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"Sample pooling of RNA extracts to speed up SARS-CoV-2 diagnosis using CDC FDA EUA RT-qPCR kit"

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ABSTRACT

Background: The CDC protocol for SARS-CoV2 RT-PCR diagnosis (2019-nCoV CDC kit) is considered a gold standard worldwide; based on three different FAM probes (N1 and N2 for viral detection; RP for RNA extraction quality control), three reactions per sample are needed for SARS-CoV-2 diagnosis.

Results: We herein describe a sample pooling protocol: pooling 3 RNA extractions into a single PCR reaction; we tested this protocol with 114 specimens grouped in 38 pools and found no significant differences for N1 and N2 Ct values between pool and single sample PCR reaction.

Conclusion: This pool of three protocol has a sensitivity of 100 % compared to the standard single sample protocol. For a typical 96-well plate, this pool assay allows 96 samples processing, speeding up diagnosis and reducing cost while maintaining clinical performance, particularly useful for SARS-CoV-2 diagnosis at developing countries.

1. Introduction

The COVID19 pandemia has challenged public health systems worldwide, not only for patient care but also for rapid enough surveillance and control programs. Although new technologies are becoming available like Loop-Mediated Isothermal Amplification (LAMP) Assays in point of care devices to speed up SARS-CoV-2 diagnosis (Nguyen et al., 2019), the gold standard for CDC or WHO is still RT-qPCR (Interim Guidelines for Collecting, Handling and Testing Clinical Specimens from Persons for Coronavirus Disease, 2019). Several in vitro diagnosis RT-qPCR kits are available on the market for the detection of SARS-CoV-2. Some of them have received emergency use authorization (EUA) from the U.S. Food & Drug Administration (FDA), like 2019-nCoV CDC EUA from the USA Center for Diseases Control and Prevention (CDC). The CDC assay is based on N1 and N2 probes to detect SARS-CoV-2 and RNaseP (RP) as an RNA extraction quality control (Interim Guidelines for Collecting, Handling and Testing Clinical Specimens from Persons for Coronavirus Disease, 2019). According to CDC protocol for 2019-nCoV CDC EUA, the 3 probes are FAM labelled so 3 PCR reactions are needed for each specimen diagnosis. With no triple PCR protocol validated for N1, N2 and RNaseP, the current CDC protocol reduces daily sampling processing capacity for a typical 96 well plate PCR device. On developing countries like Ecuador, most of clinical microbiology laboratories running SARS-CoV-2 diagnosis operates with a single Real Time PCR device. Under this scenario, pooling samples is a powerful tool to increase SARS-CoV-2 testing capacity (Eliseo and Navarro, 2020; Hogan et al., 2020; Yelin et al., 2020; Shental et al., 2020). Also, testing costs are reduced and supply shortage may be mitigated by using a pooling sample protocol, crucial to support surveillance at developing countries.

This study evaluates the performance of a sample pooling RT-qPCR protocol where samples are pooled after RNA extraction and loaded into the same RT-qPCR reaction for SARS-CoV-2 diagnosis, using 2019-nCoV CDC EUA kit (IDT, USA).

2. Methods

2.1. Study setting

114 clinical specimens (nasopharyngeal swabs collected on 0.5 mL TE pH 8 buffer) from individuals selected during SARS-CoV-2 surveillance in Galapagos Islands started on April 8th 2020, were included on
the evaluation study. Also, a negative control (TE pH 8 buffer) was included as control for carryover contamination for each set of 23 RNA extractions. 'LabGal' at 'Agencia de Regulacion y Control de la Bioseguir y Cuarentena para Galapagos' at Puerto Ayora in Galapagos Islands (Ecuador) is the only available SARS-CoV-2 diagnosis laboratory on site, operating with a single 96 well plate PCR device (CFX96 from BioRad) to cover a population above 20,000 people.

