UK Health Security Agency

PCR testing for SARS-CoV-2 in the UK during the COVID-19 pandemic

Use of PCR in the National Testing Programme

April 2023

Contents

Purpose of this document

The coronavirus (COVID-19) testing programme was developed during the pandemic when the rise in hospitalisation of COVID-19 cases threatened to overwhelm the NHS. In such a rapidly deployed and evolving programme, communication of the rationale for testing and the interpretation of test results did not always keep pace with the evolution of the programme and we attempt to redress that balance here.

Previous outbreaks of serious human coronavirus infections (SARS in 2002; MERS in 2012) produced considerable problems with nosocomial spread of infection within healthcare facilities yet there was limited community transmission of infection (1, 2). With SARS-CoV-2 infection community transmission was extensive and the early success of population based testing in control of the infection (3) led to the proposal that screening could help to limit viral transmission (4). Mass testing for SARS-CoV-2 in the UK was initially through polymerase chain reaction (PCR) testing, with alternative nucleic amplification test methods introduced later. The development of rapid tests for virus proteins ('antigens') in the form of a lateral flow allowed a further increase in capacity for population-based testing for the virus around November 2020.

This document is presented in 2 sections.

Section 1, 'UK testing context', focuses on the use of PCR testing within the National Testing Programme (NTP) with a focus on the Lighthouse Laboratory network[1]. Although the principles behind PCR are well known, there are specific aspects of its use in the NTP that require explanation. Section 1 contains information on:

- the laboratories that processed PCR tests during the COVID-19 pandemic
- quality management
- how PCR testing works
- how PCR cycle thresholds are converted to viral concentrations
- COVID-19 infection and incubation periods
- PCR and self-isolation

Section 2, 'Questions asked by the media and members of the public', provides the background and context for this section, where the more-commonly asked questions about PCR from the

[1] This document does not cover processes around quality in place within private labs procured for surge activity as part of the Lighthouse Laboratory network or private labs identified to provide testing direct to the public (for example, border testing). By illustrating relevant quality processes and other aspects of technical lab PCR functioning it hopes to inform the public of the type of processes required within a lab system.

media and members of the public over the course of the pandemic (often through ‘Freedom of Information’ requests and/or parliamentary questions) are answered. While the document is aimed at the public it is necessarily technical due to the nature of many of these common questions which often requested specific scientific and technical details.

Section 1

UK testing context within which laboratory-based PCR testing took place

The UK Health Security Agency (UKHSA), previously NHS Test and Trace under the Department for Health and Social Care, coordinated the National Testing Programme (NTP) for COVID-19 during the pandemic. Laboratory based testing, predominantly using PCR technology, was offered to control the transmission of the SARS-CoV-2 virus.

Initially testing of key workers and, as capacity increased, their household contacts was implemented to allow return to work where individuals were asymptomatic or household contacts of symptomatic individuals and it could be shown they were highly unlikely to have COVID-19. As capacity increased further, the focus changed and individuals in the community who developed any of a specified group of cardinal symptoms (fever, new continuous cough, loss of smell or taste) were encouraged to make use of free PCR testing with swabs collected via Regional Testing Sites (RTS), or through home testing (where the test was sent via post). Additional smaller, local test sites (LTS) opened from late May 2020 offering walk-in or drive-through access for individuals with symptoms. Testing of symptomatic individuals to diagnose COVID-19 infection supported identification and self-isolation of positive individuals and contact tracing. These testing delivery routes were subsequently also used for some asymptomatic testing, for example, to support testing in care homes, elective care and other settings, confirming LFD positive results, and testing of contacts of known positives.

Asymptomatic testing in most areas such as staff and patients in NHS and Adult Social Care (ASC) was initially rolled out using PCR testing from Autumn 2020. Following development of alternative testing capability in the form of lateral flow devices (LFDs) which provided a more rapid result, UKHSA were able to offer asymptomatic testing in a greater number of settings using PCR as an additional confirmatory measure. This asymptomatic testing at scale enabled the identification of infectious individuals who may not otherwise have been found, with the aim of breaking the chain of virus transmission and reducing the numbers of infections in these high-risk settings.

The focus of this document is laboratory-based PCR testing of the swabs collected from the network described above and delivered via UKHSA Labs and NHS hospitals and organised via a pillar system as shown in Table 1.

Table 1: UK SARS-CoV-2 Laboratory-based testing pillars[2]

	Pillar 1	Pillar 2
Tested population	• Some NHS/ASC staff • Patients with clinical need	• Symptomatic and latterly asymptomatic individuals in the general population • Supportive validation testing for new diagnostic capabilities (such as LFDs) • Testing to release from hotel isolations following international arrival in support of border control policy • Vaccine trials (such as Novavax and Oxford) • Vulnerable cohorts who qualified for antivirals • Some specific use cases (such as care homes, detention settings)
Swab collection site	• Hospitals and associated healthcare settings • Adult Social Care settings	• RTS • LTS • Home test kits • Organisation based (such as care homes in ASC)
Testing site	• NHS Laboratories	• UKHSA Lighthouse Laboratories (LHLs) both core and surge • LAMP van network[3]

[2] The testing pillars were originally outlined in Coronavirus (COVID-19): Scaling up our testing programmes (4 April 2020).

[3] LAMP-Loop-mediated isothermal amplification is an alternative method of detecting viral RNA. See Panel 1 for further information.

Laboratories

Lighthouse Laboratories

The network of high-throughput Lighthouse Laboratories (LHL) was inaugurated in April 2020 as a part of a government strategy to scale up the national SARS-CoV-2 testing programme[4].

Initially, laboratories were established at Milton Keynes, Alderley Park and Glasgow. In May 2020, a further laboratory was inaugurated in Cambridge. As the pandemic progressed, an additional 6 laboratories in Wales and England joined the network and a mega-laboratory, the Rosalind Franklin Laboratory at Leamington Spa, was the last laboratory to join the network in June 2021.

In addition, at times when demand rapidly grew to levels indicated to be greater than the capacity available from the LHL network (such as during Winter 2020 and during the Omicron wave in Winter 2021), short term PCR capacity was procured from private 'surge providers to supplement the core capability available from within the Lighthouse Laboratory network. The surge laboratories were managed within the Lighthouse Laboratory operational framework, which was distinct from the private to market framework used for laboratories conducting travel testing. This document focuses solely on the work conducted at Lighthouse Laboratories.

Tests used to deliver the testing strategy

Molecular tests are designed to identify the genetic material (ribonucleic acid; RNA) of the virus. These are the most sensitive tests for viral infection and can be used for both symptomatic and asymptomatic testing.

There are several types of molecular tests. In direct molecular tests, the sample is added directly to the test without extraction and purification of nucleic acid. Whereas in indirect molecular tests, the nucleic acid is extracted and purified before it is added into the test. Direct molecular tests include direct-PCR, direct-RT LAMP, and DNA Nudge which were deployed mainly in Pillar 1. Extracted molecular tests include Reverse Transcription Polymerase Chain Reaction (RT-PCR), Quantitative Real-Time PCR (qRT-PCR), Reverse Transcriptase Loop Mediated Isothermal Amplification (RT-LAMP) and End-point PCR (EPCR). These were used for testing individuals with symptoms in both Pillar 1 and Pillar 2.

[4] More information outlined in Coronavirus (COVID-19): Scaling up our testing programmes (4 April 2020). In addition a further network of surge private lab providers was also put in place.

Panel 1: Molecular tests used in diagnosis of SARS-CoV-2

Polymerase chain reaction (PCR)

Types of PCR include end-point PCR and real-time PCR.

Endpoint PCR provides a yes or no answer as to whether a specimen contains SARS-CoV-2 RNA.

Real-time PCR allows observation of positive amplification of SARS-CoV-2 RNA within a sample in 'real-time', that is as it happens. Real-time PCR has an additional advantage in that it allows a semi-quantitative assessment of the amount of SARS-CoV-2 RNA present within the sample. The earlier that amplification can be observed the greater the amount of SARS-CoV-2 RNA present in the original specimen. The detection of strongly positive samples occurs at a lower number of heating and cooling cycles (see Real-Time PCR (qRT-PCR)) than a weakly positive sample.

Transcription Mediated Amplification (TMA)

TMA differs from PCR by performing isothermal (one temperature) amplification using RNA transcription (via an RNA polymerase) and DNA synthesis to produce RNA copies from the target nucleic acid. Like endpoint PCR it generates a yes or no answer.

RT – Loop-mediated isothermal amplification (LAMP)

LAMP is a further isothermal reaction procedure that uses 4 to 6 primers recognising 6 to 8 distinct regions of the target nucleic acid. A strand-displacing DNA polymerase initiates synthesis and 2 specially designed primers form 'loop' structures to facilitate subsequent rounds of amplification through extension on the loops and additional annealing (where the temperature is lowered to enable the DNA primers to attach to the template DNA during thermal cycling) of primers.

Types include:

– RT LAMP with extraction of RNA

– RT LAMP without pre-extraction of RNA

– RT LAMP combined with sequencing (known as LamPORE)

– RT LAMP combined with CRISPR detection*

In terms of ability to detect the lowest concentrations of SARS-CoV-2 RNA, the most sensitive of these LAMP tests is LamPORE, and the least sensitive is LAMP without RNA extraction.

*Test was not available in the UK

Testing for infection using the PCR test

The PCR technique was developed in the late 1980s and has been extensively applied in diagnostic virology. Prior to its development, virologists relied upon viral culture techniques using either animals or in vitro cell culture; visualisation using electron microscopy; detection of viral proteins using techniques such as immunoassay; or the detection of specific antiviral antibodies arising post infection. The use of PCR revolutionised the world of diagnostic virology. The technique allowed specific and sensitive detection of virus at an earlier stage of infection than any of the afore-mentioned techniques, and it was instrumental in allowing more economic detection of virus infection (see Panel 2). Since its application in virology, the PCR technique has evolved and ancillary methods – generically known as nucleic acid amplification methods – have developed.

Diagnostic PCR tests are available that can detect any of the human viruses. The tests are highly selective allowing differentiation of groups of different viruses or absolutely specific identification of an individual virus or even a type of that virus.

Panel 2: Measuring a test's diagnostic accuracy

It is essential to understand how effective a test is at properly identifying the target disease or condition. Useful measures of a test's diagnostic strengths are sensitivity and specificity.

Sensitivity indicates how likely a test is to detect a condition when it is present in a patient. A test with low sensitivity may fail to identify the disease in an infected person. When a test's sensitivity is high, it is less likely to give a false negative and more likely to correctly identify the disease (a true positive).

Specificity refers to the ability of a test to rule out a disease in someone who does not have it. In a test with high specificity, a negative is truly negative. A test with low specificity may give a high number of false positives. The higher a test's specificity, the less often it will incorrectly identify a healthy individual as infected.

