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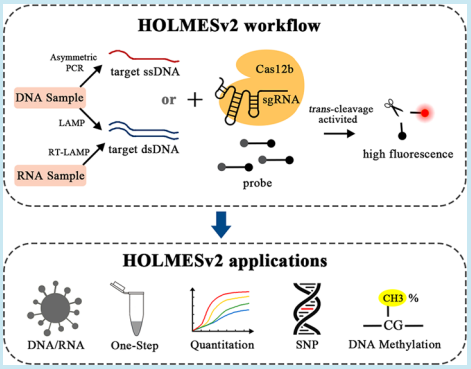
HOLMESv2: A CRISPR-Cas12b-Assisted Platform for Nucleic Acid Detection and DNA Methylation Quantitation

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Supporting Information

ABSTRACT: The next-generation CRISPR-based molecular diagnostics has the merits of rapidness, accuracy, and portability. We discovered the Cas12a *trans*-cleavage activity against collateral single-stranded DNA (ssDNA) and employed the activity to develop a rapid nucleic acid detection system, namely HOLMES (one-hour low-cost multipurpose highly efficient system). Here, with the employment of thermophilic CRISPR-Cas12b, we create HOLMESv2 for four different applications: (1) specifically discriminating single nucleotide polymorphism (SNP); (2) simply detecting virus RNA, human cell mRNA and circular RNA; (3) conveniently quantitating target nucleic acids with a one-step system combined with LAMP amplification in a constant temperature, thus avoiding cross-contamination; (4) accurately quantitating target DNA methylation degree with the combination of Cas12b detection and bisulfite treatment. These results highlight the potential of HOLMESv2 as a promising platform for both molecular diagnostics and epigenetics applications.



KEYWORDS: *CRISPR, Cas12b, nucleic acid detection, DNA methylation quantitation, HOLMES*

Because of high sensitivity, specificity, and simplicity, the recently emerged CRISPR-based nucleic acid detection methods have shown great promise in clinical diagnostics and drawn much attention.[1,2] The principle of these methods is based on the *trans*-cleavage activities of Cas proteins, including the RNA-targeting RNA-*trans*-cleaving Cas13 and DNA-targeting single-stranded DNA (ssDNA)-*trans*-cleaving Cas12a.[3−9] Combined with nucleic acid amplification procedures, Cas13 and Cas12a have been employed to develop rapid target nucleic acid detection systems, namely SHERLOCK (specific high sensitivity enzymatic reporter unlocking), HOLMES (one-hour low-cost multipurpose highly efficient system), or DETECTR (DNA endonuclease targeted CRISPR *trans*-reporter).[3−7] These CRISPR diagnostic methods rapidly detected DNA and RNA viruses, discriminated single nucleotide polymorphisms (SNP) and genotyped different strains of viruses.[3−7] However, in present systems, nucleic acid amplification and CRISPR-Cas based target detection are two separate steps, and one may need to mix the amplification product with the Cas detection system for target detection, which may cause both practical inconvenience and potential cross-contamination among samples.

Here, we employ Cas12b (previously known as C2c1),[10,11] a thermophilic RNA-guided endonuclease from type V-B CRISPR-Cas system, to create HOLMESv2 for discriminating SNP, detecting a RNA virus, human cell mRNA and circular RNA, and quantitating target DNA as well as its methylation degree in different human cell lines. Also, we integrate the nucleic acid amplification and target detection steps into one system in order to simplify operational procedures and avoid cross-contamination.

RESULTS AND DISCUSSION

Characterization of the *trans*-Cleavage Activity of Cas12b on Collateral ssDNA. We purified the *Alicycloba-*

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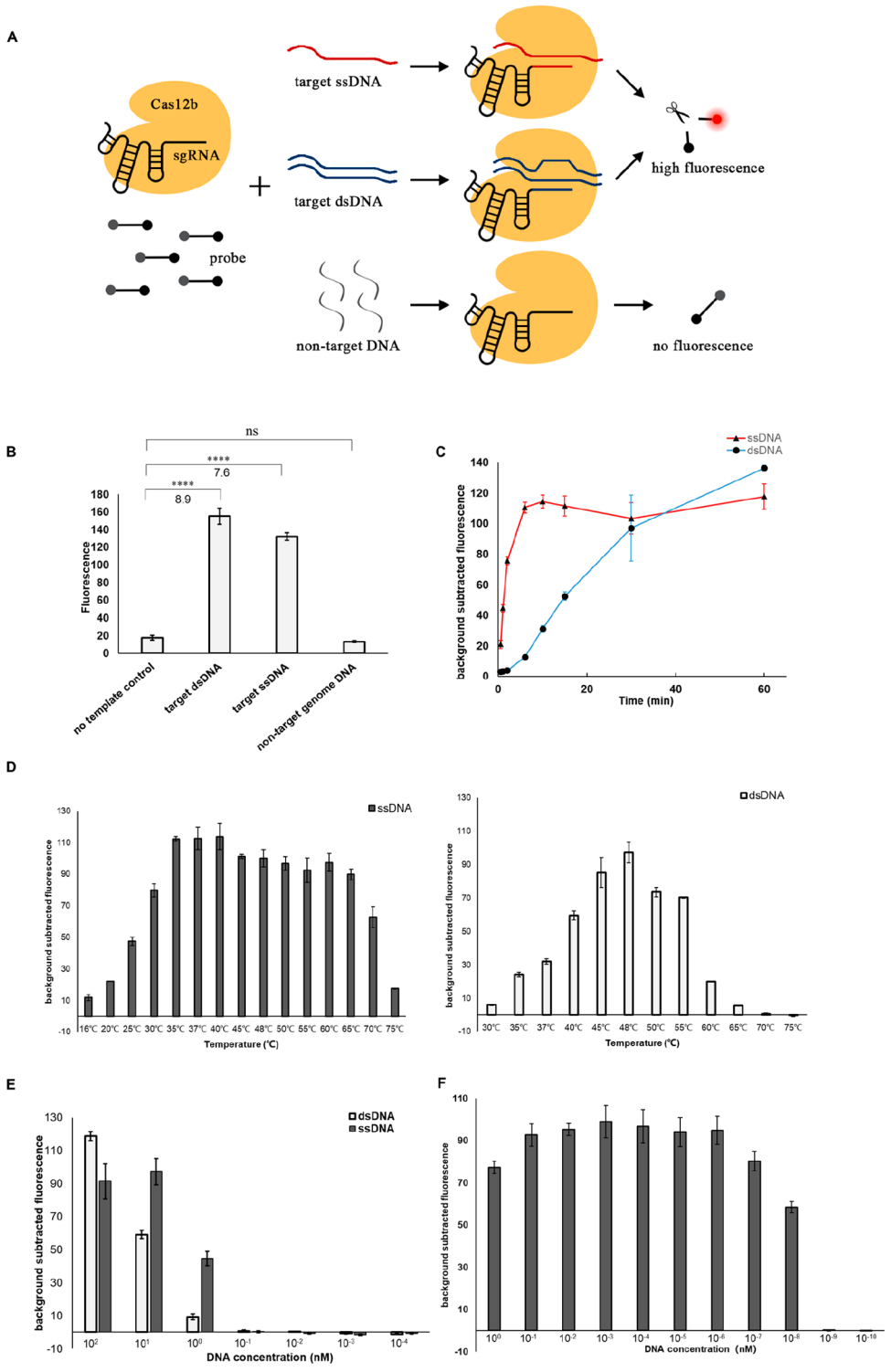


