A simple and rapid method for the quantitation of secreted hepatitis B virions in cell culture models

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Abstract

Cell culture models for hepatitis B virus (HBV) remain the mainstay for screening and testing the efficacy of anti-hepatitis B virus agents. Gradient-based ultracentrifugation followed by Southern Blotting is used for hepatitis B virion estimation in cell culture; this method has several limitations. We report the development of an assay using a commercially available HBsAg-ELISA plate for immunocapture followed by real-time PCR for quantification of hepatitis B virions in cell cultures. This assay is rapid, highly sensitive (50 copies/reaction) and highly specific for virion-associated DNA. In addition, the assay requires only 20 μL of supernatant, allowing scaling down of transfections.

Key words: ELISA, HBV, immunocapture, quantitative, real time, supernatant

Introduction

Human hepatoma-derived cell lines are widely used for the study of hepatitis B virus (HBV) replication due to lack of good small animal models. In cell culture models, HBV mutants including drug-resistant mutants influence HBV replication resulting in reduced virion production. Currently available methods for quantification of secreted hepatitis B virions in cell culture are summarized in Table 1. The classical and most widely used method is the CsCl gradient-based ultracentrifugation followed by Southern Blotting. However, gradient-based ultracentrifugation has inherent disadvantages: (a) It does not provide quantitative information, (b) It requires large volumes of cell culture supernatant, (c) Detection often requires the use of radioactivity and (d) it is labour-intensive.

Estimation of secreted hepatitis B virions in cell culture supernatant is critical for understanding replication characteristics of HBV variants, drug resistant mutants and in screening and efficacy testing of anti-HBV drugs. Therefore, the need for a simple and specific assay for absolute quantification of secreted hepatitis B virions in cell culture models is being increasingly realized.

We report the development and validation of a simple, rapid and sensitive assay for the absolute quantification of hepatitis B virions in cell culture models. Briefly, commercially available monoclonal anti-HBs coated HBsAg ELISA plates were used to capture virions followed by DNA extraction and quantification using real-time PCR. The immunocapture followed by real-time PCR assay provides high specificity for virion-associated HBV DNA and eliminates noise from non-virion associated DNA (a major problem in currently available methods using DNase I).

Materials and Methods

This work has been approved by the department and institute research committee. It does not require ethics committee approval as it does not involve human subjects.

Huh7 cells were seeded in 6-well plates and transfected with replication-competent wild type HBV constructs created as described previously. To investigate if this assay eliminated background signal from non-virion associated DNA, we created Pkex-1+2 mutant construct (mutation in the epsilon region of HBV genome) which is replication incompetent. Culture supernatants (100 μL each) from the transfected Huh7 cells (5 days post-transfection) and stably transfected HepG2.2.15 cell line were collected and subjected to DNase I (NEB, MA, USA) digestion for 30 minutes at 37°C to digest non-virion associated HBV DNA, then added to ELISA microplate wells coated with monoclonal anti-HBs antibodies (Monolisa HBsAg ULTRA ELISA kit, Bio-Rad, CA, USA) and incubated at 37°C for
90 minutes. Subsequently, the microplate wells were washed with PBS (Genetix Biotech, New Delhi, India), treated with Proteinase K and lysis buffer and then DNA was extracted as per the manufacturer’s instructions (QIAamp DNA Blood mini kit, Qiagen, Valencia, USA). Two microlitres of DNA extracted from the captured virions was quantitated in a real-time PCR assay using primers (IDT) (V-F: 5’GGTCTGCGCACCAGCACC3’ and V-R: 5’GAACCTTTAGGCATATTAGTG3’) specifically designed not to amplify transfected HBV DNA. The amplification conditions were as follows: 95°C for 2 minutes, followed by 36 cycles of 95°C for 5 seconds and 60.5°C for 5 seconds.

To test if small volumes of supernatants could be used in our assay to obtain quantitative information, we tested dilutions of the supernatant including 1:2, 1:4, 1:10 and 1:20 [Figure 1a], where 50 µl, 25 µl, 10 µl and 5 µl of the supernatant was diluted to a total volume of 100 µl (in PBS) prior to immunocapture followed by real-time PCR.

**Results**

Our assay provides absolute quantitative information on the amount of secreted virions. We demonstrate that our assay could quantitatively detect even small differences in the number of virions secreted into the cell culture medium [Figure 1a]. The specificity of our assay for virion-associated DNA was confirmed by the absence of signal from Pkex-1+2 (replication-incompetent) constructs and growth medium spiked with the wild type construct (10^{10} copies/ml) [Figure 1a]. The lower limit of detection of the real-time PCR used to quantify virions is 50 copies/per reaction. To investigate whether excess HBsAg present in the culture supernatant interferes with virion quantification, the culture supernatants from HepG2.2.15 cells were spiked with purified HBsAg (5.3 ng/mL). The difference in the number of estimated virions before and after spiking with extraneous HBsAg was not significant ($P > 0.05$; data not shown), suggesting that excess HBsAg does not interfere with immunocapture of virions.

Finally, to validate our assay with an anti-HBV drug, we transfected the wild type HBV construct with varying concentrations of lamivudine (0, 1, 5 and 10 µM) and supernatants were harvested at 72 hours post-transfection. We used 20 µL of the supernatant for immunocapture followed by real-time PCR. The dose-dependent reduction in virion secretion with increasing concentrations of lamivudine is shown in [Figure 1b].
Discussion

We believe that our assay overcomes most if not all limitations of the widely used ultracentrifugation-based methods for the quantification of virions in cell culture models. The assay we describe in this report has the following advantages: (a) high sensitivity (b) high specificity for virion-associated HBV-DNA (c) does not require the use of radioactivity and (d) high-throughput compatible. In addition, our assay requires only 20-100 μl of supernatant (as opposed to 5-20 mL required for ultracentrifugation-based methods) allowing scaling down of transfections to a 96-well plate. Furthermore, our assay requires only 5 hours as opposed to 3-5 days required for currently available methods.

Mutations in the surface gene may reduce the binding affinity of HBsAg to the anti-HBs antibody coated on the ELISA plate; this may impair immunocapture of virions with surface gene mutations. However, the Monolisa HBsAg ELISA kit that we used has been successfully used to detect 15 most common and clinically relevant HBsAg mutants. Our assay is not suitable for virion estimation in HBV clones negative for HBsAg using the commercial ELISA kit used for immunocapture; this is the major limitation of the assay we report.

The differences between HBV replication capacity (often measured by the number of pregenomic RNA/ccc DNA) and virion secretion are becoming increasingly relevant. Naturally occurring mutations in HBV genome can influence HBV virion secretion without significantly affecting the levels of replicative intermediates; strengthening the need for assays that allow quantitation of secreted virions. We believe that our assay will help shed more light on poorly understood nuances of HBV replication, virion secretion in particular. In sum, we report a method for the quantitation of secreted hepatitis B virions in cell culture models that may be particularly helpful as a research tool for assessing virion secretion in naturally occurring mutants and for high-throughput screening of anti-HBV drugs in vitro.

References