# Hepatitis B Virus Polymerase Epsilon RNA Binding Assays

HBV Biochemical Assays

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## Introduction

- HBV polymerase activity is assayed by measuring its ability to bind viral RNA at a secondary-structure motif, the epsilon (e) stem-loop structure.
- HBV polymerase is expressed in cell culture to yield its binding-active form and RNA may be supplied in culture or in vitro [1].
- This assay provides a method for evaluating levels of RNA binding by the HBV polymerase, and may include inhibitors or mutant polymerase proteins.
- This protocol was originally published in Hepatitis B Virus: Methods and Protocols (see reference 1).

## 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 and 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 are seen on RNA gels.
- 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 and 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.
- 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.

### *In vitro* and *in vivo* RNA binding

- MAXIscript SP6 kit (Ambion)
- [α-$^{32}$P] UTP (10mCi/ml, 3,000Ci/mmol) (PerkinElmer)

- Quick Spin columns for radiolabeled RNA purification (Sephadex G-25, fine) (Roche)

- RIPA buffer: 50mM Tris pH 7.0, 150mM NaCl, 1mM EDTA pH 8.0, 0.05% NP40. For 50mL,

combine 2.5mL 1M Tris pH 7.0, 1.5mL 5M NaCl, 100µL 0.5M EDTA pH 8.0, 250µL 10% NP-40, and 45.65mL DEPC-treated water. Store at room temperature, keep RNase-free.
- RIPA buffer with inhibitors: RIPA buffer plus 2mM DTT, 1mM PMSF, 1X Complete protease inhibitor cocktail, 1U/µL RNase inhibitor. To 1mL RIPA buffer add 2µL 1M DTT, 5µL 200mM PMSF, 40µL 25X Complete protease inhibitor cocktail, and 25µL 40U/ml RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.
- RIPA wash buffer: RIPA buffer plus 2mM DTT, 1mM PMSF, 28µM E-64, 5µg/mL leupeptin, and 10U/mL RNase inhibitor. To 1mL RIPA buffer add 2µL 1M DTT, 5µL 200mM PMSF, 14µL 2mM E-64, 5µL 1mg/ml leupeptin, and 0.25µL 40U/ml RNase inhibitor. Prepare just before use, use on ice, do not store, keep RNase-free.
- Gel loading buffer II (Invitrogen) or other formamide loading dye
- 10X TBE: 0.89M Tris, 0.89M boric acid, 20mM EDTA. For 1L, add 108g Tris base, 55g boric acid, and 40mL 0.5M EDTA pH 8.0. Fill to 1L with water.
- 1X TBE: 89mM Tris, 89mM boric acid, 2mM EDTA. For 1L, add 100mL 10X TBE and 900mL water.
- ULTRAhyb solution (Ambion)
- 20X SSC pH 7.0: 3M NaCl, 0.3M sodium citrate. For 1L, add 175.3g NaCl and 88.2g sodium citrate. Adjust pH and fill to 1L with water.
- Low stringency wash buffer: 2X SSC, 0.1% SDS. For 500mL, add 50mL 20X SSC, 2.5mL 20% SDS, and 447.5mL water.
- High stringency wash buffer: 0.1X SSC, 0.1% SDS. For 500mL, add 2.5mL 20X SSC, 2.5mL 20% SDS, and 495mL water.

## 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 $10^6$cells 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.

### In vitro transcription of $^{32}$P-radiolabeled ε RNA and in vitro RNA binding assay

1. Radiolabeled ε RNA is transcribed using SP6 MAXIscript kit components. The template used is a DNA oligonucleotide coding for the ε RNA sequence annealed to an SP6 promoter. Other

