A technical transfer workshop for strengthening SARS-CoV-2 detection laboratories by PCR

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Abstract:

Based on results of a SARS-CoV-2 Proficiency Test by PCR, performed in 2020 in Colombia with 120 laboratories, 14% showed difficulties at detecting viral RNA at low concentrations - 14 copies/uL, reporting in some cases a negative result or a false negative. In order to strengthen the national network of SARS-CoV-2 detection laboratories by PCR, a technical workshop was organized; for this, two RNA control materials in two concentration levels were prepared and sent to 38 laboratories, they had to prepare a calibration curve from 10^5 to 10^1 copies/uL by diluting the materials, and measure them by the implemented qPCR protocols. Based on the results, they could evaluate the precision at each concentration level, the amplification efficiency and the detection at low concentration, near the limit of detection. The development and execution of these activities made it possible to identify possible metrological weaknesses of the laboratories in the detection of SARS-CoV-2 sequences by RT-PCR and will improve the quality of the measurements from the perspective of public health surveillance.

1. Introduction

Due to the outbreak and rapid expansion of the SARS CoV-2 virus at the end of 2019, a pandemy was declared in March 2020. Based on the virus nature, a Reverse Transcription - Polymerase Chain Reaction (RT-PCR) technique was established as the reference method for its detection ([1]-[3]). Rapidly, several commercial protocols were available for SARS-CoV-2 detection; to date, there are more than 600 commercial assays (Invitro Diagnostic Kits - IVD) by PCR [7]-[8]. An important feature is that each one provides its own positive and negative quality controls; however, the controls of a particular commercial kit and their sample results are not comparable to other commercial tests in most cases [9], On the other hand, the information available on these controls is limited to specifying the nucleotide sequence of the primers supplied and, in the case of the positive control, an instrumental response for real-time PCR equipment, which is hardly equivalent to other commercial assays.

In 2020, a proficiency test (PT) was performed over the national network of laboratories for SARS-CoV-2 detection by PCR, where a panel of five samples (three positive (1400, 140 and 14 copies/uL) and two negatives) were sent to each laboratorie. 17 laboratories failed to correctly assigned the lowest concentration level sample (14 copies/uL) as positive (false negative) in assays detecting RdRp, E and N genes. The selection of the commercial kits is based on characteristics as the economic price, in the reported performance characteristics, in the number of target assays evaluated (in simplex or multiplex), in the availability, among other; however, user do not have the opportunity to evaluate the selected kits, usually because there are not available independent control

materials, with reported values in concentration instead of response units (Ct, Cq, etc)

In order to strengthen the National Network of laboratories than performed SARS-CoV-2 detection by PCR, with National Health Institute of Colombia, the National Metrology Institute of Colombia, with the support of National Institute of Standards and Technologies (NIST) and the Joint Research Center (JRC) from the European Union, a technical workshop was organized to provide the capacity to laboratories to evaluate parameters as precision, amplification efficiency, limit of detection of their implemented methods in order to guarantee confidence in their measurement results, and by the other, to get some information on the current status of the measurement methods used by participating laboratories.

For this, two control materials in two concentration levels were prepared from RGTM 10169 material (from NIST) and EURM-019 (from JRC) and named NIPC and JIPC, they were previously quantified by digital PCR with retrotranscription using the E charité gene. Laboratories had to prepare a calibration curve from 10^5 to 10^1 copies/uL by diluting the materials (JIPC or NIPC), and measure them by the implemented qPCR protocols. Based on the results, they could evaluate the precision at each concentration level, the amplification efficiency and the detection at low concentration, near to the limit of detection. The activities related with the material preparation, and the workshop results are presented below.

3. Material and methods

RT-PCR methods

One-step amplification for RT-ddPCR was performed using the One-Step RT-ddPCR Advanced Kit for Probes in the QX200 System (BioRad cat 1864001). The reaction mix consisted of 4µL of SARS CoV-2 RNA, 900nM reverse primer, 800nM forward primer 300nM for probe, 1X Supermix RT-ddPCR oneStep buffer, 20U/µL of Reverse Transcriptase 15mM DTT, and RNase- and DNase-free water to complete a final volume of 22 µL

The retrotranscription and amplification cycle were as follows: Retro-transcription at 50ºC for 60 minutes, an enzyme activation at 95ºC for 10 minutes; 45 cycles at 95ºC for 15s and 56ºC for 30s and a final enzyme inactivation at 98ºC for 10 minutes, the heating ramp was 0.5Cº/s.

The analysis of results was carried out using the R Software [11] and the QuantaSoft Analysis PRO software for the multiplex tests. A partition volume of 0.7726nL ± 0.0223nL previously determined at the INM was used.

Reagents

Two SARS-CoV-2 RNA RMs were used as starting material to produce the control materials

- Reference Material RGTM10169 provided by NIST, it is composed of two vials of synthetic RNA of the SARS-CoV-2 genome (Covering the regions of the E, RdRp and N genes) with a nominal concentration of 5 x10^6 copies/µL in a 5 ng/µL matrix of human Jurkat RNA (Invitrogen AM7858)
- Reference Material EURM-019 provided by the JRC-IRMM corresponds to a vial with 100µL, of a solution with a synthetic ss-RNA fragment of 880 bases from the SARS-CoV-2 genome (Covering the regions of the E, RdRp, N and Spike genes), transcribed in vitro and stored in Citrate buffer pH 6.4, with an average concentration of 7x10^7±0.5 copies/uL.

- Citrate buffer pH 6.4 (Invitrogen, AM 7001): dilution of the RM produced and as storage solution.
- human T-Cell Leukemia total RNA line (Invitrogen, AM7858): Spinking samples, to simulate the matrix of a human sample.

