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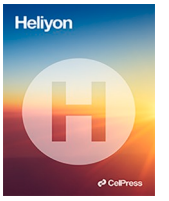

Research article

meHOLMES: A CRISPR-cas12a-based method for rapid detection of DNA methylation in a sequence-independent manner

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ABSTRACT

Aberrant DNA methylation is closely associated with various diseases, particularly cancer, and its precise detection plays an essential role in disease diagnosis and monitoring. In this study, we present a novel DNA methylation detection method (namely meHOLMES), which integrates both the TET2/APOBEC-mediated cytosine deamination step and the CRISPR-Cas12a-based signal readout step. TET2/APOBEC efficiently converts unmethylated cytosine to uracil, which is subsequently changed to thymine after PCR amplification. Utilizing a rationally designed crRNA, Cas12a specifically identifies unconverted methylated cytosines and generates detectable signals using either fluorescent reporters or lateral flow test strips. meHOLMES quantitatively detects methylated CpG sites with or without Protospacer Adjacent Motif (PAM) sequences in both artificial and real biological samples. In addition, meHOLMES can complete the whole detection process within 6 h, which is much faster than traditional bisulfite-based sample pre-treatment method. Above all, meHOLMES provides a simpler, faster, more accurate, and cost-effective approach for quantitation of DNA methylation levels in a sequence-independent manner.

1. Introduction

Mammalian DNA methylation, a pivotal epigenetic modification, frequently occurs at cytosine's fifth position (5 mC) within the CpG dinucleotides [1]. Aberrant DNA methylation has been demonstrated to be closely associated with numerous human diseases.

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Precise determination of DNA methylation levels has substantial clinical values, allowing for the surveillance of both onset and progression of many diseases, including tumors [2,3].

Dozens of technologies have been developed for DNA methylation analysis over the years, which primarily fall into two categories of PCR-based and DNA sequencing-based methods [4,5]. Although these methods are widely used, there still remain many restrictions on their applications. For instance, the PCR-based methods are critically dependent on the design and optimization of specific primers, and poorly designed primers might generate wrong results. Alternatively, DNA sequencing-based methods provide a genome-wide analysis of DNA methylation levels at a single-base resolution; however, both expensive instruments and a longer time are needed, bringing much inconvenience. Therefore, there is an urgent need for the development of a novel system that can determine DNA methylation levels in a simpler, faster and cost-effective manner.

The discovery of *trans*-cleavage activities in specific Cas proteins (i.e. Cas12 and Cas13) significantly advanced the development of CRISPR-based nucleic acid detection systems, ushering in a new era of next-generation molecular diagnostics with its promise of accurate, rapid, and portable diagnosis [6]. Previously, we characterized Cas12a single-strand DNA *trans*-cleavage activity, and subsequently developed an efficient method, namely HOLMES (an one-HOur Low-cost Multipurpose highly Efficient System), for nucleic acid detection[7,8]. In a HOLMES detection system, the Cas12a/crRNA specifically recognizes and forms a ternary complex with the target DNA, which then triggers the Cas12a *trans*-cleavage activity to cleave ssDNA reporters, generating detectable signals. Notably, mutations within either the PAM sequence or the region of the 1st-7th bases of the guide sequences completely abolish the *trans*-cleavage activity of Cas12a, which endows HOLMES with the capacity to discriminate single-base differences. On the basis of HOLMES, we later developed a one-step CRISPR system, HOLMESv2, through integrating Cas12b with isothermal amplification procedures, which supports diagnosis of both target nucleic acids and DNA methylation [9].

Besides HOLMESv2, numerous CRISPR-based biosensors, including DESCS, MSREs-Cas12a, *E*-PfRPA/Cas, CAM and *Gla*I-DC-SDA-CRISPR-Cas12a[10–14], have been successfully created for detection of DNA methylation. The above CRISPR methods employ either bisulfite or endonucleases for DNA pre-treatment, where bisulfite may result in DNA degradation and endonucleases are usually confined to specific recognition sites, hindering the detection of any target CpG sites. As a result, it is necessary to create a novel system for rapid, convenient and sensitive detection of any methylated CpG sites.

Herein, we introduce the meHOLMES system, which involves both enzyme-based cytosine deamination and CRISPR-Cas12a-based signal output processes. Our results have shown that meHOLMES can relatively quantitate DNA methylation levels and the whole procedure can be accomplished within 6 h, which is much faster than a traditional bisulfite method. In addition, PAM sequences can be designed on primers and introduced to any sites by PCR amplification, therefore enabling meHOLMES to detect DNA methylation in a sequence-independent manner.

