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## Comparative study of different SARS-CoV-2 diagnostic techniques

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### ABSTRACT

The rapid spread of SARS-CoV-2 led to the necessity of developing diagnostic tests for rapid virus detection. Many commercial platforms have appeared and have been approved for this purpose. In this study, 95 positive and 5 negative retrospective samples were analyzed by 4 different commercial RT-qPCR kits (TaqMan 2019nCoV Assay, Allplex™SARS-COV-2 Assay, FTD SARS-COV-2 Assay and qCOVID-19). The Hologic Aptima SARS-COV-2 and the Clart-COVID-19 system were also tested. serial dilutions of SARS-COV-2 standard control were included for sensitivity analysis. Among the qPCR tested qCOVID19 and Allplex™SARS-COV-2 Assay were both able to detect all the clinical samples included in the study. All four qPCR evaluated showed high sensitivity for samples with Ct<33. Clart-COVID-19 microarrays detected all samples and controls used in this study whereas Hologic Aptima Panther failed with one of the clinical samples. However, the main problem with this system was the number of invalidated samples despite avoiding the use of medium with guanidine isothiocyanate as recommended by the manufacturer. All the techniques tested were of value for SARS-CoV-2 detection.

### 1. Introduction

The appearance of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019 and its rapid spread all around the world has challenged both science and society (Sharma et al., 2020). In response to the necessity of controlling the infection and prevent pandemic progression, pharmaceutical companies have developed many commercial kits for detecting the virus. Quantitative real-time RT-PCR (RT-qPCR) is, probably, the most popular technique for SARS-CoV-2 detection. RT-qPCR is sensitivity, specific, rapid and very useful for diagnosing early infection (Wang et al., 2020; Younes et al., 2020).

However, the sensitivity and specificity of these methods are different depending on both the genes selected and the primers and probes design. Although the primers and probes are target conserved regions of the viral genomes, the variation of SARS-CoV-2 RNA sequences can produce mismatches in binding, leading to decrease in assay performance and potential false-negative results (Khan and Cheung, 2020).

Even though RT-PCR assays are the most extended diagnostic method, other molecular techniques have been developed and employed. Among these, transcription-mediated amplification (TMA)

and multiplex amplification followed by hybridization in low density microarrays are included (Prabhakar and Lakhanpal, 2020; Trémeaux et al., 2020). TMA is an isothermal amplification technology that uses a retroviral reverse transcriptase and T7 RNA polymerase for detection of two unique sequences within the ORF1ab region of SARS-CoV-2. The process involves exponential amplification of RNA and detection of the amplicon by dual kinetic acridinium ester-labeled probe hybridization. Results are reported as Relative Light Units (RLU).

TMA is an easy-to-use, fully automatic system capable of handling a large number of samples in one working day. These properties make it an attractive option for virus detection and its control spreading.

One of the main differences between RT-qPCR and TMA is that TMA detects viral RNA directly without the previous DNA synthesis needed for RT-qPCR.

Hybridization in low density microarrays after multiplex amplification is commercialized by Genómica SAU (CLART®COVID-19). Due to the automation capacity of this system, up to 96 different patient samples can be processed at the same time in less than 5 h.

We conducted a comparative study of 4 commercial RT-qPCR used for SARS-CoV-2 detection (TaqMan 2019nCoV Assay (ThermoFisher), Allplex™SARS-CoV-2 Assay (Seegene), FTD SARS-CoV-2 Assay (Siemens), qCOVID-19 (Genómica)). RT-qPCR results were also

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compared with Hologic Aptima Panther SARS-CoV-2 TMA test and CLART®COVID-19 microarrays.

## 2. Materials and methods

### 2.1. Samples

Samples were received at Microbiology Department of Hospital Clínico San Carlos in the form of nasopharyngeal swabs in several transport medium, mainly universal transport medium (UTM), viral transport medium (VTM) and liquid Amies medium. These samples were initially tested for routine diagnostic. After testing, an aliquot of each sample was kept in the laboratory collection at -70 °C. Residual sample was also conserved at -70 °C as anonymized form and marked as positive or negative according routinely diagnostic. One hundred residual samples (95 positive and 5 negative) were included in the study.

Samples were thawed only once at the time of use. Once thawed, the samples were processed in parallel both for nucleic acid extraction and for TMA analysis.