Analytical sensitivity is used to describe assay sensitivity within test laboratories. It is a more specific description of test performance and defines a test's ability to detect very low concentrations of a given substance in a biological specimen. Analytical sensitivity is often referred to as the limit of detection (LoD); the lowest concentration of an analyte in a specimen that can be consistently detected ≥ 95% of the time.

For example, a qRT-PCR such as the TaqMan™ 2019-nCoV Assay (Thermofisher, Warrington, UK), used in several LHLs, is known to have an analytical sensitivity or absolute limit of detection of around 67 dcopies/mL. In the TaqMan™ 2019-nCoV Assay using a 384 well assay format, the figure of 67 dcopies/mL represents the 95% confidence interval for the SARS-CoV-2 N gene target within the assay. This equates to as little as 2.7dcopies in a single sample reaction tube.

The PCR mixture has 5 key components which are:

- the DNA template to be copied – in the case of a virus, such as SARS-CoV-2, the genetic material of which is ribonucleic acid (RNA), the RNA must first be processed to produce a deoxyribose nucleic acid (DNA) copy of the RNA in a process known as reverse transcription; the DNA copy subsequently forms the DNA template for the PCR reaction
- primers (also known as oligonucleotides), designed to bind to either side of the section of DNA that is to be copied and used to initiate the PCR reaction – they are short stretches of DNA made synthetically that are specific for the template to be detected, in this case specific for SARS-CoV-2
- DNA nucleotide bases (also known as dNTPs), the building blocks of DNA (Adenine, Cytosine, Guanine and Thymidine) and needed to construct the new strand of DNA
- Taq polymerase[5] enzyme which adds the DNA nucleotide bases in sequence using the original DNA template as a pattern
- buffer, used to ensure the conditions are optimal for the enzyme to copy the DNA

The reaction mixture is put through a process of heating and cooling to allow the repetitive copying of the DNA template. The process is known as thermal cycling and the number of cycles of heating and cooling will vary according to the type of PCR.

The thermal cycling usually comprises 3 steps (as shown in Figure 1) which are:

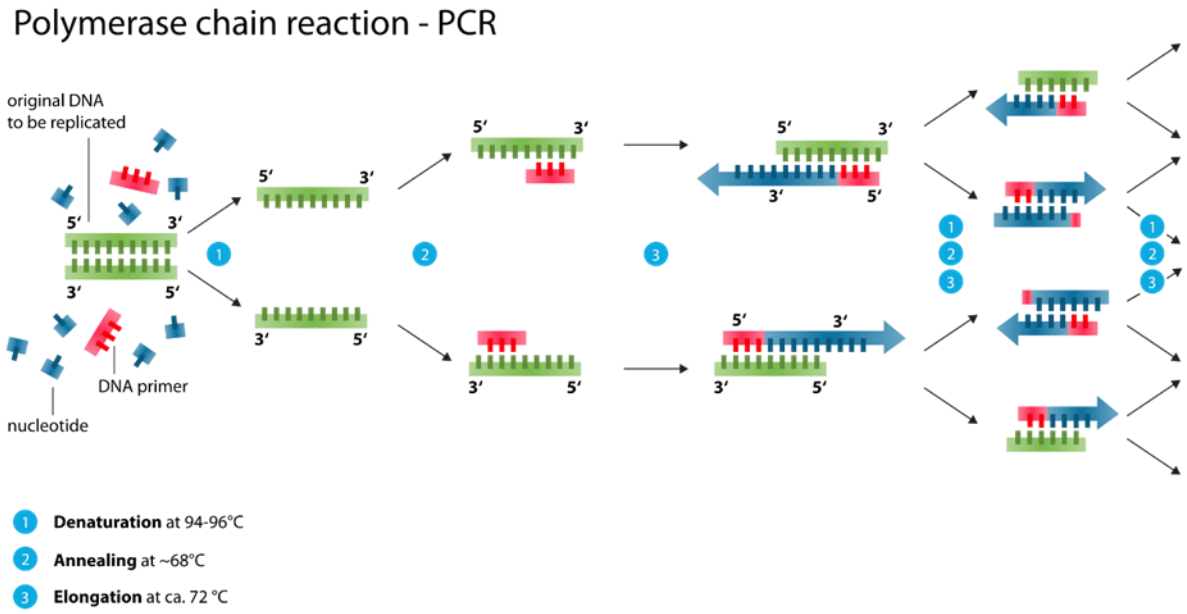
1. Denaturing – where the double-stranded template DNA is heated to separate it into 2 single strands.

2. Annealing – where the temperature is lowered to enable the DNA primers to attach to the template DNA.

3. Extension – where the temperature is raised to allow copying of the new strand of DNA by the Taq polymerase enzyme.

[5] Taq polymerase is a heat resistant enzyme prepared from a thermostable bacterium *Thermus aquaticus*. Thermophilic bacteria such as *Thermus aquaticus* show best growth at 65 to 70°C.

Figure 1. Schematic drawing of PCR thermal cycling[6]



The number of cycles used in a PCR are set during the development of the PCR. In the case of commercially supplied PCR tests, the number of cycles are defined by the test manufacturer to comply with regulatory approval of the test (EU CE marking, MHRA and/or FDA approval).

Real-Time PCR (qRT-PCR)

Real-Time chemistry technology allows the detection of PCR amplification during the early and exponential phases of the PCR reaction. Traditional thermocycler block gel-electrophoresis PCR is measured at the endpoint of the PCR, while real-time PCR collects data in the exponential growth phase of the PCR. An increase in the reporter fluorescent signal is directly proportional to the number of copies of DNA generated and does not require post-PCR processing.

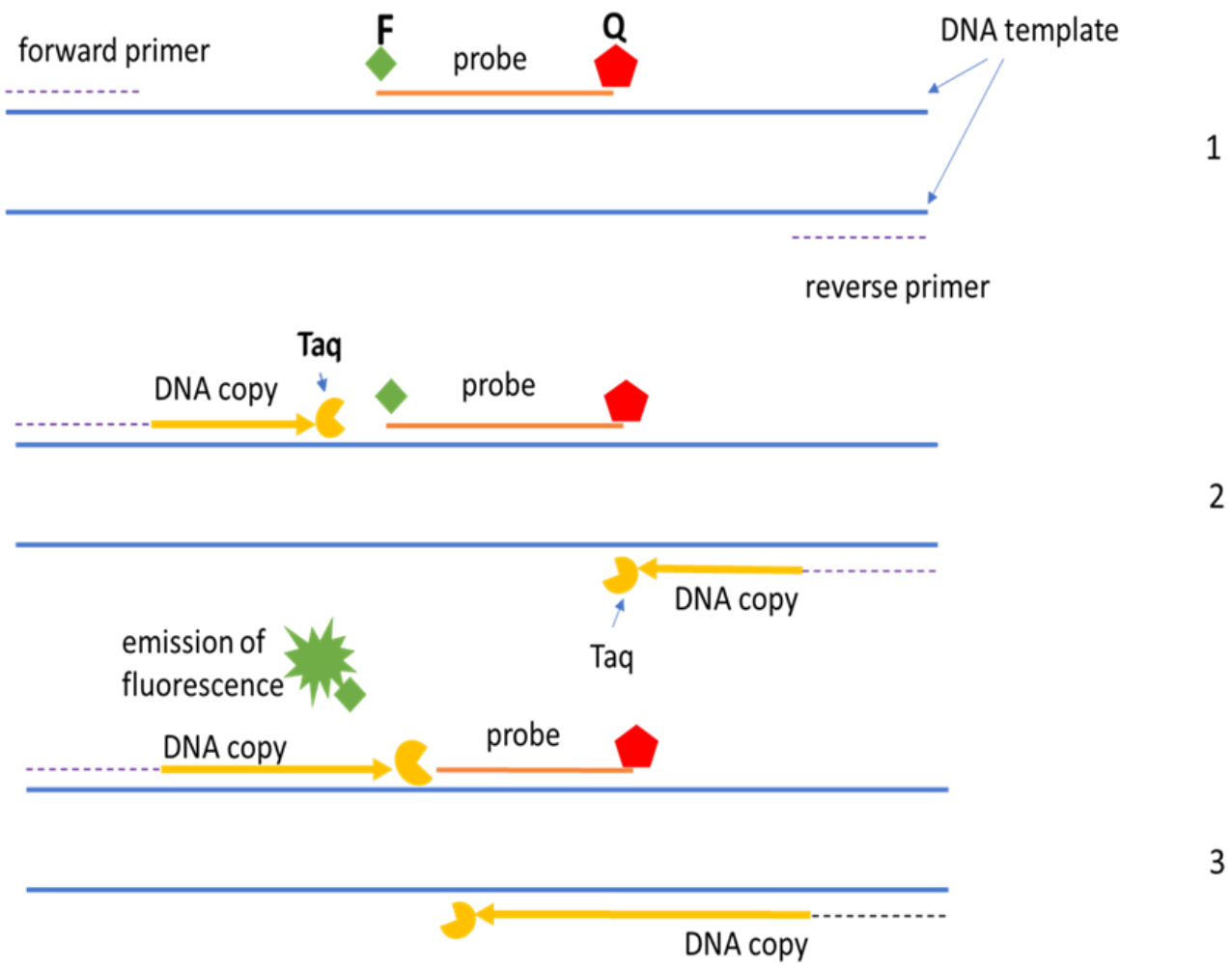
While a wide variety of different procedures are available for qRT-PCR, a popular one, Taqman probe real-time PCR is illustrated in Figure 2. In step 1, heating of the DNA template causes the double stranded DNA to separate into 2 strands, the primer sequences anneal to the dissociated DNA together with a 'probe' sequence, which anneals to a region between the forward and reverse primer binding regions. The probe bears a fluorescent dye marker, F, at one end and at the opposite end a quencher molecule Q. The quencher molecule quenches any fluorescence emitted by the fluorescent dye as long as it remains in close proximity to the fluorescent dye.

[6] Image by Enzoklop (2014) Creative commons Attribution-ShareAlike 3.0 Unported Licence CC BY-SA 3.0 (Creative Commons – Attribution-ShareAlike 3.0 Unported – CC BY-SA 3.0) accessed via Wikimedia Commons January 2022

In step 2, Taq polymerase enzyme binds to the primer-annealed DNA and begins to copy the DNA template by adding nucleotide bases that complement the existing base within the strand of DNA being copied, moving in opposite directions on the 2 strands of DNA being copied.

In step 3, as the Taq polymerase proceeds in copying the DNA, it encounters the probe DNA bound to the DNA strand. The Taq takes advantage of a second enzymatic activity associated with it – an exonuclease activity – and cuts the probe DNA free from the DNA strand, one nucleotide at a time. The cutting action releases the fluorescent dye molecule from the probe. In the process, it separates the fluorescent dye from the quencher molecule and the fluorescent dye begins to emit fluorescence. The process is repeated, and the amount of fluorescence released per heating and cooling cycle doubles with each cycle.

