Considerations for Diagnostic Assays in Phase IIB/III Vaccine Trials for SARS-CoV-2

Version 1; October 2020

COVID-19 Enabling Sciences SWAT Team

Disclaimer: This document provides a summary of the current status of the available molecular and serology assays for SARS-CoV-2 detection as of October 2020. The document also summarizes relevant literature on diagnostic assay performance evaluations conducted to date. We have used the diagnostic pipeline that FIND has developed to track the available diagnostic assays for SARS-CoV-2. In addition, we have consulted internal and external experts on the subject matter in the preparation of the document. The main purpose of the document is to provide an overview of the available diagnostic assays and support vaccine developers to make informed decisions in their choice of diagnostic assays for use in Phase IIB/III trials for COVID-19.

This document is by no means exhaustive. Given the rapidly evolving development of diagnostic assays and related comparative performance evaluation studies, it is highly likely that the information collated in this document will change over time. Therefore, updated version will be released as new evidence is available. This document does not intend to be prescriptive or substitute the recommendations that have already been made or will be provided by regulatory authorities on the use of diagnostic assays for SARS-CoV-2. We thus encourage each vaccine developer to review available evidence and follow the relevant guidelines and recommendations on the subject matter from regulatory agencies as needed.

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COVID-19 Enabling Sciences SWAT Team

1. Introduction

CEPI is funding and facilitating the development of vaccines through partnerships for its target diseases selected from the wider range of priority pathogens identified under the WHO R&D Blueprint. To date, CEPI has established partnership agreements with more 30 vaccine developers among which nine are working on SARS-CoV-2 vaccine candidates. CEPI anticipates that approximately 50% of these SARS-CoV-2 candidate vaccines will advance to Phase IIB/III vaccine efficacy trials before end of 2020.

Diagnostic assays are crucial for vaccine development, in particular for pivotal advanced stage clinical trials establishing vaccine efficacy to support licensure. CEPI is committed to making sure that reliable diagnostics are available to implement CEPIs clinical vaccine development strategy. Phase IIB/III trials would need validated diagnostic assays with high sensitivity and specificity for case confirmation and differentiating between vaccine induced versus natural infection.

The objective of this note is to provide an overview of the available molecular and serological assays for the detection of SARS-CoV-2 and provide considerations for use in Phase IIB/III efficacy trials.

1.1 SARS-CoV-2 structural components

Knowledge on the structural components of SARS-CoV-2 is important to understand the target genes and antigens used in diagnostic assays. The SARS-CoV-2 is composed of four main structural proteins including the (Spike) glycoprotein, nucleocapsid (N) protein, small envelope (E) glycoprotein and the membrane (M) glycoprotein (1) (Figure 1). The spike protein contains S_1 and S_2 subunits. The S_1 domain has a receptor-binding domain (RBD) which is crucial for attaching to the host cell receptor angiotensin converting enzyme (ACE2). The S_2 domain contains the stalk of the spike protein and facilitates fusion between viral envelope and host cell membrane. The "S" protein is highly immunogenic. The "N" protein is the most abundant protein in the SARS-CoV-2 virion and is highly expressed at the time of host cell infection (2), Both the "N" and "S" proteins are important targets for the antibody-based detection of SARS-CoV-2 (3).

Figure 1. Structure of severe acute respiratory syndrome coronavirus 2 (4)
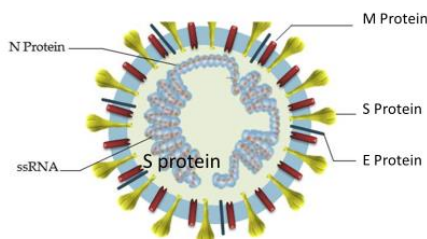
1.2 Study population and clinical endpoints for SARS-CoV-2 Phase IIB/III trials

In line with feedback from regulatory agencies as well as WHO, CEPI's clinical development team has defined the primary and secondary endpoints for Phase IIB/III trials. The endpoints' main targets are to identify both laboratory-confirmed symptomatic disease (primary endpoint) and asymptomatic infection (secondary endpoint). Diagnostic assays will be used at baseline to confirm eligibility (PCR) and serostatus upon trial entry, and for follow up of trial participants throughout the study period. Therefore, the main application of the diagnostic assays will be: 1) to rule out active SARS-CoV-2 infection/disease at the time of inclusion of trial participants; 2) to define previous exposure to SARS-CoV-2 at the time of inclusion of trial participants, and 3) continue testing trial participants for infection and also for differential seroconversion to distinguish vaccine-elicited immunity from infection-triggered immune responses, and disease at a defined time interval throughout the study period, or as required. Infection can be assessed directly by demonstration of the viral nucleic acid using reverse transcriptase (RT)-PCR, or indirectly by measurement of specific immunoglobulins using a suitable serology assay. The trial population will include individuals who are at risk of SARS-COV-2 infection and contracting severe COVID-19 in a defined geographic area. According to the vaccine developers' plans, the estimated number of trial participants may range from less than 10,000 to 30,000 or more. This indicates that the diagnostic assays to be used in vaccine trials will have to consider high capacities / throughput as well as rapid turnaround times related to several molecular and serology assay formats including rapid diagnostic tests (RDT)/point-of care testing (POCT).

2. **What types of diagnostic assays are needed for SARS-CoV-2 clinical vaccine development?**

The types of diagnostic assays needed for the SARS-CoV-2 vaccine trials mainly depend on the clinical endpoints and study design. Other factors include number of trial participants, study site /country, testing time intervals and duration of follow up period, and availability, performance, and cost of diagnostic assays. In principle, case confirmation is best achieved through detection and isolation of the virus in a patient / trial participant. In addition, defining the use of serological assays requires strategies to distinguish between vaccine-induced immune response and immune response following natural infection with the community acquired virus among the trial participants. In order to address the diagnostic assay's needs, the following specifications for molecular and serology assays are recommended to efficiently run the Phase IIB/III trials.

2.1 Molecular

2.1 RT-PCR: Optimally, a 100% sensitive and specific, and validated test for case confirmation is needed. This test should be available at reference labs/clinical trial sites. Use of an assay with a performance of less than a 100% sensitivity and specificity may have a significant effect in the results of a Phase IIB/III trials given the high number of tests foreseen. If, for example, it was planned to assess asymptomatic infection on a weekly basis in a trial with a sample size of 20,000 subjects and a follow up duration of 12 months, an RT-PCR test with a specificity of 99,9% would produce approximately n=1,040 false positive test results, which could potentially even exceed the true number of SARS-CoV-2 infections in the trial. If RT-PCR tests are applied in symptomatic patients, the positive predictive value will be much higher as compared to testing asymptomatic trial participants.

2.2 Point-of-care test (POCT) preferably an RDT (lateral flow) test that can be used for self-testing is highly required. This test should be 100% sensitive and more than 95% specific.

This will help trial participants to self-test and report from home to the trial coordinators. Samples from those with positive results must be confirmed by RT-PCR at a reference lab.

2.3 Immunoassays (ELISA, IgG/ IgM) – The best immunoassay would be to use a 100% sensitive and specific ELISA for quantifiable antibody evaluation (to predict prior infection) and assess seroconversion. However, this may be difficult to achieve. The Medicines and Healthcare Products Regulatory Agency (MHRA) in UK has developed a target product profile (TPP) for enzyme immunoassays. In this TPP, the immunoassays are expected to achieve a cut-of-point of ≥98% sensitivity and specificity.

