Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Pyrococcus furiosus Argonaute-mediated nucleic acid detection

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Experimental Section

Expression and purification of recombinant *Pyrococcus furiosus* Argonaute protein

The open reading frame (ORF) of *Pyrococcus furiosus* Argonaute (*Pf*Ago) was optimized based on the codon usage bias of *Escherichia coli* and synthesized by Genecreate (China, Wuhan). The ORF was cloned into a pET23a plasmid to obtain the pET23a-6×His-*Pf*Ago expression vector (Figure S8A). The sequence of the *Pf*Ago coding region in plasmid pET23a-6×His-*Pf*Ago is detailed in Table S1. The recombinant expression vector was transformed into *E. coli* BL21 (DE3) pLySs, and the transformant inoculated in Luria-Bertani (LB) media containing 100 μg/ml ampicillin followed by incubation at 37°C, 220rpm for 16 h. Cells were harvested by centrifugation at 3000 × g for 5 min, and the supernatant was removed. The pellet was resuspended in Terrific Broth containing 50 μg/ml ampicillin and incubated at 37°C until the optical density (OD_{600}) reached 0.6−0.8. Expression of the target protein was induced with 1 mM of isopropyl β-d-1-thiogalactopyranoside (IPTG) at 18°C for 20 h. Cells were collected and resuspended in Buffer I (20 mM Tris/HCl, pH 8.0, 300 mM NaCl, 2 mM $MnCl_2$), followed by disruption with Ultrasonic Homogenizer (Scientz, China, Ningbo). The crude cell lysate was centrifuged at 30,000 × g for 30 min, 4°C. The supernatant was collected and incubated at 80°C for 30 min, followed by centrifuging at 30,000 × g for 30 min and 4°C to remove denatured protein. The supernatant was applied to nickel-charged (Ni-NTA) beads for affinity purification. The column was washed twice with two column volumes of wash buffer (20 mM Tris/HCl pH8.0, 300 mM NaCl, 2 mM $MnCl_2$, 50 mM imidazole), then one column volume of elution buffer (20 mM Tris/HCl pH8.0, 300 mM NaCl, 2 mM $MnCl_2$, 200 mM imidazole) to recover the target protein. The sample was then collected and dialyzed with a Millipore (Darmstadt, Germany) 50-kDa-cut-off membrane at 4°C to remove ions and salts, followed by resuspension in storage buffer (20 mM Tris-HCl, pH8.0, 300 mM NaCl, 0.5 mM $MnCl_2$, 15% [v/v] glycerol). The final solution was stored at −80°C until analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S8B).

Primers, enzymes, and reagents

All the double-stranded (ds)DNA targets used in this study were synthesized by Genecreate and are detailed in Table S2. The single-stranded (ss)DNAs (guide DNA, ssDNA targets, and molecular beacons) were synthesized by Sangon (China, Wuhan), and the sequences are listed in Tables S3−S9. All primers used for amplification of the targets in *Pf*Ago-mediated nucleic acid detection (PAND) were designed with melting temperatures of approximately 58°C and their sequences are detailed in Table S10. The DNA-PAGE gel extraction kit (70908-30) was purchased from TIANDZ (China, Beijing). We obtained SYBR

Gold from Invitrogen (United States), PrimerSTAR Max DNA Polymerase was purchased from Takara Biomedical Technology (Beijing). The IsoAmp®II Universal thermophilic helicase-dependent DNA amplification (tHDA) Kit and T4 polynucleotide kinases (PNK) were purchased from New England Biolabs (NEB, United States). Reagents for cell culturing, Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin-streptomycin were obtained from Hyclone (United States). For clinical sample detection, the Blood Directed Polymerase Chain Reaction (PCR) Kit V2 was purchased from Vazyme (China, Nanjing).

Protocol for *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection

Before the procedure, ssDNA was treated with T4 PNK to obtain 5'-phosphorylated ssDNA. About 10 pmol of *Pf*Ago, 1 pmol of 5'-phosphorylated ssDNA (total concentration), 5 pmol of molecular beacon, and 1−4 pmol target DNA were added into the reaction buffer (which contained 20 mM HEPES pH7.5, 250 mM NaCl, and 0.5 mM $MnCl_2$) in a total volume of 10 μl, to which 10 μl mineral oil was added. The reaction mixture was incubated at 95°C for 30 min in a dry bath and it was cooled to room temperature for fluorescence intensity detection using a fluorescence spectrometer (Hitachi, F-2700, Tokyo). To improve the sensitivity of this nucleic acid detecting method, PCR or tHDA were employed to amplify target DNA fragments of approximately 70−300 bp before endonuclease digestion. The following program of PCR was used: initial denaturation at 95°C for 3 min; 30 thermo-cycles of denaturation at 95°C for 10 s, annealing at 58°C for 15 s, and extension at 72°C for 10 s; followed by extension at 72°C for 5 min. We performed tHDA according the manufacturer's instructions included in the IsoAmp®II Universal tHDA Kit.

Gel electrophoretic analysis

PrimerSTAR Max DNA Polymerase amplification and tHDA amplification were conducted as per the manufacturers' protocols. Analysis of dsDNA was conducted with 1% (w/v) agarose gel electrophoresis. We mixed 1 μl of 6 × loading buffer with 5 μl nucleic acid mixture, and gel electrophoresis was performed by running the prepared solution through the gel in 1 × TAE at 120 V for 50 min. The ssDNA was analyzed by 16% urea-denaturing PAGE (4.2 g urea, 4 ml 40% acrylamide, 1 ml 10 × TBE, 2 ml formamide, 9 μl 10% APS and 1 μl TEMED in a total volume of 10 ml). Next, 10 μl of 2 × loading buffer (7 M urea, 0.3 M sodium acetate, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol FF) was mixed with 10 μl of the nucleic acid mixture, and the mixture incubated at 95°C for 5 min to prepare for electrophoresis. Gel electrophoresis was performed by running the prepared solutions through the gel

in 0.5 × TBE at 150 V for 90 min. After electrophoresis, gels were stained with SYBR Gold (Invitrogen) and visualized on the Gel Doc XR+ system (BioRad, USA).

Extration of HEK293T genomic DNA

HEK293T cells were cultured at 37℃ under 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum. Genomic DNA of HEK293T cells was extracted as described in manufacturer's instructions included with the TIANamp kit (Tiangen Biotech, Beijing)

Determining the sensitivity and specificity of *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection

To investigate the sensitivity of PAND, the concentration of the HEK293T genomic DNA was measured with the Nanodrop 8000 (Thermo Fisher Scientific) and adjusted to by successive 10-fold serial concentrations from 1.6×10^5 aM to a final concentration of 1.6×10^0 aM. The diluted samples were utilized as targets for PAND. The rs12516 single nucleotide polymorphism (SNP) was detected using three guide DNAs; M1-gf (rs12516), M1-gr (rs12516), and M1-gt (rs12516T); and a molecular beacon, MB-rs12516T (Table S4). To analyze the specificity of PAND, we designed 17 guide DNAs (Table S5) including one wild-type and 16 single-base mismatch mutants. These guides were incubated with the corresponding molecular beacons in the presence of *Pf*Ago at 95°C for 30 min, and the intensity of the fluorescence signal of each sample was measured.

