Biotechnol Lett (2021) 43:2105–2110
https://doi.org/10.1007/s10529-021-03177-z

ORIGINAL RESEARCH PAPER

Rapid and cost-effective screening of CRISPR/Cas9-induced mutants by DNA-guided Argonaute nuclease

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Received: 1 June 2021 / Accepted: 26 August 2021 / Published online: 17 September 2021

Abstract
Objective With the widespread application of CRISPR/Cas9 gene editing technology, new methods are needed to screen mutants quickly and effectively. Here, we aimed to develop a simple and cost-effective method to screen CRISPR/Cas9-induced mutants.
Result We report a novel method to identify CRISPR/Cas9-induced mutants through a DNA-guided Argonaute nuclease derived from the archaeon *Pyrococcus furiosus*. We demonstrated that the *Pyrococcus furiosus* Argonaute (PfAgo)-based method could distinguish among biallelic mutants, monoallelic mutants and wild type (WT). Furthermore, this method was able to identify 1 bp indel mutations.
Conclusion The PfAgo-based method is simple to implement and can be applied to screen biallelic mutants and mosaic mutants generated by CRISPR-Cas9 or other kinds of gene editing tools.

Keywords CRISPR/Cas9 · Biallelic mutants · Monoallelic mutations · Argonaute nuclease · *Pyrococcus furiosus*

Introduction

CRISPR/Cas9 has been widely applied for genome editing in many fields. The engineered CRISPR-Cas9 cleaves the target DNA and forms a double-strand break (DSB) (Ran et al. 2013). Subsequent DNA repair by the error-prone nonhomologous end-joining (NHEJ) pathway may lead to indels that disrupt open reading frames (Le et al. 2013). The T7 endonuclease I (T7EI) assay and Surveyor nuclease assay are frequently used methods to identify engineered nuclease-induced mutants. However, they are unable to distinguish biallelic mutants from monoallelic mutants. They also cannot distinguish homozygous biallelic mutants from wild type (WT). Several mutation detection methods have been developed for the screening of CRISPR/Cas9-induced mutants in labs, such as high-resolution melting curve analysis (HRM) (Thomas et al. 2014), heteroduplex mobility assay (Chenouard et al. 2016), annealing at critical temperature polymerase chain reaction (ACT–PCR) (Hua et al. 2017), single-strand conformational

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10529-021-03177-z.

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polymorphism (SSCP) (Zheng et al. 2016), mutation sites-based specific primers PCR (MSBSP–PCR) (Guo et al. 2018), and multiplex ligation dependent probe amplification (MLPA) (Biswas et al. 2020), However, each method has limitations, such as being complicated, tedious, and time consuming and having poor efficiency.

We previously reported a CRISPR/Cas12a-based biosensing platform that can simply and efficiently screen CRISPR/Cas9-induced biallelic mutants (Xiao et al. 2020). However, crRNA, which guides the Cas12a protein to cleave target DNA, is unstable and readily degrades in vitro. In addition, the synthesis of crRNA is expensive. Moreover, CRISPR-Cas requires the presence of a protospacer-adjacent motif (PAM) to carry out cleavage activity. These features are barriers to the wide application of CRISPR/Cas12a-based biosensing platforms in the screening of mutants. Analogous to CRISPR–Cas, Argonaute (Ago) proteins are nucleic acid-guided endonucleases that have been discovered in both eukaryotes and prokaryotes (Swarts et al. 2014b; Hegge et al. 2018). Eukaryotic Agos exclusively mediate RNA-guided RNA interference (Bohmert 2014; Ketting 2011). Some prokaryotic Ago proteins are able to employ short DNA instead of RNA as guides to cleave cognate DNA targets (Cao et al. 2019; Kuzmenko et al. 2019; Swarts et al. 2014a, 2015). Unlike CRISPR-Cas, Ago proteins do not require the presence of a PAM, offering more versatility. Ago protein from *Pyrococcus furiosus* is a well-studied DNA-guided nuclease that targets cognate DNA. Behnam Enghiad and Huimin Zhao reported the first application of *P. furiosus* Argonaute (PfAgo) to develop programmable DNA-guided artificial restriction enzymes that can recognize and cleave DNA sequences at any random site and generate defined sticky ends of varying length (Enghiad and Zhao 2017). Recently, Ruyi He and his colleagues developed PfAgo-mediated nucleic acid detection (PAND), which can detect DNA at attomolar sensitivities and distinguish single-nucleotide mutants (He et al. 2019). PAND was successfully applied to identify SARS-CoV-2 and SARS-CoV-2 D614G mutants (Wang et al. 2020). Given that PfAgo possesses single-nucleotide discrimination and does not require the presence of a PAM, we hypothesized that DNA-guided PfAgo could be developed to screen CRISPR/Cas9-induced mutants.