2. Material and methods

2.1. Construction of plasmid

The plasmid pClone007s-COL1A2p-TCGA was constructed from pClone007s-COL1A2p using COL1A2p-TCGA-F/R primers and FastPfu high-fidelity DNA polymerase (Tolo Biotech). The amplicon was *Dpn*I-digested and transformed into *E. coli* DH5α competent cells (Tolo Biotech). Clones were verified via Sanger DNA sequencing. The pET28a-Septin9 plasmid was constructed by integrating the synthesized Septin9 DNA fragment into the pET28a vector.

2.2. Generation of dsDNA target

The dsDNA sequences of FAM-COL1A2p-TCGA, COL1A2p-CG, and COL1A2p-TG were amplified from their respective plasmids using M13-F and M13-R-FAM primers. The PCR products were purified through agarose gel electrophoresis and quantified with NanoDrop 2000 (ThermoFisher).

2.3. Preparation of methylated DNA sequences

One microgram FAM-COL1A2p-TCGA fragment was methylated using *M.SssI* (ThermoFisher). Briefly, the reaction system was incubated at 37 °C for 2 h, then heat-inactivated at 65 °C for 20 min. The DNA methylation level was confirmed by DNA digestion using *Hpa*II (ThermoFisher), followed by PAGE analysis.

2.4. TET2/APOBEC-mediated cytosine deamination

The TET2/APOBEC-mediated cytosine deamination assay was performed following the *NEBNext® Enzymatic Methyl-seq Conversion Module* protocol. Briefly, DNA treatment included both TET2 reaction and AMPure XP Beads purification. Oxidation levels were evaluated through *Msp*I digestion of TET2-converted DNA, followed by PAGE analysis. The DNA was later denatured with formamide, treated with APOBEC, and purified with AMPure XP Beads (Beckman Coulter). Deamination levels were measured by PCR amplification and *Taq*I-*v2* digestion, and then analyzed by agarose gel electrophoresis and Sanger DNA sequencing.

2.5. DNA methylation detection with meHOLMES

DNA methylation was detected using the meHOLMES method with either fluorescence reporters or lateral flow strips. The meHOLMES reaction system contained 0.5 μM LbCas12a (Tolo Biotech), 12.5 nM crRNA, 0.5 μM FQ-reporters (Tolo Biotech), and 1.5 ng target DNA in 1 × NEB buffer 3.1. Reaction was initiated by incubation at 37 °C, and fluorescence intensities were recorded for 30 min at intervals of 15 s, using a real-time PCR machine (λex: 488 nm, λem: 535 nm). For the lateral flow assay, a similar reaction system was prepared, containing 0.5 μM LbCas12a, 0.5 μM crRNA, 0.5 μM 15C FB-reporters (Tolo Biotech) and 20 ng target DNA. Reaction was first performed at 37 °C before the products being analyzed with CRISPR nucleic acid test strips.

2.6. Genomic DNA Extraction

Genomic DNA were extracted from cancer cells with commercial kits (Vazyme), following the manufacturer's instructions. Briefly, the process involved adding ingredients, vortexing, incubating at specific temperatures, washing, purifying, and quantifying using NanoDrop 2000.

3. Results and discussion

3.1. Principles of meHOLMES

The meHOLMES experiments consist of two primary steps: DNA pre-processing and signal readout. As depicted in Fig. 1, TET2 first modifies 5 mC to 5-carboxycytosine (5caC) via oxidation, leaving unmethylated cytosine unaffected. This is followed by formamide-induced denaturation of double-stranded DNA to generate single-stranded DNA (ssDNA) molecules. Subsequently, APOBEC deaminates cytosines to uracils, which will be further converted to thymines by PCR amplification. While 5caC cannot be deaminated by APOBEC and changes back to cytosine after the PCR step. Consequently, the pre-processing procedure merely transforms unmethylated cytosines to thymines, leaving 5 mC within the CpG sites unconverted (Fig. 1A).

The signal readout step of meHOLMES relies on the CRISPR-Cas12a-based specific recognition of the pre-processed DNA sequences, where the Cas12a/crRNA complex recognizes target sequences with unconverted cytosines only. Specifically, a crRNA designed to recognize unconverted methylated sequences ("C'' sequence) neither recognizes nor forms a complex with sequences comprising converted thymines ("T'' sequence). Moreover, meHOLMES intensities may reflect the methylation levels of the CpG sites within a target sequence.

