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Hepatitis C Virus: Propagation, Quantification, and Storage

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Abstract

Hepatitis C Virus (HCV) is a leading cause of chronic liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It is well known for its restricted tropism and does not replicate well in animal species other than humans and chimpanzees. Since classical in vitro propagation of natural HCV isolates is not possible, we provide a protocol for the rescue of infectious virus from cDNA clones (genotype 1a pH77S and genotype 2a pJFH-1) transfected as RNA into permissive cells. Because these two molecular clones behave differently in their ability to propagate and produce infectious virus, we describe different methods for propagation of these two viral strains. We also provide methods for infectious virus titration, which can be accomplished by counting foci of infected cells following immunostaining for viral antigen expression in cells infected with serial dilutions of a virus harvest (fluorescent focus, or FFU, assay).

INTRODUCTION

Hepatitis C Virus (HCV) is a leading cause of chronic liver diseases that include chronic hepatitis, cirrhosis and hepatocellular carcinoma. It is well known for its restricted tropism and does not replicate well in animal species other than humans and chimpanzees. Almost all HCV strains isolated from infected individuals cannot replicate in conventional cell cultures. Accordingly, since classical in vitro propagation of natural HCV isolates is not possible, we will begin with a protocol that describes the rescue of infectious virus from cDNA clones (Basic Protocol 1). Currently there are two different molecular clones of HCV that produce readily detectable infectious virus when transfected as RNA into permissive cells (typically Huh-7 human hepatoma cells or their derivatives). These are the genotype 1a pH77S and its derivatives and genotype 2a pJFH-1 and related chimeric derivatives containing sequences encoding structural regions derived from other HCV genotypes. These two molecular clones behave differently in their ability to propagate and produce infectious virus. H77S RNA replicates slowly and produces about 100-fold less infectious virus than JFH-1 RNA. It also shows only a limited ability for cell-free transmission and further adaptation to cell culture. On the other hand, JFH-1 replicates efficiently, and produces virus that is readily capable of cell-free transmission, cell culture adaptation, and serial cell-free passage. Therefore, we describe different methods for propagation of these two viral strains (Basic Protocol 2 for H77S and Basic Protocol 3 for JFH-1). Infectious virus titration can be accomplished by counting foci of infected cells following immunostaining for viral antigen expression in cells infected with serial dilutions of a virus harvest (fluorescent focus, or FFU, assay; Basic Protocol 4).

CAUTION: HCV is considered a Biosafety Level 2 (BSL-2) virus, according to the *Biosafety in Microbiological and Biomedical Laboratories* manual published by the Centers for Disease Control and Prevention. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms, including use of aa class II biological safety cabinet (BSC). See UNIT 1A.1 and other pertinent resources (APPENDIX 1B) for

more information. Since HCV is a blood-borne pathogen, researchers should take extra precautions with sharp objects when handling materials potentially containing HCV.

CAUTION: Infectious HCV may be decontaminated by applying 10% bleach solution. The virus-inactivated solution may be discarded in a laboratory sink. Other contaminated materials should be placed in an autoclavable bag and autoclaved before discarding as regular trash. The surface of the BSC cabinet and plastic ware used in experiments with infectious HCV should be decontaminated by using disinfectant Calvicide or its equivalent.

BASIC PROTOCOL 1

Generation of virus from cell culture infectious cDNA clones—Since HCV is a single-stranded, positive-sense RNA virus, transfection of genomic RNA into the appropriate cell lines results in infectious virus production. RNA transfection can be achieved by using commercially available RNA transfection reagents or electroporation. Below, we describe an electroporation method. Currently the only cell type that readily produces cell-culture infectious HCV is human hepatocellular carcinoma cell line Huh-7 and its derivatives.

Materials—In vitro-transcribed, full-length H77S or JFH-1-derived RNA

Huh-7 cell line and its derivatives, such as S5C-500-3, 8C1, FT3-7, Huh7.5

PBS, ice-cold

Bio-Rad Gene Pulser Xcell electroporation apparatus or equivalent

Complete medium: DMEM with 10% FBS and other supplements (see recipe)

Additional reagents and equipment for culture of Huh-7 cell lines and performing electroporation (CPCB UNIT 20.5).

