Applied Cell Biology

ISSN : 2320-1991 Online
ISSN : 2320-1983 Print
Volume 10 Issue 2
DOI: https://dx.doi.org/10.53043/2320-1991.acb90024

Research Article
Appl Cell Biol, 10(2), 2022 [45-50]

An in house qPCR System to Evaluate Transcripts Stimulated by an Immune Innate Agonist (CIGB2020) during the Covid19 Pandemic Scenario

Jorge Aguiar*, José Angel Silva, Omar Gell, Regla Estrada, Freya Freyre, Chabeli Rodríguez, Eduardo Pentón, Gerardo Guillén and Julio Cesar Aguilar

Biomedical Research Division, Center for Genetic Engineering and Biotechnology (CIGB); Ave 31 between 158 and 190. Cubanacan, Playa, P.O. Box 6162, Havana 10600, Cuba

***Corresponding author:** Jorge Aguiar, Titular Researcher, Center for Genetic Engineering and Biotechnology, Department of Vaccine, Havana, Cuba, E-mail: jorge.aguiar@cigb.edu.cu

ABSTRACT

Background: A low cost and simple real-time PCR method to quantify different transcripts in human samples is a need in most developing countries. A quantitative real-time PCR assay to evaluate the relative RNA expression stimulation of specific transcripts during the Pandemic COVID19 scenario was developed at the Center for Genetic Engineering and Biotechnology in Havana, using a commercial amplification system with standards and specific primers that are produced in house.

Methods: Oro-pharyngeal scrape samples from patients that were treated previously with an innate immune agonist, the CIGB2020, were employed to RNA purification, cDNA synthesis, and evaluation of different gene transcripts by the in house qPCR assay using different standard curves constructed with specific primers that amplify a pool of the own evaluated transcripts.

Results: Preliminary results showed a typical standard curve from the in house qPCR system developed. A typical standard curve during the evaluation of the TLR3 gene transcript was described as example, and was calculated automatically by plotting the Ct values against each standard of known concentration, and the linear regression of this curve was also calculated as: M=-3.31641, B=42.73084, R=0.9516. It was also observed the influence of the CIGB2020 on the stimulation of the three toll like receptors from the innate immune system.

Conclusions: This study demonstrated that the in house qPCR system is useful to quantify the relative induction of the expression of several genes transcripts considered as immunological markers of the immune innate system.

Keywords

in house qPCR
Gene transcripts
CIGB2020
COVID19

INTRODUCTION

The innate immune system is the target of several immune pathological mechanisms of CoV and other respiratory infections. Innate immune sensing serves as the first line of antiviral defense. To overcome this defensive system, coronaviruses (CoVs) develop multiple evasive strategies that affect their detection, a common property among RNA viruses causing respiratory infections [1,2].

HeberNasvac is a mucosal/ parenteral therapeutic vaccine for chronic hepatitis B codified as CIGB2020 and comprise inactivated antigens (HBsAg + HBcAg) with immune-modulatory and antiviral activities. CIGB2020 antigens are well known for their capacity to safely stimulate TLRs and their capacity to increase the antigen presentation [3,4].

High prices characterize most of the specific commercial system for PCR quantification. A low cost and simple real-

time PCR method to quantify different transcripts is a need in most countries from third world. The high reduction of the final volume of one qPCR reaction from our in house qPCR system until 15 uL, and the generation of several standard curves originated from a pool of the own samples in study and amplified with the produced primer from CIGB, leading to the minimization of the cost and the simplicity of the in house qPCR reaction. Our laboratory has experience to create in house qPCR systems simple and cheap [5].

The present article is aimed to describe the characteristics of a simple quantitative PCR method, using a combination of an unspecific commercial amplification kit:"LightCycler 480 SYBR Green I Master" (Roche, Germany), and standard curves based on a pool of transcripts amplified with specific primers produced at CIGB, to measure in cDNA samples the relative concentration of several important genes uses as makers of the stimulation of the innate immune systems, like the RNA receptors known as "toll like receptors" (TLR), for example TLR3, TLR7 and TLR8.

