Nanoscale

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Nanoscale*, 2022, **14**, 13500

Received 30th June 2022,
Accepted 11th September 2022

DOI: 10.1039/d2nr03590a

rsc.li/nanoscale

Recombinase amplified CRISPR enhanced chain reaction (RACECAR) for viral genome detection†

Mahera J. Kachwala, [a] Christopher W. Smith, [a] Nidhi Nandu [a] and Mehmet V. Yigit *[a,b]

We have developed a 'recombinase amplified CRISPR enhanced chain reaction' (RACECAR) assay that can detect as little as 40 copies of hepatitis B virus (HBV) genome using a benchtop spectrofluorometer. The limit of detection was determined to be 3 copies of HBV genome. The specificity of RACECAR was confirmed against hepatitis A virus (HAV). This assay can detect the genomic targets directly in serum samples without an extraction step. The 4 h-long fluorometric assay was developed by combining three tiers of isothermal amplification processes and can be repurposed for any target of choice. This highly modular reaction setup is an untapped resource that can be incorporated into the front-runners of molecular diagnostics.

1. Introduction

As evidenced by the disease outbreaks in recent times, viral infections continue to pose a significant threat to human health and global economy. Owing to high virus mutability and transmissibility, efforts for early intervention and disease management are critical.[1,2] Highly sensitive and precise nucleic acid-based diagnostics are key to bolster these efforts to mitigate the viral threat.[3] With that intent, here we report a diagnostic assay which was designed and tested against hepatitis B virus (HBV) genome. The detection assay encompasses three unique isothermal amplification steps, *i.e.*, hybridization chain reaction (HCR), CRISPR-Cas12a and recombinase polymerase amplification (RPA), which make the resulting assay highly sensitive for genome detection.

Hybridization chain reaction (HCR), first presented by Dirks and Pierce, is a cascade reaction between two complementary hairpin DNAs (H1 & H2) that is initiated by a single-stranded (ss-) *initiator* DNA (Scheme S1†).[4] Without the *initiator*, H1 and H2 remain in their hairpin conformation (Scheme S1b†). However, in the presence of the *initiator*, the hairpins begin to hybridize to each other, forming a long, nicked double-stranded (ds-) DNA (Scheme S1a†).[5] Due to opening of many hairpins with a single *initiator*, HCR has been employed as an enzyme-free amplification step for many biosensing and environmental screening studies.[6–8]

CRISPR-Cas12a, on the other hand, consists of a CRISPR RNA (crRNA) that has a Cas12a specific sequence and a target specific sequence. Once the Cas complex (Cas12a-crRNA) recognizes and binds to its target, the Cas endonuclease gets activated and consequently cleaves all DNA in its vicinity (Scheme S2a†).[9] As one target recognition event induces numerous DNA trans cleavage reactions, incorporating CRISPR-Cas12a with HCR reinforces the amplification feature of both reactions (Scheme S2†). Previously we have demonstrated that combining HCR with CRISPR-Cas12a enhanced the sensitivity and programmability of a gel-based short DNA detection assay developed in our lab.[10] Now, we have designed a fluorescence-based approach, which is refined with advanced preclinical applicability, by coupling them to a third isothermal reaction named recombinase polymerase amplification (RPA).[35]

RPA, introduced in 2006, is a replication reaction carried out by the strand-displacing polymerase – *Sau*DNA polymerase, *Escherichia coli* RecA recombinase, and single-strand DNA binding proteins (SSBs). These proteins along with other accessory proteins and cofactors facilitate the denature-free amplification process.[11] CRISPR diagnostics (CRISPR-Dx) namely DETECTR and SHERLOCK constitute a new wave of molecular biosensing which blends characteristic traits of Cas endonucleases with isothermal amplification reactions like RPA.[9,12] Although PCR has long been the gold standard of nucleic acid detection, the engineering of the aforementioned approaches has prompted the assimilation of more streamlined protocols

[a]*Department of Chemistry, University at Albany, State University of New York, 1400 Washington Avenue, Albany, New York 12222, USA. E-mail: myigit@albany.edu; Tel: (+1) 518-442-3002*

[b]*The RNA Institute, University at Albany, State University of New York, 1400 Washington Avenue, Albany, New York 12222, USA*

†Electronic supplementary information (ESI) available: The experimental mechanisms (Schemes S1 and S2) and details, list of materials, oligonucleotide sequences (Table S1), and additional data (Fig. S1–S5). See DOI: **https://doi.org/10.1039/d2nr03590a**

that are less reliant on heavily equipped laboratories and well-trained laboratory personnel.

