Rijksinstituut voor Volksgezondheid
en Milieu
*Ministerie van Volksgezondheid,
Welzijn en Sport*

RIVM

A. van Leeuwenhoeklaan 9
3721 MA Bilthoven
Postbus 1
3720 BA Bilthoven
www.rivm.nl

T 030 274 91 11
info@rivm.nl

Toelichting op vraag van Haga aan Jaap van Dissel

Naar aanleiding van de update van de webpagina 'Aanvullende informatie diagnostiek COVID-19 Bijlage bij de LCI-richtlijn COVID-19 | Versie 25 september 2020' (https://lci.rivm.nl/covid-19/bijlage/aanvullend) zijn een aantal vragen gesteld. Updates van deze bijlage naar de huidige stand van ontwikkelingen en inzichten vinden regelmatig plaats.

Vraag: waarom is de afkapwaarde voor de Ct-waarde bij de PCR aangepast?
RIVM 15 oktober 2020

Deze vraag refereert naar de volgende zinsnede in het document:

> RdRP-gen-PCR SARS-CoV-2 door diverse laboratoria specifiek en vergelijkbaar gevoelig als de E-gen-PCR gemaakt. De amplificatiecurve dient goed te worden beoordeeld bij de hogere Ct-waardes (zeker bij een Ct van >35). Is de curve afwijkend, onbetrouwbaar of moeilijk te interpreteren, dan is zeker bij een epidemiologisch onverwachte positieve uitslag confirmatie nodig. Afhankelijk van de lokale implementatie met dit gen kan dat met de RdRP-PCR of andere eigen PCR op het RNA, of door her-testen van hetzelfde monster door het eigen of samenwerkingslab, of door de patiënt opnieuw te bemonsteren.

Daar stond in de voorgaande versie van het document de Ct-waarde >30. De onderliggende vraag bij de vraag naar aanpassing van deze waarde suggereert dat het hier gaat om een afkapwaarde die onderscheid maakt tussen positief en negatief. Dat is niet zo. Het gaat hier om een waarschuwingswaarde. Ct-waarden van COVID-19 patiënten kunnen typisch variëren van circa 15 tot 40, met de meerderheid tussen 20 en 35 (grenzen afhankelijk van gebruikte apparatuur en chemie voor de PCR-test). Tegen de limiet van detectie van de PCR-test die zich typisch tussen Ct 35 en 40 bevindt, moeten de amplificatiecurves extra goed bekeken worden of ze aan de S-curve eis voldoen. De tekst gaat over de Ct-waarde waarboven men extra alert moet zijn bij beoordelen van de amplificatiecurves. Het betreft een aanwijzing die kan helpen bij de beoordeling van de diagnostiek.

Sommige laboratoria gebruiken om te bepalen of de PCR-test positief of negatief is een Ct afkapwaarde of andere afkapwaarde voor methoden waarbij geen Ct-waarde wordt gegenereerd. Andere laboratoria gebruiken geen afkapwaarde, maar beoordelen amplificatiecurves van het genetisch materiaal van het virus of een combinatie van beiden gebruiken. De keuze voor methode van beoordelen van een PCR- test wordt per laboratorium onderbouwd in de eigen validatie en gebruikte testalgoritmen of vastgelegd in de 'Instructions for use' (IFU) van de gebruikte commerciële kit. Daarom verschillen de beoordelingsmethoden tussen laboratoria en gebruikte testen en kan er geen generiek specifieke richtlijn voor gegeven worden. Voor "laag-positieve" resultaten waarbij getwijfeld wordt aan de juistheid, meestal een amplificatie signaal in de buurt van de detectiegrens van de PCR-test, geldt dat elk laboratorium een eigen algoritme heeft hoe hiermee om te gaan. Dit staat ook in de IFU van commerciële testen. Het vervolg op zo'n signaal kan inhouden dat er een bevestigingstest gedaan wordt op hetzelfde materiaal of dat nieuw materiaal gevraagd wordt van enkele dagen later.

Toelichting op wijzigingen

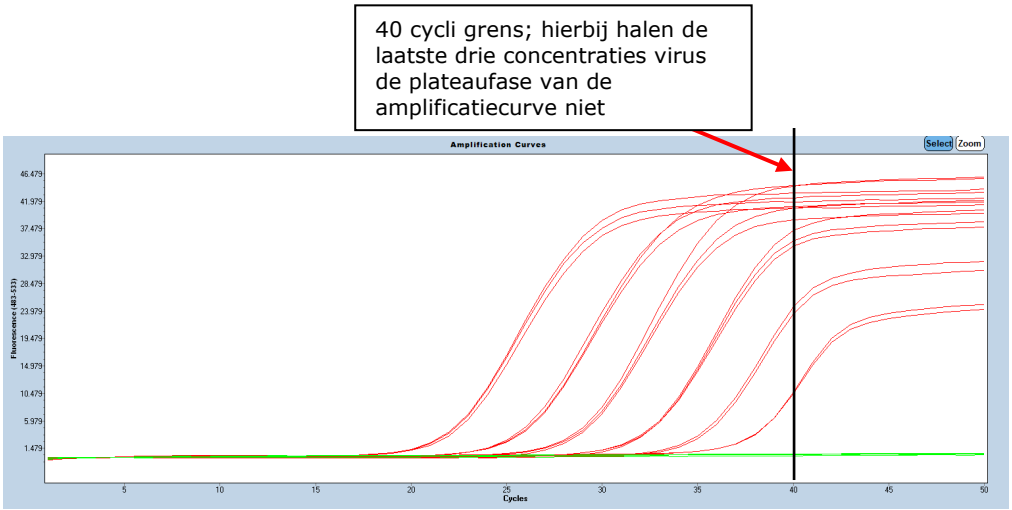
Het landschap van PCR testen voor SARS-CoV-2 heeft zich van het begin met twee in-house protocollen met één set primers en probes met twee target genen (E-gen en RdRP-gen) uitgebreid naar een scala aan protocollen gebaseerd op in-house ontwikkeling en in gebruik nemen van commerciële PCR testen en testen gebaseerd op andere amplificatietechnieken. Die hebben allemaal hun eigen optimale temperatuur waarbij de amplificatie plaatsvindt en eigen optimale aantal amplificatiecycli. Het optimum aantal cycli ligt voor het overgrote aantal testen tussen de 40 en 45 en is niet in de tijd verandert. De WHO COVID-19 referentielaboratoria ErasmusMC en RIVM gebruiken voor het originele protocol respectievelijk 45 en 50 cycli.

Het verschil in aantal cycli voor waarschuwings Ct-waarde van eerst 30 naar nu 35 in het document maakt niet dat er meer monsters positief worden of dat er meer foutief positieven bij zouden komen. Meer cycli draaien kan helpen om monsters die laat in het aantal cycli positief worden een nog duidelijkere S-vormige amplificatiecurve te geven. Maar daar gaat het niet om bij de wijziging van 30 naar 35 zoals hiervoor beschreven. Door kwaliteitscontrole weten we dat alle PCR testen en andere amplificatietesten die nu in Nederland gebruikt worden een vergelijkbare limiet van detectie hebben.

We moesten met eerst weinig positieven in het begin van de epidemie in Nederland nog ervaren hoe de ontwikkelde en uitgerolde PCR-test voor SARS-CoV-2 zich gedroegen en wat de dynamiek van de virale load tijdens het ziektebeloop van patiënten was. Daarom was in de voorgaande versie van het document in de concluderende paragraaf over de beoordeling van resultaten van de originele PCR-test aan de veilige kant geschreven om amplificatiecurves boven Ct 30 die er niet typisch uitzien (geen duidelijke S curve hebben die op exponentiële vermeerdering wijst) nader te bekijken en te confirmeren. Confirmeren kan dan met een andere test op hetzelfde monster als er een andere test beschikbaar is in het lab, of door de patiënt opnieuw enkele dagen later te laten bemonsteren. De verandering van Ct 30 naar Ct 35 als waarschuwingswaarde is ingegeven omdat gebleken is dat beneden Ct 35 de curves altijd wel typisch waren en dat er boven Ct 35 tegen de limiet van detectie van de PCR-test er pas niet typische curves konden ontstaan. Dit is eigenlijk niet anders dan bij PCR testen voor andere pathogenen. Maar nu door ervaring ook voor SARS-CoV-2 bevestigd.

Omdat veel labs niet meer de eerste uitgerolde PCR opzet gebruiken is die Ct 35 ook weer ‘vloeibaar’ geworden. Commerciële kits definiëren in hun IFU eigen Ct waarschuwingswaardes, als ze dat al doen. Elke implementatie zou volledig gekarakteriseerd moeten worden om een waarschuwingswaarde te definiëren. Met een algemene Ct 35 als waarschuwingswaarde om daarboven nauwkeuriger naar amplificatiesignalen te kijken zit je nog steeds aan een veilige kant.

Hoe zit het dan met de veelbesproken afkapwaarde voor positief of negatief? Die is er eigenlijk niet voor een goed ontwikkelde PCR-test. Minder dan 1 molecuul kan niet gedetecteerd worden en 1 molecuul in een PCR-test heeft na pakweg 35-36 amplificatiecycli voldoende kopieën gegenereerd om boven de fluorescentie achtergrond waarde uit te komen. Typisch voorbeeld van SARS-CoV-2 detectie met protocol wat gebruikt wordt op het RIVM hieronder. Dit is een efficiënt verlopende PCR reactie. Zoals te zien is verlengd meer dan 40 cycli draaien de laatste curve in de grafiek verder naar rechts waardoor de S-vorm zichtbaar wordt, maar de Ct waarde verschuift daardoor niet.



Figuur. Amplificatiecurves van RdRP-gen van 10-voudige verdunningsreeks SARS-CoV-2 gesimuleerde klinische monsters in drievoud getest (rood) in de achtergrond van negatieve klinische monsters (groen). De laatste curves rechts zijn bij een concentratie virus op de detectiegrens van de PCR-test waarbij de PCR-test in 2 van de 3 herhalingen een amplificatiecurve geeft. Zou 40 cycli gedraaid zijn ipv 50 cycli dan zouden de laatste curves voordat ze de S-vorm krijgen afgekapt zijn. Dat geldt ook voor de 10-voudig en 100-voudig hogere concentraties daarvoor. 50 cycli draaien maakt het beoordelen van de curves een stuk gemakkelijker. Bij minder dan 40 cycli draaien zou er sensitiviteit verlies optreden.

Voorbij de absolute limiet van detectie van 1 molecuul per PCR reactie komen er geen specifieke curves bij. Zou je meer dan 50 cycli draaien dan krijg je artefacten van primerdimeren en andere troep. Daarom wordt regulier bijna nooit meer dan 40-45 cycli gedraaid. Maar, zoals getoond voor het RIVM protocol levert 50 cycli draaien geen problemen op en het vergemakkelijkt het beoordelen van de amplificatiecurves.

Bijgevoegd de Standard Operating Procedure (SOP) en het validatierapport van de PCR-test die we bij het RIVM gebruiken. Deze, of de ErasmusMC variant van de originele PCR-test, wordt nog steeds door labs in Nederland gebruikt. Bijgevoegd ook de IFU van drie commerciële kits die door labs in Nederland worden gebruikt. In de Ridagene kit IFU staat een Ct waarde genoemd waarboven er extra naar curves

gekeken moet worden of herhaling aangevraagd om meer zekerheid te krijgen. In de Seegene kit IFU gaat het om welke targets positief zijn wanneer er extra aandacht aan de uitslag gegeven moet worden. Idem voor de PCR sneltest van Cepheid die veel gebruikt wordt in Nederland. Dit geeft ongeveer de variatie aan waarmee we nu in Nederland te maken hebben.

Een andere aanpassing in het document is de aandacht die gevraagd wordt voor het gebruik van testen met één of met meer dan één target gen. Het landschap aan op het moment van schrijven beschikbare testen is in de update weergegeven. In de voorgaande versie was dit vooral uitgebreid gericht op gebruik van de originele E-gen en RdRP-gen PCR-testen. In de huidige versie is de aandacht voor het originele protocol teruggebracht naar één alinea en meer aandacht gegeven aan de ontwikkelingen over de laatste maanden op het gebied van PCR testen. Waar eerst vanwege materiaal en reagentia tekorten van twee targets (E-gen en RdRP-gen) overgaan kon worden naar één target (E-gen) zijn door introductie van commerciële kits (zoals hiervoor genoemd en voorbeeld IFU bijgesloten) en betere beschikbaarheid van materialen en reagentia steeds meer tests met meerdere targets in gebruik genomen. Ook hierdoor zijn er niet meer of minder foutief positieve resultaten. Omdat verschillende targets niet 100% vergelijkbaar in sensitiviteit zijn kan het aantal zwak positieven toenemen, omdat bij zwak positieven niet altijd alle targets positief zijn. Dit is normaal en het gevolg van een statistisch proces om een lage concentratie virus in een monster rond de detectielimiet van de test op te kunnen pikken. Voor het beoordelen van niet alle targets positieve resultaten hebben laboratoria een algoritme of dit ligt vast in de IFU van de commerciële PCR-test kit (zie bijgevoegde voorbeelden).

Kortom, het aantal amplificatiecycli wat in PCR testen of andere amplificatietechnieken gebruikt wordt is optimaal voor een sensitieve test en het aantal amplificatiecycli is sinds de start van SARS-CoV-2 circulatie in Nederland niet verhoogd om meer monsters positief te vinden. Daarnaast is het gebruik van meer dan één target ingegeven om minder patiënten met een erg lage hoeveelheid virus te missen en het risico te spreiden dat ondanks zorgvuldige ontwikkeling van de testen door mutaties in het virus genoom een test op één deel van het virus genoom opeens minder gevoelig zou kunnen worden en er patiënten gemist zouden kunnen worden. Dit is tot nu toe niet opgetreden met de veel gebruikt E-gen PCR omdat die gebruik maakt van een zeer stabiel deel van het erfelijk materiaal van het virus.

Wanneer is de aanpassing gemaakt en waarom?

De tekst op de website is 25 september 2020 aangepast. De aanpassing is gemaakt om de laatste stand van zaken in ontwikkelingen van PCR-testen, andere type testen en ontwikkelingen op diagnostiek gebied te communiceren. De aanpassing van de waarschuwings Ct-waarde is gemaakt omdat de opgedane ervaring leert dat onder Ct 35 er geen twijfelcurves zijn. De aanpassing aan aantal target genen in PCR-testen en andere typen testen is gemaakt om het veranderde landschap aan

beschikbare en gebruikte testen in Nederland te beschrijven.

Wie schrijft en beoordeelt aanpassingen en hoe wordt het gecommuniceerd?

Een team van artsen-microbiologen, virologen, medisch moleculair biologen, artsen openbare gezondheidzorg, en epidemiologen van binnen en buiten het RIVM schrijven updates van het document. Communicatie hierover is gedaan via (Lab)Inf@ct: COVID-19 (nieuw coronavirusinfectie) (68), 29 september 2020. De vaststellingsprocedure via de subcommissie Diagnostiek LCI-richtlijnen in samenwerking met de Nederlandse Vereniging voor Medische Microbiologie loopt.

Eén target of meerdere targets en andere Ct waarde: wat is het effect op het aantal en percentage positieven?

In het begin van de epidemie werd in Nederland getest op het E-gen en het RdRP-gen. Omdat het E-gen het gevoeligst was van deze twee is er vanwege schaarste en efficiënt gebruik van middelen en reagentia ervoor gekozen om het testen tot één gen, het E-gen, te beperken. Dit heeft geen invloed gehad op het aantal en het percentage positieven omdat de E-gen PCR-test de gevoeligste test was.
De Ct waarde waarboven bij beoordeling extra aandacht gegeven moet worden heeft geen invloed op het aantal PCR positieven, niet absoluut en niet relatief. Onterecht wordt de suggestie gewekt dat de genoemde waarschuwingswaarde samenhangt met daaronder positief en daarboven negatief. Het gaat slechts om een waarde waarboven bij de beoordeling van de amplificatiecurves extra aandacht wordt gegeven. Het gebruik van één of meerdere target genen voor de PCR-test heeft zich vloeiend voltrokken en kan daarom een plotselinge stijging in het percentage positieven niet verklaren. Daarnaast geeft het kwaliteitsprogramma aan dat de gebruikte PCR-testen tussen laboratoria en in laboratoria een vergelijkbare gevoeligheid hebben. Zoals hierboven beschreven heeft het verhogen van de waarschuwings Ct waarde van 30 naar 35 geen invloed op het aantal positieven en fout-positieven en kan daarom een plotselinge stijging in het percentage positieven niet verklaren.

1) Rijksinstituut voor Volksgezondheid en Milieu, COVID-19 Richtlijn (https://lci.rivm.nl/richtlijnen/covid-19).

Bijlagen:

1. V040 Validatierapport Coronavirus 2019 n-CoV detectie PCR_met_ref
2. SOP deel 1_M900 Het isoleren en zuiveren van nucleinezuur uit klinische monsters mbv MagNA Pure 96 versie 7
3. SOP deel 2_M012_Lightcycler-qPCR_versie_9
4. SOP deel 3_F927 coronavirus detectie PCR
5. EUA-Seegene-allplex2-ifu
6. pg6815_ridagene_sars-cov-2_2020-07-27_en_final
7. Xpert Xpress SARS-CoV-2 Assay ENGLISH Package Insert 302-3750 Rev. C

Verification of qRT-PCR assay for detection of SARS-CoV-2

Version 2; 20-05-2021: concentration dPCR copies SARS-CoV-2 updated and some additional information added on quantitation limit. This only affects the description of the absolute minimum amount that can be detected. The test remains having the highest sensitivity possible in national and international comparisons.

Method: qPCR – One step RT-PCR with Fast Virus Master Mix
Manufacturer/Origin: Biolegio
Type of validation: verification of improved primers/probe set specific for detection of SARS-CoV-2
Application test: patient diagnostics, detection of SARS-CoV-2 in clinical material
Conclusion: Based on the results, we conclude that using the improved primers/probe set the RT-PCR meets the predefined criteria of the following parameters: measurement trueness, measurement accuracy, quantitation limit, analytical sensitivity and detection limit. The RT-PCR is more sensitive than with the original primers/probe set and now has similar sensitivity as the E-gene RT-PCR. Therefore the improved primers/probe set has been accepted for implementation in routine diagnostics of SARS-CoV-2.
Author: Lisa Wijsman
Date: 6-7-2020

Introduction & purpose
At this moment when a request is made for corona diagnostics the sample is tested on 2 genes of SARS-CoV-2. The E-gene and RdRp-gene are detected in 2 separate PCRs. The E-gene PCR is more sensitive than the RdRp-gene PCR. With an adjustment of the primers and probe set for the RdRp-gene, the idea is that the sensitivity of RdRp-gene PCR improves, so that it provides additional confirmation whether a sample is SARS-CoV-2 positive. Especially when the viral load is low.

Material & method
Wobbles are removed from the original primers to match the target sites in SARS-CoV-2 exactly, the reverse primer is extended to better match the Tm of the forward primer and the probe has been extended by 1 nt for increased Tm and better binding compared to the original one for an annealing/extension temperature of 60°C (adjustment based on work from Mirjam Hermans JBZ, personal communication). The adjusted primers and probe were analysed against a representative (geographic origin and distributed over time in the pandemic) set of sequences for match; no mismatched nucleotides were found. Samples for testing were extracted on the MagNa Pure 96 instrument (Roche) using the MagNa Pure 96 DNA and Virual NA Small volume kit (Roche) and eluted in a volume of 50 µl. RdRp-assay primers and probes as described in the provided protocol by Corman et al.[1] (Table 1) were used to compare the performance of the new specific SARS-CoV2 RdRp gene primers and probe (Table 2). Reagents and temperature protocol are described in Tables 3 and 4 respectively.

Table 1: Original RdRp-gene primers and probes Corman et al.[1]

Oligonucleotide	Sequence	Label	Target
RdRp_SARSr-F	GTG ARA TGG TCA TGT GTG GCG G		Sarbeco
RdRp_SARSr-R	CAR ATG TTA AAS ACA CTA TTA GCA TA		Sarbeco
RdRp_SARSr-P1	CCA GGT GGW ACR TCA TCM GGT GAT GC	TXR-BHQ2	Sarbeco
RdRp_SARSr-P2	CAG GTG GAA CCT CAT CAG GAG ATG C	FAM-BHQ1	SARS-CoV-2

Table 2: Adjusted primers and probe specific for SARS-CoV2 RdRp gene

Oligonucleotide	Sequence	Label
RdRp_SARS-F2	GTG AAA TGG TCA TGT GTG GCG G	
RdRp_SARS-R2	CAA ATG TTA AAA ACA CTA TTA GCA TAA GCA	
RdRp_SARS-P2.2	CCA GGT GGA ACC TCA TCA GGA GAT GC	FAM-BHQ1

Table 3: Composition reagents mixture for RdRP-gene SARS-CoV-2 RT-PCR. The primers and probes obtained from Biolegio were premixed at a final concentration of 10 µM primers and 5 µM probe.

RdRp qRT PCR	µl
4x Taqman Fast Virus MM	5
Primers/probe Mix (10 µM)	3
PCR grade water	7
Specimen nucleic acid	5
Total volume	20 µl

Table 4: Amplification temperature protocol.

PCR Program	Segment number	Temp Target (°C)	Hold Time (sec.)	Slope (°C/sec.)	Acquisition mode	
Reverse Transcription	**1**	**50**	**900**	**EXTERNAL**		
Denaturation/Inactivation	**1**	**95**	**120**	**EXTERNAL**		
Denaturation	1	95	60	4.4	None	**LC 480**
Amplification	1	95	10	4.4	None	
(cycles:50)	2	**60**	30	2.2	Single	
Cooling	1	40	30	4.4	None	

Analytic parameters:

The detection qPCR is not used as a pure quantitative method. Results are scored positive/negative to confirm if a patient is infected with SARS-CoV-2. Ct values are used as semi-quantitative information when useful for interpretation of the result. Only those parameters are tested that need verification compared to the original validation

Measurement uncertainty (Meetonzekerheid):	– not tested
Measurement trueness (Juistheid):	– tested: Saliva panel (page 5)
Measurement accuracy (Accuraatheid):	– tested: Saliva panel (page 5)
Measurement precision including measurement repeatability (Precisie inclusief herhaalbaarheid):	– not tested
Analytical sensitivity (Analytische sensitiviteit):	– tested: LOD95 determination (page 4)
Analytical specificity, including interfering substances (Analytische specificiteit met inbegrip van interfererende substanties):	– not tested
Detection limit (Detectielimiet, bij kwantitatieve methoden):	– tested: LOD95 determination (page 4)
Quantitation limit (Kwantificatielimiet, bij kwantitatieve methoden):	– tested: 10-fold dilution of quantified control (page 3)
Measuring interval (Meetinterval, kwantitatieve methoden):	– not tested

Clinical parameters:

Diagnostic sensitivity (Diagnostische Sensitiviteit):	– not tested
Diagnostic specificity (Diagnostische Specificiteit):	– not tested

Results

Quantitation limit

The SARS-CoV-2 RNA used for this verification is an 1:100 dilution of the hCoV-19/Netherlands/NoordBrabant_10003/2020 (4732000117 P2) stock obtained from cell culture. To establish PCR efficiency we first ran a triplicate 10-fold dilution series of viral RNA for each assay. Viral RNA was isolated by using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche). After Nucleic Acid extraction the concentration is $5.58*10^5$ digital copies RdRp-gene SARS-CoV-2 positive strand RNA/µl extract.

Table 5: 10-fold dilution series both primers and probe sets

Dilution	Current nCoV detection			New nCoV detection		
	I	II	III	I	II	III
SARS-CoV-2 10-1*	23.81	24.31	24.55	22.07	22.17	22.04
SARS-CoV-2 10-2	27.93	28.16	28.27	25.77	26.04	25.94
SARS-CoV-2 10-3	30.63	31.17	30.9	28.97	28.95	28.89
SARS-CoV-2 10-4	33.23	33.27	32.9	32.41	32.45	32.5
SARS-CoV-2 10-5	34.91	34.92	34.55	34.92	36.2	34.97
SARS-CoV-2 10-6	Negative	Negative	Negative	Negative	37.52	Negative
SARS-CoV-2 10-7	Negative	Negative	Negative	37.25	37.19	Negative
SARS-CoV-2 10-8	Negative	Negative	Negative	Negative	Negative	Negative

* Starting concentration is $5.58*10^4$ RNA copies/µl extract.

We determined the slope in Excel and defined the required levels for PCR efficiency (E) and R2 as >95% and >0.95, respectively. The new specific SARS-CoV-2 RdRp gene primers and probe meet the requirements (Figure 1, Table 6).

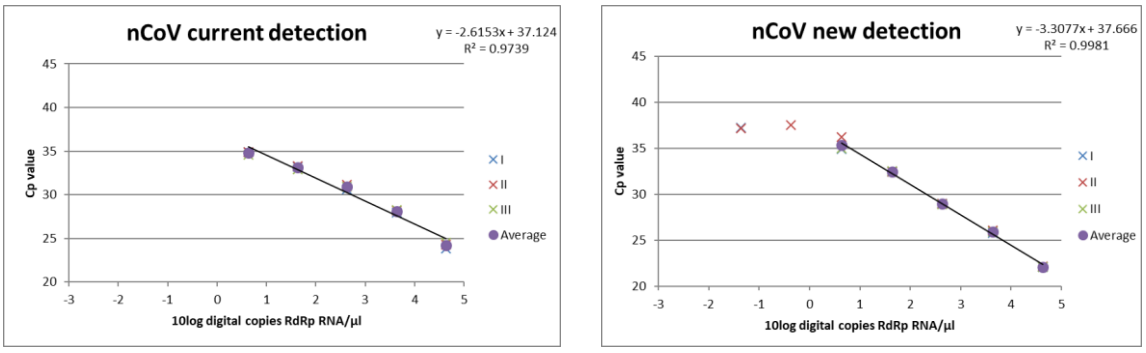


Figure 1: Linearity and amplification efficiency both primers and probes sets.

Table 6: The amplification efficiency and linearity calculated using the slope of the tangets in figure 1 (calculated in Excel)

	Slope	$E = 10^{-1/slope}$	$E(\%) = 100 \times [10^{-1/slope} - 1]$	R^2
Theorie	**-3.322**	**2.00**	**100**	**0.9500**
Current nCoV	-2.615	2.41	141.19	0.9739
New nCov	-3.308	2.01	100.60	0.9981

Analytical sensitivity and Detection Limit

Next, we ran four replicates of a 2-fold dilution series (diluted in yeast carrier RNA in water) to determine the LOD95% by Probit analysis using SPSS Statistics (IBM, version 24) (Figures 2 and 3).

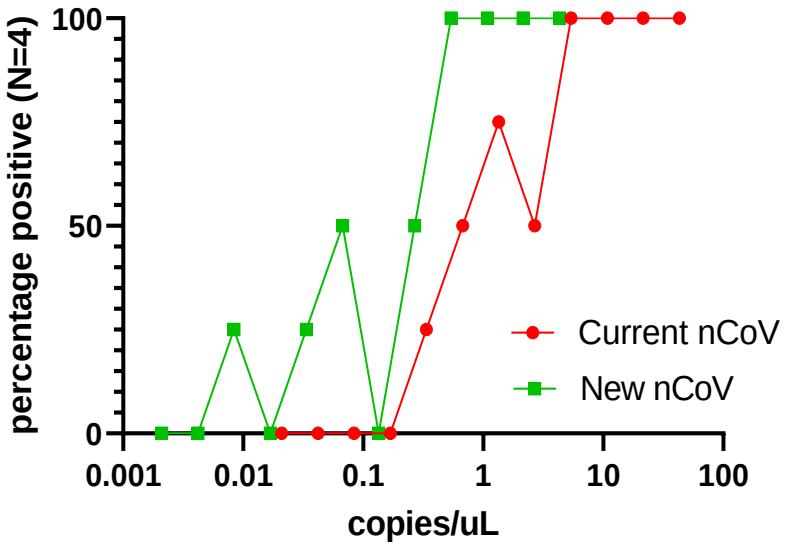
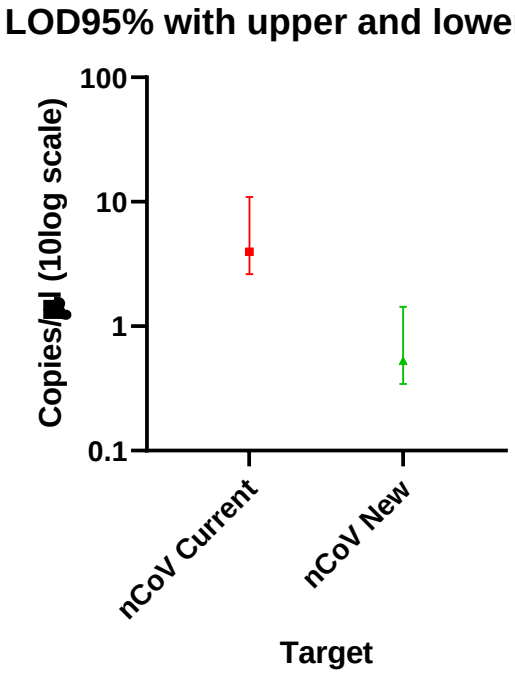


Figure 2: The results in percentages from the four replicates of the 2-fold dilution series against copies RdRp RNA/µl extract.



SPSS	Current nCoV	New nCoV
LOD 95%	3.976	0.528
lower bound	2.616	0.344
upper bound	10.952	1.435

Figure 3: The LOD95% in RdRp RNA copies/µl extract of the current nCoV-RdRp-gene and the new nCoV-RdRp-gene RT-qPCR.

Measurement trueness and Measurement accuracy

The measurement trueness and accuracy has been tested using the saliva EQA panel. The panel specimens were extracted in triplicate before and after storage at -80 °C. The sensitivity of the new nCoV PCR is better than the current test. In the current test the 10-7 dilution, virus particles equivalent to 1395 RdRp-gene digital PCR copies/ml saliva, is negative. In the new nCoV test the 10-7 dilution is positive (Table 7 and Figure 4). A freeze/thaw cycle did not result in reduction of detectable RNA.

Table 7: Saliva panel with cp-value*

Name	Dilution Stockvirus 4732000117 P2	Cp nCoV new			Cp nCoV current			Comment
Sen.Saliva_CoV20-1	10-4	25.46	25.48	25.21	25.86	26.02	25.75	Before -80 °C storage
Sen.Saliva_CoV20-2	10-7	32.01	31.91	32.04				Before -80 °C storage
Sen.Saliva_CoV20-3	10-5	28.84	28.7	28.62	27.76	27.62	28.44	Before -80 °C storage
Sen.Saliva_CoV20-4	negative							Before -80 °C storage
Sen.Saliva_CoV20-5	10-6	29.42	29.15	30.75	28.19	28.02	28.72	Before -80 °C storage
Sen.Saliva_CoV20-6	10-8			32.55				Before -80 °C storage
Sen.Saliva_CoV20-1.1	10-4	25.2	25.94	26	25.86	26.21	26.23	After -80 °C storage
Sen.Saliva_CoV20-2.1	10-7	31.82	31.98	31.95				After -80 °C storage
Sen.Saliva_CoV20-3.1	10-5	29.7	28.18	28.82	28.11	27.56	28	After -80 °C storage
Sen.Saliva_CoV20-4.1	negative							After -80 °C storage
Sen.Saliva_CoV20-5.1	10-6	30.98	30.88	31	29.46	29.12	28.94	After -80 °C storage
Sen.Saliva_CoV20-6.1	10-8							After -80 °C storage

* Concentration of the dilution series starts at a virus concentration equivalent to $1.40*10^6$ (10-4 dilution) dPCR RdRp-gene copies/ml in specimen.

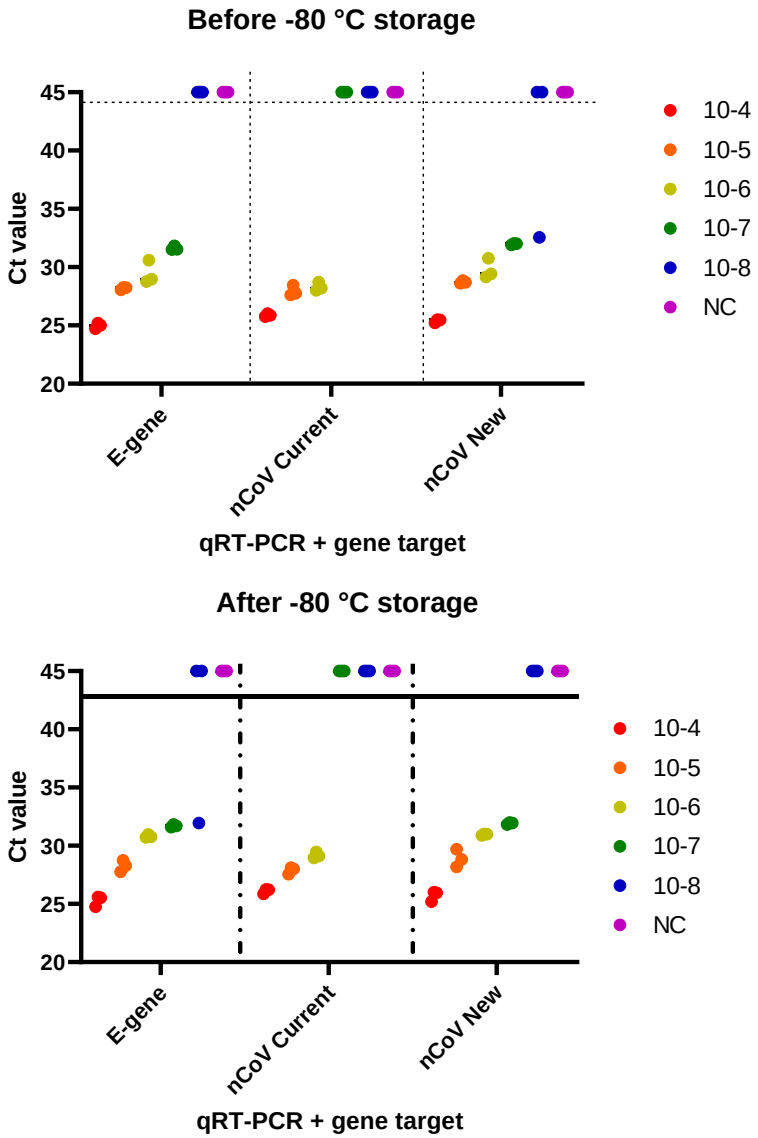


Figure 4: E-gene and RdRp-gene qRT-PCR results of saliva panel before and after -80 °C storage

Conclusion
Based on the results of the measurement trueness, measurement accuracy, quantitation limit, analytical sensitivity and detection limit the test can be implemented to detect SARS-CoV-2 in clinical specimens.

Literature

1. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):2000045. doi:10.2807/1560-7917.ES.2020.25.3.2000045

Archive raw data
R:\IDS\Afdeling EID\Projecten\AA Corona\Wuhan-CoV\Validatie_PCR\nCoV validatie resultaten.xlsx

Linked documents:
IDS_PRO_0701 'Validatie van methoden en apparatuur – versie 13
Leidraad parameters en criteria validatie (IDS_PRO_0701_bijlage 1) – versie 2
IDS/VIR/P014 Uitpakprotocol diagnostiekaanvraag BSL-2plus en BSL-3 respiratoire virussen – versie 6
IDS/VIR/M900 Het isoleren en zuiveren van nucleinezuur uit klinische monsters m.b.v. de MagNA Pure 96 – versie 7
IDS/VIR/M012 Lightcycler- qPCR – versie 9
IDS/VIR/F927 Coronavirus detectie PCR – versie 3
V040 Validatierapport Coronavirus 2019 n-CoV detectie PCR (Versie 2)

Bijlage 1:

Validatierapport Coronavirus 2019 n-CoV detectie PCR

NB Het validatierapport is in het engels vanwege internationale samenwerking bij het ontwikkelen van deze test

Validation of qRT-PCR assays for detection of novel coronavirus 2019-nCoV

Version 2; 20-05-2021: number of copies SARS-CoV-1/µl harmonized and some additional information added on quantitation limit. This only affects the description of the absolute minimum amount that can be detected. The test remains having the highest sensitivity possible in national and international comparisons.

Methode: qPCR – Onestep RT-PCR met behulp van Fast Virus Master Mix
Fabrikant/herkomst: Eurogentec, Thermofisher
Soort validatie: validatie van test specifiek voor het aantonen van coronavirus 2019 n-CoV
Toepassing test: patiëntendiagnostiek, detecteren van coronavirus 2019 n-CoV in klinisch materiaal
Conclusie: Op basis van de resultaten is gebleken dat de resultaten voldoen aan de vooraf gestelde critera van de volgende parameters: juistheid, analytische sensitiviteit en detectielimiet en (analytische) specificiteit.
Auteur: Adam Meijer
Datum: 22-1-2020

Introduction & goal

Background surveillance acute respiratory infections

The clinical specimens for the clinical validation of the E-assay and GdRp-assay were selected from the biobank containing combined nose swab and throat swab specimens in virus transport medium from the national surveillance of influenza-like illness (ILI) and acute respiratory infections (ARI) in the Netherlands. General practitioners from the Nivel Netherlands institute for health services research Primary Care Database report the incidence of ILI on a weekly basis and take nasopharygeal and oropharyngeal swabs from patients consulting with ILI or ARI. These specimens are submitted to the National Institute for Public Health and the Environment (RIVM) for in house developed qRT-PCR diagnosis of influenza virus, respiratory syncytial virus, rhinovirus and enterovirus. When questions arise on unsuspected increases in ILI incidence accompanied by a low detection rate of pathogens the FTD Respiratory pathogens 21 assay (Fast Track Diagnostics) is used.

Material & methods

Selection of specimens

One-hundred specimens have been selected from the surveillance biobank (Table 1). For influenza viruses, RSV and rhinovirus about ten specimens with Ct values below 30 and representative for the range of Ct values in clinical practice. For enterovirus the range of types detected in respiratory surveillance; for most a low and higher Ct value per type. For the other pathogens those detected with a Ct value below 30 during increased ILI incidence at the start of the 2018/2019 respiratory season that could not be fully explained by routinely detected viruses.
Additional were tested, CoV-HKU1 RNA from highly positive specimen, CoV-NL63 virus isolate, CoV-229E virus isolate, *Chlamydia psittaci* culture, *Chamydophila pneumoniae* culture, *Mycoplasma*

pneumoniae culture and the 2019 MERS-CoV QCMD EQA panel containing different dilutions of MERS-CoV in 5 specimens, CoV-OC43 (n=1) and CoV-NL63 (n=1) virus and 1 negative specimen. Except for CoV-HKU1 RNA all specimens were freshly extracted.

***Table 1**. Tests of known respiratory viruses and bacteria in clinical specimens*

Clinical specimens with known viruses/bacteria	Number of samples tested in both assays	Ct range
CoV-HKU1	1	NA
CoV-OC43	5	22.7 – 29.9
CoV-229E	3	25.1 – 28.5
Influenza virus A(H1N1)pdm09	10	18.9 – 28.1
Influenza virus A(H3N2)	10	17.4 – 27.7
Influenza virus B/Victoria	7	19.8 – 29.9
Influenza virus B/Yamagata	10	23.5 – 29.1
Rhinovirus	13	13.4 – 28.0
Enterovirus (for types see supplementary excel file)	18	19.6 – 28.9
Respiratory syncytial virus A	10	18.0 – 29.8
Respiratory syncytial virus B	11	21.0 – 29.4
Parainfluenza virus 3	1	26
Human metapneumovirus	3	26.7 – 27.6
Adenovirus	2	21.0 – 27.3
Human Bocavirus	1	35 (borderline)
Mycoplasma spp.	1	26.8
Total	106	

qRT-PCR

Per clinical specimen 200 µl was extracted on a MagNA Pure 96 Instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) and eluted in a volume of 50 µl. E-assay and RdRP-assay primers and probes as described in the provided protocol by Corman et al. were kindly provided by Corman and ordered from Eurogentec (Belgium). As control material SARS-CoV-1 full virus RNA (Strain Frankfurt 1, concentration 1000 copies RNA/µl) (Ct value 29-30 at 5 µl/rx) was also kindly provided by Corman.

Reaction conditions were as shown in Tables 2-4.

***Table 2**. For primers and probes provided by Corman.*

E-gene qRT PCR	µl	RdRP-gene qRT-PCR	µl
4x Taqman Fast Virus MM	5	4x Taqman Fast Virus MM	5
SJVE-F (10 µM)	1	SJVR-F (10 µM)	1.5
SJVE-R (10 µM)	1	SJVR-R (10 µM)	2.0
SJVE-P (10 µM)	0.5	SJVR-P1/SJVR-P2 (10 µM)	0.5
PCR grade water	7.5	PCR grade water	6
Specimen nucleic acid	5	Specimen nucleic acid	5
Total volume	20 µl	Total volume	20 µl

***Table 3**. For primers and probes obtained from Eurogentec primers and probes were premixed at a final conancentration of 10 µM each except for the SJVR probes for 5 µM each.*

E-gene qRT PCR	µl	RdRP-gene qRT-PCR	µl
4x Taqman Fast Virus MM	5	4x Taqman Fast Virus MM	5
SJVE Mix (10 µM)	3	SJVR Mix (10 µM)	3
PCR grade water	7	PCR grade water	7
Specimen nucleic acid	5	Specimen nucleic acid	5
Total volume	20 µl	Total volume	20 µl

***Table 4.** Amplification temperature protocol.*

PCR Program	Segment number	Temp Target (°C)	Hold Time (sec.)	Slope (°C/sec.)	Acquisition mode	
Reverse Transcription	**1**	**50**	**900**	**EXTERNAL**		
Denaturation/Inactivation	**1**	**95**	**120**	**EXTERNAL**		
Denaturation	1	95	60	4.4	None	LC 480
Amplification	1	95	10	4.4	None	
(cycles:50)	2	**58**	30	2.2	Single	
Cooling	1	40	30	4.4	None	

Standard EAV external control qRT-PCR was performed to control for inhibitors. (IDS/VIR/F351)

Analytic parameters:

The detection qPCR is not used as a quantitative method. Results are scored positive/negative to confirm if a patient is infected with the novel coronavirus 2019-nCoV. Therefore all analytic parameters, used for validation of quantitative methods, are not tested.

Measurement uncertainty (Meetonzekerheid): – not tested
Measurement trueness (Juistheid): – tested: MERS-CoV panel and Frankfurt-1 SARS-CoV-1 control RNA (page 13)
Measurement accuracy (Accuraatheid): – not tested, no golden standard available
Measurement precision including measurement repeatability (Precisie inclusief herhaalbaarheid): – not tested
Analytical sensitivity (Analytische sensitiviteit): – tested: LOD95 determination (page 12)
Analytical specificity, including interfering substances (Analytische Specificiteit met inbegrip van interfererende substanties): – tested: clinical validation including negative controls (page 13)
Detection limit (Detectielimiet, bij kwantitatieve methoden): – not tested
Quantitation limit (Kwantificatielimiet, bij kwantitatieve methoden): – not tested

Measuring interval (Meetinterval, kwantitatieve methoden): – not tested

Clinical parameters:

Diagnostic sensitivity (Diagnostische Sensitiviteit): – not tested, no clinical specimens of SARS-CoV-2 positive patients available

Diagnostic specificity (Diagnostische Specificiteit): – not tested, no clinical specimens of SARS-CoV-2 positive patients available

Results

LOD95 determination

10-fold dilution series of SARS-CoV-1 control material were amplified using the reference primers and probes and the Eurogentec ordered primers and probes. Based on this dilution series a starting point for 2-fold dilution series in 4-fold was chosen, prepared and subjected to amplification to determine the LOD95.

Table 5. *RT-qPCR results of 10-fold dilution series of SARS-CoV-1 RNA with both primer and probe sets*

Name	E-gene		RdRP-gene	
Dilution	Eurogentec (EuroE) Ct	Duitsland (DuitE) Ct	Eurogentec (EuroR) Ct	Duitsland (DuitR) Ct
SARS-CoV-1 undiluted	26.09	25.15	27.08	28.77
SARS-CoV-1 10-1	29.18	28.32	30.49	31.48
SARS-CoV-1 10-2	31.84	30.85	33.23	32.98
SARS-CoV-1 10-3	33.99	34.01	35.68	34.5
SARS-CoV-1 10-4	neg	35.92	35.75	34.97
SARS-CoV-1 10-5	neg	36.12	neg	neg
SARS-CoV-1 10-6	neg	35.18	neg	neg
SARS-CoV-1 10-7	neg	35.26	neg	neg
Neg extraction control 1	neg	34.44	neg	neg
Neg extraction control 2	neg	ND	neg	neg

nd = not done

Eurogentec E-gene primers and probe give a cleaner picture (Figure 1). German E-gene primers and probe generate 'ghost' curves in high dilutions and negative control (Figure 1). For the RdRP-gene qRt-PCR primers and probes of both sources the qRT-PCRs generated similar results. For both qRT-PCR assays the Eurogentec primers and probes generated higher fluorescence values than the German primers and probes.

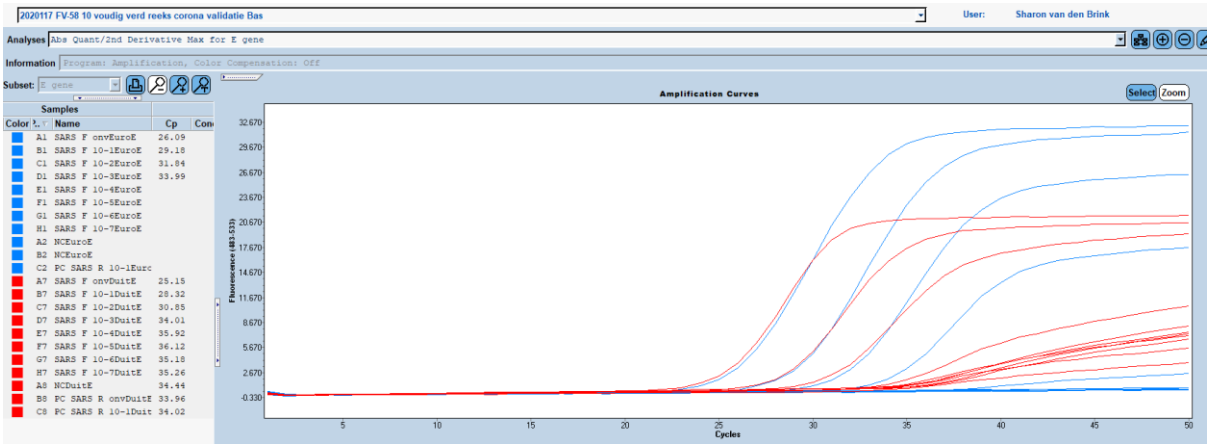


***Figure 1**. Amplification curve E-gene qRT-PCR. Blue Eurogentec primers and probe; red German primers and probe.*

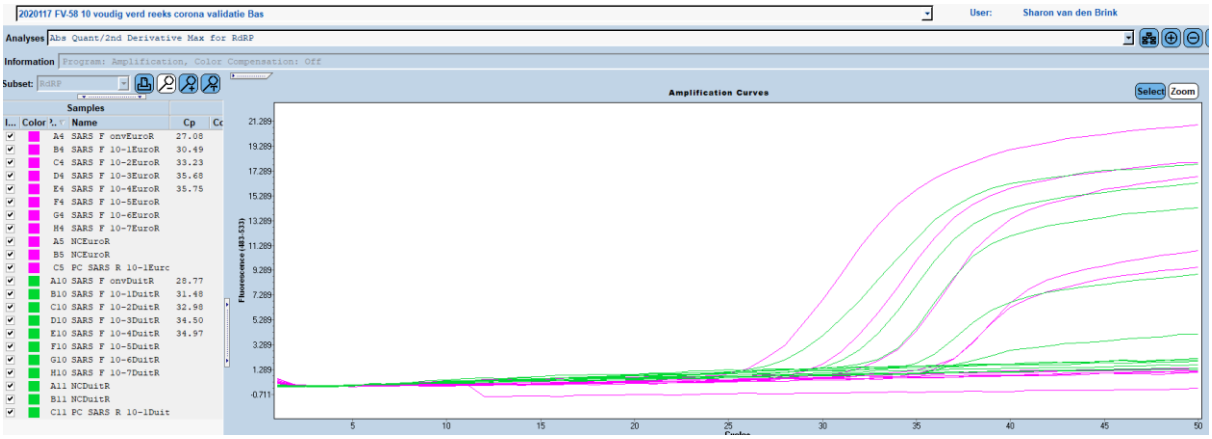
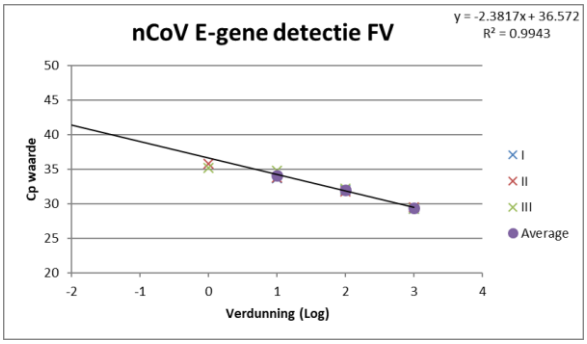
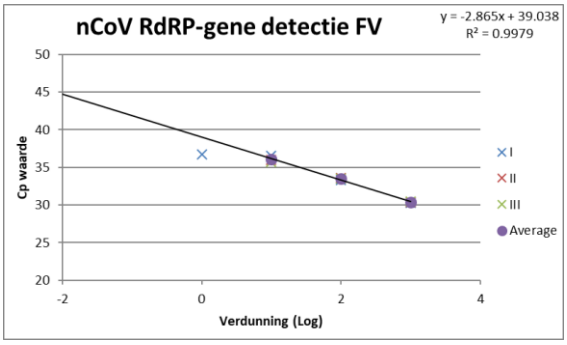


***Figure 2.** Amplification curve RdRP-gene qRT-PCR. Pink Eurogentec primers and probe; green German primers and probe.*

Based on the 10-fold dilution series the linearity was determined and the efficiency calculated (Figure 3). Result of the LOD95% determination is shown in Figures 4 and 5.

A



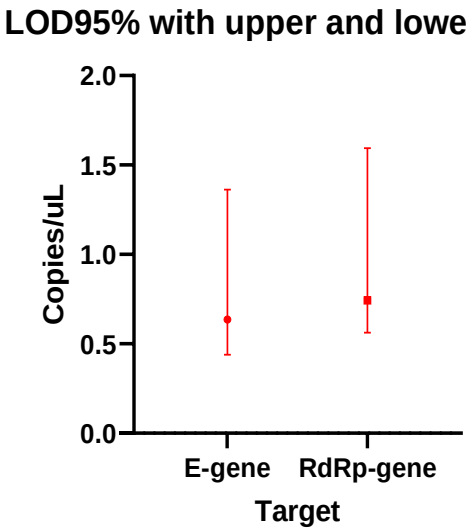


B

	Slope	$E = 10^{-1/slope}$	$E\ (\%) = 100 \times [10^{-1/slope} - 1]$	R^2
Theoretical	**-3.322**	**2.00**	**100**	**0.9500**
nCoV E-gene	-2.382	2.63	162.95	0.9943
nCov RdRP-gene	-2.865	2.23	123.38	0.9979

Figure 3. *Linearity (panel A) and amplification efficiency (panel B) with Eurogentec primers and probes*

Linearity and efficiency were excellent.



SPSS	E-gene	RdRp-gene
LOD 95%	0.636	0.742
lower bound	0.439	0.563
upper bound	1.363	1.595

Figure 5: *The LOD95% copies/µl RNA of the current E-gene and RdRp-gene RT-qPCR.*

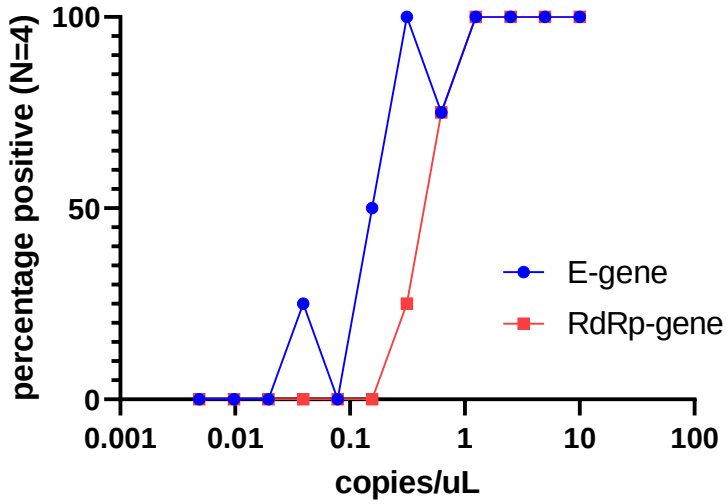


Figure 4*. 95% limit of detection curves with calculated LOD95; Eurogentec primers and probes*

Clinical specimens

Except for two of the clinical specimens and other cultured viruses, the cultured bacteria and the 2019 MERS-CoV QCMD EQA panel specimens were negative in the qRT-PCR with Eurogentec primers and probes.

One (with Ct 28.1) of 10 influenza A(H1N1)pdm09 positive specimens and one (with Ct 29.3) of 11 RSV-B positive specimens were positive in the E-gene qRT-PCR only with high Ct values of 37.7 and 39.1 respectively (Figure 5). Both specimens have been repeated in the E-gene RT-PCR with annealing temperatures of 58°C and 60°C and turned out negative. None of the other A(H1N1)pdm09 and RSV-B positive specimens with higher viral load generated a positive signal in the E-gene qRT-PCR.

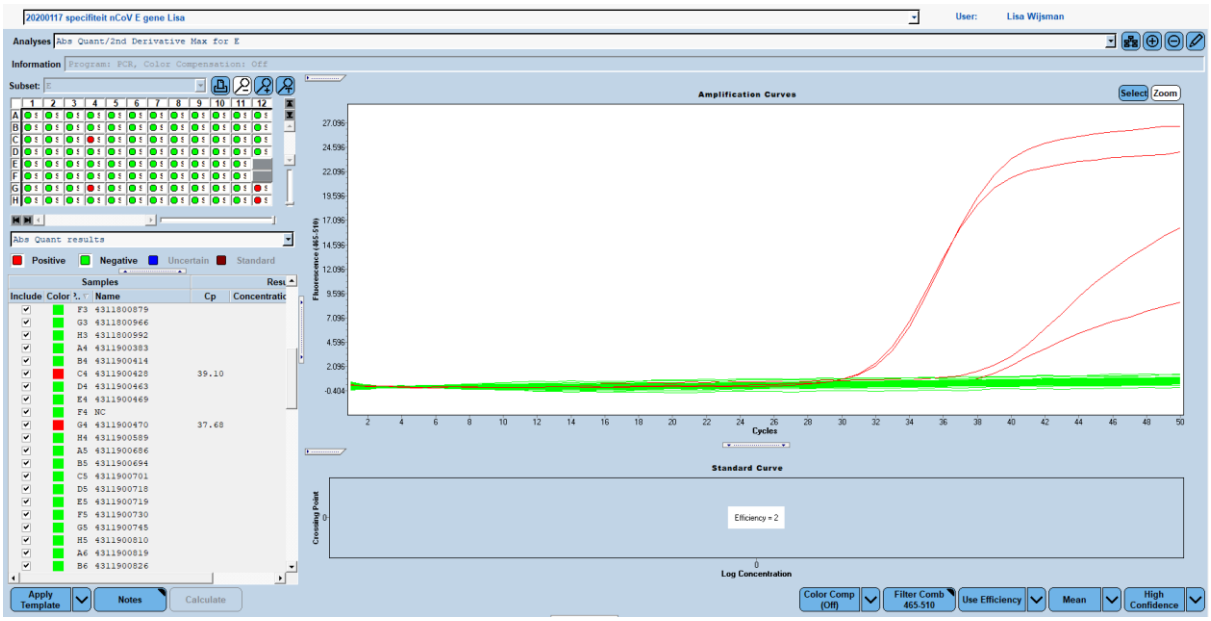


Figure 6. *Amplification curves of clinical specimens with E-gene qRT-PCR*

Discussion

Except for two of the clinical specimens and other cultured viruses, the cultured bacteria and the 2019 MERS-CoV QCMD EQA panel specimens were negative in the qRT-PCR with Eurogentec primers and probes. Both specimens have been repeated in the E-gene RT- and turned out negative. The measurement trueness is 100%.

