Cell Insight 2 (2023) 100080

Contents lists available at ScienceDirect

Cell Insight

journal homepage: www.journals.elsevier.com/cell-insight

Research Article

Cas12a-based one-pot SNP detection with high accuracy

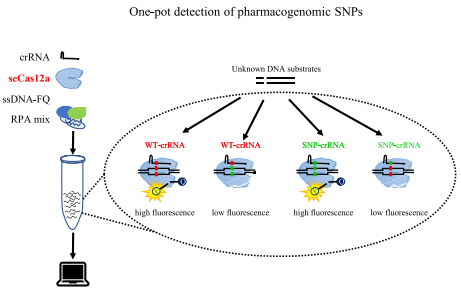
Hong-Xia Zhang [a,1], Caixiang Zhang [a,1], Shuhan Lu [a,b], Xiaohan Tong [a,b], Kun Zhang [a,b], Hao Yin [a,b,**], Ying Zhang [a,*]

[a] Department of Rheumatology and Immunology, Medical Research Institute, Frontier Science Center for Immunology and Metabolism, Zhongnan Hospital of Wuhan University, Wuhan University, Wuhan, China
[b] Department of Pathology, Zhongnan Hospital of Wuhan University, Wuhan University, Wuhan, China

HIGHLIGHTS

- Engineered LbCas12a, referred as seCas12a, exhibited enhanced SNP discrimination in one-pot reaction.
- SeCas12a functions to slow down *cis*-cleavage kinetics, thereby conferring improved sensitivity to SNP.
- SeCas12a-based one-pot reaction faithful detect pharmacogenomic SNPs of clinical samples with high accuracy in 30 min.

GRAPHICAL ABSTRACT



ABSTRACT

CRISPR-Cas12a based one-pot detection system has been used in nucleic acid detection and diagnosis. However, it is not sensitive enough to distinguish single nucleotide polymorphisms (SNP), which has greatly restricted its application. To overcome these limitations, we engineered a LbCas12a variant with enhanced sensitivity against SNP, named seCas12a (sensitive Cas12a). SeCas12a-based one-pot SNP detection system is a versatile platform that could use both canonical and non-canonical PAM, and was almost not limited by mutation types to distinguish SNPs located between position 1 to 17. The use of truncated crRNA further improved SNP specificity of seCas12a. Mechanistically, we found only when the *cis*-cleavage was at low level between 0.01min^{-1} and 0.0006 min^{-1}, a good signal-to-noise ratio can be achieved in one-pot test. SeCas12a-based one-pot SNP detection system was applied to detect pharmacogenomic SNPs in human clinical samples. Of thirteen donors tested in two different SNPs, the seCas12a mediated one-pot system could faithfully detect the SNPs in 30 min with 100% accuracy.

1. Introduction

Single nucleotide polymorphisms (SNP), including base transition, transversion, deletion and insertion, are the most common type of human heritable variation with an average frequency of one SNP in every 1–1.9 kilobase pairs (Altshuler et al., 2015; Sachidanandam et al., 2001). SNPs are involved in many biological processes, including genetic disease, tumorigenesis, drug metabolism and so on. Pharmacogenomic SNPs, often categorized as SNPs related to drug absorption, distribution, metabolism and excretion, play a critical role in defining the response of an individual's susceptibility to drug treatment (Zhao et al., 2021). Cytochrome P450 (CYP) family are the most important drug metabolism enzymes and mediate multiple drug metabolism. More than 2000 variations in CYP have been reported, and some of these variations have

* Corresponding author.
** Corresponding author. Department of Rheumatology and Immunology, Medical Research Institute, Frontier Science Center for Immunology and Metabolism, Zhongnan Hospital of Wuhan University, Wuhan University, Wuhan, China.
E-mail addresses: haoyin@whu.edu.cn (H. Yin), ying.zhang84@whu.edu.cn (Y. Zhang).
[1] These authors contributed equally to the work.

https://doi.org/10.1016/j.cellin.2023.100080
Received 5 December 2022; Received in revised form 26 January 2023; Accepted 29 January 2023
Available online 1 February 2023

serious effects on enzyme activity. For example, CYP2C9 is one of the critical enzyme involved in the metabolism of warfarin, an anticoagulant drug that requires careful personalized prescription to reach a delicate balance of avoiding over-anticoagulation associated clotting and under-anticoagulation associated bleeding (Furuya et al., 1995; Stefanovic et al., 2005). Two common variants of *CYP2C9*, *CYP2C9**2 (3608 C > T) and *CYP2C9**3 (42,614 A > C), have been identified to cause reduced enzymatic activity (Ablin et al., 2002). Individuals who carry one or two copies of variants are more sensitive to warfarin, showing increased risk of bleeding during warfarin treatment. CYP2C19 is the key metabolizer of clopidogrel, an effective antiplatelet drug which can inhibit ADP-induced platelet aggregation. Clopidogrel is metabolized into 2-oxygen-clopidogrel by CYP2C19, and finally becomes an active mercaptan metabolite to execute its anticoagulant function. Patients with *CYP2C19**2 allele are ineffective in response to clopidogrel treatment due to the impaired enzyme activity caused by *CYP2C19**2 allele (Hulot et al., 2006). Therefore, when using drugs that can cause severe side effects due to individual genomic differences, fast and accurate detection of key pharmacogenomic SNPs is crucial to personalized medication. In our study, we focused on a fast and accurate SNP detection of CYP2C9 and CYP2C19, two important enzymes involved in drug metabolism, such as warfarin, clopidogrel, phenytoin, siponimod, and valproate (Agrawal et al., 2020; Gardin et al., 2019; Liao et al., 2018; Liu & Obeng, 2020).

Many SNP detection methods have been developed and can be categorized into electrophoresis or high throughput based assays (Yang et al., 2020). Electrophoresis based SNP detection, such as denatured gradient gel electrophoresis, allele-specific PCR and cleaved amplified polymorphic sequence labeling, all shared a common characteristics of time-consuming, low accuracy, being able to detect whether there was a SNP, but unable to specify the detailed nucleotide changes. High throughput methods, such as DNA sequencing and high resolution melting, have an advantage of high accuracy but they are also time-consuming and relative expensive. Due to the limitation of current assays, new approaches using highly tailored DNA polymerase and duplex binding protein or clustered regularly interspaced short palindromic repeats (CRISPR)–associated (Cas) proteins have been developed (Gootenberg et al., 2018; Mitani et al., 2007; Paul & Montoya, 2020). DNA polymerase based SNP detection strongly depends on the complex primer design process, making the assay development complicated and restraining its broad application.

