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Uracil-Mediated New Photospacer-Adjacent Motif of Cas12a To Realize Visualized DNA Detection at the Single-Copy Level Free from Contamination

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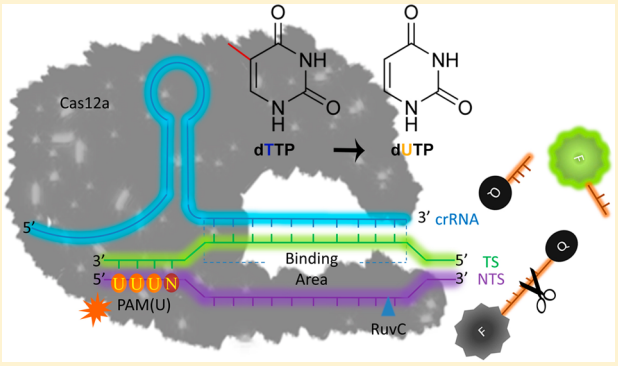
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Supporting Information

ABSTRACT: The CRISPR/Cas12a (cpf1) system was reported to indiscriminately cleave single-stranded DNA after binding with target DNA strands. This usually required the target DNA strands to contain the protospacer-adjacent motif (PAM) sequence of TTTN. Herein, we found Cas12a can also recognize another PAM sequence of UUUN resulting in activation of its ssDNA collateral cleavage effect. To make this finding useful, by combining with LAMP, we first realized CRISPR/Cas12a for directly visualized DNA detection at the single-copy level. By treating with UDG enzyme, we made this system free from residual amplicon contamination, which is a big problem in this field. Thus, an ultrasensitive and anticontaminant DNA detection platform, namely, UDG and LAMP and CRISPR (ULC). This new finding would help us better understand the mechanism of Cas12a and expand its application.



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The clustered regularly interspaced short palindromic repeats (CRISPR) system, which was developed from the antiviral defense of prokaryote, has been employed as a programmable and highly specific genome editing tool for the past few years.[1,2] The CRISPR-associated protein (CRISPR-Cas) family is getting larger since more and more new types of Cas enzymes are being discovered. Because of the two catalytic domains, RuvC and HNH, these proteins showed accurate double-stranded DNA (dsDNA) cleavage activity.[3,4] Researchers have already successfully employed the CRISPR system for single-cell imaging, single-nucleotide variation analysis, and DNA/RNA sensing, etc.[5−7] They demonstrated the great capabilities and promising prospects of the CRISPR system in the field of molecular diagnosis and biosensing. CRISPR-Cas12a (cpf1), which belongs to the class 2 type V-A CRISPR-Cas system, was reported to cleave dsDNA by utilizing a single RuvC catalytic domain under the guidance of the guide RNA.[8−11] Cas12a recognizes a unique protospacer-adjacent motif (PAM) sequence of TTTN which is different from Cas9.[11] In addition to the common dsDNA cutting activity, on the basis of Doudna's report, Cas12a enzyme was found to indiscriminately cleave single-stranded DNA (ssDNA) after binding with target DNA strands in the system.[12,13] This effect is defined as collateral cleavage on nontargeted ssDNA, which makes Cas12a enzyme have a great potential in target DNA analysis.[13,14] However, according to the existing reports using CRISPR/Cas for DNA detection, there are still defects such as amplicon contamination, low sensitivity, and time consumption.[15,16] These shortcomings are supposed to be optimized. For this purpose, we further explored the Cas12a enzyme and found that it could recognize another new PAM (UUUN) in addition to the widely reported old PAM (TTTN). This feature helps us make an improvement on the flaws mentioned above and realize a highly sensitive and visualized DNA diagnosis.

