Hybridization kinetics analysis of an oligonucleotide microarray for microRNA detection

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MicroRNA (miRNA) microarrays have been successfully used for profiling miRNA expression in many physiological processes such as development, differentiation, oncogenesis, and other disease processes. Detecting miRNA by miRNA microarray is actually based on nucleic acid hybridization between target molecules and their corresponding complementary probes. Due to the small size and high degree of similarity among miRNA sequences, the hybridization condition must be carefully optimized to get specific and reliable signals. Previously, we reported a microarray platform to detect miRNA expression. In this study, we evaluated the sensitivity and specificity of our microarray platform. After systematic analysis, we determined an optimized hybridization condition with high sensitivity and specificity for miRNA detection. Our results would be helpful for other hybridization-based miRNA detection methods, such as northern blot and nuclease protection assay.

Keywords microRNA microarray; microRNA

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Introduction

MicroRNAs (miRNA) are a class of small regulatory non-coding RNAs, which have been shown to play an essential role in a large number of biological and pathological processes [1–3]. Abnormal miRNA expression usually results in developmental defects, cancer generation, and other diseases in human and other organisms [4–7]. Recent studies have shown that miRNAs are a class of potential diagnostic molecules and could be useful as therapeutic targets in the future [5, 8–10]. Therefore, a reliable detection system for miRNAs is very important for miRNA research. Till date, a range of techniques are available for miRNA quantification based on various strategies and detection methods [11, 12].

Microarray, originally widely used in cDNA analysis, has been applied to detect mature miRNA [13–15]. It is, in principle, based on nucleic acid hybridization between target molecules and their complementary probes. Unlike mRNA microarray, the problem of potential cross-hybridization for miRNA microarrays is more significant, because mature miRNAs belonging to the same family exhibit a high degree of similarity [14]. It is more difficult to distinguish as little as one single nucleotide difference in miRNAs with much shorter sequences than mRNAs. Therefore, the primary point for miRNA detection by microarray is to evaluate and control the hybridization specificity of the targets and probes.

We have developed a cost-effective miRNA microarray that enabled us to profile miRNA expression on a global scale in various samples [16–18]. However, when we applied small RNA samples from total RNA to our microarrays, we could not determine the specificity of the hybridization reaction because the exact amount of some similar miRNAs in a certain total RNA sample was unknown. To test the hybridization specificity of our miRNA microarray, we developed a small array consisting of three groups of miRNAs with high similarity and equal Tm value. The results showed that high temperature could improve the specificity but would reduce the hybridization efficiency. Furthermore, we determined the detection sensitivity of the microarray by using serially diluted targets. The miRNA expression pattern in rice seedlings was also profiled using this optimized condition.

Materials and Methods

Construction of small RNA cDNA library

Construction of small RNA cDNA library was performed as described [16]. Briefly, total RNA was extracted from rice seedlings, and separated on a 15% denaturing
polyacrylamide gel. Gel slides containing small RNAs of 18–26 nt were excised and immersed in water overnight. Small RNAs were precipitated and ligated to adaptors on both ends with T4 RNA Ligase (Fermentas, Lithuania). The adaptors sequences were 5'-ACCGAATTCACGTCA GACC-3' and 5'-pGCAGATCGACAATTCGAt-3' (p, phosphate; t, 2',3'-dideoxythymidine). The ligation product was further purified as above and used for RT-PCR amplification (AccessQuick™ RT-PCR System; Promega, Madison, USA).

**Preparation of fluorescence targets**

To prepare fluorescence targets from rice samples, small RNA libraries from rice total RNA were subjected to PCR amplification by the primers: Cy5-5'ACCGAATTCACGTCA GACC-3' and 3'-pGCAGATCGACTTGCC-5'. For fluorescence targets for miR-156a, miR-164a, and miR-169a, a positive control, 5'-ACCGAATTCACAGTCA GACC-3' and 3'-pGCAGATCGACTTGCC-5' were amplified by PCR with positive control, 5'-ACCGAATTCACAGTCA GACC-3' and 3'-pGCAGATCGACTTGCC-5'. These templates were amplified by PCR with primers listed above separately. Fluorescence targets from PCR reaction were precipitated, resuspended in 10 μl ddH2O, and cleaned up by a sephadex G-50 column. All oligonucleotides were synthesized by Invitrogen (Invitrogen, Carlsbad, USA).

