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Quantitative PCR of Small Nucleic Acids: Size Matters

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Abstract

Quantitative dysregulation in small nucleic acids (NA), such as microRNA (miRNA), extracted from minimally invasive biopsies, such as, blood, stool, urine, nose, throat, are promising biomarker for diseases diagnosis and management. We quantify the effect of the extra step of poly(A) ligation for cDNA synthesis and small size of the NA on the limit of quantification (LOQ) of quantitative PCR (qPCR), the gold standard to measure copy number. It was discovered that for small NA, the cycle threshold, Ct that is proportional to −log[c], where [c] is the concentration of the target NA exhibits a sharp transition. The results indicate that although the limit of detection (LOD) of qPCR can be in femtomolar range, the LOQ is significantly reduced by well over three orders of magnitude, in picomolar range. Specifically, the study reveals that the PCR product length is the primary reason the limitation on LOQ and is explicitly shown to be an important consideration for primer design for qPCR in general.

Keywords

Intercalations; MicroRNA; Nucleic acids; Polymerase chain reaction; Reverse transcription

Introduction

Quantitative genomics to measure copy number of specific nucleic acid (NA) sequences in non-invasively obtained bio-specimens, i.e., liquid biopsy,[1] is rapidly coming to center stage[2] as a diagnostic and prognostic tool for cancer,[3] heart,[4] psychiatric,[5] and infectious[6] diseases, among others. The viral load of SARS-CoV2 in nasal swab samples by measuring copy number of viral RNA is central to discerning contagiousness and

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Authors Contribution

R.F.S and R.T. conceived the project and provided guidance on data analysis and interpretation. J.M.L. and R.T. performed experiments, analyzed, and interpreted data. R.F.S., R.T. and J.M.L. wrote the manuscript. R.F.S obtained the financial support for the project.

Supporting Information

Supporting information contains information on miRNA sequences, primers detail, limit of quantification (LOQ) and limit of detection (LOD) of qPCR, amplification and melt curves for Luciferase Control mRNA, miR-34a and miR-155 nucleic acids.

Supporting information for this article is available on the WWW under https://doi.org/10.1002/slct.202100807

Conflict of Interest

The authors declare no conflict of interest.

contact tracing.[7] Dysregulation in copy number of circulating noncoding RNA (ncRNA),[8] particularly, microRNA (miRNA), are shown to be effective biomarkers for cancer for early stage detection and disease management.[9] An important need emerging is quantification of small, less than ~50 nucleotide (nt) long NA's which does not have a poly(A)-tail present at the 3′-end for mRNA.

Quantitative Polymerase Chain Reaction (qPCR) is considered the gold standard for relative or absolute measurement of copy number of specific sequence in a biospecimen.[10] Complimentary DNA (cDNA) are synthesized for each NA molecule using a reverse transcriptase (RT) enzyme in a thermal cycler; followed by qPCR where the cDNA is replicated in a cyclic process.[11] The number of copies nominally double during each cycle controlled by periodic temperature excursion leading to amplification curves that are measured by fluorescence that is exclusively caused by a dye that intercalates in the PCR product.[11] In qPCR, the copy number of the original NA molecules is calculated from the number of cycles needed in the qPCR to obtain a threshold number of copies, cycle threshold Ct.[11] The Ct is proportional to −klog[N], where N are the number of target (cDNA) molecules in qPCR reaction mixture and k is the slope of the so called qPCR standard curve. From simple geometric growth at efficiency, E for each amplification cycle, $E=10^{(-1/k)}-1$, thus for E=1, k ~ −3.32.[12] The quantitative range of qPCR is defined as the range where the exponential relationship holds, i.e., k is constant. Furthermore, to obtain quantification of the copies in the original sample, i.e., number of copies in the RT mix to form cDNA, it is generally assumed that the efficiency of cDNA synthesis is close to 100%, i.e., for each copy of target NA one copy of cDNA is synthesized. An aspect of the study reported is quantitatively access the efficiency of NA to cDNA conversion.