The CRISPR-Cas systems are widely used in genome editing and nucleic acid detection (Kim et al., 2021; Li et al., 2019; Shi et al., 2022; Wang et al., 2022; Yin et al., 2021; Zhang et al., 2019). Type V and VI Cas proteins, including Cas12a, Cas12b, Cas13a, and Cas14 have the ability to initiate non-specific trans cleavage (also known as collateral cleavage) upon target recognition (Li, Cheng, Liu, et al., 2018). Upon crRNA-guided target DNA recognition, collateral cleavage activity is turned on, which allows signaling amplification by cleaving nucleic acid reporter (Kim et al., 2021). Among these proteins, Cas13a and Cas14 have the ability to discriminate SNPs when targeting RNA or single-stranded DNA (ssDNA) substrates, respectively (Harrington et al., 2018; Myhrvold et al., 2018). The limitation to RNA or ssDNA substrates requires an additional step of reverse transcription or generation of ssDNA to amplify substrates for effective detection in one-pot content (Gootenberg et al., 2017; Harrington et al., 2018). Cas12a recognizes dsDNA as substrate, and has been applied to detect nucleic acid. But the lack of sensitivity to SNP restrains its usage in SNP detection.

Here, we developed a Cas12a-based one-pot SNP detection method by combining protein engineering, crRNA truncation, and an optional non-canonical PAM. The engineered sensitive Cas12a (seCas12a) system was able to detect pharmacogenomic SNPs in *CYP2C9* and *CYP2C19* within 30 min. The newly developed one-pot seCas12a detection system allowed SNP detection with high specificity and accuracy in a short sample-to-answer time.

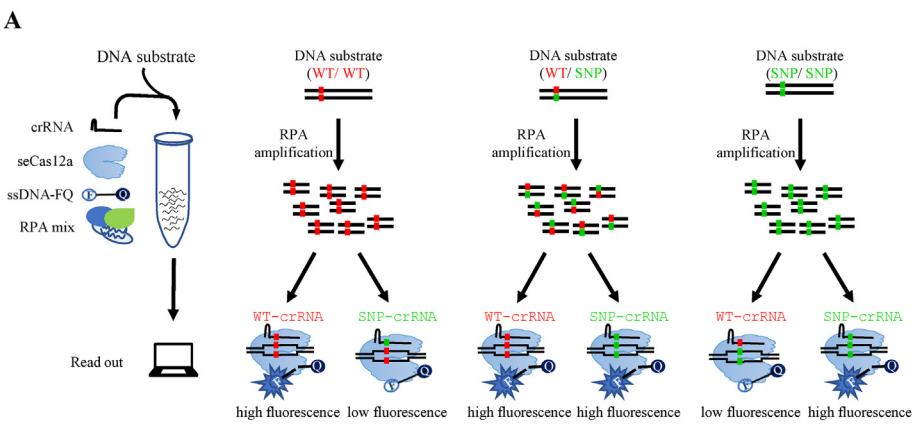
2. Results

2.1. Structure-guided protein engineering of LbCas12a with enhanced sensitivity to single nucleotide polymorphism

Cas12a has been used for nucleic acid detection (Paul & Montoya, 2020). Recently, we have established a Cas12a-based one-pot detection system named "sPAMC", which has shown enhanced specificity and sensitivity in SARS-CoV-2 detection (Lu et al., 2022). Cas12a based nucleic acid detection lacks the sensitivity to differentiate SNP, thereby limiting its application in one-pot detection of SNP. In this study, we aimed to engineer a simplified SNP detection system, whereby Cas12-mediated detection and RPA amplification are conducted at the same time and allele specific crRNA is added in separate reactions to guide the allele specific fluorescence readouts (Fig. 1A).

We first designed and screened crRNA recognizing pharmacogenomic SNP in *CYP2C19**2 (681 G > A) and *CYP2C9**3 (42,614 A > C), two clinically relevant targets. We compared allele specific crRNA with their respective matched genotype or unmatched genotype. The fluorescence produced by matched or unmatched genotype were defined as signal or noise respectively (Fig. 1B). Of 42 crRNAs screened in *CYP2C19**2, crRNA #17 and crRNA #30 had highest signal-to-noise ratio against the *CYP2C19**2 SNP (Fig. S1A) and were chosen for further optimization. In *CYP2C9**3 (42,614 A > C) locus, there was only one crRNA for each allele due to the limited PAM sequence of TTTV. Previous study has indicated truncating the length of crRNA could help increase the specificity (Fu et al., 2014). We then compared variable length of crRNA targeting A allele at 42,614 SNP (Fig. S1B). CrRNA truncation improved its specificity to a certain extent, but the effect was not ideal with compromised on-target activity when truncated to 18 nt length, suggesting that crRNA truncation alone cannot be used as an effective way to distinguish variations.

We then speculated that the binding strength between Cas12a RNP and substrate may be crucial to its specificity. To test the hypothesis, we examined the protein structure of LbCas12a (Yamano et al., 2017) and focused on sites that have close interaction with crRNA and DNA substrates. Amino acids responsible for crRNA binding and DNA recognition, such as Asn160, Lys167, Arg174, Asn260, Ser286 in the REC1 domain, Lys897, Ile896, Gln944, Lys945, Phe983, Lys984 in the RuvC domain, Tyr542 in the wedge domain and Arg883 in the Bridge helix were individually mutated to alanine or lysine to modulate the binding affinity. In addition, Glu174, Asn282, Ser542 and Ser548 in AsCas12a were reported to be important for its activity and specificity (Kleinstiver et al., 2019). Their corresponding amino acids in LbCas12a (Asp156, Asn260, Gly532 and Lys538, respectively) were also mutated. We used crRNA #17 targeting G allele in *CYP2C19**2 (681 G > A) SNP in the first round screening (Fig. 1B). As shown in Fig. 2A and B, LbCas12a variants carrying R174A, R174K, K538R/R174K, K897A/R174A or K945A/R174A exhibited enhanced SNP specificity without sacrificing its on-target activity. To determine whether these variants function similar for A allele in *CYP2C19**2 SNP, we performed second screening using crRNA #30 (Fig. 1B). Of five variants tested, only K538R/R174K variant showed high signal against matched genotype and low noise signal to unmatched genotype (Fig. 2C and D). To assess whether the enzyme activities of these variants were affected, we examined the *trans*-cleavage of the variants, in which RPA amplification was not included. CrRNA #17 and crRNA #30 targeting *CYP2C19**2 (681) G or A allele respectively were used with their respective matched genotype (Fig. S1C & D). The results



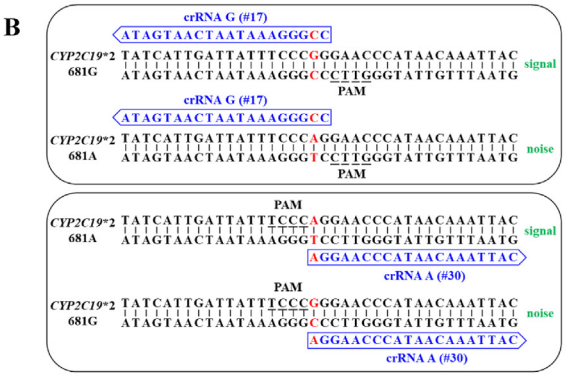


Fig. 1. Schematic diagram of seCas12a-based SNP detection system.
(**A**) Schematic illustration of seCas12a-based SNP detection method. In one-pot SNP test, RPA and Cas12a-based detection were performed simultaneously. Allele specific crRNA was added to each reaction and fluorescence readout was used to deduct the SNP (**B**) Schematic of crRNA design. CrRNA G/681 G or crRNA A/681A refers to crRNA fully complementary to *CYP2C19**2681 G or A allele respectively. Signal or noise refers to fluorescence produced by matched or unmatched genotype respectively.

