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PCR-amplification of wild type receptor binding domain coding gene of SARS CoV-2 spike protein for cloning into a bacterial expression vector

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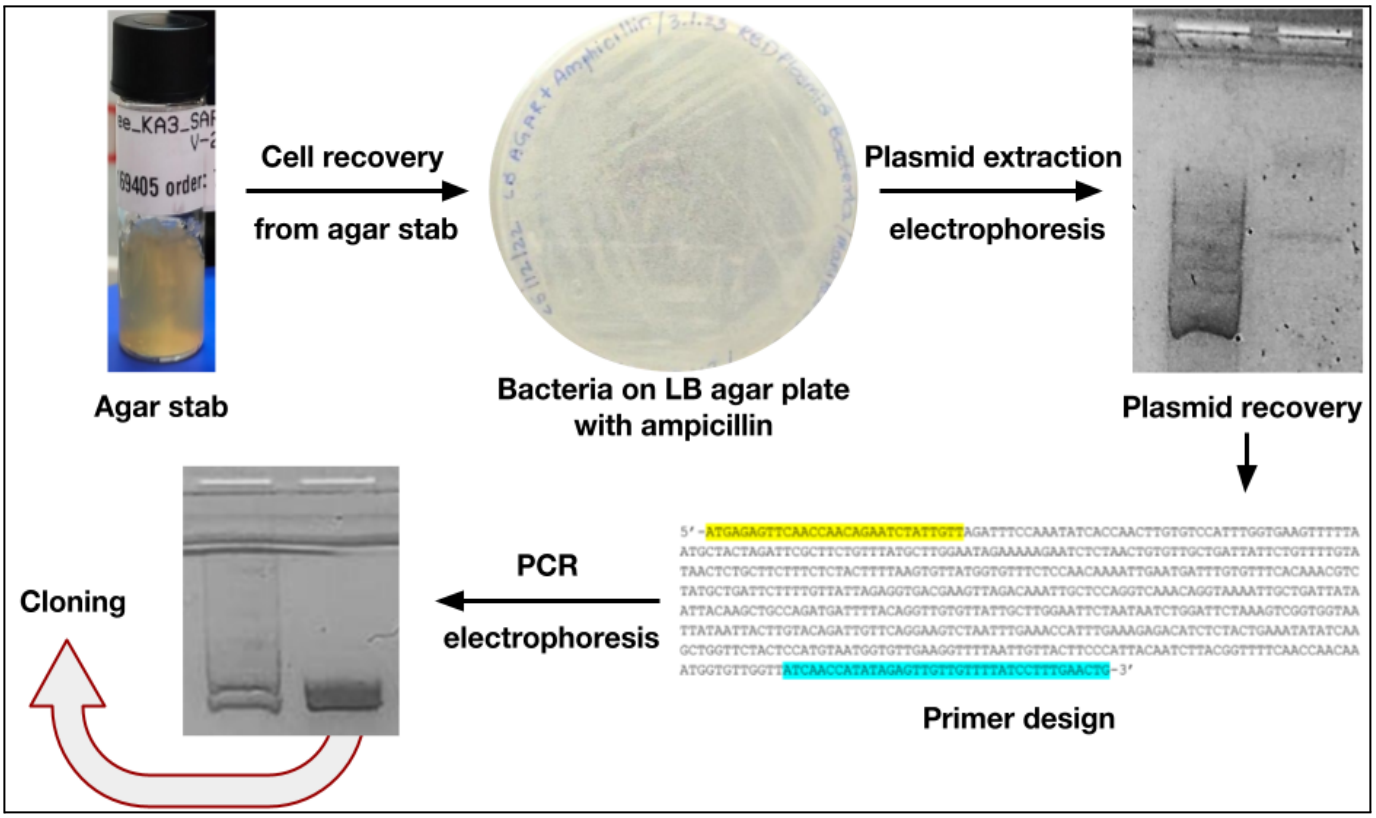
Keywords: SARS CoV-2, Spike protein, receptor binding domain, PCR, primer design, agarose gel electrophoresis.



Graphical abstract outlining the overall process of this study.