Control materials production

There were prepared four control materials: NIPC-1 and NIPC-3 from RM RGTM 10169 (label yellow) and JIPC-1 and JIPC-3 from RM EURM-019 (label red); by gravimetric dilutions with 1 mM sodium citrate buffer pH 6.5

The dilutions were shaken in an orbital for 40 min to ensure their homogenization before to dispense 50 uL into 65 vials of 500 µL polypropylene, screw cap and seal, DNase- and RNase-free cryovial (Biologix Cat 81-7054) for each material and each concentration level.

A panel composed of 5 vials: NIPC-1, NIPC-3, JIPC-1, JIPC-3, and 1mM citrate buffer (NTC blue label) were packed in a plastic blister, a plastic bag and in an aluminized bag. 65 panels were produced (Figure 1)

Figure 1: Presentation of each panel with control materials

Reference Materials characterization

For the homogeneity study, 8 vials per level were randomly selected, according to ISO-35 guide [7], they were measured in triplicate by RT-dPCR and RT-qPCR, using the E Charité assay. From a one factor ANOVA, The homogeneity uncertainty (u_{hom}) was calculated according to Equation 1, where $MS_{between}$ is the mean square between vials and MS_{within} is the mean square within the vials and n the degrees of freedom

$$u_{hom} = u_{bb} = s_{bb} = \sqrt{\frac{MS_{between} - MS_{within}}{n}} \quad \textbf{Equation 1.}$$

For the stability study, an isochronous design was used, at two concentration levels (high and low for NIPC and JIPC) at 4ºC and -20ºC, using -70ºC as reference temperature, for eigth weeks; for each time/temperature point, two units of each control material were measured in triplicate. The stability

evaluation was calculated through a simple linear regression analysis, where the uncertainty (u_{lts}) was calculated based on the slope standard deviation $s(b)$ and the time(t) (in weeks) according to Equation 2.

$$u_{lts} = s(b) * t \quad \textbf{Equation 2.}$$

The assigned value for each control material was calculated as the mean of five units randomly selected; they were measured by RT-ddPCR using the N1-China assay in triplicate. The standard uncertainty was calculated from the combination of characterization, homogeneity and stability uncertainties according to Equation 3

$$u_{RM} = \sqrt{u_{char}^2 + u_{hom}^2 + u_{stab}^2} \quad \textbf{Equation 3.}$$

The characterization uncertainty (Equation 4) was derived from the mathematical model expression(Equation 5) for the quantification of each control material by dPCR, it is function of the sample dilution (d), the droplet volume (V), the lambda (copies per partition) parameter (λ), calculated from the positives (p) and the total number of partitions (R).

$$u_{CMR} = C_{MR} * \sqrt{\left(\frac{u_\lambda}{\lambda}\right)^2 + \left(\frac{u_d}{V}\right)^2 + \left(\frac{u_d}{d}\right)^2} \quad \textbf{Equation 4.}$$

$$[M] = \frac{\lambda}{V*d} \quad \lambda = -ln\left(1 - \frac{P}{R}\right) \quad \textbf{Equation 5.}$$

Workshop

38 laboratories from different cities in Colombia received:

- A panel with four vials with RNA from SARS-CoV-2 and one with a NTC (citrate buffer, pH 6.5).
- A safety data sheet for the control materials
- An information sheet with material description, the controls intended use, the assigned values (in copies/uL), informative values (Ct values for E, S and RdRp genes), a description of the measurement process for value assignment, handling, storage and transport instructions, among others.
- A detail technical protocol than includes instructions for reception and storage of samples, gravimetric or volumetric dilution for calibration curve preparation for NIPC and JIPC material with the citric buffer (they were asked to select JIPC or NIPC material depending the implemented protocols in the laboratory), samples measurement, analysis of results
- A digital form to register and analyze data from sample measurements, it allows laboratories to calculate the concentration value for each dilution, the precision by repeatability of each concentration level in the calibration curve, the amplification efficiency, and a control chart if the laboratory uses a dilution several times. On the other hand, this form allowed us to collect some information related to measurement platforms, commercial kits, most common genes used for SARS-CoV-2 detection, experimental volumes, among others used by participating laboratories.

4. Results and discussion

Homogeneity Study

Table 1 shows the relative homogeneity uncertainty results for each one of the prepared control materials. JIPC 1 and JIPC 3 showed the higher values, while NIPC 1 and NIPC-3 the smallest; all of them are below 2.0%; taking into consideration than similar materials may have uncertainties around 10 to 20% [4]-[5], 2.0% (the fifth part) was considered suitable for this process.

