

Variability of the Reverse Transcription Step: Practical Implications

Stephen Bustin,^{1*} Harvinder S. Dhillon,¹ Sara Kirvell,¹ Christina Greenwood,¹ Michael Parker,¹
Gregory L. Shipley,² and Tania Nolan³

BACKGROUND: The reverse transcription (RT) of RNA to cDNA is a necessary first step for numerous research and molecular diagnostic applications. Although RT efficiency is known to be variable, little attention has been paid to the practical implications of that variability.

METHODS: We investigated the reproducibility of the RT step with commercial reverse transcriptases and RNA samples of variable quality and concentration. We quantified several mRNA targets with either singleplex SYBR Green I or dualplex probe-based real-time quantitative PCR (qPCR), with the latter used to calculate the correlation between quantification cycles (C_q) of mRNA targets amplified in the same qPCR assay.

RESULTS: RT efficiency is enzyme, sample, RNA concentration, and assay dependent and can lead to variable correlation between mRNAs from the same sample. This translates into relative mRNA expression levels that generally vary between 2- and 3-fold, although higher levels are also observed.

CONCLUSIONS: Our study demonstrates that the variability of the RT step is sufficiently large to call into question the validity of many published data that rely on quantification of cDNA. Variability can be minimized by choosing an appropriate RTase and high concentrations of RNA and characterizing the variability of individual assays by use of multiple RT replicates.

© 2014 American Association for Clinical Chemistry

The reverse transcription (RT)⁴ of RNA to cDNA is an essential first step for many research and molecular diagnostic applications. One of its most important uses involves the identification of RNA-based biomarkers, for example, those associated with environmentally induced diseases (1) or prognostic stratification and ad-

juvant therapy selection in cancer (2). Despite this central role, relatively few publications have explored the reliability and reproducibility of results and conclusions on the basis of the use of RT. Those that have investigated this matter with regard to real-time quantitative RT-PCR (qPCR) have reported that the RT step results in considerable variability (3, 4), particularly at low template concentrations (5), and that results depend significantly on the choice of priming strategy, reaction conditions, and RNA concentration (6) as well as the choice of reverse transcriptase (RTase) (7). The same is true for digital RT-PCR, where results are both RTase and target dependent (8). Other reports have called attention to the many methods used to generate cDNA as a source of RT variability (9) or described priming-specific problems, for example, the generation of truncated cDNAs through internal oligo-dT priming (10). There is also a measurable impact of RNA quality on reference gene variation (11) and on the differential expression of prognostic marker genes between cancer patient risk groups (12), as well as inconsistencies arising owing to inherent biological variability associated with the transcriptome (13).

Variability can be up to 100-fold (7), raising serious concerns about the validity of the huge number of publications that record relatively modest differences in the expression levels of mRNA and miRNAs and claim biological and clinical significance (2). When in combination with qPCR, there are additional well-publicized, but usually ignored, problems associated with normalization of RNA fold-changes against reference genes (14). Not only are most reference genes never validated (15), making it difficult to assess the reliability of any conclusions (16), but their RT efficiencies also can suffer from poor reproducibility, resulting in inadequate RT robustness and lack of covariance with RT efficiencies of the genes of interest (17). The effects of this variability are exacerbated when re-

¹ Postgraduate Medical Institute, Faculty of Medical Science, Anglia Ruskin University, Cambridge, UK; ² Shipley Consulting, LLC, Austin, TX; ³ Institute of Population Health, Faculty of Medical and Human Sciences, University of Manchester, Manchester, UK.

* Address correspondence to this author at: Postgraduate Medical Institute, Bishop Hall Lane, Chelmsford, UK CM1 1SQ. E-mail stephen.bustin@anglia.ac.uk.

Received July 25, 2014; accepted September 23, 2014.

Previously published online at DOI: 10.1373/clinchem.2014.230615

⁴ Nonstandard abbreviations: RT, reverse transcription; qPCR, real-time quantitative RT-PCR; RTase, reverse transcriptase; C_q, quantification cycle; MIQE, Minimum Information for the Publication of Real-Time Quantitative PCR Experiments; tRNA, transfer RNA; RIN, RNA integrity.

sults from different laboratories are compared, making it essential to consider appropriate quality assurance measures (18). Disregard of technical issues is not confined to qPCR: unreliable quantification and false-positive results are also observed with microarrays (19, 20), and estimating relative transcript abundance by next-generation sequencing is inconsistent not just at low levels of coverage but even when coverage levels are high (21).

To ascertain the reliability of currently available RTases, we set out to quantify the variability inherent in the RT step by assessing the performance of different RTases in qPCR assays carried out with separate RT and qPCR steps. Our results, although based on a limited number of RTases, target genes, and sample types, lead us to conclude that reporting of results on the basis of RT should include information on empirically determined RT variability, and future experiments involving cDNA synthesis require detailed statistical evaluation of analysis methods to improve the reliability of RNA-associated data.

Materials and Methods

In-depth details of materials and methods are provided in Supplemental Methods, which accompanies the online version of this article at <http://www.clinchem.org/content/vol61/issue1>. The investigational workflow was as follows. (a) We assessed uniformity of qPCR instrument blocks and reproducibility of pipetting by 48 replicate qPCR assays carried out on the Bio-Rad CFX and Illumina Eco (now PCRmax Exo48) instruments. (b) We evaluated RT variability by carrying out qPCR assays targeting *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase)⁵ #1 on 10 cDNA replicates from each of 6 RTases. Enzymes were scored by calculating a change in quantification cycle (ΔCq) range, which measures the difference between the highest and lowest Cq recorded for each set of replicates; the best-performing (smallest ΔCq) and worst-performing (highest ΔCq) RTases were compared further with qPCR assays targeting additional markers. (c) We assessed concentration-dependent variability by qPCR assays comparing the ΔCq ranges from 10 replicate RT reactions transcribed at 2 different concentrations. (d) We analyzed assay-dependent variability by qPCR assays comparing the ΔCq ranges from 10 replicate RT reactions

targeting different regions of 2 mRNAs. (e) We investigated sample-dependent variability by qPCR assays comparing the ΔCq ranges from 10 replicate RT reactions from 4 RNA samples. (f) We measured the effect of RNA quality on RT variability by comparing the ΔCq ranges from 10 replicate RT reactions of 2 poor-quality RNA samples.

MINIMUM INFORMATION FOR PUBLICATION OF qPCR EXPERIMENTS

Relevant information demonstrating compliance with the Minimum Information for the Publication of Real-Time Quantitative PCR Experiments (MIQE) guidelines (22) is provided within the appropriate tabs of the online Supplemental Methods; a MIQE checklist was submitted during electronic submission of the article.

RNA

Total RNA was prepared from five 30-mg breast cancer biopsy samples with the RNeasy lipid tissue mini-kit (Qiagen). Sample details as well as RNA quantity and quality assessments are shown in online Supplemental Tab 1.

PRIMERS AND PROBES

Oligonucleotide sequences, mRNA targets, and PCR efficiency details are shown in online Supplemental Tab 2.