**Microarray methods**

Microscope glass slides (Sigma, St. Louis, USA) were activated as described [19]. The antisense probes of microRNAs were printed at a concentration of 20 μM [16]. The probe sequences are listed in Supplementary Table S1. The printed microRNA microarrays were further chemically covalently coupled under 60% humidity overnight at room temperature. Before hybridization, printed glass slides were treated with quenching buffer (0.1 M Tris-HCI/50 mM ethanolamine, pH 8.0) to block unprinted surface. The fluorescence targets were denatured at 95°C for 5 min and mixed with equal volume of 2× hybridization buffer (8× SSC/0.2% SDS, pH 7.0). Apply the mixture to hybridization chambers to allow hybridization overnight at different temperatures as indicated [16]. After hybridization, slides were washed (2× SSC/0.03% SDS, 2 min; 0.2× SSC, 2 min; 0.1× SSC, 1 min) at 25°C, and then scanned with ScanArray 5000 (PerkimElmer, Boston, USA) with a scan resolution of 5 μm. Images were analyzed by QuantArray (PerkimElmer) using the fixed circle quantification methods. Signal intensities of the spots were calculated by subtracting local background from total intensities. For each probe at one temperature, an arithmetic mean of 20 replicates from four independent hybridizations was calculated.

**Results and Discussion**

To determine the specificity of the microarray, a small array containing probes to three sets of rice miRNAs (miR-156a, k, l; miR-164a, d; and miR-169a, e, q) and a positive control were designed. The selected miRNAs from

<table>
<thead>
<tr>
<th>microRNAs</th>
<th>Probe</th>
<th>Hybridization temperature (°C)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>39</td>
<td>42</td>
</tr>
<tr>
<td>156a</td>
<td>52</td>
<td>1.000 ± 0.167</td>
</tr>
<tr>
<td>156k</td>
<td>52</td>
<td>0.390 ± 0.083</td>
</tr>
<tr>
<td>156l</td>
<td>52</td>
<td>0.876 ± 0.234</td>
</tr>
<tr>
<td>164a</td>
<td>58</td>
<td>1.000 ± 0.186</td>
</tr>
<tr>
<td>164d</td>
<td>58</td>
<td>0.595 ± 0.116</td>
</tr>
<tr>
<td>169a</td>
<td>56</td>
<td>1.000 ± 0.175</td>
</tr>
<tr>
<td>169e</td>
<td>56</td>
<td>0.495 ± 0.056</td>
</tr>
<tr>
<td>169q</td>
<td>56</td>
<td>0.009 ± 0.003</td>
</tr>
</tbody>
</table>
the same family have the different types of sequence variation (Fig. 1) and the same melting temperature (Table 1). Four fluorescence-labeled PCR target products (against miR-156a, miR-164a, miR-169a, and positive control) were mixed and hybridized on the small array at the indicated temperatures (Table 1). The signals from the probes complementary to miR-156a, miR-164a, and miR-169a were normalized to 1. The signals from other probes were considered as cross-hybridization and normalized to miR-156a, miR-164a, and miR-169a, respectively. The results of the small array were summarized in Table 1. Compared with miR-169a, miR-169q has one missing nucleotide and several mismatched nucleotides in the middle and the end of its sequence. The results showed almost no cross-hybridization between the miR-169q probe and the miR-169a target (Table 1). For miR-156k, there was one mismatched nucleotide in the middle and two mismatches in the 3’ end of the sequence with miR-156a. The cross-hybridization between the miR-156k and the miR-156a probe was very low and even lower at higher hybridization temperature (Table 1). For the probes of miR-156l and miR-169e, they only have mismatches with their targets on both ends of the sequence. The signal of miR-156a probe was 52°C and that of miR-169a probe was 56°C, which indicated that a hybridization temperature of 54°C was more stringent for the miR-156a probe than for the miR-169a probe. The cross signals of miR-156l and miR-169e were 0.180 and 0.306 at 54°C (Table 1). Thus, it is expected that a stringent-enough condition with higher hybridization temperature would reduce cross-hybridization, when mismatches are only at both ends of probes and targets. For miR-164d, there was only one mismatched nucleotide at one end of the miR-164a probe. In this case, increasing hybridization temperature showed little effect to reduce cross-hybridization (Table 1). In summary, our microarray could clearly distinguish those miRNAs containing mismatches in the middle of the sequence. For those miRNAs with the mismatches at the end of the sequences, it was difficult to distinguish them clearly. In fact, this problem is unsolved by all nucleic acid detection methods based on hybridization. Raising the hybridization temperature could improve the specificity, especially when the probes have a lower melting temperature, given that the hybridization efficiency was acceptable at such a temperature (Table 1).

