HBV RNA detection in liver tissues by in situ hybridization

HBV Nucleic Acid Analyses

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Introduction

- HBV RNA detection at the cellular level in the liver
- Multiplex detection of two RNA targets in tissue sections derived from formalin fixed and paraffin embedded tissue as well as frozen tissue
- Chromogenic signal detection also amenable to fluorescence detection and analysis
- Probe set combination and branched DNA signal amplification enable highly sensitive RNA detection and a very high signal to noise ratio.
- Commercial kit reagents facilitating reproducible assay performance and data comparison in the field. No specific instrumentation required.
- This protocol was originally published in Hepatitis B Virus: Methods and Protocols (see reference 1).

Materials and Reagents

A. Equipment, buffers and disposables

1. ThermoBrite StatSpin oven (Abbott Laboratories, IL, USA). ***Note:*** *Alternatively, a humidized cell culture incubator or other humidified and temperature controlled oven can be used*
2. Temperature controlled water bath.
3. Hot plate stirrer (max setting ≥ 200°C).
4. Glass Coplin Jar.
5. 1,000 ml Pyrex glass beaker.
6. Vertical metal slide rack fitting into the Pyrex glass beaker.
7. Plastic slide box.
8. StainTray slide staining system.
9. Tap water.
10. Water (Bi-distilled).
11. 10 x PBS (molecular biology grade, nuclease free).
12. 1 x PBS (molecular biology grade, nuclease free).
13. Fixation buffer: 4% Formaldehyde in 1 x PBS, freshly prepared from 37% Formaldehyde; store at 4°C.
14. Ethanol 100%.
15. Ethanol 50%, 75% (200 ml each, V/V in water), prepared from 100% ethanol.
16. Xylene
17. Gill’s Hematoxylin 1.
18. Water based, DAPI containing mounting media.
19. Hydrophobic barrier pen (Vector Laboratories, Burlingame, CA, USA).
20. Superfrost Plus Gold glass slides (Thermo-Scientific, Waltham, MA, USA).
21. 24x50 mm nr.1 (0,13 - 0,16 mm) coverslip glass (MEDITE GmbH, Burgdorf, Germany).

22. Transparent nail polish.
23. ViewRNA™Tissue Assay Core Kit (ref. 19931, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA).
24. ViewRNA™ Tissue Assay Blue Module (ref. 19932, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA).
25. Appropriate type 1 and type 6 probe sets (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA).
26. Pre-warmed (90°-95°C) 1 x pre-treatment solution prepared according to the manufacturer's instructions (ViewRNA™, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA).
27. 1 x working protease solution prepared according to the manufacturer's instructions (ViewRNA™, Invitrogen, Thermo Fisher Scientific, Waltham, MA USA).
28. Pre-warmed (40°C) Probe Set Diluent QT (ViewRNA™ Kit component).
29. Pre-warmed (40°C) PreAmplifier Mix QT (ViewRNA™ Kit component).
30. Pre-warmed (40°C) Amplifier Mix QT (ViewRNA™ Kit component).
31. Pre-warmed (40°C) Label Probe Diluent QF (ViewRNA™ Kit component).

B. Preparing and processing frozen tissue embedded in OCT

1. Benchtop liquid nitrogen container (Thermo-Scientific).
2. Plastic beaker fitting inside the liquid nitrogen container.
3. Liquid nitrogen.
4. Methylbutane (106056; Merck).
5. Optimal cutting temperature compound (OCT; Sakura Tissue-Tek, Sakura Finetek, Torrance, CA, USA).
6. Disposable plastic molds for tissue embedding (Sakura Tissue-Tek, Sakura Finetek).
7. Dry-ice.
8. Cryostat (Leica, Wetzlar, Germany).
9. Carbon blades C35 for cryosectioning (FEATHER Safety Razor Co., Ltd., Osaka, Japan).

C. Processing formalin fixed and paraffin embedded tissue

1. Formalin fixed and paraffin embedded liver tissue.
2. Ice or cooling plate.
3. Microtome
4. Stainless steel blades S35 for sectioning (FEATHER Safety Razor Co., Ltd., Osaka, Japan).
5. General purpose lab oven set at 50°C.

Experimental Procedures

The following protocol outlines the protocol for duplex in situ hybridization (ISH) for localization of hepatitis B virus RNA and any other (eg. human albumin) mRNA in liver tissue sections. This protocol has been tested and verified for human and mouse specimens.