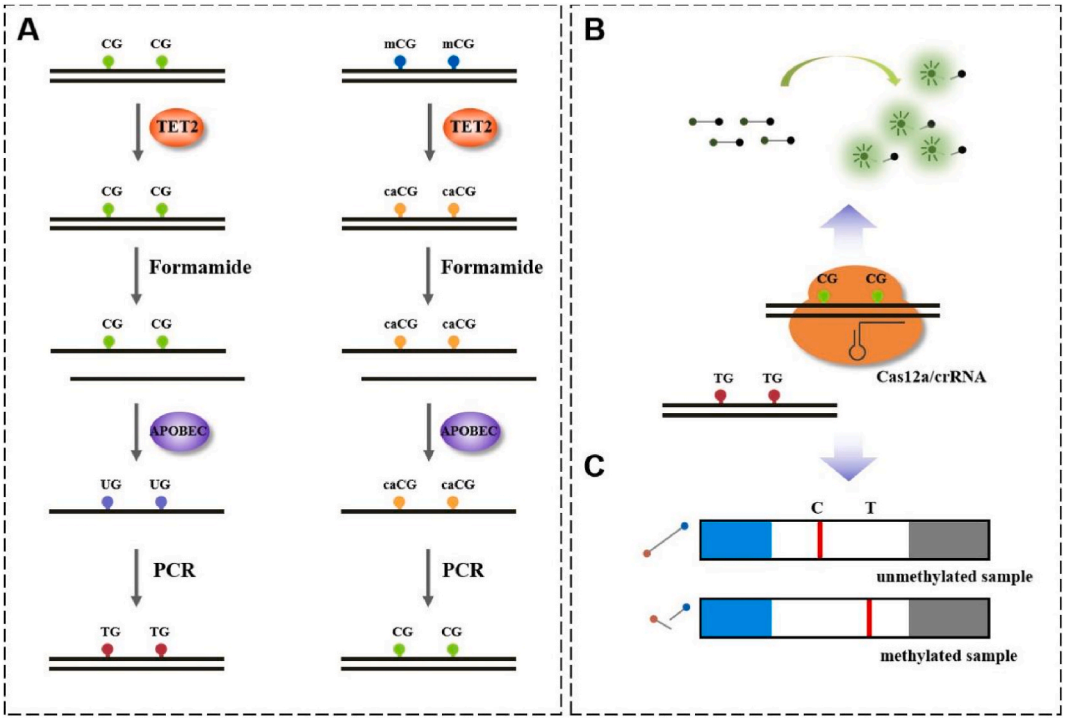

Fig. 1. Schematic illustration of meHOLMES system.
(A) The pre-treatment procedure of meHOLMES. (B) Readout of meHOLMES signals using the fluorescence method. (C) Readout of the meHOLMES signals using the LFA method.

Signals of meHOLMES system can be released from FQ-reporters, which are labeled with fluorescent and quencher units on the 5′- and 3′-ends, respectively, and recorded by a fluorescence reader (Fig. 1B). Alternatively, meHOLMES can be integrated with lateral flow assay (LFA) with the employment of ssDNA reporters labeled with FITC and Biotin (i.e., FB-reporters), which is more convenient and does not require any instruments (Fig. 1C).

3.2. DNA pre-processing step of meHOLMES

To demonstrate the feasibility of DNA pre-processing step of meHOLMES, the COL1A2 promoter containing 13 CpG sites (M1-M13) was chosen (Fig. S1). The "ACGA" sequence at the M5 site was mutated to "TCGA", the *TaqI-v2* restriction site, generating "COL1A2p-TCGA" sequence. To assess the oxidation efficacy of TET2, both unmethylated COL1A2p-TCGA and fully methylated COL1A2p-mTCGA were treated by TET2, followed by *Msp*I digestion, which cuts the 5′-CCGG-3′ site but not the 5′-C(caC)GG-3′ site. The results showed that *Msp*I efficiently cut the TET2-treated COL1A2p-TCGA, but not COL1A2p-mTCGA (Fig. S3A), indicating that only methylated cytosines in the CpG sites were converted to 5caC by TET2 treatment.

