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Optimization of the pseudoparticle system for standardized assessments of neutralizing antibodies against hepatitis C virus

Ana Chumbe[1], Richard A. Urbanowicz[2,3,4,5], Kwinten Sliepen[1], Sylvie M. Koekkoek[1], Richard Molenkamp[6], Alexander W. Tarr[2,3,4], Jonathan K. Ball[2,3,4], Janke Schinkel[1,*] and Marit J. van Gils[1,*]

Abstract

A better understanding of the antibody response during natural infection and the effect on disease progression and reinfection is necessary for the development of a protective hepatitis C virus (HCV) vaccine. The HCV pseudoparticle (HCVpp) system enables the study of viral entry and inhibition by antibody neutralization. A robust and comparable neutralization assay is crucial for the development and evaluation of experimental vaccines.

With the aim of optimizing the HCVpp–murine leukaemia virus (MLV) system, we tested the neutralization of HCVpp-harbouring E1E2 from 21 HCV isolates representing 6 different genotypes by several monoclonal antibodies (mAbs). HCVpps are generated by expressing functional envelope glycoproteins (E1E2) onto pseudoparticles derived from env-deleted MLV. Adjustments of E1E2, gag–pol and luciferase plasmid ratios resulted in increased yields for most HCVpps and recovery of one non-infectious HCVpp. We simplified and improved the protocol to achieve higher signal/noise ratios and minimized the amount of HCVpps and mAbs needed for the detection of neutralization. Using our optimized protocol, we demonstrated comparable results to previously reported data with both diluted and freeze–thawed HCVpps.

In conclusion, we successfully established a simplified and reproducible HCVpp neutralization protocol for studying a wide range of HCV variants. This simplified protocol provides highly consistent results and could be easily adopted by others to evaluate precious biological material. This will contribute to a better understanding of the antibody response during natural infection and help evaluate experimental HCV vaccines.

INTRODUCTION

Although highly effective hepatitis C virus (HCV) treatment has been available since 2015, with cure rates approaching 100%, almost 290 000 people die each year due to complications of chronic HCV infection [1, 2]. In addition, an estimated 1.5 million new infections occur each year [3] and incidence in some countries is increasing, as exemplified by the USA, where between 2012 to 2019, the number of new HCV infections has increased every year, resulting in an estimated 57 500 new cases [4]. Therefore, to eliminate HCV by the year 2030, as envisioned by the World Health Organization (WHO) [5], a vaccine is urgently needed. The extreme viral diversity of HCV and the limited knowledge of protective immune responses against HCV [6] are major obstacles for the development of a vaccine.

A better understanding of the antibody response during natural infection and the effect on disease progression and reinfection is necessary for the development of a protective HCV vaccine. This calls for a robust and convenient virus neutralization assay. The HCV pseudoparticle (HCVpp) system is a fast and simple method to study neutralizing antibodies against HCV. Pseudoparticles are viral particles usually based on retroviral expression systems that have the capacity to incorporate foreign glycoproteins on

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Author affiliations: [1]Department of Medical Microbiology and Infection Prevention, Amsterdam UMC, University of Amsterdam, Amsterdam Institute for Infection and Immunity, Amsterdam, Netherlands; [2]School of Life Sciences, Faculty of Medicine and Health Sciences, The University of Nottingham, Nottingham, UK; [3]Wolfson Centre for Global Virus Research, The University of Nottingham, Nottingham, UK; [4]National Institute for Health Research Nottingham Biomedical Research Centre, Nottingham University Hospitals National Health Service Trust, Nottingham, UK; [5]Department of Infection Biology and Microbiomes, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool L3 5RF, UK; [6]Department of Viroscience, Erasmus MC, Rotterdam, Netherlands.
*Correspondence: Janke Schinkel, j.schinkel@amsterdamumc.nl; Marit J. van Gils, m.j.vangils@amsterdamumc.nl
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their surface [7, 8] creating viral mimics. Pseudoparticles are widely used in serological tests for Ebola [9], influenza [10], severe acute respiratory syndrome coronavirus (SARS-CoV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [11], Middle East respiratory syndrome (MERS) [7], and human immunodeficiency virus 1 (HIV-1) [12], among others. The HCVpps can be made using several retroviral systems, such as murine leukaemia virus (MLV) and HIV, and use an expression plasmid for functional HCV E1E2 envelope glycoprotein complexes, a packaging construct coding for MLV or HIV gag-pol under the action of a CMV promoter, plus an internal transcriptional unit, which includes the luciferase reporter gene [13, 14]. Even though a HCV cell culture (HCVcc) system [15–18] based on the genotype 2 a JfH1 strain has been developed, production of new isolates with an E1E2 variant of interest is challenging, mainly due to genotype incompatibilities that require specific mutations in order to work. Due to its rapid and efficient way to test diverse glycoproteins and the comparability between HCVpp and HCVcc neutralization data [19], the HCVpp system remains the first choice to evaluate antibody responses.

After the establishment of the HCVpp system, many laboratories adopted this neutralization assay [20–22]. Nevertheless, variability in assay signal has been observed between laboratories, making it difficult to compare results. Recently a protocol to test neutralization using HCVpps has been published with the aim of establishing general guidelines to study neutralization using the HCVpp system [23]. Independently, a fine-tuned ratio between the E1E2 and the MLV gag–pol plasmids has increased the success rate of obtaining patient E1E2-derived HCVpps [21] and, more recently, production of HCVpps in a hepatoma kidney cell lacking of CD81 receptor was introduced [24]. Additional studies have revealed that HCVpps can be generated expressing E1E2 from six genotypes [22]. However, it was also shown that not all complete E1E2 open reading frames generated infectious HCVpps [25]. Therefore, further optimization of this system is needed to improve reproducibility and make it easy to implement by others, more cost-effective and high throughput.

