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TITLE:

A Cell Culture Model for Producing High Titer Hepatitis E Virus Stocks

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SUMMARY:

Described here is an effective method on how to produce high viral titer stocks of hepatitis E virus (HEV) to efficiently infect hepatoma cells. With the presented method, both non-enveloped, as well as enveloped viral particles can be harvested and used for inoculating various cell lines.

ABSTRACT:

Hepatitis E virus is the leading cause of liver cirrhosis and liver failure with increasing prevalence worldwide. The single-stranded RNA virus is predominantly transmitted by blood transfusions, inadequate sanitary conditions and contaminated food products. To date the off-label drug ribavirin (RBV) is the treatment of choice for many patients. Nonetheless, a specific HEV treatment remains to be identified. So far, the knowledge about the HEV life cycle and pathogenesis has been severely hampered because of the lack of an efficient HEV cell culture system. A robust cell culture system is essential for the study of the viral life cycle which also includes the viral pathogenesis. With the method described here one can produce viral titers of up to 3 x 10^6 focus forming unit/mL (FFU/mL) of non-enveloped HEV and up to 10^4 FFU/mL of enveloped HEV. Using these particles, it is possible to infect a variety of cells of diverse origins including primary cells and human, as well as animal cell lines. There is no need for inoculation with limited patient isolates, as the production of infectious HEV particles from plasmids poses an infinite source, which makes this protocol exceedingly efficient.

INTRODUCTION:

Hepatitis E is a fairly an underestimated disease with increasing prevalence worldwide. About 20

million infections result in more than 70,000 deaths per year[1]. The underlying agent, the Hepatitis E virus (HEV), was reassigned recently and is now classified within the family *Hepeviridae* including the genera Orthohepevirus and Piscihepevirus. HEV of various origins are classed within the species Orthohepevirus A-D including isolates from humans, swine, rabbits, rats, birds and other mammals[2]. At present, eight different genotypes (GT) of the positive-orientated, single-stranded RNA virus have been identified[2]. Although they differ in their sequence identity, routes of transmission and geographical distribution, their genomic structure is highly conserved. More specific, the 7.2 kbp HEV genome is divided into 3 major open reading frames (ORF1-3). While ORF1 encodes all enzymes needed for a successful replication within the host cell, ORF2 encodes the capsid protein, and the ORF3 protein operates as a functional ion channel required for assembly and release of infectious particles[3]. Once released into the basal or apical lumen HEV exists in both, quasienveloped and non-enveloped/naked species depending on whether the virus originates from blood or feces, respectively[4,5].

While GT1 and GT2 are mainly found in developing countries solely infecting humans[6] via the fecal-oral route, GT3, GT4 and GT7 predominantly occur in developed countries[1,7] with a variety of species serving as reservoirs, e.g., swine[8], rat[9], chicken[10,11], deer[12], mongoose[13], bat[14], rabbit[15,16], wild boar[17] and many more[7,18,19], providing evidence of zoonosis[7,20–22]. In addition to inadequate sanitary conditions[23] and contaminated food products[12,24–26], transmission via blood transfusion and organ transplantations is also possible[27,28]. HEV is a common cause of liver cirrhosis and liver failure[29] especially in patients with pre-existing liver disease, immunocompromised individuals (genotype 3, 4 and 7) and pregnant women (genotype 1). Of note, there are also extrahepatic manifestations such as hematopoietic disease[30–32], neurological disorders[33] and renal injury[34].

To date, the off-label drug ribavirin (RBV) is the treatment of choice for many infected patients[35,36]. However, cases of treatment failure and poor clinical long-term outcomes have been reported. Treatment failure has been linked to viral mutagenesis and increased viral heterogeneity in chronically infected patients[37–39]. On the contrary, a recent European retrospective multicenter study was not able to correlate polymerase mutations to RBV treatment failure[40]. In clinical observations and in vitro experiments, interferon[41–43], sofosbuvir[44,45], zinc salts[46] and silvestrol[47,48] have also shown antiviral effects. Nonetheless, a specific HEV treatment remains to be found, hampered by the lack of knowledge about the HEV life cycle and its pathogenesis. Therefore, a robust cell culture system for virological studies and the development of new antiviral drugs is urgently needed[49].

Unfortunately, like other hepatitis viruses, HEV is difficult to propagate in conventional cell lines and usually progresses very slowly leading to low viral loads. Although some approaches boosted viral loads by the generation of cell line subclones[50] or the adjustment of media supplements[51]. Recently the generation of cDNA clones[52] and the adaption of primary patient isolates by passaging[53,54] further improved HEV propagation in cell culture[55]. In this protocol, we used the genome of a cell culture adapted Kernow-C1 strain (referred to as p6_WT)[54]. Later, an RNA-dependent RNA polymerase mutant of the same strain (p6_G1634R) was identified with enhanced viral fitness, which also contributed to treatment failure[37]. Kernow-C1 is the most

frequently used strain in HEV cell culture and is capable to produce high viral loads. By assessing viral RNA copy numbers, HEV replication can be monitored in vitro. Nevertheless, these techniques do not allow assessment of the number of infectious particles being produced. Therefore, we have established an immunofluorescence staining to determine Focus Forming Units (FFU/mL).

The here described method[56] can be used to produce full-length infectious viral particles that are capable to infect a variety of cell types from diverse origins including primary cells and mammalian cell lines. This is a fundamental prerequisite to decipher important aspects of HEV infection and tropism. There is no need for inoculation with usually limited patient isolates. The production of infectious HEV particles from plasmids poses an infinite source, which makes this protocol comparably efficient. In addition, this system can be used for reverse genetics enabling the study of in vivo identified genome alteration and their impact on HEV replication and fitness. This technique overcomes many limitations and, can path the way for drug development, mutagenesis studies and the evaluation of virus-host interactions such as restriction or entry factors.

PROTOCOL:

NOTE: All experiments are performed under BSL-2 condition. All materials that get in contact with Hepatitis E virus RNA or infectious virus must be rinsed properly with 4% Kohrsolin FF from a waste container inside the hood prior to disposal.

1 Plasmid preparation

1.1 Inoculate 200 mL LB medium containing 100 µg/mL ampicillin with transformed *Escherichia coli* JM109 incorporating a plasmid encoding for the full-length HEV gt3 Kernow-C1p6 sequence (pBluescript_SK_HEVp6 [JQ679013][54] or pBluescript_SK_HEVp6-G1634R[37]). Incubate for 16 h at 37 °C under permanent agitation (170 rpm).

NOTE: Plasmid isolation was carried out using a plasmid extraction kit (see **Table of Materials**).

1.2 Following the manufactures protocol, spin down 200 mL of overnight culture at 6,000 *x g* and 4 °C for 10 min and discard the supernatant. The bacterial pellet can be frozen and stored at -20 °C.

1.2.1 Resuspend the bacterial pellet in 4 mL of Resuspension buffer by pipetting up and down with a 10 mL serological pipette and a pipette man. Additionally, vortex rigorously.

1.2.2 Add 4 mL of prewarmed Lysis buffer (30-40 °C). Invert gently for several times and incubate at room temperature (RT) for 5 min.

1.2.3 Add 4.8 mL of Neutralization buffer, invert gently and make sure that the lysate is quantitatively neutralized (see manufacturer's description). Then centrifuge at 11,000 *x g* and 4

°C for 20 min.

1.2.4 Place a 2 cm x 2 cm mull piece inside a 1 mL non-filter tip, load resuspended, lysed and neutralized supernatant in 10 mL serological pipette, add 1 mL tip with mull to tip of the serological pipette and filter the supernatant in a new 50 mL tube.

NOTE: Supernatant can be stored at 4 °C for up to 30 min if necessary.

1.2.5 In several steps apply 750 µL of the filtered supernatant on a total of 4 filter columns (provided in the kit) and centrifuge at 11,000 *x g* for 30 s. Discard the flow-through and repeat this sequence until all supernatant is applied onto the filter columns.

1.2.6 Wash each filter column twice with 500 µL of prewarmed (50 °C) wash buffer AW at 11,000 *x g* for 30 s and discard the flow-through afterwards.