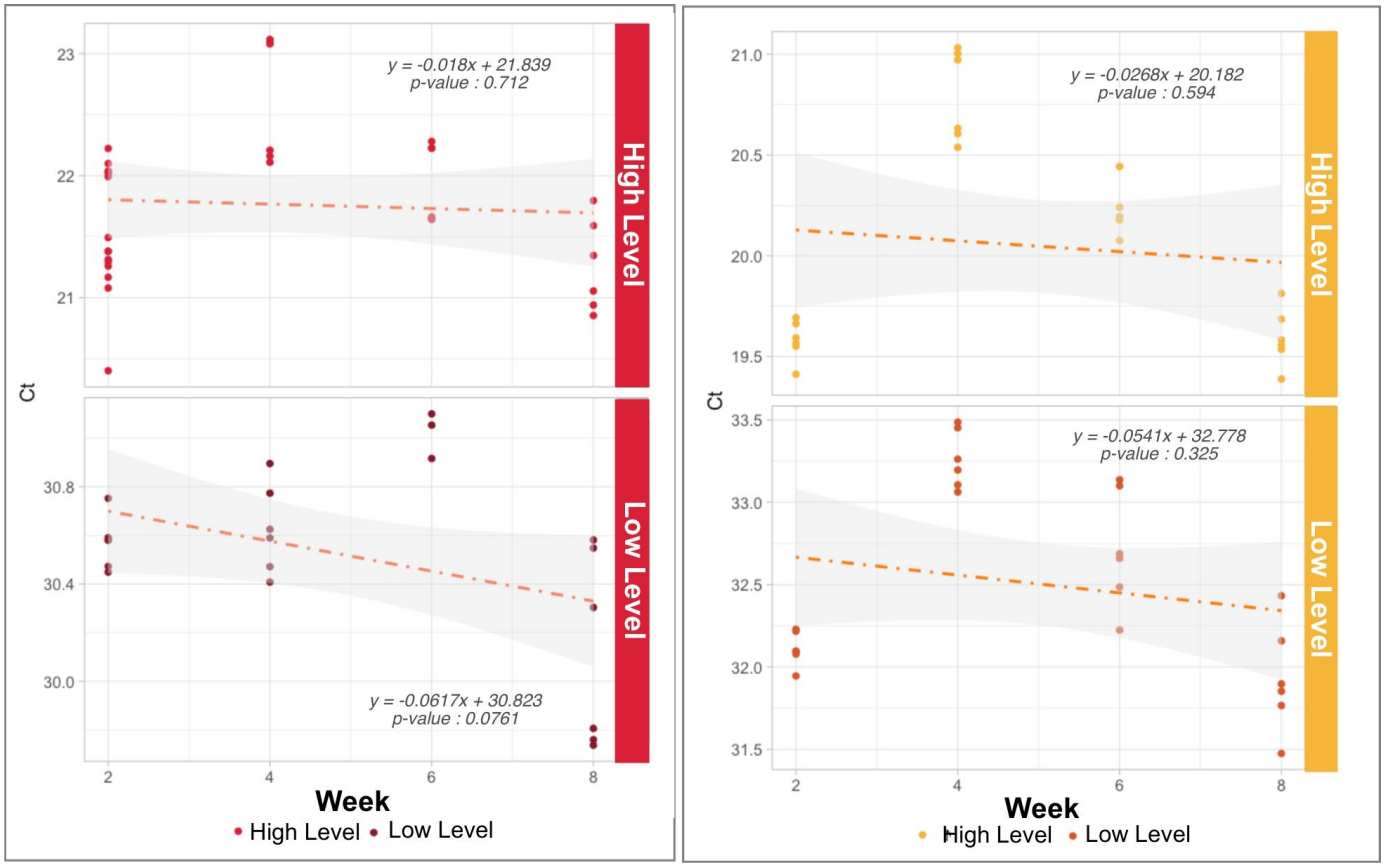
Table 1: Relative homogeneity uncertainty

Control material	Mean Ct value	rel uncertainty (%)
JIPC-1	20.62	2.0
JIPC-3	29.62	1.4
NIPC-1	19.17	0.7
NIPC-3	31.86	0.9

Stability Study

Figure 2 shows the stability study results for each control material at 4°C, according to p-values, there is not a significative trend between the instrumental response (Ct) and the time (in weeks), indicating that NIPC and JIPC control materials at both concentration levels are stable for eighth weeks, enough time to perform the technical workshop.

Figure 2: Stability Study - SARS CoV-2 RM



In relation to the uncertainty due to stability, it was obtained values of 0.40 (1.9%), 0.43 (1.32%) for the NIPC-1 and NICP-3 material, and 0.38 (1.8%) and 0.43 (1.4%) for the JIPC-1 and JIPC-3 materials respectively.

Control material Characterization

Table 2 shows the results for the characterization and the assigned value process for each control material. From the five vials selected, a one factor ANOVA was performed in order to include the precision component. As the measurement was performed in digital PCR, the precision component was higher than the component from the homogeneity study, measured by RT-qPCR.

Table 2: Value assignment for prepared control materials

Control material	Assigned value	rel u_ Characterization (%)	rel u_hom (%)	rel u_stab (%)	Relative standard uncertainty (%)	standard uncertainty
JIPC-1	45344	19.6	2.0	1.8	19.8	8958
JIPC-3	99	13.3	1.4	1.4	13.5	13
NIPC-1	169019	4.5	0.7	1.9	5.0	7704
NIPC-3	33	18.2	1.9	1.32	18.4	6

The precision value obtained for JIPC-1 was higher than expected, a great variability was observed, this is consistent with the high value for the homogeneity study for this material, indicating that homogenization of the material was not sufficient, which significantly influenced a variation in the concentration of the vials. value assignment for JIPC-1 and JIPC-3 was (45344±8958) copies/µL and (99±13) copies/µL respectively, while for NIPC-1 and NIPC-3, it was (169019 ±7704) and (33±6) copies/µL respectively.

Table 3: Informative Ct values for MR SARS CoV-2

Property	E Gene	S Gene	RdRp Gene
Ct Value RT-PCR JIPC 1	21.04 ± 0.07	20.66 ± 0.09	-
Ct Value RT-PCR JIPC 3	29.76 ± 0.04	29.45 ± 0.19	-
Ct Value RT-PCR NIPC 1	19.55 ± 0.18	-	20.36 ± 0.06
Ct Value RT-PCR NIPC 3	32.06 ± 0.34	-	32.47 ± 0.24

On the other hand, as laboratories use more than one detection target in their tests, the material was measured with the E Charité, Spike and RdRp targets in RT-qPCR, to obtain information on the behavior of the materials produced with these detection targets. Table 3 shows the results obtained, the Ct values are consistent between them and there is no variation greater than one Ct unit, which indicates very close concentration values.