### 2.2. Viral RNA extraction

SARS-CoV-2 RNA was extracted from selected samples after thawing them by the NucliSENS® easyMAG™ method (BioMérieux, Madrid, Spain) following manufacturer's instructions. Two hundred microliters were used as input volume. Elution was done in 50 µL of elution buffer. Extracted RNA was stored at -70 °C until use.

### 2.3. RT-qPCR assays

Nucleic acid extract from the 100 selected samples were investigated by four commercially available COVID-19 RT-qPCR kits: TaqMan 2019nCoV Assay (ThermoFisher, Spain), Allplex™SARS-CoV-2 Assay (Seegene, Werfern, Spain), FTD SARS-CoV-2 Assay (Siemens, Spain), qCOVID-19 (Genómica, Spain). All the assays were performed according to the manufacturer's protocol.

### 2.4. TMA

TMA testing were carried out with the Hologic Aptima SARS-CoV-2 transcription-mediated amplification test (Hologic, Spain) on a Hologic Panther system according to the manufacturer's package insert.

### 2.5. CLART®COVID-19

CLART®COVID-19 (Genómica, Coslada, Spain) was performed and interpreted automatically by a reader (CAR® or CLINICAL ARRAY READER) running tailor-made software. Amplification reactions were performed on an Eppendorf 6333 Nexus MasterCycler Thermal Cycler (Eppendorf, Spain) using a template volume of 5 µL and an internal control. Only one half of the samples was tested by CLART®COVID-19.

### 2.6. Sensitivity of each commercial kit

Serial dilutions (1:10) of commercial SARS-CoV-2 standard control (SARS-CoV-2 Standard, BioRad, Spain) were used to evaluate the sensitivities of SARS-CoV-2 detection assays. The synthetic RNA quantified control contains E, N, ORF1ab, RdRP and S genes that are each quantitated at 200,000 cp/mL using Bio-Rad Digital Droplet PCR.

Four different dilutions were prepared and two replicates of each one were assayed. The limit of detection was defined as the lowest dilution at which both replicates were positive.

### 2.7. Statistical analysis

The normal distribution of the data was analyzed by Shapiro-Wilk

test. The data were expressed as mean ± standard error of mean (SEM) with 95 % confidence intervals. Statistical analysis were performed using a Student's *t*-test for each statistical comparison. A critical value for significance of  $P < 0.05$  was used throughout the study.

## 3. Results

### 3.1. Sensitivity of the commercial platforms studied

All qPCR commercial platforms were able to detect the first two 10-fold dilutions and the undiluted control. Remaining dilution was detected only in one of the two assays performed (Table 1).

Aptima assay showed less sensitivity and it informed as positive only the undiluted and first diluted control. In contrast, CLART®COVID-19 appeared like the most sensitive technique detecting all dilutions tested.

### 3.2. Comparative evaluation of the qPCRs tested

Ninety-two of the hundred samples tested were positive for all the commercial qPCRs evaluated according manufactures' recommendation when N probe was considered. The other three previously positive samples showed different results depending on the technique examined (Table 2). Two of them were negative with Allplex™SARS-CoV-2 Assay and positives with the other three techniques, one sample was positive with qCOVID-19 and Allplex™SARS-CoV-2 and negative with TaqMan 2019nCoV Assay and FTD SARS-CoV-2 Assay and the remaining discordant sample was positive with qCOVID-19 and negative with the rest of techniques. The five negative samples were confirmed as negative by all the qPCR used.

To compare the sensitivity of the four qPCR, the Ct values of N probe were considered (Fig. 1). The lowest mean value of N probe was obtained with TaqMan 2019nCoV Assay (21.9) despite the two samples that could not be detected with this technique. Differences between TaqMan 2019nCoV Assay and FTD SARS-CoV-2 Assay (24.4) were statistically significant ( $p > 0.05$ ) although not with qCOVID-19 (mean N value 23.26) or Allplex™SARS-CoV-2 Assay (mean N value 23.91).

### 3.3. Relative efficiency of the different probes

Two of the 4 qPCR evaluated (TaqMan 2019nCoV Assay and Allplex™SARS-CoV-2 Assay) employ three different probes for SARS-CoV-2 detection.

Since one sample can be reported as positive with a single positive probe, the Cts of each technique were compared with each other for the three probes (Fig. 2).

