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Two-Temperature Hybridization for Microarray Detection of Label-Free MicroRNAs with Attomole Detection and Superior Specificity**

*Jeong Min Lee and Yongwon Jung**

MicroRNAs (miRNAs) are short (19–24 nucleotides, nt), single stranded, non-protein-coding RNAs that serve as potent regulators of gene expression in viruses, plants, and animals.[1] Evidence accumulated from many studies has uncovered distinct patterns of miRNA expression in the tissue and blood samples of patients with various human diseases.[2] miRNA profiling methods have shown great potential in basic research as well as for clinical applications, such as diagnosis and the evaluation of drug efficiency.[3] Among the most common techniques that are used for miRNA profiling, which include microarrays, sequencing, and real-time PCR, microarray-based detection is particularly effective for profiling large numbers of miRNAs, often several hundred at a time.[4] At present, many companies offer microarray platforms for miRNA profiling.[5] However, a major issue in the field of microarray-based miRNA profiling is the inability to compare results between different techniques, which greatly reduces the credibility of these methods.[5,6] As a result of the small size and often low abundance of miRNAs, many strategies have been developed to improve the design of probes and the labeling of the miRNAs for reliable detection with microarrays.[7] Chemical or enzymatic labeling or amplification reactions as well as various enrichment steps often introduce quantitative biases and experimental errors.[5,8] Furthermore, the design of short, complementary probes that are capable of discriminating miRNAs with a single base mismatch from the perfectly matched miRNAs at a given hybridization temperature is a difficulty faced by most profiling methods (except for deep sequencing). The obvious solution would be to greatly simplify the method of profiling and thus generate the least technical variance while maintaining high sensitivity and specificity.

Herein, we describe a new array-based miRNA profiling method that requires only two hybridization steps between the miRNAs and two short locked nucleic acid (LNA)-modified probes. This method can achieve detection at attomolar concentrations and ultrasensitive single mismatch discrimination. Trapping miRNAs between two short probes (approximately 10 nt) allows the use of label-free miRNAs for profiling because various labels can be introduced to the detection probe (Figure 1). When properly executed, this two-probe strategy offers an extremely simple and reliable way to profile miRNAs without the need for chemical and enzymatic reactions. However, the practical use of short capture probes in multiplex miRNA array detection has been severely limited as a result of low and variable melting temperatures (T_m), which create difficulties in differentiating between similar miRNA sequences. In general, almost the entire miRNA sequence is used for the design of the capture probe and modified nucleic acids, such as LNAs, are used to enhance profiling sensitivity and specificity by equalizing the T_m values.[5,9] Even so, it is still generally believed that complete elimination of cross-hybridization between genome-wide miRNAs and short probes is highly unlikely, even at the optimal hybridization temperature.[10]

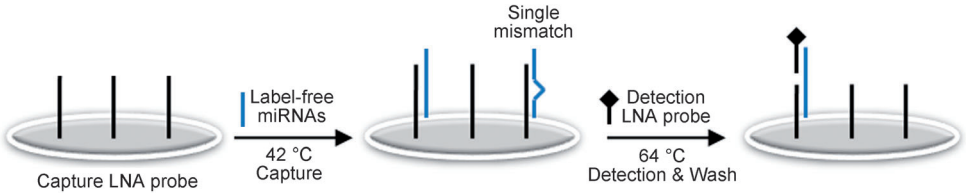

Figure 1. Representation of miRNA array detection by two short, LNA-modified probes in a two-temperature hybridization procedure. The miRNAs and miRNAs with a single base mismatch are hybridized with the surface-bound capture probe during the low-temperature capture step (42 °C). Only the mismatched miRNAs are removed from the surface during the high-temperature detection and wash step (64 °C). A detectable label (black diamond; biotin) is present in the detection probe.