The method is adapted from the ViewRNA ISH Tissue Assay system (Invitrogen, Thermo Fisher Scientific, Waltham, MA USA). Modifications were introduced to the manufacturer's instructions to achieve optimal viral RNA visualization while maintaining sensitivity and specificity of host mRNA detection.

The ISH method is suitable for both formalin fixed, paraffin embedded (FFPE) and frozen tissue (OCT embedded). However, as we have previously described [1-3], frozen tissue is superior for quantitative detection of RNA. Thus, the protocol below also includes a detailed description of collection, embedding, freezing and cryosectioning of frozen liver tissue samples.

FFPE tissue is typically processed in ny standard procedures in pathology departments and other labs. We therefore omit description of tissue harvesting and processing, and focus on describing optimal sectioning and ISH procedures using FFPE specimens.

Probe Set Design:The extremely high signal to noise ratio that is a prerequisite for detection of low level viral and/or cellular RNAs is achieved by simultaneous binding of multiple (≥ 20) adjacent probe set pairs to the target RNA. The target recognition size of each probe is around 20 nucleotides in length. Thus, optimal target recognition requires a perfect match of each probe set to the target. Unfortunately, neither the HCV nor HBV genome contains long enough regions that are sufficiently conserved between or within viral genotypes suitable for probe set design. Therefore, as we have shown previously [1-3], it is well advised to design isolate (i.e. patient)-specific probe sets and use frozen tissue specimens whenever possible to achieve optimal assay performance.

D. Tissue collection and ISH procedure for frozen tissue specimens

1. Prepare a benchtop liquid nitrogen container half-way filled with liquid nitrogen.
2. Pour 250 ml of methylbutane in a plastic beaker that closely fits into the liquid nitrogen tank.
3. Chill the methylbutane by floating the beaker on the liquid nitrogen.
4. Wash freshly harvested tissue twice in sterile saline or 1 x PBS to remove excess blood. ***Note:*** *Minimize the time from tissue harvest to freezing (steps D.4-6) to prevent RNA degradation.*
5. Cut 1 cm long sections from a needle liver biopsy cylinder or blocks of 0.5 cm diameter from resection tissue, place them on the bottom of disposable plastic molds, immediately cover the tissue and completely fill the mold with OCT avoiding any bubble formation.
6. Dip the filled mold into the chilled methylbutane (use long forceps) and wait until the OCT turns completely white. Transport the molds containing the frozen OCT blocks on dry ice and store samples at -80°C.
7. Set the cryostat chamber to -17°C, the sample holder to -15°C and let the temperature equilibrate for 30 minutes before sectioning. If dual temperature control is not available, set the cryostat chamber to -15° C ± 2°C.
8. Set the blade holder inclination in the range of 2° to 10°.
9. Use C35 disposable blades for cryosectioning.
10. Set up the cryostat for 10µm sections.
11. Carefully clean the blade holder and cryostat chamber with 80% ethanol and let them air-dry.
12. Remove the OCT block from the disposable plastic mold inside the cryostat chamber and keep the mold for re-embedding in step D.18. ***Note:*** *Perform steps D.12 through D.17 inside the cryostat chamber to avoid any exposure of the OCT block to room temperature.*
13. Add a small amount of fresh OCT on the cryostat's removable specimen holder and put the frozen OCT block on it with the tissue facing up. Distribute the fresh OCT between frozen OCT block and specimen holder by carefully pressing on the frozen OCT block. Transfer the holder and the block to the cryostat's freezing station and wait until the fresh OCT turns completely white.
14. Lock the specimen holder, fitted with the tissue containing OCT block, into the cryostat specimen head and wait 10 minutes to allow the sample to reach the correct cutting temperature.
15. Start sectioning.
16. Mount the sections onto Superfrost Plus Gold glass slides.
17. Immediately transfer slides into a pre-chilled slide storage box. Keep the box in the cryostat chamber during sectioning. When finished, transfer the slide boxes on dry ice and store them at -80°C until use. ***Note:*** *Use slides within two weeks. For longer-term storage, add some dried silica gel crystals into the slide storage boxes as desiccant.*
18. After sectioning, add a few drops of fresh OCT into the corresponding mold, re-embed the sectioned OCT block in the mold and place it on the cryostat freezing station. After a few

minutes, remove the OCT block from the metal specimen holder. Store re-embedded OCT blocks at -80°C. ***Note:*** *Re-embedding improves the quality of tissue preservation.*
19. Clean the cryostat with 80% ethanol and run a long UV-light decontamination cycle if possible. ***Note:*** *Discard used blades; do not reuse them to avoid sub-optimal blade performance and cross-contamination.*