1.2.7 Wash each filter column with 600 µL of wash buffer A4 by centrifuging at 11,000 *x g* for 30 s. Discard the flow-through and dry the filter columns by centrifugation at 11,000 *x g* for 2 min.

1.2.8 Elute the plasmid DNA from each filter column by transferring 60 µL of elution buffer onto the center of the filter columns. Incubate for 1 min at RT and centrifuge at 11,000 *x g* for 1 min.

1.2.9 Combine eluates and measure the concentration of the extracted plasmid DNA using a spectrophotometer.

2 Linearization and DNA purification

[Place **Figure 1** here]

NOTE: The linearization increases RNA yield during in vitro transcription (step 3)

2.1 To linearize the plasmid DNA (**Figure 1**) mix 10 µg of the template DNA (extracted in step 1), 10 µL of the buffer, 2 µL of *MluI* and adjust to a volume of 100 µL with H_2O.

2.1.1 Incubate for 1 h at 37 °C and confirm linearization of the plasmid by agarose gel electrophoresis (e.g., load non-digested and digested plasmid DNA [1 µL of DNA each] on a 1% agarose gel and run electrophoresis at 120 V constant current).

2.2 Following the manufacturers' protocol for DNA extraction (see **Table of Materials**, **Figure 1**), mix 500 µL of Binding buffer, provided in the kit, with 100 µL of linearized DNA. Apply the sample to a filter column, and centrifuge at 17,800 *x g* for 30 s. Discard the flow-through and place the filter columns back in the same tube.

2.2.1 Wash the filter column by adding 650 µL of Wash buffer and centrifugation at 17,800 *x g*

for 30 s. Discard the flow-through and place the filter column back in the same tube. To remove the residual Wash buffer, dry centrifuge at 17,800 *x g* for 60 s and place each filter column in a clean 1.5 mL microcentrifuge tube afterwards.

2.2.2 Elute DNA by transferring 60 µL of prewarmed H_2O (PCR grade, 70 °C) into the center of the filter. Incubate for 1 min at 70 °C and centrifuge at 18,000 *x g* for 1 min. Measure the concentration of the purified DNA using a spectrophotometer.

NOTE: Store DNA at -20 °C until in vitro transcription.

3 In-vitro transcription of full-length HEV genotype 3 p6 DNA and RNA purification

[Place **Figure 2** here]

NOTE: In vitro transcription is necessary to produce viral genomic RNA from plasmid DNA.

3.1 For in vitro transcription mix 20 µL of 5x T7 transcription buffer, 10 mM DTT, 100 U of ribonuclease inhibitor, 25 mM ATP, CTP, and UTP, 12.5 mM GTP, 5 mM Ribo m^7G Cap Analog, 2 µg of linearized DNA template, and 80 U of T7 RNA polymerase. Fill up to 100 µL with nuclease free H_2O, mix well and incubate for 2 h at 37 °C.

NOTE: Short term storage at -20 °C is possible after step 0 to 0.

3.1.1 Add 2 µL of T7 RNA polymerase, mix well and incubate for another 2 h at 37 °C.

3.1.2 To digest the initial DNA template, add 7.5 µL of DNase (RNase free, 1 U/µL), mix well and incubate at 30 min at 37 °C.

NOTE: Clean all surfaces and pipettes with surface decontaminant for RNase when working with RNA to avoid degradation. Also, ensure that all reaction tubes are RNase-free and sterile. Only use tips with filters and dilute solely with RNase-free and sterile water.

3.2 Following the manufacture's protocol for RNA extraction (see **Table of Materials**, **Figure 2**), prepare a premix of Lysis buffer and 100% ethanol by mixing 330 µL of Lysis buffer and 330 µL of 100% ethanol for each in-vitro transcription reaction. Add 660 µL of Lysis buffer-ethanol premix to 110 µL of RNA and vortex. Load sample on a filter column and centrifuge at 8000 *x g* for 30 s. Discard the flow-through and place the filter column back in the same tube.

3.2.1 Add 600 µL of Wash buffer and centrifuge at 8000 *x g* for 30 s. Discard the flow-through and place filter column back in the same tube. Add 350 µL of Wash buffer and centrifuge at 8000 *x g* for 2 min to remove the residual wash buffer. Place each filter column in a clean 1.5 mL microcentrifuge tube. Open the lid of the filter column and let the column dry for 3 min.

3.2.2 Elute RNA by placing 50 µL of RNase free H_2O into the center of the filter column. Incubate

for 1 min at RT and subsequentially centrifuge at 8,000 *x g* for 1 min. Check RNA by loading 1 µL of RNA on to an agarose gel and measure the concentration of extracted RNA using a spectrophotometer.

NOTE: Store RNA at -80 °C until electroporation and exclusively thaw RNA on ice to avoid degradation.

4 Preparation of HepG2 cells for cell culture derived HEV (HEVcc) production

[Place **Figure 3** here]

NOTE: To avoid contamination, preparation of cells, electroporation, infection, harvesting and cell fixation were carried out under sterile conditions in a biosafety level 2 facility. Incubation steps at 37 °C that involve cells were accomplished in a 5% CO_2 incubator.

4.1 To prepare HepG2 cells for HEVcc production (**Figure 3**), seed cells in complete Dulbecco's Modified Eagle Medium (DMEM) (supplemented with 10 % fetal bovine serum (FBS), 1% MEM Non-Essential Amino Acids solution (NEAA), 1% penicillin/streptomycin and 1 % L-glutamine onto a 15 cm collagen (see **Table 1**, sterile filtered through 0.2 µm mesh) coated culture dish. Incubate at 37 °C until cells are 90% confluent.

NOTE: Each 15 cm dish containing 90% confluent cells will generate enough cells for up to 4 electroporation.

4.2 Gently remove the medium from plate and wash cells once with 10 mL of 1x PBS. Trypsinize cells by adding 3 mL of 0.05% trypsin-EDTA (see **Table 1**) onto the cells and incubate at 37 °C until cells are detached completely. Resuspend cells in 10 mL DMEM complete medium and transfer the cell suspension into a 50 mL tube. Determine total number of cells.

4.3 Since each electroporation requires 5 x 10^6 cells, transfer the appropriate volume in a new 50 mL tube and fill up to at least 35 mL with 1x PBS. Centrifuge cells at 200 *x g* for 5 min and carefully discard the supernatant without disturbing the cell pellet.

4.4 Wash cells once again with 35 mL of 1x PBS at 200 *x g* for 5 min. Place cells on ice and do not remove 1x PBS yet, to keep the cells in suspension.

5 Electroporation of HepG2 cells

[Place **Figure 4** here]

5.1 For electroporation of HepG2 cells (**Figure 4**) prepare 400 µL of Cytomix complete per electroporation by supplementing 384 µL of Cytomix (see **Table 1**) with 2 mM ATP and 5 mM Glutathione. Prepare fresh right before use and place directly on ice.

NOTE: A correct yet quick execution of the following steps is crucial. Therefore, make sure that everything is prepared properly.

5.2 Carefully remove 1x PBS without disturbing the cell pellet from step 0. Resuspend 5 x 10^6 cells in 400 µL of Cytomix complete and add 5 µg RNA from step 0. Transfer the solution into a 4 mm cuvette and pulse once with 975 µF, 270 V for 20 ms with an electroporation system.

5.2.1 After electroporation transfer cells as quickly as possible with a Pasteur pipette into 11 mL DMEM complete per electroporation.

NOTE: To make sure that RNA is successfully transfected into HepG2 cells, a transfection control (TC) will be performed. Expected transfection rates vary between 40-60% of ORF2-positive cells.

5.3 Transfer 10 mL of electroporated cells into a 10 cm culture dish coated with collagen. Add 1.3 x 10^5 cells (300 µL) into one well of a collagen-coated 24-microtitre plate carrying a cover slip (later used as a transfection control). Distribute cells evenly and incubate at 37 °C.

5.3.1 After 24 h, change the medium of the 10 cm dish and replace with 10 mL fresh DMEM complete. Do not change the medium of the transfection control. Incubate for another 6 days at 37 °C.

