

# Chapter 2

## Development and Characterization of an Infectious cDNA Clone of Equine Arteritis Virus

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### Abstract

Development and characterization of several infectious cDNA clones of equine arteritis virus (EAV) have been described in the literature. Here we describe the assembly of the full-length infectious cDNA clone of the virulent Bucyrus strain (VBS; ATCC VR-796) of EAV in a plasmid vector. This system allows generation of infectious in vitro-transcribed (IVT) RNA from the linearized plasmid that can be transfected or electroporated into mammalian cells to produce infectious recombinant progeny virus. This is an efficient reverse genetics system that allows easy manipulation of EAV genomes to study molecular biology of the virus and pathogenesis of equine viral arteritis.

**Key words** Equine arteritis virus, EAV, Equine viral arteritis, EVA, Arteriviruses, Infectious cDNA clone, Reverse genetics

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### 1 Introduction

It has long been known that positive-sense viral RNA is infectious and can generate progeny virus following its introduction into cells. Alexander and colleagues first demonstrated the infectivity of poliovirus RNA in HeLa cells [1, 2]. Subsequently, Racaniello and Baltimore developed the first infectious cDNA clone of poliovirus by cloning the full-length RNA genome into a bacterial plasmid vector [3, 4]. The advent of reverse transcription polymerase chain reaction (RT-PCR) technology in the mid-1980s, along with other recombinant DNA techniques, expedited the development of infectious cDNA clones of other RNA viruses [5, 6]. It was subsequently shown in numerous virus systems that in vitro transcripts of cDNA clones, and in some instances the cDNA itself, can initiate a complete productive infectious cycle in susceptible mammalian cells. As a result, genetic manipulation (reverse genetics) of full-length cDNA clones has become the most important tool with which to study the biology, pathogenesis, and virulence determinants of both positive- and negative-stranded RNA viruses.

Reverse genetic strategies are especially useful for identification and functional characterization of specific viral genes because they demonstrate phenotypic effect(s)/consequences of introducing defined nucleotide change(s) to the gene of interest.

EAV is included within the order *Nidovirales*, and it is the prototype virus of the genus *Arterivirus*, family *Arteriviridae*. Similar to other positive-stranded RNA viruses, the genomes of Arteriviruses are infectious to cells [7, 8]. The first full-length infectious cDNA clone of EAV was developed in 1996 by cloning 12 fragments from a cDNA library spanning the entire genome of a highly cell culture-adapted laboratory strain of EAV downstream of the T7 RNA polymerase promoter in the pUC18 plasmid vector (pEAV030 [GenBank accession number Y07862]) [9]. This was also the first full-length infectious cDNA clone constructed from a member of the order *Nidovirales*. A second infectious cDNA clone of a very similar, highly cell culture-adapted laboratory strain of EAV was described soon thereafter [10–12]. Subsequently, we developed two infectious cDNA clones of EAV: the first from the highly virulent, horse-adapted virulent Bucyrus strain (VBS) of EAV (pEAVrVBS [DQ846751]) [13] and the other from the MLV vaccine strain of EAV (ARVAC®, Zoetis, Kalamazoo, MI, USA, pEAVrMLV [FJ798195]) [14] that was originally developed by extended cell culture passage of the VBS virus.

Here we describe the assembly of the full-length infectious cDNA clone of the virulent Bucyrus strain (VBS; ATCC VR-796) of EAV in the pTRSB plasmid under the control of T7 RNA promoter. The EAV genome is in vitro transcribed (IVT) into RNA using the T7 RNA-dependent RNA polymerase enzyme. At the 3'-end, a 20 bp poly (A) tail is incorporated downstream of the EAV genome followed by a unique restriction site (Xho-I) for linearization of the plasmid to generate runoff IVT RNA. For cloning purposes, another unique restriction enzyme site (Xba-I) is incorporated upstream of the 5'-end of the T7 promoter. This system allows generation of infectious IVT RNA from the linearized plasmid for subsequent electroporation into a mammalian cell line to generate infectious progeny virus.

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## 2 Materials

### 2.1 Assembly of the Infectious cDNA Clone

1. Plasmid and *E. coli* strain.
  - (a) The pTRSB plasmid is available upon request from the authors of this chapter. It carries ampicillin-resistant gene for selection of recombinant clones.
  - (b) *E. coli* DH5α™ competent cells: These bacterial cells can be either purchased from Life Technologies (MAX Efficiency®

DH5 $\alpha$ <sup>TM</sup> Competent Cells) or prepared in the laboratory following the protocol described in Subheading 3.6.