showed that while their specificity improved, their enzyme activity decreased accordingly. To investigate the sensitivity of seCas12a-based one-pot SNP detection system, we detected double-stranded DNA (dsDNA) substrates with different concentrations. The detection limit of seCas12a-based SNP detection system was about 0.11 pM against *CYP2C19**2 SNP or 0.22 pM against *CYP2C9**3 SNP in the one-pot reaction (Fig. S1E). Together, through structure-guided protein engineering, we identified a variant K538R/R174K of LbCas12a, renamed as seCas12a (sensitive Cas12a), showing enhanced sensitivity to discriminate SNPs.

2.2. Truncated crRNA further enhanced the sensitivity of seCas12a

We next determined whether the specificity of seCas12a-based one-pot SNP detection system can be improved by crRNA truncation. CrRNA A or crRNA C targeting *CYP2C9**3 (42,614) A or C allele respectively were tested. While truncating the spacer region of crRNA to 18 nt failed to produce sufficient fluorescent signal, truncating to 19 nt help improve the specificity of crRNA C when paired with seCas12a (Fig. 3A). In comparison, the 19 nt crRNA C when paired with wildtype Cas12a still developed substantial noise signal against target bearing A allele (Fig. 3A). Quantification of fluorescent signal at endpoint also confirmed that crRNA C developed highest specificity when using 19 nt truncated version with seCas12a variant and crRNA A exhibited high specificity at all three lengths when paired with seCas12a (Fig. 3B). Normalization of signal-to-noise (SNR) of each crRNA suggested that only when the SNR is above four, the assay is sensitive enough to detect the SNP (Fig. 3E).

Our previous study has suggested that a delicate balance between CRISPR activity and RPA amplification was the key to a successful one-pot reaction (Lu et al., 2022). On the one hand, CRISPR detection requires sufficient target DNA enrichment via RPA to generate enough signal. On the other hand, CRISPR itself consumes target DNA to produce signals. To solve the incompatibility, the kinetics of *cis*-cleavage has to be finely tuned to allow sufficient RPA-mediated target enrichment in the early stage in one-pot reaction (Lu et al., 2022). In the SNP detection, the target DNA and non-target DNA only have one nucleotide difference, requiring a more rigid balance of CRISPR kinetics between on-target and non-target. To determine the delicate balance, we quantified *cis*-activity of seCas12a with their respective crRNAs (Fig. 3C). Consistent with one-pot readout, crRNAs that has good signal-to-noise fluorescence ratio also developed good signal-to-noise cleavage with their respective targets (Fig. 3E). For example, 20 nt or 19 nt crRNA C when paired with seCas12a produced low but detectable cleavage products against C allele but no cleavage against A allele (Fig. 3C, bottom panel). When the length of crRNA is truncated to 18 nt, no *cis*-cleavage activity was detected when paired with seCas12a, consistent with no fluorescent signal using the same crRNA and Cas pair. Since *cis*-activity determines the final *trans*--activity, we then measured the *cis*-cleavage kinetics of each crRNA to their on-target allele. As revealed in Fig. 3D and E, only when *cis*-kinetics was within the range of $0.0007\ min^{-1}<K_{cleave}<0.01\ min^{-1}$, the crRNA with seCas12a exhibited a signal-noise ratio greater than 4 in one-pot reaction.

2.3. SeCas12a-based one-pot SNP detection system is a versatile platform

In our seCas12a-based one-pot SNP detection system, we used non-canonical PAM (VTTV, TCTV, TTVV, CCTV, TCCV, CCCV, V = non T) which has enabled significantly more sites to choose crRNA compared with the canonical PAM (TTTV, V = non T) (Fig. 4A). Further analysis of human genome that can be edited with non-canonical PAMs showed the editable sites was 4.62 times that of canonical PAM (Fig. 4A). To test whether canonical PAM was suitable for seCas12a-based one-pot SNP detection system, we changed non-canonical PAM of crRNA A or crRNA C targeting *CYP2C9**3 (42,614) A or C allele from TCCA to TTTA. CrRNA A exhibited comparable specificity on substrates with canonical or non-canonical PAM, while crRNA C developed increased noise signal against non-matched genome using canonical PAM (Fig. 4B), indicating that the PAM selection has to be individually tested and tailored for each

