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Image: Test plate from experimenting on coronavirus.
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IN-HOUSE TEST DEVELOPMENT FOR MOLECULAR DETECTION OF SARS-COV-2

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ABBREVIATIONS

bp	Base pairs
BSC	Biosafety cabinet
BSL	Biosafety level
cDNA	Complementary deoxyribonucleic acid
COVID-19	Coronavirus 2019
Cq	Cycle quantification value
CT	Cycle threshold value
DNA	Deoxyribonucleic acid
DNTP	Deoxynucleoside triphosphate
dsDNA	Double-stranded deoxyribonucleic acid
DTT	Dithiothreitol
DUTP	Deoxyuridine triphosphate
EQA	External quality assessment
EVAg	European Virus Archive – GLOBAL
gDNA	Genomic deoxyribonucleic acid
G	Guanine
GITC	Guanidinium isothiocyanate
IAC	Internal amplification control
IQC	Internal quality control
ISO	International Organization for Standardization
IVD	*In vitro* diagnostic
KCl	Potassium chloride
KPI	Key performance indicator
LDT	Laboratory-developed test
LMICs	Low- and middle-income countries
LoD	Limit of detection
MgCl2	Magnesium chloride
NAAT	Nucleic acid amplification test
NEC	Negative extraction control
No-RT	No reverse-transcriptase control
NTC	No template control
PCR	Polymerase chain reaction
PT	Proficiency testing
PTC	Positive template control
QA	Quality assurance
QC	Quality control
QMS	Quality management system
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SOP	Standard operating procedure
ssRNA	Single strand ribonucleic acid
TAT	Turnaround time
Tm	Melting temperature
UNG	Uracil-N-glycosylase
WHO	World Health Organization

INTRODUCTION

The SARS-CoV-2 global pandemic has thrust testing and diagnostic laboratory services into the global spotlight. Aggressive, sustained testing is the cornerstone of the test-trace-isolate strategies that are central to today's SARS-CoV-2 response, and critical to mitigating both the health and economic impact of the pandemic.

Effective testing strategies rely on the quick turnaround of results from reliable, accurate tests. Testing provides critical information for disease surveillance and targeted interventions for communities most in need. It can also help weak health systems manage scarce resources such as hospital beds.

Looking forward, effective testing will also underpin the success of future SARS-CoV-2 vaccines and therapeutics. Test data are already informing clinical trials that are currently underway. Once therapies or vaccines become available, diagnostics will enable roll out strategies and help ensure our most vulnerable populations can be reached first.

Despite its widely accepted importance, SARS-CoV-2 diagnostic testing has been a critical failure in many countries. In the case of a new human pathogen like SARS-CoV-2, diagnostics are not available immediately and must be created "from scratch". This means that there is a lag time between when the need for tests is identified and when those tests are available for use. In low- and middle-income countries (LMICs), the challenges are drastically magnified and span the entire diagnostic value chain. Accurate, rapid diagnostic tests are not available; personal protective equipment and necessary testing reagents are backlogged, unavailable, or unaffordable; and cold-chain systems necessary for preserving the integrity of diagnostic reagents are fragile. The development of tests "in-house" to detect emerging pathogens is one strategy that can help mitigate some of the challenges associated with relying on kits produced elsewhere in the region or world.

The World Health Organization (WHO) recommends testing for SARS-CoV-2 using nucleic acid amplification tests (NAATs). This technology is based on the detection of unique sequences of viral ribonucleic acids (RNA) using techniques such as real-time reverse transcription polymerase chain reaction (real-time RT-PCR). There are two types of testing platforms for the detection of SARS-CoV-2:

+ **Closed systems:** These are proprietary testing systems, where all accessories need to be sourced from the test manufacturer. Closed testing systems have standardized procedures and cannot be programmed to use test accessories sourced from a different manufacturer. Examples include the Abbott m2000 RealTime System, BioFire® FilmArray® System, Cepheid GeneXpert® Systems, and Roche cobas® systems.

+ **Open systems:** Unlike closed systems, open systems can accommodate different kinds of tests from multiple manufacturers as well as laboratory-developed tests (LDTs). Standards depend on the various components. Examples include Bio-Rad's CFX systems, the ABI 7500 DX, and Qiagen's Rotor-Gene.

The process of developing a new diagnostic molecular assay is challenging, and requires the investment of time, money, and resources to accomplish. For closed systems in particular, the resources available to produce and distribute supplies are highly constrained and during large outbreaks of disease, test manufacturers can meet only a small fraction of the global demand for their products. Ultimately, the ability to develop and validate tests "in-house" positions a laboratory to respond more quickly to outbreaks of emerging pathogens than it would be able to if it were reliant on commercial assay development and approval.

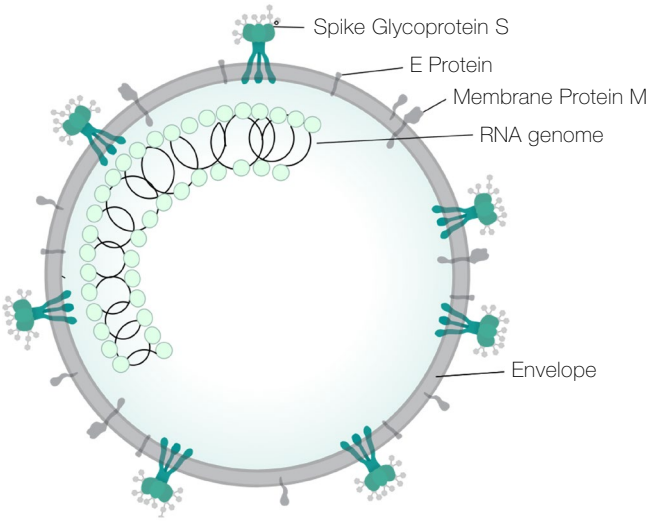
PURPOSE AND SCOPE

This document provides guidance on the development of a new, in-house, real-time RT-PCR test for SARS-CoV-2 for national laboratories, university laboratories, and private laboratories with established molecular biology capabilities. The information contained herein will also provide a roadmap for the creation of new molecular diagnostic tests for other emerging pathogens as well. Throughout the document, we will take into consideration the constraints of resource-limited laboratories, as well as provide an outline of requirements for good molecular biology practices including quality control (QC) and quality assurance (QA).

ABOUT SARS-COV-2

SARS-CoV-2 is a positive-sense single-stranded RNA virus with a single linear RNA segment. The causative agent of coronavirus diseases 2019 (COVID-19), it is the seventh known coronavirus and fifth from the Betacoronavirus genus (including; OC43, HKU1, SARS-CoV, and MERS-CoV) to infect people (1). Like other coronaviruses, SARS-CoV-2 has four structural proteins, known as the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins. The N protein coats and protects the RNA genome, and the S, E, and M proteins together create the viral envelope. The S protein is the protein responsible for allowing the virus to attach to and fuse with the membrane of a host cell (2). The emergence of the virus in the Wuhan province of China was first reported in December 2019 (3). WHO declared the virus pandemic on 11 March 2020 (4), and as of the time of writing this document, the pandemic is ongoing.

Figure 1: SARS-CoV-2 Structure. From SPQR10 Binte altaf / CC BY-SA



For reliable information on the latest SARS-CoV-2 research, see:

+ https://www.thelancet.com/coronavirus?dgcid=kr_pop-up_tlcoronavirus20
+ https://www.nature.com/collections/hajgidghjb
+ https://www.nejm.org/coronavirus
+ https://www.sciencemag.org/collections/coronavirus?intcmp=ghd_cov

INTRODUCTION TO PCR/RT-PCR

Polymerase chain reaction (PCR) is a method used to rapidly copy a specific target gene or gene region by making use of various components such as primers, deoxynucleoside triphosphates (dNTPs), thermostable deoxyribonucleic acid (DNA) polymerase, and magnesium chloride ($MgCl_2$) buffer. In its conventional form, PCR consists of three steps:

1. Denaturation of double-stranded DNA into single strands at 95°C for 15 – 60 seconds;
2. Annealing of the primers to specific regions on the single stranded DNA between 47°C and 60°C for 30 – 60 seconds; and
3. Elongation, facilitated by the DNA polymerase enzyme, of the single stranded DNA to from double stranded DNA again at 72°C for 30 – 180 seconds (depending on the processivity, or speed, of the enzyme and the length of the strand of nucleic acid to be elongated).