Nucleic acid amplification-based assays such as PCR and LAMP have been widely used for DNA sensing.[17−19] However, amplicon contamination is a big problem in this field. Unexpected tube opening, test strip detection, or performing gel electrophoresis experiments may all cause contamination of the amplicon, which leads to a false positive interference in subsequent experiments. At present, no thorough anticontamination measures have been developed. So, it is of great sense to optimize existing methods to conquer this problem.

Uracil usually exists in RNA which binds to adenine via two hydrogen bonds. It may also occur in DNA due to either cytosine deamination or thymine (T) replacement incorporation.[20] Besides cytosine deamination, uracil can accumulate

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in DNA if dTTP biosynthesis is disturbed. Abnormally elevated dUTP/dTTP ratios will lead to thymine replacing uracil incorporation since most DNA polymerases do not distinguish between thymine and uracil.[21−25] Uracil is a demethylated form of thymine, and there is only one methyl difference in chemical structure between them (Supporting Information Figure S1A). The uracil-DNA-glycosylase (UDG) enzymes can degrade dsDNA-containing uracil without thymine. Several reports showed that this feature was utilized to prevent contamination of DNA amplicon in nucleic acid amplification assays.[26−28] However, the digestion process may certainly make the whole workflow of nucleic acid testing tedious.

Herein, by successfully combining the CRISPR and UDG system with loop-mediated isothermal amplification (LAMP), we developed this DNA sensing platform named ULC (UDG and LAMP and CRISPR). The whole DNA sensing process was free from amplicon contamination and bulky machines.

MATERIALS AND METHODS

Preparation of Primers, Probe, and DNA Template. LAMP primers and probe were synthesized commercially (Sangon Biotech Co., Ltd., Shanghai, China). RNA was synthesized commercially (Sunya Biotech Co., Ltd., Shanghai, China). Las DNA template for sensitivity evaluation was from a pUC57 plasmid synthesized commercially (Sangon Biotech). The DNA template was extracted from the plasmid. The extraction was performed with a 10-fold gradient dilution for sensitivity evaluation. For the real sample detection, the DNA templates extracted were from a half-inch by a half-inch leaf mid-rib region of 60 diseased leaves by the TIANamo Genomic DNA Kit (TIANGEN Biotech (Beijing) Co., Ltd.). Specific sequences of all of the oligonucleotides can be found in the Supporting Information.

Normal LAMP and Uracil−LAMP Assay. LAMP was performed in a total of 25 μL of reaction mixture containing 1.6 μM FIP and BIP, 0.2 μM F3 and B3, 0.4 μM LF and LB, 16 units of Bst DNA polymerase large fragment (New England Biolabs, Inc., Ipswich, MA, USA), 1.4 mM dNTPs (0.35 mM dATP, 0.35 mM dTTP, 0.35 mM dGTP, 0.35 mM dCTP), 2.5 μL of 10× Thermopol reaction buffer (New England Biolabs), 4.0 mM $MgSO_4$, 0.8 M betaine, 2.0 μM SYTO 9 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 2.5 μL of target DNA. The reaction tube was incubated at 63 °C for 40 min via a QuantStudio 3 real-time PCR system (Thermo Fisher). Uracil−LAMP was performed in a total of 25 μL of reaction mixture containing 1.6 μM FIP and BIP, 0.2 μM F3 and B3, 0.4 μM LF and LB, 16 units of Bst 2.0 DNA polymerase (New England Biolabs), 1.4 mM dUTPs (0.35 mM dATP, 0.35 mM dUTP, 0.35 mM dGTP, 0.35 mM dCTP), 1× isothermol reaction buffer (New England Biolabs), 4.0 mM $MgSO_4$, 0.8 M betaine, 2.0 μM SYTO 9 (Thermo Fisher), and 2.5 μL of target DNA. The reaction tube was incubated at 63 °C for 40 min via the QuantStudio 3 real-time PCR system (Thermo Fisher). The fluorescence value was recorded every 30 s.