Figure 1. Characterization of the *trans*-cleavage activity on collateral ssDNA by Cas12b. (A) Schematic of Cas12b-mediated ssDNA *trans*-cleavage activity. (B) Detection of the fluorescence signal of Cas12b reaction systems. The systems contained Cas12b, sgRNA (sgRNA-T1), collateral ssDNA probe (HEX-N12-BHQ1), and different target DNA substrates, and were incubated at 48 °C for 30 min. (C) Time-course analysis of the Cas12b *trans*-cleavage activity with either target ssDNA or target dsDNA. The reaction systems were incubated at 48 °C. (D) Analysis of the optimal temperature range required for Cas12b *trans*-cleavage with either target ssDNA or target dsDNA. The reaction systems were incubated at the designated temperature for 30 min. (E) Determination of the target concentration required for triggering the Cas12b *trans*-cleavage activity. Target dsDNA (DNMT1−3) and target ssDNA (DNMT1−3) were serially diluted to concentrations ranging from 100 nM to 10^{-4} nM, which were then directly detected by Cas12b *trans*-cleavage without amplification. (F) Determination of HOLMESv2 sensitivity. Target DNA (pUC18-DNMT1−3) was serially diluted, amplified by LAMP, and then *trans*-cleaved by Cas12b, followed by fluorescence determination with a fluorescence reader. Error bars represented standard deviation (s.d.) from n = 3 replicates.

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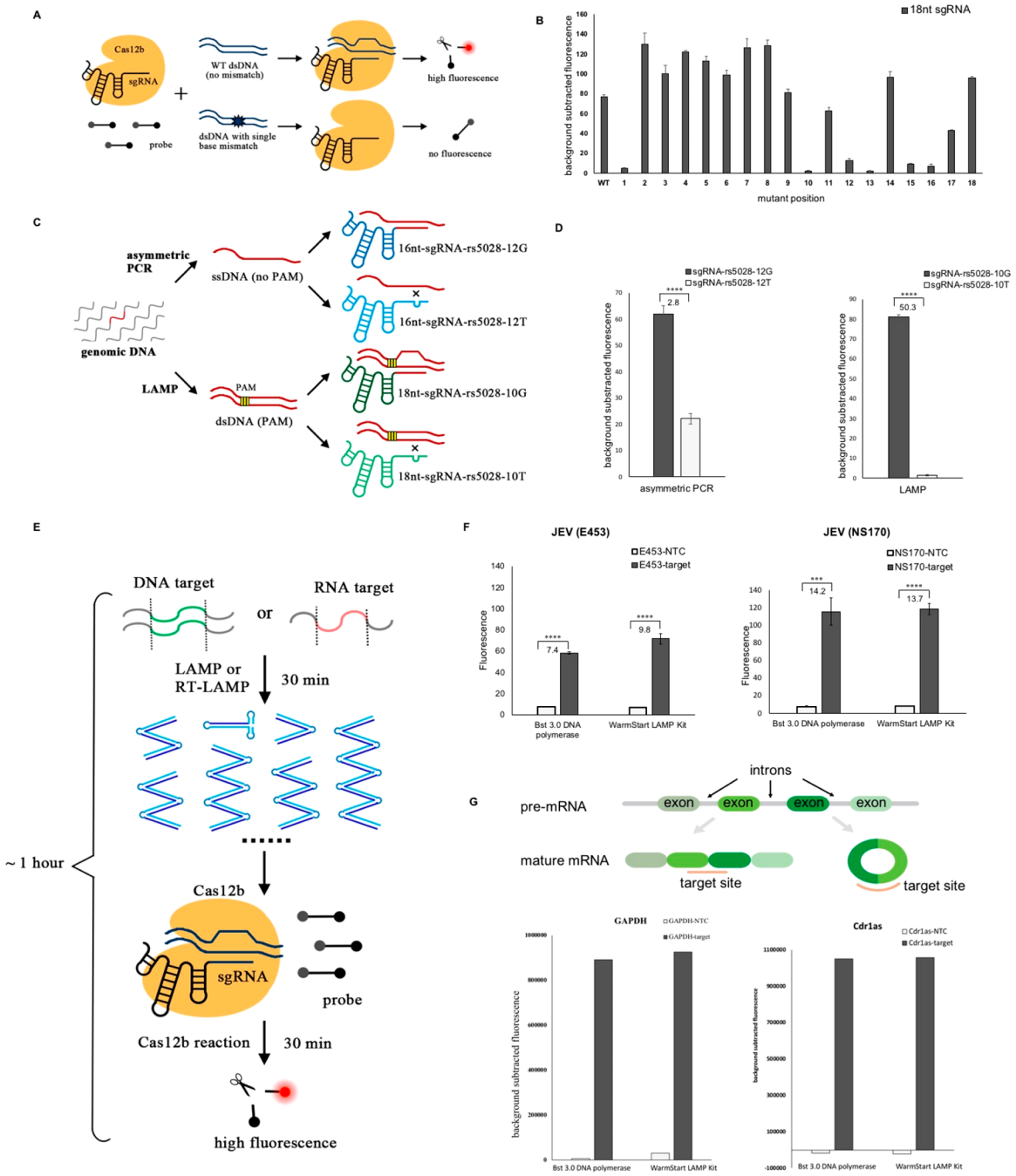


Figure 2. Distinguishing target DNA and target RNA by Cas12b-mediated ssDNA *trans*-cleavage activity. (A,B) Distinguishing target dsDNA with single-base mismatch. The schematic was illustrated (A), and results (B) were obtained with the use of an sgRNA with 18-nt wild-type guide sequence. Mutated target sequences were listed in part C, and mutated sequences of No. 1 to No. 18 were analyzed with the 18-nt sgRNA. (C,D) Genotyping the rs5028 locus in the human cell HEK293T by HOLMESv2. Target DNA containing the SNP locus (e.g., rs5028) was either amplified by asymmetric PCR or LAMP. For asymmetric PCR, the sgRNA was designed to align the mismatch at the 12th position of the guide sequence, counting from the PAM sequence. For LAMP amplification, a PAM sequence was introduced by the amplification primers, and the mismatch was aligned at the 10th position. (E) Schematic of nucleic acid detection by HOLMESv2 combining either LAMP or reverse transcription (RT)-LAMP. (F) Detection of RNA virus by HOLMESv2. Japanese encephalitis virus (JEV) RNA was amplified by either RT-LAMP or Bst 3.0 DNA polymerase, followed by Cas12b detection. Reactions with no template added were designated as NTC. Two different sgRNAs were designed, targeting E453 and NS170 sites in JEV, respectively. Error bars represented s.d. from $n = 3$ replicates. (G) Detection of mature mRNA (GAPDH) and circular RNA (CDS1as) by HOLMESv2. HEK293T cell RNA was amplified by either RT-LAMP or Bst 3.0 DNA polymerase, followed by Cas12b detection. Reactions with no template added were designated as NTC.

