DENUNCIA QUERELA

Io sottoscritta/o _____ nato/a a _____ il _____cod. fisc. _____ residente in _____ - mail _____

PREMESSA

1) Con una dichiarazione congiunta il Dott. Fabio Franchi Medico, infettivologo Esperto di virologia, la dott.ssa Antonietta Gatti Scienziata Esperta di nanopatologie, il dott. Stefano Montanari, Farmacista Ricercatore scientifico e nanopatologo, ed il Prof. Stefano Scoglio, Ricercatore Scientifico, Candidato Premio Nobel per la Medicina 2018 hanno rilevato che i risultati dei tamponi sono del tutto inattendibili e che ***"proseguire nell'utilizzo dei tamponi da cui ricavare dati utili a determinare proclami sullo stato di emergenza, quarantene individuali o di gruppo, e per imporre limitazioni e lockdown, dalle scuole alle imprese alle famiglie, è pratica senza nessun fondamento scientifico"***.
2) In particolare il Prof. Stefano Scoglio (Ph.D., B.Sc.) ha coordinato le attività peritali e realizzato lo studio *"I TAMPONI COVID-19 PRODUCONO FINO AL 95% DI FALSI POSITIVI : CERTIFICATO DALL'ISTITUTO SUPERIORE DI SANITÀ Con l'analisi più completa sui tamponi Covid-19" (doc. 4).*
3) **IN PARTICOLARE I PREDETTI ESPERTI HANNO EVIDENZIATO**:
    a) di aver appurato, da un **documento della Commissione Europea e da uno dell'Istituto Superiore di Sanità**, che i tipi di tampone circolanti in Europa al 16 Maggio 2020 erano 78, nessuno dei quali autorizzato, valutato o validato;
    b) di aver riscontrato dai medesimi documenti che gran parte dei medesimi tamponi sono altresì privi della dichiarazione delle sequenze geniche contenute nei tamponi;
    c) per stessa ammissione del Centers for Disease Control and Prevention americano (doc. 3) e del Gruppo di Lavoro Covid della Commissione EU, il virus SARS-Cov2 (doc. 2), presunto responsabile del Covid, non è mai stato isolato fisio-chimicamente;
    d) I liquidi patogeni usati come modello per il sequenziamento genico non avevano nessuna titolazione del virus, il che implica che in tali liquidi erano contenute miliardi di altre particelle simil-virali (incluse le non patogeniche vescicole extracellulari naturalmente presenti nel nostro organismo);
    Ciò comporta che non esiste a tutt'oggi nessun marker specifico del virus, e dunque nessuno standard che possa rendere i tamponi affidabili.
    e) I tamponi attualmente circolanti, oltre 100, sono esentati dai controlli previsti dalla legge europea sui dispositivi medici del 1997;
    f) Al contempo non sono ancora assoggettati alla nuova norma europea del 2017, che entrerà in vigore solo nel Maggio 2022;
    g) Tale limbo normativo rende i produttori liberi di far circolare qualsiasi tipo di dispositivo senza nessun controllo;
    Ciò comporta che la conformità dei tamponi prodotti a degli standard che ne possano accertare la corretta efficacia non è oggi verificata.
    h) Ci sono numerosi studi che attestano la continua mutazione del virus, e le stesse autorità sanitarie riconoscono che se il virus continua a mutare i tamponi diventano inutili.
    i) Ci sono quasi 150.000 diversi sequenziamenti del virus presso la banca dati dei virus GISAID; erano 70.000 ad Aprile; e continuano a crescere, perché si trovano sempre nuove mutazioni, e ciò rende i tamponi circolanti del tutto inutili. Sul punto si allega la ricerca scientifica del dott. Scoglio che analizza anche questo elemento essenziale.
    Ciò comporta che I tamponi circolanti, anche qualora si volessero ritenere astrattamente efficaci e nel concreto conformi alla normativa, sono del tutto inutili in quanto non possono accertare le mutazioni.
    j) Esiste inoltre un problema sostanziale legato alla metodologia utilizzata nei tamponi, la RT-PCR. Come sottolineano i massimi esperti di questa metodologia, per funzionare correttamente tale metodologia idealmente dovrebbe utilizzare tra i 20 e i 30 cicli di PCR; e non si dovrebbe comunque mai superare i 35 cicli, perché sopra tale soglia la PCR inizia a creare sequenze casuali. Ebbene, come confermato anche da diversi documenti che alleghiamo, quasi tutti i tamponi superano i 35-40 cicli di media, e sono dunque da considerare del tutto inefficaci e produttivi di falsi positivi.
    k) Da ultimo, come spiegato in un recente documento dell'Istituto Superiore di Sanità, l'efficacia dei tamponi dipende da 3 fattori: la sensibilità, la capacità di rilevare la presenza di RNA, la specificità, la capacità di limitare tale RNA a quello specifico del virus che si cerca; e la prevalenza, ovvero la presenza della patologia virale nella popolazione. Questo perché maggiore è la prevalenza, maggiore è la circolazione del virus, e dunque maggiore è la possibilità di rilevarlo. Ad oggi, la prevalenza in Italia, che nelle vere pandemie può arrivare anche al 30% della popolazione, è allo 0.1%; e anche se aumentasse di 10 volte sarebbe sempre un livello di prevalenza irrisorio; il che significa, in base ad una tabella della autorevole organizzazione internazionale FIND ripresa dall'ISS, che i tamponi di media performance, in Italia, producono attorno all'85%-90% di falsi positivi.

Nella sostanza **IL TAMPONE FARINGEO NON HA VALORE DIAGNOSTICO.**

CONSIDERAZIONI

A) **Sulla base dei test in parola** negli ultimi mesi **SONO STATI DIFFUSI DATI ALLARMANTI** relativi a:
   1) numero di decessi per Covid;
   2) numero di contagiati da Covid ricoverati;
   3) numero di contagiati da Covid asintomatici;

B) **Sulla base dei risultati dei medesimi test SONO STATI EMESSI PROVVEDIMENTI**:
   1) limitativi della libertà personale prevedendo l'obbligo di quarantena;
   2) limitativi della libertà di circolazione tra regioni e nell'ambito del territorio nazionale;
   3) limitativi della libertà di entrare nel territorio nazionale ovvero nel territorio Regionale;
   4) che hanno gravato pesantemente sulla spesa pubblica e sull'economia nazionale;

C) **Sulla base dei medesimi test si comprime**:
   1) il diritto all'istruzione;
   2) il diritto di accedere alla sanità pubblica;
   3) il diritto di accedere agli asili ed i nidi d'infanzia;
   4) il diritto al lavoro;
   5) la libertà di iniziativa economica privata.

Tali circostanze hanno infatti generato pesanti ripercussioni sulla generale economia dell'Italia ed hanno compromesso irrimediabilmente le attività produttive ed in particolare la piccola e media impresa così come tutte le partite iva. Parimenti i dipendenti, in via diretta o riflessa, hanno subito importanti ripercussioni economiche. In generale ogni cittadino ha subito ingenti danni sia di carattere economico che di carattere relazionale così come biologico personale in riferimento allo stato ansioso determinato in tutta la popolazione.

Tanto premesso e considerato, per i fatti sopra esposti il sottoscritto, come sopra meglio generalizzato,

CHIEDE

che codesta Autorità adita Voglia disporre gli opportuni accertamenti in ordine ai fatti così come esposti dettagliatamente in narrativa, valutando gli eventuali profili d'illiceità penale degli stessi e, nel caso, individuare i possibili soggetti responsabili al fine di procedere nei loro confronti.

Con il presente esposto si intende formulare denuncia-querela, sempre in relazione ai fatti sovra descritti, nell'ipotesi in cui dagli accertamenti svolti dalle Autorità competenti dovessero emergere fattispecie di reato per i quali la legge richiede la procedibilità a querela di parte e **ciò al fine di ottenere la condanna di chi sarà ritenuto responsabile alle pene ritenute di giustizia ed AL RISARCIMENTO DEI DANNI PERSONALMENTE SUBITI in ragione delle circostanze indicate sub A), B) e C) delle considerazioni**.

Il sottoscritto chiede, inoltre, di essere avvertito ai sensi degli artt. 405 e 408 c.p.p. nel caso in cui la S.V. voglia richiedere la proroga dei termini delle indagini preliminari o l'archiviazione del presente esposto.

Il sottoscritto si riserva, inoltre, di integrare la prova orale e documentale e nomina difensore di fiducia l'Avv. _____, del Foro di _____, con studio in _____.

Si depositano i seguenti in formato informatico:

1) EU test validation in Working document test performance 16 April 2020;
2) ISS Covid tests Part 2 https---www.epicentro.iss.it-coronavirus-pdf-rapporto-covid-19-46-2020;
3) CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel aggiornato al 13-07-2020;
4) Prof. Stefano Scoglio - Sui Tamponi Covid-19;
5) FIND evaluation update- SARS-CoV-2 molecular diagnostics – FIND;
6) Dichiarazione congiunta;

_____, lì _____

Firma (_____)

La presente denuncia querela potrà essere compilata e depositata presso qualsiasi Commissariato, stazione dei Carabinieri o ufficio ricezione atti della Procura della Repubblica.

I documenti indicati nel modello di denuncia potranno essere allegati in formato informatico salvandoli su di un CD oppure su una chiavetta. In alternativa potranno essere stampati.

Qualora lo desideriate potrà essere indicata nella denuncia, indicandone il nominativo nell'apposito campo, la nomina dell'avv. Nicola Massafra, del foro di Roma, con studio in Roma Largo Ecuador n. 6 oppure potrà essere indicata nel medesimo la nomina di un qualsiasi altro legale di Vostra fiducia.

La predisposizione del presente modello di denuncia e la messa a disposizione della documentazione da allegare non prevede alcun tipo di costo e compenso essendo stata predisposta su incarico del Dott. Mariano Amici con il fine di metterla a disposizione di chiunque vi abbia interesse.

Al fine di poter archiviare tra le pratiche dello studio le denunce depositate si prega, nel caso di nomina dell'Avv. Nicola Massafra, di inviare una copia della denuncia, con il timbro del depositato rilasciato dall'Autorità, direttamente all'indirizzo mail info@studiomassafra.com indicando nell'oggetto "denuncia tamponi" ed allegando anche l'informativa sulla privacy riportata a pagina 4 e 5 unitamente alla copia del proprio documento e codice fiscale.

Per le successive fasi, volte alla costituzione di parte civile per poter richiedere il risarcimento dei danni subiti ed ottenere la condanna di chi sarà ritenuto responsabile, l'aderente potrà contattare lo Studio Legale Massafra o qualsiasi altro Studio Legale incaricato.

Per ogni informativa sulle iniziative dello studio si prega di visitare il sito dello studio legale www.studiomassafra.com ed iscriversi alla pagina Facebook https://www.facebook.com/StudioLegaleMassafra

INFORMATIVA AI SENSI DELL'ART. 13 DEL REGOLAMENTO EUROPEO 679/2016 E CONSENSO, D.LVO N.56/2004 (NORMATIVA ANTIRICICLAGGIO) E D.LVO 28/2010 (MEDIAZIONE OBBLIGATORIA)

Ai sensi dell'art. 13 del Regolamento europeo (UE) 2016/679 (di seguito GDPR), e in relazione ai dati personali di cui lo studio entrerà nella disponibilità con l'affidamento della Sua pratica, Le comunichiamo quanto segue:

Titolare del trattamento e responsabile della protezione dei dati personali

Titolare del trattamento l'avv. Nicola Massafra (di seguito indicato anche come "professionista") con domicilio eletto in [Largo Ecuador 6. Il Titolare può essere contattato mediante PEC all'indirizzo nicolamassafra@ordineavvocatiroma.org. Lo studio legale non ha nominato un responsabile della protezione dei dati personali (RPD ovvero, data protection officer, DPO).

Finalità del trattamento dei dati

Il trattamento è finalizzato alla corretta e completa esecuzione dell'incarico professionale ricevuto, sia in ambito giudiziale che in ambito stragiudiziale. I suoi dati saranno trattati anche al fine di:

1. adempiere agli obblighi previsti in ambito fiscale e contabile;
2. rispettare gli obblighi incombenti sul professionista e previsti dalla normativa vigente;
3. trattamento finalizzato a newsletter, comunicazione dei social network (Facebook, twitter, instagram ..) iniziative sociali, culturali, solidaristiche; informazioni commerciali; marketing e referenze; invio di materiale pubblicitario/ informativo/promozionale e di aggiornamenti su servizi e iniziative dello studio legale, e/o di Società/professionisti terzi che operano o meno in collaborazione con la Studio Legale altresì in relazione a programmi e promozioni, anche on line, volti a premiare i clienti;

I dati personali potranno essere trattati a mezzo sia di archivi cartacei che informatici (ivi compresi dispositivi portatili) e trattati con modalità strettamente necessarie a far fronte alle finalità sopra indicate.

Base giuridica del trattamento

Lo studio del professionista tratta i Suoi dati personali lecitamente, laddove il trattamento:

- sia necessario all'esecuzione del mandato, di un contratto di cui Lei è parte o all'esecuzione di misure precontrattuali adottate su richiesta;
- sia necessario per adempiere un obbligo legale incombente sul professionista;
- sia basato sul consenso espresso;

Il conferimento dei dati per le finalità indicate ai punti 1) e 2) è obbligatorio ed essenziale per la legge e/o al fine dell'esecuzione del contratto. Il consenso è facoltativo per le finalità di cui al punto 3). In tale ultimo caso non sussistono pertanto conseguenze in caso di un Suo rifiuto, se non l'impossibilità di assicurarle una maggiore informazione sugli sviluppi dei nostri Servizi e un maggiore adeguamento degli stessi alle Sue esigenze

Conservazione dei dati

I Suoi dati personali, oggetto di trattamento per le finalità sopra indicate, saranno conservati per il periodo di durata del contratto e, successivamente, per il tempo in cui il professionista sia soggetto a obblighi di conservazione per finalità fiscali o per altre finalità, previste, da norme di legge o regolamento.

Comunicazione dei dati

I Suoi dati personali potranno essere comunicati a:

1. consulenti e commercialisti o altri legali che eroghino prestazioni funzionali ai fini sopra indicati;
2. istituti bancari e assicurativi che eroghino prestazioni funzionali ai fini sopra indicati;
3. soggetti che elaborano i dati in esecuzione di specifici obblighi di legge;
4. Autorità giudiziarie o amministrative, per l'adempimento degli obblighi di legge.

Profilazione e Diffusione dei dati

I Suoi dati personali non sono soggetti a diffusione né ad alcun processo decisionale interamente automatizzato, ivi compresa la profilazione.

Diritti dell'interessato

Tra i diritti a Lei riconosciuti dal GDPR rientrano quelli di:

a) chiedere al professionista l'accesso ai Suoi dati personali ed alle informazioni relative agli stessi; la rettifica dei dati inesatti o l'integrazione di quelli incompleti; la cancellazione dei dati personali che La riguardano (al verificarsi di una delle condizioni indicate nell'art. 17, paragrafo 1 del GDPR e nel rispetto delle eccezioni previste nel paragrafo 3 dello stesso articolo); la limitazione del trattamento dei Suoi dati personali (al ricorrere di una delle ipotesi indicate nell'art. 18, paragrafo 1 del GDPR);

b) richiedere ed ottenere dal professionista - nelle ipotesi in cui la base giuridica del trattamento sia il contratto o il consenso, e lo stesso sia effettuato con mezzi automatizzati - i Suoi dati personali in un formato strutturato e leggibile da dispositivo automatico, anche al fine di comunicare tali dati ad un altro titolare del trattamento (c.d. diritto alla portabilità dei dati personali);

c) opporsi in qualsiasi momento al trattamento dei Suoi dati personali al ricorrere di situazioni particolari che La riguardano;

d) revocare il consenso in qualsiasi momento, limitatamente alle ipotesi in cui il trattamento sia basato sul Suo consenso per una o più specifiche finalità e riguardi dati personali comuni (ad esempio data e luogo di nascita o luogo di residenza), oppure particolari categorie di dati (ad esempio dati che rivelano la Sua origine razziale, le Sue opinioni politiche, le Sue convinzioni religiose, lo stato di salute o la vita sessuale). Il trattamento basato sul consenso ed effettuato antecedentemente alla revoca dello stesso conserva, comunque, la sua liceità;

e) proporre reclamo a un'autorità di controllo (Autorità Garante per la protezione dei dati personali – www.garanteprivacy.it).

CONSENSO AL TRATTAMENTO DEI DATI PERSONALI REGOLAMENTO (UE) 2016/679

In relazione all'informativa, nel prendere atto che i dati personali fornitivi vengano trattati, diffusi e comunicati per lo svolgimento degli adempimenti relativi alle finalità di cui al punto ai punti 1), 2), e 3) dell'informativa, fornisco il consenso per la finalità di cui ai punti:

□ 1) □ 2) □ 3)

Data _____ Firma del Cliente _____

La presente informativa viene redatta e comunicata anche ai sensi della normativa vigente in materia di antiriciclaggio, essendo il professionista sottoposto agli obblighi di identificazione, registrazione e segnalazione di cui al D.Lvo 56/2004. Inoltre ai sensi del D.lvo 04.03.2010 n. 28 La informiamo: 1. della facoltà di avvalersi del procedimento di mediazione previsto dal d.lgs. n. 28/2010 e dal decreto legislativo 8 ottobre 2007, n. 179 (Procedure di conciliazione e arbitrato presso la Consob e sistema di indennizzo), per le materie ivi regolate, nonché del procedimento di conciliazione istituito in attuazione dell'articolo 128-bis del testo unico delle leggi in materia bancaria e creditizia di cui al decreto legislativo 1° settembre 1993, n. 385, e successive modificazioni, per le materie ivi regolate; 2. dell'obbligo di utilizzare il procedimento di mediazione previsto dal d.lgs. n. 28/2010, in quanto condizione di procedibilità del giudizio, nel caso che la controversia sia relativa a diritti disponibili in materia di diritti reali, divisione, successioni ereditarie, patti di famiglia, locazione, comodato, affitto di aziende, risarcimento del danno da responsabilità medica e da diffamazione con il mezzo della stampa o con altro mezzo di pubblicità, contratti assicurativi, bancari e finanziari; 3. della possibilità, qualora ne ricorrano le condizioni, di avvalersi del gratuito patrocinio a spese dello Stato per la gestione del procedimento nei casi in cui il tentativo di conciliazione è condizione di procedibilità del giudizio; 4. dei benefici fiscali connessi all'utilizzo della procedura ed in particolare della possibilità di giovarsi di un credito d'imposta commisurato all'indennità che sarà corrisposta all'Organismo di mediazione, fino a concorrenza di 500 euro, in caso di successo della mediazione (credito ridotto della metà in caso di insuccesso della stessa); nonché del fatto che tutti gli atti, documenti e i provvedimenti relativi al procedimento di mediazione sono esenti dall'imposta di bollo e da ogni spesa, tassa o diritto di qualsiasi specie e natura e della circostanza che il verbale di accordo è esente dall'imposta di registro entro il limite di valore di 50.000 (cinquantamila) euro e che in caso di valore superiore l' imposta è dovuta solo per la parte eccedente
Per ricevuta della suddetta comunicazione

Roma, lì _____

Nome e Cognome _____

Codice Fiscale _____

Residenza _____

Mail/ PEC _____

Telefono/cell _____

Firma _____

Si allega: 1) copia documenti identificativi del cliente e codice fiscale.

Working document of Commission services

Current performance of COVID-19 test methods and devices and proposed performance criteria

16 April 2020

Table of contents

DISCLAIMER

This working document has been produced as output of a dedicated project group consisting of representatives of the Commission services (DG SANTE, DG JRC, DG RTD), the European Centre for Disease Prevention and Control (ECDC) and several experts from *in vitro* diagnostics competent authorities and health technology assessment. DG JRC conducted the literature review and developed the proposed performance criteria. All other parties contributed input to the project and reviewed the document. DG SANTE coordinated the project group.

The information included in this working document is limited to what could be retrieved from online resources, following the strategy indicated in section 3 of the report, including information provided by members of the project group, up to 06 April 2020.

The correctness of information, such as listed performance data of the test methods and devices, has not been directly confirmed by checking raw experimental data or full technical documentation of the manufacturer (not accessible) or by own laboratory verification or any clinical validation studies. Therefore, the authors should not be deemed responsible for the validity of such data.

The evaluation has been conducted based on information available to the authors on 06 April 2020. Considering the rapidly evolving situation in relation to the development and commercialisation of test methods and diagnostic devices for COVID-19, the completeness of this report is limited to this date.

Because of the urgency of the request to provide this working document, it was not possible to consult with external experts and to perform an independent review of its content.

Executive summary

The coronavirus disease 2019, abbreviated as COVID-19, is a global pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Timely and accurate COVID-19 testing is an essential part of the management of the COVID-19 crisis.

In the EU, commercial *in vitro* diagnostic tests for COVID-19 are currently regulated by Directive 98/79/EC on *in vitro* diagnostic devices (the IVDD). As of 26 May 2022, the Directive will be replaced by Regulation (EU) 2017/746 on in vitro diagnostic devices (IVDR)[1]. When assessing conformity with the legislation and prior to affixing the CE-mark, the manufacturer must evaluate the performance of the device and report the performance information in the instructions for use and technical documentation of the device. This is usually achieved by conducting performance studies. In addition to this, after being placed on the market the performance of devices may be validated, i.e. confirmed by additional testing that the manufacturer's specifications are indeed satisfied, e.g. in reference laboratories, academic institutions or national regulatory agencies. Such validation is not legally obligatory but highly recommended for public health decision making, especially in the context of the current COVID-19 crisis. Validation can be done not only for CE-marked devices but should also be performed for in-house laboratory protocols.

The aim of this working document was to collect and review publicly available information from manufacturers on commercially available devices for COVID-19[2] and to review performance assessment studies of test methods and devices for COVID-19 that have been performed by academic institutions, national regulatory agencies, international organisations, health technology assessment (HTA) bodies, reference laboratories, and similar organisations. Moreover, this report proposes performance criteria for different types of COVID-19 test methods and devices.

The tests used for COVID-19 can be classified into two groups. The first group contains tests that can **detect the presence of the virus itself (RNA and antigen tests)**. The main purposes of these tests are to support the diagnosis of patients with COVID-19-like symptoms, to screen for infections in crucial target groups like healthcare workers, and to test whether an individual recovered from COVID-19 is still infectious. The second group of tests **detect the immune response of the body against the SARS-CoV-2 virus, i.e. they report on past or ongoing infection with the virus (antibody tests)**. The immunity conferred by the antibodies is still under investigation. Once this is clarified, such antibody tests would be, together with the direct virus detection, an essential tool in the development of de-escalation strategies in which mobility and contact restrictions could be removed for people with proven immunity.

Literature review

RNA tests, based on a reverse transcriptase polymerase chain reaction (RT-PCR), are usually laboratory-based and need special equipment. Published in-house protocols (i.e. not based on commercial devices) generally perform well, in particular with low limits of detection and high analytical specificity. From the review of the literature, although several new methods are being developed, it is still recommended to use tests that explicitly declare the implementation of a WHO protocol, of which also validated versions are available. As for CE-marked devices, 78

[1] In the currently ongoing transition period, devices may also be placed on the market if they comply with the Regulation, according to its Article 110.

[2] It should be noted that full information on the manufacturer's performance evaluation of the device is contained in the technical documentation required by Annex III of the IVDD. Manufacturer technical documentation is not publicly available and was outside the scope of this study.

CE-marked RNA tests were identified. The performance parameters reported by the manufacturers were also overall good, in line with those in the published literature. Nevertheless it is difficult to link scientific publications to specific CE-marked devices as the latter do not disclose the RNA sequences detected by the test.

Antigen tests are available generally in a rapid test form that could be used at the point of care. These can potentially offer practical advantages compared to RNA tests for the purpose of reporting on the infectious status. However, the field of antigen tests for COVID-19 appears to be still relatively immature and information on their performance in the scientific literature is scarce. Only 13 antigen tests are CE-marked to date.

As regards information on **antibody tests** 101 CE-marked antibody devices were identified. Overall good levels of sensitivity and specificity are claimed (however not validated by third parties), with the exception of early infection when antibodies are only beginning to be produced. It is not clear how the performance of protocols reported in the literature translates to CE-marked tests. Tests that detect two antibody types at the same time (IgG and IgM) are superior to the ones testing for only one antibody.

In general, on the basis of the literature review it is difficult to recommend particular tests on the basis of independent studies, also because those usually do not mention specific devices.

Performance criteria

The proposed performance criteria for different types of COVID-19 devices are intended as additional guidance to the legally obligatory requirements defined in the IVDD (or IVDR). The proposals cover both analytical performance (relating to how well the marker of interest is detected) and clinical performance (relating to how well the device actually informs on patient status). They also include additional elements on descriptive information, quality control and safety measures. The **most critical performance parameters** for reliable decision-making are:

- **for identifying if a person is infected with SARS-CoV-2**: the **diagnostic sensitivity** of the RNA or antigen test, as false negative test results have to be avoided;
- **for identifying the persons who have developed an immune response** against SARS-CoV-2: the **diagnostic specificity** of the antibody test, as false positive test results have to be avoided.

The information collected in the working document clearly shows that currently available evidence on the reliability and comparability of most COVID-19 tests is limited and has to be expanded as soon as possible to ensure that these tests demonstrate suitability for their intended use. It should be kept in mind that in particular clinical studies are time and resource consuming and therefore an immense benefit would come from **pooling efforts** for test validation, including both sharing data and organising joint studies. A prerequisite for effective assessment of performance, both for the manufacturer in carrying out performance evaluation and for the laboratories validating that performance, is the **availability of necessary control samples and reference materials**. Some of these may be challenging to develop, such as positive virus samples that would be needed for antigen tests. In addition, it is necessary to discuss approaches to **enable more meaningful comparisons and standardisation**, including both methods to achieve this and availability of comparator data sets. Discussions among regulators and all stakeholders are necessary to tackle these issues in a pragmatic way to ensure that COVID-19 devices of the highest reasonably achievable performance level are available in the EU.

1. Introduction

The coronavirus disease 2019, abbreviated to COVID-19, is a global pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). *In vitro* diagnostic tests play an essential role for a rapid and effective response to this crisis as they contribute to patient screening, diagnosis, monitoring/treatment, as well as epidemiologic recovery/surveillance (see Figure 1).

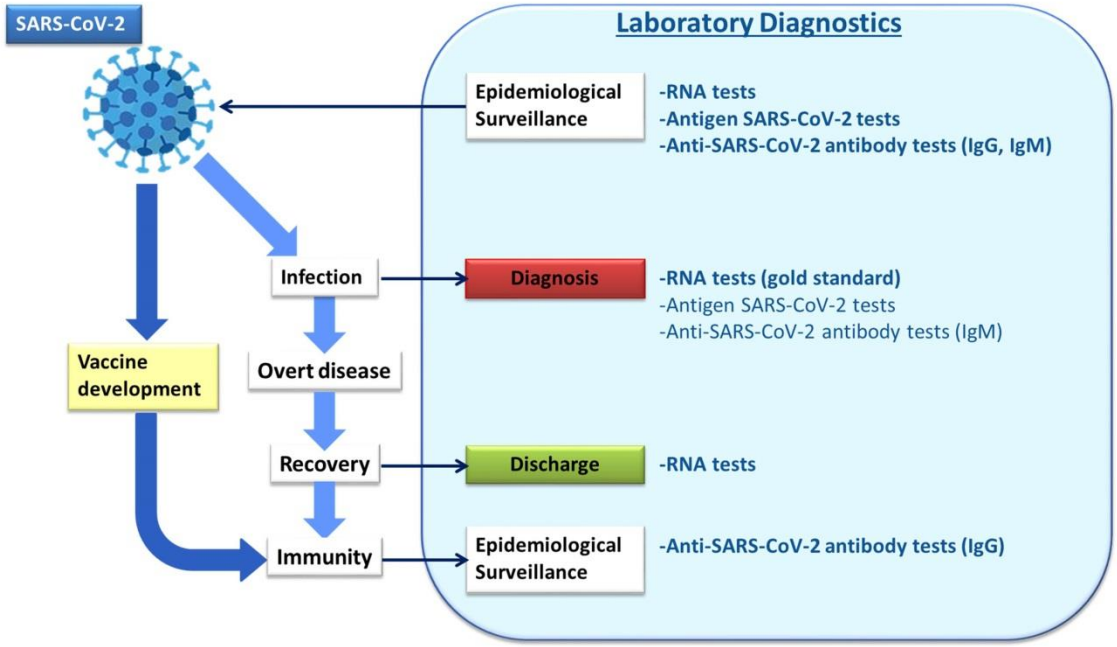

Figure 1: Testing in the context of the COVID-19 disease

At present real-time reverse transcription polymerase chain reaction (RT-PCR) is the gold standard for diagnosing suspected cases of COVID-19. These methods, targeting viral ribonucleic acid (RNA), are currently used as the preferred approach to identify the SARS-CoV-2 virus directly.

The RNA contained in this virus is generally detectable in respiratory specimens during the early and acute phases of infection. Whilst positive results are indicative of the presence of SARS-CoV-2 RNA, a clinical correlation with the patient history and other diagnostic information is necessary to determine the infection status of the patient. Positive results do not rule out an additional bacterial infection or a co-infection with other viruses.

Negative results do not preclude a SARS-CoV-2 infection and should not be used as the sole basis for treatment or other patient management decisions. For instance, negative RT-PCR results from throat swabs occur in the later infection phase when the virus has migrated into the lung. Negative results should be combined with clinical observations, patient history and epidemiological information.

Parts of the coronavirus, in particular several proteins on its surface, are able to act in the body of an infected person as antigens, i.e. they can cause an immunological reaction. In the context of a test, the detection of those 'foreign' proteins can also be used as a signal of a viral infection. The antibodies produced by the patient's adaptive immune system to recognise and neutralise

the antigens can also be detected. These antibodies are secreted into the blood and mucosa. Antigens are likely detectable at a much earlier stage in the COVID-19 infection process than the antibodies produced as part of an immune response. Antigen tests give information on the patient's coronavirus infection status and determine whether a person should be isolated. Antibody tests provide information on a patient's immune status. After infection, the first antibodies to appear in the blood are of the IgM type. They initiate the first line of defence. IgA antibodies, which appear around the same time as the IgM, are mainly present in the mucosa and at lower concentrations in the blood.[3] IgG antibodies appear later, and further control the infection. Typically IgM antibodies disappear in several weeks to months, but IgG could remain present in the blood for many years, or even the rest of the person's life, and play a role in protective immunity. It has been found that COVID-19 patients produce antibodies in a similar pattern. Figure 2 presents results from a study on anti-SARS-CoV-2 which shows IgM and IgG levels over time. Antibody tests would give negative results in the early stages of disease.

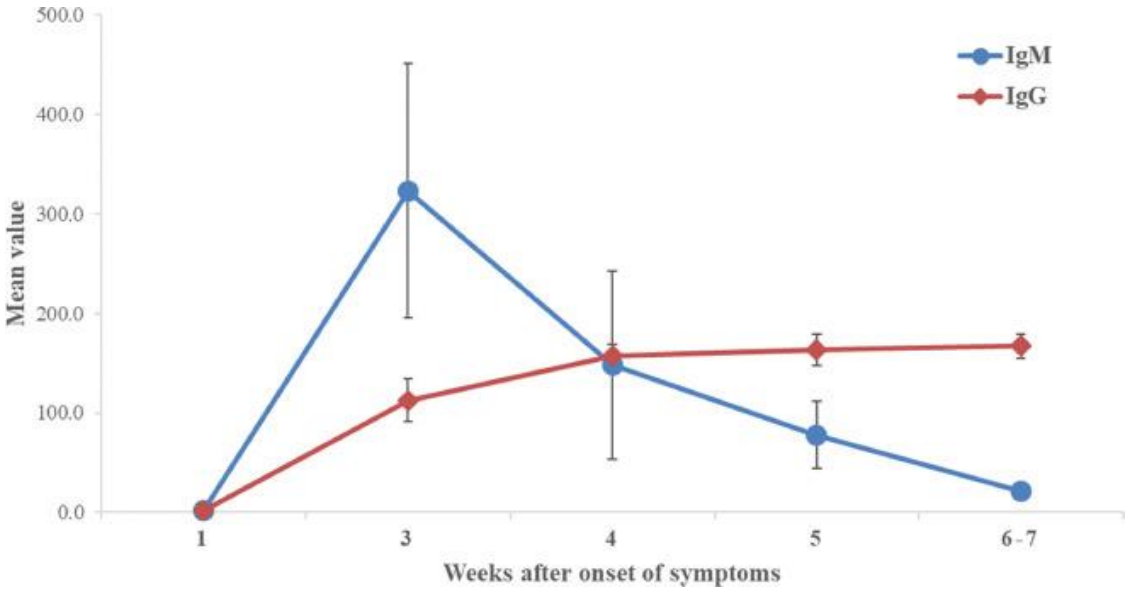

Figure 2: Estimated timeline of IgM and IgG antibody levels to SARS-CoV-2 from the onset of symptoms (adapted from [4])

Both antigen and antibody testing is based on the application of immunological reactions to capture and detect either SARS-CoV-2 components or the patient's antibodies, respectively. Such tests are collectively called immunoassays. The present value of immunoassays (both antibody and antigen tests) for COVID-19 diagnosis and monitoring is heavily debated. These tests are currently not recommended for the diagnosis of suspected COVID-19 cases by the European Centre for Disease Prevention and Control (ECDC)[5], the World Health Organization

[3] Li Guo, Lili Ren, Siyuan Yang et al. Profiling Early Humoral Response To Diagnose Novel Coronavirus Disease (COVID-19) Clinical Infectious Diseases (2020), doi: https://academic.oup.com/cid/article/doi/10.1093/cid/ciaa310/5810754

[4] Ai Tang Xiao, Chun Gao, Sheng Zhang, Profile of Specific Antibodies to SARS-CoV-2: The First Report, Journal of Infection (2020), doi: https://www.sciencedirect.com/science/article/pii/S0163445320301389?via%3Dihub

[5] ECDC Rapid risk assessment: Coronavirus disease 2019 (Covid-19) pandemic: increased transmission in the EU/EEA and the UK, eighth update, https://www.ecdc.europa.eu/en/publications-data/rapid-risk-assessment-coronavirus-disease-2019-covid-19-pandemic-eighth-update

(WHO), the Centers of Disease Control and Prevention (CDC) in the United States[6] and other public health organizations. The ECDC has indicated that clinical trials are needed for the clinical validation of COVID-19 immunoassays before they can be safely and reliably used for medical or public health decision making.[7,8] In addition, the Food and Drug Agency (FDA) of the United States has stated that results from immunoassays should not be used as the sole basis to diagnose or exclude a SARS-CoV-2 infection.[9]

It is too early for sound scientific evidence on the long-term protective immunity against SARS-CoV-2. However, the ECDC has advised the EU that immunoassays detecting specific antibodies against SARS-CoV-2 will play an important role in the future for epidemiological surveillance, evaluation of immunity and the outcome of vaccination studies.[5]

Table 1 illustrates in a simplified manner the correlation between different test results and the phase of infection. It describes an envisaged 'ideal decision' situation that is so far not validated and achieved by currently available tests and data therefrom.

Table 1: Envisaged indications on a person's COVID-19 status from testing different targets

COVID-19 phase	**RNA test***	**Antigen test**	**IgM test**	**IgG test**
before infection	negative**	negative**	negative	negative
first phase of infection	positive	positive later than RNA test	negative	negative
second phase of infection	positive	positive	positive***	negative
last phase of infection	positive	positive	positive***	positive***
after infection	negative**	negative**	positive and later negative	positive***

*with optimal specimen sampled
**diagnostic sensitivity of the test very important (see 4.1.1 & 4.1.2)
***diagnostic specificity of the test very important (see 4.2.1)

The combination of test results from RT-PCR, antigen and antibody testing can provide a clearer picture on the status of a patient. When the RT-PCR and the antigen test results are positive (independently from the presence of symptoms), this indicates that the person is

[6] Centers of Disease Control and Prevention (CDC): Information for laboratories website: https://www.cdc.gov/coronavirus/2019-nCoV/lab/index.html
[7] European Centre for Disease Prevention and Control (ECDC): An overview of the rapid test situation for COVID-19 diagnosis in the EU/EEA https://www.ecdc.europa.eu/en/publications-data/overview-rapid-test-situation-covid-19-diagnosis-eueea
[8] In the EU regulatory framework for in vitro diagnostic medical devices, these trials, when carried out by the manufacturer for the purposes of CE-marking, are called 'performance studies'
[9] FDA Policy for Diagnostic tests for Coronavirus Disease-2019 during the public health emergency: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/policy-diagnostic-tests-coronavirus-disease-2019-during-public-health-emergency

infected and the virus is present and replicating in the body. The presence of IgM and IgG, as detected by immunological tests, shows that the patient's immune system is reacting to a recent infection (IgM) and that the patient has developed (temporary) immunity to the virus (IgG).

The screening of persons with or without symptoms can be differentiated based on the purpose and the testing techniques. Testing based on RT-PCR or an antigen test is important for the identification of infectious cases, as it confirms that the virus is present in the body. This information is essential for assessing the risk of spreading of the virus and to understand the chains of infection.

Antibody testing, coupled with RT-PCR, will be important for surveillance and provides a tool for developing an exit strategy with selective restrictions as a reaction to the pandemic[5]. Antibody tests can indicate who has had the virus and therefore may be immune, although as stated previously sound scientific evidence on the long-term protective immunity against SARS-CoV-2 is still lacking. Surveillance based on antibody testing may reduce the burden on direct virus testing. In the short term, it could also contribute to inform whether people with a demonstrated immunity could be exempt from confinement measures.

