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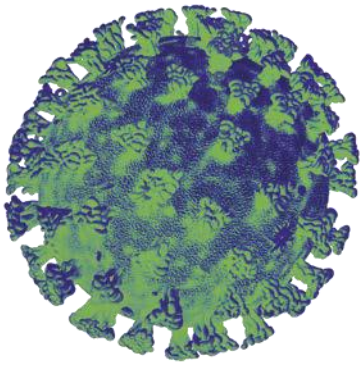
Technical Advisory Cell

Core principles for utilisation of RT-PCR tests for detection of SARS-CoV-2



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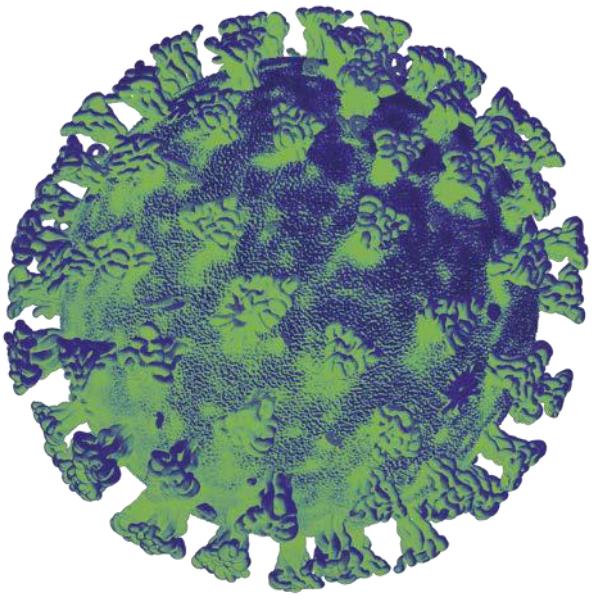

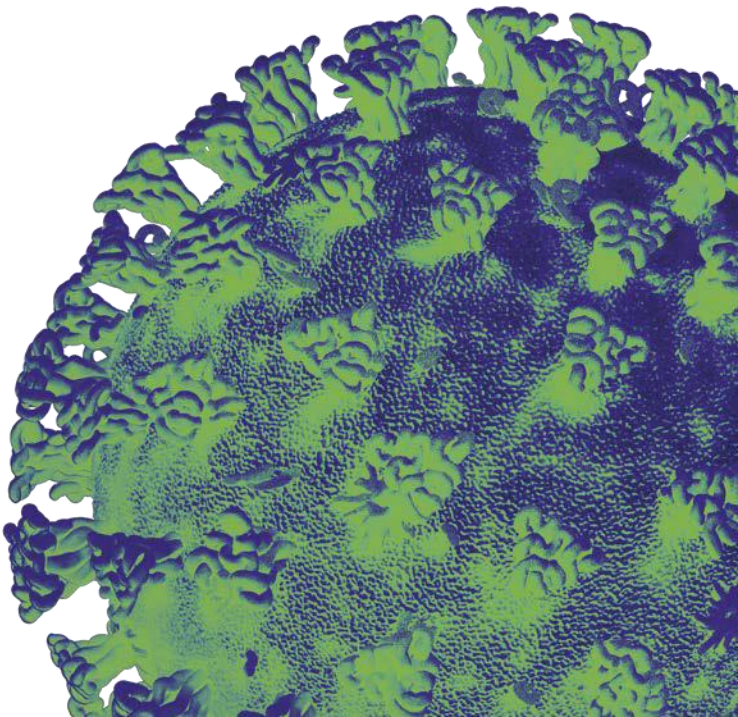

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COVID-19 Technical Advisory Cell: Core principles for utilisation of RT-PCR tests for detection of SARS-CoV-2

Purpose

This paper presents key information about the RT-PCR for the detection of SARS-CoV-2 virus. It then applies this information to propose core principles and recommendations to help guide the use of this test in Wales. It is intended that this paper should act as a 'foundation' on which other papers focused on specific question can be provided.

Scope

- In scope:
    - Reverse-transcription polymerase chain reaction (RT-PCR) tests for the detection of SARS-CoV-2 virus.
- Out of scope:
    - Other tests including tests for antibody and antigen

Summary

- RT-PCR tests have been developed for the detection of SARS-CoV-2 RNA to support the diagnosis of COVID-19 in symptomatic individuals.
- The analytical performance of the test is defined by the Analytical Sensitivity and Specificity. This is a constant for each test.
- The clinical performance of the test is defined by the Diagnostic Sensitivity and Specificity, and may vary between different clinical scenarios.
- Integration of the prevalence of the clinical target condition with the Diagnostic Sensitivity and Specificity can determine the positive and negative predictive values and false positive and false negative rates for the test in that scenario.
- Testing strategies should always consider the rationale for testing, and the individual or population actions arising from positive or negative results.
- Tests may be used for
    - Diagnostic testing in symptomatic individuals
    - Screening in asymptomatic individuals

