Chapter 5

Bacterial Artificial Chromosome-Based Lambda Red Recombination with the I-SceI Homing Endonuclease for Genetic Alteration of MERS-CoV

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Abstract

Over the past two decades, several coronavirus (CoV) infectious clones have been engineered, allowing for the manipulation of their large viral genomes (~30 kb) using unique reverse genetic systems. These reverse genetic systems include targeted recombination, in vitro ligation, vaccinia virus vectors, and bacterial artificial chromosomes (BACs). Quickly after the identification of Middle East respiratory syndrome-CoV (MERS-CoV), both in vitro ligation and BAC-based reverse genetic technologies were engineered for MERS-CoV to study its basic biological properties, develop live-attenuated vaccines, and test antiviral drugs. Here, I will describe how lambda red recombination can be used with the MERS-CoV BAC to quickly and efficiently introduce virtually any type of genetic modification (point mutations, insertions, deletions) into the MERS-CoV genome and recover recombinant virus.

Key words Coronavirus, MERS-CoV, Bacterial artificial chromosome (BAC), Lambda red recombination, Reverse genetics, Infectious clone

1 Introduction

Coronaviruses are large, enveloped, single-stranded positive-sense RNA viruses that cause both significant human and veterinary disease. Prior to the severe acute respiratory syndrome-CoV (SARS-CoV) outbreak in 2003, human CoVs were only known to cause mild, self-limiting upper respiratory diseases. Approximately 10 years after the emergence of SARS-CoV in 2012, Middle East respiratory syndrome (MERS)-CoV emerged in the Middle East where it then spread to 27 different countries, and to date (December 2018, WHO) there have been 2278 laboratory-confirmed cases and 806 associated deaths for a case fatality rate of 35%. Most of these cases have occurred in the Middle East, aside from an outbreak of ~200 infected individuals in South Korea in 2015 [1].

Infectious clones are highly valuable research tools that enable modification of viral genomes to better understand their fundamental biology, develop novel vaccine candidates, and test antiviral therapeutics. Soon after identifying MERS-CoV as the causative agent of MERS, two distinct infectious clones were reported for MERS-CoV [2, 3]. These infectious clones were engineered using in vitro ligation or bacterial artificial chromosomes (BACs), each of which had been used previously for CoVs [4–6]. In vitro ligation uses unique type II restriction endonucleases that cleave several bases away from their recognition site, allowing for the reassembly of authentic CoV genomes from smaller fragments. Each fragment is separately maintained in its own small plasmid for efficient genetic modification using traditional molecular cloning methods. Separating specific nucleotide sequences in ORF1A helped to eliminate the problem of these sequences being toxic for bacteria. A T7 promoter is inserted at the 5′ end of the genome, allowing for in vitro transcription of the viral RNA and subsequent transfection into mammalian cells for virus production. In contrast, BACs allow for the stable propagation of full-length CoV cDNA in bacteria, due to the ability to restrict their copy number to 1 or 2 plasmids per cell. Different restriction fragments of these BACs can be sub-cloned into smaller vectors for efficient modification, or the full-length genome can be modified using lambda red recombination, which will be discussed here. CoV BAC plasmids contain a CMV promoter 5′ of the viral genome, allowing for transcription of the viral genome following transfection of BAC DNA into mammalian cells. In addition, the CoV BACs contain a polyA tail, a Hepatitis D Virus (HDV) ribozyme, and bovine growth hormone (BGH) termination and polyadenylation signals to create genomic RNA with an authentic 3′ end. The full-length nature of BAC DNA and the CMV promoter subvert the need for in vitro ligation or transcription to recover infectious virus. BACs were initially developed in the early 1990s, and by the mid-late 1990s they were utilized by Herpes virologists for modification of these large DNA viruses, which revolutionized the field. A few years later a BAC for a CoV, transmissible gastroenteritis virus (TGEV), was engineered, and since then BACs have been successfully developed for several CoVs including feline infectious peritonitis virus (FIPV), OC-43, SARS-CoV, MERS-CoV, murine hepatitis virus strain JHM (MHV-JHM), porcine epidemic diarrhea virus (PEDV), and the SARS-like CoV WIV-1 [2, 4, 7–12]. Thus, it is likely that BAC-based reverse genetics could be useful for any novel or emergent CoV.

