A technical guide to identifying miRNA normalizers using TaqMan Advanced miRNA Assays

Introduction

MicroRNAs (miRNAs) represent a class of regulatory biomolecules with roles in diverse processes such as cell proliferation, differentiation, apoptosis, and oncogenesis [1]. In recent years, technological advances in research tools including qPCR, microarrays, and next-generation sequencing (NGS) have enabled sensitive detection of miRNAs. However, accurate quantification of miRNAs using qPCR is largely dependent on proper normalization techniques, the absence of which can lead to misinterpretation of data and incorrect conclusions [1]. The goal of most miRNA experiments using qPCR is to identify differences in expression between two groups of samples, typically a normal (control) and a mutated (test) sample group. The purpose of normalization is to remove any differences between these two groups other than that which is a true representation of expression levels of the miRNAs in the mutated state.

Differences could arise from sample procurement, stabilization, RNA extraction or quantification methods, or sample-to-sample inconsistencies. A proper normalization strategy is one of the essential key elements included in the MIQE guidelines [2]. An ideal normalization strategy uses 2 or 3 miRNAs that are uniformly expressed in the tissue or cell type of interest and demonstrate storage stability, extraction, and quantification efficiency equivalent to that of the target of interest [3]. However, identifying miRNAs that fulfill these criteria has been challenging. Small RNA sequencing and other profiling studies have revealed that expression of miRNAs varies greatly within different tissues and body fluids, and therefore no single miRNA can be used as a "global normalizer" [4,5].

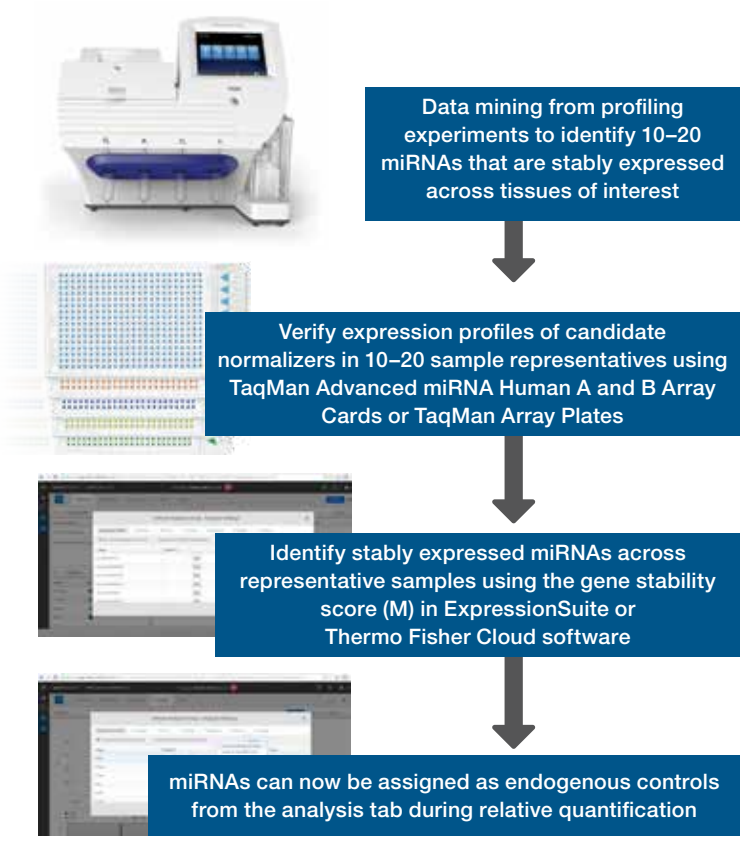

Figure 1. Workflow to identify the 2 or 3 most stable miRNAs in a set of samples and use them as endogenous controls to normalize miRNA expression in qPCR studies. We offer a complete solution for profiling (next-generation sequencing, Applied Biosystems™ Megaplex™ PreAmp Primers), verification (custom Applied Biosystems™ TaqMan® Array Cards and Plates), and data analysis. Thermo Fisher Cloud offers the geNorm algorithm [16] to identify the most stably expressed genes in a set of samples.

