Supporting Information

Recombinase Amplified CRISPR Enhanced Chain Reaction for Viral Genome Detection

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Materials and methods, supporting schemes, figures and tables are provided in this supporting information.

EXPERIMENTAL SECTION

Materials and Methods. The template hepatitis B DNA i.e., 'HBV' (catalog #45020D, batch #70019870) and hepatitis A DNA i.e., 'HAV' (catalog #VR-3257SD, batch #70008704) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All other oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). The RPA kit (catalog #TALQBAS01) was purchased from TwistDx Limited (Cambridge, UK). EnGen Lba Cas12a (Cas12a) (catalog #M0653) and dNTP solution mix (catalog #N0447S) were purchased from New England Biolabs (Ipswich, MA, USA). Bovine serum albumin (BSA) (catalog #9048-46-8); and Magnesium chloride hexahydrate (catalog #7791-18-6) were purchased from Amresco (Solon, OH, USA). Purelink Genomic DNA extraction kit (catalog #K182001), Fetal Bovine Serum (FBS, catalog # A4766801), 10X Tris-borate-EDTA (TBE) buffer, and RNase-free distilled water were purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Designing primers, truncated target, and crRNA for HBV. The RPA primers were designed by aligning 51 HBV sequences of genotypes A to J - AB194951, AF090842, AJ309369, AY217375, U87742, X51970, MK355500 (genotype A); AB010292, AB031266, AB033554, AB033555, AF100309, D00329, U87747, D00330, AB073858 (genotype B); AB014381, AB048704, AB049609, AB241110, AY123041, X01587, X04615, X75665, M12906, AB033556 (genotype C); AY090453, M32138, V01460, X85254, X02496, X65259 (genotype D); AB032431, DQ060823, X75657 (genotype E); AB036905, AB036910, AB086397, AF223962, AF223965, AY090455, X69798 (genotype F); AB064310, AF160501, AF405706 (genotype G); AB059661, AY090454, AY090457, AY090460 (genotype H); AF241407 (genotype I); AB486012 (genotype J).[1–4] NCBI Multiple Sequence Alignment Viewer and SnapGene software (from Insightful Science; available at snapgene.com) were used for the sequence alignment. A highly conserved 24 base sequence, in the core protein reading frame (HBcAg),[2,5] and having a PAM sequence of TTTG was selected as the target sequence to be amplified (**Fig. S1**). Nucleic acid amplification strategies like RPA are prone to false positive results because of non-specific off-target amplicons being generated from interfering DNAs. To address the possibility of this systemic error, we screened three pairs of primers to ensure the amplification of a very specific target. These three sets of forward and reverse primers were designed for the aforementioned 24 nucleotide-long target site. All crucial features of the primers mentioned in the TwistDx design manual were retained. The crRNA for the

identification of the target amplicon was designed with a recognition sequence that was complementary to the target sequence, and a scaffold that associates with the Cas12a enzyme.[6,7]

Designing the modified hairpin probe. The decisive aspect while designing fluorogenic hairpin probes is the efficiency of energy transfer between the fluorophore and quencher. The design of the hairpin must not only guarantee the retention of its ability to change its conformation, i.e., from hairpin form (intramolecular base pairing) to linear form (intermolecular base pairing), but also induce a notable change in fluorescence upon said conformational change. Previously published reports on labelling nucleotides with fluorophores were used as a guide for designing the modified harpin sequence.[8–10] In a measure to alleviate cost, we added terminal oligo modifications, where the Fluorescein or FAM (F) and Iowa Black FQ (Q) molecules were added to the 5' and 3' end respectively. All nucleotide sequences are listed in **Table S1**.