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PCR-based amplification of genes is routinely done these days due to the availability of new varieties of DNA polymerases with high fidelity. COVID-19 is a pandemic that has claimed millions of lives across the globe in recent years. Vaccines were designed and were employed in time as a rescue mechanism. However, the viral evolution through mutations in its spike protein is out competing the vaccine design strategies that exist today. Newer ways are needed for vaccine design. In this study, we PCR-amplified the SARS CoV-2 spike protein receptor binding domain (RBD) using specific primers. The PCR product size was confirmed using agarose gel electrophoresis. The annealing temperatures were thoroughly scanned to identify the best one. This PCR product shows the correct size on the gel compared to the standard 1kb DNA ladder. In future, this PCR product will be cloned into an expression vector (pET) in order to express and evaluate the protein for a better vaccine design.

COVID-19, a global pandemic, was caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) during 2019 - 2020. SARS-CoV-2 was first reported in Wuhan, China in December 2019. SARS-CoV-2 virus belongs to the coronaviridae family and was found to be closely related to human pathogenic corona viruses like SARS-CoV-1 which caused the 2002 - 2004 outbreak and MERS. Properties like high reproductive number, stronger interaction with the host cells and efficient spreading capacity have turned SARS-CoV-2 a more infectious one compared to the other viruses of the same family [1][2]. Corona virus has four important structural proteins, out of which Spike protein [S] is found to play a major role in the processes of - entry of the viral particle, attachment and fusion of the viral membranes with the host cell membrane [3][4]. Spike protein is a homotrimeric membrane protein having two subunits S1 and S2 in each monomer. RBD is present in the S1 subunit and is of 200 amino acids length [5][6].

The receptor binding domain (RBD) of the spike protein is an important region of the spike protein structure which facilitates the interaction of the spike protein with the human ACE2 receptor. RBD of the spike protein is also an important target for the production of antibodies and in the development of drugs and vaccines [7][8][9]. Hence, RBD has been the major target for therapeutic development based studies on COVID-19 caused by SARS-CoV-2. But in the process of evolution, many variants of SARS-CoV-2 have emerged which pose a challenge for the existing drugs and vaccines working against the RBD in the spike protein of SARS-CoV-2. Owing to the above mentioned necessity, we are in the process of development of newer vaccine strategies for COVID-19 pandemic that aims to target multiple existing and emerging variants of SARS-CoV-2 and to provide safer and less invasive vaccine administration. As the preliminary requirement of the research is the availability of the RBD gene in desired quantities, we believe that the optimization of PCR-based amplification of RBD gene will help to generate multiple copies of the same for further use. In this study, we have focussed on optimization of PCR-based amplification of the receptor binding domain gene from SARS-CoV-2 spike protein in order to use it for cloning purposes.

Materials & Methods:

Bacterial strains and plasmids: Escherichia coli, DH5α strain cells were used in this study. *E. coli*, DH5α strain cells containing the RBD plasmids were purchased from Addgene (#169405) as agar stabs. The SARS-CoV-2 RBD plasmid used for transformation was pmCellFree_KA3_SARS-COV-2_RBD was a gift from Kirill Alexandrov (Addgene plasmid #169405; http://n2t.net/addgene:169405; RRID: Addgene_169405) [10].

Media preparation and bacterial cell culture: Luria Bertani (LB) Broth and LB Agar plates were used for culturing the *E. coli*, DH5α cells. LB Broth was purchased from HiMedia (catalog no. M1245). The liquid broth and agar plates were prepared according to the manufacturer's instructions. One hundred ml of broth was prepared by adding 2.5 grams of LB broth powder to 100 mL of di-water. Similarly for LB Agar plate preparation, to 300 mL of di-water, 7.5 grams of LB Broth powder and 4.5 grams of Agar-Agar (Bacteriological grade) were added. Both the solutions were mixed well and autoclaved at 121 °C temperature and 15 lb pressure for 20 minutes. Ampicillin (final conc. 50 mcg) was added to the nutrient media. LB Agar solution was poured into petri plates and cooled to solidify. The *E. coli* cells containing the RBD plasmid which were obtained as agar stab were initially streaked on LB Agar + Amp plates and incubated at 37 °C overnight (Figure 1). The following day, a loopful of colonies were inoculated into 5 ml of LB Broth with ampicillin and incubated at 37 °C overnight.

