

An efficient and cost-effective method for directed mutagenesis at multiple dispersed sites—a case study with Omicron Spike DNA

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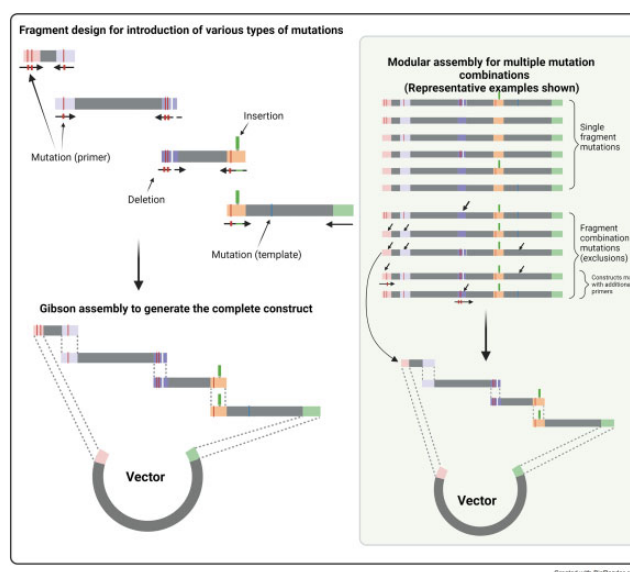
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Abstract

Site-directed mutagenesis is an invaluable technique that enables the elucidation of the contribution of specific residues to protein structure and function. The simultaneous introduction of mutations at a large number of sites (>10), singly and in multiple combinations, is often necessary to fully understand the functional contributions. We report a simple, efficient, time and cost-effective method to achieve this using commonly available molecular biology reagents and protocols, as an alternative to gene synthesis. We demonstrate this method using the Omicron Spike DNA construct as an example, and create a construct bearing 37 mutations (as compared to wild-type Spike DNA), as well as 4 other constructs bearing subsets of the full spectrum of mutations. We believe that this method can be an excellent alternative to gene synthesis, especially when three or more variants are required.

Graphical Abstract



Keywords: cost-effective method, directed multiple mutagenesis, Omicron Spike, PCR based

Introduction

The method of choice for the introduction of one or a few mutations located in close proximity in recombinant DNA constructs is site-directed mutagenesis. In contrast, for introducing a large number of mutations, especially those not clustered together

within the region covered by a typical primer and spread over several kilobases, commercial gene synthesis is preferred [1]. However, this may be too expensive, may take too long and may not be an option in some geographical locations or for some projects. In this scenario, an alternative method combining

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polymerase chain reaction (PCR), overlap extension PCR, and Gibson cloning can be used to create the desired construct in a short duration and at a cost lower than that of commercial synthesis. This alternate strategy will provide a significant cost advantage especially when a series of constructs with combinations of mutations needs to be created.

The basic principle of the method lies in assembling the complete construct using a series of smaller, mutation-containing fragments. The construct is split into fragments in such a way that forward and reverse primers used to amplify a fragment will contain all of the mutations located in that fragment. The mutation-containing fragments are then seamlessly assembled using a homology-based cloning method such as Gibson assembly [2]. Mutations located within a 50 bp region are introduced using a single primer; fragment sizes typically range from 100 bp to 3 kb and an unlimited number of fragments can be combined into the final construct in one or more rounds of cloning. Due to the modular nature of construct assembly, a combinatorial library of constructs including or excluding any combination of mutations can be generated with minimal additional effort, time and cost.

We illustrate this method using the example of the Omicron Spike gene which contains 37 mutations (dispersed over nearly 3 kb) as compared to the wild-type Spike gene. We followed a similar strategy to create the Delta Spike construct previously, which was successfully used in the pseudovirus neutralization assay reported by Kumar *et al.* [3], validating this approach.

Overall strategy

The comprehensive list of mutations incorporated into the Omicron Spike was based on the GISAID sequence EPI_ISL_6814922 (27 November 2021) [4] and is listed below:

Spike A67V, Spike H69del, Spike V70del, Spike T95I, Spike G142D, Spike V143del, Spike Y144del, Spike Y145del, N211del, Spike L212I, Spike ins214EPE, Spike G339D, Spike S371L, Spike S373P, Spike S375F, Spike K417N, Spike N440K, Spike G446S, Spike S477N, Spike T478K, Spike E484A, Spike Q493R, Spike G496S, Spike Q498R, Spike N501Y, Spike Y505H, Spike T547K, Spike D614G, Spike H655Y, Spike N679K, Spike P681H, Spike N764K, Spike D796Y, Spike N856K, Spike Q954H, Spike N969K and Spike L981F.