The temperatures and time described above will vary depending on the polymerase used and the gene target. Reverse transcription PCR, or RT-PCR, is the process of converting RNA into single-stranded complementary DNA (cDNA) using reverse transcriptase (polymerase) and DNA primers; the cDNA is then amplified by PCR. This process can be used to amplify viral RNA gene targets, such as gene targets for SARS-CoV-2. Real-time RT-PCR (real-time RT-PCR) is a variation of RT-PCR that combines RT-PCR amplification with real-time detection of the amplified gene target. This process eliminates the need for time-intensive detection methods like gel electrophoresis and allows for data monitoring in real-time, as the amplification cycles are typically observable in the provided software. Real-time assays can be either qualitative or quantitative.

Looking for a more detailed refresher on real-time RT-PCR? Check out this helpful review at:

+ https://geneticeducation.co.in/real-time-pcr-principle-procedure-advantages-limitations-and-applications/

and a useful video on how real-time RT-PCR works at

+ https://www.youtube.com/watch?v=iiXisgizkxs.

Free training on performing molecular testing for SARS-CoV-2 is available at

+ https://www.futurelearn.com/courses/laboratory-training-for-covid-19-molecular-testing/1/todo/78814

QUALITATIVE VS. QUANTITATIVE ASSAYS

A qualitative PCR assay is designed to detect the presence or absence of the target nucleic acids. Quantitative PCR assays (relative or absolute quantification) determine how much of the specific target nucleic acid is present in the test sample. The assays used for COVID-19 diagnosis are primarily qualitative assays, while quantitative RT-PCR (qRT-PCR) assays are used for more research-related applications where the virus load in a sample needs to be determined.

Qualitative PCR assays are sometimes called semi-quantitative PCR assays, as multiple results can be compared to determine which samples had relatively more target nucleic acids present. This comparison is based on the amplification cycle at which the target signal becomes stronger than the background signal, a point known as the cycle threshold value (C_T). For example, two samples containing SARS-CoV-2 are tested by real-time RT-PCR: sample 1 has a C_T value of 24 and sample 2 has a C_T value of 30. Sample 1 has more viral nucleic acid relative to sample 2, but the absolute amount of viral nucleic acids present in both samples remains unknown.

To determine the absolute amount of viral nucleic acids present by qRT-PCR, samples are tested along with a series of standards of known concentration. These standards are used to establish a standard curve from which the quantity of the target sequence in the test sample can be calculated. Quantitative assays are not often used for diagnostics, as they are more time consuming and use more reagents than qualitative assays do.

VIRAL RNA EXTRACTION METHODS

The daily sample throughput in the laboratory normally determines if manual nucleic acid extraction is to be done or if an automated nucleic acid extraction system will be implemented. Most laboratories prefer to use automated nucleic acid extraction systems if they are available; however, it is suggested that a manual method also be included as it serves as a back-up system should the automated nucleic acid extraction system be faulty or should there be a shortage of the reagents used in these devices.

Most automated nucleic acid devices are large and cannot fit into a biosafety cabinet (BSC), thus the method used to inactivate the virus-containing sample should also be considered when choosing extraction reagents. It is important that both the inactivation methods and the extraction methods used do not degrade the viral nucleic acid, yet it should be effective enough to inactivate and lyse the virus particles, respectively. There are multiple manual viral RNA extraction and purification kits and automated nucleic acid extraction systems available globally (see **Appendix 1**).

The success of RT-PCR and real-time RT-PCR depends on the quality of the extracted RNA. RNA extraction entails the lysis of the viral particles in the patient sample to release the nucleic acid. At this stage, the sample contains the nucleic acid of interest (the RNA), contaminating DNA and proteins, as well as nucleoprotein complexes and RNAses and thus still needs to be purified for the PCR reaction. It is critical that the RNAses be inactivated and the nucleoprotein complexes degraded during the extraction process as these will destroy the RNA. All solutions and consumables used must be RNAse free. The two most used organic methods of RNA extraction make use of either 4 M Guanidinium thiocyanate (GITC) or phenol and sodium dodecyl sulfate. The guanidinium thiocyanate-phenol-chloroform RNA extraction method is most used to extract SARS-CoV-2 RNA from a sample derived from a person under investigation for COVID-19 (**Table 1**).

Table 1: Basic steps of RNA extraction using organic methods.

Step 1	Cell lysis & dissolution (homogenization of cells/ particles to release the RNA) by means of buffers & centrifugation, respectively	Cell lysis is done by using buffers or reagents containing chaotropic agents such as guanidinium isothiocyanate, guanidinium chloride, sodium dodecyl sulphate (SDS), sarcosyl, urea, phenol or chloroform. Commercial agents such as TRIzol, Realter or Qiazol can be used to maintain RNA integrity during lysis.
Step 2	Denaturation of DNA & proteins	DNase is to be used to degrade DNA, and proteinase K is can be added to aid in the digestion of proteins. Alternatively, repeated organic extraction using phenol and chloroform, or dissolving the sample in buffers containing guanidinium salts, can also be used to remove proteins.
	Inactivation of RNases	This can be achieved using any of the chaotropic agents mentioned above, such as phenol and chloroform.
Step 3	RNA isolation (removal/ separation of cellular components)	RNA can be separated from other cellular components by adding chloroform and centrifuging the solution. This separates the solution into two phases: organic and aqueous phases. The aqueous phase contains RNA.
Step 4	Precipitation	RNA is often recovered from the aqueous phase using isopropyl alcohol and centrifugation. RNA can also be selectively precipitated from DNA through the use of ammonium acetate. Alternatively, lithium chloride can be used to selectively precipitate RNA from DNA as well as proteins.
Step 5	Transfer of purified RNA	Storage of the pellet in RNase-free water.

There are two main types of nucleic acid extraction technologies that can be used instead of the organic method outlined in **Table 1**:

- **Silica-membrane based technology** is a solid phase extraction method used to purify nucleic acids. RNA will bind to the silica under certain conditions, whilst other molecules such as proteins and DNA will not. After the cells have been lysed and then genetic material is accessible, a buffer solution and ethanol or isopropanol are added to the sample. This forms the binding solution. This solution is then centrifuged in a spin column, which forces the binding solution through a silica gel membrane in the spin column. If the conditions such as pH and salt concentrations of the binding solution are optimal, the RNA will bind to the silica gel as the solution passes through.

- In **paramagnetic particle technology**, the use of magnetic beads that have an individual coating that allows them to have affinity for specific molecules within a sample, such as DNA, RNA or proteins, administrates the isolation and separation of different particles within a sample. Only the RNA binds to the coated magnetic beads, leaving any remaining contaminants in the solution.

Owing to global shortages of laboratory equipment, reagents and consumables required for RNA extraction due to the SARS-CoV-2 pandemic, some molecular diagnostic laboratories have stretched the alternative approach to RNA extraction by simply boiling the sample (5).

PCR PROTOCOL DESIGN

RESOURCE CONSIDERATIONS FOR DEVELOPING IN-HOUSE TESTS

Design and implementation of a new open-platform diagnostic RT-PCR assay requires a number of resources, including but not limited to: reliable electricity and cold storage capability, specialized equipment, the ability to produce or procure high-quality reagents, an established quality management system (QMS) including a QMS officer or dedicated staff member, and the capability to perform long-term monitoring and documentation of assay performance. Staff involved in the development process must possess an in-depth understanding of how each step and component of the assay is supposed to perform, and must be capable of troubleshooting issues with the assay reactions as well as the equipment being used. Having access to individuals capable of performing the statistical analysis required during the protocol validation process is also essential. Meticulous records of the development process must be maintained, and the final protocol itself should be clearly written. The written protocol should be tested by staff trained to perform molecular diagnostics who were not involved in the development process, to ensure that it is complete and easy to follow.

