An *in vitro* workflow to create and modify novel infectious clones using replication cycle reaction

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Abstract

Reverse genetics systems are a critical tool in combating emerging viruses by enabling a better understanding of the genetic mechanisms by which they cause disease. Traditional cloning approaches are fraught with difficulties due to the bacterial toxicity of many viral sequences. Many attempts have been made to develop a simplified workflow to produce a simple-to-use infectious clone without the concerns of host toxicity; however, none offers the advantages of bacterial cloning without the various disadvantages. This paper demonstrates a novel in vitro workflow that leverages gene synthesis and replication cycle reaction—an innovative technology that reconstitutes the bacterial replication machinery in a tube—to develop a supercoiled infectious clone plasmid that is easy to rescue. Using this workflow, we developed two infectious clones, one of a low passage dengue virus serotype 2 isolate (PUO-218) and the Washington strain of SARS-CoV-2, which behaved similarly to their respective parental viruses. Furthermore, we generated a medically relevant mutant of SARS-CoV-2, Spike D614G. These results indicate that this novel in vitro workflow is a viable method to generate and manipulate infectious clones, specifically for viruses that are notoriously difficult for traditional bacterial-based cloning methods. This work represents a paradigm shift in producing infectious clones and lowers the barrier of entry for scientists to develop such tools.

1. Introduction

The development of reverse genetics systems for emerging viruses is critical in responding to global health threats as it enables studies to dissect the mechanisms by which these viruses cause disease [1]. The infectious cDNA clone allows researchers to explore many different aspects of virology, including viral kinetics [2, 3], evolution [4, 5], tropism [6–8], and vaccinology [9–12]. The traditional approach to produce infectious clones involves generating fragments from viral RNA by RT-PCR and then cloning these fragments into bacterial plasmids [13]. However, traditional cloning approaches can be difficult due to viral genomic instability in living hosts, which often arises when the genomes of flaviviruses [14], alphaviruses [15], and coronaviruses [16] are introduced into bacteria. The proposed mechanism for this instability is cryptic bacterial promoters in the viral genome, driving the production of toxic viral genes [14, 17–23]. In response to the plasmids’ toxicity, a selective pressure is imposed on the bacteria to propagate plasmids containing viral genomes with deletions, mutations, and recombination events that mitigate the toxicity [17–19], making mechanistic studies extremely challenging.

Several approaches have been published to mitigate the effects of host-specific toxicity on infectious clone stability. One approach involves maintaining the viral genome across several plasmids, combining the genomic fragments in vitro, and then using that product for direct transfection for CMV-based clones or in vitro transcription followed by transfection [8, 16, 18, 24–28]. This approach is effective but often requires maintaining up to 7 plasmids, making these systems cumbersome. Several groups have developed reverse genetics systems in which the products of circular polymerase extension cloning/reaction (CPEC/CPER), Gibson assembly, or infectious subgenomic amplicons (ISAs) are directly transfected into permissive cells [1, 29–34]. While these approaches remove the need for bacteria
because the assembly occurs molecularly or within permissive eukaryotic cells, the full-length cDNA clone cannot be re-used for manipulation, like generating mutant viruses, or easily shared.

Other attempts have been made to reduce the toxicity of the full-length clones in bacteria. One approach was to insert introns into toxic transcripts to disrupt the open reading frame (ORF) [20–22, 35–37]. Another approach is introducing synonymous mutations into sequences believed to contain cryptic bacterial promoters to prevent transcription by bacterial polymerases [14]. Others involved altering the plasmid architecture, like using low copy number bacterial artificial chromosomes [38–42], heavily repressed promoters [43, 44], or using yeast-based systems [45–49]. Nonetheless, these systems still struggle to remove the toxicity of viral genomes [1].

In summary, there have been many attempts to develop a workflow that can ameliorate the inherent toxicity of the viral genomes in bacteria while also producing a recoverable full-length cDNA clone that is straightforward to work with; unfortunately, these attempts have only modest success. However, an alternative and emerging idea is to shift from ameliorating the host issue to removing the host from the system entirely.