To test the feasibility of detecting multiple miRNAs with two short probes, five sets of short LNA-modified probes were designed for five target miRNAs[11] (see the Supporting Information for sequences). These capture and detection probes were 10–12 nt in length with different numbers of G, C, and LNA residues (4–6 G or C residues and 4–6 LNAs). The theoretical T_m values between the probes and the

[*] J. M. Lee, Prof. Dr. Y. Jung
BioNanotechnology Research Center
Korea Research Institute of Bioscience and Biotechnology
P.O. Box 115, Yuseong, Daejeon 305-806 (Korea)
E-mail: ywjung@kribb.re.kr
J. M. Lee, Prof. Dr. Y. Jung
Nanobiotechnology, School of Engineering
Korea University of Science and Technology (UST)
P.O. Box 115, Yuseong, Daejeon 305-806 (Korea)

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selected miRNAs were only partially equalized because some level of T_m variation is inevitable in genome-wide miRNA profiling. Moreover, at present, theoretical T_m calculations for short probes are highly inaccurate and are even more complicated for LNA-modified probes.[9] In these experiments, we monitored the surface hybridization between the two short LNA probes and the miRNAs at different temperatures. Synthetic miRNAs were hybridized with the capture probes, which were spotted on a glass surface (16 h, capture step). Surface-bound miRNAs were then identified by treating the slides with a mixture of singly biotinylated detection probes (5 nM, 1 h, detection step), then with Cy3-labeled streptavidin (Figure 1). The miRNA capture and detection steps were performed at different temperatures, which each ranged from 25 °C to 58 °C. For the four target miRNAs (miR206, miR96, Let-7a, and miR21) maximum fluorescence signals were generally detected below 42 °C, whereas highly diverse signal patterns were obtained at higher temperatures (47 °C–58 °C) (Figure 2a). The surface hybridization stability of each probe pair with its corresponding target miRNA was greatest for Let-7a, and then decreased in the order miR206, miR21, and miR96.

We examined the surface target selectivity at different temperatures by introducing a single base mismatch into the capture-probe binding site in each miRNA (Figure 2a). The discrimination of single base mismatches varied widely and depended on the hybridization temperature and the LNA probe used. Let-7a and Let-7c sequences were only moderately discriminated even at a high hybridization stringency (52 °C/52 °C), which is consistent with previous reports.[10,11] However, under these conditions the signals for

