


Design considerations for point-of-need devices based on nucleic acid amplification for COVID-19 diagnostics and beyond

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Current COVID-19 diagnostics

Currently, the ongoing pandemic has created an unprecedented pressure for the development of various analytical assays and especially their immediate practical applications. A number of tests based on different principles such as immunoassay [1], electrochemistry [2], nucleic acid amplification tests (NAATs) [3] and others have been reported [4]. Immunoassays and NAATs are currently being utilized clinically, while systems based on several other principles did not go further than the acceptance of manuscripts for publication.

Rapid immunoassays detecting specific antigens are popular because potential patients can conduct those tests by themselves, and the results are obtained in a few minutes or less (Figure 1A). Nevertheless, the complexity of both the viral biology and the immune response presents a problem, leading to commercial tests with different sensitivities of SARS-CoV-2 antigen detection and frequent false-negative results. The antigen dose required to exceed the limit of detection is significantly higher compared with NAATs [1,5], making the window for antigen-based false-negative results larger (Figure 1B). Thus NAATs are the heart of any meaningful portable system such as point-of-need (PON) tests with the required high specificity and selectivity, as shown in Figure 1A.

The main type of NAAT used is qRT-PCR, which became the gold standard of SARS-CoV-2 NAATs [7]. Single-stranded RNA of the virus is typically extracted before qRT-PCR through sample collection, virus inactivation and viral RNA release, isolation and purification. Then the single-stranded RNA is converted to cDNA via reverse transcription. The number of cDNA copies is subsequently amplified by thermal cycling in the presence of nucleotides, polymerase and either fluorescent probe(s) or fluorescent intercalating dye. Sufficient specificity is achieved by melting curve analysis at the end of the PCR [8], the use of a sequence-specific probe [9], or multiplexing to also handle partially degraded RNA samples or a mutated virus genome [10].

Although qRT-PCR possesses sufficient sensitivity and a superb limit of detection [11], the reaction requires temperature cycling. This seems to be simple; nevertheless, it prevents the sample holder from being thermally insulated for an acceptable cooling rate; thus the thermal cycler demands a large quantity of power due to periodic temperature changes and it is therefore difficult to power a thermal cycler via a battery for long-term use. Isothermal amplification (IA) techniques [12] represent an advance in NAAT. Isothermal systems do not require temperature cycling; thus they can be thermally insulated, resulting in low power consumption and suitability for PON applications [13]. Furthermore, the IA assay results can also be evaluated by simple methods – such as photometric determination of sample turbidity, pH measurement and color evolution, even using the naked eye – as well as standard fluorescence analysis, making the system design less demanding.

Loop-mediated isothermal amplification is one of the most widely used IA methods due to its specificity, and it has been adopted for detection of the SARS-CoV-2 genome [14] in less than 30 min. That is not spectacularly fast in comparison with ultrafast PCR systems capable of performing 40 cycles in less than 30 s [15], but it is still acceptable for the PON systems. The other variant of IA techniques, recombinase polymerase amplification (known as RPA) [16], was recently used for SARS-CoV-2 detection and showed promise for use in other low-power PON devices.

In summary, the IA techniques bring advantages such as simplification of thermal management as well as simplification of product detection by measurement of either colorimetry or turbidity, while RT-PCR requires more demanding fluorescence measurement.

Unfortunately, there are also significant disadvantages of IA, such as its complex chemistry, the utilization of six primers for loop-mediated isothermal amplification in comparison with two for RT-PCR (or three if a probe is used), the slow amplification and challenges

