Immunofluorescent Staining for the Hepatitis B Core Antigen in frozen Liver Sections

Animal Models, HBV Antigen Analyses, HBV Biochemical Assays

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Introduction

- The HBV core antigen (HBcAg) is the structural protein of the viral nucleocapsids but is also recruited to the nuclear HBV persistence form. Thus, HBcAg can be detected in the cytoplasm and nucleus of infected hepatocytes.
- Here, we report a sensitive immunofluorescence staining method to detect HBcAg in cryopreserved liver sections from HBV-infected human liver chimeric mice (1, 2). The method combines conventional immunofluorescence staining with the Tyramide Signal Amplification (TSA) system, and has previously been described as a chapter in the book *Methods in Molecular Biology: Hepatitis B Virus* (3).
- The TSA technology can be included in a standard staining protocol involving a primary and a secondary antibody coupled with horseradish peroxidase. To amplify the signal, the peroxidase activity converts fluorescein-coupled tyramides into highly reactive, short-lived tyramide radicals that bind to electron-rich regions of adjacent proteins (mostly tyrosine residues) which generates a stable and strong labeling (4, 5).
- This method has previously been described in greater detail as a chapter in the book *Methods in Molecular Biology: Hepatitis B Virus* (see reference 3).

Materials and Reagents

Preparation of cryo-preserved liver sections:

- Isopentane on dry ice
- Cryotome
- Embedding media, i.e. Tissue-Tek O.T.C. compound, Sakura, catalog number 4583
- microtome blades, i.e. Feather C35 type
- Superfrost microscope slides (75 x 25 mm)

HBcAg staining:

- Staining jars
- Acetone
- Wash buffer: 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5. To prepare a 10x stock solution dissolve 121.14 g Tris and 87.66 g NaCl in deionized water, adjust pH with HCl and fill up to 1L. Dilute 1:10 with water before use. Store at RT.
- PBS and H_2O_2 for the peroxidase quenching buffer
- Humidifying chamber, i.e. a covered box with wet paper towels
- lab wipes

- Blocking buffer: 0.5% blocking reagent (supplied in the TSA kit) in 1x washing buffer. Dissolve the blocking reagent by warming up to 60 °C and continuous stirring. Aliquot and store at -20 °C.
- Primary antibodies: Polyclonal rabbit anti-HBcAg antibody, Dako, catalog number B0586 and monoclonal mouse anti-cytokeratin 18 antibody (clone DC-10), Santa Cruz, catalog number sc-6259
- Secondary antibodies: horseradish peroxidase conjugated goat anti-rabbit antibody, i.e. Jackson, catalog number 111-035-003, and Alexa Fluor 555 conjugated goat anti-mouse antibody, i.e. Life Technologies, catalog number A32727
- TSA™ Fluorescence Systems Tyramide Signal Amplification (PerkinElmer, catalog number NEL701A001KT)
- Dimethyl sulfoxide (DMSO)
- Hoechst 33258, 10 mg/ml in H_2O
- Fluorescent mounting medium
- High precision glass cover slips (i.e. 24 x 50 mm and 170µm thickness, Marienfeld, catalog number 0107222)

Fluorescence microscope including the appropriate filters: Fluorescein: excitation 494 nm, emission 517 nm, common filter set FITC, and Alexa Fluor 546, peak excitation 556 nm, peak emission 573 nm, common filter set: TRITC. Alternatively, a confocal microscope with the appropriate laser lines.

Experimental Procedures

This method has previously been described in greater detail as a chapter in the book *Methods in Molecular Biology: Hepatitis B Virus.* **Preparation of cryo-preserved liver sections**

- Sacrifice the mouse, excise the liver and cut the lobes into small pieces of around 1 x 0.5 x 0.5 cm size. Snap freeze the liver pieces by dropping them piece by piece in pre-chilled isopentane (fill a glass beaker with isopentane and keep on dry ice), then store at -80 °C.

Note: It is important to freeze the liver pieces very quickly and to keep them frozen for all time. Thawing and slow freezing processes will destroy the tissue and lead to suboptimal staining results.

- Place a piece of liver in embedding medium on the object holder of a cryotome set at -20 °C. Cut 12 µm thick sections. Mount two sections on each microscope slide. Let the sections dry for 10 min at room temperature and store them at -80°C. Allow the slides to stay at -80° for at least one night before using them.

HBcAg staining

- Use appropriate controls to test for the validity of the signal. For instance, use a section of a highly infected chimeric mouse liver as a positive control and one of a non-infected mouse as a negative control. In addition to these biological controls, it is advisable to use a technical negative control slide (without the primary antibody).
- All steps are carried out at room temperature. Make sure the section don´t dry out during the entire procedure.
- Fill a staining jar with acetone. Remove the slides from -80°C and immerse them immediately in the fixative. Fix for 10 min.

Note: Fixation needs to be performed with acetone. Using other fixatives such as methanol or paraformaldehyde will lead to loss of HBcAg (6).

- Transfer the slides to a new jar filled with 1x wash buffer. Wash three times. Each time for 5 min.