Similar validation/optimization studies have moved the HIV field forward to test vaccine efficacy and study natural responses in HIV+ cohort participants [26]. More recently, in SARS-CoV-2, establishment of consistent, robust and comparable neutralization assays proved to be crucial for tthe development and comparison of vaccines [27–29]. In this study we optimized the HCVpp system further using the existing standard protocol as a starting point. We were able to increase the luminescence reporter signal for many HCVpps considerably, facilitating the successful use of more diverse E1E2s and the ability to use not only freshly prepared or diluted but also frozen stored HCVpps after one freeze–thaw cycle. In addition, we reduced the total volume of HCVpp and antibodies or serum needed in the neutralization assay itself to use the HCVpps more efficiently and save valuable patient material and monoclonal antibodies (mAbs).

METHODS

Cell lines

Human embryonic kidney (HEK) 293 T cells (ATCC, CRL-11268) were maintained with Dulbecco's modified essential medium (DMEM; Gibco by Thermo Fisher Scientific) supplemented with 10% foetal bovine serum and 0.1% penicillin–streptomycin. Huh-7 cells, a gift from François-Loïc Cosset, a hepatocyte-derived carcinoma cell line, were kept in DMEM supplemented with 10% foetal bovine serum, 0.1% penicillin–streptomycin, 1% nonessential amino acids and 1% HEPES buffer (Huh-7 medium). Both cell lines were incubated at 37 °C with humidified 5% CO_2 and passaged twice per week using 0.25% trypsin, 1 mM ethylenediaminetetraacetic acid (EDTA). 293 F cells (Invitrogen, cat no. R79009) were maintained in FreeStyle medium (Life Technologies). They were incubated at 37 °C, humidified with 8% CO_2 on an orbital shaker platform at 125 r.p.m and passaged twice per week.

HCV pseudoparticles

Plasmids encoding the HCV envelope glycoprotein E1E2 sequences of a panel of 22 HCV variants from six genotypes were used, including 9 HCVpps from locally collected patient samples [30]: AMS0229, AMS0230, AMS0231, AMS0232, AMS.1b.k2, AMS.2b.k21, AMS.3a.k26 and AMS.4d.k9 (GenBank IDs: OL855838.1, OL855838.1, OL855837.1, OL855836.1, KR094962.1, KR094963.1, KR094964.1 and ON623878, respectively). In addition, the following HCVpp plasmids from the Nottingham panel [25] were used: UKNP1.2.3, UKNP2.1.1, UKNP2.2.1, UKNP2.4.1, UKNP3.2.1, UKNP3.2.2, UKNP4.1.1, UKNP5.2.1, UKNP6.1.1 and UKNP6.1.2 (GenBank IDs: KU285154.1, KU285209.1, KU285211.1, KU285213.1, KU285218.1, KU285219.1, KU285220.1, KU285226.1, KU285227.1 and KU285228.1, respectively), and the reference strain H77 (GenBank ID: AAB67037; including three amino acid changes at the following positions: R564C, V566A and G650E), a gift from Dr Jean Dubuisson, and a JFH1-AM120 (GenBank ID: KF700370.1)-derived HCVpps. Sequences were not codon-optimized and did not undergo any kind of modification.

Generation of HCVpps

A 10 cm dish was seeded with 1.5×10^6 293 T cells a day prior to transfection. Together with E1E2 plasmids, a MLV gag–pol plasmid with proteins responsible for RNA packaging and encapsidation and a plasmid encoding firefly luciferase (both plasmids a gift from Jean Dubuisson) were used [13]. These three plasmids were tested in three different ratios (E1E2 : gag–pol : luc at 1:1:1, 1:2.6:2.6 and 1:26:34) with a total amount of 6 µg of DNA and 12 µl of Lipofectamine 2000 (Invitrogen by Thermo Fisher

Scientific) per 10 cm dish. It was previously suggested that empirical optimization of the E1E2 : gag–pol : luc ratio is necessary for successful HCVpp production [21]. For that, variable amounts of gag–pol and E1E2 plasmid were tested for several HCVpps with a fixed amount of luciferase. Based on those infectivity matrixes, we chose three amounts of E1E2 and gag–pol that have worked before: (1) the usual 1:1 ratio of E1E2 and gag–pol; (2) a ratio that previously worked in the publication by Urbanowicz *et al.* [21], 1:26 E1E2 and gag–pol; and (3) a more conserved approach that also showed great success, 1:2.6 E1E2 and gag–pol. This resulted in the ratios E1E2 : gag–pol : luc of 1:1:1, 1:26:34 and 1:2.6:2.6.

Twenty-four hours after transfection, Opti-MEM was replaced with DMEM supplemented with 10% foetal bovine serum and 0.1% penicillin–streptomycin DMEM (Gibco by Thermo Fisher Scientific). Two days later the supernatant containing the HCVpps was harvested and passed through a 0.45 µm filter. HCVpps were stored at 4 °C or −80 °C.

HCVpp infectivity assays

Several experiments were performed to optimize infectivity of the HCVpps on Huh-7 (3×10^3 cells/well in a white 96-well plates, unless stated otherwise). In a first experiment, different inoculation methods were tested. After 4 h of incubation, HCVpp inoculum was either removed or left in the well before adding fresh Huh-7 medium on top. In a second experiment, different incubation times were tested, up to 7.5 h. As a third step, the impact of cell density on infectivity was explored by seeding 5×10^3 to 2×10^4 Huh-7 cells per well. In a fourth experiment, the maximum dilution that still provided reliable relative light units (r.l.u.) was tested. Finally, the impact of one freeze–thaw cycle on HCVpp infectivity was tested using 15×10^3 Huh-7 cells/well. For all experiments, the incubation time before readout was 72 h at 37 °C in 5% CO_2.