Here we have compiled a list of candidate miRNAs that can potentially be used as normalizers. It is highly recommended that any group of miRNAs identified as normalizers from such a study be empirically verified for each sample type used in qPCR [1,3]. Ideally, candidate normalizers are first checked for their stability across 10–20 representative samples of interest [1]. Here we briefly describe a workflow that could be used to identify and verify miRNAs as normalizers for qPCR experiments (Figure 1). High tissue-specific conservation among human, mouse, and rat miRNAs has been reported [4,5]; based on these reports, we recommend the use of mouse and rat Applied Biosystems™ TaqMan® Advanced miRNA Assays that correspond to human miRNA controls listed in this paper.

Tissue normalizers

The miRNA expression atlas based on microarray technology [4] and small RNA sequencing [5] revealed that while most miRNAs show extremely highly tissue-specific expression patterns, hsa-miR-16-5p was uniformly expressed across most tissues in the 250 samples analyzed. Further, Liang et al. reported stable expression of hsa-mir-30e-5p and hsa-miR-24-3p across 40 normal tissues [6]. Peltier and Latham reported consistent expression of hsa-miR-191-5p, hsa-miR-17-5p, and hsa-miR-103a-3p across 13 normal tissues and 5 pairs of distinct tumor and normal adjacent tissues [1]. In an inter-laboratory, inter-platform study, Bargaje et al. found hsa-miR-92, hsa-miR-93-5p, hsa-miR-191-5p, and hsa-miR-103a-3p to be constitutively expressed across several different tissues [7]. Available TaqMan Advanced miRNA Assays for candidate tissue normalizers are listed in Table 1.

Table 1. miRNAs reported to have relatively stable expression across most human tissues.

miRNA name	TaqMan Advanced miRNA Assay
hsa-miR-16-5p	477860_mir
hsa-miR-30e-5p	479235_mir
hsa-miR-103a-3p	478253_mir
hsa-miR-191-5p	477952_mir
hsa-miR-24-3p	477992_mir

Serum normalizers

Cell-free miRNAs are emerging as important noninvasive biomarkers because of their stability and mutation-specific expression patterns in serum and plasma. One challenge in studying miRNAs from serum and plasma, however, is their relatively low abundance and lack of reliable endogenous controls. Recently, Marabita et al. found hsa-miR-24, hsa-miR-126, and hsa-miR-484 to be the most stable miRNAs from a profiling study on serum [8]. Song et al. reported hsa-miR-16-5p and hsa-miR-93-5p as stably expressed miRNAs in serum from mutated and control samples of gastric cancer [9], while Hu et al. successfully used hsa-miR-484 and hsa-miR-191-5p as normalizers in serum from breast cancer tissues [10]. Available TaqMan Advanced miRNA Assays for candidate serum normalizers are listed in Table 2.

Table 2. miRNAs reported to have stable expression in serum and plasma.

miRNA name	TaqMan Advanced miRNA Assay
hsa-miR-24	477992_mir
hsa-miR-484	478308_mir
hsa-miR-93-5p	478210_mir
hsa-miR-191-5p	477952_mir
hsa-miR-126-3p	477887_mir
hsa-miR-16-5p	477860_mir

Hemolysis markers

Hemolysis causes release of miRNAs that dramatically alter the miRNA profile of serum and plasma. While several techniques such as spectrophotometry and analytical analysis are routinely used to detect hemolysis, Shah et al. found that the ratio of hsa-miR-451a to hsa-miR-23a-3p was the most sensitive method to detect down to 0.001% hemolysis in serum [11].

