

Video Article

Bacterial Artificial Chromosomes: A Functional Genomics Tool for the Study of Positive-strand RNA Viruses

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Abstract

Reverse genetics, an approach to rescue infectious virus entirely from a cloned cDNA, has revolutionized the field of positive-strand RNA viruses, whose genomes have the same polarity as cellular mRNA. The cDNA-based reverse genetics system is a seminal method that enables direct manipulation of the viral genomic RNA, thereby generating recombinant viruses for molecular and genetic studies of both viral RNA elements and gene products in viral replication and pathogenesis. It also provides a valuable platform that allows the development of genetically defined vaccines and viral vectors for the delivery of foreign genes. For many positive-strand RNA viruses such as Japanese encephalitis virus (JEV), however, the cloned cDNAs are unstable, posing a major obstacle to the construction and propagation of the functional cDNA. Here, the present report describes the strategic considerations in creating and amplifying a genetically stable full-length infectious JEV cDNA as a bacterial artificial chromosome (BAC) using the following general experimental procedures: viral RNA isolation, cDNA synthesis, cDNA subcloning and modification, assembly of a full-length cDNA, cDNA linearization, *in vitro* RNA synthesis, and virus recovery. This protocol provides a general methodology applicable to cloning full-length cDNA for a range of positive-strand RNA viruses, particularly those with a genome of >10 kb in length, into a BAC vector, from which infectious RNAs can be transcribed *in vitro* with a bacteriophage RNA polymerase.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53164/>

Introduction

For RNA virologists, the advent of recombinant DNA technology in the late 1970s made it possible to convert viral RNA genomes into cDNA clones, which could then be propagated as plasmids in bacteria for the genetic manipulation of RNA viruses.¹ The first RNA virus to be molecularly cloned was bacteriophage Q β , a positive-strand RNA virus that infects *Escherichia coli*. A plasmid containing a complete cDNA copy of the Q β genomic RNA gave rise to infectious Q β phages when introduced into *E. coli*.² Shortly thereafter, this technique was applied to poliovirus, a positive-strand RNA virus of humans and animals. A plasmid bearing a full-length cDNA of the poliovirus genomic RNA was infectious when transfected into mammalian cells and capable of producing infectious virions.³ In this "DNA-launched" approach, the cloned cDNAs should be transcribed intracellularly to initiate viral RNA replication; however, it is unclear how the transcription is initiated and how the transcripts are processed to the correct viral sequence. This concern has led to the development of an alternative "RNA-launched" approach, whereby a complete cDNA copy of the viral RNA genome is cloned under a promoter recognized by an *E. coli* or phage RNA polymerase for the production of synthetic RNAs *in vitro* with defined 5' and 3' termini, which undergo the complete viral replication cycle when introduced into host cells.^{4,5} The first success with this approach was reported for brome mosaic virus,^{6,7} a positive-strand RNA virus of plants. Since then, the RNA-launched approach has been developed for a wide range of positive-strand RNA viruses, including caliciviruses, alphaviruses, flaviviruses, arteriviruses, and coronaviruses.^{1,4,5,8}

In both the DNA- and RNA-launched reverse genetics systems, the construction of a full-length cDNA clone is the key to generating infectious DNA or RNA of positive-strand RNA viruses, but it becomes a considerable technical challenge as the size of the viral genome increases.⁹⁻¹⁷ In particular, a large RNA genome of ~10-32 kb presents three major obstacles to the cloning of a full-length functional cDNA.¹⁸ The first difficulty is the synthesis of a faithful cDNA copy, since the fidelity of RT-PCR is inversely proportional to the length of the viral RNA. The second hurdle is the presence of potentially toxic sequences, since long RNA molecules are more likely to contain unexpected sequences capable of making the cDNA fragment in plasmids unstable in *E. coli*. The third and most critical issue is the availability of a suitable vector, since it is difficult to find a cloning vector that can house a viral cDNA insert of >10 kb. Over the past three decades, these barriers have been overcome by several advances in enzymology, methodology, and vectorology.^{1,4,5,8} Of these, the most promising and innovative development is the cloning of large positive-strand RNA viruses as infectious bacterial artificial chromosomes (BACs). The BAC vector is a low-copy cloning plasmid (1-2 copies/cell) based on the *E. coli* fertility factor, with an average DNA insert size of ~120-350 kb.¹⁹⁻²¹ A DNA fragment is inserted into the BAC vector in a similar fashion to cloning into general cloning vectors; the resulting BAC clones are stable over many generations in *E. coli*.^{22,23} To

date, the BAC technology has been used to create infectious cDNA clones for >10 members of three positive-strand RNA virus families, *i.e.*, *Flaviviridae*,²⁴⁻²⁹ *Arteriviridae*,³⁰ and *Coronaviridae*.^{9,16,17,31,32}

Using Japanese encephalitis virus (JEV) as an example, the present work reports the detailed procedures that can be used to construct a genetically stable full-length infectious BAC for a variety of positive-strand RNA viruses. JEV is a zoonotic flavivirus³³ that is transmitted in nature between birds, pigs, and other vertebrate hosts by mosquito vectors.^{34,35} In humans, JEV infection can cause the severe often fatal neurological disease Japanese encephalitis (JE),³⁶ which occurs in Asia and parts of the Western Pacific,^{37,38} with an estimated annual incidence of ~50,000-175,000 clinical cases.^{39,40} The genome of JEV is an ~11-kb, single-stranded, positive-sense RNA molecule and consists of a single open reading frame (ORF) flanked by two non-coding regions (NCRs) at the 5' and 3' ends.^{41,42} The ORF encodes a polyprotein that is cleaved by host and viral proteases to generate 10 individual proteins, designated C, prM, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 in the N- to C-terminal direction.^{34,43,44} Also, an extended form of NS1 (NS1') is expressed by -1 ribosomal frameshifting at codons 8-9 of NS2A.^{45,46} Of these 11 proteins, the three structural proteins (C, prM, and E) are essential for the formation of infectious virions,^{47,48} and the remaining eight nonstructural proteins (NS1 to NS5, and NS1') are crucial for viral RNA replication,⁴⁹⁻⁵¹ particle assembly,⁵²⁻⁵⁶ and innate immunity evasion.⁵⁷⁻⁵⁹ Both the 5' and 3' NCRs contain conserved primary sequences and form RNA secondary/tertiary structures,⁶⁰⁻⁶² which are important for modulating viral RNA replication.^{63,64}

This protocol describes the tools, methods, and strategies for generating a full-length infectious BAC of JEV SA₁₄₋₁₄₋₂.²⁸ This functional BAC clone contains a complete cDNA copy of the JEV genomic RNA,⁶⁵ which is encompassed by a promoter for the SP6 RNA polymerase upstream of the viral 5'-end and a unique *Xba* I restriction site downstream of the viral 3'-end for *in vitro* run-off transcription. This BAC technology is applicable to constructing a fully functional cDNA molecular clone for an array of positive-strand RNA viruses.

Protocol

Note: **Figure 1** presents a strategy for the construction of a full-length infectious JEV cDNA as a BAC.²⁸ **Table 1** provides a list of the oligonucleotides used in this protocol.²⁸

1. Extract Viral RNA from JEV Particles in Cell Culture Supernatants

1. Start with the cell culture medium containing JEV SA₁₄₋₁₄₋₂, a live JE vaccine virus that requires Biosafety Level 2 containment.
Note: The viral titer is approximately $1-3 \times 10^6$ plaque-forming units/ml.
2. Take the biosafety training necessary for all standard microbiological practices, safety equipment, and laboratory facilities prior to working with JEV SA₁₄₋₁₄₋₂.
3. Purify viral RNA from an aliquot of the virus-containing cell culture medium using a monophasic solution of phenol and guanidine isothiocyanate⁶⁶ (**Figure 1A**).
 1. Add 600 μ l of the monophasic reagent to 200 μ l of the culture supernatant in a 1.7 ml microtube. Homogenize the mixture by hand-shaking the tube vigorously for 30 sec and incubating for 5 min at RT.
 2. Add 160 μ l of chloroform to the homogenized sample. Mix thoroughly by hand-shaking the tube vigorously for 15 sec and leaving it for 2-3 min at RT.
 3. Centrifuge the total lysate at $13,400 \times g$ for 15 min at 4 °C, which results in a separation of two liquid phases, *i.e.*, a lower organic phase and an upper aqueous phase. Transfer the upper aqueous phase (less than 200 μ l) containing the RNA to a new microtube.
 4. Precipitate the RNA by adding 1 μ l of 5 μ g/ μ l glycogen and 400 μ l of 100% isopropanol and incubating for 10 min at RT.
 5. Centrifuge the mixture at $13,400 \times g$ for 10 min at 4 °C. Discard the supernatant and wash the RNA pellet with 1 mL of 75% ethanol by pulse-vortexing three to five times and centrifuging at $13,400 \times g$ for 5 min at 4 °C.
 6. Air-dry the RNA pellet for 10 min and dissolve it in 40 μ l of dH₂O. Store the extracted RNA at -80 °C until use.