Luciferase measurement

The Luciferase Assay System (E1500, Promega) was used to quantify infectivity. Briefly, supernatant was removed using a vacuum pump and 50 µl of cell lysis buffer (E1531, Promega) was added immediately to each well. After 5 min of shaking (600 r.p.m) incubation at room temperature, readout was performed with the GloMax Luminometer from Promega. The machine was set to dispense 50 µl of luciferase reagent and r.l.u. were recorded for 1 s (integration time) after 0 s delay.

Monoclonal antibodies

Reference mAbs targeting the main antigenic regions in E1E2 – IgH505 [31], AR4A [32], AR3B [7], AP33 [33], HC84.26 [34], AT1209, AT1618 and AT1211 [30] – were produced in-house and used in the HCVpp neutralization assay. Monoclonal antibody production was performed as follows. One day prior to transfection, 5×10^9 293 F cells l^{-1} were seeded with prewarmed FreeStyle 293 Expression Medium (Life Technologies by Thermo Scientific) in a 2 l disposable Nalgene flask (VWR) at 37 °C, 8% CO_2 and 125 r.p.m. For 1 l transfection, 937 µl PEImax (1 mg ml^{-1}) in 25 ml Opti-MEM (Life Technologies by Thermo Scientific) was mixed with 156 µg Heavy Chain IgG plasmid and 156 µg Light Chain IgG plasmid in 25 ml Opti-MEM and left to incubate for 30 min at room temperature. The transfection mix was subsequently added to 1 l 293 F cells and the supernatant was collected 5 days later.

The mAbs were isolated from the supernatant using protein A/G columns (Thermo Fisher Scientific). After the supernatant was run over the protein A/G column, the columns were washed with three column volumes of phosphate-buffered saline (PBS). Next, the mAbs were eluted with 9 ml of 0.1 M glycine pH 2.5 (elution buffer) in 1 ml of 2 M Tris pH 8.6 (neutralization buffer). For buffer exchange, Vivaspin6 100000 kDa filters (GE Healthcare) were used at 3000 r.p.m and mAbs were resuspended in PBS, aliquoted and stored at −20 °C.

HCVpp neutralization assays

One day before the neutralization assays were performed, 3×10^3 and 15×10^3 Huh-7 cells per well were seeded in 100 µl Huh-7 medium to test whether changes in Huh-7 cell density or HCVpp inoculum alter 50% inhibitory concentration (IC_{50}) neutralization values.

Then 25 µl of 1:10 diluted or undiluted HCVpps were incubated with 5 µl of serial diluted mAb for 1 h at 37°C in 5% CO_2. In addition, both fresh and frozen HCVpps were used to evaluate the impact of freeze–thaw cycles on observed IC_{50} values. After removing the Huh-7 cells supernatant, the mixture of HCVpp and mAb was placed on top of the Huh-7 cells and after 4 h, unless stated otherwise, fresh Huh-7 medium was added. After 72 h at 37 °C in 5% CO_2, medium was removed and the readouts were performed as described above.

Statistical analysis

Neutralizing antibody titres were calculated by measuring the reduction in r.l.u. We calculated the IC_{50} using log (inhibitor) versus response (variable slope) considering 0 and 100% as bottom and top constraints. Pairwise comparisons were performed using analysis of variance (ANOVA) and a non-parametric Friedman test and Dunn's test for multiple comparisons. For comparisons of slopes and intercepts analysis of covariance (ANCOVA) was used. Linear regression was performed to evaluate correlations

