RT-qPCR Diagnostics: the "Drosten" SARS-CoV-2 Assay Paradigm

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Abstract: The reverse transcription quantitative polymerase chain reaction (RT-qPCR) is an established tool for the diagnosis of RNA pathogens. Its potential for automation has caused it to be used as a presence/absence diagnostic tool even when RNA quantification is not required. This technology has been pushed to the forefront of public awareness by the COVID-19 pandemic, as its global application has enabled rapid and analytically sensitive mass testing, with the first test targeting three viral genes published within days of the publication of the SARS-CoV-2 genomic sequence. One of those, targeting the RNA-dependent RNA polymerase gene, has been heavily criticised for supposed scientific flaws at the molecular and methodological level and this criticism has been extrapolated to doubts about the validity of RT-qPCR for COVID-19 testing in general. We have analysed this assay in detail and our findings reveal some limitations, but also highlight the robustness of the RT-qPCR methodology for SARS-CoV-2 detection. Whilst our data show that some errors can be tolerated, it is always prudent to confirm that primer and probe sequences complement their intended target, since when errors do occur, they may result in a reduction in the analytical sensitivity. However, in this case it is unlikely that a mismatch will result in poor specificity or significant number of false positive SARS-CoV-2 diagnoses, especially as this is routinely checked by diagnostic laboratories as part of their quality assurance.

Keywords: COVID-19; reverse transcription; qPCR; SARS-CoV-2; molecular diagnosis

1. Introduction

The reverse transcription quantitative polymerase chain reaction (**RT-qPCR**) has long been the research tool of choice for detection and quantification of a wide variety of RNAs [1]. Nonetheless, the problems associated with reproducibility caused by a range of parameters including workflow complexity, protocol differences as well as a diversity of analytical approaches and calibration as well as unitage issues led to an early recognition of the limitations of the technology as an aide to therapeutic decision making [2]. Moreover, operator- and reagent-associated variability also contribute appreciably to the reliability of RT-qPCR data [3]. There have been numerous publications addressing **PCR**-related challenges [4–11], culminating in the publication of guidelines to encourage better experimental practice to allow more reliable and unequivocal interpretation of qPCR results [12–14]. Uptake of these recommendations has been patchy, at best [15–17], and until recently little notice was taken of the significant challenges posed by poor assay design, lack of optimisation and validation and variable data interpretation [18]. One area where **RT-qPCR** based diagnosis and reproducible quantification guiding therapeutic decisions has been successful is that of infectious diseases [19] and **RT-qPCR** has become the first line diagnostic test for many different microorganisms [20], including viruses using a range of chemistries, instruments and protocols [21–26].

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the aetiological agent for coronavirus disease in 2019 (**COVID-19**) has enhanced the role of **RT-qPCR** as an essential tool for the early diagnosis of this disease, but also highlighted the previously ignored challenges [27]. Initial difficulties have been early and openly acknowledged [28], especially with regards to the

divergence of performance between assays, RT-PCR kits and laboratories [29–31] and as the emergence of variants refractory to amplification by a commercial assay [32]. However, a substantial body of opinion has emerged, propagated mainly through the press and social media, maligning its use and questioning the validity and utility of RT-qPCR-based testing, in particular. This is in part due to criticism of the first such test, which was released with remarkable alacrity only a day or so after the viral genomic sequence was made available and published in a peer-reviewed journal a week later [33]. This test consisted of three assays targeting genes specifying the RNA-dependent RNA polymerase (RdRp), envelope small membrane protein (E) and nucleoprotein (N). The reverse primer sequence of the RdRp assay contains an incorrect degenerate base S, defined as C or G [34], whereas the SARS-CoV-2 RNA sequence at that position is a T. Whilst the amplification efficiency of the RdRp assay in question can be 100% [35], the quantification cycles (Cq) are higher when compared to a range of other SARS-CoV-2-specific assays [34–37] affecting the sensitivity, but not the specificity of the assay [38]. Ironically, the current debate with regards to the interpretation of RT-qPCR test results does not focus on sensitivity but on the clinical relevance of detecting the virus, often at very low copy numbers. Nonetheless, given the importance of maintaining confidence in the ability of molecular testing to detect SARS-CoV-2 accurately, reliably and sensitively, we have dissected the performance of the RdRp assay to determine just how well it performs and to remind the scientific community that mismatches between primers and target do not necessarily affect the performance of an assay.