In this diagnostic assay, we have demonstrated that a simple benchtop spectrofluorometer (Fig. S5†) can be used to detect as little as 40 copies of HBV genome through fluorescence readings. This approach, named Recombinase Amplified CRISPR Enhanced Chain Reaction (RACECAR), is demonstrated to be highly specific for HBV detection. However, it is important to note that RACECAR can be rapidly repurposed for the detection of any genomic target by simply replacing the crRNA content of the assay.

HBV is a circular, partially double stranded DNA of the *Hepanaviridae* family which is the major causative agent of viral hepatitis.[13,14] If left undiagnosed, HBV infections culminate in chronic hepatitis and other complications like hepatocellular carcinoma and liver cirrhosis.[15] In 2019 alone there were 1.5 million new HBV infections and 0.82 million deaths as a result of hepatitis and its associated causes.[16] Despite an efficacious vaccine and extensive immunization drives, HBV remains a burden that the WHO hopes to eradicate by 2030.[17]

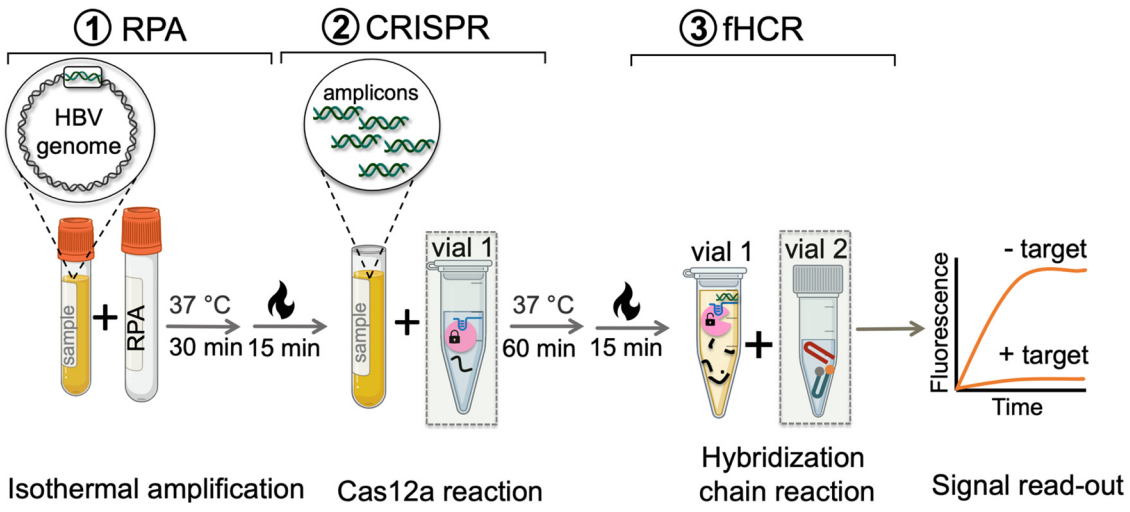
HBV diagnosis is commonly accomplished by using immunoassays that screen for hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg) or the viral capsid protein (HBcAg), which are secreted into the blood from virus infected liver cells.[18] Recently, nucleic acid based tests, where HBV DNA is directly detected in serum and plasma, are also being developed.[19,20] The viral load in the serum of HBV positive patients that are inactive carriers (carriers are also $HBsAg^+$) is typically less than 10^6 copies per mL because of reduced viral replication.[21] Due to the serum HBV DNA levels being so low, highly sensitive DNA-based diagnostic assays are essential.