5.4 Stop the transfection control in 24 well plate 5-7 d post electroporation depending on the cell density by continuing with the immunofluorescence staining protocol (step 0). The transfection efficiency is calculated by counting the number of ORF2 positive cells normalized to the total number of cells.

6 Harvesting of intra- and extracellular HEVcc

[Place **Figure 5** here]

6.1 To harvest extracellular HEVcc (**Figure 5**), filter the supernatant, obtained from the 10 cm dish after 6 days (step 5.3.1), through a 0.45 µm mesh to remove any cell debris. Store harvested extracellular HEVcc at 4 °C for the same day infection, otherwise store at -80 °C.

6.2 To harvest intracellular HEVcc (**Figure 5**), wash cells with 1x PBS and trypsinize by adding 1.5 mL of 0.05% trypsin-EDTA. Incubate at 37 °C until cells are detached completely. Add 8.5 mL of DMEM complete, flush plate to detach cells and transfer cells suspension into 50 mL tube. Centrifuge at 200 *x g* for 5 min.

6.2.1 Discard the supernatant and resuspend the cell pellet in 1.6 mL of DMEM complete per electroporation. Transfer the cell suspension into a 2 mL reaction tube.

NOTE: Do not use larger volumes as it would dramatically decrease viral loads.

6.2.2 Freeze (in liquid nitrogen) and thaw cells. Repeat this sequence 3 times.

NOTE: Do not pool more than one electroporation (1.6 mL) for the 3 freeze-and-thaw cycles as the lysis efficiency would be impaired. Do not vortex the cell suspension in between the cycles. Make sure to thaw cell suspension slowly (e.g., room temperature or on ice) to maximize viral loads.

6.2.3 High-speed centrifuge the lysed cells for 10 min at 10,000 *x g* to separate cell debris. Transfer the supernatant in a new tube. Take the supernatant for infection, otherwise store at -80 °C.

6.2.4. Optionally, concentrate the extra- and intracellular HEVcc using a concentrator to increase viral loads (according to manufacturer's protocol).

7 Infection of HepG2/C3A cells with intra- and extracellular HEVcc

[Place **Figure 6** here]

NOTE: The infection of HepG2/C3A cells shall ensure that infectious particles were produced. Additionally, the titration of the harvested intracellular and extracellular HEVcc is used to calculate the virus titers in FFU/mL. This will be later referred to as infection control (IC)

7.1. To prepare HepG2/C3A cells for HEVcc infection (**Figure 6**), prewarm Minimal Essential Medium (MEM) complete (see **Table 1**) to 37 °C.

7.1.1 Seed 2 x 10^4 cells/well in 100 µL onto the collagen coated 96 well microtiter plate one day prior to step 0. Make sure to fill the outermost wells with 1x PBS to prevent evaporation of the medium inside the internal 60 wells. Incubate at 37 °C for 24 h.

7.1.2 Infect with extracellular HEVcc by adding 50 µL of the supernatant (from step 0) to the HepG2/C3A cells seeded the day before. Mix well by pipetting up and down and serially dilute six times 1:3 by transferring 50 µL into the next well. Perform duplicates of serial dilution for technical replicates.

7.1.3 Infect with intracellular HEVcc by adding 25 µL supernatant (from step 0) to the HepG2/C3A cells seeded the day before. Mix well by pipetting up and down and serially dilute six times 1:5 by transferring 25 µL into the next well. Perform duplicates serial dilution for technical replication.

7.1.4 After 7 d at 37 °C stop the infection by continuing with the immunofluorescence staining protocol (step 0).

8 Immunofluorescence staining of transfection- and infection control

[Place **Figure 7** here]

8.1 For immunofluorescence staining (Figure 7), wash 3x with 1x PBS and fix cells by dispensing 350 µL (transfection control [TC]) or 50 µL (infection control [IC]) of 4% Fixation solution per well. Incubate for 15 min at RT and carefully wash twice with 1x PBS afterwards.

NOTE: The protocol can be paused here for the transfection control until the infection of HepG2/C3A cells is stopped as well. Store well plate at 4 °C. Also seal with parafilm to prevent evaporation.

8.2 Permeabilize cells by adding 350 µL (TC) or 50 µL (IC) of 0.2% Triton X-100 (in 1× PBS) for 5 min at RT. Then carefully wash twice with 1x PBS and block with 350 µL (TC) or 50 µL (IC) of 5% Horse Serum (in 1x PBS) for 1 h at RT under constant agitation on an orbital shaker (30 rpm).

NOTE: Prepare small plastic dish (30 mm) by placing a moist tissue inside and a paraffin film layer on top, creating a humid chamber. Then transfer the TC cover slip facing up onto the paraffin film. The following steps for the TC will be executed in the humid chamber.

8.3 Add 70 µL (TC) or 25 µL (IC) of primary antibody anti-ORF2 8282 (HEV-specific rabbit hyperimmune serum,1:5,000 in 5% Horse Serum) per well and incubate overnight at 4 °C under constant agitation on an orbital shaker (30 rpm).

8.3.1 Carefully wash twice with 1x PBS and add 70 µL (TC) or 25 µL (IC) of secondary antibody goat anti-rabbit 488 (1:1,000 in 5 % Horse serum). Incubate for 1 h under constant agitation on an orbital shaker (30 rpm) in the dark. Again, carefully wash twice with 1x PBS

8.4 Add 70 µL (TC) or 25 µL (IC) of DAPI (1:10,000 in H_2O) and incubate for 1 min. Carefully wash twice with H_2O. Take out the cover slip from the humid chamber and mount with 6 µL of the mounting reagent upside down on a cover slide (only for TC). Store IC in water and seal the plate with paraffin film to avoid drying due to evaporation.

8.5 Take images to confirm successful transfection and infection.

NOTE: Comparable results were obtained using the commercially available anti-ORF2 1E6 antibody[57] (1:200 in 5 % Horse serum) and a secondary antibody donkey anti-mouse (1:1,000 in 5 % Horse serum)

9 FFU determination

NOTE: One FFU is defined as one or more ORF2-positive cells separated from another FFU by at least three negative cells.

9.1 For extracellular HEVcc start to count all ORF2-positive foci of the two wells in the two lowest dilutions. A total of four wells should be counted. The focus forming units (FFU) per

milliliter can be calculated with the following equation:

$$Number\ of\ ORF2\ positive\ foci\ x\ Dilution\ factor\ x\ 20 = FFU/mL$$

NOTE: Expected titers vary between 10^3 and 5 x 10^4 FFU/mL.

9.2 For intracellular HEVcc start to count all ORF2-positive foci in wells where between 50 to 100 foci are observed. Additionally, count the two wells in the next higher dilution. A total of four wells should be counted. The focus forming units (FFU) per milliliter can be calculated with the following equation:

$$Number\ of\ ORF2\ positive\ foci\ x\ Dilution\ factor\ x\ 40 = FFU/mL$$

NOTE: Expected titers vary between 10^5 and 3 x 10^6 FFU/mL. The factors 20x and 40x are used to extrapolate the number of FFU per 50 µL and 25 µL to 1 mL and can be adapted accordingly.

REPRESENTATIVE RESULTS:

In this protocol, we describe the production of high titer infectious HEVcc. The first step is to isolate plasmid DNA (pBluescript_SK_HEVp6[54] and pBluescript_SK_HEVp6-G1634R[37], **Figure 8a**), which then is linearized by restriction digestion and purified for in vitro transcription (**Figure 1**). A successful linearization can be verified by comparing the non-digested plasmid-DNA to the digested plasmid-DNA using gel electrophoresis. In addition to a size-shift, only one DNA band should be visible representing the linear form. The linearization is complete when the two other bands above and below the linear form, representing the nicked circle and the supercoiled form, respectively, are completely diminished (**Figure 8b**). The yield of the purified DNA should exceed 150 ng/µL. Only if these characteristics hold true the linearized DNA should be used for in vitro transcription. The in vitro transcribed RNA should be as well checked using gel electrophoresis and in case of low RNase abundancy should show distinct bands rather than a blurred smear (**Figure 8c**). Additionally, the purified RNA yield should exceed 500 ng/µL The in vitro transcribed RNA (**Figure 2**) eventually is electroporated into HepG2 cells for virus production (**Figure 3** and **Figure 4**). Successful electroporation is monitored by the immunofluorescence staining of the transfection control (**Figure 9a**). The transfection efficiency should exceed 40% (**Figure 9b**). A replication deficient mutant serves as a negative control, to ensure specificity of the ORF2 staining, as no ORF2 expression is expected (**Figure 9c**). After 7 days of incubation the enveloped (extracellular) HEVcc are harvested by collecting and filtering the cell culture supernatant. Non-enveloped (intracellular) HEVcc is released from the cells by several freeze and thaw cycles. To remove any cell debris the cell lysate is centrifuged at high speed (**Figure 5**). Subsequently, both HEVcc species are utilized to infect HepG2/C3A cells by serial dilution (**Figure 6 and Figure 9d**). According to the equations above (see step 0) viral titers are determined by FFU calculation.

