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REVIEW

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# Reverse genetics systems for SARS-CoV-2

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## Abstract

The ongoing pandemic of coronavirus disease 2019 (COVID-19) has caused severe public health crises and heavy economic losses. Limited knowledge about this deadly virus impairs our capacity to set up a toolkit against it. Thus, more studies on severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) biology are urgently needed. Reverse genetics systems, including viral infectious clones and replicons, are powerful platforms for viral research projects, spanning many aspects such as the rescues of wild-type or mutant viral particles, the investigation of viral replication mechanism, the characterization of viral protein functions, and the studies on viral pathogenesis and antiviral drug development. The operations on viral infectious clones are strictly limited in the Biosafety Level 3 (BSL3) facilities, which are insufficient, especially during the pandemic. In contrast, the operation on the noninfectious replicon can be performed in Biosafety Level 2 (BSL2) facilities, which are widely available. After the outbreak of COVID-19, many reverse genetics systems for SARS-CoV-2, including infectious clones and replicons are developed and given plenty of options for researchers to pick up according to the requirement of their research works. In this review, we summarize the available reverse genetics systems for SARS-CoV-2, by highlighting the features of these systems, and provide a quick guide for researchers, especially those without ample experience in operating viral reverse genetics systems.

**KEYWORDS**

BAC, CPER, reverse genetics systems, SARS-CoV-2, TAR

## 1 | INTRODUCTION

The global pandemic of coronavirus disease 2019 (COVID-19) has caused more than 400 million confirmed cases and more than 5.7 million deaths as of February 11, 2022 (https://covid19.who.int/). With a rapidly growing number of infections and mortality, COVID-19 has already been among the most severe documented pandemics in human history.[1] SARS-CoV-2 was identified as the causative pathogen of COVID-19, which is highly transmissible and pathogenic.[2–4] Similar to the patients with severe acute respiratory syndrome (SARS) in 2002/2003 and Middle East respiratory syndrome (MERS) in 2012, the patients with COVID-19 usually present symptoms of viral pneumonia, such as fever, sore throat, chest and muscle pain, cough, and dyspnea.[5,6] The effective strategies for clinical therapy are still limited, and more research projects on this deadly virus are urgently needed.[7]

SARS-CoV-2 belongs to the *Betacoronavirus* genus of *Coronaviridae* family, which also includes SARS-CoV and MERS-CoV.[3,6] The viral particle of SARS-CoV-2 is composed of host-cell derived membrane bearing spike (S) protein, envelope (E) protein, and membrane (M) protein, and a linear positive-sense single-stranded RNA (+ssRNA) wrapped with nucleocapsid (N) protein.[3,6] The adhesion of virion to a cell depends on Brownian motion, diffusion, static electricity.[8,9]

Wenhao Wang and Xiaoxue Peng contributed equally to this study.

  

After binding to receptors, such as the human angiotensin-converting enzyme 2 (ACE2), the S glycoprotein of SARS-CoV-2 undergoes proteolysis by transmembrane protease serine 2 (TMPRSS2) and other proteases, triggering the conformational changes of S2 subunit and the fusion of viral and cellular membranes.[10] Once entering into the permissive cell, the coronavirus releases its genomic RNA from nucleocapsid structure, ready for translation and following transcription/replication.[11,12]

The 30-kb genomic RNA of SARS-CoV-2 comprises more than nine open-reading frames (ORFs) and is flanked by two untranslated regions (UTRs) at 5′ and 3′ terminus, respectively. Similar to the host messenger RNA (mRNA), the viral genomic RNA possesses a 5′ cap and a 3′ ploy (A) tail structure. The viral genomic RNA uses its ORF1a and ORF1ab to express polyproteins 1a and 1ab (pp1a and pp1ab). The latter's expression requires a −1 ribosomal frameshift process. The polyproteins are processed into 16 nonstructural proteins (nsps) with their own papain-like proteinase activity in nsp3 and 3C-like proteinase activity in nsp5.[2,13,14] Although many details are still missing, it is well recognized that the many nsps are assembled into a replication/transcription complex (RTC) to synthesize the viral negative- or positive-sense genomic and subgenomic RNAs.[16,17]

Through the mechanism of discontinuous transcription/replication, the viral RTC generated the viral subgenomic RNAs.[3,18] Nine transcriptional regulatory sequences (TRSs), one in 5′ UTR and eight in the body sequence of 3′ terminal part of the genomic RNA, play essential roles in the discontinuous replication of viral genomic RNA, by forming the junctures between the sequence before the TRS in 5′ UTR (named leader sequence), and the sequence after the TRSs in 3′ terminal art of genomic RNA (named body sequences).[12,14,18] Using the subgenomic RNAs, the ORFs in 3′ terminus of genomic RNA encode four well-known structural proteins, S, E, M, and N, and a set of accessory proteins, whose functions still await further investigation.[15]

The coronaviruses adopt a delicate and complex replication mechanism, in which a number of viral component proteins are involved. However, this mechanism also makes the virus vulnerable to the inhibitory effect caused by drugs or other factors targeting the component proteins or the association between them.[19] The inhibitory effect of the drugs can be verified with the model of virus-infected cells, which is strictly required to be carried out in BSL3 facilities. The limited availability of BSL3 facility, especially in ongoing pandemics, becomes an obstacle to live virus-associated research.[20] Furthermore, the model of virus-infected cells is not an ideal system for investigating the mechanistic details, which are more conveniently and feasibly investigated using the biosafe replicon, modified from the infectious reverse genetics system.[14]

The reverse genetic systems for emerging coronaviruses, including SARS-CoV-2, SARS-CoV, MERS-CoV, and many coronaviruses, have been developed through various strategies, which can be categorized into RNA- and cDNA-recombination based strategies. In this report, we review and discuss various strategies for the construction of reverse genetics systems, especially those for SARS-CoV-2 (Table 1), and share the experience in the operation of the reverse genetics systems.

## 2 | REVERSE GENETICS SYSTEMS BASED ON RNA RECOMBINATION

The first reverse genetics system for coronavirus was successfully constructed with a targeted RNA recombination strategy.[21–23] Homologous RNA recombination is common in regions with high sequence similarity during viral RNA replication.[24] Lai et al. first reported that RNA recombination mediates the fusion of RNA of different mouse hepatitis virus (MHV) strains during genome replication.[25] Moreover, targeted RNA recombination is applied in introducing site-specific mutations into the genome of coronavirus by using defective interfering (DI) RNA as the donor.[26,27]

A host range-based positive selection strategy was developed to expand the application of targeted RNA recombination.[28] In this system, a mutant MHV was created, in which the ectodomain of the S protein was replaced with its counterpart of other coronaviruses, leading to an expanded range of hosts (Figure 1).[28] Besides MHV, the targeted RNA recombination strategy has been employed to construct reverse genetics systems of other coronaviruses, including feline infectious peritonitis virus (FIPV) and porcine epidemic diarrhea virus (PEDV).[29,30]

Targeted RNA recombination is very useful for studying the approximately 9 kb sequence at 3′ terminus of the coronaviral genome. However, this strategy becomes incompetent to manipulate the replicase gene sequence upstream of the S gene. The first reason is the large size of the replicase gene (more than 20 kb). The second more important reason is the strategy's dependence on viral passage, which is mediated by the viral replicase gene. The viral passage could be impaired when the viral replicase gene is manipulated by targeted RNA recombination.[31] Thus, targeted RNA recombination is ineffective in dealing with the questions about the viral replication/transcription mechanism, which is usually more feasibly investigated with the viral cDNA recombination-based strategies.

