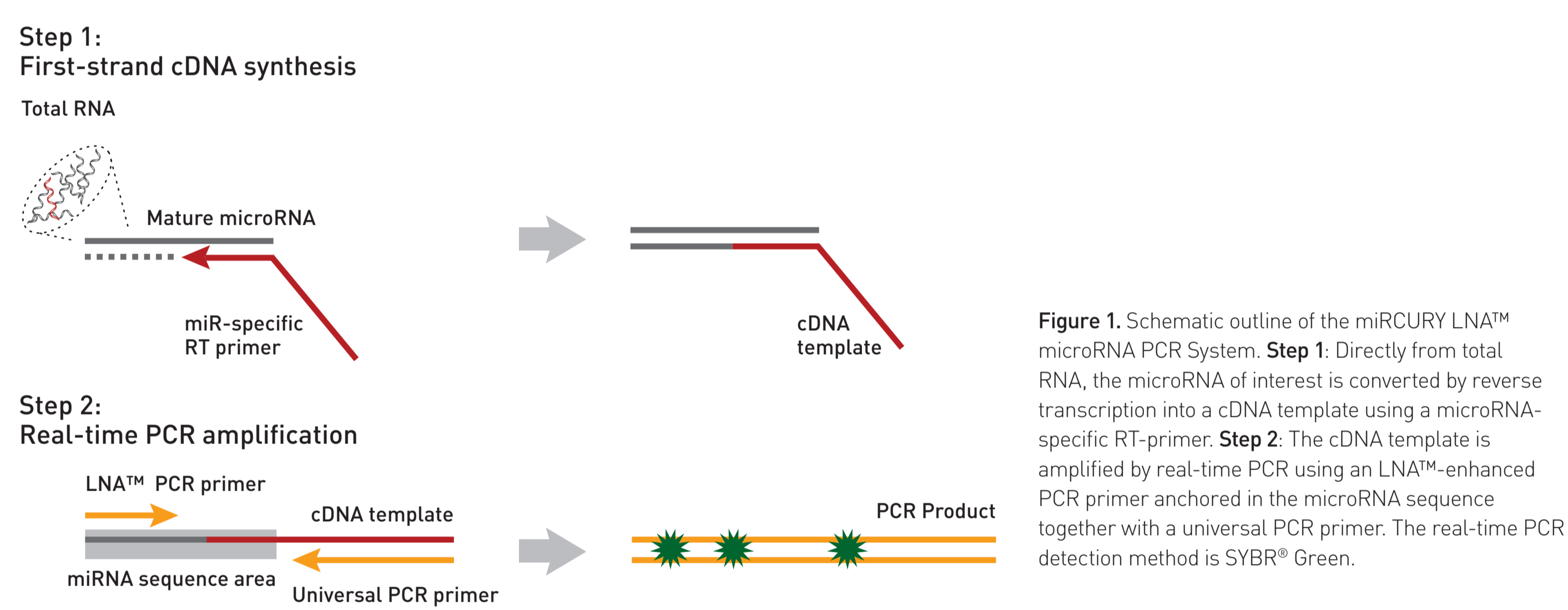


Figure 1. Schematic outline of the miRCURY LNA™ microRNA PCR System. **Step 1:** Directly from total RNA, the microRNA of interest is converted by reverse transcription into a cDNA template using a miRNA-specific RT primer. **Step 2:** The cDNA template is amplified by real-time PCR using an LNA™-enhanced PCR primer anchored in the microRNA sequence together with a universal PCR primer. The real-time PCR detection method is SYBR® Green.