Representative results are depicted in **Figure 9**. The transfection control should comprise around 50% ORF2-positive cells to guarantee an efficient amount of virus being produced (**Figure 9a,b**). The less ORF2-positive cells the lower the titer will be. Precisely following the steps mentioned in the protocol will generate titers that vary between 10^5 and 3 x 10^6 FFU/mL for the non-

enveloped (intracellular) HEVcc. For the enveloped (extracellular) HEVcc titers between 10^2 and 10^4 FFU/mL are expected (**Figure 9e**). Additionally, elevated FFU counts were observed for the G1634R mutant. When calculating the ratio between genome copies and infectious viral particles the produced intracellular HEVcc for both p6_WT and p6_G1634R was found to be lower compared to the extracellular HEVcc, suggesting a higher specific infectivity of the non-enveloped HEV species (**Figure 9f**).

[Place **Figure 9** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic experimental setup for the plasmid linearization and DNA purification.

Figure 2: Schematic experimental setup for the *in vitro* transcription and RNA purification.

Figure 3: Schematic experimental setup for the preparation of HepG2 cells for cell culture derived HEV (HEVcc) production.

Figure 4: Schematic experimental setup for the electroporation of HepG2 cells.

Figure 5: Schematic experimental setup for harvesting intra- and extracellular HEVcc.

Figure 6: Schematic experimental setup for the infection of HepG2/C3A cells with intra- and extracellular HEVcc.

Figure 7: Schematic experimental setup for the immunofluorescence staining of transfection and infection control.

Figure 8: Generation of *in vitro* transcribed RNA. (**A**) Example of an absorbance spectrum of isolated Plasmid-DNA. (**B**) Gel electrophoresis of non-digested and digested Plasmid-DNA. (**C**) Gel electrophoresis of in vitro transcribed RNA.

Figure 9: Representative results of high titer HEVcc production. (**A**) Transfection control of electroporated HepG2 cells. Transfected cells were stained with anti-ORF2 antibody (green, LS-Bio) and DAPI (blue). (**B**) The transfection efficiency was calculated by the number of ORF2-positive cells normalized to the number of total cells. (**C**) A replication-deficient mutant serves as a negative control for immunofluorescence staining, to ensure ORF2 specificity. (**D**) For FFU determination serial dilutions of the produced virus stocks were performed. ORF2 positive cells are depicted in white. (**E**) Viral titers were calculated by FFU counting and (**F**) viral loads were determined by qPCR and normalized to FFU/mL. Bars show the mean and standard deviation of 38 and 10 independent experiments, respectively.

Table 1: Table of Buffer Composition.

DISCUSSION:

Starting with the plasmid preparation, DNA yields should exceed 500 ng/µL to be able to do multiple linearization from the same plasmid stock, which minimizes the risk of bacteria-induced mutagenesis of crucial genome sequences. Furthermore, it is important to check the restriction digest for the complete plasmid linearization by gel electrophoresis (**Figure 8b**). A lack of linearized plasmid DNA would induce rolling circle amplification causing the in vitro transcription to be less efficient. In addition, RNA integrity should be confirmed to evaluate the abundance of RNases within the sample (**Figure 8c**). Only then an electroporation of target cells should be considered. Of note, before starting with the preparation of HepG2 cells, make sure everything is at hand and well-prepared avoiding long waiting periods. Especially, assure short-time storage of the cells and RNA in the Cytomix until electroporation. To circumvent heating of the cells during electroporation it is essential to cool the buffer on ice beforehand. The duration of the pulse should not exceed 20-25 ms and 270 V. After electroporation the cells require a quick transfer into fresh medium to ensure cell viability. Before infection of target cells, the TC should be checked for the percentage of ORF2-positive cells. In case the TC shows no ORF2-positive cells it is most likely that the electroporation has failed or the staining did not work, and the experiment should be repeated.

When harvesting intracellular HEVcc special attention should be payed to the speed of the freeze and thaw cycles. Viral titers can be increased by executing the freezing in liquid nitrogen rather than at -80 °C. On the contrary, the thawing should take place the slowest way possible suggesting the storage on ice until the cell suspension is liquidated completely. Nonetheless, it is also possible to thaw the cell suspension at room temperature, in a 37 °C incubator or water bath, however the faster the thawing will be executed the more the titers will decrease.

Depending on the following experiments it is also possible to do the freeze and thaw cycles in MEM complete or 1x PBS without dramatic loss of viral titers, however, one should bear in mind that this causes the extracellular HEVcc to be in a different medium than the intracellular HEVcc. Following these crucial steps of the protocol, expected titers vary between 10^5 and 3 x 10^6 FFU/mL for the non-enveloped (intracellular) HEVcc. For the enveloped (extracellular) HEVcc titers between 10^2 and 10^4 FFU/mL are awaited (**Figure 9e**). So far studies employing HEV cell culture systems monitor viral replication and propagation predominantly by qPCR. The assessment of RNA genome copies yet provides no insight into assembly and release of infectious particles.

A previous study[58] successfully propagated patient isolates in cell culture with maximum titers of 10^3 TCID50/mL. As shown recently, it is also possible to successfully passage HEVcc in cell culture and adapt our protocol to other strains, such as 47832c and 83-2 which does not harbor an insertion in the hypervariable region[56]. Although, whether patient derived sequences can be cloned into the Bluescript vector backbone and still yield high viral titers, was not tested. With the introduced method, non-enveloped as well as enveloped viral particles can be harvested and used to inoculate a variety of naïve cell lines such as A549, Huh7.5, Jeg-3 and primary human and porcine hepatocytes, providing a benefit for future applications such as the investigation of HEV tropism, pathogenesis, drug development, viral and host interactions, inactivation studies,

neutralizing antibodies and many more.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

