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RNA Strand Displacement Assay for Hepatitis E Virus Helicase

Vidya P. Nair and Milan Surjit*

Virology Laboratory, Vaccine and Infectious Disease Research Centre, Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana, India
*For correspondence: milan@thsti.res.in

[Abstract] The hepatitis E virus (HEV) helicase uses ATP to unwind the RNA duplexes. This is an essential step for viral replication. This protocol aims to measure the double strand RNA unwinding activity of the HEV helicase.
Keywords: Hepatitis E virus, RNA virus, Helicase, Helicase assay, Viral replication, Nonradioactive helicase assay

[Background] The RNA unwinding activity of HEV helicase has been measured using radiolabeled double stranded RNA (dsRNA, Karpe *et al.*, 2010). We have established a non-radioactive assay protocol for measuring the dsRNA unwinding activity of the HEV helicase. This assay utilizes a fluorescently tagged RNA to measure the activity of the HEV helicase protein purified from human hepatoma cell, thus eliminating the need to handle radioactive material.

Materials and Reagents

1. 1.5 ml microcentrifuge tube
2. 60 mm plates
3. 1.6 ml RNase free microcentrifuge tube
4. PVDF (Polyvinylidene fluoride) membrane (Pall, catalog number: BSP0149)
5. Mammalian expression plasmids (details in Nair *et al.*, 2016):
   a. pCDNA5 Helicase-flag [HEV helicase sequence was PCR amplified using primers: pCDNA5 HEL-flag FP, pCDNA5 HEL-flag RP; digested with *Hind*III and ligated into pCDNA5 vector digested with *Hind*III and *Eco*RV]
   b. pUNO ORF2-flag [ORF2 with C-terminal Flag tag was PCR amplified with primers: pUNO ORF2-flag FP, pUNO ORF2-flag RP; digested with *Bgl*II and ligated into pUNO vector digested with *Nhe*I [blunted] and *Bam*HI]
6. Huh7 human hepatoma cells (obtained from Dr. C. M. Rice; Blight *et al.*, 2000)
7. RNA oligos: HRNA1 5'-UUUUUUUUUUUCGCUGAUGUCGCCUGG-3' and HRNA2 5'-[6FAM] CCAGGCGACAUCAGCG-3' (Sigma-Aldrich, USA)
8. Nuclease-free water (Thermo Fisher Scientific, Ambion™, catalog number: AM9937)
9. Ethidium bromide solution (Sigma-Aldrich, catalog number: E1510)
10. Glycogen (Sigma-Aldrich, catalog number: G0885)

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2198

11. Ethanol (EMD Millipore, catalog number: 100983)
12. Lipofectamine 2000 (Thermo Fisher Scientific, Invitrogen™, catalog number: 11668019)
13. DMEM
14. 10% fetal bovine serum (FBS)
15. Phosphate-buffered saline (PBS) (Bio Basic, catalog number: PD0100)
16. Flag M2 agarose resin (Sigma-Aldrich, catalog number: A2220)
17. Flag peptide (Sigma-Aldrich, catalog number: F4799)
18. DEPC-treated water (Thermo Fisher Scientific, Ambion™, catalog number: AM9922)
19. Octa-probe antibody (Santa Cruz Biotechnology, catalog number: sc-807)
20. Skimmed milk (Sigma-Aldrich, catalog number: 70166)
    Note: This product has been discontinued.
21. Anti-rabbit IgG Horseradish peroxidase(HRPO) (Santa Cruz Biotechnology, catalog number: sc-2004)
22. Clarity Western ECL blotting substrate (Bio-Rad Laboratories, catalog number: 1705061)
23. Silver stain kit (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 24612)
24. Bradford assay reagent (Bio-Rad Laboratories, catalog number: 5000002)
25. RNAsin
26. ATP
27. Proteinase K (Sigma-Aldrich, catalog number: P2308)
28. Glycogen (Sigma-Aldrich, catalog number: G0885)
29. Tris (Sigma-Aldrich, catalog number: T6066)
30. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
31. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E6758)
32. Ammonium acetate (Sigma-Aldrich, catalog number: A1542)
33. SDS (Sigma-Aldrich, catalog number: L3771)
34. Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*,-tetraacetic acid (EGTA) (Sigma-Aldrich, catalog number: 03777)
35. Triton X-100 (Sigma-Aldrich, catalog number: 93418)
    Note: This product has been discontinued.
36. Sodium pyrophosphate tetrabasic decahydrate ($Na_4P_2O_4{\cdot}10H_2O$) (Sigma-Aldrich, catalog number: S6422)
37. β-glycerol phosphate (Sigma-Aldrich, catalog number: 50020)
38. Sodium orthovanadate (Na_3VO_4) (Sigma-Aldrich, catalog number: S6508)
39. Protease inhibitor cocktail tablet (Roche Diagnostics, catalog number: 04693132001)
40. HEPES (Sigma-Aldrich, catalog number: H8651)
41. Magnesium chloride ($MgCl_2$) (Sigma-Aldrich, catalog number: M8266)
42. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
43. BSA
44. DL-dithiothreitol (DTT) (Sigma-Aldrich, catalog number: D0632)

