Application of nucleic acid amplification tests in managing COVID-19 pandemic

Serhiy Souchelnytskyi1, Nazariy Souchelnytskyi2,3

1 College of Medicine, QU Health, Qatar University, Doha, Qatar,
2 Angstrom Laboratory, Polacksbacken, Uppsala University, Uppsala, Sweden,
3 Oranta Cancer Diagnostics AB, Uppsala, Sweden

Background. COVID-19 pandemic highlighted the importance of sensitive and specific tests that would be cost-efficient, fast and scalable. There are more than 200 COVID-19 detection tests available worldwide, with every country developing its own assays. Sample collection, preparing for a test, the test itself and interpretation of results have a strong impact on the clinical value of testing. The diversity of tests and workflows requires the analysis of their performance in clinics.

Methods. Literature review, analysis of clinical reports, online resources, public and commercial reports were used to collect information about tests. The collected information was processed to obtain information relevant to this review.

Results. COVID-19 tests based on the amplification of nucleic acids are reviewed. Tests employ polymerase chain reaction (PCR) or loop-mediated isothermal amplification (LAMP). The clinical value of these tests depends on the technologies used, as they differ for LAMP, real-time and standard PCR methods. The diversity of sample preparation protocols, different designs of the tests, used chemicals and protocols have a significant impact on tests. Tailoring a testing workflow to available infrastructure and selecting the most efficient combination of tests and protocols for each step in a testing workflow is crucial for the success.

Conclusion. Strengths and weaknesses of different test systems and protocols that were reviewed herein can be helpful in selecting a testing workflow to achieve maximum clinical utility.

Keywords: COVID-19, detection test, PCR, LAMP
Використання тестів базованих на ампліфікації нуклеїнових кислот для контролю коронавірусної (COVID-19) пандемії

Сергій Сушельницький1, Назарій Сушельницький2,3

1 Медичний Коледж, КУ Здоров’я, Н12, Катарський Університет, Аль-Тарфа, Доха, Катар
2 Ангстрем лабораторія, Упсальський Університет, Упсала, Швеція
3 Оранта Діагностика Раку АБ, Упсала, Швеція

Коронавірусна пандемія показала необхідність чутливих та специфічних тестів, які були б також недорогі, швидкі та могли б застосовуватись у великих об’ємах. На серпень 2020, заздекларованими є більше, ніж 200 різних тестів на визначення коронавірусу. Кожна країна розробляє власні тести. Відмінності між тестами та способами їх застосування вимагають детального аналізу.

Представлено огляд літератури, аналіз звітів з клінік, документації від державних, громадських та комерційних організацій, які використано для аналізування ефективності коронавірусних тестів, що грунтуються на ампліфікації нуклеїнових кислот.

Проаналізовано робочі протоколи тестувань зі застосуванням полімеразної ланцюгової реакції (ПЛР) або ізотермічної кільцевої ампліфікації (LAMP). Різноманітність протоколів підготовки експрес-тестів, відмінності у виконанні тестів та протоколів оцінки результатів мають істотний вплив на чутливість та вібріковість тестів. Цю різноманітність узагальнено з акцентом на позитивні та критичні параметри робочих протоколів кожного з етапів тестування. Таке аналізування може бути корисним при виборі тестування.

Представленний огляд дозволить оцінити та вибрати протокол для тестування на коронавірус відповідно до клінічних вимог та наявної інфраструктури.

Ключові слова: коронавірус, тести на детекцію, ПЛР, ЛАМП

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Introduction. The workflow of COVID-19 testing includes the collection of a sample, testing on-site at the point of care (POC) or transportation to a laboratory, testing with the use of advanced tools, interpreting results. By August 1, 2020, 125 COVID-19 test systems have been approved by the Food and Drug Administration (FDA) in the USA (https://www.fda.gov/medical-devices/ coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas), and more than 100 test systems were registered in China (see references at http://ph.china-embassy.org/eng/sgd/PhP0200200324570010409522.pdf) and the European Union countries (see references at https://ec.europa.eu/jrc/en/news/coronavirus-testing-information-test-devices-and-methods-single-place). The official website of the Ukrainian government reports the use of German and Chinese test systems, not specifying their number and types (https://covid19.gov.ua/en). The number of reported test systems is most probably an underestimation, taking into account numerous developments in research laboratories and the legal recognition of laboratory-developed tests on the same level as in-vitro diagnostics [1-3]. Moreover, an analysis of the logistic of COVID-19 testing showed the importance of performing tests at core laboratories in the hospitals and points of care instead of outsourcing them to central laboratories [4]. Hospitals’ core laboratories decreased the turn-around time from 21 to 3.7 days as compared to the outsourcing of testing [4].