1. Prepare logarithmically growing Huh-7–derived cells for electroporation. Wash the cells twice with PBS and remove them from the culture vessel by treatment with trypsin (e.g., see CPCB UNIT 1.1).
2. Wash the cells with ice-cold PBS twice.
3. Resuspend the cells in cold PBS at a density of 1×10^7 cells/mL.
4. Mix 10 μg of RNA with 500 μL of cell suspension and transfer the mix to a 0.4-cm gap-width electroporation cuvette.
5. Place the cuvette in the Gene Pulser Xcell electroporation apparatus and pulse once at 270V and 950 °F.
6. Transfer the cells to a 25-cm^2 flask containing 10 mL of medium and incubate at 37°C in a 5% CO_2 environment.

   Transfection efficiency can be determined at day 2 after transfection by seeding of cells in an 8-well tissue-culture-chamber slide at 2×10^4 cells per well at the time of electroporation and detecting HCV antigen by indirect immunofluorescence as described below (Basic Prooicol 4).
7. Collect the cell-culture supernatants at time points later than 48 h after electroporation.
8. Remove the cell debris by low-speed centrifugation (2000 *g* for 5 min).

9. If desired, filter virus through a 0.2-μm PES filter.

   Wash the filter with medium before use to remove residual chemical on the filter.

   This step may reduce the virus titer up to 90%.

10. Store virus at this point at 4°C in a refrigerator for several days.

    This will result in only a small incremental reduction of virus titer for up to a week.

11. Alternatively, supplement the virus solution with FBS to 20% before aliquoting and storing the virus in a −80°C freezer.

    Under these conditions, loss of infectivity is minimal after multiple freeze-thaw cycles.

    Storage of virus at room temperature or higher is not recommended, since this may result in rapid decrease in infectivity.

    Also, do not directly freeze virus in the 10% serum medium at −20 or −80°C since this will rapidly decrease infectious virus titer.

    The virus titer may be measured using a thawed sample as described in Basic Protocol 4.

BASIC PROTOCOL 2

Propagation of genotype 1a H77S and its derivatives—There are limitations in the propagation of the currently available genotype 1a H77S and its derivatives by using cell-free virus due to its relatively low replication efficiency in cell culture. Therefore, a higher-titer virus stock should be obtained by amplifying H77S RNA-transfected cells, followed by concentrating the supernatant bathing the cells. Not all Huh-7 derivative cell lines are suitable for propagating H77S. Relatively speaking, H77S RNA replicates at a high level in the 8C1 cell line followed by S5C-500-3 and Huh7.5 cell lines. These three cell lines were derived from Huh-7-derived HCV replicon cell lines following the removal of replicating HCV replicon RNA. H77S-induced cytotoxicity is more prominent in Huh7.5 cells than in 8C1 cells, making 8C1 cells more suitable to amplify H77S during long-term culture. Since the 8C1 cell line lacks HCV receptor CD81, this cell line could be used only for virus production.

Materials—In vitro-transcribed, full-length H77S or its derivative RNA

Huh-7 cell line and its derivatives such as S5C-500-3, 8C1, Huh7.5.

PBS, ice-cold

Bio-Rad Gene Pulser Xcell electroporation apparatus or equivalent

Complete medium; DMEM with 10% FBS and other supplements (see reagents)

Serum-free medium

Centricon Plus-20 (UFC2BHK08), Centricon Plus-70 (UFC710008) (Millipore).

1. Electroporate H77S RNA into 8C1 or other Huh-7 derivative cell lines as described in Basic Protocol 1. Put cells from 3× electroporation cuvettes into one 175-cm flask.

   At the time of electroporation, seed the electroporated cells in an 8-well, tissue-culture chamber slide at 2×10^4 cells per well and detect HCV antigen at day 2

post-electroporation by indirect immunofluorescence to determine the RNA transfection efficiency (Basic Protocol 4).