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2198

45. Tween 20
46. Bromophenol blue (Sigma-Aldrich, catalog number: B0126)
47. Xylene cyanol FF (Sigma-Aldrich, catalog number: X4126)
48. Annealing buffer (see Recipes)
49. Soaking buffer (see Recipes)
50. IP buffer (see Recipes)
51. Strand displacement buffer (see Recipes)
52. 5x termination buffer (see Recipes)
53. 2x proteinase K buffer (see Recipes)
54. Phosphate buffered saline with 0.1% Tween 20 (PBST) (see Recipes)
55. 50 mg/ml proteinase K (see Recipes)
56. 10 mg/ml glycogen (see Recipes)
57. RNA loading dye (see Recipes)

Equipment

1. Vertical electrophoresis mini apparatus
2. Gel documentation system (Bio-Rad Laboratories, model: ChemiDoc™ MP)
3. NanoDrop (Thermo Fischer Scientific, USA)
4. Heat block
5. Water bath
6. Refrigerated table top centrifuge
7. -80 °C deep freezer
8. Flip flop rocker
9. 5% CO_2 incubator

Procedure

A. Preparation of fluorescently labeled dsRNA
   1. All steps should be performed using nuclease free reagents.
   2. Resuspend the RNA oligos to 100 pmol/µl in nuclease free water. Resolve 1 µl on 20% non-denaturing PAGE (Polyacrylamide gel electrophoresis), stain with ethidium bromide (2 µl of 10 mg/ml stock in 50 ml running buffer) for 15 min at room temperature and visualize the signal using gel documentation system under UV set.
      Note: This step is necessary to rule out occasional degradation of the oligo from the source itself.
   3. Mix 100 pmole (100 pmole/µl stock) of each oligo in 40 µl annealing buffer, incubate at 95 °C for 30 sec and cool gradually to room temperature.

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2198

4. Resolve the annealed oligos in 20% non-denaturing PAGE and excise the band corresponding to the RNA duplex.
5. Transfer the excised bands to a fresh 1.5 ml microcentrifuge tube, mince and soak in 500 µl soaking buffer overnight (16 h) in a flip flop rocker at room temperature.
6. Next day, centrifuge the tubes at 13,000 *x g* for 5 min at room temperature.
7. Collect the supernatant into a fresh microcentrifuge tube, add 5 µg glycogen (5 mg/ml stock in water) and 1 ml ice cold absolute ethanol and incubate at -80 °C for 45 min.
8. Centrifuge at 14,000 *x g* for 30 min at 4 °C.
9. Wash the pellet with 500 µl 75% ethanol, centrifuge at 14,000 *x g* for 10 min at 4 °C.
10. Remove 75% ethanol and dry the pellet at room temperature.
    Note: Be careful while removing the 75% ethanol, pellet may be lost.
11. Dissolve the RNA pellet in 30 µl nuclease-free water and incubate at 55 °C for 10 min.
12. Run 1 µl dsRNA in 20% non-denaturing PAGE. Visualize the fluorescent RNA using the gel documentation system using appropriate setting (6-FAM fluorescent dye setting, excitation: 492 nm, emission: 518 nm).
13. Quantitate the dsRNA by spectrophotometry using NanoDrop and store in aliquots in RNase free microcentrifuge tubes at -80 °C.