INSTRUMENT BLOCK UNIFORMITY/PIPETTING ACCURACY

We tested block uniformity of the Bio-Rad CFX Connect (Bio-Rad) and Illumina Eco (now PCRmax Exo48) qPCR instruments by running identical 10- μ L *GAPDH* reactions containing 300 nmol/L primers and approximately 50 ng cDNA in each alternate well of a 96-well plate (CFX) or all wells of a 48-well plate (Eco 48).

RT REACTIONS

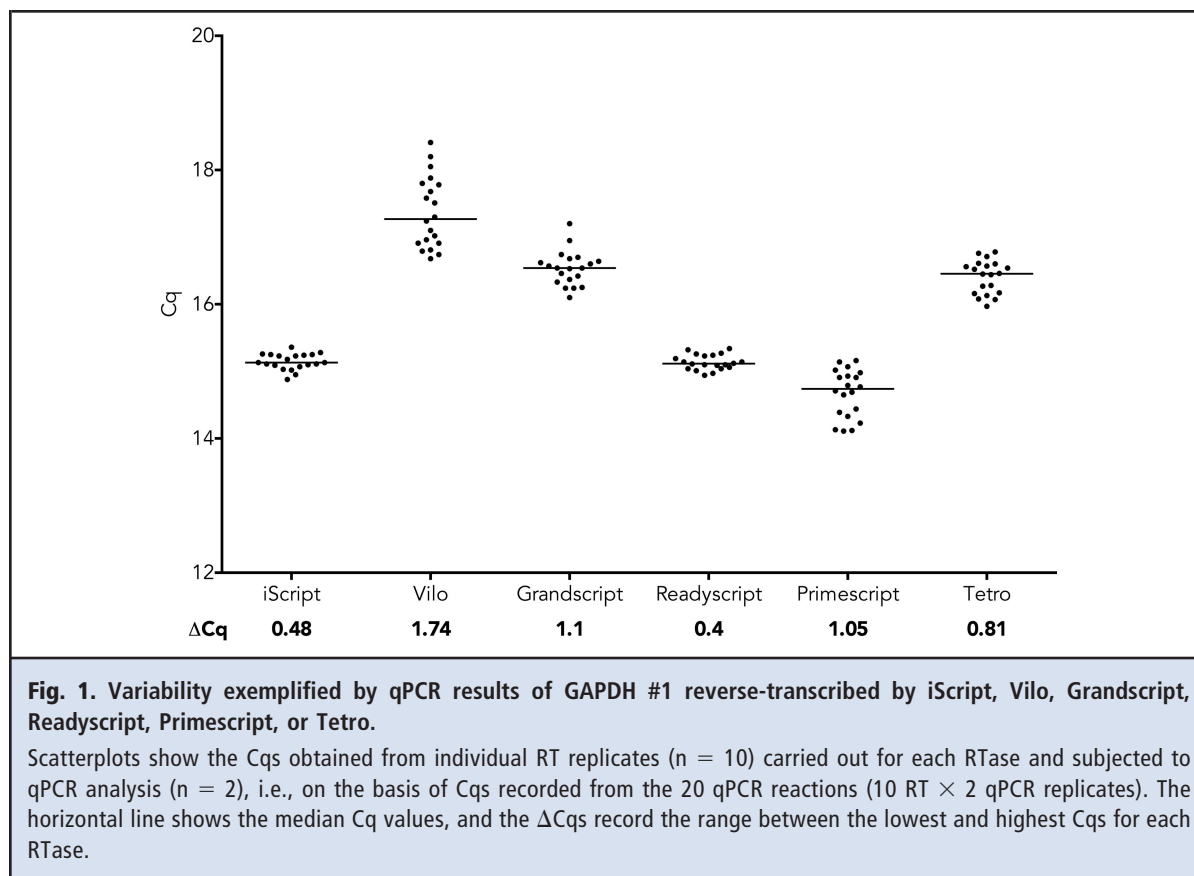
RT reactions were carried out by use of random priming within the recommended range of RNA concentrations of either 50 or 12.5 ng/ μ L as specified by the manufacturers' RT protocols in 10 individual 5- μ L reactions.

One set of experiments was carried out by use of replicate 1- μ L RT reactions containing 50 ng/ μ L RNA. These were subsequently subjected to dualplex qPCR analysis targeting *CDH1* [cadherin 1, type 1, E-cadherin (epithelial)]/*CTNNB* [catenin (cadherin-associated protein), β 1, 88 kDa] and *CDH1/MAX* (MYC associated factor X).

qPCR

SYBR Green I qPCR assays were carried out in 10- μ L reactions containing 1 \times KAPA SYBR Fast reaction mix (Anachem), primers at 300 nmol/L final concentration, and 1 μ L cDNA on the CFX qPCR instru-

⁵ Human genes: *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *CDH1*, cadherin 1, type 1, E-cadherin (epithelial); *CTNNB*, catenin (cadherin-associated protein), β 1, 88 kDa; *MAX*, MYC associated factor X; *CDK2*, cyclin-dependent kinase 2; *RBL1*, retinoblastoma-like 1; *MYC*, v-myc avian myelocytomatosis viral oncogene homolog; *UBC*, ubiquitin C; *KRT19*, keratin 19; *HMBS*, hydroxymethylbilane synthase; *TP53B3*, tumor protein p53 inducible protein 3.



ment (Bio-Rad) programmed as follows: 95 °C for 5 s, followed by 40 cycles of 95 °C for 2 s, 60 °C for 1 s, and 72 °C for 1 s, with fluorescence collection at 72 °C.

Dualplex assays were carried out in 10- μ L reactions containing 1 \times NuPCR reaction mix (Illumina), NuPCR primer/probe mix (0.5 μ L/assay), and 1 μ L cDNA on the Eco thermal cycler programmed as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 30 s, with fluorescence collection at 50 °C.

ANALYSIS

Standard curves were prepared with 10-, 5-, or 2-fold (depending on abundance of target) serial dilutions of C10 cDNA, diluted into 100 ng/ μ L yeast transfer RNA (tRNA) (Life Technologies). These were run for each assay, and the slopes and y -intercepts derived from the regression equations were used to calculate sample target copy numbers. Amplification efficiencies for each assay are shown in online Supplemental Tab 2.

The statistical analyses for most data sets were analyzed and graphed with Prism 6 for Macintosh, version 6.0e (Graphpad Software), and are shown in the appropriate online supplemental tabs. The statistical

analysis of the data variance shown in online Supplemental Tab 9 is provided in the online Supplemental Methods.

Results

INSTRUMENT BLOCK UNIFORMITY/PIPETTING ACCURACY

The uniformity of the thermal cycler blocks in both qPCR instruments was high, as was the accuracy of pipetting (see online Supplemental Tab 3), with the 48 qPCR assays targeting *GAPDH* #1 resulting in median Cqs of 20.17 (range 19.96–20.39) or 21.11 (range 20.89–21.34), respectively, for the Eco and CFX instruments.