Lambda red recombination utilizes bacteriophage enzymes Exo, Beta, and Gam (Red proteins) to mediate homologous recombination near the ends of linear double-stranded DNA [13, 14]. PCR products containing positive selection markers are suitable substrates for these enzymes, so long as they bear

extensions of 40–50 bases that are homologous to the target sequence. A major advancement in this technique came with the development of an *E. coli* strain, DY380, where the Red proteins were placed under the control of a temperature-inducible promoter [15]. Several methods for removing the positive selection markers from the viral genomes have been developed, including flanking sequences with FRT or loxP sites [16], or utilizing positive and negative selection markers on a single gene cassette, such as the Galactose Kinase (GalK)-Kan^R gene cassette [17]. These methods both have certain downfalls, including the retention of small FRT or loxP sites following removal of the marker, or the unintended removal of negative selection markers by repeat sequences in the BAC plasmid. To improve the efficiency of removing the positive selection marker, a unique method utilizing the I-SceI homing endonuclease under an arabinose-inducible promoter was developed (Fig. 1) [18]. I-SceI is an endonuclease with an 18 bp recognition site that is not present in the *E. coli* genome, making it safe to express in *E. coli*. In the method described here, this recognition site is engineered on a plasmid (pEP-KanS) just outside of the positive selection marker, and its cleavage with the I-SceI enzyme allows for the removal of the positive selection marker by intramolecular Red recombination utilizing sequence duplication introduced in the original PCR primers. This method can be utilized to introduce any type of modification into the BAC DNA, including mutations, deletions, and insertions. Here I will outline the procedure for this highly efficient method to engineer markerless modifications, focusing on single point mutations in the full-length MERS-CoV BAC.

2   Materials

2.1   Manipulation of the MERS-CoV BAC

2.1.1   Plasmids and Bacterial Strains

1. pBAC-MERS-CoV^{FL}. This MERS-CoV BAC was first engineered by the Luis Enjuanes lab [2]. The full protocol for creating this BAC was subsequently published by the same group in a previous Methods in Molecular Biology book [19]. This plasmid contains the parA, parB, and parC genes derived from the *E. coli* F-factor to prevent more than one or two BACs from coexisting in the same cell. It also contains genes involved in the initiation and orientation of DNA replication and the chloramphenicol resistance gene (*Cml^r*).

2. pEP-KanS. This plasmid contains the AphAI-I-SceI cassette containing a kanamycin resistance marker (*Kan^r*) and an I-SceI restriction site [18]. This plasmid also contains an ampicillin resistance marker.

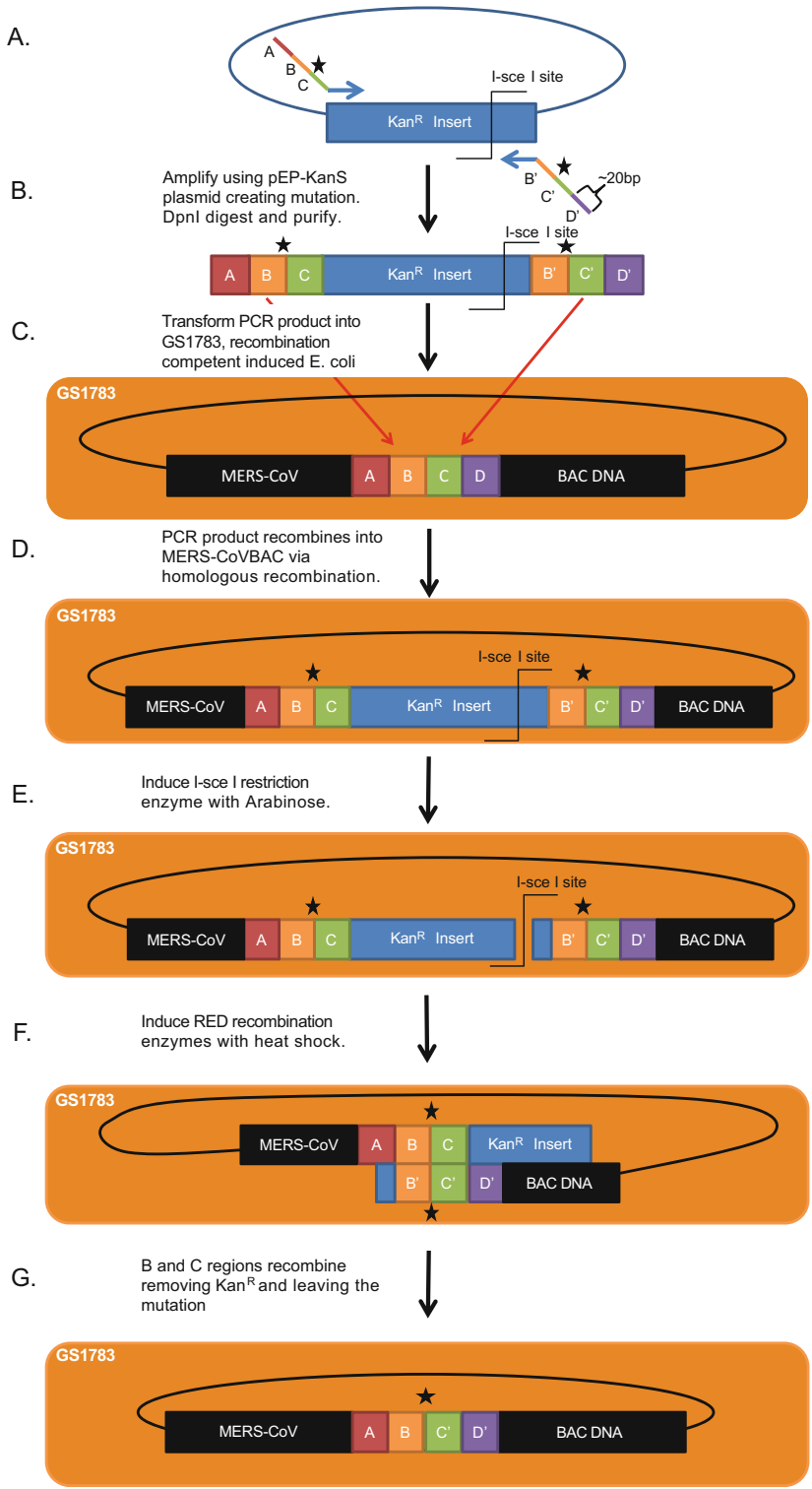3. *E. coli* strains DH10B (*see* **Note 1**) and GS1783 (*see* **Note 2**) cells.