Under normal conditions, any positive result detected during screening tests should undergo confirmatory testing using more reliable methods. In case of COVID-19, two different scenarios based on the epidemiological situation are proposed by WHO[10]: in areas with no or low virus circulation[11], suspected cases should be confirmed by a positive RT-PCR result for at least two different targets of the virus genome or a positive RT-PCR result for the presence of a beta-coronavirus that is further identified by partial or whole sequencing of the virus genome. However, in the situation where the virus is widespread and where a second confirmatory test might not be feasible or delay the medical decision, screening by RT-PCR of a single discriminatory target is considered sufficient.[5]

In the light of infrastructure limitations and supply shortages, access to reliable rapid diagnostic tests could alleviate the pressure on laboratories and expand testing capacities[5]. Rapid tests, as defined in the common technical specifications for IVD (revised Commission Decision 2002/364/EC[12]) are qualitative or semi-quantitative tests, which involve non-automated procedures and have been designed to provide a fast result. Both antigen and antibody tests can be found on the market in the form of rapid tests. Moreover, such rapid tests can be designed either for use by health professionals or by lay users. In the latter case they would fall under the category of self-tests, which have to undergo additional verification by a notified body before being placed on the market. Self-testing performed by non-professionals could be a tool to reduce the burden on testing laboratories, but despite efforts by many companies to provide these tests, the EU notified bodies have not yet issued certificates for any such test, and Member State competent authorities are generally not in favour of their use for COVID-19 at this stage.

Point-of-care tests (POCT), also termed near-patient tests, carried out by professionals can provide quicker results than laboratory tests. Some tests of this kind have been CE-marked.

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[10] https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117

[11] According to ECDC daily reports, low circulation EU countries (less than 0.2% of reported cases/1000 habitants) are at present: Greece, Poland, Slovakia, Hungary, Bulgaria

[12] Commission Decision 2002/364/EC on common specifications for in vitro diagnostic devices, OJ L 131, 16.5.2002, p. 17

2. Scope

This report has been established under the mandate to collect and review performance assessment studies of test methods and devices for COVID-19 relevant to the EU that have been performed by academic institutions, national regulatory agencies, international organisations, health technology assessment (HTA) bodies, reference laboratories, and similar organisations, and to suggest performance criteria for different types of COVID-19 test methods and devices. The report does not provide a quality ranking of specific tests or devices with respect to their reliability or performance. Moreover, it does not rank commercial devices regarding their usefulness for the different tasks in COVID-19 diagnostics.

Readers should bear in mind that this report is based on the information available to the authors up to 06 April 2020.

Devices placed on the market in the EU must comply with the relevant requirements of Directive 98/79/EC on *in vitro* diagnostic medical devices (IVDD)[13]. As of 26 May 2022, the Directive will be replaced by Regulation (EU) 2017/746 (IVDR)[14] and during the currently ongoing transition period it is also possible to place on the market devices which comply with the IVDR according to its Article 110. The performance criteria proposed in this document are intended as additional temporary emergency guidance, in view of the COVID-19 pandemic, that cannot replace the essential requirements given in the IVDD or IVDR. Ultimately, manufacturers are responsible for bringing the corresponding devices in full conformity with all requirements of the IVDD or IVDR.

[13] OJ L 331, 7.12.1998, p.1
[14] OJ L 117, 5.5.2017, p. 176

3. Methodology used

The evidence collected regarding the performance of test methods and devices for COVID-19 testing and diagnostics included:

- published peer-reviewed journal articles and non-peer-reviewed manuscript preprints;
- industry documentation on product technical specifications or instructions for use;
- non-peer reviewed published studies or assessment reports by national/international regulatory agencies, including WHO EUL prequalification of IVD;
- preliminary results from validation studies by clinical or reference laboratories reported to open access clinical research registries.

Search strategy for scientific literature

The literature search to identify documents describing the use (or evaluation) of methods for SARS-CoV-2 detection was performed on April 4th, 2020, in three resources:

- Scopus (https://www.scopus.com), peer-reviewed articles;
- bioRxiv (https://www.biorxiv.org/), preprints;
- Europe PMC (https://europepmc.org/), peer-reviewed articles and preprints.

Scopus

The search was performed using the following string:

TITLE-ABS-KEY (covid OR "sars-cov-2" OR "2019-nCoV")

The results (1028 articles) were downloaded as a tab-separated document.

Using a script, each entry was scanned (title, abstract, author keywords and index keywords) for any of the following strings: “detection”, “diagnos”, “polymerase”, “immuno".

The results (218 articles) were extracted and reviewed one by one to identify relevant articles based on the title (and, when necessary, the abstract). The final selection contained 13 articles.

bioRxiv

The bioRxiv site provides a link listing COVID-19 SARS-CoV-2 preprints from both medRxiv and bioRxiv (https://connect.biorxiv.org/relate/content/181).

The JSON version of this list (1146 articles, 886 medRxiv, 260 bioRxiv on April 4th) was retrieved by a script and each entry was scanned (title and abstract) for any of the following strings: “detection”, “diagnos”, “polymerase”, “immuno".

The results (312 articles) were extracted and reviewed one by one to identify relevant articles based on the title (and, when necessary, the abstract). The final selection contained 62 articles.

Europe PMC

The search was performed using the following string:

(“2019-nCoV” OR “COVID-19” OR “SARS-CoV-2") AND (detect* OR diagnos*) AND (FIRST_PDATE:2020)

The results (1391) were downloaded as a tab-separated document. Each entry was reviewed one by one to identify relevant articles based on the title. The final selection contained 83 articles.

Final list

The final list was obtained by combining the results of the three searches, removing the duplicates (based on the DOI and/or title). The final list contained 101 unique articles (publications and preprints). Additional articles were suggested by the members of the Project Group. The total number of scientific articles assessed was 120 (see Annex 2).

Documentation on test methods and devices

The search was performed trying to cover the broader space and therefore different approaches were applied.

At first, depository websites were investigated which are dedicated to collect information on existing and emerging tests developed for the purpose or adapted to the detection of COVID-19:

- Tests commercially available or in development for the diagnosis of COVID-19 from the FIND[15] webpage https://www.finddx.org/covid-19/pipeline/;
- 360Dx[16] web page Coronavirus Test Tracker: Commercially Available COVID-19 Diagnostic Tests (https://www.360dx.com/coronavirus-test-tracker-launched-covid-19-tests).

Secondly, the EMM (Europe Media Monitor)-finder was used to identify news articles as of 1st January 2020 containing 'COVID-19' or 'SARS-CoV-2' in combination with 'detection' or 'test' or 'diagnostics' or 'method' or 'measurement'. The EMM-finder is a specific adaptation of the JRC tool for text mining of media news and allows searching also back in time in the database.

Finally, similar searches were performed using Google to identify the largest possible number of Covid-19 diagnostic tests available in the market.

A parallel search was conducted using the PitchBook[17] tool. The search, for deals from 15 November 2019, was made by looking for "covid" OR "covid-19" OR "covid 19" OR "SARS-CoV-2" OR "novel coronavirus" OR "novel corona virus" OR "2019-ncov" OR "2019ncov". The above search was expanded with "sars" OR "mers" OR "ace2" OR "soluble ace 2" OR "sars-cov" OR "mers-cov". Lastly, a search was performed in "diagnostic equipment" AND "virus" with deals from 15 November 2019.

The manufacturers obtained from the financial tool were manually screened to verify if they were producing COVID-19 devices. When this was the case and if they were not already in the list, they were added.

[15] FIND (https://www.finddx.org/) is the Foundation for Innovative New Diagnostics, a global non-profit organization driving innovation in the development and delivery of diagnostics to combat major diseases affecting the world's poorest populations.

[16] 360Dx (https://www.360dx.com/) is published by GenomeWeb, an independent online news organization based in New York. 360Dx was launched in 2016, to cover emerging economic and technological trends in the clinical diagnostic market.

[17] PitchBook (https://pitchbook.com/) is a financial data and software company that provides comprehensive data on the private and public markets (venture capital, private equity, mergers and acquisitions).

The final compilation of devices, together with the additional information that was gathered for each is found in Annex 1. The table contains the name of the manufacturer, the name of the device, the type of method (PCR, immunological, digital, control panel, CRISPR), the status of the device ("commercialised" or "in development"), an indication of the speed and the regulatory frame for which they are fit for. The indication of the speed is using the 7 categories defined by FIND, "rapid diagnostic test" that include immunoassays providing results in less than 20 min, the classical immunoassays without a label, the "manual NAT" are PCR methods based on nucleic acid amplification tests that are more time consuming, "Automated lab-based, near-POC NAT or POC NAT" are automatic PCR tests that allow near point-of-care or near-patient laboratory testing, finally those classified as "manual or automatic" because they exist in both versions. Finally, the regulatory frame classified the devices as "research use only", "in development", "proof of concept" and approved by the different country/region regulatory bodies (US FDA, China, Korea, India or CE-IVD (EU compliance label according to the IVD Directive).

From this list, each method/device obtained an internal unique number. A selection was made for further analysis based on criteria to focus on those devices accessible in the EU. For this reason, products labelled as for "research use only" or under development have been ignored as well as products fulfilling other regulatory frameworks than the EU IVD Directive. Those with the CE-IVD marking were further classified and split in three tables depending on the methodology (Annex 2). In total, 78 devices based on RT-PCR (or variants e.g. CRISPR and LAMP), 101 for the detection of antibodies and 13 for the detection of antigens were assessed.

4. Overview of information on performance & proposed performance criteria

4.1. Detection of viral status

4.1.1. RT-PCR

A) Evaluation of evidence

The most crucial information concerning RT-PCR based methods developed for the detection of SARS-CoV-2 are the sequences of the oligonucleotides (primers and probe) used for the amplification of the cDNA. That information allows to establish a RT-PCR based method, as it targets a precise sequence in the genome of the virus.

For many of the RT-PCR devices matching the selection criteria described in Section 3, information supplied by the manufacturer could be found in the form of technical specifications or instructions for use. This information contained some relevant information on the device performance, compiled in the annexed table and summarised below.

It is important to note that, except for a few cases, no information on the actual sequences of the primers and probes in the device could be found. This makes the complementation of performance information from what is published in the scientific literature for an individual device conditional on the explicit mentioning of the device in the 'materials and methods' part of a publication.

Literature review – general observations

A significant number of new real-time PCR protocols to detect the presence of SARS-CoV-2 RNA have been published recently in addition to those recommended by WHO[18]. Most of the studies tested and compared RT-PCR methods also on clinical samples from patients, some on different multiple samples coming from large numbers of patients.

Instances of new or improved methods have also been described. For example, ART69 (Annex 2) compared a new set of primers for the RdRp gene with the method recommended by WHO developed at the Charité Universitätsmedizin Berlin, Germany. The method shows *in vitro* a very low limit of detection (LOD) with viral RNA transcripts (11.2 RNA copies/reaction). In patient samples, the new assay detected SARS-CoV-2 RNA in 42 out of 273 (15.4%) additional specimens that were tested negative by the Charité assay. The new assay was significantly more sensitive than the Charité assay for the detection of SARS-CoV-2 RNA in nasopharyngeal swab, saliva, and plasma specimens.

A particularity of the assays described in the scientific literature is that attempts are being made to develop and implement methods based on novel techniques, such as digital chamber PCR, digital droplet PCR, LAMP and CRISPR.

A few papers applied the RT-PCR protocol from the CDC method based on the ORF1Ab and N gene to digital PCR (dPCR) (ART90, ART68 and ART42) or used a commercial kit for dPCR (ART104) to test patient samples (close to 100 in each study) previously tested by real-time PCR. In these studies, among all the confirmed positive samples, some that were tested false negative by RT-PCR were corrected by the more sensitive dPCR assay. The digital PCR

[18] WHO, 2020. https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance

shows an improved lower limit of detection, sensitivity and accuracy compared to real-time PCR for low viral load diagnosis. This methodology showed to reduce false negative detection in samples from the lower respiratory tract, especially in cases of low viral load samples. This technique requires more sophisticated instruments.

CRISPR technology is widely known for its use in gene editing techniques. But in recent years, CRISPR has been also used for the *in vitro* detection of nucleic acids, thereby emerging as a powerful technology for molecular diagnostics. [19,20]

What made CRISPR revolutionary was its ability to recognise specific sequences of DNA. The same can be done for viral genetic material so that the reaction emits a fluorescence signal on cleavage or could be alternatively detected on a paper strip by lateral flow in a portable manner.[21]

However, it is important to note that all of the published studies on CRISPR are only at a proof of concept stage, where the technique is only tested on plasmid positive controls or spiked human samples. None of the published studies have analysed real patient samples. At present, only two kits are available using this technology but both are still "in development" phase; one of them is made available for "Research Use Only".

LAMP is an isothermal variation of PCR (meaning it does not require dedicated and expensive thermal cycling equipment) and is therefore commonly used for point-of-care testing (POCT) due to its high sensitivity, rapid reaction and simple operation. The result is evaluated by a colour change that does not require specialised personnel.

All the published papers presented LAMP combined with a reverse transcription assay (RT-LAMP) which had been developed for the detection of multiple respiratory RNA viruses to avoid the need for expensive technologies. The LAMP technique is highly specific through the combined detection of three genes of the coronavirus. It is claimed by manufacturers that this would possibly increase the accuracy of detection to almost 99%. However, it is reported that the use of one of the three genes alone would reduce the diagnostic sensitivity or specificity of the test, causing false positive or false negative results.

Application aspects

RT-PCR methods require specific laboratory equipment and various sets of reagents. As a rule, these tests need to be performed by trained laboratory personnel who are proficient in performing the methods. In addition, several scientific publications underline the importance of combining CT scans and clinical observation with RT-PCR results for the follow-up of COVID-19 patients, in order to improve the discovery rate of the disease and to evaluate a patient discharge from the hospital. Furthermore, the testing of different kinds of swabs and samples from different body fluids is recommended in order to increase the potential detection of the virus by RT-PCR at different stages of the disease.

The time needed to perform the test, when reported, has been compiled in the annexed table, and varies from one to a few hours. Part of the reported differences comes from the different starting point, i.e. whether the method includes the steps to extract and purify the viral RNA from the original sample or only starts from a previously purified sample. The assay protocols

[19] Gootenberg JS, Abudayyeh OO, Lee JW, et al. Nucleic acid detection with CRISPRCas13a/C2c2.2017. Science;356(6336):438–442

[20] Li SY, Cheng QX, Wang JM, et al. CRISPR-Cas12a-assisted nucleic acid detection. 2018. Cell Discov. 4:20

[21] Myhrvold C, Freije CA, Gootenberg JS, et al. Field-deployable viral diagnostics using CRISPR-Cas13. 2018. Science 360(6387):444-448

generally rely on preparing tube-by-tube reactions; how many can then be run at once is varying according to the instrumental setup (e.g. 96 wells and more). Proofs of concept of testing workflows for lab-based surveillance have been proposed using pools of several RNA samples from different patients in order to increase the throughput (ART04, ART59). This approach needs further validation, but it would reduce costs while monitoring the epidemic in the population.

Despite the high analytical sensitivity and specificity of real-time PCR (see below), a study on 610 hospitalized patients from Wuhan in February 2020 (ART97) showed that RT-PCR test results of pharyngeal swab specimens were variable. This study concluded that this method should not be considered as the only indicator for the presence of the coronavirus.

Quality control

The information relevant to the controls included for each device, based on the components and protocol, are compiled (when found) in the annexed table.

In general devices include a positive control material, i.e. a purified template known to contain the amplified target(s) (e.g. a pseudovirus RNA). Many also include an internal control method, i.e. a method that amplifies a target and produces a signal whether or not the sample contains SARS-CoV-2 RNA (e.g. human RNase P gene, Rpp30 gene). Negative controls are usually described as reactions performed without adding a sample to the reaction mix (e.g. distilled water, IVT synthetic mix).

Analytical performance

Limit of Detection (LOD)

Some information made public by the manufacturers includes indication of the LOD of the methods, sometimes providing additional details on how the determination was performed. Identified values are compiled in the annexed table, reaching as low as a few copies of the viral RNA per reaction. This is in line with the generally very low LOD achievable by RT-PCR-based methods and is a main advantage of these approaches.

For most of the publications reviewed, only a partial in-house validation of the method was performed. Sensitivity measurements are not always done when testing a method or comparing different method performances. Moreover, when sensitivity is tested, the LOD assessment is rarely performed according to the minimum performance criteria described below.

(Analytical) Specificity

Depending on the devices, information was sometimes included in the public documentation regarding specificity, describing:

- bioinformatics analysis to confirm that the oligonucleotides would bind correctly to all known SARS-CoV-2 RNA isolates whose sequences have been compiled in repositories;
- bioinformatics analysis to confirm that the oligonucleotides would not bind to nucleic acid sequences of other microorganisms, including viruses close to SARS-CoV-2;
- laboratory experiments with samples from other microorganisms to demonstrate the lack of signal produced by the device in these instances.

The annexed table reports when such information was available, in particular for laboratory experiments that are of most practical relevance. The compiled information is in line with the generally very high analytical specificity achievable by RT-PCR-based methods and is a main advantage of these approaches.

In the literature, specificity of the methods is only sometimes tested with panels (often commercially available) of human viruses and pathogen RNA spiked into clinical samples, in order to exclude cross-reactivity. It should be noted that a study has highlighted some nucleotide mismatches (that may adversely affect the reactions efficiency) on the primers' annealing sites of methods listed by WHO (Annex 2, ART31).

PCR efficiency, robustness, precision

With very rare exceptions, no information was found on efficiency, robustness and precision as defined below.

Exceptions include Novacyt's genesig Real-Time PCR COVID-19 device, whose instructions for use include a description of the reproducibility and repeatability analysis performed in the laboratory, as well as the two devices from Genomica Sau, for which it is reported that a validation study was performed at the National Center for Microbiology (Instituto de Salud Carlos III, Spanish National Reference Center) with a panel of 80 samples. It is unclear for the latter whether the validation included considerations of robustness.

Similarly, the efficiency of RT-PCR reactions with synthetic target RNA transcripts is often not reported as measured in scientific studies and the effect of potential interfering substances has only been reported in one publication (Annex 2, ART37). The reproducibility of the method is sometimes assessed using different RNA extraction kits, which may lead to a different sensitivity of the method influencing the results with false negatives, especially in samples with low viral load.

Clinical performance

For a few of the devices assessed, information was found about the results of clinical performance studies, compiled in the annexed table.

When available, manufacturer claims on both the diagnostic sensitivity and specificity were very optimistic (in the range of 96-100%).

Linking the scientific literature with specific commercial devices

Finding additional information in the scientific literature on the performance of specific commercial devices is difficult due to the lack of information regarding the actual sequences of the oligonucleotides that are comprising the devices' main components, as explained above. Without this information, it is not possible to link relevant scientific studies, even if the sequences are almost always made explicit in the publications, unless the article explicitly mentions the device used in the study.

Exceptions to this include manufactured devices that specifically mention the implementation of one of the WHO recommended methods, as the primers and probes for those have been made public and have been extensively used in research and testing laboratories until now. These are:

Manufacturer	**Test**	**WHO method explicitly referenced**	
1drop Inc.	1copy™ COVID-19 qPCR Kit	Charité protocol (2nd version)	Reference
AB ANALITICA Srl	REALQUALITY RQ-2019-nCoV	Charité protocol (2nd version)	Reference
Diatheva SRL	COVID-19 PCR DIATHEVA Detection kit	Charité protocol (2nd version)	Reference
Sentinel CH	STAT-NAT® Covid-19 B	Charité protocol (2nd version)	Reference
Sentinel CH	STAT-NAT® Covid-19 HK	Hong Kong Faculty of Medicine protocol	Reference
BioGX	SARS-CoV-2 Open System Reagents for BD MAX	US CDC protocol	Reference

The protocols suggested by WHO are currently the most recurrent ones in the literature, because some of their primer/probe sets are commercially available. However, many of them present background noise that render the interpretation of results difficult at low viral load and a proper validation of these RT-PCR methods is recommended. So far, among WHO suggested protocols only the ones from the Institut Pasteur, the Hong Kong Faculty of Medicine and the Charité were in-house validated and good analytical specificity and LODs were reported (Institute Pasteur, LOD: 100 cp/reaction; Charité for target E, LOD: 3.9 cp/reaction and for target RdRp, LOD: 3.6 cp/reaction). The performances of all WHO suggested protocols (except the one from Institut Pasteur) have been evaluated in several papers and, although all methods showed high analytical specificity, the Charité method targeting E and RdRp genes and the method of CDC (US) targeting N1 and N2 regions have the best sensitivity. However, not all methods were compared under the same conditions and not for all of them the analytical specificity and sensitivity were properly assessed.

Some scientific publications include studies evaluating or comparing explicitly named commercial CE marked devices found in the attached table. In one study, devices from DAAN Gene Co., Sansure Biotech, Chaozhou Hybribio Biochemistry Ltd were analysed and shown to perform slightly better than another device, Bioperfectus (Annex 2, ART37). It should, however, be noted that the resulting LOD values were higher than the ones provided by the manufacturers.

Commercial devices are sometimes used for comparison purposes in studies showing the performance of novel methods. For comparisons with new LAMP-based methods, ART47 uses a SARS-CoV-2 device from Shanghai BioGerm Medical Biotechnology Co. Ltd and a device from DAAN Gene Co., while ART49 uses the Liferiver Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCRT device. In ART38, 57, 68 and 69 devices are mainly used in comparison with other developed RT-PCR and dPCR methods or to confirm the results coming from the newly developed methods. In these studies, no particular evaluation of the devices is included since it was not part of the main objectives of the work.

B) Proposed performance criteria

Both commercial diagnostic devices and test methods (e.g. protocols) based on the RT-PCR principle have been taken into account for this report. Test methods require a more detailed consideration, as each individual reagent (e.g. Master Mix, primers/probe, control samples) has to be purchased or prepared by the user, whereas such reagents will often be included as parts of the devices so that the corresponding information does not have to be necessarily disclosed.

The analytical and clinical performance criteria are described below. In addition to this, guidance on descriptive information, quality control and safety measures is given.

Descriptive information

Essential requirements are laid down in Annex I of the IVDD and IVDR. More specifically, for the testing of SARS-CoV-2 virus RNA, the following descriptive information is of particular importance:

- the nature of the test (qualitative, semi-quantitative);
- the measured target (the specific RNA fragment from SARS-CoV-2 amplified);
- the type of specimen that can be tested, e.g. oropharyngeal swab sample, its pretreatment (e.g. required dilution), stability and storage
- the purpose (e.g. the results are for the identification of SARS-CoV-2 RNA);
- the indication whether any particular training is required (e.g. the test is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays).

The general principle of the method should be described (e.g. RNA isolated and purified from upper and lower respiratory specimens is reverse transcribed to cDNA and subsequently amplified in a real-time PCR instrument).

If the RNA extraction step is not part of the device, a list of the RNA extraction kits (name of the instrument/manufacturer, name of the extraction kit, catalogue no.) for which the devices/tests have been proven to perform reliably should be provided.

A complete list of reagents either provided (in case of devices) or needed (in case of test methods) should be provided as well as the necessary consumables and equipment. Information concerning the control of efficacy of the sample preparation and the absence of inhibitors in the PCR reaction should be provided.

For reagents the expiration date and instructions concerning their storage and handling (e.g. reconstitution of lyophilised material and reagents) should be known.

Quality control

The required quality controls should be described. Extraction and amplification should be controlled by an internal control in each specimen. Positive and negative controls should be analysed in parallel with the specimens in each run to guarantee the validity of the test and the correct interpretation of the results.

Safety measures

It is of the utmost importance that proper biosafety guidelines are followed by clinical laboratories when handling samples from suspected COVID-19 patients. The laboratory

biosafety guidance related to the coronavirus disease issued by the World Health Organization should be taken into account where applicable.[22]

Analytical performance

Limit of detection (LOD)

The LOD can be determined by limiting dilution studies using sufficiently characterised samples and should be provided.

Since no virus isolates with a quantified amount of the SARS-CoV-2 are currently available, assays designed for detection of the SARS-CoV-2 RNA could be tested with characterised stocks of *in vitro* transcribed RNA containing the target of interest of a calculated titer (RNA copies/µL) spiked into a diluent consisting of a suspension of human cells in viral transport medium (VTM) to mimic a clinical specimen. Such studies are generally performed in two steps. In a preliminary step, an approximate LOD for an assay is determined by testing triplicate samples of RNA purified using a defined extraction method. The approximate LOD is determined by extracting and testing 10-fold serial dilutions of characterized stocks of *in vitro* transcribed RNA. A confirmation of the predetermined LOD is then performed at a chosen dilution of the spiked RNA samples with a minimum of 20 extracted replicates. The LOD is determined as the lowest concentration where ≥ 95% (19/20) of the replicates are positive. More commonly, this is done by at least five half-logarithmic dilutions around the pre-determined LOD, tested in replicates of at least 24 samples.

(Analytical) Specificity

The analytical specificity of the assay (also named inclusivity testing) is assessed *in silico* by aligning the oligonucleotide primer and probe sequences to all publically available nucleic acid sequences of SARS-CoV-2 in GenBank as of a particular date. The percentage of identity of the amplicon should be evaluated and a potential mismatch should be reported. The risk of a single mismatch resulting in a significant loss in reactivity and potentially a false negative result should be evaluated. Experience has shown that a proper design of the primers and probes with melting temperatures > 60 °C and PCR run conditions of the assay with annealing temperature at 55 °C could tolerate one to two mismatches.

A search for significant homologies with other SARS coronaviruses, the Bat SARS-like coronavirus genome and the human genome and human microflora should be performed to evaluate and predict potential false positive RT-PCR results.

The *in silico* assessment should be complemented by testing the assays against RNA extracted from real samples (isolates/clinical specimen of other human coronaviruses, MERS coronaviruses, SARS coronavirus, human influenza, etc.) to confirm a negative result.

Robustness

The effect of other potential interfering substances should be investigated. The equivalence of the LOD of the assays should be evaluated with different enzyme master mixes on serial dilutions of *in vitro* transcribed RNA.

[22] https://www.who.int/publications-detail/laboratory-testing-for-2019-novel-coronavirus-in-suspected-human-cases-20200117

Retrospective positive and negative clinical respiratory specimens (when available), extracted by different extraction kits, should be tested to evaluate the equivalent performance of the extraction kits.

Precision

Both repeatability (i.e. testing the same sample under the same conditions) and reproducibility (i.e. testing the same sample under variable conditions such as different reagent kits, days, different analysts, or different instruments) should be assessed. In the case of qualitative test results the precision parameters can be expressed as the percentage of agreement.

Clinical performance

The analytical performance criteria are usually evaluated on a number of well-defined laboratory samples and extreme patient samples. The next step should be the clinical performance evaluation of the diagnostic test accuracy. The determination of diagnostic accuracy should be performed in (clinical) studies using head-to-head comparison between results from one or more RT-PCR tests under assessment and those of the reference RT-PCR test in the target population intended to be tested. The diagnostic accuracy is composed of:

- Diagnostic sensitivity: Proportion of those individuals with the target condition (infected individuals with reference SARS-CoV-2 RNA true positive specimen) who test positive with the RT-PCR test;
- Diagnostic specificity: Proportion of those individuals without the target condition (infection-free individuals with reference SARS-CoV-2 RNA true negative specimen) who test negative with the RT-PCR test.

The diagnostic sensitivity of a specified RT-PCR test is the most crucial parameter for the identification of persons who are infected by SARS-CoV-2, as false negative test results have to be as low as possible.

The number of patients (sample size) to be included in the trial should be determined by statistical power calculation for a desired precision level of the accuracy estimates. The study report should provide the estimates of diagnostic sensitivity and specificity with confidence intervals. The STARD 2015 (STAndards for Reporting Diagnostic accuracy studies) should be followed[23]. It is recommended to report the 95 % confidence interval for the estimates of both the diagnostic sensitivity and the diagnostic specificity.

The clinical performance of a test method or device should be evaluated for all the intended conditions of use, e.g. each type of specimen mentioned should be evaluated.

4.1.2. Antigen tests

A) Evaluation of evidence

There are two main types of immunological tests, namely ELISA (enzyme-linked immunosorbent assay) and LFA (lateral flow assays). ELISA tests are more complex and require trained technicians operating under sterile laboratory conditions, but tend to be more

[23] Cohen JF, Korevaar DA, Altman DG, et al. STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. BMJ Open 2016;6:e012799. doi:10.1136/bmjopen-2016012799

sensitive. LFA are point-of-care formats that have been developed with simplicity and portability in mind. They can be simple and easy-to-produce devices.

Literature and product review

At present rapid antigen tests are not fully developed and the literature is scarce. A few papers have described an antigen microarray, but this was rather fundamental research and not an available commercial device (Annex 2, ART05 and ART111).

We have identified very few (13) commercial antigen tests and, according to our analysis, they are currently at a too early stage of development, i.e. they do not meet the proposed performance criteria described in Section 4.2.1B.

B) Proposed performance criteria

The proposed performance criteria on the descriptive information and the analytical performance are identical to the ones for antibody tests and are therefore presented together in Section 4.2.1B. The clinical performance criteria described below are specific for the antigen tests:

Clinical performance

The proposed clinical performance criteria for SARS-CoV-2 antigen detection are similar to those for SARS-CoV-2 RNA detection by RT-PCR (Section 4.1.1B). The determination of diagnostic accuracy should be performed in clinical studies using head-to-head comparison between results from one or more antigen tests under assessment and those of the reference RT-PCR test in the target population intended to be tested. The clinical performance criterion for a SARS-CoV-2 antigen test under validation is the diagnostic accuracy, composed of:

- Diagnostic sensitivity: Proportion of those individuals with the target condition (infected individuals with reference SARS-CoV-2 RNA true positive specimen) who test positive with the antigen test;
- Diagnostic specificity: Proportion of those individuals without the target condition (infection-free individuals with reference SARS-CoV-2 RNA true negative specimen) who test negative with the antigen test.

For antigen tests the diagnostic sensitivity is the most crucial parameter for the correct identification of persons who are infected by SARS-CoV-2, as false negative test results must be as low as possible.

The number of patients (sample size) to be included in the trial should be determined by statistical power calculation for a desired precision level of the accuracy estimates. The study report should provide the estimates of diagnostic sensitivity and specificity with confidence intervals. The STARD 2015 (STAndards for Reporting Diagnostic accuracy studies) should be followed[26]. It is recommended to report the 95 % confidence interval for the estimates of both the diagnostic sensitivity and the diagnostic specificity.

The clinical performance of a test method or device should be evaluated for all the intended conditions of use, e.g. each type of specimen mentioned should be evaluated.

4.2. Detection of immunological status

4.2.1. Antibody tests

A) Evaluation of evidence

The majority of the devices are targeting the immunoglobulins IgG and IgM in a combined manner (54 devices). These devices were designed to display the presence/absence of one of the two immunoglobulins or of both in one single test. The remaining devices are targeting only IgG or only IgM. We found only one case where the test was directed to IgA.

Literature review

Many of the currently available tests have been used on larger numbers of patients and their results tend to be similar (e.g. regarding sensitivity and specificity). Tests using the virus spike protein to capture the antibodies seem to be more sensitive than the ones using the nucleocapsid protein. Tests that detect both IgG and IgM at the same time are superior to the ones testing for only one antibody. One study (Annex 2, ART108) even claimed to measure total antibodies (TotAb) using a commercially available assay and reached an even better sensitivity. Overall, ELISA-type tests seem to provide better results than flow-strip tests. Although the latter are faster and potentially suitable for point-of-care, the former seem to be more sensitive and reliable.

Most of the early studies reported high sensitivity and specificity for the method, whereas the only EU study found (Annex 2, ART75) reported disappointing results when using the tests on the hospital floor. This may be related to the rather late antibody response (see Section 1) making the method less suitable for the triage of patients. In addition, earlier publications are often vague on the device they used and often do not mention how long after symptom onset the samples were taken.

Application aspects

In contrast to RT-PCR methods, the vast majority of antibody test methods do not require complex laboratory equipment and various sets of reagents. Only a few of them require large instruments for reading the results. Therefore, they are often suitable for applications outside a laboratory.

The reported time to perform a test has been compiled in the annexed table. In the majority of specifications for the analysed devices, there is a claim of being "quick" and the time of execution to obtain the final qualitative result (test positive/test negative) was reported to be between 8 and 20 minutes.

The main difference among the rapid tests is the format: either a dipstick enclosed in a cassette or a simple strip or deep strips, fingertips and cards that have to be either inserted in a portable reader or in a tube with reagents to reveal the results. These all appear to be practical formats, easy to use in terms of portability, size, and practicability.

The assay protocols generally include a few steps: sample taking, sample reaction, result visualization and interpretation. Usually a drop of sample is taken (e.g. whole blood and serum/plasma) and is brought to the reacting strip. After some minutes, the qualitative result is visualized and interpreted (positive/negative).

In the minority of evaluated devices, the time range was up to 120 minutes because the test (the classical ELISA) procedure was more demanding, meaning that it required additional laboratory resources (e.g. anticoagulants, non-portable reader, plate incubator, trained personnel).

For many of the searched devices the time of operation was not explicitly reported or found. However, it was claimed that they are "quick".

In all cases, the cost was only available upon request for the devices.

Quality Control

For the majority of devices an internal control was included. The control consisted of a built-in line to monitor procedural mistakes and reagent defects. In the case of the classical ELISA (up to 120 min), a positive and a negative control are included.

Analytical performance

Limit of Detection (LOD)

Only very limited information was available on the LOD of the analysed devices. It should be noted that almost all of them provided a qualitative (positive/negative for presence/absence) result and do not quantify the antibody amount further.

Specificity

Cross-reactivity was not reported for the majority of the analysed devices.

Efficiency, robustness, precision

No information on the efficiency, robustness and precision as defined in Section 4.2.1B was found.

Clinical performance

No information was found in the sources searched about the results of clinical performance studies. When available, claims about both the diagnostic sensitivity and specificity were variable in the overall range of 81-98%.

The annexed table lists the available information.

B) Proposed performance criteria

The analytical and clinical performance criteria are described below. In addition to this, guidance on descriptive information, quality control and safety measures is given.

Descriptive information

Essential requirements are laid down in Annex I of the IVDD. More specifically, for the testing of human antibodies against SARS-CoV-2 or SARS-CoV-2 specific antigen respectively, the following descriptive information is of particular importance:

- Test type: e.g. manual heterogeneous immunoassay, automated immunoassay, rapid test, point-of-care test (POCT);
- nature of the test result: quantitative, semi-quantitative or qualitative ;
- measured target: SARS-CoV-2 specific antigen or human antibodies against SARS-CoV-2. In case of antibodies the immunoglobulin class should be specified, i.e. IgG, IgM or IgA;
- individuals intended to be tested: e.g. patients with suspected SARS-CoV-2 infection, individuals who have been vaccinated, general population;
- type of specimen that can be tested: e.g. oropharyngeal swab sample, whole blood, serum or plasma (EDTA or Heparin), its pretreatment (e.g. required dilution), stability and storage ;
- required qualification level of the staff needed, indication whether any particular training is required;
- guidance on the interpretation of results: e.g. cut off, grey zone, result not being suitable as a sole basis of diagnosis, further testing needed to obtain a reliable result, testing on follow-up samples taken after a recommended time period;
- potential limitations: e.g. possible reasons for false negative or false positive results, known cross-reactions.

Moreover, a short description of the method principle should be provided including the following information:

- Solid support and vessel where the immunoassay takes place (e.g. microplate strips with pre-coated wells, test cassette);
- detection principle (e.g. enzyme-linked colorimetry, fluorescence, colloidal gold);
- if necessary, the reading interval time, i.e. first time point when a reliable result can be read until the time point beyond which the read result is no longer reliable.

A complete list of reagents, either provided (in case of devices) or needed (in case of test methods and devices), should be described as well as the necessary consumables and equipment. The composition of this list depends on the test type:

- For manual immunoassays:

- Reagent kits including calibrators and positive and negative controls
- Plate type
- ELISA washers and readers
- Common laboratory equipment like tips, pipettes, tubes…

- For automated tests:

- Automated platform or instrument (including software)
- Reagent kits
- 96 well plates and/or dedicated sample cups

- For rapid tests:

- Test cassettes
- Reagents
- Detection system (if applicable)

For reagents, the expiration date and instructions concerning their storage and handling (e.g. reconstitution of lyophilised material and reagents) should be stated.

Quality control

The design of the quality controls depends on the test format and the type of test results:

- For manual and automated immunoassays tests:

Positive and negative controls should be analysed in parallel with the specimens in each run. In case of quantitative or semi-quantitative immunoassays the measurement results obtained for the controls shall fall within predetermined limits. In case of devices, these controls should be included in the device or clear information about required controls should be provided.

- For rapid tests and POCT:

These tests should include a migration control line. A test result is only valid, if the control line is visible. In addition, positive and negative controls should be tested under specific circumstances (e.g. a new lot of tests, a new operator, a new test environment).

Safety measures

It is of the utmost importance that proper biosafety guidelines are followed by clinical laboratories when handling samples from suspected COVID-19 patients. The laboratory biosafety guidance related to the coronavirus disease issued by the World Health Organization should be taken into account where applicable.[9]

Analytical performance

Limit of detection (LOD)

The LOD can be determined by testing a dilution series of one or more samples with a known amount of the measured target. Ideally, certified reference materials should be used. As long as these are not available for the SARS-CoV-2 antigen and SARS-CoV-2 antibodies, respectively, in-house developed control materials should be used to allow a consistent LOD determination over various batches of the devices or reagents.

Tests that provide qualitative test results do not often use a numerical value for their assay cut-off. In the absence of a suitable reference material it might not be feasible to estimate the concentration of the measured target at the assay's cut-off. Nevertheless, the way in which the cut-off was selected to obtain a reliable differentiation between positive and negative specimens should be described.

(Analytical) Selectivity

Cross-reactivity refers to the potential of false positive results due to present (for antigen and antibody tests) or past (for antibody tests only) infections or vaccinations (for antibody tests only) that are not linked to the SARS-CoV-2 virus.