RTase-DEPENDENT VARIABILITY

qPCR targeting *GAPDH* #1 resulted in Δ Cqs for the 10 replicates reverse-transcribed by iScript, Vilo, Grandscript, Readyscript, Primescript, and Tetro ranging from 0.4 to 1.74 (Fig. 1; online Supplemental Tab 4A). In contrast, the Δ Cqs attributable to qPCR/pipetting variability were lower and ranged from 0.07 to 0.16 (see online Supplemental Tab 4B).

A more detailed analysis of the least (Readyscript) and most (Vilo) variable RTases confirmed that Ready-

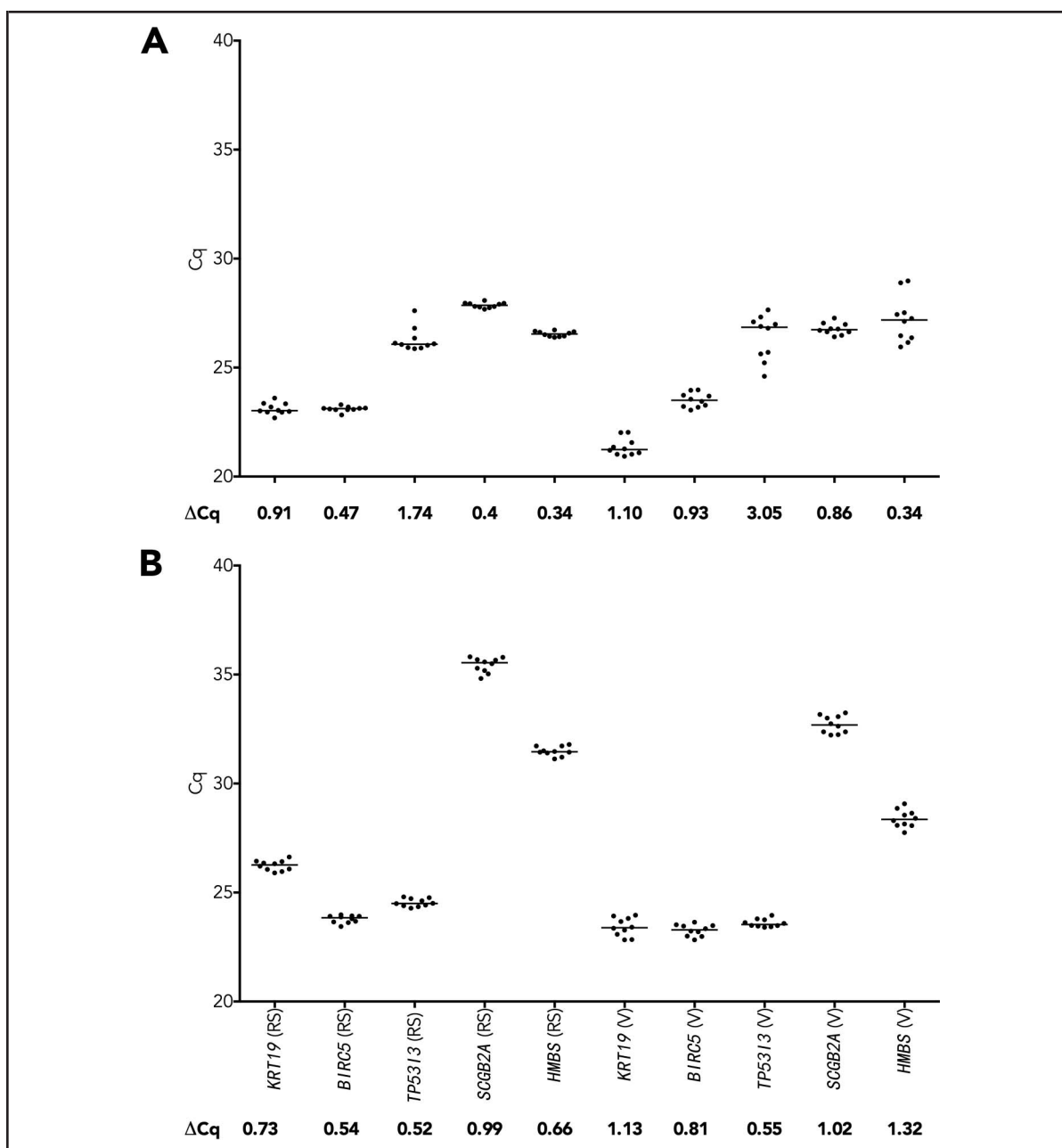


Fig. 2. Variability of cDNA synthesis targeting 5 mRNAs with Readyscript (RS) or Vilo (V) RTases.

(A), Sample C6. (B), Sample C10. Scatterplots show the Cqs obtained from individual RT replicates ($n = 10$) carried out for RS or V and subjected to qPCR analysis ($n = 1$), i.e., on the basis of Cqs recorded from 10 qPCR reactions. The horizontal line shows the median Cq values. Markers shown on the x axis are the same for (A) and (B). *BIRC5*, baculoviral IAP repeat containing 5; *SCGB2A*, secretoglobin, family 2A (containing members 1 and 2).

script showed consistently less variation, with RT-dependent ΔCq s ranging from 0.34 to 1.74 and 0.86 to 3.05, respectively (Fig. 2A; online Supplemental Tab 5A). A similar result was obtained when a different sample (C10) was used, with Readyscript and Vilo re-

ording median RT-dependent ΔCq s of 0.52–0.99 and 0.55–1.3, respectively (Fig. 2B; online Supplemental Tab 5B). On the other hand, Vilo recorded consistently lower Cqs for most of the mRNA targets (see online Supplemental Tab 5, C and D).