The immunoassays to be used for SARS-CoV-2 vaccine trials should include the "full-length S" protein-based ELISA relevant for the immune response to vaccine, and an "N" protein ELISA appropriate for determining seroconversion due to natural infection.

These tests could either be done at a reference lab or at the clinical trial site. Another alternative could be to train subjects to provide the proper clinical specimens (nasal swab or saliva) which are sent to the site / lab via a courier.

The immunoassays should have no cross-reactivity with other human coronaviruses or common respiratory pathogens. It is also important to note that a N-protein based test will only work on samples taken from subjects enrolled in studies where the test vaccine does not contain the "N" protein (not suitable for e.g., inactivated whole virion-based candidates).

3. Current status of available molecular assays for the detection of SARS-CoV-2

3.1 Molecular assays
The majority of molecular-based detection kits made available for SARS-CoV-2 are real-time reverse transcriptase (RT-PCR) assays. The kits target various gene components: the open reading frame (Orf1ab) gene including the RNA dependent RNA polymerase (RdRP) gene which is situated within Orf1ab (5), the N-gene, the S-gene and the E-gene. While the E gene is highly conserved among all beta coronaviruses (6), the N gene may cross-react with other coronaviruses (6). The S gene is known for its high divergence from other coronaviruses, which is important for differentiating SARS-CoV-2 (Figure 2).

To date, there are more than 371 RT-PCR assays registered with different platforms (7): RT-PCR, RDT and POCT. The assays target between one to three genes. Having more than two target genes is an advantage. If the virus mutates, the chances of all three targets mutating is very low. The majority of the assays in the pipeline have received Emergency Use Authorization (EUA) from the FDA and CE-marking for sale in Europe. A considerable number of assays have research use only markings. There are also many in-house assays relied upon for institutional research methods.

The Foundation for Innovative New Diagnostics (FIND) has done independent performance evaluations on 21 selected manual RT-PCR assays (8). The evaluation has included assays targeting different numbers of genes (1-3 genes) (Table 1). The evaluation was conducted at the University Hospitals of Geneva (HUG). The main objective of the evaluation was to validate the limit of detection (LOD) – as defined by the developers. In order to measure the LOD, cultured viral stocks that was quantified using an E gene standard was used. The clinical performance analysis was done using specimens (50 PCR positive and 100 PCR negative) collected from persons suspected of having COVID-19 (8).

The majority of the assays showed sensitivity of (93-100%) and specificity ranging from 95-100%. The limit of detection (LOD) was found to be low (1-10 copies/mL) A lower LOD

means a more sensitive test (i.e., the higher the likelihood of detecting samples that have smaller amounts of virus).

FIND has also evaluated automated molecular tests that can be used at POC level (decentralized laboratories and/or clinics) Table 2. One of the concerns of these evaluations conducted by FIND is that they were performed in only one reference laboratory in Geneva, Switzerland. Furthermore, the number of assays evaluated (N=21) is very small compared to the high number of available molecular assays (N=3371).

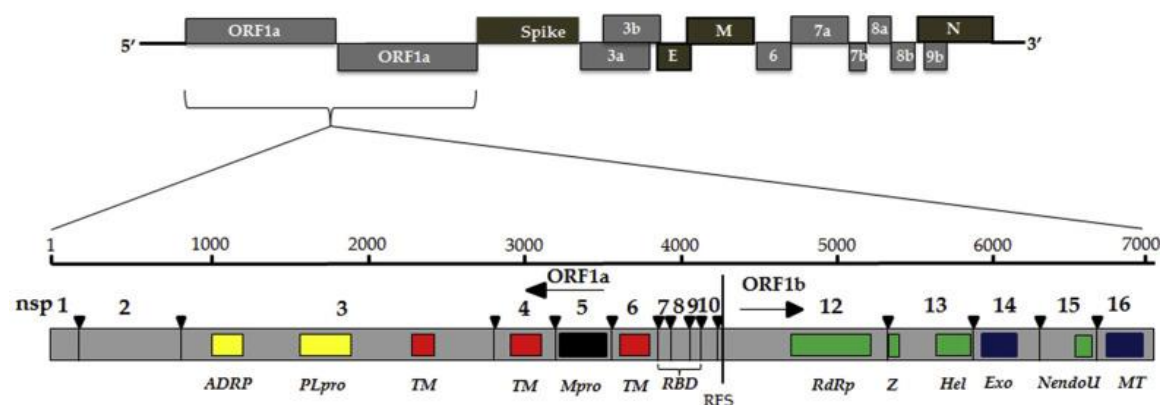**Figure 2. Genome and nonstructural proteins of SARS-CoV-2. Dandekar et al., 2005**

Table 1. Results for 21 manual (open) molecular tests included in the round 1 evaluation

