

Guidelines and Recommendations

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IFCC Interim Guidelines on Molecular Testing of SARS-CoV-2 Infection

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Abstract: The diagnosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection globally has relied extensively on molecular testing, contributing vitally to case identification, isolation, contact tracing, and rationalization of infection control measures during the coronavirus disease 2019 (COVID-19) pandemic. Clinical laboratories have thus needed to verify newly developed molecular tests and increase testing capacity at an unprecedented rate. As the COVID-19 pandemic continues to pose a global health threat, laboratories continue to encounter challenges in the selection, verification, and interpretation of these tests. This document by the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) Task Force on COVID-19 provides interim guidance on: (A) clinical indications and target populations, (B) assay selection, (C) assay verification, and (D) test interpretation and limitations for molecular testing of SARS-CoV-2 infection. These evidence-based recommendations will provide practical guidance to clinical laboratories worldwide and highlight the continued importance of laboratory medicine in our collective pandemic response.

Keywords: COVID-19; molecular testing; SARS-CoV-2; virology.

Introduction

Molecular testing for diagnosing acute severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has played an essential role in case identification, isolation, contact tracing, and rationalization of infection control measures during the coronavirus disease 2019 (COVID-19) pandemic. Since the first SARS-CoV-2 genetic sequence was uploaded to the Global Initiative on Sharing All Influenza Data (GISAID) platform on January

10, 2020, diagnostic companies and manufacturers have rapidly developed nucleic acid amplification tests (NAATs), mostly reverse-transcription polymerase chain reaction (RT-PCR)-based molecular tests to detect SARS-CoV-2 RNA in various clinical specimens, most notably nasopharyngeal and/or oropharyngeal swabs. The development of these molecular assays has been of paramount importance to our collective pandemic response, guiding patient care and public health decisions globally [1].

This document by the IFCC Task Force on COVID-19 provides interim guidance on: (A) clinical indications and target populations, (B) assay selection, (C) assay verification, and (D) test interpretation and limitations for molecular testing of SARS-CoV-2 infection. It is aimed to assist laboratories in selecting, validating, and implementing regulatory approved molecular assays.

Taskforce recommendations – molecular assays

A Clinical indications and target population

[A1] Key clinical indications for molecular testing of SARS-CoV-2 infection

Molecular tests can be broadly defined as NAATs for identification of viral RNA in various specimens [1]. Throughout the COVID-19 pandemic, the testing strategies for molecular testing of SARS-CoV-2 infection have varied by region and over time, depending on accessibility and epidemiological concerns. Key clinical indications are provided below.

Recommendation [A1]: Key clinical indications for molecular testing of SARS-CoV-2 infection.

The following indications should be regarded as supported by current evidence and of clinical value:

- To diagnose viral infection in the acute phase of symptomatic illness (0–<14 days).
- To assist in clinical assessment of asymptomatic, pre-symptomatic or mildly symptomatic patients with known exposure to positive COVID-19 cases.
- To assist in screening of asymptomatic, pre-symptomatic or mildly symptomatic individuals in various contexts, including but not limited to: prior to scheduled surgery or delivery, travel, hospital discharge, return to work/school and to manage small outbreaks (retesting should be considered).

[A2] Populations that should be prioritized for molecular testing

Test accessibility has been an issue worldwide. Inadequate access to testing has resulted in prioritization strategies at the public health level. Key populations that should be prioritized for molecular testing of SARS-CoV-2 infections are described below.

Recommendation [A2]: Populations that should be prioritized for molecular testing.

- Patients with acute respiratory illness (fever and at least one sign/symptom of respiratory disease, e.g. cough, shortness of breath) and all individuals having been in contact with a confirmed or probable COVID-19 case in the last 14 days (in resource limiting settings) [2].
- Higher risk groups and settings, including the elderly and patients with pre-existing conditions (e.g. cardiovascular disease, diabetes, cancer, hypertension, etc.).

B Assay selection

Assay selection is a crucial step in SARS-CoV-2 molecular assay implementation that will likely depend on which commercially available assays are accessible to the clinical laboratory and complementary to their current instrumentation. To assist clinical laboratories with selection, potential variables for consideration are provided below. It is critical that laboratories consider the importance of balancing the desired clinical performance to meet the intended use of the assay.