COVID-19 detection tests are classified into 2 types - nucleic acid detection and serological/immunological tests. Herein, we focus on nucleic acid amplification-based tests. All reported workflows of COVID-19 testing include sample collection, preparation for a test, the test itself and interpretation of results (Figure 1). Variations are in the origin of the sample, the time for collection, conditions of preparation and transportation of the sample, test type, logistics, e.g. POC or outsourcing, and clinical interpretation of results.

There is no test that would secure 100% sensitivity and specificity. The efficacy of testing depends on the probability of detecting the infection and is the main concern concerns the selection of a workflow. For example, viral nucleic acid detection is dependent on the viral load in a selected type of the sample over the course of infection (Figure 2) [5,6]. The highest viral load
in nasal and oropharyngeal swabs was observed upon the onset of symptoms. However, even at the points of the highest viral load, the probability of virus detection has never been 100%. It was reported that only 42% of people who died from COVID-19 tested positive for COVID-19 [7]. Failure to detect COVID-19 could be due to variability in the efficacy at any stage of the workflow. Examples of test specificity and sensitivity are reported to range from 90% to 80% [6-9]. Therefore, biomedical variables, e.g. viral load and technical suitability of test workflow are crucial for the interpretation of results. To mitigate potential failure, multiple tests are ordered for a patient suspected to be infected; e.g. reportedly, the conclusion on the absence of infection may require up to 4 tests per person and at least 2 subsequent negative tests [6-9].

Therefore, accumulated clinical experience shows that there is a need for multiple testing and a clinical decision should include the interpretation of the patient’s clinical condition (Figure 3). The following sections focus on each step of the workflow starting with a sample collection, followed by the point of care tests and tests in centralized laboratories, and then by the interpretation of results with emphasis on lessons learned from the tests based on the amplification of COVID-19 nucleic acid.

Search strategy
The PubMed database was searched with the Medical Subject Headings (MeSH) search terms “COVID-19”, “detection”, “test”, “PCR” and “LAMP”. Collected publications were screened manually for a description of COVID-19 detection methods. The same terms were used to search Google, as there are many publications deposited online but not represented on PubMed, e.g. bioRxiv.org. The third source of searches were websites of agencies involved in fighting COVID-19, e.g. who.int, www.fda.gov, www.ema.europa.eu and moz.gov.ua. The fourth source of information were online resources of companies producing COVID-19 detection kits. Inclusion criteria were 1) a description of types and performance of COVID-19 detection kits, 2) description of technologies, reagents and protocols used in COVID-19 testing and/or 3) analysis and comparison of different kits and protocols. The exclusion criterion was the lack of detailed information about the kit, e.g. no description of the technology, no information about reagents and a lack of detailed protocol. This search was last updated on the 15 August 2020.

Sample collection
The best option for sample collection would be self-collection at the time of the highest
viral load using a sample solution preserving COVID-19 RNA during storage and transportation. The importance of sample collection is emphasized by the report stating that suboptimal sampling contributes significantly to an increase in false-negative COVID-19 testing results [10].

Reportedly, COVID-19 was detected in all tested sources, i.e. nasopharyngeal, bronchoalveolar lavage (BAL), sputum, saliva, cerebrospinal fluid (CSF), plasma and stool [11-16]. Multiple sample sources reflect a broad range of tissues and cells directly affected by the virus [11,17]. Endothelial, epithelial, myoepithelial, smooth muscle, hepatocytes, neurons and glial cells were identified as targeted directly by COVID-19 [17].