cillus acidoterrestris Cas12b protein (AacCas12b)[10] and confirmed that Cas12b had both *cis*-cleavage activity on target double-stranded DNA (dsDNA) (Figure S1, Table S1−S3) and *trans*-cleavage activity on ssDNA. Consistent with previous findings,[3,12] the nonspecific ssDNA *trans*-cleavage activity could be triggered by either target dsDNA or target ssDNA (Figure 1A,B). Besides, we found that the Cas12b ternary complex with target ssDNA showed higher *trans*-cleavage

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activity (Figure 1C). Notably, Cas12b showed different target preference in *trans*-cleavage from Cas12a, which exhibited a higher nonspecific ssDNA *trans*-cleavage rate with target dsDNA than with target ssDNA.[3]

On the basis of previous studies,[11] the protospacer adjacent motif (PAM) site of 5′-TTN-3′ is required by AacCas12b to cleave target dsDNA. To test whether PAM is required in Cas12b-mediated ssDNA *trans*-cleavage, we designed several mutant PAM sequences (e.g., 5′-TTC-3′, 5′-TAC-3′, 5′-ATC-3′, 5′-AAC-3′, 5′-GGC-3′, and 5′-CCC-3′) for *trans*-cleavage assay (Figure S2A). Judging by the fluorescence intensity generated by *trans*-cleavage, the Cas12b-mediated *trans*-cleavage was sensitive to the PAM sequence when targeting dsDNA, and showed high *trans*-cleavage activity with PAM sequences of 5′-TTC-3′ and 5′-TAC-3′ (Figure S2B). However, when Cas12b targeted ssDNA substrates, it *trans*-cleaved the ssDNA probe in a PAM-independent manner (Figure S2B).

We then tested different reaction temperatures and found the optimal temperature range for Cas12b *trans*-cleavage was from 35 to 65 °C with target ssDNA and from 45 to 55 °C with target dsDNA (Figure 1D). Next, the lowest target concentration required for triggering the Cas12b *trans*-cleavage was also determined, which was as low as 1 nM for either target ssDNA or target dsDNA (Figure 1E). However, when combined with loop-mediated isothermal amplification (LAMP),[13] the required target concentration decreased to 10^{-8} nM (Figure 1F), which was equivalent to that of Cas12a.[7] Therefore, considering the high sensitivity, Cas12b was employed for nucleic acid detection, and the new method combining amplification and Cas12b *trans*-cleavage detection was named HOLMESv2.

Target DNA Detection by HOLMESv2. To check the target specificity of HOLMESv2, we then tested whether HOLMESv2 could distinguish target DNA with single-nucleotide mismatch (SNM). Considering the ability to distinguish SNM by Cas12a is determined by both the length of the CRISPR RNA (crRNA) guide sequence and the mismatch position within the guide sequence,[7] we analyzed sgRNAs with distinct guide sequence lengths and target ssDNA (or dsDNA) sequences with SNMs at different positions. We tested target ssDNA (DNMT1−3), and found only sgRNA with 16-nt or 17-nt guide sequences (abbreviated as 16-nt or 17-nt sgRNA thereafter) showed great differences in fluorescence intensities when the mismatch was aligned within the 10th−16th positions (Figure S3A−C). Otherwise, Cas12b either failed to distinguish targets with SNM (e.g., all showed high fluorescent signals when using 18-nt−20-nt sgRNAs) or showed remarkably reduced *trans*-cleavage activity (e.g., using 14-nt and 15-nt sgRNAs) (Figure S3C). In addition, we also tested the 16-nt sgRNA for more ssDNA targets and demonstrated it effective for discriminating SNMs but with distinct requirements of mismatch positions for different targets (e.g., 8th and 10th−12th for rs5028, 11th for rs1467558, and 8th−15th for rs2952768) (Figure S3D,E). We then tested target dsDNA of DNMT1−3(TTC PAM) (Figure S4A), and found 18-nt−20-nt sgRNAs were more effective for Cas12b to discriminate target with SNM (Figure 2A,B, Figure S4B). However, different guide lengths led to different requirements of the mismatch positions. For example, with the 18-nt sgRNA, Cas12b was able to distinguish target dsDNA of DNMT1−3 with SNM positioned at the 1st, 10th, 12th, 13th, 15th, or 16th base of the guide sequences, but positions altered when either 19-nt or 20-nt sgRNAs was used (Figure 2A,B, Figure S4B). On the basis of the results above, the mismatch position for detecting SNMs should be further away from the PAM site, with a 16-nt or 17-nt sgRNA for target ssDNA and an sgRNA with 18-nt−20-nt targeting sequences for target dsDNA.

We then used HOLMESv2 to examine the single nucleotide polymorphism (SNP) locus of rs5028 from human cell HEK293T. As there is no suitable PAM sequence near the rs5028 locus, we employed two distinct approaches to amplify the target, including asymmetric PCR and LAMP. When combined with asymmetric PCR, which generates ssDNA products, Cas12b successfully distinguished the SNP locus without a PAM sequence, where only with perfectly matched sgRNA did the Cas12b show high *trans*-cleavage activity (Figure 2C,D). However, when LAMP was employed for target nucleic acid amplification, the PAM sequence was required for the target double-stranded amplicons and was introduced by primers (Table S3) to successfully assist Cas12b in distinguishing the SNP locus (Figure 2C,D).

In addition, to test the practicability of using HOLMESv2 for detecting target DNA from body fluid such as urine, purified plasmid pUC18-rs5028 was serially diluted by fresh urine to prepare the urine spike-in of target DNA. The results clearly showed that the detection sensitivity of HOLMESv2 for target DNA from urine was 10^{-8} nM (Figure S6A), demonstrating that the HOLMESv2 sensitivity was unaffected by urine.

Target RNA Detection by HOLMESv2. Besides DNA, RNA targets can also be detected with CRISPR-based methods; however, RNA needs to be first reverse transcribed into cDNA, which is then used as the template for either *in vitro* transcription (e.g., SHERLOCK or SHERLOCKv2) or DNA polymerase-based amplification (HOLMES or DETECTR).[3−8] Therefore, the whole process for RNA detection is complicated and usually requires more than one enzyme involved. To simplify the procedure by omitting an extra reverse transcription step, we used Bst 3.0 DNA polymerase, which contained the 5′ → 3′ DNA polymerase activity with either DNA or RNA templates, to directly amplify the RNA target.[14] Similar to the reverse transcription LAMP (RT-LAMP) reaction system, HOLMESv2 with LAMP that used Bst 3.0 DNA polymerase successfully detected the Japanese encephalitis virus (JEV) within 1 h (Figure 2E,F), highlighting HOLMESv2 as a convenient platform for target RNA detection. Furthermore, the RNA detection sensitivity of HOLMESv2 was determined, and the lower detection limit was 10^{-8} nM for target RNA (Figure S5), which was the same as that for target DNA. However, when target RNA existed in urine samples, the detection sensitivity was largely reduced. For example, the lower limit was 10^{-3} nM when the urine concentration was above 4%, and reached 10^{-5} nM in 1% urine condition (Figure S6B).

