All-in-One™ miRNA qPCR Primer Validation Report

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All-in-One™ miRNA qPCR Primers Sample Validation Report

I. Materials and Methods

1. Instrument
   iQ5 Real Time PCR Detection System: Bio-Rad

2. Reagents
   All-in-One™ miRNA qRT-PCR Detection Kit (Catalog Nos. AOMD-Q020 or AOMD-Q050)
   Validation templates: cDNAs extracted from 10 different human tissues
   Detection primers: Total 6 pairs

<table>
<thead>
<tr>
<th>Catalog number</th>
<th>Primer ID</th>
<th>Mature_Acc</th>
<th>Mature_ID</th>
<th>PCR size</th>
</tr>
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<tbody>
<tr>
<td>miRQP0227</td>
<td>hsmq-0031</td>
<td>MIMAT0000069</td>
<td>hsa-miR-16</td>
<td>75bp</td>
</tr>
<tr>
<td>miRQP0074</td>
<td>hsmq-0032</td>
<td>MIMAT0000422</td>
<td>hsa-miR-124</td>
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<td>miRQP0096</td>
<td>hsmq-0034</td>
<td>MIMAT0000423</td>
<td>hsa-miR-125b</td>
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<tr>
<td>miRQP0175</td>
<td>hsmq-0035</td>
<td>MIMAT0000429</td>
<td>hsa-miR-137</td>
<td>76bp</td>
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<tr>
<td>miRQP9901</td>
<td>hsnRNA-U6</td>
<td>NR_002752.1</td>
<td>RNU6B</td>
<td>80bp</td>
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<tr>
<td>MiRQP0293</td>
<td>hsmq-0655</td>
<td>MIMAT0000073</td>
<td>hsa-miR-19a</td>
<td>72bp</td>
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</tbody>
</table>

II. Procedure

RNA Extraction

1. Sample preparation
   Place a small amount of material from a human tissue sample into a pre-cooled mortar. Add a small amount of liquid nitrogen and grind the tissue to a fine powder. Transfer the powder to a centrifuge tube containing 1 ml of TRIzol (Invitrogen). Vortex for 5 minutes. (If the samples are cultured cells, use about $10^6$-10$^7$ cells with 1 ml of TRIzol). Pipette up and down until the cells are completely lysed.

2. Phase separation
   Leave the cell or tissue samples at room temperature for about 10 minutes. Add 200 μl of chloroform per 1 ml of TRIzol. Close the cap and vortex vigorously for 1 minute. Let the samples settle down at room temperature for 2-5 minutes. Centrifuge at 12,000 g for 15 minutes at 6°C. Remove tubes from the centrifuge being careful not to disturb the liquid. The samples should be separated into 3 layers with RNA in the top layer.

3. RNA Precipitation
   Carefully transfer about 450 μl (per 1 ml TRIzol) of the supernatant to a new centrifuge tube containing 600 μl of cold 2-propanol. Mix well and keep at −20°C for 10 minutes. Then centrifuge at 12,000 g for 10 minutes (at 6°C).
4. **Washing**

   Remove the supernatant. Add 500 μl of cold 75% ethanol. Vortex until a pellet forms at the bottom of the tube. Centrifuge at 12,000 g for 5 minutes (6°C). Remove the supernatant. Centrifuge briefly again and remove the remaining supernatant.

5. **Dissolve RNA**

   Air-dry the samples for 5 to 10 minutes. (Do not over dry the samples. The samples are dry when they turn white). Dissolve the samples in 30 μl of DEPC water. Label the samples properly and store them at –80°C.

6. **Determine RNA Concentration**

   Take 1 μl of RNA sample. Dilute the sample 1:10 in DEPC water. RNA concentration can be measured with a NanoDrop (Thermo Scientific). DEPC water should be used as a blank. Record both the RNA concentration and the ratio of $A_{260}$/A$_{280}$.

7. **RNA electrophoresis**

   7.1. **Prepare denaturing gel**

      Add 1 g of agarose into 75 ml of de-ionized water. Boil the agarose for 1–2 minutes and cool it down to about 70°C. Add 10 ml of 10X MOPS, 15 ml of formaldehyde and 5 μl of ethidium bromide (EB). Pour the gel into a tray with big combs. Cover the tray.

   7.2. **Prepare electrophoresis buffer (1X MOPS)**

      Take 50 ml of 10X MOPS and dilute 1:10 to the final volume of 500 ml with de-ionized water. Pour the buffer into a gel box, and add some EB to it.