    - Testing for infectivity in recently infected individuals
- Testing for screening in asymptomatic individuals is likely to generate a high rate of false positive tests when the prevalence of infection is low.
    - In the context of pre-surgical screening, this may lead to significant unnecessary postponement of surgery
    - In the context of critical worker screening, this may lead to significant unnecessary exclusion from work.
- Evidence is emerging that testing for infectivity may be refined by the level of test positivity (Ct value) and the presence of an antibody response.
- It is recommended that all testing plans use the embedded template to define the rationale, and actions dependent on testing, and triangulate against the likely false positive and negative rates at the predicted condition prevalence.

Recommendations

- Clinically-led testing associated with the medical needs of an individual should have primacy over testing for other purposes.
- The utility of additional RT-PCR testing be considered in the wider context of other testing that is taking place, including the ‘test, trace and protect’ programme.
- The performance of the existing RT-PCR is at its best when its use is targeted, for example, when used to support diagnosis in symptomatic individuals. It is unsuited to the non-targeted screening of asymptomatic individuals, especially in populations with a low prevalence of infection. Use in asymptomatic individuals should, wherever possible, be on the basis of effective targeting, for example following tracing that has indicated a high-probability of exposure and thus likely infection.
- The purpose of testing (either ‘single’ or ‘repeat’) within defined target settings or cohorts should be clearly defined and agreed prior to the implementation of that testing.
- Criteria for the cessation of repeat testing within defined target setting or cohorts should be clearly defined and agreed prior to the implementation of that testing.

Key Principles for a National Strategy (RCPATH)

The following seven principles underpin any form of diagnostic assessment and must be applied to a national testing strategy.

1. The test is the right one, at the right time, and with the correct result. This result includes the appropriate clinical interpretation and, where not specifically designed and validated for home use, a test carried out by skilled trained laboratory professionals to recognised and accredited quality and service standards.

2. Testing must be carried out for a purpose: for diagnosis, for screening or for gathering data to understand the spread, or level, of disease in a population. Any testing programme must be clear as to its purpose, and the tests chosen appropriate for that purpose.

3. Problems in testing result in problems with care. With an infectious disease, this can have significant impact on disease spread, risk assessment, morbidity, mortality and population health. These problems arise from a range of issues including poor specimen taking, poor labelling or poor transcription of details, slow turnaround of results, poor quality control, ineffective communication of the result, inappropriate application of the result, and lack of clinical input or oversight. Many of these issues have been seen in recent times, all of which must be urgently addressed.

4. Data connectivity is a cornerstone of testing. It is a key aspect of improving quality, and great strides have been made in this area through the rapid connection of labs via NPex across much of the UK. Consistent test coding will aid this, and should be rolled out at speed. Links that connect primary and secondary care and public health bodies should be strengthened to ensure all results are available to clinicians when required, form a part of individuals' permanent health records and can be used, in an appropriate and legal framework, for public health purposes.

5. Testing standards must be upheld. Testing will be carried out in many settings, but must be carried out as part of a quality assured system, meeting accredited standards in regulated or approved settings. Accredited standards will apply to both laboratory and point-of-care testing. Different technologies will be used, depending on the clinical setting, clinical pathways and public health need.

6. People being tested should be informed about why they are being tested, and the implications and limitations of their results. They should have access to those results. Individuals should be informed if their data becomes part of a research

programme and of their rights to be excluded if they so wish, within the context of public health needs.

7. At societal level, the more people who understand about the testing being performed, the more informed their consent will be. Information needs to be in plain language and accessible to all in a range of formats. National and international awareness is raised by media, professional bodies and programmes such as Lab Tests Online, but broader education, including in schools, has a role also. It is important that sampling is not mistaken for testing – the language used must be accurate to give the public confidence in test results.

Key Definitions

Pivotal performance characteristics of a test are defined in terms of its sensitivity and specificity and each has two key types.

The **analytical sensitivity (ASe)** of a test relates to the smallest detectable amount of analyte that can be measured with a defined certainty. In the case of the RT-PCR that analyte is viral RNA.

The **analytical specificity (ASp)** of a test is the degree to which the assay distinguishes between the target analyte and other components in the sample matrix; the higher the analytical specificity, the lower the level of false-positive

The **diagnostic sensitivity (DSe)** of a test is the likelihood that a sample from a person who truly fulfils the target condition will yield a positive result when tested with that test.

The **diagnostic specificity (DSp)** of a test is the likelihood that a sample a person who does not fulfil the target condition will yield a negative result when tested with that test.