Figure 2: Rigorous validation of primer sets for the miRCURY LNA™ PCR system

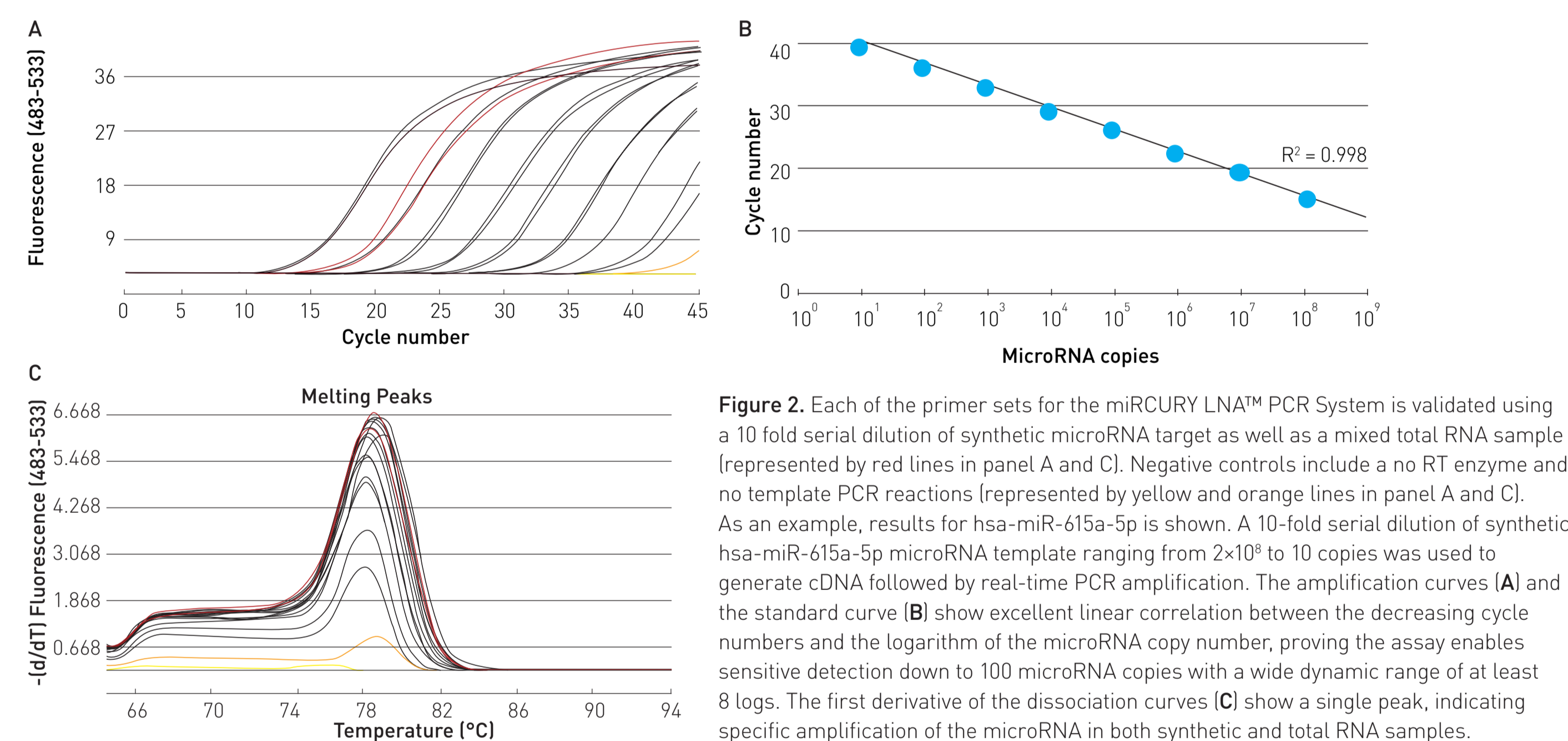


Figure 2. Each of the primer sets for the miRCURY LNA™ PCR System is validated using a 10 fold serial dilution of synthetic microRNA target as well as a mixed total RNA sample (represented by red lines in panel A and C). Negative controls include a no RT enzyme and no template PCR reactions (represented by yellow and orange lines in panel A and C). As an example, results for hsa-miR-615a-5p is shown. A 10-fold serial dilution of synthetic hsa-miR-615a-5p microRNA template ranging from  $2 \times 10^8$  to 10 copies was used to generate cDNA followed by real-time PCR amplification. The amplification curves (A) and the standard curve (B) show excellent linear correlation between the decreasing cycle numbers and the logarithm of the microRNA copy number, proving the assay enables sensitive detection down to 100 microRNA copies with a wide dynamic range of at least 8 logs. The first derivative of the dissociation curves (C) show a single peak, indicating specific amplification of the microRNA in both synthetic and total RNA samples.