The important observation is that virus detection is more dependent on the course of disease than on the sample source [3,6,7]. The stage of symptom appearance may have the highest viral load, while the load is lower at the stages of initial infection, recovery and after-recovery (Figure 2). The detection limit of most tests ranges from 5 to 10 viral RNA molecules per reaction, which corresponds to the viral load in the range of $3 \times 10^4$ RNA copies/ml [18]. If the load of the viral RNA is below $1 \times 10^3$ copies/ml in a sample, this would require reconsideration of the source of sampling and/or a need for concentration of the RNA.

Currently, the most frequent source of samples includes nasal and oropharyngeal swabs. More than 160 designs of swabs have been reported [12]. The use of these swabs was similar, provided that its material did not interfere with the extraction of nucleic acids and PCR reaction, e.g. swabs must not contain cotton, wood or calcium alginate [12].

Collection solutions, on the other side, may have a significant impact on the preservation of the viral RNA and compatibility with subsequent testing. For example, the use of Variplex system without RNA extraction in a LAMP test led to 83% false-negative rate [13]. Therefore, a sample solution must be compatible with transportation, storage conditions and the type of test to be used. Before embarking on the full-scale testing, compatibility of the sample solution with the planned sample type should be tested using control virus-contain-
ing samples, positive and negative controls with defined concentrations of the viral RNA molecules.

There have been numerous attempts to minimize variability in sample collection. These include heating of samples and adding organic solvents and detergents. The rationale is that heating would lead to denaturation of molecules in the sample, including RNAases [19-21]. Organic solvents and detergents are expected to produce a similar result, i.e. inactivation of RNA degradation enzymes [19-21]. The additional effect is the dissociation of RNA-containing viral particles in the presence of detergents and subsequently, the release of RNA into a solution.

The report by Pan et al. showed that sample heating increased Ct of detection, indicating decreased sensitivity [5]. Another heating testing (at 56°C to 65°C) showed no differences as compared to non-treaded sample [22]. Adding ethanol to a sample solution also had an inhibitory effect on the microbial growth in the nasal, oropharyngeal swabs and saliva [20,22]. Strong detergents may inhibit the PCR reaction. For example, even low concentrations of sodium dodecyl sulfate, i.e. 0.1%, strongly inhibited PCR reaction. The inhibitory effect was also observed with detergents of Triton X-100 type at concentrations higher than 1.0% [20,21]. The use of additives, like ethanol or detergents, should be pre-tested for every workflow to take advantage of blocking RNA/DNAases, sterilization and solubilization of viral RNA, and to avoid any negative impact on the reactions of reverse transcriptase and nucleic acid amplification.

Therefore, when selecting a sample collection protocol, consideration should be given to a) the type of sample and collection method, e.g. swab, saliva, stool and sample solution compatibility; b) expected viral load in collected samples, to ensure that LOD of the test would allow detecting the infection; and c) preservation of viral nucleic acids in the sample solution upon collection and transportation.

Sample preparation
RNA in collected samples has to be accessible for amplification. The most common approach is to purify RNA and then use it in reverse transcriptase and amplification reactions. There are many commercial kits for RNA purification. The quality of kits is generally good, no serious issues have been reported. The only consideration in terms of selecting a purification protocol is the cost of the kit, requirements concerning the tools, reagents and personnel.

For example, the cost of kits vary from 4 to 10 USD per sample (see an example of Sigma Aldrich/ Merck at: https://www.sigmaaldrich.com/life-science/molecular-biology/molecular-biology-products.html?TablePage=9618834; an example of Qiagen available at: https://www.qiagen.com/kr/products/discovery-and-translational-research/dna-rna-purification/rna-purification/total-rna/measy-mini-kit/?clear=true#orderinginformation). Kits vary by mechanisms of RNA purification, reagents and consumables. However, most kits deliver good results when used strictly following recommended protocols.

RNA purification can be performed manually or in an automated way. The best solution is to use the kit and the purification device from the same manufacturer. QIAcube or QIAcube HT can serve as examples of Qiagen purification kits (https://www.qiagen.com/kr/search/products?query=qiacube). Automation comes with a price tag and may add from 15,000 to 25,000 USD to the cost of the testing investment.