Having previously demonstrated the ability to rescue and manipulate infectious clones using rolling circle amplification (RCA), an \textit{in vitro} process [2, 13, 50, 51], we set out to use an emerging technology called replication-cycle reaction (RCR) [52–55] to develop a pipeline to produce novel infectious clones. The RCR system works by reconstituting 14 proteins and 25 polypeptides critical for chromosomal replication in \textit{E. coli} bacteria [52]. This process produces supercoiled DNA and acts with high fidelity, with an error rate of \( \sim 10^{-8} \) per base per replication cycle [52]. This approach removes the need for a living host and the risk of selection due to sequence-specific toxicity [14–16]. Leveraging RCR, we developed a novel workflow to generate and manipulate viral infectious clones (Fig. 1). Briefly, this process uses the high efficiency and rapidly decreasing costs of chemical gene synthesis [16, 24, 45], high fidelity PCR, \textit{in vitro} assembly, and \textit{in vitro} amplification. The resulting clonal supercoiled plasmid population can then be rescued and manipulated identically to a bacterial-derived plasmid.

In this paper, we demonstrate that the above-described workflow could be used to generate infectious clones of medically relevant viruses. For this work, we selected dengue virus serotype 2 (DENV), strain PUO-218, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), strain USA-WA1/2020. Based on sequencing and viral kinetics, we concluded that our innovative workflow could effectively generate new infectious clones. Furthermore, we demonstrate that the infectious clones, specifically the SARS-CoV-2 clone, can be used as a template to generate medically relevant mutant viruses. This workflow represents a paradigm shift in developing and manipulating infectious clones for emerging pathogens.

2. Materials And Methods

2.1 Cells and viruses
Vero (CCL-81), BHK-21 clone 13 (CCL-10), and Calu-3 (HTB-55) cells were obtained from American Type Culture Collection Vero E6 cells expressing transmembrane protease, serine 2, and human angiotensin-converting enzyme 2 (VeroE6 hACE2-TMPRSS2) cells were obtained from BEI (Catalog No. NR-54970). HEK293A cells were kindly provided by Dr. Jamie Smyth from the Fralin Biomedical Research Institute. Vero and BHK-21 clone 13 cells were cultured at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% nonessential amino acids, and 0.1% gentamicin. For VeroE6 hACE2-TMPRSS2 cells, the media described above was supplemented with puromycin to a 0.01 mg/mL final concentration. Dengue virus serotype 2 (DENV2) strain PUO-218 (GenBank: ON398847) was obtained from the Center for Disease Control and Prevention (Lot: TC00838). SARS-CoV-2 USA-WA1/2020 strain (GenBank: MN985325.1) was acquired from BEI resources (NR-52281; Lot: 70034262). Before testing, both parental viruses were passaged once in Vero (DENV2) and VeroE6 hACE2-TMPRSS2 (SARS-CoV-2).

2.2 Construction of novel infectious clones

The infectious clone was designed in silico using SnapGene 6.0.2 software (GSL Biotech). When designing the infectious clones, one (DENV2) or two (SARS-CoV-2) restriction enzyme sites were ablated by synonymous mutation to allow downstream differentiation between the parental and clone-derived virus [56, 57]. Fragments with 40 bp overlaps were designed for clonal gene synthesis either by Twist Bioscience (DENV2) or Bio Basic Inc. (SARS-CoV-2). These plasmids were then used as templates for PCRs using the SuperFi II Master Mix (Catalog number: 12368010). The bands were then extracted and purified using the Machary-Nagel NucleoSpin Gel and PCR Clean-up kit (Item Number 740609). Fragments were quantified by Qubit prior to assembly and then mixed in equimolar concentrations to a final concentration of 8 ng/µL, which was then assembled using the OriCiro 2x Recombination Assembly (RA) mix and incubated for 30 minutes at 42°C, followed by a 65°C step for 2 minutes to eliminate misassembled products. The amplification mixture was prepared according to the kit's protocol during the assembly step. The amplification mixture was primed by incubating at 33°C for 15 minutes. After priming, the assembly reaction was mixed into the amplification mix and incubated for 6 hours at 33°C and then held at 12°C. The next day, the product was supercoiled by diluting the product two-fold in 1x Amplification Buffer and incubating at 33°C for 30 minutes. The supercoiled product was then tested by restriction digestion to confirm proper assembly. The remaining supercoiled product was diluted to a final concentration of 20 mM EDTA.

2.3 Rescue of infectious clones

For the DENV2 infectious clone, viral rescue was performed similarly as previously described [13]. Briefly, we used the EquiPhi29™ DNA Polymerase (Catalog number: A39390) to amplify the RCR product by RCA and then used HEK293A cells for transfection. The virus, representing the passage zero (p0) stock, was harvested seven days post-transfection and titered by plaque assay on Vero cells [13]. The p0 stock was then used to passage the virus once in Vero cells at an MOI of .01 to produce the p1 stock, which was used for downstream testing. For the SARS-CoV-2 infectious clone, supercoiled plasmid obtained by RCR was co-transfected with pUC19 [58] into BHK-21 clone 13 cells. The supernatant was collected two- and
three days post-transfection, pooled, and used to blindly infect VeroE6 hACE2-TMPRSS2 [45] to produce a p1 stock used for downstream testing.