Analysis of the effect of gn sequence on the sensitivity of *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection

Nine groups of guides (Table S6) were designed to target the HPV16L1 gene. The dsDNA target was amplified using primer pair F (HPV16)/R (HPV16) (Table S10), followed by splitting into nine portions and incubation at 95°C with nine groups of guides, *Pf*Ago and MB-HPV16-1, or MB-HPV16-2 (Table S6) for 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, or 22 min. The intensity of the fluorescence signal of each sample was detected using the CFX96 Touch Real-Time PCR Detection System.

Detecting circulating tumor DNA with *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection

Mock-tumor mutant *KRAS* G12D and *EGFR* T790M were synthesized, and mixed in ratios of 0:100, 1:999, 1:99, or 5:95 with wild-type DNA fragments. The fluorescence intensity of mutants was detected using PAND. Sequences of the DNA guides and molecular beacons are provided in Table S7.

Multiple channel detection with *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection

For five-channel multiplexed detection, five types of genomic fragments from five human papillomavirus sub-types were synthesized and used as targets. We carried out PCR as described above

with five pairs of primers (10 in total) added into the same reaction mixture. Then, five groups of guide DNAs (15 in total) and five molecular beacons labeled with different fluorophores were mixed and reacted with the PCR products in the presence of *Pf*Ago in one tube. Target detection was carried out as described above. Sequences of the guides and molecular beacons are listed in Table S8.

Clinical sample detection with *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection

To test the detection of *BRCA*1 SNPs, serum samples from patients with breast cancer were amplified with the Blood Directed PCR Kit V2 using primer pairs F (rs12516)/R (rs12516) and F (rs16941)/R (rs16941) (Table S10) to obtain DNA fragments bearing the *BRCA*1 rs12516 (T) or rs12516 (C) alleles, and SNP rs16941 (A) or SNP rs16941 (G) alleles, respectively. The SNP rs12516 was detected when three gDNAs; gf (rs12516), gr (rs12516), and gt (rs12516); and two molecular beacons, MB-rs12516T and MB-rs12516C, were used (Table S4). The SNP rs16941 was detected when three gDNAs; gf (rs16941), gr (rs16941), and gt (rs16941); and two molecular beacons, MB-rs16941G and MB-rs16941A, were used (Table S9).

To detect HPV sub-types, genomic DNA was extracted from serum samples of patients infected with multiple HPV types with Takara MiniBEST Whole Blood Genomic DNA Extraction Kit (Takara Biomedical Technology). The procedure was the same as described in multiple channel detection assay. The gDNAs and molecular beacons used for this assay were the same as five-channel multiplexed detection.

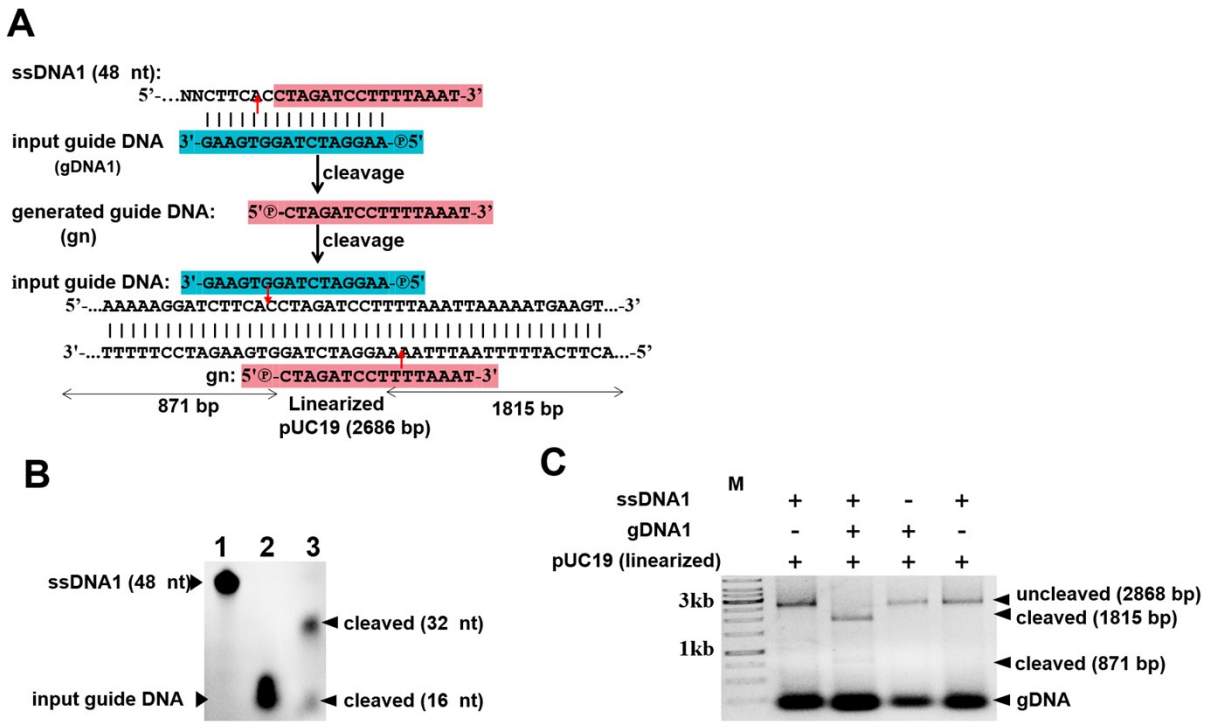
Analysis of fluorescence data from *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection

To calculate background-subtracted fluorescence data, the initial fluorescence of samples was subtracted. This enabled comparison between different conditions. Background fluorescence (in conditions of either no targets or no guide DNA) was subtracted from sample data to produce background-subtracted fluorescence data (BKgd-subtracted fluorescence).

Statistical analysis

Statistical analyses were carried out using Microsoft Excel 2016. The two-tailed Student's t-test was used to compare differences between two groups, with a p-value < 0.05 taken as the threshold for significance. All data are shown as mean ± standard deviation from three technical replicates.

Supplemental Figures



Figures S1. Identification of the cleavage-guidance function of single-stranded DNA produced from *Pyrococcus furiosus* Argonaute-mediated cleavage of long single-stranded DNA. (A) Schematic of the generation of short 5′-phosphorylated single-stranded DNA (gn) by *Pyrococcus furiosus* Argonaute (*Pf*Ago) cleavage of the long ssDNA target (ssDNA1), which meditates subsequent PfAgo cleavage of double-stranded DNA (linearized pUC19). (B) Generation of gn from ssDNA1 from guide DNA. Lane 1: ssDNA target; Lane 2: input guide DNA; Lane 3: the two short ssDNA fragments that resulted from cleavage. (C) Detection of gn-mediated cleavage of linearized pUC19 by agarose gel electrophoresis. The 5′-phosphorylated ssDNA generated by *Pf*Ago-cleavage of ssDNA mediated a second round of *Pf*Ago cleavage. Key: M, 1 kb molecular weight standard.