Once an LDT has been developed, it should undergo rigorous validation at the central or national laboratory before being sent to regional or satellite labs. The consequences of validation failure can be severe—during the United States' SARS-CoV-2 response, a vital reagent in the CDC assay was contaminated, but the contamination was not discovered until after the kits were sent to numerous regional labs. This failure in the validation process resulted in major delays in testing at the outset of the pandemic. Once the LDT is validated at the central or national laboratory, it will need to be validated at every additional laboratory where it is to be used. The results of those validation exercises should be reviewed by the design team to determine if additional modifications to the protocol or additional training at the other sites are necessary.

NOTE: Most laboratories will not be positioned to design closed-platform LDTs. Most closed-platform systems are proprietary, which means that development of assays for these systems requires working directly with the system manufacturer. In the event that your facility is in a position to work with one of these manufacturers, the development process will be very similar to what is described in this document for open platform LDT design.

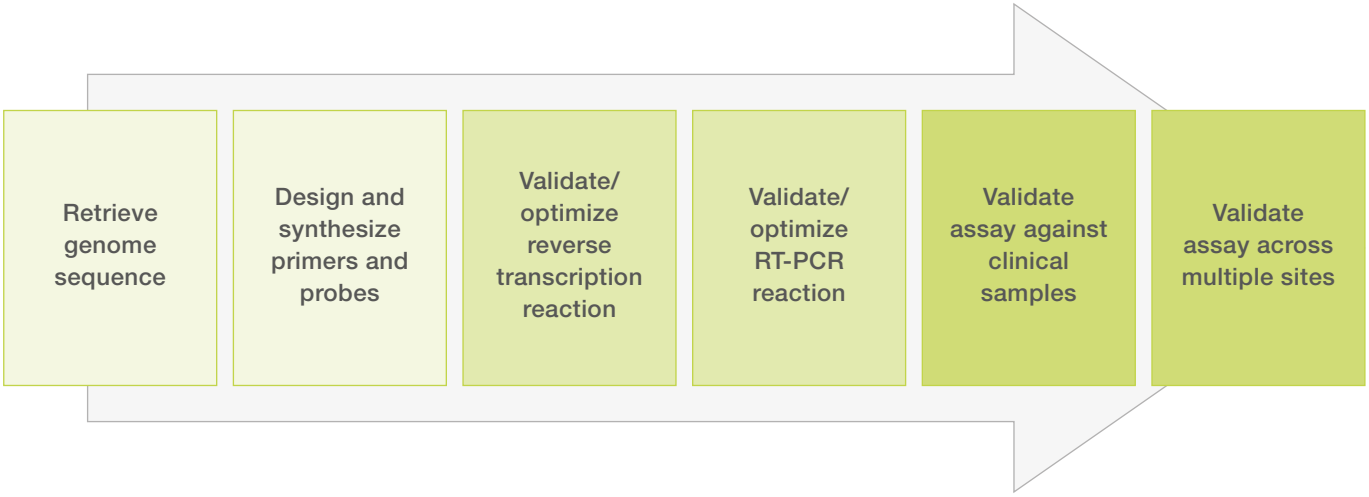
ASSAY DEVELOPMENT EQUIPMENT REQUIREMENTS

The LDT development and validation process requires a variety of specialized equipment. A brief list of the minimum equipment requirements is provided in Appendix 2. Minimum Required Equipment. Real-time PCR systems can be procured from various suppliers. A list of potential suppliers is provided in Appendix 3. Select real-time RT-PCR System Manufacturers. The choice of a system is guided by the speed at which the system can reach the programmed temperatures, the maintenance and calibration requirements, the number of channels for color detection in relation to the complexity of the PCR assay, the signal per target, and the dye selection for a specific instrument's filters. If the assay is designed to detect a single target (e.g. SARS-CoV-2), including various internal controls, an entry-level instrument can be used to detect the target. Laboratories looking to meet International Organization for Standardization (ISO) requirements should consider systems rated for clinical diagnostic or *in vitro* diagnostic (IVD) use.

OVERVIEW OF THE ASSAY DESIGN PROCESS

The assay design process involves multiple planning, validation, and optimization steps. A general diagram of the process is shown in **Figure 2**.

Figure 2: LDT design process.



PRIMER AND PROBE DESIGN

PRIMER DESIGN

It is important to ensure proper amplification of genomic regions specific to the SARS-CoV-2 virus when designing an LDT. SARS-CoV-2 sequences have been published (6) and validated primer sequences for SARS-CoV-2 detection are available (7).

When designing primers for LDTs, it is important to note that the SARS-CoV-2 virus shares 95% homology with other coronaviruses, thus creating a high risk of cross-reactivity. Therefore, it is essential that primers and probes are designed to specifically amplify only the RNA of the genes of the SARS-CoV-2 virus and not related viruses.

The melting temperature (Tm) of a primer is by definition "the temperature that the one half of the DNA duplex dissociates and becomes single stranded, thereby indicating the duplex stability". The optimal Tm range is normally between 52°C and 58°C and is directly linked to the guanine-cytosine (GC) content of the primer. Care should be taken when designing and using primers with higher melting temperatures (above 65°C) as these have a proclivity to form secondary structures.

The following should be considered when designing primers:

- Design primers that are 18-27 base pairs (bp) long and which will produce amplicons that are 70–150 bp long. The short length ensures easy binding to the template nucleic acid during the annealing step in the PCR cycle.
- Avoid homology within or between primers, especially at the 3' end to avoid primer-dimer formation
- Design primers that have a GC content of 40–60% and avoid long stretch (4+ repeats) of GC as well as G or C. Try to include a GC clamp at the 3' end of the primer: to promote specific binding there should be a G or C nucleotide present within five bases of the 3' end of the primer.
- Ensure that the primers have a melting temperature between 55 and 65°C. Tm of the two primers should be as close as possible.

- Avoid designing primers for regions with secondary structures. Complementarity within a primer will favor hairpin loop formation.
- Check the sequences of the forward and reverse primers for 3' complementarity. This can result in primer-dimer or cross-dimer formation. A primer dimer is formed when two primers in the same direction bind because of intermolecular interactions.
- Verify specificity for the target sequence and pathogen by running a nucleotide BLAST search against available sequences (8). BLAST compares the designed primer sequences to published sequences and determines the potential of hybridization of the primers chosen with sequences from other SARS viruses as well as other organisms.

Online tools such as the United States National Center for Biotechnology Information's Primer-BLAST tool allow users to design their primers based on publicly available genome sequences. Many primer synthesis companies also offer free primer design tools on their websites. Examples of some of these tools are provided in Appendix 4. Once the primers and probes are designed and synthesized, the concentrations as well as those of the other PCR components should be optimized to ensure maximum amplification efficiency (9).

HYBRIDIZATION/HYDROLYSIS PROBE DESIGN

There are many fluorescence detection probe chemistries that can be used to create real-time RT-PCR assays for SARS-CoV-2. The three most common chemistries include SYBR® Green-based detection, dual-labeled fluorogenic oligonucleotide probes, and molecular beacon probes.

SYBR® Green I, is a fluorescent dye that intercalates, or is bound up, into dsDNA and can be used in real-time RT-PCR reactions. With each amplification cycle, more dye is bound up, leading to an increase in fluorescence. SYBR Green is not sequence-specific which makes this method susceptible to false positives due to the formation of non-specific PCR products or primer-dimers. Primer specificity is usually confirmed by evaluating whether the amplified product is the correct size and whether it melts at the anticipated temperature, which serves as an indication that the sequence of the product has the expected composition. This confirmation process is called a melting curve analysis.

Dual-labeled fluorogenic oligonucleotide probes (e.g., TaqMan® probes) have a fluorescent reporter dye at their 5' end and a quencher dye at their 3' end. The probes are added to the master mix along with the specific primer set. During PCR, if the target sequence is present, the probe anneals downstream from a primer site and is cleaved by the 5' nuclease activity of Taq DNA polymerase during polymerization. This cleavage releases the reporter dye from the probe and separates it from the quencher dye, resulting in fluorescence that is detected by the instrument.