The overall strategy was to split the Spike-coding sequence into fragments, each to be amplified by PCR, incorporating the desired mutations in the primer binding regions. The fragments would then be stitched together by overlap PCR, and then assembled by Gibson assembly [2] to create the complete sequence (Fig. 1). Gibson assembly uses a combination of three enzymes to ligate fragments with homologous ends and leaves no scar in the final ligated product, an essential requirement while assembling a large number of fragments. The process efficiently ligates up to five fragments, after which the efficiency drops. Since this involved a large number of fragments, at least two rounds of assembly and verification by sequencing were anticipated.

We used the Spike-coding sequence, with the 19 amino acid deletion at the C-terminal end, as our template since this was the construct to be used in the Pseudovirus neutralization assay [5]. We used the wild-type Spike, or the Delta-plus variant Spike DNA as the template for PCR, based on the mutations required to be incorporated in a specific fragment. Three mutations were incorporated from the Delta-plus Spike DNA template and the rest were introduced using primers. We divided the Spike sequence into 16 fragments, each demarcated by the location of one or more Omicron-specific mutations, and each overlapping with the preceding and following fragments by at least 27 bp. We designed primers for the amplification of each fragment incorporating the required mutation(s) at the centre of the primer, with at least

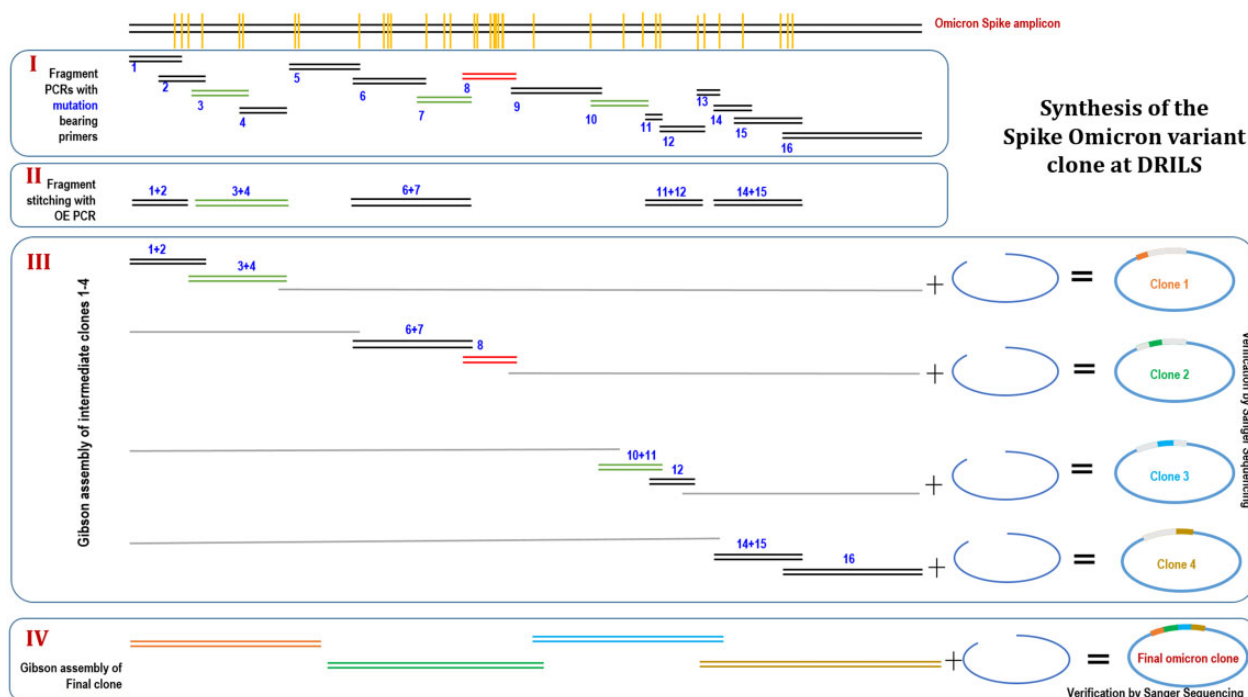


Figure 1. Strategy for the construction of Omicron Spike incorporating 37 mutations.

12 bp of perfectly matched sequence to promote proper annealing and amplification during PCR (Fig. 1).

