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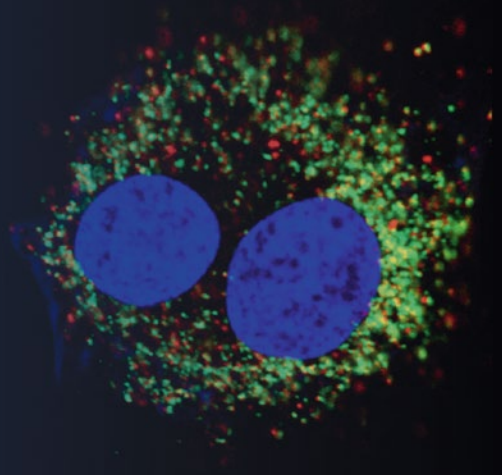

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Coronaviruses

Methods and Protocols

Second Edition

Humana Press

METHODS IN MOLECULAR BIOLOGY

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Edited by

Helena J. Maier and Erica Bickerton

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-0716-0899-9 ISBN 978-1-0716-0900-2 (eBook)
https://doi.org/10.1007/978-1-0716-0900-2

Cover illustration: Infectious bronchitis virus-infected chicken DF1 cell labelled to show viral RNA species in red and green.

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The registered company address is: 1 New York Plaza, New York, NY 10004, U.S.A.

Preface

In December 2019, a novel Coronavirus, SARS-CoV-2, emerged into the human population following a cross-species transmission event from an animal source. Since then it has spread globally resulting in the largest pandemic in a century, causing significant numbers of fatalities. Following widespread use of social distancing measures, the SARS-CoV-2 pandemic has had unprecedented impacts on day-to-day life worldwide. The wider impacts on the global economy and how emergence of this virus will alter society in the long term remain to be seen after the current pandemic ends. Coronaviruses have long been recognized to have the potential to cause such devastating outbreaks, and the current situation highlights the importance of studying this family of viruses to understand how they emerge, evolve, replicate, and cause disease. Only with this knowledge will it be possible to identify and control viruses with increased potential to cross the species barrier and to develop the diagnostics, vaccines, and antiviral therapeutics that are required to manage future outbreaks in both humans and animals.

Although it has not been possible to include chapters describing protocols for working with SARS-CoV-2 specifically in this current book, the protocols described here are applicable to all members of the *Coronavirinae* subfamily. Indeed, we aim with this book to provide a comprehensive collection of protocols being used in the coronavirus research field currently and that are also transferrable to other fields of virology. We hope it will be informative for both long-standing coronavirus researchers and those joining the field in the wake of the SARS-CoV-2 pandemic.

We would like to thank the authors who have contributed to this book for the time they have taken to prepare detailed methods as well as for providing practical hints and tips that are often essential to get a new protocol working. We would also like to give a special thanks to Nicole Doyle who has been a tremendous help with final edits during a very challenging time.

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Chapter 1

Coronaviruses: An Updated Overview of Their Replication and Pathogenesis

Yuhang Wang, Matthew Grunewald, and Stanley Perlman

Abstract

Coronaviruses (CoVs), enveloped positive-sense RNA viruses, are characterized by club-like spikes that project from their surface, an unusually large RNA genome, and a unique replication strategy. CoVs cause a variety of diseases in mammals and birds ranging from enteritis in cows and pigs, and upper respiratory tract and kidney disease in chickens to lethal human respiratory infections. Most recently, the novel coronavirus, SARS-CoV-2, which was first identified in Wuhan, China in December 2019, is the cause of a catastrophic pandemic, COVID-19, with more than 8 million infections diagnosed worldwide by mid-June 2020. Here we provide a brief introduction to CoVs discussing their replication, pathogenicity, and current prevention and treatment strategies. We will also discuss the outbreaks of the highly pathogenic Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle Eastern Respiratory Syndrome Coronavirus (MERS-CoV), which are relevant for understanding COVID-19.

Key words *Nidovirales*, Coronavirus, Positive-sense RNA viruses, SARS-CoV, MERS-CoV

1 Classification

CoVs, the largest group of viruses within the *Nidovirales* order, comprises *Coronaviridae*, *Arteriviridae*, *Roniviridae*, and *Mesoniviridae* families. The *Coronavirinae* include one of two subfamilies in the *Coronaviridae* family, with the other subfamily being *Torovirinae*. The *Coronavirinae* are further subdivided into four genera: the α, β, γ, and δ-CoVs. The viruses were initially sorted into these groups based on serology but now are divided by phylogenetic clustering and pairwise evolutionary distances in seven key domains of the replicase–transcriptase polyprotein. The arteriviruses consist of five genera of mammalian pathogens. The roniviruses, which infect shrimp and the mosquito-borne mesoniviruses have invertebrate hosts.

All viruses in the *Nidovirales* order are enveloped, nonsegmented positive-sense RNA viruses. They share a significant number of common features including (a) a highly conserved genomic

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_1,

organization, with a large replicase gene upstream of structural and accessory genes; (b) expression of many nonstructural protein genes by ribosomal frameshifting; (c) several unique or unusual enzymatic activities encoded within the large replicase–transcriptase protein product; and (d) expression of downstream genes by synthesis of 3′ nested subgenomic mRNAs. In fact, the *Nidovirales* order name is derived from these nested 3′ mRNAs as *nido* is Latin for "nest." The major differences within the four Nidovirus families are in the numbers, types, and sizes of their structural proteins and significant alterations in the structure and morphology of their virions and nucleocapsids.

2 Genomic Organization

Nidoviruses, which include CoVs, have the largest identified RNA genomes; CoVs contain approximately 30 kilobases (kb). The genome contains a 5′ cap structure along with a 3′ poly(A) tail, allowing it to act as a mRNA for translation of the replicase polyproteins. The replicase gene encoding the nonstructural proteins (nsps) occupies two-thirds of the genome, about 20 kb, as opposed to the structural and accessory proteins, which make up about 10 kb of the viral genome. The 5′ end of the genome contains a leader sequence and untranslated region (UTR) that contains multiple stem loop structures required for RNA replication and transcription. Additionally, at the beginning of each structural or accessory gene are transcriptional regulatory sequences (TRSs) that are required for expression of each of these genes. The 3′-UTR also contains RNA structures required for replication and synthesis of viral RNA. The organization of the CoVs genome is 5′-leader–UTR–replicase–S (Spike)–E (Envelope)–M (Membrane)–N (Nucleocapsid)–3′-UTR–poly(A) tail with accessory genes interspersed within the structural genes at the 3′ end of the genome (*see* Fig. 1). As shown using reverse genetics with deletion of these accessory genes, accessory proteins are almost exclusively nonessential for replication in tissue culture; however, some have been shown to have profound roles in viral pathogenesis [1–5]. In some cases, accessory proteins inhibit the host defense response, especially innate immune mechanisms. For example, during MERS-CoV infection, accessory ORFs 3–5 antagonize the innate immune response [6]; ORF4a binds to dsRNA, inhibiting type I interferon (IFN-I) expression and prevents the antiviral stress response [7, 8]; ORF4b inhibits IFN-I expression [9] and blocks NF-kB signaling [10]. ORF4b also encodes a cyclic phosphodiesterase, which blocks RNaseL activation [11].

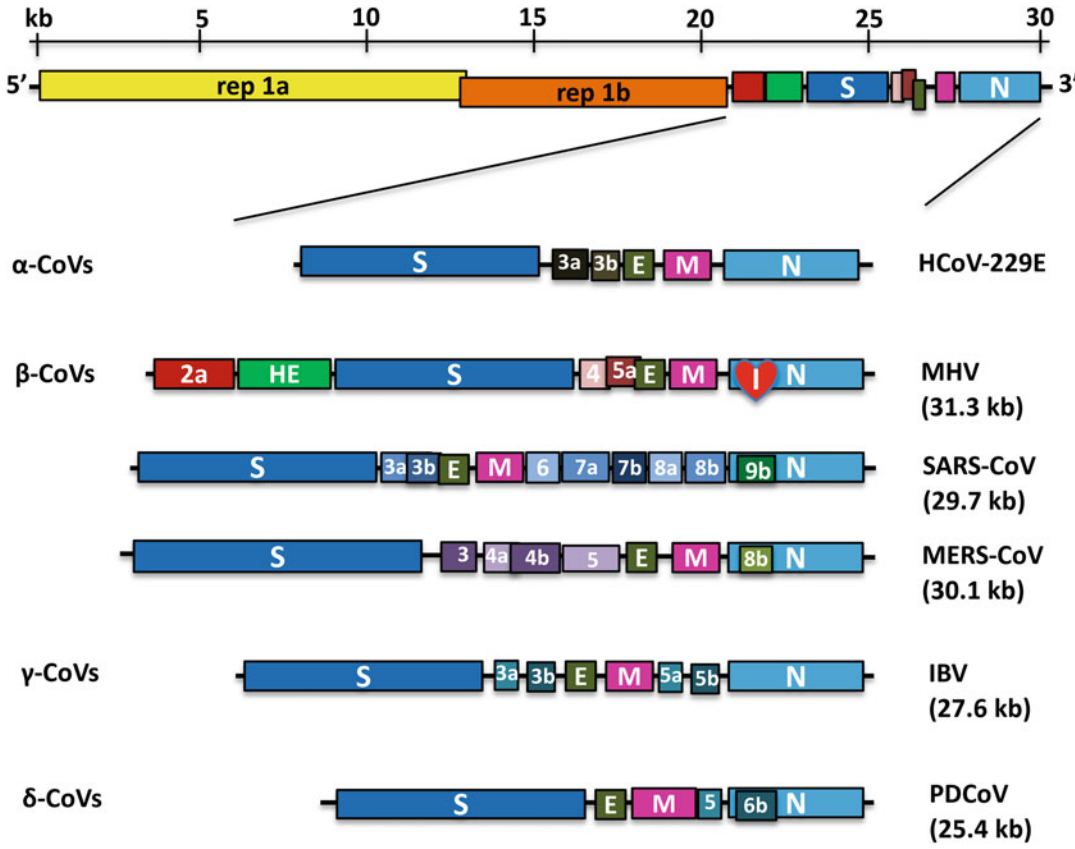

Fig. 1 Genome organization of representative α, β, γ, and δ-CoVs. An illustration of the MHV genome is shown on top. The replicase gene constitutes two ORFs, rep 1a and rep 1b, which are expressed by a ribosomal frameshifting mechanism. The expanded regions below show the structural and accessory proteins in the 3′ regions of α-CoVs (HCoV-229E), β-CoVs (MHV, SARS-CoV, and MERS-CoV), γ-CoVs (IBV), and δ-CoVs (PDCoV). The total genome size is given for each virus. The sizes and positions of accessory genes are indicated, relative to the basic genes S, E, M, and N. The size of the genome and individual genes are approximated using the legend at the top of the diagram but are not drawn to scale

3 Virion Structure

CoVs virions are spherical with diameters of approximately 125 n as depicted in studies by cryo-electron tomography and cryo-electron microscopy [12, 13]. The most prominent feature of CoVs is the club-shape spike projections emanating from the surface of the virion. These spikes are a defining feature of the virion and give them the appearance of a solar corona, prompting the name CoVs. Within the envelope of the virion is the nucleocapsid. CoVs have helically symmetrical nucleocapsids, which is uncommon among positive-sense RNA viruses but far more common for negative-sense RNA viruses.

CoVs virus particles contain four main structural proteins. These are the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins, all of which are encoded within the 3′ end of the viral genome. The distinctive spike structure on the surface of CoV is comprised of trimers of S molecules [14, 15]. The S protein is a class I viral fusion protein [16]. It binds to host cell receptors and mediates the earliest infection steps. In some case it also induces cell–cell fusion in late infection. The S monomer is a transmembrane protein with mass ranging from 126 to 168 kDa and is heavily N-linked glycosylated, increasing the apparent molecular weight by some 40 kDa. In most, but not all CoVs S is cleaved by a host cell furin-like protease into two separate polypeptides, S1 and S2 [17–19]. The S protein contains a very large ectodomain and a small endodomain. The ectodomain structures of representative viruses from each genus of CoV, MHV [20], HCoV-HKU1 [21], HCoV-NL63 [22], MERS-CoV [23], PDCoV [24], and IBV [25], have been determined by high resolution cryo-electron microscopy (cryo-EM) and were found to share a common architecture.

The M protein is the most abundant structural protein in the virion [26] and is thought to give the virion its shape. The M monomer, which ranges from 25 to 30 kDa, is a polytopic protein with three transmembrane domains [27]. It has a small N-terminal ectodomain, and a C-terminal endodomain that accounts for the major part of the molecule and is situated in the interior of the virion or on the cytoplasmic face of intracellular membranes [28]. M is usually modified by N-linked glycosylation [29, 30], although a subset of β-CoVs and δ-CoVs M proteins exhibit O-linked glycosylation [31]. M protein glycosylation has been shown to affect both organ tropism and the IFN inducing capacity of certain CoVs [32, 33]. Despite being cotranslationally inserted in the ER membrane, most M proteins do not contain a signal sequence. Rather, the first or the third transmembrane domain of the MHV and IBV M proteins suffices as a signal for insertion and anchoring of the protein in its native membrane orientation [34, 35]. M proteins of the α-CoVs species do contain cleavable amino-terminal signal peptides, but it is still not clear if these are necessary for membrane insertion [36]. One study suggested that the M protein exists as a dimer in the virion and adopts two different conformations, allowing it to promote membrane curvature as well as bind to the nucleocapsid [37]. The M protein of MHV binds to the packaging signal in nsp15 and in conjunction with the N protein is likely the primary determinant of selective packaging [38].

The E protein is a small protein with a size 8–12 kDa and is found in small quantities within the virion [39]. E proteins from different CoVs are highly divergent but share a common architecture: a short hydrophilic N-terminal, followed by a large

hydrophobic region, and, lastly, a large hydrophilic C-terminal tail. The membrane topology of E protein is not completely resolved [40–42], but most data suggest that it is a transmembrane protein. The E protein has ion channel activity and was observed to assemble into homooligomers, ranging from dimers through hexamers [43], and a pentameric α-helical bundle structure has been solved for the hydrophobic region of SARS-CoV E protein [44]. An oligomeric form is consistent with the ion channel activity of the E protein, but the monomeric form of E may also play a separate role. As opposed to other structural proteins, recombinant viruses lacking the E protein are not always lethal, although this is virus type dependent [45, 46]. The E protein facilitates assembly and release of the virus but also has other functions. For instance, SARS-CoV E protein is not required for viral replication but is required for pathogenesis [47, 48].

The N protein constitutes the only protein present in the helical nucleocapsid. It is composed of two independently folding domains, an N-terminal domain (NTD) and a C-terminal domain (CTD), both capable of binding RNA in vitro, but each domain uses different mechanisms to bind RNA. It has been suggested that optimal RNA binding requires contributions from both domains [49, 50]. N protein is heavily phosphorylated [51], which may be important for triggering a structural change enhancing the affinity for viral versus nonviral RNA, and is ADP ribosylated [52]. N protein binds the viral genome in a beads-on-a-string type conformation. Two specific RNA substrates have been identified for N protein: the transcription-regulating sequences (TRSs) [53] and the genomic packaging signal. The genomic packaging signal has been found to bind specifically to the second, or C-terminal RNA binding domain [38]. N protein also binds to nsp3 [50, 54], a key component of the replicase–transcriptase complex (RTC), and to the M protein [26]. These protein interactions serve to tether the viral genome to the RTC and subsequently package the encapsidated genome into viral particles.

Hemagglutinin-esterase (HE), a fifth structural protein, is present only in a subset of β-CoVs, which include MHV, BCoV, HCoV-OC43, and HCoV-HKU1. The protein acts as a hemagglutinin, binds sialic acids on surface glycoproteins, and contains acetylesterase activity [55]. These activities are thought to enhance S protein-mediated cell entry and virus spread through the mucosa [56]. Interestingly, HE enhances murine hepatitis virus (MHV) neurovirulence [1]; however, it is selected against in tissue culture for unknown reasons [57].

4 Coronavirus Life Cycle

4.1 Attachment and Entry

The initial attachment of the virion to the host cell is initiated by interactions between the S protein and its receptor. This interaction is the primary determinant controlling CoVs host species range and tissue tropism. Individual CoVs usually infects one or a few closely related hosts. S protein includes two subunits, the comparatively variable S1 subunit mediates the binding to receptor and the more conserved S2 subunit undergoes large conformational changes that results in fusion of virion and cell membranes. The sites of receptor binding domains (RBD) within the S1 region of a CoVs S protein vary depending on the virus: the RBD located at the N-terminal of S1 (MHV) in some cases [58] while it is present in the C-terminal of S1 in the case of SARS-CoV [59], MERS-CoV [60, 61], HCoV-229E [62], HCoV-HKU1 [63], HCoV-NL63 [64], and TGEV [65].

MHV enters cells by binding to its receptor (carcinoembryonic antigen-related adhesion molecule 1, CEACAM1), the CoVs receptor that was first discovered [66–68]. CEACAM1 has different isoforms which contains two and four Ig-like domains. The diversity of the receptor isoforms expressed in different genetic backgrounds results in a wide range of pathogenicity of MHV in mice [69]. Many α-CoVs and δ-CoVs utilize aminopeptidase N (APN) as their cellular receptor [70–75]. APN (also called CD13), a heavily glycosylated homodimer, is a cell-surface, zinc-binding protease that is resident in respiratory and enteric epithelia and in neural tissue. The α-CoVs receptor activities of APN homologs are not interchangeable among species [76, 77], while the δ-CoV PDCoV can use APN homologs from multiple mammalian and avian species as a receptor [73].

SARS-CoV uses angiotensin-converting enzyme 2 (ACE2) as its receptor [78]. ACE2 is mainly expressed in epithelial cells of the lung and the small intestine, the primary targets of SARS-CoV, and also in heart, kidney, and other tissues [79]. ACE2 is a cell-surface, zinc-binding carboxypeptidase and plays a role in regulation of cardiac function and blood pressure. ACE2 also serves as the receptor for the α-CoV HCoV-NL63 [80].

MERS-CoV uses dipeptidyl-peptidase 4 (DPP4) as its cellular receptor [81]. DPP4, also called CD26, is a membrane-bound exoprotease with a wide tissue distribution; it cleaves dipeptides from hormones, chemokines, and cytokines and plays multiple other physiological functions [81]. DPP4 includes an N-terminal eight-blade β-propeller domain and a C-terminal catalytic domain. Structure analysis of the MERS-CoV RBD-DPP4 complex indicates that the receptor binding surface of the RBD is a four-stranded β-sheet that contacts blades 4 and 5 of the DPP4 propeller domain [61, 82]. Further, key residues of camel and human DPP4

Table 1
The known receptors for coronaviruses

Virus	Receptor	References
α-CoVs		
CCoV	Canine APN (cAPN)	[76]
FCoV I	Unknown but not fAPN	[224]
FCoV II, FIPV	Feline APN (fAPN)	[72]
HCoV-229E	Human APN (hAPN)	[71]
HCoV-NL63	ACE2	[80]
PEDV	Unknown but not pAPN	[225, 226]
PRCoV	Porcine APN (pAPN)	[75]
TGEV	Porcine APN (pAPN)	[70]
β-CoVs		
MHV	Murine CEACAM1	[67]
BCoV	Neu5,9Ac2	[227]
HCoV-OC43	Neu5,9Ac2	[228]
SARS-CoV	ACE2	[78]
MERS-CoV	DPP4	[81]
γ-CoVs		
IBV	Alpha-2,3-linked sialic acid	[229]
δ-CoVs		
PDCoV	Porcine APN	[73, 74]

APN aminopeptidase N, *ACE2* angiotensin-converting enzyme 2, *BCoV* bovine coronavirus, *CCoV* canine coronavirus, *CEACAM* carcinoembryonic antigen-related adhesion molecule 1, *DPP4* dipeptidyl peptidase 4, *HCoV* human coronavirus, *PRCoV* porcine respiratory coronavirus, *TGEV* transmissible gastroenteritis virus, *PEDV* porcine epidemic diarrhea virus, *FIPV* feline infectious peritonitis virus, *MHV* murine hepatitis virus, *SARS-CoV* severe acute respiratory syndrome coronavirus, *MERS-CoV* Middle East respiratory syndrome coronavirus, *Neu5,9Ac2* *N*-acetyl-9-*O*-acetylneuraminic acid, *PDCoV* Porcine Delta-Coronavirus

critical for binding to the RBD are highly conserved facilitating zoonotic transmission of MERS-CoV [82] (*See* Table 1 for a list of known CoVs receptors).

Many CoVs S proteins are cleaved during exit from the producer cells, often by a furin-like protein [17]. This cleavage separates the RBD and fusion domains of the S protein [83]. Following receptor binding, the virus must next gain access to the host cell cytosol. This is generally accomplished by a second proteolytic cleavage of the S protein by TMPRRS2, a cathepsin or another protease [84, 85]. Following cleavage at S2′, a fusion peptide is exposed, which is followed by joining of two heptad repeats in S2

forming an antiparallel six-helix bundle [16]. The formation of this bundle allows for the mixing of viral and cellular membranes, resulting in fusion and ultimately release of the viral genome into the cytoplasm. Fusion generally occurs at the plasma membrane or in some cases, within acidified endosomes [86] .

The S protein is the major target for antivirus neutralizing antibodies and its binding to host cell receptor is critical for a productive infection and for cross-species transmission. This was illustrated during the SARS epidemic, when the S protein showed extensive adaptation to the human ACE2 receptor [87]. In contrast, the MERS S protein has changed little during the course of the MERS outbreak [88], except during the Korean outbreak when there was a single point introduction of virus [89]. Remarkably, virus mutated so that the S protein exhibited reduced affinity of the DPP4 receptor [90]. This may have been driven by the antivirus neutralizing antibody response, but this is not certain. One interpretation is that, in the case of MERS-CoV, receptor binding is less important than other parts of the entry process, such as S protein cleavage by host cell proteases, particularly TMPRSS2 [91].

4.2 Replicase Protein Expression

The next step in the CoVs lifecycle is the translation of the replicase gene from the virion genomic RNA. The replicase gene encodes two large ORFS, rep1a and rep1b, which express two coterminal polyproteins, pp1a and pp1ab (Fig. 1). In order to express both polyproteins, the virus utilizes a slippery sequence (5′-UUUAAAC-3′) and an RNA pseudoknot that cause ribosomal frameshifting from the rep1a reading frame into the rep1b ORF. In most cases, the ribosome unwinds the pseudoknot structure and continues translation until it encounters the rep1a stop codon. Occasionally the pseudoknot blocks the ribosome from continuing elongation, causing it to pause on the slippery sequence, changing the reading frame by moving back one nucleotide (−1 frameshift) before the ribosome is able to melt the pseudoknot structure and extend translation into rep1b, resulting in the translation of pp1ab [92, 93]. In vitro studies predict the incidence of ribosomal frameshifting to be as high as 25%, but this has not been determined in the context of virus infection. Viruses probably utilize frameshifting to control the precise ratio of rep1b:rep1a proteins or delay the production of rep1b products until the products of rep1a have created a suitable environment for RNA replication [94].

Polyproteins pp1a and pp1ab contain the nsps 1–11 and 1–16 respectively. In pp1ab, nsp11 from pp1a becomes nsp12 following extension of pp1a into pp1b. However, γ-CoVs do not contain a comparable nsp1. These polyproteins are subsequently cleaved into individual nsps [95]. There are two types of polyprotein cleavage activity. One or two papain-like proteases (PLpro), which are situated within nsp3, carry out the relatively specialized separations

of nsp1, nsp2, and nsp3. Many PLpro also have deubiquitinase activity, which counters some host antiviral defenses [96]. nsp5, the main protease (Mpro), performs the remaining 11 cleavage events [97, 98]. Mpro is often designated the 3C-like protease (3CLpro) to denote its distant relationship to the 3C proteins of picornaviruses. Since PLpro and Mpro have pivotal roles early in infection, they present attractive targets for antiviral drug design [96, 99].

Next, many of the nsps assemble into the replicase–transcriptase complex (RTC) to create an environment suitable for RNA synthesis including replication and transcription of subgenomic RNAs [100]. Notably, products of rep 1a, nsp3, nsp4, and nsp6 each contain multiple transmembrane helices which anchor the RTC to intracellular membranes [101, 102]. They are responsible for remodeling membranes to form organelles which are dedicated to viral RNA synthesis [103]. Among them, nsp3 is the largest RTC proteins by far [104]. It contains a hypervariable acidic N-terminal region that is a ubiquitin-like domain (Ubl1) and a highly conserved C-terminal region which is designated the Y domain and contains three metal-binding clusters of cysteine and histidine residues [105]. Ubl1 interacts with the serine and arginine-rich region (SR region) of the N protein; this interaction may tether the genome to the RTC, facilitating formation of the RNA synthesis initiation complex [54, 106, 107]. Also located within nsp3 is a conserved macrodomain (Mac1), that exhibits ADP-ribose-protein hydrolase activity [108]. The macrodomain nonessential for viral replication but critical for viral pathogenesis [109]. The nsps also have other functions important for RNA replication. For example, nsp10, a small nonenzymatic viral protein contributes to CoV replication fidelity by regulating nsp14 and nsp16 activity during virus replication [110]; nsp12 encodes the RNA-dependent RNA polymerase (RdRp); nsp13 encodes the RNA helicase and RNA 5′-triphosphatase; nsp14 encodes the exoribonuclease (ExoN) involved in replication fidelity [111–113] and N7-methyltransferase activity [114]; and nsp16 harbors 2′-*O*-methyltransferase activity [115]. In addition to roles in replication, nsp1 blocks innate immune responses by direct inhibition of translation or by promoting degradation of host IFN mRNA by nsp1 [116]; nsp15 contains an endoribonuclease domain that mediates evasion of host dsRNA sensors [117, 118]. For a list of nonstructural proteins and their putative functions, *see* Table 2. Ribonucleases nsp15-NendoU and nsp14-ExoN activities are unique to the *Nidovirales* order and are considered genetic markers for these viruses [119].

4.3 Replication and Transcription

Viral RNA synthesis follows the translation and assembly of the viral replicase complexes. Viral RNA synthesis produces both genomic and subgenomic RNAs. Subgenomic RNAs serve as mRNAs for

Table 2
Functions of coronaviruses nonstructural proteins

Protein	Function	References
nsp1	· Blocks host cell mRNA translation or promotes cellular mRNA degradation of host mRNA, including IFN mRNA · Inhibits IFN induction and signaling	[116, 230, 231]
nsp2	· No known function, binds to prohibitin proteins	[232, 233]
nsp3	· Ubiquitin-like1 (Ubl1) and acidic domains, interact with N protein · Papain-like protease (PLpro)/Deubiquitinase domain blocks IRF-3 activation and NF-κB signaling · Mac2, Mac3, and PLpro block p53 action · Mac1, Mac2, Mac3 (macrodomains have ADP-ribosylhydrolase activity), interferes with IFN-induced antiviral activity, promotes host proinflammatory cytokine expression · Ubl2, NAB, G2M, Y domains, unknown functions	[105, 234–240]
nsp4	· Potential transmembrane scaffold protein, important for proper structure of DMVs	[241, 242]
nsp5	· Main protease (Mpro), cleaves viral polyprotein · Inhibits IFN induction	[243, 244]
nsp6	· Potential transmembrane scaffold protein	[101]
nsp7	· Acts as cofactor with nsp8 to bind to nsp12 · Is responsible for the replication and transcription of the viral genome	[245, 246]
nsp8	· Acts as a cofactor with nsp7 to bind to nsp12 · Is responsible for the replication and transcription of the viral genome	[245, 246]
nsp9	· RNA binding protein	[247, 248]
nsp10	· Cofactor for nsp16 and nsp14, forms heterodimer with both and stimulates ExoN and 2-*O*-MT activity; · Contributes to CoV replication fidelity	[110, 249, 250]
nsp12	· RNA-dependent RNA polymerase (RdRp) · Binds to its essential cofactors, nsp7 and nsp8 to assemble RNA-synthesis complex	[246, 251]
nsp13	· RNA helicase · 5′ triphosphatase	[252, 253]
nsp14	· N7 methyltransferase, adds 5′ cap to viral RNAs · Viral exoribonuclease activity (ExoN), proofreading activity · Interferes with IFN-induced antiviral activity	[111–114, 254, 255]
nsp15	· Viral endoribonuclease, NendoU · Evades RNA sensing	[117, 118, 256–258]
nsp16	· 2′-*O*-methyltransferase (2′-*O*-MT) · Shields viral RNA from MDA5 recognition	[115, 259]

NAB nucleic acid binding, *DMVs* double-membrane vesicles, *MDA5* Melanoma differentiation associated protein 5

the structural and accessory genes which reside downstream of the replicase genes in Orf1. All positive-sense subgenomic RNAs are 3′ coterminal with the full-length viral genome and thus form a set of nested RNAs, a distinctive property of the order *Nidovirales*. Both genomic and subgenomic RNAs are produced through negative-strand intermediates. These negative-strand intermediates are only about 1% as abundant as their positive-sense counterparts and contain both polyuridylate and antileader sequences [120].

Many cis-acting sequences are important for the replication of viral RNAs. Within the 5′ UTR of the genome are seven stem-loop structures that may extend into the replicase 1a gene [121–124]. The 3′ UTR contains a bulged stem-loop, a pseudoknot, and a hypervariable region [125–128]. The stem-loop and the pseudoknot at the 3′ end overlap, and thus cannot form simultaneously [126, 129]. Therefore, these different structures are proposed to regulate alternate stages of RNA synthesis, although exactly which stages are regulated and their precise mechanism of action are still unknown.

Perhaps the most novel aspect of CoV replication is how the leader and body TRS segments fuse during production of subgenomic RNAs. Leader-TRS joining occurs during the discontinuous extension of negative-strand RNA [130]. The current model proposes that the RdRp pauses at body TRS sequences (TRS-B); following this pause, the RdRp either continues elongation to the next TRS or switches to amplifying the leader sequence at the 5′ end of the genome guided by complementarity of the TRS-B to the leader TRS (TRS-L). Furthermore, nucleocapsid phosphorylation and RNA helicase DDX1 recruitment was shown to facilitate the transition from discontinuous to continuous transcription [131]. However, many questions remain. For instance, how does the RdRp bypass all of the TRS-B sequences to produce full-length negative-strand genomic RNA? Also, how are the TRS-B sequences directed to the TRS-L and how much complementarity is necessary? Answers to these questions and others will be necessary to gain a full perspective of how RNA replication occurs in CoVs. Eventual development of an in vitro replication system will be required to fully understand these processes.

Finally, CoVs are also known for their ability to recombine by both homologous and nonhomologous recombination [132, 133]. The ability of these viruses to recombine is tied to the strand switching ability of the RdRp. Recombination likely plays a prominent role in viral evolution and is the basis for targeted RNA recombination [134], a reverse genetics tool used to engineer viral recombinants at the 3′ end of the genome.

4.4 Assembly and Release

Following replication and subgenomic RNA synthesis, the viral structural proteins, S, E, and M are translated and inserted into the endoplasmic reticulum (ER). These proteins move along the

secretory pathway into the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) [135, 136]. There, viral genomes encapsidated by N protein bud into membranes of the ERGIC containing viral structural proteins, forming mature virions [137].

The M protein directs most protein–protein interactions required for assembly of CoVs. M protein is not sufficient for virion formation as virus-like particles (VLPs) cannot be formed by M protein expression alone. However, when M protein is expressed along with E protein, VLPs are formed, suggesting these two proteins function together to produce CoV envelopes [138]. N protein enhances VLP formation, suggesting that fusion of encapsidated genomes into the ERGIC enhances viral envelopment [139]. The S protein is incorporated into virions at this step but is not required for assembly. The ability of the S protein to traffic to the ERGIC and interact with the M protein is critical for its incorporation into virions.

While the M protein is relatively abundant, the E protein is only present in small quantities in the virion. Thus, it is likely that M protein interactions provide the impetus for envelope maturation. E protein may assist the M protein in virion assembly either by inducing membrane curvature [46, 140, 141], preventing the aggregation of M protein [142], or by an uncharacterized mechanism. The E protein may also have a separate role in promoting viral release by altering the host secretory pathway [143].

The M protein also binds to the nucleocapsid, and this interaction promotes the completion of virion assembly. These interactions have been mapped to the C-terminus of the endodomain of M with CTD 3 of the N-protein [144]. However, it is unclear exactly how the nucleocapsid complexed with virion RNA traffics from the RTC to the ERGIC to interact with M protein and become incorporated into the viral envelope. Another outstanding question is how the N protein selectively packages only positive-sense full-length genomes among the many different RNA species produced during infection. A packaging signal for MHV is present in the nsp15 coding sequence [38]. Mutation of this signal drastically increases sgRNA incorporation into virions, and while virus production in cultured cells is negligibly affected, the mutant virus elicits a stronger IFN response in mice [145]. Furthermore, most CoVs do not contain similar sequences at this locus, indicating that packaging may be virus specific.

Following assembly, virions are transported to the cell surface in vesicles and released by exocytosis. It is not known if the virions use a traditional pathway for transport of large cargo from the Golgi or if the virus has diverted a separate, unique pathway for its own exit. Genome-wide screening has identified a host protein, valosin-containing protein (VCP/p97) that is required for release of CoV from endosomes [146]. In several CoVs, S protein that does not assemble into virions transits to the cell surface where it

mediates cell–cell fusion between infected cells and adjacent, uninfected cells. This leads to the formation of multinucleated cells, which allows the virus to spread within an infected organism without being detected or neutralized by virus-specific antibodies.

5 Pathogenesis

5.1 Animal Coronaviruses

CoVs cause a large variety of diseases in animals, and their ability to cause severe disease in livestock and companion animals such as pigs, cows, chickens, dogs, and cats led to significant research on these viruses in the last half of the twentieth century. For instance, Transmissible Gastroenteritis Virus (TGEV) and Porcine Epidemic Diarrhea Virus (PEDV) cause severe gastroenteritis in young piglets, leading to significant morbidity, mortality, and ultimately economic losses. Recently a novel HKU2-related bat CoV, Swine Acute diarrhea Syndrome Coronavirus (SADS-CoV), was shown to cause an outbreak of fetal swine disease [147]. Porcine Hemagglutinating Encephalomyelitis Virus (PHEV) causes enteric infection but has the ability to infect the nervous system, causing encephalitis, vomiting and wasting in pigs. Feline enteric coronavirus (FCoV) causes a mild or asymptomatic infection in domestic cats, but during persistent infection, mutation transforms the virus into a highly virulent strain of FCoV (Feline Infectious Peritonitis Virus, FIPV) that leads to development of a lethal disease called feline infectious peritonitis (FIP). FIPV is macrophage tropic and is believed to cause aberrant cytokine and/or chemokine expression and lymphocyte depletion, resulting in lethal disease [148]. Bovine CoV, Rat CoV, and Infectious Bronchitis Virus (IBV) cause mild to severe respiratory tract infections in cattle, rats, and chickens respectively. Bovine CoV causes significant losses in the cattle industry and also has spread to infect a variety of ruminants, including elk, deer, and camels. In addition to severe respiratory disease, the virus causes diarrhea ("winter dysentery" and "shipping fever"), all leading to weight loss, dehydration, and decreased milk production [149]. Some strains of IBV, a γ-CoV, also infect the urogenital tract of chickens causing renal disease. IBV significantly diminishes egg production and weight gain, causing substantial losses in the chicken industry each year [150]. Interestingly, a novel CoV (SW1) was identified in a deceased Beluga whale [151] and shown to be a γ-CoV based on phylogenetic analysis. This is the first example of a nonavian γ-CoV, but it is not known whether this virus actually causes disease in whales.

In addition, there has been intense interest in identifying novel bat CoVs, since these are the likely ultimate source for most CoV, including SARS-CoV and MERS-CoV [152, 153]. Hundreds of novel bat CoV have been identified over the past decade [154], including the agent of SADS, described above. Another novel,

non-CoVs group of nidoviruses, *Mesoniviridae*, were recently identified as the first ones to exclusively infect insect hosts [155, 156]. These viruses are highly divergent from other nidoviruses but are most closely related to the roniviruses. In size, they are ~20 kb, falling in between large and small nidoviruses. Consistent with this relatively small size, these viruses do not encode for an endoribonuclease, which is present in large nidoviruses. Recently a novel nidovirus, planarian secretory cell nidovirus (PSCNV), was identified and shown to have a 41.1 kb genome, making it the largest RNA viral genome yet discovered. The genome contains the canonical nidoviral genome organization and key replicative domains. It encodes a predicted 13,556 aa polyprotein in an unconventional single ORF [157].

The most heavily studied animal CoV is murine hepatitis virus (MHV), which causes multiple diseases in mice, including respiratory, enteric, hepatic, and neurologic infections. For instance, MHV-1 causes severe respiratory disease in susceptible A/J and C3H/HeJ mice, A59 and MHV-3 induce hepatitis, and JHMV causes encephalitis and acute and chronic demyelinating diseases. MHV-3 induces cellular injury through the activation of the coagulation cascade via a fgl2/fibroleukin dependent way [158]. A59 and attenuated versions of JHMV cause chronic demyelinating diseases that bears similarities to multiple sclerosis (MS), making MHV infection one of the best models for this debilitating human disease. Early studies suggested that demyelination was dependent on viral replication in oligodendrocytes in the brain and spinal cord [159, 160]; however, more recent reports clearly demonstrate that the disease is immune-mediated. Irradiated mice or immunodeficient (lacking T and B cells) mice do not develop demyelination, but addition of virus-specific T cells restores the development of demyelination [161–163]. Additionally, demyelination is accompanied by a large influx of macrophages and microglia that can phagocytose infected myelin [164]. Microglia are especially important in the initial host defense to MHV since mice succumb to the infection if these cells are depleted [165].

5.2 Human Coronaviruses

Prior to the SARS-CoV outbreak, CoVs were only thought to cause mild, self-limiting respiratory infections in humans. Two of these human CoVs are α-CoVs (HCoV-229E and HCoV-NL63), while the other two are β-CoVs (HCoV-OC43 and HCoV-HKU1). HCoV-229E and HCoV-OC43 were isolated nearly 50 years ago [166–168], while HCoV-NL63 and HCoV-HKU1 were only identified following the SARS-CoV outbreak [169, 170]. HCoV-229E and HCoV-NL63 arose from a common ancestor and diverged 200 years ago [171]. HCoV-OC43 is closely related to BCoV and may have crossed species from bovids, or alternatively, may have been transmitted from humans to cows. These viruses are endemic in the human populations, causing 15–30% of upper

respiratory tract infections each year. They cause more severe disease in neonates, the elderly, and in individuals with underlying illnesses, with a greater incidence of lower respiratory tract infection in these populations [172]. HCoV-NL63 is also associated with acute laryngotracheitis (croup) [173]. One interesting aspect of these viruses is their differences in tolerance to genetic variability. HCoV-229E isolates from around the world have only minimal sequence divergence [174], while HCoV-OC43 isolates from the same location but isolated in different years show significant genetic variability [175]. Based on the ability of MHV to cause demyelinating disease, it has been suggested that human CoVs may be involved in the development of multiple sclerosis (MS) [176]. However, no evidence to date suggests that human CoVs play significant roles in MS.

SARS-CoV, a group 2b β-CoV, was identified as the causative agent of the severe acute respiratory syndrome (SARS) epidemic that originated in 2002–2003 in the Guangdong Province of China. During the 2002–2003 outbreak, approximately 8098 cases occurred with 774 deaths, resulting in a mortality rate of 9%. This rate was much higher in aged individuals, with mortality rates approaching 50% in individuals over 60 years of age while no patients under 24 years died from the infection [177]. Furthermore, the outbreak resulted in the loss of nearly $40 billion dollars in economic activity as the virus nearly completely shut down many activities in Southeast Asia and Toronto, Canada for several months. The epidemic began in wet markets in Guangzhou, likely originating in bats. It is widely accepted that SARS-CoV is a bat virus as a large number of Chinese horseshoe bats contain sequences of SARS-related CoVs and contain serologic evidence for a prior infection with a related CoV [178, 179]. Further, two novel bat SARS-related CoVs were later identified that are more similar to SARS-CoV than any other virus identified to date, further supporting a bat origin for SARS-CoV [180].

SARS-CoV then spread from infected bats to intermediate animals such as Himalayan civet cats and raccoon dogs present in the markets and then to humans [181]. An individual was infected in Guangzhou and then stayed at a hotel in Hong Kong, spreading the infection to others staying at the hotel, and ultimately, throughout the world. Although some human individuals within wet animal markets had serologic evidence of SARS-CoV infection prior to the outbreak, these individuals had no apparent symptoms [181]. Thus, it is likely that SARS-like CoV circulated in the wet animal markets for some time before a series of factors facilitated its spread into larger human populations.

Transmission of SARS-CoV was relatively inefficient, as it largely spread through large droplets and direct contact with infected individuals and transmission only occurred after the onset of clinical illness. Thus, the outbreak mostly occurred within

households and healthcare settings [182], except in a few cases of superspreading events where one individual was able to infect multiple contacts due to high viral burdens or an ability to aerosolize virus. As a result of the relatively inefficient transmission of SARS-CoV, the outbreak was controllable through the use of quarantining. Only a small number of SARS cases occurred after the outbreak was controlled in June 2003 [183].

SARS-CoV primarily infects epithelial cells within the lung [184]. The virus is capable of entering macrophages and dendritic cells but only causes an abortive infection [185, 186]. Despite this, infection of these cell types may be important in inducing proinflammatory cytokines that may contribute to disease [187]. In fact, many cytokines and chemokines are produced by these cell types and are elevated in the serum of SARS-CoV infected patients [188]. Viral titers decrease when severe disease develops in both humans and in several animal models of the disease, suggesting that the host response is responsible for much of the clinical signs and symptoms. Furthermore, animals infected with rodent-adapted SARS-CoV strains show similar clinical features to the human disease, including an age-dependent increase in disease severity [189]. These animals also show increased levels of proinflammatory cytokines and reduced T-cell responses, consistent with a possible immunopathological mechanism of disease [190, 191].

While the SARS-CoV epidemic was controlled in 2003, and the virus has not since returned, a novel human CoV emerged in the Middle East in 2012. This virus, named Middle East Respiratory Syndrome-CoV (MERS-CoV), is a group 2b β-CoV and was found to be the causative agent of a highly lethal respiratory tract infection in Saudi Arabia and other countries in the Middle East [153, 192]. Since its emergence, the virus has spread to over 27 countries, including to South Korea in 2015, where it caused 186 cases and 38 deaths [193]. Among those cases, 83% were transmitted from five super spreading events and 44% were due to nosocomial transmission at 16 hospitals [194]. As of September 2019, there have been a total of 2468 laboratory-confirmed cases of MERS-CoV, with 851 associated deaths and a case fatality rate of approximately 35%, as reported to the World Health Organization (https://www.who.int/emergencies/mers-cov/en/). The majority of cases early in the outbreak resulted from nosocomial transmission. As better infection control measures were instituted, approximately 50% of cases are considered primary, with infection resulting from direct or indirect contact with camels, the zoonotic source of the infection [195]. Serological studies have identified MERS-CoV antibodies in dromedary camels in the Middle East and Africa from samples obtained as early as 1983 [196]. Supporting evidence for camel to human transmission comes from studies identifying nearly identical MERS-CoVs in camels and humans in nearby proximities in Saudi Arabia [5, 23, 197]. In one of these

studies the human case had direct contact with an infected camel and the virus isolated from this patient was nearly identical to the virus isolated from the camel [5]. MERS-CoV likely originated in bats because it is related to two previously identified bat CoV, HKU4 and HKU5 [198]. Furthermore, new evidence has emerged to support the hypothesis that bats are the evolutionary source of MERS-CoV since a MERS-like CoV was identified from a *Pipistrellus* cf. *hesperidus* bat sampled in Uganda [199].

MERS-CoV utilizes Dipeptidyl peptidase 4 (DPP4) as its receptor [81]. The virus is only able to use the receptor from certain species such as bats, humans, camels, rabbits, and horses to establish infection. While the virus is unable to naturally infect mouse cells due to differences in the structure of DPP4, several mouse models expressing human DPP4 have been developed which can successfully be infected with MERS-CoV [200–203].

6 Diagnosis, Treatment, and Prevention

In most cases of self-limited infection, diagnosis of CoVs is unnecessary. However, it is important in certain clinical and veterinary settings or in epidemiological studies to identify an etiological agent. Diagnosis is also important in locations where a severe CoV outbreak is occurring, such as, at present, in the Middle East, where MERS-CoV continues to circulate. The identification of cases will guide the development of public health measures to control outbreaks. It is also important to diagnose cases of severe veterinary CoV-induced disease, such as PEDV and IBV, to control these pathogens and protect food supplies. The primary methods to diagnose CoV infection use molecular techniques such as RT-PCR. RT-PCR has become the method of choice for diagnosis of human CoV, as multiplex real-time RT-PCR assays, such as RT-RTPA, and RT-LAMP, have been developed. They are able to detect all four respiratory human CoVs and could be further adapted to detect novel CoVs [204, 205]. Serologic assays are important in cases where RNA is difficult to isolate, virus is no longer present, and for epidemiological studies. Because rapid and accurate diagnosis of MERS is important, several diagnostic tests including one in which RT-LAMP is combined with vertical flow visualization (RT-LAMP-VF) [206] have been developed.

To date, there are no antiviral therapeutics that specifically target human CoVs, so treatments are only supportive. IFNs have been used in some patients, without evidence of therapeutic benefit [207]. Studies in mice indicate that the relative timing of IFN administration and virus replication are critical to either protective or pathogenic effects after infection with SARS-CoV [208] or MERS-CoV [209], which may explain the variable results that are observed in patients. The SARS and MERS outbreaks have

stimulated research on these viruses, and this research has identified a large number of suitable antiviral targets, such as viral proteases, polymerases, and entry proteins, but so far no specific treatment has been licensed. Multitarget treatment should be a priority as for antiviral treatment [210, 211]. Significant work remains to develop drugs that target these processes and are useful in infected patients.

Many CoV-specific vaccines have been developed and some targeting veterinary CoV pathogens have been licensed. Vaccines have been approved for IBV [212], TGEV [213], and canine CoV, but these vaccines are not always used because they are either not very effective, or in some cases, have resulted in the selection of novel pathogenic CoVs via recombination of circulating strains [214]. In general, it is thought that live attenuated vaccines are most efficacious in targeting CoVs. This was illustrated in the case of TGEV, where an attenuated naturally appearing variant, porcine respiratory coronavirus (PRCoV), appeared in Europe in the 1980s. This variant only caused mild disease and protected swine from lethal TGEV. This attenuated virus has prevented the recurrence of severe TGEV in Europe and the USA over the past 30 years [215].

In the case of SARS-CoV, several potential vaccines have been developed. The spike protein, which elicits a neutralizing antibody response, has been a major target of vaccine development [216, 217]. Therapeutic SARS-CoV neutralizing human monoclonal antibodies have been generated and stockpiled [218]. These antibodies would be useful for passive immunization of healthcare workers and other high-risk individuals in the event of another SARS outbreak [219]. Similarly, efforts have been made to develop vaccines against MERS-CoV. Several vaccine approaches have been tried, including subunits vaccines, DNA vaccines, viral vector vaccines and live attenuated and inactivated vaccines [220, 221]. Some of these have shown efficacy in animal testing and several are in clinical trials. For example, a MERS-CoV DNA vaccine recently underwent phase I Clinical Trials [222, 223]; while it induced MERS-CoV-specific neutralizing antibody titers, these tended to decline substantially by 60 weeks after immunization.

Owing to the lack of effective therapeutics or vaccines, the best measures to contain human CoVs outbreaks remain a strong public health surveillance system coupled with rapid diagnostic testing and quarantine when necessary. For international outbreaks, cooperation of governmental entities, public health authorities, and health care providers is critical. During outbreaks of veterinary CoV that are readily transmitted, such as PEDV, more drastic measures such as culling of entire herds of pigs may be necessary to prevent transmission of these deadly viruses.

7 Conclusions

Over the last half century, several varieties of CoVs have emerged to cause human and veterinary diseases. It is likely that these viruses will continue to emerge and evolve, and cause both human and veterinary outbreaks owing to their ability to recombine, mutate, and infect various animal species and cell types.

Critical problems remain to be resolved in future research. One focus should be to understand viral replication and pathogenesis in greater detail. Another is to explore the propensity of these viruses to cross species and the features that facilitate or inhibit cross-species transmission and to identify CoVs reservoirs, which will enhance our ability to predict potential future epidemics. So far, bats seem to be a primary reservoir for these viruses, but they do not develop clinically evident disease, for reasons that require further investigation. Additionally, many of the nonstructural and accessory proteins encoded by CoVs are only partly characterized, and it will be important to identify their mechanisms of action and their role in viral replication and pathogenesis. These studies will help identify more suitable therapeutic targets. Finally, additional studies should probe CoV-induced immunopathological disease and delineate the relationship between CoVs and the host immune response. These will guide efforts to design vaccines and drugs that prevent and treat CoV infections.

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Part I

Coronavirus Detection, Discovery, and Evolution

Chapter 2

Discovery of Novel Coronaviruses in Rodents

Theocharis Tsoleridis and Jonathan K. Ball

Abstract

The recent emergence of SARS, SARS-CoV2 and MERS and the discovery of novel coronaviruses in animals and birds suggest that the Coronavirus family is far more diverse than previously thought. In the last decade, several new coronaviruses have been discovered in rodents around the globe, suggesting that they are the natural reservoirs of the virus. In this chapter we describe the process of screening rodent tissue for novel coronaviruses with PCR, a method that is easily adaptable for screening a range of animals.

Key words Discovery, Rodents, PCR, Coronavirus detection, Alphacoronavirus, Betacoronavirus

1 Introduction

Rodents are known to be an important source of emerging viral infections [1]. Rodentia include approximately 2200 species such as voles, mice, and rats and is the single largest mammalian order comprising ~40% of all mammals [2, 3].

Historically, virus discovery relied on relatively inefficient in vitro or in vivo virus isolation methods. These led to the identification of two rodent coronaviruses (CoVs); rat sialodacryoadenitis coronavirus (SADV) [4] and murine hepatitis virus (MHV) [5], both of which are from the same viral species within the *Betacoronavirus* genus. However, the advent of degenerate primer PCR and unbiased viral metagenomics has significantly enhanced our ability to detect novel viruses, and their use has led to the discovery of numerous alpha- and betacoronaviruses in a range of rodent species, including field voles, bank voles, rats, and mice in East Asia and Europe [6–11]. These findings are paving the way toward a better understanding of the longer-term evolution and origins of these important viral species [12].

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_2,

In this chapter we describe a degenerate primer PCR method that we have used [10] to detect coronaviruses in a variety of tissues obtained from rodent species sampled postmortem.

2 Materials

2.1 Sample Acquisition

1. Ethics approval.
2. Live traps and snap traps of different sizes to collect wild rodents (voles, rats).
3. −80 °C freezers.
4. RNA preservative such as RNAlater or DNA/RNA shield.
5. Class I biosafety cabinet.
6. Disposable scalpels.

2.2 Total RNA Extraction from Mammalian Tissue

1. Class I biosafety cabinet.
2. Plastic tweezers.
3. Petri dishes.
4. Homogenizer.
5. Rodent tissue sample (preferably liver) in a preservative such as RNAlater stored at −80 °C (*see* **Note 1**).
6. Total RNA extraction kit from mammalian tissues such as GenElute™ Mammalian Total RNA Miniprep Kit (*see* **Note 2**).
7. Thermocycler.
8. NanoDrop® spectrophotometer or similar.

2.3 cDNA Synthesis with Random Hexamers

1. Random hexamers, such as RNA to cDNA EcoDry (*see* **Note 3**).
2. PCR-grade water.
3. Thermocycler.

2.4 PCR

1. Taq polymerase and buffer, such as HotStarTaq DNA Polymerase.
2. Thermocycler.
3. PCR-grade water.
4. 10 mM deoxynucleotide mix.
5. Primers (*see* Table 1; *see* **Notes 4** and **5**).

2.5 Agarose Gel Electrophoresis

1. Gel electrophoresis tank.
2. 2% agarose gel with 0.5 μl/ml ethidium bromide (from a 10 mg/ml stock).
3. 6× loading buffer: 10 mM Tris–HCl (pH 7.6) 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM

Table 1
List of primers used

Primer name	Sequence 5′–3′	References
GAPDH_F	CCATCTTCCAGGAGCGAGA	[10]
GAPDH_R	GCCTGCTTCACCACCTTCT	
CoV_F	GGTTGGGACTATCCTAAGTGTGA	[13]
CoV_R	CCATCATCAGATAGAATCATCATA	

EDTA (or equivalent commercial ones such as Thermo Scientific 6× DNA Loading Dye).

4. Tris–acetate–EDTA (TAE): 40 mM Tris–acetate and 1 mM EDTA (pH 8.3) buffer containing 0.5 μl/ml ethidium bromide (from a 10 mg/ml stock) (*see* **Note 6**).
5. UV transilluminator.

3 Methods

3.1 Sample Acquisition

1. Acquire ethical approval from relevant committees to trap, euthanize, and handle wild rodents.
2. Wild rodents can either be live trapped and euthanized later, or snap-killed by the trap. In either case, the carcasses should be stored as soon as possible at −80 °C.
3. In a Class I biosafety cabinet, the carcasses should be dissected using disposable scalpels and collect the organs of interest (in this case liver and intestine).
4. The organs should be placed in a tube containing a preservative such as RNAlater and stored immediately at −80 °C until they are processed for RNA extraction.

3.2 RNA Extraction from Mammalian Tissues

1. All the extractions from mammalian tissues should be performed inside a Class I biosafety cabinet to reduce any safety risk while handling the tissues and to avoid contamination.
2. Remove the tissue samples from the freezer, and once thawed remove from the preservative and place the sample in a petri dish (*see* **Note 7**).
3. Using a pair of tweezers, tease a section of tissue measuring approximately 1 mm^3 in volume from the tissue sample. Put the remaining tissue back in the tube with the preservative and store it at −80 °C.
4. Proceed with the extraction following the steps of the extraction kit protocol.

5. Measure the quality and the concentration of the extracted RNA using a NanoDrop® or similar spectrophotometer. Use the elution buffer of the extraction kit as a blank to calibrate the spectrophotometer. A pure RNA sample should have A_{260}/A_{280} and A_{260}/A_{230} ratios between 1.80 and 2.2 (*see* **Note 8**). One A_{260} unit equals 40 μg/ml single stranded RNA (ssRNA).

3.3 cDNA Synthesis with Random Hexamers

1. Add up to 5 μg of the extracted RNA from Subheading 3.2 above to a tube of EcoDry™ cDNA lyophilized premix (random hexamers). If necessary, add molecular biology–grade (RNase/DNase-free) water to yield a total volume of 20 μl.
2. Incubate the reaction at 42 °C for 60 min and then stop the reaction by heating at 70 °C for 10 min.

3.4 PCR Amplification of the GAPDH "Housekeeping Gene" to Assess cDNA Quality

1. For each of the cDNA samples, make up a 12.5 μl PCR reaction containing 1× PCR Buffer, 2.5 units HotStarTaq Polymerase, 0. 2 mM of each dNTP, 0.4 μM Forward Primer, 0.4 μM Reverse Primer, molecular biology–grade water, and cDNA template (*see* **Notes 9** and **10**). Also include negative control reactions containing water as template.
2. Transfer the reaction tubes to a thermal cycler and perform the amplification using the following cycling parameters: 1 cycle of 15 min at 95 °C; 55 cycles of 20 s at 95 °C, 20 s at 60 °C, 30 s at 72 °C; and a final extension cycle of 60 s at 72 °C.
3. Add 1 μl of 6× gel loading buffer to 5 μl of the PCR reaction and load into the wells of a preprepared 2% agarose gel in 1× TAE-EtBr. Load a suitable DNA ladder (e.g., GeneRuler DNA Ladder mix) to allow size estimation of any PCR products.
4. Resolve the DNA products by electrophoresis in 1×TAE-EtBr at 90 V for 40 min.
5. The expected product size for successful amplification of the *GAPDH* gene is 571 bp (*see* **Note 11**).

3.5 PCR Screening for Coronaviruses

1. Samples of cDNA that yield a *GAPDH* PCR product can then be screened for the presence of coronavirus using a degenerate primer PCR.
2. Similar to Subheading 3.4, for each GAPDH positive cDNA sample, make up a 12.5 μl Coronavirus-specific PCR reaction containing 1× PCR Buffer, 2.5 units HotStarTaq Polymerase, 0.2 mM of each dNTP, 0.4 μM Forward Primer, 0.4 μM Reverse Primer, molecular biology–grade water, and cDNA template (*see* **Notes 10** and **12**).
3. Transfer the reaction tubes to a thermal cycler and perform the amplification using the following cycling parameters: 1 cycle of 15 min at 95 °C; 55 cycles of 20 s at 95 °C, 20 s at 48 °C, 30 s at 72 °C; and a final extension cycle of 10 min at 72 °C.

4. Analyse the PCR products by electrophoresis through a 2% agarose gel in 1×TAE-EtBr at 90 V for 40 min. Coronavirus-specific PCR products should appear as a band of approximately 440 bp.
5. Any PCR positive samples should be sent for Sanger sequencing using the sense and/or antisense PCR primers (*see* **Note 13**).

3.6 Agarose Gel Preparation and Electrophoresis

1. Seal the casting tray and add the combs of interest.
2. To prepare a 2% agarose gel add 2 g of agarose in 100 ml of 1× TAE buffer (*see* **Note 6**).
3. Place the flask containing the mixture into a microwave oven until it starts boiling. Take the flask out, stir it gently while holding it and place it back to the oven. Repeat the procedure until the mixture is transparent and there is no visible agarose.
4. Cool the flask briefly under the tap with cold water until it is cold enough to place it on your hand.
5. Add 5 μl of EtBr (from a 10 mg/ml stock) and star stirring gently.
6. Pour the mixture into the casting tray steadily and remove any bubbles with a pipette tip.
7. Let it rest for 20–30 min until it becomes solid.
8. Load the gel as described in Subheading 3.4, **step 3** and run it at 90 V for 40 min.
9. Visualize the gel on the UV transilluminator.
10. Always be careful when handling EtBr and dispose of as per local regulations. There should be a dedicated space or room for preparing and running agarose EtBr gels.

4 Notes

1. For best quality RNA and virus retrieval, the rodent carcasses should be immediately frozen after their death. As soon as they are dissected, each tissue should be placed in a tube containing a preservative such as RNAlater (to preserve the RNA and prevent degradation) and stored immediately at −80 °C. The tissues and the RNA should always be placed on ice when handled. Avoid repeated freeze–thaw cycles as they contribute to the rapid degradation of the nucleic acid.
2. There are several methods for RNA extraction, such as phenol–chloroform and TRIzol. However, it is much easier to use a commercial extraction kit. We routinely use GenElute™

Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) which gives us good RNA recovery.

3. For the purpose of coronavirus discovery, cDNA synthesis with either random hexamers or a specific primer is acceptable. Random hexamers provide the flexibility of performing several PCRs directed at different targets such as different viruses or housekeeping genes for quality control. Primer-specific cDNA, on the other hand, is less flexible but is reportedly more sensitive.
4. A PCR targeting the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene (or any other housekeeping gene) is essential to assess the quality of the cDNA.
5. There are several published primer-sets for coronavirus screening [13–17]. However, in this chapter we describe the use of Woo et al. primer-set [13]. Although it was designed to detect the Human HKU-1 CoV, which is a betacoronavirus, it has been shown to detect alphacoronaviruses [10].
6. It is easier to buy a commercially available 10× TAE buffer and dilute to a final concentration of 1×.
7. RNA is very sensitive and degrades at room temperature; therefore, all the RNA work should be done on ice to keep it stable.
8. The purity of the RNA sample will impact on downstream applications such as cDNA synthesis. Therefore, it is essential to aim for an RNA sample free from contaminants.
9. All the PCR preparation should be performed in a dedicated pre-PCR room where there are no traces of amplicons or plasmids. The amplification and all the post-PCR handling should be done in separate rooms as far away from the pre-PCR room as possible. Thus, the risk of contamination is minimized.
10. The maximum amount of cDNA used in a PCR should not be more that 1/10th of the total PCR volume. If the user wants to add more cDNA template, then the reaction volume should be increased accordingly.
11. In this chapter we describe the conventional PCR method. Quantification of the template is possible with quantitative real-time PCR by doing serial dilutions and using a housekeeping gene as a reference.
12. Sometimes virus titers in a sample can be very low and might not be detected using low levels of template. Larger-volume PCRs, utilizing increased template can be beneficial in these

cases. This approach can also be applied to rescreen potential hits that have yielded low amounts of product (as indicated by the presence of a faint band of the expected size following agarose gel electrophoresis) in the initial screening PCR.