Molecular beacon probes use a variation of the dual-label process described above. For molecular beacon probes, reporter and quencher dyes are held together by a hairpin structure in the probes but become sufficiently separated by linearization of the probe after annealing with the template to allow the reporter dye fluorescence to be detected.

Not all RT-PCR systems support all probe chemistries. Before selecting a chemistry and designing your probes, check the user manual of your RT-PCR system to determine what chemistries will work most efficiently. If you do not have the user manual, you can usually download one from the manufacturer's website. Another consideration when designing an LDT with multiple probes and primers is that probes absorb and emit at specific wavelengths of light, and these wavelengths overlap on some probes. Therefore, if you are not careful with your selections, false positives could occur to do "incidental stimulation" of a probe.

For more information on probe chemistry and fluorophore selection, check out the tips provided at

- https://www.bioradiations.com/tips-to-make-fluorophore-picking-easier/

SUGGESTIONS FOR THE SELECTION OF PRIMERS AND PROBES

Most companies that produce primers and probes for real-time RT-PCR assays have tools available online to help with the design process. If you are looking to purchase primers and probes from a company located in a different country, make sure you talk to someone at the company to confirm whether they will be able to ship your materials to you properly. Some companies are able to provide their primers and probes in a lyophilized, or freeze-dried, form. Lyophilized materials should be considered if there are concerns that your purchase may take several days to pass through Customs, or if there are concerns about whether the cold-chain can be successfully maintained during shipping and delivery. Some examples of custom primer and probe vendors are provided in Appendix 4.

ADDITIONAL COMPONENTS OF THE REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTIONS

The RT-PCR process requires two sets of reagents—one set for the reverse transcription step, and one for the PCR. Diagnostic assays may be designed as two-step processes, where the product of the reverse transcription process is added to the PCR reagents or as one-step processes where the reverse transcription and PCR steps take place within the same tube. In addition to the initial template RNA, the typical components of the reverse transcription reaction are shown in **Table 2**; the basic components of the PCR portion of the assay required to replicate the cDNA are shown in **Table 3**. Some PCR systems/enzymes may require use of magnesium sulfate ($MgCl_2$), bovine serum albumin, dimethylsulfoxide, and glycerol for optimal conditions. Most commercial suppliers of reverse transcriptase and DNA polymerase will supply the enzyme with the buffer in which the enzyme works optimally.

Table 2: Reverse transcription reaction components.

Component	Typical concentration per reaction	Notes
Buffer	50 mM Tris HCl pH8.3 75 mM KCl 10 mM DTT 3 mM $MgCl_2$	This should be prepared as 5x concentration
dNTPs	1 mM	All four dNTPs (adenine, guanine, cytosine, and thymine triphosphates) must be included
Reverse transcriptase	1 Unit	Optimal concentration should be determined during optimization

Table 3: PCR reaction components.

Component	Typical concentration per reaction	Notes
Buffer	20 mM Tris HCl pH8.3 50 mM KCl	This should be prepared as 5x concentration
dNTPs mix	0.2- 0.5 mM	Optimal concentration should be determined during optimization
$MgCl_2$	0.5 - 5 mM	Optimal concentration should be determined during optimization
Forward primer	0.1 - 0.5 µM	Optimal concentration should be determined during optimization
Reverse Primer	0.1 - 0.5 µM	Optimal concentration should be determined during optimization
Probe	0.1 - 0.5 µM	Optimal concentration should be determined during optimization
DNA Polymerase	0.25 - 0.5 Unit	Optimal concentration should be determined during optimization

The mixture of PCR components primers and probes, DNA polymerase, dNTPs, $MgCl_2$ and buffer is called the **master mix**, and it is generally prepared at the concentration needed for use, and then aliquoted into PCR tubes or 96-well PCR plates. Commercial suppliers of PCR reagents offer ready mixes that include all necessary PCR components except the primers and probes. The ready mixes present the advantage that they can be used to set up and optimize PCR for the detection of any pathogens if specific primers and probes are available. This reduces the risk of contamination and pipetting errors, and most importantly, saves time.

To develop in-house laboratory test for SARS-CoV-2 or any other pathogens, the following steps should be taken:

1. Design pathogen-specific primers and probes. You will need to know the sequence of the pathogen.
2. Place the order at companies who synthesized oligonucleotides and request at least SDS-PAGE purification scale. Remember to add your 5' and 3' ends modifications (attaching the desired fluorophore) during the ordering.
3. Obtain enzymes, buffers, dNTPs mix, and magnesium chloride from commercial suppliers. It would be time saving to buy ready mixes from commercial suppliers such as those listed in Appendix 5.

ENZYMES

Reverse transcriptases are the enzymes used make cDNA from RNA. Taq DNA polymerase, isolated from thermophilic bacterium *Thermus aquaticus*, is the primary enzyme used in the amplification of DNA.

When selecting an enzyme type for a method, the designer must evaluate the different strengths and weaknesses of the DNA polymerases available to determine which individual polymerase, or combination of polymerases, will work with their template nucleic acid. Records of lot numbers of all reagents should be captured and stored. Reagents

should be aliquoted into single use aliquots to avoid excessive freeze-thawing and to protect stock reagents from contamination. These reagent tubes should be clearly labeled. All reagents containing fluorescent probes should be protected from excessive light to prevent degradation by photo-bleaching. It is recommended to incorporate dUTP and an RNAse inhibitor in the master mix to prevent degradation by uracil-DNA glycosylase (UNG) and to minimize the loss of the RNA template material, respectively.

SUGGESTIONS FOR THE SELECTION OF ENZYMES

The selection of reverse transcriptases and DNA polymerases should consider the reaction temperatures at which they will be used, the size of the cDNA or PCR product that will produced, the desired sensitivity and accuracy of the reaction, and the ability to work with degraded or inhibitor-containing RNA. The variety of enzyme choices is higher when designing two-step assays, while the reverse transcriptases and polymerases for use in one-step assays typically come pre-mixed from the vendor. As with primers and probes, some vendors sell lyophilized master mixes that include the reverse transcriptase and DNA polymerase already present.

CONTROLS

During the development of an LDT, it is important to consider what controls will be needed for the test. Controls are used to monitor the performance of the complete diagnostic process of the molecular assay. Inclusion of the proper controls allows the laboratorian to confirm that the RNA extraction process was successful; to identify instances of possible PCR inhibition; to verify the functionality of the equipment; to detect the introduction of contaminating genomic material into the workflow; and to validate the test results. The use of the appropriate controls for every diagnostic run is therefore essential to ensuring the quality of the test results. An example of a control scheme developed for a SARS-CoV-2 LDT, including additional details regarding the source of the controls and their purpose, is shown in **Table 4. Table 5** provides additional details regarding controls and their purpose.

Table 4: Example of a control scheme for a SARS-CoV-2 real-time RT-PCR. From (10).

Control	Controls for:	Control requirement:
NTC (No Template Control)	Contamination in master mix/ plate set up	One per real-time RT-PCR plate; Control passed
COV_Pos (Positive Assay Control)	real-time RT-PCR process control	One per real-time RT-PCR plate; Control passed
COV_Neg (Negative Extraction Control)	Extraction control, real-time RT-PCR Process for RNase P	One per extraction; Control passed
RNAse P in Negative Sample (Internal Amplification Control)	Confirms full process for negative samples	Built in for all real-time RT-PCR wells; Pass when sample negative

Table 5: Control sources and interpretations.