RNA purification stage may also be prone to failures, especially when the viral load is low. Therefore, there were attempts to develop protocols that would omit the nucleic acid purification step. Circumventing RNA purification significantly improves and facilitates on-site POC testing. Several reports show COVID-19 markers in nasopharyngeal swabs [15,21] and saliva [14,20]. A direct comparison with the protocol including RNA purification showed similar detection accuracy and reliability. The only concerns were about the potential decrease of LOD in presence of strong denaturants in the sample solution, e.g. SDS, and interfering components in the sample itself, e.g. mucin, enzymes, etc.

Therefore, the stage of sample preparation offers options with or without RNA purification. If the sample is used for further studies of COVID-19, e.g. sequencing, RNA purification is
recommended. This would add additional cost and require specialized equipment and trained personnel. If the sample is used only to detect the virus, RNA purification may be omitted. Omitting RNA purification makes testing faster, cheaper and more reliable. However, the compatibility of the sample type with a direct detection test should be evaluated due to the potential interference of sample components. This is performed by spiking intended samples with controlled quantities of the viral RNA and measuring the limit of detection.

**Nucleic acid amplification tests, general comment**

Monitoring COVID-19 pandemic requires tests to be used at points of care (POC) and tests requiring advanced laboratory infrastructure. POC tests could be performed on-site by the personnel with minimum training and without advanced laboratory infrastructure. These tests would be employed for testing a large number of people in a short time. Examples include airports, ports of entry, and places of large people gatherings, e.g. industrial areas or rallies. The second type of tests is performed in a laboratory. Laboratory-based tests are essential in order to monitor infected people, confirm their recovery, purify, sequence and study the virus.

There is no sharp discrimination of these two application types by technologies employed in the tests. PCR and LAMP amplification can be employed in POC and laboratory-based tests. The design of devices and instruments defines whether the test is suitable for POC or central laboratory-based detection. Small tools even allow real-time PCR using a small benchtop instrument with minimum requirements to sample preparation. An example of such approach is reported by Wee and colleagues [23]. LAMP is usually used for POC tests, as it does not require expensive tools. LAMP detection can be performed using any device that maintains a constant temperature, e.g. a heating block or a thermostat.

Tools are becoming cheaper and more compact. On the contrary, the cost of consumables and reagents is a significant part of testing expenses. In addition, the miniaturized and automated tools use dedicated consumables. It limits the use of these tools to these unique consumables and minimizes flexibility of assays.

**Loop-mediated isothermal amplification (LAMP) tests**

The application of LAMP to detect COVID-19 has been successful. Some publications reported and review LAMP assays to detection COVID-19 [24-29]. Herein, we focus on criteria to consider when selecting a LAMP test (Figure 4). Recognition and amplification of the targeted viral sequences are dependent on the specificity of primers, the temperature of the reaction, buffer composition, pH, and presence of interfering substances from the sample. The efficacy of reverse transcriptase and a DNA polymerase also affects test performance.

There are no reported warnings for targeting specific COVID-19 genes and avoiding others. The consensus is that the targeted region is not crucial, as long as the sequence is unique for COVID-19 [29]. Similarity search tools, e.g. BLAST of NCBI, are a good option to find primers that would be unique to COVID-19 with no overlap with other species and genes, as they detect only COVID-19.

LAMP methodology is based on the recognition of 6 sequences of the targeted gene, followed by a building and amplification of a nucleic structure representing targeted sequences, and the detection of this amplified structure [27-29]. The positioning of target sequences allows LAMP primers to build a structure that would be self-amplified. The key to performance of a LAMP test is primers design (Figure 4).

It is almost impossible to manually design LAMP primers targeting 6 sequences in the viral genome in LAMP-required positions that would ensure comparable annealing parameters. Many dedicated tools used for the design of LAMP primers are available online. Examples can be accessed at https://primerexplorer.jp/e/v4_manual/pdf/PrimerExplorerV4_Manual_1.pdf, or http://www.premierbiosoft.com/isothermal/lamp.html, or http://loopamp.eiken.co.jp/e/lamp/primer.html. Similar annealing properties of primers are essential for the initial amplification and formation of double-loop structures. These double-loop structures would be then amplified. When the
structure is formed, the amplification from the viral template is not maintained any more. The amplification is dominated by the DNA synthesis from the formed structure. Therefore, the applicability of the LAMP test is strongly dependent on the recognition of targeted viral sequences by primers during the initial phase of double-loop structure formation.

