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Original Research Article

Design multiplex PCR primer and probe gen ORF1ab and gen N on COVID-19

Hasna Mirda Amazan[1], Andani Eka Putra[2]*, Rauza Sukma Rita[3]

[1]Master Program in Biomedical Sciences, Faculty of Medicine, Andalas University, Padang, Indonesia
[2]Department of Microbiology, Faculty of Medicine, Andalas University, Padang, Indonesia
[3]Department of Biochemistry, Faculty of Medicine, Andalas University, Padang, Indonesia

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***Correspondence:**
Dr. Andani Eka Putra,
E-mail: Andani1508@med.unand.ac.id

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ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the SARS-CoV-2 virus which is a new strain of the Coronavirus, the primary and probe designs were carried out to find candidate primers and probes to be used as the detection of COVID-19. The purpose of this study was to design multiplex PCR on primers and ORF1ab and N gene probes in COVID-19 examination using the multiplex PCR method and it is expected to have good validity in the examination.
Methods: The research design is in the form of explorative descriptive with a cross sectional approach. Starting from April to December 2021. The samples used in this study were 221 COVID-19 gene sequences downloaded from the NCBI and GISAID gene databases.
Results: The design results of the ORF1ab gene primer and probe with forward primer: 5'-CGCAATTTACAACACAGAC -3' reverse primer: 5'- GTTCTTTATGCTAGCCACT -3' amplicon length 183 bp, and probe sequence: FAM 5'-AAACACACAACAGCATCGTCA-3' BHQ-1, on the N gene with forward primer: 5'-AATTCAACTCCAGGCAG -3' and reverse primer: 5'- CTCTCTCAAGCTGGTTCAATC -3' amplicon length 111 bp, and probe sequence: HEX 5'-CAGCAAAGCAAGAGCAGCA-3' BHQ-1 primary pair sequence and ORF1ab gene probe and N gene conjecture qualified for q-PCR.
Conclusions: The obtained primary and probe pair sequences can be used as COVID-19 detection using the multiplex PCR method.

Keywords: COVID-19, Primary and Probe, ORF1ab gene, N Gene

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by the SARS virus CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) which is a new strain of Coronavirus.[1] On July 11, 2021, the Government of the Republic of Indonesia reported as many as 2,527,203 positive cases with deaths reaching 66,464 people, COVID-19 cases continue to increase and are difficult to control. COVID-19 spread to different countries of the world and became public healththreatts globally in a relatively short period.[2]

Over time, the coronavirus will undergo genetic mutations. Gene mutation is a spontaneous genetic change from a parent virus particle to a derivative viral particle. The coronavirus gene is composed of a series of ribonucleic acid (RNA), the genetic sequence in the coronavirus is the genome of the coronavirus.[3] The genome in SARS-CoV-2 consists of a single strand of RNA with a size of 29,903 base pairs, two-thirds of the viral genome sequence is a non-structural protein encoder in the ORF1ab gene that plays a role in RNA synthesis by amplifying the target gene in SARS-CoV-2.[4] The virulence mechanism of the coronavirus is related to

structural proteins and non-structural proteins. Coronavirus provides messenger RNA (mRNA) that can help the translation process of the replication/ transcription process. The genes that play a role in this replication and transcription process include two-thirds of the 5'-end RNA sequence and two overlapping Open Reading Frames (ORFs), namely ORF1a and ORF1b.[5] There are 16 non-structural proteins encoded by ORF. The other 1/3 part of the viral RNA circuit, which does not play a role in the replication/transcription process, plays a role in encoding 4 structural proteins, namely the S protein (spike), protein E (envelope), protein M (membrane), and protein N (nucleocapsid).[6]

The N gene is a structural protein that binds to the virus's genetic material, encodes the formation of nucleocapsid protein, and is a conserved region, so there is very little chance of it mutating.[7] The N gene is one of the targets that have a very high analytical sensitivity, making the N gene the most sensitive polymerase chain reaction (PCR) diagnostic test target recommended by the CDC, United States.[8] Along with the development of molecular biotechnology, bioinformatics which is a computational technique for managing and analyzing biological information has developed rapidly. In this study, by utilizing bioinformatics, the design of primers and probes with the multiplex PCR method on the ORF1ab gene and N genes is expected to be able to become good validity if there are certain mutations or variants in SARS-CoV-2 in the future, that it can be used as a specific primary candidate and probe in the detection of COVID-19.