2. Results

2.1. One step RT-qPCR

The results for the six assays (A-F) amplified with each of the five master mixes are shown in Figure 1, A-E). Amplification patterns are similar, with assays B (correct specific R primer) and D (correct specific F and R primers) consistently recording the lowest Cqs. Interestingly, combining the specific F primer ALT Fsp with the mismatched reverse primer resulterd in an assay (C) that performed less well than the original assay A (ΔCq=0.88 (95% CI 0.71 to 1.05)). Substituting specific primers with wobble primers (assays E and F) also resulted in poorer performance with all five master mixes. Replacing the original mismatched RdRp SARSr-R primer with the correct, specific primer ALT-Rsp increased the sensitivity of assay B by around 4-fold (95% CI 3.05-5.2, with ΔCqs of -1.96±0.66, -2.05±0.09, -1.45±0.26, -1.5±0.11 and -2.5±0.95 for PrimeScriptIII, Novaprime, Toughmix, Taqpath and Luna, respectively (Figure 1F). All Cqs are listed in the supplementary data file tab 2.
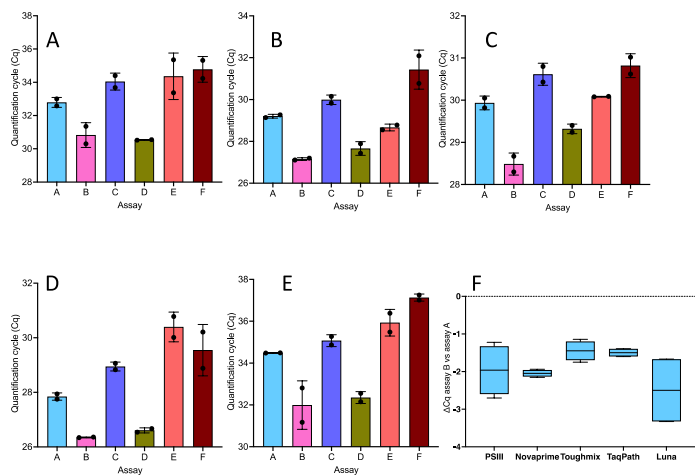
Figure 1. Performance of the six assays listed in Table 2 (A: original assay; B: original forward and correct specific reverse primers; C: correct specific forward and original reverse primers; D: correct specific primers; E: original forward and correct wobble reverse primers; F: correct specific forward and wobble reverse primers). Each of the assays was used with different one-step RT-qPCR different master mixes. **A.** Takara PrimeScript III. **B.** GSD Novaprime. **C.** Quanta 1-step Toughmix. **D.** ABI TaqPath. **E.** NEB Luna. Plots are shows as bars with individual Cqs and standard deviations. Each replicate is an independent RT-qPCR reaction. **F.** Effect of replacing the incorrect original reverse porimer with a correct specific one as shown by the ΔCq values of assay B compared with assay A for the five master mixes. The box and whiskers plot shows the minimum and maximum ΔCq values, together with the median (solid line).

2.2. RT temperature effect

The effects of modifying the RT conditions were analysed using three 1-step master mixes: all six assays were reverse transcribed and amplified with GSD Novaprime, whilst PCRBio and Quanta Toughmix were used to assess assays A, B and D. Assay A with the mismatched reverse primer recorded lower Cqs at higher RT temperatures, although the increase in sensitivity was modest, with the PCRBio assay showing the smallest Cq range at 1.2 and NovaPrime the largest at 1.42 (Figure 2A). Between 42°C and 50°C, the Cq range was even smaller, for example PCRBio recording only a Cq difference of 0.5. Assays B and D were affected even less by the RT temperatures, with only the NovaPrime master mix showing a clear effect (Figures 2B and C). Whilst the PCRBio and Quanta Toughmix master mixes recorded similar results, GSD NovaPrime consistently recorded the lowest Cqs. All Cqs, together with those recorded for assays C, E and F amplified by the NovaPrime master mix, are listed in supplementary data file tab 3.
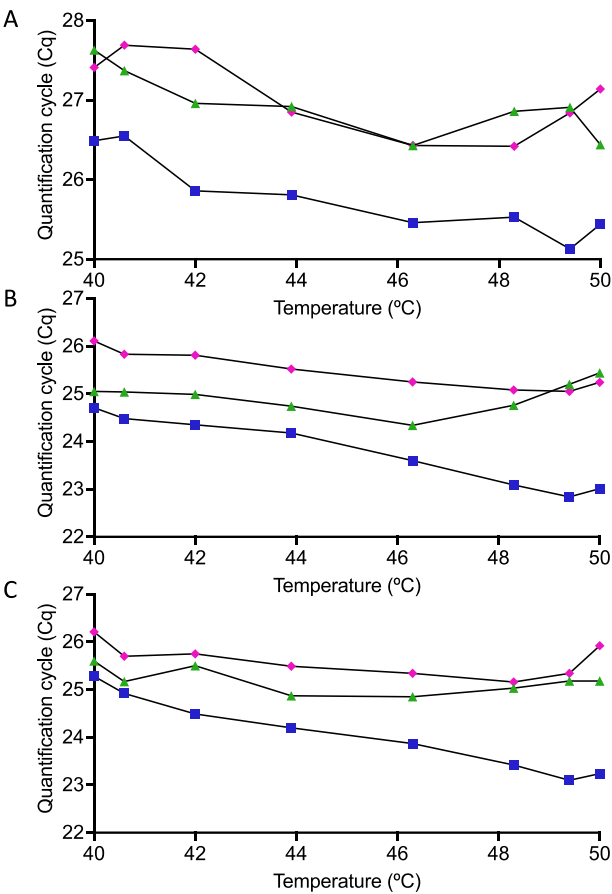
Figure 2. Effect of a reverse transcription temperature gradient on the sensitivity recorded by three assays amplified using different one step RT-qPCR master mixes. **A.** Assay A with original primers **B.** Assay B with corrected reverse primer. **C.** Assay D with specific, correct forward and reverse primers. Quanta Toughmix results are shown with purple diamonds, PCRBio results witg green triangles and GSD Novaprime results with blue squares.

2.3. Separate RT primed by random primers followed by qPCR

The relative contributions of the RT and qPCR steps were further investigated by reverse transcribed RNA using random primers with SS4 or iScript and subjecting the cDNA to qPCR amplification. This resulted in a rather different amplification pattern compared with each other as well as the 1-step methodology for both RTs (Figure 3A and B). First, the Cqs recorded by SS4-transcribed cDNA were consistently lower than those from iScript. Second, assays A and B gave broadly comparable results, with the ΔCqs similar at 0.93 (95% CI 0.26 to 1.59) for SS4 (Figure 3C) and 0.1 (95% CI -0.44 to 0.63) for iScript (Figure 3D). Third, assays A and B performed much worse with SS4 than assay D, recording a ΔCq$_{A:D}$ of 3.96 (95% CI -0.44 to 0.63) and ΔCq$_{B:D}$ of 3.04 (95% CI 2.42 to 3.66). In contrast, the ΔCqs with cDNA transcribed with iScript were 1.30 (95% CI 0.85 to 1.74), and 1.19 (95% CI 0.69 to 1.69) respectively. Finally, assays C, D, E and F recorded similar results with both cDNAs, suggesting that the presence of wobble bases in the primers did not affect the PCR reaction.

