In Vitro Hepatitis B Virus Polymerase Priming Assay

HBV Biochemical Assays

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Introduction

- HBV polymerase activity is assayed by measuring the first step in DNA synthesis, protein priming, wherein the polymerase becomes nucleotide-labelled.
- This *in vitro*assay uses a plasmid-based expression system performed in cell culture, producing a chaperone-bound, epsilon-dependent polymerase [1].
- Priming levels are determined by supplying radionucleotides, and measuring autoradiography after polyacrylamide gel electrophoresis.
- This assay provides a direct test of effects on priming by HBV polymerase inhibitors, and provides a method to screen for functional mutants.
- An additional assay uses the enzyme Tdp2, which is able to remove, and to allow subsequent visualization of primed products from HBV polymerase.
- This protocol was originally published in Hepatitis B Virus: Methods and Protocols (see reference).

Materials and Reagents

Cell culture and transfection

- Human embryonic kidney (HEK) 293T cells
- Complete DMEM/F12 (1:1) medium, supplemented with 10% FBS, 100 U/mL penicillin, 10 µg/mL streptomycin
- pcDNA-3FHP: Expresses a triple FLAG-tagged HBV polymerase (HP) under the human cytomegalovirus (CMV) promoter in the pcDNA3 (Invitrogen) backbone. Three in-frame copies of the FLAG epitope tag are N-terminal to the HBV polymerase coding sequence, strain ayw (GenBank accession number X59795.1 which is phylogenetically HBV genotype D as in pCMV-HBV [2]).
- pCMV-HE: For ε RNA expression in human cells, pCMV-HE has a 0.5 kb fragment of the CMV promoter plus HBV sequence from 1,801-1,993 from pCMV-HBV (NdeI to XbaI) substituting for the CMV and T7 promoter in pcDNA3 (NdeI to XbaI fragment). pCMV-HE will produce, upon RNA Pol II transcription in transfected mammalian cells, capped and polyadenylated HBV RNA initiating at the authentic pgRNA initiation site (1,814) and containing the 5’ DR1 (1,822-1,832) and ε RNA sequences (1,845-1,905). Polyadenylation can occur from native or BGH poly(A) sites, and both can be visualized on a urea-polyacrylamide gel by electrophoresis.
- Calcium phosphate transfection kit (Clontech)

Preparation of FLAG antibody-bound beads

- Anti-FLAG M2 antibody (Sigma)