Oligonucleotide preparation. As per the ATCC certificate of analysis, the 336 ng of HBV was dissolved in 33.6 µL of RNase-free DI water (RDI) to form a 1x solution having 1.2 x 10^9 copies/µL, while the 1x solution of HAV had 3.8 x 10^5 copies/µL. All other oligonucleotides were dissolved in RDI to form 1 mM stock solutions. These 1 mM stocks were diluted using RDI to form 100 µM stocks and their concentration was confirmed using Nanodrop Spectrophotometer. The 100 µM oligonucleotides were further diluted to form 10 µM solutions. 10 µM of all primers was prepared in RDI. 10 µM of crRNA was prepared in 1X Tris-EDTA buffer, pH 7.9 at 25 °C. 10 µM of $tDNA_{HBV}$ and $cDNA_{HBV}$, was prepared in **Cas buffer** - 10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl2, 100 µg/ml BSA, pH 7.9 at 25 °C (same as commercially available 1X NE Buffer r2.1). 10 µM of H1*, H1, H2 and *initiator* was prepared in **HCR buffer** - 50mM Na2HPO4・H2O, 500mM NaCl, pH 6.8 at 25 °C.

Testing efficiency of modified H1* probe (fHCR). To form hairpins, 100 µL of 1 µM H1*, 1 µM H1 and 1 µM H2 (diluted from the 10 µM solutions) were heated separately in 0.6 mL microtubes at 95ºC on a heat block for 4 minutes. The microtubes were then snap cooled in an ice bath for 4 minutes and incubated for 2 hours at room temperature (RT) before use. 1 µM *initiator* was prepared in HCR buffer (50mM Na_2HPO_4, 500mM NaCl, pH 6.8). Post 2 hours, H1*, H1, and H2 were mixed in a ratio of 1:3:4 such that the solution contained equimolar ratio of total H1 and H2. This solution of H1*/H1/H2 was added to two 0.6 mL microtubes. Next, HCR buffer was added to tube 1, and *initiator* to tube 2 ensuring that the amount of *initiator* was half the corresponding amount of H2, as previously demonstrated by us.[11] Final concentration of H1*/ H1/ H2 was 20 nM: 60 nM: 80 nM (1:3:4) and *initiator* was 40 nM. In a self-triggered reaction like fHCR, the ratio and concentration of the components i.e., H1*/H1/H2 and *initiator* determines the overall responsiveness and sensitivity of the reaction. We limited the concentration of H1* to <100 nM because it is enough to generate a detectable fluorescence signal

while having minimal background fluorescence. The concentrations of H1 and H2 were determined based on the concentration of H1*. Finally, the selected concentration of *initiator* (40nM) is just enough to initiate fHCR. Both tubes were incubated at RT overnight. Next day, the reaction mixture was added to a microplate to measure its fluorescence signal (λ_{ex} 485 nm, λ_{em} 520 nm) using a microplate reader, BioTek Synergy H1 Hybrid Multi-Mode Reader (**Fig. S5**).

Sensitivity of CRISPR-fHCR against different concentrations of dsDNA$_{HBV}$. Cas12a was mixed with crRNA in a 1:1.2 ratio in Cas buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 100 μg/ml BSA, pH 7.9) to form a 1 μM:1.2 μM Cas12a-crRNA complex. The complex was incubated in a 37ºC water bath for 30 minutes for its formation. tDNA$_{HBV}$ and cDNA$_{HBV}$ were hybridized in Cas buffer to form 1 μM of dsDNA$_{HBV}$ for detection of a fragment of HBV target. dsDNA$_{HBV}$ (0.5, 5, and 50 pM; and 0.5, 5, and 50 nM) was added to 6 tubes containing 60 nM of *initiator* and 60 nM of the prepared Cas12a-crRNA complex. To the 7th tube having the complex and *initiator*, Cas buffer was added instead. All tubes were incubated in a PCR Thermocycler for the following cycle – 60 minutes at 37 ºC for enzymatic activity and 15 minutes at 65 ºC for thermo-inactivation of Cas12a according to the manufacturer's instructions. During the incubation period, H1*, H1, and H2 (in hairpin form) were mixed to form a 120 nM:240 nM:360 nM H1*/H1/H2 solution. Post inactivation, 30 μL of the reaction was combined with 30 μL of the H1*/H1/H2 solution in a microplate. Final concentration of H1* was 60 nM. The fluorescence release was observed at RT for 2 hours at λ_{ex} 485 nm and λ_{em} 520 nm. Although, the '*signal turn off*' nature of H1* generates an inverse proportionality between the target concentration and signal response (**Fig. 1b inset**), the end point fluorescence can be processed to flip the generated response to '*signal turn on*' (**Fig. 1b)**. This was done by using the formula f2 = |f1-x|; where f2 = new fluorescence, f1 = original fluorescence, and x = 11,100. It must be noted here that the value of 'x' is selected based on 'f1' for '0 copies', 'x' should be such that the 'f2' for '0 copies' is > 500. To compare with a CRISPR-only reaction, 60 nM of traditional ssprobe (5' FAM-TTATT-3' Iowa BlackFQ,) was used instead of *initiator* and the enzymatic activity was observed in an incubated (37 ºC) plate reader for an hour. Here the response generated is '*signal turn on*', hence there is no need for further processing the fluorescence response (**Fig. S2**).