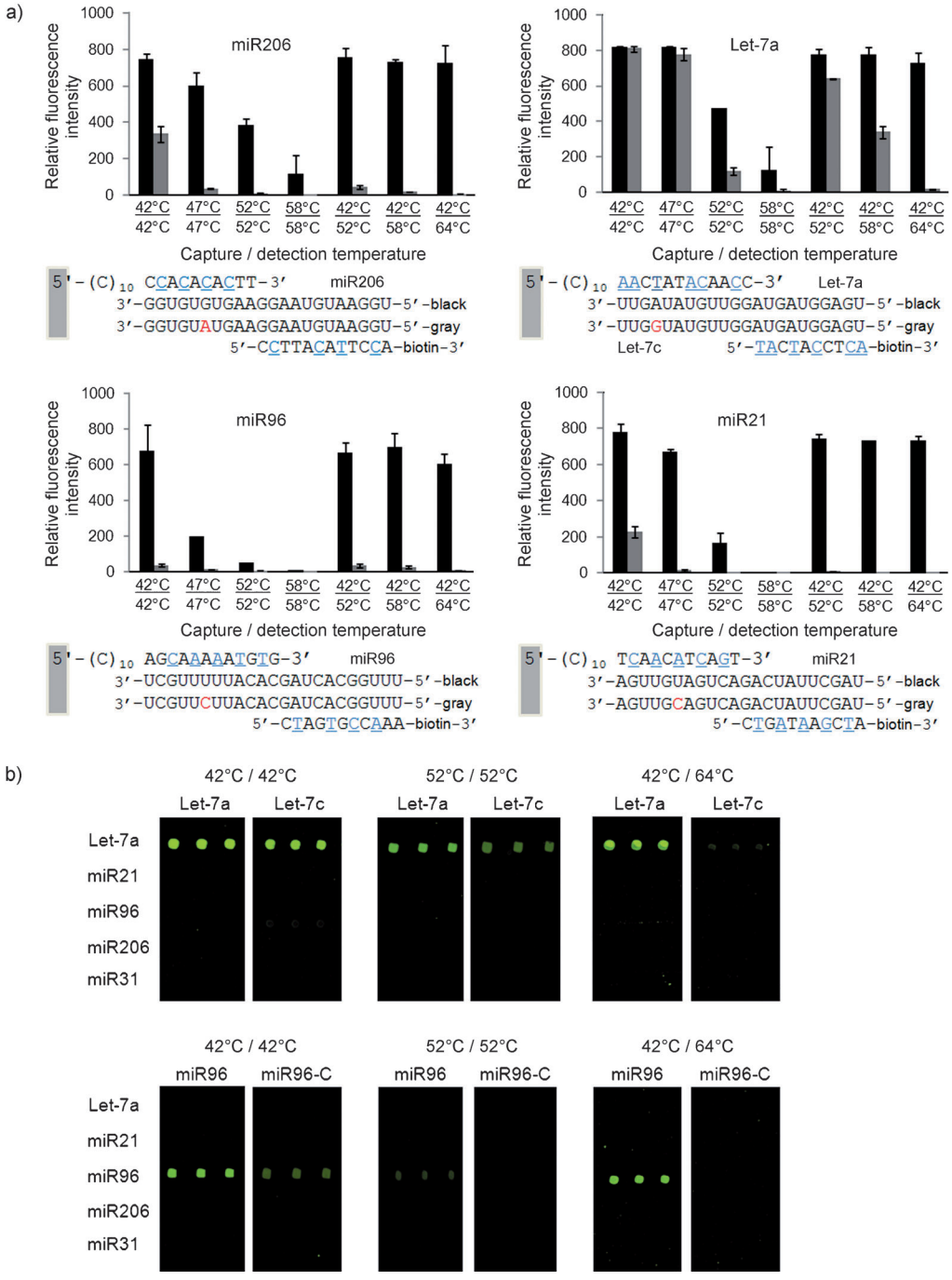

Figure 2. Two-temperature hybridization for microarray detection of label-free miRNAs. a) Target specific fluorescence signals for four target miRNAs (black bars) and miRNAs with a single base mismatch (gray bars) obtained by two-temperature hybridization. The relative signal intensities for target miRNAs were calculated by dividing the fluorescence intensity of each miRNA spot by that of the same capture-probe spot without target miRNA. Error bars = standard deviation, $n = 3$ samples. Capture and detection probe (LNAs in blue), target miRNA, and mismatched sequences (red) are given under each graph. b) Fluorescence images of microarray detection of human Let-7a (top) and miR96 (bottom), and the corresponding miRNAs with a single base mismatch (Let-7c and miR96-C, sequences in Figure 2a) under various two-temperature hybridization conditions.

miR96 and miR21 were vastly diminished. These experiments verified that obtaining both high sensitivity and specificity in array-based profiling of multiple miRNAs at a single hybridization temperature is an extremely challenging task, particularly with shorter probes.

Interestingly, the discrimination of mismatches in the detection-probe binding site of the miRNAs was generally less effective than mismatch discrimination in the capture-probe binding site (see the Supporting Information). The binding characteristics between the capture probes and miRNAs (16 h) are likely to be distinct from those of the surface-captured miRNAs and the detection probes in solution (1 h). Therefore, the hybridization temperature was changed in the detection step, but maintained at 42 °C during the capture step. Gradual increases in the temperature during the detection step resulted in visibly reduced fluorescence signals for the miRNAs which contained single-base mismatches (Figure 2b). By conducting the detection step at 64 °C, the fluorescence signals of all of the mismatched miRNAs decreased to near background levels. Surprisingly, the fluorescence signals of all the perfectly matched miRNAs were almost unaffected by the increased detection temperature, even after the one hour incubation at 64 °C, which is higher than all of the observed surface T_m values (Figure 2). The two-temperature hybridization procedure, 42 °C for the capture step and 64 °C for the detection step, gave the most potent single mismatch discrimination. This assay performed better than all of the single-temperature hybridization assays that were tested at any temperature. Furthermore, close to the maximum sensitivity was maintained for all of the miRNAs tested in the two-temperature approach with short LNA probes, in spite of the wide range of experimental T_m values for these combinations of miRNAs and probes. The short detection probe is critical for the unexpected stability of the perfectly matched miRNAs on the array surface during the high temperature detection step (see the Supporting Information).

