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Molecular diagnostics in the era of COVID-19

Harindi Jayakody,^a Guy Kiddle,^b Semali Perera,^a Laurence Tisi^{*b}
and Hannah S. Leese^{*a}

As the COVID-19 pandemic continues to escalate globally and acquires new mutations, accurate diagnostic technologies continue to play a vital role in controlling and understanding the epidemiology of this disease. A plethora of technologies have enabled the diagnosis of individuals, informed clinical management, aided population-wide screening to determine transmission rates and identified cases within the wider community and high-risk settings. This review explores the application of molecular diagnostics technologies in controlling the spread of COVID-19, and the key factors that affect the sensitivity and specificity of the tests used.

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1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the virus responsible for causing the COVID-19 pandemic that was first identified in Wuhan, China in 2019.¹ According to data published by the John Hopkins Coronavirus Research Centre, there have been over 190 million cases globally and over 4 million deaths, as of July 2021.²

SARS-CoV-2 is a positive sense RNA virus made of four main structural proteins and other accessory proteins.³ The spike protein binds to the angiotensin-converting enzyme 2

(ACE2) receptor found on human host cells, enabling entry.^{4,5} COVID-19 most commonly spreads *via* direct exposure to respiratory secretions of an infected person, but it can also be spread by airborne transmission, through contact with contaminated surfaces and *via* the faecal-oral route.⁶⁻¹³ Infected individuals may present mild to severe symptoms of the infection, such as fever or chills, a persistent cough, shortness of breath, and headaches amongst others, and these are typically displayed 2–14 days following initial exposure.^{6,14}

Testing for SARS-CoV-2 aids for a timely diagnosis and helps to control transmission within different settings, thereby preventing the re-introduction of SARS-CoV-2 into settings where the virus is under control (*i.e.* testing visitors travelling into a given jurisdiction).¹⁵ In addition to tests performed on

^aMaterials for Health Lab, Department of Chemical Engineering, University of Bath, Bath, UK. E-mail: h.s.leese@bath.ac.uk

^bErba Molecular, Ely, Cambridgeshire, UK. E-mail: ltisi@erbamannheim.com



Harindi Jayakody is a second year PhD student in the Department of Chemical Engineering, University of Bath, UK. She is a graduate in molecular biology (MBioSci) from the University of Sheffield. She is also working as a scientist at Erba Molecular, Ely developing diagnostics platforms for COVID-19.



Dr Guy Kiddle is a principle scientist that has worked as a Biotechnologist for over 30 years, specialising in advancing the scope and applications of biochemical and molecular methodologies. In his early career, he applied molecular technologies such as microarray analysis to elucidate changes in gene expression induced by oxidative stress and vitality.

More recently he worked on developing novel amplification technologies and platforms for diagnosing contaminants and infectious disease. Guy currently heads up a team of synthetic biologists who are in the early stages of developing an automated desk-top device capable of synthesising high quality error free DNA.



individuals, environment monitoring for contamination through surface swabbing, wastewater testing, and air sampling have all proven advantageous in managing the spread of SARS-CoV-2.^{13–19}

Sensitive molecular tests are essential to ensure that individuals infected with SARS-CoV-2 are accurately identified and quarantined quickly. Whilst molecular tests, in particular reverse transcription polymerase chain reaction (RT-PCR), are widely used due to their high sensitivity, many factors can impede the accuracy of these tests. This review explores the use of molecular tests in different contexts and discusses the technologies that have been widely deployed, exploring the key factors affecting a tests sensitivity and specificity.



Prof. Semali Perera is a Professor of Chemical Engineering at the University of Bath with 25 years of experience in research on the manufacture, characterisation and testing of low-pressure drop, antimicrobial/air purification filters such as foams, hollow fibres and functionalised membranes for applications that recently have included bulk separations, environmental management and personal

protection. Currently she is working on antiviral filters for air conditioning systems and for use in enclosed spaces such as aircrafts. Prof Perera's research on the development of energy-efficient air purification filters led to her recognition by The Royal Society with the award of their prestigious Brian Mercer Award for Innovation in 2007 and winning the 2017 FDM everywoman in Technology Awards.



Dr Laurence Tisi is currently President Molecular Division at Erba Mannheim. Previously, CEO of Lumora ltd until 2015 which he co-founded in 2003 from the University of Cambridge. At Lumora, he developed novel molecular diagnostic technologies now endorsed by the USDA for food safety. He is author on numerous patents and was a winner of the UK's Biotechnology and Biological

Sciences Research Council (BBSRC) innovator of the year 2012. In 2015 Lumora was acquired by Erba Mannheim and Dr Tisi runs the molecular diagnostic division responsible for novel diagnostics platforms including, this year, a freeze-dried PCR test for Covid-19 perfect for low-resource settings without cold-chain capabilities.

2. Diagnosis

Two main types of testing are used to aid the diagnosis of COVID-19 during an active infection; molecular tests that detect the presence of the RNA genome, and antigen tests that detect the presence of viral antigens, such as the viral protein coating.²⁰ Additionally, serological tests (antibody tests) that target the immune response of an individual post-infection, have also been deployed during this pandemic.²¹ As COVID-19 is still a relatively new disease, clinicians have monitored host antibody levels to determine how sustained the natural immune response is, post-infection. Such investigations have revealed that most COVID-19 patients develop antibodies against SARS-CoV-2, one to three weeks following symptom onset, with high levels of neutralising antibodies detected in severe cases.^{22–24} The World Health Organization (WHO) does not recommend the use of antibody tests, in isolation, for the diagnosis or clinical management of acute infection.^{25,26}

Molecular testing is conducted to identify COVID-19 positive individuals who are: symptomatic and develop mild (paucisymptomatic) to severe disease; asymptomatic (may not express symptoms); and presymptomatic (infected individuals that may develop symptoms after a positive test result). The WHO recommends a testing cascade for the clinical management of patients suspected of COVID-19 infection (Fig. 1).

The management of the COVID-19 disease burden has relied upon molecular testing within specialised laboratories and the rapid transportation of these samples from collection points within communities. Nasopharyngeal (NP) swabs are by far the most routinely collected samples, although other sample types have been used. The Centers for Disease Control & Prevention (CDC) specifies the range of upper respiratory tract (URT) and lower respiratory tract (LRT) specimens that are compatible with a given commercial test kit.²⁷ Since prolonged detection of SARS-CoV-2 RNA has been reported in faecal samples, the testing of this sample type during the later stages of this disease



Dr Hannah S. Leese is an assistant professor in the Department of Chemical Engineering at the University of Bath and leading the Materials for Health Lab. She received her Ph.D. in Chemical Engineering from the University of Bath and was post-doctoral research associate at Imperial College London (2013–2017) and the University of Manchester (2017–2018). Hannah's current research focus

includes microneedle biosensors, molecularly imprinted polymers for disease detection, and therapeutic textiles supported by EPSRC, the Royal Society and CRUK.





