Real-time PCR: Advancing RNA Interference and MicroRNA Studies

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MicroRNAs

The endogenous small RNAs known as microRNAs (miRNAs) found in the genomes of both animals and plants are highly conserved by evolution, suggesting a fundamental biological function. MicroRNAs are cleaved from larger hairpin precursors (Figure 1), and it is the mature, cleaved, approximately 22-nucleotide miRNA that currently is the focus of most research. The inherent homology between the longer pre-miRNA and the mature, active miRNA makes it difficult to preferentially detect the active molecule using traditional hybridization-based methods. This has contributed to the challenges of accurately quantifying and elucidating the role of miRNAs in different tissues, developmental stages and disease states.

A total of approximately 850 unique miRNAs have been discovered so far, including 222 human miRNAs (about 250 miRNAs for each species have been predicted). Although miRNAs were only recently discovered, they are relatively abundant, accounting for approximately 1% of the predicted genes in animals and plants (1). Despite their general abundance, the level of individual miRNAs varies dramatically between cell type and developmental stage, ranging from a few to as many as 50,000 molecules per cell (2). As with mRNA gene transcripts, these differences in miRNA levels are believed to be a key indicator of miRNA activity. This large variability in the amount of miRNAs in different tissues makes it imperative that methods for identifying and quantifying miRNAs are accurate over an unusually large dynamic range.
MicroRNAs are not translated into proteins; instead, they regulate the expression of other genes by either cleaving or repressing the translation of their messenger RNA (mRNA) targets (translational repression, not cleavage, is the mechanism of miRNAs that usually occurs in animals, although the mechanism of action appears to be driven by the level of base complementarity between the miRNA and its target). The discovery that miRNAs play an important role in the post-transcriptional regulatory process has added a new layer to our understanding of the complexity of gene regulation.

Recent studies have implicated miRNAs in such fundamental processes as cell development, differentiation, communication and death. Therefore, it is not surprising that miRNAs have been found to be involved in such disparate areas as hematopoiesis, insulin secretion, nervous system patterning and human cancer development. Current evidence suggests that thorough studies of gene regulation will increasingly include miRNA analysis, and thus the development of more efficient laboratory tools to quantify microRNAs is becoming increasingly important.

**Current Methods for Assaying MicroRNAs**

Today, many researchers are using hybridization-based methods, such as Northern blots and microarrays, to identify and quantitate miRNAs in tissues and cells. Yet, in the face of the special challenges presented by miRNAs, these methods are not ideal.

**Northern analysis.** Northern analysis was one of the first methods commonly used for miRNA analysis because it generally is a homegrown technology that most labs can readily access without an investment in new capital equipment and without learning new protocols. Because it is such a labor-intensive, manual process, Northern analysis is not amenable to large-scale screening experiments. However, it was adequate for the first phase of miRNA biology, in which researchers focused on discovering the general mechanisms of miRNA function. For example, researchers have used Northern analysis to elucidate details about miRNA maturation (3) and to determine that complementarity between a miRNA and its RNA target directs the miRNA's cellular function (4).

The dynamic range of Northern blot analysis typically only reaches two orders of magnitude — a significant problem given the wide range in the number of miRNA molecules in cells. For example, blot conditions that can measure 40,000 miRNA molecules per cell will not accurately detect 10 miRNA molecules per cell. Further, because Northern blots are based on hybridization, they often cannot distinguish well between miRNAs that only have small sequence differences — a common situation among closely related miRNA family members. For example, the miRNA let-7c differs from family members let-7a and let-7b by only one base. In addition, the reproducibility of Northern blots is known to be relatively low.

When miRNAs are rare, successful detection of the precursor and mature miRNA signals can require exposure times as long as three days, which not only makes experimentation slow but also increases the risk of losing the signal in the background on the blot. Another major disadvantage of Northern blots is the enormous amount of starting sample required. Researchers typically only hybridize one miRNA probe to a Northern blot at a time, and it is not uncommon to need 5 to 10 micrograms of total RNA per lane to successfully detect miRNAs — a situation that makes experiments impossible or prohibitive with scarce samples such as stem cells or primary human tumor cells, and that makes profiling multiple miRNAs impractical.

**Microarrays.** Microarrays provide advantages over Northern blots in that they tend to have a slightly larger dynamic range and greatly improved throughput. Therefore, they can enable researchers to conduct cost-effective large-scale screening experiments. For example, researchers recently used high-density oligonucleotide microarrays to conduct a global analysis of miRNA expression levels in...
multiple eukaryotes, including human, mouse and rat (5).

Yet, microarrays still require enough RNA (approximately 5 µg per array) that their usefulness is limited to experiments where the sample is relatively abundant. This limitation is compounded by the fact that several array replicates are usually required for statistical validity. Further, because microarrays do not inherently include size-separation like Northern blots, the identical sequence shared between precursor and mature, active miRNAs can make it difficult or impossible to distinguish between the two; when the assessment of biological activity is the goal, this limitation can confound interpretation. Similarly, as with Northern blots, the hybridization basis of microarrays also means that they do not distinguish well between miRNAs that have only small differences in sequence.

