Helicase Assays

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[Abstract] Helicases are a class of enzymes which are motor proteins using energy derived from ATP hydrolysis to move directionally along a nucliec acid phosphodiester backbone (such as DNA, RNA and DNA-RNA hybrids) and separate two annealed nucleic acid strands. Many cellular processes, such as transcription, DNA replication, recombination and DNA repair involve helicase activity. Here, we provide a protocol to analyze helicase activities *in vitro*. In this protocol, the DNA helicase protein Merkel cell polyomavirus large T-antigen was expressed in the mammalian cell line HEK293 and immoblized on an IgG resin. The helicase assay is performing while the protein is immoblized on IgG resin.

Materials and Reagents

1. HEK293
2. Dulbecco's modified eagle medium high glucose (DMEM) (Life Technologies, catalog number: 11965-084)
3. Fetal bovine serum (FBS) (Hyclone, catalog number: SH30071.03)
4. DPBS without Ca^{2+} and Mg^{2+} (Life Technologies, catalog number: 14190136)
5. Trypsin/EDTA (Life Technologies, catalog number: 25300054)
6. IgG sepharose6 fast flow (GE, catalog number : 17-0969-01)

7. M13mp18 DNA (New England Biolab, catalog number: N4040S)
8. DNA oligo: CCAGGGTTTTCCCAGTCACGACGTTGTAAAC
9. DNA polymerase I klenow fragment (New England Biolab, catalog number: M0210S)
10. 100 mM dCTP (Promega Corporation, catalog number: U1221)
11. 100 mM dGTP (Promega Corporation, catalog number: U1211)
12. 100 mM dATP (Promega Corporation, catalog number: U1201)
13. 100 mM ATP (Roche Diagnostics, catalog number: 11140965001)
14. [gamma-^{32}P] dATP
15. DNA loading dye
16. Polyacrylamide gel
17. 1 M Tris (pH 7.6) (see Recipes)
18. 0.1 M Tris (pH 7.6) (see Recipes)
19. 5 M NaCl stock (see Recipes)
20. 1 M $MgCl_2$ stock (see Recipes)
21. 10 mM $MgCl_2$ (see Recipes)
22. 0.1 M PMSF (Sigma-Aldrich, catalog number: 78830) (see Recipes)
23. 0.1 M DTT (Sigma-Aldrich, catalog number: 43819) (see Recipes)
24. 1 mg/ml Aprotinin (Roche Diagnostics, catalog number: 10236624001) (see Recipes)
25. 1 mg/ml Leupeptin (Roche Diagnostics, catalog number: 11017101001) (see Recipes)
26. 1 mg/ml Pepstatin A (Roche Diagnostics, catalog number: 10253286001) (see Recipes)
27. 10% NP40 stock (see Recipes)
28. 1% Bovine serum albumin (BSA) (Sigma-Aldrich) (see Recipes)
29. 10 mg/ml BSA (see Recipes)
30. 10x TBE buffer (see Recipes)
31. IPP400 buffer (see Recipes)
32. Helicase assay buffer (see Recipes)
33. Stop buffer (see Recipes)

34. Acrylamide gel (see Recipes)

Equipment

1. 20 G needle (BD, catalog number: 305175)
2. PowerPac basic power supply (Bio-Rad Laboratories, catalog number: 164-5050)
3. Mini-Protean tetra cell (Bio-Rad Laboratories, catalog number: 165-8000)
4. Sorvall RC 6 plus centrifuge and Sorvall SA-600 rotor (Thermo Fisher Scientific)
5. 37 °C, 5% CO_2 cell culture incubator
6. Typhoon 9410 variable mode Imager
7. Storage phosphor screens and cassettes (GE)
8. Fisher Vortex Genie 2 (Thermo Fisher Scientific, catalog number: 12-812)

Procedure

A. Protein purification and immobilization

Note: Before this procedure, it is suggested to pre-cool centrifuge to 4 ˚C and cool down all buffers used in this procedure on ice. Performing all steps at a cold condition is optimal.

1. 48 h post transfection (calcium phosphate assay is recommended for HEK293 cell transfection. In our experiment, a plasmid which encodes Merkel cell polyomavirus large T-antigen fusion to an IIT tag (IgG-IgG-TEV tag) was used in either 15 cm dishes or 10 cm dishes),wash transfected cells with cold PBS, scrape and re-suspend in cold PBS, and pellet at 500 *x g* for 5 min, 4 °C.
2. Wash cells once with cold PBS and re-pelleted at 500 *x g* for 5 min, 4 °C.
3. Wash cell pellet with IPP400 (100 µl buffer for each 1 x 10^7 cells. The ratio is adjustable dependent on cell line. For HEK293, a confluent 10 cm dish contains 1 x 10^7 cells and a confluent 15 cm dish contains 2.5 x 10^7 cells).