There were two regions that required a slightly different design. Fragment 8 contained nine mutations spread over ~200 bp region, which did not align with the aforementioned strategy. Therefore, two long oligonucleotides with a 23 bp overlap were designed to generate Fragment 8 by routine overlap extension. Fragment 15 contained two mutations spread over 48 bp, which was too short to split into separate fragments, and too long for a single primer (~74 bp). Hence, two consecutive reverse primers were designed to be used sequentially for Fragment 15, and the second PCR was done using the first PCR as a template.

A few additional modifications were employed to simplify the assembly and screening process:

- 1) Plasmids (pCMV-Spike (wt) and pCDNA-Spike (Delta-plus)) used as a template for PCRs were digested with at least two restriction enzymes in order to reduce the background from inserts during transformation.
- 2) pCMV-Spike was used as the template for PCRs, and fragments were cloned into pCDNA3.1. This allowed for the correct identification of clones by colony PCR using a combination of Spike-specific internal primers and pCDNA3.1-specific primers at each end.
- 3) The last mutation L981F was incorporated only in the final round of assembly, so as to be able to amplify one end from a pCMV-Spike template and thus allowing colony PCR-based screening as mentioned in #2 above.
- 4) The assembly was planned in such a way as to generate intermediate chimeric clones, which would contain the Omicron mutations in just the NTD, RBD or CTD (with the rest being wild-type Spike sequence).

Methods and results

The complete Omicron Spike DNA construct was split into four parts for ease of synthesis, based on the technical limitation of the Gibson assembly process. The mutation containing fragments of each part was amplified first and assembled into a clone in the first round. A simple workflow for the generation of each clone is shown below (Fig. 2). The four parts were then combined into the complete Omicron Spike DNA construct, by following the same workflow in subsequent rounds.

PCR amplification of fragments

Each of the fragments (1–7 and 9–16) was amplified from the appropriate template in a standard 25 µl PCR reaction (Fig. 3A, representative gel images of select PCR products are shown). Taq polymerase was used for PCR amplification of fragments for the first part (fragments less than 1000 bp). For later rounds, Q5 polymerase was used to avoid PCR errors. Additional fragments to

complete the assembly of each clone were also amplified using the pCMV-Spike (wild-type) template (labelled fragments 17–23, examples shown in Fig. 3A). For example, clone 1 would include mutation containing fragments 1, 2, 3, 4 and the rest (5–16) amplified from wild-type Spike (Fragment 17). The list of primers used in this study can be found in Table 1, and details of the fragments used, along with the primers and template DNA used to

PCR with Taq polymerase

Input DNA	10 ng (0.5 µl)
FP (10 µM)	0.75 µl
RP (10 µM)	0.75 µl
10X Taq Buffer	2.5 µl
2.5 mM dNTPs	2.0 µl
Taq DNA Pol	0.5 µl
Water	18 µl
Total	25 µl

PCR Programme for Taq

95°C: 4 min; [95°C: 20 s; 48°C: 20 s; 72°C: 20 s] 5 cycles; [95°C: 20 s, 58°C: 20 s, 72°C: 20 s] 25 cycles; 72°C: 10 min; Hold at 10°C

PCR with Q5

Input DNA	10 ng (0.5 µl)
FP (10 µM)	0.75 µl
RP (10 µM)	0.75 µl
2 × Q5 mix	12.5 µl
Water	10.5 µl
Total	25 µl

PCR Programme for Q5:

98°C: 5 min; [98°C: 10 s, 72°C: 1.30 min] 28 cycles; 72°C: 10 min, Hold at 10°C

amplify them, can be found in Table 2. A standard PCR reaction was assembled as follows:

Challenge: The overlap PCR to generate Fragment 8 was inefficient, and prevented efficient assembly into Clone 2. This was corrected in the subsequent round of assembly by using the long oligonucleotides as just primers.

Fragment stitching

In order to improve the efficiency of Gibson assembly by reducing the number of inserts (to a maximum of three excluding vectors), we stitched short PCR fragments using the method of Hilgarth et al. [6]. Briefly, each PCR fragment was purified on a DNA cleanup column to get rid of primers, combined in equimolar amounts and stitched together in overlap extension 1 (OE1). Subsequently, the stitched fragment was amplified using the forward primer of the first fragment, and the reverse primer of the second fragment in OE2 (Fig. 3B).



Figure 2. A simple workflow for the assembly of each part of the complete Omicron construct.

