

Data Normalization Strategies for MicroRNA Quantification

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BACKGROUND: Different technologies, such as quantitative real-time PCR or microarrays, have been developed to measure microRNA (miRNA) expression levels. Quantification of miRNA transcripts implicates data normalization using endogenous and exogenous reference genes for data correction. However, there is no consensus about an optimal normalization strategy. The choice of a reference gene remains problematic and can have a serious impact on the actual available transcript levels and, consequently, on the biological interpretation of data.

CONTENT: In this review article we discuss the reliability of the use of small RNAs, commonly reported in the literature as miRNA expression normalizers, and compare different strategies used for data normalization.

SUMMARY: A workflow strategy is proposed for normalization of miRNA expression data in an attempt to provide a basis for the establishment of a global standard procedure that will allow comparison across studies.

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MicroRNAs (miRNAs)⁵ are small noncoding RNA transcripts of approximately 22 nucleotides in length that exert an important regulatory role on cell activity at the transcriptional level. miRNAs are initially transcribed as immature pri-miRNAs, being processed in the cell nucleus and cytoplasm by the RNAase III enzymes Drosha and Dicer and loaded into Ago proteins to form the RNA-induced silencing complex (RISC) (1). Mature functional miRNAs loaded in the RISC complex interact with complementary sequences usually located at the 3'

untranslated region (3'-UTR) of mRNAs present in the cell cytoplasm, promoting mRNA cleavage or repressing their translation (2). Ultimately, this regulates gene expression by decreasing the production of effector proteins. miRNAs can repress multiple targets within the same pathway, resulting in amplification of their biological effects (3).

In physiological conditions, miRNAs regulate cell differentiation, cell proliferation and survival, and metabolism, among many other functions (2). Additionally, disruption of their expression patterns implicates miRNAs in disease onset and progression (4), such as cancer (5), and their potential role as prognostic and predictive biomarkers in patient management has been described (6). Beyond the functions they exert in the cells that produce them, miRNAs may also be secreted and transferred to other cells, circulating in virtually all body fluids, either in protein complexes or enclosed inside extracellular vesicles, such as microvesicles and exosomes (7).

Because the collection of miRNAs produced by cells reflects their physiological state, these noncoding RNAs have been much explored as disease biomarkers (8). Different methodologies have been applied to characterize qualitatively and quantitatively the expression patterns of miRNAs associated with pathological vs normal conditions, including quantitative real-time PCR (qPCR), microarray screening, Northern blotting, ultrahigh-throughput miRNA sequencing (e.g., small RNA-seq, next generation sequencing), in situ hybridization with locked nucleic acids probes, and hybridization in solution with tagged probes (e.g., nCounter® nanoString technology), among many others (9). To accurately determine the levels of analyzed miRNAs, their expression data are usually normalized relatively to endogenous and/or exogenous reference genes. However, different studies use different normalization strategies to report miRNA expression levels. This leads to ambiguous data interpretation, misleading conclusions, and erroneous biological predicted effects, impairing comparisons between studies; consequently, no one optimal normalization strategy seems to have reached consensus status for the scientific community so far.

Technical Challenges

The outcomes of miRNA analysis depend on several aspects of the overall process, beginning with the nature of

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⁵ Nonstandard abbreviations: miRNA, microRNA; RISC, RNA-induced silencing complex; 3'-UTR, 3' untranslated region; qPCR, quantitative real-time PCR; Cq, cycle threshold; snRNA, small nuclear RNA; IPA, ingenuity pathway analysis.

the sample, the way it is collected, preserved, and processed, the technical method applied for miRNA detection, and the strategy followed for data normalization and analysis (10).

The accurate comparison of miRNA expression between samples requires that equal amounts of total miRNAs are used as input for the detection method applied. Usually, the determination of the quantity of sample input for miRNA detection techniques is based on total RNA quantification, but the real proportion of miRNAs may vary from sample to sample, especially if they have a different origin. Additionally, the integrity of miRNAs is rarely determined, with microfluidic capillary electrophoresis being currently the best method to assess miRNA quality, but even with results being compromised by mRNA degradation in the samples (11).

miRNAs may be isolated by different methods from cultured cells, fresh tissues, frozen and fixed tissues, or as cell-free circulating RNAs from conditioned cell culture media and body fluids, including, for instance, whole blood, plasma, serum, urine, and cerebrospinal fluid (12–16). The collection of fresh human samples requires their preservation by different methods, such as coagulation prevention, freezing, fixing, and paraffin embedding. The preservation process induces molecular changes that may lead to global miRNA instability or enrichment/depletion of specific miRNAs in the samples (17). As an example, Kim et al. demonstrated that the capacity to detect endogenous and exogenous miRNAs in plasma samples strongly depends on the method used to prevent blood coagulation (18). Likewise, Farina et al. showed that freeze and thawing cycles differently affected the levels of specific miRNAs in serum samples (19). The time selected for sample collection is also important when analyzing circulating miRNAs, because their physiological levels may vary according to the circadian rhythm, meal ingestion, and overall lifestyle (for instance, smoking and drug consumption) (20, 21). Another factor to consider when analyzing the levels of miRNAs in pathological conditions is the variation of their levels according to disease stage and progression (22, 23), as well as medical interventions and treatment course (24, 25).

Currently, the study of circulating miRNAs strongly focuses on RNA isolated from microvesicles and exosomes actively secreted by cells. For the particular case of exosomes, different methods can be employed for their isolation, most commonly, ultracentrifugation (using or not a density gradient), filtration, size-exclusion chromatography, and precipitation (using polymeric solutions or beads with immunoaffinity to an exosomal protein marker). Considering their working principle, the different methods lead to an enrichment of specific vesicle subpopulations that likely carry different cargo (21, 26). Notably, large and dense complexes of proteins associated with other biomolecules, such as different types of

RNA, have been detected as coprecipitants in exosome pellets isolated by ultracentrifugation. Consequently, miRNA analysis of these samples does not reflect the real intraexosomal content. Size-exclusion chromatography has been proposed as a good isolation alternative to circumvent this issue (27). Immunoaffinity-based methods have been widely used to isolate exosomes from body fluids, targeting proteins described as disease-specific biomarkers and carried on vesicle surfaces. This strategy may bias the definition of specific circulating miRNAs as disease biomarkers compared to the relative miRNA expression in exosomes isolated in healthy conditions by a method of diminished specificity (28). Conversely, disease-specific miRNA biomarkers may be missed because of their absence in vesicles selected by the particular protein biomarker chosen (29).

Ultimately, the global analysis of miRNA expression, especially for the confident discovery and validation of disease biomarkers, strongly depends on the size of cohorts/sets of samples analyzed. Very frequently, only small-sized test populations are studied, leading to an erroneous or biased misidentification of biomarkers. In a recently published metaanalysis reviewing miRNA biomarker discovery in nonneoplastic diseases, it was revealed that the majority of the studies published rely on populations under 100 individuals (median size, 69 study participants) (30).