With RACECAR, we first use RPA to amplify a conserved region in the core protein (HBcAg) reading frame of HBV genome. This was done by aligning 51 HBV sequences of genotypes A to J[22–25] using NCBI Multiple Sequence Alignment Viewer and SnapGene software (from Insightful Science; available at snapgene.com) (Fig. S1†). After 30 minutes of RPA, the reaction mixture containing the resulting amplicons is added to a vial containing Cas12a, a crRNA complementary to the target, and the *initiator* for 60 min. If the sample contains the HBV genome, amplicons are generated, crRNA binds to the amplicons and activates Cas12a which then cleaves the *initiator* in reaction mixture (Scheme 1). The cleaved *initiator* is unable to initiate HCR in the proceeding reaction vial containing H1 & H2 hairpins. In contrast, in the absence of an HBV genome, amplicons will not be generated, therefore Cas12a cannot be activated, which in turn cannot cleave the *initiator*. Consequently, the intact *initiator* initiates HCR in the proceeding reaction vial with the hairpin pairs.

As a result, the absence of HBV genome is confirmed by occurrence of HCR (fluorescence recovery) after three amplification steps, whereas its presence is verified by the impediment of HCR. The occurrence of HCR is monitored through fluorescence measurements (fHCR, Scheme S1†). In fHCR, H1 is labelled with a fluorophore (F) and quencher (Q) pair (H1*) such that, when closed, the fluorescence of H1* remains quenched. However, when H1* is open (as a result of HCR) the fluorescence recovers (Scheme S1c†).

2. Results and discussion

First, we tested whether the CRISPR and fHCR couple functions by itself as intended without RPA. The detection capacity of CRISPR–fHCR was tested by using different concentrations of a truncated HBV genome, $dsDNA_{HBV}$ (0.5 pM, 5 pM, 50 pM, 0.5 nM, 5 nM, 50 nM; Fig. 1a). As the amount of target increased, more *initiator* got cleaved. Upon subsequent addition of H1* and H2, less HCR takes place as evidenced with a lower fluorescence recovery (Scheme S2a,† and Fig. 1a). Although, the '*signal turn off*' nature of H1* generates an



Scheme 1 Reaction protocol for recombinase amplified CRISPR enhanced chain reaction *i.e.*, RACECAR.

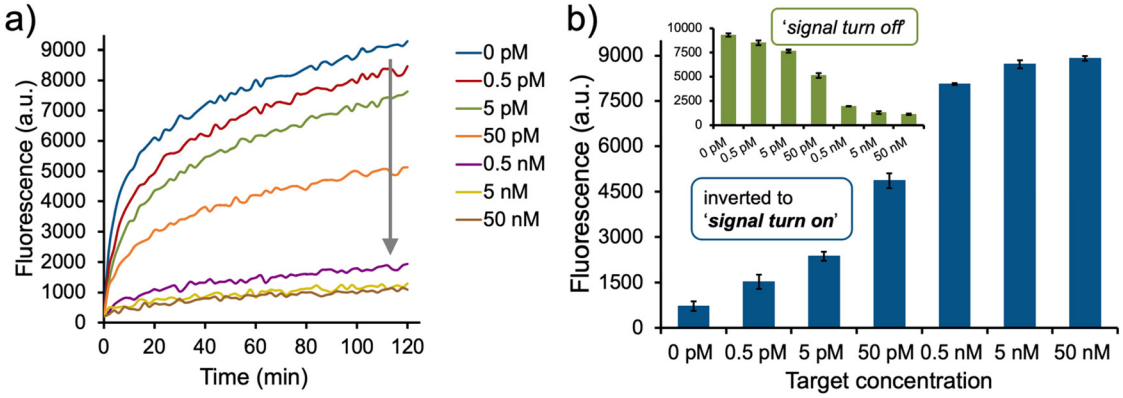


Fig. 1 Sensitivity of CRISPR–fHCR against different concentrations of $dsDNA_{HBV}$ depicted by (a) kinetic study *'signal turn off'* and (b) end point fluorescence *'signal turn on'*.

