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Target-independent hybridization chain reaction-fluorescence resonance energy transfer for sensitive assay of ctDNA based on Cas12a

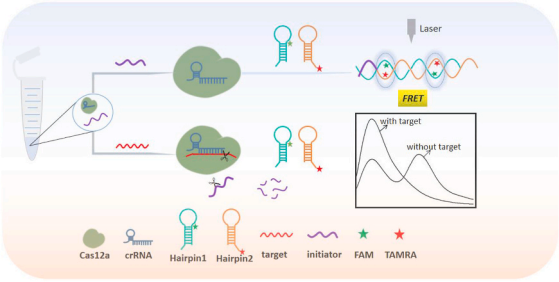
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HIGHLIGHTS

- A general fluorescent signal system, Hybridization chain reaction-Fluorescence resonance energy transfer (HCR-FRET) is proposed and optimized for sensitive assay of ctDNA.
- Theindependent of target HCR-FRET system, could be simply transplanted to the assay of other targets in parallel, and explanted into other assay protocols.
- This assay strategy is a post-amplification mode.

GRAPHICAL ABSTRACT



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ABSTRACT

Circulating tumor DNA (ctDNA) is a noninvasive biomarker which offer valuable information for cancer diagnosis and prognosis. In this study, a target-independent fluorescent signal system, Hybridization chain reaction-Fluorescence resonance energy transfer (HCR-FRET) system, is designed and optimized. Combined with CRISPR/Cas12a system, a fluorescent biosensing protocol was developed for sensing assay of T790 M. When the target is absent, the initiator remains intact, opens the fuel hairpins and triggers the following HCR-FRET. At presence of the target, the Cas12a/crRNA binary complex specifically recognizes the target, and the Cas12a *trans*-cleavage activity is activated. As a result, the initiator is cleaved and subsequent HCR responses and FRET processes are attenuated. This method showed detection range from 1 pM to 400 pM with a detection limit of 316 fM. The target independent property of the HCR-FRET system endows this protocol a promising potential to transplant to the assay of other DNA target in parallel.

1. Introduction

Statistics reveal that in 2020, lung cancer has the second highest incidence rate and the highest mortality among of all malignancies in China and worldwide [1,2]. Non–small cell lung cancer (NSCLC), one main type of lung cancer, accounts for the majority (approximately 80%) of the cases and has proven difficulty to treat due to its high incidence of postoperative recurrence and metastasis [3]. Studies have shown that patients harboring Epidermal growth factor receptor (EGFR) mutations, one of the most common driver gene in NSCLC, benefit from

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targeted therapy with EGFR-tyrosine kinase inhibitor (TKIs), such as gefitinib and erlotinib. However, acquired resistance develops usually after 10–14 months of TKI treatment for NSCLC patients. Among them, patients with the EGFR T790 M mutation (Thr790Met), a gatekeeper mutation, contribute about half of the resistant cases [4–6]. With the recent availability of the third-generation of EGFR inhibitor, such as osimertinib, the presence of EGFR T790 M identifies a clinical subset with a good prognosis and indolent progression [6].

In 1977, Leon first reported the correlations of cell free DNAs in serum and therapy effect against cancers [7]. Since then, more researches revealed that the levels of circulating tumor DNA (ctDNA), free DNA fragments released from primary tumors cells, are closely related to disease progression, metastasis and clinical outcome [8,9]. With the development of assay technologies, several methods, ddPCR [10], flow cytometry [11], electrochemistry [12], fluorescence [13], have been developed for selective analysis of ctDNAs. However, considering the low abundance of ctDNAs, sensitive, robust and cost/time effective recognition methods of ctDNAs, especially T790 M, are still in need [14,15].

Clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein (Cas)-based diagnostics have the potential to fulfil these unmet needs. CRSIPR systems are an essential part of microbial adaptive immune system against virus invasion [16]. To date, CRISPR-Cas systems including Cas9、Cas12a、Cas13a etc, have been repurposed for a wide range of applications, including diagnosis [17–20], cell imaging [21,22], transcription modulation [23] and gene therapy [24]. Its ability to accurately identify and slice specific nucleic acid sequences has endowed CRISPR-based detection and diagnostics one of the promising disruptive technologies of 2022 [25].

Also, various amplification systems, such as RCA [26], LAMP [27], NASBA [28], were employed to furtherly improve the sensitivity of the CRISPR/Cas system in bio-assay application. These new technologies based on CRISPR/Cas systems have demonstrated their potential to provide low-cost and practical diagnostic tools for the detection of pathogens and diseases [29–32], and have been widely used in biomedical detection [33]. However, most of them are pre-amplification mode, amplifications before the enzymatic digestion of probes via Cas proteins. The pre-amplification mode mainly depends on the duplication of target sequence, so that the design of the amplification unit has to modulate according the target.

Hybridization chain reaction (HCR), first developed by Dirks and Piece [34] is a kinetics-controlled process involving a series of repeated hybridization events between metastable DNA probes. The final product is usually a long double-stranded DNA (dsDNA), which provides excellent signal amplification efficiency. With advantages like great assembly kinetics, facile operation, and enzyme-free and isothermal reaction, HCR are integrated with mainstream methods, like fluorescence [35], electrochemistry [36], and colorimetry [37] for ultrasensitive detection of abundant targets [38].