MATERIALS AND METHODS

Formulation and antigens

CIGB2020 contains 100 µg of each antigen in a final volume of 1.0 mL in saline-phosphate buffer, pH 7.0. No other additives, preservatives or stabilizers are included.

Subjects

The study was planned to enroll two groups, treatment and control, with a total 26 patients, of both sexes, 60 years or older, defined as risk contacts of SARS-CoV2 infected patients or suspected SARS-CoV-2 infection according to symptoms, arriving to the outdoor facilities of the Navy Hospital "Luis Diaz Soto" (Havana, Cuba). Patients unable to provide informed consent were excluded.

Samples

Oro-pharyngeal scraping samples were taken in day 0 (Time 0 without treatment) and in day 4 (Time 1 or treated with CIGB2020) from the tonsils region of patient using Ayre's spatulas. These samples were conserved in sample preservation solution (Miltenyi, USA). In general, mucosal samples were taken according to dedicated procedures approved by the Biosafety Department at CIGB and after training of healthcare workers at the clinical site.

RNA purification and reverse transcription for TLR gene expression

RNA purification was performed using the RNeasy Mini Kit (250) purification kit (Qiagen, Germany), following kit instructions. RNA was quantified using a Nanodrop spectrophotometer (Thermo-Scientific, US). The reverse transcription (RT) reaction to obtain the cDNA was carried out according to kit instructions (Quantitect Reverse Transcription Kit, Qiagen, Germany). Patient samples with paired RNA from days 0 (T0) and 4 (T1) were selected for qPCR assessment.

Genes and primers

Previously reported primers were used to amplify fragments from the cDNA of the human TLR3 gene (sense: 5′ CGAGTTAAGAGGCTGGAATGGT 3′, antisense: 5′ GCCAGGAATGGATGAGGTCAGA 3′), which generates an amplicon of 176 base pairs (bp); TLR7 (sense: 5′ TGTCGACGCATCAAAAGCAT 3′, antisense: 5 'GTGGAAATTGCCCTCGTTGT 3′), which generates an amplicon of 101bp, and TLR8 (sense: 5′ TCTGCATGAGGTTGTCGTGA 3′, antisense: 5′GTCGTCGTCGTCGTGCGTGCGTGA 3') which generates an amplicon of 103bp [6]. As a constitutive gene for normalization, the GusB gene (sense: 5′ CGTGGTTGGAGAGCTCATTTGGAA 3′, antisense: 5′ ATTCCCCAGCACTCTCGTCGGT 3′) was used, which generates a 73 bp amplicon [7].

In house quantitative polymerase chain reaction (qPCR) design for TLR gene expression studies

The relative concentrations of the TLR3, TLR7 and TLR8 was detected using a standard curve for each TLR according to the methodology developed in-house (CIGB, Havana, Cuba), using the house-keeping gene GusB as a control gene for normalization of the results, following the methodology previously reported by Nolan and cols, and others authors [8-10].

Quantitative analysis of the gene expression using the in house qPCR

After the reverse transcription (RT) assay, samples were diluted 1/10 with water from the qPCR kit before using for qPCR. In agreement with RNA concentration inside of each RT sample, it was calculated the volume per each sample that represent the same RNA/cDNA amount to start similarly in the qPCR reactions.

In house qPCR reaction

In general, the qPCR reactions were prepared using the SYBR Green from Roche kit (Light Cycler 480 SYBR Green I Master, Germany), also were added the specifics primers (synthesized at the Primers Synthesis Department of CIGB) and the final volume it was completed with water from the Roche kit. The qPCR reaction was stablished in 15 uL final volume as follow: 7.5 uL of SYBR Green Master mix from Roche kit, 1 uL of each sense and antisense primers at the concentration of 10 pmoL/uL, and 0.5 ul water. Finally, it was added to the qPCR reaction until 5 uL of each RT or cDNA sample, according to

the calculated quantity to start with approximately the same amount of cDNA in all qPCR reactions, the rest of the volume was completed with water from the Roche kit.