CRISPR/Cas12 Introduced Uracil−LAMP and Visualized Diagnosis. UDG digestion was performed in a total of 25 μL of reaction mixture containing 1× Taq reaction buffer (New England Biolabs), 1 unit of uracil DNA glycosylase (New England Biolabs), 2.0 μM SYTO 9 dye, and 10 μL amplicons. The reaction tube was incubated at 37 °C for 30 min via the QuantStudio 3 real-time PCR system (Thermo Fisher). CRISPR reaction was performed in a total of 20 μL of reaction mixture containing 1× NEB Buffer 2.1 (New England Biolabs), 0.015 μM Cas12a enzyme (New England Biolabs), 0.6 μM crRNA, 2.5 μM probe, 0.4 U of recombinant RNase inhibitor (Takara Biomedical Technology (Beijing) Co., Ltd.), and 2 μL of target DNA. The reaction tube was incubated at 37 °C for 5 min. A 25 μL aliquot of uracil−LAMP reaction mixture was added at the bottom of the tube, covered with 20 μL of mineral oil. Then, 20 μL of CRISPR reagent was added inside the tube cover. The CRISPR reagent was separated from the high-temperature zone at the bottom by a section of the air column and oil phase (Figure S4). After a 40 min LAMP reaction, the CRISPR-Cas12a system inside the tube cover was mixed with the LAMP reaction product by centrifugation. And then the CRISPR reaction was carried out at 37 °C for 5 min. Reaction tubes were observed with a homemade tiny device consisting of two filters (470 and 530 nm), one LED (480 nm), one heating module, one reaction chamber, and one observation chamber (video integrated). This device can provide the proper temperature for the LC or ULC assay and allows us to observe or record the reaction situation in real time. A simple workflow of our sensing work is described in Figure S3.

RESULTS AND DISCUSSION

New U-Rich PAM Analysis of Cas12a Enzyme and Visual DNA Detection Strategy. Usually, uracil only exists in RNA. Here, to verify whether the Cas12a enzyme could recognize U-rich DNA, dUTP was used as the amplification reagent instead of dTTP in LAMP assay. The real-time curve of normal LAMP was shown to have a 5 min lead ahead of the uracil−LAMP. After about 30 min of amplification, uracil−LAMP also reached the plateau, and the amounts of amplicons by these two LAMP assays were comparable in terms of fluorescence intensity (Figure S1A). The amplicons were verified by gel electrophoresis (Figure. S1B). Then, we specifically designed a crRNA and used a single-stranded fluorescent probe as the indicator to prove whether this U-rich dsDNA can be specifically recognized and whether the collateral cleavage effect of Cas12a enzyme will be activated. These probes were supposed to be cleaved by Cas12a enzyme and release fluorescence, which can be recorded in real-time. The specific sequence of crRNA can be found in the Supporting Information.

And then the CRISPR reaction was carried out at 37 °C for 5 min. The real-time fluorescence values were recorded every 30 s. We tested the fluorescence changes of the CRISPR reaction with different concentrations of Cas12a enzyme (0.05−0.5 μM), crRNA (0.6−1.5 μM), and probe (1.0−5.0 μM). As shown in Figure 2A, under our modified condition, in the positive group, the real-time fluorescence accumulated rapidly, reaching the plateau only in about 3 min. However, the fluorescence value of the negative sample group seemed to have no change. Theoretically, the generation of the fluorescent signal was only possible from the broken single-stranded probe. That is to say, Cas12a enzyme did recognize this new PAM, namely, UUUN (Figure 1). And the collateral cleavage function was still available. We simultaneously videoed the live pictures listed along the real-time curve (Figure 2A). As can be seen from about 1.5 min, the positive group and the negative group gradually produced significant brightness differences. Further, to make this difference of brightness more apparent, we doubled the concentration of the

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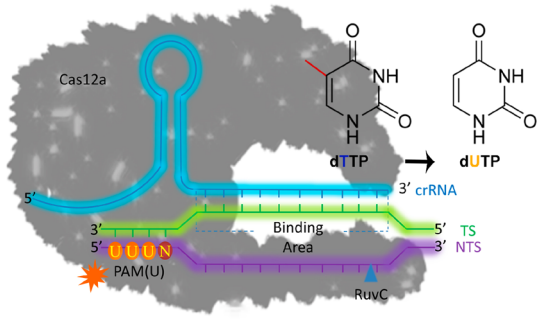


Figure 1. Scheme of the uracil-mediated new PAM sequence (UUUN) of Cas12a enzyme.