Fig. 1 Schematic for making point mutations in MERS-CoV using Lambda Red Recombination. This diagram illustrates and describes each individual step (**a–g**) in the protocol for creating individual point mutations in the MERS-CoV BAC

2.1.2 Culture and Freezing Reagents for E. coli

1. LB medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl. Sterilize by autoclaving on liquid cycle.

2. LB agar plates: LB medium containing 15 g/L agar. After preparing LB medium add the agar. Sterilize by autoclaving as above. Allow the medium to cool to ~45–50 °C, add appropriate antibiotics (1 mL of 1000× stocks/L) or arabinose (40 mL of 25% arabinose/L) to the medium, then dispense in Petri dishes.

3. LB freezing medium: 40% (v/v) glycerol in LB medium. Add glycerol, water, and dry LB ingredients to desired volume (i.e., 200 mL of glycerol for 500 mL total LB freezing medium). Sterilize by autoclaving on liquid cycle.

4. LB cml media: LB medium with chloramphenicol (25 mg/mL).

5. LB cml/kan media: LB media with chloramphenicol (25 mg/mL) and kanamycin (40 mg/mL).

6. SOC medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose. Sterilize by autoclaving on liquid cycle.

7. Antibiotics: Make 1000× stock solutions of ampicillin (100 mg/mL in ethanol), kanamycin (40 mg/mL in H_2O), and chloramphenicol (25 mg/mL in ethanol).

8. Arabinose: Make 25% (w/v) solution in H_2O. Sterilize by passing it through a 0.22 μm disposable filter.

9. Glycerol: Make a 10% (v/v) solution and sterilize by autoclaving on liquid cycle.

2.1.3 Enzymes

1. Restriction endonucleases, Taq DNA polymerase, high-fidelity thermostable DNA polymerase, and reverse transcriptase can be purchased from several different commercial sources.

2.1.4 DNA Oligomers

1. DNA oligomers can be purchased from several different commercial sources. Long DNA oligos (>80 nt) are needed, so identifying a commercial source that can make long oligos at a reasonable price is important (*see* **Note 3**).

2. Recombination Primers.

Forward: 5′----------60bp_homology------AGGATGACGAC-GATAAGTAGGG-3′.

Reverse: 5′----------60bp_homology------GCCAGTGTTACA ACCAATTAACC-3′.