In house standard curves

Standard curves were created with a previously prepared pool of all cDNA samples in the study and serial dilutions 1/5, 1/10, 1/20 and 1/50 were used as curve standards, that after were amplified with the TLRs primers pairs (TLR3, TLR7 and TLR8). For each point of the curves was assigned an arbitrary concentration value: 2000, 1000, 500 and 200 relative units/mL to establish the relative quantification system.

qPCR machine

The qPCR experiments were conducted in a "Rotor Gene 3000 Machine" from Corbbett Research, Australia, using a 72 wells rotor. In each qPCR experiment a total of 10 cDNA samples were analyzed by triplicate with the specific primers for a particular gene under study, and a four point's standard curve in which, each point was also amplified by triplicate, with exception of 1/5 dilution point that only was amplified by duplicate for rotor space reason.

qPCR program

The program of the qPCR assay was as follow: 95oC per 5´, 45X (94oC -10", 60oC -10" and 72oC -10"). The qPCR results were calculated using the Corbett software. The final value of each sample was obtained through the ratio of each gene (TLR3, TLR7 and TLR8) and the mean of the GusB gene. The results were compared between the same group assessments (control or treated) at days 0 vs day 4 after treatment start.

Statistics

Statistical analysis was conducted using the Graphpad Prism Version 5.0. Differences were considered as statistically significant ($p<0.05$).

RESULTS AND DISCUSSION

General performance of the in house qPCR assay

Firstly, the base pair size of the PCR products obtained with the primers selected (6,7) and synthesized at CIGB using the in house qPCR were verified in agarose gel and compared with the appropriate molecular weight standards. Secondly, the performance of the standard curves for each gene evaluated with the in house qPCR were assayed and studied in order to characterize our in house qPCR method.

Calculation of the in house qPCR standard curves: An example of the standard curve performance for TLR3 gene transcript evaluation

The standard curve from the in house qPCR for TLR3 gene transcript evaluation was calculated automatically by plotting the cycle threshold (Ct) values against each standard of known concentration and the linear regression of this curve was also calculated as: slope (M)=-3.31641, intercept (B)=42.73084, correlation coefficient (R)=0.9516 (Figures 1 and 2).

