

Technical note: LNA™-based systems are superior for profiling of microRNAs displaying 3'-end heterogeneity

Abstract

It has been demonstrated that a particular microRNA can occur in a cell or tissue as more than one variant. The difference in sequence between the variants is primarily found at the 3'-end of the molecule. The functional significance of this 3'-end heterogeneity is still not fully understood, but it is likely that the regulatory effect of microRNAs is not restricted to one particular variant. When analyzing microRNA expression, it is, therefore, important that all variants are detected. In this study, we have evaluated the ability of microarray and real-time PCR platforms based on different technologies to detect microRNAs independent of 3'-end heterogeneity. The LNA™-based miRCURY LNA™ microRNA Array and miRCURY LNA™ microRNA PCR System were found to be less vulnerable to common microRNA 3'-end heterogeneity than platforms utilizing loop DNA or full-length DNA probes/primers. The LNA™-based systems, therefore, provide the most accurate data and the most biologically relevant microRNA expression profiles.

Introduction

MicroRNAs are an important class of non-coding, short, endogenous RNAs that act as posttranscriptional regulators of mRNAs by base pairing to their 3' untranslated regions. The mature microRNAs (19-25 nucleotides long) are processed from longer, hairpin transcripts by the RNase III ribonucleases Drosha¹ and Dicer^{2,3}.

The miRBase database at the Wellcome Trust Sanger Institute annotates microRNAs based on large-scale cloning studies that define sequence boundaries of annotated microRNAs⁴. Recently it has been demonstrated that many of the annotated microRNAs can occur as different variants than the one annotated in miRBase. The difference between such variants most often lies at the 3'-end of the molecule and typically varies 1-3 nucleotides in length. Such 3'-end heterogeneity variation has been demonstrated both by Northern blotting⁵⁻¹⁰ and in cloning studies¹¹⁻¹³. The microRNA sequence annotated in miRBase will often be that of the predominant variant. However, it may not be the predominant variant in all cells and tissues¹³ and the polymorphic profile can vary from tissue to tissue¹². The biological relevance of 3'-end heterogeneity has been discussed in several publications, and it has been suggested that it might play a role in subcellular localization or functional efficacy¹³ and that untemplated heterogeneity might be a signal for microRNA degradation¹⁴. Wu *et al.*¹³ predict that enzymes with end-modifying activity that act downstream of the Drosha/Dicer processing might be the cause of the end heterogeneity, while others suggest that it might be due to inaccurate Dicer processing or degradation of the 3'-end of the microRNA^{11,15}.

Figure I

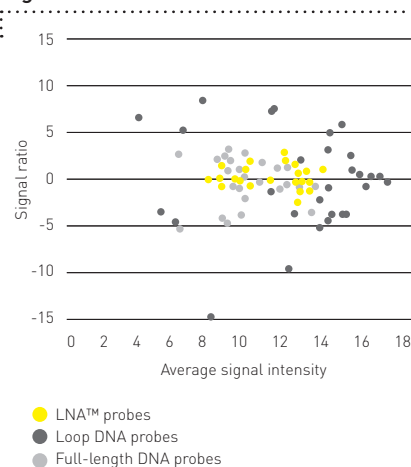


Figure I. Variation in array signal ratios between miRBase-annotated sequences and 3'-end variants. Twenty-nine synthetic microRNA pairs of a miRBase annotated sequence and a selected 3'-end variant were hybridized to three different array platforms, and the annotated/variant signal ratios were calculated. The results are presented in an MA-plot in which the x-axis= $\frac{1}{2} \times \text{Log}_2(\text{signal from annotated sequence} \times \text{signal from variant})$ and the y-axis= $\text{Log}_2(\text{signal from annotated sequence} / \text{signal from variant})$. The full-length DNA capture probes (grey) failed to detect both the annotated sequence and the 3'-end variant in two cases. The LNA™-based array (yellow) is clearly less vulnerable to 3'-end variation in the target microRNA than the full-length DNA and loop DNA (black) capture probe-based arrays.