Company	Product name	Gene target	Verified LOD (copies/reaction	Avg Ct (lowest dilution 10/10)	Clinical sensitivity (50 positives)	Clinical specificity* (100 negatives)
altona Diagnostics	RealStar®SARS-CoV-2RT-PCR Kit 1.0	E	1–10	35.45	92% (95%CI: 81, 97)	100% (95%CI: 96, 100)
		S	1–10	35.99	92%(95%CI:81, 97)	100%(95%CI: 96, 100)
Atila BioSystems Inc.	Atila iAMP COVID-19 Detection (isothermal detection)	ORF1ab	50–100	N/A	100%(95%CI:93, 100)	99%*(95%CI:95, 100)
		N	1–10	N/A	100%(95%CI:93, 100)	100%(95%CI:96, 100)
Beijing Wantai Biological Pharmacy	WantaiSARS-CoV-2 RT-PCR Kit	ORF1ab	1–10	36.20	100% (95%CI:93, 100)	100%(95%CI:96, 100)
		N	1–10	37.12	100%(95%CI:93, 100)	100%(95%CI:96, 100)
BGI Health (HK) Co. Ltd	RT Fluorescent, RT-PCR kit for COVID 19 (CE-IVD)	ORF1	1–10	32.43	100%(95%CI:93, 100)	99%*(95%CI:95, 100)
bioMérieux SA	ARGENE® SARS-COV-2 R-GENE®	N	10–50	36.44	100%(95%CI:93, 100)	100%(95%CI:96, 100)
		RdRP	10–50	32.44	96%[a](95%CI:87, 99)	100%(95%CI:96, 100)
Bioneer Corporation	AccuPower® SARS-CoV-2 Real-TimeRT-PCR Kit	E	10–50	35.85	100%(95%CI:93, 100)	100%(95%CI:96, 100)
		RdRP	10–50	36.18	100%(95%CI:93, 100)	100%(95%CI:96, 100)
Boditech Med. Inc.	ExAmplar COVID-19 real-time PCR kit	E	10–50	34.9	100%(95%CI:93, 100)	100%(95%CI:96, 100)
		RdRP	50–100	33.46	90%(95%CI:79, 96)	100%(95%CI:96, 100)
CerTest Biotec S.L.	VIASURE SARS-CoV-2 Real Time PCR	ORF1ab	10–50	35.16	98% (95%CI:90, 100)	100%(95%CI:96, 100)
		N	1–10	35.46	100% (95%CI:93, 100)	100%(95%CI:96, 100)
DAAN Gene Co. Ltd of Sun Yat-Sen University	Detection Kit for 2019 Novel Coronavirus (2019-nCoV) RNA (PCR-Fluorescence Probing)	ORF1	1–10	38.76	100%(95%CI:93, 100)	96%*(95%CI:90, 98)
		N	1–10	36.97	100%(95%CI:93, 100)	98%*(95%CI:93, 99)
EUROIMMUN AG	EURORealTime SARS-CoV-2	ORF1ab/N	1–10	37.88	100%(95%CI:93, 100)	98%*(95%CI:93, 99)
GeneFirst Ltd	The Novel Coronavirus (2019-nCoV) Nucleic Acid Test Kit	ORF1	1–10	35.45	100%(95%CI:93, 100)	99%*(95%CI:95, 100)
		N	1–10	36.72	98%(95%CI:90, 100)	100%(95%CI:96, 100)
KH Medical Co. Ltd	RADI COVID-19 Detection Kit	S	1–10	37.94	100%(95%CI:93, 100)	100%(95%CI:96, 100)
		RdRP	10–50	36.74	100% (95%CI:93, 100)	100%(95%CI:96, 100)
PerkinElmer Inc.	PerkinElmer® SARS-CoV-2 Real-time RT-PCR Assay[c,d]	N	1–10	39,43	100%(95%CI:93, 100)	99%*(95%CI:95, 100)
		ORF1	1–10	38,99	100%(95%CI:93, 100)	100%(95%CI:96, 100)
Primerdesign Ltd	Coronavirus COVID-19 genesig® Real-Time PCR	RdRP	1–10	36.7	100%(95%CI:93, 100)	100%(95%CI:96, 100)
R-Biopharm AG	RIDA®GENE SARS-CoV-2 RUO	E	1–10	37.99	100% (95%CI:93, 100)	100%(95%CI:96, 100)
Sansure Biotech Inc.	Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing)	ORF1	10–50	35.16	100%(95%CI:93, 100)	100%(95%CI:96, 100)
		N	10–50	34.96	100%(95%CI:93, 100)	95%*(95%CI:89–98)
SD Biosensor Inc.	STANDARD M nCoV Real-Time Detection Kit	E	1–10	37.43	100%(95%CI:93, 100)	97%*(95%CI:92, 99)
		ORF1	1–10	36.99	100%(95%CI:93, 100)	99%*(95%CI:95, 100)
Seegene Inc.	Allplex™ 2019-nCoV Assay	E	1–10	33.3	100% (95%CI:93, 100)	100%(95%CI:96, 100)
		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)
Shanghai Kehua Bio-Engineering Co. Ltd	KHB Diagnostic kit for SARS-CoV-2 Nucleic Acid (Real-time PCR)	ORF1	1–10	30.39	100%(95%CI:93, 100)	100%(95%CI:96, 100)
		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)
ThermoFisher Scientific	TaqPath™ COVID-19 CE-IVD RT-PCR Kit	ORF1ab; S, N	1–10	NA	100%(95%CI:93, 100)	100%(95%CI:96, 100)
Vela Diagnostics	ViroKey™ SARS-CoV-2 RT-PCR Test	RdRP	10–50	30.95	94%(95%CI:84, 98)	100%(95%CI:96, 100)
		ORF1	1–10	35.57	100%(95%CI:93, 100)	100%(95%CI:96, 100)
Tib Molbiol/Roche Diagnostics	ModularDx Kit SARS-CoV (COVID19) E-gene (Tib Molbiol) + LightCycler Multiplex RNA Virus Master (Roche)	E	1–10	33.34	100% (95%CI:93, 100)	100% (95%CI:96, 10)

3.2 RDT/POC testing

Molecular tests in the form of an RDT and POC test have been developed. A multi-centre evaluation of Cepheid x-pert Xpress SARS-CoV-2 was performed (9). The evaluation showed that the test allowed highly sensitive and accurate detection of SARS-CoV-2 in a number of upper and lower respiratory tract specimens. The assay has short turnaround time to results (45 minutes) (7). This test has recently been evaluated by FIND (Table 2).

Table 2. Near-POC automated tests included in the round 1 evaluation

Company	Product name	Gene target	Clinical sensitivity (50 positives)	Clinical specificity* (100 negatives)
Cepheid Inc.	Xpert® Xpress SARS-CoV-2	N2	100% (95%CI: 92,100)	99% * (95%CI: 95, 100)
		E	97.7%(95% CI: 88, 100)	100% (95%CI: 96, 100)
Molbio Diagnostics Pvt Ltd	TrueNat SARS-CoV-2	E+RdRP	98% (95% CI: 90.98)	96% * (95% CI:90,98)

There are few RDTs developed that can be used for self-administered (home-based) testing. The Redeemers University in Nigeria in collaboration with partners at Harvard have developed a molecular RDT, a lateral flow test that showed high sensitivity and specificity which is comparable to PCR. This test uses both nasal swab and saliva specimens. According to the developer, the test is very inexpensive has very short turn-around time (30 minutes) and has been rolled out in Nigeria (10). However, the performance of this test has not yet been evaluated by independent laboratories. In addition, there are other similar tests developed (8); however, none of these tests have also been evaluated by independent laboratories. Self-administered molecular tests are particularly crucial to assess asymptomatic cases in SARS-CoV-2 Phase IIB/III trials.

4.Which clinical specimens are suitable for SARS-COV-2 detection?
The types of clinical specimens used for testing may influence the amplification and detection of viral RNA and the validation of clinical sensitivity and specificity of the assays for SARS-CoV-2. Understanding the biodistribution of SARS-CoV-2 in the various types of body fluids (tissues) is useful to determine the types of specimens that are best to use for SARS-COV-2 testing. A study that analyzed eight types of clinical specimens which were collected from hospitalized patients in China revealed that the bronchoalveolar lavage (83%) and sputum (75%) samples showed higher viral loads compared to other specimens (Table 3) (11). In this analysis, most patients provided pharyngeal swabs one to three days after they were admitted. Other body fluids including blood, sputum, feces, urine, and nasal specimens were collected throughout the patients' admission periods. All the clinical specimens were tested using an RT-PCR assay targeting the open reading frame 1ab gene of SARSCoV-2.

Table 3. Results of the various types of clinical specimens tested for SARS-CoV-2 using RT-PCR assay

| Specimen type	N	Positive N(%)
Broncho alveolar Lavage	15	14 (93)
Fibrinobroscope brush biopsy	13	6 (46)
Sputum	104	75 (72)
Nasal swab	8	5 (63)
Pharyngeal swab	398	126 (32)
Feces	153	44 (29)
Blood	307	3 (1)
Urine	72	0

A related study assessed the presence of SARS-CoV-2 in the anal swab, blood, and oral swabs obtained from 16 hospitalized patients in Hubie, China (12). The findings showed more anal swab positives than oral swab positives at a later stage of infection. The result also revealed that viral nucleotide can be found in anal swab or blood even if it cannot be detected in oral swabs. In this study, the RT-PCR assay targeting the "S" gene was applied for testing the specimens.

The time of detection of viral RNA in the various clinical specimens of symptomatic patients was investigated. In the majority of the patients, viral RNA in the nasopharyngeal route became detectable as early as day one of symptoms and peaks in the first seven days of symptom onset. This positivity started to decline by the 3rd week and subsequently became undetectable (13) (Figure 3).

