

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. Na₂HPO₄ (Sigma-Aldrich, catalog number: S3264)
9. KH₂PO₄ (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% CO₂, 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 2×10^5 in 2 ml of complete DMEM.
2. Day two: Before transfection, media was replaced with DMEM not containing Penicillin/Streptomycin.
3. 4 μ M 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 μ g/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 μ g/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 dH₂O 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 Na₂HPO₄
- 2 mM KH₂PO₄
- Add dH₂O 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 dH₂O 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 dH₂O 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 dH₂O to 15 ml

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References

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