METHODS

The research design used is descriptive research in the form of cross-sectional studies. The population is a sequence of the ORF1ab gene and the N gene with a total sample of 221 COVID-19 gene sequences downloaded from the NCBI and GISAID gene databases. The sampling technique used is in the form of non-random purposive sampling. The research was conducted at the Bioinformatics Research Laboratory of the Faculty of Medicine, University. Data analysis of the results of the study was carried out descriptively.

RESULTS

Primary and probe design analysis on ORF1ab and N genes. The design of primers and probes was carried out to search for the best primary candidates and probes to be used for COVID-19 detection using the multiplex PCR method. Primers and probes were designed using the CLC Genomic Workbench 21 program, a total of 221 sequences in the ORF1ab gene and N genes collected from several countries in the NCBI and GISAID database downloads can be seen in Table 1. The ORF1ab gene is at 266 to 21,555 nucleotide bases. encoded more than 16 non-structural proteins, primers, and orf1ab gene probes designed to measure 183 bp, on specific forward primers amplifying from sequences 15601 to 15619, reverse primers 15765 to 15783 and probes in sequences 15717 to 15737 as well as specific attachment sites to primary attachment sites and ORF1ab gene probes on SARS-CoV-2 RNA can be seen in Figure 1.

The N gene is at 28,274 to 29,533 nucleotide bases, the N gene primer and probe are designed to measure 111 bp, the forward primer on the specific gene amplifies from the order 28858 to 28875, the reverse primer 28950 to 28968 and the specific probe amplifies from sequence 28921 to 28939. The primary attachment site and N gene probe on SARS-CoV-2 RNA can be seen in Figure 2.

Table 1: COVID-19 sequences from several countries.

No	Sequence	Country	No	Sequence	Country
1	NC_045512.2	Wuhan	41	MW704357.1	Bahrain
2	MT324062.1	South Africa	42	MT876607.1	Bangladesh
3	MT653082.1	Benin	43	MT607248.1	
4	MT653081.1		44	MT581413.1	
5	MT653080.1		45	MT775570.1	
6	MT653079.1		46	MW577029.1	Netherlands
7	MW582699.1	Morocco	47	MT705205.1	
8	MW803167.1		48	MT705206.1	
9	MT513758.1		49	MW674675.1	Belarus
10	MW453084.1		50	MT747438.1	Belgium
11	MW739943.1	Ghana	51	MW368439.1	
12	MW739939.1		52	MW368440.1	
13	MW739937.1		53	MT844027.1	Belize
14	MW739942.1		54	MT844026.1	
15	MW739934.1		55	MT844024.1	
16	MT576584.1	Nigeria	56	MT844023.1	
17	MT994707.1		57	MT126808.1	Brazil
18	MT630432.1	United Arab Emirates	58	MT807936.1	
19	MT630431.1		59	MT738173.1	
20	MT630429.1		60	MT350282.1	

Continued.

No	Sequence	Country	No	Sequence	Country
21	MT820489.1		61	MW642249.1	
22	MW633906.1	Argentina	62	MW593153.1	
23	MW633909.1		63	MW642248.1	
24	MW633908.1		64	MT371570.1	
25	MT745749.1		65	MT371572.1	Czech Republic
26	MT745748.1		66	MT517434.1	
27	MT745746.1	Australia	67	MT517433.1	
28	MT007544.1		68	MW064264.1	Chile
29	MT906650.1		69	MT670023.1	
30	MT882022.1		70	MW672157.1	
31	MW321434.1		71	MT919536.1	Denmark
32	MW672391.1		72	MT919534.1	
33	MW672390.1		73	MW342708.1	Ecuador
34	MW672389.1	Austria	74	MW342706.1	
35	MW672376.1		75	MW703383.1	Ethiopia
36	MW672372.1		76	MW703382.1	
37	MW672361.1		77	MW735440.1	Philippine
38	MW672353.1		78	MW735424.1	
39	MW704388.1	Bahrain	79	MW735442.1	
40	MW704385.1		80	MT844049.1	Guatemala
81	MT434757.2		127	MT428554.1	Kazakhstan
82	MT434760.1	India	129	MT428551.1	
83	MT416725.2		130	MW332241.1	Russia
84	MT396243.2		131	MW332232.1	
85	MT811569.1	Jordan	132	MW332237.1	
86	MT811566.1		133	MW494127.1	Demonican Republic
87	MT811552.1		134	MW556288.1	
88	MT907520.2	Venezuela	135	MW556269.1	Portugal
89	MT907516.1		136	MT775833.1	
90	MT907518.1		122	MT775829.1	
91	MW853569.1	Uzbekistan	123	MW273892.1	
92	MW853562.1		124	MT263074.1	
93	MW298643.1	Uruguay	125	MW185825.1	Peru
94	MT466071.1		126	MW030268.1	
95	MW306668.1	Turkey	127	MW938104.1	
96	MW340911.1		128	MT594402.1	France
97	MW320722.1		129	MT320538.2	
98	MT499220.1	Tunisia	130	MW322968.1	
99	MT499216.1		131	MW447645.1	Pakistan
100	MW452539.1		132	MT730114.1	
101	MT093571.1	Sweden	133	MT470219.1	Colombia
102	MW769775.1		134	MT256924.2	
102	MW769742.1	Spain	135	MT072688.1	
104	MW769741.1		136	MT810119.1	
105	MW769736.1		122	MT730002.1	Korea
106	MW769717.1		123	MW466796.1	
107	MW012279.1	Serbia	124	MT020782.1	
108	MT883500.1		125	MT134009.1	
109	MT706050.1	New Zealand	126	MT873897.1	Cuba
110	MW898809.1	Iran	127	MT873896.1	
111	MT646036.1		128	MW720771.1	Lebanon
112	MW674874.1	Israel	129	MT801003.1	
113	MW674840.1		130	MT801000.1	
114	MW672647.1		131	MW494315.1	USA
115	MT077125.1	Italy	132	MW494308.1	
116	MT066156.1		127	MW494187.1	
117	MW530510.1		128	MT339040.1	

