Chapter 13

In-Yeast Assembly of Coronavirus Infectious cDNA Clones Using a Synthetic Genomics Pipeline

Tran Thi Nhu Thao, Fabien Labroussaa, Nadine Ebert, Joerg Jores, and Volker Thiel

Abstract

The *Escherichia coli* and vaccinia virus-based reverse genetics systems have been widely applied for the manipulation and engineering of coronavirus genomes. These systems, however, present several limitations and are sometimes difficult to establish in a timely manner for (re-)emerging viruses. In this chapter, we present a new universal reverse genetics platform for the assembly and engineering of infectious full-length cDNAs using yeast-based transformation-associated recombination cloning. This novel assembly method not only results in stable coronavirus infectious full-length cDNAs cloned in the yeast *Saccharomyces cerevisiae* but also fosters and accelerates the manipulation of their genomes. Such a platform is widely applicable for the scientific community, as it requires no specific equipment and can be performed in a standard laboratory setting. The protocol described can be easily adapted to virtually all known or emerging coronaviruses, such as Middle East respiratory syndrome coronavirus (MERS-CoV).

**Key words** Coronavirus, RNA virus, Reverse genetics, Full-length cDNA clone, Transformation-associated recombination (TAR) cloning, Homologous recombination, *Saccharomyces cerevisiae*, Synthetic genomics

1 Introduction

Reverse genetics platforms for viruses allow the generation of infectious viral cDNA clones, and hence the reconstitution of corresponding viruses and their mutants for characterization studies to gain insights into the basis of viral replication and pathogenesis and to foster vaccine development. However, coronavirus reverse genetics is challenging due to their relatively large genome sizes and the instability of certain viral sequences when propagated in bacterial hosts. Accordingly, coronavirus reverse genetics makes use of unconventional methods such as in vitro ligation of cDNA fragments [1], vaccinia virus as a vector [2, 3] or cloning cDNA as
Section 3.1

Section 3.2

Section 3.3

Section 3.4

Section 3.5

Fig. 1 A schematic workflow of coronavirus reverse genetics system. Yeast-based TAR cloning, a synthetic genomics-derived assembly platform, is employed to assemble full-length viral cDNAs which are maintained as yeast artificial chromosomes (YACs). Yeast clones carrying correctly assembled viral genomes are identified via PCR-based screening of assembly junctions. Reconstruction of viruses starts with in vitro transcription of sequence-confirmed YACs to generate full-length viral mRNA bearing authentic 5’-end cap and 3’-end poly (A) tail. The viral mRNA is delivered to appropriate mammalian cells via electroporation to allow the recovery of infectious viruses.

bacterial artificial chromosome (BAC) in E. coli [4]. Constant epidemic/pandemic threats by (re-)emerging RNA viruses necessitate a fast and easy-to-implement reverse genetics platform that enables the reliable assembly of viral genomes in order to rapidly rescue viruses and their mutants for characterization studies.

Here, we describe a synthetic genomics-derived assembly platform as a part of a new reverse genetics system for coronaviruses (Fig. 1). It uses the transformation-associated recombination (TAR) cloning strategy that exploits the inherently robust homologous recombination system of the yeast Saccharomyces cerevisiae (S. cerevisiae) to generate and maintain full-length viral cDNAs as
yeast artificial chromosomes (YACs). This method, originally used to isolate eukaryotic DNA fragments in yeast [5], was later adapted for the construction of large double-stranded DNA viruses [6, 7] as well as entire bacteria genomes (Mycoplasmas, ~1 Mb) [8–10]. Compared to other stepwise assembly methods, TAR cloning simplifies the accurate assembly of virtually any full-length coronavirus cDNA within a single yeast transformation event. It requires co-transformation of overlapping DNA fragments into yeast, irrespective of their sizes and/or their incompatibility with other intermediate hosts. Applied to coronaviruses, this significantly reduces the timeframe required to build infectious clones and rescue recombinant viruses. In addition, it opens up the possibility to simultaneously modify different viral genomic sequences of interest, thus providing a more versatile and rapid pipeline for genome engineering. An equally noteworthy characteristic is the easy establishment of this method in different lab settings, for it does not require any special equipment or infrastructure.

2 Materials

2.1 Preparation of Overlapping DNA Fragments and TAR Cloning Vector for Viral Genome Assembly

1. pCC1BAC-his3 [10] (see Note 1).
2. One-step RT-PCR system, e.g., SuperScript™ IV One-Step RT-PCR System.
3. Hot start DNA polymerase, e.g., KOD Hot Start DNA Polymerase.
4. DpnI restriction enzyme and provided buffer.
5. Nuclease-free water.
6. PCR thermal cycler.
7. Gel electrophoresis system.
8. Gel visualization and documentation system.
9. Gel and PCR clean-up kits, e.g., Nucleospin.
10. DNA quantification device, e.g., NanoDrop OneC.