2. Culture medium for *E. coli*.

- (a) LB medium (Luria-Bertani medium; 1 L): Deionized water 1000 mL, Bacto-tryptone 10 g, Bacto-yeast extract 5 g, and NaCl 10 g. Stir until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Sterilize by autoclaving for 20 min on liquid cycle.
- (b) LB agar plates: Prepare LB medium according to the above recipe. Just before autoclaving, add 15 g of Bacto agar/1000 mL of LB medium. Sterilize by autoclaving for 20 min on liquid cycle. After the medium is removed from the autoclave, swirl it gently to distribute the melted agar throughout the solution. Allow the medium to cool to 45–50 °C before adding antibiotics (ampicillin 50 µg/mL). To avoid air bubbles, mix the medium by swirling. Pour 20–25 mL of medium into a petri dish (90 mm). After medium has solidified completely, invert the plates, wrap in aluminum foil, and store them at 4 °C until needed. The plates should be removed from storage 1–2 h before they are used in order to allow them to dry.
- (c) LB freezing buffer: 40% (v/v) glycerol in LB medium. Sterilize by passing it through a 0.45 µm disposable filter.
- (d) SOB medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, and 2.5 mM KCl. Adjust the pH to 7.0 with 5 N NaOH and sterilize by autoclaving on liquid cycle (*see Note 1*).
- (e) SOC medium: SOB medium containing 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose. After autoclaving the SOB medium, cool to 45 °C and add the MgCl<sub>2</sub>, MgSO<sub>4</sub>, and glucose from filter-sterilized 1 M stock solutions.

3. Media and solutions for preparing competent *E. coli* cells.

- (a) Glucose-supplemented LB medium (500 mL): Bacto-tryptone 5.0 g, Bacto-yeast extract 2.5 g, NaCl 2.5 g, and glucose 0.5 g. Bring the volume to 500 mL with distilled water. Autoclave for 30 min on liquid cycle. Store at 4 °C.
- (b) Glycerol 100 mL: Autoclave for 30 min on liquid cycle. Store at 4 °C.
- (c) 1 M MgCl<sub>2</sub> stock (100 mL): MgCl<sub>2</sub>·6H<sub>2</sub>O (FW 203.30) 20.33 g in 100 mL of distilled water. Autoclave for 30 min on liquid cycle. Store at room temperature.
- (d) 1 M CaCl<sub>2</sub> stock (100 mL): CaCl<sub>2</sub>·2H<sub>2</sub>O (FW 47.02) 14.70 g in 100 mL of distilled water. Autoclave for 30 min on liquid cycle. Store at room temperature.

- (e) Prepare working solutions: 0.1 M  $\text{MgCl}_2$  working solution (100 mL) and 0.1 M  $\text{CaCl}_2$  working solution (100 mL; *see Note 2*).
- 4. Special buffers and solutions.  
Ampicillin stock (50 mg/mL): Dissolve solid ampicillin in sterile water to a final concentration of 50 mg/mL and filter through a 0.45  $\mu\text{m}$  filter. Store the solution in the dark at  $-20^\circ\text{C}$ .
- 5. Enzymes and buffers.  
Restriction endonucleases, T4 DNA ligase, high-fidelity DNA polymerase, and reverse transcriptase. These enzymes can be purchased from various commercial sources (*see Note 3*). Use the buffer supplied with the enzyme by the manufacturer.
- 6. Other Molecular Biology Kits, Reagents, and Other Materials
  - (a) QIAamp Viral RNA Mini Kit (Qiagen).
  - (b) QIAprep Spin Miniprep Kit (Qiagen).
  - (c) QIAgen Plasmid Maxi Kit (Qiagen).
  - (d) QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies Inc.).
  - (e) MagMAX<sup>TM</sup>-96 Viral RNA Isolation Kit (Life Technologies).
  - (f) Magnetic-Ring Stand (Life Technologies) for 96-well plates.
  - (g) U-bottom plates and lids (Evergreen Scientific).
  - (h) Orbital shaker (Multi-Microplate Genie, Scientific Industries Inc.).
  - (i) Proteinase K (Life Technologies).
  - (j) 100% Ethanol, molecular biology grade (Sigma).
  - (k) 100% Isopropanol, molecular biology grade (Sigma).
  - (l) Single-channel and multichannel pipets.
  - (m) RNase-free filter tips (aerosol-resistant tips).
  - (n) Protective gear: lab coat, gloves, and goggles.
  - (o) RNaseZap Solution (Life Technologies).
  - (p) Ice buckets and trays.
  - (q) Sterile autoclave bottles (250 mL) or tubes.
  - (r) RNase/DNase-free microcentrifuge tubes.
  - (s) Sterile screw-cap tubes.
  - (t) 0.45  $\mu\text{m}$  Filters.
  - (u) Sterile 15 and 50 mL conical tubes.
  - (v) Falcon 15 mL polypropylene tubes.
  - (w) Amicon Ultra<sup>®</sup> concentration columns (EMD Millipore).