Materials and methods

Cell materials

Individual *CXCL9* and *GBP4* THP-1 cell lines generated by CRISPR/Cas9 and maintained in our lab were used to test the feasibility of the PfAgo-based method. Genomic DNA was extracted from each individual clone using a TIANamp Genomic DNA Kit (TIANGEN Biotech, Shanghai, China).

PfAgo expression and purification

The *PfAgo* gene was codon-optimized for *Escherichia coli* BL21 (DE3) and inserted into a pET-28a expression vector. Target recombinant expression plasmids were transformed into *E. coli* BL21 (DE3) and cultivated on LB agar at 37 °C. The transformed bacteria were inoculated into 10 mL LB medium with antibiotics and incubated overnight at 37 °C with shaking. Cultures were diluted in 1 L fresh LB medium and incubated at 37 °C with shaking until the OD_{600} reached 0.8. PfAgo expression was induced by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. Subsequently, 0.5 mM IPTG was added, and cultures were incubated at 18 °C for 12 h. Bacteria were harvested by centrifugation at 12,000 ×g for 10 min and frozen at 80 °C. The recombinant proteins were purified using a His-tag Protein Purification Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Diafiltration and concentration of the purified proteins were performed using Amicon® Ultra15 10 K Centrifugal Filter Devices (Millipore, Shanghai, China) according to the manufacturer's instructions. The purified proteins were analyzed by SDS–PAGE. The concentrations of the purified proteins were determined using a BCA protein assay reagent kit (Beyotime, Shanghai, China).

Design of guided DNA (gDNA)

gDNAs were designed to target CRISPR-Cas9 editing sites. CRISPR-Cas9 usually mediates DSBs ∼ 3 bp upstream of the PAM. Most mutations are either insertions or deletions located close to the DSB site. A previous study demonstrated that the cleavage activity of PfAgo decreased dramatically when the single mismatch was located 3–5 nt or 8–15 nt from the 5′

end (He et al. 2019). Therefore, the gDNA should be designed to cover the DSB site. 5'-Phosphorylated gDNA targeting the desired location was synthesized by IDT Sangon Biotech (Shanghai, China) and is listed in supplementary table 1. PCR primers used for amplification of the target region are listed in supplementary table 1.

PfAgo cleavage assay

PCR was performed in 50 µl volumes using 2 × Phanta Max Master Mix (Vazyme, Shanghai, China) and the indicated primers. The following amplification program was used: one cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 60–68 °C for 30 s, and 72 °C for 30 s; followed by one cycle at 72 °C for 10 min; and then holding at 4 °C. For the PfAgo cleavage assays, PfAgo and gDNA were premixed at a 1:2:2 ratio (500 nM PfAgo, 1 µM forward-gDNA, 1 µM reverse-gDNA) in reaction buffer (20 mM HEPES pH = 7.5, 250 mM $NaCl_2$, 0.5 mM $MnCl_2$) at 75 °C for 10 min. Then, 3–5 µl unpurified PCR products were added to premixed PfAgo-gDNA complexes and incubated at 95 °C for 10 min (unless stated) followed by slow cooling. All the cleavage products were directly examined by 1% agarose gel electrophoresis.