**Quantitative Real-time PCR**

Because of the limitations associated with both microarray and Northern experiments, researchers typically validate results obtained by these methods using quantitative real-time polymerase chain reaction (PCR), which is considered to be the gold standard for quantifying both DNA and RNA levels. Therefore, it is reasonable to expect that researchers will increasingly use quantitative real-time PCR as their primary assay, rather than using it as a secondary validation step, especially when ultra high throughput is not required.

Quantitative, real-time PCR is based on the quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed (6, 7). The specificity of TaqMan® (Roche Molecular Systems Inc., Basel, Switzerland) assays (quantitative real-time PCR) relies on the combined action of two PCR primers and a sequence-specific TaqMan probe (8). These fluorogenic-labeled probes take advantage of the 5’ nuclease activity of Taq DNA polymerase (TaqMan reagent-based chemistry), greatly improving real-time PCR detection and making it possible to eliminate post-PCR processing for the analysis of probe degradation (e.g., signal generation). This enables a simple, easily performed, homogeneous assay for gene expression profiling (8).

Despite the fact that quantitative real-time PCR requires dramatically less sample than Northern analysis or microarrays and has significantly improved dynamic range and sensitivity, this technology also has encountered challenges when faced with miRNA detection. For example, the short length of mature miRNAs has made it difficult to design effective primer and probe sets that are specific for mature miRNAs. Because it

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<thead>
<tr>
<th>Table 1. MiRNA assay methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Quantitative real-time PCR</strong></td>
</tr>
<tr>
<td>Starting material</td>
</tr>
<tr>
<td>Sensitivity</td>
</tr>
<tr>
<td>Specificity</td>
</tr>
<tr>
<td>Dynamic range</td>
</tr>
<tr>
<td>Throughput</td>
</tr>
<tr>
<td>Time to results</td>
</tr>
<tr>
<td>Radiolabel required?</td>
</tr>
<tr>
<td>Reliably distinguishes mature miRNA from precursor</td>
</tr>
</tbody>
</table>

Figure 2. Assay scheme.
Step 1 — Looped-primer RT: Looped primers are annealed to miRNA targets and extended by reverse transcriptase. Step 2 — Real-time PCR: miRNA-specific forward primer, a TaqMan probe and universal reverse primer are used for PCR reactions. Quantitation of miRNAs is estimated based on measured CT values.
is possible, with careful design, to create quantitative real-time PCR assays for the longer precursor molecules, some researchers have used the level of miRNA precursors as surrogate markers for the mature, active miRNA (9). Yet, the level of precursor miRNA present in cells is not necessarily a valid indication of the level of the corresponding mature miRNA, making this method less than ideal.

Recently, however, a new miRNA quantitation method has been developed that overcomes these challenges (Table I). The method uses stem-looped primers for reverse transcription (RT) of the miRNA, followed by quantitative real-time PCR (Figure 2). The stem-loop structure, which is specific to the 3′ end of the mature miRNA, extends the very short mature miRNA molecule and adds a universal 3′ priming site for real-time PCR. The stem-loop also is believed to create steric hindrance that prevents priming of the precursor miRNA. Real-time PCR then provides a highly specific and quantitative method to screen miRNA expression levels across various biological contexts using a very small amount of starting material (1 to 10 ng of total RNA or equivalent). For example, the stem-loop method has been found to have a dynamic range of up to seven orders of magnitude (Figure 3), making it ideal for detecting both high- and low-abundance miRNAs. In addition, this method enables researchers to discriminate between miRNAs that differ by only one base (Figure 4). Finally, as with traditional quantitative real-time PCR assays, carrying out these new stem loop assays in the lab is fast and simple.

RNA Interference

The discovery that cells possess a mechanism for gene regulation that employs small RNAs led to the development of methods for introducing synthetic small RNAs to harness the power of that mechanism. This technique is called RNA interference (RNAi) and can be used to determine gene function, study signaling pathways, identify and validate drug targets and generate animal knockdown models. RNAi is carried out in the lab by introducing small, double-stranded, non-coding RNAs to "silence" the expression of targeted genes in a sequence-specific manner. The short, non-coding RNAs used in RNAi experiments include both short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs).

Once the si/shRNAs are delivered to the cells, researchers routinely measure the expression level of the target mRNA to determine the extent of transcript silencing. Although methods like Northern blots and ribonuclease protection assays are
available, quantitative real-time PCR with fluorescently labeled probes provides the most sensitive and accurate method for detecting and measuring mRNA (8, 10). It is thus ideal for correlating the extent of gene knockdown with results from a follow-up assay or with an RNAi-induced phenotype. Finally, as the design technology for quantitative real-time stem-loop PCR assays advances, in the future these assays also should be applicable to direct quantitation of si/shRNA levels in cells. This capability will enable researchers to directly measure the success of their RNAi delivery efforts and correlate these levels to experimental results.

References

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The PCR process and 5' nuclease process are covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd.

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