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Letter

# Development of an HSV-1 F strain BAC system using the synthetic platform

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*Dear Editor,*

Herpes simplex virus type 1 (HSV-1) is a large double-stranded DNA virus capable of establishing infections at diverse anatomical sites including the ocular mucosa, orofacial epithelium, genital tract, and neural tissue. As a globally prevalent human pathogen, sustained research on HSV-1 has driven advances in related therapies and basic virology. Moreover, HSV-1 exhibits significant oncolytic potency as an anti-tumor therapeutic vector (Russell et al., 2012). The oncolytic viruses Talimogene Laherparepvec (T-VEC) (Shalhout et al., 2023) and G47Δ (Frampton, 2022) have been approved for treating malignancies including melanoma and glioma. To optimize safety and therapeutic efficacy, oncolytic viruses of HSV-1 typically require targeted genetic modifications, including deletion of virulence factors and insertion of exogenous immunomodulatory genes. However, HSV-1 contains a linear genome of approximately 152 kb with high GC content (68%), which presents a major challenge for the genetic modifications (Dotto-Maurel et al., 2025).

HSV bacterial artificial chromosome (BAC) allows stable maintenance of HSV genomes in *Escherichia coli* and the modification of HSV genome using the bacterial recombination machinery. Infectious virus is then rescued by transfection of BAC plasmid into mammalian cells, thus facilitating manipulation of HSV. However, HSV BACs containing required sequences (*e.g.* mini *F* and antibiotic genes for replication in bacteria) usually exceed the packaging limit of HSV and result in impaired growth of the rescued viruses in mammalian cells (Gierasch et al., 2006). Therefore, significant efforts have been made to improve the HSV BAC system. One of the most commonly used methods is to flank the BAC sequence with *LoxP* sites, enabling its subsequent removal via *in vivo* or *in vitro* Cre-mediated excision. Recently, synthetic biology has emerged as a powerful alternative for manipulation of HSV-1 genome. In 2017, *de novo* synthesis of KOS strain established a key platform for HSV-1 engineering (Oldfield et al., 2017), and later on, a KOS BAC was generated based on the platform and *in vitro* Cre-mediated recombination (Grzesik et al., 2018). In 2024, a synthetic virus F-Syn based on the Fisher (F) strain was generated in our laboratory (Xiao et al., 2024). Oncolytic viruses derived from F strain have been successfully used in clinical application (Frampton, 2022; Ling et al., 2023). In this study, we report the use of a synthetic platform to generate a synthesized HSV-1 F strain BAC (F-BAC) carrying an inserted BAC sequence flanked by *LoxP* sites. Cre-*loxP* recombination was used to produce F-BACΔ *in vitro*, thereby restoring the viral replication proficiency that had been blocked by the BAC sequence.

Previously, F-Syn with a GFP cassette between *UL22* and *UL23* (Fig. 1A) was generated by synthetic biology (Xiao et al., 2024). The GFP cassette (eGFP-T2A-eGFP), which comprises two eGFP genes linked by the T2A self-cleavage peptide, was derived from H129-G4, an engineered HSV-1 designed for neuronal circuit tracing (Zeng et al., 2017). For the construction of F-BAC, a BAC cassette with two *LoxP* sites at both ends was inserted adjacent to the left of the GFP cassette (Fig. 1A). The 10.2 kb BAC sequence was derived from pGF, a plasmid which has been used as a transfer-vector for synthetic biology (Hou et al., 2016; Xiao et al., 2024). The BAC sequence contains the following elements (Fig. 1A): the *oriV*, *Ori2*, *repE*, and *cos* sites ensure the replication of the genome in *E. coli*; the CEN-ARS cassette guarantees gene replication in yeast and facilitates transformation associated recombination (TAR) based assembly; the $Cm^R$ and *His3* markers are used for screening procedures in *E. coli* and yeast, respectively; and *SopA*, *SopB*, and *SopC* cassettes provide the plasmid partition systems that ensure the stability and genetic accuracy of the genome. The BAC sequence was first inserted into the A4 fragment which was used to generate F-Syn (Xiao et al., 2024), then the A4-BAC was generated by PCR and TAR (Supplementary Materials, Supplementary Fig. S1). Then B1-BAC was assembled from linearized A1, A2, A3 and A4-BAC by TAR in yeast (Fig. 1B). Subsequently, the B1-BAC, B2 and B3 were linearized and co-transfected into Vero cells. Total cellular DNA was extracted after 72 h and transformed into *E. coli* to screen for F-BAC clones. The correct F-BAC clone was confirmed by restriction enzyme digestion analysis (Supplementary Materials, Supplementary Fig. S2) and next-generation sequencing (GenBase and GenBank accession numbers are C_AA129720.1 and PX776338, respectively). The F-BAC DNA was used