Based on the 10-fold dilution series in both E-gene and RdRP-Gene the efficiency excellent.

Based on the 2-fold dilution series in both E-gene and RdRP-Gene the analytical sensitivity is below 1 copy per µl input RNA and 3.2 and 3.7 copies RNA/reaction, respectively.

Conclusion

Based on the results of the measurement trueness, efficiency and analytical specificity and analytical sensitivity the test can be implemented to detect the new coronavirus 2019-nCoV.

Literature

R:\IDS\Afdeling EID\Projecten\AA Corona\Wuhan-CoV\Publicaties over 2019-nCoV

Archive raw data

R:\IDS\Afdeling EID\Projecten\AA Corona\Wuhan-CoV\Validatie_PCR\nCoV validatie resultaten.xlsx

Linked documents:
IDS_PRO_0701 'Validatie van methoden en apparatuur – versie 13
Leidraad parameters en criteria validatie (IDS_PRO_0701_bijlage 1) – versie 2
IDS/VIR/P014 Uitpakprotocol diagnostiekaanvraag BSL-2plus en BSL-3 respiratoire virussen – versie 6
IDS/VIR/M900 Het isoleren en zuiveren van nucleinezuur uit klinische monsters m.b.v. de MagNA Pure 96 – versie 7
IDS/VIR/M012 Lightcycler- qPCR – versie 9
IDS/VIR/F927 Coronavirus detectie PCR – versie 1

Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR

Victor M Corman[1], Olfert Landt[2], Marco Kaiser[3], Richard Molenkamp[4], Adam Meijer[5], Daniel KW Chu[6], Tobias Bleicker[1], Sebastian Brünink[1], Julia Schneider[1], Marie Luisa Schmidt[1], Daphne GJC Mulders[4], Bart L Haagmans[4], Bas van der Veer[5], Sharon van den Brink[5], Lisa Wijsman[5], Gabriel Goderski[5], Jean-Louis Romette[7], Joanna Ellis[8], Maria Zambon[8], Malik Peiris[6], Herman Goossens[9], Chantal Reusken[5], Marion PG Koopmans[4], Christian Drosten[1]

1. Charité – Universitätsmedizin Berlin Institute of Virology, Berlin, Germany and German Centre for Infection Research (DZIF), Berlin, Germany
2. Tib-Molbiol, Berlin, Germany
3. GenExpress GmbH, Berlin, Germany*
4. Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands
5. National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
6. University of Hong Kong, Hong Kong, China
7. Universite d Aix-Marseille, Marseille, France
8. Public Health England, London, United Kingdom
9. Department of Medical Microbiology, Vaccine and Infectious Diseases Institute, University of Antwerp, Antwerp, Belgium

Correspondence: Christian Drosten (christian.drosten@charite.de)

Citation style for this article:
Corman Victor M, Landt Olfert, Kaiser Marco, Molenkamp Richard, Meijer Adam, Chu Daniel KW, Bleicker Tobias, Brünink Sebastian, Schneider Julia, Schmidt Marie Luisa, Mulders Daphne GJC, Haagmans Bart L, van der Veer Bas, van den Brink Sharon, Wijsman Lisa, Goderski Gabriel, Romette Jean-Louis, Ellis Joanna, Zambon Maria, Peiris Malik, Goossens Herman, Reusken Chantal, Koopmans Marion PG, Drosten Christian. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):pii=2000045. https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045

Article submitted on 21 Jan 2020 / accepted on 22 Jan 2020 / published on 23 Jan 2020

Background: The ongoing outbreak of the recently emerged novel coronavirus (2019-nCoV) poses a challenge for public health laboratories as virus isolates are unavailable while there is growing evidence that the outbreak is more widespread than initially thought, and international spread through travellers does already occur. **Aim:** We aimed to develop and deploy robust diagnostic methodology for use in public health laboratory settings without having virus material available. **Methods:** Here we present a validated diagnostic workflow for 2019-nCoV, its design relying on close genetic relatedness of 2019-nCoV with SARS coronavirus, making use of synthetic nucleic acid technology. **Results:** The workflow reliably detects 2019-nCoV, and further discriminates 2019-nCoV from SARS-CoV. Through coordination between academic and public laboratories, we confirmed assay exclusivity based on 297 original clinical specimens containing a full spectrum of human respiratory viruses. Control material is made available through European Virus Archive – Global (EVAg), a European Union infrastructure project. **Conclusion:** The present study demonstrates the enormous response capacity achieved through coordination of academic and public laboratories in national and European research networks.

Introduction

According to the World Health Organization (WHO), the WHO China Country Office was informed of cases of pneumonia of unknown aetiology in Wuhan City, Hubei Province, on 31 December 2019 [1]. A novel coronavirus currently termed 2019-nCoV was officially announced as the causative agent by Chinese authorities on 7 January. A viral genome sequence was released for immediate public health support via the community online resource virological.org on 10 January (Wuhan-Hu-1, GenBank accession number MN908947 [2]), followed by four other genomes deposited on 12 January in the viral sequence database curated by the Global Initiative on Sharing All Influenza Data (GISAID). The genome sequences suggest presence of a virus closely related to the members of a viral species termed severe acute respiratory syndrome (SARS)-related CoV, a species defined by the agent of the 2002/03 outbreak of SARS in humans [3,4]. The species also comprises a large number of viruses mostly detected in rhinolophid bats in Asia and Europe.

As at 20 January 2020*, 282 laboratory-confirmed human cases have been notified to WHO [5]. Confirmed cases in travellers from Wuhan were announced on 13 and 17 January in Thailand as well as on 15 January in Japan and 19 January in Korea. The extent of human-to-human transmission of 2019-nCoV is unclear at the time of writing of this report but there is evidence of some human-to-human transmission.

Among the foremost priorities to facilitate public health interventions is reliable laboratory diagnosis. In acute respiratory infection, RT-PCR is routinely used to detect causative viruses from respiratory secretions. We have previously demonstrated the feasibility of introducing robust detection technology based on real-time RT-PCR in public health laboratories during international

Table 1

Primers and probes, real-time RT-PCR for 2019 novel coronavirus

Assay/use	Oligonucleotide	Sequence[a]	Concentration[b]
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG	Use 600 nM per reaction
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ	Specific for 2019-nCoV, will not detect SARS-CoV. Use 100 nM per reaction and mix with P1
	RdRP_SARSr-P1	FAM-CCAGGTGGWACRTCATCMGGTGATGC-BBQ	Pan Sarbeco-Probe will detect 2019-nCoV, SARS-CoV and bat-SARS-related CoVs. Use 100 nM per reaction and mix with P2
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA	Use 800 nM per reaction
E gene	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	Use 400 nm per reaction
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	Use 200 nm per reaction
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA	Use 400 nm per reaction
N gene	N_Sarbeco_F	CACATTGGCACCCGCAATC	Use 600 nm per reaction
	N_Sarbeco_P	FAM-ACTTCCTCAAGGAACAACATTGCCA-BBQ	Use 200 nm per reaction
	N_Sarbeco_R	GAGGAACGAGAAGAGGCTTG	Use 800 nm per reaction

[a] W is A/T; R is G/A; M is A/C; S is G/C. FAM: 6-carboxyfluorescein; BBQ: blackberry quencher.

[b] Optimised concentrations are given in nanomol per litre (nM) based on the final reaction mix, e.g. 1.5 µL of a 10 µM primer stock solution per 25 µL total reaction volume yields a final concentration of 600 nM as indicated in the table.

health emergencies by coordination between public and academic laboratories [6-12]. In all of these situations, virus isolates were available as the primary substrate for establishing and controlling assays and assay performance.

In the present case of 2019-nCoV, virus isolates or samples from infected patients have so far not become available to the international public health community. We report here on the establishment and validation of a diagnostic workflow for 2019-nCoV screening and specific confirmation, designed in absence of available virus isolates or original patient specimens. Design and validation were enabled by the close genetic relatedness to the 2003 SARS-CoV, and aided by the use of synthetic nucleic acid technology.

Methods

Clinical samples and coronavirus cell culture supernatants for initial assay evaluation

Cell culture supernatants containing typed coronaviruses and other respiratory viruses were provided by Charité and University of Hong Kong research laboratories. Respiratory samples were obtained during 2019 from patients hospitalised at Charité medical centre and tested by the NxTAG respiratory pathogen panel (Luminex, S´Hertogenbosch, The Netherlands) or in cases of MERS-CoV by the MERS-CoV upE assay as published before [10]. Additional samples were selected from biobanks at the Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven, at Erasmus University Medical Center, Rotterdam, at Public Health England (PHE), London, and at the University of Hong Kong. Samples from all collections comprised sputum as well as nose and throat swabs with or without viral transport medium.

Faecal samples containing bat-derived SARS-related CoV samples (identified by GenBank accession numbers) were tested: KC633203, Betacoronavirus BtCoV/Rhi_eur/BB98–98/BGR/2008; KC633204, Betacoronavirus BtCoV/Rhi_eur/BB98–92/BGR/2008; KC633201, Betacoronavirus BtCoV/Rhi_bla/BB98–22/BGR/2008; GU190221 Betacoronavirus Bat coronavirus BR98–19/BGR/2008; GU190222 Betacoronavirus Bat coronavirus BM98–01/BGR/2008; GU190223, Betacoronavirus Bat coronavirus BM98–13/BGR/2008. All synthetic RNA used in this study was photometrically quantified.

RNA extraction

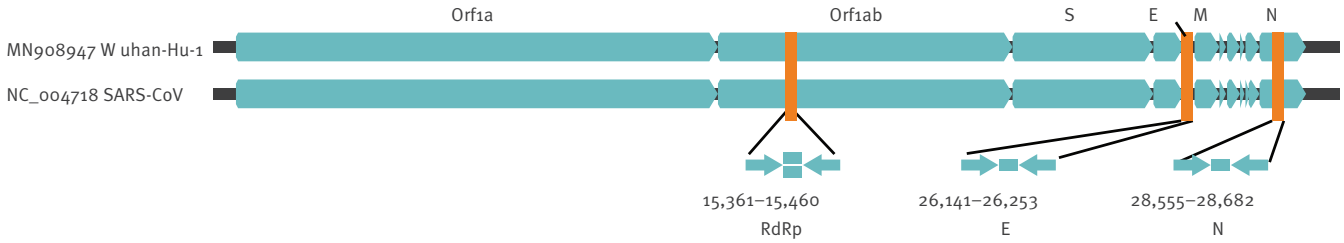
RNA was extracted from clinical samples with the MagNA Pure 96 system (Roche, Penzberg, Germany) and from cell culture supernatants with the viral RNA mini kit (QIAGEN, Hilden, Germany).

Real-time reverse-transcription PCR

A 25 µL reaction contained 5 µL of RNA, 12.5 µL of 2 × reaction buffer provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen, Darmstadt, Germany; containing 0.4 mM of each deoxyribont triphosphates (dNTP) and 3.2 mM magnesium sulphate), 1 µL of reverse transcriptase/Taq mixture from the kit, 0.4 µL of a 50 mM magnesium sulphate solution (Invitrogen), and 1 µg of nonacetylated bovine serum albumin (Roche). Primer and probe sequences, as well as optimised concentrations are shown in Table 1. All oligonucleotides were synthesised and provided by Tib-Molbiol (Berlin,

Figure 1

Relative positions of amplicon targets on the SARS coronavirus and the 2019 novel coronavirus genome



E: envelope protein gene; M: membrane protein gene; N: nucleocapsid protein gene; ORF: open reading frame; RdRp: RNA-dependent RNA polymerase gene; S: spike protein gene.

Numbers below amplicons are genome positions according to SARS-CoV, GenBank NC_004718.

Germany). Thermal cycling was performed at 55 °C for 10 min for reverse transcription, followed by 95 °C for 3 min and then 45 cycles of 95 °C for 15 s, 58 °C for 30 s. Participating laboratories used either Roche Light Cycler 480II or Applied Biosystems ViiA7 instruments (Applied Biosystems, Hong Kong, China).

Protocol options and application notes

Laboratories participating in the evaluation used the TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher) with the same oligonucleotide concentrations and cycling conditions. The QIAGEN One-Step RT-PCR Kit was also tested and found to be compatible.

The intended cross-reactivity of all assays with viral RNA of SARS-CoV allows us to use the assays without having to rely on external sources of specific 2019-nCoV RNA.

For a routine workflow, we recommend the E gene assay as the first-line screening tool, followed by confirmatory testing with the RdRp gene assay. Application of the RdRp gene assay with dual colour technology can discriminate 2019-nCoV (both probes positive) from SARS-CoV RNA if the latter is used as positive control. Alternatively, laboratories may choose to run the RdRp assay with only the 2019-nCoV-specific probe.

Ethical statement

The internal use of samples for diagnostic workflow optimisation was agreed under the medical ethical rules of each of the participating partners.

Results

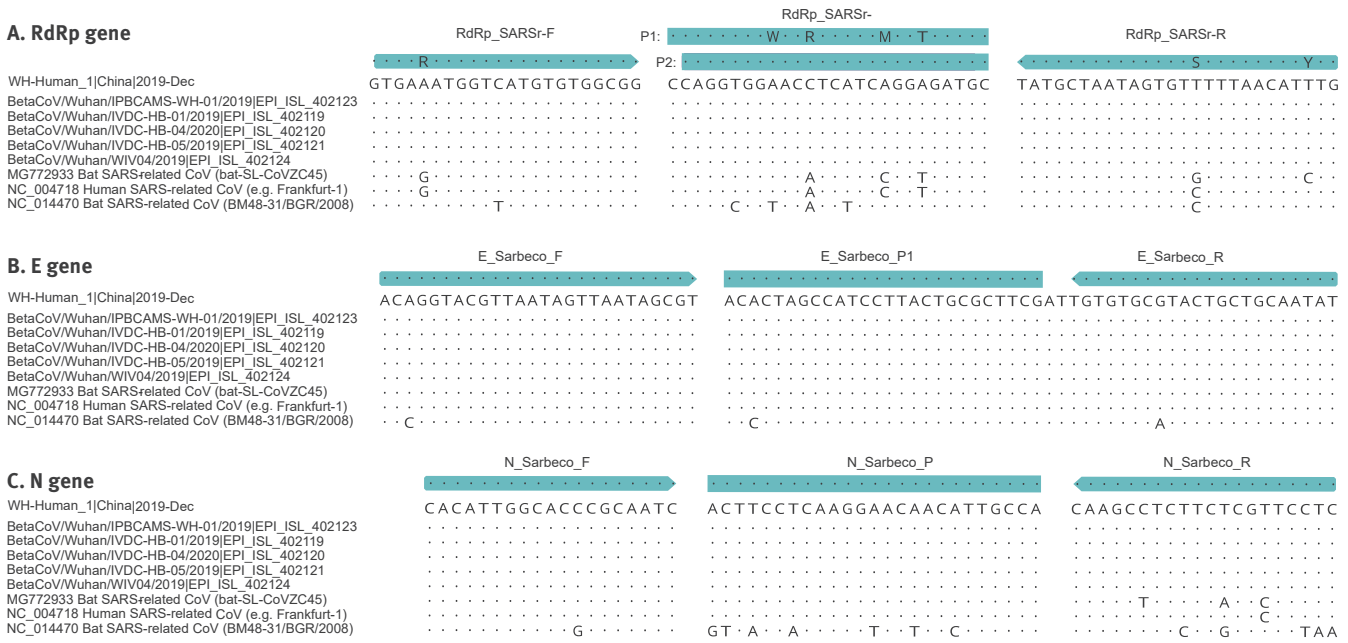
Before public release of virus sequences from cases of 2019-nCoV, we relied on social media reports announcing detection of a SARS-like virus. We thus assumed that a SARS-related CoV is involved in the outbreak. We downloaded all complete and partial (if>400 nt) SARS-related virus sequences available in GenBank by 1 January 2020. The list (n=729 entries) was manually checked and artificial sequences (laboratory-derived, synthetic, etc), as well as sequence duplicates were removed, resulting in a final list of 375 sequences. These sequences were aligned and the alignment was used for assay design (Supplementary Figure S1). Upon release of the first 2019-nCoV sequence at virological.org, three assays were selected based on how well they matched to the 2019-nCoV genome (Figure 1). The alignment was complemented by additional sequences released independently on GISAID (https://www.gisaid.org), confirming the good matching of selected primers to all sequences. Alignments of primer binding domains with 2019-nCoV, SARS-CoV as well as selected bat-associated SARS-related CoV are shown in Figure 2.

Assay sensitivity based on SARS coronavirus virions

To obtain a preliminary assessment of analytical sensitivity, we used purified cell culture supernatant containing SARS-CoV strain Frankfurt-1 virions grown on Vero cells. The supernatant was ultrafiltered and thereby concentrated from a ca 20-fold volume of cell culture supernatant. The concentration step simultaneously reduces the relative concentration of background nucleic acids such as not virion-packaged viral RNA. The virion preparation was quantified by real-time RT-PCR using a specific in vitro-transcribed RNA quantification standard as described in Drosten et al. [8]. All assays were subjected to replicate testing in order to determine stochastic detection frequencies at each assay's sensitivity end point (Figure 3A and B). All assays were highly sensitive, with best results obtained for the E gene and RdRp gene assays (5.2 and 3.8 copies per reaction at 95% detection probability, respectively). These two assays were chosen for further evaluation. One of the laboratories participating in the external evaluation used other basic RT-PCR reagents (TaqMan Fast Virus 1-Step Master Mix) and repeated the sensitivity study, with equivalent results (E gene: 3.2 RNA copies/reaction (95% CI: 2.2–6.8); RdRP: 3.7 RNA copies/reaction (95% CI: 2.8–8.0). Of note, the N gene assay also performed well but was not subjected

Figure 2

Partial alignments of oligonucleotide binding regions, SARS-related coronaviruses (n = 9)



The panels show six available sequences of 2019-nCoV, aligned to the corresponding partial sequences of SARS-CoV strain Frankfurt 1, which can be used as a positive control for all three RT-PCR assays. The alignment also contains a closely related bat virus (Bat SARS-related CoV isolate bat-SL-CoVZC45, GenBank accession number MG772933) as well as the most distant member within the SARS-related bat CoV clade, detected in Bulgaria (GenBank accession number NC_014470). Dots represent identical nucleotides compared with the WH_Human_1 sequence. Nucleotide substitutions are specified. Blue arrows: oligonucleotides as specified in Table 1. More comprehensive alignments can be found in the Supplement.

to intensive further validation because it was slightly less sensitive (Supplementary Figure S2)

Sensitivity based on in vitro-transcribed RNA identical to 2019 novel coronavirus target sequences

Although both assays detected 2019-nCoV without polymorphisms at oligonucleotide binding sites (Figure 2), we additionally generated in vitro-transcribed RNA standards that exactly matched the sequence of 2019-nCoV for absolute quantification and studying the limit of detection (LOD). Replicate reactions were done at concentrations around the detection end point determined in preliminary dilution experiments. The resulting LOD from replicate tests was 3.9 copies per reaction for the E gene assay and 3.6 copies per reaction for the RdRp assay (Figure 3C and D). These figures were close to the 95% hit rate of 2.9 copies per reaction, according to the Poisson distribution, expected when one RNA molecule is detected.

Discrimination of 2019 novel coronavirus from SARS coronavirus by RdRp assay

Following the rationale that SARS-CoV RNA can be used as a positive control for the entire laboratory procedure, thus obviating the need to handle 2019-nCoV RNA, we formulated the RdRp assay so that it contains two probes: a broad-range probe reacting with SARS-CoV and 2019-nCoV and an additional probe that reacts only with 2019-nCoV. By limiting dilution experiments, we confirmed that both probes, whether used individually or in combination, provided the same LOD for each target virus. The specific probe RdRP_SARSr-P2 detected only the 2019-nCoV RNA transcript but not the SARS-CoV RNA.

Detection range for SARS-related coronaviruses from bats

At present, the potential exposure to a common environmental source in early reported cases implicates the possibility of independent zoonotic infections with increased sequence variability [5]. To show that the assays can detect other bat-associated SARS-related viruses, we used the E gene assay to test six bat-derived faecal samples available from Drexler et al. [13] und Muth et al. [14]. These virus-positive samples stemmed from European rhinolophid bats. Detection of these phylogenetic outliers within the SARS-related CoV clade suggests that all Asian viruses are likely to be detected. This would, theoretically, ensure broad sensitivity even in case of multiple independent acquisitions of variant viruses from an animal reservoir.

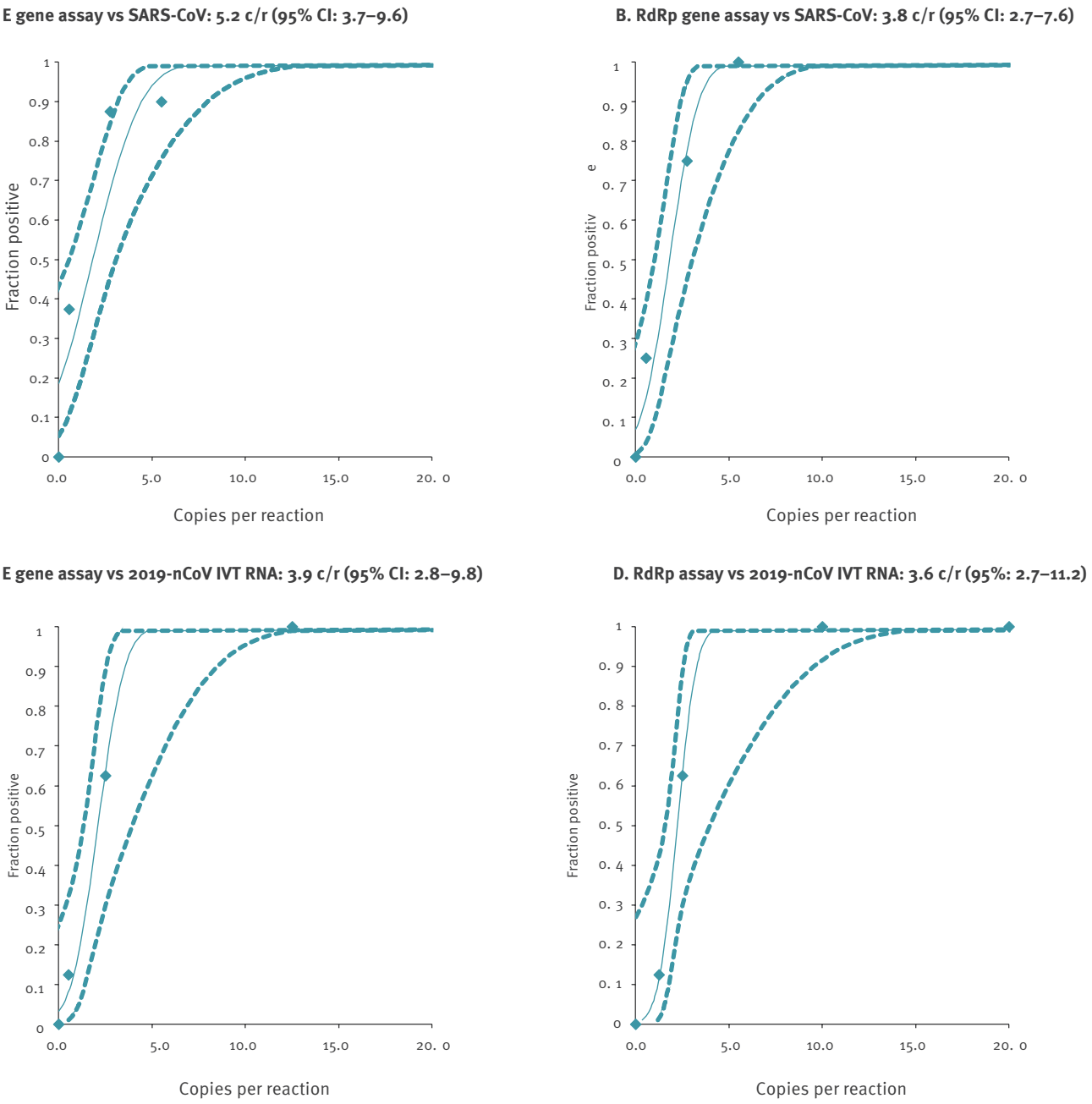
Specificity testing

Chemical stability

To exclude non-specific reactivity of oligonucleotides among each other, causing artificial fluorescent

Figure 3

Determination of limits of detection based on SARS coronavirus genomic RNA and 2019 novel coronavirus-specific in vitro transcribed RNA



CI: confidence intervals; c/r: copies per reaction; IVT: in vitro-transcribed RNA.

A: E gene assay, evaluated with SARS-CoV genomic RNA. B: RdRp gene assay evaluated with SARS-CoV genomic RNA. C: E-gene assay, evaluated with 2019-nCoV-specific in vitro-transcribed RNA standard. D: RdRp gene assay evaluated with 2019-nCoV-specific in vitro-transcribed RNA standard.

The x-axis shows input RNA copies per reaction. The y-axis shows positive results in all parallel reactions performed, squares are experimental data points resulting from replicate testing of given concentrations (x-axis) in parallels assays (eight replicate reactions per point).

Technical limits of detection are given in the panels headings. The inner line is a probit curve (dose-response rule). The outer dotted lines are 95% CI.

Table 2

Tests of known respiratory viruses and bacteria in clinical samples and cell culture preparations for cross-reactivity in 2019 novel coronavirus E and RdRp gene assays (n = 310)

Clinical samples with known viruses	Clinical samples[a]	Virus isolates[b]
HCoV-HKU1	14	1[c]
HCoV-OC43	16	2[d]
HCoV-NL63	14	1[e]
HCoV-229E	18	2[f]
MERS-CoV	5	1[g]
Influenza A(H1N1)pdm09	17	1
Influenza A(H3N2)	16	1
Influenza A (untyped)	11	NA
Influenza A(H5N1)	1	1
Influenza A(H7N9)	0	1
Influenza B (Victoria or Yamagata)	31	1
Rhinovirus/enterovirus	31	NA
Respiratory syncytial virus (A/B)	33	NA
Parainfluenza 1 virus	12	NA
Parainfluenza 2 virus	11	NA
Parainfluenza 3 virus	14	NA
Parainfluenza 4 virus	11	NA
Human metapneumovirus	16	NA
Adenovirus	13	1
Human bocavirus	6	NA
Legionella spp.	3	NA
Mycoplasma spp.	4	NA
Total clinical samples	**297**	**NA**

[a] For samples with multiple viruses detected, the virus with highest concentration is listed, as indicated by real-time PCR Ct value.

[b] Directly quantified or spiked in human negative-testing sputum.

[c] 1×10^5 RNA copies/mL, determined by specific real-time RT-PCR. Isolated from human airway epithelial culture.

[d] 1×10^{10} RNA copies/mL, determined by specific real-time RT-PCR of one isolate. The other isolate was not quantified but spiked in human negative-testing sputum.

[e] 4×10^9 RNA copies/mL, determined by specific real-time RT-PCR.

[f] 3×10^9 RNA copies/mL, determined by specific real-time RT-PCR of one isolate. The other isolate was not quantified spiked in human negative-testing sputum.

[g] 1×10^8 RNA copies/mL, determined by specific real-time RT-PCR.

signals, all assays were tested 120 times in parallel with water and no other nucleic acid except the provided oligonucleotides. In none of these reactions was any positive signal detected.

Cross-reactivity with other coronaviruses

Cell culture supernatants containing all endemic human coronaviruses (HCoV)229E, NL63, OC43 and HKU1 as well as MERS-CoV were tested in duplicate in all three assays (Table 2). For the non-cultivable HCoV-HKU1, supernatant from human airway culture was used. Viral RNA concentration in all samples was determined by specific real-time RT-PCRs and in vitro-transcribed RNA standards designed for absolute quantification of viral load. Additional undiluted (but not quantified) cell culture supernatants were tested as summarised in Table 2. These were additionally mixed into negative human sputum samples. None of the tested viruses or virus preparations showed reactivity with any assay.

Exclusivity of 2019 novel coronavirus based on clinical samples pre-tested positive for other respiratory viruses

Using the E and RdRp gene assays, we tested a total of 297 clinical samples from patients with respiratory disease from the biobanks of five laboratories that provide diagnostic services (one in Germany, two in the Netherlands, one in Hong Kong, one in the UK). We selected 198 samples from three university medical centres where patients from general and intensive care wards as well as mainly paediatric outpatient departments are seen (Germany, the Netherlands, Hong Kong). The remaining samples were contributed by national public health services performing surveillance studies (RIVM, PHE), with samples mainly submitted by practitioners. The samples contained the broadest range of respiratory agents possible and reflected the general spectrum of virus concentrations encountered in diagnostic laboratories in these countries (Table 2). In total, this testing yielded no false positive outcomes. In four individual test reactions, weak initial reactivity was seen but they were negative upon retesting with the same assay. These signals were not associated with any particular virus, and for each virus with which initial positive reactivity occurred, there were other samples that contained the same virus at a higher concentration but did not test positive. Given the results from the extensive technical qualification described above, it was concluded that this initial reactivity was not due to chemical instability of real-time PCR probes but most probably to handling issues caused by the rapid introduction of new diagnostic tests and controls during this evaluation study.

Discussion

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. Effective assay design was enabled by the willingness of scientists from China to share genome information before formal publication, as well as the availability of broad sequence knowledge from ca 15 years of investigation of SARS-related viruses in animal reservoirs. The relative ease with which assays could be designed for this virus, in contrast to SARS-CoV in 2003, proves the huge collective value of descriptive studies of disease ecology and viral genome diversity [8,15-17].

Real-time RT-PCR is widely deployed in diagnostic virology. In the case of a public health emergency, proficient diagnostic laboratories can rely on this robust technology to establish new diagnostic tests within their routine services before pre-formulated assays become available. In addition to information on

reagents, oligonucleotides and positive controls, laboratories working under quality control programmes need to rely on documentation of technical qualification of the assay formulation as well as data from external clinical evaluation tests. The provision of control RNA templates has been effectively implemented by the EVAg project that provides virus-related reagents from academic research collections [18]. SARS-CoV RNA was retrievable from EVAg before the present outbreak; specific products such as RNA transcripts for the here-described assays were first retrievable from the EVAg online catalogue on 14 January 2020 (https://www.european-virus-archive.com). Technical qualification data based on cell culture materials and synthetic constructs, as well as results from exclusivity testing on 75 clinical samples, were included in the first version of the diagnostic protocol provided to the WHO on 13 January 2020. Based on efficient collaboration in an informal network of laboratories, these data were augmented within 1 week comprise testing results based on a wide range of respiratory pathogens in clinical samples from natural infections. Comparable evaluation studies during regulatory qualification of in vitro diagnostic assays can take months for organisation, legal implementation and logistics and typically come after the peak of an outbreak has waned. The speed and effectiveness of the present deployment and evaluation effort were enabled by national and European research networks established in response to international health crises in recent years, demonstrating the enormous response capacity that can be released through coordinated action of academic and public laboratories [18-22]. This laboratory capacity not only supports immediate public health interventions but enables sites to enrol patients during rapid clinical research responses.

*Author's correction

The sentence As at 20 January 2020, 282 laboratory-confirmed human cases have been notified to WHO was originally published with a wrong date (As at 20 January 2019...). This mistake was corrected on 8 April 2020.

On 29 July 2020 the correct affiliation of Marco Kaiser was added and the remaining affiliations were renumbered.

**Addendum

The Conflict of interest section was updated on 29 July 2020.

Acknowledgements

This work was funded by European Union DG Research through projects Prepare (GA602525), Compare (GA643476), and EVAg (GA653316); by European Union DG SANCO through EVD-LabNet, as well as by the German Ministry of Research through projects RAPID (01KI1723A) and DZIF (301-4-7-01.703).

We gratefully acknowledge the authors, the originating and submitting laboratories for their sequence and metadata shared through GISAID, on which this research is based.

All authors of data may be contacted directly via www.gisaid.org: National Institute for Viral Disease Control and Prevention, China CDC (Wenjie Tan, Xiang Zhao, Wenling Wang, Xuejun Ma, Yongzhong Jiang, Roujian Lu, Ji Wang, Weimin Zhou, Peihua Niu, Peipei Liu,Faxian Zhan, Weifeng Shi, Baoying Huang, Jun Liu, Li Zhao, Yao Meng, Xiaozhou He, Fei Ye, Na Zhu, Yang Li, Jing Chen, Wenbo Xu, George F. Gao, Guizhen Wu); Wuhan Institute of Virology, Chinese Academy of Sciences (Peng Zhou, Xing-Lou Yang, Ding-Yu Zhang, Lei Zhang, Yan Zhu, Hao-Rui Si, Zhengli Shi); Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College (Lili Ren, Jianwei Wang, Qi Jin, Zichun Xiang, Yongjun Li, Zhiqiang Wu, Chao Wu, Yiwei Liu); and National Institute for Communicable Disease Control and Prevention (ICDC), China CDC (Zhang Y-Z, Wu, F, Chen Y-M, Pei Y-Y, Xu L, Wang W, Zhao S, Yu B, Hu Y, Tao Z-W, Song Z-G, Tian J-H, Zhang Y-L, Liu Y, Zheng J-J, Dai F-H, Wang Q-M, She J-L and Zhu T-Y)

We thank Marta Zuchowski, Sigrid Kersten, and Joerg Hofmann for help with sample logistics. In vitro-transcribed control RNA for the E gene assay can be acquired from author C. D. through the European Virus Archive platform (www.european-virus-archive.com),

Conflict of interest **

Olfert Landt is CEO of Tib-Molbiol; Marco Kaiser is senior researcher at GenExpress and serves as scientific advisor for Tib-Molbiol.

Authors' contributions

VMC: Planned and conducted experiments, conceptualised the laboratory work

OL: Planned and conducted experiments, conceptualised the laboratory work

MK: Planned and conducted experiments

RM: Planned and conducted experiments, conceptualised the laboratory work

AM: Planned and conducted experiments, conceptualised the laboratory work

DKWC: Planned and conducted experiments

TB: Planned and conducted experiments

SB: Planned and conducted experiments

JS: Planned and conducted experiments

MLS: Planned and conducted experiments

DGJCM: Planned and conducted experiments

BLH: Planned and conducted experiments

BvdV: Planned and conducted experiments

SvdB: Planned and conducted experiments

LW: Planned and conducted experiments

GG: Planned and conducted experiments

JLR: Contributed to overall study conceptualization

JE: Planned and conducted experiments, conceptualised the laboratory work

MZ: Planned laboratory work, contributed to overall study conceptualization

MP: Planned laboratory work, contributed to overall study conceptualization

HG: Contributed to overall study conceptualization

CR: Planned experiments, conceptualised the laboratory work

MPGK: Planned experiments, conceptualised the laboratory work

CD: Planned experiments, conceptualised the laboratory work, conceptualised the overall study, wrote the manuscript draft.

References

1. World Health Organization (WHO). Coronavirus. Geneva: WHO; 2020 [Accessed 21 Jan 2020]. Available from: https://www.who.int/health-topics/coronavirus
2. Zhang Y-Z. Novel 2019 coronavirus genome. Virological. [Accessed 21 Jan 2020]. Available from: http://virological.org/t/novel-2019-coronavirus-genome/319
3. de Groot RJ, Baker SC, Baric R, Enjuanes L, Gorbalenya AE, Holmes KV, et al. Family Coronaviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ. Virus taxonomy: classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses. London; Waltham: Academic Press; 2012. p. 806-20.
4. Peiris JS, Yuen KY, Osterhaus AD, Stöhr K. The severe acute respiratory syndrome. N Engl J Med. 2003;349(25):2431-41. https://doi.org/10.1056/NEJMra032498 PMID: 14681510
5. World Health Organization. (WHO. Novel Coronavirus (2019-nCoV). Situation report – 1. Geneva: WHO; 21 Jan 2020. Available from: https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200121-sitrep-1-2019-ncov.pdf
6. Abbott A. SARS testing: First past the post. Nature. 2003;423(6936):114. https://doi.org/10.1038/423114a PMID: 12736651
7. Corman VM, Müller MA, Costabel U, Timm J, Binger T, Meyer B, et al. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. 2012;17(49):20334. https://doi.org/10.2807/ese.17.49.20334-en PMID: 23231891
8. Drosten C, Günther S, Preiser W, van der Werf S, Brodt HR, Becker S, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003;348(20):1967-76. https://doi.org/10.1056/NEJMoa030747 PMID: 12690091
9. Corman VM, Eickmann M, Landt O, Bleicker T, Brünink S, Eschbach-Bludau M, et al. Specific detection by real-time reverse-transcription PCR assays of a novel avian influenza A(H7N9) strain associated with human spillover infections in China. Euro Surveill. 2013;18(16):20461. PMID: 23611031
10. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17(39):20285. https://doi.org/10.2807/ese.17.39.20285-en PMID: 23041020
11. Panning M, Charrel RN, Donoso Mantke O, Landt O, Niedrig M, Drosten C. Coordinated implementation of chikungunya virus reverse transcription-PCR. Emerg Infect Dis. 2009;15(3):469-71. https://doi.org/10.3201/eid1503.081104 PMID: 19239767
12. Corman VM, Rasche A, Baronti C, Aldabbagh S, Cadar D, Reusken CB, et al. Assay optimization for molecular detection of Zika virus. Bull World Health Organ. 2016;94(12):880-92. https://doi.org/10.2471/BLT.16.175950 PMID: 27994281
13. Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, Goettsche M, et al. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. J Virol. 2010;84(21):11336-49. https://doi.org/10.1128/JVI.00650-10 PMID: 20686038
14. Muth D, Corman VM, Roth H, Binger T, Dijkman R, Gottula LT, et al. Attenuation of replication by a 29 nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. Sci Rep. 2018;8(1):15177. https://doi.org/10.1038/s41598-018-33487-8 PMID: 30310104
15. Corman VM, Muth D, Niemeyer D, Drosten C. Hosts and sources of endemic human coronaviruses. Adv Virus Res. 2018;100:163-88. https://doi.org/10.1016/bs.aivir.2018.01.001 PMID: 29551135
16. Drexler JF, Corman VM, Drosten C. Ecology, evolution and classification of bat coronaviruses in the aftermath of SARS. Antiviral Res. 2014;101:45-56. https://doi.org/10.1016/j.antiviral.2013.10.013 PMID: 24184128
17. Cui J, Li F, Shi ZL. Origin and evolution of pathogenic coronaviruses. Nat Rev Microbiol. 2019;17(3):181-92. https://doi.org/10.1038/s41579-018-0118-9 PMID: 30531947
18. Romette JL, Prat CM, Gould EA, de Lamballerie X, Charrel R, Coutard B, et al. The European Virus Archive goes global: A growing resource for research. Antiviral Res. 2018;158:127-34. https://doi.org/10.1016/j.antiviral.2018.07.017 PMID: 30059721
19. Alleweldt F, Kara S, Osinski A, Van Baal P, Kellerborg K, Aarestrup FM, et al. Developing a framework to assess the costeffectiveness of COMPARE - a global platform for the exchange of sequence-based pathogen data. Rev Sci Tech. 2017;36(1):311-22. https://doi.org/10.20506/rst.36.1.2631 PMID: 28926006
20. Domingo C, Ellerbrok H, Koopmans M, Nitsche A, Leitmeyer K, Charrel RN, et al. Need for additional capacity and improved capability for molecular detection of yellow fever virus in European Expert Laboratories: External Quality Assessment, March 2018. Euro Surveill. 2018;23(28):1800341. https://doi.org/10.2807/1560-7917.ES.2018.23.28.1800341 PMID: 30017021
21. Pas SD, Patel P, Reusken C, Domingo C, Corman VM, Drosten C, et al. First international external quality assessment of molecular diagnostics for Mers-CoV. J Clin Virol. 2015;69:81-5. https://doi.org/10.1016/j.jcv.2015.05.022 PMID: 26209385
22. Gobat N, Amuasi J, Yazdanpanah Y, Sigfid L, Davies H, Byrne JP, et al. Advancing preparedness for clinical research during infectious disease epidemics. ERJ Open Res. 2019;5(2):00227-2018. https://doi.org/10.1183/23120541.00227-2018 PMID: 31123684

License, supplementary material and copyright

Any supplementary material referenced in the article can be found in the online version.

Veiligheid / Caution

Bijlage 2. SOP deel 1_M900 Het isoleren en zuiveren van nucleinezuur uit klinische monsters mbv MagNA Pure 96 versie 7

Er wordt gewerkt met patiënten-materialen. Werk daarom volgens de algemene veiligheidsvoorschriften voor microbiologische laboratoria. De regels die hierop betrekking hebben, zijn te lezen in IDS_DOC_0502 en KAM regel 008. Voor werken in de PCR ruimtes zijn aparte voorschriften, deze zijn te lezen in IDS_DOC_0503

Specifieke waarschuwingen:
Guanidinium thiocyanate (zit in lysisbuffer) : CAS-no. 593-84-0; CHS05 schadelijk CHS07 irriterend. Is toxisch bij inademing, opname door de mond en via de huid. Voorkom direct contact. Bij aanraking langdurig spoelen met water zo nodig arts waarschuwen. Voorkom dat het in contact komt met chloor of zuren, want dan wordt het giftige blauwzuur gas gevormd.
Kit bevat verder: Guanidine chloride, 20% triton X-100, <2% proteinase K, 38% ethanol, Isopropanol en Glycerol. CHS05 schadelijk; CHS07 irriterend; CHS02 brandbaar. Voorkom direct contact en inname met deze vloeistoffen. Bij aanraking langdurig spoelen met water zo nodig arts waarschuwen.

- Gebruik bij overgieten van het gele 10 liter vat naar een vijf liter vat, handschoenen, gelaatsscherm en de lekbak. Het MagNA Pure afvalvat moet worden afgevoerd in een vijf liter vat met een sticker met Uncode **UN 2924**.
- Draag bij alle werkzaamheden disposable handschoenen.

01 Inleiding / Introduction

1.1 Doel

Het isoleren en zuiveren van nucleïnezuur uit klinische monsters m.b.v. de MagNA Pure 96. Het opgezuiverde nucleïnezuur dient als template voor de (RT-)PCR's.

1.2 Principe van de bepaling

Het nucleïnezuur wordt geïsoleerd uit het monster met behulp van een chaotrope lysisbuffer en "Magnetic Glass Particles" (MGP), waarbij het nucleïnezuur na lysis aan de glaspartikels bindt. Na een aantal wasstappen wordt het nucleïnezuur van de partikels geëlueerd, waarna het vervolgens gebruikt kan worden voor de DNA amplificatie (PCR), eventueel voorafgegaan door cDNA synthese bij RNA.

1.3 Doelgroep

Laboratoriummedewerkers met ervaring in moleculair biologische technieken.

1.4 Afkortingen

MP96:	MagNA Pure 96
PCR:	Polymerase Chain Reaction
RNA:	Ribonucleic Acid
DNA:	Desoxyribonucleic acid
EAV:	Equine Arthritis Virus
RT-PCR:	Reverse Transcription Polymerase Chain Reaction
cDNA:	Copy DNA
PhHV:	Phocine Herpesvirus
MEM:	Minimum Essential Medium
GLY:	gelatine, lactalbuminehydrolysaat en extract van gist (yeast) in Hank's zoutoplossing met antibiotica

1.5 Anti-contaminatie

Algemene waarschuwingen ter voorkoming van contaminatie:

- Draag altijd een (schone) laboratoriumjas die alleen in het RNA-lab gebruikt wordt en draag steeds disposable handschoenen.
- Gebruik altijd Rnase/ Dnase vrije eppen, pipetpuntjes met filter, buffers, water, etc.
- Kweken altijd eerst verdunnen voordat deze naar het RNA lab worden gebracht. (zie bijvoorbeeld ook IDS/VIR/M006 paragraaf 5.3 punt 5)
- RNA-lab (onderdruk): In deze ruimte wordt nucleïnezuur uit klinische monsters geïsoleerd. In deze ruimte mag nooit met amplificaat gewerkt worden.

02 Chemicaliën, reagentia en media

2.1 Chemicalien en reagentia voor de MP96

- MagNa Pure 96 DNA and Viral NA Small Volume kit**

- Lysis/Binding Buffer refill*
- MagNa Pure 96 System Fluid (external) container

*Zie voor de ingangscontrole van deze reagentia het digitale labjournaal. (R:\IDS\Discipline VIR\Analistenpool\Labjournaal\ingangscontrole reagentia) Zie Hoofdstuk 07 "Samenhangende documenten" de link naar de betreffende bijsluiters.

**De MagNa Pure 96 DNA and Viral NA Small Volume kit is een kritisch reagens. Bij het binnenkomen van een nieuw batchnummer van kritische reagentia dient normaliter een ingangscontrole te worden uitgevoerd om de werking te verifiëren. Echter, omdat het niet mogelijk is om twee kits van verschillende batchnummers in een run mee te nemen, en dus een aparte run uitgevoerd zou moeten worden, zou de kit hierdoor lang ongebruikt open staan, waardoor deze expireert. Om deze reden wordt voor deze kit geen ingangscontrole uitgevoerd.

Bij het in gebruik nemen van een nieuwe batch, LET OP: Zorg er voor dat er enkel materialen aan boord staan waarvan genoeg spijt-materiaal aanwezig is om de extractie te kunnen herhalen.

2.2 Andere reagentia

- Yeast tRNA, Invitrogen.
- PCR-grade, Rnase/ Dnase vrij water
- Voor negatieve controle:
  - GLY-medium
  - MEM-medium
  - Fysiologisch zout

03 Biologisch materiaal

De MagNA Pure 96 kan voor extractie van verschillende biologische materialen dienen, waaronder klinisch materiaal (oa serum, fecessuspensies, urine) en (verdund) gekweekt materiaal (virus- en bacterie-kweken).
Er bestaan verschillende kits (zie 2.1) waarmee de diverse biologische materialen geëxtraheerd kunnen worden.

Interne controle materiaal: EAV (RNA-virus), PhHV (DNA-virus)
Cellijnen t.b.v. Viruskweken.

04 Apparatuur en hulpmiddelen

4.1 Apparatuur

· MagNA Pure 96 (Roche) zie ook IDS/A801

4.2 Hulpmiddelen

- Draaidop buizen (0,5 en 2 ml)
- Pipet 10-20-100-1000 µl
- MagNa Pure 96 Needle set (4)
- MagNa Pure 96 Output Plate
- MagNa Pure 96 Processing Cartridge
- MagNa Pure 96 Tips (1000µl) (8*480)

05 Werkwijze / Method

Opmerking:

1. Ook DNA wordt op het RNA lab met de MP96 geïsoleerd omdat er slechts 1 MP96 aanwezig is.
2. Gebruik altijd het “externe lysis”-protocol. Externe lysis bestaat uit het inactiveren van klinische materiaal door middel van het toevoegen van lysisbuffer voordat het materiaal in de MP96 wordt gezet. Om te voorkomen dat het lab of de MP96 wordt blootgesteld aan infectieus materiaal.
3. De plastic disposables, (tips en platen), zijn tot 3 jaar na de productie-datum te gebruiken. De productie datum staat aangegeven op de grootverpakking en ook op de kleine sticker op de individuele plastic verpakkingen.
4. De MP96 software heeft drie tellers voor de reagentia:

a. Time after first use: Deze telt vanaf de eerste keer dat de verpakking opengeprikt wordt door de naald en blijft lopen, ook wanneer de reagentia van boord gehaald worden.
Voor de DNA and Viral NA small volume kit is de maximale tijd om de kit te gebruiken 28 dagen.

b. Onboard time: zoals de naam al suggereert, telt deze teller de daadwerkelijke tijd aan boord van de MP96. Wanneer niet aan boord, dienen reagentia geseald in de koelkast te
staan. Voor de DNA and Viral NA small volume kit is de maximum onboard time bijvoorbeeld 32 uur.

c. Number of re-uses: Een kit kan maar een beperkt aantal runs gebruikt worden. Voor de DNA and Viral NA small volume kit is het aantal runs ingesteld op maximaal 8.

6. Wanneer kit in koelkast heeft gestaan deze eerst op kamertemperatuur laten komen.
7. Het is niet mogelijk verschillende lotnummers van de kits door elkaar te gebruiken. Geopende kits zijn 1 maand te gebruiken.
8. Wanneer een nieuwe kit in gebruik genomen wordt: sticker alle onderdelen zodat de kit bij elkaar blijft met een gekleurde sticker, noteer dit in de map.

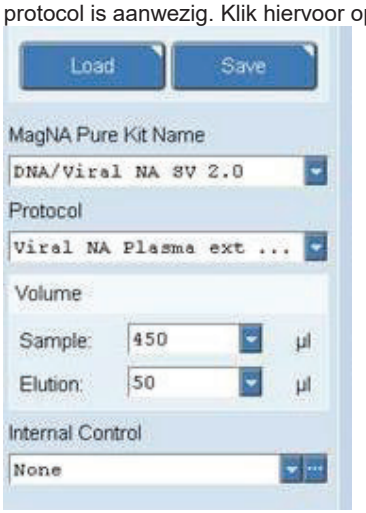
5.1 Monster voorbewerking

1. Voeg 200 µl klinisch materiaal toe aan 250 µl lysisbuffer en voeg daarbij eventueel interne controle en yeast-tRNA(zie bijlage3). Indien er te weinig monster-materiaal voorhanden is, kan het volume worden aangevuld met negatief controle-materiaal, zoals fysiologisch zout, GLY-medium of MEM-medium). Bij een totaalvolume minder dan 450 µl vindt de extractie namelijk niet efficiënt plaats.
   - NB: lysisbuffer heeft enige tijd nodig om aanwezige virussen te inactiveren (polioviruskweken = 20 minuten). Zorg dat het klinisch materiaal voldoende tijd geincubeerd heeft met lysisbuffer om er mee te werken buiten de BHK.
   - NB: Als er gewerkt wordt met kweken, moeten deze verdund worden voordat ze toegevoegd worden aan lysisbuffer. Verdunning hangt af van de te verwachten virale/bacteriele load. Zie de betreffende kweek-SOPs voor deze verdunning. (IDS/VIR/M199 Enteroviruskweek, IDS/VIR/M006 Influenzaviruskweek)
2. Negatieve controle: Voeg aan 250 µl lysisbuffer en de eventuele interne controle 200 µl medium toe (bijvoorbeeld gly, MEM, fysiologisch zout). Geen water als negatieve controle gebruiken. Dit heeft een negatief effect op de extractie.
3. Vortexen, kort afdraaien.
4. Pipetteer 450 µl van deze vloeistof in de Processing Cartridge of sla op bij -20°C.

5.2 Bediening MP96

Onderstaande uitleg wordt geïllustreerd aan de hand van screenshot-plaatjes van de menuschermen van de MagNA Pure 96 in bijlage 1.

1. Zet de PC en de MagNA Pure 96 aan door op de aan-knoppen te drukken. (MagNa Pure 96 zal standaard controles uitvoeren neemt ± 5 minuten in beslag).
2. Inloggen PC= **.**\MP96User, wachtwoord= MPDev09
3. Klik op het desktop icoon `MagNa Pure 96` (Bijlage 1 figuur 1: overview is zichtbaar)
4. Klik rechts op ``Logon`` ID= Admin, Password= Master1
5. Klik in de bovenste balk op ``Instrument``, gevolgd door ``Maintenance & Service``, (Bijlage 1 figuur 2: Instrument-Maintenance & Service is zichtbaar)
   Voer de Daily Maintenance uit door deze aan te vinken en op start te drukken.
   Check of er ``On demand Maintenance`` noodzakelijk is, voer zo nodig uit.
6. Klik in de bovenste balk op ``Workplace`` gevolgd door ``order``. (Bijlage 1 figuur 3: Workplace-Order) Een standaard protocol is aanwezig. Klik hiervoor op ``Load``, selecteer ``Pool 50 external lysis``. **Controleer onderstaande instellingen:**



7.
8. Wanneer ander protocol noodzakelijk is, raadpleeg de beheerder van het apparaat.
9. Selecteer het gewenste aantal monsters door per monster een naam of monsternummer in te vullen, of een 1, onder 'Sample Name'. Dit moet per 8 monsters.Save het experiment door op de gele disk te klikken en het experiment een naam te geven.
10. Klik onderaan in het scherm op ``>>``, (Bijlage figuur 4: Workplace-Stage) Open de load flap en breng de aangegeven disposables, kits in het apparaat, zo ook de lege tip trays (lege rekjes waar 1000µl tips in hebben gezeten) en waste cover (zwart afvalrekje) in de waste rack (= rek op meest rechtse positie. Dit rek wordt de inhoud niet gecheckt op aanwezigheid).
11. Sluit de load flap. Indien alle disposables en kits op de juiste plaats en in juiste hoeveelheid aan boord zijn gebracht, worden deze in het overzicht in groen weergegeven. Rechts in het scherm, Onder status, moeten alle opties groen zijn. (Bijlage figuur 4: Workplace-Stage) Alleen dan kan de extractie gestart worden door op start te drukken. (Wanneer een plek wit is geworden, betekent dit dat er op deze plek iets staat dat het apparaat voor deze run niet nodig heeft maar mag blijven

staan). Bij een rode positie check wat er mis is. Bv. Klep niet goed gesloten, afval vat vol, reagentia niet aan boord of twee verschillende kits.

12. Na ongeveer een uur is de extractie voltooid, haal het eluaat uit het apparaat.

5.3 Afvoer van afval en schoonmaak

1. Gooi de gebruikte disposables weg m.u.v. lege tip trays en de waste rack. Deze **goed afspoelen** in de waterbak (om blauwzuurgasvorming te voorkomen) en vervolgens in een chloorbad (van ongeveer 2 liter) met 1 tablet (elke week vers te maken) leggen en overnacht laten staan. Afspoelen met water en laten drogen.
2. **Bij het uitvoeren van meerdere runs achter elkaar, de waste-cover steeds vervangen, dit voorkomt contaminatie tussen runs.**
3. Zet de gebruikte kit in de koelkast nadat deze is afgeplakt met folie.
4. Maak het dek schoon met een doekje hypochloriet, gevolgd door ethanol.
5. Klik op ``Instrument`` gevolgd door ``Maintenance & Service``, vink ``UV decontaminatie`` aan. Vul 60 minuten in, open en sluit de MP96-klep, en klik op start. (Bijlage Figuur 2: Instrument- Maintenance & Service)
6. Apparaat nooit definitief uitzetten voordat de kits van boord zijn en het apparaat gescand heeft. (Het apparaat moet weten dat de kits van boord zijn betreffende de houdbaarheid).
7. Wanneer het apparaat bij ``Waste`` aangeeft dat het gele 10 liter vat vol is, giet de vloeistof over in 10 liter vaten met een sticker **categorie V**.
8. **Gebruik bij het overgieten een gelaatsscherm.**

06 Kwaliteitscontrole

Als de run klaar is, wordt er een melding weergegeven dat de run is geslaagd. (Bijlage figuur5: Workplace result). Indien deze status niet weergegeven wordt, dient de run herhaald te worden.

