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Limit of Detection (LOD) of in-house N1N2 CDC real-time RT-PCR assay and commercial kits to detect SARS-CoV-2-

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ABSTRACT

Introduction: There are two types of SARS-CoV-2 real-time RT-PCR (rRT-PCR) kits used in the laboratory in Indonesia, in-house and commercial kits. Our laboratory developed an in-house kit based on N1N2 CDC. In this study, we reported the In-house kit's Limit of Detection (LOD) compared with several commercial kits.

Method: This report was an experimental study conducted in Clinical Microbiology Laboratory, Microbiology Department, FMUI in Jakarta. Commercial SARS-CoV-2 RNA (Vircell, Granada, Spain, Lot No. 20MBC137004-R) was used. The LoD was determined using a 2-fold dilution of the RNA in DNase/RNase-free water (Vircell®). The diluted RNA(s) were used as templates for in-house and commercial rRT-PCR kits.

Result: The LOD of in-house rRT-PCR and three commercial kits (BioCoV-19 [Bio Farma], Standard M [SD Biosensor], and Real-Q [BioSewoom]) showed higher sensitivity (3.5 copies/reaction) than Power Chek [Kogenebiotech] (7 copies/reaction).

Conclusion: The LOD of our In-house kit showed high performance in sensitivity and comparable with other commercial kit.

Keywords: Limit of Detection, LOD, SARS-CoV-2, Coronavirus.

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INTRODUCTION

COVID-19 (Coronavirus Disease 2019) is a disease caused by SARS-CoV-2 and was declared a pandemic by the World Health Organization (WHO) on March 12, 2020.[1–3] WHO recommended a real-time reverse transcription polymerase chain reaction (rRT-PCR) to detect SARS-CoV-2. [4] PCR is a method amplifying small samples of DNA, amplified to quantities that are large enough for analysis. In real-time PCR, the newly amplified DNA is tagged with a fluorescent dye, so fluorescence levels can be quantified after every PCR cycle. Reverse-transcription PCR uses reverse transcriptase enzyme to make DNA from the RNA template (or from a cell's mRNA), and the DNA is then amplified.[5]

Targets of the SARS-CoV-2 gene currently used by various countries for detecting this virus include the United States (CDC) on the N1 gene. and N2; China on ORF1ab and N; Germany at RdRp, E and N; and Japan on NIID_2019-nCoV_N.[2,6,7] In this study, the tested rRT-PCR was based on N1N2 CDC.

Numerous SARS-CoV-2 kits and assays are being used for clinical testing across various laboratories worldwide, including Indonesia. The limit of detection (LOD) across the kits and assays has shown variation in many studies.[6,9,10] The LOD is defined as the lowest actual concentration of an analyte that can be consistently detected in at least 95% of specimens tested.[9,11]

There were some studies about LOD of N1N2 CDC. Nalla et al. reported LOD of N1 was 63 copies/reaction (mean Ct 33.7 with 20 positives from 20 duplications) and N2 was 31.5 copies/reaction (mean Ct 35.1 with 20 positives from 20 duplications).[12] In other hand, LOD of N1N2 studied by CDC was 3,16 ($10^{0.5}$) copies/reaction (mean Ct 34.15).[6] Although the LOD of N1N2 CDC has been studied, we could not say that the LOD of our In-house N1N2 kit is the same since the reagent used was different. Because of that, we conduct this study to identify our kit's LOD and assess its usefulness in the clinical laboratory. We also assessed the LOD of several commercial kits used in our laboratory.

METHODS

The study design is laboratory experimental and performed in March 2021. The research was carried out at the Clinical Microbiology Laboratory, Faculty of Medicine, University of Indonesia.

The Limit of Detection (LOD) test was determined by dilution of the standard SARS-CoV-2 genome RNA from Vircell, Granada, Spain (Amplirun SARS-CoV-2 RNA Control) with a known RNA copy number (14000 copies/µL, Lot No. 20MBC137004-R). The standard genome was diluted 10 times and 2 times.