Primer screening for HBV. 25 μL of 2x Reaction Buffer, 9.2 μL of 10 mM dNTP mix, 5 μL 10x Basic E-mix, and 2.5 μL of 20x Core Reaction Mix, per RPA reaction, were combined to form an RPA master mix. 2.4 μL of primer 2F was added to PCR tubes 1-6. 2.4 μL of primers 1R, 2R, and 3R was added to tubes 1-3 and 4-6 in that order. 41.8 μL of the RPA master mix and 2.5 μL of 280 mM of magnesium acetate (MgOAc) was added to all 6 tubes. 1x HBV solution (1.2 x 10^9 copies/μL) was diluted to a 5000 copies/μL solution, and 1 μL was added to

tubes 1-3. 1 µL RDI was added to tubes 4-6. All tubes were incubated in a PCR Thermocycler for the following cycle – 30 minutes at 37 ºC for amplification and 15 minutes at 65 ºC for thermo-inactivation of all RPA enzymes. 1 µM:1.2 µM Cas12a-crRNA complex was prepared as before. A 75 nM:75 nM Cas mix was prepared by mixing the prepared Cas12a-crRNA complex with 10 µM of ssprobe. Post amplification, 12 µL of amplicon solution and 48 µL of the prepared Cas mix (final concentration of Cas12a-crRNA complex:ssprobe is 60 nM:60 nM) was added to a half-volume microplate. The microplate was quickly transferred to a preheated plate reader to record fluorescence recovery at 37 ºC for over 2 hours. The entire reaction was duplicated using 3R with 1F, 2F, and 3F as the forward primers (**Fig. S3**). Aseptic techniques and extensive disinfection steps were carried out to maintain a contamination-free work area. Two separate workstations were utilized for pre-RPA and post-RPA reactions.

Sensitivity of RACECAR against different amounts of HBV genome. 1x HBV solution was diluted to 8, 40, 200, 1000, 5000, and 25000 copies/µL, and 1 µL of each was added to 6 PCR tubes containing primers 2F/3R, RPA master mix, and MgOAc in the same quantities as before. 1 µL of RDI was added to 7th tube containing the same. All tubes were incubated at 37 ºC and 65 ºC for 30 minutes and 15 minutes respectively. 20 µL of amplicons from each tube was added to *initiator* and Cas12a-crRNA complex (both 60 nM). The tubes were filled up to 100 µL with Cas buffer and incubated at 37 ºC (60 minutes) and 65 ºC (15 minutes) for Cas12a activity and inactivation. 30 µL of each reaction was then combined with 30 µL of 120 nM:240 nM:360 nM H1*/H1/H2 solution in a microplate. Real-time HCR was observed at RT for 2 hours using the same wavelengths as before (**Fig. 2a**). Here too the fluorescence end-point reads were flipped to '*signal turn on*' using the previously explained method (**Fig. 2b-c**). To compare to an RPA and CRISPR-only reaction, everything was repeated with 60 nM ssprobe, and real-time CRISPR activity was observed at 37 ºC for 1 hour (**Fig. S4**).