Note: It is not recommended to use detergents such as Tween or Triton for the washing steps or dilution of the antibodies as HBcAg is extremely sensitive to detergents and will be washed out of the tissue.

- Prepare a 0.01% H_2O_2/PBS solution in a new staining jar, transfer the slides, and let incubate for 10 min to quench endogenous peroxidase activity, which might otherwise interfere with the horseradish peroxidase of the secondary antibody.

Note: High H_2O_2 concentrations will lead to the detachment or destruction of the tissue, and optimal time and H_2O_2 concentrations will probably need to be determined in every lab.

- Wash the slides as indicated above.
- Block unspecific protein binding sites on the section with the blocking buffer. Remove the slides from the wash buffer and pipet 200 µl of blocking buffer directly on the sections. Incubate in a humidifying chamber for 30 min.

Note: When applying a solution directly on the slide, remove excess liquid and carefully wipe around the sections always leaving the same small amount of liquid on the sections. Then add the new solution and pipet up and down on the slide to mix the liquid. Make sure that the sections are entirely covered and that the rim of the liquid is beyond the rim of the tissue section in order avoid artifacts at the edges of the section. Hydrophobic barrier pens for circling the sections can be used but are not necessary since the surface tension is usually enough to keep the bubble of liquid on the section.

- Without washing move on to the incubation with the primary antibodies. In addition to the anti-HBcAg antibody, we use a mouse antibody that specifically detects human cytokeratin 18 to distinguish between human and murine hepatocytes in the liver of human liver chimeric mice.

Note: HBcAg staining can be combined with other primary antibodies. For instance, the delta antigen of the Hepatitis Delta virus (HDV) can be co-stained in an HBV/HDV-infected mouse (7), or a proliferation marker such as KI-67 can be used to co-stain dividing cells (8).

- Remove the blocking buffer and add the antibody solution. The rabbit anti-HBcAg antibody should be diluted 1:2000 in wash buffer and the mouse anti-cytokeratin 18 antibody 1:400. Use around 200 µl antibody solution per slide. Incubate the slides in a humidifying chamber for 1 h at room temperature or at 4°C overnight. Overnight incubation usually results in enhanced specific staining and less unspecific background staining.

Note: When the amount of HBcAg-positive cells is expected to be low, the anti-HBcAg antibody can be diluted down to 1:5000. A high concentration of primary antibody together with a small number of positive cells might otherwise lead to considerable background staining. **Note**: The anti-HBcAg antibody from Dako, which we and others have routinely used for the HbcAg staining, has been discontinued and is not available any more. We would be grateful to the users, to post any antibody that was found to achieve a similar staining pattern as a possible substitute in the comments section.

- Wash the slides as indicated above.
- Incubate the slides with the secondary antibodies. Dilute the HRP-conjugated anti-rabbit antibody 1:200 and the Alexa Fluor 555-conjugated anti-mouse antibody 1:1200 in wash buffer. Add 200 µl of the antibody solution to each slide and incubate in a humidifying chamber for 1 h. From now on slides should be kept in the dark.
- Wash the slides as indicated above.
- Proceed to the TSA step. The fluorophore tyramide amplification reagent can be prepared ahead of time by dissolving the fluorescein tyramide in DMSO as indicated in the TSA

manual. Freshly prepare a 1:50 working solution of the fluorophore tyramide amplification reagent using the amplification diluent provided in the kit. Remove the wash buffer and pipet 100 µl of the working solution on every slide. Incubate for 5 min in a humidifying chamber in the dark.

Note: When staining intensities are to be compared in a quantitative manner, special care has to be taken to ensure conformity in the staining procedure. For instance, the incubation step with the fluorophore tyramide amplification reagent should last for exactly 5 min as the time of incubation influences the staining intensity. It is also advisable to run all samples from the experimental groups to be compared in parallel as the age of the TSA kit, i.e. the number of times the fluorophore tyramide amplification reagent was used and exposed to light, may likewise influence the staining intensity.

- Wash the slides as indicated above.
- Perform nuclear staining by diluting Hoechst 1:20,000 in a staining jar filled with wash buffer. Immerse the slides and incubate for 2 min.
- Remove the slides from the staining jar one by one, remove any liquid, apply one drop of fluorescent mounting medium and cover with a glass cover slip. Avoid trapping air bubbles under the cover slip. Store the slides in the dark, flat and cool.

HBcAg staining can now be assessed with a fluorescence light microscope or confocal microscope to quantify the number of HBV-infected cells or compare staining patterns and intensities between experimental groups of mice. The figure below shows an example of a human chimeric mouse in the spreading phase of HBV infection showing scattered HBcAg-positive hepatocytes and one fully HBV-infected mouse where all human hepatocytes stain positive for HBcAg.