The methodology chosen for miRNA detection also influences the outcomes of miRNA quantification. Currently, the most used methodologies are qPCR and hybridization on microarray platforms, with the former being the gold standard for detection of specific sets of miRNAs of interest and the later mostly applied for large-scale profiling.

Measurement of miRNAs by qPCR is very specific and sensitive, allowing the detection of very small quantities of miRNAs, and relatively inexpensive, and commercial ready-to-use kits are widely available. The most common qPCR detection techniques are stem-loop-shaped RT-primer Taqman assays (Applied Biosystems), assays using locked nucleic acid primers (Exiqon), and assays with poly-A tailing primers (QIAGEN, Stratagene). With these approaches, only a limited set of miRNAs can be tested in a single reaction, and detection is greatly influenced by the specificity of the primers designed (31).

Conversely, microarrays allow the probing of a large set of miRNAs simultaneously, currently at much more competitive costs, because several off-the-shelf platforms are commercially available from companies such as Affimetrix, Agilent, and Exiqon, among others. This technique requires an input of higher amounts of RNA, and assay performance may sometimes be compromised by hybridization conditions that are not optimal to the whole probes in the test. Likewise, the design of the probes to include in the platform may be troublesome, because they have to be specific enough for the target

miRNAs and, at the same time, share similarities in the conditions required for hybridization (32).

Both approaches have associated advantages and drawbacks, but one of the most striking pitfalls is the low correlation between different techniques. In a comparative study by Sato et al. (33), miRNAs in liver and prostate human tissues were profiled using microarray platforms from different companies and expression log-ratios were ranked. Surprisingly, the median rank correlation across platforms was only 0.55, and the highest correlation found was 0.87. Interestingly, the correlation between microarray- and Taqman-based expression data was higher, with a median correlation coefficient of 0.7, and only 1 of the platforms with a correlation coefficient lower than 0.5. The latter data support the common practice of using qPCR-based techniques as a confirmatory method for the validation of microarray expression data.

In an attempt to minimize the effect imposed by the factors previously described on miRNA expression levels, an accurate data analysis should be performed, using appropriate reference genes for external and internal variation correction (34).

Relative Data Normalization to Endogenous Reference Genes

Quantification of miRNAs has come to the fore and produced a wealth of literature, particularly during the last 5 years (35). Here, we focus on the issues that may arise with the data normalization in the gold-standard method qPCR. There are 2 most commonly used methods to analyze data from qPCR: absolute and relative quantification. Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control (36). For the relative miRNA quantification, the PCR-derived cycle threshold (Cq) of target miRNAs is compared with that of a stably expressed endogenous miRNA from the same sample. The difference between these values is the ΔCq value (37). This normalization approach aims at removing differences due to sampling and quality of the samples. The guidelines for QC and standardization of qPCR imply the use of an optimal normalization method (38); however, there are no universally accepted reference genes or so-called “housekeeping” transcripts for miRNA data normalization. This lack of consensus has resulted in various normalization strategies (39). Some studies have even proposed the erroneous notion of completely abandoning an endogenous normalization (40–44), and should of course be approached with some caution. In fact, it was reported that qPCR can be used without endogenous controls to analyze miR-371-3 in the serum of patients with testicular cancer, if the tech-

nical procedure is performed under controlled conditions. This study compared the Cq and ΔCq values of miR-371a-3p, miR-372, and miR-373-3p by real-time PCR with and without 18S rRNA (ribosomal RNA) as a reference gene for data normalization. The miRNA transcript levels were measured in 25 testicular germ cell tumor patients, 4 nontesticular germ cell tumor patients, and 17 age-matched male controls. A highly positive correlation between Cq and ΔCq values was found in all samples. The highest correlation was found for miR-371a-3p (R^2 , 0.956) (45). Despite this positive correlation between Cq and ΔCq values detected in all serum samples (45), relative data quantification is absolutely indispensable to substantiate robust miRNA data (46). In this particular study (45), the detected significant correlation was likely related to the similar (good) quality and handling of the serum samples, but there is no guarantee that this will always be the case. There is no evidence to support the notion that good sample quality will always be obtained, because total RNA can be partly degraded and bias the results. Therefore, miRNA quantifications without an internal control should be considered critically.

Fortunately, a majority of studies have carried out a relative quantification that compares the expression levels of target genes with the expression of an endogenous reference gene. Before starting an experiment, the choice of an endogenous reference gene is a critical step to avoid misinterpreted data and to identify true changes in miRNA expression levels. Usually, researchers select their endogenous reference gene for miRNA quantification according to reports in the literature or based on a distinguishable low SD in miRNA microarrays. Considering the most recent and significant reports (see Table 1 in the Data Supplement that accompanies the online version of this review at <http://www.clinchem.org/content/vol61/issue11>), numerous reference genes, such as small nuclear/nucleolar RNAs, have been commonly used for miRNA quantification (47–50). These small RNA molecules share similar properties, such as RNA stability and size, and are abundantly expressed. Although these transcripts display constant expression in single analyses, their expression levels can change under different experimental conditions and may be affected by specific types of disease (49). Therefore, proposed normalizers first should be established across various sample types, and the combination of several normalizers might be more appropriate than a single universal normalizer (51).

RNU6 and Other RNUs as Normalizers

The small noncoding RNA RNU6 genes RNU6A and RNU6B are the reference genes most frequently used as normalizers (see online Supplemental Table 1). However, RNU6 is not an miRNA, and, consequently, does not reflect the biochemical character of miRNA molecules in terms of their transcription, processing, and

tissue-specific expression patterns (52, 53). In addition, the efficiency of their extraction, reverse transcription, and PCR amplification may differ from that of miRNAs. It has been argued that it is best to normalize miRNAs with reference genes belonging to the same RNA class, thus to miRNAs (39). In this regard, Gee et al. showed that the use of small nuclear RNAs (snRNAs) as reference genes can introduce bias when quantifying miRNA expression, and that this bias can be quite important in cancer prognosis (53).

Gee et al. (53) measured RNU6B, RNU44, RNU48, and RNU43 for data normalization in qPCR analyzing breast cancer and head and neck squamous cell carcinoma patients. The expression of these snRNAs was as variable as the expression of the target miRNAs miR-21, miR-210, and miR-10b, and data normalization to these recommended reference snRNAs introduced bias in the associations between miRNA and pathology or outcome (53). Using microarray-based serum miRNA profiling followed by qPCR, Xiang et al. screened and compared the expression levels of reference RNAs in patients with different tumors and healthy controls. They found large fluctuations in RNU6 expression and a relatively stable expression of miR-16. The difference of ΔC_q values of RNU6 between the highest and lowest expression level was 3.29, and that of miR-16 was 1.23. Xiang et al. also subjected the serum samples to different freeze-thaw cycles and showed that RNU6 expression gradually decreased after 1, 2, and 4 cycles of freezing and thawing, whereas the expression of miR-16 and miR-24 remained relatively stable (54).

