Interpreting SARS-CoV-2 RT-PCR Test Results: Recommendations From Experiences and Lessons Learnt from the KEMRI-CGMRC/ KEMRI-Wellcome Trust Research Programme Laboratory in Kilifi

Key messages

- There are more than one hundred commercial real-time reverse transcription PCR kits (RT-PCR) for SARS-CoV-2 diagnosis with varied performance.
- Specifically, there are significant differences in the accuracy of available kits implying false positives/ false negatives can happen, especially in a sample with low virus quantities.
- Among the factors that affect diagnostic accuracy are sample type, sample quality, timing of sample collection from the patient, RNA purification procedures and the RT-PCR kit used.
- Sample quality is dependent on sample collection skills, transportation and storage conditions e.g. temperature and transport media.
- For accurate interpretation of the test results, initial optimisation and validation of all diagnostic steps is important.
- SARS-CoV-2 status may change rapidly over time, hence a positive or negative laboratory result does not mean a future sample from the same individual will have the same result as the initial acute infection, and infection can occur any time during or after exposure to the virus.
- The natural history of infection is that positive and negative results may alternate.
- There is no current SARS-CoV-2 diagnostic test that is 100% accurate.
- Test results are only part of the evidence that should be interpreted together with clinical and epidemiological evidence.

Background

Detection of SARS-CoV-2 genetic material (viral RNA) using real-time reverse transcription PCR (RT-PCR) is the gold standard method for COVID-19 diagnosis. Globally, there are over 100 SARS-CoV-2 RT-PCR diagnostic kits approved by various authorities. The diagnostic accuracy of a SARS-CoV-2 test in a laboratory is a sum total of the integrity and robustness of the all the steps of the diagnostic process. The key factors that may influence a patient SARS-CoV-2 result include sample type, timing of sample collection during the course of infection (from pre-symptoms to recovery), sample quality (dependent on sample collection skills, transportation and storage conditions e.g. temperature and transport media), viral nucleic acid material (RNA) purification efficiency and the RT-PCR protocol/kit used. Throughout the SARS-CoV-2 testing process it is important to remember that:

1. If an incorrect technique is used for a nasal swab, and only superficial material is sent, then virus may not be present on the swab sent to the laboratory despite an active infection in the patient.
2. Virus RNA is very sensitive and degrades if stored/transported at sub-optimal conditions;
3. Detection depends on the stage at which a patient is sampled; virus levels are highest around the time of onset of symptoms/ or for the first 5-days post-infection if the patient is asymptomatic;
4. Levels of virus vary between patients. Patients with symptoms tend to have heavier infections with higher virus levels in the samples taken.

KEMRI-Kilifi diagnostic experience

The KEMRI-CGMRC/KEMRI-Wellcome Trust Research Programme (KWTRP), Kilifi has been providing SARS-CoV-2 diagnostics for Coastal Kenya since 17th March 2020. As at 7th July 2020, the laboratory had conducted over 28,000 RT-PCR tests for suspected patient samples. For viral RNA purification we have used 3 different commercial RNA extraction kits from QIAGEN: QIAamp, QIACUBE HT and QIASYMPHONY, while for RT-PCR, we have used four kits/protocols to date: the Berlin (Charité) protocol, European Virus Archive – GLOBAL (EVAg) protocol, the DAAN RT-PCR Kit and the Beijing Genomic Institute (BGI) RT-PCR kit. The first two protocols provide only primer/probe mixes, that require the separate addition of the other RT-PCR components (e.g. buffers, enzymes, water), while the latter two come with all RT-PCR components

pre-mixed, ready for sample testing after inclusion of viral RNA extract from the patient sample. There are 3 genes in the SARS-CoV-2 that are commonly the target of PCR tests. At the beginning of the COVID-19 outbreak, the WHO recommended testing for at least two SARS-CoV-2 genes to determine the true status of a sample. Subsequently recommendations have changed, and many laboratories including ours screen based on a single gene. This allows many more samples to be tested, but increases the risk of inaccurate test results.

Our laboratory optimization process focused on both the virus nucleic-acid purification and the RT-PCR steps in the SARS-CoV-2 diagnostic process. The RT-PCR cycle threshold (Ct) indicates the amount of virus, normally ranging from ~10.0 to 40.0. The higher the Ct, the lower the virus concentration. Generally, it is easier to miss individuals with extremely low amounts of SARS-CoV-2 genetic material than those with high amount of virus (ie Ct above 35). The optimisations were conducted with a positive control sample with a known concentration that was diluted to generate a series of highly concentrated and very dilute control, this allows the determination of test kit performance and at what point the test does not detect the most dilute sample. The dilute sample is important as it gives an indication of the point at which a patient sample with similar low viral loads may not be detected and it provides confidence in the level at which cycle threshold cut-off is defined to identify a true positive result.