The In-house N1N2 rRT-PCR assay was performed using primers and probes according to CDC guidelines (Table 1).[8] The rRT-PCR enzyme for the In-house N1N2 kit was SensiFAST SYBR No-ROX One-Step Kit (Meridian, Bioscience, London, UK, Lot No. SF619-B091080) and the rRT-PCR composition with 20 µl reaction was 10 µL of 2x SensiFAST Probe No-ROX one-step mix, 0.4 µL of RNase inhibitor, 0.2 µl of reverse transcriptase, 1.5 µL of pre-mixed primer (N1 or N2) and probe (2019-nCoV RUO Kit, IDT Integrated

DNA technologies, Cat. No:10006713), and 7.9 µL of RNA template for N1 or N2. The exception of the composition was 5 µL of RNA template and added 2.9 µL DNase/RNase-free-water for RNase P. The thermal cycling (MA-6000 Real-Time PCR System (Molarray, Suzhou, China) was performed at 50°C for 30 min; 95°C for 2 min; 45 cycles of 95°C for 15 sec and 55°C for 30 sec.

The RT-PCR enzymes and protocols for commercial kits were used according to the kit's instructions. The commercial kits tested were the BioCoV-19 RT-PCR kit (Bio Farma, Indonesia), Standard M nCoV Real-Time Detection kit (SD Biosensor, Korea), and Real-Q 2019-nCoV Detection Kit (BioSewoom, Korea) and Power Chek 2019-nCoV Real-time PCR Kit (Kogenebiotech, Korea).

The rRT-PCR is reported as positive results if Ct ≤ 40 for both N1 and N2. The LOD is the minimal dilution that still gives a positive value in two gene duplications.

The ethical commission approved this research of the Faculty of Medicine, University of Indonesia with the number KET-395UN2.F1/ETIK/PPM.00.02.2020.

Table 1. In-house N1N2 SARS-CoV-2 primer and probe.[8]

N1 gene	Forward primer	GAC CCC AAA ATCAGCGAA AT
	Reverse primer	TCTGGTTACTGCCAGTTGAATCTG
	Probe	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
N2 gene	Forward primer	TTACAA ACATTGGCCGCA AA
	Reverse primer	GCGCGACATTCCGAAGAA
	Probe	FAM-ACA ATTTGCCCCCAGCGTTAG-BHQ1
RNase P gene (RP) [Internal Control]	Forward primer	AGATTTGGACCTGCGAGCG
	Reverse primer	GAGCGGCTGTCTCCACAAGT
	Probe	FAM -TTCTGACCTGAAGGCTCTGCGCG-BHQ-1

Table 2. The rRT-PCR results of the In-house N1N2 CDC kit.

No.	RNA copies/reaction	N1 (Ct Value)		N2 (Ct Value)		Interpretation
1	14	34.14	34.74	33.66	33.66	Positive
2	7	35.76	35.59	35.01	34.51	Positive
3	**3.5**	**35.54**	**35.50**	**35.13**	**34.66**	**Positive**
4	1.75	-	-	36.02	36.16	Inconclusive
5	0.88	-	-	37.98	-	Negative
6	0.44	-	-	-	-	Negative

RESULTS

The LOD of the In-house N1N2 CDC kit was 3.5 copies/reaction (mean of Ct 35.21). The dilution below 3.5 was not detected for the N1 gene. In contrast, the N2 gene was still detected at 1.75 copies/reaction (two duplications) and 0.88 copies/reaction (only one of two duplications). Still, according to the CDC guidelines, it was interpreted as inconclusive and negative (Table 2).

The LODs of several commercial kits used in our laboratory were also studied. The LODs of BioCoV-19 (Bio Farma), Standard M (SD Biosensor), and Real-Q (BioSewoom) were the same, which were 3.5 copies/reaction; while the LOD of Power Chek (Kogenebiotech) was 7 copies/reaction (Table 3).

The dilution below 3.5 was still detected in all four commercial kits but was interpreted as inconclusive and negative according to the CDC guidelines. For BioCoV-19 (Bio Farma), the N2 gene was still detected at 0.88 copies/reaction in two duplications, but the RdRp gene was only detected one of the duplications at 1,75 copies/reaction. For Standard M (SD Biosensor), the ORF-1ab gene was still detected at 1,75 copies/reaction in two duplications, but the E gene was only detected one of the duplications at 1,75 copies/reaction. For Real-Q (BioSewoom), the E gene was still detected at 1,75 copies/reaction in two duplications, but the RdRp gene was only detected one of the duplications at 1,75 copies/reaction. For Power Chek (Kogenebiotech), the E gene was still detected at 0,88 copies/reaction in two duplications, but the RdRp gene was only detected one of the duplications at 1,75 copies/reaction.