To further evaluate APOBEC's function, the TET2-treated DNA samples were denatured to ssDNA by formamide before being treated by APOBEC. The APOBEC-treated products were then employed as the template for PCR amplification, followed by *TaqI-v2* digestion and agarose gel electrophoresis analysis. The results clearly showed that only sample COL1A2p-mTCGA could be digested by *TaqI-v2*, while no cleavage was observed for sample COL1A2p-TCGA (Fig. S3B). Above findings suggested that methylated CpG sites in COL1A2p-mTCGA remained unconverted after TET2/APOBEC treatment, while unmethylated "TCGA" sequence was converted to "TTGA", thereby inhibiting *TaqI-v2* cleavage. The conversion of the DNA sequences was further validated by Sanger DNA sequencing (Figs. S3C and S4).

3.3. Signal readout step of meHOLMES

Feasibility of the meHOLMES signal readout was demonstrated using four CpG sites in the COL1A2 gene promoter. Noticeably, M3 and M9 sites had adjacent PAM sequences, while M2 and M6 did not (Fig. S5). Consequently, to facilitate Cas12a-based target detection, a PAM site (5′-TTN-3′) was incorporated via PCR amplification (Figs. S6–8). Take the M3 site detection as an example, a crRNA was designed to perfectly match the M3 sequence, guiding Cas12a to specifically recognize pre-processed methylated instead of unmethylated M3 site. As expected, fluorescent intensities increased over time when detecting COL1A2p-mTCGA, but were negligible for the COL1A2p-TCGA sequence (Fig. S9).

To facilitate convenient operation, meHOLMES was also tested with LFA with the employment of FB-reporters (Fig. S10). Similarly, sample DNA was treated first by the pre-processed step followed by Cas12a *trans*-cleavage reaction. Then, instead of the employment of a fluorescence machine, the colloidal gold test strip was directly immersed in the reaction solution. A clear band appeared at the test line region when targeting COL1A2p-mTCGA; however, only the control line was visible for unmethylated COL1A2p-TCGA (Fig. S11). Taken together, the above evidences clearly confirmed the feasibility of meHOLMES-based DNA methylation detection, employing either a fluorescence machine or a test strip for signal readout.

3.4. Performances of meHOLMES

To test the performances, parallel meHOLMES experiments were performed, and the results showed that meHOLMES had high repeatability with a relative standard deviation (RSD) of 6.36 % (Fig. S12). We then evaluated the repeated freeze-thaw cycles on the stability of Cas12a protein, which is the key component of meHOLMES system, and found no significant fluctuation in fluorescence intensities after 20 cycles' treatment, where the RSD was only 2.76 % (Fig. S13).

The quantitative capability of meHOLMES was also verified, using two COL1A2p-CG and COL1A2p-TG fragments, mimicking fully methylated and unmethylated sample respectively, were proportionally mixed. The resulting fluorescence intensities increased linearly with the increase of the methylated DNA ratios (Fig. 2A–S14), achieving a coefficient of determination (R^2) of 0.98 (fig. 2B).

Similarly, to check the quantitative performance of meHOLMES, COL1A2p-TCGA and COL1A2p-mTCGA were treated by pre-processing procedures including TET2-and APOBEC-mediated treatment and PCR amplification. After that, pre-processed products were mixed with different ratios and then detected with Cas12a-mediated HOLMES test, and concentrations of the input COL1A2p-mTCGA were calculated based on the measured fluorescence intensities and the equation obtained in Fig. 2B. Noticeably, albeit the measured concentrations were slightly higher than the input, there was a strong positive correlation between them and the R^2 value reached 0.97 (Fig. S15). Therefore, one could conclude that meHOLMES can be employed for quantitative analysis of DNA methylation at specific sites.

The specificity of the meHOLMES was also analyzed with the employment of different target sequences, which included unmethylated COL1A2p-TCGA, apramycin resistance cassette and Septin 9. Among these targets, only methylated COL1A2p-mTCGA triggered the Cas12a *trans*-cleavage activities, illuminating fluorescence signals and demonstrating the high specificity of meHOLMES (fig. 2C–S16).

meHOLMES was also integrated with LFA to establish a low-cost point-of-care approach for DNA methylation detection, where a fluorescence machine was replaced with test strips to get rid of the requirement of an expensive instrument. A mixture of DNA fragments containing different ratios of methylated COL1A2p-mTCGA *versus* unmethylated COL1A2p-TCGA was prepared and detected with meHOLMES. The results clearly showed a positive correlation between the contents of methylated COL1A2p-mTCGA and the intensities of the test line, which represented the Cas12a *trans*-cleavage activities against the FB-reporters (Fig. 2D). When

the ratio of methylated DNA was more than 60 %, meHOLMES intensities reached a plateau and all FB-reporters were cleaved, and only the test line appeared. In addition, the LFA-assisted meHOLMES was found to be of high specificity when tested with different target sequences, which was the same as that of the fluorescence-based meHOLMES (Fig. S17).