Figure 3: Superior assay sensitivity of the miRCURY LNA™ PCR System

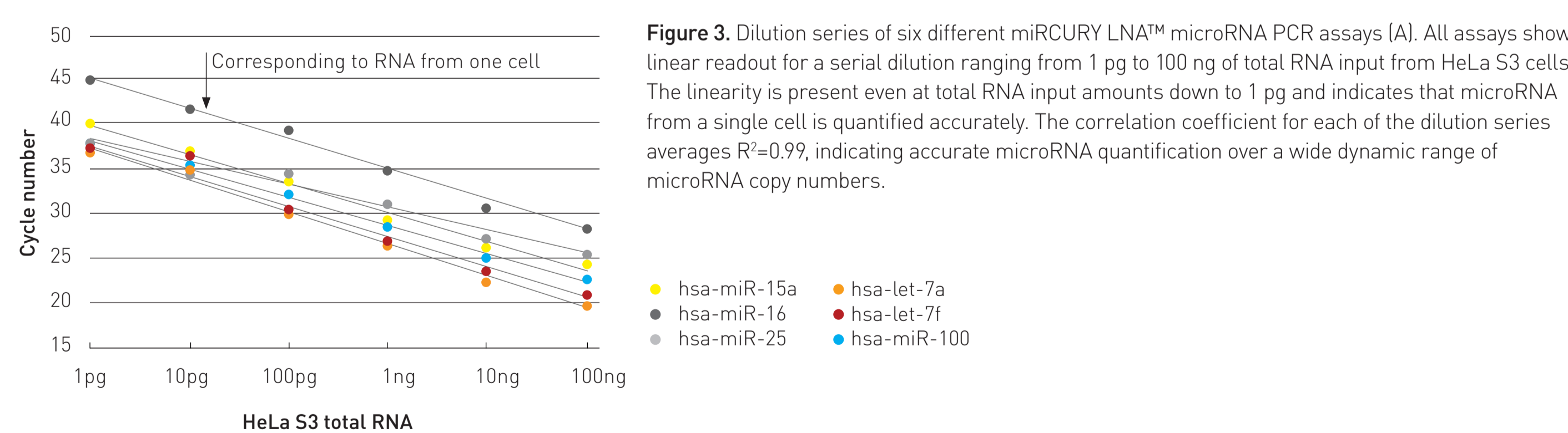


Figure 3. Dilution series of six different miRCURY LNA™ microRNA PCR assays (A). All assays show linear readout for a serial dilution ranging from 1 pg to 100 ng of total RNA input from HeLa S3 cells. The linearity is present even at total RNA input amounts down to 1 pg and indicates that microRNA from a single cell is quantified accurately. The correlation coefficient for each of the dilution series averages  $R^2=0.99$ , indicating accurate microRNA quantification over a wide dynamic range of microRNA copy numbers.