N.B. Sensitivity and specificity are independent of the prevalence of infection in the population that the tested individual comes from.

The **positive predictive value (PPV)** of a test is the likelihood that, given that a sample from an individual has yielded a positive result to that test, the individual truly fulfils the target condition.

The **negative predictive value (NPV)** of a test is the likelihood that, given that a sample from an individual has yielded a negative result to that test, the individual truly does not fulfil the condition.

N.B. Importantly predictive values are a function of DSe, DSp and the prevalence of infection in the population that the tested individual comes from. Given that in the operational circumstances in which tests are used it is the test result that is known, rather than the true status of the subject, it is very important to understand predictive values in the context of the test use. The numbers of false positive and false negative results for a given test population can be calculated from the PPV, NPV, and prevalence of the condition within the population.

The table exemplifies the inter-relationship between prevalence, DSe, DSp, PPV, and NPV, and shows the impact on false positive and false negative rates.

DSp	DSe	Prev (%)	PPV	Positive Results/10,000 tests			NPV	Negative Results/10,000 tests		
				Total positive results	True positives	False positives		Total negative results	True negatives	False negatives
0.98	0.9	0.1	0.04	209	9	200	1.00	9791	9790	1
		1	0.31	288	90	198	1.00	9712	9702	10
		5	0.70	640	450	190	0.99	9360	9310	50
		10	0.83	1080	900	180	0.99	8920	8820	100
0.98	0.8	0.1	0.04	208	8	200	1.00	9792	9790	2
		1	0.29	278	80	198	1.00	9722	9702	20
		5	0.68	590	400	190	0.99	9410	9310	100
		10	0.82	980	800	180	0.98	9020	8820	200
0.8	0.9	0.1	0.00	2007	9	1998	1.00	7993	7992	1
		1	0.04	2070	90	1980	1.00	7930	7920	10
		5	0.19	2350	450	1900	0.99	7650	7600	50
		10	0.33	2700	900	1800	0.99	7300	7200	100

Core Information

The reverse transcription polymerase chain reaction (RT-PCR) is an enzymatic and chemical process by which short strands of ribonucleic acid (RNA) are converted to deoxyribonucleic acid (DNA) and copied in a doubling time reaction (amplification) to concentrations that can be detected and visualised by the human eye.

This method has been in use for over two decades for the detection of viruses which have an RNA genome in a range of clinical samples, and most recently it is the primary method to confirm the presence of SARS-CoV-2, the virus that causes COVID19, in suspected cases during the pandemic.

Following the discovery of the SARS-CoV-2 virus in China, the full genome sequence was released globally and this allowed for the development of RT-PCR tests to detect the virus. This was a vital step, as to specifically detect any virus using RT-PCR prior knowledge of the sequence is required, as it is short genome fragments that the test targets to amplify.

The test works by using small fragments of DNA (primers) added into a reaction mix that complements the target region of the viral genome. Performed at a low temperature, the complementary DNA specifically binds to the correct region in the viral genome. An enzyme present in the mix automatically adds individual nucleotides to the strand making a complementary DNA (cDNA) strand.

Once this is complete, the test enters a heating and cooling cycling process firstly to separate the strands - destroying the initial RNA genome leaving behind the cDNA strand. The primer then attaches again (annealing) and a second enzyme in the mixture automatically adds complementary nucleotides to complete the now double stranded DNA.

The mixture is heated again, the strands separate and now both DNA strands can act as a target for the reaction – therefore two become four, four become eight and so on.

Most tests that are used are real-time RT-PCR tests - that is the amplification product can be visualised as it happens using specialised laboratory equipment

(thermocyclers) and the addition of a complementary DNA sequence that sits between the primers in the target viral genome. This is called the probe. This is labelled with a light emitting dye at one end and a light quencher at the other. This works by annealing to the target DNA at the same time the primer also anneals to the single stranded DNA. As the enzyme works from the primer adding the nucleotides to the DNA strand it destroys the probe releasing the light dye away from the quencher, emitting a light in doing so.

This light is read by the thermocycler, and this light increases as more product is produced by the reaction. This appears an amplification curve on the thermocyler software.

SARS-CoV-2 test targets

The RT-PCR tests that are available target one or more of three different parts of the virus genome, as specified by WHO.

- The envelope gene – specific for the wider family the SARS-CoV-2 virus belongs to and can also cross react with SARS-CoV-1 and bat betacoronaviruses.
- The open reading frame (ORF) – including the RNA dependant RNA polymerase gene
- The nucleoprotein – specific for SARS-CoV-2

In addition there will be an internal control that will ensure the assay has worked effectively throughout the whole process.