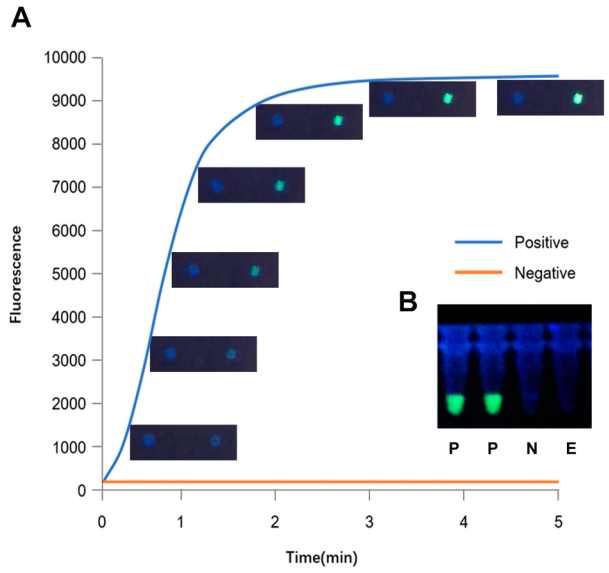


Figure 2. (A) Real-time fluorescence (CFU) record of the positive (blue) group and negative (orange) group during CRISPR reaction. Real-time images of the two groups observed by naked eye were listed along the real-time curve. (B) End point image of two positive templates "P", one negative template "N", and one empty tube "E" after the 5 min CRISPR reaction.

single-stranded probe. As shown in Figure 2B, four reaction tubes represented for two experimental groups (positive template), one control group (negative template) and one empty tube (set for background brightness reference). The brightness of the two positive tubes was significantly higher than that of the negative group and the empty one. This apparent difference was sufficient for visual detection under normal light condition.

It is worth noting that the working temperature of the LAMP reaction is 63 °C, while the optimal temperature of the Cas12a enzyme is 37 °C. In order to prevent the Cas12a enzyme from being deactivated at a high temperature, we added the LAMP reaction mixture at the bottom of the tube and then added the CRISPR reagents inside the tube cover. By this separation, we realized coexistence of the CRISPR reagent and LAMP reaction mixture at an inappropriate temperature for Cas12a enzyme. The specific layer situation is described in Figure S4. After a 40 min LAMP reaction, the CRISPR reagents inside the tube cover were then mixed with the LAMP reaction product by centrifugation. The further CRISPR reaction was carried at 37 °C. And the results can be directly observed by the naked eye. In this way, the LAMP and CRISPR (LC) were successfully coupled.