2. Incubate electroporated cells at 37°C in a 5% CO_2 environment.
3. After 3 or 4 days when the cells reach confluence, amplify them the by splitting1:3.
4. One day after splitting the cells replace the medium with FBS-free medium to facilitate virus concentration via a centrifugal concentrator.
5. At day 2 post-split, collect the medium using a pipet, add complete medium to cells, and allow them to grow for one or two more days until cells reach confluence before repeating steps 2–4.

   The percentage of H77S antigen-positive cells will be decreased at a slow rate.

   Huh-7.5 cells secrete low levels of infectious virus for up to 21 days after transfection of H77S RNA. However, it may not be effective to try to harvest virus after 2 weeks post-electroporation due to low levels of virus in the supernatant.
6. Remove cell debris from the collected medium by low-speed centrifugation (2,000 *g* for 5 min).

   Further clearing of supernatant by filtration may lead to a significant loss of virus titer due to low starting titer.
7. To increase the virus titer, concentrate collected supernatant by using a Centricon Plus-20 (up to 15 mL) or Centricon Plus-70 (up to 50 mL) centrifugal concentrator or equivalent. Add the supernatant to the filtration device, and centrifuge at 2000 *g*, 4°C, for 20 min or until the desired volume of the virus concentrate solution is reached.
8. Supplement the virus solution with FBS to 20% before aliquoting and storing the virus in a −80°C freezer.

   Under these conditions, loss of infectivity is minimal after multiple freeze-thaw cycles.

   The virus titer may be measured by using a frozen and thawed sample by the method described in Basic Protocol 4.

BASIC PROTOCOL 3

Propagation of genotype 2a JFH-1 and its derivatives—Due to its relatively high-level replication rate in *in vitro* cell culture, genotype 2a JFH-1, especially cell culture-adapted JFH-1 and its derivatives (including intra- and inter genotypic chimeras) can be propagated by the conventional cell-free transfer of virus in addition to the method shown for H77S. Since JFH-1 is more cytopathic than is H77S, the long-term culture of highly infectious JFH-1-replicating cells may result in massive cell death. FT3-7 and Huh7.5 cells are suitable to amplify JFH-1-derived virus. Initially, nonadapted JFH-1 could be propagated by the protocol shown in Basic Protocol 2 to make virus stock. However, a high enough virus titer may be obtained by day 2 post-electroporation of highly cell culture-adapted JFH-1 or its derivatives to allow its use in further propagation of the virus by cell-free infection.

Materials—JFH-1-derived virus stock, frozen or stored in 4°C refrigerator.

Huh-7 cell line or its derivatives such as FT3-7, Huh7.5.

Complete medium: DMEM with 10% FBS and other supplements (see recipe)

Serum-free medium or low percentage serum medium

Centricon Plus-20 (UFC2BHK08), Centricon Plus-70 (UFC710008) (Millipore).

1. Prepare confluent monolayers of naïve FT3-7 or Huh7.5 cells by adding ~7× 10^6 cells to a 25-cm flask one day before virus inoculation.

   Cell number can be adjusted in keeping with the size of the cell culture flasks or plates.

2. Thaw the −80°C stored frozen virus stock quickly at 37°C water bath.

3. Dilute the thawed virus stock solution with complete medium, before inoculating it into naïve FT3-7 or Huh7.5 cells grown to confluence. To increase the infection efficiency, use minimal amounts of diluted virus solution, enough to cover the cell layer grown in the cell culture flask (e.g., 2.5 ml for a 25-cm flask).

4. Gently roll the flask side to side a few times to prevent drying of the cells and to spread infection. Incubate the virus-inoculated cells at least 4 hrs at 37°C for adsorption to the cells before adding 5 ml of complete medium.

   It is not necessary to remove the inocula unless it is required for a particular application.

