Reports

A comparison of miRNA isolation and RT-qPCR technologies and their effects on quantification accuracy and repeatability

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MicroRNAs (miRNAs) are short (~22 nucleotides), non-coding RNA molecules that post-transcriptionally regulate gene expression. As the miRNA field is still in its relative infancy, there is currently a lack of consensus regarding optimal methodologies for miRNA quantification, data analysis and data standardization. To investigate miRNA measurement we selected a panel of both synthetic miRNA spikes and endogenous miRNAs to evaluate assay performance, copy number estimation, and relative quantification. We compared two different miRNA quantification methodologies and also assessed the impact of short RNA enrichment on the miRNA measurement. We found that both short RNA enrichment and quantification strategy used had a significant impact on miRNA measurement. Our findings illustrate that miRNA quantification can be influenced by the choice of methodology and this must be considered when interpreting miRNA analyses. Furthermore, we show that synthetic miRNA spikes can be used as effective experimental controls for the short RNA enrichment procedure.

MicroRNAs (miRNAs) are short (~22 nucleotides), non-coding, RNA molecules that control diverse biological processes, including cell fate determination, cell proliferation, cell differentiation, and cell death (1,2). miRNAs regulate gene expression post-transcriptionally by interacting with and down-regulating target mRNA molecules (3–6). There has been a growing interest in their use for clinical applications, largely due to their high stability and cell/tissue-specificity (7,8). Most notably, the recent discovery of miRNAs in the circulating bloodstream led to extensive investigations into their suitability as biomarkers for diseases including cancer (8–12) and cardiovascular disease (13,14).

If the recent advances in miRNA research are to be successfully implemented, there are still many challenges that need to be addressed, particularly those associated with the accuracy and reproducibility of miRNA quantification measurements. There is currently little consensus as to which are the optimal methodologies for sample collection, miRNA isolation, miRNA quantification, and data analysis. Furthermore, repeatability and reproducibility, which are especially important for successful clinical application of miRNAs as biomarkers, require the development of robust tools to enable the standardization of miRNA data. There are a range of techniques used to quantify miRNA expression (15–21), but RT-qPCR is currently considered to be the gold standard due to its unparalleled sensitivity and specificity (22–26). There are several commercially available miRNA RT-qPCR assays that employ diverse approaches to address the challenges of achieving accurate miRNA quantification (20,21,25). Although these assays have been described in previous publication, they have not been extensively compared experimentally. The effects of upstream variables, such as sample preparation, on downstream miRNA quantification have received less attention in previous investigations (27–33). For example, RNA samples are routinely prepared for miRNA analysis by enriching the short RNA (<200 nucleotides) fraction. To our knowledge the effects of enrichment on RT-qPCR measurement, however, have not yet been extensively investigated. In addition to issues affecting the accuracy of miRNA quantification, possibly one of the most important and difficult challenges that needs to be addressed by the field is the standardization of miRNA data. Typically, miRNA profiling involves a series of steps that are highly sensitive to technical manipulations; therefore, there is an urgent need for methods to standardize various procedures for within-platform or cross-platform comparisons. Considerable work has been done to facilitate data normalization through the identification of stable reference genes (34) and global mean strategies (35). Several studies have also investigated the use of external standards to control for variability in RNA extraction between samples (8,36–38). However the use of standards to control for downstream RNA processing procedures, such as short RNA enrichment, has not previously been evaluated.

In the present study we compare two of the most prominent commercially available miRNA RT-qPCR assays — Life Technologies' Taqman miRNA Assay (20) and Exiqon's miRCURY LNA Universal RT microRNA PCR assay — and evaluate the impact of a short RNA enrichment method on RT-qPCR measurement. Furthermore, we investigate the application of external *Arabidopsis thaliana* miRNAs standards for use as experimental procedure controls.