2. Synthesize a Set of Four Overlapping cDNA Fragments (F1 to F4) Spanning the Entire Viral Genomic RNA by Reverse Transcription (RT)-PCR

1. Perform a 20 μ l RT reaction with a 10 μ l aliquot of the purified viral RNA as a template and a modified form of Moloney murine leukemia virus reverse transcriptase.⁶⁷
 1. Set up a 13 μ l mixture containing 10 μ l of the purified RNA, 1 μ l 10 mM dNTP mix, 1 μ l 4 pmol/ μ l primer, and 1 μ l dH₂O. Use the fragment-specific primer for each RT reaction: 1RT for F1, 2RT for F2, 3RT for F3, and 4RT for F4.
 2. Incubate the mixture at 65 °C for 5 min, place on ice for 1 min, and then quick-spin to collect the contents at the bottom of the tube.
 3. Add 4 μ l 5 \times RT buffer, 1 μ l 0.1 M DTT, 1 μ l 40 U/ μ l RNase inhibitor, and 1 μ l 200 U/ μ l reverse transcriptase. Mix by pipetting up and down three to five times.
 4. Let the reaction proceed at 50 °C for 1 hr, then heat-inactivate the sample at 70 °C for 15 min. Store the synthesized first-strand cDNA at -20 °C until use.
2. Perform a 100 μ l PCR reaction with a 5 μ l aliquot of the heat-inactivated RT reaction as a template and a high-fidelity thermostable DNA polymerase⁶⁸ (**Figure 1B**).
 1. Set up a 100 μ l PCR reaction on ice, containing 5 μ l of the RT reaction, 20 μ l 5 \times PCR buffer, 4 μ l 10 mM dNTP mix, 5 μ l 10 μ M forward primer, 5 μ l 10 μ M reverse primer, 1 μ l 2 U/ μ l DNA polymerase, and 60 μ l dH₂O. Use the fragment-specific primer pair for each PCR reaction: 1F+1R for F1 (2573 bp), 2F+2R for F2 (4171 bp), 3F+3R for F3 (3922 bp), and 4F+4R for F4 (1798 bp).
 2. Mix gently by finger-flipping the tube three to five times and centrifuge briefly to collect its contents at the bottom.
 3. Begin thermocycling with an initial denaturation step of 30 sec at 98 °C, followed by 25-30 cycles with the following PCR profile: 10 sec at 98 °C, 30 sec at 60 °C, and 1-2 min (30 s/kb) at 72 °C. Store the PCR products at 4 °C until analyzed.

3. Run a 2-5 μ l aliquot of each PCR reaction on a 0.8% agarose gel containing 0.5 μ g/ml ethidium bromide (EtBr) (**Figure 2**).
CAUTION: EtBr is a potent mutagen and requires lab coats, safety glasses, and gloves to be worn and extreme caution to be observed during its use, storage, and disposal.

3. Subclone Each of the Four cDNA Fragments (F1 to F4) into a BAC Vector to Create pBAC/F1 to pBAC/F4 by Molecular Cloning Techniques

1. Digest the vector and insert DNAs with two appropriate restriction endonucleases, as follows:
 1. Perform a sequential digestion of pBAC/PRRSV/FL (vector),³⁰ a derivative of the pBeloBAC11 plasmid (7507 bp, GenBank accession number U51113), with *Pme* I and *Not* I in a total volume of 60 μ l (containing ~500 ng DNA, 10 U enzyme, 1x digestion buffer, and 1x BSA) at 37 °C for 12-15 hr, which yields the 15426-bp vector fragment.
 2. Perform a sequential digestion of each of the four cDNA amplicons (insert) with *Sma* I and *Not* I in a total volume of 60 μ l (containing ~1 μ g DNA, 20 U enzyme, 1x digestion buffer, and 1x BSA) at 25 °C (*Sma* I) or 37 °C (*Not* I) for 12-15 hr, which yields the following insert fragments of the desired size: F1 (2559 bp), F2 (4157 bp), F3 (3908 bp), and F4 (1784 bp).
2. Purify the desired vector and insert DNA fragments by gel extraction.
 1. Separate the doubly digested products on a 1% low-melting point agarose gel containing 0.5 μ g/ml EtBr. Cut out a band of the desired DNA fragment with a minimal amount of agarose (usually ~200 μ l) under long-wave ultraviolet light.
 2. Add an equal volume of TEM buffer (10 mM Tris-Cl, 1 mM EDTA, and 10 mM MgCl₂) to the excised agarose in a 1.7 ml microtube. Incubate the sample at 72 °C for 10-15 min, with vortexing every 2-3 min until the agarose has completely melted.
 3. Add an equal volume of pre-warmed buffer-saturated phenol, vortex vigorously for 1 min, and centrifuge at 13,400 \times g for 10 min at RT. Note: The mixture separates into a lower organic phase and an upper aqueous phase. Transfer the upper aqueous phase containing the DNA to a new microtube.
 4. Add an equal volume of chloroform:isoamyl alcohol (24:1), vortex for 1 min, and centrifuge at 13,400 \times g for 10 min at RT. Transfer the upper aqueous phase to a new microtube.
 5. Add 0.5 μ l of 10 μ g/ μ l yeast tRNA, 1/10 volume of 3 M sodium acetate, and 2.5 volumes of 100% ethanol. Keep the mixture on ice for 20 min.
 6. Centrifuge the mixture at 13,400 \times g for 10 min at RT. Remove the supernatant and wash the DNA pellet with 1 ml of 70% ethanol by pulse-vortexing three to five times and centrifuging at 13,400 \times g for 10 min.
 7. Air-dry the DNA pellet for 10 min and dissolve it in 20 μ l of TE buffer (10 mM Tris-Cl and 1 mM EDTA [pH 7.6]).
3. Ligate the desired vector and insert DNA fragments using T4 DNA ligase.
 1. Set up a 20 μ l ligation reaction containing 50-100 ng of the vector DNA, a ~3-fold molar excess of the insert DNA, 400 U T4 DNA ligase, and 1x ligation buffer. Incubate the ligation reaction at 16 °C for 12-15 hr.
Note: Include a negative control reaction, *i.e.*, vector only without the insert, in parallel.
 2. Perform four separate ligations, each joining the 15426-bp *Pme* I-*Not* I fragment of pBAC/PRRSV/FL with the 2559-bp (for F1), 4157-bp (for F2), 3908-bp (for F3), or 1784-bp (for F4) *Sma* I-*Not* I fragment of one of the four cDNA amplicons, to generate subclones pBAC/F1 to pBAC/F4.
4. Transform the ligated DNA into *E. coli* DH10B by the CaCl₂-heat shock method.
 1. Take 100 μ l aliquots of the CaCl₂-treated competent DH10B cells⁶⁹ stored at -80 °C and thaw them on ice.
Note: Use 100 μ l of cells per transformation.
 2. Add a 10 μ l aliquot of the DNA ligation reaction to 100 μ l of the thawed cells in a 1.7 ml microtube, mix gently by tapping the tube, and keep on ice for 30 min.
 3. Heat-shock the DNA-cell mixture for 45 sec in a 42 °C water bath, place on ice for 2 min, and then add 900 μ l of LB broth pre-warmed to RT.
 4. Incubate the heat-shocked cells at 35 °C for 1 hr, with shaking at 225-250 rpm.
 5. Spread 50 to 200 μ l aliquots of the cultured cells on LB agar plates containing 10 μ g/ml chloramphenicol (Cml). Keep the plates at RT, right side up, until they are dry.
 6. Turn the plates upside down and incubate at 35 °C for 15 hr.
5. Recover the cloned BAC DNA from the host cells by a column-based purification method (**Figure 1C**).
 1. Pick six to eight bacterial colonies from the LB-Cml agar plates and inoculate them into 3 ml of 2xYT broth containing 10 μ g/ml Cml. Incubate the cultures at 35 °C for 10 hr with vigorous shaking (225-250 rpm).
 2. Isolate recombinant BAC DNAs from 1-1.5 ml of the bacterial cultures using spin columns, as directed by the manufacturer.⁷⁰ Elute the extracted DNA (typically 100-200 ng) in 20 μ l of TE buffer.
 3. Perform two analytical restriction enzyme digestions of the isolated BACs for ~6 hr in a total volume of 10 μ l, one to identify the presence of the vector with a correct insert using the same enzymes used for cloning (see Protocol 3.1), and the other to test the integrity of the cloned BACs with an appropriate enzyme (*e.g.*, *Bgl* II, *Nco* I, or *Pst* I), generating a unique restriction fragment pattern.
 4. Propagate the correctly cloned BACs by inoculating 500 μ l of the positive bacterial cultures (from Protocol 3.5.1) in 500 ml of 2xYT-Cml medium and cultivating the inoculum for 6 hr at 35 °C while shaking at 225-250 rpm. Purify the BAC DNA (typically 10-20 μ g) from the 500 ml culture using filter columns, as recommended by the manufacturer.⁷¹
Note: The initial four BAC subclones, each containing a cDNA fragment of JEV genomic RNA, may prove to have one or more unwanted mutation(s) when compared to the consensus sequence of the viral genome⁴² (which serves as a reference sequence). Any such mutation needs to be corrected by PCR-based site-directed mutagenesis prior to the assembly of a full-length JEV cDNA.²⁷