2.1.5 DNA Preparation Kits	1. DNA Miniprep Kit.
	2. Machery-Nagel Nucleobond Xtra Midi Kit (*see* **Note 4**).
	3. Invitrogen PureLink PCR Purification Kit (*see* **Note 5**).
2.1.6 Special Software and Equipment	1. Electroporator and 1 mm cuvettes.
	2. 42 °C shaking water bath (*see* **Note 6**).
	3. 30–32 °C incubator and shaking incubator.
	4. DNA analysis software.
2.2 Rescue of BAC-Derived Recombinant Viruses	1. Human liver-derived Huh-7 (JCRB Cell Bank, JCRB0403) or Vero cells (ATCC, CCL-81).
	2. Cell growth medium: Dulbecco's Modified Eagle Medium (DMEM) + 10% FBS.
	3. Cell growth medium: DMEM + 2% FBS.
	4. Opti-MEM I Reduced Serum Medium.
	5. Trypsin-EDTA: 0.05% (w/v) trypsin, 0.02% (w/v) EDTA.
	6. Lipofectamine 2000 (Life Technologies, Invitrogen) (*see* **Note** 7).

3 Methods

3.1 Transformation and Storage of MERS-CoV BAC DNA into DH10B or GS1783 Cells	1. Precool electroporation cuvette at 4 °C or on ice.
	2. Prepare labeled 14 mL culture tube(s) with 1 mL SOC.
	3. Add 1–10 ng of BAC DNA in a sterile Eppendorf tube on ice. Add 24 µL of electrocompetent DH10B or GS1783 *E. coli*.
	4. Carefully transfer the mixture into the groove of an electroporation cuvette on ice.
	5. Pulse the cuvette at 25 µF, 1750 V, and 200 Ω.
	6. Recover by adding 0.5–1.0 mL SOC to the cuvette, and transfer the mixture to a 14 mL culture tube. Incubate at 32 °C and 220 rpm for 1 h. Pre-warm LB-agar-cml plate.
	7. Add 25–50 µL of culture to LB-cml plate. Incubate at 30–32 °C overnight (o/n) (*see* **Note 8**).
	8. Next day pick 1–2 colonies and incubate in 2 mL LB-cml broth at 30–32 °C o/n.
	9. Next day mix 0.5 mL o/n culture with 0.5 mL bacterial freezing medium. Store in negative 80 °C freezer.
3.2 Prepare pBAC-MERS-CoV Competent Cells for Lambda Red Recombination	Day 1
	1. Streak glycerol stock of GS1783 pBAC-MERS-CoV bacteria generated in 3.1 on LB-cml plate to generate isolated colonies. Incubate at 30–32 °C o/n.

Day 2

2. Pick one colony from the plate and transfer to 2 mL LB-cml in tube. Incubate at 30–32 °C and 220 rpm o/n. Put ddH$_2$O (200 mL) and 10% glycerol in ddH$_2$O (100 mL) at 4 °C to precool.

Day 3

3. Dilute 1 mL of o/n culture into 49 mL LB-cml in a 250 mL Erlenmeyer flask. Protocol can be scaled up to 100 mL (use 500 mL Erlenmeyer flask). Incubate at 32 °C and 220 rpm. In the meantime, turn on shaking water bath, check water level, and set to 42 °C. Label 10 Eppendorf tubes per 50 mL culture and precool them in a plastic tube rack at −80 °C. Precool the tabletop centrifuge with the swinging bucket rotor and a microcentrifuge to 4 °C.

4. Monitor the OD$_{600}$ of culture starting at 2 h of incubation. When OD$_{600}$ = 0.6–0.8 (~3–4 h), incubate at 42 °C and 200 rpm (*see* **Note 9**) in shaking water bath for *15 min*. In the meantime, prepare an ice water slurry in an autoclave bin or other suitable container and get a bucket of ice. Place two 50 mL conical tubes on ice.

5. Swirl the Erlenmeyer flask in the ice water slurry for *10 min*. From here on, keep bacteria cold at all times.

6. Aliquot 25 mL into each 50 mL conical tube on ice. Centrifuge at 1800 × *g* for 10 min at 4 °C in a tabletop centrifuge.

7. Pour off supernatant with one quick motion. While pouring, position the bacterial pellet away from the liquid to limit the amount of bacteria lost. Add 5 mL ice-cold sterile ddH$_2$O and resuspend pellet by swirling and tapping the tube to the bottom of the ice-cold autoclave bin. Once resuspended, add an additional 20 mL ice-cold sterile ddH$_2$O and centrifuge at 1800 × *g* for 10 min at 4 °C. Repeat 1×.

8. Following the second water wash, resuspend each pellet with 10% glycerol, first in 5 mL, then add an additional 15 mL. Centrifuge at 1800 × g for 10 min at 4 °C.

