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RESEARCH ARTICLE

Clear or White? A RT-PCR plate comparison for SARS-CoV-2 diagnosis

Elena Cristina Preda[1#], Valeriu Moldovan[1#*], Oana Roxana Oprea[2]

1. County Emergency Clinical Hospital, Târgu Mureș, Romania
2. George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Targu Mures, Romania

Objective: During the COVID-19 pandemic, laboratories have used various extraction and amplification kits, associated with different auxiliary materials. This study aims to investigate how different types of plates may affect RT-PCR performance. **Methods**: Data on the positive controls (PCs) of RT-PCR runs for SARS-CoV-2 detection between December 2020 and April 2022 was collected retrospectively in the Molecular biology department of the County Emergency Clinical Hospital of Târgu Mureș's clinical laboratory. The materials used consisted in MOLgen SARS-CoV-2 (Adaltis) and EliGene COVID19 CONFIRM 500 R amplification kits, 96-well full-skirted white and clear plates, and clear films. Microsoft Excel was used for the database and it included information about Cycle threshold (Ct) and maximum fluorescence. Statistical analysis, performed in MedCalc, consisted of Grubbs test, Kolmogorov–Smirnov Test, F test, T student test, and Mann-Whitney test to compare central tendencies. The significance threshold was set at $p<0.05$. **Results**: The Ct comparison for MOLgen kit white plates vs clear plates: FAM channel- U=1052.5, Z=2.07, p=0.038, medians for white plates and clear plates were 22.80 and 23.25, respectively; ROX channel- U=784, Z=3.21, p=0.001, medians 21.93 and 21.43, respectively; Cy5 channel- U=1028.5, Z=1.95, p=0.518, medians 22.12, 21.75, respectively. For EliGene kit: U=848.5, Z=3.27, p=0.001, medians 28.26 (white plates) and 28.0 (clear plates). Comparison of the maximum fluorescence reached on both kits with white and clear plates computed p values <0.0001. **Conclusions**: Between white and clear plates there are statistically significant differences considering Ct values and maximum read fluorescence, but with no impact on test outcome.

Keywords: RT-PCR, Cycle threshold, white plates, clear plates

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Introduction

The COVID-19 pandemic, caused by the SARSCoV-2 virus, raised major challenges on healthcare facilities and medical infrastructure. The devastating effects on populations, social structures, and economic growth urged the development of rapid diagnostic tools, effective treatment protocols, and most importantly, vaccines against the pathogen [1]. Some of the innovations in diagnosis were: reliably detecting the virus presence in nasopharyngeal swab samples without any RNA extraction steps and novel targets which improved the sensitivity and accuracy of detection [2,3].

If the laboratory tests, radiological characteristics, clinical ensemble and the exposure history rise suspicion for COVID-19, a definitive diagnosis is confirmed by real time reverse transcription polymerase chain reaction (RT-PCR) testing. The method implies reverse transcription of viral ribonucleic acid (RNA) found in patient samples, to complementary deoxyribonucleic acid (cDNA), continuing with the amplification of specific cDNA regions depending on the kit [4]. Some components of the master mix are the probes and primers. Probes, which are marked DNA sequences, are used to identify genetic targets, while primers are used to support the replication of the identified genetic material. For the SARS-CoV-2 genome, four targets are usually identified by RT-PCR: RdRp gene (RNA-dependent RNA polymerase), genes from structural proteins E (virus envelope) and N (virus nucleocapsid), and ORF1ab gene (open reading frame 1a and 1b) [5]. Nowadays, this method is considered the gold standard for the diagnosis of SARS-CoV-2 infection, characterized by 70% sensitivity and 95% specificity [6].

The cycle threshold (Ct) represents the number of replication cycles required to produce a detectable fluorescent signal. Regarding the interpretation of results, the lower the Ct values, the higher the viral RNA loads, and in most cases, a Ct <40 alongside with an exponential amplification curve (sigmoidal shape) announces a positive result [7], fact also indicated in the insert of the amplification kit [8]. However, for Ct values close to 40, the difference between a positive and a negative result is made depending on the utilized materials and the shape of the amplification curve, therefore molecular diagnostic laboratories may set a lower threshold in order to declare real positive samples [9].

Like any other assay, RT-PCR needs to meet quality control standards. Positive (PC), negative and no template controls provide information on the process of reverse transcription and amplification steps and on the availability of adequate intact target RNA and the availability of the master mix components [10]. To achieve the best performance of the extraction kit, the laboratory must supplement the kit with wisely chosen auxiliary materials. For instance, the microplates, tubes, sealing cups, and films, are a critical yet often overlooked component for PCR.