Figure 4: The advantage of LNA™ primers compared to DNA primers

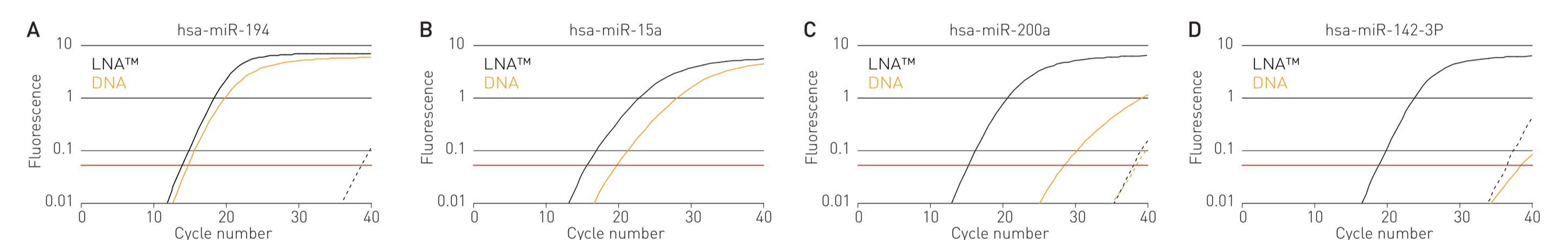


Figure 4. LNA™ in microRNA-specific PCR primers improve the performance or rescue the microRNA real-time PCR assay. The sensitivity and dynamic range of assays using LNA™-substituted primers (gray) versus DNA primers (blue) is shown for hsa-miR-194 (A), hsa-miR-15a (B), hsa-miR-200a (C) and hsa-miR-142-3p (D). The enabling effect of using LNA™ primers becomes more important as the GC content of the microRNA sequence decreases (E). Hence the assay for hsa-miR-200a was considerably improved by LNA™ substitutions while the hsa-miR-142-3p assay was rescued by using an LNA™ primer. Synthetic microRNAs were spiked into a complex background of MS2 bacteriophage total RNA. The microRNA signals are shown as solid lines and the no RNA template controls displayed as dotted lines.

MicroRNA	%GC	sequence [5'-3']
miR-194	45	UGUACAGCAACUCCAUUGUGGA
miR-15a	41	UAGCAGCACAUAAUGGUUUGUG
miR-200a	41	UAACACUGUCUGGUAACGAUGU
miR-142-3p	35	UGUAGUGUUUUUCCUUAUUUGGA

Figure 5: Excellent single nucleotide mismatch discrimination

Assay	Target	Sequence	Cycle number differences: Perfect match - mismatch	
			miRCURY LNA™ PCR	Leading competitor
hsa-miR-27b	hsa-miR-27b	UUCACAGUGGCUAAGUUCUGC		
hsa-miR-27b	hsa-miR-27a	UUCACAGUGGCUAAGUUCGCG	9	9
hsa-let-7c	hsa-let-7c	UGAGGUAGUAGGUUGUAGUGGU		
hsa-let-7c	hsa-let-7a	UGAGGUAGUAGGUUGUAGUGGU	8	10
hsa-miR-100	hsa-miR-100	AACCCGUAAGUCCGAACUUGUG		
hsa-miR-100	hsa-miR-99a	AACCCGUAAGUCCGAUCUUGUG	4	1
hsa-miR-196a	hsa-miR-196a	UAGGUAGUUUCCUUGUUGUGG		
hsa-miR-196a	hsa-miR-196b	UAGGUAGUUUCCUUGUUGUGG	>12	3
hsa-let-7a	hsa-let-7a	UGAGGUAGUAGGUUGUAGUGGU		
hsa-let-7a	hsa-let-7f	UGAGGUAGUAGGUUGUAGUGGU	8	3

Figure 5. The miRCURY LNA™ PCR system allows superior discrimination of single nucleotide mismatches. In the above table, primer sets have been used to detect the perfect match and single nucleotide mismatch targets for a selection of related microRNAs with mismatches in the 3' end and the middle of the sequence. The difference in cycle numbers (delta Ct or Cp) obtained between the perfect match and mismatch target is shown for the miRCURY LNA™ primer set and a leading competitor. A cycle number difference of 8 represents a 250 fold difference in detection.