## 3 | REVERSE GENETICS SYSTEMS BASED ON VIRAL cDNA RECOMBINATION

The feasibility of constructing a full-length cDNA clone of coronavirus, the largest known RNA virus, was questioned until Enjuanes et al. and Baric et al. successfully constructed the viral full-length cDNA clones of porcine transmissible gastroenteritis coronavirus (TGEV) using bacterial artificial chromosome (BAC) vector and the in vitro cDNA ligation, respectively.[32,33] Later, the viral full-length cDNA was successfully cloned using a vaccinia virus vector,[34] the yeast-based recombination system,[35] and the circular polymerase extension reaction strategy (CPER).[36,37] Among these five known strategies, BAC and the in vitro ligation are mostly adopted for the construction of reverse genetics systems of coronaviruses.

**TABLE 1** Summary of reverse genetics systems of SARS-CoV-2

| System | Strain | Vector | Promoter | Number of fragments | Fragment generation | Reporter genes | Genetic identity | Tested cell lines | Testedanimal models | Applicable to replicon | Sensitivity to antiviral agents | Refs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BAC | USA-WA1/2020 | pBeloBAC11 | CMV | 5 | Synthesized | / | Full-length | Vero E6 | Golden Syrian hamsters | No | / | [21] |
| | Wuhan-Hu-1 | pSMART-BAC | CMV | 6 | PCR | Nanoluciferase (NLuc) | Full-length | Vero E6 | / | Yes | Remdesivir | [22] |
| | Hu/DP/Kng/19-020 | pSMART-BAC | CMV | 8 | PCR | Renilla luciferase (RLuc) | ORF1a and ORF1b | HEK 293T, Huh-7, VeroE6 | / | Yes | Remdesivir, IFN-β, EIDD-2801 | [23] |
| | Wuhan-Hu-1 | pSMART-BAC | T7 | 5 | Synthesized | Firefly luciferase (FLuc), GFP | Deletion of S, E, M genes | HEK 293T, A549, Calu-1, Huh-7.5 | / | Yes | GS-441524, GC376 | [24] |
| | nCoV-SH01 | pSMART-BAC | T7 | 20 | PCR | secreted *Gaussia* luciferase (sGLuc) | Deletion of S, E, M genes | Huh-7, Huh-7.5, BHK-21, Vero E6 | / | Yes | Remdesivir, IFN-α | [25] |
| | Wuhan-Hu-1 | pCC1-4K | CMV | 5 | Synthesized | mCherry, ZsGreen, NLuc | Full-length | Vero E6, A549 | / | No | / | [26] |
| | Wuhan-Hu-1 | pBeloBAC11 | CMV | 7 | Synthesized | mNeonGreen, FLuc | Deletion of S gene | A549, DLD-1, Huh-7, SY-SH5H, HEK 293 T | / | Yes | Remdesivir, Ritonavir, Lopinavir, Carmofur | [15] |
| In vitro ligation | nCoV-SH01 | / | T7 | 4 | Plasmid | sGLuc | Deletion of S, E, M genes | Huh-7 | / | Yes | / | [25] |
| | USA-WA1/2020 | / | T7 | 7 | RT-PCR or synthesized | mNeonGreen | Full-length | Vero E6 | / | No | IFN-α | [27] |
| | USA-WA1/2020 | / | T7 | 7 | RT-PCR | GFP, GFP-fused NLuc | Full-length | Vero E6 | / | No | / | [28] |
| | 2019-nCoV-WIV04 | / | T7 | 11 | Synthesized | RLuc | Deletion of genes in 21593-28213 nt | BHK-21 | / | Yes | Remdesivir, Brusatol, Dehydrodiisoeugenol, Bruceine A | [29] |
| | Wuhan-Hu-1 | / | T7 | 5 | RT-PCR | GFP | Deletion of N gene | Caco-2 | / | No | IFN-β, Remdesivir, Ritonavir, Lopinavir, GC376 | [30] |

(Continues)

**TABLE 1** (Continued)

| System | Strain | Vector | Promoter | Number of fragments | Fragment generation | Reporter genes | Genetic identity | Tested cell lines | Testedanimal models | Applicable to replicon | Sensitivity to antiviral agents | Refs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yast-based TAR cloning | BetaCoV/Germany/BavPat1/2020 | pVC604, pCC1BAC-His | T7 | 12/14/19 | RT-PCR or synthesized | -/GFP/GFP | Full-length | Vero E6 | / | No | Remdesivir | 31 |
| | MN908947 | pYES1L | CMV | 9 | PCR | tGFP-BlaR, FLuc | Deletion of nsp1 | HEK 293T | / | Yes | Remdesivir, E64-D | 32 |
| | USA-WA1/2020 | pCC1-BAC-His3 | T7 | 13 | PCR | mNeonGreen, GLuc | Deletion of S gene | BHK-21, Huh-7.5 | / | Yes | Remdesivir, AM580 | 33 |
| CPER | SARS-CoV-2/Hu/DP/Kng/19-020 | / | CMV | 10 | PCR | sfGFP, NLuc binary technology (NanoBiT) | Full-length | BHK-21, HEK 293T, Vero E6 | / | No | / | 34 |
| | QLD02 | / | CMV | 6 | PCR | ZsGreen | Full-length | Vero E6 | / | No | / | 35 |
| Others | Wuhan-Hu-1 | / | CMV | 3 | Synthesized | FLuc | 3 Fragments derived from ORF1ab | HEK 293T | / | Yes | Remdesivir, Ritonavir, Lopinavir, Emetine, Disulfiram, Masitinib, Alectinib, Rociletinib, Mitoxantrone, Adriamycin | 36 |

Abbreviations: CMV, cytomegalovirus; RT-PCR, reverse-transcription polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

