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Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes

Anna Válóczi, Csaba Hornyik, Nóra Varga, József Burgyán, Sakari Kauppinen[1] and Zoltán Havelda*

Agricultural Biotechnology Center, Szent-Györgyi Albert ut 4, Gödöllő H-2100, Hungary and [1]Department of Functional Genomics, Exiqon, Bygstubben 9, DK-2950 Vedbaek, Denmark

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ABSTRACT

We describe here a new method for highly efficient detection of microRNAs by northern blot analysis using LNA (locked nucleic acid)-modified oligonucleotides. In order to exploit the improved hybridization properties of LNA with their target RNA molecules, we designed several LNA-modified oligonucleotide probes for detection of different microRNAs in animals and plants. By modifying DNA oligonucleotides with LNAs using a design, in which every third nucleotide position was substituted by LNA, we could use the probes in northern blot analysis employing standard end-labelling techniques and hybridization conditions. The sensitivity in detecting mature microRNAs by northern blots was increased by at least 10-fold compared to DNA probes, while simultaneously being highly specific, as demonstrated by the use of different single and double mismatched LNA probes. Besides being highly efficient as northern probes, the same LNA-modified oligonucleotide probes would also be useful for miRNA *in situ* hybridization and miRNA expression profiling by LNA oligonucleotide microarrays.

INTRODUCTION

MicroRNAs (miRNAs) are 19–25 nt non-coding RNAs that are processed from longer endogenous hairpin transcripts by the enzyme Dicer (1,2). To date, >1100 microRNAs have been identified in invertebrates, vertebrates and plants according to the miRNA registry database and many miRNAs that correspond to putative genes have also been identified (3). Some miRNAs have multiple loci in the genome (4) and occasionally, several miRNA genes are arranged in tandem clusters (5). Growing evidence suggests that miRNAs play crucial roles in eukaryotic gene regulation. The first miRNAs genes to be discovered, lin-4 and let-7, base-pair incompletely to repeated elements in the 3′ untranslated regions (UTRs) of other heterochronic genes in *Caenorhabditis elegans*, and regulate the translation directly and negatively by antisense RNA–RNA interaction (6,7). Other miRNAs are thought to interact with target mRNAs by limited complementarity and suppressed translation as well (5,8). Many studies have shown, however, that given a perfect complementarity between miRNAs and their target RNA, could lead to target RNA degradation rather than inhibit translation (9,10), suggesting that the degree of complementarity determines their functions. By identifying sequences with near complementarity, several targets have been predicted, most of which appear to be potential transcriptional factors that are crucial in cell growth and development. The high percentage of predicted miRNA targets acting as developmental regulators and the conservation of target sites suggests that miRNAs exhibit a wide variety of regulatory functions and exert significant effects on cell growth, development and differentiation (11,12), including human development and disease. For example, a recent study determined that two different human miRNA genes (miR15a and miR16a) are clustered and located within the intron of LEU2, which, in turn, lies within the deleted minimal region of the B-cell chronic lymphocytic leukaemia (B-CLL) tumour suppressor locus, and that both miRNA genes are deleted or down-regulated in the majority of CLL cases (13).

The current view that miRNAs represent a newly discovered, hidden layer of gene regulation has resulted in high interest among researchers in the discovery of miRNAs, their targets, expression and mechanism of action. Most miRNA researchers use northern blot analysis combined with polyacrylamide gels to examine the expression of both the mature and precursor miRNAs, since it allows both quantitation of the expression levels as well as miRNA size determination (5,7,8,13). A major drawback of this method is its poor sensitivity, especially when monitoring expression of low-abundant miRNAs. Consequently, a large amount of total RNA per sample is required for northern analysis, which is not feasible when the cell or tissue source is limited. We describe here a new method for highly efficient detection of miRNAs by northern blot analysis using locked nucleic acid (LNA)-modified oligonucleotide probes, and demonstrate its significantly improved sensitivity and high specificity by

*To whom correspondence should be addressed. Tel: +36 28 526100; Fax: +36 28 526145; Email: havelda@abc.hu
Correspondence may also be addressed to Sakari Kauppinen. Tel: + 45 45 66 08 88; Fax: + 45 45 66 18 88; Email: sk@exiqon.com

detection of different microRNAs in mouse, *Arabidopsis thaliana* and *Nicotiana benthamiana*.