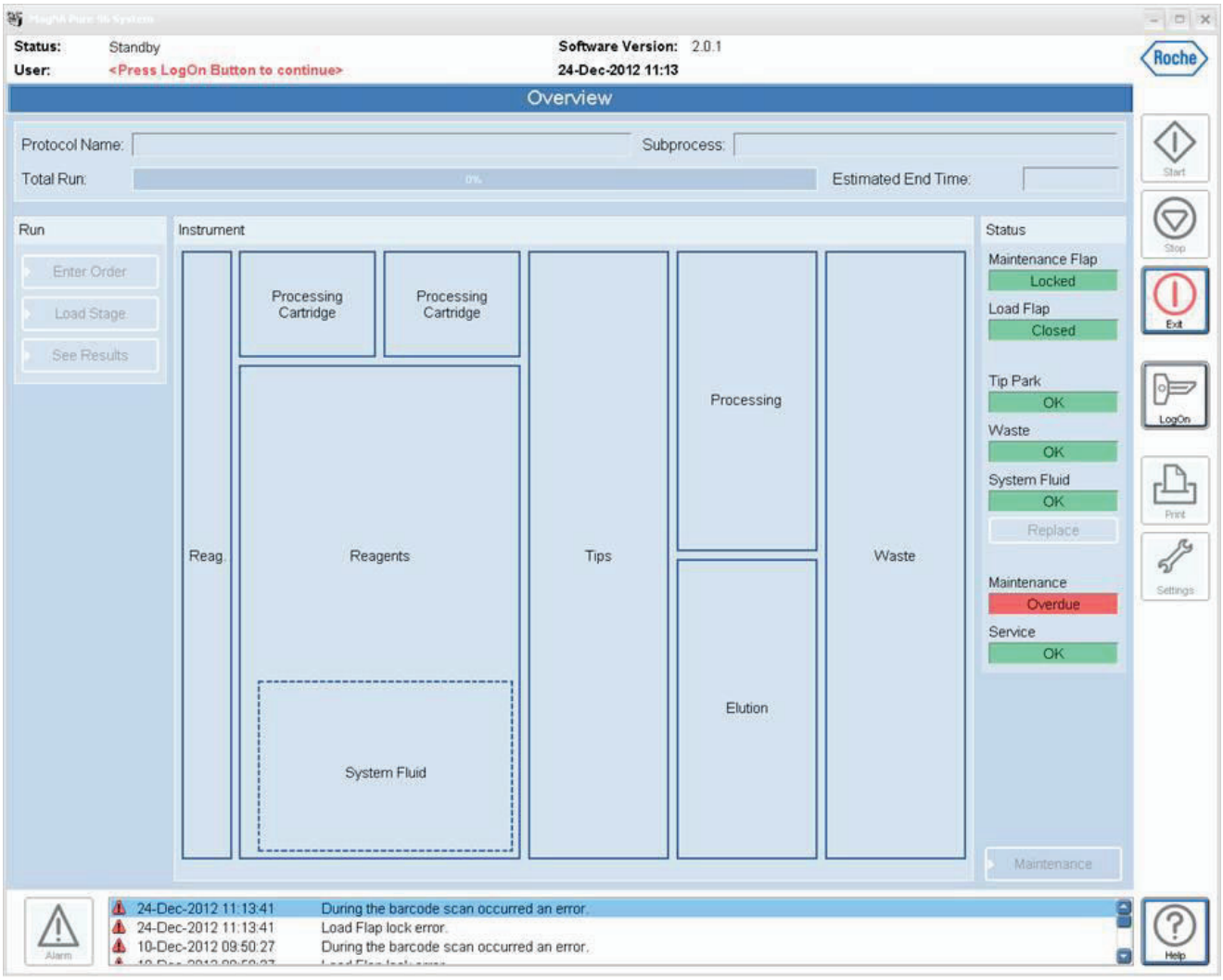
07 Samenhangende documenten

- IDS_DOC_0502
- IDS_DOC_0503
- KAMregel 008
- IDS/VIR/M006
- IDS/VIR/F897 MagNA Pure 96 Werklijst
- MagNA Pure 96 Operator's Manual, Roche
- IDS/A801 Gebruikershandleiding MagNA Pure 96
- R:\IDS\Discipline VIR\Analistenpool\Labjournaal\ingangscontrole reagentia
- IDS/VIR/B008 Bijsluiter MP96 lysisbuffer
- IDS/VIR/B009 Bijsluiter MagNa Pure 96 DNA and Viral NA Small Volume kit
- Validatierapport MagNA Pure 96 extractie

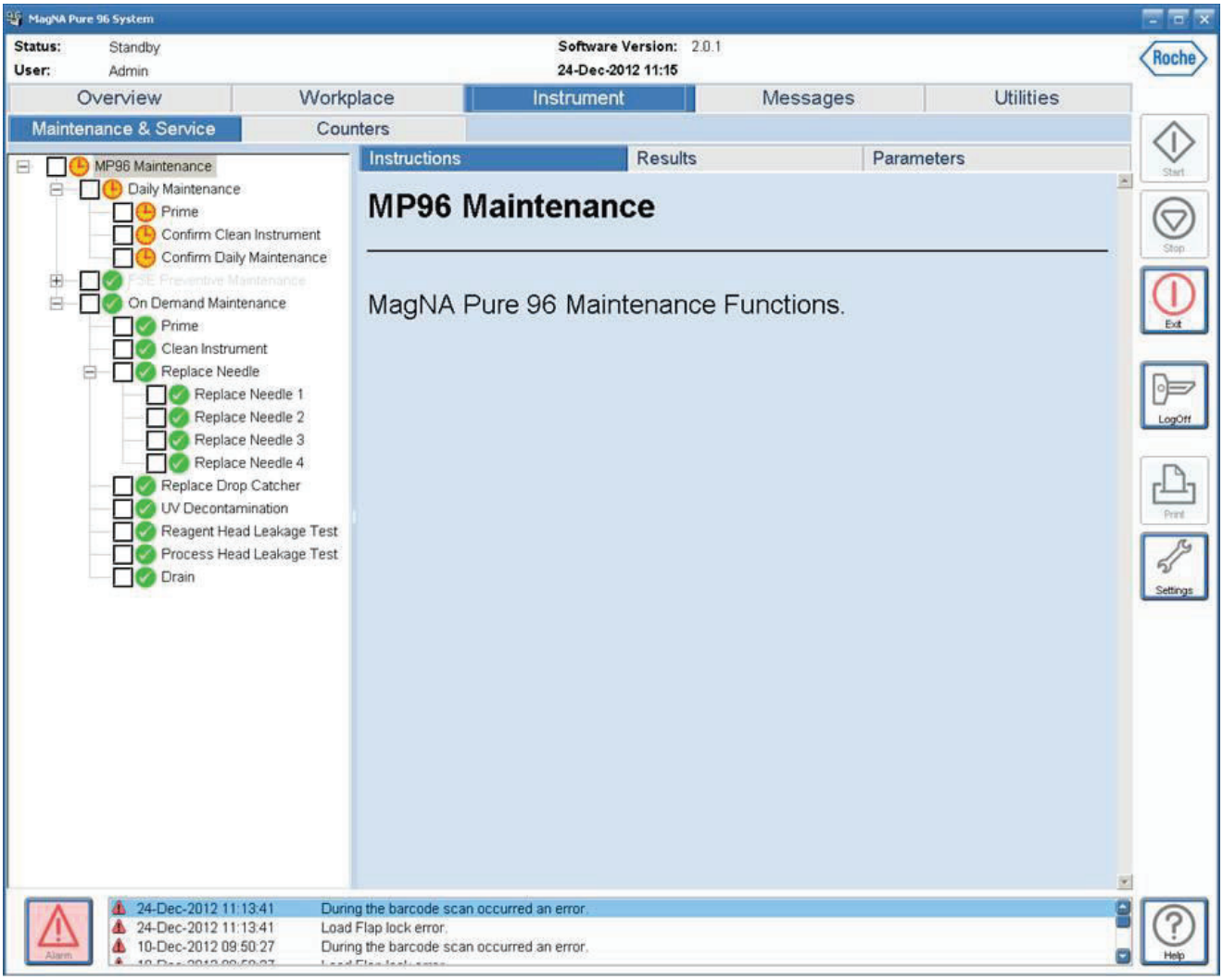
Literatuur / References

1. MagNA Pure 96 Operator's Manual, Roche
2. http://technical-support.roche.com/product.aspx?productID=9965 (Background Information- help Corner)

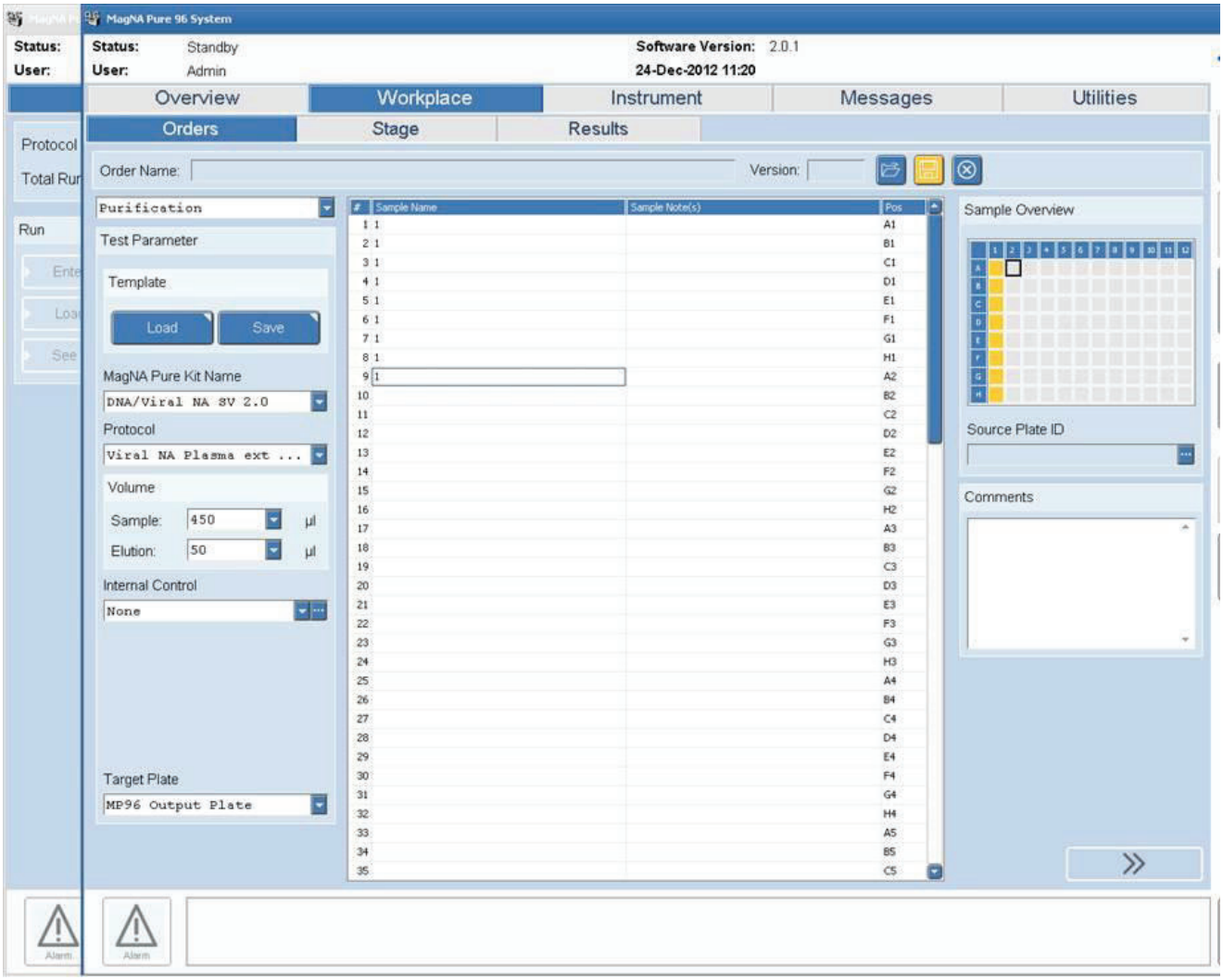
Bijlage 1



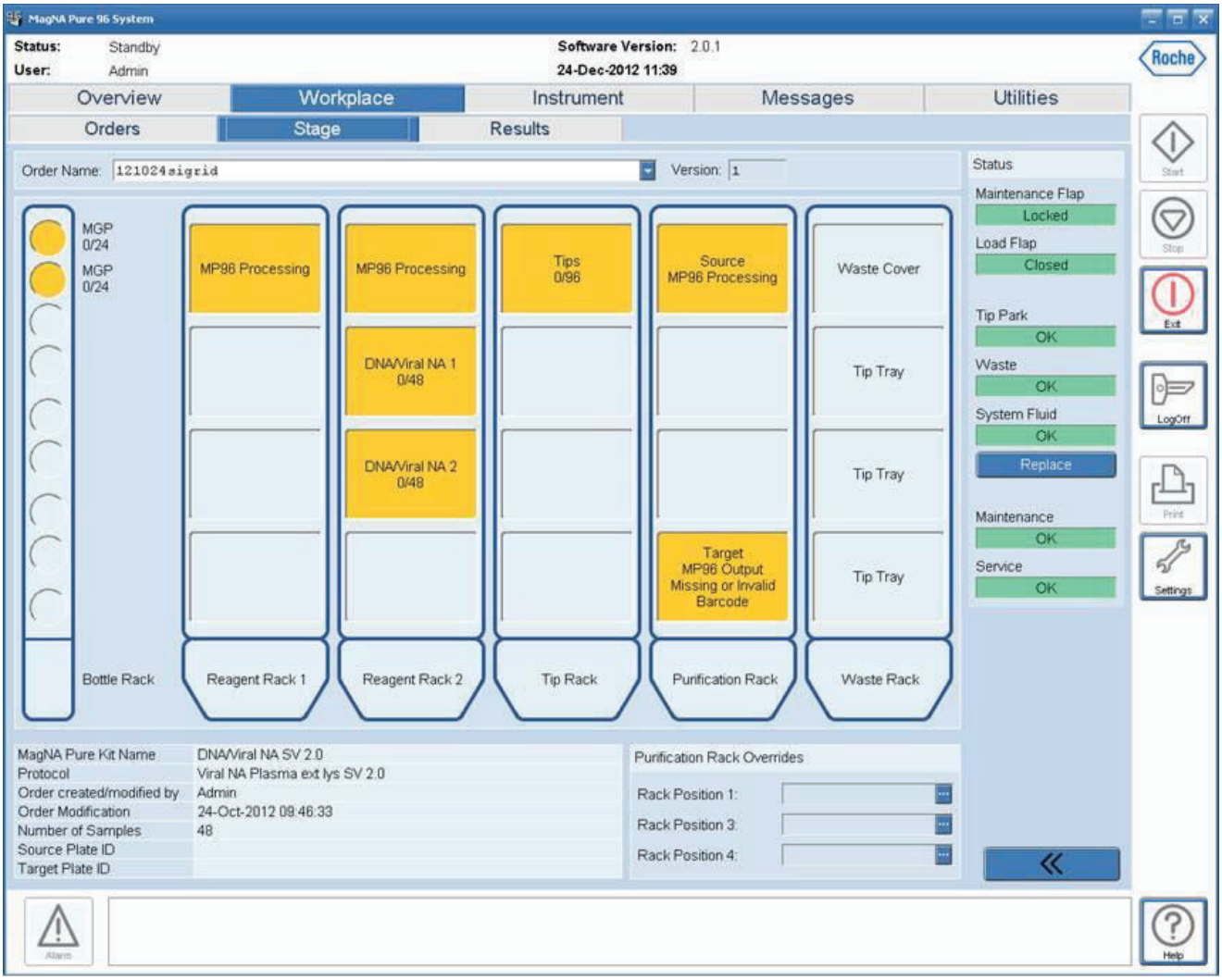
Figuur 1: Overview



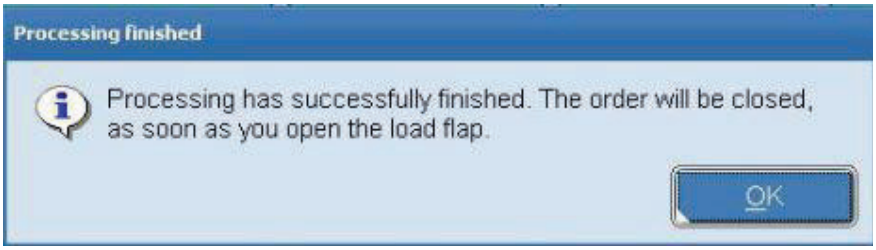
Figuur 2: Instrument- Maintenance & Service



Figuur 3: Workplace-Order



Figuur 4: Workplace-Stage



Figuur 5: Workplace- Result

Bijlage 2

Trouble shooting

1. Vaak kunnen problemen opgelost worden door het systeem opnieuw op te starten. Sluit hiervoor eerst het systeem af (eerst computer, dan instrument). Controleer de volgende punten:

   ·Controleer of barcodes door condensatie of andere verontreinigingen misschien niet goed gescand kunnen worden. Controleer ook de positie van de barcode (scan gebeurt vanaf links)

·Controleer de bottle shaker, het rek met de magnetic beads, of de shaker kan bewegen en of het rek ver genoeg naar binnen geschoven is.
·Controleer of er obstakels in de incubatorplaat zitten, de roterende plaat onder het purificatie rek. Controleer ook of de processing cartridge die hierop past vrij is van obstakels.
·Controleer of de Tip park positie, achterin het instrument, leeg is voor het starten van een nieuwe run.
·Controleer de naalden en/of het gevorkte klemmetje bovenaan de naald goed is aangesloten.
·Controleer of er ergens op het platform van de MP96 misschien obstructies zoals verloren tips zijn.

Start hierna het instrument opnieuw op, zet eerst het instrument aan, daarna pas de computer. Wacht na het opstarten van de computer een minuut voordat de software gestart wordt. De database moet eerst op de achtergrond gestart worden, te snel starten van de software kan een error geven.

2. Twee andere punten die aan de orde kunnen komen:
   ·Na het doen van een leakage test moeten de disposables die hiervoor aan boord geplaatst zijn, weggegooid worden. Bij een leakage test worden deze namelijk niet getraceerd, waardoor het systeem bij het beginnen van de volgende extractie run niet ziet dat deze leeg zijn.
   ·De Magna Pure 96 plate holder is bedoeld voor verschillende typen platen die niet in de standaard adapter passen op de gekoelde output positie. Deze plate holder moet geplaatst worden op positie drie van het purification rack, dit is de tweede positie van vooraf gezien.
3. Probleem nog niet verholpen, herstart nog minimaal 2x. Wanneer het probleem niet is opgelost, raadpleeg MagNA Pure 96 Operator's Manual, Roche , http://technical-support.roche.com/product.aspx?productID=9965 Hierna kan de Roche helpdesk worden gebeld.

Bijlage 3

Virologische bepalingen worden verwerkt door de virologische moleculaire pool (VIR-pool). Indien de monsters door de VIR-pool verwerkt worden, geldt dat eppen met Lysis buffer en met EAV (spike) en Yeast tRNA vooraf worden uitgevuld. Deze oplossing wordt als volgt gemaakt:
- Pipetteer 80 µl EAV (10^{-1} verdunning van kweek dd. 27-03-2006) en 1,818 mL Yeast tRNA in 100 ml lysisbuffer (MagNa Pure Lysis/Binding Buffer, Roche).
- Vul deze oplossing uit door in elke 2 ml microtube (Sarstedt 72.694.006) 275 µl te pipetteren met een multistepper.
- Plaats de microtubes in de -20°C. Ontdooi de buizen direct voor gebruik voordat 200 µl monster wordt toegevoegd

Yeast-tRNA:
Werkoplossing = 1 mg/ml, 25 mg oplossen in 25 ml PCR-grade,Rnase/ Dnase vrij water, door eerst in 2 ml op te lossen, na een uur dit toevoegen aan 23 ml)

Veiligheid / Caution

Bijlage 3. SOP deel 2_M012_Lightcycler-qPCR_versie_9

Zie IDS_DOC_0502

01 Inleiding / Introduction

1.1 Doel

Deze SOP beschrijft het inzetten van een qPCR met behulp van de Lightcycler 480 (realtime thermocycler) voor de realtime detectie van (copy) DNA m.b.v. Taq polymerase .

1.2 Principe van de bepaling

DNA, gemaakt via reverse transcriptase reactie of rechtstreeks geïsoleerd, wordt geamplificeerd met target specifieke primers en het enzym Taq polymerase .
De detectie van de gevormde amplificaten gebeurt realtime met behulp van het meten van fluorescentie. Hiervoor wordt gebruik gemaakt van hydrolyse probes . De probe heeft een target specifieke sequentie en bevat een fluorescente label en een Quencher (deze onderdrukt het fluorescente signaal).Tijdens de PCR reactie wordt de probe door het Taq polymerase gehydrolyseerd en komt het Fluorescente label vrij en ongequenched in het medium. Aanstraling van het molecuul in vrije toestand leidt tot licht-emissie met een label afhankelijke golflengte. Dit licht wordt gedetecteerd en softwarematig omgezet in een signaal. Het moment waarop het geëmitteerde signaal boven de achtergrond uit komt wordt softwarematig berekend. Een positief fluorescentiesignaal betekend dat het beoogde target aanwezig is. Het moment waarop de fluorescentie boven het achtergrondsignaal uitkomt, is een maat voor de hoeveelheid target.
Deze maat wordt uitgedrukt in Crossing Point (Cp) eenheden. De Cp waarde is de cyclus van de PCR waarbij het fluorescentiesignaal significant boven de achtergrond uitkomt. (zie de Lightcycler handleidingen bij hoofdstuk 7: Samenhangende documenten)

1.3 Doelgroep

Laboratoriummedewerkers van het IDS, met ervaring in moleculair biologische technieken.

1.4 Afkortingen

DNA:	Deoxyribo Nucleic Acid.
PCR:	Polymerase Chain Reaction.
RNA:	Ribo Nucleic Acid.
RT-PCR:	Reverse Transcriptase Polymerase Chain Reaction.
Cp	Crossing Point

02 Chemicaliën, reagentia en media

Reagentia:

- Taqman FastVirus 1-step mastermix (4x mix, klaar voor gebruik) Life Technologies.*
- Lightcycler® Taqman master, Roche*
  - *Let op: Bij in gebruik name van de mix(1a) moet 10 µl enzym(1b) worden toegevoegd aan elke ep mix.*
- Primers en probes (zie de betreffende F-formulieren)**
- Probes Master, Roche

** Zie voor de ingangscontrole van deze reagentia het digitale labjournaal. Zie Hoofdstuk 07 "Samenhangende documenten" de link naar de betreffende bijsluiters.*
*** Zie voor ingangscontrole van primers en probes IDS/VIR/P013 en/of de betreffende labjournaals*

Opmerkingen:

- *Gebruik nooit chemicaliën, reagentia en/of H_2O welke gebruikt zijn in ruimten waar ook met amplificaat wordt of is gewerkt.*
- Tenzij anders vermeld, worden reagentia bij –20 °C bewaard.
- Werkoplossingen van primers en probes worden in de -20°C bewaard, en hebben een houdbaarheid van een jaar.

03 Biologisch materiaal

Geëxtraheerd /gezuiverd RNA of DNA, en gesynthethiseerd cDNA.
Zie voor extractiemethoden IDS/VIR/M900, IDS/VIR/M007.

04 Apparatuur en hulpmiddelen

- Lightcycler 480 apparaat + software, Roche (zie IDS/A801)
- 96-wellsplaten-centrifuge
- Lightcycler 480 multiwell 96 platen, Roche
- Thermoblokken (zie IDS/A922)

05 Werkwijze / Method

Algemene opmerkingen:
- Controleer of alle instellingen van gebruikte apparatuur juist staan ingesteld. Dit geldt ook voor alle formulieren vermeld in dit analysevoorschrift.
- Volg de richtlijnen die opgesteld zijn in IDS_DOC_0503: Werken in de vier V3 PCR laboratoria en de LightCyclerruimte V307

5.1 Uitvoering van de qPCR

1. De extractie van Nucleïnezuren worden uitgevoerd volgens de betreffende SOP\'s (IDS/VIRM007, IDS/VIR/M900). Alle nucleïnezuur extracties worden op de daartoe ingerichte labs V3.13 of V3.22 uitgevoerd.
2. Maak reagentia-mixen voor de PCR. Deze worden op het reagenslab V312 gemaakt volgens de betreffende werkformulieren (zie hoofdstuk 7)
3. Voeg RNA of (c)DNA aan de reagentia-mixen toe volgens de werkformulieren. Dit gebeurt op het toevoeglab V316.
4. Volg de specifieke werkformulieren voor de uitvoering van de cDNA-synthese en de PCR om verschillende organismen en genen/gen-sequenties aan te tonen.(zie hoofdstuk 7).
5. Run het PCR-programma dat vermeld staat op het werkformulier. Voor gebruik van de lightcyclers en de lightcycler software, zie de lightcycler handleiding IDS/A801 en de handleiding van de fabrikant.
6. Tenzij amplificaten voor andere doeleinden nodig zijn, worden na gebruik de 96-wellsplaten eerst in een afvalzakje gedaan en vervolgens in een wiva-vat gedeponeerd.

5.2 Analyse van de resultaten

- De ruwe data van elk experiment wordt, na voltooien van het PCR-programma, automatisch geëxporteerd worden naar S:\Labdata\Flow. Er kan vanaf de uitwerkplek ingelogd worden op de LightCycler software met eigen inlognaam. Hierna kan vanaf S:\Labdata\Flow het experiment geimporteerd worden. Ook kan er vanaf de uitwerkplek ingelogd worden op elke LightCycler door de LightCycler software op te starten, in te loggen met Username: “admin” en Password: “Master1” en door het selecteren van de juiste LightCycler.

LightCycler	Locatie
LC1	162.132.241.94:20482
LC2	162.132.241.95:20482
LC3	162.132.241.96:20482
LC4	162.132.241.97:20482
LC5	162.132.241.98:20482
LC6	162.132.241.99:20482

5.2.1 Invoeren van monsternummers en subsets

1. Open de LightCycler software en log in met gebruikersnaam en wachtwoord. (te verkrijgen bij de administrator)
2. Open het experiment, door op **Navigator** te klikken en via **import** het bestand te importeren.
3. De monsternummers kunnen worden toegevoegd door (in het scherm links) op **Sample Editor** te klikken, en vanaf F900 deze te kopiëren en plakken (met CTRL-C en CTRL-V). Let er op dat de lijst juist gesorteerd staat (deze staat standaard op horizontale plaat invoer). Ook kunnen de monsternummers handmatig worden ingetypt.
4. Maak de subsets aan die op het werkformulier genoemd staan. Klik op **Subset Editor**.
5. Klik op **“+”** voor het aanmaken van een nieuwe subset.
6. Geef deze de exacte naam vernoemd op het werkformulier.
7. Selecteer de wells die bij deze subset horen en klik op **Apply**.

8. Let op bij het analyseren van de subset EAV: indien er van meerdere PCR's EAV geanalyseerd wordt, moet de EAV-analyse de toevoeging van de betreffende PCR krijgen.

5.2.2 Analyse

1. Klik op Analysis.
2. Klik bij Create New Analysis op **Abs Quant/2nd Derivative Max**.
3. Selecteer de juiste subset en op OK(vinkje).
4. Selecteer het filter dat bij deze subset hoort, zie werkformulier, door op **FilterComb** te klikken.
5. Indien bij het experiment colorcompensatie vereist is, staat dit vermeld op het werkformulier. Klik dan op **Color Comp** en kies **In Database**. Selecteer de color compensatie die vermeld staat op het werkformulier. Indien geen color compensatie vereist is, gebruik dan **Color Comp (Off)**.
6. Klik op **Calculate**.

Uitleg: De software geeft nu een figuur van alle amplificatie curves en eventueel een Cp-waarde. (Let op, anders dan bij Sybrgreen 1, is door het gebruik van een probe in de PCR iedere toename van fluorescentie een specifiek en dus positief signaal.) Hier de mogelijkheden op een rij;
Als er een Cp-waarde wordt gegeven dan is er een specifiek PCR-produkt gevormd. Deze well wordt in rood weergegeven. De monsters met een Cp-waarde moeten dan ook als positief worden beschouwd. Meestal ligt de Cp-waarde bij een positief monster tussen de 10 en 35 cycli.
Er wordt geen toename van het fluorescentie signaal gemeten. Deze well wordt in groen weergegeven. Er wordt dan geen Cp-waarde toegekend en het monster is negatief.
Als een well in blauw wordt weergegeven, samen met de melding "? - Detector call uncertain", wordt er wel een Cp-waarde berekend, maar is de exponentiële curve niet goed gevormd. Deze monsters zijn waarschijnlijk zwak positief, of hebben last van remming. Bij de twijfelachtige resultaten moet het resultaat worden gecontroleerd door de curve visueel te beoordelen. Dit kan het best worden gedaan door een negatieve controle samen met dit twijfelachtige monster in een figuur te zetten (dit kan door beide monsters aan te klikken waarbij de "control" toets ingedrukt blijft). Er moet dan een duidelijke toename zijn in de curve van het twijfelachtige monster die niet te zien is bij de negatieve controle. Is dit het geval dan is het monster positief, verschilt de curve van het monster niet van de negatieve controle dan is dit monster negatief.
Beoordeel de positieve, negatieve en interne controle (zie ook hoofdstuk 6)

7. Sla het experiment op (Analistenpool/Experiments/*jaar*/*week*)

5.2.3 Koppelen van data naar unilab

1. Ga naar **Navigator**.
2. Klik op **Results Batch Export** en zoek in de folder het experiment op.
3. Selecteer de locatie voor het export file. **Browse**: **S:Unilab/Instrumentenkoppeling/LC480/LC480_Invoer/in**, en geef het experiment een naam.
4. *Klik op **Next***
5. *Selecteer de analyse die in het experiment gebruikt wordt **Abs Quant/2nd Derivative Max**. Als in het experiment ook **Abs quant /Fit points analyses** zijn gedaan wordt hiervoor een aparte export file gemaakt. De export file van de **Abs Quant/2nd Derivative Max** en de export file van de **Abs quant /Fit points analyses** moeten verschillende namen krijgen.*
6. *Klik 2x op **Next** en wacht op het scherm **Export done**.*
7. *Klik op **Done**.*
8. Ga naar programma Koppelen. *Voor koppelen vanaf LC480 S:\Unilab\Instrumentenkoppeling\LC480*
9. Klik op **LC480_Invoer** en selecteer de **Input file** en klik op **Koppel**. *Koppel altijd eerst de Abs quant /Fit points analyses en daarna de Abs Quant/2nd Derivative Max.*
10. Bij het koppelen wordt een logfile getoond, met wel en niet gekoppelde resultaten. Hierin is te zien of alle gegevens goed zijn gekoppeld. Nadat de input file is gekoppeld, klik op **OK**.

06 Kwaliteitscontrole

6.1 Eerste lijns controle

Negatieve controles mogen geen Cp waardes geven, behalve bij het target EAV. Als de negatieve controle hier niet aan voldoet, overleg met de vakinhoudelijk deskundige (zie IDS_DOC_0404 voor bevoegd persoon) en herhaal zo nodig de gehele test met toevoeging van een andere negatieve controle.

Positieve controles moeten een Cp waarde geven. Deze Cp-waarde wordt genoteerd in de betreffende Shewhart kaart. De Cp waarde van de positieve controle is afhankelijk van de batch van het materiaal.
Shewhart controle kaarten worden gebruikt om positieve controles te monitoren. Bij afwijkende Cp waarden worden de goed-afkeur criteria gehanteerd, zoals beschreven in IDS_PRO_5602.

Als de positieve controle volgens deze criteria wordt afgekeurd, overleg met de vakinhoudelijk deskundige (zie IDS_DOC_0404 voor bevoegd persoon) en herhaal zo nodig de gehele test met toevoeging van een andere positieve controle, of met gebruik van andere reagentia.

Interne controles geven altijd een Cp waarde. De Cp waarde van interne controle is afhankelijk van de batch.
Bij afwijkende Cp waarden worden de goed-afkeur criteria gehanteerd, zoals beschreven in IDS_PRO_5602.
Indien EAV als interne controle wordt gebruikt, zie ook IDS/VIR/F001.

6.2 Derde lijns controle

Voor de in hoofdstuk 7 vermelde qPCRs wordt meegedaan aan een 3e lijns rondzending (interlaboratoriumvergelijkingsprogramma) . De informatie over deze rondzendingen is te vinden op R:\IDS\Discipline VIR\Afdelingszaken\Kwaliteit\Rondzendingen.

Zie ook: IDS_PRO_5601

6.3 Interferenties en kruisreacties

Er zijn factoren die remming kunnen geven in de PCR, zoals de aanwezigheid van hemoglobine of vetten in het oorspronkelijke materiaal. Deze remming wordt gemeten met behulp van de interne controle. Zie hiervoor 6.1.
Bij de validatie van de PCR's is geen kruisreactie met andere micro-organismen gemeten. Zie de betreffende validatierapporten (hoofdstuk 7).

07 Samenhangende documenten

Extractie DNA/RNA:

- **IDS/VIR/M900** : het isoleren en zuiveren van nucleïnezuur uit klinische monsters mbv MagnaPure 96
- **IDS/VIR/M007** : RNA extractie mbv High Pure isolation kit

Apparaat beschrijving:

- **LightCycler480_OperatorsManual_V1.5.1**
- **IDS/A801**: Gebruikershandleiding LC480
- apparaat validatierapport **LightCycler480 achter**
- apparaat validatierapport **LightCycler480 midden**
- IDS/VIR/V032 Verificatierapport vergelijkbaarheid LightCycler apparaten
- **IDS/A922**: gebruikershandleiding thermoblokken

F-formulieren virus detectie PCR\'s:

- **IDS/VIR/F350**: Bijzondere subtyperingen Influenza A virus
- **IDS/VIR/F351**: Respiratoire PCR’s FastVirus mastermix
- **IDS/VIR/F326**: Mazelen Rubella virus detectie en typerings PCR
- **IDS/VIR/F321**: Bof virus detectie en typerings PCR
- IDS/VIR/F336: Parvo virus detectie PCR
- **IDS/VIR/F131**: Gastro enteritis detectie en typerings PCR’s
- IDS/VIR/F202: Blaasjes diagnostiek detectie PCRs
- **IDS/VIR/F250:** Hepatitis A detectie en typerings PCR
- **IDS/VIR/F143**: Hepatitits E detectie en typerings PCR
- **IDS/VIR/F896**: Chikungunya virus detectie PCR
- **IDS/VIR/F919**: Zika virus detectie PCR
- **IDS/VIR/F917**: Parecho Entero virus detectie PCR
- **IDS/VIR/F921**: Polio Virus detectie-PCR
- IDS/VIR/F923 Feline Calicivirus detectie PCR
- **IDS/VIR/F021:** Dengue virus group-specific RT-PCR
- **IDS/VIR/F022**: Dengue virus serotype-specific RT-PCR
- **IDS/VIR/F267**: Hepatitis B detectie en typerings PCR
- IDS/VIR/F927: Coronavirus detectie PCR

Validatierapporten methoden:

- Validatierapport **bof virus** detectie PCR fast virus
- Validatierapport **Chikungunya virus** detectie PCR fast virus
- Validatierapport **Zika virus** detectie PCR
- Validatierapport **Hepatitis A virus** detectie PCR fast virus
- Validatierapport **Hepatitis E virus** detectie PCR fast virus
- V035 Validatierapport **gastro-enteritis virus** detectie PCR fast virus

- Validatierapport **rhino entero virus** detectie PCR
- Validatierapport **MERS-Corona virus** detectie PCR fast virus
- Retrospectief validatierapport **Influenza A virus H7N9** detectie PCR
- Retrospectief validatierapport **Influenza A virus H5N1** detectie PCR
- Retrospectief validatierapport **Equine Arteritis virus** RT-PCR
- V006 Validatierapport **Hepatitis B** detectie en typerings PCR
- V007 Validatierapport **dengue virus** groep specific en serotype specific RT-PCR
- V014 Validatierapport **Mazelen-Rubella virus** detectie PCR
- V016 Validatierapport **entero en parecho virus** detectie PCR
- V017 Validatierapport **polio-sabin virus** detectie PCR
- V018 Validatierapport **influenza A en EAV virus** detectie PCR
- V019 Validatierapport **influenza A virus H1V H3 N1V N2** subtyperings PCR
- V020 Validatierapport **RSV en influenza B, subtype VIC-YAM, virus** detectie PCR
- V027 Validatierapport Influenza A virus SNP-PCR: aanpassing **H275 probe**
- V029 Validatierapport influenza virus detectie PCR: influenza A en EAV en haemagglutinine subtyperins PCR
- V030 Validatierapport influenza A H3N2 SNP-PCR
- V036 Validatierapport Parvo virus detectie PCR
- V037 Validatierapport blaasjes virussen detectie PCR
- V040 ValidatierapportCoronavirus 2019 n-CoV detectie PCR

Procedures:

- **IDS/VIR/F001:** EAV interne controle
- **IDS_PRO_5602**: Procedure gebruik Shewhart kaart
- **IDS/VIR/P013**: ingangscontrole reagentia
- **IDS_DOC_0502**: Veilig werken in het laboratorium
- **IDS_DOC_0503** Werken in de vier V3 PCR laboratoria en de LightCyclerruimte V307
- **IDS_PRO_5601**: Leidraad toepassing 1e, 2e en 3e lijnscontroles binnen IDS

Bijsluiters kits:

- **IDS/VIR/B007:** Bijsluiter LightCycler Taqman Mastermix
- **IDS/VIR/B016:** Bijsluiter Taqman fast virus 1-step master mix

nCoV 2019 RT-PCR: detection of SARS-like betacoronavirussen

F927

IDS/VIR/M012

DATE:	15-10-2020	WEEK:	42	NAME TECHNICIAN:

Mastermix Worksheet Wuhan CoV/SARS Detection

		Amount Samples	10			
PCR-mix E-gene:	**STOCK**	**µl**				**µl**
4x Taqman Fast Virus MM		5.0	X	12.25	=	**61.3**
E+EAV Mix	**[10 µm]**	3.0	X	12.25	=	**36.8**
PCR Grade Water		7.0	X	12.25	=	**85.8**
		15.0				**183.8**
PCR-mix RdRP gene (confirmation):	STOCK					
4x Taqman Fast Virus MM		5.0	X	12.25	=	**61.3**
nCoV	**[10 µm]**	3.0	X	12.25	=	**36.8**
PCR Grade Water		7.0	X	12.25	=	**85.8**
		15.0				**183.8**

Detection Format:	Multi Color Hydrolysis Probe					
PCR Program	**Segment number**	**Temp Target (°C)**	**Hold Time (sec.)**	**Slope (°C/sec.)**	**Acquisition mode**	
Reverse Transcription	**1**	**50**	**900**	**EXTERNAL**		
Denaturation/Inactivation	**1**	**95**	**120**	**EXTERNAL**		
Denaturation	1	95	60	4.4	None	LC 480
Amplification	1	95	10	4.4	None	
(cycles:50)	2	**60**	30	2.2	Single	
Cooling	1	40	30	4.4	None	

TECHNICIAN: checklist analysis in LC-software			
Inlog:	admin	Pasword:	Master1
Program LC480:	FV-EXT-60		
CC	-		
Check Positive Control:			
Check Negative Control:			
Fill in Shewhart Chart:			
Save and export experiment:			
Export data to Unilab:			
Turn in handouts:			
Remarks:			
SUPERVISOR			
Check Internal Control (EAV)			

1 Fill in form IDS/VIR/F900: position of the samples at the 96-wells plate
2 Fill in the amount of samples
3 Preheat two thermoblocks on 50°C and 95°C (toevoeglab)
4 Make PCR-mixes (reagenslab)

Toevoeglab:

1 Aliquot **15** µl portions of PCR-mixes in 96-wellsplate according to IDS/VIR/F900
2 Add **5** µl RNA to PCR-mix
3 Seal the 96-well plate
4 Centrifuge briefly
5 Incubate directly 15 minutes at 50°C (heating block)
6 Incubate directly 2 minutes at 95°C (heating block)
7 Centrifuge briefly
8 Keep plate at 4°C if you can't run directly
9 Run PCR program on LightCycler 480
10 Analyse results using SOP IDS/VIR/M012

Analysis in LC-software:

target	Label	Filter (nm) LC3,LC5	Filter (nm) LC1,LC2,LC4,LC6	Subset name	PCR
PAN-SARBECO-E	FAM-BHQ1	483-533	465-510	**Sarbe**	**E+EAV**
internal control EAV	TXR-BHQ2	558-610	533-610	**EAV**	
nCoV 2019	FAM-BHQ1	483-533	465-510	**nCoV**	**RdRP**

info primers and probes: see tab "primers-probes"

control	Name	Isolate	Gene
positive control combi	PC SARS-CoV-1 10-5	Strain Frankfurt-1	E
	PC SARS-CoV-2 10-4	hCoV-19/Netherlands/Diemen_1363454/2020	RdRP&E
negative&internal control	NC	EAV culture 10-3 (IDS/VIR/F001)	EAV

nCoV / SARS RT-PCR

E gene	Sequence	Label	Filter (Nm)	pmol in PCR-mix
E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT			**10**
E_Sarbeco_R	ATATTGCAGCAGTACGCACACA			**10**
E_Sarbeco_P1	ACACTAGCCATCCTTACTGCGCTTCG	FAM-BHQ1	483-533	**5**
EAV-2043F	CTG TCG CTT GTG CTC AAT TTA C			**10**
EAV-2193R	AGC GTC CGA AGC ATC TC			**10**
EAV 2102P-2	TGC AGC TTA TGT TCC TTG CAC TGT GTT C	TXR-BHQ2	558-610	**5**
RdRP gene	**Sequence**	**Label**	**Filter (Nm)**	**pmol in PCR-mix**
RdRp_SARSr-F2	GTG AAA TGG TCA TGT GTG GCG G			**10**
RdRp_SARSr-R2	CAA ATG TTA AAA ACA CTA TTA GCA TAA GCA			**10**
RdRP_SARS_P2.2	CCA GGT GGA ACC TCA TCA GGA GAT GC	FAM-BHQ1	483-533	**5**

Bijlage 5. EUA-Seegene-allplex2-ifu

Allplex™ 2019-nCoV Assay (version 2.0; July 24th,2020)

(Cat no. RP10243X / RP10252W)

Instructions for Use

For *in vitro* diagnostic use

For Emergency Use Authorization Only

Prescription Use only

Seegene

Table of Contents

CHAPTER 1: Intended Use

The Allplex™ 2019-nCoV Assay is an *in vitro* diagnostic (IVD) real-time reverse transcriptase polymerase chain reaction (RT-PCR) test intended for the qualitative detection of SARS-CoV-2 viral nucleic acids in human nasopharyngeal swab, oropharyngeal swab, anterior nasal swab, mid-turbinate nasal swab, nasopharyngeal wash/aspirate, nasal aspirate, bronchoalveolar lavage (BAL) and sputum specimens from individuals who are suspected of COVID-19 by their health care provider. Testing is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, that meet requirements to perform high complexity tests.

Results are for the identification of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in respiratory specimens during the acute phase of infection. Positive results are indicative of the presence of SARS-CoV-2 RNA; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status.
Positive results 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 SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions. Negative results must be combined with clinical observations, patient history, and epidemiological information.

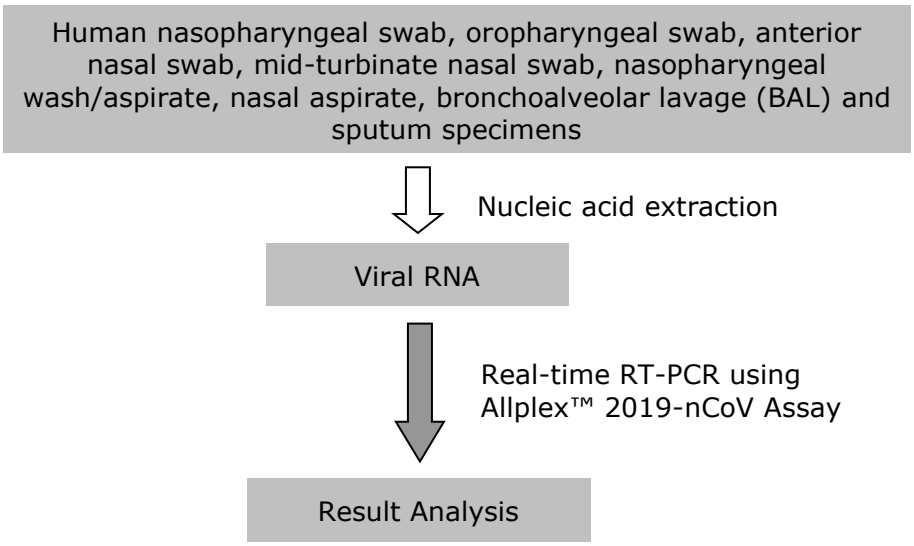
The Allplex™ 2019-nCoV Assay is intended for use by qualified, trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time RT-PCR and *in vitro* diagnostic procedures. The Allplex™ 2019-nCoV Assay is only for use under the Food and Drug Administration's Emergency Use Authorization (EUA).

CHAPTER 2: Summary and Explanation of the Test

The technology of the Allplex™ 2019-nCoV Assay is a real-time reverse transcription polymerase chain reaction (RT-PCR) test. The 2019-nCoV primer and probe set(s) is designed to detect RNA from the 2019-nCoV in human nasopharyngeal swab, oropharyngeal swab, anterior nasal swab, mid-turbinate nasal swab, nasopharyngeal wash/aspirate, nasal aspirate, bronchoalveolar lavage (BAL) and sputum specimens.

CHAPTER 3: Principle of the Procedure

Nucleic acids are isolated and purified from specimen using a manual or an automated nucleic acid extraction system. 10 µL of Internal Control (RP-V IC) must be added before the extraction. Follow detailed extraction procedures in manufacturer's instructions. 8 µL of purified nucleic acid is reverse transcribed using 5X Real-time One-step Buffer/Real-time One-step Enzyme into cDNA which is then subsequently amplified in a CFX96™, CFX96 Touch™, Applied Biosystems 7500, or Applied Biosystems 7500 Fast Dx real-time PCR systems. During 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 the CFX96™, CFX96 Touch™, Applied Biosystems™ 7500, Applied Biosystems™ 7500 Fast Dx real-time PCR detection systems. The result of amplification is reported through 'Seegene viewer' analysis. The 'Seegene viewer' shows whether the exported data is 2019-nCoV Detected, Presumptive positive, or Negative for easy retrieval of result by the user.



■ CHAPTER 4: Assay Materials

Materials provided

The reagents contained in one Allplex™ 2019-nCoV Assay kit are sufficient for 100/124 reactions.

Table 1. Allplex™ 2019-nCoV Assay Composition

Contents	Volume (RP10243X/ RP10252W)	Description
2019-nCoV MOM	500 µL / 620 µL	MuDT* Oligo Mix (MOM): - Amplification and detection reagent *MuDT is the brand name of Seegene's oligo mixture
Real-time One-step Enzyme	200 µL	Enzyme mix for one-step RT-PCR
5X Real-time One-step Buffer	500 µL	Buffer for one-step RT-PCR - Buffer containing dNTPs
2019-nCoV PC	80 µL	Positive Control (PC) for PCR control: - Mixture of pathogen and IC clones
RP-V IC	1,000 µL	Exogenous Internal Control (IC) of Allplex™ 2019-nCoV Assay
RNase-free Water	1,000 µL	Ultrapure quality, PCR-grade RNase-free Water provided for: 1. Negative Control (NC) for PCR control 2. RT-PCR Mastermix (Refer to Table 6)

Materials required but not provided

Additional materials and equipment required

- Disposable powder free gloves (latex or nitrile)
- Pipettes (adjustable) and sterile pipette tips
- 1.5 mL microcentrifuge tubes
- Clean bench
- Ice
- Desktop centrifuge
  (1.5 mL microcentrifuge and 96 well plate centrifuge)
- Vortex mixer
- Instruments and kits for nucleic acid extraction

Manufacturer	Instrument	Extraction Kit	Catalog No./Reaction No.
Seegene	Seegene STARlet (65415-03)	STARMag 96 X 4 Universal Cartridge Kit	384 reactions (744300.4.UC384)
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	384 reactions (EX00013C)
Hamilton	Microlab STARlet IVD (173000-075)	STARMag 96 X 4 Universal Cartridge Kit	384 reactions (744300.4.UC384)
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	384 reactions (EX00013C)
Seegene	Seegene NIMBUS (67930-03)	STARMag 96 X 4 Universal Cartridge Kit	384 reactions (744300.4.UC384)
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	384 reactions (EX00013C)
Hamilton	Microlab NIMBUS IVD (65415-02)	STARMag 96 X 4 Universal Cartridge Kit	384 reactions (744300.4.UC384)
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	384 reactions (EX00013C)
LG Chem	AdvanSure E3 System (YETS0001EG)	AdvanSure NA EX Kit	96 reactions Reagent Plate (RPE0001K01)
			Proteinase K Tube (RPK0001K01)
			Strip (E3 System) (YSTP0500KG)
GeneAll	N/A (Manual)	Ribospin vRD (Viral RNA/DNA Extraction Kit)	50 extractions (302-150 SG1701)
QIAGEN	N/A (Manual)	QIAmp DSP Viral RNA Mini Kit	50 extractions (61904)
Roche	Roche MagNA Pure 96 (MP96)	DNA and Viral NA Small Volume Kit	576 extractions (06 543 588 001)
ThermoFisher Scientific	KingFisher Flex automated extraction	MagMAX Viral/Pathogen Nucleic Acid Isolation Kit	200 extractions (A42352) 2,000 extractions (A48310)

NOTE:
(1) All extraction options are commercially available.
(2) The Seegene, Hamilton, LG Chem, and GeneAll extraction reagents/instrumentation can be purchased through Seegene Technologies (CA, US), support@seegenetech.com
(3) The Seegene and Hamilton extraction reagents/instrumentation are validated with Seegene Launcher V6 software.

- PCR Instrument & Consumables
  - ⊖ Applied Biosystems™ 7500 (ThermoFisher Scientific), Applied Biosystem™ 7500 Fast Dx (ThermoFisher Scientific)

Consumables (Cat. No.)
For Applied Biosystems™ 7500; • MicroAmp™ Optical 96-Well Reaction Plate (Cat. No. N8010560, ThermoFisher Scientific) • MicroAmp™ Optical 96-Well Reaction Plate with Barcode (Cat. No. 4306737, ThermoFisher Scientific) For Applied Biosystems™ 7500 Fast Dx. • MicroAmp™ Fast 96-Well Reaction Plate (0.1mL) (Cat. No. 4346907, ThermoFisher Scientific) • MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL (Cat. No. 4346906, ThermoFisher Scientific) For both Applied Biosystems™ 7500/7500 Fast Dx; • Optical Adhesive Covers (Cat No. 4360954, ThermoFisher Scientific)

Software	
Applied Biosystems™ 7500	Applied Biosystems™ 7500 Fast Dx
SDS software v2.0.5	SDS software v1.4.1 (Windows XP) / 1.5.1 (Windows 7 64-bit)

  - ⊜ CFX96™ Real-time PCR Detection System-IVD (Bio-Rad), CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad)

Consumables (Cat. No.)
• Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/white (Cat. No. HSP9655, Bio-Rad) • Hard-Shell® 96-Well PCR Plates, low profile, thin wall, skirted, white/white, barcoded (Cat. No. HSP9955, Bio-Rad) • Optical Flat 8-Cap Strips (Cat. No. TCS0803, Bio-Rad) • Low Tube Strip, WHT (Cat. No. TLS0851, Bio-Rad) • Optically Clear Heat Seal (Cat. No. 1814030, Bio-Rad) • Permanent Clear Heat Seal (Cat. No. 1814035, Bio-Rad)***

• PX1 PCR plate sealer (auto-sealer, Cat. No. 181-4000, Bio-Rad)*** • MicroAmp™ Optical 8-Cap Strip (Cat. No. 4323032, ThermoFisher Scientific) • MicroAmp™ Optical 8-Tube Strip (0.2 mL) (Cat. No. 4316567, ThermoFisher Scientific) • EU 8-Single Attachable Indented Cap (Cat. No. B79501, BIOplastics) • EU 0.2 ml Thin-wall 8-Tube Strip (Cat. No. B77009, BIOplastics) • Mini-centrifuge (Cat. No. Mini-6K, Protagen) • PCR plate centrifuge (Cat. No. MPC-P25, Powerlab) *** The Permanent Clear Heat Seal must be used with the PX1 PCR Plate Sealer when running the Allplex™ assay
Software
CFX Manager™ Software V3.1 or CFX Maestro™ Software V1

NOTE: All consumables for CFX96™ Real-time PCR Detection System-IVD and CFX96 Touch™ Real-Time PCR Detection System can be purchased through Seegene Technologies (California, US).

CHAPTER 5: Warnings and Precautions

The Allplex™ 2019-nCoV Assay should be performed by qualified, trained personnel.

- For *in vitro* diagnostic use only.
- For Emergency Use Authorization Only
- For Prescription Use Only
- This test has not been FDA cleared or approved; the test has been authorized by FDA under an Emergency Use Authorization (EUA) for use by laboratories certified under CLIA of 1988, 42 U.S.C. §263a, that meet requirements to perform high complexity tests.
- This test has been authorized only for the detection of nucleic acid from SARS-CoV-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 Federal Food, Drug, and Cosmetic Act, 21 U.S.C. § 360bbb-3(b)(1), unless the authorization is terminated or revoked sooner.
- Reliability of the results depends on adequate specimen collection, storage, transport, and processing procedure.
- This test has not been validated for any other types of specimens other than those indicated in the intended use.
- If not tested immediately, store extracted RNA at ≤ -70℃ until use and keep on ice during testing.
- Sensitivity of the assay may decrease if samples are repeatedly frozen and thawed for more than 7 times.
- Workflow in the laboratory should proceed in a unidirectional manner.
- Wear disposable gloves and change them before entering different areas. Change gloves immediately if contaminated or treat them with DNA decontaminating reagent.
- Supplies and equipment must be dedicated to working areas and should not be moved from one area to another.
- Do not pipette by mouth.
- Do not eat, drink, or smoke in laboratory work areas. Wear disposable powder-free gloves, laboratory coats and eye protections when handling specimens and reagents. Wash hands thoroughly after handling specimens and test reagents.
- Avoid contamination of reagents when removing aliquots from reagent tubes. Use of sterilized aerosol resistant disposable pipette tips is recommended.

- Do not pool reagents from different lots or from different tubes of the same lot.
- Do not use the product after its expiry date.
- Do not reuse any disposable items.
- Use screw-capped tubes and prevent any potential splashing or cross-contamination of specimens during preparation.
- Avoid possible contamination of reagents with extracted nucleic acids, PCR products, and positive control. To prevent contamination of reagents, use of filter-tips is recommended.
- Use separated and segregated working areas for each test run.
- To avoid contamination of working areas with amplified products, open PCR reaction tubes or strips only in designated working areas after amplification.
- Store positive materials separated from the kit's reagents.
- Handle all specimens as if infectious. Laboratory safety procedures (refer to Biosafety in Microbiological and Biomedical Laboratories & CLSI Documents) must be taken when handling specimens. Thoroughly clean and disinfect all work surfaces with 0.5% sodium hypochlorite (in de-ionized or distilled water). Product components (product residuals, packaging) can be considered as laboratory waste. Dispose of unused reagents and waste in accordance with applicable federal, state, and local regulations.
- Manipulation of potentially infected specimens should be performed in a certified Class II BSC in a BSL-2 facility or higher. This includes aliquoting and/or diluting specimens and nucleic acid extraction procedures involving potentially infected specimens.
- Use appropriate personal protective equipment including but not limited to disposable gloves, laboratory coat/gown, and eye protection when handling specimens, reagents, pipettes, and other equipment.
- Keep extracted RNA on cold block or on ice during reaction set-up.
- Keep PCR reagents on cold block or on ice during reaction set-up.
- Expiry date is 8 months from the date of manufacture when product is stored at ≤ -20℃. Please refer to label for expiry date.
- Seegene STARlet and Seegene NIMBUS are private label devices and are the same as the Microlab STARlet IVD and Microlab NIMBUS IVD. There is no change in the device other than labeling. All four devices can be used interactively and generate equivalent results. Instruments indicated share the same software application ("Seegene Launcher") and extraction kit ("STARMag 96 X 4 Universal Cartridge Kit" and "STARMag 96 X 4 Viral DNA/RNA 200 C Kit").
- This Allplex™ 2019-nCoV Assay is a qualitative *in vitro* test for the single or multiple detection of 3 target genes (E gene, RdRP gene, and N gene)

CHAPTER 6: Storage and Handling Conditions

Reagent storage and handling

- All reagents of the Allplex™ 2019-nCoV Assay kit must be stored at -20 °C or below.
- Completely thaw all reagents on ice prior to use
- Do not store reagents in a frost-free freezer.
- Do not use kits or reagents beyond indicated expiry date.
- Always check the expiry date on the reagent tubes prior to use.