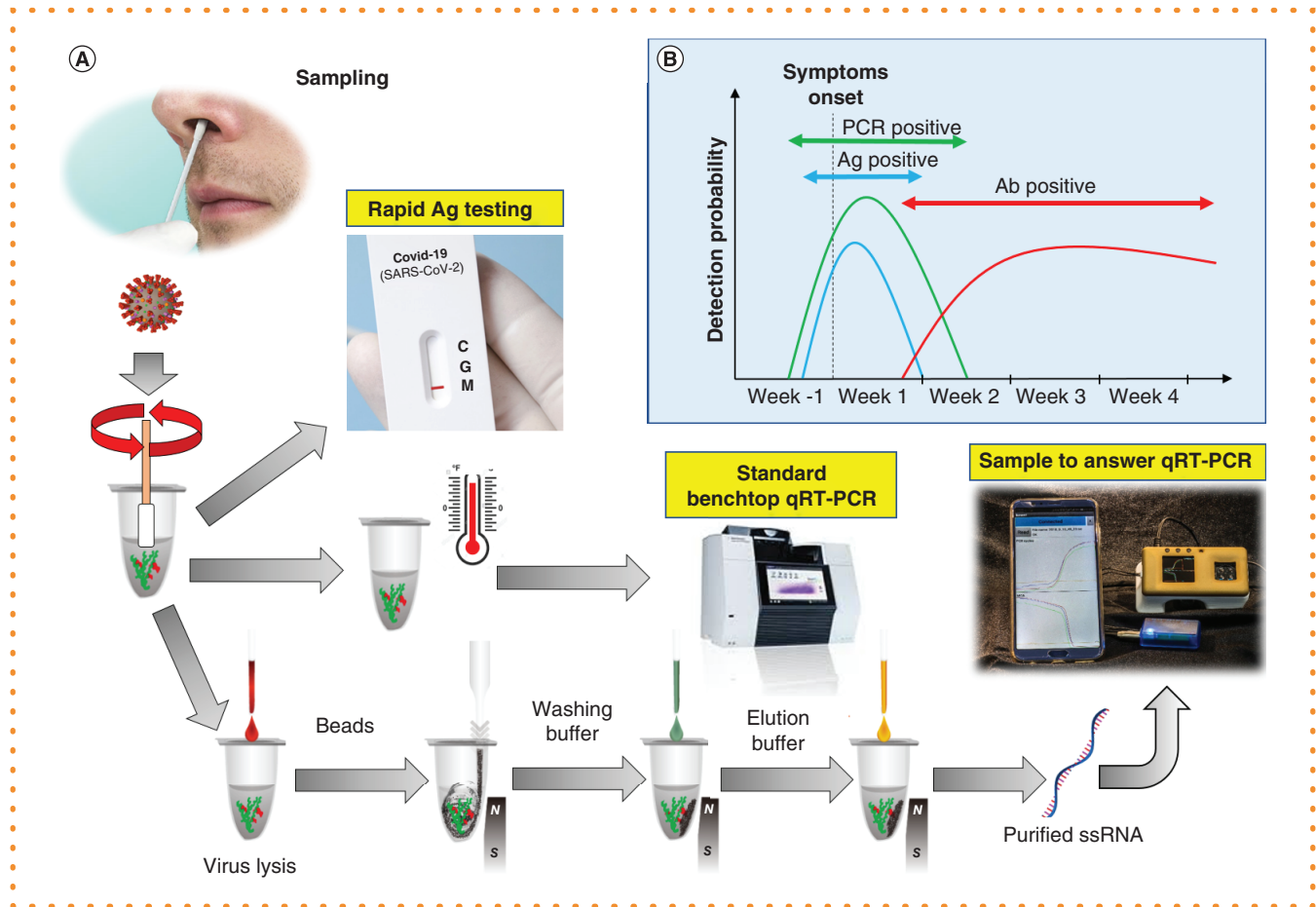


Figure 1. (A) Scheme of the sample-to-answer system using either antigen testing by directly introducing the sample into the testing system, processing sample by an automated system with qRT-PCR, or performing step-by-step sample preparation starting with virus lysis and RNA release and then RNA pre-concentration and purification with a qRT-PCR system at the final stage. Reprinted from [7], with permission from Elsevier. (B) COVID-19 detection probability for qRT-PCR, antigen and antibody tests showing superiority of the qRT-PCR testing method. Inspired by Figure 1 in [6].

in target multiplexing. Overall, only the simplification of thermal management is an important benefit for PON systems and it is questionable whether the advantage is strong enough to push system development toward isothermal amplification.