The earliest testing strategies used a single gene test to screen often the one exhibiting the highest sensitivity. For most this was the E gene, followed by confirmation with a more specific gene target. Increasingly, tests were developed that detected more than one gene target with an internal control in a single tube. Whilst this afforded the quickest and potentially the most sensitive strategy, adding more targets within a single assay adds an extra layer of difficulty in both ensuring the test remain specific whilst not compromising sensitivity.

Additionally, the virus itself introduces mutations at a rate of around 2 changes per month that occur anywhere along the viral genome. Should any of these occur in the primer or probe detection sites, sensitivity can be significantly compromised.

ECDC and WHO recommend that assays used for RT-PCR are based either on WHO recommended tests or that the commercial companies ensure that target sites remain free from mutations that might affect assay sensitivity.

Platforms currently available in Wales

Platform	**'E' Gene**	**RNAse P**	**RdRp (ORF1)**	**'N' gene**	**External IC**	**LLOD**	**Comments**	**Low Positives**
In House	Yes	Yes			No	~ 10 copies/ml	Based on WHO protocol (our Gold standard)	CT value > 37
Seegene CE	Yes	No	Yes	Yes	Yes	TBC *	Manufacturers 10 copies/ml	Only **ONE** target positive Or 1 target CT > 37
Roche EU	Yes	No	Yes	No	Yes	TBC *		Only **ONE** target positive Or 1 target CT > 37
M2000 EU	No	No	Yes	Yes	Yes	TBC *	Manufacturer 100 copies/ml	Difficult to record Ct value.
Luminex EU	Yes	No	Yes	Yes	Yes	TBC *	False positives at low level – needs careful interpretation	Low levels reviewed & repeated on alternative
Perkin Elmer EU	No	Yes	Yes	Yes	Yes	Under evaluation	Manufactures 20 copies/ml Based on Chinese CDC assay	Only **ONE** target positive Or 1 target CT > 37 Reports Ct > 40
Hologic	No	No	Yes x2 targets	No	Yes	Under evaluation	Ct not reportable	Ct not reportable
Cepheid	Yes	No	No	Yes	Yes	TBC*		If only **ONE** target positive Or 1 target has CT > 37 Reports Ct > 40

*- Comparison data very favourable to in – house

Determining lower level of detection (LLOD) and specificity

The lower level of detection refers to the lowest concentration of virus reliably detected by the RT-PCR. This is normally quoted as the 95% confidence level. Whilst this level normally refers to sensitivity, it also impacts on specificity as it takes into account the level at which the positive is called above the background noise in the test itself. This is particularly important at the lowest levels of detection. It is at this point where there is clearance between background noise and signal that the threshold is set to call a positive (see diagrams A and B).

Once this is established the LLOD of the test can be determined accurately. (diagram C)

Diagram A – the blue line relates to the threshold where the signal crosses to become positive (Ct value) the background noise of the test is very close to the amplification signal, making it difficult to determine a true positive from background noise