MATERIALS AND METHODS

Experimental material

Six-week-old *N.benthamiana* and *A.thaliana* plants grown in soil under normal growth conditions were used for collecting flowers and leaves. Dissected organs (brain and liver) from 4-week-old mice were used for preparing mouse total RNA samples.

Synthesis of the LNA-modified oligonucleotide probes

The LNA-modified and DNA oligonucleotide probes (Table 1) were synthesized in 0.2 μmol scale on an Expedite 8909 synthesizer (Applied Biosystems, Foster City, CA) using the phosphoramidite method (14). Commercially available DNA and LNA phosphoramidite monomers and reagents were used according to the conventional protocols (for details, see www.exiqon.com). The oligonucleotides were subsequently removed from the solid support and deprotected by treatment with concentrated NH_4OH and purified by RP-HPLC. The purity of the oligonucleotides was assessed by SAX–HPLC analysis (LC2010A, Shimadzu, USA), and the molecular masses were confirmed by mass spectrometry using a MALDI-TOF Voyager DE-PRO (Applied Biosystems).

RNA extraction and northern blot analysis

Total RNA was extracted from plant and mouse tissues by using the TRI Reagent (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. Total RNA was fractionated on a denaturing 12% polyacrylamide gel containing 8 M urea, transferred to Nytran N membrane (Schleicher & Schuell, Germany) by capillary method and fixed by ultraviolet cross-linking. Membranes were probed with ^{32}P-labelled standard DNA and LNA-modified oligonucleotides, complementary to the mature microRNAs (Table 1): 10 pmol of each oligonucleotide probe was end-labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Prehybridization of the filters was carried out in 50% formamide, 0.5% SDS, 5× SSPE, 5× Denhardt's solution and 20 μg/ml sheared, denatured salmon sperm DNA. Hybridizations were performed in the same solution at 34–45°C. The labelled probes were heated for 1 min at 95°C before addition to the filters in the prehybridization solution. After hybridization, the membranes were washed at low stringency in 2× SSC, 0.1% SDS at 34–45°C twice for 5 min or at high stringency in 0.1 SSC, 0.1% SDS at 65°C twice for 5 min.

RESULTS AND DISCUSSION

LNAs comprise a new class of bicyclic high-affinity RNA analogues in which the furanose ring in the sugar–phosphate backbone is chemically locked in an N-type (C3′-*endo*) conformation by the introduction of a 2′-*O*,4′-*C* methylene bridge (15,16). Several studies have demonstrated that LNA-modified oligonucleotides exhibit unprecedented thermal stability when hybridized with their DNA and RNA target molecules (16–22). Consequently, an increase in melting temperature (T_m) of +1–8°C per introduced LNA monomer against complementary DNA, and of +2–10°C per monomer against complementary RNA compared to unmodified duplexes have been reported. Structural studies of different LNA–RNA and LNA–DNA heteroduplexes based on NMR spectroscopy and X-ray crystallography have shown that LNA is an RNA mimic, which fits seamlessly into an A-type Watson–Crick duplex geometry (23–26) similar to that of dsRNA duplexes. Furthermore, in heteroduplexes between LNA oligonucleotides and their complementary DNA oligonucleotides, an overall shift from a B-type duplex towards an A-type duplex has been reported resulting in increased stability of the heteroduplexes. Another important observation is that LNA monomers are also able to twist the sugar conformation of flanking DNA nucleotides from an S-type (C2′-*endo*) towards an N-type sugar pucker in LNA-modified DNA oligonucleotides (25–26). The unprecedented thermal stability of LNA oligonucleotides together with