Cas12a is an endonuclease of the Class II typeV type in the CRISPR/Cas system. Once the target-binding crRNA integrated into the Cas12a, Cas12a/crRNA/target complex exhibits *trans*- and *cis*-cleavage activity and chops up any ssDNA present in the vicinity [39,40]. Herein, a post-amplification mode, a hybridization-chain-reaction fluorescence-resonance-energy-transfer Cas12a-based assay strategy (HCR-FRET CRISPR assay), is raised and verified. Generally, the targets (T790 M positive ctDNAs), hybridize with the crRNA and trigger the *trans*-cleavage activity of Cas12a. As a result, the initiator sequence will be digested and no FRET signal is detected. At the absence of target, the initiator survived and threshold the following HCR so that FRET happens. Fluorescence resonance energy transfer (FRET) are employed as signals to monitor and characterize the target concentrations. The FRET system employs the ratio of fluorescent signal between acceptor and donor moiety as illustrator, so that the false positive could be avoided. The developed signal-amplification unit HCR-FRET system is independent of the target sequence and can be extend to the assay of other DNA targets.

Table 1
Base sequences used in this work.

Name	Sequence (5′-3′)
Target	CTCCACCGTGCAGCTCATCATGCAGCTCATGCCCTTCG
Initiator	AAGAGAGAACAAGCAGGAATT
crRNA	UAAUUUCUACUAAGUGUAGAUGCAUGAGCUGCAUGAUGAGCUG
NTC	TGTACTCACCGGTTCCGCAGACCACCTGATCCGGACTA
H1	AATTCCTGCTTGTTCTCTCTTAAGAGAAAGAGAGAACAAGCA
H2	AAGAGAGAACAAGCAGGAATTTGCTTGTTCTCTCTTTCTCTT
H3	AATTCCTGCTTGTTCTCTCTT(FAM)AAGAGAAAGAGAGAACAAGCA
H4	AAGAGAGAACAAGCAGGAATTTGCTTGTTCTCTCTTTCTCTT (TAMRA)
H5	AAGAGAGAACAAGCAGGAATTTGCTTGTTCTCTCTTTCT(TAMRA) CTT
H6	AAGAGAGAACAAGCAGGAATTTGCTTGTTCTCTCTT(TAMRA) TCTCTT
H7	AAGAGAGAACAAGCAGGAATTTGCTTGTTCTCT(TAMRA) CTTTCTCTT
H8	AAGAGAGAACAAGCAGGAATTTGCTT(TAMRA) GTTCTCTCTTTCTCTT
MT1 (WT)	CTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCG
MT2	CTCCACCGTGCAGCACATGATGCAGCTCATGCCCTTCG
MT3	CTCCACCGTGCAGCACATGATGCAGGTCATGCCCTTCG
probe	5′6-FAM-TTATT-3′BHQ1

Instruments: Fluorescence spectrum was performed on a F97 fluorescence spectrometer (Shanghai, China). Tanon electrophoresis analyzer (Tanon 2500R, Shanghai, China) was used for electrophoresis analysis.

2. Materials and method

2.1. Material and instruments

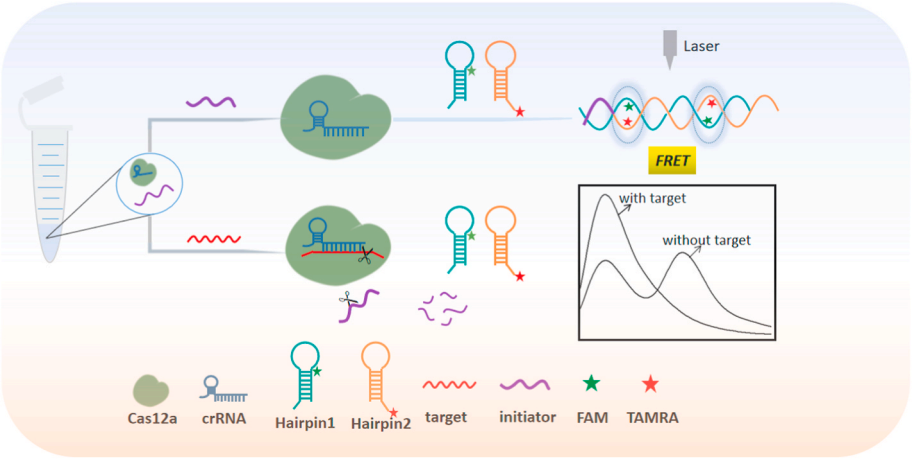
Materials: LbCas12a protein was purchased from Magigen Co., Ltd. (Guangzhou, China). QIAamp Circulating Nucleic Acid Kit (50) was ordered from QIAGEN Co., Ltd (Shanghai, China). Magnesium sulfate was obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and DEPC-Treated water were purchased from Thermo Fisher (Shanghai, China). Other chemicals were of analytical grade and used without further purification. All solutions were prepared with double distilled water (18 MΩ cm^{-1}) from a Milli-Q purification system (Millipore Corp, Milford, MA) and stored at 4 °C. All the nucleic acid sequence synthesis was provided by Sangon Biotech (Shanghai, China). The nucleic acid sequences are shown in Table 1.

2.2. Cas12a collateral cleavage activity

To form annealed hairpins, crRNA was denatured at 76 °C for 5 min, and then slowly cooled to room temperature. Cas12a collateral activity was investigated by using 50 nM Cas12a, 50 nM crRNA, 125 nM reporters, and varying concentrations of target in reaction buffer. The solutions were incubated at 37 °C for 30 min before fluorescent assay or gel electrophoresis.

2.3. Gel electrophoresis

To verify the Cas12a collateral cleavage activity and HCR, polyacrylamide gel electrophoresis (PAGE) analysis were performed. A 15% polyacrylamide gel was prepared with 10 x TBE buffer. The reaction products were diluted by using 6 x loading buffer with a volume ratio of 5:1 for gel electrophoresis. The electrophoresis was performed at 80 V for 100 min in 1 x TBE buffer. The gel was stained with Gel-Red Stain and imaged by the UV transilluminator (Bio-Tanon, Shanghai, China).