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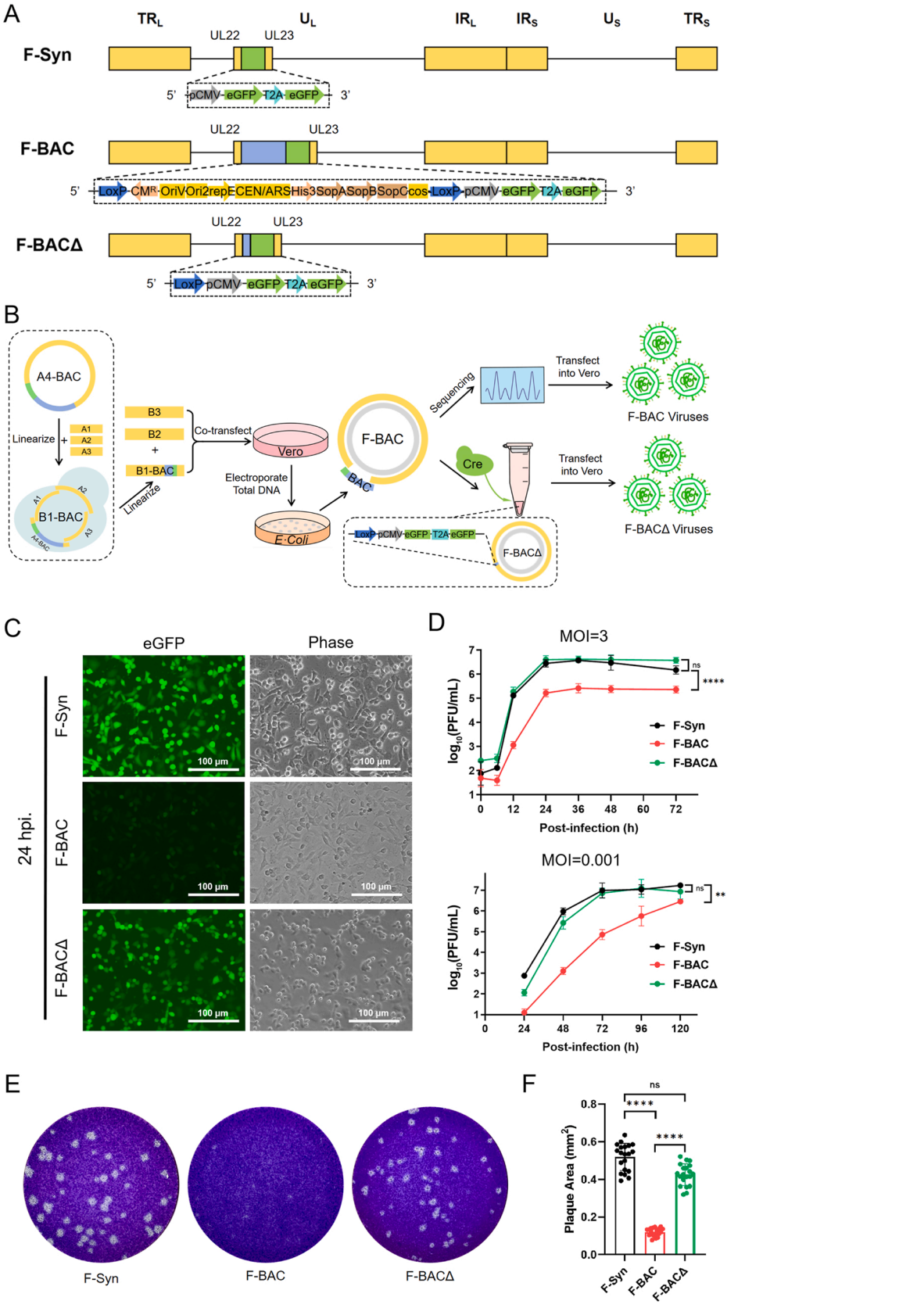


