Highly performing point-of-care molecular testing for SARS-CoV-2 with RNA extraction and isothermal amplification.

P.Garneret*(1) , E.Coz*(1), E.Martin(1), J.C.Manuguerra(2), E.Brient-Litzler(2), V. Enouf(2), DF.González Obando(2), JC Olivo-Marin(2), F.Monti(1), S.Van der Werf(2), J.Vanhomwegen(2), P. Tabeling(1)†

(1) ESPCI PSL, CBI, IPGG, 6 rue Calvin, Paris 75005 (France)
(2) Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 1

**** These authors contributed equally to the work.***
† Corresponding author: email patrick.tabeling@espci.fr

In order to respond to the urgent request of massive testing, developed countries perform nucleic acid amplification tests (NAAT) of SARS-CoV-2 in centralized laboratories. Real-time RT - PCR (Reverse transcription - Polymerase Chain Reaction) is used to amplify the viral RNA and enable its detection. Although PCR is 37 years old, it is still considered, without dispute, as the gold standard. PCR is an efficient process, but the complex engineering required for automated RNA extraction and temperature cycling makes it incompatible for use in point of care settings. In the present work, by harnessing progress made in the past two decades in DNA amplification, microfluidics, and membrane technologies, we succeeded to create a portable test, in which SARS-CoV-2 RNA is extracted, amplified isothermally by RT - LAMP (Loop mediated Amplification), and detected using intercalating dyes or highly fluorescent probes. Depending on the viral load, the detection takes between twenty minutes and one hour. Using pools of naso-pharyngal clinical samples, we estimated a sensitivity comparable to RT-qPCR (up to a Cycle threshold of 38, equivalent to virus titers <0.1 TCID50 per mL) and a 100% specificity, for other human coronaviruses and eight respiratory viruses currently circulating in Europe. We designed and fabricated an easy-to-use portable device called "COVIDISC" to carry out the test at the point of care. The low cost of the materials along with the absence of complex equipment paves the way towards a large dissemination of this device. The perspective of a reliable SARS-CoV-2 point of care detection, highly performing, that would deliver on-site results in less than one hour opens up a new efficient approach to manage the pandemics.

Nucleic Acid Amplification Tests (NAATs) detect, through amplification of the nucleic acids, the presence of pathogens in infected samples. They are characterized by high sensitivities (10-100 viral particles) and excellent specificities. There are different ways of performing NAATs but today, PCR (Polymerase Chain Reaction), coupled to extraction, is considered, as the gold standard.

The main problem of PCR is that, due to an inherent complexity, the only way to obtain high throughputs is to perform highly parallelized tests in centralized laboratories. This generates logistics issues, long delivery times, (several days, in practice) and costs

unaffordable for developing countries. Over the last ten years, with the advent of isothermal amplification technologies *(2)*, a new area of research has opened, raising hope to perform NAATs at the point of care, with portable devices much cheaper than extraction/PCR platforms and showing comparable performances. A new generation of NAATs has grown along the years, in laboratories, often coupling isothermal amplification to paper microfluidics, a field pioneered by G.Whitesides *(3-15).*

Unfortunately, this second generation of testing, despite its potential, did not succeed to take off as a point of care (POC) product, for several reasons (low practicability of the devices, absence of sample preparation, in particular RNA extraction, clinical and analytical performances still to be assessed, costly surrounding equipment, competitiveness,…). Thereby, as SARS-COV2 started to propagate around the world, the isothermal technology was not ready to face the urgent request of global massive testing. Over these last weeks, stimulated by the crisis, isothermal commercial machines have been proposed *(16).* However, because of cost, throughput and performance limitations, they do not represent yet an alternative to the current extraction/RT-PCR technology. Laboratory isothermal detection of SARS-CoV-2 have been reported in the literature, but only in tubes *(17)(18)(19)(20).*

By carrying out series of improvements and optimizations over the last six years, we made a technological leap in the domain. The new generation of molecular tests we report here combines membrane extraction, RT-LAMP testing *(21)* and paper microfluidics. It has performances comparable to the extraction/RT-PCR method, with a potential to be used promptly for the detection of SARS-CoV-2 at the point of care.

