Generation of a molecular standard for circulating HBV RNA detection and quantification assays in CHB patients

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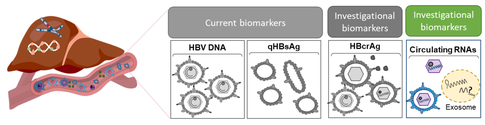
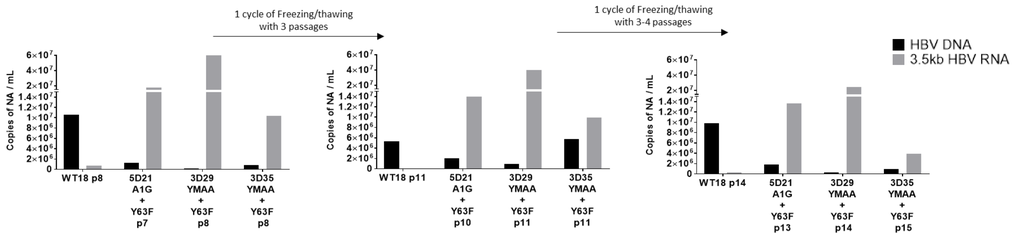
Background

The quantification of serum HBV RNA (mostly, but not exclusively, 3.5 kb RNAs) is increasingly recognized as a biomarker to evaluate HBV treatments activity and predict patient outcomes. In untreated patients, serum HBV RNA reflects cccDNA transcriptional activity. In addition to several homemade assays, 2 PCR-based research use only (RUO) investigational assays (IA) have been developed (Butler et al., 2018; Liu et al., 2019; Scholtès et al., 2022). The WHO HBV-DNA standard or a synthetic armored RNA (arRNA) have been used for calibration. Here, we have generated a stable clonal cell line producing large quantities of secreted viral RNAs amenable to be used as an RNA standard to calibrate PCR-based circulating HBV RNA quantification assays.



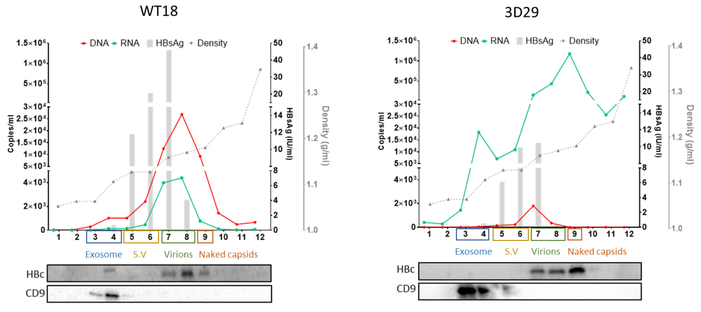
Generation of a stable clonal cell line secreting high quantities of HBV RNAs

HepG2- and HuH7-derived clonal cell lines transduced with HBV genomes carrying mutations in the Pol catalytic site (YMAA), the Pol TP-domain (Y63F), the pgRNA ε-loop (mutation A1G) that are expected to strongly reduce / abolish HBV rc-DNA synthesis have been generated. The clonal cell line Huh7-3D29, carrying a double YMAA/Y63F mutation, displayed the desired RNA secretory phenotype (e.g., inversion of the secreted HBV DNA/RNA ratio). Although a slight reduction of HBV RNA secretion has been observed, after several freeze-thawing and amplification cycles Huh7-3D29 cells maintained their RNA secretory phenotype with a high RNA/DNA ratio in cell supernatants and minimal residual DNA (~5 $\log_{10}$).



HBV RNAs Standard

The majority of Huh7-3D29 secreted HBV RNAs are found in naked capsids, and virion-like particles



Huh7-WT18, and Huh7-3D29 cells supernatants were analyzed by iodixanol/sucrose density gradient ultracentrifugation followed by HBsAg quantification by ELISA, HBV DNA, and total HBV RNA quantification by ddPCR, HBc and CD9 detection by Western blot in each fraction.

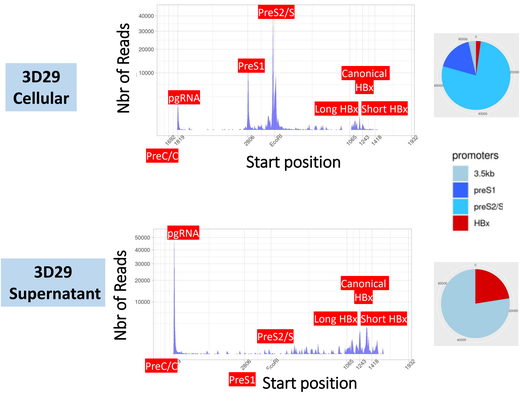
In the HBV mutant clonal cell line Huh7-3D29, the HBV RNA was found in RNA particles (fraction 7 and 8), in naked capsids (fractions 9) and to a lower extent in exosomes (fractions 3 and 4), while the residual HBV DNA peak was found in virions (fraction 7). Notably, as compared to the Huh7-WT18 cell, a larger proportion of HBV RNA was found in naked capsids.