Fig. 1 WHO testing cascade for diagnosis of COVID-19 (adapted from WHO, 2020,²⁶ created with BioRender.com). If a patient meets the clinical criteria for COVID-19 as specified by the WHO, a NAAT (Nucleic Acid Amplification Test) test is recommended. Antigen testing can be used alongside the NAAT depending on the prevalence of disease and the sensitivity and specificity of the antigen test. Following a positive result by the NAAT, the patient is classified as a positive case. Confirmation of weak positive results either through repeat NAAT testing or in some cases, viral sequencing is recommended. Negative results do not rule out COVID-19 infection and in the event of sustained clinical suspicion due to an epidemiological link and other clinical findings such as radiological signs (chest CT scans), repeat testing is recommended. A positive result on repeat testing is identified as a confirmed case. A negative result upon retesting can be further evaluated using one serum sample collected in the acute phase and one sample collected in the convalescent phase, to explore seroconversion or a rise in antibodies.²⁶

has been proposed by the WHO.²⁸ Irrespective of the sample type, laden swabs are placed within viral transport medium (VTM) for storage during transportation, followed by nucleic acid extraction using commercially available kits, and then amplification and detection of RNA in the case of molecular testing.^{27,29} There are currently over 240 molecular amplification chemistries that have been approved for emergency use (EUA) by the U.S. Food & Drug Administration (FDA).³⁰ Most approved assays are based on reverse transcription polymerase chain reactions (RT-PCR).

2.1 Polymerase chain reaction

All RT-PCR amplifications are initiated by a reverse transcriptase, which is an enzyme that typically converts two or more targeted regions of the SARS-CoV-2 viral RNA into complementary DNA

(cDNA); this component of the reaction is performed at a constant temperature for a few minutes prior to the PCR reaction. The resultant cDNA is then exponentially amplified *via* a series of thermal cycles (Ct; normally 35–40), in a reaction that has the potential to double the targeted DNA load within each consecutive cycle. The increasing amount of amplified product generated during the thermal cycling is typically detected by exciting fluorophores that are coupled to target specific nucleotide sequences, which become unquenched during the amplification process.³¹ The quantity of initial SARS-CoV-2 RNA is inversely proportional to the Ct in which fluorescence is detected (Fig. 2). A typical RT-PCR assay will use a maximum of 40 Ct's and when fluorescence is detected above a given signal intensity, this would be regarded as a positive result.³²

Many factors can affect the efficiency of a given RT-PCR, particularly the choice of sample and the method of





Fig. 2 Ct thresholds (adapted from Public Health England, 2020,³² created with BioRender.com). The Ct value refers to the cycle number at which the fluorescence level exceeds a set threshold. In this case, the Ct value is 26. The Ct cut off is set at 40 Ct's and therefore all samples yielding a Ct greater than 40 are considered as negatives.

extraction, as well as the timing of sample collection. Individuals experiencing prolonged viral shedding with low viral loads (Ct values greater than 40 Ct's), can be inaccurately called out as negative. Similarly, borderline qualitative positive results cannot adequately differentiate between naked, sheared or encapsulated viral RNA and these tests currently do not indicate an individual's potential to transmit the disease.^{33,34}

RT-PCR is incredibly sensitive and enables a high throughput analysis of samples; each run can typically allow the comparison of positivity for at least 90 presumptive samples. However, this amplification is slow compared to alternative polymerase chain reactions. RT-PCR also requires experienced operators, complex automated extraction technologies and logistical pipelines. There are however variants of the PCR technology that utilise the reverse transcriptase, but do not rely on thermal cycling to amplify the cDNA but can instead be amplified at a constant temperature. These so-called 'isothermal technologies' all use sophisticated priming mechanisms and strand displacement polymerases and are more tolerant to sample derived inhibitors. These isothermal amplifications can normally achieve a similar sensitivity to RT-PCR with the advantage of being maintained on a simple heating block with the potential for faster turnaround times.²⁹

2.2 Isothermal technologies

Many examples of isothermal PCR amplifications have now been developed and commercialised for SARS-CoV-2 RNA amplification: loop mediated isothermal amplification (RT-LAMP), recombinase polymerase amplification (RPA), nicking endonuclease amplification reaction (RT-NEAR), and transcription mediated amplification (RT-TMA).^{30,35,36} These isothermal technologies offer the advantage of shorter turnaround times compared to RT-PCR (less than 1 hour in most cases) and most can be deployed at the point-of-care (POC) and within limited resource settings.³⁷

RT-LAMP typically uses 6 primers to target 8 positions within a conserved portion of the SARS-CoV-2 genome.³⁸ The amplification process takes place at a steady temperature and makes use of strand displacement polymerases, which are more resistant to typical PCR inhibitors and afford the invasion of the duplex DNA molecules that are primed from the cDNA.³⁹ During the propagation of RT-LAMP, some of the primers fold over after polymerisation forming open loop structures in the amplicon that can be effortlessly re-amplified.

Several RT-LAMP based assays have been developed for the detection of SARS-CoV-2 including the rapid, affordable, easy-to-use multiplex RT-LAMP assay with a turnaround time of 1 hour among others.^{40–42} Despite the benefits offered, designing multiple primers for the assay is complex and therefore multiplexing the assay is difficult.²⁹

NEAR uses a strand displacement polymerase and nicking endonucleases that exponentially amplify the SARS-CoV-2 target sequences.⁴³ One of the main limitations of NEAR, is the formation of non-specific products, which affect the amplification efficiency and sensitivity; despite this, recent optimisations have been shown to limit the impact of nonspecific amplification.⁴⁴ NEAR forms the basis of the ID NOW test, developed by Abbott Diagnostics for the detection of SARS-CoV-2 and is complete within 15 minutes.²⁹

NEAR is similar to RPA, as this method only requires a pair of primers to amplify any given sequence, and it can be performed at lower temperatures compared to most other isothermal amplifications, with equivalent turnaround times. However, RPA suffers from unwanted priming interactions and requires multiple enzymes that can impact the specificity and complicate the manufacturing process.²⁹ Examples of these RPA based SARS-CoV-2 assays have been published, but to date, no tests have been commercialised.^{45,46}