- Protein A/G agarose beads
- Low retention pipet tips

HBV polymerase immunoprecipitation and purification

- Diethylpyrocarbonate (DEPC)-treated water or other nuclease-free water
- RNaseZap or similar RNase removal product (Ambion)
- Dithiothreitol (DTT), 1M, make fresh for each use, keep RNase-free
- β-mercaptoethanol, 12.8M, keep RNase-free
- Complete EDTA-free protease inhibitor, 25X concentration, store prepared solution for up to 3 months at -20°C, keep RNase-free (Roche)
- Complete protease inhibitor, 25X concentration, store prepared solution for up to 3 months at -20°C, keep RNase-free (Roche)
- E-64 protease inhibitor, 2mM, store prepared solution for up to 9 months at -20°C, keep RNase-free
- Leupeptin protease inhibitor, 1mg/ml, store prepared solution for up to 6 months at -20°C, keep RNase-free
- Phenylmethanesulfonyl fluoride (PMSF) protease inhibitor, 200mM in isopropanol, store at room temperature for up to 9 months, keep RNase-free. PMSF is always added last to solutions and not placed on ice since it precipitates out of solution at colder temperatures.
- RNasin Plus (Promega) or RNaseOUT (Thermo Fisher) RNase inhibitors
- RNase-free Tris, 1M, pH 7.0 (Ambion)
- RNase-free NaCl 5M (Ambion)
- KCl, 1M, keep RNase-free
- Glycerol, 80%, keep RNase-free
- NP-40, 10%, keep RNase-free
- EDTA, 0.5M, pH 8.0, store at room temperature, keep RNase-free
- TN buffer: 50mM Tris pH 7.0, 100mM NaCl. For 50mL, combine 2.5mL 1M Tris pH 7.0, 1mL 5M NaCl, and 46.5mL nuclease-free water. Store at room temperature.
- 10X RNase-free PBS (Ambion). Prepare 1X PBS with nuclease-free water, keep RNase-free.
- 1X PBS with protease inhibitors: 28μM E-64, 5μg/mL leupeptin, 1mM PMSF. To 50mL 1X PBS, add 350μL 2mM E-64, 125μL 1mg/mL leupeptin, and 125μL 200mM PMSF. Prepare just before use, do not store, keep RNase-free.
- FLAG lysis buffer: 50mM Tris pH 7.0, 100mM NaCl, 50mM KCl, 10% glycerol, 1% NP-40, 1mM EDTA pH 8.0. For 50mL, combine 2.5mL 1M Tris pH 7.0, 1mL 5M NaCl, 2.5mL 1M KCl, 6.25mL 80% glycerol, 5mL 10% NP-40, 100μL 0.5M EDTA pH 8.0, and 32.65mL nuclease-free water. Store at room temperature, keep RNase-free.
- FLAG lysis buffer with inhibitors: 1X Complete protease inhibitor cocktail, 10mM β-mercaptoethanol, 2mM DTT, 1mM PMSF, 250U/mL RNase inhibitor. To 1mL FLAG lysis buffer, add 40μL 25X Complete protease inhibitor cocktail, 0.78μL 12.8M β-mercaptoethanol, 2μL 1M DTT, 5μL 200mM PMSF, and 6.25μL 40U/μL RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.
- FLAG wash buffer with inhibitors: 28μM E-64, 5μg/mL leupeptin, 1mM PMSF, 10mM β-mercaptoethanol, 2mM DTT, 10U/μL RNase inhibitor. To 1mL FLAG lysis buffer, add 14μL 2mM E-64, 5μL 1mg/mL leupeptin, 5μL 200mM PMSF, 0.78μL 12.8M β-mercaptoethanol, 2μL 1M DTT, and 0.25μL 40U/μL RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.

Downstream analysis of immunopurified HBV polymerase complexes

- 2X SDS lysis buffer: 125mM Tris pH 6.8, 20% glycerol, 4.6% SDS, 0.1% bromophenol blue. Store at room temperature. For 50mL, combine 6.94mL 1M Tris pH 6.8, 11.1mL glycerol, 25.56mL 10% SDS, 1.1mL 5% bromophenol blue, and 5.28mL distilled water. Before use,

add one-tenth volume 12.8M β-mercaptoethanol.

Assay of *in vitro* protein priming and transferase activity

- [α-^{32}P] dATP, dCTP, dGTP, and/or TTP (10mCi/ml, 3,000Ci/mmol) (PerkinElmer). Note the initiating nucleotide strand begins with dGAA. TTP gives the strongest signal when priming with manganese, ideal for the Tdp2 cleavage assay.
- 100mM deoxynucleotide triphosphates (dNTPs) (Invitrogen)
- 1M $MgCl_2$, store at -20°C, keep RNase-free. Magnesium priming supports the type of priming thought to occur in vivo.
- 1M $MnCl_2$, store at -20°C, keep RNase-free. Manganese priming activity supports the HBV polymerase's terminal transferase activity, which is epsilon-independent.
- 10X TMgNK buffer: 200mM Tris pH 7.0, 150mM NaCl, 100mM KCl, 40mM $MgCl_2$. For 500µL, combine 315µL nuclease-free water, 100µL 1M Tris pH 7.0, 15µL 5M NaCl, 50µL 1M KCl, and 20µL 1M $MgCl_2$. Store at -20°C, keep RNase-free.
- TMgNK priming buffer: 1X TMgNK, 1X Complete EDTA-free protease inhibitor cocktail, 4mM DTT, 1U/µL RNase inhibitor, 1mM PMSF. For 100µL, combine 82.6µL nuclease-free water, 10µL 10X TMgNK, 4µL 25X Complete EDTA-free protease inhibitor cocktail, 0.4µL 1M DTT, 2.5µL 40U/µL RNase inhibitor and 0.5µL 200mM PMSF. Prepare just before use, use on ice, do not store, keep RNase-free.
- 10X TMnNK buffer: 200mM Tris pH 7.0, 150mM NaCl, 100mM KCl, 20mM $MnCl_2$. For 500µL, combine 325µL nuclease-free water, 100µL 1M Tris pH 7.0, 15µL 5M NaCl, 50µL 1M KCl, and 10µL 1M $MnCl_2$. Store at -20°C, keep RNase-free.
- TMnNK priming buffer: 1X TMnNK, 1X Complete EDTA-free protease inhibitor cocktail, 4mM DTT, 1U/µL RNase inhibitor, 1mM PMSF. For 100µL, combine 82.6µL nuclease-free water, 10µL 10X TMnNK, 4µL 25X Complete EDTA-free protease inhibitor cocktail, 0.4µL 1M DTT, 2.5µL 40U/µL RNase inhibitor and 0.5µL 200mM PMSF. Prepare just before use, use on ice, do not store, keep RNase-free.

