

# Public Health Laboratory Network Guidance on Nucleic Acid Test Result Interpretation for SARS-CoV-2

## Revision history

<i>Version</i>	<i>Date Endorsed by PHLN</i>	<i>Revision note</i>
1.2	13 July 2020	Update to clarify the terms 'false positives' and 'inconclusive results'. Amend the title of the document from 'Public Health Laboratory Network Statement on Nucleic Acid Test False Positive Results for SARS-CoV-2'.
1.1	8 June 2020	Initial document

## Overview of diagnostic testing for SARS-CoV-2

Diagnostic testing for SARS-CoV-2 (the virus that causes COVID-19) is vital to containing the COVID-19 pandemic in Australia. Nucleic acid tests (NAT), predominantly reverse transcriptase polymerase chain reaction (RT-PCR), are the primary methods used to detect SARS-CoV-2 in Australia. It is also the benchmark for acute diagnosis of COVID-19 infection. There is a range of NAT used in Australian laboratories, including:

- In-house and commercial assays;
- Assays targeting different parts of the SARS-CoV-2 genome, including the N gene, E gene, S gene, RdRp gene and Orf-1ab gene;
- Single gene target assays, or assays that detect multiple SARS-CoV genes in one test.

## SARS-CoV-2 NAT Reporting

Due to the range of commercial and in-house RT-PCR tests available and the different viral genome targets for each, several reporting outcomes from a RT-PCR test for SARS-CoV-2 test are possible.

- Positive or detected:
  - the detection of SARS-CoV-2-specific target
- Negative or not detected:
  - no detection of SARS-CoV-2-specific target
- Presumptive positive:

- Several commercial and in-house RT-PCR tests for SARS-CoV-2 include both Sarbeco subgenus and SARS-CoV-2-specific targets.
- The Sarbeco subgenus includes SARS-CoV-2, SARS-CoV-1 and several bat-derived SARS-like viruses.
- Several commercial assays report the combination of a Sarbeco target detection with a negative SARS-CoV-2-specific target as a presumptive positive.
- As it is extremely unlikely SARS-CoV-1 or other SARS-like bat viruses are circulating in the human population, a presumptive positive report most likely indicates SARS-CoV-2 detection.
- Sarbeco subgenus only detections are most commonly found when the amount of SARS-CoV-2 target is near the lower limit of detection for the assay.
- Indeterminate or equivocal:
  - Testing has produced discrepant results that cannot be resolved as either a negative or positive despite repeat or further testing of the sample (e.g. testing using a different RT-PCR assay or genetic sequencing).
  - In this situation, the sample may be sent to a reference laboratory for further testing and a repeat sample collection is recommended.
- Invalid:
  - There has been a failure of one or more test's internal controls, such as inhibition of the PCR reaction.
  - In this situation, a repeat sample collection is recommended.

## Test performance of SARS-CoV-2 RT-PCR

PCR assays are extraordinarily high-fidelity assays which are designed for maximal sensitivity and minimal off-target reactivity that benefit from a design process. This includes accurate in silico design of PCR primers and probes and use of extensive databases to identify high-fidelity target sequences with minimal potential off-target interactions. The formats used for PCR enhance fidelity, such as the use of nested, or hemi-nested PCR formats, or formats that include binding of a target-specific probe, potentially boosts specificity. The use of multiple gene targets within the same test, or reflex testing of positives samples with a second PCR test using a different gene target also increases specificity. Cumulatively, all these factors contribute to high specificity and make PCR assays the highest fidelity assays used in infectious disease diagnostics. Although PCR tests are the most accurate tests for detection of SARS-CoV-2, no test has 100% sensitivity or specificity in all clinical circumstances.

Australian laboratories performing NAT are required to:

- implement the National Pathology Accreditation Advisory Council's (NPAAC) Requirements for Medical Testing of Microbial Nucleic Acids quality framework. This includes procedures to minimise the risk of false positive and false negative tests,
- document procedures for reviewing suspected incorrect results, and
- retain records documenting contamination including the identified source of contamination (if known), and measures taken to reduce the risk of such events in the future.

