Acta Biochim Biophys Sin 2024, 56(7): 1089–1092
https://doi.org/10.3724/abbs.2024071
Advance Access Publication Date: 27 May 2024
Lab Note

Lab Note

Highly sensitive detection of a long-COVID-related SNP in LZTFL1 allele with *Pyrococcus furiosus* Argonaute in point-of-care settings

Yixin Tang[1,†], Miao Xu[1,†], Bo Luo[1,†], Yue Wang[1], Yukang Chen[1], Guangxi Yu[1], Guang Yang[1,*], Song Gao[1,*], and Pei Wang[2,*]

[1]Jiangsu Key Laboratory of Marine Biological Resources and Environment, Co-Innovation Center of Jiangsu Marine Bio-industry Technology, Jiangsu Ocean University, Lianyungang 222005, China, and [2]School of Marine and Biological Engineering, Yancheng Teachers University, Yancheng 224007, China

[†]These authors contributed equally to this work.

*Correspondence address. Tel: +86-518-85895919; E-mail: 2020000050@jou.edu.cn (G.Y.) / Tel: +86-518-85895370; E-mail: gaos@jou.edu.cn (S.G.) / Tel: +86-515-88213885; E-mail: wangp04@yctu.edu.cn (P.W.)

Received 11 March 2024 Accepted 22 April 2024

The leucine zipper transcription factor-like 1 (LZTFL1) gene is active in respiratory and lung epithelia. Mutation of base G to A at the rs17713054 locus on the LZTFL1 allele causes an increase in the expression of the gene, leading to an increase in viral load after SARS-CoV-2 infection [1]. This mutation significantly increases the risk of developing severe cases and long-term symptoms of COVID-19 (long-COVID) [2]. This SNP, the G > A mutation at the rs17713054 locus on the LZTFL1 allele, is carried by 60% of South Asian, 15% of European, 2.4% of African and 1.8% of East Asian populations. This long-COVID-related SNP may also be related to the risk of tumors, *e.g.*, kidney renal clear cell carcinoma (KIRC), rectum adenocarcinoma (READ), and uveal melanoma (UVM) [3].

SNP detection methods mainly include DNA sequencing, TaqMan fluorescence quantitative PCR, and denaturing high-performance liquid chromatography [1,4,5]. These methods are accurate with high sensitivity. However, these methods all rely on specialized laboratory equipment and well-trained operators and have a relatively long turn-around time, making it difficult for them to play a good role in point-of-care settings. *Pyrococcus furiosus* Argonaute (*Pf*Ago) is a programmable nucleic acid endonuclease. With the guidance of 5′ phosphorylated single-stranded (ss) guide DNA (gDNA), targeted cleavage of ssDNA or dsDNA complementary to gDNA can be achieved. Importantly, the cleavage activity of *Pf*Ago exhibits single-base specificity, and a single base of noncomplementary pairing between the gDNA and the target affects the cleavage efficiency [6]. This property can be naturally exploited for the development of more convenient methods for SNP detection that require the identification of base differences. The combination of *Pf*Ago and PCR has been used for the detection of SARS-CoV-2 mutations and tumor-associated rare mutations [7,8], which are similar to SNP from the perspective of nucleic acid detection.

In this study, we combined *Pf*Ago with recombinase polymerase amplification (RPA), a nucleic acid isothermal amplification technology that is better suited to point-of-care settings than PCR, to achieve highly sensitive detection of the G > A SNP at the rs17713054 locus on the LZTFL1 allele (Figure 1A). The targets, wild-type (WT, G) or mutant (MT, A), are specifically amplified by RPA, and the column-purified amplicons are tested in *Pf*Ago reactions with WT- or MT-specific gDNA. The specific gDNA-guided 1st cleavage generates a new 5′-phosphorylated ssDNA (gn) that serves as the gDNA for the 2nd cleavage, which cleaves the fluorescence-labeled molecular beacon (MB) specifically designed for WT (HEX) or MT (FAM) target, producing the signal. The entire SNP detection can be completed within 1.5 h, and single-copy targets can be distinguished. Since the RPA amplification and *Pf*Ago cleavage reactions are performed at constant temperatures, the detection does not require thermal cycling equipment, and the fluorescence results can be observed with the naked eye under blue light. This study is the first application of *Pf*Ago combined with RPA for human SNP detection. It achieves highly sensitive detection of the long-COVID-related SNPs in the LZTFL1 allele in point-of-care settings and provides a new technological pathway for the development of human SNP detection methods suitable for conditions with limited resources.