Figure 2. Taqman probe real-time PCR

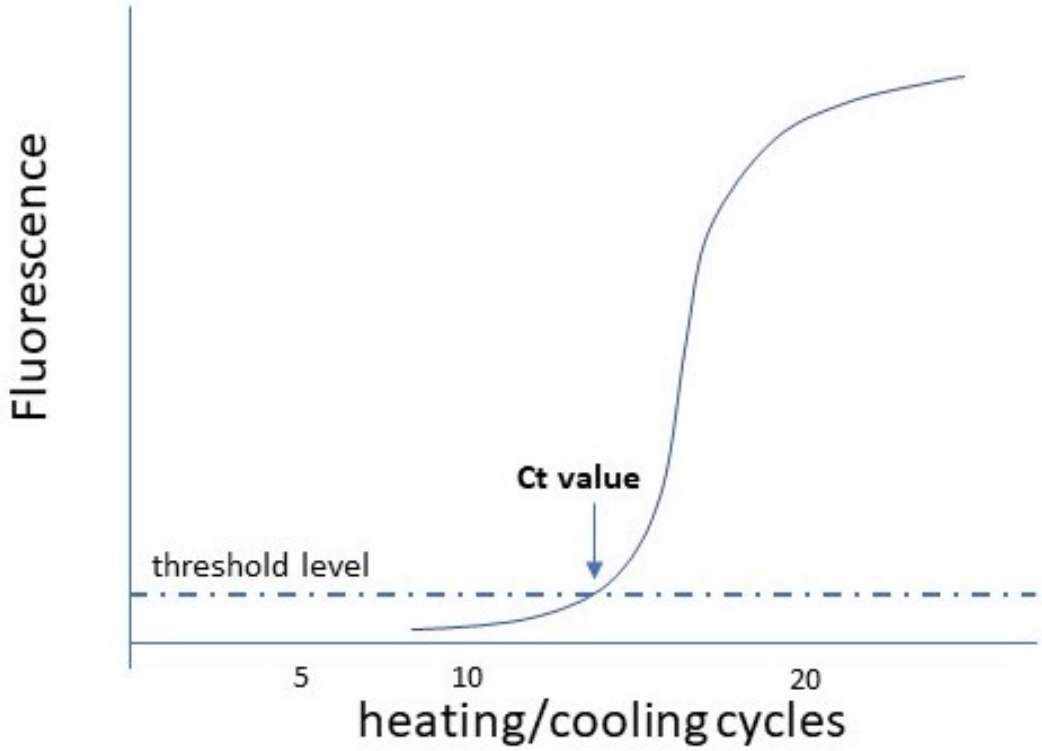


The fluorescence released in each cycle is measured. Once the fluorescence level has increased to a greater level than the background fluorescence within the measuring instrument and the environment, the machine will then register that the fluorescence has crossed the threshold level. The heating and cooling cycle in which this crossing of the threshold occurs is recorded and is known as the cycle threshold value or Ct value. The term Ct (cycle threshold) may also be referred to as Cq (cycle quantification), Cp (crossing point), or even TOP (take-off point).

The Ct value is thus defined as the PCR cycle number at which a sample's reaction curve intersects the threshold line. The threshold line is a line across a graph that represents a level above background fluorescence as demonstrated in Figure 3. This line intersects with the reaction curve somewhere at the beginning of its exponential phase. In Figure 3, the y-axis shows the fluorescence detected within an individual reaction tube. Values are Delta Rn where ΔRn is the normalisation of the Rn obtained by subtracting the baseline (ΔRn = Rn - baseline), Rn being the ratio of the fluorescence emission intensity of the reporter dye (indicating amplification of the target nucleic acid) to the fluorescence emission intensity of a passive reference dye included within the chemical components of the reaction mixture within the sample tube. The x-axis indicates the number of heating and cooling cycles used in the assay. When amplification of the target nucleic acid occurs, the amount of fluorescence detected increases exponentially.

Real-time PCR also allows the amount of input nucleic acid to be measured. The time of appearance of positive results in this technique is proportional to the amount of input nucleic acid (in this case the SARS-CoV-2 genetic material). The larger the amount of input nucleic acid the earlier in the process of testing that a positive result will be produced.

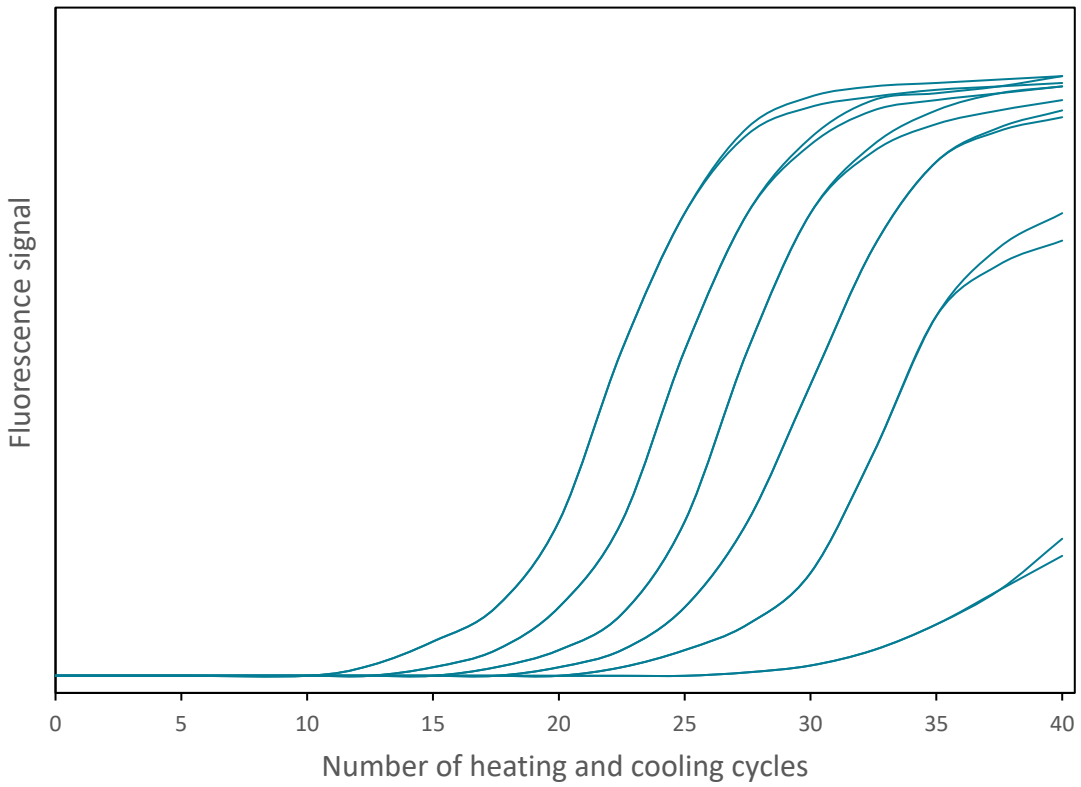
Figure 3. Example of a sample's reaction curve showing the intersection with the threshold level (Ct value)



Determination of the quantity of virus specific genetic material present in a sample uses a series of quantitative standards (measured amount of virus specific nucleic acids) added to the test as illustrated below in Figure 4. The y-axis shows the magnitude of the fluorescence signal being generated within an individual sample reaction tube. The x-axis shows the number of heating and cooling cycles used in the assay (in the illustration, up to 40 heating and cooling cycles are shown).

The most concentrated sample (leftmost amplification curve) begins to show a fluorescent signal generated between 10 and 15 heating and cooling cycles. The least concentrated sample (rightmost amplification curve) begins to show a fluorescent signal after 30 to 35 heating and cooling cycles. The instrument performing the heating and cooling (the thermocycler) uses mathematical interpolation to determine the exact point at which the amplification curve takes off exponentially, that is, is distinguishable from the normal environmental background of fluorescence and the electronic 'noise' within the instrument.

Figure 4. Determining quantity of virus genetic material present in a sample



Real-Time PCR (qRT-PCR) – measuring the amount of virus present in a sample

As there is a relationship between the Ct value produced within the real-time PCR assay and the amount of virus present in the original test sample, it is possible to exploit this to measure the quantity of virus present in the sample. In the LHLs, to quantify this, samples of SARS-CoV-2 assay were prepared by growing virus in cell culture. The virus was deactivated by gamma irradiation and heat (5, 6) followed by quantitation in a different PCR – droplet digital PCR (ddPCR).

ddPCR provides an absolute count of target DNA copies per input sample and is currently recognised as being the most accurate means of measurement of copies of DNA. Using samples containing known amounts of virus it is possible to generate a standard curve by plotting the Ct value generated by each of the samples against concentration in droplet digitally determined copies/mL. This thereby makes it possible to determine the formula to convert Ct to viral concentration (RNA dcopies per mL of sample).

Figure 5. A quantitative standard curve generated for the N gene target of a SARS-CoV-2 qRT-PCR

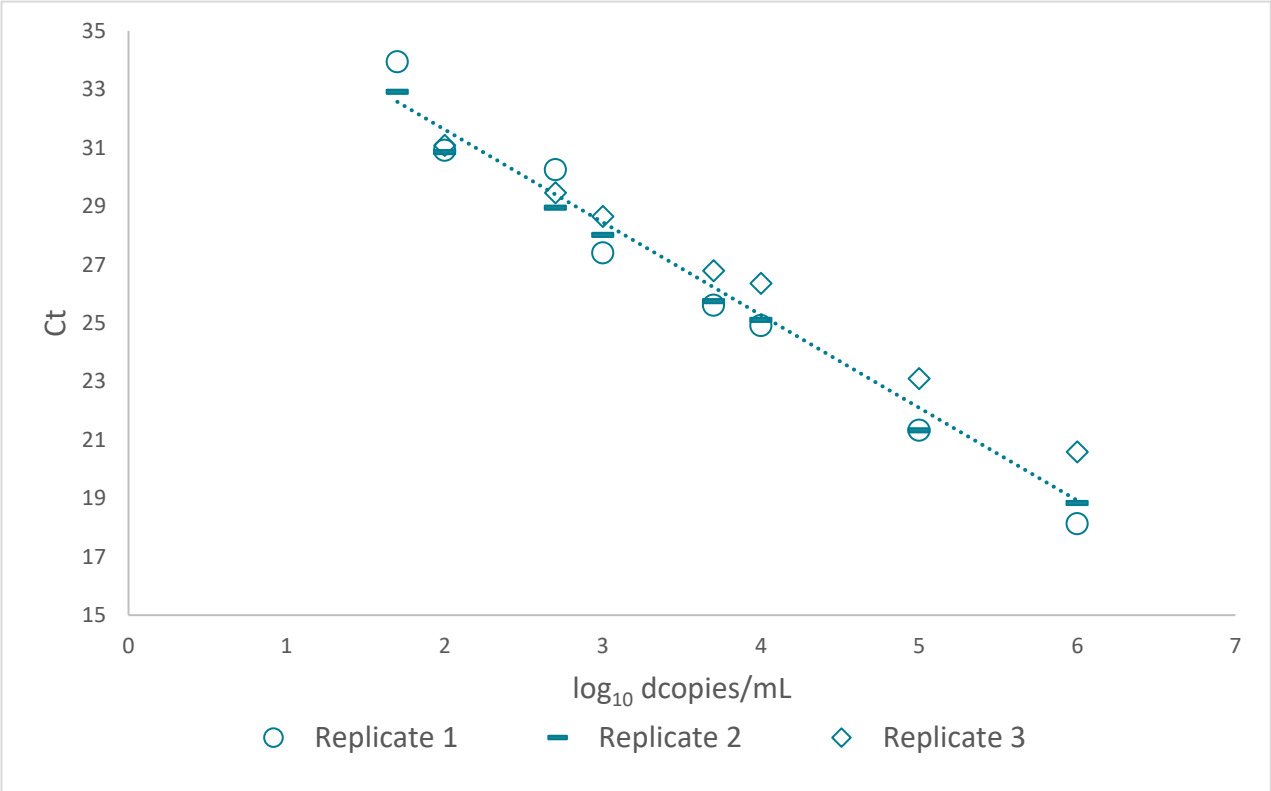


Figure 5 shows a quantitative standard curve generated for the N gene target of a SARS-CoV-2 real-time PCR. Samples containing known amounts of digitally determined copies per mL of inactivated virus were tested in triplicate and concentrations ranged from $\log_{10}1.7$ dcopies/mL (50 dcopies/mL) to $\log_{10}6.0$ dcopies/mL (1million dcopies/mL).