Technical transfer workshop for SARS-CoV-2 measurements by RT-qPCR

Based on the results reported by laboratories, 68% of the laboratories measured the NIPC material, with the targets E, N, N2, RdRp and Orf1ab; 26% used JIPC with targets E and N; and 5% reported

data for both materials. The most reported gene was E, followed by RdRp and N (Table 4).

Table 4: Assays used by laboratories in Technical transfer workshop

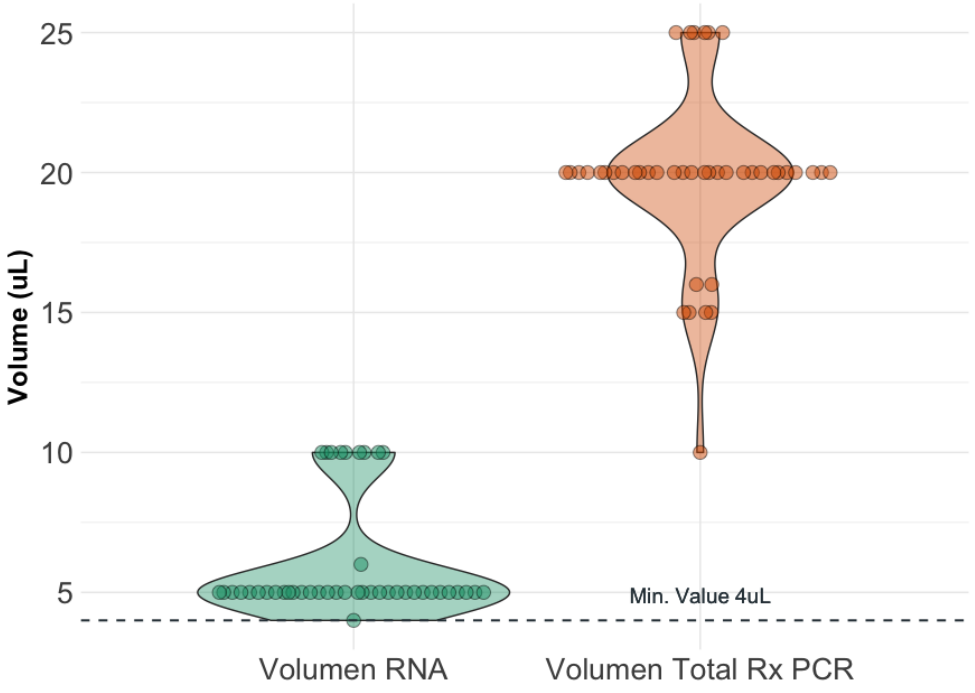
Material	Laboratories	E	N1-china	N2-CDC	RdRp	OR1ab
NIPC	26	17	12	2	14	3
JIPC	10	11	-	-	3	-
NIPC/JIPC	2	-	-	-	-	-

Based on the results reported by the laboratories, the following characteristics related to the implementation of the different protocols for the detection of the SARS-CoV-2 virus by RT-PCR as the reference method were determined:

Total reaction volume of RNA used in the PCR

Figure 3 presents the distribution of the RNA volume, as well as the reaction mix reported by the laboratories, the first ranging from 4 µL to 10 µL, with 5 µL being the most used (30 laboratories). In relation to total or master mix volume, the range is between 10 µL and 25 µL, being 20 µL the most common, followed by 15 µL.

Figure 3: RNA volume and total volume of PCR reaction used.



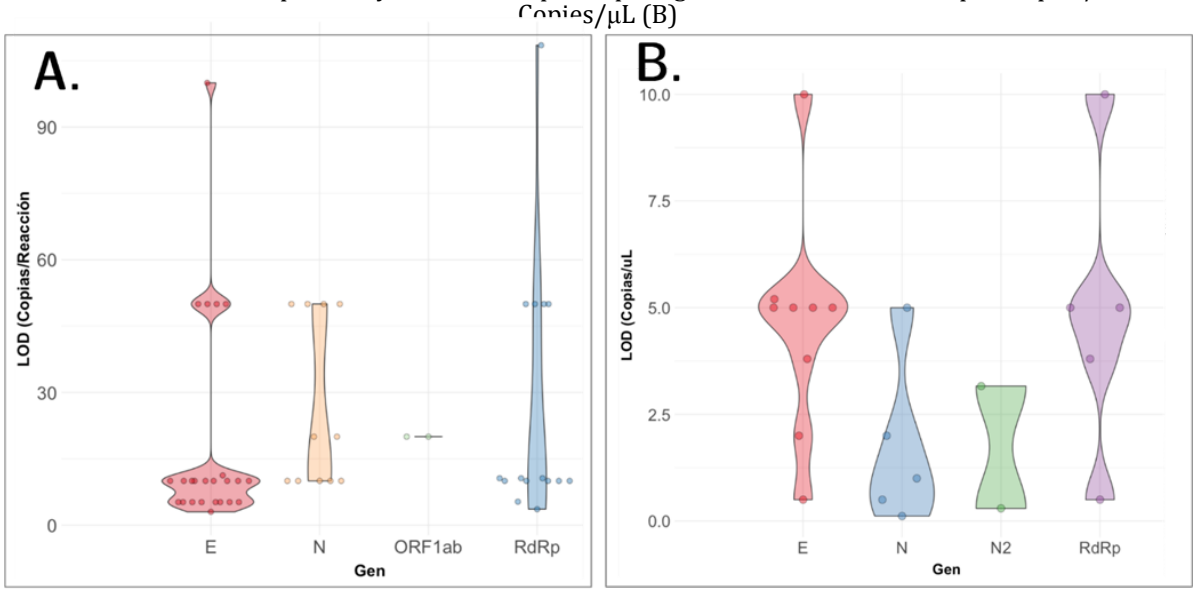
Detection limits associated with measurement methods