   7.3. **Preparation of RNA sample**

      Take 3 μl of the RNA sample and add DEPC to a total volume of 18 μl. Heat the sample at 65°C for 10 minutes. Cool it down immediately. Add 2 μl of 1X RNA loading buffer into the sample.

   7.4. **RNA electrophoresis**

      Place the RNA gel in a gel box with 1X MOPS electrophoresis buffer. Pre-run the gel at 100 V for 5 minutes. Load the treated RNA samples into the gel and run at 100 V until the bromophenol blue runs to about one third the length of the gel. Take a picture using a UV scanner.

8. **RNA detection results**

   Electrophoresis results of RNAs from 10 different tissues (3 μl RNA each well). See table 8.2 for the specific lane information.
Table 8.2. RNA sample source, concentration and the ratio of OD260/OD280.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Tissue</th>
<th>Concentration (ng/µl)</th>
<th>OD A&lt;sub&gt;260&lt;/sub&gt;/A&lt;sub&gt;280&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Brain</td>
<td>2385</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>Lung</td>
<td>2430</td>
<td>1.94</td>
</tr>
<tr>
<td>3</td>
<td>Liver</td>
<td>2521</td>
<td>1.91</td>
</tr>
<tr>
<td>4</td>
<td>Kidney</td>
<td>3444</td>
<td>1.94</td>
</tr>
<tr>
<td>5</td>
<td>Breast</td>
<td>2785</td>
<td>1.87</td>
</tr>
<tr>
<td>6</td>
<td>Testis</td>
<td>2972</td>
<td>1.9</td>
</tr>
<tr>
<td>7</td>
<td>Placenta</td>
<td>3515</td>
<td>1.91</td>
</tr>
<tr>
<td>8</td>
<td>Spleen</td>
<td>3344</td>
<td>1.91</td>
</tr>
<tr>
<td>9</td>
<td>Heart</td>
<td>3394</td>
<td>1.91</td>
</tr>
<tr>
<td>10</td>
<td>Pancreas</td>
<td>3101</td>
<td>1.91</td>
</tr>
</tbody>
</table>

Note: In order to detect miRNAs, the extracted RNA must contain small molecular weight RNAs. The kit used for RNA extraction must be applicable for isolation of total RNA or small RNA. The quality of RNA is critical to the success of downstream experiments. Follow the instructions of the RNA extraction kit exactly. Examine RNA samples by electrophoresis to ensure high quality.

miRNA reverse transcription

1. Thaw all the reagents needed for miRNA reverse transcription. Mix reagents well by gently inverting the tubes. Spin down briefly and keep on ice.

2. Prepare miRNA reverse transcription reaction: Add the following reagents into an RNase-free reaction tube which is pre-cooled on ice. The final volume should be 25 µl.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA</td>
<td>2 µg</td>
<td></td>
</tr>
<tr>
<td>2.5 U/µl Poly A Polymerase</td>
<td>1 µl</td>
<td>2.5 U</td>
</tr>
<tr>
<td>RTase Mix</td>
<td>1 µl</td>
<td></td>
</tr>
<tr>
<td>5X Reaction Buffer</td>
<td>5 µl</td>
<td>1X</td>
</tr>
<tr>
<td>ddH&lt;sub&gt;2&lt;/sub&gt;O (RNase/DNase free)</td>
<td>to 25 µl</td>
<td></td>
</tr>
</tbody>
</table>

Note: The total RNA in the reaction must contain small molecular RNA. If total RNA is used, the amount of RNA should be between 1 ng ~ 5 µg. If the purified small molecular RNA is used, the amount of RNA should be between 0.1 ng ~ 1 µg.
3. Reverse Transcription Reaction: Mix reaction solution well. Spin down briefly. Incubate the reaction solution at 37°C for 60 minutes. Terminate the reaction by heating at 85°C for 5 minutes. The products of reverse transcription can be diluted 1:5 with sterile water for the downstream qPCR reaction.

**qPCR to detect miRNA**

1. Thaw 2X All-in-One qPCR mix from the All-in-One miRNA qRT-PCR Detection Kit. Mix well by inverting the tube several times. Spin down briefly and keep on ice.

2. Prepare qPCR reaction solution on ice. All miRNAs are tested in duplicate. NTC (No template control) is tested singly.

### Reagents | Volume | Final Concentration
--- | --- | ---
2×All-in-One qPCR Mix | 10 μL | 1X
All-in-One miRNA qPCR Primer (2 μM) | 2 μL | 0.2 μM
Universal Adaptor PCR Primer (2 μM) | 2 μL | 0.2 μM
First strand cDNA (diluted 1:5) | 2 μL | 
ddH2O | 4 μL | 
Final volume | 20 μL |

3. Mix the qPCR reaction solution well. Transfer the solution to a PCR tube. Spin briefly to make sure that the reaction solution is at the bottom of the tube.