Table 1. Design of the LNA-modified oligonucleotide probes used in this study

Probe name	Probe sequence (5′→3′)[a]	T_m (°C)[b]	miRNA target sequence (5′→3′)[a]
miR171DNA	gatattggcgcggctcaatca	66	ugauugagccgcgccaauauc
miR171LNA2	gAtAtTgGcGcGgCtCaAtCa	83	ugauugagccgcgccaauauc
miR171LNA3	gAtaTtgGcgCggCtcAatCa	78	ugauugagccgcgccaauauc
miR171LNA3/2MM	gAtaTtgGcgAagCtcAatCa	ND	ugauugagccgcgccaauauc
miR171LNA3/MM11	gAtaTtgGcgAggCtcAatCa	ND	ugauugagccgcgccaauauc
miR171LNA3/MM8	gAtaTtgAcgCggCtcAatCa	ND	ugauugagccgcgccaauauc
miR171LNA3/MM14	gAtaTtgGcgCggAtcAatCa	ND	ugauugagccgcgccaauauc
miR122aLNA3	acAaaCacCatTgtCacActCca	78	uggagugugacaaugguguuugu
miR124LNA3	tgGcaTtcAccGcgTgcCttAa	80	uuaaggcacgcggugaaugcca
miR128LNA3	aaAagAgaCcgGttCacTgtGa	77	ucacagugaaccggucucuuuu
miR161LNA3	cCccGatGtaGtcActTtcAa	73	uugaaagugacuacaucgggg
miR167LNA3	tAgaTcaTgcTggCagCttCa	79	ugaagcugccagcaugaucua
miR319DNA	gggagctcccttcagtccaa	66	uuggacugaagggagcuccc
miR319LNA3	ggGagCtcCctTcaGtcCaa	78	uuggacugaagggagcuccc
TCVLNA3	gAacTtcCggActCtaGgaTc	74	gauccuagaguccggaaguuc

[a]LNA nucleotides in uppercase, RNA and DNA nucleotides in lowercase. C denotes LNA methylcytosine.
[b]The melting temperatures (T_m) were predicted using a thermodynamic nearest neighbour model based on the determination of T_m values of a large set of LNA oligonucleotide duplexes (>1000) by UV spectroscopy (23). The LNA T_m prediction tool is accessible at www.exiqon.com.

their improved mismatch discrimination has made them well suited for highly accurate genotyping assays (20,27–29). In addition, LNA-substituted oligonucleotides have been used to increase the sensitivity and specificity in gene expression profiling by spotted oligonucleotide microarrays (30), in potent and selective gene knock-down by LNA antisense (18–19,31–33) and more recently, in efficient isolation of intact poly(-A)$^+$RNA from lysed cell and tissue extracts by LNA oligo(T) affinity capture (21).

