New Microbiologica, 44, 3, 173-176, 2021, ISSN 1121-7138

SHORT COMMUNICATION

Molecular diagnosis of SARS-CoV-2, performances and throughput by Direct RT-PCR

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SUMMARY

The Covid-19 pandemic has required all laboratories to rapidly and unexpectedly reorganize to cope with the increase in requests for tests in rapid response times and, not least, to provide the shortening of molecular reagents.

In order to validate an accurate, faster and cheaper method suitable for large-scale diagnosis of SARS-CoV-2, we evaluated a simplified workflow by Direct RT-PCR on 181 nasopharyngeal swabs on Seegene's automated platform.

Direct RT-PCR ensured 99% overall concordance versus standard RNA RT-PCR in samples with Ct values under 35, saving 100% on extraction reagents and providing an approximately three-fold increase in productivity in 24 hours.

Received April 12, 2021 *Accepted* August 18, 2021

The Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) pandemic, started in China in December 2019 (Guan *et al.*, 2020), has quickly and globally revolutionized the approaches and needs of microbiological molecular diagnostics (Corman *et al.*, 2020; WHO, 2020). The urgent need to diagnose and screen on a large scale by molecular detection of SARS-CoV-2 RNA on nasopharyngeal swabs, to be able to control the spread of the virus, has engaged diagnostic laboratories in an unprecedented challenge (Reusken *et al.*, 2020). The need to process thousands of samples a day, guaranteeing response times of a few hours within 24 hours, taking into account the instrumental and human resources available, maintaining analytical accuracy, has forced microbiological diagnostic laboratories to make numerous and decisive choices (Tang *et al.*, 2020).

The nucleic acid (NA) purification before reverse transcription PCR is the gold standard for molecular diagnostics (RNA RT-PCR). Authoritative and numerous literature data support the Direct RT-PCR approach (Bruce *et al.*, 2020; Smyrlaki *et al.*, 2020; Merindol *et al.*, 2020; Fomsgaard *et al.*, 2020; Pan *et al.*, 2020; Freppel *et al.*, 2020), which is found to be necessary in moments of disproportionate diagnostic request compared to the resources available in a Laboratory and the need to detect positive cases quickly, as in a pandemic (Larremore *et al.*, 2020).

Several simplified approaches, which involve minimal handling of the samples before the RT-PCR for SARS-CoV-2, were employed to avoid the NA purification step. One of the most used methods is the extraction-free/pre-heated RT PCR (Merindol *et al.*, 2020; Fomsgaard *et al.*, 2020; Pan *et al.*, 2020), also suggested by Seegene Inc, directly on the primary sample, after thermal treatment (thermolysis of the sample at 98°C for 3 minutes, and cooling at 4°C for 5 minutes). However, in our opinion, the pre-heated approach on Seegene's automated platform, which involves the preparation, manual capping, vortexing, movement in the thermal block for thermolysis and then cooling, vortexing, manual decapping, repositioning in the machine for PCR set up of each 96-well plate, slows the flow of laboratory work by about 20 minutes per run, i.e., 6 hours per day, with a high risk of contamination. In a pilot study on 30 positive samples, we successfully compared analytical results of the pre-heated approach suggested by Seegene Inc, with a not-heated approach (Direct RT PCR) (Grant *et al.*, 2020; Brown *et al.*, 2020), with the aim of further accelerating the response times and streamlining the work flow. In light of these preliminary results, in the present study, we evaluated SARS-CoV-2 direct RT-PCR on 181 respiratory samples compared to the classic RNA RT-PCR method on Seegene's automated platform, in order to verify its accuracy and its possible implementation in our routine diagnostic pathways.

A total of 181 consecutive nasopharyngeal swabs for

Key words:
SARS-CoV-2, Direct RT-PCR, Extraction-free.