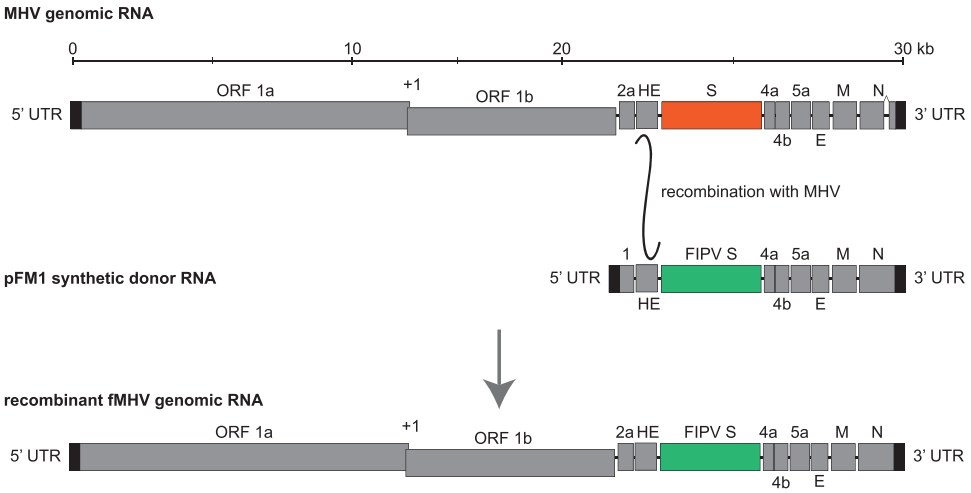


**FIGURE 1** Reverse genetics systems based on RNA recombination. Targeted RNA recombination was employed to generate the recombinant mouse hepatitis virus (MHV) genomic RNA. The synthetic donor RNA containing the S gene of feline infectious peritonitis virus (FIPV) is transcribed from the pFM1 vector, in which the FIPV S gene is flanked by the sequence derived from MHV. Then the donor RNA was transfected into mouse L2 cells, which were infected with the thermolabile MHV N gene deletion mutant. A crossover event within the HE gene fragment of the donor RNA happened, leading to the generation of the recombinant MHV genomic RNA with the FIPV S gene.[28]

## 3.1 | Reverse genetics system based on BAC

The bacterial vectors containing viral cDNA are convenient and efficient for the genetic manipulation of viral genomes. However, the instability of viral cDNA sequence in bacteria hampered the construction of full-length cDNA clones of various viruses, especially for coronavirus with the largest size of viral genomic RNA. The instability issue was overcome in 2000 by Enjuanes et al. by assembling the in vitro generated full-length TGEV cDNA into BAC plasmid, pBeloBAC11.[33,38,39] The copy number of BAC is strictly controlled by *Escherichia coli* F (fertility)-factor to one to two copies per cell, thus minimizing the toxicity of coronavirus sequences to the host cells.[39] This BAC-based strategy has been successfully utilized to construct the full-length cDNA clones of various coronaviruses, including TGEV,[33] HCoV-OC43,[40] FIPV,[41] SARS-CoV,[42] MERS-CoV,[43] and the recently emerged SARS-CoV-2[14,44–46] (Table 1 and Figure 2).

BAC plasmid is widely used for cloning large DNA fragments (up to 300 kb).[47] By assembling the essential elements for a cloning vector, including F-factor, unique cloning sites, suitable promoters to drive the gene expression, a chloramphenicol selectable marker, a lambda cos site for phage package, and *LoxP* site for cre cleavage, Shizuya et al. constructed pBAC108L and pBeloBAC11 vectors.[39,47,48] The latter contains pGEM vector-derived *LacZ*α reporter gene for screening the clones with inserts.[39] The F-factor in BAC plasmid ensures that each daughter cell obtains one copy of BAC plasmid during the bacteria division,[49] and maintains the stable propagation of complex DNA inserts in *Escherichia coli*.[47] However, the low copy number of BAC vector also has disadvantage, because of the low yield of plasmid recovery and a high chance of the contamination of host DNA.

To solve this issue, Wild et al. inserted the *ori*V replication element into the pBeloBAC11 vector to generate the pCC1-BAC vector. As a high-copy origin of DNA replication, the function of *ori*V is regulated by TrfA replication protein, whose expression is very tightly controlled by inducible promoter $P_{BAD}$ promoter and its regulatory protein AraC.[50] Upon the induction, *ori*V can increase the copy of the pCC1-BAC vector per host cell from one to approximately 100 copies, thus drastically increasing the yield of vector DNA.[50] Besides the pCC1-BAC vector, another inducible BAC vector, pSMART® BAC vector (Lucigen), is also widely used for cloning large DNA fragments, including the cDNA of SARS-CoV-2. pSMART® BAC vector is claimed to be able to protect inserts from the destabilizing influence of transcription by altering the orientation of the chloramphenicol promoter, which faces the cloning sites in pCC1-BAC vector pBeloBAC11, and thus may drive the transcription of insert and lead to the unexpected influence of transcripts.

Bacteriophage T7 promoter and human cytomegalovirus (CMV) promoter are used to drive the expression of viral cDNAs cloned in BAC.[33,44] The T7 promoter strategy needs the synthesis of the capped full-length RNA transcript on the linearized vector with T7 RNA polymerase, and electroporation of viral RNA transcript into the permissive cells to rescue the virus. Unlike T7 promoter approach, the CMV promoter approach saves the in vitro transcription step. The delivery of the DNA plasmid containing the full-length viral cDNA in the permissive cells can efficiently rescue the viral particles. Furthermore, DNA is more stable and easier to manipulate compared with RNA. The most popular settings for CMV promoter approach are described as follows. The CMV promoter upstream of the full-length viral cDNA initiates the synthesis of viral transcripts using mammalian RNA polymerase II mechanism, which is terminated by the bovine growth hormone (BGH) terminator. Between BGH and viral poly (A) tail, the hepatitis delta virus ribozyme (HDVr) sequence was inserted to precisely remove the nonviral 3′-terminal sequence generated by BGH terminator and to expose the authentic viral poly (A) tail.[33]