Materials and methods

RNA samples

Synthetic miRNA oligonucleotides used for spike-in material and standard curves were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and quantified by measuring absorbance at A_{260} nm (Nanodrop, Thermo Scientific, Waltham, MA, USA) and purity assessed by calculation of the A_{260}/A_{280} ratio. 50 ng/µl of human fetal brain total RNA (Cat. No. 540157, Agilent Technologies, Santa Clara, CA, USA) was spiked to make the following concentrations of synthetic *Arabidopsis* miRNAs (miR-159a: 1.2×10^7 copies/µl, miR-172a: 6.4×10^5 copies/µl, miR-394a: 1.6×10^6 copies/µl). For standard curves, synthetic miRNA molecules were spiked into yeast transfer RNA (tRNA) (10ng/µL) (Cat. No. R5636, Sigma, St. Louis, MO, USA) carrier solution at the following miRNA copy number ranges per reaction: 10^5 - 10^8 for let-7a, let-7c, miR-16, -394a, -26b, -159a; 10^4 – 10^7 for miRs-21 and -172a. All RNA was stored in RNA Storage Solution (Cat. No. AM9937, Life Technologies, Carlsbad, CA, USA) after dilution at -80°C.

Short RNA enrichment

Short RNA enrichment was performed using the miRVana miRNA isolation kit (Cat. No. AM1560, Life Technologies) according to the manufacturer's instructions and ethanol from Sigma (Cat. No. 1147662). In addition to the extraction of total RNA in which the short RNA fraction is retained and optionally enriched, the miRVana miRNA isolation kit also enables the user to enrich the short RNA fraction that was isolated by another method. The latter procedure, which we performed in this study, involves the separation of the larger (>200 nucleotides) and shorter (<200 nucleotides) RNA species in a sample. Briefly, total RNA samples were mixed with 5 volumes of Lysis/Binding buffer and 1/10 volume of miRNA homogenate additive and left on ice for 10 min. A low concentration of ethanol (25% v/v) was then added to the samples which were subsequently mixed and bound to a filter cartridge by centrifugation. The relatively low concentration of ethanol in this first treatment allows the binding of the larger RNAs to the column while the shorter, more soluble RNAs pass through and are collected. In the second step, a higher concentration of ethanol was added to the eluant (40% v/v), allowing the shorter RNAs to be immobilized to filter cartridges during centrifugation and subsequently eluted. This procedure was performed on 50 µL of total human fetal brain RNA (2.5 µg) (Cat. No. 540157, Agilent Technologies, Santa Clara, CA, USA) containing synthetic *Arabidopsis* miRNA spike-ins. Short RNA was eluted in 50 µL elution buffer so that the effective volume was identical before and after the enrichment.

Reverse transcription

Reverse transcription reactions for the Taqman miRNA Assays (Life Technologies) were performed using the Taqman microRNA Reverse Transcription Kit (Cat. No. 4366596, Life Technologies) with 10 ng total RNA containing Arabidopsis miRNA spike-ins, per 7.5 µL reaction. Ten µl reverse transcription reactions for the miRCURY LNA Universal RT microRNA PCR assays (Exiqon, Vedbaek, Denmark) were performed using the Universal cDNA synthesis kit (Cat. No. 203300, Exiqon) with 20 ng total RNA with *Arabidopsis* miRNA spike-ins per reaction. The differences in RNA used between the two kits reflect the recommended working concentrations. All reverse transcription reactions were performed in accordance with the manufacturers' protocols (Life Technologies: Cat. No. 4364031, Rev. E, 01/2011; Exiqon: Cat. No. 203300, Version 4.1, 08/2011) with the exception that half volume reactions were used. Reverse transcription thermocycling parameters were as follows: for Life Technologies assay: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min; for Exiqon assay: 42°C for 60 min, 95°C for 5 min. For short RNA enriched samples, the same volumes of RNA eluant were added to each reaction as used for the corresponding non-enriched samples. Reactions were performed on a DNA Engine Tetrad 2 Thermocycler (BioRad, Hercules, CA, USA). All reactions were performed in triplicate and included the following controls: no template (RT NTC), no reverse transcriptase enzyme (no RT) and yeast carrier tRNA (carrier only). cDNA was stored at -20°C for less than one week prior to qPCR analysis.

Quantitative real-time PCR (qPCR)

qPCR was performed in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Supplementary Table S1); however, primer sequences used for the different methods are not currently provided by the kit providers. Prior to qPCR reactions being performed, cDNA was diluted 1 in 5 and 1 in 80 for the Life Technologies and Exiqon assays respectively. The Taqman miRNA assays (Cat. No. 4427975, Life Technologies) and miRCURY LNA Universal RT microRNA PCR assays (Cat. No. 206999, Exiqon) were performed using the Taqman Universal PCR mastermix (Cat. No. 4304449, Life Technologies) and Sybr Green mastermix, Universal RT (Cat. No. 203450, Exiqon) respectively as per manufac-