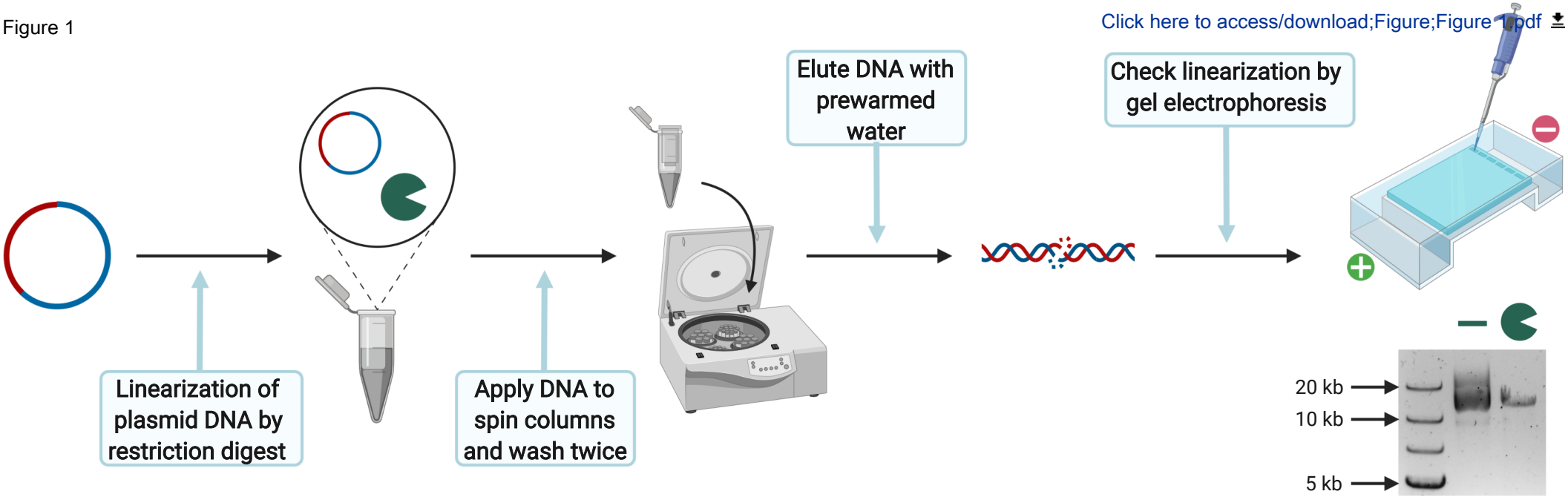


Figure 2

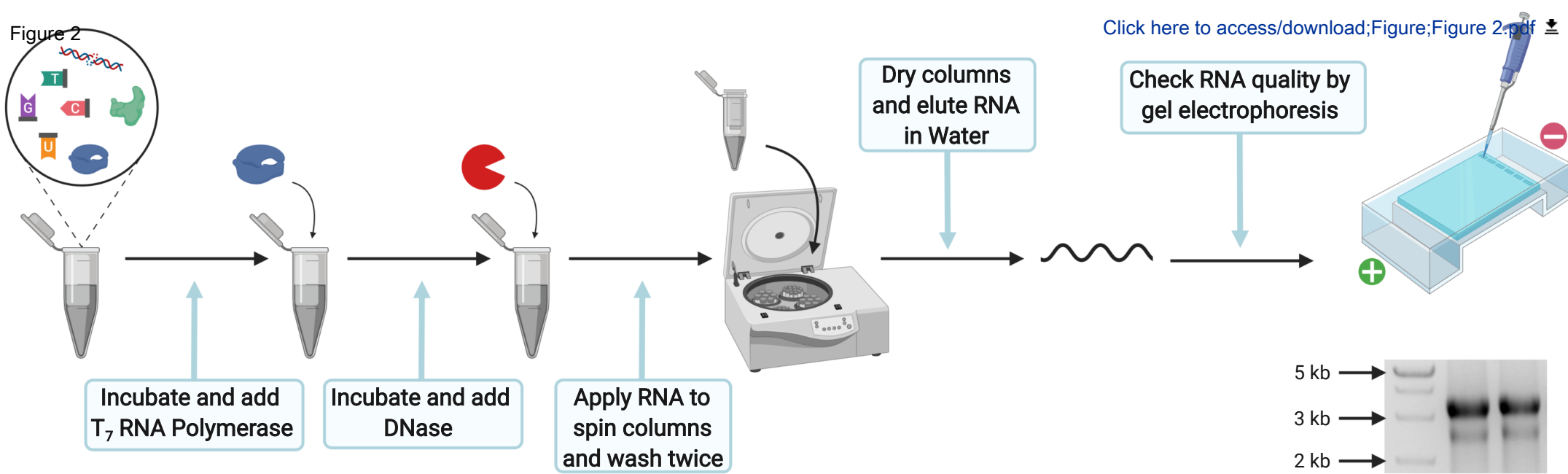
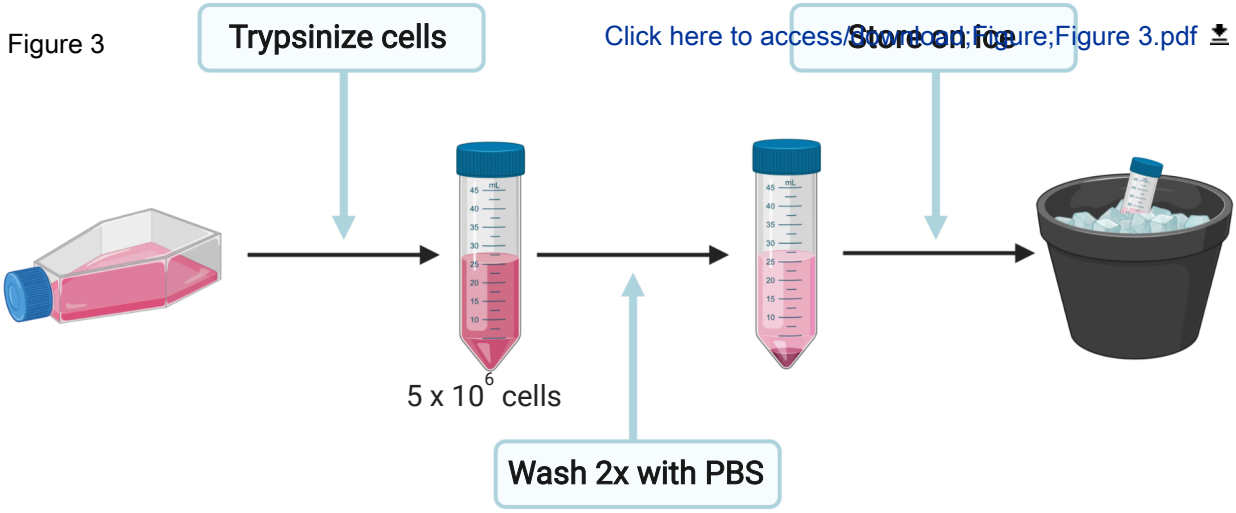


Figure 3



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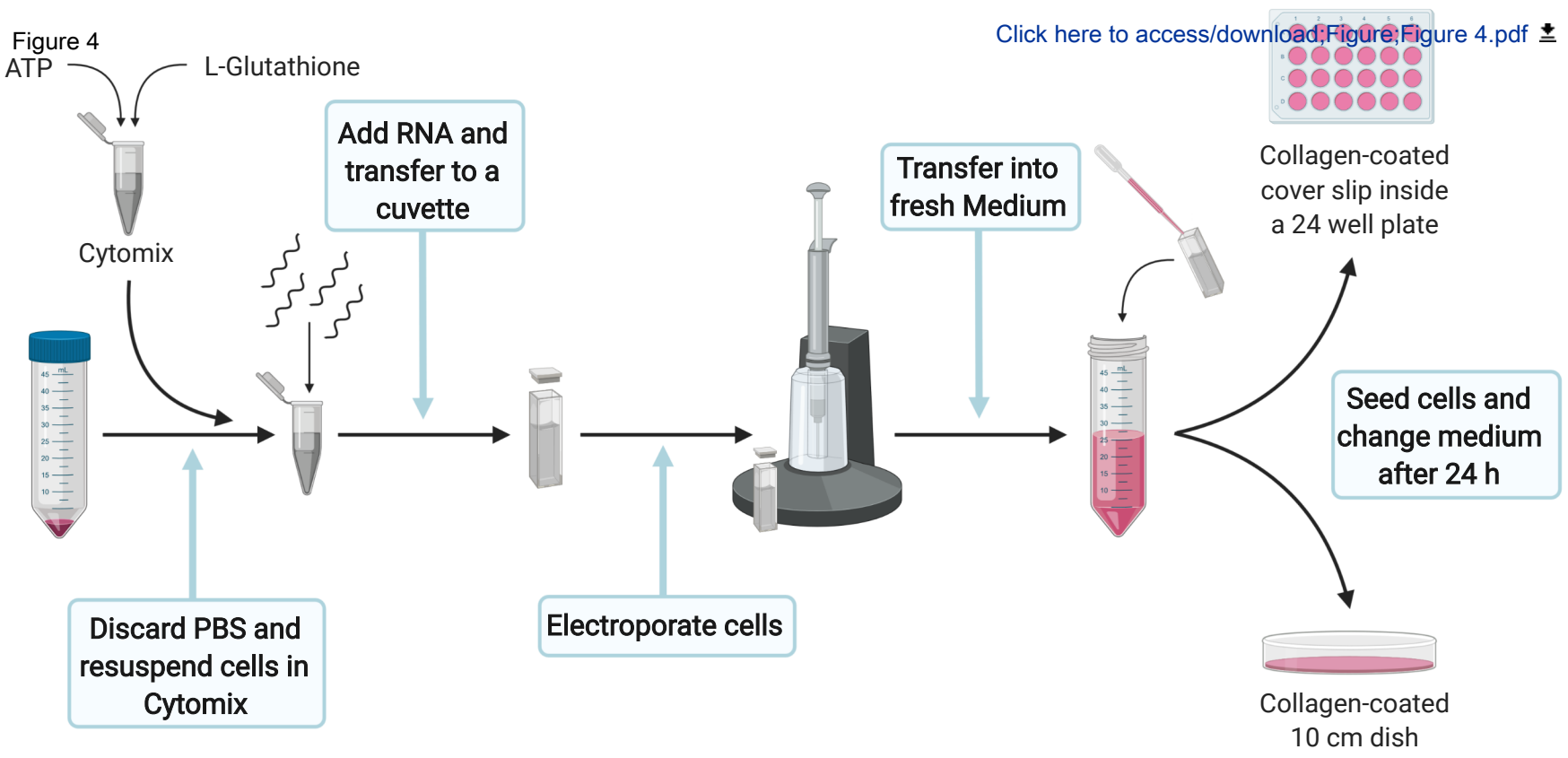


