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## Supplemental Information

# One-Vector System for Multiplexed CRISPR/Cas9 against Hepatitis B Virus cccDNA Utilizing High-Capacity Adenoviral Vectors

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**Supplemental information**

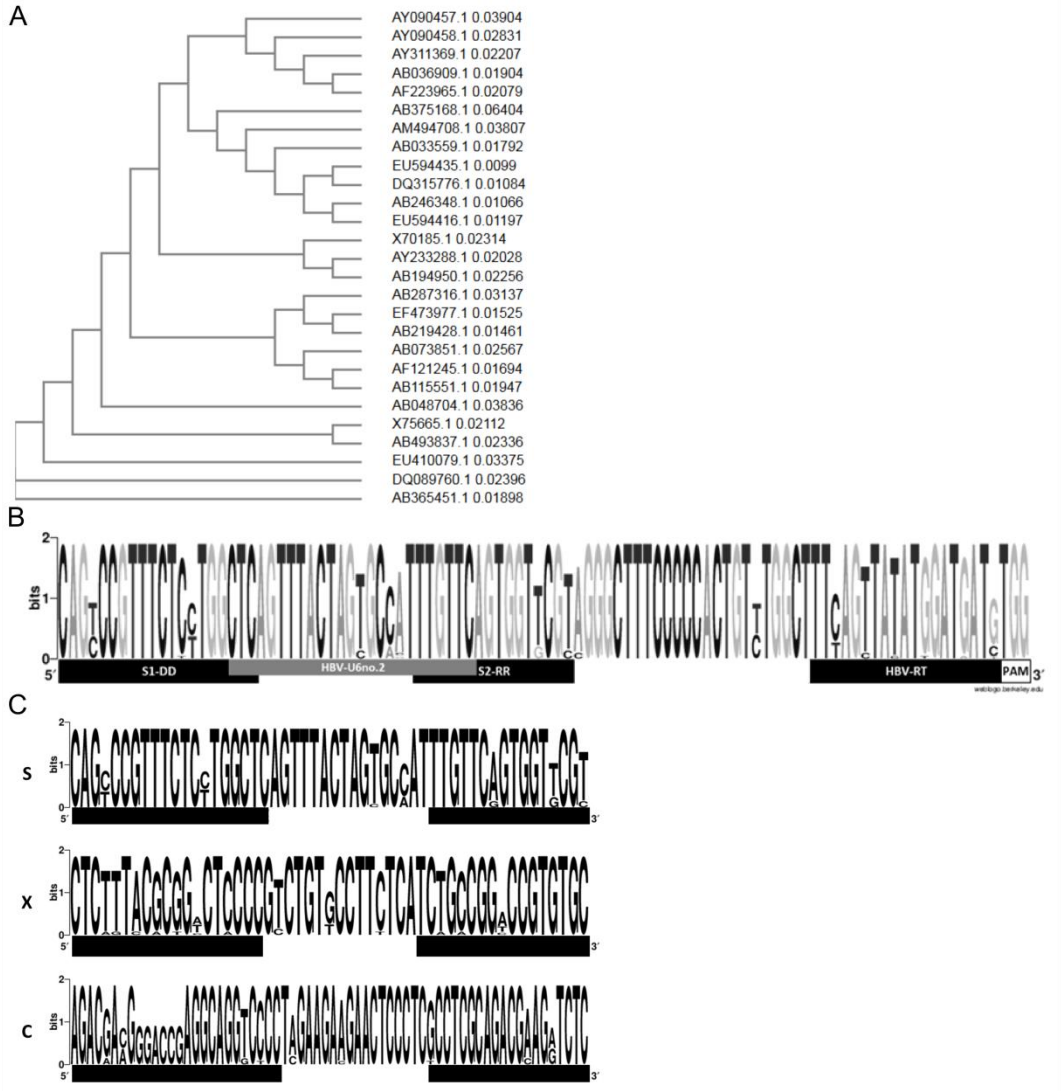


**Figure S1. Schematic illustration of sequence conservation profile of target sites.** (A) Phylogenetic tree from HBV sequence reference panel used for creation of conservation profile showing accession numbers and phylogenetic distances. Analysis was performed using Clustal Omega 1.2.4 (1). (B) Conservation profile of target sites in the surface antigen ORF. TALEN binding sites [TALEN S1 / TALEN S2] which were based on the previously published RNAi target