2.2 Assembly of Full-Length cDNA Via TAR in Yeast

1. *Saccharomyces cerevisiae* (S. cerevisiae) strain VL6-48N (MATα trp1-Δ1 ura3-Δ1 ade2-101 his3-Δ200 lys2 met14 cir/C14) [11], derived from the strain VL6-48 [12] (see Note 2).
2. YPDA broth: dissolve 25 g YPDA powder in 500 ml of distilled water. Adjust pH to 6.5 using sodium hydroxide or hydrochloric acid and autoclave at 121 °C for 15 min. Store at room temperature.
3. SD-His agar dishes: 46.7 g of SD agar base, 0.77 g of dropout (DO) supplement –His. Add distilled water up to 1 l. Adjust pH to 5.8 using sodium hydroxide or hydrochloric acid and autoclave at 121 °C for 15 min. Pour 30 ml per plate, and store plates at 4 °C.
4. Dimethyl sulfoxide (DMSO): filter-sterilize using a 0.22 μm filter and store at room temperature.

5. 0.1 M LiAc/1X TE solution: 1 ml of 1 M Tris–HCl pH 7.5, 20 μl of 0.5 M EDTA pH 7.5, 1.02 g lithium acetate dehydrate. Add distilled water up to 100 ml. Filter-sterilize using a 0.22 μm filter and store at room temperature.

6. Single-stranded deoxyribonucleic acid from salmon testes: prepare solution at 10 mg/ml in sterile water. Store at −20 °C.

7. 40% (wt/vol) PEG3350: 20 g of polyethylene glycol 3350, 0.1 M LiAc/1X TE solution up to 50 ml. Filter-sterilize using a 0.22 μm filter and store at room temperature.

8. Sterile water.

9. Inoculation spreader.

10. Tubes 50 ml.

11. Tubes 13 ml.

12. Digital lab scales.

13. Shaking incubator.

14. Water bath.

15. Spectrophotometer with semi-micro disposable plastic cuvettes.

16. Table-top microcentrifuge.

2.3 Identification of Yeast Clones Harboring Correctly Assembled Viral cDNA

1. SD-His agar dishes: 46.7 g of SD agar base, 0.77 g of dropout (DO) supplement –His. Add distilled water up to 1 l. Adjust pH to 5.8 using sodium hydroxide or hydrochloric acid and autoclave at 121 °C for 15 min. Pour 30 ml per plate, and store plates at 4 °C.

2. SD-His broth: 26.7 g of SD base, 0.77 g of dropout (DO) supplement –His. Add distilled water up to 1 l. Adjust pH to 5.8 using sodium hydroxide or hydrochloric acid and autoclave at 121 °C for 15 min. Store at room temperature.

3. 5% Chelex®100 in deionized water supplemented with acid-washed glass beads.

4. Nuclease-free water.

5. DNA LoBind 1.5 ml microtube.

6. 32-Square petri dish stickers.

7. Optional: 12-Sector petri dish stickers.

8. DNA polymerase master mix, e.g., GoTaq® G2 Green Master Mix.

9. Multiplex PCR kit, e.g., QIAGEN® Multiplex PCR Kit (see Note 3).
10. TE buffer: 10 mM Tris–HCl, 1 mM EDTA. Adjust pH to 8.0 and store at room temperature.
11. Digital lab scales.
12. Thermal mixer.
13. Table-top microcentrifuge.
14. PCR thermal cycler.
15. Gel electrophoresis system.
16. Gel visualization and documentation system.

2.4 Large-Scale Preparation of Recombinant Plasmids in Yeast: Midiprep

1. SD-His broth: 26.7 g of SD base, 0.77 g of dropout (DO) supplement –His. Add distilled water up to 1 l. Adjust pH to 5.8 using sodium hydroxide or hydrochloric acid and autoclave at 121 °C for 15 min. Store at room temperature.
2. β-Mercaptoethanol,
3. Isopropanol.
4. 70% ethanol
5. Zymolyase®-100 T from Arthrobacter luteus.
6. Tubes 50 ml.
7. Erlenmeyer culture flasks 1000 ml.
8. Digital lab scales.
9. Spectrophotometer with semi-micro disposable plastic cuvettes.
10. Shaking incubator.
11. Refrigerated bench-top centrifuge.
12. Nuclease-free water.

2.5 Recovery of Infectious Coronaviruses from TAR-Cloned Full-Length Viral cDNA

2.5.1 Generation of Infectious Full-Length Viral RNA and N Gene RNA by In Vitro Transcription

1. Restriction enzymes and provided buffers, e.g., PacI.
2. Large-scale T7 polymerase in vitro transcription system, e.g., T7 RiboMax™ large-scale RNA production system.
3. Phenol-chloroform-isoamylalcohol (25:24:1).
4. Chloroform-isoamylalcohol (24:1).
5. 3 M sodium acetate, pH 5.2
6. Absolute ethanol.
7. 70% ethanol.
8. Nuclease-free water.
9. m7G(5′)ppp(5′)G RNA Cap Structure Analog.
10. Lithium chloride (LiCl) precipitation solution: 7.5 M lithium chloride, 50 mM EDTA.
11. RNA loading dye 2×: 95% formamide, 0.02% sodium dodecyl sulfate, 0.02% bromophenol blue, 0.01% xylene cyanol, 1 mM EDTA or NEB.

