Chapter 13

Engineering Infectious cDNAs of Coronavirus as Bacterial Artificial Chromosomes

Fernando Almazán, Silvia Márquez-Jurado, Aitor Nogales, and Luis Enjuanes

Abstract

The large size of the coronavirus (CoV) genome (around 30 kb) and the instability in bacteria of plasmids carrying CoV replicase sequences represent serious restrictions for the development of CoV infectious clones using reverse genetic systems similar to those used for smaller positive sense RNA viruses. To overcome these problems, several approaches have been established in the last 13 years. Here we describe the engineering of CoV full-length cDNA clones as bacterial artificial chromosomes (BACs), using the Middle East respiratory syndrome CoV (MERS-CoV) as a model.

Key words Coronavirus, MERS, Reverse genetics, Infectious clones, Bacterial artificial chromosomes

1   Introduction

Coronaviruses (CoV) are enveloped, single-stranded, positive-sense RNA viruses relevant in animal and human health [1, 2]. Historically, CoV infection in humans has been associated with mild upper respiratory tract diseases [1]. However, the identification of the severe acute respiratory syndrome CoV (SARS-CoV) in 2003 [3] and the recently emerged (April 2012) Middle East respiratory syndrome CoV (MERS-CoV) [4], which has been associated with acute pneumonia, redefined historic perceptions and potentiated the relevance of CoVs as important human pathogens. In this sense, the development of CoV infectious clones provides a valuable molecular tool to study fundamental viral processes, to develop genetically defined vaccines, and to test antiviral drugs. However, the generation of CoV infectious clones has been hampered for a long time due to the huge size of the CoV genome (around 30 kb) and the toxicity of some CoV replicase gene sequences during its propagation in bacteria. Recently, these problems were overcome using nontraditional approaches based
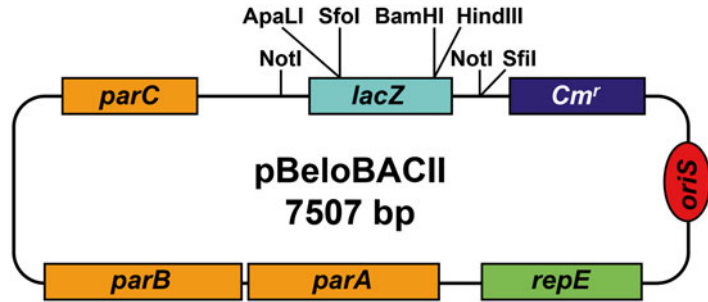
Fig. 1 Schematic of plasmid pBeloBAC11. The regulatory genes *parA*, *parB*, *parC*, and *repE*, the F-factor replication origin (*OriS*), the chloramphenicol resistance gene (*Cmr*), the *lacZ* gene, and the restriction sites that can be used to clone foreign DNAs are indicated

on the use of bacterial artificial chromosomes (BACs) [5], in vitro ligation of cDNA fragments [6], and vaccinia virus as a vector for the propagation of CoV full-length cDNAs [7, 8].

In this chapter we describe the protocol to assemble CoV full-length cDNAs in BACs using the MERS-CoV EMC12 strain [9] as an example. In this system, the full-length cDNA copy of the viral genome is assembled in the BAC plasmid pBeloBAC11 [10] (Fig. 1), a low-copy-number plasmid based on the *Escherichia coli* (*E. coli*) F-factor [11] that presents a strictly controlled replication leading to one or two plasmid copies per cell. This plasmid minimizes the instability problem of several CoV sequences when amplified in high-copy-number plasmids, allows the stable maintenance of large DNA fragments in bacteria [11], and its manipulation is similar to that of conventional plasmids. The cDNA of the CoV genome is assembled in the BAC under the control of the cytomegalovirus (CMV) immediate-early promoter and it is flanked at the 3′-end by a 25-bp synthetic poly(A) followed by the sequences of the hepatitis delta virus (HDV) ribozyme and the bovine growth hormone (BGH) termination and polyadenylation signals to produce synthetic RNAs bearing authentic 5′- and 3′-ends of the viral genome. This DNA-launched system couples expression of the viral RNA in the nucleus from the CMV promoter [12] with a second amplification step in the cytoplasm driven by the viral polymerase, allowing the recovery of infectious virus from the cDNA clone without the need for in vitro ligation and transcription steps. Although some splicing events could occur during the nuclear expression of the viral genome, the efficiency of this phenomenon is very low and does not affect the recovery of infectious virus [5].

The BAC approach, originally applied to the transmissible gastroenteritis coronavirus (TGEV) [5], has been successfully used to engineer the infectious clones of the feline infectious peritonitis virus (FIPV) [13] and the human CoVs (HCoVs): HCoV-OC43 [14],

SARS-CoV [15], and MERS-CoV [16], and it is potentially applicable to the cloning of other CoV cDNAs, other viral genomes, and large-size RNAs of biological relevance.

2   Materials

To reach optimal results, all solutions should be prepared using pure Milli-Q grade water (resistivity of 18.2 MΩ/cm at 25 °C) and analytical grade reagents.

2.1   Assembly and Manipulation of BAC Clones

2.1.1   Plasmids and Bacterial Strains

1. Plasmid pBeloBAC11 [10]. This plasmid contains genes *parA*, *parB*, and *parC* derived from the *E. coli* F-factor to ensure the accurate partitioning of plasmids to daughter cells, avoiding the possibility of coexistence of multiple BACs in a single cell. In addition, this plasmid carries gene *repE* and the element *oriS* involved in initiation and orientation of DNA replication, the chloramphenicol resistance gene (*Cmr*), the *lacZ* gene to allow color-based identification of recombinants by α-complementation, and the restriction sites ApaLI, SfoI, BamHI, HindIII, and SfiI to clone large DNA fragments (Fig. 1).