Figure 3. Estimated variation over time in diagnostic tests for detection of SARS-CoV-2 infection relative to symptom onset
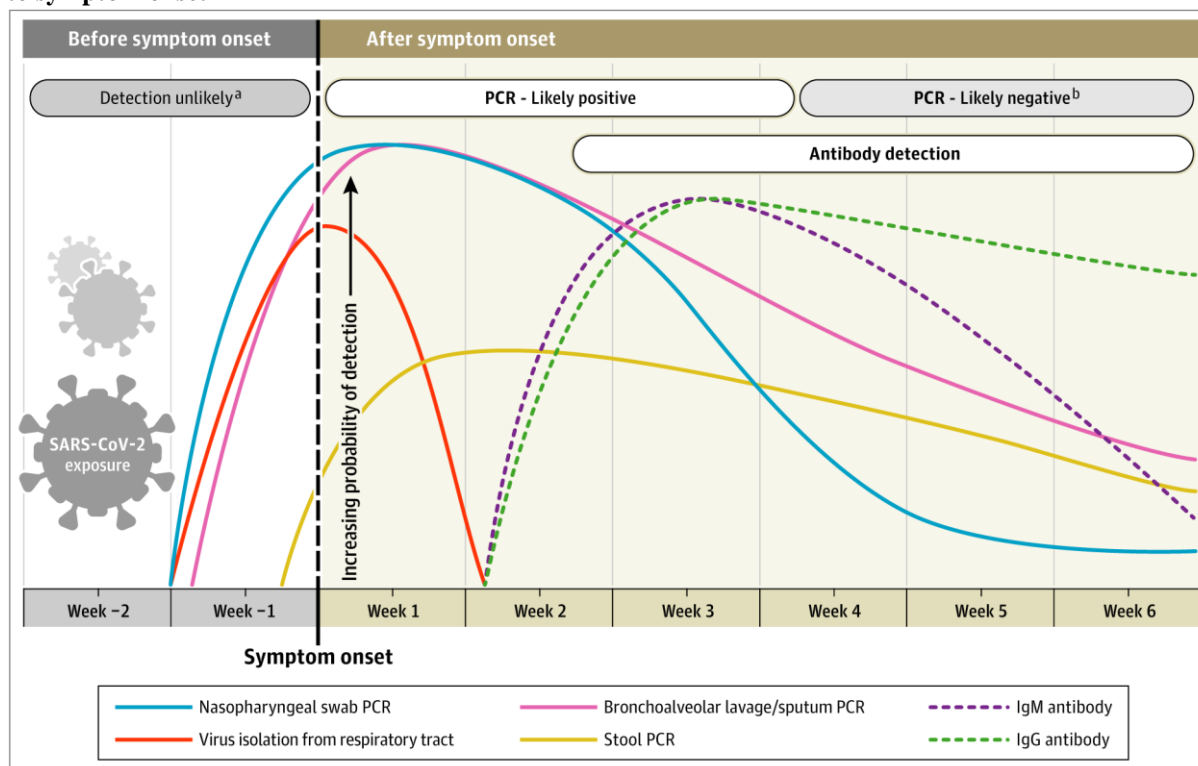
WHO has recommended nasopharyngeal swabs, oropharyngeal swabs, and nasopharyngeal or endotracheal washes as upper respiratory specimens in ambulatory patients for clinical specimen collection and testing for SARS-CoV-2 (14). Lower respiratory specimens including sputum, endotracheal aspirate, and bronchoalveolar lavage are also possible sampling options for testing. However, for asymptomatic and pre-symptomatic patients, getting sputum sample may be difficult as these group of individuals do not have productive coughs. From the vaccine development perspective, the clinical specimens to be collected for testing should be suitable enough to rule out the presence of SARS-CoV-2 among the various groups of trial participants (healthy, asymptomatic, and symptomatic cases) who can be involved during screening and actual vaccine trial periods.

5. Can saliva be used and an alternative clinical specimen for SARS-CoV-2 detection?
Several studies have been conducted evaluating the suitability of nasopharyngeal specimens and saliva for SARS-CoV-2 testing. A study that compared nasopharyngeal/throat swab and saliva samples collected from 200 persons seeking care at a respiratory clinic in China showed that the sensitivity and specificity of saliva samples were 84.2% (95% CI 79.2%-89.3%), and 98.9 (95% CI 97.5-100.3%), respectively. The observed agreement between the two clinical specimens was 97.5% (15) (Figure 4). An RT-PCR assay targeting both ORF 1ab and "N" gene was used to test the specimens.

Figure 4. Detection of SARS-COV-2 in nasopharyngeal/ throat and saliva samples by RT-PCR
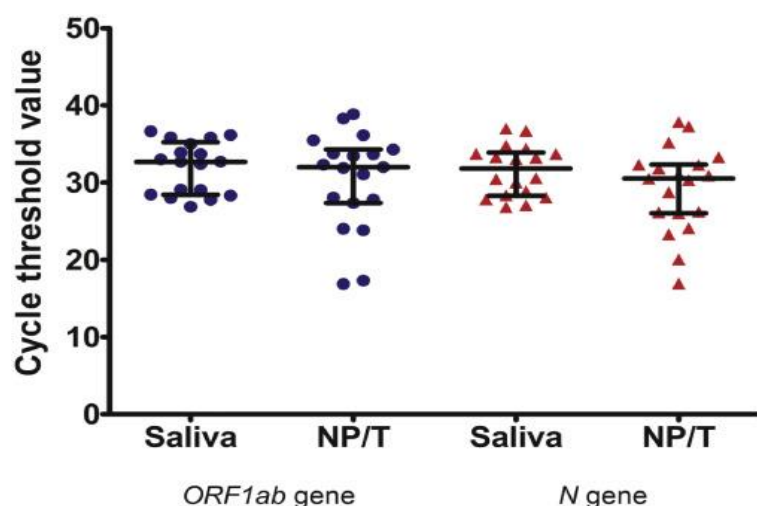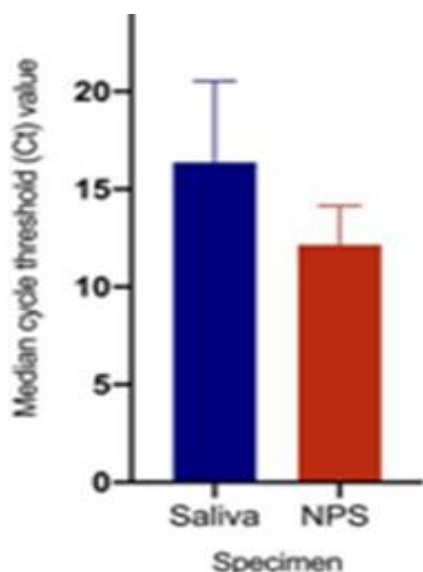

Figure 5. Detection of SARS-COV-2 in nasopharyngeal and saliva samples

Another study that tested 622 patients for SARS-CoV-2 at a screening clinic in Australia reported that 39/622 (6.3%; 95% confidence interval [CI], 4.6% to 8.5%) patients had PCR-positive nasopharyngeal swabs, and 33/39 patients (84.6%; 95% CI, 70.0% to 93.1%) had SARS-CoV-2 detected in saliva (16). The median CT value was significantly lower in the nasopharyngeal swab than saliva, suggestive of higher viral loads in nasopharyngeal swab (Figure 5). The authors concluded that saliva testing may be a suitable alternative first-line screening test in low-resource settings.

A recent study from Canada that enrolled 1939 participants including asymptomatic, high-risk persons and those with mild symptoms of COVID-19 showed that SARS-CoV-2 was detected in 70 samples, 80.0% with nasopharyngeal swabs and 68.6% with saliva (17). Thirty (48.6%) tested positive for SARS-CoV-2 on both swab and saliva samples. In this study, discordant findings were observed: 22 (31.4%) who tested positive with swab alone and in 14 (20%) who tested positive with saliva alone. The evaluation was performed using an RT-PCR assay targeting the envelope (E) gene. Finally, considering the 20% COVID-19 cases detected by saliva alone, the authors noted that standard nasopharyngeal testing may be an unreliable reference standard.