12. DNA LoBind 1.5 ml microtube.

13. Gel electrophoresis system.

14. Gel visualization and documentation system.

15. RNase AWAY solution or similar.

2.5.2 Recovery of Infectious MHV

1. Baby hamster kidney cells (BHK-21).

2. Murine 17 clone 1 cells (17Cl-1).

3. Minimal essential medium \((1 \times) + \text{GlutaMAX}^\text{TM}-\text{I}\) supplemented with 10% fetal bovine serum and 1% Pen Strep.

4. Cell dissociation medium, e.g., TrypLE\textsuperscript{TM} Express \((1 \times)\).

5. Phosphate-buffered saline (PBS).

6. 0.4% Trypan Blue.

7. T75 cell culture flasks.

8. Improved Neubauer chamber.

9. Electroporation cuvettes, 0.4 cm.

10. CO\textsubscript{2} incubator maintained at 37 °C and under a 5% CO\textsubscript{2} atmosphere.

11. Refrigerated bench-top centrifuge.

12. Electroporation system.

3 Methods

The TAR cloning method makes use of the natural ability of \textit{S. cerevisiae} to recombine overlapping DNA fragments via homologous recombination. In addition to a centromere sequence and a yeast selectable marker, the TAR vector also contains two targeting sequences called “hooks” at both ends overlapping with the 3′ and 5′ ends of the viral DNA sequences. The recombination between the TAR vector and its respective homologous sequences after yeast transformation will result in a circular YAC which is able to freely replicate and segregate in the yeast. Free-end viral DNA fragments and a TAR vector should be properly designed and generated to contain appropriate overlaps for efficient in-yeast homologous recombination (Fig. 2).
Fig. 2 (a) Representative design of TAR cloning vector and overlapping viral DNA fragments exemplified by mouse hepatitis virus (MHV). Nine overlapping fragments cover the entire MHV genome with the T7 promoter preceding the viral 5’-UTR, and poly (A) tail and unique restriction site PacI following the viral 3’-UTR. TAR cloning vector pCC1BAC-his3 contains overlapping sequences with fragments 1 and 9. pT7 T7 promoter; UTR untranslated region; 1a, 1b, NS2, HE, S, 5a, E, M, and N viral genes; J junction, Cm(R) Chloramphenicol resistance gene; ori origin of replication; repA, parA, parB and parC regulatory genes; HIS3 histidine gene; CEN6 yeast centromeric sequence; ARS autonomously replicating sequence. (b) Primer design strategy to introduce homologous regions to MHV fragment 1 and the 3’ end of TAR vector. Green and blue shades indicate primer binding sites and overhangs, respectively. Primer 1 binds to the viral 5’-UTR...
3.1 Preparation of Overlapping DNA Fragments and TAR Cloning Vector for Viral Genome Assembly

3.1.1 Design and Generation of Overlapping Viral DNA Fragments from Viral RNA

1. Design viral genome-covering DNA fragments using DNA analysis software, e.g., Geneious, to contain at least 50 bp of overlapping sequences with their neighboring fragments (see Notes 4–6).

2. To generate the DNA fragments from viral RNA templates, use SuperScript™ IV One-Step RT-PCR System. For each fragment, set up a 50 μl PCR using 0.01 pg to 1 μg total RNA, 0.5 μM each primer, 25 μl 2× Platinum™ SuperFi™ RT-PCR Master Mix, and 0.5 μl SuperScript™ IV RT Mix. Cycling parameters are 45–60 °C for 10 min, 98 °C for 2 min; 30–40 cycles at 98 °C for 10 s, 55–72 °C (depending on the primers used) for 10 s, and 72 °C for 30 s/kb; and a 5-min incubation at 72 °C (see Notes 6 and 7).

3. Separate the PCR products on 1% agarose gel and subsequently extract the fragments of correct sizes, and purify them using a commercially available gel extraction kit if necessary (see Note 8).

4. Quantify the extracted DNA fragments individually.

5. Combine all fragments to equimolar amounts to obtain approximately a concentration of 1 μg in 50 μl, e.g. 100 ng per fragment for 10 fragments including the TAR vector (see Note 9).

3.1.2 Design and Generation of TAR Cloning Vector

1. Design primers to amplify TAR cloning vector and introduce at least 50 bp of overlapping sequences with the first and last viral DNA fragments (see Note 5).