### 2.4 Library Preparation and NGS Sequencing

For the DENV2 infectious clone, libraries were prepared from the RCA product using the sparQ DNA Frag & Library Prep Kit from Quantabio (Cat. 95194-024). After assessing the library size by Tapestation, the samples were sequenced using 150 bp paired-end reads on the Illumina Novaseq 6000. To analyze the resulting data, a previously described workflow was used [5]. Briefly, paired-end reads were trimmed to a quality score of 30 using BBduk [59] before being mapped to the parental sequence using the Burrows-Wheeler aligner (BWA) [60], and variants were identified using LoFreq [61]. The consensus sequence of the clone was then produced using Genome Analysis Toolkit (GATK) [62] and then aligned to the in silico to confirm the correct sequence.

For SARS-CoV-2, clone-derived viral RNA was sequenced using the plexWell ARTIC protocol V3 and the plexWell 384 Library Prep Kit protocol. Briefly, cDNA was prepared from RNA samples using SuperScript IV Reverse Transcriptase (Invitrogen, Waltham, MA). PCR amplification was performed using Q5 Polymerase (NEB, Ipswitch, MA) and primers from ARTIC nCoV-2019 Amplicon Panel, V4.1 (Integrated DNA Technologies, Coralville, IA). Amplicons were quantitatively assessed using a Qubit 2.0 fluorometer (Thermo/Fisher) and then barcoded and pooled using the plexWell™ 384 Library Preparation Kit (seqWell, Beverly, MA). Pooled libraries were amplified using a Kappa HiFi Hot Start Ready Mix kit (Roche, CA). Libraries were sequenced using the 300-cycle Miseq Sequencing Kit (Illumina, San Diego, CA).

Bioinformatic analysis was performed identically to the DENV samples. To determine if the minority variants in the viral stocks were present in the plasmid stocks, the NSP3 region of the plasmid was amplified by PCR and purified. Samples were submitted for Sanger sequencing to the Virginia Tech Genomic Sequencing Center. Sanger sequencing reads were aligned to the reference sequence using SnapGene.

### 2.5 Comparing viral kinetics of clone-derived and parental viruses

Vero (DENV2) or VeroE6 hACE2-TMPRSS2 (SARS-CoV-2) cells were plated to an 80–90% confluency in 24-well plates. Viral stocks were diluted in Roswell Park Memorial Institute medium (RPMI 1640) with 25 mM HEPES and 1% FBS. Cells were infected at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU) per cell and incubated at 37°C for one hour. After the adsorption period, the cells were washed in phosphate-buffered saline (PBS), and fresh media was added. The supernatant was harvested every 24 hours until 50–75% CPE was observed. Harvested samples were stored at -80°C until they were tested by plaque assay. Viral RNA was used to generate cDNA for both viruses using the Maxima H-RT kit (Catalog number: EP0752). The resulting cDNA was used to amplify the region of the genome containing the ablated restriction site. The amplicons were then digested with either EcoRI (DENV2) or HindIII (SARS-CoV-2) and tested by gel electrophoresis to observe the banding differences between parental- and clone-derived viruses.
2.6 Generating mutant viruses and comparing fitness by competition assay.

To generate the mutant SARS-CoV-2 Spike D614G virus, mutagenic primers were designed bearing the desired point mutation. The SARS-CoV-2 infectious clone served as a template for PCRs and was serially diluted to the lowest concentration that still resulted in amplification. The mutants were then constructed and rescued identically to the infectious clones via a bacteria-free cloning strategy [2, 13, 50, 51]. To confirm that the new viral stocks contained the appropriate mutation, the viral stocks were first confirmed by Sanger sequencing, followed by whole genome next generation sequencing (NGS) as described above. After confirmation, the mutant virus was mixed with the wild-type virus at a 1:1 PFU ratio. The virus mix was then used to infect VeroE6 hACE2-TMPRSS2 cells at an MOI of 0.01. Viral RNA was extracted from the inoculum and viral supernatant at 1 dpi, which was used to generate cDNA using the Maxima H- RT kit. The resulting cDNA was used to amplify the region of the genome containing the mutation of interest. The amplicon was then submitted for Sanger sequencing, and the relative ratios of wild-type and mutant virus were determined using EditR [63]. Relative fitness of the mutant compared to the wild-type virus was defined as \( W = \frac{F(t)}{F(0)} \), where \( F(x) \) represents the ratio of mutant virus (either at baseline or 1 dpi) [64].

