

SHORT COMMUNICATION

The accuracy of reverse genetics systems for SARS-CoV-2: Circular polymerase extension reaction versus bacterial artificial chromosome

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Abstract

Background: Reverse genetics systems to rescue viruses from modified DNA are useful tools to investigate the molecular mechanisms of viruses. The COVID-19 pandemic prompted the development of several reverse genetics systems for SARS-CoV-2. The circular polymerase extension reaction (CPER) method enables the rapid generation of recombinant SARS-CoV-2; however, such PCR-based approaches could introduce unwanted mutations due to PCR errors.

Methods: To compare the accuracy of CPER and a classic reverse genetics method using bacterial artificial chromosome (BAC), SARS-CoV-2 Wuhan/Hu-1/2019 was generated five times using BAC and five times using CPER. These 10 independent virus stocks were then deep sequencing, and the number of substitutions for which the frequency was greater than 10% was counted.

Results: No nucleotide substitutions with a frequency of greater than 10% were observed in all five independent virus stocks generated by the BAC method. In contrast, three to five unwanted nucleotide substitutions with a frequency of more than 10% were detected in four of the five virus stocks generated by the CPER. Furthermore, four substitutions with frequencies greater than 20% were generated in three virus stocks by using the CPER.

Conclusions: We found that the accuracy of the CPER method is lower than that of the BAC method. Our findings suggest care should be used when employing the CPER method.

KEYWORDS

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

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as a novel human pathogen in China at the end of 2019, and it continues to circulate globally. Since the World Health Organization (WHO) declared a pandemic on March 11, 2020, many variants of

concern including omicron have emerged. Reverse genetics is a useful tool with which to study the functional effects of mutations. Since the emergence of SARS-CoV-2, several reverse genetics systems for SARS-CoV-2 have been reported.¹⁻⁵ One of them, the circular polymerase extension reaction (CPER) method, which is a PCR-based, bacterium-free method, is widely used because of its speed and

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simplicity.^{1,2,6-9} However, PCR-based methods come with the risk of PCR errors. In contrast, the reverse genetics system that uses bacterial artificial chromosome (BAC) is a classical method to rescue recombinant viruses possessing a large genome such as coronaviruses and herpes viruses.¹⁰⁻¹² Because BACs are amplified by *Escherichia coli*, the BAC method takes longer to prepare recombinant viruses than PCR-based methods. However, the BAC method using cloned and sequence-verified BACs yields recombinant viruses with a very low genetic mixture. To compare the two methods, here, we generated SARS-CoV-2 five times using the BAC method and five times using the CPER method and assessed the sequence accuracy by deep sequencing.

2 | MATERIALS AND METHODS

2.1 | Cells

VeroE6/TMPRSS2 (JCRB 1819) cells¹³ were propagated in the presence of 1 mg/mL geneticin (G418; Invivogen) and 5 µg/mL plasmocin prophylactic (Invivogen) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Human embryonic kidney 293T (HEK293T) cells were cultured in DMEM supplemented with 10% FCS. VeroE6/TMPRSS2 cells and HEK293T cells were maintained at 37 °C under 5% CO₂. The cells were regularly tested and confirmed to be negative for mycoplasma contamination by using PCR.

2.2 | BAC construction

The full-genome nucleotide sequence of SARS-CoV-2 Wuhan/Hu-1/2019 was assembled into the pBeloBAC11 vector to generate infectious cDNA clones under the control of a cytomegalovirus (CMV) promoter by using Gibson Assembly Master Mix (NEB, E2611) as described previously¹⁴ with some modifications. Briefly, five fragments (Fa to Fe) covering the full-length SARS-CoV-2 genome with a 30-bp overlap and restriction sites and linearized pBeloBAC11 (linker

fragment) were amplified by PCR using PrimeSTAR GXL DNA Polymerase (TaKaRa), which is a high fidelity PCR enzyme,¹⁵ and six primer sets (Table 1). The pBeloBAC11 vector was used as a template for PCR to amplify the linker fragment. The artificially synthesized DNA of SARS-CoV-2 Wuhan/Hu-1/2019 was used as a template for PCR to amplify the five fragments, Fa to Fe. First, Fa and Fe were ligated with the linker by using Gibson Assembly Master Mix (NEB) resulting in a BAC containing Fa and Fe. Fb and Fc were subsequently ligated into the BAC digested with FseI and Ascl. Finally, Fd was cloned into the BAC digested with FseI and Ascl resulting in a BAC carrying the full-length SARS-CoV-2 genome. The constructed BACs were introduced into DH10B *E. Coli* (NEB) by electroporation. Four to six clones were sequenced in each step to select a clone without mutations. The *E. Coli* was amplified at 37°C, and BACs were extracted by using NucleoBond Xtra Maxi (TaKaRa).