## 2.2 Rescue of Recombinant Virus

1. Cells.
  - (a) Equine endothelial cells (EECs) are available upon request from the corresponding author of this chapter.
  - (b) Baby hamster kidney cells (BHK-21; ATCC, CCL-10, Manassas, VA, USA).
  - (c) Rabbit kidney cells (RK-13; ATCC, CCL-37, Manassas, VA, USA).
2. Cell culture medium.
  - (a) The EECs are maintained in Dulbecco's modified essential medium (Mediatech, Manassas, VA) with sodium pyruvate, 10% fetal bovine serum (FBS; HyClone Laboratories, Inc.), 100 U/mL penicillin-100 µg/mL streptomycin, and 2 mM L-glutamine (Mediatech).
  - (b) BHK-21 and RK-13 cells are maintained in Eagle's minimum essential medium (EMEM; Mediatech) supplemented with 10% ferritin-supplemented bovine calf serum (HyClone Laboratories, Inc), and 100 U/mL penicillin-100 µg/mL streptomycin (Gibco).
  - (c) Trypsin-EDTA solution: 0.25% (w/v) trypsin, 0.02% (w/v) EDTA.
3. In Vitro transcription reagents.
 

In vitro-transcribed (IVT) RNA synthesis from linearized plasmid can be performed either with a commercial kit (mMESSAGE mMACHINE® kit (Life Technologies)) or in-house assembly of the reaction using individually purchased reagents (m<sup>7</sup>G[5']PPP[5']G RNA cap structure analogue (New England BioLabs)), recombinant RNasin® ribonuclease inhibitor [40 U/µL], 5 µL of rATP, rCTP, rGTP, and rUTP [10 mM mix], 2.5 µL of 100 mM DTT, 2.5 µL of T7 RNA polymerase, and 1× transcription buffer (Promega).
4. Miscellaneous molecular biology-grade reagents.
 

Agarose, 10% SDS, 0.5 M EDTA (pH = 8.0), TE buffer (pH = 7.2), and gel-loading buffer (6×).
5. Special equipment.
 

Gene Pulser Xcell™ Electroporation Systems (Bio-Rad) or BTX electroporation system (Harvard Apparatus) fitted with electrodes spaced 0.4 cm.

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## 3 Methods

### 3.1 General Strategy for the Assembly of Full-Length EAV VBS cDNA Clone

The basic strategy for the generation of EAV infectious cDNA clone is described using the EAV VBS (GenBank accession number DQ846751) as a model. The assembly of the full-length infectious cDNA clone of EAV VBS is facilitated by the construction of two

**EAV VBS Genomic RNA 12.7kb**

5' 3' An

a b c d

T7 4,629 bp 8,527 bp

*Xba*-I *Eco*-RV *Xho*-I

RE digest with *Xba*I & *Eco*RV

RE digest with *Eco*-RV & *Xho*-I

pSin4.6 pSin8.5

AB CD

RE digestion of pSin4.6 and pSin8.5 with *Xho*I and *Eco*RV, and sub-clone the *Eco*RV-*Xho*I fragment from pSin8.5 into pSin4.6 plasmid

pEAVrVBS

T7Promoter

IVT RNA (infectious rVBSRNA transcripts)

BHK-21 or equine endothelial cells

Assembly

Replication Complex

rVBS

[illegible]

common restriction site (e.g., EcoRV) to be assembled into a full-length infectious cDNA clone of the virus (*see Note 4*). The full-length cDNA clone has the unique restriction sites selected in the first step, a T7 promoter sequence at the 5'-end followed by the cDNA sequence of the EAV VBS strain, and a 20 nt synthetic poly(A) at the 3'-end which is followed by a unique restriction site to be used for the linearization of the plasmid to generate infectious IVT RNA. All these elements have to be precisely assembled to produce synthetic IVT RNA bearing authentic 5'- and 3'-end of the viral genome (Fig. 1; *see Note 5*).

1. Cloning strategy.

The first step in developing an infectious cDNA clone is the careful design of the RT-PCR amplification and cloning of the full-length cDNA copy of desired EAV strain into a bacterial plasmid under a promoter (e.g., T7, SP6, or CMV; in this protocol, we describe the use of T7 promoter). Ideally, the master sequence of the EAV strain that is to be cloned should be obtained by sequencing the entire genome using high-fidelity reverse transcriptase and DNA polymerase enzymes. The primers for PCR amplification and sequencing could be designed for the conserved regions of the EAV sequences available in GenBank. Even though the sequence of the virus strain that is intended to be cloned is available in GenBank, it is a good idea to sequence the RNA from virus stock that is available in the laboratory. The entire genome sequence including the 5'- and 3'-end of the genome should be determined using 5'- and 3'-RACE technology. This will provide the master sequence of the virus available in the laboratory for the given strain, and help to determine whether it is identical to the published sequence available in GenBank. Once you confirm the sequence of your strain, that sequence should be used to design cloning strategy. Sequence analysis and primer design could be performed using Vector NTI (Life Technologies) or Genius 7.0.6 software (Biomatters Ltd.)