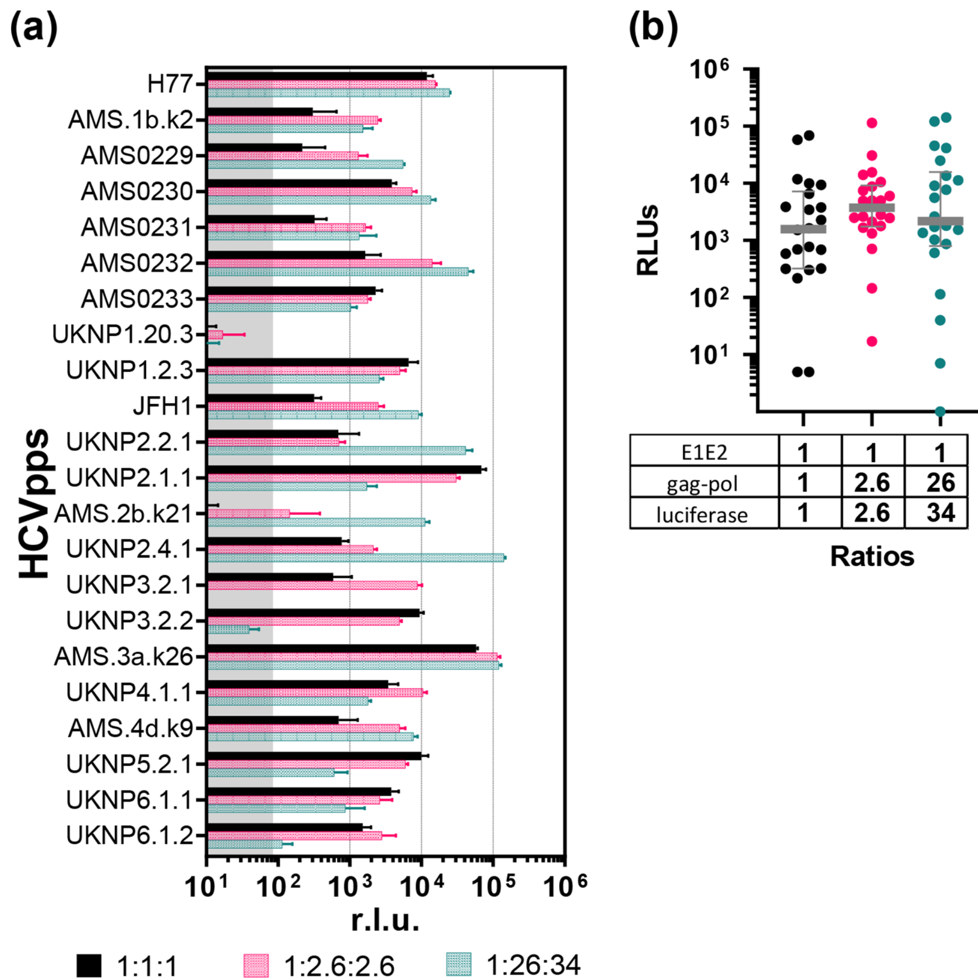


Fig. 1. r.l.u. obtained after transfection with three ratios. (a) Twenty-two HCVpps were generated by transfecting with plasmids E1E2:gag–pol:luc at ratios of 1:1:1, 1:2.6:2.6 and 1:26:34. The three different transfection ratios were evaluated in triplicate per HCVpp (*n*=1–3; Fig. S1). Mean and standard deviation (SD) is shown. A light grey area indicates the highest background signal by non-E1E2pp among all transfection ratios. (b) Median with interquartile range of r.l.u. grouped per ratio are shown. No significant differences were found among groups. r.l.u., relative luciferase units.

between luminescence and HCVpp dilutions, luminescence before and after freezing, and also between IC_{50} values of non-frozen and frozen HCVpps. GraphPad Prism version 8.3.0 was used for all statistical analysis.

RESULTS

Plasmid transfection ratios affect HCVpp infectivity

Previous studies showed that the optimal ratio between the E1E2 and the MLV gag–pol plasmids needs to be established per HCVpp E1E2 sample to maximize infectious particle production [21]. We produced HCVpps by transfecting cells with 1:1:1, 1:2.6:2.6 or 1:26:34 ratios of E1E2:gag–pol : luc, respectively, to evaluate the extent to which transfection ratios affect infectivity. Indeed, a diverse range of infectivity was observed (Fig. 1a), with no general superior ratio that resulted in high yields for all HCVpps (Fig. 1b). UKNP1.20.3 did not work with any ratio tested. The change in infectivity was plotted by comparing the r.l.u. of the HCVpps obtained using the alternative plasmid ratios to the r.l.u. of the 1:1:1 ratio (Fig. S1a, available in the online version of this article). Some HCVpps (H77, AMS0230 and AMS.3a.k26) worked equivalently with all ratios tested. When considering a threefold improvement as a cutoff, four and seven HCVpps worked best with ratios 1:2.6:2.6 and 1:26:34, respectively. For the seven HCVpps that had the highest infectivity using the 1:26:34 ratio, the fold improvement was surprisingly high (80× on average), with one HCVpp (AMS.2b.k21) that could only produce infectious particles using the 1:26:34 ratio (Fig. S1a). Highly reproducible results were obtained after duplication of the experiment with different plasmid ratios with several HCVpps, especially the ones that performed best with the most extreme ratios (Fig. S1b and c). We continued with the optimal ratio for each HCVpp in subsequent experiments.

Decreasing HCVpp volume and increasing incubation time enhances HCVpp infectivity

Testing of antibody neutralization of HCVpp involves several steps (Fig. 2a). We attempted to improve the overall infectivity by evaluating various conditions at different steps in the protocol (modes 1–5 as illustrated in Fig. 2b). First, cell culture media (100 µl) were either left (mode 1 and 2) or removed from the Huh-7 cells (modes 3–5) before adding the HCVpp mix (HCVpps with antibodies, sera or purified immunoglobulins). Second, the effect on infectivity of total volume of the HCVpp mix per well (100 µl in modes 1, 2 and 5 or 30 µl in modes 3 and 4) was tested. Third, the HCVpp mix was either left on the cells (modes 2 and 4) or removed after ~4 h of incubation (modes 1, 3 and 5). For optimal cell maintenance, 200 µl of Huh-7 medium was added when possible (modes 1, 3, 4 and 5) after this 4 h incubation. These five modes were all tested side by side and scored on HCVpp infectivity, background signal of non-E1E2 particles and practicality. Mode 2 (100 µl HCVpp mix directly on cell culture and left for the duration of the experiment) was the least time-consuming protocol and also yielded the highest infectivity of the HCVpps. However, this protocol also resulted in the highest background r.l.u. In contrast, the other modes showed lower background signals, but also lower HCVpp r.l.u. (Fig. S2a). Mode 4, in which only 30 µl HCVpp mix was added to Huh-7 cells after supernatant removal and additional media after 4 h, resulted in the highest signal-to-noise ratio and the second highest HCVpp infectivity (Fig. 2b and Fig. S2b). Subsequently, multiple independent side-by-side comparison experiments showed robust results and support mode 4 as the best option.

To further optimize the protocol, we determined whether the incubation time of the HCVpp mix on top of the cells would affect the HCVpp infectivity. For mode 4, the media were added 1–7 h after the start of incubation and for mode 5, the mix was replaced with media after 1–7 h of incubation on the cells. Mode 4 only showed a small increase in r.l.u. in the first hours and no further increase after 4 h, while for mode 5 (standard protocol) the infectivity increased over time up to 4 h and even increased further over time for some HCVpps (Fig. 2c). Therefore we selected 4 h as the optimal incubation time because of practicality and high infectivity.

Cell density, and HCVpp dilutions and freeze–thawing influence HCVpp infectivity

The confluence of Huh-7 cells could be another source of variability in the assay, therefore infectivity of two highly infectious HCVpps (AMS0232 and UKNP2.4.1) was tested on either 5×10^3, 1×10^4, 1.5×10^4 or 2×10^4 cells per well in 100 µl. Cell counts between 10×10^3 and 15×10^3 gave similar higher results compared to 5×10^3 (Fig. 3a). At the higher 20×10^3 cells/well density, we observed the lowest signal. We continued using between 10×10^3 and 15×10^3 cells/well for further experiments.