For good amplification of the target (a fragment containing the rs17713054 locus of the *LZTFL1* gene, GenBank accession no. NG_033917.1), RPA primers were screened (Supplementary Table S1 and Figure S1). PCR screening suggested that the primer pairs F1R1, F1R3 and F2R1 had better amplification efficiency. Further screening with RPA revealed that the primer pair F1R3 was the best

© The Author(s) 2024. This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License (https://creativecommons.org/licenses/by-nc-nd/4.0/).

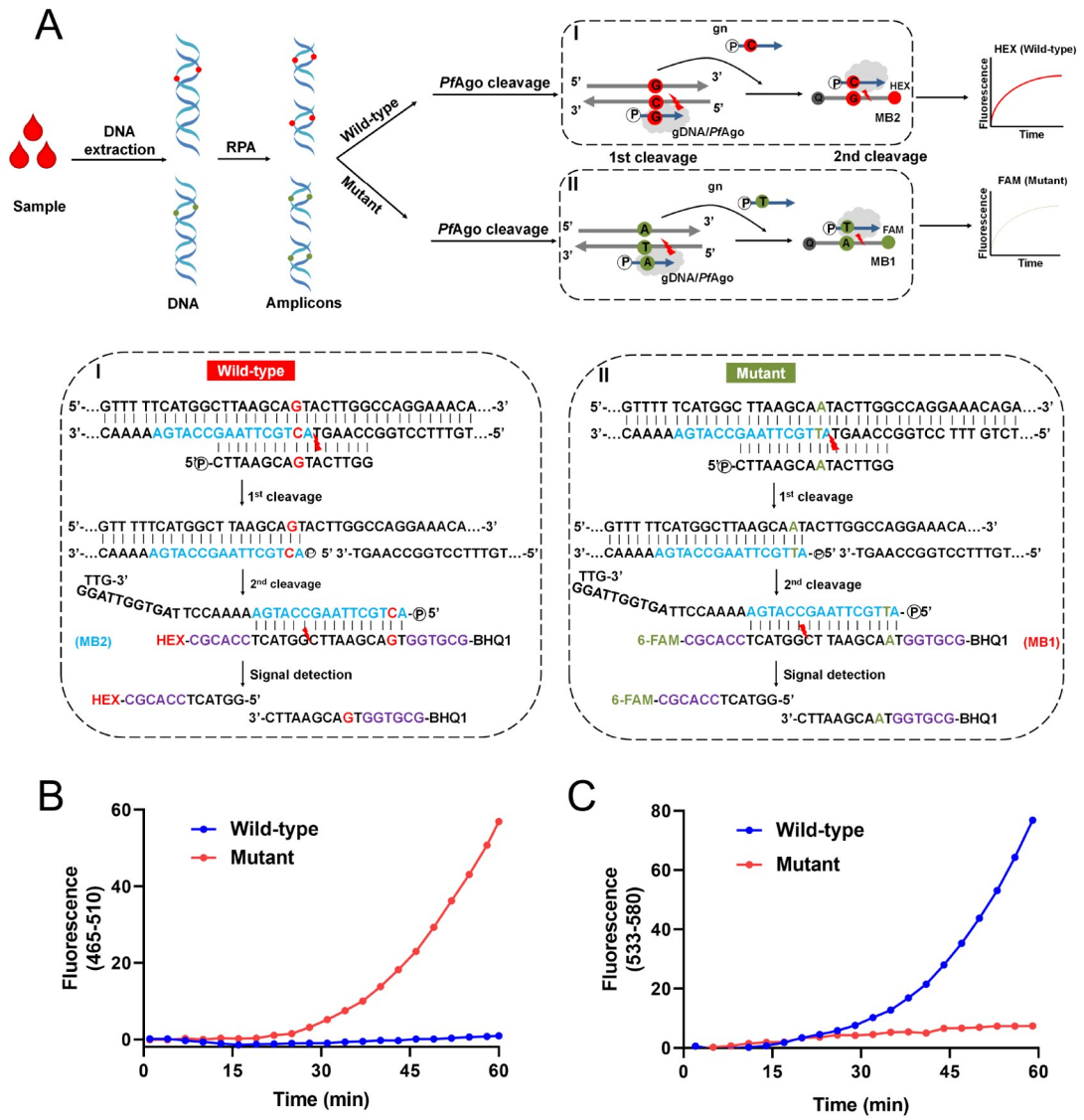


Figure 1. Establishment of the SNP detection method based on *Pf*Ago and RPA (A) Method principle. The key elements of the method are indicated with different shapes and colors. The wild-type base G and mutant base A at the rs17713054 locus on the *LZTFL1* allele are indicated by red and green dots, respectively. Some dots are further labeled with letters corresponding to the bases. "P" represents the 5′-phosphate of the DNA fragments. The *Pf*Ago cleavages are indicated by red lightning symbols. Boxes I (wild-type) and II (mutant) display the detailed sequences of the targets, gDNAs, cleavage products, and molecular beacons (MBs). HEX and FAM are the fluorescence labels. BHQ1 is the quenching group. The sequences of gDNAs and MBs are determined in the study. (B, C) Fluorescence curves showing the detection of mutant (B) and wild-type (C) targets with the RPA-*Pf*Ago method. A total of 10^{10} copies of the standard plasmids were used as the targets of the respective detections. The detection wavelength ranges (in nm) are indicated at the y-axis.