The effect of the following infections or vaccinations should be evaluated:

- Infections with the common human pathogenic coronaviruses like HCoV-HKU1, -NL63, -OC43, or -229E;
- infections with influenza viruses and other respiratory viruses;

- vaccination against influenza viruses;
- acute bacterial pneumonia.

Moreover, the effect of past infections with the closely related virus strains SARS-CoV (-1) and MERS-CoV could also be investigated.

The potential for wrong testing results (both false negative and false positive) arising from interferences from at least the substances/conditions listed below should be investigated:

- Samples with autoantibodies such as rheumatoid factor and anti-nuclear antibodies (ANA);
- samples with elevated IgG and IgA levels;
- samples from pregnant women, especially multipara (women who had more than one pregnancy);
- samples with high concentrations of haemoglobin (haemolytic), triglycerides (lipaemic) and bilirubin (icteric);
- samples with human antibodies against components of the expression system used to produce the antigens or antibodies present in the reagents of the immunoassay;
- samples of individuals treated with relevant medicines like
    - antiviral and antibacterial drugs,
    - common anti-inflammatory drugs (acetylsalicylic acid, paracetamol, ibuprofen)
    - common anti-hypertensive drugs,
    - common anti-diabetic drugs,
    - drugs currently used against COVID-19 in clinical studies (e.g. hydroxychloroquine)

Robustness

Robustness refers to the capacity of an immunoassay to remain unaffected by small variations in the test parameters. Therefore, the effects of the following parameters should be evaluated:

- incubation time;
- temperature.

Clear limits should be set for those parameters that have a significant impact on the outcome of the testing results.

Precision

Both repeatability (i.e. testing the same sample under the same conditions) and reproducibility (i.e. testing the same sample under variable conditions such as different reagent kits, days, different analysts, or different instruments) should be assessed.

For the presence/absence testing these precision parameters can be expressed as the percentage of agreement.

Clinical performance

The analytical performance criteria are usually evaluated on a number of well-defined laboratory samples and extreme patient samples. The next step in the validation process should be a clinical performance study which mimics as much as possible a real-life situation. In this study a number of both known positive and negative samples are tested and the following parameters should be determined:

- Diagnostic sensitivity, i.e. the percentage of the true positive samples that gave a positive result with the antibody test;
- Diagnostic specificity, i.e. the percentage of the true negative samples that gave a negative result with the antibody test.

The determination of the diagnostic sensitivity and specificity requires well-characterized samples, i.e. true negative and true positive samples. This is challenging in the specific case of antibodies against the SARS-CoV-2 virus, as there is no reference antibody test available at the time of writing this report. Currently, it is recommended to evaluate the diagnostic sensitivity in a group of individuals with a present infection (several days after onset of COVID-19 symptoms) or a past infection with the SARS-CoV-2 virus. Both the present and the past infection should have been proven by a positive result with a reference RT-PCR method during the infection. The diagnostic specificity of antibody tests would require the availability of specimens from individuals that had never been in contact with the SARS-CoV-2 virus. Considering the worldwide spread of the virus it is recommended to use specimens which were collected before November 2019.

Once a reference anti-SARS-CoV-2 antibody immunoassay would be established, the next step in the validation process should be the clinical performance evaluation of the diagnostic accuracy of the antibody test under assessment for the two distinct intended uses: (a) SARS-CoV-2 infection detection/diagnosis and (b) determination of specific antiviral immunity.

(a) SARS-CoV-2 antibody tests used for indirect detection of a SARS-CoV-2 infection:

The determination of diagnostic accuracy should be performed in clinical studies using head-to-head comparison between results from one or more antibody tests under assessment and those of the reference RT-PCR test in the intended to be tested target population.

The diagnostic accuracy is composed of:

- Diagnostic sensitivity: Proportion of those individuals with the target condition (infected individuals with reference SARS-CoV-2 RNA true positive specimen sampled at least several days after onset of the COVID-19 symptoms) who test positive with the antibody test;
- Diagnostic specificity: Proportion of those individuals without the target condition (infection-free individuals with reference SARS-CoV-2 RNA true negative specimen and without any history of SARS-CoV-2 infection) who test negative with the antibody test.

For SARS-CoV-2 antibody tests intended for indirect detection of a SARS-CoV-2 infection the diagnostic sensitivity is the most crucial parameter, as the false negative rate should be as low as possible.

(b) SARS-CoV-2 antibody tests used for determination of the immune status against SARS-CoV-2:

The determination of diagnostic accuracy of antibody tests of interest should be performed in clinical studies using head-to-head comparison between results from one or more antibody tests under assessment and those of the reference antibody test in the intended to be tested target population. The diagnostic accuracy is composed of:

- Diagnostic sensitivity: Proportion of those individuals with the target condition (immune individuals with reference SARS-CoV-2 antibody test true positive specimen) who test positive with the antibody test;
- Diagnostic specificity: Proportion of those individuals without the target condition (non-immune individuals with reference SARS-CoV-2 antibody test true negative specimen) who test negative with the antibody test.

For SARS-CoV-2 antibody tests intended for the determination of the immune status against SARS-CoV-2 the diagnostic specificity is the most crucial parameter, as the false positive rate should be as low as possible.

The number of patients (sample size) to be included in the study should be determined by statistical power calculation for a desired precision level of the accuracy estimates. The study report should provide the estimates of diagnostic sensitivity and specificity with confidence intervals. The STARD 2015 (STAndards for Reporting Diagnostic accuracy studies) should be followed[26]. It is recommended to report the 95 % confidence interval for the estimates of both the diagnostic sensitivity and the diagnostic specificity.

5. Conclusions and recommendations

Literature review of RT-PCR tests

Performance parameter information summarised in this report was self-reported by the manufacturer or distributor of the device, with no access to details or raw data for the studies that created these quality parameters.

Unless and until the results of independent validation studies are made available for some of the other devices in the annexed table, we would recommend the use of the ones that explicitly declare the implementation of a WHO protocol, in view of the availability of information on the previous use of methods based on the same sets of primers and probes.

Literature review of antigen tests

From the information that was retrievable from the literature and other sources, current antigen tests are so far not accompanied by sufficient proof of evidence regarding their performance characteristics.

Literature review of antibody tests

Currently, a comparison of the available antibody tests (devices) is not possible, because there is an almost complete lack of proper validation and standardisation among antibody targeting methods. Reported performance parameters are difficult to compare. Even in the literature analysed it is, for example, not always mentioned how long after infection the samples were taken, and 'true positive' and 'true negative' samples are often chosen in a different manner. Information on performance parameters is usually self-reported by the manufacturer or distributor of the device, with no access to details or raw data for the studies that produced these quality parameters.

It was found in the analysis of close to 100 immunoassay devices that data on false negatives, false positives and cross-reactivity are practically never reported. Only a few of them mentioned 'cross reactivity' with other viruses that could be associated with false positive results.

General

- Currently a rapidly expanding number of SARS-CoV-2 RNA tests (mostly by RT-PCR) and tests for antibodies against the coronavirus (mostly immunoassays) are appearing in the literature and on the market. A small number of antigen tests are also available ;
- There is a clear mismatch between the currently existing or reported quality assurance information about the COVID-19 tests/devices and the performance criteria proposed above, which are based on the principles of good analytical (testing) practice and corresponding international standards such as ISO/IEC 17025 and ISO 15189 ;
- There is an urgent need to properly assess (i.e. validate) the performance of existing and emerging test methods targeting the viral RNA, SARS-CoV-2 as antigen or its antibodies (see also Annex 3);
- The most critical performance parameters for reliable diagnostic decisions are:

  - for identifying if a person is infected with SARS-CoV-2: the diagnostic sensitivity of the RNA or antigen test, as false negative test results have to be avoided;
  - for identifying the persons who have developed an immune response against SARS-CoV-2: the diagnostic specificity of the antibody test, as false positive test results have to be avoided.

- For that purpose, as well as for the comparison of the performance of different tests, the required quality benchmarks, i.e. well-characterised reference (control) materials mimicking real patient samples, and reference test methods have to be inventoried, verified or established as soon as possible. In addition, more proficiency testing exercises should be organised allowing laboratories to demonstrate their COVID-19 testing competence.

Scientific terminology used

Limit of detection (LOD)

The LOD represents the lowest concentration of the measured target (number of virus RNA fragments, antigens from the SARS-CoV-2 virus or antibodies against the SARS-CoV-2 virus) at which approximately 95% of the replicate measurements on samples containing the target give a positive result.

Precision

The term precision refers to the closeness of agreement between independent test results obtained under stipulated conditions. Both repeatability (i.e. measuring the same sample under the same conditions) and reproducibility (i.e. measuring the same sample under variable conditions such as different reagent kits, days, different analysts, or different instruments) are important performance characteristics.

Selectivity

Selectivity refers to the extent to which the test can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behaviour.

(from: IUPAC in Pure Appl. Chem. 73, 1381-1386 (2001))

(analytical) Specificity

Capability of a measuring system, using a specified measurement procedure, to provide measurement results for one or more measurands which do not depend on each other nor on any other quantity in the system undergoing measurement.

(from EN ISO 18113-1:2011)

(analytical) Sensitivity

Quotient of the change in an indication of a measuring system and the corresponding change in a value of a quantity being measured.

(from ISO/IEC Guide 99:2007)

Robustness

Capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters.

(from ISO 18158:2016)

Diagnostic sensitivity

Ability of an *in vitro* diagnostic examination procedure to identify the presence of a target marker associated with a particular disease or condition.

It is also defined as percent positivity in samples where the target marker is known to be present.

Diagnostic sensitivity is expressed as a percentage (number fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of the number of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/(TP + FN). This calculation is based on a study design where only one sample is taken from each subject.

(from EN ISO 18113-1:2011)

Diagnostic specificity

Ability of an IVD examination procedure to recognise the absence of a target marker associated with a particular disease or condition.

It is also defined as percent negativity in samples where the target marker is known to be absent.

Diagnostic specificity is expressed as a percentage (number fraction multiplied by 100), calculated as 100 × the number of true negative values (TN) divided by the sum of the number of true negative plus the number of false positive (FP) values, or 100 × TN/(TN + FP). This calculation is based on a study design where only one sample is taken from each subject.

(from EN ISO 18113-1:2011)

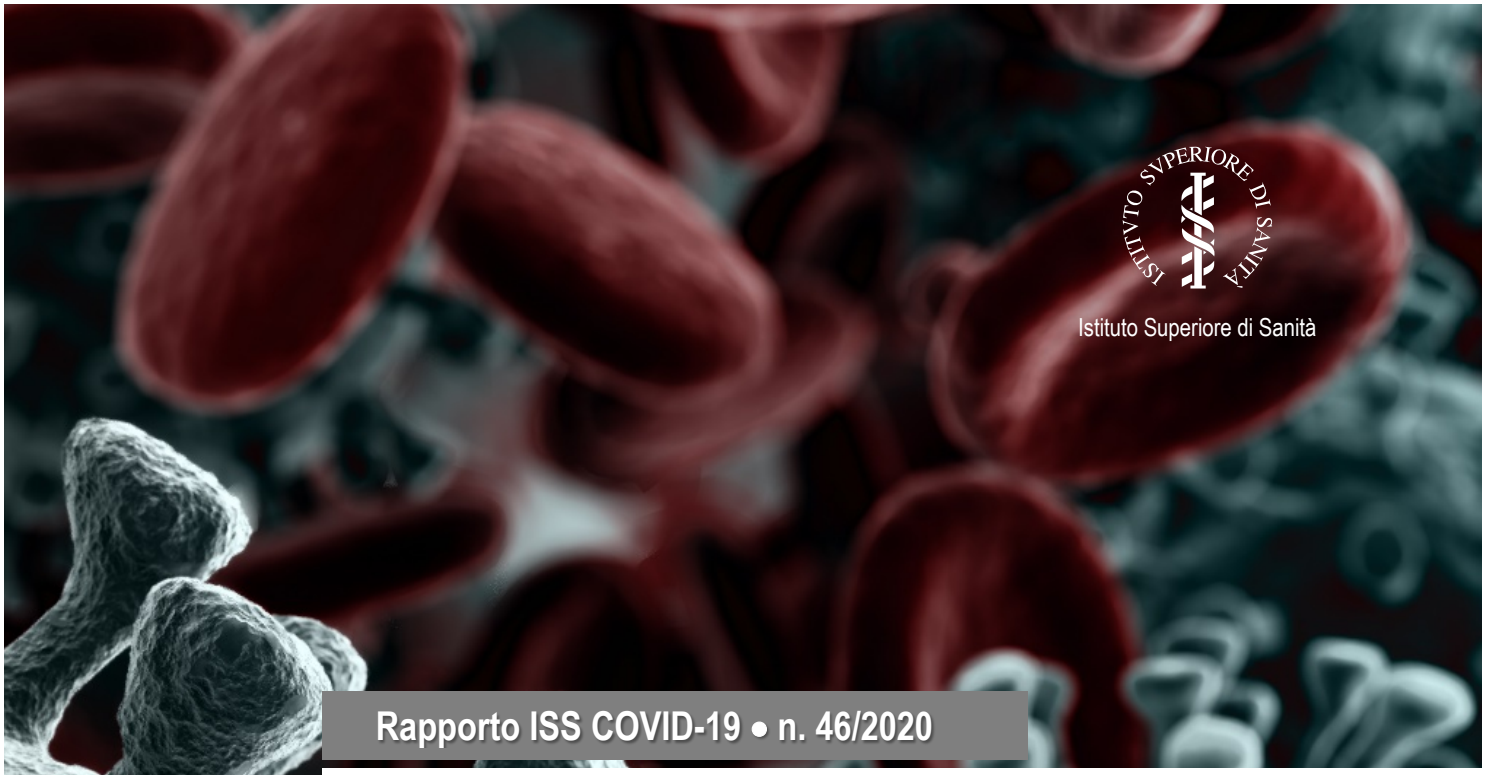
Please see also Commission Decision 2002/364/EC on common specifications for *in vitro* diagnostic devices, OJ L 131, 16.5.2002, p. 17.

Annexes

Annex 1: Commercial devices

Annex 2: Scientific literature

Annex 3: Search on validation studies



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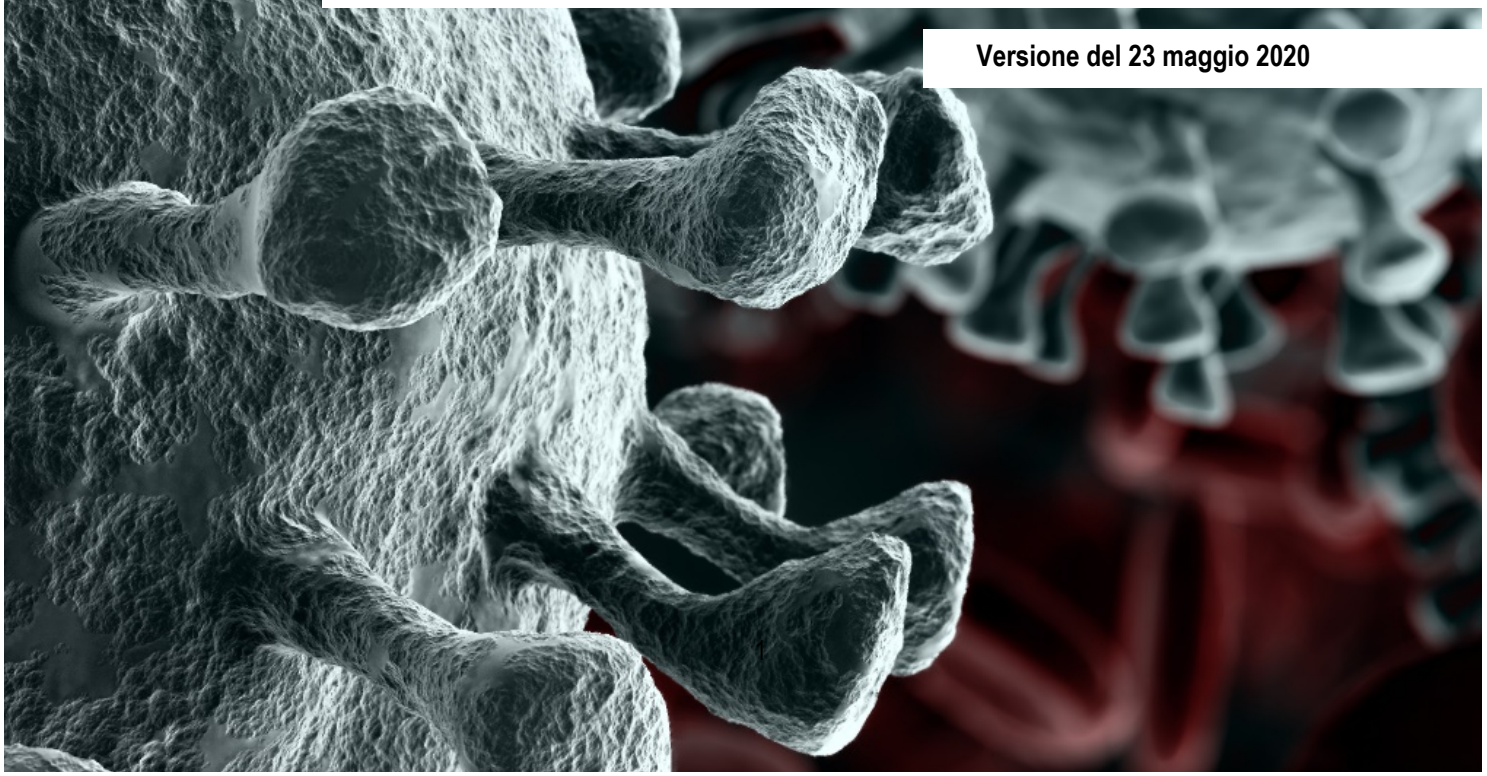
Rapporto ISS COVID-19 • n. 46/2020

Dispositivi diagnostici *in vitro* per COVID-19. Parte 2: evoluzione del mercato e informazioni per gli stakeholder

Gruppo di Lavoro ISS Test Diagnostici COVID-19
Gruppo di Lavoro ISS Dispositivi Medici COVID-19



Versione del 23 maggio 2020

Dispositivi diagnostici *in vitro* per COVID-19. Parte 2: evoluzione del mercato e informazioni per gli stakeholder

Versione del 23 maggio. 2020

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Dispositivi diagnostici *in vitro* per COVID-19. Parte 2: evoluzione del mercato e informazioni per gli stakeholder. Versione del 23 maggio 2020.
Gruppo di Lavoro ISS Test Diagnostici COVID-19 e Gruppo di Lavoro ISS Dispositivi Medici COVID-19
2020, 9 p. Rapporto ISS COVID-19 n. 46/2020

La Commissione Europea ha pubblicato in data 15 aprile 2020 le linee guida sui test diagnostici *in vitro* (IVD) per COVID-19. In un primo Rapporto ISS COVID-19 (n. 28/2020) sono stati già affrontati i contenuti relativi agli aspetti normativi che regolano l'uso e la commercializzazione degli IVD nei paesi dell'Unione Europea e le diverse tipologie di IVD che possono essere rinvenute sul mercato. Il presente rapporto illustra ulteriori argomenti presenti nelle linee guida. Viene in particolare descritta l'evoluzione del mercato degli IVD specifici per COVID-19 e vengono fornite informazioni per gli *stakeholders* (utilizzatori, distributori e fabbricanti) in merito al corretto utilizzo degli IVD in relazione alla destinazione d'uso ed alle prestazioni indicate dal fabbricante.

Istituto Superiore di Sanità
COVID-19 *in vitro* diagnostic devices. Part 2: market development and information to stakeholders. Version of May 23, 2020.
ISS Working group Diagnostic Tests COVID-19 and ISS Working group Medical Devices COVID-19
2020, 9 p. Rapporto ISS COVID-19 n. 46/2020 (in Italian)

On April 15, 2020 the European Commission published the guidelines on COVID-19 *in vitro* diagnostic devices (IVD). A first report (Rapporto ISS COVID-19 n. 28/2020) described the regulatory context governing the use and marketing of IVDs in European Union countries and the different types of tests on the market. The present report illustrates further issues addressed by the above guidelines. In particular, the report describes the market development of the IVDs for COVID-19 and provides information to stakeholders (users, distributors, manufacturers) about the proper use of IVDs according to the intended purpose and performance specified by the manufacturer.

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Citare questo documento come segue:

Gruppo di Lavoro ISS Test Diagnostici COVID-19 e Gruppo di Lavoro ISS Dispositivi Medici COVID-19. *Dispositivi diagnostici in vitro per COVID-19. Parte 2: evoluzione del mercato e informazioni per gli stakeholder. Versione del 23 maggio 2020.* Roma: Istituto Superiore di Sanità; 2020. (Rapporto ISS COVID-19 n. 46/2020)

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Indice

Introduzione

I dispositivi diagnostici *in vitro* (*In Vitro Diagnostic Devic*e, IVD) rappresentano uno strumento essenziale per la gestione dell'epidemia di COVID-19. La Commissione Europea ha pubblicato in data 15 aprile 2020 le linee guida sui test diagnostici *in vitro* per COVID-19 e sulle loro prestazioni (1).

I contenuti di tali linee guida relativi al contesto legislativo ed alla tipologia di dispositivi circolanti sul mercato sono già stati specificamente trattati nel precedente **Rapporto ISS COVID-19 n. 28/2020 "Dispositivi diagnostici *in vitro* per COVID-19. Parte 1: normativa e tipologie".**

Nel presente rapporto vengono affrontati i restanti contenuti relativi all'**evoluzione del mercato** degli IVD per COVID-19 nonché **informazioni per gli stakeholder** (utilizzatori, distributori e fabbricanti) **riguardanti il corretto utilizzo dei dispositivi in relazione con la destinazione d'uso e con le prestazioni** indicate dal fabbricante.

Mercato degli IVD per COVID-19 nell'Unione Europea

Dall'inizio dell'epidemia i dispositivi presenti sul mercato dell'Unione Europea sono in continuo aumento. In base ad un'indagine effettuata all'inizio di aprile, i dispositivi diagnostici per COVID-19 marcati CE ai sensi della Direttiva 98/79/CE sono risultati almeno 78 per i saggi NAT, almeno 13 per i test rapidi per l'antigene ed almeno 101 per i test sugli anticorpi, la maggioranza di questi ultimi della tipologia test rapidi (1). Tali dati verosimilmente rappresentano una sottostima dei test realmente in circolazione in Europa, questo dipende dalle differenti modalità adottate dai singoli paesi dell'Unione Europea in merito al tracciamento dei test registrati nel territorio nazionale.

La Commissione Europea ha redatto il documento "Current performance of COVID-19 test methods and devices and proposed performance criteria - Working document of Commission services" (16/04/2020; ultimo aggiornamento: 17/04/2020), consultabile al link: https://ec.europa.eu/docsroom/documents/40805, che riporta sia la situazione del mercato europea che considerazioni in merito alle performance dei dispositivi, del quale se ne raccomanda la consultazione (2).

Per quanto riguarda l'Italia, una stima minima degli IVD per COVID-19 commercializzati è stata effettuata monitorando quelli registrati nella banca dati "Elenco dei dispositivi medici" del Ministero della Salute (3). Ciò è stato possibile in quanto i fabbricanti e i mandatari stabiliti in Italia che immettono in commercio dispositivi medico-diagnostici i*n vitro* a proprio nome hanno l'obbligo di comunicazione al Ministero della salute (4). Inoltre i Fabbricanti/Mandatari che non risiedono in Italia possono su base volontaria registrare i propri dispositivi nella Banca dati del Ministero della salute. Questi dati rappresentano in realtà una sottostima degli IVD in commercio, in quanto fabbricanti e mandatari situati in altri paesi europei non hanno obbligo di registrazione in Italia. Tali dati tuttavia restituiscono un quadro dell'evoluzione del mercato nazionale di questi dispositivi (Figura 1).

I primi IVD per COVID-19 registrati in Italia sono stati i test per RNA virale e test sierologici rapidi (Figura 1 A e C), a partire dalla seconda metà di febbraio, mentre i primi test sierologici di laboratorio (ELISA, CLIA, CMIA) sono stati registrati solo a partire dalla metà di marzo (Figura 1 B). Nel giro di due mesi le registrazioni hanno raggiunto valori considerevoli per i test per RNA virale (n. 83) e, soprattutto, per i test sierologici rapidi (n.157 all'ultimo aggiornamento del 23 maggio), mentre la crescita delle registrazioni dei test sierologici di laboratorio (che comprendono test per le IgM, o le IgG, o le IgA, o per anticorpi totali) è stata più contenuta.

A partire dall'emergenza pandemica, in un ristretto periodo di tempo, vi è stato un incremento improvviso di richiesta a livello mondiale di reagenti, soprattutto per le metodiche molecolari, che ha messo a dura prova le capacità produttive dei fabbricanti, la filiera della distribuzione e l'organizzazione logistica per la messa a disposizione di tali dispositivi. Di conseguenza, i laboratori si sono trovati di fronte a difficoltà nell'approvvigionamento dei dispositivi necessari durante la pandemia COVID-19. A livello Europeo la disponibilità dei dispositivi può variare ampiamente tra i differenti Stati Membri: ciò è dovuto al fatto che il fabbricante può destinare la propria produzione contemporaneamente a mercati UE od extra UE oppure perché possono non esserci distributori che vendono questi dispositivi in tutti gli Stati Membri.

Nel contesto della situazione emergenziale di necessità relativa alla reperibilità dei dispositivi, si è inserita la problematica delle frodi: un certo numero di dispositivi contraffatti, immessi sul mercato illegalmente, è stato identificato dalle Autorità nazionali competenti, che hanno riscontrato la presenza in alcuni casi di false prove di registrazione e certificati falsificati. Gli Stati Membri hanno preso provvedimenti per rimuovere tali dispositivi dal mercato. In questo contesto è quindi raccomandabile che gli importatori, i distributori ed i laboratori, pongano particolare attenzione alle offerte che vengono loro proposte, verificando per quanto possibile la loro provenienza e la regolarità della marcatura CE e collaborino con le Autorità Competenti nell'identificare il commercio dei dispositivi contraffatti.

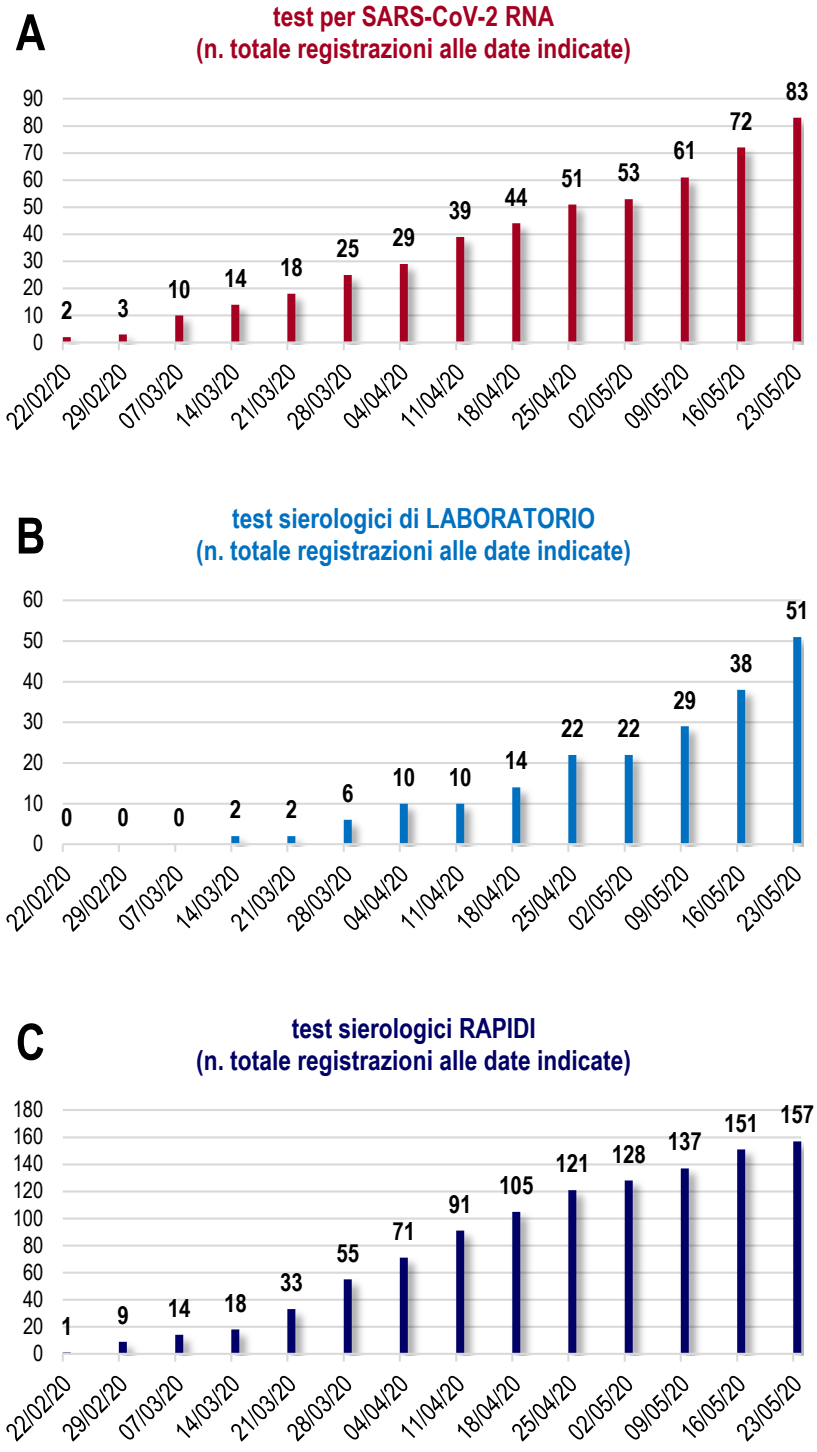

Figura 1. Numero di registrazioni di IVD per COVID-19 risultanti nella banca dati "Elenco dispositivi medici" del Ministero della Salute alle date indicate per tre diverse tipologie di test

Nota: Il numero totale di registrazioni non corrisponde esattamente al numero di test, in quanto alcune registrazioni riguardano aggiornamenti o nuovi formati di test già in precedenza registrati.

Particolare attenzione va posta nella scelta di dispositivi idonei alle esigenze diagnostiche, che presentino delle prestazioni adeguate relativamente all'uso a cui devono essere destinati ed una conformità documentata in modo più esteso possibile.

Le informazioni che vengono rese disponibili dal fabbricante, consistono essenzialmente in:

- istruzioni per l'uso accluse al dispositivo fornite all'utilizzatore;
- immagini relative al confezionamento del dispositivo;
- materiale pubblicitario;
- eventuali report riassuntivi in merito agli studi di validazione condotti;
- dichiarazione CE di conformità alla direttiva 98/79/CE;
- eventuali certificazioni volontarie rilasciate da enti privati accreditati;
- eventuali prove di registrazione del dispositivo alle autorità competenti degli Stati Membri.

È importante prima di procedere alla scelta di un dispositivo diagnostico avere a disposizione tutte le possibili informazioni per ponderare l'affidabilità, la disponibilità e la trasparenza del fabbricante e/o del distributore; è opportuno valutare attentamente la esaustività delle informazioni presenti nelle Istruzioni per l'uso del dispositivo (che per il nostro mercato devono essere tra l'altro redatte in lingua Italiana), oltre che la serietà delle dichiarazioni rese con la pubblicità del dispositivo.

In merito ai messaggi pubblicitari, la normativa italiana prevede inoltre che il fabbricante od il responsabile dell'immissione in commercio di un dispositivo medico-diagnostico *in vitro* chieda il rilascio dell'autorizzazione ad effettuare una pubblicità sanitaria presentando una domanda al Ministero della Salute. L'autorizzazione deve essere richiesta anche per quanto riguarda la pubblicità diffusa via web (5).

È inoltre opportuno **richiedere l'evidenza che il dispositivo oltre ad essere corredato di una dichiarazione CE di conformità sottoscritta dal legale rappresentante del fabbricante, sia stato registrato presso l'Autorità Competente di uno stato membro dell'Unione Europea**.

In merito ai dispositivi IVD registrati in Italia, è possibile consultare la banca dati del Ministero della Salute "Elenco dei dispositivi medici" (3). Tuttavia se il dispositivo è registrato presso un'altra Autorità Competente dell'UE potrebbe non essere presente nel data-base Italiano, sebbene ugualmente commercializzabile in Italia.

Relativamente ai diagnostici *in vitro* per COVID-19, al sito web https://www.finddx.org/covid-19/ si possono ottenere informazioni utili per avere una panoramica generale dei kit commerciali circolanti a livello mondiale, anche se tali dati non sono ufficiali.

Direttiva 98/79/CE: informazioni per gli stakeholder

Destinazione d'uso prevista dal fabbricante

La Direttiva 98/79/CE stabilisce che i dispositivi devono essere progettati e fabbricati in modo tale da essere idonei alla destinazione d'uso specificata dal Fabbricante, tenendo conto dello stato dell'arte generalmente riconosciuto.

La destinazione d'uso deve essere specificata nelle istruzioni per l'uso e/o sull'etichetta, a meno che non sia ovvio per l'utente (7). Ciò dovrebbe essere **completo e preciso, compresi l'utente previsto e gli aspetti clinici come la popolazione target**. Bisogna verificare che i test siano coerenti con la destinazione d'uso prevista: ad esempio diagnosi o screening; deve essere tenuto conto della popolazione target specifica sulla quale il saggio deve essere eseguito (es. operatori sanitari, popolazione generale, ecc.), la fase della malattia per cui sono destinati (es. soggetti asintomatici/sani, casi confermati, dimissione dopo ricoveri ospedalieri, ecc.) e la decisione clinica da prendere sulla base dei risultati del test.

Una particolare attenzione nella scelta deve essere inoltre posta in merito ai campioni di controllo inseriti nel kit dal fabbricante: questi dovrebbero essere in grado di rilevare qualsiasi problema che possa intercorrere durante la prova analitica: ad esempio per i saggi NAT (*Nucleic Acid Test*) è preferibile che siano inseriti dei controlli interni (all'interno di ciascun campione) per verificare che non vi sia inibizione durante la reazione. Tali controlli dovrebbero simulare il più possibile il campione da analizzare per verificare ognuna delle fasi che portano al risultato: ad esempio un controllo di "Armored RNA" (ovvero RNA stabilizzato rivestito da proteine), che permette il controllo delle fasi di estrazione, retrotrascrizione e amplificazione, è preferibile sia ad un "RNA nudo" (che non permette il controllo della rimozione delle proteine durante l'estrazione), sia ad un "cDNA" (che non permette di controllare le fasi di estrazione e retrotrascrizione).

Prestazioni indicate dal fabbricante

Un aspetto da non trascurare è che le prestazioni del dispositivo devono essere adeguate alla tipologia di test: ad esempio per la sensibilità e la specificità sono richiesti valori più elevati per la diagnosi rispetto allo screening. La popolazione target a cui si applica il test è anch'essa fondamentale: le prove per la validazione eseguite dal Fabbricante dovrebbero dimostrare l'efficacia del dispositivo sulla determinata popolazione che si intende saggiare. Infatti uno stesso dispositivo, con una determinata sensibilità analitica, se applicato a gruppi di pazienti con caratteristiche differenti (es. all'esordio dei sintomi piuttosto che in pazienti che hanno sviluppato la malattia) potrebbe fornire risultati diversi in termini di sensibilità diagnostica-clinica a causa, ad esempio, di una differente concentrazione nel campione analizzato dell'analita che il dispositivo identifica.

La Direttiva 98/79/CE prevede che i test debbano raggiungere le prestazioni pertinenti, in particolare in termini di: **sensibilità analitica, sensibilità diagnostica, specificità analitica, specificità diagnostica, accuratezza, ripetibilità, riproducibilità, compreso il controllo di interferenze rilevanti note e limiti di rilevazione, dichiarati dal fabbricante** (6). I fabbricanti sono tenuti a riassumere gli studi delle prestazioni effettuati ed in base ai risultati di tali studi determinare la destinazione d'uso specifica del dispositivo. Sono inoltre obbligati ad identificare la popolazione target.

Le istruzioni per l'uso devono includere i livelli di prestazione per i parametri di cui sopra (8). Nelle istruzioni per l'uso devono essere inoltre chiaramente specificate le tipologie di campioni utilizzabili con il test ed essere definite le performance per ciascuna tipologia di campione applicato, dato che queste

possono differire tra loro. Ad esempio, per un test sugli anticorpi, se nel foglietto illustrativo è specificato che il test può essere utilizzato su campioni di sangue, siero e plasma, deve essere fornita la prova che il test funziona come previsto su tutte le diverse tipologie di campione. Nel caso dei saggi basati su metodica NAT il test deve essere validato per ogni metodo di estrazione dichiarato applicabile per il dispositivo e per ogni strumento amplificatore dichiarato utilizzabile nelle istruzioni per l'uso in combinazione con il kit. Sarebbe opportuno inoltre inserire, tra le avvertenze, che le *performance* dichiarate non sono garantite nel caso di utilizzo di campioni, metodiche di estrazione e strumentazione per il rilevamento differenti da quelle indicate dal fabbricante.

In merito alle *performance* il fabbricante dovrebbe progettare il dispositivo tenendo conto che possono esserci compromessi tra i parametri, ad esempio tra la sensibilità del test (rilevamento del numero massimo di individui positivi) e la sua specificità (capacità di distinguere tra veri e falsi positivi) a seconda dell'uso a cui il dispositivo è destinato. Un test molto sensibile nel rilevare il bersaglio di interesse ha maggiori probabilità di rilevare anche bersagli correlati ma distinti che non sono di interesse, vale a dire che può essere meno specifico.

