Measuring "Truth": Disparities in RNA Quantitation by Two-Step Reverse Transcription-Droplet Digital PCR (RT-ddPCR)

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INTRODUCTION

Digital PCR, including droplet digital PCR, is widely accepted as a gold standard approach for absolute quantitation of DNA. Its usage for sensitive and specific detection of DNA has increased in a wide range of biological applications, including food and water quality testing, and medical diagnostics. In the clinic, digital PCR is especially useful for defining allelic copy number variation and rare gene mutation detection, due to its sensitivity advantages over qPCR.[1,2]

A variety of pathological states can be defined and tracked via transcriptomic changes, therefore digital PCR for disease detection and monitoring of RNA could also offer significant advantages. However, an ongoing challenge lies in the uncertainty in reverse transcription (RT) chemistry[2,3] – the initial reaction step to convert RNA to cDNA as prerequisite for PCR amplification. There is a wide variety of commercially available RT kits, but inadequate data on how they perform with respect to accurate and precise quantitation of transcripts. This, coupled with a lack of primary RNA reference standards present significant obstacles to fully leveraging digital PCR for transcript quantitation.

In this study, we performed ddPCR on cDNAs generated from synthetic *ABL1* transcript using a dozen commercial RT kits, and observe considerable differences in RNA quantitation. To provide a reference point, we compared UV-spectrophotometry (UV-spec) measured quantities of purified *ABL1* RNA to RT-ddPCR measurements, and through RT optimization and reproducibility studies, narrow down to several RT kits that most closely align with UV-spec quantitation. We further demonstrate some differences in RNA quantitation by RT-ddPCR using the same *ABL* assay on different synthetic purified transcripts. Our results show that estimation of RNA levels via RT-ddPCR is largely governed by the choice of RT kit/enzyme, alterations to RT chemistry, and potentially sequence-specificity of transcripts. Approaches aimed at optimizing RT chemistries and components for greater reproducibility and utility for RT-ddPCR are strongly urged before adoption of RT-ddPCR for clinical use.

METHODS

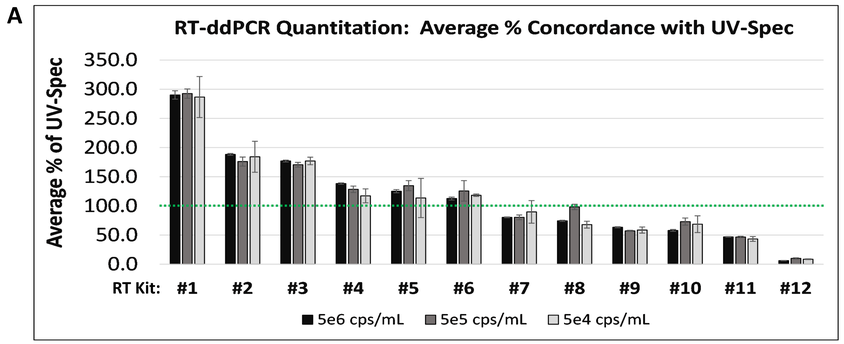
ABL1 synthetic transcript was generated via in vitro transcription, purified, quantified by A260/A280 UV-spec, and serially diluted to a concentration range between $1x10^3$ to $5x10^6$ copies/mL (cps/mL). One-time use aliquots of the serially diluted *ABL1* RNAs were then stored at -70°C and used within three months. For Figure 4, these same methods were used to generate NPM1 Type A mutant transcript, and BCR-ABL (p190 or p210) fusion transcripts with the same *ABL1* assay sequence downstream.

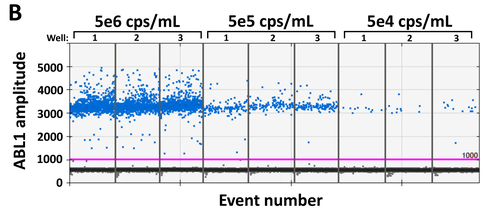
ABL1 RNA was converted to cDNA using 12 different RT kits from 9 companies, amplified using the same lot of ddPCR kit (Bio-Rad, ddPCR Supermix for Probes (No dUTP); cat.#186-3024) and analyzed using the QX200 Droplet Digital PCR System (Bio-Rad). For Figure 1B, the scatterplot was generated using QuantaSoft Analysis Pro (Bio-Rad, version 1.0.596). All other graphs and data shown were generated in Microsoft Excel.