Next, meHOLMES was employed to detect the methylation level of the M3 site in real biology samples, including cervical cancer cells (HeLa), colorectal adenocarcinoma cells (SW480 and HCT116), human breast cancer cells (MDA-MB-231), hepatoma cells (Huh 7 and 97H), and human normal live cells (LO2). Extracted genomic DNA was first treated by TET2/APOBEC and PCR amplification, and was then detected by Cas12a, in combination with either a fluorescence machine (Fig. 3A) or test strips (Fig. 3B). The results showed that the methylation level of the M3 site in COL1A2 promoter was significantly higher in tumor cells than that in a normal cell, which was consistent with previous reports [15]. Based on above results, one could conclude that meHOLMES facilitates convenient, quantitative, and accurate detection of DNA methylation levels.

4. Conclusions

In summary, we here employed both the TET2/APOBEC-mediated cytosine deamination and Cas12a-based HOLMES methods and developed a simple, rapid, accurate, and cost-effective DNA methylation detection system, namely meHOLMES. meHOLMES can be integrated with either fluorescence or LFA for signal readout, and has been demonstrated here to be of high stability (RSD = 2.76 %) and repeatability (RSD = 6.36 %). When combined with PCR-mediated PAM introduction step, meHOLMES can detect target DNA methylation in a sequence-independent manner. In addition, compared to the bisulfite-based pre-treatment in other CRISPR-based DNA methylation detection methods[10–14], the enzyme-based pre-treatment in meHOLMES is much simpler and milder and causes less DNA damage, which endows meHOLMES with rapidness and high accuracy in DNA methylation detection in both synthesized DNA and genomic DNA from tumor cells (Table S5). Although meHOLMES is unable to achieve absolute quantification or amplification-free detection in DNA methylation, and it cannot simultaneously detect multiple methylation sites within a single reaction system, it still holds great potential in both basic research and clinical applications for disease diagnosis, prognosis, and treatment monitoring.