Single mismatch discrimination by the optimized two-temperature strategy (42 °C/64 °C) was tested further by using many miR96, miR206, miR31, and miR21 sequences with single base mismatches (see the Supporting Information). Various single base mismatches were systematically introduced into the capture-probe binding site of the target miRNAs. Most mismatches were completely discriminated, as was the background signal, although discrimination of mismatches at the very ends of the binding site was less effective. Our miRNA microarray assay also effectively discriminated between five closely related sequences from the Let-7 family of miRNAs (Figure 3). The highest cross-hybridization rate was only 3.2 % (Let-7c), which is significantly lower than that of any previously reported, single probe based assay with a single hybridization temperature (ca. 25 %).[10] Moreover, a single-temperature hybridization approach with a commercially available Let-7a LNA probe was clearly less efficient for the discrimination between the Let-7 sequences under our experimental conditions (see the Supporting Information). The sensitivity of the two-temperature assay for synthetic human miRNAs was also examined. A wide range of miRNA concentrations (1 fM–1 nM) of

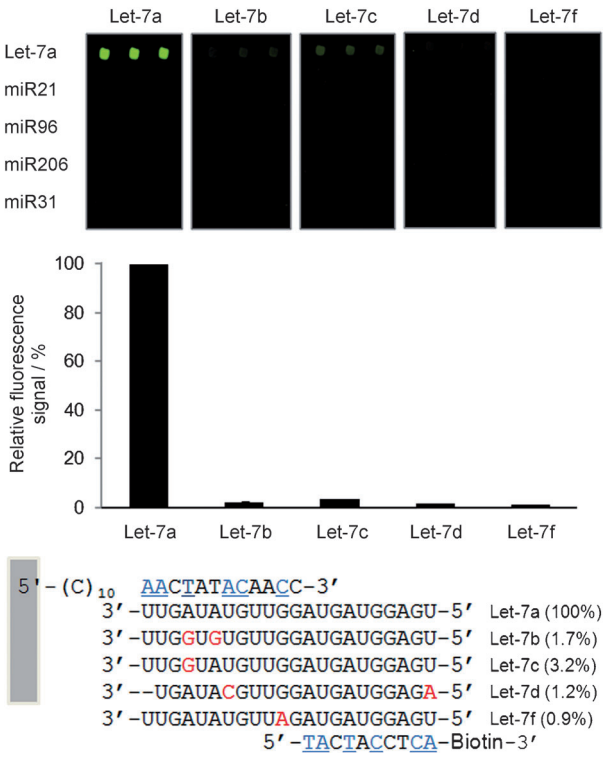

Figure 3. Target specificity of two-temperature array detection of miRNAs with two LNA probes. The sequences of the five Let-7 miRNAs are given under the graph.

miR21, miR96, miR206, and Let-7a sequences were tested (Figure 4a). A good signal linearity was detected over a concentration range of 10 fM–100 pM (1 attomole–10 femtomoles in 0.1 mL hybridization volume) for all miRNAs. A detection limit of 10 fM–20 fM is significantly lower than that of any label-free microarray miRNA detection method[12] and is comparable to some of the most sensitive detection methods that use various labeling and amplification reactions (see the Supporting Information).[10,13]