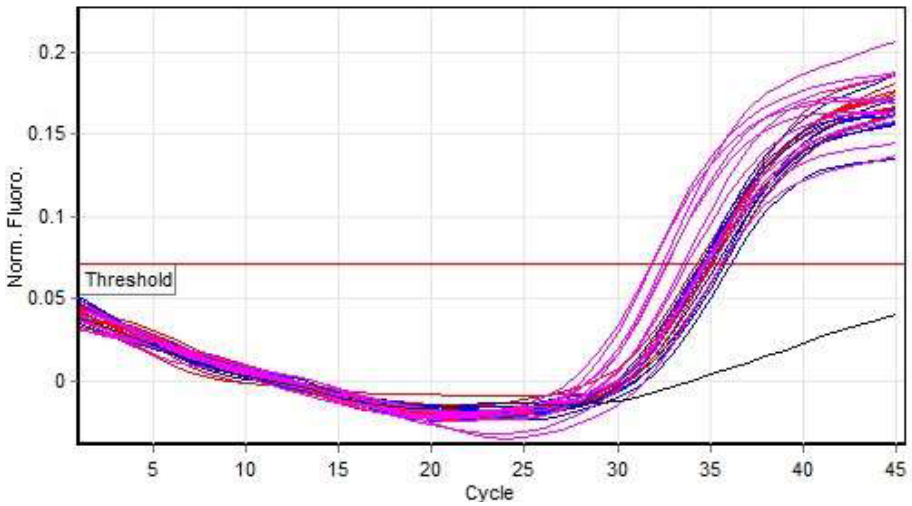

Figure 1: The figure represents the quantification data of a qPCR experiment using an in house TLR3 standard curve of four points by triplicate each one, and also contains as example of quantifications, data from three patient samples of the study analyzed too by triplicate. In this qPCR experiment, the lowest point of the in house qPCR standard curve to evaluate the TLR3 gene transcripts relative expression is the major dilution from the cDNA pool of the study (1/50). Negative controls of water, instead cDNA, do not produce detectable PCR reaction in up to 45 PCR cycles.

Research Article

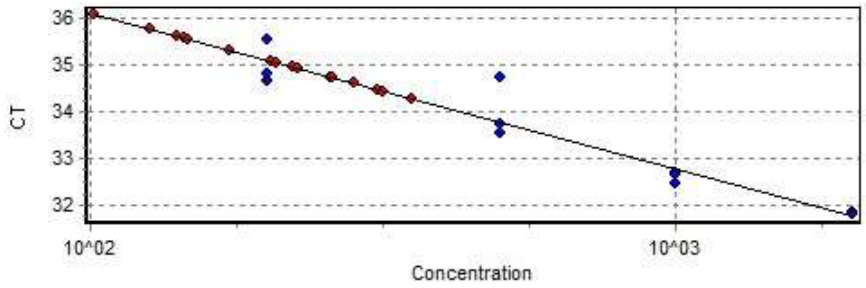

Figure 2: This figure shows the standard curve graph of the same experiment presented in figure 1. The relative quantification of the TLR3 gene transcripts in three individual samples of the treated group with CIGB2020 comparing the times T0 (not yet treated) vs T1 (treated) using a standard curve amplified from a pool of cDNA with the selected primers for TLR3 gene transcripts, shows the linearity of the different points of the curve when analyzing the values obtained by three replicates per point. M=-3.31641, B=42.730843, R=0.9516, R2=0.90554. The efficiency of the in house qPCR reaction was high: 1.00231.

The values of M and B observed in this experiment are repeated in the same range in all qPCR experiments carried out with the in house qPCR system developed at CIGB, evidencing good efficiency and sensitivity. This result also evidenced the accuracy of the in house qPCR system, since majority values obtained from the evaluation of the standard curves were within the interval of expected values ± 0.5 log on analyzing the values obtained by three replicates per point (Table 1). Table 1 shows an example of the quantification results by three replicates of three samples from the study comparing T0 (not yet immunized) and T1 (after treatment) from the treated group with CIGB2020, and also shows how the Ct (cycle threshold) varies as a function of the concentration of the standards curve points.

We consider that the in house qPCR system reported and discussed in this paper is reliable because of the correct size observed in agarose gel electrophoresis for the four PCR products from the gene transcripts studied (TLR3, TLR7, TLR8 and GusB) according to the reported primer pair [6,7] employed and described in the Material and Methods section (data not shown), and also because of the accurate results found in the quantification of the standard curve and the three patient's samples by triplicate (see the example in table 1), and finally because of the repeatability of the calculated parameters of the standard curves obtained for the four genes studied as commented before (data not shown). The methodology of qPCR quantification reported by other authors [8-10] and employed by us, resulted useful in the evaluation of the relative concentration of transcripts as we described here in this work.

Highlights of the TLRs (TLR3, TLR7 and TLR8) gene transcript stimulation of the Cuban patients evaluated after CIGB2020 application by nasal spray

The 26 Cuban subjects evaluated in this study are from Havana. These patients are of age more than 60 and of both sexes. Figure 3 represents the influence of the CIGB2020 on the activation or stimulation of the three RNA receptors of the innate immune system (TLR3, TLR7 and TLR8) evaluated as a whole at the CIGB2020 treated group of the study, comparing T1 or treated patients at day 4, with T0 (not yet treated or not yet immunized patients). Also other authors have described recently the influence of CIGB2020 on the stimulation of the gene transcripts synthesis and proteins from the innate immune system in the pandemic COVID19 scenario [11,12].