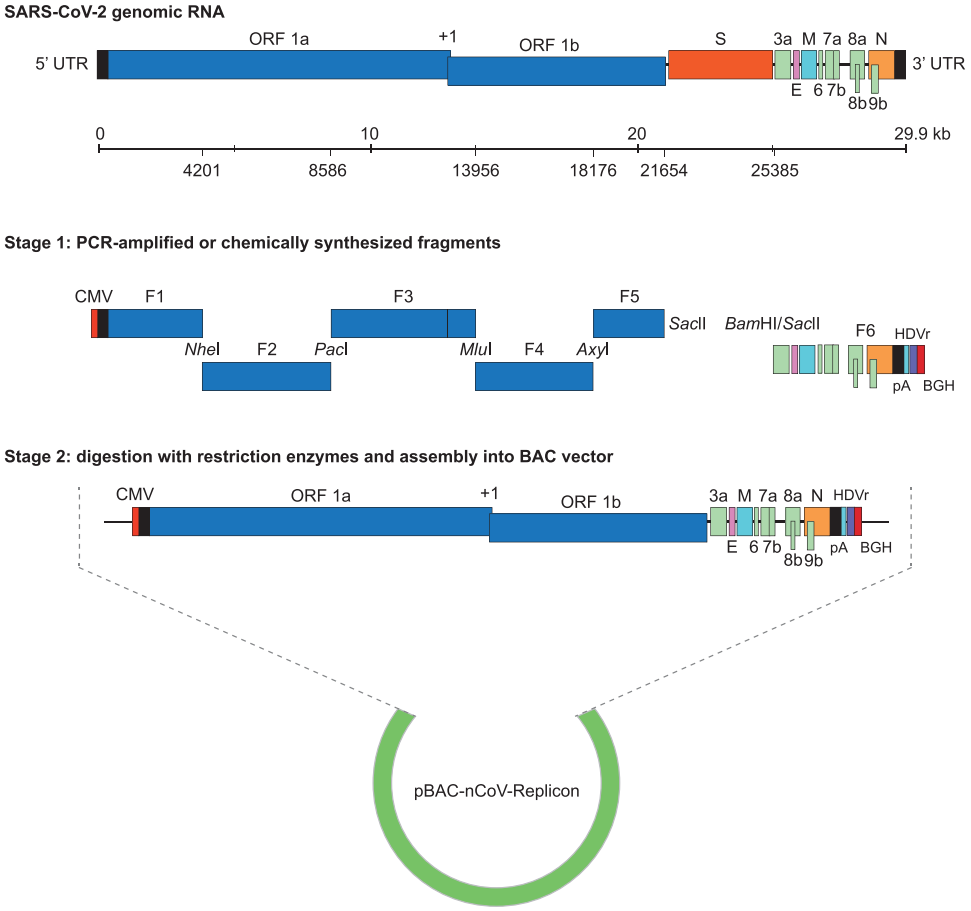


**FIGURE 2** Reverse genetics system based on bacterial artificial chromosome (BAC). The genomic complementary DNA (cDNA) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) without the S gene was separated into six component fragments. Fragment 1 (F1) was fused with a cytomegalovirus (CMV) promotor at its 5′ terminus, and the F6 was fused with poly(A) (pA), HDVr and BGH terminator at its 3′ terminus. *Sac*II restriction site was inserted downstream of the TRS of S gene. All fragments have unique restriction sites at both ends. With these unique restriction sites, all the fragments were assembled into a pBAC-MCS plasmid containing the designed restriction sites.[14]

Although long-range polymerase chain reaction (PCR) has successfully produced fragments of up to 40 kb (such as QIAGEN LongRange PCR Kit), cloning the full-length viral cDNA of coronaviruses into BAC vector in one step is still not feasible. The full-length viral cDNA is usually separated into a few fragments for assembly, which is usually lower than 6 kb, mostly due to the cloning efficiency.[51] All the adjacent fragments are ligated at the unique restriction sites, the selection of which is critical for both construction and future reconstruction of full-length viral clones.[14] When the unique restriction sites are not enough to separate the viral cDNA into the desired size of fragments, new restriction sites are generated by introducing the silent mutations, which can also be used as the genetic marker for viral full-length cDNA clones.[14] It is worthy to note that the ethanol precipitation method can achieve a decent recovery efficiency and fewer damages on digested fragments and BAC plasmids compared with many DNA recovery approaches. BAC plasmid containing multiple cloning sites, including all the designed restriction sites for fragment assembly, is helpful for the downstream cloning work, though it is not indispensable.

In theory, the fragments could be inserted in the BAC plasmid in any order with the unique restriction sites. However, due to the potential toxicity of viral sequence around nsp8-nsp12 for many coronaviruses, the fragments covering this region could be left last to handle.[14,38] In our laboratory practice, we find that the positive colony percentage notably drops while assembling this region into viral cDNA clones. Besides BAC, while constructing the full-length clone for SARS-CoV, we successfully assembled the full-length viral cDNA into the pBR322-derived vector, which generates 10–20 copies per *E. coli* cell. However, pBR322 cannot maintain the fidelity of cDNA, for the sequence covering nsp8-nsp12 is prone to bear the unexpected insertions or deletions, which orchestrate the increased yield of plasmids while performing the DNA extraction from the bacteria (unpublished data).

Although the viral cDNA is usually maintained stably in the BAC vector, sequencing is still recommended to ensure no undesired mutations, especially at those unstable regions in ORF1, which could happen during the cloning steps or amplification in the bacteria. The instability of ORF1 region could be ameliorated by using introns to separate the instable regions into two or more segments.[38] During

the transcription of viral RNA in the nucleus, the introns are precisely removed by RNA splicing and the authentic viral RNA sequences are exported into the cytoplasm to initiate the viral transcription/replication.[52,53]

The infectious clones in BAC for SARS-CoV, HCoV-OC43, FIPV, MERS-CoV, PEDV, and SARS-CoV-2 have successfully rescued the viruses in the permissive cells. The rescued viruses for SARS-CoV-2 have equivalent growth characteristics and plaque sizes as the natural isolate in cultured cells and SARS-CoV-2-infected mice models, supporting the great potential of BAC-based strategy in SARS-CoV-2 research works.[35,45,54,55]

A homologous recombination strategy is developed to introduce the point mutations, insertions or deletions. In this strategy, two steps combining the red recombination system and the homing endonuclease I-SceI mediated counterselection are designed.[56–60] In our laboratory practice, we produce the fragments containing wanted mutations, insertions, or deletions with Gibson Assembly system and amplify the mutant fragments with PCR. The fragment is flanked with two unique restriction sites, between which the corresponding wild-type fragment in the BAC clone is replaced with the mutant one. This strategy was successfully applied to all the cDNA fragments of SARS-CoV-2.[14]

While employing the BAC-based strategy in constructing the full-length viral clones, the researchers should be aware of several disadvantages and approaches to overcome them. First, the fragments larger than 8 kb are difficult to clone into intermediate constructs. Our experience supports that 4-5 kb fragments are the equilibrium point of cloning success rate and time (unpublished data). Second, the intermediate clones should be sequenced to detect unexpected mutations whenever PCR is performed in various steps of cloning or amplification, or the plasmid yield for DNA extraction is abnormally increased. Third, BAC-specific DNA extraction kits are helpful to improve the plasmid yield. Finally, electroporation instead of chemical or lipid-based transfection strategy can significantly increase the expression of viral clones in mammalian cells.

## 3.2 | Reverse genetics system based on the in vitro ligation

The in vitro ligation-based strategy is to generate the full-length viral cDNA by ligating viral cDNA fragments in vitro, which can be used as the template to synthesize viral RNA transcripts using bacterial RNA polymerase. This strategy was first developed by Rice et al., while producing the infectious RNAs of yellow fever virus.[61] Because the building blocks are a series of clones containing viral cDNA segments, this strategy is less likely influenced by the instability of viral cDNA sequence. Parallel to Enjuanes et al., who developed the BAC-based reverse genetics system for TGEV in 2000, Baric et al. successfully constructed infectious RNA of TGEV using in vitro ligation-based strategy.[32,33]

In their design, the full-length cDNA of TGEV was separated into five contiguous fragments (Fragment A-F), which is generated by specific primer-guided RT-PCR. Each fragment is flanked with desired *Bgl*I restriction sites and then cloned into the individual plasmid for unlimited production of materials.[62] To "detoxify" poisonous TGEV sequences in *E. coli*, Baric et al. bisected fragment B by inserting a *Bst*XI site at 9949 nt of TGEV and obtained the intact fragment B from two separate clones. In this multi-plasmid system, the toxicity of the viral genomic cDNA to bacteria was attenuated, thus improving the clone propagation in bacteria. The fragments flanked by the specific nonpalindromic sequences are separated from the plasmids by type IIS restriction endonucleases and purified from an agarose gel. The fragments with 3 or 4-nucleotide overhangs can specifically anneal with adjoining fragments with the complementary overhangs, allowing high specific and sensitive assembly of viral genomic cDNA from continuous small fragments.[32]