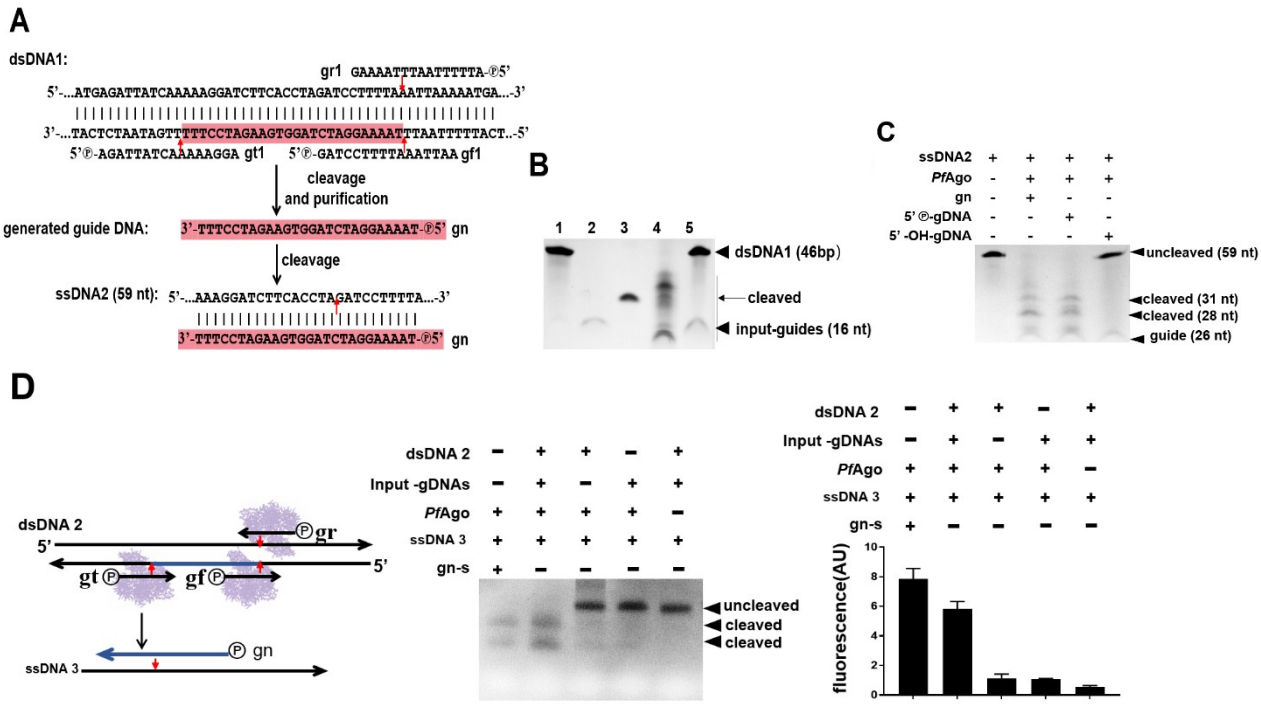


Figure S2. Identification of the cleavage-guidance function of single-stranded DNA produced from *Pyrococcus furiosus* Argonaute cleavage of double-stranded DNA. (A) Schematic of the generation of 5′-phosphorylated single-stranded DNA (gn) by *Pyrococcus furiosus* Argonaute (*Pf*Ago) cleavage of the double- stranded DNA target (dsDNA1) which induced a second round of *Pyrococcus furiosus* Argonaute (*Pf*Ago) cleavage. (B) Analysis of *Pf*Ago-cleavage of double-stranded DNA by 16% urea polyacrylamide gel electrophoresis. Lane 1: dsDNA1; Lane 2: input guides (gt1, gf1, gr1); Lane 3: synthesized 26-nucleotide ssDNA (control); Lane 4: *Pf*Ago-cleaved dsDNA; Lane 5: un-cleaved dsDNA and input guide DNA. (C) Analysis of *Pf*Ago cleavage of the downstream target under guidance of the newly generated 5′-phosphorylated ssDNA by 16% urea polyacrylamide gel electrophoresis. The gn was generated by *Pf*Ago-mediated cleavage of dsDNA, and initiated the second round of *Pf*Ago cleavage. The 5′-phosphorylated and 5′-hydroxyl guide DNA were used positive and negative controls, respectively. (D) We carried out *Pf*Ago-mediated nucleic acid detection in a single tube. Left: Integrated generation of new guide DNA and the second round of cleavage of downstream targets were combined into one reaction. Middle: 16% urea polyacrylamide gel electrophoresis analysis of the cleavage product. Right: fluorometric analysis of the detection result.

