**Hepatitis B Antiviral Drug Development**

**Multi-Marker Single-Plate Screening Assay**

## Introduction

The hepatitis B virus (HBV) is one of a few known non-retroviral viruses which use reverse transcription as a part of its replication process. After the virus has entered the cell, the partially double-stranded viral DNA is transferred to the nucleus where it is made fully double stranded and transformed into covalently closed circular DNA (cccDNA) that serves as a template for transcription of four mRNAs. (Figure 1) The largest mRNA (pre-genomic RNA or pgRNA) is translated to produce the hepatitis B core antigen (HBcAg or nucleocapsid), the soluble and secreted HBeAg, and the polymerase. The pgRNA is also used to make new copies of the viral genome by reverse transcription. The other mRNAs are translated to produce the viral envelope proteins and the HBV X protein.

In light of the complexity of HBV replication, ImQuest BioSciences has developed and qualified a single-plate method to expedite the screening of antiviral agents against multiple markers of HBV replication.

The antiviral activity of 2',2'-dideoxy-3'-thacytidine (3TC) and tenofovir disoproxil fumarate (TDF), both nucleoside reverse transcriptase inhibitors (NRTI), on HBV replication in Hep AD38 and HepG2 2.2.15 was evaluated as a means of development and qualification of the single-plate assay.

## Methodology

The method was developed using Hep AD38 and HepG2 2.2.15 cells. HepG2 2.2.15 cells are derived from the human hepatoblastoma cell line HepG2 by stable transfection with full length HBV plasmid. Hep AD38 cells express proteins, RNA, and DNA intermediates characteristic of HBV replication. The Hep AD38 cells were derived from HepG2 stably transfected with an expression vector in which HBV replication is regulated by tetracycline.

Cells were seeded into 96-well plates and treated with serial half-log dilutions of TDF or 3TC for six days. Cell culture supernatants were collected for quantification of extracellular HBV markers. The cells were lysed and fractionated into nuclear and cytoplasmic components for evaluation of intracellular markers of viral replication. (Figure 2)
Results

**cccDNA Accumulation:** A dose-dependent reduction in accumulation of cccDNA was observed in both cell lines with relative cccDNA levels reduced to 50% of untreated cells in HepG2 2.2.15 cells and to undetectable levels in Hep AD38 cells at the high 3TC concentration (10 μM). The reduction in cccDNA accumulation is consistent with the inducible nature of HBV replication in Hep AD38 cells and the chronic infection of HepG2.2.15 cells. (Figure 3)

Figure 3. Analysis of cccDNA in the nuclear fraction from HepG2 AD38 and HepG2.2.15 cells treated with 3TC.
**Viral DNA Synthesis:** The results depicted in Figures 4a & 4b were obtained by diluting cell culture supernatants 1:10 in PCR dilution buffer. The samples were boiled and amplified with specific primers and TaqMan probes.

![Graph showing viral DNA synthesis](image)

**Evaluation of the effect of TDF and 3TC on HBV replication in both HepG2 2.2.15 and Hep AD38 cells from a single 96-well plate demonstrated a dose-dependent reduction in extracellular HBV DNA in both cell lines.**

**Encapsidated Viral DNA Production:** To determine the effect of 3TC on viral DNA replication in Hep AD38 cells, cell culture supernatant and cytoplasmic fractions from cell lysates were treated with micrococcal nuclease to digest unencapsidated nucleic acids. The enzyme was then inactivated with EGTA. Nuclease-treated samples were boiled in PCR buffer and subjected to qPCR with HBV primers and probe. The effect was dose dependent. (Figure 5)

![Graph showing encapsidated viral DNA production](image)

**HBV pgRNA Transcription:** To determine the effect of 3TC on synthesis of pgRNA in Hep AD38 and HepG2.2.15 cells, cytoplasmic nucleic acids were treated with DNase prior to RT-qPCR with HBV specific primers and TaqMan probes. Reduction of pgRNA transcription by 3TC was non-specific in both cell lines. (Figures 6a and 6b)
HBV Antigen Production: To evaluate the effect of 3TC on synthesis of HBV antigens, cell culture supernatant and cell cytoplasm were analyzed for the presence of HBsAg and HBcAg by ELISA. Reduction of HBsAg and HBcAg by 3TC was not observed at the highest test concentration (10 μM). (Figure 7)

Antiviral Drug Efficacy: EC_{50} values for inhibition of extracellular HBV DNA accumulation were comparable in the two lines; EC_{50} values for inhibition of extracellular DNA were lower than those for cytoplasmic DNA in both cell lines. See table at left.

Summary

ImQuest has developed a broad spectrum method to screen the effect of antiviral agents on multiple markers of HBV replication from a single 96-well plate. The method was developed and qualified by evaluating the effect of reverse transcriptase inhibitors on multiple HBV markers in both HepG2 2.2.15 and Hep AD38 cells. Results demonstrated a dose-dependent 3TC-mediated reduction in extracellular HBV DNA, cytoplasmic HBV DNA and cccDNA consistent with the inhibition of HBV replication by an inhibitor of reverse transcription.