Since the discovery of qPCR over 25 years ago,[13] continual effort on developing better algorithms to analyze the measured Ct to accurately and reliably quantify the copy number of NA is a testimony to the complexity and challenges.[14] For example, "kit-dependent" conditions lead to high variability and data bias making intra- and inter-lab results irreproducible causing great debate and frustration.[15] Thus, the need for standardization of PCR assay is well recognized.[16] There is ample evidence that the choice of primer is crucial in designing proper quantitative analysis by qPCR.[12] Here we focus on two additional consideration for qPCR analysis to quantify copy number particularly of small NA. The premise of the study is our central observation that the limit of quantification (LOQ) of small NA compared to (long) standard mRNA as a control, is orders of magnitude lower (discussed in Figure 2). To explain this drastic reduction in LOQ we identify A-tail ligation and qPCR amplicon length as two primary factors, where the second one is more significant and has not been explicitly realized. First, the ligation of poly(A)-tail that is essential for cDNA synthesis that is missing in small ncRNA, including a range of long noncoding RNA (lncRNA).[17] Second, the small size of the NA will limit the binding site of the primer molecules for amplification is roughly (only) 6–8 nt.[18] The length of the binding site may limit the specificity to differentiate miRNA families with only a few changes in bases,[19] that may not be in the primer binding site. The key finding is that the small product size during the qPCR amplification also called the amplicon, rather than the ligation step for cDNA synthesis, is the primary reason to significantly decrease the LOQ of qPCR by over three orders of magnitude. Although, our analysis is focused on SYBR Green based

method that are significantly more inexpensive and pervasive than the TaqMan method,[20] the key observation on the limitations and recommendations are similar.

Results and Discussion

The result for the first consideration, effect of cDNA synthesis which includes A-tail ligation, is discussed. To quantify the effect of poly(A)-tail ligation on small NA, two approaches were adopted to relate the Ct value to the copy number (N) of starting ssDNA or RNA (Figure 1): Method ① (Target Dilution): The original stock, $N_1=10^{13}$ copies is diluted to different amounts, d_1N_1, where d_1 is dilution amount followed by cDNA synthesis (which includes poly(A)-tail ligation) at efficiency, η_1. The qPCR is performed on $(N_1d_1)\eta_1$. Method ② (cDNA Dilution): The cDNA is synthesized from $N_2=10^{13}$ copies in RT mix at efficiency, η_2, and then diluted by d_2. The qPCR is performed on $(N_2\eta_2)d_2$. (To note is that, for clarity, the order of the variables is to reflect the sequence of process steps). To quantitatively examine if the overall efficiency of poly(A)-tail ligation and cDNA synthesis changes at lower number of molecules in RT mix, η_1 and η_2 are compared by setting $N_1d_1=N_2d_2$. If the efficiency of A-tail ligation and cDNA synthesis is larger in cDNA dilution approach, i.e., $\eta_2 > \eta_1$, then Ct_1 will be larger than Ct_2, and vice versa. In case the cDNA conversion efficiency is unchanged for all number of molecules in RT mix, i.e., both methods have same efficiency ($\eta_1=\eta_2$), then, $Ct_1=Ct_2$.

The standard curves for miR-34a and miR-155, detailed in SI, Table S1, were measured by both the dilution methods (Figure 2). The standard curves for miR-34a and miR-155 in both RNA and DNA form are significantly different from Luciferase Control mRNA (as this is the only mRNA in our study, from now on, we will refer it as just ‘mRNA’) (Figure 2). The sequences of the primer pair targeting mRNA (Primer Set 4) is in SI, Table S2. The mRNA shows an expected standard curve with slope, k=−3.76 which is slightly lower than the expected range,[12] corresponding to E ~ 85%. The LOQ for mRNA defined as the lowest measurable copies in the exponential region was Ct ~ 35 corresponds to $N \sim 10^2$ copies in the RT mix which is consistent with the “best practice” methods.[21] In terms of concentration, the LOQ corresponded to [c] ~ 16.7 fM in the (starting) sample. Furthermore, as expected, the results are virtually identical for the two dilution approaches. The high coincidence for the two dilution approaches over eight orders of magnitude clearly indicates that for mRNA, $\eta_2=\eta_1=1$, i.e., the efficiency of cDNA synthesis is 100%. The corresponding melt curves for the qPCR shows the required single peak of the product at Tm ~ 84.8°C (SI Figure S1). Thus, the behavior for mRNA serves as a control indicating that the two dilution methods are equivalent and there are no spurious errors in sample handling and processing, such as, pipetting, NA storage, buffer exchanges between various processes.