2. Selection of restriction endonuclease sites in the viral genome.

The next step in the assembly of the full-length cDNA clone is the selection of appropriate restriction endonuclease sites in the viral genome. These restriction sites must be present in the cloning vector (e.g., pTRSB plasmid). In the case of EAV VBS the restriction sites selected are EcoRV (natural site at nucleotide position 4228), Xba I (an engineered site upstream of T7 promoter), and XhoI (an engineered site downstream of poly(A) tail). Synthetic primers are designed according to the published sequences of EAV VBS, GenBank accession # DQ846750), and used for RT-PCR amplification of the EAV VBS genome in two overlapping fragments. Two synthetic oligonucleotide primers A and B (Fig. 1) are used to RT-PCR amplify the 5'-end of the EAV genome. The



positive-sense primer A (62 nt's) consists of 30 nucleotides of the extreme 5'-end of the EAV VBS genome with an engineered overhanging Xba I site and a T7 promoter sequence. The negative-sense primer B is 309 nucleotides downstream of the natural EcoRV site (at nt 4228). The PCR segment is 4629 base pairs and has a 5'-Xba I site as well as a T7 promoter sequence, and a natural EcoRV site at the 3'-end. Two synthetic oligonucleotide primers C and D (Fig. 1) are used for the RT-PCR amplification of the 3'-end of the EAV genome. The negative-sense primer D is complementary to the extreme 3'-end of the genome (last 24 nt of the EAV genome) and has an overhanging poly(T) tail (20 nt) and a XhoI restriction enzyme site. The positive-sense primer C is 33 nucleotides upstream of the natural EcoRV site (at nt 4228). The PCR segment is 8527 base pairs (Fig. 1) and has a 3'-poly(A) tail followed by a XhoI site, and a natural EcoRV site at the 5'-end.

### 3.2 RT-PCR

#### ***Amplification of EAV VBS Genome into Two Overlapping Fragments***

1. cDNA synthesis, PCR amplification, and cloning of EAV VBS
  - (a) Isolate genomic viral RNA from the tissue culture fluid containing EAV VBS strain using QIAamp viral RNA purification columns (QIAamp® viral RNA mini kit, Qiagen) according to the manufacturer's instructions. Alternatively, genomic viral RNA could also be isolated using MagMAX™-96 Viral RNA Isolation Kit (Life Technologies) according to the manufacturer's instructions.
  - (b) The first-strand cDNA is synthesized using the Superscript™ II RNase H-reverse transcriptase (Life Technologies [formerly Invitrogen]) according to the manufacturer's instructions. Two first-strand cDNAs are synthesized using primers B and D, respectively.
  - (c) Amplification of two long PCR fragments covering the EAV VBS entire genome is carried out according to the manufacturer's instructions with the Expand™ Long Template PCR System (Roche, Indianapolis, IN, USA) using the aforementioned primer pairs A and B, and C and D (*see Note 6*). This system utilizes a unique enzyme mixture containing Taq DNA polymerase (5'–3' polymerase activity) and Pwo DNA polymerase (3'–5' proofreading ability). The PCR products are concentrated (Amicon Ultra®, EMD Millipore, Billerica, MA, USA) and agarose gel purified using a commercial kit (QIAquick Gel Extraction Kit, Qiagen; *see Note 7*).

### 3.3 Assembly of the Full-Length Infectious cDNA Clone of EAV

1. Construction of intermediate plasmids containing the 5'- and 3'-EAV sequences.
 

The PCR segment containing the 5'-end of the genome is digested with XbaI and EcoRV and cloned into the pTRSB vector, which also has been cut with the same restriction enzymes.



This recombinant plasmid containing the 5'-end of the EAV VBS genome is named pSin4.6. The PCR segment containing the 3'-end of the genome is cut with EcoRV and XhoI and cloned into pTRSB vector, which also has been cut with the same restriction enzymes. This recombinant plasmid containing the 3'-end of the EAV VBS genome is named pSin8.5. These plasmids are transformed into *E. coli* (DH5 $\alpha$  or HB101; Life Technologies); the authenticity of each insert was confirmed by restriction enzyme digestion, and sequencing of multiple plasmids. Two plasmids with authentic 5'- and 3'-sequences of EAV are selected to be assembled into the full-length genome.

2. Amalgamation of 5'- and 3'-sequences to generate the full-length infectious cDNA clone of EAV.

The 3'-end of the EAV VBS genome from pSin8.5 plasmid is subcloned into the pSin4.6 plasmid. Briefly, the pSin8.5 recombinant plasmid is digested with EcoRV and XhoI restriction enzymes and the restriction fragment containing the 3'-end of the EAV VBS is gel-purified. This fragment is then subcloned into the pSin4.6 plasmid that has also been cut with the same restriction enzymes. This new recombinant plasmid contains the complete copy of the viral cDNA downstream of a T7 promoter. This plasmid is transformed into *E. coli* (DH5 $\alpha$ ; Life Technologies) and authenticity of the insert is confirmed by restriction enzyme digestion. One full-length plasmid clone is selected for nucleotide analysis, and the complete sequence is determined from both strands using automated sequencing. Recombinant plasmid DNA containing the authentic EAV genome (cDNA copy) is stored at  $-20^{\circ}\text{C}$ .