Experimental Procedures

Cell culture and transfection

1. HEK293T cells are maintained in complete DMEM/F12 (1:1) media in a humidified cell culture incubator at 37°C, 5% CO_2. Transfection efficiency is higher when cells are between 3 weeks and 3 months age, post-thaw.
2. Passage cells one day before transfection, plating approximately 1.25 x 10^6cells per 6 cm dish, or an amount that yields 60-90% confluence the next day. The procedure may be scaled: 6 cm dishes yield 2 bead aliquots, 10cm dishes yield 5 bead aliquots, and 15 cm dishes yield 12 bead aliquots.
3. Change medium 2-3 hours before transfection.
4. Transfect each plate with half pCDNA-3FHP and half pCMV-HE (by weight) using calcium phosphate transfection (or any other suitable method). Use 10 µg total weight of DNA for 6 cm dishes, 20 µg for 10 cm dishes, and 50 µg for 15 cm dishes. Include any desired controls.
5. Calculate volumes for water, DNA, calcium solution, and 2X HBS phosphate solution. Total volume should be 500 µL for 6 cm dishes, 1 mL total for 10 cm dishes, or 2.5 mL for 15 cm dishes.
6. Add in the following order: sterile water, DNA, and calcium chloride (calcium chloride volume is 31 µL for 6 cm, 62 µL for 10 cm, or 155 µL for 15 cm dishes).
7. To the DNA-calcium tube, add 2X HBS dropwise (250 µL for 6 cm, 500 µL for 10 cm, or 1.25 µL for 15 cm dishes) while agitating the receiving tube by flicking or agitating on a vortexer set low enough that no splashing occurs.
8. After 5 to 20 minutes, apply transfection reagent dropwise onto labeled plates.

9. Incubate at 37°C, 5% $CO_2$for 8 hours to overnight with transfection reagent. Wash cells once with 1X PBS and apply fresh medium.
10. Allow cells to grow for two days, then lyse according to the procedure below. Alternatively, cells can be frozen at -80°C in parafilm-wrapped dishes after removing growth medium and a PBS rinse.

Preparation of FLAG antibody-bound beads

1. Resuspend immobilized protein A/G beads by inverting several times. Transfer 20 µL of the bead suspension per 6 cm plate (50 µL per 10 cm plate, 125 µL per 15 cm plate) into a single tube, which will be split into bead groups later.
2. Pellet beads by centrifugation and remove storage buffer. Bead centrifugation steps should be at 350 x g for 2 minutes. Wash beads three times with TN buffer. Wash by adding the buffer to resuspend the beads, then centrifuge and remove the buffer.
3. After washing, resuspend beads in TN buffer at half the original bead volume but at least 200 µL.
4. Bind anti-FLAG IgG antibody onto washed beads by adding 2.8 µL of anti-FLAG antibody per each 6 cm dish (7 µL per 10 cm dish, or 17.5 µL per 15 cm dish).
5. Rotate at room temperature for 3 to 4 hours. Proceed to cell lysis during incubation.
6. After the anti-FLAG antibody is bound to beads, spin and remove unbound antibody. Wash beads three times with 500 µL FLAG lysis buffer (protease inhibitors are not necessary in this buffer). When adding wash buffer the final time, resuspend beads with low retention tips, and divide equal volumes into separate tubes for each condition used in the transfection. Place tubes on ice.

