# Colony Forming Assay for HCV-Replicon Cell Line

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**[Abstract]** Hepatitis C virus (HCV) is the main causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Since the HCV genome is present exclusively in RNA form during replication, a number of anti-HCV drugs show appearance of rapid drug-resistant viruses. Therefore, it is important to test generation of drug-escape mutant viruses by developed antiviral drugs for their validity. Here, we describe a colony formation assay-based method to observe appearance of drug-resistant viruses against nucleic acid based anti-HCV drugs in genotype 1b based subgenomic replicon cell culture system (Lee *et al*., 2013).

## Materials and Reagents

1. Cell line: HCV-replicon Huh-7 human hepatoma cell line (HCV genotype 1b subgenomic replicon pFKI389neo/NS3–3'/5.1 containing the neomycin resistant gene, provided by R. Bartenschlager, Heidelberg University, German) (Lohmann *et al*., 1999; Krieger *et al*., 2001)
2. 0.1% Trypsin-EDTA (WELGENE, catalog number: LS015-01)
3. Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Hyclone™, catalog number: SH30919.03)
4. 100x Penicillin/Streptomycin solution (Thermo Fisher Scientific, Hyclone™, catalog number: 3V30010)
5. Lipofectamine 2000 (Life Technology, catalog number: 11668-019)
6. NaCl (Sigma-Aldrich, catalog number: S5886)
7. KCl (Sigma-Aldrich, catalog number: P5405)
8. $Na_2HPO_4$ (Sigma-Aldrich, catalog number: S3264)
9. $KH_2PO_4$ (Sigma-Aldrich, catalog number: P9791)
10. NaOH (Sigma-Aldrich, catalog number: S5881)
11. 1x Phosphate Buffered Saline (PBS) (see Recipes)
12. Complete Dulbecco's modified Eagle medium with high glucose (DMEM) (Thermo Fisher Scientific, Hyclone™, catalog number: SH30243.01) (see Recipes)
13. 50 mg/ml G418 (Merck KGaA, catalog number: 345810) (see Recipes)
14. 2% Paraformaldehyde solution (Sigma-Aldrich, catalog number: 158127) (see Recipes)

15. 1% Methylene blue (Duksan Scientific, catalog number: MEE0-22002) (see Recipes)

## Equipment

1. 35 mm cell culture plate (Corning, catalog number: 430165)
2. 100 mm cell culture plate (BD Bioscience, catalog number: 353003)
3. 5% $CO_2$, 37 °C Incubator (Thermo Fisher Scientific, catalog number: 311)

## Procedure

1. Day one: HCV-replicon cells are sub-cultured and are seeded on 35 mm cell culture dish at density of 2 x $10^5$ in 2 ml of complete DMEM.
2. Day two: Before transfection, media was replaced with DMEM not containing Penicillin/Streptomycin.
3. 4 μM of test or control nucleic acid-based drugs were transfected using Lipofectamine 2000 reagent according to manufacturer's instruction.
4. Four hours after transfection, cells were replaced with 2 ml of fresh complete DMEM containing 500 μg/ml G418, and incubated for 2 days.
5. Step 2 to step 4 was repeated every 2 day during about two weeks.
6. About two weeks later, cells were trypsinized and sub-cultured, and one-tenth of the cells were replated to 35 mm dishes with 2 ml of 500 μg/ml G418 containing complete DMEM.
7. Step 2 to step 6 was repeated until visible colonies were obtained (usually, colonies formed one month after first transfection).
8. After acquiring colonies, media was removed and cells were washed 1 time with 1ml of 1x PBS.
9. Cells were treated with 1 ml of 2% paraformaldehyde for 15 min at room temperature.
10. Remove the paraformaldehyde from the cells and add 1 ml of 1% methylene blue for 10 min.
11. Remove the methylene blue and wash cells with 1 ml of $dH_2O$ three times.
12. Cells were air-dried for 15 min, and analyze appearance of drug-resistant cells by counting the number of G418-resistant cell colonies. You can find examples of picture showing drug-resistant colonies in the Figure 4C of Reference 1 (Lee *et al*., 2013).

## Recipes

1. 1x PBS
   137 mM NaCl

2.7 mM KCl
10 mM $Na_2HPO_4$
2 mM $KH_2PO_4$
Add $dH_2O$ to 1 L and sterilize using autoclave

2. Complete DMEM medium (505 ml)
   50 ml FBS
   5 ml 100x Penicillin/Streptomycin solution
   450 ml DMEM
3. 50 mg/ml G418
   1 g G418 powder
   Add $dH_2O$ to 20 ml and filter (0.45 μm) for sterilization
   Aliquote
   Store at -20 °C
4. 2% Paraformaldehyde solution (15 ml)
   0.3 g paraformaldehyde
   75 μl 10 N NaOH
   Add to $dH_20$ to 13.5 ml and incubate 60 °C until paraformaldehyde is dissolved
   Add 1.5 ml of 10x PBS and store at 4 °C
5. 1% methylene blue (15 ml)
   0.15 g methylene blue
   Add to $dH_2O$ to 15 ml

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## References

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