Real-Time PCR (qRT-PCR) – quantitation by interpolation from the standard curve

Using the standard curve, it is possible to estimate the number of dcopies/mL of virus present in an unknown sample by measuring the Ct value of that sample and interpolating the number of dcopies present. This estimates the amount of virus in the sample at the point of processing and is not an absolute value for the amount of virus in the nose or throat of the patient.

The amount of virus in the sample will be a product of the efficiency of collection of the nose and throat swab from the test subject, the dilution of the virus within the medium used to transport the specimen to the laboratory (the virus transport medium) and the preservation of the virus during its transport from test subject to the point of processing within the laboratory.

Comparing Ct values across labs and PCR assays

The effect of using different PCR assays

It is not possible to directly compare Ct values obtained using tests in different laboratories, there are a number of reasons why this is the case, which are explored below.

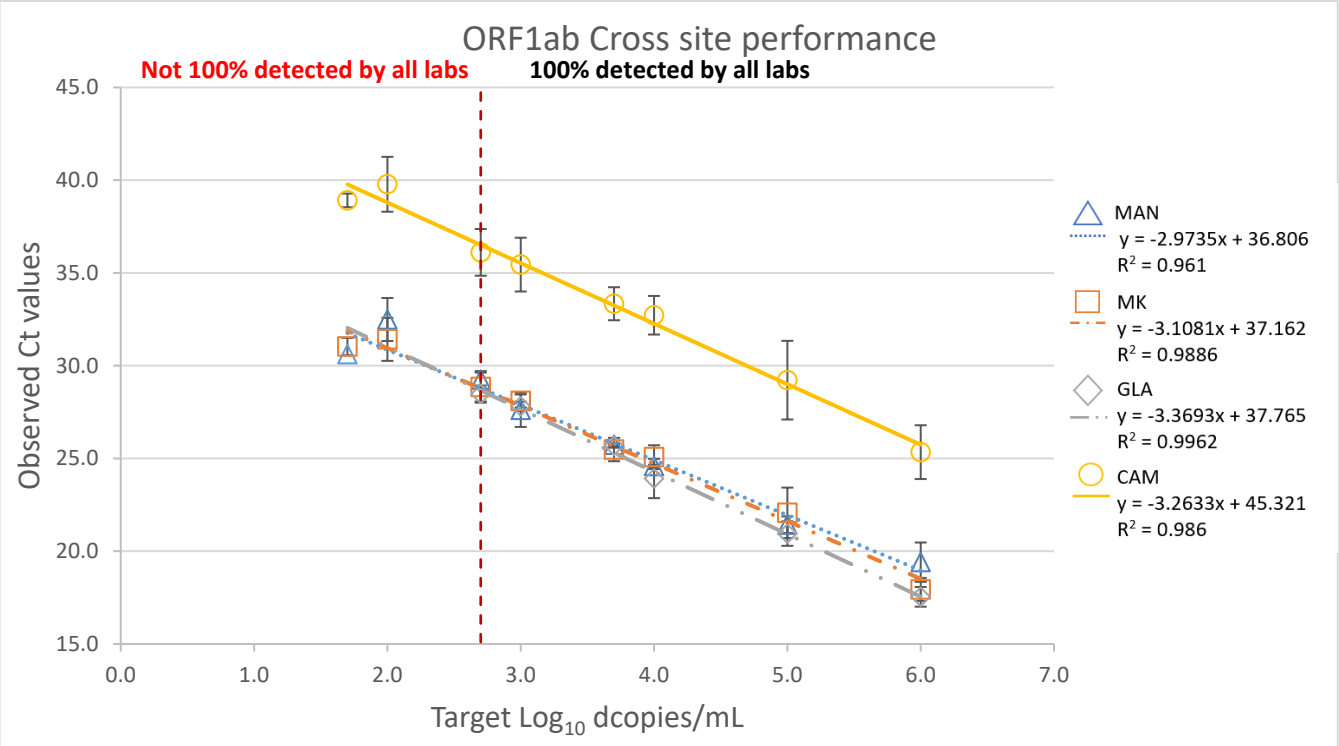
The effect of different test procedures was examined soon after the inception of the LHLs. A panel of SARS-CoV-2 samples quantified by ddPCR was run in each of 4 laboratories which were:

- Manchester Alderley Park (MAN)
- Milton Keynes (MK)
- Glasgow (GLA)
- Cambridge (CAM)

Three of the laboratories (MAN, MK, GLA) ran the same assay (Thermofisher Taqpath) and one (CAM) ran an alternative assay (Primerdesign). Using the same specimens (with the same quantities of virus) the different Ct values produced by 2 different PCR assays is clearly illustrated in Figure 6.

Each laboratory tested 3 replicates of each dilution of the standards panel, and mean Ct values are shown for each laboratory. All laboratories detected targets in each of the 3 replicates down to the sample indicated by the dotted red line. This is equivalent to 2.7$\log_{10}$dcopies/mL or 500dcopies/mL (viral RNA was detectable below this threshold in all 3 laboratories using Taqpath, but not for all 3 replicates). The Primerdesign assay reports much higher Ct values at each concentration than the assay used in the other 3 laboratories.

Figure 6. Orf1ab gene results for the Qnostics SCV2AQP01 quantitative SARS-CoV-2 standards panel run in 4 LHLs



Using the same concentration of virus, minor differences are seen for the laboratories using the same assay which is explained in differences in method of extraction and equipment differences between each of the laboratories. However, there is a major difference between these 3 laboratories and the fourth laboratory using a different PCR technique. The Primerdesign assay reported much higher Ct values than the Thermofisher assay at each concentration of the virus. This does not mean the sensitivity or dynamic range of the Primerdesign assay is inferior to the Thermofisher assay, it merely reflects a different design approach by the assay manufacturer.

In addition, not all assays target the same regions of the SARS-CoV-2 genome; the Thermofisher TaqPath assay, for example, uses 3 discrete genomic targets to define infection. The Primerdesign assay uses a single genomic target. Other assays target other regions of the virus and, depending upon the genomic target, may produce different Ct values for the same amount of virus. While all of these approaches to diagnosis are valid, this does produce complexity when attempting to define a threshold or cut-off for PCR (see Panel 4). These differences in Ct value generated for the same amounts of virus can have significant practical implications for managing the public health response to the virus infection. Results reported by Ct values from individual laboratories cannot be directly compared.

Comparing Ct values between test methodologies

As a wide range of qRT-PCR methods are available to test for SARS-CoV-2 it is not possible to define the amount of virus present in a sample in terms of a Ct value alone. Different PCR techniques will report different Ct values for the same amount of virus.

Hence each qRT-PCR must be calibrated using standardised samples in order for comparisons to be made between different assay procedures. An International Standard for SARS-CoV-2 RNA became available early in 2021. Normalisation of assay results can be achieved by comparing this standard in different assays and relating an individual assay reported Ct value for samples of known viral concentration and reporting results harmonised to this International Standard. An alternative is to use a standardised sample of inactivated virus calibrated in dcopies/mL (as described above).

Panel 3. qRT-PCR – International standards

In the early stages of the development of population-based testing for SARS-CoV-2 RNA, no standardised controls were available. Development of independent positive controls was prioritised. The independent, not for profit, QCMD worked with the German provider of Quality Assurance (QA) materials, Instand, to develop materials for the first International EQA of SARS-CoV-2 RNA testing (5).The material used in this EQA was calibrated using droplet digital PCR. The material from this exchange was made available for use as an independent positive control for testing by the sister company of QCMD, Qnostics Ltd for use within assays and a series of standard samples containing differing amounts of SARS-CoV-2 RNA were made available to allow inter-laboratory comparison of assays.

In addition to EQA schemes, a variety of standard preparations of virus are used for the calibration and standardisation of molecular diagnostic assays. Several of these preparations have been developed as a part of a WHO-sponsored initiative.

As outlined in the publication of Vierbaum and others (6) “the reliability of individual RT-qPCR test systems, as well as a good comparability of interlaboratory test results, is crucial for interpreting results and for making appropriate clinical decisions, for example, for estimating the infectivity of a patient for developing criteria for discharging patients from isolation. Data from the literature suggests that the probability of virus cultivation (especially from diagnostic samples taken after symptom onset) is low for diagnostic samples with a viral load below ~106 to ~107 dcopies/ml (conservatively estimated at about 20%). This implies viral RNA quantity could be used as a surrogate to guide patient stratification in terms of risk of transmission or as for criteria for discharging patients from isolation.”

The authors continue: “Furthermore, some groups have suggested using viral quantitative cut offs, using Ct values as units of measure, for this purpose. However, the interlaboratory variation outlined above suggests Ct values alone may not be a reliable measure to guide

patient stratification." In the publication they showed: "an indispensable prerequisite for linking Ct values to SARS-CoV-2 viral loads is that they are treated as being unique to an individual laboratory… For this reason, clinical guidance based on viral loads should not cite Ct values."

The work of Vierbaum and others (6) preceded the development of the WHO International Standard for SARS-CoV-2 RNA (NIBSC code 20/146), and clearly showed that individual laboratories should use specified standard material (such as the International Standard) in their individual test systems to link the specified RNA viral concentration of reference materials to the corresponding Ct values for the respective gene region. Only in this way can test system- and gene-dependent interpretation of results be made. Clinical decisions made based on Ct values should not be made when considering results derived from different laboratories. Clinical decisions may be made based upon data from a single laboratory and not across multiple laboratories unless viral concentration estimates are based upon reporting relative to the International Standard in units of International Units/mL.

