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Standardized Method for the Study of Antibody Neutralization of HCV Pseudoparticles (HCVpp)

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Abstract

Hepatitis C virus (HCV) pseudoparticles (HCVpp) are generated by cotransfection of HCV envelope (E1 and E2) genes along with a retroviral packaging/reporter construct into HEK293T cells. Enveloped particles bearing HCV E1E2 proteins on their surface are released through a retroviral budding process into the supernatant. Viral E1E2 glycoproteins facilitate a single round of receptor-mediated entry of HCVpp into hepatoma cells, which can be quantified by reporter gene expression. These HCVpp have been employed to study mechanisms of HCV entry into hepatoma cells, as well as HCV neutralization by immune sera or HCV-specific monoclonal antibodies.

Keywords

Hepatitis C virus; Flaviviridae; HCV pseudoparticles; Neutralizing antibodies; Neutralizing breadth

1 Introduction

The development of HCVpp [1, 2] has advanced studies of HCV entry and neutralization by antibodies for more than a decade. HCVpp were first used to identify HCV cell surface receptors required for viral entry [3–6]. More recently, studies using HCVpp have also advanced understanding of HCV neutralization by antibodies [7–13], establishing that neutralizing antibodies can drive evolution in vivo of HCV E1E2, leading to neutralization escape [11, 14]. These particles have been produced with a variety of E1E2 variants from multiple isolates and genotypes [15]. Diverse HCVpp panels have been used to define

neutralizing breadth of antibodies and to show that development of broadly neutralizing antibodies is associated with spontaneous clearance of HCV infection [10, 11], and reduced liver fibrosis in chronic infection [16].

Key issues to allow comparison of HCVpp neutralization among different laboratories are quality of pseudoparticles that are generated from clinical isolates and use of appropriate controls in neutralization assays. The following protocol addresses these issues.

2 Materials

Recommended vendors are listed in parentheses. Unless otherwise specified, products of equal or better quality than the recommended ones can be used whenever necessary.

1. HEK293T cells (ATCC).
2. Huh7 (APATH, L.L.C.) or Hep3B2.1–7 (“Hep3B”, ATCC) hepatoma cells.
3. HEK293T/Huh7 growth medium: Dulbecco’s modified Eagle medium (DMEM, Gibco), supplemented with 2 mM L-glutamine (Gibco), 1× nonessential amino acids (Sigma-Aldrich), 1 mM Sodium Pyruvate (Gibco), and 10% heat-inactivated fetal bovine serum (Gibco).
4. HEP3B growth medium: Modified Eagle medium (MEM, Gibco), supplemented with 2 mM L-glutamine (Gibco), 1× nonessential amino acids (Sigma-Aldrich), 1 mM sodium pyruvate (Gibco), and 10% heat-inactivated fetal bovine serum (Gibco).
5. Opti-MEM medium (Invitrogen).
6. 6-well or 10 cm Primaria-coated tissue culture dishes (Corning).
7. 96-well white flat bottom tissue culture plates (Corning).
8. 0.45 micron filters (Millipore).
9. 37 °C, 5% CO₂ incubator.
10. 5× Cell Culture Lysis Reagent (Promega).
11. Luciferase Assay System reagent (Promega).
12. Luminometer (Berthold or BMG Labtech).
13. HCV E1E2 in pcDNA3.1 or pcDNA3.2 expression plasmids (Invitrogen).
14. Murine Leukemia Virus (MLV) Envelope expression plasmid or Vesicular Stomatitis Virus-G (VSV-G) Envelope expression plasmid (NIH AIDS Reagent Program).

2.1 For HIV Gag-Packaged HCVpp Production

1. Lipofectamine 2000 (Invitrogen).
2. pNL4.3.Luc.R-E-plasmid (NIH AIDS Reagent Program).
3. pAdvantage plasmid (Promega).

2.2 For MLV Gag-Packaged HCVpp Production

1. Polyethylenimine (PEI), MW 25,000 (Polyscience).
2. phCMV MLV Gag/Pol packaging construct (phCMV-5349).
3. pTG126 Luciferase encoding reporter.

3 Methods

3.1 Generation of HCVpp by Transfection

Two different transfection protocols in Subheading 3.1 have been optimized for generation of either HIV Gag-packaged or MLV Gag-packaged pseudoparticles.