4. The qPCR reaction, using the standard 3-step method to detect the DNA amount (these experiments were designed based on Bio-Rad iQ5 instrument). Melting analysis can be done immediately after the amplification reactions.

### Number of Cycles | Step | Temperature | Time | Detection
--- | --- | --- | --- | ---
1 | Pre-denature | 95 °C | 10min | No
40 | Denature | 95 °C | 10sec | No
| Annealing | See reference below | 20sec | No |
| Extension | 72 °C | 10sec | Yes |
## III. Test Results

1. **hsa-miR-16 (hsmq-0031 primer)**

   Recommended annealing temperature: 60°C

   ![hsa-miR-16 Amplification Plot](image1)

   ![hsa-miR-16 melting analysis curve](image2)

2. **hsa-miR-124 (hsmq-0032 primer)**

   Recommended annealing temperature: 60°C

   ![hsa-miR-124 Amplification Plot](image3)

   ![hsa-miR-124 melting analysis curve](image4)
3. **hsa-miR-125b (hsmq-0034 primer)**

   Recommended annealing temperature: 60°C

   ![hsa-miR-125b Amplification Plot](Image)
   ![hsa-miR-125b melting analysis curve](Image)

4. **hsa-miR-137 (hsmq-0035 primer)**

   Recommended annealing temperature: 60°C

   ![hsa-miR-137 Amplification Plot](Image)
   ![hsa-miR-137 melting analysis curve](Image)

5. **hsnRNA U6 primer testing**

   Recommended annealing temperature: 60°C

   ![hsnRNA U6 Amplification Plot](Image)
   ![hsnRNA U6 melting analysis curve](Image)
6. Electrophoresis Results

5 µl of the PCR of products, run on 3% agarose gel. Each set contains two positive controls and one NTC
Lane 1: hsa-miR-16; Lane 2: hsa-miR-124; Lane 3: hsa-miR-125b; Lane 4: hsa-miR-137; Lane 5: hsnRNA-U6.

7. hsa-miR-19a (hsmq-0655 primer)

7.1 Validation.

The following link may be used to view a larger amplification plot and melting curve as well as a
gel electrophoresis image of this primer’s validation results. Contact GeneCopoeia at
inquiry@genecopoeia.com or call 866-360-9531 to have an electronic document sent to you.


7.2 Discrimination assay of two primers with single-base differences in their sequences

7.2.1 has-miR-19a BLAST

<table>
<thead>
<tr>
<th>hsa-miR-19a</th>
<th>UGUCAACCAUGCCAAAUCUGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-19b</td>
<td>UGUCAACCAUGCCAAAUCUGA</td>
</tr>
</tbody>
</table>

The BLAST alignment shows that a single-base difference exists between the two
miRNAs: hsa-miR-19a and miR-19b. To discriminate and validate the primer of interest
(has-miR-19a) two plasmids were constructed for each of the two miRNAs.
7.2.2 Sequence result of the miRNA qPCR plasmids

hsa-miR-19a sequence result:

hsa-miR-19b sequence result:

7.2.3 qPCR validation

Using the All-in-One miRNA qRT-PCR Detection Kit, the hsa-miR-19a-specific primer was validated using miRNA expression plasmids for has-miR-19a and has-miR-19b as templates (about $10^7$ molecules/reaction).

The percent relative discrimination was calculated using the differences between the Ct values of the mismatched template and the matching template with respect to the hsa-miR-19a-specific primer (Relative discrimination = $2^{-\Delta Ct} \times 100\%$). The results are shown in the table below:

<table>
<thead>
<tr>
<th>Annealing temperature °C</th>
<th>Ct value for miRNA expression plasmid amplification</th>
<th>Relative discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hsa-miR-19a</td>
<td>hsa-miR-19b</td>
</tr>
<tr>
<td>63.3</td>
<td>20.30</td>
<td>30.89</td>
</tr>
<tr>
<td>61.4</td>
<td>18.40</td>
<td>25.81</td>
</tr>
<tr>
<td>58.9</td>
<td>17.50</td>
<td>21.57</td>
</tr>
</tbody>
</table>

7.2.4 Conclusion

Based on these results, a qPCR annealing temperature between 59.9 and 61.4 °C is recommended. This range gives the highest amplification efficiency as well as the best relative discrimination between the two single-base-different miRNAs.
IV. Limited Use License and Warranty

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