NOTE: The performance of kit components is unaffected for up to 7 cycles of freeze and thaw. If the reagents are used only intermittently, they should be stored in aliquots.

Specimen storage and transport

- Specimen types: human nasopharyngeal swab, oropharyngeal swab, anterior nasal swab, mid-turbinate nasal swab, nasopharyngeal wash/aspirate, nasal aspirate, bronchoalveolar lavage (BAL) and sputum specimens

NOTE: Sample collection devices are not provided with the assay. All testing for COVID-19 should be conducted in consultation with a healthcare provider. Refer to CDC guidelines for sample collection (Nasopharyngeal swab (NP) /oropharyngeal swab (OP) / anterior nasal swab / mid-turbinate nasal swab and sputum) and storage at: https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html

Once the swabs have been collected in accordance with CDC guidelines, it is recommended to use Universal Transport Medium (UTM) for collection of nasopharyngeal, oropharyngeal anterior nasal and mid-turbinate nasal swabs.

- After collection, the specimen should be stored at 2-8°C and processed within 72 hours.
- If delivery and processing exceed 72 hours, specimens should be transported in dry ice and once in laboratory frozen at -70°C or colder.

NOTE:
(1) Performance may be affected by prolonged storage of specimens.
(2) Specimens transport should adhere to local and national instructions for transport of pathogenic material.
(3) Specimens should be collected and handled according to the swab collection device manufacturer's recommended procedures.

■ CHAPTER 7: Assay Control Material(s)

PCR controls

The PCR controls below are provided with the Allplex™ 2019-nCoV Assay to confirm the validity of each PCR run on the same plate.

In prior to determining of the validity of each PCR run, the user must confirm the results of the negative control and positive control on the 'Well Plate' on the upper left corner of the Seegene viewer.

The results of the negative control and positive control are displayed under the 'Auto Interpretation' column on the bottom half of the Seegene viewer. If the positive and/or negative control results are invalid, the corresponding PCR run must be repeated.

1. **Negative Control (NC)** is used as a PCR control to confirm test validity, and the absence of any contaminants during testing. The "No template" control is prepared using RNase-free Water added to the Master Mix prior to PCR. NC must be included in each test run. No signal should be detected with the NC.

2. **Positive Control (PC)** is used to confirm test validity, and functions as the validation control for PCR amplification and the test detection steps. The PC is constructed using plasmids encoding Allplex™ 2019-nCoV Assay target sequences and must be included in each test run.

NOTE: The Positive Control included in this kit is a high concentration PCR control. Dilute the PC with TE buffer by 1:10 before use.

The real-time PCR results of the positive and negative control can be viewed from the Seegene Viewer as shown in Picture 1 and Picture 2.

Picture 1. Example of valid positive/negative control results

Well	Name	Type	FAM		Cal Red 610		Quasar 670		HEX		Auto Interpretation	Comment
			E gene	C(t)	RdRP ...	C(t)	N gene	C(t)	IC	C(t)		
B11		NC	-	N/A	-	N/A	-	N/A	-	N/A	Negative Control(-)	
B12		PC	+	20,64	+	20,97	+	19,09	+	19,96	Positive Control(+)	

Picture 2. Example of invalid positive/negative control results

Well	Name	Type	FAM		Cal Red 610		Quasar 670		HEX		Auto Interpretation	Comment
			E gene	C(t)	RdRP ...	C(t)	N gene	C(t)	IC	C(t)		
F01		PC	+	38,99	+	38,77	+	39,05	-	N/A	Positive Control(Invalid)	
F02		NC	-	N/A	+	37,23	+	36,85	-	N/A	Negative Control(Invalid)	

Table 2. Allplex™ 2019-nCoV Assay; Control Acceptance Criteria

Control	Seegene Viewer Result (Ct value)				
	IC (HEX)	E gene (FAM)	RdRP gene (CalRed 610)	N gene (Quasar 670)	Auto Interpretation
2019-nCoV Positive Control	≤ 40	≤ 40	≤ 40	≤ 40	Positive Control (+)
	>40 or N/A	>40 or N/A	>40 or N/A	>40 or N/A	Positive Control (Invalid)
Negative Control	>40 or N/A	>40 or N/A	>40 or N/A	>40 or N/A	Negative Control (-)
	≤ 40	≤ 40	≤ 40	≤ 40	Negative Control (Invalid)

Internal Control

The Allplex™ 2019-nCoV Assay includes a full process Internal Control (RP-V IC) which is composed of MS2 phage genome. This Internal Control material verifies all steps of the analysis process, including sample extraction, reverse transcription, and PCR to demonstrate proper specimen processing and test validity of each specimen.

A positive signal for the Internal Control indicates that all processing steps performed by the Allplex™ 2019-nCoV Assay were successful.

A negative signal of all targets including the Internal Control invalidates all negative results in the analysis. Repeat testing if an invalid result is reported. Refer to section 'Interpretation of Results' for more details.

External Control

External controls are not provided with the Allplex™ 2019-nCoV Assay. Quality control requirements should be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard quality control procedures.

The following external controls are available:
AccuPlex™ SARS-COV-2 reference material (Seracare Life Sciences, Inc., Cat no. 0505-0126; this kit includes positive & negative reference material.) The positive reference material may be used as an external extraction control.

■ CHAPTER 8: Procedure

Sample collection, transport, and storage

Collect Nasopharyngeal swab (NP) /oropharyngeal swab (OP)/nasal swab/mid-turbinate s nasal swab, nasopharyngeal wash/aspirate, nasal aspirate, bronchoalveolar lavage (BAL) and sputum according to CDC guidelines and/or manufacturer's protocol for sample collection, storage and handling.

Nucleic acid extraction

The assay was validated with the extraction options listed below. Perform the RNA extraction on samples according to the manufacturer's instructions for use. For the Microlab STARlet IVD, Seegene STARlet, Microlab NIMBUS IVD and Seegene NIMBUS extraction methods, follow the detailed instruction provided in the section of 'Preparation on Microlab STARlet IVD, Seegene STARlet, Microlab NIMBUS IVD and Seegene NIMBUS'.

Seegene STARlet / Seegene NIMBUS / Microlab STARlet IVD / Microlab NIMBUS IVD (STARMag 96 X 4 Universal Cartridge Kit; Cat No. 744300.4.UC384) See Operation Manual of each instrument or the section under 'preparation' for details. - Sample volume: 300 µL, Elution volume: 100 µL
Seegene STARlet / Seegene NIMBUS / Microlab STARlet IVD / Microlab NIMBUS IVD (STARMag 96 X 4 Viral DNA/RNA 200 C Kit; Cat No.EX00013C) See Operation Manual of each instrument or the section under 'preparation' for details. - Sample volume: 300 µL, Elution volume: 100 µL
AdvanSure E3 System **(AdvanSure NA EX Kit; Cat No.RPE0001K01,RPK0001K01)** See AdvanSure NA EX Kit User Manual for details. - Sample volume: 200 µL, Elution volume: 100 µL
QIAamp® DSP Virus Spin Kit **(Cat No.61704) (QIAGEN)** Follow the 'Protocol Purification of viral nucleic acids from plasma or serum' of the QIAamp® DSP Virus Spin Kit Handbook. - Specimen volume: 190 µL, Elution volume: 40 µL
Ribospin™ vRD **(Viral RNA/DNA Extraction Kit (Cat No.302-150, SG1701) (GeneAll)** See Ribospin™ vRD Manual for details. - Specimen volume: 290 µL, Elution volume: 40 µL
KingFisher™ Flex automated extraction **(MagMAX Viral/Pathogen Nucleic Acid Isolation Kit, Cat No. A42352)** See MagMAX Viral/Pathogen Nucleic Acid Isolation Kit Manual for details. - Specimen volume: 200 µL, Elution volume: 50 µL
Roche MagNA Pure 96 (MP96) **(DNA and Viral NA Small Volume Kit; Cat No.06 543 588 001)** See DNA and Viral NA Small Volume Kit Manual for details. - Specimen volume: 200 µL, Elution volume: 50 µL

Preparation on Microlab STARlet IVD, Seegene STARlet, Microlab NIMBUS IVD and Seegene NIMBUS:

Hardware installation, Seegene Launcher software for operation and customer training (on site and/or video tutorial) are provided by Seegene Technologies (California, US), support@seegenetech.com.
The Seegene Launcher is an application software that controls functions and protocols of the Microlab STARlet IVD/Seegene STARlet/Microlab NIMBUS IVD/Seegene NIMBUS.
The user manual of 'Seegene Launcher V6' containing detailed descriptions on instrument maintenance and experimental procedures of nucleic acid extraction using Microlab STARlet IVD, Seegene STARlet, Microlab NIMBUS IVD and Seegene NIMBUS will be provided.

The purification procedure is designed to ensure safe and reproducible handling of potentially infectious samples and comprises of 4 steps: sample lysis, nucleic acids binding to magnetic beads, debris washing and elution of purified nucleic acids.

Below instructions describe the procedures for Microlab STARlet IVD and Seegene STARlet. For Microlab NIMBUS IVD and Seegene NIMBUS, the same Seegene launcher software is used. Please follow exactly the same procedure as below after selecting NIMBUS in the setting during installation of the launcher.

For STARMag 96 X 4 Universal Cartridge Kit:

1. Take out 1 cartridge from the STARMag 96 X 4 Universal Cartridge Kit. 1 cartridge contains reagents for 96 tests, and the STARMag 96 X 4 Universal Cartridge Kit contains 4 cartridges (384 tests).

Picture 3. 1 cartridge from the STARMag 96 X 4 Universal Cartridge Kit

Table 3. Components of STARMag 96 X 4 Universal Cartridge Kit

Reagents	Volume
Lysis Buffer Universal LB	4 X 23 mL

Reagents	Volume
Binding Buffer Universal BB	4 X 68 mL
Wash Buffer 1 Universal WB1	4 X 55 mL
Wash Buffer 2 Universal WB2	4 X 10 mL
Wash Buffer 3 Universal WB3	4 X 55 mL
Elution Buffer Universal EB	4 X 18 mL
Universal Magnetic Beads	4 X 1.8 mL
Lysis Buffer Universal LB	200 mL
Universal Proteinase K (lyophilized)	4 X 75 mg
Proteinase Buffer Universal PB	4 X 3 mL
Tub Cover	25 ea.
User Manual	2 ea.

NOTE:

(1) Lysis Buffer (LB), Binding Buffer (BB), and Wash Buffer 1 (WB1) contain chaotropic salt. Wear gloves and goggles always when handling buffers.
(2) Store all the components of extraction reagent kit at room temperature (18 - 25℃). In case of dissolved Proteinase K, store at -20℃.
(3) The expiration date of the product is indicated on the label. The cartridge remains effective for up to 15 months prior to its opening and for up to 4 months after its opening.
(4) All buffers are delivered ready-to-use.
(5) Lysis Buffer (LB) may form a salt precipitate during storage. To re-dissolve the precipitate, incubate the buffer bottle at 40℃ until the precipitate is re-dissolved completely.

2. Before placing the cartridge on the Microlab STARlet IVD, Seegene STARlet, Microlab NIMBUS IVD or Seegene NIMBUS prepare the following:

- Proteinase K: When using the kit for the first time, add 2.6 mL Proteinase Buffer Universal PB to the lyophilized Proteinase K. Dissolved Proteinase K solution is stable at - 20 °C for at least 6 months. Transfer the Proteinase K solution into a 1.5mL microtube according to the number of samples. The volume of Proteinase K solution is automatically calculated by the Launcher software if the number of samples is entered into the software.
- Wash Buffer 2 Universal WB2: Prepare 48mL of absolute ethanol (Cat. No. 1.00983.1011, Merck). After removing the film on the WB2 tub, add 48 mL of absolute ethanol into the WB2 tub. The WB2 tub should be covered after use and should be stored at room temperature (18 - 25°C).

- Magnetic Bead: Suspend the magnetic bead by manually tapping the tube, and then quick vortexing.

For STARMag 96 X 4 viral DNA/RNA 200 C Kit;

1. Take out 1 cartridge from the STARMag 96 X 4 viral DNA/RNA 200 C Kit. 1 cartridge contains reagents for 96 tests, and the STARMag 96 X 4 viral DNA/RNA 200 C Kit contains 4 cartridges (384 tests).

Picture 4. 1 cartridge from the STARMag 96 X 4 viral DNA/RNA 200 C Kit

Table 4. Components of STARMag 96 X 4 viral DNA/RNA 200 C Kit

Reagents	Volume
Lysis Buffer LB	4 X 23 mL
Binding Buffer BB	4 X 68 mL
Wash Buffer 1 WB1	4 X 55 mL
Wash Buffer 2 WB2	4 X 10 mL
Wash Buffer 3 WB3	4 X 55 mL
Elution Buffer EB	4 X 18 mL
Magnetic Beads	8 mL
Bead Tube (2 mL tube)	4 ea.
Tub Cover	25 ea.
User Manual	1 ea.

NOTE:

(1) Store all the components of extraction reagent kit at room temperature (18 - 25℃).
(2) The expiration date of STARMag 96 X 4 Viral DNA/RNA 200 C Kit is indicated on the box label and store up to 1 month after its opening.
(3) All buffers are delivered ready-to-use.

2. Before placing the cartridge on the Microlab STARlet IVD or Seegene STARlet, prepare the following:

- Add 48 mL of absolute ethanol into WB2 tub before use. WB2 tub should to be covered with Tub Cover after using and stored at room temperature (18 ~ 25°C).
- After sufficiently vortexing the Magnetic beads in the bottle, transfer 1.8 ml of Magnetic beads to bead tube(2 mL tube) before use.

Table 5. Materials required, but not provided

Basic Item
Absolute EtOH
Disposable powder free gloves (latex or nitrile)
Desktop centrifuge
Ice or cooler box
Pipettes (adjustable) and sterile aerosol resistant pipette tips
Vortex mixer

Purchasing Item	Cat. No.	Manufacturer
SMP-CAR-24-Tube Carrier Set-4 (24 sample carrier)	173440	Hamilton
SMP CAR 12 D35 (12 sample carrier)	185052	Hamilton
1.5 mL sterile microtubes	MCT-150-C	Axygen
96 Deep Well Micro Plate	SDP0096	Supercon
Deep well plate, 96 wells with Barcode label	SDP0096B	Supercon
MicroAmp® Optical 8-Tube Strip (0.2 mL)	4316567	Applied Biosystems
EU 0.2 ml Thin-wall 8-Tube Strip	B77009	BIOplastics
Hard-Shell® PCR plates 96-well WHT/WHT	HSP9655	Bio-Rad
Hard-Shell® PCR plates 96-well WHT/WHT,	HSP9955	Bio-Rad
Low Tube Strip, WHT	TLS0851	Bio-Rad
MicroAmp® Optical 8-Cap Strip	4323032	Applied Biosystems
EU 8-Single Attachable Indented Cap	B79501	BIOplastics
Optical Flat 8-Cap Strips	TCS0803	Bio-Rad
Optically Clear Heat Seal	1814030	Bio-Rad
Permanent Clear Heat Seal	1814035	Bio-Rad
PX1 PCR plate sealer (auto-sealer)	1814000	Bio-Rad
Mini-centrifuge	Mini-6K	Protagen
PCR plate centrifuge	MPC-P25	Powerlab
UPS	HP 910	Sampoongpower

NOTE: All purchasing items listed above can be purchased through Seegene Technologies (California, US).

Operation

NOTE:

(1) Prior to running the Seegene launcher, inspect the deck and carriers for cleanliness and empty the tip waste/liquid waste if there are any.

(2) A minimum of 300µL specimen volume is required to ensure 200µL of specimen pipetting by Microlab STARlet/Seegene STARlet. This will result in 100µL elution volume of nucleic acids (RNA) necessary to run the Allplex™ 2019-nCoV Assay.

(3) Only 12mm tubes, 16mm tubes and 1.5mL micro centrifuge tubes can be directly loaded to the Microlab STARlet/Seegene STARlet.

(4) For information on maintenance, refer to the Seegene Launcher V6 manual.

1. Open the Seegene launcher software installed on the laptop connected to the Microlab STARlet IVD/Seegene STARlet for operation of the Microlab STARlet IVD/Seegene STARlet.

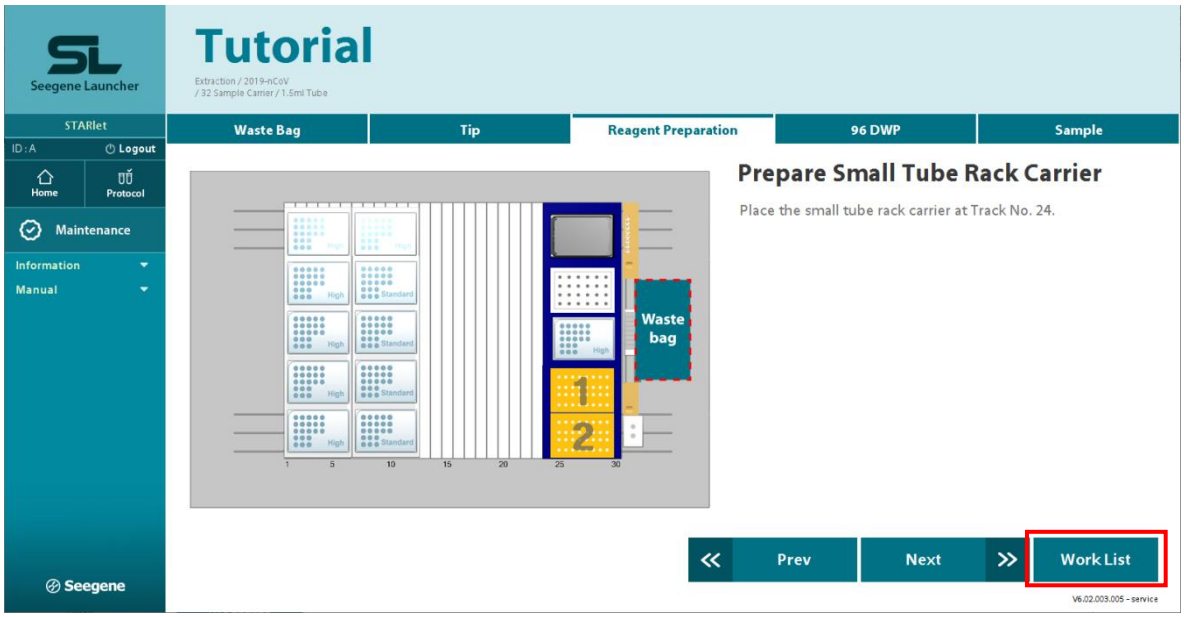
2019-nCoV
Launcher

2. Click on **LAUNCHER RUN** on the main page.

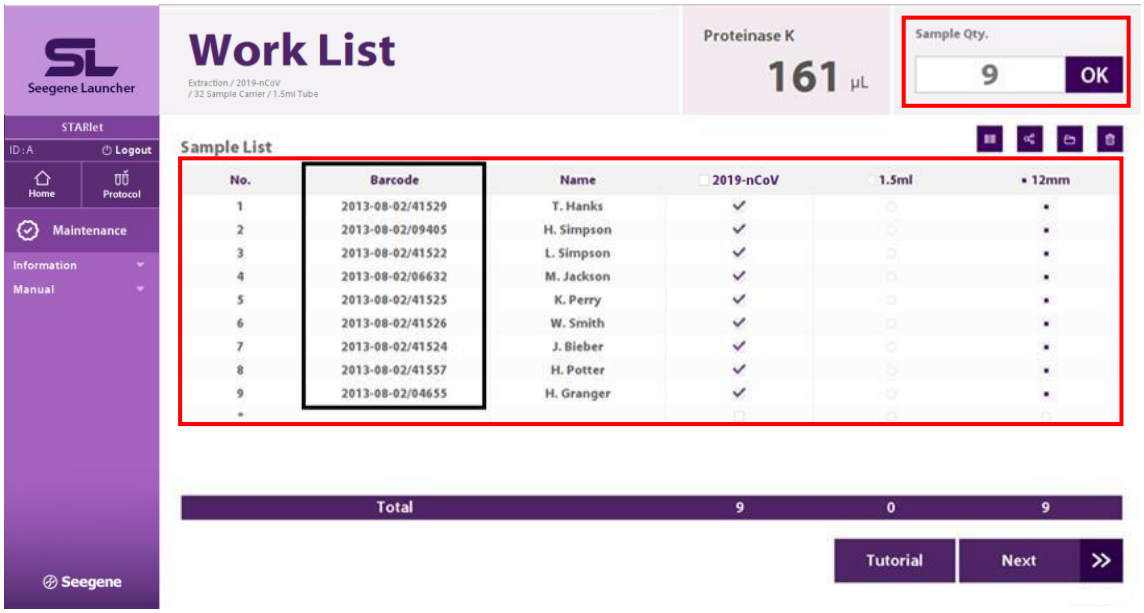
3. Select **2019-nCoV** (protocol for Allplex™ 2019-nCoV Assay) to begin the protocol. All following steps are included in a step by step instruction included in the software.

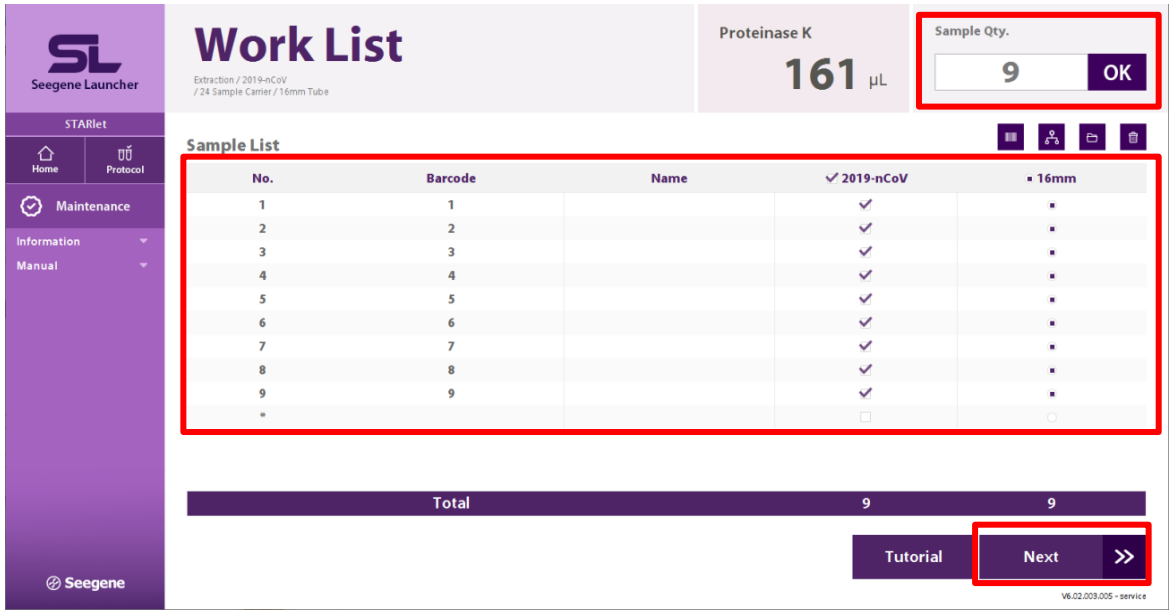
4. Check and follow the instructions carefully and then click on **Work List**. Samples, Internal Control, consumables, and 1 cartridge from the STARMag 96 X 4 Universal Cartridge Kit are placed on the Microlab STARlet IVD/Seegene STARlet while following step by step instructions guided by the Seegene Launcher software.

   NOTE: After equilibrating specimens to room temperature, vortex each specimen briefly.

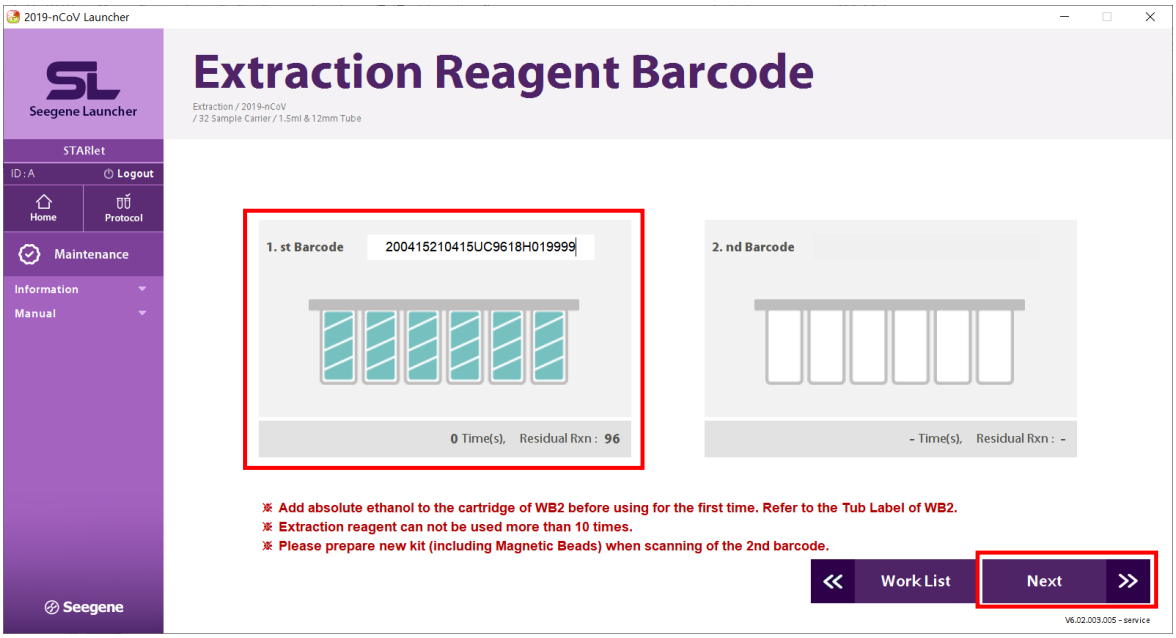


5. A barcode reader installed inside the Microlab STARlet IVD/Seegene STARlet automatically reads sample information. The sample information can also be manually entered, if necessary.
   Click on **Next**, once **Sample Quantity, Barcode, Name** (optional) and labware (1.5ml or 12mm or 16mm) information are entered correctly.

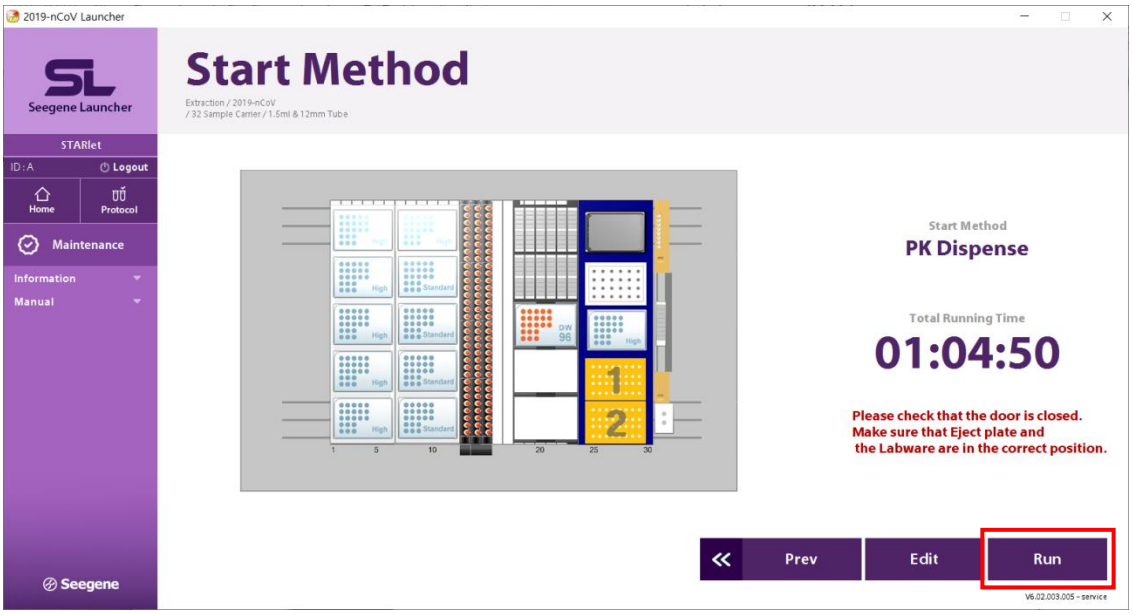




6. Using a hand-held barcode reader provided with the Microlab STARlet IVD/Seegene STARlet, read barcode label attached on the side of the cartridge. After the **Extraction Reagent Barcode** information is entered, click on **Next**. If the remaining volume of the existing cartridge is insufficient to run the desired number of samples, a second cartridge needs to be barcoded and placed.

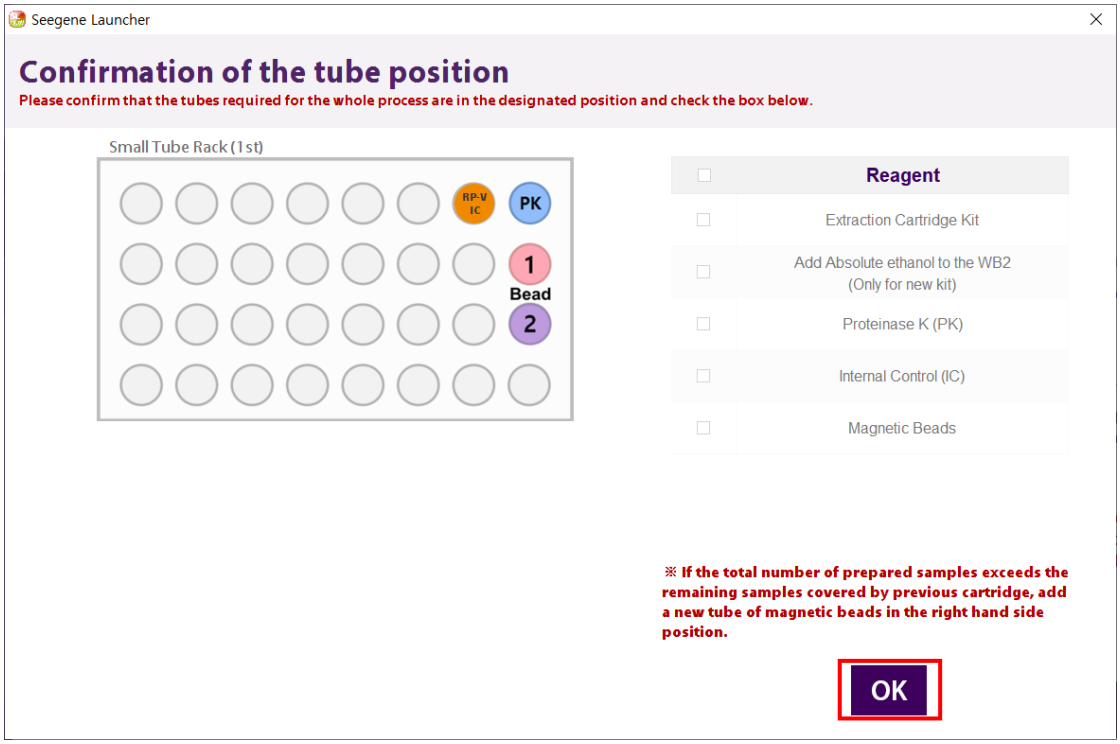


7. Ensure that the Microlab STARlet IVD/Seegene STARlet door is firmly closed, and that the eject plate and labware are in their correct positions as shown below. Click on **Run** after all preparations are done. Do not open the door of the Microlab STARlet IVD/Seegene STARlet during operation.

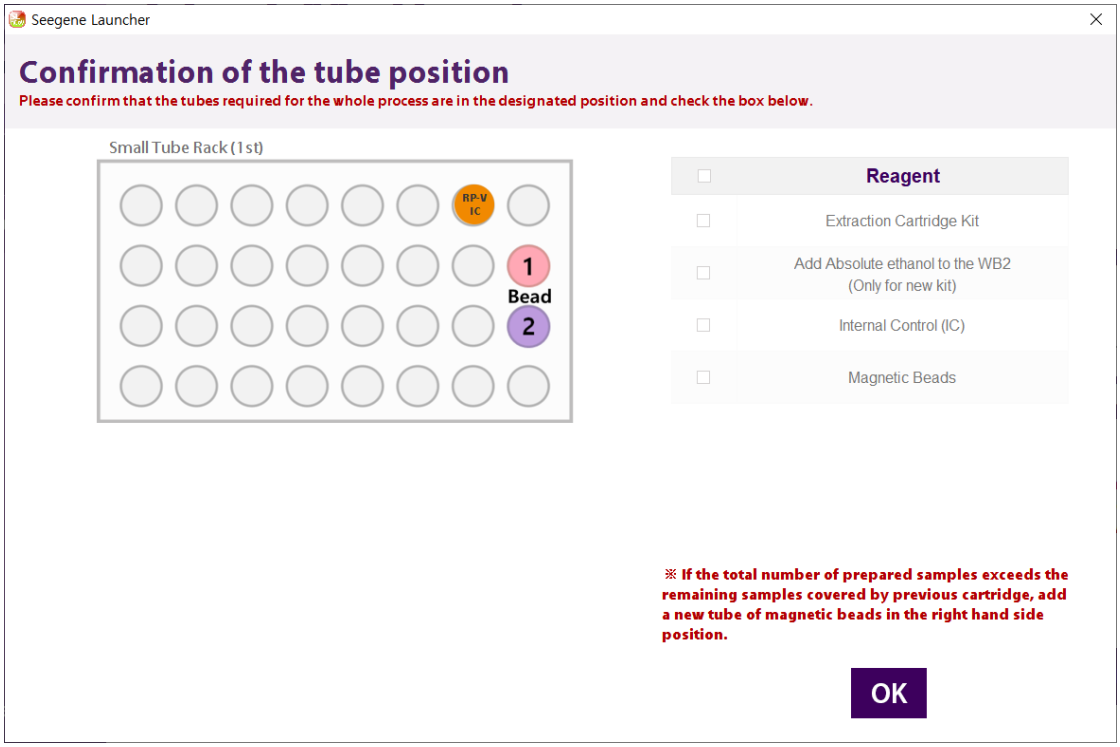


8. Check that the reagents are in the right position and click on **OK** to start run.

For STARMag 96 X 4 Universal Cartridge Kit;



For STARMag 96 X 4 viral DNA/RNA 200 C Kit;



For further inquiries regarding the extraction procedure, contact Seegene Technologies (California, US) at support@seegenetech.com.

Please refer to the user manual of 'Seegene Launcher V6' for detailed description on experimental procedures of nucleic acid extraction using Microlab STARlet IVD and Seegene STARlet.

Amplification and detection: CFX96™ and CFX96 Touch™

A video tutorial is available upon request to Seegene Technologies (California, US, support@seegenetech.com) for training on all experimental procedures related to amplification and detection under this section. Seegene Viewer v 3.20 for auto-interpretation of results is provided by Seegene Technologies (California, US), support@seegenetech.com.

Preparation for real-time PCR

NOTE:

(1) To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and samples, and always use aerosol barrier pipette tips.
(2) Extracted RNA handling and PCR reagent preparation must be performed at different areas.
(3) Remove all reagents from ≤ -20℃ storage. After thawing them completely, spin down each reagent for quick spin.
(4) The provided positive control (PC, PCR control) and clinical sample RNA extracts require special caution in handling to avoid carry-over contamination.
(5) Include one Positive Control and one Negative Control on each plate.

1. Prepare following reagents in a labeled sterile 1.5 mL tube. Set up all reagents on ice.

Table 6. One-step RT-PCR Mastermix for different number of reactions (unit: µL)

No. of Reactions	1	2	3	4	5
2019-nCoV MOM	5	10	15	20	25
RNase-free Water	5	10	15	20	25
5X Real-time One-step Buffer	5	10	15	20	25
Real-time One-step Enzyme	2	4	6	8	10

2. Mix by inverting each reagent tube 5 times or quick vortex, and briefly centrifuge.

   In 96-well PCR plate, Aliquot 17 µL of the One-step RT-PCR Mastermix into PCR tubes. NOTE: Prior to adding specimen extract/positive

controls to PCR plate, move from the reagent prep area to a specimen processing area.

3. Add 8 µL of each sample's extracted nucleic acids, 2019-nCoV PC and NC (RNase-free Water; Negative Control (NC) for PCR control) into the tubes containing aliquot of the One-step RT-PCR Mastermix.

4. Cover with Permanent Clear Heat seal for 96-Well Skirted PCR Plates on PX1™ PCR Plate sealer, and briefly centrifuge the PCR tubes.

   NOTE: The PCR tubes must be centrifuged before running PCR reaction. It needs to force the liquid to the bottom and to eliminate air bubbles.

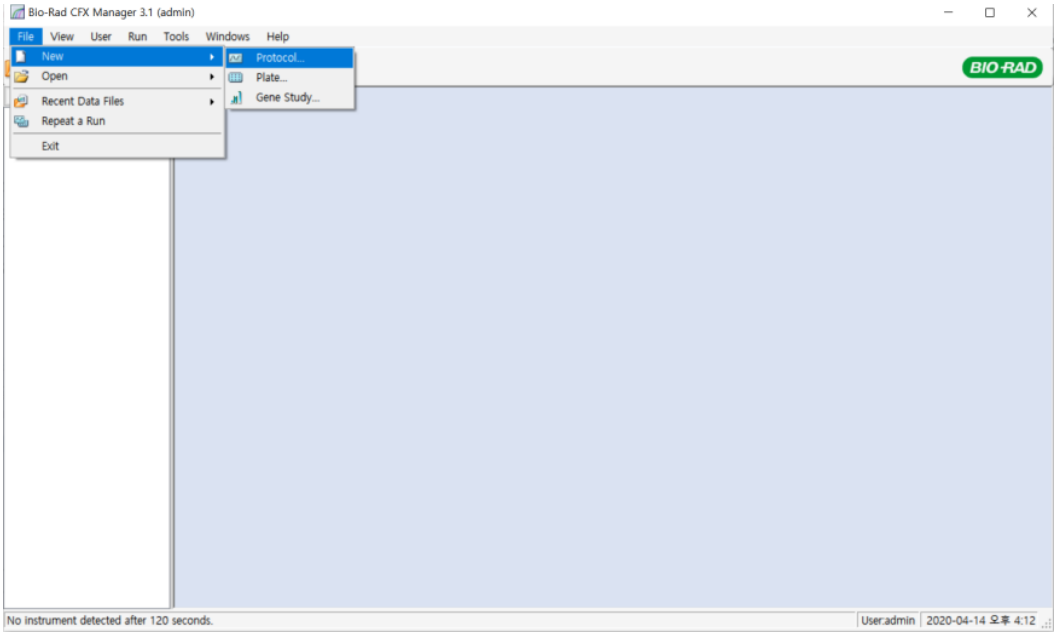
5. Verify that the liquid containing all PCR components is at the bottom of each PCR tube. If not, centrifuge again at a higher rpm and for a longer time.

6. Immediately initiate the PCR on the Bio-Rad CFX or Bio-Rad CFX96 instruments. See details on PCR instrumentation set-up below.

Real-time PCR Instrument Set Up

Protocol Setup

1. In the main menu, select File → New → Protocol to open Protocol Editor.



2. In Protocol Editor, define the thermal profile as table below.

Step	No. of cycles	Temperature	Duration
1	1	50℃	20 min
2	1	95℃	15 min
3	45	94℃	15 sec
4		58℃	30 sec
5	GOTO Step 3, 44 more times		

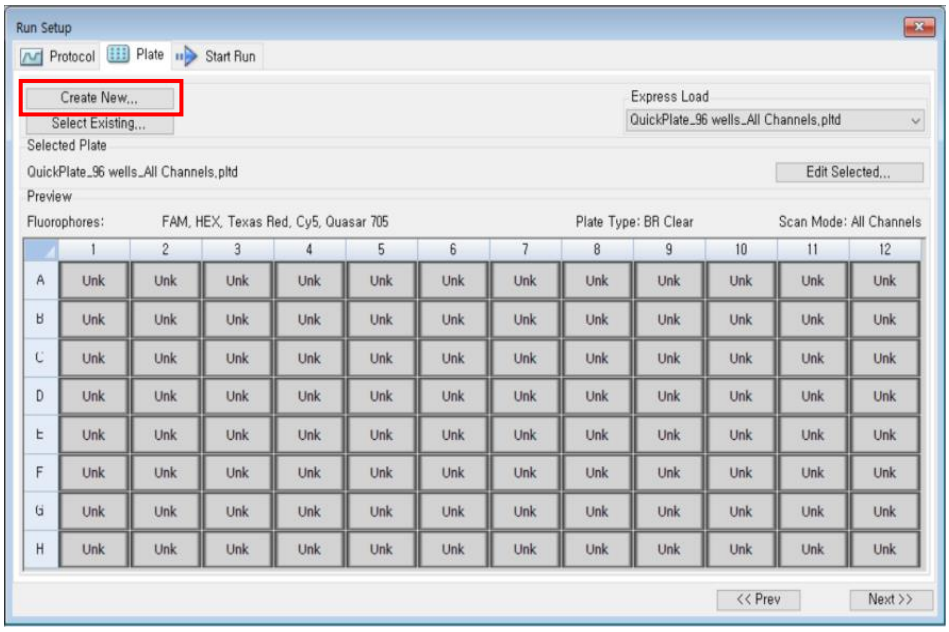
NOTE: Plate Read at Step 4. Fluorescence is detected at 58°C.

3. Click the box next to Sample Volume to directly input 25 µL.

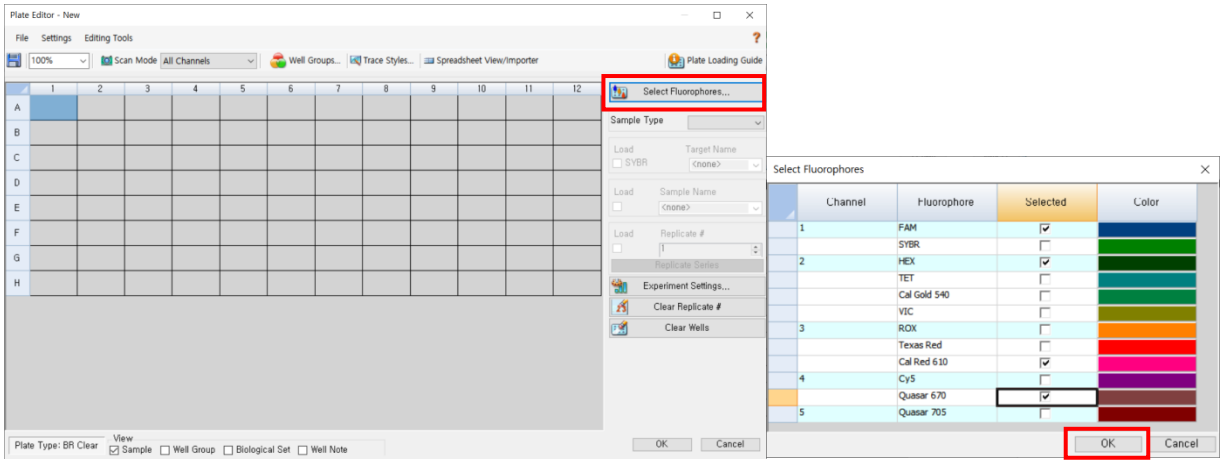
4. Click **OK** and save the protocol to open the Experiment Setup window.

Plate Setup

1. From **Plate** tab in **Experiment Setup**, click **Create New** to open **Plate Editor** window.

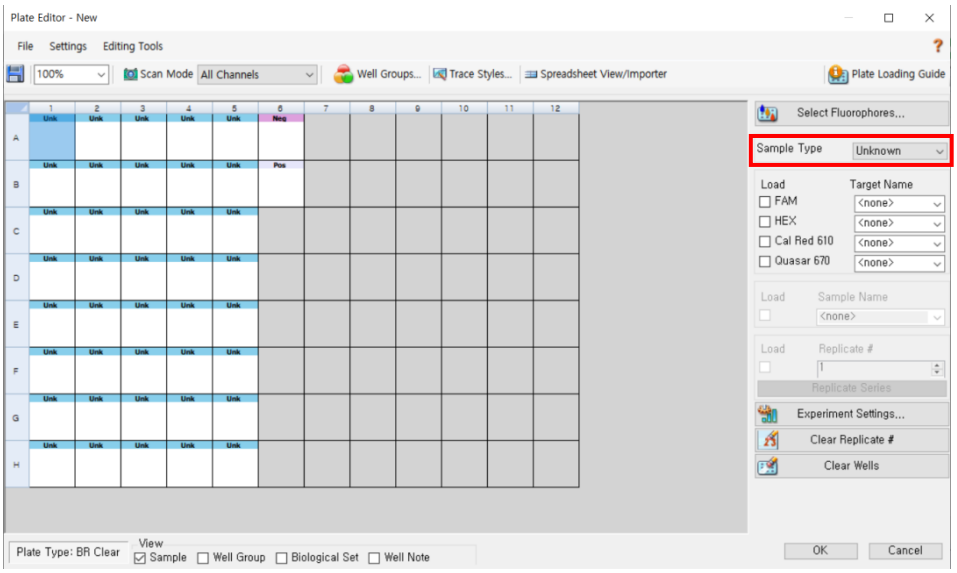


2. Click **Select Fluorophores** to indicate the fluorophores **(FAM, HEX, Cal Red 610** and **Quasar 670)** that will be used and click **OK**.

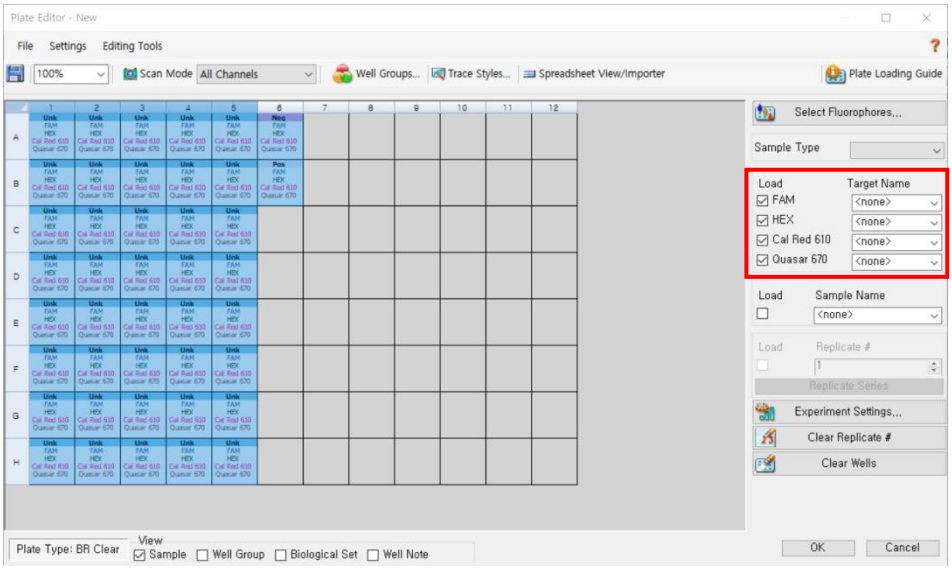


3. Select the desired well(s) and then its sample type from the **Sample Type** drop-down menu.

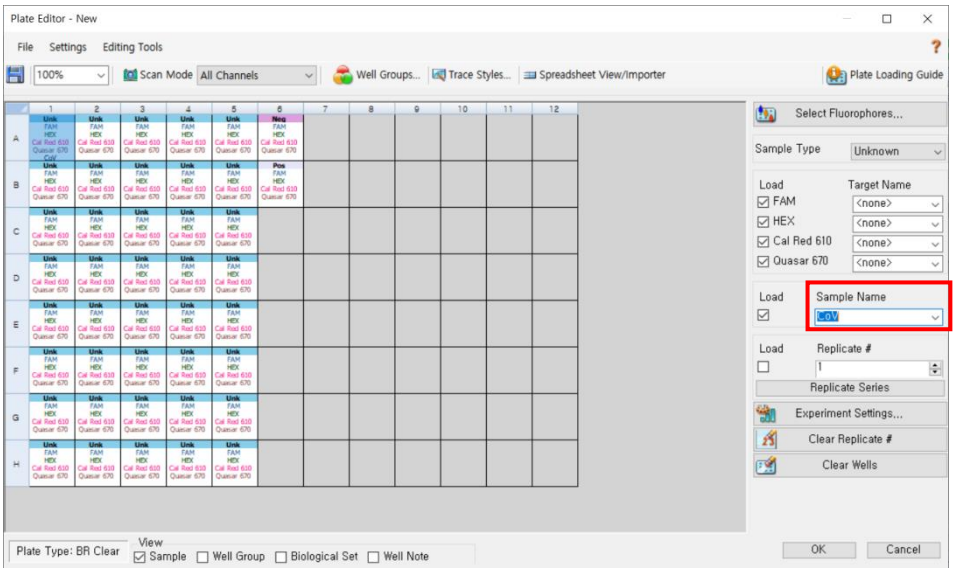
- **Unknown**: Clinical samples
- **Negative Control**
- **Positive Control**



4. Click on the appropriate checkboxes (**FAM, HEX, Cal Red 610** and **Quasar 670**) to specify the fluorophores to be detected in the selected wells.

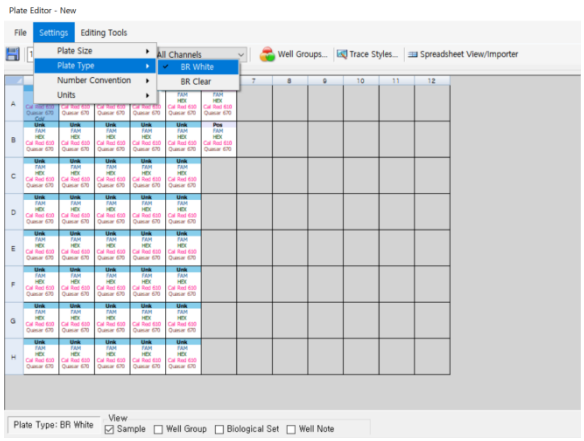


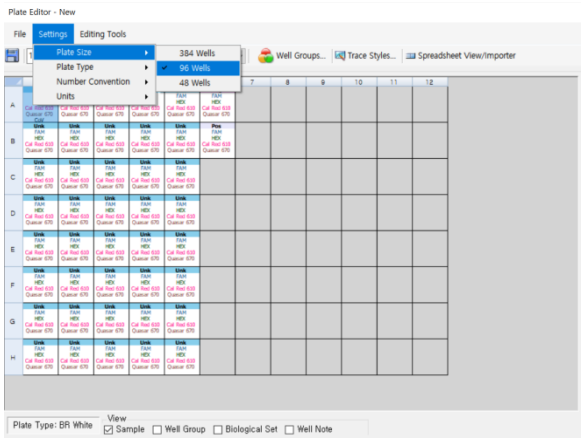
5. Type in **Sample Name** and press enter key.



6. In **Settings** of the **Plate Editor** main menu, choose **Plate Size (96 wells)** and **Plate Type (BR White)**.

7. Click **OK** to save the new plate.





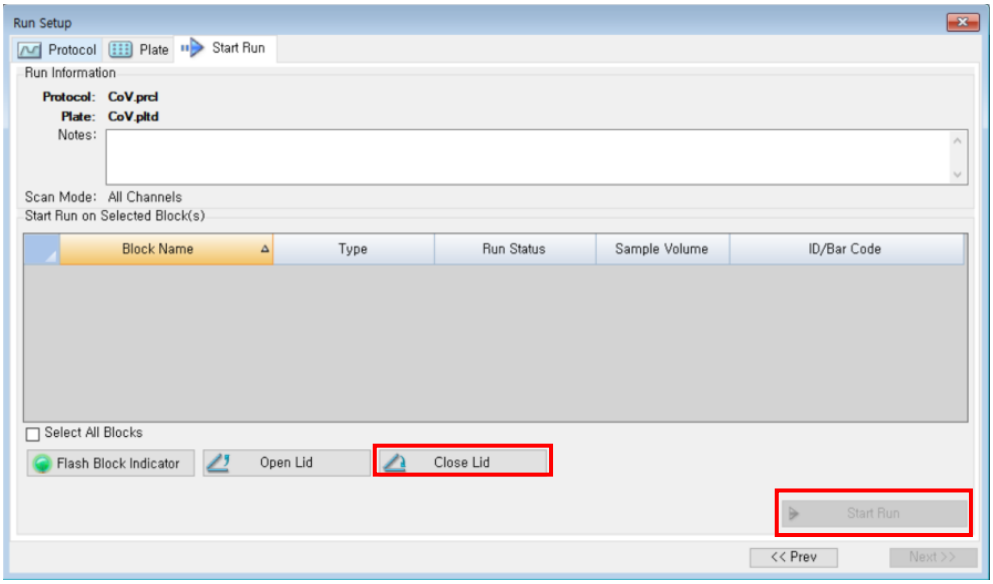
8. You will be returned to the Experiment Setup window.

Real-time PCR run

Start Run

1. From **Start Run** tab in **Experiment Setup**, click **Close Lid** to close the instrument lid.

2. Click **Start Run**.



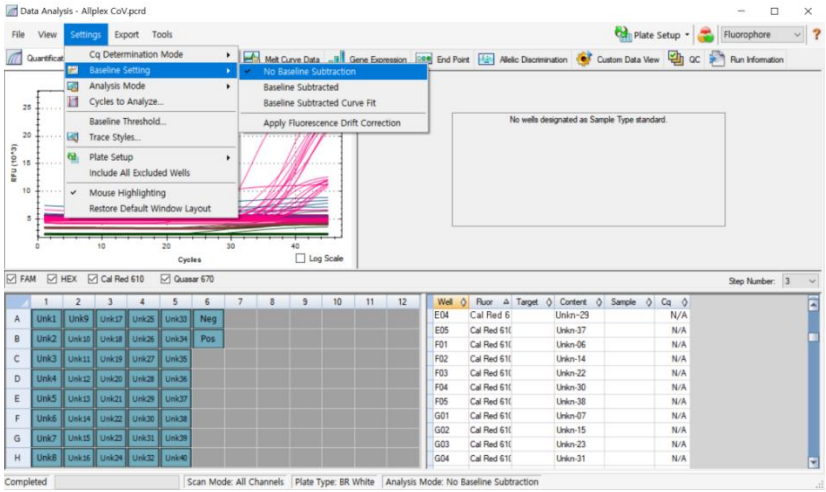
3. Store the run file either in My Documents or in a designated folder. Enter the file name, click **SAVE**, and the run will start.