Mean values for each probe were 23.7 (E probe), 24.05 (probe S/Rpd) and 23.9 (probe N) for Allplex™SARS-CoV-2 Assay and 21.96 (ORF1 probe), 22.67 (probe S) and 21.9 (probe N) for TaqMan 2019nCoV Assay. Differences observed were not statistically significant in any case. However, the three samples with N probe negative in Allplex 2019-nCoV assay, showed positive results with some of the other probes of this assay. This is not the case with TaqMan 2019nCoV Assay (Table 3).

### 3.4. Concordance between qPCR and TMA assays

Six positive qPCR samples (included both TaqMan 2019nCoV Assay false negative) were informed as invalid for the Hologic Aptima SARS-CoV-2 and one positive sample was reported as negative (Table 2). The remaining samples (88 positive and 5 negative by qPCR) showed concordant results. It was not possible to establish any relationship between the Hologic Aptima SARS-CoV-2 RLU and qPCR Ct values.

### 3.5. CLART®COVID-19 results

Forty-five positive and three negative qPCR samples were tested by

**Table 1**  
Analytical sensitivity comparison of molecular kits included in the study.

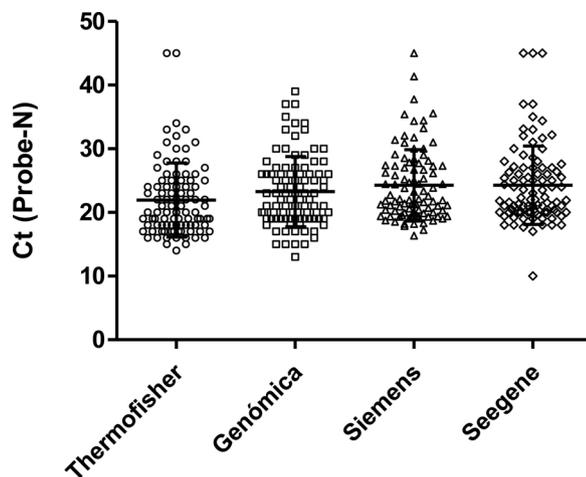
N probe Ct values						
Control dilution	TaqMan 2019nCoV	qCOVID-19	FTD SARS-CoV-2	Allplex SARS-CoV-2	Hologic Aptima SARS-CoV-2	Clart COVID19
Undiluted	26,48 / 26,7	31,7 / 31,32	29,6 / 30,1	29,4 / 29,5	Positive / Positive	Positive / Positive
1:10	30,3 / 29,85	34,4 / 35,23	32,4 / 33,3	33 / 33,4	Positive / Positive	Positive / Positive
1:100	34,3 / 32,82	38,3 / 39,71	37,1 / 37,9	37,4 / 37,7	Negative / Negative	Positive / Positive
1:1000	>40 / 35,66	39,6 / >40	38,6 / >40	37,8 / >40	Negative / Negative	Positive / Positive

Abbreviations: Ct, Cycle threshold; N, nucleocapsid protein of SARS-CoV-2.

**Table 2**  
Individual results of discordant samples.

N probe Ct values						
Sample ID	TaqMan 2019nCoV	qCOVID-19	FTD SARS-CoV-2	Allplex SARS-CoV-2	Hologic Aptima SARS-CoV-2	Clart COVID19
HCSC-5	27	29	30	30	Invalid	ND
HCSC-13	23	26	28	27	Invalid	ND
HCSC-23	25	26	28	27	Invalid	ND
HCSC-48	33	37	37.8	>40	Positive	ND
HCSC-52	33	34	35.6	>40	Positive	Positive
HCSC-66	30	30	32.5	33	Invalid	Positive
HCSC-70	32	33	34.5	37	Negative	Positive
HCSC-71	>40	37	>40	>40	Invalid	Positive
HCSC-72	>40	39	>40	34.4	Invalid	Positive

Abbreviations: Ct, Cycle threshold; N, nucleocapsid protein of SARS-CoV-2; ND, no determined.



**Fig. 1.** Efficiency comparison of four commercial RT-qPCR according to N probe cycle threshold. Ninety-five positive clinical samples were used for the analysis.

TaqMan 2019nCoV Assay (Thermofisher), qCOVID-19 (Genómica), FTD SARS-CoV-2 Assay (Siemens), Allplex™SARS-CoV-2 (Seegene).