Exogenous controls

In the absence of reliable endogenous controls in serum, exogenous or spike-in controls can be used to normalize miRNA expression data. Exogenous controls are also used to monitor extraction efficiency or sample input amount for difficult samples (e.g., serum, plasma, or other biofluids). Exogenous controls are synthetic RNA oligonucleotides that match the target sequence of the detection assay. TaqMan Advanced MicroRNA Assays are available for a number of miRNAs that can be used as spike-in controls with human, mouse, and rat samples as listed in Table 3. The final concentration of the spike-in control in your test sample (during cDNA synthesis) should be within the range of 1–10 pM. TaqMan Advanced miRNA chemistry requires that the RNA oligo has a 5´ phosphate.

Table 3. Recommended exogenous controls for human, mouse, and rat samples.

miRNA name	Assay ID	Target sequence
ath-miR159a	478411_mir	UUUGGAUUGAAGGGAGCUCUA
cel-miR-2	478291_mir	UAUCACAGCCAGCUUUGAUGUGC
cel-miR-238	478292_mir	UUUGUACUCCGAUGCCAUUCAGA
cel-miR-39	478293_mir	UCACCGGGUGUAAAUCAGCUUG
cel-miR-54	478410_mir	UACCCGUAAUCUUCAUAAUCCGAG
cel-miR-55	478295_mir	UACCCGUAUAAGUUUCUGCUGAG

Tissue-specific normalizers

Profiling studies by small RNA sequencing or high-throughput miRNA expression platforms such as TaqMan Array Cards or OpenArray™ plates have shown that miRNAs exhibit an extremely high tissue-specific index. Selection of endogenous controls in tissues therefore requires extensive verification in specific tissues of interest. This section compiles data on miRNA expression patterns in various tissues.

In a cross-platform analysis of 32 early breast cancer samples, Rinnerthaler et al. found miR-16-5p and miR-29a-3p to be stably expressed [12]. In an independent study by Davoren et al., miR-16-5p and let-7a-5p also emerged as stable miRNAs across malignant, benign, and normal breast tissues [13]. In the same study, miR-21, miR-26b-5p, and miR-10b-5p were also found to be relatively stable. Bargaje et al. found miR-133a-3p, miR-26a-5p, and miR-1-3p to be specifically associated with heart, miR-155 with normal blood, miR-125b and miR-191-5p with testis, miR-122-5p with liver, and miR-30c, miR-203, and miR-10b with kidney [7]. Das et al. found miR-1228-3p and miR-25-3p to be stable across all cancer cell lines tested [14]. Sood et al. found miR-122-5p to be extremely specific to liver and miR-204 to testis [15]. Ludwig et al. found miR-514a-3p to be exclusively expressed in testis, miR-449c-5p and miR-449b-5p in kidney, and miR-449a in lung, kidney, and brain [4]. Table 4 provides a summary of recommended endogenous controls for these tissues.

Table 4. Recommended tissue-specific miRNA normalizers.

Tissue	Recommended normalizers	TaqMan Advanced miRNA Assay
Breast	hsa-let-7a-5p	478575_mir
	hsa-miR-10b-5p	478494_mir
	hsa-miR-16-5p	477860_mir
	hsa-miR-21	477975_mir
	hsa-miR-26b-5p	478418_mir
Kidney	hsa-miR-215-5p	478516_mir
	hsa-miR-30c-5p	478008_mir
	hsa-miR-449b-5p	479528_mir
	hsa-miR-449c-5p	479367_mir
Testis	hsa-miR-191-5p	477952_mir
	hsa-miR-204	478491_mir
	hsa-miR-514a-3p	479397_mir
Heart	hsa-miR-133a-3p	478511_mir
	hsa-miR-1-3p	477820_mir
	hsa-miR-26a-5p	478418_mir
Liver	hsa-miR-122-5p	477855_mir
	hsa-miR-192-5p	478262_mir
Cancer cell lines	hsa-miR-1228-3p	478643_mir
	hsa-miR-25-3p	477994_mir
	hsa-miR-93-5p	478210_mir
Lung	hsa-miR-221-3p	477981_mir
	hsa-miR-449a	478561_mir