13. Nowadays, Sanger sequencing is commercially available and very affordable. The only requirement usually is to dilute the PCR products (usually 1 in 10 if the gel band is clear and bright) and to provide your primer of interest at a certain concentration.

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Chapter 3

Detection and Discovery of Coronaviruses in Wild Bird Populations

Chantal J. Snoeck and Siamak Zohari

Abstract

Wild birds are natural hosts of multiple microbial agents, including a wide diversity of coronaviruses. Here we describe a pan-Coronavirus detection RT-PCR method to identify those viruses regardless of the coronavirus genus or nature of the specimen. We also describe a protocol using high-throughput sequencing technologies to obtain their entire genome, which overcomes the inherent difficulties of wild bird coronavirus sequencing, that is, their genetic diversity and the lack of virus isolation methods.

Key words Wild birds, Gammacoronavirus, Deltacoronavirus, Sample collection, Pan-Coronavirus, RT-PCR, Next-generation sequencing, Illumina MiSeq

1 Introduction

Wild birds are natural hosts of multiple microbial agents such as low pathogenic avian influenza viruses or avian avulaviruses but also avian coronaviruses (CoV). Historically, avian CoV mainly referred to CoV from poultry that have been long studied, especially infectious bronchitis virus (IBV). However, the discovery of the first gamma-CoV in wild birds in Norway in 2003 [1], followed by the first delta-CoV in Hong Kong in 2007 [2] changed our perspective on CoV epidemiology in birds. The significant challenges in understanding their ecology include the large numbers of potential host species and the usually short period of viral shedding, combined with the difficulty of catching and sampling representative numbers of individual species. Although some surveillance has started to target CoV in wild birds, more research is necessary to determine which species may serve as reservoirs, carrying and shedding the disease asymptomatically.

In addition, IBV-like strains are occasionally detected in wild birds, and conversely, wild bird gamma- or delta-CoV strains are occasionally identified in domestic poultry [3]. This promiscuity is also raising questions regarding the origin of some genetic

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_3,

fragments observed in new CoV species such as Turkey Coronavirus and Guinea fowl Coronavirus. The genome of these two poultry viruses is closely related to IBV, except for the S gene, which is of unknown origin [4, 5]. Efforts toward full genome characterization of wild bird CoV are therefore warranted to understand and prevent potential recombination events.

Here we describe a pan-Coronavirus RT-PCR detection method to identify CoV in wild birds, regardless of the expected CoV genus of interest. Given the challenge of sequencing CoV with classical methods due to their genetic variability and the lack of in vitro culture methods, high-throughput sequencing technologies offer faster, cheaper, and less laborious alternatives to obtain complete wild bird CoV genome information. Here we provide a protocol coupling metagenomics approach and high throughput sequencing technology, taking advantage of metagenomics unbiased survey of the genetic material within a sample.

2 Materials

2.1 Specimen Collection (See Note 1)

1. Virus transport medium (VTM; *see* **Note 2**).
2. Flocked swabs (*see* **Note 3**).
3. 70% alcohol.
4. Screw-cap tubes.
5. Personal protective equipment (*see* **Note 4**).

2.2 RNA Extraction and Amplification of the RNA-Dependent RNA Polymerase (RdRp) Gene

1. RNA extraction kit (e.g., QIAamp viral RNA minikit or similar).
2. RNase/DNase-free water.
3. One-step RT-PCR kit (e.g., Qiagen One-Step RT-PCR kit).
4. Taq polymerase 5 U/μl (e.g., Platinum Taq DNA Polymerase).
5. 10 mM dNTPs.
6. 50 mM $MgCl_2$.
7. RNase inhibitor (e.g., RNaseOUT 40 U/μl).
8. Fwd primer for first round RT-PCR: 5′-GGKTGGGAYT AYCCKAARTG-3′ [6].
9. Rev. primer for first round RT-PCR: 5′-TGYTGTSWRCARAA YTCRTG-3′ [6].
10. Fwd primer for nested PCR: 5′-GGTTGGGACTATCCT AAGTGTGA-3′ [6].
11. Rev. primer for nested PCR: 5′-CCAACAYTTNGARTCWG CCAT-3′ [7] (*see* **Note 5**).
12. Thermocycler.

13. Gel electrophoresis equipment.
14. 50× TAE buffer: 242 g tris base, 57.1 ml acetic acid, 100 ml 0.5 M EDTA (pH 8.0), add double distilled water to reach 1 l.
15. 1.5% agarose gel: 1.5 g of agarose, 100 ml 1× TAE.
16. Molecular weight marker (e.g., 1 kb plus DNA ladder or similar).
17. Gel purification kit (e.g., QIAquick Gel Extraction Kit or similar).
18. PCR product purification kit (e.g., QIAquick PCR Purification Kit or similar).

2.3 NGS Sequencing

1. RNA extraction kit (e.g., QIAamp RNeasy Mini Kit or similar).

2.3.1 RNA Extraction Prior to NGS Sequencing

2. TRIzol LS Reagent.
3. Chloroform.
4. Molecular biology-grade ethanol.
5. DNase mix: 5 μl DNase (6 U/μl), 75 μl DNase Digestion Buffer mixed in a nuclease-free tube.
6. Round bottom 2 ml collection tube.
7. Chemical fume hood.
8. Refrigerated microfuge.
9. Vortex.

2.3.2 Quantification and RNA Integrity Check

1. Qubit® 2.0 Fluorometer (Invitrogen).
2. Qubit RNA HS Assay Kit (Invitrogen).
3. Thin-wall, clear 0.5 ml PCR tubes Qubit® assay tubes (Invitrogen) or Axygen PCR-05-C tubes.

2.3.3 cDNA Synthesis and Quantification

1. SuperScript IV First Strand Synthesis Reaction or similar.
2. 3′→5’exo$^-$ Klenow DNA polymerase.
3. Thermocycler.
4. PCR plate.
5. Qubit® 2.0 Fluorometer (Invitrogen).
6. Qubit dsDNA HS (High Sensitivity) Assay Kit (Invitrogen).
7. Thin-wall, clear 0.5 ml PCR tubes Qubit® assay tubes (Invitrogen) or Axygen PCR-05-C tubes.

2.3.4 Library Preparation for Sequencing with Illumina MiSeq

1. Nextera® XT Library Preparation Kit (Illumina).
2. Nextera XT Index Kit (Illumina).
3. TruSeq Index Plate Fixture Kit (Illumina).
4. MiSeq Reagent Kit (Illumina).
5. PhiX Control (Illumina).

6. Hard-Shell skirted PCR plate or similar.
7. Microseal adhesive film.
8. 96-well 0.8 ml polypropylene deep-well plate.
9. Agencourt AMPure XP beads.
10. Molecular biology-grade ethanol.
11. 0.1 N NaOH.
12. Ultrapure water.
13. High Sensitivity DNA chip (Agilent).
14. Thermocycler.
15. Magnetic stand.
16. Microplate centrifuge.
17. Agilent Technology 2100 Bioanalyzer.
18. Illumina® MiSeq System.

3 Methods

3.1 Specimen Collection [8, 9]

1. Swabs taken from the cloaca, oropharynx, and trachea or fresh dropping samples are suitable for CoV detection (*see* **Note 1**).
2. Wear appropriate personal protective equipment when handling birds and samples (*see* **Note 4**).
3. Unwrap a swab, according to the size of the bird, from the stem end of the packaging without touching the swab tip.
4. *Tracheal swab*: open the bird's mouth and gently pull the tongue forward to expose the trachea at the rear end of the tongue.
5. Wait until the bird breathes and the cartilage protecting the trachea is open.
6. Insert the swab into the trachea and gently touch the sides and back of the trachea.
7. *Oropharyngeal swab*: open the bird's mouth and gently roll the swab tip around the inside of the bird's mouth and behind the tongue.
8. *Cloacal swab*: insert the entire tip of the swab into the cloaca and swab with two to four circular motions while applying gentle pressure against the mucosal surfaces.
9. *Fresh droppings*: insert the entire tip of the swab into the fresh droppings, swab with two to four circular motions, taking care to not touch the underlying surface (*see* **Note 6**).
10. Place the swab tip into a screw cap vial containing VTM, ensuring the swab tip is fully immersed into VTM.

11. Cut or snap the stem of the swab so that the swab remains in the vial and the cap can be screwed on tightly.
12. Wipe the tube with 70% alcohol.
13. Label the tube with appropriate information.
14. Record sample tube number on data sheet.
15. Immediately store all samples on ice (preferably dry ice), transferring to a −70 °C freezer as soon as possible (*see* **Note 7**).

3.2 RNA Extraction and Amplification of the RdRp Gene

1. Prior to the extraction, vortex the swab or fecal material 20% w/v in VTM for 1 min.
2. Centrifuge for 5 min at 2000 rpm (400 × *g*).
3. Extract RNA from 140 μl of clarified supernatant on a silicate column with a RNA extraction kit, such as QIAamp viral RNA minikit according to the manufacturer's instructions, including all necessary positive and negative controls.
4. RNA samples can be stored up to 48 h at +4 °C or immediately at −80 °C for later use.
5. Perform the RT-PCR by mixing 13.9 μl of RNase/DNase-free water, 5 μl of 5× enzyme buffer, 0.5 μl $MgCl_2$, 0.5 μl dNTPs, 1 μM of each primer (final concentration), 0.1 μl RNase inhibitor, 1 μl enzyme, and 2 μl RNA.
6. Perform the RT-PCR using the following cycle conditions: 50 °C for 30 min, 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min.
7. Analyze the PCR products on a 1.5% agarose gel, let samples migrate for 45 min at 130 V. The expected size of the fragment is 602 bp (*see* **Note 8**).
8. Perform the nested PCR reaction by mixing 16.9 μl of RNase/DNase-free water, 2.5 μl of 10× enzyme buffer, 1 μl $MgCl_2$, 0.5 μl dNTPs, 0.7 μM of each primer (final concentration), 0.2 μl enzyme, and 2.5 μl of first round PCR products (*see* **Note 9**).
9. Amplify the PCR products using the following cycle conditions: 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min, followed by a final elongation at 72 °C for 10 min.
10. Analyze the PCR products on a 1.5% agarose gel, let samples migrate for 45 min at 130 V. The expected size of the fragment is 555 bp (*see* **Note 10**).
11. The samples with a band at the expected size can be directly purified using a PCR product purification kit if specific, or perform a gel extraction using a commercial kit if more than one band are visible.

12. Sequence purified PCR fragments in both directions using your preferred Sanger sequencing platform to confirm positivity.

3.3 NGS Sequencing (See Note 11)

3.3.1 RNA Extraction Prior to NGS Sequencing

This protocol making use of QIAamp RNeasy Mini Kit is suitable for the purification of RNA from 250 μl sample volumes. Sample material can be liquid samples (serum/plasma, cell culture supernatant, EDTA-blood diluted 1:2 in PBS, supernatant from swabs) or samples homogenized and lysed (tissue and stool homogenates).

1. Thaw the samples on ice and keep them on ice during the entire procedure to prevent degradation.
2. Mix each sample by pulse-vortexing for 20 s.
3. Working in a chemical hood, pipet 750 μl TRIzol LS Reagent into a 2 ml tube, add 250 μl sample and thoroughly shake manually for 15 s.
4. Briefly centrifuge for 5 s at 8000 rpm and incubate 5 min at room temperature.
5. Add 200 μl chloroform.
6. Thoroughly shake manually for 15 s and incubate for 10 min at room temperature. Centrifuge for 10 min at 13,000 rpm (16,000 × *g*) in a precooled refrigerated microfuge set at 4 °C.
7. Carefully transfer the upper aqueous phase without disturbing the DNA containing interphase into a new sterile 2 ml tube.
8. Add 600 μl 75% ethanol, mix by pulse-vortexing and briefly centrifuge for 5 s at 8000 rpm (6000 × *g*).
9. Apply 600 μl of the sample from **step 8** to the column, and centrifuge at 10,000 rpm (9600 × *g*) for 20 s.
10. Discard the collection tube and place the column into a clean 2 ml collection tube, apply residual sample from **step 8** to the column and centrifuge at 10,000 rpm for 20 s.
11. Discard the collection tube and place the column into a clean 2 ml collection tube, add 350 μl buffer RW1 to the column and centrifuge at 10,000 rpm for 20 s.
12. Discard the collection tube and place the column into a clean 2 ml collection tube, apply 80 μl DNase mix solution to the center of the membrane without touching the membrane.
13. Incubate for 15 min at room temperature, add 400 μl buffer RW1 to the column and centrifuge at 16,000 rpm (>21,000 × *g*) for 30 s.
14. Discard the collection tube and place the column into a clean 2 ml collection tube, add 500 μl buffer RPE and centrifuge at 16,000 rpm for 30 s.

15. Discard the collection tube and place the column into a clean 2 ml collection tube, add 500 µl buffer RPE and centrifuge at 13,000 rpm for 1 min.
16. Discard the collection tube and place the column into a clean 2 ml collection tube, centrifuge for 2 min at 13,000 rpm (to let the column dry).
17. Discard the collection tube and place the column into a clean 1.5 ml tube, add 50 µl RNase-free water to the center of the membrane, incubate 1 min at room temperature and centrifuge for 1 min at 10,000 rpm.
18. RNA can be used immediately or stored at ≤70 °C.

3.3.2 Quantification and RNA Integrity Check

1. Store the dye and the buffer at room temperature. Store the DNA, RNA, and protein standards at 4 °C. Ensure that all assay reagents are at room temperature before starting.
2. Set up two Assay Tubes for the standards and one tube for each user sample.
3. Prepare the Qubit™ Working Solution by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. Prepare 200 µl of Working Solution for each standard and sample.
4. Prepare the Assay Tubes according to Table 1.
5. Vortex all tubes for 2–3 s.
6. Incubate the tubes for 2 min at room temperature.
7. Insert the tubes in the Qubit® 2.0 Fluorometer and take readings.

3.3.3 cDNA Synthesis and Quantification

1. Prepare a mastermix 1 and 2 for the first strand synthesis using the SuperScript IV First Strand Synthesis Reaction according to Table 2.
2. Add 5 µl of mastermix 1 to all necessary wells, then add 8 µl of samples.
3. Perform the primer annealing step by placing the plate on a thermocycler at 65 °C for 5 min, then place the plate directly on ice for 1 min.
4. Add 7 µl of mastermix 2 to all necessary wells.
5. Perform the reverse transcription by placing the plate on a thermocycler at 23 °C for 10 min, followed by 50 °C for 10 min, then 80 °C for 10 min. At the end, place the plate directly on ice for 1 min.
6. Add 0.5 µl 3′→5’exo$^-$ Klenow DNA polymerase for synthesis of dsDNA.
7. Perform the dsDNA synthesis by placing the plate in a thermocycler at 37 °C for 60 min, 75 °C for 10 min, then hold at 4 °C.

Table 1
Volume of specific reagents to prepare standards and samples to be quantified on the Qubit® 2.0 Fluorometer

Reagent	Volume to add to prepare Standard Assay Tubes	Volume to add to prepare Sample Assay Tubes
Working solution (from **step 3**)	190 µl	180–199 µl
Standard (from kit)	10 µl	–
Sample	–	1–20 µl
Total volume	200 µl	200 µl

Table 2
Composition of mastermixes for cDNA synthesis

Mastermix	Reagent	Volume for 1 sample
Mastermix 1	DEPC-treated water Random hexamer [50 ng/µl] 10 mM dNTPs RNA (up to 5 µg) Total volume	3 µl 1 µl 1 µl 8 µl 13 µl
Mastermix 2	5×SSIV buffer DTT [0.1 M] RNaseOUT [40 U/µl] SuperScript IV [200 U/µl] Total volume	4 1 1 1 7 µl

8. Store cDNA at −20 °C or continue directly with the library preparation.
9. Quantify the cDNA on a Qubit® 2.0 Fluorometer, similarly to RNA quantification, using the Qubit dsDNA HS Assay Kit.
10. Use 2 µl of each DNA sample with 198 µl of the Qubit working solution (prepared by diluting the Qubit™ reagent 1:200 in Qubit™ buffer).
11. Target a 260/230 ratio of 2.0–2.2 values.

3.3.4 Illumina MiSeq Library Preparation—DNA Tagmentation

Fragment DNA and then tag DNA with adapter sequences in a single step as follows (*see* **Note 12**):

1. Record information about your samples before beginning library preparation.
2. Thaw the reagents on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly before use. Add the following volumes in the order listed to each well of a new PCR plate:

5 μl Tagment DNA Buffer (TD), 2.5 μl DNA (0.2 ng/μl per sample), 2.5 μl Amplicon Tagment Mix (ATM) to each well. Pipet to mix.

3. Centrifuge at 280 × *g* at 20 °C for 1 min.
4. Run the tagmentation program on a thermocycler with pre-heated lid (55 °C for 5 min, hold at 10 °C). When the sample reaches 10 °C, immediately proceed to the next step because the transposome is still active.
5. Add 5 μl Neutralize Tagment Buffer (NT) to each well. Pipette to mix (at room temperature).
6. Centrifuge at 280 × *g* at 20 °C for 1 min.
7. Incubate at room temperature for 5 min.

3.3.5 Illumina MiSeq Library Preparation—Library Amplification

This step amplifies the tagmented DNA using a limited-cycle PCR program.

1. Setup of TruSeq Index Plate Fixture according to the provided kit protocol and arrange the tubes and tips provided with the kit.
2. Add the following reagents and mix by pipetting after adding each reagent: 7.5 μl Nextera PCR Master Mix (NPM), 2.5 μl Index 2 primers (S5XX), 2.5 μl Index 1 primers (N7XX).
3. Centrifuge at 280 × *g* at 20 °C for 1 min.
4. Place the plate on a thermocycler and run the following program: 72 °C for 3 min, 95 °C for 30 s, 12 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s followed by a final step at 72 °C for 5 min, and hold at 10 °C.
5. Seal the plate and store at 2–8 °C for up to 2 days. Alternatively, leave on the thermocycler overnight.

3.3.6 Illumina MiSeq Library Preparation—Library Cleanup

This step uses AMPure XP beads to purify the library DNA and remove short library fragments.

1. Centrifuge at 280 × *g* at 20 °C for 1 min.
2. Transfer 50 μl PCR product from each well of the PCR plate to corresponding wells of a deep-well plate.
3. Add 15 μl AMPure XP beads to each well.
4. Shake at 1800 rpm for 2 min then incubate at room temperature for 5 min.
5. Place on a magnetic stand and wait until the liquid is clear (approx. 2 min).
6. Remove and discard all supernatant from each well.

7. Wash two times as follows: add 200 µl fresh 80% ethanol to each well, incubate on the magnetic stand for 30 s, remove and discard all supernatant from each well.
8. Using a 20 µl pipette, remove residual 80% ethanol from each well. Then let dry on the magnetic stand for 15 min before removing the plate from the stand.
9. Add 52.5 µl Resuspension Buffer (RSB) to each well, and shake at 1800 rpm for 2 min.
10. Incubate at room temperature for 5 min.
11. Place on a magnetic stand and wait until the liquid is clear (approx. 2 min).
12. Transfer 25 µl supernatant from the midi plate to a new PCR plate.
13. Seal the plate and store at −25 °C to −15 °C for up to 7 days.

3.3.7 Illumina MiSeq Library Preparation—Library Check and Pooling

1. Run 1 µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip. A typical library shows a broad size distribution (Fig. 1).
2. Pooling libraries combines equal volumes of normalized libraries in a single tube. After pooling, dilute and heat-denature the library pool before loading libraries for the sequencing run.
3. Centrifuge at 280 × *g* at 20 °C for 1 min.
4. Transfer 5 µl of each library from the new 1.5 ml tube. Invert to mix.

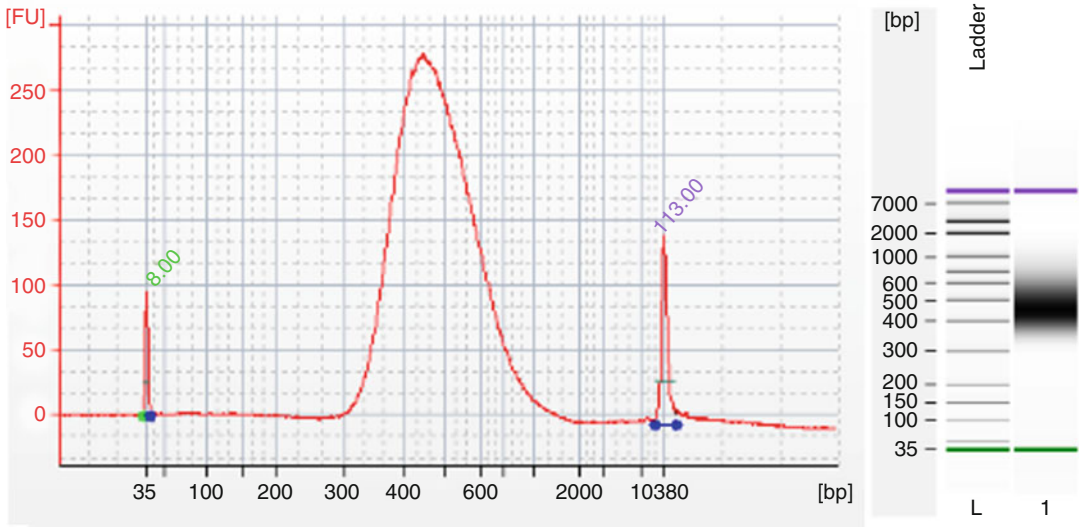

Fig. 1 Typical fragment size distribution of a library

5. Dilute pooled libraries to the loading concentration for your sequencing system. For instructions, see the denaturing and dilution library guide for your system.
6. Store unused pooled libraries at −25 °C to −15 °C for up to 7 days.

3.3.8 Sequencing of Pooled Libraries with the MiSeq Instrument

1. Mix 2.5 μl of diluted NaOH (0.1 N NaOH, max. 7 days old) and 7.5 μl of a library pool for 5 min at room temperature.
2. Add 940 μl of hybridization buffer to the denatured library and 50 μl PhiX Control.
3. Load 600 μl of the library mix onto the reagent cartridge.
4. Run the MiSeq system.
5. Wash the instrument with incorporation buffer PR2.
6. Inspect cluster density for the data output quality.

4 Notes

1. Before planning studies in wild birds necessitating capture and sampling, all the necessary permits from local and national wildlife authorities in the country should be obtained. Handling endangered species may require additional permits. Wild birds may be captured using several methods, including nets, live traps, and spotlighting. Once wild birds are captured, handling by authorized personnel should be done according to the recommendations of the local wildlife authorities. Captured birds are kept in a well-ventilated and quiet environment to prevent them from overheating and to minimize the stress.
2. A variety of viral transport media are commercially available. However, viral transport media can also be prepared as following: Hanks balanced salt solution supplemented with 10% glycerol, 200 U/ml penicillin, 200 μg/ml streptomycin, 100 U/ml polymyxin B sulphate, 250 μg/ml gentamicin, and 50 U/ml nystatin.
3. If you are planning to sample different species of birds, consider purchasing different sizes of swabs for large and small bird species to avoid injuries.
4. When handling wild birds, the presence of an infectious agent should always be assumed. It is therefore critical to take standard precautions to prevent exposure and the spread of pathogens. Visiting several sampling sites must be avoided unless microbial decontamination of equipment and clothing has been carried out.

5. Several protocols for the detection of coronaviruses in (wild) birds have been published (reviewed in [10]). The primers presented here offer the advantage of a pan-Coronavirus detection, not only amplifying gammacoronaviruses but the four genera of coronaviruses. The use of such pan-Coronavirus assays has led to the characterization, among other, of a plethora of gamma- and deltacoronaviruses in wild birds. The existence of betacoronaviruses in wild birds [11] is still debated. No alpha- or betacoronaviruses were detected in our cohorts using those primer sets [3] while they were successfully used to detect and characterize alpha- and betacoronavirus strains in bats [7].
6. Alternatively, fresh droppings can be collected using a spoon and later resuspended 20% w/v in VTM in the laboratory.
7. Samples should be kept in an undisrupted cold chain. Fast freezing and rapid handling of the samples for the laboratory investigations are critical for the conservation of nucleic acid in the samples. If using dry ice for sample shipment, enough dry ice should be foreseen to ensure that some is remaining until the samples arrive at the laboratory. This requires a minimum of 1 kg dry ice for every kg of samples.
8. Samples with a band at the expected size (602 bp) can be sequenced directly, without the need for a nested PCR. Refraining from performing a nested reaction on the first round PCR product that is already quite concentrated prevents cross contamination during the subsequent steps.
9. Dilution (e.g., 1:5 in RNase/DNase-free water) of the first round PCR products prior to the nested PCR is often useful to remove leftover reagents, diminishing carry-over into the nested reaction, which then often gives better results.
10. Due to the degenerate nature of the primer sequences to allow for a pan-Coronavirus detection, nonspecificity is expected. Therefore, confirmation of positivity by Sanger sequencing is often useful and advisable. In addition, the genetic information generated is useful to infer the coronavirus genus and perform phylogenetic analyses.
11. The risk for cross contamination is high during next generation sequencing steps due to clonal amplification. To minimize the risk for carryover of the amplified library DNA into original sample material or sequencing ready libraries, use separate areas for sample processing/library construction and run preparation and never mix materials from the sequencing and library preparation area.
12. To avoid contamination, do not use the same barcode as in the sequencing run before.

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Chapter 4

Competitive ELISA for the Detection of Serum Antibodies Specific for Middle East Respiratory Syndrome Coronavirus (MERS-CoV)

Shuetsu Fukushi

Abstract

Middle East respiratory syndrome coronavirus (MERS-CoV) is the etiological agent of MERS, a severe respiratory disease first reported in the Middle East in 2012. Serological assays are used to diagnose MERS-CoV infection and to screen for serum antibodies in seroepidemiological studies. The conventional enzyme-linked immunosorbent assay (ELISA) is the preferred tool for detecting serum antibodies specific for pathogens; however, the utility of conventional ELISA with respect to detection of MERS-CoV antibodies is limited due to the number of false-positives caused by cross-reactivity of serum antibodies with antigens that are conserved among coronaviruses. The competitive ELISA (cELISA) uses a pathogen-specific monoclonal antibody (MAb) that competes with serum antibodies for binding to an antigen; therefore, it is used widely for serological surveillance of many pathogens. In this chapter, I describe detection of serum antibodies using cELISA based on MAbs specific for MERS-CoV.

Key words MERS-coronavirus, Serological assay, Competitive ELISA, Monoclonal antibody, Neutralizing antibody

1 Introduction

Middle East respiratory syndrome (MERS) is a severe respiratory illness first reported in the Middle East in 2012; it is caused by a newly recognized coronavirus called MERS-CoV [1]. Humans are infected with MERS-CoV through direct or indirect contact with dromedary camels, indicating that dromedary camels are the amplifying host and a major source of zoonotic infection [2, 3]. Since the discovery of MERS-CoV, serological antibody assays have been developed to assess antibody responses of infected patients and to investigate the seroprevalence of MERS [4–6]. Virus neutralization assays are the gold standard for detecting antibodies specific for MERS-CoV because they are both specific and sensitive [7]. However, conventional virus neutralization assays require handling of infectious MERS-CoV under biosafety level (BSL)-3 conditions.

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_4,

Furthermore, it takes several days to obtain results because detectable levels of virus replication in virus infected cells are required.

General laboratories lacking heightened biocontainment facilities can use replication-incompetent pseudotyped viruses bearing viral glycoproteins to detect neutralizing antibodies and to investigate the mechanism underlying virus entry into host cells. Indeed, studies show the utility of a high-throughput pseudotyped virus system for detecting neutralizing antibody responses against MERS-CoV and to search for drugs that inhibit entry of MERS-CoV into cells [8–10]. The major advantage of the MERS-CoV pseudotype is that it can be handled without the need for BSL-3 conditions. However, the pseudotype system is not always a versatile approach since it requires equipment for cell culture, along with machines able to measure fluorescence or chemiluminescence to detect pseudotype infection; such a system is not readily adaptable to rural areas or developing countries in which the availability of expensive equipment is limited. Furthermore, the method used to generate the pseudotype virus is tricky; for example, carboxyl-terminal truncation of the MERS-CoV spike (S) protein might be needed to generate a high titer of the vesicular stomatitis virus-based MERS-CoV pseudotype [10].

Enzyme-linked immunosorbent assay (ELISA) is used widely in general clinical laboratories to measure serum antibody responses. Binding of serum antibodies to an antigen attached to a microplate generates a colorimetric reaction that is detected by a microplate reader; most importantly, ELISAs do not require specialized techniques. However, the usefulness of these assays for detecting anti-MERS-CoV antibodies is limited by the fact that antibodies that bind to conserved proteins expressed by coronaviruses are often cross-reactive; therefore, these assays often yield false-positive reactions [7, 11, 12].

The competitive ELISA (cELISA) was developed to detect serum antibody responses against many viruses [13–16]. It is based on a labeled monoclonal antibody (MAb) that is specific for a target antigen; this antibody competes with serum antibodies for binding to the antigen, thereby enabling detection and measurement of pathogen-specific antibodies (Fig. 1). Furthermore, since cELISA does not require a species-specific secondary antibody, it has an advantage over conventional ELISA in that it can detect serum antibodies in any animal species, making it a useful tool for seroepidemiological surveillance of MERS. Neutralizing responses to MERS-CoV in test serum can be measured using cELISA when the labeled MAb recognizes neutralizing epitopes in the MERS-CoV S protein [17]. Comparison with the results generated by a neutralization assay using live MERS-CoV shows that the results of cELISA correlate well with neutralization antibody titers [17]. The following protocol describes detection of serum antibodies using a cELISA based on a MAb specific for MERS-CoV.

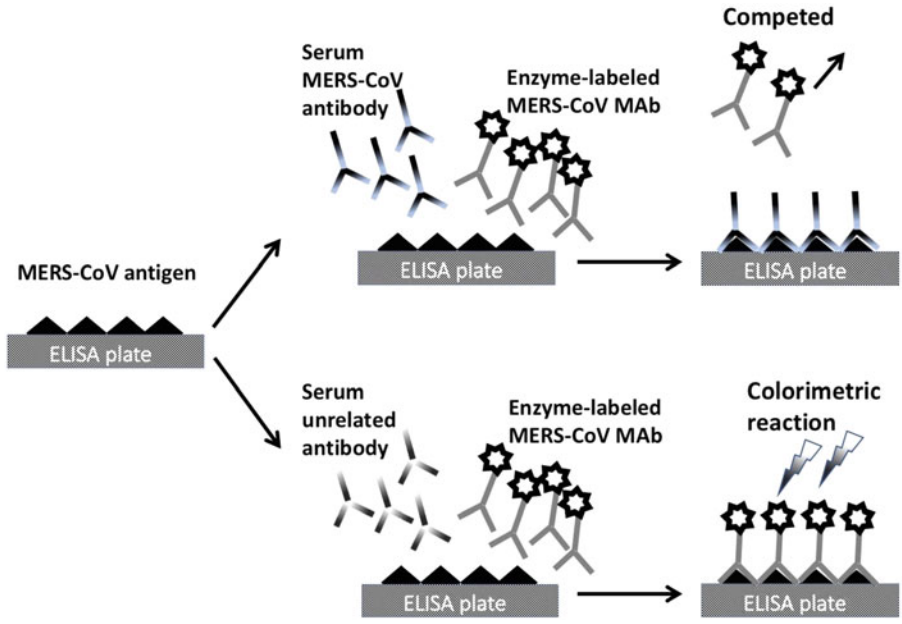


Fig. 1 Schematic representation of the cELISA. A labeled MAb competes with test serum antibodies for binding to the target antigen. The method does not require a species-specific secondary antibody; therefore, it detects antibodies in a species-independent manner

2 Materials

1. MAb 45C2 (750 μg/ml) recognizing the receptor binding domain (RBD) of the MERS-CoV S protein, biotinylated using Biotin Labeling Kit-NH2 (*see* **Note 1**).
2. Positive control antibody (serum from a rabbit immunized with the MERS-CoV S protein expressed in a recombinant baculovirus expression system, inactivated at 56 °C for 30 min) [10].
3. Test serum (inactivated at 56 °C for 30 min).
4. MERS-CoV antigen cell slurry (*see* **Note 2**) or purified recombinant RBD antigen (rRBD, *see* **Note 3**).
5. Wash buffer: Phosphate-buffered saline (PBS, pH 7.4) containing 0.05% tween 20 (PBS-T).
6. Blocking buffer: PBS-T containing 2% bovine serum albumin.
7. High Sensitivity Streptavidin-HRP.
8. Substrate: 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) tablets dissolved in ABTS™ buffer. One ABTS tablet (50 mg) is dissolved in 50 ml ABTS™ buffer working solution.
9. 96-well ELISA plate.

10. 96-well U-bottom microplate.
11. 12-channel pipette.
12. Dispenser trays.
13. ELISA plate washer.
14. ELISA plate reader capable of reading at wavelengths of 405 and 495 nm.

3 Methods

3.1 cELISA

1. Coat the ELISA plate with 100 µl MERS-CoV antigen cell slurry (diluted 1:800 in PBS) or 100 µ1 of purified recombinant RBD antigen (47 ng/ml in PBS) (Fig. 2). Seal the plate and incubate overnight at 4 °C.
2. Wash the ELISA plate twice with 300 µl PBS-T.
3. Add 200 µl blocking buffer to each well and seal the plate. Incubate at 37 °C for 2 h.
4. Prepare the series of twofold dilutions in the U-bottom microplate (Fig. 3) (*see* **Note 4**). Add 82.5 µl blocking buffer to line A and 55 µl blocking buffer to lines B–H.
5. Mix 27.5 µl test serum with 82.5 µl blocking buffer to make a fourfold dilution (line A).
6. Take 55 µl of the fourfold diluted serum sample (line A) and mix with 55 µl blocking buffer to make an eightfold dilution (line B).
7. Take 55 µl of the eightfold diluted serum sample (line B) and mix with 55 µl blocking buffer to make a 16-fold dilution (line C).

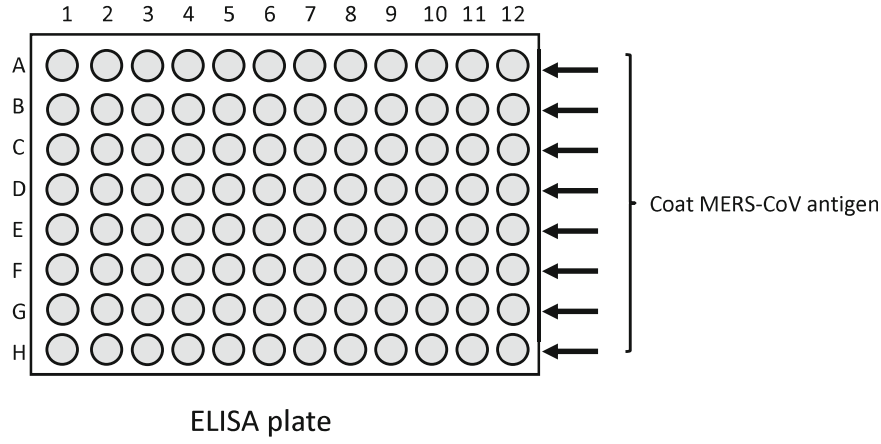


Fig. 2 Coating of the 96-well ELISA plate with MERS-CoV antigen

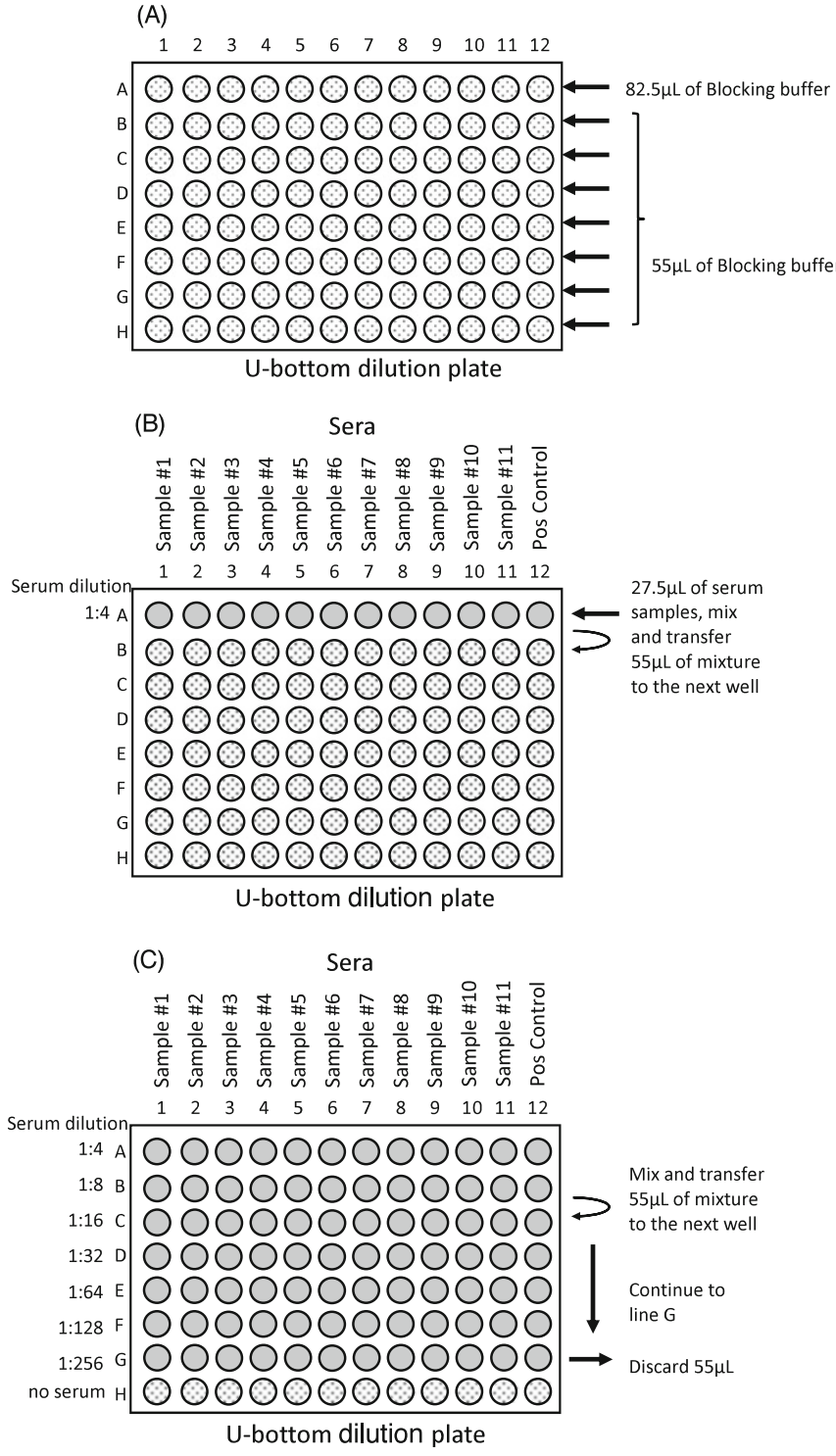


Fig. 3 Dilution of test serum in the U-bottom microplate. (**a**) Blocking buffer is added to the U-bottom microplate. (**b**) A fourfold dilution of each serum sample is added to line A. (**c**) Serum is diluted serially from line A to G. The nonserum control is placed in line H

8. Take 55 μl of the 16-fold diluted serum sample (line C) and mix with 55 μl blocking buffer to make a 32-fold dilution (line D).
9. Take 55 μl of the 32-fold diluted serum sample (line D) and mix with 55 μl blocking buffer to make a 64-fold dilution (line E).
10. Take 55 μl of the 64-fold diluted serum sample (line E) and mix with 55 μl blocking buffer to make a 128-fold dilution (line F).
11. Take 55 μl of the 128-fold diluted serum sample (line F) and mix with 55 μl blocking buffer to make a 256-fold dilution (line G). Discard 55 μl of the mixture.
12. Place the nonserum control in line H.
13. In the dispenser tray, prepare a 2340-fold dilution of biotinylated MAb 45C2 (e.g., mix 6 μl of biotinylated 45C2 with 14 ml of blocking buffer).
14. Using the 12-channel pipette, take 55 μl of diluted biotinylated 45C2 (prepared in 13 above) and mix with 55 μl of serially diluted serum sample (final serum dilutions range from 8 to 512) and nonserum control in a U-bottom microplate (prepared in 12 above). Pipette tips should be replaced after mixing each sample (Fig. 4).
15. After blocking, wash the ELISA plate twice with 300 μl PBS-T.

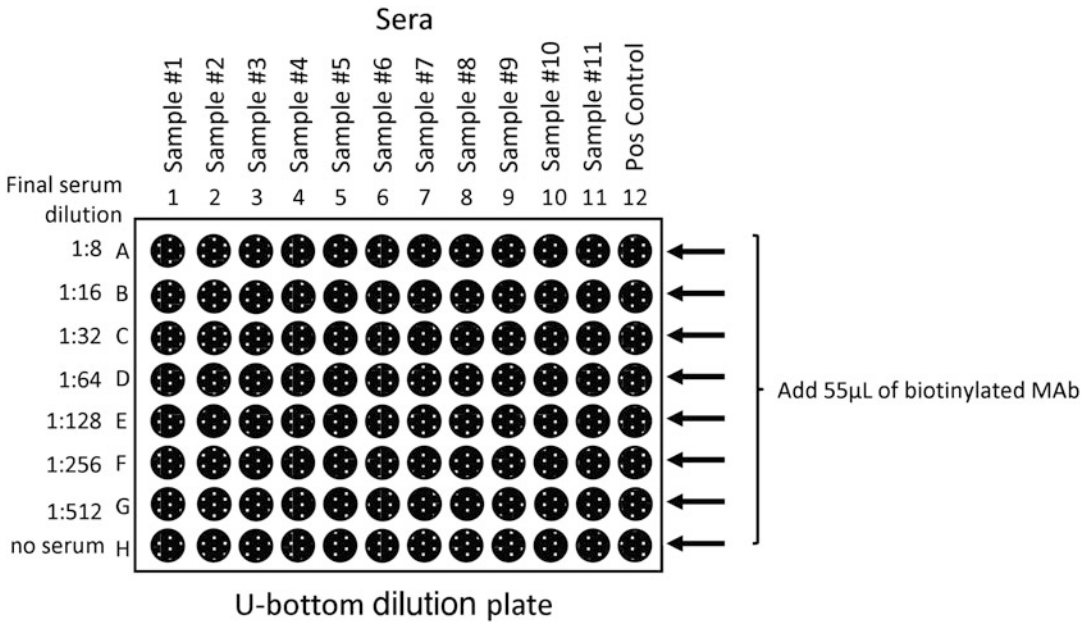


Fig. 4 Biotinylated monoclonal antibody 45C2 is added to the U-bottom microplate plate containing diluted serum samples

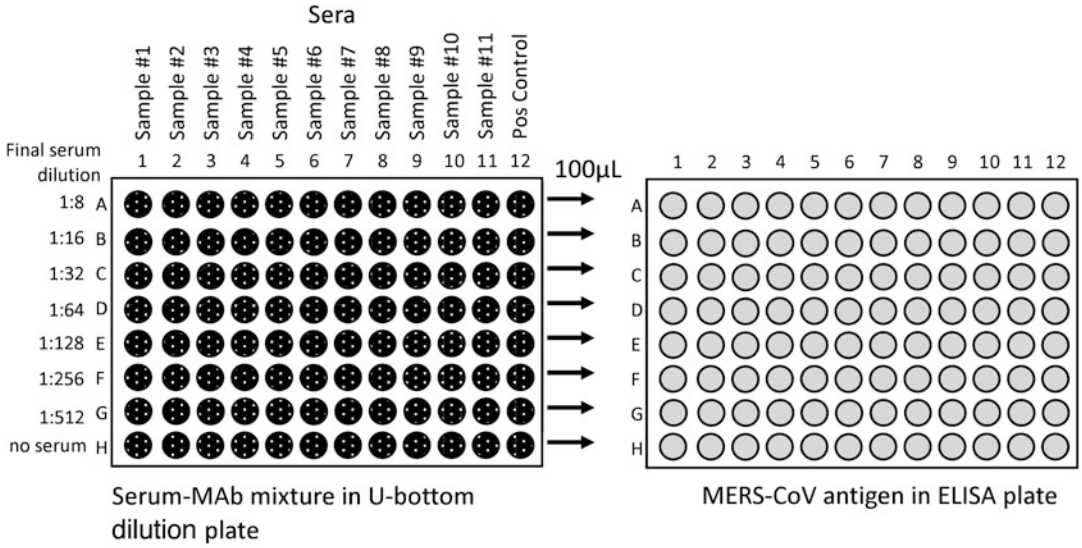


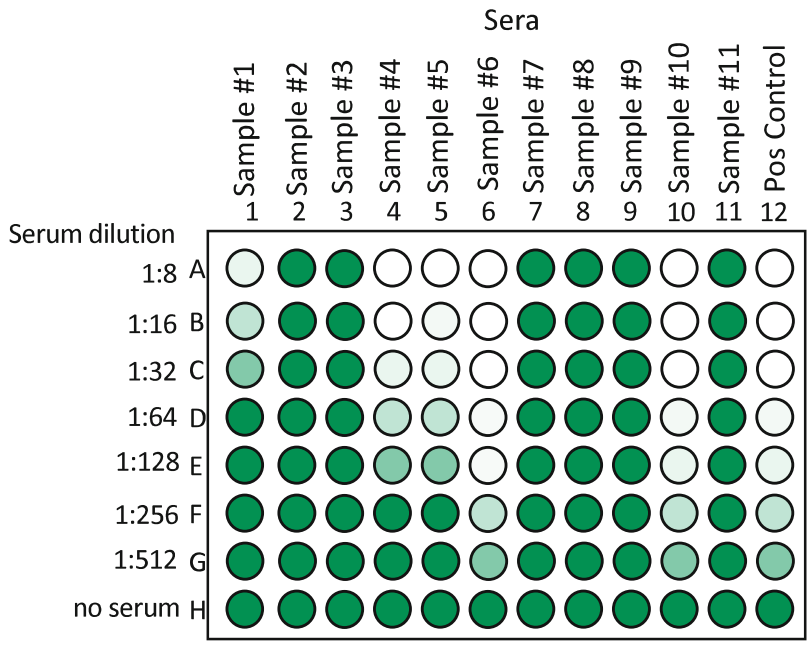
Fig. 5 The serum–MAb mixture is transferred to the MERS-CoV antigen-coated microplate

16. Transfer 100 µl of the serum–antibody mixture from the U-bottom microplate (prepared in 14 above) to the ELISA plate (prepared in 15 above) (Fig. 5). The mixture can be transferred from line A to line G without changing tips, but for line H the mixture should be transferred using new tips. Seal the plate and incubate at 37 °C for 1 h.
17. Dilute the High Sensitivity Streptavidin-HRP with blocking buffer (final dilution, 1:8000).
18. Wash the ELISA plate three times with 300 µl PBS-T.
19. Add 100 µl of the diluted High Sensitivity Streptavidin-HRP to each well. Seal the plate and incubate at 37 °C for 1 h.
20. Wash the ELISA plate three times with 300 µl PBS-T.
21. Add 100 µl ABTS substrate. Incubate the plate at room temperature for 15–30 min. The O.D. values in the control non-serum wells should not be higher than the limit of the microplate reader (e.g., O.D. 405 nm >3.5) (Fig. 6).
22. Measure the O.D. at 405 nm at the reference wavelength of 495 nm.

3.2 Evaluate cELISA Results

1. Calculate the inhibition rate (%) of each serum sample as follows:

   % inhibition = (1 − O.D. value with serum/O.D. value without serum) × 100 (Fig. 6).
2. The cutoff values of % inhibition for each serum dilution can be determined using at least ten negative serum samples.



Example: for the 1:8 dilution of sample A

$$\% \text{ inhibition} = \left[1 - \frac{\text{O.D. value in 1A}}{\text{O.D. value in 1H}} \right] \times 100$$

Fig. 6 Schematic representation of the colorimetric reaction after addition of ABTS solution to the ELISA plate. After measurement of O.D. at 405 nm (reference wavelength, 495 nm), the percent inhibition at each serum dilution can be determined

3. The antibody titer is determined by performing cELISA with twofold serial dilutions (from 8- to 512-fold) of serum samples and is expressed as the reciprocal of the highest dilution at which % inhibition is above the cut-off value for each serum dilution.
4. Comparison with the results of a neutralization assay using live MERS-CoV allows for confirmation that the results of the cELISA correlate well with neutralizing antibody titers [17].

4 Notes

1. To generate MAb 45C2 (specific for the MERS-CoV antigen), BALB/c mice are immunized with purified, UV-inactivated MERS-CoV particles [17]. Hybridoma cells are produced by fusing mouse myeloma cells with splenic cells isolated from the

immunized mice. ELISA was used to screen culture supernatants of hybridoma cells for antibodies specific for the MERS-CoV antigen. Neutralizing activity of the MAb is confirmed in a plaque-reduction assay using live MERS-CoV. Epitope mapping experiments have indicated that MAb 45C2 recognized the RBD of MERS-CoV. The detailed characterization of MAb 45C2 has been published by Fukushi et al. [17]. Ask the author to share the MAb; this can be done formally using a material transfer agreement (MTA). A MAb specific for the MERS-CoV RBD is also available commercially (Absolute antibody, Wilton, UK); however, detailed characterization (specificity, epitopes, etc.) of this MAb might be required.

2. To prepare the MERS-CoV antigen cell slurry, Vero cells are grown in a T75 flask and inoculated with MERS-CoV at a multiplicity of infection (m.o.i.) of 1.0. After 26 h, the cells are lysed in 1 ml of PBS containing 1% NP40 to extract viral antigens from infected cells. After centrifugation at 8000 × *g* for 10 min, the supernatant is collected and used as the source of MERS-CoV antigen in a cELISA. The MERS-CoV was inactivated by UV irradiation (312 nm, 2.5 mW/cm^2) for 10 min in a trans-illuminator before use. Viral inactivation is confirmed to be complete by inoculating an aliquot of antigen onto Vero cells, followed by cultivation of cells for at least 3 weeks. A preliminary experiment determines the optimum dilution of the antigen used for the cELISA as 1:800 (in PBS).
3. Recombinant RBD (rRBD) can be used as an alternative antigen for the cELISA. To prepare rRBD from MERS-CoV, the mammalian expression plasmid pCAGGS-RBD, which encodes histidine-tagged MERS-CoV RBD (amino acid 358-588), is transfected to 293T cells. At 2 days post-transfection, the rRBD is purified from the supernatant using a His-Bind Purification Kit. The amount of purified rRBD protein is determined using a BCA protein assay kit. Ask the author to share the rRBD; this can be done formally using an MTA. A commercial MERS-CoV S antigen (e.g., MERS-CoV S1 subunit protein; CD Creative Diagnostics, Shirley, NY, or Sino Biological, Beijing, China) might be available for use in the cELISA.
4. To screen serum antibodies from a large number of samples, it is better to test first at a single serum dilution (fourfold dilution) and then serially dilute only samples that are antibody-positive to measure antibody titers.

Acknowledgments

This work was supported, in part, by a grant from the Japan Agency for Medical Research and Development (AMED) (# JP19fk0108058).

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Chapter 5

Whole-Genome Sequencing Protocols for IBV and Other Coronaviruses Using High-Throughput Sequencing

Graham L. Freimanis and Michael S. Oade

Abstract

This chapter reports the high-throughput sequencing protocol for sequencing Coronaviruses and other positive strand viruses to produce a dataset of significant depth of coverage. The protocol describes sequencing of infectious bronchitis virus propagated in embryonated eggs and harvested in the allantoic fluid. The protocol is composed of three main steps—enrichment of the allantoic fluid using ultracentrifugation, extraction of total RNA from allantoic fluid, and library preparation from total RNA to DNA sequencing libraries. The workflow will be suitable for all coronaviruses using high-throughput sequencing platforms.

Key words Quasispecies, High-throughput sequencing, RNA, Virus, Infectious bronchitis virus, Coronavirus

1 Introduction

High-throughput sequencing (HTS) technologies provide the opportunity to rapidly obtain full genome sequence data of pathogenic organisms. In the recent COVID-19 pandemic, HTS was been used to quickly identify the causative agent as a coronavirus, which has subsequently been named SARS-COV-2 [1]. The subsequent dissemination of sequence information online has allowed laboratories worldwide to study the virus and begin the development of vaccines eliminating the requirement of access to live virus samples. HTS and whole-genome sequencing (WGS) are therefore powerful, versatile tools for the development of novel treatments and vaccines, for studying virus evolution and genetic associations to disease or for tracking outbreaks with notable recent publications investigating viral outbreaks in health-care settings [2, 3].

The depth of data and high sequence quality obtained by such HTS also affords the possibility to dissect and study virus populations leading to the identification of low frequency mutations that

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_5,

would otherwise be undetected by conventional Sanger-based sequencing. This is particularly relevant when working with RNA viruses, like coronaviruses, which have multiple variant viruses within a viral swarm due to factors including rapid replication rates, an error prone RNA-dependent RNA polymerase and large population sizes [4]. Despite the relatively small size of virus genomes, their sequencing is complicated by the atypical low qualities of viral nucleic acid template extracted from samples. Sample preparation therefore is the key to deep sequencing, in that it is important to enrich for viral RNA whist maintaining the integrity of input material, resulting in a DNA-free high-quality preparation.

The protocol described here can produce high coverage depth datasets for Coronavirus genomes, such as Infectious Bronchitis Virus (IBV) grown in ovo from around 50 ng of total RNA. The growth of IBV in embryonated eggs has been described previously and is not discussed in this protocol [5]. Allantoic fluid must been free of membrane or other solid masses and ideally be free from blood. If the IBV causes hemorrhaging of the blood vessels, it is important to harvest the allantoic fluid before this happens. Due to the low sample matrix content in allantoic fluid and the polyA enrichment component of this protocol, some host mRNAs are also captured during sequencing, however this is dependent of the purity of the original sample.

This protocol is suitable for the capture of both viral gRNA and mRNA (combined) from allantoic fluid, is robust in terms of coverage depth, producing >99% genome recovery.

2 Materials

2.1 Purification of IBV

1. 50 ml centrifuge tubes.
2. 30% sucrose (w/v) in PBS adjusted to pH 7.2 with HCl, filtered through 0.22 μm syringe.
3. Refrigerated benchtop centrifuge.
4. Ultracentrifuge and rotor (e.g., Beckman Coulter SW55Ti rotor and Sorvall WX Ultra 80 ultracentrifuge).
5. Ultracentrifuge tubes compatible with rotor (e.g., Beckman polypropylene (13 × 51 mm) ultracentrifuge tubes).
6. Cannula.

2.2 RNA Extraction

1. RNase cleaning agent (e.g., RNaseZAP).
2. RNA extraction kit (e.g., RNeasy kit) or RNA isolation reagent (e.g., TRIzol).
3. Nuclease-free water.
4. DNA Lobind tubes.
5. 80% ethanol.

2.3 QC of Extracted Total RNA

1. High sensitivity DNA quantitation kit (e.g., Qubit high sensitivity RNA kit or equivalent).
2. Agilent Bioanalyzer 2100 high sensitivity RNA kit (or equivalent).

2.4 IBV Sequencing Library Preparation

1. NEBNEXT Ultra II stranded mRNA RNA-Seq (or equivalent).
2. NEBNEXT Ultra II indices for multiplexing (or equivalent).
3. SPRI paramagnetic beads (e.g., Beckman Coulter AMPure XP beads or equivalent).
4. 80% ethanol.
5. 10 mM Tris–HCl, pH 8.

2.5 Normalization of Sequencing Libraries

1. Agilent Bioanalyzer DNA 1000 kit.
2. DNA quantitation kit (e.g., Qubit DNA BR kit).

2.6 Quantification of Sequencing Library Pool

1. NEBNEXT Illumina library quantitation kit (or equivalent).
2. Fast PCR thermocycler (e.g., ABI 7500 fast thermocycler).

2.7 Denaturation of Sequencing Pool and Loading of Libraries

1. 0.2 N sodium hydroxide.
2. Control library (e.g., PhiX).
3. MiSeq reagent cartridge v3 600 cycles.
4. Illumina MiSeq benchtop sequencer.

3 Methods

Perform all activities prior to library preparation in a clean RNase free environment with dedicated RNA pipettes, RNase-free wipes, and other consumables. Always clean workspace with RNase cleaning agents prior to starting protocol. A schematic of the whole protocol is shown in Fig. 1.

3.1 Purification of IBV (2 h)

1. Place 3–10 ml of IBV infected allantoic fluid into a 15 ml tube and centrifuge for 10 min, 1150 × *g* at 4 °C in a benchtop centrifuge in order to remove large solid matter from the IBV infected allantoic fluid.
2. Take the supernatant and add it to a 5 ml ultracentrifuge tube. A cushion of 0.5 ml 30% Sucrose is placed at the base of the tube under the allantoic fluid using a canula being careful not to disturb the allantoic fluid. Tubes must be balanced carefully.
3. Ultracentrifuge for 60 min, 236,880 × *g* at 4 °C in an ultracentrifuge.

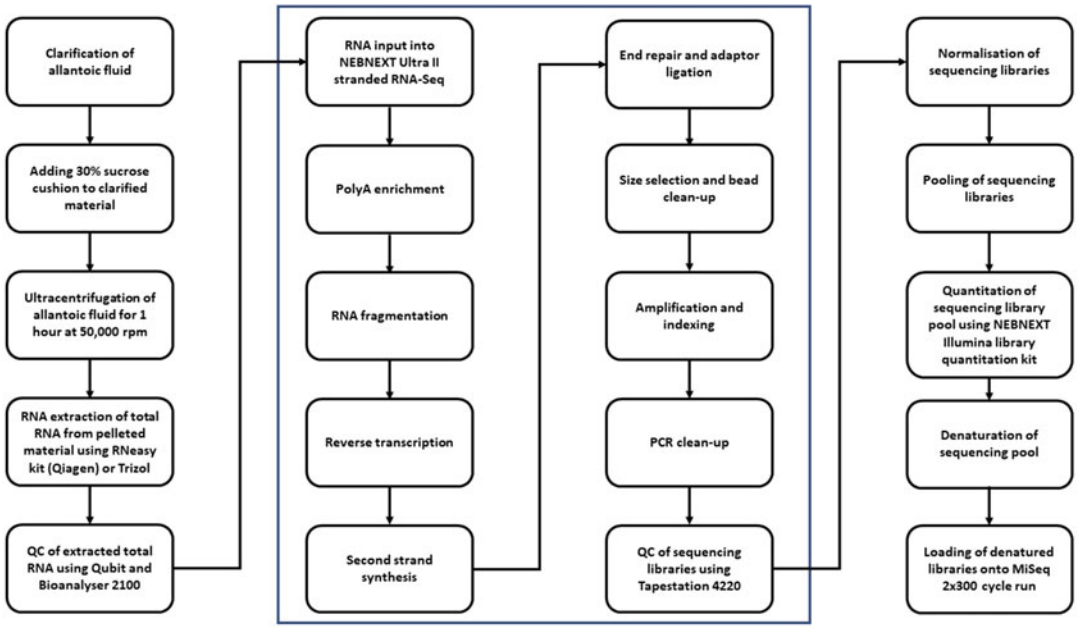

Fig. 1 Schematic for the overall IBV library preparation pipeline

4. Remove supernatant in layers, taking care not to disturb the pellet (*see* **Note 1**).
5. Resuspend the pellet in 350 μl of RLT lysis buffer or TRIzol.
6. Either store at −20 °C or progress directly onto total RNA extraction.