TMA has been commercialised by a few companies and a few have been approved for EUA, including the Aptima SARS-CoV-2 assay developed by Hologic and Pacific Diagnostics, among others.³⁰ The TMA based Panther Fusion assay was reported to be simpler to use and expressed higher sensitivity with 100% detection down to 5.5×10^3 copies per ml, which was an order of magnitude greater compared to RT-PCR.⁴⁷

Despite the advantages that these isothermal technologies afford, RT-PCR is still more widely used for SARS-CoV-2 testing for several reasons. For instance, RT-PCR has been implemented for decades and is now considered to be the gold standard molecular amplification method. Access to RT-PCR machines is common in hospitals and centralised laboratories throughout the world, as are the consumables, support networks and infrastructure. For many of these reasons RT-PCR has dominated the testing landscape during the COVID-19 pandemic.^{29,48}

2.3 The need for sensitive and specific diagnostic tests

Sensitive and specific molecular amplification technologies are essential and have helped to inform clinicians on the accuracy of a given respiratory diagnosis.⁴⁹ Manufacturers and end-users are required to determine the analytical and clinical performance of their test with the use of a reference panel.^{30,50,51}



The limit of detection (LoD) is a measure of an RT-PCR's analytical sensitivity and is defined as the lowest concentration of the target that can be detected with a probability of detection of 95% at a stated level of confidence.⁵² A test with a low LoD or high analytical sensitivity will be able to detect individuals with low viral loads, thereby limiting the number of false negatives.⁵³ Although the exact definition for a high or low viral load has not been defined for SARS-CoV-2, studies do report that the average viral load from an NP or oropharyngeal (OP) swab is approximately 10^5 cp per ml up to 5 days post symptom onset, with a maximum load of 7.11×10^8 cp per swab; higher viral loads have been reported for sputum samples (2.35×10^9 cp per ml).⁵⁴ Another study investigating viral loads in throat and sputum samples revealed that viral loads ranged from 641 to 10^{11} cp per ml.⁵⁵

According to the WHO and the Medicines and Healthcare products Regulatory Agency (MHRA), an analytical LoD of 1000 cp per ml for a diagnostic test for any respiratory tract specimen type is considered as an acceptable LoD, whilst an analytical LoD of less than 100 cp per ml in upper/lower respiratory tract specimens and stool samples is desirable.^{56,57} Another important consideration in the reporting of the LoD is that values are reported using different units such as cp per ml, genome equivalents per ml, copies/rxn.^{58,59} According to the target product profile for COVID-19 testing developed by the WHO, there is currently no international standard unit. Nonetheless, the use of a standard metric may enable better comparisons to be drawn between workflows.^{49,59} Currently, the most sensitive tests that have received FDA EUA approval include the PerkinElmer New Coronavirus Nucleic Acid Detection Kit and the

Viracor SARS-CoV-2 assay, which both achieve an LoD of 180 NAAT NDU per ml (NAAT detectable units per ml).⁵⁰

2.4 Factors affecting test sensitivity and specificity

Several factors affect the accuracy of molecular tests including the site and quality of sampling, the stage of disease, rate of viral clearance, and prevalence. In addition, design features of the molecular test are also important to consider *e.g.*, genes targeted and the reliance on amplification from multiple targets. For these reasons, a test is never 100% accurate and the lack of a gold standard for benchmarking performance, further compounds this problem.⁶⁰

False negatives and false positives can be derived from the factors described in Fig. 3.^{61,62} False negatives can lead to infected individuals circulating within the community, unwittingly propagating the pandemic; these misleading results could arise from inadequate sample loads, sampling individuals too early or too late within the disease cycle, and/or result from the degradation of the viral genome.⁶³ False positives, which affect a test specificity, can result from the contamination of reagents such as primers, or contamination occurring during sample collection and processing, and this in turn could lead to an overestimation of COVID-19 incidences.^{61,64,65} This review will now explore some of the other key factors that have been reported, which influence the sensitivity of these molecular tests.

2.4.1 Specimen type. The CDC reports a range of acceptable specimen types for the diagnosis and surveillance of COVID-19 in addition to NP swabs, including oropharyngeal swabs, saliva, and nasal washes.^{27,54,66,67} NP swabs are invasive and



Fig. 3 Factors determining the accuracy of molecular tests (created with BioRender.com). Most PCR tests are recommended as an aid for diagnosis and therefore the WHO recommends that clinicians consider the factors described above when interpreting test results, as a measure to reduce the occurrence of false negatives and false positives.



uncomfortable for the recipient, and therefore in the event of self-testing, individuals may not collect their sample adequately. If the testing is performed by healthcare professionals (HCPs), there is a greater risk of transmission, logistical frameworks and personal protective equipment (PPE).⁶⁸

Saliva samples, which are easy to collect, enable much more simplistic self-sampling, thereby reducing the risk of transmission to HCPs; this sample type has been proposed as a more suitable alternative.⁶⁸ Several studies support the use of saliva, with evidence to prove that similar sensitivities were achieved compared to NP swabs.^{69–71} Some reports also demonstrate that saliva was a more viable alternative compared to NP swabs for the diagnosis of severely infected patients, and for the screening of asymptomatic and presymptomatic individuals,⁷² whilst other reports are contradictory.⁶⁸ Although the CDC recommends saliva as an acceptable sample type, the WHO does not recommend the use of saliva as the sole specimen type for diagnosis and screening of SARS-CoV-2.⁷²

The use of faecal samples for the detection of SARS-CoV-2 has also been explored.^{28,54,67,73} A review by van Doorn *et al.*⁷⁴ revealed that 43% of patients tested positive for SARS-CoV-2 *via* anal swabs and faecal samples, up to 70 days following symptom onset. Viable virus was found in 35% of the patients examined and positive faecal samples remained positive for up to a maximum of 33 days following negative results obtained from a respiratory tract sample.⁷⁵ In line with this finding, Chinese authorities have recently adopted the use of anal swabs for mass screening of individuals as a more stringent effort to mitigate transmission.⁷⁶

Given these findings, it is clear that the distribution of SARS-CoV-2 viral load varies markedly between different sample types, as well as between the collection method *i.e.*, self-collection, clinician collection or supervised self-collection.⁷⁷ For these reasons, obtaining specimens from multiple sample types may help to improve the sensitivity of testing and limit the occurrence of false negative results.^{67,78}