Pre-mRNA alternative splicing is a key post-transcriptional regulatory event in high eukaryotes and plays important roles in human diseases such as tumor progression. We next employed HOLMESv2 to detect mRNA splicing by specifically targeting to the splicing sites, and with the combination of RT-LAMP amplification, the spliced mature mRNA of the *GAPDH* gene was successfully detected by HOLMESv2 (Figure 2G). Besides, with a similar strategy for the sgRNA design, we also successfully detected circular RNA of Cdr1as in HEK293T (Figure 2G), which is likely involved in various cancers.[15]

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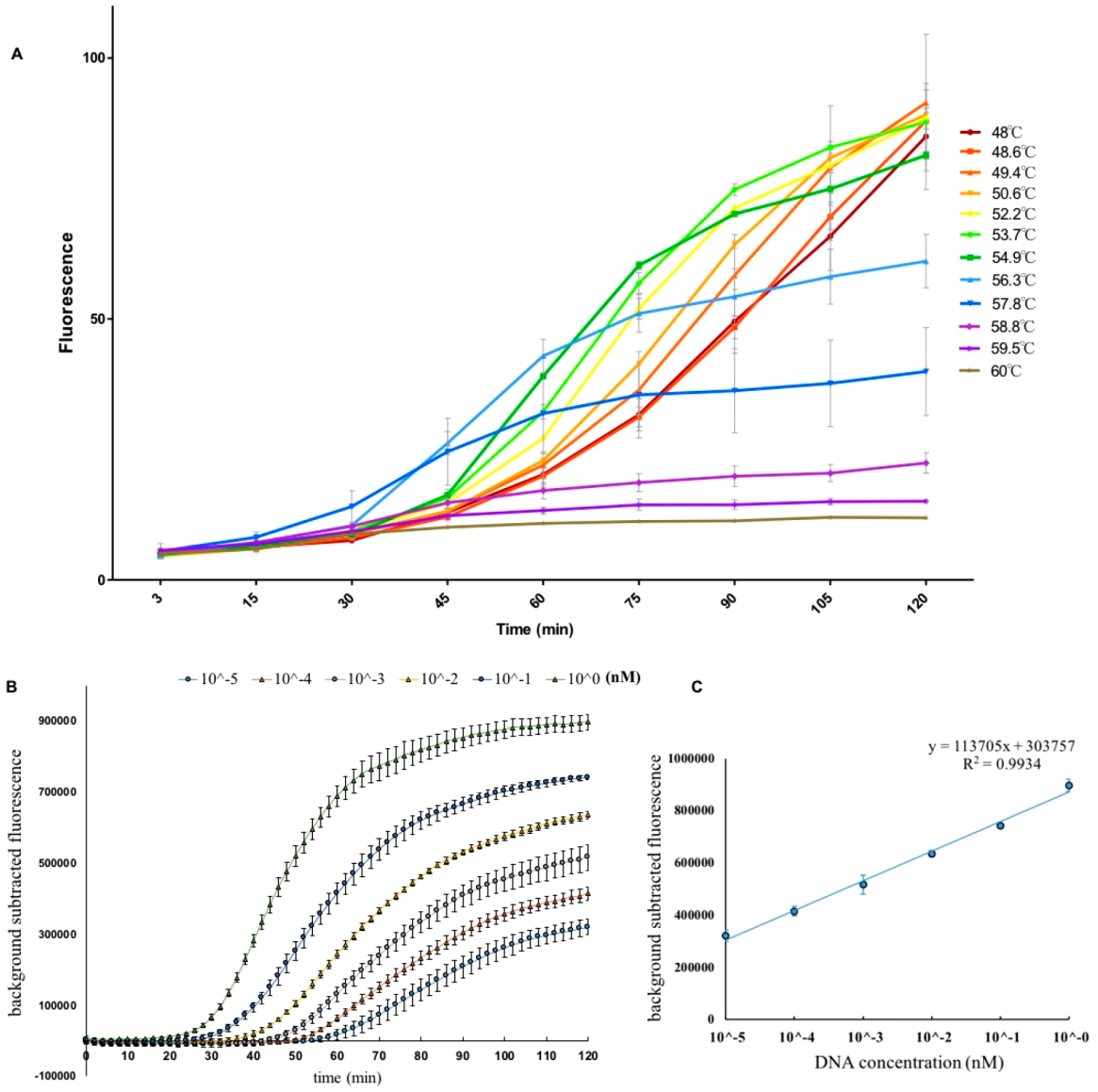


Figure 3. Target DNA quantitation by one-step HOLMESv2. (A) One-step HOLMESv2 analysis under reaction temperatures ranging from 48 to 60 °C. Fluorescence signal was measured at different time points from 3 to 120 min. (B) Real-time detection of target dsDNA by HOLMESv2 using a real-time machine. Plasmid pUC18-rs5028 containing the target rs5028 locus was serially diluted and used as the target. (C) Standard curve analysis of the one-step HOLMESv2 analysis. Fluorescence intensities used in the standard curve were collected from targets ranging from 1 nM to 10^{-5} nM at the time point of 120 min from Figure 3B. Error bars represented s.d. from n = 3 replicates.

Target DNA Quantitation by One-Step HOLMESv2. As stated above, cross-contamination between samples would be fatal for molecular diagnostics, especially for the highly sensitive CRISPR-based technologies. Therefore, to reduce the chance of cross-contamination, we tested the practicability of integrating two steps of isothermal LAMP amplification and Cas12b detection into one reaction system. Because both steps have a wide temperature range, the integrated system was tested under temperatures ranging from 48 to 60 °C to find out the most optimal temperature for the one-step HOLMESv2. As shown in Figure 3A, with the increase of the reaction temperature, although the lag phase reduced, the fluorescence intensity reflecting the Cas12b *trans*-cleavage activity also reduced, and the signal was hardly detectable when the temperature rose above 57.8 °C. Considering both the lagging time and the signal intensity, we chose the 55 °C reaction temperature for the one-step HOLMESv2 system. Assisted with a real-time fluorescence reader, we real-time monitored the one-step HOLMESv2 reaction and quantitated the target DNA (i.e., plasmid pUC18-rs5028), which was serially diluted to concentrations ranging from 1 nM to 10^{-8} nM. As expected, higher target concentration would lead to less takeoff time and higher fluorescence intensity (Figure 3B), and with the fluorescence intensities at the time point of 120 min, a standard curve was generated with the R^2 value of 0.9934 (Figure 3C).