These results are consistent with the reduction of HBV DNA synthesis in the immature capsid imposed by the HBV mutations and the resulting blockade of capsids maturation.

Huh7-3D29 secreted HBV RNAs are strongly enriched in pgRNA and HBx RNAs

Characterization of the RNA species secreted by the Huh7-WT18 and mutant Huh7-3D29 clone has been performed by single molecule long reads Nanopore sequencing. The sequencing results show that:

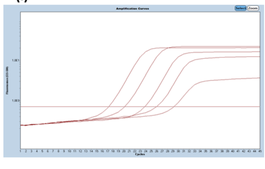
a) secreted HBV RNAs sequence is identical to the HBV genome used for the establishment of the different cell lines; and
b) the majority of HBV RNAs detected are full-length transcripts (and pgRNA-derived spliced RNAs) whereas only a minority of PreS/S RNAs and HBx RNAs were detected.



Huh7-3D29 derived HBV RNA standard is suitable for both Roche automated and manual assays

We have then tested Huh7-3D29 SNs as a calibrator for the Roche MWR and the related HBV RNA cobas® 6800/8800 automated investigational assay (Scholtès et al., 2022). We have previously shown that the cobas® 6800/8800 IA assay and the related MWR assay tolerate up to ~10^6 DNA:RNA ratios without losing specificity and linearity (Scholtès et al., 2022). The HBV RNA standard produced and secreted by the Huh7-3D29 cell line fulfill this criterion. Serial dilution experiments indicate that Huh7-3D29 SN performs as the synthetic arRNA. Notably, HBV RNA quantification of Huh7-3D29 supernatants with the Roche MWF showed no significant difference with and without DNase treatment, supporting the use of the Huh7-3D29 derived HBV RNA standard with both the manual assay (that includes DNAse treatment) and the DNAse free cobas® 6800/8800 automated assay.

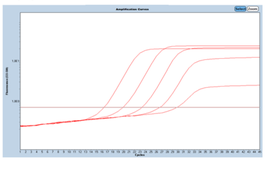
(i) Armored RNA (Roche) 10 fold dilution amplification curves from MWF

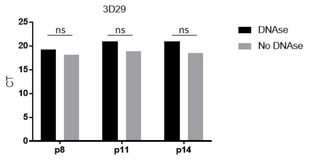


(ii)

Ct of diluted armored RNA	Copies / mL	Ct of diluted 3D29 RNA
19,52	10^5	18,70
22,59	10^4	22,80
26,44	10^3	26,07
29,38	10^2	29,96
32,05	10^1	34,09

(iii) 3D29 HBV RNA 10 fold dilution amplification curves from MWF





Conclusion

We generated a clonal cell line that secretes high amounts of HBV RNAs with very low quantities of HBV DNAs. Huh7-3D29 SN represents a stable source of RNA standard for the calibration of all HBV RNA assays.

References

Butler, E.K., Gersch, J., McNamara, A., Luk, K.-C., Holzmayer, V., de Medina, M., Schiff, E., Kuhns, M., and Cloherty, G.A. (2018). Hepatitis B Virus Serum DNA andRNA Levels in Nucleos(t)ide Analog-Treated or Untreated Patients During Chronic and Acute Infection. Hepatol. Baltim. Md 68, 2106–2117. https://doi.org/10.1002/hep.30082.

Liu, Y., Jiang, M., Xue, J., Yan, H., and Liang, X. (2019). Serum HBV RNA quantification: useful for monitoring natural history of chronic hepatitis B infection. BMC Gastroenterol. 19, 53. https://doi.org/10.1186/s12876-019-0966-4.

Scholtès, C., Hamilton, A.T., Plissonnier, M.-L., Charre, C., Scott, B., Wang, L., Berby, F., French, J., Testoni, B., Blair, A., et al. (2022). Performance of the cobas® HBV RNA automated investigational assay for the detection and quantification of circulating HBV RNA in chronic HBV patients. J. Clin. Virol. 150–151, 105150. https://doi.org/10.1016/j.jcv.2022.105150.