For small NA targets, processed in parallel with mRNA on the same well plate, the standard curve is remarkably different from mRNA in two salient aspects (Figure 2): (i) the LOQ is significantly reduced by well over three to six orders of magnitude as signified by the large shift to the right of the exponential line; and (ii) the Ct value plateaus at lower NA target copies. Although the LOQ is low, the limit of detection (LOD), defined as the highest measurable Ct (SI, Figure S2) where the melt and amplification curves are (still) reasonable (SI, Figure S3–S6), was ~ 10^2 to 10^3 copies that is comparable to LOQ and LOD of

mRNA (Figure 2). Furthermore, the slopes of the standard curves are practical reflecting reasonable amplification efficiencies.[12] Importantly, the significantly lower LOQ due to shift and the plateau-like characteristics at high Ct were also observed for TaqMan method further supporting the two above-mentioned aspects for small NA (Figure 3). Although the LOQ for TaqMan was two orders of magnitude lower than SYBR Green method, it was still significantly lower than that for mRNA in Figure 2. The LOD for both the methods was comparable and similar to that for mRNA. Lastly, it should be mentioned that the tight error bars based on running each reaction in triplicate for each condition and one peak in melt curve indicate that artifacts due to processing and chemical contamination and any possibility of primer-dimer formation was unlikely for both mRNA and small NA's (SI, Figure S3–S6).

The cDNA synthesis has been recognized as a major source of error depending on the RNA quality[22] and kit utilized for the RT reaction.[23] However, for the pristine mRNA studied here, the characteristics for the two dilution processes for mRNA was virtually identical for over eight orders of magnitude, up to LOQ of $N \sim 10^2$ molecules indicating that cDNA synthesis was virtually perfect corresponding to same number of target molecules in the whole (target) concentration range. In contrast to mRNA, the standard curve for the two dilution methods is not coincident for small NA's (Figure 2). To quantitatively compare the discrepancy, Ct value for the known initial starting copy number (N_1 or N_2) times the respective dilution (d_1 and d_2) are compared, i.e., Ct values for $N_1d_1=N_2d_2$. If $\eta_1=\eta_2$ then the number of copies in qPCR mix, $(N_1d_1)\eta_1$ or $(N_2\eta_2)d_2$ will be the same leading to $Ct_1=Ct_2$. At lower dilution, indeed $\eta_1=\eta_2$; however, at larger dilution, Ct_1 for $N_1d_1\eta_1$ copies in the qPCR mix is larger than Ct_2 for $N_2d_2\eta_2$ copies implying, $\eta_1<\eta_2$ (Figure 4). The discrepancy suggests that when the efficiency for cDNA conversion decreases (from 100%) as the number of copies in the RT mix reduces. At the extreme, for $Ct_1 \sim 35$ the corresponding $Ct_2 \sim 30$ indicating a reduction in copy number of roughly 10-fold in the qPCR mix for the former (as per Figure 2(b) for miR-155 DNA). Although, the error of 10-fold is significant, it does not account for three to six order of shift in the standard curve compared to mRNA observed in Figure 2. Nevertheless, it should be noted that the cDNA synthesis efficiency for low copy number of small NA can decrease by 10-fold.

To explain the large shift in standard curve and consequently the LOQ for small NA's compared to mRNA, we consider the nature of primers. It is well documented in the literature that selection of the primer is critical in developing an optimum qPCR measurement.[12] However, the effect of primer selection that exclusively affects the length of the amplicon product rather than its location on the mRNA has not been reported. We chose primer set for mRNA to systematically affect the length of the qPCR product length (see SI, Table S2). For fixed number of mRNA copies in RT mix, the resulting Ct value changed significantly (Figure 5(a)). The number of copies in the RT mix was 10^{10} which was well in the exponential region of the standard curve (Figure 2). The sudden and remarkable increase in the Ct value for small product length may be explained by considering the fluorescence signal generation in the qPCR to determine the Ct value. SYBR Green measures dsDNA formation (i.e., amplicons) during qPCR reaction due to 10^3-fold enhancement in fluorescence on binding to the duplex.[24] Therefore, the (threshold) fluorescence signal that determines the Ct value will depend on the SYBR Green-dsDNA