CLART-COVID-19. Microarray results showed fully agreement with qCOVID-19 (Genómica).

#### 4. Discussion

This study compared the analytical sensitivities and technical

performance of 6 molecular assays for SARS-CoV-2 detection: four qPCR, one commercial DNA-microarray and the Aptima™ SARS-CoV-2 TMA assay. All of them showed satisfactory sensitivity and seem suitable tools for the diagnosis of coronavirus disease 2019.

Since the beginning of pandemic, the majority of commercially available test for SARS-CoV-2 diagnosis are based on real-time RT-PCR assays (Shen et al., 2020). In our study the concordances among the qPCR techniques tested was 96 %. We use the Ct values of probe N to compare the four qPCRs with each other as this is the only common target for all of them. The used a Ct value for viral load calculation is being debated. However, it should be noted that, even if the same gene is examined, all other reaction properties are different (primers, probes, reaction buffer, enzymes, reaction conditions). Also, factors such the software definitions or the calculation method can affect the results, not only the reaction sensitivity itself.

The used a Ct value for viral load calculation is being debated. Differences in Ct values for the same calculated viral load have been observed from different assays (Carroll and McNamara, 2021). These differences can be associated with the technique employed but there are other factors that should be also taken into account. So, RNA extraction system or technical expertise cannot be ruled out. To avoid interference of RNA extraction system, the same EasyMag extraction was used in our study for all commercial qPCR assays and only persons with high molecular experience were responsible for testing.

Our comparative analysis showed different results with four samples. All discordant results were false negatives compared to previous data. The false negative results are especially worrisome, since they can lead to community transmission.

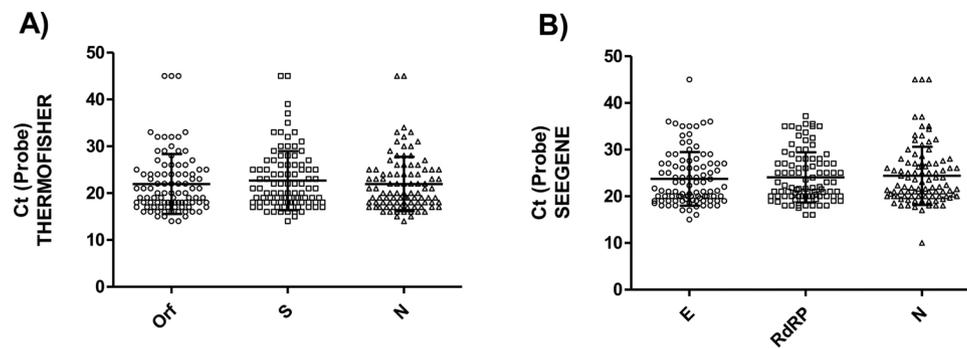
The use of frozen specimens for analysis can affect sensitivity of PCR but since the same sample was used for all the assays, they should all be affected equally.

Two out of the four reagents used for SARS-CoV-2 RNA assay employ triple-target genes, being necessary only one to consider a sample as positive. Detection of the E reaction only is not theoretically sufficient to render the sample positive because envelope gene is common to all Sarbecovirus, in practice, as at present no other SARS-like coronaviruses are circulating, the test can be considered also specific. The use of three genes could complicate the interpretation of the results but increases the possibility of detection since the chances that more than one gene is mutated at the same time, in the target regions, are very low.

Although the assays for comparing sensitivity of the probes reveal that differences seen among all the three probes were not statistically significant in any case, the results obtained using one or all probes are not the same, at least with Allplex™SARS-CoV-2 kit. Considering only N probe, three out of the 95 positive samples testing were negative by Allplex™SARS-CoV-2. Nevertheless, when all probes of this technique were considered all three became positive as at least one of the other two targets tested positive. This is in agreement with previous findings (Le Blanc et al., 2020) that have shown that the use of more than one probe improves virus detection even in samples with low viral loads.

A closer analysis showed that the two samples missed by the TaqMan 2019nCoV Assay were also missed by at least one of the other qPCR assays. These two samples were closed to the limit detection.