Results and discussion

CRISPR/Cas9-induced mutations in diploid cells may have three genotypes, including biallelic mutations (in which both alleles are mutated), monoallelic mutations (in which one of the two alleles is a single mutation), and WT (in which neither allele is mutated). Figure 1 shows a schematic overview of the PfAgo-based cleavage method. Target sequences were amplified by PCR and then incubated with PfAgo preloaded with gDNAs at 95 °C for 10–30 min. The reaction was cooled to room temperature and examined by agarose gel electrophoresis. If both alleles were WT, the agarose gel displayed two bands. If one of the two alleles was mutated, the agarose gel displayed three bands. When both alleles were mutated, the gel displayed a single band. In general, small indels are always generated at target sites after CRISPR/Cas9 gene editing, while large deletions are much less common. When a large deletion occurs in biallelic mutants, it is evidenced in agarose gels as a single, smaller band or even as no band compared to the mock. When a large deletion occurs in monoallelic

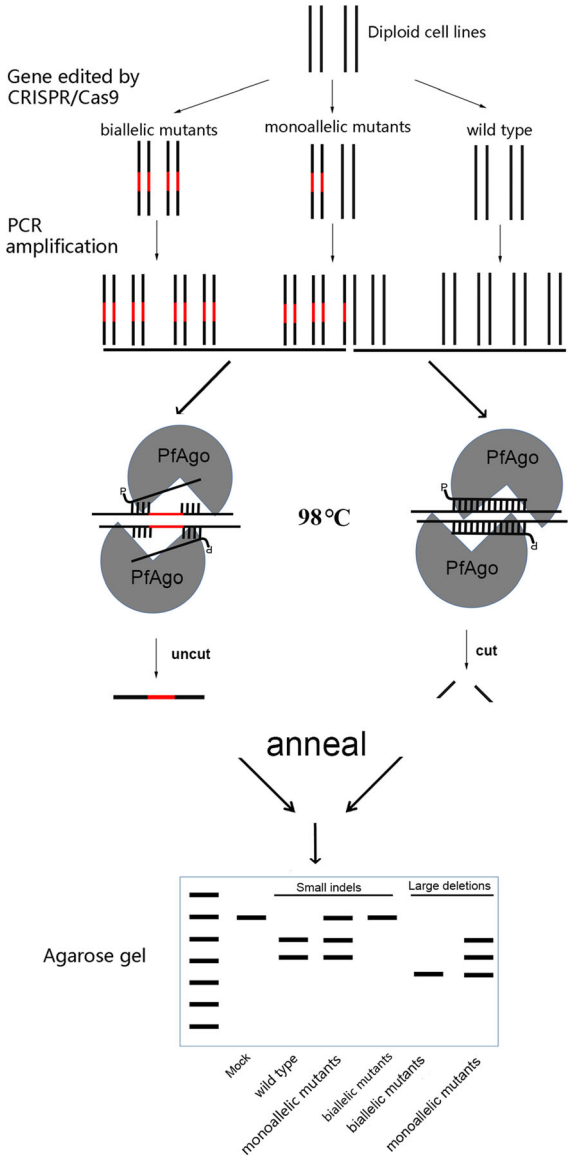

Fig. 1 Schematic overview of the PfAgo-based method. Diploid cells edited by CRISPR/Cas9 may have one of three genotypes: biallelic mutant, monoallelic mutant, and wild type. Mutations in genomic regions are shown in red. Target sequences containing mutation sites were first amplified by PCR. PfAgo with paired gDNAs was incubated with PCR products at 98 °C. After cooling, the reaction mixtures were examined by agarose gel electrophoresis. Wild type, monoallelic mutants, and biallelic mutants show two, three, and one band, respectively, in gel

mutants, it appears in agarose gel as three smaller bands compared to the mock.