Scheme 1. Schematic illustrated the principle of the HCR-FRET CRISPR assay strategy.

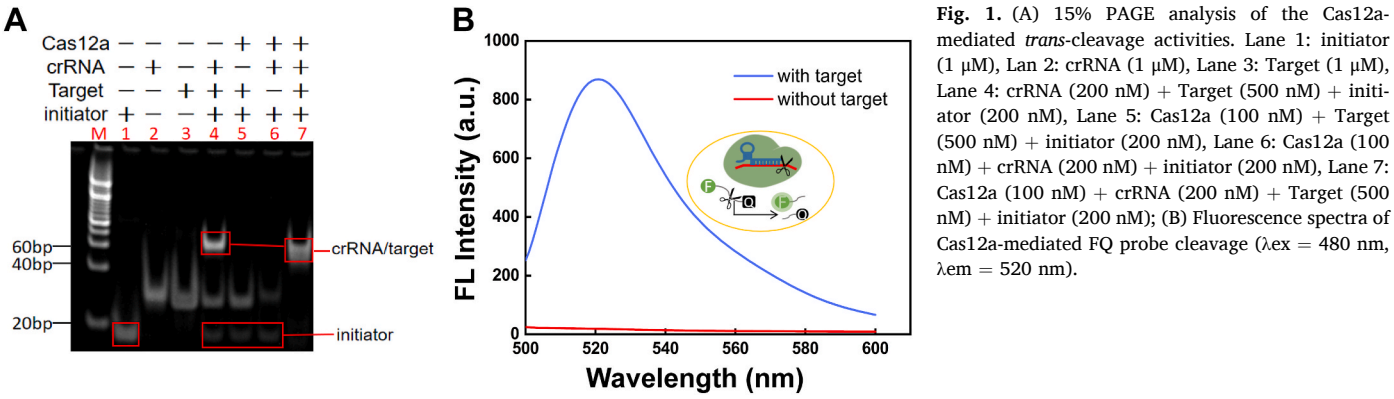


Fig. 1. (A) 15% PAGE analysis of the Cas12a-mediated *trans*-cleavage activities. Lane 1: initiator (1 μM), Lan 2: crRNA (1 μM), Lane 3: Target (1 μM), Lane 4: crRNA (200 nM) + Target (500 nM) + initiator (200 nM), Lane 5: Cas12a (100 nM) + Target (500 nM) + initiator (200 nM), Lane 6: Cas12a (100 nM) + crRNA (200 nM) + initiator (200 nM), Lane 7: Cas12a (100 nM) + crRNA (200 nM) + Target (500 nM) + initiator (200 nM); (B) Fluorescence spectra of Cas12a-mediated FQ probe cleavage (λex = 480 nm, λem = 520 nm).

2.4. Detection of ctDNA in buffer/clinical specimen using the Cas-FRET biosensing technique

Cas 12a cleavage reaction: To form annealed hairpins, crRNA was denatured at 76 °C for 5 min, and then slowly cooled to room temperature. Cas12a and crRNA, were mixed with different concentrations of target and initiator/probe sequences. The final concentration of Cas12a was 50 nM as instructed and the final concentration of crRNA was 50 nM for cleavage activity confirmation and 100 nM for other reactions. Then, the solutions were incubated at 37 °C for 30 min for Cas12a cleavage before HCR, fluorescent assay or gel electrophoresis.

HCR reaction: All the H# (1–8) sequences were incubated at 95 °C for 5 min and slowly cooled to room temperature to form annealed hairpins before HCR. The incubation time and temperatures for HCR were detailed explored. All H1∼H8 were diluted in 10 mM PBS buffer (pH = 7.4). Other were diluted in reaction buffer (1 x reaction buffer).

Fluorescence assay: Fluorescence spectrum were employed to explore performance of the HCR-FRET-Cas12a system. The reaction products were excited with 480 nm laser and the emission spectrum were recorded from 500 nm to 650 nm.

3. Results and discussion

3.1. General mechanism

Based on the nonspecific *trans*-cleavage activity, a HCR-FRET Cas12a assay strategy is proposed in Scheme 1 for the detection of ctDNA (T790 M). Generally, an efficient and universal HCR-FRET sensing system is designed. When the target is absent, the Cas 12a protein remains inactive and the initiators survive to trigger the downstream HCR amplification. As a result, the donor and acceptor moieties are pulled close to each other so that the fluorescence energy transfers between them, and the fluorescence of acceptor moiety is recorded. At the presence of the target, the target/crRNA complex is formed and activates the *trans*-cleavage activity of Cas12a/crRNA. As a result, the initiator is digested, and the downstream HCR amplification could not be initiated and no fluorescence signal of acceptor moiety could be detected.

3.2. CRISPR-Cas12a system verification

Polyacrylamide gel electrophoresis (PAGE) and Fluorescence Spectrum were employed to confirm the cleavage activity of CRISPR/Cas12a. As shown in Fig. 1A, strips in lane 1 to 3 represented initiator, crRNA and target respectively. The new strip around 60 bps indicates the formation of crRNA/target duplex (lane 4 and 7) and specifically recognized by Cas12a protein (lane 7). As no additional strip appears in Lane 7, this may ascribe to the activation of the nonspecific *cis*-cleavage ability of Cas12a and all the ssDNAs were digested. To further confirm the *cis*-cleavage activity of the Cas12a/crRNA complex, a fluorophore quencher (FQ)-labeled probes were designed and introduced into the system. As shown in Fig. 1B, the fluorescence signal was released significantly in the presence of target, while no signal appears without the target. The consistence of these results demonstrated that the target could effectively activate Cas12a *trans*- and *cis*-cleavage ability.