2. pBeloBAC11^{-StuI}, a pBeloBAC without the StuI restriction site.

3. *E. coli* DH10B strain [F⁻ *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) Ø80d*lacZ* ΔM15 Δ*lacX*74 *deo*R *rec*A1 *end*A1 *ara*D139 (*ara, leu*)7697 *gal*U *gal*K λ⁻ *rps*L *nup*G] (*see* **Note 1**).

4. DH10B electrocompetent cells. These bacterial cells could be purchased or prepared following the procedure described in Subheading 3.2.3.

2.1.2   Culture Media for E. coli

1. LB medium: 1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl. Adjust the pH to 7.0 with 5 N NaOH. Sterilize by autoclaving on liquid cycle.

2. LB agar plates: LB medium containing 15 g/l of Bacto Agar. Prepare LB medium and just before autoclaving add 15 g/l of Bacto Agar. Sterilize by autoclaving on liquid cycle and dispense in 90-mm petri plates.

3. LB agar plates containing 12.5 μg/ml chloramphenicol. After autoclaving the LB agar medium, allow the medium to cool to 45 °C, add the chloramphenicol to a final concentration of 12.5 μg/ml from a stock solution of 34 mg/ml, and dispense in 90-mm petri plates.

4. SOB medium: 2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.05 % (w/v) NaCl, 2.5 mM KCl. Adjust the pH to 7.0 with 5 N NaOH and sterilize by autoclaving on liquid cycle (*see* **Note 2**).

5. SOC medium: SOB medium containing 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose. After autoclaving the SOB medium, cool to 45 °C and add the $MgCl_2$, $MgSO_4$ and glucose from filter sterilized 1 M stock solutions.

2.1.3 Enzymes and Buffers

1. Restriction endonucleases.
2. Shrimp alkaline phosphatase.
3. T4 DNA ligase.
4. Taq DNA polymerase.
5. High-fidelity thermostable DNA polymerase.
6. Reverse transcriptase.
7. dNTPs.
8. Enzyme reaction buffers. Use the buffer supplied with the enzyme by the manufacturer.

2.1.4 Special Buffers and Solutions

1. LB freezing buffer: 40 % (v/v) glycerol in LB medium. Sterilize by passing it through a 0.45-μm disposable filter.
2. Chloramphenicol stock (34 mg/ml). Dissolve solid chloramphenicol in ethanol to a final concentration of 34 mg/ml and store the solution in a light-tight container at –20 °C. This solution does not have to be sterilized.
3. Ice-cold 10 % glycerol in sterile water.

2.1.5 Reagents

1. Qiagen QIAprep Miniprep Kit.
2. Qiagen Large-Construct Kit.
3. Qiagen QIAEX II Kit.

2.1.6 Special Equipment

1. Equipment for electroporation.
2. Cuvettes fitted with electrodes spaced 0.2 cm.

2.2 Rescue of Recombinant Viruses

2.2.1 Cells

1. Baby hamster kidney cells (BHK-21).
2. Human liver-derived Huh-7 cells (*see* **Note 3**).

2.2.2 Cell Culture Medium, Solutions and Reagents

1. Cell growth medium: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 % nonessential amino acids, gentamicin (50 mg/ml), and 10 % fetal calf serum (FCS).
2. Opti-MEM I Reduced Serum Medium.
3. Trypsin–EDTA solution: 0.25 % (w/v) trypsin, 0.02 % (w/v) EDTA.
4. Lipofectamine 2000.

3   Methods

3.1   Assembly of Full-Length CoV cDNAs in BACs

The basic strategy for the generation of CoV infectious clones using BACs is described for the MERS-CoV EMC12 strain (GenBank accession number JX869059) [9] as a model (Fig. 2).