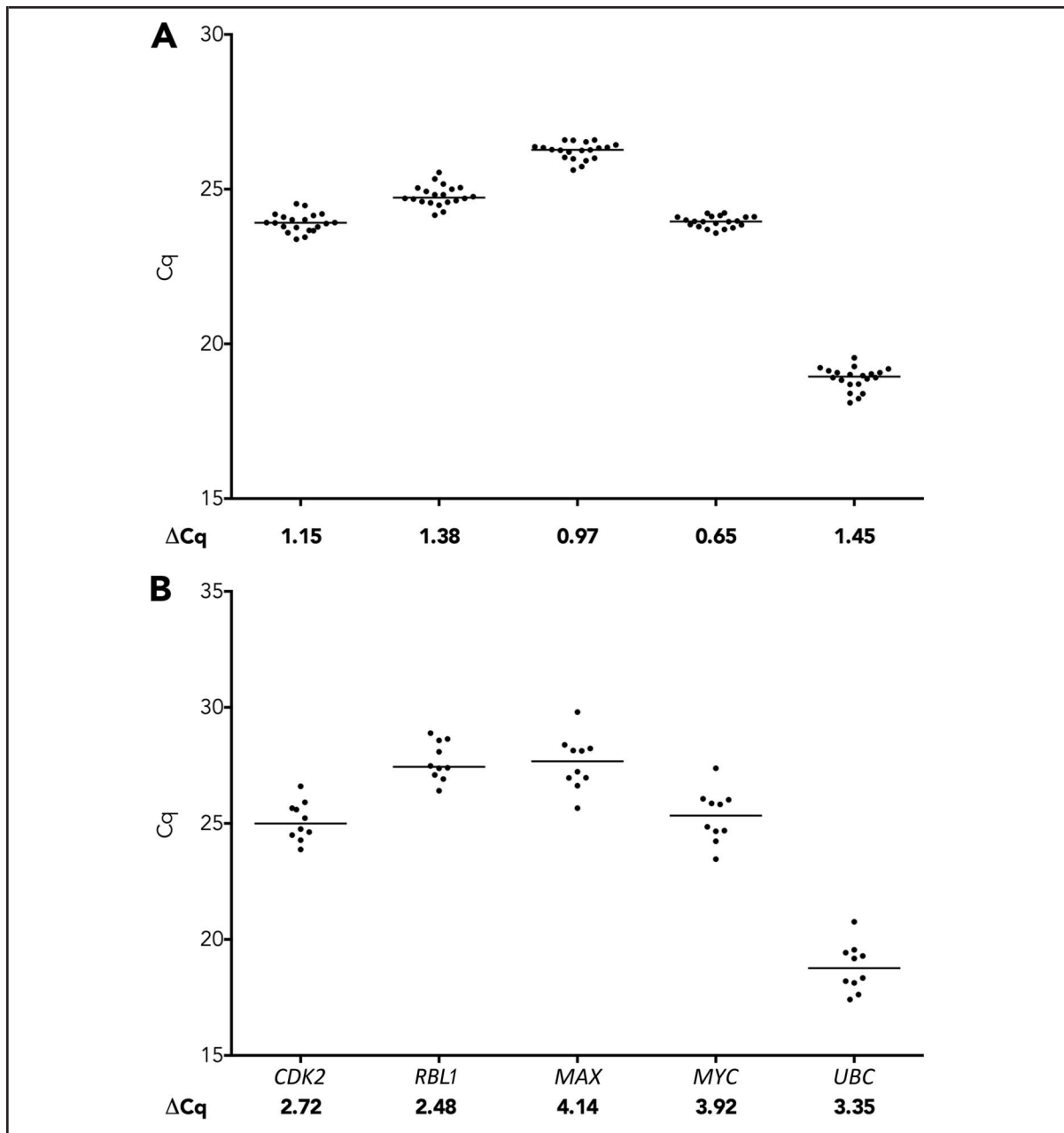


Fig. 3. Concentration-dependent variability.

(A), Scatterplots show the Cqs obtained from individual RT replicates ($n = 10$) carried out with $50 \text{ ng}/\mu\text{L}$ RNA, followed by subsequent qPCR assays ($n = 2$) for *CDK2/RBL1* and *MAX/MYC* carried out as dualplex assays with Illumina's probe-based assays; *UBC* was detected with SYBR. (B), RT reactions ($n = 10$) were repeated with $12.5 \text{ ng}/\mu\text{L}$ RNA, but this time each cDNA was subjected to a single qPCR assay ($n = 1$). Horizontal lines indicate the median Cq determined from 20 (A) or 10 (B) data points. Markers shown on the x axis are the same for (A) and (B).

CONCENTRATION-DEPENDENT VARIABILITY

A third RNA sample ($50 \text{ ng}/\mu\text{L}$) was reverse-transcribed by Reagentscript and subjected to dualplex *CDK2* (cyclin-dependent kinase 2)/*RBL1* (retinoblastoma-like 1) or

MAX/MYC (v-myc avian myelocytomatosis viral oncogene homolog) and singleplex *UBC* (ubiquitin C) #1 qPCR analysis. This resulted in RT-dependent ΔCqs ranging from 0.65 to 1.45 (Fig. 3A; online Supplemental

Tab 6A), with qPCR/pipetting-associated ΔCq s from 0.25 to 0.34 (see online Supplemental Tab 6B). There was significant correlation between *CDK2* and *RBL1* as well as *MAX* and *MYC*, although it was higher for the former ($r^2 = 0.84$ vs 0.58, respectively) (see online Supplemental Tab 6, C and D).

When the experiment was repeated with the a 1:5 dilution of the same RNA, the RT-dependent ΔCq range increased to 2.48–4.14 (Fig. 3B; online Supplemental Tab 7), with high correlation between *CDK2/RBL* and *MAX/MYC* ($r^2 = 0.94$ vs 0.99, respectively) (see online Supplemental Tab 7, B–D).

ASSAY-DEPENDENT VARIABILITY

qPCR analysis of assays designed against different regions of *UBC* and *GAPDH* mRNA from 2 RNA samples reverse-transcribed by Readyscript and Vilo gave similar patterns. Cq values for *UBC* #1 and *GAPDH* #1 were lower than those for *UBC* #2 and *GAPDH* #2, despite similar amplification efficiencies (Fig. 4; online Supplemental Data Tab 8, A and B). As before, the RT variability across all target mRNAs was higher for Vilo than Readyscript. A repeat with Primescript of the replicate RT reactions for the *GAPDH* assay also gave a similar result (see online Supplemental Tab 8C).

SAMPLE-DEPENDENT VARIABILITY

Expression patterns of qPCR assays targeting *CDK2/RBL1*, *MAX/MYC*, or *UBC* #1 were similar across 4 different RNA samples reverse-transcribed by Readyscript (see online Supplemental Tab 9A). However, there was significant heterogeneity of ΔCq variance among sample types ($P = 0.005$) (see online Supplemental Statistical Analysis), suggesting that the observed intramarker variability is sample dependent (see online Supplemental Tab 9B). The 2 samples with RNA integrity (RIN) values of 10 showed less variability, with median RT-dependent ΔCq s of 2.2 and 2.4, as opposed to 2.8 and 3.4 for the samples with RIN values 8 and 9.5.

There was significant correlation for *CDK2/RBL1* in all 4 samples, although the variability of individual RT reactions was apparent and indicated by the 95% CIs shown, which range from 0.87 to 0.99 (see online Supplemental Tab 10). Correlations for *MAX/MYC* were similarly significant for 3 of the RNA samples, with greater variability recorded by 1 (C10). The relative expression levels [4.1 (0.7)] and differences in fold change [1.5 (0.1)] of *CDK2/RBL1* were similar in the 4 RNA samples, whereas they differed widely for *MAX/MYC* at [0.8 (0.5)] and [3.5 (3.0)] of *MAX/MYC*, respectively (Fig. 5; online Supplemental Tab 11).

We further analyzed the correlation between mRNAs expressed by that sample by subjecting 35 RT replicates to amplification with dualplex qPCR target-

ing either *CDH1/CTNNB* or *CDH1/MAX*. The results are consistent with those observed previously: although there was significant correlation within both sets of markers, 1 set (*CDH1/MAX*) showed more variability as revealed by the lower correlation coefficient (see online Supplemental Tab 12).