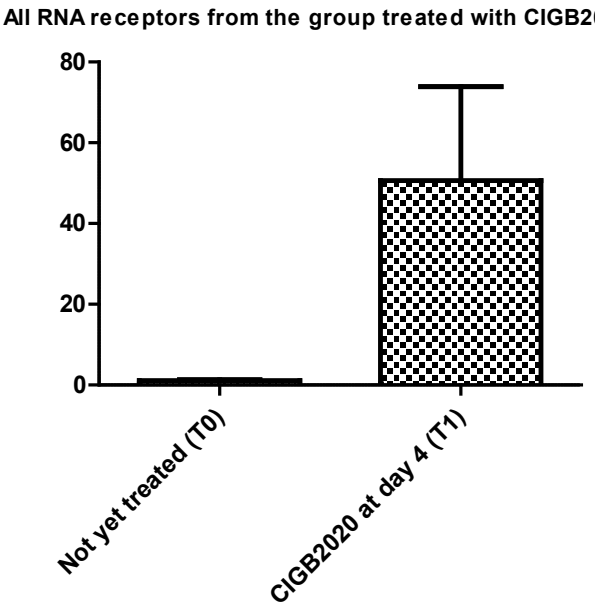

Figure 3: Influence of CIGB2020 on all three RNA receptors studied (TLR3, TLR7 and TLR8) as a whole in the study at day 4. Significant statistical differences were observed (p=0.0367) between not treated (T0) and treated group with CIGB2020

Research Article

Table 1: The table represents the quantification of TLR3 gene transcripts by triplicate of all samples corresponding to the four standard curve points and three patient's samples from the experiment represented in Figures 1 and 2.

No.	Name	Type	Ct	Given Conc	Calc Conc)	% Var
1	cDNA Sample 1 (S1) time 0 (T0), first attempt	Unknown	34.28		353.18	
2	S1 (T0) second attempt	Unknown	34.73		258.68	
3	S1 (T0) third attempt	Unknown	34.44		315.73	
4	S1 (T1)	Unknown	35.55		146.62	
5	S1 (T1)	Unknown	36.08		101.36	
6	S1 (T1)	Unknown	35.77		126.01	
7	S2 (T0)	Unknown	34.74		256.10	
8	S2 (T0)	Unknown	34.96		220.66	
9	S2 (T0)	Unknown	34.61		281.59	
10	S2 (T1)	Unknown	34.93		225.45	
11	S2 (T1)	Unknown	35.31		172.36	
12	S2 (T1)	Unknown	35.57		144.55	
13	S3 (T0)	Unknown	35.61		140.07	
14	S3 (T0)	Unknown	35.08		203.38	
15	S3 (T0)	Unknown	35.05		207.54	
16	S3 (T1)	Unknown	34.44		315.21	
17	S3 (T1)	Unknown	34.47		308.88	
18	S3 (T1)	Unknown	34.61		281.59	
19	1/5 only two replicates	Standard	31.85	2,000.00	1,907.84	4.6%
20	1/5	Standard	31.80	2,000.00	1,970.66	1.5%
21	1/10 three replicates	Standard	32.46	1,000.00	1,254.13	25.4%
22	1/10	Standard	32.69	1,000.00	1,069.07	6.9%
23	1/10	Standard	32.67	1,000.00	1,079.08	7.9%
24	1/20 three replicates	Standard	33.75	500.00	510.42	2.1%
25	1/20	Standard	34.75	500.00	255.51	48.9%
26	1/20	Standard	33.55	500.00	585.39	17.1%
27	1/50 three replicates	Standard	35.56	200.00	145.66	27.2%
28	1/50	Standard	34.66	200.00	270.71	35.4%
29	1/50	Standard	34.81	200.00	244.27	22.1%
30	Negative Control using water instead cDNA	NTC				

Ct: Cycle threshold; Given Conc: Given concentration; Calc Conc: Calculated concentration; %Var: Percentage of variation=[(Calc. Conc-Given Conc)/ Given Conc)] x 100; Unknown: patient cDNA samples. Standard: in house standard curve points.