3.1.1   Selection of Restriction Endonuclease Sites in the Viral Genome

1. The first step for 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 absent in the BAC plasmid (*see* **Note 4**). In the case of MERS-CoV, the restriction sites BamHI (genomic position 806), StuI (genomic positions 7,620 and 9,072), SwaI (genomic position 20,898), and PacI (genomic position 25,836) were selected to assemble the infectious clone (Fig. 2).
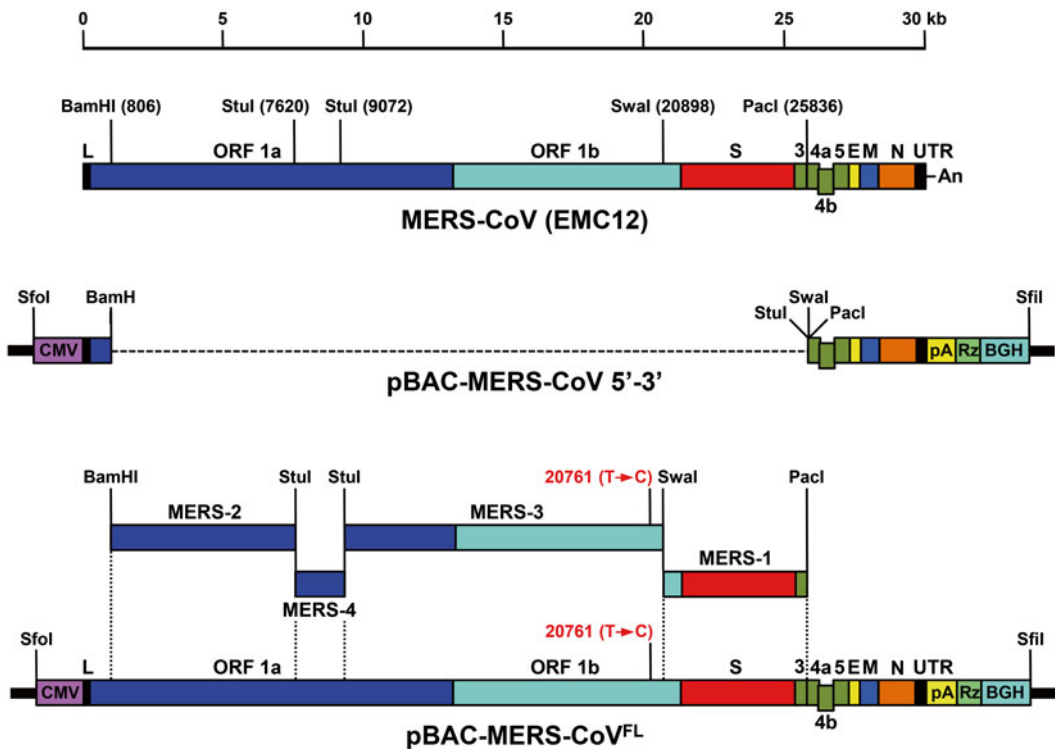
Fig. 2 Strategy to assemble a MERS-CoV infectious cDNA clone as a BAC. After selection of appropriate restriction sites in the genome of the MERS-CoV EMC12 strain (*top* of the figure), the intermediate plasmid pBAC-MERS-CoV 5′–3′ was generated and used as the backbone to assemble the full-length cDNA clone (pBAC-MERS-CoV^FL) by sequential cloning of four overlapping cDNA fragments (MERS-1 to MERS-4) covering the entire viral genome. The full-length clone is assembled in BAC under the control of the CMV promoter and it is flanked at the 3′-end by a 25-bp poly(A) tail (pA) followed by the HDV ribozyme (Rz) and the BGH termination and polyadenylation sequences (BGH). The viral genes (ORF 1a, ORF 1b, S, 3, 4a, 4b, 5, E, M, and N), relevant restriction sites (genomic positions in *brackets*) and the genetic marker (T to C) introduced at position 20,761 to abrogate the SwaI restriction site at position 20,760 are indicated. *L* leader sequence, *UTR* untranslated region. Figure adapted from ref. [16]

2. In case that no adequate restriction sites were available in the viral genome, new restriction sites, appropriately spaced in the viral genome, could be generated by the introduction of silent mutations. In addition, natural restriction sites could be eliminated following the same approach to facilitate the assembly of the infectious clone (*see* **Note 5**).

3.1.2 Construction of an Intermediate BAC Plasmid as the Backbone to Assemble the Full-Length cDNA Clone

The assembly of the infectious clone in a BAC is facilitated by the construction of an intermediate BAC plasmid containing the 5′-end of the genome (until the first restriction site selected) under the control of the CMV promoter, a multicloning site containing the restriction sites selected in the first step, and the 3′-end of the genome (from the last restriction site selected to the end of the genome) followed by a 25-nt poly(A) tail, the HDV ribozyme, and the BGH termination and polyadenylation sequences. All these elements have to be precisely assembled to produce synthetic RNAs bearing authentic 5′- and 3′-ends of the viral genome. A detailed protocol for the generation of the MERS-CoV intermediate plasmid, pBAC-MERS-CoV 5′-3′, is described next (Fig. 2).

1. Generate by chemical synthesis a DNA fragment containing the CMV promoter [12] precisely fused to the first 811 nt of the viral genome (from the first nucleotide to the restriction site BamHI), flanked at the 5′- and 3′-ends by restriction sites SfoI and BamHI, respectively. Alternatively, this DNA fragment could be generated by PCR using two overlapping PCR fragments as template (*see* **Notes 6** and **7**). One of these fragments should contain the CMV promoter flanked at the 5′-end by the restriction site SfoI and at the 3′-end by the 20 first nucleotides of the genome as overlapping sequence. The second overlapping PCR fragment should expand from the first nucleotide to the restriction site BamHI.

2. The generated DNA fragment is digested with SfoI and BamHI, and cloned into pBeloBAC11[-StuI] digested with the same restriction enzymes to generate the plasmid pBAC-MERS-CoV 5′.

3. Generate a second DNA fragment, containing the last 4,272 nt of the viral genome (from the restriction site PacI at genomic position 25,836 to the end of the genome) precisely joined to a 25-nt poly(A) tail, the HDV ribozyme, and the BGH termination and polyadenylation sequences, flanked at the 5′-end by a multicloning site with the restriction sites selected before (BamHI, StuI, SwaI, and PacI) and at the 3′-end by the restriction site SfiI. This DNA fragment could be generated by chemical synthesis or by overlapping PCR as described before (*see* **Notes 6** and **7**).

4. Digest with BamHI and SfiI the second DNA fragment (containing the multicloning site, the viral 3′-end followed by the

poly(A) tail, and the HDV-BGH sequences) and clone it into the plasmid pBAC-MERS-CoV 5′ digested with the same restriction enzymes to generate the intermediate plasmid pBAC-MERS-CoV 5′-3′ (Fig. 2).