   Since the maximum titer will be equal or less than ~10^7 FFU/ml even after concentration of adapted JFH-1-derived viruses, a high multiplicity of infection (MOI) may be infeasible to uniformly infect 100% of cells with JFH-1-derived viruses. However, in most cases, inoculation of MOI even lower that 0.01 will lead to an amplification of virus due to the efficient nature of viral spread.

5. Incubate infected cells at 37°C in a 5% CO_2 environment.

6. Monitor the virus spread by checking the percentage of HCV-positive cells by using the immunofluorescence assay described in Basic Protocol 4.

   The differently adapted viruses (whether it is JFH-1 or chimeras, and whether they have certain adaptive mutations on them or not) may show differences in their ability to spread. Therefore the optimal protocol to amplify virus should be determined empirically for individual virus clones. To obtain the maximal yield, wait until ~100% of cells become positive for HCV antigen. The speed of virus propagation will be dependent on the initial size of the inocula as well as cell culture adaptation level of JFH-1 derivatives.

7. Change the medium either with serum-free or low percentage serum medium (up to 3 %). Collect the supernatant on the following day.

   The use of a low percentage of serum may allow virus harvest for up to 3 days for JFH-1- derived virus by repeating the process of adding medium and collecting the supernatant on the next day. However, the higher the percentage of serum in the medium the stickier the stock solution becomes following concentration. Also, the longer the cells were maintained in low serum media, the more cells may show signs of crisis (detachment of cells from the flasks probably due to severe cytotoxicity).

8. Collect supernatant and remove cell debris by low-speed centrifugation (2,000 *g* for 5 min).

9. Filter virus through a 0.2-μm PES filter if desired.

Wash the filter with medium to remove residual chemical on the filter before use.

10. To increase the virus titer, concentrate collected supernatant by using a Centricon Plus-20 (up to 15 mL) or Centricon Plus-70 (up to 50 mL) centrifugal concentrator or equivalent. Add the supernatant to the filtration device, and centrifuge at 2000 *g*, 4°C, for 20 min or until the desired volume of the virus concentrate solution is reached.

11. Supplement the virus solution with FBS to 20% before aliquoting and storing the virus in a −80°C freezer.

    The virus titer may be measured by using a frozen and thawed sample by the method described in Basic Protocol 4.

BASIC PROTOCOL 4

Titration of HCV—HCV titer may be determined following the immunostaining of infected cells with HCV-specific antibodies. The infection of cell culture by infectious HCV often results in infection foci formation. The foci appear as clusters of antigen-positive cells (typically 2–10 cells for H77S-derived viruses, and few to more than 100 cells for JFH-derived viruses). Following immunostaining, these foci can be counted under the microscope. Titers are calculated from the number of foci of infection produced per 1 mL of the starting inoculum (focus-forming units, FFU/ml). Alternatively, HCV titers could be calculated by the Reed-Muench equation following immunostaining of multiple replicates of dilutions of a given sample are employed (end point dilution). However, latter method is not suitable for genotype 1a-derived virus due to its lower titer.

8-well tissue culture chamber slide.

Huh-7.5 or S5C-500-3 cells in culture

Methanol:acetone 1:1 (vol:vol) solution.

Mouse monoclonal anti-core antibody (Affinity Bioreagent, C7-50, or any other HCV antibodies suitable for immunostaining of HCV antigen).

Alexa 488-conjugated, goat-anti-mouse IgG antibody (Invitrogen).

Bovine serum albumin (7.5% in DPBS, Sigma).

Hoechst 33258, 10 mg/mL (Bisbenzimide).

VECTASHIELD® mounting fluid (Vector Labs).

1. 24 h before virus inoculation, seed the 8-well chamber slide with Huh-7.5 or S5C-500-3 cells at a density of 1×10^5 cells per well.

   The deduced virus titer will vary depending on cell density: decreasing the cell density may result in a decrease in titer, however increasing the cell density more than described above does not increase the titer, but does lessen the core antigen signal intensity probably due to a decrease in viral replication.