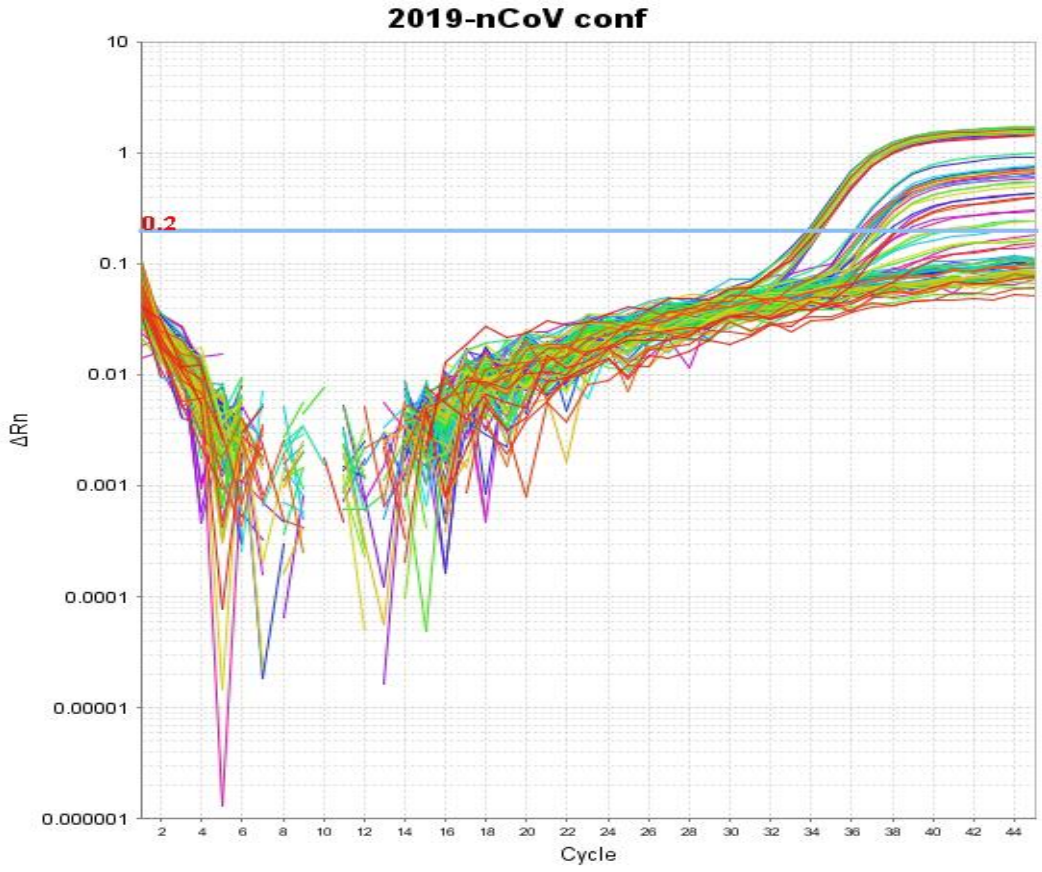


Diagram B – using the same threshold, the signal to noise ratio is much clearer in this test, meaning a low level positive can be clearly identified

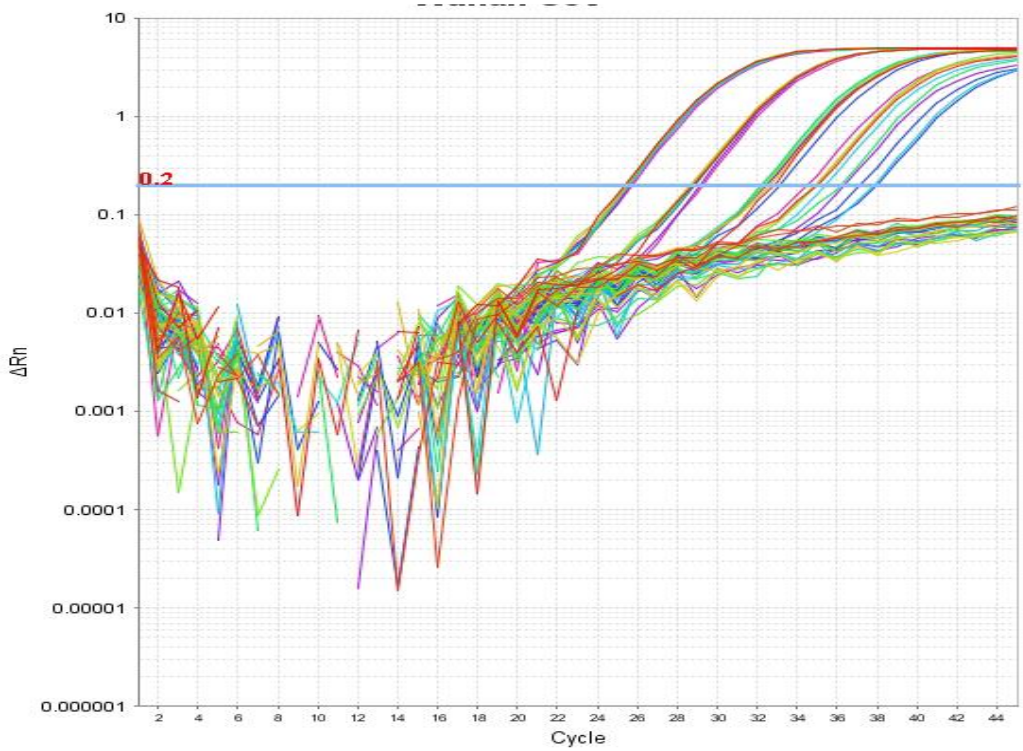
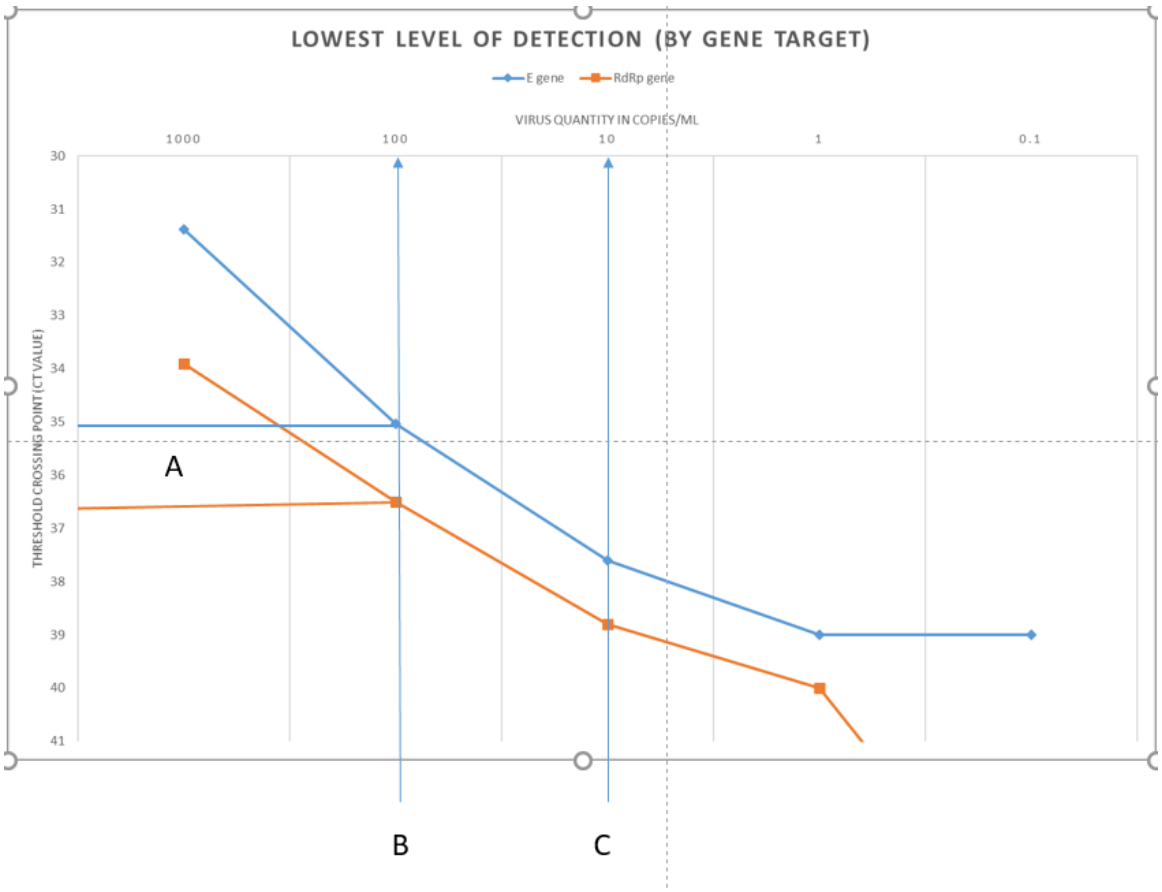


