Design Pipeline for TaqMan® Small RNA Assays

MicroRNAs are short ~22-nucleotide sequences that have recently emerged as important global regulators of gene expression. These RNAs were overlooked for many years because of their small size, which also presents a significant challenge to their detection using existing qPCR technologies. In 2005, Applied Biosystems introduced TaqMan® MicroRNA Assays, which utilize a novel miRNA-specific stem-loop primer approach to overcome the difficulty of amplifying small nucleic acids. We now offer the most extensive catalogue of inventoried real-time PCR assays for the detection and quantitation of miRNA. Our design knowledge is captured in an exclusive, automated small RNA assay design pipeline: the miRPipe™ design process, resulting in unparalleled sensitivity, specificity, and dynamic range of small RNA detection. In this article, we describe the development of the design pipeline and describe its application beyond miRNAs to the design of TaqMan® Assays for any small RNA.

Featured Products
TaqMan® MicroRNA Assays
Early Access: Custom TaqMan® Small RNA Assays
Coming Soon! TaqMan® siRNA Assays

Applied Biosystems® TaqMan® MicroRNA Assays incorporate a target-specific, stem-loop reverse transcription (RT) primer to address the fundamental problem of miRNA quantitation: the short length of mature miRNAs (~22 nt). The stem-loop structure provides specificity for the mature miRNA target and, after reverse transcription, forms an RT primer/mature miRNA chimera that extends the 3′ end of the miRNA (Figure 1). This longer RT product presents a template amenable to standard TaqMan® real-time PCR.

Initial Design-Pipeline Development

The first TaqMan® MicroRNA Assays were designed manually by harnessing the principles of TaqMan® Assay design and incorporating the stem-loop RT primer. This initial effort required selection of multiple assays for each miRNA transcript followed by rigorous testing in the lab. The assay that met stringent assay performance requirements was then selected. Although tedious, this approach served a 2-fold purpose: to make TaqMan® MicroRNA Assays commercially available for known human and rodent miRNAs and to generate a training set used to develop an initial assay design algorithm predictive of TaqMan® Assay performance for real-time quantitation of miRNAs.

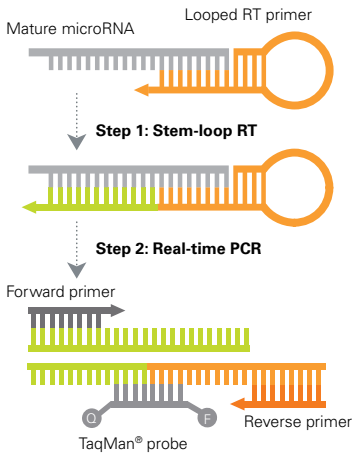

Figure 1. TaqMan® MicroRNA Assays. An innovative stem-loop RT primer brings the advantages of real-time PCR to the study of small noncoding RNA.

Availability of this first-generation version of the miRPipe™ design pipeline enabled rapid assay design for new miRBase database content. Furthermore, data from confirmatory wet lab testing of assays has been used to further refine the design algorithm. This intensive development effort has resulted in the *in silico* design and experimental verification of over 1,800 high performing TaqMan® MicroRNA Assays.

Scoring-Optimization and Validation

To automate the design process and enable design of assays for other small noncoding RNAs such as siRNA, piRNA, and rasiRNA, a large test set of small RNAs was used for scoring-optimization and validation of the design pipeline. The goal was to balance excellent assay performance, reasonable assay design times, and the ability to design assays for virtually any small RNA sequence.

More than 10 possible configurations of the design pipeline were evaluated using a validation set of 1,500 target sequences composed of 500 miRNA sequences from the miRBase database, 500 piRNA from the NCBI database, and 500 siRNA from the Ambion® *Silencer*® Select siRNA product line. The design mode with the best balance of turnaround time and design success was selected as the default mode for the design pipeline. Next, a subset of assays from the validation set were selected for experimental testing to further validate the high correlation between the design scores and performance.

The miRPipe™ pipeline is designed for flexibility; each step of the scoring process can be customized, and the combining of individual scores to yield an overall assay quality measurement can be optimized. The benefit of this architecture is that the design process can be kept current as new information on small noncoding RNAs becomes available.

Overview of the Completed miRPipe™ Design Pipeline

The miRPipe™ design pipeline consists of two major parts: assay design and *in silico* quality control (QC) (Figure 2). As targets are processed through the pipeline, potential RT and PCR primers, TaqMan® probes, and complete assays are assigned quality scores for a long list of attributes. These scores are then combined to assess overall assay quality and identify the best assay design. The process consists of the following steps:

1. Generate all potential oligonucleotides (primers and probe) needed for the assay based upon the input target sequence and assign scores based on thermodynamic properties and assay design rules.

Figure 2. Overview of the Small RNA Assay Design Pipeline.

2. Combine compatible RT primers, forward and reverse PCR primers, and TaqMan® probes into assay sets.

3. Score potential assays using an *in silico* QC module focusing on two main areas:
   — Oligonucleotide interaction: All pairwise oligonucleotide interactions are analyzed using an algorithm that incorporates a customized substitution matrix and a position-dependent scoring matrix derived from experimental data. In addition, primer extension products of all potential interactions are analyzed. The results are used to assign scores for the likelihood of nonspecific amplification and other undesirable oligonucleotide interactions.
   — Specificity for intended target: Assays are evaluated and scored for specificity of both the RT primer and the TaqMan® probe/PCR primer set using proprietary bioinformatics tools developed for the design of TaqMan® Gene Expression Assays [1].