B. Preparation of flag-affinity purified proteins

1. Transfect 3 µg each of pUNO RdRp-flag and pUNO ORF2-flag plasmids into ten 60 mm plates (each plasmid transfected into 10 plates, not cotransfected), containing Huh7 human hepatoma cells, using Lipofectamine 2000 in 1:1 ratio, following manufacturer's instructions. Incubate cells at 37 °C in 5% CO_2 incubator. 24 h post-transfection, replace media with 2 ml DMEM supplemented with 10% fetal bovine serum.
2. Forty eight hours post transfection, wash the cells with 1x PBS and resuspend in 800 µl IP buffer.
3. Generate a homogenous suspension by repeated pipetting and vortexing, and incubate overnight (16 h) on ice at 4 °C (cold room or fridge).
4. Next day, clarify the lysate by centrifugation at 13,000 *x g*, 4 °C, 10 min and collect the supernatant into a fresh 1.6 ml RNase free microcentrifuge tube.
5. Add 100 µl of flag agarose beads, incubate on a rocker at 4 °C for 4 h, wash 3 x in 1 ml IP buffer each by centrifuging at 800 *x g*, 1 min, at 4 °C. Add 200 µl flag peptide (0.2 mg/ml in PBS) and incubate on a rocker for 15 min at 4 °C. Centrifuge at 800 *x g*, 1 min, at 4 °C and collect the supernatant, which contains the eluted protein.
6. Check an aliquot of the purified protein by Western blotting using octa-probe antibody and silver staining, as mentioned below.
7. Resolve the proteins by 10% SDS-PAGE and transfer onto a PVDF (Polyvinylidene fluoride) membrane.

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2198

8. Block the membrane using 5% skimmed milk in 1x PBS for 45 min at room temperature, incubate overnight (16 h) with 1:1,000 octa-probe primary antibody diluted using 5% skimmed milk in 1x PBST at 4 °C, wash 3 x in PBST, incubate with 1:5,000 anti-rabbit IgG HRPO secondary antibody diluted using 5% skimmed milk in 1x PBST at room temperature, followed by detection of the signal by enhanced chemiluminescence using 'clarity Western ECL blotting substrate'.
9. Acquire the Images and analyze using a gel documentation system. A single band of approximately 27 kDa should be obtained, as illustrated in Nair *et al.* (2016).
10. For silver staining, resolve the proteins in 10% SDS-PAGE.
11. Proceed for silver staining using silver stain kit, following the manufacturer's protocol (Thermo Fisher Scientific, USA). A major band of approximately 27 kDa (corresponding to the size of the band obtained in Western) should be obtained, as illustrated in Nair *et al.* (2016). Few additional bands are also detected above 27 kDa, which does not affect the efficiency of the assay in our experience. If expected bands are obtained in Western and silver staining, estimate the respective protein concentration by serial dilutions by Bradford assay and store the protein in single use aliquots at -80 °C.

C. RNA strand displacement assay

1. Incubate 1 pmol dsRNA and 2 µg flag affinity purified HEV helicase protein or ORF2 protein in strand displacement buffer at 37 °C for 2 h. Following order may be followed.

Water	24 µl
10x buffer	4 µl
RNAsin	1 µl
ATP (10 mM)	4 µl
Protein	5 µl
dsRNA	2 µl
Total volume	40 µl

2. Use appropriate negative controls such as dsRNA without protein or dsRNA with an unrelated protein (ORF2).
3. Terminate the reaction by adding 10 µl 5x termination buffer.
4. Add 50 µl 2x proteinase K buffer and 1 µl proteinase K (50 mg/ml stock solution in water) and incubate for 10 min at 37 °C.
5. Add 1 µl glycogen (10 mg/ml stock solution in water) and 1 ml of ice-cold absolute ethanol to the samples; incubate at -80 °C for 45 min.
6. Centrifuge the samples at 14,000 *x g*, 15 min, 4 °C.
7. Wash the pellet with 1 ml 75% ethanol and resuspend in 10 µl nuclease free water.
   Note: Be careful while removing the 75% ethanol, pellet may be lost. Although not necessarily a better alternative, it may be possible to minimize pellet loss by gently pipetting out 75%

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2198

ethanol instead of directly decanting. Irrespective of whatever handling technique preferred, attention should be given not to disturb the pellet.