Quantitation of Target DNA Methylation Degree by HOLMESv2 Combined with Bisulfite Treatment. As an important epigenetic modification, the changes of DNA methylation are often linked to human diseases such as cancer,[16] and quantitative measurement is a key step. Considering the merits of accurate target quantitation and SNM discriminating abilities by HOLMESv2, we further used HOLMESv2 to rapidly quantitate the degree of specific DNA

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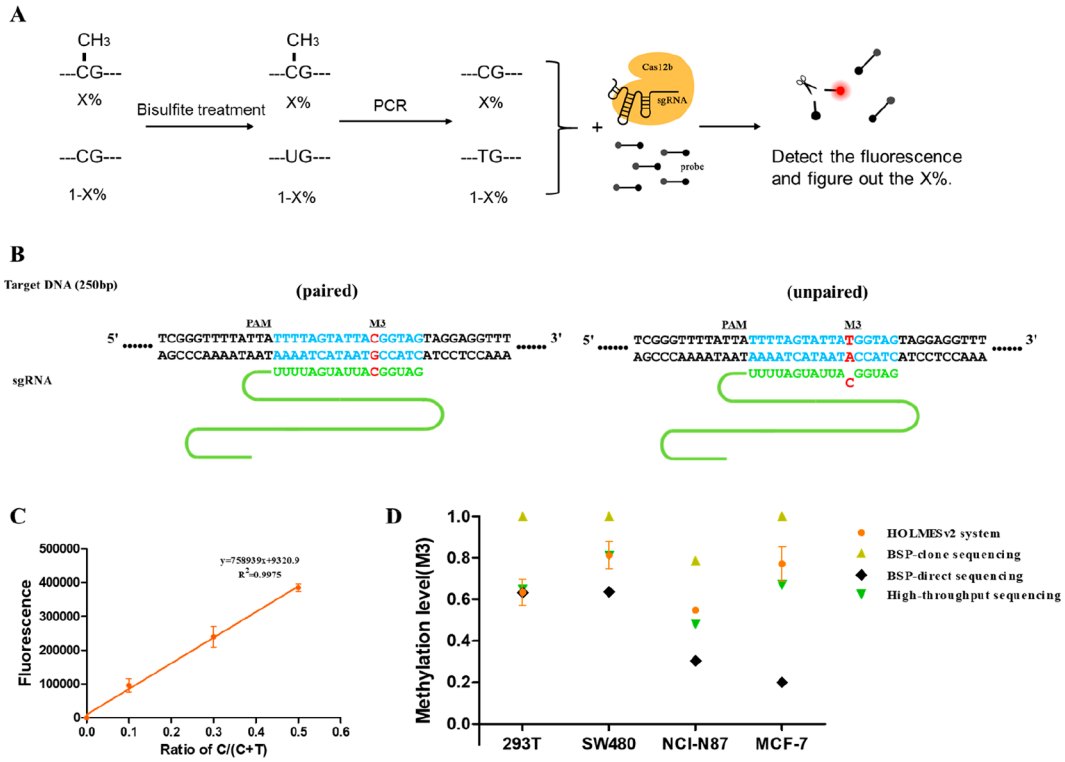


Figure 4. Quantitation of target DNA methylation degree by HOLMESv2 combined with bisulfite treatment. (A) Schematic of the workflow of HOLMESv2-mediated quantitation of target DNA methylation degree. Target DNA was treated with bisulfite, PCR amplified, quantitated, and finally subjected to HOLMESv2 detection. With paired sgRNA and target DNA, Cas12b *trans*-cleaved nonspecific ssDNA probes and illuminated fluorescence. (B) Presentation of target sequence with its sgRNA. The sgRNA guide sequence was either paired (left panel) or unpaired (right panel) with the target DNA sequences. (C) Standard curve for quantitation of the methylation degree of M3 site in COL1A2 promoter region. The *X*-axis value represented the ratio of C/(C+T), mimicking the ratio of methylated M3 in *COL1A2* promoter region, where unmethylated C was transformed into T after bisulfite treatment. The *Y*-axis showed the corresponding fluorescence intensity of each sample detected by HOLMESv2, employing Cas12b and sgRNA-COL1A2m3-C12. (D) Quantitation of the methylation degree of M3 in *COL1A2* promoter region in four cell lines (i.e., 293T, SW480, NCI-N87, and MCF-7). After bisulfite treatment, the methylation status was analyzed by four different methods, including HOLMESv2 system, BSP-clone sequencing, BSP-direct sequencing and the NGS-based high-throughput sequencing. The degree of M3 methylation in 293T, SW480, NCI-N87, and MCF-7 was 63.3%, 81.3%, 54.8%, and 77.1% by HOLMESv2 (Figure S8); 92.3% (12/13), 100% (15/15), 78.6% (11/14), and 71.4% (10/14) by BSP-clone sequencing (Figure S9); 63.2%, 63.6%, 30.4%, and 20% by BSP-direct sequencing, and 65.3%, 81.1%, 48.3%, and 67.3% by NGS-based high-throughput sequencing (Table S4), respectively. Error bars represented s.d. from n = 3 replicates.

methylation. We chose the collagen α2(I) gene (*COL1A2*), which encodes the protein collagen and plays an important role in tumor development,[17] and analyzed the methylation degree of the third CpG site (M3) in its promoter region using HOLMESv2 (Figure 4A, Figure S7). First, two fragments of COL1A2 (BSP)-C and COL1A2 (BSP)-T that covered the M3 site were cloned and PCR amplified, which mimicked the bisulfite-treated samples with 0% and 100% methylated status, respectively. Then, the two fragments were mixed in different proportions and measured by HOLMESv2 using Cas12b and sgRNA-COL1A2m3-C12, generating a standard curve with the R^2 value of 0.9975 (Figure 4B). Next, the genomic DNAs from four different cell lines (i.e., 293T, SW480, NCI-N87, MCF-7) were treated by bisulfite, and the M3 site was subsequently PCR-amplified for HOLMESv2 measurement (Figure 4A). On the basis of the standard curve, the M3 methylation degree was quantitatively determined and found to be varied in different cell lines (Figure 4, Figure S8), which was consistent with previous studies.[17]

Besides, to assess the accuracy of the methylation quantitation results by HOLMESv2, samples were also measured with traditional methods, including the cloning-based bisulfite sequencing (BSP-clone sequencing), BSP-direct sequencing, and next generation sequencing (NGS)-based methylation sequencing (Figure 4C, Figure S9 and Table S4). On the basis of the paired t test results, the HOLMESv2 method was highly consistent with the NGS method (Figure S10), which has been well recognized as an accurate approach for DNA methylation quantitation.[18] The BSP-clone sequencing method, which is the most commonly used for methylation quantitation, obtained an obviously higher methylation degree, and the methylation degree by BSP-direct sequencing was significantly lower than the NGS method, indicating relative inaccuracy of these two methods. Therefore, considering the merits of high accuracy, rapid speed, and much less cost by HOLMESv2, HOLMESv2 may serve as a potential platform for efficient detection of DNA methylation degree.