However, higher hybridization temperature always results in poorer hybridization efficiency and lower signal intensity. To determine a compromising hybridization temperature for the hybridization efficiency and specificity, we tested series of hybridization temperatures with a pair of positive control probe and target (Fig. 2). The melting temperature of the positive control is 55°C. The signal intensities from the
positive control reached peak (saturation) at 45°C, which is 10°C lower than the $T_m$ of positive control (Fig. 2). When hybridization temperature was <45°C, signal intensity decreased a little. When hybridization temperature was >45°C, signal intensity became lower along with the increase in hybridization temperature (Fig. 2). To balance the specificity and the signal intensity, we considered 48°C as the best temperature for a pair of probe and target with a

![Figure 5 miRNA expression pattern in rice seedling](image)

The miRNAs with intensity >100 were plotted by a histogram. Signals from negative and random probes were indicated.
$T_m$ of 55°C. Therefore, for a real array analysis, we routinely set a hybridization temperature 7°C lower than the mean melting temperature of all probes on array.

Sensitivity is another evaluation criterion for a microarray. To consummate the sensitivity evaluation in our optimized hybridization condition as above, 0.1, 0.5, 2.5, and 12.5 fMole PCR-labeled products in 20 µl hybridization buffer were hybridized to the microarray. The images and quantitative results were shown in Fig. 3. The mean signal from 0.1 fMole labeled targets was about 700 (higher than the generally accepted cutoff value). Thus, our miRNA microarray could reliably detect as little as 0.1 fMole labeled targets in 20 µl hybridization buffer (5 pM) in the optimized hybridization condition.

After a systematic optimization on our miRNA microarray, we applied a real array to analyze the expression of a set of rice miRNAs. As shown in Fig. 4, the original scan image showed quite high quality. According to the results of hybridization specificity presented in this study, the same probe was used for those similar miRNAs that had different nucleotides only in their ends. For other similar miRNAs with different nucleotides in their middle, different probes were used. Quantitative results are listed in Supplementary Table S2. The miRNAs with intensity >100 were plotted in Fig. 5. Two negative probes to rice ini-tRNA and one random sequence were indicated. They showed very low signal intensities. MiR-166 was the most highly expressed family in rice seedlings. There were five probes to these miR-166 members. Four of them (miR-166k, l; miR-166a, b, c, d, e, f, n; miR-166 m; and miR-166g, h) along with miR-168a probe showed the top five signal intensity values. However, the signal intensity of miR-166i, j was only about 10% of that of the other miR-166 members, although their sequences were highly similar, which indicated that little cross-hybridization occurred in our condition. Therefore, our data showed a reliable miRNAs express pattern in rice seedlings by using the optimized hybridization condition. Our results would be helpful for miRNA detection by microarray and other methods based on nucleic acid hybridization.

Supplementary Data

Supplementary data are available at ABBS online.

Funding

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