Table 1. Comparison of miRNA RT-qPCR assay efficiency

miRNA assay	Life Technologies		Exiqon	
	Amp. Efficiency (%)	± SD	Amp. Efficiency (%)	± SD
mir-16	98.54	2.69	104.47	6.72
mir-26b	98.01	0.98	97.66	6.24
mir-21	99.93	3.70	97.82	6.76
let-7a	97.48	5.65	100.17	8.34
let-7c	99.27	1.86	93.89	0.75
mir-159a	97.36	3.80	96.74	9.03
mir-172a	100.44	6.34	109.59	3.43
mir-394a	100.95	2.57	107.07	6.21

Comparison of the average qPCR amplification (amp.) efficiencies for the Life Technologies and Exiqon miRNA assays measured in 3 independent experiments and showing ± % standard deviations (SD)

turers' instructions (see Reverse transcription for references to manufacturers' protocols) with the exception that 10 µL reactions (half volume) were used for the Life Technologies assays, as was also used for the Exiqon assays. Supplementary Table S3 gives details of the RT-qPCR assays used. All assays were available off-the-shelf with the exception of the Exiqon *Arabidopsis* miRNA assays, which were custom designed. Details of the design of the *Arabidopsis* miRNA assays can be found at www.exiqon.com/miRNA-qPCR-primers. Reactions were performed according to the manufacturers' instructions using a Prism 7900HT Real Time PCR system (Life Technologies). qPCR thermocycling conditions were as follows: for Life Technologies assay: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min; for Exiqon assay: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 1 min, melt curve analysis performed between 60–95°C for 15 min at a ramp-rate of 1.6°C/s. Quantification was performed using the standard curve method with four serial dilutions (see RNA samples for concentrations) and three replicates at each dilution. Interpolated miRNA copy number values were normalized for differences in input RNA between the two RT-qPCR technologies. PCR NTCs were run as controls on all plates in addition to the reverse transcription controls (RT NTCs, no RTs and carrier only). The SDS software v2.4 (Life Technologies) was used to calculate the quantification cycle (Cq) value, which is defined as the number of cycles at which the fluorescence signal is significantly above the threshold, which was converted to copy number using the relevant standard curve.

Statistical analysis

The data were analyzed using linear mixed effects models with maximum likelihood estimation. The R statistical programming environment (build 2.13.0) (www.r-project.org/), running under Microsoft Windows XP, was used to perform all statistical analysis, and to produce graphical output. Sample replicate and qPCR plate were modeled as random effects, while the miRNA target assay technologies used and enrichment status (total RNA or enriched) were modeled as fixed effects. The inclusion of all eight miRNAs in the same model entails pooling all the residuals; however, given the within group variances, this was justifiable and increased the number of residual degrees of freedom. The random effects were, as with linear models of this type, assumed to be normally distributed with a mean of 0 and variance representing the unit-to-unit variability (run-to-run or plate-to-plate in this case). Copy numbers were log-transformed in order to stabilize the within-group variances and to better approximate a normal distribution. Further details describing the statistical analysis can be found in the Supplementary Material.

Results and discussion

RT-qPCR is the method of choice for the accurate quantification of miRNA expression (22–26), and there are several commercially available miRNA RT-qPCR methods that employ distinct approaches to prime the miRNA for reverse transcription and then amplify the cDNA. A previous study compared two of these assays, one of which employs a universal tailing reverse transcription primer platform and another, a sequence-specific stem-loop reverse transcription primer platform (26). These two assays were compared in terms of their sensitivity and specificity, revealing that the former assay was less specific as it generated more non-specific products but was generally more sensitive as it detected more low abundance miRNAs successfully. The majority of the low abundance products detected by the universal tailing method, however, were non-specific (26). In our study, however, we do not assess the sensitivity or specificity of miRNA RT-qPCR analysis, but investigate measurement precision, accuracy, and linearity, and the effect of short RNA enrichment on these parameters, as these important performance characteristics have not been previously evaluated. We quantified miRNA levels using two popular RT-qPCR technologies: Life Technologies' Taqman miRNA Assay (20) and Exiqon's miRCURY LNA Universal RT microRNA PCR assay, as these have not to our knowledge been previously compared. The Taqman miRNA assay uses a miRNA-specific stem-loop reverse transcription primer to generate cDNA for subsequent hydrolysis probe qPCR amplification (20). In contrast, the miRCury LNA Universal RT PCR assay involves the poly(A) tailing of mature miRNAs and the subsequent use of a poly(T) reverse transcription primer containing a 3′ degenerate anchor and 5′ universal tag to generate cDNA for