HBV polymerase immunoprecipitation and purification

1. Wash cells once with 2 mL 1X PBS per 6 cm plate (4 mL per 10 cm plate, or 10 mL per 15 cm plate), being careful not to detach cells.
2. Wash cells once with 2 mL cold 1X PBX with protease inhibitors per 6 cm plate (4 mL per 10 cm plate, or 10mL per 15 cm plate). From here, keep samples RNase-free and on ice.
3. To each 6 cm plate, add 0.4 mL cold FLAG lysis buffer with inhibitors, (1 mL to 10 cm plates, or 2.5 mL to 15 cm plates). Free cells from the dish by scraping with a cell scraper or spraying with the buffer from a pipet tip.
4. Collect cells from the same treatment condition into a single chilled tube, and rotate for 20 minutes at 4°C.
5. Centrifuge lysate at 4°C for 10 minutes at maximum speed in a microcentrifuge ~18,000 x g. Supernatants represent the cytoplasmic fraction, which contains HBV polymerase.
6. Transfer supernatants to chilled tubes of prepared anti-FLAG antibody-bound beads. The pellet and some lysate (~100 µL) may be frozen together for troubleshooting.
7. Rotate antibody-bound beads and cell lysate supernatant at 4°C overnight to allow immunoprecipitation to occur.
8. The next day, spin beads at 4°C for 2 minutes at 350 x g. Remove unbound supernatant (supernatant may be saved for troubleshooting).
9. Wash beads five times with 500 µL FLAG wash buffer. When adding wash buffer the final time, resuspend beads with low retention tips, and divide equal volumes into separate chilled labeled tubes for each assay to be performed. Assays usually include a western blot (polymerase protein levels), northern blot (epsilon RNA levels), and the priming assay itself.
10. Store bead aliquots at -80°C, removing wash buffer before further experiments. Approximate bead volume is 10 µL per tube.

Downstream analysis of immunopurified HBV polymerase complexes

1. For western blotting or protein staining (Coomassie blue, silver stain):
    1. Keeping samples on ice throughout, remove the wash buffer, and add 20 µL 2X SDS lysis buffer to the ~10 µL beads.
    2. Boil for five minutes, vortex, boil another five minutes, and place samples back on ice. Spin briefly (~4,000 x g for one second) to collect condensate.
    3. Mix samples and load 20 µL on a 9% SDS-PAGE gel.
    4. If analyzing/troubleshooting each step, load lysate supernatant (30 µL lysate + 30 µL 2X SDS lysis buffer, load 30 µL) and load insoluble pellet (add 50 µL TE, vortex to re-suspend pellet, then add 200 µL 2X SDS lysis buffer, mix, boil, load 50 µL).
    5. At this point, the gel can be used for staining or transferred to a membrane for western blotting, with a 1:2000 dilution of anti-FLAG antibody and a 1:20,000 dilution of anti-mouse secondary antibody.
2. For analyzing epsilon RNA bound to immunoprecipitated HBV polymerase, please see the related protocol “Hepatitis B Virus Polymerase Epsilon RNA Binding Assays”.

Assay of *in vitro* protein priming and transferase activity

1. To a bead aliquot (approximately 10 µL beads and 10 µL residual buffer), add 19 µL of TMgNK (or TMnNK) priming buffer.
2. Add 1 µL of radiolabeled nucleotide triphosphates or 1 µL of a 100 µM solution of unlabeled nucleotide triphosphates. Shake at room temperature 4 hours, such as a vortexer set to 3.
3. Alternatively, 2-step incubations can be performed with nucleotide analogs (triphosphate form) or other RT inhibitors shaken for 2 hours, then dNTPs are added and samples are shaken for 2 more hours.
4. Pellet beads at 350 x g for 1 minute. Remove supernatant to radioactive waste. (Nucleotides may be removed with the Tdp2 cleavage assay at this point).
5. To visualize priming levels, wash each sample once with 500µl of TNK wash buffer. Remove the supernatant to radioactive waste. Add 20 µL 2X SDS lysis buffer. Boil 5 minutes, vortex, and boil another 5 minutes. Spin briefly to collect condensate (~4,000 x g for one second). Load 20 µL of each sample on a 9% SDS-PAGE gel.
6. Place gel onto filter paper and cover with plastic wrap. Dry for 3 hours on a vacuum gel drying apparatus at 75°C if available, otherwise proceed with imaging directly.
7. Expose the gel to film or phosphorimager screen.