Control type	Example material	Expected resultment:	Possible reasons for a positive result	Possible reasons for a negative result
Positive extraction	A sample known to contain the assay sequences, e.g., RNA/ gDNA expressing/ containing the target	Positive	Correct	Assay or extraction failure. Any positive data from other samples is unreliable.
Positive assay (PTC)	Any nucleic acid compatible with the PCR assay design that contains the target sequence	Positive	Correct	Assay failure. Any positive data from other samples is unreliable.
Negative assay	Any nucleic acid compatible with the PCR assay design known not to contain the target sequences	Negative	The assay is non-specific or there was contamination of the control during PCR or reagent preparation	Correct
No template (NTC)	Water	Negative	The primers are self-dimerizing resulting in primer dimer product or there was contamination of the control during PCR or reagent preparation	Correct
Minus RT enzyme negative (No-RT)	RNA sample and all components of the RT reaction with the exception of RT enzyme. This should be performed on all samples to verify that they do not contain gDNA contamination that amplifies under the PCR conditions without the need for RT enzyme	Negative	The sample contains gDNA. The reaction became contaminated during set up. The primers formed primer dimers. Analyze in conjunction with NTC.	Correct
Negative extraction (NEC)	Water	Negative	Contamination during extraction	Correct
Internal amplification control (IAC)	Occur naturally in test specimens, e.g., 16s, Beta-Actin, RNAse-P	Positive (All samples and controls should contain the IAC except the NTC)	Correct	Assay or extraction failure. Any positive data from other samples is unreliable.

NOTE: The tables on the previous page controls only address the nucleic acid extraction and PCR assay positive and negative controls. It is required that all the activities from sample collection to results reporting are subject to QC and QA.

Where LDTs are used or when a test kit fails to include a positive control template, positive control material can be obtained in the form of cDNA from Charité in Berlin, via the Global European Virus Archive (EVAg - https://www.european-virus-archive.com), or in the form of single stranded RNA (ssRNA) fragments of SARS-CoV-2 from the Joint Research Centre in Belgium (https://crm.jrc.ec.europa.eu/p/EURM-019).

OPTIMIZING RUN CONDITIONS

The optimization of PCR run conditions involves determining 1) the ideal concentrations for each primer and probe used in the assay, 2) the annealing temperature to be used for the assay, and 3) refining the concentrations of buffer components (particularly when more than one target will be assayed at a time in a single tube, a process referred to as multiplexing). After successful nucleic acid extraction and amplification, the assay should be validated by separating the PCR products on a gel and correlating the melting temperatures with the correct amplicon sizes. The no-reverse transcriptase (No-RT) control also needs to be verified to determine whether an RNA digestion step needs to be added to the sample before the RT-PCR reaction.

USING LDTS DEVELOPED BY OTHER LABORATORIES

National laboratories and non-government organizations are increasingly sharing their own LDT protocols online for use and adaptation by other groups. These protocols provide valuable information regarding which regions within the viral genome may provide suitable targets for primers and probes, what run conditions provide a strong signal, whether a ready-mixed master mix may be suitable for use, and what enzymes perform well in the assay. If a laboratory chooses to adopt an LDT developed by another laboratory, whether that laboratory is part of their national laboratory network or is located in a different country, it must validate that test to confirm that it performs as expected in their facility with the strains of the virus present in their local population.

Examples of LDT protocols for SARS-CoV-2 that are currently available online include the following:

+ https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c_2
+ https://www.fda.gov/media/139743/download
+ https://www.who.int/docs/default-source/coronaviruse/whoinhouseassays.pdf?sfvrsn=de3a76aa_2

LDT PROTOCOL VALIDATION

The performance characteristics of a COVID-19 LDT PCR assay must be determined prior to implementing the assay for use in routine testing at each laboratory location. This process is called validation. The principles of validation exist to ensure standards of laboratory practice and accuracy of test results generated by clinical laboratories and were defined by the International Organization for Standardization (11, 12). Performance characteristics include an array of parameters such as accuracy, precision, reproducibility, analytical sensitivity and specificity, linearity, and lot specific testing and requirements will vary according to national regulations.

The samples to be used in the validation are especially important. These should consist of positive samples with known cycle quantification values (Cq; known viral loads) as well as negative samples that were tested on validated systems. If possible, representative samples collected in the different transport media (e.g. saline, viral transport media, or universal transport media) and the different anatomical sites of sample collection (e.g. nasopharyngeal swab, oropharyngeal swab, sputum), should be subjected to the same validation process. The results obtained from these variables will allow conclusions to be drawn on the limitations of assay. When selecting the samples for a validation the following guidelines should be followed:

+ Irrespective of the prevalence of SARS-CoV-2, the number of samples should be consistent across tests.
+ All sample types expected for the assay should be included.
+ Samples with all the possible results (CT ranges) should be included.
+ Controls and calibration materials should be obtained and included.

SENSITIVITY

Sensitivity is the ability of an assay under evaluation to identify correctly true positive (reference assays positive) samples. Therefore, sensitivity is the number of positive samples correctly identified by the assay under evaluation divided by the total number of true positive samples (for example those positive by the reference assays), expressed as a percentage as indicated: Sensitivity = [True Positive / (True Positive + False Negative)] X 100%

SPECIFICITY

Specificity is the ability of an assay to identify correctly true negative (reference assays negative) samples. Therefore, specificity is the number of true negative samples correctly identified by the assay under evaluation, divided by the total number of true negative samples (for example those negative by the reference assays), expressed as a percentage as indicated: Specificity = [True Negative / (True Negative + False Positive)] X 100%

ANALYTICAL SENSITIVITY

The analytical sensitivity of an assay is the ability of the assay to detect very low concentrations of a given substance in a biological specimen. It is also known as the “limit of detection” (LoD). To estimate the LoD, repeated measurements are obtained from low concentration samples, for example positive samples with known Cqs that have been serially diluted. Experiments to determine the LoD should be carried out over several days to reflect performance of the assay over a range of typical laboratory conditions but without a change in reagent lots. The LoD should be determined for each type of specimen that will be tested in the laboratory and each genetic variant of the intended target (13, 14).

ANALYTICAL SPECIFICITY

Analytical specificity is the ability of an assay to detect only the intended target and ensure that the quantification of the target is not affected by cross-reactivity from related or potentially interfering nucleic acids or specimen-related conditions. Interference studies will determine any potential cross-reactivity and/or interference. This screening process will involve testing an array of samples with or without different concentrations of potentially interfering substances and cross-reacting agents, the choice of which will be determined by the type of assay, samples, and intended target (12, 14)

PRECISION

Precision is the ability of an assay to perform consistently when the sample is tested multiple times over many days by different operators using different batches of reagents. It reflects the impacts on the results due to random variances that occur while the assay is performed under normal conditions. Unlike other validation parameters, precision is a qualitative measure of performance; assays are generally rated as having high, medium, or low precision. Experiments to determine assay precision are performed in duplicate over multiple days (typically about 20) on at least three concentrations of sample (LoD, 20% above the LoD, and 20% below the LoD). Running the samples in duplicate allows the repeatability, or within-run imprecision, of the assay to be evaluated, while running samples over multiple days with multiple operators and reagent lots allows the reproducibility, or run-to-run imprecision, to be evaluated.

Table 6 illustrates one possible protocol for validating the sensitivity, specificity, LoD, and precision of a qualitative LDT, using samples with a range of concentrations of target viral RNA present (the analyte). For a more in-depth discussion of how to perform validation testing and the associated statistical analyses, see (14).

Table 6: Sample protocol for determining LDT performance characteristics. Adapted from (14).

Performance characteristic	Analyte concentration tested											Comments
	Low					Medium			High			
	1	2	3	4	5	6	7	8	9	10	11	
Sensitivity & specificity	x	x	x	x	x	x	x	x	x	x	x	Test 50 positive, 100 negative samples, with approximately 30% of the positives in the low range, 30% in the medium range, and 30% of the high range of concentrations.
LoD	x	x	x	x	x							Run 8-12 replicates each of 4-5 samples at the low end of the analyte concentration over at least 5 days.
Precision		x			x					x		Prepare multiple aliquots each of 3 samples representing the LoD, 20% below the LoD, and 20% above the LoD and store at -20°C or colder; test the three samples in duplicate for 20 days, using different operators and different reagent lots as possible.

DIAGNOSTIC TESTING IN THE CLINICAL LABORATORY

SETTING UP THE LABORATORY

Implementing real-time RT-PCR assays for SARS-CoV-2 (both commercially available and in-house) often necessitates laboratory scale-up and strengthening.