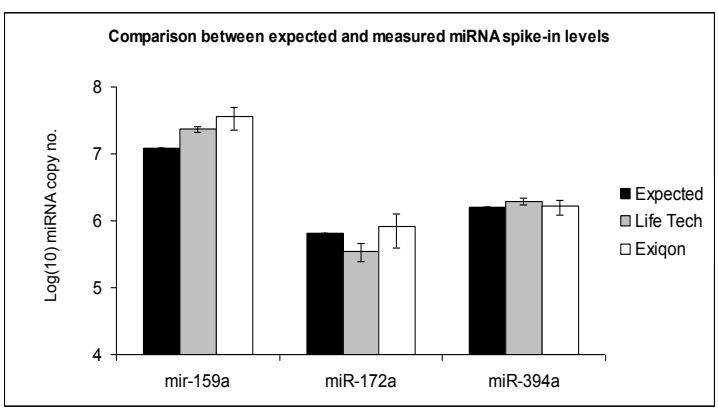

Figure 1. Comparison of the expected and measured copy numbers for the Arabidopsis thaliana spike-in miRNAs in human brain total RNA. Copy number values have been normalized to account for differences in input RNA between the RT-qPCR technologies. Error bars are equal to the standard error in the measurement.

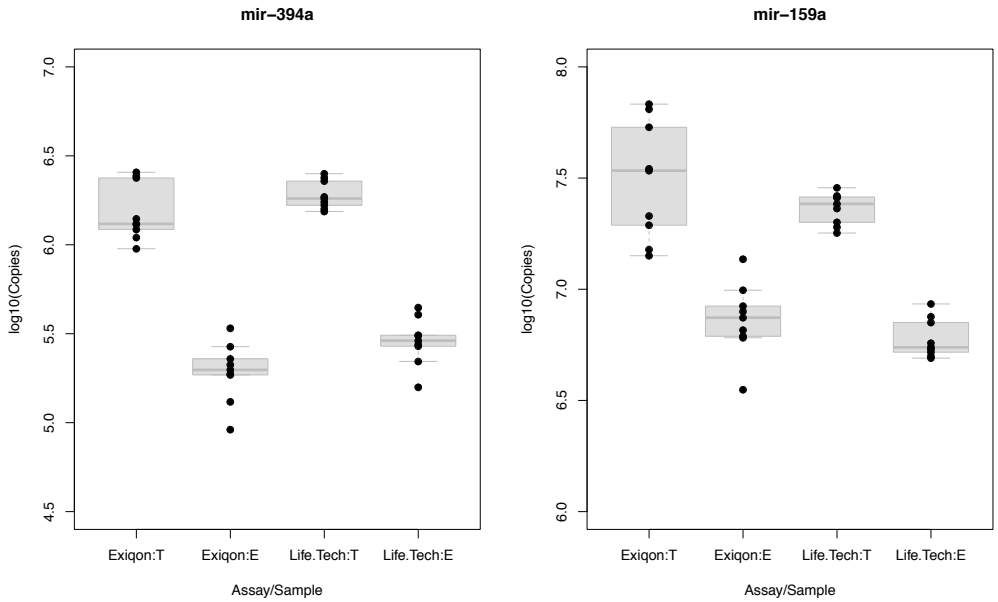

Figure 2. The effects of RT-qPCR technology and enrichment on miRNA levels in human brain RNA. RT-qPCR was performed using the Life Technologies and Exiqon assays on total (T) and enriched (E) RNA as indicated. Log(10) copy numbers of miRNA levels are shown for miRs-394a and -159a as boxplots, with the constituent data points overlaid in order to illustrate distribution. Copy number values have been normalized to account for differences in input RNA between the RT-qPCR technologies.

amplification by SYBR Green qPCR using miRNA-specific forward and reverse primers containing locked nucleic acids (LNAs). The use of miRNA-specific forward and reverse primers offers an advantage in terms of the specificity of amplification, compared with other assays of similar design. For example, Qiagen's miScript PCR System uses a miRNA-specific forward primer but with a universal reverse primer for the qPCR, possibly leading to a comparatively lower specificity of amplification. The Exiqon system also offers the potential advantage of using LNA containing primers. LNA molecules are modified RNA nucleotides that are mixed with DNA or RNA nucleotides to increase hybridization properties (39). Levin et al. (40) demonstrated, however, that LNA containing primers can display poorer amplification efficiencies than those that do not contain LNA molecules (40).