### **3.4 Transformation of Competent *E. coli* and Purification of Plasmid DNA**

1. Remove DH5 $\alpha$  cells from  $-80^{\circ}\text{C}$  and thaw on ice.
2. Gently mix DH5 $\alpha$  cells with pipet and aliquot 100  $\mu\text{L}$  into chilled polypropylene tubes (Falcon).
3. Dilute 1  $\mu\text{L}$  of plasmid DNA into 19  $\mu\text{L}$  sterile nuclease-free water. Add 2.5  $\mu\text{L}$  of 1:20 diluted plasmid DNA into 100  $\mu\text{L}$  of bacterial cells.
4. Incubate cells on ice for 30 min.
5. Preheat SOC medium in a  $42^{\circ}\text{C}$  water bath for use in **step 8** below.
6. Heat-shock cells for 45 s in a  $42^{\circ}\text{C}$  water bath (*see Note 8*).
7. Place on ice for 2 min.
8. Add 0.9 mL of preheated SOC medium.
9. Incubate the tubes at  $37^{\circ}\text{C}$  with 1 h shaking at 240 rpm.
10. After incubation at  $37^{\circ}\text{C}$  for 1 h with shaking, the bacteria and medium are transferred to a microcentrifuge tube, and centrifuged at  $4000 \times g$  for 3 min. Decant the supernatant and leave about 100  $\mu\text{L}$  supernatant in the tube. Resuspend the

cells and plate 70  $\mu\text{L}$  of cells onto one LB agar plate with 100  $\mu\text{g}/\text{mL}$  ampicillin. Plate the remaining 30  $\mu\text{L}$  of cells onto another LB agar plate with 100  $\mu\text{g}/\text{mL}$  ampicillin. Incubate the plates overnight at 37 °C.

11. The amplification and isolation of plasmid DNA are performed using standard procedures described for conventional plasmids. Select individual bacterial colonies (2–6 colonies) for screening (*see* **Note 9**). Inoculate 2 mL of LB broth containing ampicillin with an individual bacterial colony and incubate at 37 °C overnight in a shaker incubator (240 rpm). Purify the plasmid DNA using QIAprep Spin Miniprep Kit according to the manufacturer's instructions. Authenticity of the plasmid is confirmed by restriction digestion and sequencing. Prepare large-scale working plasmid stock by inoculating fresh LB broth medium (250 mL) containing ampicillin. Briefly dilute 0.1 mL of the culture into 250 mL of selective LB medium pre-warmed to 37 °C and grow the cells with vigorous shaking (250 rpm) in a 1 l flask at 37 °C for 12–16 h (overnight) until an OD value of 1.2–1.5 at 550 nm is reached. This cell density typically corresponds to the transition from a logarithmic to a stationary growth phase. Harvest the bacterial cells by centrifugation at  $6000 \times g$  for 15 min at 4 °C and purify the plasmid DNA with the QIAGEN Plasmid Maxi Kit.

### 3.5 Storage of Bacterial Cultures

1. Mix 0.5 mL of LB freezing medium with 0.5 mL of an overnight bacterial culture in a cryotube with a screw cap (*see* **Note 10**).
2. Vortex the culture to ensure that the glycerol is evenly dispersed, freeze in ethanol-dry ice, and transfer to –80 °C for long-term storage.
3. Alternatively, a bacterial colony can be stored directly from the agar plate without being grown in a liquid medium. Using a sterile pipet tip, scrape the bacteria from the agar plate, and resuspend the cells into 200  $\mu\text{L}$  of LB medium in a cryotube with a screw cap. Add an equal volume of LB freezing medium, vortex the mixture, and freeze the bacteria as described above.
4. To recover the bacteria, scrape the frozen surface of the culture with a sterile inoculating needle and then immediately streak the bacteria that adhere to the needle onto the surface of an LB agar plate containing appropriate antibiotics (e.g., ampicillin; *see* **Note 11**). Incubate the plate at 37 °C overnight. Return the original frozen culture to storage at –80 °C.

### 3.6 Preparation of DH5 $\alpha$ Competent Cells for Electroporation

Here we describe the protocol for preparing electrocompetent *E. coli* DH5 $\alpha$  cells from 500 mL of bacterial culture. All the steps of this protocol should be carried out under sterile conditions.

1. Streak out *E. coli* (DH5 $\alpha$ , HB101, or other strain) on an LB agar plate (without antibiotics) and incubate overnight at 37 °C.