Test reliability and timing in relation to infection

Transmission of SARS-CoV-2 depends on infected individuals encountering non-infected individuals. The higher the rate of dispersion of the contacts in the population the more the infection will spread. It is important to diagnose the infection as early as possible so that intervention (such as self-isolation) can be put in place to help confine the infection and prevent its spread. Diagnostic tests used to identify infection must therefore be very sensitive and very specific.

The PCR technique provides an extremely sensitive and specific method for the detection of virus and for this reason is an ideal test for early identification of infection. Real-time PCR is capable of detecting infection during the incubation period this can be up to 2 days before the appearance of symptoms in the case of COVID-19. Studies of infectiousness – that is the ability to transmit the infection – suggest that the ability to transmit infection increases as the amount of virus detected in a nose and throat swab increases (7, 8). The viral concentration level above which transmission is possible has not been determined, though it has been estimated that the majority of transmissions occur from cases with viral concentration above 10^6 dcopies/mL, however, transmission itself depends on many other factors such as length of exposure time and distance from the contact. Levels of virus during acute infection are often much higher than this.

Using real-time PCR to estimate the amount of virus present in a sample we can define a 'zone of infectivity' during acute infection (illustrated in Figure 7) when the infected individual is most likely to transmit infection (≥106 dcopies/mL). The aim of testing is to identify individuals before they become infectious and isolate (quarantine) those individuals.

Figure 7. A notional 'zone of infectivity' – an illustration of the appearance of PCR positive results in nose and throat swabs when testing patients

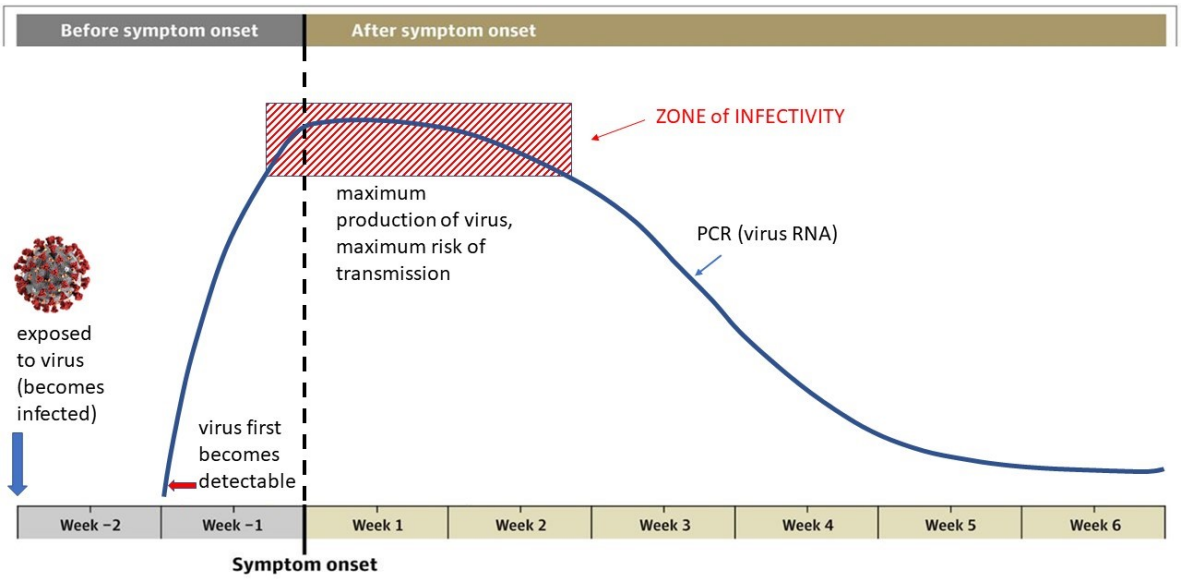
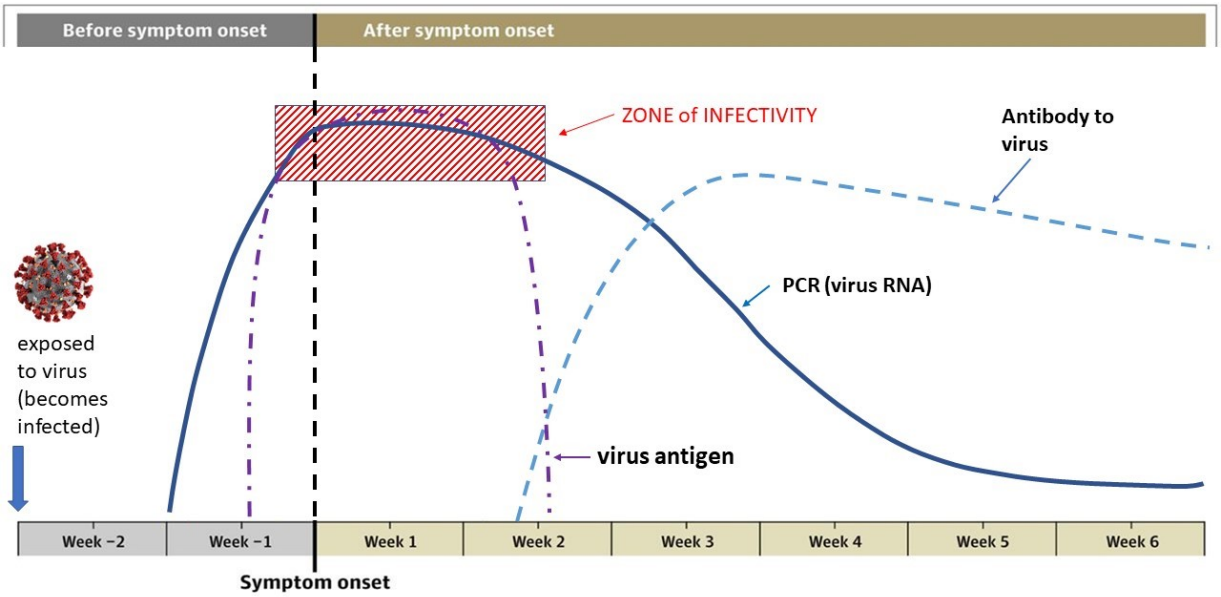


Figure 7 illustrates the appearance of PCR positive results in nose and throat swabs when testing patients. The incubation period is shown as 7 days although the average incubation period of SARS-CovCoV-2 variant strains can be shorter (for example, Omicron has an incubation period of 3.42 days) and the peak viral load occurs 1 to 3 days before symptom onset (8).

The zone of infectivity is shorter in relation to the duration of detection of SARS CoV 2 RNA because of the development of an immune response which is indicated by the appearance of antibodies in the blood. As the amount of virus reduces, the infectivity of the individual rapidly declines. The PCR however, continues to remain positive at higher Ct values indicating steady clearance of the virus from the individual.

Serendipitously, an alternative test for SARS-CoV-2, the viral antigen test (commonly called Lateral Flow Device or LFD) is good at identifying those in the zone of infectivity. Figure 8 shows that in the early stages of infection the viral antigen test remains negative when the PCR becomes positive. As the amount of virus produced in the nasopharynx increases to levels of $\geq 10^6$ dcopies/mL in nose and throat swabs the antigen test becomes positive. The antigen test (and the PCR test) remains positive during the acute infection, but the antigen test becomes negative coincident with the decline in VC observed in recovery from infection and the appearance of antibodies in the blood. With the appearance of antibodies in the blood the infected individual becomes non-infectious. The positive test results from the antigen test are therefore an almost perfect marker of the beginning of the zone of infectivity and subsequent negative results the marker of the end of this zone. The combination of the 3 tests – PCR, antigen and antibody – allows clear definition of the stage of a patient's illness and recovery from infection.

Figure 8. Illustration of the infection cycle, showing when viral antigen and PCR tests are likely to return a positive result



PCR testing and self-isolation

In the early stage of the pandemic, a quarantine period of 14 days after a positive PCR test was recommended. This was based upon knowledge that the incubation period was on average 5 days and 97% of persons who contracted the virus would show symptoms within 11 days. By setting 14 days for the self-quarantine period there would be certainty that infected individuals would not spread the infection to others once identified. As the pandemic progressed and greater understanding of the infection and its disease COVID-19 evolved, the impact of a reduced isolation period could be considered and weighed against the impact of asking people to self-isolate. The quarantine period was accordingly reduced to 10 days in the immunocompetent.

Concerns have been expressed that up to one third of individuals who were advised to isolate were never infectious (9). As illustrated in Figure 8, infectiousness (the zone of infectivity) comes to an end with the appearance of antibodies to the virus in the blood of the infected individual. A person who tested during the incubation period was required to isolate before they became infectious. Testing in the period of convalescence from infection when the person was developing antibodies and was no longer contagious might be viewed as leading to unnecessary self-isolation. However, the 'tailing' of PCR positive results (when PCRs still detect the virus despite the individual no longer being infectious) in the period of convalescence from infection was recognised at an early stage of the pandemic and led to advice not to re-test by PCR for 90 days after a PCR positive test result unless the individual had new onset of symptoms. Most of the testing of those with symptoms of infection occurred whilst they still had symptoms or soon thereafter, that is while they were still infectious.

Some SARS-CoV-2 infections are believed to be clinically silent, that is, asymptomatic (10). Immunocompetent individuals who tested and did not have symptoms may have tested positive while incubating the infection or while recovering from infection when they were not infectious. In population testing, as opposed to testing within healthcare facilities, it is important to note that testing has a subtly different purpose. In population testing, testing guides intervention to reduce the spread of infection. Theoretically, in population testing, absolute accuracy is not essential for achievement of public health impact, however, as discussed above, accuracy is required at an individual level. Testing for the purpose of clinical care on the other hand, guides therapeutic interventions which must take account of the overall clinical picture.

Panel 4. Thresholding for clinical relevance

A number of studies have been able to investigate the level of virus associated with risk of transmission. The consensus is that where the amount of virus found in the transport medium falls below 106 dcopies/mL then transmission is unlikely to occur (8, 11 to 15).

In theory, a Ct value for infectivity could be defined for each individual assay. It would be reasonable to assume an individual is non-infectious where the viral concentration of the sample is below a threshold of 106 dcopies/mL. In SARS-CoV-2 infection, the PCR test is likely to be positive before the patient becomes infectious. A PCR test is capable of detection of less than 250 dcopies/ml. Peak infectivity is seen at or just after the appearance of symptoms and is maintained at a high level for 4 to 5 days and then rapidly declines as a result of the developing immune response (heralded by the appearance of antibodies in blood) as per Figure 7 and Figure 8.

However, if those with less than 106 dcopies/mL were declared non-infectious this would include those to the left of the curve who are incubating infection and are about to become infectious and whom public health control measures seek to isolate.