E. Fixation, Pretreatment and Hybridization

1. Cool down 80 ml of fixation buffer to 4°C in a Coplin Jar (≥ 1 hour).
2. Directly submerge the frozen slides in the chilled fixation buffer and incubate over night at 4ºC (16-18 hours).
3. Turn on the ThermoBrite and let equilibrate to 37°C.
4. Insert two water soaked humidifier strips into the ThermoBrite lid.
5. Heat 400 ml pre-treatment solution on a hot plate stirrer to between 85°C-95°C.
6. Pre-warm the following reagents in a water bath set to 40°C:

- 40 ml of 1 x PBS buffer
- Probe Set Diluent QT
- PreAmplifier Mix QT
- Amplifier Mix QT
- Label Probe Diluent QF

7. Thaw probe sets on ice.
8. Spin down Label Probe 6-AP and Label Probe 1-AP and keep on ice.
9. Bring Naphthol Buffer, AP Enhancer solution and Blue Buffer to room temperature. Keep Fast Red Tablets and Fast Blue reagent on ice.
10. Equilibrate fixation buffer containing slides to room temperature (10-15 minutes), decant fixation buffer and keep it for later.
11. Wash the slides twice for 1-5 minutes in 1 x PBS (shake vigorously).
12. Sequentially soak slides for 10 minutes in 50%, 75% and 100% ethanol.
13. Bake the slides in the open ThermoBrite oven for 5 minutes at 37°C.
14. Draw hydrophobic barrier around tissue section with the hydrophobic barrier pen.
15. Bake slides for an additional 5 minutes at 37°C.
16. Remove slides from the ThermoBrite oven and let them cool to room temperature.
17. Start HYB program on ThermoBrite oven (i.e. constant 40°C) and close lid for good humidification.
18. Place slides in a vertical metal rack and submerge them in the pre-heated (85°-95°C) pre-treatment solution for 1 minute.
19. Transfer slides to a coplin jar and briefly wash twice in 1 x PBS.
20. Prepare working protease solution by diluting Protease QF stock solution 1:100 in pre-warmed 1 x PBS.
21. Remove the slides from PBS, tap them to remove the excess fluid, place them on the Thermobrite oven, add 400 µl working Protease solution and incubate for 10 minutes at 40°C. ***Note:*** *Although the manufacturer's instructions typically suggest to add 400 µl of solution (protease, hybridization reagents etc.) to each section on a slide, in our experience 200-300 µl are sufficient without affecting the assay performance.*
22. Decant protease solution and wash slides once with water, followed by two washes in 1 x PBS in a coplin jar.
23. Transfer slides into fixation buffer and incubate for 3 minutes at room temperature. ***Note:*** *Incubation in fixation buffer for more than 3 minutes (step E.23) may result in reduced hybridization efficiency.*
24. Wash slides twice with 1 x PBS.
25. Prepare working hybridization mix by diluting probe sets 1:40 in pre-warmed (40°C) Probe Set Diluent QT per the manufacturer's instructions. ***Note:*** *Steps E.25 through E.27 and F.1 through F.19 are per the manufacturer's*

26. Hybridize sections with 400µl working hybridization mix for 2.5 hours (increase to 3 hours for very low abundance RNA detection) at 40°C in the ThermoBrite oven.
27. Decant hybridization solution, transfer slides in a vertical rack in a coplin jar containing 1 x Wash Buffer, wash slides 3 times (2 minutes per wash) under vigorous agitation.