2.4.2 Transport media. Viral transport medium (VTM) ensures that the virus is preserved during sample transportation to a centralised testing facility.⁷⁹ Studies have also highlighted the capacity for collection sites and clinics to store NP swabs in alternative preservatives that include phosphate buffered saline (PBS), minimal essential media, saline, universal transport media, M4 medium, ESwab. Once collected, samples are stored at temperatures ranging from ambient to -30 °C. It was noted that a slight decrease in RNA yield, represented by an increase in Ct value was measured for samples stored in saline (the reason for this increase is still unknown).^{80–82} An alternative transport medium called molecular transport medium (MTM) offers several advantages over VTM, including inactivation of the virus, which reduces infection risk when handling samples, and it enables samples to be stored at ambient temperatures for several days.⁸³ VTM often requires cold storage and may contain nucleases that degrade the SARS-CoV-2 RNA.^{83,84} For these reasons, it is essential to refer to the manufacturer's instructions for molecular assays when determining the process recommended for sample

collection and transport, in order to minimise the potential for reporting false results.⁸⁵

2.4.3 Time of sampling. The time-of-sampling relative to the time-since-exposure is another crucial factor that affects perceived viral loads within samples. The percentage of false negative results has been reported to decrease from 100% for samples collected on day 1 of exposure, to 68% for samples collected on day 4, to 38% for samples collected on day 5 (day of symptom onset) and to 20% by day 8.⁸⁶ This data suggests that the optimal time of testing, is around day 8 after the initial exposure or 3 days after symptom onset. Whilst viral loads may be high in the URT following the initial exposure to SARS-CoV-2 and during the time of symptom onset, loads do tend to decline thereafter. Therefore, it may be more appropriate to use LRT samples to ensure adequate detection in individuals presenting late within the disease cycle.^{67,87}

2.4.4 Sample preparation method. Efficient RNA extraction and purification determine the yield of RNA introduced into the RT-PCR reaction and therefore the sensitivity of the test.⁸⁸ Several studies have evaluated the efficiency of existing automated *vs.* manual extraction platforms. For instance, the King Fisher exhibited lower Ct values in comparison to the easyMAG and EZ1 extraction platforms, which may be attributed to differences in the final elution volumes.⁸⁹ In addition, the sample type, the method employed for extraction (magnetic beads, spin column), the sample input volume, and the composition of buffers all affect the overall sensitivity of the workflow.⁹⁰ The sample processing times also vary greatly between extraction platforms, which will alter the overall test turnaround time.⁸⁹

The RNA extraction step is the rate-limiting step in any workflow, due to the challenges associated with liquid handling, the cost of reagents, consumables, and machines. Some examples of methods are now emerging that have the potential to allow the direct addition of crude RNA to a given amplification.⁹¹

2.4.5 Molecular assay design. The product profile recommended by the UK government for laboratory-based tests should make use of multiple targets and must include appropriate internal controls.⁵⁶ The inclusion of positive and negative controls within an array of tested samples helps the analyst determine the accuracy of the test result, by highlighting potential contamination events, RT-PCR inhibition and problems associated with nucleic acid extraction and degradation.⁹² The inclusion of efficient internal controls also identifies any inefficiencies in sample extraction and sample collection.⁹³

The RNase P (RP) internal control used in the CDC SARS-CoV-2 assay amplifies both the human genomic DNA (gDNA) and reverse transcribed RNA. Therefore, in the presence of human gDNA, the RP control yields a positive signal even if the RNA extraction has failed or if the sample processing resulted in RNA degradation; in these instances, the negative SARS-CoV-2 results obtained would be interpreted as false negative results.⁹⁴ The potential for these types of false negative results has prompted a redesign of the RP internal control to specifically detect human RP mRNA and not the genome.⁹⁵



The inclusion of multiple target regions into an RT-PCR can help to minimise the proportion of false negatives that occur because of mutations arising in the regions of the genome that are targeted by these tests and gives information about genome integrity.^{96,97} For instance, the emergence of the variant (B.1.1.7; 501Y.V1), which harbours several new mutations have caused concern with regards to the sensitivity of certain diagnostic tests. In fact, the 69–70 deletion has resulted in the spike (S) gene dropout when using the TaqPath COVID-19 Combo Kit (ThermoFisher). Given that the TaqPath RT-PCR test targets multiple sequences, the overall impact of the mutated spike gene is minimal.^{98–100} For instance, the TaqPath is currently being used by PHE to monitor the prevalent Delta variant (B.1.617.2) through detection of the S gene. Other variants of concern include the B.1.351, P.1, P.2, B.1.427, B.1.429, B.1.525, B.1.526, A.23.1 with E484K, B.1.1.7 with E484K.¹⁰¹ Whilst RT-PCR tests and isothermal assays tend to be less affected by the current mutations of concern, as they typically target multiple regions spanning the entire SARS-CoV-2 genome, the effect of these mutations on the rapid antigen tests may become insidious, as the virus evolves and the target antigen degenerates.^{102,103} The frequent occurrence of such variants also highlights the importance of sequencing technologies that rapidly identify any mutations occurring at the PCR test target region.⁶¹ Understanding the operational false positive and negative rates within centralised testing facilities is also a key determinant that affects the accurate reporting of results. In line with this, Public Health England has put forward recommendations that may help in the determination of these values.^{61,104}

2.4.6 Reporting Ct values. The Ct value of RT-PCR is inversely proportional to the starting concentration of the target nucleic acid. For a 100% efficient PCR reaction, each 3.3 Ct's or cycles represents a 10-fold change in starting concentration.^{105,106} Some researchers suggest that current RT-PCR tests, which have high cycle time thresholds (between 37–40 Ct's) may be too sensitive and whilst detecting infectious individuals, these borderline positive samples may also detect individuals experiencing prolonged viral shedding, but who are no longer infectious.¹⁰⁷ However, such highly sensitive tests are important for the identification of acute patients with low viral loads.¹⁰⁸

The reporting of Ct values may enable clinicians to distinguish between patients with high or low viral loads, and these results can help clinicians make patient-specific interventions, prioritise the clinical management of individuals who are likely to be more infectious, determine the need for isolation, help with contact tracing strategies to prioritise the search for contacts of cases with low Ct's and to aid epidemiologists pinpoint COVID-19 outbreaks.¹⁰⁷