So far, over 1.5 million SNPs have been found within the human genome and many are related to personalities and disease risks. Because of the importance of SNP characterization, a number of genotyping methods have been developed for it,[19] among which the recently emerged CRISPR-Dx technologies demonstrated the merits of simplicity, speed, and high sensitivity.[5,7] Here, with the characterization of Cas12b,

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we developed the Cas12b-based HOLMESv2 method, allowing for multipurpose applications such as SNP discrimination, RNA detection, one-step DNA quantification, and quantitation of DNA methylation degree. Similar to HOLMES, HOLMESv2 also detected target ssDNA without the need of a PAM sequence. Therefore, combined with the amplification of target DNA with asymmetric PCR, which generates ssDNA amplicons, HOLMESv2 can be used for convenient detection of any sequences, regardless of the presence of PAM sequences. Alternatively, the PAM site can be introduced during amplification by PCR or LAMP, where the PAM can be designed in the primers. In the future, with the availability of engineered Cas12b mutants that recognize various PAM sequences, it will become more convenient to design suitable crRNAs for target nucleic acid detection.

As a thermophilic Cas protein, Cas12b can be integrated with the isothermal LAMP amplification procedure to generate a one-step system for convenient target detection. More importantly, the one-step system not only avoids cross-contamination among samples, but also facilitates point-of-care testing (POCT), where only simple instruments are required and the detection results can be obtained within a short time. However, although we have found a reaction buffer suitable for both LAMP amplification and Cas12b detection, the buffer was not further optimized in this study, and both the sensitivity and speed of the one-step HOLMESv2 is not as good as that with separate steps (Table S5). Therefore, we speculate that a better reaction buffer can improve the detection efficiency in future studies. Considering LAMP has been successfully tested for amplification of a single nucleic acid molecule,[13] HOLMESv2 therefore has great potential for further clinical diagnostics at the single-molecule level. In addition, with its good performance in DNA quantification, HOLMESv2 was also used for accurate quantitation of DNA methylation degree in this study. Combined with bisulfite treatment, HOLMESv2 specifically discriminated methylated and unmethylated DNA sites. Noticeably, the HOLMESv2 method is very simple, rapid, and inexpensive, but has great accuracy in DNA methylation quantitation, which is comparable to the well-recognized NGS method.

Besides DNA detection, we here also proved that HOLMESv2 could be used in successful detection of RNA targets, including RNA viruses and the RNA splicing events. Compared with other CRISPR-Dx technologies, HOLMESv2 combines the LAMP reaction and uses the Bst DNA polymerase to efficiently amplify the RNA target, generating DNA amplicons for rapid Cas12b detection. Noticeably, HOLMESv2 also successfully detected target DNA or target RNA from body fluid such as urine, although the lower detection limit for target RNA was affected, which might be caused by less stability of RNA in the urine environment. Therefore, considering the wide application scenarios of DNA/RNA detection, HOLMESv2 may have great potential in clinical detection of a large number of pathogens.

Overall, HOLMESv2 can be conveniently coupled with any nucleic acid amplification methods, including the isothermal LAMP or recombinase polymerase amplification (RPA),[20] to improve the detection sensitivity. Besides, HOLMESv2 can also be integrated into present diagnostic instruments and platforms, including the portable lateral flow assay devices for convenient detection, the microfluidic platform for multiple targets, the quantitative PCR machine for relative quantitation, and the digital PCR system for absolute quantitation.

METHODS

Generation of DNA Targets. Single-stranded target DNA was commercially synthesized (Table S1). For short double-stranded target DNA, two complementary oligonucleotides were commercially synthesized (Table S1), and were then annealed to form the dsDNA. Specifically, paired oligonucleotides (1 μM) were mixed in 1 × PCR buffer (Transgen Biotechnology.) to a total volume of 20 μL, and then an annealing procedure was performed: initial denaturation at 95 °C for 5 min and then decrease 1 °C per minute from 95 to 20 °C using a thermocycler.

To verify the *cis*-cleavage activity of Cas12b, the PCR fragment was amplified from plasmid pUC18-T1,[6] using PCR primers of T1-(TTC)PAM-F and T1-(TTC)PAM-R. The PCR product was treated with DpnI (NEB), phosphorylated with T4 PNK (Tolo Biotechnology), and then self-ligated with T4 DNA ligase (Tolo Biotechnology). After that, the ligation product was transformed into the *Escherichia coli* DH10b competent cell to obtain the plasmid pUC18-T1-(TTC)PAM, where the PAM site was changed from 5′-TTG-3′ in pUC18-T1 to 5′-TTC-3′ in pUC18-T1-(TTC)PAM. Then, the obtained plasmid was used as the template to produce a PCR fragment of pUC18-T1-600 that contained the target T1 sequence, using PCR primers of T1-300-F and T1-300-R. The fragment was subsequently used as the substrate for Cas12b *cis*-cleavage assay (Figure S1).

To construct pUC18-rs5028, a 198-bp fragment was PCR amplified from HEK293T genomic DNA with primers of rs5082-F-198bp and rs5082-R-198bp, using the high-fidelity FastPfu DNA polymerase (Tolo Biotechnology). Similarly, the vector was prepared by PCR amplification with primers of pUC18-5082-F and pUC18-5082-R, employing plasmid pUC18 as the template, which was finally eliminated by DpnI digestion. The two fragments were the assembled using the Ezmax seamless assembly kit (Tolo Biotechnology), and the obtained clone was verified by DNA sequencing with the correct one named as pUC18-rs5028.

To verify the necessity of PAM sequence for Cas12b-mediated *trans*-cleavage activity when targeting dsDNA (Figure S2), a PCR fragment from plasmid pDNMT1-3-2016bp was used. First, a 2016-bp DNA fragment containing the target sequence of DNMT1-3 was PCR amplified, treated with DpnI, and then cloned into pEASY-Blunt (Transgen Biotechnology) to obtain pDNMT1-3-2016bp. With pDNMT1-3-2016bp as the template, six pairs of primers corresponding to different PAM sequences were used to PCR amplify six 276-bp DNA fragments, all of which contained the target DNMT1-3 with the corresponding PAM sequences of TTC, TAC, ATC, AAC, GGC, and CCC, respectively. The six fragments were then used as substrates to verify the necessity of PAM sequences required for Cas12b to *trans*-cleave ssDNA.

For DNA methylation detection, a 250-bp promoter region of gene *COL1A2* was PCR amplified from human cell 293T genome with primers of COL1A2-F and COL1A2-R, using 2× Tolo FastPfu Mix (Tolo Biotechnology), and the PCR amplicon was then inserted into a T-vector to obtain pClone007S-COL1A2. Then, pClone007S-COL1A2 was methylated by M.Sss I CpG methylase (NEB), following the manufacturer's instructions. Both methylated and unmethylated plasmids were then treated by bisulfite, PCR was amplified with primers of COL1A2(BSP)-F and COL1A2-(BSP)-R, and inserted into a T-vector to obtain two plasmids

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for standard curve analysis, namely pClone007S-COL1A2 (BSP)-C (all CpG sites were C) and pClone007S-COL1A2 (BSP)-T (all CpG sites were T), respectively. The above plasmids were verified by Sanger sequencing. To prepare a standard curve for quantitation of target DNA methylation degree, fragments of COL1A2 (BSP)-C and COL1A2 (BSP)-T were PCR amplified from the two plasmids mentioned above, using primers of COL1A2(BSP)-F and COL1A2(BSP)-R (Figure S7).