4. Create a Full-length JEV cDNA with the 5' SP6 Promoter and the 3' Run-off Site

- Make three genetic modifications (see below Protocols 4.1.1-4.1.3) in the cloned cDNAs to allow *in vitro* run-off transcription of genome-length RNAs with the authentic 5' and 3' ends of the viral genome.
 - Introduce an SP6 promoter directly upstream of the 5' end of the viral genome by overlap extension PCR (**Figure 1D**, pBAC/F1^{SP6}).
 - Amplify two overlapping DNA fragments via the first standard PCR of pBAC/F1 with the two primer pairs SP6F+SP6R (product size, 173 bp) and F1F+F1R (product size, 676 bp), each in a 50 μ l reaction containing 1 μ l template DNA (~200 pg/ μ l), 10 μ l 5 \times PCR buffer, 2 μ l 10 mM dNTPs, 2.5 μ l each of 10 μ M forward and reverse primers, 0.5 μ l 2 U/ μ l DNA polymerase,⁶⁸ and 31.5 μ l dH₂O. Perform the PCR using the following cycling profile: 98 °C for 30 sec, and 25 cycles of 98 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 20 sec.
 - Gel-purify the two PCR-amplified DNA fragments after running each of the two PCR products on a 1.5% low-melting point agarose gel, as described in Protocol 3.2.
 - Fuse the two gel-purified DNA fragments via the second fusion PCR using the outermost primers SP6F+F1R (product size, 821 bp) in a 100 μ l reaction including 1 μ l each of the two purified DNA fragments (~100 pg/ μ l), 20 μ l 5 \times PCR buffer, 4 μ l 10 mM dNTPs, 5 μ l each of 10 μ M forward and reverse primers, 1 μ l 2 U/ μ l DNA polymerase,⁶⁸ and 63 μ l dH₂O. Use the following thermal cycling profile: 98 °C for 30 sec, and 25 cycles of 98 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 30 sec.
 - Gel-purify the fused PCR amplicon from a 1% low-melting point agarose gel, as described in Protocol 3.2.
 - Perform the five-step cloning procedure described in Protocols 3.1-3.5 to ligate the 760-bp *Pac* I-*Bs*W I fragment of the gel-purified fused PCR amplicon with the 9532-bp *Pac* I-*Bs*W I fragment of pBAC/F1 to generate pBAC/F1^{SP6}.
 - Remove the pre-existing, internal *Xba* I site at nucleotide 9131 by introducing a silent point mutation (A⁹¹³⁴→T) via overlap extension PCR (**Figure 1D**, pBAC/F3^{KO}).
 - Amplify two overlapping DNA fragments by the first standard PCR of pBAC/F3 with the two primer pairs X1F+X1R (product size, 746 bp) and X2F+X2R (product size, 316 bp) in a 50 μ l reaction under the experimental conditions described in Protocol 4.1.1.1.
 - Gel-purify the two PCR-amplified DNA fragments after electrophoretic separation on 1.5% low-melting point agarose gels, as detailed in Protocol 3.2.
 - Fuse the two gel-purified DNA fragments via the second fusion PCR using the outermost primers X1F+X2R (product size, 1033 bp) in a 100 μ l reaction under the experimental conditions described in Protocol 4.1.1.3.
 - Gel-purify the fused PCR amplicon from a 1% low-melting point agarose gel, as detailed in Protocol 3.2.
 - Perform the five-step cloning procedure described in Protocols 3.1-3.5 to ligate the 949-bp *Avr* II-*Not* I fragment of the gel-purified fused PCR amplicon with the 16245-bp *Not* I-*Bs*W I and 2141-bp *Bs*W I-*Avr* II fragments of pBAC/F3 to produce pBAC/F3^{KO}.
 - Engineer a new artificial *Xba* I run-off site just downstream of the 3' end of the viral genome by PCR-based site-directed mutagenesis (**Figure 1D**, pBAC/F4^{RO}).
 - Generate one DNA fragment by PCR of pBAC/F4 with primers ROF+ROR (product size, 324 bp) in a 100 μ l reaction containing 1 μ l template DNA (~200 pg/ μ l), 20 μ l 5 \times PCR buffer, 4 μ l 10 mM dNTPs, 5 μ l each of 10 μ M forward and reverse primers, 1 μ l 2 U/ μ l DNA polymerase,⁶⁸ and 64 μ l dH₂O. Perform the PCR using the following cycling profile: 98 °C for 30 sec, and 25 cycles of 98 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 15 sec.
 - Gel-purify the PCR-amplified DNA fragment from a 1% low-melting point agarose gel, as detailed in Protocol 3.2.
 - Perform the five-step cloning procedure described in Protocols 3.1-3.5 to ligate the 283-bp *Sfi* I-*Not* I fragment of the gel-purified PCR amplicon with the 16933-bp *Sfi* I-*Not* I fragment of pBAC/F4 to create pBAC/F4^{RO}.
- Assemble a set of the four modified, overlapping cDNAs into a single full-length SA₁₄-14-2 BAC (pBAC/SA₁₄-14-2) by joining at three natural restriction sites (*Bsr*G I, *Bam*H I, and *Ava* I) in a sequential manner using the five-step cloning procedures detailed in Protocols 3.1-3.5 (**Figure 1E**): F1^{SP6} → F1^{SP6}F2 (by replacing the *Bsr*G I-*Not* I fragment of pBAC/F1^{SP6} with that of pBAC/F2) → F1^{SP6}F2F3^{KO} (by replacing the *Bam*H I-*Not* I fragment of pBAC/F1^{SP6}F2 with that of pBAC/F3^{KO}) → F1^{SP6}F2F3^{KO}F4^{RO} (by replacing the *Ava* I-*Not* I fragment of pBAC/F1^{SP6}F2F3^{KO} with that of pBAC/F4^{RO}).

5. Prepare a High-purity Maxi-prep of the Full-length SA₁₄-14-2 BAC

- Grow a single colony of *E. coli* DH10B carrying pBAC/SA₁₄-14-2 in 3 ml of 2 \times YT broth containing 10 μ g/ml Cml for 10 hr at 35 °C with shaking at 225-250 rpm, and then scale up by inoculating 500 μ l of the 10 hr bacterial culture into 500 ml of 2 \times YT-Cml medium and cultivating the inoculum for 6 hr at 35 °C with vigorous shaking.
- Centrifuge the bacterial culture in two 250 ml bottles at 3,107 \times g for 15 min at 4 °C. Resuspend each pellet in 30 ml of GTE solution (50 mM glucose, 25 mM Tris-Cl, and 10 mM EDTA [pH 8.0]), and then add 500 μ l of 60 mg/ml lysozyme. Incubate the two cell suspensions on ice for 10 min.
- Add 60 ml of freshly made Lysis solution (0.2 N NaOH and 1% SDS) to each cell suspension, mix well until clear, and keep the lysates at RT for 10 min.
- Add 45 ml of Neutralization solution (100 ml, consisting of 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml dH₂O) to each bottle, mix thoroughly by inverting the bottles, and incubate the neutralized lysates on ice for 10 min.
- Centrifuge the neutralized lysates at 18,566 \times g for 20 min at 4 °C. Transfer the supernatant of both bottles into two new 250 ml bottles; add 0.6 volume of 100% isopropanol to each, and keep them on ice for 20 min.
- Spin down the precipitates at 18,566 \times g for 20 min at 4 °C. Dissolve each pellet in 5 ml of TE buffer, combine them in a 50 ml tube (10 ml total), and precipitate the RNA by adding an equal volume of 5 M lithium chloride. Incubate the mixture on ice for 10 min.
- Centrifuge the RNA precipitate at 14,636 \times g for 20 min at 4 °C. Transfer the supernatant to a new 250 ml tube and precipitate DNA by adding 2 volumes of 100% isopropanol. Incubate the mixture on ice for 20 min.

8. Spin down the DNA precipitate at $18,566 \times g$ for 20 min at 4 °C. Aspirate the supernatant, resuspend the DNA pellet in 9.5 ml of TE buffer (pH 7.6), and add 10 g of cesium chloride (CsCl) and 390 μ l of 10 mg/ml EtBr.
9. Load the DNA-CsCl-EtBr solution into a 16 \times 76 mm sealable polypropylene tube using a syringe equipped with an 18 G needle. Spin the sealed CsCl gradient in an ultracentrifuge at $401,700 \times g$ for 16 hr at 20 °C (**Figure 3**).
10. Collect a DNA band of BAC plasmid from the CsCl gradient using an 18 G needle to create an air vent at the top of the gradient and a 20 G needle-equipped syringe to retrieve the BAC DNA from the side of the gradient.
11. Add 2.5 volumes of dH₂O-saturated butanol to the EtBr-stained BAC DNA sample and mix by vortexing. Centrifuge the mixture at $13,400 \times g$ for 1 min and transfer the lower aqueous phase to a new 1.7 ml microtube. Repeat this procedure six times.
12. Precipitate the EtBr-free BAC DNA by adding 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol to the butanol-extracted BAC DNA and incubating for 10 min on ice. Centrifuge the precipitate at $13,400 \times g$ for 10 min, wash the DNA pellet with 1 ml of 70% ethanol, and re-pellet it by centrifugation.
13. Air-dry the DNA pellet for 10 min and dissolve it in 200 μ l of TE buffer (pH 7.6).
Note: **Figure 4** shows an overview of the reverse genetics system for JEV SA₁₄₋₁₄₋₂.

6. Transcribe Synthetic RNAs *In Vitro* from a Linearized Full-length JEV BAC DNA

1. Perform a large-scale restriction enzyme digestion of pBAC/SA₁₄₋₁₄₋₂ with *Xba* I in a total volume of 100 μ l (containing 3 μ g DNA, 60 U enzyme, 1 \times digestion buffer, and 1 \times BSA) at 37 °C for 12-15 hr. Examine a 3 μ l aliquot of the digestion reaction on a 0.8% agarose gel containing 0.5 μ g/ml EtBr.
2. Incubate the digestion reaction further with 25 U of mung bean nuclease (MBN) at 30 °C for 2 hr (**Figure 4A**).
3. Bring the volume of the *Xba* I-digested, MBN-treated sample up to 300 μ l with dH₂O. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the diluted sample, vortex vigorously for 1 min, spin at $13,400 \times g$ for 10 min, and then transfer the upper aqueous phase to a new 1.7 ml microtube. Add an equal volume of chloroform and repeat the extraction procedure.
4. Recover the phenol/chloroform-extracted, linearized BAC by ethanol precipitation: Add 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol, and incubate on ice for 20 min. Centrifuge the precipitate at $13,400 \times g$ for 10 min, wash the DNA pellet with 1 ml of 70% ethanol, and then spin it down by re-centrifugation.
5. Air-dry the DNA pellet for 10 min and dissolve it in 30 μ l of dH₂O. Examine a 1 μ l aliquot of the recovered BAC on a 0.8% agarose gel with 0.5 μ g/ml EtBr (**Figure 5A**).
6. Perform a run-off transcription of the linearized BAC DNA in a total volume of 25 μ l (containing ~200 ng template DNA, 0.8 mM cap analog [m⁷G(5')ppp(5')A], 1 mM rNTPs, 40 U RNase inhibitor, 20 U SP6 RNA polymerase,⁷² and 1 \times transcription buffer) at 37 °C for 1 hr (**Figure 4B**). Include 0.5 μ M [³H]UTP for RNA quantification on the basis of [³H]UTP incorporation, as monitored by adsorption to DE-81 filter paper.⁶⁹
7. Run a 1-2 μ l aliquot of the run-off transcription reaction on a 0.6% agarose gel containing 0.5 μ g/ml EtBr (**Figure 5B**).