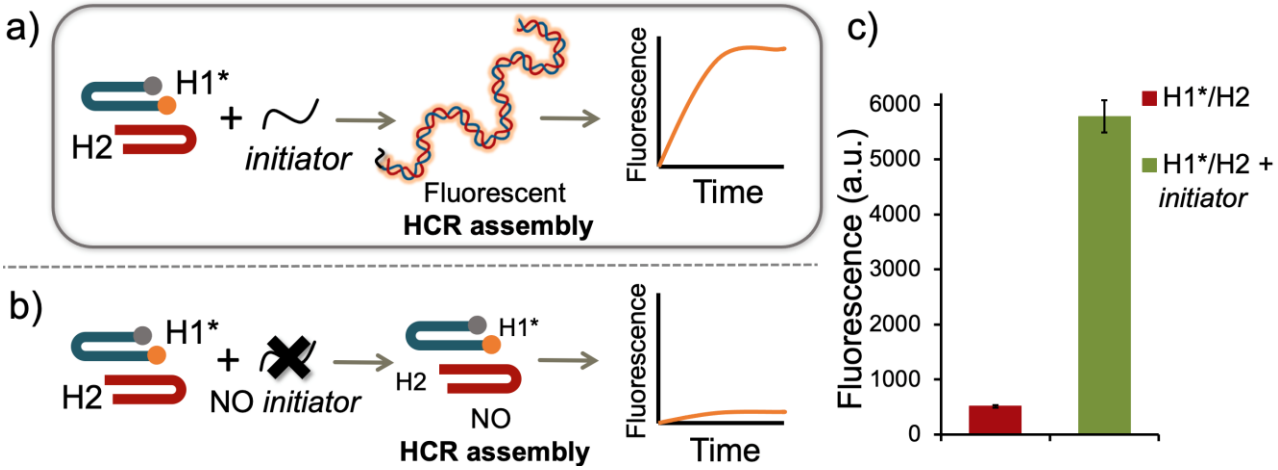
Specificity of and in-serum detection using RACECAR. 1x HBV solution and 1x HAV solution (3.8 x 10^5 copies/µL) was diluted to 200 and 5000 copies/µL. RPA was carried out exactly as before using 1 µL of HBV and HAV (200 and 5000 copies/µL) as template. The same reaction protocol and conditions were implemented for performing CRISPR-fHCR on the amplicons.

1x HBV solution was diluted to 200 µL solution of 5000 copies/µL HBV in fetal bovine serum (FBS). The protocol for genomic DNA extraction followed was as provided by the kit manufacturer. 100 µL of serum with 5000 copies/µL was used for extraction, ensuring 100 µL of RDI was used to elute the extracted DNA. This means that the concentration of HBV in serum after extraction is same as before i.e., 5000 copies/µL. The remaining 100 µL of the serum was left as is (no extraction performed). RACECAR was performed using the same protocol as

before, but with extracted and unextracted HBV (5000 copies/μL) as the template. The results from both these experiments were flipped to '*signal turn on*' like before (**Fig. 3**).

Statistical Analysis. The experiments were performed in triplicate and the error bars were calculated by standard deviation (SD). Data are represented as mean±SD. Regression analysis was performed using the Analysis Toolpak in Microsoft Excel to calculate the limit of detection (LOD = 3.3*[σ/s]) and limit of quantitation (LOQ = 10*[σ/s]), where 'σ' is the standard deviation of response and 's' is the slope of the calibration curve.[12–15] A paired t test was performed using RStudio (version 1.3.1073) to compare means and measure statistical differences. A p-value of < 0.05 at 95% confidence was considered statistically significant.

Scheme S1. Hybridization chain reaction (HCR) using fluorescent hairpin probes (fHCR) (a) with *initiator* (b) without *initiator* and (c) fluorescence end point reads for the same**.**



Scheme S2. Reaction mechanism of CRISPR-fHCR in the (a) presence and (b) absence of target DNA