3.2 RNA Extraction (1 h)

In our experience although TRIzol produced higher-quality RNA, RNeasy extractions by comparison yielded more RNA. A greater RNA yield and the easier scalability of RNeasy versus TRIzol resulted in RNeasy being our preferred method of RNA extraction for this protocol. Other RNA extraction kits and methods may also be used.

1. Extract total RNA from the 350 μl of nucleic acid dissolved in lysis buffer (RLT) using the Qiagen RNeasy RNA extract kit (or equivalent) according to the manufacturer's protocol, following the optional additional spin to remove excess RPE. Elute in a total of 30 μl of elution buffer. Sometimes running elution buffer, prewarmed at 37 °C, through the column twice can improve the yield of the kit.

3.3 Quality Control (QC) of Extracted Total RNA (1 h)

1. Quantify total RNA content using Qubit and the Qubit High sensitivity RNA kit (or equivalent) following the manufacturer's instructions with each sample measured in duplicate.

2. Identify size distribution of RNA using the Bioanalyzer 2100 and Bioanalyzer RNA nano chip (or equivalent) following the manufacturer's protocol (*see* **Note 2**).

3.4 Library Preparation (1–2 Days Dependent Upon Kit)

1. Calculate a minimum input of 50–200 ng of total RNA and dilute into a total volume of 50 μl to satisfy input requirements for the NEBNEXT Ultra II stranded mRNA RNA-Seq kit (or equivalent) (*see* **Note 3**).
2. The remainder of the protocol is carried out following the manufacturer's instructions, fragmenting for 200–300 bp fragments in terms of fragmentation and size selection protocols. *However, if the amount of input material is low (<50 ng) then 200 bp is recommended.*
3. Elute the final libraries in 20 μl of 10 mM Tris–HCl, pH 8. Store at −20 °C for a *maximum* of 4 weeks (*see* **Note 4**).

3.5 Library QC and Normalization of Sequencing Libraries (3 h)

1. Run generated libraries on a Bioanalyzer 2100 using Bioanalyzer DNA 1000 kit (or equivalent) following the manufacturer's instructions (*see* **Note 5**). A typical library profile is as below (Fig. 2). Record the average library size for each library using smear analysis to increase accuracy of average size.
2. Manually quantitate each sample using the Qubit high sensitivity kit measuring each sample in duplicate to get an accurate reading.
3. Convert the average Qubit reading from ng/μl to nM using the average library size from the Bioanalyzer results using the formula below;

$$\frac{\text{concentration in ng/μl}}{660\ \text{g/mol} \times \text{average library size(in bp)}} \times 10^6 = \text{concentration in nM}$$

4. For longer term storage, dilute in 10 mM Tris–HCl, pH 8.5. Do not pipette volumes <2 μl (*see* **Note 6**). Otherwise, proceed to **step 5**.
5. Dilute libraries to a concentration of 5 nM using the formula below. A minimum of 4 nM is required before addition to the pool.

$$C_1 V_1 = C_2 V_2$$

where

C_1 = initiation concentration of solution.

V_2 = volume of the initiation solution that will be diluted.

C_2 = concentration of final solution.

V_2 = desired volume of final solution.

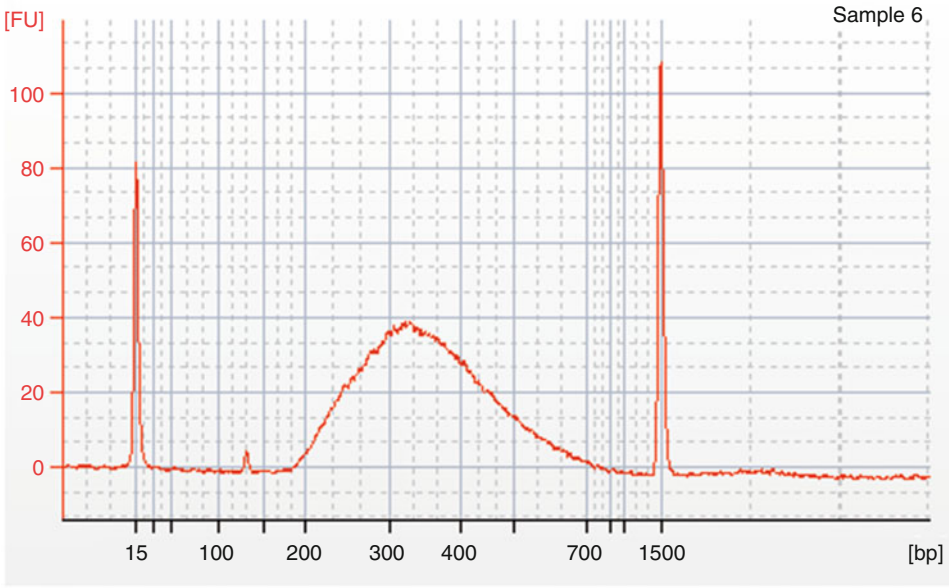

Fig. 2 Typical output example of final libraries using Agilent Bioanalyzer

6. Take 5 µl from all samples and pool in a single 1.5 ml LoBind tube and store at −20 °C. If you do not intend to progress to the next step immediately then ensure sequencing is performed within 2–3 weeks for optimal cluster densities where possible.

3.6 Quantitation of Sequencing Pool Using qPCR, Sample Denaturation and Loading onto Illumina MiSeq (1 Day)

1. Quantitate the pooling using the NEBNEXT Quantitation for Illumina Libraries kit (or equivalent) using the manufacturer's protocol.
2. On confirmation that the sequencing pool is at a concentration of approximately 4–5 nM, proceed to denaturing libraries with NaOH, for loading on the MiSeq.
3. Denature libraries with 0.2 N NaOH and dilute according to the manufacturer's guidelines. Samples are then loaded at a concentration of 15 pM onto a 2 × 300 cycle v3 MiSeq reagent cartridge, using a 1% PhiX spike (*see* **Note 7**).

4 Notes

1. This protocol can be very sensitive to input material. Depending on the yield of virus grown in eggs, the viral pellet may not be prominent after ultracentrifugation. Insufficient yield of virus at this stage will greatly hamper the quality of obtained reads. It may therefore be beneficial to repeat this stage as necessary until a greater yield of virus is achieved.

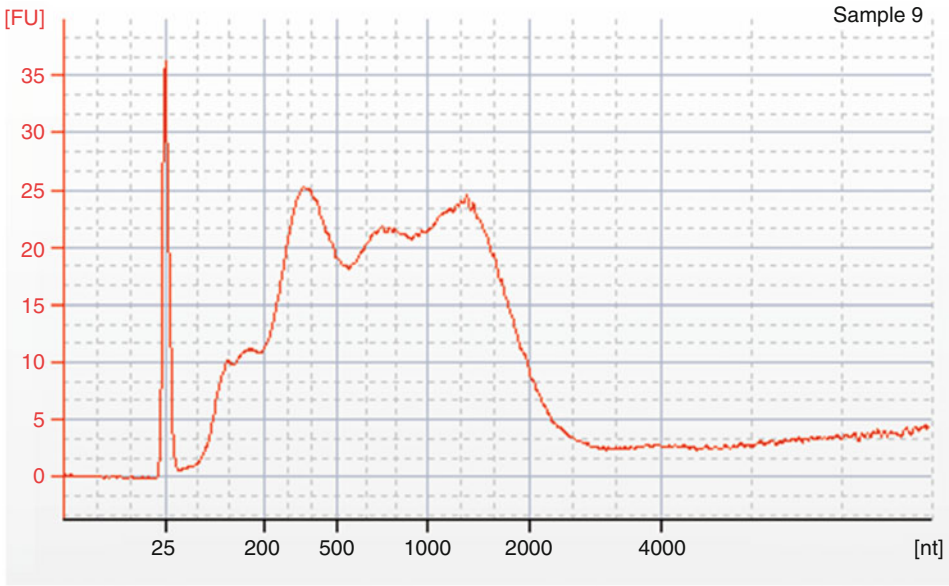

Fig. 3 Typical output using extracted RNA from Agilent Bioanalyzer

2. The purpose of running the Bioanalyzer is to check that RNA is present within the sample. The RNA Integrity Number (RIN) will not be informative to the final library yield in this case. An example of a typical trace is shown in Fig. 3.
3. Using the poly-A enrichment module will directly enrich for both genomic vRNA and viral mRNAs due to the presence of a poly-A tail at the 3′ end of the IBV genome.
4. Libraries should not be left for longer than 4 weeks at −20 °C or this can result in a reduction in clustering and sequencing quality.
5. Performing library QC is important for indicating inefficient library preparation/low library yield.
6. Where possible, do not dilute values less than 2 μl. Pipetting smaller volumes is highly inaccurate and will unduly bias the proportion of this library on the flow cell.
7. Loading concentrations may need to be optimized for individual machines.

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Part II

Propagation and Titration of Coronaviruses

Chapter 6

Isolation and Tissue Culture Adaptation of Porcine Deltacoronavirus: A Case Study

Hui Hu, Kwonil Jung, Scott P. Kenney, and Linda J. Saif

Abstract

Porcine deltacoronavirus (PDCoV) has emerged as a novel, contagious swine enteric coronavirus that causes watery diarrhea and/or vomiting and intestinal villous atrophy in nursing piglets. PDCoV-related diarrhea first occurred in the USA in 2014 and was subsequently reported in South Korea, China, Thailand, Vietnam, and Lao People's Democratic Republic, leading to massive economic losses and posing a threat to the swine industry worldwide. Currently, no treatments or vaccines for PDCoV are available. The critical step in the development of potential vaccines against PDCoV infection is the isolation and propagation of PDCoV in cell culture. This chapter provides a detailed protocol for isolation and propagation of PDCoV in swine testicular (ST) and LLC porcine kidney (LLC-PK) cell cultures supplemented with pancreatin and trypsin, respectively. Filtered clinical samples (swine intestinal contents or feces) applied to ST or LLC-PK cells produce cytopathic effects characterized by rounding, clumping, and detachment of cells. PDCoV replication in cells can be quantifiably monitored by qRT-PCR, immunofluorescence assays, and immune-electron microscopy. Infectious viral titers can be evaluated by using plaque assays or 50% tissue culture infectious dose ($TCID_{50}$) assays. The ST or LLC-PK cells efficiently supported serial passage and propagation of PDCoV. After serial passage of PDCoV in either ST or LLC-PK cells, the virus can be purified further in ST cells by plaque assays.

Key words *Porcine deltacoronavirus* (PDCoV), Isolation, Propagation, $TCID_{50}$, Plaque assay

1 Introduction

Porcine deltacoronavirus (PDCoV), a member of the genus *Deltacoronavirus* in the family *Coronaviridae* of the order *Nidovirales*, is a novel, contagious swine enteropathogenic coronavirus that causes watery diarrhea and/or vomiting, dehydration, and intestinal villous atrophy in nursing pigs [1]. PDCoV was first detected in pig feces during a molecular surveillance of coronaviruses in mammals and birds in Hong Kong in 2012 [2]. However, the first outbreak of PDCoV-related diarrhea in pigs was identified in the USA in 2014 [3]. Since then, PDCoV-related diarrhea has been reported in many countries, including China, Canada, South Korea, Lao People's Democratic Republic, Thailand, and Vietnam [4–8].

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_6,

PDCoV is enteropathogenic in young pigs [1]. Wild-type or cell culture–adapted strains of PDCoV caused severe gastrointestinal disease in gnotobiotic (Gn) and conventional, 5–19-day-old pigs [9–11]. Apart from pigs, PDCoV was also reported to have a limited ability to infect Gn calves [12]. This was evident by the detection of high titers of fecal PDCoV RNA and serum PDCoV-specific IgG antibody in inoculated calves (but with a lack of clinical signs and histological lesions). Similarly, PDCoV infected and replicated within various cell lines of human and chicken origin in vitro [13]. In in vivo settings, PDCoV could also infect and serially propagate in chicken embryos, and orally inoculated chickens showed mild diarrhea and low viral RNA titers in the feces, providing evidence of the possibility of interspecies transmission of deltacoronavirus between birds and pigs [14].

Cell culture–adapted and plaque-purified strains of PDCoV are useful for studies of PDCoV pathogenesis, and development of virological and serological assays and vaccines. Many cell culture–grown strains of PDCoV have been reported in the USA, China and Korea. The strains USA/IL/2014 [10], Michigan/8977/2014 [11], and OH-FD22 [15] were isolated in the USA, and the strain OH-FD22 has been serially passaged more than 100 times in LLC porcine kidney (LLC-PK) cells. The Chinese strains HNZK-02 [14], NH [16], CHN-HN-2014 [17, 18], CHN-GD-2016 [19], and CHN-HG-2017 [20] and Korean strain KNU16-07 [21] were all isolated in cell culture primarily utilizing methods first reported by our laboratory [15].

In our earlier studies, various cell lines of swine and monkey origin, including Vero cells, commonly used for isolation and propagation of porcine epidemic diarrhea virus, were tested for primary isolation of PDCoV using cell culture medium supplemented with different additives (small intestinal contents, trypsin, and pancreatin). However, only the LLC-PK or swine testicular (ST) cells of swine origin efficiently supported the isolation and serial propagation of PDCoV in cell cultures supplemented with exogenous trypsin or pancreatin, respectively [15]. Recently, porcine, human, and avian aminopeptidase N (APN) was identified as a major cell entry receptor for PDCoV in vitro [17, 22, 13]. Therefore, possible differences in APN expression levels among the cell lines used could influence their susceptibility to infection with PDCoV. In our studies, PDCoV replicated in LLC-PK cells without trypsin treatment, but it did not induce obvious cytopathic effects (CPE), and the virus titer was lower when compared with the trypsin supplemented cell cultures [15]. We also found that PDCoV could be serially propagated in ST cells supplemented with pancreatin or small intestinal contents from Gn piglets, but not with trypsin [15]. The addition of trypsin and pancreatin in PDCoV-inoculated LLC-PK and ST cells, respectively, might be essential for both growth of PDCoV and induction of CPE [15, 23]. Similarly, a

porcine small intestinal epithelial cell line, IPEC-J2, was also susceptible to PDCoV infection, accompanied by CPE, when the cell culture medium was supplemented with 10 μg/ml of trypsin [24]. The cell culture systems currently available for PDCoV are useful for an understanding of the mechanisms related to PDCoV infection and generation of live or inactivated vaccine strains of PDCoV.

This chapter is focused on the description of basic protocols for the isolation and propagation of PDCoV in LLC-PK or ST cells, titration of infectious virus by $TCID_{50}$ or plaque assays, and purification of PDCoV by plaque assay, based on our experience in isolating and adapting US PDCoV OH-FD22 or Chinese HNZK-02 strains in these cell lines. Details regarding quantification of viral RNA by TaqMan real-time quantitative RT-PCR (qRT-PCR), immunofluorescence assay for detection of PDCoV antigen in cells, and immune-electron microscopy for identification of viral particles in the culture medium have been published previously [15].

2 Materials

2.1 Sample Collection and Preparation

1. Clinical swine samples, such as feces or intestinal contents, are collected from diarrheic pigs positive for PDCoV RNA by RT-PCR or qRT-PCR and stored at −80 °C (*see* **Note 1**).

2.2 Cell Culture, Virus Propagation, and $TCID_{50}$

1. LLC-PK cell growth medium: Minimal essential media (MEM) supplemented with 5% heat inactivated fetal bovine serum (FBS), 1% MEM nonessential amino acids (NEAA), 1% antibiotic–antimycotic, and 1% HEPES.
2. LLC-PK cell maintenance medium (LLC-PK cell MM): MEM is supplemented with 1% NEAA, 1% antibiotic–antimycotic, 1% HEPES, and 5 μg/ml of trypsin (*see* **Note 2**).
3. ST cell growth medium: Advanced MEM supplemented with 5% heat-inactivated FBS, 1% antibiotic–antimycotic, 1% HEPES, and 1% L-glutamine.
4. ST cell maintenance medium (ST cell MM): Advanced MEM supplemented with 1% antibiotic–antimycotic, 1% HEPES, and 1% (v/v) pancreatin (*see* **Note 3**).
5. Cell dissociation medium for LLC-PK cells: 0.05% trypsin with 0.02% EDTA (*see* **Note 4**).
6. Cell dissociation medium for ST cells: 0.25% trypsin with 0.02% EDTA (*see* **Note 4**).
7. Dulbecco's modified Eagle's medium (DMEM).
8. Phosphate buffered saline (PBS).

9. Tissue culture flasks.
10. 15 ml conical centrifuge tubes.
11. Benchtop centrifuge.
12. 6-well cell culture plates.
13. 96-well cell culture plates.
14. Cell culture incubator set at 37 °C with 5% CO_2.

2.3 Plaque Assay for Purification of PDCoV

1. 2% (v/v) of pancreatin (*see* **Note 3**).
2. Dulbecco's PBS (DPBS) without Mg^{2+} and Ca^{2+}.
3. 2× MEM: 2× MEM supplemented with 1% antibiotic–antimycotic, 1% HEPES, 1% NEAA, and 2% pancreatin.
4. 2% (w/v) agarose (*see* **Note 5**).
5. Microwave.
6. 0.33% neutral red.
7. 1.5 ml microcentrifuge tube.

3 Methods

3.1 Isolation and Propagation of PDCoV in LLC-PK or ST Cells

3.1.1 Passage or Preparation of LLC-PK Cells

1. Wash confluent LLC-PK cell monolayers grown in T75 cell culture flasks once with 3–5 ml of 0.05% trypsin–EDTA, and then immediately aspirate the wash fluid.
2. Add 3 ml of 0.05% trypsin–EDTA and incubate cells for 5–20 min at 37 °C, dependent on the extent of cell detachment.
3. Terminate digestion by adding 3 ml of FBS-containing growth medium and transfer cells to a sterile 15 ml conical centrifuge tube.
4. After centrifugation of the cell suspension medium at 200 × *g* for 5 min, remove the medium and resuspend the cell pellet in 6 ml of growth medium.
5. 1 ml of precipitated cells (approximately 1–2 × 10^6 cells/flask) and 14 ml of growth medium are added to a new T75 cell culture flask that is then incubated at 37 °C in 5% CO_2.

3.1.2 Passage or Preparation of ST Cells

1. Wash confluent ST cell monolayers grown in T75 cell culture flasks once with 3–5 ml of 0.25% trypsin–EDTA, and then immediately aspirate the wash fluid.
2. Add 3 ml of 0.25% trypsin–EDTA and incubate cells for 10–20 min at 37 °C, dependent on the extent of cell detachment.
3. Aspirate 2 ml of trypsin–EDTA when cell detachment begins.

4. Digest cells in the remainder (1 ml) of trypsin–EDTA until they are completely detached.
5. Terminate digestion by adding 5 ml of FBS-containing growth medium and transfer to a sterile 15 ml conical centrifuge tube.
6. After centrifugation of the cell suspension medium at 200 × *g* for 5 min, remove the medium and resuspend the cell pellet in 6 ml of growth medium.
7. 1 ml of precipitated cells (approximately 1–2 × 10^6 cells/flask) and 14 ml of growth medium are added to a new T75 cell culture flask that is then incubated at 37 °C in 5% CO_2.

3.1.3 Isolation of PDCoV in LLC-PK or ST Cells

1. Prepare 1–2-day-old, 80–90% confluent cell monolayers in 6-well cell culture plates for inoculation with filtered clinical samples.
2. Wash cells twice with LLC-PK or ST cell MM (without 5 μg/ml of trypsin or 1% pancreatin) or DPBS (*see* **Note 6**).
3. To prepare inoculum for viral isolation, tenfold serially dilute PDCoV RNA-positive intestinal contents or feces in PBS or DMEM. Vortex for 2 min, and then centrifuged at 1847 × *g* at 4 °C for 10 min.
4. Filter supernatants through 0.22 μm filters. These filtered supernatants are used as the inoculum for viral isolation in cell culture.
5. Add 300 μl of filtered samples to each well and incubate for 1 h at 37 °C in 5% CO_2 (*see* **Note 7**).
6. Remove the inoculum, and wash cells three times with MM or DPBS (*see* **Note 6**).
7. Add 2 ml of MM supplemented with 5 μg/ml of trypsin (LLC-PK cells) or 1% pancreatin (ST cells) to each well.
8. Incubate cells at 37 °C in 5% CO_2 until CPE, such as rounding, clumping, or detachment of cells (Fig. 1), is observed usually by 5 days postinoculation (*see* **Note 8**).
9. Freeze the cell plates at −80 °C once CPE is observed in 80% of the cell monolayer.
10. Thaw and freeze the cells twice at −80 °C and harvest the cell supernatants for qRT-PCR or titration of infectious virus.

3.1.4 Serial Passage of PDCoV in LLC-PK or ST Cells

1. Prepare 1–2 day-old, 80–90% confluent cell monolayers in T25 cell culture flasks for inoculation with the cell supernatants.
2. Remove the old growth medium and wash cells twice with LLC-PK or ST cell MM (without 5 μg/ml of trypsin or 1% pancreatin) (*see* **Note 9**).
3. If the titer of infectious virus in the inoculum is known, a desired multiplicity of infection (MOI) of the virus

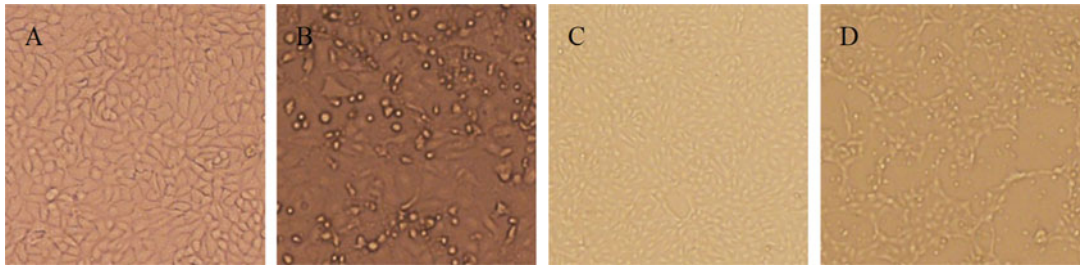

Fig. 1 Cytopathic effects (CPE) of PDCoV HNZK-02 in inoculated ST and LLC-PK cells. Cells were inoculated with mock (**a**, **c**) or PDCoV HNZK-02 (**b**, **d**). (**a**) Mock-inoculated ST cells with the addition of 1% pancreatin at 24 h postinoculation (hpi), showing no CPE. (**b**) PDCoV-inoculated ST cells with the addition of 1% pancreatin at 24 hpi, showing rounded and clumped cells. (**c**) Mock-inoculated LLC-PK cells with the addition of 5 μg/ml trypsin at 18 hpi, showing no CPE. (**d**) PDCoV-inoculated LLC-PK cells with the addition of 5 μg/ml trypsin at 18 hpi, showing rounded and detached cells. Original magnification, all ×200

(0.5–0.6 ml per T25 cell culture flask) should be inoculated. If not, a sufficient volume (0.5–0.6 ml) of inoculum is added to a T25 cell culture flask (*see* **Note 10**).

4. Incubate cells at 37 °C for 1 h.
5. Remove the inoculum, and wash cells three times with MM or DPBS.
6. Add 5 ml of MM supplemented with 5 μg/ml of trypsin (LLC-PK cells) or 1% pancreatin (ST cells) per T25 cell culture flask and incubate at 37 °C in 5% CO_2 until CPE, such as rounding, clumping, or detachment of cells, is observed.
7. Freeze the cell flasks at −80 °C once CPE is observed in more than 80% of the cell monolayer.
8. Thaw and freeze the cells once at −80 °C and harvest the cell supernatants for qRT-PCR or titration of infectious virus by $TCID_{50}$ or plaque assays.

3.2 Titration of Infectious PDCoV by $TCID_{50}$ Assay

1. Add 200 μl of LLC-PK cells (approximately 5×10^4 cells/well) to each well in 96-well cell culture plates and incubate cells at 37 °C in 5% CO_2 until reaching 80–90% confluence.
2. Wash 80–90% confluent cell monolayers once with LLC-PK cell MM either with or without 5 μg/ml of trypsin.
3. Serially dilute samples or virus (tenfold) with LLC-PK cell MM either with or without 5 μg/ml of trypsin.
4. Inoculate 100 μl of diluted samples or virus per well in eight replicates per dilution (*see* **Note 11**).
5. Incubate cells at 37 °C in 5% CO_2 for 1 h.
6. Add 100 μl of the MM supplemented with 5 μg/ml of trypsin (MMT) (*see* **Note 12**).

7. Monitor CPE for 5–7 days postinoculation. If CPE is observed, virus titers are calculated by using the Reed–Muench method [25] and expressed as $TCID_{50}$ per ml (*see* **Note 13**).

3.3 Titration and Purification of PDCoV in ST Cells by Plaque Assay (See Note 14)

1. Prepare 100% confluent ST cell monolayers in 6-well cell culture plates (*see* **Note 15**).
2. Discard and replace the old growth medium with ST cell MM without 1% pancreatin. Then, incubate cells for 1 h at 37 °C in 5% CO_2 (*see* **Note 16**).
3. Prepare viral inoculum by tenfold serial dilution in ST cell MM without 1% pancreatin.
4. Wash cells twice with ST cell MM or DPBS.
5. Add 300 μl of tenfold serially diluted viral inoculum in duplicate to each well and incubate for 1 h at 37 °C in 5% CO_2.
6. Prepare agarose. Warm 2× MEM in a water bath at 37 °C. Approximately 7 ml is needed per plate.
7. Heat the agarose gel in a microwave oven until fully melted, and then keep it in a water bath at 42 °C. Approximately 7 ml is needed per plate.
8. Mix equal volumes of 2× MEM (**step 6**) and agarose (**step 7**) (*see* **Note 17**).
9. Remove virus inoculum and wash cells twice with DPBS.
10. Add 2 ml of the agarose-MEM mixture to each well (*see* **Note 18**).
11. Cool the plates for 10 min to solidify the agarose.
12. Incubate the plates upside down at 37 °C in 5% CO_2 for 2–3 days postinoculation (*see* **Note 19**).
13. Prepare the working solution of neutral red (0.01%). Dilute 1 ml of the stock neutral red (0.33%) in 25 ml of DPBS.
14. Add 2 ml of 0.01% neutral red solution to each agarose-coated well.
15. Incubate the plates at room temperature for 2–4 h, and then remove the neutral red solution. Plaques are visualized as clean spots in the red background (Fig. 2).
16. Count plaques under oblique light. Plaques should be confirmed by light microscopy. The plaque titers are expressed as plaque forming units (PFU) per ml. The titer of PFU/ml is calculated by using the following formula: Y (viral titer, PFU/ml) = X (mean numbers of plaques from the duplicate wells)/300 × 1000 × viral dilution factor.
17. Pick a uniform and clear plaque by using a sterile pipette tip, and then place the agarose plug into a 1.5 ml microcentrifuge tube containing 0.5 ml of ST cell MM (*see* **Note 20**).

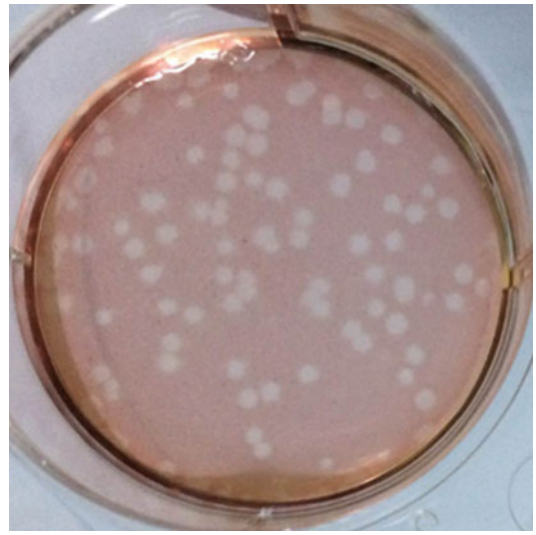

Fig. 2 PDCoV plaques in ST cells. ST cells in a 6-well cell culture plate were inoculated with PDCoV HNZK-02 (passage number 15). The plate was stained with 0.01% neutral red at 3 days postinoculation

18. Inoculate the selected plaque clones (0.5 ml) onto ST cell monolayers prepared in a 6-well cell culture plate.
19. Incubate the plate for 1 h at 37 °C in 5% CO_2.
20. Add 1.5 ml of ST cell MM supplemented with 1% pancreatin and incubate the plate at 37 °C in 5% CO_2 for 4–5 days until CPE is observed.
21. Harvest CPE-positive clones and store them at −80 °C (*see* **Note 21**).

4 Notes

1. Detailed procedures, including isolation of viral RNA from clinical samples and qRT-PCR for the detection of the membrane (M) gene of PDCoV were published previously [15, 26]. The qRT-PCR was conducted by using Qiagen One-step RT-PCR kit (Qiagen Inc., Valencia, CA, USA) and a real-time thermocycler (RealPlex; Eppendorf, Germany) [15]. The forward and reverse oligonucleotide primers and probe used to detect the M gene of PDCoV are as follows: PDCoV MF: 5′-ATCGACCACATGGCTCCAA-3′, PDCoV MR: 5′-CAGCTCTTGCCCATGTAGCTT-3′, and PDCoV M-Probe: FAM-CACACCAGTCGTTAAGCATGGCAAG CT-IABkFQ (5 μM).
2. Trypsin (2.5%) without phenol red and EDTA is used. The presence of EDTA could enhance cell detachment, hindering infection and replication of PDCoV and observation of CPE.

The stock of trypsin (2.5%) is diluted (1:5000) in MEM (e.g., 10 μl of 2.5% trypsin is added to 50 ml of MEM supplemented with 1% NEAA, 1% antibiotic–antimycotic, and 1% HEPES), and the working concentration of trypsin in LLC-PK MM is 5 μg/ml.

3. A 10× pancreatin stock is made by dissolving 2.50 g of pancreatin and 0.85 g of NaCl in 100 ml of MilliQ water followed by sterilizing through a 0.22 μm filter. The 10× pancreatin solution is further diluted (1:10) in sterile PBS or MEM (1× pancreatin). Aliquot into 1 ml vials, and store at −20 °C until use. Store at 4 °C after thawing. Finally, 1 ml of the 1× pancreatin solution is added to 99 ml of the maintenance medium.
4. Aliquot into sterile 15 ml conical centrifuge tubes, and store at −20 °C until use. Prior to use, thaw and warm at room temperature.
5. 2 g agarose is added to 100 ml of distilled water, autoclaved, and stored at room temperature. Prepare the agarose overlay by melting the agarose gel in a microwave oven. Keep it in liquid form by incubation at 42 °C.
6. Trypsin-containing MM solutions can also be used for washing the LLC-PK cells.
7. Frequently, the filtered clinical samples can be toxic to cells, in which case the early detachment of cells is observed at 4–6 h postinoculation. To reduce their cell cytotoxicity, the samples can be diluted by 1:10, 1:100, or 1:1000 (v/v) in MM, or MM with trypsin (LLC-PK cells).
8. If CPE is not observed by 5 days postinoculation, the cell plates are frozen at −80 °C and thawed three times, and the cell culture medium is harvested and further inoculated onto fresh LLC-PK or ST cells according to the steps described. After three continuous passages of the samples, if there is no CPE and cell culture supernatants are negative for increased PDCoV RNA during the serial passages, the samples are considered as negative for growth in cell culture.
9. Cells should not be allowed to dry out before virus inoculation.
10. The MOI can also be estimated by the extent of CPE observed and is associated with the extent of adaptation of PDCoV to the LLC-PK or ST cells. After viral inoculation, flasks are also gently shaken by hand every 15 min.
11. Each plate should include one row of negative control (MM alone).
12. If the samples are already diluted with the MM supplemented with 5 μg/ml of trypsin, **step 6** is not necessary. The final concentration of trypsin in the cell culture medium in each well should be 5 μg/ml.

13. The $TCID_{50}$ assay using ST cells is also similar to the $TCID_{50}$ assay using LLC-PK cells, except for use of ST MM supplemented with 1% pancreatin, instead of LLC-PK MM. The CPE observed can be further confirmed as PDCoV-specific by immunofluorescence staining with PDCoV-specific antibodies.
14. ST cells can be used to titrate PDCoV grown in either ST or LLC-PK cells by plaque assay.
15. For virus inoculation, 100% confluence cell monolayers are further allowed to attach to the plate for 24–36 h.
16. We believe that this step where cells are incubated without FBS will contribute to an enhanced adsorption of PDCoV to cells.
17. Prepare agarose only prior to use. Dissolve agarose completely. Be sure that the temperature of the agarose used for overlay is around 37 °C.
18. Dispense the agarose mixture thoroughly throughout the well.
19. Duration of the incubation is dependent on the extent of plaque formation.
20. The plaques can be stored in the maintenance medium at −80 °C until use or promptly mixed using a vortex mixer for 1 min and then used to inoculate cells prepared in 6-well cell culture plates.
21. Viral titers of the plaque clones can be determined by qRT-PCR, $TCID_{50}$, or plaque assays. Based on the titers, some clones can be selected for further passage.

Acknowledgments

Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio, USA.

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Chapter 7

The Preparation of Chicken Kidney Cell Cultures for Virus Propagation

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Abstract

Chicken kidney (CK) cells have been widely utilized in virus research studies for many years. The optimized technique of primary CK cell culture production involving both mechanical and enzymatic disaggregation is described. This updated method proved to consistently give high cell yields and resultant cultures are readily used for virus assays.

Key words Chicken kidney cells, Primary cultures, Virus propagation, Viral assays

1 Introduction

Primary chicken kidney cultures have always been widely used for research purposes and propagation of various viruses [1, 2]. Chicken embryonic kidney (CEK) cultures are known to have high susceptibility to enzymatic disaggregation and great proliferation potential. However, kidneys from a few days old embryos are hard to excise and cell yields are quite low. Young chicken kidneys contain more connective tissue and are generally more difficult to dissociate as compared to embryonic tissues. Nevertheless, they give higher cell yields and resulting cultures are susceptible to infection with different animal viruses [1, 3].

Kidney cultures from young birds are routinely mentioned in literature as a successful system for virus studies and even primary virus isolation [1, 4–6], occasionally showing higher sensitivity to viruses than CEK cells [7]. In particular, primary CK cells are an important tool in avian *Gammacoronavirus* infectious bronchitis virus (IBV) research. The ability of CK cells and ex vivo chicken tracheal organ cultures (TOCs) to support the growth of various IBV strains enabled the comprehensive study conducted by Maier et al. [8] on IBV induced membrane rearrangements described in previous studies [9, 10] and their potential role in the process of

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_7,

virus replication. This work focused on an in-depth comparison of types, shape and size, abundance, dynamics and temporal and spatial co-occurrence of membrane rearrangements induced by a substantial set of IBV strains, including pathogenic and apathogenic field and vaccine strains.

The use of CK cells has also allowed for comparisons of the growth kinetics of naturally occurring strains against recombinant IBVs [11]. It has previously been discovered that spike (S) glycoprotein of the virus envelop is responsible for extended cellular tropism exhibited by particular IBV strains [12]. Recombinant IBV viruses were constructed and screened to expand on this finding and determine which S protein subunit is exactly imparting this feature [11]. Another study focused on RT-qPCR as a method for quick detection of early IBV infection. Authors showed that choosing the genes of appropriate transcriptional stability is one of the key factors in correct analysis of RT-qPCR data as transcriptional profiles vary in different IBV-infected cell types, which was demonstrated by using CK cells along with DF1 cell line as two model systems [13].

In this chapter, we describe an improved protocol for primary CK cells isolation, a modification of method described previously by Hennion and Hill [14]. The introduced changes doubled the yield of primary chicken kidney cells, improved quality of obtained monolayers and reduced the overall laboriousness of the method.

2 Materials

2.1 Extraction of Kidneys

1. 2–3 week old chicken(s) from specific pathogen free (SPF) flock culled by cervical dislocation and death confirmed by cessation of the heart.
2. Sterile phosphate buffered saline without calcium and magnesium (PBSa).
3. 70% ethanol and decontaminating wipes.
4. Sterile instruments to include two pointed scissors and one round ended forceps.
5. Skirted falcon tubes and rack.
6. Sterile bioassay dish.
7. Clinical waste bags and cable ties.
8. Class II microbiological safety cabinet (MBSC).

2.2 Isolation and Culture of Kidney Cells

1. Sterile instruments to include tweezers and two disposable scalpels.
2. Sterile Petri dish.

3. Sterile glassware to include two 1 l and one 500 ml baffled flasks, 250 ml beaker, 250 ml wide-necked conical flask and two 100 ml measuring cylinders.
4. Sterile magnetic stirrer bar of suitable size for 500 ml baffled flask.
5. Sterile 50 ml syringe.
6. Sterile glass funnel with two layers of muslin to act as a filter.
7. Sterile wire mesh (mesh No. 50 × 0.200 mm diameter wire, 0.308 mm aperture) folded into a filter shape to fit a funnel (*see* **Note 1**).
8. Sterile Swinnex 25, reusable, syringe driven polypropylene filter unit fitted with metal gauze mesh No. 100 × 0.100 mm diameter wire, 0.154 mm aperture.
9. Sterile 250 ml plastic centrifuge tubes.
10. Sterile serological pipettes and pipette gun.
11. Tissue culture grade flasks or plates.
12. Pipettes and sterile filtered tips.
13. Centrifuge.
14. Magnetic stirring platform with heating.
15. Haemocytometer.
16. Trypan blue.
17. Inverted microscope suitable for observing cell cultures.
18. Incubator set at 37 °C and 5% CO_2.
19. Sterile PBSa.
20. Newborn calf serum (NBCS).
21. Trypsin-EDTA solution: 0.5 g/l porcine trypsin and 0.2 g/l EDTA·4Na(HBSS).
22. Growth medium: Eagles Minimum Essential Medium (EMEM) supplemented with 10% NBCS, 10% Tryptose phosphate broth (TPB), 2 mM L-glutamine, 10 mM HEPES, 20 U/ml penicillin and 0.02 mg/ml streptomycin.

3 Method

The kidneys are removed immediately after confirmation of death to minimise the build-up of blood clots and maintain viability of the cells. We have consistently observed the average yield of 4×10^8 to 5×10^8 CK cells from a 2–3 week old Rhode Island Red bird, which is more than a two-fold increase compared to the previously reported yields [14].

3.1 Extraction of Kidneys

1. After culling, place the birds on a bioassay dish and spray the back and under the wings with ethanol to clean and dampen the feathers before placing inside the MBSC.
2. Insert the blade of a clean pair of scissors just below where the wing attaches to the body and sever across the body through the spinal cord. From one end of this cut, angle scissors towards the legs and cut through the rib cage stopping just before the femur (leg bone), taking care to avoid piercing any internal organs.
3. Repeat this from the opposite end of the first cut to cut down other side of bird towards the other femur (*see* **Note 2**).
4. Using your hands, place thumb on the severed part of the spine and pull until the inner organs are exposed and the carcass lays open on its own. Push the bird's legs (by the knees) backwards until the ends of the femurs pop out, which enables the legs to stay flat.
5. Carefully fold back the cut section of the bird to reveal the internal organs. Gently move intestines aside to expose the kidneys. Use another pair of clean scissors to cut any fatty tissue or membrane to minimize resistance and the chance of tearing intestines.
6. To extract cut along both sides of each kidney, then cut along underneath whilst pulling the kidney up with forceps. Place kidneys into falcon tube containing PBSa.
7. Repeat **steps 1–6** for every bird.
8. When all the required kidneys have been removed from the birds, agitate them in the falcon and discard the PBSa. Repeat this process until the wash PBSa looks clear.

3.2 Isolation and Culture of Kidney Cells

1. Prior to processing the kidney tissues, prepare the digestion solution by diluting Trypsin-EDTA in a 3:1 ratio with PBSa. While kidneys are being extracted, pre-warm PBSa, Trypsin-EDTA solution and growth medium at 37 °C. In particular, ensure that the Trypsin-EDTA digestion solution is continuously maintained at 37 °C (even in between digestions).
2. Perform the next steps in a clean lab in a separate MBSC from the one used for kidney extractions.
3. Transfer the falcon tube with the freshly extracted kidneys inside a clean MBSC along with the sterile beaker, tweezers, Petri dish and two disposable scalpels.
4. Pour the contents of the falcon into a beaker before tipping one or two kidneys at a time onto the Petri dish. Using two scalpels remove the kidneys tissue from the organ membrane with gentle "sweeping" movements. Shred this kidney core finely teasing away obvious blood clots and white connective tissue.

5. Transfer the minced tissue into a sterile 500 ml baffled flask with approximately 100 ml of warm PBSa.
6. Once all the kidneys have been processed, rinse the tissue several times with approximately 100 ml PBSa until the supernatant runs clear. During each wash, discard the PBSa by gently swirling and allowing the fragments to settle for approximately 30 s (*see* **Note 3**).
7. Add 80 ml of warm diluted Trypsin-EDTA solution to drained tissue along with the magnetic stirrer bar and digest in a 37 °C incubator on the heated stirring platform, set to 36 °C, at a moderate speed for 4 min (*see* **Note 4**).
8. Allow the tissue to settle and pour the supernatant into a conical 1 l flask containing 100 ml of cold NBCS (*see* **Note 5**). Gently swirl the flask to ensure Trypsin-EDTA neutralisation in the serum.
9. Repeat **steps 7** and **8** until no more tissue remains (*see* **Note 6**).
10. Position the metal gauze filter on top of the glass funnel with muslin and filter the cell suspension/NBCS mix collected in **step 8** through this into a fresh sterile conical flask.
11. Distribute filtered suspension equally into sterile 250 ml centrifuge bottles and spin at 300 × *g* for 10 min to pellet the cells.
12. Carefully discard the supernatant from the centrifuge bottles and resuspend the cell pellets in warm growth medium, mixing enough to ensure a single cell suspension.
13. Pass the cell suspension through a 50 ml syringe connected to the Swinnex filter, collecting the filtrate in a fresh 250 ml wide-necked conical flask (*see* **Note 7**).
14. Using measuring cylinder, determine the volume of resulting cell suspension. Perform a cell viability count using trypan blue (*see* **Note 8**).
15. Following the cell count, dilute to required concentration in complete medium and place CK cells in culture flasks or plates in a 37 °C incubator to grow until the required confluency. Change the media 3 days after seeding to remove unattached cells and facilitate the formation of an even monolayer.

4 Notes

1. Wire mesh is obtainable from Locker Wire Weavers, www.wiremesh.co.uk.
2. Should any internal organ be punctured, stop work immediately, discard carcass into the appropriate waste stream and discard/clean tray.

3. During this step, some kidney cells may be lost but it is an effective way of excluding red blood cells from the final product.
4. Increasing the speed of the stirrer gradually during latter repeats ensures a thorough mechanical breakdown/digestion of the kidney tissues.
5. This NBCS volume is given for digestion of 5 pairs of kidneys and a maximum of 10 digestion steps. If by the end of tenth digestion, there is still significant amount of tissue left or in the case of more than 10 kidneys being used, the volume of NBCS should be increased correspondingly (10 ml NBCS per each additional digestion step).
6. During the first few steps, digestion occurs quite slowly while later, after the tissue bits are saturated with the Trypsin-EDTA solution, significantly higher numbers of cells are released.
7. Consecutive filtrations in **steps 10** and **13** are aimed at removing any of the undigested tissue aggregates and larger cell clumps, correspondingly. By doing so, better quality of CK cell monolayers is achieved, which is essential for plaque assays.
8. During the cell count, red blood cells, which can be distinguished from the kidney cells by their size and shape, and dead blue cells should be excluded.

Acknowledgements

Elena Lokhman and Srijana Rai contributed equally to this work.

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Chapter 8

The Preparation of Chicken Tracheal Organ Cultures and Their Application for Ciliostasis Test, Growth Kinetics Studies, and Virus Propagation

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Abstract

Chicken tracheal organ cultures (TOCs) provide a simple ex vivo system that makes use of transverse section of tracheal rings extracted from embryos or adult birds to perform classical virological techniques for virus isolation, propagation and titrations, alongside with gene-expression analysis and virus-host interaction studies. Most IBV strains replicate well in TOCs, thus conveniently allowing growth kinetics analysis. Viral replication is revealed by observation of ciliostasis as marker of infection in tracheas extracted from birds ex vivo, as well as in vitro analysis providing a reliable infection model and a useful tool for titration.

Key words Tracheal organ cultures, Ciliostasis, Virus titration, Virus passaging, Respiratory virus

1 Introduction

Tracheal organ cultures (TOCs) are largely used in microbiology studies across multiple disciplines [1], finding their place in virology, bacteriology, parasitology, toxicology and immunology due to the mimicking of in vivo conditions applicable in in vitro experiments to investigate respiratory pathogens [2, 3]. Use of TOCs in avian virology is well established, having provided in vitro infection models to extensively study morphological and functional effects on the respiratory epithelium for avian influenza (IAV), Newcastle disease virus (NDV), avian metapneumoviruses (aMP), infectious laryngotracheitis virus (ILV), avian adenoviruses and, particularly, infectious bronchitis virus (IBV) [4–6]. TOCs allow for virus isolation, quantitative titrations and growth kinetic studies, providing a valid method for isolating field or recombinant viral strains, given the high sensitivity and a targeted set of cellular receptors. More recently, pathogenicity studies and host interaction analysis have been conducted using ex vivo TOCs, extending the use of TOCs in

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_8,

the field of vaccinology, as they offer a subsidiary method to test virus pathogenicity and assess virus protection [7–10].

In this chapter, we first describe the preparation of TOCs from tracheas of 19-day-old embryos, as those are reported to be equally susceptible as at 9-day-old [4]. However, the same protocol with specific modifications applies to adult TOCs when extracted from 2 to 3-week-old birds. Age of birds does slightly affect sensitivity when comparing the ciliostasis activity of IBVs at 3 days post infection (p.i.), however, use of adult TOCs allow for greater viral yield, as quantitatively more tissue is susceptible to virus replication, therefore, based on the purpose of the study, use of adult rather than embryo TOCs may be more appropriate. Cultivation of TOCs may be successful in multiwell plates [11], however use of a tissue culture rolling drum with associated racks is preferred as it reduces accumulation of debris preserving viability of the TOCs and allowing for clearer observation of the cilia beating. Following preparation of TOCs, protocols for ciliostasis tests, growth kinetics analysis and propagation of IBV in TOCs for passaging are provided in detail. Ciliostasis tests are commonly carried out during in vivo experiments to evaluate tracheas ex vivo to examine vaccine protection [10, 12] and correlation with virus pathogenicity [7]. Additionally, observation and scoring of ciliary activity following infection of TOCs in vitro correlates with virus infectivity and helps define virus characteristics, thus allowing further applications, such as the use of TOCs for titrations by determining the ciliostatic dose (CD_{50}). Some IBV strains may be poorly ciliostatic, thus precluding the use of TOCs for titrations by scoring the cilia activity. Most IBV strains replicate in TOCs regardless of their ability to cause ciliostasis, therefore TOCs can still be used to investigate replication dynamics and for propagation and passaging of IBV to assess viral genetic stability. Following preparation of TOCs, screening and selection of TOCs suitable for use is fundamental before proceeding with any technique. TOCs must always be viable and clear of any bacterial contamination (*see* **Note 1**). TOCs work may be labour intensive but, at the same time, it represents an invaluable in vitro model of infection for IBV studies.

In this chapter, we describe updated protocols for preparation of embryonic TOCs, a modification of method described previously by Hennion [13]. We also include methods for preparation of TOCs from 2 to 3-week old chickens, and the application of TOCs for the study of viral growth kinetics, ciliostasis and virus propagation.

2 Materials

2.1 Preparation of Tracheal Section

1. 19- to 20-day-old embryonated eggs from specific pathogen free (SPF) chicken flock or tracheas extracted from 2 to 3-week-old chickens.
2. Tissue chopper e.g. a McIlwain mechanical tissue chopper (Mickle Laboratory Engineering Co. Ltd.).
3. Double-edged razor blades.
4. Sterile curved scissors (small).
5. Sterile scissors (large).
6. Sterile forceps.
7. Sterile scalpel.
8. Sterile Whatman filter paper discs 55 mm diameter (*see* **Note 2**).
9. 70% industrial methylated spirits (IMS).
10. Penicillin + streptomycin (100,000 U of each per ml).
11. 1 M HEPES buffer prepared from HEPES (free acid) and tissue culture grade water, sterilized in an autoclave at 115 °C for 20 min.
12. Culture medium: Modified Eagle's Medium (MEM), 40 mM HEPES buffer, 250 U/ml penicillin, and 250 U/ml streptomycin.
13. Sterile Bijou bottles or similar.
14. Sterile 100- and 150-mm-diameter petri dishes.
15. Sterile phosphate buffered saline (PBS).
16. Large bore Pasteur pipette.

2.2 Culture of Tracheal Section and All Applications

1. Tissue culture roller drum capable of rolling at approximately 8 revolutions/h at 37 °C.
2. Associated rack suitable for holding 16 mm tubes on roller drum.
3. Sterile, extra-strong rimless soda glass tubes 150 mm long and 16 mm outside diameter, suitable for bacteriological work (*see* **Note 3**).
4. Sterile silicone rubber bungs 16 mm diameter at wide end, 13 mm diameter at narrow end, and 24 mm in length (*see* **Note 4**).
5. Inverted microscope.

3 Methods

To calculate the number of embryonated eggs required for an assay, assume that each trachea will yield 17–20 rings. For adult TOCs about the same number of rings is expected for a single trachea as the proportions are conserved and the rings are thicker. The same procedures apply in both cases with a few modifications specified in the protocol. Expect a loss of up to 20% of the TOCs during the preliminary incubation step, due to damage to the rings during preparation or spontaneous cessation of ciliary activity. Those TOCs that have reduced visibility of the lumen but are clearly viable need to be excluded from ciliostasis tests but may be included in other studies, for example growth kinetic analysis or passaging.

3.1 Preparation of Tracheal Sections

1. On a clean workbench spray or wipe the top of the eggs with 70% IMS (*see* **Note 5**).
2. Using curved scissors or forceps remove the top of the shell, lift the embryo out by the wing and cut off the yolk sac. Place the embryo in a 150 mm petri dish and discard the egg and yolk sac.
3. With a sharp pair of scissors decapitate the bird, severing the spinal cord just below the back of the head and angling the cut to just below the beak (*see* **Note 6**).
4. Position the embryo on its back and, using small forceps and scissors, cut the skin along the length of the body from the neck to the abdomen. Care must be taken not to damage the underlying structures.
5. Locate the trachea and using small scissors and forceps, dissect it away from the surrounding tissues (*see* **Note 7**).
6. Cut the trachea at the levels of the carina and larynx (the larynx may have been removed on decapitation) and remove it from the embryo, placing the tissue in a Bijou bottle containing culture medium or a petri dish (*see* **Note 8**).
7. Repeat **steps 2–6** for all available embryos.
8. Place one trachea at a time on a disc of filter paper and, using two pairs of fine forceps, gently remove as much fat as possible (*see* **Note 9**).
9. For adult TOCs, as the size allows, wash them with PBS, flushing through with a Pasteur pipette.
10. Place the cleaned tracheas in a 100 mm petri dish containing culture medium.
11. Swab the tissue chopper with 70% IMS.
12. Place two filter paper discs on top of the plastic cutting table disc and slide the assembled discs under the cutting table clips on the tissue chopper.

13. Raise the chopping arm of the of the tissue chopper and attach the razor blade.
14. Position the arm over the center of the cutting table (*see* **Note 10**).
15. Place the tracheas on to the filter paper under, and perpendicular to, the raised blade and moisten with a small amount of culture medium (*see* **Note 11**).
16. Adjust the machine to cut sections 0.5–1.0 mm thick and activate the chopping arm. Do not forget to set the settings for embryo or adult TOCs, accordingly (*see* **Note 12**).
17. Once the arm has stopped moving, discard the first few rings from each end of the cut tracheas; then with a scalpel, scrape the remaining rings into a 150-mm petri dish containing culture medium.
18. With a large bore Pasteur pipette or similar gently aspirate the medium to disperse the cut tissue into individual rings (*see* **Note 13**).
19. Repeat **steps 11–17** until all the tracheas have been sectioned (*see* **Note 14**).

3.2 Culture of Tracheal Sections

1. With a large bore Pasteur pipette or similar dispense one to five TOC rings into a glass tube previously filled with 1 ml culture medium (*see* **Note 15**).
2. Seal the tube with silicone bung or lid and check that each tube is filled with the exact number of rings selected (*see* **Note 16**).
3. Put the tubes in the roller tube rack, place on the roller apparatus and set to roll at approximately 8 revolutions/h, at approximately 37 °C. Leave the tubes rolling for 1–2 days (*see* **Note 17**).

3.3 TOC Assessment and Selection

1. Check each tube for complete TOC rings and the presence of ciliary activity, using a low power inverted microscope.
2. Discard any tubes in which less than 100% of the luminal surface has clearly visible ciliary activity.
3. For tubes containing more than one ring the luminal surface may not be fully accessible to inspection, in that case observing the approximate viability of the rings and carefully evaluating the medium for bacterial contamination will be sufficient.

3.4 Ciliostasis Test During In Vitro Infection

1. Prepare the viruses for the infections accordingly to the titre set in advance. The small volume for the infections (100–200 μl) may be a limiting factor to consider when deciding the infection titres.

2. For each virus select 10 replicates of 1×TOC tubes and arrange the tubes in rows on a standing rack. Include 10 tubes as negative control for mock infections and at least 1 positive control virus of known ciliary activity.
3. Wash the TOCs rings with PBS (*see* **Note 18**).
4. Inoculate each tube with 100–200 μl of virus, or with TOC medium for the mock infected tubes. Make sure the TOCs are immersed in the infection liquid.
5. Apply the lids or silicone bungs.
6. Mark the top of the lids or the tubes for identification.
7. Incubate the tubes at 37 °C for 1 h standing upright in the rack without rotation.
8. At the end of the incubation time top up with 900 μl of TOC culture medium and incubate on the rotor in the rolling drum at 37 °C, approximately 8 revolutions/h.
9. Starting at day 3 post-infection assess the ciliary activity of all the TOCs infected and the controls by light microscopy. Take note of the score until the ciliary activity is completely abrogated in the test samples. Ciliary activity is scored as 4, 3, 2, 1 or 0 corresponding to 100, 75, 50, 25 or 0% residual ciliary activity. Mock infected TOCs should retain close to 100% ciliary activity until the end of the experiment.
10. For most accurate results, the ciliostasis test should be repeated three times with the same controls and the average trend recorded.

3.5 Assessment of Ciliary Activity Ex Vivo

1. Tracheas extracted from chickens experimentally infected with IBV are collected in PBS during necropsy examinations and taken to the lab for processing.
2. Wash and flush the TOC rings through with PBS.
3. Remove the excess of tissues and fat using forceps and scalpel.
4. Arrange the trachea lengthwise and use a scalpel to cut four rings approximately 1–2 mm thick from the centre and three rings from each end. Place all the ten rings on a microscope slide keeping them hydrated with PBS.
5. Assess ciliary activity using the same scoring method as described in Subheading 3.4, **step 9**.
6. The same tissue can be re-used to assess presence of viral genome by RT-PCR following RNA extraction, or other non-sterile techniques.

3.6 Growth Kinetics Study

1. Determine the number of tubes required with the appropriate number of TOC rings per tube based on the time points selected and number of viruses to test, always including enough

control tubes for mock infection and ideally a control virus with known growth kinetics. Generally, we use three to five tubes, each containing three TOCs, per time point per virus.

2. Prepare the viruses for the infections at a known titre. As it is not easy to determine the multiplicity of infection (MOI) with TOCs, a higher/lower viral titre may be selected based on the purpose of the study.
3. Arrange the number of required TOCs tubes in rows on a standing rack (*see* **Note 19**).
4. Wash the TOC rings with PBS (*see* **Note 18**).
5. Remove any PBS and inoculate each tube containing TOCs with 500 μl of virus, or with TOC medium for the mock infected TOCs. Make sure the TOCs are immersed in the infection liquid.
6. Apply the lids or the silicone bungs to close the tubes.
7. Mark the top of the lids or the tubes for identification.
8. Incubate the tubes at 37 °C for 1 h standing upright in the rack without rotation.
9. At the end of the incubation time, wash the TOC rings with PBS twice.
10. Remove the final wash and add 1 ml of TOC medium per tube.
11. Incubate the tubes on the rotor in the rolling drum at 37 °C, approximately 8 revolutions/h.
12. At each time point collect the supernatants, and the TOCs if required, for titrations and further analysis.

3.7 Virus Propagation in TOCs

1. Determine the number of tubes required with the appropriate number of TOC rings per tube. Generally, we use 3 to 5 replicate tubes per virus, containing each 3 to 5 TOC rings, including a mock infected control and ideally, a control virus with a known ability to propagate in TOCs.
2. Prepare the viruses for the infections, diluting to a particular titre in TOC medium if required.
3. Arrange the number of required TOCs tubes in rows on a standing rack.
4. Wash the TOC rings with PBS (*see* **Note 18**).
5. Remove the PBS and inoculate each tube with 200–500 μl of virus/TOC medium per tube. Make sure the TOCs are immersed in the infection liquid.
6. Close the tubes by applying the lids or the silicone bungs.
7. Mark the top of the lids or the tubes for identification.
8. Incubate the tubes at 37 °C for 1–2 h standing upright in the rack without rotation.

9. At the end of the incubation time, wash the TOCs with PBS.
10. Remove the PBS and add 1 ml TOC medium per tube.
11. Incubate the tubes on the rotor on the roller drum at 37 °C, approximately 8 revolutions/h, for 24 h or longer, based on the individual growth kinetics of the selected virus strain.
12. Harvest the supernatant, and the TOC rings if required, and proceed with further passages following the same methods (*see* **Note 20**).

4 Notes

1. When assessing TOCs for ciliostasis tests, TOCs are selected if they display a very neat and clear lumen to allow further inspections at the light microscope. However, this criterion is not as strict when selecting TOCs for growth kinetics tests or virus propagation, as including several TOCs per tube prevents a clear observation of the whole lumen.
2. Batches of sterile Whatman filter papers can be prepared by interleaving individual discs with slips of grease-proof paper and placing them in a glass petri dish. Wrap the dish in aluminium foil or in paper bags and sterilize in a hot air oven (160 °C for 1 h) or autoclave (120 °C for 20 min).
3. Batches of sterile tubes can be prepared by placing them, open end down, in suitable sized lidded tins lined with aluminium foil or in paper bags. Sterilize in a hot air oven or autoclave as in **Note 2**.
4. Batches of sterile silicone rubber bungs can be prepared by placing them, narrow end down, in shallow, lidded tins. Sterilize by autoclaving at 120 °C for 20 min. Alternatively, plastic lids compatible with the tubes may be purchased for single use. In this case always be careful to fit the lids tightly and stabilise with some autoclave tape if required.
5. Preparation of TOCs can be performed on the open laboratory bench after cleaning the surfaces with 70% IMS or any other suitable disinfectant.
6. Care must be taken at this stage not to damage the trachea.
7. The trachea can be identified by the presence of transverse ridges seen down its length owing to the underlying rings of cartilage.
8. The carina and larynx can be identified by the increased diameter at either end of the trachea.
9. To avoid damage to the trachea hold it as close to one end as possible with the first pair of forceps and use the second pair to strip away the fatty tissue.