binding per bp of the amplicon. For higher binding per bp of amplicon, fluorescence threshold will be obtained at low Ct value. The interaction of SYBR Green and dsDNA is by intercalation and minor groove.[25] The electrostatic minor groove binding site occupies about 3–4 bases[25] which may be unaffected by binding per bp of amplicon. However, due to well-known requirement of duplex unwinding to accommodate intercalation in the π-π stacks of bases,[26] the effective inclusion of number of SYBR Green will be limited as the size of the amplicon reduces. The latter is the well-studied neighbour-exclusion principle.[27] As a result, the binding of SYBR Green per dsDNA base-pair, and hence the fluorescence, from small duplex chains will be lower than longer chains. From Figure 5(a), for product length >50 bp the intercalation and binding is adequate to efficiently count the number of duplex formations. However, below 50 bp the efficiency starts to rapidly decrease attributed to neighbor-exclusion requiring more number of cycles to make adequate product for substantial increase in fluorescence leading to higher Ct value. The corresponding melt curve indicates single product formation with no side reactions, such as primer-dimer effects (Figure 5(b). The melt curve for longer products, P3 to P6 for product length >50 bp falls within T_m of within 80–85°C (Figure 5(b)) that is comparable Luciferase Control mRNA at T_m of ~ 84.5°C (SI, Figure S1). The no particular trend in T_m as a function of product sizes is attributed to non-systematic differences in GC content and product length. However, the shorter product lengths, have a T_m of 73.2°C (40 bp) and 74.8°C (44 bp) (Figure 5(b)) which resemble the melt-curves and Tm values of miR-34a (T_m of ~75°C in SI, Figures S3 and S4) and miR-155 (T_m of ~73°C in SI Figure S5 and S6). This indicates that the shorter product length limits the miRNA amplification. Importantly, the higher Ct value for smaller product size leads to significant shift right of the standard curve for larger product size of mRNA. Furthermore, plateau region begins to appear at high Ct value for smaller primer length, P1 primer set (Figure 5(c)). Thus, we can reasonably attribute the lower LOQ and plateau effect observed in Figure 2 for small NA to the small qPCR product length. Although not explicitly mentioned in the study, the significant improvement in sensitivity by ligating artificially longer, such as stem-loop chains to miRNA,[18] may be attributed to the chain length effect discovered in this study.

Conclusions

The standard curves of synthetic small NA (~22–25 nt) and reference Luciferase Control mRNA were measured. The standard curves were compared for samples of known dilution of the target NA before cDNA synthesis (i.e., target dilution) to samples where synthesis of cDNA at high concentration was followed by dilution after the RT reaction (cDNA dilution). The standard curve of long mRNA exhibited the expected behavior with LOQ of ~ 100 copies in the RT mix. The standard curve for target or cDNA dilution was virtually identical over eight orders of magnitude indicating that efficiency of cDNA synthesis from target mRNA was virtually 100%. The qPCR behavior for small NA's was remarkably different. First, the standard curve significantly shifted to the right with respect to mRNA by three to six orders of magnitude leading to well over 10^3-fold higher LOQ than for mRNA. Second the standard curve exhibited a plateau at low concentrations. Third the target and cDNA dilution were different such that at low target concentrations leading to Ct below 20, the efficiency of cDNA synthesis was significantly reduced. The cDNA copies were 10-fold