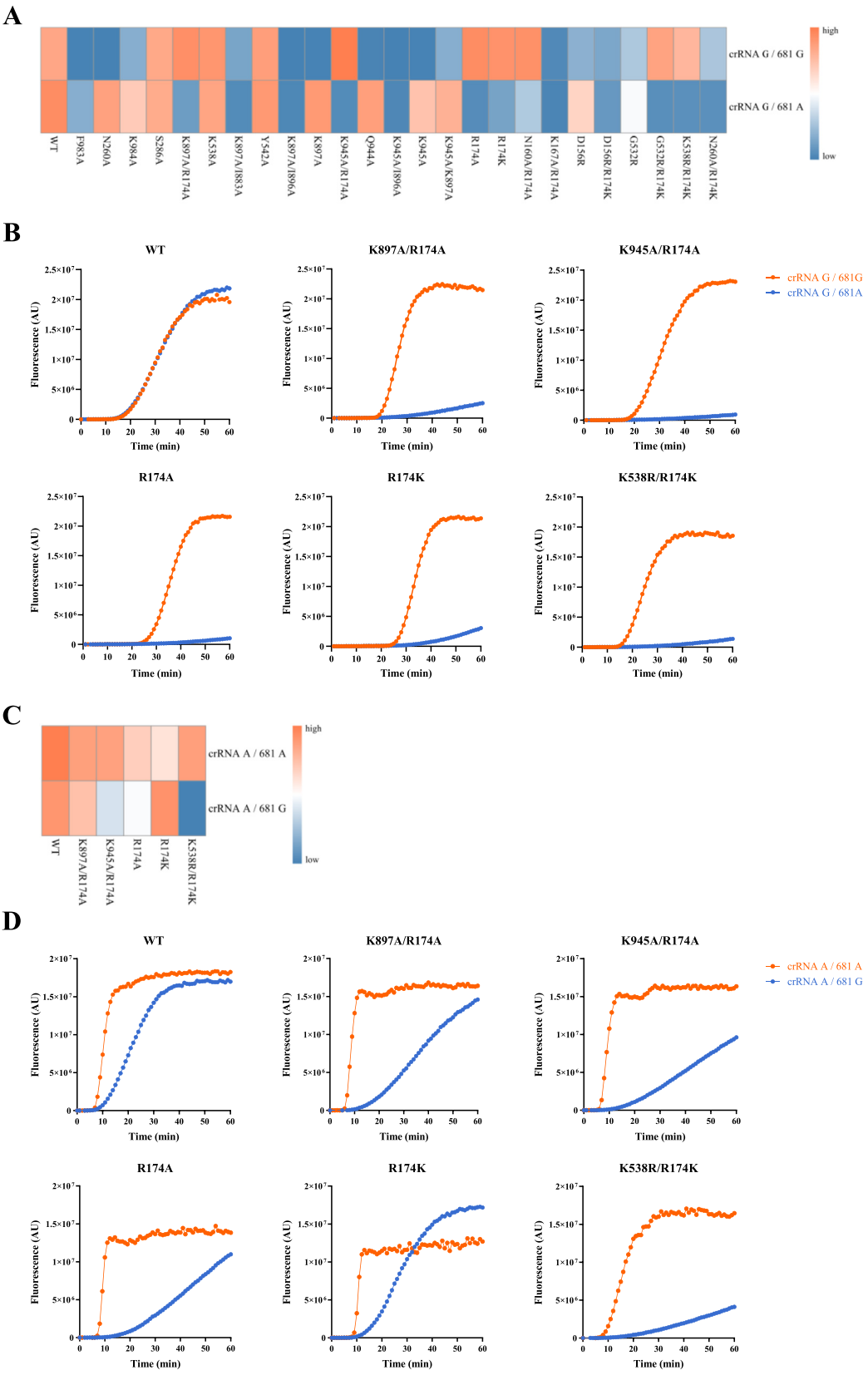
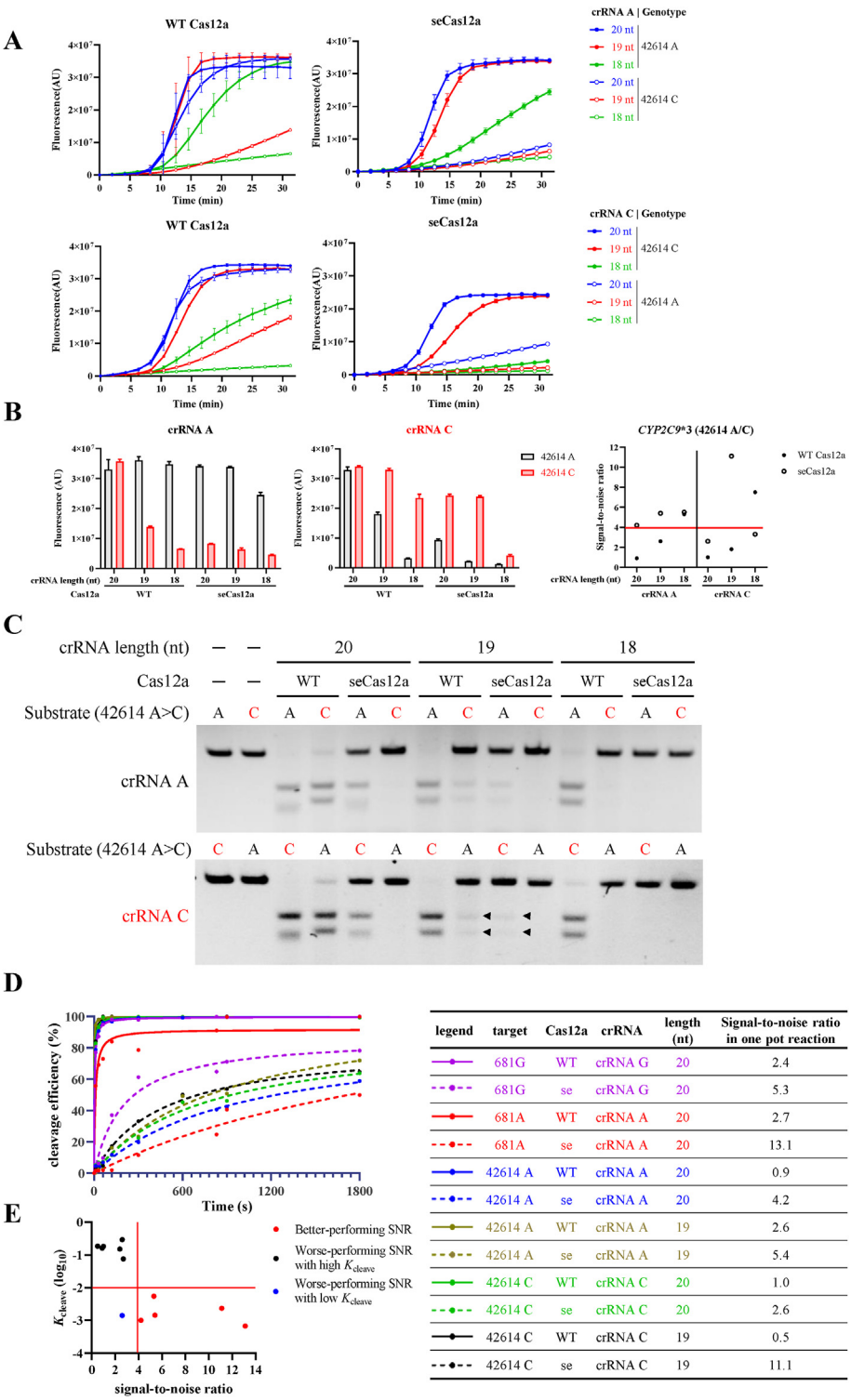


Fig. 2. Genetically engineered LbCas12a to allow SNP detection in one-pot test.
(**A**) Heatmap of end point fluorescence signals (collected at 60 min). First round of screening of LbCas12a variants was tested in one-pot SNP detection of *CYP2C19**2. CrRNA G was used and tested in *CYP2C19* *2 SNP bearing G or A allele. (**B**) Representative real-time fluorescence curves of LbCas12a variants with enhanced sensitivity. Fluorescence signals were read with a 1-min intervals (λ_{ex} = 485 nm; λ_{em} = 528 nm). (**C**) Heatmap of end point fluorescence signals using crRNA A in *CYP2C19**2 SNP. (**D**) Real-time fluorescence curve of LbCas12a variants in (**C**). Fluorescence signals were read with a 1-min intervals (λ_{ex} = 485 nm; λ_{em} = 528 nm).



legend	target	Cas12a	crRNA	length (nt)	Signal-to-noise ratio in one pot reaction
	681G	WT	crRNA G	20	2.4
	681G	se	crRNA G	20	5.3
	681A	WT	crRNA A	20	2.7
	681A	se	crRNA A	20	13.1
	42614 A	WT	crRNA A	20	0.9
	42614 A	se	crRNA A	20	4.2
	42614 A	WT	crRNA A	19	2.6
	42614 A	se	crRNA A	19	5.4
	42614 C	WT	crRNA C	20	1.0
	42614 C	se	crRNA C	20	2.6
	42614 C	WT	crRNA C	19	0.5
	42614 C	se	crRNA C	19	11.1