8. Heat denature an equal amount of dsRNA containing sample at 95 °C for 10 min.
9. Mix the samples with 10 µl RNA loading dye and resolve in 20% non-denaturing PAGE to differentiate the migration between single strand (ss) RNA and dsRNA.
10. Visualize the fluorescent bands using a gel documentation system.

Data analysis

While performing the assay, appropriate controls should be included in order to interpret the data and rule out non-specific signals. Negative controls should include omission of helicase protein and nucleotides from the reaction mixture. A titration experiment using increasing quantities of helicase protein in the assay should also be performed to rule out non-specific signals. An assay using an unrelated protein instead of helicase will also rule out a possible non-specific signal. A sample assay has been illustrated in Figure 1. The effect of other factors or compounds on helicase activity may be evaluated by adding them to the reaction mixture.

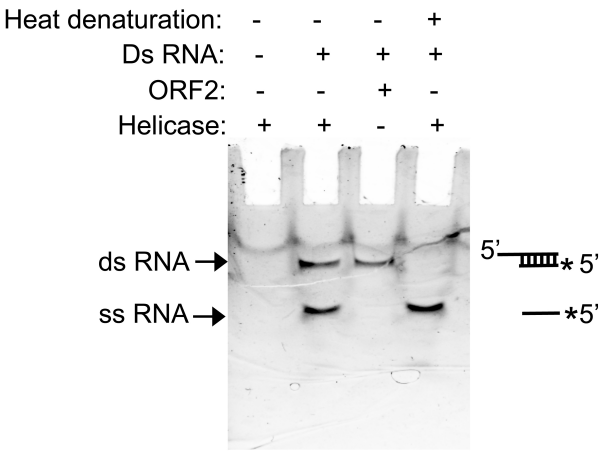

Figure 1. Assay of RNA strand displacement by helicase. dsRNA: double strand RNA, ssRNA: single strand RNA, Heat denaturation: the sample was incubated at 95 °C for 10 min before loading into the gel.

Recipes

1. Annealing buffer
   10 mM Tris-Cl (pH 7.4)
   100 mM NaCl
   1 mM EDTA
2. Soaking buffer
   0.5 M ammonium acetate

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2198

10 mM EDTA
1% SDS

3. IP buffer
   20 mM Tris-Cl (pH 7.4)
   150 mM NaCl
   1 mM EDTA (pH 8.0)
   1 mM EGTA (pH 8.0)
   1% Triton X-100
   2.5 mM sodium pyrophosphate
   1 mM β-glycerol phosphate
   1 mM sodium orthovanadate
   1x protease inhibitor cocktail (25x stock prepared by dissolving one tablet in 2 ml 1x PBS)
4. Strand displacement buffer
   50 mM HEPES (pH 7)
   2 mM $MgCl_2$
   10 mM KCl
   0.05 mg BSA/ml
   2 mM DTT
   1 mM ATP
5. 5x termination buffer
   1% SDS
   100 mM EDTA
6. 2x proteinase K buffer
   300 mM NaCl
   100 mM Tris-Cl (pH 7.5)
   1% SDS
7. PBST
   1x phosphate-buffered saline
   0.1% Tween 20
8. 50 mg/ml proteinase K
   50 mg/ml stock solution prepared in nuclease free water
9. 10 mg/ml glycogen
   10 mg/ml stock solution prepared in nuclease free water
10. RNA loading dye
    0.5 mM EDTA
    0.025% bromophenol blue
    0.025% xylene cyanol FF

Vol 7, Iss 07, Apr 05, 2017
DOI:10.21769/BioProtoc.2198

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