Unlike BAC-based strategy, the in vitro ligation-based strategy adopts No See'm cloning technology to generate the overhangs of fragments for the assembly.[62] In No See'm cloning technology, the recognition sites, linker sequence between recognization sites and cleavage sites, and cleavage sites flanking the viral sequence are removed after cleavage by type IIS restriction endonucleases. Thus, it does not need to generate new restriction sites in authentic viral sequences by introducing mutations in the absence of available sites. The manipulation of the viral cDNA could be performed on plasmids containing the target sites and save the effort, which is invested in the BAC-based strategy in replacing the wild-type fragments with the fragments containing desired mutations. Unlike the BAC-based strategy, T7 promoter transcription system with capping activity was used to synthesize viral infectious RNAs *in vitro*, which were then delivered into permissive cells to rescue the viral particles.[54,55]

Besides TGEV, the in vitro ligation-based strategy has been successfully applied to constructing the reverse genetics systems of many coronaviruses, including IBV,[63] SARS-CoV,[64] bat SARS-like CoV,[65] HCoV-NL63,[66] MERS-CoV,[67] and SARS-CoV-2 (Figure 3).[54] These reverse genetics systems achieved the rapid identifications of coronavirus protein function, such as the dispensable roles of IBV ORF5a[63] and HCoV-NL63 ORF3[66] in viral replication and the essential role of consensus T1015N mutation[67] in the S glycoprotein of MERS-CoV in viral propagation in cell culture.

After the outbreak of SARS-CoV-2, Baric et al. and Shi et al. use the in vitro ligation-based strategy to rapidly generate the infectious full-length viral clone[54,55] (Table 1 and Figure 3). Chemical synthesis was utilized to obtain the potential toxic sequences, thus contributing to the rapid progress in cloning work. In their studies, the acquired SARS-CoV-2 exhibited similar replication kinetics and plaque sizes to the original clinical isolates.[54,55] They modified the SARS-CoV-2 infectious clone with the insertion of the reporter genes such as nLuc[54,55] and mNeonGreen.[54] The modified clones were more suitable for antiviral drug screening, as the expressions of reporter genes were sensitive to the treatment of known viral inhibitors.

Compared with the BAC-based strategy, the in vitro ligation-based strategy has obvious advantages. This strategy is relatively simple and straightforward, as most operations are performed using conventional cloning skills on regular high or medium copy plasmids,

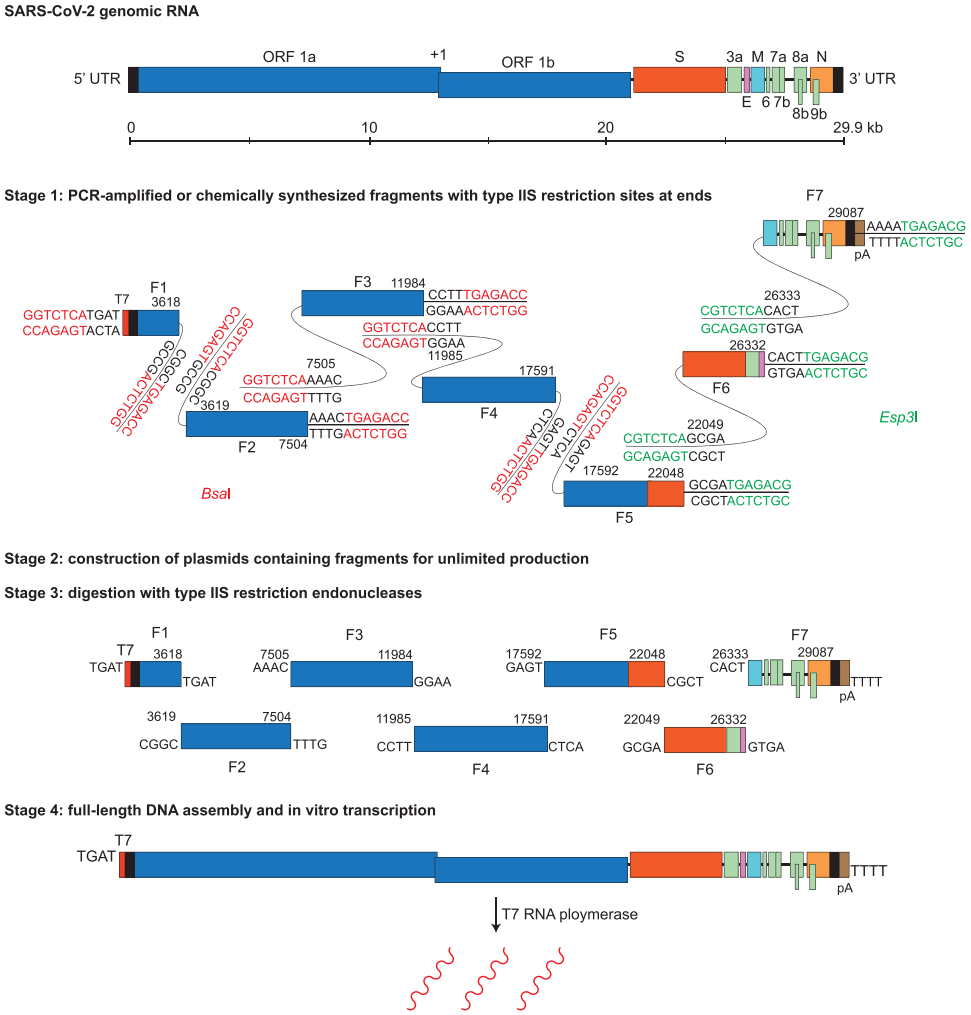


**FIGURE 3** Reverse genetics system based on the in vitro ligation. Seven contiguous complementary DNA (cDNA) fragments covering the entire severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genome were flanked by unique type IIS restriction endonucleases sites, which was adopted by No See′m cloning technology. F1 was fused with T7 promoter at its 5′ terminus. The fragments were cloned into a high-copy plasmid for unlimited production. Each fragment was cleaved out of the plasmid with designed type IIS restriction endonucleases, purified from the gel, and ligated to each other to assemble the full-length cDNA of SARS-CoV-2, which was transcribed with T7 polymerase to generate the infectious full-length viral genomic RNA.[54]

with which amplification and mutagenesis are easily carried out. This strategy can overcome the instability issue of some coronavirus cDNA sequences by bisecting the toxic sequences into multiple segments. Moreover, this strategy can bypass the obstacles of limited available restriction sites for assembling the cDNA component fragments in BAC vector. In theory, the assembly sites could be freely selected in the viral cDNA with the No See'm technology.