Study results

Since March 2020, we have undertaken several optimization experiments to understand SARS-CoV-2 laboratory results of the RT-PCR kits/protocols we use in our laboratory. Here we highlight a few of those experiments. First, the amount of patient material used in the RNA purification step can influence the final RT-PCR result. We compared the performance of the diagnostic assay using two different volumes of the patient sample (140 µl vs 280 µl). It was clear that using more patient material gives a clearer positive result for a patient with "light infections" or low virus levels **(Table 1)**. Also, different RT-PCR protocols have different primers/probe sequences that target different regions of the SARS-CoV-2 genomes. Their detection efficiencies are different as was observed in the experiment depicted in **Table 1**.

Table 1. Comparison of example sample results applying 3 gene targets using the Berlin Charité Protocol with different sample volumes used in extraction.

Protocol (target gene)	Sample Volume analyzed		Interpretation
	140 ul	280 ul	
Berlin Charite (E)	37.59 (+ve)	32.167 (+ve)	+ve
Berlin Charite (RdRp)	Undetermined (-ve)	36.26 (+ve)	+ve
Berlin Charite (N)	40.05 (-ve)	34.89 (+ve)	+ve

Notes: (a) E ; Envelope, RdRp; RNA-dependent RNA- Polymerase; N, Nucleocapsid. (b) The larger sample volume (280 uL) provided a clear positive result and (c) There was a different in virus titer detected by the different assays influence by gene assayed.

Second, some kits (e.g. DAAN) come with an internal control (IC) assay which helps monitor the diagnostic process. The internal control is normally a housekeeping human gene that is expected to be present in all samples collected from human tissue. The internal control assesses the source of the sample, efficiency of sample collection and validity of the extraction and the RT-PCR steps. When the internal control fails, the patient RT-PCR result is invalid and the diagnostic process should be repeated or a fresh sample collection requested for re-analysis.

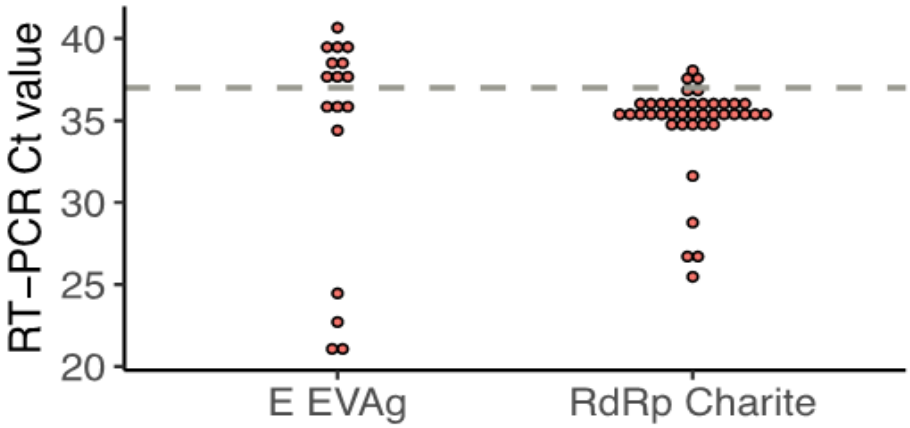
Table 2. Interpretation of DAAN RT-PCR kit results

	IC	N target	ORF1ab target	Interpretation
Sample 1	30.16	Undetermined	Undetermined	Negative
Sample 2	29.44	Undetermined	Undetermined	Negative
Sample 3	26.17	36.13	34.89	Positive
Sample 4	Undetermined	Undetermined	Undetermined	invalid
Sample 5	Undetermined	24.34	24.09	Invalid

Notes: (a) IC, Internal Control; ORF, Open Reading Frame; N, Nucleocapsid.

Third, some primer/probe sets e.g. the Charité protocol RdRp are prone to false positives that identify negative samples as weak positives. In one run where we analyzed 43 patient samples, the Charité protocol RdRp identified 32 (74.1%) as positive while European Virus Archive global (EVAg) E assay confirmed only 6 (13.9%) samples as positive, Figure 1. Most of the false positives identified by the RdRp Charite protocol had a Ct >34.0 and were difficult to distinguish from true weak negatives.

Figure 1. Example result of where processing samples by two assays helped identify false positives that were showing up as weak positives. This occurrence was common with the Charite RdRP assay. The dashed horizontal line indicates Ct 37.0 below which we would consider a sample RT-PCR positive if a single assay is used.