To exploit the improved hybridization properties of LNAs against complementary RNA targets and the ability of LNA monomers to perturb neighbouring DNA nucleotides in chimeric LNA–DNA oligonucleotides toward the A-type geometry, we designed several LNA-modified oligonucleotide probes for detection of different microRNAs in animals and plants with regular spacing in the LNA substitution pattern (Table 1). First, we synthesized LNA-modified oligonucleotides complementary to the relatively low-abundant mature *A.thaliana* miR171, by substituting every second and every third nucleotide position with an LNA monomer, designated here as miR171LNA2 and miR171LNA3, respectively (Table 1). Substitution of the miR171 DNA probe with regularly spaced LNAs resulted in significantly increased sensitivity in detecting the mature miR171 in *A.thaliana* flowers and leaves by 5′-labelled probes, when low stringency washing conditions were employed (Figure 1) in the northern blot analysis, in accordance with their increased thermal duplex stability (Table 1). The larger RNAs detected by both LNA-modified miR171 probes as well as with the DNA probe did not correspond to the expected size of the 123 nt miRNA 171 stem–loop precursor, but most likely represented unspecific hybridization to other *A.thaliana* RNAs (Figure 1). Although the use of the radioactively labeled miR171LNA2 probe showed increased sensitivity compared to the DNA probe, it also resulted in a high background under the given conditions, presumably due to the high LNA substitution degree (50% LNA) of the probe, which, in turn, resulted in extremely high duplex stability of the LNA–RNA duplexes. In contrast, the miR171LNA3 probe showed significantly improved sensitivity, while simultaneously having a low background on the northern blot comparable to that of the DNA control probe. Consequently, a specific hybridization signal corresponding to the mature *A.thaliana* miR171 could be obtained after 3 h of exposure with the miR171LNA3 oligonucleotide probe, whereas the DNA control probe required 48 h of exposure in order to obtain a comparable hybridization signal (Figure 1). As expected, the prolonged exposure of the miR171LNA2 and miR171LNA3-probed northern blots resulted in highly overexposed autoradiograms. Similar results were obtained by comparing LNA2- and LNA3-modified probes in the detection of mature miR159 of *A.thaliana*, except that strong miRNA159-specific signals were obtained with the labeled miR159LNA3 probe already after 1 h of exposure (data not shown). Our findings with the LNA-modified microRNA probes are supported by the NMR spectroscopic studies of different LNA–RNA hybrid structures (25,26). Substitution of a complementary 9mer DNA strand with three LNAs in the resulting nonamer LNA3–RNA hybrid inferred that the number of LNA modifications had reached a saturation level with regard to the structural change into an A-like duplex geometry. Hence, the structural alteration introduced by a fully modified LNA nonamer in the LNA–RNA duplex was found to be small compared to the LNA3–RNA duplex, while the T_m of this duplex was significantly increased (26). Similarly, the calculated T_m values of our *A.thaliana* LNA2-modified miRNA probes were higher compared to the LNA3 probes, requiring further optimization of the hybridization and washing conditions in northern blot analysis, as demonstrated by the high background on the northerns. In contrast, the LNA3-modified miRNA probes could be readily used in standard northern blots without further adjustment of the hybridization conditions. We thus decided to use LNA3-substituted microRNA probes in all subsequent comparative experiments.

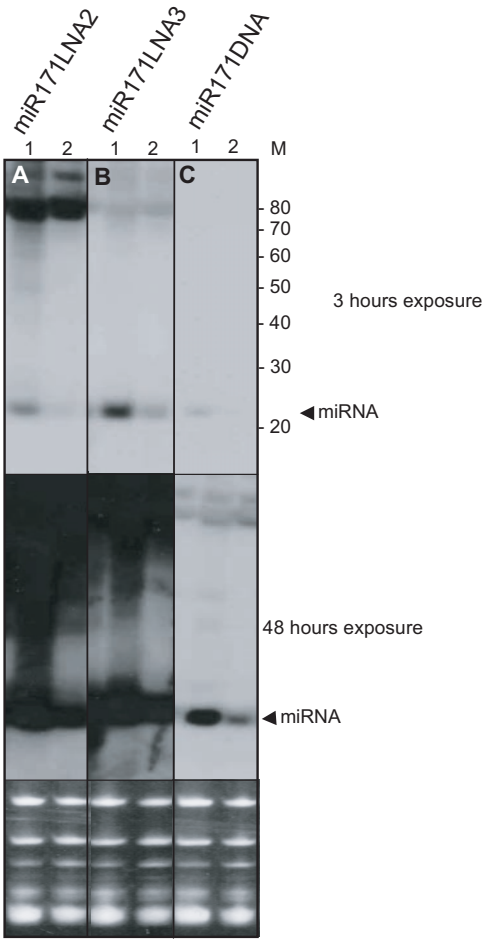

Figure 1. Comparison of LNA2- and LNA3-modified oligonucleotide probes with DNA probes in the detection of the low-abundant miR171 in *A.thaliana* flowers and leaves by northern blot analysis. Total RNAs (40 μg/sample) from *A.thaliana* flowers (lane 1) or leaves (lane 2) were electrophoresed on 12% polyacrylamide gel under denaturing conditions, blotted and hybridized with ^{32}P-labelled miR171LNA2 (**A**), miR171LNA3 (**B**) and miR171DNA (**C**) oligonucleotide probes at 37°C. The membranes were washed at low stringency. The gel loading controls are shown from ethidium bromide staining of the rRNAs (bottom panels). M denotes the RNA molecular weight marker of 20–80 nucleotides.