However, compared with the BAC-based strategy, the in vitro ligation-based strategy has some disadvantages. Both the BAC-based strategy and the in vitro ligation-based strategy use transfection or electroporation to rescue viruses in the mammalian cells, while the in vitro ligation-based strategy needs extra steps before electroporation, including purification of component fragments, the in vitro ligation of DNA fragments, and further purification of full-length cDNA, and RNA synthesis using T7 transcription system combined with capping activity. These extra steps could become obstacles for researchers without ample experience in these operations. The in vitro ligation-based strategy is fit for quickly rescuing live viruses, whose titer could be built up from a relatively low yield of viral particles after passages in permissive cells. Due to the deletion of essential viral structural genes, the biosafe replicon generated by in vitro ligation-based strategy or BAC-based strategy is not capable to produce the live virus. The replication of viral genomic/subgenomic RNAs relies largely on the stability and the original input of reverse genetics systems. In terms of the stability of the transfected material, the BAC-based strategy that starts with DNA material has an apparent advantage over the in vitro ligation-based strategy transfects the viral RNA. Furthermore, the homogeneity of RNA products from the in vitro ligation-based strategy cannot be guaranteed, because it could include various transcripts from abortive transcriptions or misassembled templates. Thus, the in vitro ligation-based strategy could perform poorly in the assays related to the quantitative comparison between the wild-type and mutant

replicons. For those studies in the conditions with critical biosafety concerns and lack of BSL3 facility, BAC-based strategy has apparent advantages in the assays related to the quantitative comparison between the wild-type and mutant replicons and high-throughput screening work, which are easily performed with a regular transfection on mammalian cells.

### 3.3 | Reverse genetics system using yeast-based TAR cloning

The size limit and instability of viral sequence are the major obstacles to cloning the full-length cDNA of coronaviruses into a replicable vector. The BAC-based strategy has successfully cloned the full-length cDNA of many coronaviruses, as mentioned above, but the toxicity of some viral sequences to bacteria hinders the cloning work. Compared with bacteria, the yeast *Saccharomyces cerevisiae* is less sensitive to toxic viral sequences. Moreover, the cloning strategies in yeast, such as transformation-associated recombination (TAR) cloning, have a higher capacity to clone and maintain the large DNA fragments up to 250 kb.[68]

TAR cloning is successfully used to clone large DNA virus genomes like CMV and herpes simplex virus type 1 (HSV-1).[69,70] TAR enables the direct cloning of clinical isolates through synthetic biology tools without excess tissue culture in vitro.[68,69] In TAR, the overlapping fragments generated by chemical synthesis or RT-PCR, including linearized TAR vector and genomic DNA fragments containing homologous regions, are delivered into the yeast *S. cerevisiae*.[71] TAR occurs among all the homologous regions in various fragments and linearized TAR vector, which contains a centromere (CEN) sequence, a yeast histidine selectable marker and two highly-conserved targeting sequences termed "hooks" at both ends, overlapping with the 5′ and 3′ ends of the viral DNA fragments.

The suitability of yeast *S. cerevisiae* to assemble and maintain genomes of coronaviruses was proven by the successful construction of an infectious full-length cDNA clone of MHV-A59 using yeast-based TAR cloning.[35] The genomic RNA of MHV-A59 obtained from MHV-infected mouse 17Cl-1 cells was used as the template to amplify seven overlapping DNA fragments with RT-PCR.[35] The fragment containing the 5′ terminus of viral genome was fused with an upstream sequence of T7 promoter. The fragment containing the 3′ terminus of viral genome ends with *Pac*I cleavage site, which is essential for the termination of T7 RNA transcription and generation of an authentic viral 3′-terminal sequence. After being delivered into the yeast with transformation, all viral DNA fragments and linearized pVC604, a TAR vector, were assembled based on the homologous ends. pVC604 containing an MHV genome cDNA was examined with multiplex PCRs, and the positive rate of clones reached more than 90%, indicating a highly efficient assembly in yeast. The positive clones are used as the template for the synthesis of viral RNA transcripts after being amplified in yeast and linearized with *Pac*I.[35]

Except for the study by Wang et al.,[72] most of the studies of yeast-based TAR cloning use T7 promoter to drive the synthesis of viral full-length RNA transcripts,[35] which were later transfected into BHK-MHV-N cells together with mRNA transcript of MHV N protein for the rescue of viral particles. N protein was shown to increase the replication/transcription of viral RNA genome and thus contributed to the increased yield of viral particles, though the mechanistic details are still obscure. Similar to the in vitro ligation-based strategy, the viral particles produced after transfections of viral infectious full-length RNA should be amplified in MHV-susceptible 17Cl-1 cells before the yield of viral particles is sufficient for downstream assays. Besides MHV clones, the full-length cDNA clones of coronaviruses including SARS-CoV, MERS-CoV, and SARS-CoV-2 have been constructed.[35] Combined with chemical synthesis technology, this strategy enables the rapid generation of different virus strains.[35]

For the full-length cDNA clone of SARS-CoV-2, Thi Nhu Thao et al. separated the viral genome into 12 overlapping fragments or 14 fragments by replacing fragment 11 with three sub-fragments to insert a GFP reporter gene for antiviral drugs screening[35] (Table 1 and Figure 4). With the help of synthetic genomics platform, fragments except fragments 5 and 7 were quickly generated without the guidance of viral RNA genome. The amplification of fragments 5 and 7 by RT-PCR depends on the availability of SARS-CoV-2 clinical isolate (BetaCoV/Germany/BavPat1/2020). The full-length cDNA clones of SARS-CoV-2 with/without GFP reporter gene were assembled through the yeast-based TAR cloning strategy. The linearized clones are served as the template for the synthesis of viral genomic RNA by T7 RNA polymerase and capping activity in vitro. The viral RNA transcripts together with mRNA of SARS-CoV-2 N protein were then delivered into BHK-21 cells by electroporation to rescue the first-generation recombinant virus, which was further amplified to reach decent titers in the SARS-CoV-2 permissive cells like Vero E6. As expected, the recombinant virus possesses comparable replication kinetics with parental isolates.[35]

Compared with the BAC-based strategy and the in vitro ligation-based strategy, the yeast-based TAR cloning strategy possesses many advantages. The yeast-based TAR cloning seems to achieve higher efficiency in assembling component fragments. The full-length clones can be amplified in the yeast system, providing sufficient materials for the further research and saving a large amount of effort in the preparation of fragments for the in vitro ligation. The availability of restriction sites does not constrain the fragment designs. The assembly efficiency of TAR cloning is not sensitive to the number of fragments like the in vitro ligation-based strategy. Yeast is not sensitive to the toxicity of viral cDNA like bacteria, and thus the full-length clones are well maintained. It is worthy to note that the homologous sequence should be carefully chosen because some factors like the high GC content can decrease the recombination efficiency in the yeast.[73]

### 3.4 | Reverse genetics system based on CPER

One of the major aims of various cloning strategies is to obtain full-length cDNA with transcription elements. The aforementioned