Lamba et al. compared the stability of RNU6 and RNU6B in hepatic tissue and found that both snRNAs were not suitable for the use as endogenous controls for normalizing miRNA data in this tumor type. They used Taqman-based qPCR to quantify the expression levels of 22 miRNAs along with RNU6 and RNU6B in 50 human liver samples (55). The software programs NormFinder (56) and GeNormplus both identified RNU6 to be among the least stable of all candidate snRNAs analyzed, and RNU6B was also not among the top genes in stability. In their analyses, miR-152 and miR-23b were identified to be the 2 most stable candidates and to be eligible as endogenous controls for data normalization.

Benz et al. analyzed RNU6B levels in the serum samples of healthy volunteers, intensive care unit patients, and patients with liver fibrosis. They demonstrated that serum RNU6B levels displayed a high variability between the cohorts and, consequently, were dysregulated in a disease-specific manner. Most importantly, the expression levels were significantly upregulated in the serum of patients with critical illness and sepsis compared with controls and were correlated with established markers of inflammation. In contrast, in patients with liver fibrosis, RNU6B levels were significantly

downregulated (52). Furthermore, Ratert et al. also showed that, notably, RNU6B is unsuitable for miRNA normalization. On the basis of miRNA microarray data, a total of 16 miRNAs were identified as putative reference genes. After validation by qPCR, RNU6B, RNU48, miR-101, miR-125a-5p, miR-148b, miR-151-5p, miR-181a, miR-181b, miR-29c, miR-324-3p, miR-424, miR-874, and Z30 were evaluated by the programs geNorm (57), NormFinder, and BestKeeper. These algorithms recommended the combinations of 4 (miR-101, miR-125a-5p, miR-148b, and miR-151-5p) and 3 (miR-148b, miR-181b, and miR-874) reference miRNAs for data normalization (58).

In miRNA expression studies on renal cell carcinoma, RNU6B was also unsuitable as a normalizer. Validation experiments were performed on 4 miRNAs (miR-28, miR-103, miR-106a, and miR-151) together with RNU6B, RNU44, and RNU48. miR-28, miR-103, miR-106a, and RNU48 were proven to be the most stably expressed genes, but RNU6B was differentially expressed. If only a single reference gene can be used, miR-28 was recommended as the normalizer, although the combinations of miR-28 and miR-103 or of miR-28, miR-103, and miR-106a were preferred (59).

Torres et al. used qPCR to investigate the expression of 12 candidate snRNAs (RNU6, RNU44, RNU48, RNU75, RNU54, RNU49, RNU6B, RNU38B, RNU18A, miR-16, miR-26b, and miR-92a) in tissue samples of 30 endometrioid endometrial carcinoma patients and 15 normal endometrium samples. The stability of candidate endogenous controls was also evaluated using the algorithm programs and an equivalency test. The results were then validated using a larger number of samples. RNU48, RNU75, and RNU44 were identified as the most stably and equivalently expressed snRNAs between malignant and normal tissues. Both the NormFinder and geNorm programs indicated that these 3 snRNAs were optimal for qPCR data normalization in endometrioid endometrial tissues. The authors suggested that the average values of these snRNAs could be used as a reliable endogenous control in studies on endometrioid endometrial cancer (60).

In a study on Parkinson disease, RNU24 ranked at the top of the list of reference genes, followed by Z30. In contrast, miR-103a-3p was ranked as the worst reference gene, so that in combination with other reference genes this miRNA led to biased results. It is important to underline that miR-103a-3p alone or in combination with the other reference genes reversed the direction of the expression levels of the target miRNAs miR-29a-3p and miR-30b-5p. Also, RNU6B was not considered to be a reliable reference gene for Parkinson disease blood samples, because the efficiency, the r^2 , and the stability values were too low (47).

These findings suggest that RNU6 may be unsuitable as an endogenous reference gene in the research of

miRNA quantification. In contrast, the et al. recommended RNU6 as reference gene for the relative quantification of miRNA expression levels in pleural effusion. Following miRNA microarray, the expression levels of candidate reference miRNAs, together with RNU6B, RNU44, and RNU48, were validated in 46 benign pleural effusion samples and 45 lung adenocarcinoma-associated malignant pleural effusion samples by qPCR, and the results were verified using the NormFinder and BestKeeper algorithms. RNU6B and miR-192 were identified as single reference genes, and the combination of these genes was preferred for the relative quantification of miRNA expression levels in pleural effusion (61).

miR-16 as Normalizer

miR-16 is also frequently used as a normalizer, because it is highly expressed and relatively invariant across various samples (62). To normalize qPCR data, McDermott et al. demonstrated that the combined use of miR-16 together with miR-425 generated more reliable results than the use of either one of these miRNAs alone, or the use of RNU6. Following miRNA profiling of approximately 380 miRNAs, qPCR was performed in 40 breast cancer patients and 20 healthy women. The analysis by geNorm and NormFinder algorithms showed that miR-16 and miR-425 were the most stable combination, achieving the lowest V-value of 0.185 (63).

Song et al. used qPCR to analyze the expression levels of miR-16 together with let-7a, miR-93, miR-103, miR-192, miR-451, and RNU6B in the serum samples of 40 gastric cancer patients and 20 healthy volunteers. The geNorm, NormFinder, and Bestkeeper algorithms were used to select the most stably expressed reference gene from the 7 candidates. The algorithms revealed miR-16 and miR-93 to be the most stably expressed reference genes, with stability values of 1.778 and 2.213, respectively, for serum miRNA quantification across all the patients and healthy controls. The effects of different normalization strategies were also compared. When the data were normalized to the serum volume, there were no significant differences of miRNA levels between patients and controls. However, when the data were normalized to miR-16 or miR-93, or the combination of miR-93 and miR-16, significant differences were detected. These results demonstrated that the use of reference genes for qPCR data normalization has a great effect on the study outcome, and that miR-16 and miR-93 can be recommended as suitable reference genes for serum miRNA quantification in gastric cancer patients (50).

In contrast, Schaefer et al. reported that data normalization to miR-16 may lead to biased results using tissue and normal adjacent tissue sample pairs from men with untreated prostate carcinoma collected after radical prostatectomy. In this study (64), the expression levels of

4 putative reference genes (miR-16, miR-130b, RNU6-2, and SNORD7) were examined with regard to their use as normalizers. Candidate miR-130b and RNU6-2 showed no significantly different expression levels between the matched malignant and nonmalignant tissue samples, whereas miR-16 was significantly downregulated in malignant tissue. GeNorm and NormFinder algorithms predicted miR-130b and the geometric mean of miR-130b and RNU6-2 as the most stable reference genes (64). To date, the expression of miR-16 has also been described to be deregulated in different diseases by several other studies (65–70). For example, in osteoclast differentiation, the expression of miR-16 is increased, and miR-16 was characterized as a regulator of osteolytic bone metastasis (66).