Figure 5

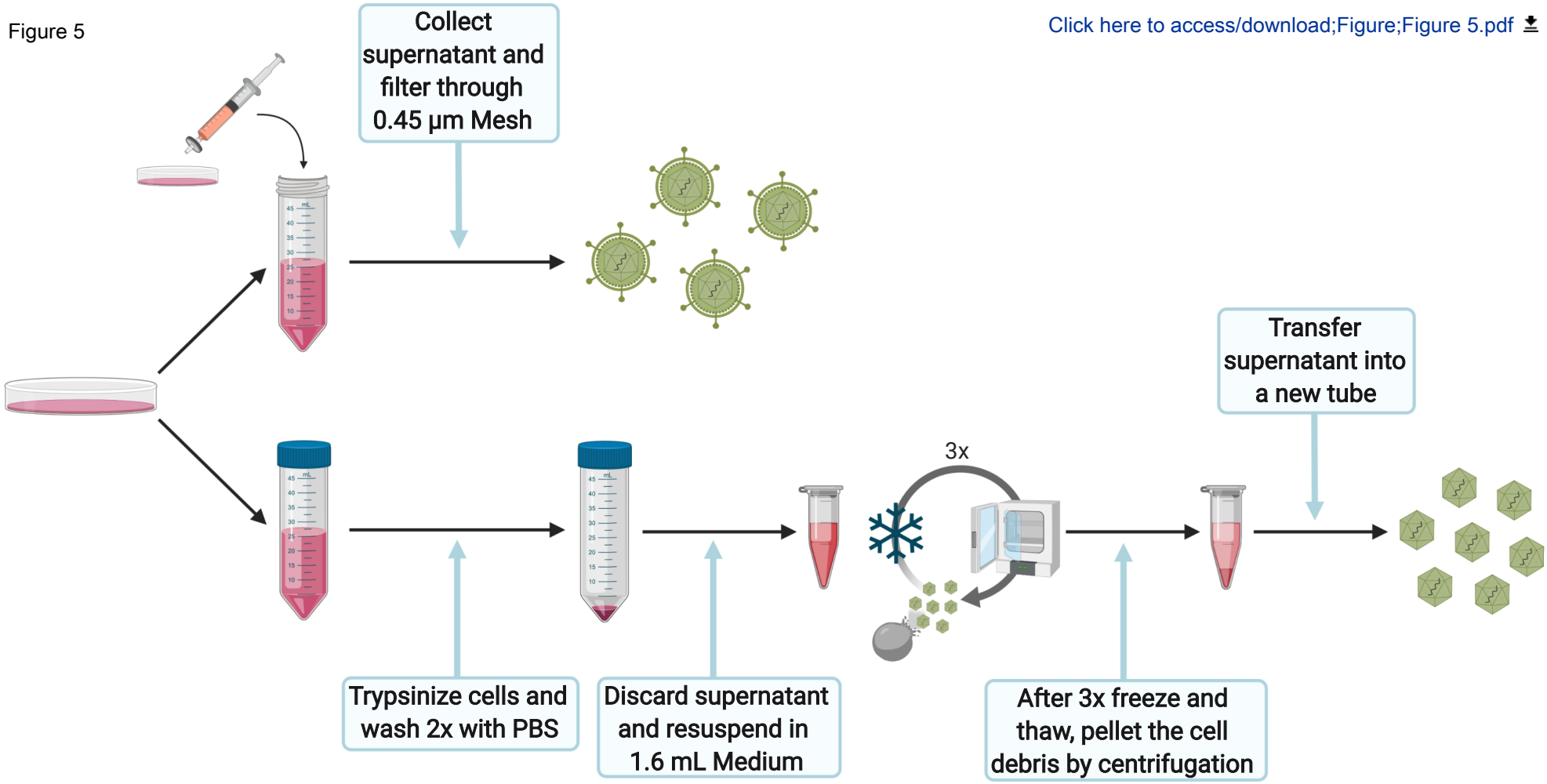
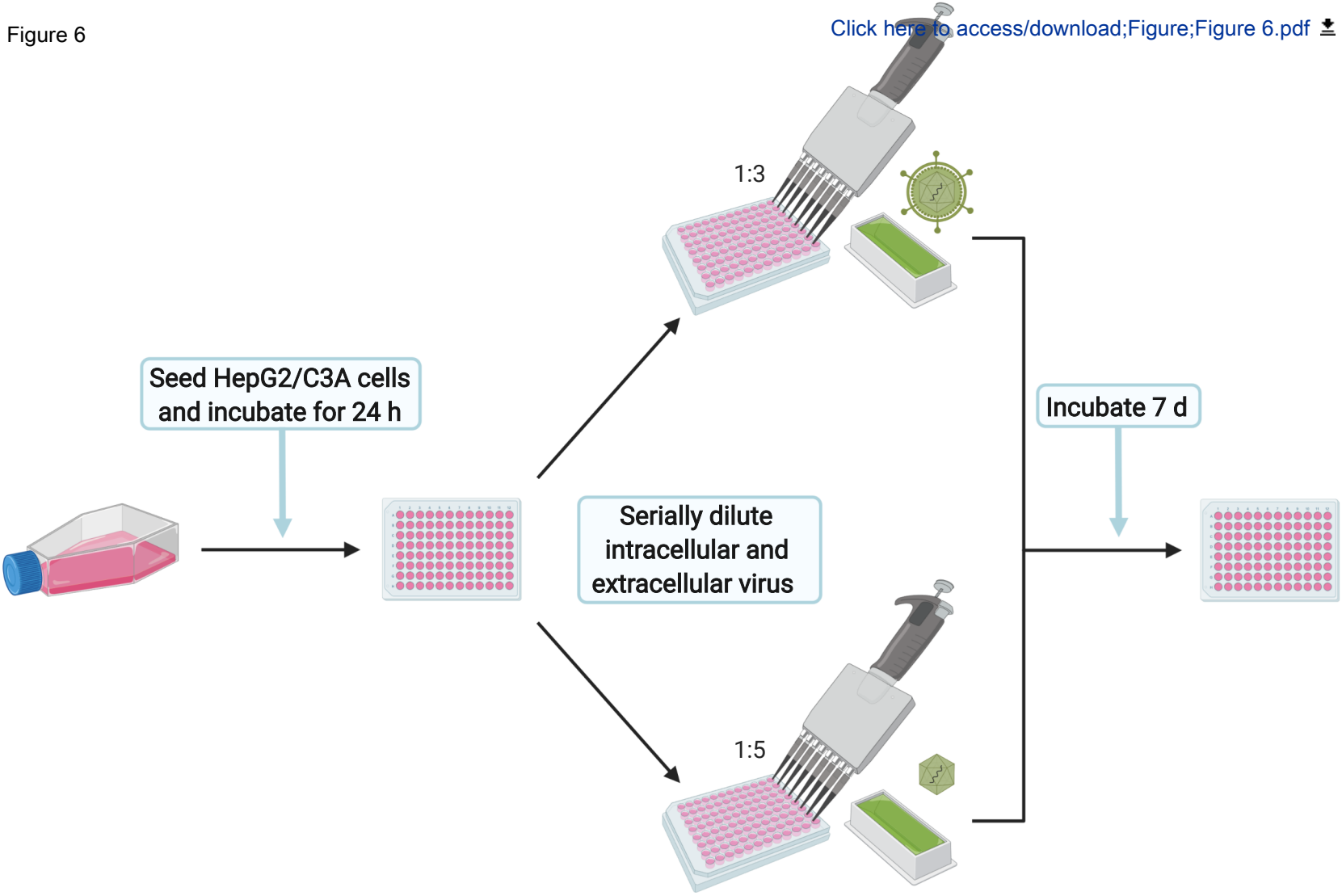