We examined the expression patterns of five miRNAs (Let-7a, miR21, miR96, miR206, and miR31) in human tissue. Total RNA extracts (2 μg) from tissue were dissolved in hybridization solution and applied directly to the capture-probe microarray chips. The resulting array data clearly indicated that human skeletal muscle and heart tissues contain different levels of miRNAs (Figure 4b). As previously reported, Let-7a was found to be the most abundant of the five miRNAs tested, with an estimated concentration of approximately 30 pM in human skeletal muscle tissue and approximately 32 pM in heart tissue.[11] MiR96 And miR31 were not detected in the total RNA samples obtained from either tissue, which is also consistent with a previous report.[11] A distinct expression pattern was detected for miR206 in skeletal muscle tissue (approximately 16 pM) and in heart tissue (less than 10 fM).[10,11] The expression of miR21 in these tissue types (approximately 8 pM in both tissues) also correlated well with previous results.[14] Various concentrations of miR96 or miR206, which were spiked into the total

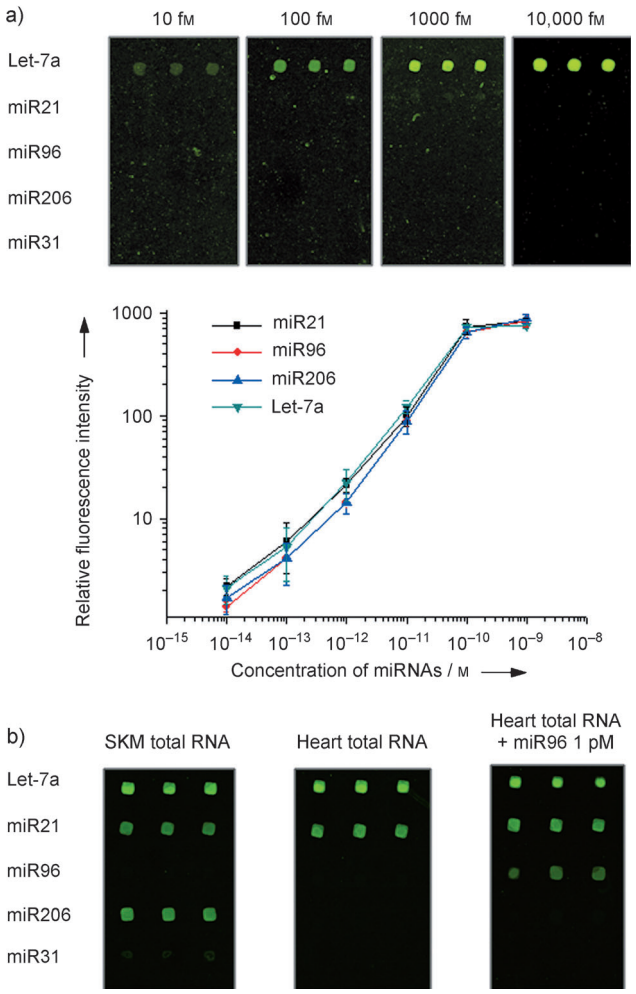

Figure 4. a) Concentration-dependent fluorescence signals for four miRNAs (human Let-7a, miR21, miR96, and miR206). Fluorescence images of human Let-7a (10 fM–10 pM) array detection are shown. Error bars = standard deviation, $n = 3$ samples. b) Fluorescence microarray detection of five human miRNAs from human skeletal muscle (left) or heart (middle) total RNA extracts (2 μg). Synthetic miR96 (1 pM) was added to the heart RNA extract (right).

RNA sample, (2 μg) were also effectively detected (Figure 4b and the Supporting Information).

Our approach relies only on hybridization between short oligonucleotides, which is among the most consistent and robust of biomolecular interactions. An interaction between perfectly matched miRNA and two LNA probes could withstand short but high stringency incubation (64 °C, 1 h) on solid surfaces, whereas even one mismatch could severely disrupt this cooperative interaction. Although each LNA probe showed highly diverse surface hybridization stability with target miRNAs, the three-piece interaction and two-temperature hybridization strategy afforded surprisingly uniform sensitivity and exceptional selectivity for the detection miRNA on this array. This method may be the simplest and most effective microarray technique for miRNA profiling. Moreover, the present principle of a three-piece interaction can be applied to numerous hybridization-based detection methods for DNA and RNA molecules on solid supports. Future development of this strategy for use with other modified nucleic acids (or unmodified DNA) and solid supports, such as bead-based or microfluidic platforms, will further expand the potential applications of the method.

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