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clinical routine diagnostics of SARS-CoV-2, collected in 3 ml of universal transport medium (UTM®,COPAN Diagnostics Inc.), were evaluated at arrival in laboratory (within 8-10 hours after collection), as our standard laboratory practice (RNA-Extraction followed by RT-PCR), or, in parallel, by-passing RNA-extraction step (Direct RT-PCR). All experiments were adapted to a same commercial amplification method performed as per the manufacturer's instruction (Allplex SARS-CoV-2 Assay, Seegene Inc.). According to safe laboratory procedures, manipulation of potentially infected specimens was performed in a certified Class II BSC in a BSL-2 facility.

Viral RNA from patient samples was extracted and purified using STARMag 96 X 4 Universal Cartridge Kit (Seegene Inc.) on the Seegene NIMBUS IVD automated NA extraction platform, as standard method for viral NA extraction, according to the manufacturer's instructions. STARMag 96 X 4 Universal Cartridge Kit is intended to be used for the isolation of nucleic acid from nasopharyngeal swabs and other specimens using automated NA extraction instruments. The procedure is based on reversible adsorption of nucleic acids to paramagnetic beads under appropriate buffer conditions. 200 µL of nasopharyngeal swab UTM® tube-derived medium was extracted and eluted in 100 µL of Elution Buffer Universal. At the end of extraction, the RT-PCR mix was automated prepared and distributed on a 96-well plate. The reaction was performed in a 20 µL reaction volume containing 15 µL of RT-PCR real time master mix and 5 µL of sample/positive control or nuclease-free water (as negative control). The well plate was transferred on CFX96 Real-Time-PCR System (Bio-Rad).

For Direct RT-PCR, in a 96 deep-well plate, 45 µL of an Ultrapure Distilled water DNase/RNase free (Invitrogen) was added to 15 µL of UTM® tube-derived medium; subsequently 5 µL of each diluted sample, positive control or nuclease-free water (as negative control) were added by automated platform Seegene NIMBUS IVD to 15 µL of RT-PCR real time master mix.

All specimens were reverse transcribed into cDNA and subsequently amplified in a CFX96 using Allplex SARS-CoV-2 Assay Kit. The Allplex SARS-CoV-2 Assay Kit is an in vitro diagnostic real-time RT-PCR test intended for the qualitative detection of SARS-CoV-2 viral nucleic acids in a human nasopharyngeal swab. Is a multiplex real-time PCR assay to detect 4 target genes of SARS-CoV-2, causing COVID-19 in a single tube. The assay is designed to detect RdRP, S and N genes specific for SARS-CoV-2, and E gene for all Sarbecoviruses including SARS-CoV-2. The Allplex SARS-CoV-2 Assay includes a full process Internal Control (RP-V IC) which is composed of MS2 phage genome. This Internal Control material verifies all steps of the analysis process to demonstrate proper specimen processing and test validity of each spec-

