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Functional studies of microRNA based on knockdown using Locked Nucleic Acid probes

Here we present a microRNA (miRNA) knockdown application, based on the use of Locked Nucleic Acid (LNA™)-modified probes. The use of LNA for the knockdown of miRNA is particularly effective because these probes have high affinity for their short RNA targets and excellent mismatch-discrimination capability. Here we demonstrate that LNA-modified probes, termed miRCURY™ LNA Knockdown probes, are highly specific and potent tools for functional analysis of miRNAs.

The importance of microRNAs

Mature miRNAs are 16–29 nucleotides (nt) long and are made by sequential processing from longer hairpin transcripts by the RNase III ribonucleases Drosha and Dicer. miRNAs have emerged as an important class of short endogenous RNAs that act as regulators of gene expression by base-pairing with their target mRNAs. This results in repression of translation of the target mRNAs or in their degradation, depending on the degree of sequence complementarity between the miRNA and its mRNA target.

The study of microRNAs has become of considerable interest to researchers who study the control of gene expression, or search for biomarkers and therapeutic targets. The explosion of interest in miRNAs in the past two years necessitates effective tools for functional analysis of these molecules. For this purpose, LNA-based miRNA knockdown has been demonstrated to be very efficient and reliable[1–5].
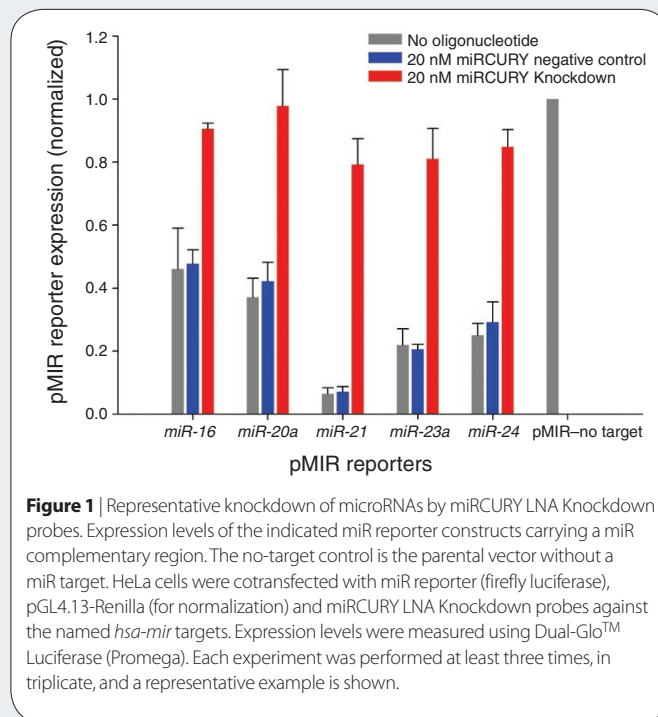
Locked Nucleic Acids

LNAs are a class of nucleic acid analogs in which the ribose ring is 'locked' with a methylene bridge connecting the 2'-O atom with the 4'-C atom. LNA nucleosides containing the six common nucleobases (thymidine, cytosine, guanosine, adenosine, uridine and methyl cytosine) that appear in DNA and RNA are able to form base pairs with their complementary nucleosides according to standard Watson-Crick base-pairing rules.

The inclusion of LNAs in oligonucleotides dramatically increases the affinity for the target. The addition of a single LNA nucleotide typically increases the melting temperature (T_m) of the oligonucleotide -target duplex by 2–6 °C, in sequences below 30 nt in length. Furthermore, it has been demonstrated that LNA-containing probes have excellent mismatch discrimination capabilities, expressed as a
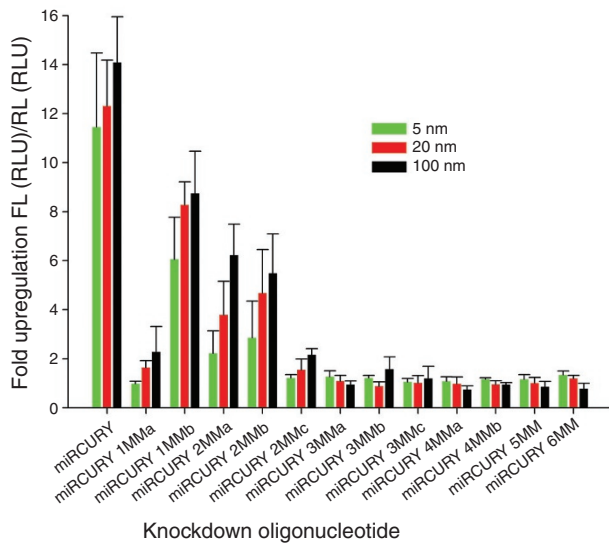

Figure 1 | Representative knockdown of microRNAs by miRCURY LNA Knockdown probes. Expression levels of the indicated miR reporter constructs carrying a miR complementary region. The no-target control is the parental vector without a miR target. HeLa cells were cotransfected with miR reporter (firefly luciferase), pGL4.13-Renilla (for normalization) and miRCURY LNA Knockdown probes against the named *hsa-mir* targets. Expression levels were measured using Dual-Glo™ Luciferase (Promega). Each experiment was performed at least three times, in triplicate, and a representative example is shown.

substantial perturbation in T_m against a mismatched target[6]. These LNA-containing oligonucleotides have low cytotoxicity and are easy to transfect.