Figure 6



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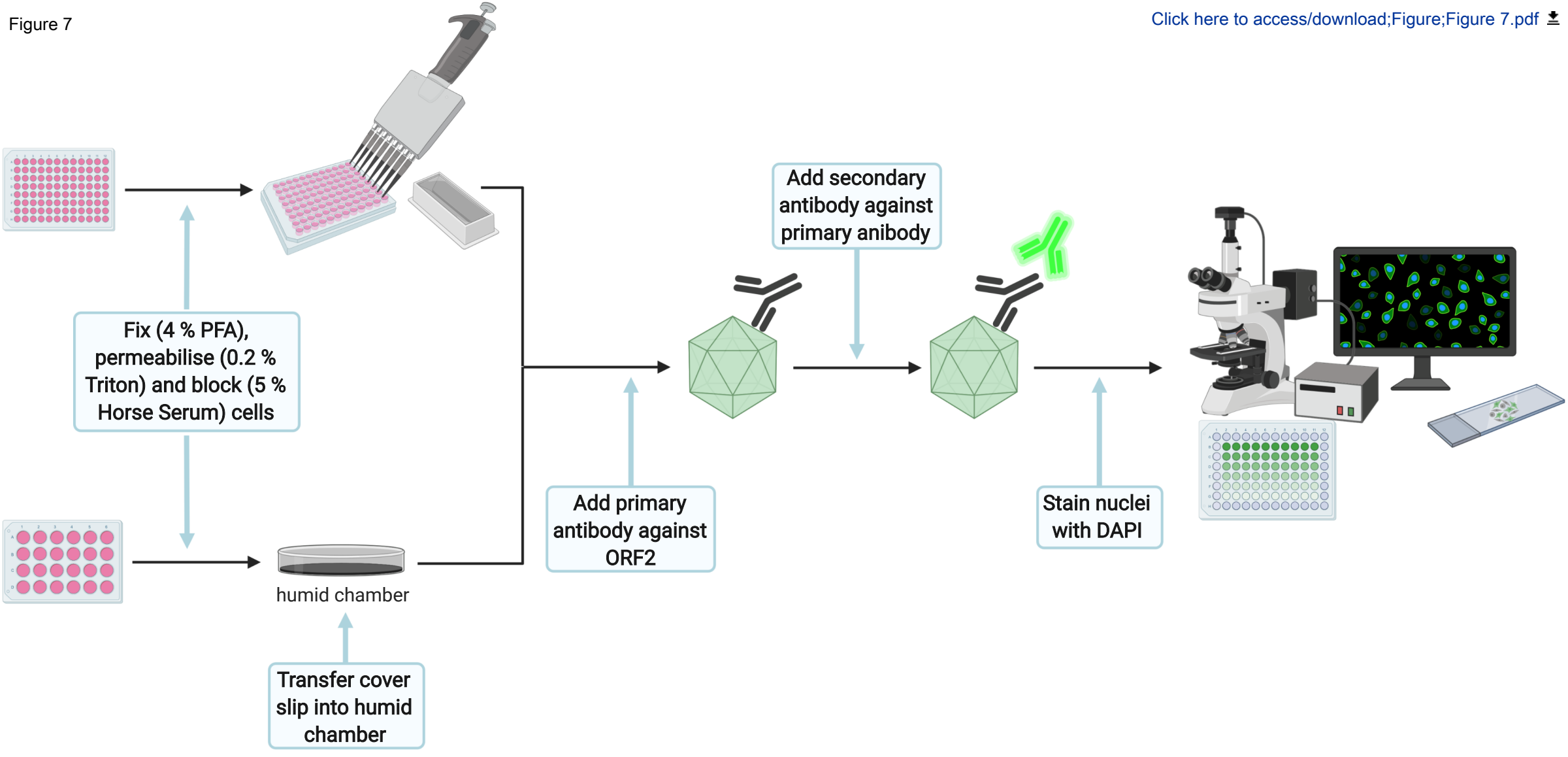