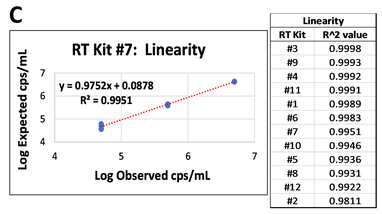
For reproducibility studies (Figure 3), two different operators used four RT kits of the same manufactured lots as those used to produce data in Figures 1 & 2. Each operator generated cDNAs from *ABL1* RNA on the same day, while ddPCR testing was performed over multiple days.

RESULTS

Figure 1. Evaluation of commercially available RT kits through cDNA quantitation comparison with UV-spectrophotometry (UV-Spec). (A) A dozen RT kits were purchased and *ABL1* transcript was converted to cDNAs using each kit based on suggested protocols by the company. Differences of nearly two-fold (or greater) in calculated starting RNA quantity relative to UV-spec were observed for nearly half of kits used in this study. Five RT kits (kits #4-8) demonstrated greater agreement with UV-spec and ranged from -25% to +25% of UV-spec measurements. **(B)** Representative ddPCR scatterplot (RT Kit #6) with triplicate-well droplet reads of 5e6, 5e5 and 5e4 copies/mL *ABL1* RNA. All kits showed similar droplet signal amplitudes and distributions. **(C)** (*Left panel*) Example simple linear regression plot of the *ABL1* dilution series for a representative ddPCR dataset from one RT kit, and (*right panel*) list of R^2 Pearson's correlation coefficient for all ddPCR serial dilution datasets from all RT kits.

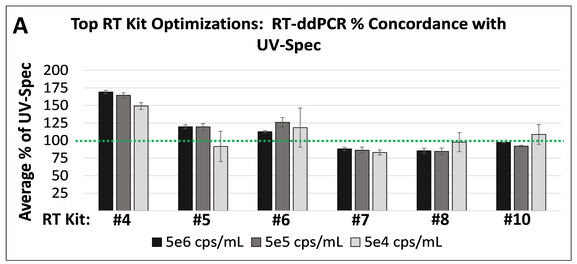






Linearity	
RT Kit	R^2 value
#3	0.9998
#9	0.9993
#4	0.9992
#11	0.9991
#1	0.9989
#6	0.9983
#7	0.9951
#10	0.9946
#5	0.9936
#8	0.9931
#12	0.9922
#2	0.9811

Figure 2. Alterations to reaction chemistries of reverse transcription kits can sometimes increase ddPCR quantitation agreements with UV-spec. (A) Changes to RT kit reactions included titrating $MgCl_2$ and RT enzyme concentrations, as well as various thermocycling parameters. From these studies, 5 RT kits (same lots of kits used in Figure 1) resulting in +/- 20% alignment with UV-spec were chosen for reproducibility studies. **(B)** Table of average number of positive ABL1 signal droplets observed starting from 5e4 cps/mL *ABL1* RNA, as measured by UV-spec.



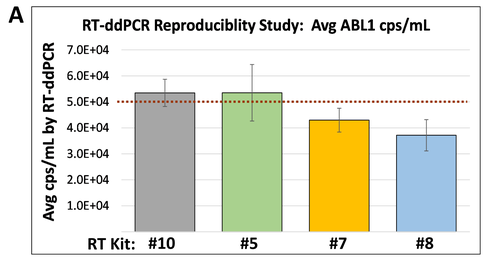
B

5e4 cps/mL ABL1 RNA

RT Kit	Avg # Droplets	StDev	%CV
#4	73.0	2.6	3.6
#5	45.7	9.9	21.6
#6	136.0	19.3	14.2
#7	40.3	5.5	13.7
#8	47.3	13.2	27.9
#10	55.0	2.6	4.8