10. At this stage gently lower the arm on to the cutting area disc, loosen the screw holding the blade slightly, check that the blade is aligned correctly (the full length of the blade must be in contact with the cutting area), tighten the screw again, and raise the arm.
11. A maximum of five tracheas can be laid side by side on the cutting bed at any one time. Gently stretch each trachea as it is placed on the cutting area, and when all five are in the correct position, wet them with a few drops of culture medium.
12. Settings of the chopper require to be adjusted for TOCs extracted from embryos or adult birds. Using the McIlwain mechanical tissue chopper set the control knob at 2–3 for embryo TOCs, and 8 for adult TOCs.
13. TOCs prepared from adult birds cannot be separated through a Pasteur pipette as they are too large, instead use a pair of forceps to separate each individual ring and to place them in the tubes.
14. It is important to use a fresh blade and paper discs for each set of five tracheas to be sectioned and ensure used blades are disposed of in an appropriate sharps bin.
15. The number of rings that can allocated per tube depend on the assay performed, however it also changes based on the TOC size. For growth kinetics, when more than one TOC per tube is required, we usually dispense 3–5 embryo TOCs or a maximum of 3 TOCs from 2 to 3 week old chickens. The total number of TOCs available should also be considered.
16. Check for damaged glass tubes at this stage, particularly around the rims. Discard any with cracks as these can fail when bungs are inserted, leading to injured fingers. Make sure the tracheal rings are fully submerged in culture medium and not stuck on the wall of the tube. Discard any that are ragged or incomplete. Check the lids fit perfectly and seal properly those that are loose and might come off during the rolling with tape.
17. Make sure that the drum is aligned correctly on the apparatus and that the roller is actually rotating before leaving the cultures to incubate; the speed of the roller apparatus is slow.
18. A quick and easy way to speed up the washing step is to add few ml of PBS without removing the media in the tube, then with a rapid and confident rotation of the hand pour it on a stack of tissues being careful not to lose the TOC ring. Finally, remove the excess of PBS by aspiration or using a pipette. This step is useful when taking into account that a large number of tubes need to be processed in the shortest time possible.

19. When multiple viruses are tested at the same time, we suggest working in a team so that the washing step and the addition of virus or medium are carried out in the quickest time for all the samples.
20. The volume generated through passaging is limited, so take it into consideration when planning for further assays. The volume of medium can be reduced to increase the titre and replicates may be pooled together to increase the volume available.

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Chapter 9

Isolation and Propagation of Coronaviruses in Embryonated Eggs

James S. Guy

Abstract

The embryonated egg is a complex structure comprised of an embryo and its supporting membranes (chorioallantoic, amniotic, and yolk). The developing embryo and its membranes provide a diversity of cell types that allow for the successful replication of a wide variety of different viruses. Within the family *Coronaviridae* the embryonated egg has been used as a host system primarily for two avian coronaviruses within the genus *Gammacoronavirus*, infectious bronchitis virus (IBV) and turkey coronavirus (TCoV). IBV replicates well in the embryonated chicken egg, regardless of inoculation route; however, the allantoic route is favored as the virus replicates well in epithelium lining the chorioallantoic membrane, with high virus titers found in these membranes and associated allantoic fluids. TCoV replicates only in epithelium lining the embryo intestines and bursa of Fabricius; thus, amniotic inoculation is required for isolation and propagation of this virus. Embryonated eggs also provide a potential host system for detection, propagation, and characterization of other, novel coronaviruses.

Key words Embryonated egg, Allantoic, Amniotic, Chicken, Turkey

1 Introduction

Embryonated eggs are utilized as laboratory host systems for primary isolation and propagation of a wide variety of different viruses, including the well-characterized avian coronaviruses, infectious bronchitis virus (IBV), a cause of a highly contagious disease of chickens, and turkey coronavirus (TCoV), a cause of enteritis in turkeys [1–3]. This host system also has been utilized to isolate coronaviruses from a wide variety of other avian species, including pheasants, pigeons, peafowl, and teal, and they provide a potential host system for studies aimed at identifying other, novel coronaviruses [4–6]. Embryonated eggs are routinely utilized for propagation of IBV and TCoV for research purposes and, in the case of IBV, for commercial production of vaccines.

The embryonated egg is comprised of the developing embryo and several supporting membranes which enclose cavities or "sacs"

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_9,

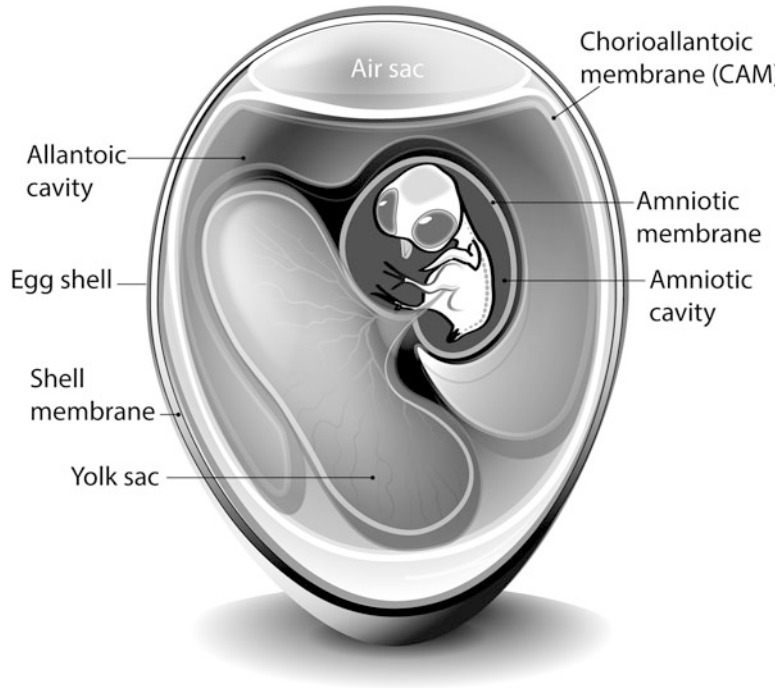

Fig. 1 Anatomical features of embryonated chicken egg at approximately 10 days of incubation (*Alice MacGregor Harvey, illustrator*)

within the egg [7]. The shell membrane lies immediately beneath the shell (Fig. 1). This membrane is a tough, fibrinous membrane that forms the air sac in the region of the blunt end of the egg. In contrast to the shell membrane, chorioallantoic, amniotic, and yolk membranes are comprised largely of epithelium, and these represent potential sites for coronaviral replication. The chorioallantoic membrane (CAM) lies directly beneath the shell membrane; this is a highly vascular membrane that serves as the respiratory organ of the embryo. The CAM is the largest of the embryo membranes, and it encloses the largest cavity within the egg, the allantoic cavity; in the embryonated chicken egg, this cavity contains approximately 5–10 ml of fluid, depending upon the stage of embryonation. The amniotic membrane encloses the embryo and forms the amniotic cavity; in the embryonated chicken egg, this cavity contains approximately 1 ml fluid. The yolk sac is attached to the embryo and contains the nutrients the embryo utilizes during embryonic development and the immediate post-hatch period.

The developing embryo and its membranes (CAM, amniotic, yolk) provide the diversity of cell types that are needed for successful replication of a wide variety of different viruses. Embryonated eggs may be inoculated by depositing virus directly onto the CAM, or by depositing virus within allantoic, amniotic, and yolk sacs [8]. For avian coronaviruses, inoculation of eggs by allantoic or

amniotic routes has been shown to provide these viruses with access to specific cell types that support their replication [1–6]. IBV is an epitheliotropic virus that replicates in a variety of epithelial tissues in the post-hatch chicken including respiratory tract, gastrointestinal tract, kidney, bursa of Fabricius and oviduct [9]. In the embryonated chicken egg, IBV replicates well regardless of inoculation route; however, the allantoic route is favored as the virus replicates extensively in epithelium of the CAM and high titers are shed into allantoic fluid [10]. Coronaviruses obtained from pheasants, pigeons and peafowl have been isolated and propagated in embryonated chicken eggs using procedures similar to those utilized for IBV (allantoic route inoculation) [4–6]. TCoV also is epitheliotropic in post-hatch chickens and turkeys, but replicates only in epithelium lining the intestinal tract and bursa of Fabricius [1, 3, 11]. These same cellular tropisms of TCoV occur in the embryonated egg; the virus replicates only in embryonic intestines and bursa of Fabricius, sites that are reached only via amniotic inoculation.

2 Materials

2.1 Preparation and Collection of Samples for Egg Inoculation

1. Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% fetal bovine serum (FBS) and antibiotics: penicillin 1000 U/ml, gentamicin 0.05 mg/ml, amphotericin B 5 μg/ml. Adjust pH to 7.0–7.4 using either 1 N NaOH or 1 N HCl. Other cell culture basal media (minimal essential medium, RPMI 640, etc.) or tryptose phosphate broth may be substituted for DMEM.
2. Sterile cotton-tipped swabs are used for collection of antemortem samples (respiratory secretions, feces, etc.). Type 4 Calgiswabs (Puritan Medical Products) are useful for collection of respiratory secretions from small birds.
3. Sterile Whirl-pak® bags (Fisher Scientific) are used for collection of tissues.
4. Tissue homogenizer (e.g., mortar and pestle, Ten Broeck homogenizer, or Stomacher® [Fisher]).

2.2 Egg Inoculation and Incubation

1. Fertile eggs are obtained, preferably, from specific-pathogen-free (SPF) flocks (e.g., Charles River/SPAFAS). Alternatively, fertile eggs may be used that are from healthy flocks free of antibody to the virus of interest (*see* **Note 1**).
2. Disinfectant: 70% ethanol, 3.5% iodine, 1.5% sodium iodide.
3. A vibrating engraver (Fisher Scientific), drill (e.g., Dremel) or similar is used to prepare holes in egg shells. Prior to use, disinfect the tip of the engraving tool/drill to prevent contamination of the egg.

4. Plastic cement, glue, tape or nail varnish are used to seal holes in eggshells after inoculation.
5. Egg flats.
6. Egg candlers are available from a variety of commercial sources.
7. A suitable egg incubator is needed; these are available from a variety of commercial sources. Commercially available egg incubators generally are equipped with heat source, humidifier, and a timer-based, mechanical turning system.

2.3 Collection of Specimens from Inoculated Eggs

1. Sterile scissors and forceps.
2. Sterile pipettes or 5 ml syringes with 1 in., 18 gauge needles.
3. Sterile plastic tubes e.g. 12 × 75 mm snap-cap tubes or micro-centrifuge tubes.

3 Methods

Embryonated chicken and turkey eggs are extensively utilized for isolation and propagation of IBV and TCoV, respectively [2, 3]. These same eggs and techniques may be useful for amplification of other coronaviruses [4–6]. However, many viruses exhibit host specificity and this should be a consideration when attempting to isolate and propagate novel coronaviruses.

Embryonated eggs from avian species other than chickens and turkeys may be utilized; these are inoculated essentially as described for chicken and turkey eggs, primarily by making adjustments in the length of time embryos are incubated before inoculation. Embryonated chicken eggs are inoculated by the allantoic route at approximately the middle of the 21-day embryonation period, at 8–10 days of embryonation; they are inoculated by the amniotic route late in the incubation period, at 14–16 days of embryonation. Turkey and duck eggs have a 28-day embryonation period and generally are inoculated by the allantoic route at 11–14 days of embryonation, and by the amniotic route at 18–22 days of embryonation.

Embryonated chicken and turkey eggs are incubated at a temperature of 38–39 °C with a relative humidity of 83–87%. They should be turned several times per day to ensure proper embryo development and to prevent development of adhesions between the embryo and its membranes. Fertile eggs may be stored for brief periods with minimal loss of viability [12]. Ideally, fertile eggs are stored at a temperature of 19 °C with a relative humidity of approximately 70%. Alternatively, eggs may be stored at room temperature. Eggs should be tilted at 45 °C, with daily alternation from side to side to minimize loss of embryo viability.

Indirect evidence of coronavirus replication in inoculated embryonated eggs may consist of embryo mortality or lesions in the embryos such as hemorrhage, edema or stunting; however,

virus replication may occur in the absence of readily discernible effects on the embryo. Methods for specific detection of coronaviruses in inoculated embryonated eggs include electron microscopy, immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) procedures [2, 3, 13, 14]. Electron microscopy is a particularly useful tool as this method depends solely on morphologic identification of the virus and does not require specific reagents [15]. The characteristic electron microscopic morphology of coronaviruses allows their presumptive identification in embryonic fluids (e.g., allantoic fluid) or embryo intestinal contents. A variety of immunohistochemical and RT-PCR procedures have been developed for detection of coronaviruses, and these same procedures may be useful for detection of novel coronaviruses due to antigenic and genomic similarities among coronaviruses, particularly those within the same genus [2, 3, 11, 13, 14, 16–18].

3.1 Collection of Samples for Egg Inoculation

1. Swabs used to collect clinical samples such as respiratory secretions and feces are placed in 2–3 ml of DMEM supplemented with FBS and antibiotics.
2. Tissues are collected using aseptic technique and placed in clean, tightly sealed bags (e.g. Whirl-pak bags).
3. Clinical samples should be chilled immediately after collection and transported to the laboratory with minimal delay. Samples may be shipped on ice, dry ice, or with commercially available cold packs (*see* **Note 2**).

3.2 Preparation of Samples for Egg Inoculation

1. Use a vortex mixer to expel material from swabs, then remove and discard swab. Clarify by centrifugation (1000–2000 × *g* for 10 min) in a refrigerated centrifuge. Filter, if needed, through a 0.45 μm filter, and store at −70 °C (*see* **Note 3**).
2. Prepare tissues and feces as 10–20% suspensions in DMEM supplemented with FBS and antibiotics. Homogenize tissues using a mortar and pestle, Ten Broeck homogenizer, or Stomacher® (Fisher). Clarify tissue and fecal suspensions using centrifugation (1000–2000 × *g* for 10 min) in a refrigerated centrifuge; this removes cellular debris and most bacteria. Filter, if needed, through a 0.45 μm filter, and store at −70 °C (*see* **Note 3**).

3.3 Allantoic Sac Inoculation

1. Chicken eggs (21 day embryonation period) are generally inoculated by the allantoic route at 8–10 days of embryonation (Fig. 2). Turkey and duck eggs (28 day embryonation period) generally are inoculated by this route at 11–14 days of embryonation. Eggs from other avian species may be used by making adjustments to the ages at which embryos are inoculated.

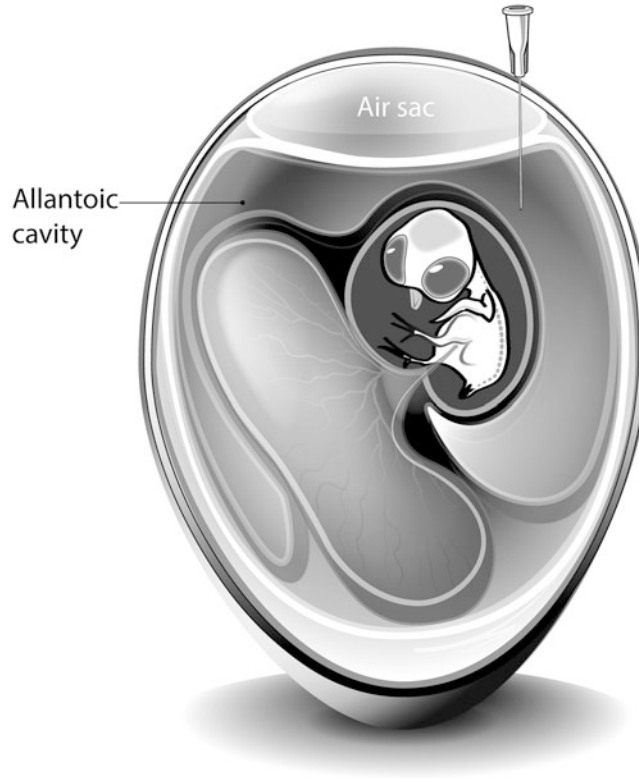

Fig. 2 Allantoic inoculation of embryonated chicken egg. Needle placement is illustrated for inoculation into allantoic cavity of embryonated chicken egg at approximately 10 days of incubation. (*Alice MacGregor Harvey, illustrator*)

2. Place eggs in an egg flat with the air sac up. Candle eggs to ensure viability and mark the edge of the air sac.
3. Disinfect the area marked on the shell and drill a small hole just above the mark so that the hole penetrates the air sac, but not the portion of the egg below the air sac.
4. A 1-ml syringe with a 25-gauge, 0.5 in. (12 mm) needle is used to inoculate eggs. Insert the needle to the hub while holding the syringe vertically and inject 0.1–0.3 ml of inoculum into the allantoic cavity (Fig. 2).
5. Seal holes and return eggs to incubator.
6. Incubate eggs for 3–7 days. Evaluate embryos and allantoic fluid for the presence of virus as described below.

3.4 Amniotic Sac Inoculation (Method A)

1. Fertile embryonated eggs are inoculated late in the incubation period. Chicken eggs are inoculated at 14–16 days of embryonation; turkey and duck eggs are inoculated at 18–22 days of embryonation (Fig. 3).

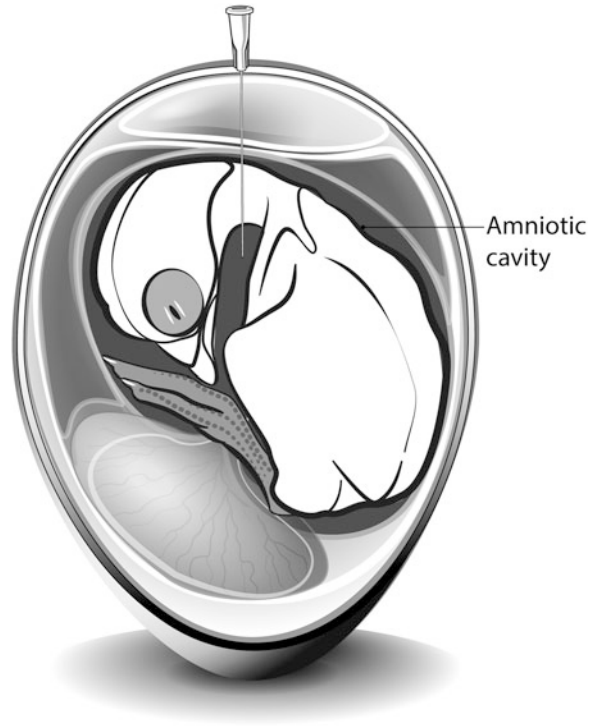

Fig. 3 Amniotic inoculation of embryonated chicken egg. Needle placement is illustrated for inoculation of embryonated chicken egg at approximately 18 days of incubation. (*Alice MacGregor Harvey, illustrator*)

2. Candle eggs to ensure embryo viability. Place eggs in an egg flat with the air sac up.
3. Disinfect the shell at the top of the egg, over the center of the air sac. Drill a small hole through the shell at center of air sac using a vibrating engraver or drill.
4. A 1-ml syringe with a 22-gauge, 1.5 in. (38 mm) needle is used to inoculate chicken, duck and turkey embryos. Insert the needle to the hub while holding the syringe vertically and inject 0.1–0.2 ml of inoculum into the amniotic cavity (*see* **Note 4**).
5. Seal holes and return eggs to incubator.
6. Inoculated embryos generally are examined for the presence of virus after incubation for 2–5 days. Evaluate inoculated embryos for the presence of virus as described below.

3.5 Amniotic Sac Inoculation (Method B)

1. Fertile embryonated chicken eggs are inoculated, as above, at 14–16 days of embryonation; turkey and duck eggs at 18–22 days of embryonation. Using an egg candler, visualize and mark the general location of the embryo (*see* **Note 5**).

2. Place eggs in an egg flat with the air sac up. Disinfect the shell at the top of the egg, over the center of the air sac. Drill a small hole through the shell at center of air sac.
3. A 1-ml syringe with a 22-gauge, 1.5 in. (38 mm) needle is used to inoculate chicken, duck and turkey embryos. Inoculate eggs in a darkened room, as the embryo must be visualized for this method of amniotic inoculation. Hold the egg against an egg candler and insert the needle into the egg and toward the shadow of the embryo. As the tip of the needle approaches the embryo, a quick stab is used to penetrate the amniotic sac. Penetration of the amniotic sac is verified by moving the needle sideways; the embryo should move as the needle moves (*see* **Note 4**).
4. Seal holes and return eggs to incubator. Inoculated embryos generally are examined for the presence of virus after incubation for 2–5 days.

3.6 Collection of Allantoic Fluid from Eggs Inoculated by Allantoic Route

1. Candle eggs once daily after inoculation. Discard all eggs with embryos that die within the first 24 h after inoculation (*see* **Note 6**).
2. Collect allantoic fluid from all eggs with embryos that die >24 h after inoculation and from eggs with embryos that survive through the specified incubation period. Eggs with live embryos following the specified incubation period are refrigerated at 4 °C for at least 4 h, or overnight, prior to collection of allantoic fluid (*see* **Note 7**).
3. Place eggs in an egg flat with the air sac up. Disinfect the portion of the egg shell that covers the air sac, and use sterile forceps to crack and remove egg shell over air sac.
4. Use forceps to gently dissect through the shell membrane and CAM to expose the allantoic fluid. Use forceps to depress membranes within the allantoic cavity so that allantoic fluid pools around the tip of forceps. Use a pipette or syringe with needle to aspirate fluid. Place fluid in sterile, 12 × 75 mm snap-cap tubes, or other vials. Store at −70 °C (*see* **Note 8**).
5. Examine allantoic fluid for the presence of coronavirus using electron microscopy, immunohistochemistry, or RT-PCR (*see* **Note 9**).

3.7 Collection of Embryo Tissues from Eggs Inoculated by Amniotic Route

1. Candle eggs once daily after inoculation. Discard all eggs with embryos that die within the first 24 h after inoculation (*see* **Note 6**).
2. Examine all eggs with embryos that die >24 h after inoculation and eggs with embryos that survive through the specified incubation period (*see* **Note 10**).

3. Euthanatize live embryos by placing eggs in a plastic bag or plastic bucket filled with carbon dioxide gas, or refrigerate (4 °C) overnight. Alternatively, embryos may be euthanatized by cervical dislocation upon removal from eggs using the handles of a pair of scissors (*see* **Note 11**).
4. Place eggs in an egg flat with the air sac up. Disinfect the portion of the egg shell that covers the air sac, and use sterile forceps to crack and remove the egg shell over air sac.
5. Use forceps to dissect through the shell membrane and CAM.
6. Grasp the embryo with sterile forceps and gently remove from the egg.
7. Remove selected tissues and/or intestinal contents from embryo for coronavirus detection using electron microscopy, immunohistochemistry, or RT-PCR (*see* **Note 12**).

4 Notes

1. Fertile eggs from non-SPF flocks may be used; however, the presence of antibodies may interfere with isolation and propagation, and the presence of egg-transmitted infectious agents may result in contamination of any viruses obtained with these eggs.
2. If dry ice is used, place samples in tightly sealed containers to prevent inactivation of viruses from released carbon dioxide.
3. The supernatant fluid should be filtered if the specimen is feces or other sample that likely is contaminated with high concentrations of bacteria. Filtration of samples will reduce virus titer, and is only done when necessary.
4. The accuracy of delivering an inoculum into the amniotic sac using this method may be determined by sham inoculation of embryos with a dye such as crystal violet (0.2% crystal violet in 95% ethanol), then opening eggs and determining site of dye deposition.
5. A distinct advantage of Method A is that visualization of the embryo is not required. Method B requires visualization of the embryo and this may not be possible for embryonated eggs having a dark shell color (e.g., turkey eggs, brown chicken eggs). Method A also requires less skill for delivery of inoculum into the amniotic cavity, but is more prone to error than Method B with the possibility that inoculum is deposited at sites other than the amniotic cavity. If the embryo can be visualized, a potential advantage of Method B is more precise delivery of inoculum into the amniotic cavity as compared with Method A.

6. Embryo deaths that occur <24 h after inoculation generally are due to bacterial contamination, toxicity of the inoculum, or injury to the embryo or its supporting membranes.
7. Refrigeration kills the embryo and causes the blood to clot. This prevents contamination of allantoic fluid with blood.
8. Multiple passages in embryonated eggs may be necessary for initial isolation of coronaviruses; allantoic fluid is used as inoculum for additional passages in embryonated eggs. Embryos at each passage should be evaluated for gross lesions. For IBV, embryo-lethal strains generally result in embryos with cutaneous hemorrhage; non–embryo-lethal strains result in stunting, curling, clubbing of down, and/or urate deposits in the mesonephros of the kidney. In some cases, virus replication in embryonated eggs may not be associated with readily detectable embryo lesions.
9. Allantoic fluids commonly are examined for the presence of coronavirus using electron microscopy or RT-PCR procedures. Alternatively, immunohistochemical detection may be accomplished by staining either sections of CAM, or the epithelial cells that are present in allantoic fluid (these should be collected by centrifugation prior to freezer storage of allantoic fluid).
10. TCoV rarely results in embryo mortality. Typically, only those eggs with live embryos are examined following the specified incubation period; however, the possibility of embryo-lethal viruses should not be overlooked.
11. The method of euthanasia employed will depend upon the method used to detect virus in inoculated embryos. Fresh tissues are required if immunohistochemistry is to be employed; for this, embryos should be euthanatized by cervical dislocation or exposed briefly to carbon dioxide gas.
12. Intestinal contents commonly are examined for the presence of coronaviruses using electron microscopy or RT-PCR procedures. Alternatively, immunohistochemical detection may be accomplished by staining sections of intestines or bursa of Fabricius.

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Chapter 10

Well-Differentiated Primary Mammalian Airway Epithelial Cell Cultures

Mitra Gultom, Laura Laloli, and Ronald Dijkman

Abstract

Well-differentiated primary airway epithelial cell (AEC) cultures have been widely used for the characterization of several human respiratory viruses including coronaviruses. In recent years, there has been an increase in interest toward animal AEC cultures and their application to characterize veterinary viruses with zoonotic potential, as well as studying host–pathogen interactions in animal reservoir host species. In this chapter, we provide a revised and improved protocol for the isolation and establishment of well-differentiated AEC cultures from diverse mammalian species and the use of the cultures for the characterization of veterinary coronavirus. We also describe immunohistochemistry protocols with validated antibodies for the visualization and identification of viral cell tropism in well-differentiated AEC cultures from human, swine, bovine, and feline origin.

Key words Veterinary coronavirus, Animal airway epithelial cell cultures, Cell tropism, Virus characterization

1 Introduction

Coronaviruses are important respiratory pathogens that can cause a large variety of diseases in mammalian species including humans, bovine, porcine, feline, and nonmammalians such as avian species. In humans, coronavirus infections are mainly associated with the common cold, however more recently also with several outbreaks of life-threatening respiratory infections, as illustrated by Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV) cases [1, 2]. In addition to humans, coronaviruses are also notable for causing respiratory illnesses in animals, as in the case of porcine respiratory coronavirus (PRCoV) [3, 4], bovine respiratory coronavirus (BCoV) [5, 6], and feline coronavirus (FCoV) [7]. From a genetic perspective, many human and animal coronaviruses share a high degree of similarity; for instance, BCoV and human coronavirus OC43 (HCoV-OC43) share a nucleotide identity of 96%

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_10,

[8]. Moreover, it is evident that some of the emerging human coronaviruses originate from animal coronaviruses that crossed the species barrier [9, 10]. This explains the increasing interest in veterinary coronavirus infections and efforts to study the pathogenesis of coronavirus in the authentic animal hosts.

One of the most commonly used in vitro platforms to study coronavirus infections is well-differentiated airway epithelial cell (AEC) cultures grown at an air–liquid interface. These organotypic cell cultures recapitulate many aspects of the respiratory tract, including airway cell heterogeneity, mucus production, ciliary beating activity, and the dynamics of the innate immune system toward pathogens. In the veterinary research field, there is an increasing demand for the use of more physiologically relevant systems in the study of veterinary pathogens. Hence, the availability of animal AEC cultures for the study of animal respiratory pathogens, especially coronaviruses, is highly desirable.

Previously our group has described a protocol to establish human AEC cultures and its use for human coronavirus replication studies [11]. In this chapter, we revise and adapt the previous version for the establishment of AEC cultures from various animal species. We outline optimized protocols on how to generate AEC cultures from bovine, porcine, and feline species, which have been successfully cultivated and are in use for veterinary coronavirus studies in our lab (*see* Figs. 1 and 2). We highlight that these methods are versatile and can be easily adapted for other animal species. In addition, we describe an updated immunofluorescence protocol with validated antibodies to visualize virus in the animal AEC cultures and to identify coronavirus tropisms. Other virus detection methods that are useful for virus detection such as quantitative reverse transcriptase (RT-qPCR) and plaque assays are not discussed in this chapter as they have been previously described [11]. Hopefully, these techniques will provide a more realistic model for coronavirus pathogenesis and host–virus interaction studies in the veterinary field and also contribute to reducing animal use (3Rs principle) in the veterinary virology field.

2 Materials

2.1 AEC Cultures

1. Whole tracheobronchial tissue resections for the isolation of primary tracheobronchial epithelial cells from mammalians can be obtained postmortem, whereas for human origin, this can also be done from partial tracheobronchial tissue resections or bronchoscopy samples. It is mandatory that the procurement of the tissue material is in accordance with local ethical guidelines.

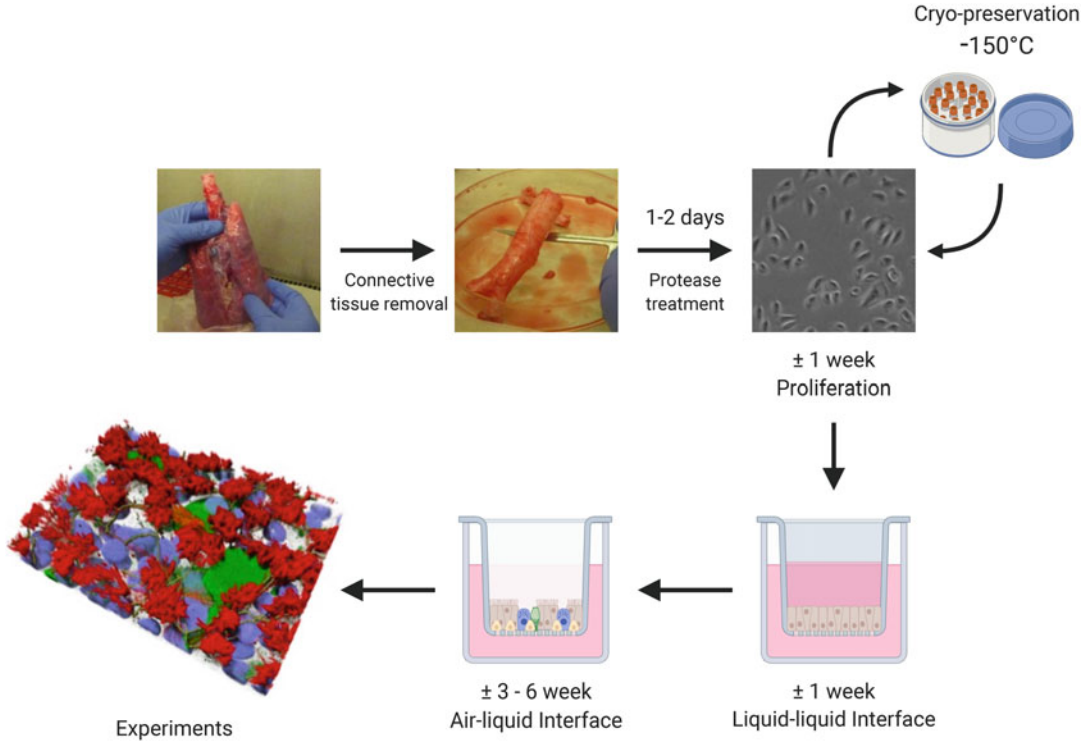

Fig. 1 Schematic representation of the establishment of mammalian AEC cultures. Chronological representation of the different procedural steps for the establishment of AEC cultures from mammalian tracheal/bronchial tissue material that can be used for the characterization of respiratory coronaviruses. The experimental analysis shows a three-dimensional projection of Z-stacks from human AEC infected with HCoV-229E expressing GFP (green) for 48 h at 33 °C, and the visualization of nuclei (blue), tight junctions (yellow), and cilia (red)

2. Isolating/transfer medium: minimum essential medium (MEM), 100 U/ml penicillin, 100 μg/ml streptomycin, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).
3. Sterile phosphate-buffered saline (PBS).
4. 10× Digestion solution: 1% (w/v) protease from *Streptomyces griseous* type XIV, 0.01% deoxyribonuclease I from bovine pancreas in isolating/transfer medium.
5. Bronchial epithelial growth medium (BEGM): LHC basal medium, supplemented with the required additives (*see* Tables 1 and 2).
6. Air–liquid interface (ALI) medium: LHC basal medium and Dulbecco's modified MEM (DMEM) mixed in a 1:1 ratio, supplemented with the required additives (*see* Tables 1, 2, and 3).

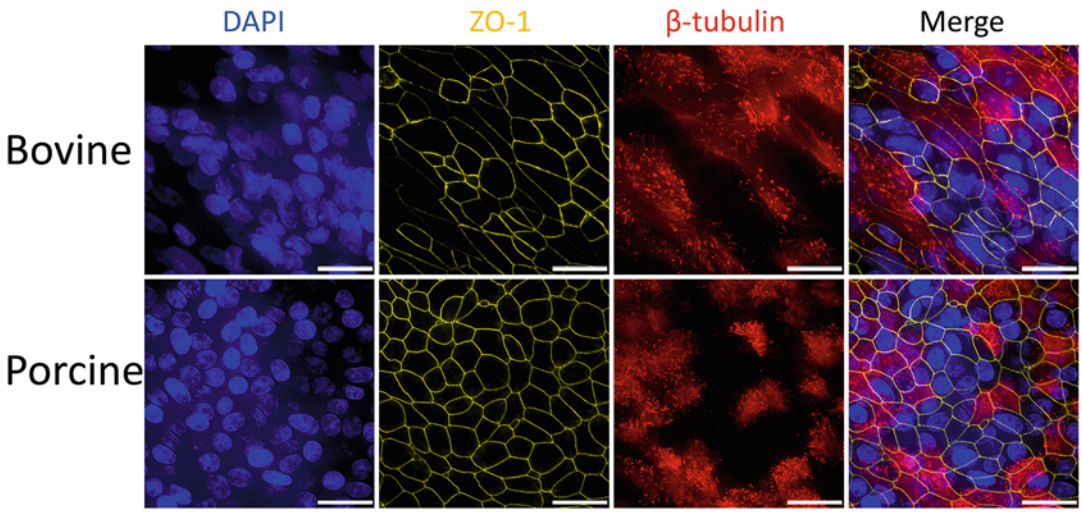

Fig. 2 Immunofluorescence staining of bovine and porcine AEC cultures. Maximum intensity projections of Z-stacks from bovine and porcine AEC cultures that were grown at air–liquid interface for 4 weeks. The cells were formalin-fixed and stained for the visualization of nuclei (blue), tight junctions (yellow), and cilia (red). Scale bar = 20 μm

7. 0.4 μm Pore polyester membrane inserts (diameter 6.5 mm) and 24-well cluster plates.
8. 3 mg/ml Collagen type I and III solutions from human fibroblasts.
9. 0.5 mg/ml Collagen type IV from human placenta. Reconstitute 10 mg in 5 ml filter-sterilized water with 0.25% acetic acid. Dissolve for a few hours at 37 °C, occasionally swirling. Once dissolved, add sterile water containing 0.25% acetic acid to the total volume of 20 ml, mix gently by pipetting. Filter-sterilize the solution through a 0.22 μm polyethersulfone (PES) filter, and store at −20 °C in aliquots of 800 μl per microfuge tube. The stock solution is stable for at least 1 year at −20 °C.
10. TEER solution: dissolve 4.5 g NaCl, 91.89 mg $CaCl_2$, and 1.194 g of HEPES in 400 ml of distilled water and stir for 10 min at room temperature. Add water up to 500 ml and sterilize the solution via autoclaving, 20 min at 121 °C.
11. Dissection sets: forceps, tweezers, scissors (*see* **Note 9**).
12. Cover sponge/drape.
13. Rocking platform/tube roller or magnetic stirrer.

Table 1
Common stock additives for BEGM and ALI medium

Component	Stock concentration	Comment
Bovine serum albumin (BSA)	300×, 150 mg/ml	*See* **Note 1**
Bovine pituitary extract (BPE)	1000×, ~14 mg/ml	
Insulin	2000×, 10 mg/ml	Store at 4 °C
Transferrin (TF)	1000×, 10 mg/ml	Dissolve in PBS
Triiodothyronine (T3)	1000×, 0.067 mg/ml	Dissolve in 1 mM sodium chloride (NaOH)
Epinephrine (EP)	1000×, 0.6 mg/ml	Dissolve in 10 mM hydrochloric acid (HCl)
Phosphorylethanolamine (PE)	1000×, 70 mg/ml	Dissolve in PBS
Ethanolamine (EA)	1000×, 30 μl/ml	Dissolve in PBS
Stock 11 (S11)	1000×, 0.863 mg/ml	*See* **Note 2**
Stock 4 (S4)	1000×	*See* **Note 3**
Trace elements (TR)	1000×	*See* **Note 4**
Penicillin/streptomycin (P/S)	100× 10,000 U/ml penicillin and 10.000 μg/ml of streptomycin	Store at 4 °C

All additives should be aliquoted and stored at −20 °C unless stated otherwise

Table 2
Stock additives for BEGM and ALI medium added differently (*see* Note 5)

Component	Stock concentration	BEGM	ALI	Comment
Epidermal growth factor (EGF)	1000×–50,000×, 25 μg/ml	25 ng/ml	Vary	Dissolve in 0.25 mM HCl
Hydrocortisone (HC)	720×–1000×, 0.48 mg/ml	0.48 μg/ml	Vary	Dissolve in PBS
Retinoic acid (RA)	720× to 1000×, 5×10^{-5} M	50 nM	Vary	*See* **Note 6**
Y-27632	2500×, 20 mM	Yes	No	
Isoprenaline	333,000×, 100 mM	Yes	No	Dissolve in sterile water
A83-01	10,000×, 10 mM	Yes	No	
Primocin™	500×, 50 mg/ml	Yes	No	*See* **Note 7**
DAPT	1000×, 5 mM	No	Vary	

All additives should be aliquoted and stored at −20 °C unless stated otherwise

Table 3
Optimized EGF, RA, HC, and DAPT concentration in the ALI medium for differentiation of AEC cultures from several species (*see* Note 8)

Components	Stock concentration	Working concentration			
		Human	Bovine	Porcine	Feline
EGF	25 μg/ml	0.5 ng/ml	25 ng/ml	25 ng/ml	25 ng/ml
RA	5×10^{-5} M	50 nM	50 nM	70 nM	50 nM
HC	0.48 mg/ml	0.48 μg/ml	0.48 μg/ml	0.072 μg/ml	0.072 μg/ml
DAPT	5 mM	No	2.5 μM	No	No

2.2 Propagation of Coronaviruses from Mammalian Origin

1. Apical wash solution: Hank's Balanced Salt Solution (HBSS), without calcium and magnesium.
2. Virus transport medium (VTM): MEM, 25 mM HEPES-buffered, 0.5% gelatin (*see* **Note 10**), 100 U/ml penicillin, and 100 μg/ml streptomycin.
3. Aerosol barrier pipette tips and 1.5 ml locking lid microfuge tubes.

2.3 Immuno-fluorescence Analysis

1. Fixation solution: 4% formalin solution, neutral buffered.
2. Confocal staining buffer (CB): 50 mM ammonium chloride (NH_4Cl), 0.1% saponin, and 2% IgG and protease-free BSA dissolved in 500 ml of PBS (pH 7.4). Filter-sterilize (0.2 μm PES filter) solution, prepare aliquots of 40 ml, and store at −20 °C (*see* **Note 11**).
3. Primary antibodies: *see* Table 4.
4. Secondary antibodies: *see* Table 5.
5. Fluorescent DNA dyes: 4′,6-diamidino-2-phenylindole (DAPI) 1 mg/ml (1000×).
6. Wash solution: PBS, pH 7.4, without calcium and magnesium.
7. Scalpel (No.10).
8. Rat-tooth forceps.
9. Aluminum foil or nontransparent plastic container.
10. Microscope slides and coverslips.
11. Hard setting mounting medium.
12. Gyro rocker.

Table 4
List of example primary antibodies

Antibody	Target	Dilution	Host	Clone	Supplier	Species reactivity
β-Tubulin IV	Cilia	1:100	Rabbit	EPR16775	Abcam	H, B, P, F
β-Tubulin	Cilia	1:100	Rabbit	Clone 9F3	Cell signaling	H, B, P, F
ZO-1	Tight junctions	1:100	Mouse	Clone 1A12	Thermofisher	H, B, P, F
Cytokeratin-5	Basal cells	1:100	Rabbit	EP1601Y	Abcam	H, P
Uteroglobine	Club cells	1:200	Rabbit		Abcam	H, P
Muc-5 AC	Goblet cells	1:200	Rabbit	EPR16904	Abcam	H, P
Vimentin	Non-epithelial cells	1:200	Mouse	RV202	Abcam	H
dsRNA	dsRNA	1:500–1000	Mouse		Scicons	
CD13	CD13/APN	1:200	Sheep		RnDsystems	H
CD26	CD26/DPP4	1:100	Goat		RnDsystems	H
CD26	CD26/DPP4	1:100	Mouse	OTI11D7	Origene	H, B
ACE2	ACE2	1:200	Rabbit		Abcam	H
Intravenous immunoglobulins (IVIG)	Viral proteins	1:1000	Human			

H human, *B* bovine, *P* porcine, *F* feline
Antibodies listed used successfully in our laboratory. Alternative antibodies and suppliers may be used after validation

Table 5
List of example secondary antibodies

Antibody	Conjugate	Dilution	Host	Supplier
Anti-mouse	Alexa Fluor® 488	1:400	Donkey	Jackson Immunoresearch
Anti-goat	Alexa Fluor® 647	1:400	Donkey	
Anti-rabbit	Alexa Fluor® 594	1:400	Donkey	
Anti-human	Cy3	1:400	Donkey	

Antibodies listed are used successfully in our laboratory. Alternative antibodies and suppliers may be used after validation

3 Methods

Unless stated otherwise, carry out all the procedures in a biosafety cabinet according to local biosafety regulations.

3.1 AEC Cultures

3.1.1 Collagen Coating of Cell Culture Flasks for Cell Expansion

Cell culture flasks are coated with a mixture of type I and III collagen from fibroblasts. This is necessary to promote attachment and proliferation of primary AECs.

1. Use filter-sterilized water (0.22 μm PES membrane) to prepare a 1:75 dilution of collagen type I and III stock.
2. Use 6 ml per 75 cm^2, distribute the collagen solution by gently shaking the flask to make sure the entire surface is covered.
3. Incubate for 2 h at 37 °C or overnight at room temperature.
4. Aspirate remaining liquid and wash twice with 10 ml of PBS to remove traces of acetic acid.
5. Culture flasks can be directly used. Optional: store coated flasks at 4 °C for a maximum of 6–8 weeks.

3.1.2 Collagen Type IV Coating of Inserts

The inserts need to be coated with collagen type IV, which will support the development and long-term maintenance of differentiated AEC cultures.

1. Prepare 1:10 collagen solution by mixing 7.2 ml of filter-sterilized water with 800 μl of collagen type IV solution stock.
2. Apply 50 μl of diluted collagen type IV per insert. After completing one plate, make sure that the entire surface of each well is covered.
3. Incubate the plates for 2 h at 37 °C.
4. To remove traces of acetic acid, wash inserts twice with 200 μl of PBS.
5. After these steps, coated inserts can be used directly. Optional: store at 4 °C for a maximum of 6–8 weeks.

3.1.3 Isolation of Primary Tracheal and/or Bronchial Cells

Isolation of primary epithelial cells from tracheobronchial tissue resections from animals can be done in the same manner as for primary epithelial cells of human origin.

1. Transport of the tracheal/bronchial tissue resections should be done in isolating/transport medium. The tissue should be kept on ice to prevent degradation and to minimize the bacterial growth (*see* **Note 12**).
2. Isolation can be done in a flow cabinet or workbench in the Biosafety Level 2 (BSL-2) lab. Prepare a sterile working environment cleaned with 70% ethanol. Subsequently, cover the surface with a large sterile cover drape/sponge. For large animal tissues, first cut the large tissue into 5–10 cm segments.

3. Transfer the tracheal/bronchial tissue to a large petri dish and remove all excess of connective tissue, fat, and lymph nodes.
4. Wash the cleaned tissue three times with PBS.
5. Before the digestion step, take a sample of the trachea for histological analysis. Cut a 0.5 cm segment of the trachea tissue and fix the segment with 40 ml of fixation solution overnight. Wash the fixed tissue three times in PBS. For long-term storage, this sample can be stored in PBS at 4 °C.
6. Fill the desired number of sterile containers (50 ml tube for small trachea tissue or 1 L wide neck glass bottle with screw cap for large trachea tissue) with isolating/transfer medium up to 60% of its capacity (30 ml or 600 ml). Transfer as many tissue segments as possible into a single container, until the volume reaches 36 ml or 720 ml, respectively. Then add 10% (v/v) of 10× digestion solution to each tube/bottle, to a final volume of 40 ml or 800 ml, respectively.
7. Place 50 ml tubes on a rocking platform/tube roller at 4 °C and incubate for 48 h (*see* **Note 13**). For the trachea tissue of large animals, place the 1 L bottles on magnetic stirrers with a low speed in a 4 °C cold room.
8. Place the 50 ml tube/1 L bottle containing the digested tissue on ice and add heat-inactivated FBS to each tube to a final concentration of 10% (v/v), to neutralize the protease activity. Invert tubes/bottle three times.
9. Pour solution along with the tissue into a large petri dish. Cut open the trachea cartilage side and gently scrape off the epithelium from the collagen–cartilage surface, using a scalpel in a parallel angle to the surface. Pool solutions containing dissociated cells into a 50-ml conical tube and wash the petri dish once with PBS to collect remaining cells.
10. Centrifuge for 5 min at 500 × *g*. Wash cells once with HBSS and resuspend cells in warm BEGM to a concentration of, approximately, 5×10^6 cells/ml.
11. If not expanded directly, the cells can be cryopreserved for long-term storage.

3.1.4 Maintenance of AEC on Plastics for Expansion

1. To generate passage 1 and 2 cell lineages for subsequent subculture on porous supports, primary cells can be plated in BEGM on collagen-coated flasks at a density of 1–3 × 10^6 cells per 75 cm^2.
2. Change the growth media 24 h after seeding to remove red blood cells and any unattached epithelial cells. First, remove the old medium and wash the cell monolayer twice with 12 ml of HBSS. Lastly, add 15 ml of pre-warmed BEGM to the monolayer.

3. Incubate cultures at 37 °C, humidified with 5% CO_2.
4. BEGM growth media should be changed every 2–3 days until the cells reach 80–90% confluence.

3.1.5 Subcultivation of Primary Epithelial Cells on Porous Inserts for Differentiation

To generate differentiated AEC culture, the expanded AECs can be seeded on collagen type IV-coated porous supports once the cells in the collagen-coated flask reach 80–90% confluence.

1. Collect medium and wash the cell monolayer twice with 12 ml of HBSS.
2. Dissociate the cells with 5 ml of trypsin–EDTA solution per T75 cell culture flask and incubate the cells for 5–10 min at 37 °C, in a humidified 5% CO_2 incubator (*see* **Note 14**).
3. Pellet cells in previous collected medium by centrifugation for 5 min at 500 × *g*.
4. Remove supernatant and wash cells once with 25 ml of HBSS.
5. Pellet cells by centrifugation for 5 min at 500 × *g*.
6. Remove supernatant and resuspend the cell pellet in 6 ml of BEGM medium.
7. To generate differentiated AEC cultures, seed cells at a density of 3.3–8.25 × 10^5 cells per cm^2, which is equivalent to 1.0–1.5 × 10^5 cells in 200 µl per 6.5 mm insert. Count cells with trypan blue method and dilute the cells accordingly.
8. Fill the basolateral compartment of the culture plates with 500 µl of BEGM medium and transfer 200 µl of the diluted cell suspension to the upper chamber of the collagen-coated inserts. The cells are now in liquid–liquid interface.
9. Incubate cultures at 37 °C, in a humidified 5% CO_2 incubator.
10. The next day, change the media. Changing the media should be done in the following order: remove the medium in the apical compartment to remove any unattached cells. Wash the apical surface with 200 µl HBSS. Next, remove the old medium from the basolateral side. Apply 500 µl of pre-warmed BEGM medium to the basolateral side. Lastly, apply 200 µl of pre-warmed BEGM medium on the apical side.
11. Change the BEGM medium every 2–3 days until the cells reach confluence on the inserts (*see* **Note 15**).
12. Monitor the resistance of the cells using a Volt/Ohm meter for tissue culture. To measure the resistance, add 200 µl of TEER solution to the apical sides. Once the resistance reaches 500 Ω. cm^2, replace BEGM with ALI medium.
13. Different animal species require a different proportion of several components necessary for differentiation. Adjust the concentration of several additives in the ALI medium as listed in

Table 3. Keep the cells at liquid–liquid interface in ALI medium for at least 2 days.

14. To establish air–liquid interface, aspirate the apical side medium, and wash the surface twice with HBSS. Remove the basolateral medium and add 500 µl of pre-warmed ALI (*see* **Note 16**).
15. To prevent acidification, the basolateral medium should be changed every 2–3 days.
16. The mucus, although protecting the cells, can accumulate over time and then can affect the cells negatively. Therefore, the apical surface should only be washed with HBSS every 7–14 days to maintain the homeostatic balance of the mucus and cells in the ALI. Differentiation of the animal AEC cultures can be assessed by the cilia appearance, mucus production, and the stability of the measured TEER resistance. After 3–4 weeks, depending on the donor, the well-differentiated animal AEC cultures are ready to be used for further experiments.

3.2 Virus Propagation

1. Wash the apical surface of the culture twice with 200 µl of HBSS solution prior to inoculation with the veterinary coronavirus specimen to remove excess mucus.
2. Dilute the clinical material or virus supernatant in HBSS and inoculate 100 µl dropwise to the apical surface and incubate for 2 h at either 33 °C or 37 °C (*see* **Note 17**), in a humidified 5% CO_2 incubator. Optional: centrifuge inoculum solution for 4 min at 1500 × *g* at room temperature to remove cell debris prior to inoculation.
3. Collect the inoculum, transfer it to a container and store at −80 °C for later analysis. Optional: transfer the collected inoculum into an equal volume of VTM.
4. Wash the apical surface three times with 100 µl HBSS. Incubate the infected cultures for the desired amount of time at the appropriate temperature in a humidified 5% CO_2 incubator, e.g., 48 h at 33 °C.
5. Apply 100 µl of HBSS dropwise to the apical surface 10 min prior to the desired collection time and incubate in the humidified 5% CO_2 incubator. After the 10 min incubation, collect the apical wash containing progeny virus and transfer it to a container. Store at −80 °C for later analysis. Optional: transfer the collected progeny virus into an equal volume of VTM.
6. Wash the apical surface once with 200 µl of HBSS solution.
7. To quantify the cellular viral RNA yield, apply a total of 350 µl of lysis buffer to the cells, divided over two steps, and incubate for 10 min at room temperature. Collect the cell lysate and store it at −80 °C if not analyzed directly.

3.3 Immunofluorescence Analysis

All incubation steps are performed at room temperature on a gyro rocker (20–30 rpm), unless stated otherwise.

1. After the apical wash containing progeny virus has been collected, the apical surface needs to be washed twice with 200 µl of HBSS (*see* Subheading 3.2, **step 6**), prior to cell fixation with formalin solution for later immunofluorescence analysis.
2. Apply 200 µl of 4% formalin solution to the apical compartment and 500 µl to the basolateral. Incubate for 15–30 min.
3. Remove the formalin solution and wash both compartments three times with equal volumes of PBS. This material can also be used for histology analysis together with the previously fixed ex vivo tissue (*see* **Note 18**).
4. Transfer the fixed AEC cultures to a new 24-well cluster plate.
5. Discard washing solution and apply 200 µl and 500 µl of confocal buffer (CB) solution to apical and basolateral compartments, respectively.
6. Incubate fixed cultures for 30–60 min to block non-specific binding of antibodies (*see* **Note 19**).
7. Remove the CB solution from the apical and basolateral compartments.
8. From this stage, one should only apply CB solution to the apical compartment.
9. Wash the apical surface once with 200 µl of CB solution for 5 min.
10. Apply primary antibodies (*see* Table 4) diluted in 50 µl CB solution dropwise to the apical surface and incubate for 120 min.
11. Wash the apical surface three times with 100 µl of CB solution for 5 min.
12. Apply the appropriately diluted conjugated secondary antibodies (*see* Table 5) in 50 µl CB solution dropwise to the apical surface and incubate for 60 min. From this step, cover the plates with aluminum foil to prevent bleaching of the fluorophores (*see* **Note 20**).
13. Wash the apical surface twice with 100 µl of CB solution for 5 min.
14. Incubate cells with nucleic acid counter stain solution (DAPI) diluted in 50 µl of CB solution for 5 min.
15. Wash the apical surface once with 100 µl of CB solution for 5 min.
16. Lastly, wash the apical surface twice with 100 µl of PBS for 5 min to remove residual saponin and restore cell membrane integrity.

17. Before removing the washing solution, apply mounting medium on a glass slide (use 1–2 drops). Remove any air bubbles.
18. Excise the membrane from the plastic holder and carefully place the basolateral side of the membrane on top of the mounting medium, without generating air bubbles.
19. Then slowly add one drop of mounting medium on top of each membrane.
20. Slowly place the coverslip, in a tilted fashion, on top of the membrane without generating air bubbles.
21. Allow the mounting medium to cure overnight at RT, after which the slide can be analyzed. At all times protect the slides from direct sunlight exposure, and keep at 4 °C for long-term storage.

4 Notes

1. Dissolve 5 g of BSA, globulin-free, powder in 20 ml PBS in a 50 ml tube (do not vortex). Place the tube on a shaker/roller bank for 2–4 h (max 24 h) at 4 °C, until BSA is completely dissolved. Add the volume up to 34 ml, mix gently by inverting the tube three times. Filter-sterilize the solution through a 0.22 μm PES filter, and store at −20 °C in aliquots of 3.5 ml in 15 ml tubes. Invert the tube three times before usage.
2. Dissolve 43.2 mg zinc sulfate in 50 ml water. Filter-sterilize the solution through a 0.22 μm filter, and store at −20 °C in aliquots of 1100 μl per microfuge tube.
3. Dissolve 42 mg ferrous sulfate heptahydrate, 12.2 g magnesium chloride hexahydrate, and 1.62 g calcium chloride dihydrate in 80 ml water, add 0.5 ml concentrated HCl, and fill up to 100 ml. Filter-sterilize the solution through a 0.22 μm filter, and store at −20 °C in aliquots of 1100 μl per microfuge tube.
4. Prepare seven separate 25 ml stock solutions (*see* Table 6) in water. Filter-sterilize (0.22 μm) each component after preparation. Afterwards, transfer from each separate component an aliquot of 50 μl in 49.6 ml filter-sterilized water (0.22 μm) and add a volume of 50 μl concentrated HCl solution. Mix the solution well through gentle vortexing, filter-sterilize the solution through a 0.22 μm filter, and store at −20 °C in aliquots of 1100 μl per microfuge tube.
5. Y-27632 and A83-01 are small molecules that act as a Rho-kinase and TGF-β pathway inhibitors, respectively. These molecules have been shown to enhance the proliferation of primary cells [12]. Dissolve 10 mg Y-27632 in 1.56 ml filter-

Table 6
Stock solutions for trace elements

Component	Formula	Amount/25 ml	Comment
Selenium	$NaSeO_3$	130.0 mg	Solution stable for 30 days at 4 °C
Silicone	$Na_2SiO_3 \cdot 9H_2O$	3.55 g	
Molybdenum	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	31.0 mg	
Vanadium	NH_4VO_3	14.75 mg	Heat up to >100 °C to dissolve
Nickel	$NiSO_4 \cdot 6H_2O$	6.5 mg	
Tin	$SnCl_2 \cdot 2H_2O$	2.75 mg	
Manganese	$MnCl_2 \cdot 4H_2O$	5 mg	

sterilized water and store at −20 °C in aliquots of 250 μl per microfuge tube. To make the A83-01 stock, dissolve 10 mg A83-01 in 2.37 ml DMSO. Store the stock at −20 °C in aliquots of 100 μl per microfuge tube. DAPT is a Notch pathway inhibitor molecule that has been shown to promote ciliary differentiation of epithelial cells [13]. DAPT stock should also be dissolved in DMSO. Add 2.31 ml DMSO to 5 mg DAPT. Store the DAPT stock at −20 °C in aliquots of 100 μl in dark microfuge tubes.

6. We recommend using EC23, a stable retinoic acid receptor ligand, to replace the commonly used all-trans retinoic acid in the BEGM and ALI medium. Prepare the stock solution by dissolving 25 mg EC23 in 2.5 ml dimethyl sulfoxide (DMSO). Store this stock at −20 °C in aliquots of 100 μl in 1.5 ml tubes. To prepare the 1000× stock, dilute 100 μl of EC23 stock in 60 ml of PBS containing BSA 7.5 mg/ml. The 1000× stock solution should be stored at −20 °C in aliquots of 1 ml per microfuge tube without the need to protect from light.
7. Additional antibiotics are important for the first 5 days after plating to minimize microbial contamination. Thereafter, the media should only be supplemented with P/S as prolonged use of the antibiotic combination can interfere with primary cell differentiation.
8. The concentration of the additives in the ALI medium has been optimized to generate animal AEC cultures with 40–80% of ciliated cells after 4 weeks of differentiation. To define the optimum conditions for other animal species, test several dilutions of EGF, HC, RA, and DAPT in the ALI medium.
9. Isolation of primary tracheal/bronchial cells from large animal such as bovine requires a larger size of dissection kit set.

10. Prepare a 10× stock solution of 5% gelatin by dissolving 5 g gelatin in 100 ml of distilled water. Autoclave the solution for 30 min at 121 °C. Cool down the 10× solution to RT before mixing with other VTM components.
11. Alternatively, autologous commercially available serum can be used although not recommended, due to the presence of potential virus-directed IgGs.
12. One can acquire tracheal/bronchial tissues from other geographical locations. For shipment, the tissue should be kept in isolating/transport medium in a sealed container and packed in an insulated box with cold packs.
13. For the trachea tissue of small animals (e.g., cats), protease/DNAse treatment can be reduced to 24 h.
14. Cells might take longer to dissociate from the bottom of the flask due to the collagen coating. If the cells are not dissociated after 5–10 min, additional rounds of 1 min incubations can be performed until all cells have detached.
15. The seeded primary cells in BEGM should reach confluence on the inserts within 1 week. If this takes longer, the success rate of establishing well-differentiated AEC cultures declines exponentially.
16. If the day after no leakage of medium to the apical side is observed, the cells can be maintained at air–liquid interface. Otherwise, put the cells back to liquid–liquid interface for another day. If at some point the cell layers form holes or a relatively large scratch is accidentally introduced during washing, the cells can be put back at liquid–liquid interface.
17. The temperatures of 33 °C and 37 °C are the incubation conditions of the upper and lower respiratory tract of humans, respectively. However, the incubation temperature can be adapted as some animals have a higher body and respiratory tract temperature.
18. For morphological comparison, vertical sections of both fixed animal ex vivo tissue and AEC culture from the same animal can be stained with hematoxylin and eosin using standards histological techniques. Sectioning 10–20 μm thickness of fixed AEC inserts is recommended to prevent the detachment of the insert membrane from the cells.
19. Formalin-fixed AEC cultures can be kept for 1–3 months at 4 °C if the CB is filter-sterilized (0.2 μm), and all the procedures are performed under sterile conditions. After storage, it is preferential to acclimatize the fixed cultures for 15 min to room temperature on a gyro rocker (20–30 rpm) prior to continuation of the staining protocol.

20. Alternatively, already conjugated primary antibodies for immunofluorescence staining are commercially available. In this case, **steps 12** and **13** can be omitted from the immunofluorescence protocol.

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Chapter 11

Quantification of Coronaviruses by Titration In Vitro and Ex Vivo

Giulia Dowgier and Helena J. Maier

Abstract

Several techniques are currently available to quickly and accurately quantify the number of virus particles in a sample, taking advantage of advanced technologies improving old techniques or generating new ones, generally relying on partial detection methods or structural analysis. Therefore, characterization of virus infectivity in a sample is often essential, and classical virological methods are extremely powerful in providing accurate results even in an old-fashioned way. In this chapter, we describe in detail the techniques routinely used to estimate the number of viable infectious coronavirus particles in a given sample. All these techniques are serial dilution assays, also known as titrations or end-point dilution assays (EPDA).