OE1

PCR fragments after column clean-up (100 ng/kb each)	10 µl
2 × Q5 mix	12.5 µl
Water	2.5
Total	25 µl

PCR Programme for OE1:

94°C: 30 s; [94°C: 15 s; 72°C: (−0.5°C/cycle) 30 s/kb] for 9 cycles; [94°C: 15 s; 67°C: (−0.5°C/cycle) 15 s; 72°C: 30 s/kb] for 5 cycles; 72°C: 2 min; Hold at 10°C

OE2

OE1 product	2 µl
2 × Q5 mix	12.5 µl
2 µM FP	0.35 µl
2 µM RP	0.35 µl
Water	9.8 µl
Total	25 µl

PCR Programme for OE2:

94°C: 30 s; [94°C: 15 s; 67°C: (−0.5°C/cycle) 15 s; 72°C 30 s/kb] for 17 cycles; [94°C: 15 s; 59°C: 15 s; 72°C: 30 s/kb] for 23 cycles; 72°C: 2 min; Hold at 10°C

Construct assembly Part 1—Gibson assembly of Clones 1, 2, 3 and 4

pCDNA3.1 was digested with BamHI and HindIII, treated with Antarctic Phosphatase and purified using a DNA cleanup column, and checked for background by transformation before using in the assembly reaction. Each of the clones was assembled with equimolar amounts of cut vector and inserts, except in the case of fragments shorter than 400 bp, for which a 3- to 5-fold molar excess was used. The fragments that were incorporated into each of the clones are listed below and the details of the fragments can be found in Table 2. A typical Gibson assembly reaction was assembled as described below, incubated at 50°C for 1 h, and transformed into 200 µl competent cells. At least 40–50 colonies were obtained for each of the 4 reactions.

Gibson assembly

Clone number	Fragments used
Clone 1	1 + 2OE, 3 + 4OE, 17 and vector
Clone 2	18, 6 + 7OE, 8, 19 and vector
Clone 3	20, 10, 11 + 12OE, 21 and vector
Clone 4	22, 14 + 15'OE, 16, vector
Reaction:	
Cut vector:	0.4 µl (50 ng)
Insert:	Equimolar amount of inserts (0.5–1.5 µl of each unpurified PCR product as estimated from gel images)
Gibson mix:	15 µl
Water:	Remaining
Total	20 µl

Colony PCR screening

For each clone, five colonies were screened by colony PCR using two sets of primers: AS145 and AS645/Omi F4Rev (Front), and AS575 and AS507 (End), to confirm insertion of the entire Spike sequence (Fig. 3C, colony PCR results for clones 1 and 4 as examples). Each colony was resuspended in 20 µl of water, lysed by heating and 1.5 µl of the supernatant was used as input for a 10 µl PCR reaction. Plasmid was prepared from at least two positive

clones, size-verified on a gel (Fig. 3D) and sent for sequencing with primers expected to cover the mutated region.

Challenge: Since Fragment 8 could not be generated efficiently, 10 colonies were screened for clone 2, and 4 plasmids were sequenced. However, no mutations in Fragment 8 were incorporated. In addition, for clone 3, all of the sequencing reactions failed. Therefore, we altered the strategy to include repeats of these two segments in the subsequent steps.

Construct assembly Part 2—Gibson assembly to generate clone 1.2 (combining clones 1 and 2) and Clone 3.4 (clones 3 and 4)

For Parts 2 and 3 of the construct assembly, protocols identical to those described for part 1 above, were followed. The fragments assembled for each are listed below.

Gibson assembly

Clone number	Fragments used
Clone 1.2	1, 23, 6 + 7OE, 25, 19 and vector
Clone 3.4	10, 11 + 12OE, 26 and vector

We obtained positive clones for both in this round, as confirmed by sequencing. A six-fragment assembly (five inserts and vector) proved to be successful as seen in the generation of clone 1.2.

Construct assembly Part 3—Gibson assembly to generate full-length clone

Gibson assembly

Clone number	Fragments used
Full-length clone	29, 27, 28 and vector
NTD alone	30, 17 and vector
RBD alone	31, 32, 19 and vector
NTD+RBD	29, 19 and vector
CTD alone	20, 33 and vector

We obtained at least one positive clone for each of the above constructs, as confirmed by sequencing.

Challenges: In some instances, we observed errors in the primer binding regions which we attribute to errors in the oligonucleotides during synthesis, especially for long oligonucleotides (such as those for Fragment 8). We overcame this by using a shorter oligonucleotide for a second round of PCR to eliminate specific errors.