* Correspondence to: Valeriu Moldovan. E-mail: valeriumoldovan@gmail.com
# These authors have contributed equally and share first authorship.

Microplates variability of properties like differences in the microplate plastic, format, desired throughput, and even the "whiteness" of the plate can significantly affect assay performance [11,12].

In this study, we aimed to investigate how different types of plates may affect RT-PCR performance in terms of Ct values and detected fluorescent signals.

Materials and method

The study was performed in the Molecular biology department of the County Emergency Clinical Hospital of Târgu Mureș's clinical laboratory. Data on the PCs of RT-PCR runs for SARS-CoV-2 detection between December 2020 and April 2022 was collected retrospectively. Throughout this period, two different amplification kits were used in our laboratory: MOLgen SARS-CoV-2 (Adaltis, Italy) and EliGene COVID19 CONFIRM 500 RT (Elisabeth Pharmacon, REF: 90078-RT-500, Czech Republic). Also, different types of 96-well full-skirted plates were used: white plates (Eppendorf Twin-tec 96 real-time PCR, Eppendorf, United Kingdom) and clear plates (Azenta 96 Well Skirted PCR, Azenta, United States), all covered with clear adhesive PCR films (Eppendorf Masterclear real-time PCR Film, United Kingdom). Inclusion criteria: first run of the day and runs that respected validation criteria according to the kit's manufacturer. Exclusion criteria: runs other than the first run of the day, runs that did not meet the validation criteria, or runs performed with other amplification kits. Following this selection, a number of 214 PCs were selected for further statistical analysis.

MOLgen SARS-CoV-2 real time RT-PCR Kit was designed to detect SARS-CoV-2 genes N, E, and RdRP using FAM, ROX and Cy5 fluorimeter channels, respectively. The fluorescence was measured at 60°C, at the fourth step (primer annealing) every amplification cycle and the threshold line was set above any background signal.

EliGene COVID19 CONFIRM 500 RT detected SARS-CoV-2 using a mixture of 3 sets of primers and 3 TaqMan probes, labeled with FAM (excitation at 494 nm, emission at 512 nm). Emission signals were collected at 55°C every cycle, at the fourth step (primer annealing).

For reverse transcription and amplification, CFX96 Touch Real-Time PCR Detection System (BIO-RAD Laboratories) was used. CFX Maestro Software for CFX Real-Time PCR Systems 2.0 was used for data collection, data analysis, and graphing of real-time PCR data. Ct values were determined with the Base Line Subtracted Curve Fit analysis method of the software, using fluorescein normalized data.

For database and statistical analysis, we used Microsoft Excel 2018 (Microsoft Corporation) and MedCalc Statistical software version 20.104 (MedCalc Software Ltd, Ostend, Belgium). The following statistical tests were applied, based on data characteristics: Grubbs test for outlier detection, Kolmogorov–Smirnov to verify data distribution, F test for parametric data, T student test for unpaired data, and Mann-Whitney test to compare central tendencies. The significance threshold was set at $p<0.05$.

Results

From the 214 PCs selected, we found 55 MOLgen PCs on white plates, 51 MOLgen PCs on clear plates, 53 EliGene PCs on white plates, and 55 EliGene PCs on clear plates. The descriptive statistics after the exclusion of outliers is presented in Tables I and II.

For MOLgen PCs, the Mann-Whitney test was applied for white vs clear plates and the following results were obtained:

- Ct comparison for FAM: U=1052.5, Z=2.07, p=0.038.
- Ct comparison for ROX: U=784, Z=3.21, p=0.001.
- Ct comparison for Cy5: U=1028.5, Z=1.95, p=0.518.

Comparison of the maximum fluorescence reached on FAM, ROX and Cy5 channels, returned U values of 0, 4, and 0, respectively, and Z values of 8.83, 8.80, and 8.82, respectively. The p value was <0.0001 in all three cases.

Analyzing the EliGene PCs, the Ct comparison for the FAM channel between white and clear plates, revealed U=848.5, Z=3.27, p=0.001. Comparison of the maximum fluorescence on the FAM channel between different plates indicated $p<0.0001$.

Table I. Descriptive statistics for MOLgen PCs on clear (top) and white (bottom) plates. Data is presented as Ct values as well as RFU (relative fluorescence units) for each of the three channels: FAM, ROX, and Cy5.