4. Pass cells through a 20-G needle 10 times and rotate at 4 °C for 1 h.
5. During rotation, wash IgG Sepharose6 Fast flow beads with at least 5x resin volume of PBS once. Sit on ice until most of the resin settles down. Usually, it will take 10-20 min.
6. Remove PBS and pre-block IgG Sepharose6 Fast flow in 1% (w/v) BSA solution in PBS for at least 30 min at 4 °C with rotation. Settle down resin on ice.
7. Spin cell lysate from step A4 at 32,572 *x g* for 15 min, 4 °C to get rid of debris.
8. Remove 1% BSA from resin in step A6, mix supernatant from step A7 with pre-blocked resin from step A6. Usually, 10-15 µl resin was used for cell lysate from each 15 cm dish in our experiment.
9. Rotate for 2 h at 4 °C.
10. Settle down resin on ice and gently remove supernatant with a fine tip. Add at least 5 volumes of IPP400 supplemented with NP-40 at a final concentration of 0.01% (v/v). Re-suspend resin.
11. Repeat step A10 at least twice.
12. Wash resin with IPP400 without NP40 twice as in step A10.
13. Settle resin down on ice and remove supernatant.
14. Spin down resin at 500 *x g* for 5 min, 4 °C. Gently remove all liquid with a fine tip.
15. Equilibrate with 10 resin volumes of 1x helicase assay buffer. Settle resin down on ice. Use immobilized protein on resins as fresh as possible. Usually, use 10 µl beads and about 1 µg protein for each reaction (to measure how many recombinant protein you get from each dish, purify protein by IgG resin following manufacturer's protocol and briefly quantify the purified protein). The usage of protein in each reaction is adjustable.

B. Substrate labeling

Note: Radioactive materials are used in this procedure. Please follow standard guidelines to safely handle radioactive materials.

1. Dissolve synthesized oligo DNA in sterilized water to a final concentration 35 ng/µl.

2. Prepare annealing reaction as follows:

Reagent	Volume	Final conc.
M13mp18 single strand DNA (250 ng/μl)	4 μl	
Oligo (35 ng/μl)	1 μl	
0.1 MTris-Cl (pH7.6)	4.3 μl	10 mM
10 mM MgCl2	4.3 μl	1 mM

Add H_2O to final volume 43 μl.

3. Heat to 95 °C for 15 min.
4. Briefly spin down. Incubate at 50 °C for 1 h.
5. Briefly spin down. Slowly cool down to room temperature.
6. Add 5 μl NEB restriction enzyme buffer 2.
7. Add dCTP and dGTP to a final concentration 0.1 mM and 1 μl [α-^{32}P]-dATP (3000 Ci/mM, 1 mCi/ml)
8. Add 1 μl DNA polymerase I Klenow fragment.
9. Incubate at room temperature for 20 min.
10. Add 0.5 μl 10 mM dATP. Incubate at room temperature for another 20 min.
11. Use labeled substrate as fresh as possible. Avoid freezing and thawing.
12. Titrate labeled substrate as following: take 1, 2, 3, 4, 5 μl substrate, add sterilized water to 9 μl and add 1 μl 10x DNA loading dye. Boil 5-10 min. Load on an 11% non-denaturing polyacrylamide gel and electropherose. Dry gel and expose for autoradiography. Adjust the usage of substrate in the helicase assay depending on labeling efficiency and storage phosphor sensitivity (10-40 ng labeled DNA was used in our experiments).

C. Helicase activity detection

Note: Radioactive materials are used in this procedure. Please follow standard guidelines to safely handle radioactive materials.

1. Prepare reaction buffer as follows:

10x helicase assay buffer	5 µl
100 mM ATP	1 µl
H_2O	44-x µl

Gently remove 1× helicase buffer from resins in step A15. Add reaction buffer to resin.

2. Add x µl labeled substrate (dependent on your titration) to the reaction.
3. Incubate at 37 °C for 30 min (during this process, keep shaking tubes on a Fisher vortexer with the speed 1.5).
4. Add 5 µl stop buffer.
5. Briefly vortex, shortly spin down resin at room temperature and take the supernatant. Mix with 10x DNA loading dye and 5-10 µl supernatant is loaded onto an 11% non-denaturing polyacrylamide gel. Electrophorese in 1x TBE buffer.
6. Dry gel and subject to autoradiography.