There is no visualization of amplification products in the standard LAMP test, e.g. the size of generated DNA products cannot be controlled. The LAMP signal is dependent on the quantity of synthesized DNA and the type of DNA detection. For example, for detection using pH-sensing dyes, a buffering capacity of the reaction should be not higher than 1 mM for a Tris buffer [30]. Frequently used pH-sensing dyes, e.g. phenol red, cresol red, neutral red, hydroxy napthol blue, could detect the accumulation of DNA at an initial level of 3 to 30,000 copies in a reaction mix. This level of detection is comparable to real-time and classical PCR [30]. Direct comparison of the quantities of generated DNA in a LAMP and PCR assays is not relevant because the limit of detection plays a more important role, e.g. sensitivity of the detection method is crucial.

To detect the virus using DNA-interacting dyes, the capacity of dyes to inhibit the amplification reaction has to be considered. Quyen and colleagues tested 23 dyes and showed that some of DNA fluorescence dyes can inhibit LAMP reaction. The high inhibitory effect was reported for POPO3, DCS1, SYBR Green I, BOBO 3, Pico 488, and TOTO 3 dyes. Dyes SYTO 9, SYTO 13, SYTO 16, SYTO 64, SYTO 82, Boxto, Miami Green, Miami Yellow, and Miami Orange were found not to interfere with the amplification of DNA [31]. Frequently used cresol red, neutral red and phenol red dyes have not been reported as inhibitors of the LAMP reaction. For the use of other dyes, a comparison test is recommended adding dyes before and after the reaction, followed by monitoring of generated DNA products by an agarose gel electrophoresis.

LAMP was successfully used to detect COVID-19 in versions with and without RNA purification [32]. The authors targeted N-gene of the virus. Detecting a positive signal with the LAMP test was comparable with Ct below 30 cycles for a real-time PCR [32]. This indicates that the LAMP assay can be as sensitive as the real-time PCR.

Therefore, to develop an efficient LAMP test, optimization trials have to address: a) the design of primers, e.g. computer-assisted design is required, b) sample collection conditions should be optimal and composition of the sample collection solution should not interfere with LAMP, e.g. no detergents or nucleases, c) selecting amplification conditions (buffers, enzymes, additives and the protocol should allow efficient amplification and detection), and d) the detection system should allow efficient detection, e.g. by selecting DNA dyes/fluorescence, pH-sensing or pyrophosphate precipitation (Figure 4).

**PCR tests: real-time reverse transcriptase and standard reverse-transcriptase tests**

PCR tests are the golden standard for COVID-19 detection. PCR reaction is highly specific, has high fidelity, solid technology development and ensures high detection specificity and sensitivity. A real-time reverse transcriptase (qRT-PCR) and standard reverse transcriptase (RT-PCR) use the same PCR principle, but different combinations of primers and different methods of signal generation and detection (Figure 5).

Real-time PCR (qRT-PCR) is the most frequently used technique to detect COVID-19. It is explained by robust development of its theory, reagents, protocols and tools. The success of qRT-PCR is also dependent on the automation and simultaneous amplification and detection of the product. The majority of approved COVID-19 detection tests are based on qRT-PCR (to see examples, see www.fda.gov/medical-devices and ec.europa.eu). They provide a good balance of high-quality PCR-based detection and a reasonable level of automation. However, some issues must be controlled to ensure high performance of tests, which are discussed in this section.

Standard RT-PCR is more laborious as compared to qRT-PCR. To assess RT-PCR result, the generated product must be visualized. Agarose gel electrophoresis is a standard technique for visualization. When the analysis quality has to be the highest, RT-PCR is
the first choice. The visualized product shows the size and can be sequenced for validation. Sequencing of the generated product is also used for monitoring of mutations in the viral genome. The sequencing of RT-PCR products provides data for monitoring viral strains and subsequent spreading of the disease. Viral mutations may affect treatment strategies too. Therefore, if COVID-19 testing requires the highest quality and/or is to be combined with a study of COVID-19 virus, standard RT-PCR is the method of choice (Figure 5).

When selecting a qRT-PCR or RT-PCR test for a clinical application, the entire workflow must be designed. The test must be compatible with sample collection and preparation protocols. The specificity of primers, conditions of the reaction, specificity of tools, and available laboratory infrastructure are other concerns.