Fig. 3. Optimization of crRNA can improve sensitivity in one-pot SNP detection.
(**A-B**) Real-time fluorescence curve (**A**) and endpoint comparison (**B**) of WT or seCas12a based one-pot SNP detection targeting *CYP2C9**3 (42,614 A/C) SNP. Experiments were performed independently for three times and data were plotted as mean ± S.D. (**C**) *In vitro cis*-cleavage activity of WT or seCas12 with indicated crRNA targeting *CYP2C9**3 (42,614 A/C) SNP. (**D**) *Cis*-cleavage kinetic analysis of Cas12a-based SNP detection. Corresponding signal-to-noise ratio was calculated using endpoint fluorescence signals measured at 30 min in one-pot reaction. (**E**) Correlation analysis of cleavage kinetics and one-pot SNP signal-to-noise ratios.

crRNA to fit into the kinetic window (Fig. 3D and E).

Next, we determined the effect of SNP position in our one-pot reaction by generating single mutation in the perfect paired crRNA at different positions. In brief, M0 refers no mutation, M1 to M19 refers to the mutation at indicated position (Fig. S1F). As revealed in Fig. 4C and D, while M18 and M19 produced high fluorescent signal, M1 to M7 or M14-M17 produced weak fluorescent signal that could be distinguished from wildtype substrate (M0), and M8 to M13 showed background signal. Together, it suggested that seCas12a-based one-pot reaction could discriminate mutations at spacer region from M1-M17, but not in PAM-distal region. In short, when designing crRNA targeting SNPs, as long as SNPs fall into the position 1–17 counting from PAM side, a good separation of signal verse noise could be achieved. To investigate whether seCas12a-based one-pot SNP detection system could discriminate both base transversion and substitution, we mutated *CYP2C9**3 (42,614) A allele to C, G and T, respectively. Both crRNA A and crRNA C could discriminate base transversion and substitution (Fig. 4E). Quantification of fluorescent signal at endpoint also confirmed that crRNA A

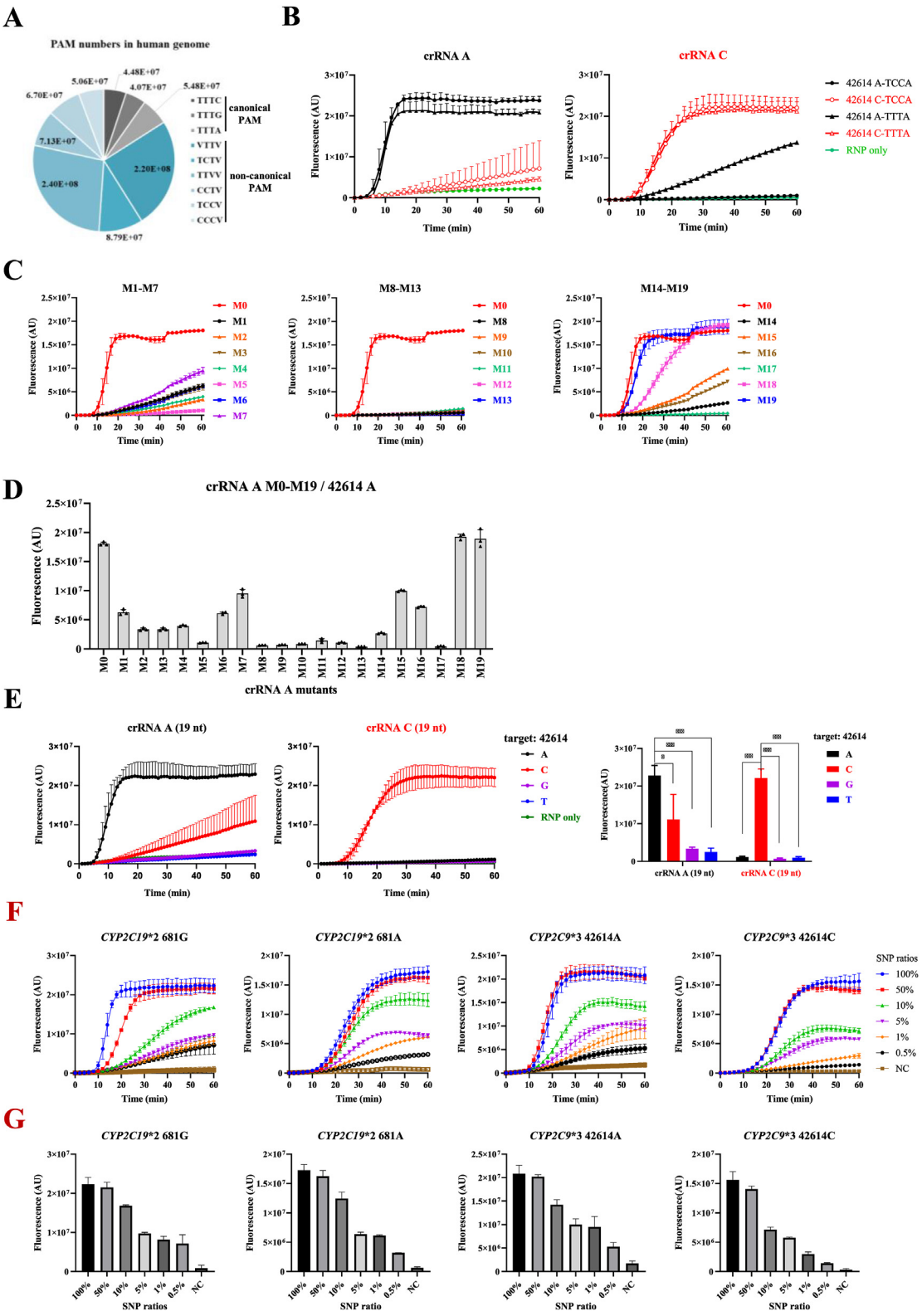


Fig. 4. SeCas12 mediated one-pot SNP detection is flexible with PAM selection.
(**A**) Canonical (gray) and non-canonical (blue) PAM of LbCas12a in human genome. (**B**) Real-time fluorescence curve of non-canonical (5'-TCCA-3') and canonical PAMs (5'-TTTA-3') in one-pot reactions. Truncated crRNA A (19 nt) or crRNA C (19 nt) targeting *CYP2C9**3 42,614 A or C allele respectively were used ($n = 3$, mean ± S.D.). (**C-D**) Real-time fluorescence curve (**C**) and endpoint (**D**) representation of the effects of SNP positions on one-pot sensitivity. 19 nt crRNA A targeting 42,614 A was used.$N = 3$, mean ± S.D. (**E**) Real-time fluorescence curve and endpoint representation of the effects of SNP types on one-pot sensitivity. 19 nt crRNA A and crRNA C targeting 42,614 A or C allele were used. $N = 3$, mean ± S.D. (**F-G**) Real-time fluorescence curve (**F**) and endpoint (**G**) representation of substrates with indicated SNP ratios. $N = 3$, mean ± S.D.

