

Safe Operating Procedure (SOP)	School of Molecular & Biomedical Science	
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Procedure: Propagation, concentration, titration and experimental use of cell culture propagated hepatitis C virus (HCVcc) and generation of cell lines harbouring autonomous replication of HCV RNA ('replicons')		
Approved by:	Signature:	

PPE required: Gown, closed shoes, double glove & eye protection-Centrifuge & Spill Procedures may apply. **The following procedures involve the production of infectious hepatitis C virus (HCV). All personnel working with this material must be trained in the handling of this material.**

Materials

- Plasmid DNA containing HCV clone (e.g. pJFH-1) downstream of T7 promoter
- Appropriate restriction enzyme and buffers (XbaI for pJFH-1).
- Equipment and reagents for agarose gel electrophoresis and gel documentation.
- Spectrophotometer.
- MAXIscript® T7 in vitro transcription kit (Ambion).
- Gene Pulser® Cuvette (4mm gap) and Gene Pulser® electroporation system (Bio-Rad).
- Cell culture reagents (media [DMEM and Opti-MEM {Invitrogen}], foetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, trypsin/EDTA, plasticware, haemocytometer, trypan blue, inverted microscope, humidified 37°C/5% CO₂ incubator, biosafety cabinet).
- 40% (w/v) polyethylene glycol (PEG) MW:8000 (Sigma) in PBS.
- Benchtop centrifuge (refrigerated).
- 12.5% sodium hypochlorite solution.
- 80% (v/v) ethanol solution.
- Reagents for immunofluorescent staining of HCV antigens and inverted fluorescent microscope.

Methods

Preparation of HCV RNA

1. Digest 5 µg of plasmid DNA containing HCV clone (e.g. pJFH-1) using appropriate restriction enzyme (XbaI for pJFH-1) at 37°C overnight.
2. Subject 1 µg of digested DNA to 1% agarose gel electrophoresis to confirm presence of a band of the expected size (~13 kb for pJFH-1).
3. Add the following reagents (provided in the MEGAscript® T7 in vitro transcription kit [Ambion]) to a sterile PCR tube:
 - 2 µl ATP
 - 2 µl CTP
 - 2 µl GTP
 - 2 µl UTP
 - 2 µl 10x reaction buffer
 - X µl digested/purified plasmid DNA (1 µg)
 - X µl nuclease-free water

20 µl total

4. Incubate at 37°C for 3 h.
5. Transfer samples to 1.5ml microcentrifuge tubes, add 1 µl TURBO DNase (provided), mix and incubate at 37°C for 15 min.
6. Add 30 µl of RNase-free water, mix, and add 30 µl of cold LiCl solution (provided).
7. Incubate at -20°C for at least 30 mins.
8. Centrifuge at maximum speed (18,000×G) for 15 mins at 4 °C.
9. Carefully remove supernatant and wash RNA pellet by addition of 1ml of cold 70% ethanol, vortexing, and centrifugation as above for 5 mins.
10. Carefully remove supernatant and air-dry RNA pellet (~2 mins).
11. Resuspend pellet in 10-20 µl of RNase-free water (RNA can be stored at -80 °C).
12. Measure RNA concentration using spectrophotometer and subject 1 µl to agarose gel electrophoresis to check RNA integrity (expect bands at ~4.3 and ~9.6kb).

HCV RNA transfection

1. Culture at least two 175cm² flasks of Huh-7 cells (in DMEM +10% FBS and antibiotics) to near confluence, harvest by trypsinization and wash twice in 10 ml Opti-MEM.
2. Resuspend cells in Opti-MEM to 1 × 10⁷ cells/ml.
3. Aliquot 0.4 ml of cells per electroporation cuvette (on ice), add 10 µg of RNA and mix gently.
4. Electroporate cells with a single pulse at 0.27 kV, 100 ohms and 960µF.
5. Immediately plate cells into 175cm² flasks (one per electroporation) in complete culture medium (DMEM +10% FBS and antibiotics). Also plate some cells into wells of a 96-well culture tray (1-2 × 10⁴ cells/well) to allow monitoring of HCV antigen expression in transfected cells by immunofluorescence microscopy at 2-3 days post-transfection (see below). * **N.B. Henceforth treat all samples as infectious; i.e. wear two pairs of latex gloves, full-length laboratory gowns and appropriate eye protection, do not use sharps (including glass pipettes), use only disposable single-use plasticware and treat all used plasticware by passing with 12.5% (w/v) sodium hypochlorite solution and treat unwanted culture fluids with sodium hypochlorite solution to >2.5% (w/v) (final concentration). Dispose of all used plasticware and containers of hypochlorite-treated culture media in autoclave bags, seal bags and transfer in locked biohazard 'wheelie' bins to outside containment area (lockable) for collection for incineration. Tubes and cell culture flasks/trays containing infectious material must only be opened in type II biosafety cabinets unless contents are inactivated by treatment with fresh aqueous hypochlorite solution (≥2.5% w/v final concentration), the detergents Triton X100 (≥1% w/v final concentration), sodium dodecyl sulphate (≥1% w/v final concentration), n octyl glucoside (≥1% w/v final concentration) or DECON (≥10% v/v final concentration), fixed with acetone:methanol (1:1) for 15 minutes (cell monolayers) or inactivated with Trizol (Invitrogen; a monophasic solution of phenol and guanidine isothiocyanate used for isolating total cellular RNA). Culture vessels that do not have liquid-tight seals should be bunged where practical. After use work surfaces within type II biosafety cabinets must be swabbed with 1.25%(w/v) sodium hypochlorite solution and then 80% (v/v) ethanol solution. Closed biosafety cabinets should then further sterilized under UV light (~200 microwatts/cm² at 254nm). Hands must be washed before and after handling cell lines or viral preparations.**