2.7 Statistical Analysis

Statistical analysis was performed in Prism 8 (GraphPad, San Diego, CA, USA). A 2-way ANOVA test was performed with a Šidák correction for multiple comparisons for the viral kinetics assays. A one-sample t-test was used for the competition assays after a Shapiro-Wilk test confirmed normality.

3. Results

3.1. Generating a Medically Relevant DENV2 Infectious Clone

DENV represents one of the most significant global health threats, infecting 400 million people yearly (roughly 5% of the global population) [65]. Unfortunately, the high concentration of cryptic bacterial promoters within the DENV genome makes the development of infectious clones extremely challenging [17, 23]. Here, we sought to develop a new, recoverable, full-length DENV serotype 2 infectious clone using an \textit{in vitro} approach, which has yet to be done. We selected DENV2 strain PUO-218, a low passage virus isolated in Thailand in 1980 [66] that is also a component of Sanofi's Dengvaxia vaccine [67–69]. To generate the clone, the viral genome was synthesized in four clonal fragments (Fig. 2). We created a synonymous mutation that ablated an EcoRI site within the NS3 sequence to allow for differentiation between the clone and the parental virus. The four synthesized fragments and a donor plasmid containing the CMV promoter, hepatitis delta virus ribozyme (HDVr) sequence, and the OriC sequence were used as templates for high fidelity PCR. The CMV promoter allows for direct recovery of infectious virus through cellular RNA polymerase II while the HDVr removes extra nucleotides at the 3' end of the
3.2. Comparison of DENV2 Infectious Clone to the Parental Virus

We compared the growth of the parental DENV2 PUO-218 isolate to the infectious clone-derived virus in Vero cells to ensure similar growth kinetics. Vero cells were selected as they are highly susceptible to DENV [70]. The cells were infected at an MOI of 0.01 PFU/cell, allowing for multiple rounds of replication. Supernatants from the infected cells were collected until 50–75% of cells showed cytopathic effect (CPE). We found no significant differences in viral titer between the parental and the clone on any day (Fig. 3A). To confirm the identity of each virus, we performed RT-PCR on the viral stocks and digested the amplicons with EcoRI. We expected two bands at 400 bp and 732 bp for the parental virus and only a single band in the clone-derived virus at roughly 1.1 kb, which we observed (Fig. 3B). Finally, next-generation sequencing revealed no variants above consensus level within the clone, indicating no unwanted mutations were generated during the cloning or viral rescue process (data not shown). These data indicate that the DENV2 clone's sequence and behavior match the parental virus.

3.4. Generating a SARS-CoV-2 Infectious Clone

From its emergence in late 2019 to June 2022, SARS-CoV-2 has infected over 500 million people, and at least 6 million have died due to the virus or associated conditions [71]. During the early stages of the pandemic, there was a significant push to develop an infectious clone of SARS-CoV-2. However, the difficulty of manipulating the SARS-CoV-2 genome in bacteria—and coronaviruses as a whole [16, 24]—led to a near six-month lag between the virus's first identification [72] and the first clone being reported [57], which limited our ability to understand the emergence of SARS-CoV-2 and to develop countermeasures. Towards generating a workflow to develop infectious clones more quickly, we sought to produce an infectious clone of an early isolate of SARS-CoV-2, USA-WA1/2020 strain. The SARS-CoV-2 genome was synthesized as four clonal fragments (Fig. 4). Two HindIII sites within Orf1a and the Spike protein were ablated during the design phase to distinguish between parental- and clone-derived viruses. As with the DENV2 clone, the clonal fragments and a donor plasmid were used as templates for PCR, and those amplicons were used in an RCR to produce a supercoiled infectious clone plasmid.

3.5. Comparison of SARS-CoV-2 Clone-derived virus to the Parental Virus

We tested the growth of the parental and clone-derived virus in VeroE6 hACE2-TMPRSS2 since they are highly susceptible to SARS-CoV-2 [73]. When quantifying the viral kinetics by plaque assay (Fig. 5A), we
found the clone-derived virus was attenuated on day 1 ($P = .0005$) and day 2 ($P = .003$). RT-PCR confirmation was performed on the stocks, and the amplicons were digested with HindIII. We expected to see a band at 1.1kb (representing two overlapping bands of equal size) for the parental virus and 2.2 kb for the clonal virus, which we observed (Fig. 5B).