Key words Titration, $TCID_{50}$, CD_{50}, Plaque-forming units, End-point dilution assay (EPDA)

1 Introduction

With the new-generation technologies progressing at a fast pace, high-throughput techniques are becoming available straight onto the bench, providing fast and accurate methods for routine tests in the lab. Detection of virus particles or selected antigens and their quantification are made possible in a short time in an easy way, thus establishing new laboratory standards [1, 2]. Detection of viral particles based on physical properties is commonly performed using specialized techniques that combine advanced optics and microfluidics, such as flow cytometry [1, 3], light scattering [4], capillary electrophoresis [5], or fluorescence correlation spectroscopy [6], providing fast results with high sensitivity. However, questions regarding the biological properties of viral particles analyzed through physical detection methods or nucleic acid amplification techniques (NAAT) remain a conundrum when identifying infectious particles is key. Combining analysis of physical particles and biochemical properties may answer this question, but this is not widely applicable. Classic virological techniques are therefore still needed to quantify virus infectivity. These techniques exploit the

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_11,

fact that viruses propagate in biological systems, such as cell culture, embryonated eggs, or organ cultures, and replication is generally accompanied by morphological or functional changes dictated by the number of infectious particles [7]. In this chapter we provide protocols for the quantification of coronaviruses using different methods that are applicable depending on the sample, virus, and, inevitably, laboratory capability. The tissue culture infectious dose/$_{50}$ ($TCID_{50}$) or plaque assay titrations are described in detail for coronaviruses that have been adapted to grow in cultures of primary cells or continuous cell lines, providing results respectively in tissue culture infectious dose/$_{50}$ ($TCID_{50}$) or plaque-forming unit (PFU) per volume of sample. Additionally, we describe a titration method for avian infectious bronchitis virus (IBV) adapted from Cook et al. [8], using trachea organ cultures (TOCs). This is applicable for viruses that cause ciliostasis, providing results in ciliostatic dose$_{50}$ (CD_{50}), and representing an alternative to titrations carried out in embryonated eggs (EID_{50}) for those viruses not adapted to cell culture, thus limiting the required number of animals in compliance with the 3R principle.

All these methods provide accurate titrations; however, they are not always applicable for every virus. Although propagation of a virus in cell culture is generally accompanied by changes in cell morphology (referred to as cytopathic effect or CPE), which can be visualized using a microscope, some viruses do not induce CPE. In addition, use of TOCs for titration of respiratory viruses relies on observation and scoring of the cilia beating, as viruses replicating within the epithelia generally cause ciliostasis. However, some virus strains may be poorly ciliostatic; therefore, other techniques need to be used in these cases to quantify the virus.

To assess viral titer, a sample containing virus is diluted tenfold or twofold, depending on the expected virus concentration, and is used to infect tissue cultures or TOCs. Several days later or during the course of the infection, the cytopathic effect or the ciliostasis is recorded. From these data, the virus titer is calculated using the methods described by Spearman and Kaerber [9, 10] or Reed and Muench [11]. Importantly, these calculations apply to mismatched group sizes, as may happen when TOCs are lost due to bacterial infections or aspecific death. The virus titer is defined as the reciprocal of the dilution at which 50% of the inoculated tissue cultures show CPE or at which 50% of the inoculated TOCs show no residual beating of cilia.

Current limitations in adopting cell-based techniques are related to strain specificity in terms of host range and tropism. As many field strains of coronaviruses do not grow in cell culture, this limits the application of some techniques in principle. However, most isolates can be adapted to propagate in vitro in primary cells or continuous cell lines upon serial passage, selecting for the fittest subpopulations, eventually acquiring mutations related to

cell-culture adaptation and as a result altering virus characteristics in vivo [12–14]. Here we describe protocols for titrating IBV by plaque assay or CD_{50} and porcine deltacoronavirus (PDCoV) by $TCID_{50}$. These protocols can easily be adapted for use with different coronaviruses and different cells, depending on the culture conditions required for the virus in use.

2 Materials

2.1 Titration of PDCoV by Tissue Culture Infective Dose ($TCID_{50}$)

1. Ninety-six-well plates containing 80–100% confluent LLC-PK1 cells.
2. Medium −trypsin: EMEM supplemented with 1% HEPES, 1% nonessential amino acids, and 1% antibiotic antimycotic.
3. Medium +trypsin: Medium −trypsin supplemented with 10 μg/ml trypsin (*see* **Note 1**).
4. Microfuge tubes.
5. Multichannel aspirator.
6. Multichannel pipette.
7. 37 °C cell culture incubator with 5% CO_2.
8. Inverted microscope.

2.2 Titration of IBV by Plaque-Forming Units

1. Six-well or twelve-well plates containing 70–90% confluent CK cells.
2. 1× BES medium: EMEM, 10% w/v tryptose phosphate broth, 0.2% w/v bovine serum albumin (BSA), 20 mM N,N bis (2-hydroxethyl)-2-aminoethanesulfonic acid (BES), 0.4% w/v sodium bicarbonate, 2 mM L-glutamine, 250 U/ml nystatin, 100 U/ml penicillin, 100 μg/ml streptomycin.
3. 2× BES medium: 2× EMEM, 20% w/v tryptose phosphate broth, 0.4% w/v BSA, 40 mM BES, 0.8% w/v sodium bicarbonate, 4 mM L-glutamine, 500 U/ml nystatin, 200 U/ml penicillin, 200 μg/ml streptomycin.
4. Sterile phosphate-buffered saline (PBS).
5. 2% w/v agarose in water (autoclaved).
6. 10% w/v formaldehyde in PBS.
7. 0.1% w/v crystal violet in water.
8. 37 °C cell culture incubator with 5% CO_2.
9. Microwave.
10. Water bath.
11. Small spatula.

2.3 Titration of IBV by Ciliostatic Dose (CD50) in Tracheal Organ Cultures (TOCs)

1. Tissue culture roller drum capable of rolling at approximately 8 revolutions/hour at 37 °C.
2. Associated rack suitable for holding 16 mm tubes on roller drum.
3. Sterile, extra-strong rimless soda glass tubes 150 mm long, 16 mm outside diameter, suitable for bacteriological work.
4. Sterile silicone rubber bungs 16 mm diameter at wide end, 13 mm diameter at narrow end, and 24 mm in length.
5. Inverted microscope.
6. TOCs culture medium: MEM, 40 mM HEPES buffer, 250 U/ml penicillin, and 250 U/ml streptomycin.
7. Sterile PBS.

3 Methods

3.1 Titration of PDCoV by Tissue Culture Infective Dose ($TCID_{50}$)

1. Seed LLC-PK1 cells into 96-well plates 1 or 2 days before the titration. At the time of titration, the monolayer should be nearly confluent. Each virus to be titrated requires ½ a 96-well plate (*see* **Note 2**).
2. Prepare two- or tenfold serial dilutions of the samples in medium −trypsin (*see* **Note 3**).
3. Aspirate the medium from the 96-well plate.
4. Add 100 μl of medium −trypsin to the wells in row H including five replicates. These provide a negative control.
5. Add 100 μl of diluted virus including five replicates with the most dilute in row G and the most concentrated in row A (Fig. 1).
6. Incubate the plate at 37 °C for 1 h.
7. After 1 h, add an additional 100 μl of medium +trypsin.
8. Incubate the plate at 37 °C for 5 days.
9. Quantify wells as positive or negative for CPE.
10. Calculate the viral titer using the Reed and Muench equation, as follows:

    (Log dilution above 50%) + (proportionate distance × log dilution factor) = log ID_{50}

    where the proportionate distance is calculated as follows:

$$\text{Proportionate distance} = \frac{(\%\text{positive above } 50) - 50\%}{(\text{positive above } 50\% - \text{positive below } 50\%)}$$

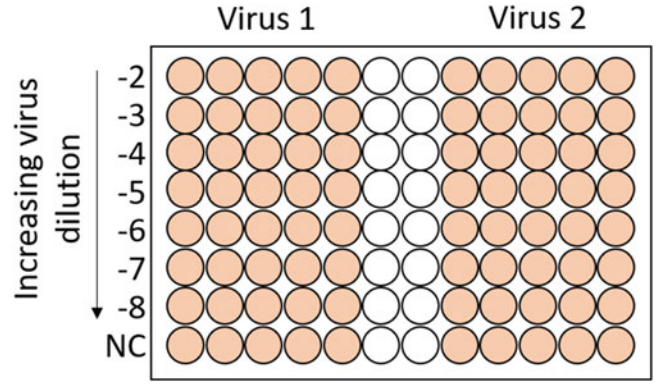

Fig. 1 Layout of sample dilutions in the 96-well titration plate. *NC* negative control

3.2 Titration of IBV by Plaque-Forming Units

3.2.1 Infection of Cells

1. Seed CK cells into 6- or 12-well plates 3 days prior to titration. When performing the titration, the monolayer should be 70–90% confluent.
2. Prepare tenfold or twofold serial dilutions of virus in 1× BES.
3. Remove media from cells and wash once with sterile PBS.
4. Remove PBS from the cells and add 500 or 250 μl of diluted virus per well for 6- or 12-well plate, respectively. Duplicate wells should be inoculated for each dilution in a six-well plate or triplicate wells in a 12-well plate.
5. Incubate cells at 37 °C for 1–2 h to allow virus attachment.
6. Melt 2% agar in a microwave and then transfer to a 42 °C water bath. Allow the agar to equilibrate in temperature.
7. Mix the partially cooled agar with 2× BES pre-warmed to 37 °C to generate 1× BES + 1% agar. Keep at 42 °C until needed to prevent premature setting (*see* **Note 4**).
8. Remove virus inoculum and overlay cells with 2.5 or 2 ml of the 1× BES/agar mix for the 6- or the 12-well plates, respectively.
9. Leave cells at room temperature for approximately 5 min until agar has solidified.
10. Incubate at 37 °C and 5% CO_2 for 3 days to allow plaques to develop.

3.2.2 Staining Cells and Determining Titer

1. After 3 days, overlay agar with 1 ml of 10% formaldehyde in PBS per well.
2. Incubate at room temperature for 1 h.
3. Remove formaldehyde and ensure disposal according to local regulations.
4. Using a small spatula, flick off the agar from the cells (*see* **Note 5**).

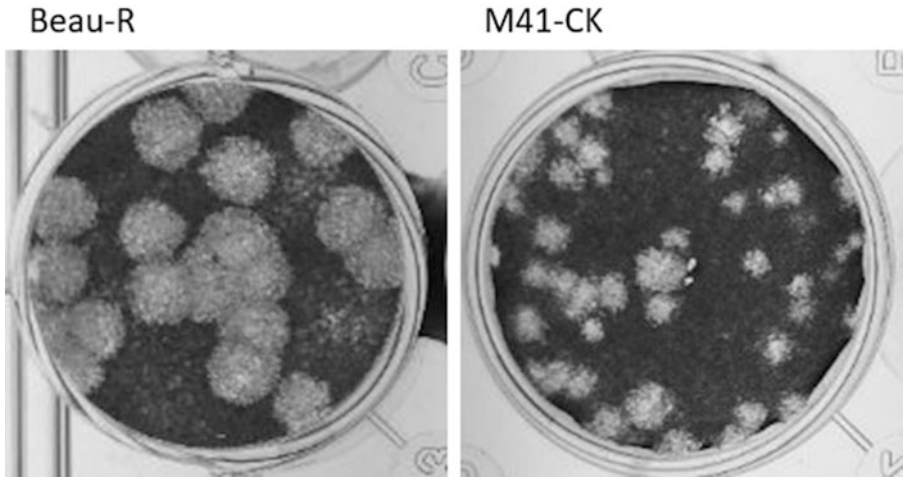

Fig. 2 Example plaques generated by IBV strains. CK cells were infected with Beau-R or M41-CK strains of IBV and incubated at 37 °C for 3 days. Cells were then fixed and stained with 0.1% crystal violet

5. Wash cells by shaking the plate upside down in a sink full of water.
6. Add 0.5 ml of 0.1% crystal violet to each well or the minimum volume to just cover each well.
7. Incubate at room temperature for 10 min.
8. Remove crystal violet and dispose of according to local regulations.
9. Wash the plate by shaking upside down in a sink of water.
10. Pat plate dry and leave upside down at room temperature to fully dry.
11. Plaques should be clearly visible as holes in the monolayer varying in size and morphology based on the IBV strain (Fig. 2). Count the number of plaques per well at the dilution with clearly defined, individual (not over-lapping) plaques (typically 10–50 plaques/well). Ensure duplicate wells are counted and an average taken.
12. Determine titer using the following equation:

$$\text{Titer (PFU/ml)} = \frac{\text{average number of plaques}}{\text{dilution factor} \cdot \text{inoculum volume (ml)}}$$

13. For the most accurate results, the plaque assay should be repeated three times and the average titer determined.

3.3 Titration of IBV by Ciliostatic Dose Method

3.3.1 Screening and Selection of TOCs

1. After 2 days recovery after preparation, TOCs are screened individually for vitality and any tubes in which less than 60% of the luminal surface has clearly visible ciliary activity are discarded.
2. The selected tubes are used for the titration, calculating a number of five tubes per dilution per virus and an additional group of mock infected TOCs as negative control. At least one virus of a known titer should always be included as positive control.

3.3.2 Infecting TOCs

1. Prepare tenfold serial dilutions of virus in TOC culture medium.
2. Remove media from the tubes and wash once with sterile PBS.
3. Remove PBS from the tubes and add 500 μl of diluted virus to each tube selected for that dilution (*see* **Note 6**).
4. Incubate the tubes at 37 °C for 6 days before assessing the titer by scoring the ciliary activity under the light microscope. TOCs are scored positive for infection when cilia activity is completely abrogated with a tolerance of 5% cilia still beating, whereas negative when residual activity is recorded up to 95% (*see* **Note 7**).
5. Determine the titers using the Reed and Muench calculations looking at the log dilutions and TOCs scores as follows:

   (Log dilution above 50%) + (proportionate distance × log dilution factor) = log ID_{50}

   where the proportionate distance is calculated as follows:

$$\text{Proportionate distance} = \frac{(\%\text{positive above } 50) - 50\%}{(\text{positive above } 50\% - \text{positive below } 50\%)}$$

   The log ID_{50} represents the end-point dilution at which the 50% of the TOC score positive. The dilution factor is finally applied accordingly to what applied, generating the final log CD_{50}/ml.

6. For most accurate results, the CD_{50} titration should be repeated three times and the average titer determined.

4 Notes

1. Trypsin/EDTA used for dissociating cells for passage can be used.
2. Titrations are performed using five replicate wells over eight rows (seven virus dilutions plus mock), requiring ½ and 96-well plate. If additional virus dilutions or additional replicate wells are preferred, plate cells accordingly.

3. If the likely titer of the virus is not known, use tenfold serial dilutions to identify the best range. If required, subsequently using twofold serial dilutions can provide a more accurate titer.
4. Alternative methods also exist for mixing media and agar. If there is concern regarding the overlay setting too quickly or risk of contamination from the water bath, hot agar can be mixed directly with cold media (4 °C). Once the mixture feels warm to the touch, rather than hot, it can be added to cells.
5. The simplest method for removing agar from the cells is to hold the plate upside down with the lid removed. The small spatula is inserted between the agar and the wall of the well. Once the base of the well is reached, a small amount of pressure is applied to remove the agar, being careful not to scrape off the cells. The whole agar plug should then fall out easily.
6. A quick and easy way to speed up the washing step during TOC titration is to add a few milliliters of PBS without removing the media in the tube, then with a rapid and confident rotation of the hand, pour the media/PBS mix onto a stack of tissues being careful not to lose the TOC ring. Finally, remove the excess PBS by aspiration or using a pipette. This step speeds up the procedure taking into account that many tubes, often more than 100, may need to be processed.
7. Assessing cilia activity during TOC titration may seem a subjective interpretation; however, the main effect on the TOC lumen is in reality quite striking at 6 days postinfection for viruses that are ciliostatic, usually leaving no doubt about the results. However, if a virus is poorly ciliostatic, this test should not be the first choice for quantification unless virus is detected by other techniques, such as antibody-based assays or probe-based techniques.

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Part III

Manipulating the Genomes of Coronaviruses

Chapter 12

Transient Dominant Selection for the Modification and Generation of Recombinant Infectious Bronchitis Coronaviruses

Sarah Keep, Paul Britton, and Erica Bickerton

Abstract

We have developed a reverse genetics system for the avian coronavirus *infectious bronchitis virus* (IBV) in which a full-length cDNA corresponding to the IBV genome is inserted into the vaccinia virus genome under the control of a T7 promoter sequence. Vaccinia virus as a vector for the full-length IBV cDNA has the advantage that modifications can be introduced into the IBV cDNA using homologous recombination, a method frequently used to insert and delete sequences from the vaccinia virus genome. Here, we describe the use of transient dominant selection as a method for introducing modifications into the IBV cDNA that has been successfully used for the substitution of specific nucleotides, deletion of genomic regions, and the exchange of complete genes. Infectious recombinant IBVs are generated in situ following the transfection of vaccinia virus DNA, containing the modified IBV cDNA, into cells infected with a recombinant fowlpox virus expressing T7 DNA-dependent RNA polymerase.

Key words Transient dominant selection (TDS), Vaccinia virus, Infectious bronchitis virus (IBV), Coronavirus, Avian, Reverse genetics, Nidovirus, Fowlpox virus, T7 RNA polymerase

1 Introduction

Avian infectious bronchitis virus (IBV) is a gamma-coronavirus that is the etiological agent of infectious bronchitis (IB); an acute and high contagious disease of poultry. Coronaviruses are enveloped viruses which replicate in the cell cytoplasm. Coronavirus genomes consist of single-stranded positive-sense RNA and are the largest of all the RNA viruses ranging from approximately 27 kb to 32 kb; the genome of IBV is 27.6 kb. Molecular analysis of the role of individual genes in the pathogenesis of RNA viruses has been advanced by the availability of full-length cDNAs, for the generation of infectious RNA transcripts that can replicate and result in infectious viruses. The assembly of full-length coronavirus cDNAs was hampered due to regions from the replicase gene being unstable in bacteria. We therefore devised a reverse genetics strategy for IBV

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_12,

involving the insertion of a full-length cDNA copy of the IBV genome, under the control of a T7 RNA promoter, into the vaccinia virus genome in place of the thymidine kinase (TK) gene. This was followed by the in situ recovery of infectious IBV in cells both transfected with vaccinia virus DNA and infected with a recombinant fowlpox virus expressing T7 RNA polymerase [1].

One of the main advantages of using vaccinia virus as a vector for IBV cDNA is its ability to accept large quantities of foreign DNA without loss of integrity and stability [2]. A second and equally important advantage is the ability to modify the IBV cDNA within the vaccinia virus vector through transient dominant selection (TDS), a method taking advantage of recombinant events between homologous sequences [3, 4]. The TDS method relies on a three-step procedure. In the first step, the modified IBV cDNA is inserted into a plasmid containing a selective marker under the control of a vaccinia virus promoter. In our case, we use a plasmid, pGPTNEB193 (Fig. 1; [5]), which contains a dominant selective marker gene, *Escherichia coli guanine phosphoribosyltransferase* (*Ecogpt*; [6]), under the control of the vaccinia virus P7.5K early/late promoter.

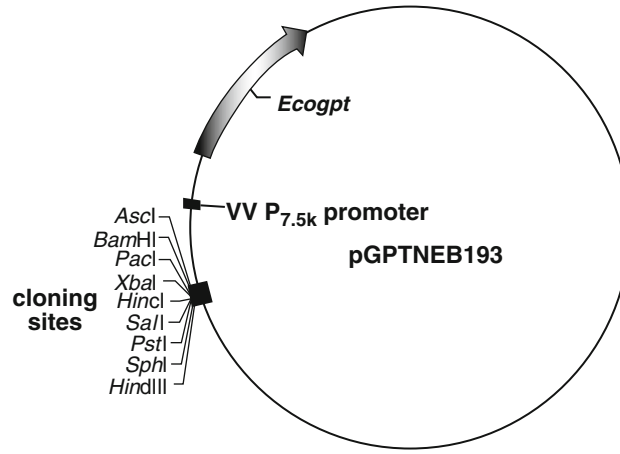

Fig. 1 Schematic diagram of the recombination vector for insertion of genes into a vaccinia virus genome using TDS. Plasmid pGPTNEB193 contains the *Ecogpt* selection gene under the control of the vaccinia virus early/late $P_{7.5K}$ promoter, a multiple cloning region for the insertion of the sequence to be incorporated into the vaccinia virus genome and the *bla* gene (not shown) for ampicillin selection of the plasmid in *E. coli*. For the modification of the IBV genome, a sequence corresponding to the region being modified, plus flanking regions of 500–800 nucleotides for recombination purposes is inserted into the multiple cloning sites using an appropriate restriction endonuclease. The plasmid is purified from *E. coli* and transfected into Vero cells previously infected with a recombinant vaccinia virus containing a full-length cDNA copy of the IBV genome

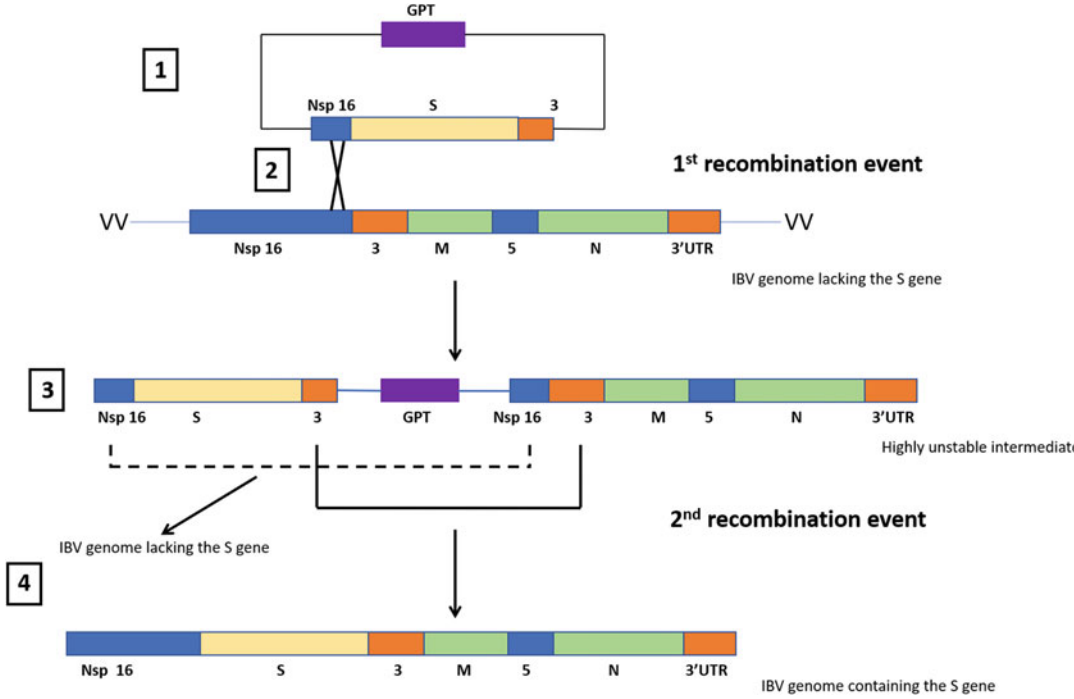

Fig. 2 The incorporation of the IBV S gene using transient dominant selection. Transient dominant selection is used to modify the cDNA copy of the IBV genome encoded by an rVV [1]. To modify the IBV genome, a plasmid is required that contains a GPT gene as well as the desired IBV-derived sequence. For the insertion of the S gene into an IBV genome missing the S gene, the plasmid contains the S gene flanked by neighboring sequence in nsp 16 and gene 3 [2]. Recombination occurs between the homologous IBV sequences in the plasmid vector and the IBV cDNA, resulting in the complete plasmid sequence inserting into the rVV genome, thereby creating, due to the presence of duplicate sequences, a highly unstable intermediate [3]. The presence of the GPT gene allows for the selection and isolation of this highly unstable intermediate through plaque purification in the presence of selection agents MPA, xanthine and hypoxanthine [4]. Once the selective pressure is removed, a second homologous recombination event occurs that either results in the generation of an rVV containing the original unmodified IBV sequence or the generation of an IBV cDNA containing the desired modification; in this example the insertion of the S gene

In the second step, this complete plasmid sequence is integrated into the IBV sequence within the vaccinia virus genome (Fig. 2). This occurs as a result of a single cross-over event involving homologous recombination between the IBV cDNA in the plasmid and the IBV cDNA sequence in the vaccinia virus genome. The resulting recombinant vaccinia viruses (rVV) are highly unstable due to the presence of duplicate sequences and are only maintained by the selective pressure of the *Ecogpt* gene, which confers resistance to mycophenolic acid (MPA) in the presence of xanthine and hypoxanthine [3]. In the third step, the MPA-resistant rVVs are grown in the absence of MPA selection, resulting in the loss of the *Ecogpt* gene due to a second single homologous recombination event between the duplicated sequences (Fig. 2). During this

third step two recombination events can occur; one event will result in the generation of the original (unmodified) IBV sequence and the other in the generation of an IBV cDNA containing the desired modification (i.e., the modification within the plasmid sequence). In theory these two events will occur at equal frequency; however, in practice this is not necessarily the case.

To recover infectious rIBVs from the rVV vector, rVV DNA is transfected into primary chick kidney (CK) cells previously infected with a recombinant fowlpox virus expressing T7 RNA polymerase (rFPV-T7; [7]). In addition, a plasmid, pCi-Nuc [1, 8], expressing the IBV nucleoprotein (N), under the control of both the cytomegalovirus (CMV) RNA polymerase II promoter and the T7 RNA promoter, is co-transfected into the CK cells. Expression of T7 RNA polymerase in the presence of the IBV N protein and the rVV DNA, containing the full-length IBV cDNA under the control of a T7 promoter, results in the generation of infectious IBV RNA, which in turn results in the production of infectious rIBVs (Fig. 3).

The overall procedure is a multistep process that can be divided into two parts: the generation of an rVV containing the modified IBV cDNA (Fig. 4) and the recovery of infectious rIBV from the rVV vector (Fig. 4). The generation of the *Ecogpt* plasmids, based on pGPTNEB193, containing the modified IBV cDNA, is by standard *E. coli* cloning methods [9, 10] and will not be described here. General methods for growing vaccinia virus have been published by Mackett et al. [11] and for using the TDS method for modifying the vaccinia virus genome by Smith [12].

2 Materials

2.1 Homologous Recombination and Transient Dominant Selection in Vero Cells

1. Vero cells.
2. Phosphate-buffered saline (PBS)a: 172 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, adjusted to pH 7.2 with HCl.
3. Eagle's minimum essential medium (E-MEM) with Earle's salts supplemented with 2 mM L-glutamine.
4. CK cell culture medium or otherwise commonly referred to as BES cell culture medium: 1× E-MEM, 10% tryptose phosphate broth (TPB), 0.2% bovine serum albumin (BSA), 20 mM N, N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES), 0.21% sodium bicarbonate, 2 mM L-glutamine, 50 U/ml nystatin, 10 U/ml penicillin and 10 μg/ml streptomycin.
5. Reduced serum medium for transfection, e.g., OPTIMEM 1 with GLUTAMAX-1.
6. Transfection reagent, e.g., lipofectin.

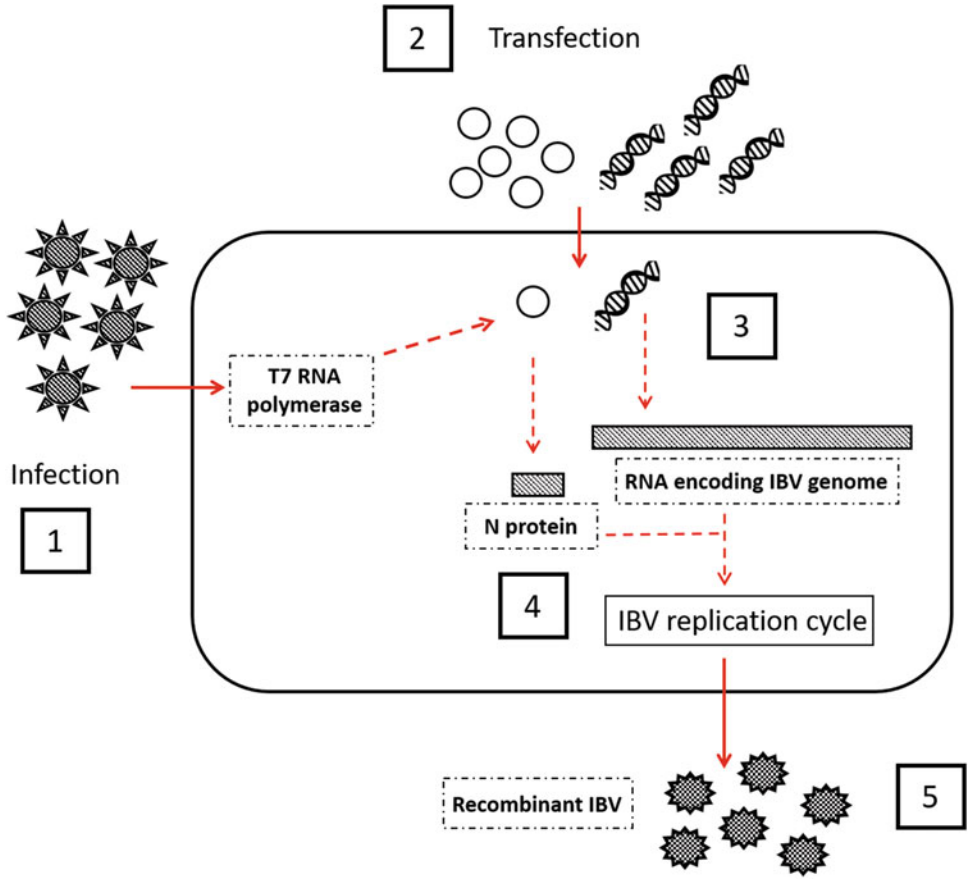

Fig. 3 Schematic detailing of the recovery of infectious rIBV in CK cells [1]. Primary CK cells are infected with a recombinant fowlpox virus which expresses a T7 RNA polymerase (rFPV-T7) [2]. Cells are subsequently co-transfected with recombinant vaccinia DNA encoding the desired IBV cDNA under the control of a T7 RNA promotor, and a plasmid (pCi-Nuc) expressing the IBV N protein under the control of both a cytomegalovirus (CMV) RNA polymerase II promoter and a T7 RNA promoter [3]. The rFPV-T7 derived RNA polymerase generates both the N protein from pCi-Nuc, and infectious IBV RNA from the rVV DNA. This in turn [4] initiates an IBV replication cycle resulting [5] in the generation of recombinant IBV (rIBV). This rIBV is amplified through passaging in either CK cells or embryonated hen's eggs

7. 10 mg/ml mycophenolic acid (MPA) in 0.1 M NaOH (30 mM); 400× concentrated.
8. 10 mg/ml xanthine in 0.1 M NaOH (66 mM); 40× concentrated. Heat at 37 °C to dissolve.
9. 10 mg/ml hypoxanthine in 0.1 M NaOH (73 mM); 667× concentrated.
10. Screw-top 1.5 ml microfuge tubes with gasket.
11. Cup form sonicator.

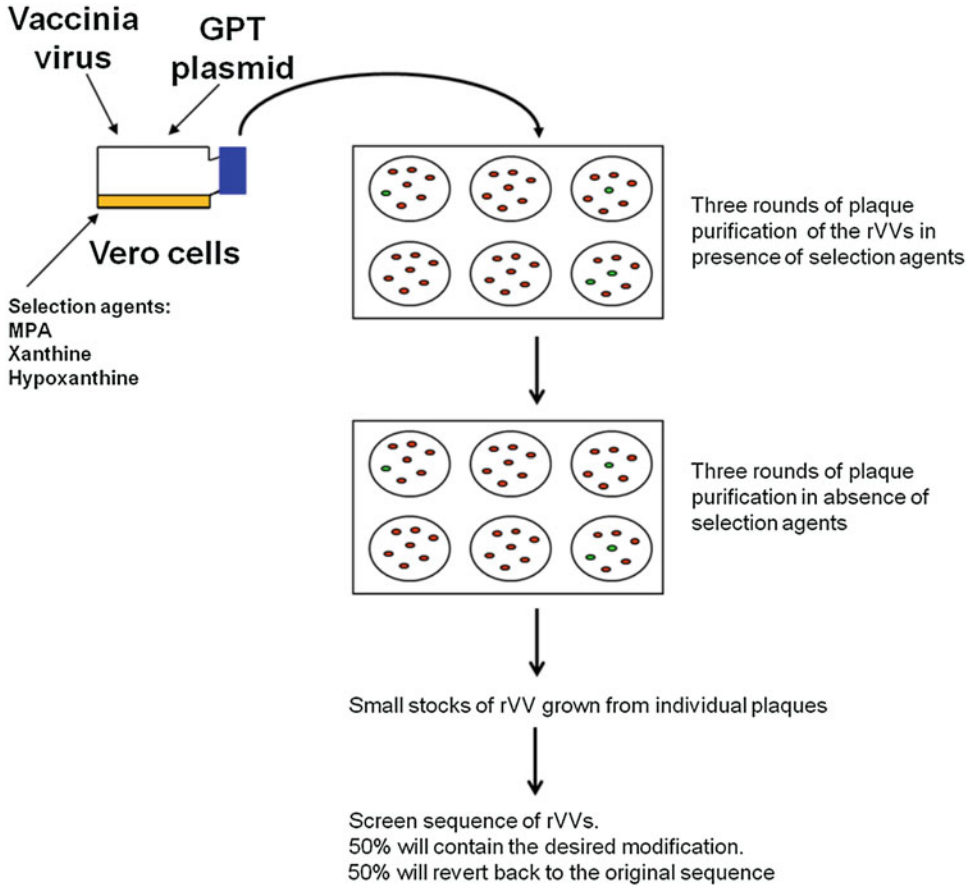

Fig. 4 Schematic detailing of the multi-step process of constructing a recombinant Vaccinia virus. Vero cells are infected with rVV containing IBV cDNA and then transfected with a plasmid containing the IBV sequence to be inserted and the selective marker gene GPT. Selection agents, MPA, xanthine and hypoxanthine, are added to the culture. Homologous recombination occurs, and the complete plasmid sequence is inserted into the rVV. The GPT gene allows positive selection of these rVV as it confers resistance to MPA in the presence of xanthine and hypoxanthine. The viruses are plaque purified three times in the presence of selection agents ensuring no wild-type VV is present. The removal of the selection agents results in a second recombination event with the loss of the GPT gene. Plaque purification in the absence of selection agents three times not only ensures the loss of the GPT gene but also ensures the maintenance of a single virus population. Small stocks of rVV are grown from individual plaques which are screened through PCR for the desired modification; this is found in theoretically 50% of rVVs

12. 2× E-MEM: 2× E-MEM, 10% fetal calf serum, 0.35% sodium bicarbonate, 4 mM L-glutamine, 50 U/ml nystatin, 20 U/ml penicillin and 20 μg/ml streptomycin.
13. 2% agar.
14. *Ecogpt* selection medium: E-MEM, 75 μM MPA, 1.65 mM xanthine, 109 μM hypoxanthine, 1% agar.
15. 1% Neutral red solution.

2.2 Extraction of DNA from Recombinant Vaccinia Virus

1. 20 mg/ml proteinase K.
2. 2× proteinase K buffer: 200 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.4% SDS, 400 mM NaCl.
3. Phenol/chloroform/isoamyl alcohol (25:24:1).
4. Chloroform.
5. Absolute ethanol.
6. 70% ethanol.
7. DNA extraction kit, e.g., QlAamp DNA mini kit.
8. 3 M Sodium acetate.
9. Cold (−20 °C) absolute ethanol and 70% ethanol.
10. Bench top centrifuge.
11. Spectrophotometer, e.g., nanodrop.

2.3 Production of Large Stocks of Vaccinia Virus

1. BHK-21 maintenance medium: Glasgow-modified Eagle's medium (G-MEM), 2 mM L-glutamine, 0.96% fetal calf serum, 9.68% TPB, 44 U/ml nystatin, 8.8 U/ml penicillin and 8.8 μ/ml streptomycin.
2. TE buffer: 10 mM Tris–HCl pH 9, 1 mM EDTA.
3. BHK-21 cells.
4. 50 ml tubes.

2.4 Vaccinia Virus Partial Purification

1. 30% sucrose (w/v) in 1 mM Tris–HCl pH 9, filtered through 0.22 μm filter.
2. Ultracentrifuge and rotor, e.g., Superspin 630 rotor and Sorvall OTD65B.
3. Ultracentrifuge tubes.

2.5 Analysis of Vaccinia Virus DNA by Pulse Field Agarose Gel Electrophoresis (PFGE)

1. 10× TBE buffer: 1 M Tris, 0.9 M boric acid pH 8, and 10 mM EDTA.
2. Pulsed field-certified ultrapure DNA-grade agarose.
3. DNA markers, e.g., 8–48 kb markers, Biorad.
4. 70% ethanol.
5. 0.5 mg/ml ethidium bromide.
6. Pulsed field gel electrophoresis (PFGE) equipment, e.g., CHEF-DR® II pulsed field gel electrophoresis apparatus.
7. 6× sample loading buffer: 62.5% glycerol, 62.5 mM Tris–HCl pH 8, 125 mM EDTA and 0.06% bromophenol blue.

2.6 Preparation of rFPV-T7 Stock Virus

1. CEF growth medium: 1× 199 medium with Earle's salts, 8.29% TPB, 8.29% FCS, 16.6 U/ml penicillin, 16.6 μg/ml streptomycin, and 41.4 U/ml nystatin.

2. CEF maintenance medium for FPV infections: 1× 199 medium with Earle's salts, 1% TPB, 2% new-born calf serum, 2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, and 50 U/ml nystatin. For serum-free media, new-born calf serum is removed, and the volume replaced with sterile water.
3. Chicken embryo fibroblast (CEF) cells.

2.7 Recovery of rIBV and Serial Passage on CK Cells

1. Chick kidney (CK) cells.
2. Stock of rFPV-T7 virus.
3. The rVV DNA prepared from large partially purified stocks of rVV.
4. Plasmid pCi-Nuc which contains IBV nucleoprotein under the control of the CMV and T7 promoters (*see* **Note 1**).
5. 0.22 μm syringe-driven filters.
6. 5 ml syringes.

3 Methods

3.1 Infection/ Transfection of Vero Cells with Vaccinia Virus

1. Freeze-thaw the vaccinia virus containing the full-length IBV cDNA genome to be modified three times (37 °C/dry ice) and sonicate for 2 min using a cup form sonicator, continuous pulse at 70% duty cycle, seven output control (*see* **Notes 2–5**).
2. Infect six-well plates of 40% confluent monolayers of Vero cells with the rVV at an MOI of 0.2. Use two independent wells per recombination (*see* **Notes 2–5**).
3. Incubate at 37 °C 5% CO_2 for 2 h to allow the virus to infect the cells.
4. After 1 h of incubation, prepare the following solutions for transfection:
   Solution A: For each transfection, dilute 5 μg of modified pGPTNEB193 (containing the modified IBV cDNA) in 1.5 ml of OPTIMEM medium.
   Solution B: Dilute 12 μl of lipofectin in 1.5 ml of OPTIMEM for each transfection.
5. Incubate solutions A and B separately for 30 min at room temperature, then mix the two solutions together and incubate the mixture at room temperature for 15 min.
6. During the 15 min incubation, remove the inoculum from the vaccinia virus-infected cells and wash the cells twice with OPTIMEM.
7. Add 3 ml of the transfection mixture (prepared in **step 5**) to each well.
8. Incubate for 60–90 min at 37 °C, 5% CO_2 (*see* **Note 6**).

9. Remove the transfection mixture from each well and replace it with 5 ml of BES medium.
10. Incubate the transfected cells overnight at 37 °C, 5% CO_2.
11. The following morning add the MXH selection components, MPA 12.5 μl, xanthine 125 μl, and hypoxanthine 7.4 μl, directly to each well (*see* **Note 7**).
12. Incubate the cells at 37 °C, 5% CO_2 until they display extensive vaccinia virus-induced CPE (normally 2 days).
13. Harvest the infected/transfected cells into the cell medium of the wells and centrifuge for 3–4 min at 300 × *g*. Discard supernatant and resuspend the pellet in 400 μl 1× E-MEM and store at −20 °C.

3.2 Plaque Purification in the Presence of GPT Selection Agents: Selection of MPA-Resistant Recombinant Vaccinia Viruses (GPT⁺ Phenotype)

1. Freeze-thaw the vaccinia virus produced from Subheading 3.1 three times and sonicate as described in Subheading 3.1, **step 1**.
2. Remove the medium from confluent Vero cells in six-well plates and wash the cells once with PBSa.
3. Prepare 10^{-1} to 10^{-3} dilutions of the recombinant vaccinia virus in 1× E-MEM.
4. Remove the PBSa from the Vero cells and add 500 μl of the diluted virus per well.
5. Incubate for 1–2 h at 37 °C, 5% CO_2.
6. Remove the inoculum and add 3 ml of the *Ecogpt* selection medium (*see* **Note 8**).
7. Incubate for 3–4 days at 37 °C, 5% CO_2, and stain the cells by adding 2 ml of 1× E-MEM containing 1% agarose and 0.01% neutral red.
8. Incubate the cells at 37 °C, 5% CO_2 for 6–24 h, and pick 2–3 well-isolated plaques for each recombinant, by taking a plug of agarose directly above the plaque. Place the plug of agarose in 400 μl of 1× E-MEM.
9. Perform two further rounds of plaque purification for each selected recombinant vaccinia virus in the presence of selection medium, as described in **steps 1–8** (*see* **Note 9**).

3.3 Plaque Purification in the Absence of GPT Selection Agents: Selection of MPA Sensitive Recombinant Vaccinia Viruses (Loss of GPT⁺ Phenotype)

1. Take the MPA-resistant plaque-purified rVVs which have been plaque purified a total of three times as described in Subheading 3.2 and freeze-thaw and sonicate as described in Subheading 3.1, **step 1**.
2. Remove the medium from confluent Vero cells in six-well plates and wash the cells with PBSa.
3. Prepare 10^{-1} to 10^{-3} dilutions of the recombinant vaccinia virus in 1× E-MEM.

4. Remove the PBSa from the Vero cells and add 500 µl of the diluted virus per well.
5. Incubate for 1–2 h at 37 °C, 5% CO_2.
6. Remove the inoculum and add 3 ml of the overlay medium (*see* **Note 10**).
7. Incubate for 3–4 days at 37 °C, 5% CO_2 and stain the cells by adding 2 ml 1× E-MEM containing 1% agarose and 0.01% neutral red.
8. Incubate the cells at 37 °C, 5% CO_2 for 6–24 h and pick 3–6 well-isolated plaques for each recombinant, by taking a plug of agarose directly above the plaque. Place the plug of agarose in 400 µl of 1× E-MEM (*see* **Note 9**).
9. Perform two further rounds of plaque purification for each selected recombinant vaccinia virus in the presence of selection medium, as described in **steps 1–8**.

3.4 Production of Small Stocks of Recombinant Vaccinia Viruses

1. Take the MPA-sensitive plaque-purified rVVs which have been plaque purified a total of three times as described in Subheading 3.3 and freeze-thaw and sonicate as described in Subheading 3.1, **step 1**.
2. Remove the medium from confluent Vero cells in six-well plates and wash the cells with PBSa.
3. Dilute 150 µl of the sonicated rVVs in 350 µl of BES cell culture medium.
4. Remove the PBSa from the Vero cells and add 500 µl of the diluted rVVs per well.
5. Incubate at 37 °C, 5% CO_2 for 1–2 h.
6. Add 2.5 ml per well of BES cell culture medium.
7. Incubate the infected Vero cells at 37 °C, 5% CO_2 until the cells show signs of extensive vaccinia virus-induced CPE (approximately 4 days).
8. Scrape the Vero cells into the medium and harvest into 1.5 ml screw cap tubes with gaskets.
9. Centrifuge for 3 min at 16,000 × *g* in a bench top centrifuge.
10. Discard the supernatants and resuspend the cells in a total of 400 µl of BES cell culture medium and store at −20 °C.

3.5 DNA Extraction from Small Stocks of Recombinant Vaccinia Virus for Screening by PCR

There are two methods for DNA extraction:

3.5.1 DNA Extraction Using Phenol/Chloroform/ Isoamyl Alcohol

1. To 100 μl of rVV stock produced in Subheading 3.4, add 100 μl 2× proteinase K buffer and 2 μl of the proteinase K stock. Gently mix and incubate at 50 °C for 2 h.
2. Add 200 μl of phenol/chloroform/isoamyl alcohol to the proteinase K-treated samples and mix by inverting the tube 5–10 times and centrifuge at 16,000 × *g* for 5 min (*see* **Note 11**).
3. Take the upper aqueous phase and repeat **step 2** twice more.
4. Add 200 μl of chloroform to the upper phase and mix and centrifuge as in **step 2**.
5. Take the upper phase and precipitate the vaccinia virus DNA by adding 2.5 volumes of absolute ethanol; the precipitated DNA should be visible. Centrifuge the precipitated DNA at 16,000 × *g* for 20 min. Discard the supernatant.
6. Wash the pelleted DNA with 400 μl 70% ethanol and centrifuge at 16,000 × *g* for 10 min. Discard the supernatant carefully and remove the last drops of 70% ethanol using a capillary tip.
7. Air dry the pelleted DNA for 5–10 min.
8. Resuspend the DNA in 30 μl of water and store at 4 °C (*see* **Note 12**).

3.5.2 Extraction of rVV DNA Using the Qiagen QIAamp DNA Mini Kit

1. Follow the blood/bodily fluid spin protocol and start with 200 μl of rVV stock produced in Subheading 3.4.
2. Elute the rVV DNA in 200 μl buffer AE (provided in the kit) and store at 4 °C.

At this stage, the extracted rVV DNA is analyzed by PCR and/or sequence analysis for the presence/absence of the *Ecogpt* gene and for the modifications within the IBV cDNA sequence. Once an rVV is identified that has both lost the *Ecogpt* gene and also contains the desired IBV modification, large stocks are produced. Typically, two rVVs will be taken forward at this stage, which ideally have been generated from different wells of the infection/transfection of Vero cells stage previously described in Subheading 3.1. Once the large stocks of the chosen rVVs have been produced, rVV DNA will be extracted and prepared for the recovery of rIBV.

3.6 Production of Large Stocks of Vaccinia Virus

1. Freeze-thaw and sonicate the chosen rVV stocks from Subheading 3.4 as described in Subheading 3.1, **step 1**.
2. Add G-MEM to the sonicated virus and infect 11 × T150 flasks of confluent monolayers of BHK-21 cells using 2 ml of the diluted vaccinia virus per flask at an MOI of 0.1–1.
3. Incubate the infected cells for 1 h at 37 °C, 5% CO_2.

4. Add 18 ml of pre-warmed (37 °C) G-MEM and incubate the infected cells at 37 °C, 5% CO_2 until the cells show an advanced CPE (normally about 2–3 days postinfection). At this stage, the cells should easily detach from the plastic.
5. Either continue to **step 6** or freeze the flasks in Tupperware boxes lined with absorbent material and labeled with biohazard tape at −20 °C until further use.
6. If prepared from frozen, the flasks need to be defrosted by leaving them at room temperature for 15 min and then at 37 °C until the medium over the cells has thawed.
7. Tap the flasks to detach the cells from the plastic, if necessary, use a cell scraper.
8. Transfer the medium containing the cells to 50 ml tubes and centrifuge at 750 × *g* for 15 min at 4 °C to pellet the cells.
9. Discard the supernatant (99% of vaccinia virus is cell-associated) and resuspend the cells in 1 ml of TE buffer per flask.
10. Pool the resuspended cells, then aliquot into screw top microfuge tubes with gasket and store at −70 °C.
11. Use one 1 ml aliquot of the resuspended cells as a virus stock. Use the resuspended cells from the remaining 10 flasks for partial purification.

3.7 Vaccinia Virus Partial Purification

1. Freeze-thaw and sonicate the resuspended cells generated from Subheading 3.6 as described in Subheading 3.1, **step 1**.
2. Centrifuge at 750 × *g* for 10 min at 4 °C to remove the cell nuclei.
3. Keep the supernatant and add TE buffer to give a final volume of 16 ml.
4. Add 20 ml of the 30% sucrose solution into a Beckman ultra-clear (25 × 89 mm) ultracentrifuge tube and carefully layer 13 ml of the cell lysate from **step 3** on to the sucrose cushion.
5. Centrifuge the samples using an ultracentrifuge at 36,000 × *g*, 4 °C for 60 min.
6. The partially purified vaccinia virus particles form a pellet under the sucrose cushion. After centrifugation, carefully remove the top layer (usually pink) and the sucrose layer with a pipette. Wipe the sides of the tube carefully with a tissue to remove any sucrose solution.
7. Resuspend each pellet in 5 ml TE buffer and store at −70 °C.

3.8 Extraction of Vaccinia Virus DNA from Large Partially Purified rVV Stocks

1. Defrost the partially purified vaccinia virus from Subheading 3.7 at 37 °C.
2. Add 5 ml of pre-warmed 2× proteinase K buffer and 100 μl of 20 mg/ml proteinase K to the partially purified vaccinia virus in a 50 ml tube. Incubate at 50 °C for 2.5 h (*see* **Notes 2–5**).
3. Transfer into a clean 50 ml tube.
4. Add 5 ml of phenol/chloroform/isoamyl alcohol, mix by inverting the tube 5–10 times, and centrifuge at 1100 × *g* in a bench-top centrifuge for 15 min at 4 °C. Transfer the upper phase to a clean 50 ml tube using wide-bore pipette tips (*see* **Notes 11** and **12**).
5. Repeat **step 3**.
6. Add 5 ml chloroform, mix by inverting the tube 5–10 times, and centrifuge at 1100 × *g* for 15 min at 4 °C. Transfer the upper phase into a clean 50 ml tube.
7. Precipitate the vaccinia virus DNA by adding 2.5 volumes of −20 °C absolute ethanol and 0.1 volumes of 3 M sodium acetate. Centrifuge at 1200 × *g*, 4 °C for 60–90 min. A glassy pellet should be visible.
8. Discard the supernatant and wash the DNA using 10 ml −20 °C 70% ethanol. Leave on ice for 5 min and centrifuge at 1200 × *g*, 4 °C for 30–45 min. Discard the supernatant and remove the last drops of ethanol using a capillary tip. Dry the inside of the tube using a tissue to remove any ethanol.
9. Air dry the pellet for 5–10 min.
10. Resuspend the vaccinia DNA in 100 μl of water. Do not pipette to resuspend as shearing of the DNA will occur.
11. Leave the tubes at 4 °C overnight. If the pellet has not dissolved totally, add more water.
12. Measure the concentration of the extracted DNA using a nanodrop or equivalent.
13. Store the vaccinia virus DNA at 4 °C. DO NOT FREEZE (*see* **Note 12**).

3.9 Analysis of Vaccinia Virus DNA by PFGE

1. Prepare 2 l of 0.5× TBE buffer for the preparation of the agarose gel and as electrophoresis running buffer; 100 ml is required for a 12.7 × 14 cm agarose gel, and the remainder is required as running buffer.
2. Calculate the concentration of agarose that is needed to analyze the range of DNA fragments to be analyzed. Increasing the agarose concentration decreases the DNA mobility within the gel, requiring a longer run time or a higher voltage. However, a higher voltage can increase DNA degradation and reduce

resolution. A 0.8% agarose gel is suitable for separating DNA ranging between 50 and 95 kb. A 1% agarose gel is suitable for separating DNA ranging between 20 and 300 kb.

3. Place the required amount of agarose in 100 ml 0.5× TBE buffer and microwave until the agarose is dissolved. Cool to approximately 50–60 °C.
4. Clean the gel frame and comb with MQ water followed by 70% ethanol. Place the gel frame on a level surface, assemble the comb and pour the cooled agarose into the gel frame. Remove any bubbles using a pipette tip and allow the agarose to set (approximately 30–40 min) and store in the fridge until required.
5. Place the remaining 0.5× TBE buffer into the electrophoresis tank and switch the cooling unit on. Leave the buffer circulating to cool.
6. Digest 1 μg of the DNA with a suitable restriction enzyme such as *Sal I* in a 20 μl reaction.
7. Add the sample loading dye to the digested vaccinia virus DNA samples and incubate at 65 °C for 10 min.
8. Place the agarose gel in the electrophoresis chamber; load the samples using wide bore tips and appropriate DNA markers (*see* **Note 12**).
9. The DNA samples are analyzed by PFGE at 14 °C in gels run with a 0.1–1.0 s switch time for 16 h at 6 V/cm at an angle of 120° or with a 3.0–30.0 s switch time for 16 h at 6 V/cm depending on the concentration of agarose used.
10. Following PFGE, place the agarose gel in a sealable container containing 400 ml 0.1 μg/ml ethidium bromide and gently shake for 30 min at room temperature.
11. Wash the ethidium bromide-stained agarose gel in 400 ml MQ water by gently shaking for 30 min.
12. Visualize DNA bands using a suitable UV system for analyzing agarose gels. An example of recombinant vaccinia virus DNA digested with the restriction enzyme *Sal I* and analyzed by PFGE is shown in Fig. 5.

3.10 Preparation of rFPV-T7 Stock

Infectious recombinant IBVs are generated in situ by co-transfection of vaccinia virus DNA, containing the modified IBV cDNA, and pCi-Nuc (a plasmid containing the IBV N gene) into CK cells previously infected with a recombinant fowlpox virus expressing the bacteriophage T7 DNA dependent RNA polymerase under the direction of the vaccinia virus P7.5 early–late promoter 8 (rFPV-T7). This protocol covers the procedure for preparing a stock of rFPV/T7 by infecting primary avian chicken embryo fibroblasts (CEFs).

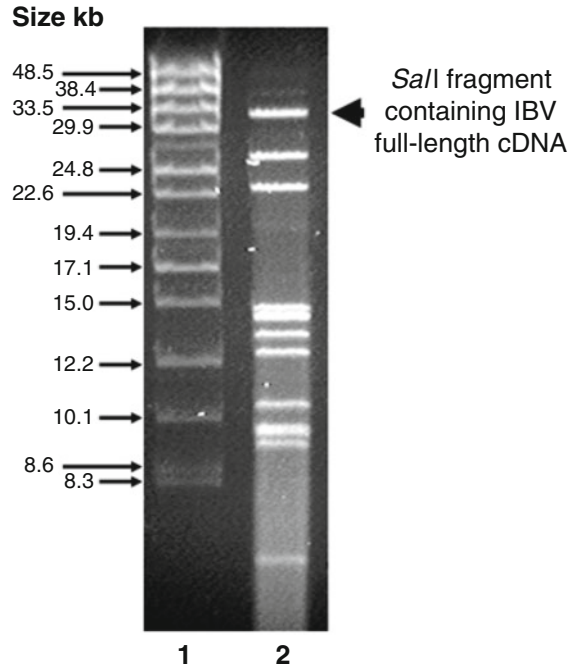

Fig. 5 Analysis of *Sal* I digested vaccinia virus DNA by PFGE. Lane 1 shows DNA markers and Lane 2 the digested vaccinia virus DNA. The IBV cDNA used does not contain a *Sal I* restriction site; therefore, the largest DNA fragment (~31 kb) generated from the recombinant vaccinia virus DNA represents the IBV cDNA with some vaccinia virus-derived DNA at both ends

Preparation of 200 ml stock of rFPV-T7 uses ten T150 flasks containing confluent monolayers of CEFs.

1. Remove the culture growth medium from the cells, wash cells with PBSa, and infect with 2 ml rFPV/T7 at an MOI of 0.1, previously diluted in CEF maintenance medium that does not contain serum.
2. Incubate the infected cells for 1 h at 37 °C, 5% CO_2, then without removing the inoculum, add 20 ml of CEF maintenance medium (containing serum).
3. After 4 days postinfection, check for CPE (90% of the cells should show CPE). Tap the flasks to detach the cells from the plastic and disperse the cells into the medium by pipetting them up and down.
4. Harvest into 50 ml tubes and freeze-thaw the cells three times as described in Subheading 3.1, **step 1**.
5. Centrifuge at 750 × *g*, 4 °C for 5 min to remove the cell debris. Take the supernatant containing the virus stock and store at −70 °C until required.
6. Determine the titer of the virus stock by plaque assay using CEF cells. The titer should be in the order of 10^6–10^7 PFU/ml.

3.11 Infection and Transfection of CK Cells for the Recovery of rIBV

1. Wash the 40% confluent CK cells in six-well plates once with PBSa (*see* **Note 13**).
2. Infect the cells with rFPV-T7 at an MOI of 10 in 1 ml of BES medium. Typically, we carry out 10 replicates per recovery experiment.
3. Incubate for 1 h at 37 °C, 5% CO_2.
4. During this infection period, prepare the transfection reaction solutions.
   Solution A: 1.5 ml OPTIMEM, 10 μg rVV DNA and 5 μg pCi-Nuc per replicate (*see* **Note 1**).
   Solution B: 1.5 ml OPTIMEM and 30 μl lipofectin.
5. Incubate solutions A and B at room temperature for 30 min.
6. Mix solutions A and B together producing solution AB and incubate for a further 15 min at room temperature.
7. Remove the rFPV-T7 from each well and wash the CK cells twice with OPTIMEM and carefully add 3 ml of solution AB per well.
8. Incubate the transfected cells at 37 °C, 5% CO_2 for 16–24 h.
9. Remove the transfection medium from each well and replace with 5 ml of BES medium and incubate at 37 °C, 5% CO_2.
10. Two days after changing the transfection media, when FPV/IBV-induced CPE is extensive, harvest the cell supernatant from each well and using a 5 ml syringe, filter through 0.22 μm to remove any rFPV-T7 virus present (*see* **Note 14**).
11. Store the filtered supernatant, referred to as passage 0 (P_0 CKC) supernatant at −70 °C.

3.12 Serial Passage of rIBVs in CK Cells

To check for the presence of any recovered rIBVs, the P_0 CKC supernatant is passaged three times, P_1 to P_3, in CK cells (Fig. 4b) (*see* **Note 15**). At each passage, the cells are checked for any IBV-associated CPE and for further confirmation RNA is extracted from P_3 CKC supernatant and is analyzed by RT-PCR (*see* **Note 16**).

1. Wash the confluent CK cells in six-well plates once with PBSa.
2. Add 1 ml of the P_0 CKC supernatant per well and incubate at 37 °C, 5% CO_2 for 1 h.
3. Without removing the inoculum, add 2 ml of BES medium per well.
4. Check cells for IBV-associated CPE over the next 2–3 days using a bright-field microscope.
5. Harvest the supernatant from each well and store at −80 °C.
6. Repeat **steps 1–6** for passages P_2 and P_3 in CK cells.
7. At P_3 any recovered virus is used to prepare a large stock for analysis of the virus genotype and phenotype.

4 Notes

1. While the M41 strain can be rescued using an N plasmid encoding the Beau-R N gene and vice-versa, it is unknown to how specific the match must be between the N gene encoded in the IBV cDNA and the N gene expressing plasmid. It is therefore advisable to match exactly the N gene encoded in the IBV cDNA and the N encoded in the plasmid.
2. Vaccinia virus is classified as a category 2 human pathogen, and its use is therefore subject to local regulations and rules that have to be followed.
3. Always discard any medium or solution containing vaccinia virus into a 1% solution of Virkon, leave at least 12 h before discarding.
4. Flasks of cells infected with vaccinia virus should be kept in large Tupperware boxes, which should be labeled with the word vaccinia and biohazard tape. A paper towel should be put on the bottom of the boxes to absorb any possible spillages.
5. During centrifugation of vaccinia virus-infected cells, use sealed buckets for the centrifugation to avoid possible spillages.
6. After 2 h of incubation with the transfection mixture, the cells begin to die. It is best therefore not to exceed 90 min incubation.
7. It is important that after the addition of each selection agent, the medium is mixed to ensure the selection agents are evenly distributed. This can be achieved by *gently* rocking/swirling the plate.
8. Add an equal volume of 2% agar to the 2× EMEM containing MPA, xanthine and hypoxanthine and mix well before adding it to vaccinia virus-infected cells. There is skill to making the overlay medium and adding it to the cells before the agar sets. There are a number of methods including adding hot agar to cold medium or pre-warming the medium to 37 °C and adding agar which has been incubated at 50 °C. Despite the method chosen, it is important that all components of the overlay medium are mixed well, and the medium is not too hot when it is added to the cells. If there are problems, 1% agar can be substituted with 1% low melting agarose.
9. The first recombination event in the TDS system will not necessarily occur in the same place in every rVV. It is therefore important to pick a number of plaques from the first round of plaque purification in presence of GPT selection agents and take a variety of them forward. The following two rounds of plaque purification in the presence of GPT selection agents ensure a single virus population and also that no carry through of the input receiver/wild-type vaccinia virus has occurred.