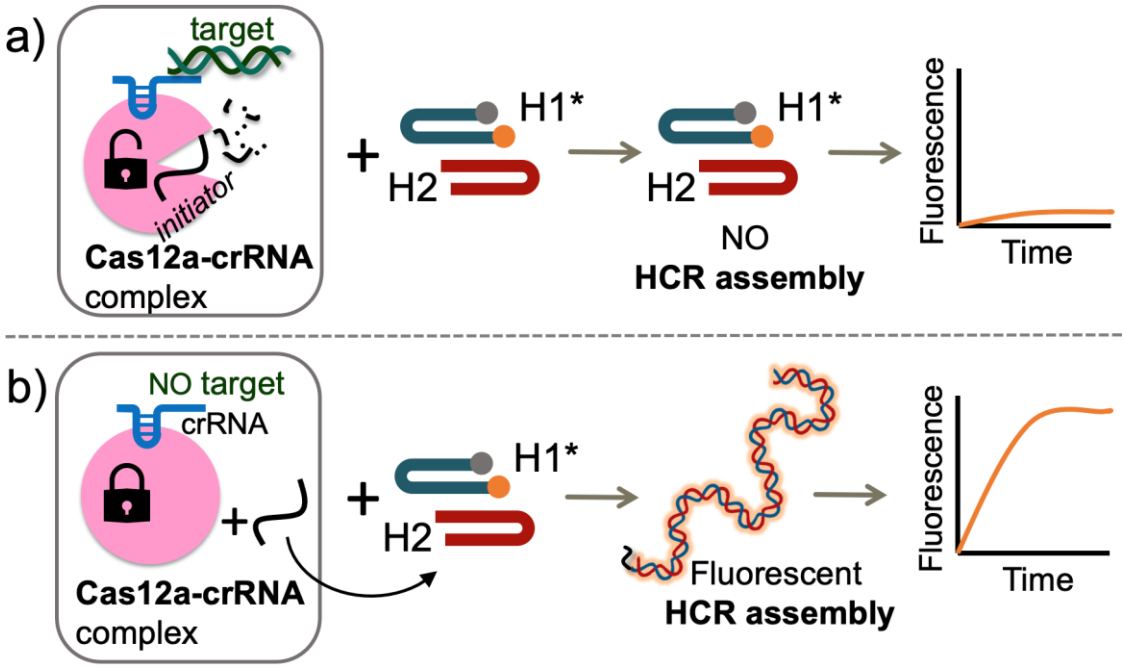


Table S1. List of oligonucleotide sequences. For crRNA, the recognition region is in bold and underlined. For truncated HBV DNA, target recognition site is in bold and underlined, with PAM highlighted in yellow.

Label	**Sequence**
1F (forward primer 1)	5'- TCC CAC TTT TCA AGC CTC CAA GCT GTG CCT TGG G -3'
2F (forward primer 2)	5'- CTT TTC AAG CCT CCA AGC TGT GCC TTG GGT GG -3'
3F (forward primer 3)	5'- TTC AAG CCT CCA AGC TGT GCC TTG GGT GGC TTT -3'
1R (reverse primer 1)	5'- GCA AAA ACG AGA GTA ACT CTA CAG TAG CTC CAA AT -3'
2R (reverse primer 2)	5'- CGA GAG TAA CTC TAC AGT AGC TCC AAA TTC T -3'
3R (reverse primer 3)	5'- AAA AAC GAG AGT AAC TCT ACA GTA GCT CCA AAT TC -3'
crRNA	5'- UAA UUU CUA CUA AGU GUA GAU **GGG CAU GGA CAU UGA CCC UU** -3'
$tDNA_{HBV}$ (truncated HBV target)	5'- ATT CTT TAT **AAG GGT CAA TGT CCA TGC CCC AAA** GCC ACC CAA GGC ACA GC -3'
$cDNA_{HBV}$ (complementary to $tDNA_{HBV}$)	5'- GCT GTG CCT TGG GTG GC**T TTG GGG CAT GGA CAT TGA CCC TT**A TAA AGA AT -3'
ssprobe	5'- /56-FAM/TTA TT/31ABkFQ/ -3'
initiator	5'- AGT CTA GGA TTC GGC GTG GGT TAA -3'
H1* (modified H1)	5'- /56-FAM/TTA ACC CAC GCC GAA TCC TAG ACT CAA AGT AGT CTA GGA TTC GGC GTG /31ABkFQ/ -3'
H1 (unmodified H1)	5'- TTA ACC CAC GCC GAA TCC TAG ACT CAA AGT AGT CTA GGA TTC GGC GTG -3'
H2	5'- AGT CTA GGA TTC GGC GTG GGT TAA CAC GCC GAA TCC TAG ACT ACT TTG -3'

Figure S1. Sequence logo of consensus sequence from alignment of 51 HBV sequences ranging from genotype A to J. The selected conserved region is in the core protein (HBcAg) reading frame and has a PAM sequence of TTTG.