The near equivalence in the results obtained for assays A and B was corroborated by repeating the RT reactions for both RTases using a different RNA sample, this time carrying out eight separate RT reactions with SS4 and four with iScript. (Figure 3E). The SS4 results (ΔCq= 0.3 (95% CI 0.16 to 0.45)) were equivalent to the previous experiment, whereas the iScript results showed a slightly larger ΔCq (ΔCq=0.91 (95% CI=0.65 to 1.16)), but nevertheless in line with the previous results. All Cqs are listed in the supplementary data file tab 4.
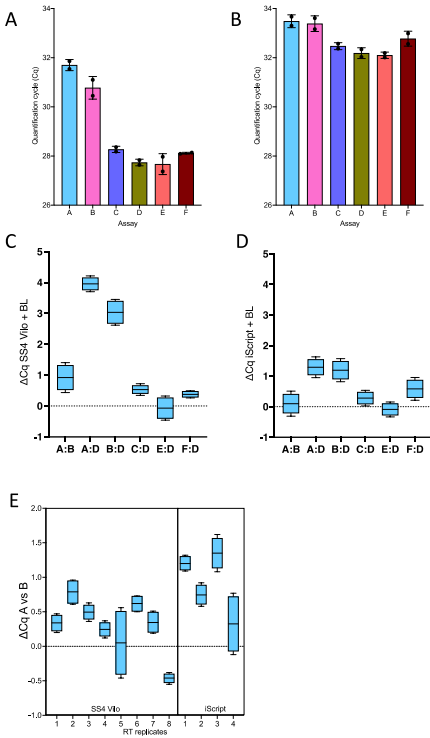
Figure 3. Two step RT-qPCR of the six assays listed in Table 2 with separate random priming of followed by amplification with SensiFast. **A.** Scatterplot with bars and SD for Cqs recorded with SS4 Vilo RT. **B.** Scatterplot with bars and SD for Cqs recorded BioRad iScript. **C.** Plot showing the frequency distribution of the SS4 Vilo ΔCq data, together with the median (solid line). A:B compares the original assay to the one with a correct specific reverse primer. The others compare each of the assays against D, which uses specific forward and reverse primers. **D.** Plot showing the frequency distribution of the iScript ΔCq data, together with the median (solid line) for the same assay combinations. **E.** Plot showing the frequency distribution of the 2x4 replicate SS4 Vilo and for replicate iScript ΔCq data, together with the median (solid line).

2.4. qPCR temperature effect

Compared with SS4 Vilo, the ΔCqs between assays A and D with the iScript-derived cDNA were unexpectedly small (3.83±0.31 vs 0.96±0.34). Hence qPCR analysis of both assays was repeated with another sample of cDNA synthesised by iScript. The first three cycles were carried out using a 45.0°C to 60.0°C annealing/polymerisation gradient, followed by standard amplification at 60°C, with either SYBR Green (Figure 4A) or RdRp_SARSr-P2 (Figure 4B) as reporters. In both cases, the Cqs recorded by assay A were comparable to those for assay D (Figure 4C) and, notably, despite the supposedly incompatible melting temperatures of the primers, both assays amplified equally well at all temperatures even above 58°C, with melt curves showing single amplicons (Figure A, insert). All Cqs are listed in the supplementary data file tab 5.
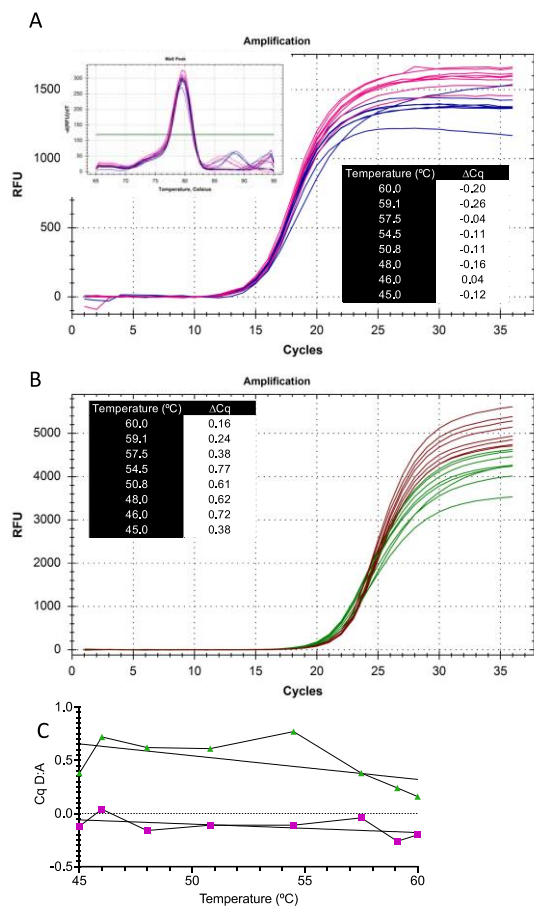
Figure 4. Effect of annealing temperature on RT-qPCR. cDNA synthesised by BioRad iScript was used to amplify assays A and D with Bioline SensiFast master mix. **A.** SYBR-green reported amplification plots and ΔCq values for assays A (blue) and D (pink), with the melt curves shown in the insert. **B.** Amplification plots and ΔCq values for assays A (green) and D (brown). **C.** Plot of ΔCq vs temperature with trend lines for the SYBR Green (pink) and probe-based (green) assays.