Fourth, our experience with the BGI RT-PCR kit is that it was prone to false-positives and there was considerable batch-to-batch variation of its performance. While following the protocol provided, our first use of this kit detected >75% samples analyzed to be positive. As this was epidemiologically impossible, we repeated the test and detected only very few samples to be positive (**Figure 2**).

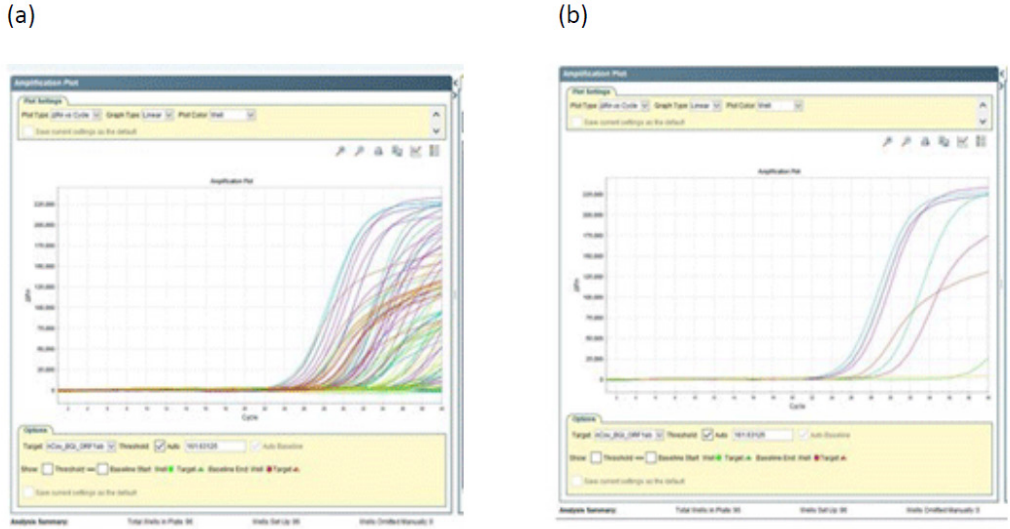


Figure 2. BGI Kit RT-PCR. (a) First use of BGI using 50% of the manufacturer's recommended reaction volumes. (b) Following the use of 75% of neat volumes and 0.25µl of enzyme there was improved sensitivity in the assay as indicated from the 96 sample test run with 4 samples positives meeting the <37.0 cut-off criteria

Conclusions

1. To tackle false negatives, assay sensitivity may be optimised by increasing sample volumes taken into the viral RNA purification process and the RT-PCR steps especially for weak positives.
2. False positive results may be a result of contaminated reagents or the inherent characteristics of certain virus detection assays. As a result, interpretation of low level positives needs to be made carefully to distinguish false positives from weak negatives.
3. The cycle threshold cut-off for calling positive and negative samples can be subjective especially when the patient has very low virus quantities, which happens when the patient is almost recovered. The cycle threshold cut-off for every assay should be determined through optimisation experiments in every laboratory as it can vary between assays and laboratories.
4. The duration of SARS-CoV-2 shedding can range from 7 to 20 days or even longer. RT-PCR is most sensitive when the patient is shedding high virus quantities and thus timing of sampling influences test sensitivity.
5. All tests will be prone to false negative and false positives, and patients results will vary from day to day. It is to be expected the discrepant results will emerge from time to time.

Recommendations

a. A two or more target gene protocol is better in deciding the sample SARS-CoV-2 status than a single assay protocol. The combinations required for identifying a positive result should be pre-determined. For any inconclusive or invalid result, it is best to retest the sample preferably targeting a different viral gene.
b. In comparing test results between laboratories, an agreed set of standard samples should be developed and circulated to all participating laboratories. It should be noted that even when the same samples are compared, other factors can still influence the test result outcome including sample transport conditions and storage history, and that no test is foolproof. A single discrepant result between laboratories does not indicate a failing in one or other laboratory.
c. In deploying a new test kit, it is important to undertake a well-documented optimization process and compare results with already working kits.
d. Throughout the testing process quality control measures should be included (Positive Control, Negative Control, Internal Control) for test result validity.

Related Publication

This brief is adapted from two recent research papers from our laboratory submitted under the titles, (a) "An optimisation of four SARS-CoV-2 qRT-PCR assays in a Kenyan laboratory to support the national COVID-19 rapid response teams" and (b) Pooled testing conserves SARS-CoV-2 laboratory resources and improves turn-around time: experience at KEMRI-Wellcome Trust Programme, Kenya

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