Il Fabbricante è tenuto a predisporre una documentazione tecnica dettagliata, relativa alla dimostrazione della conformità del dispositivo alla direttiva, che deve essere conservata dal Fabbricante e dal suo eventuale mandatario e tenuta a disposizione delle Autorità Competenti. La documentazione tecnica del dispositivo deve contenere adeguati dati di valutazione delle prestazioni che dimostrino le prestazioni dichiarate dal produttore e devono essere supportate da un sistema di misurazione di riferimento (se disponibile), con informazioni sui metodi di riferimento, i materiali di riferimento, i valori di riferimento noti, l'accuratezza e le unità di misura utilizzate.

I dati dovrebbero provenire da studi in un ambiente clinico o di altro tipo o risultanti da riferimenti pertinenti (9). Le informazioni sulle prestazioni dovrebbero essere complete per consentire una valutazione della sua qualità. Ad esempio, i fabbricanti dovrebbero identificare chiaramente i metodi di confronto, il numero di soggetti che hanno preso parte allo studio delle prestazioni, il modo in cui tali soggetti sono stati qualificati come positivi o negativi.

Il fabbricante deve dimostrare che il dispositivo sia stato fabbricato tenendo conto dello "stato dell'arte" in termini dei parametri di prestazione elencati sopra; questo non significa che il dispositivo deve essere il migliore della sua categoria. Tuttavia, il dispositivo non dovrebbe essere inferiore al livello che può essere ragionevolmente raggiunto, od è raggiunto dalla maggior parte dei dispositivi* (10).

Un aspetto particolare che merita un approfondimento riguarda le prestazioni dei dispositivi in merito ai parametri di sensibilità e di specificità, parametri notoriamente antitetici tra loro. Possono esserci svantaggi sia da un'insufficiente sensibilità diagnostica (che porterebbe a non rilevare una parte delle persone infette) sia da un'insufficiente specificità diagnostica (che potrebbe fornire dei risultati falsi positivi aspecifici). Tale dato deve essere preso in considerazione **insieme allo stadio della pandemia** in relazione alla prevalenza, in una particolare popolazione. In questo contesto è utile considerare che in situazioni epidemiologiche differenti le caratteristiche di sensibilità e di specificità di un determinato test possono restituire dei valori differenti in termini di «valore predittivo», ovvero la probabilità che una persona risultata positiva a un test condotto con un dispositivo presenti una determinata malattia oggetto di indagine o che una persona

* Come riferimento, si veda la "Commission Decision 2008/932/EC of 2 December 2008, C(2008)7378, OJ L 333, 11.12.2008 p. 5" su uno specifico test per HIV del fabbricante M.B.S. Nel caso esaminato, i dati combinati di vari istituti nazionali mostravano un quadro complessivo secondo il quale tutti i test per HIV potevano essere raggruppati in tre fasce, grossolanamente corrispondenti a tre generazioni di test. In quel caso, il test per HIV in questione è stato ritenuto non adeguato allo "stato dell'arte" poiché cadeva nella fascia più bassa.

risultata negativa a tale test non presenti una determinata malattia. È quindi importante considerare il valore predittivo positivo (*Positive Predictive Value*, PPV) ed il valore predittivo negativo (NPV) di un test*.

Nella tabella che segue, tratta dal documento Rapid diagnostic tests for COVID-19 (11), viene mostrato con un esempio numerico come la capacità di identificare correttamente i positivi (colonna PPV) sia correlata sia alla sensibilità e specificità del test, sia alla prevalenza del marcatore nella popolazione target, esemplificata da quattro coorti di 1.000 individui con quattro diversi valori di prevalenza: 2%, 5%, 10% e 30%.

Cohort	Pre-test probability (prevalence)	Sensitivity	Specificity	Cases	Non-cases	True positive (TP)	False negative (FN)	True negative (TN)	False positive (FP)	PPV	NPV
High performance											
1,000	2.0%	95%	98%	20	980	19	1	960	20	49.2%	100%
1,000	5.0%	95%	98%	50	950	48	2	931	19	71.4%	100%
1,000	10.0%	95%	98%	100	900	95	5	882	18	84.1%	99%
1,000	30.0%	95%	98%	300	700	285	15	686	14	95%	98%
Mid performance											
1,000	2.0%	85%	90%	20	980	17	3	882	98	14.8%	100%
1,000	5.0%	85%	90%	50	950	43	8	855	95	30.9%	99%
1,000	10.0%	85%	90%	100	900	85	15	810	90	48.6%	98%
1,000	30.0%	85%	90%	300	700	255	45	630	70	78%	93%
Low performance											
1,000	2.0%	75%	85%	20	980	15	5	833	147	9.3%	99%
1,000	5.0%	75%	85%	50	950	38	13	808	143	20.8%	98%
1,000	10.0%	75%	85%	100	900	75	25	765	135	35.7%	97%
1,000	30.0%	75%	85%	300	700	225	75	595	105	68%	89%

Impiegando su una popolazione con bassa prevalenza (2%) un saggio *low performance,* con sensibilità 75% e specificità 85%, il saggio rileva 15 + 147 = 162 positivi, dei quali però solo 15 sono veri positivi (PPV: 9,3%), **quindi sostanzialmente il test non è di alcuna utilità pratica per identificare i positivi su tale popolazione**.I risultati migliorano molto impiegando sulla stessa popolazione un saggio *high performance,* con sensibilità 95% e specificità 98%: in tal caso il test rileva un totale di 19 + 20 = 39 positivi, dei quali 19 sono veri positivi (PPV: 49,2%). I valori riportati in tabella mostrano che al crescere dei valori di prevalenza nella popolazione aumenta la proporzione di veri positivi identificati con tutti i tipi di test, con le differenze tra i valori di PPV che si attenuano. Poiché però i valori di prevalenza in una popolazione sono spesso poco noti o del tutto ignoti, è chiaro che è sempre preferibile l'impiego di test con i valori più alti possibile di sensibilità e specificità; diventa poi indispensabile l'impiego di test con alta/altissima sensibilità e specificità se già ci si attende una bassa prevalenza.

* PPV (valore predittivo positivo): la capacità di un dispositivo di separare i risultati veri positivi da quelli falsi positivi per un determinato attributo in una determinata popolazione; NPV (valore predittivo negativo): la capacità di un dispositivo di separare i risultati veri negativi da quelli falsi negativi per un determinato attributo in una determinata popolazione

Un importante aspetto da considerare, relativamente alle validazioni condotte per determinare le prestazioni dei dispositivi per COVID-19, è la limitata disponibilità di campioni clinici relativi alle differenti categorie di pazienti, della mancanza di metodi standardizzati, di materiali di riferimento certificati, di pannelli di sieroconversione, che rendono difficile la comparazione delle *performance* tra i differenti dispositivi che rilevano lo stesso analita; bisogna anche tener conto che non è ancora disponibile una "*robusta*" letteratura scientifica in merito a SARS-CoV-2. Questi fattori determinano uno stato dell'arte iniziale, "*pionieristico*", che si rispecchia nella qualità dei dati sulle prestazioni dichiarate dai fabbricanti.

Per i laboratori europei, il Centro comune di ricerca della Commissione ha recentemente sviluppato un materiale di controllo positivo per i test NAT (RNA stabilizzato) (12). Il materiale di controllo è disponibile a questo indirizzo https://crm.jrc.ec.europa.eu/p/EURM-019.

Tuttavia sarebbe opportuno sviluppare e rendere disponibile, sia per i laboratori che per i fabbricanti, una preparazione standard di riferimento internazionale di SARS-CoV-2 inattivato, che consenta la validazione e la verifica dei kit NAT a partire dalla fase di estrazione, fino al rilevamento conclusivo, oltre che consentire la validazione dei kit progettati per la ricerca dell'antigene virale.

Per quanto riguarda i kit sierologici, i pannelli di sieroconversione e i pannelli di campioni positivi a titolo noto sono esempi di ulteriori materiali necessari.

La mancanza di dati comparativi disponibili pubblicamente rende difficile confrontare le prestazioni tra i diversi dispositivi. Per generare tali dati, sono necessari programmi di valutazione esterna della qualità. Lo European Centre for Disease Prevention and Control (ECDC) e l'Organizzazione Mondiale della Sanità (OMS) (13) stanno organizzando uno schema esterno di valutazione della qualità per i test NAT.

È evidente che nel caso di COVID-19 lo stato dell'arte stia evolvendo rapidamente assieme alla letteratura scientifica che viene pubblicata: i fabbricanti dovrebbero sforzarsi di aggiornare continuamente, con il progredire delle informazioni disponibili, i dati sulle prestazioni, l'analisi dei rischi e la documentazione tecnica dei propri dispositivi, per quanto ragionevolmente possibile, integrando quando opportuno anche le "Istruzioni per l'uso" del dispositivo.

Un ulteriore aspetto che il fabbricante dovrebbe tenere in considerazione, ad esempio per i test NAT, è lo stato di aggiornamento delle banche dati relative alle sequenze di SARS-CoV-2 che il dispositivo identifica per l'amplificazione: il virus infatti può mutare e nuove sequenze nucleotidiche depositate nelle banche dati possono rivelare se queste mutazioni possano a loro volta rendere un particolare test meno efficace o addirittura inefficace. È quindi importante monitorare le mutazioni del virus, anche studiando le sequenze dei campioni che hanno dato risultati falsi negativi con il proprio dispositivo o con dispositivi che amplificano la stessa sequenza bersaglio.

È importante puntualizzare che per la diagnostica di questo virus emergente, con uno stato dell'arte in evoluzione, le reali prestazioni del dispositivo osservate possano differire rispetto a quelle determinate dallo studio iniziale delle prestazioni condotto dal fabbricante ai fini della marcatura CE, in uno stato dell'arte precedente. Al sito https://finddx.shinyapps.io/COVID19DxData/ è disponibile una panoramica sulle prestazioni (sensibilità e specificità) effettivamente rilevate da laboratori che, da tutto il mondo, volontariamente rendono disponibili i risultati ottenuti con i test che hanno provato.

È essenziale, quindi, che il fabbricante abbia un ruolo proattivo nell'aggiornare il dispositivo allo stato dell'arte durante la fase post-market, raccogliendo dati dal mercato, disponendo nuove convalide delle prestazioni su un numero sufficientemente ampio di soggetti della popolazione target (quando disponibili) ed utilizzando i materiali di riferimento standard che saranno resi disponibili, ai fini di confermare ed aggiornare le performance del dispositivo. Tutto ciò a garanzia dell'efficacia e sicurezza dei dispositivi e, di conseguenza, della tutela della salute pubblica.

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CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel

For Emergency Use Only

Instructions for Use

Catalog # 2019-nCoVEUA-01
1000 reactions

For *In-vitro* Diagnostic (IVD) Use

Rx Only

Centers for Disease Control and Prevention
Division of Viral Diseases
1600 Clifton Rd NE
Atlanta GA 30329

CENTERS FOR DISEASE CONTROL AND PREVENTION

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Intended Use

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the 2019-nCoV in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals who meet 2019-nCoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with 2019-nCoV infection, contact with a probable or confirmed 2019-nCoV case, history of travel to geographic locations where 2019-nCoV cases were detected, or other epidemiologic links for which 2019-nCoV testing may be indicated as part of a public health investigation). Testing in the United States is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.

Results are for the identification of 2019-nCoV RNA. The 2019-nCoV RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with 2019-nCoV but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization.

Summary and Explanation

An outbreak of pneumonia of unknown etiology in Wuhan City, Hubei Province, China was initially reported to WHO on December 31, 2019. Chinese authorities identified a novel coronavirus (2019-nCoV), which has resulted in millions of confirmed human infections globally. Cases of asymptomatic infection, mild illness, severe illness, and deaths have been reported.

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is a molecular *in vitro* diagnostic test that aids in the detection and diagnosis 2019-nCoV and is based on widely used nucleic acid amplification technology. The product contains oligonucleotide primers and dual-labeled hydrolysis probes (TaqMan®) and control material used in rRT-PCR for the *in vitro* qualitative detection of 2019-nCoV RNA in respiratory specimens.

The term "qualified laboratories" refers to laboratories in which all users, analysts, and any person reporting results from use of this device should be trained to perform and interpret the results from this procedure by a competent instructor prior to use.

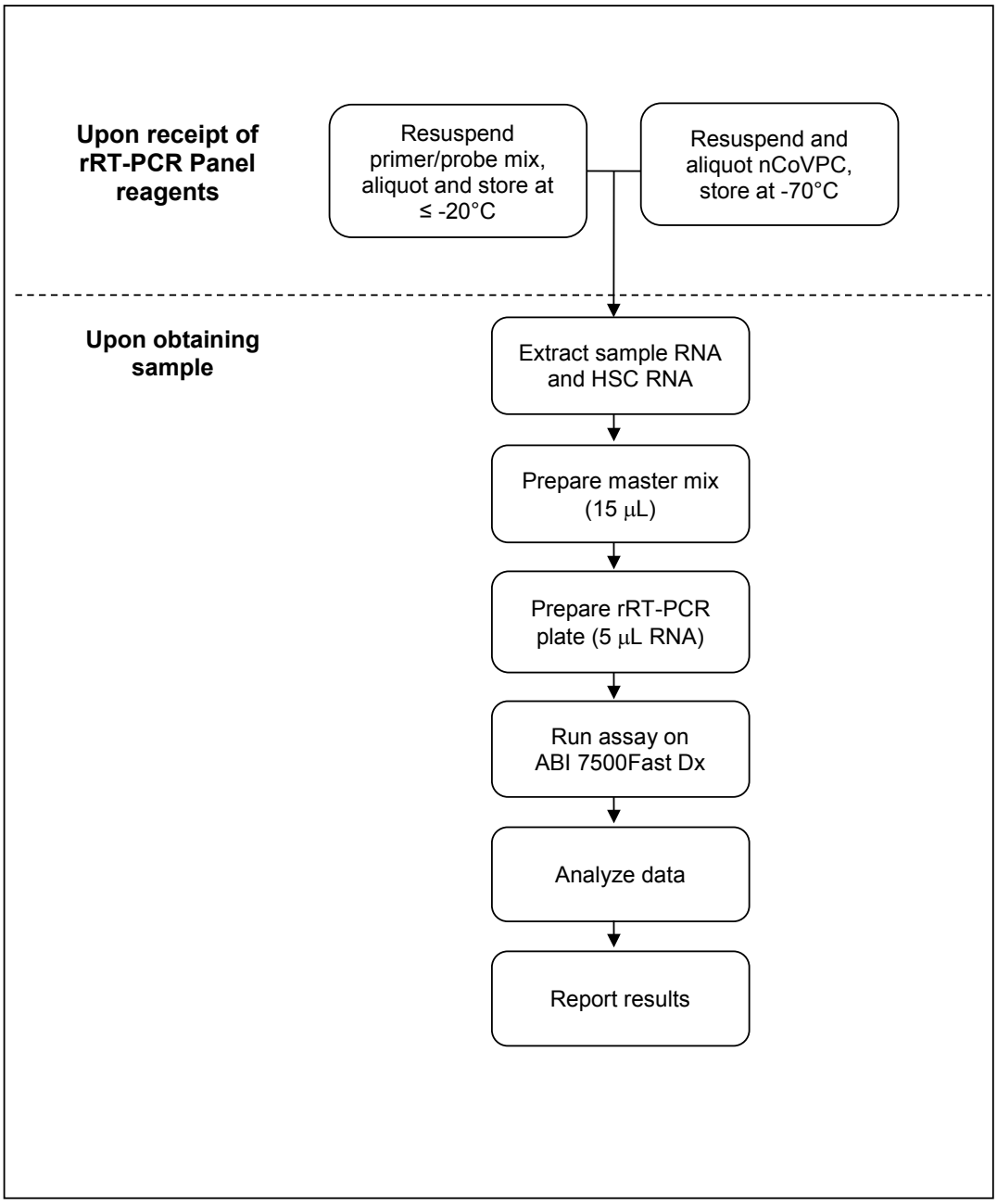
Principles of the Procedure

The oligonucleotide primers and probes for detection of 2019-nCoV were selected from regions of the virus nucleocapsid (N) gene. The panel is designed for specific detection of the 2019-nCoV (two primer/probe sets). An additional primer/probe set to detect the human RNase P gene (RP) in control samples and clinical specimens is also included in the panel.

RNA isolated and purified from upper and lower respiratory specimens is reverse transcribed to cDNA and subsequently amplified in the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS version 1.4 software. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by Applied Biosystems 7500 Fast Dx Real-Time PCR System with SDS version 1.4 software.

Detection of viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.

Summary of Preparation and Testing Process



Materials Required (Provided)

Note: CDC will maintain on its website a list of commercially available lots of primer and probe sets and/or positive control materials that are acceptable alternatives to the CDC primer and probe set and/or positive control included in the Diagnostic Panel. Only material distributed through the CDC International Reagent Resource and specific lots of material posted to the CDC website are acceptable for use with this assay under CDC's Emergency Use Authorization.

This list of acceptable alternative lots of primer and probe materials and/or positive control materials will be available at:
https://www.cdc.gov/coronavirus/2019-nCoV/lab/virus-requests.html

Primers and Probes:

Catalog #2019-nCoVEUA-01 Diagnostic Panel Box #1:

Reagent Label	*Part #*	*Description*	*Quantity / Tube*	*Reactions / Tube*
2019-nCoV_N1	RV202001 RV202015	2019-nCoV_N1 Combined Primer/Probe Mix	22.5 nmol	1000
2019-nCoV_N2	RV202002 RV202016	2019-nCoV_N2 Combined Primer/Probe Mix	22.5 nmol	1000
RP	RV202004 RV202018	Human RNase P Combined Primer/Probe Mix	22.5 nmol	1000

Positive Control (either of the following products are acceptable)

Catalog #2019-nCoVEUA-01 Diagnostic Panel Box #2:

Reagent Label	*Part #*	*Description*	*Quantity*	*Notes*
nCoVPC	RV202005	2019-nCoV Positive Control (nCoVPC) For use as a positive control with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel procedure. The nCoVPC contains noninfectious positive control material supplied in a dried state and must be resuspended before use. nCoVPC consists of *in vitro* transcribed RNA. nCoVPC will yield a positive result with each assay in the 2019-nCoV Real-Time RT-PCR Diagnostic Panel including RP.	4 tubes	Provides (800) 5 µL test reactions

Catalog #VTC-04 CDC 2019-nCoV Positive Control (nCoVPC)

Reagent Label	*Part #*	*Description*	*Quantity*	*Notes*
nCoVPC	RV202005	2019-nCoV Positive Control (nCoVPC) For use as a positive control with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel procedure. The nCoVPC contains noninfectious positive control material supplied in a dried state and must be resuspended before use. nCoVPC consists of *in vitro* transcribed RNA. nCoVPC will yield a positive result with each assay in the 2019-nCoV Real-Time RT-PCR Diagnostic Panel including RP.	4 tubes	Provides (800) 5 µL test reactions

Materials Required (But Not Provided)

Human Specimen Control (HSC)

Description	Quantity	CDC Catalog No.
Manufactured by CDC. For use as a nucleic acid extraction procedural control to demonstrate successful recovery of nucleic acid as well as extraction reagent integrity. The HSC consists of noninfectious (beta-Propiolactone treated) cultured human cell material supplied as a liquid suspended in 0.01 M PBS at pH 7.2-7.4.	10 vials x 500uL	KT0189

Acceptable alternatives to HSC:

- Negative human specimen material: Laboratories may prepare a volume of human specimen material (e.g., human sera or pooled leftover negative respiratory specimens) to extract and run alongside clinical samples as an extraction control. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.
- Contrived human specimen material: Laboratories may prepare contrived human specimen materials by suspending any human cell line (e.g., A549, Hela, or 293) in PBS. This material should be prepared in sufficient volume to be used across multiple runs. Material should be tested prior to use as the extraction control to ensure it generates the expected results for the HSC listed in these instructions for use.

CDC will maintain on its website a list of commercially alternative extraction controls, if applicable, that are acceptable for use with this assay under CDC's Emergency Use Authorization, at: https://www.cdc.gov/coronavirus/2019-nCoV/lab/virus-requests.html

rRT-PCR Enzyme Mastermix Options

Reagent	Quantity	Catalog No.
Quantabio qScript XLT One-Step RT-qPCR ToughMix	100 x 20 µL rxns (1 x 1 mL)	95132-100
	2000 x 20 µL rxns (1 x 20 mL)	95132-02K
	500 x 20 µL rxns (5 x 1 mL)	95132-500
Quantabio UltraPlex 1-Step ToughMix (4X)	100 x 20 µL rxns (500 µL)	95166-100
	500 x 20 µL rxns (5 x 500 µL)	95166-500
	1000 x 20 µL rxns (1 x 5 mL)	95166-01K
Promega GoTaq® Probe 1- Step RT-qPCR System	200 x 20 µL rxns (2 mL)	A6120
	1250 x 20 µL rxns 12.5 mL	A6121
Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix, CG	1000 reactions	A15299
	2000 reactions	A15300

RNA Extraction Options

For each of the kits listed below, CDC has confirmed that the external lysis buffer is effective for inactivation of SARS-CoV-2.

Instrument/Manufacturer	Extraction Kit	Catalog No.
QIAGEN	[2]QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
	[2]QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
QIAGEN EZ1 Advanced XL	[2]EZ1 DSP Virus Kit	48 extractions (62724) Buffer AVL (19073 or 19089) EZ1 Advanced XL DSP Virus Card (9018703)
	[2]EZ1 Virus Mini Kit v2.0	48 extractions (955134) Buffer AVL (19073 or 19089) EZ1 Advanced XL Virus Card v2.0 (9018708)
Roche MagNA Pure 24	[2]MagNA Pure 24 Total NA Isolation Kit	96 extractions (07 658 036 001) External Lysis Buffer (06 374 913 001, 12 239 469 103, 03 246 779 001 or 03 246 752 001)
Roche MagNA Pure 96	[2]DNA and Viral NA Small Volume Kit	576 extractions (06 543 588 001) External Lysis Buffer (06 374 913 001, 12 239 469 103, 03 246 779 001 or 03 246 752 001)
[1]Roche MagNA Pure LC	[2]Total Nucleic Acid Kit	192 extractions (03 038 505 001)
[1]Roche MagNA Pure Compact	[2]Nucleic Acid Isolation Kit I	32 extractions (03 730 964 001)
Promega Maxwell® RSC 48	[3]Maxwell® RSC Viral Total Nucleic Acid Purification Kit	48 extractions (AS1330) 144 extractions (ASB1330)
[1]QIAGEN QIAcube	[2]QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
	[2]QIAamp Viral RNA Mini Kit	50 extractions (52904) 250 extractions (52906)
[1, 3]bioMérieux NucliSENS® easyMAG® and [1, 3]bioMérieux EMAG® (Automated magnetic extraction reagents sold separately. Both instruments use the same reagents and disposables, with the exception of tips.)		EasyMAG® Magnetic Silica (280133) EasyMAG® Lysis Buffer (280134) EasyMAG® Lysis Buffer, 2 mL (200292) EasyMAG® Wash Buffers 1,2, and 3 (280130, 280131, 280132) EasyMAG® Disposables (280135) Biohit Pipette Tips (easyMAG® only) (280146) EMAG®1000µL Tips (418922)

[1]Equivalence and performance of these extraction platforms for extraction of viral RNA were demonstrated with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (K190302). Performance characteristics of these extraction platforms with 2019-nCoV (SARS CoV-2) have not been demonstrated.

[2] CDC has confirmed that the external lysis buffer used with this extraction method is effective for inactivation of SARS-CoV-2.
[3] CDC has compared the concentration of inactivating agent in the lysis buffer used with this extraction method and has determined the concentration to be within the range of concentrations found effective in inactivation of SARS-CoV-2.

Alternative to Extraction:
If a laboratory cannot access adequate extraction reagents to support testing demand due to the global shortage of reagents, CDC has evaluated a heat treatment procedure for upper respiratory specimens using the Quantabio UltraPlex 1-Step ToughMix (4X), CG. Though performance was comparable, this method has been evaluated with a limited number of clinical specimens and a potential reduction in sensitivity due to carryover of inhibitory substances or RNA degradation cannot be ruled out. It should only be used when a jurisdiction determines that the testing need is great enough to justify the risk of a potential loss of sensitivity. Heat-treated specimens generating inconclusive or invalid results should be extracted with an authorized extraction method prior to retesting. Details and procedure for the heat treatment alternative to extraction may be found in Appendix A.

Equipment and Consumables Required (But Not Provided)

- Vortex mixer
- Microcentrifuge
- Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
- Multichannel micropipettes (5-50 µl)
- Racks for 1.5 mL microcentrifuge tubes
- 2 x 96-well -20°C cold blocks
- 7500 Fast Dx Real-Time PCR Systems with SDS 1.4 software (Applied Biosystems; catalog #4406985 or #4406984)
- Extraction systems (instruments): QIAGEN EZ1 Advanced XL, QIAGEN QIAcube, Roche MagNA Pure 24, Roche MagNA Pure 96, Promega Maxwell® RSC 48, Roche MagNA Pure LC, Roche MagNA Pure Compact, bioMérieux easyMAG, and bioMérieux EMAG
- Molecular grade water, nuclease-free
- 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
- DNA*Zap*™ (Ambion, cat. #AM9890) or equivalent
- RNase AWAY™ (Fisher Scientific; cat. #21-236-21) or equivalent
- Disposable powder-free gloves and surgical gowns
- Aerosol barrier pipette tips
- 1.5 mL microcentrifuge tubes (DNase/RNase free)
- 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
- MicroAmp Optical 8-cap Strips (Applied Biosystems; catalog #4323032)

Qualifying Alternative Components:

If a laboratory modifies this test by using unauthorized, alternative components (e.g., extraction methods or PCR instruments), the modified test is not authorized under this EUA. FDA's Policy for Diagnostic Tests for Coronavirus Disease-2019 during the Public Health Emergency, updated May 11, 2020, does not change this. As part of this policy, FDA does not intend to object when a laboratory modifies an EUA-authorized test, which could include using unauthorized components, without

obtaining an EUA or EUA amendment, where the modified test is validated using a bridging study to the EUA-authorized test .

Warnings and Precautions

- For *in vitro* diagnostic use (IVD).
    - This test has not been FDA cleared or approved; this test has been authorized by FDA under an EUA for use by laboratories certified under CLIA, 42 U.S.C. § 263a, to perform high complexity tests.
    - This test has been authorized only for the detection of nucleic acid from SARSCoV-2, not for any other viruses or pathogens.
    - This test is only authorized for the duration of the declaration that circumstances exist justifying the authorization of emergency use of in vitro diagnostic tests for detection and/or diagnosis of COVID-19 under Section 564(b)(1) of the Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.
- Follow standard precautions. All patient specimens and positive controls should be considered potentially infectious and handled accordingly.
- Do not eat, drink, smoke, apply cosmetics or handle contact lenses in areas where reagents and human specimens are handled.
- Handle all specimens as if infectious using safe laboratory procedures. Refer to Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with 2019-nCoV https://www.cdc.gov/coronavirus/2019-nCoV/lab-biosafety-guidelines.html.
- Specimen processing should be performed in accordance with national biological safety regulations.
- If infection with 2019-nCoV is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions.
- Performance characteristics have been determined with human upper respiratory specimens and lower respiratory tract specimens from human patients with signs and symptoms of respiratory infection.
- Perform all manipulations of live virus samples within a Class II (or higher) biological safety cabinet (BSC).
- Use personal protective equipment such as (but not limited to) gloves, eye protection, and lab coats when handling kit reagents while performing this assay and handling materials including samples, reagents, pipettes, and other equipment and reagents.
- Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.
    - Maintain separate areas for assay setup and handling of nucleic acids.
    - Always check the expiration date prior to use. Do not use expired reagents. Do not substitute or mix reagents from different kit lots or from other manufacturers.
    - Change aerosol barrier pipette tips between all manual liquid transfers.
    - During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples and the inadvertent

introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.
    - Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.
    - Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.
    - Change gloves between samples and whenever contamination is suspected.
    - Keep reagent and reaction tubes capped or covered as much as possible.
    - Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on a cold block at all times during preparation and use.
    - Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, DNA*Zap*™, or RNase AWAY™ to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.
- RNA should be maintained on a cold block or on ice during preparation and use to ensure stability.
- Dispose of unused kit reagents and human specimens according to local, state, and federal regulations.

Reagent Storage, Handling, and Stability

- Store all dried primers and probes and the positive control, nCoVPC, at 2-8°C until re-hydrated for use. Store liquid HSC control materials at ≤ -20°C.
  Note: Storage information is for CDC primer and probe materials obtained through the International Reagent Resource. If using commercial primers and probes, please refer to the manufacturer's instructions for storage and handling.
- Always check the expiration date prior to use. Do not use expired reagents.
- Protect fluorogenic probes from light.
- Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on a cold block at all times during preparation and use.
- Do not refreeze probes.
- Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use.

Specimen Collection, Handling, and Storage

Inadequate or inappropriate specimen collection, storage, and transport are likely to yield false test results. Training in specimen collection is highly recommended due to the importance of specimen quality. CLSI MM13-A may be referenced as an appropriate resource.

- Collecting the Specimen
  - Refer to Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Patients Under Investigation (PUIs) for 2019 Novel Coronavirus (2019-nCoV) https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html
  - Follow specimen collection device manufacturer instructions for proper collection methods.
  - Swab specimens should be collected using only swabs with a synthetic tip, such as nylon or Dacron®, and an aluminum or plastic shaft. Calcium alginate swabs are unacceptable and cotton swabs with wooden shafts are not recommended. Place swabs immediately into sterile tubes containing 1-3 ml of appropriate transport media, such as viral transport media (VTM).
- Transporting Specimens
  - Specimens must be packaged, shipped, and transported according to the current edition of the International Air Transport Association (IATA) Dangerous Goods Regulation. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential 2019-nCoV specimens. Store specimens at 2-8°C and ship overnight to CDC on ice pack. If a specimen is frozen at -70°C or lower, ship overnight to CDC on dry ice.
- Storing Specimens
  - Specimens can be stored at 2-8°C for up to 72 hours after collection.
  - If a delay in extraction is expected, store specimens at -70°C or lower.
  - Extracted nucleic acid should be stored at -70°C or lower.

Specimen Referral to CDC

For state and local public health laboratories:

- Ship all specimens overnight to CDC.
- Ship frozen specimens on dry ice and non-frozen specimens on cold packs.
- Refer to the International Air Transport Association (IATA - www.iata.org) for requirements for shipment of human or potentially infectious biological specimens. Follow shipping regulations for UN 3373 Biological Substance, Category B when sending potential 2019-nCoV specimens.
- Prior to shipping, notify CDC Division of Viral Diseases (see contact information below) that you are sending specimens.
- Send all samples to the following recipient:

Centers for Disease Control and Prevention
c/o STATT
Attention: Unit 66
1600 Clifton Rd., Atlanta, GA 30329-4027
Phone: (404) 639-3931

The emergency contact number for CDC Emergency Operations Center (EOC) is 770-488-7100.

All other laboratories that are CLIA certified and meet requirements to perform high complexity testing:

- Please notify your state and/or local public health laboratory for specimen referral and confirmatory testing guidance.

Reagent and Controls Preparation

NOTE: Storage information is for materials obtained through the CDC International Regent Resource. If using commercial products for testing, please refer to the manufacturer's instructions for storage, handling, and preparation instructions.

Primer and Probe Preparation:

1) Upon receipt, store dried primers and probes at 2-8°C.
2) Precautions: These reagents should only be handled in a clean area and stored at appropriate temperatures (see below) in the dark. Freeze-thaw cycles should be avoided. Maintain cold when thawed.
3) Using aseptic technique, suspend dried reagents in 1.5 mL of nuclease-free water and allow to rehydrate for 15 min at room temperature in the dark.
4) Mix gently and aliquot primers/probe in 300 µL volumes into 5 pre-labeled tubes. Store a single, working aliquot of primers/probes at 2-8°C in the dark. Store remaining aliquots at ≤ -20°C in a non-frost-free freezer. Do not refreeze thawed aliquots (stable for up to 4 months at 2-8°C).

2019-nCoV Positive Control (nCoVPC) Preparation:

1) Precautions: This reagent should be handled with caution in a dedicated nucleic acid handling area to prevent possible contamination. Freeze-thaw cycles should be avoided. Maintain on ice when thawed.
2) Resuspend dried reagent in each tube in 1 mL of nuclease-free water to achieve the proper concentration. Make single use aliquots (approximately 30 µL) and store at ≤ -70ºC.
3) Thaw a single aliquot of diluted positive control for each experiment and hold on ice until adding to plate. Discard any unused portion of the aliquot.

Human Specimen Control (HSC) (not provided)

1) Human Specimen Control (HSC) or one of the listed acceptable alternative extraction controls must be extracted and processed with each specimen extraction run.
2) Refer to the Human Specimen Control (HSC) package insert for instructions for use.

No Template Control (NTC) (not provided)

1) Sterile, nuclease-free water
2) Aliquot in small volumes
3) Used to check for contamination during specimen extraction and/or plate set-up

General Preparation

Equipment Preparation

Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use. Decontamination agents should be used including 10% bleach, 70% ethanol, and DNA*zap*™, or RNase AWAY™ to minimize the risk of nucleic acid contamination.

Nucleic Acid Extraction

Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is dependent upon the amount and quality of template RNA purified from human specimens. The following commercially available RNA extraction kits and procedures have been qualified and validated for recovery and purity of RNA for use with the panel:

Qiagen QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit

Recommendation(s): Utilize 100 µL of sample and elute with 100 µL of buffer or utilize 140 µL of sample and elute with 140 µL of buffer.

Qiagen EZ1 Advanced XL

Kit: Qiagen EZ1 DSP Virus Kit and Buffer AVL (supplied separately) for offboard lysis
Card: EZ1 Advanced XL DSP Virus Card
Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

Kit: Qiagen EZ1 Virus Mini Kit v2.0 and Buffer AVL (supplied separately) for offboard lysis
Card: EZ1 Advanced XL Virus Card v2.0
Recommendation(s): Add 120 µL of sample to 280 µL of pre-aliquoted Buffer AVL (total input sample volume is 400 µL). Proceed with the extraction on the EZ1 Advanced XL. Elution volume is 120 µL.

Roche MagNA Pure 96
Kit: Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit
Protocol: Viral NA Plasma Ext LysExt Lys SV 4.0 Protocol or Viral NA Plasma Ext Lys SV Protocol
Recommendation(s): Add 100 µL of sample to 350 µL of pre-aliquoted External Lysis Buffer (supplied separately) (total input sample volume is 450 µL). Proceed with the extraction on the MagNA Pure 96. (**Internal Control = None)**. Elution volume is 100 µL.

Roche MagNA Pure 24
Kit: Roche MagNA Pure 24 Total NA Isolation Kit
Protocol: Pathogen 1000 2.0 Protocol
Recommendation(s): Add 100 µL of sample to 400 µL of pre-aliquoted External Lysis Buffer (supplied separately) (total input sample volume is 500 µL). Proceed with the extraction on the MagNA Pure 24. (**Internal Control = None**). Elution volume is 100 µL.

Promega Maxwell® RSC 48
Kit: Promega Maxwell® Viral Total Nucleic Acid Purification Kit
Protocol: Viral Total Nucleic Acid
Recommendation(s): Add 120 µL of sample to 330 µL of pre-aliquoted External Lysis Buffer (300 µL Lysis Buffer plus 30 µL Proteinase K; supplied within the kit) (total input volume is 450 µL). Proceed with the extraction on the Maxwell® RSC 48. Elution volume is 75 µL.

Equivalence and performance of the following extraction platforms were demonstrated with the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (K190302) and based on those data are acceptable for use with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

QIAGEN QIAcube
Kit: QIAGEN QIAamp® DSP Viral RNA Mini Kit or QIAamp® Viral RNA Mini Kit
Recommendations: Utilize 140 µL of sample and elute with 100 µL of buffer.

Roche MagNA Pure LC
Kit: Roche MagNA Pure Total Nucleic Acid Kit
Protocol: Total NA External_lysis
Recommendation(s): Add 100 µL of sample to 300 µL of pre-aliquoted TNA isolation kit lysis buffer (total input sample volume is 400 µL). Elution volume is 100 µL.

Roche MagNA Pure Compact
Kit: Roche MagNA Pure Nucleic Acid Isolation Kit I
Protocol: Total_NA_Plasma100_400
Recommendation(s): Add 100 µL of sample to 300 µL of pre-aliquoted TNA isolation kit lysis buffer (total input sample volume is 400 µL). Elution volume is 100 µL.

bioMérieux NucliSENS® easyMAG® Instrument
Protocol: General protocol (not for blood) using "Off-board Lysis" reagent settings.
Recommendation(s): Add 100 µL of sample to 1000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 1100 µL). Incubate for 10 minutes at room temperature. Elution volume is 100 µL.

bioMérieux EMAG® Instrument
Protocol: Custom protocol: **CDC Flu V1** using "Off-board Lysis" reagent settings.
Recommendation(s): Add 100 µL of samples to 2000 µL of pre-aliquoted easyMAG lysis buffer (total input sample volume is 2100 µL). Incubate for 10 minutes at room temperature. Elution volume is 100 µL. The custom protocol, **CDC Flu V1**, is programmed on the bioMérieux EMAG® instrument with the assistance of a bioMérieux service representative. Installation verification is documented at the time of installation. Laboratories are recommended to retain a record of the step-by-step verification of the bioMérieux custom protocol installation procedure.

Manufacturer's recommended procedures (except as noted in recommendations above) are to be followed for sample extraction. HSC must be included in each extraction batch.

Disclaimer: Names of vendors or manufacturers are provided as examples of suitable product sources. Inclusion does not imply endorsement by the Centers for Disease Control and Prevention.

Assay Set Up

Reaction Master Mix and Plate Set Up

Note: Plate set-up configuration can vary with the number of specimens and workday organization. NTCs and nCoVPCs must be included in each run.

1) In the reagent set-up room clean hood, place rRT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.
2) Mix buffer, enzyme, and primer/probes by inversion 5 times.
3) Centrifuge reagents and primers/probes for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
4) Label one 1.5 mL microcentrifuge tube for each primer/probe set.
5) Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the NTC, nCoVPC, HSC (if included in the RT-PCR run), and RP reactions and for pipetting error. Use the following guide to determine N:
   - If number of samples (n) including controls equals 1 through 14, then N = n + 1
   - If number of samples (n) including controls is 15 or greater, then N = n + 2
7) For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N = # of reactions).

Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	TaqPath™ 1-Step RT-qPCR Master Mix (4x)	N x 5.0 µL
	Total Volume	**N x 15.0 µL**

Promega GoTaq® Probe 1- Step RT-qPCR System

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 3.1 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	GoTaq Probe qPCR Master Mix with dUTP	N x 10.0 µL
4	Go Script RT Mix for 1-Step RT-qPCR	N x 0.4 µL
	Total Volume	**N x 15.0 µL**

Quantabio qScript XLT One-Step RT-qPCR ToughMix

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 3.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	qScript XLT One-Step RT-qPCR ToughMix (2X)	N x 10.0 µL
	Total Volume	**N x 15.0 µL**

Quantabio UltraPlex 1-Step ToughMix (4X)

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	UltraPlex 1-Step ToughMix (4X)	N x 5.0 µL
	Total Volume	**N x 15.0 µL**

8) Dispense reagents into each respective labeled 1.5 mL microcentrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. ***Do not vortex***.
9) Centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
10) Set up reaction strip tubes or plates in a 96-well cooler rack.
11) Dispense 15 µL of each master mix into the appropriate wells going across the row as shown below (**Figure 1**):

Figure 1: Example of Reaction Master Mix Plate Set-Up

	1	2	3	4	5	6	7	8	9	10	11	12
A	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1	N1
B	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2	N2
C	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP	RP
D												
E												
F												
G												
H												

12) Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions for column #1 in the assay preparation area.
13) Pipette 5 µL of nuclease-free water into the NTC sample wells (**Figure 2**, column 1). Securely cap NTC wells before proceeding.
14) Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

Nucleic Acid Template Addition

1) Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2) Centrifuge for 5 seconds to collect contents at the bottom of the tube.
3) After centrifugation, place extracted nucleic acid sample tubes in the cold rack.
4) Samples should be added to columns 2-11 (column 1 and 12 are for controls) to the specific assay that is being tested as illustrated in **Figure 2**. Carefully pipette 5.0 µL of the first sample into all the wells labeled for that sample (i.e. Sample "S1" down column #2). *Keep other sample wells covered during addition. Change tips after each addition.*
5) Securely cap the column to which the sample has been added to prevent cross contamination and to ensure sample tracking.
6) Change gloves often and when necessary to avoid contamination.
7) Repeat steps #4 and #5 for the remaining samples.

8) If necessary, add 5 µL of Human Specimen Control (HSC) extracted sample to the HSC wells (**Figure 2**, column 11). Securely cap wells after addition. NOTE: Per CLIA regulations, HSC must be tested at least once per day.
9) Cover the entire reaction plate and move the reaction plate to the positive template control handling area.

Assay Control Addition

1) Pipette 5 µL of nCoVPC RNA to the sample wells of column 12 (**Figure 2**)**.** Securely cap wells after addition of the control RNA.
   NOTE: *If using 8-tube strips, label the TAB of each strip to indicate sample position.* ***DO NOT LABEL THE TOPS OF THE REACTION TUBES!***
2) Briefly centrifuge reaction tube strips for 10-15 seconds. After centrifugation return to cold rack.
   NOTE*: If using 96-well plates, centrifuge plates for 30 seconds at 500 x g, 4°C.*

Figure 2. 2019-nCoV rRT-PCR Diagnostic Panel: Example of Sample and Control Set-up

	1	2	3	4	5	6	7	8	9	10	11[a]	12
A	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
B	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
C	NTC	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	nCoV PC
D												
E												
F												
G												
H												

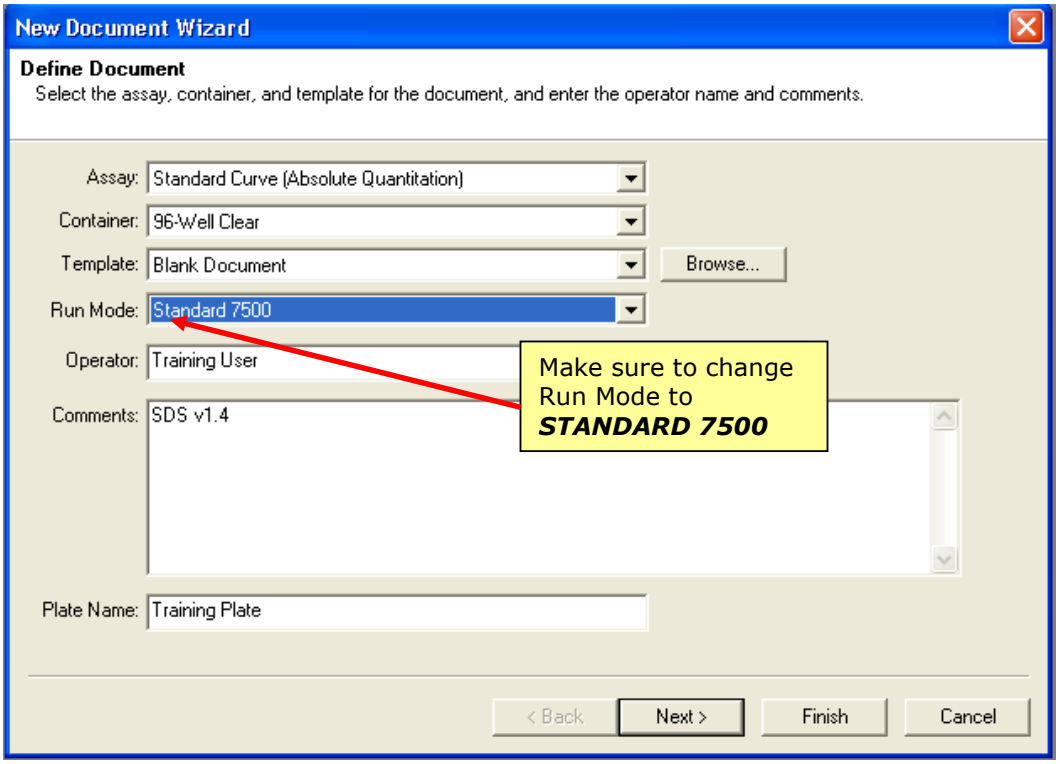
[a]Replace the sample in this column with extracted HSC if necessary

Create a Run Template on the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument (Required if no template exists)

If the template already exists on your instrument, please proceed to the **RUNNING A TEST** section.

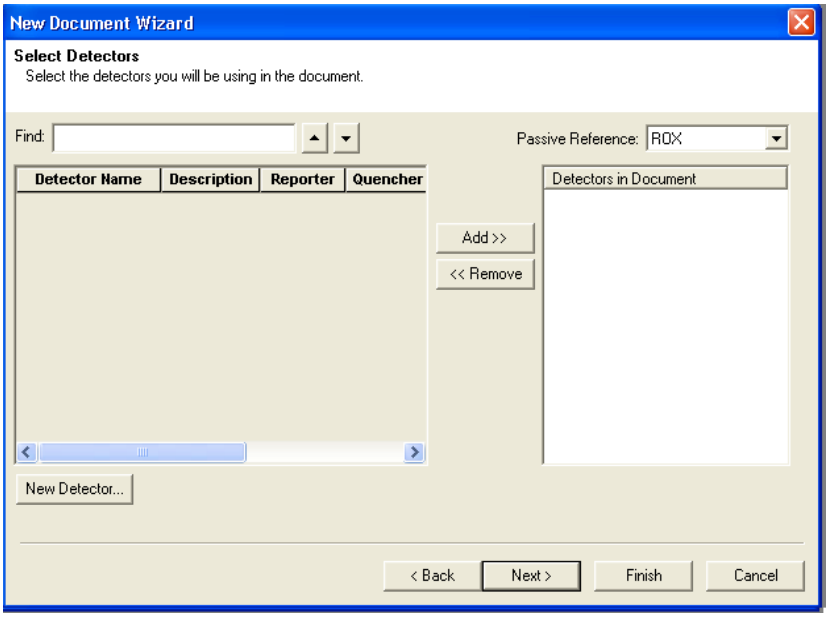
1) Launch the Applied Biosystems 7500 Fast Dx Real-time PCR Instrument by double clicking on the Applied Biosystems 7500 Fast Dx System icon on the desktop.
2) A new window should appear, select **Create New Document** from the menu.

Figure 3. New Document Wizard Window



3) The **New Document Wizard** screen in **Figure 3** will appear. Select:
   a. Assay: **Standard Curve (Absolute Quantitation)**
   b. Container: **96-Well Clear**
   c. Template: **Blank Document**
   d. Run Mode: **Standard 7500**
   e. Operator: ***Your Name***
   f. Comments: **SDS v1.4**
   g. Plate Name: ***Your Choice***
4) After making selections click **Next** at the bottom of the window.

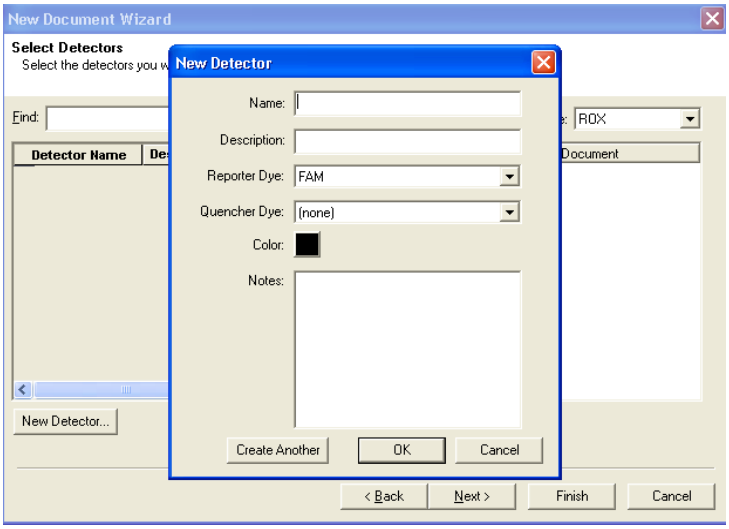
Figure 4. Creating New Detectors



NOTE: ROX is the default passive reference. This will be changed to "none" in step 12.

5) After selecting next, the ***Select Detectors*** screen (**Figure 4**) will appear.
6) Click the **New Detector** button (see **Figure 4**).
7) The **New Detector** window will appear (**Figure 5**). A new detector will need to be defined for each primer and probe set. Creating these detectors will enable you to analyze each primer and probe set individually at the end of the reaction.

Figure 5. New Detector Window

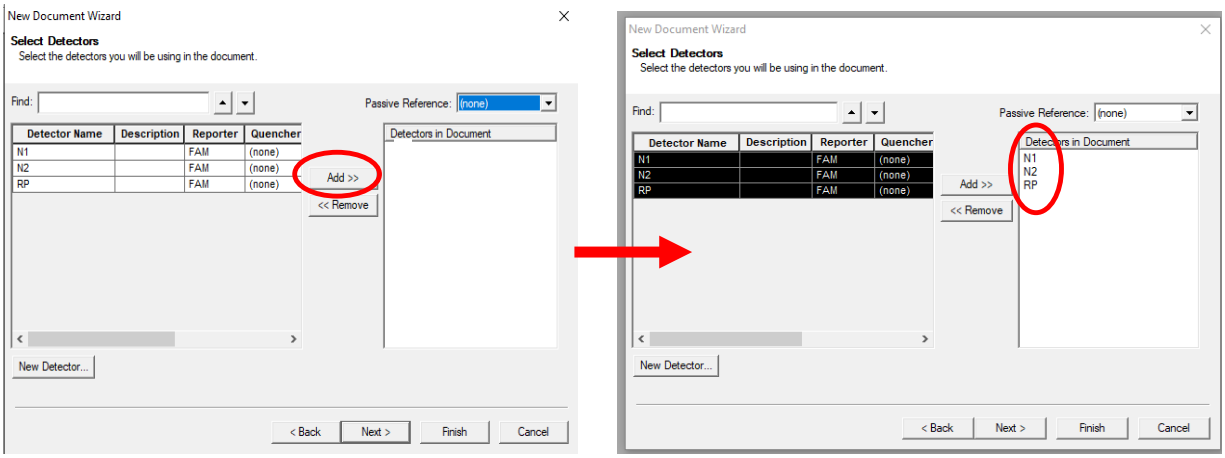


8) Start by creating the N1 Detector. Include the following:
   a. Name: **N1**
   b. Description: *leave blank*
   c. Reporter Dye: **FAM**
   d. Quencher Dye: **(none)**
   e. Color: *to change the color of the detector indicator do the following:*
      ⇒ Click on the color square to reveal the color chart
      ⇒ Select a color by clicking on one of the squares
      ⇒ After selecting a color click **OK** to return to the New Detector screen
   f. Click the **OK** button of the New Detector screen to return to the screen shown in **Figure 4**.
9) Repeat step 6-8 for each target in the panel.

Name	Reporter Dye	Quencher Dye
N1	FAM	(none)
N2	FAM	(none)
RP	FAM	(none)

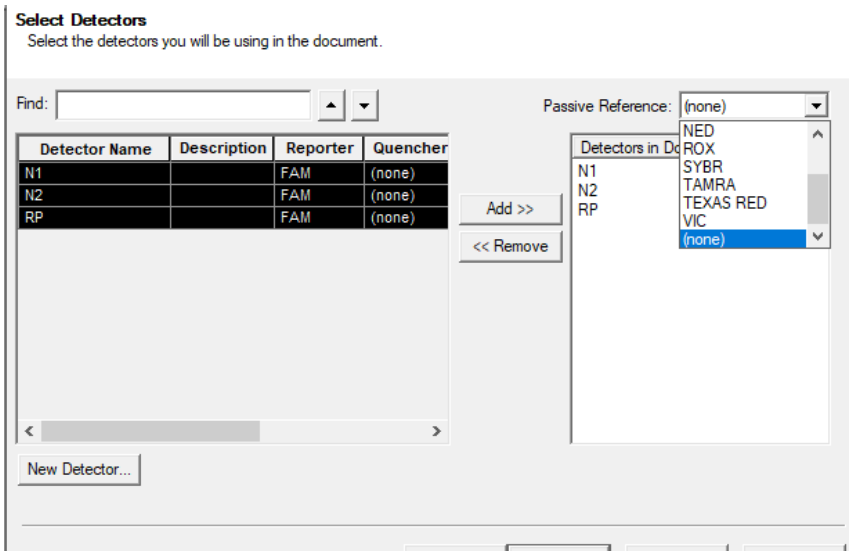
10) After each Detector is added, the **Detector Name**, **Description**, **Reporter** and **Quencher** fields will become populated in the **Select Detectors** screen (**Figure 6**).
11) Before proceeding, the newly created detectors must be added to the document. To add the new detectors to the document, click **ADD** (see **Figure 6**). Detector names will appear on the right-hand side of the **Select Detectors** window (**Figure 6**).

Figure 6. Adding New Detectors to Document



12) Once all detectors have been added, select **(none)** for **Passive Reference** at the top right-hand drop-down menu (**Figure 7**).

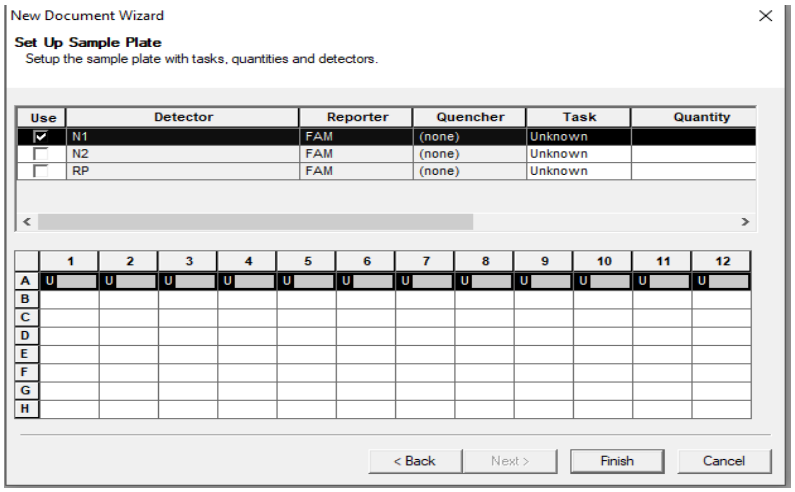
Figure 7. Select Passive Reference



Passive reference should be set to "(none)" as described above.

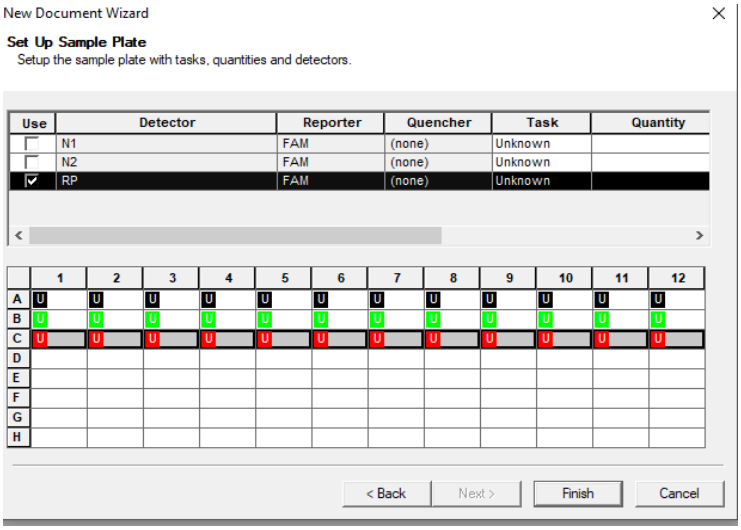
13) Click **Next** at the bottom of the **Select Detectors** window to proceed to the **Set Up Sample Plate** window (**Figure 8**).
14) In the **Set Up Sample Plate** window (**Figure 8**), use your mouse to select row A from the lower portion of the window, in the spreadsheet (see **Figure 8**).
15) In the top portion of the window, select detector **N1**. A check will appear next to the detector you have selected (**Figure 8**). You will also notice the row in the spreadsheet will be populated with a colored "U" icon to indicate which detector you've selected.
16) Repeat step 14-15 for each detector that will be used in the assay.

Figure 8. Sample Plate Set-up



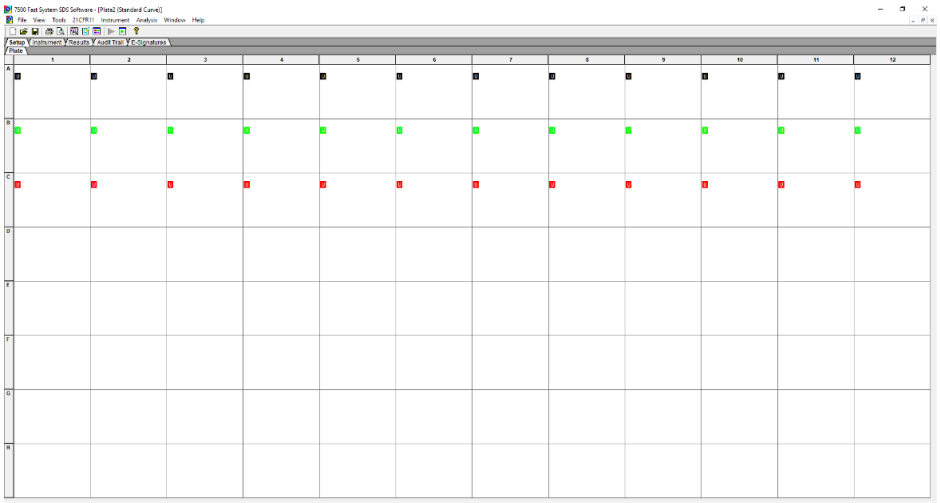
17) Select **Finish** after detectors have been assigned to their respective rows. (**Figure 9**).

Figure 9. Finished Plate Set-up



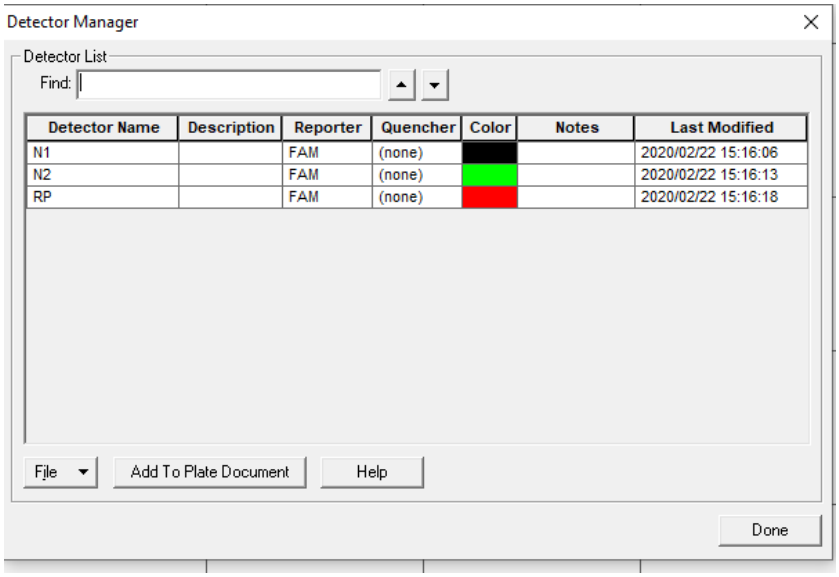
18) After clicking “Finish”, there will be a brief pause allowing the Applied Biosystems 7500 Fast Dx to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***
19) After initialization, the **Plate** tab of the Setup (**Figure 10)** will appear.
20) Each well of the plate should contain colored U icons that correspond with the detector labels that were previously chosen. To confirm detector assignments, select **Tools** from the file menu, then select **Detector Manager.**

Figure 10. Plate Set-up Window



21) The Detector Manager window will appear (**Figure 11**).

Figure 11. Detector Manager Window



22) Confirm all detectors are included and that each target has a **Reporter** set to **FAM** and the **Quencher** is set to **(none)**.
23) If all detectors are present, select **Done**. The detector information has been created and assigned to wells on the plate.

Defining the Instrument Settings

1) After detectors have been created and assigned, proceed to instrument set up.
2) Select the **Instrument** tab to define thermal cycling conditions.
3) Modify the thermal cycling conditions as follows (**Figure 12**):

Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix, CG

a. In Stage 1, Set to 2 min at **25°C**; **1 Rep**.
b. In Stage 2, Set to 15 min at **50°C**; **1 Rep**.
c. In Stage 3, Set to 2 min at **95°C, 1 Rep.**
d. In Stage 4, Step 1 set to **3 sec** at **95°C**.
e. In Stage 4, Step 2 set to **30 sec** at **55.0°C.**
f. In Stage 4, Reps should be set to **45.**
g. Under **Settings** (**Figure 12**), bottom left-hand box, change volume to 20 µL.
h. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
i. Step 2 of Stage 4 should be highlighted in yellow to indicate data collection (see **Figure 12**).

OR

Quantabio qScript™ XLT One-Step RT-qPCR ToughMix or UltraPlex 1-Step ToughMix (4X)

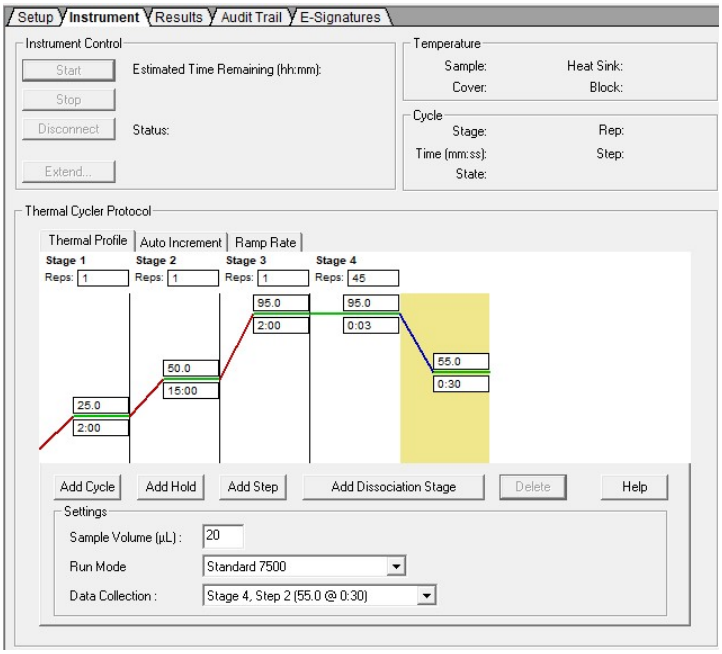
a. In Stage 1, Set to 10 min at **50°C**; **1 Rep**.
b. In Stage 2, Set to 3 min at **95°C, 1 Rep.**
c. In Stage 3, Step 1 set to **3 sec** at **95°C**.
d. In Stage 3, Step 2 set to **30 sec** at **55.0°C.**
e. In Stage 3, Reps should be set to **45.**
f. Under **Settings** (**Figure 12**), bottom left-hand box, change volume to 20 µL.
g. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
h. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 12**).

OR

Promega GoTaq® Probe 1-Step RT-qPCR System

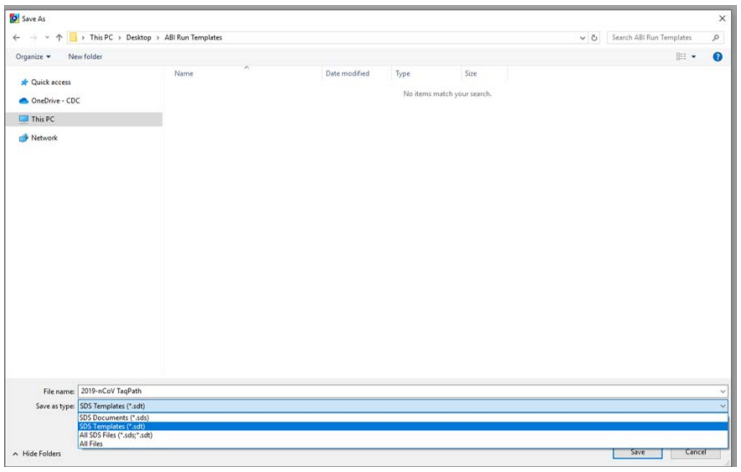
a. In Stage 1, Set to 15 min at **45°C**; **1 Rep**.
b. In Stage 2, Set to 2 min at **95°C, 1 Rep.**
c. In Stage 3, Step 1 set to **3 sec** at **95°C**.
d. In Stage 3, Step 2 set to **30 sec** at **55.0°C.**
e. In Stage 3, Reps should be set to **45.**
f. Under **Settings** (**Figure 12**), bottom left-hand box, change volume to 20 µL.
g. Under **Settings**, **Run Mode** selection should be **Standard 7500**.
h. Step 2 of Stage 3 should be highlighted in yellow to indicate data collection (see **Figure 12**).

Figure 12. Instrument Window



4) After making changes to the **Instrument** tab, the template file is ready to be saved. To save the template, select **File** from the top menu, then select **Save As**. Since the enzyme options have different instrument settings, it is recommended that the template be saved with a name indicating the enzyme option.
5) Save the template as **2019-nCoV Dx Panel TaqPath** or **2019-nCoV Dx Panel Quanta** or **2019-nCoV Dx Panel Promega** as appropriate in the desktop folder labeled "***ABI Run Templates***" (*you must create this folder*). Save as type should be SDS Templates (*.sdt) (**Figure 13**).

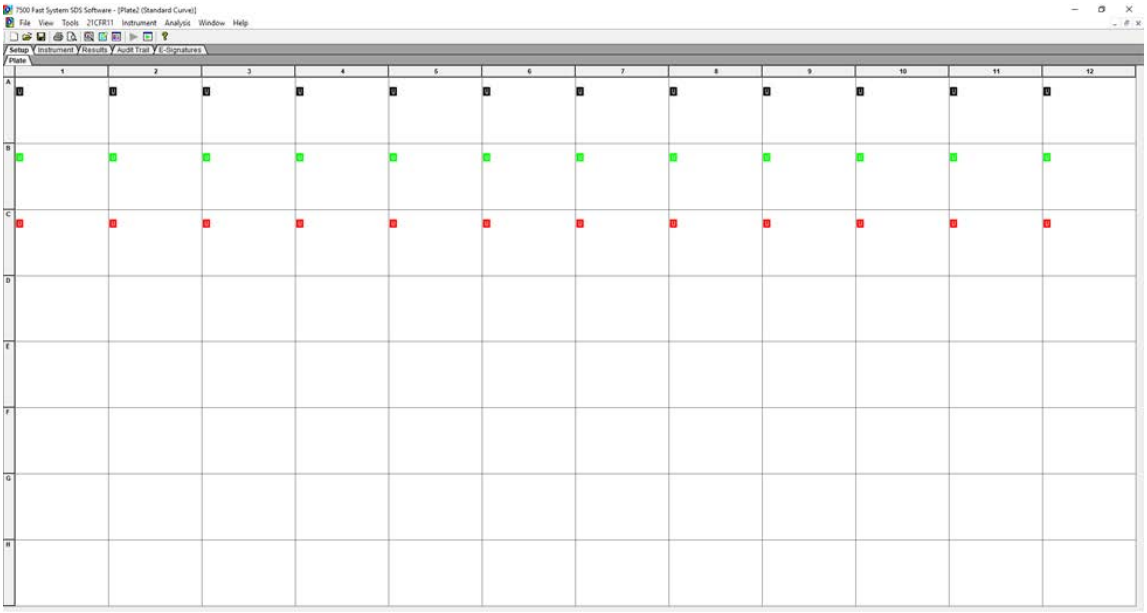
Figure 13. Saving Template



Running a Test

1) Turn on the ABI 7500 Fast Dx Real-Time PCR Instrument.
2) Launch the Applied Biosystems 7500 Fast Dx Real-time PCR System by double clicking on the 7500 Fast Dx System icon on the desktop.
3) A new window should appear, select **Open Existing Document** from the menu.
4) Navigate to select your ABI Run Template folder from the desktop.
5) Double click on the appropriate template file (**2019-nCoV Dx Panel TaqPath** or **2019-nCoV Dx Panel Quanta** or **2019-nCoV Dx Panel Promega)**
6) There will be a brief pause allowing the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument to initialize. This initialization is followed by a clicking noise. ***Note: The machine must be turned on for initialization.***

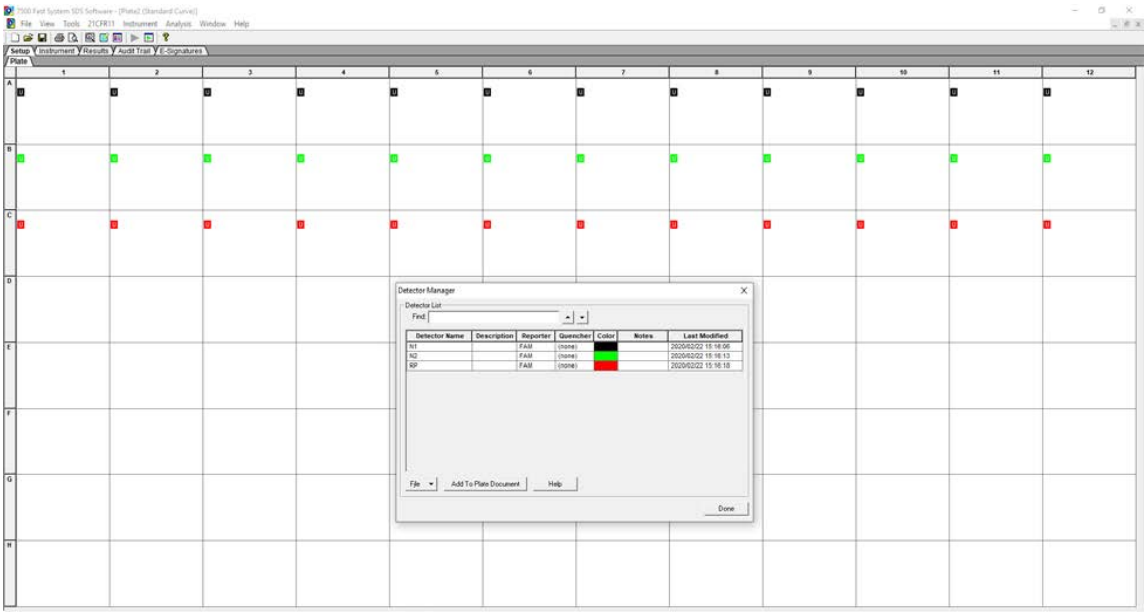
Figure 14. Plate Set-up Window



7) After the instrument initializes, a plate map will appear (**Figure 14**). The detectors and controls should already be labeled as they were assigned in the original template.

8) Click the **Well Inspector** icon  from the top menu.
9) Highlight specimen wells of interest on the plate map.
10) Type sample identifiers to **Sample Name** box in the **Well Inspector** window (**Figure 15**).

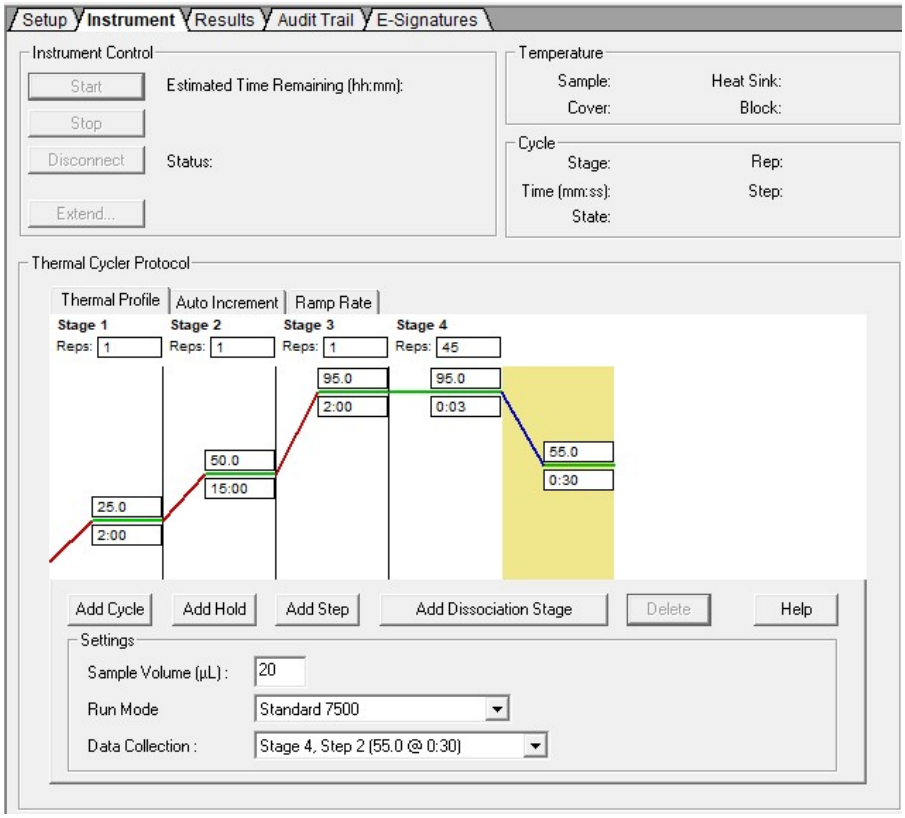
Figure 15. Labeling Wells



11) Repeat steps 9-10 until all sample identifiers are added to the plate setup.

12) Once all specimen and control identifiers are added click the **Close** button on the **Well Inspector** window to return to the **Plate** set up tab.
13) Click the **Instrument** tab at the upper left corner.
14) The reaction conditions, volumes, and type of 7500 reaction should already be loaded (**Figure 16**).