9. Pour off supernatant as described above. Following the pour, resuspend the pellet in the remaining 10% glycerol (~500 μL). If the combined amount of cells and 10% glycerol is greater than 550 μL, transfer the suspension to a cold Eppendorf tube and pellet the cells for 2 min at 5000 × g and 4 °C. After spinning the cells, remove an appropriate amount of supernatant such that ~500 μL of cell suspension remains. Resuspend the cells to a homogenous solution and aliquot 50 μL to prefrozen Eppendorf tubes and flash-freeze tubes in liquid nitrogen or a dry ice-methanol bath. Use immediately or store at −80 °C. Cells are typically good for 6–12 months, but may be useful even after several years.

3.3 Create PCR Cassette Containing the Viral Genome Mutation or Insertion of Interest with the AphAI-I-SceI (Herein Termed Kan^r-I-Scel) Gene Cassette

1. Design and order Kan^r-I-SceI primers with 60 bp homology to the region of interest flanking the desired site to be modified (Fig. 1a). To design simple point mutations, start by developing a 60 bp flanking sequence, calling each 20 base pair section as A, B, and C. Then incorporate the desired mutation at the end of section B (40th base pair, or the 39th and 40th base pair if two changes are required). Finally, attach this sequence to the 22 bp Kan^r-I-SceI sequence below to create your forward recombination primer. To create the reverse recombination primer, create a new block of 20 bp we will call section D′ that is the reverse complement of the sequence immediately downstream from section C. These 20 bp will be followed on your primer by sections C′ and B′, the reverse complements to sections B and C, with B′ containing the desired mutations. Finally, add the 23 bp Kan^r-I-SceI sequence to finish the reverse primer. During negative selection, sections B/C will recombine with B′/C′ leading to the loss of the Kan^R-I-SceI cassette (Fig. 1g). For deletion mutants, leave out the desired sequence from your primers. For instance, to delete sections D/E/F, simply create the forward primer with sections A/B/C, and the reverse primer with sections G′/C′/B′. Insertions of small sequences can be achieved by adding the entire insertion sequence at the 3′ end of the forward primer, and at least 50 bp of reverse complement sequence at the 3′ end of the reverse primer (Fig. 2). Larger insertions may require the development of a full plasmid, or potentially the use of nested PCRs. For additional details, *see* ref. [18]. While designing recombination primers (indicated below), remember to also order short primers about 100–200 bp outside of the insertion site to check for the proper insertion of the gene cassette by PCR.

2. Set up PCR reaction and perform reaction according to manufacturer's protocol with following modifications (Fig. 1b).

Fig. 2 Model of the PCR product used for inserting specific sequences into BACs using lambda red recombination. The full sequence for insertion is incorporated at the 5′ end of the Kan^R-I-SceI cassette while at least 50 nt of sequence homologous to the 3′ end of the insertion sequence is incorporated at the 3′ end of this cassette. Surrounding these sequences are 50 nt of sequence homologous to the viral sequence where the sequence is to be inserted

3. For a PCR template, use ~50 ng of the pEP-KanS plasmid.

4. Use 1 μL high-fidelity polymerase (*see* **Note 10**).

5. For the annealing temperature, I prefer to use a step-down procedure lowering the annealing temperature by 1 °C starting at 68 °C and continuing the PCR reaction for 25 cycles. Using this method we rarely see spurious PCR products.

6. Analyze the PCR product on an agarose gel with ethidium bromide and image it using a UV-gel box and gel-imaging software. PCR product should be ~1.2 kb.

7. Purify the PCR product using the PureLink PCR purification kit (*see* **Note 5**). Use binding buffer B3 according to manufacturer's protocol to remove primer dimers from the mixture. Elute DNA into 44 μL of water.

8. DpnI digest the pEP-KanS plasmid in the purified PCR product (Fig. 1b). DpnI specifically cleaves methylated DNA and is needed to digest the pEP-KanS plasmid used in the PCR reaction. It has a 4 bp recognition site so it should cleave DNA approximately every 250 bp. Without this digestion all of your transformants will maintain the pEP-KanS plasmid as its transformation is much more efficient than the recombination of the PCR product. Incubate for 1–3 h at 37 °C.

   1 μL DpnI.

   5 μL Buffer.

   44 μL PCR product.