Data export and analysis

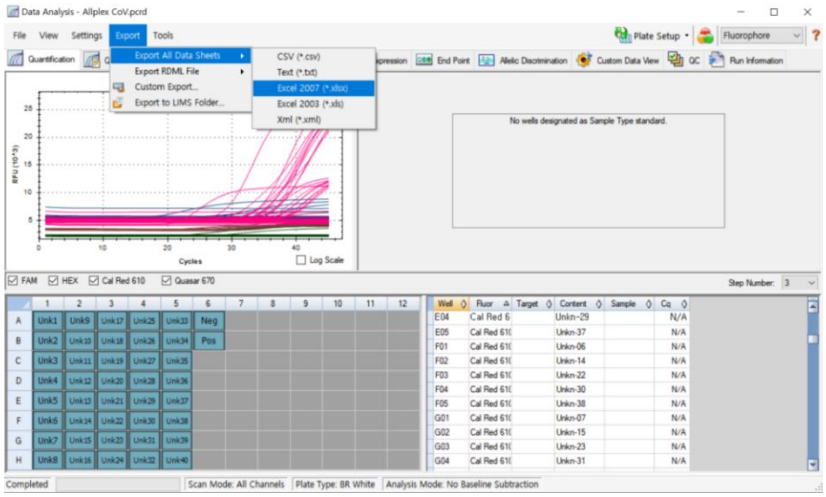
Data export
(CFX96 Touch™, CFX Manager™ Software V3.1 & CFX Maestro™ Software)

1. Create folders for data export
   - Create a folder to save amplification curve detection results.
   - The location and name of the folder is specified by user, but in case of using 'Seegene Export' function, folder named "QuantStep4" is created automatically in selected location.

2. After the PCR reaction, select **No Baseline Subtraction** from **Baseline Setting** of **Settings** menu.



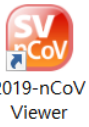
3. Select **Excel 2007** from **Export All Data Sheets** from **Export** menu.



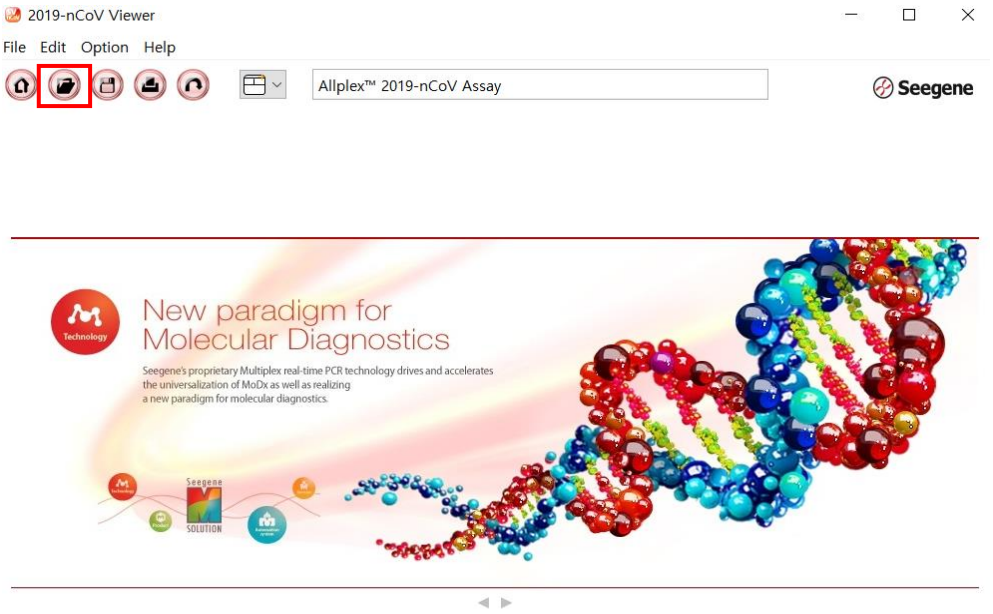
4. Choose a location to save data and click **OK**.

Data analysis

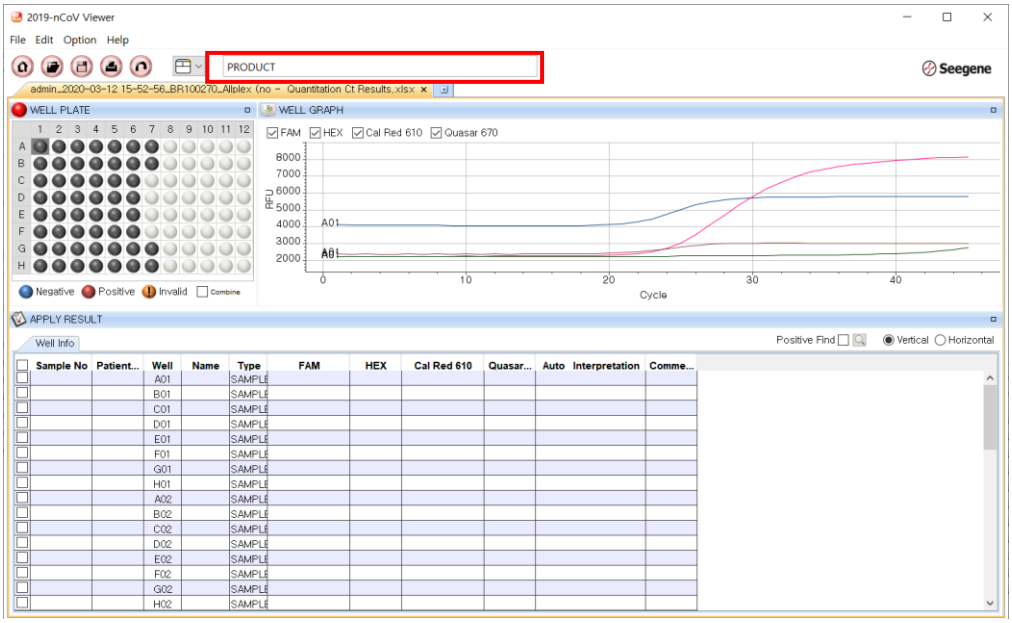
1. Open the **Seegene Viewer** software installed on the laptop connected to the Bio-Rad CFX96™.



2. Click on Open icon and find CFX96™ export data on location where CFX96™ data was saved.



3. After opening the results file, select '**Allplex™ 2019-nCoV Assay**' from the **PRODUCT** menu.



4. View test results. The results for each sample can be viewed by clicking on each well.

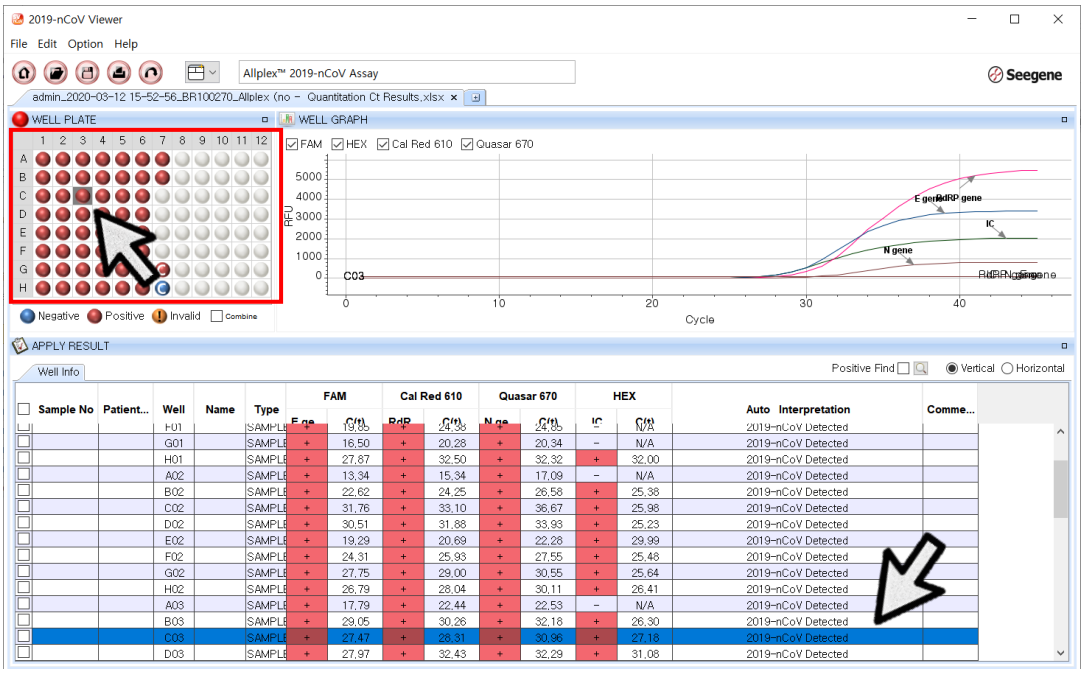


Table 7. Analytes of the Allplex™ 2019-nCoV Assay

Fluorophore	Analyte
FAM	E gene
HEX	Internal Control (IC)
Cal Red 610	RdRP gene
Quasar 670	N gene

Amplification and detection: Applied Biosystems™ 7500

Preparation for real-time PCR

NOTE:

(1) To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and samples, and always use aerosol barrier pipette tips.
(2) Extracted RNA handling and PCR reagent preparation must be performed at different areas.
(3) Remove all reagents from ≤ -20℃ storage. After thawing them completely, spin down each reagent for quick spin.
(4) The provided positive control (PC, PCR control) and clinical sample RNA extracts require special caution in handling to avoid carry-over contamination.
(5) Include one Positive Control and one Negative Control on each plate.

1. Prepare following reagents in a labeled sterile 1.5 mL tube. Set up all reagents on ice.

Table 6. One-step RT-PCR Mastermix for different number of reactions (unit: µL)

No. of Reactions	1	2	3	4	5
2019-nCoV MOM	5	10	15	20	25
RNase-free Water	5	10	15	20	25
5X Real-time One-step Buffer	5	10	15	20	25
Real-time One-step Enzyme	2	4	6	8	10

2. Mix by inverting each reagent tube 5 times or quick vortex, and briefly centrifuge. In 96-well PCR plate, Aliquot 17 µL of the One-step RT-PCR Mastermix into PCR tubes.

   NOTE: Prior to adding specimen extract/positive controls to PCR plate, move from the reagent prep area to a specimen processing area.

3. Add 8 µL of each sample's extracted nucleic acids, 2019-nCoV PC and NC (RNase-free Water; Negative Control (NC) for PCR control) into the tubes containing aliquot of the One-step RT-PCR Mastermix.

4. Cover with adhesive covers for 96-Well PCR Plates, and briefly centrifuge the PCR tubes.

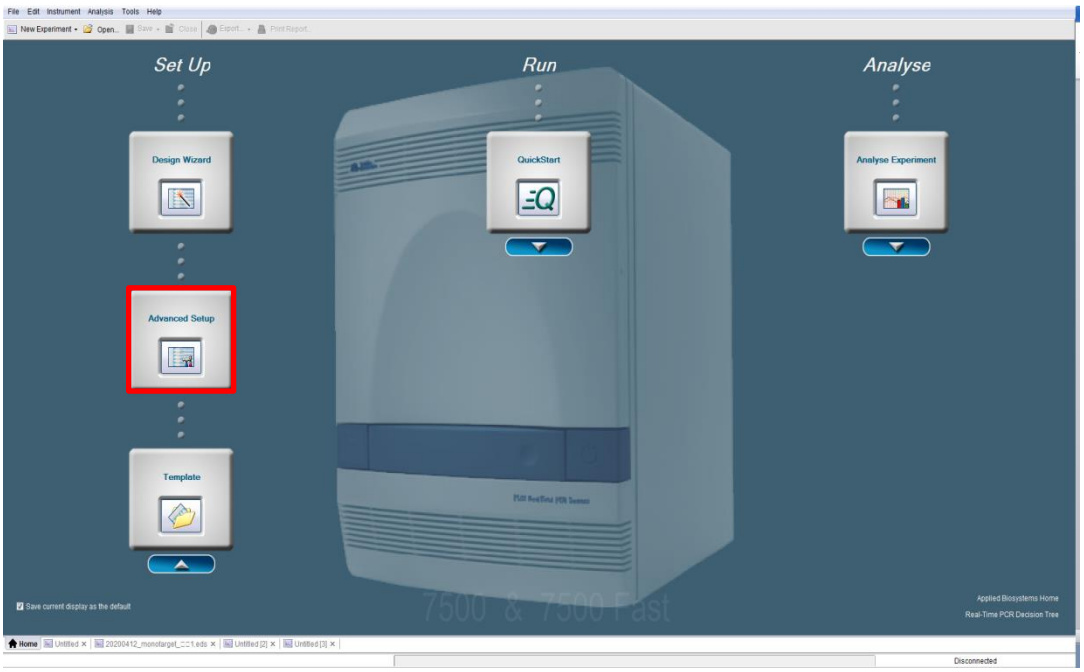
5. Verify that the liquid containing all PCR components is at the bottom of each PCR tube. If not, centrifuge again at a higher rpm and for a longer time.

6. Immediately initiate the PCR on the Applied Biosystems™. See details on PCR instrumentation set-up below.

Real-time PCR Instrument set up

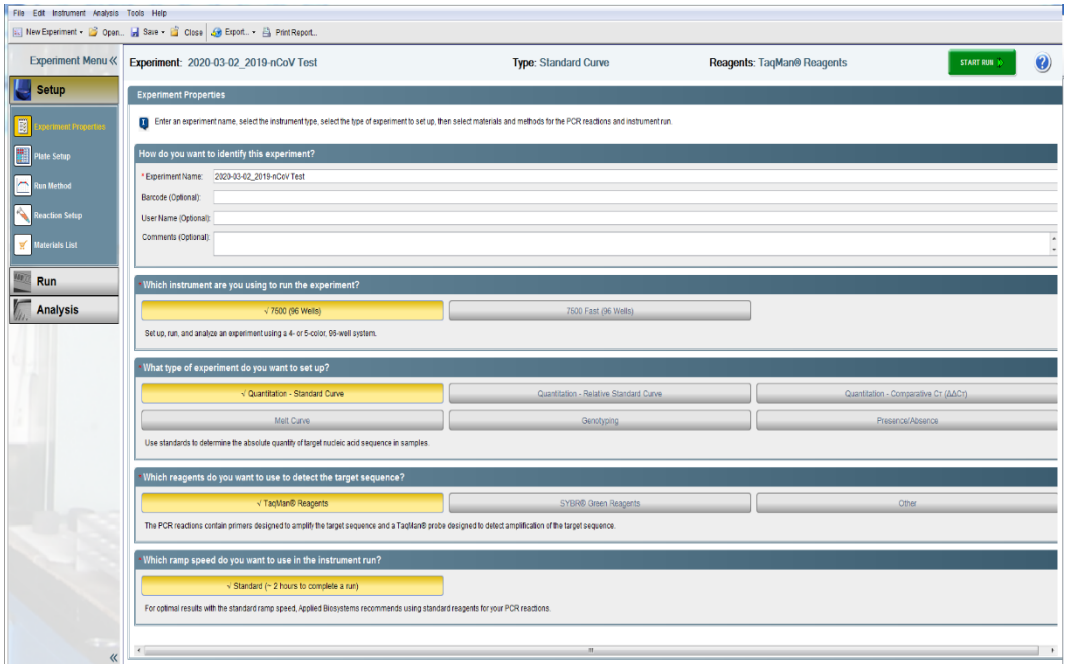
NOTE: The instrument must be calibrated before use.

1. In the Applied Biosystems™ 7500 software, click on **Setup** → **Advanced set up**.



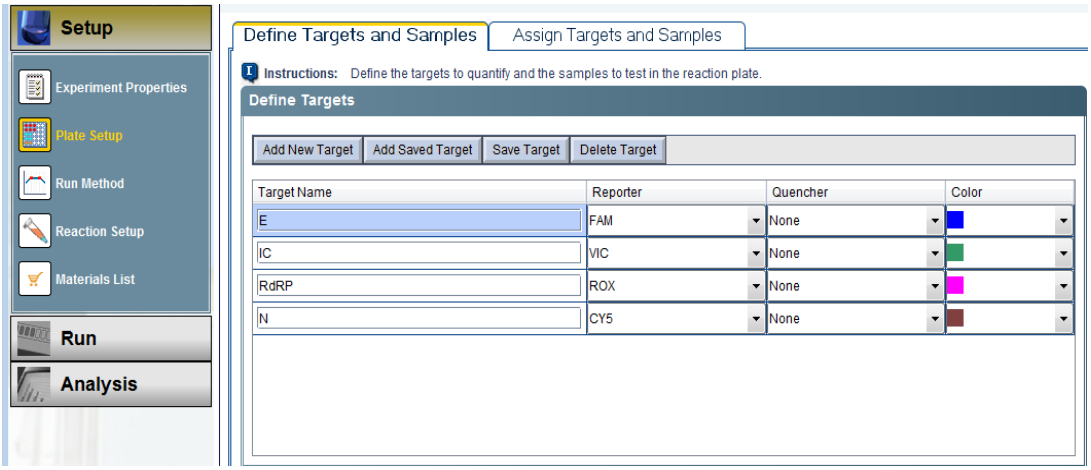
2. In the **Experiment properties tab**, enter **Experiment Name** and select **Instrument**, **Experiment type**, **Reagents**, and **Ramp speed** as follows.

Instrument	7500 (96 Wells)
Experiment type	Quantitation – Standard Curve
Reagents	Taqman® Reagents
Ramp speed	Standard



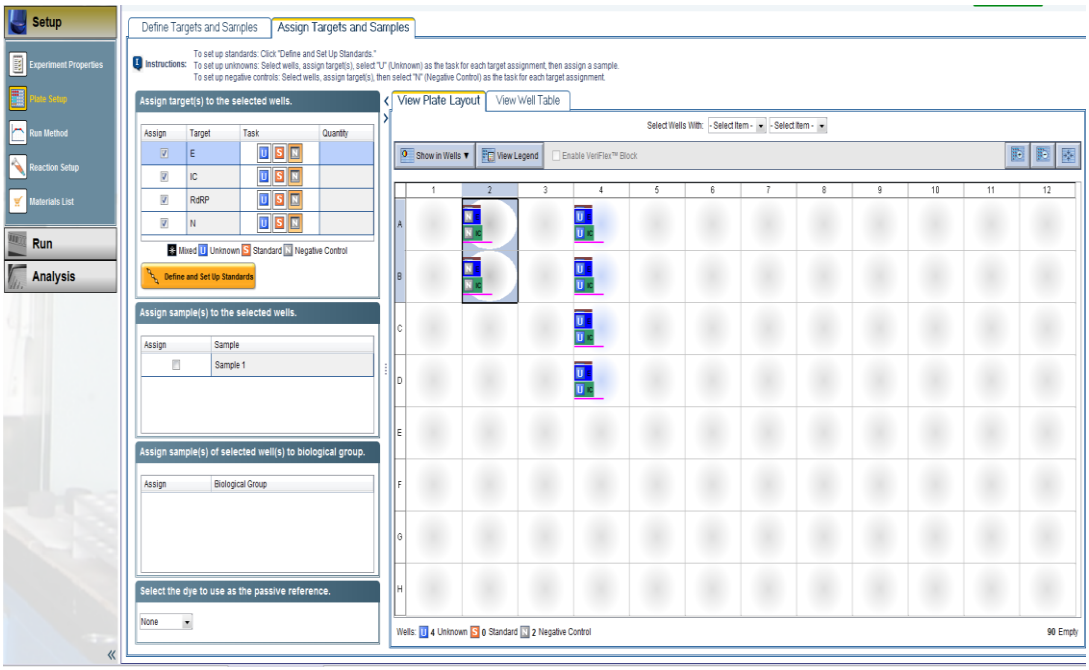
3. Click on **Plate setup** tab. In the **Define Targets** and **Samples** tab, enter **Target Name** and select **Reporter** and **Quencher** as follows.

Target Name	Reporter	Quencher
E	FAM	None
IC	VIC	None
RdRP	ROX	None
N	CY5	None



4. Click on **Assign Targets and Samples** tab, select wells where the PCR tube will be placed and assign targets. Select None for Passive reference.

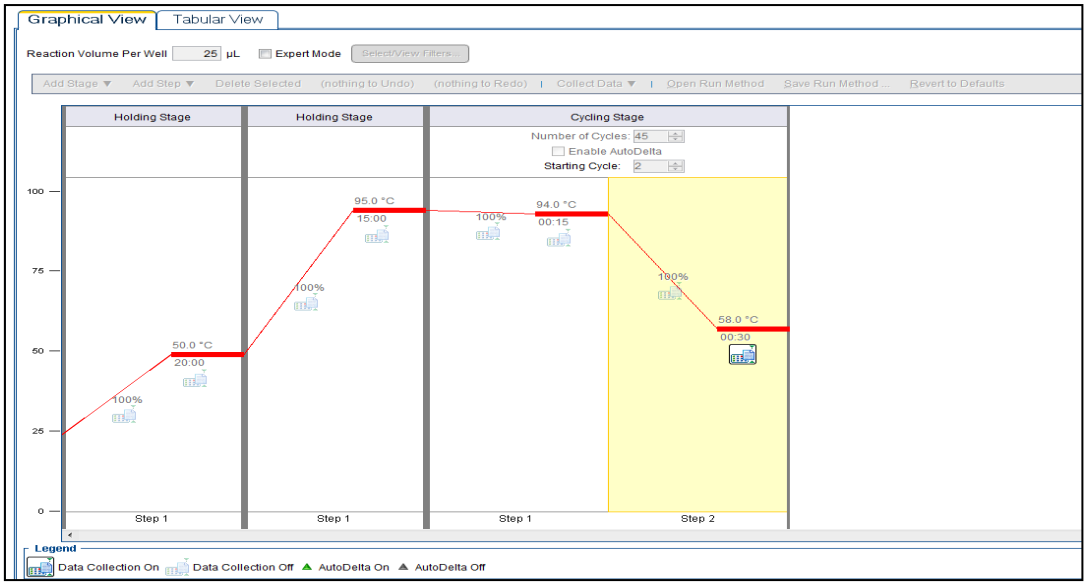
NOTE: If a well without sample or Mastermix is selected, signal noise may be observed. Ensure that only wells containing samples or Mastermix are selected.



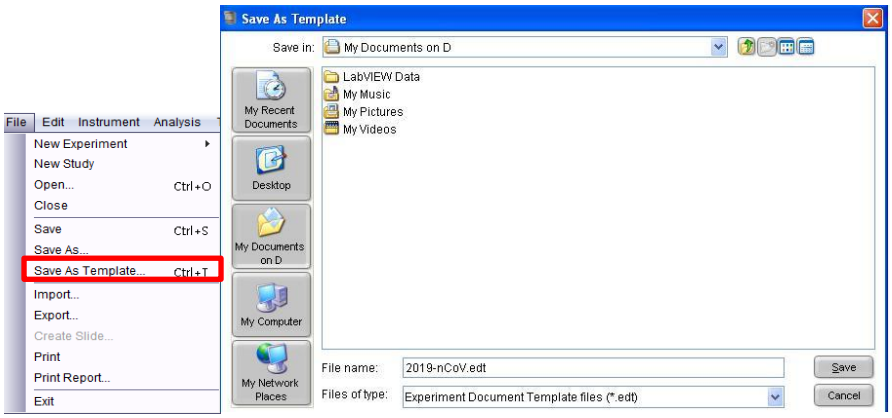
5. Click on Run Method. In the Graphical View or Tabular View tab, enter **25 µL** as the **Reaction Volume** per Well field. Define the thermal profile as table below.

Step	No. of cycles	Temperature	Duration
1	1	50°C	20 min
2		95°C	15 min
3	45	94°C	15 sec
4		58°C	30 sec
5	GOTO Step 3, 44 more times		

NOTE: Plate Read at Step 4. Fluorescence is detected at 58°C.



6. Click on **File** → **Save as Template** to save the new template file in **.edt** format. Enter the file name, select a location for the template, then click **Save**. The saved template can be used for future testing.



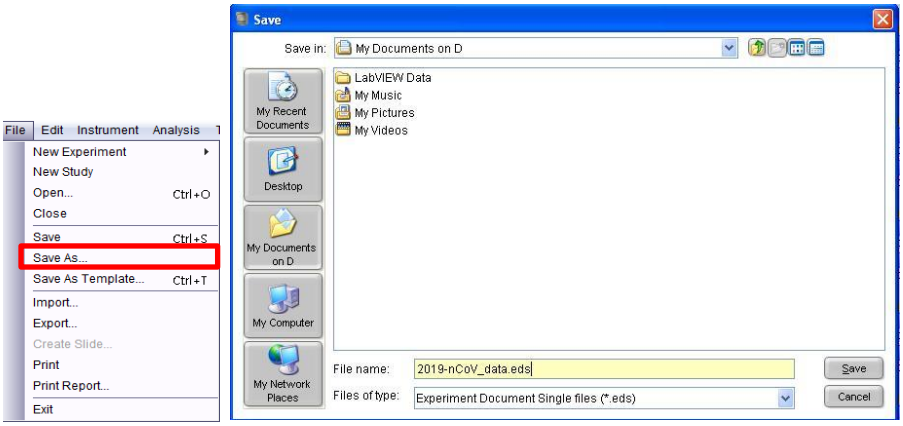
Start the Run

1. Turn on the laptop and Applied Biosystems™ 7500 real-time PCR system. Ensure that the laptop is connected to the instrument.

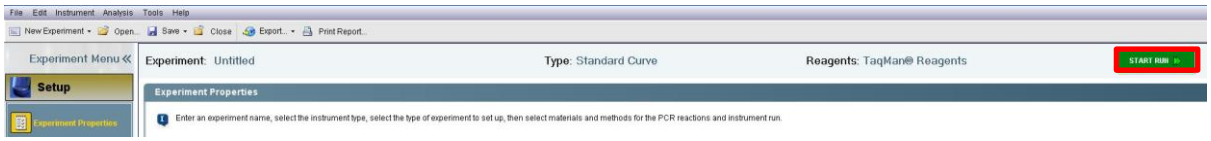
2. Push the tray door to open the instrument. Load the PCR plate onto the plate holder of the instrument.

3. Push the tray door to close the instrument.

4. Click on **File** → **Save as** to save experiment in .eds format.



5. Click **START RUN**.



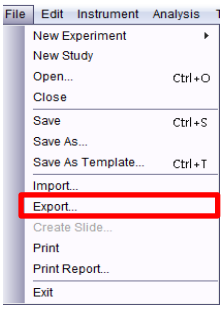
Data export and analysis

Create folders for data export

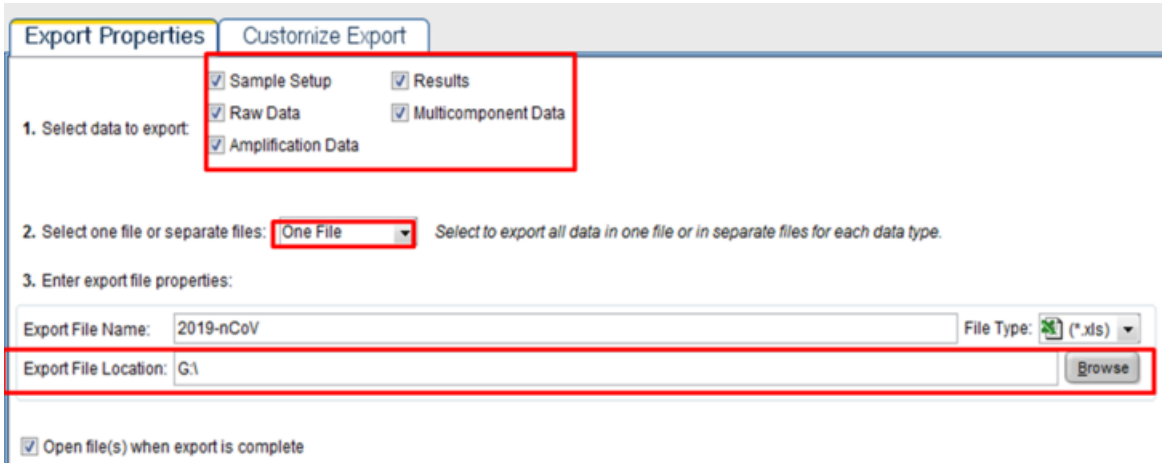
1. Create a folder to save data for all of amplification curve detection steps from the result file.
2. Enter folder name as necessary.

Data export

1. Click on **File** → **Export**



2. Click on the Export Properties tab (default) and select Sample Setup, Raw data, Amplification Data, Results, and Multicomponent Data under 1. Select data to export.

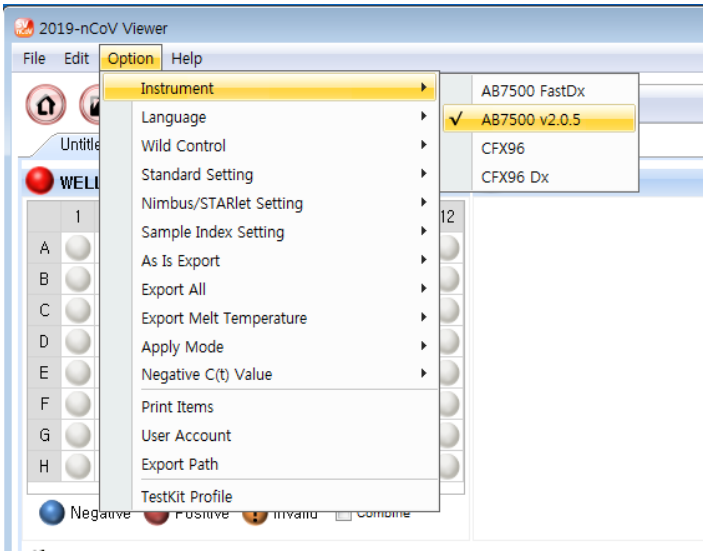


3. Select 'One File' under 2. Select one or separate files:
4. Enter Export File Name, then select Export File Location.
5. Select .xls in the File Type drop-down list.
6. Click Start Export.

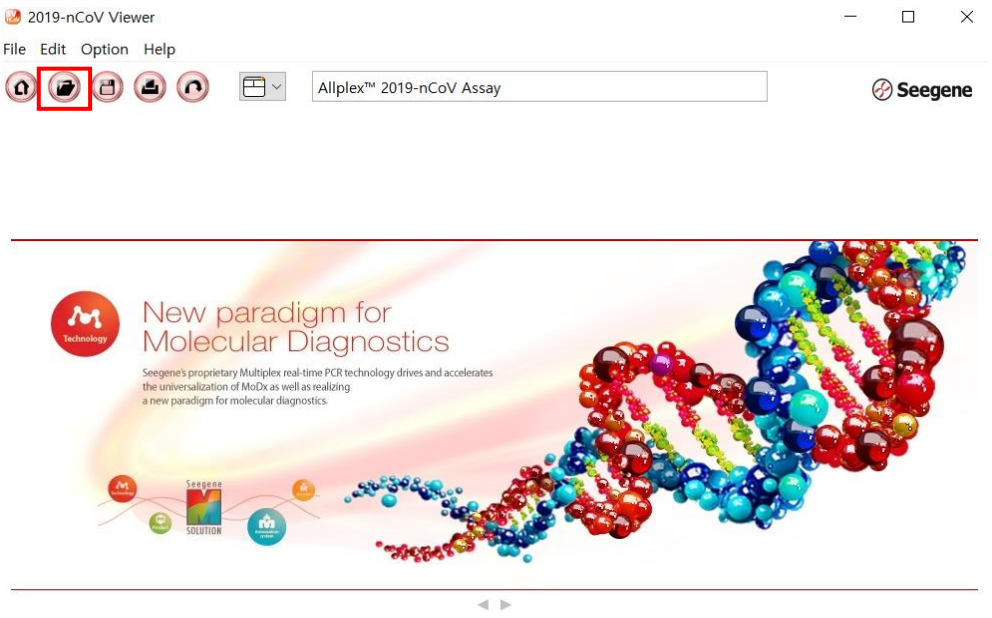
Data analysis

1. Open the Seegene Viewer software installed on the laptop connected to the Applied Biosystems™ 7500.

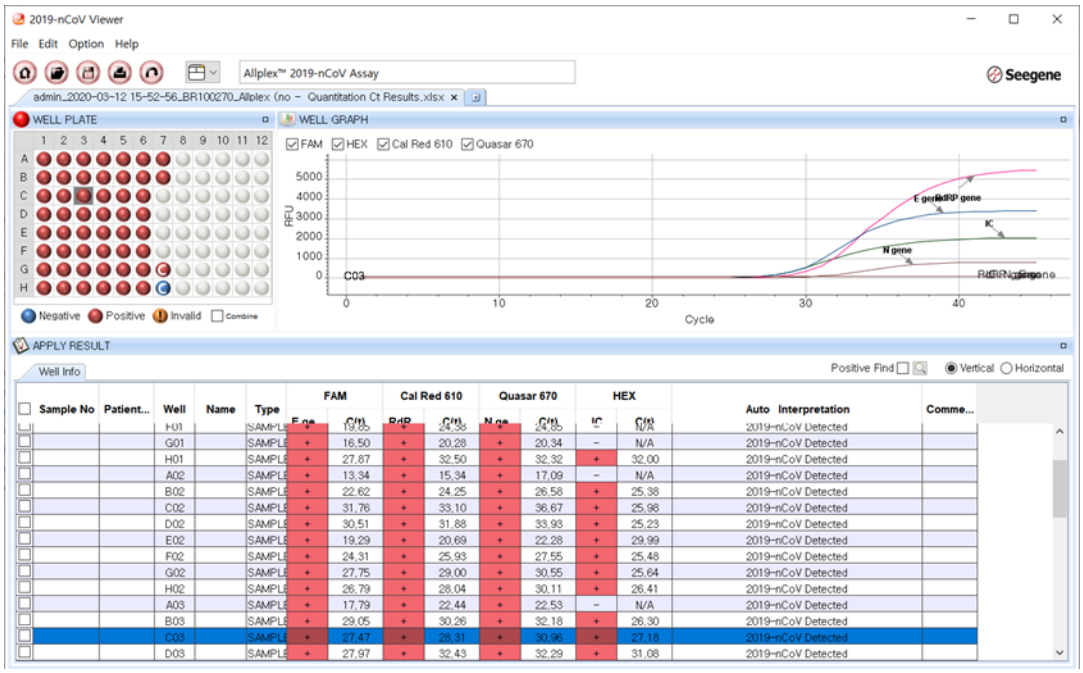
2. Click on **Option** to select AB7500 v2.0.5 from the **Instrument** menu.



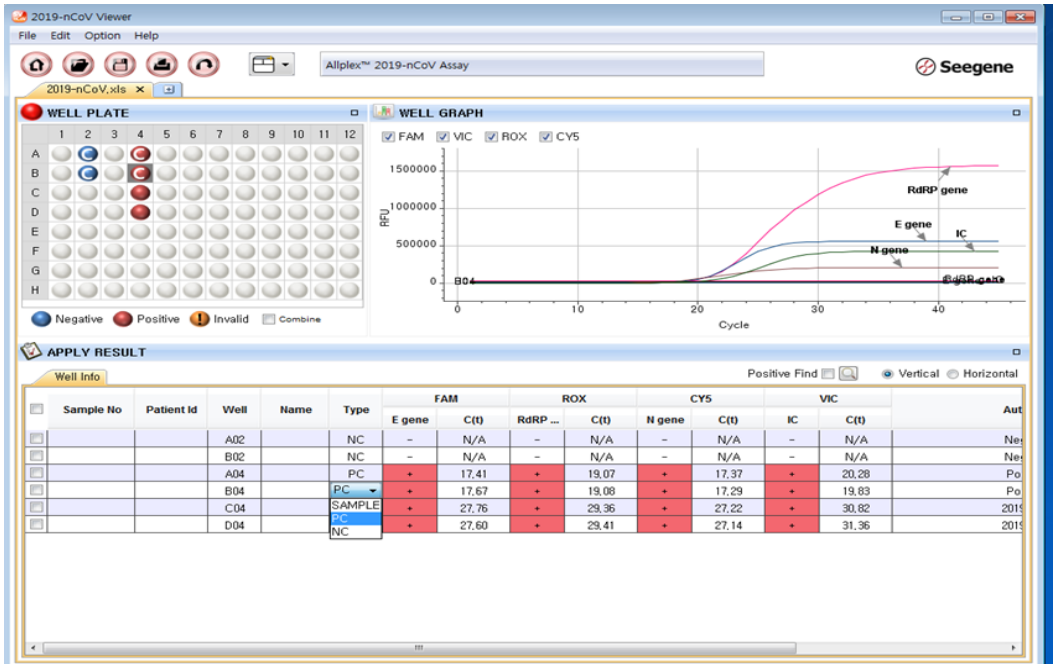
3. Click on the **Open** icon and locate the Applied Biosystems™ 7500 export data where the Applied Biosystems™ 7500 data was saved.



4. After opening the results file, select 'Allplex™ 2019-nCoV Assay' from the PRODUCT menu.



5. Assign Positive and Negative control accordingly by selecting PC, NC under the Type drop-down menu.



6. View test results. The auto-interpreted results for each sample can be viewed by clicking on each well.

Amplification and detection: Applied Biosystems™ 7500 Fast Dx

Preparation for real-time PCR

NOTE:

(1) To prevent contamination, prepare reagents in a PCR workstation or equivalent amplicon-free area. Do not use the same pipette for controls and samples, and always use aerosol barrier pipette tips.
(2) Extracted RNA handling and PCR reagent preparation must be performed at different areas.
(3) Remove all reagents from ≤ -20℃ storage. After thawing them completely, spin down each reagent for quick spin.
(4) The provided positive control (PC, PCR control) and clinical sample RNA extracts require special caution in handling to avoid carry-over contamination.
(5) Include one Positive Control and one Negative Control on each plate.

1. Prepare following reagents in a labeled sterile 1.5 mL tube. Set up all reagents on ice.

Table 6. One-step RT-PCR Mastermix for different number of reactions (unit: µL)

No. of Reactions	1	2	3	4	5
2019-nCoV MOM	5	10	15	20	25
RNase-free Water	5	10	15	20	25
5X Real-time One-step Buffer	5	10	15	20	25
Real-time One-step Enzyme	2	4	6	8	10

2. Mix by inverting each reagent tube 5 times or quick vortex, and briefly centrifuge.

   In 96-well PCR plate, Aliquot 17 µL of the One-step RT-PCR Mastermix into PCR tubes. NOTE: Prior to adding specimen extract/positive controls to PCR plate, move from the reagent prep area to a specimen processing area.

3. Add 8 µL of each sample's extracted nucleic acids, 2019-nCoV PC and NC (RNase-free Water; Negative Control (NC) for PCR control) into the tubes containing aliquot of the One-step RT-PCR Mastermix.

4. Cover with adhesive covers for 96-Well PCR plates, and briefly centrifuge the PCR tubes.

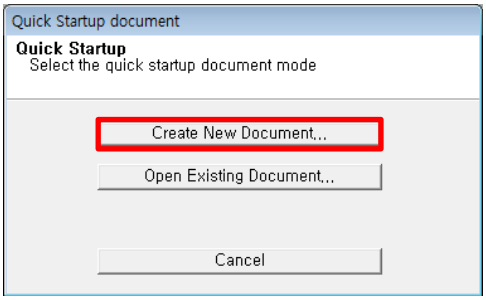
5. Verify that the liquid containing all PCR components is at the bottom of each PCR tube. If not, centrifuge again at a higher rpm and for a longer time.

6. Immediately initiate the PCR on the Applied Biosystems™ 7500. See details on PCR instrumentation set-up below.

Real-time PCR Instrument set up

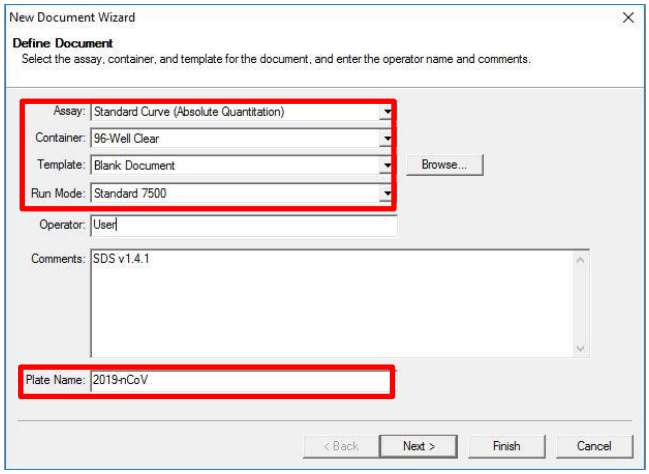
NOTE: The instrument must be calibrated before use.

1. In the SDS software, click on **Quick Startup → Create New Document.**

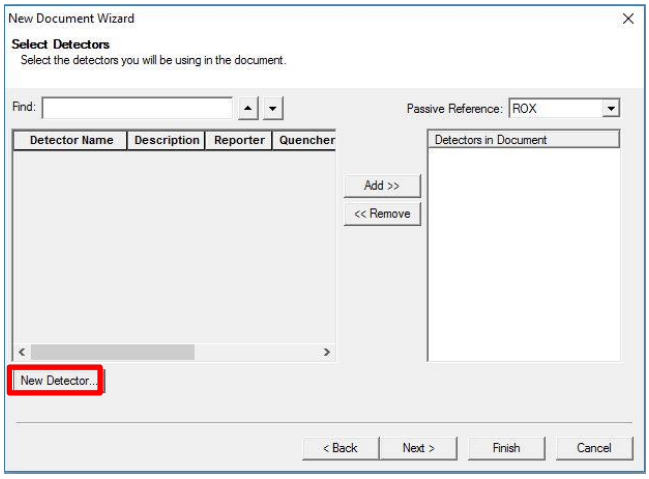


2. In **New Document Wizard**, select **Assay**, **Container**, **Template**, and **Run mode** as below then enter **Plate Name**.

Assay	Standard Curve (Absolute Quantitation)
Container	96-Well Clear
Template	Blank Document
Run mode	Standard 7500

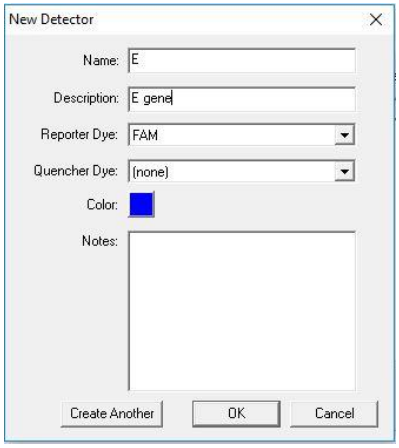


3. In **Select Detectors**, click on **New Detector** to add reporter and quencher information of analytes.

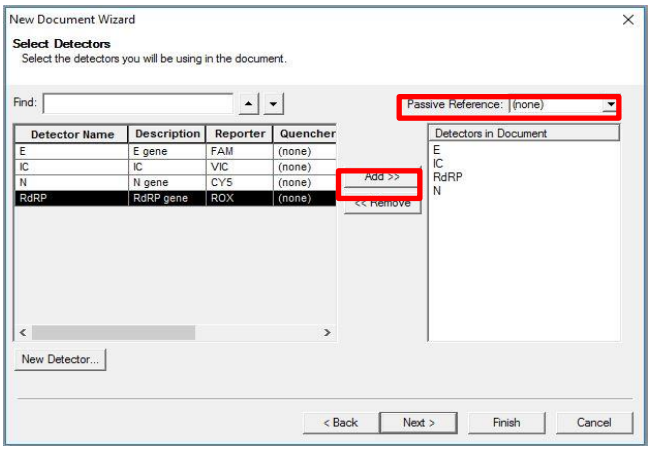


4. In **New Detector**, enter **Detector Name**, **Description** (optional) and select **Reporter, Quencher** as table below.

Detector Name	Reporter	Quencher
E	FAM	None
IC	VIC	None
RdRP	ROX	None
N	CY5	None

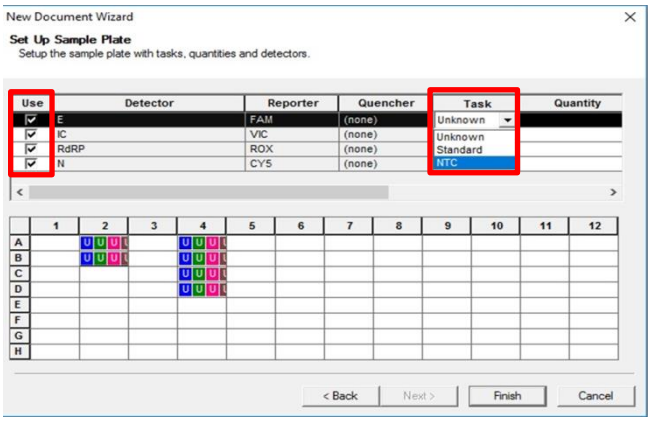


5. In **Select Detectors**, add E, IC, RdRP, and N to **Detectors in Document** field by clicking on **Add >>**. Select **none** for **Passive Reference.**

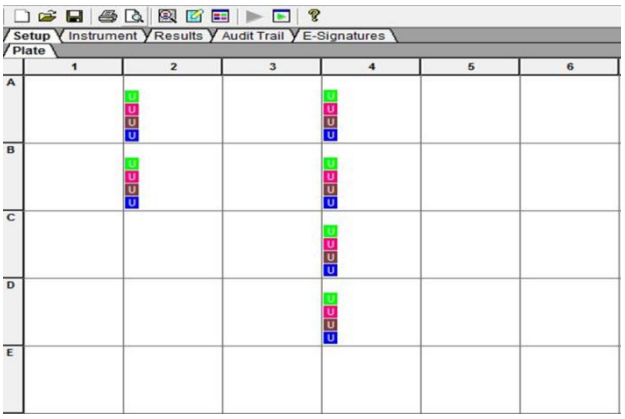


6. In **Set Up Sample Plate**, drag and select the wells where the PCR tube will be placed and assign targets by clicking on the check boxes next to each **Detector** and select **Unknown** from **Task**.

NOTE: If a well without sample or Mastermix is selected, signal noise may be observed. Ensure that only wells containing samples or Mastermix are selected.



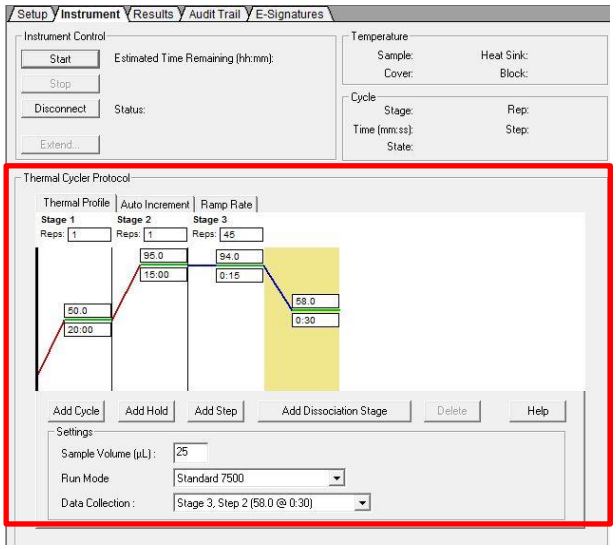
7. In the **Setup** – **Plate** tab, confirm the run plate information.



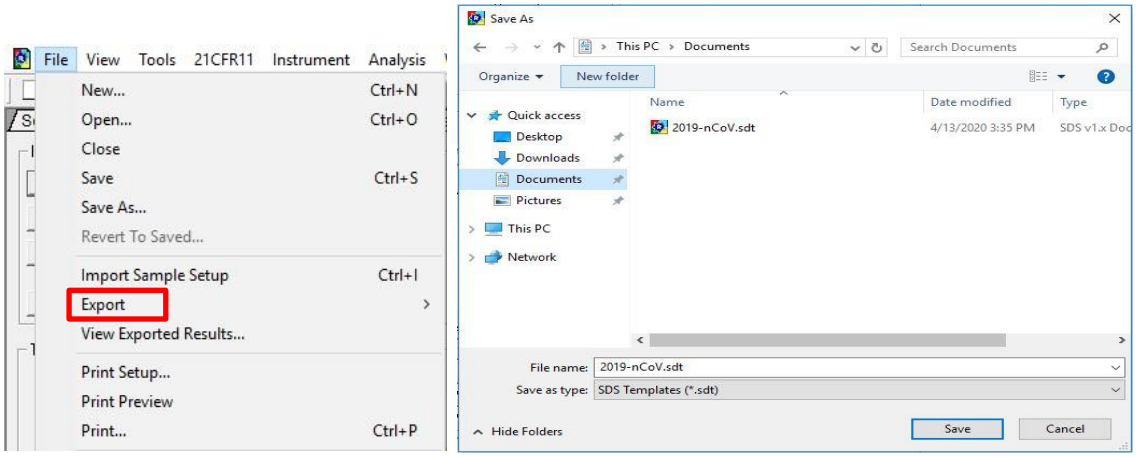
8. In the **Instrument** tab – **Thermal Cycler Protocol**, define the thermal profile as below. Enter **25 µL** in the **Sample Volume (µL)** field, select Stage 3, Step 2 [58.0°C @ 0:30] for **Data Collection** (Plate Read)

Step	No. of cycles	Temperature	Duration
1	1	50°C	20 min
2		95°C	15 min
3	45	94°C	15 sec
4		58°C	30 sec
5	GOTO Step 3, 44 more times		

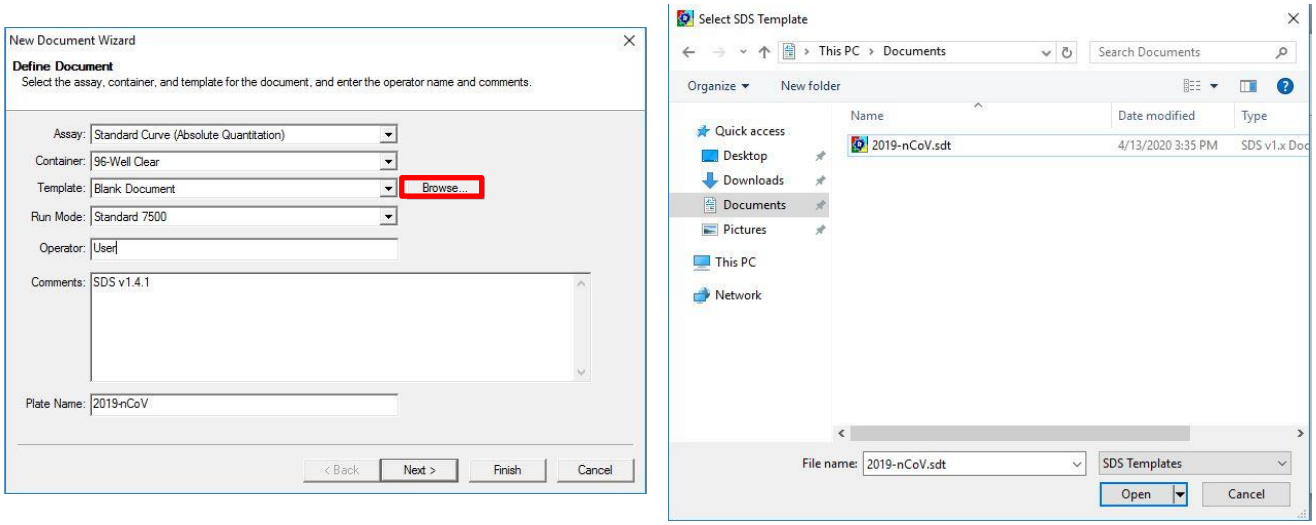
NOTE: Plate Read at Step 4. Fluorescence is detected at 58°C.



9. Click on **File → Save As** to save the new template file in **.sdt** format. Enter **File name**, select a location for the template, then click on **Save**. The saved template can be used for future testing.



10. To open the saved run protocol file, click on **Browse** in the **Template** field to open the template file.



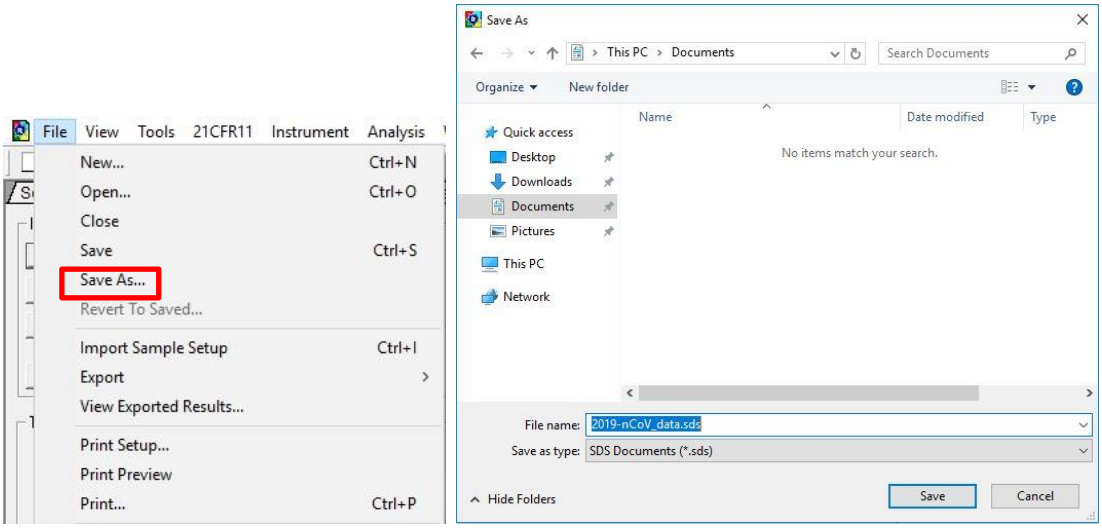
Start the Run

1. Turn on the laptop and Applied Biosystems™ 7500 Fast Dx real-time PCR system. Ensure that the laptop is connected to the instrument.

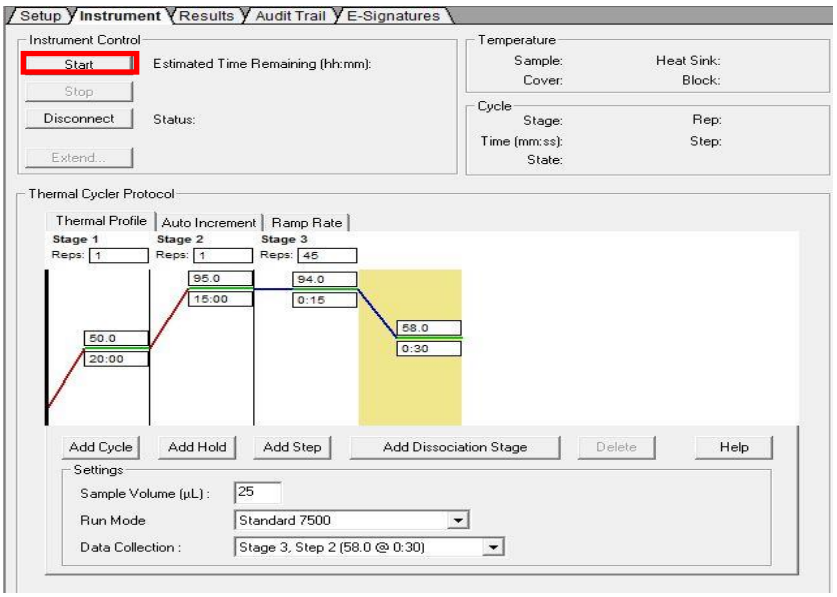
2. Push the tray door to open the instrument. Load the PCR plate onto the plate holder of the instrument.