10. As an alternative approach to confirm whether recombination has occurred resulting in MPA-sensitive recombinant vaccinia virus, during plaque purification in the absence of GPT selection agents, plate 10^{-1} rVV dilution in the presence of GPT selection medium and rVV dilutions 10^{-2} and 10^{-3} in the absence. When there are no plaques in the 10^{-1} dilution, it means that the rVV has lost the GPT gene, and the plaques are ready to amplify and check for the presence of mutations.
11. There are risks associated with working with phenol/chloroform/isoamyl alcohol and chloroform. It is important to check the local guidelines and code of practices.
12. Vaccinia virus DNA is a very large molecule that is very easy to shear; therefore, when working with the DNA, be gentle and use wide bore tips or cut the ends off ordinary pipette tips. In addition, always store vaccinia virus DNA at 4 °C; do not freeze as this leads to degradation. However, there is an exception to this if the vaccinia virus DNA has been extracted using the Qiagen QlAamp DNA mini kit, as this DNA will have already been sheared (the kit only purifies intact DNA fragments up to 650 bp). This DNA can be stored at −20 °C, but it is only suitable for the analysis of the rVV genome by PCR and is *not* suitable for the infection and transfection of CK cells for the recovery of rIBV.
13. While CK cells remain the cell type of choice for the recovery of infectious rIBV, it is possible to recover Beau-R [1] in DF1 cells, an avian cell line derived from fibroblasts isolated from 10-day old chicken embryos [13].
14. Not all IBV strains can be propagated in CK cells, and this is dictated by the S gene [14–16]. When performing a rescue using rIBVs that cannot be propagated in CK cells, the infected cell lysate must also be harvested at this stage. To harvest the cell lysate, add 500–600 μl CK maintenance media per well, and freeze at −80 °C. Immediately before passaging, thaw the lysate and titurate, no more than five times, using a needle and syringe, and filter to remove the rFPV-T7. This technique to break open the cells is harsh and will potentially damage any recovered rIBV. It is therefore strongly advised that the processed cell lysate is immediately taken forward for passaging, without a further freeze-thaw.
15. For rIBVs that cannot be propagated in CK cells, it is also possible to passage in 10-day old SPF embryonated hen's eggs. This has been successfully tried for several rIBVs [15]. Typically, for the first passage, each egg would be inoculated with up to 300 μl of P_0 supernatant, or P_0 cell lysate and incubated for up to 48 h at 37 °C. The embryonated eggs are chilled at 4 °C for at least 4 h before the allantoic fluid is

harvested. The harvested allantoic fluid is then taken forward to the next passage.

16. There is always the possibility that the recovered rIBV is not cytopathic. In this case, check for the presence of viral RNA by RT-PCR at P_3. It is quite common even with a cytopathic rIBV not to see easily definable IBV-induced CPE at P_1 and P_2. The recovery process is a low probability event and the serial passage of rIBVs in CK cells or embryonated eggs acts as an amplification step.

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Chapter 13

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

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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

Tran Thi Nhu Thao, Fabien Labroussaa, Joerg Jores, and Volker Thiel contributed equally to this work.

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_13,

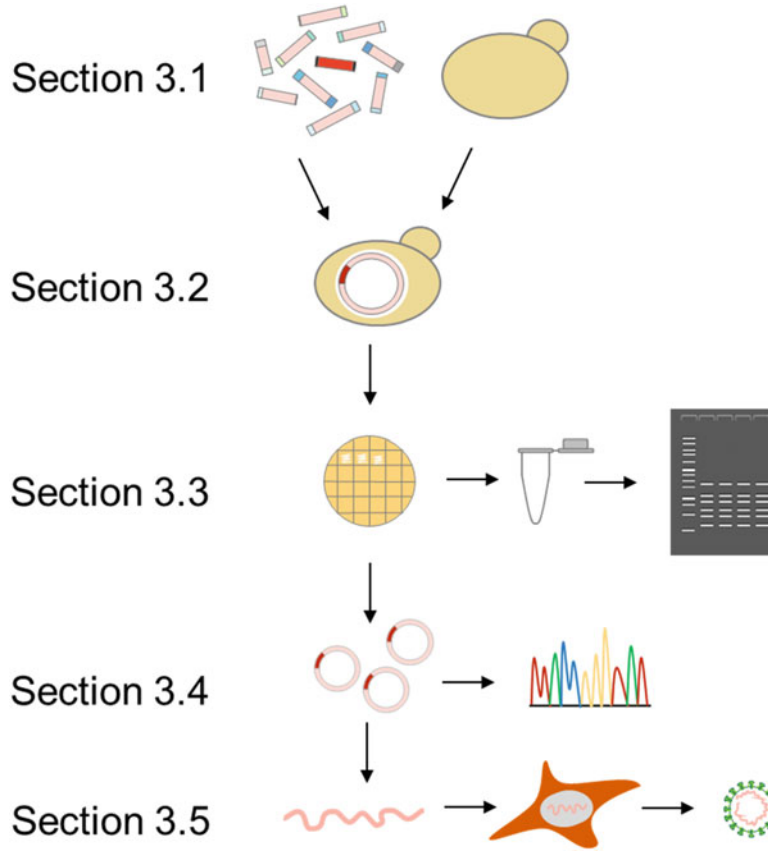

Fig. 1 A schematic workflow of coronavirus reverse genetics system. Yeast-based TAR cloning, a synthetic genomics-derived assembly platform, is employed to assemble full-length viral cDNAs which are maintained as yeast artificial chromosomes (YACs). Yeast clones carrying correctly assembled viral genomes are identified via PCR-based screening of assembly junctions. Reconstitution 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. *Dpn*I 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 One^{C}.

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°) [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×) + GlutaMAX™-I supplemented with 10% fetal bovine serum and 1% Pen Strep.
4. Cell dissociation medium, e.g., TrypLE™ Express (1×).
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_2 incubator maintained at 37 °C and under a 5% CO_2 atmosphere.
11. Refrigerated bench-top centrifuge.
12. Electroporation system.

3 Methods

The TAR cloning method makes use of the natural ability of *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).

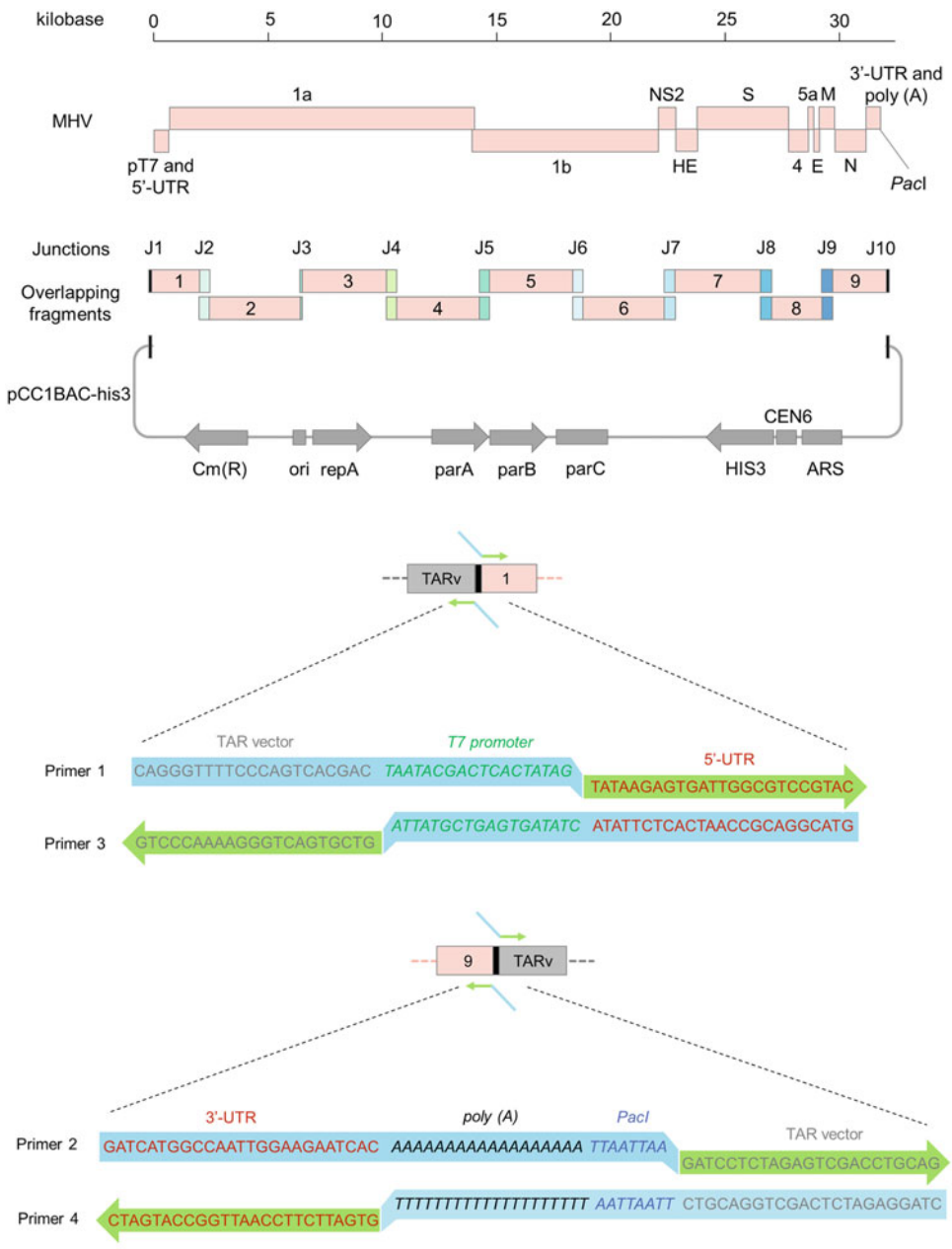

Fig. 2 (**a**) Representative design of TAR cloning vector and overlapping viral DNA fragments exemplified by mouse hepatitis virus (MHV). Nine overlapping fragments cover the entire MHV genome with the T7 promoter preceding the viral 5′-UTR, and poly (A) tail and unique restriction site *PacI* following 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 $MgSO_4$, 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., *Pac*I 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 *Dpn*I 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 1×10^7 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 × *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 × *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

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).

3.3.1 Growth of Yeast Transformants on Agar Dishes

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 × *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 Junctions 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 OD_{600nm} 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., *Pac*I 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.

Table 1
Reaction mix for in vitro transcription

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

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.

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 × *g* for 5 min at 4 °C.
5. Resuspend 1×10^7 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 = ∞.
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 clean-up 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 (OD_{600nm} ~ 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.

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Part IV

Studying Virus-Host Interactions

Chapter 14

Proximity Labeling for the Identification of Coronavirus–Host Protein Interactions

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Abstract

Biotin-based proximity labeling circumvents major pitfalls of classical biochemical approaches to identify protein–protein interactions. It consists of enzyme-catalyzed biotin tags ubiquitously apposed on proteins located in close proximity of the labeling enzyme, followed by affinity purification and identification of biotinylated proteins by mass spectrometry. Here we outline the methods by which the molecular microenvironment of the coronavirus replicase/transcriptase complex (RTC), i.e., proteins located within a close perimeter of the RTC, can be determined by different proximity labeling approaches using $BirA_{R118G}$ (BioID), TurboID, and APEX2. These factors represent a molecular signature of coronavirus RTCs and likely contribute to the viral life cycle, thereby constituting attractive targets for the development of antiviral intervention strategies.

Key words Coronavirus, Replication transcription complex RTC, Proximity labeling, Biotin ligase, Ascorbate peroxidase APEX2, BioID, TurboID, Affinity purification, Mass spectrometry, Proteomics, Replicase microenvironment

1 Introduction

Coronaviruses cause a wide range of diseases in animals and humans. In recent years, particular attention has been drawn by severe acute respiratory syndrome coronavirus (SARS-CoV), MiddleEast respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which can cause severe and lethal respiratory disease in humans [1–5]. Moreover, in the veterinary field, coronaviruses such as, feline infectious peritonitis virus, porcine epidemic diarrhea virus, or the avian infectious bronchitis virus, severely affect companion animals and livestock [6–8].

Viral pathogenicity is determined by the virus tissue and cell tropism and the host (inflammatory) responses to the infection, as well as viral countermeasures evading the host immune defense system.

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_14,

On the cellular level, the expression of up to 15–16 non-structural proteins carrying various enzymatic functions result in the establishment of viral replication and transcription complexes (RTC) and the generation of membranous structures that host the RTC in the infected host cell cytosol [9, 10]. This stage is considered a major determinant of pathogenicity and infection outcome. Indeed, host proteins may be recruited to the RTC to promote viral replication [11, 12] or may display antiviral functions to restrict virus replication [13]. Knowledge on the composition of viral and host proteins located at the RTC is therefore of crucial importance to understand critical virus–host interactions taking place at the site of viral RNA synthesis.

Proximity labeling approaches have been implemented in an increasing number of investigations during the past years [14]. The strong enthusiasm of the scientific community is best exemplified by the constant improvement and adaptation of proximity labeling enzymes to a wide range of applications [15].

The hallmark of enzyme-catalyzed proximity labeling is the promiscuous and covalent biotin labeling of proteins located within a close perimeter (few nanometers). As such, the labeling is not dependent on the protein–protein interaction affinity. The high affinity between biotinylated residues and streptavidin, however, allows stringent and efficient affinity purification using streptavidin-coated beads. Eventually, affinity-purified proteins are sensitively identified by mass spectrometry. Additionally, proximity labeling is also suited to capture transient interactions, as the labeling time can vary from 1 min to several hours depending on the enzyme [15–18]. Of note, the labeling by most enzymes is not toxic and can be performed in live cells.

In this context, $BirA_{R118G}$ (BioID) was the first proximity labeling enzyme successfully employed in different assays [16]. $BirA_{R118G}$ is a promiscuous *E. coli* biotin ligase that uses free biotin in labeling reactions that require several hours of biotin incubation to obtain sufficient amounts of biotinylated proteins for further analysis. During viral infections, this feature can be advantageous when recording protein–protein interactions occurring at any stage during the entire course of infection in an unbiased screening approach [19].

The slow labeling kinetics of BioID were first improved by the development of BioID2 [18]. Recently, Branon et al. used directed evolution of BioID to engineer TurboID and miniTurbo, which contained few key amino acid substitutions conferring similar labeling capacities within 10 min instead of the 15–18 h required by BioID [15]. This significant improvement allows retaining the intrinsic advantages of proximity labeling enzymes while narrowing the labeling window. When applied to virus infections, TurboID enables to distinguish protein–protein interactions relevant at defined stages of infection.

Another popular proximity labeling enzyme is the soy bean-derived ascorbate peroxidase APEX2 [17, 20]. APEX2 catalyzes biotin-phenol substrates into biotin-phenoxyl radicals that react and tag neighboring proteins. This reaction is triggered by the addition of hydrogen peroxide to cells for 1 min. APEX2 can thereby be used to provide a "snapshot" of factors surrounding the protein of interest to which it is fused.

Alternatively, APEX2 catalyzes the polymerization of 3,3-diaminobenzidine (DAB) resulting in the deposition of insoluble DAB polymers at the site of production [21]. Given that DAB precipitates generate high contrast upon heavy-metal staining, this feature allows detecting, in a specific manner, the localization of an APEX2 fusion protein by electron microscopy.

Lastly, APEX2 has been demonstrated to label closely associated RNAs [22]. Upon purification of biotinylated RNAs, sequencing reveals the RNA population associated with a particular protein complex.

Here, we describe the use of BioID, TurboID, and APEX2 in the context of viral infections (Fig. 1). More specifically, by incorporating these proximity labeling enzymes into the RTC of a prototype coronavirus, these strategies enable the identification of critical host factors comprised within the coronavirus RTC microenvironment. The proximity labeling procedures described here are largely adapted from detailed protocols available for BioID [23],

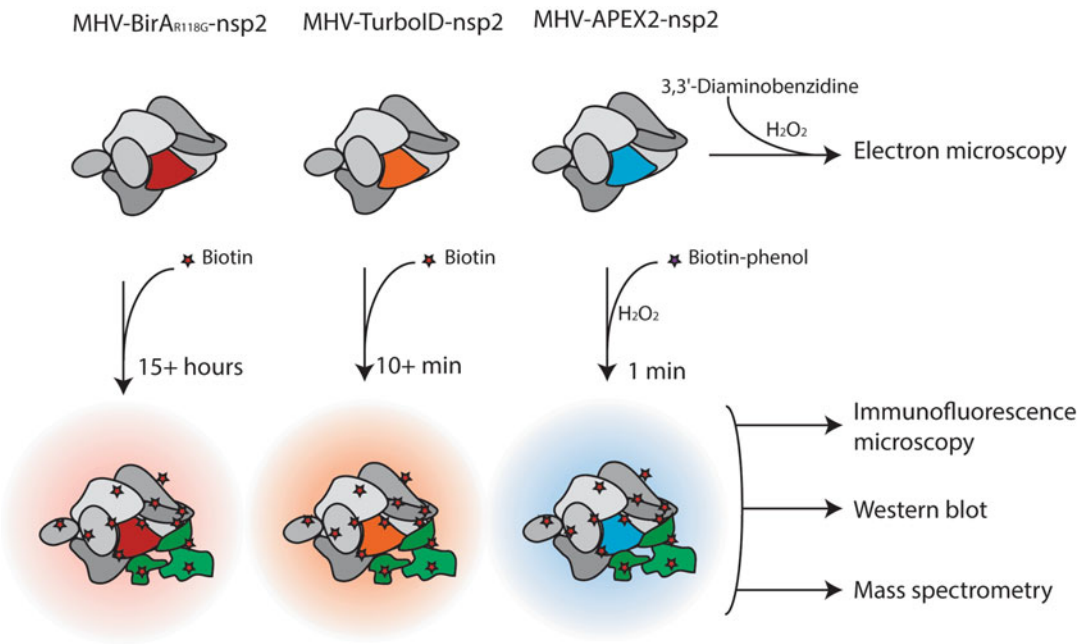

Fig. 1 Overview of proximity labeling assays using MHV-BirA$_{R1118G}$-nsp2, MHV-TurboID-nsp2, and MHV-APEX2-nsp2

TurboID [15], and APEX2 [17, 21]. Further procedures such as mass spectrometry of affinity purified proteins, electron microscopy, or data analysis are not covered in this chapter.

2 Materials

2.1 General Reagents and Equipment

1. 4% neutral buffered formalin.
2. Antibodies/reagents (*see* Table 1).
3. Branson Sonifier 250.
4. Cell culture plates, 6-well and 24-well.
5. Cell scrapers.
6. Confocal buffer (CB): phosphate-buffered saline (PBS) supplemented with 50 mM NH_4Cl, 0.1% (w/v) saponin and 2% (w/v) BSA. Filter-sterilize and store 50 ml aliquots at −20 °C.
7. Coomassie Brilliant Blue G250.
8. Coverslips #1.5H glass, 12 mm diameter, 0.170 mm thick.
9. Dimethyl sulfoxide (DMSO).
10. Western Blotting Transfer System, e.g., eBlot L1 Wet Transfer (Genscript).
11. Glass slides.
12. Hard setting IFA mounting medium containing DAPI.
13. L929 cells.

Table 1
Antibodies and reagents used in this protocol

Antibodies/reagents	Supplier	Reference	Dilution
Anti-dsRNA J2 (mouse monoclonal IgG2a, kappa chain)	English and Scientific Consulting		1:200
Anti-myc (mouse monoclonal)	Cell signaling		1:8000
Anti-MHV nsp2/3 (rabbit polyclonal)	Gift from S. Baker	[24]	1:200
Anti-MHV nsp8 (rabbit polyclonal)	Gift from S. Baker	[25]	1:400
Donkey anti-mouse 488	Jackson ImmunoResearch		1:400
Donkey anti-rabbit 594	Jackson ImmunoResearch		1:400
Donkey anti-rabbit 647	Jackson ImmunoResearch		1:400
AlexaFluor 594-conjugated streptavidin	ThermoFisher Scientific		1:300
HRP-conjugated streptavidin	Dako		1:2500
Anti-V5	Abcam		1:1000

Antibodies stated here have been used successfully by us, other antibodies may also be suitable after validation

14. Laemmli buffer (5×): 125 mM Tris–HCl pH 6.8, 20% (v/v) glycerol/glycerin (v/v), 5% (v/v) SDS, 10% (v/v) β-mercaptoethanol, 0.1% (w/v) bromophenol blue.
15. Magnetic tube holder, e.g., DynaMag-2.
16. MEM+/+: Gibco minimum essential media (MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 IU/ml penicillin.
17. M-PER mammalian protein extraction reagent.
18. Nitrocellulose blotting membrane, supported 0.45 μm NC.
19. Phosphate-buffered saline (PBS).
20. PBS supplemented with 0.5% (v/v) Tween 20.
21. 1 M potassium chloride (KCl).
22. Protein-free blocking buffer, e.g., AdvanBlock-PF (Advansta).
23. 2% (w/v) sodium dodecyl sulfate (SDS).
24. SDS–PAGE equipment, e.g., Mini PROTEAN Tetra Cell (Biorad).
25. 0.1 M sodium carbonate (Na_2CO_3).
26. Streptavidin-coated magnetic beads, e.g., Dynabeads MyOne Streptavidin C1 or Pierce Streptavidin Magnetic Beads.
27. 50 mM Tris–HCl pH 7.4.
28. 10 mM Tris–HCl pH 8.0.
29. 20% (v/v) Triton X-100.
30. 2 M Urea, 10 mM Tris–HCl pH 8.0.
31. Widefield fluorescence microscope or a laser scanning confocal microscope.

2.2 BioID and TurboID Reagents

1. 100 mM Biotin stock in DMSO, sterile filtered, store aliquots at −20 °C.
2. BioID lysis buffer: 50 mM Tris–HCl pH 7.4, 500 mM NaCl, 0.2% (w/v) SDS, 1 mM DTT, and 1× protease inhibitor cocktail. Prepare fresh before use.
3. BioID wash buffer 2: 0.1% (w/v) deoxycholic acid, 1% (v/v) Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES pH 7.5. Prepare fresh before use.
4. BioID wash buffer 3: 0.5% (w/v) deoxycholic acid, 0.5% NP40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris–HCl pH 7.4. Prepare fresh before use.
5. RIPA buffer: 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS (v/v), 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 1× protease inhibitor cocktail. Store 10–20 ml aliquots at −20 °C.

2.3 APEX Reagents

1. APEX lysis buffer: 50 mM Tris–HCl pH 7.4, 500 mM NaCl, 0.2% SDS, 1× protease inhibitor cocktail, 10 mM sodium ascorbate, 5 mM Trolox, 10 mM sodium azide. Prepare immediately before use.
2. APEX elution buffer: 75 mM Tris–HCl (pH 6.8), 6% 2-mercaptoethanol, 12% glycerol, 3% SDS, 0.06% bromophenol blue, 2 mM biotin, and 20 mM DTT.
3. APEX wash 1: 2% SDS in H_2O.
4. APEX wash 2: 2 M Urea, 10 mM Tris–HCl pH 7.4.
5. APEX wash 3: 0.1% (w/v) deoxycholate, 1% (v/v) Triton X-100, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES pH 7.5.
6. APEX wash 4: 0.5% (w/v) deoxycholate, 0.5% (vol/v) NP-40, 1 mM EDTA, 250 mM LiCl, 10 mM Tris–HCl pH 7.4.
7. APEX wash 5: 50 mM Tris–HCl pH 7.4.
8. Biotin-phenol (1000×): 500 mM biotin-phenol in DMSO. Sonicate to dissolve. Filter-sterilize the solution and store as 50 μl aliquots at −80 °C for several months.
9. H_2O_2 solution, 100 mM in PBS (100×). Prepare immediately before use (*see* **Note 1**).
10. Quencher solution, in PBS: 10 mM sodium ascorbate, 5 mM Trolox, and 10 mM sodium azide in PBS. Prepare immediately before use.
11. Sodium ascorbate (100×): 1 M sodium ascorbate in H_2O. Prepare fresh before mixing the Quencher solution.
12. Sodium azide (100×): 1 M sodium azide in H_2O. Aliquots can be stored at −20 °C for several months.
13. Trolox (100×): 500 mM Trolox in DMSO. Sonicate to dissolve if necessary. Prepare fresh before mixing the Quencher solution.

2.4 APEX Electron Microscopy Reagents

1. DAB stock (10×): Dissolve 50 mg 3,3′-diaminobenzidine (DAB) in 10 ml 0.1 M HCl. If DAB remains insoluble after prolonged vortexing at room temperature, pellet at 12,000 × *g* for 5 min and aliquot (10× 1 ml) the supernatant. Flash freeze aliquots in liquid nitrogen and store at −80 °C.
2. EM fixative: 2% (v/v) glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, supplemented with 2 mM calcium chloride. Prepare fresh before use.
3. Sodium cacodylate buffer: 100 mM sodium cacodylate, pH 7.4, supplemented with 2 mM calcium chloride. Store at 4 °C.
4. 20 mM glycine in sodium cacodylate buffer.
5. 0.5 mg/ml DAB, 10 mM H_2O_2 in sodium cacodylate buffer.

3 Methods

3.1 Generation of Recombinant MHV, General Considerations Prior to Commencing Proximity Labeling

1. Cloning strategies and generation of recombinant viruses (Fig. 2) are adapted from Freeman et al. [26]. Coding sequences for $BirA_{R118G}$, TurboID and APEX2 are derived from Addgene cat. no. 74223, 116904, and 72480, respectively, and cloned into a pGPT-1 vector as described previously [19]. Recombinant MHV viruses are generated using an established vaccinia virus-based reverse genetic system [27, 28]. N-terminally tagged proximity labeling enzymes are inserted in the MHV genome within ORF1a. Note the preserved polyprotein cleavage sites ensuring the release of the nsp2 fusion protein (Fig. 2, black arrows). Additionally, a flexible $(SGG)_3$ linker is placed between proximity labeling enzymes and nsp2 to provide structural flexibility and avoid potential steric hindrance of the fusion protein when embedded in the MHV RTC (*see* **Note 2**).
2. Generate and aliquot a virus working stock with sufficient titer to perform the planned experiments (if possible do not exceed 2–3 virus passages). Assess virus titers by plaque titration assays.
3. Ensure genetic integrity of every working stock and verify sequence identity by RT-PCR sequencing of the modified genomic region. Analytical agarose gel electrophoresis of RT-PCR products indicates potential truncations in the region of interest, or mixed populations in the virus stock (*see* **Note 3**).
4. Assess whether recombinant viruses have similar growth phenotype as compared to the parental strain. For this, perform growth curves and assess virus titers in the supernatant of infected cells.

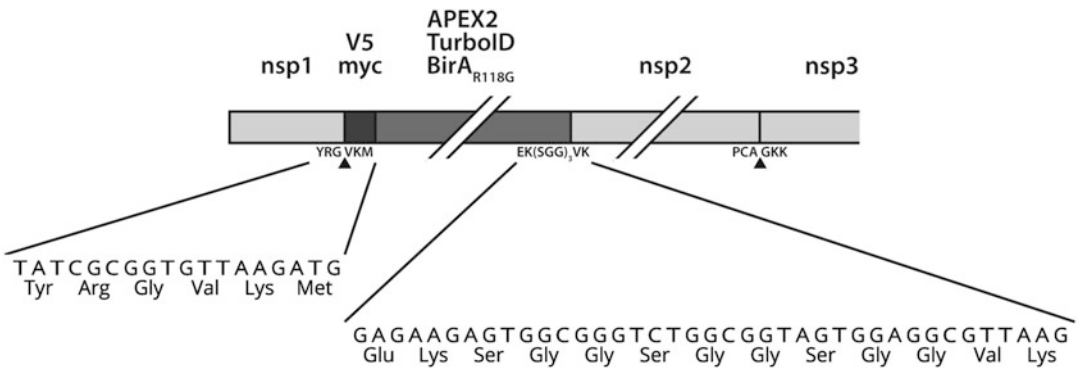

Fig. 2 Schematic overview of recombinant MHV-$BirA_{R118G}$-nsp2, MHV-TurboID-nsp2, and MHV-APEX2-nsp2. ORF1a nsp1, nsp2, and nsp3 are represented by gray boxes; $BirA_{R118G}$ (BioID), TurboID, and APEX2 by a dark gray box; and molecular V5 and myc tag by a black box. The amino acid and nucleotide sequence at the junction between nsp1 and the tagged proximity labeling enzyme are highlighted. The junction between the proximity labeling enzyme and nsp2, in which a flexible linker is incorporated is highlighted as well. Black arrows indicate proteolytic cleavage sites

5. Establish that a functional fusion protein is expressed at the desired subcellular location by performing immunofluorescence microscopy and colocalization analyses and by detecting biotinylated proteins by both immunofluorescence microscopy and western blot analysis (*see* Subheadings 3.6 and 3.7).

3.2 Labeling Procedure Using MHV-BirA$_{R118G}$-nsp2

1. Seed L929 cells (8×10^4 cells/ml) in 6-well cell culture plates or 24-well cell culture plates, using 2 ml/well or 0.5 ml/well, respectively. L929 cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO_2, for 16–18 h.
2. Infect L929 cells with MHV-BirA$_{R118G}$-nsp2 (MOI = 1). Use 2 ml/well for 6-well plates and 0.5 ml/well for 24-well plates. MHV-BirA$_{R118G}$-nsp2 is diluted in MEM+/+ supplemented with 67 μM biotin.
3. Optional: At 1 h post infection (h.p.i.), wash the cells 2–3 times with PBS and incubate cells in fresh MEM+/+ supplemented with 67 μM biotin (*see* **Note 4**).
4. At 15 h.p.i., wash the cells three times with PBS to remove free biotin contained in the cell culture medium.
5. Proceed by fixating the cell for immunofluorescence microscopy analysis or prepare lysates for western blot analysis as described in Subheadings 3.6 and 3.7.

3.3 Labeling Procedure Using MHV-TurboID-nsp2

1. Seed L929 cells (8×10^4 cells/ml) in 6-well cell culture plates or 24-well cell culture plates, using 2 ml/well or 0.5 ml/well, respectively. L929 cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO_2, for 16–18 h.
2. Infect L929 cells with MHV-TurboID-nsp2 (MOI = 1). Use 2 ml/well for 6-well plates and 0.5 ml/well for 24-well plates. MHV-TurboID-nsp2 is diluted in MEM+/+.
3. At 1 h.p.i., wash the cells 2–3 times with PBS and incubate cells in fresh MEM+/+.
4. At desired times post infection, add 500 μM biotin to the cell culture supernatant and incubate further. Labeling times can vary between 10 min up to several hours.
5. After labeling, wash the cells three times with PBS to remove free biotin contained in the cell culture medium.
6. Proceed by fixating the cell for immunofluorescence microscopy analysis or prepare lysates for western blot analysis as described in Subheadings 3.6 and 3.7.

3.4 Labeling Procedure Using MHV-APEX2-nsp2

1. Seed L929 cells (8×10^4 cells/ml) in 6-well cell culture plates or 24-well cell culture plates, using 2 ml/well or 0.5 ml/well, respectively. L929 cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO_2, for 16–18 h.

2. Infect L929 cells with MHV-APEX-nsp2 (MOI =4). Mock controls receive fresh MEM+/+. Use 2 ml/well for 6-well plates and 0.5 ml/well for 24-well plates. MHV-APEX-nsp2 is diluted in MEM+/+.
3. After 1 h remove the inoculum, wash the cells three times with PBS and add fresh MEM+/+.
4. 30 min before the desired time point, exchange the medium with MEM+/+ supplemented with 500 μM biotin-phenol (BP).
5. At the desired time-point, add appropriate volume of 100× H_2O_2 solution to the culture medium to obtain a 1× H_2O_2 solution. Shake gently to mix.
6. Incubate for exactly 1 min at RT.
7. Quickly aspirate the labeling solution and wash the cells immediately three times with Quencher solution (*see* **Note 5**).
8. Proceed by fixating the cell for immunofluorescence microscopy analysis or prepare lysates for western blot analysis as described in Subheadings 3.6 and 3.7.

3.5 Controls

1. Appropriate control samples are of crucial importance and should be included in every experiment.
2. Parental recombinant viruses that do not express the proximity-labeling enzyme are used as negative control.
3. Alternatively, incubating cells in medium supplemented with DMSO instead of biotin (during MHV-BirA_{R118G}-nsp2 or MHV-TurboID-nsp2 infections) will result in "background" biotinylation.
4. During MHV-APEX2-nsp2 infections, the omission of biotin-phenol or the H_2O_2 should be used as negative controls. In case of BP omission, supplement the medium with DMSO instead.
5. Mock infections followed by the labeling procedure can serve as additional negative controls.

3.6 Immunofluorescence Microscopy Analysis (IFA) of Biotinylated Proteins

1. Perform infections for IFA in 24-well cell culture plates.
2. Prior to seeding cells, place sterile glass coverslips in 24-well plates.
3. Perform infections and labeling procedures as indicated in Subheadings 3.2, 3.3, or 3.4.
4. Fix cells with 4% (v/v) formalin for 20 min at room temperature. Use 1 ml per well.
5. Wash cells three times with PBS.

6. Permeabilize, block unspecific antibody binding, and quench PFA by incubating the coverslips in confocal buffer (CB; 1 ml) for 1 h at room temperature. Gentle shaking is recommended.
7. Incubate coverslips, cells facing down, on a 30–50 µl drop of primary antibodies diluted in CB to label antigens of interest (*see* **Note 6**). 60 to 120 min incubation at room temperature is recommended. Antibodies used in our laboratory are listed in Table 1.
8. Place coverslips into a 12-well plate and wash coverslips three times with 1 ml CB for 5 min.
9. Incubate coverslips, cells facing down, on a 30–50 µl drop of secondary antibodies diluted in CB for 60 min at room temperature (*see* **Note 6**). Protect from light. Add fluorophore-conjugated streptavidin during this step to label biotinylated proteins.
10. Place coverslips into 12-well plate and wash coverslips twice with 1 ml CB for 5 min, wash once with 1 ml PBS, and once, briefly, with 1 ml dH_2O.
11. Mount coverslip on glass slide using DAPI-containing mounting medium.
12. Cure by incubating glass slide overnight at room temperature. Protect from light.
13. Image cells using a widefield fluorescence microscope or a laser scanning confocal microscope. Use identical imaging setting for all samples and controls of same experiment. Further deconvolve 3D images if necessary.
14. For display purposes, adjust brightness and contrast to appropriate controls during post-acquisition processing of immunofluorescence microscopy images.

3.7 Western Blot Analysis of Biotinylated Proteins (Analytical Scale)

1. Perform infections and labeling procedures for analytical western blots in six-well cell culture plates as indicated in Subheadings 3.2, 3.3, or 3.4.
2. Prepare lysates using M-PER mammalian protein extraction reagent, RIPA, or other lysis buffers (*see* Subheadings 3.9–3.11) containing protease inhibitors. Typically, 100–200 µl/well lysis buffer is used, and cells are scraped off using an inverted pipette tip.
3. Collect lysate in 1.5 ml tube, centrifuge at ~14,000 × *g* for 5–10 min to pellet the cell debris and transfer supernatant to new tube. Add Laemmli buffer and boil for 5–10 min.
4. To assess biotinylated proteins in affinity-purified fractions, *see* Subheadings 3.9–3.11.
5. Separate proteins on a 10% (w/v) SDS–polyacrylamide gel.

6. Electroblot onto a nitrocellulose membrane.
7. Incubate nitrocellulose membrane in protein-free blocking buffer for 60 min at room temperature (*see* **Note 7**).
8. Incubate nitrocellulose membrane with horseradish peroxidase (HRP)-conjugated streptavidin diluted in protein-free blocking buffer. Incubate at 4 °C overnight.
9. Wash the nitrocellulose membrane three times with PBS supplemented with 0.5% (v/v) Tween 20 and once with PBS.
10. Visualize biotinylated proteins using an enhanced chemiluminescence (ECL) HRP substrate and a chemiluminescence CCD detector system.

3.8 Preparation of Cells Infected with MHV-APEX2-nsp2 for Electron Microscopy

1. Seed L929 cells (8–12 × 10^4 cells/ml) in 24-well cell culture plates, using 0.5 ml/well. L929 cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO_2, for 16–18 h. Cells should be 90–100% confluent at the time of fixation.
2. Infect cells with MHV-APEX2-nsp2 at MOI = 3. MHV-APEX2-nsp2 is diluted in MEM+/+ (*see* **Note 8**).
3. At 1 h.p.i., wash the cells 2–3 times with PBS and incubate cells in fresh MEM+/+.
4. At desired time post infection, wash cells once with prewarmed (37 °C) PBS.
5. Fix cells using prewarmed (37 °C) EM fixative.
6. Place on ice for 60 min.
7. The following incubations are performed on ice in ice-cold buffers. Wash cells three times with sodium cacodylate buffer.
8. Quench with 20 mM glycine in sodium cacodylate buffer for 5 min.
9. Wash three times with sodium cacodylate buffer.
10. Stain cells in sodium cacodylate buffer containing 0.5 mg/ml DAB and 10 mM H_2O_2 for approximately 20 min until DAB precipitates are visible by light microscopy (*see* **Notes 9** and **10**). For this, use an inverted cell culture microscope and compare to a side-by-side control condition.
11. Wash three times with cacodylate buffer to stop the staining reaction.
12. Refer to electron microscopy facility's guidelines for sample submission.

3.9 Sample Preparation for Mass Spectrometry, Infections with MHV-BirA$_{R118G}$-nps2

1. Seed L929 cells in 4 × 150 cm^2 tissue culture flasks, 1 × 10^7 cells per flask. Cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO_2, for 16–18 h.
2. Infect L929 cells with MHV-BirA$_{R118G}$-nsp2 (MOI = 1) in 25 ml MEM +/+ supplemented with 67 μM biotin. Do not wash the cells after infection (*see* **Note 11**).

3. At 15 h.p.i., wash cells three times with a large excess of PBS (50 ml per tissue culture flask) (*see* **Note 12**).
4. Lyse the cells by adding 1 ml ice-cold BioID lysis buffer per flask and detach the cells using a cell scraper.
5. Keep the cells on ice for the rest of the procedure.
6. Pool the lysates of the four flasks into one 50 ml conical tube.
7. Add 400 μl 20% Triton X-100 to each tube.
8. Sonicate cells with two rounds of 20 pulses using a Branson Sonifier 250 (at 30% constant, 30% power). Put cells on ice during the sonication rounds.
9. Add 3.6 ml 50 mM Tris–HCl pH 7.4 to each tube.
10. Sonicate cells for one additional round of 20 pulses.
11. Transfer lysates to 4 × 2 ml tubes and centrifuge 10 min at 18,000 × *g*, 4 °C.
12. Wash streptavidin-coated magnetic beads (800 μl beads per condition) with 1 ml lysis buffer diluted 1:1 with 50 mM Tris–HCl pH 7.4. Place the cells on a magnetic tube holder and remove the wash solution.
13. Repeat wash with 1 ml of lysis buffer diluted 1:1 with 50 mM Tris–HCl pH 7.4, and distribute the solution to four 2 ml tubes. Remove wash solution using a magnetic tube holder.
14. Take an aliquot of 100 μl from the lysate (total 8 ml) before incubation with the magnetic beads as control and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.
15. Distribute the remaining lysate to the four 2 ml tubes containing the washed streptavidin-coated beads and incubate on a rotator at 15 rotations per minute at 4 °C overnight.
16. Place the samples on a magnetic tube holder and collect the "flowthrough" (non-bound protein lysate). Combine the beads into one tube.
17. Take an aliquot of 100 μl from the flowthrough and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.
18. Wash the beads as described below. After each washing step, place the tube on the magnetic tube holder and remove the washing solution.
19. Wash beads twice with 1.5 ml 2% (w/v) SDS by incubating beads for 5–8 min on a rotator.
20. Wash once with 1.5 ml BioID washing buffer 2 by incubating beads for 5–8 min on a rotator.

21. Wash once with 1.5 ml BioID washing buffer 3 by incubating beads for 5–8 min on a rotator.
22. Wash once with 1 ml 50 mM Tris–HCl pH 7.4.
23. Elute proteins from beads by the addition of 100 µl 1× Laemmli SDS-sample buffer supplemented with 0.5 mM biotin and heating at 95 °C for 10 min in a heating block while shaking at 700 rpm.
24. Keep 10% of eluate for western blot analysis.
25. Refer to the mass spectrometry facility's guidelines for sample submission. Samples are typically separated 1 cm into an SDS-polyacrylamide gel, stained with Coomassie, and extracted from the gel for mass spectrometry analysis (*see* **Note 13**).

3.10 Sample Preparation for Mass Spectrometry, Infections with MHV-Turbo-nsp2

1. Seed L929 cells in 150 cm^2 tissue culture dishes, 1×10^7 cells per dish. Cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO_2, for 16–18 h.
2. Infect L929 cells with MHV-Turbo-nsp2 (MOI = 1) in 25 ml MEM +/+.
3. At 1 h.p.i., wash cells twice with PBS and incubate cells in 25 ml fresh MEM +/+ (*see* **Note 14**).
4. At a chosen time post infection, add biotin to a final concentration of 500 µM.
5. Incubate cells at 37 °C, 5% CO_2 for 10 min up to multiple hours.
6. At chosen time after biotin addition, wash the cells five times with 25 ml ice-cold PBS (*see* **Note 12**).
7. Lyse the cells in 1.5 ml RIPA buffer containing protease inhibitors. After addition of RIPA buffer, incubate for 1–2 min before using a 1 ml pipette to detach the cells from the surface of the dish.
8. Transfer the lysate to 2 ml tubes and spin at 12,000 × *g*, 4 °C.
9. Using a magnetic tube holder, wash streptavidin-coated magnetic beads twice with 1 ml RIPA buffer. Use 350 µl beads per condition (*see* **Note 15**).
10. Take a 100 µl aliquot of the lysate before incubation with the magnetic beads as control and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.
11. Add the lysate to the streptavidin-coated beads and incubate on a rotator at 4 °C overnight.
12. Place the samples on a magnetic tube holder and collect the "flowthrough" (non-bound protein lysate).

13. Take an aliquot of 100 µl from the flowthrough and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.
14. Wash the beads as described below. After each washing step, place the tube on the magnetic tube holder and remove the washing solution.
15. Wash the beads twice with 1 ml RIPA buffer for 2–5 min.
16. Wash the beads with 1 ml 1 M KCl for 2–5 min.
17. Wash the beads with 1 ml 0.1 M Na_2CO_3. Homogenize the beads with a 1 ml pipette and continue immediately with next washing step.
18. Wash the beads with 1 ml 2 M urea, 10 mM Tris–HCl (pH 8.0). Homogenize the beads with a 1 ml pipette and continue immediately with next washing step.
19. Wash the beads with 1 ml 10 mM Tris–HCl (pH 8.0).
20. Elute proteins from beads by the addition of 100 µl 1× Laemmli SDS-sample buffer supplemented with 0.5 mM biotin and heating at 95 °C for 10 min in a heating block while shaking at 700 rpm.
21. Place the beads on the magnetic tube holder and collect eluates in fresh 1.5 ml tubes.
22. Refer to the mass spectrometry facility's guidelines for sample submission. Samples are typically separated 1 cm into an SDS–polyacrylamide gel, stained with Coomassie and extracted from the gel for mass spectrometry analysis (*see* **Note 13**).

3.11 Sample Preparation of Cells Infected with MHV-APEX2-nsp2 for Mass Spectrometry

1. Seed 2×10^6 L929 cells in a 10 cm^2 dish. Cells are cultured in MEM+/+. Incubate at 37 °C, 5% CO_2 for 16–18 h.
2. Infect the cells with MHV-APEX2-nsp2 or MHV-WT at MOI = 4 (*see* **Note 16**).
3. After 1 h remove the inoculum and wash the cells three times with PBS. Add fresh MEM+/+ (*see* **Note 12**).
4. Thirty minutes before the desired time point, exchange the medium with 10 ml MEM+/+ supplemented with 500 µM biotin-phenol (BP). Add fresh medium to APEX-negative controls.
5. At the desired time-point, add 100 µl H_2O_2 solution (100×) directly to the dish containing the 10 ml MEM+/+ supplemented with BP. Shake gently to mix.
6. Incubate for exactly 1 min at RT.
7. Quickly aspirate the labeling solution and wash the cells immediately three times with 10 ml of Quencher solution (*see* **Note 17**).

8. Remove the remaining Quencher solution and add 1 ml of APEX lysis buffer directly onto the cells. Use a cell scraper to detach all cells off the dish (*see* **Note 18**).
9. Collect the lysate and transfer it into a 50 ml tube and add 100 μl 20% Triton X-100.
10. Sonicate cells with two rounds of 30 pulses using a Branson Sonifier 250 (at 30% constant, 30% power). Put cells on ice during the sonication rounds.
11. Add 900 μl of 50 mM Tris–HCl pH 7.4 to each sample.
12. Sonicate samples one more time for 30 pulses.
13. Transfer samples into a 2 ml tube and centrifuge at 16,000 × *g* at 4 °C for 10 min.
14. Transfer the supernatant into a fresh 2 ml tube. This is considered the Lysate (*see* **Note 19**).
15. Prepare 100 μl streptavidin-coated magnetic beads per sample in a 2 ml tube.
16. Add 1 ml of APEX lysis buffer to the beads and place the tube on a rotator at 15 rotations per minute for 8 min.
17. Place the tube on a magnetic tube holder for 1 min and subsequently remove all liquid from the tube without touching the beads on the wall.
18. Repeat the washing step again for a total of two washes with APEX lysis buffer.
19. Take an aliquot of 100 μl from the lysate and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.
20. Add the remaining lysate to the beads and incubate the samples on a rotator at 15 rotations per minute at 4 °C overnight.
21. Place the samples on a magnetic tube holder and collect the "flowthrough" (non-bound protein lysate).
22. Take an aliquot of 100 μl from the flowthrough and add Laemmli buffer. Boil 10 min at 98 °C and store at −20 °C for further western blot analysis.
23. Subject the beads to a series of washing steps, described below. Each step consists of 1 ml of the respective washing buffer followed by 8 min on the rotator and 1 min on magnetic tube holder. Always remove the wash buffers before the next washing step.
24. Wash the beads twice with APEX wash 1.
25. Wash the beads once with APEX wash 2.
26. Wash the beads twice with APEX wash 3.
27. Wash the beads twice with APEX wash 4.

28. Wash the beads once with APEX wash 5.
29. Remove the remaining wash buffer with a 20 µl pipette tip.
30. Add 50 µl of APEX elution buffer to the beads and elute biotinylated proteins by boiling each sample at 95 °C for 10 min while shaking at 700 rpm.
31. Vortex the beads briefly and cool the samples on ice. Quickly spin the samples to bring down condensation.
32. Place the beads on the magnetic tube holder and collect eluates in fresh 1.5 ml tubes.
33. Use 10 µl eluate for western blot analysis and 35 µl for mass spectrometry (*see* **Note 20**).
34. Refer to the mass spectrometry facility's guidelines for sample submission. Samples are typically separated 1 cm into a SDS-polyacrylamide gel, stained with Coomassie and extracted from the gel for mass spectrometry analysis (*see* **Note 13**).

4 Notes

1. Keep H_2O_2 stock in small aliquots, cold and protected from light at all times.
2. The use of longer linkers can extend the biotinylation range [18].
3. One unique band, which is migrating at the expected molecular weight, is expected.
4. A washing step at 1 h.p.i. ensures that the cells are found in a similar stage of infection at any given time point. Also *see* **Note 11**.
5. Be careful not to detach too many cells during the washing. Remove the remaining liquid from the washes.
6. Alternatively, coverslips can be left in 24-well plate. In this case, dilute antibodies in a sufficient volume to cover coverslips.
7. Milk is to be avoided as it contains biotin, which competitively inhibits biotinylated protein recognition by HRP-coupled streptavidin [29].
8. Increase MOI if necessary in order to obtain 70–90% of infected cells.
9. Prepare 10 ml using a 10× DAB stock. Prepare fresh before use and discard unused buffer.
10. Add H_2O_2 to the buffer as a last step and just before use. Also *see* **Note 1**.
11. Do not wash the cells after infection. This will allow the cells to be infected in a non-synchronized fashion and enable to record

cells during different stages of the viral life cycle at the moment of lysis. Also *see* **Note 4**.

12. Repeated washing is important to completely remove free biotin, which might interfere with binding of proteins to streptavidin-coated beads.
13. Alternatively, proteins are not eluted from the beads and are submitted to on-bead tryptic digestion for mass spectrometry analysis.
14. If precise synchronization of the virus infection is desired, infect the cells at 4 °C and subsequently incubate them 60–90 min at 4 °C. This allows the virus particles to attach to the receptor but prevents entry. Afterwards place the cells in the incubator at 37 °C. Add 25 mM HEPES to the infection medium to buffer the cells.
15. The use of streptavidin-coated magnetic beads from Pierce are recommended when using the washing conditions described in Subheading 3.10. The use of MyOne Streptavidin C1 beads has proven to result in clumping and strong adherence to the walls of tubes, thus rendering washing and elution steps difficult. Lysis and affinity purification of biotinylated proteins in MHV-TurboID-nsp2-infected cells can alternatively be performed using the conditions described in Subheading 3.9. Nevertheless, most recent publications favor the use of buffers described in Subheading 3.10.
16. Prepare one extra 10 cm dish, which can be used to count the cells and calculate the MOI for the others.
17. Be careful not to detach too many cells during the washing. Remove the remaining liquid from the washes.
18. If you need to process multiple samples, store the falcon tubes containing the lysates on ice in the meantime.
19. The lysate can also be frozen at −80 °C until further processing.
20. Eluates can be immediately processed or frozen at −80 °C for storage.

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Chapter 15

Using Yeast to Identify Coronavirus–Host Protein Interactions

Stuart Weston and Matthew Frieman

Abstract

We have developed a screening system using the yeast *Saccharomyces cerevisiae* to identify eukaryotic genes involved in the replication of mammalian viruses. Yeast come with various advantages, but in the context of coronavirus research and the system outlined here, they are simple and easy to work with and can be used at biosafety level 2. The system involves inducible expression of individual viral proteins and identification of detrimental phenotypes in the yeast. Yeast knockout and overexpression libraries can then be used for genome-wide screening of host proteins that provide a suppressor phenotype. From the yeast hits, a narrowed list of candidate genes can be produced to investigate for roles in viral replication. Since the system only requires expression of viral proteins, it can be used for any current or emerging virus, regardless of biocontainment requirements and ability to culture the virus. In this chapter, we will outline the protocols that can be used to take advantage of *S. cerevisiae* as a tool to advance understanding of how viruses interact with eukaryotic cells.

Key words Yeast, Suppressor screening, Host–virus interaction, Host factors

1 Introduction

Yeast have a long-standing history of use in cell biology research. The model organisms *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been utilized to great effect in developing understanding of higher order eukaryotes owing to a high degree of homology in the context of a simplified genomic system. In the Frieman lab, we have developed a set of protocols that utilize *S. cerevisiae* for the identification of novel eukaryotic factors involved in viral replication. An example of this can be found in the work presented in Weston *et al.* that identified a host factor involved in replication of Middle East respiratory syndrome coronavirus (MERS-CoV) [1]. The system is amenable for use with any other virus through the series of protocols that will be outlined here. Any currently studied or newly emerging coronavirus could potentially be assessed in this system to allow for rapid

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_15,

identification of host factors involved in viral replication. A major advantage of the yeast system is that it allows for research into viral replication in a biosafety level 2 condition, avoiding potential constraints on certain known and novel coronaviruses. In addition, yeast provide a simple and easy tool with many well-established protocols to aid in understanding of coronavirus replication in a simplified genetic system.

The yeast screening platform utilizes phenotypes induced in *S. cerevisiae* through the overexpression of viral proteins. Our work with MERS-CoV identified four viral genes capable of causing slow growth of the yeast. All other viral systems we have tested (SARS-CoV being another coronavirus) have at least one, if not multiple proteins capable of causing yeast to grow slowly. As will be detailed below, the expression of viral proteins in yeast requires standard cloning procedures with yeast vectors that allow inducible expression. This slow growth phenotype can be leveraged to screen for host factors that may have a role in viral replication. Yeast knockout and overexpression libraries are well established, easily obtainable semi-genome to genome-wide screening platforms that can be used in conjunction with the viral protein induced phenotypes for suppressor screening. A suppressor phenotype in this context is one whereby the knockout or overexpression of a host gene causes a loss of the original slow growth phenotype induced by the viral protein. This suppression is the readout utilized to identify eukaryotic proteins that have a functional genetic interaction with the viral protein of interest. From this point of identifying yeast gene suppressors, mammalian cell culture system can be used to validate the homologous genes using standard techniques (e.g., RNAi/CRISPR) for roles in viral replication.

Throughout this chapter, we will use the example of a slow growth phenotype in the context of a galactose inducible (GAL1) system using uracil (URA)-based selection. However, there are numerous other phenotypes that can be caused by exogenous expression of viral proteins in yeast and used for suppressor screening. Additionally, there are various other inducible expression systems and metabolic selection systems. For the sake of brevity, we will keep the discussion here focused on the system we have developed using MERS-CoV that can be extended to any other virus. As mentioned previously, an advantage of utilizing yeast as an initial screening platform is the wealth of established protocols available. We direct the reader to other sources should a deeper investigation in the way yeast can be used for studying mammalian viruses be desired [2, 3].

One may consider using the protocols provided here as a means to identify genes involved in coronavirus replication that may not be identified in other screening systems. In more complex mammalian cell culture systems, genome wide screens can be biased toward the strongest hits such as entry factors or innate immune response

modulators. The yeast system avoids these issues as it focuses only on a single protein and does not require any viral entry to take place, and yeast do not have an interferon response. Moreover, while yeast share a high degree of genetic homology with mammalian cells, they are significantly simpler with approximately 6000 open reading frames. Therefore, using yeast may allow for the identification of interactions that could be masked in the more complex genetic pathways of mammalian cells. Ultimately, we propose that yeast may be a powerful tool to advance the understanding of coronavirus (and other viral families) replication and identify potential antiviral targets.

2 Materials

2.1 Yeast Strains

1. *S. cerevisiae* strain BY4742 or BY4741 (*see* **Note 1**).
2. Yeast knockout library (*see* **Note 2**).

2.2 Yeast Culture Media

1. Yeast extract-peptone-dextrose (YEPD or YPD) medium: 1% yeast extract, 2% peptone, and 2% glucose/dextrose. For 1 l dissolve 10 g yeast extract and 20 g peptone in 700 ml ddH_2O. Make volume to 900 ml and autoclave for 20 min. Then add 100 ml 20% glucose solution that has been run through a 0.2 μM filter.
2. YPD agar plates: 1% yeast extract, 2% peptone, 2% glucose/dextrose, and 2% agar. For 1 l, prepare as media above with the addition of 20 g agar prior to autoclaving. Add the filtered 20% glucose after autoclaving and pour plates.
3. Casamino acid/yeast nitrogen base (CAA/YNB) yeast minimal medium: 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 0.6% casamino acids, 2% sugar (*see* **Note 3**).
4. CAA/YNB agar plates: CAA/YNB medium, 2% agar (*see* **Note 3**).
5. 30–50% glycerol solution.

2.3 Plasmid Transformation

2.3.1 Small-Scale and Large-Scale Transformation

1. Plasmids for expression of viral proteins in yeast (*see* **Note 4**).
2. Molecular biology grade water.
3. 10× TE: 100 mM Tris–Cl, pH 7.6, 10 mM EDTA.
4. 1 M lithium acetate.
5. 50% polyethylene glycol (PEG).
6. Yeast carrier DNA or salmon sperm DNA (either 10 mg/ml stock or 2 mg/ml stock can be used).
7. Sterile glass tubes.
8. YNB/CAA agar plates.

9. Bench top centrifuge.
10. Microfuge.
11. Water baths or heat blocks set at 30 °C and 42 °C.

2.3.2 Transformation in a 96-Well Format

1. Plasmids for expression of viral proteins in yeast (*see* **Note 4**).
2. Molecular biology grade water.
3. 1× TE: 10 mM Tris-Cl, pH 7.6, 1 mM EDTA.
4. 1 M lithium acetate.
5. 50% PEG.
6. 2 mg/ml yeast carrier DNA or salmon sperm DNA.
7. Deep well 96-well plates (1 ml culture volume).
8. 96-well microplate replicator or multichannel pipette.
9. 15 cm YNB/CAA agar plates.
10. Bench-top centrifuge.

2.4 Culturing Yeast

1. Sterile glass tubes.
2. Sterile glass flasks.
3. 96-well plates.
4. 30 °C incubator with a shaker set to 230–270 rpm.
5. An automated plate reader that can incubate at specified temperatures and read OD600 (e.g., BioTeK Synergy HTX) or a spectrophotometer (e.g., a nanodrop).
6. Multichannel pipette.

2.5 NaOH Protein Extraction

1. 0.1 M NaOH.
2. Western blot loading buffer (e.g., NuPAGE loading dye).
3. Beta-mercaptoethanol.

2.6 Genomic DNA Extraction

1. Yeast lysis buffer: 10 mM Tris–Cl, pH 8, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton-X100.
2. TE buffer: 10 mM Tris–Cl, pH 8, 1 mM EDTA.
3. 1:1 phenol:chloroform.
4. Glass beads.
5. Bead beater homogenizer (e.g., MagNA Lyser).
6. 100% ethanol.
7. 70% ethanol (at 4 °C).
8. RNase.
9. Microfuge.
10. Microfuge tubes.
11. Microfuge tubes suitable for use in bead beater.

3 Methods

In order to take advantage of the yeast system for the identification of eukaryotic factors involved in viral replication, a certain series of steps must be undertaken. Initially, expression plasmids must be produced to exogenously express viral proteins in yeast. The yeast strain and expression plasmids must be chosen to allow for the selection of transformants through auxotrophic selection from the culture media and plates. Following production of expression plasmids for viral proteins of interest through standard cloning procedures (not described here), yeast must be transformed. Successful transformation is best confirmed through western blotting for exogenous proteins along with the auxotrophic selection. Once expression has been confirmed, analysis of the yeast for phenotypes that can be used in suppressor screening must be performed. Here, we will use a discussion of slow growth phenotypes as these are easily analyzed. We direct the reader to other sources for more in-depth discussion of methods for suppressor screening [2, 3]. Once these phenotypes have been identified, library transformations must be performed using knockout libraries, as described here, or overexpression libraries in a large-scale format. These libraries can be screened for suppressor phenotypes, which in the discussion here will be determined by a loss of the slow-growth phenotype. This allows identification of suppressor yeast clones which can be determined as hits once they have been found to have enhanced growth, while maintaining expression of the exogenous viral protein. Finally, genomic extractions and DNA sequencing allow for the identification of the knockout/overexpressed eukaryotic gene that is having a functional genetic interaction with the viral protein. Identification of the mammalian homologs of these suppressor hits will generate a list of candidate genes to investigate for roles in replication of the virus of interest.