FAM		Ct			Fluorescence (RFU)		
		ROX	CY5	FAM	ROX	Cy5	
Clear plates	N	55	50	54	54	54	55
	Data distribution	Non-parametric	Parametric	Non-parametric	Non-parametric	Non-parametric	Non-parametric
	Mean	23.17	21.41	22.12	1956.87	1410.04	1348.60
	Median	22.80	21.43	21.75	1924.50	1383.00	1394.00
	SD	1.30	0.75	1.21	158.08	230.96	313.10
	CV %	5.60	3.48	5.49	8.08	16.38	23.22
White plates	N	50	50	49	51	51	51
	Data distribution	Parametric	Non-parametric	Parametric	Non-parametric	Non-parametric	Non-parametric
	Mean	23.25	22.02	22.21	21569.22	11001.14	10300.18
	Median	23.20	21.93	22.12	20475.00	10059.00	9568.50
	SD	0.70	0.97	0.67	3834.13	3614.63	2698.92
	CV %	3.03	4.42	3.02	17.77	32.86	26.20

Abbreviations: N – number of PCs, Ct – cycle threshold, SD – standard deviation, CV – coefficient of variation.

Table II. Descriptive statistics for EliGene PCs on clear (left) and white (right) plates. Data is presented as Ct values as well as RFU (relative fluorescence units) for the FAM channel.

	Clear plates		White plates	
	FAM Ct	FAM Fluorescence (RFU)	FAM Ct	FAM Fluorescence (RFU)
N	51	51	53	55
Data distribution	Parametric	Parametric	Non-parametric	Parametric
Mean	28.05	3528.84	28.41	35201.78
Median	28.01	3471.00	28.26	35428.00
SD	0.28	407.57	0.56	2695.05
CV %	1.01	11.55	1.97	7.66

Abbreviations: N – number of PCs, Ct – cycle threshold, SD – standard deviation, CV – coefficient of variation.

Discussion

During the COVID-19 pandemic, many laboratories have used various extraction and amplification kits, associated with different auxiliary materials. In PCR reactions it is important to acknowledge that the poor choice of microplate color can result in lower signal-to-background ratios (compared to the optimal microplate color) and decreased assay performance [12].

Descriptive statistics

The collected MOLgen PC data showed homogeneous and small dispersion of Ct values, with parametric and non-parametric distribution regardless of the plate type. The maximum fluorescence was a relatively homogenous group indicating a higher degree of variation to its mean. The Ct and maximum fluorescence data collected from runs using the EliGene kit had, in most cases, normal and homogenous distribution with narrow variation to its mean, regardless of the plate used.

The intra-group variation can be explained by human pipetting errors (imprecision), the time needed to pipette the plate, and by the stability of the master mix components that could be altered by the number of freeze-thaw cycles.

Fluorescence analysis

As expected, white plates generated a statistically significant stronger fluorescence compared to the clear plates for both MOLgen and EliGene detection kits. White plates have a higher amplification efficiency. This theory is explained by the effect of white pigment in plastic on optic phenomena. The light reflection towards the fluorescence detector is enhanced by the white pigment. Also, the signal transmission through plastic is diminished, resulting in a more constant and intense reflection [10]. It is considered that white pigment plates with optimized amount of light returned to the detector, increase the intensity and sensitivity of real-time PCR assays [13,14]. This outcome was also observed in another study where the higher autofluorescence of white plates had positive effects on qPCR sensitivity, but not directly on qPCR efficiency [15].

Comparison of Cts

For MOLgen, the comparison of Cts obtained with different plates revealed statistically significant differences between Ct medians for FAM and ROX channels, and a statistically irrelevant difference in the case of Cy5 channel. Thus, it was shown that the kit has a higher detection capacity when combined with clear plates. The EliGene Ct median obtained on the FAM channel was statistically higher for the white plates compared to the clear plates. Regardless of the detection kit that was being used, the Ct values were lower for clear plates, indicating a better sensitivity to amplificated viral cDNA when this type of plate is used.

Despite the statistical findings from our data, by analyzing the central tendencies of Ct values, it can be observed that all medians differ by one another with a less than 0.5 cycles. Hence, the differences between white and clear plates when using EliGene and MOLgen SARS-CoV-2 detection kits, are unlikely to alter the outcome of an RT-PCR test result and affect clinical decision-making. In a study run by the company Analytik Jenna, a 2-cycle difference was detected between Ct values read on white and clear plates [13]. (Figure 1)

The PCR interpretation is not a matter of black and white, there are many variables that must be taken into consideration. Therefore, the laboratory diagnosis of SARS-CoV-2 infection is formulated not only based on Ct values, but also after verifying if the shape of the amplification curve has the specific sigmoidal fitting of the exponential equation [16]. Also, when Ct values are very close to the diagnostic threshold, either below or above, communication with the clinicians can provide additional data that may support the formulation or exclusion of a SARS-CoV-2 infection diagnosis. Moreover, in such uncertain cases, laboratory doctors may request an additional sampling while emphasizing the importance of the preanalytical phase.