RNA QUALITY DEPENDENCE OF VARIABILITY

The RNA sample with the higher integrity (C71A) recorded lower Cq s for *KRT19* (keratin 19), *UBC* #1, and *HMBS* (hydroxymethylbilane synthase), but not for *TP53I3* (tumor protein p53 inducible protein 3) and *GAPDH* (see online Supplemental Tab 13A). In each series of 10 RT replicates, there was 1 (C71A sample 1, C71B sample 3) that generated significant outlier results for *KRT19*, *UBC* #1, *TP53IP*, and *HMBS* (Grubbs test, $P < 0.05$, 2-sided) but not for *GAPDH* (see online Supplemental Tab 13, B and C). The RT-dependent ΔCq range reverse-transcribed from the higher-quality RNA was narrower for all 4 targets compared to the lower-quality one (median 2.42 vs 3.70, respectively).

The probe-based assays targeting *CDK2* and *RBL1* recorded similar patterns, with the Cq s recorded for both targets lower in C71A (see online Supplemental Tab 14). There was 1 outlier (C71A, *CDK2* sample 10) as calculated by Grubbs test, with significant correlation between the targets assessed in the dualplex reaction in sample C71A (Pearson $r = 0.715$, 95% CI 0.155–0.927), but none in C71B (Pearson $r = 0.208$, 95% CI –0.485 to 0.741). The RT-dependent ΔCq range was lower in the higher-quality RNA sample for *CDK2* but not for *RBL1*.

Discussion

We have studied the real-world variability of commercial RTases by use of qPCR to quantify several mRNA targets with different RNA samples of varying concentration, integrity, and purity. Although the variability between the different enzymes was never as high as that reported previously (7), our data show that there is experimental variation and that it is sufficiently large to have important implications with regard to reproducibility, robustness, and accuracy of any molecular technique using cDNA. Variability was also sample and concentration dependent and was always higher than that of qPCR/pipetting-associated variability of technical replicates. The sample-dependent differences may be linked to differential RNA integrity and may also be affected by organic contaminants. Although the amounts of RNA used in the RT step were well within the manufacturers' specifications, we observed a significant concentration-dependent variability. A similar effect has also been identified in next-generation sequencing, where the use of limiting amounts of mRNA

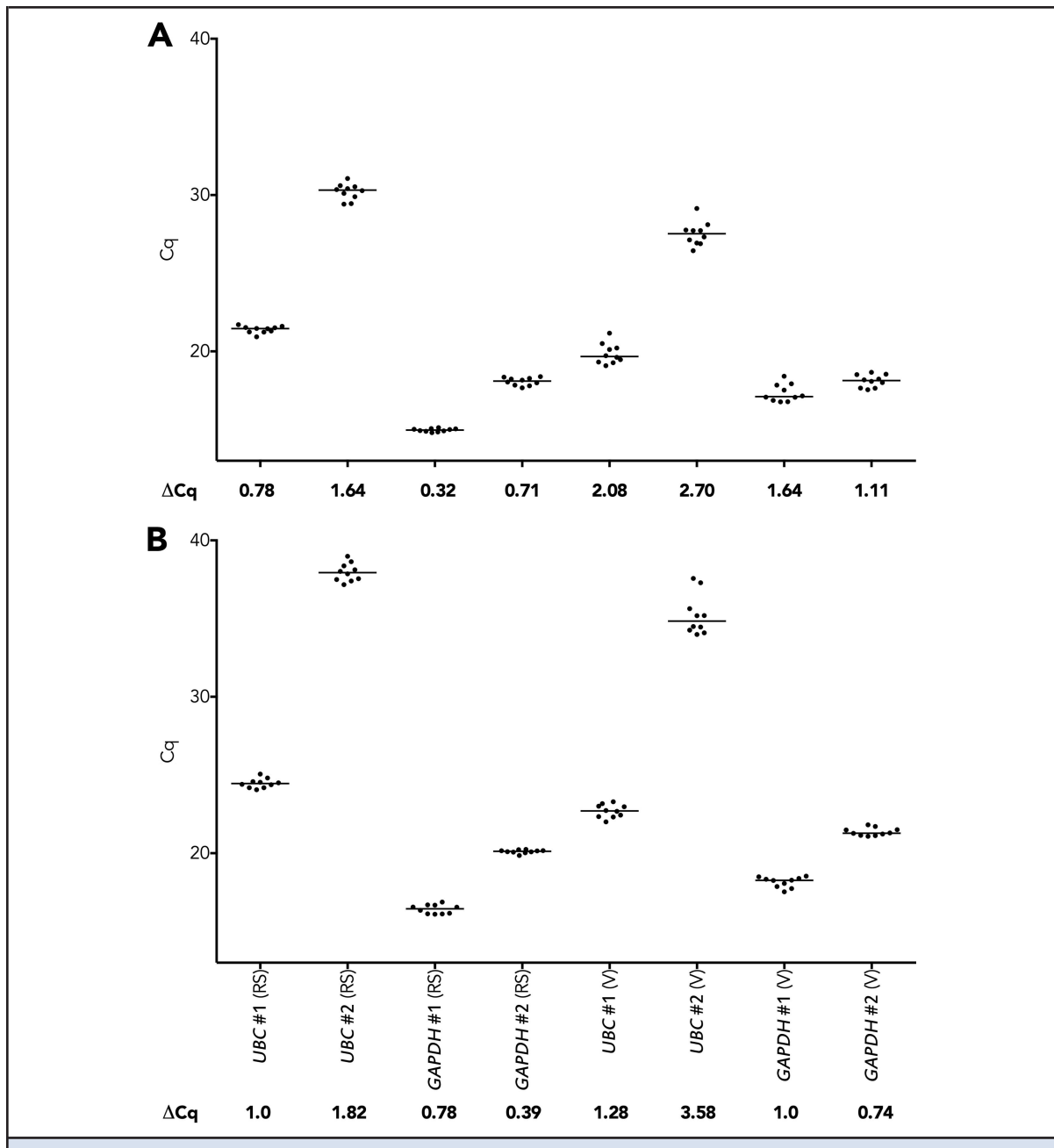


Fig. 4. Assay-dependent variability.

Replicate RT-qPCR assays targeting 2 different locations on *UBC* (*UBC* #1 and *UBC* #2) or *GAPDH* (*GAPDH* #1 and *GAPDH* #2) mRNA were carried out with either Readyscript (RS) or Vilo (V). (A), Sample C6. (B), Sample C10. Scatterplots show the C_q s obtained from individual RT replicates ($n = 10$) carried out for each RTase and subjected to qPCR analysis ($n = 1$), i.e., on the basis of C_q s recorded from 10 qPCR reactions. The horizontal line shows the median C_q values. Markers shown on the x axis are the same for (A) and (B).

results in significant technical variation, with inefficient amplification of the majority of low to moderately expressed transcripts masking subtle biological differences (23). This is rather disconcerting, given the

increasing interest in carrying out RNA expression analysis on single cells.