5. After each cloning step, the integrity of the cloned DNA fragments is verified by restriction analysis and sequencing.

3.1.3 Assembly of the Full-Length cDNA Clone

1. The full-length cDNA clone (pBAC-MERS-CoV^FL) is assembled by sequential cloning of overlapping DNA fragments (MERS-1 to MERS-4), covering the entire viral genome, into the intermediate plasmid pBAC-MERS-CoV 5′-3′ using the restriction sites selected in the first step of the cloning strategy (Fig. 2) (*see* **Note 8**).

2. The overlapping DNAs flanked by the appropriated restriction sites are generated either by chemical synthesis or by standard reverse transcriptase PCR (RT-PCR) (*see* **Note 6**) using specific oligonucleotides and total RNA from infected cells as template. In the case of fragment MERS-3, a silent mutation (T to C) was introduced at position 20,761 to abrogate the SwaI restriction site at position 20,760. This mutation facilitates the cloning process and can be used as a genetic marker to identify the virus recovered from the full-length cDNA clone.

3. The genetic integrity of the cloned DNAs is verified throughout the subcloning and assembly process by extensive restriction analysis and sequencing.

3.2 Generation and Manipulation of BAC Clones

One of the major advantages of using BAC vectors to generate infectious clones is that the manipulation of BAC clones is relatively easy and essentially the same as that of a conventional plasmid with slight modifications due to the huge size of the BAC clones and the presence of this plasmid in only one or two copies per cell [11]. The amplification and isolation of BAC plasmids is performed using standard procedures described for conventional plasmids but using large volumes of bacterial cultures.

3.2.1 Isolation of BAC Plasmids from Small-Scale Cultures

Small amounts of BAC DNAs are prepared from 5 ml cultures of BAC transformed DH10B cells by the alkaline lysis method. Any commercial kit could be used, but we suggest the QIAprep Miniprep Kit (Qiagen) following the recommendations for purification of large low-copy plasmids.

1. Streak the bacterial stock containing the BAC plasmid onto a LB agar plate containing 12.5 μg/ml chloramphenicol and incubate for 16 h at 37 °C (*see* **Note 9**).

2. Inoculate a single colony in 5 ml of LB medium plus 12.5 μg/ml chloramphenicol in a flask with a volume of at least four times the volume of the culture and incubate for 16 h at 37 °C with vigorous shaking (250 rpm) (*see* **Note 10**).

3. Harvest the bacterial cells in 15 ml centrifuge tubes by centrifugation at $6,000 \times g$ for 10 min at 4 °C and pour off the supernatant fluid.

4. Purify the BAC plasmid following the manufacturer's instructions. Owing to the size of BAC DNAs and the need to use large culture volumes, we recommend duplicating the volume of buffers P1, P2, and N3, performing the optional wash step with buffer PB, and eluting the DNA from the QIAprep membrane using buffer EB preheated at 70 °C (*see* **Note 11**).

5. Depending of the BAC size, yields of 0.1–0.4 µg can be obtained. Although the BAC DNA prepared by this method is contaminated with up to 30 % of bacterial genomic DNA, it is suitable for analysis by restriction enzyme digestion or PCR.

3.2.2 Isolation of Ultrapure BAC Plasmids from Large-Scale Cultures

Large-scale preparation of ultrapure BAC DNA suitable for all critical applications, including subcloning, DNA sequencing or transfection experiments, is performed by alkaline lysis with the Qiagen Large-Construct Kit, which has been specifically developed and adapted for BAC purification. This kit integrates an ATP-dependent exonuclease digestion step that enables efficient removal of bacterial genomic DNA contamination to yield ultrapure BAC DNA.

1. Inoculate a single colony from a freshly streaked plate (LB agar plate containing 12.5 µg/ml chloramphenicol) (*see* **Note 9**) in 5 ml of LB medium containing 12.5 µg/ml chloramphenicol and incubate for 8 h at 37 °C with vigorous shaking (250 rpm).

2. Dilute 1 ml of the culture into 500 ml of selective LB medium (*see* **Note 10**) pre-warmed to 37 °C and grow the cells with vigorous shaking (250 rpm) in a 2 l flask at 37 °C for 12–16 h, to an OD at 550 nm between 1.2 and 1.5. This cell density typically corresponds with the transition from a logarithmic to a stationary growth phase (*see* **Note 12**).

3. Harvest the bacterial cells by centrifugation at $6,000 \times g$ for 15 min at 4 °C and purify the BAC DNA with the Qiagen Large-Construct Kit according to the manufacturer's specifications (*see* **Note 13**). Depending of the BAC size, yields of 20–35 µg of ultrapure BAC DNA can be obtained.

3.2.3 Preparation of DH10B Competent Cells for Electroporation

Owing to the large size of BAC plasmids, the cloning of DNA fragments in BACs requires the use of DH10B competent cells with transformation efficiencies higher than 1×10^8 transformant colonies per µg of DNA. These efficiencies are easily obtained by the electroporation method, which is more reproducible and efficient than the chemical methods. Here we described the protocol for preparing electrocompetent DH10B cells from 1 l of bacterial culture. All the steps of this protocol should be carried out under sterile conditions.

1. Inoculate a single colony of DH10B cells from a freshly streaked LB agar plate into a flask containing 10 ml of SOB medium and incubate the culture overnight at 37 °C with vigorous shaking (250 rpm).