Figure 8

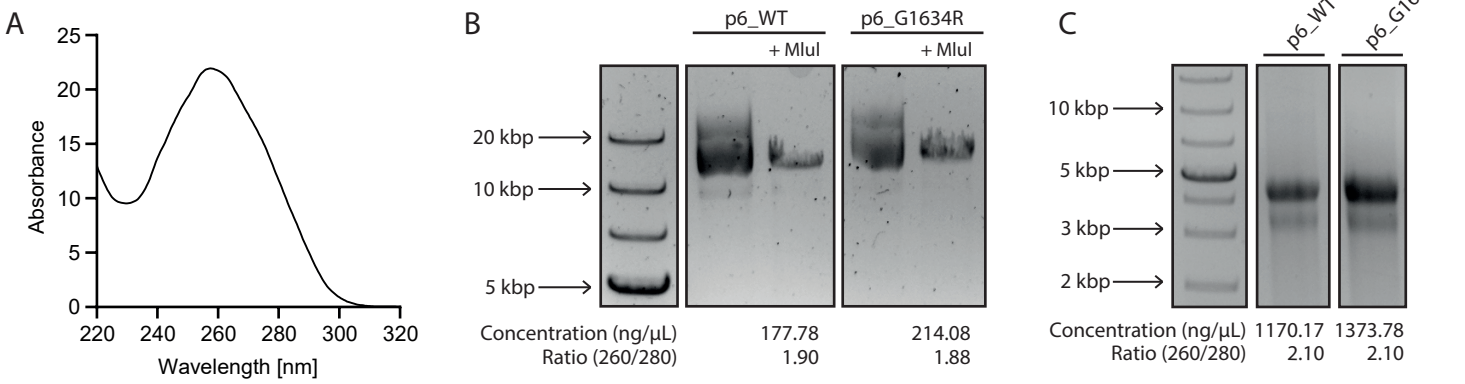

Figure 9

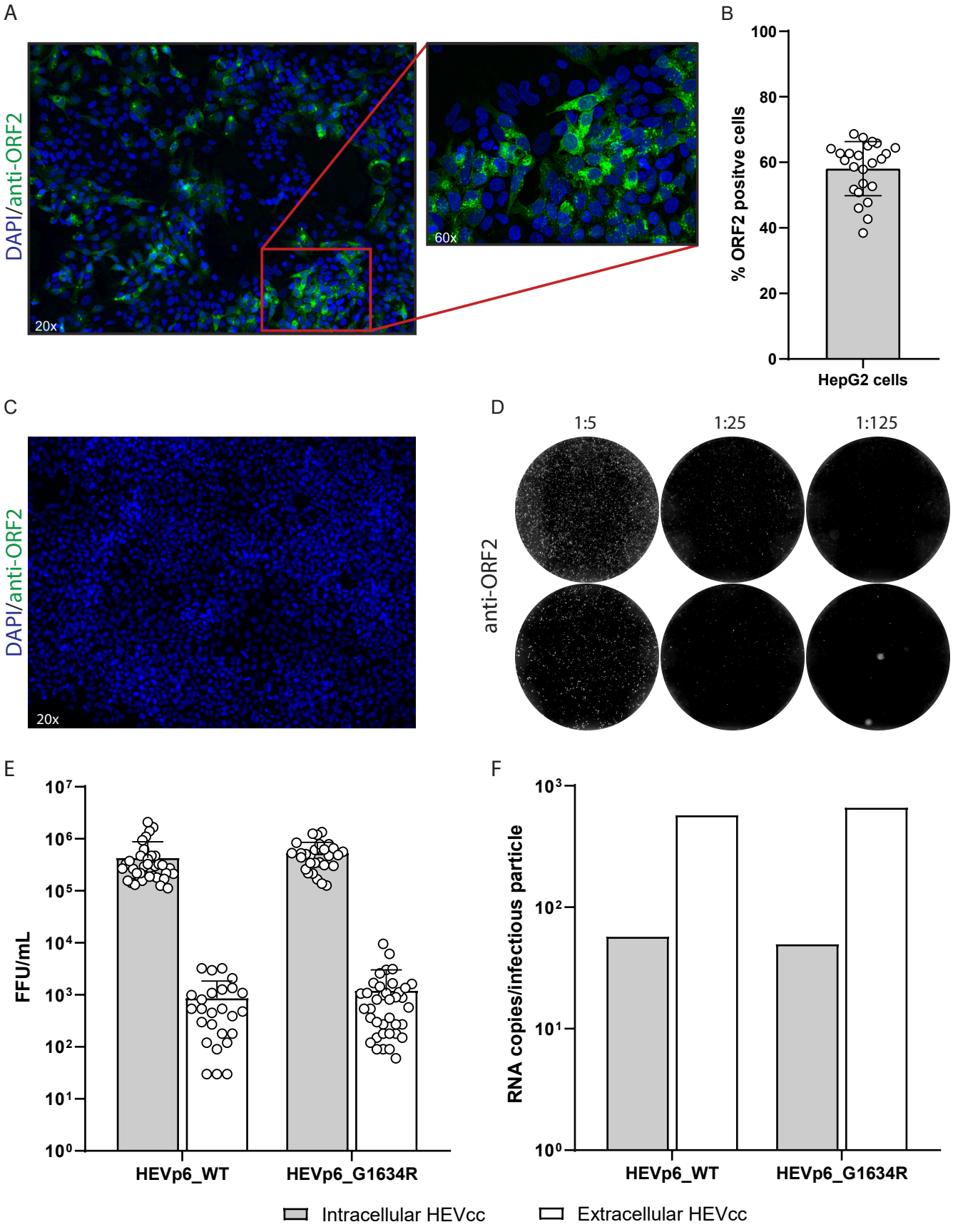

Table of buffer compositions

Working solution	Concentration	Components
Collagen	40 mL	PBS
	40 µl	Acetic acid
	1 mL	Collagen R solution 0.4% sterile
Cytomix	120 mM	KCl
	0.15 mM	$CaCl_2$
	10 mM	K_2HPO_4 (pH 7.4)
	25 mM	HEPES
	2 mM	EGTA
DMEM complete	500 mL	DMEM
	5 mL	Pen/Strep
	5 mL	MEM NEAA (100X)
	5 mL	L-Glutamin
	50 mL	Fetal bovine serum
MEM complete	500 mL	MEM
	5 mL	Sodium Pyruvat
	5 mL	Gentamycin
	5 mL	MEM NEAA (100X)
	5 mL	L-Glutamin
	50 mL	ultra-low IgG

Table of materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number
0.45 µm mesh	Sarstedt	83.1826
4 % Histofix	CarlRoth	P087.4
Acetic acid	CarlRoth	6755.1
Amicon Ultra-15	Merck Millipore	UFC910024
Ampicillin	Sigma-Aldrich	A1593
ATP	Roche	11140965001
BioRender	BioRender	
$CaCl_2$	Roth	5239.2
Collagen R solution 0.4 % sterile	Serva	47256.01
CTP	Roche	11140922001
Cuvette	Biorad	165-2088
DAPI	Invitrogen	D21490
DMEM	gibco	41965-039
DNAse	Promega	M6101
DTT	Promega	included in P2077
EGTA	Roth	3054.3
Escherichia coli JM109	Promega	L2005
Fetal bovine serum	gibco	10270106
Fluoromount	SouthernBiotech	0100-01
GenePulser Xcell Electroporation System	BioRad	1652660
Gentamycin	gibco	15710049
GTP	Roche	11140957001
H_2O	Braun	184238001
Hepes	Invitrogen	15630-03
Horse serum	gibco	16050122
K_2HPO_4	Roth	P749.1
KCL	Roth	6781.3
KH_2PO_4	Roth	3904.2
L-Glutamin	gibco	25030081
L-Glutathione reduced	Sigma-Aldrich	G4251-5G
MEM	gibco	31095-029

MEM NEAA (100×)	gibco	11140-035
$MgCl_2$	Roth	2189.2
Microvolume UV-Vis spectrophotometer NanoDrop One	Thermo Fisher	ND-ONE-W
MluI enzyme	NEB	R0198L
NEB buffer	NEB	included in R0198L
NucleoSpin Plasmid kit	Macherey & Nagel	740588.250
NucleoSpin RNA Clean-up Kit	Macherey & Nagel	740948.250
PBS	gibco	70011051
Pen/Strep	Thermo Fisher	15140122
Plasmid encoding full-length HEV genome (p6_G1634R)	Todt et.al	
Plasmid encoding full-length HEV genome (p6_WT)	Shukla et al.	GenBank accession no. JQ679013
Primary antibody 1E6	LS-Bio	C67675
Primary antibody 8282		
QIAprep Spin Miniprep Kit	Qiagen	27106
Ribo m^7G Cap Analog	Promega	P1711
RNase away	CarlRoth	A998.3
RNasin (RNase inhibitor)	Promega	N2515
Secondary antibody donkey anti-mouse 488	Thermo Fisher	A-21202
Secondary antibody goat anti-rabbit 488	Thermo Fisher	A-11008
Sodium Pyruvat	gibco	11360070
T_7 RNA polymerase	Promega	P2077
Transcription Buffer	Promega	included in P2077
Triton X-100	CarlRoth	3051.3
Trypsin-EDTA (0.5 %)	gibco	15400054
ultra-low IgG	gibco	1921005PJ
UTP	Roche	11140949001

Comments/Description
Harvest extracellular Virus
Immunofluorescence
Collagen working solution
Virus harvesting
Selection of transformed bacteria
in vitro transcription and electroporation
Figure Generation
Cytomix
Collagen working solution
in vitro transcription
Electroporation
Immunofluorescence
Cell culture
in vitro transcription
in vitro transcription
Cytomix
Transformation
Cell culture
Immunofluorescence
Electroporation
Cell culture
in vitro transcription
Immunofluorescence
Cytomix
Immunofluorescence
Cytomix
Cytomix
Cytomix
Cell culture
Cytomix
Cell culture

Cell culture
Cytomix
DNA/RNA concentration
Linearization
Linearization
Plasmid preparation
RNA purification
Cell culture
Cell culture
Virus production
Virus production
Immunofluorescence
Rainer Ulrich, Friedrich Loeffler Institute, Germany
DNA extraction
in vitro transcription
RNA purification
in vitro transcription
Immunofluorescence
Immunofluorescence
Cell culture
in vitro transcription
in vitro transcription
Immunofluorescence
Cell culture
Cell culture
in vitro transcription

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March 30th 2020

Revision JoVE61373R1

Dear Dr. Vineeta Bajaj,

Thank you for your reply and the helpful editorial comments concerning our manuscript now entitled "**A Cell Culture Model to Produce High Titer Hepatitis E Virus Stocks**".

Uploaded to the **JoVE** online submission site we now provide a revised version of the manuscript with all changes tracked. Please find the detailed response and explanation to each comment as individual answer in the document.
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Thank you very much for the help with formatting. We used the attached file for changes.

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3. Once done, please ensure that the highlight is no more than 2.75 pages including headings and spacings.

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We carefully proofread the manuscript and hope to have identified all typos.

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Dr. Daniel Todt