2. To amplify TAR vector using KOD Hot Start DNA Polymerase, set up a 50 μl PCR containing 1 pg to 1 ng of the pCC1BAC-his3, 0.3 μM each primer, 1.5 mM of MgSO4, 0.2 mM of dNTPs (each), 5 μl of 10× Buffer for KOD Hot Start DNA Polymerase, and 0.02 U/μl of KOD Hot Start DNA Polymerase. Cycling parameters are 95 °C for 2 min; 35 cycles at 95 °C for 20 s, 55 °C for 30 s, and 68 °C for 10 min; and a 10 min incubation at 68 °C (see Notes 6 and 7).

---

**Fig. 2** (continued) belonging to the MHV fragment 1; its overhang introduces T7 promoter sequence and 21 nucleotides from the 3’ end of TAR vector. Primer 3 binds to the 3’ end of TAR vector and introduces overlapping sequences with MHV fragment 1. (c) Primer design strategy to introduce homologous regions to MHV fragment 9 and the 5’ end of TAR vector. Green and blue shades indicate primer binding sites and overhangs, respectively. Primer 4 binds to the viral 3’-UTR belonging to the MHV fragment 9; its overhang introduces a poly (A) tail followed by a unique restriction site (e.g., PacI for MHV) and 23 nucleotides from the 5’ end of TAR vector. Primer 2 binds to the 3’-UTR belonging to MHV fragment 9; it contains an overhang that adds an extra sequence overlapping with TAR vector.
3. Optional: If template plasmid is prepared from bacteria, a DpnI digestion is recommended to remove the DNA template, i.e., pCC1BAC-his3.

4. Separate the PCR-amplified TAR vector on a 1% agarose gel and purify it using a commercially available gel extraction kit if necessary (see Note 8).

5. Quantify the amount of purified TAR vector (see Note 9).

### 3.2 Assembly of Full-Length cDNA Via TAR in Yeast

All procedures regarding the inoculation of media and the transformation of yeast cells should be performed in a biosafety cabinet or in the immediate vicinity of a laboratory standard Bunsen burner to prevent contamination of cultures.

1. Inoculate the yeast strain VL6-48N from a −80 °C glycerol stock in 10 ml of YPDA broth and incubate overnight at 30 °C under agitation at 200 rpm (see Note 10).

2. The next day, determine the cell count of the yeast culture. Using a spectrophotometer, measure OD$_{600nm}$, i.e. a suspension containing $1 \times 10^7$ cells per ml will give an OD$_{600nm}$ of 1.

3. Dilute the culture to an OD$_{600nm}$ ~ 0.2–0.25 with pre-warmed YPDA broth into an adequate volume (see Note 11). Incubate the culture at 30 °C under agitation until the OD$_{600nm}$ reaches ~1. It usually takes about 4 h.

4. Centrifuge 3 ml of the culture per condition at 4250 × $g$ for 5 min at room temperature.

5. Discard the supernatant, resuspend the pellet in 1 ml of 0.1 M LiAc/1× TE buffer, and transfer the mixture to a 1.5 ml sterile microtube.

6. Centrifuge at 16,000 × $g$ for 1 min at room temperature.

7. Repeat step 5.

8. Incubate yeast cells at 30 °C for 30–60 min without agitation.

9. Denature DNA carrier (single-stranded deoxyribonucleic acid from salmon testes) by heating at 100 °C for 10 min and subsequent cooling in ice water for at least 10 min.

10. Harvest the yeast cells by centrifuging at 2500 × $g$ for 3 min at room temperature.

11. Discard the supernatant and gently resuspend the yeast cells in 50 μl of 0.1 M LiAc/1× TE buffer. Add 5 μl of denatured DNA carrier and DNA (max. 50 μl), i.e., overlapping DNA fragments and TAR vector (see Note 9). Mix carefully.

12. Add 500 μl of 40% PEG3350 solution to the DNA mixture.

13. Add DMSO to 10% of the final volume of the DNA/PEG3350 mixture, and gently resuspend.

14. Incubate at 30 °C for 30 min without agitation.
15. Incubate the mixture for 25 min at 42 °C in a water bath.
16. Centrifuge the cells at $2500 \times g$ for 3 min at room temperature.
17. Discard the supernatant, and gently resuspend cells in 1 ml of YPDA medium.
18. Transfer the cell suspension to a 13 ml tube and incubate at 30 °C for 60 min under agitation at 200 rpm.
19. Pre-warm SD-His agar plates at 30 °C.
20. Harvest cells by centrifuging at $2500 \times g$ for 3 min at room temperature.
21. Completely remove the supernatant, and gently resuspend cells in 300 μl of sterile water.
22. Carefully spread the cell mixture evenly over the agar surface using sterile spreader.
23. Incubate agar plates for 3–4 days at 30 °C.

### 3.3 Identification of Yeast Clones Harboring Correctly Assembled Viral cDNA

#### 3.3.1 Growth of Yeast Transformants on Agar Dishes

Transformed yeast colonies are transferred onto new SD-His agar plates to allow further growth of yeast for subsequent screening (see Subheadings 3.3.3 and 3.3.4) and plasmid preparation (see Subheading 3.4).