The quote obtained for the commercial synthesis of this construct from a reputed vendor was INR 1 Lakh (USD 1330) and the time from order to delivery of the sequence verified construct was 45 days at our location, possibly due to import restrictions and pandemic-related delays. The in-house synthesis of the final sequence verified construct was completed in under 29 days at an estimated cost of INR 50 000 (USD 665) (Tables 3 and 4). In addition to the complete Omicron Spike gene, four additional variants were synthesized using no additional reagents. The time required for in-house synthesis was primarily limited by the time required for commercial oligo synthesis/delivery and turnaround time for sequencing, which required 50% of the total time to synthesize and

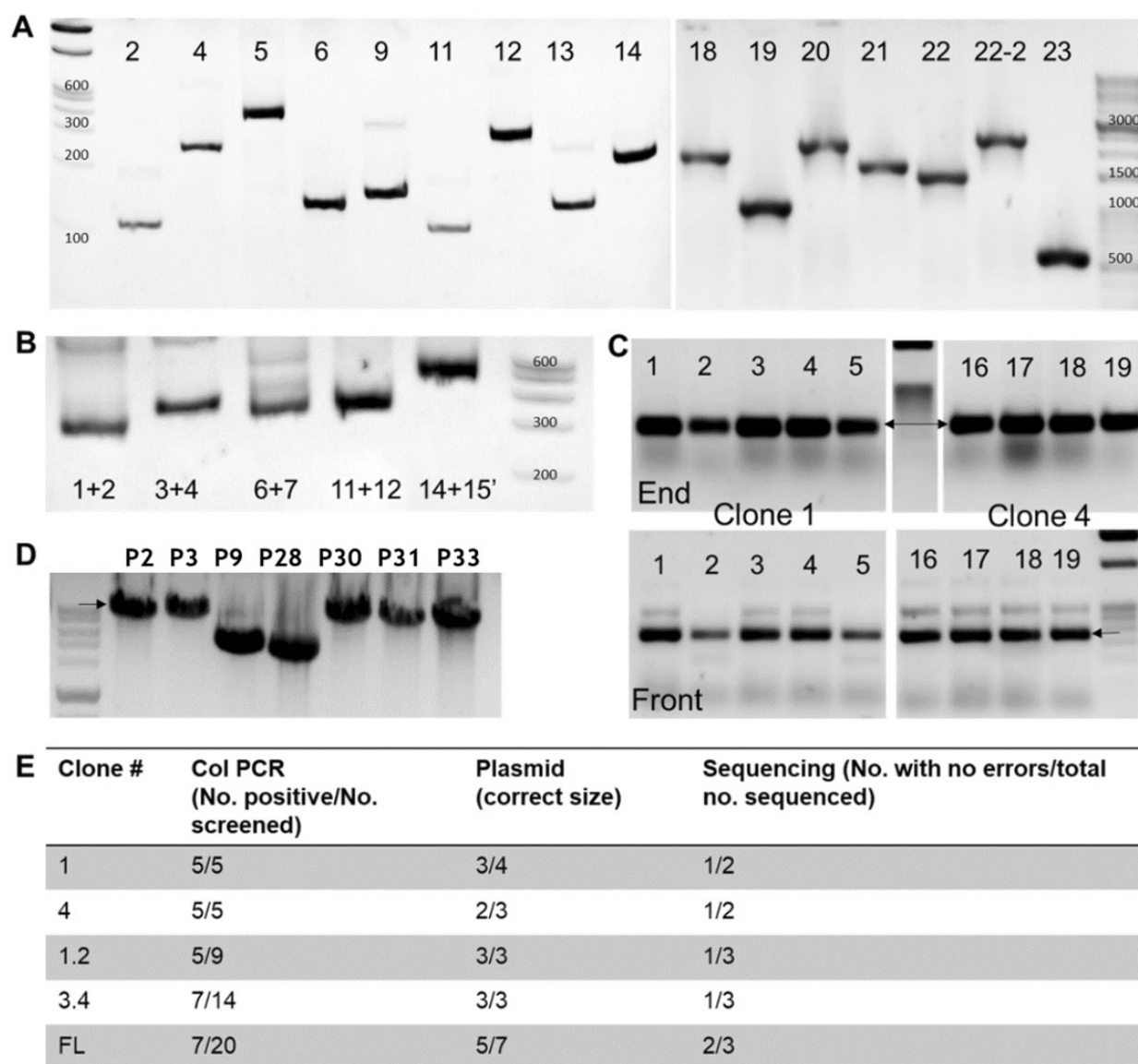


Figure 3. Representative gel images at various stages of Omicron synthesis. (A) Individual fragments were amplified by PCR using Taq Polymerase or Q5 and 8 μ l was visualized on a native polyacrylamide gel (fragments <800 bp) or agarose gel. (B) Fragments stitched together by OE were visualized by native polyacrylamide gel electrophoresis (PAGE). (C) Colony PCR was used to confirm the presence of the complete insert using two primer sets, one across the vector-insert junction at the N-terminal end of the Spike ORF (Front), and the second at the junction near the C-terminal end (End). Two representative clones are shown. (D) Plasmids amplified from positive colonies were checked for size to confirm the presence of all insert fragments, by resolving on an agarose gel. (E) For each clone assembled using Gibson assembly, the fraction of positive colonies by colony PCR, by plasmid size and finally by sequencing are indicated as a measure of efficiency of the cloning process.

sequence-validate the full-length construct. The cost saving was in part due to the use of lab-made enzymes and cloning reagents.

Discussion

In this study, we report a strategy for simultaneous site-directed mutagenesis at multiple sites spread over several kilobases, with the added option of being able to generate a combinatorial library of mutant constructs. The method uses simple laboratory reagents and protocols and the strategy and methods are widely applicable. In addition to being an alternative for gene synthesis for complex constructs bearing multiple mutations, this technique may be used to synthesize a large collection of single as well as modular combinations of mutations. Constructs generated in this manner could be useful in a wide array of

applications including but not limited to (alanine) scanning mutagenesis for identification of sites of post-translation modifications [7], fitness, function, activity or structure contributions [8–10], as well as for functional analysis of a spectrum of patient mutations in genes implicated in monogenic disorders [11].