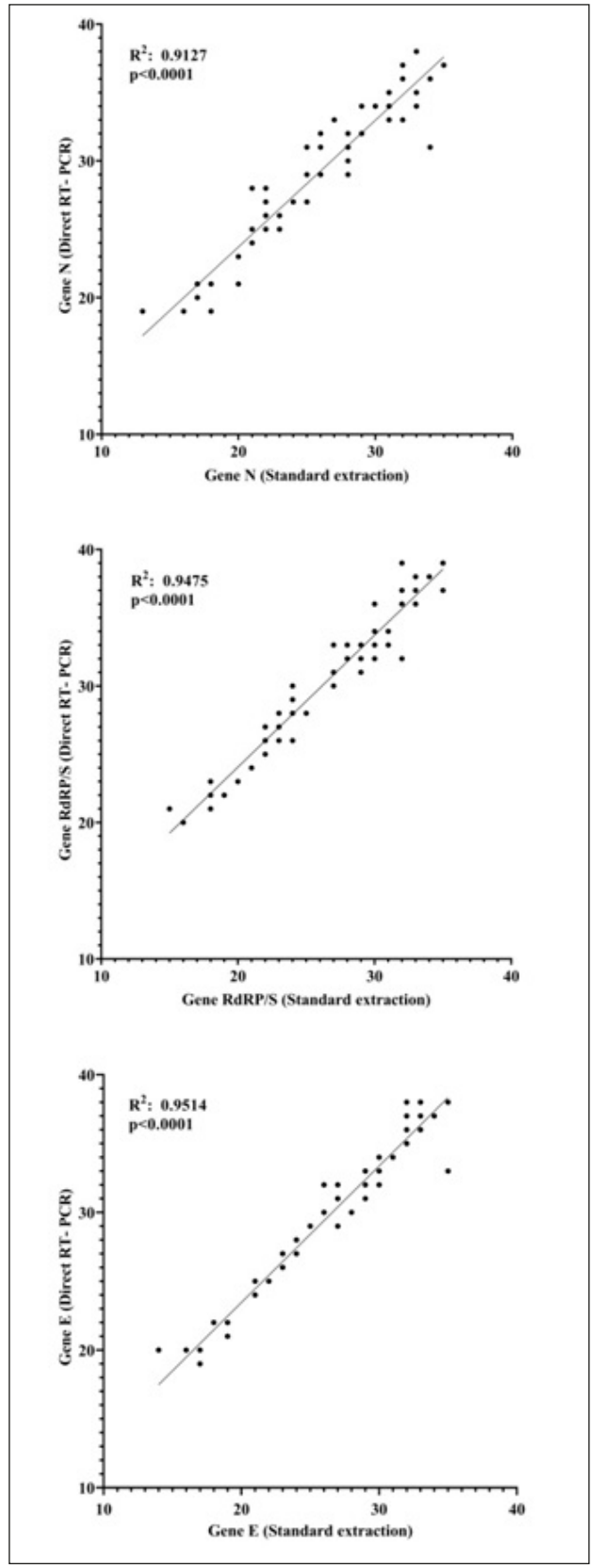

Figure 1 - *Correlation of Ct values obtained between Direct RT-PCR and RNA RT-PCR (Standard extraction) for the detection of SARS-CoV-2 (*n=*57,* n=*53,* n=*55, for N, RdRP/S and E genes, respectively).*

imen. In particular, for Direct RT-PCR the RP-V IC was automatically added to Mastermix during preparation for PCR, while for standard extraction RP-V IC was automatically added to each sample during the extraction phase.

The thermal cycling conditions were as follows: 20 minutes at 50°C, 15 minutes at 95°C and 45 cycles of 10 seconds at 95°C, 15 seconds at 60°C and 10 seconds at 72°C. The analysis was performed using a real-time CFX96 Real-Time-PCR System.

The results of amplification are reported through 'Seegene viewer' analysis. Ct from FAM (E gene), Cal Red 610 (RDRP/S gene), Quasar 670 (N gene) and HEX (internal control) were acquired. Samples were considered positive when a signal was detected at Cycle threshold (Ct) <40 for any gene. A sample was considered negative if the internal control was amplified but not the viral genes.

All statistical analyses were performed by GraphPad Prism 6.0 software.

Direct RT-PCR results were compared to those obtained by RNA RT-PCR for clinical diagnosis of SARS-CoV-2 to assess its accuracy in term of sensitivity and sensibility. Out of 181 samples examined by RNA RT-PCR, 88 (48.6%) were negative and 93 (51.4%) were positive (among positives, *n* 57 at Ct <35, *n* 36 at Ct >35) for SARS-CoV-2. Out of 181 samples examined by Direct RT-PCR, 119 (65.2%) were negative, 62 (34.2%) were positive (among positives, *n* 55 had Ct values < 35, *n* 7 had Ct values > 35), compared to RNA RT-PCR results. More in detail, 29/31 false negatives by Direct RT-PCR had Ct values >35 by RNA RT-PCR (Ct values range 35-40, median 37), as noted in other studies (Merindol *et al*., 2020; Lubke *et al*., 2020), while 2/31 had Ct values <35. One sample was detected by Direct RT-PCR, but negative by RNA RT-PCR. No sample tested showed an invalid result (internal control lack of amplification).