Sample collection problems

The proper collection of samples from the patient is a critical prerequisite for successful analysis. In general, sample collection can be more or less accurate and invasive. Accordingly, the collection procedure may or may not require the assistance of trained personnel [17].

For NAAT-based analysis, the most conclusive samples should be taken as nasopharyngeal and/or oropharyngeal swabs. These samples are the preferred choice (the gold standard) for swab-based SARS-CoV-2 testing. However, these can only be performed by a trained healthcare provider. Sputum, bronchoalveolar lavage and endotracheal aspirate specimens have even higher sensitivity but are not collected routinely due to increased invasiveness and inconvenience. Alternatively, saliva, sputum or nasal swabs can be readily self-collected by the person being tested. However, these samples are prone to lower sensitivity, either due to improper collection procedure or improper pre-collection activities such as eating, tooth brushing or mouth washing.

Other sources of sample are stool and blood. While stool samples are fully valid and easy to collect, the kinetics of viral appearance in stool could be slightly different than in swabs from the respiratory tract. The blood is not considered a useful source for NAATs of any type due to lack of genetic information in the blood related to the disease. Blood is, however, the preferred sample for antibody testing [18].

Importantly, the PON testing that is enabled by the portable systems discussed here provides a significant advantage by eliminating the logistics problems with sample storage and transport before analysis.



Figure 2. From portable to hand-held systems. (A) The Convergy's® POC RT-PCR COVID-19 Testing Platform [22]. (B) BOSCH vivalytic analyzer [23]. (C) Roche Cobas® Liat® System [24]. (D) Mesa Biotech's Accula™ system [25]. (E) Personal PCR device for detection of COVID-19 made by Visby Medical Inc. [26].

PON instrumentation

The qRT-PCR system consists of the following steps: sample collection, lysis, RNA binding, purification, elution, mixing eluate with qRT-PCR master mix and performing qRT-PCR (Figure 2).

A number of PON microfluidic systems have been proposed but, as of now, only a few of them are used. The sample collection is conducted at level of a few hundred or more microliters, but this quantity cannot be processed, as the sample volume range for NAAT is typically from a few microliters up to 10 μl . Thus a sample pre-concentration step has to be adopted, otherwise the PON system's sensitivity will be compromised. This feature is demonstrated by a few systems approved by the US FDA that have a detection limit of 100,000 copies· μl .

Additionally, the sample has to be treated to make the virus inactivated to protect personnel from getting contaminated after collection. Alternatively, the system has to be fully enclosed for protection. Next, the sample has to be loaded into a microfluidic system and eventually processed by qRT-PCR or another NAAT. The qRT-PCR requires the sample to be combined with a master mix in the volume ratio determined by the master mix manufacturer. Once the master mix is preloaded into the system in a lyophilized form, problems often occur with mixture dilution in water. Finally, in the chamber with qRT-PCR, the master mix containing the template is thermally cycled to perform PCR or kept at constant temperature to perform IA. PCR then requires a thermoelectric cooler or a heater alone (with passive cooling) or in conjunction with a fan (active cooling), fluorescent detection systems, a central processing unit, a communication module, a display and a power supply.

Methods of improvement

A few key parameters have already been noted. First, the PON system needs to increase its capability to handle a rather large sample volume while performing sample preparation to eventually pre-concentrate the sample into a volume of $\approx 1 \mu\text{l}$, or a similar one, suitable to be mixed with the qRT-PCR master mix.

The system's heating rate is limited by the power available for heating and the heating system's thermal capacity (H), while its cooling effectiveness depends on either the active cooling capability (such as the amount of air blown onto the heated system by a fan) or by the system's thermal time constant (τ) and thermal conductance (G) for passive cooling:

$$\tau = H/G \quad (\text{Eq. 1})$$

The power consumption (P) is:

$$P = G \Delta T \quad (\text{Eq. 2})$$

where ΔT is the temperature difference the system is heated to.

Thus the use of a simple method of improving the system cooling rate without compromising the value of P can lower its H value, using a smaller volume of a PCR sample. qRT-PCR samples of 0.2 μl have been processed without compromising the system efficiency requiring sample pre-concentration [19].