Transcription of sgRNAs. To prepare the template for transcribing sgRNA for target T1, plasmid pUC18-sgRNA-T1 was constructed using pUC18 as the plasmid backbone, which was inserted with the T7 promoter and target T1 sequence, allowing for subsequent transcription of sgRNA with T7 RNA polymerase. In detail, two rounds of PCR were performed. For the first round of PCR, plasmid pUC18 was used as the template, with pUC18-1-F and pUC18-1-R as the amplification primers. After amplification, the PCR product was purified and self-ligated by T4 DNA ligase before being transformed into *E. coli* DH10b. The correct clone was verified by Sanger sequencing and named pUC18-sgRNA-T1-pre, which was subsequently used as the template for the second round of PCR with primers of pUC18-2-F and pUC18-2-R. Following the same procedure, the PCR product was self-ligated and transformed in *E. coli* DH10b to obtain the correct plasmid pUC18-sgRNA-T1.

Then, to prepare the DNA template for *in vitro* transcription, pUC18-sgRNA-T1 was employed as a template for PCR amplification with primers of T7-crRNA-F and ZL-sgRNA-T1-R (Table S2). The PCR product was first treated with DpnI (10 U DpnI per 50 μL of PCR system) at 37 °C for 30 min, and then purified by Wizard SV Gel and PCR Clean-Up System (Promega). Quantified purified PCR product was used as the template for sgRNA biosynthesis with the T7 High Yield Transcription Kit (ThermoFisher), and the reaction was performed at 37 °C overnight (12−16 h).

For target DNMT1-3, plasmid pUC18-sgRNA-DNMT1-3 was obtained by replacing the 20-bp sequence targeting T1 in plasmid pUC18-sgRNA-T1. Then, with plasmid pUC18-sgRNA-DNMT1-3 as the template and T7-crRNA-F and ZLsgRNA-DNMT1-3-R as the PCR primers, the PCR products were used as the template for *in vitro* transcription. Similarly, plasmids containing sgRNA sequences for other targets (including the target M3 CpG site in *COL1A2* promoter region) were constructed on the basis of plasmid pUC18-sgRNA-DNMT1-3, and *in vitro* transcription was performed in the same procedure as described above, using T7-crRNA-F as the upstream primer and corresponding target sequences as the downstream primers to prepare the transcription templates (Table S2).

Finally, DNase I was added into the transcription system (10 U DNase I per 50 μL of transcription system) and incubated at 37 °C for 30 min to remove the template DNA. Then, transcribed RNA was purified using the RNA Clean & ConcentratorTM-5 (Zymo Research), quantitated by NanoDrop 2000C (ThermoFisher), and stored at −80 °C until use.

Purification of Cas12b Protein. The full-length gene encoding *Alicyclobacillus acidoterrestris* Cas12b (AacCas12b) was codon optimized and synthesized by Tolo Biotechnology, and then cloned into a pET28a vector. The N-terminal his-tagged recombinant Cas12b protein was produced in *E. coli* BL21(DE3). When OD_{600} reached 0.6, cells were induced by 0.25 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 14−18 h at 16 °C before being harvested by centrifugation. Cell pellets were resuspended in buffer A (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 20 mM imidazole, and 1/500 phenylmethylsulfonyl fluoride (v/v)), lysed by a high-pressure homogenizer (Avestin), and centrifuged at 15 000 rpm for 30 min. The supernatant was incubated with Ni Sepharose (GE Healthcare) which was pre-equilibrated in buffer A. The resin was washed with 20 column volumes of buffer B (50 mM Tris-HCl (pH 7.6), 200 mM NaCl, and 30 mM imidazole), and then the recombinant protein was eluted with buffer B supplemented with 270 mM imidazole. Eluted fractions containing AacCas12b were collected and concentrated to about 5 mL by ultrafiltration before being loaded onto a HiLoad 16/600 Superdex 200 pg column (GE Healthcare) that had been equilibrated with buffer C (40 mM Tris-HCl (pH 7.6), 200 mM NaCl, 2 mM DTT, and 5% glycerol (v/v)). The washing fractions containing AacCas12b protein were collected and concentrated to a final concentration of 4 mg/mL and stored at −20 °C with the addition of 50% glycerol.

HOLMESv2 Method. *1. Asymmetric PCR and LAMP.* For asymmetric PCR amplification, genomic DNA of human cell HEK293T was used as the template with primers of ASP-primer (0.2 μM), ASP-rs5028-F (0.2 μM) and ASP-rs5028-R (1 μM) (Table S3), and KOD FX DNA polymerase (ToYoBo) was employed, using the procedure: 98 °C, 10 s; 55 °C, 20 s; 68 °C, 20 s; with 30 cycles.

For LAMP amplification, the total volume of LAMP amplification reaction system is 25 μL, containing primer pairs of LAMP-FIP (1.6 μM) and LAMP-BIP (1.6 μM), LAMP-F3 (0.2 μM) and LAMP-B3 (0.2 μM), and LAMP-LoopF (0.4 μM) and LAMP-LoopB (0.4 μM) (Table S3). LAMP amplification was performed using the WarmStart LAMP Kit (NEB) for most reactions. While for JEV RNA virus detection, Bst 3.0 DNA polymerase (NEB) was also tested. The reaction was performed following the manufacture's instruction, for example, incubation at 65 °C for 30 min before being terminated at 85 °C for 10 min.

2. Preparation of sgRNA and Cas12b-Mediated trans-Cleavage Reaction. Purified sgRNA was diluted to an appropriate concentration (5 μM) and self-annealed by a PCR machine, following the procedure: denature at 75 °C for 5 min, and then cool from 75 to 20 °C with a decrease rate of 1 °C per minute. Annealed sgRNA was then mixed with equimolar Cas12b and incubated at 30 °C for 20−30 min.

In a 20 μL Cas12b reaction system, both sgRNA and Cas12b were added to a final concentration of 250 nM, and then the fluorescence quenching probe (HEX-N12-BHQ1, final concentration of 500 nM), 2 μL of 10 × NEB Buffer 3.1, and 0.5 μL of RNase inhibitor (40 U/μL) were subsequently added. The system was first incubated at 48 °C for 30 min and then stopped by heating at 98 °C for 5 min. The 20 μL system was then transferred to a 96-well plate and detected with a Varioskan Flash (ThermoFisher) with the excitation at 535 nm and emission at 556 nm.

3. Determination of the Sensitivity for Target RNA. A 335 bp region containing the E453 site was amplified from the cDNA of the JEV virus with primers of JEV-E-T7-F and JEV-E-R, and the PCR amplicon was gel purified, quantitated, and then used as the template for RNA preparation with the T7 RNA polymerase. The *in vitro* transcription system was then treated with DNase I to remove the DNA template, and the transcribed RNA was then purified with the RNA Clean & ConcentratorTM-5 (Zymo Research), followed by quantita-

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tion with NanoDrop 2000C (ThermoFisher). Serially diluted target RNA was first treated with the WarmStart LAMP Kit for 1 h at 65 °C, and then 2 μL of LAMP product was added into a 20 μL Cas12b reaction system for further incubation at 48 °C for 30 min. The fluorescence intensity was detected following the procedures described above.