Next-generation sequencing was first performed on the viral RNA to compare the clone-derived virus sequence to the parental virus. The consensus sequences of both viruses were identical. A minority variant at genome position 13,169 was identified in the clone-derived virus and was detected at a percentage of 45.9%, resulting in a mutation at NSP3-P125Q. We also sequenced the infectious clone plasmid and could not detect the mutation, suggesting that this mutation was generated during the viral rescue process. These data indicate that the clone sequence matches the parental isolate.

### 3.5. Generation and characterization of medically relevant early pandemic mutant using site-directed mutagenesis

Having demonstrated that the *in vitro* clone development workflow produces a full-length, recoverable product, the next critical step was demonstrating that the new clones could serve as templates for further manipulation. One of the advantages of using infectious clones is that they allow connecting viral genotypes and phenotypes through the generation of viral mutants [2–8]. To that end, using the SARS-CoV-2 infectious clone, we engineered a virus bearing a medically significant mutation, Spike D614G, hereafter called D614G, an early variant of SARS-CoV-2 [74]. D614G quickly became the dominant genotype globally [74–77] and has been shown to have increased infectivity [78, 79] and fitness [80, 81] when compared to earlier genotypes.

The mutant virus was generated by site-directed mutagenesis and assembled similarly to the parental infectious clone. Specifically, the mutagenic primers were designed to replace the A with a G at position 23,403 and the T with a C at position 23,404, which resulted in the D614G mutation. After confirming that the mutant virus bore the mutation and titration, the mutant was prepared for a competition assay (Fig. 6).

Briefly, the mutant was mixed at a 1:1 PFU ratio with the wild-type SARS-CoV-2 infectious clone-derived virus. These mixes were then used to infect VeroE6 hACE2-TMPRSS2, using the same methods as the infectious clone validation. Viral RNA was extracted from the inoculum and the viral supernatant at 1 dpi and was used for reverse transcription. The viral cDNA was amplified by PCR to capture the regions of the genome bearing the mutations. The amplicons were submitted for Sanger sequencing, and relative fitness was assessed by comparing the starting and ending proportions for each virus (Fig. 7). The D614G mutant generated using the *in vitro* workflow has significantly increased fitness ($P = .0005$), which aligns with previous findings regarding this mutation [80, 81]. These data demonstrate that the *in vitro* workflow can effectively be used to engineer mutations into infectious clones.

### 4. Discussion


Infectious clones enable mechanistic studies to understand the impacts of mutations and studies to generate vaccines and diagnostic tools. However, the currently available systems struggle to mitigate host toxicity, making generating infectious clones for many viruses extremely cumbersome [14, 17–22, 37–48]. To address these issues, we leveraged several emerging in vitro technologies, including gene synthesis and RCR, to develop a novel workflow to develop infectious clones (Fig. 1). Using this system, we successfully produced infectious clones for DENV2 and SARS-CoV-2, two viruses of great medical health importance.

An important consideration when producing an infectious clone is that clone-derived and parental viruses replicate similarly and have the same amino acid sequence. For the DENV clone, the parental- and clone-derived sequence and growth kinetics were identical. For SARS-CoV-2, the sequence of the infectious clone plasmid matched the parental virus; however, we detected a significant minority variant in the viral stock (nsp3 P125Q). Since the mutation was not detected in the infectious clone plasmid, we hypothesize that the mutation is a byproduct of the low-efficiency transfection of the clone, a known problem with large plasmid transfections, which caused a bottleneck, resulting in changes in the variant frequencies in the stock [58, 82, 83]. We view this as a limitation of the transfection system we use and not the cloning workflow. Electroporation, which has been shown to have higher efficiency for large plasmids [58, 84], represents a viable alternative to the current transfection approach used in this paper and will be explored in the future for rescuing large infectious clones. The difference in kinetics that we observe with the SARS-CoV-2 infectious clone could be due to the mutation mentioned above; however, a reduction in fitness has also been shown in several other clones with correct consensus sequences, though the cause has not been experimentally defined [57, 85]. We further demonstrated that the infectious clone could be easily manipulated, as we were able to generate a medically relevant mutant variant of SARS-CoV-2, Spike D614G [74–81], and characterize it using a competition assay. In its totality, this report represents a significant shift in developing infectious clones. The ease of access to the technologies that underpin this workflow provides ample ability for other researchers to use this system, thus lowering the barrier of entry for researchers to develop and manipulate infectious clones for emerging viruses.