Figure II

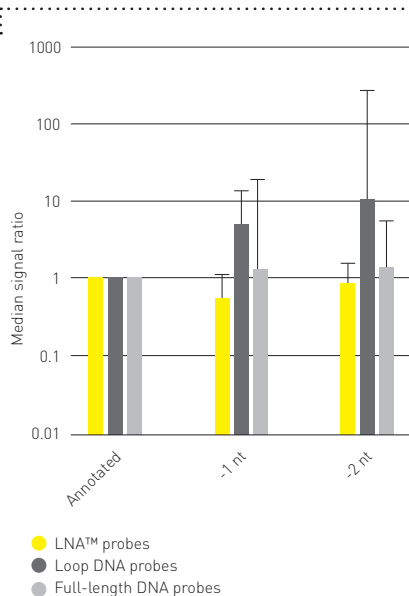


Figure II. Median array signal ratios of the annotated microRNA to variants with shorter 3'-ends. Each bar represents median ratios for 5-9 different microRNAs. The LNA™-based miRCURY LNA™ Array and the full-length DNA-based array detect all variants with equal sensitivity, while the loop DNA-based array is significantly affected by 3'-end variation with drastically reduced sensitivity towards the shorter variants.

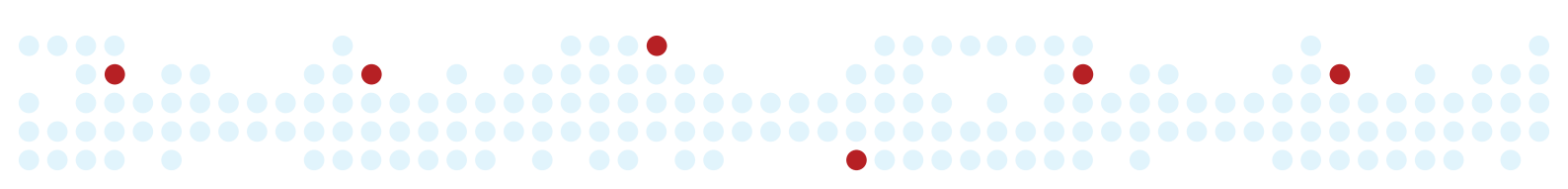
It is likely that the regulatory effect of a particular microRNA is not limited to the sequence annotated in miRBase. Therefore, it is important to detect all variants in order to obtain a functionally relevant microRNA expression profile. However, techniques for detection of microRNAs often have different sensitivity towards such variants because the techniques were designed to specifically detect the sequence annotated in miRBase. This can be a problem for detection of microRNAs for which the annotated sequence only constitutes a small fraction of all the variants or if regulation in the variant profile occurs. Such a situation has been described by Wu *et al.*¹³ who found that the mmu-miR-21 sequence annotated in miRBase matched only 24-57% of the variants found in Naive, Effector and Memory T cells. In addition, Ruby *et al.*¹¹ found that for some microRNAs in *C. elegans* the annotated sequence only constitutes 16% of the total pool of variants.

Results - microarray analysis

In order to evaluate the ability of different microRNA detection platforms to detect 3'-end variants of microRNAs, 29 synthetic microRNAs that were identical to miRBase annotated sequences were made. For each of the 29 microRNAs, a synthetic sequence that differed from the miRBase sequence by 1 to 3 nucleotides at the 3'-end was also obtained. The chosen variants had been identified by 454 Sequencing of human breast cancer samples prior to this study. The synthetic microRNAs were hybridized to three different microRNA microarray platforms that utilize either LNA™-, loop DNA or full-length DNA capture probes. For each microarray platform, the signal ratios between the miRBase sequence and the 3'-end variant were calculated and used to assess differences in sensitivity towards the two sequences.

The LNA™-based array (miRCURY LNA™ microRNA Array) had the least difference in signal from the two variants tested for each capture probe as visualized in the MA plot in Figure I. The signal ratios between the miRBase sequence and the 3'-end variants averaged 2.0 for the LNA™-based array. In comparison, the ratios for the loop DNA- and full-length DNA-based arrays were >1000-fold and 6.7-fold, respectively, demonstrating that these arrays are strongly affected by 3'-end heterogeneity in the target microRNAs.

When comparing median signal ratios from variants that were one or two nucleotides shorter than the corresponding miRBase-annotated sequence, the loop DNA-based array clearly has difficulty detecting the shorter variant (Figure II). In contrast, the LNA™-based and full-length DNA-based arrays were found to be less sensitive to such truncations. However, the full-length DNA-based array produces maximum ratios between the annotated microRNA and the variant of >40, whereas the LNA™-based array only produces ratios of up to 2.7.



Results - real-time PCR analysis

The miRCURY LNA™ microRNA PCR System is based on the use of primers that contain a number of LNA™-monomers. The ability of this system to detect 3'-end microRNA variants was compared to that of a competitor real-time PCR system utilizing loop DNA primers. While the two systems were equally sensitive to the miRBase-annotated sequences, it was clear that the loop DNA primers were not able to detect shorter variants with the same sensitivity as the LNA™-based system (Figure III). When the target microRNA was shortened by only 1 nucleotide in the 3' end, the average cycle number increased by more than 5, corresponding to a greater than 40-fold drop in sensitivity.