3.1.1 Generation of HIV Gag-Packaged Pseudoparticles

1. *Day 1:* For each HCVpp to be produced, seed 1×10^6 HEK293T cells into each well of a 6-well tissue culture plate in 3 mL of HEK293T growth medium and incubate overnight at 37 °C and 5% CO₂. Culture size can be scaled up as needed.
2. *Day 2:* Check the density of cells seeded the day before. They should be well spaced over the entire dish and at a confluency of around 70%.
3. For each HCVpp to be produced, dilute 2 µg pNL4.3.Luc.R-E- plasmid, 1 µg E1E2 expression plasmid, and 0.5 µg pAdvantage plasmid in 250 µL Opti-MEM medium.
4. For each HCVpp to be produced, dilute 12 µL Lipofectamine 2000 in 250 µL Opti-MEM.
5. Generation of control pseudoparticles:
 - a. To generate mock pseudoparticles without E1E2 (a control for nonspecific entry) repeat **steps 3 and 4**, but do not add any E1E2 expression plasmid.
 - b. To generate MLV or VSV-G-enveloped pseudoparticles (a control for nonspecific neutralization), repeat **steps 3 and 4**, but replace HCV E1E2 with MLV or VSV-Genvelope expression plasmid.
6. Mix diluted plasmids with diluted Lipofectamine and incubate for 20–30 min at room temperature, then add drop-wise to HEK293T cells without removing growth medium.
7. Incubate overnight at 37 °C in a humidified incubator, then remove medium and replace with 2 mL of fresh HEK293T growth medium.
8. Incubate at 37 °C in a humidified incubator for 48 h. Harvest media and store at 4 °C. Replace media in wells with fresh growth medium and incubate at 37 °C in a humidified incubator for 24 more hours.

9. *Day 5:* Pool 48 h and 72 h supernatants from each well and pass it through a 0.45 μ M filter. This supernatant contains the pseudoparticles and can be used either immediately, stored at 4 °C for a maximum of 2 weeks, or frozen at –80 °C indefinitely (*see* Note 1).

3.1.2 Generation of MLV Gag-Packaged Pseudoparticles

1. *Day 1:* For each HCVpp to be produced, seed 1.2×10^6 HEK293T cells into a 10 cm diameter Primaria coated dish (Corning) in 10 mL of HEK293T growth medium and incubate overnight at 37 °C and 5% CO₂.
2. *Day 2:* Check the density of cells seeded the day before. They should be well spaced over the entire dish and at a confluency of around 50%.
3. For each HCVpp to be produced, mix 2 μ g of phCMV-5349 (MLV Gag/Pol packaging construct), 2 μ g of pTG126 Luciferase reporter construct and 2 μ g of E1E2 expression plasmid together in a 1.7 mL Eppendorf tube, topped up to 300 μ L with Opti-MEM.
4. In a separate tube mix 276 μ L of Opti-MEM with 24 μ L cationic polymer transfection reagent (Polyethylenimine, PEI; Polyscience) (*see* Note 2).
5. Add the 300 μ L PEI–Opti-MEM mix to the Eppendorf containing the plasmid mix to give a total of 600 μ L.
6. Generation of control pseudoparticles:
 - a. To generate mock pseudoparticles (control for nonspecific entry), repeat **steps 3–5**, but do not add any E1E2 construct.
 - b. To generate MLV or VSV-G-enveloped pseudoparticles (control for nonspecific neutralization), repeat **steps 3–5**, but replace HCV E1E2 expression plasmid with MLV or VSV-G-envelope expression plasmid.
7. Incubate at RT for 1 h.
8. During this hour, remove the complete media with a serological pipette, taking care not to disrupt the cell layer. Add 7 mL of Opti-MEM by tilting the dish and pipetting into the bottom so as not to disturb the cells.
9. Add all 600 μ L of the plasmid–PEI mix to the cells, dropwise across the top to ensure even spread.
10. Incubate for 6 h at 37 °C and 5% CO₂.
11. Remove the Opti-MEM and replace with 10 mL of complete medium.
12. Incubate at 37 °C in a humidified incubator for 72 h.

¹-HCVpp infectivity decreases by two- to fivefold with each free-ze–thaw cycle.

²-In MLV-Gag pseudoparticle transfection protocol, add PEI to Opti-MEM, not the other way around.

13. *Day 5:* Remove the supernatant from the dish and pass it through a 0.45 μM filter. This supernatant contains the pseudoparticles. This can be used either immediately or stored at 4 °C for a maximum of 2 weeks.