for amplification of the target. The detailed experimental procedures of this study are included in Supplementary Information.

For good *Pf*Ago cleavage, *Pf*Ago was prepared by recombinant expression [6] and appropriate gDNAs were extensively screened. *Pf*Ago can cleave the target only if the gDNA recognizes a target sequence that is complementary to it. The gDNAs g1-g16 for MT and g1′-g16′ for WT were designed by placing the base of the mutation ("A" or "G") of the rs17713054 locus at different positions (nt 1-16) of the gDNA (Supplementary Figure S3). The screening results showed that *Pf*Ago was able to efficiently cleave MT targets when using g9 or g16, and g9 could not guide the cleavage on the WT targets, indicating that g9 was the gDNA for MT targets. Similarly, *Pf*Ago was able to cleave WT targets when using g9′ or g16′, and g9′ could not guide the cleavage on the MT targets, indicating that g9′ was the gDNA for WT targets (Supplementary Figure S3). The reason that g16 or g16′ cannot distinguish between MT or WT targets could be that *Pf*Ago performs two rounds of cleavage in the detection process. The first round of cleavage is a specific cleavage guided by the specially designed gDNA, which produces a 5′ phosphorylated ssDNA as the guide DNA (gn) for the second round of cleavage. As *Pf*Ago cleaves the target between the 10^{th} and 11^{th} nucleotides from the 5′ end complementary to the guide DNA, the first-round cleavage guided by g16 or g16′ produces sequence-identical ssDNA (gn) that does not include the mutation site, resulting in nonspecific second-round cleavage.