produced significantly higher fluorescent signal on A target when compared with C, G and T target. Similarly, crRNA C produced significantly higher fluorescent signal on C target but only background-level fluorescent signal on A, G and T target (Fig. 4E). To determine limit of SNP ratio in dsDNA substrate, we detected fluorescent signals produced by substrates with different SNP ratio. The results showed that the limit of SNP ratio in seCas12a-based reaction was 5% against CYP2C9*3 SNP or 1% for CYP2C19*2 SNP when the SNR threshold is set at four (Fig. 4F–G and Fig. S1G). Collectively, seCas12a-based one-pot SNP detection system could use both canonical and non-canonical PAM, and was almost not limited by mutation types to distinguish SNPs located between position 1 to 17.

2.4. Detection of pharmacogenomic SNPs using seCas12a-based one-pot reaction

Finally, we applied seCas12a-based one-pot reaction to detect *CYP2C9*3* (42,614 A > C) and *CYP2C19*2* (681 G > A) in human samples. Human oral epithelial cells from volunteers were quick lysed and used as substrates directly. No purification was required to enable a fast and simple processing. For each donor, we sequenced the SNP and indicated the genotypes in the legend (Fig. S2A & B). For *CYP2C9*3* SNP, 19 nt crRNA A and crRNA C were used. Of thirteen donors tested in *CYP2C9*3* SNP, SeCas12 with allele specific crRNA faithfully detected their corresponding genotype within 30 min (Fig. 5A). For *CYP2C19*2* SNP, crRNA #17 and crRNA #30 targeting G and A allele respectively were used. Of thirteen donors tested, both crRNA #17 and crRNA#30 successfully produced allele specific fluorescent signal within 30 min of reaction (Fig. 5B). Together, our new assay based on seCas12a could detect two pharmacogenomic SNPs with high accuracy in human crude lysates.

3. Discussion

CRISPR-Cas based detection have been used in nucleic acid detection and diagnosis, but its application in SNP detection was limited due to the insensitive to distinguish the one nucleotide difference. In this study, we engineered a sensitive LbCas12a variant, named seCas12a. When combined with non-canonical PAM and crRNA truncation, seCas12a could discriminate genomic SNPs within 30 min in one-pot reaction. Protein engineering greatly improved SNP discriminate ability of LbCas12a, making it possible to develop a LbCas12a based one-pot SNP detection system. Considering LbCas12a has a large number of non-canonical PAM, the usage of non-canonical PAM greatly expanded its detection range on genome. In addition, for different targets, we can adjust the crRNA length to achieve the best detection effect. As a result, our system can detect SNPs distributed in a large portion of the genome. The sample preparation in our system is very simple with one-step reaction, which help avoid cross contamination, reduce operation difficulty and detection cost.

Among various Cas proteins, Cas12a recognizes dsDNA as substrates, while Cas13 or Cas14 recognizes RNA or ssDNA respectively (Harrington et al., 2018; Kaminski et al., 2021). The amplification of ssDNA is complex, therefore it is difficult for Cas14a to be applied in one-pot reaction. For Cas13a, transcription process in substrates amplification is time-consuming and the ssRNA reporter is unstable and expensive. In comparison, dsDNA can be quickly amplified via RPA and the amplification and cleavage could be combined in one reaction (Lu et al., 2022). In addition, the ability to use non-canonical and canonical PAMs in seCas12 system greatly expanded its targeted region within human genome. Both canonical and non-canonical PAMs performed well in seCas12a based one-pot reaction, opening the opportunity to design crRNAs targeting almost all SNPs in the genome.

Wild type Cas12a can't well discriminate single-nucleotide mismatch in substrates (Kleinstiver et al., 2019; Li, Cheng, Wang, et al., 2018). To solve the issue, we engineered Cas12a to improve its specificity. Among multiple variants, single amino acid mutant of Arg174 (R174A and R174K) exhibited the highest signal-to-noise ratio, indicating that Arg174 may play a key role in mediating the sensitivity of Cas12 to its DNA target. In support, Arg174 located in RECdomain of Cas12a and had been proved to be intact with crRNA (Yamano et al., 2017). Mutation of Arg174 may interfere the binding ability of Cas12a and render a more sensitive interaction with matched SNP. In addition to Arg174, compound variants K538R/R174K, K897A/R174A and K945A/R174A also exhibited good signal-to-noise fluorescence readout, while K538R, K897A and K945A alone exhibited poor sensitivity. This further highlighted importance of Arg174. Because K538R/R174K variant is the most stable, producing reliable signals on different crRNAs tested, we selected this variant (also renamed as seCas12a) to the one-pot SNP detection. We also performed mechanistic analysis by analyzing the *cis*-kinetics and plotted against the one-pot behavior in SNP detection. We found that a delicate balance between CRISPR activity and RPA amplification was the basis of one-pot reaction for discriminating SNP. When the *cis*-kinetics of CRISPR detection is too strong, the fast cleavage outcompetes the RPA-mediated amplification, thereby leading to reduced signal-to-noise ratio; when the *cis*-kinetics of CRISPR detection is within a tight window range of 0.01min^{-1} and 0.0006 min^{-1}, it favors RPA-mediated amplification but with little compromise on signal production (Fig. 3D and E). More importantly, balance of CRISPR kinetics between on-target and non-target was the key to a successful SNP detection (Fig. 3C).

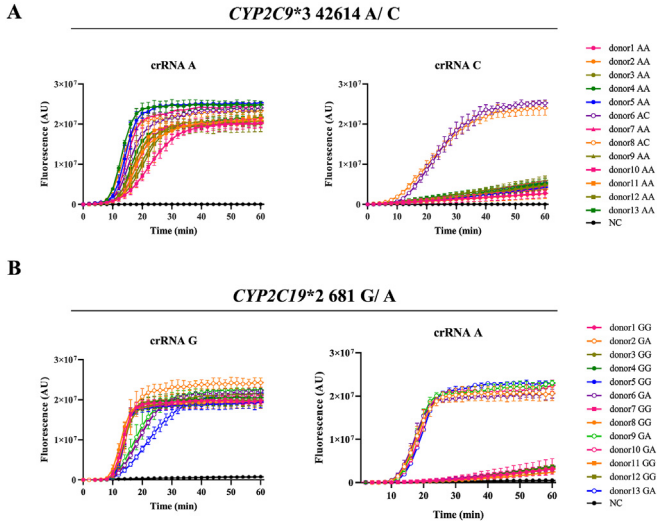


Fig. 5. Application of seCas12-mediated one-pot test in clinical samples. (**A**) Detection of *CYP2C9*3* (42,614 A/C) SNP using truncated crRNAs in 13 donors tested. Truncated crRNA A (19 nt) and crRNA C (19 nt) could faithfully detect the respective genotype.(**B**) Detection of *CYP2C19*2* (681 G/A) allele using full length crRNAs in 13 donors. CrRNA G (20 nt) and crRNA A (20 nt) could faithfully detect the respective genotype. NC is a negative control without any DNA substrate. Data are mean ± S.D. (n = 3).