Failure to develop a proper workflow design may lead to low sensitivity and specificity. Recent reports show that qRT-PCR tests may not always detect positive cases, giving a false-negative value in 80% of cases [9]. This means that many positive cases are missed. Such a test may subsequently fail in preventing the infection spread. The analysis shows that the reason could be in a non-optimal workflow, and not in the performance of qRT-PCR reaction itself. Negative results may be the result of sample collection and preparation, where the viral RNA has low stability and losses of RNA during purification and interference with the efficacy of PCR reaction [9]. This calls for positive controls in samples too, not only a positive technical control of the detection system. In clinical practice, it is ensured by spiking a sample upon collection with a known quantity of COVID-19 genomic marker, e.g. adding an aliquot of the sequence probe targeted in the test DNA.

To ensure successful completion of qRT-PCR and RT-PCR tests, different combinations of primers and multiplexing have been tested [33]. Primers targeting nucleocapsid (N), membrane protein (M), spike (S), envelop (E), nsp2, RNA-dependent RNA polymerase / helicase (RDRP/Hel) and orf1a regions have been reported [20,23,34,35,36]. The conclusion is that the location of targeted sequences in COVID-19 genome does not influence detection. The design of primers to ensure COVID-19 specificity is crucial. Primers' specificity is easy to secure with available online tools, e.g. BLAST of NCBI (blast.ncbi.nlm.nih.}

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**Fig. 4. Critical steps of LAMP in the application to COVID-19 testing.**

The impact on LAMP performance of primer design, optimization of the conditions of LAMP reaction, evaluation of interfering substances in a sample and the quality of enzymes are annotated. The size of arrows indicates relative impact, e.g. large arrow indicates a strong impact. For description, see the text.
The second important point of securing primer detection specificity is an optimization of PCR reaction. An example of such optimization was demonstrated by Liu et al who reported the workflow of selecting well-performing primers for RT-PCR and digital droplet-RT-PCR [37].

Multiplexing improves testing by detecting multiple regions of COVID-19 genome. Two to four gene regions have been targeted in the multiplex PCR [34,36]. Park et al reported detection optimization by targeting RDRP, N, E, and S genes in a single qRT-PCR reaction [36]. The success of this optimization was due to product visualization by standard RT-PCR used for optimization, as qRT-PCR does not visualize products. 3-plexing detection limit reported by Ishige and colleagues was calculated as 25 copies of COVID-19 RNA per reaction [34]. Simultaneous detection of 4 genes (with 8 primers in one reaction) was reported by Liu et al [37]. Thus, the reported developments of COVID-19 PCR tests showed that primers can target all regions of the viral genome, and multiplexing up to 4 gene markers in one reaction is possible. The design of primers can be performed with available online tools while securing COVID-19 specificity. Primers for qRT-PCR have to be validated using RT-PCR and amplified product visualization.

The issues with PCR tests have been attributed to sample collection, RNA preparation and interference with PCR reactions. Storing viral particles and RNA upon collection, losses of RNA during purification and PCR reaction inhibiting substances are the main concerns (Figure 5).

RNA is highly sensitive to degradation. Stabilization of RNA upon collection has to be validated for sample collection solution. It is reported that Universal (UTM) and Viral (VTM) transport media are designed to preserve or lye virus particles. If the testing workflow presupposes RNA purification step that would remove all components of the transportation media, then there are no serious precautions to consider. RNA purification for COVID-19 tests is performed with the use of commercial kits. These kits are used for an automated or semi-automated procedure. The optimization of RNA purification step includes the evaluation of the lowest concentration of RNA in the
sample that the kit can recover from the sample to ensure the acceptable limit of detection.

The prevention of RNA degradation by following the collection and transportation protocol would be the only other requirement. The technical control over the purification and PCR reaction includes the detection of household human genes, e.g. RNAse P gene. If the workflow circumvents RNA purification step, the direct detection would require lysis of the sample, release and stabilization of RNA. It was reported that detergents, e.g. Triton X-100, Tween 20, in concentrations of up to 1% in the transportation medium were tolerated in a reverse transcriptase and PCR reactions. Snap-heating of the collected sample to 70°C and up to 120°C may be considered for sample preservation [19-21]. Thus, optimizing the testing workflow may require the evaluation of the transportation media (preserving or lysing), transportation conditions (frozen or +4°C), and direct detection or purification of RNA steps followed by the PCR reaction [19-21].