[B1] Importance of assay methodology (Lab-based vs. POCT)

NAATs are currently the gold standard for diagnosing suspected SARS-CoV-2 infections [1]. RT-PCR is the most common type of NAAT applied in authorized molecular assays and is used by both the Centres for Disease Control and Prevention (CDC)-developed assay and the World Health Organization (WHO)-endorsed assays [1–3]. Isothermal nucleic acid amplification such as reverse transcription loop-mediated isothermal amplification, transcription-mediated amplification, and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-based assays represent the second most common type of NAAT [1]. However, very few authorized assays are currently based on this principle. Due to the time-consuming nature of RT-PCR testing, there is increasing

interest in rapid diagnostic tests (e.g. without RNA extraction and purification), that can be used at the point-of-care (POC), particularly for assessment of ambulatory patients or when urgent triage is needed. Laboratory-based assays outnumber currently available point-of-care testing (POCT) assays by far and there is concern regarding the diagnostic sensitivity and specificity of POC testing assays [4]. The diminished diagnostic performance of POC tests is mostly attributable to the fact that these assays differ in their molecular targets and especially in their relative limit of detection of the gene target [5–9].

Recommendation [B1]: Importance of assay methodology (Lab-based vs. POCT).

- Clinical laboratories should select an appropriate NAAT-based assay based on the desired clinical application (screening, diagnosis, monitoring), keeping in mind that performance of current POC assays have not been well demonstrated.

[B2] Importance of viral gene target selection

The main gene targets employed by currently available molecular assays to detect SARS-CoV-2 include the nucleocapsid (*N*), envelope (*E*), spike (*S*), RNA-dependent RNA polymerase (*RdRP*) and open reading frame 1ab (*ORF1ab*) genes. Global Institutions from various countries select different gene targets for SARS-CoV-2 molecular testing, including: China (*ORF1ab* and *N* genes), Germany (*RdRP*, *E* and *N* genes), United States (three targets in *N* gene), France (two targets in *RdRP*), Thailand (*N* gene), and Japan (pancorona and multiple targets, spike protein) [10]. Some publications have compared the analytical and clinical performance of molecular assays targeting different SARS-CoV-2 genes, demonstrating inconclusive findings [10–12]. In assay selection, the gene targets and primers used by manufacturers should be reviewed to ensure they considered robustness to at least the most common mutant strains [13], and are targeted to highly conserved regions. In addition to gene target specificity, lack of harmonization between primer and probe sets limits robust comparison of assay sensitivity between different platforms, and also jeopardizes patient management when longitudinal monitoring is carried out in different laboratories, using different methods.

Recommendation [B2]: Importance of viral gene target selection.

- There is currently insufficient evidence to suggest a definitive advantage of selecting an assay based on a specific SARS-CoV-2 gene target (i.e. *N*, *E*, *R*, *RdRp*, or *ORF1ab* genes).
- Assays for molecular diagnosis should employ a minimum of two gene targets to minimize the risk of false negatives [30, 35].

[B3] Importance of specimen type in test performance

SARS-CoV-2 viral RNA has been detected in nasopharyngeal swabs, oropharyngeal swabs, throat swabs, sputum, bronchoalveolar lavage fluid, whole blood, serum, stool, urine, saliva, rectal swabs, conjunctival swabs, as well as in some tissues [10]. The WHO recommends that, at minimum, upper respiratory specimens (nasopharyngeal and oropharyngeal swabs in ambulatory patients) and/or lower respiratory specimens (sputum (if produced) and/or endotracheal aspirate or bronchoalveolar lavage in patients with more severe respiratory disease) should be collected for molecular testing [2]. Importantly, between 20 and 30% false negative results may occur when using upper respiratory tract specimens, and this is potentially due to issues with sample collection [14]. Thus, lower respiratory specimens are desired for molecular testing, but may not be clinically realistic. On the other hand, disappearance of SARS-CoV-2 from the upper respiratory tract, but shedding of infected cells or viral material (e.g. RNA fragments) from the lower respiratory tract may cause a certain number of positive test results not necessarily correlating to active viral replication and viable virus, which could be misleadingly interpreted as reinfections [15]. Nonetheless, not all manufacturers have validated lower respiratory tract and alternate specimens.