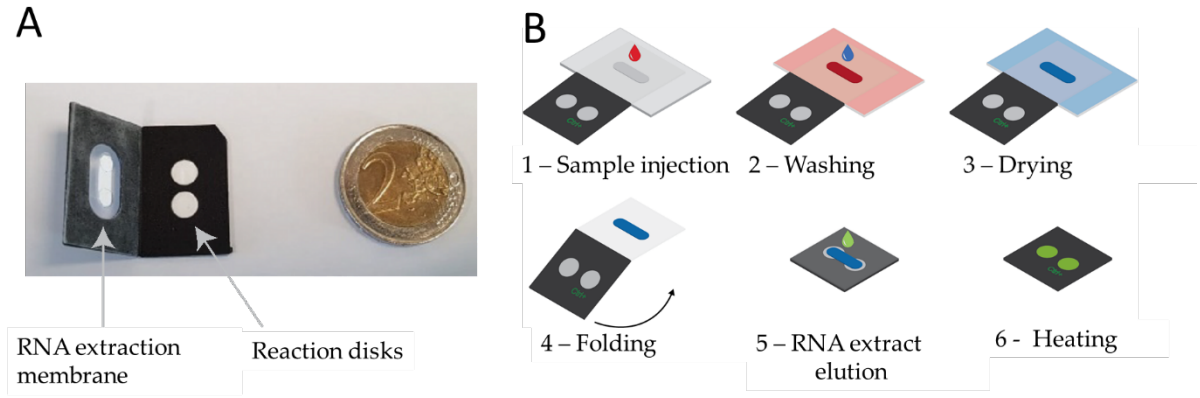

Fig. 1: A: Two sheets of polypropylene (black), in which pretreated pieces of glass fiber (in white) are incorporated. B displays the mode of operation. 1 - The sample, in which the virus has been lysed, is injected onto the extraction membrane; 2- Washing. 3- Drying of the extraction membrane. 4 – Folding of the device in such a way that the extraction membrane comes into contact with the two reaction disks. On the first disk, the freeze-dried mix and primers permit reverse transcription and amplification, on the second one, an internal control (human 18S RNA) is reverse transcribed and amplified. 5 – Elution on the reaction disks. 6 – Heating at 65°C. Read-out in real time with an intercalating agent (SYTO82).

The laboratory device we used is displayed on Fig 1A. Two sheets of polypropylene (black), two centimeters in size, incorporate pretreated laser-cut disks, one (oblong form) for nucleic acid extraction, and the two others (circular disks) for the RT-LAMP reactions. The oblong disk is a commercial silicon membrane optimized for RNA extraction, the others are made in fiber glass (Watman). Figure 1B decomposes the workflow. The lysed sample is injected onto the extraction membrane and washed *(22)(23).* After drying, the device is folded so as to place the oblong extraction membrane onto the two reaction disks, in tight contact. Then RNA elution is achieved, using the RT-LAMP reaction buffer. In this process, the eluate is driven towards the reaction disk, owing to the action of capillarity. On the first disk, the reaction mix gets hydrated and, upon temperature raise to 65°C, reverse transcription and amplification are performed. The second reaction disk serves as a positive control. We use real-time RT-LAMP, whose kinetics is measured by tracking the fluorescence emission of a DNA intercalating dye.