The performance of assays A and D at different annealing/polymerisation temperatures (58°C, 60°C, 62°C and 64°C, Figure 5A) was further investigated using four additional master mixes and a different qPCR instrument. Results for assay A were equivalent to those of assay D at an annealing/polymerisation temperature of 58°C for three of the master mixes, with the PrimerDesign, KAPA and Quanta master mixes giving similar results, with $\Delta Cqs_{A:D}$ of 0.17±0.25, -0.72±0.23 and 0.01±0.30, respectively (Figure 5B). The ThermoFisher master mix performed significantly worse (ΔCq= 5.23±0.73). Increasing the annealing/polymerisation temperature to 60°C, resulted in a deterioration of the performance of assay A relative to that of assay D, especially for the PrimerDesign master mix, a trend exacerbated by further increases to 62°C and 64°C although assay A was able to amplify its target even at 64°C. The ThermoFisher master mix continued to be the worst performer and failed to amplify either assay at 64°C. All Cq values are listed in the supplementary data file, tab 6.
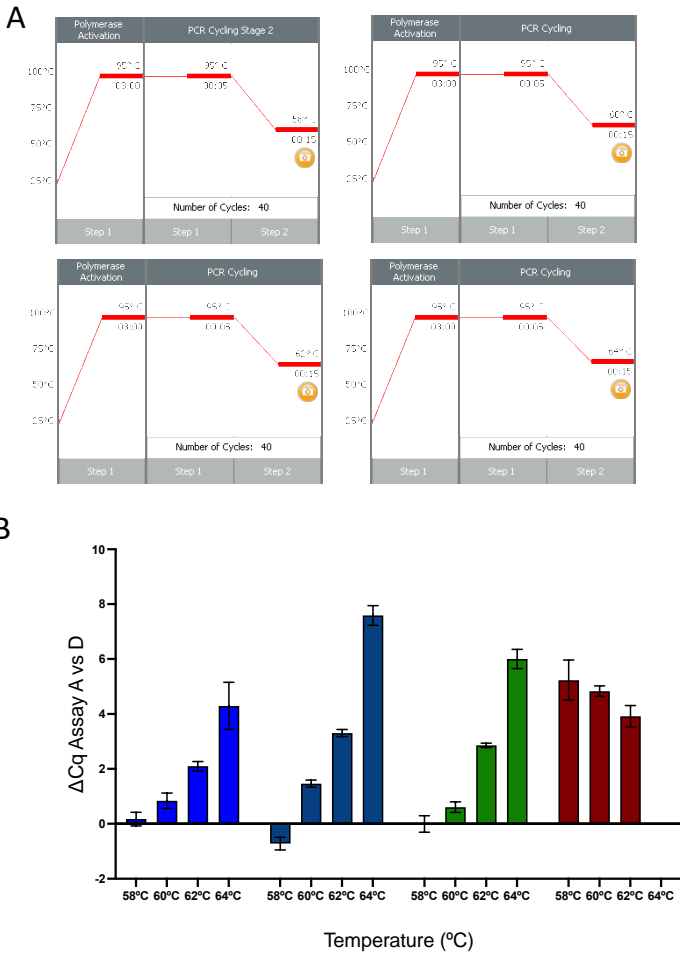
Figure 5. Influence of master mix on qPCR performance of assays A and D with cDNA synthesised by BioRad iScript. **A.** Protocols of the four annealing/polymerisation conditions. **B.** Plots of the ΔCqs between assays A and D, carried out in duplicate at the four temperatures recorded with PrimerDesign (blue), KAPA (turquois), Quanta (green) and ABI (brown) master mixes. The error bars show standard deviations.

2.5. Comparison of mismatched and corrected RdRp probes

The performance of assay G, which uses the original mismatched RdRP_SARSr-P1 probe, was compared to assay H, which substitutes that probe with the corrected ALT-P1dg sequence. The performance of both was compared to that of a published SARS-CoV-2 assay (CoV2-ID) [39]. Three replicate assays using the PCRBio 1-step RT-qPCR master mix recorded equivalent Cqs, with G:H ΔCqs of 0.42±0.71, 1.01±0.50 and -0.05±0.45, respectively (Figure 6A). As expected, and in line with the previously reported lower sensitivity of this assay, both assays were less sensitive than CoV2-ID, with average ΔCqs of 5.09±0.64 and 5.54±0.75 for G and H, respectively. Repeating the comparison between assays G and H using two step RT-qPCR assays with cDNA synthesised by Ultrascript (Figure 6B) or SS4 (Figure 6C) and amplified using Bioline's SensiFast qPCR master mix confirmed the equivalence of the two probes, indicating that the two mismatches did not affect the ability of the probe to bind to the PCR amplicon. Cqs are listed in supplementary data file tab 7.
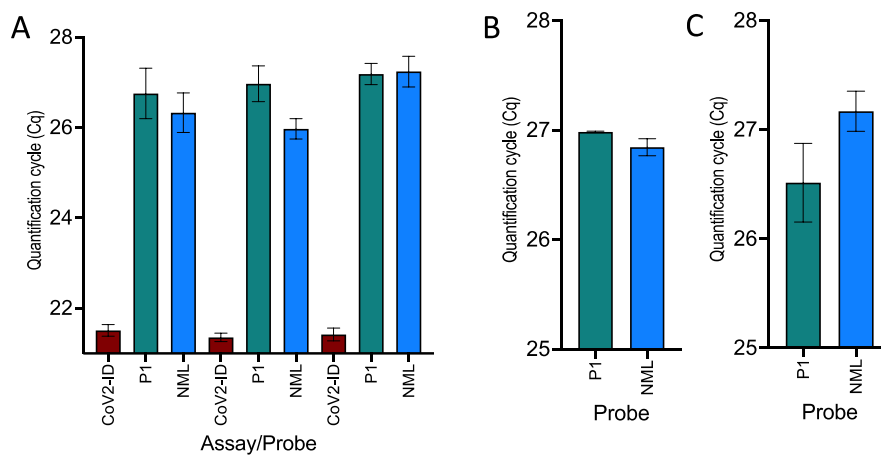
Figure 6. Comparison of an efficient SARS-CoV-2 assay (CoV2-ID) to assays with mismatched RdRp_SARSr-P2 (assay G) and perfectly matched ALT P1 dg (assay H) probes. Column bar charts are shown plotted as the mean Cq± SD. CoV2-ID is shown in brown, assay G results in green and assay H in blue. **A.** Cqs from three replicate PCRBio 1-step RT-qPCR assays demonstrate that whilst the RdRp assay is less sensitive than CoV2-ID, the mismatched probe assay records approximately the same Cqs as the non-mismatched one. **B.** Cqs were recorded using a two step protocol with RNA reverse transcribed by UltraScript and the cDNA amplified by SensiFast qPCR master mix. **C.** Cqs were recorded using a two step protocol with RNA reverse transcribed by SS4 and amplified by SensiFast qPCR master mix.