2. Prepare serial dilutions of the virus inoculum in complete medium: 2-fold serial dilutions for low-titer virus and up to 10- fold serial dilutions for high-titer virus.

3. Remove medium from the chamber slide and add 100 μL of the serially diluted inoculum to each well of the chamber slide.

4. Incubate the slide at 37°C in a 5% CO_2 environment.
5. Remove the inoculum at 4 h and add 200 μL of fresh medium to each well of chamber slide.
6. Incubate the slide for 3 d at 37°C in 5% CO_2 environment.

   For H77S-derived virus, a 4-day incubation may result in a stronger immunofluorescence signal intensity.
7. Aspirate medium from the well, wash cells twice with PBS, and fix the cells in methanol: acetone (1:1) solution for 9 min at room temperature.
8. Remove methanol:acetone solution and wash cells twice with PBS.
9. Dilute primary C7-50 mouse anti-core antibody 1:300 in PBS with 3% BSA, and add 100 μL to each well.

   If using other HCV antibodies, dilution should be determined empirically.
10. Incubate for 2 h at room temperature.
11. After washing twice with PBS, apply 100 μL Alexa 488-conjugated secondary antibody diluted 1:100 in PBS with 3% BSA.
12. Incubate for 1 h at room temperature.
13. Wash cells twice with PBS and apply Hoechst 33258 (diluted 1:1000 in PBS) for 5 min.
14. Wash cells twice with PBS and mount a cover slip on the slide after applying a small amount of VECTASHIELD®.
15. Examine slides under an epifluorescence microscope and count foci of infection, which appear as clusters of antigen-positive cells. Titers are calculated from the number of foci of infection produced per 1 mL of the starting inoculum (*see* Note 12).

    To assess the infectious titer of an inoculum accurately, use FFU counts generated by dilutions of the inoculum that produce neither too many (overlapping) nor too few (random variation) infectious foci. The ideal is FFU counts derived from two replicate wells showing between 20 and 60 foci of infection.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2A; for suppliers, see SUPPLIERS APPENDIX.

Complete medium

Supplement 440 ml of Dulbecco’s modified Eagle’s medium (DMEM, high-glucose) with: 50 ml heat inactivated Fetal Bovine Serum (10%),

5 ml sterile penicillin/streptomycin stock solution (100 U/ml penicillin and 100 μg/ml streptomycin)

5 ml sterile 1M HEPES (10 mM).

Store less than 2 months at 4°C.

Methanol:acetone 1:1 (vol:vol) solution

Mix equal volumes of methanol and acetone.

Store at room temperature.

COMMENTARY

Background Information

HCV has been originally known as non-A, non-B (NANB) hepatitis virus since this agent caused viral hepatitis in the absence of the serologic makers from such known hepatotropic agents as hepatitis A virus or hepatitis B virus. Since the mid-1970s, HCV has been suspected as the main causative agent of blood transfusion-associated hepatitis (Feinstone et al. 1975). The major difficulty in identifying the etiologic agent for NANBH (non-A, non-B hepatitis) was due to the difficulty in growing this agent in *in vitro* cell culture and the inability to transfer the infection to any organism other than the chimpanzee. Despite these difficulties, findings in physicochemical studies have led to suggestions that HCV is a small, enveloped RNA virus (Bradley et al. 1985). HCV has been recognized to cause chronic infection in more than 70% of all cases with a range of liver diseases that include chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Alter et al. 1978, Hoofnagle 1997). The only currently available therapy for HCV infection is standard of care with a combination of pegylated interferon-α and ribavirin (Shimakami et al. 2009).