The detection limits reported by the laboratories, and which correspond to those listed in the protocols for the use of commercial assays or to the information reported for in-house methods, are found in two types of units: copies/µL and copies/reaction. Figure 4 presents its distribution through violin-type graphs, according to the detection target. For the assays reported in copies/µL, the range of values is between 0.26-10 copies/µL, with 5 copies/µL being the value with the highest incidence

or mode (10 laboratories), only target N presenting lower LOD's with values less than 2.5 copies/µL.

In the case of the detection limit in copies/reaction (Figure 4-B), there is a higher density in discrete values, mainly 5, 10 and 50 copies/reaction, due to the values reported by commercial kits, which normally report the same value for all targets in the multiplex assay.

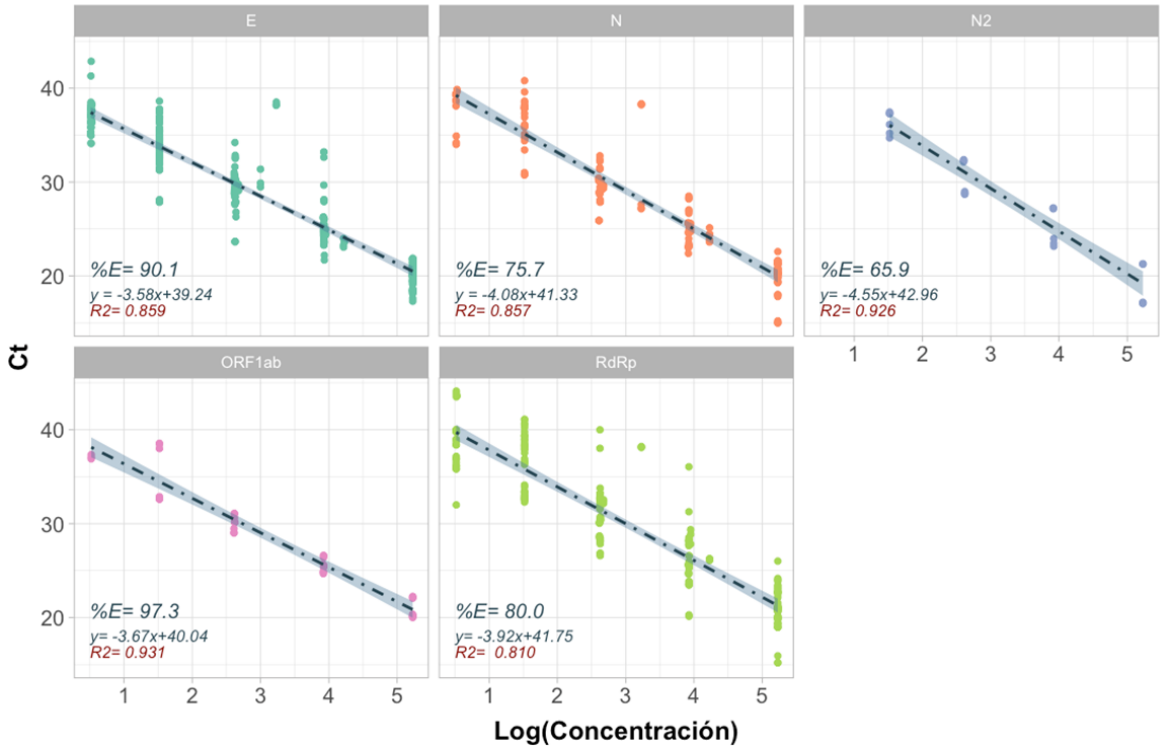
Figure 4: Detection limits reported by laboratories participating in the transfer workshop in Copies/reaction (A), Copies/µL (B)



Descriptive data analysis

Figures 5 and 6 present the consolidated experimental results (in Ct) for each of the NIPC and JIPC materials measured by the laboratories depending on the detection target. The value in the box of each graph corresponds to the efficiency of the reaction calculated from the global slope of the data.

Figure 5: Instrumental response Ct vs Log concentration for each of the targets evaluated by the laboratories for the NIPC material.(B)



It is observed that for the NIPC material (Figure 5), the greatest dispersion of the data is in the E, N and RdRp genes, since they are the ones with the greatest number of data, while those with apparently few data have less dispersion. Similar trends are presented by the results associated with the JIPC material for the E gene (Figure 6), while the dispersion increases for RdRp, which only has 3 data sets.

If 90-110% are taken as confidence limits for the efficiency value, it is the E gene in both materials that presents the best behavior; orf1ab and RdRp, which may be directed to the same region in the genome, present an intermediate value greater than or equal to 80%, while N1-china and N2-CDC, which also detect a similar region, present the lowest efficiency. For the JIPC RdRp gene, the result is the opposite, presenting a higher efficiency, although also outside the range.

Figure 6: Instrumental response Ct vs Log concentration for each of the targets evaluated by the laboratories for the JIPC material.