2.6. Effect of increased reverse primer concentration

Finally, the potential for increasing the efficiency of the RT step was investigated by doubling the concentrations of the reverse primers in assays A, C and D and carrying out RT-qPCR assays with PrimeScript 3 and PCRBio 1-step master mixes. This resulted in lower Cqs for assays A and C, especially with the PrimeScript master mix (Figure 7). There was no real effect on Cqs recorded by assay D, run with specific primers. Cqs are listed in supplementary data file tab 8.
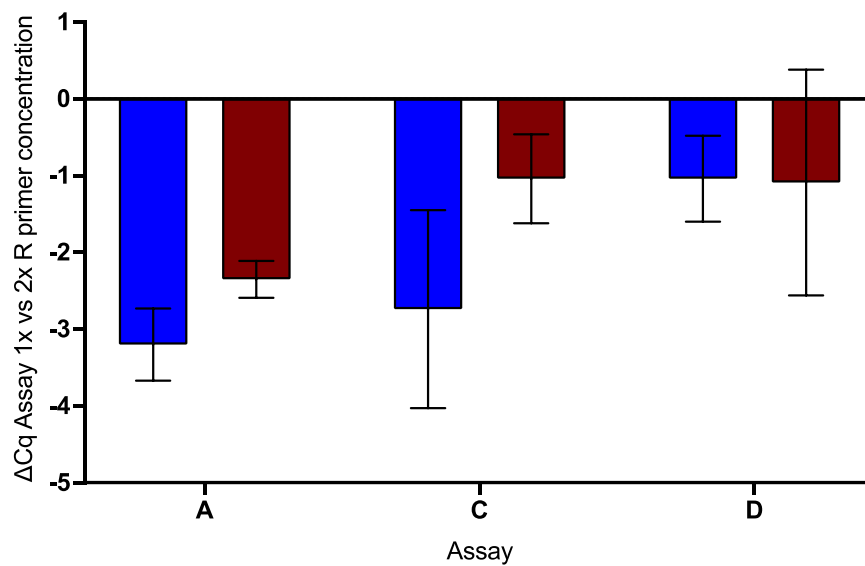
Figure 7. Effect of doubling R primer concentration for RT-qPCR analysis of RNA with assays A, C and D. The lower Cqs recorded by the higher primer concentration are apparent for assays A and C carried out using PrimeScript III (blue), compared with assay D, where doubling the concentration of ALT R sp made little difference. Assay A was also enhanced with the PCRBio (brown) master mix, whereas assays C and D were not. The error bars show the standard deviations recorded by two independent replicate reactions carried out with each assay.

3.   Discussion

This investigation clarifies and restates a number of issues with regards to the Charité RdRp assay:

1.   The single base mismatch in the reverse primer reduces the sensitivity of the assay by affecting the RT step.
2.   The qPCR step is less affected by the primer mismatch than had been suggested.
3.   In one step RT-qPCR reactions specific primers perform better than those incorporating wobble bases.
4.   The two mismatches in the RdRp_SARS-P1 probe do not affect the performance of the assay.
5.   Although there is a significant difference in Tm between the forward and reverse primers, our data show that the RdRp assay performs reliably at a broad range of annealing temperatures and well above the calculated Tm for the R primer.

The performance of this assay has been evaluated previously in several publications and shown to result in higher Cqs and reduced sensitivity when compared with assays targeting other viral genes [34–38]. The actual reduction, however, is not clear. This is because the ΔCqs vary considerably between reports and the protocols used are also significantly different (Table S1, supplementary data). Critically, there is no agreement on which of the most commonly used assays is the most sensitive one. The overall quality of a molecular diagnostic test is dependent on an optimised, complete workflow starting with sample selection and collection and ending with appropriate data, rather than individual components of the workflow [31]. The performance differences of the various SARS-CoV-2 RT-qPCR kits are due to both the different viral sequences being targeted and also the different reagents and master mix formulations including kit production quality [30]. Certainly, the conclusion from one of the papers is worth repeating that "thanks to this [the Charité RdRp] assay an important number of COVID-19 diagnoses were made, which contributed to limiting the spread of the outbreak" [38].