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, Methods in ESI†). The data show that as little as 0.5 pM (~3.0×10^8 copies per mL; *p*-value <0.05 with 0 pM) can be detected using CRISPR–fHCR. This is an improvement to typical CRISPR-alone methods where detection limit is about 10 pM.[26–28] To demonstrate the amplification validity of HCR, we re-run the study with traditional F and Q-labeled ssDNA probe (5′ FAM-TTATT-3′ Iowa BlackFQ, *i.e.* 'ssprobe') instead of fHCR, where we were only able to detect 50 pM of $dsDNA_{HBV}$, a 100-fold depreciation than CRISPR–fHCR (Fig. S2†). This signifies the vigor of HCR amplification and its importance for our assay's sensitivity.

To detect (1) intact real HBV genome and (2) increase the sensitivity beyond clinical levels of detection (<10^6 copies per mL), down to less than hundred copies, we acquired whole HBV genome from American Type Culture Collection (ATCC). The target genome was tested with our three-step amplification reaction and was compared to hepatitis A genome (HAV, negative control). The target genome was first processed with RPA, prior to CRISPR–fHCR reactions, with a select pair of primers. For accurate amplification of the conserved region of HBV, all primer combinations were screened beforehand and the optimum combination of 2F and 3R was selected and used for the RACECAR assay (Fig. S3†).

To test the sensitivity of RACECAR, the detection was performed using different amounts of HBV genome (8, 40, 200, 1000, 5000, and 25 000 copies). As expected, an increasing amount of genomic target resulted in more amplicons being generated. These amplicons then activated more Cas complex and caused multiple *initiators* being cleaved. Since less *initiators* were left intact, the rate of fHCR and H1* linearizing was low. Consequently, a lower fluorescence signal was generated (Fig. 2a). The end-point fluorescence signal was inverted ('*signal turn on*') like before for ease of demonstration and the graph indicates that as little as 40 copies (~160 copies per mL; *p*-value <0.05 with 0 copies) of HBV can be detected by this method (Fig. 2b). This makes detection of HBV in even inactive carriers highly plausible as it is well below the threshold of 10^6 copies per mL.

To further evaluate the preclinical significance of the assay, a regression analysis was performed on the data obtained in the previous experiment. The linear graph had an R^2 of 0.9906, while the limit of detection and quantitation (LOD, LOQ) was 3 copies (~12 copies per mL) and 26 copies (~104 copies per mL) respectively (Fig. 2c).

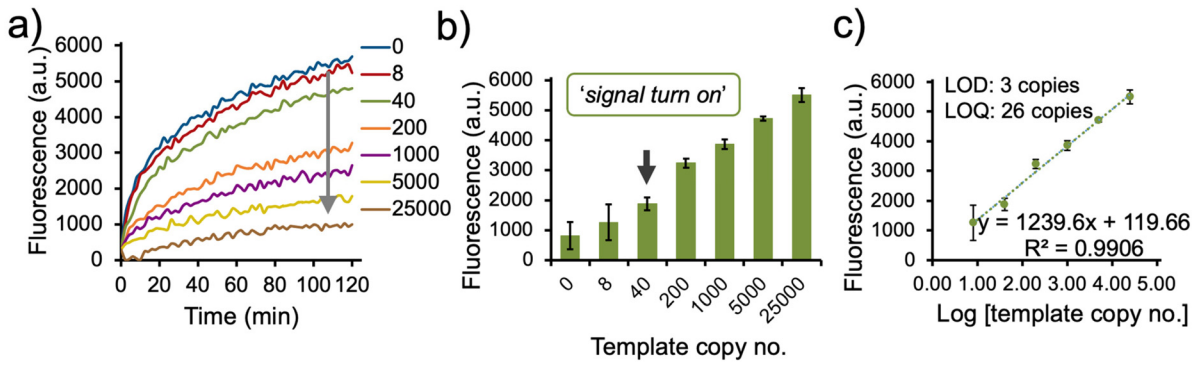


Fig. 2 Sensitivity of RACECAR against different amounts of HBV template depicted by (a) kinetic study *'signal turn off'*, (b) end point fluorescence *'signal turn on'* and (c) linear regression for LOD and LOQ.