To evaluate possible saturation of the system and to see if HCVpps could be further diluted, we examined the upper and lower limits of the r.l.u. HCVpps with high (UKNP2.4.1), intermediate (H77) and low infectivity (UKNP4.1.1 and AMS.2b.k21) were serially diluted before inoculating the Huh-7 cells (Fig. 3b). Infectivity inversely correlated with the inoculum at all dilutions for all HCVpps tested (Pearson r=−0.9573, P<0.0001; r=−0.9304, P=0.0003; r=−0.9981, P<0.0001; r=−0.9536, P<0.0001, H77, AMS.2b.k21, uknp2.4.1 and uknp4.1.1, respectively). r.l.u. decay due to HCVpp dilutions showed no significant difference between HCVpps.

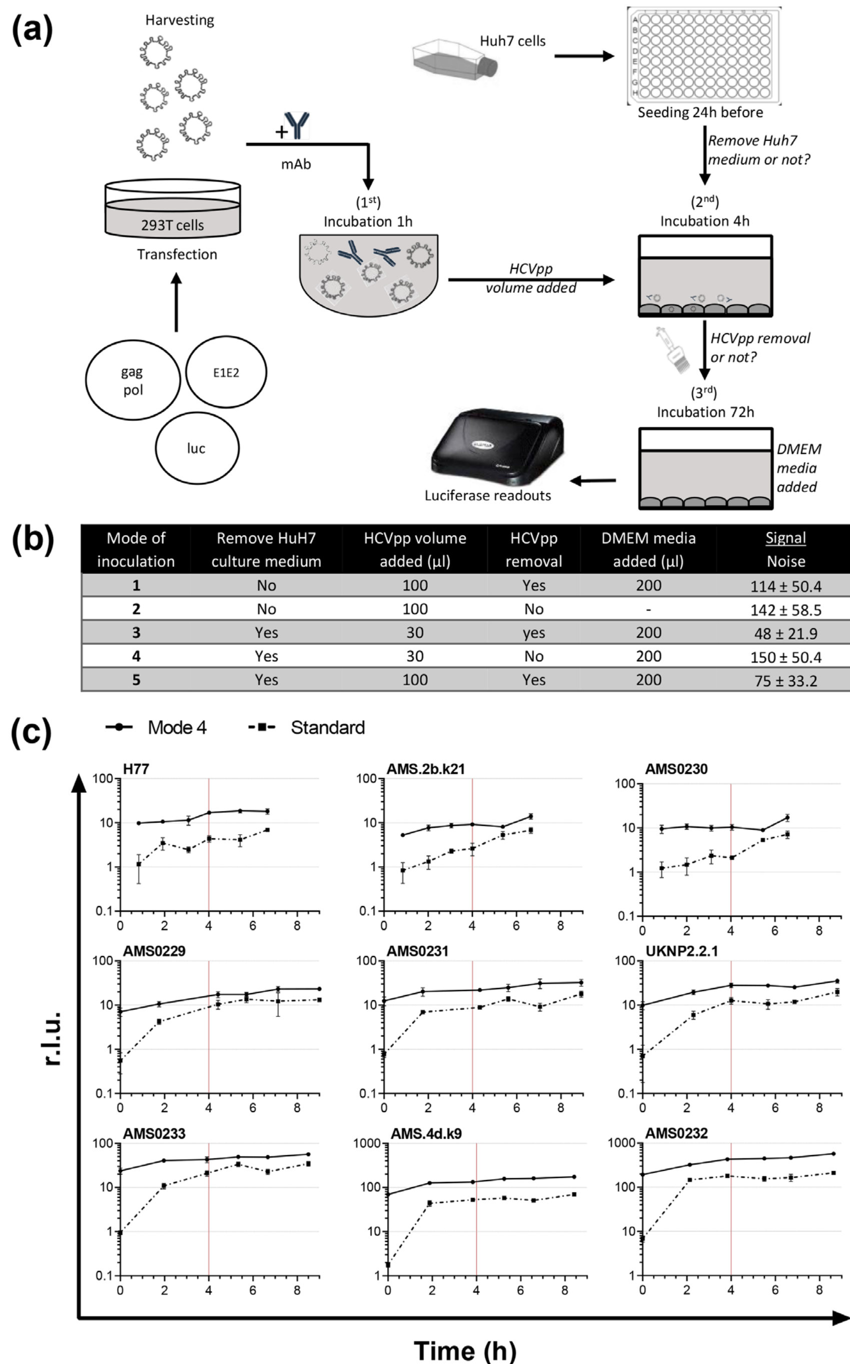
In addition, the r.l.u. variation between triplicates should be small to measure infectivity in neutralization assays reliably. Therefore, r.l.u. were plotted against the coefficient of variation (CV) at different dilutions to determine a minimum infectivity threshold that results in reproducible r.l.u. (Fig. 3c). Sample triplicates with r.l.u. above 3000 r.l.u. had an acceptable CV% (<30%) . As a result, a simple quality control for further HCVpp batch productions and dilutions was set at >3000 r.l.u.

Subsequently, we compared the infectivity of freshly produced and frozen material from 10 representative HCVpp preparations. After harvesting the 10 HCVpps, half of the volume was frozen at −80 °C and the other half was kept at 4 °C. One hour later, we thawed the HCVpps and measured the r.l.u. using mode 4 protocol. After one freeze–thaw cycle on average 36% of r.l.u. was recovered compared to the freshly produced HCVpp (Fig. S3) affecting all HCVpps proportionally (Fig. 3d). All 21 tested HCVpps were successfully recovered from the freezing process, provided that the prefreezing signal exceeded 10 000 r.l.u.

To provide some hints about stability, HCVpps kept at −80ºC <1 month or >30 months were tested on Huh-7 cells (Fig. S4a). HCVpps stored from 31 up to 49 months showed a positive correlation (R^2=0.9643). Overall, mean r.l.u. were somewhat variable but independent of storage time with increased variation of the replicates with HCVpps that show lower r.l.u. (Fig. S4b).