HCV is a positive-stranded enveloped RNA virus belonging to Flaviviridae. Due to the lack of a fidelity mechanism of encoded NS5B replicase, HCV replicates in a quasispecies manner. Currently, HCV is classified in 6 major genotypes and multiple subtypes (Simmonds et al. 2005). Genotype 1 is considered to be the most ancient strain of HCV and resistant to current therapy. On the other hand, genotype 2 is considered to be youngest variant and responds well to the current therapy (Pang et al. 2009). The molecular cloning of HCV in 1989 led to the first breakthrough in HCV research in molecular level (Choo et al. 1989). However, it was not until 1999 that the study of viral RNA replication *in vitro* became possible, with the development of drug-selectable subgenomic replicons that forced the adaptation of replicating HCV RNA in cell culture (Lohmann et al. 1999, Blight et al. 2000, Yi & Lemon 2004). Even with the availability of this drug-selectable replicon system, establishing HCV RNA replication system in cell culture turned out to be extremely difficult, and, consequently, only a few stains of HCV have been shown to replicate in vitro. The short list includes genotype 1a H77 strain, genotype 1b HCV-N and con1 strain, genotype 2a JFH-1 strain and a few other genotype 1 strains (Kato et al. 2003, Yi & Lemon 2004, Ikeda et al. 2002, Lohmann et al. 1999). Also, the Huh-7 cell line or its derivatives are the only practical cell lines to support HCV replication, although there are reports that JFH-1 may have extended tropism in different cell lines, including Hela and mouse cells (Uprichard et al. 2006, Kato et al. 2005, Zhu et al. 2003). In 2005, an *in vitro* HCV infection system was established with JFH-1, followed by H77S derived from H77 (Wakita et al. 2005, Zhong et al. 2005, Lindenbach et al. 2005, Yi et al. 2006), allowing the study of the entire life cycle of HCV. However, even after the establishment of infectious HCV systems, limitations in HCV research still remain, since there are only two infectious clones that produce infectious virus in *in vitro* cell culture and the development of convenient small animal model is far out of sight at present.

JFH-1 is unique in that it can automatically replicate efficiently in cell culture without any cell culture-adaptive mutations (Kanda et al. 2006). This is different from the most of the clinical isolates of HCV that were established to replicate in vitro, since they did not show detectable levels of RNA replication without adaptive mutations (Blight et al. 2000, Yi & Lemon 2004, Lohmann et al. 2001). However, even with JFH-1, initial infectious virus production following introduction of its RNA into Huh7 cells was inefficient, although it has

been found to rapidly adapt to a cellular environment with concomitant accumulation of adaptive mutations to produce high levels of infectious virus (Wakita et al. 2005, Zhong et al. 2005, Zhong et al. 2005). Since adapted JFH-1 virus can efficiently spread from cell-to-cell, cell-free virus stock could be used to amplify the virus stock.

On the other hand, the H77S infection system is relatively inefficient for producing infectious virus compared to the JFH- based infection system. There are two potential reasons for this. First, even after adaptation to in vitro cell culture, the replication efficiency of H77S is lower than that of JFH-1. Second, as exemplified by genotype 1b con1 stain, in vitro adaptive mutations may have a negative effect on virus production (Bukh et al. 2002). The specific infectivity of H77S virus is relatively low, probably due to the inefficient cell-free transfer of virus. Therefore H77S virus stock should be made by amplifying cells replicating H77S rather than from virus stock. Despite these limitations, the H77S infection system is genuine and important due to its clinical relevance. A recent study showed that cell culture-derived H77S.2 successfully infected chimpanzees when introduced intravenously and caused chronic infection, which is the typical clinical outcome of HCV infection in humans (Yi and Lemon, in preparation). This is a striking contrast from the clinical phenotype of cell culture-derived JFH-1 or its derivatives, which only ended up in an acute infection in the chimpanzee. Although more studies are need to determine the cause of different clinical outcomes of these two cell culture-derived virus infections of the chimpanzee, the H77S system is a valuable resource in HCV research despite its current limitation of propagating inefficiently in cell culture.