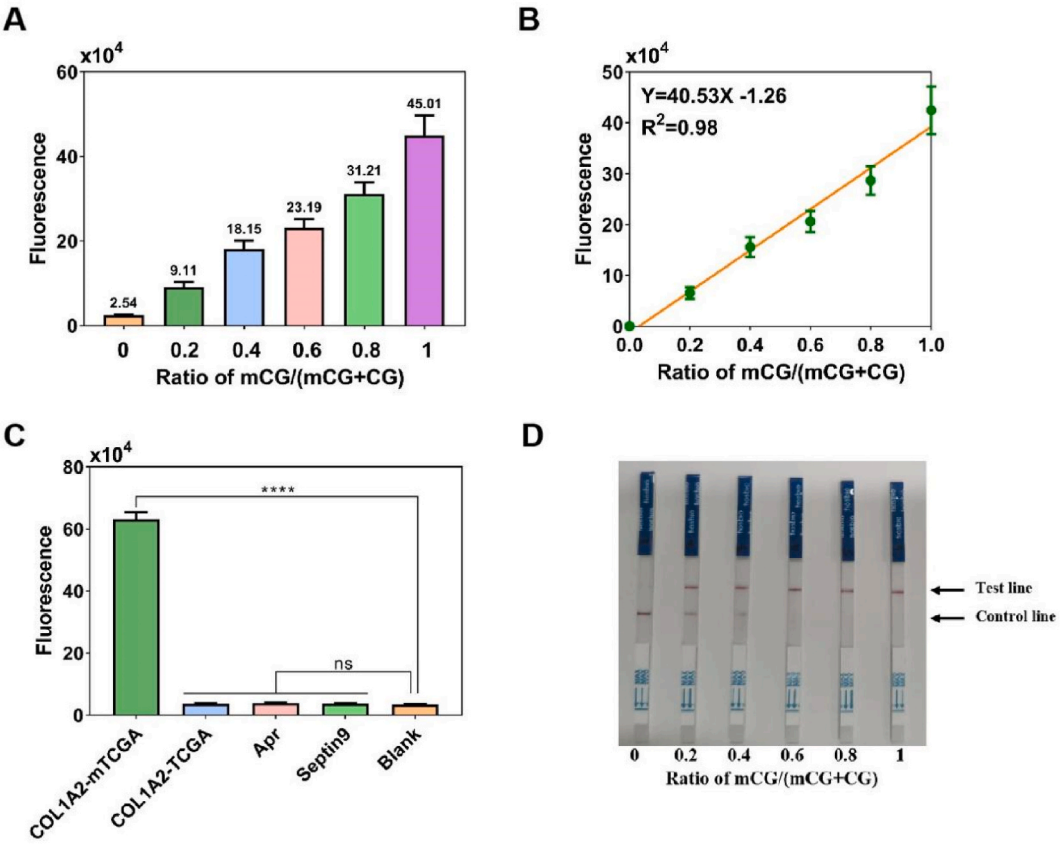

Fig. 2. Performances of meHOLMES.
(A) Fluorescence intensities released by Cas12a *trans*-cleavage were collected at 30 min (mean ± SD, n = 3). (B) Correlation of the fluorescence intensities and the ratios of methylated DNA sequences. The solid line was a linear regression line ($R^2 = 0.98$; n = 3). (C) Fluorescence intensities of the meHOLMES system with different target sequences. Fluorescence intensities at 30 min were shown (mean ± SD, n = 3, two-tailed Student's t-test, ****$p < 0.0001$). (D) Intuitive results using the LFA-assisted meHOLMES system.

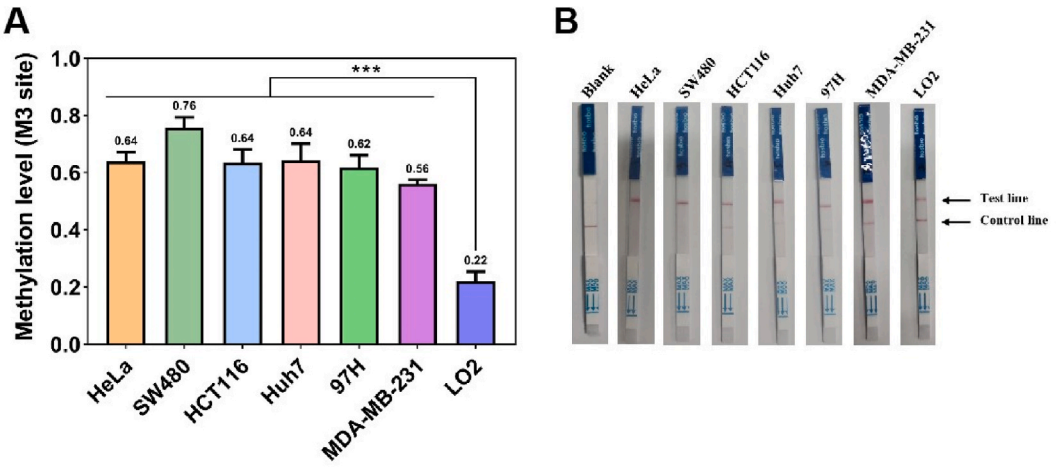

Fig. 3. Measurement of methylation levels at the COL1A2p M3 site in biological samples.
(A) Results of fluorescence-assisted meHOLMES (mean ± SD, n = 3, two-tailed Student's t-test, ***p < 0.001). (B) Results of LFA-assisted meHOLMES.

Data availability

Data will be made available on request.

CRediT authorship contribution statement

Songkuan Zhuang: Writing – original draft, Validation, Methodology, Funding acquisition, Data curation, Conceptualization. **Tianshuai Hu:** Validation, Conceptualization. **Xike Zhou:** Resources, Data curation. **Hongzhong Zhou:** Resources, Data curation. **Shiping He:** Resources, Data curation. **Jie Li:** Validation. **Long Qiu:** Validation. **Yuehui Zhang:** Supervision, Funding acquisition. **Yong Xu:** Supervision, Funding acquisition. **Hao Pei:** Writing – review & editing, Supervision, Funding acquisition. **Dayong Gu:** Writing – review & editing, Supervision, Funding acquisition. **Jin Wang:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e24574.

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