Analytical accuracy of the PCR is therefore very high but clinical accuracy of the PCR is a function of the prevalence of SARS-CoV-2 in the population being tested. A higher prevalence of SARS-COV-2 in the population, increases the pretest probability and the likelihood for detecting SARS-COV-2 RNA. This is reflected in a higher positive predictive value for the test. Conversely, even a very specific test will have a reduced positive predictive value if the population being tested has a very low prevalence of SARS-CoV-2. For example, if a test with a specificity of 99% is used to test symptomatic passengers on a cruise ship

where the likelihood of infection is 50%, the positive predictive value is 99% (i.e. for every 100 people with a positive test result, 99 people will have SARS-CoV-2 infection but 1 person without infection will have a false positive result). However, using the same test, if a low risk asymptomatic population is tested where the likelihood of infection is 5 in 10,000 (i.e. 0.05%), the positive predictive value is 4.3% (i.e. for every 100 people with a positive test result, four to five will have SARS-CoV-2 infection but 95-96 people without infection will have a false positive result).

The SARS-CoV-2 RT-PCR tests used in Australia have very high specificities and the strategy of using a second and/or third SARS-CoV-2 PCR assay with different gene targets increases the specificity of the PCR even further. The combined SARS-CoV-2 PCR testing experience of the PHLN laboratories is that the false positive rate is extremely low.

Test performance can be measured by:

- Analytical sensitivity (the ability of the test to detect a pathogen when it is present).
- Analytical specificity (the ability of a test to be negative when a pathogen is not present).
- Clinical sensitivity (the proportion of people with infection who will have a positive test).
- Clinical specificity (the proportion of people without the infection who will have a negative test).

## **Clinical sensitivity**

All PCR tests have a lower limit of detection for SARS-CoV-2, below which they will return a negative result. The amount of virus in a SARS-CoV-2 infected patient's upper respiratory tract increases over several days before symptom onset to peak around the time of COVID-19 illness onset, and then reduce through the first week of illness. Upper respiratory tract specimens often become PCR negative toward the end of the first week of COVID-19 illness, but some remain PCR positive for several more weeks and a few remain PCR positive, usually with high cycle threshold (Ct) values, for several months. Therefore a SARS-CoV-2 PCR test can be negative despite a person being symptomatic with COVID-19 and the longer into the illness, the more likely the SARS-CoV-2 PCR will be negative. Also, when the amount of virus in the sample is near the limit of detection for the SARS-CoV-2 RT-PCR test, the result is very dependent on the sampling site and technique. Therefore, either a positive, presumptive positive, indeterminate or negative result can be recorded for the sample, or differing PCR results can be found between different samples.

## **False negative and false positive SARS-CoV-2 RT-PCR results**

PHLN emphasises the likelihood of false positive and false negative results occurring is very low. Within the laboratory, false positive and false negative results may not always be easily identified, and laboratory staff, clinicians and/or public health physicians should remain vigilant. Indicators of false positive and false negative results include:

- discrepant clinico-epidemiological findings;
- unexpected laboratory results (such as when an entire batch of or consecutive samples test positive);
- erroneous results from external quality assurance programs;
- warnings from diagnostic companies about potential contaminated assays or reagents; or
- when supplemental NATs or other diagnostics tests such as genomic sequencing or serology do not concur with the initial NAT result.

## Causes of false positive and false negative SARS-CoV-2 RT-PCR results

Table 1 outlines the potential sources of false positive and false negative SARS-CoV-2 RT-PCR results. These can be broadly separated into preanalytical (pre-test) factors, analytical (factors related to test performance such as false-positive reactive RT-PCR) and post-analytical (errors related to incorrect reporting of the result).

The main reasons for false positive and false negative results are:

1. Laboratory error resulting in an incorrect PCR test result assigned to a person's sample.
2. Off-target (non-specific) reactivity in the SARS-CoV-2 RT-PCR test producing a falsely positive result for that person's sample.

