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MicroRNA quantitation from a single cell by PCR using SYBR® Green detection and LNA-based primers

We describe a new, highly sensitive and specific PCR approach for quantitation of microRNAs (miRNAs): the miRCURY™ LNA microRNA PCR system. The method, which allows detection of 10 copies of miRNA, is enabled by the use of Locked Nucleic Acids (LNA™). The LNA-conferred sensitivity facilitates accurate detection of miRNA expression in a single cell.

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miRNAs are small, noncoding RNAs (~22 nt) that have key roles in regulation of many biological processes. miRNAs may regulate as much as 30% of all genes in mammalian genomes, and the number of miRNAs being identified in animals, plants and viruses is rapidly increasing.

More recently, miRNAs have been implicated in a wide range of pathological conditions, including heart disease, neurological disorders and human cancers, in which they act as tumor suppressors and oncogenes[1–3]. The latter role indicates that miRNAs may serve as therapeutic targets and as biomarkers for disease diagnosis and prognosis, emphasizing the importance of the study of miRNA expression and function. In these studies, miRNA analysis is often restricted by limited sample availability. Here we present a new LNA-based PCR system for quantitation of miRNAs from very small samples of total RNA. It is a three-component system comprising all reagents needed for the miRNA quantification: miRCURY LNA First-strand cDNA synthesis kit, miRCURY LNA SYBR Green master mix and a variety of miRCURY LNA microRNA primer sets.

LNA improves miRNA analysis

miRNAs are highly conserved across different organisms, and miRNA families display close sequence identity. This presents major challenges for the development of accurate and sensitive tools for miRNA analysis.

LNAs are a class of high-affinity nucleic acid analogs. Insertion of LNAs into oligonucleotides increases the binding affinity of that oligonucleotide to its complementary DNA or RNA target. The higher affinity is shown by marked increases in the melting temperature (T_m) of the duplex. Additionally, LNA-modified oligonucleotides considerably improve specificity, allowing single-nucleotide-mismatch discrimination that enables optimal differentiation between closely related members of the same miRNA family.

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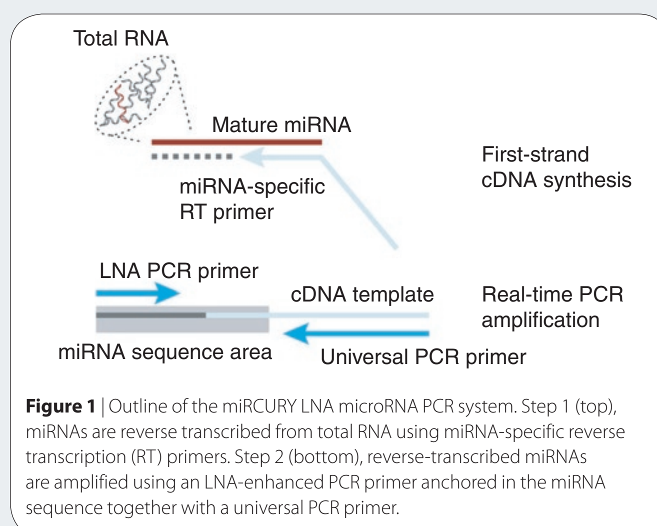

Figure 1 | Outline of the miRCURY LNA microRNA PCR system. Step 1 (top), miRNAs are reverse transcribed from total RNA using miRNA-specific reverse transcription (RT) primers. Step 2 (bottom), reverse-transcribed miRNAs are amplified using an LNA-enhanced PCR primer anchored in the miRNA sequence together with a universal PCR primer.

These unique properties of LNA oligonucleotides have been shown to enable sensitive and specific detection of miRNAs in several different applications, including northern blot analysis[4], *in situ* hybridization[5] and microarray profiling[6].

miRNA quantitation by LNA-enhanced PCR

Real-time PCR is one of the standard methods for detection and quantitation of gene expression in small samples. However, for detection of miRNA, conventional DNA-based PCR techniques are often not sufficiently sensitive and specific. The short length of miRNAs complicates the design of specific primers, as conventional primers would be equivalent in size to the full-length miRNA target. Design of PCR primers for such short sequences introduces a great risk of primer-dimer generation.