With the specific example of the Omicron Spike gene, we were able to generate the construct containing 37 mutations spread across a ~3.7 kb DNA fragment using simple methods and reagents, accessible to most basic molecular biology labs in the world. Despite delays in sequencing turnaround and strategic/experimental failures, we were able to complete this synthesis in half the time that it took to receive a commercially synthesized construct at our location. As suggested by the numbers in Fig. 3E, the efficiency at each step was quite high and this complex construct with the desired sequence was assembled by screening

Table 1. List of primers used in this study

S.no	Primer	Sequence
1.	F1.2 pCDNA	CCCAAGCTGGCTAGCGTTTAACTTAAGCT-TCACCATGTTTCGTTTCTCTGTTCT
2.	R3.2 pCDNA	ATCTGCAGAATTCACCACACTGGACTAGT-GGATCCTTAGCAACATGATCCGCAAGAG-CA
3.	Omi F1Rev	AGTTCCAGAGATAACATGAAACCAAGT
4.	Omi F2Fwd	ACTTGGTTTCATGTTATCTCTGGAAC
5.	Omi F2Rev	TATGTTTGACTTTTCAATGGAAGCGAAGTA
6.	Omi F3Fwd	TACTTCGCTTCCATTGAAAAGTCAAACATA
7.	Omi F3Rev	GTTATTCTTATGATCCAGGAAGGGATC
8.	Omi F4Fwd	GATCCCTTCCCTGGATCATAAGAATAAC
9.	Omi F4Rev	TCCCTGGGGCAGGTCTTCCGGTTCGCGCAC-AATTATTGGAGTGTG
10.	Omi F5Fwd	CACACTCCAATAATTGTGCGCGAACCGGAA-GACCTGCCCCAGGGA
11.	Omi F5Rev	GTTGAACACCTCATCGAAGGGACACAG
12.	Omi F6Fwd	CTGTGTCCTTCGATGAGGTGTTCAAC
13.	Omi F6Rev	GCACCTTGAAGGTGAAGAACGGGGCCAGGT-TGTAGAGGAC
14.	Omi F7Fwd	GTCCTCTACAACCTGGCCCCGTCTTCAAC-TTCAAGTGC
15.	Omi F7Rev	ATTGTAGTTACCGCTGACCTTAGAATCGAG-TTTATTAGAGTTCCA
16.	Omi F8FwdOE	AAGGTCAGCGGTAACATACTACCTGTAC-CGCTTGTTTAGGAAGTCAAACCTGAAGC-CTTTCGAGAGGGATATTTCAACCGAAAT-CTATCAAGCGGGTAATAAACCGTGTA
17.	Omi F8RevOE	CACGCGATAAGGTTGATGACCCACGCCATA-GGTTGGACGGAAGCTGTAAGACCGCAGG-GGGAAGTACGAGTTAAATCCTGCCACAC-CGTTACACGGTTTTATTACCGCTTGA
18.	Omi F9Fwd	GGCGTGGGTCATCAACCTTATCGCGTG
19.	Omi F9Rev	CACTCCAGTTCTTTGAGGCCATTGAA
20.	Omi F10Fwd	TTCAATGGCCTCAAAGGAACCTGGAGTG
21.	Omi F10Rev	GGAGTTATTACATATTTCCGCTCCGAT
22.	Omi F11Fwd	ATCGGAGCGGAATATGTGAATAACTCC
23.	Omi F11Rev	TCTCCTGTGTGATTTAGTTTGTGTCTG
24.	Omi F12Fwd	CAGACACAACTAAATCACACAGGAGA
25.	Omi F12Rev	TGTAAGGGCGCGTTTCAATTGGGTACA
26.	Omi F13Fwd	TGTACCCAATTGAAACGCGCCCTTACA
27.	Omi F13Rev	GAAGCCTCCGAAATACTTAATGGGCGG
28.	Omi F14Fwd	CCGCCCATTAAGTATTTTCGAGGCTTC
29.	Omi F14Rev	GACTGTACGGCTTTAAACTTCTGGGC
30.	Omi F15Fwd	GCCCAGAAGTTTAAAGGCCTGACAGTC
31.	Omi F15Rev1	GCTTACCAGAGTATTCAAAGCCTGAGCAT-TATGGTTCACACGTC
32.	Omi F15Rev2	GATAGCGCGAATTTGAAGAGAGTTGCTT-CACCAGAGTATTCAAA
33.	Omi F16Fwd	CAACTCTCTTCAAAATTCGGCGCTATC
34.	Omi F16.1Fwd	TTGAACGACATCTTTAGTCGCCTTGAT
35.	Omi F16.1Rev	ATCAAGGCGACTAAAGATGTCGTTCAA
36.	AS507	GGGCAACAACAGATGGCT
37.	AS575	gttgggtttatcgccggt
38.	AS645	tccttgctgcctcaagatcca
39.	AS145	GCGAAATTAATACGACTCACTATAGG