There are further problems with this strategy: each laboratory would need to calibrate their assays and define for their laboratory, with their individual PCR test, and their individual methodology and equipment what the cut-off would be. This might be possible for those laboratories using qRT-PCR but not all PCR tests are performed using qRT-PCR. Some assays cannot be calibrated as a qRT-PCR. Some PCRs are not quantitative. An example is the end-point PCR used at the Rosalind Franklin UKHSA Laboratory. The use of technologies other than qRT-PCR was of course essential to increasing the capacity of testing, a strategy essential to public health control of the pandemic.

A further issue with setting a threshold relates to the sample. The sample used for the calibration would be a nose and throat combined swab placed in a transport medium. This is different to collection of a fluid such as blood, urine or cerebrospinal fluid. In the latter samples, a consistent volume may be collected and measurement of an analyte in the specimen can allow accurate measurement of the concentration of that analyte in a set volume of the fluid.

When a nose and throat swab sample is collected from an individual, there are 2 variables that are difficult to control. These are:

(i) The quality of the swab taken, that is how efficiently virus and/or virus infected cells were collected.

(ii) The time at which the swab was collected after contracting the virus. As illustrated in Figure 7, at different stages of the infection, different amounts of virus will be present in the nose and the throat.

The amount of virus present on the swab is thus not controlled and quantitative assessment of samples is thus less reliable than quantitative sampling of a sample such as blood.

In the light of these considerations, the setting of a standardised threshold for diagnosis would not be appropriate.

Infection and the incubation period

The incubation period is defined as the number of days between being infected by the virus and developing symptoms. The incubation period is important because it defines when a person will become symptomatic and is most likely to spread the disease. The time from first infection to symptom onset describes the speed of replication of the virus. It gives information about the cause and source of an infection when these factors are unknown.

Panel 5. Are viruses alive?

Viruses have to replicate within a host cell, and they use the host cell machinery for this. They do not contain the full range of required metabolic processes and are dependent on their host to provide many of the requirements for their replication. If a virus is not considered to be alive it cannot be killed. Thus, it is perhaps better to describe a virus as being active (and capable of infecting a host cell) or inactive (and not capable of infection). A virus would be inactivated if for example the nucleic acid were damaged either through environmental or chemical conditions.

During incubation, it might appear that the virus is inactive or dormant; however, this is not the case. During this period, the virus undergoes cycles of reproduction during which thousands of new viral particles are produced within each infected cell.

The new virus particles are released from the cell and spread to infect neighbouring cells or are released into the fluid surrounding these cells and then into the environment where they can infect other people. The number of cells that are initially infected by the virus determines how much virus is produced during each 24-hour period and therefore the duration of the incubation period.

Knowing the incubation period is important as it can be used to predict the spread of the infectious disease and, in turn, to define quarantine measures used for disease control.

Panel 6. Incubation period and the reproductive number

The average incubation period is often used to calculate the basic reproductive number (R0) (an epidemiological measure used to describe the contagiousness or transmissibility of infectious agents) and the maximum incubation period is used to determine the duration of quarantine.

In SARS-CoV-2 virus infection the incubation period is estimated to be between 2 and 14 days. The average for the Alpha variant was around 5 to 6 days; for Delta and Omicron variants, incubation ranged from 3 to 5 days (16). However, ranges of up to 27 days have been reported (although these may represent infections in which more than one exposure to the virus occurred and it was the second or third exposure which caused the infection).

A particular problem of many viral infections is that the infection may not produce any clear symptoms. These asymptomatic infections may have similar incubation periods to infections that produce symptoms though this is challenging to determine (10) However, unless a person is deliberately infected with virus and tested for evidence of infection each day, it is not possible to be certain. When such experiments are conducted, often referred to as ‘human challenge experiments’ such as (17), it becomes apparent that other factors are involved:

Firstly, not every person who is exposed to a virus will become infected. In some individuals, this is because they have existing immunity against that virus due to previous exposure to it or a related virus. However, in other cases, no evidence of previous infection or infection with a related virus can be found and yet infection does not occur suggesting some other natural defence mechanism.

Secondly, the dose, or amount, of virus delivered to an individual can influence whether infection occurs. A high dose of virus means that infection is more likely to occur than if a low dose of virus is delivered to an individual.

Lighthouse Laboratories – diversity of PCR test procedures

The LHL network used several different PCR tests or ‘assays’ and a variety of targets within the SARS-CoV-2 genome. In addition, the individual laboratory workflows used different procedures for the isolation of viral RNA (the genome of the virus) prior to nucleic acid amplification (PCR), based on assay manufacturer. The equipment differed between laboratories; some tested samples in batches of 96, others in batches of 384. All assays were required to be validated for

diagnostic COVID-19 testing prior to use in any of the Pillar 1 or Pillar 2 laboratories and were subject to standard laboratory practices for performance monitoring (including the use of positive and negative controls).

Between 31 March 2020 and 30 August 2022 more than 209 million PCR tests were conducted across the NTP (Pillar 1 and Pillar 2), the vast majority of which were real-time PCR. At their peak, the LHLs in Pillar 2 processed over half a million tests a day (January 2022).

Assurance around testing used for clinical care and for public health

Pillar 1 laboratories were the clinical laboratories of the NHS and Public Health England (now UKHSA), including Porton Down and Colindale, that pre-dated the LHLs. Staff were predominantly professionals registered with the Health Care Professions Council (HCPC) or the General Medical Council (GMC). Information available to staff within the Pillar 1 laboratories alongside the specimen included clinical details pertaining to the individual, as well as their name and the testing source.

In Pillar 2 laboratories, clinical professionals such as HCPC registered clinical virologists or registered clinical advisors oversaw the work of staff trained up to process the tests. In Pillar 2 laboratories (LHLs), no patient demographics were available; specimens were only tested under a test specimen identifier. The demographics information was held centrally and was not available to personnel working within the Pillar 2 laboratories.

The differences between Pillar 1 and Pillar 2 laboratories reflect the difference in purpose of testing of the 2 pillars: in Pillar 1, testing was primarily for healthcare support; in Pillar 2, testing was primarily for the purposes of public health control of infection.

In Pillar 1 laboratories, consultant virologists or microbiologists were able to review and interpret individual results in relation to the clinical features, stage of disease and location of the test subject (for example whether the individual was in Intensive Care). In addition, they could link specimens to an individual and determine whether the individual had been previously tested (which could give information on the stage of disease).

For Pillar 2 laboratories, where no clinical information, no patient identifier, and no location information was available, interpretation of test results was more challenging. Furthermore, it was not possible to link previous testing of that individual. In these circumstances the quality system underpinning testing was essential to ensure that a positive result was meaningful, that

is, reduce to an absolute minimum the possibility of reporting a false positive or false negative test result.

In terms of communicating these results to the public, educational messaging was made available to explain test results and necessary actions consequent on the test result. The results message wording was constantly reviewed and revised in light of behavioural studies and changing test, trace and isolation policies.

Standard practices and quality management

In each laboratory, test procedures were defined in a series of standard operating procedures (SOPs) which covered all aspects of the operation of the laboratory. Outside of the laboratory, a similar series of SOPs governed the collection, transport and delivery of specimens to test centres (a 'chain of custody') and the return of results and messaging to test subjects. Each laboratory developed a quality management system in compliance with ISO standard 15198 (ISO 15189:2012; Medical laboratories – requirements for quality and competence). Laboratories were independently inspected, and re-inspected, by the UK Accreditation Service (UKAS). At the start of the pandemic, all laboratories in the network either had, or were in the process of acquiring, accreditation to this standard.

A summary of quality assurance measures used in LHLs

A range of laboratories were included within Pillar 2 and not all laboratories followed the same processes. However, the 3 main laboratories (Milton Keynes, Alderley Park and Glasgow) were closely aligned in their approach, and their procedures are illustrative of the approach followed in all Pillar 2 laboratories. This approach is summarised below.

The laboratories each had an experienced Clinical Adviser who was either a Consultant Clinical Virologist or Consultant Medical Microbiologist (General Medical Council (GMC) registered) or a Consultant Clinical Virologist (HCPC registered). These individuals bore responsibility for the clinical relevance of results issuing from their respective laboratories.

As the members of the workforce were not all HCPC registrants in virology, comprehensive training and competency assessment were put in place for all personnel.

Independent assay controls (positive and negative) were sourced in line with UKAS recommended practice. SARS-CoV-2 RNA in the form of heat inactivated and gamma irradiated tissue culture grown virus in these samples was quantified in droplet digital PCR copies/mL (dcopies/mL) and was obtained from Qnostics Ltd, Glasgow. These controls were run on each

assay plate and acted as go/no-go control (on failure of either control, results for the 90 test specimens on the plate were held and tests were repeated). The level of the positive control (5,000 dcopies/mL of SARS-CoV-2) was deliberately set close to the limit of detection of the assay to ensure there was no drift in the sensitivity of the assay.

A ‘run control’ was sourced from the National Institute of Biological Standards and Quality Control (NIBSC, Potters Bar, UK) and this control (5,000 dcopies/mL of SARS-CoV-2 genetic material enclosed in a lentivirus construct) was run once per shift. The results of this control were used to develop a ‘control chart’ and the individual values were compared with the mean and standard deviation of an average of 20 values to monitor systematic errors within test processing.

To ensure consistency and accuracy in the reading of results, an artificial intelligence (AI) analytical programme was used in front-line analysis of results (Ugentec Fastfinder, Hasselt, Belgium). Results which the software could not interpret were flagged for manual review by experienced data scientists on a 24-hour basis. The software performance was verified by experienced staff with expertise in reading real-time PCR results who also helped train the AI software to improve performance. This approach also mitigated the workforce challenges of a lack of experienced staff available to read real-time PCR results.

All laboratories performed quality assurance of batches of supplied test reagents before these reagents were introduced into testing. Early in the programme it became apparent that there was unacceptable variation in the quality of reagents supplied to the programme despite the manufacturer’s quality assurance processes. It was soon realised that the laboratories would need their own layer of quality assurance of reagents for accurate clinical diagnostics.

A continuous quality improvement programme was maintained through internal and external audit procedures mandated by the quality management systems of the individual laboratories.

Inter-laboratory exchanges of test specimens were performed to ensure consistency of results produced by the different laboratories. The initial laboratories participated in the 1st International External Quality assurance exchange for SARS-CoV-2 and performed well (5).

A ‘chain of custody’ was established between the test subject and the arrival of a specimen within the test laboratory.

Discrete, functionally independent areas were established within laboratories including specific procedures and areas for: specimen unpacking (where leaked specimens could threaten the integrity of other specimens); extraction of nucleic acid from specimens; preparation of reagents for

the PCR (the building blocks – nucleotides – enzyme for the amplification process); combination of purified nucleic acid and test reagents; and the amplification and post-amplification procedures.

Laboratory personal protective equipment was restricted to discrete areas of work and gloves were changed regularly. Areas of work, including safety cabinets, were subject to chemical cleaning and ultra-violet irradiation several times a day, after each batch of samples or in the event of a spillage, in order to degrade and remove any contamination. After each clean down, environmental contamination monitoring was conducted and if necessary the process was repeated.