Continued.

No	Sequence	Country	No	Sequence	Country
118	MT528235.1		129	MT510718.1	
119	MT525950.1		130	MW493773.1	
120	MT748758.1		131	MW079429.1	
121	MT507276.1	Jamaica	132	MW757203.1	
122	MT507793.		133	MW757191.1	
123	LC573289.2		134	MT372480.1	Malaysia
124	LC606022.1	Japang	135	MW757192.1	
125	LC606017.1		136	MW757197.1	
126	LC606020.1		137	MT372482.1	
138	EPI_ISL_1660474		180	EPI_ISL_984277	
139	EPI_ISL_1660416		181	EPI_ISL_1534694	
140	EPI_ISL_1622410		182	EPI_ISL_1534693	
141	EPI_ISL_1622427		183	EPI_ISL_1533095	
142	EPI_ISL_747194		184	EPI_ISL_538502	
143	EPI_ISL_576387		185	EPI_ISL_910014	
144	EPI_ISL_574613		186	EPI_ISL_1469235	
145	EPI_ISL_757287		187	EPI_ISL_1469236	
146	EPI_ISL_791981		188	EPI_ISL_1469233	
147	EPI_ISL_538503		189	EPI_ISL_1469232	
148	EPI_ISL_775596		190	EPI_ISL_1447361	
149	EPI_ISL_1265168		191	EPI_ISL_1447354	
150	EPI_ISL_747237		192	EPI_ISL_1447351	
151	EPI_ISL_707696		193	EPI_ISL_1447347	
152	EPI_ISL_707778		194	EPI_ISL_1447343	
153	EPI_ISL_707890		195	EPI_ISL_1533098	
154	EPI_ISL_791979		196	EPI_ISL_775594	
155	EPI_ISL_1533101		197	EPI_ISL_1257864	
156	EPI_ISL_525492		198	EPI_ISL_1170955	
157	EPI_ISL_576383_		199	EPI_ISL_467375	
158	EPI_ISL_610161	Indonesia	200	EPI_ISL_538500	
159	EPI_ISL_538502		201	EPI_ISL_1072986	
160	EPI_ISL_538498		202	EPI_ISL_516829	
161	EPI_ISL_538511		203	EPI_ISL_574603	
162	EPI_ISL_791983		204	EPI_ISL_753699	
163	EPI_ISL_467374		205	EPI_ISL_775598	
164	EPI_ISL_1391013		206	EPI_ISL_833501	
165	EPI_ISL_1533098		207	EPI_ISL_791986	
166	EPI_ISL_775594		208	EPI_ISL_956316	
167	EPI_ISL_1257864		209	EPI_ISL_956313	
168	EPI_ISL_1170955		210	EPI_ISL_1257832	
169	EPI_ISL_467375		211	EPI_ISL_1265451	
170	EPI_ISL_1263323		212	EPI_ISL_1447347	
171	EPI_ISL_1263322		213	EPI_ISL_1447343	
172	EPI_ISL_984277		214	EPI_ISL_1469236	
173	EPI_ISL_1534694		215	EPI_ISL_1469233	
174	EPI_ISL_1534693		216	EPI_ISL_1469232	
175	EPI_ISL_1533095		217	EPI_ISL_1447361	
176	EPI_ISL_538502		218	EPI_ISL_1447354	
177	EPI_ISL_910014		219	EPI_ISL_1447351	
178	EPI_ISL_1469235		220	EPI_ISL_1447347	
179	EPI_ISL_1469236		221	EPI_ISL_1447343	

Table 2. Sequences of primary and probe design results on ORF1ab gene and N.