1. Apply a 32-square sticker to an SD-His agar plate.
2. Use a 20 μl pipette tip to pick a single isolated yeast colony and transfer it onto the plate, filling the area of a sticker-divided square. Repeat until 32 colonies have been picked per construct (see Notes 12–14).
3. Incubate agar plates for 1–2 days at 30 °C.

#### 3.3.2 Extraction of Yeast DNA with GC Prep Method (Chelex100 Preparation) for Colony Screening

The GC prep, or Chelex100 preparation, is a fast and easy method to extract yeast genomic DNA and was adopted from a recent publication [13]. This method ensures that the quality and yield of extracted yeast DNA will suffice for PCR-based screening.

1. From 1 cm$^2$ patch, use a 20 μl pipette tip to collect yeast cells (see Note 14).
2. Resuspend yeast cells in a 1.5-ml microtube containing 100 μl of 5% Chelex100 solution and glass beads.
3. Vortex at high speed for 4 min at room temperature.
4. Heat the mixture at 100 °C for 2 min.
5. Centrifuge at $15,000 \times g$ for 1 min at room temperature.
6. Carefully transfer 50 μl of the supernatant to a new microtube without disturbing the pellet.
3.3.3 Screening for the Presence of Desired Construct by Simplex PCR

Yeast transformants carrying the correctly assembled genome can firstly be identified via PCR-based screening that targets the presence of a specific DNA sequence in the final construct, i.e., a sub-fragment of one of the PCR fragments via simplex PCR.

1. Set up a 25 µl PCR using 1 µl of yeast DNA obtained by GC prep method (see Subheading 3.3.2), 0.75 µM each primer, and 12.5 µl GoTaq® G2 Green Master Mix. Cycling parameters are 95 °C for 1 min, 35 cycles at 95 °C for 10 s, 55–60 °C (depending on the primers used) for 30 s, and 72 °C for 1 min/kb; and a 10-min incubation at 72 °C.

2. Visualize the PCR products on 1% agarose gel.

3.3.4 Verification of Assembly Juncions by Multiplex PCR

Yeast transformants carrying the correctly assembled genome should subsequently be identified via multiplex PCR targeting all the junctions where overlapping fragments recombine. In multiplex PCR, all primers should be carefully designed, so that (a) each pair should span across an overlapping region of two neighboring fragments, ensuring that amplicons contain the recombined region and fragment-specific sequences; and (b) the PCR amplicons of a single PCR are of sufficiently different sizes to be easily distinguished and visualized by gel electrophoresis.

1. Normalize all primer stocks to a concentration of 100 µM. Prepare a 10× primer by mixing 2 µM of each primer in TE buffer to reach the final volume of 500 µl (see Notes 15 and 16).

2. Set up a 50 µl multiplex PCR using 1 µl of yeast DNA obtained by GC prep method (see Subheading 3.3.2), 5 µl of 10× primer mix, and 25 µl of 2× QIAGEN Multiplex PCR Master Mix. Cycling parameters are 95 °C for 15 min; 30–45 cycles at 94 °C for 30 s, 55–63 °C for 90 s (depending on the primers used), and 72 °C for 90 s; and a 10 min incubation at 72 °C.

3. Analyze the PCR products on 1% agarose gel (see Note 17).

3.4 Large-Scale Preparation of Recombinant Plasmids in Yeast: Midiprep

This section describes the preparation of TAR plasmids containing cloned viral cDNAs in preparative amounts to provide sufficient template for in vitro transcription reactions (see Subheading 3.5). The below protocol is basically a midi plasmid preparation; yet it can easily be scaled up if necessary. Additionally, it is important to note that yeast genomic DNA will be extracted alongside the TAR plasmids.

1. Inoculate 200 ml of SD-His medium with a yeast pre-culture containing the TAR clone of interest (see Note 10). The yeast doubling time should be estimated for the pre-culture (usually 2- to 3-h doubling time). Based on this, the inoculum can be adjusted to the amount of yeasts that will result in an OD_{600nm} of ~2 within 12–16 h.
2. Incubate for 12–16 h at 30 °C under agitation at 200 rpm until the OD600nm reaches ~2.

3. Centrifuge the yeast culture at 24,000 × g for 30 min at 4 °C and discard the supernatant.

4. Resuspend the cell pellet thoroughly in a freshly prepared lysis solution containing 16 ml of buffer RES supplemented with RNase A, 1600 µl of zymolyase solution, and 160 µl of β-mercaptoethanol (see Note 18).

5. Incubate the mixture at 37 °C for 1 h without agitation.

6. Follow the Macherey-Nagel Plasmid DNA purification kit protocol (see Note 19). Add 16 ml of lysis buffer LYS to the suspension. Mix gently by inverting the tube 5–10 times.