Remarkable, the evaluation of the in house qPCR system we presented here is relatively cheap, simple and accurate, and it has repeatability. Through an example is shown the performance of the standard curve for TLR3 gene transcript quantification, and also it is observed the relative quantification of three samples from de study (Table 1). Finally by the graph of figure 3, it is observed the influence of CIGB2020 on the stimulation of the three receptors from the innate immune system (TLR3, TLR7 and TLR8) studied as a whole, that were estimated using the in house qPCR system proposed in this communication.

REFERENCES

1. Kikkert M (2020) Innate immune evasion by human respiratory RNA viruses. Journal of Innate immunity 12: 4-20.

2. Vabret N, Britton GJ, Gruber C, Hegde S, Kim J, et al. (2020) Immunology of COVID-19: Current State of the Science. Immunity 52: 910-941.

3. Pentón-Arias E, Aguilar-Rubido JC (2021) Cuban Prophylactic and Therapeutic Vaccines for Controlling Hepatitis B. MEDIC Review 23: 21-29.

4. Al-Mahtab M, Akbar SM, Aguilar JC, Uddin MH, Khan MS, et al. (2013) Therapeutic potential of a combined hepatitis B virus surface and core antigen vaccine in patients with chronic hepatitis B. Hepatol Int 7: 981-989.

5. Aguiar J, Rodriguez L, León Y, Freyre F, Silva JA, et al. (2014) Hepatitis B Virus DNA Quantification Using a Cost Effective and Simple Real Time PCR in Cuban Carriers. Journal of Infectious Diseases and Therapeutics 2: 49-58.

6. Xu J, Yang Y, Sun J, Ding Y, Su L, et al. (2006) Expression of Toll-like receptors and their association with cytokine responses in peripheral blood mononuclear cells of children with acute rotavirus diarrhea. Clinical & Experimental Immunology 144: 376-381.

7. Wisskirchen K, Metzger K, Schreiber S, Asen T, Weigand L, et al. (2017) Isolation and functional characterization of hepatitis B virus-specific T-cell receptors as new tools for experimental and clinical use. PLoS One 12: e0182936.

8. https://www.gene-quantification.de/national-measurement-system-qpcr-guide.pdf

9. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M (2015) How good is a PCR efficiency estimate: Recommendations for precise and robust qPCR efficiency assessments. Biomolecular Detection and Quantification 3: 9-16.

10. https://www.qiagen.com/us/resources/faq?id=411524f1-fe07-41bc-bbd2-32e1ce16b497&lang=en

11. Fleites YA, Aguiar J, Cinza Z, Bequet M, Marrero E, et al. (2021) HeberNasvac, A Therapeutic Vaccine for Chronic Hepatitis B, stimulates Local and Systemic Markers of Innate Immunity: Potential Use in SARS-CoV-2 Postexposure Prophylaxis. Euroasian Journal of HepatoGastroenterology 11: 59-70.

12. Akbar SMF, Mahtab MA, Aguilar JC, Uddin MH, Khan SI, et al. (2021) Repurposing NASVAC, a hepatitis B therapeutic vaccine, for pre- and postexposure prophylaxis of SARS-CoV-2 infection. Journal of Antiviral &Antiretroviral 13: 1-7.

Citation: Aguiar J, Silva JA, Gell O, Estrada R, Freyre F, et al. (2022) An in house qPCR System to Evaluate Transcripts Stimulated by an Immune Innate Agonist (CIGB2020) during the Covid19 Pandemic Scenario. Appl Cell Biol, 10(2), 2022 [34-50]