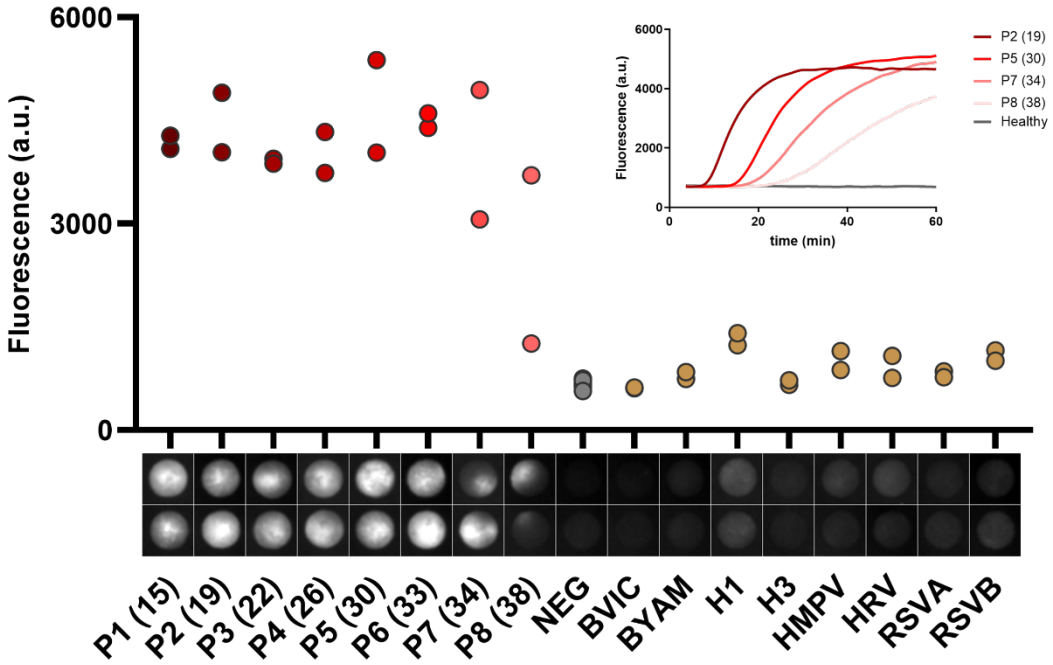

Fig 2. Sensitivity and Specificity measurements obtained on SARS-CoV-2 positive samples and negative controls (individuals with a negative RT-PCR or patients diagnosed with other respiratory infections). The low intensity level measured on the negative is due to natural disk fluorescence. The internal sample controls (RNA 18s) are shown in detail in Supp Mat 2. At the bottom, series of disk images obtained at the end point, i.e. t=60 min, each vertical pair corresponding to duplicate assays. (Insert) RT-LAMP amplification curves obtained by real-time monitoring of the fluorescence produced by an intercalating dye (SYTO82).

We now use the device of Fig 1 for the detection of SARS-CoV-2. The primers are displayed in Supp Mat 1 *(20).* Figure 2 shows end point results, sixty minutes after sample

injection. We used eight pools of naso-pharyngal samples infected with different SARS-CoV-2 viral loads, in duplicate. All were characterized with the real-time duplex RT-PCR (targeting the RdRP gene) developed by the French National Reference Centre for Respiratory Viruses *(24).* In terms of cycle thresholds (Ct), they span a range extending from 12 to 38, i.e down to a few viral particles per milliliter. For the specificity, we used clinical naso-pharyngal samples infected by respiratory pathogens (RSV A and B, influenza A H1 and H3, influenza B Victoria and Yamagata lineages, HRV and HMPV). In addition, testing of high-titer solutions of human coronaviruses in tubes, including SARS-CoV, MERS-CoV, OC43 and 229E demonstrated a 100% specificity towards these closely-related viruses.

Below the abscissa of Fig 2, we show the fluorescence images of the reaction disks corresponding to the end point measurements. Heterogeneities of the fluorescence field are visible on most of them, a phenomenon we attribute, speculatively, to heterogeneous nucleation and low diffusion of the amplicons inside the porous medium. The fluorescence signal on Figure 2 is the average intensity of the 25% brighter pixels in the reaction disk, a simple approach to take into account profiles of low Ct samples, where the reaction is confined in a subpart of the disk. This signal heterogeneity within the disk can be leveraged as a specific marker for positives to improve the interpretation of the test. (see SupMat 4).

Fig. 2 shows that we clearly detect patient samples 1-7, i.e up to a cycle threshold of 35. The sample n°8 at Ct=38 is also detected, but with a substantial dispersion. We conclude that the limit-of-detection of the method is equivalent to the extraction/RT-PCR performances.

The insert of Fig 2 shows the kinetics of the reactions, for different viral loads, using a fluorescent intercalating agent (SYTO82) *(25).* The negative control remains at a level corresponding to the natural fluorescence of the reaction disk. Amplification curves have the usual sigmoid shape. The sample with the highest viral load, (Ct=16-18, equivalent to an infectious titer of 10^6 TCID50 per mL of sample), can be detected after 8 – 10 minutes. At higher Ct values, i.e. as dilution increases, the signal takes off at later times. Plateaus are observed for the patients 1-6, i.e up to Ct=33. For the samples with the lowest viral loads (pools 7 and 8), i.e. with viral loads less than 10 genome copies per reaction, a plateau is not reached, but still, the virus can be detected without ambiguity.

The right part of Figure 2 addresses the question of the specificity. The images show that for non-SARS-CoV-2 pathogens, fluorescence is barely visible. This is confirmed by intensity measurements, which show that the fluorescence level is indistinguishable from the background. One may conclude that the specificity of the test, based on this limited set of pathogens, is 100%.

In order to transform the foldable paper system into a practical POC device , we created the “COVIDISC”, shown in Fig 3.