One of the main obstacles for using Ct thresholds to manage COVID-19 relates to differences in the LoD for a given workflow, as these often use different reagents and sample preparation methods (extraction and elution volumes). In addition, some assays do not report Ct values, whilst others directly report the results.³² For instance, the Cepheid Xpert Xpress SARS-CoV-2 kit

and the Abbot Real Time assay have an identical workflow LoD as reported by the FDA. However, due to higher thresholds set for the Cepheid assay, several negative samples called by the Abbott assay were reported as positive with the Cepheid assay.^{109,110} For these reasons, some researchers recommend that Ct values are only provided to clinicians on a case-by-case basis, while others advise against the use of Ct values for patient management.^{108,111–114} As Ct values have also been used as a marker to determine viral loads associated with transmission, the implications of quantification will be explored below.¹¹⁵

All the factors highlighted above form part of the quality management system that is required when manufacturing, performing and reporting results of SARS-CoV-2 tests. Quality control of COVID-19 molecular tests is essential and must be included in every step of the diagnostic testing scheme. This includes but is not limited to the validation and verification of the molecular test using reference panels such as those provided by the FDA to ensure the test performs as intended and thereby gain regulatory approval, and the inclusion of external and internal quality controls to validate nucleic acid extraction and PCR.⁵⁰ Quality control also encompasses aspects of the testing laboratory including external quality assessments to monitor the performance of the laboratory and international accreditations.^{116,117} The need for a standardised quality management system is clear by the efforts of the International Organisation for Standardisation (ISO) who are now drafting a quality management standard for NAATs that detect SARS-CoV-2.¹¹⁸

3. Viral load quantification

Whilst an understanding of viral load has proved particularly important in treating and monitoring HIV disease progression, the importance of quantifying COVID-19 has not yet been clearly defined. As discussed earlier, the presence of SARS-CoV-2 RNA in a sample does not necessarily indicate the presence of viable virus.¹¹⁹ Viral cultures prepared from clinical samples generate a more accurate representation of a sample's potential for viral replication; despite this, positive viral culture results do not always concur with a qualitative positive RT-PCR result.⁵⁴ Due to the challenges and the obvious risks associated with routinely preparing and propagating live viral cultures, this technique is not recommended for the routine diagnosis of infectivity, and viral load determined by RT-PCR is instead regarded as the best surrogate for determining an individual's propensity to transmit the virus.^{26,34,120}

The relationship between high nasopharyngeal/saliva/plasma viral loads and disease outcomes, such as intubation, disease progression, systemic inflammation and death has been reported.^{121–127} Some studies demonstrate that high viral loads in nasopharyngeal swabs are not necessarily related to disease outcomes, such as hospital admission, length of oxygen support and mortality, but instead the data accumulated links mortality directly to other factors, such as an uncontrolled inflammatory response, underlying co-morbidities and a patient's age.^{127,128} Undoubtedly, viral load quantification *via* RT-PCR could be



a useful tool for identifying individuals with a high burden of disease, which in turn, would enable rapid triaging of patients to appropriate medical departments.

Viral load monitoring can also be used to explore the rate of secondary transmission, which may help with implementing effective control measures and inform a clinician when a patient can be released from isolation, predicting future outbreaks and determining COVID-19 incidence within communities.^{67,129–132}

Viral load in respiratory specimens is often higher in patients with mild disease compared to patients with severe disease, whereas no such difference was reported for stool and serum samples.³⁴ Furthermore, viral loads in respiratory samples obtained from patients with mild disease have been reported to increase in the first week after symptom onset, peaking within 2 weeks, then declining thereafter. Whereas those with severe disease had high viral loads even 3–4 weeks after symptom onset. This study also suggests that older individuals over the age of 60 have prolonged infections (viral loads of 1.40–2.19 log₁₀ RNA cp per test) up to 2 weeks post symptom onset, whilst children under 10 experienced peak viral loads (2.50 log₁₀ RNA cp per test) during the first 2 days following symptom onset declining rapidly thereafter.¹³³

Recent reports published by bodies such as the AACC (American Association for Clinical Chemistry) and PHE have outlined the challenges with using Ct values for estimating viral loads thereby clearly highlighting the need for quantitative molecular technologies for viral load monitoring, which would help to support both clinical and public health decisions (Fig. 4).^{113,114,129,133,134}

4. Mass screening

Mass screening refers to the process of testing large numbers of individuals regardless of their symptoms. The objective of this testing strategy is to identify individuals who are positive for SARS-CoV-2 and to use this information to determine the most effective intervention for managing transmission. Such testing regimes can be implemented at a household level, at an individual level (testing before international travel & returning to work), and for monitoring of disease in crowded indoor spaces such as schools and healthcare settings. Screening can also be coupled with contact tracing to inform individuals who have come into close contact with individuals who have tested positive upon screening.¹³⁵

The main considerations for controlling the spread of COVID-19 are to account for the possibility of asymptomatic or pre-symptomatic transmission.^{43,136–145} Presymptomatic transmission has been defined as transmission from a COVID-19 positive individual (index) to another before the recipient displays any symptoms for COVID-19. The potential to infect is highest when an individual is close to symptom onset (Fig. 4).^{54,146–150} However, the potential for asymptomatic transmission in comparison to symptomatic transmission is still unclear, some reports suggest similar transmission potentials due to similar viral loads observed between asymptomatic and symptomatic cases,^{148,149} whilst others suggest that the potential to transmit is low from asymptomatic individuals.^{151,152}

Early studies investigating the transmissibility of SARS-CoV-2 among children reported that children were less susceptible to infection, and experienced milder disease compared to adults. However, more recent studies have determined that



Fig. 4 SARS-CoV-2 viral load and testing (Adapted from Cevik *et al.*,¹⁴⁷ Gugleimi,¹⁶⁶ Larremore *et al.*,¹⁶⁷ created with BioRender.com). Following exposure to the virus, SARS-Cov-2 viral load in the upper respiratory tract peaks during week 1 since symptom onset. *Individuals can continue shedding infectious virus for approximately 14 days. Highly sensitive PCR tests can detect the presence of virus prior to symptom onset (** pre infectious), through the infectious phase and even post-infection. Antigen tests with their low sensitivity threshold can only detect high viral loads when individuals are likely to be most infectious.



children of all ages are prone to infection, mortality, and transmission.¹⁵³ Yonker *et al.*, reported that one-third of school children displaying symptoms were positive for SARS-CoV-2.¹⁵⁴ Although younger children are less susceptible and pose a lower risk of transmission due to lower expression of ACE2 receptors.^{154–157}