S.no	Component	Volume
1	Emerald Amp GT PCR Master Mix (2X Premix) (Takara #RR310)	25 µl
2	DI Water	21 µl
3	RBD Plasmid DNA (Template DNA)	2 µl
4	Forward Primer	1 µl
5	Reverse Primer	1 µl
	Total	50 µl

Table 1. Composition of PCR for 2 µl of template DNA.

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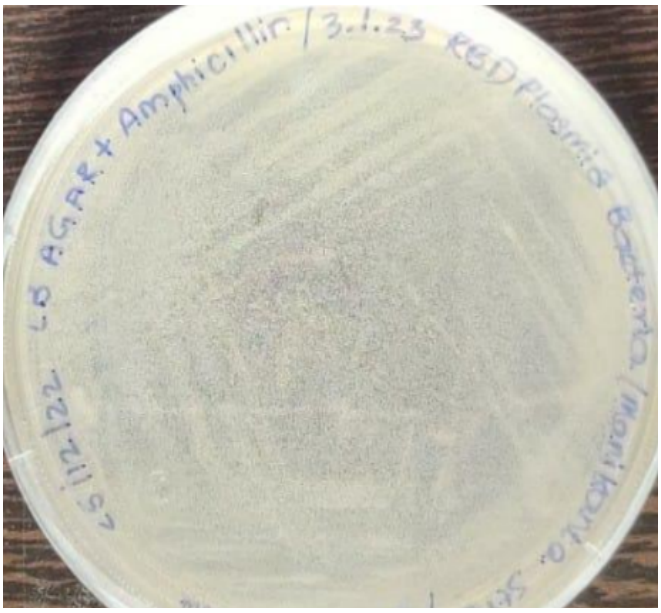

Figure 1. LB agar plate showing cell recovery from agar stab.

Plasmid DNA isolation: The overnight culture which was inoculated with *E. coli* cells containing the RBD plasmid was used to perform plasmid DNA extraction. Plasmid DNA Extraction kit (Column based) purchased from HiMedia (catalog no. MB505) was used to perform plasmid DNA extraction, according to manufacturer's instructions. Cells from the overnight culture were harvested by centrifugation. The culture was centrifuged at 12,000 rpm for 1 min. The culture media was discarded and the pellet containing the bacterial cells was resuspended in 250 µl of resuspension solution containing RNase. The pellet was resuspended without any clumps by mixing thoroughly by pipetting. To the resuspended bacterial cells, 250 µl of Lysis solution was added and mixed gently by inverting the tube 4-5 times. Next, 350 µl of neutralization solution was added and mixed gently by inverting the tube 4-5 times. A cloudy white precipitate was observed after adding the neutralization solution. The cells were centrifuged at 12,000 rpm for 10 minutes. The clear supernatant was transferred to a spin column carefully and centrifuged at 12,000 rpm for 1 minute. The flowthrough was discarded. To the spin column, 500 µl of wash solution (HPB) was added and centrifuged at 12,000 rpm for 1 minute. The flow-through was discarded. To the spin column 700 µl of wash solution containing ethanol (HPE) was added and centrifuged at 12,000 rpm for 1 minute. The flow-through was discarded and the spin column was given a dry spin by centrifugation at 12,000 rpm for 1 minute. The collection tube attached to the spin column was replaced with a new one. To the spin column, 50 µl of elution buffer was added and incubated at room temperature for 3-5 minutes. The spin column was centrifuged at 12,000 rpm for 1 min. The eluate containing the plasmid DNA was stored in a capped polypropylene tube at -20 °C until further use.

PCR primer design: For amplification of the RBD region in the plasmid, primers were designed such a way targeting the 5' and 3' ends of the RBD gene sequence mentioned in Figure 2. The primers designed included necessary restriction sites for cloning purposes. The sequence of the RBD gene and the primers are shown in Figure 2. The primers were ordered from IDT Inc. (Coralville, IA. USA) and their respective data sheets disclosing the properties of forward and reverse primers were displayed in Figure 3.

PCR optimization: To identify the suitable annealing temperature for amplifying the RBD sequence, PCR optimization was performed for a range of annealing temperatures from 50 °C to 60 °C. PCR was performed using Emerald Amp GT PCR Master Mix (2X Premix) (Takara #RR310), forward and reverse primers from IDT and RBD plasmid DNA as template DNA. The required components were added to a 0.2 ml PCR tube (Table 1). The PCR tube was placed in a thermal cycler in which the following PCR protocol was programmed and run to amplify the RBD gene. The protocol was mentioned in Table 2.