Figure 16. Instrument Settings

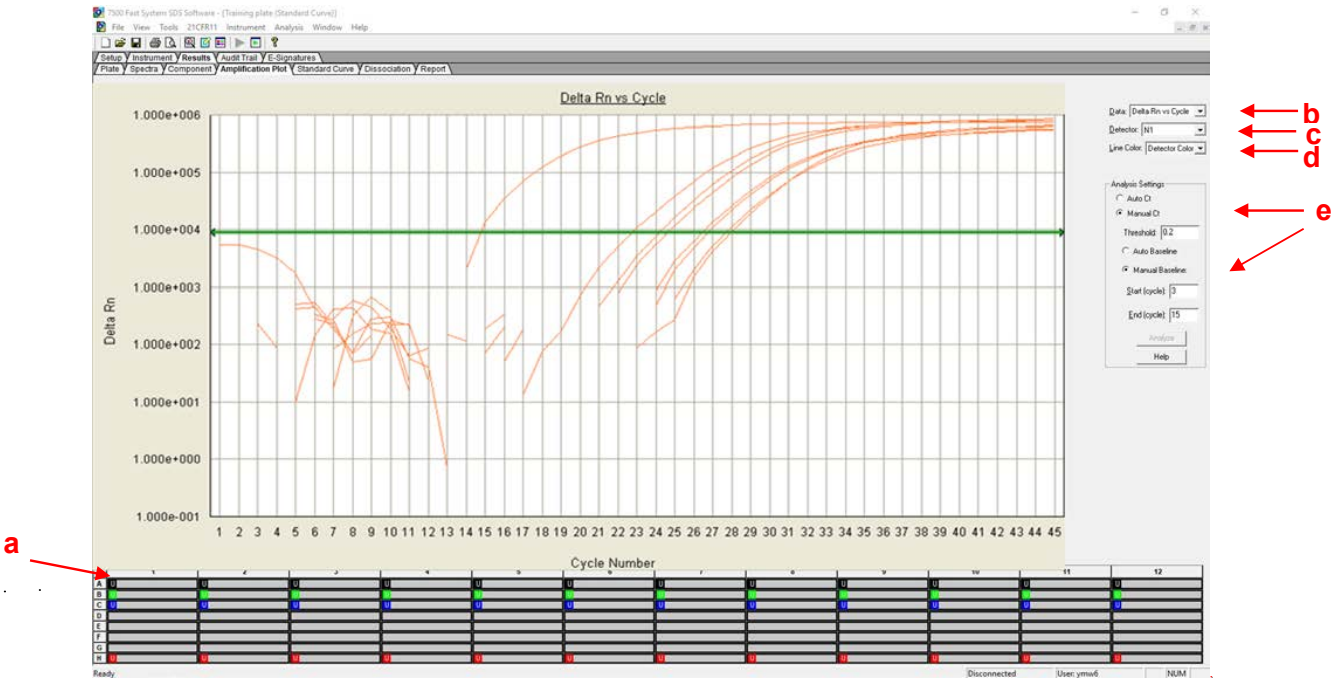


15) Ensure settings are correct (refer to the *Defining Instrument Settings*).
16) Before proceeding, the run file must be saved; from the main menu, select **File,** then **Save As**. Save in appropriate run folder designation.
17) Load the plate into the plate holder in the instrument. Ensure that the plate is properly aligned in the holder.
18) Once the run file is saved, click the **Start** button. *Note: The run should take approximately 1 hour and 20 minutes to complete.*

Data Analysis

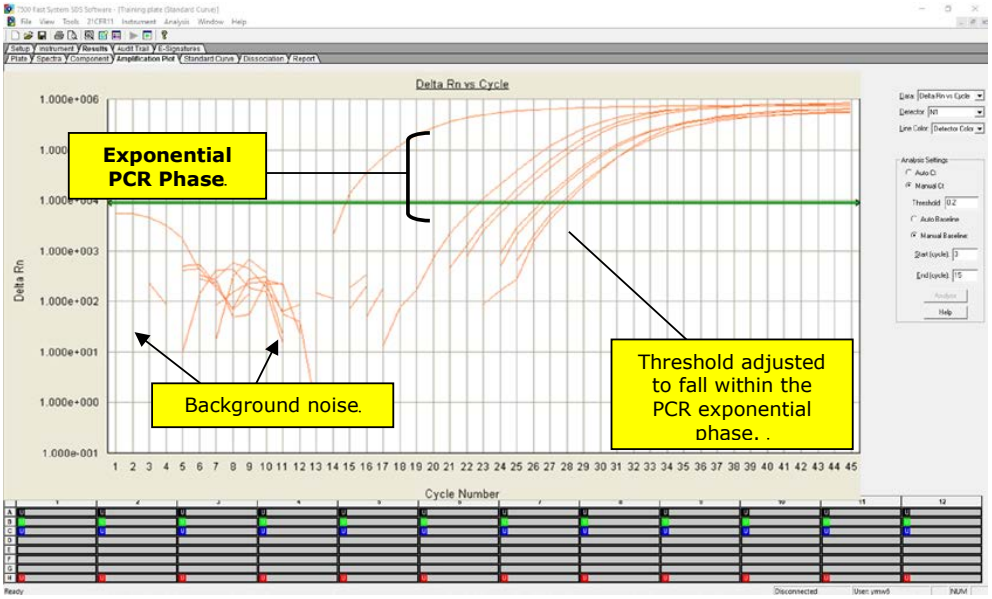
1) After the run has completed, select the **Results** tab at the upper left corner of the software.
2) Select the **Amplification Plot** tab to view the raw data (**Figure 17**).

Figure 17. Amplification Plot Window



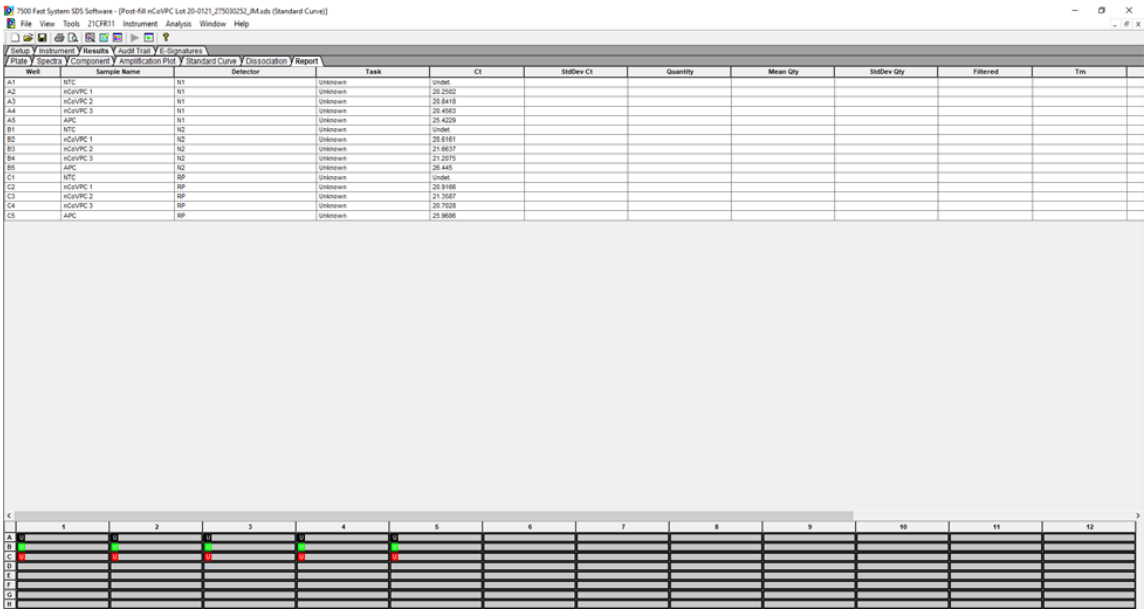
3) Start by highlighting all the samples from the run; to do this, click on the upper left-hand box **(a)** of the sample wells (**Figure 17**). All the growth curves should appear on the graph.
4) On the right-hand side of the window **(b)**, the **Data** drop down selection should be set to **Delta Rn vs. Cycle**.
5) Select **N1** from **(c)**, the **Detector** drop down menu, using the downward arrow.
    a. Please note that each detector is analyzed individually to reflect different performance profiles of each primer and probe set.
6) In the **Line Color** drop down **(d)**, **Detector Color** should be selected.
7) Under **Analysis Settings** select **Manual Ct (e)**.
    b. Do not change the **Manual Baseline** default numbers.
8) Using the mouse, click and drag the red threshold line until it lies within the exponential phase of the fluorescence curves and above any background signal (**Figure 18)**.

Figure 18. Amplification Plot



9) Click the **Analyze** button in the lower right corner of the window. The red threshold line will turn to green, indicating the data has been analyzed.
10) Repeat steps 5-9 to analyze results generated for each set of markers (N1, N2, RP).
11) Save analysis file by selecting **File** then **Save As** from the main menu.
12) After completing analysis for each of the markers, select the **Report** tab above the graph to display the Ct values (**Figure 19**). To filter report by sample name in ascending or descending order, simply click on **Sample Name** in the table.

Figure 19. Report



Interpretation of Results and Reporting

Extraction and Positive Control Results and Interpretation

No Template Control (NTC)

The NTC consists of using nuclease-free water in the rRT-PCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence growth curves that cross the threshold line. If any of the NTC reactions exhibit a growth curve that crosses the cycle threshold, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines.

2019-nCoV Positive Control (nCoVPC)

The nCoVPC consists of in vitro transcribed RNA. The nCoVPC will yield a positive result with the following primer and probe sets: N1, N2, and RP.

Human Specimen Control (HSC) (Extraction Control)

When HSC is run with the CDC 2019-nCoV rRT-PCR Diagnostic Panel (see previous section on Assay Set Up), the HSC is used as an nucleic acid extraction procedural control to demonstrate successful recovery of nucleic acid as well as extraction reagent integrity. The HSC control consists of noninfectious cultured human cell (A549) material. Purified nucleic acid from the HSC should yield a positive result with the RP primer and probe set and negative results with all 2019-nCoV markers.

Expected Performance of Controls Included in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Control Type	External Control Name	Used to Monitor	2019 nCoV_N1	2019 nCoV_N2	RP	Expected Ct Values
Positive	nCoVPC	Substantial reagent failure including primer and probe integrity	+	+	+	< 40.00 Ct
Negative	NTC	Reagent and/or environmental contamination	-	-	-	None detected
Extraction	HSC	Failure in lysis and extraction procedure, potential contamination during extraction	-	-	+	< 40.00 Ct

If any of the above controls do not exhibit the expected performance as described, the assay may have been set up and/or executed improperly, or reagent or equipment malfunction could have occurred. Invalidate the run and re-test.

RNase P (Extraction Control)

- All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 40.00 cycles (< 40.00 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:
  - Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
  - Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
  - Improper assay set up and execution.
  - Reagent or equipment malfunction.
- If the RP assay does not produce a positive result for human clinical specimens, interpret as follows:
  - If the 2019-nCoV N1 and N2are positive even in the absence of a positive RP, the result should be considered valid. It is possible, that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RP signal does not preclude the presence of 2019-nCoV virus RNA in a clinical specimen.
  - If all 2019-nCoV markers AND RNase P are negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

2019-nCoV Markers (N1 and N2)

- When all controls exhibit the expected performance, a specimen is considered negative if all 2019-nCoV marker (N1, N2) cycle threshold growth curves DO NOT cross the threshold line within 40.00 cycles (< 40.00 Ct) AND the RNase P growth curve DOES cross the threshold line within 40.00 cycles (< 40.00 Ct).
- When all controls exhibit the expected performance, a specimen is considered positive for 2019-nCoV if all 2019-nCoV marker (N1, N2) cycle threshold growth curves cross the threshold line within 40.00 cycles (< 40.00 Ct). The RNase P may or may not be positive as described above, but the 2019-nCoV result is still valid.
- When all controls exhibit the expected performance and the growth curves for the 2019-nCoV markers (N1, N2) AND the RNase P marker DO NOT cross the cycle threshold growth curve within 40.00 cycles (< 40.00 Ct), the result is invalid. The extracted RNA from the specimen should be re-tested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the re-tested sample is negative for all markers and RNase P, the result is invalid and collection of a new specimen from the patient should be considered.
- When all controls exhibit the expected performance and the cycle threshold growth curve for any one marker (N1 or N2, but not both markers) crosses the threshold line within 40.00 cycles (< 40.00 Ct) the result is inconclusive. The extracted RNA should be retested. If residual RNA is not available, re-extract RNA from residual specimen and re-test. If the same result is obtained,

report the inconclusive result. Consult with your state public health laboratory or CDC, as appropriate, to request guidance and/or to coordinate transfer of the specimen for additional analysis.

- If HSC is positive for N1 or N2, then contamination may have occurred during extraction or sample processing. Invalidate all results for specimens extracted alongside the HSC. Re-extract specimens and HSC and re-test.

2019-nCoV rRT-PCR Diagnostic Panel Results Interpretation Guide

The table below lists the expected results for the 2019-nCoV rRT-PCR Diagnostic Panel. If a laboratory obtains unexpected results for assay controls or if inconclusive or invalid results are obtained and cannot be resolved through the recommended re-testing, please contact CDC for consultation and possible specimen referral. See pages 13 and 50 for referral and contact information.

2019 nCoV_N1	2019 nCoV_N2	RP	Result Interpretation[a]	Report	Actions
+	+	±	2019-nCoV detected	Positive 2019-nCoV	Report results to CDC and sender.
If only one of the two targets is positive		±	Inconclusive Result	Inconclusive	Repeat testing of nucleic acid and/or re-extract and repeat rRT-PCR. If the repeated result remains inconclusive, contact your State Public Health Laboratory or CDC for instructions for transfer of the specimen or further guidance.
-	-	+	2019-nCoV not detected	Not Detected	Report results to sender. Consider testing for other respiratory viruses.[b]
-	-	-	Invalid Result	Invalid	Repeat extraction and rRT-PCR. If the repeated result remains invalid, consider collecting a new specimen from the patient.

[a]Laboratories should report their diagnostic result as appropriate and in compliance with their specific reporting system.

[b]Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens from the same patient may be necessary to detect the virus. The possibility of a false negative result should especially be considered if the patient's recent exposures or clinical presentation suggest that 2019-nCoV infection is possible, and diagnostic tests for other causes of illness (e.g., other respiratory illness) are negative. If 2019-nCoV infection is still suspected, re-testing should be considered in consultation with public health authorities.

Quality Control

- Quality control requirements must be performed in conformance with local, state, and federal regulations or accreditation requirements and the user's laboratory's standard quality control procedures. For further guidance on appropriate quality control practices, refer to 42 CFR 493.1256.
- Quality control procedures are intended to monitor reagent and assay performance.
- Test all positive controls prior to running diagnostic samples with each new kit lot to ensure all reagents and kit components are working properly.
- Good laboratory practice (cGLP) recommends including a positive extraction control in each nucleic acid isolation batch.
- Although HSC is not included with the 2019-nCov rRT-PCR Diagnostic Panel, the HSC extraction control must proceed through nucleic acid isolation per batch of specimens to be tested.
- Always include a negative template control (NTC) and the appropriate positive control (nCoVPC) in each amplification and detection run. All clinical samples should be tested for human RNase P gene to control for specimen quality and extraction.

Limitations

- All users, analysts, and any person reporting diagnostic results should be trained to perform this procedure by a competent instructor. They should demonstrate their ability to perform the test and interpret the results prior to performing the assay independently.
- Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel has only been established in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate).
- Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Optimum specimen types and timing for peak viral levels during infections caused by 2019-nCoV have not been determined. Collection of multiple specimens (types and time points) from the same patient may be necessary to detect the virus.
- A false-negative result may occur if a specimen is improperly collected, transported or handled. False-negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- Positive and negative predictive values are highly dependent on prevalence. False-negative test results are more likely when prevalence of disease is high. False-positive test results are more likely when prevalence is moderate to low.
- Do not use any reagent past the expiration date.
- If the virus mutates in the rRT-PCR target region, 2019-nCoV may not be detected or may be detected less predictably. Inhibitors or other types of interference may produce a false-negative result. An interference study evaluating the effect of common cold medications was not performed.
- Test performance can be affected because the epidemiology and clinical spectrum of infection caused by 2019-nCoV is not fully known. For example, clinicians and laboratories may not know

the optimum types of specimens to collect, and, during the course of infection, when these specimens are most likely to contain levels of viral RNA that can be readily detected.

- Detection of viral RNA may not indicate the presence of infectious virus or that 2019-nCoV is the causative agent for clinical symptoms.
- The performance of this test has not been established for monitoring treatment of 2019-nCoV infection.
- The performance of this test has not been established for screening of blood or blood products for the presence of 2019-nCoV.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

Conditions of Authorization for the Laboratory

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Letter of Authorization, along with the authorized Fact Sheet for Healthcare Providers, the authorized Fact Sheet for Patients, and authorized labeling are available on the FDA website:
https://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm
Use of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel must follow the procedures outlined in these manufacturer's Instructions for Use and the conditions of authorization outlined in the Letter of Authorization. Deviations from the procedures outlined are not permitted under the Emergency Use Authorization (EUA). To assist clinical laboratories running the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, the relevant Conditions of Authorization are listed verbatim below, and are required to be met by laboratories performing the EUA test.

- Authorized laboratories[1] will include with reports of the results of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.

- Authorized laboratories will perform the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel as outlined in the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use. Deviations from the authorized procedures, including the authorized RT-PCR instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to perform the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel are not permitted. [2]

- Authorized laboratories that receive the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel must notify the relevant public health authorities of their intent to run the test prior to initiating testing.

[1]Authorized Laboratories: For ease of reference, the Letter of Authorization refers to "laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests" as "authorized laboratories."

[2]If an authorized laboratory is interested in implementing changes to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel that are not in the scope (Section II) of this letter of authorization FDA recommends you discuss with FDA after considering the policy outlined in *Immediately in Effect Guidance for Clinical Laboratories and Food and Drug Administration Staff: Policy for Diagnostics Testing in Laboratories Certified to Perform High Complexity Testing under CLIA prior to Emergency Use Authorization for Coronavirus Disease-2019 during the Public Health Emergency* (https://www.fda.gov/media/135659/download).

- Authorized laboratories will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.

- Authorized laboratories will collect information on the performance of the test and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and CDC (respvirus@cdc.gov) any suspected occurrence of false-positive or false-negative results and significant deviations from the established performance characteristics of the test of which they become aware.

- Authorized laboratories will report adverse events, including problems with test performance or results, to MedWatch by submitting the online FDA Form 3500 (https://www.accessdata.fda.gov/scripts/medwatch/index.cfm?action=reporting.home) or by calling 1-800-FDA-1088

- All laboratory personnel using the test must be appropriately trained in RT-PCR techniques and use appropriate laboratory and personal protective equipment when handling this kit and use the test in accordance with the authorized labeling.

- CDC, IRR, manufacturers and distributors of commercial materials identified as acceptable on the CDC website, and authorized laboratories will ensure that any records associated with this EUA are maintained until otherwise notified by FDA. Such records will be made available to FDA for inspection upon request.

Performance Characteristics

Analytical Performance:

Limit of Detection (LoD):

LoD studies determine the lowest detectable concentration of 2019-nCoV at which approximately 95% of all (true positive) replicates test positive. The LoD was determined by limiting dilution studies using characterized samples.

The analytical sensitivity of the rRT-PCR assays contained in the CDC 2019 Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel were determined in Limit of Detection studies. Since no quantified virus isolates of the 2019-nCoV are currently available, assays designed for detection of the 2019-nCoV RNA were tested with characterized stocks of in vitro transcribed full length RNA (N gene; GenBank accession: MN908947.2) of known titer (RNA copies/µL) spiked into a diluent consisting of a suspension of human A549 cells and viral transport medium (VTM) to mimic clinical specimen. Samples were extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit (Cat# 62724) and manually with the QIAGEN DSP Viral RNA Mini Kit (Cat# 61904). Real-Time RT-PCR assays were performed using the ThemoFisher Scientific TaqPath™ 1-Step RT-qPCR Master Mix, CG (Cat# A15299) on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR Instrument according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel instructions for use.

A preliminary LoD for each assay was determined testing triplicate samples of RNA purified using each extraction method. The approximate LoD was identified by extracting and testing 10-fold serial dilutions of characterized stocks of in vitro transcribed full-length RNA. A confirmation of the LoD was determined using 3-fold serial dilution RNA samples with 20 extracted replicates. The LoD was determined as the lowest concentration where ≥ 95% (19/20) of the replicates were positive.

Table 4. Limit of Detection Confirmation of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with QIAGEN EZ1 DSP

Targets	2019-nCoV_N1			2019-nCoV_N2		
RNA Concentration[1]	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$
Positives/Total	20/20	19/20	13/20	20/20	17/20	9/20
Mean Ct[2]	32.5	35.4	NA	35.8	NA	NA
Standard Deviation (Ct)	0.5	0.8	NA	1.3	NA	NA

[1] Concentration is presented in RNA copies/µL

[2] Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results.

NA not applicable

Table 5. Limit of Detection Confirmation CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with QIAGEN QIAmp DSP Viral RNA Mini Kit

Targets	2019-nCoV_N1			2019-nCoV_N2			
RNA Concentration[1]	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$
Positives/Total	20/20	20/20	6/20	20/20	20/20	20/20	8/20
Mean Ct[2]	32.0	32.8	NA	33.0	35.4	36.2	NA
Standard Deviation (Ct)	0.7	0.8	NA	1.4	0.9	1.9	NA

[1] Concentration is presented in RNA copies/µL

[2] Mean Ct reported for dilutions that are ≥ 95% positive. Calculations only include positive results.

NA not applicable

Table 6. Limit of Detection of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Virus	**Material**	**Limit of Detection (RNA copies/µL)**	
		QIAGEN EZ1 Advanced XL	**QIAGEN DSP Viral RNA Mini Kit**
2019 Novel Coronavirus	N Gene RNA Transcript	$10^{0.5}$	10^{0}

FDA Sensitivity Evaluation: The analytical sensitivity of the test will be further assessed by evaluating an FDA-recommended reference material using an FDA developed protocol if applicable and/or when available.

In Silico Analysis of Primer and Probe Sequences:

The oligonucleotide primer and probe sequences of the CDC 2019 nCoV Real-Time RT-PCR Diagnostic Panel were evaluated against 31,623 sequences available in the Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org) database as of June 20, 2020, to demonstrate the predicted inclusivity of the 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Nucleotide mismatches in the primer/probe regions with frequencies > 0.1% are shown below. With the exception of one nucleotide mismatch with frequency > 1% (2.00%) at the third position of the N1 probe, the frequency of all mismatches was < 1%, indicating that prevalence of the mismatches were sporadic. Only one sequence (0.0032%) had two nucleotide mismatches in the N1 probe, and one other sequence from a different isolate (0.0032%) had two nucleotide mismatches in the N1 reverse primer. No sequences were found to have more than one mismatch in any N2 primer/probe region. The risk of these mismatches resulting in a significant loss in reactivity causing a false negative result is extremely low due to the design of the primers and probes, with melting temperatures > 60°C and with annealing temperature at 55°C that can tolerate up to two mismatches.

Table 7. In Silico Inclusivity Analysis of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Among 31,623 Genome Sequences Available from GISAID as of June 20, 2020

Primer/probe	N1 probe	N1 reverse		N2 probe
Location (5'>3')	3	15	21	13
Mismatch Nucleotide	C>T	G>T	T>C	C>T
Mismatch No.	632	34	71	46
Mismatch Frequency (%)	2.00	0.11	0.22	0.15

Specificity/Exclusivity Testing: In Silico Analysis

BLASTn analysis queries of the 2019-nCoV rRT-PCR assays primers and probes were performed against public domain nucleotide sequences. The database search parameters were as follows: 1) The nucleotide collection consists of GenBank+EMBL+DDBJ+PDB+RefSeq sequences, but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences and sequences longer than 100Mb; 2) The database is non-redundant. Identical sequences have been merged into one entry, while preserving the accession, GI, title and taxonomy information for each entry; 3) Database was updated on 10/03/2019; 4) The search parameters automatically adjust for short input sequences and the expect threshold is 1000; 5) The match and mismatch scores are 1 and -3, respectively; 6) The penalty to create and extend a gap in an alignment is 5 and 2 respectively.

2019-nCoV_N1 Assay:
Probe sequence of 2019-nCoV rRT-PCR assay N1 showed high sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. However, forward and reverse primers showed no sequence homology with SARS coronavirus and Bat SARS-like coronavirus genome. Combining primers and probe, there is no significant homologies with human genome, other coronaviruses or human microflora that would predict potential false positive rRT-PCR results.

2019-nCoV_N2 Assay:
The forward primer sequence of 2019-nCoV rRT-PCR assay N2 showed high sequence homology to Bat SARS-like coronaviruses. The reverse primer and probe sequences showed no significant homology with human genome, other coronaviruses or human microflora. Combining primers and probe, there is no prediction of potential false positive rRT-PCR results.

In summary, the 2019-nCoV rRT-PCR assay N1 and N2, designed for the specific detection of 2019-nCoV, showed no significant combined homologies with human genome, other coronaviruses, or human microflora that would predict potential false positive rRT-PCR results.

In addition to the *in silico* analysis, several organisms were extracted and tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel to demonstrate analytical specificity and exclusivity. Studies were performed with nucleic acids extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit. Nucleic acids were extracted from high titer preparations (typically ≥ 10^5 PFU/mL or ≥ 10^6 CFU/mL). Testing was performed using the ThemoFisher Scientific TaqPath™ 1-Step RT-qPCR Master Mix, CG on the Applied Biosystems™ 7500 Fast Dx Real-Time PCR instrument. The data demonstrate that the expected results are obtained for each organism when tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Table 8. Specificity/Exclusivity of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Virus	Strain	Source	2019-nCoV_N1	2019-nCoV_N2	Final Result
Human coronavirus	229E	Isolate	0/3	0/3	Neg.
Human coronavirus	OC43	Isolate	0/3	0/3	Neg.
Human coronavirus	NL63	clinical specimen	0/3	0/3	Neg.
Human coronavirus	HKU1	clinical specimen	0/3	0/3	Neg.
MERS-coronavirus		Isolate	0/3	0/3	Neg.
SARS-coronavirus		Isolate	0/3	0/3	Neg.
bocavirus	-	clinical specimen	0/3	0/3	Neg.
Mycoplasma pneumoniae		Isolate	0/3	0/3	Neg.
Streptococcus		Isolate	0/3	0/3	Neg.
Influenza A(H1N1)		Isolate	0/3	0/3	Neg.
Influenza A(H3N2)		Isolate	0/3	0/3	Neg.
Influenza B		Isolate	0/3	0/3	Neg.
Human adenovirus, type 1	Ad71	Isolate	0/3	0/3	Neg.
Human metapneumovirus	-	Isolate	0/3	0/3	Neg.
respiratory syncytial virus	Long A	Isolate	0/3	0/3	Neg.
rhinovirus		Isolate	0/3	0/3	Neg.
parainfluenza 1	C35	Isolate	0/3	0/3	Neg.
parainfluenza 2	Greer	Isolate	0/3	0/3	Neg.
parainfluenza 3	C-43	Isolate	0/3	0/3	Neg.
parainfluenza 4	M-25	Isolate	0/3	0/3	Neg.

Endogenous Interference Substances Studies:

The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel uses conventional well-established nucleic acid extraction methods and based on our experience with CDC's other EUA assays, including the CDC Novel Coronavirus 2012 Real-time RT-PCR Assay for the presumptive detection of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and the CDC Human Influenza Virus Real-Time RT-PCR Diagnostic Panel-Influenza A/H7 (Eurasian Lineage) Assay for the presumptive detection of novel influenza A (H7N9) virus that are both intended for use with a number of respiratory specimens, we do not anticipate interference from common endogenous substances.

Specimen Stability and Fresh-frozen Testing:

To increase the likelihood of detecting infection, CDC recommends collection of lower respiratory and upper respiratory specimens for testing. If possible, additional specimen types (e.g., stool, urine) should be collected and should be stored initially until decision is made by CDC whether additional specimen sources should be tested. Specimens should be collected as soon as possible once a PUI is identified regardless of symptom onset. Maintain proper infection control when collecting specimens. Store specimens at 2-8°C and ship overnight to CDC on ice pack. Label each specimen container with the patient's ID number (e.g., medical record number), unique specimen ID (e.g., laboratory requisition number), specimen type (e.g., nasal swabs) and the date the sample was collected. Complete a CDC Form 50.34 for each specimen submitted.

Clinical Performance:

As of February 22, 2020, CDC has tested 2071 respiratory specimens from persons under investigation (PUI) in the U.S. using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Specimen types include bronchial fluid/wash, buccal swab, nasal wash/aspirate, nasopharyngeal swab, nasopharyngeal/throat swab, oral swab, sputum, oropharyngeal (throat) swab, swab (unspecified), and throat swab.

Table 9: Summary of CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Data Generated by Testing Human Respiratory Specimens Collected from PUI Subjects in the U.S.

Specimen Type	2019 nCoV Negative	2019 nCoV Positive	Inconclusive	Invalid	Total
Bronchial fluid/wash	2	0	0	0	2
Buccal swab	5	1	0	0	6
Nasal wash/aspirate	6	0	0	0	6
Nasopharyngeal swab	927	23	0	0	950
Nasopharyngeal swab/throat swab	4	0	0	0	4
Oral swab	476	9	0	0	485
Pharyngeal (throat) swab	363	10	0	1	374
Sputum	165	5	0	0	170
Swab (unspecified)[1]	71	1	0	0	72
Tissue (lung)	2	0	0	0	2
Total	2021	49	0	1	2071

[1]Actual swab type information was missing from these upper respiratory tract specimens.

Two thousand twenty-one (2021) respiratory specimens of the 2071 respiratory specimens tested negative by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Forty-nine (49) of the 2071 respiratory specimens tested positive by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Only one specimen (oropharyngeal (throat) swab) was invalid. Of the 49 respiratory specimens that tested positive by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, seventeen (17) were confirmed by genetic sequencing and/or virus culture (positive percent agreement = 17/17, 95% CI: 81.6%-100%)

During the early phase of the testing, a total of 117 respiratory specimens collected from 46 PUI subjects were also tested with two analytically validated real-time RT-PCR assays that target separate and independent regions of the nucleocapsid protein gene of the 2019-nCoV, N4 and N5 assays. The nucleocapsid protein gene targets for the N4 and N5 assays are different and independent from the nucleocapsid protein gene targets for the two RT-PCR assays included in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel, N1 and N2. Any positive result from the N4 and/or the N5 assay was further investigated by genetic sequencing.

Performance of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel testing these 117 respiratory specimens was estimated against a composite comparator. A specimen was considered comparator negative if both the N4 and the N5 assays were negative. A specimen was considered comparator positive when the N4 and/or the N5 assay generated a positive result, and the comparator positive result(s) were further investigated and confirmed to be 2019-nCoV RNA positive by genetic sequencing.

Table 10: Percent Agreement of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel with the Composite Comparator

CDC 2019-nCoV Panel Result	**Composite Comparator Result**	
	Positive	Negative
Positive	13[1]	0
Inconclusive	0	0
Negative	0	104

[1]Composite comparator results were available for 13 of 49 CDC 2019-nCoV Panel positive specimens only.

Positive percent agreement = 13/13 = 100% (95% CI: 77.2% - 100%)
Negative percent agreement = 104/104 = 100% (95% CI: 96.4% - 100%)

Enzyme Master Mix Evaluation:

The limit of detection equivalence between the ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix and the following enzyme master mixes was evaluated: Quantabio qScript XLT One-Step RT-qPCR ToughMix, Quantabio UltraPlex 1-Step ToughMix (4X), and Promega GoTaq® Probe 1- Step RT-qPCR System. Serial dilutions of 2019 novel coronavirus (SARS CoV-2) transcript were tested in triplicate with the CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel using all four enzyme master mixes. Both manufactured versions of oligonucleotide probe, BHQ and ZEN, were used in the comparison. The lowest detectable concentration of transcript at which all replicates tested positive using the Quantabio qScript XLT One-Step RT-qPCR ToughMix and Quantabio UltraPlex 1-Step ToughMix (4X) was similar to that observed for the ThemoFisher TaqPath™ 1-Step RT-qPCR Master Mix. The lowest detectable concentration of transcript when using the Promega GoTaq® Probe 1- Step RT-qPCR System was one dilution above that observed for the other candidates when evaluated with the BHQ version of the CDC assays. The candidate master mixes all performed equivalently or at one dilution below the ThemoFisher TaqPath™ 1-Step RT-qPCR Master Mix when evaluated with the ZEN version of the CDC assays.

Table 11: Limit of Detection Comparison for Enzyme Master Mixes – BHQ Probe Summary Results

Copy Number	ThemoFisher TaqPath™ 1-Step RT-qPCR Master Mix		Quantabio qScript XLT One-Step RT-qPCR ToughMix		Quantabio UltraPlex 1-Step ToughMix (4X)		Promega GoTaq® Probe 1- Step RT-qPCR System	
	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2
10^2 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^1 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^0 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3
10^{-1} copies µL	2/3	0/3	1/3	1/3	1/3	1/3	0/3	0/3

Table 12: Limit of Detection Comparison for Enzyme Master Mixes – ZEN Probe Summary Results

Copy Number	ThemoFisher TaqPath™ 1-Step RT-qPCR Master Mix		Quantabio qScript XLT One-Step RT-qPCR ToughMix		Quantabio UltraPlex 1-Step ToughMix (4X)		Promega GoTaq® Probe 1- Step RT-qPCR System	
	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2	2019-nCoV_N1	2019-nCoV_N2
10^2 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^1 copies/µL	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
10^0 copies/µL	3/3	2/3	3/3	3/3	3/3	2/3	3/3	3/3
10^{-1} copies µL	1/3	1/3	0/3	0/3	0/3	1/3	1/3	1/3

Retrospective positive (18) and negative (17) clinical respiratory specimens were extracted using the QIAGEN EZ1 Advanced XL instrument and EZ1 DSP Virus Kit and were tested with the CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel using the Quantabio qScript XLT One-Step RT-qPCR ToughMix, Quantabio UltraPlex 1-Step ToughMix (4X), and Promega GoTaq® Probe 1- Step RT-qPCR System master mixes. All three enzyme master mixes performed equivalently, demonstrating 100% positive and 100% negative agreement with expected results and a 95% confidence interval of 82.4%-100% and 81.6%-100%, respectively.

Table 13: Clinical Comparison – Retrospective Study Summary Results

CDC 2019-nCoV Real-time RT-PCR Diagnostic Panel Result	Quantabio qScript XLT One-Step RT-qPCR ToughMix		Quantabio UltraPlex 1-Step ToughMix (4X)		Promega GoTaq® Probe 1-Step RT-qPCR System	
	Positive	Negative	Positive	Negative	Positive	Negative
Positive	18	0	18	0	18	0
Negative	0	17	0	17	0	17

Roche MagNA Pure 24 and MagNA Pure 96 Extraction Platform Evaluation:

Performance of the 2019-CoV Real-time RT-PCR Diagnostic Panel using the Roche MagNA Pure 24 and MagNA Pure 96 extraction platforms was compared to performance with an authorized extraction method. Serial dilutions of quantified inactivated SARS-CoV-2 virus (USA-WA1/2020; 100 RNA copies/µL) in lysis buffer were added to pooled negative upper respiratory tract specimen matrix. Five samples of each dilution were extracted in parallel with the QIAGEN EZ1 Advanced XL (EZ1 DSP Virus Kit Cat# 62724) and the Roche MagNA Pure 24 (MagNA Pure 24 Total NA Isolation Kit Cat# 07658036001) and Roche MagNA Pure 96 (MagNA Pure 96 DNA and Viral Nucleic Acid Small Volume Kit Cat# 06543588001) extraction platforms and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix. The observed LoD was defined as the lowest concentration at which 100% (5 out of 5 total) of all replicates tested positive for both primer/probe sets (N1 and N2) in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. The acceptance criteria for equivalence were defined as demonstrating an observed LoD either at the same endpoint or within a 3-fold dilution. The results showed that both the MagNA Pure 24 and MagNA Pure 96 extraction platforms performed equivalently or within one 3-fold dilution of the LoD observed when using the QIAGEN EZ1 Advanced XL extraction platform.

Table 14. Limit of Detection Comparison between the QIAGEN EZ1 Advanced XL, Roche MagNA Pure 96, and Roche MagNA Pure 24 Extraction Platforms using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Platform	Parameter	2019-nCoV_N1 Assay			2019-nCoV_N2 Assay			Observed LoD[1]
QIAGEN EZ1 Advanced XL	RNA copies/µL	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{0.5}$
	# pos./total	5/5	5/5	5/5	5/5	5/5	3/5	
	Mean Ct[2]	34.0	35.0	36.3	33.9	36.6	NA	
	Std. Deviation	0.2	0.8	0.2	0.4	0.9	NA	
Roche MagNA Pure 96	RNA copies/µL	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{0.5}$
	# pos./total	5/5	5/5	5/5	5/5	5/5	2/5	
	Mean Ct[2]	33.3	34.6	36.1	33.2	35.7	NA	
	Std. Deviation	0.5	0.5	0.3	0.3	0.4	NA	
Roche MagNA Pure 24	RNA copies/µL	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{1.0}$
	# pos./total	5/5	3/5	3/5	5/5	5/5	5/5	
	Mean Ct[2]	34.4	NA	NA	35.2	36.9	36.2	
	Std. Deviation	0.6	NA	NA	0.5	1.0	0.8	

[1]Concentration is presented in RNA copies/µL. The observed LoD is the lowest concentration where both assays showed 100% positive detection.
[2]Mean Ct reported for dilutions that show 100% positivity. Calculations only include positive results.
NA = not applicable

Previously characterized clinical remainder specimens (14 positive and 15 negative) were extracted using both the Roche MagNA Pure 96 and MagNA Pure 24 extraction platforms and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix. Acceptance criteria for clinical equivalence was defined as demonstrating 100% concurrence with qualitative results shown with the authorized comparator method (QIAGEN EZ1 Advanced XL). Results from this study showed 100% concurrence with the comparator method for both the Roche MagNA Pure

96 and Roche MagNA Pure 24 extraction platforms when used with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic panel.

Table 15. Clinical Comparison Results – Retrospective Study Results

Test Platform	Test Platform Result	QIAGEN EZ1 Advanced XL Result		Positive % Agreement (CI)[1]	Negative % Agreement (CI)[1]
		Positive	Negative		
Roche MagNA Pure 96	Positive	14	0	100.0 (78.5 – 100.0)	100.0 (79.6 – 100.0)
	Negative	0	15		
Roche MagNA Pure 24	Positive	14	0	100.0 (78.5 – 100.0)	100.0 (79.6 – 100.0)
	Negative	0	15		

[1] CI = 95% confidence interval

Promega Maxwell® RSC 48 Extraction Platform Evaluation:

Performance of the 2019-CoV Real-time RT-PCR Diagnostic Panel using the Promega Maxwell® RSC 48 extraction platform was compared to performance with an authorized extraction method. Serial dilutions of quantified inactivated SARS-CoV-2 virus (USA-WA1/2020; 100 RNA copies/µL) in VTM were added to pooled negative upper respiratory tract specimen matrix. Five samples of each dilution were extracted in parallel with the QIAGEN EZ1® Advanced XL (EZ1 DSP Virus Kit Cat# 62724) and the Promega Maxwell® RSC 48 (Promega Maxwell® Viral Total Nucleic Acid Purification Kit Cat# AS1330) extraction platforms and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix. The observed LoD was defined as the lowest concentration at which 100% (5 out of 5 total) of all replicates tested positive for both primer/probe sets (N1 and N2) in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. The acceptance criteria for equivalence were defined as demonstrating an observed LoD either at the same endpoint or within a 3-fold dilution. The results showed that the performance of the Maxwell® RSC 48 extraction platform performed equivalently or within one 3-fold dilution of the LoD observed when using the QIAGEN EZ1® Advanced XL extraction platform.