2. The next day, pick a single bacterial colony and grow in 2 mL of LB medium (without antibiotics), with vigorous shaking (250 rpm) at 37 °C overnight. Take 0.5 mL of the prepared LB medium to use as a blank for the OD. Transfer overnight bacterial prep to a 2 L flask containing 500 mL of glucose-supplemented LB medium. Incubate at 37 °C with vigorous shaking (250 rpm). During this time, chill all solutions, centrifuge bottles and tubes on ice, turn on spectrophotometer, and set wavelength at 550 nm. After several hours (2.5–3 h), remove 0.5 mL of bacteria using a sterile pipet and check OD<sub>550</sub> (use LB/glucose as blank). Continue to check the culture until the OD<sub>550</sub> reaches 0.5–0.7 (bacteria double about every 20 min; *see Note 12*). As soon as the correct OD is achieved, immediately transfer the culture flask from the shaker to an ice/water bath. Swirl the culture flask occasionally for 5–20 min to ensure that cooling occurs uniformly. From this point on, it is crucial that the temperature of the bacteria does not rise above 4 °C.
3. Pour the bacteria into two chilled (ice-cold) 500 mL centrifuge bottles and spin at  $6000 \times g$  (6000 rpm in a Sorvall GS3 rotor) for 15–20 min at 4 °C.
4. Carefully decant the supernatant and place bottle with bacteria pellet on ice. Resuspend the pellet in 10 mL of ice-cold 0.1 M MgCl<sub>2</sub> using sterile 10 mL pipet. Once resuspended, add the remaining 90 mL of chilled 0.1 M MgCl<sub>2</sub>. Set on ice for 5 min and spin at  $6000 \times g$  (6000 rpm in a Sorvall GS3 rotor) for 20 min at 4 °C.
5. While spinning, transfer 8.6 mL of 0.1 M CaCl<sub>2</sub> to a 15 mL conical tube. Add 1.4 mL glycerol. Mix well and let sit on ice.
6. Carefully decant the supernatant and place the bottle on ice. Resuspend in 10 mL of ice-cold 0.1 M CaCl<sub>2</sub> using sterile 10 mL pipet. Once resuspended, add the remaining 81.4 mL of ice-cold 0.1 M CaCl<sub>2</sub>. Set on ice for 5 min and spin at  $6000 \times g$  (6000 rpm in a Sorvall GS3 rotor) for 20 min at 4 °C.
7. Decant supernatant well and place bottle on ice. Resuspend the bacteria in a chilled solution of 8.6 mL 0.1 M CaCl<sub>2</sub> containing 1.4 mL glycerol. Mix well and transfer 0.25 mL aliquots to microcentrifuge or screw-cap tubes (approximately 40 tubes) that have been placed in dry ice-methanol bath or drop tubes directly into liquid nitrogen container for quick freeze. Store bacteria at –80 °C until use. Once thawed, the cells should not be frozen again.

### 3.7 Linearization and Purification of the Plasmid DNA

1. Linearize approximately 10 µg of plasmid DNA per restriction digestion reaction. An example of restriction digestion reaction is given below.

Plasmid DNA	32.0 µL <sup>a</sup>
XhoI	4.0 µL
Buffer (10×)	4.0 µL
Sterile nuclease-free water	To 40.0 µL final volume <sup>b</sup>
Incubate the reaction tube at 37 °C for 2–4 h	

<sup>a</sup>Plasmid volume depends on the DNA concentration

<sup>b</sup>Reaction volume can be adjusted to 40 µL using nuclease-free water

2. Run 1 µL digested plasmid DNA on 1% agarose gel to make sure that the plasmid DNA is linearized. Use an appropriate DNA molecular weight marker on the gel (e.g., 1 kb DNA ladder, Life Technologies [formerly Invitrogen]).

Digested plasmid DNA	1.0 µL
6× loading buffer	2.0 µL
Sterile nuclease-free water	To 12.0 µL final volume

3. Add 1 µL of 20 mg/mL proteinase K, bring the volume to 100 µL with nuclease-free water, and incubate at 37 °C for 30 min.
4. Perform phenol:chloroform extraction twice and precipitate the DNA with 100% ethanol as follows:
  - (a) Add 100 µL of phenol:chloroform:isoamyl alcohol (25:24:1) and mix until an emulsion is formed.
  - (b) Centrifuge at  $16,000 \times g$  (13,000 rpm) for 4 min at room temperature.
  - (c) Remove 90 µL of the upper aqueous phase and transfer into a new tube.
  - (d) Add 90 µL of phenol:chloroform:isoamyl alcohol (25:24:1) and repeat the extraction.
  - (e) Remove 80 µL of the upper aqueous phase and transfer to a new tube.
  - (f) Add 250 µL of 100 or 96% ethanol and 8 µL of 3 M sodium acetate to the DNA sample.
  - (g) Mix and then centrifuge at  $13,000 \times g$  for 10 min.
  - (h) Wash with 70 µL of 70% ethanol.
  - (i) Spin at  $13,000 \times g$  for 4 min.
  - (j) Aspirate the 70% ethanol and dry on the bench for 4–5 min.

5. Resuspend the pellet in 16  $\mu\text{L}$  of nuclease-free water and run 1  $\mu\text{L}$  on 1% agarose gel.
6. Store the linearized plasmid at  $-20^\circ\text{C}$  until further use.

### **3.8 *In Vitro* Transcription of Linearized Plasmid DNA**

1. The transcription of cDNA to RNA is carried out with T7 RNA polymerase (MEGAscript kit; Life Technologies). The reaction is performed according to the manufacturer's instructions. The recombinant RNA produced will be capped and have a poly(A) tail; thus when it is transfected into the cells, it will be treated as messenger RNA.
2. Alternatively, the in vitro transcription reaction can be set up by combining various commercial reagents purchased individually.