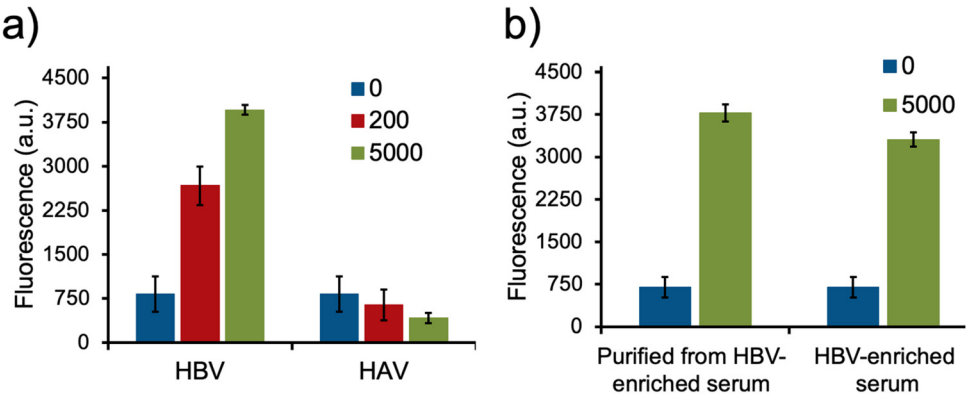


Fig. 3 (a) Specificity of RACECAR against 200 and 5000 copies of HBV and HAV genomes. (b) Application of RACECAR against serum samples with 5000 copies of HBV genome after and before extraction step.

Our assay has a greater LOD and is point-of-care friendly compared to a previously reported real-time PCR assay having a detection limit of 10 HBV copies,[29] a recent electrochemistry-based DNA diagnostic reporting an LOD of 10 copies per μL,[30] and another assay also employing an isothermal amplification reaction that managed to achieve an LOD of 93.4 copies.[31]

For further internal comparison, an RPA and CRISPR-only protocol (control assay), where ssprobe was used to examine Cas cleavage activity, was also performed. In this study, where fHCR was excluded, we were able to detect down to 200 copies of HBV (~800 copies per mL; p-value <0.05 with 0 copies; Fig. S4†). In contrast to our 40 copies detection performance, this depreciation in the detection capacity is substantial. Although incorporating fHCR increases the assay time by 2 hours, the 5-fold increase in sensitivity is very significant. Compared to previously reported assays, RACECAR is particularly advantageous for detection of pathogens that require fewer copy numbers to cause severe infections.[9,32,33] The RACECAR assay is a highly modular setup, by changing the crRNA element of the assay only, RACECAR can be programmed for any specific target of choice including bacterial and viral plant, animal and human pathogens.

Nucleic acid amplification strategies like RPA are often susceptible to false positives as a result of non-specific amplification of contaminating or interfering DNAs.[34] Hence, in addition to high sensitivity, a diagnostic must also illustrate high selectivity in its detection. To check the specificity of RACECAR, hepatitis A virus (HAV) genome from ATCC was used as an off-target DNA. From our previous sensitivity study (Fig. 2), we picked two of the higher copy numbers, *i.e.*, 200 and 5000, to ensure that HAV can't generate a false-positive signal even at copy numbers greater than the LOD of the assay. The specificity studies were performed using both HBV and HAV. For HAV, insignificant signal variation was obtained between the control (0 copies) and experimental wells for both 200 and 5000 copies. On the other hand, for HBV, the signals obtained for both copy numbers were significantly higher than the control (Fig. 3a). Therefore, despite the increased possibility of false positives by way of amplification, RACECAR can successfully differentiate between the two types of viral hepatitis.

Ultimately, we tested serum samples with our diagnostic to estimate the preclinical relevance of RACECAR. To mimic a preclinical scenario, two serum samples were prepared by enriching the serum with HBV genome. One sample was processed using a genomic extraction kit prior to the assay while the other one was tested without the extraction step. The data indicate that regardless of the extraction step, RACECAR was able to detect HBV in the serum sample (Fig. 3b). This is noteworthy because the robustness of our system has eliminated a genomic extraction step, thus reducing human error, reaction time, cost, and expertise required to perform the protocol.