Test efficiency depends on primers, enzymes and reaction buffers. The design of primers was discussed above. Reverse transcriptase and DNA polymerases with and without exo-nuclease activity and a strand-displacement activity (e.g. Bst DNA polymerase for LAMP, Pfu and Taq DNA polymerases for PCR) are available from many suppliers. To select the enzyme, it is important to select the reaction mix, too. Enzyme suppliers offer the reaction mix to be used with their enzymes. As this master mix is already optimized with enzymes, it is recommended to evaluate proposed combinations first. If the proposed enzyme-master mix combination is not performing well, an alternative combination must be considered and tested. In some cases, it is possible to develop a special master mix, but it requires significant efforts to produce in-house enzymes.

For qRT-PCR tests, positive and negative controls are standard. In addition, to optimize tests with these controls, it is recommended to include the acquisition of the melting curve. The analysis of amplified products by electrophoresis is not performed, as the product is smaller and can be misinterpreted as primer dimers. qRT-PCR curves provide quantitative information, e.g. Ct values, which facilitates the interpretation of results.

Interpretation of RT-PCR results is straightforward using gel electrophoresis. The detection of amplification products of the expected size, and, if required, sequencing of these products provide a secured interpretation. Standard RT-PCR is semi-quantitative. However, visualization of the amplified products makes quantification less important for the interpretation of results. For the clinic, the result must be “positive” or “negative”, and the visualization of the product is sufficient for such a conclusion (Figure 5).

To optimize the testing workflow, it is recommended to include the detection of the endogenous human gene(s) in the testing, e.g. RNAse P gene [38]. This allows monitoring the entire workflow, while PCR positive and negative controls allow monitoring a PCR reaction.

The detection efficiency is dependent on the stage of the disease (Figure 1). An example of a low consistency between COVID-19 detection and CT changes in lungs may indicate that virus detection does not correlate with specific clinical symptoms [39]. This is a strong indication that COVID-19 detection must be interpreted in combination with all clinical information, e.g. symptoms, history of a patient’s health and travel pattern (Figure 3).

The financial drawback of PCR tests is the requirement for advanced tools and infrastructure. To take PCR-based tests to the bedside and clinics and healthcare providers on-site, portable devices (POC devices) are under development. Wee et al reported the development of the PCR tester for nucleocapsid (N) gene detection with LOD 6 copies of RNA per reaction from sputum and nasal exudate [23]. The readers are directed to the review of POC devices by Cheng et al. [8]. The performance of these devices is currently under evaluation, and if validated, it would significantly ease the load on laboratories.

To sum up, PCR-based tests are and will be the main standard in the detection and study of COVID-19. Multiplexing of qRT-PCR will increase its clinical value. RT-PCR is indispensable in the development of PCR-based tests and the study of COVID-19. Both qRT-PCR and
RT-PCR deliver reliable performance. However, workflow optimization is essential. Selecting the sample type, sample collection medium, storage and transportation conditions, RNA purification step, PCR test itself and interpretation of results must be performed with patients’ and control samples.

Conclusion
The success of the fight against COVID-19 is dependent on detection tests. Today, more than 200 tests are available in the market. Most of these tests perform well when manuals and recommended protocols are followed [40]. In addition to nucleic acid amplification tests discussed herein, immunological/serological tests, novel variants of testing by massive parallel sequencing [41] and digital droplet PCR [42] are coming into the market. However, testing does not consist in the tests only. Testing is a workflow that includes sample selection, collection, transportation, preparation for a test, the test itself and result interpretation (Figure 1). Moreover, the results of testing should be interpreted together with the examination and clinical symptoms (Figure 3). Tests detecting genetic material of COVID-19 are and will be used in the foreseen future, as they ensure the most reliable virus detection. Tailoring the testing workflow to the specifics of every healthcare provider would require the optimization of sample collection, detection and interpretation processes. This review highlighted some of the concerns of such optimization.

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