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to transfect Vero cells to rescue the F-BAC viruses (Fig. 1B). To generate the F-BACΔ virus, the BAC sequence was excised from the F-BAC genome by performing Cre-*loxP* recombination *in vitro*. The modified genome was then transfected into Vero cells to rescue the virus (Fig. 1B). The resulting F-BACΔ virus was plaque-purified and its DNA was subjected to PCR and sequencing to confirm the removal of the BAC sequence.

To compare the growth properties of the viruses, Vero cells were individually infected with the viruses of F-Syn, F-BAC, and F-BACΔ with a multiplicity of infection (MOI) of 3. At 24 h post infection (p.i.), F-BAC virus-infected cells exhibited markedly lower GFP fluorescence intensity and an attenuated cytopathic effect (CPE) compared to those exhibited by cells infected with the parental virus F-Syn. In contrast, the fluorescence and CPE of cells infected by F-BACΔ viruses were indistinguishable from those of F-Syn (Fig. 1C). These findings suggested that the GFP expression was affected by the reduced viral replication due to the BAC insertion in F-BAC, and it was restored following the excision of the BAC sequence. Viral growth kinetics further corroborated these observations: at both high (MOI = 3) and low (MOI = 0.001) viral challenges, F-BAC viruses exhibited significantly reduced titers compared to those of F-Syn, while the growth kinetics of F-BACΔ viruses exhibited no significant difference from those of F-Syn (Fig. 1D). More specifically, at an MOI of 3, the average titer of F-BAC viruses was $2.4 \times 10^5$ PFU/mL at the peak of replication, while those of F-Syn and F-BACΔ virus were $1.6 \times 10^6$ PFU/mL and $3.87 \times 10^6$ PFU/mL, respectively. At a lower MOI of 0.001, virus titers of F-Syn and F-BACΔ reached a plateau at 72 h p.i., while the titer of F-BAC viruses was significantly lower and did not reach a plateau even at 120 h p.i. (Fig. 1D). The viral protein (gD) expression was detected by Western blotting, and the result confirmed that the protein level produced by F-BAC was significantly lower than those of F-Syn, while that of F-BACΔ showed no obvious difference compared to F-Syn (Supplementary Fig. S3). Plaque assay results showed that under similar infection conditions, plaques formed by F-BAC viruses were significantly smaller than those generated by the F-Syn control ($P < 0.001$), while those of F-BACΔ viruses showed no significant difference ($P = 0.0531$) (Fig. 1E and F). Collectively, these findings demonstrated that the impairment in replication kinetics of F-BAC viruses was rescued after removing the BAC sequence by Cre-*loxP* recombination.