Diagram C – determining the LLOD of the tests by gene target – lower numbers for Ct value relate to higher concentrations of virus detected. In this diagram the lowest levels of virus concentrations are plotted by mean detection from 8 replicates at each concentration. Both gene targets had a 100% detection level at 100 copies/ML with the E gene showing more sensitivity overall demonstrated by lower Ct values recorded at each level (A and B).



At dilution C relating to 10 copies/ML, the E gene detected the virus at this concentration 80% of the time, whereas the RdRp gene only detected virus at this concentration 50% of the time.

Discrepancy of gene targets to fail to detect a positive result are problematic at lower levels as it isn't always clear whether the single signal is a true detection, especially if a commercial assay is being used where the raw data can't be analysed to determine noise to signal ratio.

Highly complex assays with multiple targets are more prone to non-specific noise that can be reported as positive.

Principles of Use

Purpose

The purpose of any testing activity should be clearly defined and agreed prior to the implementation.

For diagnostic purposes the context of the test is important and the limitations of the test should be fully understood.

The clinical history and examination is extremely important. Diagnostic investigative tools are used to provide additional information to inform patient management and confirm or refute the initial clinical diagnosis.

When tests are used as screening tool, where there is an absence of history and results from other investigations, there is a requirement to understand the limitations of the test as the positive and negative predictive values will change as the prevalence of the infection changes. Test used in this context are ‘unfocused’ and therefore interpretation is more difficult, if the purpose is ill defined.

Test Utility

The clinical utility of both a positive and a negative test result should be clearly understood and defined prior to testing.

Limitations of the current RT-PCR as a diagnostic tool

Initial Diagnosis

It is very clear that the RT-PCR for SARS CoV 2 is negative in a proportion of cases that present with classic COVID 19 symptoms. The diagnosis in these instances is made based on the history, examination and the results of other investigations. The absence of a positive PCR does not change the diagnosis, but when positive confirms the clinical diagnosis.

In the situation above the absence of detectable RNA in the throat is a result of the clinical limitations of the assay and not the limitations of the assay itself. A deep

sample, such as BAL (broncho alveolar lavage) will, in some circumstances, be positive for SARS-CoV 2 RNA, which supports the clinical diagnosis.