3. Conclusion

In conclusion, we have designed a detection system that is fortified with three signal amplifying reactions. This isothermal process called RACECAR, has been shown to detect even low copies of HBV genome using a single instrument – a standard benchtop microplate fluorometer. The sensitivity of RACECAR is matched by its high specificity. While we were able to distinguish between the two hepatitis types – HBV and HAV with high accuracy, RACECAR can be programmed for detecting HAV or other pathogens by simply redesigning the crRNA. Finally, RACECAR can also detect genomic DNA in serum samples, without requiring a DNA purification step. Thus, a significant advantage of this approach is that it is a point-of-care test that can also be utilized in endemic prone regions that often have limited resources.

Author contributions

M.V.Y and M.J.K conceived the idea and deliberated over the experimental set up for the study. M.J.K performed all the experiments and analyzed the pertaining data. C.W.S and N.N

provided intellectual contribution. M.V.Y and M.J.K wrote the paper.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

This work is supported in part by the USDA National Institute of Food and Agriculture (NIFA), AFRI project (2018-67021-27973, 2017-07822) and National Institute of Health (NIH) 1R15GM12811501.

References

1 M. M. Ling, C. Ricks and P. Lea, *Expert Rev. Mol. Diagn.*, 2007, **7**, 87–98.
2 K. M. Abu-Salah, M. M. Zourob, F. Mouffouk, S. A. Alrokayan, M. A. Alaamery and A. A. Ansari, *Sensors*, 2015, **15**, 14539–14568.
3 N. R. Y. Ho, G. S. Lim, N. R. Sundah, D. Lim, T. P. Loh and H. Shao, *Nat. Commun.*, 2018, **9**, 1–11.
4 R. M. Dirks and N. A. Pierce, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 15275–15278.
5 J. Ikbal, G. S. Lim and Z. Gao, *Trends Anal. Chem.*, 2015, **64**, 86–99.
6 M. Rana, M. Balcioglu, M. Kovach, M. S. Hizir, N. M. Robertson, I. Khan and M. V. Yigit, *Chem. Commun.*, 2016, **52**, 3524–3527.
7 M. Balcioglu, M. Rana, M. S. Hizir, N. M. Robertson, K. Haque and M. V. Yigit, *Adv. Healthcare Mater.*, 2017, **6**, 1600739.
8 M. Rana, M. Balcioglu, N. M. Robertson, M. S. Hizir, S. Yumak and M. V. Yigit, *Chem. Sci.*, 2017, **8**, 1200–1208.
9 J. S. Chen, E. Ma, L. B. Harrington, M. da Costa, X. Tian, J. M. Palefsky and J. A. Doudna, *Science*, 2018, **360**, 436–439.
10 M. J. Kachwala, C. W. Smith, N. Nandu and M. V. Yigit, *Anal. Chem.*, 2021, **93**, 1934–1938.
11 R. K. Daher, G. Stewart, M. Boissinot and M. G. Bergeron, *Clin. Chem.*, 2016, **62**, 947–958.
12 C. Myhrvold, C. A. Freije, J. S. Gootenberg, O. O. Abudayyeh, H. C. Metsky, A. F. Durbin, M. J. Kellner, A. L. Tan, L. M. Paul, L. A. Parham, K. F. Garcia, K. G. Barnes, B. Chak, A. Mondini, M. L. Nogueira, S. Isern, S. F. Michael, I. Lorenzana, N. L. Yozwiak, B. L. MacInnis, I. Bosch, L. Gehrke, F. Zhang and P. C. Sabeti, *Science*, 2018, **360**, 444–448.