Based on the above results, the sequences of gn (MT or WT)

produced by the first round of *Pf*Ago cleavage were used to design molecular beacons (MBs) that were complementary to the gn sequences (Supplementary Table S1). The results of the validation reactions with MBs showed that only the FAM fluorescence signal could be detected with the MT target, while only the HEX fluorescence signal could be detected with the WT target (Figure 1B,C). These results revealed that combinations of g9-MB1 (for MT) and g9′-MB2 (for WT) could be used for SNP detection of rs17713054. After the determination of the gDNA and corresponding MB, the *Pf*Ago cleavage reaction system was optimized. The optimal concentrations of gDNA, *Pf*Ago and MB were 2 μM, 2 μM and 1.5 μM, respectively (Supplementary Figure S4).

The sensitivity of this RPA-*Pf*Ago method was characterized. By using MT and WT standard plasmids with different copy numbers as targets, the results showed that the method was able to effectively detect the rs17713054 G > A SNP with as low as 1 copy of the targets (Figure 2A–D). This indicated that the sensitivity of this method was 1 copy, which meant that if 1 copy of the target fragment was present in the sample loaded for testing (up to 5 μL), the SNP could be detected.

The G > A SNP at the rs17713054 locus of the LZTFL1 allele is a heterozygous mutation. To verify the detection efficiency of the RPA-*Pf*Ago method for heterozygous samples, we prepared simulated heterozygous samples by mixing the MT and WT standard plasmids at molar ratios of 1:9, 3:7 and 5:5. The RPA-*Pf*Ago method detected both WT and MT targets in the hetero-