Assay-dependent variability was apparent from the results obtained with multiple assays targeting the

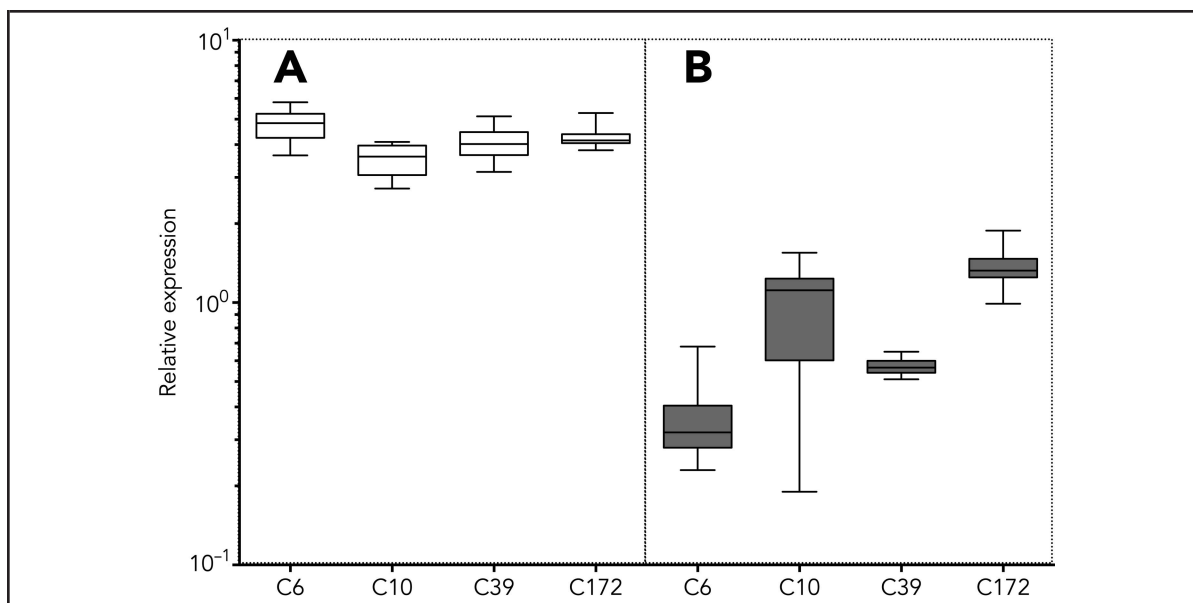


Fig. 5. Expression levels of *CDK2* relative to *RBL1* (A) or *MAX* relative to *MYC* (B) calculated for RT-qPCR reactions carried out on 10 replicates of cDNA (Readyscript) from samples C6, C10, C39, and C172.

Copy numbers for each target were calculated on the basis of individual standard curve regressions with the slopes and y-intercepts shown in online Supplemental Tab 1. Relative expression was calculated by dividing the copy numbers for *CDK2* and *MAX* by the copy numbers of *RBL1* or *MYC*, respectively. The variation in the fold change in each sample was calculated by dividing the sample with the highest relative expression by the sample with the lowest relative expression. The upper hinge of the boxes represents the 75th percentile, the middle horizontal line the median value, and the lower hinge the 25th percentile. Minimum and maximum expression levels are also shown, as are the fold changes.

same mRNA: RT variability differed between *UBC* #1 and #2 as well as between *GAPDH* #1 and #2. This is not easily explained by differences in amplicon secondary structures, since although there are secondary structures at the forward or reverse primer binding sites of *UBC* #1 and *GAPDH* #2, there are none in those generated by *UBC* #2 and *GAPDH* #1. Assay- and sample-dependent variability of the RT step was also apparent when comparing the expression of 5 mRNA markers across 4 RNA samples. There was statistically significant heterogeneity of ΔCq variance among sample types (Brown and Forsythe test for heterogeneity of variance, $P = 0.005$), which may be partly explained by differences in RNA integrity, with high RIN value samples recording lower RT-dependent ΔCqs than those with lower RIN values. Integrity-associated variability was also evident from the results obtained from a comparison of 2 RNA preparations extracted from the same sample at different times. One looked acceptable on an electropherogram although it did not yield a RIN value; the other was degraded, with a RIN value of 4.9. The latter sample recorded higher Cqs and showed more variability across all but 1 of the targets quantified by qPCR. This observation of assay-dependent contribu-

tion to data variability reemphasizes the need for empirical assay design and validation before carrying out qPCR analysis (24) and indicates that choosing optimal assays can reduce data variability. It also substantiates the original MIQE requirement of submitting primer sequences with each publication, since even a small difference in primer sequence can change the performance of an RT-qPCR assay (25).

Dualplex qPCR assays targeting *CDK2/RBL1* and *MAX/MYC* in high-quality RNA preparations indicate that the RT reactions are highly correlated, although again there is random variability apparent, as demonstrated by variable correlation coefficients and 95% CIs. For example, the r^2 value between *CDK2* and *RBL1* in 4 RNA samples is around 0.94, but is similarly high for *MAX/MYC* in only 3 of the samples, dropping to 0.76 in 1 (see online Supplemental Tab 10). An analysis of the correlation of *CDK2/RBL1* Cqs in 2 poor-quality samples shows significantly lower correlation, in line with the absence of an RIN value for 1 sample and the low RIN value for the other (see online Supplemental Tab 14). This is probably associated with differential stability of different transcripts and emphasizes again the need for care when comparing RNA samples with