### 1. Laboratory Error

Laboratory error does not vary with prevalence in the tested population, although it may increase with pressure on laboratories due to episodes of increased throughput. Such episodes may coincide with testing of large numbers of samples with low pre-test probability has occurred at some stages of the current COVID-19 pandemic. Common categories of laboratory error include: (a) clerical error (b) wrong sample tested (c) cross-contamination from positive samples (d) reagent contamination with amplicon.

### 2. Off-target test reactivity

Off-target reactivity is relatively uncommon but is an inherent characteristic of a PCR assay, the relative robustness or otherwise of assay design and of clinical implementation. Off-target reactivity may include (a) cross reaction with non-target genetic material, and (b) self-priming phenomena in the absence of target. In some cases, it is difficult to confirm or exclude off-target reactivity, particularly in samples with high Ct values.

A PCR reaction amplifies a highly specific target region of the SARS-CoV-2 genome. Each amplification reaction is known as a cycle, and usually 35-45 cycles are undertaken. The Ct value of a reaction is the cycle number when the fluorescence of a PCR product can be detected above the background signal (Figure 1). Whilst extending the number of cycles beyond 40 may increase the sensitivity of the assay, it may also increase the risk of false positivity due to non-specific amplification. This may be identified by assessing the Ct value (usually high), shape of the amplification curve and/or performing a melt curve analysis.

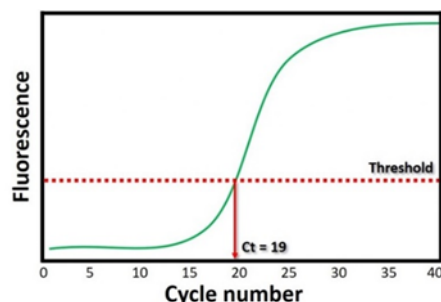


Figure 1: Typical amplification curve of a positive (detected) result

Commercial assays often have in-built analysis systems to interpret the PCR result. To comply with TGA requirements, the laboratory must report the results according to the commercial manufacturer's recommendations. For in-house assays, the parameters for determining a result have been determined by the laboratory through the assay development and evaluation process.

## **Recommended mitigation strategies to prevent false positive and false negative PCR results**

PHLN recommends the below mitigation strategies in the laboratory setting to limit the clinical and/or public health impact of false positive and false negative PCR results. Full consideration of available mitigation strategies, including preventative measures and laboratory actions is described in Table 1.

- Maintain a quality framework in accordance with the NPAAC Requirements for Medical Testing of Microbial Nucleic Acids, which includes procedures to minimise the risk of false positive and false negative tests.
- Maintain high standards of quality control at all stages of testing – including pre-analytical, analytical and post-analytical components.
- In a low prevalence setting, samples with a high Ct value should be interpreted with caution; and repeat testing of the same sample and re-sampling considered.
- Maintain laboratory protocols and procedures to manage low level amplifications (high Ct values) and/or unexpected positive or negative results, such as:
  - Re-extraction and repeat testing of the same sample using the same assay.
  - Confirmatory testing of the same sample using different assays with a different SARS-CoV-2 target (of equal or better sensitivity).
  - Patient specimen re-collection and testing.
  - Ensure the clinical microbiologist (pathologist) providing clinical governance (including direct liaison with clinicians and public health officers), is available to discuss unusual and/or unexpected cases.
  - Ensure close collaboration between the laboratory, local public health units and clinicians to:
    - identify potential false positives,
    - ensure clear understanding of test results and potential limitations, and
    - if required, to initiate further testing.

Laboratories should also determine the process, persons responsible and timelines for resolution of indeterminate or discrepant results, including:

- Repeat testing from the original sample.
- The expected time frame for retesting (preferably with 48 hours).
- The assays used for retesting depending on the target of the primary assay.
- The point at which Clinical Microbiologists and Public Health Units are notified.
- When a repeat specimen is to be collected from the patient (preferably within 48 hours).
- Who is responsible for contacting the patient to arrange repeat sample collection for NAT.
- The role of serology testing.