BIOSAFETY REQUIREMENTS

When setting up novel diagnostic testing for SARS-CoV-2 or any new pathogen, it is critical to consider the biohazard risks. Biohazard risks include the potential for infection of a laboratory worker (laboratory technician, cleaner, student, etc.), and furthermore the potential for release of infectious SARS-CoV-2 virus in the community/environment. A risk assessment should be performed to identify the appropriate risk mitigations necessary to ensure the safety of laboratory personnel, the community, and the environment. The WHO recommends that SARS-CoV-2 "specimen handling for molecular testing would require [biosafety level 2] BSL-2 or equivalent facilities" and that "attempts to culture the virus require BSL-3 facilities at minimum" (15). In a BSL-2 laboratory, access should be limited, and the entrance labeled with a biohazard sign (preferably made from fire resistant material). The WHO Laboratory Biosafety Manual describes three components to biosafety in laboratories (16). These components are:

> Looking for additional information on safe handling and processing of specimens associated with COVID-19? Check
>
> + https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html

1. The facility layout (design of the laboratory working areas);
2. Essential equipment, purchased according to standard specifications with a serviceable maintenance plan (including [re-]certification); and
3. Work practices (laboratory procedures, personal protection and personal protective equipment, health and medical surveillance, training, waste handling, and chemical, fire, electrical, radiation and equipment safety.

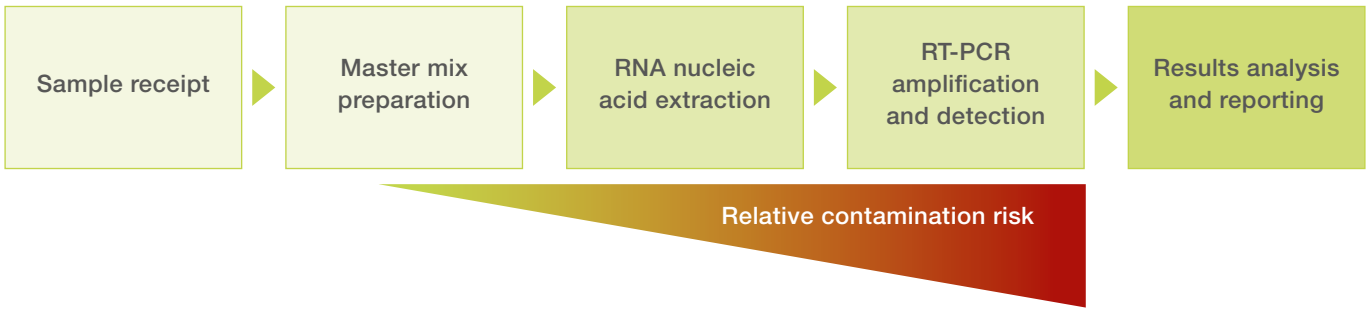
Good laboratory practices and good microbiological techniques are essential to minimize the generation of infectious aerosols and the spread of nucleic acids (genomic products and amplicons). If an open platform nucleic acid extraction device is to be used during the PCR process, the samples must first be inactivated inside the BSC before the extraction process is performed. The PCR master mix preparation area and the amplification and detection area only require BSL-1 precautions.

PCR TESTING WORKFLOW

In addition to a sample reception area, the molecular laboratory must consist of at least three different dedicated areas: the PCR reagent preparation area, the nucleic acid extraction area, and the amplification and detection area. Physical separation of the pre-amplification areas (or "clean areas") and the post-amplification areas (or "dirty areas") is a requirement to mitigate the risk of contamination during testing.

To further minimize the risk of contamination of samples, reagents and laboratory surfaces and equipment, testing must be performed following a unidirectional workflow. The "clean" work is to be done before the "dirty" work, without going back into the clean areas once work in the dirty areas has been done. The table below illustrates the diagnostic workflow, procedural steps, specific inputs, required materials needed for each step in the process and the specific output of each step in the process in the molecular laboratory for SARS-CoV-2 testing. As the steps of the workflow progresses the contamination risk increases, as shown in **Figure 3**.

Figure 3: Molecular diagnostics workflow and relative genomic contamination risk.



A summary of the diagnostic workflow is shown in **Table 7**. A number of resources are available online that provide additional guidance on setting up a new molecular diagnostics laboratory (17–19).

Table 7: Summary of a SARS-CoV-2 Diagnostic Workflow.

Testing Phase	Procedural Step	Input	Material needed	Output	Area
Pre-Analytical	Sample collection, packaging, transportation, and receipt at the laboratory	A sample from the patient to test for the presence of SARS-CoV-2	+ A swab to collect the sample + Transport Media in a collection tube to transport the sample without degradation + Triple packaging	A sample for PCR analysis	General sample reception area
Analytical	RT-PCR master mix preparation	PCR reagents (in-house or kits)	+ Primers and Probe (SARS-CoV-2 specific) + dNTPs (building blocks of nucleic acids) + Polymerase (Enzyme) + Reverse Transcriptase (RT) + PCR buffer + Positive Control (specific to SARS-CoV-2) + No Template Control	RT-PCR Master Mix	Pre-amplification (ultra-clean)
	RNA nucleic acid extraction	A sample for PCR analysis	+ Extraction System + Extraction Reagents including: - Lysis Buffer - RNA Extraction Control - Human Specimen Control - Positive Control specific to SARS-CoV-2	Extracted nucleic acids	Sample processing area (clean)
	RT-PCR amplification and detection	Extracted nucleic acids & RT-PCR master mix	RT- PCR instruments	Fluorescence output signal indicating presence or SARS-CoV-2 RNA	Amplification/ post-amplification (dirty)
Post-analytical	Results reporting	Fluorescence output signal indicating presence of SARS-CoV-2 RNA	COVID-19 IVD Software	Report of output indicating presence of SARS-CoV-2 virus	Amplification/ post-amplification (dirty)

PREVENTING PCR CONTAMINATION

Contamination refers to the erroneous introduction of viral RNA, or previously amplified nucleic acid products (amplicons) or other contaminants or inhibitors into the sample or the PCR reaction tube. Whatever the source of the contaminant, an incorrect diagnosis is likely to occur. Prevention of PCR contamination is equally critical during the assay development process. Examples of good contamination-prevention practices are provided in **Appendix 6**.

MEASURE OF UNCERTAINTY FOR SARS-COV-2 MOLECULAR ASSAYS

The ability of the assay to detect the target sequence(s) are hindered by various factors and thus the limitations of each LDT for COVID-19 detection must be determined. The semi-quantitative nature of SARS-CoV-2 assays allows for the detection of the SARS-Cov2 specific gene(s) of interest for example, but what is the LoD of the assay and what factors could influence the reliability of the newly designed assay?

The viral load in the sample determines the amount of cycles needed for the time-to-detection during the exponential phase of the PCR reaction and can be used deductively to estimate viral shedding (grade of infection). What is the impact on the assay's usefulness if the sample is older than 5 days, or if proper temperatures were not maintained during sample transport? Such deviations from the intended protocol may negatively affect the accuracy of the assay. Sources of uncertainty can occur in both the pre-analytical and analytical phases of the diagnostic process, as described below.

PRE-ANALYTICAL SOURCES OF UNCERTAINTY

+ Sample collection and storage prior to testing
  - The assay was only validated on certain collection devices and certain types of transport media. For example, the transport media may contain guanidine thiocyanate and thus these samples cannot be used if the nucleic acid extraction procedure makes use of sodium hypochlorite as cyanide gas will be produced when the guanidine thiocyanate containing transport media is exposed to bleach.
  - Blood-stained swabs will inhibit the PCR reaction.
  - Samples collected using the wrong collection device. For example, samples collected using calcium alginate swabs, cotton-tipped swabs or swabs with wooden shafts are not suitable for use with SARS-CoV-2 testing.
  - Samples collected outside of viraemic phase could result in false negatives due to the level of viral RNA being below the assay's LoD.
  - Samples delivered late or stored improperly before testing, i.e. samples older than 5 days or which were not aliquoted into cryogenic vials and frozen at or below -70°C prior to testing, could result in false negatives due to RNA degradation.