3.3. Design and optimization of the HCR-FRET system

Firstly, the feasibility of HCR was explored via 15% PAGE. As shown

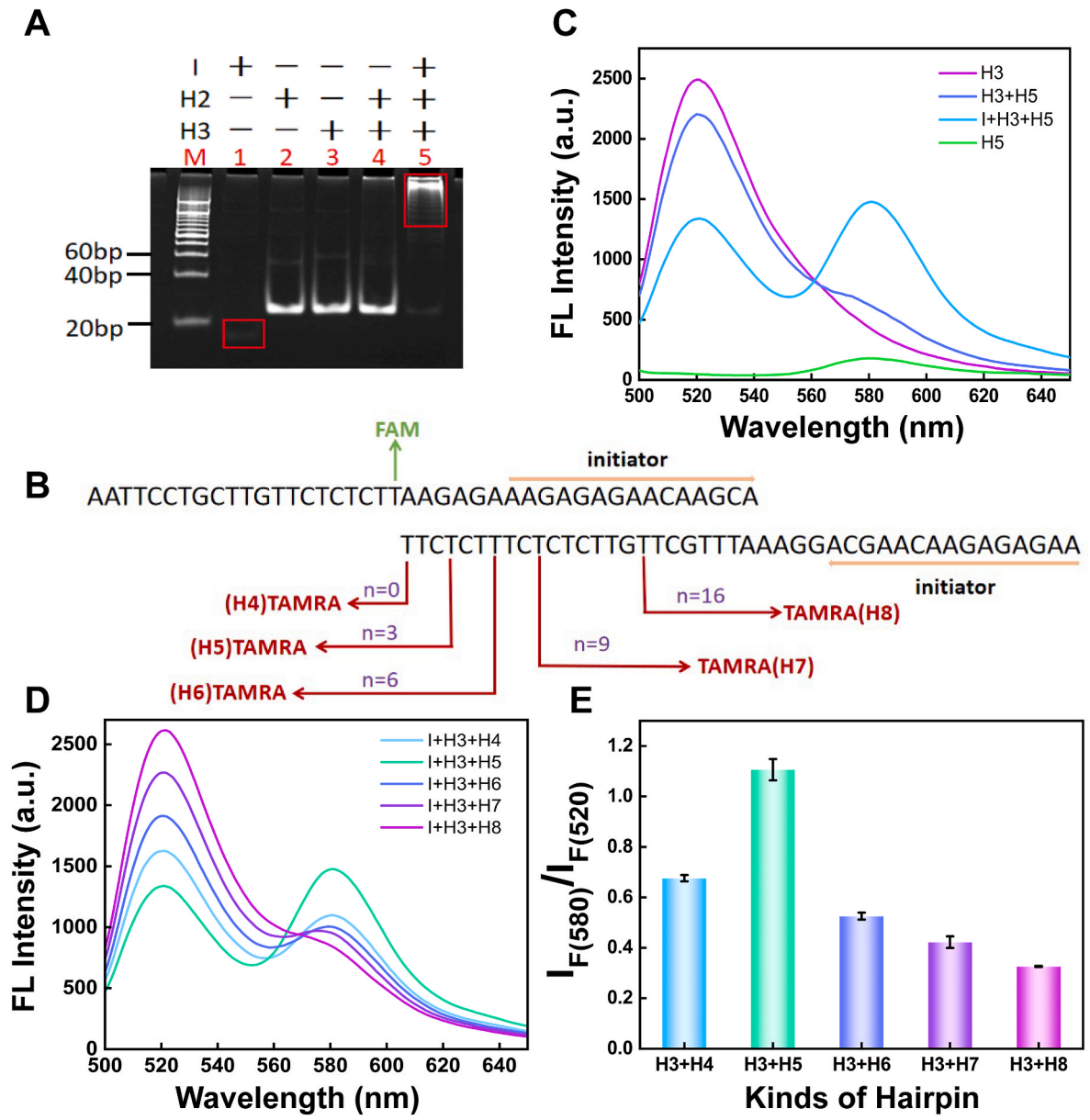


Fig. 2. Confirmation and optimation of HCR-FRET. (A) PAGE result of HCR, Lane 1–5 represents: initiator (100 nM), H1 (1 μM), H2 (1 μM), H1 + H2 (1 μM), initiator (100 nM) + H1 (1 μM) + H2 (1 μM) respectively; (B) Design of five different ssDNA sequences H4∼H8 (TAMRA moiety marked in different position); (C) The fluorescence spectrum of HCR; (D) The fluorescence spectrums of products of HCR with H4∼H8 respectively; (E) The energy transferring efficiency index of H4∼H8.

in Fig. 2A, the deep strips in lan5 showed a serial of DNA sequence appears. Also, only slightly light strips between 10–30 bps illustrated few initiator and fuel sequences remain. This result indicated the hairpin sequences hybridized with each other and the HCR reaction worked well. As the distance between the fluorescence donor (FAM) and acceptor (TAMRA) is the key factor that affect the energy transfer efficiency, five different ssDNA sequences (H4∼H8, TAMRA moiety marked in different position in H2) were specially designed to select the most effective distance for fluorescent signal transmission (Fig. 2B). The distance between FAM and TAMRA were 0, 3, 6, 9, 16 nucleotides respectively when the H# sequence (# = 4–8) hybridized with H3 sequence (H1 modified with FAM moiety). In the fluorescence spectrum (Fig. 2C and D), all these groups showed a signal decrease in the emission of the donor (520 nm) and an enhancement in the emission intensity of the acceptor (580 nm) in different levels. The ratios of the two fluorescence intensities were calculated as the energy transferring efficiency index ($\varepsilon = I_{F(580)}/I_{F(520)}$) [41]. The results in Fig. 2E illustrated that the H3/H5 sequence pair had the highest energy transfer efficiency. The HCR-FRET system worked efficiently when the donor and acceptor were separated by 3 bases, this might contribute to the formation of DNA double helix in the HCR product, which turned the two fluorescent probes into appropriate space distance. The performance of the H3/H5 sequence pair is even better than the previously reported HCR-FRET design [42,43]. As a result, the H3/H5 hairpin-pairs were taken for further experiments. The fluorescence spectrum data (Fig. 2C) also furtherly stand up for the feasibility of HCR-FRET.