2. Dilute 1 ml of the overnight culture into 1 l of SOB medium pre-warmed at 37 °C and grow the cells with vigorous shaking (250 rpm) in a 2 l flask at 37 °C until the OD at 550 nm reaches 0.7 (this can take 4–5 h) (*see* **Note 14**).

3. Transfer the flask to an ice-water bath for about 20 min. Swirl the culture occasionally to ensure that cooling occurs evenly. From this point on, it is crucial that the temperature of the bacteria not rise above 4 °C.

4. Divide the bacteria culture in two ice-cold 500 ml centrifuge bottles and harvest the cells by centrifugation at $6,000 \times g$ for 10 min at 4 °C. Discard the supernatant and resuspend each cell pellet in 500 ml of ice-cold 10 % glycerol in sterile water.

5. Harvest the cells by centrifugation at $6,000 \times g$ for 15 min at 4 °C. Carefully pour off the supernatant and resuspend each cell pellet in 250 ml of ice-cold 10 % glycerol (*see* **Note 15**).

6. Repeat **step 5** reducing the resuspension volume to 125 ml from each cell pellet.

7. Harvest the cells by centrifugation at $6,000 \times g$ for 15 min at 4 °C. Carefully pour off the supernatant (*see* **Note 15**) and remove any remaining drops of buffer using a Pasteur pipette attached to a vacuum line.

8. Resuspend the cells in a final volume of 3 ml ice-cold 10 % glycerol, avoiding the generation of bubbles. This volume has been calculated to reach an optimal cell concentration of $2–4 \times 10^{10}$ cells/ml.

9. Transfer 50 µl of the suspension to an ice-cold electroporation cuvette (0.2-cm gap) and test whether arcing occurs when an electrical discharge is applied with the electroporation apparatus using the conditions described in Subheading 3.2.6, **step 4**. Arcing is usually manifested by the generation of a popping sound in the cuvette during the electrical pulse. If arcing occurs, wash the cell suspension once more with 100 ml 10 % glycerol and repeat **steps 7** and **8**.

10. Dispense 100 µl aliquots of the final cell suspension into sterile, ice-cold 1.5 ml microfuge tubes, freeze quickly in a dry ice–methanol bath, and transfer to a –70 °C freezer. Electrocompetent DH10B cells could be stored at –70 °C for up to 6 months without loss of transforming efficiency.

3.2.4 Cloning of DNA Fragments in BACs: Preparation of BAC Vectors and DNA Inserts

The same standard techniques used for the cloning of DNA in conventional plasmids are applied to BACs with special considerations owing to the large size of BAC plasmids.

1. Digest the BAC vector and foreign DNA with a two- to three-fold excess of the desired restriction enzymes for 3 h using the buffers supplied with the enzymes and check a small aliquot of the digestions by agarose gel electrophoresis to ensure that the entire DNA has been cleaved. Use an amount of target DNA sufficient to yield 2 μg of the BAC vector and 0.25–0.5 μg of the desired DNA insert.

2. When two enzymes requiring different buffers are used to digest the DNA, carry out the digestion sequentially with both enzymes. Clean the DNA after the first digestion by extraction with phenol–chloroform and standard ethanol precipitation or by using the Qiagen QIAEX II Gel Extraction Kit following the manufacturer's instructions for purifying DNA fragments from aqueous solutions (*see* **Note 16**).

3. Purify the digested BAC vector and the DNA insert by agarose gel electrophoresis using the Qiagen QIAEX II Gel Extraction Kit following the manufacturer's instructions (*see* **Note 16**).

4. Determine the concentration of the BAC vector and the insert by UV spectrophotometry or by quantitative analysis on an agarose gel.

5. If the BAC vector was digested with only one restriction enzyme or with restriction enzymes leaving compatible or blunt ends, the digested BAC vector has to be dephosphorylated prior to its purification by agarose gel electrophoresis to suppress self-ligation of the BAC vector. We recommend cleaning the DNA before the dephosphorylation reaction as described in **step 2** and using shrimp alkaline phosphatase following the manufacturer's specifications.

3.2.5 Ligation Reaction

1. For protruding-ended DNA ligation, mix in a sterile microfuge tube 150 ng of purified digested BAC vector, an amount of the purified insert equivalent to a molar ratio of insert to vector of 3:1, 1.5 μl of 10× T4 DNA ligase buffer containing 10 mM ATP, 3 Weiss unit of T4 DNA ligase, and water to a final volume of 15 μl. In separate tubes, set up two additional ligations as controls, one containing only the vector and the other containing only the insert. Incubate the reaction mixtures for 16 h at 16 °C (*see* **Note 17**).

2. In the case of blunt-ended DNAs, to improve the ligation efficiency use 225 ng of vector, the corresponding amount of insert, 6 Weiss unit of T4 DNA ligase, and incubate the reaction mixtures for 20 h at 14 °C.

3.2.6 Transformation of DH10B Competent Cells by Electroporation

1. Thaw the electrocompetent DH10B cells at room temperature and transfer them to an ice bath.

2. For each transformation, pipette 50 μl of electrocompetent cells into an ice-cold sterile 1.5 ml microfuge tube and place it on ice together with the electroporation cuvettes.

3. Dilute 2.5 μl of the ligation reaction (about 25 ng of DNA) in 47.5 μl of sterile water, mix with the competent cells and incubate the mixture on ice for 1 min. For routine transformation with supercoiled BACs, add 0.1 ng of DNA in a final volume of 2 μl. Include all the appropriate positive and negative controls.