only 5–10 colonies at each intermediate stage. Along the way, we were also able to generate constructs containing mutations only in smaller domains in the full-length gene.

A previous report described the LFEAP mutagenesis method which also relied on PCR and ligation-mediated assembly to incorporate multiple mutations [12]. However, this method involved the amplification of the entire plasmid, bringing with it the increased possibility of errors, and T4 DNA ligase-mediated assembly which is usually less efficient than overlap-mediated assembly. Traditional scanning mutagenesis methods involve elegant but technically more complex strategies using specially modified templates [13]. IVA cloning is also a comparable simple

Table 2. A list of fragments generated during the construction of the Omicron Spike clone, the primers and templates used for their generation

Fragment	Primers Used	Input source
1	F1.2 pCDNA+ Omi F1Rev	Wild-type
2	Omi F2Fwd+ Omi F2Rev	Wild-type
3	Omi F3Fwd+ Omi F3Rev	Delta-plus
4	Omi F4Fwd+ Omi F4Rev	Wild-type
5	Omi F5Fwd+ Omi F5Rev	Wild-type
6	Omi F6Fwd+ Omi F6Rev	Wild-type
7	Omi F7Fwd+ Omi F7Rev	Delta-plus
8	Omi F8FwdOE+ Omi F8RevOE	–
9	Omi F9Fwd+ Omi F9Rev	Wild-type
10	Omi F10Fwd+ Omi F10Rev	Delta-plus
11	Omi F11Fwd+ Omi F11Rev	Wild-type
12	Omi F12Fwd+ Omi F12Rev	Wild-type
13	Omi F13Fwd+ Omi F13Rev	Wild-type
14	Omi F14Fwd+ Omi F14Rev	Wild-type
15	Omi F15Fwd+ Omi F15Rev	Wild-type
15'	Omi F15Fwd+ Omi F15Rev2	Fragment 15
16	Omi F16Fwd+ 3.2 pCDNA	Wild-type
17	Omi F5Fwd + R3.2 pCDNA	Wild-type
18	F1.2 pCDNA+ Omi F5Rev	Wild-type
19	Omi F9Fwd + R3.2 pCDNA	Wild-type
20	F1.2 pCDNA+ Omi F9Rev	Wild-type
21	Omi F13Fwd + R3.2 pCDNA	Wild-type
22	F1.2 pCDNA+ Omi F13Rev	Wild-type
23	Omi F2Fwd + Omi F5Rev	Clone 1 plasmid 3
24	Omi F8FwdOE + R3.2 pCDNA	Wild-type
25	Omi F8FwdOE+ Omi F8RevOE	Wild-type
26	Omi F13Fwd + R3.2 pCDNA	Clone 4 plasmid 25
27	Omi F9Fwd + Omi F16.1Rev	Clone 25
28	Omi F16.1Fwd + R3.2 pCDNA	Wild-type
29	F1.2 pCDNA+ Omi F8RevOE	Clone 1.2, plasmid 13
30	F1.2 pCDNA + Omi F4Rev	Clone 1.2, plasmid 13
31	F1.2 pCDNA+ Omi F4Rev	Wild-type
32	Omi F5Fwd+ Omi F8RevOE	Clone 1.2, plasmid 13
33	Omi F10Fwd + R3.2 pCDNA	OmicronFL Clone 30