The human microRNA hsa-let-7b is shown here as a specific example of the difference between the two PCR systems. The common 3'-end variants found for hsa-let-7b (Figure IV) were quantified in both systems. While the miRCURY LNA™ microRNA PCR System was resistant to changes in the 3'-end, the loop primer system clearly had difficulties detecting the shorter variants (Figure V).

Conclusion

The LNA™-based systems (miRCURY LNA™ microRNA Arrays and miRCURY LNA™ microRNA PCR System) are less vulnerable to 3'-end heterogeneity than competitor platforms utilizing pure DNA or stemloop probes/primers. The LNA™-based systems are therefore likely to provide researchers with the most biologically relevant microRNA expression profiles.

LNA™ makes it possible to design capture probes or primers that have the optimal T_m while at the same time avoiding that the probe/primer efficiency is dependent on the most 3'-end positions of the microRNA. This makes the use of loop probes/primers or full-length probes superfluous, and provides the LNA™-based systems with a superior advantage.

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Figure III

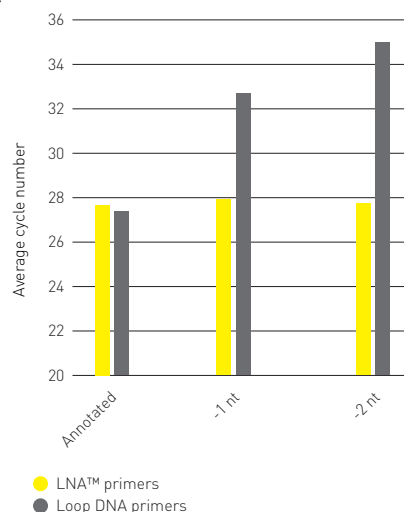


Figure III. Average real-time PCR cycle number values of annotated microRNAs and variants with shorter 3'-ends. Synthetic microRNAs were used as templates and quantification was done using the miRCURY LNA™ microRNA PCR System (yellow) and a loop DNA primer based real-time PCR system (grey). The values are an average of 3-6 microRNAs in different 3'-end length variants. It is clear, that the LNA™-based system is not affected by 3'-end heterogeneity, while the loop DNA-based system is unable to detect the shorter variants satisfactorily.

Figure IV

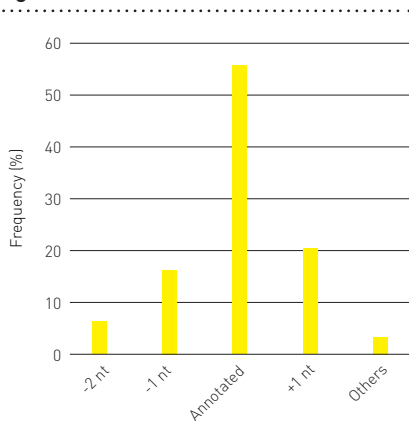


Figure IV. Distribution of hsa-let-7b 3'-end variants in breast cancer tissue. Data are extracted from 454 Sequencing (1597 counts in total were detected from all hsa-let-7b variants).

Figure V

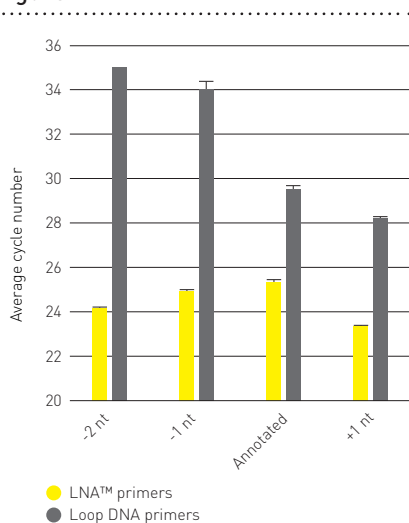


Figure V. Quantitation of hsa-let-7b variants by real-time PCR. Cycle numbers obtained from quantitation by the miRCURY LNA™ microRNA PCR System (yellow) and a loop primer based real-time PCR system (grey) are shown. Synthetic 5'-phosphorylated microRNAs were used as target molecules. The loop DNA probe design was very vulnerable to a reduction of target length, whereas the miRCURY LNA™ microRNA PCR System was unaffected by 3'-end variation.

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Concerning the miRCURY LNA™ microRNA PCR System:

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