3.2 Single Round Infection of Hepatoma Cells

1. *Day 1:* Seed 1.5×10^4 Huh7 cells or 8×10^3 Hep3B cells per well into a 96-well white flat bottom tissue culture plate (Corning) in 100 μL of growth medium and incubate overnight at 37 °C and 5% CO_2 (see Note 3).
2. *Day 2:* Serially dilute HCVpp and mock pseudoparticles twofold into Huh7 or Hep3B growth medium, depending on the target cells to be infected.
3. Remove growth medium from cells and replace it with 100 μL of diluted HCVpp or mock pseudoparticles in duplicate or triplicate.
4. Incubate for 4–6 h at 37 °C and 5% CO_2 .
5. Replace medium with a phenol-free growth medium and incubate cells at 37 °C and 5% CO_2 for 72 h.
6. *Day 5:* Discard the medium from each well and add 50 μL of 1 $\times$ cell lysis buffer (Promega) to each well.
7. Incubate at RT for 15 min.
8. Detect luminescence by adding 50 μL of luciferase reagent (Promega) per well and quantitate relative light units (RLU) for 1–5 s after a 0.2 s delay using a luminometer.
9. *Quality control of HCVpp:* HCVpp with functional E1E2 generally produce RLU values at least 10 $\times$ greater than mock pseudoparticles, but some HCVpp produced using clinical isolate E1E2 may have lower infectivity. Neutralization results obtained using these low-infectivity HCVpp may be unreliable and should be interpreted with caution (see Notes 4 and 5).

3.3 Neutralization Assay

1. *Day 1:* Seed 1.5×10^4 Huh7 cells or 8×10^3 Hep3B cells per well per into a 96-well white flat bottom tissue culture plate (Corning) in 100 μL of growth medium and incubate overnight at 37 °C and 5% CO_2 .
2. *Day 2:* If plasma or serum samples are to be tested for neutralizing activity, heat inactivate complement by incubating plasma/serum at 56 °C for 30 min. If white precipitate forms after heat inactivation, samples can be clarified by centrifugation at 1200 $\times g$ for 5 min.

³A confluent T175 flask has $\sim 1 \times 10^7$ Hep3B cells, enough to seed ~ 10 –12 96-well plates.

⁴Infectivity can vary significantly between different preparations (transfections) of the same HCVpp, and some E1E2 variants are more likely to produce functional HCVpp when packaged with MLV-Gag rather than HIV-Gag, or vice versa. Some E1E2 variants also require nonstandard ratios of E1E2 plasmid and packaging construct to produce functional pseudoparticles [19].

⁵Inadequate infectivity of HCVpp can sometimes be improved through codon optimization of E1E2 genes. Infectivity may also be improved by switching from HIV Gag-packaged pseudoparticles to MLV Gag-packaged pseudoparticles, or vice versa.

3. Based on result of prior HCVpp infection, dilute HCVpp into HEK293T growth medium to a concentration that will result in infection in the linear range of the infectivity assay. Generally, all but the most infectious HCVpp supernatants can be used undiluted.
4. Dilute MLV-enveloped or VSV-G-enveloped pseudoparticles to similar level of infectivity to HCVpp to use as a control for nonspecific neutralization.
5. Neutralization assays are performed in duplicate or triplicate. Each well has a total volume of 100 μ L, made up of 90 μ L of pseudoparticle and 10 μ L of mAb or plasma/serum. Once the highest concentration and dilution series has been calculated, dilute the mAb or plasma/serum accordingly into PBS or complete growth media. MAbs are generally tested at a maximum concentration of 50 μ g/mL, and serum is generally tested at a minimum dilution of 1:50.
6. Dilute an isotype control mAb or nonspecific polyclonal human IgG as a noninhibiting control for neutralizing mAbs. Dilute HCV-negative plasma/serum as a noninhibiting control for neutralization by plasma/serum.
7. Mix diluted antibody and pseudoparticles and incubate for 1 h at 37 °C and 5% CO₂.
8. After 1 h, remove the media from the 96-well white plate seeded with Huh7 or Hep3B cells on Day 1, and add 100 μ L of the pseudoparticle/antibody mixture per well, in duplicate or triplicate.
9. Incubate for 4–6 h at 37 °C and 5% CO₂.
10. Replace media with phenol-free growth media and incubate cells at 37 °C and 5% CO₂ for 72 h.
11. *Day 5:* Discard the media from each well and add 50 μ L of 1 $\times$ Cell Lysis Buffer (Promega) to each well.
12. Incubate at RT for 15 min.
13. Detect luminescence by adding 50 μ L of luciferase reagent (Promega) per well and quantitating relative light units (RLU) for 1–5 s after a 0.2 s delay using a luminometer.
14. Neutralization at each mAb concentration, serum dilution, or plasma dilution can be calculated using HCVpp infectivity in the presence of test mAb, test serum, or test plasma (RLU_{test}) relative to infectivity of HCVpp alone (RLU_{control}). Neutralization values can be expressed as percent neutralization [Percent neutralization = $(1 - \text{RLU}_{\text{test}}/\text{RLU}_{\text{control}}) \times 100$]. Fifty-percent inhibitory concentration (IC₅₀) of an inhibitor can be calculated from measurements of percent neutralization by serial dilutions of antibody (see Note 6).