The detailed reaction conditions, including the ratio of two kinds of hairpins, concentration of initiator, reaction time and temperature, were furtherly explored and optimized, as shown in Fig. 3.

Concentration of H5: Previous studies have concluded that the HCR reacts most effectively when the two hairpins had the same concentration. However, the ratio of the two hairpins' concentration has a certain relationship with the efficiency of HCR-mediated FRET. We set the concentration of H3 as a constant (400 nM) and compare the final FRET

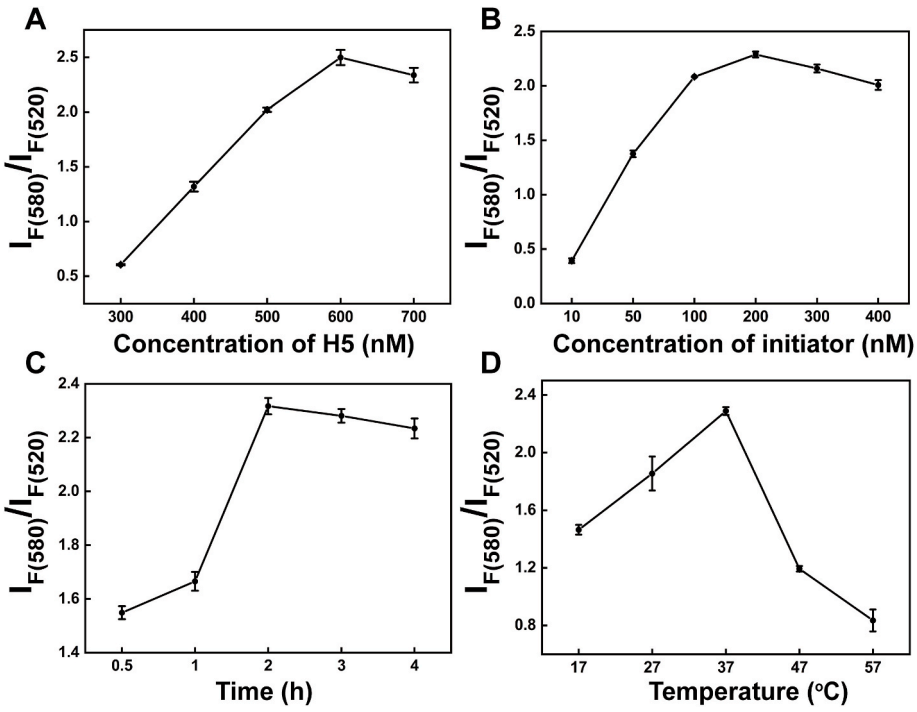


Fig. 3. Optimization of concentration of H5 (A), initiator (B), reaction time (C) and reaction temperature (D). Error bars show standard deviations of three repetitive measurements.

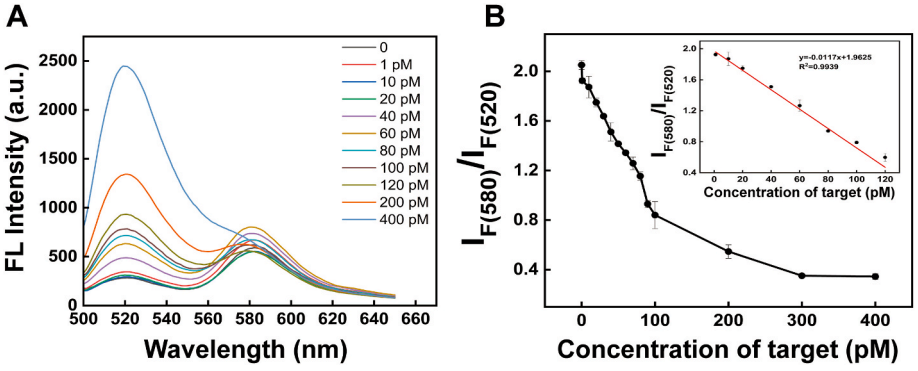


Fig. 4. (A) Fluorescence spectra of different target concentrations; (B)The relationship between fluorescence energy transferring efficiency and different ctDNA concentrations. Error bars mean SD and every experiment was adopted three times.

effect with different concentrations of H5. As shown in Fig. 3A, the ε value rises with the increase in H5' concentration, when it up to 600 nM the FRET efficiency reaches the maximum.

Concentration of initiator: The initiator, another key element which starts the following HCR responses, will be cleaved nonspecifically by the activated Cas12a. As shown in Fig. 3B, the energy transferring efficiency index (ε) raised with the initiator concentration and achieved the highest value when the initiator concentration reached 200 nM, this means more initiator molecules starts more HCR reactions. The ε value slightly decrease when more initiators were added. For this stage, too much initiators and more HCR will relatively dilute the FRET signals in the final products and impact the final energy transfer efficiency.

Reaction time and Temperature: Different reaction time points and temperatures were also taken into considerations, the results are shown in Fig. 3C and D. The most efficient reaction time is 2 h and optimal reaction temperature is 37 °C, both of which are consistent with previous research [34,41].

Table 2
Comparison between this work and other reported methods for ctDNA detection.