3. Push the tray door to close the instrument.

4. Click on **File → Save as** to save the experiment in **.sds** format.



5. Click on **Start**.



Data export and analysis

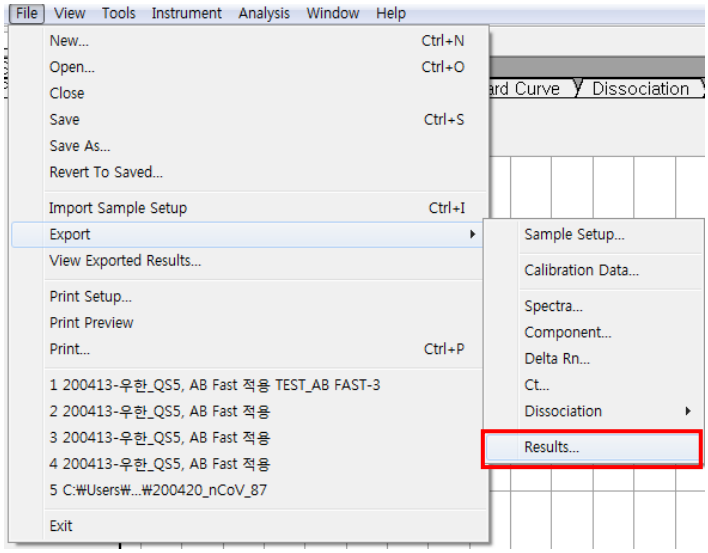
Create folders for data export

1. Create a folder to save data for all of amplification curve detection steps from the result file.

2. Enter folder name as necessary.

Data export

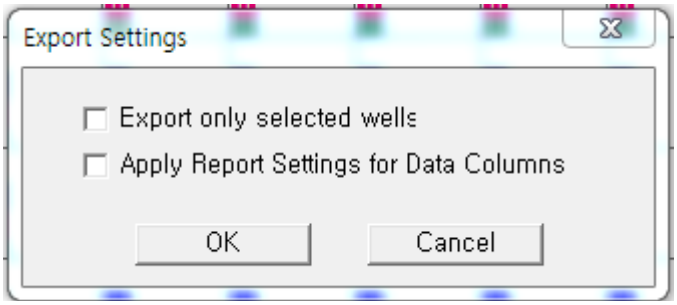
1. Click on **File** → **Export** → **Results** and select data file to export.



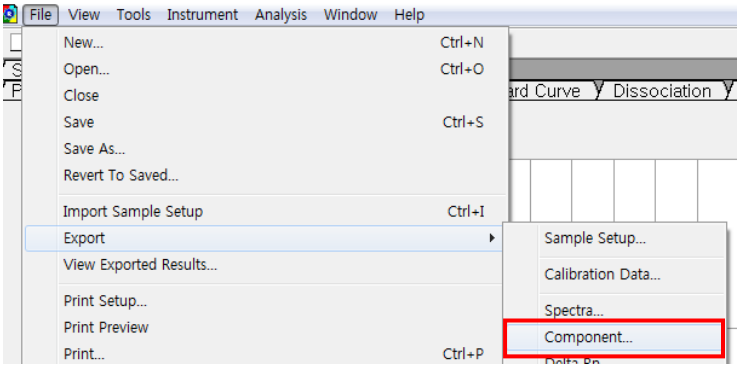
2. Select location to save exported files and enter '_Result' at the end of the file name. Then click on **Save**.

e.g. 200420-Allplex 2019-nCoV Assay_AB FAS_Result

3. In Export Settings, click on **OK** without checking the boxes.



4. After exporting the Ct file, export data containing graphs by clicking on **File → Export → Component.**



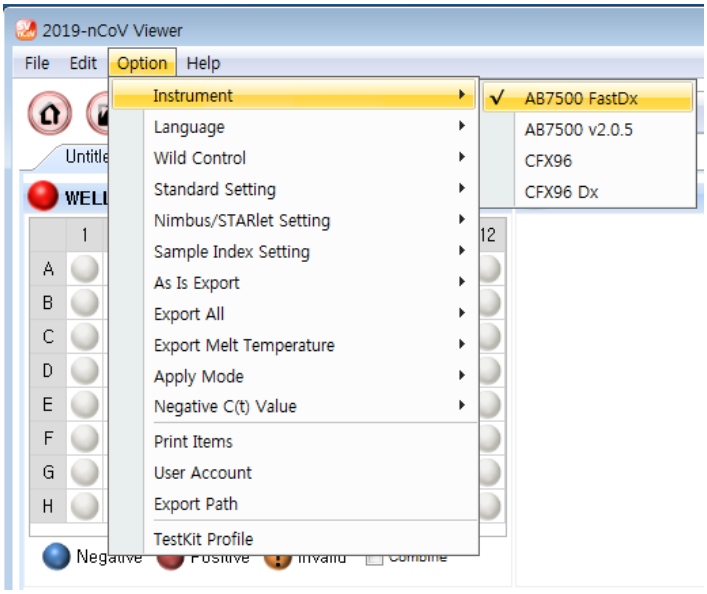
5. Select location to save exported files, and enter '_Result-g' at the end of the file name. Then click on **Save.**

e.g. 200420-Allplex 2019-nCoV Assay_AB FAS_Result-g

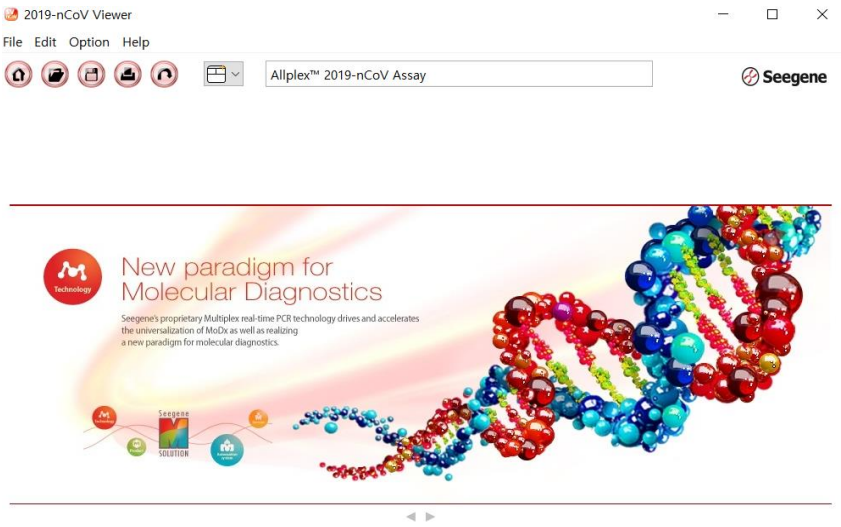
Data analysis

1. Open the Seegene Viewer software installed on the laptop connected to the Applied Biosystems™ 7500 Fast Dx

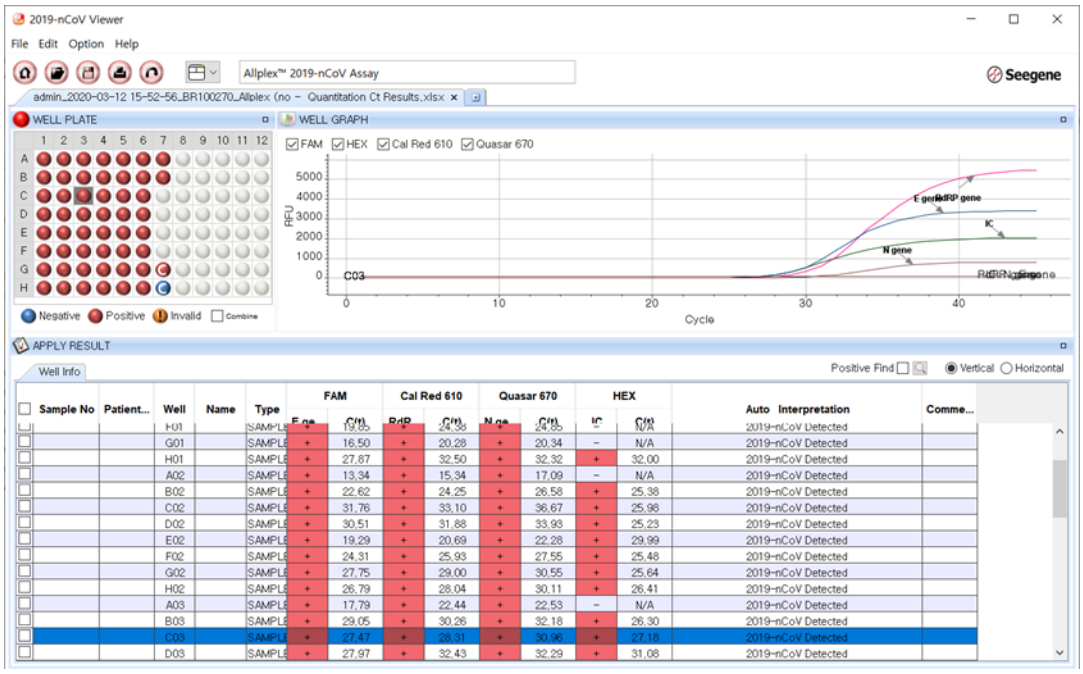
2. Click on **Option** to select AB7500 FastDx from the **Instrument** menu.



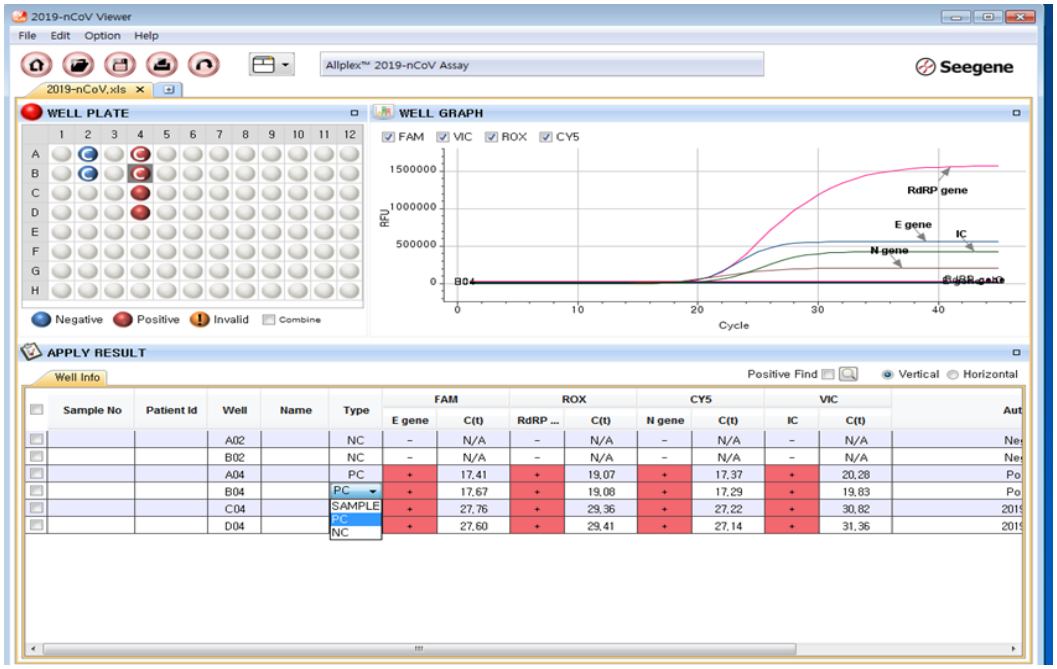
3. Click on the **Open** icon and locate the Applied Biosystems™ 7500 Fast Dx export data where the Applied Biosystems™ 7500 data was saved. The name of the file should end in '_Result'



4. After opening the results file, select 'Allplex™ 2019-nCoV Assay' from the PRODUCT menu.



5. Assign Positive and Negative Control accordingly by selecting PC, NC under the Type drop-down menu.



6. View test results. The auto-interpreted results for each sample can be viewed by clicking on each well.

CHAPTER 9: Interpretation of Results

All PCR controls should be examined prior to interpretation of patient results. If the controls are invalid, the patient results cannot be interpreted and reported.

One Negative Control and one Positive Control are processed with each run.

The results are analyzed by the Seegene Viewer software. Assessment of clinical specimen test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. The results are validated using the Seegene Viewer auto-interpretive software based on performance of the Positive Control and Negative Control. In cases of validity failure, the sample results should not be interpreted or reported, and the run must be repeated.

The Seegene Viewer software is installed on a separate computer that is interfaced with the Bio-Rad CFX96™ or Thermofisher Scientific Applied Biosystems™ 7500/7500 Fast Dx. The results are exported and transferred to the Seegene Viewer according to instructions under the section of 'Procedure: application and detection' provided for each instrument.

The auto-interpreted results can be exported to obtain a report in a preferred format (such as excel or pdf).

Seegene Viewer software (V 3.20) is provided by Seegene Technologies (California, US), support@seegenetech.com.

Result interpretation for clinical specimens is presented in Table 8.

Table 8. Result interpretation, clinical specimens

Ct value	Result
≤ 40	Detected (+)
> 40 or N/A	Not detected (-)

Potential Result Type	IC (HEX)	E gene (FAM)	RdRP gene (CalRed 610)	N gene (Quasar 670)	Auto-Interpretation	Interpretation/Further Actions
Case 1	+/-	+	+	+	2019-nCoV Positive	All Target Results are valid. 2019-nCoV (SARS-CoV-2) RNA is Detected.
Case 2	+/-	+	-	+	2019-nCoV Positive	All Target Results are valid. 2019-nCoV (SARS-CoV-2) RNA is Detected. Missing amplification of individual targets may be due to: 1) a sample at concentrations near or below the limit of detection of the test, 2) a mutation in the corresponding target region, or 3) other factors.
Case 3	+/-	+	+	-		
Case 4	+/-	-	+	+		
Case 5	+/-	-	-	+		
Case 6	+/-	-	+	-		
Case 7	+/-	+	-	-	Presumptive positive for 2019-nCoV	All Target Results are valid. Sarbecovirus RNA is detected but 2019-nCoV (SARS-CoV-2) specific RNA targets are not detected. Repeat testing. For samples with the same result on a repeated test, additional confirmatory testing may be conducted, if it is necessary to differentiate between 2019-nCoV (SARS-CoV-2) and SARS-CoV-1 or other Sarbecovirus currently unknown to infect humans, for epidemiological purposes or clinical management. Missing amplification of the 2019-nCoV (SARS-CoV-2) specific targets may be due to: 1) a sample at concentrations near or below the limit of detection of the test, 2) a mutation in the corresponding target region, or 3) other factors.
Case 8	+	-	-	-	Negative	All Target Results are valid. 2019-nCoV (SARS-CoV-2) RNA is Not Detected.
Case 9	-	-	-	-	Invalid	Results are invalid. Repeat test. If the result is still invalid, a new specimen should be obtained.

CHAPTER 10: Assay Limitations

- The use of this assay as an *in vitro* diagnostic under the FDA Emergency Use Authorization (EUA) is limited to laboratories that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high complexity tests.

- Use of this assay is limited to personnel who are trained in the procedure. Failure to follow these instructions may result in erroneous results.

- The performance of the Allplex™ 2019-nCoVAssay was established using nasopharyngeal swab, oropharygeal swab and sputum samples. Anterior nasal swabs and mid-turbinate nasal swabs are also considered acceptable specimen types for use with the Allplex™ 2019-nCoV Assay but performance has not been established. Testing of nasal and mid-turbinate nasal swabs (self-collected or collected by a healthcare provider) is limited to patients with symptoms of COVID-19. Please refer to FDA's FAQs on Diagnostic Testing for SARS-CoV-2 for additional information.
  https://www.fda.gov/medical-devices/emergency-situations-medical-devices/faqs-diagnostic-testing-sars-cov-2

- SARS-CoV-2 may mutate in one or more of the target regions of the Allplex™ 2019-nCoV Assay. If this occurs, then SARS-CoV-2 may not be detected.

- Based on the *in silico* analysis, SARS-CoV and other SARS-like coronaviruses in the same subgenus (Sarbecovirus) as SARS-CoV-2 may cross-react with the Allplex™ 2019-nCoV Assay. SARS-CoV is not known to be currently circulating in the human population, therefore is highly unlikely to be present in patient specimens.

- Samples must be collected, transported, and stored using appropriate procedures and conditions.

- False negative results may arise from improper specimen collection, handling, and degradation of the viral RNA during shipping/storage.

- Detection of viral RNA may not indicate the presence of infectious virus or that 2019-nCoV is the causative agent for clinical symptoms.

- Extraction and amplification of nucleic acid from clinical samples must be performed according the specified methods listed in this procedure. Other extraction approaches and processing systems have not been evaluated.

- Avoid contamination by adhering to good laboratory practices and to the procedures specified in this package insert.

- False positive results may happen from cross- contamination between patient samples, specimen mix-up and RNA contamination during product handling.

- False-negative results may arise from:
    - Improper sample collection
    - Degradation of the viral RNA during shipping/storage
    - Specimen collection after nucleic acid can no longer be found in the specimen matrix
    - The presence of RT-PCR inhibitors
    - Mutation in the SARS-CoV-2 virus
    - Failure to follow instructions for use

- Negative results do not preclude infection with SARS-CoV-2 virus and should not be the sole basis of a patient management decision.

- A positive result indicates the detection of nucleic acid from the relevant virus. Nucleic acid may persist even after the virus is no longer viable.

CHAPTER 11: Conditions of Authorization for Laboratory

The Allplex™ 2019-nCoV Assay 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/medical-devices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas. However, to assist clinical laboratories using the Allplex™ 2019-nCoV Assay, the relevant Conditions of Authorization are listed below.

1. **Authorized laboratories[1] using the Allplex™ 2019-nCoV Assay will include with result reports of the Allplex™ 2019-nCoV Assay, all authorized Fact Sheets. Under exigent circumstances, other appropriate methods for disseminating these Fact Sheets may be used, which may include mass media.**

2. **Authorized laboratories using the Allplex™ 2019-nCoV Assay will perform the Allplex™ 2019-nCoV Assay as outlined in the Allplex™ 2019-nCoV Assay Instructions for Use. Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to perform the Allplex™ 2019-nCoV Assay are not permitted.**

3. **Authorized laboratories that receive the Allplex™ 2019-nCoV Assay must notify the relevant public health authorities of their intent to run the test prior to initiating testing.**

4. **Authorized laboratories using the Allplex™ 2019-nCoV Assay will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.**

5. **Authorized laboratories will collect information on the performance of Allplex™ 2019-nCoV Assay and report to DMD/OHT7-OIR/OPEQ/CDRH (via email: CDRH-EUA-Reporting@fda.hhs.gov) and Seegene Technologies (support@seegenetech.com) 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.**

6. All laboratory personnel using The Allplex™ 219-nCoV Assay 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.

7. Seegene Inc., its authorized distributor(s) and authorized laboratories using the Allplex™ 2019-nCoV Assay 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.

[1] The letter of authorization refers to, “Laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, 42 U.S.C. §263a, that meet requirements to perform high complexity tests” as “authorized laboratories.”

CHAPTER 12: Performance Evaluation

Limit of Detection (LoD) - Analytical Sensitivity

1. A study was conducted to evaluate the LoD of the Allplex™ 2019-nCoV Assay on different real time PCR instruments using SARS-CoV-2 reference RNA material (AccuPlex SARS-COV-2 Reference Material Kit, Seracare Life Sciences, Inc., Cat no. 0505-0126). All sample replicates were prepared by spiking the reference RNA material into negative clinical sputum matrix. An initial- range-finding study was performed and included five replicates at each of four different analyte concentrations (i.e., 1.2X LoD, 1X LoD, 0.1X LoD, and 0.01X LoD based on preliminary LoD testing using an alternate RNA material). An additional 20 replicates were evaluated at a concentration level where all targets were detected in the range finding study as well as at a 3-fold lower concentration to establish the LoD. The final LoD for each target was confirmed to be the lowest concentration for which at least 19/20 replicates were detected.

2. Specimen extraction was performed using the STARMag 96 X 4 Universal Cartridge Kit and the Microlab STARlet IVD instrument. Real-time RT-PCR was performed using the CFX96™, and CFX96 Touch™ Real-time PCR Detection Systems (Bio-Rad), and the Applied Biosystems™ 7500 and 7500 Fast Dx (ThermoFisher Scientific) real-time PCR systems. The LoD of each SARS-CoV-2 target is shown in Table 9.

Table 9. LoD of each target gene

PCR instrument	Target	Positive Rate	Limit of Detection	Unit
CFX96™	E gene	20/20	4,167	Copies/mL
	RdRP gene	19/20	1,250	Copies/mL
	N gene	20/20	4,167	Copies/mL
CFX96 Touch™	E gene	20/20	4,167	Copies/mL
	RdRP gene	20/20	4,167	Copies/mL
	N gene	20/20	4,167	Copies/mL
Applied Biosystems™ 7500	E gene	20/20	4,167	Copies/mL
	RdRP gene	20/20	4,167	Copies/mL
	N gene	20/20	4,167	Copies/mL
Applied Biosystems™	E gene	20/20	4,167	Copies/mL

PCR instrument	Target	Positive Rate	Limit of Detection	Unit
7500 Fast Dx	RdRP gene	20/20	4,167	Copies/mL
	N gene	20/20	4,167	Copies/mL

3. The final LoD of the Allplex™ 2019-nCoV Assay on CFX96™, and CFX96 Touch™ Real-time PCR Detection Systems (Bio-Rad), Applied Biosystems™ 7500, and Applied Biosystems™ 7500 Fast Dx (ThermoFisher Scientific) is confirmed as in Table 10 following the result interpretation criteria in Table 8.

Table 10. LoD Summary of the Allplex™ 2019-nCoV Assay on different real time PCR instruments

PCR instrument	Limit of Detection	Unit
CFX96™	4,167	Copies/mL
CFX96 Touch™	4,167	Copies/mL
Applied Biosystems™ 7500	4,167	Copies/mL
Applied Biosystems™ 7500 Fast Dx	4,167	Copies/mL

4. A study was conducted to evaluate the LoD of the Allplex™ 2019-nCoV Assay using additional extraction methods. Samples were prepared by spiking the same Accuplex SARS-CoV-2 reference material (catalog no. 0505-0126) into pooled lower (sputum) respiratory negative sample matrix. As the RNA target concentration of the Seracare material is 5,000 copies/mL, an initial- range-finding study was performed and included five replicates at each of four different analyte concentrations (i.e., 1.2X LoD, 1X LoD, 0.1X LoD, and 0.01X LoD). The tentative LoD was determined followed by confirmatory LoD evaluation (20 replicates spiked at 1X LoD) for lower (sputum) respiratory negative sample matrix. If 20/20 replicates were detected in the confirmatory LoD testing, the next lower concentration, using 3-fold dilution, was tested until <100% detection was observed. The confirmed LoD, defined as the lowest SARS-CoV-2 target concentration with ≥95% detection, is presented in Table 11 for each extraction method.

Table 11. LoD Summary of the Allplex™ 2019-nCoV Assay using different extraction methods

Manufacturer	Instrument	Extraction Kit	Limit of Detection (Copies/mL)

Manufacturer	Instrument	Extraction Kit	Limit of Detection (Copies/mL)
Seegene	Seegene STARlet (65415-03)	STARMag 96 X 4 Universal Cartridge Kit	4,167
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	4,167
Hamilton	Microlab STARlet IVD (173000-075)	STARMag 96 X 4 Universal Cartridge Kit	4,167
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	4,167
Seegene	Seegene NIMBUS (67930-03)	STARMag 96 X 4 Universal Cartridge Kit	4,167
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	4,167
Hamilton	Microlab NIMBUS IVD (65415-02)	STARMag 96 X 4 Universal Cartridge Kit	4,167
		STARMag 96 X 4 Viral DNA/RNA 200 C Kit	4,167
LG Chem	AdvanSure E3 System (YETS0001EG)	AdvanSure NA EX Kit	4,167
GeneAll	N/A (Manual)	Ribospin vRD (Viral RNA/DNA Extraction Kit)	4,167
QIAGEN	N/A (Manual)	QIAmp DSP Viral RNA Mini Kit	4,167
Roche	MagNA Pure 96 (MP96)	DNA and Viral NA Small Volume Kit	4,167
ThermoFisher Scientific	KingFisher Flex automated extraction	MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (200uL of sample is used)	4,167

Inclusivity (Analytical Sensitivity)

In silico analysis for all sequences of SARS-CoV-2, available from NCBI and GISAID databases, was conducted by mapping the primers and probes of the Allplex™ 2019-nCoV Assay. If the *in silico* analysis for a target sequence revealed < 100% homology between the SARS-CoV-2 sequences and assay primers/probes and the mismatches have the potential to affect assay sensitivity, the target sequence containing the mismatch was evaluated in a wet test. As of May 13, 2020, *in silico* analysis through GISAID (n = 16667) and NCBI (n = 3490) sequences, generated data as shown in Table 12 below. Of these, 9 cases with homology of '< 100%' in the primer / probe region were identified (Table 13).

Table 12: *In silico* analysis for detection of SARS-CoV-2 sequences, percent homology (as of May 13, 2020)

Data Base	Target Gene	Percent Homology (Total)		
		F' Primer	Probe	R' Primer
GISAID (n=16667)	RdRP	99.73 %	99.95 %	99.99 %
	E	99.97 %	99.86 % Case 1)	99.97 % Case 2)
	N	80.87 % Case 3)	99.98 %	99.81 %
NCBI (n=3490)	RdRP	99.85 %	99.91 %	100.00 %
	E	100.00 %	99.91 % Case 1)	100.00 %
	N	92.28 % Case 3)	100.00 %	99.88 %
Data Base	Target Gene	Percent Homology (Exclude*)		
		F' Primer	Probe	R' Primer
GISAID (n=16667)	RdRP	100.00 %	99.99 %**	100.00 %
	E	100.00 %	100.00 %	100.00 %
	N	82.01 % Case 4)	100.00 %	99.99 % Case 5)
		Case 6~10) F primer 3mer + R primer 1mer combination case Case 11) F primer 3mer + Probe 1mer combination case		
NCBI (n=3490)	RdRP	100.00 %	99.97 %**	100.00 %
	E	100.00 %	100.00 %	100.00 %
	N	93.31 % Case 4)	100.00 %	99.97 % Case 5)

*** Exclude table section excluded 1mer mismatch cases with the assumption that such cases are predicted to have less impact on sensitivity compared to mismatch cases with two or more mismatches.**

**** Only one case was confirmed to have a 2mer mismatch for RdRP probe. (GISAID: EPI_ISL_417919, NCBI: MT372483)**

Since quantified virus isolates of the 2019-nCoV variants (Cases 1-11) are not currently available, characterized stocks of *in vitro* transcribed RNA containing the specific variant/mismatch sequence were used

(Case 1): NCBI accession no. MT039890, Case 2): GISAID accession no. EPI_ISL_412459, Case 3): NCBI accession no. MT163714, Case 4): GISAID accession no. EPI_ISL_427043, Case 5): GISAID accession no. EPI_ISL_418898, Case 6): GISAID accession no. EPI_ISL_423172, Case 7): GISAID accession no. EPI_ISL_423553, Case 8): GISAID accession no. EPI_ISL_423270, Case 9): GISAID accession no. EPI_ISL_422657, Case 10):

GISAID accession no. EPI_ISL_422300, Case 11): GISAID accession no. EPI_ISL_425742).

In vitro transcription RNA of known titer (Unit: Copies/mL, Concentration: 3X LoD = 12,500 Copies/mL) was spiked into negative sample matrix (lower respiratory specimen; sputum) to mimic clinical specimens.

The Allplex™ 2019-nCoV Assay was tested for the 11 cases of mismatch types. Testing was performed in triplicate under the same condition, and all cases were detected (Table 13).

Table 13: Allplex™ 2019-nCoV Assay testing of 11 cases of mismatch types

No.	Type	Rep.	E gene	IC	RdRP gene	N gene
1	Case 1; E gene probe region 1mer mismatch	1	31.23	29.61	33.15	35.45
		2	31.27	29.47	32.37	35.3
		3	31.09	29.38	32.32	34.6
2	Case 2; E gene R' primer region 1mer mismatch	1	30.6	29.35	32.26	35.91
		2	31.39	29.41	32.73	34.91
		3	31.23	29.22	32.8	35.39
3	Case 3; N gene F' primer region 3mer mismatch	1	30.93	29.45	32.72	35.5
		2	31.13	29.55	32.71	35.15
		3	30.7	29.51	32.25	35.1
4	Case 4; N gene F' primer region 4mer mismatch.	1	32.06	32.73	36.21	27.67
		2	32.44	32.76	37.73	27.8
		3	31.91	32.36	37.04	27.67
5	Case 5; N gene F' primer region 2mer mismatch	1	32.13	32.24	36.39	27.74
		2	31.51	32.1	37.75	27.61
		3	31.27	32.06	34.87	28.07
6	Case 6; N gene F' primer region 3mer mismatch & N gene R' primer region 1mer	1	31.33	32.19	36.78	27.8
		2	31.86	31.87	38.15	27.71

No.	Type	Rep.	E gene	IC	RdRP gene	N gene
	match	3	31.99	32.89	36.7	27.74
7	Case 7; N gene F' primer region 3mer mismatch & N gene R' primer region 1mer mismatch	1	31.8	32.39	36.23	27.78
		2	31.82	32.18	35.52	27.7
		3	31.32	32.67	36.3	27.5
8	Case 8; N gene F' primer region 3mer mismatch & N gene R' primer region 1mer mismatch	1	32.07	32.08	36.68	27.7
		2	31.91	31.68	36.94	27.68
		3	31.79	32.49	37.21	27.61
9	Case 9; N gene F' primer region 3mer mismatch & N gene R' primer region 1mer mismatch	1	31.62	32.4	35.41	27.69
		2	31.85	32.29	35.98	27.61
		3	31.26	32.49	37.85	27.66
10	Case 10; N gene F' primer region 3mer mismatch & N gene R' primer region 1mer insertion	1	31.72	32.42	37.37	27.5
		2	31.89	31.9	35.59	27.74
		3	31.26	32.07	36.75	27.51
11	Case 11; N gene F' primer region 3mer mismatch & N gene probe region 1mer mismatch	1	31.08	32.05	N/A	27.45
		2	31.65	32.31	36.84	28.03
		3	31.79	32.64	37.94	27.71

Results from this testing demonstrated that all samples were detected at concentrations of 3X LoD; therefore, the base mismatches discovered by *in silico* analysis are not expected to affect assay performance.

Cross-reactivity (Analytical Specificity)

Evaluation of Cross-reactivity with high priority pathogens

In silico analysis was performed to evaluate the potential for cross-reactivity of the Allplex™2019-nCoV Assay targets with pathogens listed in Table 14 that may be encountered in clinical respiratory specimens. In addition, the pathogens listed in Table 16, were also wet tested.

Table 14. List of pathogens analyzed *in silico*

Other high priority pathogens from the same genetic family	High priority pathogens likely in the circulating area
Human coronavirus 229E	Adenovirus (e.g. C1 Ad. 71)
Human coronavirus OC43	Human Metapneumovirus (hMPV)
Human coronavirus HKU1	Parainfluenza virus 1-4
Human coronavirus NL63	Influenza A & B
SARS-coronavirus	Enterovirus (e.g. EV68)
MERS-coronavirus	Respiratory syncytial virus
	Rhinovirus
	Chlamydia pneumoniae
	Haemophilus influenzae
	Legionella pneumophila
	Mycobacterium tuberculosis
	Streptococcus pneumoniae
	Streptococcus pyogenes
	Bordetella pertussis
	Mycoplasma pneumoniae
	Pneumocystis jirovecii (PJP)
	Candida albicans
	Pseudomonas aeruginosa
	Staphylococcus epidermis
	Streptococcus salivarius

In silico analysis test results

Cross-reactivity of the Allplex™ 2019-nCoV Assay was evaluated by *in silico* analysis and cross-reactivity was defined as greater than 80% homology between 'oligo set' and any sequence present in the targeted microorganism as table above. Cross-reaction is likely to occur when first, the amplicon size is below 500 bp, and second, when the homology of the binding site between the oligo set (forward primer, reverse primer, and probe) and the microorganism is greater or equal to 80% (Table 15. *In silico* analysis results of targeted pathogens).

Table 15. *In silico* analysis results of targeted pathogens

Pathogen	RdRP gene	E gene	N gene	Complex
Human coronavirus 229E	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Human coronavirus OC43	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Human coronavirus HKU1	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Human coronavirus NL63	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
SARS-coronavirus	Amp. Mis. #	100% Match*	Amp. Mis. #	Amp. Mis. #
MERS-coronavirus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Adenovirus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Human Metapneumovirus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Parainfluenza virus 1	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Parainfluenza virus 2	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Parainfluenza virus 3	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Parainfluenza virus 4	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Influenza A virus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Influenza B virus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Enterovirus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Respiratory syncytial virus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Rhinovirus	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Chlamydia pneumoniae	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #

Pathogen	RdRP gene	E gene	N gene	Complex
Hemophilus influenzae	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Legionella pneumophila	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Mycobacterium tuberculosis	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Streptococcus pneumoniae	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Streptococcus pyogenes	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Bordetella pertussis	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Mycoplasma pneumoniae	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Pneumocystis gynoecia (PJP)	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Candida albicans	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Pseudomonas aeruginosa	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Staphylococcus epidermis	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #
Streptococcus salivarius	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #	Amp. Mis. #

NOTE:
(*) E gene convers 100% of SARS-coronavirus (taxonomy ID: 694009)
(#) Amp. Mis: Amplicon mismatch. Amplicon is not predicted to be formed. The combination of assay oligos with each microorganism did not achieve above 80% homology.

As a result of analysis, there were no microorganisms with potential non-specific or cross-reactive sequences except for E gene target sequences that showed a 100% match with SARS-coronavirus. E gene is a target gene for Sarbecovirus, so the results of the *in silico* analysis is expected (see Table 8 for result interpretation).

The Allplex™ 2019-nCoV Assay was further evaluated for potential cross-reactivity by wet-testing a total of 60 pathogens as well as pooled human nasal wash (Table 16). The bacterial species were tested at ≥ 1 x 10^6 CFU/mL, and viral species at ≥ 1 x 10^5 PFU/mL or 1 x 10^6 genome copies/rxn.

Testing with the Allplex™ 2019-nCoV Assay was performed in triplicate for each organism under the same conditions. None of the 60 pathogens or

the pooled human nasal wash generated detectable signals with SARS-CoV-2 targets of the Allplex™ 2019-nCoV Assay.

Table 16. List of pathogens evaluated by wet testing

No.	Usage	Pathogen	Source	Isolate No.
1	Exclusivity	human coronavirus HKU1	Korean isolate	
2	Exclusivity	human coronavirus OC43	ATCC	VR-1558
3	Exclusivity	human coronavirus NL63	Korean isolate	
4	Exclusivity	human coronavirus 229E	ATCC	VR-740
5	Exclusivity	human Severe Acute Respiratory Syndrome, SARS	Korean isolate	
6	Exclusivity	human Middle East Respiratory Syndrome Coronavirus: MERS-CoV	Korean isolate	
7	Exclusivity	influenza A virus (H1N1)	ATCC	VR-95
8	Exclusivity	Influenza A virus (H3N2)	ATCC	VR-547
9	Exclusivity	influenza B virus	ATCC	VR-523
10	Exclusivity	Human Rhinovirus 1	KBPV	VR-81
11	Exclusivity	Rhinovirus 21	KBPV	VR-40
12	Exclusivity	Human rhinovirus type 90	ATCC	VR-1291
13	Exclusivity	Human rhinovirus type 16	ATCC	VR-283
14	Exclusivity	Human rhinovirus type 42	ATCC	VR-338
15	Exclusivity	Human rhinovirus type 8	ATCC	VR-488
16	Exclusivity	Human rhinovirus type 14	ATCC	VR-284
17	Exclusivity	Human enterovirus type 68	ATCC	VR-1826
18	Exclusivity	Human enterovirus type 70	ATCC	VR-836
19	Exclusivity	Human enterovirus type 71	ATCC	VR-784
20	Exclusivity	human respiratory syncytial virus A	ATCC	VR-26
21	Exclusivity	human respiratory syncytial virus B	ATCC	VR-955
22	Exclusivity	Parainfluenza 1 virus	ATCC	VR-1380
23	Exclusivity	Human parainfluenza virus 2	ATCC	VR-92
24	Exclusivity	Human parainfluenza virus 3	ATCC	VR-93
25	Exclusivity	human parainfluenza 4 virus 4a	ATCC	VR-1378
26	Exclusivity	Human parainfluenza virus 4b	ATCC	VR-1377
27	Exclusivity	Human Metapneumovirus (MPV)	KBPV	VR-87
28	Exclusivity	Human adenovirus 1	ATCC	VR-1
29	Exclusivity	Human adenovirus 11	KBPV	VR-63
30	Exclusivity	Human adenovirus 18	ATCC	VR-1095
31	Exclusivity	Human adenovirus 23	ATCC	VR-1101
32	Exclusivity	Human adenovirus 3	ATCC	VR-3
33	Exclusivity	Human adenovirus 4	ATCC	VR-1572

No.	Usage	Pathogen	Source	Isolate No.
34	Exclusivity	Human adenovirus 8	ATCC	VR-1368
35	Exclusivity	Human adenovirus type 31	ATCC	VR-1109
36	Exclusivity	Human adenovirus type 40	ATCC	VR-931
37	Exclusivity	Human adenovirus type 5	KBPV	VR-61
38	Exclusivity	Human adenovirus type 35	ATCC	VR-718
39	Exclusivity	*Legionella pneumophila Serotype 2*	ATCC	33154
40	Exclusivity	*Legionella pneumophila subsp. fraseri Serotype 4*	ATCC	33156
41	Exclusivity	*Legionella pneumophila Serotype 7*	ATCC	33823
42	Exclusivity	*Legionella pneumophila Serotype 10*	ATCC	43283
43	Exclusivity	*Legionella pneumophila Serotype 11*	ATCC	43130
44	Exclusivity	*Legionella pneumophila Serotype 12*	ATCC	43290
45	Exclusivity	*Legionella pneumophila Serotype 13*	ATCC	43736
46	Exclusivity	*Legionella pneumophila Serotype 14*	ATCC	43703
47	Exclusivity	*Legionella pneumophila subsp. fraseri Serotype 15*	ATCC	35251
48	Exclusivity	*Mycoplasma pneumoniae*	ATCC	15293
49	Exclusivity	*Mycoplasma pneumoniae M129-B7*	ATCC	29342
50	Exclusivity	*Chlamydophila pneumoniae*	ATCC	53592
51	Exclusivity	*Bordetella pertussis*	ATCC	BAA-589
52	Exclusivity	*Pseudomonas aeruginosa (Z139; VIM-1)*	Zeptometrix	801908
53	Exclusivity	*Mycobacterium tuberculosis*	ATCC	25177
54	Exclusivity	*Haemophilus influenzae*	ATCC	51907
55	Exclusivity	*Streptococcus pneumoniae*	KCCM	40410
56	Exclusivity	*Streptococcus pyogenes*	ATCC	19615
57	Exclusivity	*Staphylococcus epidermidis*	KCCM	40416
58	Exclusivity	*Candida albicans*	KCCM	11282
59	Exclusivity	*Pneumocystis pneumonia jirovecii (PJP)*	Korean isolate	
60	Exclusivity	*Staphylococcus salivarius*	Korean isolate	
61	Exclusivity	Pooled human nasal wash	Clinical sample	

Clinical Evaluation

In the clinical evaluation study, selected left-over archived samples from symptomatic patients suspected of COVID-19 infection. Specimens were previously subjected for SARS-CoV-2 testing and then stored at a clinical laboratory in South Korea prior to including in this study. A total of 300 samples (150 upper respiratory samples, 150 lower respiratory samples); 100 positive samples (50 upper respiratory samples (NP/OP swabs in UTM), 50 sputum samples) and 200 negative samples (100 upper respiratory samples (NP/OP swabs in UTM), 100 sputum samples) were tested. The purpose of this clinical study was to assess the clinical performance of Seegene's Allplex™ 2019-nCoV Assay.

For this study, extraction was performed using the STARMag 96 X 4 Universal Cartridge Kit and the Microlab STARlet IVD instrument. Real-time RT-PCR was performed using the CFX96 Real-time PCR Detection System (Bio-Rad).

All specimens were evaluated with the Allplex™ 2019-nCoV Assay and a validated real-time PCR comparator assay. The comparator assay primers and probes were identical to the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel but used alternate extraction and PCR instrumentation. The LoD of the comparator assay was shown to be equivalent to the CDC assay and therefore adequate for evaluation of clinical performance for the Allplex™ 2019-nCoV Assay.

The results from testing upper respiratory specimens including nasopharyngeal + oropharyngeal swabs shown in Table 17 generated a Positive Percent Agreement (PPA): 100.00% (49/49) [95% CI: 92.75% ~ 100.00%], and a Negative Percent Agreement (NPA): 93.07% (94/101) [95% CI: 85.76% ~ 96.93%].

The results from testing lower respiratory specimens (sputum) shown in Table 18, generated Positive Percent Agreement (PPA): 100.00% (49/49) [95% CI: 92.75% ~ 100.00%], and a Negative Percent Agreement (NPA): 96.84% (92/95) [95% CI: 90.39% ~ 99.18%]

Table 17. Upper respiratory samples (nasopharyngeal + oropharyngeal swab) n=150

Final results		Comparator assay			
		2019-nCoV Detected	Inconclusive	2019-nCoV Not Detected	Total
Allplex™ 2019-nCoV Assay	SARS-CoV-2 Positive	49	1[1)]	6[2)]	56
	Presumptive Positive for SARS-CoV-2	0	0	0	0
	Negative	0	0	94	94
	Total	49	1	100	150

NOTE: (1) Sequencing result was SARS-CoV-2 positive (Comparator assay: N1 positive / N2 negative)
(2) Sequencing results were SARS-CoV-2 positive for 5 cases, and SARS-CoV-2 negative for 1 remaining case.

A. Positive Percent Agreement (PPA): 100.00% (49/49)
[95% CI: 92.75% ~ 100.00%]
B. Negative Percent Agreement (NPA): 93.07% (94/101)
[95% CI: 85.76% ~ 96.93%]

Table 18. Lower Respiratory samples (Sputum) n=150

Final results		Comparator assay			
		2019-nCoV Detected	Inconclusive	2019-nCoV Not Detected	Total
Allplex™ 2019-nCoV Assay	SARS-CoV-2 Positive	49	1[1)]	2[2)]	52
	Presumptive Positive for SARS-CoV-2	0	0	0	0
	Negative	0	0	92	92
	Total	49	1	94	144

NOTE: (1) Sequencing result was SARS-CoV-2 positive. (Comparator assay: N1 negative / N2 positive)
(2) Sequencing results were all SARS-CoV-2 positive for 2 cases.

A. Positive Percent Agreement (PPA): 100.00% (49/49)
[95% CI: 92.75% ~ 100.00%]
B. Negative Percent Agreement (NPA): 96.84% (92/95)
[95% CI: 90.39% ~ 99.18%]

CHAPTER 13: Key to Symbols

Symbol	Explanation
IVD	*In vitro* diagnostic medical device
LOT	Batch code
REF	Catalog number
	Use-by date
	Upper limit of temperature
PRIMER	Oligonucleotide mix for amplification and detection
ENZYME	Enzyme Mix
BUFFER	Buffer
WATER	RNase-free Water
CONTROL +	Positive Control (PC)
CONTROL IC	Internal Control (IC)
	Consult instructions for use
	Manufacturer
	Date of manufacture
	Caution
	Contains sufficient for <n> tests
Rx Only	Prescription Use only
EUA	Emergency Use Authorization

CHAPTER 14: Ordering Information

The product will be distributed by Seegene Inc., located at Taewon Bldg., 91. Ogeum-ro, Songpa-gu, Seoul, Republic of Korea, 05548, and Seegene Technologies located at 325 N Wiget Ln #140, Walnut Creek, CA 94598 U.S.A.

Seegene Inc., Taewon Bldg., 91. Ogeum-ro, Songpa-gu, Seoul, Republic of Korea, 05548

Customer Support & Technical Support: support@seegenetech.com

For more contact information visit www.seegene.com

RIDA®GENE SARS-CoV-2

REF PG6815

R-Biopharm AG, An der neuen Bergstraße 17, 64297 Darmstadt, Germany
Phone: +49 (0) 61 51 81 02-0 / Fax: +49 (0) 61 51 81 02-20

1. Intended use

For *in vitro* diagnostic use. The RIDA®GENE SARS-CoV-2 test, which will be performed on the Roche LightCycler® 480II, is a multiplex real-time RT-PCR for the direct qualitative detection of coronavirus (SARS-CoV-2) RNA from human throat and nasopharyngeal swabs of people with symptoms of a respiratory infection.
The RIDA®GENE SARS-CoV-2 test is designed to support the differential diagnosis of SARS-CoV-2 infections in patients with symptoms of respiratory infection in conjunction with other clinical and laboratory findings.
Negative results do not rule out infection with SARS-CoV-2 and should not be used as the sole basis for diagnosis.
The product is intended for use by professional users in hospital laboratories, reference laboratories, private laboratories or state laboratories.

2. Summary and explanation of the test

At the end of December in the Chinese metropolis of Wuhan, numerous cases of pneumonia of unknown cause occurred.[1] At the beginning of January, Chinese authorities identified a new type of corona virus (SARS-CoV-2) as the cause.[1] The disease caused by SARS-CoV-2 is officially named COVID-19 („Corona Virus disease 2019“) and is transmissible from person to person.[2]

Worldwide, 3,925,815 cases have been reported (as of May 10, 2020).[3] The initial cases in Germany were confirmed at the end of January 2020.[4] In Germany 169,575 cases have been reported (as of May 11, 2020).[4,5] The WHO declared an international health emergency on January 31, 2020.[1,5]

The original WHO guidelines recommended a three-target strategy.[6] When this guideline was prepared, there was insufficient sequence information for SARS-CoV-2, so that the sequence ranges published by the WHO in the individual target gene regions were too non-specific for reliable detection of SARS-CoV-2 RNA. In order to increase the specificity of the tests for SARS-CoV-2, a three-target strategy was pursued accordingly. On 17 January 2020, this three-target strategy was reduced to a two-target strategy because the third target had insufficient sensitivity.
On 17 March 2020, the WHO issued interim guidance for the diagnosis of suspected cases of coronavirus disease (COVID-19), caused by SARS-CoV-2, with regard to the requirements for molecular biological laboratory tests, which states: “In areas where the COVID-19 virus is widely spread, a simpler algorithm might be adopted in which, for example, screening by real-time RT-PCR of a single discriminatory SARS-CoV-2 target (single-target strategy) is considered sufficient.”

In developing the RIDA®GENE SARS-CoV-2 real-time RT-PCR assay, R-Biopharm AG has followed the single-target strategy right from the start. Our goal was to establish sensitive and specific detection of SARS-CoV-2 with an optimised workflow, i.e. without any further necessary confirmation. This was possible even at this point in

time because a large number of genome sequences of the novel coronavirus SARS CoV-2 were available. In the course of genome analyses, we were able to identify a region within the E gene which is highly specific to SARS-CoV-2 and which has high sensitivity for screening purposes. This region is located about 200 base pairs downstream of the non-specific region of the E gene of SARS-CoV-2 published by the WHO. In detecting this specific E gene region, we were thus able to reduce the detection of SARS-CoV-2 RNA to only one target.

3. Test principle

RIDA®GENE SARS-CoV-2 is a multiplex real-time RT-PCR for the direct qualitative detection of coronavirus (SARS-CoV-2) RNA from human throat and nasopharyngeal swabs of people with symptoms of a respiratory infection.
Detection is done in a one-step real-time RT-PCR format: reverse transcription (RT) and subsequent PCR take place in one reaction vial. In the process, the isolated RNA is transcribed into cDNA with the help of a reverse transcriptase. The specific gene fragments for SARS-CoV-2 (E gene) are then amplified using real-time PCR. The amplified target sequences are detected using hydrolysis probes that are labeled at one end with a quencher and a fluorescent reporter dye (fluorophore) at the other. The probes hybridize to the amplicon in the presence of a target sequence. During extension, the Taq-Polymerase separates the reporter from the quencher. The reporter emits a fluorescent signal that is detected by the optical unit of a real-time PCR device. The fluorescent signal increases with the quantity of formed amplicons. The RIDA®GENE SARS-CoV-2 test contains an Internal Control RNA (ICR) to be able to control sample preparation and/or any potential PCR inhibition.

4. Reagents provided

Table 1: Reagents provided (The reagents provided in the kit are sufficient for 100 determinations.)

Kit code	Reagent	Amount		Lid color
1	Reaction Mix	2x	1050 µl	yellow
2	Enzyme Mix	1x	80 µl	red
R	Internal Control RNA	2x	1700 µl	brown
N	No Template Control	1x	450 µl	white
P	Positive Control	1x	200 µl	blue

5. Storage instructions

- All reagents must be stored away from light at -20 °C and, if unopened, can be used until the expiration date printed on the label. After the expiration date, the quality guarantee is no longer valid.
- All reagents should be carefully thawed prior to use (e.g., in a refrigerator at 2 °C - 8 °C).
- Repeated freezing/thawing of up to 20 times does not have an impact on the test property (if necessary, create aliquots after the first thaw and refreeze reagents immediately).
- Cool all reagents appropriately during PCR preparation (2 °C - 8 °C).

6. Additional necessary reagents and necessary equipment

The RIDA®GENE SARS-CoV-2 multiplex real-time RT-PCR test was verified with the following extraction platform and real-time PCR device combination:

Table 2a: Necessary equipment (verified)

Extraction platforms	
Promega	Maxwell®RSC
Real-time PCR devices	
Roche	LightCycler®480II

Furthermore, the RIDA®GENE SARS-CoV-2 multiplex real-time RT-PCR test is compatible for use with the following extraction platforms and real-time PCR devices:

Table 2b: Necessary equipment (compatible)

Extraction platforms	
R-Biopharm AG	RIDA®Xtract
Roche	MagNA Pure 96[7]
Real-time PCR devices	
R-Biopharm AG	RIDA®CYCLER
Agilent Technologies	Mx3005P
Applied Biosystems	ABI 7500 Fast Dx
Bio-Rad	CFX96™ Dx
QIAGEN	Rotor-Gene Q

Note: When using Rotor-Gene Q (QIAGEN), use only 0.1 ml tubes.

Should you have to use other extraction procedures or real-time PCR instruments, please contact R-Biopharm AG to check the compatibility at mdx@r-biopharm.de.

- RIDA®GENE Color Compensation Kit IV (PG0004) when using LightCycler® 480II
- Real-time PCR consumables (plates, tubes, foil)
- Centrifuge with rotor for reaction vials or plates
- Vortexer
- Pipettes (0.5 - 20 µl, 20 - 200 µl, 100 - 1,000 µl)
- Pipette tips with filters
- Powder-free disposable gloves
- PCR water (nuclease-free)

7. Precautions for users

- This test must be carried out only by trained laboratory personnel. The guidelines for working in medical laboratories must be followed.
- Always adhere strictly to the user instructions for carrying out this test.
- Do not pipette samples or reagents using your mouth. Avoid contact with broken skin and mucous membranes.
- Wear personal protective equipment (appropriate gloves, lab coat, safety glasses) when handling reagents and samples, and wash hands after completing the test.
- Do not smoke, eat, or drink in areas where samples are handled.
- Ensure that the extraction, PCR preparation, and PCR are carried out in different rooms in order to avoid cross-contaminations.
- Clinical samples must be viewed as potentially infectious and must be disposed of appropriately, like all reagents and materials that come into contact with potentially infectious samples.
- Dispose of test kit once the expiration date has lapsed.
- Users are responsible for proper disposal of all reagents and materials after use. For disposal, please adhere to national regulations.

For further details, see the safety data sheets (SDSs) at www.r-biopharm.com.

8. Collection and storage of samples

8.1 RNA preparation from human respiratory samples

A commercially available nucleic acid extraction kit (e.g., RIDA®Xtract (R-Biopharm AG)) or nucleic acid extraction system (e.g., Maxwell®RSC (Promega)) is recommended for RNA preparation from human respiratory samples. The manufacturer's instructions must be observed.

The RIDA®GENE SARS-CoV-2 test contains an Internal Control RNA that indicates potential PCR inhibition, checks the integrity of the reagents, and confirms successful nucleic acid extraction. The Internal Control RNA can be used either only as an

inhibition control or as an extraction control for sample preparation and as an inhibition control.

If the Internal Control RNA is used only as an inhibition control, 1 µl of the Internal Control RNA must be added to the master mix for each reaction (see Table 4).

If the Internal Control RNA is used as an extraction control for sample preparation **and** as an inhibition control, 20 µl of the Internal Control RNA must be used for each sample during extraction. The Internal Control RNA should be added to the sample/lysis buffer mix and should **not** be added directly to the sample material. We recommend adding 1 µl for each reaction of the Internal Control RNA to the PCR mix of the negative control and the positive control.

For more information on the collection, storage and transport of samples, see WHO Interim guidance, 2 March 2020 https://apps.who.int/iris/handle/10665/331329.[6]

9. Test procedure

9.1 Master Mix preparation

The total number of the reactions needed for PCR (samples and control reactions) must be calculated. One positive control and one negative control must be included in each test run.

Adding an additional 10 % volume to the master mix is recommended in order to balance out the pipette loss (see Table 3, Table 4). Before using the Reaction Mix, thaw the Enzyme Mix, Positive Control, No Template Control, and Internal Control RNA, mix thoroughly and centrifuge for a short time. Always cool reagents appropriately during work steps (2 °C - 8 °C).

Table 3: Example of the calculation and preparation of the master mix for 10 reactions (ICR as extraction and inhibition control)

Kit code	Components of the master mix	Quantity per reaction	10 reactions (plus 10 %)
1	Reaction Mix	19.3 µl	212.3 µl
2	Enzyme Mix	0.7 µl	7.7 µl
	Total	**20 µl**	**220 µl**

Mix the master mix and then centrifuge for short time.

Table 4: Example of the calculation and production of the master mix for ten (10) reactions (ICR only as inhibition control)

Kit code	Components of the master mix	Quantity per reaction	10 reactions (plus 10 %)
1	Reaction Mix	19.3 µl	212.3 µl
2	Enzyme Mix	0.7 µl	7.7 µl
R	Internal Control RNA	1.0 µl	11 µl
	Total	**21.0 µl**	**231.0 µl**

Mix the master mix and then centrifuge for short time.

9.2 Preparation of the PCR Mix

Pipette 20 µl of the master mix into each reaction vial (vial/plate).

Negative control: Pipette 5 µl of the No Template Control into the pre-pipetted master mix.

Note: **If the Internal Control RNA is used as an extraction control for sample preparation and as an inhibition control, we recommend adding 1 µl of the Internal Control RNA to the RT-PCR mix of the negative control.**

Samples: Add 5 µl eluate to the pre-pipetted master mix.

Positive control: Add 5 µl of the Positive Control to the pre-pipetted master mix.

Note: **If the Internal Control RNA is used as an extraction control for sample preparation and as an inhibition control, we recommend adding 1 µl of the Internal Control RNA to the RT-PCR mix of the positive control.**

Seal the reaction vials or plates, briefly centrifuge at slow speed, and transfer into the real-time PCR device. Start PCR according to PCR instrument set-up (see Table 5, Table 6, Table 7).

9.3 PCR instrument set-up

9.3.1 Universal real-time PCR profile

Table 5: Universal real-time RT-PCR profile for LightCycler® 480II and RIDA®CYCLER

Reverse transcription	10 min, 58 °C
Initial denaturation	1 min, 95 °C
Cycles	45 cycles
PCR Denaturation Annealing/Extension	10 sec, 95 °C 15 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and extension take place in the same step.

Table 6: Universal real-time RT-PCR profile for Mx3005P, ABI7500 Fast Dx, Rotor-Gene Q, and CFX96™ Dx

Reverse transcription	10 min, 58 °C
Initial denaturation	1 min, 95 °C
Cycles	45 cycles
PCR Denaturation Annealing/Extension	15 sec, 95 °C 30 sec, 60 °C
Temperature Transition Rate / Ramp Rate	Maximum

Note: Annealing and extension take place in the same step.

Note: The universal real-time PCR profile can also be used for DNA tests if RIDA®GENE DNA and RIDA®GENE RNA real-time PCR tests are combined in one run.