Other miRNAs as Normalizers

Using geNorm and NormFinder, Peltier and Latham found that miR-191 was the most consistently expressed miRNA across different human tissues, followed by miR-93, miR-106a, miR-17-5p, and miR-25. In contrast, RNU6 and snRNA5S were the least stable. Indeed, the difference in stability between miR-191 and snRNA5S was an SD of nearly ± 1 Cq or a difference of ± 2 -fold. Normalization to total RNA mass was also evaluated, but this reference approach ranked behind miR-191 and miR-93 in stability (71).

Hu et al. designated miR-1228 as a promising stable endogenous control for quantifying circulating miRNAs in cancer patients. In this report, circulating miRNAs were quantified in controls (healthy individuals and those with chronic hepatitis B and cirrhosis) and cancer patients (hepatocellular, colorectal, lung, esophageal, gastric, renal, prostate, and breast cancer). GeNorm and NormFinder algorithms as well as CV were used to select the most stable endogenous control, whereas ingenuity pathway analysis (IPA) was adopted to explore the signaling pathways involved. miR-1228, with CV = 5.4% and minimum M and S values, presented as the most stable endogenous control across 8 cancer types and 3 controls. IPA showed miR-1228 to be involved extensively in metabolism-related signal pathways and organ morphology, implying that miR-1228 functions as a housekeeping gene. Additionally, functional network analysis found that miR-1228 was associated with hematological system development, explaining its steady expression in the blood (72).

On the basis of their use of a TaqMan low-density array and the NormFinder algorithm, Zhu et al. recommended the combination of miR-26a, miR-221, and miR-22* as the most stable set of reference genes for the evaluation of circulating miRNA in hepatitis B virus-infected patients and healthy individuals (73). To determine the levels of candidate reference genes (RNU1-4,

RNU6-2, SNORD43, SNORD44, SNORD48, SNORA74A, miR-let-7a-1, and miR-106a) for urological malignancies, Sanders et al. used qPCR to analyze (in *Caenorhabditis elegans*) cel-miR-39–spiked serum of prostate cancer patients, bladder cancer patients, renal cell carcinoma patients, and controls. Recovery of cel-miR-39 (mean, 11.6%; range, 1%–56%) was similar in controls and cancer patients. SNORD44 and SNORD74A levels were around the detection limit of the assay. Using the NormFinder and geNorm algorithms, SNORD43 was the most stable reference gene. A combination of 2 genes (SNORD43 and RNU1-4) increased the stability somewhat, indicating that SNORD43 may be a suitable reference gene for the quantification of circulating miRNA in uro-oncological patients (74). For uterine cervical tissues, Shen et al. suggested that miR-23a and miR-191 are the optimal reference miRNAs. Following a microarray assay, the stability of candidate reference genes (miR-26a, miR-23a, miR-200c, let-7a, and miR-1979) was assessed by qPCR in a cohort of 108 clinical uterine cervical samples. miR-23a was identified as the most reliable reference gene, followed by miR-191 (75). To screen suitable reference genes for hepatocellular carcinoma, gastric carcinoma, hepatic cirrhosis, and hepatitis B, Tang et al. used GeNorm, Normfinder, BestKeeper, and comparative ΔC_q algorithms integrated in RefFinder and measured plasma concentrations of RNU6, let-7a, miR-21, miR-106a, miR-155, miR-219, miR-221, and miR-16 in these patients and healthy volunteers. RefFinder revealed miR-106a and miR-21 as the most stably expressed reference genes, with comprehensive stability values of 1.189 and 1.861, respectively, whereas RNU6 was the most unstable miRNA (76). However, this study should be considered critically, because the upregulation of miR-21 was reported in a variety of human malignancies. In fact, it is one of the first discovered oncomiRs and most investigated in the context of cancer (5). Following miSeq sequencing and qPCR, Wang et al. selected 5 genes (miR-193a-5p, miR-16-5p, RNU6, miR-191-5p, and let-7-d-3p) for stability analysis using geNorm and NormFinder software. These algorithms identified miR-193a-5p and miR-16-5p as the most stably expressed reference genes. One-way ANOVA indicated that no significant differences were present in the serum levels among patients with non-muscle-invasive bladder cancer, patients with muscle-invasive bladder cancer, and healthy controls. The combined use of miR-193a-5p and miR-16-5p demonstrated that normalization of miRNA data may produce reliable and accurate results for the detection of the significant upregulation of serum miR-148b-3p in bladder cancer (77). To find out the control gene for exosomal miRNA normalization, Li et al. evaluated the expression stability of 11 reference genes in circulating exosomes and found that the combination of

miR-221, miR-191, let-7a, miR-181a, and miR-26a can be an optimal gene reference set for normalizing the expression of liver-specific miRNAs. This combination enhanced the robustness of the relative quantification analyses (78).

Upshot of Relative Data Normalization

Taken together, these findings summarize the endeavors of developing an optimal endogenous miRNA control to normalize miRNA expression levels. The suggested normalizers for target miRNAs are tissue and species specific. So far, the studies also demonstrate that no consensus exists regarding the normalization to a standard reference gene in various diseases, making the miRNA results incomparable. On the one hand, some studies have evaluated and suggested convenient miRNAs, snRNA, or rRNAs as ideal candidate reference genes for data normalization in different diseases using specific algorithms, whereas on the other hand, other studies have shown their deregulation, even for the same disease. In this regard, normalization to a standard reference is still in its infancy. Furthermore, the selection of a normalizer should always follow validation screening tests on a subset of the samples under analyses.

However, these studies also demonstrate that the use of more than 1 reference gene increases the accuracy of quantification compared to the use of a single reference gene. More than 10 years ago, Vandesompele et al. evaluated 10 housekeeping genes from different abundance and functional classes in various human tissues and demonstrated that the conventional use of a single gene for normalization leads to relatively large errors in a substantial proportion of samples tested. The geometric mean of multiple carefully selected housekeeping genes was validated as an accurate normalization factor (57). Chugh and Dittmer described the potential pitfalls in microRNA profiling and showed that the best way to approach the analysis of miRNA expression data is via global mean normalization of a set of reference genes that may be tissue specific. This method takes a minimum of 3 stable housekeeping genes and takes the geometric mean to provide a reliable normalization factor that can control for outliers and differences in abundance between genes (39).