We analyzed the expression of five endogenous human miRNAs and three *Arabidopsis thaliana* synthetic spikes. The human miRNAs were selected on the basis that they are well characterized and show a high abundance across a wide range of cell and tissue types. For the synthetic *Arabidopsis* spike-ins, well-characterized miRNAs were also chosen as opposed to synthetic or re-assorted existing miRNAs because artificial oligonucleotides may not exhibit the same physiochemical properties of natural miRNA species. Furthermore, the three *Arabidopsis* miRNAs were selected because they displayed no homology with any known human miRNAs. The suitability of the *Arabidopsis* miRNAs was confirmed by experiments that showed there was an absence or a very low-level of cross-reactivity (Cq >35) with each other, background human brain total RNA, or a yeast tRNA carrier (data not shown).

The performance of the assays, in terms of efficiency and variability of efficiency, were evaluated by measuring serial dilutions of synthetic miRNAs in three independent experiments and miRNAs quantified using both qPCR technologies (Table 1). The RT-qPCR technologies did not differ significantly in their assay efficiency (Table 1, all R^2 values >0.988), but the Exiqon assay efficiency measurement appeared to be more variable between runs when compared with the Life Technologies assay (Table 1), as the latter usually showed a lower average standard deviation across the three repeats. However, these differences in standard deviation were not statistically significant.

The precision and accuracy of miRNA RT-qPCR quantification was evaluated by investigating variability of miRNA copy

Table 2. miRNA copy number in human brain RNA measured by RT-qPCR

miRNA	Life Technologies					Exiqon				
	Total RNA		Short RNA enriched			Total RNA		Short RNA enriched		
	Copy no.	% CV	Copy no.	% CV	% yield	Copy no.	% CV	Copy no.	% CV	% yield
mir-16	1.01E+07	31.89	2.62E+06	29.51	25.92	1.19E+07	13.30	3.01E+06	21.58	25.35
mir-26b	1.81E+06	9.51	4.52E+05	9.65	24.98	1.49E+06	19.53	3.76E+05	16.55	25.24
mir-21	4.85E+05	8.15	1.36E+05	48.81	28.13	5.59E+05	33.66	1.23E+05	28.96	22.08
let-7a	3.90E+07	30.42	1.33E+07	11.79	34.12	2.26E+07	6.71	6.52E+06	16.91	28.85
let-7c	1.87E+07	12.64	5.83E+06	14.39	31.12	3.00E+07	30.00	9.77E+06	53.51	32.61
mir-159a	2.34E+07	16.57	6.10E+06	23.60	26.03	3.61E+07	65.00	7.74E+06	35.24	21.43
mir-172a	3.53E+05	54.17	1.06E+05	78.48	30.00	8.20E+05	88.98	2.07E+05	78.36	25.24
mir-394a	1.93E+06	20.93	2.98E+05	25.90	15.41	1.63E+06	44.10	2.04E+05	34.04	12.55

miRNA copy number is presented as the average of 3 measurements performed in independent experiments; short RNA enrichment was performed independently in each experiment; % yield represents the relative miRNA levels after enrichment for each RT-qPCR technology; CV = Coefficient of Variation

number measurements in human brain total RNA, which was spiked with the three *Arabidopsis* miRNAs across a range of physiologically relevant levels and subsequently short RNA enriched in three independent replicate experiments using the miRVana miRNA isolation kit (Table 2). The enrichment of short RNA (<200 nucleotides) molecules from total RNA preparations is commonly performed prior to miRNA expression analysis. However, to our knowledge the effects of this procedure on miRNA quantification by RT-qPCR have not been extensively investigated. As described in detail in the materials and methods section, the miRVana miRNA isolation kit can be used to isolate total RNA in which the short RNAs are retained and optionally enriched or, alternatively, it can be performed on total RNA material that has already been isolated, possibly by a different method. The latter procedure, which we perform in this study, involves the separation of short RNAs from larger (>200 nucleotides) RNA species in the sample. The short RNA molecules isolated as a result of this process should have a higher concentration relative to the remaining RNA present in the sample compared with the relative levels of short RNAs to total RNA in the non-enriched starting material. However, this process may also result in the loss of short RNAs compared to the amount present in the starting material, which is something we investigate in this study. RT-qPCR measurements were performed with the Life Technologies and Exiqon assays on the non-enriched total RNA and short RNA enriched material. Serial dilutions of synthetic miRNA molecules were run on each plate to estimate miRNA copy number values. Measurement accuracy was determined by comparing expected miRNA copy number to that measured for the *Arabidopsis* spike-ins (Figure 1).