Linear plasmid DNA (1 $\mu\text{g}$ )	15.0 $\mu\text{L}$
rNTPs (10 mM each)	5.0 $\mu\text{L}$
BSA 1 mg/mL	5.0 $\mu\text{L}$
100 mM DTT	2.5 $\mu\text{L}$
5 $\times$ Transcription buffer	10.0 $\mu\text{L}$
Cap analogue	5.0 $\mu\text{L}$
RNase inhibitor guard	2.5 $\mu\text{L}$
T7 RNA polymerase	2.5 $\mu\text{L}$
Nuclease-free water	To 50.0 $\mu\text{L}$ final volume
Incubate at $37^\circ\text{C}$ for 1 h	

3. Run 2  $\mu\text{L}$  of in vitro-transcribed RNA on a 0.8% agarose gel.

TE buffer (pH = 7.2)	8.0 $\mu\text{L}$
10% SDS	1.0 $\mu\text{L}$
0.5 M EDTA (pH = 8.0)	0.25 $\mu\text{L}$
RNA	2.0 $\mu\text{L}$
Place mix at $70^\circ\text{C}$ for 2 min and then place on ice for 2 min	
Add 2.25 $\mu\text{L}$ of 6 $\times$ gel-loading buffer	

### **3.9 *Transfection* of Mammalian Cells with IVT RNA and Rescue of the Recombinant Virus**

Infectious virus is recovered by transfection of susceptible mammalian (equine endothelial or BHK-21) cells with the IVT RNA derived from the full-length cDNA clone. The following protocol is indicated for a 35 mm diameter dish and can be scaled up or down if desired.

1. Preparation of Mammalian Cells for Electroporation of IVT RNA

- (a) Split cells (EECs or BHK-21 cells) on day before to be 90–95% confluent by next day. As rule of thumb, propagate cells in 150 cm<sup>2</sup> flasks, and usually this will give enough cells to perform 3–4 electroporations per flask of cells ( $2.5 \times 10^7$ ; *see* **Note 13**).
  - (b) Treat cells with trypsin following standard laboratory protocol.
  - (c) After cells slough off, add 10 mL MEM containing FBS to inactivate residual trypsin.
  - (d) Use a pipet with a wide bore to transfer cells to a 50 mL conical centrifuge tube. Place on ice immediately.
  - (e) Spin cells at 4 °C,  $300 \times g$  (700–800 rpm), for 6 min. Place cells back on ice.
  - (f) Remove medium with a pipet and add 25 mL sterile ice-cold PBS (pH = 7.4). Resuspend the cell pellet by gently shaking the tube. Cells also can be resuspended by using a wide-bore pipet.
  - (g) Repeat step (e).
  - (h) Remove PBS as before and again wash by the addition of 25 mL ice-cold PBS. At this time, take a small sample of cells for counting (i.e., make a 1:20 dilution by adding 50  $\mu$ L of cells into 950  $\mu$ L of PBS for counting).
  - (i) Repeat step (e). While cells are spinning, conduct a cell count with a hemocytometer or an automated cell counter.
  - (j) After cells have been spun down, place them back on ice and remove PBS with a pipet. Resuspend cell pellet in ice-cold PBS to a final concentration of  $1 \times 10^7$  cells/mL using the count.
2. Electroporation of Mammalian Cells with IVT RNA.
- (a) Set the electroporator to desired voltage (*see* **Note 14**). Gene Pulser (Bio-Rad, Hercules, CA, USA): 1500 V, capacitance at 25  $\mu$ F, and resistance at infinity Ohms or BTX 600 (Harvard Apparatus, Holliston, MA): 260 V, capacitance at 950  $\mu$ F, and 13  $\Omega$ .
  - (b) Place 10  $\mu$ L of freshly thawed transcription mix (~10–20  $\mu$ g IVT RNA) into each electroporation cuvette (0.2 cm, Bio-Rad, Hercules, CA, USA, or 0.4 cm, BTX, Harvard Apparatus, Holliston, MA, USA) to be used (*see* **Note 15**).
  - (c) Place 500  $\mu$ L of cells ( $5 \times 10^6$ ) into each cuvette. Addition of cells will mix with the IVT RNA. Do not mix the cells and IVT RNA by inverting the cuvette because this will generate bubbles.