7. Determine RNA Infectivity and Virus Yield

1. Cultivate BHK-21 cells in 150 mm culture dishes at a density of 3×10^6 cells/dish for 24 hr at 37 °C with 5% CO₂.
Note: Maintain the BHK-21 cells in alpha minimal essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, vitamins, and penicillin/streptomycin.
2. Rinse the cell monolayer with 10 ml of cold Solution A (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄). Detach the cells from the dishes by treatment with 4 ml of trypsin-EDTA (0.25%), and collect them by centrifugation at $270 \times g$ in a desktop centrifuge for 2 min.
3. Resuspend the cell pellet with 50 ml of cold Solution A in a 50 ml conical tube and centrifuge the cell suspension at $270 \times g$ for 2 min. Repeat this wash procedure three times; after the last wash, resuspend the cell pellet at a density of 2×10^7 cells/ml in Sol A.
4. Mix a 400 μ l aliquot of cell suspension with 2 μ g of synthetic RNA in a 2-mm gap cuvette, and promptly electroporate the mixture with an electroporator under optimal electroporation conditions: 980 V, 99 μ sec pulse length, and 5 pulses (**Figure 4C**).
Note: Use the ³H-labeled RNA synthesized in Protocol 6.5 directly for electroporation without further purification.
5. Leave the electroporated cells at RT for 10 min and transfer to a 1.7 ml microtube containing 600 μ l of complete culture medium.
6. Prepare a 10-fold serial dilution of the electroporated cells in 1 ml of complete culture medium and plate a 100 μ l aliquot of each dilution on the monolayers of unelectroporated BHK-21 cells (5×10^5) in a 6-well plate.
7. After 4-6 hr of incubation, overlay the cells with 0.5% agarose in minimal essential medium containing 10% fetal bovine serum. Incubate the plates for 4 days at 37 °C with 5% CO₂.
8. Visualize the infectious centers (plaques) by fixation with 7% formaldehyde and staining with 1% crystal violet in 5% ethanol²⁷ (**Figure 6A**).
1. Optional: Examine RNA-electroporated cells at 18-20 hr post-transfection for JEV protein expression by immunofluorescence assays^{27,73} (**Figure 6B**), and harvest the supernatants from the RNA-electroporated cells at 22 and 40 hr post-transfection for virus titration by plaque assays^{27,63} (**Figure 6C**).

Representative Results

For all positive-strand RNA viruses, the reliability and efficiency of a reverse genetics system depend on the genetic stability of a cloned full-length cDNA, whose sequence is equivalent to the consensus sequence of viral genomic RNA.²⁷ **Figure 1** shows a five-step strategy for the construction of a full-length infectious cDNA as a BAC for JEV SA₁₄₋₁₄₋₂²⁸: Step 1, purification of viral RNA from the cell culture supernatant of JEV-infected BHK-21 cells (**Figure 1A**); Step 2, synthesis of four overlapping cDNA amplicons (F1 to F4) spanning the whole viral genome (**Figure 1B**); Step 3, subcloning of each of the four contiguous cDNA fragments into a BAC vector, creating pBAC/F1 to pBAC/F4 (**Figure 1C**); Step 4, modification of the cloned cDNAs for *in vitro* run-off transcription with SP6 RNA polymerase, *i.e.*, placing an SP6 promoter sequence immediately upstream of the viral 5'-end (pBAC/F1^{SP6}), eliminating a pre-existing internal *Xba* I site at nucleotide 9131 by introducing a silent point mutation, A⁹¹³⁴→T (pBAC/F3^{KO}), and inserting a new artificial *Xba* I run-off site immediately downstream of the viral 3'-end (pBAC/F4^{RO}) (**Figure 1D**); and Step 5, assembly of a full-length SA₁₄₋₁₄₋₂ cDNA BAC, pBAC/SA₁₄₋₁₄₋₂ (**Figure 1E**). **Table 1** lists the oligonucleotides used in this cloning procedure.²⁸

For the construction of a functional JEV cDNA, the first important step is the synthesis of the four overlapping cDNA fragments using the purified viral RNA as a template for RT-PCR. **Figure 2** provides a representative result for the four RT-PCR products that were electrophoresed on a 0.8% agarose gel. This gel demonstrates clearly that a full-length JEV cDNA is amplified into four overlapping cDNA fragments. Occasionally, RT-PCR reactions might yield one or more additional virus-specific or nonspecific products that are mostly smaller than the expected product, because of the nonspecific annealing of primers during cDNA synthesis/amplification. On the other hand, little or no expected RT-PCR product would be amplified because of accidental RNase contamination during the viral RNA isolation or improper RT-PCR performance.

The next key step is the cloning and modification of a partial- or full-length JEV cDNA in BAC, which is a relatively straightforward procedure that uses standard recombinant DNA techniques.⁶⁹ **Figure 3** presents a representative outcome for the purification of the BAC clone containing a full-length cDNA of JEV SA₁₄₋₁₄₋₂ by banding in a CsCl-EtBr gradient. In this experiment, after centrifugation for 16 hr at 401,700 × g, two distinct bands, *i.e.*, the *E. coli* chromosomal DNA above and the supercoiled BAC plasmid DNA below, are visible in the middle of the tube under long-wave ultraviolet light. A minimal volume (~400 µl) of the lower BAC DNA band was carefully collected by poking a hole with a syringe on the side of the tube. Subsequently, the EtBr was extracted from the BAC DNA by butanol extraction, and the EtBr-free BAC DNA was concentrated by ethanol precipitation.

The final step is the determination of the specific infectivity of the synthetic RNAs transcribed *in vitro* from the full-length SA₁₄₋₁₄₋₂ BAC (pBAC/SA₁₄₋₁₄₋₂) after RNA transfection into permissive cells (**Figure 4**). This step involves three sequential steps: Step 1, linearization of the full-length SA₁₄₋₁₄₋₂ cDNA at the 3'-end of the viral genome (**Figure 4A**); Step 2, production of synthetic RNAs from the linearized cDNA by run-off transcription (**Figure 4B**); and Step 3, rescue of the recombinant viruses in BHK-21 cells transfected with the synthetic RNAs (**Figure 4C**). Experimentally, two independent clones of pBAC/SA₁₄₋₁₄₋₂ were linearized with *Xba* I digestion and treated with MBN to remove the four-base 5' overhang generated by the *Xba* I digestion. The linearized BACs were cleaned up by phenol-chloroform extraction, followed by ethanol precipitation. The linearization of the two purified BACs was demonstrated on a 0.8% agarose gel (**Figure 5A**). The phenol-chloroform extraction must be done carefully to ensure that the linearized BACs are RNase-free. Each of the two linearized BACs served as a cDNA template for run-off transcription using SP6 RNA polymerase in the presence of the m⁷G(5')ppp(5')A cap analog. The integrity of the synthetic RNAs was shown by running aliquots of the two transcription reaction mixtures on a 0.6% agarose gel, along with a reference 1 kb DNA ladder (**Figure 5B**). In this simple assay, the major prominent RNA band always migrated just below the 3 kb reference DNA band and appeared to be sharp. However, degraded RNA would have a smeared appearance on the same gel.

An infectious center assay is the gold standard for determining the specific infectivity of the synthetic RNAs. This assay was done by electroporating BHK-21 cells with RNA samples, seeding equal aliquots of the 10-fold serially diluted electroporated cells in 6-well plates containing naïve BHK-21 cells (3 × 10⁵ cells/well), and overlaying agarose onto the cell monolayers. After incubation for 4 days, surviving cells were fixed with formaldehyde and stained with a crystal violet solution to quantify the number of infectious centers (plaques), which corresponds to the number of infectious RNA molecules delivered into the cells (**Figure 6A**). Since the cDNA template used for *in vitro* transcription has been proven to be non-infectious,²⁷ an aliquot of the transcription reaction mixture was directly used for electroporation. Electroporation is the preferred method for RNA transfection; alternatively, RNAs can be transfected by other methods using DEAE-dextran and cationic liposomes. RNA electroporation is very effective, but "arcing" of the electric pulse occurs rarely if salts are present in the electroporation reaction or if the electroporation cuvette is reused. The expression of viral proteins in RNA-transfected cells was examined by immunofluorescence assays using an anti-NS1 rabbit antiserum (**Figure 6B**). The production of viral particles accumulated in the supernatants of RNA-transfected cells was analyzed by plaque assays (**Figure 6C**). The results of these experiments show clearly that the cDNA-derived synthetic RNAs are infectious in permissive BHK-21 cells, generating a high titer of recombinant viruses.

Oligonucleotide	Sequence ^a (5' to 3')	Position ^b	Polarity
1RT	TAGGGATCTGGGCGTTTCTG GCAAAT	2578–2603	Antisense
1F	aatccccgggAGAAGTTTATC TGTGTGAACCT	1–22	Sense
1R	attgcgccgcCCACGTCGT TGTGCACGAAGAT	2532–2553	Antisense
2RT	TTCTGCCTACTCTGCCCTC CGTTGA	5975–6000	Antisense
2F	aatccccgggTCAAGCTCAGT GATGTTAACAT	1800–1821	Sense
2R	attgcgccgcGATGGGTTT CCGAGGATGACTC	5929–5950	Antisense
3RT	ACGGTCTTTCTTCTGCTGC AGGTCT	9426–9451	Antisense
3F	aatccccgggGAGGATACATT GCTACCAAGGT	5500–5521	Sense
3R	attgcgccgcGTAAGTCAG TTCAATTATGGCT	9380–9401	Antisense
4RT	AGATCCTGTGTTCTTCTCA CCACCA	10952–10977	Antisense
4F	aatccccgggAGTGAAGGCT CAGGCGTCCAA	9200–9221	Sense
4R	attgcgccgcAGATCCTGT GTTCTTCTCACC	10956–10977	Antisense
SP6F	catacccccggtattccac ta		Sense
SP6R	ACAGATAAACTTCTctatag tgtcccctaaa	1–14	Antisense
F1F	aggggacactatagAGAAGT TTATCTGTGTG	1–17	Sense
F1R	TGGATCATTGCCCATGGTAA GCTTA	638–662	Antisense
X1F	CGAATGGATCGCACAGTGTG GAGAG	8403–8427	Sense
X1R	AAAGCTTCAAACCAAGATA CCGTGCTCC	9120–9148	Antisense
X2F	GGAGCACGGTATCTTGAGTT TGAAGCTTT	9120–9148	Sense
X2R	cacgtggacgagggcatgcc tgcag		Antisense
ROF	CCAGGAGGACTGGGTTACCA AAGCC	10670–10694	Sense
ROR	agggcgccgctctagAGAT CCTGTGTTCTTCTCACCAC	10954–10977	Antisense

^aJEV sequences are shown in uppercase letters, and BAC sequences are indicated in lowercase letters.