site [HBVU6no.2] and Cas9 localization site [sgRNA HBV RT] lie in close proximity. (C) Conservation profile of all TALEN open reading frames attacked in this study (S: HBsAg; X: X-Protein; C: HBcAg).


1. Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J. *et al.* (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology*, 10.1038/msb.2011.75.

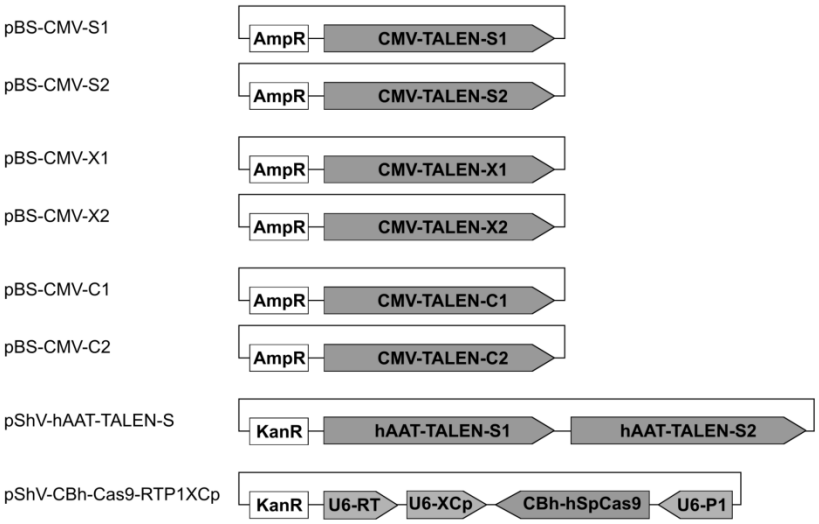


**Figure S2. Plasmid constructs for transfection experiments.** Expression of TALEN units from separate plasmids was driven by CMV promoter and for the merged construct from tissue-specific human alpha-1-antitrypsin (hAAT) promoter. Cas9 endonuclease in the context of the CRSIPR/Cas9 system was expressed from the CBh promoter. gRNAs RT, XCp and P1 were expressed under the control of the U6 promoter.