Recently, saliva has been proposed as reliable specimen for SARS-CoV-2 viral detection [16]. Saliva may be particularly recommended in patients or subjects who have tuberculosis in order to prevent cross infection. However, further research in larger patient cohorts is necessary before this sample type can be broadly applied. In addition, the prospect of self-collection has been proposed to improve test accessibility. There are various pre-analytical issues with self-collection and thus results should be interpreted with caution [17, 18].

Sample pooling is another emergent issue in COVID-19 diagnostics. This concept refers to the practice of pooling a variable number of clinical specimens (typically between 5 and 30 nasopharyngeal swabs), which will then be tested altogether [19]. When the pool tests positive, the individual samples are then assayed separately to identify that/those which generated the positivity of the pool. There are some critical issues in adopting this strategy, which can be summarized as follows: (i) sample pooling shall only be used for SARS-CoV-2 screening in low resource or low prevalence (i.e. <5%, preferably <1%) environments, but not for diagnosing a suspected infection in an individual; (ii) the number of clinical specimens in the pool shall be decided according to the analytical sensitivity of the

method and likelihood of positive results; (iii) the presence of interfering substances (e.g. anti-retroviral therapy) should be accurately ruled out (if possible); and (iv) original sample traceability shall be ensured throughout the total testing process. Pool testing requires robust sample handling, sample labelling and result tracking. These steps are to be well documented, particularly in instances where the pooled samples need to be ‘unbundled’ for individual identification.

Recommendation [B3]: Importance of specimen type in test performance.

- The acceptable specimen type for molecular testing should follow manufacturers’ recommendations.
- At minimum, an upper respiratory tract specimen should be collected for molecular testing of SARS-CoV-2 infection.
- Additional evidence is needed to support the use of saliva as a sample type for molecular testing of SARS-CoV-2 infection.
- Self-collection kits are not recommended unless there is appropriate instruction or patient education. Results should always be interpreted with caution.
- Pooling specimens should only be used in low prevalence (<5%, preferably <1%) or low resource settings after appropriate validation.

C Verification of regulatory-approved assays

Verification of a laboratory assay is a procedure that provides objective evidence that the performance characteristics of a test fulfil specified requirements, while validation confirms whether the performance characteristics of the test are adequate for the intended use. The following recommendations are meant to provide general guidance to clinical laboratories on method verification carried out prior to clinical testing for assays that have regulatory approval. This guidance is not meant for validation of laboratory developed tests or for validation of new tests by manufacturers. Individual laboratories should consider local resource availability, as well as regulatory and accreditation requirements, which may differ from those stated below, and modify their evaluation plans accordingly. During evaluation, the selected assay should be assessed by verifying whether the assay meets the manufacturer’s claim and whether it meets the laboratory’s set requirements based on assay use. Ideally, the assay should be evaluated in two parts:

- (1) Evaluation of analytical performance in the context the assay will be used.

- (2) Evaluation of clinical performance in the context the assay will be used.

[C1] Specifications for analytical performance verification of molecular tests for SARS-CoV-2

It is desirable to verify the performance of the testing system on all sample matrices that will be encountered during routine testing. It is anticipated that some laboratories will not have direct access to the samples required for evaluation. This access may be overcome by close collaboration with peers, or with a reference laboratory. All samples used in the evaluation should be stored in conditions that ensure high stability and should be thoroughly homogenised prior to testing. An example analytical assay evaluation protocol is provided in Table 1 for regulatory approved molecular tests for SARS-CoV-2 infection.

Participation in a recognised Quality Assurance Program (QAP) for SARS-CoV-2 molecular testing is also essential [20]. Further, inclusion of a positive quantitative control in each reaction is highly recommended. This step allows continuous monitoring of assay reproducibility, estimation of individual sample viral load from Ct values, and early notification of loss of analytical sensitivity.

Recommendation [C1]: Specifications for analytical performance verification of molecular tests for SARS-CoV-2.

- Laboratories should verify the analytical performance claims of regulatory-approved molecular tests, including the parameters described in Table 1, before routine use.
- Laboratories should participate in a relevant Quality Assurance Program, where possible.