Comparison of the two RT-qPCR assays revealed that the variability of measurement was higher for Exiqon compared with Life Technologies (Table 2), which was supported by statistical analysis (detailed in Supplementary Material). As these measurements were performed on RNA material for which the spiking of the *Arabidopsis* miRNAs and subsequent short RNA enrichments were replicated, the standard deviation values presented in Table 2 demonstrate the variability associated with the preparation of the human brain RNA material in addition to that associated with the RT-qPCR assays. However, given that the same template material was used for both RT-qPCR assays, the differences in measurement precision between them can be due only to the variability inherent in the performance of the assays themselves. In order to eliminate any additional operational variability, all experiments in this study were performed by a single analyst. One possible explanation for the difference in measurement variability between the two RT-qPCR technologies could be differences in the assay methodologies. The Exiqon assay requires the cDNA reaction product to be diluted 80-fold. Since the reverse transcription reaction volume is 20 µL, this does not allow the diluting water (1580 µL or 790 µL for 10 µL half volume reactions) to be added directly to the reverse transcription reaction because this would exceed the volume of most standard PCR reaction tubes. Therefore, the cDNA needs to be transferred to a second, larger vessel, thus introducing an additional pipetting step and increasing the likelihood of variability between repeats. By contrast, the Life Technologies assay protocol does not require any dilution of the cDNA (or a small amount of diluent can be added directly to the reverse transcription reaction tube if desired) and thus there is less manipulation. miRNA copy number estimates varied considerably between the two RT-qPCR assays, but there was no general trend. For example Let-7a showed a mean 1.73-fold lower measured copy number in the total RNA sample with the Exiqon compared to the Life Technologies assay (Table 2). In the majority of cases these differences were statistically significant (detailed model output shown in Supplementary Material). This would therefore suggest that the technical error (or measurement uncertainty) associated with RT-qPCR technology could have a significant impact on miRNA quantification. This therefore needs to be considered, particularly when comparing experiments where absolute quantification, such as in our study where miRNA copy number values have been obtained by interpolation from standard curves of serially diluted synthetic miRNAs, has been performed using different approaches, e.g., meta-analyses. In addition to the effects on absolute miRNA quantification, our findings also have implications for relative miRNA quantification strategies in which the relative expression of multiple miRNAs has been determined in comparison to each other or the same miRNAs in different experimental conditions, due to the significant uncertainty associated with the measurement of both experimental and 'housekeeping' miRNAs**.** Indeed, previous studies have reported significant differences in relative miRNA expression of as low as 1.5-fold (41,42), but our findings suggest that such small differences in expression should be treated with caution.

The use of external miRNA standards of a known copy number revealed that both methods possess a comparable estimation of the respective quantity of the three *Arabi-*

dopsis spike-ins as the measurements of the spike-ins in non-enriched total RNA were within ± log(10) 0.5 miRNA copies of the expected values for both RT-qPCR technologies (Figure 1). As the *Arabidopsis* miRNAs were spiked into human total RNA and the serially diluted synthetic miRNAs used to generate the standard curves for interpolating miRNA copy number were in a yeast tRNA background, this would suggest that there was no significant impact of the different background RNA matrices for either assay. It would be interesting to investigate whether our results are consistent with other assays of a comparable design, for example whether Qiagen's miScript PCR System yields similar results to the Exiqon, since they both use a similar RT and qPCR priming strategy, or whether these findings are specific to the two assays we have assessed. This is something we will be addressing in future experiments. An important note is that we performed these experiments by replicating the whole reverse transcriptase and PCR process. The Life Technologies protocol advises a single reverse transcription to be performed, followed by replicate PCRs; however, this does not provide any estimation of the variation associated with the reverse transcription step (43). We therefore elected to replicate the reverse transcription step for both the Life Technologies and Exiqon assays.