2.2. RNA extraction and RT-qPCR for SARS-CoV-2 diagnosis

Samples were tested following an adapted version of the CDC protocol: using PureLink Viral RNA/DNA Mini Kit (Invitrogen, USA) as an alternate RNA extraction method; using CFX96 BioRad instrument (Freire-Paspuel et al., 2020a, b). We performed this protocol for 38 SARS-CoV-2 positive and 76 negative samples individually, but also pooling one positive sample with two negative samples at the RT-PCR reaction mix. While 4 μL of a single RNA was added to a single reaction (in both cases, final volume of reaction was 15 μL). The reaction mix was run periodically RNA extraction quality control tests. This protocol is an easy way to speed up SARS-CoV-2 diagnosis when the sample reaction and the 3 sample pool RT-qPCR reaction: 31.30 ± 3.69 vs 31.16 ± 4.04 for N1 (p = 0.72); 34.09 ± 3.83 vs 33.25 ± 3.96 for N2 (p = 0.14). Results are detailed on Table 2. Moreover, Altman Bland analysis confirmed that all Ct values for 3 samples pools reactions were included within the 95 % CI, with the only exceptions of samples 11 and 12. These samples were pooled with sample 10 (all 3 samples were SARS-CoV2 positive) and so the Ct values for N1 and N2 for the pool reaction is the same for all these samples (See Table 1) and higher for samples 11 and 12 than single sample Ct values.

2.3. Statistics

For statistical analysis of Ct values, t-student and Altman-Bland analysis were performed.

2.4. Ethics statement

All samples have been submitted for routine patient care and diagnostics. Ethical approval for this study was not required since all activities are according to legal provisions defined by the "Comité de Operaciones Especiales Regional de Galápagos" that is leading the Covid19 surveillance in Galapagos Islands. No extra specimens were specifically collected for this validation study. All data used in the current study was anonymized prior to being obtained by the authors.

3. Results

The 114 samples included on this study were tested for SARS-CoV2 following the standard protocol described on the methods. 38 of this samples tested positive for N1 and N2 viral probes, and Ct values are detailed on Table 1. These 114 samples were also pool on group of 3 samples after RNA extraction and prior to RT-PCR following the pool protocol detailed on the methods. The Ct values for N1 and N2 for the 38 positives samples on the RT-PCR pool reaction are detailed on Table 1. All positive pools included only a positive sample and two negative samples, with exception of a pool that included 3 positives samples (samples 10, 11 and 12 on Table 1). All the 36 pools including at least a positive sample tested positive on the pool RT-PCR reaction, so the sensitivity obtained for our 3 sample pooling protocol was 100 %.

We found no significant differences for Ct values between the single sample reaction and the 3 sample pool RT-qPCR reaction: 31.30 ± 3.69 vs 31.16 ± 4.04 for N1 (p = 0.72); 34.09 ± 3.83 vs 33.25 ± 3.96 for N2 (p = 0.14). Results are detailed on Table 2. Moreover, Altman Bland analysis confirmed that all Ct values for 3 samples pools reactions were included within the 95 % CI, with the only exceptions of samples 11 and 12. These samples were pooled with sample 10 (all 3 samples were SARS-CoV2 positive) and so the Ct values for N1 and N2 for the pool reaction is the same for all these samples (See Table 1) and higher for samples 11 and 12 than single sample Ct values.

