HBV Ribonuclease H assay resolved by denaturing PAGE

HBV Biochemical Assays, HBV Nucleic Acid Analyses

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Introduction

- This assay characterizes the products of HBV RNase H activity on a DNA:RNA heteroduplex.
- Products are resolved by denaturing PAGE to assess specificity of cleavage and effects of inhibitory compounds on the enzyme

Materials and Reagents

- HBV Ribonuclease H: This protocol requires purified recombinant HBV RNaseH. Purification of the enzyme is a lengthy procedure and the protocol is undergoing refinement. Please contact John Tavis (john.tavis@health.slu.edu) for the current protocol and RNaseH expression plasmid.]
- Substrate: Any RNA of 200-300 nt annealed to a complementary oligo that binds ~30% from one end will work. We use an in vitro transcribed RNA called “DRF+” together with complementary and non-complementary DNA oligos. These sequences are: DRF+ = GAACA AAAGCUUGCAUGCCUGCAGGUCGACUCUAGAGGAUCCCCACUUUGUCCCGAGCAAAUAUAAUCC UGCUGACGGCCCAUCCAGGCACAGACCGCCUGAUUGGACGGCUUUUCCAUACACCCCUCUCUCG AAAGCAAUAUAUAUUCCACAUAGGCUAUGUGGAACUUAAGAAUUACACCCCUCUCCUUCGGAGCU GCUUGCCAAGGUAUCUUUACGUCUACAUUGCUGUUGUCGUGUGUGACUGUGGGUACCGAGCUC GAAUU (1 ug/uL) Complementary DNA oligo = 5’ GTCCGTACGTTCGAAAACAAG (1 ug/uL) Non-complementary DNA oligo (negative control) = 5’ CAGGCATGCAAGCTTTTGTTC (1 ug/uL)
- **Reagents**
    - -Nuclease free H2O, RNaseOUT (Thermo Fisher)
    - -10x RNase H Buffer (1 M NaCl, 500 mM HEPES pH 8.0)
    - -50 mM MgCl2, DMSO
    - -SYBR Gold (Thermo Fisher)
    - -9% sequencing acrylamide (8M Urea, 1x TBE, 6% acrylamide [19:1 acrylamide: bis-acrylamide])
    - -10% Ammonium Persulfate
    - -Tetramethylethylenediamine
    - -Sequencing loading buffer (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol)

Experimental Procedures

RNaseH reactions: Per reaction in an RNaseH free tube or well of a plate:

- -Combine 3.5 uL nuclease free H2O, 2 uL 10x RNaseH Buffer, and 0.5 uL RNaseOUT

- -Then add to each reaction (in the following order): 3 uL DNA oligo, 2 uL test compound (diluted to 10x desired concentration) or water if an inhibitor is not being tested, 6 uL HBV RNaseH (adjust concentration as needed to balance activity), and 1 uL substrate RNA.
- - Incubate reactions for 90 minutes at 37oC
- - Stop reactions by adding 80 uL sequencing loading buffer

Electrophoresis:

- - Pour a denaturing 9% Urea PAGE/TBE gel.
- - Boil samples for 5 minutes.
- -Load 50 uL sample per lane (remaining sample can be stored at -80C).
- - Run the gel until the bromophenol blue dye front is near the bottom. Rinse gel in water to remove urea 2-3x for 15 minutes.
- -Stain gel with SYBR gold: Rock gel at RT in 1:1000 dilution of SYBR Gold in 1x TBE for 20 minutes
- - Image on a high-sensitivity imaging system such as a GE Typhoon or equivalent instrument.

Interpretation: RNaseH activity will be evident as cleavage of the RNA at the site where the DNA oligonucleotide bound. This results in 3 bands: uncleaved substrate, the larger product, and a smaller product **Notes**:

- - Include a reaction lacking a DNA oligo and one containing the incorrect polarity DNA oligo as specificity controls. No RNA:DNA heteroduplex will form under those conditions, so any RNA degradation under those conditions will be background.
- - Adjust the molar ratio of DNA to RNA as needed to get adequate cleavage. Most RNAs have substantial secondary structure, and a high ratio of DNA:RNA is needed for the DNA to bind to RNA sites in strong secondary structures.

References

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