7. Incubate the mixture for 5 min at room temperature.

8. Meanwhile, apply 12 ml of equilibration buffer EQU onto the rim of the filter inserted in Nucleobond Xtra Column and make sure to wet the entire filter. Allow the column to empty by gravity flow and do not leave the column to dry out.

9. Incubate for 5 min and add 16 ml of neutralization buffer NEU to the suspension. Mix gently by inverting the tube 5–10 times, strictly avoid vortexing.

10. Centrifuge the mixture at 24,000 × g for at least 30 min (see Note 20).

11. Apply the supernatant to the filter. Allow the column to empty by gravity.

12. Apply 5 ml of equilibration buffer EQU onto the rim of the filter to wash away any applied supernatant that is remaining in the filter (see Note 21).

13. Remove the filter prior to applying wash buffer WASH to avoid low purity.

14. Add 8 ml of wash buffer WASH.

15. Meanwhile, warm up elution buffer ELU at 50 °C.

16. Add 5 ml of pre-warmed buffer ELU (50 °C) to elute DNA into a new tube.

17. Add 3.5 ml of room-temperature isopropanol to the eluate to precipitate DNA.

18. Mix thoroughly, but avoid vortexing.

19. Centrifuge at 24,000 × g for 30 min at 4 °C.

20. Carefully discard the supernatant without disturbing DNA pellet.

21. Add 2 ml of room-temperature 70% ethanol to wash the DNA pellet.

22. Centrifuge at 24,000 × g for 15 min at room temperature.
23. Carefully discard the ethanol completely. Allow the DNA pellet to dry at room temperature (see Note 22).

24. Dissolve DNA pellet in appropriate amount (depending on DNA pellet size) of TE buffer or nuclease-free water (see Notes 23 and 24). DNA concentrations can be expected in the range of 50–100 ng/μl in a 50 μl volume.

3.5 Recovery of Infectious Coronaviruses from TAR-Cloned Full-Length Viral cDNA

In this protocol, the reconstitution of infectious viruses starts with generating an mRNA encoding the viral N gene and a full-length viral RNA bearing authentic 5'-end cap and 3'-end poly(A) tail via in vitro transcription. Subsequently, the RNAs are delivered to mammalian cells via electroporation. Once the transfected full-length viral RNA is translated to produce coronavirus replicase, the virus replication cycle is initiated. For coronaviruses, it has been shown that co-transfection of full-length RNA and N gene RNA helps to increase the rescue efficiency [1]. The protocol below outlines the rescue procedure for mouse hepatitis virus (MHV) and can be adapted when applied to other coronaviruses, especially in terms of target cell lines.

3.5.1 Generation of Infectious Full-Length Viral RNA and N Gene RNA by In Vitro Transcription (see Note 25)

1. In a 1.5-ml microtube, linearize purified plasmid DNA (1–10 μg) at a unique restriction site, i.e., PacI for MHV, located downstream of the 3'-end poly(A) tail.

2. Extract linearized plasmid DNA following the phenol–chloroform extraction protocol. Add phenol–chloroform–isoamylalcohol (25:24:1) to DNA mixture at the ratio of 1:1 volume. Mix gently.

3. Centrifuge the mixture at 16,000 × g for 5 min at room temperature. Transfer the upper aqueous phase to a new 1.5-ml microtube.

4. Add chloroform–isoamylalcohol (24:1) at the ratio of 1:1 volume. Mix gently.

5. Repeat step 3.

6. Precipitate DNA by adding 1:20 volume of 3 M sodium acetate (pH 5.2), and 2.5 × volume of absolute ethanol. Mix gently.

7. Leave DNA to precipitate at −20 °C for 30 min.

8. Centrifuge at 16,000 × g for 30 min at 4 °C. Carefully remove the supernatant to avoid disturbing DNA pellet.

9. Wash DNA pellet with 70% ethanol.

10. Centrifuge at 16,000 × g for 5 min at room temperature.

11. Completely remove the supernatant. Allow DNA pellet to dry at room temperature.

12. Dissolve DNA in 10–20 μl of nuclease-free water.
13. Set up a 50-μl in vitro transcription reaction using the T7 RiboMax™ Large Scale RNA Production System with m^7G (5')ppp(5')G RNA Cap Structure Analog (see Table 1).

14. Incubate at 30 °C for 3 h.

15. Add 2 μl of RNase-free DNase. Incubate at 37 °C for 20 min.

16. Purify synthesized RNA using lithium chloride (LiCl) precipitation protocol. Add 1:2 volume of LiCl solution. Mix gently and thoroughly.

17. Leave RNA to precipitate at −20 °C for 30 min.

18. Centrifuge at 16,000 × g for 15 min at 4 °C. Carefully remove the supernatant without disturbing the RNA pellet.

19. Wash RNA pellet with 70% ethanol.

20. Centrifuge at 16,000 × g for 15 min at 4 °C. Completely remove the supernatant. Allow the pellet to dry at room temperature.