Table 1: Sources of False Positive and false negative PCR Tests, Preventative Measure and Laboratory Responses

Potential Sources of False Positivity	Preventative Measures	Laboratory Responses
<b>1. Laboratory Errors</b>		
Specimen mislabelling – specimen was not collected from the patient identified on the request form or the specimen	<ul style="list-style-type: none"> <li>Standard procedures at point of collection for positive patient identification and specimen labelling</li> <li>Identification of error at data entry/specimen reception prior to testing</li> <li>Note: if there is complete mislabelling of both the request form and specimen, the error is not identifiable by the laboratory and may only be resolved by re-collection due to investigation of an unexpected result</li> </ul>	<ul style="list-style-type: none"> <li>Specimen rejected and repeat collection performed</li> </ul>
Data entry/specimen receipt transcription errors – specimen is entered under the incorrect patient	<ul style="list-style-type: none"> <li>Standard procedures at data entry/specimen reception to minimise errors</li> <li>Identification of error within the laboratory prior to testing</li> </ul>	<ul style="list-style-type: none"> <li>Re-check patient details on specimen and repeat testing to determine true result</li> </ul>
Contamination of primary specimen with positive specimen	<ul style="list-style-type: none"> <li>Standard laboratory procedures to minimise contamination of equipment, solutions and surfaces which may lead to specimen contamination</li> <li>Use of negative controls to identify contamination</li> <li>Identification of error at time of analysis and/or reporting due to low level amplification and inconsistent clinical history</li> </ul>	<ul style="list-style-type: none"> <li>Repeat testing on a newly collected specimen</li> </ul>
Contamination of specimen extract with positive specimen extract or positive control material	<ul style="list-style-type: none"> <li>Standard laboratory procedures to minimise contamination of equipment, solutions and surfaces which may lead to specimen contamination</li> <li>Use of negative controls to identify contamination</li> <li>Identification of error at time of analysis and/or reporting due to low level amplification and inconsistent clinical history</li> </ul>	<ul style="list-style-type: none"> <li>Repeat extraction and re-testing of original specimen on same and/or different assay</li> <li>Repeat testing on a newly collected specimen</li> </ul>
180 degree rotation of 96 well plates during extraction or assay setup	<ul style="list-style-type: none"> <li>Plate markers; second checkers; strategic placement of controls</li> </ul>	<ul style="list-style-type: none"> <li>Repeat extractions and testing as required</li> </ul>
Data transcription errors – wrong result entered or result entered into wrong patient record	<ul style="list-style-type: none"> <li>Standard laboratory procedures to minimise transcription errors</li> <li>Identification of error may occur at time of reporting</li> </ul>	<ul style="list-style-type: none"> <li>Re-check results and repeat testing from primary specimen or extracted specimen if necessary</li> </ul>
Patient mis-identification following verbal notification of result	<ul style="list-style-type: none"> <li>Standard procedures for positive patient identification during verbal notification procedures</li> <li>Identification by clinician on correlation with formal written/electronic report</li> </ul>	<ul style="list-style-type: none"> <li>Provision of formal written/electronic report</li> </ul>
Misinterpretation of result by the clinician	<ul style="list-style-type: none"> <li>Design of laboratory reports to reduce ambiguity and inclusion of interpretive comments to assist in clinician interpretation</li> </ul>	<ul style="list-style-type: none"> <li></li> </ul>

Potential Sources of False Positivity	Preventative Measures	Laboratory Responses
<b>2. Off-target Reactivity</b>		
Amplification of non-specific PCR products	<ul style="list-style-type: none"> <li>• Assay design</li> <li>• Result analyses and interpretation</li> <li>• Identification at time of analysis and/or reporting due to low level amplification, poor amplification curve and/or inconsistent clinical history</li> </ul>	<ul style="list-style-type: none"> <li>• Repeat extraction and re-testing of original specimen on same and/or different assay and/or genomic sequencing</li> <li>• Repeat testing on a newly collected specimen</li> </ul>