^bNucleotide position refers to the complete genome sequence of JEV SA₁₄-14-2 (Genbank accession number JN604986).

Table 1: Oligonucleotides used for cDNA synthesis, PCR amplification, and BAC mutagenesis.

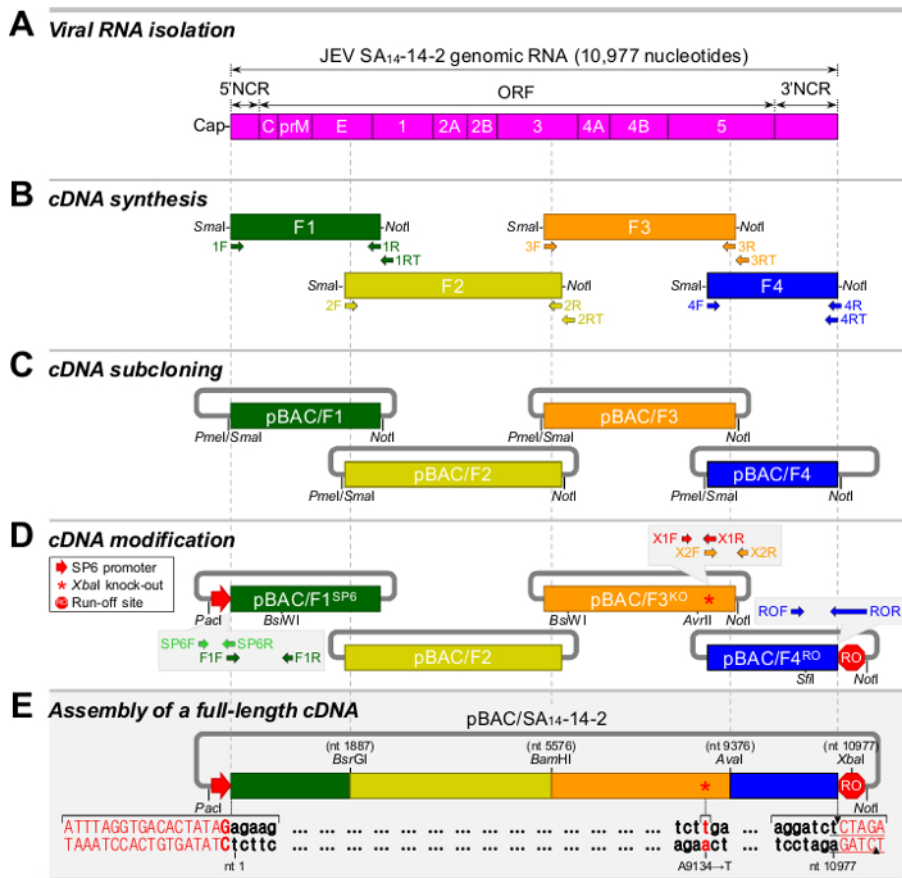


Figure 1. Strategy for the construction of a full-length cDNA of JEV SA₁₄₋₁₄₋₂ as a BAC. (A) Isolation of viral RNA from JEV particles. Shown is a schematic diagram of the genomic RNA of JEV SA₁₄₋₁₄₋₂. (B) Synthesis of four overlapping cDNA fragments (F1 to F4) covering the entire viral genome. (C) Subcloning of four overlapping cDNA fragments into a BAC vector, creating pBAC/F1 to pBAC/F4. (D) Modification of the cloned cDNAs for run-off transcription *in vitro*. pBAC/F1^{SP6} is a derivative of pBAC/F1 that contains the SP6 promoter sequence upstream of the viral 5'-end. pBAC/F3^{KO} is a derivative of pBAC/F3 that contains a silent point mutation (A⁹¹³⁴→T, asterisk). pBAC/F4^{RO} is a derivative of pBAC/F4 that contains an artificial *Xba* I run-off site downstream of the viral 3'-end. (E) Assembly of a full-length SA₁₄₋₁₄₋₂ BAC (pBAC/SA₁₄₋₁₄₋₂). Please click here to view a larger version of this figure.

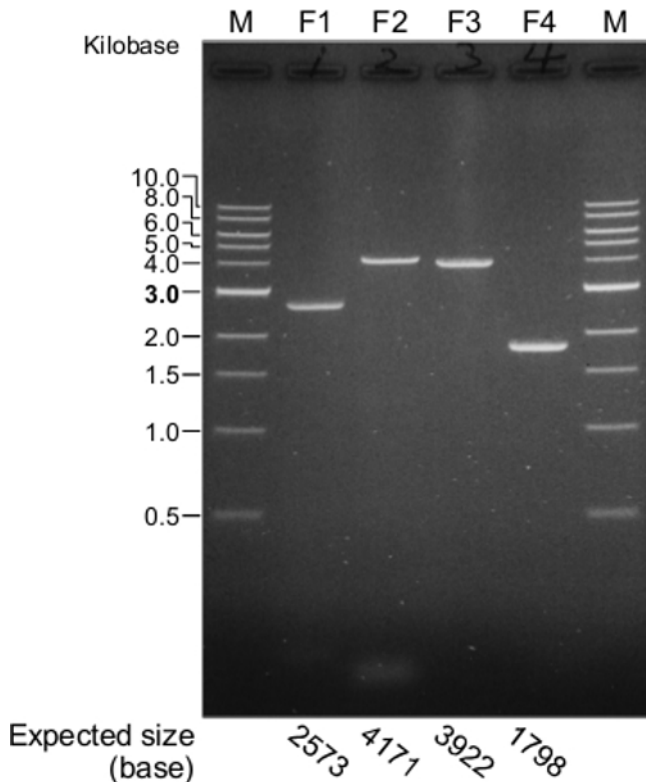


Figure 2. Synthesis of four overlapping cDNA fragments (F1 to F4) spanning the full-length genomic RNA of JEV SA₁₄-14-2. The four RT-PCR products are evaluated by electrophoresis in a 0.8% agarose gel. M, 1 kb DNA ladder. The expected sizes of the four cDNA fragments are indicated at the bottom of the gel image. [Please click here to view a larger version of this figure.](#)

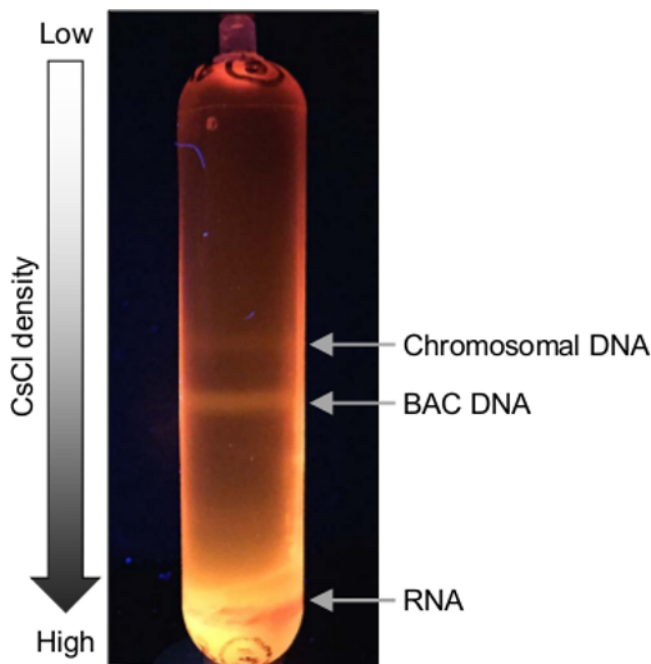


Figure 3. Purification of the BAC containing a full-length cDNA of JEV SA₁₄-14-2. The BAC plasmid is isolated from *E. coli* DH10B by the SDS-alkaline lysis method and further purified by banding in a CsCl-EtBr gradient. Presented is an example of the CsCl-EtBr gradient using a 16 × 76 mm sealable polypropylene tube. [Please click here to view a larger version of this figure.](#)