The sensitivity in detection of mature microRNAs by northern blots by LNA3-modified oligonucleotide probes was assessed by loading serial dilutions of the same *A.thaliana* total RNA sample from 100 to 2.5 μg on the northern gels, followed by hybridization with 5′ end-labelled miR171LNA3,

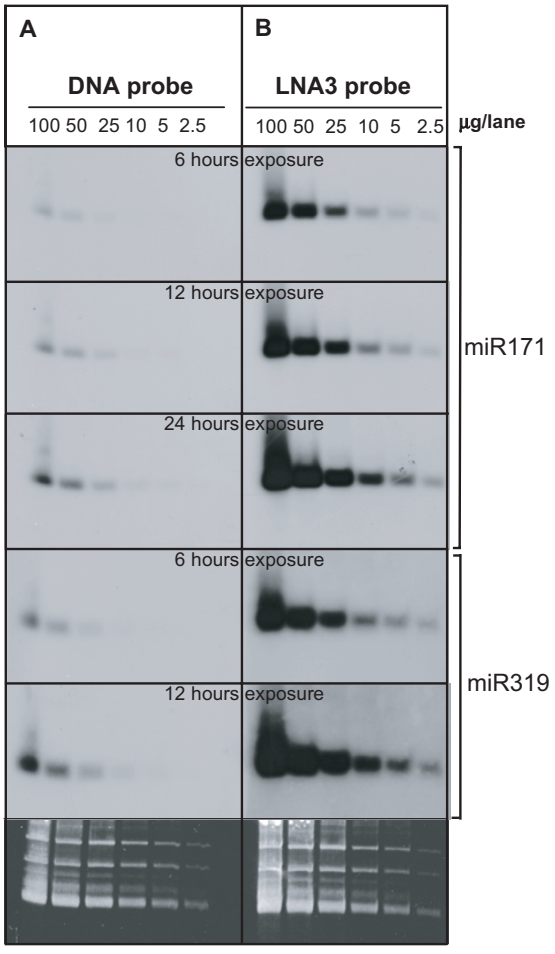

Figure 2. Assessment of the sensitivity of LNA3-modified probes compared with DNA probes in the detection of miR171 and miR319 in *A.thaliana* flowers by northern blot analysis. Two duplicate dilution series of *A.thaliana* total RNA from 100 to 2.5 µg were electrophoresed on 12% polyacrylamide gel under denaturing conditions, blotted and hybridized with ^{32}P-labelled DNA (**A**) and LNA3 (**B**) oligonucleotide probes, respectively, at 34°C. The filters were first hybridized with LNA3 and DNA probes specific for mature mir171, washed with low stringency and exposed as indicated. The filters were stripped, exposed for checking that removal of the probes was complete, and then re-hybridized with LNA3 and DNA probes specific for mature miR319, washed with low stringency and exposed as indicated. The gel loading controls are shown from ethidium bromide staining of the rRNAs (bottom panels).