Table 16. Limit of Detection Comparison Between the QIAGEN EZ1® Advanced XL and Promega Maxwell® RSC 48 Extraction Platforms Using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel

Platform	Parameter	2019-nCoV_N1 Assay			2019-nCoV_N2 Assay			Observed LoD[1]
QIAGEN EZ1® Advanced XL	RNA copies/µL	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.0}$
	# pos./total	5/5	5/5	0/5	5/5	5/5	3/5	
	Mean Ct[2]	32.27	33.80	NA	35.13	36.41	NA	
	Std. Deviation	0.81	0.40	NA	0.81	0.40	NA	
Promega Maxwell® RSC 48	RNA copies/µL	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{0.0}$
	# pos./total	5/5	5/5	3/5	5/5	5/5	5/5	
	Mean Ct[2]	31.11	32.97	NA	31.89	33.95	35.17	
	Std. Deviation	0.24	0.34	NA	0.24	0.35	0.65	

[1]Concentration is presented in RNA copies/µL. The observed LoD is the lowest concentration where both assays showed 100% positive detection.

[2]Mean cycle threshold (Ct) reported for dilutions that show 100% positivity. Calculations only include positive results.

NA = not applicable

Previously characterized clinical remainder specimens (15 positive and 15 negative) were extracted using the Promega Maxwell® RSC 48 extraction platform alongside the currently authorized QIAGEN EZ1® Advanced XL extraction platform and evaluated using the 2019-nCoV Real-Time RT-PCR Diagnostic Panel and ThermoFisher TaqPath™ 1-Step RT-qPCR Master Mix. Results from the Maxwell® RSC 48 were compared with the QIAGEN EZ1® Advanced XL extraction performed in parallel showing 100% (15/15) qualitative concurrence on positive samples and 93.3% (14/15) qualitative concurrence on negative samples. This evaluation showed that two originally negative (QIAGEN QIAamp® DSP Viral RNA Mini Kit) specimens (Specimens 16 and 24) yielded an inconclusive result after extraction using the QIAGEN EZ1® Advanced XL. Repeat of the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel resolved one of the two specimens (Specimen 24, negative result). The second specimen (Specimen 16) remained inconclusive. Both these specimens yielded a negative result on the Maxwell® RSC 48.

Table 17. Clinical Comparison Results – Retrospective Study Results

Test Platform		Promega Maxwell® RSC 48			Positive % Agreement (CI)[1]	Negative % Agreement (CI)[1]
	Result	Positive	Negative	Inconclusive		
QIAGEN EZ1® Advanced XL	Positive	15	0	0	100.0 (79.6-100.0)	93.3 (70.2-98.9)
	Negative	0	14	0		
	Inconclusive	0	1	0		

[1] CI = 95% confidence interval

Disposal

Dispose of hazardous or biologically contaminated materials according to the practices of your institution.

References

1. Ballew, H. C., *et al*. “Basic Laboratory Methods in Virology,” DHHS, Public Health Service 1975 (Revised 1981), Centers for Disease Control and Prevention, Atlanta, Georgia 30333.

2. Clinical Laboratory Standards Institute (CLSI), “Collection, Transport, Preparation and Storage of Specimens for Molecular Methods: Proposed Guideline,” MM13-A

3. Lieber, M., *et al*. "A Continuous Tumor Cell Line from a Human Lung Carcinoma with Properties of Type II Alveolar Epithelial Cells." *International Journal of Cancer* 1976, 17(1), 62-70.

Revision History

Revision #	Effective Date	Summary of Revisions
1	February 4, 2020	Original Instructions for Use
2	March 15, 2020	• Intended use update • Removal of N3 primer and probe set from Diagnostic Panel • Performance data update • Addition of alternative nucleic acid extraction platforms • Addition of acceptable alternatives to HSC and addition of QIAGEN RUO extraction reagents • Positive results no longer presumptive. No confirmation of positive results required
3	March 30, 2020	• Addition of alternative enzyme master mix options
4	June 12, 2020	• Addition of MagNA Pure 24 extraction method • Addition of performance data for the MagNA Pure 96 extraction method with SARS-CoV-2 • Addition of heat treatment alternative to specimen extraction • Addition of Roche and QIAGEN external lysis buffer alternatives • Acknowledgment of FDA policy permitting end users to qualify alternative components without seeking an EUA or EUA amendment
5	July 13, 2020	• Addition of Promega Maxwell® RSC 48 extraction method • Update to *in silico* inclusivity analyses

Contact Information, Ordering, and Product Support

For technical and product support, contact the CDC Division of Viral Diseases directly.

Send email to: respvirus@cdc.gov

Note: If your laboratory is using reagents sourced from someone other than the CDC International Reagent Resource, please refer to the manufacturer's instructions provided with the commercial materials.

Appendix A: Heat Treatment Alternative to Extraction
UltraPlex 1-Step ToughMix (4X)

This procedure is only for use by public health laboratories.

Purpose:

In response to a global shortage of nucleic acid extraction reagents causing significant delays in testing, the CDC has investigated the use of a heat treatment method requiring minimal reagents as a specimen processing alternative to nucleic acid extraction for use with the 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Where possible, laboratories should use qualified RNA or total nucleic acid extraction methods for processing of specimens for subsequent testing by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel. Extraction removes inhibitory substances from specimens that could negatively impact PCR performance.

This procedure for use of heat treatment for specimen processing is only recommended when a shortage of qualified extraction reagents is a limiting factor in a laboratory's ability to meet urgent COVID-19 testing demand.

Precautions/Warnings/Limitations:

- CDC has evaluated this heat treatment process and has determined that this process is effective for inactivation of SARS-CoV-2 in patient specimens.
- Performance was evaluated with only upper respiratory specimens. Heat treatment of lower respiratory specimens for subsequent testing by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel has not been evaluated.
- This procedure for heat treatment of specimens is only for use with the Quantabio UltraPlex 1-Step ToughMix (4X).
- Heat treatment should only be conducted when a lab is ready to test the specimens by PCR. Testing of heat-treated specimens must be conducted the same day.

Acceptable Specimens:

- Upper respiratory specimens
  Note: Do not use heat treatment to process specimens that appear bloody or that contain particulate matter. Such specimens should be extracted using a qualified RNA or TNA extraction method prior to testing.

Materials Required (not provided):

- 70% ethanol
- 10% bleach, freshly prepared
- 96-well PCR reaction plates (Applied Biosystems catalog # 4346906, 4366932, 4346907, or equivalent)
- Optical strip caps (Applied Biosystems 4323032, or equivalent)
- 1.5 mL Sarstedt tubes or equivalent

- Aerosol resistant micropipette tips
- Micropipettes
- 96-well cold block
- Cold blocks for 1.5 mL - 2.0 mL tubes
- Vortex mixer
- 96-well plate centrifuge or equivalent
- Thermal cycler or equivalent
- Class II Biological Safety Cabinet (BSC)

Procedure:

Sample Preparation

1) Decontaminate BSC with 10% bleach followed by 70% ethanol.
2) If samples are frozen, thaw on ice or at 4°C. Wipe the outside of the sample tube with 70% ethanol. Place thawed sample on cold rack or ice in BSC.
3) Pulse vortex each sample and briefly spin down in a centrifuge to collect the liquid at the bottom of the tube.

Heat Treatment

1) Place a thermal cycler in the BSC, turn on, and program for 95°C for 1 min followed by 4°C hold.
2) Place a 96-well PCR plate onto a cold rack or ice in the BSC.
3) Transfer 100 µL of each sample to the 96-well PCR plate and securely cap each well using optical strip caps.
   NOTE: Ensure that an HSC extraction control is included in each batch run as required under CLIA.
4) Place this 96-well PCR plate on the pre-heated thermal cycler and start run. Leave plate on thermal cycler at 4°C, or place on ice or a cold block.
5) Remove plate and centrifuge for 1 minute at 500 x g to pellet cellular debris.
6) Place plate on a cold rack or ice and proceed to testing the supernatant by rRT-PCR.
7) Testing of heat-treated specimens must be conducted the same day heat treatment is performed. For long term storage, keep the original specimen at ≤-70°C.

Special Testing Considerations for Heat Treated Specimens:

- **Enzyme Master Mix**
  Testing of specimens that have been processed with heat treatment should be conducted with the **Quantabio UltraPlex 1-Step ToughMix (4X)**, which demonstrated the best performance with heat treated specimens. PCR testing of heat-treated specimens should follow the instructions in the main body of this Instructions for Use document.
- **Resolution of Inconclusive and Invalid Results**
  Retesting of heat-treated specimens that generated an inconclusive or invalid result must include extraction of the original specimen with a qualified RNA or total nucleic acid (TNA) extraction method, if available. Do not re-test the heat-treated specimen material to resolve inconclusive or invalid test results.

Verification:

CDC recommends performance of verification studies for the heat treatment method prior to diagnostic use that includes side-by-side preparation of a panel of positive and negative clinical specimens using a qualified extraction method and this heat treatment method with subsequent testing by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.

Performance Characteristics:

Quantabio UltraPlex 1-Step ToughMix (4X)

Limit of Detection Comparison

Serial dilutions of inactivated SARS-CoV-2 [SARS-CoV-2 USA-WA1/2020] were prepared in simulated specimen material (human A549 cells suspended in viral transport medium). Each concentration was prepared side-by-side five times by both EZ1 extraction and by heat treatment. Each extracted or heat-treated sample was subsequently tested by the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel using the Quantabio UltraPlex 1-Step ToughMix (4X) on the Applied Biosystems 7500 Fast Dx instrument. Observed detection was similar between the two specimen preparation methods.

Table B1: UltraPlex Limit of Detection Comparison between QIAGEN EZ1 Advanced XL extraction and heat treatment (95°C for 1 min) method – Summary Results

Enzyme	Platform	Parameter	2019-nCoV_N1 Assay					2019-nCoV_N2 Assay					Observed LoD[1]
Quantabio UltraPlex 1-Step ToughMix (4X) 5 µL Template Addition	QIAGEN EZ1 Advanced XL	RNA copies/µL	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$	$10^{0.5}$
		# pos./total	5/5	5/5	4/5	4/5	3/5	5/5	5/5	5/5	2/5	2/5	
		Mean Ct[2]	34.11	34.59	NA	NA	NA	32.97	33.76	34.70	NA	NA	
		Std. Deviation	0.75	0.99	NA	NA	NA	0.33	0.72	0.98	NA	NA	
	Heat Treatment 95°C for 1 min	RNA copies/µL	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$	$10^{1.0}$	$10^{0.5}$	$10^{0.0}$	$10^{-0.5}$	$10^{-1.0}$	$10^{0.5}$
		# pos./total	5/5	5/5	4/5	5/5	1/5	5/5	5/5	4/5	2/5	1/5	
		Mean Ct[2]	33.41	34.32	NA	36.73	NA	33.45	35.25	NA	NA	NA	
		Std. Deviation	0.62	0.40	NA	0.82	NA	0.40	0.80	NA	NA	NA	

[1]Concentration is presented in RNA copies/µL. The observed LoD is the lowest concentration where both assays showed 100% positive detection.

[2]Mean Ct reported for dilutions that show 100% positivity. Calculations only include positive results.

NA = not applicable

Clinical Comparison

A panel of 39 upper respiratory specimens were tested side-by-side using extraction with the Qiagen EZ1 extraction instrument and heat treatment. Extracted and heat-treated specimens were subsequently tested with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel using the Quantabio UltraPlex 1-Step ToughMix (4X). Qualitative results were compared to demonstrate agreement.

Table B2: Clinical Comparison Results Summary – Heat Treatment versus QIAGEN EZ1 Advanced XL

	Test Result	Heat Treatment			Total	Positive % Agreement (CI)[1]	Negative % Agreement (CI)[1]
		Positive	Inconclusive	Negative			
QIAGEN EZ1 Advanced XL	Positive	18	1	0	19	94.7 (75.4-99.1)	100 (83.9-100)
	Inconclusive	0	0	0	0		
	Negative	0	0	20	20		
	Total	18	1	20	39		

[1] CI = 95% confidence interval

Questions and Comments:

If you have questions or comments about this procedure, please send by email to: respvirus@cdc.gov

****** DO NOT DISCARD: Important product-specific information ******

CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel – Verification Requirements

Please consult the following guidance from the Centers for Medicare & Medicaid Services (CMS) regarding diagnostic tests under Emergency Use Authorization (EUA): https://www.cms.gov/Medicare/Provider-Enrollment-and-Certification/SurveyCertificationGenInfo/Policy-and-Memos-to-States-and-Regions-Items/QSO18-19-CLIA

INTENDED USE

The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is a real-time RT-PCR test intended for the qualitative detection of nucleic acid from the 2019-nCoV in upper and lower respiratory specimens (such as nasopharyngeal or oropharyngeal swabs, sputum, lower respiratory tract aspirates, bronchoalveolar lavage, and nasopharyngeal wash/aspirate or nasal aspirate) collected from individuals who meet 2019-nCoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with 2019-nCoV infection, contact with a probable or confirmed 2019-nCoV case, history of travel to a geographic locations where 2019-nCoV cases were detected, or other epidemiologic links for which 2019-nCoV testing may be indicated as part of a public health investigation). Testing in the United States is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.

Results are for the identification of 2019-nCoV RNA. The 2019-nCoV RNA is generally detectable in upper and lower respiratory specimens during infection. Positive results are indicative of active infection with 2019-nCoV but do not rule out bacterial infection or co-infection with other viruses. The agent detected may not be the definite cause of disease. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.

Negative results do not preclude 2019-nCoV infection and should not be used as the sole basis for treatment or other patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

Testing with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is intended for use by trained laboratory personnel who are proficient in performing real-time RT-PCR assays. The CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel is only for use under a Food and Drug Administration's Emergency Use Authorization.

REQUIRED MATERIALS

The 2019 novel coronavirus positive control (nCoVPC) is provided with the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel and should be prepared according to the Instructions for Use. The nCoVPC consists of an RNA transcript of the 2019-nCoV N gene as well as human RNase P gene segment. nCoVPC will yield a positive result with the following primer and probe sets: 2019-nCoV_N1, 2019-nCoV_N2, and RP.

Approximately 2 mL of an upper respiratory specimen (e.g. nasopharyngeal swab (NPS) in transport media) are needed for testing. Specimens may be pooled if less than 2 mL of one specimen is available.

Refer to CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel package insert (manufacturer instructions) for additional reagents, materials, and instructions.

PRECAUTIONS

This reagent should be handled in an approved biosafety level 2 (BSL-2) handling area to avoid contamination of laboratory equipment and reagents that could cause false positive

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results. This product is an RNA transcript and is non-infectious. However, the nCoVPC should be handled in accordance with Good Laboratory Practices.

Store reagent at appropriate temperatures (see Instructions for Use) and hold on ice when thawed.

Please use standard precautions when handling respiratory specimens.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE QIAamp® DSP VIRAL RNA MINI KIT OR THE QIAamp® VIRAL RNA MINI KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 560 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 140 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 14 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 14 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 14 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE QIAGEN EZ1® ADVANCED XL

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 280 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 120 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE TOTAL NUCLEIC ACID KIT OR THE ROCHE MagNA PURE NUCLEIC ACID ISOLATION KIT I

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 300 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.

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- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE 24 AND TOTAL NUCLEIC ACID ISOLATION KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 400 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE ROCHE MagNA PURE 96 DNA AND VIRAL NA SMALL VOLUME KIT

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 350 µL of lysis buffer into each of nine tubes labeled 1-9.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE PROMEGA MAXWELL® RSC 48

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 330 µL of lysis buffer (300 µL of lysis buffer + 30 µL Proteinase K, included in the kit) into each of nine tubes labeled 1-9.

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- Add 120 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

INSTRUCTIONS FOR PREPARING SAMPLES BEFORE EXTRACTION WITH THE BIOMÉRIEUX NucliSENS easyMAG OR THE BIOMÉRIEUX EMAG

- Refer to the 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for reconstitution of the materials for use. RNA should be kept cold during preparation and use.
- Make a 1/10 dilution of nCoVPC by adding 5 µL of nCoVPC into 45 µL of nuclease-free water or 10 mM Tris.
- Aliquot 1000 µL or 2000 µL of pre-aliquoted easyMAG lysis buffer into each of nine tubes labeled 1-9 for the easyMAG or eMAG, respectively.
- Add 100 µL of upper respiratory specimen (e.g. NPS in viral transport media) into each of the nine labeled tubes with lysis buffer.
- To prepare samples at a moderate concentration, spike 12 µL of undiluted nCoVPC (rehydrated as described in the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use) into each tube labeled 1-3 containing lysis buffer and specimen.
- To prepare samples at a low concentration, spike 12 µL of 1/10 dilution of nCoVPC into each tube labeled 4-6 containing lysis buffer and specimen.
- To prepare negative samples, spike 12 µL of nuclease-free water into each tube labeled 7-9 containing lysis buffer and specimen.
- Perform extractions of all nine samples according to the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use.

PROCEDURE

Follow the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Instructions for Use for testing the nine extracted samples at least once.

EXPECTED RESULTS

Moderate nCoVPC samples should be positive for 2019-nCoV.
Low nCoVPC samples should be positive for 2019-nCoV.
Negative upper respiratory samples should be negative for 2019-nCoV.

≥90% of test results should be in agreement with the expected results. If test results are <90% in agreement with expected results, contact CDC at respvirus@cdc.gov.

QUESTIONS

Please send questions or comments by email to respvirus@cdc.gov.

DISTRIBUTION

Distributed to qualified laboratories by Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA, 30329 USA

I TAMPONI COVID-19 PRODUCONO FINO AL 95% DI FALSI POSITIVI : CERTIFICATO DALL'ISTITUTO SUPERIORE DI SANITÀ

Con l'analisi più completa sui tamponi Covid-19

Dr. Stefano Scoglio, Ph.D., B.Sc.

Ho già scritto alcuni post e articoli su come i tamponi e i test sierologici per il Covid-19 siano inaffidabili, di fatto senza alcun significato perché senza nessun vero legame con un presunto virus SARS-Cov2, che non è mai stato isolato.

Abbiamo anche visto come tale inaffidabilità sia stata addirittura certificata dalla Commissione Europea e dall'Istituto Superiore di Sanità, che nell'Aprile-Maggio scorso hanno pubblicato documenti dove affermavano che in Europa circolavano 78 tamponi diversi, di cui nessuno validato da organismi indipendenti, nessuno valutato o autorizzato preventivamente, e addirittura la stragrande maggioranza dei quali non dichiarava neppure quali sequenze geniche utilizzasse, e quindi potenzialmente contenenti qualsiasi cosa. A questo punto ho voluto approfondire la cosa, e ho scoperto ulteriori elementi, sia scientifici che legali.

La situazione normativo-regolatoria

Innanzitutto, va detto che i tamponi rientrano nella nuova normativa REGOLAMENTO (UE) 2017/746 DEL PARLAMENTO EUROPEO E DEL CONSIGLIO del 5 aprile 2017 relativo ai dispositivi medico-diagnostici in vitro e che abroga la direttiva 98/79/CE.

Nella normativa precedente abrogata, in generale bastava l'apposizione del marchio CE, che è un marchio relativo soprattutto alla sicurezza; e solo per alcuni dispositivi diagnostici in vitro elencati nell'Allegato II, e aventi a che fare con i virus già conosciuti (HIV 1 e 2, HTLV I e II e dell'epatite B, C e D), si richiede la valutazione tecnica e di efficacia da parte di un Organismo Notificato, ovvero un organismo di validazione riconosciuto dalla EU. Ora, sappiamo dal Documento della Commissione Europea del 16 Aprile scorso che nessuno dei 78 modelli di test tampone in circolazione a quella data sono stati valutati o sottoposti a qualsiasi organismo di valutazione riconosciuto, e che addirittura ciò non sarebbe stato neppure possibile dato che quasi nessuno di quei 78 tamponi mette a disposizione una adeguata scheda tecnica, inclusa la specifica delle sequenze geniche utilizzate. Come è possibile? In fondo, il SARS Cov2 dovrebbe essere un virus anche più importante di quelli dell'epatite o dell'HIV, che non hanno mai portato alla chiusura dell'economia e della vita sociale di intere nazioni. E' possibile perché il Regolamento della Direttiva 98/79 CE elenca solo i virus suddetti, ed essendo il SARS Cov 2 un nuovo virus non è incluso.

Già, ma abbiamo appena visto che tale regolamento è stato abrogato dal regolamento del 2017, che a sua volta pone requisiti ancora più stringenti di quello precedente, richiedendo valutazioni preliminari di efficacia da parte di organismi di validazione riconosciuti per tutti i dispositivi diagnostici in vitro in cui rientrano anche i tamponi Covids-19. E allora perché sono stati autorizzati in commercio test tampone privi di qualsiasi validazione o anche solo valutazione preliminare, e addirittura privi delle specifiche sulle sequenze geniche utilizzate?

Perché l'Italia ha fatto scuola, e il motto "fatta la legge trovato l'inganno" è diventato motto europeo: **il Regolamento 2017/46 del 5 Aprile 2017 entrerà in vigore, per i dispositivi diagnostici in vitro, solo il 26 Maggio 2022!** E con questo i tamponi Covid-19 hanno goduto dell'interregno, non essendo inclusi, in quanto relativi a un virus nuovo, nel Regolamento del '98; e non essendo ancora sottoposti a un Regolamento del 2017 che li avrebbe messi tutti fuori legge, ma che non entrerà in vigore se non a metà del 2022!

La domanda che occorre porsi, e che non può non avere rilevanza anche giuridica, è: questi tamponi sono del tutto privi di valutazione e validazione, e sono in circolazione solo grazie al fatto che si è creato un vuoto normativo tra Regolamento del 1998, che limitava la lista dei virus solo a quelli conosciuti (ma che per analogia dovrebbe applicarsi anche ai nuovi emergenti) e Regolamento del 2017, che abroga quello del '98 ma non entra in vigore solo nel 2022; **se insomma questi tamponi Covid-19 sono utilizzati solo grazie ad una anomalia legislativa, e nel 2022 sarebbero del tutto illegali; è ammissibile che a tali tamponi, in vita per puro miracolo o distorsione giuridica, si affidino le sorti di intere nazioni e dell'intera economia mondiale?** Ovviamente no, non dovrebbe essere ammissibile, e se lo sarà, sarà solo perché la forma giuridica viene fatta prevalere sulla sostanza giuridica.

Veniamo però alla sostanza scientifica dei tamponi. Il primo argomento è che sono del tutto senza significato perché il virus non è mai stato isolato, e dunque non esiste nessun marker realistico che ne supporti l'azione. Questo è discorso che ho affrontato in dettaglio altrove; ma sembra che su questo punto le orecchie di chi dovrebbe intervenire tendono a restare chiuse (anche se noi continueremo a gridare la verità). Facciamo dunque finta che non sia questo il problema, che il virus sia stato isolato. Vedremo che anche da questo punto di vista, i tamponi restano del tutto inaffidabili e privi di significato.

La questione della mutazione del virus

Uno dei problemi fondamentali è la continua mutazione del virus. Come scrive lo stesso Istituto Superiore di Sanità (confermando quello che vado dicendo da sempre):

> "…il virus infatti può mutare e nuove sequenze nucleotidiche depositate nelle banche dati possono rivelare se queste mutazioni possano a loro volta rendere un particolare test meno efficace o **addirittura inefficace**…È importante puntualizzare che **per la diagnostica di questo virus emergente, con uno stato dell'arte in evoluzione**, **le reali prestazioni del dispositivo osservate possano differire rispetto a quelle determinate dallo studio iniziale delle prestazioni condotto dal fabbricante ai fini della marcatura CE**, in uno stato dell'arte precedente." [1]

[1] Gruppo di Lavoro ISS Test Diagnostici COVID-19, *Dispositivi diagnostici in vitro per COVID-19. Parte 2: evoluzione del mercato e informazioni per gli stakeholder ,* Rapporto ISS COVID-19 n. 46/2020, 23 Maggio 2020, p. 8

Come ho sempre sostenuto anch'io: se al GISAID, dove si raccolgono le sequenze geniche del SARS-Cov 2, ci sono oltre 70.000 sequenze diverse, e aumentano costantemente, **che valore ha un tampone messo a punto nel febbraio 2020 e utilizzato nel Luglio 2020, quando il virus era certamente modificato**?

Per capire ciò, basterebbe dire che la gran parte dei tamponi in circolazione sono stati strutturati (se lo sono stati) sul virus sequenziato dai Cinesi a Wuhan. Ma in Italia sono stati sia lo Spallanzani che il San Raffaele a fornire sequenziamenti genici diversi, ed entrambi, oltre a pseudo-isolare il virus con le stesse metodiche farlocche che ho descritto altrove[2], hanno subito messo in chiaro che si trattava di virus modificati rispetto a quello isolato in Cina[3]; e in uno studio organizzato da diversi centri medici italiani (Sacco, San Raffaele, etc.), quando hanno analizzato 59 campioni di liquido da pazienti Covid-19 da diversi centri del Centro e Nord Italia, hanno trovato una notevole mutazione, al punto da trovare :

> "*A mean of 6 nucleotide substitutions per viral genome was observed, without significant differences between synonymous and non-synonymous mutations, indicating genetic drift as a major source for virus evolution."*[4]
>
> *"Una media di 6 sostituzioni nucleotidiche per ogni genoma virale, senza differenze significative tra mutazioni sinonime e non sinonime, delineando così una deriva genetica come importante fonte dell'evoluzione del virus."*

Da questo studio si evince che non solo il virus muta da continente a continente, da nazione a nazione, ma addirittura da provincia a provincia, e di fatto da persona a persona! Ci sono dunque 7 miliardi di virus diversi che solo si assomigliano? Esiste un virus talmente magico da incorporare 7 miliardi di mutazioni? E soprattutto: a cosa serve, in questo quadro, un test tampone universale, che ha solo una o al massimo 3 sequenze geniche?

Come afferma lo stesso ISS, "…queste mutazioni possano a loro volta rendere un particolare test meno efficace o addirittura inefficace", e tuttavia nessuno, tra le autorità politiche o giuridiche, si preoccupa di verificare se i tamponi che sostengono e mantengono la pseudo-pandemia, siano o no corrispondenti alle innumerevoli mutazioni di questo super-virus!

La costante mutazione del SARS-Cov2, tale da renderlo di fatto irriconoscibile, è stata confermata anche a livello internazionale: un articolo americano, che include anche Robert Gallo tra gli autori, ha riscontrato decine di mutazioni crescenti nel tempo in parallelo con la presunta diffusione del virus

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[2] https://www.byoblu.com/2020/09/12/lo-studio-in-esclusiva-su-byoblu-virus-mai-isolato-una-dittatura-basata-su-tamponi-non-convalidati-stefano-scoglio-candidato-premio-nobel-per-la-medicina-nel-2018/

[3] Capobianchi M.R. et al., *Molecular characterization of SARS-CoV-2 from the first case of COVID-19 in Italy*, Clin Microbiol Infect, 2020 Jul;26(7):954-956.

[4] Lai A. et al., *Molecular Tracing of SARS-CoV-2 in Italy in the First Three Months of the Epidemic,* Viruses 2020, 12, 798; doi:10.3390/v12080798.

dall'Asia all'Europa agli USA[5]; mentre un autore asiatico ha analizzato 85 diverse [6]sequenze genomiche SARS-Cov2 disponibili presso GISAID, e ha trovato ben 53 diversi ceppi SARS-Cov2 provenienti da varie aree della Cina, dell'Asia, dell'Europa e degli Stati Uniti.[6]

Insomma, **se il virus muta costantemente, allora il test tampone è inutile, perché va a cercare un virus sempre precedente** e sempre diverso rispetto a quello attualmente in circolazione. Basterebbe questo da solo per capire che **il tampone Covid-19 il test è completamente, al 100%, fallace!**

Questo è davvero ciò che accade nella realtà. Il "Drosten PCR Test" e il test dell'Institute Pasteur, i due test considerati i più affidabili (sebbene nessuno dei due lo sia stato convalidato esternamente), entrambi utilizzano un test del gene E, anche se il test di Drosten lo utilizza come test preliminare, mentre l'Institut Pasteur lo utilizza come test definitivo. Secondo gli autori del Drosten test, il test E-gene è in grado di rilevare tutti i virus asiatici, essendo così al contempo molto aspecifico (tutti i ceppi viruali) e limitato ad un'area geografica (Asia). Ancora, il test I**nstitute Pasteur, uno dei più adottati in Europa, utilizza il test E-Gene come test finale**, anche se è ormai noto che il virus (o virus) SARS-Cov2 che si ritiene circolino in Europa sarebbero diversi da quelli asiatici. E poi ad aprile, **l'OMS ha cambiato l'algoritmo "... raccomandando che da ora in poi un test può essere considerato positivo anche se solo il dosaggio del gene E** (che probabilmente rileverà tutti i virus asiatici!) dà un risultato positivo".[7] Insomma, **per OMS ed epigoni, tutto fa brodo pur di mantenere la tragica farsa della pandemia**!

La questione dei cicli (runs) della RT-PCR

Un'altro grave problema dei tamponi, che utilizzano la metodica della RT-PCR, è che l'affidabilità di tale metodica dipende dal numero di cicli (replicazioni) che vengono usati per trovare il virus SARS-Cov2. Prof. Stephen Bustin, una delle autorità mondiali di PCR, ha scritto in un recente articolo relativamente alla identificazione della presenza di SARS-Cov 2:

> "…the most widely used method is quantitative fluorescence-based reverse transcription polymerase chain reaction (RT-qPCR). Despite its ubiquity, there is a significant amount of uncertainty about how this test works, potential throughput and reliability."[8]

> "…il metodo più utilizzato è la Reazione a catena delle polimerasi quantitativa a trascrizione inversa basata sulla fluorescenza (RT-qPCR).

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[5] Pachetti M. et al., *Emerging SARS-CoV-2 mutation hot spots include a!novel RNA-dependent RNA polymerase variant*, J Transl Med (2020) 18:179 https://doi.org/10.1186/s12967-020-02344-6.

[6] Phan Tung, *Genetic diversity and evolution of SARS-CoV-2*, Infection, Genetics and Evolution, 81 (2020), 104260.

[7] Engelbrecht T, Demeter K., COVID19 PCR Tests are Scientifically Meaningless, Jun 27 2020, p.21. https://off-guardian.org/2020/06/27/covid19-pcr-tests-are-sc

[8] Bustin S.A, Nolan T., *RT-qPCR Testing of SARS-CoV-2: A Primer,* Int. J. Mol. Sci. 2020, 21, 3004; doi:10.3390/ijms21083004, p. 1

Nonostante la sua ubiquità, c'è un significativo livello di incertezza su come funziona questo test, sulla sua potenziale produzione e affidabilità."

Probabilmente questo è dovuto anche e soprattutto alla questione dei cicli di PCR che vengono normalmente effettuati coi tamponi. In una intervista al compianto David Crow, prezioso ricercatore canadese, Bustin afferma:

"…the cycle number per se is not a good measure…most instruments, when you get above a cycle number of 35, then you start worrying about the reliability of your result…so, you want to be sure that your results are within the 20 to 30 cycles…"

"…il numero di cicli di per sé non è una buona misura…la maggioranza degli strumenti, **quando sali oltre il numero di 35 cicli, cominci a preoccuparti sull'affidabilità dei tuoi risultati**…quindi, vuoi assicurarti **che i tuoi risultati siano prodotti dai 20 a un massimo di 30 cicli**…".

E dato che la maggioranza dei tamponi sale fino e oltre i 40 cicli, Crow domanda a Bustin:

"…if you get up to 40 cycles, you could get a ghost, the PCR could string bases together casually…"

"…se sali a 40 cicli, potresti produrre un fantasma, la PCR può iniziare a raccordare assieme basi nucleotidiche in modo casuale…"

E Bustin risponde: "I would be very unhappy about 40 cycles…";

"Sarei molto scontento a 40 cicli…". [9]

Vediamo quindi quanti cicli vengono normalmente usati nei tamponi. Forse vi ricordate della recente polemica, alimentata dal dr. Remuzzi del San Raffaele, per cui i tamponi che trovano il virus solo con un'alto numero di cicli si riferiscono a casi di bassissima viralità, considerata non infettiva:

"Remuzzi riferisce che la positività nei tamponi dello studio del Mario Negri emergeva solo dopo 34-38 cicli di amplificazione. Ma più si amplifica, più il segnale si fa debole e incerto, facendo pensare a tracce di Rna virale ormai residuali e inattive. Niente infezione, insomma."[10]

Quest è in accordo con ciò che sostiene il Prof. Bustin: sopra i 35 cicli, l'affidabilità del tampone crolla, e al massimo, per salvare la baracca, si può sostenere che si

[9] David Crow, The Infectious Myth: https://infectiousmyth.podbean.com/e/the-infectious-myth-stephen-bustin-on-challenges-with-rt-pcr/

[10] Luca Carra, *Debolmente positivi: realtà o illusione?,* Internazionale, 23 Giugno 2020.

tratta di presenza di virus talmente debole da non essere più infettivo. La sostanza non cambia: **che il virus venga creato dalla PCR come un "fantasma", come sostengono Crow e Bustin, o che esso sia senza nessuna carica virale, perché si conti una a utilizzare questi risultati da tampone per terrorizzare la gente e prorogare vari tipi di lockdown?**

E che i tamponi utilizzino normalmente sopra i 35 cicli di PCR è confermato da questa tabella che riporta una serie di diversi tamponi e la media del loro numero di cicli :

Azienda	Nome prodotto	Numero prodotto	Gene		Cycle threshold
altona Diagnostics	RealStar® SARS-CoV-2 RT-PCR Kit 1.0	821003/ 821005	E	1–10	35.45
			S	1–10	35.99
CerTest Biotec S.L.	VIASURE SARS-CoV-2 Real Time PCR Detection Kit	VS-NC0112L VS-NC0212L	ORF1ab	10–50	35.16
			N	1–10	35.46
DAAN Gene Co. Ltd of Sun Yat-Sen University	Detection Kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence	DA0930-DA0932	ORF1	1–10	38.76
			N	1–10	36.97
BGI Health (HK) Co. Ltd	Real-time Fluorescent RT-PCR kit for	MFG030010	ORF1	1–10	32.43
Beijing Wantai Biological Pharmacy Enterprise	Wantai SARS-CoV-2 RT-PCR Kit	WS-1248	ORF1ab	1–10	36.20
			N	1–10	37.12
Bioneer Corporation	AccuPower® SARS-CoV-2 Real-Time RT-PCR Kit	SCV-2122	E	10–50	35.85
			RdRP	10–50	36.18

KH Medical Co. Ltd	RADI COVID-19 Detection Kit	RV008	S	1–10	37.94
			RdRP	10–50	36.74
bioMérieux SA	ARGENE® SARS-COV-2 R-GENE®**[b]**	423720 (CE-IVD) 423717 (RUO)	N	10–50	36.44
			RdRP	10–50	32.44
EUROIMMUN AG	EURORealTime SARS-CoV-2**[c]**	MP 2606-0425	ORF1ab/N	1–10	37.88
Boditech Med. Inc.	ExAmplar COVID-19 real-time PCR kit (L)	UFPK-4	E	10–50	34.9
			RdRP	50–100	33.46
GeneFirst Ltd	The Novel Coronavirus (2019-nCoV) Nucleic Acid Test Kit	MPA-COVID19	ORF1	1–10	35.45
			N	1–10	36.72
PerkinElmer Inc.	PerkinElmer® SARS-CoV-2 Real-time RT-PCR Assay**[c,d]**	SY580	N	1–10	39,43
			ORF1	1–10	38,99
Primerdesign Ltd	Coronavirus COVID-19 genesig®	Z-Path-COVID-19-CE	RdRP	1–10	36.7
QuantumDx	QuantuMDx SARS-CoV-2	Q22003	Orf1, N, S	1–10	36.8
R-Biopharm AG	RIDA®GENE SARS-CoV-2	PG6815RUO	E	1–10	37.99

Questa tabella riprende i primi 16 dei 22 tamponi analizzati e testati da FIND (Foundation for Innovative New Diagnostics), un organizzazione spesso indicata anche dalle autorità sanitarie come un affidabile strumento per la valutazione degli

strumenti diagnostici. Come si vede dalla tabella da me ricostruita (per la tabella completa vedi: https://www.finddx.org/covid-19/sarscov2-eval-molecular/), a parte un paio di casi al limite, tutti gli altri utilizzano più di 35 cicli, a volte anche vicino ai 40. **E si consideri che queste sono le medie, il che significa che nel 30-35% dei casi si sale oltre i 40 cicli.**

E la cosa è confermata anche per il test Xpert Xpress di Cepheid, che la FDA americana ha ritenuto così importante e affidabile da emettere un'autorizzazione di emergenza[11], saltando tutti i passaggi di verifica. Ebbene, anche questo test così importante, adotta un numero di cicli eccessivo:

Tabella 3. Concordanza del test Xpert SARS-CoV-2 con i risultati attesi per concentrazione del campione

Concentrazione target	Numero di concordanti/numero di analizzati	Media Ct di E	Media Ct diN2	% concordanza [IC 95%]
2x LoD	20/20	35,4	38,4	100% [83,9% - 100%]
3x LoD	5/5	34,2	37,2	100% [NA*]
5x LoD	5/5	33,9	37,0	100% [NA*]
Negativo	30/30	NA	NA	100% [88,7% - 100%]

La media riferita al gene E, che è comunque aspecfifico e tipico di tuti i coronavirus, è attorno ai 34-35 cicli; ma la media riferita al gene N2, che dovrebbe essere più specifico del SARS-Cov2 (vedremo che non è così neppure per questo gene), si attesa **attorno a 37-38 cicli**!

Questo significa che nella maggioranza dei casi i tamponi danno o risultati fantasma, o se anche "beccano" il virus, lo trovano in uno stato talmente indebolito da non costituire più nessun pericolo. Questo significa anche che dunque **non c'è più nessuna motivazione per terrorizzare con lo spettro dei positivi asintomatici**, perché come minimo si tratta di individui incapaci di infettare alcunché. Ma la verità, come stiamo per vedere, è che i tamponi producono risultati senza nessun significato, risultati fantasma o comunque non indicativi della presenza del SARS-Cov 2 .