4. Detection of Urine Spike-ins of Target DNA or Target RNA with HOLMESv2. A fresh urine sample was taken from a healthy volunteer, and serially diluted target DNA of pUC18-rs5028 and target RNA of the above *in vitro* transcribed E453 RNA fragment were added into the urine to obtain urine spike-in samples. Then, target DNA or RNA was first amplified from the samples using the WarmStart LAMP Kit (NEB), followed by the same 20 μL reaction system as shown above. Besides, to test the influence of urine on the sensitivity of target RNA detection, fresh urine was also diluted to different folds by RNase-free water before the HOLMESv2 analysis as indicated above.

One-Step HOLMESv2. The total volume of each reaction system was 20 μL, containing primer pairs of 1.6 μM LAMP-rs5028-FIP/LAMP-rs5028-BIP, 0.2 μM LAMP-rs5028-F3/LAMP-rs5028-B3, and 0.4 μM LAMP-rs5028-LoopF/LAMP-rs5028-LoopB (Table S3), 10 μL of a mixture of WarmStart LAMP Kit (NEB), 500 nM sgRNA, and 500 nM Cas12b, 500 nM fluorescent probe (HEX-N12-BHQ1 or FAM-N12-Eclipse), 25 ng/μL sheared salmon sperm DNA, and 0.5 μL of RNase inhibitor (40 U/μL). Salmon sperm DNA was added in the one-step HOLMESv2 reaction system in order to minimize the experimental errors among tested groups.

To test different reaction temperatures for one-step HOLMESv2 (Figure 3A), HEX-N12-BHQ1 was used as the probe, and pUC18-rs5028 plasmid (0.1 nM) was used as the template. Fluorescence was detected by a Varioskan Flash (ThermoFisher).

To quantitate target DNA with one-step HOLMESv2 (Figure 3B), the FAM-N12-Eclipse probe was used, and reactions were performed using serially diluted pUC18-rs5028 plasmid as the template. The LAMP system was then added into a 96-well plate, and the reaction was performed at 55 °C for 120 min in the StepOnePlus Real-Time PCR System (ThermoFisher), followed by real-time detection of fluorescence with the instrument.

DNA Methylation Detection. *1. Bisulfite Treatment, BSP-Clone Sequencing, BSP-Direct Sequencing and NGS High-Throughput Sequencing.* Genomic DNA from different human cell lines (i.e., 293T, SW480, NCI-N87, and MCF-7) was extracted and treated with EZ DNA methylation-direct kit (ZYMO RESEARCH), where unmethylated base C would be converted to U and methylated base C would remain unchanged. Then, a 250-bp fragment covering CpG islands in the promoter region of *COL1A2* was PCR amplified with primers COL1A2(BSP)-F and COL1A2(BSP)-R.

For BSP-clone sequencing, the 250-bp fragments were then inserted into a T-vector and transformed into *E. coli* DH10b competent cells, obtaining positive clones for Sanger sequencing analysis. The methylation degree was calculated by ratio of the number of clones with C to the total clones (i.e., C and T).

For BSP-direct sequencing, the purified 250-bp fragments were directly sequenced by Sanger sequencing, and the methylation degree was calculated by the ratio of the peak area of C to the peak areas of C and T.

For NGS high-throughput sequencing, purified PCR products were sent to Sinotech Genomics Co., Ltd. (Shanghai, China) for library construction and sequencing to analyze the methylation degree of 13 CpG sites. Paired-end sequencing library with an insert size of 370 bps was constructed using the KAPA Hyper Prep Kit (Kapa Biosystems), following the manufacturer's instructions. Purified libraries were quantified by Qubit 2.0 fluorometer (ThermoFisher) and validated by Agilent 2100 bioanalyzer (Agilent Technologies) to confirm the inset size and calculate the mole concentration. Qualified libraries were sequenced on the Illumina HiSeq X-ten platform, and the results were analyzed with Bismark[21] (version 0.19.0).

2. Methylation Degree Quantitation with HOLMESv2. To detect the methylation degree of the M3 site in the promoter region of *COL1A2*, the sulphite treatment of genomic DNA and PCR amplification were the same as described above.

To quantitate the methylation degree in the M3 CpG site of *COL1A2* promoter (refer to Figure S7), a standard curve was first prepared. In detail, fragments of COL1A2 (BSP)-C and COL1A2 (BSP)-T were PCR amplified from templates as described above, representing full methylated and unmethylated targets, respectively. Then, methylated and unmethylated fragments were mixed in a series of proportions, obtaining templates that contained 0%, 10%, 30%, and 50% of COL1A2 (BSP)-C, respectively. The sgRNA for the *COL1A2* M3 site was prepared as described above, then purified sgRNAs were diluted to 10 μM before being annealed by the PCR machine. Cas12b was diluted to 5 μM with the storage buffer, mixed with the same volume of 10 μM annealed sgRNA, and incubated at 30 °C for 15−20 min. Preincubated Cas12b and sgRNA of final concentrations of 250 nM and 500 nM, respectively, were added into a 20 μL reaction system containing fluorescence quenching probe (FAM-N12-Eclipse, final concentration of 500 nM), 2 μL of 10 × NEBuffer 3.1, 0.25 μL of Recombinant RNase Inhibitor (40 U/μL), 500 ng of sheared salmon sperm DNA, and a certain amount of target DNA. Systems containing the above components were prepared on ice and transferred to a 96-well plate and were then processed at 48 °C for 1 h in the StepOnePlus Real-Time PCR system, followed by fluorescent kinetics measured every 2 min. The fluorescence at the time point of 30 min was chosen, and simple linear regression was used to establish a standard curve as shown by the following equation: $y = ax + b$, where the Y axis represented the fluorescence intensity and the X axis represented the ratio of methylated DNA. For each methylation site, a distinct standard curve was prepared.

To quantitate the methylation degree of the *COL1A2* M3 site in unknown samples (e.g., different human cell lines), procedures of bisulphite treatment, PCR amplification, and DNA quantitation as well as HOLMESv2 reaction were the same as those described in the standard curve preparation. As the linear range of the methylated substrate DNA in the standard curve was 0% to 50%, PCR products from unknown samples were diluted with equimolar unmethylated fragment of COL1A2 (BSP)-T before being subjected to HOLMESv2 analysis. Then, the methylation degree of the M3 site in the sample could be calculated as $x_{\text{unknown}} = 2(y_{\text{unknown}} - b)/a$.

■ ASSOCIATED CONTENT

Supporting Information

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Oligonucliotides used in this study, comparison of HOLMESv2 and HOLMES; supplementary Figures S1−S10; Tables S1−S5 (PDF)

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Notes

The authors declare the following competing financial interest(s): L.L, S.L. and J.W. are co-inventors on patent applications filed by Shanghai Tolo Biotechnology Company Limited and Shanghai Institutes for Biological Sciences relating to the work in this manuscript.

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