Limitations of this work include our use of only two viruses from two viral families: Flaviviridae and Coronaviridae. However, these families represent the most challenging positive-sense RNA viruses to manipulate in bacteria; thus, we are confident that the workflow could be easily transferred to other positive-sense RNA viruses. Future studies involving negative-sense RNA viruses, multipartite viruses, and DNA viruses should be performed to expand the usage of this technology. Another limitation is the genome size that was explored in this report. While coronaviruses have the largest RNA viral genomes [86, 87], they are by no means the largest viral genomes, particularly when compared to the giant aquatic viruses, like Megavirus [88] and Mimivirus [89]. Given that the largest reported genome amplified with the OriC system was 1 Mb [54], slightly below the genome size of some large aquatic viruses [88, 89], further characterizations may be needed for these giant viruses.

**Declarations**

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Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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Conflicts of Interest: J.M.M has received financial support from OriCiro in travel reimbursement to present the above work. The other authors have no conflicts to report.

References


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Figures
**Figure 1**

**Novel in vitro infectious clone generation workflow.** To produce a new clone, chemical gene synthesis is performed, followed by PCR, Gibson assembly, and replication cycle reaction (RCR) to produce a supercoiled infectious clone. Infectious virus is then recovered by transfection into susceptible cells. The clone and/or viral stock is then assessed by deep sequencing and growth kinetics to ensure matching sequences and behaviors with the parental virus.

**Figure 2**

**Schematic for Producing DENV2 Infectious Clone.** To produce the DENV2 PU0-218 infectious clone, the viral genome was synthesized in four clonal fragments. The four resulting plasmids and a donor plasmid containing the necessary components for expression and replication were used as templates for PCRs. The amplicons from these reactions were then assembled and amplified by RCR to produce a supercoiled infectious clone.
**Figure 3**

**Comparison of DENV2 Infectious Clone to the Parental Virus.** (A) Growth curve of DENV2 PUO-218 isolate and infectious clone in Vero cells. Data represent two biological replicates, each containing three technical replicates, and the error bars represent the standard deviation. No significant difference was detected at any time by a 2-way ANOVA with a Šidák correction for multiple comparisons. (B) A representative gel image of an EcoRI digest of the genetically marked region of the infectious clone. The 100bp DNA ladder from NEB (#N3231) was used for reference, with the first sample lane containing the viral isolate and the second containing the clone.
Figure 4

**Schematic for Producing SARS-CoV-2 Infectious Clone.** To produce the SARS-CoV-2 USA-WA1/2020 infectious clone, the viral genome was synthesized in four clonal fragments. The four resulting plasmids, along with a donor plasmid that contained the necessary components for expression and replication, were used as templates for PCRs. The amplicons from these reactions were then assembled and amplified by RCR to produce a supercoiled infectious clone.
Figure 5

Comparison of SARS-CoV-2 Infectious Clone to the Parental Virus. (A) Growth curve of SARS-CoV-2 USA-WA1/2020 strain isolate and the infectious clone in VeroE6 hACE2-TMPRSS2 cells. Data represent two biological replicates, each consisting of three technical replicates. Statistical analysis was performed using a 2-way ANOVA test with a Šidák correction for multiple comparisons (** P = .003 *** P = .0005). (B) A representative gel image of a HindIII digest of the genetically marked region of the infectious clone. The 1kb Plus DNA Ladder from NEB (#N3200) was used for reference, with the first sample lane containing the viral isolate and the second containing the clone.
Figure 6

SARS-CoV-2 Mutant Competition Assay Workflow. To assess the fitness of Spike D614G, wild-type (WT) and D614G were mixed at a 1:1 PFU ratio. After confirming the mix composition by Sanger sequencing and plaque assay, the mix was used to infect VeroE6 hACE2-TMPRSS2 cells. RNA was extracted from viral supernatant at 1 dpi and used to synthesize cDNA for downstream
Figure 7

Comparing Fitness of Early Pandemic SARS-COV-2 Mutants Using a Novel Infectious Clone. Competition assays using SARS-CoV-2 USA-WA1/2020 wild-type infectious clone and mutants bearing Spike-D614G in VeroE6 hACE2-TMPRSS2 cells. Data represent two biological replicates, each consisting of three technical replicates, and the error bars represent the standard deviation from the mean. Statistical analysis was performed using a one-sample t-test with a null value of 1 (*** P = .0005).