21. Dissolve RNA in 20–40 μl of RNase-free water.

22. Analyze the RNA product by gel electrophoresis. Mix 5 μl of RNA product with 5 μl of RNA Loading Dye 2×.

23. Denature the RNA by heating the mixture at 65 °C for 5 min. Immediately incubate on ice for at least 5 min.

24. Analyze denatured RNA on 0.8% agarose gel.

---

**Table 1**

| **5X T7 transcription buffer** | **10 μl** |
|--------------------------------|-----------|
| m^7G(5')ppp(5')G RNA cap structure analog (30 mM) | 5 μl |
| GTP (100 mM) | 0.75 μl |
| ATP, CTP, UTP (100 mM), each | 3.75 μl |
| Extracted DNA (1–10 μg) | X μl |
| Enzyme mix (RNasin, T7 RNA polymerase) | 5 μl |
| RNase-free water | Up to 50 μl |
| **Total** | **50 μl** |

180 Tran Thi Nhu Thao et al.

---

**3.5.2 Recovery of Infectious MHV**

1. On day 1, seed BHK-21 cells in order to have 1 × 10^7 cells per electroporation condition (see Note 26).

2. On day 2, collect all BHK-21 cells in a 50-ml tube by trypsinizing and centrifuging at 430 × g for 5 min at 4 °C.

3. Resuspend cell pellet in 10 ml of ice-cold phosphate-buffered saline (PBS). Ensure that cells are well separated and determine the cell count.
4. Pellet cells by centrifuging at $430 \times g$ for 5 min at 4 °C.

5. Resuspend $1 \times 10^7$ cells in 0.8 ml of ice-cold PBS and transfer all cells to a 0.4-cm electroporation cuvette.

6. Add 10 μg of full-length viral RNA and 2 μg of N gene transcript to the cell suspension.

7. Electroporate cells three times with two pulses using the following settings on BioRad Gene Pulser: 0.85 kV, 25 μF, resistance = $\infty$.

8. Gently transfer the electroporated BHK-21 cells to a T75 flask containing a monolayer of 17Cl-1 cells (70–80% confluent) by allowing the cell suspension to run slowly to the bottom of the flask. The rescued viruses will be released into cell culture medium and termed passage 0 viruses.

9. Collect supernatant every 24 h and store at −80 °C for further analysis.

10. Optional: To further confirm the recovery of infectious viruses, transfer the supernatant containing passage 0 viruses to fresh target cells for MHV, e.g., 17Cl-1 or L929 cells.

### 4 Notes

1. This plasmid derives from the pCC1BAC plasmid (Epicenter) and has been modified for the purpose of DNA isolation in yeast using the TAR cloning method. As it stands, the pCC1BAC-his3 is a yeast/E. coli shuttle vector containing bacterial artificial chromosome (BAC) and yeast centromeric plasmid (YCP) sequences for efficient replication in both organisms. It also contains a histidine selectable marker and a centromere (CEN) to be maintained in yeast as a yeast artificial chromosome (YAC).

2. This yeast strain is highly transformable. *S. cerevisiae* is grown in rich YPD media supplemented with adenine (YPDA). Yeast transformed with the pCC1BAC-his3 is first plated on minimal synthetic defined (SD) agar media without histidine (SD-His). Yeast colonies are subsequently propagated in SD-His broth at 30 °C under agitation at 200 rpm. Yeast culture reaching an optical density at 600 nm (OD$_{600nm}$) of ~2 is aliquoted in cryovials containing glycerol (15% final concentration) or, e.g., Roti®-Store yeast cryovials (Carl Roth), and stored at −80 °C.

3. NucleoBond Xtra Midi Resuspension buffer RES is supplemented with RNase A and stored at 2–8 °C. Buffer LYS should be checked for precipitation of SDS before use. White precipitate can be dissolved by warming the buffer at 30–40 °C for several minutes.
4. The sizes of viral DNA fragments obtained by PCR amplification can be greatly flexible depending on the polymerase being used.

5. In case of the rescue strategy described here, a T7 promoter sequence and a poly (A) tail followed by a unique restriction site are introduced upstream of the 5′-UTR and downstream of the 3′-UTR of the viral genome, respectively. In addition, the TAR vector pCC1BAC contains a T7 promoter sequence which is removed after PCR amplification of the vector.

6. To minimize the likelihood of introducing undesired mutations, the amplification of input DNA fragments and TAR vector should be performed using high-fidelity polymerases according to the manufacturer’s recommendations.

7. Polyacrylamide gel electrophoresis (PAGE) purification is generally necessary for long oligonucleotides (more than 50 bases) and critical 5′ sequences.

8. If no unspecific PCR amplifications are observed, a PCR cleanup is sufficient.

9. Concentration of each DNA fragment and TAR vector should not be too low to keep the volume of input DNA (overlapping DNA fragments, TAR vector, and DNA carrier) to around 55 μl during yeast transformation, i.e., less than 10% of the final volume of a transformation reaction (see Subheading 3.2, step 2).