2.3 | CPER

Viruses were rescued by CPER as previously described.² In brief, the artificially synthesized DNA of SARS-CoV-2 Wuhan/Hu-1/2019 was used as a template for PCR. The six DNA fragments covering the full-length SARS-CoV-2 genome and a linker fragment encoding hepatitis delta virus ribozyme, bovine growth hormone polyA signal, and the CMV promoter were amplified by PCR using PrimeSTAR GXL DNA Polymerase, a high fidelity PCR enzyme. The seven DNA fragments were mixed and used for CPER.²

2.4 | SARS-CoV-2 rescue

To rescue recombinant SARS-CoV-2, BACs or CPER products were transfected into HEK293T cells by using TransIT-293 (TaKaRa) according to the manufacturer's protocol. At 3 days post-transfection, the supernatant containing viruses was collected and inoculated onto VeroE6/TMPRSS2 cells for virus propagation, and the supernatant

TABLE 1 Primers used for bacterial artificial chromosome construction.

Fragment	Direction	Sequence (5' → 3')
Linker	Forward	TAGCTTCTTAGGAGAATGACAAAAAAAAAAAAAAAAAAAAAAAAAAGGGTCGGCATGGCAT
	Reverse	CTGGGAAGGTATAAACCTTTAATACGGTTCCTAAACGAGCTC
Fa	Forward	ATTAAAGGTTTATACCTTCCCAG
	Reverse	AGATTCTCATAAACAAATCCATAAGTTCGTGGCGCGCCGATGGCCGGCCGATGAAATGGTAATTTGTATAGTTTCTAAA
Fb	Forward	TTTAGAACTATACAAATTACCATTTCATCTTTTAAATGGGATTTAACTG
	Reverse	AAACATTAAGTTTGCACAATGCAGAATGCATCTGTCTATCCAAACAGTTA
Fc	Forward	GCATTCTGCATTGTGCAAACTTTAATGTTTTATTCTCTACAGTGTCCCA
	Reverse	AGATTCTCATAAACAAATCCATAAGTTCGTGGCGCGCCGATGGCCGGCCTGTTCTGTTTAGTTGTTAACAAGAACATCAC
Fd	Forward	GTGATGTTCTTGTAAACAACAACTAAACGAACAATGTTTGTGTTTCTTGTGTTT
	Reverse	AGATTCTCATAAACAAATCCATAAGTTCGTTTATGTGTAATGTAATTTGA
Fe	Forward	ACGAACTTATGGATTGTTTATGAGAATCTTCACAATTGGAAGTAACT
	Reverse	TTTTTTTTTTTTTTTTTTTTTTTTTTTGTCTTCTCCTAAGAAGCTA

was stored as a virus stock after full cytopathic effect appearance. For the BAC method, the BAC transfection was performed five times independently, and five independent virus stocks were prepared. For the CPER method, PCR amplification of DNA fragments and the CPER and transfection were performed five times independently, and five independent virus stocks were prepared. All experiments with SARS-CoV-2 were performed in enhanced biosafety level 3 containment laboratories at the University of Tokyo.

2.5 | Sequencing analysis

The stock viruses were subjected to next-generation sequencing using iSeq100 according to a previous report.¹⁶ The number of substitutions whose frequency was higher than 10% was counted.

3 | RESULTS AND DISCUSSION

SARS-CoV-2 Wuhan/Hu-1/2019 was generated five times by using BAC and five times using CPER. In this way, we generated 10 independent virus stocks and subjected them to deep sequencing. As shown in Table 2, no nucleotide substitutions with a frequency of greater than 10% were observed in all five independent virus stocks generated by the BAC method. In contrast, 3–5 unwanted nucleotide substitutions were detected in four of the five virus stocks generated by the CPER. Although these substitutions were not dominant, four substitutions with frequencies greater than 20% were observed in three virus stocks. These results clearly demonstrate that reverse genetics by BAC is highly accurate and that the CPER method comes with a high risk of unwanted mutations.

Although the fidelity of the DNA polymerase for PCR has been improving, it is still lower than that of bacterial polymerase. Therefore, unwanted substitutions in rescued SARS-CoV-2 have been observed

in some previous studies.^{1,7,9} Unwanted substitutions due to PCR errors are introduced randomly and, therefore, their effects on viral characteristics cannot be estimated. This makes it difficult to explore the functions of an amino acid substitution because we want to generate a parental virus and its mutant with only that single amino acid substitution. Such an experiment requires completely identical genome sequences other than at the position of interest. Therefore, such unwanted and uncontrollable mutations might lead to incorrect conclusions that would not be drawn had the viruses been generated by using the BAC method or cloned and sequenced DNA.

The CPER method is a powerful and rapid tool to generate recombinant viruses and has been frequently used in several studies. However, we need to pay close attention to unwanted mutations in stock viruses prepared by the CPER method or other PCR-based and non-cloned methods.