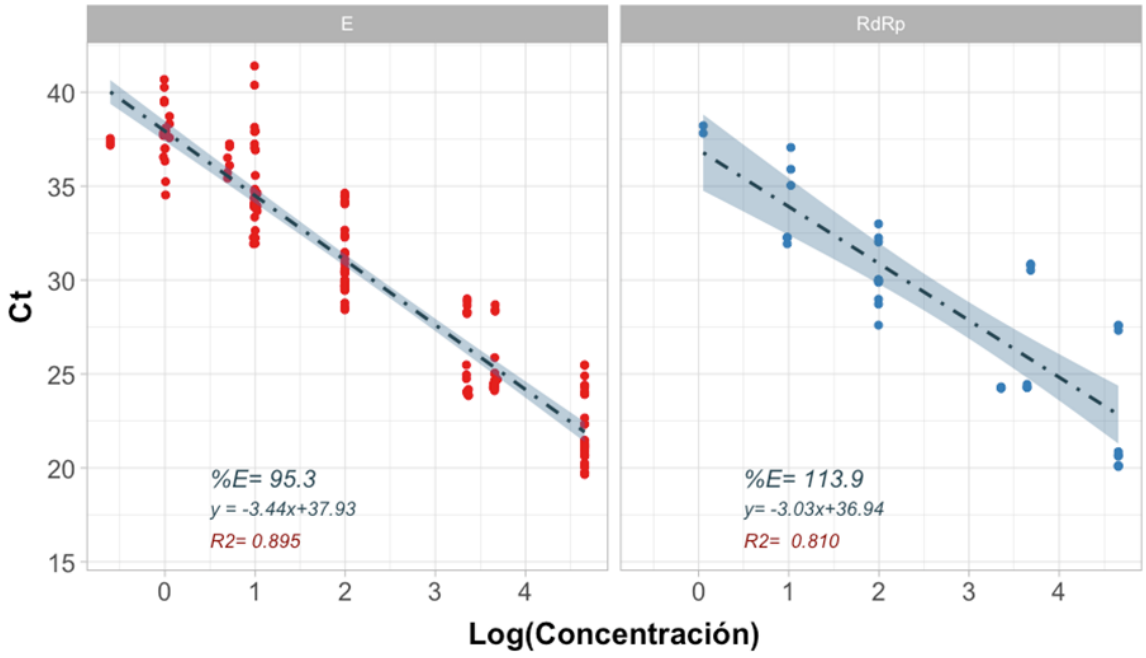
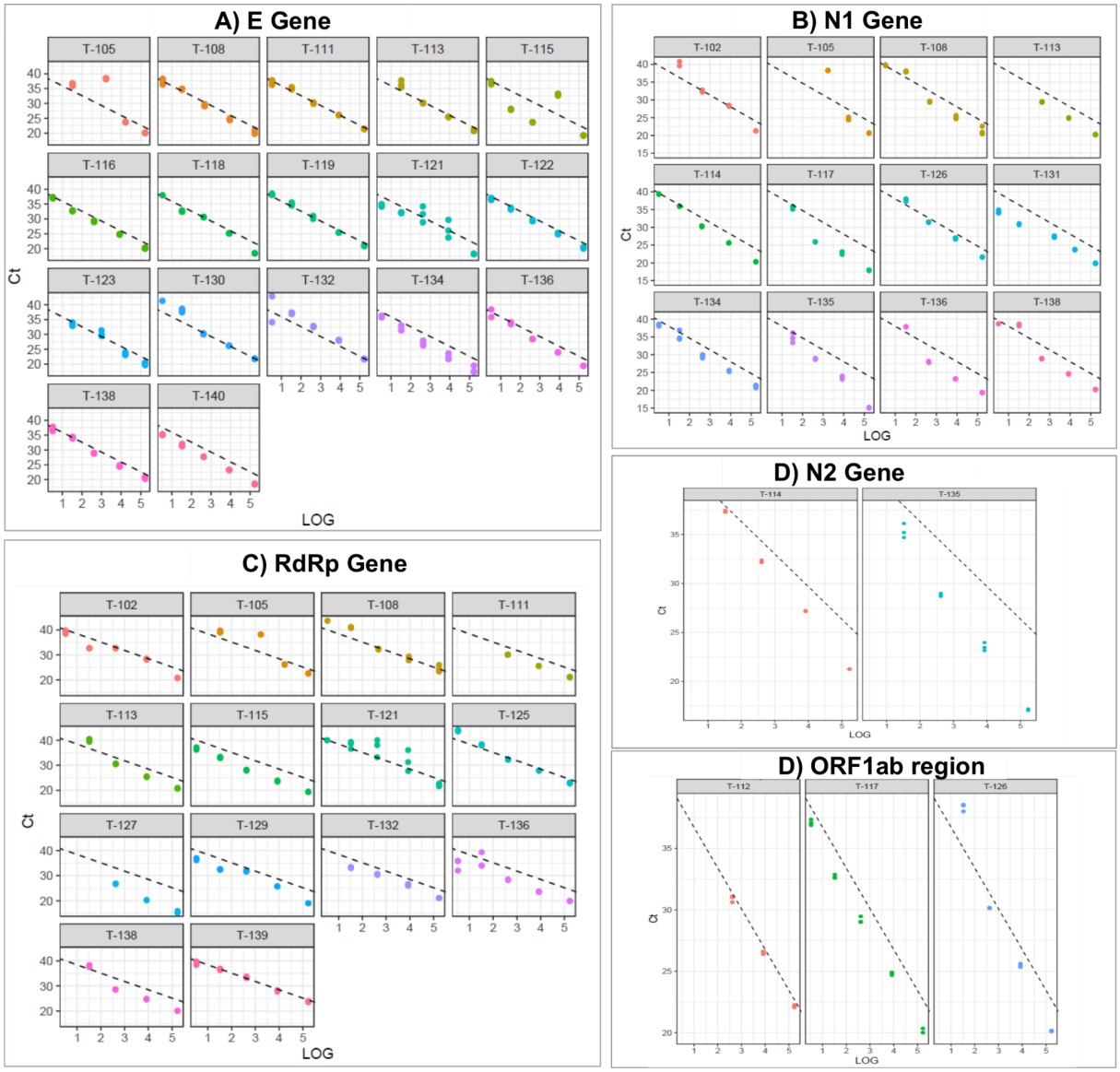