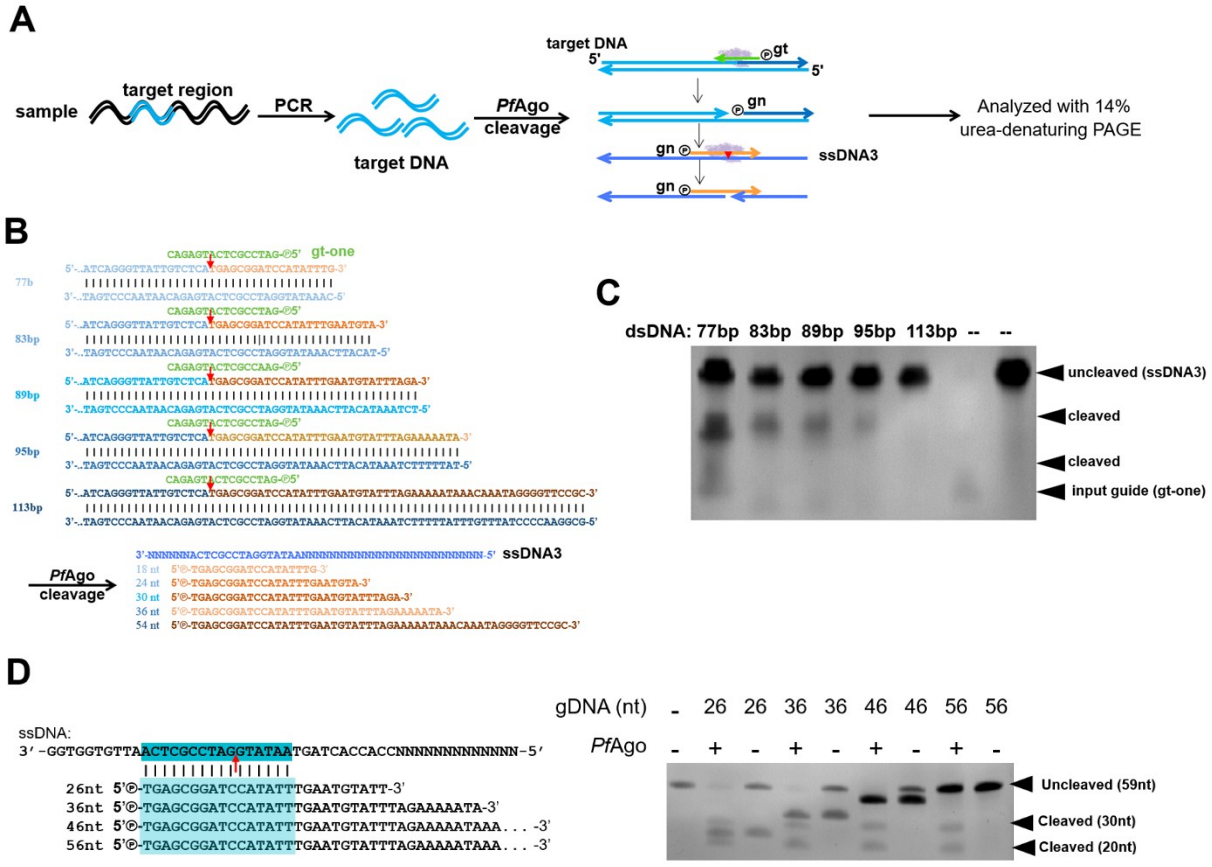


Figure S3. Cleaving target double-stranded DNA with a single guide DNA to generate gn. (A) Schematic illustrating the procedure of generation of the 16-nt single-stranded DNA fragment (gn) via cleavage of double-stranded (ds)DNA by the *Pyrococcus furiosus* Argonaute (*Pf*Ago)/guide DNA (gt) complex, and initiation of the second round of cleavage (of ssDNA3) by the newly assembled *Pf*Ago/gn complex. (B) Sequences of gt, different length dsDNA targets (77, 83, 89, 95, and 113 bp), newly generated gn, and ssDNA3. Cleavage sites on the dsDNA are indicated with red arrows. The gn lengths were extended from 18 to 55 nucleotides with increasing dsDNA length from 77 to 113 base pairs, respectively. (C) Evaluation of the efficiency of *Pf*Ago-mediated cleavage of ssDNA3 under the guidance of gn produced from dsDNA of different lengths. The cleavage products of ssDNA3 were analyzed with 14% urea polyacrylamide gel electrophoresis. The efficiency of ssDNA3 cleavage decreased with increasing length of the dsDNA. (D) Investigation of different length guide DNA to mediate enzymatic digestion of ssDNA by pfAgo; each guide DNA forms 16 base pairs with the ssDNA target.

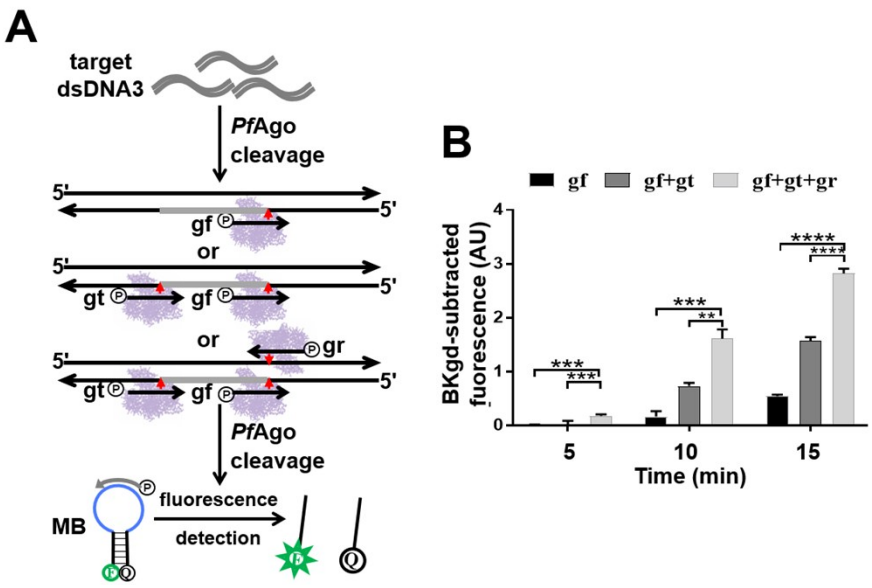


Figure S4. Determining the optimal number of guide DNAs for *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection. (A) Schematic illustrating the procedure of nucleic acid detection with one (gf), two (gt and gf), or three guides (gt, gf, and gr). (B) Analysis of fluorescence intensity generated by cleavage of the molecular beacon. The use of three guide DNAs resulted in more intense signals than two or one guide DNA $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ from the two-tailed Student's t-test. Data are presented as mean ± standard deviation from three technical replicates.

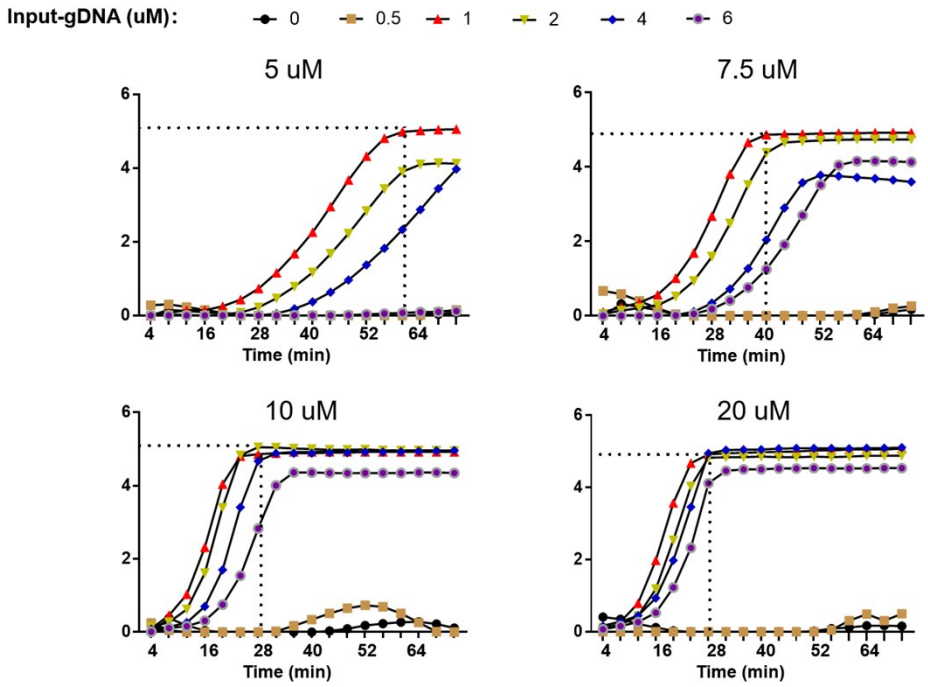


Figure S5. Determining the concetration of *Pyrococcus furiosus* Argonaute and input guide DNA for *Pyrococcus furiosus* Argonaute-mediated nucleic acid detection. We incubated 5, 7.5, 10 and 20 μM *Pyrococcus furiosus* Argonaute with increasing concentrations of input guide DNA from 0 to 6 μM, respectively. The dotted line indicates the time (min) and fluorescence value (AU) when the signal was stable.