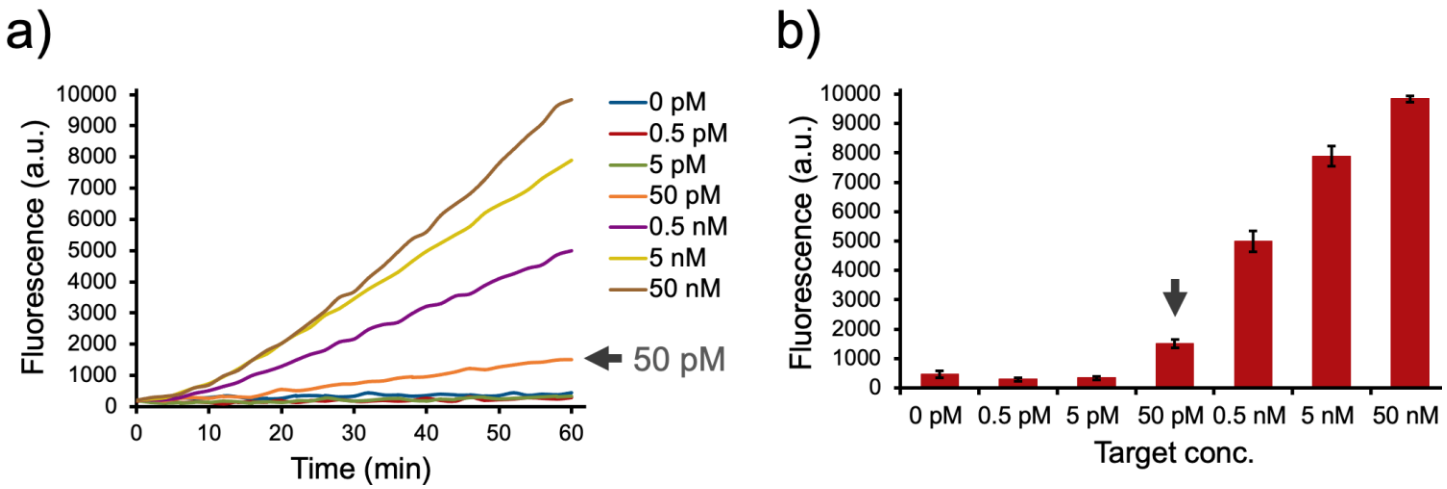


Figure S2. Sensitivity of CRISPR-only using ssprobe against different concentrations of $dsDNA_{HBV}$ depicted by (a) kinetic study and (b) end point fluorescence.