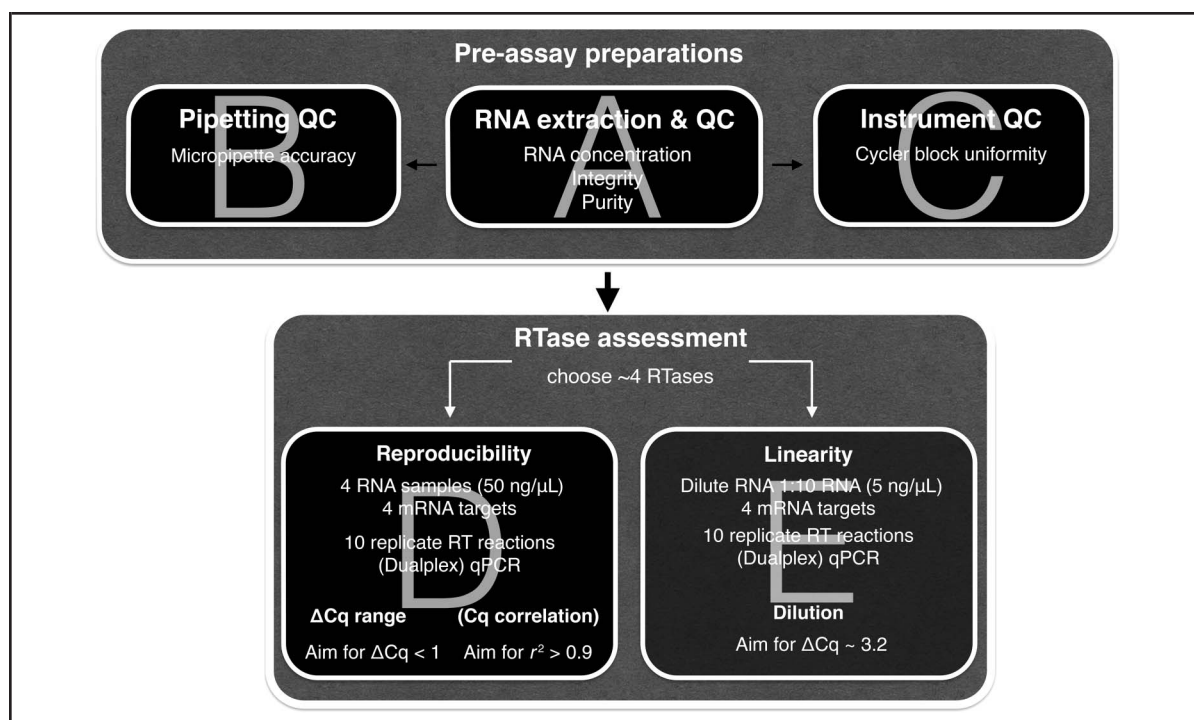


Fig. 6. Suggested workflow for initial selection of most appropriate RTase.

Steps (A)–(C) are carried out before cDNA synthesis is carried out. Steps (D) and (E) involve selection and optimizing of reagents for cDNA synthesis. (A), Careful extraction and quality control of RNA are 2 essential initial steps. It is worth bearing in mind that the less RNA is used in the RT step, the higher the variability is likely to be. (B), The use of calibrated micropipettes of suitable volume and with appropriate tips is essential to ensure pipetting accuracy. (C), Once-yearly instrument QC is advisable, as lack of uniformity across a block will result in distorted and inaccurate results. (D), Reproducibility is tested by analyzing the ΔCq range of 4 RNA targets, aiming to pick an RTase that consistently records ΔCq values of < 1 . If probes are being used, the correlation between targets should also be assessed, and r^2 values should ideally be > 0.9 . (E), Linearity is assessed by diluting RNA and repeating the experiments carried out for (D). In addition, the ΔCq between dilutions should correspond to the dilution factor (i.e., 3.2 for a 1:10 dilution). It is important to note that even careful selection of RT cannot guarantee optimal efficiency and linearity for every sample or target analyzed.

different integrity values. The data confirm the view that a universal RIN value provides no definitive information about the integrity of individual mRNA species, and hence must be treated with caution. They also suggest that variability for RNA samples is not constant and that minimal variability and high correlation between markers in 1 sample cannot be extrapolated to other samples. This irregularity is also apparent when the correlation is analyzed between *CDH1* mRNA and 2 mRNAs specifying proteins CTNNB1 and MAX, previously reported as being associated with *CDH1* (26, 27). The correlation between *CDH1* and *CTNNB1* mRNA is much better than between *CDH1* and *MAX*, with respective correlation coefficients of 0.96 and 0.83 and 95% CIs of 0.93–0.98 and 0.69–0.91 (see online Supplemental Tab 12).

Expression levels of genes of interest are usually reported as fold changes relative to those of 1 or more

reference genes. The results reported in online Supplemental Tab 9 were used to calculate the expression levels of *CDK2*, *RBL1*, *MAX*, and *MYC* relative to the reference gene *UBC #1* (see online Supplemental Tab 15). It is apparent that the relative fold changes are sample and assay dependent, as 5 of 16 assays (31%) record fold changes that are < 2 -fold, another 7 (44%) have changes that are between 2- and 3-fold, and 4 (25%) record fold changes > 3 -fold, with a maximum of nearly 7-fold. This sample- and target-dependent variability is further emphasized by the results shown in Fig. 5, which imply that small fold changes for *CDK2/RBL1* can be reliably quantified, whereas the much greater differences in fold change for *MAX/MYC* suggest that small fold changes for these markers cannot be reliably quantified.

The previously documented variability of the RT step (6, 7) is acknowledged in the MIQE guidelines,

which require a detailed description of the protocol and reagents used to convert RNA into cDNA (22). Our results extend the earlier data by demonstrating that the RT step is defined by its dependence on enzyme/buffer formulation, sample, assay, and RNA concentration, that this dependence results in significant variability in RT efficiency, and that this variability is inconsistent. For these reasons, it is essential that the reporting of results involving cDNA syntheses is as transparent and complete as possible and includes the amount of RNA reverse-transcribed, priming strategy, enzyme type, volume, temperature, and duration of the reverse transcription step. Our data also provide further support for the suggestion that the reverse-transcription step be carried out in duplicate or triplicate (22, 28). Unfortunately, the research community continues to disregard the existence of RT variability, and this recommendation remains unheeded.

In addition, there is a lack of transparency in the reporting of experimental detail (15), which calls into question the reliability of conclusions on the basis of data obtained from RT-qPCR (16, 29). The implications with regard to reproducibility, robustness, and accuracy of RTase-based assays are clear. Most published RNA biomarkers report small fold changes between healthy and diseased samples, with the vast majority between 2- and 8-fold (for example, (30–37)). However, virtually none turn out to be of clinical significance (38), and around 80% of the results cannot be reproduced independently (39), likely because of serious technical issues (2), consistent with an estimate that around 85% of research funding is wasted (40). For the first time, we demonstrate that the variability of the RT step is 1 reason for this, as the variability inherent in the RT step is well within the range reported for most biomarkers (see online Supplemental Tab 16) and so calls into question many results published with regard to RNA-based biomarkers.

These conclusions support earlier observations; however, neither the complexity and unpredictability of RT variability nor the impact of these factors on data interpretation and consequences have been demonstrated in this detail before. Although the conclusions arising from the previous publications are being universally ignored, it is essential that this information be reintroduced into the consciousness of the research community. A simple workflow designed to identify the most appropriate RTase for any sample/target combination is presented in Fig. 6. This is of particular importance when considered alongside the increasing speed of technological development that makes it more and more difficult to conduct detailed troubleshooting or quality assessment of the enormous amounts of published data.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors' Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:

Employment or Leadership: T. Nolan, Sigma Aldrich.

Consultant or Advisory Role: G.L. Shipley, Roche Applied Science.

Stock Ownership: None declared.

Honoraria: None declared.

Research Funding: S.A. Bustin, two grants from the University Faculty of Health, Social Care, and Education Support Fund.

Expert Testimony: None declared.

Patents: None declared.