Conversely, some other studies reported the superiority of saliva over nasopharyngeal swabs in detecting SARS-CoV-2 (18). For example, a study from Italy that included 25 severely ill patients reported that all patients were SARS-COV-2 positive following their saliva sample testing. The patients were initially diagnosed for COVID-19 on admission using RT-PCR assay on nasopharyngeal swabs taken from each patient. Among all the patients, eight of them underwent a second salivary swab testing after four days, and the results were consistent

with the first test analysis. Two patients showed positive salivary results on the same days when their pharyngeal swabs showed negative test result. The RT-PCR assay used for testing was the one step RT-PCR Luna Universal qPCR master mix. The authors concluded that patients should be checked for salivary viral load at time of hospital discharge. Authors also recommended that patients who had recovered should only be discharged after two sequential pharyngeal swabs and one salivary swab tested negative (18).

A recent study that analyzed saliva and nasopharyngeal specimens obtained from 44 hospitalized patients and 98 asymptomatic health workers revealed that the saliva samples yielded greater detection sensitivity. This result was found to be consistent throughout the course of infection among the cases. The 44 cases represented critically ill patients, with 19 (43%) needing intensive care without ventilation, and 10 (23%) requiring mechanical ventilation. The study finding among the 98 asymptomatic health care workers revealed that SARS-CoV-2 was found in saliva from two healthcare workers who were negative by nasopharyngeal swabs and did not report any symptoms (19). The RT-PCR test kit used in this study was the US CDC N-gene (N1 &N2) based assay. The conclusion from this study was that saliva is a viable and more sensitive alternative to nasopharyngeal swabs (Figure 6).

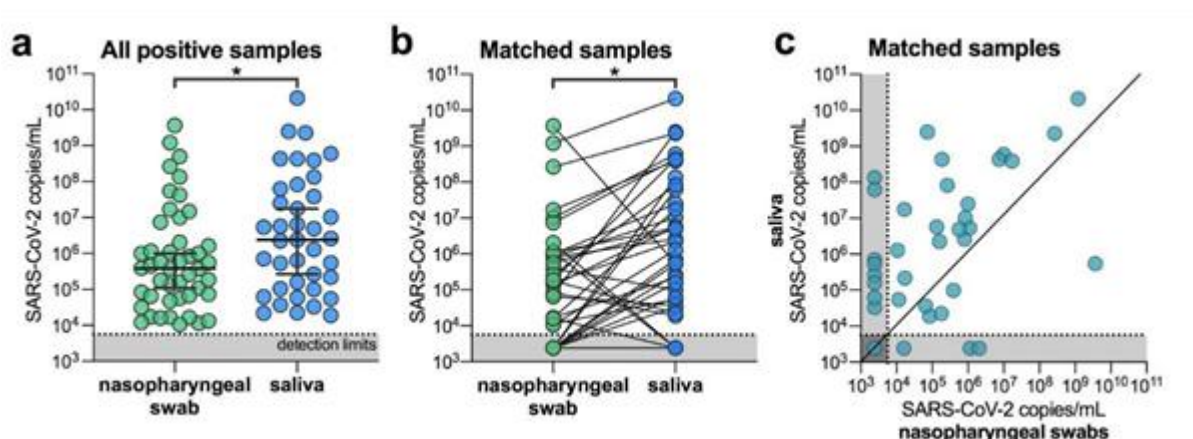**Figure 6. Comparison of saliva and nasopharyngeal samples testing for SARS-CoV-2 (19)**

Figure 6 "SARS-CoV-2 titers are higher in the saliva than nasopharyngeal swabs from hospital inpatients. (a) All positive nasopharyngeal swabs (n= 46) and saliva samples (n= 39) were compared by a Mann-Whitney test (p< 0.05). Bars represent the median and 95% CI. Our assay detection limits for SARS-CoV-2 using the US CDC "N1" assay is at cycle threshold 38, which corresponds to 5,610 virus copies/mL of sample (shown as dotted line and grey area). (b) Patient matched samples (n= 38), represented by the connecting lines, were compared by a Wilcoxon test (p< 0.05). (c) Patient matched samples (=n 38) are also represented on a scatter plot" (19).

Another recent study published in the New England Journal of Medicine reported the detection of higher SARS-CoV-2 RNA copies in the saliva specimens (mean log copies per milliliter, 5.58; 95% confidence interval [CI], 5.09 to 6.07) than in the nasopharyngeal swabs (mean log copies per milliliter, 4.93; 95% CI, 4.53 to 5.33). The study results also showed that a higher percentage of saliva samples than nasopharyngeal swab samples were positive up to 10 days after patients' diagnosis was made. In addition, the result showed that at one to five days following diagnosis, 81% (95% CI, 71 to 96) of the saliva samples were positive, as compared to 71% (95% CI, 67 to 94) of the nasopharyngeal swab specimens (20) These finding were based on the assessment of clinical specimens collected from 70 hospitalized patients. Furthermore, this study had also screened saliva and nasopharyngeal samples obtained from 495 asymptomatic health care workers for SARS-CoV-2. The finding revealed that SARS-CoV-2 RNA was detected in the saliva samples collected from 13 health care

workers who did not have any symptoms at or prior to specimen collection. Of these 13 health care workers, nine had collected matched nasopharyngeal swab specimens by themselves on the same day, and 7 of these specimens tested negative (Figure 7). The specimens were tested using the US CDC RT-PCR assay targeting the N-based (N1&N2) gene of SARS-CoV-2.

Figure 7. Comparison of saliva and nasopharyngeal samples for SARS-CoV-2 detection among hospitalized patients and asymptomatic cases (20).
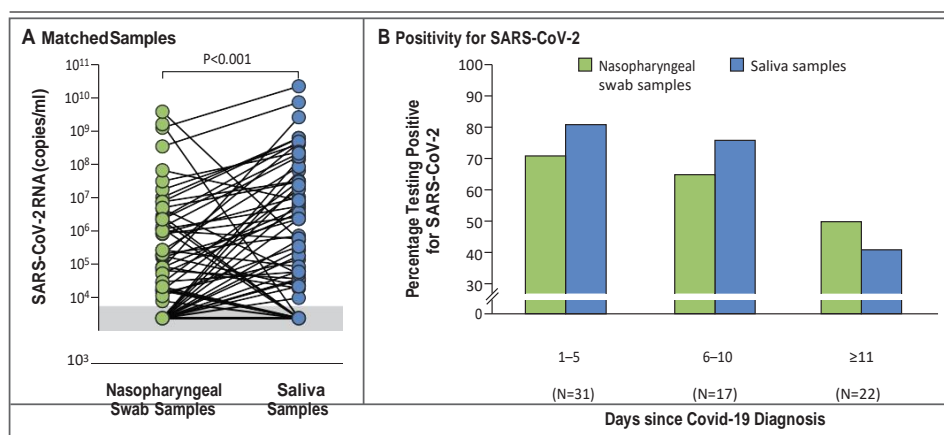

Figure 7. "Panel A shows SARS-CoV-2 RNA titers in the first available nasopharyngeal and saliva samples. The lines indicate samples from the same patient. Results were compared with the use of a Wilcoxon signed-rank test (P<0.001). Panel B shows percentages of positivity for SARS-CoV-2 in tests of the first matched nasopharyngeal and saliva samples at 1 to 5 days, 6 to 10 days, and 11 or more days (maximum, 53 days) after the diagnosis of Covid-19" (20).