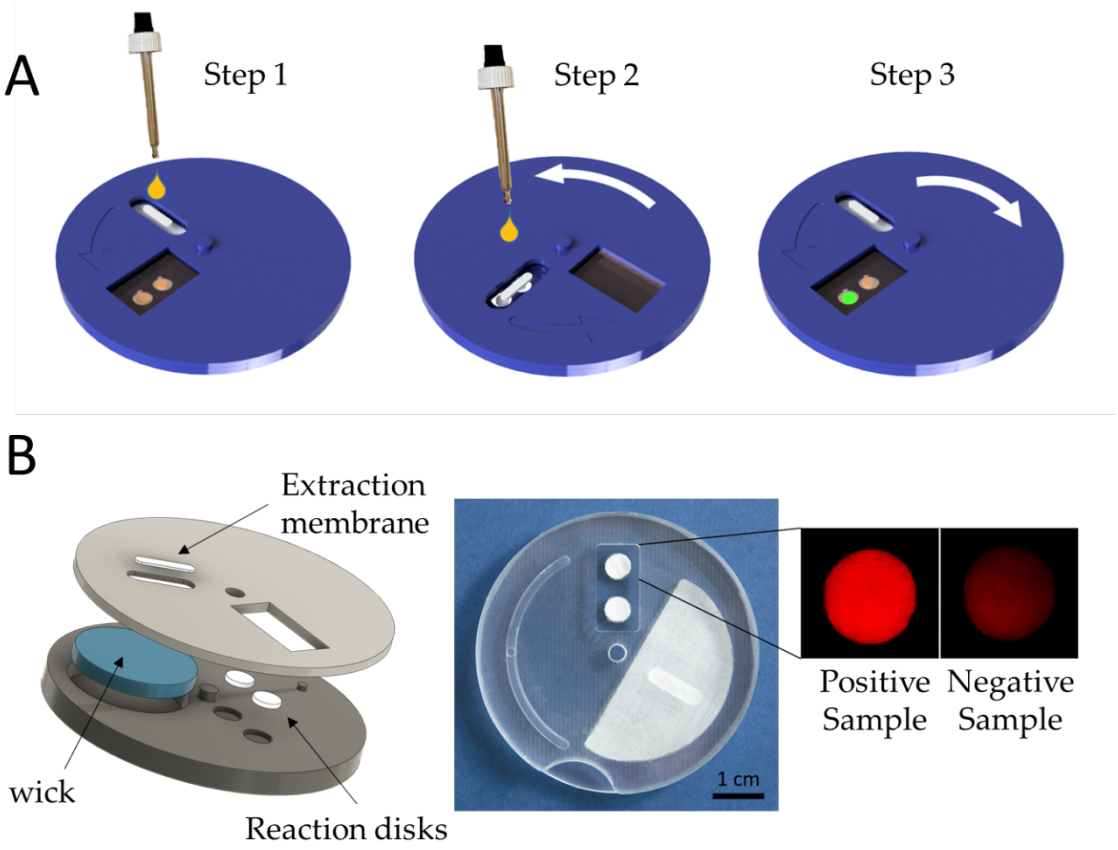

Figure 3: A –COVIDISC workflow decomposed in three steps: 1 - injection, washing, drying. 2 - Disk rotation and elution; 3 – Disk counter-rotation, coverage of the reaction zone by a PCR sealing film, heating, amplification and readout. B – Left: Exploded structure of the device. Center: Picture of a prototype. Right: QUASR readout photograph of a test on RNA extracts of SARS-CoV-2, processed as in Fig 3A; (left) sample; (right) negative control.

Fig 3A shows the COVIDISC workflow, which reproduces that of Fig 1B. The device, shown in Fig 3B consists of two plastic disks, 5 cm in diameter, able to rotate around a common axis. The extraction membrane, the wick and the reaction disks are force fitted. By performing rotations, injections and heating, one executes the workflow of Fig 1B (see SuppMat 4). On adding QUASR probes (Quenching of unincorporated amplification signal reporters) to RT-LAMP, we obtain a naked eye YES/NO answer. Fig 3B (right) shows the readout of RNA extracts of SARS-CoV-2, captured with the camera of a smart phone. We used an oven to maintain the temperature at 65°C, one LED and two gelatin filters. The equipment needed for running the test is thus minimal.

The results presented here have been obtained with 39 clinical samples. Although a confirmation on larger sets is desirable, our measurements indicate that, with a minimal equipment, one can extract, wash, elute, reverse-transcribe, amplify and measure the kinetics, with a sensitivity comparable to the gold standard RT-qPCR, i.e. 38 cycles (a few genome copies per reaction) and a specificity, based on the set of pathogens we used, of 100%. We also

created a portable device (COVIDISC) that can be used at the point of care with minimal equipment *(26).* This work paves the way toward reliable point of care testing of SARS-CoV-2, both in developing and developed countries. Performing these tests at the doctor's office, at the working place or in pharmacies could allow to isolate infected patients without delay, fasten their quarantine along with reduce logistics and costs, offering a new efficient approach to manage the pandemics. In the future, obviously, the same technology could be used for other pathogens.

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26. Industrialization of an engineered version of COVIDISC is underway.

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