Global mean normalization

In high-throughput profiling experiments or experiments with large data sets, global mean normalization is an excellent way to normalize data [16]. Global normalization first finds assays common to every sample, then uses the median C_t values of all of those assays as a normalization factor on a per-sample basis. Thermo Fisher Cloud and Applied Biosystems™ qPCR analysis modules offer an algorithm to enable global mean normalization as an option for high-throughput experiments.

applied biosystems

Conclusion

miRNAs are an important class of biomolecules with a wide range of regulatory functions. TaqMan Advanced miRNA Assays provide an extremely robust solution to study expression profiles of miRNAs in various tissues and body fluids. While qPCR by TaqMan Advanced miRNA Assays offers an extremely valuable tool to study gene expression, proper normalization is essential to avoid misinterpretation of data. In the past, miRNA gene expression data analysis relied on the use of small nucleolar RNAs (snRNAs) such as U6 as a normalizer. However, more recently researchers have indicated that it is best to normalize qPCR data using the same class of biomolecules [3]. Finding global miRNA normalizers is a challenge, since no miRNA has been found to be constitutively expressed in all tissues and biofluids. In this paper we have compiled a list of recommended endogenous controls in various tissues and biofluids, based on published literature. We also provide a brief overview of the recommended protocol to verify these miRNAs as qPCR normalizers in the tissue or body fluid of interest.

References

1. Peltier HJ and Latham GJ (2008) Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* 14(5):844–852.
2. Bustin SA, Benes V, Garson JA et al. (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611–622.
3. Vandesompele J, De Preter K, Pattyn F et al. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:34.1–34.11.
4. Ludwig N, Leidinger P, Becker K et al. (2016) Distribution of miRNA expression across human tissues. *Nucleic Acids Res* 44:3865–3877.
5. Landgraf P, Rusu M, Sheridan R et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* 129:1401–1414.
6. Liang Y, Ridzon D, Wong L et al. (2007) Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* 8:166.
7. Bargaje R, Hariharan M, Scaria V et al. (2010) Consensus miRNA expression profiles derived from interplatform normalization of microarray data. *RNA* 16:16–25.
8. Marabita F, de Candia P, Torri A et al. (2016) Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR. *Brief Bioinform* 17:204–212.
9. Song J, Bai Z, Han W et al. (2012) Identification of suitable reference genes for qPCR analysis of serum miRNA in gastric cancer patients. *Dig Dis Sci* 57:897–904.
10. Hu Z, Dong J, Wang LE et al. (2012) Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls. *Carcinogenesis* 33:828–834.
11. Shah JS, Soon PS, and Marsh DJ (2016) Comparison of methodologies to detect low levels of hemolysis in serum for accurate assessment of serum microRNAs. *PLoS One* 11:e0153200.
12. Rinnerthaler G, Hackl H, Gampenrieder et al. (2016) miR-16-5p is a stably expressed housekeeping microRNA in breast cancer tissues from primary tumors and from metastatic sites. *Int J Mol Sci* 17:156.
13. Davoren PA, McNeill RE, Lowery AJ (2008) Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer. *BMC Mol Biol* 9:76.
14. Das MK, Andreassen R, Haugen TB et al. (2016) Identification of endogenous controls for use in miRNA quantification in human cancer cell lines. *Cancer Genomics Proteomics* 13:63–68.
15. Sood P, Krek A, Zavolan M et al. (2006) Cell-type-specific signatures of microRNAs on target mRNA expression. *Proc Natl Acad Sci USA* 103:2746–2751.
16. Mestdagh P, Van Vlierberghe P, De Weer A et al. (2009) A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* 10:R64.

Ordering information

Product	Quantity	Cat. No.
TaqMan Advanced miRNA Assays	250 qPCR reactions (20 µL)	A25576
TaqMan Advanced miRNA cDNA Synthesis Kit	50 reactions	A28007
TaqMan Fast Advanced Master Mix	5 mL	4444557

Find out more at **thermofisher.com/advancedmirna**

ThermoFisher SCIENTIFIC