Currently the scientific community do not fully understand why some individuals have a negative PCR result from upper respiratory tract samples. Analysis of sequential samples from the upper respiratory tract demonstrate fluctuations in viral excretion. The peak of viral excretion is within the first 7days but individuals who present to hospital are often more than 7 days into their infection. Understanding of the pathogenesis of this infection is still evolving and the role of viral replication as part of the disease process is still to be determined.

As the tests evolve, the use of antibody may also have clinical utility in this setting as an investigative tool to confirm the diagnosis.

Sample types outside of the respiratory tract

There is increasing evidence of viral RNA being found outside of the respiratory tract including in CSF, blood and faeces. Approximately 40% of infected patients may excrete RNA in stool following the acute infection. There is limited data to support viral replication outside if the respiratory tract beyond theoretical extrapolation of the distribution of cells expressing the ACE2 receptor. It is therefore not clear whether the RNA detected from sites outside of the respiratory tract represent infectious, viable virus.

Most commercial RT-PCR systems have not been validated for these sample types and the paucity of positive material to fully validate the process complicates interpretation.

Faecal samples in particular are complex substrates and nucleic acid extraction can be difficult with potential PCR inhibiting substances being carried over on a frequent basis. This can lead to erroneous results, in particular false negative results.

The value of testing these samples must therefore be considered in the context of how the results impact management in light of the lack of data and platforms validated for these sample types.

Test of infectivity

The use of RT-PCR as a tool to inform absence of infectivity also comes with limitations as viral RNA can be detected for up to 50 days following infection. The detection of RNA does not correlate with infectivity. Evidence is accruing that following a mild infection, infectious virus is unlikely to be present after 9 days. There is also increasing evidence that a high Ct and detectable antibody suggests an absence of infectivity.

When using the test to inform discharge for individuals whose symptoms have improved, then a negative PCR, taken 14 days after onset, +/- a detectable antibody level is consistent with an absence of infectivity. A RT-PCT of Ct >37 plus the presence of detectable antibody is also consistent with the absence of infectivity.

Repeat tests in this setting have little clinical value and contribute significantly to confusion when guidelines are applied.

Test as a screening tool

There are a number of ways that RT-PCR is being used as a screening tool.

Prior to treatment

There is evidence that individuals who have a mild infection with SARS-CoV 2 prior to surgery or chemotherapy have significantly poorer outcome compared to those who are virus free at the time of their procedure of treatment. Detection of the virus will result in a delay of the surgery or chemotherapy.

In these instances a false negative results will lead to additional risk of adverse outcome following surgery or chemotherapy. A false positive result will lead to a delay in treatment, which also carries clinical risk.

When using this test in these contexts it is important that the negative and positive predictive values are carefully considered.

In this setting the RT- PCR is being used as a screening tool, to ensure absence of infection at the time of the procedure.

In addition to the negative and positive predictive value of the test, the result should also be considered in the context of the total testing pathway. If a patient self-isolates for 14 days and is tested somewhere between 10 to 12 days before admission and receives a negative result then the interpretation of that result is that this individual is highly unlikely to become infected prior to admission. The interpretation is reliant on the individual and the household's adherence to the guideline. A negative result does not guarantee absence of infection at the time of the procedure or treatment, but provides sufficient re-assurance to proceed.

A positive result, given the caveat above, at a high Ct value (>37) is more likely to be a false positive result and a repeat test should be considered, where treatment cannot be delayed.

Where treatment can be delayed then an additional period of self-isolation and monitoring of symptoms should also be considered. An antibody test after 14 days may also have value

When treatment cannot be delayed for positive results with a high Ct a repeat sample should be taken to confirm the presence of the virus. A 'blanket' recommendation should not be made for such scenarios, as risk benefit ratios require all relevant clinical information and are not based solely on isolated test results.

Testing as a population screening tool in asymptomatic populations

In this setting there may be a greater tolerance for false negative and false positives results. However the impact of the test results on epidemiology data should be considered. The positivity rate should be monitored in such a way the Ct values are recorded, to reflect the likely false positive rate, in order that accurate epidemiological data are available to inform policy decisions.

For individuals within this population there are likely to be negative impacts for both false negative and positive results. For example, a false positive result in a HCW / Key worker will negatively affect staffing levels, whereas a false negative in a HCW / key worker could lead to the unintentional spreading of the infection.

The benefit of re-testing is of limited use, but could be considered if whole services are likely to be affected by a false positive result, but to implement would require careful consideration

The identification of false negatives in an asymptomic population is not possible in the absence of symptoms developing

Testing as a population screening tool in symptomatic populations

The utility of the test is greatly improved in this setting as the presence of symptoms compatible with the infection increases the value of the result.

