EXIQON

MicroRNA expression profiling using LNA-modified probes in a liquid-phase bead-based array

Here we describe a new liquid-phase, bead-based array for in-solution expression analysis of microRNAs (miRNAs). The array combines Locked Nucleic Acid (LNA™)-modified capture probes and the xMAP® multiplexing bead technology. Incorporation of LNA in the array capture probes greatly increases their affinity for their short miRNA targets, thereby adding selectivity to the array. As a result, the FlexmiR™ system offers a highly specific, robust and fast miRNA profiling platform.

miRNAs are short, noncoding RNA molecules, 16–29 nucleotides long, that modulate gene expression by base-pairing with the 3′ untranslated region of their target mRNAs. Depending on the degree of complementarity between the miRNA and its mRNA target, the interaction leads to either inhibition of translation or degradation of the mRNA. It is estimated that miRNAs may regulate as many as 30% of all genes in mammalian genomes, and the importance of this recently discovered class of RNA molecules is well reflected by the large increase in miRNA-related scientific publications in the last 2–3 years. miRNAs are being intensively studied as regulators of gene expression, potential therapeutic targets and biomarkers. Hence, the need for effective, easy-to-use tools for miRNA analysis is steadily increasing. In particular, as the number of annotated miRNAs continues to increase, large-scale miRNA expression profiling tools have become in great demand. Several miRNA microarrays have been developed for this purpose, but the selection has so far been limited to planar arrays. However, as miRNA research continues to reveal distinct condition-related miRNA patterns, researchers will require a solution that allows for miRNA screening as well as focused multiplexing of their specific miRNA targets. The FlexmiR miRNA product line, developed by Exiqon in collaboration with the Luminex Bioscience Group, constitutes the first commercially available bead-based system for miRNA expression analysis that offers a solution to the challenges of both screening and focused profiling.

Using LNA in miRNA analysis

miRNA analysis presents several major challenges. First of all, the short nature of the target sequences makes it difficult to achieve sufficient specificity with standard DNA oligonucleotide technologies. Furthermore, some miRNAs differ from each other by as little as a single nucleotide, emphasizing the importance of good mismatch discrimination. LNAs have proven to be extremely useful in overcoming these obstacles, particularly in the areas of miRNA profiling and *in situ* hybridization[1,2].

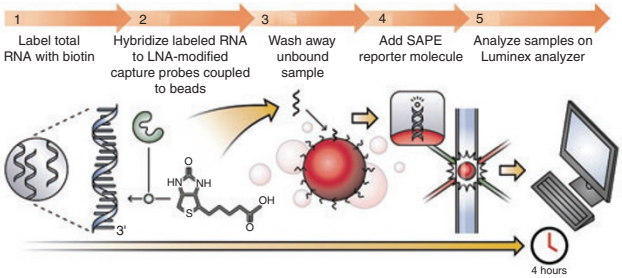

Figure 1 | The fast and simple FlexmiR workflow.

LNAs are a class of conformationally restricted nucleotide analogs. The incorporation of LNA in an oligonucleotide increases the affinity of that oligonucleotide for its complementary RNA or DNA target by increasing the melting temperature (T_m) of the duplex. Additionally, the T_m difference between a perfectly matched target and a mismatched target is substantially higher than that observed when a DNA-based oligonucleotide is used. These properties—high T_m and excellent mismatch discrimination—make LNA-modified probes ideal for analysis of short and very similar targets like miRNAs. Furthermore, by adjusting the LNA content and probe length, it is possible to design T_m-normalized probes, thereby allowing hybridization conditions that are optimal for all probes used on, for example, an array.

Developing a bead-based miRNA array with LNA-modified capture probes

Several planar microarrays are commercially available for miRNA expression profiling, including the miRCURY™ LNA Arrays from Exiqon. Although these arrays provide excellent platforms for miRNA analysis, the experimental procedures leading to generation of data are not trivial, and a typical protocol takes a minimum of 24 h.

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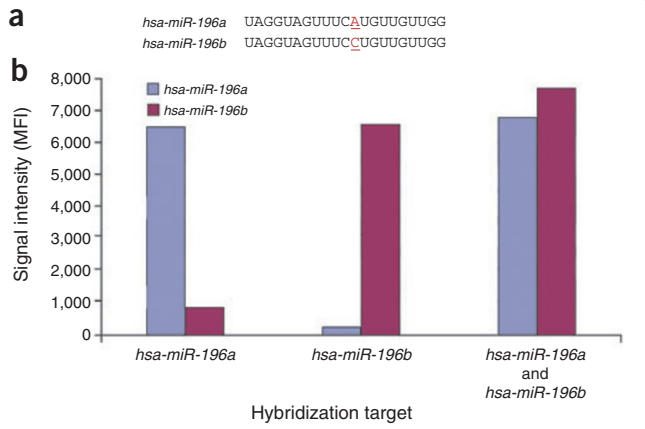