9.4 Detection channel setting

Table 7: Selection of appropriate detection channels

Real-time PCR device	Detection	Detection channel	Comment
R-Biopharm AG RIDA®CYCLER	SARS-CoV-2	Green	**-**
	ICR	Yellow	
Roche LightCycler® 480II	SARS-CoV-2	465/510	**RIDA®GENE Color Compensation Kit IV (PG0004) is required.**
	ICR	533/580	
Agilent Technologies Mx3005P	SARS-CoV-2	FAM	**Set the reference dye to none.**
	ICR	HEX	
ABI 7500 Fast Dx	SARS-CoV-2	FAM	**Set the ROX passive reference dye to none.**
	ICR	VIC	
Bio-Rad CFX96™ Dx	SARS-CoV-2	FAM	**-**
	ICR	VIC	
Qiagen Rotor-Gene Q	SARS-CoV-2	Green	**The gain settings must be set to 5 (factory default) for all channels.**
	ICR	Yellow	

10. Quality control

Samples are evaluated using the analysis software of the respective real-time PCR device according to the manufacturer's instructions. Negative and positive controls must show the correct results (see Table 8).
The Positive Control comes at a concentration of 10^3 copies/µl. It is used in a total quantity of 5×10^3 copies in every PCR run.

Table 8: A valid PCR run must meet the following conditions:

Sample	Result	ICR Ct	Target gene Ct
Positive control	Positive	N/A *[1]	See Quality Assurance Certificate
Negative control	Negative	Ct > 20	Not detectable

[1] A Ct value for the ICR is not needed to obtain a positive result of the positive control.

The positive and negative controls are valid when they meet the conditions specified in the table. The Ct range for the positive control is specified on the Quality Assurance Certificate included with the product. If one of the two controls does not meet the conditions for a valid run, all the reactions need to be re-analyzed, including the controls.

If the specified values are not met, check the following before repeating the test:

- Expiration date of the reagents used
- Functionality of the devices used
- Correct test procedure

11. Sample interpretation

The results interpretation is done according to table 9.

Table 9: Sample interpretation

Detection of		
SARS-CoV-2	**ICR**	**Result**
positive	**positive/ negative**	**SARS-CoV-2 detectable**
negative	**positive**	**Target gene not detectable**
negative	**negative**	**Invalid**

SARS-CoV-2 is detectable if the sample RNA and the Internal Control RNA show an amplification signal in the detection system.

SARS-CoV-2 is also detectable if the RNA shows an amplification signal, but no amplification signal can be seen for the Internal Control RNA in the detection system. Detecting the Internal Control RNA is not necessary in this case because high amplicon concentrations can result in a weak or absent signal of the Internal Control RNA.

SARS-CoV-2 is not detectable if the RNA shows no amplification signal, but an amplification signal can be seen for the Internal Control RNA in the detection system. Inhibition of the PCR reaction can be ruled out by the detection of the Internal Control RNA.

A sample is invalid if the sample RNA and the Internal Control RNA do not show an amplification signal in the detection system. There are PCR inhibitors in the sample, or an error occurred during the extraction process. The extracted sample should be diluted 1:10 with PCR water and re-amplified, or the isolation and purification of the sample should be improved.

The detection limit of the RIDA®GENE assay, using the LightCycler® 480II, is > 50 copies/reaction. Samples with a CT value of > 35 are within the detection limit. It should be noted that the detection limit of the RT-PCR depends on the sample matrix, the Cycler and the RNA extraction and can vary accordingly. We therefore recommend that these samples be assessed as inconclusive, examine the individual curves for sigmoidity and requesting a corresponding follow-up sample taking into account the clinical symptoms. The appropriately trained personnel are responsible for assessing these samples.

12. Limitations of the method

1. This test is intended only for human respiratory samples.
2. Improper specimen sampling, transport, storage, and handling or a pathogen load below the test's analytical sensitivity can lead to false negative results.
3. The presence of PCR inhibitors can lead to non-evaluable results.
4. Mutations or polymorphisms in the primer or probe binding sites can interfere with the detection of new or unknown variants and can lead to false negative results using RIDA®GENE SARS-CoV-2.
5. As with all PCR-based *in vitro* tests, extremely low concentrations of the target sequences under the limit of detection (LoD) can be detected. The results obtained are not always reproducible.
6. A positive test result does not necessarily indicate the presence of viable organisms. A positive result indicates that the target gene (E gene) is present.
7. This assay should be performed according to GLP (Good Laboratory Practice). The user must follow the manufacturer's instructions closely when performing the test.

13. Performance characteristics

13.1 Precision

The precision of the RIDA®GENE SARS-CoV-2 real-time PCR test was determined for the following observation levels.

Intra-assay precision: Determination of 5 control samples with 20 replicates each on the LightCycler® 480II under identical conditions.

Inter-Assay Precision: Determination of 5 control samples in duplicates over 10 working days by different operators under reproducible conditions.

Inter-Lot Precision: The tests for intra- and inter-assay precision are carried out on three different lots.

The variation coefficients obtained for the respective measurements with the RIDA®GENE SARS-CoV-2 real-time PCR test on the LightCycler® 480II were below 3 %.

13.2 Analytical sensitivity

13.2.1 Device detection limit

To determine the device detection limit, 20 replicates of a control sample (50 copies / reaction) were measured with the LightCycler® 480II. All replicas were positive.
The device detection limit is therefore 50 copies / reaction.

13.2.2 Limit of detection (LoD 95 %)

The detection limit of the RIDA®GENE SARS-CoV-2 real-time PCR test was determined in an external study using the MagNaPure 96 DNA and Viral NA Small Volume Kit and the LightCycler® 480II. The LoD 95 % determined here for the RIDA®GENE SARS-CoV-2 real-time PCR test is 4.3 copies / ml.[7]
The detection limit of the overall procedure depends on the sample matrix, the Cycler and the RNA extraction.

13.3 Analytical specificity

The RIDA®GENE SARS-CoV-2 real-time PCR test is specific for human SARS-CoV-2 from nasopharyngeal swab. Sequence matching using existing nucleotide databases (National Center for Biotechnology Information, NCBI) showed specificity for SARS-CoV-2 and no significant homology to other organisms.
Various organisms were also tested. No cross-reactivities to the following species were found (see Table 10):

Table 10: Cross-reactivity testing

Human Coronavirus 229E	-	Parainfluenza 1 (strain C35)	-	Enterovirus (Type 71)	-	*Streptococcus pyogenes*	-
Human Coronavirus OC43	-	Parainfluenza 2 (strain Greer)	-	RSV (strain Long)	-	*Bordetella pertussis*	-
Human Coronavirus NL63	-	Parainfluenza 3	-	RSV (strain 9320)	-	*Mycoplasma pneumoniae*	-
SARS	-	Parainfluenza 4 (b, strain CH19503)	-	Rhinovirus (Genogruppe A)	-	*Pneumocystis jirovecii*	-
MERS	-	Influenza A H1N1 Brisbane/59/07	-	*Chlamydia pneumoniae*	-	*Candida albicans*	-
Adenovirus 7, Human (strain Gomen)	-	Influenza A H3N2 Texas/50/12	-	*Haemophilus influenzae*	-	*Pseudomonas aeruginosa*	-
Adenovirus 1, Human (strain Adenoid 71)	-	Influenza B/Washington/02/2019	-	*Legionella pneumophila*	-	*Staphylococcus epidermidis*	-
Human Metapneumovirus	-	Influenza Virus B/Colorado/6/2017	-	*Streptococcus pneumoniae*	-	*Streptococcus salivarius*	-

13.4 Analytical reactivity

The analytical reactivity of the RIDA®GENE SARS-CoV-2 multiplex real-time RT-PCR was demonstrated using the proficiency test INSTAND e.V. RV 340059 (see Table 11).

Tab. 11: Analytical reactivity

SARS-CoV-2 RV 340059	+				

13.5 Interfering substances

The presence of RT-PCR inhibitors and interfering substances can lead to false negative or invalid results. Therefore, the effects of various substances that could be present in the corresponding samples due to widespread use in respiratory infections or widespread occurrence were examined (see Table 12).

Tab. 12: Interfering substances

Active ingredient / pharmaceutical	Concentration
Ethanol	5 % [v/v]
Guanidine hydrochloride	5 % [w/v]
Azithromycin	84 mg/mL
Mucine	60 µg/mL
Xylometazoline / nasal spray ratiopharm®	10 % [v/v]
Beclometasone dipropionate	10 % [v/v]
Paracetamol	10 mg/mL
Amoxicillin	1 mg/mL
Human blood	2 % [v/v]
Dihydrocodeine	10 % [v/v]

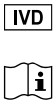
The substances listed in Table 12 showed no interfering effect at the concentrations tested.

14. Version history

Version number	Section and designation
2020-05-15	Previous version
2020-06-17	General revision: 2. Summary and explanation of the test 5. Storage instructions 11. Sample interpretation 13 Performance characteristics
2020-07-27	Revision of the spanish Instruction for Use 2. Summary and explanation of the test

15. Explanation of symbols

General symbols

IVD	For in vitro diagnostic use
	Consult instructions for use
LOT	Lot number
	Expiry
	Store at
REF	Article number
	Number of tests
	Date of manufacture

Test-specific symbols

Not applicable

16. References

1. https://www.rki.de/DE/Content/Infekt/Ausbrueche/respiratorisch/Pneumonien-China.html. Zugriff am 24.01.2020
2. https://www.spiegel.de/wissenschaft/medizin/covid-19-weltgesundheitsorganisation-verkuendet-neuen-namen-des-coronavirus-a-810ce436-7081-43d2-b8e0-f0b315503e0b. Zugriff am 12.02.2020
3. https://covid19.who.int/ Zugriff am 11.05.2020
4. https://www.rki.de/DE/Content/InfAZ/N/Neuartiges_Coronavirus/Fallzahlen.html. Zugriff am 11.05.2020
5. https://www.rki.de/DE/Content/Infekt/Ausbrueche/respiratorisch/Pneumonien-China.html. Zugriff am 03.02.2020
6. https://apps.who.int/iris/handle/10665/331329. Zugriff am 11.05.2020
7. van Kasteren, Puck B., et al. "Comparison of commercial RT-PCR diagnostic kits for COVID-19." bioRxiv (2020)

GeneXpert
Powered By CEPHEID INNOVATION

Xpert® Xpress SARS-CoV-2

Instructions for Use
For Use Under an Emergency Use Authorization (EUA) Only

℞ only

REF **XPRSARS-COV2-10**

For Use with GeneXpert Xpress System (point of care system)

Trademark, Patents and Copyright Statements

Cepheid®, the Cepheid logo, GeneXpert® and Xpert® are trademarks of Cepheid.
AccuPlex™ is a trademark of SeraCare Life Sciences.
Windows® is a trademark of Microsoft Corporation.

THE PURCHASE OF THIS PRODUCT CONVEYS TO THE BUYER THE NON-TRANSFERABLE RIGHT TO USE IT IN ACCORDANCE WITH THIS INSTRUCTIONS FOR USE. NO OTHER RIGHTS ARE CONVEYED EXPRESSLY, BY IMPLICATION OR BY ESTOPPEL. FURTHERMORE, NO RIGHTS FOR RESALE ARE CONFERRED WITH THE PURCHASE OF THIS PRODUCT.

Cepheid
904 Caribbean Drive
Sunnyvale, CA 94089 USA
Phone: +1 408 541 4191
Fax: +1 408 541 4192

Xpert® Xpress SARS-CoV-2

For use under the Emergency Use Authorization (EUA) only.

1 Proprietary Name

Xpert® Xpress SARS-CoV-2

2 Common or Usual Name

Xpert Xpress SARS-CoV-2

3 Intended Use

The Xpert Xpress SARS-CoV-2 test is a rapid, real-time RT-PCR test intended for the qualitative detection of nucleic acid from the SARS-CoV-2 in upper respiratory specimens (such as nasopharyngeal, oropharyngeal, nasal, or mid-turbinate swab and/or nasal wash/aspirate) collected from individuals suspected of COVID-19 by their healthcare provider.

Testing of nasopharyngeal, oropharyngeal, nasal, or mid-turbinate swab and nasal wash/aspirate specimens using the Xpert Xpress SARS-CoV-2 test run on the GeneXpert Dx and GeneXpert Infinity systems is limited to laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform high and moderate complexity tests.

Testing of nasopharyngeal, nasal, or mid-turbinate swab specimens using the Xpert Xpress SARS-CoV-2 test run on the GeneXpert Xpress System (Tablet and Hub Configurations) is authorized to be distributed and used in patient care settings outside of the clinical laboratory environment.

Results are for the detection of SARS-CoV-2 RNA. The SARS-CoV-2 RNA is generally detectable in upper respiratory specimens during the acute phase of infection. Positive results are indicative of active infection with SARS-CoV-2; clinical correlation with patient history and other diagnostic information is necessary to determine patient infection status. Positive results 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 SARS-CoV-2 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 Xpert Xpress SARS-CoV-2 test is intended for use by trained operators who are proficient in performing tests using either GeneXpert Dx, GeneXpert Infinity and/or GeneXpert Xpress systems. The Xpert Xpress SARS-CoV-2 test is only for use under the Food and Drug Administration's Emergency Use Authorization.

4 Summary and Explanation

An outbreak of respiratory illness of unknown etiology in Wuhan City, Hubei Province, China was initially reported to the World Health Organization (WHO) on December 31, 2019.[1] Chinese authorities identified a novel coronavirus (2019-nCoV), which has resulted in thousands of confirmed human infections in multiple provinces throughout China and exported cases in several Southeast Asian countries and more recently the United States. Cases of severe illness and some deaths have been reported. The International Committee for Taxonomy of Viruses (ICTV) renamed the virus SARS-CoV-2.[2]

The Xpert Xpress SARS-CoV-2 test is a molecular *in vitro* diagnostic test that aids in the detection and diagnosis SARS-CoV-2 and is based on widely used nucleic acid amplification technology. The Xpert Xpress SARS-CoV-2 test contains primers and probes and internal controls used in RT-PCR for the *in vitro* qualitative detection of SARS-CoV-2 RNA in upper respiratory specimens.

The term “qualified laboratories” refers to laboratories in which all users, analysts, and any person reporting results from use of this device are proficient in performing real-time RT-PCR assays.

5 Principle of the Procedure

The Xpert Xpress SARS-CoV-2 test is an automated *in vitro* diagnostic test for qualitative detection of nucleic acid from SARS-CoV-2. The Xpert Xpress SARS-CoV-2 test is performed on GeneXpert Xpress System.

The GeneXpert Xpress System automate and integrate sample preparation, nucleic acid extraction and amplification, and detection of the target sequences in simple or complex samples using real-time PCR assays. The systems consist of an instrument, computer, and preloaded software for running tests and viewing the results. The systems require the use of single-use disposable cartridges that hold the RT-PCR reagents and host the RT-PCR process. Because the cartridges are self-contained, cross-contamination between samples is minimized. For a full description of the systems, see the *GeneXpert Xpress System User's Guide*.

The Xpert Xpress SARS-CoV-2 test includes reagents for the detection of RNA from SARS-CoV-2 in nasopharyngeal, nasal, or mid-turbinate swab specimens. A Sample Processing Control (SPC) and a Probe Check Control (PCC) are also included in the cartridge utilized by the GeneXpert Xpress System instrument. The SPC is present to control for adequate processing of the sample and to monitor for the presence of potential inhibitor(s) in the RT-PCR reaction. The SPC also ensures that the RT-PCR reaction conditions (temperature and time) are appropriate for the amplification reaction and that the RT-PCR reagents are functional. The PCC verifies reagent rehydration, PCR tube filling, and confirms that all reaction components are present in the cartridge including monitoring for probe integrity and dye stability.

The nasopharyngeal, nasal, or mid-turbinate swab specimen is collected and placed into a viral transport tube containing 3 mL transport medium or 3 mL of saline. The specimen is briefly mixed by rapidly inverting the collection tube 5 times. Using the supplied transfer pipette, the sample is transferred to the sample chamber of the Xpert Xpress SARS-CoV-2 cartridge. The GeneXpert cartridge is loaded onto the GeneXpert Xpress System platform, which performs hands-off, automated sample processing, and real-time RT-PCR for detection of viral RNA.

6 Reagents and Instruments

6.1 Materials Provided

The Xpert Xpress SARS-CoV-2 kit contains sufficient reagents to process 10 specimens or quality control samples. The kit contains the following:

Xpert Xpress SARS-CoV-2 Cartridges with Integrated Reaction Tubes	**10**
• Bead 1, Bead 2, and Bead 3 (freeze-dried)	1 of each per cartridge
• Lysis Reagent	1.5 mL per cartridge
• Binding Reagent	1.5 mL per cartridge
• Elution Reagent	3.0 mL per cartridge
Disposable Transfer Pipettes	**12 per kit**
CD	**1 per kit**
• Assay Definition File (ADF)	
• Instructions to import ADF into GeneXpert software	
Flyer	**1 per kit**
• Directions to locate the Product Insert and Quick Reference Instructions on www.cepheid.com	

Note Safety Data Sheets (SDS) are available at www.cepheid.com or www.cepheidinternational.com under the **SUPPORT** tab.

Note The bovine serum albumin (BSA) in the beads within this product was produced and manufactured exclusively from bovine plasma sourced in the United States. No ruminant protein or other animal protein was fed to the animals; the animals passed ante- and post-mortem testing. During processing, there was no mixing of the material with other animal materials.

7 Storage and Handling

- Store the Xpert Xpress SARS-CoV-2 cartridges at 2-28°C.
- Do not open a cartridge lid until you are ready to perform testing.
- Do not use a cartridge that is wet or has leaked.

8 Materials Required but Not Provided

- GeneXpert Xpress System (Tablet configuration): GeneXpert Xpress II and IV instruments with proprietary GeneXpert Xpress Software Version 5.0 and 5.1, tablet computer device with touchscreen, barcode scanner, external CD drive, wireless printer, Getting Started Guide, and GeneXpert Xpress System User's Guide.
- GeneXpert Xpress System (Hub configuration): GeneXpert Xpress IV instrument, GeneXpert Hub with proprietary GeneXpert Xpress Software Version 6.1 or higher, GeneXpert Hub with integrated computer, touchscreen monitor and barcode scanner, external CD drive, Getting Started Guide, and GeneXpert Xpress System User's Guide.

9 Materials Available but Not Provided

SeraCare AccuPlex™ Reference Material Kit, catalog number 0505-0126 (Order Code CEPHEID)

10 Warnings and Precautions

10.1 General

- For *in vitro* diagnostic use.
- For emergency use only.
- Positive results are indicative of presence of SARS-CoV-2-RNA.
- Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.
- Performance characteristics of this test have been established with the specimen types listed in the Intended Use Section only. The performance of this assay with other specimen types or samples has not been evaluated.
- Treat all biological specimens, including used cartridges, as if capable of transmitting infectious agents. Because it is often impossible to know which might be infectious, all biological specimens should be handled using standard precautions. Guidelines for specimen handling are available from the U.S. Centers for Disease Control and Prevention[3] and the Clinical and Laboratory Standards Institute.[4]
- Follow safety procedures set by your institution for working with chemicals and handling biological specimens.
- Consult your institution's environmental waste personnel on proper disposal of used cartridges, which may contain amplified material. This material may exhibit characteristics of federal EPA Resource Conservation and Recovery Act (RCRA) hazardous waste requiring specific disposal requirements. Check state and local regulations as they may differ from federal disposal regulations. Institutions should check the hazardous waste disposal requirements within their respective countries.

10.2 Specimens

- Maintain proper storage conditions during specimen transport to ensure the integrity of the specimen (see Section 12, Specimen Collection, Transport, and Storage). Specimen stability under shipping conditions other than those recommended has not been evaluated.

10.3 Assay/Reagent

- Do not open the Xpert Xpress SARS-CoV-2 cartridge lid except when adding specimen.
- Do not use a cartridge that has been dropped after removing it from the packaging.
- Do not shake the cartridge. Shaking or dropping the cartridge after opening the cartridge lid may yield non-determinate results.
- Do not place the sample ID label on the cartridge lid or on the barcode label on the cartridge.
- Do not use a cartridge with a damaged barcode label.
- Do not use a cartridge that has a damaged reaction tube.
- Each single-use Xpert Xpress SARS-CoV-2 cartridge is used to process one test. Do not reuse processed cartridges.
- Each single-use disposable pipette is used to transfer one specimen. Do not reuse disposable pipettes.
- Do not use a cartridge if it appears wet or if the lid seal appears to have been broken.
- Wear clean lab coats and gloves. Change gloves between the handling of each specimen.

- In the event of a spill of specimens or controls, wear gloves and absorb the spill with paper towels. Then, thoroughly clean the contaminated area with a 10% freshly prepared household chlorine bleach. Allow a minimum of two minutes of contact time. Ensure the work area is dry before using 70% denatured ethanol to remove bleach residue. Allow surface to dry completely before proceeding. Or, follow your institution's standard procedures for a contamination or spill event. For equipment, follow the manufacturer's recommendations for decontamination of equipment.
- Biological specimens, transfer devices, and used cartridges should be considered capable of transmitting infectious agents requiring standard precautions. Follow your institution's environmental waste procedures for proper disposal of used cartridges and unused reagents. These materials may exhibit characteristics of chemical hazardous waste requiring specific disposal. If country or regional regulations do not provide clear direction on proper disposal, biological specimens and used cartridges should be disposed per WHO [World Health Organization] medical waste handling and disposal guidelines.

11 Chemical Hazards[5,6]

- Signal Word: Warning
- **UN GHS Hazard Statements**
  - Harmful if swallowed.
  - May be harmful in contact with skin
  - Causes eye irritation.
- **UN GHS Precautionary Statements**
  - **Prevention**
    - Wash hands thoroughly after handling.
  - **Response**
    - Call a POISON CENTER or doctor/physician if you feel unwell.
    - If skin irritation occurs: Get medical advice/attention.
    - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
    - If eye irritation persists: Get medical advice/attention.

12 Specimen Collection, Transport, and Storage

Proper specimen collection, storage, and transport are critical to the performance of this test. Inadequate specimen collection, improper specimen handling and/or transport may yield a false result. See Section 12.1 for swab collection procedure, Section 12.2 for nasal swab collection procedure, and Section 12.3 for mid-turbinate swab collection procedure.

Nasopharyngeal, nasal, and mid-turbinate swab specimens can be stored at room temperature (15─30 °C) for up to 8 hours and refrigerated (2─8 °C) up to seven days until testing is performed on the GeneXpert Xpress System.

Refer to the CDC Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons Under Investigation (PUIs) for Coronavirus Disease 2019 (COVID-19) https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html.

12.1 Nasopharyngeal Swab Collection Procedure

Insert the swab into either nostril, passing it into the posterior nasopharynx (see Figure 1). Rotate swab by firmly brushing against the nasopharynx several times. Remove and place the swab into the tube containing 3 mL of viral transport medium or 3 mL of saline. Break swab at the indicated break line and cap the specimen collection tube tightly.

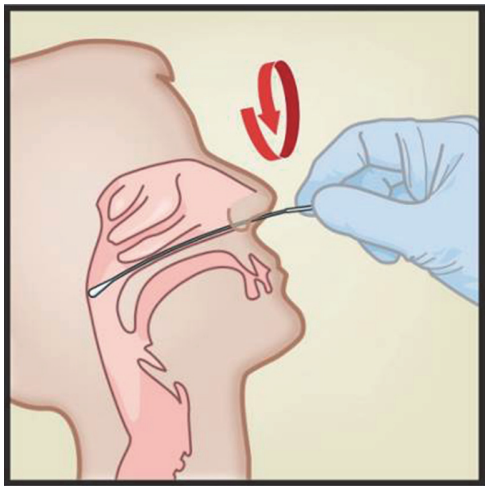


Figure 1. Nasopharyngeal Swab Collection

12.2 Nasal Swab Collection Procedure

1. Insert a nasal swab 1 to 1.5 cm into a nostril. Rotate the swab against the inside of the nostril for 3 seconds while applying pressure with a finger to the outside of the nostril (see Figure 2).

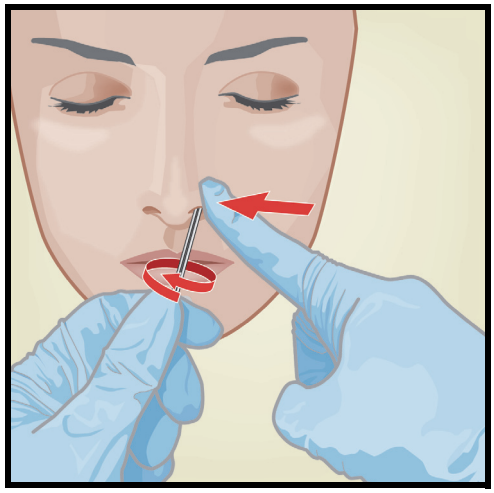


Figure 2. Nasal Swab Collection for First Nostril

2. Repeat on the other nostril with the same swab, using external pressure on the outside of the other nostril (see Figure 3). To avoid specimen contamination, do not touch the swab tip to anything other than the inside of the nostril.

Figure 3. Nasal Swab Collection for Second Nostril

3. Remove and place the swab into the tube containing 3 ml of viral transport medium or 3 mL of saline. Break swab at the indicated break line and cap the specimen collection tube tightly.

12.3 Mid-Turbinate Swab Collection Procedure

1. Insert the mid-turbinate swab into either nostril, passing it into the mid-turbinate area (see Figure 4). Rotate swab by firmly brushing against the mid-turbinate area several times.
2. Remove and place the swab into the tube containing 3 ml of viral transport medium or 3 mL of saline. Break swab at the indicated break line and cap the specimen collection tube tightly.

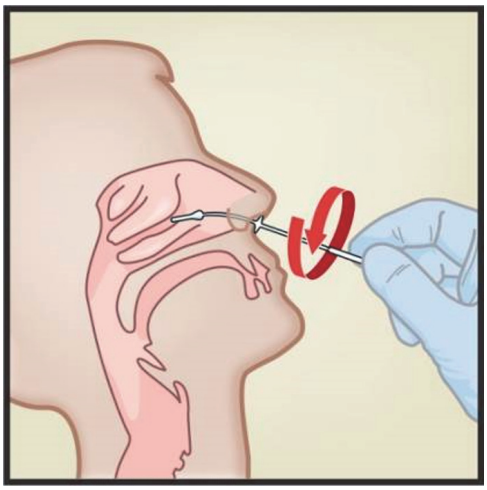


Figure 4. Mid-turbinate Swab Specimen Collection

13 Starting the System

The recommended environmental operating conditions for Xpert Xpress SARS-CoV-2 test are 15–30°C, 20–80% relative humidity.

1. Put on a clean pair of gloves.
2. Determine which system configuration you have (Figure 5).

Figure 5. Tablet and Hub System Configurations

- For the *Tablet* configuration, see Section 13.1, Starting the Tablet Configuration.
- For the *Hub* configuration, see Section 13.2, Starting the Hub Configuration.

13.1 Starting the Tablet Configuration

1. Turn on the GeneXpert Xpress instrument (GeneXpert Xpress II or GeneXpert Xpress IV).
2. Turn on the tablet computer:
   - *Windows 7*: The Windows® 7 account screen appears. Touch the **Cepheid-Admin** icon to continue.
   - *Windows 10*: The Windows Lock screen appears. Swipe up to continue.

The Windows Password screen appears.

3. Touch **Password** to display the keyboard, then type your password.
4. Touch the arrow button at the right of the password entry area.
   The GeneXpert Xpress Software starts.

13.2 Starting the Hub Configuration

1. Turn on the GeneXpert Xpress IV instrument (in two or four modules configuration).
2. Turn on the Hub computer. The Windows Lock screen appears.
3. Swipe up to continue. The Windows Password screen appears.
4. Touch **Password** to display the keyboard, then type your Windows password.
5. Touch the arrow button at the right of the password entry area. The GeneXpert Xpress Software starts and a login screen appears.
6. If enabled, you may log in by scanning a barcode on your institutional ID, using the barcode scanner (located behind the right side of the touchscreen). Then proceed to Step 9. Otherwise, follow the steps below to login manually.
7. Enter your User Name and Password (the virtual keyboard appears once you touch the entry fields).
8. Touch the **X** in the upper right of the virtual keyboard. The keyboard disappears and the **LOGIN** button appears at the bottom of the screen. Touch the **LOGIN** button to continue.
9. The Database Maintenance Reminder screen and the Archive Tests Reminder dialog boxes may appear, depending on your system configuration. For more information, see the *GeneXpert Xpress System User's Guide*.

13.3 Determining Your Software Version

When your Xpress opening screen appears, you can determine your software version and the procedure to follow, based on one of the following two screens (see Figure 6).

Software Version 5.0 or Software Version 5.1 **Software Version 6.1 or Higher**

Figure 6. Xpress Opening Screens and Software Versions

- For Software Version 5.0 or Software Version 5.1, see Section 14.
- For Software Version 6.1 or higher, see Section 16.

14 GeneXpert Xpress Software Version 5.0 or Software Version 5.1

1. On the Welcome screen, touch the **TOUCH HERE TO BEGIN** button (see Figure 7).

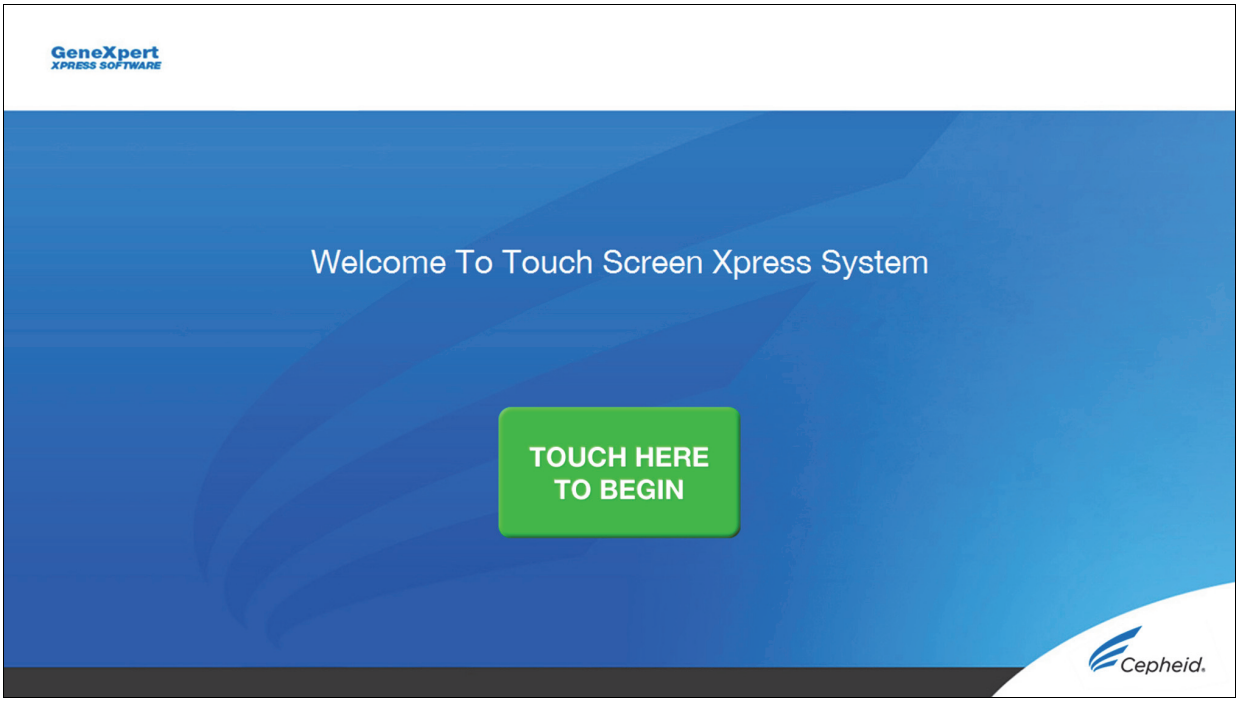


Figure 7. Welcome Screen

2. The **VIEW PREVIOUS TESTS** button appears. The **RUN NEW TEST** button will appear on the Home screen within 3 minutes.

Note If the Home screen does not display **RUN NEW TEST**, the instrument was not powered up or is no longer powered on. Exit the software using the **EXIT** button. The GeneXpert Xpress instrument must first be turned on then turn on the computer. Click on software icon to launch software and enter password.

14.1 Starting a Test

Note Instructions showing how to prepare the sample and the cartridge are shown on-screen in a video and are also described in the *Quick Reference Instructions* (QRI).

Important **Start the test within 30 minutes of adding the sample to the cartridge.**

1. Put on a new pair of gloves if performing a new test. Touch the **RUN NEW TEST** button on the Home screen (see Figure 8) to run a patient specimen or an external control.

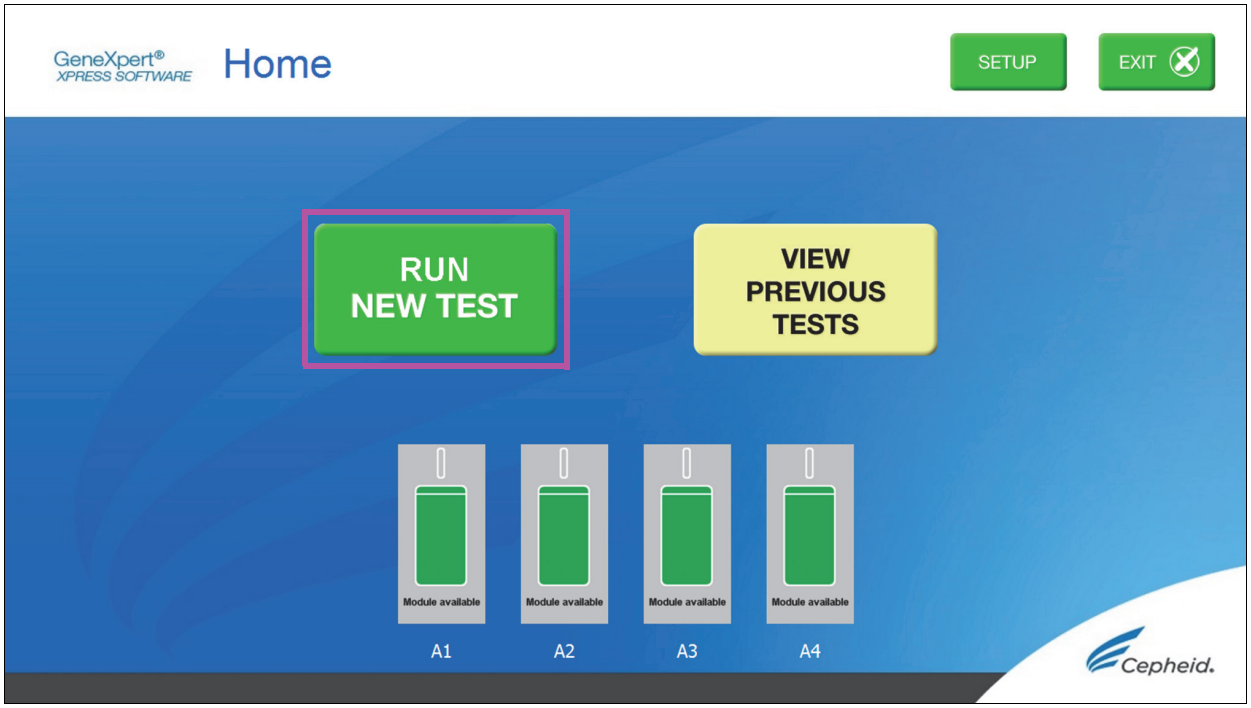


Figure 8. RUN NEW TEST button on Home Screen (GeneXpert Xpress IV screen shown)

2. Check that the specimen transport medium tube cap is closed.
3. If there is a Patient/Sample ID barcode, touch the **YES** button, then scan the Patient/Sample ID with the scanner. If there is no Patient/Sample ID barcode, touch the **NO** button, then manually enter the Patient/Sample ID and touch the **OK** button. For external control, type **Positive Control** or **Negative Control**.
4. Confirm the Patient/Sample ID. Touch **YES** if the Patient/Sample ID is correct.

14.2 Preparing the Specimen or External Control and Cartridge

It is recommended that external controls be tested at the frequency noted below.

- Each time a new lot of Xpert Xpress SARS-CoV-2 kits is received.
- Each time a new shipment of Xpert Xpress SARS-CoV-2 kits is received even if it is the same lot previously received.
- Each time a new operator is performing the test (i.e., operator who has not performed the test recently).
- When problems (storage, operator, instrument, or other) are suspected or identified.
- If otherwise required by your institution's standard Quality Control (QC) procedures.

1. Remove a cartridge and a transfer pipette from the cartridge kit box.
2. Scan the barcode on the cartridge with the scanner.

Note

If the barcode on the Xpert Xpress SARS-CoV-2 cartridge does not scan or scanning the barcode results in an error message stating the cartridge is expired, then repeat the test with a new cartridge.

If you have scanned the cartridge barcode in the Xpress software and the assay definition file is not available, a screen will appear indicating the assay definition file is not loaded on the system. If this screen appears, contact Cepheid Technical Support.

3. Confirm that the name on the cartridge is Xpert Xpress SARS-CoV-2 (shown in Figure 9 below) and touch **YES** if the displayed information is correct. Enter your user name and password if prompted.

In the following steps, keep the cartridges upright when handling or scanning. Do not rotate or tip the cartridge, because damage to the contents or injury to personnel may occur.

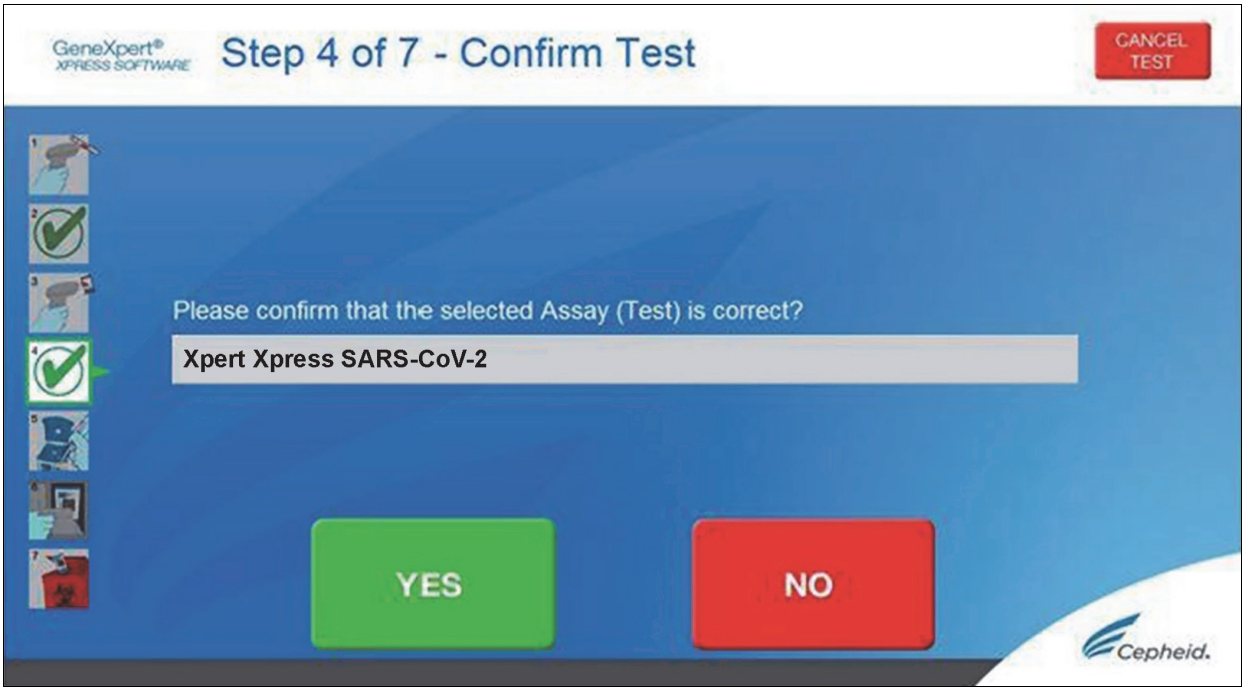


Figure 9. Confirm Test Screen

4. Watch the video before continuing. The video will repeat. Touch the **SKIP VIDEO AND CONTINUE** button to exit video. The **Load Cartridge** screen appears.
5. Mix sample by rapidly inverting the specimen transport tube or external control tube 5 times. Open cap on the specimen transport tube or external control tube.
6. Open the cartridge lid by lifting the front of the cartridge lid.
7. Remove the transfer pipette from the wrapper.

Note Do not place unwrapped pipette on the workbench.

8. Squeeze the top bulb of the transfer pipette **completely** then place the pipette tip in the specimen transport tube or the external control tube (see Figure 10).

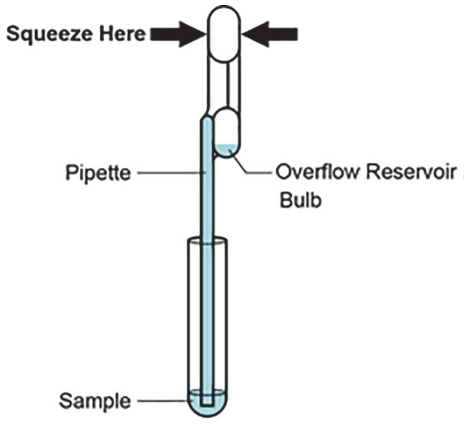


Figure 10. Transfer Pipette

9. Release the top bulb of the pipette to fill the pipette before removing from the tube. After filling pipette, excess sample can be seen in the overflow reservoir bulb of the pipette (see Figure 10). Check that the pipette does not contain bubbles.
10. To transfer the patient specimen or external control to the cartridge, squeeze the top bulb of the transfer pipette **completely** again to empty the contents of the pipette into the large opening (Sample Chamber) in the cartridge shown in Figure 11. Dispose of the used pipette.

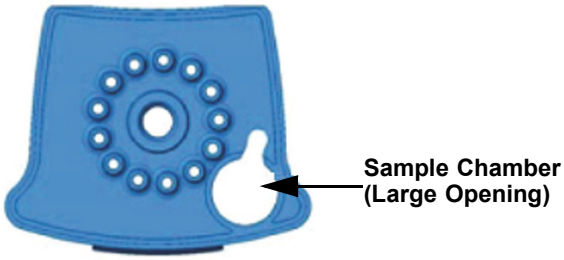


Figure 11. Xpert Xpress SARS-CoV-2 Cartridge (Top View)

Note Take care to dispense the entire volume of liquid into the Sample Chamber. False negative results may occur if insufficient sample is added to the cartridge.

11. Close the cartridge lid.

14.3 Loading the Cartridge

1. Pull open the module door with the flashing green light.
2. Load the cartridge with the barcode facing the operator onto the cartridge bay platform. Do not try to insert the cartridge past the cartridge bay platform.
3. Close the door until it clicks. The green light will stop flashing and the test starts. The **Test in Progress** screen appears. When the test is completed (green light goes out), the door will automatically unlock and the **Remove Cartridge** screen appears.
4. Follow the on-screen instructions to remove the cartridge and to reset the module for a new test.
5. Touch **CONTINUE** to view the result of the test.
6. To print results, touch the **PRINT RESULT** button.
7. Remove cartridge. Dispose of the used cartridge and gloves according to your institution's standard practices.
8. To log out, touch the **SIGN OUT** button.

Note Do not turn off or unplug the instruments while a test is in progress. Turning off or unplugging the GeneXpert Xpress instrument or computer will stop the test.

Note If the barcode on the Xpert Xpress SARS-CoV-2 cartridge does not scan or scanning the barcode results in an error message stating that the cartridge is expired, then repeat the test with a new cartridge.

Note If you have scanned the cartridge barcode in the Xpress software and the assay definition file is not available, a screen will appear indicating that the assay definition file is not loaded or that the product code was not found on the system. If this screen appears, contact Cepheid Technical Support.

14.4 Start A New Test While a Test is Running

1. Put on a clean pair of gloves if performing a new test.
2. Touch the **HOME** button to go to the Home Screen.
3. Touch the **SIGN OUT** button to log out the previous user, if applicable.
4. Start a new test following the steps in Section 14.1, Starting a Test.

15 View Status of Tests in Progress, Completed Tests, and View Results of Past Tests

15.1 Tests in Progress

1. Touch the **HOME** button to view the status of tests in progress.
2. To view a test in progress, touch the **Test in progress touch for status** button. The time remaining to complete the testing will appear on the progress bar at the bottom of the **Test in Progress** screen.

15.2 Completed Tests

1. When a test is completed, touch the **Test complete, touch to continue** button. The **Remove Cartridge** screen appears.
2. Follow the on-screen instructions to remove the cartridge. Touch the **CONTINUE** button to view the result of the test. To print results, touch the **PRINT RESULT** button.

15.3 Results of Past Tests

1. Touch the **VIEW PREVIOUS TESTS** button on the Home screen shown in Figure 12.

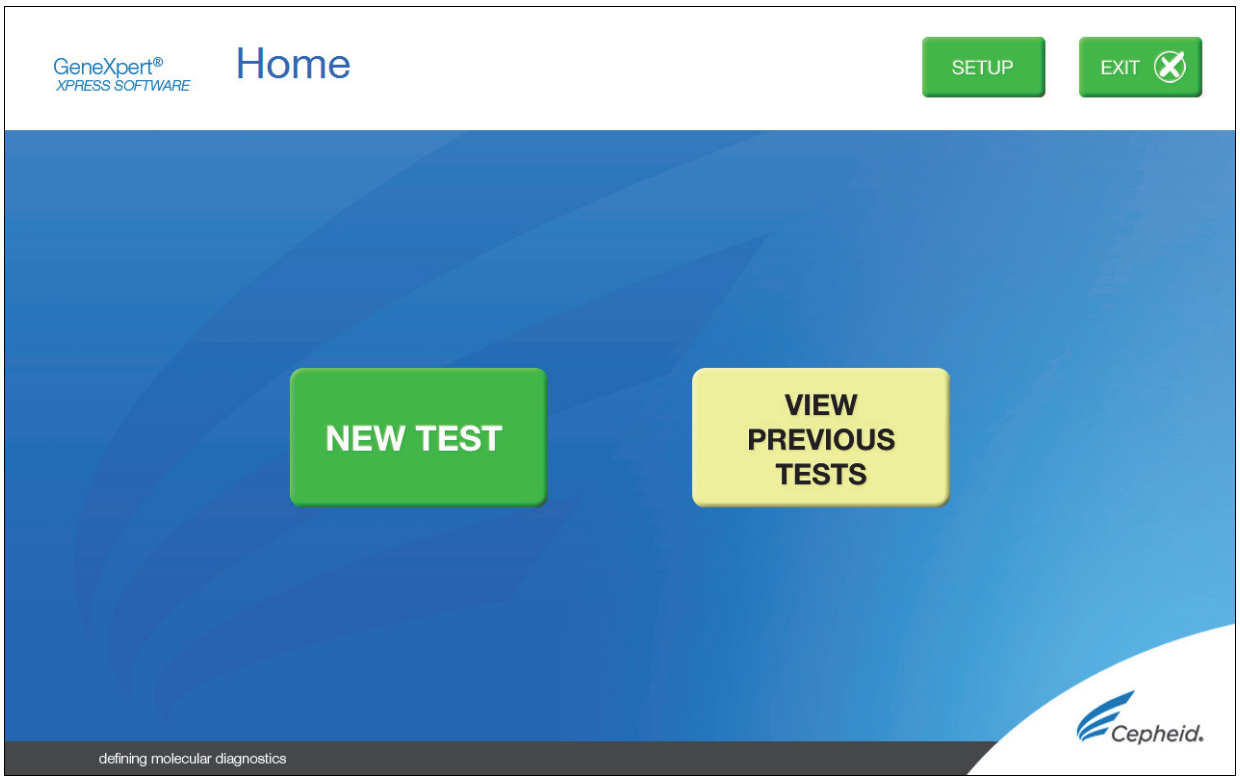


Figure 12. VIEW PREVIOUS TESTS button on Home Screen

2. Select the test by either touching the test name or using the arrows to select the test.
3. Touch the **SELECT** button shown in Figure 13 to view results.
4. To print results, touch the **PRINT RESULT** button.

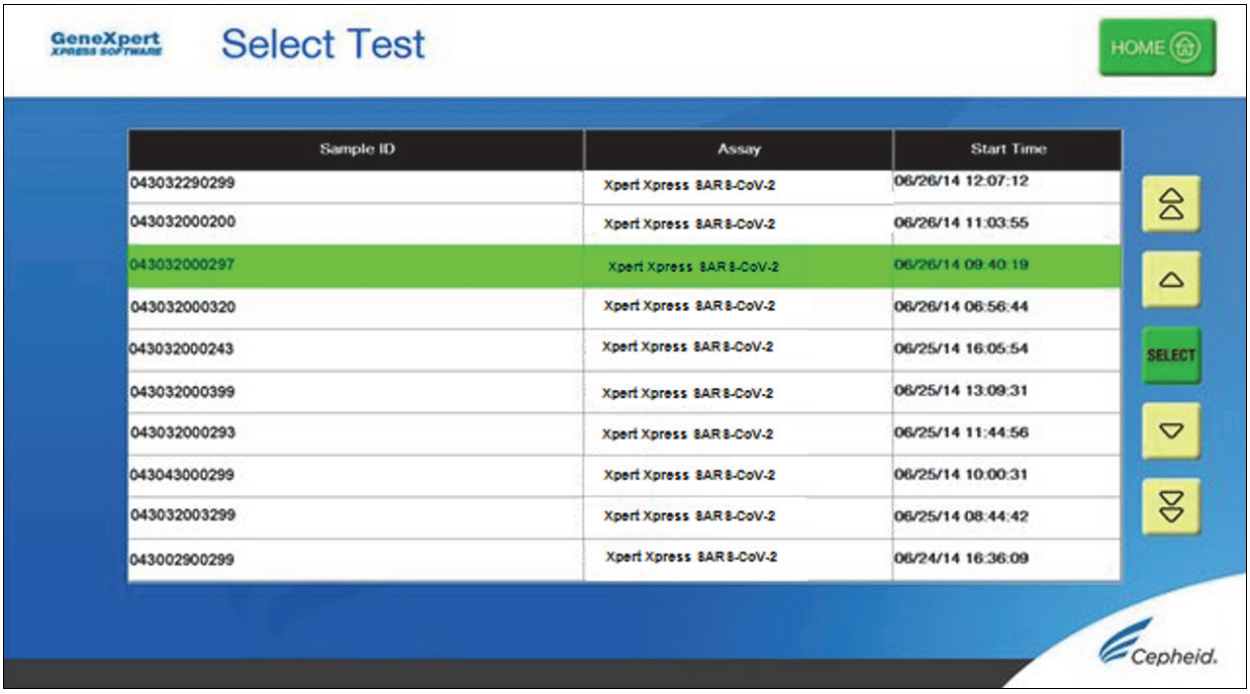


Figure 13. SELECT button

16 GeneXpert Xpress Software Version 6.1 or Higher

16.1 Starting a Test

Note Instructions showing how to prepare the sample and the cartridge are shown on-screen in videos and in the following procedure.

Important **Start the test within 30 minutes of adding the sample to the cartridge.**

1. Put on a new pair of gloves if performing a new test.
2. Touch the **NEW TEST** button on the Home screen (see Figure 14).

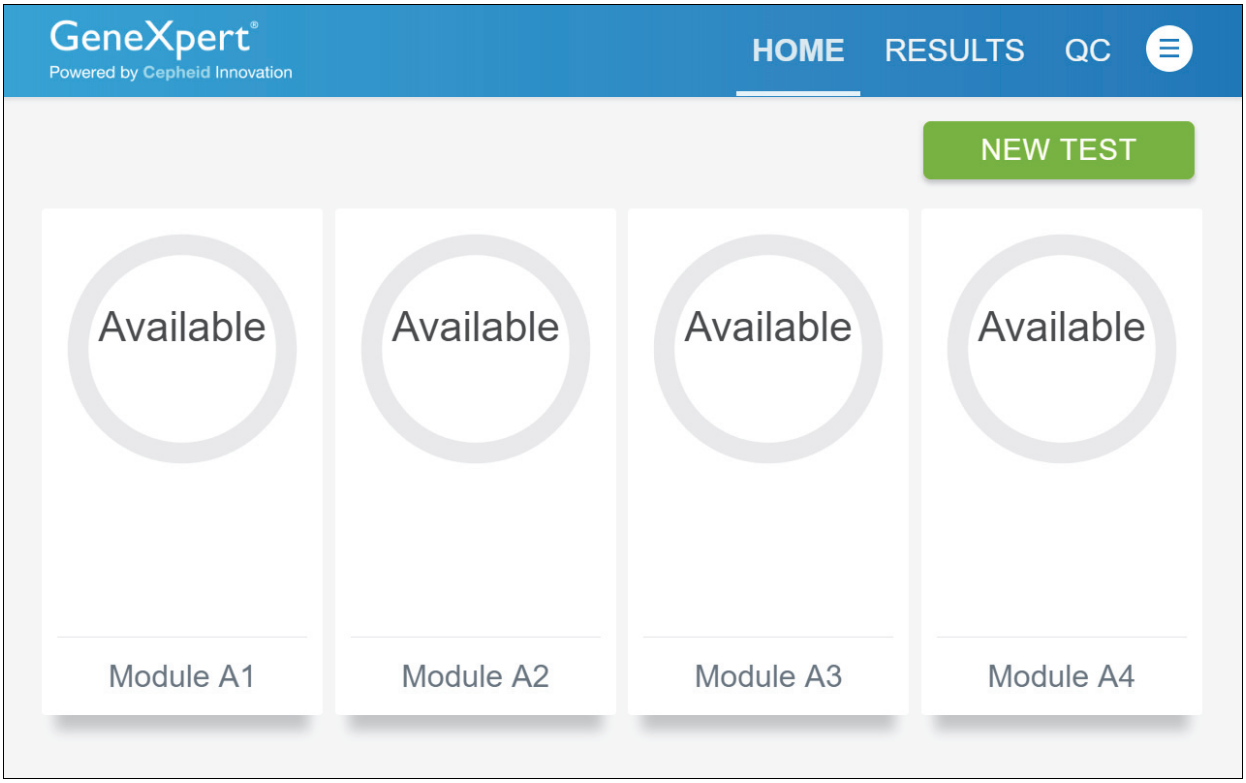


Figure 14. Home Screen

3. Check that the specimen transport medium tube cap is closed.

If Patient Information is configured by an administrator, then the Patient Information screen appears (see Figure 15). If Patient Information is not configured, the Sample ID screen appears.

4. Skip to Section 16.2 if the Sample ID screen appears.

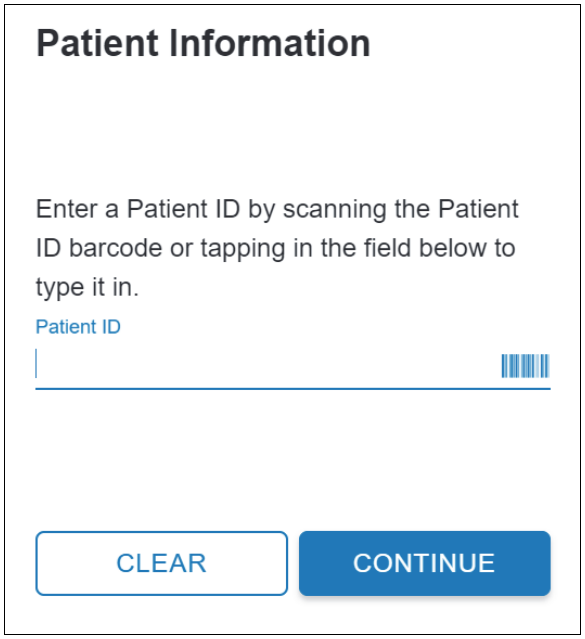


Figure 15. Patient Information Screen

5. Scan patient ID barcode or manually enter the Patient ID.
6. Touch **CONTINUE**. The Confirm Patient Information screen appears.
7. Verify the Patient ID and touch **CONFIRM**. The Sample ID screen appears.