Relative Data Normalization to Exogenous Reference Genes

To ensure that miRNA quantification is not affected by the technical variability that may be introduced at different analysis steps, synthetic, nonhuman spike-in miRNAs are frequently used to monitor RNA purification and reverse transcription efficiencies. The *C. elegans* miRNA cel-miR-39 is almost exclusively used for normalization to an exogenous reference gene (79–81), but

cel-miR-54 (44), and the synthetic miRNAs Quanto EC1 and Quanto EC2 (82), as well as the simian virus gene SV40 (83), also have been used. Because of the lack of established reference genes, some studies have carried out miRNA quantification only with data normalization to these artificial reference miRNAs. These exogenous miRNAs are usually added to the samples before reverse transcription of RNA to avoid differences in template quality and warrant efficiency of the reverse transcription reaction. This spike-in method can eliminate some deviations of the experimental process and make the results more reliable, but this method does not correct for deviations in sampling and quality of samples. A major drawback of using spike-in controls is that only the handling of experiments is considered, but not the quality of tissues, body fluids, or extracellular vesicles samples. However, age, body fluids collection, preparation, or storing of tissue or fluid samples may result in changes of miRNA levels, which may be caused by cell lysis or miRNA degradation. For example, samples with low total RNA quality showed the highest concentrations of miRNA (5, 84). Therefore, the data of this approach should be interpreted with caution. However, when normalization is based on a combination of an endogenous and an exogenous control miRNA, differences in miRNA recovery and cDNA synthesis between samples may be compensated (85).

Absolute Data Normalization

Some studies have used absolute data normalizations and calculated the miRNA expression using standard curves developed by synthetic miRNAs and melting curves (40–43). Following miRNA expression array, Yau et al. quantified 2 target miRNAs (miR-221 and miR-18a) in 40 pairs of colorectal carcinoma tissues and 595 stool samples. Their miRNA quantification was based on standard curves and normalized per nanogram of the total input RNA. Derived from standard curves plotted from known amounts of synthetic miR-221 and miR-18a, a technical detection limit of 2 copies for miR-221 resulted in a Cq value of 42, and a technical detection limit of 5 copies for miR-18a resulted in a Cq value of 47. All Cq values of >42 for miR-221, and >47 for miR-18a were assigned a value of 0. Samples with no amplification of miR-221 or miR-18a were also assigned a value of 0 (41). However, this study should be considered critically, because on the basis of MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines for qPCR, Cq values in the order of 40 and more are unreliable (86). Liu et al. examined the expression patterns of serum let-7 in 214 gastric cancer patients, 222 atrophic gastritis patients, and 202 controls. The concentration (copy number) of let-7 was calculated using a standard curve. Melting curve analysis was performed to validate the specificity of the expected PCR products

(42). Wang et al. normalized 5 serum miRNAs (miR-487a, miR-502, miR-208, miR-215, and miR-29b) to the serum volumes, because RNU6 and 5S rRNA were degraded in the serum samples and because of the lack of a consensus housekeeping miRNA for qPCR. Additionally, Wang et al. assessed the detection limits of the qPCR assay and the dynamic range and calculated the absolute concentration of target miRNAs on the basis of a calibration curve developed by synthetic miRNAs with known concentration (43).

Although these studies show interesting results, the applied absolute normalization does not consider the influence of RNA quality on the performance of qPCR. This normalization method is not optimal for an exact quantification of real miRNA amounts and reliable only for samples with a good RNA quality.

Ideal Data Normalization Models

The lack of consensus on reference gene selection for miRNA expression data normalization has led to the spread of publications screening for suitable normalizers for samples of defined origins and/or implicated in different pathological conditions. New studies performing miRNA detection by qPCR may greatly benefit from this knowledge, and thus a strategic experimental workflow is proposed (Fig. 1). Before qPCR assay performance, researchers are advised to review the literature using samples of the same origin and physiological state, processed as similarly as possible, to find candidate reference genes. Nevertheless, the suitability of these genes for the set of samples under analysis should always then be validated in a sample subset. If the candidate reference genes are stable, qPCR can be performed and data normalized using their expression levels. If not, the specific samples have to be screened for more suitable reference genes. Ideally, good reference genes should have low SDs of expression levels across samples and similar mean and median expression values and be little affected by storage conditions and sample processing, with a high efficiency of extraction. In these cases, the addition of exogenous xenogeneic or synthetic miRNAs to the samples may be beneficial, and their expression levels may be considered simultaneously with endogenous appropriate normalizers for data correction. Considering the advantages and disadvantages of the different methods for miRNA qPCR data normalization, it is evident that the standard approach of relative data normalization with endogenous and exogenous reference genes benefits from a complementary data absolute normalization. Although it should not be used alone, absolute data normalization allows data correction for limitations intrinsic to the qPCR methodology and gives more insights about the overall status of miRNA expression.

Alternatively, qPCR data for specific miRNA expression may be normalized following strategies that take