The RdRp-SARSr-R mismatch might be expected to affect either the RT or the PCR, depending on whether the cDNA protocol is a 1-step RT-qPCR protocol using specific RT primers or a 2-step protocol using random priming. *In vivo* [40] and *in vitro* [41–43]

studies established long ago that reverse transcription is a 3'-mismatch-tolerant process, a conclusion supported by more recent analyses [44]. Another study has shown that as many as four internal mismatches have no effect on RT efficiency [45] and mismatched primers are efficiently extended by Avian Myeloblastosis Virus-RT [46], again confirmed by a more recent report [47]. Importantly, the effect of a mismatch varies up to seven-fold depending on the master mix used [47]. Lastly, once a mismatched primer is reverse transcribed into a cDNA template, both are fully complementary, and no dramatic negative effect would be expected for the subsequent PCR.

The data presented here support these inferences and extend them to demonstrate that the extent of the mismatch-associated effect depends on three factors: (i) choice of RT and (ii) RT-qPCR amplification strategy as well as (iii) selection of RT-qPCR master mix reagents. As demonstrated, use of 1-step protocols results in an average variability of around four-fold across the five master mixes for the mismatch assay compared with the corrected assay. Replacing the wobble bases altogether had no beneficial effect. Combining a specific Forward primer with the mismatched Reverse primer had a slightly deleterious effect, though the corrected wobble R primer performs the least well. However, there are reagent-dependent differences; for example, assay E performs better than assay A with the GSD Novaprime master mix.

The RT temperature gradient results shed some more light on the effects of the R primer mismatch on the RT step within a 1-step RT-qPCR assay. Lower annealing temperatures have little effect on RT efficiency for all three assays, although again there are clear differences between the reagents, with the GSD Novaprime master mix recording lower Cqs and performing better at the higher temperatures. But clearly the mismatch is not significantly destabilised at higher RT temperatures and lowering the RT temperature does not restore efficient cDNA synthesis from the mismatched primer.

Whereas assay A consistently performed poorly compared to assay B in the 1-step RT-qPCR reactions, a two-step approach involving a separate RT step that includes random primers minimised the effect of the mismatch, a result observed for both RTs tested. This was reproducible, with the amplification of multiple independent RT replicates. Interestingly, there was an RT-dependent difference in the ΔCqs between A and D. The reduced sensitivity can be ameliorated by adjusting the experimental conditions, most obviously by increasing the concentration of the mismatched reverse primer. These results differ from those reported elsewhere [37], but whereas these authors did not actually increase primer concentrations above those originally reported, the concentration of RdRp_SARSr-R in this study were doubled to 1.6 µM.

cDNA obtained using random primers incorporates any initially mismatched nucleotides and although the efficiency of extension depends on how efficiently a primer hybridises onto its complementary target sequence [48] and how effective Taq polymerase binds to both [49], the consequences of mismatches during the PCR reaction are not straightforward [50]. A series of studies has established that the effects of mismatches are variable and depend on sequence context [49, 51], the nature of the mismatch [52], reaction conditions [53, 54], polymerase [55] as well as primer length [56]. Single mismatches, especially when located well away from the 3'-end of the primer generally have a small effect on PCR amplification [57–59]. An internal G:T mismatch is the least affected and lowering the annealing temperature improves the primer extension efficiency of almost all single mismatch types occurring at positions other than the last 3–5 bases from the primer 3'-end [60]. This is of course relevant since not only is the mismatch 15 nucleotides from the 3'-end of the reverse primer, but it results in the most favourable G:T pairing expected to have a minimal effect on the qPCR assay. This deduction is borne out by the comparable performance of assays A and D over a wide temperature range in qPCR reactions carried out with the first three cycles involving a 45.0°C to 60.0°C annealing/polymerisation gradient.

An analysis of the compatibility of primers and probe suggests that they are not a good match by accepted design criteria. One unanticipated feature of the two primers is their disparate Tm, with the forward primer predicted to have a significantly higher Tm than the reverse one. Conventionally, qPCR uses equimolar concentrations of two primers with similar Tm. However, this is not necessary for efficient PCR, as demonstrated by Linear-After-The-Exponential (LATE)-PCR [61], where limiting the concentration of one primer results in a decreased Tm for that primer compared to the one that is in excess [62]. The optimal annealing temperature, which is where most or all of the primers are bound to their target, is likely to be quite different from the Tm, which is defined as the temperature where 50% of primers are bound

to their target. The performance of the assay across a range of annealing conditions above the predicted Tm shows that choice of master mix is important and that even a widely mismatched primer pair can result in efficient amplification. Interestingly, mismatches in the probe that might be expected to affect its performance by reducing its Tm have little effect, as shown by a comparison of the mismatched original probe with a corrected version. It is expected that sufficient probe binds to the template prior to primer extension, maintaining adequate exonuclease digestion of the probe.

If use of a mismatched reverse oligonucleotide results in less efficient cDNA synthesis priming, one way of ameliorating this issue could be to increase its concentration, thus improving cDNA yield and hence the sensitivity of the assay. This is indeed possible, although doubling the concentration of the mismatched RdRp_SARSr-R primer improved the performance of assay A and to a lesser degree that of assay C, without significantly affecting assay D.

Lastly, no discussion of a diagnostic test would be complete without an acknowledgement that no matter how sensitive, accurate and reliable an RT-qPCR assay might be, appropriate interpretation of the results is an equally important, final component of an informed clinical decision-making process. Whilst RT-qPCR can provide a measure of the abundance of SARS-CoV-2 RNA above a technical threshold, it remains unclear how that abundance, usually expressed as a Cq value, translates as infectiousness or the need to implement rigorous public health measures. This is exemplified by the wide Cq variability observed after the amplification of the same RNA sample with a commercial primer and probe (PrimerDesigns Coronavirus Genesig assay) and nine different commercial master mixes. This is expected, and has been previously discussed, but adds weight to the argument that reporting of unqualified Cqs in the context of testing for SARS-CoV-2 is meaningless.