4. Set the electroporation machine to deliver an electrical pulse of 25 μF capacitance, 2.5 kV, and 100 Ω resistance (*see* **Note 18**).

5. Add the DNA–cells mixture into the cold electroporation cuvette avoiding bubbles formation and ensuring that the DNA–cells mixture sits at the bottom of the cuvette. Dry the outside of the cuvette with filter paper and place the cuvette in the electroporation device.

6. Deliver an electrical pulse at the settings indicated above. A time constant of 4–5 ms should be registered on the machine (*see* **Note 19**).

7. Immediately after the electrical pulse, remove the cuvette and add 1 ml of SOC medium pre-warmed at room temperature.

8. Transfer the cells to a 17×100-mm polypropylene tube and incubate the electroporated cells for 50 min at 37 °C with gentle shaking (250 rpm).

9. Plate different volumes of the electroporated cells (2.5, 20, and 200 μl) onto LB agar plates containing 12.5 μg/ml chloramphenicol and incubate them at 37 °C for 16–24 h (*see* **Note 20**).

3.2.7 Screening of Bacterial Colonies by PCR

The recombinant colonies containing the insert in the correct orientation are identified by direct PCR analysis using specific oligonucleotides and conventional Taq DNA polymerase (*see* **Note 21**).

1. For each bacterial colony prepare a PCR tube with 25 μl of sterile water.

2. Using sterile yellow tips, pick the bacterial colonies, make small streaks (2–3 mm) on a fresh LB agar plate containing 12.5 μg/ml chloramphenicol to make a replica, and transfer the tips to the PCR tubes containing the water (*see* **Note 22**). In separate tubes, set up positive and negative controls. Leave the tips inside the PCR tubes for 5 min at room temperature.

3. During this incubation time, prepare a 2× master mix containing 2× PCR buffer, 3 mM $MgCl_2$ (it has to be added only in the case that the PCR buffer does not contain $MgCl_2$), 0.4 mM dNTPs, 2 μM of each primer, and 2.5 U of Taq DNA

polymerase per each 25 μl of master mix. Prepare the appropriate amount of 2× master mix taking into consideration that the analysis of each colony requires 25 μl of this master mix.

4. Remove the yellow tip and add 25 μl of 2× master mix to each PCR tube.

5. Transfer the PCR tubes to the thermocycler and run a standard PCR, including an initial denaturation step at 95 °C for 5 min to liberate and denature the DNA templates and to inactivate proteases and nucleases.

6. Analyze the PCR products by electrophoresis through an agarose gel.

7. Pick the positive colonies from the replica plate and isolate the BAC DNA as described in Subheadings 3.2.1 and 3.2.2 for further analysis.

3.2.8 Storage of Bacterial Cultures

1. Mix 0.5 ml of LB freezing buffer with 0.5 ml of an overnight bacterial culture in a cryotube with a screw cap.

2. Vortex the culture to ensure that the glycerol is evenly dispersed, freeze in ethanol–dry ice, and transfer to –70 °C for long-term storage.

3. Alternatively, a bacterial colony can be stored directly from the agar plate without being grown in liquid media. Using a sterile yellow tip, scrape the bacteria from the agar plate and resuspend the cells into 200 μl of LB medium in a cryotube with a screw cap. Add an equal volume of LB freezing buffer, vortex the mixture, and freeze the bacteria as described in **step 2** (*see* **Note 23**).

3.2.9 Modification of BAC Clones

The modification of BAC clones is relatively easy and it is performed using the same techniques as for conventional plasmids with the modifications described in this chapter. We recommend introducing the desired modifications into intermediate BAC plasmids containing the different viral cDNA fragments used during the assembly of the full-length cDNA clone, and then inserting the modified cDNA into the infectious clone by restriction fragment exchange. Besides standard protocols, the BAC clones can be easily and efficiently modified in *E. coli* by homologous recombination using a two-step procedure that combines the Red recombination system and counterselection with the homing endonuclease I-SceI [17–20] (*see* **Note 24**).

3.3 Rescue of Recombinant Viruses

Infectious virus is recovered by transfection of susceptible cells with the full-length cDNA clone using the cationic lipid Lipofectamine 2000 as transfection reagent (*see* **Note 25**). When the transfection efficiency of the susceptible cells is very low, we recommend first transfecting BHK-21 cells and then plating these

cells over a monolayer of susceptible cells to allow virus propagation. BHK-21 cells are selected because they present good transfection efficiencies and support the replication of most known CoVs after transfection of the viral genome. The following protocol is indicated for a 35-mm-diameter dish and can be upscaled or downscaled if desired (*see* **Note 26**).

1. One day before transfection, plate 4×10^5 BHK-21 cells in 2 ml of growth medium without antibiotics to obtain 90–95 % confluent cell monolayers by the time of transfection (*see* **Note 27**). Also plate susceptible cells (Huh-7 cells in the case of MERS-CoV) at the required confluence for the amplification of the recombinant virus after transfection.

2. Before transfection, equilibrate the Opti-MEM I Reduced Serum Medium at room temperature and put the DNA (*see* **Note 28**) and the Lipofectamine 2000 reagent on ice. For each transfection sample, prepare transfection mixtures in sterile microfuge tubes as follows:

    (a) Dilute 5 μg of the BAC clone in 250 μl of Opti-MEM medium. Mix carefully, avoiding prolonged vortexing or pipetting to prevent plasmid shearing.