3.1 Yeast Culture

1. Yeast stocks can be made by mixing liquid cultured yeast with glycerol to a final concentration of 15–25% and storing at −80 °C. These stocks can be maintained indefinitely (*see* **Note 5**).
2. To recover frozen stocks, streak a small amount onto an appropriate agar plate (YPD agar when non-transformed, appropriate dropout for selection of transformed yeast). Incubate this plate at 30 °C until colonies form (2–3 days). Colonies can be picked from these plates for further culture. Seal the plates in parafilm and store at 4 °C, replacing every 1–2 months.
3. Working stock plates can be either those that were struck for single colonies or a single colony can be picked, and a patch culture made by streaking patches and growing for a further 2-3 days at 30 °C (Fig. 1).

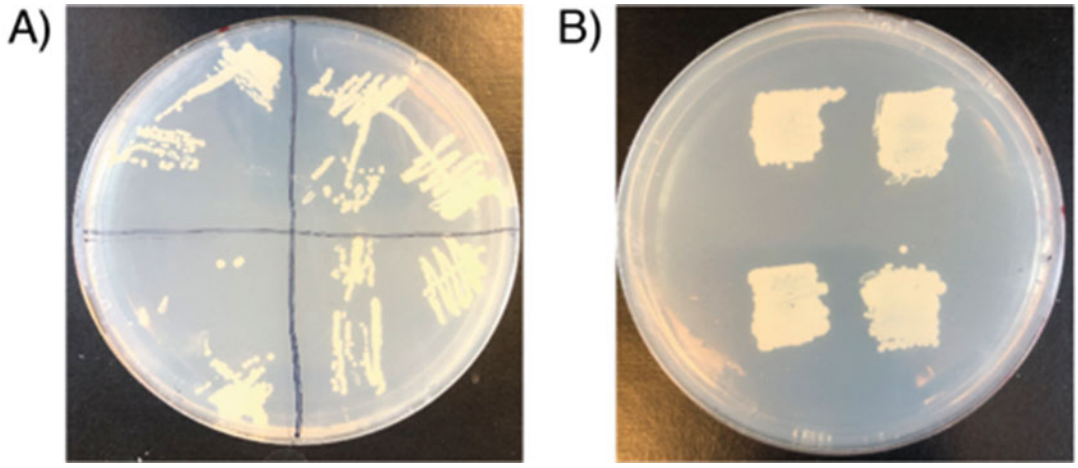

Fig. 1 Methods to prepare yeast stock culture plates. Yeast can be struck to obtain single colonies on agar plates as depicted in (**a**). Here, four separate yeast cultures have been spread out for single colonies in the quadrants of the agar plate. Single colonies by definition can only be used once. There is potential that the different single colonies may have genetic differences. Therefore, if desiring a stock culture that is clonal and can be used multiple times, patches can be made by picking a single colony and spreading it as depicted in (**b**), where four such patches are displayed. In both cases, allow 2–3 days of growth on a galactose agar plate a 30 °C

4. Pick a colony from the working stock plate and inoculate 5 ml medium in a sterile glass tube. Use YPD to culture non-transformed yeast or appropriate dropout media for transformed yeast. Briefly vortex to disperse the cells from the picked colony in the media. Incubate at 30 °C overnight with agitation. This overnight culture will yield cells in stationary phase (OD600 ~ 1–1.5).

3.2 Culturing Pooled Collections of Yeast Libraries from 96 Well Arrays

1. Thaw each 96-well plate of knockout yeast and replicator pin or multi-channel pipette (5 μl) yeast into 100 μl YPD media in a fresh 96-well plate and grow at 30 °C for 48 h.
2. Combine all wells from each plate and aliquot to make plate pools (1–2 ml aliquots).
3. Media from each plate can be further mixed and aliquoted to make full library pools.
4. Make glycerol stocks for freezing by combining pool libraries with 15–25% final concentration of glycerol and storing at −80 °C.

3.3 Small-Scale Plasmid Transformation

In order to utilize yeast for the study of coronaviruses, it is necessary to transform plasmid constructs into the yeast for exogenous protein expression. Again, for simplicity, only a lithium acetate protocol will be discussed here, but other approaches can be used. We direct the reader to [2, 3] for additional protocols.

1. Transformation is most efficient when cells are in mid-log phase (OD600 ~ 0.4–0.8). The overnight culture described above would therefore yield too much growth. Two approaches can be used to culture cells for transformation; either perform a tenfold dilution series after the initial inoculation of YPD from a single colony. Disperse cells in 10 ml YPD by briefly vortexing, transfer 1 ml of this media into 9 ml, and repeat 4–6 times. Culture all tubes overnight at 30 °C and assess OD600 the following day. Alternatively, culture cells overnight as above, then the following day dilute in fresh YPD to an OD600 around 0.2–0.3 and grow for 3–5 h at 30 °C.
2. Once cells are at the appropriate OD, pellet cells by centrifugation at 1500 × *g* for 3 min.
3. While cells are being washed (ensuing steps), heat yeast carrier DNA at 95 °C for 5 min then place on ice for at least 5 min prior to use.
4. Resuspend cells in 10 ml molecular biology-grade water.
5. Pellet cells by centrifugation again.
6. Resuspend cells in 10 ml 0.1× TE/0.1 M lithium acetate (diluted in water).
7. Pellet cells by centrifugation again.
8. Resuspend cells in 1/20th of the original growth volume (for a 10 ml culture, this would be 500 μl) of 0.1× TE/0.1 M lithium acetate.
9. Make transformation mixture: mix 50 μg yeast carrier DNA, 50–200 ng of plasmid DNA in a microcentrifuge tube, then add 50 μl of resuspended cells. Finally, add 350 μl PEG solution (40% PEG/1× TE/100 mM lithium acetate, shake to mix thoroughly before and after the addition to transformation mixture).
10. Incubate transformation mixture at 30 °C for 20 min.
11. Incubate mixture at 42 °C for 20 min.
12. Add 700 μl molecular biology-grade water, mix and centrifuge for 3 min at maximum speed in a table-top microcentrifuge.
13. Remove supernatant.
14. Resuspend pellet in 100 μl molecular biology-grade water.
15. Plate out all 100 μl to an agar plate of appropriate dropout media for the selection of transformants. Spread with a sterile glass rod or sterile glass beads.
16. Incubate transformation plate at 30 °C for 48 h.
17. Colonies will form after 2 days if transformation was successful.

18. Pick a colony or colonies and streak for singles onto a fresh agar plate. Incubate for a further 48 h to give a working stock plate that individual colonies can be picked from for experimental use.

3.4 Large-Scale Plasmid Transformation

The above plasmid transformation is best for transforming individual cultures of yeast with individual plasmids. However, it may be desirable to transform in a higher content manner, for example, to transform a yeast library or to transform multiple plasmids into different cultures of yeast. The above protocol can be adapted to this end. Two examples will be given, either transformation of a pooled collection of a yeast library or transformation of libraries in 96-well plates.

3.4.1 Large-Scale Plasmid Transformation of Pooled Yeast Library

1. Grow up overnight culture of pooled collection of a yeast library. 100 μl of glycerol stock into 50 ml of YPD. Incubate overnight with shaking at 230–270 rpm at 30 °C.
2. The following morning put this overnight culture (OD600 ~ 1–1.5) at 4 °C.
3. Produce overnight dilution series cultures (16–18 h culture). Produce 1:10 dilution series for 6–8 dilutions all of 50 ml. Culture with shaking (230–270 rpm) at 30 °C. This approach should generate yeast culture in mid-log phase of growth (OD600 ~ 0.4–0.8) at one of the dilutions.
4. Following the second overnight culture, pick the dilution level that has resulted in the best growth level for mid-log phase growth.
5. Pellet the cells by centrifugation at 1500 × *g* for 3 min.
6. While cells are being washed (ensuing steps), heat yeast carrier DNA at 95 °C for 5 min, then place on ice for at least 5 min prior to use.
7. Remove supernatant from cells and resuspend with 10 ml molecular biology-grade water.
8. Pellet again and remove supernatant.
9. Resuspend cells in 10 ml 0.1× TE/0.1 M lithium acetate in water.
10. Pellet again and remove supernatant.
11. Resuspend cells in 500 μl of 0.1× TE/0.1 M lithium acetate (a 1/100th of the original culture volume).
12. Make transformation mix in *two* separate microcentrifuge tubes (values provided are per tube): mix 125 μg yeast carrier DNA and 2.5 μg of plasmid DNA in a microcentrifuge tube, then add 125 μl of yeast cells. Finally add 875 μl of PEG solution (40% PEG/1× TE/100 mM lithium acetate, shake to mix

thoroughly before and after addition to transformation mixture).

13. Incubate transformation mixture tubes at 30 °C for 20 min.
14. Incubate mixtures at 42 °C for 20 min.
15. Add 500 μl molecular biology-grade water and mix.
16. Centrifuge at maximum speed in a bench-top centrifuge for 3 min.
17. Remove supernatant and resuspend each pellet in 1 ml molecular biology grade water. Combine the 1 ml of resuspended cells from each microcentrifuge tube in a falcon tube and make final volume up to 10 ml with further addition of water.
18. Plate all 10 ml out using 200 μl per plate on appropriate dropout and sugar agar plates (~50 plates). For example, URA dropout and glucose-containing plates. Spread with a sterile glass rod or sterile glass beads.
19. Incubate at 30 °C for 48 h.
20. Following incubation, collect colonies and make glycerol stocks: add 3 ml sterile PBS or molecular biology grade water to each plate and swirl/pipette up and down until colonies visibly detach and go into solution. Collect the 3 ml from each plate into a falcon tube(s). Wash each plate with a further 2 ml sterile PBS or molecular biology-grade water and add to the original collections. Centrifuge at 1000 × *g* for 5 min to pellet the collected cells. Resuspend in a final volume of 20 ml appropriate selection media with sugar. Mix resuspended yeast with glycerol for stocks (as discussed previously). The resuspended yeast can also be directly plated out for suppressor screening (discussed below, also depicted in Fig. 3).

3.4.2 Large-Scale Plasmid Transformation in 96-Well Format

1. Grow up overnight cultures for transformation. Thaw 96 well glycerol stock plates (as supplied with certain libraries purchased from companies such as Dharmacon). Pipette 5 μl or use a microplate replicator to transfer from the glycerol stock into 600 μl YPD media in a 96-deep-well plate.
2. Culture overnight at 30 °C with shaking at 230–270 rpm.
3. Pellet cells by centrifugation of 96-deep-well plate at 1500 × *g* for 5 min.
4. While cells are being washed (ensuing steps), heat yeast carrier DNA at 95 °C for 5 min, then place on ice for at least 5 min prior to use.
5. Remove YPD media from wells.
6. Resuspend cells in 100 μl water and transfer to a clear plastic 96 well plate.

7. Pellet cells by centrifugation again.
8. Resuspend cells in 96-well plate with 50 µl of transformation mix made in the following way: 1.5 ml 1 M lithium acetate, 2 ml of 2 mg/ml yeast carrier DNA (dilute with 1× TE if using starting with a high concentration stock), 10 µg plasmid DNA (0.1 µg per well). Make volume to 5 ml with molecular biology grade water. These values are per 96-well plate.
9. Gently vortex plate to disperse cells in the transformation mix.
10. Add 100 µl 50% PEG to the 50 µl transformation mixture in each well.
11. Put on a shaker at 230–270 rpm for 5 min at 30 °C to mix.
12. Incubate for 1 h at 42 °C.
13. Pellet cells by centrifugation at 1500 × *g* for 10 min.
14. Remove supernatant.
15. Resuspend cells in 50 µl of appropriate selection media with sugar.
16. Transfer the 50 µl of resuspended cells to new 96-deep-well plates containing 600 ul selection media with sugar per well.
17. Incubate at 30 °C for 48 h with shaking at 230–270 rpm.
18. From here, new glycerol stocks of the transformed yeast can be made by mixing cultured yeast with 30% glycerol at a 1:1 ratio and storing at −80 °C. Yeast can also be plated for suppressor screening.

3.5 Protein Extraction

To confirm expression of exogenous protein in the yeast, a protein extraction and western blot is the best approach. Here the NaOH protein extraction is discussed, but as previously, various other approaches can be used (*see* [2, 3]).

1. Grow an overnight liquid culture of yeast. Pick colonies from the struck singles plate after transformation and grow up in appropriate dropout liquid media with the appropriate induction for expression such as galactose (3–5 ml of culture). Glucose media with the transformed yeast, empty vector transformed yeast, or wild-type yeast grown in YPD can all act as negative controls.
2. Culture overnight at 30 °C to reach stationary phase as described previously (OD600 ~ 1.5).
3. From the overnight culture, take 100–500 µl into a microcentrifuge tube.
4. Pellet yeast cells by centrifugation at 900 × *g* for 3 min.
5. Remove supernatant and resuspend pellet in 100 µl of 0.1 M NaOH.
6. Incubate for 10 min at RT.

7. Pellet cells by centrifugation as above.
8. Remove supernatant and resuspend pellet in 100 μl 1× western blot buffer with 0.5% beta-mercaptoethanol.
9. Heat sample at 95 °C for 5 min.
10. Centrifuge at maximum speed in a table-top centrifuge for 10 min.
11. Use 10 μl (or more if necessary) of this sample in standard SDS–PAGE/western blotting protocols to confirm expression of exogenous protein.

3.6 Identification of Slow-Growth Phenotypes

As discussed above, there are various yeast phenotypes that can be used for suppressor screening. Here slow growth will be discussed. We direct the reader to [2, 3] for more detailed discussions of alternative screenable phenotypes in yeast. Two methods can be used to identify slow-growing yeast, growth curve analysis, or serial dilution drop cultures.

3.6.1 Growth Curve Analysis

Growth curves are most easily performed with an automated plate reader, capable of measuring OD600, with the ability to incubate at 30 °C. Alternatively OD can be measured using a spectrophotometer. Cultures can be made in either 96-well or 384-well format using clear non-tissue culture-treated plates. This protocol describes a 96-well format, which can be adjusted as necessary for a 384-well format.

1. Make a liquid culture of yeast by picking an individual colony of transformed yeast and culturing in 3–5 ml appropriate selection media with lack of induction of viral gene expression to allow equivalent growth between control and test yeast. For example, if using a GAL1 vector, use raffinose- or glucose-containing media. Culture at 30 °C for 48 h if using raffinose or 24 h if using glucose to reach stationary phase (*see* **Note 6**).
2. Dilute this liquid culture to an appropriate level for growth analysis over the next 48 h. A dilution factor of 1:100 to 1:10,000 is usually appropriate as this will give a starting OD600 close to or slightly lower than 0.1. This will allow sufficient growth to be analyzed over the following 48 h period. Dilute the starting culture in appropriate media to induce exogenous gene expression (or inhibit for control—galactose and glucose for the GAL1 system). Make enough culture to plate 100 μl per well of a 96-well plate.
3. Analyze on automated plate reader for 48 h. Set plate reader to incubate at 30 °C. Take OD600 readings at least every 30 min over this period. Prior to reading OD600, have the plate reader shake at ~230 rpm for 30 s to disperse yeast in the culture.
4. Analyze generated growth curves to identify yeast colonies with a growth defect compared to control cells.

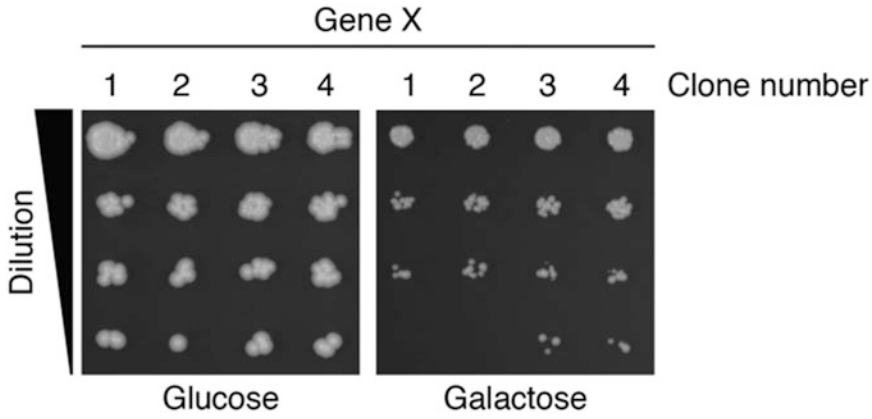

Fig. 2 Yeast serial dilution drop cultures for an example gene ("gene x") that causes a slow-growth phenotype when expressed. For this set up, four colonies of yeast with gene x expression plasmid were picked from a streak plate and struck for patches (*see* Fig. 1). Liquid cultures were made from these four patches and then serial drop dilution series made as described in the Methods section. This was plated onto glucose or galactose plates and incubated for 2–3 days. The growth inhibition is displayed by fewer colonies in the more dilute samples

3.6.2 Serial Dilution Drop Cultures

This approach involves making serial dilutions of yeast from overnight cultures and plating these onto agar plates to induce expression of the gene of interest (e.g., galactose-containing plates for GAL1 vector). *See* Fig. 2 for an example image.

1. Grow overnight culture from single transformation colonies (for control vector and gene of interest).
2. Test the OD600 and overnight culture into molecular biology-grade water or culture media to an OD 0.1.
3. Produce a twofold or fivefold dilution series from the original overnight cultures in molecular biology-grade water or culture media (up to 10 should be sufficient).
4. Plate 2–5 μl of the dilution series at even spacing onto an agar plate. This is most easily achieved using 15 cm agar plates and a multi-channel pipette (using a 96-well plate for producing the dilution series) or a 96-well microplate replicator (as was done in Fig. 2).
5. Incubate for 2–3 days until colonies form.
6. Assess growth defects by reduced growth at equivalent dilutions between gene of interest expressing yeast and control vector yeast.

3.7 Suppressor Screening

By this stage, viral genes have been tested for phenotypes that can be used in suppressor screening (e.g., slow growth), and yeast libraries have been transformed with plasmids to express those viral genes. Two formats for library transformation have been

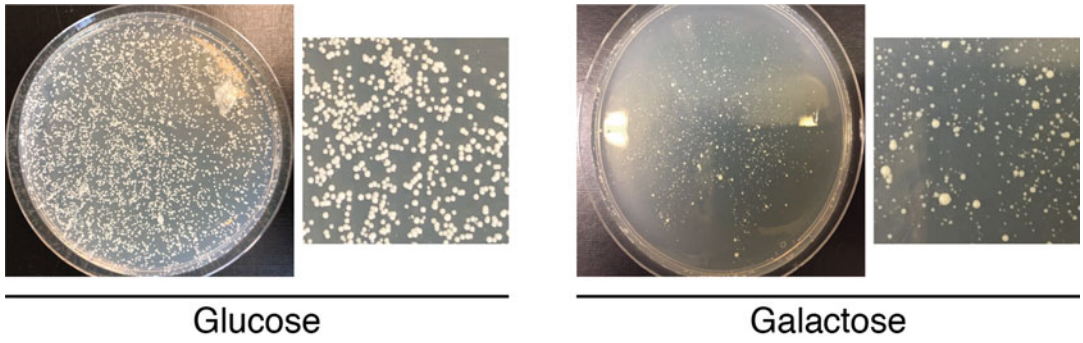

Fig. 3 Example images of a yeast knockout library transformed with a slow-growth phenotype inducing expression plasmid. On glucose plates, there is no expression of viral protein from the plasmid so all colonies can grow to a similar size. On the galactose plate, suppressors are expected to be the colonies that grow to a larger size

discussed, either transformation of a pool of the library or transformation of individual wells of an arrayed library. At this stage, the suppressor screen can be performed by plating libraries out on appropriate induction agar plates (e.g., galactose plates for a GAL1 promoter) and looking for faster growing colonies (larger colonies arising from suppression of the slow-growth phenotype). In the case of a pooled library, liquid cultures need to be grown from glycerol stocks (or from the original transformation collection) and spread onto agar plates. For the arrayed libraries, yeast can be multi-channel pipetted or multiple plate replicator plated onto 15 cm agar dishes. These plates can then be grown for 2–3 days and analyzed for fast-growing colonies which will appear as larger colonies (Fig. 3). The below-outlined protocol will discuss the approach for a pooled library, but the same principals apply for screening arrayed libraries.

1. Pick large colonies which are suspected to have a suppressor phenotype. Generate a streak plate on selection plates (e.g., galactose-containing agar plates). Allow the streak plates to grow for 48 h at 30 °C. Multiple isolated colonies from the original large colony can then be tested for higher accuracy.
2. Confirm suppressor phenotype using growth curve analysis or serial drop dilution.
3. Once a suppressor phenotype has been confirmed by growth analysis, confirm expression of the viral protein using NaOH protein extraction and western blotting. If a liquid growth curve was used for analysis, that final culture can be used in the protein extraction protocol. If serial drop dilution was used, colonies can be picked from serial drop dilutions and grown in appropriate induction media (e.g., galactose) to produce a liquid culture from which to extract protein. Alternatively, fresh colonies can be picked from the streak plate and grown up for NaOH protein extraction.

4. Once colonies have been found that express the viral protein and have enhanced growth rates, a "hit" has been found.

3.8 Identifying "Hit" Genes

In suppressor screening, a hit is defined by a yeast clone that has a suppression of the phenotype in question (e.g., slow growth), while maintaining expression of the viral protein responsible for that phenotype. In this case, the suppression is a result of loss or overexpression of a host gene depending if using a knockout or overexpression library. Each library has its own approach to determining the host gene of interest. Here, we will discuss finding host gene of interest in the knockout library. The original yeast knockout library was designed by homologous recombination of gene cassettes with uniform sequences, with the exception of a unique identifier region. Therefore, by performing a genomic DNA extraction from "hit" yeast clones and using specific primers for PCR amplification and DNA sequencing, the unique region can be found and searched within the database. For further detail, see http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html

3.8.1 Genomic DNA Extraction

1. Make a liquid culture from a single colony to test by overnight growth in glucose-containing media.
2. Centrifuge 1 ml of the overnight cultured yeast in a tube suitable for use in a bead beater homogenizer at 1500 × *g* for 3 min.
3. Aspirate supernatant and resuspend pellet in 0.2 ml yeast lysis buffer and 0.2 ml phenol:chloroform. Add a roughly similar volume of glass beads.
4. Homogenize cells in bead beater (2× 1 min at 6000 rpm).
5. Add 0.2 ml 1× TE and vortex briefly.
6. Centrifuge tubes at maximum speed in a microfuge for 5 min.
7. Take the aqueous fraction into a DNase/RNase-free microcentrifuge tube (roughly 0.38 ml). Discard the remaining sample.
8. Add 2× volume (0.76 ml) of 100% ethanol to 1 volume aqueous phase (0.38 ml). Mix thoroughly by vortexing.
9. Centrifuge at maximum speed in a microfuge for 3 min to pellet DNA.
10. Discard supernatant from the pellet.
11. Rinse the pellet with 0.5 ml of 4 °C 70% ethanol. Add slowly to the tube to minimize disruption of pellet. Centrifuge at maximum speed in a microfuge for 1 min.
12. Remove supernatant from the pellet.
13. Allow pellet to air dry.
14. Resuspend DNA pellet in 100 μl TE buffer.

15. RNase treat prior to PCR and subsequent sequencing. Use 25 μl of extracted DNA with an appropriate amount of RNase (depending on supplier).
16. From the extracted genomic DNA, a PCR can be run to amplify the unique identifier region for the knockout collection using "UPTAG" and "DOWNTAG" primers (http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html). DNA sequencing of the PCR product can be performed in any preferred manner (*see* **Note 7**).
17. Having identified yeast genes that may have roles in replication of coronaviruses, the mammalian homologs must be identified for further examination. Mammalian homologs can be found either through the yeast genome database (https://www.yeastgenome.org/) or BLAST searches. This provides a subset of genes to assess for replication of coronaviruses in mammalian cell culture through siRNA/CRISPR or other disruptive techniques.

4 Notes

1. The American Type Culture Collection (ATCC) is used as the key general laboratory source of yeast strains for many laboratories. The strains used in the protocols described here are haploid strains (BY4742 and BY4741). A diploid strain (BY4743) is also available combining these two haploid strains [4, 5]. The auxotrophic markers of the strains are: BY4741: *MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0* and BY4742 *MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*. These markers allow for dropout media to be used for selection. In the system we describe here, the plasmid has the *URA3* gene on it for selection; however, alternative auxotrophic marked plasmids are available [6].
2. Yeast libraries are commercially available in arrayed formats with individual yeast clones in 96-well plates (e.g., Horizon Discovery Inc.). These libraries can be combined into pools, which is the main screening strategy described here [7]. Single knockout screening using robotic arraying have also been performed, which will briefly be mentioned [8, 9].
3. To make CAA/YNB medium, make the following: For 1 l, dissolve 1.7 g yeast nitrogen base and 5 g ammonium sulfate in 450 ml ddH_2O in bottle 1 and 6 g casamino acids in 450 ml ddH_2O in bottle 2. Autoclave separately, combine, and then add 100 ml 20% sterile sugar solution. Sugar options (depending on the purpose as discussed in the Methods section): 20% glucose, 20% galactose, or 20% raffinose. All are sterile-filtered

through a 0.2 μM filter after dissolving sugar in water. For CAA/YNB agar plates, add 2% agar into bottle 2 prior to autoclaving. Combine bottle 1 and 2, add sugar, and pour in plates.

4. The plasmids used in these protocols are standard yeast overexpression plasmids based on those published in Mumberg et al. [6]. These plasmids can have auxotrophic markers of HIS3, TRP1, LEU2, or URA3 in combination with promoters containing a constitutively expressing promoter (TEF1) or a galactose-inducible promoter (GAL1). The galactose-inducible promoter plasmid system with a URA3 auxotrophic marker is used in the system described here. Similar to mammalian expression plasmids, it is possible to add tags to any of the viral proteins of interest in these yeast expression systems through standard cloning procedures. The galactose-inducible system is regulated by the carbon source and the yeast used for growth. In the presence of 2% glucose, the GAL1 promoter is actively repressed and no transgene is expressed. When yeast carrying the plasmid are grown in 2% galactose, the transgene is expressed to high levels. There is also the option to grow yeast in 2% raffinose which neither activates nor represses expression from the GAL1 promoter.
5. Yeast cultures can be contaminated by other yeast or bacteria. It is not necessary to work in a biosafety hood, but use of sterile equipment and reagents is advisable.
6. When inducing expression of proteins in the GAL1 system that is used here, it is worth noting that cultures may grow slower in galactose- or raffinose- containing media than glucose counterparts as these sugars are utilized less efficiently.
7. Access to DNA sequencing is required for the identification of gene knockouts if using a pooled collection of the yeast libraries rather than arrayed collections. See http://www-sequence.stanford.edu/group/yeast_deletion_project/PCR_strategy.html for information regarding required PCR primers to amplify the tag sequence that is required for the identification of knockout yeast.

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Chapter 16

Determining How Coronaviruses Overcome the Interferon and Innate Immune Response

Christopher M. Coleman

Abstract

All viruses have to overcome the innate immune response in order to establish infection. Methods have been developed to assay if, and how, viruses overcome these responses, and many can be directly applied to coronaviruses. Here, in vitro methods to determine how coronaviruses overcome this response are described.

Key words Coronaviruses, Innate immunity, Interferon, Antagonism of innate immunity, Virus protein function

1 Introduction

One of the key interactions in the pathogenesis of any viral infection is that between the virus and the host innate immune response. An effective innate immune response can clear the virus before infection is established. Therefore, most viruses have evolved mechanisms to block and/or antagonize the innate immune response.

The innate immune response is the first line of defence against an invading pathogen, including viruses. Once recognized as pathogens by pattern recognition receptors (PRRs), viruses often trigger innate immune signaling pathways that lead to the production of the type I interferons (IFNs), IFNα and IFNβ. Examples of the signaling pathways that trigger these responses are the retinoic acid-inducible gene (RIG)-I and the interferon regulatory factor (IRF)-3 pathways. RIG-I is a PRR that recognizes viral RNA (single or double stranded) carrying uncapped 5′ triphosphate groups [1]. IRF3 is a key signaling molecule in a number of virus-triggered innate signaling pathways, including the RIG-I pathway [2].

Tumor necrosis factor (TNF)-α is a cytokine induced upon viral infection and induces upregulation of various antiviral mechanisms, including apoptosis of infected cells. The TNFα signaling pathway

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_16,

signals via the transcription factor, nuclear factor kappa light chain enhancer of activated B cells (NFκB).

Coronaviruses have long been studied for their ability to induce and block innate immune responses. In recent years, we have performed methods to determine the innate immune response to Middle East respiratory coronavirus (MERS-CoV) in vitro [3] and in vivo [4]. Here, methods established for MERS-CoV will be discussed, but can be applied to any other coronaviruses.

2 Materials

2.1 Cloning of Coronavirus Genes

1. Thermocycler.
2. Sample of coronavirus RNA.
3. Optional: RNA extraction reagent, e.g., TRIzol™ reagent.
4. Optional: RNA purification kit suitable for use with TRIzol™ or similar samples.
5. High-fidelity reverse transcriptase (RT)-PCR kit to convert viral RNA to cDNA.
6. Primers, as follows:
   (a) The 5′ primer is designed as follows: [GATC][5′ restriction site (sense)]AAC**ATG**[10–15 bases of gene (sense)].
   (b) The 3′ primer is designed as follows: [GATC][3′ restriction site (antisense)][10–15 bases of gene (antisense)].
7. Taq DNA polymerase kit for the amplification of cDNA.
8. Agarose.
9. 50× TAE buffer: 50 mM EDTA, 2 M Tris, 1 M glacial acetic acid. Also commercially available.
10. Microwave.
11. Agarose gel apparatus.
12. Gel extraction kit.
13. Restriction enzymes and buffers corresponding to restriction sites designed in primers.
14. DNA Ligase.
15. Competent bacteria.
16. Materials for transformation of bacteria (e.g., by heat shock or electroporation).
17. LB-agar plates containing suitable antibiotic for selection.
18. Mini and Maxiprep kits.

2.2 Luciferase Assays for Innate Immune Signaling

1. HEK293T cells.
2. HEK293T cell media: Dulbecco's modified Eagles media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.
3. Cell culture plates.
4. 37 °C CO_2 incubator.
5. Plasmids as follows:
   (a) Reporter plasmids: a plasmid containing the luciferase gene under the control of either an IFNβ promoter or an NFκB promoter.
   (b) Plasmid containing MERS-CoV gene (or empty plasmid).
   (c) Inducer plasmids: a plasmid containing N-RIG-I or IRF-3.
6. Transfection reagent. Various methods and commercially available kits are available and HKE293T cells are relatively easy to transfect.
7. Recombinant TNFα.
8. A luciferase reporter assay. Various commercial options are available.

2.3 Virology Assay

1. HIVΔVpu containing plasmid.
2. HEK293T cells.
3. HEK293T cell media: Dulbecco's modified Eagles media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.
4. Cell culture plates.
5. 37 °C CO_2 incubator.
6. Plasmid containing MERS-CoV gene (or empty plasmid).
7. Transfection reagent.
8. HIV-1 p24 enzyme-linked immunosorbent assay (ELISA) kit.
9. Plate reader capable of reading ELISA output.

3 Methods

In any discussion of the innate immune response, it is always worth noting that the immune response is complex and can, often, only be seen in full in animal models. However, methods in this area are necessarily highly specific for specific coronaviruses and hosts. For example, we were able to use a novel mouse model to determine the immune cell infiltration and immune gene activation induced by MERS-CoV infection [4]. Here, methods for the in vitro assessment of virus: host interactions are described.

3.1 Cloning of Individual Coronavirus Genes into Plasmids

To determine specific virus: host interactions, it is usually necessary to clone and express individual coronavirus genes into expression plasmids. We have had much success in this with MERS-CoV [3]. This method used the AAV-CAGGS-EGFP expression plasmid, but others can be used.

1. Obtain a sample of MERS-CoV RNA.
   (a) Commercially available RNA samples, such as from the American Type Culture Collection.
   (b) RNA extraction from MERS-CoV infected cells using, e.g., TRIzol™ reagent (or similar) according to the manufacturers' instructions. Infection with MERS-CoV requires containment level (CL) 3 and has been described previously [5].
2. Perform cDNA synthesis using commercially available high-fidelity RT-PCR kit.
3. Perform DNA synthesis using primers designed to amplify gene of interest and allow insertion into plasmid.
4. Run DNA mixture on 1% agarose (in 1× TAE buffer) gel using electrophoresis.
5. Cut out band that corresponds to expected size of target gene and purify using a gel extraction kit, following manufacturer's instructions.
6. Cut out band and plasmid using appropriate restriction enzymes and buffers.
7. Run DNA mixtures on 1% agarose gel using electrophoresis.
8. Cut out band that corresponds to expected size of target gene and the empty plasmid and purify using a gel extraction kit, following manufacturer's instructions.
9. Ligate gene into plasmid.
10. Expand plasmid by bacterial transformation and miniprep methods using a suitable antibiotic for selection.
11. Verify plasmid by sequencing.
12. Expand plasmid using maxiprep methods.

3.2 Luciferase Assays for Innate Immune Signaling

To determine the inhibition of innate immune signaling, reporter assays can be used. We have reported assays for three innate immune signaling pathways: RIG-I-dependent IFNβ promoter activation, IRF-3-dependent IFNβ promoter activation, and TNF-α-dependent NFκB promoter activation [3].

1. Seed HEK293T cells into 48-well plates at a density sufficient to achieve 90–95% confluency the following day.
2. Culture overnight at 37 °C in 5% CO_2.

3. Co-transfect cells with the following plasmids (a total of 600 ng of plasmid DNA) using an appropriate transfection reagent (*see* **Note 1**):
   (a) 200 ng luciferase reporter plasmid.
   (b) 200 ng viral gene expression plasmid or empty vector control.
   (c) 200 ng inducer plasmid (if assaying IFNβ promoter activation).
4. Culture for 18 h at 37 °C in 5% CO_2.
5. For TNFα-dependent NFκB promoter activation only: treat cells with 30 ng recombinant TNFα for 6 h.
6. Perform luciferase assay using the luciferase reporter assay according to the manufacturers' instructions (*see* **Note 2**).
7. Calculate the relative luciferase expression in viral gene expressing cells compared to the empty plasmid controls.

3.3 Virology Assays Using Sensitive Viruses

A valuable tool in the determination of the function of viral proteins is to use reporter viruses that are blocked by the pathway of interest. A good example of this is the use of HIVΔVpu that is restricted by the anti-viral restriction factor, tetherin [6].

HIVΔVpu has been used to identify viral proteins as antagonists of tetherin, or at least that a virus encodes at least one tetherin antagonist [7]. Because tetherin tethers HIV virions to the cell surface, cell-associated HIVΔVpu will be increased, and supernatant HIVΔVpu will be lower in tetherin-positive cells compared to tetherin-negative cells. HIV concentration can be easily quantified using commercially available assays for HIV p24.

3.3.1 HIVΔVpu Stock Generation

1. Seed HEK293T cells in 10 cm plates at a density sufficient to achieve 90–95% confluency the following day.
2. Culture overnight at 37 °C in 5% CO_2.
3. Transfect cells with plasmid containing HIVΔVpu (*see* **Note 3**).
4. Replace media 6–18 h after transfection.
5. Culture for 2 days at 37 °C in 5% CO_2.
6. Harvest supernatant from transfected cells, clarify by centrifugation at 500 × *g* for 5 min, aliquot, and store at −80 °C.
7. Determine titer of HIVΔVpu stock (*see* **Notes 4** and **5**).

3.3.2 Virology Assay

1. Seed a tetherin-positive, HIV-sensitive cell line (this may require transfection/transduction of cells with tetherin and/or CD4) into 24-well plates (*see* **Note 6**).
2. Culture overnight at 37 °C in 5% CO_2.
3. Transfect cells with MERS-CoV gene expression plasmid or an empty plasmid control.

4. Culture overnight at 37 °C in 5% CO_2.
5. Infect cells with a fixed titer of HIVΔVpu (*see* **Note 7**).
6. Culture for up to 3 days at 37 °C in 5% CO_2 (*see* **Note 7**).
7. Harvest supernatant and cell lysate from infected cells.
8. Quantify the levels of HIV p24 using commercially available ELISA assays (*see* **Note 5**).
9. Compare supernatant levels of HIV-1 p24 in cells expressing coronavirus proteins to the empty vector control.

3.4 Coronavirus Infectious Clones

Instead of using a true reporter virus, such as HIVΔVpu, it is possible to use coronavirus mutants directly, as in Taylor et al. [8]. Infectious clones have been synthesized for various coronaviruses, and methods for these have been described elsewhere for MERS-CoV [9, 10]. When mutants are created, these can be used in lieu of the HIVΔVpu described above, along with appropriate methods of quantification of output [5]. However, this method replies on the ability to handle viruses at the full containment level of the parent virus (e.g., CL3 for MERS-CoV), so may be inappropriate.

4 Notes

1. Methods of transfection are deliberately not discussed as these vary significantly by laboratory. HEK293T cells are very easy to transfect and a wide range of methods work with these cells.
2. There are various commercially available luciferase assays. Luciferase reagent can also be "homemade," and recipes are available online.
3. One commercially available example of a HIVΔVpu strain is pNL-U35 available from the NIH AIDS Reagent Program.
4. There are various published methods for quantifying HIV concentration using commercially available reagents—including p24 ELISA (*see* **Note 5**) or GHOST cell titration [11].
5. When purchasing HIV-1 p24 ELISA, take note of the upper and lower limits of the assay, as some are highly sensitive, but may have too low an upper detection limit. In contrast, the AIDS Vaccine Program produces an ELISA with a wide range of detection, but is not as useful at low concentrations.
6. One example of a constitutively tetherin-positive cell line is HeLa cells [6].
7. Exact titer and time points will depend on infectibility of cells and sensitivity of p24 assay (*see* **Note 5**) and should be worked out in preliminary experiments.

Acknowledgment

The author would like to thank the laboratory of Dr. Matthew Frieman for providing background and details for some methods.

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Chapter 17

Ribopuromycylation in Coronavirus-Infected Cells

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Abstract

Ribopuromycylation enables the visualization and quantitation of translation on a cellular level by immunofluorescence or in total using standard western blotting. This technique uses ribosome catalyzed puromycylation of nascent chains followed by immobilization on the ribosome by antibiotic chain elongation inhibitor emetine. Detection of puromycylated ribosome-bound nascent chains can then be achieved using a puromycin-specific antibody.

Key words Ribopuromycylation, Translation, Puromycin, Emetine, Puromycylation

1 Introduction

Ribopuromycylation (RPM) is a method that allows visualization and quantitation of global or cellular translation via the addition of puromycin to ribosome bound nascent polypeptide chains [1]. Puromycin (PMY) is an aminonucleoside tyrosine tRNA mimetic that enters the A site of prokaryotic and eukaryotic ribosomes and is covalently incorporated by ribosome-catalyzed reaction into the nascent chain C terminus, resulting in termination of translation [2]. Eggers et al. first used puromycin as a tag for nascent proteins using polyclonal PMY antibodies for immunoblotting and immunoprecipitation of nascent proteins [3]. Following this work, Schmidt et al. produced monoclonal PMY antibodies to overcome nonspecific interactions of polyclonal antibodies for use by flow cytometry to measure relative translation rates in cells exposed to PMY [4]. David et al. improved on their initial RPM protocol by the addition of emetine. Emetine is a translation elongation inhibitor that irreversibly binds the 40S ribosomal subunit. When compared to cycloheximide, another translation elongation inhibitor, emetine was shown to increase RPM signal [5]. RPM is now a widely used technique for the study of cellular translation [6–9]. RPM is a useful technique to compare the level of translation when using translation inhibitors, viral infections, stressors,

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_17,

different cell lines, or disease models. The technique can be utilized for both immunofluorescence and western blot detection. The western blot method allows total protein translation to be visualized, similar to ^{35}S Met labeling, and is useful to visualize an overall change or differences at the population level. Importantly, the immunofluorescence method allows for translation to be visualized on a single-cell level in which individual cells, for example, infected and uninfected, can be compared.

2 Materials

2.1 RPM to Tag Nascent Polypeptides

1. Coverslips.
2. Tweezers.
3. 6- and 24-well plates.
4. Vero cells.
5. EMEM: Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum 2 mM L-glutamine and 2.2 g/l sodium bicarbonate.
6. IBV strain BeauR [10].
7. Incubator.
8. BES medium (IBV infection media): 1× Eagle's minimum essential medium (EMEM), 0.3% tryptose phosphate broth, 0.2% bovine serum albumin (BSA), 20 mM *N,N*-bis 2-hydroxethyl-2-aminoethanesulfonic acid (BES), 0.21% sodium bicarbonate, 2 mM L-glutamine, 250 U/ml nystatin, 100 U/ml penicillin and streptomycin (*see* **Note 1**).
9. 50 mM sodium arsenite.
10. 21.2 mM puromycin.
11. 180 nM emetine.

2.2 Detection of Tagged Nascent Polypeptides by Immunofluorescence (IF)

1. Phosphate-buffered saline (PBS).
2. 4% paraformaldehyde in PBS.
3. 0.1% Triton ×100 in PBS.
4. Monoclonal anti-puromycin antibody (*see* **Note 2**).
5. 0.5% BSA in PBS.
6. Fluorescently conjugated secondary antibody, e.g., anti-mouse Alexa Fluor 488.
7. 20 mg/ml 4′,6-diamidino-2-phenylindole (DAPI).
8. Mounting media.
9. Nail varnish.
10. Microscope slides.

11. Rocking platform shaker.
12. Confocal microscope.

2.3 Detection of Tagged Nascent Polypeptides by Western Blot (WB)

1. 4× Laemmli sample buffer (sample buffer): 277.8 mM Tris–HCl pH 6.8, 44.4% glycerol, 4.4% sodium dodecyl sulfate (SDS), 0.02% bromophenol blue, 10% β-mercaptoethanol.
2. Sonicator.
3. SDS-PAGE gel equipment, e.g., Biorad mini protean IV or other suitable electrophoresis equipment.
4. Western blotting equipment, e.g., Biorad Turboblot or other suitable western blotting equipment.
5. Nitrocellulose membrane.
6. Rocking platform shaker.
7. Phosphate-buffered saline (PBS).
8. PBST: 0.1% Tween-20 in PBS.
9. 5% milk in PBST (Marvel or equivalent).
10. Monoclonal anti-puromycin antibody (*see* **Note 2**).
11. Fluorescently/HRP-conjugated secondary antibody suitable for western blot.
12. Western blot imaging system.

3 Methods

3.1 RPM and Detection of Tagged Nascent Polypeptides by IF

1. Seed cells in a 24-well plate or equivalent on sterilized coverslips aiming for 60–80% confluency (*see* **Note 3**) at the time of puromycin addition [5] (*see* **Note 4**).
2. Wash cells with PBS.
3. Infect cells with 150 μl virus at the desired MOI or mock infect using 1x BES and incubate for 1 h at 37 °C (*see* **Note 5**).
4. Add 850 μl 1x BES and incubate at 37 °C for a further 23 h (*see* **Note 6**).
5. One hour prior to RPM, add 10 μl sodium arsenite to the existing media to give a final concentration of 500 μM and incubate at 37 °C (*see* **Note 7**).
6. At the time of RPM, add 8.7 μl puromycin to the existing media to give a final concentration of 18.4 μM and incubate for 30 s at room temperature (RT) (*see* **Note 8**).
7. Add 11.5 μl emetine to the existing media to give a final concentration of 208 μM and incubate for 1 min at RT (*see* **Note 9**).
8. Wash cells three times using RT 1x BES.

9. Fix cells using 4% paraformaldehyde for 15 min at RT (*see* **Note 10**).
10. All subsequent incubation steps should be performed at RT on a rocking platform shaker. Permeabilize cells using 0.1% Triton ×100 for 10 min.
11. Label cells with monoclonal anti-puromycin antibody (*see* **Note 2**) and anti-virus antibody for 1 h diluted as appropriate in 0.5% BSA in PBS.
12. Wash cells three times with PBS with no incubation between washes. After the third wash, incubate for 5 min.
13. Wash cells two further times including a 5-min incubation.
14. Label cells with fluorophore-conjugated secondary antibodies complimentary to the primary antibodies, diluted as appropriate in 0.5% BSA in PBS. Incubate for 1 h protected from light.
15. Wash cells three times with PBS with no incubation between washes. After the third wash, incubate for 5 min protected from light.
16. Wash cells two further times including incubation for 5 min protected from light.
17. Stain nuclei using DAPI diluted 1:20,000 for 4 min.
18. Replace nuclear stain with water.
19. Mount coverslips cell side down on a glass slide using mounting media and blot excess using paper towel (*see* **Note 11**).
20. Seal around coverslip using nail varnish.
21. Image on confocal microscope (*see* **Note 12**).

3.2 Immunofluorescence Quantification

1. Individual cellular puromycin signal can be quantified using image analysis software, e.g., ImageJ (Fiji) [11].
2. Puromycin signal for infected cells can be normalized using surrounding uninfected cells if using a suitably low MOI or using a mock infected well.
3. 50–100 cells for each treatment should be quantified.
4. Data can be plotted by scatter plot to represent individual values.

3.3 RPM and Detection of Tagged Nascent Polypeptides by WB

1. Seed cells in a six-well plate or equivalent aiming for 60–80% confluency (*see* **Note 3**) at the time of puromycin addition [5] (*see* **Note 4**).
2. Infect cells with 500 μl virus at the desired MOI or with BES medium for mock infected cells and incubate for 1 h at 37 °C (*see* **Note 5**).
3. Add 2.5 ml BES medium and incubate cells at 37 °C for a further 23 h (*see* **Note 6**).

4. One hour prior to RPM, add 30 µl of sodium arsenite to the existing media to give a final concentration of 500 µM and incubate at 37 °C (*see* **Note 7**).
5. At the time of RPM, add 26.1 µl of puromycin to the existing media to give a final concentration of 18.4 µM and incubate for 30 s at RT (*see* **Note 8**).
6. Add 34.5 µl of emetine to the existing media to give a final concentration of 208 µM and incubate for 1 min at RT (*see* **Note 9**).
7. Wash cells three times with PBS with no incubation between washes.
8. Add 150 µl sample buffer, scrape cells using a pipette tip and collect cells with a pipette into a microfuge tube.
9. Boil samples for 3 min (*see* **Note 13**).
10. Sonicate samples for 2 min at 70% amplitude or equivalent.
11. Boil samples again for 3 min.
12. Load samples onto an SDS-PAGE gel and run at 120 V until the loading dye has reached the bottom of the gel.
13. Transfer onto nitrocellulose using western blot transfer equipment.
14. All the subsequent incubation steps should be performed on a rocking platform shaker at room temperature. Block the membrane using 5% milk in PBST for 1 h.
15. Incubate the membrane with monoclonal anti-puromycin antibody diluted in 5% milk in PBST for 1 h (or overnight at 4 °C). It is also useful to label a housekeeping gene to allow signal normalization (e.g., GAPDH) and a viral marker to confirm infection on either separate or reused membranes. Multiple antibodies may be used on the same membrane where the western blot imaging system and primary antibody species allow.
16. Wash the membrane three times with PBST with no incubation between washes. After the third wash, incubate for 5 min.
17. Wash the membrane two further times including a 5-min incubation.
18. Incubate the membrane with a reporter-conjugated secondary antibody complimentary to primary antibody and compatible with the western blot imaging system.
19. Wash the membrane three times with PBST with no incubation between washes. After the third wash, incubate for 5 min.
20. Wash the membrane two further times including a 5-min incubation.
21. Image the membrane using western blot imaging system.

22. ImageJ (Fiji) or other analysis software can be used to quantify the band intensities for relative translation levels. At least three puromycin-stained bands should be quantified per lane for both viral and non-viral proteins if possible. Puromycin signal for non-viral (Y-axis) proteins and viral proteins (X-axis) can be plotted and the respective trendline and correlation equation can be compared between experiments to determine the level of shut off and the relative viral translation in response to the change in cellular translation.

4 Notes

1. Use infection media appropriate to the virus of interest.
2. A monoclonal puromycin antibody is suggested to prevent unwanted off-target binding.
3. It should be noted that precise puromycin and emetine addition timings will be difficult to maintain with high number of samples/wells. Therefore, we recommend a maximum of 4–6 samples at a time. If additional samples are needed; these should the processed independently.
4. Cells should be 60–80% confluent at the time of puromycin addition as when cells reach 90–100% confluent, cellular translation will decrease due to competition, lack of space, and cell stress. Therefore, ensuring cells are 60–80% confluent will provide a more accurate representation of cellular translation activity. During the experiment, the incubator door should be kept closed or opened as little as possible to reduce temperature fluctuation, which will cause cell stress and may reduce translation, especially in the time prior to RPM treatment.
5. Alter infection conditions to suit individual virus infection standard operating practice.
6. Virus infection duration can be adjusted to relevant timings specific to virus being used and to answer specific questions.
7. Translational inhibition via sodium arsenite treatment may need optimization for individual cell lines. A concentration range of 250–1000 μM is suggested. Incubation time can also be altered (e.g., 2 h).
8. Puromycin and emetine concentrations may need optimization for individual cells lines; suggested ranges include: puromycin 8–20 μM and emetine 80–210 μM.
9. Ensure puromycin and emetine volume is large enough to ensure rapid and even mixing to prevent variation in the puromycin signal throughout the well.

10. The protocol can be modified to look at localized sites of translation. This can be achieved by subjecting the cells to a coextraction/fixation procedure to remove free puromycin as detailed by Bastide et al. and V'Kovski et al. This addition allows the reduction of background puromycin signal and therefore increases the translation resolution [12, 13].
11. Use a pipette tip to carefully lever coverslip out of well and pick up using fine sharp or curved tweezers. Practice may be required to consistently remove coverslips from the wells as they are very easily broken.
12. Ensure confocal laser settings are kept constant throughout the experiment to allow accurate puromycin signal quantification and comparison across samples.
13. Samples can be frozen at −20 °C and further processed at a later stage after thawing.

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Part V

Imaging Coronavirus Infections

Chapter 18

Visualizing Coronavirus Entry into Cells

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Abstract

Coronavirus entry encompasses the initial steps of infection, from virion attachment to genome release. Advances in fluorescent labeling of viral and cellular components and confocal imaging enable broad spectrum studies on this process. Here, we describe methods for visualization of coronavirus entry into immortalized cell lines and 3D tissue culture models.

Key words Coronavirus, Entry, Endocytosis, Internalization, Confocal microscopy

1 Introduction

Coronavirus entry is initiated by interaction between the trimeric spike (S) protein and its receptor, which is expressed on the surface of the susceptible cell. During entry, the S protein undergoes structural rearrangement, which brings the cellular and viral membranes into proximity to mediate fusion. Such a structural switch may be triggered by different stimuli, including receptor binding, proteolytic cleavage of the S protein, and/or acidification of the microenvironment. The requirement for specific stimuli is species-dependent, and consequently, different coronaviruses enter cells at various subcellular sites. Some coronaviruses fuse at the plasma membrane, whereas others are believed to enter the cell through receptor-mediated endocytosis, followed by fusion within the endosomal compartments. Furthermore, recent reports show that the entry portal may vary depending on the tissue and the cell. These differences may affect the host range, pathogenicity, and cell/tissue specificity [1].

To visualize coronavirus entry into cells, we developed a confocal microscopy-based analysis method and single-virus particle-tracking tools. We believe that coronavirus entry is highly dependent on the in vitro model used, and therefore, we study coronavirus entry using susceptible cell lines, but we also confirm

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_18,

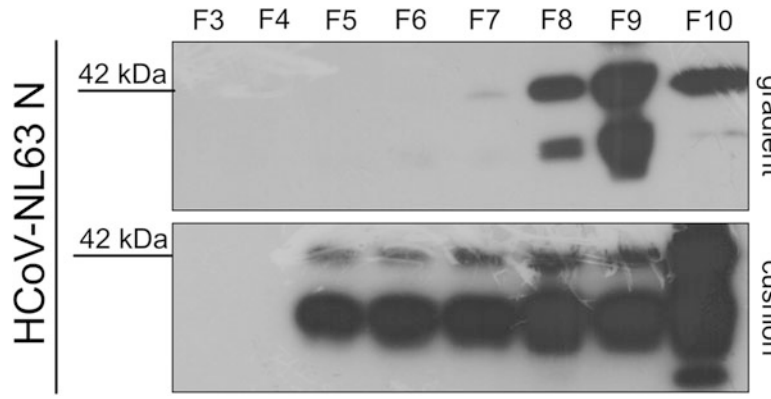

Fig. 1 Gradient purification of coronaviruses. Western blot detection of coronavirus-containing fractions obtained by gradient centrifugation of the crude virus stock. Samples from the first step of ultracentrifugation are presented in the bottom panel. Fractions F9 and F10 were further purified in the second ultracentrifugation step, and fractions are presented in the upper panel

our observations using the complex ex vivo model of human airway epithelium (HAE). HAE cultures are formed by the multilayered, fully differentiated primary human airway epithelial cells grown at the air/liquid interface built on collagen-coated plastic supports. HAE cultures mimick the natural conductive airway epithelium and serve as the most reliable model to study coronavirus infection [2–6].

To study coronavirus entry, we first prepared concentrated stocks of coronaviruses. We found the iodixanol-based medium to be optimal for coronavirus concentration and purification, as in other media (e.g., sucrose gradient), virions rapidly lose infectivity (Fig. 1). Single-step iodixanol purification was sufficient for the successful visualization of single-virus particles in the cell (Fig. 2). We visualized single coronaviral particles using immunodetection, and we decided that staining specifically for the N protein was superior due to the high availability of antibodies and abundance of the protein itself [7, 8].

In our research, we tracked virus entry from virus attachment to the cell. First, using a variety of techniques, we studied the attachment itself and made an effort to identify attachment receptors, which are usually broadly distributed molecules, e.g., sugar moieties [8–11]. Using confocal microscopy-based analysis complemented with flow cytometry appeared to be the optimal means to investigate these processes [11–13]. Further, we aimed to identify entry receptors and virus–receptor interactions. Except for proteomic analyses, which are not covered in this chapter, confocal analysis of virus–receptor interaction allowed us to delineate the process of coronavirus entry [14, 15].

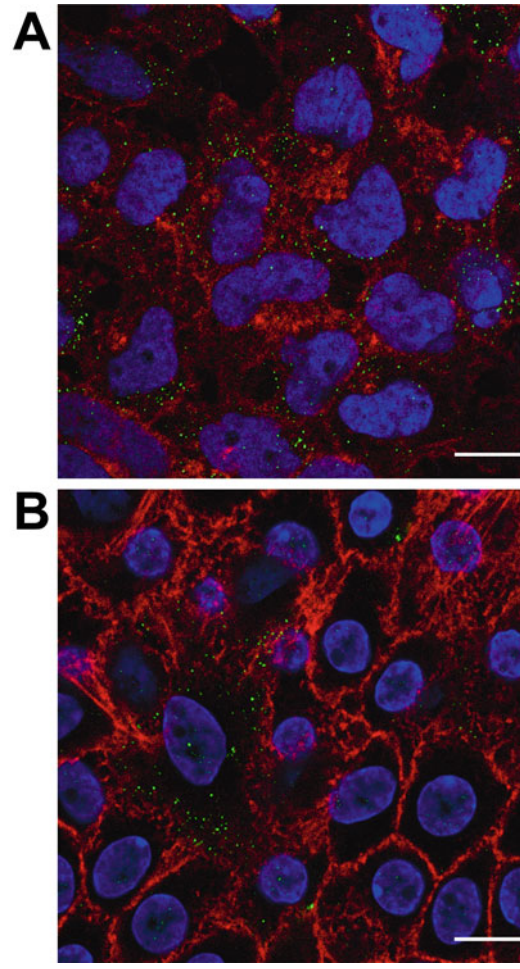

Fig. 2 Visualization of single virus particles in cells. (**a**) CRCoV strain 4182 on HRT-18G cells; (**b**) HCoV-OC43 strain 0500 on human airway epithelium. Two exemplary pictures are presented for each virus. Viral nucleocapsids are shown in green (Alexa Fluor 488), actin cytoskeleton in red (Atto 633), and nuclei in blue (DAPI). Each image is a single confocal plane. Scale bar 10 μm

Next, we checked whether the virus undergoes fusion on the surface of the cell or first requires endocytic internalization. For this we employed chemical inhibitors hampering alteration of the endosomal microenvironment (e.g., ammonium chloride, bafilomycin A), which should affect endocytic entry, but not fusion on the cell surface. Further, we tested whether the virus colocalizes with the early endosome antigen 1 (EEA-1) early after internalization. EEA-1 is a hydrophilic protein, which in cells is found exclusively in early endosomes [16] (*see* Fig. 3 for examples) [17, 18].

Subsequently, we found that identification of the endocytosis route for a given coronavirus should first rely on determining the colocalization of virions with proteins specific for endosomes

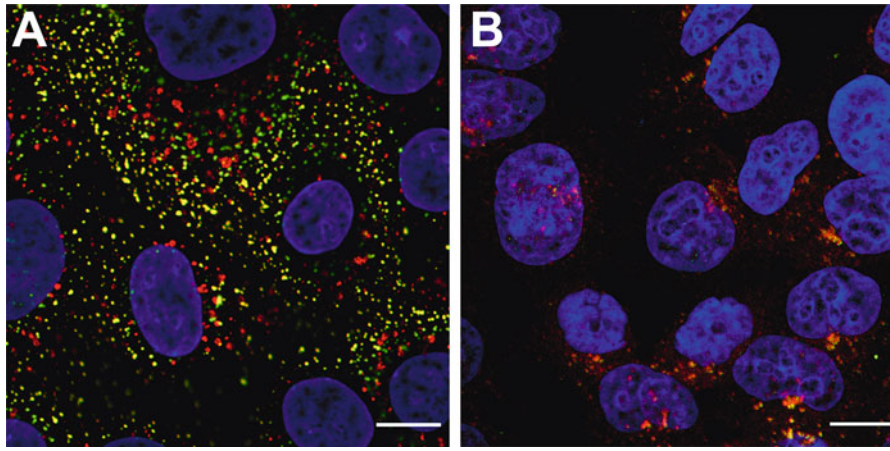

Fig. 3 Colocalization of viruses with early endosome marker EEA1. (**a**) HCoV-OC43 colocalizing with EEA1 in HCT-8 cells, (**b**) CRCoV colocalizing with EEA1 in HRT-18G cells. Viral nucleocapsids are presented in green (Alexa Fluor 488), EEA1 in red (Alexa Fluor 546). Cell nuclei are shown in blue (DAPI). Scale bar 10 μm

entering by a given pathway. For this, we incubated permissive cells with a purified coronavirus stock at 4 °C to enable virus adhesion to the cell surface and block its internalization, as at this temperature intracellular transport is inhibited. Consequently, we increased virus density on the cell surface, and by increasing the temperature, we were able to synchronize virus entry. Cultures were incubated at higher temperatures and fixed at specific time points. Samples were immunostained for coronaviral and host proteins. The colocalization rate between viral and cellular proteins was estimated using Pearson's and Manders' correlation coefficients. As controls, we used protein cargos, such as transferrin, which is endocytosed via a clathrin-mediated pathway or cholera toxin or albumin, which enter cells via caveosomes [19, 20] (Fig. 4). We verified virion colocalization with markers for specific entry routes in cells pretreated with chemical inhibitors or cells with silenced expression of proteins required for specific endocytic pathways. In these experiments, endocytosis of the virus or reference cargo was blocked, and internalization was analyzed with confocal imaging. For example, for all coronaviruses studied, we observed efficient inhibition of internalization using dynamin-2 inhibitors. On the other hand, chemicals blocking clathrin-mediated endocytosis affected only human coronavirus (HCoV)-NL63 entry, and caveolin inhibitors hampered canine respiratory coronavirus (CRCoV) and HCoV-OC43 internalization. Furthermore, as entry via endocytosis usually requires re-arrangement of the cytoskeleton, we also tested virus internalization in the presence of inhibitors affecting actin filaments and microtubules [11, 12, 21–27] (Fig. 5).