   50 μL Total

9. Purify PCR product using the PureLink PCR purification kit. Elute DNA into 30 μL pre-warmed elution buffer or water.

10. Measure DNA concentration using a spectrophotometer. A concentration of 20–80 ng/μL is typical.

3.4   Insertion of Kan^R-I-SceI Gene Cassette into MERS-CoV BAC DNA (Positive Selection)

Day 1

1. Precool the electroporation cuvette(s) on ice.

2. Prepare labeled 14 mL culture tube(s).

3. Aliquot 50–100 ng of *Kan^r*-I-SceI PCR product in a sterile Eppendorf tube on ice. This will generally be 2–4 μL. Add 23 μL competent GS1783 *E. coli* containing pBAC-MERS-CoV and mix by stirring briefly.

4. Carefully transfer the mixture into the groove of the electroporation cuvette on ice.

5. Wipe any ice water from outside of cuvette and pulse at 25 μF, 1750 V, and 200 Ω (Fig. 1c).

6. Recover by immediately adding 0.5–1 mL SOC to the cuvette, and then transfer the mixture to a 14 mL culture tube. Incubate at 32 °C and 220 rpm for 3–5 h. This is when the recombination occurs (Fig. 1d) (*see* **Note 11**). Pre-warm an LB-cml/kan plate.

7. After the recovery, transfer the bacteria to a 1.5 mL tube and centrifuge at 16,000 × g for 1 min. Remove all but ~100 μL of supernatant, resuspend the pellet, and plate the entire culture on an LB-cml/kan plate and incubate at 30–32 °C o/n. You should get anywhere from 5 to 100 colonies.

Day 2

1. Using a sterile toothpick or pipet tip, replica-plate 25 colonies from the previous step first onto an LB-amp plate (ampR = pEP-kanS plasmid background; all colonies should be negative if DpnI digest was complete), then onto an LB-cml/kan plate (should be positive if recombination occurred). A grid for this procedure is shown in Fig. 3. Incubate plates at 30–32 °C o/n.

Day 3

2. Identify bacterial clones that grew on the LB-cml/kan plate but not on the LB-amp plate. The efficiency at this step is anywhere from 50 to 100%. Inoculate culture tubes containing 6 mL of LB-cml-kan with 5–6 selected colonies (1 in each tube) from the LB-cml/kan plate and incubate at 30–32 °C and 220 rpm o/n.
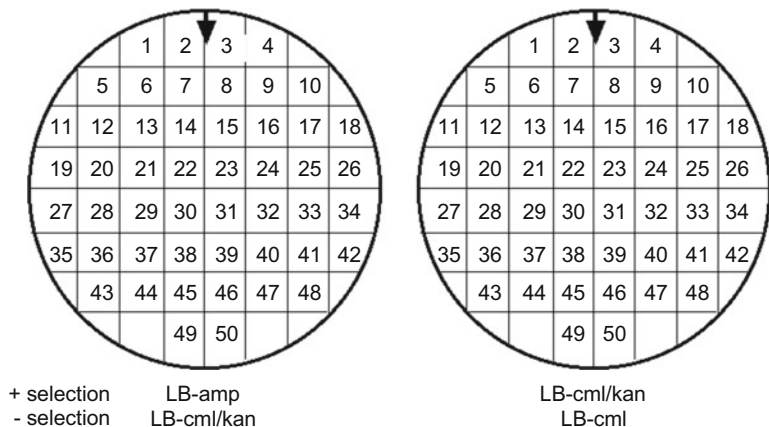

Fig. 3 Replica plate grids. These grids allow for the easy identification of identical colonies that have been plated on each plate. Using a toothpick, dot a single colony in the same spot on each plate. For both positive and negative selection, MERS-BAC clones that have successfully undergone recombination will grow on the plate on the right, but not on the plate on the left

Day 4

3. Following o/n incubation, create a freezer stock of the bacteria, then purify the BAC DNA using a standard miniprep kit. Using 1 μL of the BAC DNA, use the external primers located 100–200 bp outside the region of homology you previously designed to test for the insertion of Kan^r-I-SceI by PCR. If the insertion was successful, the DNA band from the PCR should be ~1 kb larger than the band from the MERS-CoV wild-type BAC (*see* **Note 12**). To speed this process up, colonies may be collected off the plate on day 3 and directly tested by PCR. Colonies that pass the PCR screen can then progress to the negative selection protocol (3.5) (*see* **Note 13**).

3.5 Removal of Kan^R-I-SceI from MERS-CoV BAC DNA (Negative Selection)

Day 1 (can coincide with day 4 of positive selection protocol)

1. Create two new culture tubes with 2 mL of LB-cml/kan and inoculate them with bacteria from 2 separate $kan^r+cml^r+amp^r-$ colonies. Incubate at 30–32 °C and 220 rpm o/n.