4. Materials and methods

4.1. Plasmid and dsDNA preparation

The DNA fragments containing the respective SNPs were synthesized and cloned into the pUC57 vector (GenScript Biotech, Nanjing, China). The dsDNA substrates used in cleavage and one-pot detection assay were obtained by PCR using respective PUC57 vector as templates. Primers and DNA sequences were listed in Supplementary Table 1.

4.2. In vitro transcription (IVT)

The DNA templates for *in vitro* transcription (IVT) were obtained by

overlap extension PCR. The primers were listed in Supplementary Table 1. The integrity and purity of amplicons were visualized by gel electrophoresis. CrRNAs were obtained by IVT using T7 RNA polymerase and purified with RNA cleanup kit (NewEngland Biolab).

5. Protein expression and purification

DNA fragment encoding wild-type LbCas12a was cloned into a pET28a-based expression vector with 6 × His-tag in the C-terminal. LbCas12a variant plasmids were obtained by site specific mutagenesis using wild-type Lbcas12a as a template. The *E. coli* BL21(DE3) was used for protein expression. LbCas12a proteins were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sangon) at 21 °C for about 16 h when the *E. coli* BL21 culture density reached an OD600 of 0.6–0.8. Proteins dissolved in the cell lysate were purified via Ni-NTA resin (GE Healthcare) and a gel filtration column (GE Healthcare) respectively. Then the proteins were concentrated via Amicon Ultra centrifugal filters (50 kDa, Millipore) and quantified via BCA Protein Assay Kit (Thermo Fisher Scientific). The proteins were stored at −80 °C using a storage buffer (20 mM Tris-HCl, and 200 mM NaCl, 5% glycerol, pH 7.4).

5.1. LbCas12a in vitro DNA cleavage assay

For *in vitro* cleavage assay, 3 pmol LbCas12a RNP (LbCas12a: crRNA = 1:2, molar ratio) and 50 amol (20 pg) corresponding PCR amplified substrate (about 680 bp) were incubated in 1 × NEBuffer 2.1 at 42 °C for indicated time. The reaction was terminated by adding proteinase K (20 mg/mL, Thermo Fisher Scientific) to the reaction mixture. Then the reaction mixture was incubated at 55 °C for 15 min to digest LbCas12a, at 85 °C for 10 min to inactive proteinase K. Finally, 10 × SDS DNA loading without bromophenol blue was added to visualize on 2% (or 1.5%) TAE gel.

5.2. LbCas12a collateral activity assay

To detect the collateral activity of LbCas12a variants, a 30 μL volume mixture containing 3 pmol LbCas12a RNP, 50 amol PCR amplicon substrate and 80 pmol ssDNA reporter (FAM-TTATT-BHQ1) was prepared with 1 × NEBuffer 2.1. Then the fluorescence signals were detected by SpectraMax i3x (Molecular Devices).

5.3. One-pot detection assay

One-pot detection assay was performed in a 20 μL reaction mixture containing 9 μL RPA mixture (Weifang Amp-Future Biotech, Shandong, China), 3 pmol RNP (LbCas12a: crRNA = 1:1, molar ration), 20 pmol ssDNA reporter (FAM-TTATT-BHQ1), 50 amol PCR amplicon substrate or 2 μL sample pretreatment, and 2.5 μL B Buffer in plate wells (Corning). The fluorescence signals were detected through a SpectraMax i3x at 1 or 2 min intervals (λ_{ex} = 485 nm; λ_{em} = 528 nm). All one-pot assays were measured at 37 °C. The sequences of RPA primers can be seen in Supplementary Table 1.

5.4. Human samples collection and genome DNA extraction

All human buccal epithelial cells samples were collected from voluntary donors using dental floss under biomedical ethics committee of Medical Research Institute of Wuhan University. To collect enough cells, buccal cells were collected 3 times and then suspended with 400 μL PBS buffer, centrifuged into a pellet at 200 g for 10 min. Resuspend the pellet in 50 μl PBS buffer containing 200 μg/mL proteinase K (Thermo Fisher Scientific) and incubated at 55 °C for 1 h and then 85 °C for 10 min.

Author contributions

Y.Z. and H.Y. conceived, designed and managed the project. H.Z. and C.Z. performed all experiments and analyzed the data. L.S., X.T. and K.Z. provided experimental advice. Y.Z., H.Z. and C.Z. wrote the paper.

Declaration of Competing interest

Y.Z., H.Y. and H.-X.Z. have a filed patent application on seCas12a-based SNP detection through Wuhan University (Patent No. 202211514174.0). The other authors declare no competing interests.

Acknowledgments

This work is kindly supported by National Natural Science Foundation of China (31972936 to Y.Z., 32101198 to H.-X.Z., 31871345 and 32071442 to H.Y.), the National Key R&D Program of China (2019YFA0802801 and 2018YFA0801401), the Key R&D Program of Hubei Province (to H.Y. 2022BCA089), the Fundamental Research Funds for the Central Universities (to Y.Z. and H.Y.), and the startup funding from Wuhan University (to Y.Z. and H.Y.). We thank the core facility of Medical Research Institute at Wuhan University for their technical supports.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.compbiomed.2023.106616.

References

Ablin, J., Cabili, S., Lagziel, A., & Peretz, H. (2002). Warfarin therapy in a patient homozygous for the CYP2C9*3 allele. *The Israel Medical Association Journal, 4*, 139–141.

Agrawal, S., Heiss, M. S., Fenter, R. B., Abramova, T. V., Perera, M. A., Pacheco, J. A., Smith, M. E., Rasmussen-Torvik, L. J., & George, A. L. (2020). Impact of CYP2C9-interacting drugs on warfarin pharmacogenomics. *Cts-Clin Transl Sci, 13*, 941–949.

Altshuler, D. M., Durbin, R. M., Abecasis, G. R., Bentley, D. R., Chakravarti, A., Clark, A. G., Donnelly, P., Eichler, E. E., Flicek, P., Gabriel, S. B., et al. (2015). A global reference for human genetic variation. *Nature, 526*, 68.

Furuya, H., FernandezSalguero, P., Gregory, W., Taber, H., Steward, A., Gonzalez, F. J., & Idle, J. R. (1995). Genetic polymorphism of CYP2C9 and its effect on warfarin maintenance dose requirement in patients undergoing anticoagulation therapy. *Pharmacogenetics, 5*, 389–392.