Analysis of clinical samples may be affected by several factors. Van Kasteren et al. (Van Kasteren et al., 2020), indicated that the amount of



**Fig. 2.** Comparison of the different probes of TaqMan 2019nCoV Assay (Thermofisher) and Allplex™SARS-CoV-2 (Seegene). ORF, probe for the open reading frame 1a and b; S, probe for spike protein of SARS-CoV-2; N, nucleocapsid protein; E, probe for E-gene, gene encoding the envelope protein; RdRP probe for the RNA-dependent RNA polymerase of SARS-CoV-2.

**Table 3**

Ct values of Taqman 2019nCoV and Allplex SARS-CoV-2 different probes in discordant samples.

Sample ID	TaqMan 2019nCoV			Allplex SARS-CoV-2		
	Orf1ab	S	N	E	RdRP	N
HCSC-48	>40	35	33	35.6	35	>40
HCSC-52	33	37	33	36	35.5	>40
HCSC-71	>40	>40	>40	>40	37.2	>40
HCSC-72	>40	>40	>40	36	35	34.4

subgenomic messenger RNA present inside cells of clinical samples may affect RT-PCR limit of detection. The use of standards contributes to avoid these factors and confirm the data obtained with clinical specimens.

Although qPCR offers many advantages for virus detection, this type of assays needs professionally trained staff, special equipment and is often time-consuming. Also, there are evidences that some of the available commercial RT-PCR can give false negative at the very early or very late stages of infection (Wang et al., 2020) when viral loads are low or when mutations in viral target regions are present. If multiple genes are tested, at least one out of them should still be useful for detection. The chances that all three genes (or even two genes) are mutated at the same time, in the target regions, are very low. In that sense, Thermofisher TaqMan 2019nCoV Assay shows an S-gene target failure with the new variant B.1.1.7 containing  $\Delta 69/70$  (Kidd et al., 2021). Nevertheless the use of multiple genes in the assay avoid false negative results in any case.

Moreover, false-positive results due to sample contamination can also occur and be a problem in any diagnostic laboratory whatever qPCR kit used.

This information is of great interest for laboratories who need to choose one testing platform. It is important also to take into account other factors such as cost, reagent availability or hands-on time. Many studies have carried out comparative analyzes of available commercial techniques (Rhoads et al., 2020; Zhen et al., 2020). All of them showed the usefulness of the proven assays for the detection and control of COVID-19. Not only RT-qPCR are of interest for this purpose, thus other systems like Hologic Aptima Panther SARS-CoV-2 TMA have been tested (Trémeaux et al., 2020; Zhen et al., 2020). The main conclusions of these studies indicated that the Aptima SARS-CoV-2 TMA assay data agree with those obtained with qPCR used as comparator. Our results showed that Aptima assay had slightly less agreement with the previous results mainly because of the six invalid samples. Nevertheless, Panther is easy to use and allows random access for urgent samples prioritization provide high-throughput diagnosis.

Microarray assays have showed good results with previous coronavirus (Habibzadeh et al., 2021). No data are available with

CLART®COVID-19 microarrays but CLART technology had been successfully used for detection of other respiratory viruses (Ferreira et al., 2020; Silva et al., 2019). In this study, CLART-COVID-19 correctly recognized as positive or negative all samples tested by this method.

Checking results altogether, three kits out of all tested (Allplex™SARS-CoV-2, qCOVID and CLART-COVID-19) were able to classify properly all the samples. The use of commercial SARS-COV-2 standard control confirm the results obtained with clinical samples. Only Clart-COVID-19 was able to detect all the dilutions in the assays carried on. The other techniques failed to detect the most dilute controls in some of the analysis performed. However, Clart-COVID-19 has the disadvantage that is only qualitative and it is not possible to distinguish between specimens with high or low viral load.

Considering our findings, we believe that all of the commercial assays tested can be useful for laboratory routine. For virus detection in populations with low viral loads (patients with no symptoms or patients during later stages of the infection) Clart-COVID-19 assay can be a good option while the Hologic platforms could be more appropriate for high-volume testing.

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## Ethical approval

Not required.

## CRediT authorship contribution statement

**L. Vallejo:** Investigation, Formal analysis. **M. Martínez-Rodríguez:** Investigation, Formal analysis. **M.J. Nieto-Bazán:** Investigation, Formal analysis. **A. Delgado-Iribarren:** Conceptualization, Funding acquisition, Writing - review & editing. **E. Culebras:** Data curation, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors report no declarations of interest.

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