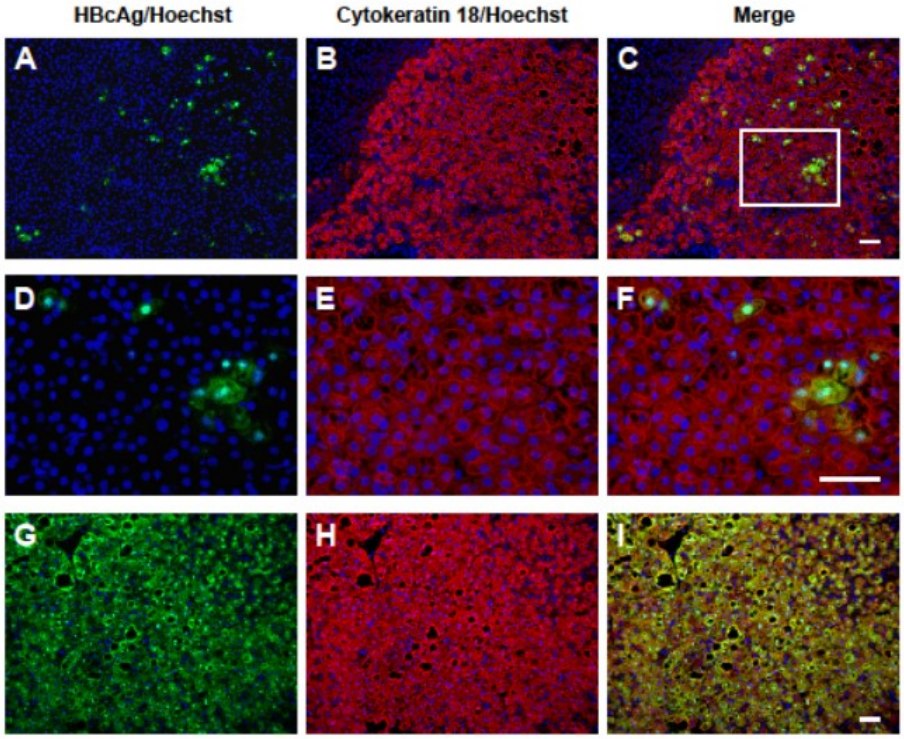

Figure Legend: HBcAg staining in HBV-infected human liver chimeric mice (uPA/SCID/beige). **A-C)** A uPA/SCID/beige mouse in the spreading phase of the infection shows scattered HBcAg-positive cells within clusters of human hepatocytes. Stained sections were analyzed by fluorescence microscopy (Biorevo BZ-9000, Keyence, Osaka, Japan) using a 20x/0.75 NA objective. **D-F)** Merged z stack image, 5 µm in total thickness, of the area in the white box in C captured with fluorescence microscopy (Biorevo BZ-9000, Keyence, Osaka, Japan) using a 60x/1.40 NA oil objective. **G-I)** A chronically HBV-infected uPA/SCID/beige mouse was analyzed as in A-C revealing HBcAg positivity in almost all human hepatocytes. In the photographs in the left column (**A, D, G**) HBcAg is shown in green, in the middle column (**B, E, H**) Cytokeratin 18, a marker for human hepatocytes, is shown in red, and in the right column (**C, F, I**) a merged picture is shown. Nuclei are counterstained with Hoechst (blue). The scale bar represents 50 µm.

References

1. Lutgehetmann M, Bornscheuer T, Volz T, Allweiss L, Bockmann JH, Pollok JM, et al. Hepatitis B virus limits response of human hepatocytes to interferon-alpha in chimeric mice. Gastroenterology. 2011;140(7):2074-83, 83 e1-2.
2. Allweiss L, Volz T, Lutgehetmann M, Giersch K, Bornscheuer T, Lohse AW, et al. Immune cell responses are not required to induce substantial hepatitis B virus antigen decline during pegylated interferon-alpha administration. J Hepatol. 2014;60(3):500-7.
3. Allweiss L, Lutgehetmann M, Dandri M. Immunofluorescent Staining for the Detection of the Hepatitis B Core Antigen in Frozen Liver Sections of Human Liver Chimeric Mice. Methods in molecular biology. 2017;1540:135-42.
4. Elmer P. 2015 [July 10, 2015]. Available from: http://www.perkinelmer.com/Resources/TechnicalResources/ApplicationSupportKnowledgebase/TSA/tsa.xhtml.
5. Krieg R, Halbhuber KJ. Detection of endogenous and immuno-bound peroxidase--the status quo in histochemistry. Progress in histochemistry and cytochemistry. 2010;45(2):81-139.
6. Gowans EJ, Burrell CJ. Widespread presence of cytoplasmic HBcAg in hepatitis B infected liver detected by improved immunochemical methods. Journal of clinical pathology. 1985;38(4):393-8.
7. Lutgehetmann M, Mancke LV, Volz T, Helbig M, Allweiss L, Bornscheuer T, et al. Humanized chimeric uPA mouse model for the study of hepatitis B and D virus interactions and preclinical drug evaluation. Hepatology. 2012;55(3):685-94.
8. Allweiss L, Volz T, Giersch K, Kah J, Raffa G, Petersen J, et al. Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo. Gut. 2017.