6. Culture cells for 2-10 days post-transfection, as required, by subculturing into new culture flasks/trays when cells approach confluence (N.B. cytopathic effects may become evident for HCV JFH-1 (infectious) RNA-transfected cells at > 5 days post-transfection). For HCV replicon-transfected cells, selection with antibiotic G418 (final concentration 800 µg/ml; Invitrogen) may be applied at 2-3 days post-transfection to select for cell populations that harbour autonomous replication of HCV RNA. HCV replicon-harboring cells may be cultured indefinitely in culture medium containing G418.

Preparation, concentration and titration of HCV viral stocks

For Huh-7 cells transfected with HCV JFH-1 RNA, virus containing supernatants may be used directly for further experimentation, amplification of viral stocks, concentration of virus and/or titration of virus infectivity. The following protocols may apply.

Preparation of viral stocks

1. Virus containing culture fluids should be cleared of cellular debris by centrifugation at 3900×G for 5 minutes. Cleared culture fluids can then be directly used experimentally for infection of naïve cells, concentrated, amplified by serial passage on naïve cells and/or titrated.

Titration of infectious HCV

1. Seed Huh-7 cells into 96-well culture trays at 2×10^4 cells/well and culture overnight.
2. Prepare serial 10-fold dilutions of virus-containing culture supernatants (or concentrated virus) in 100 µl volumes of complete culture medium (up to 1 in 10000).
3. Remove culture supernatants from near-confluent Huh-7 cells in 96 well trays and replace with 40 µl of inoculum (in duplicate for each dilution). Return to culture for 3 hours.
4. Remove inoculum wash cells once with PBS (100µl/well) and replace with 100 µl of complete medium. Return to culture for 3 days.
5. Remove culture supernatant and fix cell monolayers by addition of 100 µl of ice-cold acetone:methanol (1:1) and incubate plates at 4 °C for 15 minutes.
6. Replace fixation solution with 100 µl of PBS. Label HCV antigens by removal of PBS and addition of anti-HCV antisera (or purified antibody) diluted appropriately in PBS containing 1% bovine serum albumin (BSA) (40µl / well) and incubation at room temperature for 1 h.
7. Remove primary antibody solution and wash cell monolayers with PBS (100 µl/well). Replace with appropriately diluted fluorescent-conjugate of secondary antibody diluted (40µl / well) in PBS containing 1% BSA. Incubate at 4 °C for 1h.
8. Remove secondary antibody solution and wash cell monolayers twice with PBS (100 µl/well).
9. Visualise HCV-positive cells by fluorescence microscopy.
10. Count foci (distinct clusters) of HCV-positive cells in each well (average duplicates) and calculate virus titre using the following formula:

$$\text{Titre (focus forming units [ffu/ml])} = \text{number of foci} \times \text{dilution factor} \times 25$$

Concentration of HCV (PEG precipitation)

1. Transfer cleared virus-containing culture supernatants into 50 ml disposable conical centrifuge tubes. Where necessary, adjust volumes in each tube to 40 ml with complete culture medium.

2. Add 10 ml of 40% (w/v) polyethylene glycol (PEG) (MW:8000 [Sigma] in PBS) to give a final concentration of 8% (w/v). Mix well by inversion and incubate tubes at 4 °C overnight.
3. Centrifuge tubes at 3900×G for 30 mins at 4 °C.
4. Remove supernatant and resuspend pellet in a small volume (1-2 ml) of complete media. Aliquot samples of concentrated virus into screw cap microcentrifuge tubes. Samples can then be titrated (as above), used directly for experimental infections or stored at -80 °C (in a zip-lock bag contained within screw-capped bio-containers) for later use.

Amplification of HCV viral stocks ('up-scale')

1. Seed Huh-7 cells at 1.6×10^6 cells/75 cm² flask and culture overnight.
2. Remove culture medium and replace with 2×10^4 focus-forming units (ffu) of cell culture-propagated HCV (HCVcc) in 2-3 ml of complete culture media. Return to culture for 3 hours before adding complete media to a final volume of 10 ml.
3. Return to culture for 3 days.
4. Collect culture supernatant, harvest cells by trypsinization and sub-culture cells into 175 cm² flask.
5. Return to culture for a further 2-3 days or until cytopathic effects become evident.
6. Collect virus-containing culture supernatants and clear of cellular debris by centrifugation (3900×G for 5 minutes). Cleared culture fluids can then be directly used experimentally for infection of naïve cells, concentrated, amplified by serial passage on naïve cells and/or titrated.