Sensitivity of HCVpp to neutralization does not depend on dilution factor, cell density or a freeze–thaw step

We explored how changes in r.l.u. (due to HCVpp dilution, cell density and freeze–thawing) affected the outcome of neutralization assays. Therefore, the half maximal inhibitory concentration (IC_{50}) value of mAb AT1209 was determined using diluted (1:10) and undiluted HCVpps. The observed difference in IC_{50} values was less than threefold, even though the r.l.u. was at least 9.3 times higher for the undiluted HCVpps (Fig. S5). In addition, the IC_{50} values of AR4A after seeding 3×10^3 (low confluence) and 15×10^3 cells/well (normal confluence) were compared, showing no impact of cell density on neutralization sensitivity, although the r.l.u. was on average 2.1 times higher when using 15×10^3 cells per well (Fig. S6).



Mode of inoculation	Remove HuH7 culture medium	HCVpp volume added (µl)	HCVpp removal	DMEM media added (µl)	Signal / Noise
1	No	100	Yes	200	114 ± 50.4
2	No	100	No	-	142 ± 58.5
3	Yes	30	yes	200	48 ± 21.9
4	Yes	30	No	200	150 ± 50.4
5	Yes	100	Yes	200	75 ± 33.2

Fig. 2. Neutralization scheme and different variations (modes) tested to improve HCVpp infectivity. (a) Standard protocol indications are in regular font and intended modifications are in italic. A transient transfection of MLV gag-pol, luciferase and E1E2 envelope glycoprotein plasmids to produce HCVpps is followed by a preincubation step, where HCVpps and antibodies, sera or purified IgGs (HCVpp mix) are mixed and incubated (first) for 1 h at 37 °C. Subsequently, the supernatant of the Huh-7 cells is removed and the HCVpp mix is added to the Huh-7 cells (inoculation step). This is followed by an incubation step (second) of the HCVpp mix for 4 h to perform a single round infection of the Huh-7 cells. Next, the HCVpp mix is removed and replaced by fresh medium. After 72 h of incubation (third), cells were lysed and r.l.u. were measured [23]. (b) Five modes of inoculation were tested with 3×10^3 Huh-7 cells seeded 1 day before the experiment. In modes 1 and 2, cell culture medium was not removed before adding HCVpps. After a 4 h incubation period, the supernatant was removed (modes 1, 3 and 5) or left in the well (modes 2 and 4). Subsequently, 200 µl of fresh Huh-7 media were added in modes 1, 3, 4 and 5. Readouts were performed 72 h later as specified in the Methods section. Signal-to-noise ratios were obtained using non-E1E2 pseudoparticles. (c) Several incubation time points (*x*-axis) of a reduced amount of HCVpps (30 µl) followed by the addition of 200 µl of fresh Huh-7 medium (mode 4) are shown in solid black lines. These experiments were performed at the same time in triplicate per HCVpp (n=1). The results of different incubation times using the standard method (mode 5) are depicted using dotted lines. Infectivity was measured in r.l.u. after 72 h in all cases (*y*-axis). The red line indicates the optimal incubation time. r.l.u., relative luciferase units.