fewer when the Ct for target dilution was about 35. The inferior performance of qPCR on small NA was explained by considering the size of the amplicon in qPCR reaction. In a unique set of experiment, it was observed that if the qPCR product (i.e., amplicon) is below ~50 bp, the LOQ reduces by 10-fold even for mRNA. The effect is attributed to neighbor-exclusion principle. For small qPCR product, a plateau behavior in mRNA similar to small NA is observed. We conclude that while designing the primer sequences, it is important to ensure the length of the qPCR product is above 50 bp. Therefore, for small NA analysis the cDNA chain length should be long to obtain low LOQ. However, as the ligation chain length increases, the efficiency of cDNA synthesis may decrease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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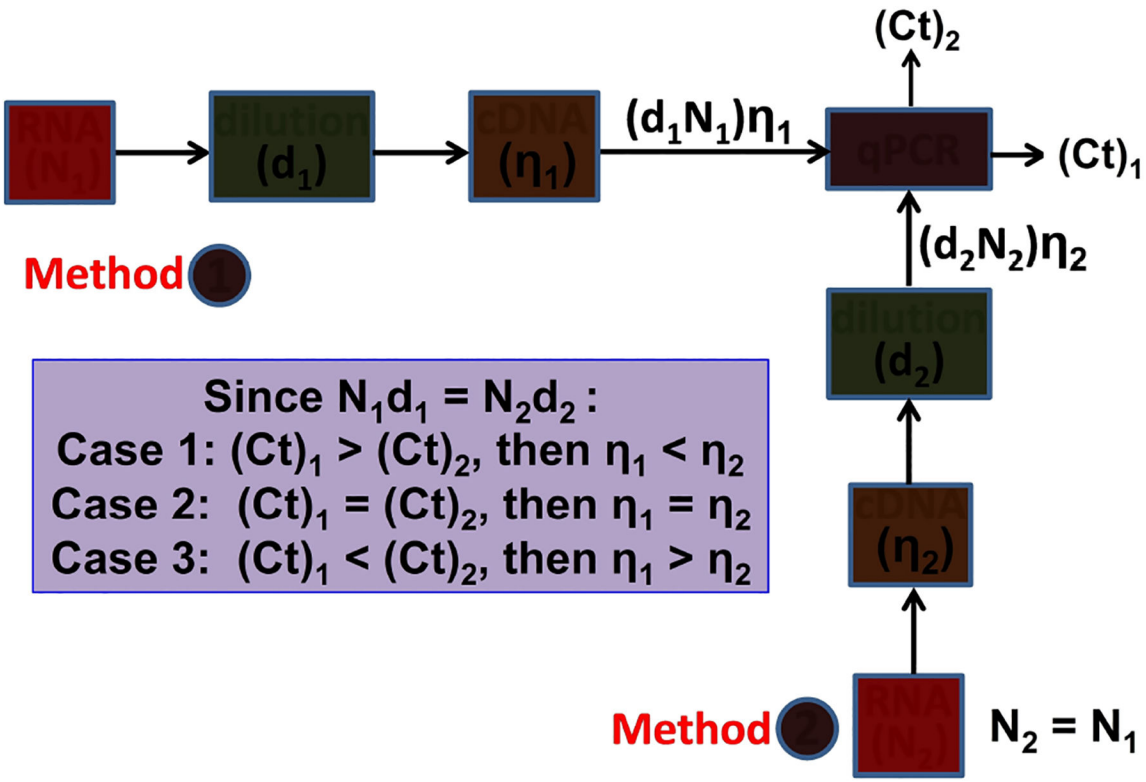


Figure 1. Flow chart for cDNA synthesis and qPCR analysis.

The copy numbers are measured by two methods: Nucleic acid dilution (Method ①); and cDNA dilution (Method ②).