Method	Detection range	LOD	Ref.
Fluorescence	1–200 nM	0.12 nM	[44]
Fluorescence	1–100 nM	0.63 nM	[45]
Colorimetry	1%–10% in WT DNA	67 pmol/L (1%)	[46]
Electrochemical	5 pM-500 nM	3 pM	[47]
Fluorescence	1 pM–400 pM	0.316 pM	This work

3.4. Sensitivity of DNA detection

To evaluate the performance of Cas12a-HCR-FRET biosensor, targets with different concentrations were measured under optimized conditions, and the fluorescence signal of reaction products was record via fluorospectro-photometer. As shown in Fig. 4, the energy transferring efficiency index showed a good linear correlation with the ctDNA concentration, in the range from 1 pM to 400 pM. The linear equation is $Y = -0.0117\,X + 1.9625$, with a correlation coefficient (R^2) of 0.9939, where

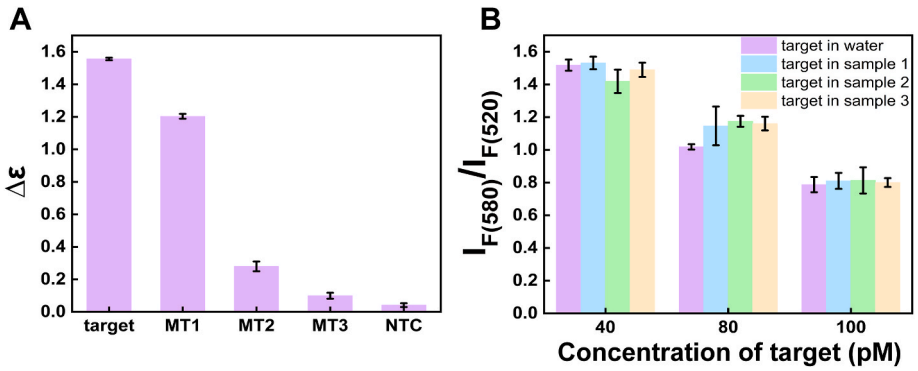


Fig. 5. (A) The specificity of this assay in the analysis of target against mismatched DNA (MT1, MT2 and MT3) and random sequence (NTC); (B) Comparison of different concentration of target DNA (40, 80 and 100 pM) prepared in water and DNA extracts. Error bars mean SD and every experiment was adopted three times.

X represents the target concentrations and Y represents the energy transferring efficiency index. The limit of the detection (LOD) was calculated to be 0.316 pM according to the 3 σ rule, which was superior to those of reported biosensors for ctDNA detection (Table 2).

3.5. Selectivity and specificity

A random sequence that could not hybridize with crRNA (NCT), and three mutation sequence were employed to explore the selectivity and specificity of this method. MT1, MT2 and MT3 represented sequences with 1, 2 and 3 nucleotides (nt) mutations respectively. Under the optimal experimental conditions, MT1, MT2, MT3 and NCT at the same concentration (120 pM) produced significantly lower FRET efficiency compared with the target. Taken the difference between the DNA groups' FRET efficiency and blank group ($\Delta\varepsilon = I_{F(580)}/I_{F(520)}$) as the vertical axis (Fig. 5A), the results demonstrate that this Cas12a-HCR-FRET assay system could discriminate the 1–3 nt mutations. The ability to distinguish mutations of this protocol ascribed to the specific recognition of the CRISPR/Cas12a system.

To furtherly verify the performance of this protocol in real sample, standard ctDNA solution were added in the total cfDNA extracts from the authors' plasma. The final concentrations were 40, 80 and 100 pM respectively. The comparison between the setting samples from plasma extraction and standard samples in water demonstrate no significant differences under the same experimental conditions (Fig. 5B) indicating the feasibility of this protocol for target DNA detection in real samples.

4. Conclusion

In summary, a general CRISPR-HCR-FRET assay protocol has been proposed and validated targeting ctDNA (T790 M). In this protocol, a HCR-FRET signaling system is specially designed. Combined with the CRISPR/Cas12a system, sensitive recognition of T790 M is realized. As the signal amplification unit (HCR-FRET system) is independent of the target recognition, the sequences design in this protocol is more general than the previously reported pre-amplification design and might be extended to other DNA targets, such as virus DNAs, cell free DNAs etc. As this reported protocol is a signal-off model, which limits its detection range and LOD, we expect a more effective and wider applications of this HCR-FRET when employed in other assay strategies.

CRediT authorship contribution statement

Xuelan Wu: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Ting Ju:** Methodology, Formal analysis. **Zeyang Li:** Writing – review & editing. **Jingwen Li:** Conducting experiments. **Xingwei Zhai:** Data analysis, Data curation, Formal analysis. **Kun Han:** Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