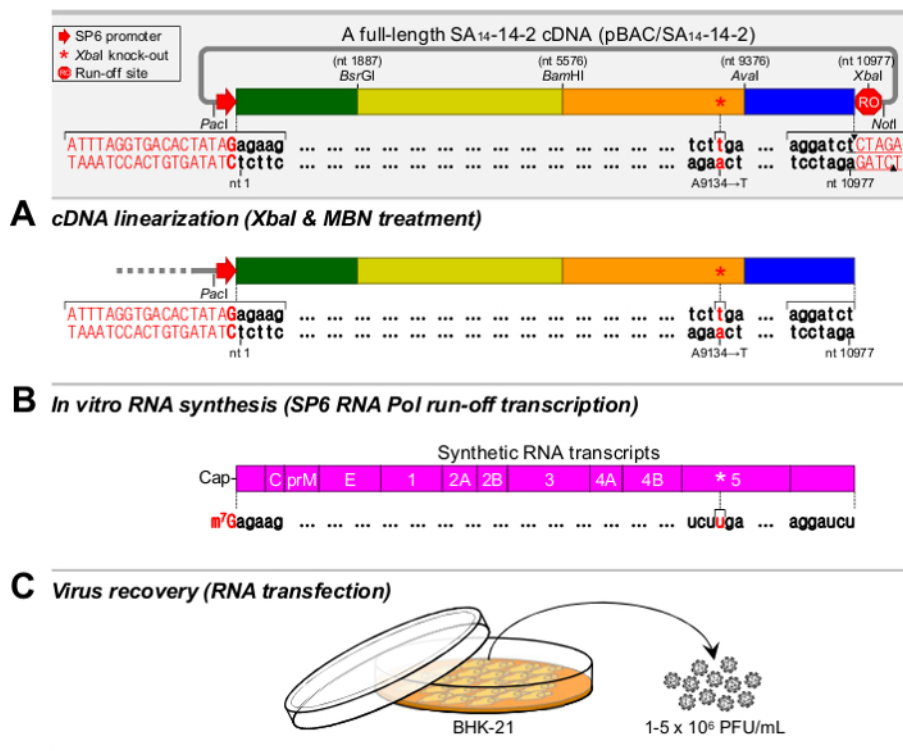


Figure 4. Overview of the recovery of infectious viruses from a full-length JEV SA₁₄₋₁₄₋₂ cDNA assembled in a BAC. (A) Linearization of the cDNA template. The full-length JEV BAC is cut with *Xba*I and treated with MBN. **(B)** Synthesis of the RNA transcripts. The linearized cDNA is transcribed by SP6 RNA polymerase in the presence of the m⁷G(5')ppp(5')A cap analog. **(C)** Recovery of the synthetic JEVs. The *in vitro* transcribed RNAs are transfected into BHK-21 cells by electroporation, which generates a high titer of synthetic virus. [Please click here to view a larger version of this figure.](#)

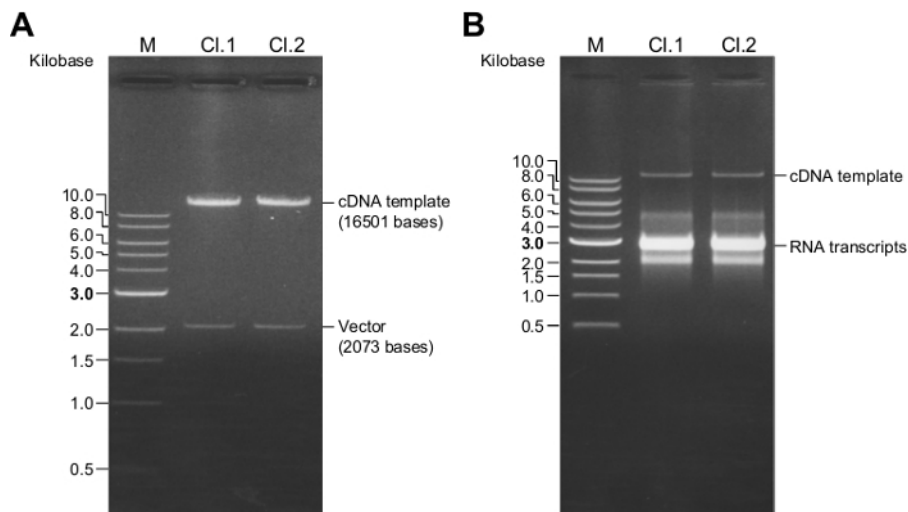


Figure 5. Synthesis of the RNAs by *in vitro* transcription using a full-length JEV BAC as a cDNA template. (A) Generation of the linearized full-length JEV BAC, pBAC/SA₁₄₋₁₄₋₂. Two independent clones of pBAC/SA₁₄₋₁₄₋₂ (Cl.1 and Cl.2) are linearized by digestion with *Xba*I and subsequent treatment with MBN. The linearized BACs are examined by electrophoresis in a 0.8% agarose gel. **(B)** Production of the synthetic RNAs by run-off transcription. Each of the two linearized BACs is used as a template for SP6 RNA polymerase run-off transcription. Aliquots of the two transcription reactions are run on a 0.6% agarose gel. M, 1 kb DNA ladder. [Please click here to view a larger version of this figure.](#)

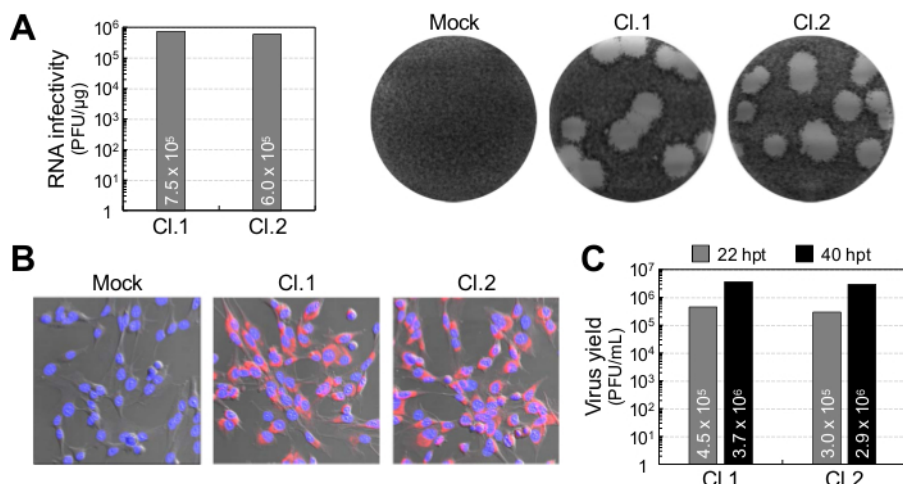


Figure 6. Specific infectivity of the synthetic RNAs transcribed from a full-length JEV BAC and the recovery of synthetic virus. BHK-21 cells are mock-electroporated (Mock) or electroporated with the RNA transcripts derived from each of the two independent clones of the full-length JEV BAC (Cl.1 and Cl.2). **(A)** RNA infectivity. The cells are overlaid with agarose and stained with crystal violet at 4 days post-transfection. RNA infectivity is determined by infectious center assays to estimate the amount of infectious RNA electroporated into the cells (left panel). Also, representative images of infectious centers are shown (right panel). **(B)** Protein expression. The cells are cultured in 4-well chamber slides. Viral protein expression in RNA-electroporated cells at 20 hr post-transfection (hpt) is analyzed by immunofluorescence assays using a primary anti-NS1 rabbit antiserum and a secondary Cy3-conjugated goat anti-rabbit IgG (red). The nuclei are counterstained with 4',6-diamidino-2-phenylindole (blue). The immunofluorescence images are overlaid on their corresponding differential interference contrast images. **(C)** Virus yield. The cells are cultured in 150 mm culture dishes. The production of infectious virions accumulated in the culture supernatants of RNA-electroporated cells at 22 and 40 hpt is examined by plaque assays. [Please click here to view a larger version of this figure.](#)

Discussion

The current protocol has been successfully used to generate full-length infectious cDNA clones for two different strains (CNU/LP2²⁷ and SA₁₄₋₁₄₋₂²⁸) of JEV, a flavivirus whose functional cDNA has proved to be inherently difficult to construct and propagate because of host cell toxicity and the genetic instability of the cloned cDNA.^{8,74-76} This protocol involves three major components: first, maximizing the synthesis/amplification of a faithful cDNA copy of the viral RNA using high-fidelity reverse transcriptase/DNA polymerase; second, cloning the viral prM-E coding region containing toxic sequences (unpublished data)^{74,77,78} in a very low-copy number vector BAC from the initial cDNA subcloning to the final full-length cDNA assembly steps; and third, utilizing a cloning vector BAC that can accommodate a foreign DNA with an average size of 120-350 kb,¹⁹⁻²¹ which apparently tolerates larger DNA inserts than do other cloning vectors. This cloning approach will be generally applicable to many other positive-strand RNA viruses, particularly those with a large RNA genome of ~10 to 32 kb. Generation of an infectious cDNA clone is a key step in developing a reverse genetics system for RNA viruses, especially for positive-strand RNA viruses, because its genome acts as viral mRNA that is translated into proteins by host cell ribosomes. Thus, viral replication can be initiated by the introduction of a cDNA-derived genome-length RNA molecule into a susceptible host cell. The availability of an infectious JEV cDNA clone, when combined with recombinant DNA technology, has increased our understanding of various aspects of the viral life cycle at the molecular level, such as gene expression^{73,79} and genome replication.^{63,64} Also, a full-length JEV cDNA clone has proven to be a valuable tool for the development of antiviral vaccines²⁸ and gene delivery vectors.^{80,81}

As with all positive-strand RNA viruses, there are multiple critical steps in constructing a reliable functional cDNA for JEV from which highly infectious RNAs can be synthesized *in vitro*. Ideally, the sequence of the synthetic RNAs transcribed from a clone of the full-length cDNA should be identical to that of the viral genomic RNA, particularly the 5'- and 3'-terminal sequences that are required for the initiation of viral RNA replication.⁶⁰⁻⁶² In the current protocol, the authentic 5'- and 3'-ends were ensured by placing the SP6 promoter sequence upstream of the first adenine nucleotide of the viral genome and positioning a unique artificial *Xba* I restriction site downstream of the last thymine nucleotide of the viral genome, respectively. Capped synthetic RNAs with the authentic 5' and 3' ends were produced by run-off transcription of an *Xba* I-linearized and MBN-treated cDNA template using SP6 RNA polymerase primed with the m⁷G(5')ppp(5')A cap analog. This protocol can be modified in several ways. For *in vitro* transcription, another bacteriophage RNA polymerase (e.g., T3 or T7) can be used in conjunction with its well-defined promoter sequence.²⁷ As a run-off site, a different restriction site can be utilized if it is not present in the viral genome and if synthetic RNA from the linearized cDNA ends with the authentic 3' end. The importance of the 3'-end nucleotide sequence has been demonstrated by a ~10-fold decrease in RNA infectivity when a synthetic RNA contains three or four virus-unrelated nucleotides at its 3' end.²⁷ In an *in vitro* transcription reaction, both the m⁷G(5')ppp(5')A and m⁷G(5')ppp(5')G cap analog can be used equally well, although the latter places an unrelated extra G nucleotide upstream of the viral 5'-end, but that addition does not alter the infectivity or replication of synthetic RNA.²⁷ Moreover, removal of the cDNA template from the RNA transcripts by DNase I digestion is not necessary for RNA infectivity tests, because the cDNA template itself is not infectious.²⁷