Out of 62 positives by both approaches, we focused on those that had an RNA RT-PCR result < 35 (*n*=57, *n*=53, *n*=55, for N, RdRP/S and E genes, respectively). As shown in *Figure 1*, a significant correlation of Ct values was obtained for N, RdRP/S and E genes (p<0.0001), with an average delay of three amplification cycles by Direct RT-PCR *versus* RNA RT-PCR.

In light of documented evidence of reduced or absent infectivity in samples with Ct values >35 (La Scola *et al*., 2020; Binnicker *et al*., 2020; Bullard *et al*., 2020), we focused on the evaluation of accuracy of Direct RT-PCR on 144/181 samples (just considering positives with Ct values <35). As shown in *Table 1*, sensitivity and overall agreement were 96.5 and 98.6%, respectively, for Ct values <35, and 100% for Ct values <30 (*Table 1*). We proceeded to evaluate accuracy after verifying that the method showed excellent repeatability and reproducibility assessed, respectively, by testing 7 positive samples at different concentrations 3 times on the same day and the same set of samples for 3 consecutive days (data not shown).

In *Table 1*, we show a projection of the Turnaound Time (TAT), time between runs and throughput in 24 hours in the hypothesis of an instrumental equipment of one Seegene NIMBUS IVD automated platform (for automated extraction and/or PCR set up), and one CFX96 Real-Time-PCR System. Considering that the maximum number manageable by the Seegene NIMBUS IVD platform is 72 batches of samples per run, approximately 493 and 1296 samples would be processed in 24 hours with RNA RT-PCR and Direct RT-PCR, respectively. Of course, the numbers would increase if the lab had even just one more thermal cycler available.

Direct RT-PCR protocol was implemented in our routine to accelerate the *sample-to-answer time* so as to promptly identify contagious patients for rapid and efficient outbreak control, during a pandemic phase

Table 1 - *Direct RT-PCR performances on 181 consecutive nasopharyngeal swabs and comparison among RNA RT-PCR and Direct RT-PCR. TAT: turnaround time from sample loading to result reporting. Time between runs: time required to begin a new run following the beginning of a previous one. Samples/24 hours: maximum number of samples that can be processed in 24 hours considering also the time required to load the samples and reagents.*

Direct RT-PCR performances		*181 samples evaluated*	
Overall Sensitivity (%)		66,7	
Sensitivity <35 ct (%)		96,5	
Sensitivity <30 ct (%)		100	
Overall Agreement (%)		82,3	
Agreement ct<35 (%)		98,6	
Agreement ct<30 (%)		100	
		RNA RT-PCR	*Direct RT-PCR*
For 72 samples/run*	*TAT (h)*	*5,20*	*3,10*
	Time between runs (h)	*3,30*	*1,20*
	Samples/24h	*493*	*1296*

*Considering an instrumental equipment of one automated platform Seegene NIMBUS IVD (for automated extraction and/or PCR set up), and one CFX96 Real-Time-PCR System.

in which the acceleration of processability and the reduction of the cost and reagents are crucial. As shown in *Table 1*, we are aware that in cases of a possible late stage of the disease a low positive result (Ct>35) may not be detected (with potential epidemiological implications, or, for the patient, with implications for the number of vaccine doses required, for example), and that a low positive result (Ct>35) could represent an individual in early stages of infections (even if in very low percentages and with a negligible contagiousness rate). These aspects should be appropriately considered by users of this Direct RT-PCR protocol.

After implementation of the Direct RT-PCR on Seegene's automated platform in our routine, we were able to process up to 1000 samples per day and consolidated a shortening of the TAT by approximately 2 hours, saving 100% on extraction reagents and achieving an approximate three-fold increase in productivity in 24 hours (critical parameters during a pandemic for the containment of infections and for the sustainability of laboratory workflows), ensuring 99% overall concordance versus standard RNA RT-PCR in samples with Ct values under 35, and therefore with a contagious viral load (La Scola *et al*., 2020; Binnicker *et al*., 2020; Bullard *et al*., 2020).

With the increasing demand for testing worldwide, accurate, fast and cheap methods for detecting acute infection can help monitor outbreaks of SARS-CoV-2. Direct RT-PCR on Seegene's automated platform is a good and reliable alternative to mass screening.

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