RESULTS

Figure 3. Reproducibility studies of four RT kits showing closest proximity to UV-spec measurements after RT optimizations. (A) (*Upper graph*) The 5e4 cps/mL concentration of *ABL1* transcript was chosen for reproducibility experiments (see Methods) with 4 RT kits. RT-ddPCR measurements from 3 kits showed high agreement with UV-spec. (*Lower table*) Average cps/mL *ABL1* RNA back-calculated from RT-ddPCR, % agreement with UV-spec quantitation, and variation. **(B)** Linear regression of sensitivity studies suggest 1e4 cps/mL RNA as a reliable quantitation limit of 3 RT kits closely aligned with UV-spec. From left to right, datasets represent 1e3, 5e3, 1e4 and 5e4 cps/mL *ABL1* RNA. Some datapoints at the 1e3 cps/mL level are missing due to detection dropout.



Kit	Avg cps/mL by RT-ddPCR	Avg % of UV-Spec	StDev	%CV
#10	5.3475E+04	106.95	10.47	9.79
#5	5.3507E+04	107.01	21.75	20.32
#7	4.2967E+04	85.93	9.20	10.70
#8	3.7142E+04	74.28	12.06	16.23

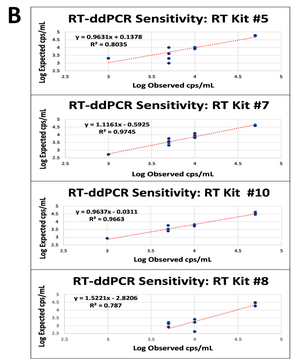
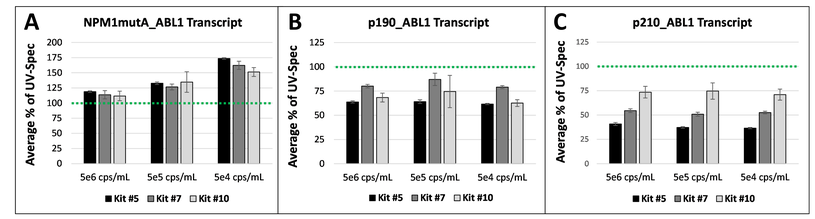


Figure 4. RT-ddPCR quantitation using the same ABL1 assay on three different synthetic RNAs reveals transcript-to-transcript differences. RT-ddPCR was performed on 3 different synthetic transcripts sharing the same ABL1 sequence and assay region towards their 3' end. The 5e6, 5e5, and 5e4 cps/mL RNA concentrations, as estimated by UV-spec, were converted to cDNAs via 3 different RT kits and ddPCR was performed using the same ABL1 probe/primers set. RT-ddPCR back-calculated cps/mL as % of UV-spec are plotted for **(A)** NPM1 Type A mutant transcript (NPM1mutA), **(B)** BCR-ABL p190/e1a2 fusion transcript, and **(C)** BCR-ABL p210/b3a2 fusion transcript.



CONCLUSIONS

- Despite evaluation of identical ABL1 transcript, we found remarkable variability among RT kits, which warrants caution for the selection of suitable RT enzymes for RT-ddPCR.
- Optimization of RT reaction chemistries occasionally improved congruence of RT-ddPCR with UV-spec measurements.
- Sensitivity studies propose RT-ddPCR concentration limits of 10 RNA copies per microliter.
- Variations in RT-ddPCR quantitation of different transcripts diluted to the same concentrations by UV-spec suggest sequence composition and/or RNA structure largely contribute to disparities in RT-ddPCR, which may resolve via further RT optimizations.

References

[1] Whale AS, Hugget JF, Cowen S, et al. Comparison of microfluidic digital PCR and conventional quantitation PCR for measuring copy number variation. *Nucleic Acids Res*, 2012; 40:e82.

[2] Hugget JF and The dMIQE Group. The digital MIQE guidelines update: Minimum information for publication of quantitation digital PCR Experiments for 2020. *Clin Chem*, 2020; 66(8):1012-1029.

[3] Levesque-Sergerie JP, Duquette M, Thibault C, et al. Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR. BMC Mol Biol, 2007; 8:93

[4] Bustin S, Dhillon HS, Kirvell S, et al. Variability of the reverse transcription step: Practical implications. *Clin Chem*, 2015; 61(1):202-12.