Figure 7 shows the results observed in Figure 6, where it is possible to show in a little more detail the data sets associated with each laboratory with higher and lower trends than expected for 100% efficiency, made more evident for example. in the case of N2, It is also observed that some laboratories present a lower degree of correlation between the value of the concentration logarithm and the instrumental response, for example T-105 and T-115, a fact that could be associated with errors in the preparation of the dilutions or in adding the samples to the reaction mixture. In other cases, it is generally observed how the precision of the results per sample, target and concentration level is similar, with the exception of some points such as T121 in the measurement of NIPC2a and 2b levels, with the E and RdRp genes, where a greater dispersion is evidenced for the replicates evaluated.

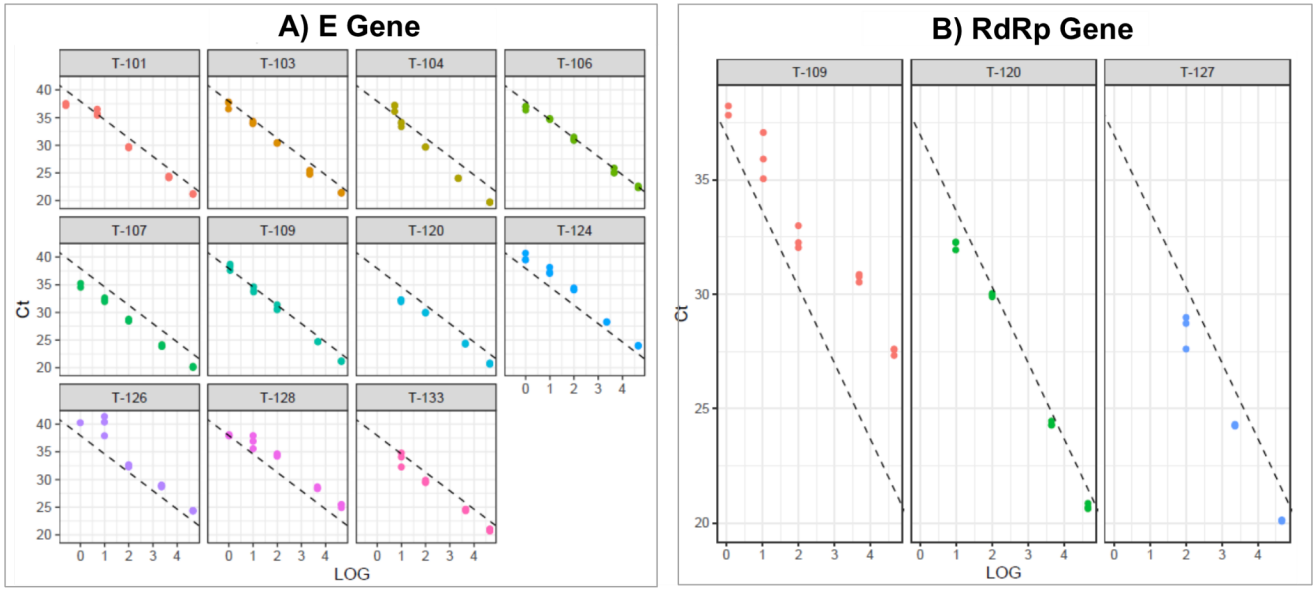
Figure 7: Instrumental response Ct vs Log concentration for each of the targets evaluated by the laboratories for the NIPC material.



Taking into account the scope of the assay under normal conditions of use, whose purpose is to determine the presence/absence of the sequences of interest, the value of the slope and therefore of the efficiency, could become more important or relevant at low concentrations, where it could avoid detecting samples of low concentration, for example, for T114 and T135 in the detection of the N2 gene.

Figure 8 presents the results obtained by gene and laboratory for the JIPC material, compared with a trend of 100%. It is observed how the data obtained by the T-109 laboratory pulls the trend and generates an efficiency of 113% (Figure 10). Data analysis is similar to that developed for the NIPC material.

Figure 8: Instrumental response Ct vs Log concentration for each of the targets evaluated by the laboratories for the JIPC material.