Notes

1. Addition of 10% of glycerol in IPP400 buffer is helpful but not necessary.
2. Immoblizing protein on other resins may also work, but the authors have not tested other resin systems.
3. Freezing-and-thawing labeled substrates will bring unexpected bands.
4. If you want to get rid of free labeled nucleic acid from the oligo substrate using ethenol precipitation, DNA purification kits, *etc*, the quality of purified substrate should be monitored to make sure the labeled short oligo is not dissociated from the M13mp18 genome.
5. If alernative single-strand DNA, DNA oligos or radiolabeled deoxyribonucleotides (such as [α-^{32}P] dCTP, *etc.*) are used, the addition of free deoxyribonucleotides in step B7 is adjustable. Noticably, your design should make sure that your radiolabeled deoxyribonucleotides have been incorporated into your substrates before the reaction is

stopped by deoxyribonucleotides deficiency (In this protocol, the reaction will be stopped by dTTP deficiency.).

Recipes

1. 1 M Tris (pH 7.6) stock

   Mix 121.14 g of Tris base with 600 ml dH_2O, pH to 7.6 with HCl

   Adjust total volume to 1 L

   Filter sterilize (0.45 μm)

   Stored at 4 °C

   1 M Tris (pH 7.5) and 1 M Tris (pH 8.0) are made at the same way
2. 0.1 M Tris (pH 7.6)

   Add 100 μl 1M Tris (pH 7.6) into 900 μl dH_2O

   Prepare fresh
3. 5 M NaCl stock

   Mix 292.2 g sodium chloride with 800 ml dH_2O

   Adjust total volume to 1 L

   Filter sterilize (0.45 μm)

   Stored at 4 °C
4. 1 M $MgCl_2$ stock

   Mix 95.2 g magnesium chloride with 800 ml dH_2O

   Add dH_2O to a final volume of 1 L

   Filter sterilize (0.45 μm)

   Stored at 4 °C
5. 10 mM $MgCl_2$

   Add 10 μl 1 M $MgCl_2$ into 990 μl dH_2O

   Prepare fresh

6. 0.1 M PMSF

   Mix 174.2 mg PMSF with 10 ml ethanol

   Stored at -20 °C

7. 0.1 M DTT

   Dissolve 1.5 g of DTT in 8 ml of H_2O

   Adjust the total volume to 10 ml

   Take 100 µl, mix with 900 µl dH_2O

   Stored at -20 °C

8. 1 mg/ml Aprotinin

   Dissolve 20 mg Aprotinin in 20 ml H_2O

   Stored at -20 °C

9. 1 mg/ml Leupeptin

   Dissolve 20 mg Leupeptin in 20 ml H_2O

   Stored at -20 °C

10. 1 mg/ml Pepstatin A

    Dissolve 20 mg Pepstatin A in 20 ml methanol

    Store at -20 °C

11. 10% NP40 stock

    Mix 50 ml NP40 with 400 ml dH_2O

    Adjust total volume to 500 ml

    Filter sterilize (0.45 µm)

    Stored at 4 °C

12. 1% BSA

    Mix 0.1 g BSA with 400 µl dH_2O

    Adjust total volume to 10 ml

    Filter sterilize (0.45 µm)

    Stored at 4 °C no more than 1 month

13. 10 mg/ml BSA

    Mix 100 mg BSA with 10 ml dH_2O

    Filter sterilize (0.45 µm)

    Stored at -20 °C

14. 10x TBE buffer

    108 g of Tris

    55 g of boric acid

    7.5 g of EDTA, disodium salt

    Add into 800 ml dH_2O then adjust total volume to 1 L

15. IPP400 buffer

Stock	20 ml	Final conc.
1M Tris-Cl [pH 8.0]	200 µl	10 mM
5 M NaCl	1600 µl	400 mM
0.1 M PMSF	40 µl	0.2 mM
1 mg/ml Aprotinin	20 µl	1 µg/ml
1 mg/ml Leupeptin	20 µl	1 µg/ml
1 mg/ml Pepstatin A	20 µl	1 µg/ml
ddH_2O	to 20 ml	

16. Helicase assay buffer

Stock	10 ml	10x Final conc.
1 M Tris-Cl (pH 7.5)	2 ml	200 mM
1 M $MgCl_2$	1 ml	100 mM
10 mg/ml BSA	1 ml	1 mg/ml
H_2O	6 ml	

    Add 5 µl 0.1 M DTT to 100 µl Helicase assay buffer for a final concentration of 5 mM

17. Stop Buffer

    3.3% SDS (w/v) and 0.1 M EDTA in dH_2O

18. Acrylamide gel

Acrylamide	Acrylamide (30%, 29:1) (ml)	10x TBE (ml)	ddH_2O (ml)	TEMED (µl)	10% APs (µl)
3.5%	0.583	0.5	3.917	2.5	25
5.0%	0.833	0.5	3.667	2.5	25
8.0%	1.333	0.5	3.167	2.5	25
11.0%	1.833	0.5	3	2.5	25
15.0%	2.50	0.5	2.0	2.5	25
20.0%	3.333	0.5	1.167	2.5	25

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