As well as scrupulous attention to the physical equipment and work procedures, environmental monitoring of the premises was performed. The procedures for environmental monitoring were designed to examine potential surface and airborne sources of contamination. Extensive monitoring was performed in each facility on an at least weekly basis. When contamination was found, clean up and recheck of the source of contamination was performed. Working with instrument manufacturers new decontamination procedures were evolved, as prior to the development of the LHLs no-one had ever worked at the level of test frequency seen in these laboratories; new engineering, physical and chemical procedures were developed in the laboratories to enhance the ability to reduce to an absolute minimum the risk of test cross-contamination.

The World Health Organization (WHO) International Standard for SARS-CoV-2 RNA testing was developed in a collaboration in which the NIBSC provided expertise and manufacturing facilities (18). The International Standard preparation was made available for use in January 2021. Prior to this, standard RNA preparations calibrated using droplet digital PCR (6) were used by the laboratories from May 2020. The LHLs used these materials to assess assay linearity and comparative sensitivity in inter-laboratory comparisons. Once the WHO International Standard became available, the assays were recalibrated using the declared International Standard in International Units per mL. Each procedural or major reagent change within the test laboratories led to re-use of the materials to verify no change in assay linearity and comparative sensitivity.

The laboratories participated in the quality assurance programmes to demonstrate proficiency in testing for SARS-CoV-2 by the International Programmes of Quality Control for Molecular Diagnostics (QCMD) (5) and UK National External Quality Assessment Service (NEQAS).

Panel 7. Quality assurance of PCR

Beginning in 1994, an EU Concerted Action on Virus Meningitis and Encephalitis ran pilot quality assurance schemes for diagnosis of viral meningitis and encephalitis. This was extended to other viral targets in a second EU Concerted Action on quality assessment of nucleic acid amplification and resulted in the creation of QCMD in June 2001. This not-for-profit limited company has since provided a wide variety of external quality assurance (EQA) schemes for diagnostic virology and organised the first EQA scheme for SARS-CoV-2 molecular diagnosis in April 2020 (5). Since the establishment of QCMD in 2001, a number of other EQA providers have been established including NEQAS in the UK. Diagnostic laboratories performing diagnostic PCR in the UK will typically participate in both QCMD and NEQAS EQA programmes. The LHLs participated in both EQA schemes.

Panel 8. Contamination, false positive reactivity and PCR

Early in the use of PCR in clinical virology, the danger of cross-contamination of PCR testing was recognised. Cross-contamination can occur when one specimen containing virus or PCR products (amplified DNA; amplicons) is inadvertently mixed with a negative specimen resulting in false positive test results. For this reason, extensive procedures to reduce, and preferably eliminate, the possibility of cross-contamination have evolved in clinical virology. The procedures developed for clinical virology precede the routine use of PCR in forensics, and can be shown to have enabled the forensic application of PCR.

These preventative procedures include many of the quality assurance measures adopted by LHLs and listed above, such as: establishment of a ‘chain of custody’ between the test subject and the arrival of a specimen within the test laboratory; the use of discrete, independent areas within a laboratory for specific procedures; sterile reagents, specimen tubes and disposable plugged tips, laboratory personal protective equipment being restricted to discrete areas of work and gloves being changed regularly; and areas of work being subject to both chemical and ultra-violet irradiation.

However, while early PCR techniques were beset with cross-contamination issues, the development of real-time PCR (as used in the LHL’s) led to a radical improvement. This was because tubes did not need to be opened post-amplification; the problem of amplicon contamination was thus greatly reduced.

Section 2

Commonly asked questions

This section answers questions raised by members of the public about COVID-19 testing. These questions were often raised through Freedom of Information (FOI) requests or parliamentary questions. The responses below are not the exact responses shared by respondents when questions were initially asked.

PCR tests

- Eating or drinking before PCR tests
- What do PCR tests do? What is their function?
- Are PCRs designed to test or to diagnose for a virus?
- What is the cycle threshold (Ct)? And how does this correlate to a positive or negative result?
- How many cycles are performed?
- Has the Ct been changed over the course of testing?
- How are variants detected (by PCR)?
- Are PCR tests specific for SARS-CoV-2?
- How accurate are PCR tests? (sensitivity and specificity)
- Request for copy of scientific evidence that the tests work
- How are the tests validated?

Eating or drinking before PCR tests

In respiratory virus infections the major site of infection (the location where the virus reproduces itself), is within living cells in the oropharynx (the part of the pharynx that lies between the soft palate and the hyoid bone). Virus is then released into the respiratory fluid overlying the cells which is then expelled from the body by respiration or coughing and sneezing. Drinking prior to collection of a swab specimen may reduce the amount of virus present in the fluid overlying the nasopharynx cells but will not reduce the amount of virus within infected cells. Provided swabbing is sufficiently vigorous to detach infected cells from the nasopharynx the accuracy of testing will not be significantly affected.

Eating prior to collection of a swab will also only have a minor effect on the efficiency of collection of virus infected cells on the swab. However, dependent on the food ingested the food may adversely affect the method used to test for the virus. In the case of PCR, excess of protein or ions such as Magnesium or Manganese can adversely affect the efficiency of the PCR. While extraction (purification) of nucleic acid prior to its use in the PCR reaction can reduce this risk, it

remains a potential problem. It is therefore wise to avoid eating and drinking for 30 minutes prior to taking a diagnostic swab sample for PCR.

What do PCR tests do? What is their function?

In the context of diagnosing SARS-CoV-2, a polymerase chain reaction (PCR) test detects genetic material from the pathogen. The technique uses short lengths of DNA-selective sequences ('primers') to 'copy' specific segments of a DNA sequence. In the SARS-CoV-2 PCR tests these primers match a segment (or segments) of the virus's genetic material. Once the primer binds, an enzyme (a polymerase) copies the sequence of DNA to which the primer binds. This allows copies of that material to be made, which can be used to detect whether the virus is present. A positive COVID-19 PCR test means that SARS-CoV-2 is present in the sample. A negative result could either mean that the sample did not contain any virus or that there was too little viral genetic material in the sample to be detected.

Are PCRs designed to test or to diagnose for a virus?

PCRs detect the presence of viral genetic material within the sample. They do not diagnose a disease in an individual.

What types of PCR tests are used?

The principal type of PCR used within the NTP has been the real-time PCR method. Although within the description of 'real-time' PCR, a number of different types of test method were used, differing principally in the methods employed to detect amplified nucleic acid. A new test methodology, 'end-point PCR', was used in two of the Lighthouse Labs: this is a high throughput variant of the original conventional PCR technique.

See Panel 1 for more details on the different PCR tests. Section 1 covers qRT-PCR in detail.

What is the cycle threshold (Ct)? And how does this correlate to a positive or negative result?

Cycle thresholds provide an indication of the amount of virus present in a test sample. Cycle thresholds vary by assay, target, and the strain or variant present in the sample meaning there are not universal thresholds across all PCR tests.

In addition, as cycle threshold will vary over the course of infection, the time taken to process the result means this level will likely no longer be accurate for the individual at time of receiving results. Cycle threshold also depends on how effectively swabbing was conducted adding a level of uncertainty to any threshold level obtained.

It is important to appreciate that interpretation of a sample's Ct value is not a 'one size fits all' science that provides a standard 'positive' or 'negative' or 'unclear' result. The positivity or

negativity indicated by the Ct level in a test sample will be interpreted differently the combination of types of equipment and analysis used in the individual laboratory that analyses the sample (19).

How many cycles are performed?

The number of heating and cooling cycles used in a PCR is determined by the manufacturer of that PCR. The physico-chemical differences in both assay equipment and individual assay components mean that some assays are completed within 35 cycles, while others are completed within 40 and others 50 or more. The actual number of cycles performed is unique to that manufacturer's assay and associated thermal cycling equipment.

See Real-Time PCR (qRT-PCR) for more details.

Has the Ct been changed over the course of testing?

The Ct value reported for an individual specimen is specific to that specimen. The range of Ct values that an individual type of real-time PCR can report, as outlined in Real-Time PCR (qRT-PCR), is unique to the particular type of assay and is defined by the assay manufacturer. There has been no change to any manufacturer's Ct range over the period of testing.

How are variants detected (by PCR)?

Variants are changes in the virus that occur over time due to the imperfect copies of the genetic material made by the virus during its replication. The variants can be deleterious to the virus or can be advantageous (for example improving its ability to spread in human populations) – in essence a form of natural selection involving the replicative fitness of the virus and its ability to transmit (20).

Variation in the genetic sequence of the virus is most readily determined by sequencing of the virus. During the pandemic, 'whole-genome' sequencing of SARS-CoV-2 was widely performed. As the name implies, in 'whole-genome' sequencing the whole genetic sequence of viruses within samples that were identified by PCR testing as being SARS-CoV-2 positive was determined using a relatively new technique known as 'next-generation' sequencing. Whilst the technique could analyse a large number of samples, it took up to 2 weeks to report results. As time went on, the processes were refined and the average time to sequence a sample was reduced to less than 4 days.

To reduce the processing time from days to hours, a new PCR-based approach was developed to allow the identification of variants. This PCR-based procedure looks for genetic changes that signify that a variant of the virus is present. The approach was highly successful in speeding up the identification of a variant strain. However, the PCR approach can only identify known

variants and not the unknown or new variants. For the latter, sequencing remains the most important method. Once a variant begins to achieve dominance in a population, a PCR can be developed for that new variant and can then be applied to identify the variant more rapidly than sequencing.

Are PCR tests specific for SARS-CoV-2? Can PCR tests detect other viruses?

The PCR tests used in the diagnosis of SARS-CoV-2 infection are specific for SARS-CoV-2. As outlined in Testing for infection using the PCR, in real-time PCR, both the primer and probe sequences detect the SARS-CoV-2 unique sequences within the genome of the virus. It is possible to target alternate genomic sequences within the genome and produce a PCR which will also detect other human coronaviruses (that is a coronavirus specific PCR rather than a SARS-CoV-2 specific coronavirus PCR) but these PCRs were not used within the National Testing Programme (NTP).

It is also possible to detect other viruses using PCR, but each PCR is specific to a particular virus or group of viruses. Assays are capable of detecting and differentiating many other viruses present within a test sample, but none of these assays were used within the NTP.

How accurate are PCR tests? (sensitivity and specificity)

The accuracy, sensitivity and specificity of PCR surpasses the accuracy, sensitivity and specificity of all other diagnostic procedures used in clinical virology (21) (see Testing for infection using the PCR above).

Request for copy of scientific evidence that the tests work

In laboratories accredited to ISO15819, only assays that comply with the EU in vitro diagnostic regulation (Regulation (EU) 2017/746 of the European Parliament and of the council on in vitro diagnostic medical devices and repealing directive 98/79/EC and commission decision 2010/227/EU) may be used in the diagnosis of human disease. All manufacturers of CE marked tests are obliged to have a technical file available detailing the scientific validity, analytical performance, and clinical performance of their assay. This information is held by the manufacturer and must be produced by the manufacturer when requested. The test manufacturer is the source of the scientific evidence that an individual test works.