[C2] Specifications for clinical performance verification of molecular tests for SARS-CoV-2

The ascertainment of clinical performance is more challenging as it requires an appropriate ‘reference’ or comparator method with sufficient analytical and clinical sensitivity and specificity [21]. Comparison of a new suboptimal assay with established but suboptimal assay may lead to erroneous conclusions regarding the clinical performance of the new method. Few publications have evaluated the clinical performance of RT-PCR assays using either repeat positive test results in a series of resampled collections, a ‘gold-standard’ assay result, or

Table 1: Analytical parameters recommended for clinical laboratories when verifying a regulatory approved commercial SARS-CoV-2 molecular assay (adapted/modified from [33, 34]). Acceptability criteria are suggestions only and should be modified depending on laboratory standards.

Consideration	Element	Quantitative assay
Limit of detection (LoD)	Design	Not required for use of EUA molecular assays. However, it is recommended that LoD be assessed, when possible. Prepare 5 samples in the range of the claimed LoD and measure 8–12 replicates over 5 days.
	Evaluation	Employ probit regression analysis to establish concentration at which 95% of samples return a positive result. Alternately, determine the concentration at which $\geq 95\%$ of samples return a positive result.
	Acceptability	$\geq 95\%$ of samples near the LoD return a positive result.
Reportable range	Design	Prepare 5–7 concentrations across stated linear range, measure 2–3 replicates at each concentration.
	Evaluation	Prepare a scatter (x–y) plot with measure and results on the y axis vs. the expected or known values on the x axis. Individual data points or mean values can be plotted for each set of replicates. Calculate slope, intercept, and correlation coefficient of linear regression for the averaged test results. Calculate observed bias for each sample from the observed mean concentration vs. the predicted concentration from the regression equation.
	Acceptability	The observed bias should be smaller than a desired allowable difference (e.g. 2–3 times the averaged claimed analytical imprecision, %CV).
Imprecision	Design	Prepare positive and negative quality control samples (if they produce quantitative signal/reading), preferably at concentrations where the imprecision claims were made by the manufacturer and run 5 times daily for five days.
	Evaluation	Calculate mean, SD and CV for repeatability and within-laboratory imprecision of the C_t values and compare against corresponding manufacturer claims. It may be necessary to employ analysis-of-variance for the calculation of each imprecision component.
	Acceptability	The imprecision should fall within manufacturer's claim, where available.
Accuracy (trueness)	Design	Prepare contrived patient samples by spiking individual negative matrix with commercial viral materials. If this is not possible, pooled samples should be used. Testing should include a minimum of 10 positive samples, including five strong positive and five moderate positive samples. Testing should also include at least 10 negative remnant patient specimens. If discordant results are obtained, the specimen should first be repeated by the test under verification. If the discordance is resolved, additional training and/or additional specimens may need to be tested to complete the verification. If the discordance is not resolved, consider testing the specimen by an alternative method, or contact the manufacturer for additional guidance.
	Evaluation	Determine the number of discordant results in study sample set.
	Acceptability	$>95\%$ concordance. If discordant results are observed, it is suggested to determine the underlying cause (e.g. contamination, technique, inhibition).
Analytical specificity	Design	Not required for use of EUA molecular assays. However, it is recommended that analytical specificity is assessed, when possible. Ideally, a panel of all four endemic strains of human coronaviruses should be assayed as well as other respiratory pathogens commonly tested in the clinical laboratory. The samples can be obtained from archived clinical samples, proficiency testing, or commercial pathogen panels.
	Evaluation	Calculate number of false positives for each species and overall negative percent agreement.
	Acceptability	No cross-reactivity observed. Overall negative percent agreement should be within the manufacturer's claim, when available, and meet the clinical performance requirement set by the lab.

EUA, emergency use authorization.

clinical criteria such as CT imaging as the reference comparator [11, 22, 23]. Due to the lack of a 'true' independent gold standard for detecting SARS-CoV-2, some have proposed the use of a composite reference standard or the WHO definition of disease that combines clinical and other test information for diagnosing SARS-CoV-2 infection. Other alternative approaches use contrived clinical samples for assessing clinical performance.

However, contrived clinical specimens in testing for SARS-CoV-2 are typically leftover specimens spiked with RNA or inactivated virus, and thus a poor proxy for actual clinical specimens [21]. Another concern is that manufacturer package inserts often include a claim of clinical performance, but do not provide sufficient information regarding the population which samples were sourced from. These limitations are important to

consider when verifying manufacturer's clinical performance claims.

Recommendation [C2]: Specifications for clinical performance verification of molecular tests for SARS-CoV-2.