constructs such as plasmid-based expression constructs should work as well. For a 20 µL reaction add 4 µL of DEPC-treated water; 1 µL each of 10 mM ATP, CTP, and GTP; 2 µL of 0.5 mM UTP (diluted from 10 mM stock 1:20 with DEPC-treated water); 1 µL of 1 µg/µL template DNA; 3 µL of SP6 enzyme; and 5 µL of [α-$^{32}$P] UTP. Controls deficient in binding may be included.
2. Incubate at 37°C for 5 hours total. Halfway through the incubation, spin briefly (~4,000 x g for one second) to collect condensate.
3. After incubation, add 1 µL DNase and incubate at 37°C for 30 minutes.
4. Inactivate the DNase by incubating at 70°C for 15 minutes.
5. Labeled samples can be stored at -80°C. If desired, transcribed labeled RNA can be visually verified on a 1.5% agarose gel in 1X TAE under RNase-free conditions. Use an appropriate RNA ladder to evaluate size.
6. Combine aliquots of immunoprecipitated HBV polymerase-bound beads with 0.5 µg in vitro-transcribed $^{32}$P-labeled ε RNA (approximately 1 to 3 µL) and 20 µL RIPA buffer with inhibitors. Inhibitory compounds to be tested should be added prior to addition of $^{32}$P-labeled ε RNA.
7. Incubate for three hours at room temperature with shaking to allow HBV polymerase to bind the ε RNA. Shake on a vortexer set to 3 or agitate by hand every 5-10 minutes to keep beads in suspension.
8. Pellet the beads and remove and save the supernatant at -80°C, which contains any unbound components.
9. Wash the beads five times with 200 µL RIPA wash buffer. Samples may be stored at -80°C for later processing. The first and fifth washes may be saved to troubleshoot binding levels.

- After removing the final wash buffer, elute bound materials by adding 60 µL of 2X SDS lysis buffer. As controls, washes, input RNA (at 1:100 dilution), and unbound RNA (at 1:100) may be included; prepared by adding 30 µL of sample to 30 µL of 2X SDS lysis buffer. Boil all samples for 5 minutes, vortex, and boil 5 more minutes. Load 30 µL of each sample onto a 15% SDS-PAGE gel.
- Cut the gel horizontally at approximately 50 kDa. The top portion of the gel which contains the HBV polymerase protein can be visualized by western blot using anti-FLAG antibody. The bottom portion of the gel containing the $^{32}$P-labeled ε RNA can be dried and directly exposed to film to detect the labeled ε RNA, which was bound to (and then disassociated from) the purified RT.

**In vivo RNA binding assay**

1. An ε RNA-specific radiolabeled probe will be used in a northern blot. This probe can be made from the same pCMV-HE plasmid used to transfect, a 1,389 bp fragment purified from an NcoI digest under RNase-free conditions. This purified fragment is used in an SP6 in vitro transcription using radiolabeled [α-$^{32}$P] UTP (see step 1 above from “In vitro transcription of $^{32}$P-radiolabeled ε RNA and in vitro RNA binding assay” for an in vitro transcription assay).
2. Using immunoprecipitated HBV polymerase (purified from cells that were transfected with both ε RNA-expressing and HBV polymerase-expressing constructs), perform a Trizol extraction of 20 µL lysate (see step 6 above from “HBV polymerase immunoprecipitation and purification”) and a ~10 µL bead aliquot. This will compare the amount of ε RNA expressed to the amount of ε RNA bound to HBV polymerase.
3. Resuspend the extracted RNA samples in a final volume of 20 µL DEPC-treated water. Add 20 µL of formamide gel loading buffer to each sample and heat to 95°C for 5 minutes by boiling or in a thermal cycler. Load the hot samples onto a 6% acrylamide 8 M urea-PAGE gel in TBE. Run the gel at 300V until Bromophenol dye front comes off (approximate time is 2hrs).
4. Rinse gel 20 minutes in 1X TBE, rinse a nylon membrane in water for 5 minutes then in 1X TBE for 5 minutes.
5. For electrophoretic transfer, run below 300 mA (6 to 7 V) for 1 hour.

6. While the transfer is running, preheat ULTRAhyb solution to 68°C to dissolve precipitated material.
7. Crosslink the RNA to the membrane by exposing to a UV transilluminator.
8. Prehybridize at least 30 minutes at 68°C. Use 10mL ULTRAhyb buffer per 100 $cm^2$of membrane.

9. Add prepared ε RNA-specific radiolabeled probe to the ULTRAhyb and allow the probe to hybridize 2 hours to overnight.

1. Make sure wash buffers have no precipitate, if so, heat to 37°C. Pre-heat high stringency wash to 68°C.
2. Wash blot twice in room temperature low stringency wash solution (20 mL per 100 $cm^2$blot) with agitation for 5 minutes, dispose of washes in $^{32}P$ waste.
3. Wash twice for 15 minute at 68°C with high stringency wash solution with agitation. Dispose of washes in $^{32}P$ waste.
4. Seal radiolabeled blot in plastic wrap and expose to film or a phosphorimager screen.

## References

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