The BAC technology has now been applied to constructing infectious cDNA clones for a handful of positive-strand RNA viruses, namely, two JEVs, CNU/LP2²⁷ and SA₁₄₋₁₄₋₂²⁸ (genome size, ~11 kb); two dengue viruses, BR/90²⁶ and NGC²⁹ (~11 kb); the bovine viral diarrhoea virus, SD1 (~12 kb);²⁵ two classical swine fever viruses, C and Paderborn (~12 kb);²⁴ the border disease virus, Gifhorn (~12 kb);²⁴ the porcine reproductive and respiratory syndrome virus, PL97-1/LP1 (~15 kb);³⁰ the transmissible gastroenteritis virus, PUR46-MAD (~29 kb);¹⁶ the feline infectious peritonitis virus, DF-2 (~29 kb);³² the severe acute respiratory syndrome coronavirus, Urbani (~30 kb);⁹ the Middle East respiratory syndrome coronavirus, EMC/2012 (~30 kb);¹⁷ and the human coronavirus, OC43 (~31 kb).³¹ The main advantage of using BACs for cDNA

construction is the high genetic stability of the large, 1- or 2-copy BAC plasmids; however, the intrinsic nature of its extremely low-copy number is also a great disadvantage, because of very low yields of BAC DNA and the consequent reduction in the purity of the BAC DNA with respect to host chromosomal DNA. In the current protocol, the yield of BAC DNA is maximized by growing *E. coli* DH10B transformed with the infectious BAC pBAC/SA₁₄-14-2 in a nutrient-rich medium, 2xYT. Despite this effort, the average yield is only ~15 µg of BAC DNA from 500 ml of 2xYT broth. Also, the purity of the BAC DNA is best achieved by using CsCl-EtBr density gradient centrifugation for purification, rather than the commonly used column-based plasmid isolation. However, it is important to keep in mind that the BAC-transformed *E. coli* should not overgrow because it might jeopardize the genetic stability of the cloned cDNA, and higher growth does not necessarily lead to greater yields or higher-purity BAC DNA.

The protocol described here is an optimized, efficient, and streamlined method for the construction and propagation of a genetically stable full-length infectious cDNA clone as a BAC for JEV, a procedure once thought practically impossible. This same cloning strategy may also be applied to many other positive-strand RNA viruses. In general, infectious cDNA clones enable us to introduce a variety of mutations (e.g., deletions, insertions, and point mutations) into a viral RNA genome to study their biological functions in viral replication and pathogenesis. This cDNA-based reverse genetics system makes it possible to develop and test vaccine and therapeutic candidates targeting a virulence factor(s) of a particular positive-strand RNA virus of interest. In addition, this infectious cDNA technology can also be utilized as a viral vector, capable of expressing a foreign gene(s) of interest for many applications in biomedical research.

Disclosures

The authors have declared that they have no competing financial interests.

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References

1. Bridgen, A. Reverse genetics of RNA viruses: applications and perspectives. West Sussex, UK, *Wiley-Blackwell*. (2013).
2. Taniguchi, T., Palmieri, M., Weissmann, C. QB DNA-containing hybrid plasmids giving rise to QB phage formation in the bacterial host. *Nature*. **274** (5668), 223-228 (1978).
3. Racaniello, V.R., Baltimore, D. Cloned poliovirus complementary DNA is infectious in mammalian cells. *Science*. **214** (4523), 916-919 (1981).
4. Stobart, C.C., Moore, M.L. RNA virus reverse genetics and vaccine design. *Viruses*. **6** (7), 2531-2550 (2014).
5. Boyer, J.C., Haenni, A.L. Infectious transcripts and cDNA clones of RNA viruses. *Virology*. **198** (2), 415-426 (1994).
6. Ahlquist, P., French, R., Janda, M., Loesch-Fries, L.S. Multicomponent RNA plant virus infection derived from cloned viral cDNA. *Proc Natl Acad Sci U S A*. **81** (22), 7066-7070 (1984).
7. Ahlquist, P., Janda, M. cDNA cloning and in vitro transcription of the complete brome mosaic virus genome. *Mol Cell Biol*. **4** (12), 2876-2882 (1984).
8. Ruggli, N., Rice, C.M. Functional cDNA clones of the Flaviviridae: strategies and applications. *Adv Virus Res*. **53**, 183-207 (1999).
9. Almazan, F., et al. Construction of a severe acute respiratory syndrome coronavirus infectious cDNA clone and a replicon to study coronavirus RNA synthesis. *J Virol*. **80** (21), 10900-10906 (2006).
10. Yount, B., Denison, M.R., Weiss, S.R., Baric, R.S. Systematic assembly of a full-length infectious cDNA of mouse hepatitis virus strain A59. *J Virol*. **76** (21), 11065-11078 (2002).
11. Yount, B., et al. Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. *Proc Natl Acad Sci U S A*. **100** (22), 12995-13000 (2003).
12. Masters, P.S., Rottier, P.J. Coronavirus reverse genetics by targeted RNA recombination. *Curr Top Microbiol Immunol*. **287**, 133-159 (2005).
13. Thiel, V., Siddell, S.G. Reverse genetics of coronaviruses using vaccinia virus vectors. *Curr Top Microbiol Immunol*. **287**, 199-227 (2005).
14. Donaldson, E.F., et al. Systematic assembly of a full-length infectious clone of human coronavirus NL63. *J Virol*. **82** (23), 11948-11957 (2008).
15. Scobey, T., et al. Reverse genetics with a full-length infectious cDNA of the Middle East respiratory syndrome coronavirus. *Proc Natl Acad Sci U S A*. **110** (40), 16157-16162 (2013).
16. Almazan, F., et al. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. *Proc Natl Acad Sci U S A*. **97** (10), 5516-5521 (2000).
17. Almazan, F., et al. Engineering a replication-competent, propagation-defective Middle East respiratory syndrome coronavirus as a vaccine candidate. *MBio*. **4** (5), e00650-00613 (2013).
18. Lai, M.M. The making of infectious viral RNA: No size limit in sight. *Proc Natl Acad Sci U S A*. **97** (10), 5025-5027 (2000).
19. O'Connor, M., Peifer, M., Bender, W. Construction of large DNA segments in *Escherichia coli*. *Science*. **244** (4910), 1307-1312 (1989).
20. Shizuya, H., et al. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. *Proc Natl Acad Sci U S A*. **89** (18), 8794-8797 (1992).
21. Stone, N.E., et al. Construction of a 750-kb bacterial clone contig and restriction map in the region of human chromosome 21 containing the progressive myoclonus epilepsy gene. *Genome Res*. **6** (3), 218-225 (1996).
22. Monaco, A.P., Larin, Z. YACs, BACs, PACs and MACs: artificial chromosomes as research tools. *Trends Biotechnol*. **12** (7), 280-286 (1994).
23. Preston, A. Choosing a cloning vector. *Methods Mol Biol*. **235**, 19-26 (2003).
24. Rasmussen, T.B., et al. Generation of recombinant pestiviruses using a full-genome amplification strategy. *Vet Microbiol*. **142** (1-2), 13-17 (2010).