Agarose gel electrophoresis: To analyze the quality of the isolated plasmid DNA, the sample was run on 1.5% agarose gel (with 8 µl SYBr Safe Gel Staining Solution). The sample was prepared by mixing 10 µl of plasmid DNA with 2 µl of 6X gel loading buffer. Along with the plasmid DNA, a 1kb ladder was loaded in one of the wells for reference. Since the Emerald Amp GT PCR Master Mix (2X Premix) contained a premixed gel loading buffer, the PCR products were directly loaded into the wells along with a 1kb ladder in one of the wells for reference. The samples were run at 75 volts for 45 minutes. The bands were visualized using a UV lamp.

S.no	PCR Stage	Temperature	Time period
1	Initial Denaturation	94°C	5 min
2	Denaturation	94°C	45 sec
3	Annealing	50°C - 60°C (variable)	30 sec
4	Extension	72°C	30 sec
	Cycles = 30 cycles		
5	Final Extension	72°C	10 min
6	Cooling	4°C	forever

Table 2. PCR protocol.

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5′-ATGAGAGTTCAACCAACAGAATCTATTGTTAGATTTCCAAATATCACCAACTTGTGTCCATTTGGTGAAGTTTTTA
ATGCTACTAGATTCGCTTCTGTTTATGCTTGGAATAGAAAAAGAATCTCTAACTGTGTTGCTGATTATTCTGTTTTGTA
TAACTCTGCTTCTTTCTCTACTTTTAAGTGTTATGGTGTTTCTCCAACAAAATTGAATGATTTGTGTTTCACAAACGTC
TATGCTGATTCTTTTGTTATTAGAGGTGACGAAGTTAGACAAATTGCTCCAGGTCAAACAGGTAAAATTGCTGATTATA
ATTACAAGCTGCCAGATGATTTTACAGGTTGTGTTATTGCTTGGAATTCTAATAATCTGGATTCTAAAGTCGGTGGTAA
TTATAATTACTTGTACAGATTGTTCAGGAAGTCTAATTTGAAACCATTTGAAAGAGACATCTCTACTGAAATATATCAA
GCTGGTTCTACTCCATGTAATGGTGTTGAAGGTTTTAATTGTTACTTCCCATTACAATCTTACGGTTTTCAACCAACAA
ATGGTGTTGGTTATCAACCATATAGAGTTGTTGTTTTATCCTTTGAACTG-3′

Figure 2. RBD template sequence with primers highlighted.

Results and Discussion:

Recovery of bacterial cells from the agar stabs: The plasmid containing RBD gene was purchased from Addgene (#169405). The plasmid contains an ampicillin resistance gene which was used as a selective marker for picking the colonies that contain the plasmid. The plasmid was received in bacterial cells as an agar stab. The cells were grown in LB media containing ampicillin. Since DH5α cells are normally not resistant to ampicillin, the formation of colonies on the LB agar plate containing ampicillin confirms the presence of the plasmid in the bacterial cells (Figure 1).

Isolation of RBD sequence containing plasmid: The quality check of the plasmid extracted was performed by running the plasmid DNA isolated on a 1.5% agarose gel and the bands were visualized using a UV lamp. The results showed that a band was observed above the third band of the 1kb DNA ladder which corresponds to 6 kb. The size of the band obtained was at 6.6 kb size, which matches with the size of the RBD plasmid, Addgene#169405 i.e., 6651 bp. The gel picture displaying the 1kb ladder and RBD plasmid DNA is shown in Figure 3. Since the bands were not very bright, the concentration of the plasmid DNA was assumed to be low. But, a crisp faint band at the required size confirmed the successful extraction of the RBD plasmid.

PCR optimization for RBD amplification: PCR was performed with varying annealing temperatures ranging from 50 °C to 60 °C. All the PCR products were run on a 1.5% agarose gel and the bands were visualized using a UV lamp.

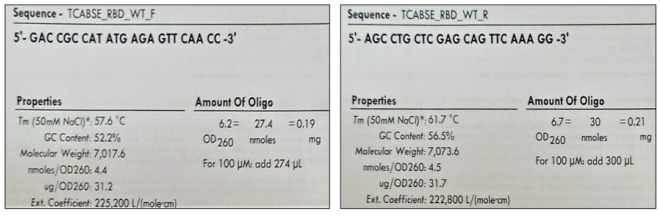

Figure 3. Fact sheets for the primers ordered from IDT Inc.