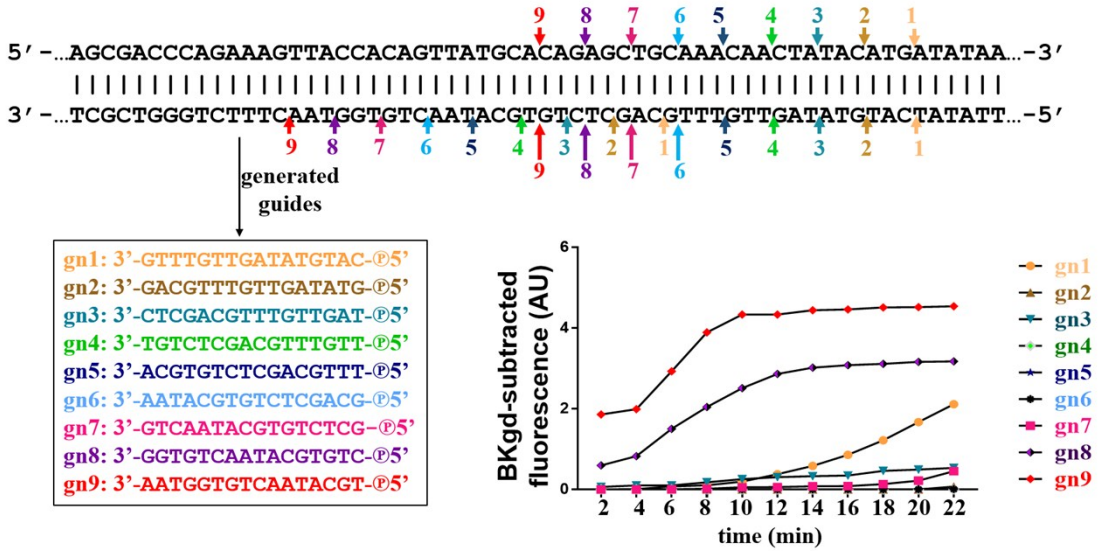


Figure S6. The effect of gn sequence on the sensitivity of *Pyrococcus furiosus* Argonaute-mediated nucleic-acid detection. (A) Schematic illustrating the cleavage sites of nine input guide DNA groups and the sequences of the nine newly generated 16-nucleotide single-stranded DNA fragments (gn). Different color arrows indicate the cleavage sites for each guide group, and numbers 1−9 relate to the nine groups of guide DNA that were used to generate gns. (B) Analysis of the fluorescence intensity generated by cleavage of the molecular beacon. Among the nine guide DNA groups.

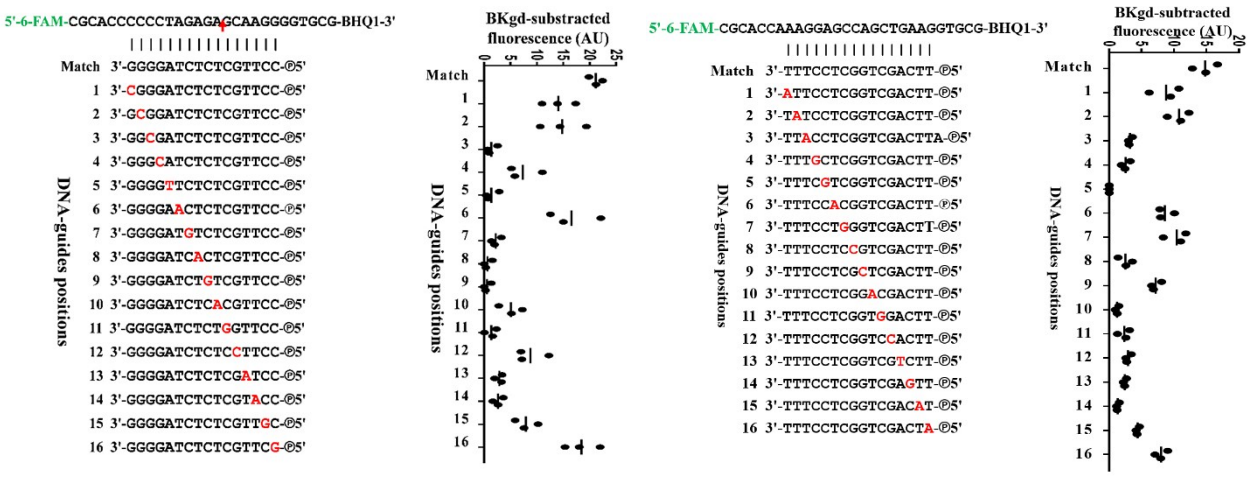


Figure S7. Single-mismatch assay analysis of the single-base specificity of *Pyrococcus furiosus* Argonaute. The sequences of three pairs of guide (bottom) and target DNA (top) are indicated in black, single-base mismatches are shown in red. Red arrowheads indicate cleavage sites. Black bars represent the average cleavage by *Pyrococcus furiosus* Argonaute from three independent replicates, black dots represent individual replicates.

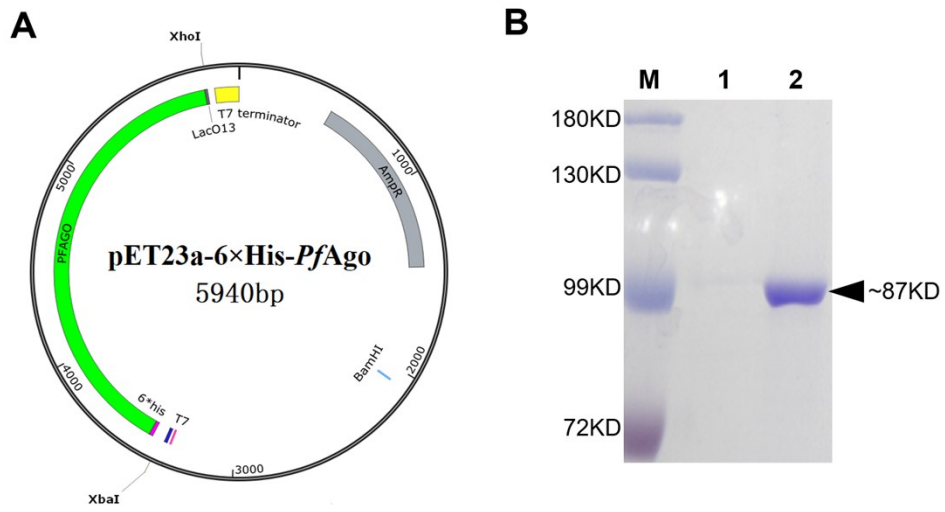


Figure S8. Expressing *Pf*Ago with *E. coli* as a host. (A) Plasmid map of pET23a-6×His-*Pf*Ago. (B) Analyzing *Pf*Ago with SDS-PAGE after purification. M: pre-stained protein marker; Lane1: flow-through; Lane2: purified *Pf*Ago. The *Pf*Ago protein (~87kD) band was indicated with a black triangle

Sequence data

Table S1. Sequences of 6×His-*Pf*Ago in plasmid pET23a-6×His-*Pf*Ago.