DISCUSSION

The LOD is commonly defined as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time.[11] The LOD can also be explained as "the lowest amount of an analyte in a sample which can be detected but not necessarily quantified as an exact value".[11] According to EURACHEM/CITAC Guide[13], the most important use of the LOD is "to show where method performance becomes insufficient for acceptable quantitation, so that improvements can be made".

The LOD in this study was 3.5 copies/reaction (mean Ct value 35.21). When analyzed per gene target, the lowest dilution detected for the N1 gene was 3.5 copies/reaction (mean Ct value 35.52); for the N2 gene, it was 1.75 copies/reaction (mean Ct value 36.09). This LOD value was lower than that of Nalla et al.[12], i.e. N1 was 63 copies/reaction (mean Ct 33.7 with 20 positives from 20 duplications) and N2 was 31.5 copies/reaction (mean Ct 35.1 with 20 positives from 20 duplications). The lower LOD from our study could be due to the difference in the PCR master mix kit and the difference in the number of duplications performed. Our study performed only two duplications for each gene, whereas in Nalla et al. were 20 duplications. Nalla et al. reported on 6.3 copies/reaction dilution of N1 showed 65% positive (13/20) with a mean Ct of 36.2, and N2 showed 90% positive (18/20) with a mean Ct of 36.8. However, since these two positive results were below the 95% threshold, the value was not considered as LOD in Nalla et al. According to this result, the LOD of the kit in our study is lower than Nalla et al. Our In-house kit showed similarity to the LOD of N1N2 studied by CDC[6], which was 3,16 ($10^{0.5}$) copies/reaction (mean Ct 34.15) using QIAGEN EZ1 DSP. Same as Nalla et al, CDC also performed twenty duplications for each gene and used the threshold of

Table 3. The rRT-PCR results of commercial kits (Ct value of each gene).

No.	RNA copies/ reaction	BioCoV-19 (Bio Farma)		Standar M (SD Biosensor)			Real-Q (BioSewoom)			Power Chek (Kogenebiotech)		
		N2	RdRp	ORF-1ab	E		RdRp	E		RdRp	E	
1	14	33.60	33.62	32.77	32.81	30.97	30.98	31.31	31.55	32.70	32.65	32.71	32.91	37.74	38.37	35.91	36.02
2	7	33.96	34.06	33.30	33.34	31.22	31.41	31.95	31.95	33.50	33.57	33.17	33.33	**38.20**	**38.55**	**36.97**	**37.01**
3	**3.5**	**35.62**	**35.66**	**34.75**	**34.85**	**32.53**	**33.05**	**33.14**	**33.23**	**33.91**	**33.97**	**33.91**	**33.97**	-	40.00	37.48	37.10
4	1.75	36.22	36.92	-	36.05	34.43	35.72	-	33.99	-	35.91	34.49	34.54	-	-	37.97	38.12
5	0.88	37.97	38.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	0.44	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

95% (19/20) as the LOD. The theoretical limit of real-time PCR detection according to Wittwer and Kusukawa, was an average of three copies to reach a 95% positive rate.[14] Our study is slightly higher than this theoretical limit, therefore is theoretically correct.

Compared to commercial kits, the LOD of our In-house N1N2 kit was the same as three (BioCoV-19, Standard M, and Real-Q) of four kits evaluated and better than one kit (Power Chek). This result demonstrated that our kit was not inferior to commercial kits, and thus can be used in a clinical laboratory. A further study is needed to evaluate our In-house kit to determine the diagnostic role in clinical specimens.

CONCLUSION

The LOD of our In-house kit was similar to that of the CDC, was not inferior to commercial kits, and was theoretically correct, therefore the In-house kit can be used in the clinical laboratory. However, it is needed to perform a further study to evaluate the kit to find out the diagnostic role in clinical specimens.

DISCLOSURES

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Conflict of Interest

There is no conflict of interest in this study.

Ethic Approval

The ethical commission approved this research of the Faculty of Medicine, University of Indonesia with the number KET-395UN2.F1/ETIK/PPM.00.02.2020.

Author Contribution

FI and AY designed the methodology and edited the manuscript. SYL searched the literature and prepared the manuscript. AY and SYL acquired and analyzed the data. All authors had reviewed the final version of the manuscript.

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