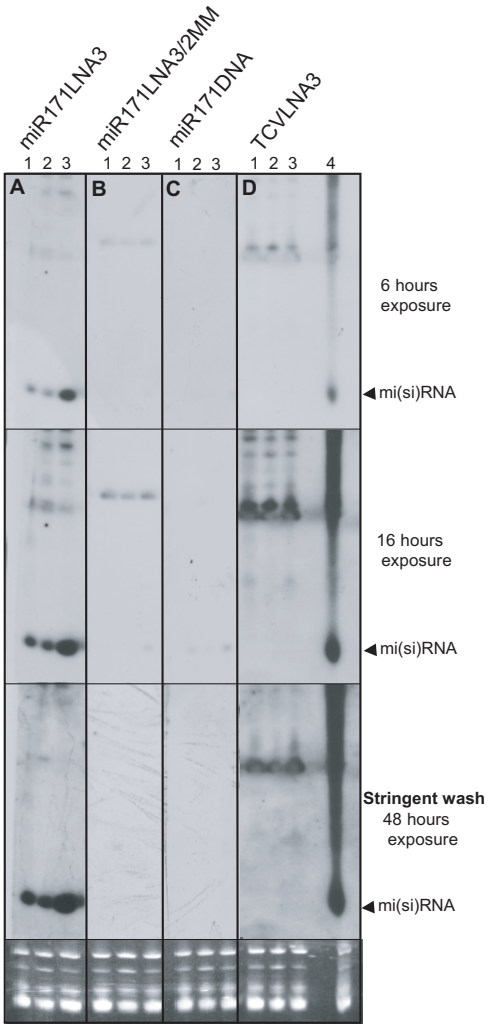

Figure 3. Improved sensitivity and specificity in the detection of miR171 in *A.thaliana* flowers and leaves by northern blot analysis using an LNA3-modified oligonucleotide probe. Total RNAs (20 µg/sample) from *A.thaliana* seedlings (lane 1), leaves (lane 2), flowers (lane 3) and TCV (Turnip crinkle virus)-infected leaves (lane 4) were electrophoresed on 12% polyacrylamide gel under denaturing conditions, blotted and hybridized with ^{32}P-labelled miR171LNA3 (**A**), miR171LNA3/2MM (**B**), miR171DNA (**C**) and TCVLNA3 oligonucleotide probes at 42°C (**D**). Stringent washes were carried out in 0.1× SSC, 0.1% SDS at 65°C twice for 5 min. The gel loading controls are shown from ethidium bromide staining of the rRNAs (bottom panels).

miR171DNA, miR319LNA3 and miR319DNA probes, respectively (Figure 2). The use of LNA3 probes resulted in significantly increased sensitivity for mature *A.thaliana* miR171 and miR319, in accordance with our initial results, showing a readily detectable signal in 2.5 µg of total RNA. The average increase in sensitivity was estimated to be at least 10-fold compared to the corresponding DNA control probes (Figure 2).

Next, we examined the possibility to use high-stringency hybridization and washing conditions that would allow improved specificity in microRNA detection by northern blots, especially when detecting highly homologous miRNAs, e.g. members of the let-7 microRNA family (34). Hybridization of the *A.thaliana* small RNA northern blots with the miR171LNA3 probe resulted in highly specific signals corresponding to the mature miR171 even when using high stringency washing conditions (0.1× SSC, 0.1% SDS at 65°C; Figure 3A). By contrast, the miR171LNA3/2MM probe with two consecutive mismatches in the central positions of the probe (Table 1) and the DNA control oligonucleotide only faintly detected the miR171 on the northern blot (Figure 3B and C). Furthermore, the TCVLNA3 control probe detected specifically the virus-derived siRNAs in *Turnip crinkle virus* (TCV)-infected *A.thaliana* plants, but no small RNAs in the uninfected plant RNA samples (Figure 3D). Taken together, these results indicate that modification of DNA oligonucleotide probes with LNA employing the LNA3 substitution pattern enables the use of high stringency hybridization conditions in the detection of mature microRNAs by northern analysis.

To further investigate the discriminatory power of LNA3-modified microRNA probes, we synthesized three additional mismatch probes for *A.thaliana* miR171, miR171LNA3/MM8, miR171LNA3/MM11, miR171LNA3/MM14, having a single mismatch at three different positions of the oligonucleotide sequence (Table 1). Hybridization of the *A.thaliana* small RNA northern blot resulted in significantly decreased signals for the mature miRNA171 by all three mismatch probes compared to the perfect match probe although at somewhat different extent, whereas no signals were detected by the double mismatch probe (Figure 4). By using high stringency washing conditions, the hybridization signal obtained by the single mismatch probes could be decreased even further, whereas the signal obtained with the perfect match probe miR171LNA3 remained unchanged under the same conditions (Figure 4). This is in good agreement with previous reports for improved mismatch discrimination by LNA probes (20,27–29), and implies that the microRNA detection by labelled, LNA3-substituted probes is highly specific.