Figure 6: Specific discrimination between precursor and mature microRNAs

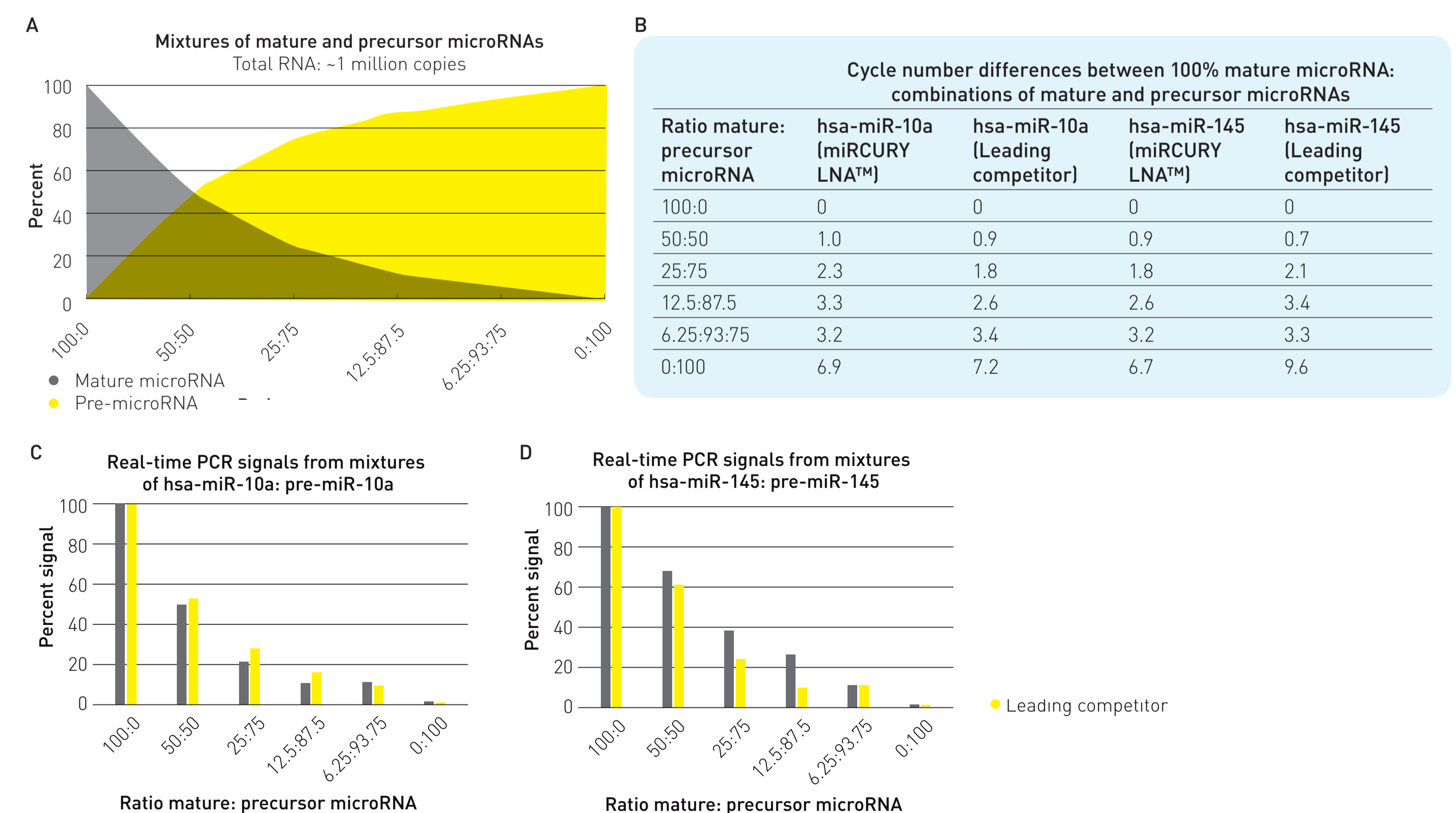


Figure 6. The miRCURY LNA™ PCR System allows discrimination between mature and precursor microRNA forms. In order to evaluate real-time PCR signals from mature and precursor microRNAs, mixtures of synthetic targets were prepared as shown in panel A where the ratios of mature and precursor forms vary from 100:0 to 0:100, and the total amount of RNA was kept constant at about 1 million copies. microRNA primer sets for hsa-miR-10a (C) and hsa-miR-145 (D) were used to quantify such mixtures. In panels C and D the signals obtained from the mixtures of precursor and mature targets are expressed as percentages of the signal obtained from the 100% mature sample. A linear decrease in signal correlating to the decrease in mature microRNA concentration is seen, despite the corresponding doubling in precursor microRNA concentration. These results show that the microRNA primer sets can discriminate between the precursor and the mature forms of the microRNAs. In panel B, a table showing the difference in cycle number (delta Ct or Cp) values between the sample containing 100% mature microRNA and various ratios of mature: precursor microRNA is shown. In each example, the discrimination afforded by the miRCURY LNA™ microRNA primer sets is equal to that of a leading competitor claiming specific detection of mature microRNAs.