Other Remuneration: S.A. Bustin, Bio-Rad, Takara, TATAA Biocentre, Bioline, Sigma, and Life Technologies provided RT enzymes free of charge.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

1. Sewall CH, Bell DA, Clark GC, Tritscher AM, Tully DB, Vanden Heuvel J, et al. Induced gene transcription: implications for biomarkers. *Clin Chem* 1995;41:1829–34.
2. Bustin SA, Murphy J. RNA biomarkers in colorectal cancer. *Methods* 2013;59:116–25.
3. Freeman WM, Walker SJ, Vrana KE. Quantitative RT-PCR: pitfalls and potential. *Biotechniques* 1999;26:112–5.
4. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol* 2002;29:23–39.
5. Chandler DP, Wagnon CA, Bolton HJ. Reverse transcriptase (RT) inhibition of PCR at low concentrations of template and its implications for quantitative RT-PCR. *Appl Environ Microbiol* 1998;64:669–77.
6. Stahlberg A, Hakansson J, Xian X, Semb H, Kubista M. Properties of the reverse transcription reaction in mRNA quantification. *Clin Chem* 2004;50:509–15.
7. Stahlberg A, Kubista M, Pfaffl M. Comparison of reverse transcriptases in gene expression analysis. *Clin Chem* 2004;50:1678–80.
8. Sanders R, Mason DJ, Foy CA, Huggett JF. Evaluation of digital PCR for absolute RNA quantification. *PLoS One* 2013;8:e75296.
9. Bustin SA, Nolan T. Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J Biomol Tech* 2004;15:155–66.
10. Nam DK, Lee S, Zhou G, Cao X, Wang C, Clark T, et al. Oligo(dT) primer generates a high frequency of truncated cDNAs through internal poly(A) priming during reverse transcription. *Proc Natl Acad Sci U S A* 2002;99:6152–6.
11. Perez-Novo CA, Claeys C, Speleman F, Van Cauwenberge P, Bachert C, Vandesompele J. Impact of RNA quality on reference gene expression stability. *Biotechniques*. 2005;39:52, 54, 56.
12. Vermeulen J, De Preter K, Lefever S, Nuytens J, De Vloed F, Derveaux S, et al. Measurable impact of RNA quality on gene expression results from quantitative PCR. *Nucleic Acids Res* 2011;39:e63.
13. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. *Nat Protoc* 2006;1:1559–82.
14. Bustin SA. Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences. *Expert Rev Mol Diagn* 2005;5:493–8.
15. Bustin SA, Benes V, Garson J, Hellems J, Hug-

- gett J, Kubista M, et al. The need for transparency and good practices in the qPCR literature. *Nat Methods* 2013;10:1063–7.
16. Dijkstra JR, van Kempen LC, Nagtegaal ID, Bustin SA. Critical appraisal of quantitative PCR results in colorectal cancer research: can we rely on published qPCR results? *Mol Oncol* 2014;8:813–8.
 17. Linden J, Ranta J, Pohjanvirta R. Bayesian modeling of reproducibility and robustness of RNA reverse transcription and quantitative real-time polymerase chain reaction. *Anal Biochem* 2012;428:81–91.
 18. Keilholz U, Willhauck M, Rimoldi D, Brasseur F, Dummer W, Rass K, et al. Reliability of reverse transcription-polymerase chain reaction (RT-PCR)-based assays for the detection of circulating tumour cells: a quality-assurance initiative of the EORTC Melanoma Cooperative Group. *Eur J Cancer* 1998;34:750–3.
 19. Miklos GL, Maleszka R. Microarray reality checks in the context of a complex disease. *Nat Biotechnol* 2004;22:615–21.
 20. Chagovetz A, Blair S. Real-time DNA microarrays: reality check. *Biochem Soc Transact* 2009;37:471–5.
 21. McIntyre LM, Lopiano KK, Morse AM, Amin V, Oberg AL, Young LJ, et al. RNA-seq: technical variability and sampling. *BMC Genomics* 2011;12:293.
 22. 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.
 23. Bhargava V, Head SR, Ordoukhanian P, Mercola M, Subramaniam S. Technical variations in low-input RNA-seq methodologies. *Sci Rep* 2014;4:3678.
 24. Bustin SA. Why the need for qPCR publication guidelines?—The case for MIQE. *Methods* 2010;50:217–26.
 25. Bustin SA. *A-Z of quantitative PCR*. La Jolla, CA: IUL Press; 2004.
 26. Hulsken J, Birchmeier W, Behrens J. E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. *J Cell Biol* 1994;127:2061–9.
 27. Batsche E, Muchardt C, Behrens J, Hurst HC, Cremisi C. RB and c-Myc activate expression of the E-cadherin gene in epithelial cells through interaction with transcription factor AP-2. *Mol Cell Biol* 1998;18:3647–58.
 28. Tichopad A, Kitchen R, Riedmaier I, Becker C, Stahlberg A, Kubista M. Design and optimization of reverse-transcription quantitative PCR experiments. *Clin Chem* 2009;55:1816–23.
 29. Bustin S. Transparency of reporting in molecular diagnostics. *Int J Mol Sci* 2013;14:15878–84.
 30. Wu X, Nguyen BC, Dziunycz P, Chang S, Brooks Y, Lefort K, et al. Opposing roles for calcineurin and ATF3 in squamous skin cancer. *Nature* 2010;465:368–72.
 31. Wang YK, Zhu YL, Qiu FM, Zhang T, Chen ZG, Zheng S, et al. Activation of Akt and MAPK pathways enhances the tumorigenicity of CD133+ primary colon cancer cells. *Carcinogenesis* 2010;31:1376–80.
 32. Plum L, Lin HV, Dutia R, Tanaka J, Aizawa KS, Matsumoto M, et al. The obesity susceptibility gene Cpe links FoxO1 signaling in hypothalamic pro-opiomelanocortin neurons with regulation of food intake. *Nat Med* 2009;15:1195–201.
 33. Koppikar P, Bhagwat N, Kilpivaara O, Manshoury T, Adli M, Hricik T, et al. Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. *Nature* 2012;489:155–9.
 34. Lardon J, Corbeil D, Huttner WB, Ling Z, Bouwens L. Stem cell marker prominin-1/AC133 is expressed in duct cells of the adult human pancreas. *Pancreas* 2008;36:e1–6.
 35. Liu Z, Shao Y, Tan L, Shi H, Chen S, Guo J. Clinical significance of the low expression of FER1L4 in gastric cancer patients. [Epub ahead of print] *Tumour Biol* June 26, 2014, as doi:10.1007/s13277-014-2259-4.
 36. Ou-Yang QH, Duan ZX, Jin Z, Lei JX. OLC1 is overexpressed in breast cancer and its expression correlates with poor patient survival. *Tumour Biol* 2014;35:8823–7.
 37. Liu T, Xia B, Lu Y, Xu Y, Lou G. TNFAIP8 overexpression is associated with platinum resistance in epithelial ovarian cancers with optimal cytoreduction. *Hum Pathol* 2014;45:1251–7.
 38. Macleod MR, Michie S, Roberts I, Dirnagl U, Chalmers I, Ioannidis JP, et al. Biomedical research: increasing value, reducing waste. *Lancet* 2014;383:101–4.
 39. Prinz F, Schlange T, Asadullah K. Believe it or not: how much can we rely on published data on potential drug targets? *Nat Rev Drug Discov* 2011;10:712.
 40. Chalmers I, Glasziou P. Avoidable waste in the production and reporting of research evidence. *Lancet* 2009;374:86–9.