Table 3. Timeline for the synthesis of the Omicron Spike construct

	Day	Activity
Bench work	1	Strategy finalized
	2	Primers ordered
	3	
	4	
	5	Primers received
	6	Fragment PCRs round 1
	7	OE and Gibson assembly round 1
	8	Col PCR 1
	9	Plasmid prep, sent for sequencing
	10	
	11	
	12	
	13	Sequencing results; Fragment PCRs round 2
	14	Round 2 cloning
	15	
	16	Col PCR 2
	17	Plasmid prep, sent for sequencing
	18	
	19	
	20	Sequencing results
	21	Fragment PCRs round 3
	22	Round 3 cloning
	23	Col PCR 3
	24	
	25	
	26	Plasmid prep, sent for sequencing
	27	
	28	
	29	Sequencing results for final clone

Table 4. Estimated cost of consumables in the study

Item	Approximate cost
Oligonucleotides	INR 33 000 (INR 18–22/base based on scale of synthesis)
Sequencing	INR 6000 (INR 500/reaction)
PCR consumables and kits	INR 6000
Cloning consumables	INR 5000 (INR 300/reaction Gibson mix, media and miscellaneous)

method for cloning that relies on recA-independent assembly in *Escherichia coli* but reports lower efficiencies for multi-fragment assemblies [14]. Both IVA cloning and POE cloning [15] involve amplification of the vector in addition to the insert fragments, which may necessitate whole-plasmid sequencing for verification. A T5 exonuclease-dependent cloning method could reduce costs further; however, efficiency with multi-fragment assemblies remains to be tested in this protocol [16].

Gibson assembly was the single most important experimental method that made the current strategy possible. This method allows for seamless assembly without the requirement for or introduction of any extra or restricted nucleotide sequences. Efficient incorporation of up to five inserts in one assembly, significantly reduces the amount of time required to assemble complex constructs. Combined with the very efficient oligo stitching method reported by Hilgarth et al., technically up to ten fragments may be assembled in one shot.

In this study, we have demonstrated single or multiple amino acid changes at consecutive or spaced locations, insertions and deletions (single or up to three residues). We expect that large deletions (>10 bp) will also be technically feasible. However, introduction of repetitive sequences, or large insertions may be slightly more challenging and may require modification of the described strategy. Despite these caveats, we believe that this strategy will be of great use in resource-constrained settings.

Author contributions

Rita Rani (Conceptualization [equal], Data curation [lead], Formal analysis [lead], Investigation [lead], Methodology [lead], Resources [supporting], Supervision [equal], Validation [supporting], Writing—original draft [supporting], Writing—review and editing [equal]), Kishore V.L. Parsa (Conceptualization [supporting], Funding acquisition [supporting], Methodology [supporting], Project administration [supporting], Resources [equal], Supervision [supporting], Writing—original draft [supporting]), Kiranam Chatti (Conceptualization [supporting], Investigation [supporting], Validation [supporting], Writing—original draft [supporting], Writing—review and editing [supporting]), and Aarti Sevilimedu (Conceptualization [equal], Data curation [supporting], Formal analysis [supporting], Funding acquisition [lead], Investigation [supporting], Methodology [supporting], Project administration [lead], Resources [equal], Supervision [equal], Validation [supporting], Visualization [lead], Writing—original draft [lead], Writing—review and editing [equal]). Rita Rani and Aarti Sevilimedu wrote the manuscript with inputs from Kishore V.L. Parsa and Kiranam Chatti.

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Conflict of interest statement. The authors declare that there is no conflict of interest.

Supplementary data

Supplementary data is available at Biology Methods and Protocols online.

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