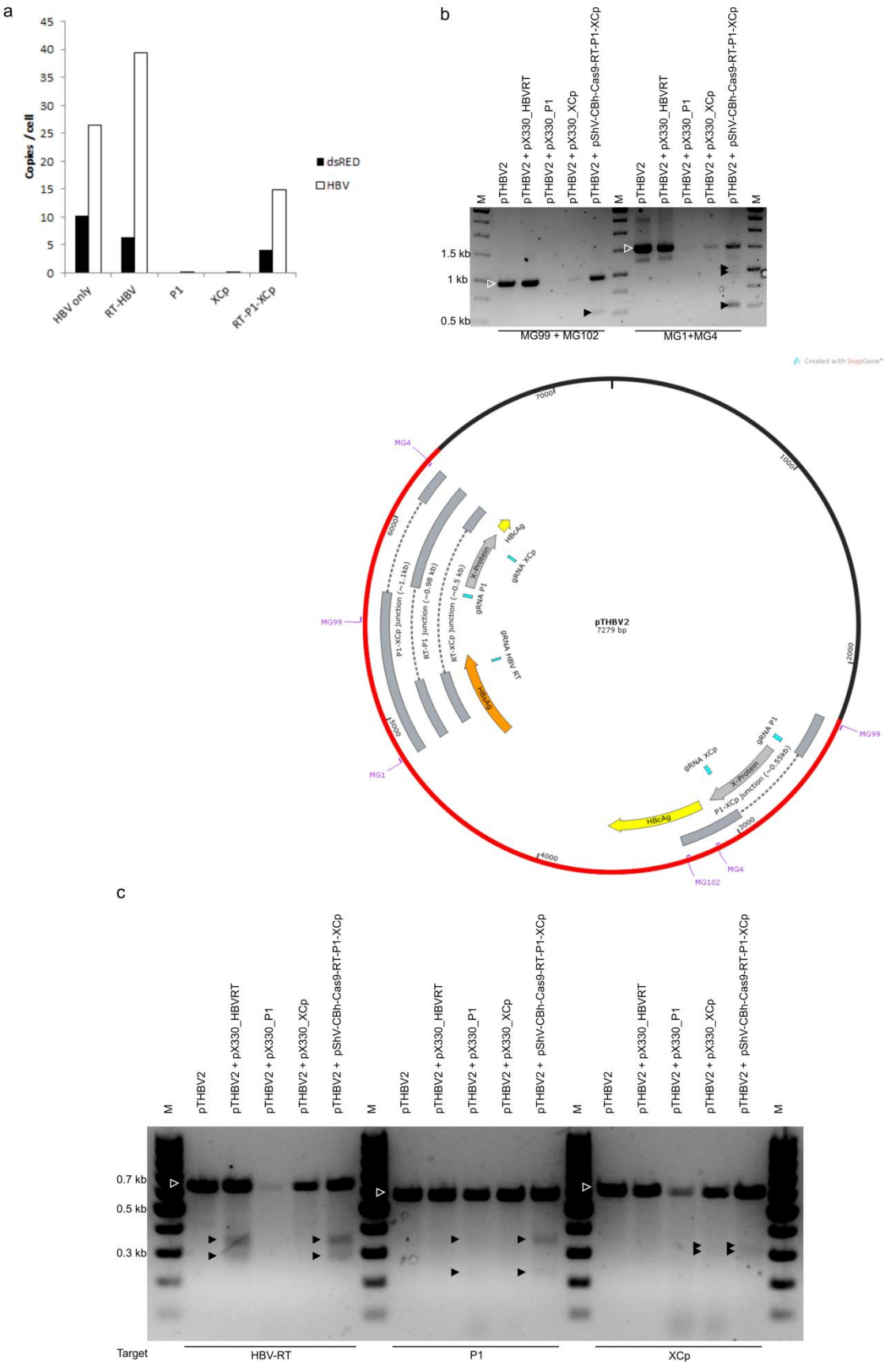
a
Copies / cell
dsRED
HBV
HBV only
RT-HBV
P1
XCp
RT-P1-XCp
b
M
pTHBV2
pTHBV2 + pX330_HBVRT
pTHBV2 + pX330_P1
pTHBV2 + pX330_XCp
pTHBV2 + pShV-CBh-Cas9-RT-P1-XCp
1.5 kb
1 kb
0.5 kb
MG99 + MG102
MG1+MG4
Created with SnapGene®
pTHBV2
7279 bp
MG4
MG99
MG1
MG102
HBcAg
X-protein
gRNA XCp
gRNA P1
gRNA HBV RT
P1-XCp junction (~1.1kb)
RT-P1 junction (~0.98 kb)
RT-XCp junction (~0.5 kb)
P1-XCp junction (~0.55kb)
c
0.7 kb
0.5 kb
0.3 kb
Target
HBV-RT
P1
XCp