Fu, Y. F., Sander, J. D., Reyon, D., Cascio, V. M., & Joung, J. K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nature Biotechnology, 32*, 279–284.

Gardin, A., Ufer, M., Legangneux, E., Rossato, G., Jin, Y., Su, Z. Z., Pal, P., Li, W. K., & Shakeri-Nejad, K. (2019). Effect of fluconazole coadministration and CYP2C9 genetic polymorphism on siponimod pharmacokinetics in healthy subjects. *Clinical Pharmacokinetics, 58*, 349–361.

Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., & Zhang, F. (2018). Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science, 360*, 439–444.

Gootenberg, J. S., Abudayyeh, O. O., Lee, J. W., Essletzbichler, P., Dy, A. J., Joung, J., Verdine, V., Donghia, N., Daringer, N. M., Freije, C. A., et al. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science, 356*, 438.

Harrington, L. B., Burstein, D., Chen, J. S., Paez-Espino, D., Ma, E., Witte, I. P., Cofsky, J. C., Kyrpides, N. C., Banfield, J. F., & Doudna, J. A. (2018). Programmed DNA destruction by miniature CRISPR-Cas14 enzymes. *Science, 362*, 839.

Hulot, J. S., Bura, A., Villard, E., Azizi, M., Remones, V., Goyenvalle, C., Aiach, M., Lechat, P., & Gaussem, P. (2006). Cytochrorne P4502C19 loss-of-function polymorphism is a major determinant of clopidogrel responsiveness in healthy subjects. *Blood, 108*, 2244–2247.

Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F., & Collins, J. J. (2021). CRISPR-based diagnostics. *Nat Biomed Eng, 5*, 643–656.

Kim, S., Ji, S., & Koh, H. R. (2021). CRISPR as a diagnostic tool. *Biomolecules, 11*.

Kleinstiver, B. P., Sousa, A. A., Walton, R. T., Tak, Y. E., Hsu, J. Y., Clement, K., Welch, M. M., Horng, J. E., Malagon-Lopez, J., Scarfo, I., et al. (2019). Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. *Nature Biotechnology, 37*, 276.

Liao, K., Liu, Y., Ai, C. Z., Yu, X., & Li, W. (2018). The association between CYP2C9/2C19 polymorphisms and phenytoin maintenance doses in asian epileptic patients: A systematic review and meta-analysis. *International Journal of Clinical Pharmacology and Therapeutics, 56*, 337–346.

Li, S. Y., Cheng, Q. X., Liu, J. K., Nie, X. Q., Zhao, G. P., & Wang, J. (2018). CRISPR-Cas12a has both cis- and trans-cleavage activities on single-stranded DNA. *Cell Research, 28*, 491–493.

Li, S. Y., Cheng, Q. X., Wang, J. M., Li, X. Y., Zhang, Z. L., Gao, S., Cao, R. B., Zhao, G. P., & Wang, J. (2018). CRISPR-Cas12a-assisted nucleic acid detection. *Cell Discovery, 4*.

Li, L., Li, S., Wu, N., Wu, J., Wang, G., Zhao, G., & Wang, J. (2019). HOLMESv2: A CRISPR-cas12b-assisted platform for nucleic acid detection and DNA methylation quantitation. *ACS Synthetic Biology, 8*, 2228–2237.

Liu, M., & Obeng, A. O. (2020). Siponimod and CYP2C9 allele prevalence among blacks. *The Journal of Clinical Pharmacology, 60*, 429–431.

Lu, S., Tong, X., Han, Y., Zhang, K., Zhang, Y., Chen, Q., Duan, J., Lei, X., Huang, M., Qiu, Y., et al. (2022). Fast and sensitive detection of SARS-CoV-2 RNA using suboptimal protospacer adjacent motifs for Cas12a. *Nat Biomed Eng, 6*, 286–297.

Mitani, Y., Lezhava, A., Kawai, Y., Kikuchi, T., Oguchi-Katayama, A., Kogo, Y., Itoh, M., Miyagi, T., Takakura, H., Hoshi, K., et al. (2007). Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology. *Nature Methods, 4*, 257–262.

Myhrvold, C., Freije, C. A., Gootenberg, J. S., Abudayyeh, O. O., Metsky, H. C., Durbin, A. F., Kellner, M. J., Tan, A. L., Paul, L. M., Parham, L. A., et al. (2018). Field-deployable viral diagnostics using CRISPR-Cas13. *Science, 360*, 444–448.

Paul, B., & Montoya, G. (2020). CRISPR-Cas12a: Functional overview and applications. *Biomedical Journal, 43*, 8–17.

Sachidanandam, R., Weissman, D., Schmidt, S. C., Kakol, J. M., Stein, L. D., Marth, G., Sherry, S., Mullikin, J. C., Mortimore, B. J., Willey, D. L., et al. (2001). A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature, 409*, 928–933.

Shi, Y. J., Duan, M., Ding, J. M., Wang, F. Q., Bi, L. L., Zhang, C. X., Zhang, Y. Z., Duan, J. Y., Huang, A. H., Lei, X. L., et al. (2022). DNA topology regulates PAM-Cas9 interaction and DNA unwinding to enable near-PAMless cleavage by thermophilic Cas9. *Molecular Cell, 82*, 4160–4175 e4166.

Stefanovic, M., Topic, E., & Samardzija, M. (2005). Genetic polymorphism of CYP2C9 and warfarin dose predicting in anticoagulation therapy. *Clinical Chemistry, 51*. A151-A151.

Wang, J., He, Z., Wang, G., Zhang, R., Duan, J., Gao, P., Lei, X., Qiu, H., Zhang, C., Zhang, Y., et al. (2022). Efficient targeted insertion of large DNA fragments without DNA donors. *Nature Methods, 19*, 331–340.

Yamano, T., Zetsche, B., Ishitani, R., Zhang, F., Nishimasu, H., & Nureki, O. (2017). Structural basis for the canonical and non-canonical PAM recognition by CRISPR-cpf1. *Molecular Cell, 67*, 633.

Yang, S., Gill, R. A., Zaman, Q. U., Ulhassan, Z., & Zhou, W. J. (2020). Insights on SNP types, detection methods and their utilization in Brassica species: Recent progress and future perspectives. *Journal of Biotechnology, 324*, 11–20.

Yin, L. J., Man, S. L., Ye, S. Y., Liu, G. Z., & Ma, L. (2021). CRISPR-Cas based virus detection: Recent advances and perspectives. *Biosensors and Bioelectronics, 193*.

Zhang, H. X., Zhang, Y., & Yin, H. (2019). Genome editing with mRNA encoding ZFN, TALEN, and Cas9. *Molecular Therapy, 27*, 735–746.

Zhao, M., Ma, J., Li, M., Zhang, Y., Jiang, B., Zhao, X., Huai, C., Shen, L., Zhang, N., He, L., et al. (2021). Cytochrome P450 enzymes and drug metabolism in humans. *International Journal of Molecular Sciences, 22*.