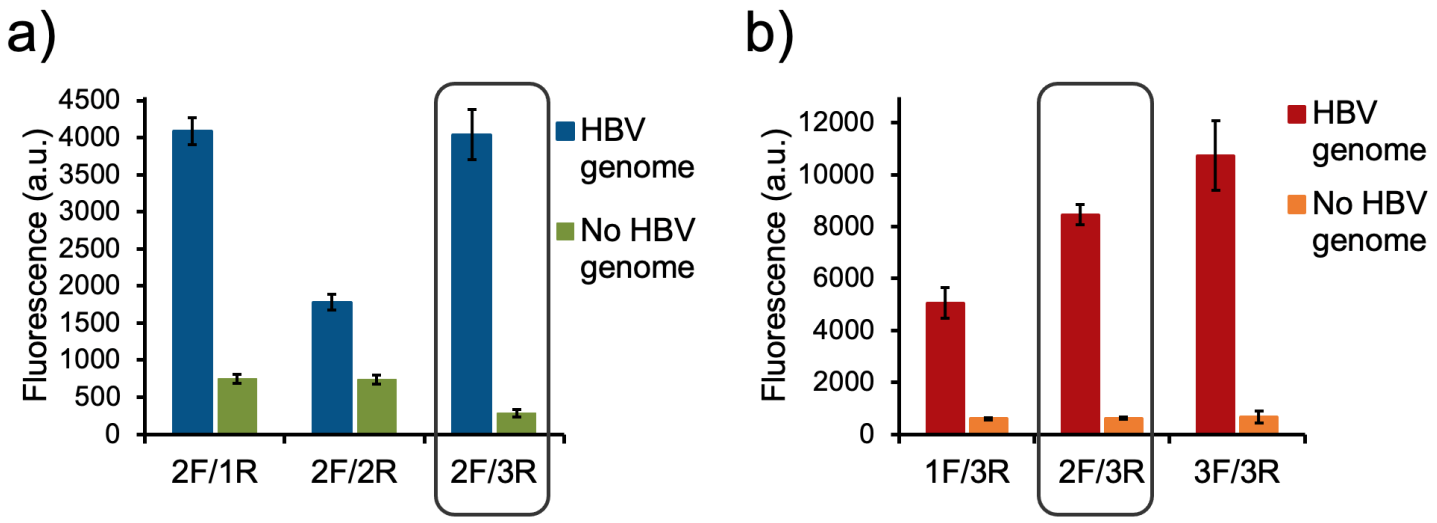


Figure S3. Primer screening with (a) different reverse primers and (b) different forward primers.

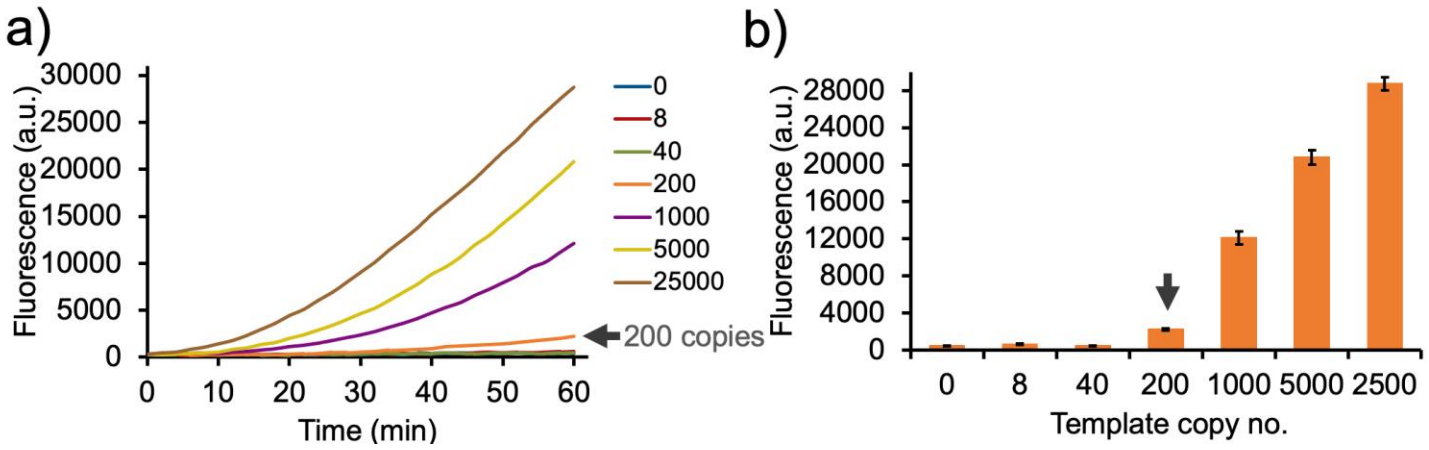


Figure S4. RPA and CRISPR-only protocol using ssprobe against different amounts of HBV template depicted by (a) kinetic study and (b) end point fluorescence.

Figure S5. Photo of the benchtop spectrofluorometer used for all fluorescence measurements.

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