25. Fan, Z.C., Bird, R.C. An improved reverse genetics system for generation of bovine viral diarrhea virus as a BAC cDNA. *J Virol Methods*. **149** (2), 309-315 (2008).
26. Suzuki, R., de Borja, L., Duarte dos Santos, C.N., Mason, P.W. Construction of an infectious cDNA clone for a Brazilian prototype strain of dengue virus type 1: characterization of a temperature-sensitive mutation in NS1. *Virology*. **362** (2), 374-383 (2007).
27. Yun, S.I., Kim, S.Y., Rice, C.M., Lee, Y.M. Development and application of a reverse genetics system for Japanese encephalitis virus. *J Virol*. **77** (11), 6450-6465 (2003).
28. Yun, S.I., *et al.* A molecularly cloned, live-attenuated Japanese encephalitis vaccine SA₁₄-14-2 virus: a conserved single amino acid in the ij hairpin of the viral E glycoprotein determines neurovirulence in mice. *PLoS Pathog*. **10** (7), e1004290 (2014).
29. Usme-Ciro, J.A., Lopera, J.A., Enjuanes, L., Almazan, F., Gallego-Gomez, J.C. Development of a novel DNA-launched dengue virus type 2 infectious clone assembled in a bacterial artificial chromosome. *Virus Res*. **180**, 12-22 (2014).
30. Choi, Y.J., Yun, S.I., Kang, S.Y., Lee, Y.M. Identification of 5' and 3' cis-acting elements of the porcine reproductive and respiratory syndrome virus: acquisition of novel 5' AU-rich sequences restored replication of a 5'-proximal 7-nucleotide deletion mutant. *J Virol*. **80** (2), 723-736 (2006).
31. St-Jean, J.R., *et al.* Recovery of a neurovirulent human coronavirus OC43 from an infectious cDNA clone. *J Virol*. **80** (7), 3670-3674 (2006).
32. Balint, A., *et al.* Molecular characterization of feline infectious peritonitis virus strain DF-2 and studies of the role of ORF3abc in viral cell tropism. *J Virol*. **86** (11), 6258-6267 (2012).
33. Thiel, H.J., *et al.* Family Flaviviridae. In: *Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses*. Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., & Ball, L.A., eds. San Diego, CA, Elsevier Academic Press, 981-998 (2005).
34. Yun, S.I., Lee, Y.M. Japanese encephalitis virus: molecular biology and vaccine development. In: *Molecular biology of the flavivirus*. Kalitzky, M., & Borowski, P., eds. Norwich, UK, Horizon Scientific Press, 225-271 (2006).
35. Gubler, D.J., Kuno, G., Markoff, L. Flaviviruses. In: *Fields virology*. Knipe, D.M., *et al.*, eds. Philadelphia, PA, Lippincott Williams & Wilkins Publishers, 1153-1252 (2007).
36. Solomon, T. Control of Japanese encephalitis-within our grasp? *N Engl J Med*. **355** (9), 869-871 (2006).
37. Halstead, S.B., Jacobson, J. Japanese encephalitis. *Adv Virus Res*. **61**, 103-138 (2003).
38. Endy, T.P., Nisalak, A. Japanese encephalitis virus: ecology and epidemiology. *Curr Top Microbiol Immunol*. **267**, 11-48 (2002).
39. Campbell, G.L., *et al.* Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ*. **89** (10), 766-774, 774A-774E (2011).
40. Tsai, T.F. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13-15 October 1998. *Vaccine*. **18 Suppl 2**, 1-25 (2000).
41. Sumiyoshi, H., *et al.* Complete nucleotide sequence of the Japanese encephalitis virus genome RNA. *Virology*. **161** (2), 497-510 (1987).
42. Yun, S.I., *et al.* Molecular characterization of the full-length genome of the Japanese encephalitis viral strain K87P39. *Virus Res*. **96** (1-2), 129-140 (2003).
43. Yun, S.I., Lee, Y.M. Japanese encephalitis: the virus and vaccines. *Hum Vaccin Immunother*. **10** (2), 263-279 (2014).
44. Lindenbach, B.D., Thiel, H.J., Rice, C.M. Flaviviridae: the viruses and their replication. In: *Fields virology*, 5th edn. Knipe, D.M., *et al.*, eds. Philadelphia, PA, Lippincott Williams & Wilkins Publishers, 1101-1152 (2007).
45. Firth, A.E., Atkins, J.F. A conserved predicted pseudoknot in the NS2A-encoding sequence of West Nile and Japanese encephalitis flaviviruses suggests NS1' may derive from ribosomal frameshifting. *Virol J*. **6**, 14 (2009).
46. Melian, E.B., *et al.* NS1' of flaviviruses in the Japanese encephalitis virus serogroup is a product of ribosomal frameshifting and plays a role in viral neuroinvasiveness. *J Virol*. **84** (3), 1641-1647 (2010).
47. Mukhopadhyay, S., Kim, B.S., Chipman, P.R., Rossmann, M.G., Kuhn, R.J. Structure of West Nile virus. *Science*. **302** (5643), 248 (2003).
48. Kuhn, R.J., *et al.* Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell*. **108** (5), 717-725 (2002).
49. Gillespie, L.K., Hoenen, A., Morgan, G., Mackenzie, J.M. The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. *J Virol*. **84** (20), 10438-10447 (2010).
50. Brinton, M.A. Replication cycle and molecular biology of the West Nile virus. *Viruses*. **6** (1), 13-53 (2014).
51. Welsch, S., *et al.* Composition and three-dimensional architecture of the dengue virus replication and assembly sites. *Cell Host Microbe*. **5** (4), 365-375 (2009).
52. Pijlman, G.P., Kondratieva, N., Khromykh, A.A. Translation of the flavivirus Kunjin NS3 gene in cis but not its RNA sequence or secondary structure is essential for efficient RNA packaging. *J Virol*. **80** (22), 11255-11264 (2006).
53. Kummerer, B.M., Rice, C.M. Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles. *J Virol*. **76** (10), 4773-4784 (2002).
54. Leung, J.Y., *et al.* Role of nonstructural protein NS2A in flavivirus assembly. *J Virol*. **82** (10), 4731-4741 (2008).
55. Patkar, C.G., Kuhn, R.J. Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions. *J Virol*. **82** (7), 3342-3352 (2008).
56. Liu, W.J., Chen, H.B., Khromykh, A.A. Molecular and functional analyses of Kunjin virus infectious cDNA clones demonstrate the essential roles for NS2A in virus assembly and for a nonconservative residue in NS3 in RNA replication. *J Virol*. **77** (14), 7804-7813 (2003).
57. Robertson, S.J., Mitzel, D.N., Taylor, R.T., Best, S.M., Bloom, M.E. Tick-borne flaviviruses: dissecting host immune responses and virus countermeasures. *Immunol Res*. **43** (1-3), 172-186 (2009).
58. Morrison, J., Aguirre, S., Fernandez-Sesma, A. Innate immunity evasion by dengue virus. *Viruses*. **4** (3), 397-413 (2012).
59. Diamond, M.S. Mechanisms of evasion of the type I interferon antiviral response by flaviviruses. *J Interferon Cytokine Res*. **29** (9), 521-530 (2009).
60. Gebhard, L.G., Filomatori, C.V., Gamamikh, A.V. Functional RNA elements in the dengue virus genome. *Viruses*. **3** (9), 1739-1756 (2011).
61. Markoff, L. 5'- and 3'-noncoding regions in flavivirus RNA. *Adv Virus Res*. **59**, 177-228 (2003).
62. Paranjape, S.M., Harris, E. Control of dengue virus translation and replication. *Curr Top Microbiol Immunol*. **338**, 15-34 (2010).
63. Yun, S.I., Choi, Y.J., Song, B.H., Lee, Y.M. 3' cis-acting elements that contribute to the competence and efficiency of Japanese encephalitis virus genome replication: functional importance of sequence duplications, deletions, and substitutions. *J Virol*. **83** (16), 7909-7930 (2009).
64. Song, B.H., *et al.* A complex RNA motif defined by three discontinuous 5-nucleotide-long strands is essential for Flavivirus RNA replication. *RNA*. **14** (9), 1791-1813 (2008).
65. Song, B.H., Yun, G.N., Kim, J.K., Yun, S.I., Lee, Y.M. Biological and genetic properties of SA₁₄-14-2, a live-attenuated Japanese encephalitis vaccine that is currently available for humans. *J Microbiol*. **50** (4), 698-706 (2012).

66. TRIzol LS Reagent [package insert]. *Life Technologies.*, Carlsbad, CA, November 15, 2010. https://tools.lifetechnologies.com/content/sfs/manuals/trizol_ls_reagent.pdf. Accessed December 01, (2014).
67. SuperScript III Reverse Transcriptase [package insert]. *Invitrogen.*, Carlsbad, CA, December 07, 2004. https://tools.lifetechnologies.com/content/sfs/manuals/superscriptIII_man.pdf. Accessed December 01, (2014).
68. PCR Using Q5 High-Fidelity DNA Polymerase [package insert]. *New England Biolabs.*, Ipswich, MA, December 13, (2013).
69. Sambrook, J., Russell, D.W. *Molecular cloning: a laboratory manual*. 3rd edn, Cold Spring Harbor, NY, *Cold Spring Harbor Laboratory Press.* (2001).
70. PureLink Quick Plasmid Miniprep Kits [Quick Reference]. *Life Technologies.*, Carlsbad, CA, April 25, 2011. https://tools.lifetechnologies.com/content/sfs/manuals/purelink_quick_plasmid_qrc.pdf. Accessed March 10, (2015).
71. PureLink HiPure Plasmid Filter Purification Kits [User Guide]. *Life Technologies.*, Carlsbad, CA, May 02, 2011. https://tools.lifetechnologies.com/content/sfs/manuals/purelink_hipure_plasmid_filter_purification_man.pdf. Accessed March 10, (2015).
72. SP6 Polymerase [product information]. *New England Biolabs.*, Ipswich, MA, January 01, (2015).
73. Kim, J.K., *et al.* Profiling of viral proteins expressed from the genomic RNA of Japanese encephalitis virus using a panel of 15 region-specific polyclonal rabbit antisera: implications for viral gene expression. *PLoS One.* **10** (4), e0124318, 10.1371/journal.pone.0124318 (2015).
74. Sumiyoshi, H., Hoke, C.H., Trent, D.W. Infectious Japanese encephalitis virus RNA can be synthesized from *in vitro*-ligated cDNA templates. *J Virol.* **66** (9), 5425-5431 (1992).
75. Mishin, V.P., Cominelli, F., Yamshchikov, V.F. A 'minimal' approach in design of flavivirus infectious DNA. *Virus Res.* **81** (1-2), 113-123 (2001).
76. Sumiyoshi, H., Tignor, G.H., Shope, R.E. Characterization of a highly attenuated Japanese encephalitis virus generated from molecularly cloned cDNA. *J Infect Dis.* **171** (5), 1144-1151 (1995).
77. Pu, S.Y., *et al.* Successful propagation of flavivirus infectious cDNAs by a novel method to reduce the cryptic bacterial promoter activity of virus genomes. *J Virol.* **85** (6), 2927-2941 (2011).
78. Yamshchikov, V., Mishin, V., Cominelli, F. A new strategy in design of +RNA virus infectious clones enabling their stable propagation in *E. coli*. *Virology.* **281** (2), 272-280 (2001).
79. Kim, J.M., *et al.* A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. *J Virol.* **82** (16), 7846-7862, 10.1128/JVI.00789-08 (2008).
80. Yun, S.I., *et al.* Engineering the Japanese encephalitis virus RNA genome for the expression of foreign genes of various sizes: implications for packaging capacity and RNA replication efficiency. *J Neurovirol.* **13** (6), 522-535, 10.1080/13550280701684651 (2007).
81. Yun, S.I., *et al.* Japanese encephalitis virus-based replicon RNAs/particles as an expression system for HIV-1 Pr55 Gag that is capable of producing virus-like particles. *Virus Res.* **144** (1-2), 298-305, 10.1016/j.virusres.2009.04.014 (2009).