AUTHOR CONTRIBUTIONS

Yuri Furusawa, Seiya Yamayoshi, and Yoshihiro Kawaoka designed the study. Yuri Furusawa and Seiya Yamayoshi performed the experiments and analyzed the data. Yuri Furusawa, Seiya Yamayoshi, and Yoshihiro Kawaoka wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

All the authors declared no conflicts of interest related to this work.

TABLE 2 Number of nucleotide substitutions in bacterial artificial chromosome (BAC) and circular polymerase extension reaction (CPER) stocks.

		Number of nucleotide substitutions detected at the indicated frequency		
		10%–20%	20%–30%	More than 30%
BAC	#1	0	0	0
	#2	0	0	0
	#3	0	0	0
	#4	0	0	0
	#5	0	0	0
CPER	#1	0	0	0
	#2	3	1	1
	#3	4	0	1
	#4	3	0	0
	#5	3	1	0

DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within the paper and from the corresponding author upon request. There are no restrictions to obtaining access to the primary data.

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PEER REVIEW

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REFERENCES

1. Torii S, Ono C, Suzuki R, et al. Establishment of a reverse genetics system for SARS-CoV-2 using circular polymerase extension reaction. *Cell Rep*. 2021;35(3):109014. doi:10.1016/j.celrep.2021.109014
2. Amarilla AA, Sng JDJ, Parry R, et al. A versatile reverse genetics platform for SARS-CoV-2 and other positive-strand RNA viruses. *Nat Commun*. 2021;12(1):3431. doi:10.1038/s41467-021-23779-5
3. Xie X, Lokugamage KG, Zhang X, et al. Engineering SARS-CoV-2 using a reverse genetic system. *Nat Protoc*. 2021;16(3):1761-1784. doi:10.1038/s41596-021-00491-8
4. Chiem K, Ye C, Martinez-Sobrido L. Generation of recombinant SARS-CoV-2 using a bacterial artificial chromosome. *Curr Protoc Microbiol*. 2020;59(1):e126. doi:10.1002/cpmc.126
5. Hou YJ, Okuda K, Edwards CE, et al. SARS-CoV-2 reverse genetics reveals a variable infection gradient in the respiratory tract. *Cell*. 2020;182(2):429-446.e14. doi:10.1016/j.cell.2020.05.042
6. Saito A, Irie T, Suzuki R, et al. Enhanced fusogenicity and pathogenicity of SARS-CoV-2 Delta P681R mutation. *Nature*. 2022;602(7896):300-306. doi:10.1038/s41586-021-04266-9
7. Kimura I, Yamasoba D, Tamura T, et al. Virological characteristics of the SARS-CoV-2 Omicron BA.2 subvariants, including BA.4 and BA.5. *Cell*. 2022;185(21):3992-4007.e16. doi:10.1016/j.cell.2022.09.018
8. Motozono C, Toyoda M, Zahradnik J, et al. SARS-CoV-2 spike L452R variant evades cellular immunity and increases infectivity. *Cell Host Microbe*. 2021;29(7):1124-1136.e11. doi:10.1016/j.chom.2021.06.006
9. Yamasoba D, Kimura I, Nasser H, et al. Virological characteristics of the SARS-CoV-2 Omicron BA.2 spike. *Cell*. 2022;185(12):2103-2115.e19. doi:10.1016/j.cell.2022.04.035
10. Ye C, Chiem K, Park J-G, et al. Rescue of SARS-CoV-2 from a single bacterial artificial chromosome. *mBio*. 2020;11(5):e02168-e02120. doi:10.1128/mBio.02168-20
11. St-Jean JR, Desforges M, Almazán F, Jacomy H, Enjuanes L, Talbot PJ. Recovery of a neurovirulent human coronavirus OC43 from an infectious cDNA clone. *J Virol*. 2006;80(7):3670-3674. doi:10.1128/JVI.80.7.3670-3674.2006
12. Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. *Proc Natl Acad Sci U S A*. 1997;94(26):14759-14763. doi:10.1073/pnas.94.26.14759
13. Matsuyama S, Nao N, Shirato K, et al. Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells. *Proc Natl Acad Sci U S A*. 2020;117(13):7001-7003. doi:10.1073/pnas.2002589117
14. He X, Quan S, Xu M, et al. Generation of SARS-CoV-2 reporter replicon for high-throughput antiviral screening and testing. *Proc Natl Acad Sci U S A*. 2021;118(15):e2025866118. doi:10.1073/pnas.2025866118
15. PrimeSTAR[®] GXL DNA Polymerase. Accessed January 31, 2023. https://catalog.takara-bio.co.jp/com/tech_info_detail.php?mode=3&masterid=M100002502&unitid=U100005215
16. Itokawa K, Sekizuka T, Hashino M, Tanaka R, Kuroda M. Disentangling primer interactions improves SARS-CoV-2 genome sequencing by multiplex tiling PCR. *PLoS ONE*. 2020;15(9):e0239403. doi:10.1371/journal.pone.0239403

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