Critical Parameters and Troubleshooting

HCV is relatively stable at 4 °C for more than 6 weeks, however, at room temperature or 37 °C it survives for only up to 48 hrs and 14 days, respectively (Song et al. 2010). Therefore, virus-containing solutions should be kept on ice/4 °C after thawing. Cell culture-derived HCV is stable for several free-thaw operations as long as the virus was concentrated and stored in the presence of 20% serum at −80 °C. The direct freezing of 10 % FBS cell culture medium containing HCV in a −20 or −80 °C freezer is not recommended, since this will cause a severe reduction in virus titer following thawing. The level of HCV RNA replication, as well as that of virus production, may vary depending on the number of passages of Huh-7 cells, and therefore it is recommended to store specifically those Huh-7 cells which showed good HCV production quality (Lohmann et al. 2003). HCV replication is shown to be inhibited by cell confluence due to a decline in the nucleoside pool (Nelson & Tang 2006), and, therefore, it is best to avoid maintaining cells in a confluent state during virus production. Culturing JFH-1-derived, virus-infected Huh7 cells for the long term may result in a cell crisis due to severe cytotoxicity. Therefore, one should harvest the virus before the crisis period is reached. In addition, long-term replication of H77S-derived RNAs in Huh7.5 cells may also cause severe cytotoxicity, although not as apparent as that of JFH-1. Remember that virus production will diminish if cells show signs of stress.

In the case of H77S, measuring FFU/ml is a relatively accurate way to assess its titer. Also, due to the low titer and low immunostaining signal, the end point dilution method may not be practical for determining its titer. However, for JFH-1-derived virus, especially for highly adapted virus, a prolonged infection period during virus titration may result in higher than expected titers due to spreading. Therefore no more than 3 days of infection is recommended. For highly infectious virus, the end point dilution method for measuring the virus titer may be more suitable than the FFU/ml method (Lindenbach 2009).

Anticipated Results

The typical titer of H77S and its derived virus following transfection of RNA into 8C1 or Huh7.5 cells will be in the range of 10^2 to 10^3 FFU/ml and that of concentrated virus 10^3 to 10^5 FFU/ml. The immunostaining signal of H77S antigen will be of a lower intensity due to its slower rate of HCV protein accumulation in cells (Yi et al. 2006). Since Huh-7 cells show significant levels of autofluorescence, it is recommended to include negative controls for immunostaining when conducting titration, such as leaving out primary antibody or inclusion of non-infected cells during immunostaining. In the case of JFH-1 and its derivatives, depending on the adaptation level, virus titers will be in the range of 10^3 to 10^5 FFU/ml. Once concentrated, viral titers will reach up to 10^7 FFU/ml. Typically JFH-1 antigen immunostaining will give a very strong signal and be easily distinguishable from background. It is anticipated that H77S and, especially, JFH-1, may accumulate mutations during the propagation of virus due to high mutation rates of HCV with its RNA polymerase lacking a fidelity mechanism. Therefore this factor should be considered when repeatedly passaging HCV, and one should be prepared to sequence the virus, if necessary.

Time considerations

To make virus stock from H77S-derived RNA, it is important to consider the best time point to harvest virus following maximal amplification of H77S RNA-transfected cells. Typically the most propitious time to harvest H77S virus is at day 5 post electroporation of RNA (day 2 of the first split of cells following electroporation of H77s RNA) and at day 8 (day 2 after second split). Although H77S virus may be harvested at day 11 post electroporation (day 2 post third-split of cells), further splitting of cells are not recommended, due to the significant reduction of virus titers. Remember that following cell splitting, the cell mass will be increased 3 fold until they are ready to be used to collect virus in the supernatant, since cells are typically split 1:3. Thus, even though the day-11 sample may have less virus per volume, total virus harvested at day 11 may be higher than the day 5 harvest.

For JFH-1, it will take 7 to 14 days to propagate JFH-1 or its derivative depending on its adaptation level and cell types (e.g, Huh7.5 vs. FT3-7), with a starting M.O.I of 0.01 to spread 100% of the cells ready to be processed for virus harvest. Using a higher M.O.I will shorten the time line for reaching 100% infection of target cells ready for virus harvesting from the supernatant bathing the virus-replicating cells. For different virus stocks, it is advisable to find an optimal time line to propagate virus empirically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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