> 6*His-*Pf*Ago

Labels: 6×His-PfAgo; 6xHis-Tag; T7 promoter; T7 terminator

TAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGCACCATCATCA
TCATCATAGCATGAAAGCCATTGTGGTGATTAACCTGGTGAAAATCAACAAAAAAATCATCCCGGATAAAATCTACGTGTACCGCCTGTTTAA
TGATCCGGAAGAAGAACTGCAGAAAGAAGGCTATAGCATTTATCGTCTGGCCTATGAAAATGTGGGCATTGTTATCGATCCGGAAAATCTGA
TTATTGCCACCACCAAAGAACTGGAATATGAAGGTGAATTTATTCCGGAAGGCGAAATCAGCTTTAGCGAACTGCGTAATGATTATCAGAGC
AAACTGGTTCTGCGTCTGCTGAAAGAAAATGGTATTGGTGAATATGAACTGTCCAAACTGCTGCGCAAATTTCGTAAACCGAAAACCTTTGGC
GATTACAAAGTTATTCCGAGCGTTGAAATGAGCGTGATCAAACACGATGAAGATTTCTATCTGGTGATCCATATCATCCATCAGATCCAGAGC
ATGAAAACCCTGTGGGAACTGGTTAATAAAGATCCGAAAGAGCTGGAAGAATTTCTGATGACCCATAAAGAAAACCTGATGCTGAAAGATAT
TGCCAGTCCGCTGAAAACCGTGTATAAACCGTGTTTTGAAGAATATACCAAAAAACCGAAACTGGACCACAACCAAGAGATCGTGAAATATT
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GCAATTCTGGCAAAATTTGCAAGCAAAATTAAAAAAAATAAAAACTACAAAATCTATCTGCTGCCGCAGCTGGTTGTTCCGACCTATAATGCA
GAACAGCTGGAAAGTGATGTGGCCAAAGAAATTCTGGAATATACAAAACTGATGCCTGAGGAACGTAAAGAGCTGCTGGAAAATATTCTGG
CAGAAGTGGATAGCGATATCATCGATAAAAGCCTGAGCGAAATCGAGGTTGAAAAAATTGCACAAGAACTGGAAAACAAAATCCGCGTGCG
TGATGATAAAGGTAATAGCGTTCCGATTAGCCAGCTGAATGTTCAGAAAAGCCAGCTGCTGCTGTGGACCAATTATTCACGTAAATATCCGGT
TATCCTGCCGTATGAAGTGCCGGAAAAATTTCGCAAAATTCGTGAAATCCCGATGTTCATTATTCTGGATAGCGGTCTGCTGGCAGATATTCA
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AGAAATCAAACGCCGTCTGTTTAACCTGAATGTTATTAGCCAGGTGGTGAATGAAGATACCCTGAAAAACAAACGCGATAAATATGATCGTA
ATCGCCTGGACCTGTTTGTTCGTCATAATCTGCTGTTCCAGGTTCTGAGTAAACTGGGTGTTAAATACTATGTGCTGGACTATCGCTTCAACTA
CGATTATATCATTGGCATTGATGTGGCACCGATGAAACGTAGCGAAGGTTATATTGGTGGTAGCGCAGTTATGTTTGATAGCCAGGGTTATAT
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AGTTCAACATCAAACTGGATAACAAAAAAATCCTGCTGCTGCGTGATGGTCGCATTACCAATAATGAAGAAGAAGGCCTGAAATATATCAGC
GAGATGTTCGATATTGAAGTGGTTACCATGGATGTGATCAAAAACCATCCGGTTCGTGCATTTGCAAACATGAAAATGTATTTTAACCTGGGT
GGTGCCATTTATCTGATTCCGCATAAACTGAAACAGGCAAAAGGCACCCCGATTCCGATTAAACTGGCGAAAAAACGCATTATCAAAAACGG
CAAAGTGGAAAAACAGAGCATTACCCGTCAGGATGTTCTGGATATCTTTATTCTGACCCGTCTGAATTATGGTAGCATTAGCGCAGATATGCG
TCTGCCTGCACCGGTTCATTATGCACATAAATTTGCCAATGCCATTCGCAACGAGTGGAAAATCAAAGAAGAATTCCTGGCCGAAGGCTTTCT
GTATTTTGTTTAAAGCGCTCACAATTCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGG
CTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCG
GAT

Table S2. Sequence information for dsDNA targets used in this study

Name	*Sequence (5’-3’)
dsDNA1	TATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATA
dsDNA 2	CCACCAGGAAGGAAGCTGTTGCTTTCTTTGAGGTGATTTTTTTCCTTTGCTCCCTGTTGCTGAAACCATACAGCTTC ATAAATAATTTTGCTTGCTGAAGGAAGAAAAAGTGTTTTTCATAAACCCATTATCCAGGACTGTTTATAGCTGTTG GAAGGACTAGGTCTTCCCTAGCCCCCCCAGTGTGCAAGGGCAGTGAAGACTTGATTGTACAAAATACGTTTTGTA AATGTTGTGCTGTTAACACTGCAAATAAACTTGGTAGCAAACACTTCCACCATGAATGACTGTTCTTGAGACTTAG GCCAGCCGACTTTCTC