10. When starting from a −80 °C glycerol stock, it is recommended to start a pre-culture in 10 ml of SD-His broth.

11. The final volume of yeast culture at this step is calculated based on the number of transformation conditions that one is planning to perform. In general, use 3 ml of yeast culture (OD₆₀₀nm ~ 1) per condition.

12. Use 2 μl of sterile water or SD-His broth for easy streaking of yeast cells on agar surface.

13. If yeast colonies are not well isolated, restreaking should be performed on 12-sector agar plates to produce isolated colonies.

14. Alternatively, 100 μl of an overnight saturated yeast culture can be used.

15. The 10× primer mix can contain up to 25 primer pairs.

16. To ensure assay performance, the primer mix should be stored at −20 °C in small aliquots to avoid multiple cycles of freezing and thawing.

17. Depending on the size difference of generated PCR products, adjust the agarose gel percentage accordingly.
18. The amount of each ingredient can be adjusted; however, the ratio Buffer RES:Zymolyase:β-Mercaptoethanol = 100:10:1 should be maintained.

19. Depending on specific preferences and settings of laboratories, other plasmid preparation kits can also be used, in which case comparable adjustments should be considered and included to ensure optimal plasmid yields.

20. If the supernatant is not yet clear, transfer it to a new tube and repeat centrifugation, preferably at a higher speed if possible.

21. As mentioned in the kit’s user manuals, failing to include this step or direct pouring of buffer EQU inside of the filter may result in lower plasmid yield.

22. DNA pellet should not be overdried as it will be more difficult to be dissolved.

23. Vortexing or pipetting with narrow tips to resuspend DNA pellet is not recommended as it causes DNA shearing.

24. DNA should be left at 4 °C for several days to be completely dissolved, and subsequently stored at −20 °C for long-term usage.

25. To avoid the possibility of degrading RNA in the following steps, it is strongly recommended that assay performers always wear gloves, and thoroughly spray working areas and pipettes with RNase AWAY solution or similar.

26. BHK-21 cells that stably express the N protein can be used if available, and, in this case, N RNA should be omitted in Subheading 3.5.2.

References

1. 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. https://doi.org/10.1128/jvi.74.22.10600-10611.2000

2. Thiel V, Herold J, Schelle B, Siddell SG (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. https://doi.org/10.1099/0022-1317-82-6-1273

3. Casais R, Thiel V, Siddell SG et al (2001) Reverse genetics system for the avian coronavirus infectious bronchitis virus reverse genetics system for the avian coronavirus infectious bronchitis virus. J Virol 75:12359–12369. https://doi.org/10.1128/JVI.75.24.12359

4. 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 97:5516–5521. https://doi.org/10.1073/pnas.97.10.5516

5. Larionov V, Kouprina N, Graves J et al (1996) Specific cloning of human DNA as yeast artificial chromosomes by transformation-associated recombination. Proc Natl Acad Sci U S A 93:491–496. https://doi.org/10.1073/pnas.93.1.491

6. Oldfield LM, Grzesik P, Voorhies AA et al (2017) Genome-wide engineering of an infectious clone of herpes simplex virus type 1 using synthetic genomics assembly methods. Proc Natl Acad Sci 114:E8885–E8894. https://doi.org/10.1073/pnas.1700534114

7. Vashee S, Stockwell TB, Alperovich N et al (2017) Cloning, assembly, and modification of the primary human cytomegalovirus isolate Toledo by yeast-based transformation-associated recombination. mSphere 2:e00331-17.
8. Gibson DG, Benders GA, Andrews-Pfannkoch C et al (2008) Complete chemical synthesis, assembly, and cloning of a mycoplasma genitalium genome. Science 319:1215–1220. https://doi.org/10.1126/science.1151721

9. Gibson DG, Benders GA, Axelrod KC et al (2008) One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic mycoplasma genitalium genome. Proc Natl Acad Sci 105:20404–20409. https://doi.org/10.1073/pnas.0811011106

10. Gibson DG, Glass JI, Lartigue C et al (2010) Creation of a bacterial cell controlled by a chemically synthesized genome creation of a bacterial cell controlled by a chemically synthesized genome. Science 329:52–57

11. Noskov V (2002) A genetic system for direct selection of gene-positive clones during recombinational cloning in yeast. Nucleic Acids Res 30:e8. https://doi.org/10.1093/nar/30.2.e8

12. Kouprina N, Annab L, Graves J et al (1998) Functional copies of a human gene can be directly isolated by transformation-associated recombination cloning with a small 3’ end target sequence. Proc Natl Acad Sci U S A 95:4469–4474. https://doi.org/10.1073/pnas.95.8.4469

13. Blount BA, Driessen MRM, Ellis T (2016) GC preps: fast and easy extraction of stable yeast genomic DNA. Sci Rep 6:1–4. https://doi.org/10.1038/srep26863