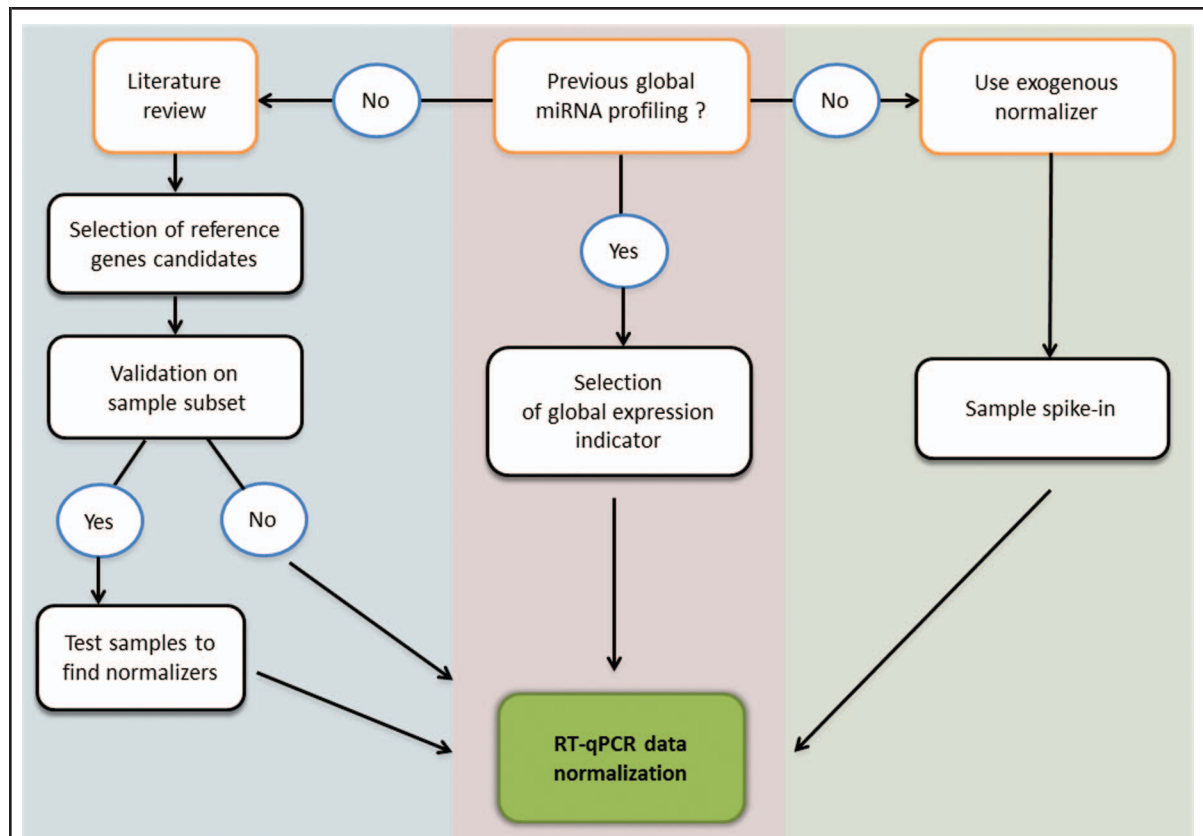


Fig. 1. Workflow for miRNA qPCR data normalization.

Candidate reference genes may be selected from the literature and validated in a subset of the samples under analysis. If these are found to be not appropriate, samples should be profiled to find adequate endogenous reference genes. Alternatively, exogenous miRNAs may be spiked-in into the samples. Also, mathematical indicators calculated from previous miRNA global expression profiles of the same or similar samples may be used for normalization.

into account the total miRNA expression in the samples. Very frequently, qPCR analysis of specific miRNAs follows global analysis of total miRNAs expressed in a sample by another technique, as is the case for microarray expression data validation. In this case, information about the whole miRNA content of the sample is available that can be used for global normalization. Mestdagh et al. proposed the use of the mean expression value of whole miRNAs in a sample to normalize miRNA qPCR data (34). In this study, a high-throughput qPCR assay that allows the detection of 430 different human miRNAs and 18 small RNA controls was performed to determine global miRNA expression levels in samples of normal and tumor tissue. The mean miRNA expression value was then calculated considering all the transcripts with a maximal Cq threshold of 35 cycles. A comparative analysis of the stability of mean expression value and common reference genes performed using geNorm clearly showed the adequate application of this strategy

for normalization, performing better than genes such as RNU48 and miR-191. Ideally, this strategy should be more widespread for qPCR data validation; nevertheless it implies that a large number of genes are always profiled, which may not be cost-effective. To circumvent this issue, the authors propose the selection of reference genes with expression levels similar to values of the global mean expression level previously reported, using their geometric mean for qPCR data normalization. In addition, researchers should bear in mind that, overall, qPCR data normalization may greatly benefit from sample preservation and stability, and thus adequate protocols for sample processing should be standardized at least for samples of the same origin in the same laboratory.

Conclusion

Normalizing to a reference gene can eliminate differences due to sampling and quality of RNA and can identify real

changes in miRNA expression levels. Therefore, careful validation of reference genes for miRNAs is of crucial importance to obtain accurate miRNA expression data. Reliability of results reported in the literature, using the wrong reference gene, or even performing no data normalization, is questionable. It should be emphasized that the applicability of reference genes in some studies does not automatically apply to other studies and that the use of a single reference gene is not sufficient to obtain reliable miRNA data. We propose the best data normalization strategy to be one that employs a combination of endogenous and exogenous control miRNAs. Routine use of such an approach will allow differences in both the preanalytical handling of samples and the analytical miRNA recovery to be considered.

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References

- Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* 2014;15:509–24.
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–97.
- Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* 2006;66:7390–4.
- Ardekani AM, Naeini MM. The role of microRNAs in human diseases. *Avicenna J Med Biotechnol* 2010;2:161–79.
- Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. *Nat Rev Clin Oncol* 2014;11:145–56.
- van Schooneveld E, Wildiers H, Vergote I, Vermeulen PB, Dirix LY, Van Laere SJ. Dysregulation of microRNAs in breast cancer and their potential role as prognostic and predictive biomarkers in patient management. *Breast Cancer Res* 2015;17:21.
- Chen X, Liang H, Zhang J, Zen K, Zhang CY. Horizontal transfer of microRNAs: molecular mechanisms and clinical applications. *Protein Cell* 2012;3:28–37.
- Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol Med* 2014;20:460–9.