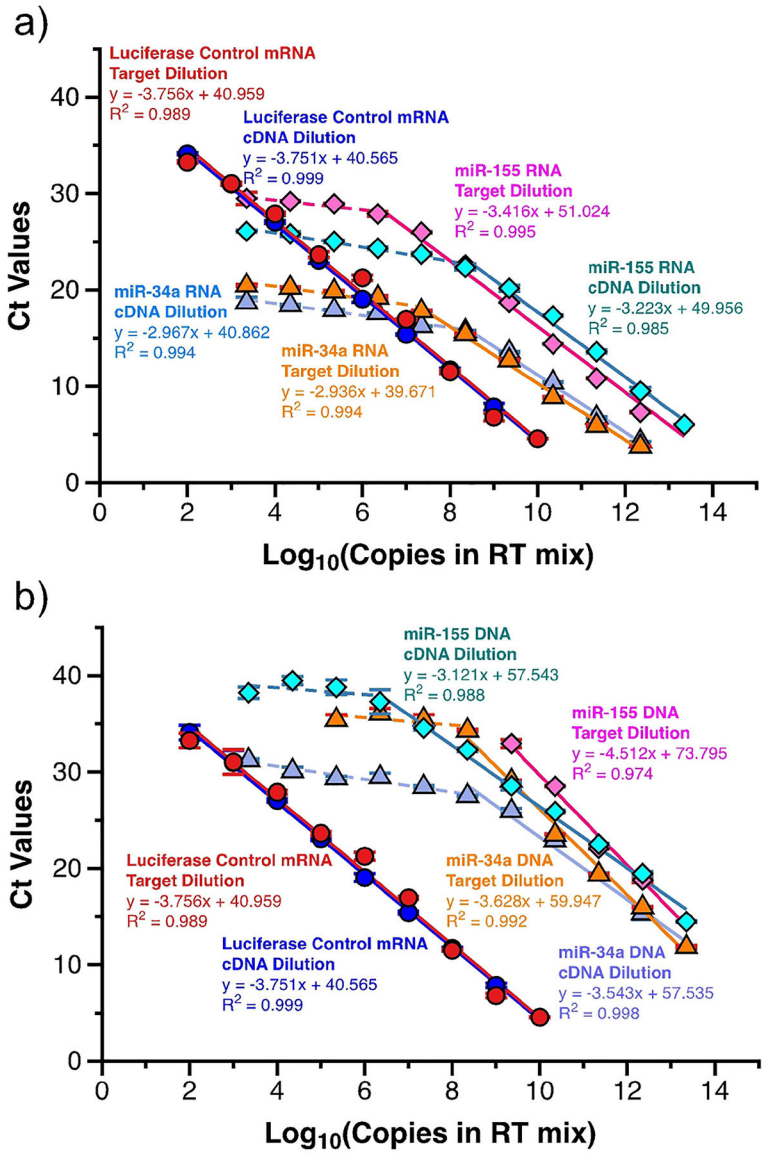
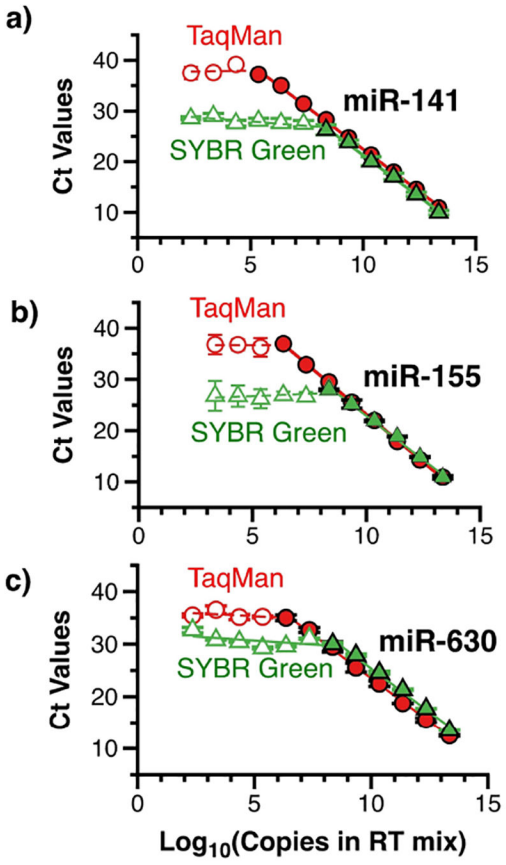


Figure 2. Comparison of standard curves for NA studied.

Standard curves based on a) RNA form, and b) DNA form of respective miRNA. Control is standard curve of Luciferase mRNA. Based on Figure 1, the number of copies in RT mix for Method ① and Method ② were N_1d_1 and N_2 for the two methods. Thus, to normalize for dilution effect in Method ②, the number of equivalent copies in the RT mix are shown, i. e., N_1d_1 and N_2d_2.



d)

Fit Parameters	TaqMan	SYBR Green
miR-141		
Slope	-3.344	-3.302
Intercept	55.875	54.300
R^2	0.998	0.997
LOQ (Copies in RT mix)	10^5	10^7
LOD (Copies in RT mix)	10^2	10^2
miR-155		
Slope	-3.734	-3.416
Intercept	60.544	57.024
R^2	0.999	0.995
LOQ (Copies in RT mix)	10^6	10^8
LOD (Copies in RT mix)	10^3	10^3
miR-630		
Slope	-3.319	-3.332
Intercept	56.715	58.68
R^2	0.998	0.991
LOQ (Copies in RT mix)	10^6	10^8
LOD (Copies in RT mix)	10^2	10^2

Figure 3. Standard curves for miRNA using TaqMan method.