Individual clones of mutated human *CXCL9* and *GBP4* THP-1 maintained in our lab were used to test the feasibility of this method. Procedures were performed as described in the Methods section. First, we tested the efficiency of PfAgo in cutting the target DNA in a time-dependent manner. As shown in Fig. 2A, PfAgo quickly and completely cut the target DNA into two bands. To determine the mismatch tolerance of the PfAgo-gDNA complex in cutting targets, we designed a series of gDNAs that contained one to six base mismatches to the target DNA. We observed that a two-base mismatch resulted in the loss of cleavage activity of PfAgo (Fig. 2B), indicating that this method can be applied to identify gene mutations. Subsequently, we used these identified biallelic mutants to test the PfAgo-based method. As shown in Fig. 2C, PfAgo failed to cleave the PCR products of the four *CXCL9* heterozygous biallelic mutants (#1–33: del 30 bp/del 14 bp, #1–63: ins 1 bp/del 37 bp, #1–8: del 4 bp/del 7 bp and #1–65: del 6 bp/del 2 bp), while the mimic monoallelic mutant (MM) and WT PCR amplicons were cleaved into three

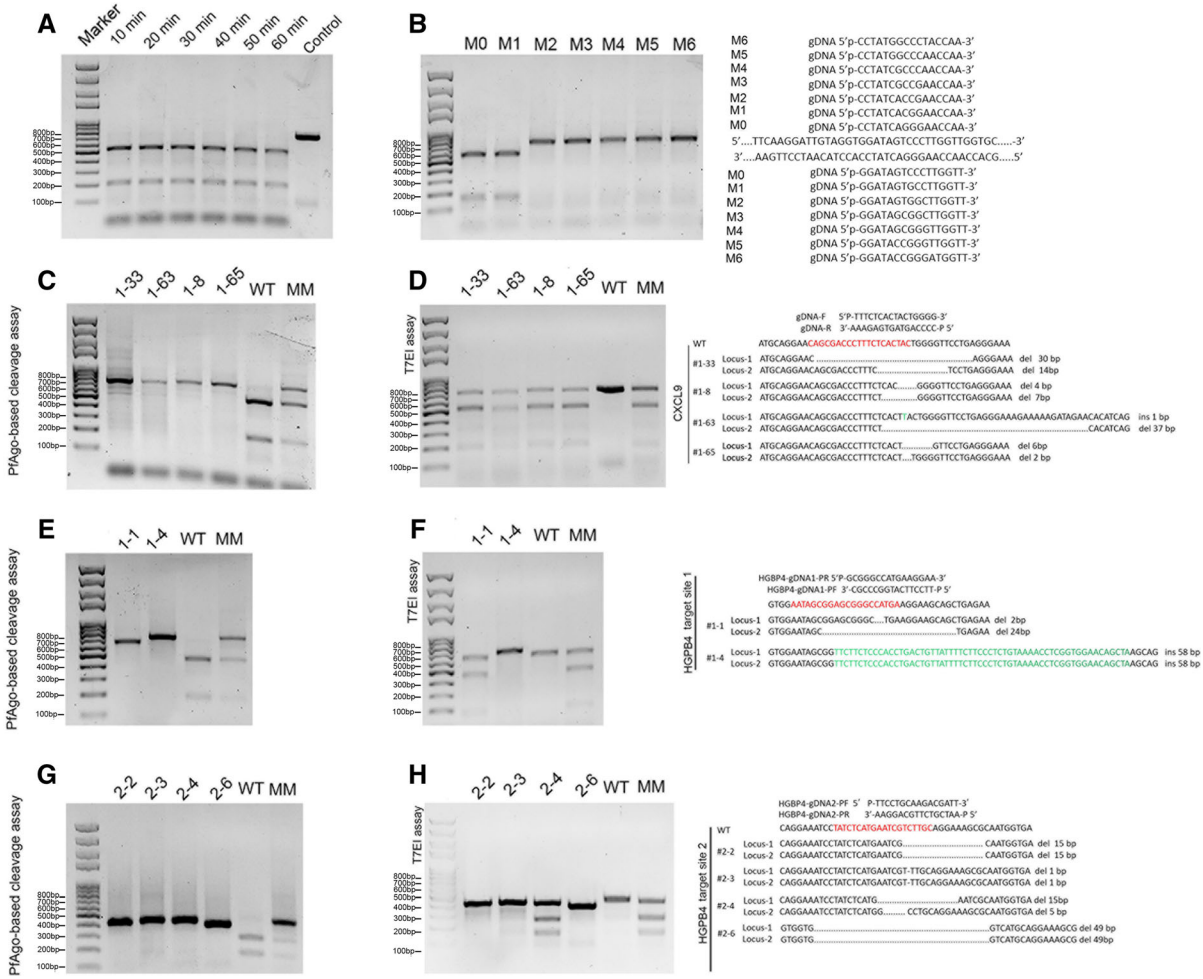

Fig. 2 Evaluation of the feasibility of the designed PfAgo-based method. **A**: Testing the efficiency of PfAgo-gDNA in cutting the target DNA at different time points. **B**: Determination of the mismatch tolerance of the PfAgo-based method. M0–M6 represent 0–6 base mismatches between gDNAs and target sites. **C**, **E**, **G**: PfAgo-based cleavage assays with identified biallelic mutants. **D**, **F**, **H**: T7EI cleavage assay with corresponding biallelic mutants. #1–33, #1–63, #1–8 and #1–65 are THP-1 *CXCL9* biallelic mutants. #1–1, #1–4, #2–2, #2–3, #2–4 and #2–6 are THP-1 *GBP4* biallelic mutants. WT represents wild type. MM containing PCR products from biallelic mutants and WT was used as a mimic monoallelic mutant. All the sequences of mutant loci are listed on the right side

and two bands, respectively (Fig. 2C). The uncut #1–63 (ins 1 bp/del 37 bp) line demonstrated that PfAgo is able to discriminate biallelic mutants with a 1 bp insertion in PCR products. In contrast, T7EI assays showed that the PCR products of the four *CXCL9* heterozygous biallelic mutants and monoallelic mutant were cut into three bands, while the PCR products of WT were uncut, suggesting that T7EI assays