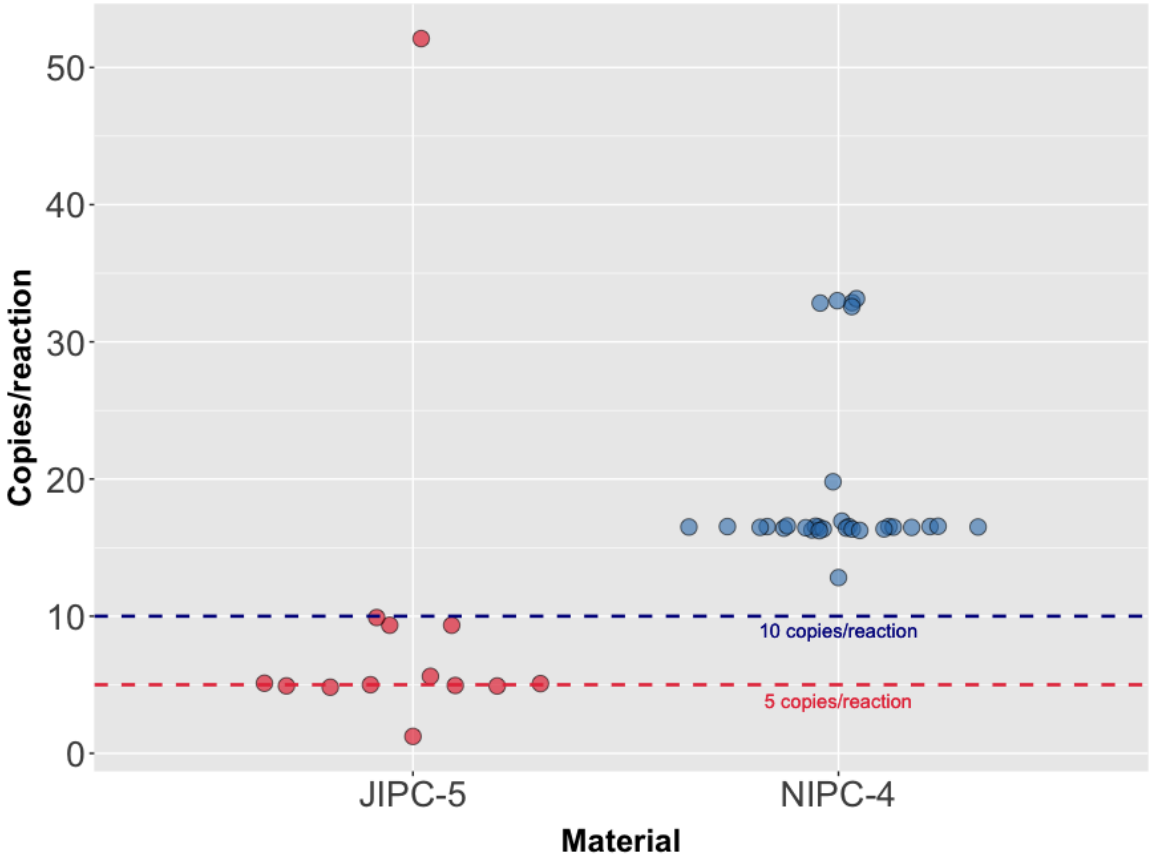
Detection limits vs. lowest prepared concentration value

From Figure 9 it can be established how with respect to the E gene, most laboratories were able to detect the 5 concentration levels, especially the lowest, which, taking into account the average volume of sample used (5 µL) , as well as the concentration of lower level in the calibration curve (3 copies/µL), corresponds to 15 copies/reaction, a value that agrees with that reported by most laboratories in terms of LoD, with values predominantly at 5 and 10 copies /reaction.

In this sense, Figure 9 presents the quantity in copies / reaction used by the laboratories in the measurement of the materials with the lowest concentration JIPC-5 (in red) and NIPC-4 (in yellow) in relation to the detection limits (majority) reported by the laboratories. In general, it could be stated that during the development of the workshop, the laboratories measured samples at concentration levels very close to the detection limit of the implemented methodologies, obtaining positive results or detection, although it is noteworthy that at these concentration levels they did not always amplify the three replicates evaluated.

Finally, from this activity it is determined that the behavior with the different genes is not systematic, it varies with the material, the dilution, the type of gene and that in general it could be affirmed that the protocols, whether commercial or in-house implemented by the laboratories are capable of detecting samples close to the detection limit.

Figure 13: Limit of detection reported by laboratories (Copies/Reaction)



5. Conclusions and future work

Based on the previously optimized and validated methodologies, and thanks to international cooperation, who supplied the starting materials, two Reference Materials were characterized at the SARS CoV-2 genomic RNA level: consisting of the materials with the JIPC and NIPC code , each starting from a different MR with the aim of covering the entire virus genome, prepared at two concentration levels: 169019 ± 7704 copies/µL and 33 ± 6 copies/µL for NIPC and 45344 ± 8958 copies/µL and 99 ± 13 copies/µL for JIPC. From the results obtained in the homogeneity, stability and characterization studies, it was determined that these were adequate for the purpose. Additionally, based on the stability study, these proved to be valid for 6 months at 4ºC, which facilitates the transport and storage processes.

Regarding the technical workshop, 38 participating laboratories prepared calibration curves from the supplied control materials and guide forms for the evaluation of the efficiency and precision parameters of their methods. The results of this activity allowed us to determine that the behavior of the assays is not systematic, and the parameters of each one are linked to the kit used, the detection target used, the detection limits of the method and even the PCR system. However, the data is not sufficient to establish statements about the causality or relationship between non-detection and the type of gene.

This exercise has allowed us to demonstrate the importance of teamwork, and the form in which in an event such as the COVID-19 pandemic, organization, cooperation, the union of the technical capacities of different institutions (National Institute of Health, National University of Colombia, National Institute of Metrology), as well as international cooperation (NIST, EU - JRC, UNIDO) have

allowed the development of technical tools that contribute to strengthening strategies and activities aimed at controlling and mitigating the pandemic. The activities carried out and the experience acquired can serve as a model or basis for the development of similar strategies in the face of the next pandemic.

6. Acknowledgments

The development of this work would not have been possible without the cooperation and joint work of various entities and programs. Initially, we would like to greatly thank the National Institute of Health for supporting the entire project, to the National Institute of Standards and Technology (NIST) and the Joint Research Center (JRC) for providing the Reference Materials used in the development of the activities of this project, and to the laboratories that participated in this exercise.

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