Our experiments were designed to address the use of RT-qPCR assays that are appropriate for the analysis of a relatively small number of miRNAs in a given study. However, for studies which aim to measure global miRNA profiles, other technologies such as global RT-qPCR arrays can also be used. Interestingly, a cross-platform analysis has previously been performed between three global miRNA profiling technologies, including the Life Technologies' Taqman Human MicroRNA Array and Exiqon's miRCURY Ready-to-use PCR, which are based on the same RT-qPCR technologies that we evaluate in this study (44). Despite this, the relative performance characteristics of the Exiqon and Life Technologies' arrays were significantly different from those identified in our study. For example, the Exiqon array displayed a superior reproducibility and linearity compared with the Life Technologies' array (44). Although the basic principles of the RT-qPCR assay and array platforms are similar, there are, however, many significant differences that could impact on the miRNA measurement. For example, the Life Technologies' array uses a pre-amplification (pre-amp) step and involves the megaplexing of >300 miRNA-specific reverse transcription primers in each reaction. Indeed, it is possible that the pre-amp step in particular might cause an increase in the variability of measurement, as has been found with other platforms (45). Comparison of the findings of the Jensen et al. (44) study with ours would suggest that the differences between the experimental design of the array platforms and the RT-qPCR assays that use similar technologies are great enough to yield very different performance characteristics. This indicates that the measurement capabilities of RT-qPCR technologies should not be generalized to their use in global array platforms.

To evaluate the effects of short RNA enrichment on the miRNA measurement, we compared equivalent volumes of total and enriched RNA material as the enrichment protocol was performed by eluting in the same volume that was used as starting material (50 µL). Consequently the same effective volumes of enriched and total RNA samples were added to the RT-qPCR reactions. This method of comparison was chosen in preference to analyzing equal amounts of total and enriched RNA as this would not allow an accurate assessment of the miRNA yield after enrichment since the accuracies of total and short RNA quantification are not comparable. The enrichment reduced the copy number of the miRNAs (endogenous and *Arabidopsis* miRNA spike-ins) to approximately 25% of that present prior to enrichment on average ($P < 0.0001$; Figure 2, Supplementary Figure S1, Table 2; detailed model output for all miRNAs is shown in the Supplementary Material). This dramatic reduction in miRNA levels represents a significant finding and highlights that it is not advisable to rely on quantifications of RNA from pre-enriched samples as this would result in a

significant underrepresentation of the miRNA amount. Importantly, it was also found that the enrichment procedure did not have an equal effect on all measured miRNAs given that the miRNA yield ranged between 12%–35% for different miRNAs, resulting in changes to relative miRNA levels and potential experimental bias. Therefore, this would suggest that miRNA levels cannot be accurately compared between total and short RNA enriched preparations using the miRVana miRNA isolation kit and this needs to be considered when using different enrichment methods. Indeed, there are several other methods for enriching short RNAs, including column-based technologies similar to the miRVana miRNA isolation kit but also techniques such as short RNA gel purification, which is often performed prior to NGS analysis (46). We focused on the miRVana miRNA isolation kit because it is one of the most commonly used, but it is important for future studies to investigate the potential bias that these other methods may also introduce and the impact of this bias on miRNA quantification. The majority of the human miRNAs we analyzed have recently been reported to be suitable endogenous controls for normalizing miRNA expression (22,47), highlighting that these findings are relevant to both relative and absolute miRNA quantification strategies as the differential enrichment of housekeeping miRNAs compared with other target miRNAs in a population could have a significant effect on relative miRNA expression after normalization. For example, let-7a shows a 22-fold lower expression relative to miR-26b in the total RNA sample when measured with the Life Technologies assay, however, after short RNA enrichment, let-7a was 29-fold lower in expression than miR-26b (Table 2).