- Moreno-Moya JM, Vilella F, Simon C. MicroRNA: key gene expression regulators. *Fertil Steril* 2014;101:1516–23.
- Tiberio P, Callari M, Angeloni V, Daidone MG, Appierto V. Challenges in using circulating miRNAs as cancer biomarkers. *Biomed Res Int* 2015;2015:731479.
- Biassoni R, Raso A, editors. *MiRNA and microRNA purity and integrity: the key to success in expression profiling*. New York: Springer; 2014.
- Li J, Smyth P, Flavin R, Cahill S, Denning K, Aherne S, et al. Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. *BMC Biotechnol* 2007;7:36.
- Monleau M, Bonnel S, Gostan T, Blanchard D, Cournaud V, Lecellier CH. Comparison of different extraction techniques to profile microRNAs from human sera and peripheral blood mononuclear cells. *BMC Genomics* 2014;15:395.
- Glynn CL, Khan S, Kerin MJ, Dwyer RM. Isolation of secreted microRNAs (miRNAs) from cell-conditioned media. *MicroRNA* 2013;2:14–9.
- Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem* 2010;56:1733–41.
- Rani S. MicroRNA profiling of exosomes isolated from biofluids and conditioned media. *Methods Mol Biol* 2014;1182:131–44.
- Ibberson D, Benes V, Muckenthaler MU, Castoldi M. RNA degradation compromises the reliability of microRNA expression profiling. *BMC Biotechnol* 2009;9:102.
- Kim DJ, Linnstaedt S, Palma J, Park JC, Ntrivalas E, Kwak-Kim JY, et al. Plasma components affect accuracy of circulating cancer-related microRNA quantitation. *J Mol Diagn* 2012;14:71–80.
- Farina NH, Wood ME, Perrapato SD, Franchlyn CS, Stein GS, Stein JL, Lian JB. Standardizing analysis of circulating microRNA: clinical and biological relevance. *J Cell Biochem* 2014;115:805–11.
- MacLellan SA, MacAulay C, Lam S, Garnis C. Pre-profiling factors influencing serum microRNA levels. *BMC Clin Pathol* 2014;14:27.
- Witwer KW, Buzas EI, Bemis LT, Bora A, Lasser C, Lotvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013;2.
- Liu R, Chen X, Du Y, Yao W, Shen L, Wang C, et al. Serum microRNA expression profile as a biomarker in the diagnosis and prognosis of pancreatic cancer. *Clin Chem* 2012;58:610–8.
- Vasilescu C, Rossi S, Shimizu M, Tudor S, Veronese A, Ferracin M, et al. MicroRNA fingerprints identify miR-150 as a plasma prognostic marker in patients with sepsis. *PLoS One* 2009;4:e7405.
- Aushev VN, Zborovskaya IB, Laktionov KK, Girard N, Cros MP, Herceg Z, Krutovskikh V. Comparisons of microRNA patterns in plasma before and after tumor removal reveal new biomarkers of lung squamous cell carcinoma. *PLoS One* 2013;8:e78649.
- Fang L, Li H, Wang L, Hu J, Jin T, Wang J, Yang BB. MicroRNA-17-5p promotes chemotherapeutic drug resistance and tumour metastasis of colorectal cancer by repressing PTEN expression. *Oncotarget* 2014;5:2974–87.
- Oksvold MP, Kullmann A, Forfang L, Kierulf B, Li M, Brech A, et al. Expression of B-cell surface antigens in subpopulations of exosomes released from B-cell lymphoma cells. *Clin Ther* 2014;36:847–62 e1.
- Boing AN, van der Pol E, Grootemaat AE, Coumans FA, Sturk A, Nieuwland R. Single-step isolation of extracellular vesicles by size-exclusion chromatography. *J Extracell Vesicles* 2014;3.
- Mathivanan S, Lim JW, Tauro BJ, Ji H, Moritz RL, Simpson RJ. Proteomics analysis of A33 immunoaffinity-purified exosomes released from the human colon tumor cell line LIM1215 reveals a tissue-specific protein signature. *Mol Cell Proteomics* 2010;9:197–208.
- Witwer KW. Circulating microRNA biomarker studies: pitfalls and potential solutions. *Clin Chem* 2015;61:56–63.
- Haider BA, Baras AS, McCall MN, Hertel JA, Cornish TC, Halushka MK. A critical evaluation of microRNA biomarkers in non-neoplastic disease. *PLoS One* 2014;9:e89565.
- Benes V, Castoldi M. Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. *Methods* 2010;50:244–9.
- Thomson JM, Parker JS, Hammond SM. Microarray analysis of miRNA gene expression. *Methods Enzymol* 2007;427:107–22.
- Sato F, Tsuchiya S, Terasawa K, Tsujimoto G. Intra-platform repeatability and inter-platform comparability of microRNA microarray technology. *PLoS One* 2009;4:e5540.
- Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol* 2009;10:R64.
- Meyer SU, Pfaffl MW, Ulbrich SE. Normalization strategies for microRNA profiling experiments: a 'normal' way to a hidden layer of complexity? *Biotechnol Lett*