Day 2

2. Transfer 100 μL of the fresh o/n culture to 2 mL of warm LB-cml and incubate at 32 °C and 220 rpm for 2 h. If not previously done, mix 0.5 mL o/n culture with 0.5 mL freezing medium as a glycerol stock.

3. After 2 h, add 2 mL of warm LB-cml with 2% arabinose to the culture tube for a final arabinose concentration of 1% (Fig. 1). Incubate at 32 °C and 220 rpm for 2 h. Warm water bath shaker to 42 °C.

4. Transfer the culture tubes to the water bath shaker at 42 °C and ~200 rpm and incubate for 30 min (Fig. 1f).

5. Transfer the culture tubes back to 32 °C and incubate for 3–4 h (Fig. 1g).

6. After the incubation, perform tenfold serial dilutions of the bacteria in LB. Plate 100 μL of 10^{-4} and 10^{-5} dilutions of original culture on pre-warmed LB-cml plates containing 1% arabinose. Incubate the plates at 30–32 °C.

Day 3

7. Pick 50 colonies and replica plate on LB-cml/kan and LB-cml plates. Colonies that underwent correct recombination should grow on LB-cml but not on LB-cml/kan. Incubate o/n at 32 °C. Efficiency is generally anywhere from 5 to 50% (*see* **Note 14**).

Day 4

8. Pick 3 separate cml$^+$ kan$^-$ colonies and culture each one in 100 mL LB-cml at 32 °C and 220 rpm o/n.

Day 5

9. Mix 0.5 mL of the o/n culture with 0.5 mL freezing solution for a glycerol stock. Purify the BAC DNA from these cultures using the NucleoBond Xtra Midi Kit according the manufacturer's protocol (*see* **Note 4**). Verify the integrity of the BAC constructs by restriction enzyme digestion of ~2 μg BAC DNA with KpnI and verify the loss of the *Kan^R*-I-SceI gene cassette by PCR (*see* **Note 15**). Verify introduced mutation(s)/insertion(s) by Sanger sequencing.

3.6   Rescue of Recombinant Virus

Day 1

1. Seed either Vero-81 or Huh-7 cells (*see* **Note 16**) in DMEM + 10% FBS into 6 well dish so that cells are 60–80% confluent at the time of transfection the next day. Incubate at 37 °C o/n.

Day 2 (BSL-3)

2. All procedures from here involve working with MERS-CoV which requires a BSL-3 containment laboratory.

3. Replace medium from cells with 2 mL DMEM +10% FBS immediately before proceeding to **step4**.

4. Prepare the transfection mixture as follows: For a single well, mix 1–2 μg of MERS-CoV BAC DNA with 10 μL Lipofectamine 2000 (*see* **Note 7**) in Opti-MEM media according to manufacturer's protocol. Scale up accordingly if multiple BAC DNAs will be transfected. Additionally, prepare the following controls:

   (a) Lipofectamine + random plasmid DNA.
   (b) Lipofectamine only.
   (c) No Lipofectamine, no DNA.

   Wait ~20 min for liposomes to form. Then add the transfection mixture dropwise to the medium in the well.

5. Approximately 3–4 h after transfection (*see* **Note 17**), replace the medium with a 2% (Vero 81 cells) or 10% (Huh-7 cells) FBS medium to slow cell growth.

Day 6 and Beyond (BSL-3)

6. Cytopathic effect (CPE) will start being visible at 3–4 days after transfection. Collect cells and supernatant when >50% of well has CPE (the more the better). To collect, scrape any

remaining cells off the well with a pipette tip or cell scraper and transfer the media and cell debris in a 2 mL microcentrifuge tube and freeze-thaw the sample. Then, centrifuge the sample at ~5000 × g to spin out the cell debris and transfer the supernatant to a new tube. We term this passage 0 (P0) virus.

7. Use ~1–2 mL of P0 virus to infect 1 T-75 flask of cells (*see* **Note 18**).

8. Collect virus at 2–3 days post-infection as describe above and aliquot. We term this as P1 virus. Titer virus on Vero/Huh-7 cells. If titer is sufficient, use for further experiments.

9. To verify whether the virus has maintained your mutation or insertion, infect a new set of cells for ~48 h, then collect the infected cells in Trizol, prepare RNA and then convert the RNA to cDNA with reverse transcriptase. Alternatively, you could prepare RNA from the supernatants and sequence these samples. Create a PCR product with the external primers that were previously used to check for the gain or loss of the insertion, and finally have that PCR product sequenced by Sanger sequencing (*see* **Note 19**).