For clinical translation, HSV-1 F strain possesses inherent advantages as an oncolytic vector backbone. As a laboratory-adapted clinical isolate, it exhibits reduced virulence (Dix et al., 1983), while retains broad tissue tropism (Demir et al., 2024) and has been successfully used for clinical oncolytic applications (Frampton, 2022; Ling et al., 2023). Over the past decades, the BAC systems derived from the HSV-1 F strain have been progressively optimized. For example, the BAC clone of F strain was initially established in which the BAC sequence was inserted in viral thymidine kinase (*TK*) gene and the resulting TK negative viruses were deficient for most animal studies (Horsburgh et al., 1999). Later on, the BAC sequence flanked by *LoxP* sites was inserted in between *UL3* and *UL4*, and it could be efficiently removed by co-transfecting mammalian cells with a recombinant adenovirus expressing Cre recombinase (Tanaka et al., 2003). In 2016, Richards et al. developed a "self-excising" F strain BAC by inserting *Cre* gene with an intron into the BAC genome, thus eliminating the need for Cre-expressing cells to generate BAC-excised virus (Richards et al., 2016). Here, we report the generation of F-BAC and F-BACΔ using a synthetic platform combined with *in vitro* Cre-mediated excision. As the synthetic platform facilitates simultaneous editing at multiple genome sites with higher efficiency and accuracy over traditional methods, we believe our results complement the existing HSV-1 F-strain BAC systems and provide useful tools for researchers to facilitate both basic research and clinical applications.

## FOOTNOTES

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## APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2026.03.003.

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**Fig. 1.** Generation and characterization of F-BAC and F-BACΔ viruses. **A** Schematic diagrams of F-Syn, F-BAC and F-BACΔ genomes. F-Syn was previously constructed (Xiao et al., 2024) containing a GFP expression cassette (eGFP-T2A-eGFP) driven by the CMV promoter, inserted between the *UL22* and *UL23* genes. F-BAC and F-BACΔ were constructed in this study based on the F-Syn backbone. F-BAC contains an insertion of a BAC cassette flanked by two identical *LoxP* sites, immediately upstream of the GFP cassette. F-BACΔ was generated from F-BAC by removing the BAC sequence and retains only a single *LoxP* site. **B** Schematic flowchart of the generation of F-BAC and F-BACΔ viruses. The BAC sequence with *LoxP* sites was inserted into the A4 fragment (for details please see Supplementary Material and Fig. S1) by TAR in yeast. The linearized A4-BAC was then combined with linearized A1, A2, and A3 fragments to assemble B1-BAC by TAR in yeast. B1-BAC was further linearized and co-transfected into Vero cells along with linearized B2 and B3. Circularized viral DNA was extracted with total cellular DNA from co-transfected cells and electroporated into *E. coli* for selecting clones containing F-BAC genome. F-BAC DNA from the correct clone confirmed by genome sequencing was used to rescue F-BAC viruses. For generating F-BACΔ viruses, F-BAC DNA was incubated with Cre recombinase *in vitro* to remove BAC sequence, and then transfected into Vero cells to rescue virus. **C** Infection assay. The purified viruses of F-BAC, F-Syn, or F-BACΔ were used to infect Vero cells with an MOI of 3, and images were taken at 24 h p.i. **D** Growth curve analysis. Vero cells were infected individually with viruses of F-BAC, F-Syn, or F-BACΔ at an MOI of 3 and 0.001. The supernatants of the infected cells were collected at the designated time points, and the titers were measured by plaque assay. Titers at each time point are represented by mean value ± standard deviation of three independent biological repeats. Statistical significance was determined using the Tukey's multiple comparisons test (**, $P < 0.01$, ****, $P < 0.0001$, ns, $P > 0.05$). **E** Plaque assay. Vero cell monolayers were infected with F-Syn, F-BAC viruses, and F-BACΔ viruses. At 80 h p.i., cells were stained with crystal violet to visualize plaques. **F** Statistical analysis of plaque size. Plaques of each virus were randomly selected and the plaque size was measured using ImageJ. Statistical significance was determined using the Dunn's multiple comparisons test (****, $P < 0.0001$, ns, $P > 0.05$, n = 20).

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