    (b) Mix Lipofectamine 2000 gently before use. Dilute 12 μl of Lipofectamine 2000 in 250 μl of Opti-MEM medium (*see* **Note 29**), mix by vortexing, and incubate the diluted Lipofectamine 2000 at room temperature for 5 min.

    (c) Combine the diluted DNA with diluted Lipofectamine 2000, mix carefully, and incubate for 20 min at room temperature.

3. During this incubation period, wash the BHK-21 cells once with growth medium without antibiotics and leave the cells in 1 ml of the same medium per dish.

4. Add the 500 μl of the DNA–Lipofectamine 2000 mixture onto the washed cells and mix by rocking the plate back and forth. Incubate the cells at 37 °C for 6 h (*see* **Note 30**).

5. Remove the transfection medium, wash the cells with trypsin–EDTA solution, and detach the cells using 300 μl of trypsin–EDTA solution.

6. Add 700 μl of growth media to collect the cells and reseed them over a confluent monolayer of susceptible cells containing 1 ml of normal growth medium.

7. Incubate at 37 °C until a clear cytopathic effect was observed.

8. Analyze the presence of virus in the supernatant by titration.

9. Clone the virus by three rounds of plaque purification and analyze the genotypic and phenotypic properties of the recovered virus.

4 Notes

1. *E. coli* DH10B strain is a recombination-defective strain used for the propagation of BACs to avoid unwanted rearrangements.

2. SOB medium should be Mg^{2+}-free to avoid arcing during the electroporation step.

3. The Huh-7 cell line has never been deposited at ATCC but it can be purchased from the Japanese Collection of Research Bioresources (JCRB) Cell Bank.

4. In case that a restriction site present in the BAC plasmid was selected, it must be removed in the plasmid by the introduction of silent mutations.

5. The silent mutations introduced in the viral genome to generate new restriction sites or to abrogate preexisting ones can be used as genetic markers to identify the virus recovered from the infectious clone.

6. To reduce the number of undesired mutations, perform all PCR reactions with a high-fidelity polymerase, according to the manufacturer's instructions.

7. The CMV promoter and the BGH termination and polyadenylation sequences can be amplified from pcDNA3.1. Alternatively, these sequences together with the HDV ribozyme could be amplified from plasmid pBAC-TGEV 5′-3′ that is available from the authors upon request.

8. In general, the cloning of CoV full-length cDNAs in BACs allows the stable propagation of the infectious clone in *E. coli* DH10B cells. If a residual toxicity, characterized by a small colony phenotype and a delay in the bacterial growth, is observed during the assembly of the infectious clone, we recommend inserting the DNA fragment responsible for this toxicity in the last cloning step to minimize the toxicity problem.

9. Cultures of BAC 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. LB broth is the recommended culture medium, since richer broths such as TB (Terrific Broth) lead to extremely high cell densities, which can overload the purification system, resulting in lower yield and less purity of the BAC DNA.

11. When other kits are used instead of the Qiagen QIAprep Miniprep Kit, equivalent modifications have to be included to optimize the recovery of BAC DNA.

12. To avoid DNA degradation and unwanted rearrangements owing to culture overaging, it is important to prevent growing the culture up to the late stationary growth phase.

13. The use of a swinging bucket rotor is recommended for the last isopropanol precipitation step to facilitate the further resuspension of the BAC DNA. After washing with 70 % ethanol, air-dry the pellet for only 5 min. Never use vacuum, as overdrying the pellet will make the BAC DNA difficult to dissolve. Carefully remove any additional liquid drop, add 250 μl of 10 mM Tris–HCl (pH 8.5) (DNA dissolves better under slightly alkaline conditions) and resuspend the DNA overnight at 4 °C. To prevent plasmid shearing, avoid vortexing or pipetting to promote resuspension of the BAC DNA. Transfer the DNA to a clean 1.5 ml microfuge tube, remove any possible resin traces by centrifugation for 1 min in a tabletop microfuge, and keep the supernatant in a clean tube at 4 °C. If the purified BAC DNA is not going to be used for a long period of time we recommend storage at –20 °C. Avoid repeated freeze-thaw cycles to prevent plasmid shearing.

14. 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.

15. Take care when decanting the supernatant as the bacterial pellets lose adherence in 10 % glycerol.

16. The Qiagen QIAEX II resin can be used to efficiently purify DNA fragments from 40 bp to 50 kb from aqueous solutions and from standard or low-melt agarose gels in TAE or TBE buffers. Other commercial kits are available, but check whether they have been optimized for purification of DNA fragments larger than 10 kb, as most BAC constructs used during the assembly of the infectious clone are larger than 10 kb.

17. The large size of the BAC vectors reduces the ligation efficiency. To increase this efficiency, it is essential to use larger amounts of vector, insert, and T4 DNA ligase than when using conventional plasmids.

18. 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.

19. The presence of salt increases the conductivity of the 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 any commercial kit or by extraction with phenol–chloroform followed by precipitation with ethanol and 2 M ammonium acetate.

20. Plating volumes higher than 200 μl of electroporated cells on a single plate may inhibit the growth of transformants owing to the large number of dead cells resulting from electroporation.

If only small numbers of transformant colonies are expected, it is recommended to spread 200 μl-aliquots of the electroporated cells on different plates.