- (d) Place the cuvette into the cuvette holder and pulse once (BTX) or twice (Gene Pulser; push the buttons until you hear a beep, then immediately push them again until you hear the second beep [time constant reading should appear within the range of 7.0–7.4]).
  - (e) After electroporation is complete, set cells aside at room temperature for a 10-min “recovery period.”
  - (f) After the recovery period is complete, transfer cells from the cuvette with a Pasteur pipet into 10 mL of cell culture medium in a 15 mL conical tube at room temperature. For immunofluorescence staining, transfer approximately 150  $\mu$ L of cells into a chamber slide. Then transfer the remaining cells and medium into a single 100 mm petri dish. Sometimes two electroporations can be combined into one petri dish.
3. Rescue and Characterization of Recombinant Virus
- (a) Examine transfected cells for expression of EAV nonstructural protein-1 (e.g., nsp-1) and/or structural proteins (e.g., GP5 and N) 12–18 h post-transfection by indirect immunofluorescence staining using protein-specific monoclonal antibodies. Immunofluorescence staining will confirm the infectivity of the transfected RNA.
  - (b) Incubate the petri dish at 37 °C for 3–5 days until a clear cytopathic effect is observed. Three to five days after transfection (or as soon as the monolayers show significant cytopathic effect), the medium is collected by centrifugation for 10 min at 2000  $\times g$ . The supernatant will contain the new virus (P0) produced by infectious recombinant RNA. Small aliquots (0.5–1.0 mL) of supernatant are stored at –80 °C.
  - (c) Analyze the presence of recombinant virus in the supernatant by titration.
  - (d) Analyze the genotypic and phenotypic properties of the recovered virus (*see* **Note 16**).

**3.10 Further  
Manipulation  
of the Infectious cDNA  
Clone Using Site-  
Directed Mutagenesis**

Genetic manipulation of full-length cDNA clones using reverse genetics has become an important and widely used tool to study the biology, pathogenesis, neutralization, and virulence determinants of EAV. Reverse genetic manipulation of EAV infectious cDNA clones can be successfully performed using QuikChange II XL Site-Directed Mutagenesis Kit (Cat # 200522, Agilent Technologies Inc., Santa Clara, CA, USA [formerly Stratagene]). The mutagenic (e.g., mutations, insertions, or deletions) oligonucleotide primers used in this protocol are designed individually following the guidelines provided by the manufacturer.



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## 4 Notes

1. To avoid arcing during electroporation, SOB medium and PBS used in this protocol should be prepared free of  $Mg^{2+}$ .
2. Stock solutions can be sterilized by autoclaving and stored at room temperature. 0.1 M solutions should be prepared fresh.
3. It is important to use high-fidelity enzymes for RT-PCR amplification. This will reduce the inadvertent introduction of nucleotide changes during RT-PCR amplification.
4. After each cloning step, the PCR-amplified fragments and cloning junctions have to be sequenced to determine that no undesired mutations are introduced.
5. Recently, we have described the in silico design and de novo synthesis of a full-length infectious cDNA clone of the horse-adapted virulent Bucyrus strain (VBS) of EAV encoding mCherry [15]. This de novo nucleotide synthesis technology facilitated innovative viral vector design without the tedium and risks posed by more complicated conventional cloning techniques described in this chapter.
6. There are several new and improved high-fidelity DNA polymerase enzymes that can be used as alternatives to the enzyme-mentioned in this protocol.
7. Ethidium bromide-DNA complex excitation by UV light may cause photo bleaching of the dye and single-strand breaks. To minimize both effects, use a long-wavelength UV illumination (302 nm instead of 254 nm) to cut the desired DNA bands from the agarose gel.
8. Instead of transformation of *E. coli* by “heat shock,” they can be transformed by electroporation following the manufacturer’s instructions. However, the presence of salt increases the conductivity of the transformation solution and could cause arcing during the electrical pulse, drastically reducing the transformation efficiency. If arcing occurs, use a smaller amount of the ligation reaction in the electroporation or remove salt from the DNA using a commercial kit or by extraction with phenol:chloroform followed by precipitation with ethanol and 2 M ammonium acetate.
9. Cultures of transformed bacteria should be grown from a single colony isolated from a freshly streaked selective plate. Subculturing directly from glycerol stocks or plates that have been stored for a long time may lead to loss of the construct.
10. Alternatively, aliquot 0.85 mL of bacterial culture medium into a freezing vial and add 0.15 mL of sterile glycerol (sterilized by autoclaving for 20 min at 15 lb./sq. inch on liquid cycle). Vortex the culture to disperse glycerol evenly.

11. Alternatively, scrape the frozen surface of the culture with a sterile plastic pipet tip and then immediately drop the tip into 2 mL of LB broth containing appropriate antibiotics (e.g., ampicillin). Incubate the plate at 37 °C overnight in a shaker incubator.
12. For efficient cell transformation, bacterial culture OD at 550 nm should not exceed 0.8. To ensure that the culture does not grow to a higher density, OD measurement every 20 min after 3 h of growth is highly recommended.
13. Cells must be subconfluent at the time of harvest. Do not overtrear cells with trypsin and do not pipet aggressively. Once cells are on ice, keep them at 4 °C during all subsequent steps and do not let cells settle in the cuvette. After the transcripts and cells are added, proceed to the electroporation within a minute or so.
14. Nucleofactor™ devices (Lonza Walkersville Inc., Walkersville, MD, USA) can provide higher electroporation efficiency compared to standard electroporation units.
15. Most electroporation machines contain programs with defined parameters for transforming specific cell types. In this case, choose the program containing the conditions closest to those described in this protocol.
16. Silent mutations introduced in the viral genome to generate new restriction sites can be used as genetic markers to identify the recombinant virus recovered from the infectious clone.

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