- 2010;32:1777–88.
36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* 2001;25:402–8.
 37. Rai SN, Ray HE, Yuan X, Pan J, Hamid T, Prabhu SD. Statistical analysis of repeated microRNA high-throughput data with application to human heart failure: a review of methodology. *Open Access Med Stat* 2012;2012:21–31.
 38. Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, et al. MIQE precis: practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Mol Biol* 2010;11:74.
 39. Chugh P, Dittmer DP. Potential pitfalls in microRNA profiling. *Wiley Interdiscip Rev RNA* 2012;3:601–16.
 40. Yan W, Li R, Liu Y, Yang P, Wang Z, Zhang C, et al. MicroRNA expression patterns in the malignant progression of gliomas and a 5-microRNA signature for prognosis. *Oncotarget* 2014;5:12908–15.
 41. Yau TO, Wu CW, Dong Y, Tang CM, Ng SS, Chan FK, et al. MicroRNA-221 and microRNA-18a identification in stool as potential biomarkers for the non-invasive diagnosis of colorectal carcinoma. *Br J Cancer* 2014;111:1765–71.
 42. Liu WJ, Xu Q, Sun LP, Dong QG, He CY, Yuan Y. Expression of serum let-7c, let-7f1, and let-7f2 microRNA with its target gene, pepsinogen C, in gastric cancer and precancerous disease. *Tumour Biol* 2015;36:3337–43.
 43. Wang J, Pei Y, Zhong Y, Jiang S, Shao J, Gong J. Altered serum microRNAs as novel diagnostic biomarkers for atypical coronary artery disease. *PLoS One* 2014;9:e107012.
 44. Kuhlmann JD, Baraniskin A, Hahn SA, Mosel F, Brede-meier M, Wimberger P, et al. Circulating U2 small nuclear RNA fragments as a novel diagnostic tool for patients with epithelial ovarian cancer. *Clin Chem* 2014;60:206–13.
 45. Spiekermann M, Dieckmann KP, Balks T, Bullerdiek J, Belge G. Is relative quantification dispensable for the measurement of microRNAs as serum biomarkers in germ cell tumors? *Anticancer Res* 2015;35:117–21.
 46. Wang X, Gardiner EJ, Cairns MJ. Optimal consistency in microRNA expression analysis using reference-gene-based normalization. *Mol Biosyst* 2015;11:1235–40.
 47. Serafin A, Foco L, Blankenburg H, Picard A, Zanigni S, Zanon A, et al. Identification of a set of endogenous reference genes for miRNA expression studies in Parkinson's disease blood samples. *BMC Res Notes* 2014;7:715.
 48. Roth C, Rack B, Muller V, Janni W, Pantel K, Schwarzenbach H. Circulating microRNAs as blood-based markers for patients with primary and metastatic breast cancer. *Breast Cancer Res* 2010;12:R90.
 49. Mahdipour M, Van Tol HT, Stout TA, Roelen BA. 225 validation of reference microRNAs for normalizing expression data generated by quantitative PCR. *Reprod Fertil Dev* 2014;27:202.
 50. Song J, Bai Z, Han W, Zhang J, Meng H, Bi J, et al. Identification of suitable reference genes for qPCR analysis of serum microRNA in gastric cancer patients. *Dig Dis Sci* 2012;57:897–904.
 51. Shen Y, Tian F, Chen Z, Li R, Ge Q, Lu Z. Amplification-based method for microRNA detection. *Biosens Bioelectron* 2015;71:322–31.
 52. Benz F, Roderburg C, Vargas Cardenas D, Vucur M, Gautheron J, Koch A, et al. U6 is unsuitable for normalization of serum miRNA levels in patients with sepsis or liver fibrosis. *Exp Mol Med* 2013;45:e42.
 53. Gee HE, Buffa FM, Camps C, Ramachandran A, Leek R, Taylor M, et al. The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis. *Br J Cancer* 2011;104:1168–77.
 54. Xiang M, Zeng Y, Yang R, Xu H, Chen Z, Zhong J, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. *Biochem Biophys Res Commun* 2014;454:210–4.
 55. Lamba V, Ghodke-Puranik Y, Guan W, Lamba JK. Identification of suitable reference genes for hepatic microRNA quantitation. *BMC Res Notes* 2014;7:129.
 56. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 2004;64:5245–50.
 57. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paeppe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034.
 58. Rater N, Meyer HA, Jung M, Mollenkopf HJ, Wagner I, Miller K, et al. Reference miRNAs for miRNAome analysis of urothelial carcinomas. *PLoS One* 2012;7:e39309.
 59. Wotschovsky Z, Meyer HA, Jung M, Fendler A, Wagner I, Stephan C, et al. Reference genes for the relative quantification of microRNAs in renal cell carcinomas and their metastases. *Anal Biochem* 2011;417:233–41.
 60. Torres A, Torres K, Wdowiak P, Paszkowski T, Maciejewski R. Selection and validation of endogenous controls for microRNA expression studies in endometrioid endometrial cancer tissues. *Gynecol Oncol* 2013;130:588–94.
 61. Han HS, Jo YN, Lee JY, Choi SY, Jeong Y, Yun J, Lee OJ. Identification of suitable reference genes for the relative quantification of microRNAs in pleural effusion. *Oncol Lett* 2014;8:1889–95.
 62. Muller V, Gade S, Steinbach B, Loibl S, von Minckwitz G, Untch M, et al. Changes in serum levels of miR-21, miR-210, and miR-373 in HER2-positive breast cancer patients undergoing neoadjuvant therapy: a translational research project within the Geparquinto trial. *Breast Cancer Res Treat* 2014;147:61–8.
 63. McDermott AM, Kerin MJ, Miller N. Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies. *PLoS One* 2013;8:e83718.
 64. Schaefer A, Jung M, Miller K, Lein M, Kristiansen G, Erbersdobler A, Jung K. Suitable reference genes for relative quantification of miRNA expression in prostate cancer. *Exp Mol Med* 2010;42:749–58.
 65. Delfino KR, Rodriguez-Zas SL. Transcription factor-microRNA-target gene networks associated with ovarian cancer survival and recurrence. *PLoS One* 2013;8:e58608.
 66. Eil B, Mercatali L, Ibrahim T, Campbell N, Schwarzenbach H, Pantel K, et al. Tumor-induced osteoclast miRNA changes as regulators and biomarkers of osteolytic bone metastasis. *Cancer Cell* 2013;24:542–56.
 67. Hu Z, Dong J, Wang LE, Ma H, Liu J, Zhao Y, et al. Serum microRNA profiling and breast cancer risk: The use of miR-484/191 as endogenous controls. *Carcinogenesis* 2012;33:828–34.
 68. Liu J, Gao J, Du Y, Li Z, Ren Y, Gu J, et al. Combination of plasma microRNAs with serum CA19–9 for early detection of pancreatic cancer. *Int J Cancer* 2012;131:683–91.
 69. Xiao G, Tang H, Wei W, Li J, Ji L, Ge J. Aberrant expression of microRNA-15a and microRNA-16 synergistically associates with tumor progression and prognosis in patients with colorectal cancer. *Gastroenterol Res Pract* 2014;364549, 2014.
 70. Zuo Z, Calin GA, de Paula HM, Medeiros LJ, Fernandez MH, Shimizu M, et al. Circulating microRNAs let-7a and miR-16 predict progression-free survival and overall survival in patients with myelodysplastic syndrome. *Blood* 2011;118:413–5.
 71. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. *RNA* 2008;14:844–52.
 72. Hu J, Wang Z, Liao BY, Yu L, Gao X, Lu S, et al. Human miR-1228 as a stable endogenous control for the quantification of circulating microRNAs in cancer patients. *Int J Cancer* 2014;135:1187–94.
 73. Zhu HT, Dong QZ, Wang G, Zhou HJ, Ren N, Jia HL, et al. Identification of suitable reference genes for qRT-PCR analysis of circulating microRNAs in hepatitis B virus-infected patients. *Mol Biotechnol* 2012;50:49–56.
 74. Sanders I, Holdenrieder S, Walgenbach-Brunagel G, von Ruecker A, Kristiansen G, Muller SC, Ellinger J. Evaluation of reference genes for the analysis of serum miRNA in patients with prostate cancer, bladder cancer and renal cell carcinoma. *Int J Urol* 2012;19:1017–25.
 75. Shen Y, Li Y, Ye F, Wang F, Wan X, Lu W, Xie X. Identification of miR-23a as a novel microRNA normalizer for relative quantification in human uterine cervical tissues. *Exp Mol Med* 2011;43:358–66.
 76. Tang G, Shen X, Lv K, Wu Y, Bi J, Shen Q. Different normalization strategies might cause inconsistent variation in circulating microRNAs in patients with hepatocellular carcinoma. *Med Sci Monit* 2015;21:617–24.
 77. Wang L, Liu Y, Du L, Li J, Jiang X, Zheng G, et al. Identification and validation of reference genes for the detection of serum microRNAs by reverse transcription-quantitative polymerase chain reaction in patients with bladder cancer. *Mol Med Rep* 2015;12:615–22.
 78. Li Y, Zhang L, Liu F, Xiang G, Jiang D, Pu X. Identification of endogenous controls for analyzing serum exosomal miRNA in patients with hepatitis B or hepatocellular carcinoma. *Dis Markers* 2015:893594, 2015.
 79. Wang P, Yang D, Zhang H, Wei X, Ma T, Cheng Z, et al. Early detection of lung cancer in serum by a panel of microRNA biomarkers. *Clin Lung Cancer* 2014;16:313–9.e1.
 80. Dong BL, Wang Z, Zhang Z, Wang J. Clinical significance of microRNA-24 expression in esophageal squamous cell carcinoma. *Neoplasma* 2015;62:250–8.
 81. Xu T, Liao Z, O'Reilly MS, Levy LB, Welsh JW, Wang LE, et al. Serum inflammatory miRNAs predict radiation esophagitis in patients receiving definitive radiochemotherapy for non-small cell lung cancer. *Radiother Oncol* 2014;113:379–84.
 82. Liu M, Liu J, Wang L, Wu H, Zhou C, Zhu H, et al. Association of serum microRNA expression in hepatocellular carcinomas treated with transarterial chemoembolization and patient survival. *PLoS One* 2014;9:e109347.
 83. Anadol E, Schiewagen R, Elmova N, Tack K, Schwarze-Zander C, Eischeid H, et al. Circulating microRNAs as a marker for liver injury in human immunodeficiency virus patients. *Hepatology* 2015;61:46–55.
 84. Becker C, Hammerle-Fickinger A, Riedmaier I, Pfaffl MW. MiRNA and microRNA quality control for RT-qPCR analysis. *Methods* 2010;50:237–43.
 85. Sourvinou IS, Markou A, Lianidou ES. Quantification of circulating miRNAs in plasma: effect of preanalytical and analytical parameters on their isolation and stability. *J Mol Diagn* 2013;15:827–34.
 86. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009;55:611–22.