ANALYTICAL SOURCES OF UNCERTAINTY

False negatives may arise from:

- Improper sample collection.
- RNA degradation
  - Degradation of the viral RNA during transportation and/or storage.
  - Degradation by RNAses if RNAse inhibitors was not added to the Reverse Transcriptase step.
- The use of unauthorized extraction or PCR reagents.
- The presence of RT-PCR inhibitors.
- Mutations in the SARS-CoV2 virus.
- Failure to follow the standard operating procedures
- The use of staff not fully trained or competent.

False positives may arise from:

- Contamination
  - Cross-contamination during sample handling or preparation.
  - Cross-contamination between patient samples.
  - Sample mix-up.
  - RNA contamination during reagent and/or PCR component handling if AmpErase UNG is not used.
  - The use of powdered gloves and non-filtered pipette tips.

Interpretation Limits

- The software used to evaluate the data might be outdated.

Limit of detection (LoD)

- The assay is dependent on the amount of initial sample material required (e.g. a minimum of 700ul) or the amount of amplicon added to the detection step.

Analytical specificity

- A negative result for any PCR test does not conclusively rule out the possibility of infection.
- The test cannot rule out diseases caused by other pathogens.

QUALITY ASSURANCE FOR PCR TESTING

Quality Assurance is a critical component of laboratory testing for diagnostic services for COVID-19. It is essential that each sample is handled (from time of collection until received in the laboratory), processed, amplified, and detected, and results recorded and reported as per the laboratory's SOPs. Each of these steps is examined frequently by means of QA activities, including:

- Specimen transport time is monitored by checking the time of collection with the time of receipt, and the cooler box, if used, is checked to see if the ice packs are cold
- Discrepancies regarding samples and sample documentation are tracked (for KPI purposes) and resolved in a timely manner.
- Laboratory instrumentation is used, maintained and calibrated per the laboratory's SOPs

+ Laboratory personnel are properly trained before handling samples without supervision. Demonstrations of capability can be used to document competence
+ Recovery of viral RNA is monitored through the use of a known positive control in the sample extraction / processing procedure
+ Performance of the extraction and PCR reagents is monitored by QC testing the reagents with a known viral load
+ Accuracy of the SARS-CoV-2 virus identification test is monitored through the use of positive and negative controls
+ Proficiency of test accuracy and technical competency are monitored by testing spiked samples in a proficiency testing (PT) and external quality assessment (EQA) panel
+ Internal quality control (IQC) samples, analyzed in the same manner as unknown samples, are used to assess the validity of the analytical results. False negative and false positive data for QC samples is analyzed routinely
+ Number of invalid results are examined daily, investigated, and tracked for trending data
+ Corrective Action processes are initiated and utilized as needed to determine root causes(s) and ensure specific errors are not repeated
+ Data and final reports are recorded on controlled forms or systems and are reviewed by at least a first and second reviewer before final approval and submission.

The measures put in place to monitor assay performance as well as the frequency thereof are done and determined internally by the laboratory itself (as described above) and by an accredited EQA service provider found to be competent to provide external PT panels as per ISO 17043 (20). Some examples of PT providers who can provide SARS-CoV-2 panels are included in Appendix 7.

QUALITY INDICATORS

In order to monitor the quality of the laboratory, the laboratory must develop, implement and maintain various quality indicators or key performance indicators (KPIs). QIs/KPIs refers to collection and analysis of data at each step of the testing process that can serve as indicator for correct performance of the whole testing process. KPIs include the following, and should be analyzed and reported on a regular basis, at least monthly:

+ Number of specimens tested, by specimen type
+ Number (%) of positive, negative and invalid test results
+ Specimen rejection rate
+ Number (%) of failed IQC results
+ EQA/PT performance (pass/fail or % score)
+ Turnaround time (TAT)
    - % of results reported within target TAT
    - average and range of TAT

CONCLUSION

When a new virus emerges, as SARS-CoV-2 did in December 2019, laboratories must rely on time-tested methods of developing diagnostic tests in order to meet their needs when commercial assays are unavailable. These LDTs are rarely perfect on the first attempt—it takes many people multiple cycles of trial, error, validation and verification before new, sensitive and specific diagnostic tests can be put into use. Ideally, these tests continue along the path of development and validation until they are formally certified for *in vitro* diagnostic use by an appropriate regulatory body, such as the United States Food and Drug Administration or a European notified body like TÜV Rheinland LGA. Most LDTs do not progress to the point of formal certification for use; however, they should still be held to the highest standard of quality possible in order to mitigate the health and economic impacts of the emerging disease and help protect our most vulnerable populations.

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9. Primer validation For Optimum Assay Performance - PCR Guide | Sigma-Aldrich. https://www.sigmaaldrich.com/technical-documents/articles/biology/assay-optimization-and-validation.html
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APPENDICES

DISCLAIMER: The systems, suppliers, services, and products listed in these appendices are a small sample of the resources available for global purchase and distribution. The lists are being provided as a convenience and for informational purposes only; they do not constitute an endorsement or an approval by the authors of any of the products, services or opinions of the corporations or organizations listed. Additional lines are provided in the tables to allow the users of this guide to make note of any additional systems, suppliers, or products they use or with which they are familiar.

APPENDIX 1: SELECT RNA EXTRACTION KITS AND AUTOMATED SYSTEMS

Manual Extraction Kits

Manufacturer	Example kit	Website
Beckman Coulter	RNAdvance Viral	https://www.beckman.com/reagents/genomic/rna-isolation/viral
Invitek Molecular	Invisorb Spin Virus RNA Mini Kit	https://www.invitek-molecular.com/products/infectious-diseases.html
Qiagen	QIAamp Viral RNA Mini Kit	https://www.qiagen.com/us/products/diagnostics-and-clinical-research/sample-processing/qiaamp-viral-rna-mini-kit/#orderinginformation
Roche	High Pure	https://lifescience.roche.com/en_us/search-results.html?searchTerm=high%20pure
Thermo Fisher Scientific	PureLink™ RNA Mini Kit	https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/rna-extraction/rna-types/total-rna-extraction/purelink-rna-mini-kit.html
Takara	NucleoSpin/ NucleoMag Virus	https://www.takarabio.com/products/nucleic-acid-purification/viral-dna-and-rna-purification-kits

Automated Extraction Systems

Manufacturer	Example kit	Website
bioMérieux	NUCLISENS® easyMAG®	https://www.biomerieux-usa.com/clinical/nuclisens-easymag
Perkin Elmer	Chemagi Prepito®	https://www.perkinelmer.com/product/chemagic-prepito-2022-0030
Qiagen	EZ1 Advanced XL, QIAcube, QIAsymphony	https://www.qiagen.com/us/products/instruments-and-automation/nucleic-acid-purification/
Roche	MagNA Pure	https://lifescience.roche.com/en_us/brands/magnapure.html#magna-pure-systems
Thermo Fisher Scientific	KingFisher	https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/automated-purification-extraction/kingfisher-instruments.html

APPENDIX 2: MINIMUM REQUIRED EQUIPMENT

Pre-Amplification	Nucleic Acid Extraction	Amplification & Detection
Dedicated micropipettes	Dedicated micropipettes	Dedicated micropipettes
-20°C freezer	-20°C freezer	-20°C freezer
PCR workstation ("dead air" box *or* laminar flow)	Biosafety cabinet (class II A2, class II B2, class II C1, or class III) or negative-pressure portable glovebox	Biosafety cabinet (class II A2, class II B2, or class II C1)
Vortex	Vortex	Real-time PCR system
Microcentrifuge	Automated nucleic acid extraction system or microcentrifuge (aerosol containment lid preferred)	Standard thermocycler (gradient temperature preferred)
		Gel electrophoresis system (power supply, running chamber, casting tray & combs or precast gels)
		Microwave (if preparing own gels)
		Balance (if preparing own gels)
		UV light table or gel imaging system