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References

[1] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 Countries, CA A Cancer J. Clin. 71 (2021) 209–249.
[2] Y.H. Luo, C.H. Chiu, C.H. Scott Kuo, T.Y. Chou, Y.C. Yeh, H.S. Hsu, S.H. Yen, Y. H. Wu, J.C. Yang, B.C. Liao, T.C. Hsia, Y.M. Chen, Lung cancer in Republic of China, J. Thorac. Oncol. 16 (2021) 519–527.
[3] R.M. Bremnes, L.T. Busund, T.L. Kilvaer, S. Andersen, E. Richardsen, E.E. Paulsen, S. Hald, M.R. Khanehkenari, W.A. Cooper, S.C. Kao, T. Donnem, The role of tumor-infiltrating lymphocytes in development, progression, and prognosis of non-small cell lung cancer, J. Thorac. Oncol. 11 (2016) 789–800.
[4] J. Wang, N. Sun, Y.T. Lee, Y. Ni, R. Koochekpour, Y. Zhu, H.R. Tseng, S. Wang, L. Jiang, H. Zhu, A circulating tumor cell-based digital assay for the detection of EGFR T790M mutation in advanced non-small cell lung cancer, J. Mater. Chem. B 8 (2020) 5636–5644.
[5] J.F. Gainor, A.T. Shaw, Emerging paradigms in the development of resistance to tyrosine kinase inhibitors in lung cancer, J. Clin. Oncol. 31 (2013) 3987–3996.
[6] N. Girard, Optimizing outcomes in EGFR mutation-positive NSCLC: which tyrosine kinase inhibitor and when? Future Oncol. 14 (2018) 1117–1132.
[7] S.A. Leon, B. Shapiro, D.M. Sklaroff, M.J. Yaros, Free DNA in the serum of cancer patients and the effect of therapy, Cancer Res. 3 (1977) 646–650.
[8] K. Jung, M. Fleischhacker, A. Rabien, Cell-free DNA in the blood as a solid tumor biomarker–a critical appraisal of the literature, Clin. Chim. Acta 411 (2010) 1611–1624.
[9] R. Zhang, F. Shao, X. Wu, K. Ying, Value of quantitative analysis of circulating cell free DNA as a screening tool for lung cancer: a meta-analysis, Lung Cancer 69 (2010) 225–231.
[10] C. Demuth, K.G. Spindler, J.S. Johansen, N. Pallisgaard, D. Nielsen, E. Hogdall, B. Vittrup, B.S. Sorensen, Measuring KRAS mutations in circulating tumor DNA by droplet digital PCR and next-generation sequencing, Transl Oncol 11 (2018) 1220–1224.
[11] T. Ju, X. Zhai, X. Liu, K. Han, A toehold-mediated strand displacement cascade-based DNA assay method via flow cytometry and magnetic separation, Anal. Methods 13 (2021) 1013–1018.
[12] D. Chen, Y. Wu, S. Hoque, R.D. Tilley, J.J. Gooding, Rapid and ultrasensitive electrochemical detection of circulating tumor DNA by hybridization on the network of gold-coated magnetic nanoparticles, Chem. Sci. 12 (2021) 5196–5201.