In theory, the use of RT-qPCR testing to determine SARS-CoV-2 viral load should be useful for clinical management of individuals, assessment of their need to self-isolate as well as launch of contact tracing. However, although samples with lower Cq values generally have more viral RNA than those with higher Cq values [63], the clinical relevance and precision associated with those differences has not been determined [64] and even the meaning of "high" Cq is undefined. The inconsistencies in using Cq cut-off values are demonstrated by a publication reporting no positive culture growth in samples with Cq >24 [65], another reporting positive culture in samples with Cqs of 34 and negative culture with Cqs of 22 [66] and yet another report of no positive culture above a Cq of 34, but 12% positive at 33, 50% at 21, 28% at 30, 70% at 29 and 53% at 27 [67]. Crucially, a single raw, not normalised Cq value is not a quantitative result. At the very least, Cq informed quantity would have to involve normalisation using some marker of the cell mass or the mucosal surface [68] and, crucially, be considered in a clinical context [69]. Sadly, the kind of results where one group reports no correlation between Cq values and severity of disease or mortality [70] and another that claims that Cq value predict disease severity and survival [71] is quite the norm. In practice there are several reasons that Cq values continue to have limited use in clinical settings. First, a positive PCR test alone does not correlate with infectivity and currently there is no standard measure of viral load in clinical samples. Second, numerous variables have a bearing on viral load and patient outcomes are determined by an additional set of variables such as co-morbidities and age. Third, Cq values are relative, as they are affected by sample collection and processing [72], targets, reagents, assays [73] and instruments [74]. Fourth, there is a statistical uncertainty around any Cq value, which for RT-qPCR can exceed 3 Cqs under some circumstances, such as when template is degraded or present at low copy number. Fifth Cq values and lower viral load may not be directly proportional because inhibitory factors within samples may cause later amplification [75]. Sixth, it has been known for a long time that testing of the same sample by different laboratories can result in huge variability in recorded Cq values. Consequently, it is not feasible to dictate a universal Cq to demarcate positive from negative test results [76], even if Cq values are translated as units of pathogen load based on a standard curve. Until suitable, clinically validated standards are available it is not possible to correlate data obtained from different testing facilities.

In conclusion, the reverse primer mismatch in the RdRp component assay of the first published SARS-CoV-2 test affects the performance of that assay. In contrast, the mismatched probe has no appreciable effect on assay sensitivity. Importantly, it is possible

to ameliorate the effects of the primer mismatch through a combination of optimal RT, reagents and protocols. Whilst we would continue to stress that it is important to design assays carefully from the start, our findings hold an important lesson for RT-qPCR assays in general, as they highlights the flexibility and robustness of this methodology, where even a sub-optimal design can be rescued by intelligent optimisation.

4. Methods

4.1 Primers and probes

This study used five oligonucleotide primers, the two RdRp gene probes (SARS RdRp_SARSr-P1 and SARS RdRp_SARSr-P2) from the Drosten paper as well as a probe correcting the mistakes in the RdRp_SARSr-P1 probe. All were HPLC purified and their sequences and locations on the 100 bp amplicon are shown in Table 1, with the IUPAC nucleotide codes indicating wobble bases highlighted in bold, underlined and italicised.

Oligonucleotide	Number	Sequence	Final concentration (nM)
RdRp_SARSr-F	1	GTGA**R**ATGGTCATGTGTGGCGG	600
ALT F sp	2	GTGAAATGGTCATGTGTGGCGG	600
RdRp_SARSr-R	3	CA**R**ATGTTAAA**S**ACACTATTAGCATA	800
ALT R sp	4	CAAATGTTAAAAACACTATTAGCATA	800
ALT R dp	5	CA**R**ATGTTAAA**V**ACACTATTAGCATA	800
RdRp_SARSr-P2	6	FAM-CAGGTGGAA-Nova-CCTCATCAGGAGATGC	100
RdRp_SARSr-P1	7	HEX-CAGGTGG**W**A-Nova-C**R**TCATC**M**GG**T**GATGC	100
ALT P1 dg	8	HEX-CAGGTGG**W**A-Nova-C**M**TCATC**M**GGAGATGC	100
IUPAC nucleotide base code		M= A or C; R=A or G; S=G or C; V= A, C or G; W=A or T	

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                                                          ATACGATTATCACAVAAAATTGTARAC  5
                                                          ATACGATTATCACASAAAATTGTARAC  3
5'- GTGAAATGGTCATGTGTGGCGGTTCACTATATGTTAAACCAGGTGGAACCTCATCAGGAGATGCCACAACTGCTTATGCTAATAGTGTTTTTAACATTTG -3'
                                                          ATACGATTATCACAAAAAATTGTAAAC  4