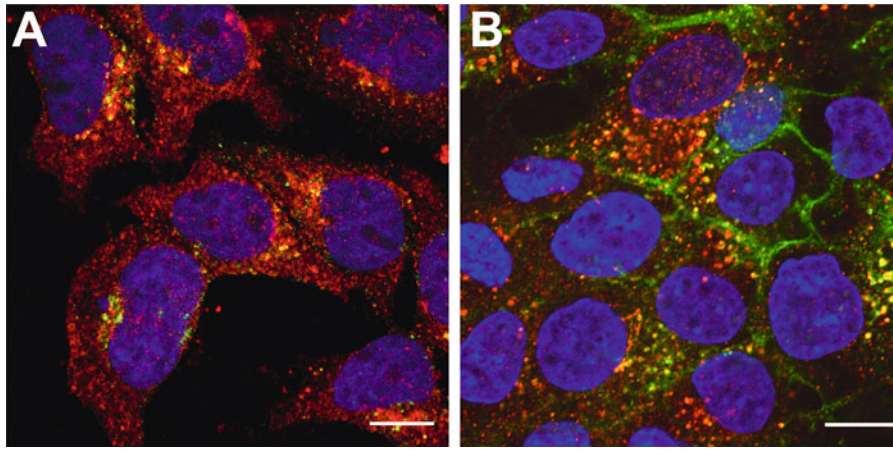

Fig. 4 Colocalization of reference cargoes with entry pathway markers. (**a**) Transferrin colocalizing with clathrin. Clathrin HC is presented in red (Alexa Fluor 546), transferrin conjugated to Alexa Fluor 488 in green, and cell nuclei are shown in blue (DAPI). (**b**) Cholera toxin colocalizing with caveolin. Caveolin 1 is presented in red, cholera toxin subunit B conjugated to FITC in green. Cell nuclei are shown in blue (DAPI). Scale bar 10 μm

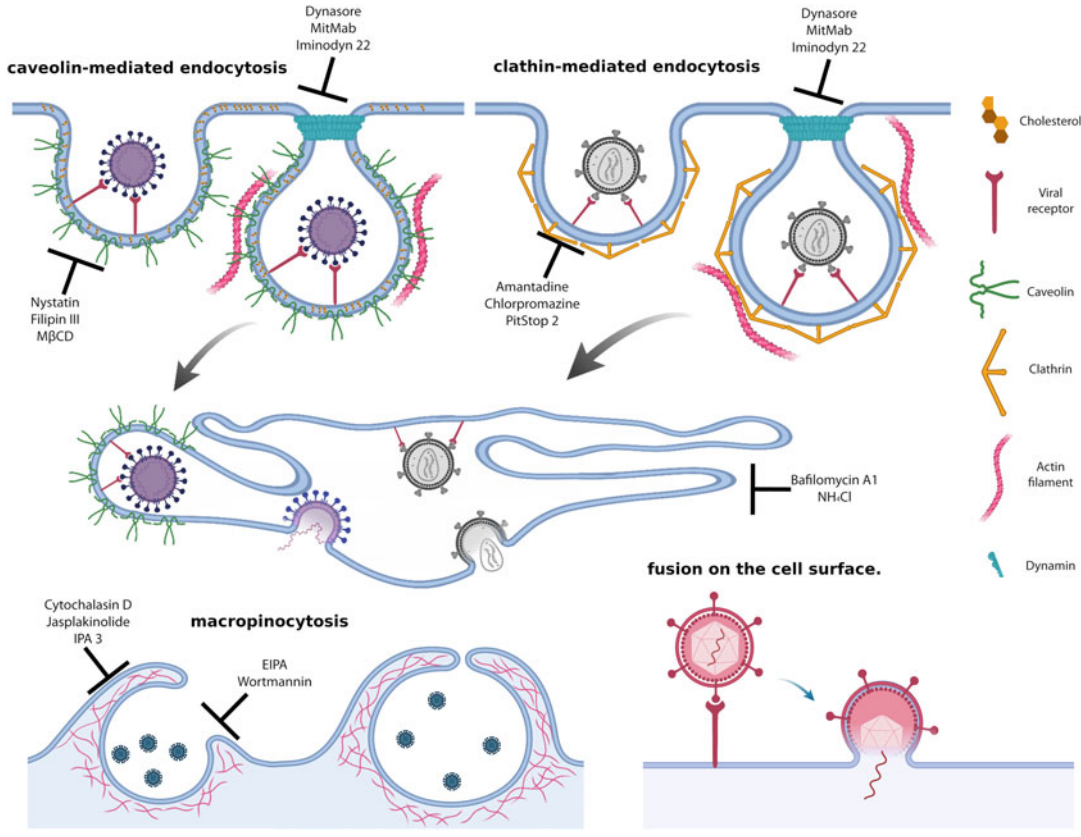

Fig. 5 Inhibition of virus entry. Compounds with a known inhibitory effect on different internalization pathways are indicated at their respective sites of action. The direct fusion of viruses with cell surface may be blocked by the application of virus-specific inhibitors

Confocal imaging of cells allows us to visualize not only the process but also to quantify it. The efficiency of entry can be estimated based on the ratio between internalized viruses and viruses attached to the surface, e.g., in the presence and absence of an inhibitor.

Virus internalization is not always linked with infectious entry. As we have shown in Owczarek et al [22], the re-direction of the virus into the micropinocytosis pathway allows effective entry but does not allow for fusion and infectious entry. For that reason, it is of importance to verify whether effective virus entry relies on specific pathways. We therefore always complement experiments described above with studies on virus replication in the presence or absence of chemical inhibitors of specific pathways [21, 22].

2 Materials

2.1 Purification of a Coronavirus Stock

1. Cell lines: LLC-Mk2 (ATCC: CCL-7), HCT-8 (ATCC: CCL-244), HRT-18G (ATCC: CRL-11663).
2. Antibiotics (1×): penicillin (100 U/ml), streptomycin (100 μg/ml), ciprofloxacin (5 μg/ml).
3. DMEM: Dulbecco's modified Eagle's medium, supplemented with 3% heat-inactivated fetal bovine serum (FBS) and antibiotics (1×).
4. MEM: two parts Hanks' MEM, one part Earle's MEM, supplemented with 3% heat-inactivated fetal bovine serum (FBS), and antibiotics (1×).
5. RPMI: RPMI-1640, supplemented with 3% heat-inactivated fetal bovine serum (FBS) and antibiotics (1×).
6. T75 tissue culture flasks.
7. Cell culture incubator set at 32 or 37 °C and with 5% CO_2.
8. Virus stock (HCoV-NL63, HCoV-OC43 or CRCoV).
9. 50 ml conical centrifuge tubes.
10. Benchtop centrifuge.
11. Syringe fitted with a 0.45 μm filter.
12. Centrifugal filters (10,000 kDa cut off).
13. Phosphate-buffered saline (PBS).
14. Iodixanol solution (Optiprep medium).
15. Ultracentrifuge vials.
16. Ultracentrifuge capable of reaching 170,000 × *g*.
17. Denaturing sample buffer for SDS–PAGE.

2.2 Cell Culture Experiments

2.2.1 In Vitro Cell Cultures

1. Cell lines (*see* Subheading 2.1, **item 1**).
2. Media (*see* Subheading 2.1, **items 3–5**).
3. Coverslips.
4. Six-well plates.
5. Purified virus stock or mock stock.
6. Wet chamber (wet paper towels in a box).
7. PBS.
8. 4% paraformaldehyde (PFA) (*see* **Note 1**).

2.2.2 Ex Vivo HAE Cultures

1. 0.4 μm ThinCert (Greiner Bio-one) or Transwell (Costar) inserts.
2. Human tracheobronchial epithelial cells obtained from airway specimens—refer to the approved protocols.
3. Bronchial epithelial growth medium (BEGM).
4. Air–liquid interface medium (ALI), as described by Fulcher et al. [4].
5. Antibiotics: penicillin (100 U/ml), streptomycin (100 μg/ml), gentamycin (10 μg/ml), amphotericin B (250 μg/ml), ciprofloxacin (5 μg/ml), Fungin (10 mg/ml).
6. Purified virus stock or mock stock preparation.
7. PBS.
8. 4% PFA (*see* **Note 1**).

2.2.3 Entry Inhibitors

The chemical inhibitors used in the studies together with the approximate concentrations in different cells are listed in Table 1, and a graphical illustration of their mechanism of action is presented in Fig. 5 (*see* **Note 2**).

2.2.4 Endocytosis Cargo Proteins

1. Clathrin-dependent cargo protein: Alexa Fluor 488-labeled transferrin (100 μg/ml).
2. Caveolin-dependent cargo protein: FITC-labeled albumin (500 μg/ml), FITC-labeled cholera toxin (40 μg/ml).
3. Macropinocytosis: dextran derivatives.

2.3 Immunostaining to Visualize Viral Entry into Cells

1. Washing buffer: 0.5% Tween 20 in PBS.
2. Blocking buffer: 5% BSA in PBS.
3. Dilution buffer: 1% BSA and 0.5% Tween 20 in PBS.
4. Primary and secondary antibodies (Table 2).
5. 0.1% Triton X-100 in PBS.
6. 0.1 μg/ml 4′, 6′-diamidino-2-phenylindole dihydrochloride (DAPI).

Table 1
Entry inhibitors used in the studies

Inhibitor	Process affected	Direct effect	Working concentration			References (PMID)
			HCT-8	HRT-18	LLC-MK2	
Bafilomycin A1	pH-dependent endocytosis	Inhibition of vacuolar-type H^+-ATPase; prevents acidification of endosomes	2.5 nM	10 nM	100 nM	[28, 29]
NH_4Cl		Acts as a proton sink, thereby inhibiting acidification of the endosome	50 mM	50 mM	50 mM	[29]
EIPA	Macropinocytosis	Inhibition of Na^+/H^+ antiport → change of submembranous pH → deactivation of the GTPases that promote actin remodeling	20 μM	10 μM	40 μM	[30, 31]
Wortmannin		Inhibition of phosphoinositide-3-kinase (PI3K)	5 μM	5 μM	5 μM	[32]
Dynasore	Dynamin-dependent entry	Inhibition of GTPase activity of dynamin 1, dynamin 2, and Drp1 (mitochondrial dynamin)	40 μM	40 μM	80 μM	[33]
Iminodyn 22		Binding of the GTPase domain at an allosteric site; uncompetitive antagonist to GTP; broad-spectrum dynamin inhibitor	40 μM	15–25 μM	20 μM	[34]
MitMab		Blocking of pleckstrin homology lipid-binding domain of the dynamin 1 and dynamin 2	10 μM	5 μM	10 μM	[35]
CT04	Actin-dependent entry	Inhibition of RhoA, RhoB, and RhoC GTPases	0.5–2 μg/ml	1 μg/ml	ND	[36]
Cytochalasin D		Disrupting actin polymerization	2 μM	10 μM	10 μM	[37]
IPA-3		Inhibition of group I p21-activated kinase (PAK1)	20 μM	20 μM		[38]
Jasplakinolide		Stabilization of actin microfilaments	150 nM	150 nM	1.5 μM	[39]
NSC23766 trihydrochloride		Inhibition of Rac1, a Rho-family GTPase	50 μM	100 μM	ND	[40]
Y27632		Inhibition of Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK)	10 μM	10 μM	10 μM	[41, 42]

Nocodazole	Microtubule-dependent entry	Depolymerization of microtubules	0.5 μM	0.5 μM	400 nM	[43]
Amantadine	Clathrin-dependent route	Stabilization of clathrin-coated pits, probably due to the interactions of the drug with the clathrin cage or the membrane structure of the vesicle	750 μM	500 μM	500 μM	[44]
Chlorpromazine		Translocation of clathrin and AP2 adaptor complexes from cell surface to endosomes → depletion of the complexes in the plasma membrane	5 μM	5 μM	5 μM	[45]
Pitstop 2		Competitively inhibits clathrin terminal domain (TD)	15 μM	5 μM	10 μM	[46]
Filipin III	Caveolin-dependent route	Alteration of membrane permeability and associated functions; binding to membrane sterols	1–10 μg/ml	2 μg/ml	2–100 μg/ml	[47]
Methyl-β-cyclodextrin		Removing cholesterol from lipid membranes	100 μM–5 mM	2 mM	5 mM	[48]
Nystatin		Sterol-binding agent	10–100 μg/ml	50 μg/ml	5 μg/ml	[49]

ND no data. Working concentration optimized for the stated cell lines in our laboratory

Table 2
Antibodies used in these studies

Target	Antibody	Dilution	Conjugate	Supplier information
Endocytosis	Anti human early endosome antigen 1	2 μg/ml	x	Santa Cruz Biotechnology; AB_2277714
	Rabbit anti-Rab 7	2 μg/ml	x	Santa Cruz Biotechnology; AB_2175483
	Rabbit anti-Rab11A	2.5 μg/ml	x	Proteintech; AB_2173458
	Rabbit anti-LAMP1	10 μg/ml	x	Thermofisher Scientific; AB_2134611
Clathrin-dependent route	Anti-human clathrin heavy chain	10 μg/ml	x	Santa Cruz Biotechnology; AB_2083170
Caveolin-dependent route	Anti-human caveolin	2 μg/ml	x	Santa Cruz Biotechnology; AB_2072042
Coronavirus proteins	Anti-HCoV-NL63 N IgG	0.25 μg/ml	x	Ingenansa; M.30.HCo.I2D4
	Anti-HCoV-OC43 N IgG	1 μg/ml	x	Merck Millpore; AB_95424
Secondary antibodies	Goat anti-mouse	10 μg/ml	Alexa Fluor® 488	Thermofisher Scientific
	Rabbit anti-mouse	10 μg/ml	Alexa Fluor® 488	
	Goat anti-rabbit	10 μg/ml	Alexa Fluor® 546	
	Donkey anti-goat	10 μg/ml	Alexa Fluor® 546	
Actin	Phalloidin	0.2 U/ml	Alexa Fluor® 633	Thermofisher Scientific

These antibodies were used successfully in our laboratory. Alternative antibodies can be used after validation

7. 0.2 U/ml Phalloidin labeled with Alexa Fluor 633.
8. Antifade medium (e.g., ProLong Gold antifade medium).
9. Immersion oil (e.g., Immersol 518 F),
10. Rotary shaker.
11. Fine tipped forceps.
12. Scalpel.
13. Glass slides.
14. Nail polish.

2.4 Image Acquisition, Processing, and Presentation

1. Confocal microscope, e.g., Zen 2.3 SP1, Carl Zeiss Microscopy GmbH.
2. Image analysis software capable of colocalization analysis and counting three dimensional objects, e.g., ImageJ1.52i [50, 51].

3 Methods

All procedures involving infectious material should be performed inside a biological safety cabinet until the fixing step.

3.1 Purification of a Coronavirus Stock

1. Obtain a stock of a given coronavirus by infecting monolayers of permissive cells growing in T75 flasks. Prepare a second T75 flask for mock-infection (negative control). Maintain the cells at 32 °C or 37 °C under 5% CO_2 for 4–5 days, depending on the virus species (*see* **Note 3**).
2. Lyse the cells by freeze-thawing at −80 °C and collect the solutions in conical 50 ml tubes (*see* **Note 4**).
3. Centrifuge at 4500 × *g* for 15 min at 4 °C.
4. Filter the supernatants using sterile syringe 0.45 μm filters.
5. Concentrate the supernatants using centrifugal filters to 8 ml each.
6. Prepare iodixanol solutions: 10%, 12.5%, 15%, 17.5%, 20% in 1× PBS, and store at 4 °C.
7. Add 2 ml of 15% iodixanol solution in the ultracentrifuge vial and overlay gently with 8 ml of mock/virus concentrate. Centrifuge at 170,000 × *g* for 3 h at 4 °C.
8. Collect ten fractions of 1 ml from the top to the bottom of the vial. The virus should be present in the fraction at the bottom of the tube, but this needs to be verified by western blot. Combine the virus-containing fraction (and corresponding mock fraction) with 1 ml of PBS and mix thoroughly.
9. In an ultracentrifuge vial, prepare gradient medium by overlaying 1.6 ml of each iodixanol solution (20% to 10%). Add 2 ml of virus/mock sample obtained in the previous step to the top of the gradient. Centrifuge at 170,000 × *g* for 18 h at 4 °C.
10. Collect ten fractions from the top to the bottom (1 ml each). Mix 15 μl of each fraction with a denaturing sample buffer for SDS–PAGE electrophoresis and perform western blot analysis to identify fractions containing the virus. Aliquot each fraction and store at −80 °C (*see* **Note 5**).

3.2 Cell Culture Experiments

3.2.1 Viral Entry In Vitro into Cell Lines

1. Seed the cells on coverslips in six-well plates and culture for 2 days at 32 or 37 °C with 5% CO_2, when they should reach 70–80% confluence. LLC-Mk2 cells are cultured in MEM, HCT-8 cells in RPMI and HRT-18G cells in DMEM.
2. Where required, incubate the cells with a given inhibitor (dissolved in cell culture medium; *see* Table 1) for 30 min at 32 or 37 °C (*see* **Note 6**).
3. Discard the supernatant and overlay the cells with 50 μl of purified virus or mock sample and incubate for 1 h at virus-specific temperature (i.e., 32 or 37 °C). To avoid evaporation put the plate in a wet chamber. For control experiments with reference proteins (cargo), incubate the cells with a given cargo dissolved in a culture medium for 1 h at virus-specific temperature (*see* **Note 7**).
4. Wash the cells three times with PBS and fix with 4% PFA diluted in PBS. Incubate the plate at RT for at least 15 min before further analysis (*see* **Note 8**).

3.2.2 Viral Entry into Ex Vivo HAE Cultures

1. Culture primary epithelial cells on cell culture inserts in multiwell plates in BEGM medium supplemented with antibiotics until they reach 100% confluence. For further culture and differentiation, maintain the cells in ALI supplemented with antibiotics for 4–8 weeks at 37 °C under 5% CO_2.
2. Maintain HAE cultures until fully differentiated. It is essential to verify the differentiation using marker proteins and phenotypic changes of cultures, e.g., mucus production, synchronized cilia movements.
3. Wash the cultures three times with 200 μl of PBS to remove the mucus. For each washing incubate the cultures for 5 min at 37 °C.
4. Where required, incubate the cultures with a given inhibitor (diluted in PBS, *see* Table 1) for 60 min at 37 °C.
5. Discard the supernatant and add 100 μl of purified virus or mock sample per insert and incubate for 2 h at 32 °C. For entry experiments of control cargo proteins, incubate the cultures with a given protein (diluted in PBS).
6. Wash the cells three times with PBS and fix with 4% PFA diluted in PBS. Incubate the plate at RT for at least 30 min before further analysis (*see* **Note 8**).

3.2.3 Synchronized Viral Entry into Cell Lines

1. Seed the cells on coverslips in six-well plates and culture for 2 days at 37 °C until they reach 70–80% confluence.
2. Cool the plate to 4 °C on ice. Washing is not necessary.

3. Overlay the cells with 50 µl of pre-cooled (4 °C) purified virus or mock preparation and incubate for 1 h at 4 °C on ice to synchronize the virions at the cell surface.
4. To avoid evaporation of the liquid, place the plate in a wet chamber, then warm up the plate to 32 or 37 °C, depending on the optimal temperature for the particular virus. Remove samples from the incubator at set time intervals ranging between 0 and 180 min to get a clear picture of the route of entry (*see* **Note 9**). For control, cargo proteins, incubate the cells with a given protein (diluted in a culture medium) at the same temperature for a given time.
5. Wash the cells three times with PBS and fix with 4% PFA in PBS. Incubate the plate at RT for at least 30 min before further analysis (*see* **Note 8**).

3.3 Immunostaining to Visualize Viral Entry into Cells

3.3.1 Immunostaining Cell Lines

1. Incubate the fixed cells on coverslips in six-well plates with 0.5 ml 0.1% Triton X-100 in PBS for 5 min at room temperature (RT) on a rotary shaker.
2. Replace Triton X-100 with 1 ml blocking buffer and incubate overnight at 4 °C. Alternatively, incubate 2 h at RT or 30 min at 37 °C.
3. To visualize coronavirus particles or endocytosis-related proteins, incubate the cells for 2 h at RT with specific antibodies diluted in dilution buffer. To limit the antibody usage, place a 50 µl drop of antibody solution on the plate cover and overlay with the inverted coverslip with cells (*see* **Note 10**).
4. Wash the coverslips three times with 1 ml washing buffer on a rotary shaker for 5 min each wash.
5. Incubate for 1 h at RT with secondary antibodies labeled with fluorophores in dilution buffer.
6. Wash three times with 1 ml washing buffer on a rotary shaker for 5 min each wash.
7. To stain actin filaments, incubate the coverslips for 60 min at RT with fluorescently labeled phalloidin in PBS (50 µl drop, as in **step 3**). Refrain from using detergents after this step.
8. To stain nuclear DNA stain the coverslips for 10 min with 0.5 ml of DAPI in PBS.
9. Wash twice in 1 ml PBS to remove residual Tween 20. Mount the coverslips onto antifade medium on glass slides. Seal the coverslips with nail polish and leave to dry overnight at RT.

3.3.2 HAE Cultures

1. Incubate the fixed cultures on inserts with 200 µl of 0.1% Triton X-100 in PBS for 15 min at RT on a rotary shaker (*see* **Note 11**).

2. Replace Triton X-100 with 0.5 ml of blocking buffer and incubate overnight at 4 °C. Alternatively, incubate 2 h at RT or 30 min at 37 °C.
3. To visualize coronavirus particles or endocytosis-related proteins, incubate the cultures for 2 h at RT with 100 µl of specific antibodies in dilution buffer (*see* **Note 10**).
4. Wash the cultures three times with 300 µl of washing buffer on a rotary shaker for 10 min per wash.
5. Incubate for 1 h at RT with 100 µl of secondary antibodies labeled with fluorescent dyes in dilution buffer.
6. Wash the cultures three times with 300 µl of washing buffer on a rotary shaker for 10 min each wash.
7. To stain actin filaments, incubate the cultures for 20 min at RT with 100 µl fluorescently labeled phalloidin in PBS.
8. To stain nuclear DNA, incubate the cultures for 15 min with 200 µl DAPI in PBS. Wash twice with 300 µl PBS to remove residual Tween 20.
9. Cut out the membrane from the insert with a scalpel or push out using a tube. Mount on a glass slide with the antifade medium. Cover the membrane with a coverslip and seal with nail polish. Let it dry overnight at RT.

3.4 Image Acquisition

1. Check the confocal microscope to determine which light sources are available (emission wavelengths).

3.4.1 Selection of Fluorescent Dyes

2. Choose fluorophores. Fluorescent dyes not only differ in the maximal excitation and emission wavelengths but also exhibit different spectrum width and quantum yield (which is a measure of the efficiency of photon emission compared to the number of photons absorbed). We have successfully visualized up to four fluorophores at the same time (cells' nuclei stained with DAPI + coronaviral proteins stained with Alexa Fluor 488 + host entry molecules stained with Alexa Fluor 546 + actin cytoskeleton stained with Atto 647 or Atto 633), but a higher number of fluorophores is possible.

To analyze the excitation and emission spectra and model compensation matrixes for evaluation of the spectral compatibility of fluorescent dyes and probes, online tools, e.g., Fluorescence SpectraViewer (www.lifetechnologies.com/handbook/spectraviewer) may be used.

3.4.2 Scanning Settings

1. Define tracks by specifying the fluorophore to be used. Set the spectrum that should be acquired. If the excitation and emission spectra are not overlapping, it is possible to detect up to three fluorophores in a single track. The configuration of a track consists of a laser line, excitation dichroic, and detector (*see* **Note 12**).

2. Choose the scanning mode. Simultaneous scanning of different wavelengths in samples labeled with multiple fluorophores is faster, but often falsifies the signal (cross talk between channels). Therefore, sequential scanning with a single laser line and a single detector activated (single fluorophore) at a time is the method of choice. The region of interest may be scanned by switching tracks line-by-line or frame-by-frame. In the first case, lasers and detectors are changed for each line, so the multicolor image is acquired at once. In the latter case, a single color is captured for the entire frame, and frames representing different fluorophores are superimposed. Scanning line-by-line while faster and favored allows modifying only channel and laser intensities between different fluorophores. In turn, frame-by-frame scanning allows also for changing beam splitters, gain, offset, and pinhole setting between the acquisition of single-color tracks.
3. Set the optical and digital zoom. To ensure adequate spatial sampling, the pixel size should be half of the microscope resolution to ensure Nyquist sampling conditions for all measurements.
4. Adjust the speed of scanning. If the sample is not prone to photobleaching, decreasing the speed will yield higher image quality; however, this may also increase the noise signal, so this step should be optimized. Otherwise, averaging may be applied while the speed of scanning remains high—such settings should improve the signal-to-noise ratio. The low scanning speed and averaging will impact the acquisition time.
5. Set image resolution at 16 bit (65,536 levels of gray, optimal for quantitative measurements).
6. Set laser power, gain, and offset values. To ensure that the signal is within the dynamic range of the detector, live mode along with range indicator should be used. It will also show under- and overexposed pixels. If there is a need to increase the fluorescence signal, it is better to modify the power of the laser or the detector's gain than to apply the digital gain boost. This step demands optimization because too high laser intensity will photobleach the sample, while too high gain will generate noisy images (*see* **Note 13**).

3.4.3 3D Imaging

To acquire a 3D image of the region of interest, activate the z-stack mode.

1. Set the top and the bottom limits of the cell of interest. It is good practice to mark it based on the fluorescence signal of the actin cytoskeleton. For HAE cultures, it is easy to set the bottom border at the ThinCert insert membrane and the top in the distant part of immunostained cilia (*see* **Note 14**).

2. Adjust the step size. It is appropriate to set a value that fits the Nyquist sampling criteria; however, for some purposes, oversampling may be recommended, e.g., it may help to separate high-frequency noise from the signal during image deconvolution. Our standard setting is 150 nm because it helps us to distinguish viruses by increasing signal to noise ratio in the z-axis.

3.5 Image Analysis

A number of image analysis software packages are available. In our research, we use the free software package ImageJ.

3.5.1 Image Processing and Presentation

It is easy to misrepresent data when adjusting the images. Therefore, it is essential to understand the difference between acceptable and unacceptable image adjustment. This is especially important for confocal microscopy or superresolution microscopy, where the presented image is always an interpretation of the signal imposed by the operator.

1. Limit adjustments as much as possible. Linear adjustments of brightness and contrast are generally considered safe and do not require an explanation as long as they are applied uniformly to all pictures in the set. Changes that do not affect each pixel in the same way (e.g., gamma) or more radical changes while allowed should be clearly described and justified in the Methods section and mentioned in the figure caption (*see* **Note 15**).
2. Even basic manipulations, as contrast adjustments, may remove some or introduce new elements into image, falsifying the message. When selecting parameters, always refer to mock-treated cells and suitable isotype controls.
3. The reader assumes that the picture presented in a figure represents a single microscope field. Therefore, always make clear divisions between elements from different images. A comparison of different images or their fragments may require resizing to obtain matching resolutions across the complete figure. While downsizing requires averaging of adjacent pixels and is acceptable, upsizing of the image introduces artificial pixels to the image and should be avoided.
4. Often, when imaging single virus particles, the number of events is low in a single field of view. To cope with this issue, imageJ Z functions allow for a combination of multiple slices into one using one of six different projection methods.
5. It is not possible to determine whether the virus entered the cell or is retained on the surface using only xy images, but it is necessary to analyze orthogonal views. The reslice option allows for the generation of xz and yz planes and also allows for downstream analyses and adjustments.

6. Never mix different color models. Doing so splits the signal into two channels, adding some of it to pre-existing channels (e.g., yellow added to RGB is split to red and green channels) and may yield false colocalization.

3.5.2 Colocalization

1. The ImageJ JACoP plugin is used for the calculation of colocalization. To use it, images should be split into single channels and loaded in pairs to be compared.
2. Select the method. There are a number of parameters that describe the colocalization of objects, and we find Pearson's and Mander's coefficients most convenient. While Pearson's correlation is easy and fast, Mander's coefficient allows to determine how object A colocalizes with object B, and how object B colocalizes with object A. To illustrate the importance of such distinction, one may imagine that while all virions (A) colocalize with clathrin (B) while entering the cell, not all clathrin-coated vesicles (B) carry a virus (A), and some clathrin is dispersed in the cell.
3. When calculating Mander's coefficient, it is essential to adjust the threshold for both channels—in contrast to Pearson's, it is necessary to define background signal before analysis (*see* **Note 16**).

3.5.3 Statistics: Particle Counting

1. Split the image into separate channels.
2. Calculate the total number of viruses using, e.g., 3D Objects Counter tool (Fiji ImageJ)—adjust the threshold value using mock-treated cells and remove noise and aggregates using minimum and maximum size filters.
3. Calculate the total number of cells using the DAPI channel and the 3D Objects Counter tool—adjust threshold value and minimum size filter to remove the signal from cell debris. As this analysis is prone to error while analyzing pictures of dense cultures (e.g., HAEs), select for the option to create a surface map, and revise obtained results.
4. Divide the number of virus particles by the number of cells to obtain an average number of particles per cell.
5. To calculate the number of particles in the nuclei of the cell, one may create a mask using the DAPI channel and subtract parts of other channels using an image calculator tool before counting. Unfortunately, masks created using the signal for actin are not sufficient to distinguish internalized and surface viral particles and they should be counted using other methods (e.g., manually). To reduce uncertainty, use gamma on the actin channel to obtain surface without gaps.

4 Notes

1. PFA preparation procedure, purity, and supplier may affect the efficacy and specificity of the immunostaining. This step requires optimization.
2. Cytotoxic activity of chemical inhibitors may affect coronavirus entry into cells in a nonspecific way. A cell viability assay for each compound should be carried out in the experimental setting before the actual experiment.
3. Obtain HCoV-NL63 stock by infecting monolayers of LLC-Mk2 cells in T75 flasks and maintaining at 32 °C. Obtain a HCoV-OC43 stock by infecting monolayers of HCT-8 cells in T75 flasks and maintaining at 32 °C. Obtain a stock of CRCoV by infecting monolayers of HRT-18G cells in T75 flasks and maintaining at 37 °C. To asses virus yield, titrate on fully confluent permissive cells, according to the method of Reed and Muench [52]. For CRCoV, additional staining is required as the virus does not produce cytopathic effect on HRT-18G cells [12].
4. To obtain HCoV stocks, lyse cells by two freeze-thaw cycles and aliquot at −80 °C. To obtain CRCoV stock, remove the supernatant, scrape the cells, and freeze-thaw them in a low volume of liquid. Mix with previously removed supernatant and aliquot at −80 °C.
5. For some coronaviral strains (e.g., clinical isolates), the stock titer may be low and insufficient for immunostaining. In such cases, virus stock should not be frozen but processed immediately after collection. It is also possible to scale up the culture and concentrate the media to 8 ml.
6. We observed that some inhibitors are active in some cells while showing no activity in others. It is essential to include reference compounds in each study.
7. The fusogenic activity of some viruses can be inhibited by acidic buffer (0.1 M NaCl, 0.1 M glycine, pH = 3; "acid wash"). This may be useful if one is willing to test which virions were on the cell surface at the time of exposition. For this, cells are washed three times with cold acidic buffer, followed by washing with PBS (pH = 7.4).
8. Importantly, coronavirus-infected samples will be treated as non-infectious after incubation in PFA. For that reason, it is important to transfer the cells on coverslips to new non-contaminated plates for fixation. For ease, fixative can be added to wells prior to the transfer of coverslips.
9. The optimal time for each route to be tested is best optimized for each cell type by testing various time points. However, a good starting point is a timecourse study, taking samples every

20–30 min from 0 to 180 min, perhaps longer depending on the endocytic marker being used. Early events are often best studied in 5-min increments. If seeking to understand whether the virus is colocalising with a marker of an endosome (e.g., EEA1), colocalisation might be expected a bit later in this timecourse.

10. Sequential immunostaining of different epitopes while lengthening the procedure allows for optimization of conditions for each antibody and allows for some atypical staining combinations (e.g., first staining with rabbit antibody specific to protein X and a secondary goat anti-rabbit antibody followed by blocking and second staining with mouse antibody specific to protein Y and secondary rabbit anti-mouse antibody).
11. Alternatively, insert membranes may be cut out after fixation, cut into 2–4 pieces and perform subsequent steps to stain for different antigens in microcentrifuge tubes.
12. Stabilize the temperature of the sample and the microscope's environment before image acquisition. All materials should be left to equilibrate the temperature for at least 15 min. This will help to avoid image deformations caused by temperature drift.
13. If slides are imaged during different sessions, acquisition settings should be identical (tip: they are usually saved as metadata in the image file). It is also essential to verify the values set by manual knobs, e.g., laser power regulation. Often they function independently of the software and will not be adjusted automatically.
14. When counting particles from cultures propagated on ThinCert insert membranes, exclude few bottom slices as high membrane autofluorescence tends to impair 3D Objects Counter algorithm function.
15. ImageJ scale bars during generation delete the fragment of the picture they cover. This process is irreversible, so it is good practice to store original versions of photos for future use.
16. The antibody size is in a range of 10–15 nm, which may severely affect the results, especially in superresolution microscopy; to improve the localization analysis, smaller labels, such as aptamers, nanobodies, or quantum-dots, may be used.

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Chapter 19

Preparation of Cultured Cells Using High-Pressure Freezing and Freeze Substitution for Subsequent 2D or 3D Visualization in the Transmission Electron Microscope

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Abstract

Transmission electron microscopy (TEM) is an invaluable technique used for imaging the ultrastructure of samples, and it is particularly useful when determining virus–host interactions at a cellular level. The environment inside a TEM is not favorable for biological material (high vacuum and high energy electrons). Also biological samples have little or no intrinsic electron contrast and rarely do they naturally exist in very thin sheets, as is required for optimum resolution in the TEM. To prepare these samples for imaging in the TEM therefore requires extensive processing which can alter the ultrastructure of the material. Here we describe a method which aims to minimize preparation artifacts by freezing the samples at high pressure to instantaneously preserve ultrastructural detail, then rapidly substituting the ice with resin to provide a firm matrix which can be cut into thin sections for imaging. Thicker sections of this material can also be imaged and reconstructed into 3D volumes using electron tomography.

Key words High-pressure freezing, Freeze substitution, Transmission electron microscopy, Sapphire discs, Electron tomography

1 Introduction

The method of preservation of samples for TEM can influence image interpretation, so it is important to stabilize the sample with as little change from the in vivo state as possible. There are two main methods of stabilizing (fixing) samples for TEM: chemical fixation and cryo-fixation. There are advantages and disadvantages to both. Chemical fixation is the most common method for preparing cells and, although time consuming, is an easy, repeatable method that requires very little specialized equipment. Alternatively, cryo-fixation methods seek to preserve samples in as near the in vivo state as possible by stabilizing instantaneously and reducing or eliminating the use of chemicals. Cryo-fixation methods are preferable to chemical fixation methods; however, they have significant technical and financial disadvantages.

Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2_19,

Standard chemical fixation protocols are readily available that give reproducible results (e.g., [1]), with minimal use of specialized equipment. These protocols are relatively quick and easy to do. The main disadvantage of using chemical fixation is that the introduction of toxic chemicals to the sample can have an unknown effect on ultrastructure. Fixative penetrates even soft biological material slowly which allows changes to occur within the sample before it is fully stabilized, for example, redistribution and extraction of ions and soluble proteins [2], extraction and rearrangement of phospholipids [3], mismatch in osmotic conditions leading to organelle blooming, and non-isotropic shrinkage [4]. There is no such thing as a "universal fixative," and fixatives do not preserve all structures within cells equally. Another important disadvantage of chemical fixation is that at every stage during the process, antigens in the sample are destroyed. Therefore, it is not possible to carry out immunogold labeling experiments using chemically fixed and epoxy resin embedded samples.

The only viable alternative to the deleterious effects of chemical fixation and dehydration is to preserve samples by freezing the water present rapidly enough to prevent ice crystals forming. If samples are frozen quickly enough, the water inside the sample is vitrified, and both soluble and non-soluble structures are held in a glass-like matrix (amorphous ice), stabilizing the sample instantaneously. Amorphous ice is non-destructive but to achieve full vitrification of cellular water, very high cooling rates are required [5]. If these cooling rates are not reached, crystalline ice forms and solutes within the cell are trapped between the crystals, forming a network of segregated compartments. When viewed in the microscope, this is known as ice segregation artifact and is particularly obvious in badly frozen nuclei where it appears as a "cracking" pattern (Fig. 1). Once frozen, the sample may be stored in liquid nitrogen (−196 °C) before further processing. The temperature cannot be allowed to approach the re-vitrification point of water (around −140 °C) or ice crystals will form. The advantage of using freezing techniques to preserve samples is that they are stabilized instantaneously without the need for chemicals. However, the disadvantages are that at atmospheric pressure good freezing only occurs within a few microns of the surface at best, and the techniques involved are difficult and time-consuming requiring dedicated equipment.

There are several different cryo-fixation methods available for use (e.g., slam freezing, jet freezing, and plunge freezing), but the depth of good preservation is limited to 20–40 μm at atmospheric pressure, whichever method is used [6]. An alternative is to freeze the sample at high pressure, an idea first postulated by Moor and Riehle in 1968 [7]. At higher pressures, water expands less during freezing; hence, less heat of crystallization is produced, so adequate cryo-fixation is achieved at reduced cooling rates [8, 9].

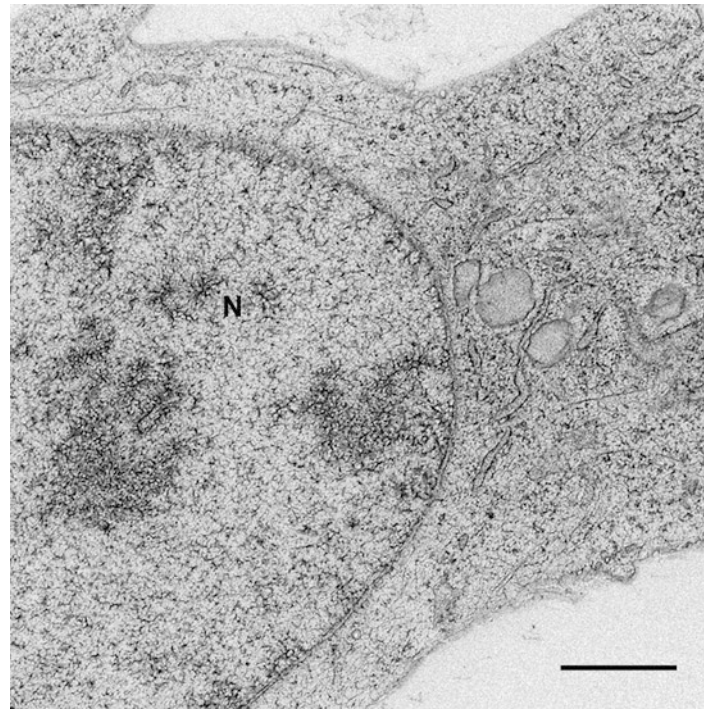

Fig. 1 Example of ice segregation artifact in a cell culture Vero cell. This appears as a "cracking" pattern and is particularly obvious in the nucleus (N) of badly frozen cells. Scale bar indicates 1 μm

At atmospheric pressure, amorphous ice is produced at a freezing rate of several 10,000 °C/s; however, if samples are frozen at 210 MPa, this required freezing rate drops to several 1000 °C/s [5]. Freezing at high pressure allows vitrification to occur to a depth of 200 μm [6, 10]. For this reason, "high-pressure freezing" (HPF) can be used to prepare many different types of sample, ranging from cell suspensions to small pieces of solid tissue and is widely regarded as the optimal cryo-fixation method for general electron microscopy [6]. For a comprehensive review of high-pressure freezing and freeze substitution, see McDonald [11].

After high-pressure freezing, samples are processed for morphological or immunocytochemical studies by freeze-substitution (FS). During FS, amorphous ice is replaced with solvent, generally acetone [12], containing one or more chemical additives at low temperature. The addition of chemicals at this stage does not affect the preservation of tissue as the sample has already been cryo-stabilized in the high-pressure freezer. After substitution, samples are infiltrated with acrylic resin at low temperature, and the resin is polymerized. Ultra-thin sections can be cut at room temperature and examined in the transmission electron microscope.

There are many published protocols for freeze substitution all of which vary considerably. One of the greatest variations is the time that samples are kept in the substitution medium prior to embedding. It is widely accepted that leaving samples in fixative and/or solvents during room temperature chemical fixation can rearrange cell components, especially lipids [3], and extract soluble cytoplasmic contents [2]. At low temperatures, this process slows but does not stop. Therefore, samples need sufficient time in substitution media for full replacement of water, but not a prolonged substitution which leads to extraction of cell components. Dedicated freeze substitution units are available which finely control the temperature changes required during substitution and in some cases can be programmed to control the addition of solvents. These units are expensive to purchase; however, a protocol has been developed which negates the need for dedicated freeze substitution units [13].

Here we describe a method for high-pressure freezing and freeze substitution of cells in culture that minimizes mechanical or chemical stress prior to freezing and gives consistent preservation of cellular architecture. The thermal load of the sample is reduced by using "naked" sapphire discs and by avoiding the use of cryo-protectants or fillers. Reducing the thermal load significantly increases the quality of freezing.

2 Materials

2.1 Chemical Reagents

1. Fetal calf serum.
2. Appropriate cell culture media.
3. Pure methanol.
4. Uranyl acetate (UA) crystals.
5. Analytical-grade acetone (99.9%).
6. 20% (w/v) solution of UA in methanol (*see* **Notes 1** and **2**).
7. Freeze substitution (FS) medium: 2% (v/v) uranyl acetate in analytical-grade acetone (*see* **Notes 1** and **2**).
8. Lowicryl HM20, made to manufacturers specification (*see* **Note 3**).
9. Plentiful supply of liquid nitrogen.
10. Epoxy resin blocks, previously polymerized in Beem capsules for mounting samples.

2.2 Hardware

1. Dark glass screw top bottle to store 2% uranyl acetate solution (at 4 °C).
2. Clear glass screw top bottle for mixing Lowicryl HM20 resin.
3. Ultra-fine forceps (long, narrow handles).
4. Cryo-forceps for transferring sample tubes to and from the liquid nitrogen dewar.

5. Liquid nitrogen dewar (1 l).
6. Polystyrene liquid nitrogen holder, shallow.
7. Adjustable, illuminated magnifying lamp.
8. High-pressure freezer (e.g., Leica EM ICE, Leica HPM100, or ABRA HPM010) with all necessary associated inserts, spacers, etc.
9. Freeze substitution unit (e.g., Leica AFS 2 or equivalent) with all necessary associated containers, embedding molds, etc.
10. Mini hacksaw.
11. Razor blade.
12. Transmission electron microscope, 120 or 200 kV (preferable for tomographical studies).

2.3 Consumables

1. 6 mm sapphire coverslips.
2. Appropriate cell culture plates.
3. 1.5 ml Eppendorf tubes, screw top, two holes punched below cap.
4. Filter paper.
5. Plastic Pasteur pipettes.
6. Formvar (or equivalent)-coated copper 200 mesh, hexagonal, thin bar grids (for thin sections, morphological imaging).
7. Formvar (or equivalent)-coated single-slot grids or copper 50 mesh, hexagonal grids (for thick sections, electron tomographical imaging).

3 Methods

Please make sure to follow local chemical safety procedures and ensure appropriate PPE is used.

3.1 Preparation of Cells

1. Choose sapphire discs appropriate to the sample holder associated with your high-pressure freezer (HPF). We use 6 mm sapphire discs designed for the Leica HPM100 (Leica Microsystems). Discs should be incubated in fetal calf serum for 60 min at 37 °C before cells are added. This provides a proteinaceous layer for cells to adhere to (*see* **Note 4**).
2. In a suitable cell culture vessel (24-, 12-, or 6-well plate), place discs on the base of each well and add the appropriate volume of cell suspension. Ensure the discs are flat on the base of each well and are not floating in the medium. Incubate at 37 °C for an appropriate amount of time, so that the sapphires are coated with an approximately 80% confluent cell monolayer.
3. Infect the cells as appropriate.

3.2 Fixation of Cells by High-Pressure Freezing

1. One hour before you want to freeze your samples, start cooling down the high-pressure freezer. Make sure you have enough liquid nitrogen available for the entire process. Fill a small (~1 l) dewar with liquid nitrogen for transporting your samples. Fill a small polystyrene box with liquid nitrogen to use to cool down your tools. Ensure that these vessels are in close proximity to the freezer and that you have the means to re-fill them as needed.
2. Once the HPF is cool and stable, do a test freezing run to check it is working correctly.
3. In an MBSC, remove cell culture medium from wells containing the sapphire discs and replace with warmed, fresh medium.
4. Take the cell culture dishes containing the sapphire discs to the HPF and load the sapphires into the appropriate holder as in Fig. 2. There is no need to add fillers/cryoprotectants (*see* **Note 5**). There is no need to encase in aluminum planchettes (*see* **Note 6**).
5. Once they are loaded, quickly freeze the samples.

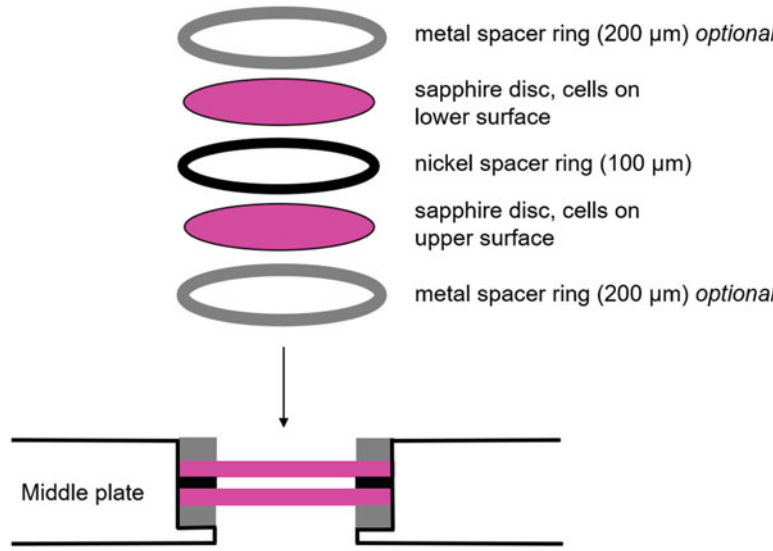

Fig. 2 Diagram illustrating the method used to load a Leica HPM100 middle plate. The method can be adapted for other manufacturers models; however, the basic "sandwich" structure should remain constant, so that the liquid nitrogen has direct access to the sapphire discs during the freezing process. Spacer rings, sapphire discs, and nickel grid all 6 mm in diameter. The use of one or two outer nickel spacers is discretionary, depending on available depth. The entire "sandwich" should fill the depth of the middle plate. Sapphire discs thickness 100 μm, nickel spacers thickness either 100 or 200 μm. Diagram adapted from [14]

6. Remove the frozen "sapphire disc sandwich" from the sample holder under liquid nitrogen in the polystyrene box, remembering to use cooled tools to do so. An illuminated magnifying lamp is useful for this step.
7. Remove the screw top and cool a labeled Eppendorf in liquid nitrogen, making sure it has had two holes punched near the top of the tube.
8. When the Eppendorf is cold, place the sapphire disc sandwich inside, replace the screw top and leave to float in liquid nitrogen until all samples have been frozen and placed in labeled Eppendorf tubes.
9. Once all samples have been frozen, quickly transfer the Eppendorf tubes, to the liquid nitrogen transfer dewar, replace the lid and transfer to appropriate liquid nitrogen storage. Samples must be stored in the liquid phase, not in the gaseous phase.

3.3 Freeze Substitution

This process is started during the late afternoon and the freeze substitution (FS) unit programmed to start substitution the following morning.

1. One hour before use, cool down the FS unit with liquid nitrogen to its lowest temperature, typically −160 °C. We have an AFS 1 and AFS 2 (Leica Microsystems); however, this FS method can be programmed into any other FS unit you may have available.
2. Ensure that the exhaust of the FS unit chamber is fed into a fume hood and that you are working in close proximity to the fume hood. It is preferable to place any waste solvent/resin straight into the hood.
3. Place an appropriate number of aluminum cups (supplied with the FS unit) into the cold chamber (one per sample) and wait 5–10 min for them to cool.
4. Using a small liquid nitrogen transfer dewar, collect the appropriate samples from the liquid nitrogen store and place the dewar in the fume hood next to the FS unit.
5. Place an Eppendorf containing one sapphire sandwich into each of the cooled aluminum cups. The samples will still be in a small amount of liquid nitrogen. This will boil off as the Eppendorf and chamber equilibrate overnight.
6. Program the FS unit to follow a short FS/short warmup cycle (Fig. 3). For this particular type of sample, this protocol was found to be superior to longer protocols (*see* **Note 7**). Here is an example of timings, adjust as necessary although keep the time intervals consistent with the protocol.
   (a) Temperature to rise from −196 °C to −160 °C overnight.

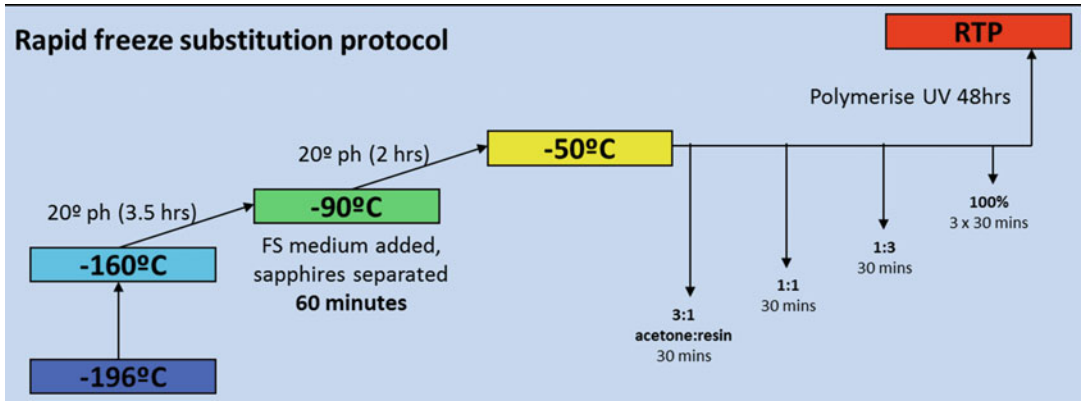

Fig. 3 Diagram summarizing short freeze substitution/short warmup cycle protocol as described in Hawes et al. [14]

(b) 05:30: temperature to rise from −160 °C to −90 °C at 20 °C per hour (3.5 h).

(c) 09:00: temperature held at −90 °C, addition of FS medium (see below) (1 h).

(d) 10:00: temperature to rise from −90 °C to −50 °C at 20 °C per hour (2 h).

(e) 12:00: temperature held at −50 °C during resin infiltration.

7. The following morning, at 08:30 ensure you have all the appropriate consumables, tools, solutions, and containers for waste within easy reach of the FS unit.
8. Cool 1–2 ml of FS medium (*see* **Note 1**) in a clean aluminum cup by placing it in the FS unit chamber and wait for at least 10 min.
9. At 09:00, undo the screw top from each Eppendorf and empty the sapphire sandwich into its aluminum cup.
10. Using a cooled plastic pipette, gently place 0.5 ml of cold FS medium in each aluminum cup, so that each sapphire sandwich is covered. Sandwiches will probably separate in the liquid; take care to note how the sapphire discs separate so you know which side the cells cover.
11. Using cooled fine forceps, ensure the discs lay in the FS medium, cells facing uppermost (*see* **Note 8**). Remove the aluminum cup containing any unused FS medium and place in fume hood to get to room temperature before disposal.
12. Leave samples in FS medium until program has reached −50 °C (12:00).
13. Before 12:00, place one clean aluminum cup in the FS unit chamber containing 1 ml Lowicryl HM20 (*see* **Note 3**) and 3 ml acetone and one empty aluminum cup for waste. Mix the

resin and acetone well and leave for a few minutes to cool. Adjust volumes according to the number of samples you have, but keep the ratio the same. The volumes described here assume four aluminum cups are present.

14. At 12:00, using a cooled plastic pipette, remove the FS medium from each sample and place in a waste aluminum cup also in the chamber.
15. Taking care to note any sapphire movement, gently add 1 ml of cold 1:3 resin:acetone mix to each aluminum cup containing a sample, using a clean cooled pipette. Leave for 30 min.
16. Remove the aluminum cup containing any leftover 1:3 resin mix and place in fume hood.
17. Place a clean aluminum cup into the chamber containing 2 ml Lowicryl HM20 and 2 ml acetone. Mix well and allow to cool.
18. After 30 min, using a cooled plastic pipette, remove the 1:3 resin mix from each sample and place in the waste aluminum cup in the chamber.
19. Taking care to note any sapphire movement, gently add 1 ml of cold 1:1 resin:acetone mix using a clean cooled pipette. Leave for 30 min.
20. Remove the aluminum cup containing any leftover 1:1 resin mix and place in fume hood.
21. Place a clean aluminum cup into the chamber and mix 3 ml Lowicryl HM20 and 1 ml of acetone. Mix well and allow to cool.
22. After a further 30 min, using a cooled plastic pipette, remove the 1:1 resin mix from each sample and place in the waste aluminum cup in the chamber.
23. Again, taking care to note any sapphire movement, gently add 1 ml of cold 3:1 resin:acetone mix using a clean cooled pipette. Leave for 30 min.
24. Remove the aluminum cup containing any leftover 3:1 resin mix and place in fume hood.
25. Place a clean aluminum cup containing 14 ml Lowicryl HM20 resin into the chamber and allow to cool.
26. After 30 min, using a cooled plastic pipette, remove the 3:1 resin mix from each sample and place in the waste aluminum cup in the chamber.
27. Taking care to note any sapphire movement, gently add 1 ml of cold resin using a clean cooled pipette. Leave for 30 min.
28. After 30 min, remove the resin and place in the waste aluminum cup in the chamber.
29. Repeat **steps 26** and **27** twice.

30. Cool a flat embedding mold (supplied with FS unit) in the chamber during the last infiltration step.
31. Using cooled tools carefully place each sapphire, cells facing uppermost, into a compartment within the embedding mold, ensuring the disc is flat, and gently cover with cold Lowicryl resin (*see* **Note 9**—handling Lowicryl resin).
32. If available, cool a UV transparent plastic cup (supplied with FS unit) and place over the embedding mold. Seal with a small amount of Lowicryl resin, so that oxygen is excluded during polymerization.
33. Place the UV light (supplied with FS unit) over the chamber, program in the polymerization protocol, and start the program:
    (a) 48 h at −50 °C, with UV.
    (b) Temperature rise from −50 °C to room temperature (20 °C) at 20 °C per hour (3.5 h), with UV.
    (c) 48 h at room temperature, with UV.
34. Dispose of waste chemicals according to local regulations.
35. After polymerization, remove the hardened blocks from the embedding mold. Place in to labeled boxes. Blocks can be stored indefinitely (*see* **Note 10**). When ready to cut, it may be necessary to first mount the Lowicryl blocks onto polymerized epoxy resin blocks from Beem capsules to provide support, as follows:
    (a) Remove the pointed end of the polymerized epoxy resin blocks with a mini hacksaw to provide a flat surface.
    (b) Carefully trim the Lowicryl resin block to the region containing the sapphire disc using a fresh razor blade.
    (c) Using epoxy glue, stick the Lowicryl resin block containing the sapphire onto the flat surface of the epoxy resin block, with the sapphire uppermost. Allow to dry.
36. With a razor blade, trim the thin layer of Lowicryl resin from around the edges of the sapphire disc.
37. Immerse the block briefly in liquid nitrogen to dislodge the sapphire disc. Discard the disc, the cells will remain embedded in the Lowicryl resin.
38. With an ultramicrotome, section the Lowicryl block *en face* to produce sections containing longitudinal views of cells in the monolayer, all in the same orientation and plane (*see* **Note 11**).
39. Collect sections on Formvar coated grids (*see* **Note 12**). When using the 2% uranyl acetate freeze substitution medium, it is not necessary to add any further contrast.

40. Collect images using a transmission electron microscope (*see* **Note 13**).
41. For immunogold labeling studies, the procedure above is very similar; however, use an FS medium containing a lower concentration of uranyl acetate, for example, 0.2%.

4 Notes

1. FS medium: first, prepare 20% (w/v) solution of UA in methanol. This will need to be kept on a stirrer in a fume hood for over an hour in order for all the UA crystals to dissolve. Then, prepare 2% (v/v) uranyl acetate by adding 0.5 ml of 20% UA in methanol to 4.5 ml of 99.9% analytical-grade acetone. Mix well. Store in dark glass bottle at +4 °C.
2. It is not necessary to store 99.9% analytical-grade acetone under a molecular sieve to keep water-free (as recommended in some older protocols). During storage under a molecular sieve, the acetone discolors over time, so it is possible the sieve is adding some form of contaminant. We have never recorded any deleterious effects of using analytical-grade acetone which has not been stored under a molecular sieve.
3. Place each resin component into a screw top glass jar and mix the resin by bubbling dry nitrogen gas through the mixture, while holding the lid close to the neck of the jar. This eliminates oxygen from the jar which is important as Lowicryl HM20 does not polymerize effectively in the presence of oxygen.
4. Preliminary experiments using "naked" sapphire discs indicated that cell adhesion was a potential weakness as cells were lost during processing. Collagen IV, fetal calf serum (FCS), Matrigel Basement Membrane Matrix (BD Biosciences), and carbon were tested as substrate pretreatments to aid cell adhesion. Discs were incubated at 37 °C for 60 min in neat FCS, Matrigel, or collagen IV solution (made to manufacturers specification), or discs were coated with 10 nm carbon using a high vacuum carbon coater (Agar Scientific). Most retention of cells was seen using FCS as the substrate pretreatment.
5. To remove intercellular air pockets within samples protocols in the past have included inert, non-penetrating "fillers," for example, 1-hexadecene. To reduce the possibility of artifact, cryo-protectant/fillers were not used during this protocol. We did not see any detrimental effect of omitting this step, indeed it could be considered advantageous as the fillers act as a heat sink and reduce the cooling rate of the sample during freezing, and prevent penetration of FS medium during substitution.

6. Many protocols protect the sapphire discs during freezing using aluminum planchettes (supplied with the high pressure freezer). These planchettes act as a barrier between liquid nitrogen and sapphire discs and act as a heat sink, reducing the cooling rate at the surface of the sample. In our protocol, liquid nitrogen jets directly onto the sapphires producing excellent freezing across the whole sapphire disc.
7. Protocols with long FS times and/or long warmup times were investigated, but the short FS and short warmup protocol described here was found to produce consistently good freezing across large areas of the sapphire, provide good ultrastructural detail, allow minimal visible extraction of cytoplasmic contents, and have the practical benefit of a shortened procedure.
8. It is possible to see the cells on the sapphire discs down the FS unit binocular eyepieces by picking the disc up, above the liquid surface and slightly angling it toward the light. Cells will appear rough on the glass, if cells are not present the disc will have a smooth, reflective surface.
9. Lowicryl HM20 resin can be difficult to work with at first. It has low surface tension and will creep along surfaces if drops escape from pipettes. It has a very strong odor, so keep in the fume hood when possible. When filling the embedding mold, allow resin to settle and then top up to make sure the volume has been filled correctly. Wipe away any drips from the FS unit chamber with paper towel or the embedding mold will become stuck to the chamber during polymerization.
10. The resin should have a pink hue which demonstrates total polymerization. However, if the resin is colorless but hard, this indicates sufficient polymerization for cutting.
11. Collect all the sections (even if incomplete) as the material will be close to the surface of the block face. Cells in culture can be very thin, so it is easy to cut through the whole cell sheet while waiting to collect a complete section.
12. If collecting thick sections for electron tomography, use Formvar (or equivalent)-coated single slot grids or -coated 50-mesh grids. We have found the 50-mesh grids to be preferable as they provide more support when imaging, although they do not allow the full area of the section to be viewed. Also collect some thin sections (on Formvar, or equivalent, -coated 200-mesh grids) for orientation. These thin sections can be grid stained with lead citrate and uranyl acetate if necessary.
13. Lowicryl HM20 is not as beam stable in the TEM as epoxy resins. The Formvar support will help with this, but avoid long beam exposures at high intensities.

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Helena J. Maier and Erica Bickerton (eds.), *Coronaviruses: Methods and Protocols*, Methods in Molecular Biology, vol. 2203, https://doi.org/10.1007/978-1-0716-0900-2, © Springer Science+Business Media, LLC, part of Springer Nature 2020

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