Elements of the literature state the proportion of infections related to asymptomatic transmission can vary from 17–20%.^{158,159} Understanding this variation, along with reports of low rates of transmission from asymptomatic individuals, some researchers claim that mass screening for asymptomatic individuals may be a waste of resources.¹⁶⁰ Instead, it has been suggested that more effort should be focused on rapidly identifying symptomatic individuals until further evidence is available to implicate the transmission rates from asymptomatic cases. Nonetheless, given that asymptomatic and presymptomatic individuals could equally pose a significant risk to public health, researchers have proposed the frequent use of rapid antigen tests with rapid turnaround times (15–30 min). Such rapid and accessible tests when performed frequently, do facilitate effective surveillance and tend to be more affordable and easier to use than their more sensitive molecular alternatives. Antigen tests have been recommended for use by the CDC for screening in high-risk congregate settings such as housing schemes, nursing facilities, universities, workplaces, airports among others and the FDA have currently issued EUA to 25 commercial antigen tests as of July 2021.^{161,162}

Whilst some reports propose the adoption of antigen tests, others have expressed concern as they tend to have a reduced sensitivity compared to RT-PCR, which may result in a high rate of false negative case reporting.^{49,162,163} However, it is important to note that in order for mass screening to be successful, speed is as important as sensitivity and in countries with limited access to resources and testing infrastructure and those facing a strain on test supply demand, these easy-to-use, affordable rapid antigen tests have helped in controlling the spread of COVID-19.^{164,165}

5. Pooling

The widespread testing of individuals to contain the spread of COVID-19 puts pressure on available testing resources, and the pooling of samples from several individuals may help to relieve some of this pressure.^{168–171} Pooling of samples has been proposed to facilitate rapid population wide screening in regions where prevalence is low.¹⁵ Pooling refers to the practice of combining multiple patient samples and performing one laboratory test on this combined sample, thereby enabling high throughput testing, rapid turnaround times and regular testing.^{168,172} Pooling can be performed at different stages of the workflow *i.e.*, during sample collection, prior to RNA extraction or by combining extracts within an RT-PCR.^{171,173–175} If the result of pooling is negative, then it is likely that all samples within the pool are negative. If the pool is positive, then all the samples within that pool are separated and tested individually. For these reasons, pooling is most effective in settings where the prevalence of infection is low.

Important factors to consider for the pooling of samples include the limit of detection, the specificity and sensitivity of the assay, prevalence of infection, the number of samples in a pool, the efficiency of the pooling strategy, the feasibility, and adaptability to change in prevalence rates.^{171,176} The FDA recommends >85% positive percentage agreement between a pooling test and an individual test, in order for the pooling strategy to receive EUA and be implemented.¹⁷⁵

Aragon-Caqueo *et al.*,¹⁷⁷ propose a model to determine the optimal pool size based on the prevalence of infection and highlight that as prevalence increases, the pooling strategy loses its effectiveness. Hence, the WHO does not recommend the routine use of pooling in clinical settings or for contact tracing.²⁶ The first EUA for a pooling test was granted by the FDA for the Quest SARS-CoV-2 RT-PCR test (Quest Diagnostics) for use of pooling up to 4 samples, in July 2020.¹⁷⁸

The occurrence of false negatives caused by the dilution of positive samples within a pool and the impact of this on RT-PCR test sensitivity remains a concern.^{179,180} Lohse *et al.*, provide evidence to support the pooling of 30 samples at a prevalence rate of 1.93%, resulting in acceptable diagnostic accuracy.¹⁷⁹ Yelin *et al.*, reports that a single positive sample can be detected in pools of up to 32 samples with a false negative rate of 10% and this investigation suggests including additional PCR cycles over 40 cycles to enable the detection of samples with low viral loads.¹⁸¹ Wacharapluesadee *et al.*, reports that the sensitivity of the test is not affected by pooling strategies, even at a prevalence rate of 20%, with the provision that the Ct value of the individual positive sample is less than 35.¹⁸² The inclusion of more than one positive sample in a pool has also been supported in studies where the inclusion of an increasing number of positive samples reduced the Ct difference between individual and pool tests and increased the sensitivity of PCR testing.¹⁷⁴

Barak *et al.*, reports a highly efficient 8 and 5 sample pooling strategy, that spared 76% of RT-PCR and extraction tests upon pooling of nearly 135 000 samples.¹⁷¹ The increase in efficiency and sensitivity of their strategy has been explained by the non-random distribution of samples. Although the assumption is that samples are randomly distributed within a pool, most samples arrive in batches, with each batch containing samples from a particular university, school, community *etc.* As a result, samples from each batch are likely to be pooled together from geographical areas of a high or low prevalence, which is thought to improve the efficiency and sensitivity of pooling. The improvement in efficiency and sensitivity and therefore a reduction in false negatives can be explained as the inclusion of a single positive sample with a high viral load results in the detection of other low positive samples in a pool using the same number of PCR tests. The same weak positive samples would have a lower chance of being identified if they were placed on their own in a pool.¹⁷¹

Sensitivity is also affected by the process of sample collection and the extraction method.¹⁸³ de Salazar *et al.*, and Lone Lim *et al.*, observe high efficiency across the different nucleic acid extraction and amplification platforms.^{180,184} Positive samples with low Ct values were detected in all volumes tested (25, 40,



60, 100 μ l), although the detection of low viral load samples was affected at low volumes.

Other key considerations for evaluating pooling strategies include ease of use, risk of contamination due to the handling of multiple samples, and risk of sample exchange which creates the risk of false positives.^{168,171,184} Clear protocols that define the procedure for sample pooling and address any losses that occur through the workflow can help minimise the risk of false negatives and simplify the pooling procedure.¹⁸⁵ Furthermore, deconvoluting large pools in the event of positive samples outweighs the time benefits offered by pooling.¹⁸³ For this reason, prevalence rates must always be estimated prior to employing a pooling strategy to reap the benefits of increased testing efficiency and shorter turnaround times. Given the effect of the factors described above, all laboratories must validate their pooling strategies based on the prevalence rate and sensitivity of the test being used and could consider the use of such strategies in settings where there is a strain on testing infrastructure.¹⁸⁶

6. Environmental contamination

The need for environmental swabbing to manage the spread of COVID-19 has been warranted upon the knowledge that SARS-CoV-2 virions remain infectious on surfaces for up to 72 hours.^{9,187,188} The overall objective of this type of testing is to determine the presence of SARS-CoV-2 RNA on surfaces following disinfection, whereby positive results may trigger further cleaning and negative results will confirm effective disinfection protocols.