When comparing the endogenous human and *Arabidopsis* synthetic miRNAs, there appeared to be no systematic differences in the performance of the *Arabidopsis* miRNAs compared with the endogenous human miRNAs. For example, the majority of the variability measurements for the synthetic miRNAs were comparable to those of the human miRNAs (Table 2), despite the samples being independently spiked in each of the three replicates. Furthermore, the percent yields of miRs-159a and -172a after short RNA enrichment were within the range of the human miRNAs; miR-394a showed the lowest yield after enrichment (15.41% when measured with the Life Technologies assay, 12.55% with the Exiqon assay, Table 2) showing that this miRNA is at the edge of the range in percent yield, but is not dramatically different from the average value (25%), when considering the spread in values for all of the miRNAs. These findings demonstrate that the *Arabidopsis* spike-ins represent effective experimental controls for the analysis of endogenous human miRNA expression when performing the short RNA enrichment procedure. Interestingly, Cui et al. (38) showed that miR-159a also serves as an effective spike-in control for RNA extraction of plasma samples, demonstrating its general suitability for RNA isolation procedures. Other previous studies have also assessed the use of other non-mammalian spike-in miRNAs to control for technical variability associated with the entire RNA extraction process (8,32,36,37). For example, Kroh et al. (37) used a pool of three *C.elegans* miRNAs to control for technical variation in sample extraction by spiking into plasma or serum after protein denaturation treatment. As some researchers have expressed concerns over the suitability of endogenous serum and plasma miRNAs as internal controls (37), synthetic non-mammalian spike-in miRNAs may be a method of choice for future studies. Therefore, further work is required to optimize the usage of miRNA spike-ins to control for technical variability in total RNA isolation and short RNA enrichment procedures, including the establishment of the most effective methods of sample normalization and the most suitable number of spike-in miRNAs to use. For example, whereas Kroh et al. (37) and Mitchell et al. (8) used multiple spike-in miRNAs, other researchers have used single spikes (32,38).

In order to ensure specific amplification of miRNA targets, we employed a range of negative controls with each miRNA assay: reverse transcription no template control (RT NTC) reactions containing the reverse transcriptase enzyme but no template to evaluate DNA and RNA contamination or non-specificity; PCR NTCs to evaluate DNA contamination during PCR set up; carrier only reactions to evaluate non-specificity; and reactions containing no reverse transcriptase enzyme (no RTs) to evaluate DNA contamination throughout the reverse transcription and PCR procedures. For three of the Exiqon assays (let-7a and –c and miR-394a), there was a low level of background signal in several of the negative control groups, typically Cq >35 (Supplementary Table S2). Derivative melt curve analysis of these assays showed that the peaks for the negative controls were generally distinct from the experimental samples (Supplementary Figure S2), suggesting non-specific amplification. The Life Technologies assays generally showed an absence of any consistent signal in the no RT controls and PCR NTCs for all of the miRNAs, but of note there was a presence of signal in the RT NTCs. One of the Life Technologies assays (miR-159a) consistently showed a signal in the RT NTCs (Supplementary Table S2), but not in the corresponding DNA controls, indicating the amplification of a potentially contaminating source at the RNA level or non-specificity by the reverse transcriptase. This finding is of note as RT NTCs are not routinely performed in addition to no RT and PCR NTCs in many laboratories. These findings indicate that RT NTCs are an important control that should be included for miRNA RT-qPCR reactions in order to determine background levels of signal associated with the reverse transcriptase step as well as the PCR.

Conclusions

Comparison of two prominent miRNA RT-qPCR technologies, Life Technologies' Taqman miRNA Assay (20) and Exiqon's miRCURY LNA Universal RT microRNA PCR assay, revealed that both assays displayed similar efficiencies but that the latter generated more variable measurements. The two assay technologies yielded significantly different copy number estimations for some of the miRNAs despite using the same standard curve templates for copy number interpolation, indicating that RT-qPCR technology can have a significant impact on the miRNA measurement. The external *Arabidopsis* spike-ins provided a useful process control for assessing technical sensitivity as they can be spiked at a known copy number and do not display significant cross-reactivity with any human miRNAs. Furthermore, the performance characteristics of the *Arabidopsis* miRNAs were comparable to those of the endogenous human miRNAs, in terms of the effects of the enrichment procedure and RT-qPCR technology. As miRNA profiling typically involves a series of steps that are sensitive to technical manipulations, the *Arabidopsis* spike-in miRNAs provide a robust method for the standardization of procedures for within or cross-platform comparisons. We have also demonstrated that short RNA enrichment of human total RNA material can result in a significant 4-fold reduction in miRNA signal when comparing equivalent volumes of total RNA and enriched material. The enrichment procedure had a variable effect on the miRNAs that were analyzed, resulting in a change in relative miRNA levels compared with the non-enriched material. These findings suggest that miRNA data from total and short RNA preparations may not be directly comparable.

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Competing interests

The authors declare no competing interests.

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