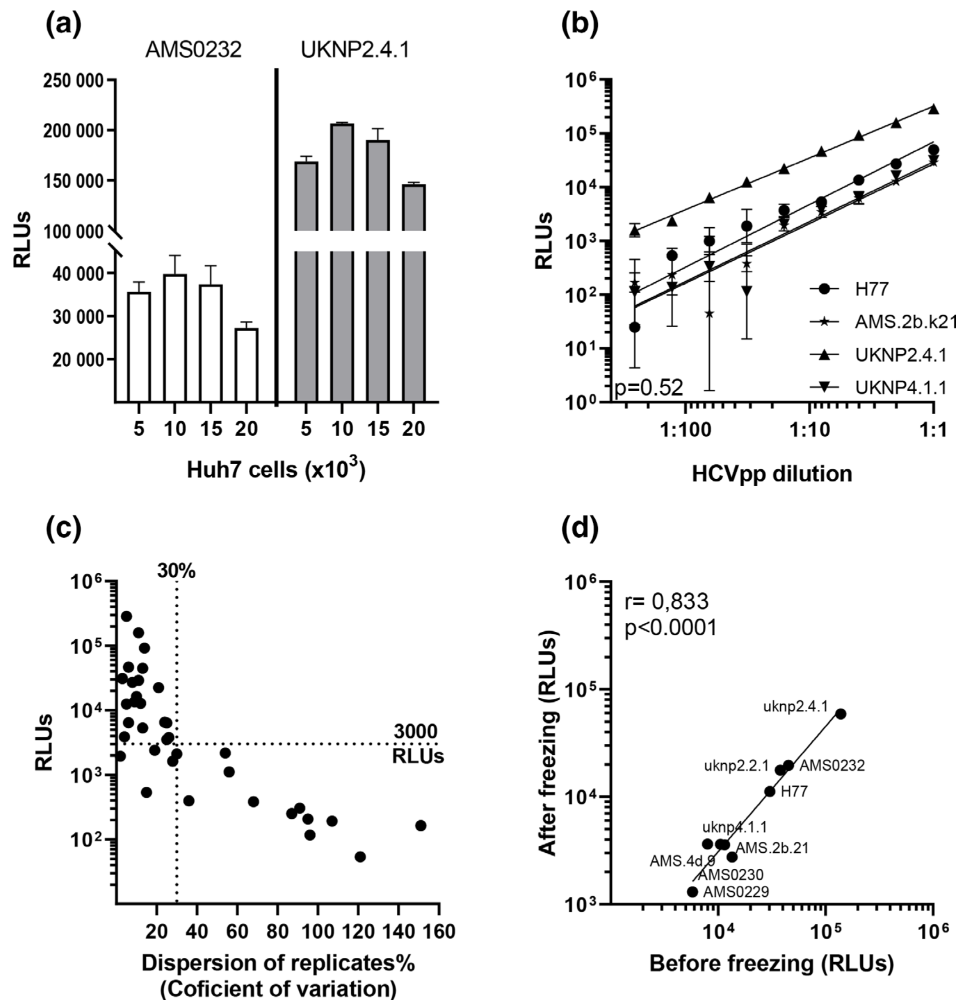


Fig. 3. Linear relationship between inoculum and infectivity. (a) r.l.u. obtained after changes in Huh-7 cell density for HCVpps (AMS0232 and UKNP2.4.1). Means and standard deviation are shown. (b) Threefold dilution curves of HCVpps (H77, AMS.2b.k21, UKNP2.4.1 and UKNP4.1.1) from 1:1 to 1:256 with mean and standard deviation per point are shown. Linear regressions were calculated in each case (*r*,Pearson correlation values). Comparisons between slopes (H77=−1.16, AMS.2b.k21=−1.1, UKNP2.4.1=−0.96 and UKNP4.1.1=−1.11) showed no significant differences (*P*=0.52). (c) Average r.l.u. per each triplicate from (b) (*y*-axes) and their coefficient of variation in percentage (%) (*x*-axes). Dotted lines indicate 30% cut-off (*x*-axes) and 3000 r.l.u. (*y*-axes). Average r.l.u. with an acceptable CV% (<30%) were higher than 3000. (d) r.l.u. of HCVpps before and after one freeze–thaw cycle (1 h at −80 °C; Spearman *r*=0.833). All these experiments were performed at the same time in triplicate for each HCVpp (*n*=1). r.l.u., relative luciferase units.

Next, we evaluated how a freeze–thaw step affected neutralization measurement by comparing the IC_{50} values of antibodies IGH505, AR3B, HC84.26, AR4A, AT1618 or AT1209 against 21 HCVpps that were either used immediately after harvesting (non-frozen) or underwent a freeze–thaw cycle (frozen). The IC_{50} values using frozen (Y) and non-frozen (X) HCVpps were very similar, as indicated by the linear regression ($Y=0.9618X-$ 0,08949). The correlation of the datasets ($r=0.9612$) indicates that the functionality of HCVpps is preserved following a freeze–thaw cycle (Fig. 4), despite the differences in r.l.u. (Fig. S7). All HCVpp-mAb combinations showed similar neutralization curves (Fig. S8a) with minor variations in IC_{50} values, which were sometimes higher and sometimes lower, independent of freezing, indicating that freezing does not reduce or increase IC_{50} values (Fig. S8b). Non-frozen HCVpps showed in general higher r.l.u. compared to frozen HCVpps and therefore had smaller CV% (Fig. S8c). Nonetheless, in both cases all triplicates above 3000 r.l.u. were below a CV of 32%, indicating that IC_{50}s generated from non-frozen or frozen HCVpps are equally reliable.

To further evaluate consistency, some HCVpp–mAb combinations were repeated several times with non-frozen and frozen HCVpps in independent experiments (Fig. S9a–d). Neutralization curves showed high similarity in shape and IC_{50} values. In addition, our results are also comparable to previously published IC_{50} values [25] for UKNP5.2.1, UKNP2.4.1 and UKNP4.1.1 with AP33 (Fig. S9e–g).

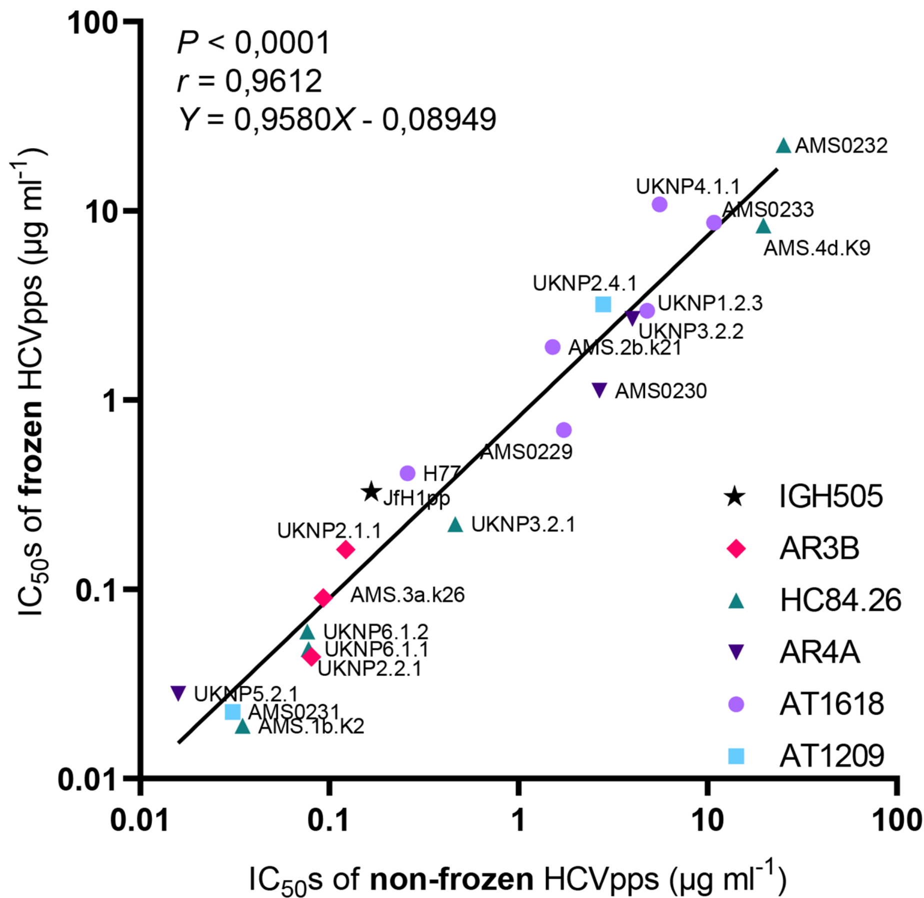


Fig. 4. Positive correlation of IC_{50} values obtained using non-frozen and frozen HCVpps. Linear regression of IC_{50} values before and after one freeze–thaw cycle of 21 HCVpps against six monoclonal antibodies targeting conformational E1E2 epitopes.