Several commercial environmental swabbing test kits are available for monitoring surface contamination in a range of different settings including, workplaces, food processing facilities, public transport hubs and vehicles, healthcare settings (dental practices and hospitals), communal areas among others.^{189,190}

The stability of SARS-CoV-2 in aerosols and surfaces has been reported, with the longest period of viability observed on stainless steel and plastic surfaces.⁹ Whilst some studies have provided evidence for environmental contamination by presymptomatic, paucisymptomatic, symptomatic and asymptomatic individuals, thereby warranting proper disinfection programmes, other reports demonstrate no such environmental contamination by infected individuals.^{191–196}

The WHO has provided guidance for surface swabbing that should sample from a surface area of no less than 25 cm³ with the use of multiple swabs.¹⁹⁷ Parker *et al.*, describe a protocol (using Isohelix swabs for sample collection, DNA/RNA shield preservatives, automated RNA extraction and RT-qPCR based amplification) that can be followed for environmental swabbing and report the need for a minimum of 1000 viable viral particles per 25 cm³ in order to ensure successful recovery and detection of the virus.¹⁷ This study assessed the effect of using swabs for surface sampling with different transport medium and preservatives, extraction methods, and inhibition caused by cleaning agents and components of building materials and concluded there were no significant differences in viral recovery rates

between the different RNA extraction methods and debris left-over by cleaning fluids. Differences in recovery rates of viral particles were observed between different surface materials and between sample collection methods, with the highest recovery recorded when swabbing from stainless steel. SARS-CoV-2 RNA and viable virus was also detected in air samples obtained in hospitalised settings, with RNA detected at higher concentrations during the early stages of infection, thereby providing evidence for acquiring COVID-19 *via* inhalation.^{198,199} As discussed earlier, SARS-CoV-2 RNA has been detected in the faeces of infected individuals and has led to the initiation of wastewater monitoring for assessing community disease burden.^{200–203}

Overall, these studies provide evidence for the SARS-CoV-2 to persist on surfaces, within the air and wastewater and highlight the importance of monitoring these environments to control the spread of COVID-19 and to broaden our understanding with respect to transmission pathways.

7. Commercially available molecular diagnostic tests

As of July 2021, over 250 molecular tests had received EUA by the FDA, of which over 100 were evaluated using the FDA reference panel.^{30,50} This section aims to compare several commercial technologies that were selected based on evidence from published literature.

As described earlier, the majority of SARS-CoV-2 *in vitro* diagnostics (IVDs) utilise RT-PCR for the amplification of viral RNA. A performance study assessing four workflows; ePlex assay (GenMark), Simplexa (DiaSorin), Panther Fusion SARS-CoV-2 (Hologic) and a CDC assay, reported that the ePlex demonstrated a higher LoD compared to the Panther Fusion and Simplexa, which failed to detect samples with viral loads of less than 1000 cp per ml. The Simplexa workflow also offers short turnaround times (approximately 1.8 hours) compared to other workflows, such as the Allplex, Quanty (Seegene, 96 samples in 4–5 hours), and Panther Fusion (120 samples in 4 hours). For these reasons, the Panther Fusion assay, Allplex and Quanty have been proposed for use in settings that require a high throughput of samples, whilst the ePlex and Simplex assays were shown to be more suited to settings that require rapid turnaround times but have fewer sample numbers to contend with.^{204–206}

Comparisons between the Cepheid Xpert Xpress SARS-CoV-2, the Roche Cobas® SARS-CoV-2 and the Roche LightMix assay revealed high concordance for samples with high viral loads with Ct <34, this, however, dropped to 22% for samples with Ct >34.²⁰⁷ For these reasons, confirmatory testing of samples that yield negatives is recommended. In contrast, other studies report excellent agreement between the Cepheid Xpert and the Roche Cobas assay and superior sensitivity for both compared to the Becton Dickinson MAX assay, the GenMark ePlex and NeuMoDx Molecular assay.^{208–210}

Given that the SARS-CoV-2 viral load in clinical samples are reported to be greater than 10⁵ cp per ml, most of the



Table 1 FDA EUA commercial molecular diagnostic tests

Name	Manufacturer	Sample type(s)	LoD (NDU per ml)	Amplification	Applications		
					POC	Screening	Pooling
Abbott RealTime SARS-CoV-2 assay	Abbott molecular	Nasal swabs – self-collection (SC) at healthcare location or by HCP. NP and OP swabs, and bronchoalveolar lavage (BAL) specimens collected by HCP	2700	RT-PCR	No	No	No
Accula SARS-CoV-2 test	Mesa Biotech Inc	Nasal or nasal mid-turbinate (NMT) swab samples collected by HCP/SC/clinician-supervised self-collected	Under review	RT-PCR	Yes	No	No
Allplex™ 2019-nCoV assay	Seegene, Inc.	NP, OP, anterior nasal swab, NMT swab, NP wash/aspirate, nasal aspirate, BAL and sputum specimens collected by HCP	— ^a	RT-PCR	No	No	No
BD SARS-CoV-2 reagents for BD MAX system	Becton, Dickinson & Company	NP, nasal, NMP, and OP swab specimens, NP wash/aspirate or nasal aspirates collected by HCP	5400	RT-PCR	No	No	No
BioFire COVID-19 test	BioFire Defense, LLC	NP swabs collected by HCP	5400	RT-PCR	No	No	Yes
BioFire respiratory panel 2.1 (RP2.1)	BioFire diagnostics, LLC	NP swabs collected by HCP	6000	RT, Nested multiplex PCR	Yes	No	Yes
Cobas SARS-CoV-2	Roche molecular systems, Inc.	Nasal, NP and OP swab collected by HCP or clinical supervised SC	1800	RT-PCR	No	No	Yes
ePlex SARS-CoV-2 test	GenMark diagnostics, Inc.	NP swabs collected by HCP	— ^a	RT-PCR	No	No	No
ID NOW COVID-19	Abbott diagnostics Scarborough, Inc.	Direct nasal, NP or throat swabs collected by HCP	300 000	RT, isothermal amplification	Yes	No	No
Lucira COVID-19 all-In-one test kit	Lucira health, Inc.	nasal swab collected by SC	— ^a	RT-LAMP	Yes	No	No
NEcov19 RT-PCR assay	Nebraska Medicine clinical laboratory	NP, OP (throat), anterior nasal, NMT swabs, nasal washes, nasal aspirates and BAL specimens collected by HCP	— ^a	RT-PCR	No	No	No
NeuMoDx SARS-CoV-2 assay	NeuMoDx molecular, Inc.	Nasal, NP, OP swabs, BAL specimens collected by HCP. Saliva by clinician supervised SC	5400	RT-PCR	No	No	No
Panther Fusion SARS-CoV-2 assay	Hologic, Inc.	Upper respiratory specimens (such as nasal, mid-turbinate, nasopharyngeal, and oropharyngeal swab specimens) and sputum by HCP	600	RT-PCR	No	Yes	Yes
Simplexa COVID-19 direct assay	DiaSorin molecular LLC	NP, nasal swabs, nasal wash/aspirate, or BAL specimens collected by HCP	6000	RT-PCR	No	No	No
Xpert Xpress SARS-CoV-2 test	Cepheid	Upper respiratory specimens (<i>i.e.</i> , NP, OP, nasal, or mid-turbinate swabs or nasal wash/aspirate) collected by HCP	5400	RT-PCR	Yes	No	No