Microplate selection is an iterative process. Factors such as automation compatibility, desired throughput, plate material and format, incubation temperature, and crosstalk must be balanced with cost considerations and availability [12]. Both types of plates, clear and white, have pros and cons: the white ones are considered to be beneficial for increasing the PCR sensitivity, while the clear ones allow the user to verify pipetting steps and volumes. When it comes to color choosing one of the most important criteria is the working principle of RT-PCR detection system, that can narrow the searching field. In our study, the instru-

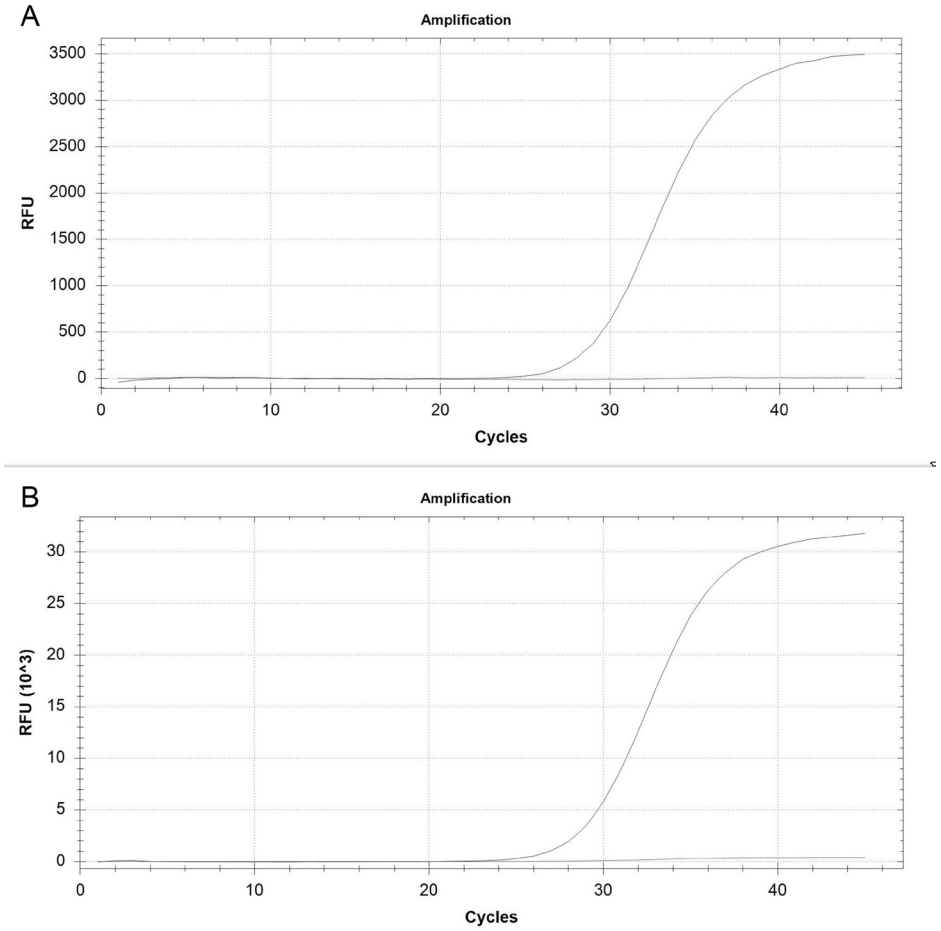

Figure 1 – An example of amplification curves of EliGene PC, A (upper curve) obtained using a clear plate and B (lower curve) using a white plate. In both examples the amplification curves have the specific sigmoidal fitting, with Ct values for A (clear) of 28.02 and for B (white) of 28.27, but despite the two similarities it can be observed the x10 difference between the measured fluorescence (A – 3027 RFU and B - 34539 RFU). The unamplified gene is represented by internal control used for nucleic acids extraction and it has no relevance in this study.

ment used had no limitation regarding the color of plates, meaning that the fluorescence detector could cover large ranges of fluorescence.

Conclusion

Between white and clear plates there are statistically significant differences considering Ct values and maximum read fluorescence, but with no impact on test outcome. Considering the equipment and detection kits used, the laboratory can use both white and clear plates interchangeably without affecting medical decision making or patient outcome.

Authors' contributions

ECP, VM, and ORO designed the study. ECP and VM collected the samples, made the statistical analysis and interpretation. All authors participated in drafting the work and revising it critically. ECP and VM have contributed equally to this work and share first authorship.

Conflict of interests

None to declare.

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