dsDNA (77bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG
dsDNA (83bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTA
dsDNA (89bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTATTTAGA
dsDNA (95bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTATTTAGAAAAATA
dsDNA (113bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTATTTAGAAAAATAAACAAATAGGGGTTCCGC
rs12516(T)	CCACCAGGAAGGAAGCTGTTGCTTTCTTTGAGGTGATTTTTTTCCTTTGCTCCCTGTTGCTGAAACCATACAGCTTC ATAAATAATTTTGCTTGCTGAAGGAAGAAAAAGTGTTTTTCATAAACCCATTATCCAGGACTGTTTATAGCTGTTG GAAGGACTAGGTCTTCCCTAGCCCCCCTAGTGTGCAAGGGCAGTGAAGACTTGATTGTACAAAATACGTTTTGTA AATGTTGTGCTGTTAACACTGCAAATAAACTTGGTAGCAAACACTTCCACCATGAATGACTGTTCTTGAGACTTAG GCCAGCCGACTTTCTC
rs12516(C)	CCACCAGGAAGGAAGCTGTTGCTTTCTTTGAGGTGATTTTTTTCCTTTGCTCCCTGTTGCTGAAACCATACAGCTTC ATAAATAATTTTGCTTGCTGAAGGAAGAAAAAGTGTTTTTCATAAACCCATTATCCAGGACTGTTTATAGCTGTTG GAAGGACTAGGTCTTCCCTAGCCCCCCCAGTGTGCAAGGGCAGTGAAGACTTGATTGTACAAAATACGTTTTGTA AATGTTGTGCTGTTAACACTGCAAATAAACTTGGTAGCAAACACTTCCACCATGAATGACTGTTCTTGAGACTTAG GCCAGCCGACTTTCTC
rs16941(A)	GAGGAACATTCAATGTCACCTGAAAGAGAAATGGGAAATGAGAACATTCCAAGTACAGTGAGCACAATTAGCCG TAATAACATTAGAGAAAATGTTTTTAAAGAAGCCAGCTCAAGCAATATTAATGAAGTAGGTTCCAGTACTAATGA AGTGGGCTCCAGTATTAATGAAATAGGTTCCAGTGATGAAA
rs16941(G)	GAGGAACATTCAATGTCACCTGAAAGAGAAATGGGAAATGAGAACATTCCAAGTACAGTGAGCACAATTAGCCG TAATAACATTAGAGAAAATGTTTTTAAAGGAGCCAGCTCAAGCAATATTAATGAAGTAGGTTCCAGTACTAATGA AGTGGGCTCCAGTATTAATGAAATAGGTTCCAGTGATGAAA
KRAS G12D wild-type	TACTGGTGGAGTATTTGATAGTGTATTAACCTTATGTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATT ATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGA CGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCAACAATAGAGG
KRAS G12D mutant	TACTGGTGGAGTATTTGATAGTGTATTAACCTTATGTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATT ATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGATGGCGTAGGCAAGAGTGCCTTGA CGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCAACAATAGAGG
EGFR T790M wild-type	AACCCCCACGTGTGCCGCCTGCTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCATGCAGCTCATGCCCTTCG GCTGCCTCCTGGACTATGTCCGGGAACACAAAGACAATATTG
EGFR T790M mutant	AACCCCCACGTGTGCCGCCTGCTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCACGCAGCTCATGCCCTTCG GCTGCCTCCTGGACTATGTCCGGGAACACAAAGACAATATTG
HPV11E6	ATTTGCGAAAGGAACAAATGTTTGCTAGACACTTTTTTAGTAGGGCCGGTACTGTGGGGGAACCTGTGCCTGATG ACCTGTTGGTAAAAGGGGGTAATAATAGATCATCTGTAGCTAGTAGTATTTATGTACATACACCTAGTGGCTCATT GGTGTCTTCCGAGG
HPV16E6	ACTGCAATGTTTCAGGACCCACAGGAGCGACCCAGAAAGTTACCACAGTTATGCACAGAGCTGCAAACAACTATA CATGATATAATATTAGAATGTGTGTACTGCAAGCAACAGTTACTGCGACGTGAGGTATATGACTTTGCTTTTCGG
HPV18L1	ATCCCCTTGGACGTAAATTTTTGGTTCAGGCTGGATTGCGTCGCAAGCCCACCATAGGCCCTCGCAAACGTTCTGC

	TCCATCTGCCACTACGTCTTCTAAACCTGCCAAGCGTGTGCGTGTACGTGCCAGGAAGA
HPV33E6	ATATTTCGGGTCGTTGGGCAGGGCGCTGTGCGGTGTGTTGGAGGTCTCGACGTAGAGAAACTGCACTGTGACGT
HPV45E6	GGACAGTACCGAGGGCAGTGTAATACATGTTGTGACCAGGCACGGCAAGAAAGACTTCGCAGACGTAGGGA
dsDNA1	TATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATA
dsDNA 2	CCACCAGGAAGGAAGCTGTTGCTTTCTTTGAGGTGATTTTTTTCCTTTGCTCCCTGTTGCTGAAACCATACAGCTTC ATAAATAATTTTGCTTGCTGAAGGAAGAAAAAGTGTTTTTCATAAACCCATTATCCAGGACTGTTTATAGCTGTTG GAAGGACTAGGTCTTCCCTAGCCCCCCCAGTGTGCAAGGGCAGTGAAGACTTGATTGTACAAAATACGTTTTGTA AATGTTGTGCTGTTAACACTGCAAATAAACTTGGTAGCAAACACTTCCACCATGAATGACTGTTCTTGAGACTTAG GCCAGCCGACTTTCTC
dsDNA (77bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG
dsDNA (83bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTA
dsDNA (89bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTATTTAGA
dsDNA (95bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTATTTAGAAAAATA
dsDNA (113bp)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATCCATATTTG AATGTATTTAGAAAAATAAACAAATAGGGGTTCCGC

*The targeted sequences are in blue. The mutation sites are in red

Table S3. Sequence information for determing the feasibility of the PAND.

Name	**Sequence (5'-3')**
g1	CCAGCTCCAACTACCA
ssDNA1	ATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTATG
g2	AAGGATCTAGGTGAAG
gf1	GATCCTTTTAAATTAA
gr1	ATTTTTAATTTAAAAG
gt1	AGATTATCAAAAAGGA
ssDNA2	NNNNNNNNNNNNNNNNNNNNNNNNNNCTTCACCTAGATCCTTTTAAAT
gf2	AAGGGCAGTGAAGACT
gr2	ATCAAGTCTTCACTGC
gt2	CTTGCACACTGGGGGG
ssDNA3	NNNNNNNNNNNNNNCGCACCCACTGCCCTTGCACACGGTGCGNNNN
gt-one	GATCCGCTCATGAGAC
g-26	TGAGCGGATCCATATTTGAATGTATT
g-36	TGAGCGGATCCATATTTGAATGTATTTAGAAAAATA
g-46	TGAGCGGATCCATATTTGAATGTATTTAGAAAAATAAACAAATAGG
g-56	TGAGCGGATCCATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGC

Table S4. Sequence information of gDNAs and MBs for detecting *BRAC*1 rs12516

Name	**Sequence (5'-3')**

gf (rs12516)	TGTGCAAGGGCAGTGA
gr (rs12516)	GTCTTCACTGCCCTTG
gt (rs12516)	TCCCTAGCCCCCCTAG
MB-rs12516T	5’-6-FAM-CGCACCcccccctagtgtgcaagggcGGTGCG-BHQ1-3’
MB-rs12516C	5’-6-FAM-CGCACCcccccccagtgtgcaagggcGGTGCG-BHQ1-3’

Table S5. Sequence information for determing the and specificity of PAND.

Name	Sequence (5’-3’)
S1-gw	AGCGGATCCATATTTG
S1-g1	TGCGGATCCATATTTG
S1-g2	ACCGGATCCATATTTG
S1-g3	AGGGGATCCATATTTG
S1-g4	AGCCGATCCATATTTG
S1-g5	AGCGCATCCATATTTG
S1-g6	AGCGGTTCCATATTTG
S1-g7	AGCGGAACCATATTTG
S1-g8	AGCGGATGCATATTTG
S1-g9	AGCGGATCGATATTTG
S1-g10	AGCGGATCCTTATTTG
S1-g11	AGCGGATCCAAATTTG
S1-g12	AGCGGATCCATTTTTG
S1-g13	AGCGGATCCATAATTG
S1-g14	AGCGGATCCATATATG
S1-g15	AGCGGATCCATATTAG
S1-g16	AGCGGATCCATATTTC
MB-S1	5’-6-FAM-CGCACCaaatatggatccgctGGTGCG-BHQ1-3’

* The point mismatch bases in gDNA sequences compared to S1-gw are in red

Table S6. Sequence information of gDNAs and MBs for determing the effect of gn sequence to the sensitivity of PAND.