^a Data unavailable, HCP – healthcare provider.

technologies (with the exception of the ID NOW assay) report LoD's of less than 5400 NDU per ml and therefore will be able to accurately identify positive samples for SARS-CoV-2 (Table 1).⁵⁰ Whilst the technologies highlighted above offer high sensitivity, most are located in centralised laboratories, they require expensive equipment, reagents and trained staff, thereby making these tests generally inaccessible to laboratories within

limited resource settings. These community-based laboratories have warranted the development of point-of-care (POC) tests that are rapid, cost-effective and robust.^{211–213} POC tests can improve infection control measures and enable better management of infected patients.²¹⁴ POC tests are also suited to home testing, which enable GPs to decide if patients can come into clinic for consultations. This type of testing can also service



nursing homes and help to determine which individuals need to isolate and engage employers to determine which employees can return to work.²¹⁵

Studies evaluating the performance of the ID NOW (Abbott) have reported that the Xpert, Cobas, Simplexa, Abbott m2000 and RealTime SARS-CoV-2 assays all have better analytical and clinical performance.²⁰⁴ However, the ID NOW, has a rapid turnaround time (15 min) compared to the Xpert, Cobas and RealTime assays; consequently, both the ID NOW and the Xpert are often deployed at POC.^{204,216–221}

A study assessing the performance of the BioFire Respiratory Panel 2.1-EZ (RP2.1-EZ) against the Xpert Xpress, Roche Cobas and BioFire Defense FilmArray, demonstrated a high agreement between all assays and showcased the capacity for the BioFire Respiratory Panel 2.1-EZ to detect low SARS-CoV-2 viral loads down to 10^3 cp per ml.²²² The ID NOW and Aptima tests in contrast were not able to detect low viral load samples that yielded Ct values which were within the upper and lower 30's when tested on the Roche Cobas and NECOV-19 platform tested.²²³

Other POC platforms include the Accula SARS-CoV-2, which exhibit high overall agreement with a laboratory developed EUA comparator test, however failed to identify some challenging low viral load samples.²²⁴ The Lucira COVID-19 All-In-One test kit is another POC platform that has a turnaround time of 30 minutes.^{224,225} The COVID Nudge was rolled out rapidly in the UK and was reported to have 94% sensitivity, 100% specificity, no sample handling, rapid turnaround time (less than 90 minutes) as well as direct storage of data in the Cloud. However, the platform is limited by its low throughput, as only one sample can be processed at a time. Nonetheless, POC platforms such as the COVID Nudge have the potential to speed up decision making, enabling better clinical management of patients.²²⁶

8. Conclusion

Ascertaining the most effective strategies for testing in a global pandemic is a complicated phenomenon that requires consideration of a plethora of factors during implementation such as the effect of sample type, sample collection time, test characteristics on the sensitivity and specificity of the test. Sound test and trace strategies are also needed to ensure that individuals are tested, results are reported in a timely manner, contacts are identified quickly and placed in isolation.

Testing for SARS-CoV-2 has many uses including diagnostic testing for identification and management of COVID-19 positive patients, population-wide screening and environment monitoring for controlling the spread of COVID-19, and informing public health decisions and viral load monitoring to promote the appropriate management of positive cases. The type of test used (POC, qualitative or quantitative molecular tests, antigen tests) should fit with the required need.²²⁷ For instance, rapid, affordable antigen tests despite their low sensitivity may prove to be more useful in the context of mass screening. The cost vs. benefits of strategies such as pooling and mass screening is also being debated. It is clear that there is not one strategy that is

sufficient to control the spread of COVID-19. Instead, what is important is ensuring that the guidelines for each of these strategies are adapted to fit the current requirements.^{228,229} Furthermore, research has proved that the adoption of combinatory strategies including testing, contact tracing and isolation reduces the effective reproductive number to a greater extent than when one strategy is employed on its own.²³⁰

Hence, the molecular testing strategies described throughout this review must be implemented alongside other measures such as contact tracing, physical distancing and isolation of positive cases to limit the spread of COVID-19.

Abbreviations

AACC	American association for clinical chemistry
ACE2	Angiotensin-converting enzyme 2
BAL	Bronchoalveolar lavage
CDC	Centers for disease control and prevention
cDNA	Complementary DNA
cp	Copies
CT	Computed tomography
Ct	Cycle threshold
ECDC	European center for disease control and prevention
EUA	Emergency use approval
FDA	U.S Food & Drug Administration
gDNA	Genomic DNA
HCP	Health care professional
ISO	International Organisation for Standardisation
IVD	Invitro diagnostics
LAMP	Loop mediated isothermal amplification
LoD	Limit of detection
LRT	Lower respiratory tract
MERS-CoV	Middle East respiratory syndrome coronavirus
MHRA	Medicines and healthcare products regulatory agency
MTM	Molecular transport medium
NAAT	Nucleic acid amplification test
NDU per ml	NAAT detectable units per ml
NEAR	Nicking endonuclease amplification reaction
NMT	Nasal mid-turbinate
NP	Nasopharyngeal
OP	Oropharyngeal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHE	Public health England
POC	Point of care
PPE	Personal protective equipment
QCMD	Quality control molecular diagnostics
RNA	Ribonucleic acid
RP	RNase P
RPA	Recombinase polymerase amplification
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SC	Self collection



TMA	Transcription mediated amplification
URT	Upper respiratory tract
VTM	Viral transport medium
WHO	World Health Organization

Author contributions

Conceptualisation: HJ and HSL; writing – original draft HJ, GK and HSL, writing – review & editing, all authors.

Conflicts of interest

There are no conflicts to declare.

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