21. A mix of small and large colonies indicates that the cloned DNA fragment presents some toxicity when amplified in *E. coli*. Choose the small colonies, which may contain the correct insert, and always grow the bacteria containing this recombinant BAC plasmid at 30 °C to minimize the toxicity problem. In this case, we strongly recommend inserting this toxic DNA fragment into the infectious clone in the last cloning step, in order to reduce the manipulation and minimize the possibility of introducing unwanted mutations. Infectious BAC cDNA clones presenting a residual toxicity should be grown at 30 °C.

22. It is important to avoid overloading the reaction by adding too much bacteria, which may alter the ionic balance of the reaction and inhibit the amplification by the Taq polymerase.

23. We recommend to use this storage method for BAC clones that present a residual toxicity and are not fully stable when amplified in *E. coli*.

24. The Red recombination system combined with counterselection with I-SceI endonuclease results in an accurate and highly efficient method to introduce insertions, deletions, or point mutations in BAC clones without retention of unwanted foreign sequences.

25. The transfection of BACs containing large inserts into mammalian cells has been optimized in our laboratory and the best transfection efficiencies were provided using Lipofectamine 2000 as transfection reagent.

26. All work involving MERS-CoV has to be performed in a Biosafety Level 3 (BSL3) laboratory, following the guidelines of the European Commission and the National Institutes of Health (NIH) of the USA.

27. Do not add antibiotics to media during transfection as this may decrease transfection activity. A healthy cell culture is critical for an efficient transfection. The use of low passage-number cells is recommended.

28. Use a BAC DNA isolated with the Qiagen Large-Construct Kit since a DNA preparation of high purity is required in the transfection step.

29. Opti-MEM I Reduced Serum Medium is recommended for dilution of the cationic lipid Lipofectamine 2000 reagent prior to complexing with DNA, although other media without serum may also be used. However, owing that some serum-free media formulations can inhibit cationic lipid-mediated transfection, test any new serum-free medium for compatibility

with the transfection reagent prior to use. Some media formulations that have been found to inhibit cationic lipid-mediated transfection are CD 293 Medium, 293 SFM II, and VP-SFM.

30. If susceptible cells are directly transfected, incubate them at 37 °C until the cytopathic effect is observed and proceed to clone and characterize the recovered virus. In this case, optimization of the transfection of the desired cells with the BAC clone using Lipofectamine 2000 should be required. For transfection optimization, use a similar size plasmid expressing GFP. This plasmid is available from the authors upon request.

Acknowledgements

References

1. Masters PS (2006) The molecular biology of coronaviruses. Adv Virus Res 66:193–292

2. Lai MMC, Perlman S, Anderson L (2007) Coronaviridae. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds) Fields virology, vol 1, 5th edn. Lippincott Williams and Wilkins, Philadelphia, pp 1305–1335

3. Stadler K, Masignani V, Eickmann M et al (2003) SARS-beginning to understand a new virus. Nat Rev Microbiol 1:209–218

4. Zaki AM, van Boheemen S, Bestebroer TM et al (2012) Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med 367:1814–1820

5. Almazán F, González JM, Pénzes Z et al (2000) Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci U S A 97:5516–5521

6. Yount B, Curtis KM, Baric RS (2000) Strategy for systematic assembly of large RNA and DNA genomes: transmissible gastroenteritis virus model. J Virol 74:10600–10611

7. Thiel V, Herold J, Schelle B et al (2001) Infectious RNA transcribed in vitro from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. J Gen Virol 82:1273–1281

8. Casais R, Thiel V, Siddell SG et al (2001) Reverse genetics system for the avian coronavirus infectious bronchitis virus. J Virol 75: 12359–12369

9. van Boheemen S, de Graaf M, Lauber C et al (2012) Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. mBio 3, e00473-12

10. Wang K, Boysen C, Shizuya H et al (1997) Complete nucleotide sequence of two generations of a bacterial artificial chromosome cloning vector. Biotechniques 23:992–994

11. Shizuya H, Birren B, Kim UJ et al (1992) 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: 8794–8797

12. Dubensky TW, Driver DA, Polo JM et al (1996) Sindbis virus DNA-based expression vectors: utility for in vitro and in vivo gene transfer. J Virol 70:508–519

13. Bálint A, Farsang A, Zádori Z et al (2012) Molecular characterization of feline infectious peritonitis virus strain DF-2 and studies of the role of ORF3abc in viral cell tropism. J Virol 86:6258–6267

14. St-Jean JR, Desforges M, Almazán F et al (2006) Recovery of a neurovirulent human coronavirus OC43 from an infectious cDNA clone. J Virol 80:3670–3674

15. Almazán F, DeDiego ML, Galán C et al (2006) Construction of a severe acute respiratory syndrome coronavirus infectious cDNA clone and a replicon to study coronavirus RNA synthesis. J Virol 80:10900–10906

16. Almazán F, DeDiego ML, Sola I et al (2013) Engineering a replication-competent, propagation-defective Middle East respiratory syndrome coronavirus as a vaccine candidate. mBio 4, e00650-00613

17. Zhang Y, Buchholz F, Muyrers JPP et al (1998) A new logic for DNA engineering using recombination in *Escherichia coli*. Nat Genet 20:123–128

18. Lee EC, Yu D, Martinez de Velasco J et al (2001) A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics 73:56–65

19. Jamsai D, Orford M, Nefedov M et al (2003) Targeted modification of a human beta-globin locus BAC clone using GET recombination and an I-SceI counterselection cassette. Genomics 82:68–77

20. Tischer BK, von Einem J, Kaufer B et al (2006) Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*. Biotechniques 40:191–197