DISCUSSION

Here, we aimed to optimize the HCVpp neutralization protocol by exploring several factors that could improve HCVpp infectivity but do not alter the outcome of the neutralization assay. The most decisive factors that contributed to our improved protocol include: (1) the use of alternative transfection E1E2 : MLV Gag–pol : Luc ratios to produce higher HCVpp infectivity; (2) reduction of the assay volume and simplification of the infection protocol; (3) the possibility to freeze HCVpps; and (4) the ability to dilute HCVpps. A combination of small optimization steps allowed us to obtain a highly reproducible protocol. Therefore, we suggest the following: first, test HCVpps with alternative DNA plasmid ratios (1:2.6:2.6 and 1:26:34); second, produce large batches of HCVpps to use (diluted) frozen–thawed HCVpps for future assays; and third, reduce the amount of HCVpps and mAb/sera in the assay to save precious material.

Approximately 45% (10/22) of the tested HCVpps could be produced with the standard plasmid ratio of 1:1:1. However, for most of the HCVpps (68%, 15/22) the signal was improved after modifying the E1E2 : MLV gag–pol : Luc ratio during transfection. To produce patient-derived HCVpp it has been suggested to test a matrix of E1E2 and structural plasmids (MLV or HIV gag–pol) [21]. However, this is quite labour-intensive, especially when the goal is to produce a panel of patient-derived HCVpps. We selected two ratios based on previous E1E2/MLV gag–pol matrices [21]. With this approach, most HCVpp yields improved and only one (UKNP1.20.3) failed to produce. We are uncertain why this HCVpp did not work in our hands. Overall, ratios of 1:2.6:2.6 or 1:24:34 represent a good first approach before testing a full matrix. In addition, differences in preferred ratios were genotype-independent. Within genotype 1, for example, we found HCVpps with highest yields for ratio 1:1:1 (UKNP1.2.3), 1:2.6:2.6 (AMS.1b.k2) and 1:24:36 (AMS0229). The changes in plasmid ratios clearly affected the infectivity of HCVpps but not their susceptibility to neutralization. In terms of E1E2 expression, all E1E2 sequences are expressed under a CMV promoter but with a different polyadenylation signal (poly-A; BGH or TKpoly-A), depending on its plasmid backbone. It has been shown that some poly-A can be more efficient than others [35] but we did not find an association between a particular poly-A and a specific ratio (data not shown). Overall, it is unclear if changing ratios modified infectivity, HCVpp production or functionality of the produced HCVpps. Previous research [21] found that

E1E2 quantity does not appear to be an effective predictor of HCVpp infectivity, since very low levels of E1E2 on particles can mediate infection and it is currently unknown how many E1E2s are required to mediate entry. Factors affecting HCVpp production after changing the ratios might be: reduced budding, competition between protein production under the same promoter, or toxic effects on producing cells. Changes in E1E2 functionality due to different interactions with packaging vectors could also not be discarded. Further studies are needed to better understand the effect of different transfection ratios.

A combination of variables rather than one specific variable may play a role in the infectivity of HCVpps. We reasoned that during the 4 h incubation period as described in the standardized protocol [23] most HCVpps in the solution (100 µl of inoculum) may not have been in contact with the Huh-7 cells and are consequently degraded before they can infect the target cells. We reduced the amount of HCVpp inoculum to less than one third and only added medium on top to keep the cells alive after 4 h. This resulted in a 2.8-fold higher signal compared to the standard protocol, indicating that a smaller volume is beneficial for infectivity and at least 4 h are required for the HCVpp to enter Huh-7 cells. We used variable amounts of HCVpps (due to non-frozen, frozen and/or different dilutions) and found that as long as they met a defined quality control threshold (r.l.u. >3000), neutralization titres remained similar. These advantages made the production and storage of HCVpps possible for later use, which contributes to increased reproducibility. We have run several independent experiments with non-frozen and (diluted) frozen HCVpps, which showed the reproducibility of the assay and the use of frozen HCVpps even after >30 months of storage. In addition, the modifications in the assay did not change the antibody IC_{50} values and our results are also comparable with previously published IC_{50} values [25], indicating that our changes do not alter the final outcome.

Our study stresses the advantage of using three main ratios to obtain higher yields per particle. The use of a high yield HCVpp production cell line (i.e. 293T CD81KO) [24] boosted the performance of our protocol considerably. We have optimized the use of HCVpp inoculum, incorporated the use of diluted/frozen HCVpps and reduced hands-on time. We tested this protocol with HCVpps from six different genotypes with conformational mAbs targeting all of the different reported antigenic regions. Therefore, we are confident that this optimized protocol can be extended to test HCVpp neutralization against both known and unknown epitopes using both serum and antibodies with a wide variety of HCV variants, especially to broaden the current scope of using patient-derived HCVpps panels [25, 36], which is highly desirable to evaluate experimental vaccines. We encourage scientists from other laboratories to test this protocol with a broader range of HCV variants. This optimized protocol is currently being used to evaluate the presence of neutralizing antibodies in a HCV patient cohort and animals, as part of ongoing efforts to develop a HCV vaccine. Our detailed protocol described here provides the scientific community with the tools to implement this protocol. This reproducible and convenient virus neutralization assay will contribute to a better understanding of the antibody response during natural infection and better evaluation of experimental HCV vaccines.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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