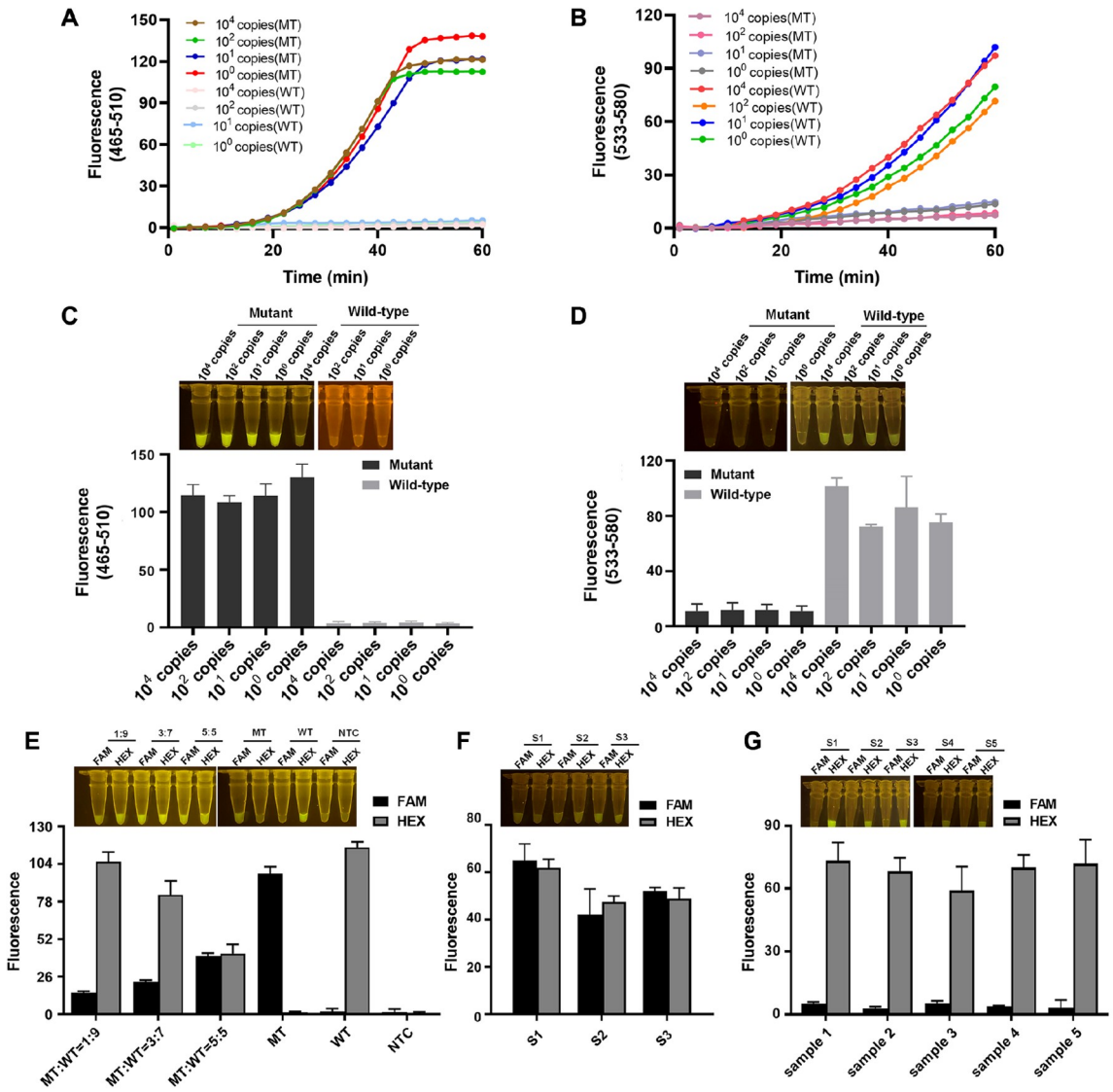


Figure 2. Characterizations of the RPA-*Pf*Ago method (A–D) Sensitivity of the method. The fluorescence curves corresponding to different amounts of the standard plasmid targets tested in the mutant-specific reaction (A) and wild-type-specific reaction (B) are shown in different colors. The end-point signal intensities of the mutant-specific reaction (C) and wild-type-specific reaction (D) are shown as bar charts. The visualized results by blue light are shown at the top of the respective bar chart. (E) Detection of simulated heterozygous samples. The end-point signal intensities and the visualized results are shown. The molar ratios of mutant to wild-type targets in the samples are indicated. A total of 10^4 copies of the standard plasmids (mutant and wild-type) were used in each tested sample. (F) Detection of three simulated heterozygous blood samples (S1–S3). Each sample contains total 10^4 copies of the target. (G) Detection of five homozygous human blood samples (samples 1–5). The error bars represent the standard errors of three independent detections.

zygous samples, and the fluorescence intensities correlated well with the mixing ratio (Figure 2E). This result indicated that the method could detect the MT copy of this SNP in a relatively large range of molar ratios between the MT and WT copies. During heterozygous sample collection and processing, the ratio between the MT and WT copies might change away from 1:1, and the method could still reliably detect the presence of the G > A mutation and determine whether the sample is heterozygous.

The RPA-*Pf*Ago method was subsequently validated using blood samples. The validation results showed that five homozygous blood samples and three simulated heterozygous blood samples were effectively identified (Figure 2F,G and Supplementary Table S2).

This study combined the highly specific recognition of single nucleotides by *Pf*Ago with the highly efficient amplification of RPA to establish a specific and sensitive SNP detection method for the rs17713054 locus in the human gene *LZTFL1*. The reported *Pf*Ago-based genotyping methods include PAND, A-Star, and MULAN [8–10]. In this study, the method combining *Pf*Ago with RPA was applied to human SNP detection for the first time and achieved high single-copy sensitivity, which is a satisfactory sensitivity as compared to the reported methods (Supplementary Table S3). The RPA reaction in this method was carried out at a constant temperature between 37°C and 42°C, and the reaction temperature for *Pf*Ago cleavage was 95°C. The temperature can be controlled by a portable metal bath. The whole detection can be completed in 1.5 h, and the fluorescence signal can be observed by the naked eye under blue light. Thus, a portable metal bath, a blue light, a mini centrifuge, and a set of pipettes can meet the condition requirements of the method, and the operation is simple enough for nonprofessionals. Column purification of the RPA amplicons before *Pf*Ago cleavage has to be performed because it seems that some of the RPA reaction components may interfere with downstream steps. Further simplifying the operation by avoiding column purification can be one of the future directions.