APPENDIX 3: SELECT REAL-TIME RT-PCR SYSTEM MANUFACTURERS

Manufacturer	Example system	Website
Agilent	Mx3000P	https://www.agilent.com/en/product/real-time-pcr-(qpcr)/real-time-pcr-(qpcr)-plastics-supplies/plastics-supplies-for-mx3000p-3005p-qpcr-system/mx3000p-qpcr-system-232710
Applied Biosystems	ABI 7500/7500 Fast Dx, QuantStudio	https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-instruments.html
Bio Molecular Systems	Mic	https://biomolecularsystems.com/mic-qpcr/
Bio-Rad	CFX96	https://www.bio-rad.com/en-us/category/real-time-pcr-detection-systems?ID=059db09c-88a4-44ad-99f8-78635d8d54db
Roche	LightCycler 2.0, LightCycler 96	https://lifescience.roche.com/en_us/brands/realtime-pcr-overview.html#qpcr-instruments

APPENDIX 4: SELECT PRIMER AND PROBE DESIGNERS AND SYNTHESIZERS

Automated Extraction Systems	
Company Name	**Website**
National Center for Biotechnology Information (United States)	https://www.ncbi.nlm.nih.gov/tools/primer-blast/

Primer and Probe Design and Synthesis	
Company Name	**Website**
BioCat	https://www.biocat.com/genomics/real-time-pcr-custom-probes-and-primers
Eurofins Genomics	https://www.eurofinsgenomics.eu/en/dna-rna-oligonucleotides/
Eurogentec	https://www.eurogentec.com/en/custom-manufacturing
GenScript	https://www.genscript.com/molecular-biology-service.html?src=pullmenu
IDT	https://www.idtdna.com/Primerquest/Home/Index
OriGene	https://www.origene.com/products/gene-expression/qpcr/primer-pairs
Sigma Aldrich	https://www.sigmaaldrich.com/technical-documents/articles/biology/oligoarchitect-online.html
Thermo Fisher Scientific	https://www.thermofisher.com/us/en/home/life-science/oligonucleotides-primers-probes-genes/applied-biosystems-custom-primers-probes.html
Tib MolBiol	https://www.tib-molbiol.com/

APPENDIX 5: SELECT MASTER MIX SUPPLIERS

Name	Website
Bio-Rad	https://www.bio-rad.com/en-us/category/pcr-reagents-qpcr-reagents?ID=M87EKA15
GeneOn	https://www.geneon.net/products/rt-pcr-reverse-transcription/lyophilized-mastermix-for-rt-pcr-1/
Norgen Biotek	https://norgenbiotek.com/category/pcr-mastermix
Promega	https://www.promega.com/products/pcr/
Takara	https://www.takarabio.com/products/pcr/pcr-master-mixes
Techne	http://www.techne.com/product.asp?dsl=7085
Thermo Fisher Scientific	https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-reagents/one-step-real-time-rt-master-mix.html

APPENDIX 6: CONTAMINATION-PREVENTION PRACTICES

AREA 1: PRE-AMPLIFICATION AREA

The following precautions are recommended:

- Daily workflows should be planned to ensure no reentry into Area 1 if Areas 2 or 3 were entered
- Absolutely no specimens or amplicons are to be brought in or allowed to enter this room
- No reagents removed from this room should be brought back into this room
- No cellphones/laptops/private belongings should be brought in or used in this room
- Pipettes and other required instruments should always be kept in this area and used exclusively for pre-PCR activities
- Each component should be set aside before working with the next component to avoid contamination of the preceding component with subsequent solutions
- If multiple rounds of PCR's on various platforms are to be conducted on the same day, all master mixes must be prepared consecutively and taken to the template addition area.
- All cleaning supplies and brooms for this room should be stored in this room
- Routine laboratory cleaning staff should not have access to this room so that contaminants from other areas are not unknowingly tracked in.

Weekly cleaning of the pre-amplification room

- At the end of each week, the pre-amplification room must be thoroughly cleaned.
- Cleaning should be top-to-bottom, starting with the bench tops and finishing with the floor.
- Once the upper surfaces have been cleaned, the floor should be swept with a broom.
- The floor should be mopped with 1% sodium hypochlorite, first using the leftover diluted bleach, and then preparing more 1% sodium hypochlorite if needed.
- Clean the mop under running water
- Spray and mop the floor with 70% alcohol.

Cleaning Bench tops including pipettes & PCR equipment

Daily method:

- Put on protective clothing, mask and gloves.
- Clean the bench tops and PCR equipment thoroughly before work is done in this room.
- As directed above, use 1% sodium hypochlorite first, followed after an interval of 30 seconds by 70% ethanol.
- After cleaning, the master mixes for the day should be prepared.
- Once the reagents have been made, the surfaces and PCR equipment should be cleaned again.

AREA 2: NUCLEIC ACID EXTRACTION AREA

Weekly cleaning

At the end of each week, the nucleic acid extraction area must be thoroughly cleaned.
Cleaning should be top-to-bottom, starting with the bench tops and finishing with the floor.
Once the upper surfaces have been cleaned, the floor should be swept with a broom.
The floor should be mopped with 1% sodium hypochlorite, first using the leftover diluted bleach and then preparing more 1% sodium hypochlorite if needed.

- Clean the mop under running water.
- Spray and mop the floor with 70% alcohol.

Biological Safety Cabinet (BSC)

Daily method:

- Put on protective clothing, mask and gloves.
- Clean the BSC thoroughly before and after each batch of specimens.
- 1% sodium hypochlorite should be used first, followed by 70% ethanol after an interval of 30 seconds.
- Clean the glass panel as well. Switch the hood's light off after cleaning.

Weekly method:

- Put on protective clothing, mask and gloves.
- Prepare sufficient paper or roller towels with 1% sodium hypochlorite and 70% ethanol.
- Remove the inner working surface of the BSC and clean it and the entire interior of the BSC thoroughly with 1% sodium hypochlorite and 70% ethanol as described above.
- The glass window of the hood should be cleaned to ensure that all smudging and smears on the glass are eliminated.
- Replace the metal inner working surface of the BSC.
- After cleaning is completed, perform a smoke test to ensure that the BSC is functioning properly, i.e. the smoke must be "sucked" into the vents and NOT escape into the outside environment.

AREA 3: AMPLIFICATION & DETECTION AREA(S)

Weekly cleaning of the amplification area(s)

- At the end of each week, the amplification room must be thoroughly cleaned.
- Cleaning should be top-to-bottom, starting with the bench tops and finishing with the floor.
- Once the upper surfaces have been cleaned, the floor should be swept with a broom.
- The floor should be mopped with 1% sodium hypochlorite, first using the leftover diluted bleach and then preparing more 1% sodium hypochlorite if needed.
- Clean the mop under running water.
- Spray and mop the floor with 70% alcohol.
- The used tips and completed PCR tubes and 96-well plates must be placed in appropriate waste buckets and autoclaved to destroy any contaminating amplicons.

Bench tops including thermal cycler, pipettes & PCR equipment

Daily method:

- Put on protective clothing, mask and gloves.
- Clean the bench tops and PCR equipment thoroughly before the work is done in this room.
- As directed above, use 1% sodium hypochlorite first, followed after an interval of 30 seconds by 70% ethanol.
- Carefully add the template material to each reaction tube and place the tubes in the thermal cycler or seal the 96-well plate with plastic foil and insert the sealed plate into the thermal cycler.
- After starting the thermal cycler run, clean the bench tops and PCR equipment as above.

Thermal cycler (PCR machine)

Weekly method:

- Put on protective clothing, mask and gloves.
- Clean the exterior of the thermal cycler thoroughly.
- Use 1% sodium hypochlorite or Contrad® first, followed after an interval of 30 seconds by 70% ethanol.

APPENDIX 7: SELECT COVID-19 PROFICIENCY TEST PROVIDERS

Name	Website
College of American Pathologists	https://www.cap.org/laboratory-improvement/international-laboratories/external-quality-assurance-proficiency-testing-for-international-laboratories
Instand (other coronaviruses)	https://www.instand-ev.de/en/eqas/eqa-program.html
Oneworld Accuracy	https://www.oneworldaccuracy.com/1wa/#/covid-19
Randox/ QCMD	https://www.randox.com/coronavirus-qcmd/
Thistle QA	http://www.thistle.co.za/coronavirus-proficiency-testing/