6. The role of RNA extraction methods in SARS-CoV- 2 detection

A crucial step in the detection of SARS-CoV-2 is to isolate high-quality viral RNA from a patient specimen. RNA is highly sensitive to degradation by ribonucleases. Inefficient RNA purification leads to poor PCR sensitivity (20). It has been reported that more than 90% of RNA is thrown away due to lack of efficient RNA extraction methods. Several RNA extraction methods have been developed (Table 4). The commonly used RNA extraction technique uses phenol-guanidine isothiocyanate (GITC). This method is able to rapidly denature nucleases and stabilize RNA. The main challenges of this method are that it is laborious and time-consuming. In addition, the extracted RNA can be contaminated by residual salts and organic solvents which inhibit downstream RT-PCR amplification. The other RNA extraction methods include use of magnetic beads and spin columns which are not commonly available in resource-poor countries. These methods can remove organic solvents and contaminants. However, the challenge here is accessing the RNA which is tightly encapsulated by the nucleocapsid protein. One possible solution for this could be preheating the samples to denature the viral proteins and release the RNA. Nonetheless this solution has been found to negatively affect the ability of RT-PCR assays to detect specimens containing low viral loads (21). Recently, RNA extraction-free techniques have been developed which are believed to be very useful for POC testing. However, the main challenge here is the need for addressing the effect of "complex specimen matrixes" that have the capacity to impede the downstream PCR amplification process. For instance, chemicals that are added to inactivate viruses at the time of sample treatment can inhibit PCR reaction (22). Overall accessing the viral RNA and extraction of high-quality RNA is a key challenge that affects the performance of RT-PCR tests. Majority of the available RNA extraction kits in the market to date are not efficient enough in ensuring high-quality RNA. Recent developments indicate that there are several in-house RNA extraction kits that are more efficient than the commercially available

kits in the market to date. Follow up the commercialization and independent evaluations results of such kits is required.

Table 4. Commercially available RNA extraction kits used for diagnosis of COVID-19 (23)

RNA extraction kit	Technology	Time per prep	Supplier
QIAamp® Viral RNA Mini Kit	spin-column	20-40 min	QIAGEN
EZ1 DSP Virus Kit	Magnetic beads	40 min	QIAGEN
Roche MagNA Pure Compact RNA Isolation Kit	Magnetic beads	30-40 min	Roche
Roche MagNA Pure Compact Nucleic Acid Isolation Kit	Magnetic beads	25 min	Roche
Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit	Magnetic beads	<1 hour	Roche
Invitrogen ChargeSwitch® Total RNA Cell Kit	Magnetic beads	15 min	Invitrogen

7. Immunoassays

Serology assays are needed for screening individuals prior to inclusion into vaccine trials, and to monitor immune responses in vaccine trial participants. According to the pipeline data from FIND, there are more than 420 immunoassays registered in the database to date. These immunoassays comprise variable platforms including RDTs such as lateral flow immunoassays (LFAs), ELISAs and chemiluminescent immunoassays (CLIA). Most of the assays are commercial, others have research use only (RUO) markings. There are also considerable number of in-house assays (24).

Most of the RDTs (antigen-and antibody-based), and antibody-based IgG, IgM, IgA ELISAs are manual and use serum or plasma. There are also automated fluorescent immunoassays which enable to measure the amounts of targeted analytes (viral antigen or IgM/IgG).

The antigen tests are designed to detect viral proteins in swab samples. They employ monoclonal antibodies specific for the viral antigens. High viral burden is important for the best outcome of antigen tests.

Several factors may affect the performance of serology assays. The earlier the stage of the infection, the higher chance of a sero-negative result. A recent study reported that the median seroconversion time following symptoms onset was between 13-14 days (25). The study also revealed that not all patients with COVID-19 developed antibodies. This means that there is a chance that some patients with severe or mild illness, or asymptomatic cases may not produce antibodies at all or may generate inadequate antibodies which may not be detected in their samples. Furthermore, current evidence shows that titers of antibodies sufficiently high for detection may not persist for the long-term hence it is highly likely that patients with high antibody titer during the acute infection period could have negative test results sometime at a later stage. Therefore, the sensitivity of a serology assay may depend on the time from acute

infection to testing. This clearly indicates that it is not logical to expect 100% sensitivity for any antibody test during the course of an infection and beyond. In areas where the prevalence of SARS-CoV-2 is low, it is highly recommended that an IgG test with a specificity of >97% must be used. Having a test with such high specificity significantly minimizes the possibility of false positive test results (26).

The performances of several immune assays were reviewed in a recent meta-analysis. A study by Cain et al., (27) showed 82% sensitivity for IgM, and 85% for IgG and total antibodies. The pooled specificity estimate was 98% for IgM and 99% for IgG and total antibodies. The authors concluded that in populations with ≤5% of seroconverted persons, the positive predictive value would be ≤88% unless the assays have a 100% specificity (27).

The main challenge with SARS-CoV-2 serologic assays is the potential for cross-reactivity with antibodies to the commonly circulating alpha- (NL63 and 229E) and beta- (OC43 and HKU1) and MERS-CoV coronaviruses which affect the specificity of the test (28). A prior study revealed that the S_1 subunit of the spike protein including the RBD showed very low cross-reactivity between epidemic coronaviruses and common human coronaviruses, whereas the S_2 domain of the spike protein and the "N" protein showed low-level cross-reactivity between the various coronavirus subtypes (29). A recent study reported that over 90% of persons aged 50 years and above had antibodies against all four-common circulating CoVs, which indicates that the potential for cross-reactivity in SARS-CoV-2 serologic assays is significant (28). This is particularly important in the application of serology assays for clinical vaccine development. An N protein-based immunoassay to be used for distinguishing vaccine induced versus natural infection must be robust, highly sensitive and specific. In order to rule out potential cross-reactivity between antibodies to SARS-COV-2 and other coronaviruses, assays validation/evaluations must be performed using well characterized samples collected from both healthy individuals, mild, moderate and severely ill COVID-19 cases and those with antibodies to common infectious pathogens and from individuals with non-infectious disease.

Despite the high number of serology assays developed to date, there have not been adequate independent comparative performance evaluations for SARS-CoV-2 immunoassays. A study that evaluated 17 rapid tests revealed that the sensitivities and specificities of the assays varied between the tests. Seven tests showed IgG sensitivity of more than 90%, and five tests had IgG sensitivity of less than 85%. Twelve rapid tests had IgG specificity of 97% or above. Among the tests with very high IgG specificity, three tests also had IgG sensitivity above 90% (30).

A recent immunoassay evaluation led by the Erasmus Medical Center in the Netherlands included three rapid tests, four IgG, IgM ELISAs, and a high throughput chemiluminescent immunoassay. The result showed the presence of wide assay performance diversity when compared to virus neutralization assay. Among the evaluated immunoassays, the Wantai ELISA detecting IgG antibodies against the RBD was found to be the best performing in detecting functional antibodies (31). The overall sensitivity and specificity were 99%. The estimated sensitivity level was maintained at the same level of measure ≥14 days post symptom onset (Figure 8). No "N" protein-based ELISA assay was included in this evaluation.