(a) to (c) The standard curve for the microRNA for TaqMan and SYBR Green method are compared. (d) Table showing the parameters from the exponential fit and the LOD.

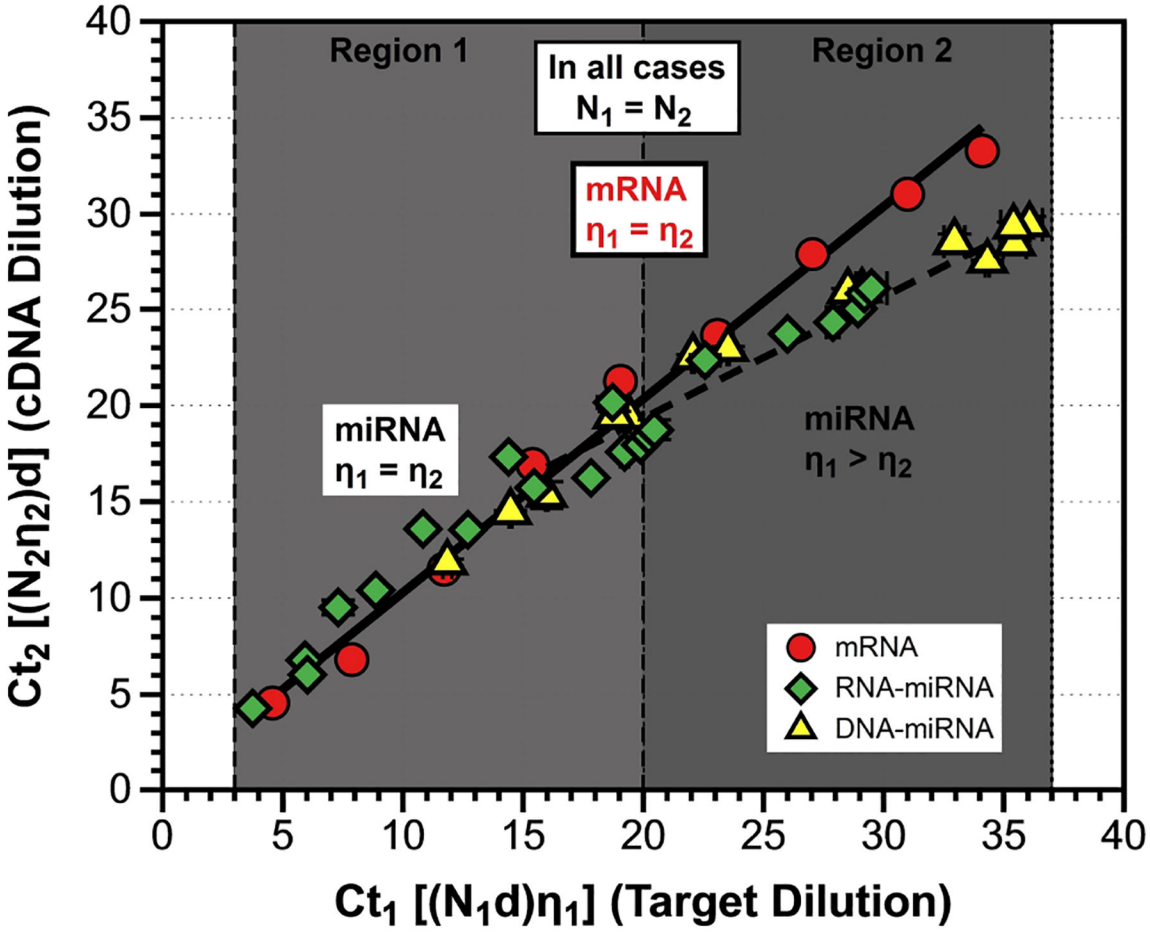
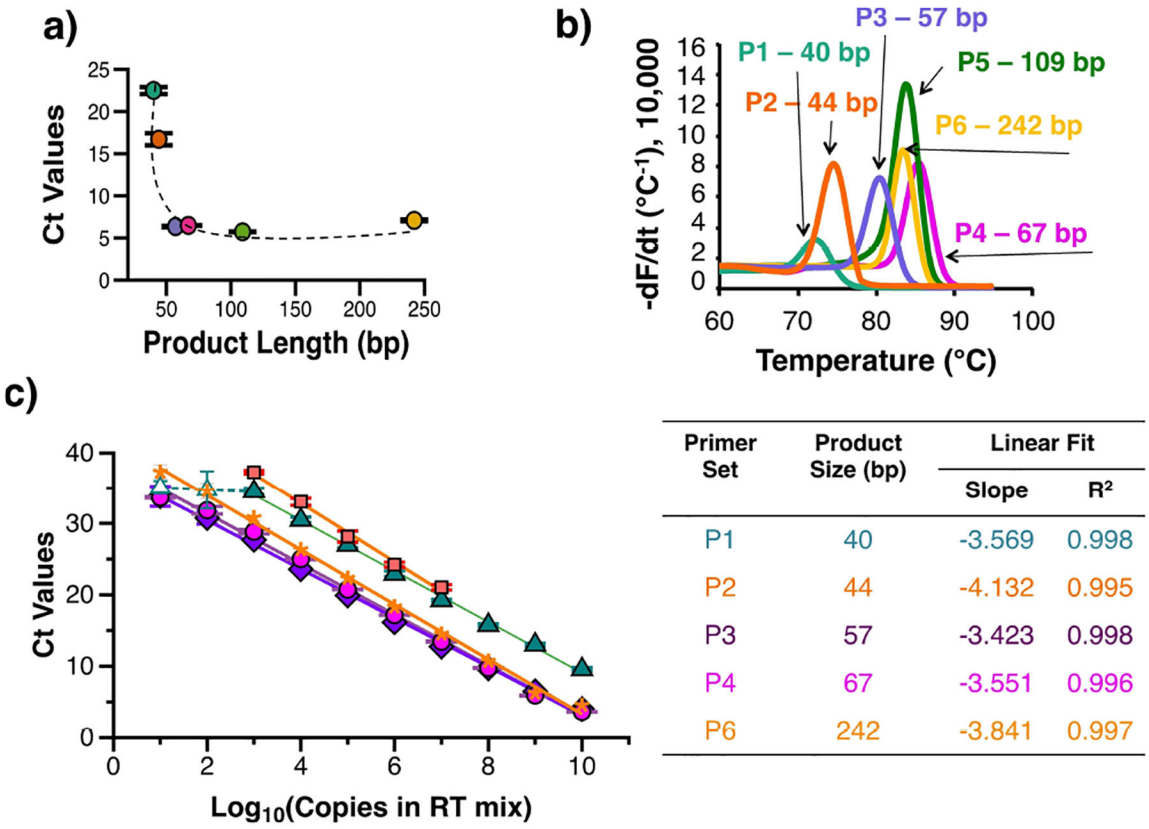


Figure 4. Effect of dilution on Ct value.

From Figure 1, the number of copies in qPCR mix to measure Ct value for Method ① and Method ② were, $N_1d_1\eta_1$ and $N_2\eta_2d_2$. Each data point in the plot is for Ct_1 and Ct_2 corresponding N_1d_1 and N_2d_2, respectively. Thus, points for $\eta_1=\eta_2$ will be on the diagonal.



Primer Set	Product Size (bp)	Linear Fit	
		Slope	R^2
P1	40	-3.569	0.998
P2	44	-4.132	0.995
P3	57	-3.423	0.998
P4	67	-3.551	0.996
P6	242	-3.841	0.997

Figure 5. Effect of qPCR product length on Ct value.

(a) For a fixed amount of Luciferase Control mRNA. (b) The corresponding melt curve of amplified products in (a). P4 and P6 shows Tm ~ 73–75 °C, similar to Tm of miRNA amplification (see Figure S3–S6, SI). (c) The corresponding standard curve for primer sets P1 to P5 (see Table S2, SI).