So far, a few systems have been introduced, most of them capable of handling only a single sample [28]. However, a reliable NAAT requires not only negative and positive controls but also three samples running concurrently. Also, the system should have simplified sample movement by using either magnets or compressed media. Magnets can be operated by direct current motors with Hall sensors for correct location determination. Small syringe pumps can also be used for pumping with pre-stored compressed air or something similar.

Conclusion & future perspective

In 2020 PCR-based devices/instruments attracted a great deal of attention due to the COVID-19 pandemic. Although PCR was a known technique before the SARS-CoV-2 spread, the pandemic witnessed this technique rightfully acknowledged around the globe as the method to diagnose COVID-19.

We can envision the development of PON systems with sample-to-answer capability. Even untrained personnel will be able to use these PON devices. The development might take a few different pathways. Most likely, all approaches will require sample treatment and pre-concentration to be able to perform NAAT in a reasonable volume, from a few to 10 μl , for a microfluidic system without losing system sensitivity [19]. According to the manufacturer's suggestion, the RT-PCR master mix with a volume of $\approx 10 \mu\text{l}$ typically includes only $\approx 1 \mu\text{l}$ of the sample; without the pre-concentration step, the system would take only 1/2000 of the original sample of $\approx 2 \text{ ml}$. This effectively lowers the system's sensitivity by 2000 times, which in qPCR would mean shifting the critical threshold by the value of ≈ 11 with an assumption of 100% efficiency of qPCR. Also, for low-viral-load diseases such as AIDS, the results would be practically always negative.

The systems determined for use in places with an available power supply, either network-based or at least 12 V car-battery type, will be based on qRT-PCR. In such cases, IA is not a real competitor for PCR due to the latter's cheaper chemicals and faster operation. The systems will be fully automated, and the operator will place the collected sample into a tube with lysis buffer and functionalized magnetic particles to destroy the virus, release its single-stranded RNA and bind it to the particles. The key part here is the virus inactivation making the sample safe for the operator. The sample will then be loaded into a microfluidic system and pumped via location with a permanent magnet; the paramagnetic particles with attached single-stranded RNA will be captured there. The particles will be moved via a washing station or directly to the area with qRT-PCR master mix under a fluorescent optical pickup head. The qRT-PCR will be conducted and the result extracted from the captured fluorescent signal. The systems will be able to conduct multiplex qPCR using either single or multiple fluorescent channels. Alternatively, a system capable of setting up the precise ratio between two or more targets would have to be based on a digital PCR [20]; this is currently under development in portable formats but, so far, without multiplexing capability [21]. Also, different systems processing between one and four samples will be designed. Four simultaneous NAATs are desired as this would allow simultaneous testing of a negative and positive control with two actual samples, making the system more robust.

Special places will have IA systems. Even though such amplification is significantly slower, its chemistry is more expensive and demanding than the one for qPCR. However, the IA systems still have their merits, as the core isothermal amplification can be well insulated and thus consumes very low power in comparison with the fast qPCR. Also, the IA chemistry can be designed in such a way that the solution will change color or pH instead of increasing fluorescence, making the product detection system less demanding.

Finally, the PON systems for infectious disease diagnosis will be part of a global network of the internet of things, communicating wirelessly with the local control center [27] and via the internet with national and international bodies tackling the pandemic. Any PON system will have to pass regular approval by standard bodies such as the FDA, European Medicines Agency and the National Medical Products Administration in China. As of now, many methods have been given emergency use authorization, but such methods are not sufficient for future usage; conventional means of approval need to be established. Once we have reliable PON systems available, we as human beings are ready for the next wave of a pandemic, which will probably occur in future. In addition, the reliable self-testing systems can be used to ease the burden of and help to tackle HIV problems in Africa, as well as potential uses for other widespread diseases elsewhere.

Author contributions

H Zhang wrote parts of isothermal amplification, PON instrumentation and methods of improvement. P Pajer wrote the section on sample collection problems. J Kudr wrote the sections on current COVID-19 diagnostics and conclusion. O Zitka participated on the review concept, on funding acquisition and revising the manuscript. P Neuzil participated on the review concept and wrote parts of methods of improvement and conclusion.

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