Additional assay - Tdp2 cleavage

Materials and Reagents

- TNK buffer: 20mM Tris pH 7.0, 15mM NaCl, 10mM KCl. For 50mL, combine 1mL 1M Tris pH 7.0, 150µL 5M NaCl, 500µL 1M KCl, and 48.35mL nuclease-free water. Store at room temperature, keep RNase-free.
- TNK wash buffer: TNK buffer plus 28µM E-64, 1mM PMSF, 5µg/ml leupeptin, and 10mM β-mercaptoethanol. To 50mL TNK buffer, add 700µL 2mM E-64, 250µL 1mg/mL leupeptin, 250µL 200mM PMSF, and 39.06µL 12.8M β-mercaptoethanol. Prepare just before use, use on ice, do not store, keep RNase-free.
- Tyrosyl DNA phosphodiesterase 2 (Tdp2/TTRAP) (Abnova). Enzyme concentration was 0.23µg/µL but may vary by lot, suspended in 50mM Tris pH 8.0 and 10mM reduced glutathione. Store at -80°C.

- Tdp2 mock buffer: 50mM Tris pH 8.0 and 10mM reduced glutathione. Store at -20°C.
- 2X Tdp2 buffer: 50mM Tris pH 8, 260mM KCl, 2mM DTT, 20mM $MgCl_2$. For 1mL, combine 50µL 1M Tris pH 8.0, 260µL 1M KCl, 2µL 1M DTT, 20µL 1M $MgCl_2$, and 668µL nuclease-free water. Make fresh, do not store.
- 1X Tdp2 wash buffer: 1X Tdp2 buffer, 28µM E-64, 1mM PMSF, 5µg/mL leupeptin. For 1mL, combine 500µL 2X Tdp2 buffer, 476µL nuclease-free water, 14µL 2mM E-64, 5µL 200mM PMSF, and 5µL 1mg/mL leupeptin. Make fresh, use on ice, do not store.
- 1X Tdp2 reaction buffer: 1X Tdp2 buffer, 1X Complete EDTA-free protease cocktail inhibitor, with Tdp2 enzyme or mock buffer. For 100µL, combine 50µL 2X Tdp2 buffer, 4µL 25X Complete EDTA-free protease inhibitor cocktail, 26µL nuclease-free water and 20µL Tdp2 enzyme (or Tdp2 mock buffer). Make fresh, do not store.
- Gel loading buffer II (Invitrogen) or other formamide loading dye.

Experimental procedures

1. Perform the priming assay as described above, with sufficient aliquots that each condition can be mock treated and Tdp2 treated.
2. Wash the priming reaction three times with 500µl TNK wash buffer.
3. Wash twice with 100 µL 1X Tdp2 wash buffer.
4. Add 10 µL of Tdp2 reaction buffer (or mock buffer) to each sample.
5. Incubate at 37°C for 1 hour with shaking.
6. After incubation, spin for 1 minute at 350 x g.
7. Separate supernatants (released nucleotides) into new tubes and save bead pellets (protein). Samples may be stored at -80°C until testing.
8. For testing supernatants (released nucleotides): Add 5µl formamide gel loading buffer to a 5µl aliquot. Heat to 95°C for 5 minutes by boiling or thermal cycler. Load all 10 µL of samples while still hot onto a 20% acrylamide 8 M urea-PAGE gel in 1X TBE. Gels should be pre-run for 30 minutes prior to loading. Run until the bromophenol blue dye front reaches 2/3 the length of the gel. Expose gel to film or phosphorimager screen.
9. For testing bead pellets (polymerase protein): Add 20µl of 2X SDS lysis buffer, boil 5 minutes, vortex, and boil another 5 minutes. Spin briefly to collect condensate (~4,000 x g for one second), and load 10 µL of each sample on a 9% SDS-PAGE gel. Expose gel to film or phosphorimager screen.

References

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