It was observed that there was successful amplification of the RBD sequence at annealing temperature of 54 °C. The gel picture in Figure 4 shows a very bright crisp band formed above the 500 bp band in the 1 kb ladder. The approximate size of the PCR product was calculated to be 600 bp which matches with the size of the RBD sequence in the plasmid.

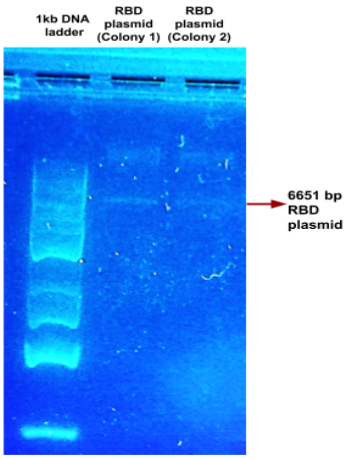

Figure 4. Agarose gel electrophoresis showing the plasmid bands.

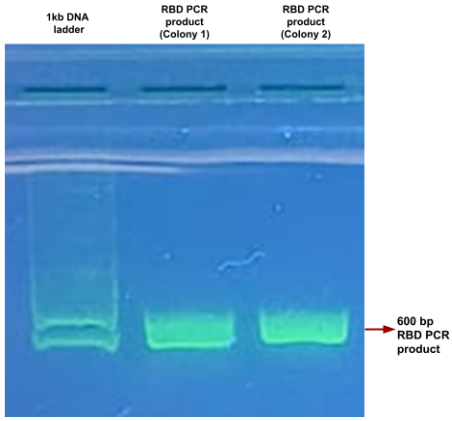

Figure 5. Agarose gel electrophoresis showing the amplified PCR products.

Conclusion and Future directions:

The primers were highly specific resulting in the expected PCR product of correct size. The wild type RBD gene was successfully amplified using PCR as confirmed by the gel electrophoresis. This PCR product can be used for cloning in the future into expression vectors and also as a template for site-directed mutagenesis for protein antigen studies.

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Conflict of interest: The authors declare no conflict of interest in this study. However, this research article is an ongoing project currently at TCABS-E, Visakhapatnam, India.

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Author contributions: M.V. recovered the bacterial cells from agar stabs, performed PCR optimization, qualitative analysis and wrote the manuscript; S.G. & S.A. performed plasmid isolation, PCR and gel electrophoresis; M.S. assisted M.V. and S.G. in bacterial recovery; M.A. assisted S.G. to repeat PCRs for confirmation; N.M. assisted in gel electrophoresis; B.P. co-supervised S.G.; S.P. co-supervised N.M.; R.S.Y. is the principal investigator, designed the project, trained M.V., S.G., M.S., M.A. and N.M., provided lab space/facilities, edited & finalized manuscript.

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Full figure legends:

Figure 1. LBagar plate showing cell recovery from agar stab. The bacterial cells were successfully recovered from the agar stab by plating on LB agar containing ampicillin as a selection marker. Bacterial lawn indicates the growth of cells containing the desired plasmid that has ampicillin-resistance.

Figure 2. RBD template sequence with primers highlighted. The template DNA that shows the RBD sequence is shown here. The forward primer was designed by taking the bases that are highlighted in yellow color and the reverse primer was designed by considering the sequence that is highlighted in cyan color. The total sequence length for RBD is 600 bp.

Figure 3. Fact sheets for the primers ordered from IDT Inc. The fact sheets for the forward and reverse primers are displayed in the left and right panels, respectively. In addition to the primer sequences, their GC content, melting temperatures, etc. are also given in these sheets.

Figure 4. Agarose gel electrophoresis showing the plasmid bands. Plasmids were isolated from two random colonies and were qualitatively checked on agarose gel. Left most lane

contains the 1kb ladder while the center and right lanes show plasmids of the expected size as indicated in the figure with an arrow.

Figure 5. Agarose gel electrophoresis showing the amplified PCR products. PCR products obtained from the two plasmids were qualitatively checked on agarose gel. The left lane shows 1kb ladder while the center and right lanes show PCR products. Both PCR products were of the same size as pointed by the arrow in the figure.