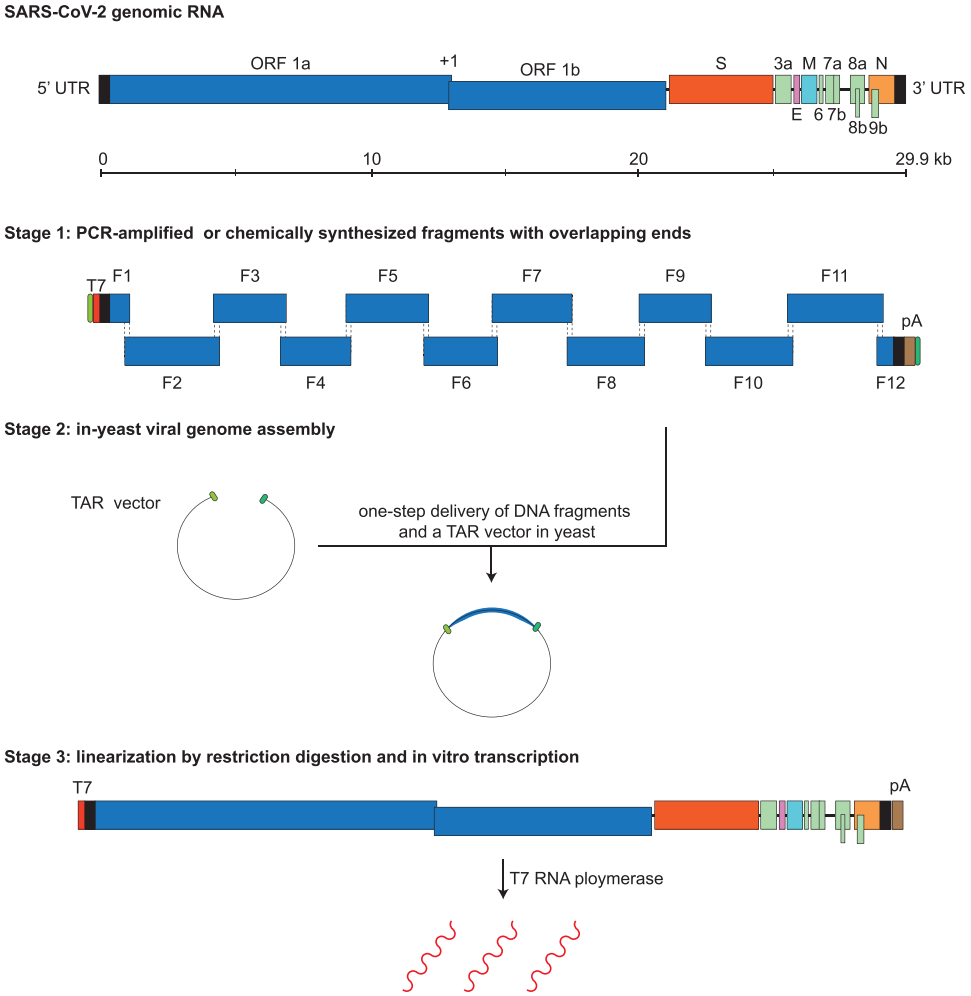


**FIGURE 4** Reverse genetics system using yeast-based TAR cloning. The genomic complementary DNA (cDNA) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was separated into twelve contiguous fragments with overlapping ends. F1 was fused with T7 promoter (red rectangle) and the overlapping sequence (light green rectangle), and F12 was fused with the other overlapping sequence (dark green rectangle). One-step delivery of all the fragments and TAR vectors with the overlapping sequences (light green rectangle and dark green rectangle) were performed on yeast cells, and all DNA fragments were assembled by homologous recombination to generate the YAC vector containing the viral full-length cDNA. The *Eag*I site at the 5′ end of F12 was cleaved to linearize the vector. The linearized vector was used as the template to synthesize the viral infectious full-length RNA.[35]

three strategies all depend on the amplification of clones containing component fragments in bacteria. Moreover, the BAC-based strategy and the yeast-based TAR cloning need bacteria and yeast, respectively, to amplify the full-length viral cDNA clones. PCR technique can promptly synthesize a sufficient amount of DNA for downstream assays. Some long-range polymerase can amplify DNA fragments as long as 40 kb, longer than the size of the coronavirus genome, indicating that PCR can be potentially used to generate the full-length viral cDNA directly without constructing the intermediate clones containing the component fragments. This idea was fulfilled firstly in constructing flavivirus infectious clones using circular polymerase extension reaction strategy (CPER).[74] Due to the big size difference between flavivirus (11 kb) and coronavirus (30 kb), whether CPER could be applied to coronaviruses, the largest RNA viruses, is not addressed until recently, when Amarilla et al. and Torii et al. successfully generated the infectious clones of SARS-CoV-2 using CPER, providing a new dish item in the menu of cloning strategies for cloning full-length of SARS-CoV-2 (Table 1 and Figure 5).[36,37]

Similar to other strategies, CPER strategy separates the viral cDNA sequence into a number of segments, containing overlapping ends with adjacent ones. Like the BAC-based strategy, the fragments containing 5′ or 3′ terminus of viral cDNA are fused with transcriptional elements, including CMV promoter, HDV ribozyme (HDVr), and transcriptional terminator sequence (polyA). The fragments are amplified on viral cDNA with specific primers containing overlapping sequences. Then, the fragments are annealed and function as primers and templates for the amplification of circular viral genome with aforementioned designed transcriptional elements. The circular full-length viral cDNA clones were transfected into tetracycline-inducible ACE2 and TMPRSS-expressing IFNAR1-deficient HEK293 (HEK293-3P6C33) cells, which are easily transfected and permissive to