Finally, we demonstrated the utility of the LNA3-modified miRNA oligonucleotide probes in characterizing expression patterns of five different microRNAs, the mouse miR122a, miR124 and miR128, and plant miR161 and miR167, respectively. Total RNA samples extracted from mouse brain and liver, and *A.thaliana* and *N.benthamiana* flowers and leaves were subjected to northern blot analyses and hybridized with the different LNA3-modified probes. Highly specific signals corresponding to the mature mouse miR124 and miR128 were detected in the mouse brain, but not in the liver (Figure 5B and C), while miR122a was detected only in the liver RNA sample (Figure 5A), which is in good agreement with previous reports (35). No unspecific background signals were detected using TCVLNA3 probe as a negative control (Figure 5D). The mature miR167 was detected both in *A.thaliana* and *N.benthamiana* flowers and leaves (Figure 5E), whereas our

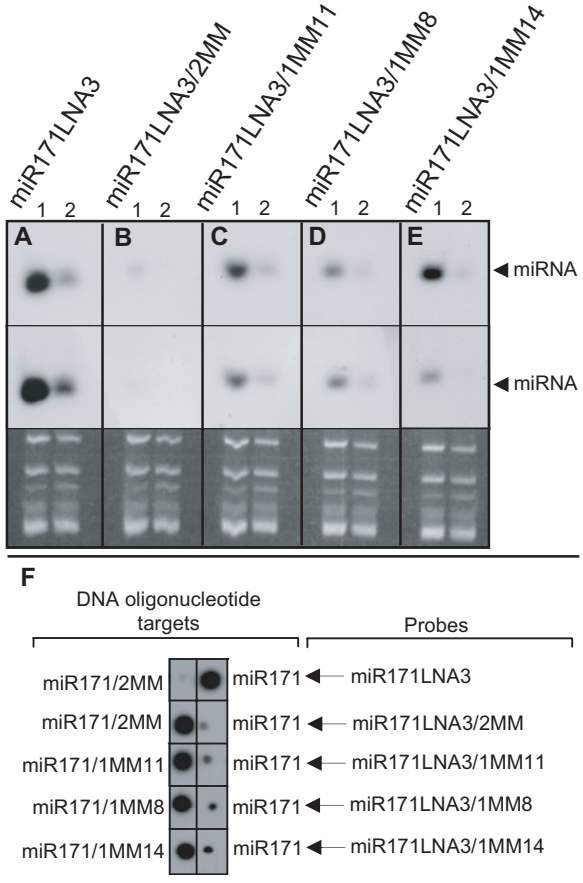

Figure 4. Assessment of the specificity of LNA3-modified probes using perfect match and different mismatched probes in the detection of miR171 in *A.thaliana* flowers and leaves by northern blot analysis. Total RNAs (20 µg/sample) from *A.thaliana* flowers (lane 1) and leaves (lane 2) were electrophoresed on 12% polyacrylamide gel under denaturing conditions, blotted and hybridized with ^{32}P-labelled miR171LNA3 (**A**), miR171LNA3/2MM (**B**), miR171LNA3/MM11 (**C**), miR171LNA3/MM8 (**D**) and miR171LNA3/MM14 (**E**) LNA-modified oligonucleotide probes at 45°C. The filters were washed at low stringency (upper panels) and high stringency (middle panels). The gel loading controls are shown from ethidium bromide staining of the rRNAs (bottom panels). (**F**) Validation of the perfect match and the different mismatched LNA probes using complementary DNA oligonucleotide targets. The end-labelled LNA-modified probes were hybridized to their respective perfect match DNA oligonucleotide targets as well as a DNA oligonucleotide corresponding to the mature miRNA171 sequence.