⁶. Percent neutralization of the same HCVpp variant by the same antibody at the same concentration can vary by about 1.5-fold between independent experiments (an independent experiment is defined by a different HCVpp transfection and different neutralization assay). Therefore, small differences in neutralization resistance between HCVpp should be confirmed with repeated independent experiments.

15. Quality control of neutralization results:

- a. Neutralization of MLV-pp or VSV-G-pp should be measured as a control for nonspecific neutralization or enhancement of infection. Neutralization by a test sample should only be considered HCV-specific if neutralization of HCVpp exceeds neutralization of MLV-pp or VSV-G-pp.
- b. Percent neutralization of HCVpp by a nonspecific control antibody (isotype control mAb or pooled human IgG), HCV negative serum pooled from multiple donors, or HCV negative plasma pooled from multiple donors, as appropriate, should also be measured as a control for nonspecific neutralization or enhancement of HCVpp infection. Optionally, RLU values in the presence of nonspecific control antibody, pooled HCV negative serum, or pooled HCV negative plasma can be used as RLU_{control} in the equation [Percent neutralization = $(1 - \text{RLU}_{\text{test}}/\text{RLU}_{\text{control}}) \times 100$] to adjust percent neutralization of test samples for nonspecific neutralization or enhancement (*see* Notes 7 and 8).
- c. Optionally, neutralization of HCVpp with H77 strain E1E2 by well-characterized mAbs can be used to validate neutralization assay methods, since many previously described broadly neutralizing mAbs have activity against this viral strain (Fig. 1 and Table 1).

3.4 Measurement of Neutralizing Breadth

Neutralization of an HCVpp is generally considered positive if it is neutralized >50% by 50 µg/mL or less of a mAb or a 1:50 dilution of plasma or serum, with appropriate controls for nonspecific neutralization. Cross-neutralization is generally defined as neutralization of at least one heterologous HCV isolate. Broad neutralization is generally defined as neutralization of the majority of isolates in a diverse panel of HCVpp (*see* Note 9). Neutralization sensitivity of distinct HCVpp isolates varies widely both within and across HCV genotypes [13, 15], and further study is needed to better define the relationship between neutralizing breadth measured using diverse isolates from a single HCV genotype and neutralizing breadth measured using isolates from multiple genotypes.

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⁷·Nonspecific enhancement or inhibition of HCVpp by sera may be corrected through the use of protein A purified serum IgG in neutralization experiments.

⁸·Heparin can inhibit HCV infection, so plasma isolated with heparin used as an anticoagulant should be avoided or used with appropriate controls.

⁹·Multiple different panels of HCVpp are commonly used for neutralization breadth testing [7, 15, 18]. A standardized panel of genotype 1 HCVpp that would allow for accurate comparison of neutralization breadth results across laboratories is currently under development.

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Fig. 1. Neutralization of H77 HCVpp by serial dilutions of three reference mAbs. Error bars indicate standard deviations between replicates. From Wasilewski et al. [17]

Table 1

Percent neutralization of strain H77 HCVpp by reference mAbs, measured at a single concentration of mAb (10 µg/mL). Values are the average of replicate wells. From Bailey et al. [13]

mAb (10 µg/mL)	H77 HCVpp % Neutralization
HC84.26	99.7
HC33.4	99.6
HC33.8	95.7
AR4A	95.4
AR5A	91.4
AR3C	86.9
AR2A	82.8
HC84.22	82.6
AR3A	76.9
HC-1	67.3
AR3D	67.1
AR4B	65.7
AR3B	62.8
CBH-7	55.2
AR1A	36.2
CBH-2	35.4
CBH-5	33
CBH-4B	11.3