Framework for assessing scenarios for testing

A framework for assessing scenarios for the use of testing is suggested. This should describe the use of the test, with impacts for positive and negative results and accounting for the false negative and false positive rates. Suggested examples are presented in Annex 1.

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Annex 1: Example frameworks for the assessment of specific defined scenarios

1.1: Diagnostic testing of education staff

COHORT	Education Staff
PURPOSE	Diagnostic
DESCRIPTION	Symptomatic education staff Excluded from work on symptoms
IMPACT OF POSITIVE RESULT	Exclude from work for 7-14 days. Possible Infectivity check. Contact trace
IMPACT OF NEGATIVE RESULT	Can return to work if well-enough
EVALUATION	Most positive results are false positives, particularly at low prevalence. Exclude ~2% of staff unnecessarily. Proportion of false negatives increases as prevalence increases

				Positive Results/10,000 tests				Negative Results/10,000 tests		
Specificity	**Sensitivity**	**Prevalence**	**PPV**	**Total positive results**	**True positives**	**False positives**	**NPV**	**Total negative results**	**True negatives**	**False negatives**
0.98	0.9	0.001	0.04	209	9	200	1.00	9791	9790	1
0.98	0.9	0.01	0.31	288	90	198	1.00	9712	9702	10
0.98	0.9	0.05	0.70	640	450	190	0.99	9360	9310	50
0.98	0.9	0.1	0.83	1080	900	180	0.99	8920	8820	100

1.2: Screening of education staff

COHORT	Education Staff
PURPOSE	Screening
DESCRIPTION	Routine testing of asymptomatic staff
IMPACT OF POSITIVE RESULT	Exclude from work for 7-14 days. Possible Infectivity check. Contact trace
IMPACT OF NEGATIVE RESULT	Can return to work if well-enough
EVALUATION	Most positive results are false positives, particularly at low prevalence. Exclude ~2% of staff unnecessarily. Proportion of false negatives increases as prevalence increases

				Positive Results/10,000 tests				Negative Results/10,000 tests		
Specificity	**Sensitivity**	**Prevalence**	**PPV**	**Total positive results**	**True positives**	**False positives**	**NPV**	**Total negative results**	**True negatives**	**False negatives**
0.98	0.8	0.001	0.04	208	8	200	1.00	9792	9790	2
0.98	0.8	0.01	0.29	278	80	198	1.00	9722	9702	20
0.98	0.8	0.05	0.68	590	400	190	0.99	9410	9310	100
0.98	0.8	0.1	0.82	980	800	180	0.98	9020	8820	200

1.3: Re-testing of previously positive education staff to assess likely infectivity (scenario 1)

COHORT	Education Staff
PURPOSE	Infectivity (Scenario 1)
DESCRIPTION	Testing of staff who were previously positive, to see if they remain infectious.
IMPACT OF POSITIVE RESULT	Exclude from work until 'negative'
IMPACT OF NEGATIVE RESULT	Can return to work if well enough
EVALUATION	Very large numbers of false positives. 20% of staff excluded unnecessarily.

Specificity	Sensitivity	Prevalence	PPV	Positive Results/10,000 tests			NPV	Negative Results/10,000 tests		
				Total positive results	True positives	False positives		Total negative results	True negatives	False negatives
0.8	0.9	0.001	0.00	2007	9	1998	1.00	7993	7992	1
0.8	0.9	0.01	0.04	2070	90	1980	1.00	7930	7920	10
0.8	0.9	0.05	0.19	2350	450	1900	0.99	7650	7600	50
0.8	0.9	0.1	0.33	2700	900	1800	0.99	7300	7200	100

1.4: Re-testing of previously positive education staff to assess likely infectivity (scenario 2)

COHORT	Education Staff
PURPOSE	Infectivity (Scenario 2)
DESCRIPTION	Testing of staff who were previously positive, to see if they remain infectious.
IMPACT OF POSITIVE RESULT	Exclude from work until CT below 37
IMPACT OF NEGATIVE RESULT	Can return to work if well enough
EVALUATION	Most positive results are false positives, particularly at low prevalence. Exclude ~2% of staff unnecessarily. Proportion of false negatives increases as prevalence increases

Specificity	Sensitivity	Prevalence	PPV	Positive Results/10,000 tests			NPV	Negative Results/10,000 tests		
				Total positive results	True positives	False positives		Total negative results	True negatives	False negatives
0.98	0.9	0.001	0.04	209	9	200	1.00	9791	9790	1
0.98	0.9	0.01	0.31	288	90	198	1.00	9712	9702	10
0.98	0.9	0.05	0.70	640	450	190	0.99	9360	9310	50
0.98	0.9	0.1	0.83	1080	900	180	0.99	8920	8820	100