Figure 8 Evaluation of COVID-19 serological assays, 2020 (31)
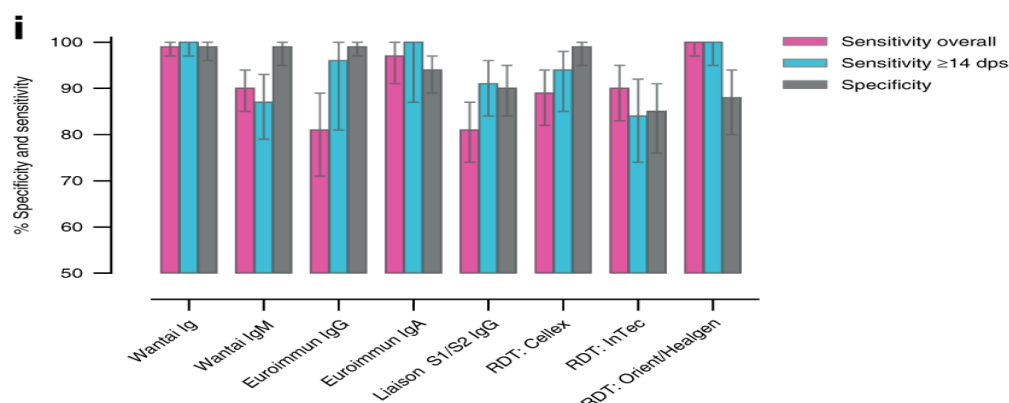
A collaborative immunoassay evaluation led by the Public Health England and University of Oxford was conducted in two independent laboratories in London, UK. In this evaluation, a total of four commercial and one in-house immunoassays were included in a head-to-head comparison. The result revealed that the Siemens and the Oxford spike protein-based immunoassays achieved sensitivity and specificity of at least 98% on samples taken 20 days post symptom onset. The results provided consistent measures in both mild and severe cases of COVID-19. The two assays target IgG antibodies.

Figure 9. Performance characteristics of five immunoassays for SARS-CoV-2: a head- to-head comparison (32)
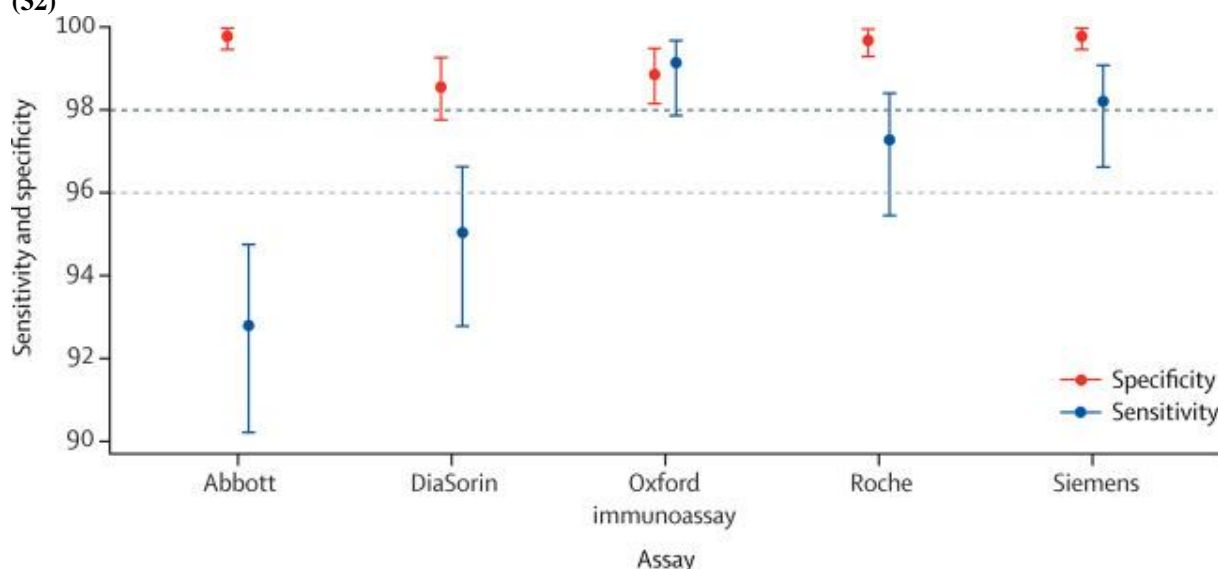
FIND is coordinating independent evaluations on selected immunoassays (33). The current evaluation has included five antigen (Ag)-based RDTs, 25 serological antibody (Ab)-based RDTs and eight serological Ab-based manual ELISAs targeting the "S" and "N" antigens. The result of the antigen tests evaluation is presented in Table 5. According to FIND, the outcome of the evaluations for the rest of the immunoassays under evaluation will be released when completed. Lack of well characterized samples has been a challenge affecting expedited assay evaluations.

Table 5. The results of four antibody RDTs evaluation

Isotype	Company	Sensitivity				Specificity
		All (n=287)	Day 0-7 (n=83)	Day 8-14 (n=70)	Day 15+ (n=134)	
IgM	BIOMEDOMICS[1]	57.0% [37.4%-74.5%]	33.7% [24.5%-44.4%]	62.9% [51.1%-73.2%]	72.4% [64.3%-79.2%]	95.1% [90.8%- 97.4%]
	BTNX[2]	85.5% [62.0%-95.5%]	59.0% [48.3%-69.0%]	87.1% [77.3%-93.1%]	95.5% [90.6%-97.9%]	84.4% [75.8%- 90.4%]
	INNOVITA	60.5% [38.3%-79.2%]	33.7% [24.5%-44.4%]	68.6% [57.0%-78.2%]	76.1% [68.2%-82.5%]	95.9% [90.3%- 98.3%]
	VIVACHEK	71.2% [44.0%-88.6%]	38.6% [28.8%-49.3%]	78.6% [67.6%-86.6%]	86.6% [79.8%-91.3%]	92.9% [87.6%- 96.0%]
IgG	BIOMEDOMICS[1]	55.4% [23.7%-83.2%]	21.7% [14.2%-31.7%]	55.7% [44.1%-66.8%]	84.3% [77.2%-89.5%]	98.4% [91.3%-99.7%]
	BTNX[2]	69.6% [36.6%-90.0%]	34.9% [25.6%-45.7%]	68.6% [57.0%-78.2%]	91.0% [85.0%-94.8%]	97.8% [94.2%-99.2%]
	INNOVITA	50.3% [26.2%-74.2%]	21.7% [14.2%-31.7%]	61.4% [49.7%-72.0%]	69.4% [61.2%-76.6%]	98.9% [95.7%-99.7%]
	VIVACHEK	68.3% [38.3%-88.2%]	32.5% [23.4%-43.2%]	77.1% [66.0%-85.4%]	85.8% [78.9%-90.7%]	96.2% [92.2%-98.2%]
IgG-IgM	BIOMEDOMICS[1]	68.7% [41.9%-87.0%]	37.3% [27.7%-48.1%]	74.3% [63.0%-83.1%]	85.8% [78.9%-90.7%]	95.1% [90.8%-97.4%]
	BTNX[2]	87.7% [65.0%-96.5%]	61.4% [50.7%-71.2%]	90.0% [80.8%-95.1%]	96.3% [91.6%-98.4%]	82.6% [74.9%-88.3%]
	INNOVITA	65.4% [40.4%-84.0%]	34.9% [25.6%-45.7%]	75.7% [64.5%-84.2%]	79.9% [72.3%-85.8%]	95.8% [89.1%-98.5%]
	VIVACHEK	71.2% [44.0%-88.6%]	38.6% [28.8%-49.3%]	78.6% [67.6%-86.6%]	86.6% [79.8%-91.3%]	92.9% [87.6%-96.0%]

[1] Generation 1 test, which supplier is replacing with a Generation 2 test.