Gen ORF 1ab Product length: 183 Bp	Length	Tm	%GC
AE.35-01*F*- GCAATTTACAACACAGAC	18	54.70	42.00
AE.35-01*R*-GTTCTTTATGCTAGCCACT	18	54.16	42.00
AE.35-01*P*- FAM AAACACACAACAGCATCGTCA-BHQ-1	21	59.00	43.00
Gen N Produk length : 111 Bp			
AE.36-01*F* - AATTCAACTCCAGGCAG	18	55.33	44.00
AE.36-01*R*- CTCTCAAGCTGGTTCAATC	18	55.09	47.00
AE.36-01*P*- VIC-CAGCAAAGCAAGAGCAGCA-BHQ-1	19	60.00	52.00

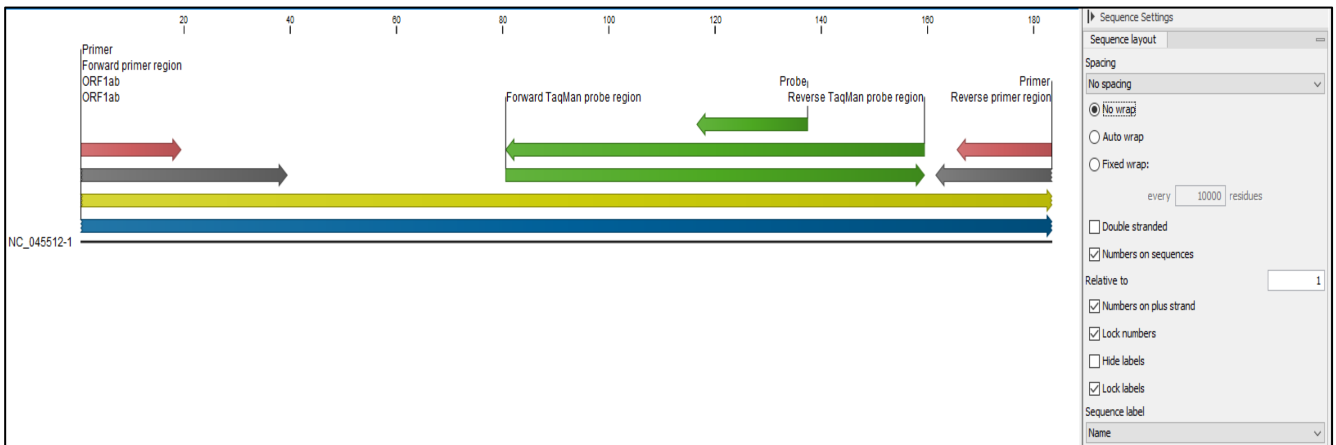

Figure 1: Primary pasting site and ORF 1ab gene probe.