4   Notes

1. DH10B cells are a standard *E. coli* strain used for general cloning. DH10B cells are recombination-deficient (recA1) and endonuclease I-deficient (endA1) and have constitutive deoxyribose synthesis for improved cloning of large plasmids. These traits make these cells ideal for the long-term storage of BAC plasmids.

2. GS1783 cells are a derivative of DH10B cells that contain the Red recombination genes under a temperature sensitive promoter and the I-SceI homing endonuclease under control of an arabinose-inducible promoter [20].

3. For creating long oligos, we prefer Invitrogen as a supplier, as they can provide oligos up to 100 nt at their standard price per base. Many companies do not make oligos longer than 60 nt at their standard rates, and thus will charge a significant amount more for the 80–90 nt oligos required for this protocol.

4. While many companies sell BAC-prep kits which will work for these purposes, these columns are often at least 3× the cost of the Nucleobond Xtra Midi Kit. We have tested the Xtra Midi Kit side by side with a BAC-prep kit and found little to no difference in CoV BAC DNA yield.

5. For purifying PCR products, we prefer the Invitrogen Pure-Link PCR Purification Kit because it includes a buffer that allows DNA products of <300 bp to go through the column.

This helps remove primer dimers from the PCR reaction that could interfere with recombination.

6. A 42 °C shaking water bath is essential for this procedure. The bacterial cultures need to heat up to 42 °C quickly to properly induce the Red enzymes. A shaking water bath is significantly better than a regular shaking incubator at quickly transferring heat to the bacterial culture.

7. We have tried several different transfection reagents, and Lipofectamine 2000 has worked the best for us. That does not mean other transfection reagents won't work, feel free to try whichever reagent you prefer.

8. It is important to always culture GS1783 cells at 32 °C or lower due to the potential for leaky expression of the Red enzymes at temperatures below the induction temperature of 42 °C.

9. Some shaking water baths cannot shake at 200 rpm; if this is the case, simply shake at a reasonable speed for the shaker.

10. We have occasionally had problems obtaining a PCR product when using the manufacturer recommended 0.5 μL of high-fidelity polymerase. Using 1 μL of polymerase provides more consistent results.

11. Unlike a typical 1 h recovery following electroporation, it is important to incubate these cultures for several hours in SOC to allow time for recombination to occur. A recovery time of 5 h or greater is preferred, with a minimal recovery time being 3 h.

12. Occasionally, we find that following PCR some clones have bands that correspond to both the WT BAC and the desired Kan^R-I-SceI insert BAC. It is likely due to at least two copies of BAC DNA being present in the same cell. Unless the molar ratio of the Kan^R-I-SceI insert to the WT BAC is very large, these clones should be avoided.

13. After positive selection, we do not check the BAC by restriction digest, because (a) we find that in many cases the amount of BAC DNA from a miniprep is insufficient for a readable digest, and (b) we find that the colonies that pass the replica plating and PCR tests rarely if ever have any significant problems concerning removing or duplicating regions of the MERS-CoV BAC DNA.

14. First, colonies may take over 1 day before they are visible. Second, while the efficiency of the negative selection can be very low, any colonies that have grown on LB-cml plates but do not grow on LB-cml/kan plates are very likely correct. Therefore, we go directly to starting large-scale cultures for these clones. That way we can do the final diagnostic tests and we can prepare for the BAC transfection and viral recovery at the same time.

15. A KpnI digest of pBAC-MERS-CoV results in DNA fragments of 19.1, 13.8, 3.9, and 1.5 kb. We have found this is the best digest for diagnostic evaluation of the MERS-CoV BAC; however other enzymes or enzyme combinations will work as well. Be sure to use a low-percentage agarose gel to effectively separate the large DNA molecules.

16. Other laboratories use BHK-21 cells for the initial transfection since these cells are highly transfectable. The transfected cells can be overlaid on the Vero-81 or Huh-7 cells for further outgrowth of the recombinant virus.

17. It is feasible to wait o/n to change the media of the transfected Vero 81 cells, but this does result in an increase in the cytotoxicity induced by Lipofectamine. This is not feasible if you are using Huh-7 cells.

18. Mutant viruses that do not replicate as well as WT virus may require increased amounts of P0 virus.

19. It is also beneficial to check the integrity of the entire MERS-CoV genome by RT-PCR after several passages as MERS-CoV tends to occasionally delete sections of the accessory proteins.

Acknowledgments

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