16.2 Preparing the Specimen

1. Remove a cartridge and a transfer pipette from the cartridge kit box.
2. Check that the transport medium tube cap is closed. Scan Sample ID barcode or manually enter the Sample ID for patient specimen.
3. Touch **CONTINUE**. The Confirm Sample ID screen appears.
4. Verify the Sample ID and touch **CONFIRM**. The Scan Cartridge Barcode screen appears (see Figure 16).

In the following steps, keep the cartridges upright when handling or scanning. Do not rotate or tip the cartridge, because damage to the contents or injury to personnel may occur.

Note

If the barcode on the Xpert Xpress SARS-CoV-2 cartridge does not scan or scanning the barcode results in an error message stating that the cartridge is expired, then repeat the test with a new cartridge.

If you have scanned the cartridge barcode in the Xpress software and the assay definition file is not available, a screen will appear indicating the assay definition file is not loaded on the system. If this screen appears, contact Cepheid Technical Support.

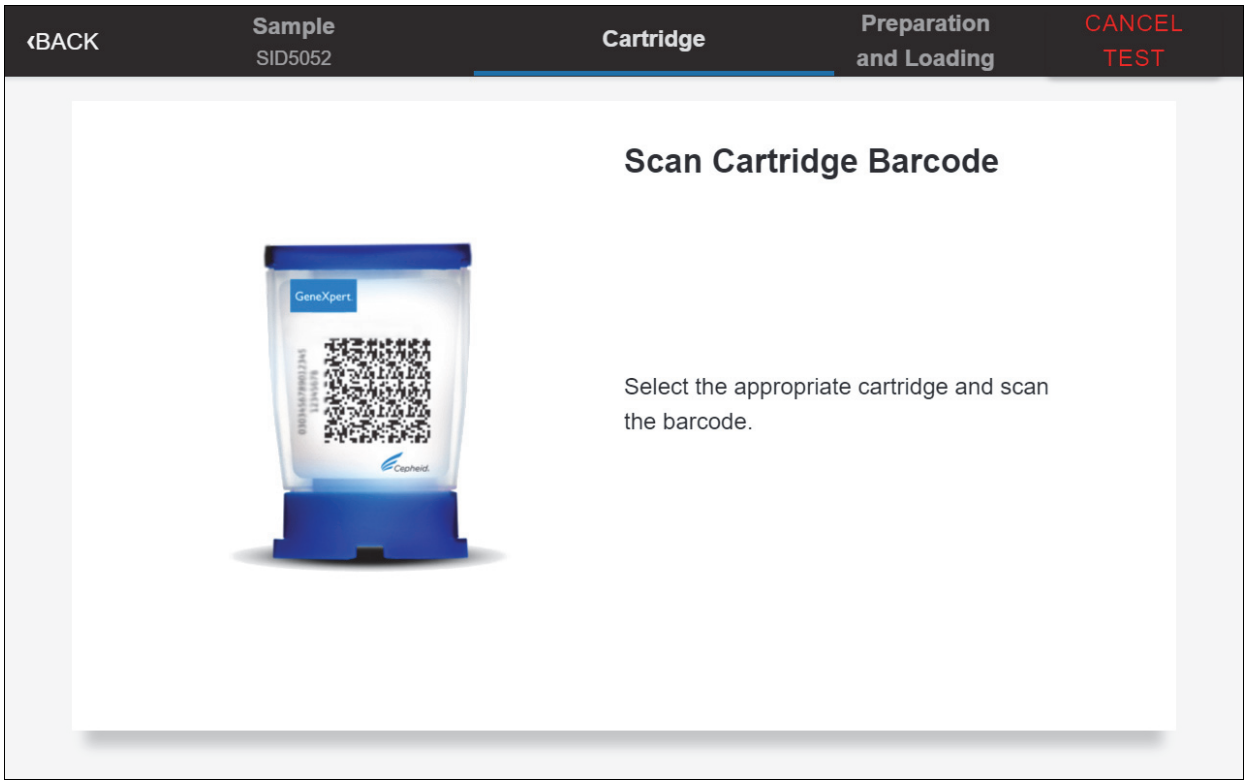


Figure 16. Scan Cartridge Barcode Screen

5. Select the appropriate cartridge with the sample and scan the cartridge barcode. After scanning, the Confirm Test Information screen appears.
6. Verify that the correct cartridge has been scanned and that the assay name matches the name of the assay on the cartridge (see Figure 17).

Figure 17. Confirm Test Information Screen

7. Touch **CONFIRM**. Depending on your configuration, the Enter Credentials to Continue screen may appear (see Figure 18). If enabled, you may log in by scanning your institutional ID. Otherwise, manually enter your User Name and Password and touch **LOGIN** to continue.

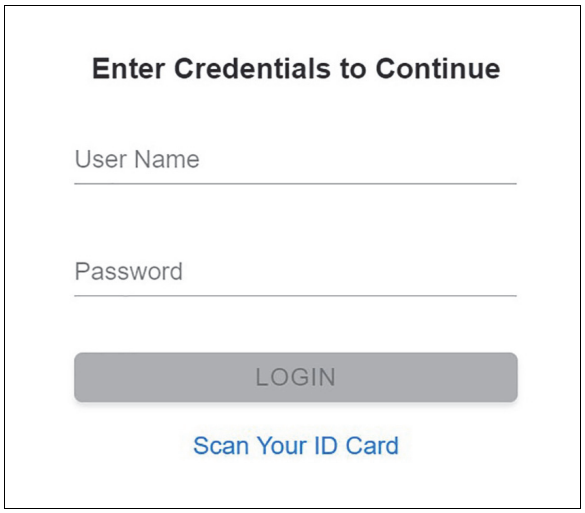


Figure 18. Enter Credentials to Continue Screen

The Cartridge Preparation screen appears (see Figure 19).

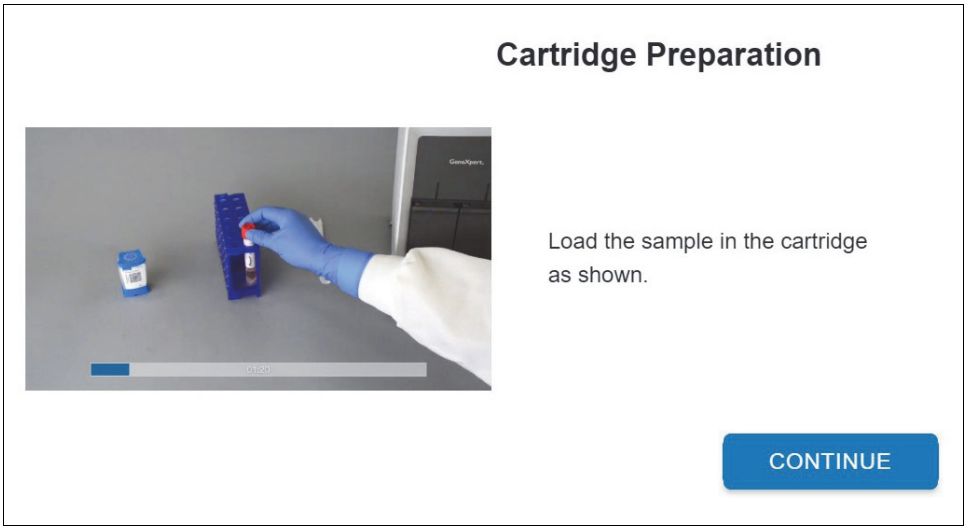


Figure 19. Cartridge Preparation Screen

8. Watch the video before continuing. The video will repeat. Touch the **SKIP VIDEO AND CONTINUE** button to exit video.
9. Mix specimen by rapidly inverting the specimen transport tube 5 times. Open the lid on the specimen transport tube.
10. Open the cartridge lid by lifting the front of the cartridge lid.
11. Remove the transfer pipette from the wrapper.

Note Do not place unwrapped pipette on the workbench.

12. Squeeze the top bulb of the transfer pipette **completely** and then place the pipette tip in the specimen transport tube (see Figure 20).

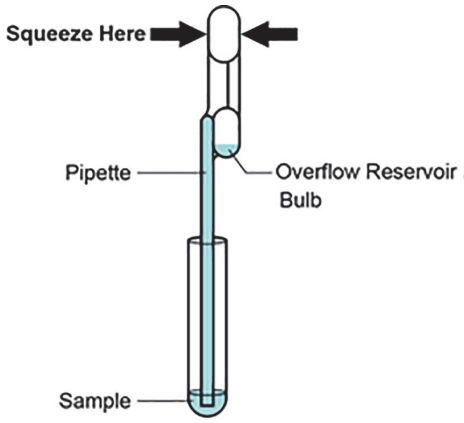


Figure 20. Transfer Pipette

13. Release the top bulb of the pipette to fill the pipette before removing from the tube. After filling pipette, excess sample can be seen in the overflow reservoir bulb of the pipette (see Figure 20). Check that the pipette does not contain bubbles.
14. To transfer the patient specimen to the cartridge, squeeze the top bulb of the transfer pipette **completely** again to empty the contents of the pipette into the large opening (Sample Chamber) of the cartridge shown in Figure 21. Dispose of the used pipette.

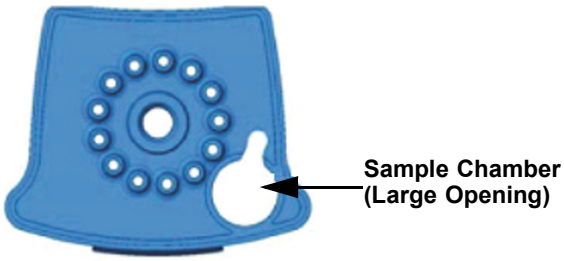


Figure 21. Xpert Xpress SARS-CoV-2 Cartridge (Top View)

Note Take care to dispense the entire volume of liquid into the Sample Chamber. False negative results may occur if insufficient sample is added to the cartridge.

15. Close the cartridge lid.
16. Go to Step 16.4, Loading the Cartridge.

16.3 Running External Controls

It is recommended that external controls be tested at the frequency noted below.

- Each time a new lot of Xpert Xpress SARS-CoV-2 kits is received.
- Each time a new shipment of Xpert Xpress SARS-CoV-2 kits is received even if it is the same lot previously received.
- Each time a new operator is performing the test (i.e., operator who has not performed the test recently).
- When problems (storage, operator, instrument, or other) are suspected or identified.
- If otherwise required by your institution's standard Quality Control (QC) procedures.

1. Put on a new pair of gloves if performing a new test. Touch the **QC** button on the Home screen (see Figure 22).

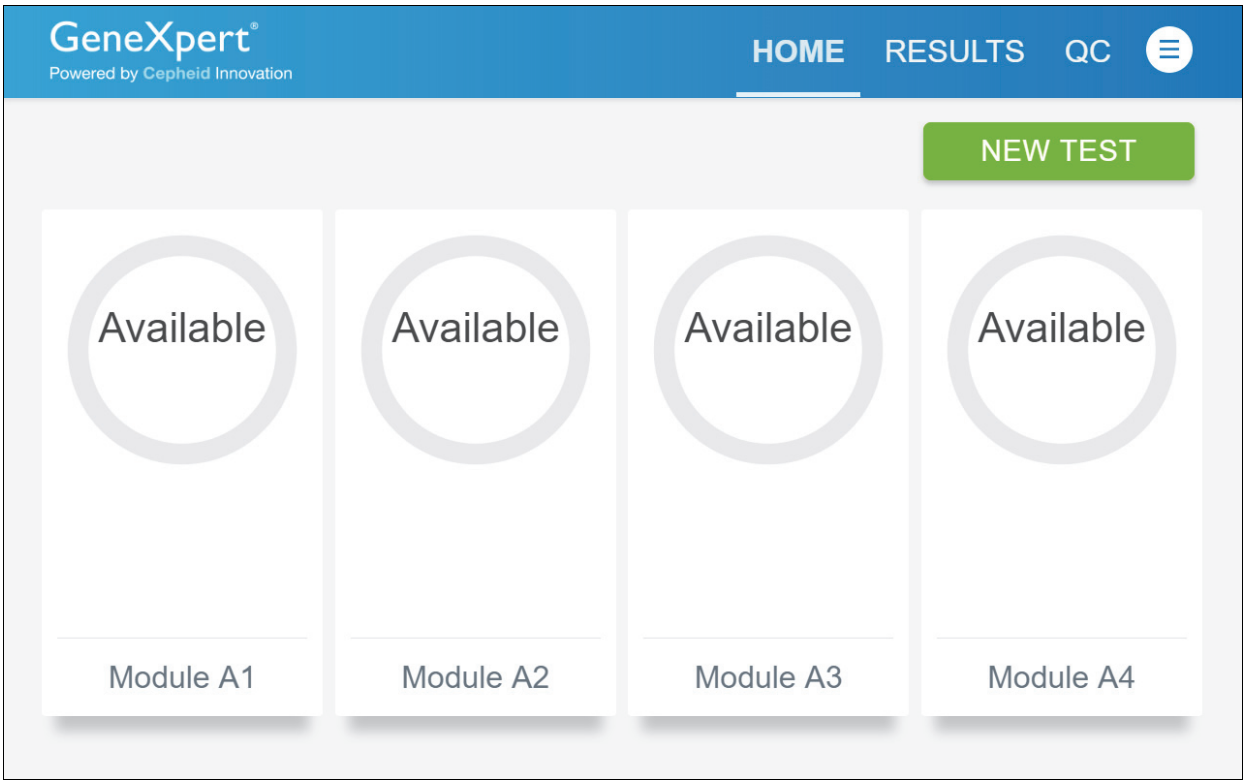


Figure 22. Home Screen

2. The Quality Control screen appears. Touch **RUN QC POSITIVE Test**, **RUN QC NEGATIVE TEST** or **RUN PROFICIENCY TEST** option (Figure 23).

Figure 23. Quality Control Screen

3. The Sample ID appears.

4. Enter the Sample ID, by typing **Positive Control** or **Negative Control** or scan the Sample ID barcode.
5. Touch **CONTINUE**. The Confirm Sample ID screen appears.
6. Verify the Sample ID and touch **CONFIRM**. The Scan Cartridge Barcode screen appears (see Figure 24).

In the following steps, keep the cartridges upright when handling or scanning. Do not rotate or tip the cartridge, because damage to the contents or injury to personnel may occur.

Note

If the barcode on the Xpert Xpress SARS-CoV-2 cartridge does not scan or scanning the barcode results in an error message stating that the cartridge is expired, then repeat the test with a new cartridge.

If you have scanned the cartridge barcode in the Xpress software and the assay definition file is not available, a screen will appear indicating the assay definition file is not loaded on the system. If this screen appears, contact Cepheid Technical Support.

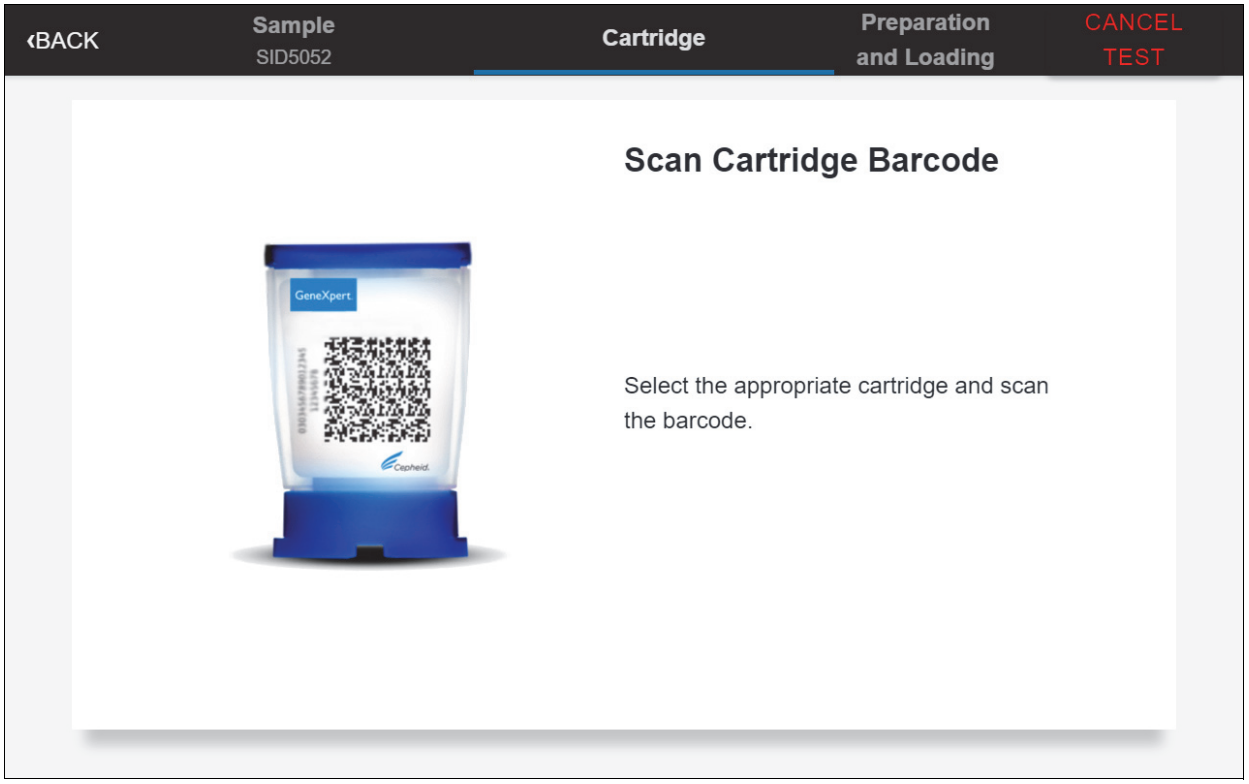


Figure 24. Scan Cartridge Barcode Screen

7. Select the appropriate cartridge with the sample and scan the cartridge barcode. After scanning, the Confirm Test Information screen appears.

8. Confirm the test information is correct then touch **CONFIRM** (see Figure 25).

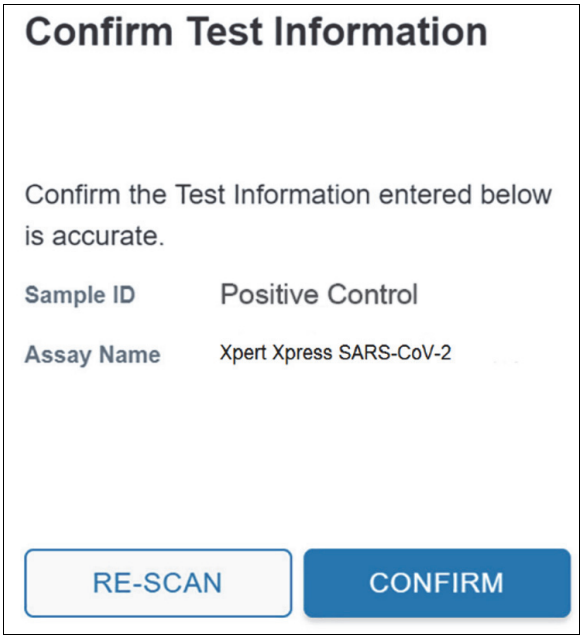


Figure 25. Confirm Test Information

9. Watch the video before continuing. The video will repeat. Touch the **CONTINUE** button to exit video.
10. Mix control by rapidly inverting the external control tube 5 times. Open the lid on the external control tube.
11. Open the cartridge lid by lifting the front of the cartridge lid.
12. Remove the transfer pipette from the wrapper.

Note Do not place unwrapped pipette on the workbench.

13. Squeeze the top bulb of the transfer pipette **completely** and then place the pipette tip in the external control tube (see Figure 26).

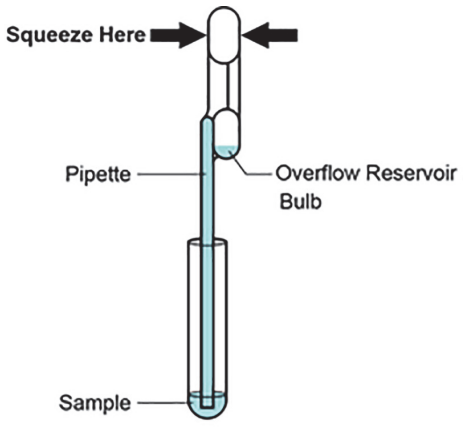


Figure 26. Transfer Pipette

14. Release the top bulb of the pipette to fill the pipette before removing from the tube. After filling pipette, excess sample can be seen in the overflow reservoir bulb of the pipette (see Figure 26). Check that the pipette does not contain bubbles.
15. To transfer the external control to the cartridge, squeeze the top bulb of the transfer pipette **completely** again to empty the contents of the pipette into the large opening (Sample Chamber) of the cartridge shown in Figure 27. Dispose of the used pipette.

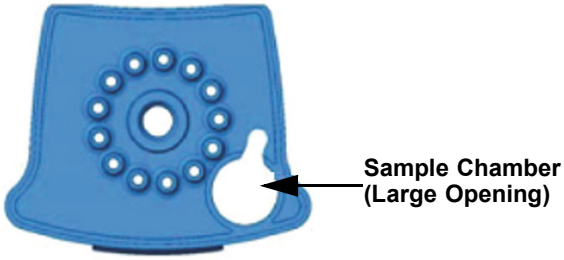


Figure 27. Xpert Xpress SARS-CoV-2 Cartridge (Top View)

Note Take care to dispense the entire volume of liquid into the Sample Chamber. False negative results may occur if insufficient sample is added to the cartridge.

16. Close the cartridge lid.
17. Go to Step 16.4, Loading the Cartridge.

16.4 Loading the Cartridge

1. Touch the **CONTINUE** button on the Cartridge Preparation screen. The Load Cartridge into Module screen appears (see Figure 28).
2. Open the module door with the flashing green light.

Figure 28. Load Cartridge into Module Screen

3. Load the cartridge with the barcode facing the operator on the cartridge bay platform. Do not try to insert the cartridge past the cartridge bay platform.
4. Close the door until it clicks. The green light will stop blinking and the test starts.
5. When the cartridge is loaded, the Test Loading screen appears, followed by the Test Running screen showing that the test is running. A circular graphic indicator at the right indicates the progress of the test and the time remaining until a test result is available.

Note While a test is running, you can start another test. See Section 16.5, Start a New Test While a Test is Running.

Note Do not turn off or unplug the instrument while a test is in progress. Turning off or unplugging the GeneXpert Xpress instrument or Hub stops the test. If necessary, touch the **STOP TEST** button to cancel a test while it is loading or running.

6. When the test is done, the green light goes out and the door automatically unlocks. The screen text changes to Test Completed. The Test Completed screen provides the results for the test just completed.

Note If an unexpected result occurs (e.g., Negative Quality Control result is positive or Positive Quality Control result is negative), test a new Quality Control sample using a new cartridge. If an unexpected result occurs upon retest, contact Cepheid Technical Support.

7. Open the module door, remove the used cartridge, and properly dispose of the cartridge according to your institution's policy.
8. Touch **HOME** to go back to the Home screen.
9. To log out, touch the **User Menu** icon, then select **Logout**.

16.5 Start a New Test While a Test is Running

You can start a new test while another test is in progress.

1. Touch the **HOME** button on the Test Running screen.
2. For a new user log in, touch the **User Menu** icon to log in.
3. Repeat the steps in Sections 16.1, Starting a Test, and 16.2, GeneXpert Xpress Software Version 6.1 or Higher and Section 16.4, Loading the Cartridge.
4. After a second test has started, touch the **HOME** button. The status of both tests appears. The Home screen displays the module(s) in use with a circular graphic indicator around each test, and Patient Identification below the module graphic (see Figure 29).

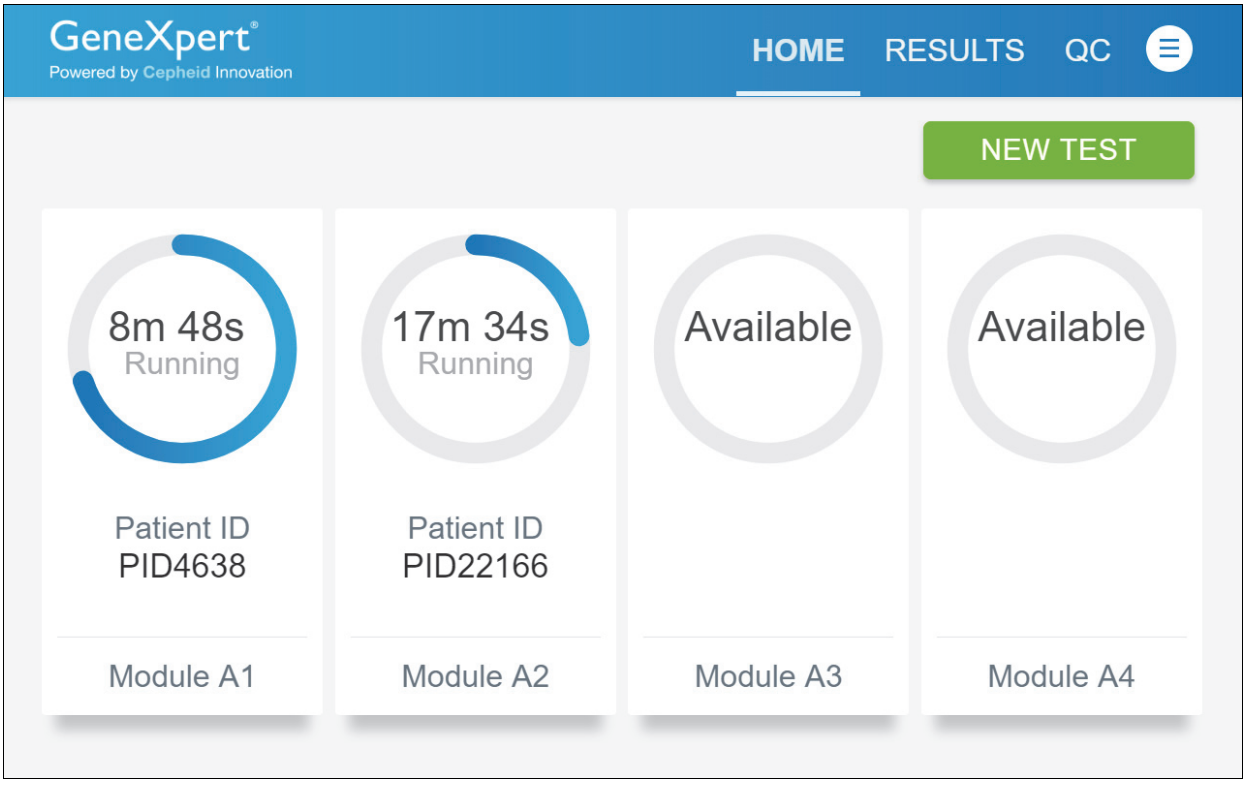


Figure 29. Home Screen showing Two Tests Running

5. After a test has completed, the module icon text changes to Complete (see Figure 30). Touch **Complete View Result** to view test results.

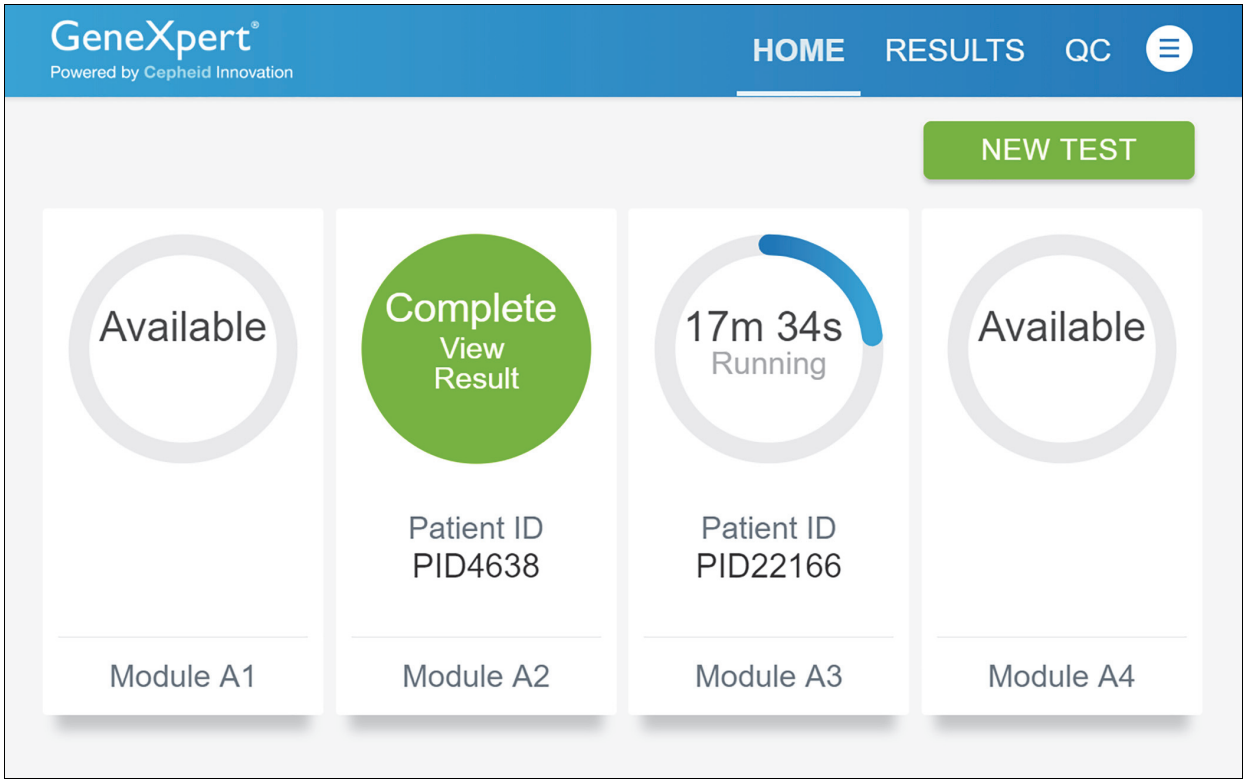


Figure 30. Home Screen with One of Two Tests Completed

16.6 Viewing Test Results

1. Touch the **RESULTS** button located on the panel at the top of the screen (see Figure 30). The Results screen appears (see Figure 31). Test results are, by default, in order of the date and time that the test was run. Navigate through the test result pages by touching the numbered buttons or arrows at the bottom of the screen.

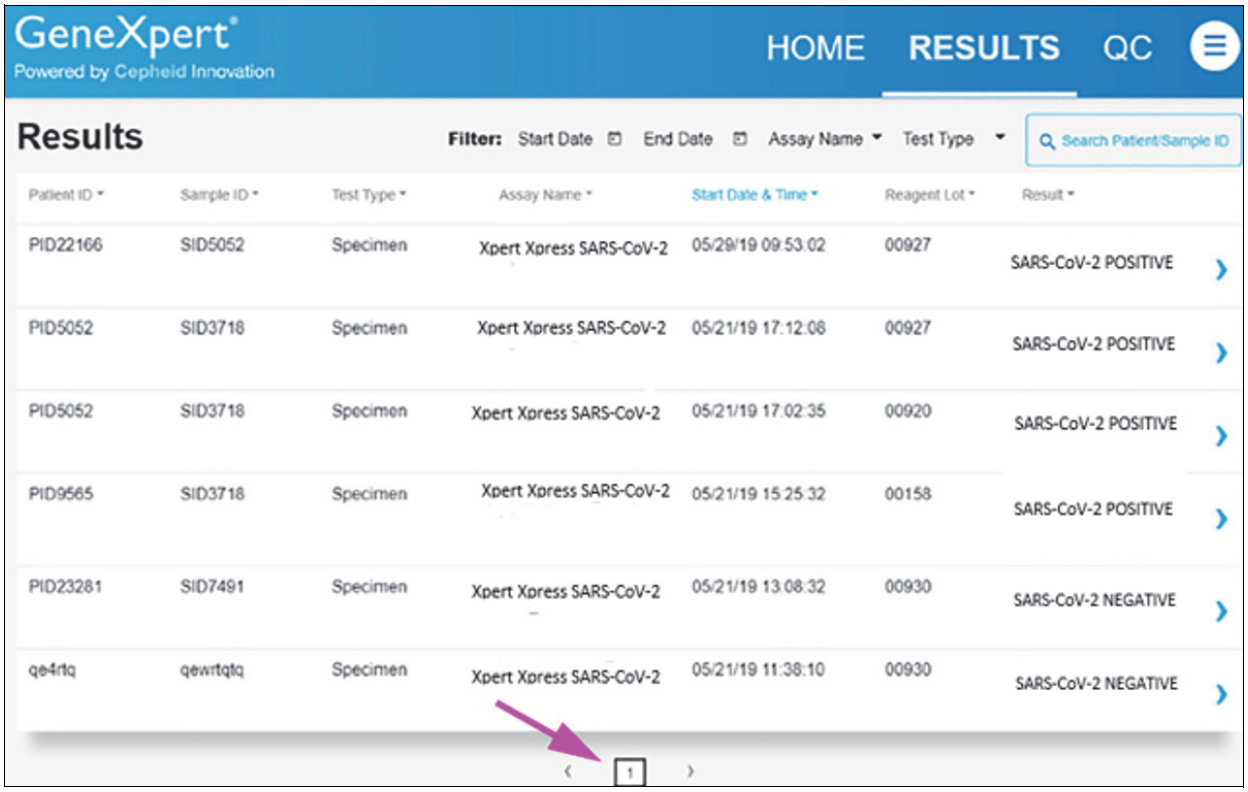


Figure 31. Results Screen

2. Touch the desired result to open the Test Result screen (see Figure 32).
3. To view test report, touch the **REPORT** button then swipe across the screen from left to right to minimize screen and view report.

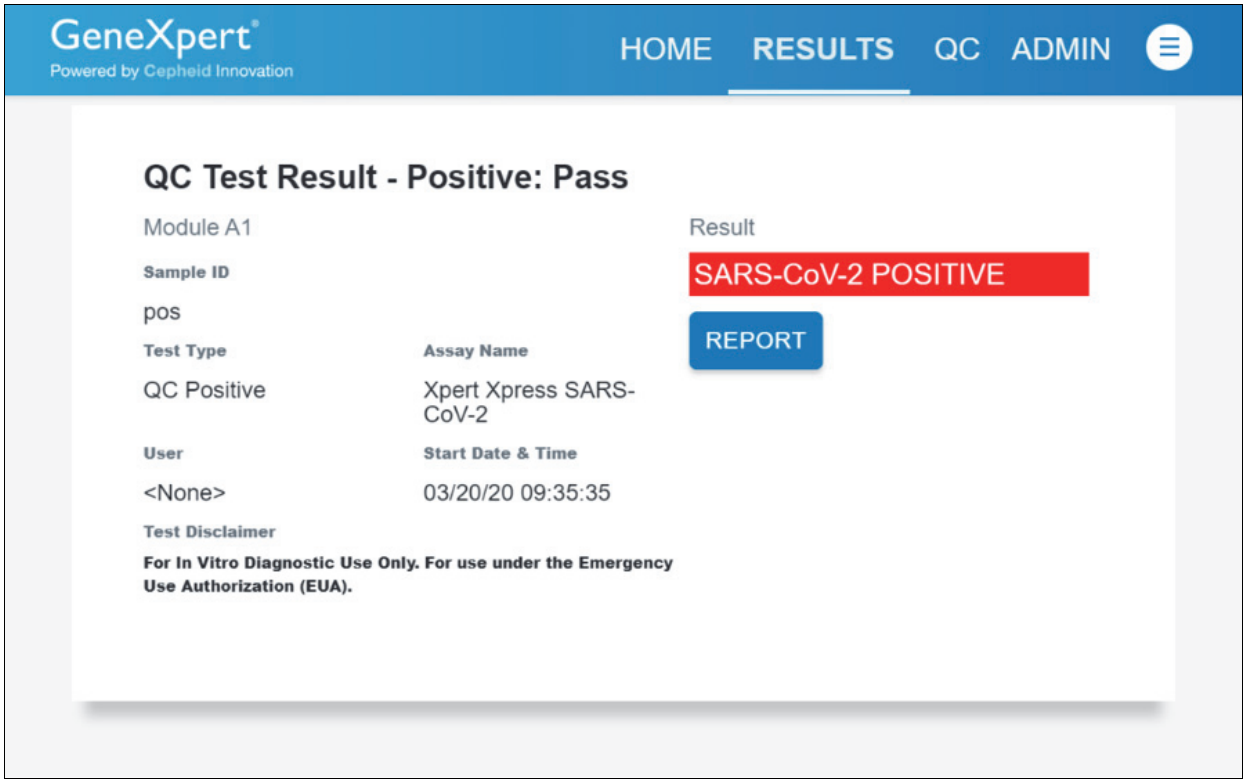


Figure 32. Test Result Screen (Example)

Note If an unexpected result occurs (e.g., Negative Quality Control result is positive or Positive Quality Control result is negative), test a new Quality Control sample using a new cartridge. If an unexpected result occurs upon retest, contact Cepheid Technical Support.

17 Quality Control

17.1 Internal Controls

CONTROL

Each cartridge includes a Sample Processing Control (SPC) and Probe Check Control (PCC).

Sample Processing Control (SPC) – Ensures that the sample was processed correctly. The SPC verifies that sample processing is adequate. Additionally, this control detects sample-associated inhibition of the real-time PCR assay, ensures that the PCR reaction conditions (temperature and time) are appropriate for the amplification reaction, and that the PCR reagents are functional. The SPC should be positive in a negative sample and can be negative or positive in a positive sample. The SPC passes if it meets the validated acceptance criteria.

Probe Check Control (PCC) – Before the start of the PCR reaction, the GeneXpert System measures the fluorescence signal from the probes to monitor bead rehydration, reaction tube filling, probe integrity, and dye stability. The PCC passes if it meets the validated acceptance criteria.

17.2 External Controls

External controls should be used in accordance with local, state and federal accrediting organizations as applicable.

18 Interpretation of Results

The results are interpreted automatically by the GeneXpert System and are clearly shown in the **View Results** window. The Xpert Xpress SARS-CoV-2 test provides test results based on the detection of two gene targets according to the algorithms shown in Table 1.

Table 1. Xpert Xpress SARS-CoV-2 Possible Results

Result Text	N2	E	SPC
SARS-CoV-2 POSITIVE	+	+/-	+/-
SARS-CoV-2 PRESUMPTIVE POS	-	+	+/-
SARS-CoV-2 NEGATIVE	-	-	+
NO RESULT - REPEAT TEST	-	-	-

See Table 2 to interpret test result statements for the Xpert Xpress SARS-CoV-2 test.

Table 2. Xpert Xpress SARS-CoV-2 Results and Interpretations

Result	Interpretation
SARS-CoV-2 POSITIVE	The 2019 novel coronavirus (SARS-CoV-2) target nucleic acids are detected. • The SARS-CoV-2 signal for the N2 nucleic acid target or signals for both nucleic acid targets (N2 and E) have a Ct within the valid range and endpoint above the minimum setting. • SPC: NA; SPC is ignored because coronavirus target amplification occurred. • Probe Check: PASS; all probe check results pass.
SARS-CoV-2 PRESUMPTIVE POS	The 2019 novel coronavirus (SARS-CoV-2) nucleic acids may be present. Sample should be retested according to the Retest Procedure in Section 19.2. For samples with a repeated presumptive positive result, additional confirmatory testing may be conducted, if it is necessary to differentiate between SARS-CoV-2 and SARS-CoV-1 or other Sarbecovirus currently unknown to infect humans, for epidemiological purposes or clinical management. • The SARS-CoV-2 signal for only the E nucleic acid target has a Ct within the valid range and endpoint above the minimum setting. • SPC – NA (not applicable); SPC is ignored because target amplification may compete with this control. • Probe Check: PASS; all probe check results pass.
SARS-CoV-2 NEGATIVE	The 2019 novel coronavirus (SARS-CoV-2) target nucleic acids are not detected. • The SARS-CoV-2 signal for two nucleic acid targets (N2 and E) do not have a Ct within the valid range and endpoint above the minimum setting. • SPC: PASS; SPC has a Ct within the valid range and endpoint above the minimum setting. • Probe Check: PASS; all probe check results pass.
NO RESULT - REPEAT TEST	If result is **NO RESULT - REPEAT TEST**, retest with a new cartridge according to the Retest Procedure in Section 19.2. If retest is **NO RESULT - REPEAT TEST**, obtain a new specimen for testing.
INSTRUMENT ERROR	If result is **INSTRUMENT ERROR**, touch **CLEAR ERROR** and follow the on-screen instructions. When the Home screen appears, repeat the test using a new cartridge according to the Retest Procedure in Section 19.2.

The Xpert Xpress SARS-CoV-2 test includes an Early Assay Termination (EAT) function which will provide earlier time to results in high titer specimens. When SARS-CoV-2 titers are high enough to initiate the EAT function, the SPC amplification curve may not be seen and its results may not be reported.

19 Retests

19.1 Reasons to Repeat the Assay

If any of the test results mentioned below occur, repeat the test once according to instructions in Section 19.2, Retest Procedure.

- An **INSTRUMENT ERROR** result could be due to, but not limited to, a system component failure, or the maximum pressure limits were exceeded.
- A **NO RESULT - REPEAT TEST** indicates that insufficient data were collected. For example, cartridge failed integrity test, Probe Check Control failure, no sample added, the operator stopped a test that was in progress, or a power failure occurred.
- A **PRESUMPTIVE POS** result indicates the 2019 novel coronavirus (SARS-CoV-2) nucleic acids may be present. Only one of the SARS-CoV-2 nucleic acid target was detected (E gene) while the other SARS-CoV-2 nucleic acid target (N2 gene) was not detected.

If an External Control fails to perform as expected, repeat external control test and/or contact Cepheid Technical Support for assistance.

19.2 Retest Procedure

To retest a non-determinate result (**NO RESULT-REPEAT TEST**, **INSTRUMENT ERROR**) or a **PRESUMPTIVE POS** result, use a new cartridge.

Use the leftover sample from the original specimen transport tube or new external control tube.

1. Put on a clean pair of gloves. Obtain a new Xpert Xpress SARS-CoV-2 cartridge and a new transfer pipette.
2. Check the specimen transport tube or external control tube is closed.
3. Mix the sample by rapidly invert the specimen transport medium tube or external control tube 5 times. Open the cap on the specimen transport tube or external control tube.
4. Open the cartridge lid by lifting the front of the cartridge lid.
5. Using a clean transfer pipette (supplied), transfer sample (one draw) to the sample chamber with the large opening in the cartridge.
6. Close the cartridge lid.

20 Limitations

- Performance of the Xpert Xpress SARS-CoV-2 has only been established in nasopharyngeal swab specimens. Use of the Xpert Xpress SARS-CoV-2 test with other specimen types has not been assessed and performance characteristics are unknown.
- Nasal swabs and mid-turbinate swabs are considered acceptable specimen types for use with the Xpert Xpress SARS-CoV-2 test but performance with these specimen types has not been established. Testing of nasal and mid-turbinate nasal swabs (self-collected under supervision of or collected by a healthcare provider) is limited to patients with symptoms of COVID-19. Please refer to FDA's FAQs on Diagnostic testing for SARS-CoV-2 for additional information.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if inadequate numbers of organisms are present in the specimen.
- As with any molecular test, mutations within the target regions of Xpert Xpress SARS-CoV-2 could affect primer and/or probe binding resulting in failure to detect the presence of virus.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.

21 Conditions of Authorization for Laboratory and Patient Care Settings

The Cepheid Xpert Xpress SARS-CoV-2 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/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#covid19ivd.

However, to assist clinical laboratories and/or Patient Care Settings using the Xpert Xpress SARS-CoV-2 (referred to in the Letter of Authorization as “Your Product”), the relevant Conditions of Authorization are listed below.

- Authorized laboratories[1] and patient care settings using your product will include with result reports of the Xpert Xpress SARS-CoV-2 test, 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 using your product will use your product as outlined in the Xpert Xpress SARS-CoV-2 Instructions for Use - For Use with GeneXpert Dx or GeneXpert Infinity systems. Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use the Xpert Xpress SARS-CoV-2 test are not permitted.
- Patient Care Settings using your product will use your product as outlined in the Xpress SARS-CoV-2 Instructions for Use - For Use with GeneXpert Xpress System and associated Quick Reference Instructions for Xpert Xpress SARS-CoV-2 and GeneXpert Xpress System (Hub configuration), and Quick Reference Instructions for Xpert Xpress SARS-CoV-2 and GeneXpert Xpress System (Tablet configuration). Deviations from the authorized procedures, including the authorized instruments, authorized extraction methods, authorized clinical specimen types, authorized control materials, authorized other ancillary reagents and authorized materials required to use your product are not permitted.
- Authorized laboratories and patient care settings that receive your product will notify the relevant public health authorities of their intent to run your product prior to initiating testing
- Authorized laboratories and patient care settings using the Xpert Xpress SARS-CoV-2 test will have a process in place for reporting test results to healthcare providers and relevant public health authorities, as appropriate.
- Authorized laboratories and patient care settings 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 Cepheid (+ 1 888.838.3222 or techsupport@cepheid.com) 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.
- All operators using your product must be appropriately trained in performing and interpreting the results of your product, use appropriate personal protective equipment when handling this kit, and use your product in accordance with the authorized labeling.
- You, authorized distributors, and authorized laboratories and patient care settings using your product 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.

[1] The Letter of Authorization refers to, "Laboratories certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. § 263a, to perform moderate or high complexity tests” as “authorized laboratories.”

22 Performance Characteristics

22.1 Clinical Evaluation– AccuPlex SARS-CoV-2 Reference Material

The performance of the Xpert Xpress SARS-CoV-2 test was evaluated using contrived clinical NP swab specimens in viral transport medium obtained from U.S. patients with signs and symptoms of respiratory infection. The samples were prepared by spiking each individual negative clinical NP swab sample with AccuPlex SARS-CoV-2 (a quantitated reference material – recombinant Sindbis virus particle containing target sequences from the SARS-CoV-2 genome) at 2x LoD, 3x LoD and 5x LoD levels. The NP swab samples were determined to be negative for SARS-CoV-2 prior to spiking. Individual negative NP swab samples were also tested in the study. All positive and negative samples in the study were tested in a randomized and blinded fashion.

Table 3 shows the number of concordant results out of the total number of samples tested for each target concentration of AccuPlex SARS-CoV-2, the mean Ct values for each of the E and N2 nucleic acid targets as well as the percent agreement with the 95% CI where appropriate. The results show 100% agreement with the expected results in the AccuPlexSARS-CoV-2 spiked samples and 100% agreement with the expected results in the negative samples.

Table 3. Xpert Xpress SARS-CoV-2 Test Agree with the Expected Results by Target Concentration

Target Concentration	Number Concordant/ Number Tested	E Mean Ct	N2 Mean Ct	% Agreement [95% CI]
2x LoD	20/20	34.8	38.0	100% [83.9% - 100%]
3x LoD	5/5	33.7	37.1	100% [NA*]
5x LoD	5/5	33.7	36.8	100% [NA*]
Negative	35/35	NA	NA	100% [90.1% - 100%]

* 95% CI not computed for sample concentrations with sample size of 5 or less.

22.2 Clinical Evaluation – Live SARS-CoV-2 Virus

The performance of the Xpert Xpress SARS-CoV-2 test was evaluated using contrived clinical NP swab specimens in viral transport medium obtained from U.S. patients with signs and symptoms of respiratory infection. The samples were prepared by spiking each individual negative clinical NP swab sample with live SARS-CoV-2 virus (USA_WA1/2020) at 2x LoD, 3x LoD and 5x LoD levels. The NP swab samples were determined to be negative for SARS-CoV-2 prior to spiking. Individual negative NP swab samples were also tested in the study. All positive and negative samples in the study were tested in a randomized and blinded fashion.

Table 4 shows the number of concordant results out of the total number of samples tested for each target concentration of live SARS-CoV-2 virus, the mean Ct values for each of the E and N2 nucleic acid targets as well as the percent agreement with the 95% confidence interval (95% CI), where appropriate. The results show 100% agreement with the expected results in the live SARS-CoV-2 virus spiked samples and 100% agreement with the expected results in the negative samples.

Table 4. Xpert Xpress SARS-CoV-2 Test Agreement with the Expected Results by Target Concentration

Target Concentration	Number Concordant/ Number Tested	E Mean Ct	N2 Mean Ct	% Agreement [95% CI]
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*]
Negative	30/30	NA	NA	100% [88.7% - 100%]

* 95% CI not computed for sample concentrations with sample size of 5 or less.

23 Analytical Performance

23.1 Analytical Sensitivity (Limit of Detection) – AccuPlex SARS-CoV-2 Reference Material

Studies were performed to determine the analytical limit of detection (LoD) of the Xpert Xpress SARS-CoV-2. The LoD of Xpert Xpress SARS-CoV-2 was established using one lot of reagent and limiting dilutions of AccuPlex SARS-CoV-2 prepared in simulated background matrix and NP swab clinical matrix and probit analysis. Verification of the estimated LoD claim was performed on one reagent lot in replicates of 35 prepared in pooled NP swab clinical matrix. The LoD is defined as the lowest concentration (copies/mL) per sample that can be reproducibly distinguished from negative samples with 95% confidence or the lowest concentration at which 19 of 20 replicates were positive. The claimed LoD for the assay is 250 copies/mL (Table 5).

Table 5. Limit of Detection of the Xpert Xpress SARS-CoV-2

Material	Claimed LoD (copies/mL)	Positives/ Replicates
SARS-CoV-2 Reference Material	250	35/35

23.2 Analytical Sensitivity (Limit of Detection) – Live SARS-CoV-2 Virus

Studies were performed to determine the analytical limit of detection (LoD) of the Xpert Xpress SARS-CoV-2. The LoD of Xpert Xpress SARS-CoV-2 was established using one lot of reagent and limiting dilutions of live SARS-CoV-2 virus (USA_WA1/2020) prepared in viral transport medium and NP swab clinical matrix and probit analysis. Verification of the estimated LoD claim was performed on one reagent lot in replicates of 22 prepared in pooled NP swab clinical matrix. The LoD is the lowest concentration (reported as PFU/mL) of live SARS-CoV-2 virus samples that can be reproducibly distinguished from negative samples ≥ 95% of the time with 95% confidence. The claimed LoD for the assay is 0.0100 PFU/mL (Table 6).

Table 6. Limit of Detection of the Xpert Xpress SARS-CoV-2

Strain	Claimed LoD (PFU/mL)	E Mean Ct	N2 Mean Ct	Positives/ Replicates
SARS-CoV-2 virus (USA_WA1/2020)	0.0100	35.9	38.9	22/22

23.3 Analytical Reactivity (Inclusivity)

The inclusivity of Xpert Xpress SARS-CoV-2 was evaluated using *in silico* analysis of the assay primers and probes in relation to 324 SARS-CoV-2 sequences available in the GISAID gene database for two targets, E and N2.

For the E target, Xpert Xpress SARS-CoV-2 had 100% match to all sequences with the exception of 4 sequences that had a single mismatch. For the N2 target, Xpert Xpress SARS-CoV-2 had 100% match to all sequences with the exception of 2 sequences that had a single mismatch. None of these mismatches found for both targets are predicted to have a negative impact on the performance of the assay, given the location of the mutations in the primer and probe regions respectively for the two variants. These mutations are not predicted to adversely affect the probe and primer binding to the sequences or reduce assay efficiency.

23.4 Analytical Specificity (Exclusivity)

An *in silico* analysis for possible cross-reactions with all the organisms listed in Table 7 was conducted by mapping primers and probes in the Xpert Xpress SARS-CoV-2 test individually to the sequences downloaded from the GISAID database. E primers and probes are not specific for SARS-CoV-2 and will detect Human and Bat SARS-coronavirus. No potential unintended cross reactivity with other organisms listed in Table 7 is expected based on the *in silico* analysis.

Table 7. Xpert Xpress SARS-CoV-2 Analytical Specificity Microorganisms

Microorganisms from the Same Genetic Family	High Priority Organisms
Human coronavirus 229E	Adenovirus (e.g. C1 Ad. 71)
Human coronavirus OC43	Human Metapneumovirus (hMPV)
Human coronavirus HKU1	Parainfluenza virus 1-4
Human coronavirus NL63	Influenza A
SARS-coronavirus	Influenza B
MERS-coronavirus	Influenza C
Bat coronavirus	Enterovirus (e.g. EV68)
	Respiratory syncytial virus
	Rhinovirus
	Chlamydia pneumoniae
	Haemophilus influenzae
	Legionella pneumophila
	Mycobacterium tuberculosis
	Streptococcus pneumoniae
	Streptococcus pyogenes
	Bordetella pertussis
	Mycoplasma pneumoniae
	Pneumocystis jirovecii (PJP)
	Parechovirus
	Candida albicans
	Corynebacterium diphtheriae
	Legionella non-pneumophila
	Bacillus anthracis (Anthrax)
	Moraxella catarrhalis
	Neisseria elongate and meningitidis
	Pseudomonas aeruginosa
	Staphylococcus epidermidis
	Staphylococcus salivarius
	Leptospira
	Chlamydia psittaci
	Coxiella burnetii (Q-Fever)
	Staphylococcus aureus

24 References

1. Centers for Disease Control and Prevention. https://www.cdc.gov/coronavirus/2019-ncov/index.html. Accessed February 9, 2020.
2. bioRxiv. (https://www.biorxiv.org/content/10.1101/2020.02.07.937862v1). Accessed March 3, 2020.
3. Centers for Disease Control and Prevention. *Biosafety in Microbiological and Biomedical laboratories* (refer to latest edition). http://www.cdc.gov/biosafety/publications/
4. Clinical and Laboratory Standards Institute. *Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline.* Document M29 (refer to latest edition).
5. REGULATION (EC) No 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008 on the classification labeling and packaging of substances and mixtures amending and repealing, List of Precautionary Statements, Directives 67/548/EEC and 1999/45/EC (amending Regulation (EC) No 1907/2007).
6. Occupational Safety and Health Standards, Hazard Communication, Toxic and Hazard Substances (March 26, 2012) (29 C.F.R., pt. 1910, subpt. Z).

25 Cepheid Headquarters Locations

Corporate Headquarters	European Headquarters
Cepheid 904 Caribbean Drive Sunnyvale, CA 94089 USA USA	Cepheid Europe SAS Vira Solelh 81470 Maurens-Scopont France
Telephone: +1 408 541 4191	Telephone: +33 563 825 300
Fax: +1 408 541 4192	Fax: +33 563 825 301
www.cepheid.com	www.cepheidinternational.com

26 Technical Assistance

Before contacting Cepheid Technical Support, collect the following information:

- Product name
- Lot number
- Serial number of the instrument
- Error messages (if any)
- Software version and, if applicable, Computer Service Tag number

Region	Telephone	Email
US	+1 888 838 3222	techsupport@cepheid.com
France	+33 563 825 319	support@cepheideurope.com
Australia New Zealand	+1800 130 821 +0800 001 028	techsupportANZ@cepheid.com

Contact information for all Cepheid Technical Support offices is available on our website: www.cepheid.com/en/CustomerSupport.

27 Table of Symbols

Symbol	Meaning
REF	Catalog number
IVD	*In vitro* diagnostic medical device
	Do not re-use
LOT	Batch code
	Consult instructions for use
	Caution
	Manufacturer
	Country of manufacture
	Contains sufficient for <n> tests
CONTROL	Control
	Expiration date
	Temperature limitation
	Biological risks
Rx only	For prescription use only

Cepheid
904 Caribbean Drive
Sunnyvale, CA 94089 USA
Phone: +1 408 541 4191
Fax: +1 408 541 4192

IVD

For use under Emergency Use Authorization (EUA) Only