Figure 2 | Single-mismatch discrimination ability of the FlexmiR system. (**a**) The two human miRNAs, *hsa-miR-196a* and *hsa-miR-196b*, differ by only one nucleotide. (**b**) Synthetic DNA oligonucleotides, with 5′ biotin modification and sequences corresponding to *hsa-miR-196a* and *hsa-miR-196b*, were hybridized to a pool of FlexmiR beads. The pool contained 73 bead sets, each coupled to an LNA-modified capture probe targeting a specific human miRNA, including one set specific for *hsa-miR-196a* and one set specific for *hsa-miR-196b*. Three hybridizations were performed: one with an *hsa-miR-196a* target, one with an *hsa-miR-196b* target and one with the two targets in combination. The signal intensities obtained demonstrate minimal cross-hybridization between the two bead sets. MFI, median fluorescence intensity.

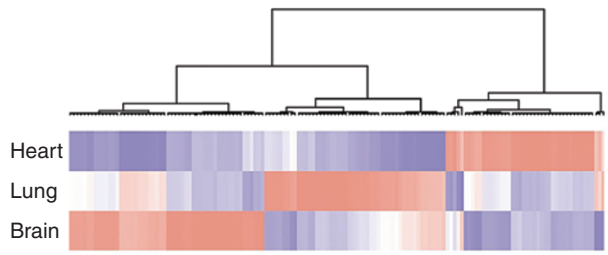

Figure 3 | Unsupervised hierarchical clustering and heat map showing that three human tissues clearly segregate from one another based on their miRNA expression profiles. Commercially available heart, lung and brain RNA was biotinylated using the FlexmiR MicroRNA Labeling kit, and miRNA profiles were obtained using the FlexmiR MicroRNA Human Panel. The time from experiment start to end of data analysis was less than 5 h. Colors indicate most unregulated (red), most downregulated (blue) and unregulated (black) miRNAs compared to the mean expression.

The FlexmiR system combines Exiqon's LNA technology with the Luminex xMAP® multiplex technology to create a bead-based profiling method that represents an attractive alternative to the planar arrays. In a fast and straightforward procedure, this system allows researchers to (i) biotinylate the 3′ ends of total RNA, (ii) hybridize the labeled RNA to T_m-normalized LNA capture probes that have been coupled to fluorescently dyed xMAP beads, (iii) stain the captured biotinylated miRNAs with streptavidin-phycoerythrin (SAPE), and (iv) analyze the samples on a Luminex analyzer capable of both identifying the fluorescent bead and measuring the SAPE intensity (**Fig. 1**).

Because the hybridization step takes place in solution, faster kinetics apply than with planar arrays, and the full protocol can therefore be performed in 4 h. The assay is performed in standard 96-well format, so analyzing multiple samples does not substantially increase assay time. Furthermore, enrichment for small RNAs is not necessary before biotinylation, which minimizes the resources spent on RNA sample preparation.

Producing fast, specific and robust data

LNA probes have proven to be superior to DNA probes with respect to specific and selective detection of miRNAs[1,2]. Furthermore, it has been suggested that the liquid-phase kinetics of a bead-based expression profiling system could offer more specific detection of closely related miRNAs than does the solid-phase kinetics of planar arrays[3]. The combination of LNA-modified probes and liquid-phase kinetics therefore provides an excellent platform for discriminating between very closely related miRNAs. Indeed, single-mismatch discrimination has been shown using this system (**Fig. 2**). This ability makes the FlexmiR system ideal for identification of distinct and characteristic miRNA profiles of different tissues (**Fig. 3**).

The fast and simple FlexmiR workflow minimizes procedure-related variation, and results in robust and reproducible data. Furthermore, correction for variation in labeling and hybridization between samples is made possible by the inclusion of several control beads in the FlexmiR panels. Each of these beads is coupled to an LNA-modified capture probe targeting either ubiquitous small nucleolar RNAs or synthetic spike-in RNAs, and can be used for evaluating assay integrity as well as intra- and inter-sample normalization.

Conclusion

By combining LNA-modified capture probes with the xMAP multiplexing beads, FlexmiR provides a highly specific and fast miRNA profiling platform that generates reliable data while saving precious time. Additionally, the flexibility of this platform opens the opportunity for customization of focused, multiplexed assays targeting a subset of miRNAs, which is ideal for those who wish to focus their research on a specific group of miRNAs.

To learn more about the FlexmiR microRNA products, please visit http://www.exiqon.com/FlexmiR or http://www.luminexcorp.com/microRNA.

ACKNOWLEDGMENTS

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