[2] Performance may have been compromised due to prolonged transit times in which temperature control may have not been maintained.

The Public Health England (PHE) (34) and the National Institute of Health (NIH) (35) have performed individual assay performance evaluations for a number of immunoassays that served as a basis for emergency use approval by regulatory bodies. These evaluations have shown very high sensitivity and specificity results for considerable number of assays. However, as the evaluations performed were not comparing the best-performing assays against each other, it may not be possible to select the best of the best assays for vaccine development purposes.

8. Sequencing

Many laboratories around the world are performing whole genome sequencing for SARS-CoV-2 isolates identified in their laboratory. This is important for diagnostic assays modification as possible mutations can be identified and primers can be redesigned. Currently, the GISAID initiative is providing a platform for the storage and rapid sharing of SARS-CoV-2 genomic data (36). Since the start of the COVID-19 outbreak, GISAID reported that 82,000 viral genomic sequences of hCoV-19.

9. Conclusion

Several CEPI awardees have planned to start Phase IIB/III trials for SARS-CoV-2 vaccine candidates before end of 2020. The availability of molecular and serology assays is essential for efficient implementation and conduct of trials. These tests need to be reliable in order to establish the candidate vaccine's efficacy against SARS-CoV-2 infection, as well as COVID-19 disease eventually supporting vaccine licensure and wide deployment within public health measures. Several molecular assays have been developed with reasonably high sensitivity and specificity. The FIND first round evaluation on RT-PCR assays has provided an excellent list of assays options to choose from for the Phase IIB/III trials. Most of the assays evaluated showed sensitivity and specificity of 100% with a confidence interval ranging from 93-100% with limit of detection ranging from 1-10 viral copy numbers which shows that the assays can even detect very low number of viral copies of SARS-CoV-2. The fact that most of the best performing assays target more than two genes in the virus is a great advantage. However, there are two concerns. Firstly, the confidence interval for the specificity of the best performing assays is estimated to be as low as 96% and as high as 100%. This shows that some trial participants may likely have false positive results. Secondly, the independent assays evaluation was only done in one laboratory. We do not know, if the current test results would be maintained if several laboratories were involved. FIND is planning to conduct additional rounds of evaluations on selected molecular assays, and we will follow up if the

next evaluations will include several labs and result in change of assays performances. However, given that several of the SARS-CoV-2 vaccines are entering/have entered in advanced clinical trials, we suggest that developers use the best performing assay as per FIND's evaluation result.

Self-administered molecular tests (RDTs) are useful to assess asymptomatic SARS-CoV-2 cases during the follow up of period of vaccine trials. At present, few promising RDTs have been developed and these need to be validated for use in the upcoming vaccine trials.

From a vaccine development perspective, the clinical specimens to be collected for testing should be suitable enough to rule out the presence of SARS-CoV-2 in the various groups of trial participants (healthy, asymptomatic, and symptomatic cases) who will be involved during screening and actual vaccine trial periods. The nasopharyngeal swab has been the recommended method for testing suspected COVID-19 cases. Recent studies have shown saliva to have had comparable results, and in some studies, to have been more sensitive than nasopharyngeal swabs in detecting SARS-CoV-2 among asymptomatic, pre-symptomatic and symptomatic individuals. This suggests the potential of saliva specimens for use in the detection of SARS-CoV-2. However, several limitations were observed in all the studies. Firstly, the studies used different types of RT-PCR assays targeting the various SARS-CoV-2 genes, making comparisons among the studies difficult. In this regard, the National Institute for Biological Standards and Control (NIBSC) has been playing a key role in leading the development of RNA standard for SARS-CoV-2. Secondly, the sample sizes included in the studies were not adequate enough to assess performance of RT-PCR assays in the various specimens. Thirdly, the studies lacked adequate representation of asymptomatic, mild, moderate and severely ill cases for measuring and comparing sensitivity and specificity among the groups during the conduct of the respective studies. Therefore, additional studies comprising adequate samples size and representation of healthy, asymptomatic, and symptomatic cases are warranted for recommending the best possible clinical specimens to use for vaccine trials.

Current reports show that the available RNA extraction kits in the market are not efficient enough in ensuring high-quality intact RNA for PCR testing of SARS-CoV-2. This challenge needs to be addressed to secure optimal RT-PCR test results during the trial periods.

Serology assays are essential for assessing previous exposure to SARS-CoV-2 and defining seroconversion in vaccine trials. Even though several serology assays have been developed, the number of comparative performance evaluations conducted to date is not adequate. Therefore, there is a need for continuing assay evaluation to ensure clinical accuracy of test results. The evaluation should include assessment of sensitivities and specificities using well characterize samples representing asymptomatic, mild moderate and severely ill COVID-19 patients. Currently, serology assays performance evaluation is undergoing by FIND. Based on the outcome of the evaluation, we will update this document.

10. Recommendations

1. For case confirmation, developers can choose the best performing RT-PCR assay from the list of assays already evaluated, for example by FIND (Table 1). The criteria for choosing the specific assay should include assay performance in terms of sensitivity and specificity, and limitation of detection (LoD), and number of targeted genes. Therefore, an assay with very high sensitivity and specificity, LoD 1-10 viral copies/mL targeting more than two genes (E, ORF1ab and N) and does not cross react

with other coronaviruses is recommended. As there could be more rounds of RT-PCR assay evaluations, it is also worth following up the result of the upcoming evaluations and revise the current recommendation accordingly.

2. An RDT (molecular) in the form of lateral flow test is crucial for home-based testing to efficiently follow up trial participants for asymptomatic infections. There is an urgent need of evaluating the available RDTs (molecular) for SARS-CoV-2 for potential use in the upcoming Phase IIB/III trials. If this is not possible, trial participants should be provided with the proper sampling device, training on specimen collection and send the samples for PCR testing at the central lab/are or at the clinical trial site lab.

3. Based on the available evidence, there is a potential for use of saliva specimens in the detection of SARS-CoV-2. Testing both nasopharyngeal swab and saliva samples at the time of trial participant recruitment, and for each participant during the trial period may have synergetic effect in detecting more SARS-CoV -2 cases there by reducing false negative results. We therefore suggest the application of both nasopharyngeal and saliva specimens at the same time for testing SARS-CoV-2 during Phase IIB/III trials.

4. In terms of choice for serology assays, there are few numbers of validated spike protein-based assays. The Wanti, Siemens and the Oxford spike protein-based immunoassays have shown very high sensitivities and specificities. Vaccine developers may use one of these assays to evaluate vaccine elicited immune responses. Immunoassays detecting "N" protein can distinguish between responses induced by vaccination with "S" based vaccines and SARS-CoV-2 infection. Currently, there are not highly specific (>98%) and validated N protein-based immunoassays that can be used to define seroconversion due to a natural infection from SARS-CoV-2. Several assays (IgG, IgM ELISAs) targeting the "S" and "N" proteins are undergoing independent evaluations by FIND. CEPI recommends selecting an N-based immunoassay for vaccine trials based on data derived from independent evaluations, when available.

Overall, for recruiting participants into a vaccine trial and defining seroconversion among trial participants during the study period, it is advisable that developers use both a N-based immunoassay and full-length "S" based immunoassay that fulfill the above described level of sensitivity and specificity. This may likely ensure high degree of confidence to rule out false positive or negatives test results.

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