4. Select highest scoring assay. Assays must meet minimal score threshold cutoffs established as described in the next section.

Easy Ordering of TaqMan® Small RNA Assays for Virtually Any Target

The final version of the small RNA assay design pipeline described above has already been in use internally at Applied Biosystems for over a year now. Over 250 customers have participated in early access collaborations; their work includes analysis of small RNAs identified via SOLiD™ System next generation sequencing, detection and quantitation of small viral RNAs, and validation of microarray results.

The pipeline continues to be used to design assays to detect newly discovered miRNAs, keeping our collection of TaqMan® MicroRNA Assays aligned with the Sanger miRBase Registry. Because of the highly conserved nature of miRNAs and other small RNAs, TaqMan® assays are not categorized according to species, unlike other commercial suppliers. We have found that, because small RNAs often share significant sequence homology, species designations are not particularly relevant.

The miRPipe™ small RNA assay design pipeline will also be used to design our new TaqMan® siRNA Assays and assays to customers' novel or proprietary small RNA sequences through the Custom TaqMan® Small RNA Assays program. TaqMan® siRNA Assays have been shown to be effective in evaluating siRNA delivery efficiency and distribution, evaluating stability of siRNAs in cells and animals, and studying specific pathways [2].

The web interface for ordering TaqMan® Assay products for detection of miRNA and other small RNA species was designed for convenience and simplicity. Find and order TaqMan® Assays for your research using the following methods:

TaqMan® MicroRNA Assays

- Go to **microrna.appliedbiosystems.com**, then click on TaqMan® MicroRNA Assays.
- Click on the Assay Search tab to search for individual or multiple assays:
  — Search for individual miRNAs listed in the Sanger miRBase database using miRBase mature identifiers, mature miRNA sequences, miRBase miRNA gene family identifiers, miRBase accession alias, assay ID, miRBase stem loop identifiers, or stem loop sequence.
  — Search by entering a list or uploading a file containing supported search terms.
- Click on the GeneAssist™ miRNA Workflow Builder to order miRNA-specific TaqMan® MicroRNA Assays, as well as related products such as Ambion® Anti-miR™ miRNA Inhibitors and Pre-miR™ miRNA Precursors, controls, and custom inhibitors and precursors.
- Search for endogenous control assays to use for your miRNA quantification experiments.

Early Access: TaqMan® Custom Small RNA Assays

- Go to **www4.appliedbiosystems.com/beta/smallma**.
- Easily submit novel and/or proprietary sequences for design:
  — Type in sequence(s) individually for the target small RNA(s), providing names for each target, or upload batch sequences in FASTA format. The Custom Design Tool incorporates automatic name parsing rules for effortless sequence uploads, from sources such as NCBI, EMBL, Sanger, DDBJ, ENSMBL, and the Applied Biosystems® SOLiD™ System for next generation sequencing.
  — When pre-designed assays are available for the target sequence entered, the Custom Design Tool will automatically identify the assay ID so that you can add it to your order.

Coming Soon: TaqMan® siRNA Assays

Currently, TaqMan® Assays for siRNAs can be ordered using the TaqMan® Custom Small RNA Assay channel.

Confident Results With TaqMan® Assay Technology

Applied Biosystems® miRPipe™ small RNA assay design pipeline is founded on our extensive background and bioinformatics expertise designing TaqMan® Gene Expression Assays. To overcome the challenges of detecting very small targets, it includes design features based on empirical data gathered over years of experiments in the lab. The resulting automated pipeline includes built-in flexibility features enabling design of assays for the broadest assortment of small RNA sequences. With the release of this new small RNA assay design pipeline, the benefits of TaqMan® Assay technology are now available for analysis of virtually any small RNA, providing results you can trust from the very first experiment.

REFERENCE

1. Lazaruk K, Wang Y, Zhong J et al. (2006) The design process for a new generation of quantitative gene expression analysis tools [white paper]. Accessible at www.appliedbiosystems.com (search for Publication 127WP02-02).
2. Cheng A, Li M, Liang Y et al. (2009) Stem-loop RT-PCR quantification of siRNAs *in vitro* and *in vivo*. *Oligonucleotides* 19(2):203–208.

ORDERING INFORMATION	P/N	SIZE
TaqMan® MicroRNA Assays	Varies*	50 RT and 150 PCR rxns
Custom TaqMan® Small RNA Assays: Early Access	4398987*	150 RT and 150 PCR rxns
TaqMan® siRNA Assays	4440878	50 RT and 150 PCR rxns
TaqMan® MicroRNA Assays (made-to-order)	4440886	50 RT and 150 PCR rxns
TaqMan® MicroRNA Reverse Transcription Kit	4366596	200 rxns
	4366597	1,000 rxns
TaqMan® Universal PCR Master Mix	Varies	200–2000 rxns

* Assays are available in different sizes, under different part numbers.