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 x 10^5 copies/µl, miR-172a: 6.4 x 10^4 copies/µl, miR-394a: 1.6 x 10^5 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: 10^3 - 10^4 for let-7a, let-7c, miR-16, -394a, -26b, -159a; 10^4 - 10^5 for miRs-21 and -172a. All RNA was stored in RNA Storage Solution (Cat. No. AM99937, 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. 147662). 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 homogenize 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 ng) (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: 15°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

<table>
<thead>
<tr>
<th>miRNA assay</th>
<th>Amp. Efficiency (%)</th>
<th>± SD</th>
<th>Amp. Efficiency (%)</th>
<th>± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-16</td>
<td>98.54</td>
<td>2.69</td>
<td>104.47</td>
<td>6.72</td>
</tr>
<tr>
<td>mir-26b</td>
<td>98.01</td>
<td>0.98</td>
<td>97.66</td>
<td>6.24</td>
</tr>
<tr>
<td>mir-21</td>
<td>99.93</td>
<td>3.70</td>
<td>97.82</td>
<td>6.76</td>
</tr>
<tr>
<td>let-7a</td>
<td>97.48</td>
<td>5.65</td>
<td>100.17</td>
<td>8.34</td>
</tr>
<tr>
<td>let-7c</td>
<td>99.27</td>
<td>1.86</td>
<td>93.89</td>
<td>0.75</td>
</tr>
<tr>
<td>mir-159a</td>
<td>97.36</td>
<td>3.89</td>
<td>96.74</td>
<td>9.03</td>
</tr>
<tr>
<td>mir-172a</td>
<td>100.44</td>
<td>6.34</td>
<td>109.59</td>
<td>3.43</td>
</tr>
<tr>
<td>mir-394a</td>
<td>100.95</td>
<td>2.57</td>
<td>107.07</td>
<td>6.21</td>
</tr>
</tbody>
</table>

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).

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 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 detected 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
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 R² 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 number expressed in each experiment; % yield represents the relative miRNA levels after enrichment for each RT-qPCR technology; CV = Coefficient of Variation.

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

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Life Technologies</th>
<th>Exiqon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total RNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Copy no. % CV</td>
<td>Copy no. % CV</td>
</tr>
<tr>
<td>mir-16</td>
<td>1.01E+07 31.89</td>
<td>1.19E+07 13.30</td>
</tr>
<tr>
<td>mir-26b</td>
<td>1.81E+06 9.51</td>
<td>1.49E+06 19.53</td>
</tr>
<tr>
<td>mir-21</td>
<td>4.85E+05 8.15</td>
<td>5.59E+05 33.66</td>
</tr>
<tr>
<td>let-7a</td>
<td>3.90E+07 30.42</td>
<td>2.26E+07 6.71</td>
</tr>
<tr>
<td>let-7c</td>
<td>1.87E+07 12.64</td>
<td>3.00E+07 30.00</td>
</tr>
<tr>
<td>mir-159a</td>
<td>2.34E+07 16.57</td>
<td>3.61E+07 65.00</td>
</tr>
<tr>
<td>mir-172a</td>
<td>3.53E+05 54.17</td>
<td>8.20E+05 88.98</td>
</tr>
<tr>
<td>mir-394a</td>
<td>1.93E+06 20.93</td>
<td>1.63E+06 44.10</td>
</tr>
</tbody>
</table>

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.
Any additional operational variability, all the assays themselves. In order to eliminate variability inherent in the performance of between them can be due only to the material was used for both RT-qPCR assays, However, given that the same template to that associated with the RT-qPCR assays. Values presented in Table 2 demonstrate 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-
dopis spike-ins as the measurements of the spike-ins in non-enriched total RNA were within \( \pm \log(10) 0.5 \) 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 \( (P < 0.0001; \text{Figure 2, Supplementary Figure S1, Table 2}; \text{detailed model output for all miRNAs is shown in the Supplementary Material}). \) 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
miRNAs were spike-ins provided a miRNA to the edge of the range in percent yield, but is Table 2) showing that this miRNA is at the Life Technologies assay, 12.55% with the Exiqon assay, 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 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.

References