**Figure S3. Contribution of three individual gRNAs to the multiplexed construct.** The functionality of the single gRNAs was also tested from single expression constructs (Cas9 plus gRNA) in the initial transfection experiment in HEK 293 cells (see Figure 2). The data is not presented in Figure 2 because of poor transfection efficiency in two groups with single constructs (P1 and XCp), the proven function of the triple construct and the fact that these gRNAs were published before. (a) The transfection efficiency was very inconsistent among the groups as can be seen by the copy numbers of a plasmid containing dsRED (as transfection control) or pTHBV2 in the isolated genomic DNA assessed by quantitative PCR. Transfection in the P1 or XCp sample appears to have failed. This is in line with the unsuccessful amplification of target sites in these groups presented in the following pictures. (b) Amplification of regions spanning over multiple gRNA target sites (see map). The amplicon in the first set of lanes was amplified with primers MG99 gctttcactttctcgccaac and MG102 gcctgagtgcagtatggtga and overlaps the P1 and XCp target sites in the HBV-replication plasmid pTHBV2. The intact amplicon is of 1.0 kb (white arrowhead) and an excision event leads to a 0.55 kb fragment, which is only visible after treatment with the triple construct, indicating a synergistic effect of these gRNAs (black arrowhead). Likewise the second set of lanes represents the amplicons amplified with primers MG1 ttcctcttcatcctgctgct and MG4 ataagggtcgatgtccatgc which cover all three gRNA target sites. The intact amplicon is of 1.5 kb in size (white arrowhead). Excision between P1 and XCp leads to a 1.1 kb fragment, excision between HBV-RT and P1 to a 0.98 kb fragment and excision between HBV-RT and XCp leads to a 0.5 kb fragment. All fragments are present in the lane of the triple construct treated samples (black arrowheads), in which the largest excision is the most prominent. (c) The T7E1 assay only showed a clear positive result for the HBV-RT single gRNA and the triple gRNA treated samples (black arrowheads). It is of note that the amount of amplicons in the T7E1 assay was set roughly to the same quantity, which means that the strength of the uncut bands in this gel picture does not resemble the actual output of the PCR which varied in accordance with the outcome of the transfection assessment.

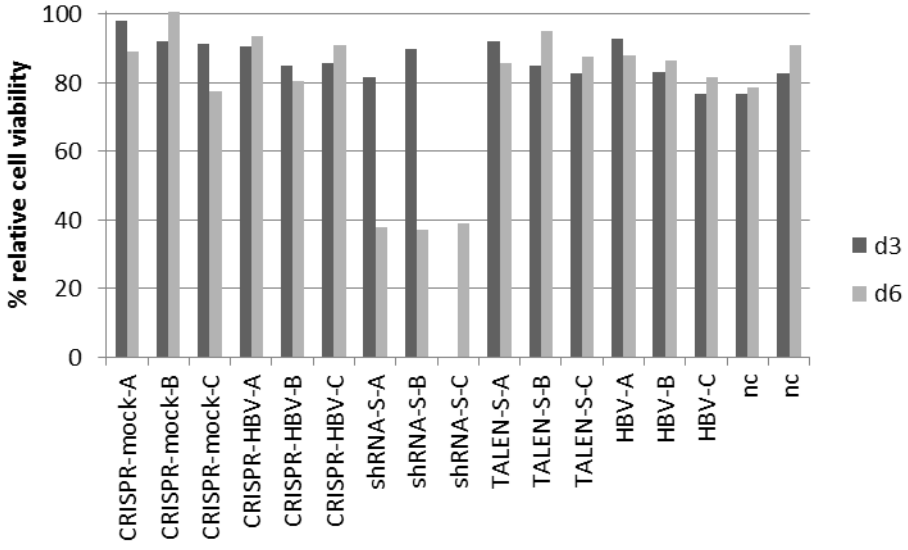


**Figure S4. Viability of HepG2-NTCP cells.** Viability of cells was tested using cell titer blue test before harvesting cells for subsequent analysis. All samples show comparable viability around 80% except for shRNA treated cells after 6 days, where high toxicity is seen and therefore these samples were excluded in further analysis.