In conclusion, the RPA-*Pf*Ago method established in this study achieves highly sensitive detection of long-COVID-related SNPs in the LZTFL1 allele in point-of-care settings and provides a new technological pathway for the development of human SNP detection methods suitable for conditions with limited resources.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Funding

This work was supported by the grants from the China Postdoctoral Science Foundation (No. 2022M721665), the Research Program of the “521 Project” of Lianyungang City of China (No. LYG06521202133), the Lianyungang Key R&D Project (No. CG2305), the Lianyungang Postdoctoral Research Funding Program (No. LYG20220022), the Open-end Funds of Jiangsu Key Laboratory of Marine Bioresources and Environment (Nos. SH20221208 and SH20231212), the “Blue Project” of Jiangsu Higher Education Institutions of China, and the Priority Academic Program Development of Jiangsu Higher Education Institutions of China.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Downes DJ, Cross AR, Hua P, Roberts N, Schwessinger R, Cutler AJ, Munis AM, *et al.* Identification of LZTFL1 as a candidate effector gene at a COVID-19 risk locus. *Nat Genet* 2021, 53: 1606–1615
2. Udomsinprasert W, Nontawong N, Saengsiwaritt W, Panthan B, Jiaranai P, Thongchompoo N, Santon S, *et al.* Host genetic polymorphisms involved in long-term symptoms of COVID-19. *Emerging Microbes Infects* 2023, 12: 2239952
3. Mo J, Zhang Z, Wang D, Wang M, Wang L. A pancancer analysis of the oncogenic role of COVID-19 risk gene leucine zipper transcription factor-like protein 1 (LZTFL1) in human tumors. *BioRxiv* 2022
4. Traspov A, Minashkin M, Poyarkov SY, Komarov A, Shtinova IA, Speshilov G, Karbyshev I, *et al.* The rs17713054 and rs1800629 polymorphisms of genes LZTFL1 and TNF are associated with COVID-19 severity. *Bulletin of RSMU* 2022, 35-40
5. Liu W, Smith DI, Rechtzigel KJ, Thibodeau SN, James CD. Denaturing high performance liquid chromatography (DHPLC) used in the detection of germline and somatic mutations. *Nucleic Acids Res* 1998, 26: 1396–1400
6. Zhao C, Yang L, Zhang X, Tang Y, Wang Y, Shao X, Gao S, *et al.* Rapid and sensitive genotyping of SARS-CoV-2 key mutation L452R with an RPA-*Pf* Ago method. *Anal Chem* 2022, 94: 17151–17159
7. He R, Wang L, Wang F, Yang J, Yu X, Wang Y, Liu Z, *et al.* Combination of ultrashort PCR and *Pyrococcus furiosus* Argonaute for DNA detection. *Analyst* 2021, 147: 35–39
8. Liu Q, Guo X, Xun G, Li Z, Chong Y, Yang L, Wang H, *et al.* Argonaute integrated single-tube PCR system enables supersensitive detection of rare mutations. *Nucleic Acids Res* 2021, 49: e75
9. Wang F, Yang J, He R, Yu X, Chen S, Liu Y, Wang L, *et al. Pf*Ago-based detection of SARS-CoV-2. *Biosens Bioelectron* 2021, 177: 112932
10. Ye X, Zhou H, Guo X, Liu D, Li Z, Sun J, Huang J, *et al.* Argonaute-integrated isothermal amplification for rapid, portable, multiplex detection of SARS-CoV-2 and influenza viruses. *Biosens Bioelectron* 2022, 207: 114169