Name	Sequence (5’-3’)
g-1f	ACTATACATGATATAA
g-1r	TACATGATATAATATT
g-1t	CACAGAGCTGCAAACA
g-2f	TTATGCACAGAGCTGC
g-2r	GTTTGCAGCTCTGTGC
g-2t	CAGAAAGTTACCACAG
g-3f	TGCACAGAGCTGCAAA
g-3r	GTTGTTTGCAGCTCTG
g-3t	AAAGTTACCACAGTTA
g-4f	ACAGAGCTGCAAACAA
g-4r	GTTACCACAGTTATGC
g-4t	ATAGTTGTTTGCAGCT

g-5f	GAGCTGCAAACAACTA
g-5r	TGTATAGTTGTTTGCA
g-5t	ACCACAGTTATGCACA
g-6f	CTGCAAACAACTATAC
g-6r	AAACAACTATACATGA
g-6t	ACAGTTATGCACAGAG
g-7f	CAAACAACTATACATG
g-7r	CAACTATACATGATAT
g-7t	GTTATGCACAGAGCTG
g-8f	ACAACTATACATGATA
g-8r	CTATACATGATATAAT
g-8t	ATGCACAGAGCTGCAA
g-9f	ACTATACATGATATAA
g-9r	TACATGATATAATATT
g-9t	CACAGAGCTGCAAACA
MB-HPV16-1	5’-6-FAM-CGCACCtgcacagagctgcaaacaactatacatgGGTGCG-BHQ1-3’
MB-HPV16-2	5’-6-FAM-CGCACCttaccacagttatgcacagagctgcaaa GGTGCG-BHQ1-3’

Table S7. Sequence information of gDNAs and MBs for detecting circulating tumor DNA (ctDNA)

Name	**Sequence (5’-3’)**
gt (*EGFR*)	CGTGCAGCTCATCATG
gf (*EGFR*)	CAGCTCATGCCCTTCG
gr (*EGFR*)	CAGCCGAAGGGCATGA
MB-*EGFR* T790M (Wild-type)	5’-6-FAM-CGCACCtcatcacgcagctcatgccGGTGCG-BHQ1-3’
MB-*EGFR* T790M (mutant)	5’-6-FAM-CGCACCtcatcatgcagctcatgccGGTGCG-BHQ1-3’
gt (*KRAS*)	GGTAGTTGGAGCTGAT
gt (*KRAS*)	GGTAGTTGGAGCTGAT
gf (*KRAS*)	GGCGTAGGCAAGAGTG
gr (*KRAS*)	AAGGCACTCTTGCCTA
MB-*KRAS* G12D (Wild-Type)	5’-6-FAM-CGCACCagctggtggcgtaggcaaGGTGCG-BHQ1-3’
MB-*KRAS* G12D (mutant)	5’-6-FAM-CGCACCagctgatggcgtaggcaaGGTGCG-BHQ1-3’

Table S8. Sequence information of gDNAs and MBs for detecting human papillomavirus (HPV)

Name	**Sequence (5’-3’)**
gf-HPV11	AACCTGTGCCTGATGA
gr-HPV11	CAGGTCATCAGGCACA
gt-HPV11	GCCGGTACTGTGGGGG
MB-HPV11	5’-ROX-CGCACCtgggggaacctgtgccGGTGCG-BHQ2-3’
gf-HPV16	GAGCTGCAAACAACTA
gr-HPV16	TGTATAGTTGTTTGCA

gt-HPV16	ACCACAGTTATGCACA
MB-HPV16	5′-CY5-CGCACCtgcacagagctgcaaaGGTGCG-BHQ1-3′
gf-HPV18	ACGTTCTGCTCCATCT
gr-HPV18	TGGCAGATGGAGCAGA
gt-HPV18	CCATAGGCCCTCGCAA
MB-HPV18	5′-6-FAM-CGCACCtcgcaaacgttctgctGGTGCG-BHQ1-3′
gf-HPV33	GTGTGTTGGAGGTCTC
gr-HPV33	CGTCGAGACCTCCAAC
gt-HPV33	GGCAGGGCGCTGTGCG
MB-HPV33	5′-CY3-CGCACCtgtgcggtgtgttggaGGTGCG-BHQ2-3′
gf-HPV45	GACCAGGCACGGCAAG
gr-HPV45	CTTTCTTGCCGTGCCT
gt-HPV45	GTGTAATACATGTTGT
MB-HPV45	5′-TET-CGCACCtgttgtgaccaggcacGGTGCG-BHQ1-3′

Table S9. Sequence information of gDNAs and MBs for detecting *BRAC*1 rs16941

Name	**Sequence (5′-3′)**
gf (rs16941)	GCCAGCTCAAGCAATA
gr (rs16941)	TTAATATTGCTTGAGC
gt (rs16941)	AAATGTTTTTAAAGAA
MB-rs16941G	5′-6-FAM-CGCACCtaaaggagccagctcaagcGGTGCG-BHQ1-3′
MB-rs16941A	5′-6-FAM-CGCACCtaaagaagccagctcaagcGGTGCG-BHQ1-3′

Table S10. Sequence information of primers used in this study

Name	**Sequence（5′-3′）**
F (rs12516)-PCR	CCACCAGGAAGGAAGCTGTTG
R (rs12516)-PCR	AGAAAGTCGGCTGGCCTAAG
F (rs12516)-tHDA	ACCCATTATCCAGGACTGTTTATAGCTG
R (rs12516)-tHDA	CAAAACGTATTTTGTACAATCAAGTCTTCACTGC
F (rs16941)	GAGGAACATTCAATGTCACCTGAAAGAGAAATG
R (rs16941)	TTTCATCACTGGAACCTATTTCATTAATACTGGAGCC
F (one)	GATACTCATACTCTTCCTTTTTCAATATTATTGAAGC
R (77)	CAAATATGGATCCGCTCATGAGACAATAAC
R (83)	TACATTCAAATATGGATCCGCTCATGAG
R (89)	TCTAAATACATTCAAATATGGATCCGCTCATG
R (95)	TATTTTTCTAAATACATTCAAATATGGATCCGCTC
R (113)	GCGGAACCCCTATTTGTTTATTTTTC
F (*KRAS*)	TACTGGTGGAGTATTTGATAGTGTATTAACC
R (*KRAS*)	CCTCTATTGTTGGATCATATTCGTCC
F (*EGFR*)	AACCCCCACGTGTGCCG
R (*EGFR*)	CAATATTGTCTTTGTGTTCCCGGACATAG
F (HPV11)	TTGCGAAAGGAACAAATGTTT
R (HPV11)	GGAAGACACCAATGAGCCACT

F (HPV16)	AGGACCCACAGGAGCGAC
R (HPV16)	AGTCATATACCTCACGTCGCAGT
F (HPV18)	GGTTCAGGCTGGATTGCG
R (HPV18)	TACACGCACACGCTTGGC
F (HPV33)	ATATTTCGGGTCGTTGGGCA
R (HPV33)	ACGTCACAGTGCAGTTTCTCTACGT
F (HPV45)	GGACAGTACCGAGGGCAGTGTAA
R (HPV45)	TCCCTACGTCTGCGAAGTCTTTC