How are the tests validated?

Verification and validation are independent procedures that are used together for checking that a test meets requirements and specifications and that it fulfils its intended purpose. Validation is the assurance that the test meets the needs of the person being tested and other identified stakeholders. It often relates to issues of acceptability and suitability with external customers. Verification is the evaluation of whether or not a product, service, or system complies with a regulation, requirement, specification, or imposed condition.

In ISO15891 accredited laboratories, test manufacturers must validate their assays in accordance with the EU in vitro Diagnostic Directive (as explained in the answer to the previous question). Laboratories using a manufacturer's test must verify that the test meets the manufacturers claims.

In the NTP a second level of validation was added. The Technical Validation Group (TVG) of the DHSC validated all tests used in the NTP for COVID-19 testing. Covid Test Device Approval (CTDA) is a legislative process which means only those tests on the CTDA register can be used in the UK; part of the process for which is ensuring assays are CE marked. CTDA is a UK wide regulation that came into force on 28 July 2021, requiring antigen and molecular detection COVID-19 tests to reach minimum performance requirements. This is done through a thorough assessment by UKHSA scientists before sale on the UK market. This process ensures that all tests sold on the UK market are fit for purpose. Successful tests are published publicly on an approved device register on gov.uk which consumers can consult.

SARS-CoV-2

Has SARS-CoV-2 been isolated or purified? Has SARS-CoV-2 been isolated or purified from test samples?

SARS-CoV-2 is the virus that causes the respiratory disease COVID-19; SARS-CoV-2 is the name of the virus, COVID-19 is the name of the disease.

The virus has been regularly isolated from patients with COVID-19. Accumulated evidence suggests the 6 criteria that are required to establish a virus as the cause of a disease (Koch's postulates, as modified by Rivers (22) have been met. Further, the Bradford Hill (23) criteria used to establish epidemiologic evidence of a causal relationship between a presumed cause and an observed effect have also been met.

Not all individuals who are infected with SARS-CoV-2 will develop symptoms (they are described as being asymptomatic). The virus has been isolated from asymptomatic and symptomatic cases. This is commonplace in respiratory disease – not all those infected with a respiratory pathogen will develop disease.

SARS-CoV-2 virus has been isolated in cell culture, and its genetic material (ribonucleic acid; RNA) and proteins have been extensively investigated.

How do you know when an individual has or has had COVID-19?

When a person is infected with SARS-CoV-2 it is possible to identify the virus by detecting its nucleic acid (the specific RNA sequence that identifies SARS-CoV-2) or by demonstrating specific proteins (antigens) of the virus. In certain cases, if required, it is possible to identify the presence of SARS-CoV-2 by isolating the virus in cell-culture, by demonstrating its presence using electron microscopy. The virus is too small to visualise using conventional light microscopes.

Following infection, immunocompetent individuals will mount an immune response to the virus. Development of this immune response can be measured by detection of virus-specific antibodies. Detection of these virus specific antibodies can be used to identify those who have been infected with the virus.

In the UK, there are different tests to check if an individual has been infected with SARS-CoV-2 coronavirus. The 2 main tests are PCR tests and rapid antigen lateral flow device (LFD) tests. The PCR test looks for the presence of viral genetic material in the patient sample, while LFDs detect SARS-CoV-2 proteins (antigens) that are present in nose and throat secretions when a person has COVID-19 infection. In addition to these there are antibody tests. An antibody test is a blood test to check if you've had COVID-19 before or been vaccinated.

How many variants have been detected or are in circulation?

A variant is declared based upon concerns about epidemiological, immunological or pathogenic properties of viruses in circulation. At this point they are designated as being a Variant Under Investigation (VUI) with a year, month, and number. Following risk assessment with the relevant expert committee, they are designated a Variant of Concern (VOC). VOCs that have had major outbreaks include the Alpha, Delta and Omicron variants. Regular updates of variant analysis are made available. A comprehensive list of variants identified to date is maintained by the European Centre for Disease Prevention and Control. The number of cases of SARS-CoV-2 infection are updated daily and the numbers and type of circulating VOC and VUI and any other variant by weeks and days is also continuously updated.

Data collected from PCR tests

Is any individual personal DNA data collected or sequenced as part of C19 testing, and is this stored anywhere?

No personal DNA data was collected or sequenced as part of COVID-19 testing.

When a nose and throat swab is collected for the purpose of testing for SARS-CoV-2 infection the sample may contain human cells which will contain human DNA. As the sample contains human tissue (in the form of cells collected on the swab) the sample is considered “Relevant Material” under the Human Tissue Act 2004 and its regulations. The Human Tissue Act 2004 created an offence of DNA ‘theft’. It is unlawful to have human tissue with the intention of its DNA being analysed, without the consent of the person from whom the tissue came. As the consent of the individual being tested for SARS-CoV-2 was for diagnosis of respiratory infection it would be a criminal offence for personal DNA data to be collected for the purpose of sequencing.

Although human DNA will be collected during the process of collection of a test sample, and that human DNA may be co-collected when viral RNA is extracted from the sample as a preparation for the PCR test, the SARS-CoV-2 test will not amplify the human DNA; it will only amplify the SARS-CoV-2 nucleic acid. When a sample which is positive for SARS-CoV-2 is subject to sequencing, only the SARS-CoV-2 nucleic acid is analysed, not the human DNA which may be present in the positive sample.

Test specimens and nucleic acids are usually discarded post-test. Retention of some samples and nucleic acid samples is necessary. Retention and any re-use of the samples is carried out in accordance with the guidelines of the Royal College of Pathologists (24). This guidance precludes the re-use of personal DNA data.

Safety and sterility of PCR swabs

How safe is it to use a PCR test swab?

There are 2 aspects to the safety of using a PCR test swab to collect a nose and throat swab. The first relates to the physical safety of the individual being swabbed. There could be accidental breakage of the swab during the process of collection of either the throat swab or the collection of the nose swab with subsequent ingestion of the swab or part thereof. This risk was addressed at an early stage of the programme by providing guidance on how to take a swab in both written and audio-visual format. In practice, there were 486 million swabs taken for PCR diagnosis between 31 March 2020 and 30 August 2022, and only 34 instances of swab breakage and ingestion of the swab were recorded. All cases were resolved without serious deterioration in state of health or death.

The second safety aspect listed here is the potential introduction of harmful microorganisms as a result of the use of a non-sterile swab. This risk is amplified if a non-sterile swab is used in an immunocompromised individual. Sterilisation of swabs is standard practice in manufacture, and swabs are generally packaged individually to maintain sterility. No instances of adverse consequences of swabbing were recorded.

How are PCR test swabs sterilised?

Methods of sterilisation are specific to the manufacturer; the main methods for sterilisation are steam at high pressure (autoclaving), ethylene oxide sterilisation, or gamma irradiation. The method used to sterilise a swab will be displayed on the packaging. The symbol is a rectangular box with the word ‘STERILE’ followed by either ‘EO’ (sterilised using ethylene oxide) or ‘R’ (sterilised using radiation)

Are any chemicals used in the process of swab sterilisation?

Ethylene oxide (EO) is a chemical that is commonly used in the sterilisation of swabs; hydrogen peroxide is also used in some manufacturing facilities. Sterilisation with ethylene oxide is the most widespread and highly standardised process in the industrial manufacture of medical products, in particular of single-use products such as bandages, sutures or syringes and catheters, but also of surgical instruments, sensitive medical products, electronically sensitive components and implants.

The sterilisation process consists of a number of highly controlled and monitored stages, including removing ethylene oxide after treating the swabs. The amount of residual EO that is allowed has been set (by the international standard ISO 10993-7:2008) according to contact time of the medical device with the person. There are 3 categories of contact time: limited, prolonged, and permanent duration. The contact time for swab sterilisation is limited.

As part of the sterilisation process the manufacturer must confirm, and document, that the residual EO level on a medical device is below the specified allowable limit before the device is packaged ready for use.

Does UKHSA hold information regarding the different chemical compounds used on PCR swabs?

The process of procurement includes the verification of swab sterilisation and the method employed. The information on chemical methods of sterilisation is thus available within UKHSA.

PCR swabs shafts are usually made of polystyrene and swab tips are made of cotton, viscose, nylon flock, rayon or polyester. The swabs are free from chemicals that may be used in the manufacturing process. The swabs are sterilised by Gamma irradiation or gas sterilised with

ethylene oxide. Post sterilisation ethylene oxide traces or residues are below limits that would be considered hazardous for health and comply with international standards.

Test kit disposal

Are test kit components recyclable?

SARS-CoV-2 is classified by UK Advisory Committee on Dangerous Pathogens as a Biohazard Group 3 organism. Organisms in this category can cause severe human disease and may be a serious hazard to those who contract it such as employees of the laboratories processing the samples; it may spread to the community, but there is usually effective prophylaxis or treatment available. So, a consequence is that any part of a test kit from a positive case where the virus has not been inactivated is regarded as infectious waste and must be disposed of with due precaution.

In relation to test kits, the swab and medium into which the swab is placed are always treated as potentially infectious and must be disposed of either through autoclaving (sterilisation under high pressure) or incineration where there is any chemical contamination. Transport bags which contain the swab and medium are also classified as infectious waste and must also be disposed of as infectious clinical waste. However, the outer cardboard container of the test kit is considered to be a low risk of infection as it has not been in contact with the primary container. After storage for 3 days (a time considered sufficient for any adherent virus to be inactivated) the cardboard is compacted and sent for recycling.

Is it safe for users to dispose of test kits into common or general waste points?

Assisted test sites were equipped with dedicated waste streams that were collected by specialist contractors and taken to appropriately permitted sites for disposal. Home test kits can be disposed of in domestic waste where potentially infected items are enclosed within plastic bags, while reusable items such as cardboard may be safely recycled. Guidance (now withdrawn) was available during the pandemic at Coronavirus (COVID-19): disposing of waste.

Abbreviations

Acronym	Description
ASC	Adult Social Care
EO	Ethylene Oxide
GMC	General Medical Council
HCPC	Health and Care Professions Council
LoD	Limit of Detection
LHL	Lighthouse Laboratories
LTS	Local Test Site
TVG	Technical Validation Group
NTP	National Testing Programme
PCR	Polymerase Chain Reaction
QCMD	Quality Control for Molecular Diagnostics
RTS	Regional Test Site
SOP	Standard Operating Procedures
UKHSA	UK Health Security Agency
WHO	World Health Organization
VOC	Variant of Concern
VUI	Variant Under Investigation

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UKHSA is responsible for protecting every member of every community from the impact of infectious diseases, chemical, biological, radiological and nuclear incidents and other health threats. We provide intellectual, scientific and operational leadership at national and local level, as well as on the global stage, to make the nation health secure.

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