La questione della cross-reattività, o mancanza di specificità.

Prendiamo i tre più importanti modelli di test-tampone, utilizzati da molti dei tamponi circolanti, quello della OMS, quello tedesco-europeo del gruppo di Drosten, e quello del CDC americano. Quello della OMS, come abbiamo già visto altrove, è talmente a rischio di aspecificità (ovvero di cogliere col tamponi virus o particelle simil-virali diverse dal SARS-Cov2) che in uno dei suoi 3 primers (le sequenze geniche con cui si va alla ricerca del virus) c'è addirittura una sequenza genica tipica del DNA umano, quella del cromosoma 8:

[11] Kubina R, Dziedzic A, *Molecular and Serological Tests for COVID-19. A Comparative Review of SARS-CoV-2 Coronavirus Laboratory and Point-of-Care Diagnostics,* Diagnostics 2020, 10, 434; doi:10.3390/diagnostics10060434, p. 6

Primers and probes

Name	Sequences (5'-3')	Length (bases)	PCR product size	Ref.
RdRp gene / nCoV_IP2				
nCoV_IP2-12669Fw	ATGAGCTTAGTCCTGTTG	17	108 bp	1
nCoV_IP2-12759Rv	CTCCCTTTGTTGTGTTGT	18		
nCoV_IP2-12696bProbe(+)	AGATGTCTTGTGCTGCCGGTA [5']Hex [3']BHQ-1	21		
RdRp gene / nCoV_IP4				
nCoV_IP4-14059Fw	GGTAACTGGTATGATTTCG	19	107 bp	1
nCoV_IP4-14146Rv	CTGGTCAAGGTTAATATAGG	20		
nCoV_IP4-14084Probe(+)	TCATACAAACCACGCCAGG [5']Fam [3']BHQ-1	19		
E gene / E_Sarbeco				
E_Sarbeco_F1	ACAGGTACGTTAATAGTTAATAGCGT	18	125 bp	2
E_Sarbeco_R2	ATATTGCAGCAGTACGCACACA	20		
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG [5']Fam [3']BHQ-1	20		

1/ National Reference Center for Respiratory Viruses, Institut Pasteur, Paris.
2/ Corman et al. Eurosurveillance[1]

Qui il rischio di far venire il tampone positivo anche senza nessun virus presente è ovviamente molto alta, visto che tutti gli esseri umani possiedono quella sequenza CTCCCTTTGTTGTGTTGT come parte del loro corredo genico.

Il CDC americano utilizza invece altre sequenze geniche, relative al gene N del virus, quello del suo nucleocapside. Questa scelta di focalizzarsi sul gene N, nelle sue due versioni N1 e N2, è dovuto al fatto che il gene E "… also detects SARS-related coronaviruses"[12] ("rileva anche altri SARS-coronavirus"). Questo mostra come il tampone OMS possa, in aggiunta a legarsi al genoma umano, identificare altri coronavirus scambiandoli per il SARS-Cov 2.

Ma che garanzie ci sono che i geni N1 e N2 siano invece più specifici? Tutti i coronavirus hanno un nucleo-capside, e dunque geni del tipo N. Il CDC sostiene che il gene N2 è specifico del SARS-Cov2; ma anche su questo non c'è accordo, dato che per alcuni ricercatori non è così:

> "…we found out that only one of them (RdRP_SARSr-P2) was **almost specific for the new coronavirus** and **the other introduced probes would detect the other types of coronaviruses**. In this regard, the false-positive test results may extend for COVID-19"[13]

> "…abbiamo trovato che solo uno di loro (il gene RdRP-SARSr-P2) è quasi specifico per il nuovo coronavirus, mentre le altre "sonde" (sequenze geniche) rilevano anche altri tipi di coronavirus. Sotto questo aspetto, **i risultati con falsi positivi possono ampliarsi in rapporto al Covid-19."**

[12] Wagginer J et al., *Triplex Real-Time RT-PCR for Severe Acute Respiratory Syndrome* Coronavirus 2, Research Letter, Volume 26, Number 7—July 2020.

[13] Kakhki RK et al, *COVID-19 target: A specific target for novel coronavirus detection,* Gene Reports 20 (2020) 100740.

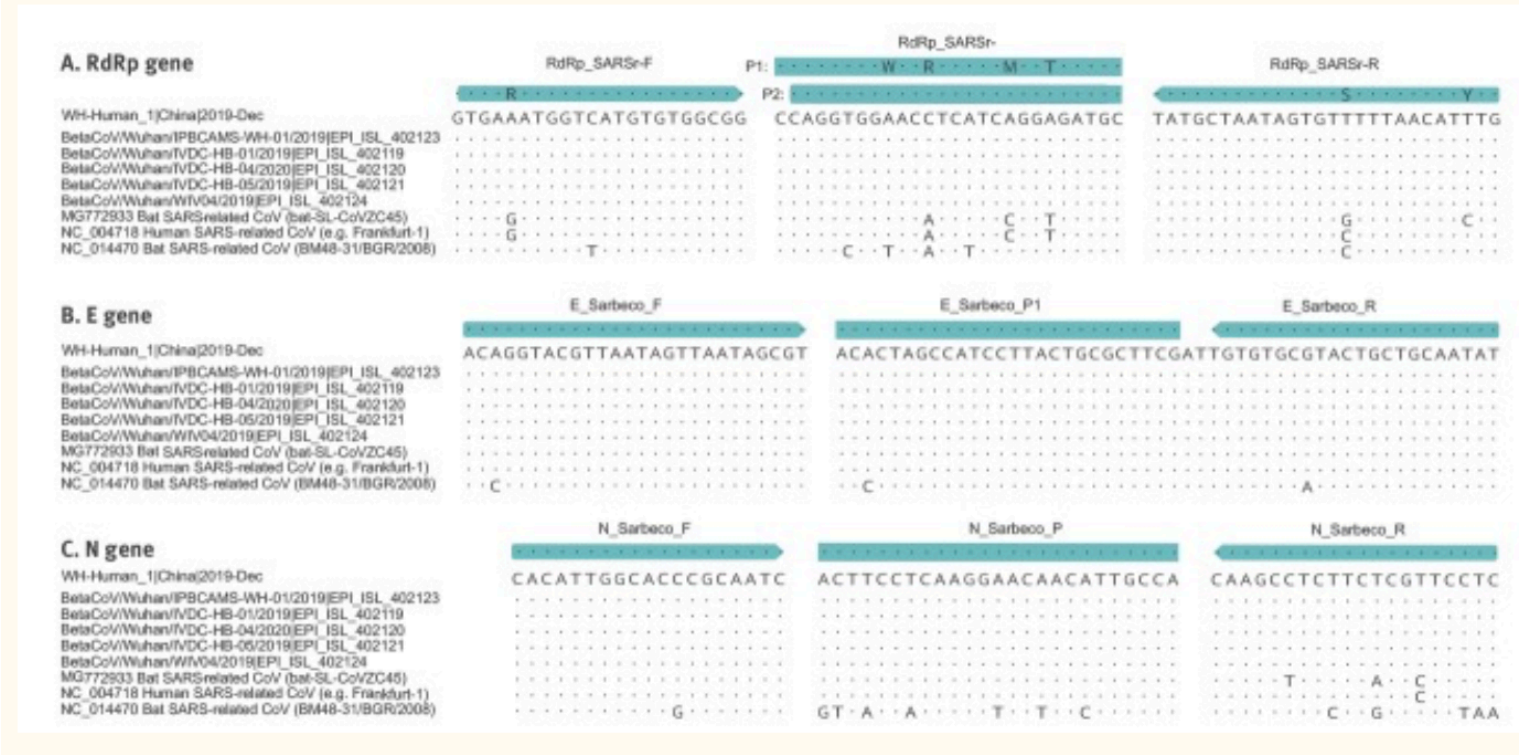
Ciò significa che non c'è alcuna sicurezza neppure sulla specificità del gene N2 usato dal modello della CDC, specie se si considera che appunto i geni N sono tipici di tutti i coronavirus. E si noti come gli autori, anche per il gene che ritengono specifico, lo definiscono "quasi" specifico, nel senso che anche quello non è completamente specifico!

E quando veniamo al test di Drosten, il test-tampone europeo, le cose diventano anche più evidenti. Innanzitutto, vediamo qui in modo apertamente dichiarato, che questi isolamenti e definizioni del virus sono tutte elaborazioni al computer, senza nessuna presenza fisica del virus:

> "The present report describes the establishment of a diagnostic workflow for detection of an emerging virus in the absence of physical sources of viral genomic nucleic acid."[14]

> "Il presente documento descrive la realizzazione di un processo diagnostico per il rilevamento di un virus emergente **in assenza delle fonti fisiche degli acidi nucleici genomici virali**".

Quindi qui l'astrazione dei tamponi dall'effettivo virus è dichiarata apertamente, e appare evidente anche dalla tabella delle sequenze geniche utilizzate dal gruppo di Drosten:



[14] Corman V et al, *Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR*, Euro Surveill. 2020 Jan 23; 25(3): 2000045, p.10.

Come si vede, il tampone di Drosten utilizza tutti e 3 i geni, E, N e RdRP. Ma se confrontiamo la sequenza genica del SARS-Cov 2 con quella del SARS-Cov originario (al penultimo posto nella lista), vediamo che:

- il gene E del SARS-Cov 2 è identico al 100% a quello del SARS-Cov1, e probabilmente a quello di tutti i SARS coronavirus (nella penultima riga non ci sono variazioni di lettere);
- Il gene N ha una sola variazione, una C invece di una T, al 15° posto della sequenza del Reverse primer. Questa è una variazione di appena 1/64esimo, ovvero di appena l'1.5%. Le possibilità di confusione e cross-reattività (rilevare un SARS virus diverso dal SARS-Cov2) è molto elevata.
- Il gene RdRP è l'unico che ha 5 variazioni su 64, di nuovo non una grande differenza, anche se meglio degli altri due (e per questo gli autori sopra lo hanno definito "quasi" specifico).

Insomma, in totale abbiamo una differenza di soli 6 nucleotidi su 214, una percentuale di appena il 2.8%. E per questo anche quando autori indipendenti hanno testato l'efficienza del test Drosten hanno concluso che il test dimostrava:

> "…a lot of cross-reactions with Coronavirus BtRs-BetaCoV (MK211374- MK211378), SARS coronavirus Urbani (MK062179-MK062184), Bat coronavirus (KY770858-KY770859), SARS coronavirus (AH013708-AH013709), and others".

> "…**elevata cross-reattività con i Coronavirus** BtRs-BetaCoV (MK211374- MK211378), SARS coronavirus Urbani (MK062179-MK062184), Bat coronavirus (KY770858-KY770859), SARS coronavirus (AH013708-AH013709, e con altri."

E anche il gene RdRP, che dovrebbe essere più specifico

> "…covers many coronavirus isolates, including Bat SARS-like Coronavirus (MG772904-MG772932), Rhinolophus pusillus Coronavirus (KY775091), Bat SARS-like Coronavirus (MG772903) and many others".

> "…copre **molti altri isolati di coronavirus**, inclusi Bat SARS-like Coronavirus (MG772904-MG772932), Rhinolophus pusillus Coronavirus (KY775091), Bat SARS-like Coronavirus (MG772903), **e molti altri**."[15]

Insomma, tutti i principali test-tamponi mancano di specificità, e sono affetti da un elevata cross-reattività, ovvero producono un elevata quantità di falsi positivi. Questa verità, che dovrebbe porre immediatamente fine alla follia della pseudo-pandemia spinta da questi tamponi farlocchi, è da ultimo, *last but not least*, apertamente confermata dallo stesso Istituto Superiore di Sanità, organismo del governo italiano.

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[15] Kakhki RK et al, *COVID-19 target: A specific target for novel coronavirus detection,* Gene Reports 20 (2020) 100740.

ISS del Governo Italiano: in questa situazione epidemica, i test-tampone danno fino al 91% di falsi positivi!

Nel documento **Dispositivi diagnostici in vitro per COVID-19. Parte 2: evoluzione del mercato e informazioni per gli stakeholder**, del 23 Maggio 2020, l'Istituto Superiore d Sanità fa una analisi già approfondita dei dispositivi test-tampone in circolazione, sottolineando la tensione esistente tra **sensibilità**, la capacità di rilevare quanto più RNA virale possibile, e la **specificità**, ovvero la necessità che tale RNA virale si riferisca solo al virus che si sta cercando, in questo caso il SARS-Cov2.

> "Un test molto sensibile nel rilevare il bersaglio di interesse ha maggiori probabilità di rilevare anche bersagli correlati ma distinti che non sono di interesse, vale a dire che può essere meno specifico."[16]

L'ISS spiega poi che tale tensione è modulata da un altro fattore, ovvero quello di "**prevalenza**". In ambito epidemiologico, la prevalenza descrive la **percentuale di popolazione affetta da una certa patologia**. Nel caso di una patologia presuntivamente virale come il Covid-19, la prevalenza indica quanti malati attuali di Covid-19 ci sono sul totale della popolazione.

Perché questo dato è importante in rapporto alla affidabilità dei test-tampone? Perché maggiore è la percentuale di popolazione affetta, maggior è la circolazione del virus, e quindi maggiore è la probabilità che il test-tampone rilevi effettivamente quel virus anziché altri, riducendo così il gap tra sensibilità e specificità.

L'ISS riprende una tabella che considera l'effetto della prevalenza sull'efficacia dei tamponi. La tabella è stata pubblicata da FIND, autorevole organizzazione internazionale già vista sopra; e così, il dato che emerge dalla tabella FIND, accettato e riproposto dal'ISS, ha valore non solo per l'Italia, ma per tutto il mondo.

Scrive l'ISS a introduzione della Tabella:

> "Nella tabella che segue, tratta dal documento Rapid diagnostic tests for COVID-19[17], viene mostrato con un esempio numerico come la **capacità di identificare correttamente i positivi (colonna PPV)** sia correlata sia alla **sensibilità e specificità** del test, sia alla **prevalenza** del marcatore nella popolazione target, esemplificata da quattro coorti di

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[16] Gruppo di Lavoro ISS Test Diagnostici COVID-19, *Dispositivi diagnostici in vitro per COVID-19. Parte 2: evoluzione del mercato e informazioni per gli stakeholder ,* Rapporto ISS COVID-19 n. 46/2020, 23 Maggio 2020, p. 6.

[17] FIND, *Rapid Diagnostic Tests for Covid-19*: https://www.finddx.org/wp-content/uploads/2020/05/FIND_COVID-19_RDTs_18.05.2020.pdf

1.000 individui con quattro diversi valori di prevalenza: 2%, 5%, 10% e 30%. “

Quindi, la capacità del test di rilevare correttamente la presenza del virus dipende da 3 fattori, tutti considerato nella tabella, ovvero **sensibilità** e **specificità**, ma alla luce della prevalenza; e la Tabella prende in considerazione 4 livelli di **prevalenza**: 2%, 5%, 10% e 30%. Prima di vedere la Tabella, vediamo a quale dei quattro gruppi appartiene la situazione Italiana (e di riflesso anche quella degli altri paesi, in cui il livello di prevalenza non si discosta molto da quello italiano). Quello che segue è la situazione Covid-19 in Italia al 25 Settembre 2020:

Il numero da considerare è quello degli attuali positivi, ovvero **47,718, che rappresenta appena lo 0.079% della popolazione italiana**, assai distante persino dal livello più basso del 2%. Anche se volessimo esagerare, e prendere in considerazione il totale dei casi che ci sono stati dall’inizio a oggi, avremmo che il numero di 306,235 è pari allo 0.5% della popolazione italiana. Utilizzare questo secondo numero è statisticamente del tutto errato, ma l’ho fatto per sottolineare come neppure prendendo tutti i casi Covid-19 ufficiali (cioè CON Covid e non PER Covid) emersi dall’inizio della pseudo-pandemia ad oggi, si arriverebbe neppure lontanamente al 2% della popolazione. Vediamo finalmente la Tabella:

Cohort	Pre-test probability (prevalence)	Sensitivity	Specificity	Cases	Non-cases	True positive (TP)	False negative (FN)	True negative (TN)	False positive (FP)	PPV	NPV
High performance											
1,000	2.0%	95%	98%	20	980	19	1	960	20	49.2%	100%
1,000	5.0%	95%	98%	50	950	48	2	931	19	71.4%	100%
1,000	10.0%	95%	98%	100	900	95	5	882	18	84.1%	99%
1,000	30.0%	95%	98%	300	700	285	15	686	14	95%	98%
Mid performance											
1,000	2.0%	85%	90%	20	980	17	3	882	98	14.8%	100%
1,000	5.0%	85%	90%	50	950	43	8	855	95	30.9%	99%
1,000	10.0%	85%	90%	100	900	85	15	810	90	48.6%	98%
1,000	30.0%	85%	90%	300	700	255	45	630	70	78%	93%
Low performance											
1,000	2.0%	75%	85%	20	980	15	5	833	147	9.3%	99%
1,000	5.0%	75%	85%	50	950	38	13	808	143	20.8%	98%
1,000	10.0%	75%	85%	100	900	75	25	765	135	35.7%	97%
1,000	30.0%	75%	85%	300	700	225	75	595	105	68%	89%

Il numero decisivo è il PPV, ovvero la capacità del test di rilevare effettivamente il virus. I numeri che ci interessano sono quelli legati al livello del 2%, che nel caso dell'Italia è in realtà molto più basso, assestandosi attorno allo 0.1%. Questo significa che i numeri di questa Tabella sono addirittura ottimisti, anche al livello del 2%, e più avanti faremo anche la proiezione della Tabella sul livello dello 0.1%.

Intanto, qui vengono considerati 3 modelli di tampone: quelli ad alta performance, a media performance, e a bassa performance. Al livello di prevalenza del 2%, questi sono i livelli di veri e falsi positivi dati dai tamponi:

Livello	Veri positivi	Falsi positivi
2% Alta performance	49.2%	**50.8%**
2% Media performance	14.8%	**85.2%**
2% Bassa performance	9.3%	**90.7%**

Quindi, nella migliore delle ipotesi, i tamponi danno il 50% di falsi positivi, e nella peggiore delle ipotesi danno quasi **il 91% di falsi positivi**! Mediamente, possiamo dire che i tamponi danno l'85,2% di falsi positivi!

In tutti i casi, l'Istituto Superiore di Sanità certifica che i tamponi sono del tutto inaffidabili! Ci sarà qualche politico che avrà voglia di ascoltare questa verità ufficiale, che più ufficiale non si può?

Qual'è il numero più probabile tra il 50% e il 91% di falsi positivi? Avendo visto in precedenza la inaffidabilità delle sequenze geniche dei principali tamponi, e soprattutto il fatto che tutti utilizzano più di 35 cicli di PCR, e dunque che i tamponi non possono che essere a bassa performance, **il numero più realistico è il 91% di falsi positivi**! Ma **se anche fossero una via di mezzo, ad esempio il risultato della "media performance" dell'85%, le cose non cambierebbero**. I tamponi sono del tutto inaffidabili, lo afferma lo stesso Istituto Superiore di Sanità e un'organizzazione autorevole internazionalmente come FIND: **cosa si aspetta a far cessare la tragica farsa dei tamponi e dei positivi asintomatici?**

E qui veniamo all'ultima considerazione, anche se non sarebbe neppure necessaria. I numeri che abbiamo visto si riferiscono al livello di prevalenza del 2%; ma in Italia oggi il livello è dello 0.1%. Un adeguato aggiustamento statistico richiederebbe un lavoro ad hoc. Ma se consideriamo che nel passaggio dal 30% di prevalenza al 2% (riduzione di 15 volte) i valori si riducono dal 95% al 49.3%, ovvero di circa la metà (50%); possiamo ragionevolmente valutare che passando dal 2% allo 0.1% (riduzione di 20 volte), i valori subiscano come minimo lo stesso dimezzamento. Questo significa che il range dei **falsi positivi passa dal 50.3 al 75% nella migliore delle ipotesi**; e **dal 90.7 al 95% circa nella peggiore delle ipotesi**.

Una ragione ancora più convincente per gridare con forza: BASTA CON LA TRUFFA DI QUESTA FALSA PANDEMIA, che genera una prevalenza di appena lo 0,1% (mentre i modelli parlano di prevalenze fino al 30%!); e che si regge su tamponi che, **secondo l'autorevole opinione di FIND ripresa dallo ISS italiano, producono fino al 95% di falsi positivi**!

FIND EVALUATION UPDATE: SARS-COV-2 MOLECULAR DIAGNOSTICS

In February 2020, FIND launched an expression of interest (EOI) for test developers of *in vitro* diagnostics (IVDs) that detect SARS-CoV-2 nucleic acid (molecular tests) to participate in independent evaluation studies. Over 200 submissions were received and applications were reviewed according to the following scoring criteria (Table 1).

Table 1: Scoring criteria	**Max score**
Limit of detection	3
Regulatory status	2
Type of organization	1
Quality management system	1
Other products available in low- and middle-income countries	3
TOTAL	**10**

As of August 2020, FIND is no longer accepting applications to evaluate molecular tests.

FIND conducted independent evaluations at the University Hospitals of Geneva (HUG) to verify the limit of detection (LOD) – as reported by the manufacturers – and the clinical performance of 22 manual molecular test kits in comparison to an in-house PCR protocol that was optimized based on the Tib Molbiol assay. The LOD analysis was performed using cultured viral stocks from a clinical isolate from Switzerland that was quantified using an E gene standard. The clinical performance analysis was conducted on extracted samples from individuals suspected to have COVID-19. 50 of which were reference PCR positive and 100 of which were reference PCR negative. Data are summarized in **Table 2**.

Additionally, a limited clinical performance evaluation of the Cepheid Xpert Xpress SARS-CoV-2 assay was also performed at HUG. A second collaborating site, the Translational Health Science and Technology Institute (THSTI) conducted a similar limited clinical performance evaluation of the Mobio TrueNat SARS-CoV-2 assay. Both studies were performed using frozen, stored respiratory samples from COVID-19 suspects. Results on the performance of these automated near-POC assays are shown in **Table 3**.

MOLECULAR ASSAY EVALUATION PROTOCOL SUMMARY

Table 2: Results for 22 manual (open) molecular tests evaluated at HUG

	Company	Product name	Product number	Gene target	Verified LOD (copies / reaction)	Avg Ct (lowest dilution 19/1)	Clinical sensitivity (50 positives)	Clinical specificity* (100 negatives)	Lot No.	PCR platform**	Supplier recommended Ct cut-off
1.	**altona Diagnostics**	RealStar® SARS-CoV-2 RT-PCR Kit 1.0	821003/ 821005	E	1–10	35.45	92% (95%CI: 81, 97)	100% (95%CI: 96, 100)	020567	BioRad CFX96 deep well	None; any signal could be considered positive
				S	1–10	35.99	92% (95%CI: 81, 97)	100% (95%CI: 96, 100)			
2.	**Atila BioSystems Inc.**	Atila iAMP COVID-19 Detection (isothermal detection)	iAMP-COVID-100-RUO	ORF1ab	50–100	N/A	100% (95%CI: 93, 100)	99%* (95%CI: 95, 100)	COVID20200320	BioRad CFX96 deep well	Any signal is considered positive (isothermal
				N	1–10	N/A	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
3.	**Beijing Wantai Biological Pharmacy Enterprise Co. Ltd**	Wantai SARS-CoV-2 RT-PCR Kit	WS-1248	ORF1ab	1–10	36.20	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	nCoVP20200305	BioRad CFX96 deep well	≤40
				N	1–10	37.12	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
4.	**BGI Health (HK) Co. Ltd**	Real-time Fluorescent RT-PCR kit for detection 2019-nCoV (DE-IVD)	MFG030010	ORF1	1–10	32.43	100% (95%CI: 93, 100)	99%* (95%CI: 95, 100)	6202020305	Roche LightCycler 480	≤38
5.	**bioMérieux SA**	ARGENE® SARS-COV-2 R-GENE® **[b]**	423720 (CE-IVD) 423717 (RUO)	N	10–50	36.44	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	1007389610 1007947520	BioRad CFX96 deep well	Any signal considered positive
				RdRP	10–50	32.44	96%**[a]** (95%CI: 87, 99)	100% (95%CI: 96, 100)			
6.	**Bioneer Corporation**	AccuPower® SARS-CoV-2 Real-Time RT-PCR Kit	SCV-2122	E	10–50	35.85	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	200931E	BioRad CFX96 deep well	<38
				RdRP	10–50	36.18	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
7.	**Boditech Med. Inc.**	ExAmplar COVID-19 real-time PCR kit (L)	UFPK-4	E	10–50	34.9	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	WLQCB02L	BioRad CFX96 deep well	≤42
				RdRP	50–100	33.46	90% (95%CI: 79, 96)	100% (95%CI: 96, 100)			
8.	**CerTest Biotec S.L.**	VIASURE SARS-CoV-2 Real Time PCR Detection Kit	VS-NC0112L VS-NC0212L	ORF1ab	10–50	35.16	98% (95%CI: 90, 100)	100% (95%CI: 96, 100)	NC02125,-023	BioRad CFX96 deep well	≤40
				N	1–10	35.46	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
9.	**DAAN Gene Co. Ltd of Sun Yat-Sen University**	Detection Kit for 2019-Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing)	DA0930-DA0932	ORF1	1–10	38.76	100% (95%CI: 93, 100)	96%* (95%CI: 90, 98)	20200107	Roche LightCycler 480	≤40
				N	1–10	36.97	100% (95%CI: 93, 100)	98%* (95%CI: 93, 99)			
10.	**EUROIMMUN AG**	EURORealTime SARS-CoV-**[c]**	MP 2606-0425	ORF1ab/N	1–10	37.88	100% (95%CI: 93, 100)	98%* (95%CI: 93, 99)	0200320AL	Light Cycler 480 II	Any signal considered positive
11.	**GeneFirst Ltd**	The Novel Coronavirus (2019-nCoV) Nucleic Acid Test Kit	MIYA-COVID19	ORF1	1–10	35.45	100% (95%CI: 93, 100)	99%* (95%CI: 95, 100)	00072	BioRad CFX96 deep well	≤37.0 positive 37-40 indeterminate >40 negative
				N	1–10	36.72	98% (95%CI: 90, 100)	100% (95%CI: 96, 100)			
12.	**KH Medical Co. Ltd**	RADI COVID-19 Detection Kit	RV008	S	1–10	37.94	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	V008.200202	BioRad CFX96 deep well	≤40
				RdRP	10–50	36.74	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
13.	**PerkinElmer Inc.**	PerkinElmer® SARS-CoV-2 Real-time RT-PCR Assay **[c,d]**	SY580	N	1–10	39.43	100% (95%CI: 93, 100)	99%* (95%CI: 95, 100)	8202020003	BioRad CFX96 deep well	≤42
				ORF1	1–10	38.99	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
14.	**Primerdesign Ltd**	Coronavirus COVID-19 genesig® Real-Time PCR assay **[c]**	Z-Path-COVID-19-CE	RdRP	1–10	36.7	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	JIN-03780-0009	LightCycler 480	Any signal regarded as positive
15.	**QuantumDx**	QuantuMDx SARS-CoV-2 RT-PCR Detection Assay	Q20003	Orf1, N, S	1–10	36.8	100% (95% CI: 92, 100)	100% (95% CI: 96, 100)	P01100	BioRad CFX96 deep well	≤40
16.	**R-Biopharm AG**	RIDABGENE SARS-CoV-2 RUO	PG6815RUO	E	1–10	37.99	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	21120N	BioRad CFX96 deep well	None; any signal could be considered positive
17.	**Sansure Biotech Inc.**	Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) **[e]**	S3102E	ORF1	10–50	35.16	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	20200072C	Thermofisher Quantstudio 5	≤40
				N	10–50	34.96	100% (95%CI: 93, 100)	95%* (95%CI: 89–98)			
18.	**SD Biosensor Inc.**	STANDARD M nCoV Real-Time Detection Kit	M-NCOV-01	E	1–10	37.43	100% (95%CI: 93, 100)	97%* (95%CI: 92, 99)	MNC00120005	Roche LightCycler 480	≤41
				ORF1	1–10	36.99	100% (95%CI: 93, 100)	99%* (95%CI: 95, 100)			
19.	**Seegene Inc.**	Allplex™ 2019-nCoV Assay	RP10244Y RP10243X	E	1–10	33.3	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	RP4320C24	BioRad CFX96	≤40
				N	1–10	36.74	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
				RdRP	1–10	34.73	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
20.	**Shanghai Kehua Bio-Engineering Co. Ltd**	KHB Diagnostic kit for SARS-CoV-2 Nucleic Acid (Real-time PCR)	KH-G-M-574-48	ORF1	1–10	30.39	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	20037410	BioRad CFX96 deep well	More than two targets detected an curve is of S shape
				N	1–10	32.95	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
				E	1–10	31.72	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
21.	**ThermoFisher Scientific**	TaqPath™ COVID-19 CE-IVD RT-PCR Kit **[f]**	A48067	ORF1ab; S protein; N protein	1–10	NA	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	2225262	Quantstudio 5	Not Applicable (Automated software interpretation)
22.	**Vela Diagnostics**	ViroKey™ SARS-CoV-2 RT-PCR Test **[c]**	300682	RdRP	10–50	30.95	94% (95%CI: 84, 98)	100% (95%CI: 96, 100)	1000003597	BioRad CFX96 deep well	≤40
				ORF1	1–10	35.57	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)			
	Tib Molbiol/Roche Diagnostics	ModulaDx Kit SARS-CoV-2 (COVID19) E-gene (Tib-Molbiol) + LightCycler Multiplex RNA Virus Master (Roche)	53-0776-96 07541105001	E	1–10	33.34	100% (95%CI: 93, 100)	100% (95%CI: 96, 100)	48202019 48274100	Roche LightCycler 480	Define the cut-off 2–4 cycles higher than obtained Cp value for 10 copies

*** Clinical specificity:** Further investigation is needed to determine if apparent false positives are truly false positives or whether they are due to a false negative reference standard result.
**** PCR platforms:** All products were evaluated on a PCR platform recommended by the supplier, listed in this table. Each test can be performed on other PCR systems, detection in the product's instructions for use.
[a] The two false negative samples tested positive with the second PCR (PCR 2) that targets E gene of SARS, SARS-COV-2 and/or SARS-like coronaviruses.
[b] Samples for both analytical and clinical analyses were from already-extracted specimen, therefore the methods varied from those recommended by the supplier as the internal control was not included.
[c] Samples for both analytical and clinical analyses were from already-extracted specimen, therefore the methods varied from those recommended by the supplier as the internal control was added to the master mix.
[d] Evaluation procedure varied from recommended protocol. In order to achieve the recommended sample input volume, a 2.5 fold dilution of the samples was used.
[e] Sansure claims a lower LOD of 6.4 cp/ml, which has been independently verified.
[f] Evaluation procedure varied from recommended protocol as source material was already-extracted RNA; extracted MS2 control was added directly to the master mix.

Table 3: Results for evaluation of two near-POC automated tests

Company	Product name	Product number	Gene target	Clinical sensitivity (50 positives)	Clinical specificity* (100 negatives)	Comparator test
Cepheid Inc.	Xpert® Xpress SARS-CoV-2	XPRSARS-COV2-10	N2	100% (95%CI: 92.100)	99% * (95%CI: 95, 100)	Roche Cobas ® SARS-CoV-2
			E	97.7% (95% CI: 88, 100)	100% (95%CI: 96, 100)	N = 44 positive N = 100 negative
Mobio Diagnostics Pvt Ltd	TrueNat SARS-CoV-2 **[1]**	601410020	E+RdRP**[2]**	98% (95% CI: 90.98)	96% * (95% CI:90.98)	altona Diagnostics (n=96) /LabGun™ (n=64) and/or Seegene, Inc. (n=12)
		601420050				N = 51 positive N = 111 negative

** Clinical specificity: Further investigation is needed to determine if apparent false positives are truly false positives or whether they are due to a false negative reference standard result*
[1] Note: evaluation performed at THSTI
[2] RdRP is only used as a reflex test; the results are for combined E+RdRP positives

MORE INFORMATION

For questions relating to the evaluation of molecular tests, please contact our Emerging Threats team.

QUICK LINKS

DIAGNOSIS OF SARS-COV-2 INFECTION AND COVID-19: ACCURACY OF SIGNS AND SYMPTOMS, MOLECULAR, ANTIGEN, AND ANTIBODY TESTS, AND ROUTINE LABORATORY MARKERS
WHO R&D ROADMAP
WHO COVID-19 LABORATORY GUIDANCE

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tutti nella loro qualità di esperti e ricercatori scientifici, con riferimento all'utilizzo dei c.d. tamponi Covid-19, che sono al centro dell'attuale gestione dello stato di emergenza collegato alla nota problematica del Covid-19.

DICHIARANO

- di aver appurato, da un documento della Commissione Europea e da uno dell'Istituto Superiore di Sanità[1] , che i tipi di tampone circolanti in Europa al 16 Maggio 2020 erano 78, nessuno dei quali autorizzato, valutato o validato;
- di aver riscontrato dai medesimi documenti che gran parte dei medesimi tamponi sono altresì privi della dichiarazione delle sequenze geniche contenute, abbiamo deciso di approfondire la cosa.

Si è pertanto ritenuto necessario approfondire la problematica attinente alla validità dei risultati dei tamponi e si è potuto accertare che:

1) per stessa ammissione del Centers for Disease Control and Prevention americano[2] (doc. 3) e del Gruppo di Lavoro Covid della Commissione EU, il virus SARS-Cov2 (doc. 2), presunto responsabile del Covid, non è mai stato isolato fisio-chimicamente

2) I liquidi patogeni usati come modello per il sequenziamento genico non avevano nessuna titolazione del virus, il che implica che in tali liquidi erano contenute miliardi di altre particelle simil-virali (incluse le non patogeniche vescicole extracellulari naturalmente presenti nel nostro organismo),

Ciò comporta che

non esiste a tutt'oggi nessun marker specifico del virus, e dunque nessuno standard che possa rendere i tamponi affidabili.

3) I tamponi attualmente circolanti, oltre 100, sono esentati dai controlli previsti dalla legge europea sui dispositivi medici del 1997;

4) Al contempo non sono ancora assoggettati alla nuova norma europea del 2017, che entrerà in vigore solo nel Maggio 2022;

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[1] Doc. 1 Current performance of COVID-19 test methods and devices and proposed performance criteria.
Doc. 2 ISS Covid tests Part 2 https---www.epicentro.iss.it-coronavirus-pdf-rapporto-covid-19-46-2020.

[2] CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel – doc.3 aggiornato al 13-07-2020.

5) Tale limbo normativo rende i produttori liberi di far circolare qualsiasi tipo di dispositivo senza nessun controllo;

Ciò comporta che

La conformità dei tamponi prodotti a degli standard che ne possano accertare la corretta efficacia non è oggi verificata.

6) Ci sono numerosi studi che attestano la continua mutazione del virus, e le stesse autorità sanitarie riconoscono che se il virus continua a mutare i tamponi diventano inutili.

7) Ci sono quasi 150.000 diversi sequenziamenti del virus presso la banca dati dei virus GISAID; erano 70.000 ad Aprile; e continuano a crescere, perché si trovano sempre nuova mutazioni, e ciò rende i tamponi circolanti del tutto inutili. Sul punto si allega la ricerca scientifica del dott. Scoglio che analizza anche questo elemento essenziale[3].

Ciò comporta che

I tamponi circolanti, anche qualora si volessero ritenere astrattamente efficaci e nel concreto conformi alla normativa, sono del tutto inutili in quanto non possono accertare le mutazioni.

8) Esiste inoltre un problema sostanziale legato alla metodologia utilizzata nei tamponi, la RT-PCR. Come sottolineano i massimi esperti di questa metodologia, per funzionare correttamente tale metodologia idealmente dovrebbe utilizzare tra i 20 e i 30 cicli di PCR; e non si dovrebbe comunque mai superare i 35 cicli, perché sopra tale soglia la PCR inizia a creare sequenze casuali. Ebbene, come confermato anche da diversi documenti che alleghiamo, quasi tutti i tamponi superano i 35-40 cicli di media, e sono dunque da considerare del tutto inefficaci e produttivi di falsi positivi.

9) Da ultimo, come spiegato in un recente documento dell'Istituto Superiore di Sanità, l'efficacia dei tamponi dipende da 3 fattori: la sensibilità, la capacità di rilevare la presenza di RNA, la specificità, la capacità di limitare tale RNA a quello specifico del virus che si cerca; e la prevalenza, ovvero la presenza della patologia virale nella popolazione. Questo perché maggiore è la prevalenza, maggiore è la circolazione del virus, e dunque maggiore è la possibilità di rilevarlo. Ad oggi, la prevalenza in Italia, che nelle vere pandemie può arrivare anche al 30% della popolazione, è allo 0.1%; e anche se aumentasse di 10 volte sarebbe sempre un livello di prevalenza irrisorio; il che significa, in base ad una tabella della autorevole organizzazione internazionale FIND[4] ripresa dall'ISS, che i tamponi di media performance, in Italia, producono attorno all'85%-90% di falsi positivi.

Ciò comporta che

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[3] Doc. 4 - Dott. Scoglio - Sui Tamponi Covid-19.

[4] Doc. 5 - FIND evaluation update- SARS-CoV-2 molecular diagnostics – FIND.

Comunque, ferme le superiori considerazioni già effettuate, i risultati dei tamponi sono del tutto inaffidabili.

Per tutte queste ragioni,
i sottoscritti ritengono che proseguire nell'utilizzo dei tamponi da cui ricavare dati utili a determinare proclami sullo stato di emergenza, quarantene individuali o di gruppo, e per imporre limitazioni e lockdown, dalle scuole alle imprese alle famiglie, è pratica senza nessun fondamento scientifico.

Roma, lì 07/10/2020

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