F. Two-Plex target detection

1. Incubate sections with 400µl of PreAmplifier Mix QT for 25 minutes (increase to 40 minutes for very low abundance RNA detection) at 40°C in the ThermoBrite oven.
2. Decant PreAmplifier Mix QT solution, transfer slides in a vertical rack in a coplin jar and wash as described in E.27.
3. Incubate sections with 400µl of Amplifier Mix QT for 15 minutes (increase to 40 minutes for very low abundance RNA detection) at 40°C in the ThermoBrite oven.
4. Decant Amplifier Mix QT solution, transfer slides in a vertical rack in a coplin jar and wash as described in E.27.
5. Prepare working label probe mix by diluting Label Probe 6-AP, 1:1,000 in pre-warmed (40°C) Label Probe Diluent QF per the manufacturer's instructions. ***Note:*** *For single target detection, incubate sections with only the Label Probe AP specific for the probe set used and only perform the corresponding target development (i.e. steps F.5 through F.10 for type 6 probe sets or steps F.13 through F.19 for type 1 probe sets).*
6. Incubate sections with 400µl of label probe mix for 15 minutes (increase to 40 minutes for very low abundance RNA detection) at 40°C in the ThermoBrite oven.
7. Decant label probe mix, transfer slides in a vertical rack in a coplin jar and wash as described in E.27.
8. Prepare Fast Blue Substrate by adding 105 µl Blue Reagent 1 to 5ml Blue Buffer and vortex. Then add 105µl Blue Reagent 2 and vortex. Finally, add 105 µl Blue Reagent 3 and vortex again. **Note:** *TheFast Blue Substrate mix decays quickly. Prepare it not more than 5-10 minutes before usage for optimal results.*
9. Incubate sections with 400µl Fast Blue Substrate mix for 30 minutes at room temperature in a horizontal slide holder (e.g. StainTray slide staining system).
10. Decant Fast Blue Substrate mix, transfer slides in a vertical rack in a coplin jar and wash as described in E.27.
11. Incubate sections with 400µl of AP Stop Buffer for 30 minutes at room temperature in a horizontal slide holder. ***Note:*** *Complete quenching of alkaline phosphatase at this point is crucial to avoid additional reaction of Label Probe 6-AP with the Fast Red Substrate in step F.18.*
12. Decant AP Stop Buffer, transfer slides in a vertical rack in a coplin jar and wash as described in E.27.
13. Prepare type 1 working label probe mix by diluting Label Probe 1-AP 1:1000 in pre-warmed (40°C) Label Probe Diluent QF per the manufacturer's instructions.
14. Incubate sections with 400µl of label probe mix for 15 minutes (increase to 40 minutes for very low abundance RNA detection) at 40°C in the ThermoBrite oven.
15. Decant label probe mix, transfer slides in a vertical rack in a coplin jar and wash as described in E.27.
16. Incubate sections with 400µl of AP Enhancer Solution for 5 minutes at room temperature in a horizontal slide holder.
17. Prepare Fast Red Substrate by dissolving one Fast Red tablet in 5 ml of Naphthol Buffer. ***Note:*** *The Fast Red Substrate mix decays quickly. Prepare it not more than 5-10 minutes before usage for optimal results.*
18. Decant the AP Enhancer Solution and incubate sections with 400µl of Fast Red Substrate mix for 30 minutes at 40°C in the humidified ThermoBrite oven.
19. Decant Fast Red Substrate mix, transfer slides in a vertical rack in a coplin jar containing 1 x PBS solution and wash slides 2 times for 1-2 minutes.

G. Counterstaining and slide mounting

1. Remove slides from PBS and incubate them in fixation buffer for 5 minutes at room temperature.
2. Briefly wash slides two times in 1 x PBS.
3. Add 500 µl Gill's hematoxylin to each slide and incubate for 5 minutes at room temperature.
4. Wash slides with water in a coplin jar.
5. Incubate the slides with running tap water for 1 minute to reveal the hematoxylin.
6. Wash slides twice with water.
7. Mount slides with water based mounting media containing DAPI and cover with Nr. 1 glass coverslip.
8. Drain excess mounting media and let the slide air-dry for 15 minutes.
9. Seal the coverslip with transparent nail polish and store at 4ºC. ***Note:*** *For best results, acquire images within a few days of performing the ISH assay.*
10. Acquire images using a brightfield and/or fluorescence microscope. ***Note:*** *The View RNA ISH system makes use of alkaline phosphatase to transform fast red and fast blue substrates into red and blue chromogenic precipitates, respectively. Accordingly, visual observation of red and blue signals using bright field microscopy represents the primary readout of the View RNA ISH assay. However, both chromogenic precipitates can also be visualized by fluorescence, but acquiring fluorescence images should always be guided by bright field analysis (see ViewRNA Kit manual). However, the fluorescence excitation and emission spectra of the chromogenic precipitates differ significantly from those of standard fluorophores used for fluorescence microscopy. Thus, special attention has to be given to selection of the best combination of probe set type and target RNA as well as fluorescence signal acquisition as we have described in detail elsewhere[1].*
11. Image analysis with for example CellProfiler[4] is performed as described [1].