[13] X. Jiang, D.-Q. Feng, G. Liu, D. Fan, W. Wang, A fluorescent switch sensor for detection of anticancer drug and ctDNA based on the glutathione stabilized gold nanoclusters, Sensor. Actuator. B Chem. 232 (2016) 276–282.
[14] C. Alix-Panabieres, K. Pantel, Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy, Cancer Discov. 6 (2016) 479–491.
[15] E. Heitzer, P. Ulz, J.B. Geigl, Circulating tumor DNA as a liquid biopsy for cancer, Clin. Chem. 61 (2015) 112–123.
[16] R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero, P. Horvath, CRISPR provides acquired resistance against viruses in prokaryotes, Science 315 (2007) 1709–1712.
[17] M. Singh, G. Bindal, C.S. Misra, D. Rath, The era of Cas12 and Cas13 CRISPR-based disease diagnosis, Crit. Rev. Microbiol. (2022) 1–16.
[18] P. Li, L. Wang, J. Yang, L.J. Di, J. Li, Applications of the CRISPR-Cas system for infectious disease diagnostics, Expert Rev. Mol. Diagn 21 (2021) 723–732.
[19] X. Wang, X. Shang, X. Huang, Next-generation pathogen diagnosis with CRISPR/Cas-based detection methods, Emerg. Microb. Infect. 9 (2020) 1682–1691.
[20] X. Xiang, K. Qian, Z. Zhang, F. Lin, Y. Xie, Y. Liu, Z. Yang, CRISPR-cas systems based molecular diagnostic tool for infectious diseases and emerging 2019 novel coronavirus (COVID-19) pneumonia, J. Drug Target. 28 (2020) 727–731.
[21] S. Chen, R. Wang, S. Peng, S. Xie, C. Lei, Y. Huang, Z. Nie, PAM-less conditional DNA substrates leverage trans-cleavage of CRISPR-Cas12a for versatile live-cell biosensing, Chem. Sci. 13 (2022) 2011–2020.
[22] S.C. Knight, R. Tjian, J.A. Doudna, Genomes in focus: development and applications of CRISPR-cas9 imaging technologies, Angew Chem. Int. Ed. Engl. 57 (2018) 4329–4337.
[23] J.W. Bryson, J.Y. Auxillos, S.J. Rosser, Multiplexed activation in mammalian cells using a split-intein CRISPR/Cas12a based synthetic transcription factor, Nucleic Acids Res. 50 (2022) 549–560.
[24] S.W. Wang, C. Gao, Y.M. Zheng, L. Yi, J.C. Lu, X.Y. Huang, J.B. Cai, P.F. Zhang, Y. H. Cui, A.W. Ke, Current applications and future perspective of CRISPR/Cas9 gene editing in cancer, Mol. Cancer 21 (2022) 57.
[25] M. Eisenstein, Seven technologies to watch in 2022, Nature 601 (2022) 658–661.
[26] G. Zhang, L. Zhang, J. Tong, X. Zhao, J. Ren, CRISPR-Cas12a enhanced rolling circle amplification method for ultrasensitive miRNA detection, Microchem. J. 158 (2020).
[27] H. Wu, X. Cao, Y. Meng, D. Richards, J. Wu, Z. Ye, A.J. deMello, DropCRISPR: a LAMP-Cas12a based digital method for ultrasensitive detection of nucleic acid, Biosens. Bioelectron. 211 (2022), 114377.
[28] A.K. Singh, S. Naskar, P.W. Ramteke, R. Kumar, Nucleic acid sequence-based amplification (NASBA) methods and CRISPR/Cas13 system to detect pig viral diseases, Protocols for the Diagnosis of Pig Viral Diseases (2022) 151–157.
[29] J.P. Broughton, X. Deng, G. Yu, C.L. Fasching, V. Servellita, J. Singh, X. Miao, J. A. Streithorst, A. Granados, A. Sotomayor-Gonzalez, K. Zorn, A. Gopez, E. Hsu, W. Gu, S. Miller, C.Y. Pan, H. Guevara, D.A. Wadford, J.S. Chen, C.Y. Chiu, CRISPR-Cas12-based detection of SARS-CoV-2, Nat. Biotechnol. 38 (2020) 870–874.
[30] X. Ding, K. Yin, Z. Li, R.V. Lalla, E. Ballesteros, M.M. Sfeir, C. Liu, Ultrasensitive and visual detection of SARS-CoV-2 using all-in-one dual CRISPR-Cas12a assay, Nat. Commun. 11 (2020) 4711.
[31] X. Xia, B. Ma, T. Zhang, Y. Lu, M.R. Khan, Y. Hu, C. Lei, S. Deng, Q. He, G. He, K. Zhang, R. Deng, G-Quadruplex-Probing CRISPR-cas12 assay for label-free analysis of foodborne pathogens and their colonization in vivo, ACS Sens. 6 (2021) 3295–3302.
[32] Y. Tao, K. Yi, H. Wang, H.W. Kim, K. Li, X. Zhu, M. Li, CRISPR-Cas12a-regulated DNA adsorption and metallization on MXenes as enhanced enzyme mimics for sensitive colorimetric detection of hepatitis B virus DNA, J. Colloid Interface Sci. 613 (2022) 406–414.
[33] Z. Wang, W. Cui, CRISPR-Cas system for biomedical diagnostic platforms, View 1 (2020).
[34] R.M. Dirks, N.A. Pierce, Triggered amplification by hybridization chain reaction, PANS (Pest. Artic. News Summ.) 101 (2004) 15275–15278.
[35] Y. Chen, L. Chen, Y. Ou, L. Guo, F. Fu, Enzyme-free detection of DNA based on hybridization chain reaction amplification and fluorescence resonance energy transfer, Sensor. Actuator. B Chem. 233 (2016) 691–696.
[36] Q. Wang, Y. Liu, J.C. Campillo-Brocal, A. Jimenez-Quero, G.A. Crespo, M. Cuartero, Electrochemical biosensor for glycine detection in biological fluids, Biosens. Bioelectron. 182 (2021), 113154.
[37] S. Xie, D. Tao, Y. Fu, B. Xu, Y. Tang, L. Steinaa, J.D. Hemmink, W. Pan, X. Huang, X. Nie, C. Zhao, J. Ruan, Y. Zhang, J. Han, L. Fu, Y. Ma, X. Li, X. Liu, S. Zhao, Rapid visual CRISPR assay: a naked-eye colorimetric detection method for nucleic acids based on CRISPR/cas12a and a convolutional neural network, ACS Synth. Biol. 11 (2022) 383–396.
[38] Y. Bai, H. Zhang, L. Zhao, Y. Wang, X. Chen, H. Zhai, M. Tian, R. Zhao, T. Wang, H. Xu, F. Feng, A novel aptasensor based on HCR and G-quadruplex DNAzyme for fluorescence detection of Carcinoembryonic Antigen, Talanta 221 (2021), 121451.
[39] J.S. Chen, E. Ma, L.B. Harrington, M. Da-Costa, X. Tian, J.M. Palefsky, J.A. Doudna, CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity, Science 360 (2018) 436–439.
[40] S.Y. Li, Q.X. Cheng, J.K. Liu, X.Q. Nie, G.P. Zhao, J. Wang, CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA, Cell Res. 28 (2018) 491–493.
[41] P. Ji, G. Han, Y. Huang, H. Jiang, Q. Zhou, X. Liu, D. Kong, Ultrasensitive ratiometric detection of Pb(2+) using DNA tetrahedron-mediated hyperbranched hybridization chain reaction, Anal. Chim. Acta 1147 (2021) 170–177.
[42] Y. Chen, L. Chen, Y.D. Ou, L.Q. Guo, F.F. Fu, Enzyme-free detection of DNA based on hybridization chain reaction amplification and fluorescence resonance energy transfer, Sensor. Actuator. B Chem. 233 (2016) 691–696.
[43] Y.S. Ang, L.Y. Yung, Rational design of hybridization chain reaction monomers for robust signal amplification, Chem. Commun. 52 (2016) 4219–4222.
[44] E. Castellanos-Rizaldos, D.G. Grimm, V. Tadigotla, J. Hurley, J. Healy, P.L. Neal, M. Sher, R. Venkatesan, C. Karlovich, M. Raponi, A. Krug, M. Noerholm, J. Tannous, B.A. Tannous, L.E. Raez, J.K. Skog, Exosome-based detection of EGFR T790M in plasma from non-small cell lung cancer patients, Clin. Cancer Res. 24 (2018) 2944–2950.
[45] Z. Fu, Y. Cui, F. Cui, G. Zhang, Modeling techniques and fluorescence imaging investigation of the interactions of an anthraquinone derivative with HSA and ctDNA, Spectrochim. Acta Mol. Biomol. Spectrosc. 153 (2016) 572–579.
[46] Y. Wang, S.L. Kong, X.D. Su, A centrifugation-assisted visual detection of SNP in circulating tumor DNA using gold nanoparticles coupled with isothermal amplification, RSC Adv. 10 (2020) 1476–1483.
[47] Y. Huang, M. Tao, S. Luo, Y. Zhang, B. Situ, X. Ye, P. Chen, X. Jiang, Q. Wang, L. Zheng, A novel nest hybridization chain reaction based electrochemical assay for sensitive detection of circulating tumor DNA, Anal. Chim. Acta 1107 (2020) 40–47.