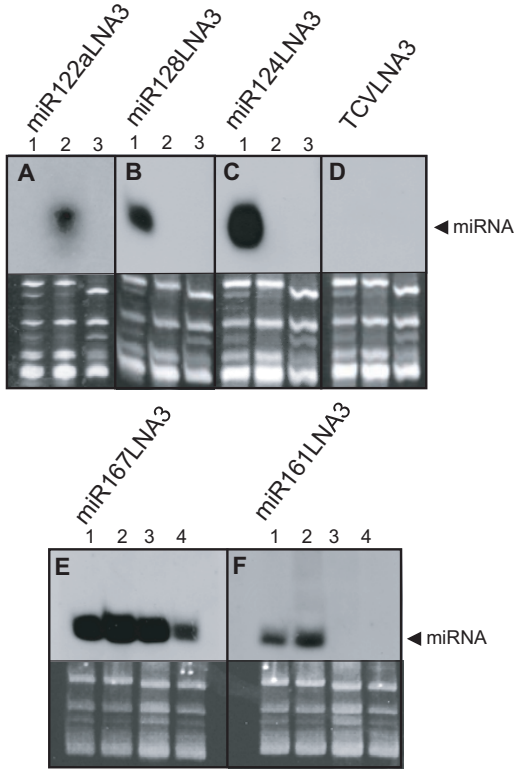

Figure 5. Northern blot analysis of three mouse and two *A.thaliana* microRNAs using LNA3-modified oligonucleotide probes. (A–D) Total RNAs (20 µg/sample) from mouse brain (lane 1), liver (lane 2) and *A.thaliana* flowers (lane 3) were electrophoresed on 12% polyacrylamide gels under denaturing conditions, blotted and hybridized with ^{32}P-labelled miR122aLNA3 (**A**), miR128LNA3 (**B**), miR124LNA3 (**C**) LNA probes as well as TCVLNA3 as a negative control probe (**D**) at 45°C. (**E** and **F**) Total RNAs (20 µg/sample) from *A.thaliana* flowers (lane 1), leaves (lane 2) and *N.benthamiana* flowers (lane 3), leaves (lane 4) were electrophoresed on 12% polyacrylamide gel under denaturing conditions, blotted and hybridized with ^{32}P-labelled miR167LNA3 (E), miR161LNA3 (F) oligonucleotide probes at 45°C. The filters were washed at low stringency. The gel loading controls are shown from ethidium bromide staining of the rRNAs (bottom panels).

results revealed that the presence of miR161 was specific for *A.thaliana* tissues since no signal was detected in samples originating from *N.benthamiana* (Figure 5F).

In conclusion, we report here that partial substitution of DNA oligonucleotides by LNA results in significantly improved sensitivity in detecting mature microRNAs by northern blot analysis, while simultaneously being highly specific. Because the electrostatic properties of LNA probes are similar to DNA and RNA oligonucleotides, and the fact that LNA chemistry is fully compatible with DNA phosphoramidite chemistry, makes it easy to synthesize LNA oligonucleotide probes using standard phosphoramidite chemistry and oligonucleotide purification methods. Another important practical advantage is that the LNA-modified oligonucleotides are fully soluble in water, which makes their labelling and use in nucleic acid hybridization experiments simple (16,17). By modifying DNA oligonucleotides with LNAs using the LNA3 substitution pattern, in which every third nucleotide position is modified by the corresponding LNA nucleotide, we were able to use the probes in northern blot analysis employing standard end-labelling methods and hybridization conditions. The sensitivity in detecting mature microRNAs by northern blots was increased by at least 10-fold compared to DNA probes, while simultaneously being highly specific as demonstrated by the use of mismatch LNA3 probes. Besides being highly efficient as northern probes, the same LNA3-modified oligonucleotide probes could also be useful for addressing the spatial expression of microRNAs by *in situ* hybridization as well as expression profiling by spotted microarrays using different LNA-modified capture probes designed to detect mature and precursor microRNAs.

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