H. Processing of formalin fixed, paraffin embedded liver tissue This section describes the tissue processing and ISH steps specific for FFPE tissue specimens.

1. Cool FFPE blocks to -20°C for at least 2 hours before sectioning. Alternatively cool down the blocks to -5°C on a cooling plate for at least 2 hours.
2. Set the blade inclination in the range of 5° to 10° (e.g. on the HM340 microtome we use 6°).
3. Use S35 disposable blades for sectioning (do not use C35 blades). ***Note:*** *Discard used blades at the end of each cutting session; do not reuse them to avoid sub-optimal blade performance and cross-contamination.*
4. Set microtome for 5µm sections.
5. Cut sections and transfer them to a water bath pre-warmed to 40°C. ***Note:*** *To maintain optimal cutting performance, don't cut more than 4 sections before re-cooling the block.*
6. Keep sections floating on water for a few minutes to allow for complete tissue/paraffin unfolding.
7. Transfer sections onto Superfrost Plus Gold glass slides.
8. Place slides vertically in a slide holder and let drain off excess water.
9. Transfer the slide holder to a lab oven pre-warmed to 50°C.
10. Incubate slides at 50°C for 2 hours.
11. Transfer slides into a slide storage box and store at -20°C until use. ***Note:*** *Do not store slides longer than two weeks.*
12. Pre-warm the ThermoBrite oven to 80°C without humidifier strips.
13. Heat 400 ml pre-treatment solution on a hot plate stirrer to between 85°C-95°C.
14. Pre-warm the following reagents in a water bath set to 40°C:

- 40 ml 1 x PBS
- Probe Set Diluent QT
- PreAmplifier Mix QT
- Amplifier Mix QT
- Label Probe Diluent QF

15. Thaw probe sets on ice.
16. Spin down Label Probe 6-AP and Label Probe 1-AP and store on ice.
17. Bring Naphthol Buffer, AP Enhancer solution and Blue Buffer to room temperature.
18. Keep Fast Red and Fast Blue reagents on ice.

I. Pretreatment and Hybridization

1. Warm up slides to 80°C in the ThermoBrite oven for 15 minutes.
2. Quickly transfer slides in a coplin jar containing xylene for paraffin removal. Incubate for 15 min at room temperature. Transfer slides into fresh xylene and incubate a second time for 30 min at room temperature.
3. Set the ThermoBrite oven to 37°C during the time of xylene treatment.
4. Wash slides twice for 2 minutes in 100% ethanol.
5. Bake slides in the ThermoBrite oven for 5 minutes at 37°C.
6. Draw hydrophobic barrier around tissue sections.
7. Bake slides in the ThermoBrite oven for 5 minutes at 37°C.
8. Transfer slides in a vertical metal rack and submerge slides in 85°-95°C Pre-Treatment solution and incubate for 10-20 minute. ***Note:*** *Optimal time of pretreatment depends on the particular tissue fixation procedure used and has to be determined experimentally.* During pre-treatment: insert two water soaked humidifier strips into the ThermoBrite lid and start the HYB program (i.e. constant 40°C).
9. Quickly transfer slides into 1 x PBS in a coplin jar and briefly wash twice with 1 x PBS.
10. Prepare working protease solution by diluting protease QF 1:100 in pre-warmed (40°C) 1 x PBS.
11. Add 400 µl working Protease QF to tissue sections and incubate for 10-20 minutes at 40°C in the ThermoBrite oven. (*see* **Note 21**). ***Note:*** *Optimal time for protease treatment depends on the particular tissue fixation procedure used and has to be determined experimentally.*
12. Wash slides once with water, then twice with 1 x PBS in a coplin jar.
13. Transfer slides to a coplin jar containing fixation buffer and incubate for 3 minutes at room temperature.
14. Wash slides twice with 1 x PBS in a coplin jar.
15. Prepare working hybridization mix by diluting the probe sets 1:40 in pre-warmed (40°C) Probe Set Diluent QT.
16. Add 400µl working hybridization mix to tissue sections and incubate for 2.5 hours (increase to 3 hours for very low abundance RNA detection) at 40°C in the humidified ThermoBrite oven.
17. Decant hybridization solution, transfer slides in a coplin jar containing 1 x Wash Buffer solution and wash slides 3 times (2 minutes per wash) with vigorous agitation.

J. Target detection, counterstaining and slide mounting

1. For pre-amplification, amplification and target detection follow the steps outlined in F.3. For counterstaining and slide mounting follow steps outlined in G.4.

References

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