can distinguish mutants from WT but not biallelic mutants from monoallelic mutants (Fig. 2D). As shown in Fig. 2E, the human *GBP4* heterozygous (#1–1: del 2 bp/del 24 bp) and homozygous biallelic mutants (#1–4: ins 58 bp//ins 58 bp) showed one band in gel, differing from the WT and monoallelic mutants. However, the T7EI assay could not distinguish the human *GBP4* homozygous biallelic mutants (#1–4: ins 58 bp/ins 58 bp) from WT or the human *GBP4* heterozygous (#1–1: del 2 bp/del 24 bp) biallelic mutants from monoallelic mutants (Fig. 2F). Figure 2G shows that PfAgo failed to cut the PCR products of the four selected *GBP4* biallelic mutants (#2–2: del 15 bp/del 15 bp, #2–3: del 1 bp/del 1 bp, #2–4: del 15 bp/del 5 bp and #2–6: del 49 bp/del 49 bp), while the WT and MM PCR amplicons were cleaved into two and three bands, respectively. The uncut #2–3 (del 1 bp/del 1 bp) line indicated that PfAgo is able to discriminate biallelic mutants with a 1 bp deletion in PCR products. As expected, T7EI failed to cut the PCR products of WT and the *GBP4* homozygous biallelic mutants (#2–2: del 15 bp/del 15 bp, #2–3: del 1 bp/del 1 bp and #2–6: del 49 bp/del 49 bp) (Fig. 2H), while both the *GBP4* heterozygous biallelic mutants (#2–4: del 15 bp/del 5 bp) and MM were cleaved into three bands (Fig. 2H).

Although the PfAgo-based method may be slightly more expensive than the T7EI cleavage assay due to the requirement of gDNA, it has a significant advantage in that it can distinguish biallelic mutants from both monoallelic mutants and WT and can identify 1 bp indels. The entire test can be performed within one hour. The use of this method to screen biallelic mutants can decrease the number of clones that require DNA sequencing and western blot confirmation, which can save time and costs.

Supplementary Information Supplementary Table 1

Funding This work was supported by the National Key Research and Development Plan (Nos. 2020YFA0907200, 2019YFC0840602), the Guangdong Foundation for Basic and Applied Basic Research (No. 2019B1515120041), the Shenzhen Scientific and Technological Foundation (No. KCXFZ202002011007083, JCYJ20180228162336873), and the Sanming Project of Medicine in Shenzhen (No. SZSM201911009).

Declarations

Conflict of interest The authors declare no conflicts of interest.

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