  2 GTGAAATGGTCATGTGTGGCGG                    6 CAGGTGGAACCTCATCAGGAGATGC
3'- CACTTTACCAGTACACACCGCCAAGTGATATACAATTTGGTCCACCTTGGAGTAGTCCTCTACGGTGTTGACGAATACGATTATCACAAAAAATTGTAAAC -5'
  1 GTGARATGGTCATGTGTGGCGG                    7 CAGGTGGWACRTCATCMGGTGATGC
                                              8 CAGGTGGWACMTCATCMGGWGATGC
```

Table 1. Sequence, concentration and location of oligonucleotides used in this study. Matched and mismatched nucleotides are shown in green or red, respectively. The final concentration of the reverse primer was doubled in one set of experiments.

Various combination of primers and probes were used to prepare eight assays designated A to H (Table 2).

Primers	A	Sequence
1	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG
3	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA
Probe	RdRp_SARSr-P2	FAM-CAGGTGGAA-Nova-CCTCATCAGGAGATGC

Primers	B	Sequence
1	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG
4	ALT-R sp	CAAATGTTAAAAACACTATTAGCATA
Probe	RdRp_SARSr-P2	FAM-CAGGTGGAA-Nova-CCTCATCAGGAGATGC

Primers	C	Sequence
2	ALT F sp	GTGAAATGGTCATGTGTGGCGG
3	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA
Probe	RdRp_SARSr-P2	FAM-CAGGTGGAA-Nova-CCTCATCAGGAGATGC

Primers	D	Sequence
2	ALT F sp	GTGAAATGGTCATGTGTGGCGG
4	ALT R sp	CAAATGTTAAAAACACTATTAGCATA
Probe	RdRp_SARSr-P2	FAM-CAGGTGGAA-Nova-CCTCATCAGGAGATGC

Primers	E	Sequence
1	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG
5	ALT R dp	CARATGTTAAAVACACTATTAGCATA
Probe	RdRp_SARSr-P2	FAM-CAGGTGGAA-Nova-CCTCATCAGGAGATGC

Primers	F	Sequence
2	ALT F sp	GTGAAATGGTCATGTGTGGCGG
5	ALT R dp	CARATGTTAAAVACACTATTAGCATA
Probe	RdRp_SARSr-P2	FAM-CAGGTGGAA-Nova-CCTCATCAGGAGATGC

Primers	G	Sequence
1	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG
3	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA
Probe	RdRp_SARSr-P2	HEX-CAGGTGGWA-Nova-CRTCATCMGGTGATGC

Primers	H	Sequence
1	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG
3	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA
Probe	ALT P1 dg	HEX-CAGGTGGWA-Nova-CMTCATCMGGAGATGC

Table 2. Assays A to H

4.2. Instruments

The two qPCR instruments used were Cole-Parmer's PCRMax instrument (WZ-93947-00 with 48 well plates (WZ-93947-99) and BioRad's CFX Connect (1855200) with white qPCR plates (HSS9665), sealed using qPCR plate heat seals (1814030).

4.3. RNA

Multiple SARS-CoV-2 RNA samples were extracted from a Seracare Accuplex SARS-CoV-2 Full Genome verification panel (505-0168) using Qiagen's QIAamp Viral RNA mini kit (52904). RNA quality and integrity were assessed using an Agilent 2100 Bioanalyser (G2939BA) and samples were stored at -80°C.

4.4. RT-qPCR reactions

4.4.1. RT reactions

SARS-CoV-2 RNA was reverse transcribed in 20μL using ThermoFisher's Superscript IV Vilo (SS4) (11756050) or BioRad's iScript (1708891), both of which use a combination of random hexamers and oligo-dT to prime cDNA synthesis. RT conditions were 5 minutes at 25°C, 5 minutes at 55°C (SSIV) or 46°C (iScript) and 5 minutes at 85°C (SSIV) or 5 minutes at 85°C (iScript). cDNA samples were diluted to 50μL with RNase-free water (Fisher Scientific 15992440).

In addition, SARS-CoV-2 RNA was reverse transcribed in 20μL using PCRBio's UltraScript (PB30.12-01) with 5μM final concentration of hexamers. RT conditions were 5 minutes at 25°C, 5 minutes at 42°C and 10 minutes at 85°C. The cDNA sample was diluted to 50μL with water

4.4.2. 1-step RT-qPCR

SARS-CoV-2 RNA was subjected to RT-qPCR amplification with the eight assays listed in Table 2 using one or more of six commercial one-step master mixes: PCRBio 1-step Go (PB10.53-10), Takara PrimeScript (RR600A), NEB Luna (E3005S), Eurofins GSD Novaprime (PCOV6033), Quanta XLT Toughmix (95132-500) and ABI TaqPath (A15299). All reactions were carried out in 5μL reactions using the oligonucleotide concentrations shown in Table 1. The accuracy and reproducibility of such a small reaction volume

is demonstrated in the supplementary file, tab 10. The protocol consisted of an RT (5 minutes at 50°C) and an activation/denaturation step (2 minutes at 95°C), followed by 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C.

4.4.3.   2-step RT-qPCR

1μL aliquots of cDNA synthesised by SS4 or iScript were amplified with the six primer combinations using Meridian Bioscience SensiFast probe master mix (BIO-86050). PCR conditions were 2 minutes at 95°C, followed by 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C. Where indicated, additional qPCR master mixes from Roche (KK4701), PrimerDesign (PPLUSXXML) and ABI (4444556) were used.

4.4.4.   Gradient RT-qPCR

The effect of varying RT temperatures was tested for all six assays using the GSD Novaprime 1-step RT-qPCR master mix. A premix of RNA in 1x master mix was added to six tubes containing the various primer combinations and probe and run on a BioRad CFX Connect using the following conditions: 5 minutes at 25°C, 10 minutes at eight different temperatures (40.0, 40.6, 42.0, 43.9, 46.3°C, 48.3°C, 49.4°C and 50°C), 2 minutes at 95°C and 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C. Assays A, B and D were also analysed using PCRBio and Quanta Toughmix 1-step reagents with the same conditions.

4.4.5.   Gradient qPCR

The effect of lowering the annealing temperature for the first three PCR cycles was analysed by amplifying cDNA samples using either SYBR Green or RdRp_SARSr-P2 as reporters with the appropriate SensiFast master mixes, SensiFast SYBR master mix (BIO-98020) or the SensiFast probe master mix. The protocol in a BioRad CFX Connect was 1 minute activation at 95°C, three cycles of 3 seconds at 95°C and 10 seconds at 45.0°C, 46.0°C, 48.0°C, 50.8°C, 54.6°C, 57.5°C, 50.1°C and 60.0°C followed by 36 cycles of 5 seconds at 95°C and 10 seconds at 60°C and a melt curve from 65°C-95°C.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Supplementary data file, supplementary Figure

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