13 T. M. Welzel, W. J. Miley, T. L. Parks, J. J. Goedert, D. Whitby and B. A. Ortiz-Conde, *J. Clin. Microbiol.*, 2006, **44**, 3325–3333.
14 M. Shariati, M. Sadeghi and S. H. R. Shojaei, *Anal. Chim. Acta*, 2022, **1195**, 339442.
15 Y. Tao, K. Yi, H. Wang, H. W. Kim, K. Li, X. Zhu and M. Li, *J. Colloid Interface Sci.*, 2022, **613**, 406–414.
16 World Health Organisation, *Global progress report on HIV, viral hepatitis and sexually transmitted infections*, 2021, vol. 53.
17 A. W. Gani, W. Wei, R. Z. Shi, E. Ng, M. Nguyen, M. S. Chua, S. So and S. X. Wang, *Sci. Rep.*, 2019, **9**, 1–11.
18 M. Krajden, G. McNabb and M. Petric, *Can. J. Infect. Dis. Med. Microbiol.*, 2005, **16**, 65–72.
19 W. M. Hassen, C. Chaix, A. Abdelghani, F. Bessueille, D. Leonard and N. Jaffrezic-Renault, *Sens. Actuators, B*, 2008, **134**, 755–760.
20 X. Li, K. Scida and R. M. Crooks, *Anal. Chem.*, 2015, **87**, 9009–9015.
21 S. Margeridon, S. Carrouée-Durantel, I. Chemin, L. Barraud, F. Zoulim, C. Trépo and A. Kay, *Antimicrob. Agents Chemother.*, 2008, **52**, 3068–3073.
22 S. Sun, S. Meng, R. Zhang, K. Zhang, L. Wang and J. Li, *Virol. J.*, 2011, **8**, 227.
23 N. Khodadad, S. S. Seyedian, A. Moattari, S. Biparva Haghighi, R. Pirmoradi, S. Abbasi and M. Makvandi, *Heliyon*, 2020, **6**, e04332.
24 T. T. T. Huy, T. T. Ngoc and K. Abe, *J. Virol.*, 2008, **82**, 5657–5663.
25 K. Tatematsu, Y. Tanaka, F. Kurbanov, F. Sugauchi, S. Mano, T. Maeshiro, T. Nakayoshi, M. Wakuta, Y. Miyakawa and M. Mizokami, *J. Virol.*, 2009, **83**, 10538–10547.
26 C. W. Smith, N. Nandu, M. J. Kachwala, Y. S. Chen, T. B. Uyar and M. V. Yigit, *Biochemistry*, 2020, **59**, 1474–1481.
27 K. Hsieh, G. Zhao and T. H. Wang, *Analyst*, 2020, **145**, 4880–4888.
28 C. W. Smith, M. J. Kachwala, N. Nandu and M. V. Yigit, *ACS Synth. Biol.*, 2021, **10**, 1785–1791.
29 A. Abe, K. Inoue, T. Tanaka, J. Kato, N. Kajiyama, R. Kawaguchi, S. Tanaka, M. Yoshiba and M. Kohara, *J. Clin. Microbiol.*, 1999, **37**, 2899–2903.
30 S. Khaliliazar, L. Ouyang, A. Piper, G. Chondrogiannis, M. Hanze, A. Herland and M. M. Hamedi, *ACS Omega*, 2020, **5**, 12103–12109.
31 X. Fan, L. Li, Y. Zhao, Y. Liu, C. Liu, Q. Wang, Y. Dong, S. Wang, T. Chi, F. Song, C. Sun, Y. Wang, D. Ha, Y. Zhao, J. Bao, X. Wu and Z. Wang, *Front. Microbiol.*, 2020, **11**, 1696.
32 M. I. Mustafa and A. M. Makhawi, *J. Clin. Microbiol.*, 2021, **59**, e00745–e00720.
33 J. S. Gootenberg, O. O. Abudayyeh, M. J. Kellner, J. Joung, J. J. Collins and F. Zhang, *Science*, 2018, **360**, 439–444.
34 A. Borst, A. T. A. Box and A. C. Fluit, *Eur. J. Clin. Microbiol.*, 2004, **23**, 289–299.
35 O. Piepenburg, C. H. Williams, D. L. Stemple and N. A. Armes, *PLoS Biol.*, 2006, **4**, 1115–1121.