Developing miRCURY LNA Knockdown probes

In addition to being short, a fair proportion of miRNAs have high sequence similarity. For example, some miRNAs differ from each other by only a single base. The short length of miRNAs and the relatedness of their sequences are recurring themes in the miRNA world. In this context, the properties of LNAs discussed above make them excellent tools for studying miRNAs—LNA-based knockdown allows for high-potency knockdown without compromising specificity.

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APPLICATION NOTES

Figure 2 | Mismatch discrimination ability of miRCURY LNA Knockdown probes. Results are from a representative experiment ($n = 3$) for perfectly matched versus mismatched knockdown probes against *hsa-mir-21*. The figure shows fold upregulation of the pMIR-21 luciferase reporter relative to the no-oligonucleotide control upon transfection with various mismatched (MM) knockdown oligonucleotides. Experiments were carried out in HeLa cells, and luciferase expression levels were measured 24 h after transfection using Dual-Glo Luciferase. FL, firefly luciferaase signal; RL, renilla luciferase signal. In each case the signal is defined in relative light units (RLU).
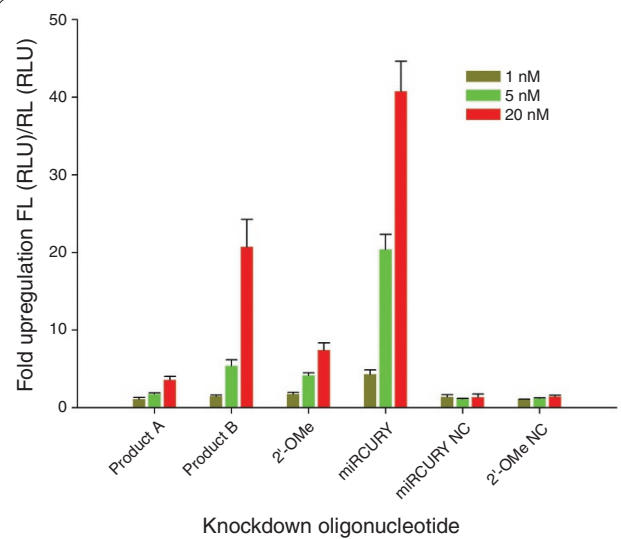
Figure 3 | Comparison of the effectiveness of different knockdown methods. The diagram shows results of a representative experiment ($n = 3$), indicating fold upregulation, relative to the untreated control of the pMIR-21 reporter (firefly luciferase) when cotransfected with various *hsa-mir-21* knockdown products. Expression levels in MCF7 cells were measured 60 h after transfection. The negative control (NC) oligonucleotides are 2′-OMe or LNA-DNA mixmers with a random sequence showing no significant complementarity to any known miRNAs.

LNAs have proven to be an excellent tool in miRNA research, particularly in the areas of miRNA profiling and *in situ* hybridization[7,8]. Research into miRNA using LNA-based tools has generated important information concerning the roles of miRNAs as biomarkers, the evolution of miRNAs in vertebrates and their role in developmental biology. LNAs, however, can also be used for more functional studies of miRNAs. We and others have found that LNA-based miRNA knockdown is very efficient and reliable for this purpose.

Exiqon uses in-house bioinformatics expertise to optimally design probes—called miRCURY LNA Knockdown probes—that result in specific knockdown of miRNAs (**Fig. 1**).

LNA-based knockdown probes have high potency without compromising specificity (**Fig. 2**). A large decrease in knockdown inhibition is demonstrated with the introduction of a single mismatch between knockdown probe and miRNA target. The introduction of two mismatches between knockdown probe and miRNA target leads to complete elimination of inhibition.

miRCURY LNA Knockdown probes are also more effective at knocking down miRNAs than standard competing methods such as DNA-based probes and 2′-O-methyl (2′-OMe)-based probes (**Fig. 3**).

Conclusion

LNA-based oligonucleotide probes have enhanced studies of miRNA by providing improved detection of these short RNAs owing to enhanced binding properties. These properties have now been exploited to develop probes that can be used to effectively knock down miRNA function in cells. These miRCURY LNA Knockdown probes work by providing specific knockdown of miRNA with much higher potency than traditional antisense technologies. Several publications demonstrate the effectiveness of these probes[1–4].

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