### Table 1

| n   | N1 Pool PCR Ct value | N1 Single PCR Ct value | N2 Pool PCR Ct value | N2 Single PCR Ct value |
|-----|----------------------|------------------------|----------------------|------------------------|
| 1   | 30.09                | 30.60                  | 33.86                | 32.28                  |
| 2   | 32.34                | 31.02                  | 36.47                | 32.46                  |
| 3   | 36.84                | 35.87                  | 40.56                | 38.72                  |
| 4   | 36.13                | 34.43                  | 38.64                | 35.84                  |
| 5   | 33.19                | 32.42                  | 36.50                | 35.08                  |
| 6   | 34.74                | 33.57                  | 38.37                | 36.03                  |
| 7   | 34.74                | 35.91                  | 38.37                | 41.72                  |
| 8   | 30.27                | 29.71                  | 33.30                | 31.39                  |
| 9   | 28.26                | 27.15                  | 32.21                | 29.33                  |
| 10  | 27.09                | 25.15                  | 30.37                | 26.59                  |
| 11  | 27.09                | 36.26                  | 30.37                | 39.87                  |
| 12  | 27.09                | 35.45                  | 30.37                | 38.08                  |
| 13  | 34.61                | 34.04                  | 37.12                | 35.83                  |
| 14  | 35.32                | 34.05                  | 35.45                | 34.43                  |
| 15  | 36.69                | 35.49                  | 36.29                | 36.24                  |
| 16  | 37.21                | 36.35                  | 36.43                | 36.45                  |
| 17  | 28.01                | 27.01                  | 30.90                | 28.19                  |
| 18  | 23.84                | 22.61                  | 25.50                | 24.02                  |
| 19  | 34.65                | 33.90                  | 36.85                | 37.34                  |
| 20  | 35.73                | 34.76                  | 36.18                | 36.80                  |
| 21  | 36.52                | 35.81                  | 37.07                | 37.05                  |
| 22  | 30.10                | 28.80                  | 30.73                | 29.98                  |
| 23  | 29.76                | 28.27                  | 31.19                | 29.82                  |
| 24  | 29.48                | 28.11                  | 31.68                | 29.54                  |
| 25  | 31.86                | 30.64                  | 33.48                | 32.08                  |
| 26  | 30.66                | 32.41                  | 32.89                | 36.46                  |
| 27  | 27.77                | 27.45                  | 28.61                | 33.80                  |
| 28  | 24.18                | 26.70                  | 25.67                | 32.92                  |
| 29  | 33.36                | 32.76                  | 35.22                | 37.49                  |
| 30  | 29.89                | 31.34                  | 38.17                | 36.15                  |
| 31  | 26.63                | 27.30                  | 29.07                | 31.96                  |
| 32  | 31.38                | 30.40                  | 34.03                | 35.31                  |
| 33  | 25.57                | 26.06                  | 27.12                | 30.08                  |
| 34  | 31.60                | 33.11                  | 33.88                | 38.40                  |
| 35  | 27.97                | 29.86                  | 29.39                | 34.04                  |
| 36  | 24.33                | 26.86                  | 25.67                | 32.46                  |
| 37  | 30.70                | 31.90                  | 33.00                | 35.00                  |
| 38  | 28.30                | 35.81                  | 38.72                | 37.05                  |

### Table 2

|    | N1 Pool PCR Ct value | N1 Single PCR Ct value | N2 Pool PCR Ct value | N2 Single PCR Ct value |
|----|----------------------|------------------------|----------------------|------------------------|
| Mean ± SD | 31.16 ± 4.04 | 33.25 ± 3.96 | 34.09 ± 3.83 |

4. Discussion

Our results support the use of a 3 samples pool RT-qPCR protocol for SARS-CoV-2 diagnosis without reducing sensitivity compared to the standard single sample RT-qPCR protocol (Interim Guidelines for Collecting, Handling and Testing Clinical Specimens from Persons for Coronavirus Disease, 2019). As no loss of sensitivity was found associated to the RT-PCR reaction (no shift on Ct values for N1 and N2), the only potential source for false negative results would be low quality of RNA extraction of sample included on a pool, where the other samples would mask the RNaseP amplification on the RT-PCR. However, on our experience, less than 0.5 % of samples does not yield a positive amplification for RNaseP. So, we recommend to improve this 3 samples pool protocol only for laboratories with high performance on RNA extraction and also run periodically RNA extraction quality control tests.

This protocol is an easy way to speed up SARS-CoV-2 diagnosis when using the CDC RT-qPCR protocol: the need of three PCR reactions per
we never found a positive pool that did not yield at least a positive single sample. Also endorses a 100% specificity for the 3 sample pooling protocol, as PCR device within a month period. Our data with over 600 pools tested showing Ct shifts and reduced sensitivity for sample pooling.

We have been successfully using this protocol during COVID19 surveillance at Galapagos Islands where more than 5% (over 2000 subjects) of the population has been tested on a single lab with only a Real Time PCR device within a month period. Our data with over 600 pools tested also endorses a 100% specificity for the 3 sample pooling protocol, as we never found a positive pool that did not yield at least a positive single sample.

The main limitation of our 3 sample pool protocol is the need for running an extra RT-PCR reaction for positives pools on a single sample mode that delays diagnosis a few hours; although overall laboratory diagnosis is clearly speed up on our experience. On one hand, this protocol would not be useful when high prevalence of SARS-CoV-2 is expected and diagnosis is expedited as for hospitalized individuals. On the other, when a low prevalence below 5% is expected and a wide screening is the goal, the 3 samples pool protocol would be of great help as less than 15% of pools would test positive.

Declaration of Competing Interest

The authors report no declarations of interest.

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