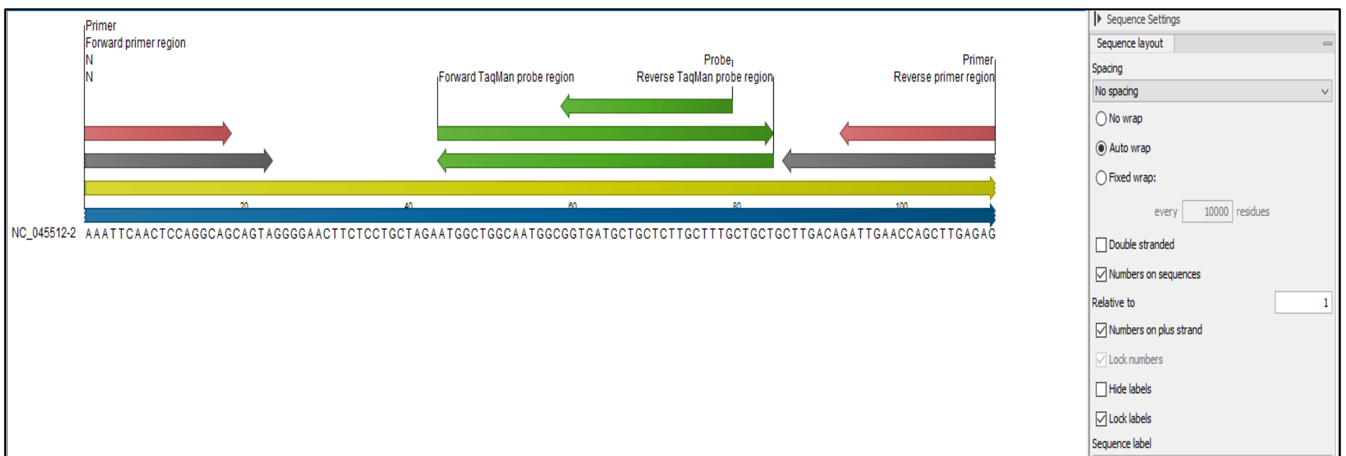

Figure 2: Primary pasting site and N gene probe.

DISCUSSION

The design of primers and probes was carried out to search for the best primary candidates and probes to be used for COVID-19 detection using the multiplex PCR method. Sequence alignment is performed to arrange DNA, RNA, or protein sequences to identify areas of similarity that may be a consequence of functional, structural, or evolutionary relationships between sequences. Alignment is carried out using sequence reference (NC_045512.2) sequences are molecularly analyzed to determine the conserved region which is a region free of mutations so that amplification can be carried out.[9] The essential quality of the ORF1ab quality, the N quality assembly the prerequisites for qRT-PCR can be seen in Table 2. The essential match within the ORF1ab quality brought about in an amplicon estimate of 183 bp and an N quality of 111 bp. The ideal measure for

PCR items is 50- 150 bp.[10] In any case, PCR items measuring up to 400 bp can moreover be well intensified.[11] so PCR products along 183 bp within the ORF1ab quality are still well opened up. The primary sequence in the ORF1ab gene, the N gene has a length of 18 bases on the forward and reverse, and the probe sequence in the ORF1ab gene has a length of 21 bases and the N gene is 19 bases. The length of the primer and probe meets the requirements in the design of the primer and probe to be used as detection in PCR The primary length meets the requirement of between 18 to 30 bases of the number of bases in the primary sequence and probe. Determines the efficiency of the PCR process.[12] Primary composition is one of the important considerations because it can reduce primary specificity, in the form of GC percentages of the amount of guanine and cytosine in a primer.[13]

The high presentation of the GC will encourage the primer to form a dimer, affecting the amplification results.[14] It can be seen in table 1. Primers designed on the ORF1ab gene have a GC percentage of 42% for primary forward and 42% for primary reverse and in gen N 44% primary forward and 47% primary reverse. Probes designed on the ORF1ab gene have a GC percentage of 43% and in the N gene of 52%, the design of primers and probes with a low GC presentation can reduce the efficiency of the PCR process caused because the primer is unable to compete to attach effectively to the template.[15] Melting temperatures in the primary orf1ab gene and N gene have a Tm that corresponds to the optimal Tm characteristics of 52-65 ℃. In the primary Tm of the ORF 1ab gene with a forward of 54.70 ℃ and reverse of 54.16 ℃, the Tm primer of the N forward gene is 55.33 ℃ and reverse of 55.09 ℃. The Tm from the probe design results in the ORF1ab gene and the N gene has a Tm that corresponds to the characteristics of 5-10 ℃ above the primary Tm, the Tm probe of the ORF1ab gene of 59 ℃, and the N gene of 60 ℃, can be seen in Table 2. The difference in the Tm value of the probe with the primer will ensure that the probe can be hybridized with the target during the primary extension process, thereby increasing the fluorescent signal in the amplification process, a stable hybridization complex will produce maximum exonuclease activity during the PCR process.[16]

CONCLUSION

Sequences of primer and probe pairs that can be used for COVID-19 detection using the multiplex PCR method. The results of the primary design of the ORF1ab gene and probe with forward primer: 5'-CGCAATTTACAACACAGAC -3' reverse primer: 5'-GTTCTTTATGCTAGCCACT -3' amplicon length 183 bp, and probe sequence: FAM 5'-AAACACACAACAACCATCGTCA-3' BHQ-1, in N gene with forward primer: 5'-AATTCAACTCCAGGCAG -3' and reverse primer: 5'-CTCTCTCAAGCTGGTTCAATC -3' amplicon length 111 bp, and probe sequence: HEX 5'-CAGCAAAGCAAGAGCAGCA -3' BHQ-1 primer pair sequence and gene probe ORF1ab and the suspected N gene were eligible for q-PCR.

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