The miRCURY LNA microRNA PCR system takes advantage of the LNA technology. It anneals short, high-affinity, LNA-based primers directly to the miRNA sequence, thereby avoiding detrimental PCR-primer overlap. The system enables a simple and accurate two-step

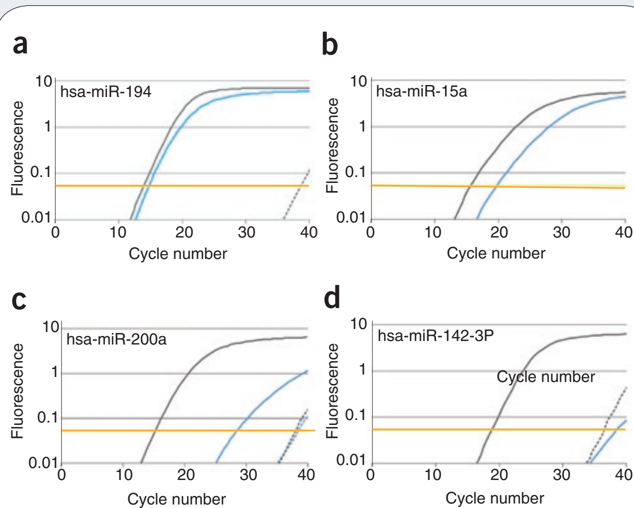

Figure 2 | LNAs in miRNA-specific PCR primers improve the performance or rescue the quantitative PCR assay. (**a**,**b**) The sensitivity and dynamic range of assays using DNA primers (blue) versus LNA-substituted primers (gray) yielded nearly similar results for the miR-194 (**a**) and the miR-15a (**b**) assays, but for miR-15a the LNA primers were slightly better. (**c**) For miR-200a, the assay was considerably improved by LNA substitutions. (**d**) The miR-142-3P assay was rescued by using LNA primer. Synthetic miRNAs were spiked into a complex background of MS2 phage total RNA. The miRNA responses are shown as solid lines, and the no-RNA template controls are displayed as dotted lines. The cycle threshold line is shown in orange.

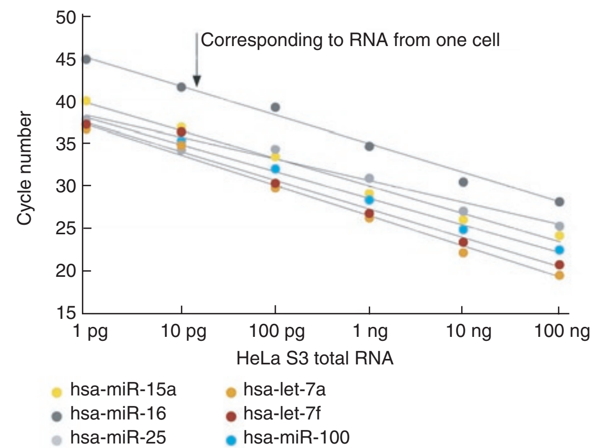

Figure 3 | Dynamic range of six different miRCURY LNA microRNA PCR assays. Results of real-time PCR analysis of endogenous miRNA expression quantified from HeLa S3 cell total RNA. All assays show linear readout for different amounts of total RNA input. The linearity is present even at total RNA input amounts of 1 pg.

quantitative PCR process based on SYBR Green detection (**Fig. 1**). The miRNA is converted by reverse transcription into a cDNA using an miRNA-specific primer and is then amplified by PCR using the miRNA-specific short LNA primer.

LNA-based primers rescue miRNA assays

LNA has proven critical for the successful quantitation of miRNA by real-time PCR. For more than 60% of the approximately 250 miRNA PCR-based assays developed so far, the use of LNAs has improved the assays, resulting in lower cycle threshold values and higher sensitivity[7]. In several cases, LNA primers have even rescued assays that did not work with DNA primers. DNA primers that failed to produce a usable PCR assay often had a T_m that was too low because of miRNA sequence characteristics (**Fig. 2**), but this problem was circumvented by the introduction of high-affinity LNAs.

The improved performance of the assays with LNA-based primers is reflected in a high sensitivity that allows detection of moderately expressed miRNAs from as little as a single cell (10 pg total RNA; **Fig. 3**). With these primers, the assays show a high dynamic range that spans 8 orders of magnitude with a linear readout and a lower detection limit of 10 copies per cell (data not shown), so that miRNAs with both high and low expression levels can be detected in the same sample. Furthermore, LNA-based primers discriminate well between miRNAs belonging to the same family that differ only by a few nucleotides (data not shown).

Conclusion

The miRCURY LNA microRNA PCR system is an accurate and sensitive PCR system for quantitation of miRNAs from small total RNA samples. Assays that use LNAs ensure accurate quantitation of miRNA concentrations spanning 8 orders of magnitude, enabling detection of as few as 10 copies per cell.

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