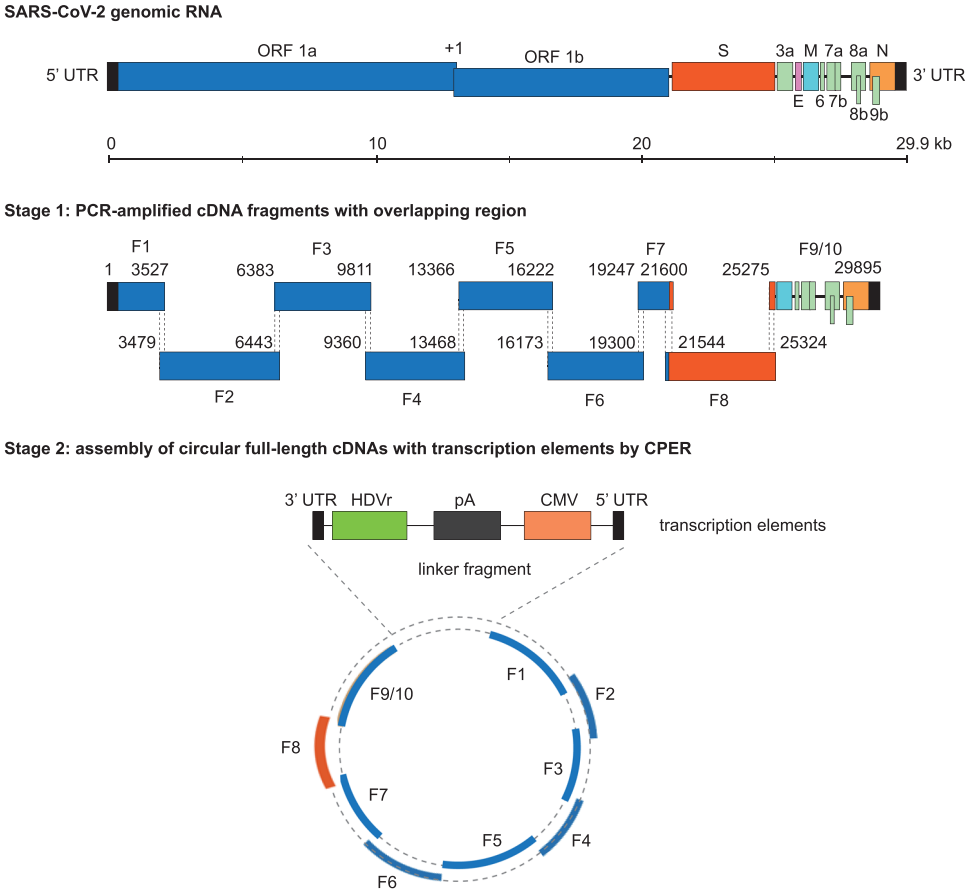


**FIGURE 5** Reverse genetics system based on circular polymerase extension reaction strategy (CPER). Ten contiguous fragments with overlapping ends covered the viral full-length complementary DNA (cDNA) and were generated by polymerase chain reaction (PCR) with specific primers. A linker fragment containing the overlapping sequence with 3′ end of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the HDV ribosome, BGH/SV40 poly (A) signal, cytomegalovirus (CMV) promoter and the overlapping sequence with 5′ end of SARS-CoV-2, was designed to facilitate the circulation and the transcription of viral RNA in mammalian cells. All the DNA fragments that functioned as primers and templates in the same reaction system were amplified by PCR to generate circular DNA, which can be used to rescue the virus after being transfected in the package cells.[37]

the infection and rescue of SARS-CoV-2.[37] Interestingly, Torii et al. found that although the co-expression of N protein promotes the rescue efficiency of the virus in many reverse genetics systems, this effect vanishes in the CPER-based system.

Torii et al. designed genetic markers in the viral genome and confirmed them through the sequence analyses of progeny viruses. They proved that the rescued SARS-CoV-2 exhibits similar biological characteristics to those of the parental virus, demonstrating that the CPER strategy is competent in the studies of the function of viral genes. Like the aforementioned strategies, reporter genes like GFP can also be integrated into the circular viral genome by the CPER strategy and expressed in a viral transcription-dependent manner. Besides SARS-CoV-2, Amarilla et al. also demonstrated the suitability of the CPER-based methodology in other positive-strand RNA viruses such as the arthritogenic alphavirus, Ross River virus (RRV), two caliciviruses, murine norovirus (MNV), and human norovirus (HuNoV). Without time-consuming steps involving the operation in bacteria or yeasts, CPER is relatively more rapid in the generation of WT, mutant and reporter gene-containing viruses compared with the aforementioned strategies. However, the CPER disadvantages, documented as follows, should be noted and still await further improvement. The in vitro synthesis using DNA polymerase in CPER can introduce unpredictable mutations in the DNA products. The efficiency in generating the circle viral genomic cDNA is unstable and is influenced by many factors, such as the design of overlapping sequence, annealing temperature, and mismatches in different rounds of amplification. Thus, compared with the BAC-based one-plasmid biosafe system, CPER is less efficient in the quantitative comparison study between WT and mutant viral replicons.

## 3.5 | Other reverse genetics systems

Due to the page limit, we cannot give comprehensive descriptions of all the works constructing the reverse genetics system of SARS-CoV-2.[75–78] Besides the reverse genetics system constructed with the strategies mentioned above, Luo et al. successfully used the fragmented replicase polyproteins to drive the replication of viral mini replicon,

consisting of viral 5′ UTR, reporter gene, and viral poly (A) in order.[79] Ju et al. and Ricardo-Lax et al. successfully rescued single-cycle-infectious virions without N and S genes, respectively.[80,81] Together, these exciting findings extend our knowledge in the design and the application of the replicon of SARS-CoV-2.

## 4 | BIOSAFETY REQUIREMENTS FOR DIFFERENT REVERSE GENETICS SYSTEMS OF SARS-COV-2

It is important to note that the operators on viral cDNA-related work should take personal protection equipment (PPE) to avoid the inhalation of plasmid aerosol generated during the DNA plasmid operation process. The operation on infectious clones should be performed under BSL3 conditions, while the noninfectious replicon can be used under BSL2 conditions. As for single-cycle-infectious virions assembled using one structural gene-deficient replicon and the structural gene, the recombination between viral replicon and the structural gene could occur, leading to the generation of WT-like infectious virions from single-cycle-infectious virion-infected cells. Although the chance for it is low, BSL3 conditions are highly recommended.

## 5 | DISCUSSION

Viral reverse genetics systems play crucial roles in many aspects of virus-associated research works. For those highly contagious viruses, biosafe replicons modified from viral reverse genetics systems provide a biosafe and convenient research platform in the regular laboratory, decreasing the reliance on limited BSL3 laboratories and the biosafety concerns.

The construction of a reverse genetics system for coronaviruses is a challenge due to the large genome of the coronavirus. Thanks to the unremitting effort of researchers, at least six effective strategies are developed and applied to the generation of coronavirus infectious clones. These six strategies can be categorized into RNA-based and DNA-based strategies. The latter includes BAC-based, the in vitro ligation-based, yeast TAR cloning, CPER strategies, and vaccinia virus vector-based strategy, which employs poxvirus vectors to clone and amplify the full-length viral genomes of coronaviruses and then provides the template for the synthesis of the infectious viral RNA genome, and the details of which can be referred to the review by Enjuanes et al.[51]

BAC-based and the in vitro ligation-based strategies are the most widely used ones in the coronavirus field. BAC-based strategies adopted the traditional cloning strategies to construct a one-plasmid system, which could be used as infectious full-length viral cDNA clones or biosafe replicons. Compared with the other strategies, the BAC-based strategy costs more effort in construction, but the clones are easily manipulated like a regular plasmid. The in vitro ligation-based strategy is a traditional strategy widely used in the virology field. Compared with the BAC-based strategy, the in vitro ligation-based strategy can achieve a rapid rescue of viral particles, but it needs more steps for each rescue assay. Although yeast TAR cloning and CPER strategies are relatively newly developed, they possess a few advantages over BAC-based and the in vitro ligation-based strategies. Yeast TAR cloning can generate a one-plasmid clone and solve the instability issue happening in bacteria. Yeast TAR cloning almost encompasses the major advantages of BAC-based and the in vitro ligation-based strategies. In theory, among all strategies, the CPER strategy should be the most rapid one in terms of the rescue of viral particles. It can be used for high-throughput mutagenesis of coronavirus, which is a crucial way of characterizing the functions of viral genes and investigating the mechanism of viral propagation and pathogenesis.

Many views in this review are based on the experience from our laboratory practice or provided by our collaborators in the construction of full-length viral cDNA clones using various strategies. Some views could be subjective and even biased. Still, this straightforward description could be helpful for the researchers previously not in the coronavirus field in choosing the appropriate full-length viral clone for their studies.

### CONSENT TO PARTICIPATE

The authors agreed to participate in the project.

### CONSENT FOR PUBLICATION

All authors read and approved the final manuscript for publication.

### AUTHOR CONTRIBUTIONS

Deyin Guo and Ji-An Pan conceived and planned the overall structure of the review. Wenhao Wang, Xiaoxue Peng, Ji-An Pan, and Deyin Guo collected the references and wrote the manuscript. Wenhao Wang and Xiaoxue Peng prepared the figures and tables. All authors contributed to the article and approved the submitted version.

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### CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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