- Laboratories should verify the clinical performance claims of the manufacturers of molecular tests in a representative local population in which the test is intended to be used.
- When clinical samples (e.g. repeat positives, positives on 'gold standard' assays, or clinical criteria) are not available, contrived specimens should be used as outlined in Table 1.
- Laboratories should follow the STARD guidelines when designing and reporting clinical performance studies.

D Test interpretation and limitations

[D1] Appropriate test result interpretation for molecular test results

It is essential that molecular test results for SARS-CoV-2 are interpreted in the context of clinical observations, including days since symptom onset and epidemiological background [14]. Current evidence suggests viral RNA can be detected in symptomatic patients as early as the first day of symptoms, peaking within the first week of symptom onset [24, 25]. Positivity is estimated to decline by week three of symptom onset, subsequently becoming undetectable with few exceptions (e.g. patients with prolonged and critical disease, who typically display longer shedding) [26]. Ultimately, considering the timing of testing and clinical context is of utmost importance in interpretation of molecular test results. In addition, patient selection based on careful clinical or epidemiological examination will enrich the pre-test probability and thereby increase post-test probability for positive results.

Reported characteristics of most molecular assays suggest high specificity and moderate sensitivity, minimizing the likelihood of false positive results and increasing confidence in reported positive findings [14]. However, a positive test result does not necessarily indicate the presence of an actively replicating virus and thus its ability to transmit to others [24]. It is important to note that cross-contamination due to handling and testing of large number of samples in a short period of time could lead to false positive results. When a false positive result occurs, it can pose inconveniences to the patient, including: recommendation for isolation,

limiting contact with family members, delaying ability to return to work, inappropriate treatment, etc. [27].

A negative test result should be interpreted with an understanding of pre-test probability (i.e. local prevalence of SARS-CoV-2 infection, exposure history, and symptoms) and test sensitivity [28]. The potential for false negative results to occur is estimated to be higher in high prevalence settings [28], or due to inappropriate timing of sample collection, insufficient collection by healthcare personnel, low viral load, presence of PCR inhibitor, preanalytical or analytical issues [29] or viral mutations [30]. Rates of false negatives have been shown to vary with time since symptom onset [31], and ranged from 2 to 29% in one systematic review [32]. Re-testing has been proposed to improve the post-test probability for negative results [28]. Based on current evidence, an optimal protocol for sampling and resampling over time cannot yet be defined.

Recommendation [D2]: Appropriate test result interpretation for molecular test results.

- **Positive test result:**
 - SARS-CoV-2 RNA has been detected in the sample and the patient should be considered presumptively infected.
 - Active viral replication and potential for viral transmission cannot be concluded. Clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status.
 - SARS-CoV-2 RNA has been detected in the sample and the patient should be considered presumptively infected.
 - Active viral replication and potential for viral transmission cannot be concluded. Clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status.
- **Negative test result:**
 - SARS-CoV-2 RNA was not present in the specimen above the limit of detection of the assay
 - SARS-CoV-2 infection cannot be ruled out and this one test result should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.
 - Re-testing should be considered if: (i) infection is still suspected after considering other differential diagnoses, (ii) molecular testing is being used for hospital release [36], or (iii) analytical inhibition is suspected.
- **Indeterminate test result:**
 - Test result cannot be interpreted, and follow-up re-testing to yield a determinate result is recommended.

Current limitations of molecular testing of SARS-CoV-2 infection

The main limitations of molecular testing in the context of COVID-19 diagnosis are the possibility of false negative results due to preanalytical/analytical factors including delayed testing after symptom onset, low viral load, the occurrence of false positive results, and the delayed time to reporting of results (i.e. from sample collection to reported result) due to limited test capabilities as well as laboratory resources. The latter can cause patient inconvenience, especially when being used as a release mechanism for various activities. All laboratories should recommend isolation for tested individuals carrying high clinical suspicion (e.g. symptomatic or having had a “strict” contact with infected people) in the period between sample collection and result release. Persistently detectable viral targets at low cycle thresholds for several weeks after infection can also complicate interpretation and may justify serological evaluation. Taken together, clinical laboratories should clearly communicate to clinicians that a negative result does not rule out SARS-CoV-2 infection. However, a positive test result can be used to rule in diagnosis when supported by clinical and/or epidemiological findings.

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