Development of UDG−LAMP−CRISPR Platform for Anticontamination. Considering that, in the LAMP assay, amplicon contamination is very troublesome, it is of great sense to make a modification. Since we have validated that Cas12a enzyme can also recognize this U-rich PAM, namely, UUUN, resulting in activation of its ssDNA collateral cleavage function. Further, we intended to use the UDG enzyme to degrade the U-rich amplicons to make the whole process free from contamination. It is known that uracil-containing DNA can be digested by UDG enzyme utilizing each uracil as the cleavage site.[26] Here, in this LAMP and CRISPR system described above, we have replaced all thymine with uracil by using dUTP instead of dTTP as the amplification reagent. In this case, the amplicons were all U-rich and they could be digested which can prevent the contamination of residual amplicons. However, the normal DNA templates without uracil would not be degraded. Here, the Antarctic Thermolabile UDG enzyme which is sensitive to heat and can be rapidly and completely inactivated at a temperature above 50 °C was employed for the digestion.[28] To prove the feasibility of degradation by UDG enzyme, first, we used the diluted U-rich amplicons as the target and treated them with the UDG enzyme at 37 °C for 30 min. Then, the temperature was raised to 63 °C for the 40 min LAMP reaction. During this time, the UDG enzyme would be completely inactivated at such a higher temperature. The control group was not treated with UDG enzyme. The time threshold (T_t) value (min) means the time that fluorescence reached the threshold. After a 40 min LAMP amplification, the untreated control group showed a curve with a T_t value of about 21.3. However, the treated experimental group could not perform a good amplification any more (Figure S2). In this way, the anticontamination strategy by UDG enzyme was successfully coupled with the LC assay. Thus, a complete UDG and LAMP and CRISPR (ULC) platform for visual DNA analysis was basically built up.

Then, we further evaluated the detection limit of this ULC platform. Plasmid DNA was performed with a 10-fold gradient dilution as the DNA template. A brief schematic of sensitivity evaluation was described in Figure 3A. In the normal LAMP group, the DNA templates of each concentration were only amplified by normal LAMP for 40 min. In the ULC group, they were first subjected to the 40 min uracil−LAMP, then following with a 5 min CRISPR reaction. Three repeated parallel reaction tubes were set at each concentration, and the average fluorescence value at the end of the reaction was recorded. As shown in Figure 3B,C, in the experiment group, the normal LAMP assay could only detect the sample with an initial concentration of about 1,000 copies. As for the lower initial concentration (<1,000 copies), it showed almost no accumulation of fluorescent signals, while, in the ULC group, as is shown in Figure 3C, the detection limit was lower than 10 copies, which was 100 times more sensitive than the normal LAMP assay. Also, we recorded the brightness at the end of the reaction of each sample. It can be seen that when the initial template concentration decreased to the single-copy level (<10 copies), the tube still showed visible brightness. Compared to the normal LAMP, the sensitivity could be improved to the single-copy level. This feature provides a great impact on ultrasensitive DNA detection. As we thought, after LAMP amplification, each amplicon in the system can be specifically

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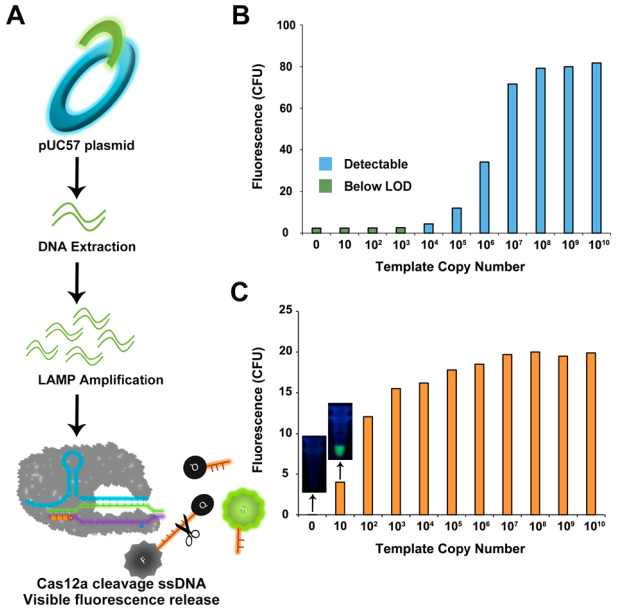


Figure 3. (A) Scheme of UDG−LAMP−CRISPR (ULC) platform for its sensitivity evaluation. (B) Normal LAMP reaction showed a detection limit of about 1,000 copies (blue), and the lower concentrations were below its LOD (green). (C) LAMP−CRISPR reached a detection limit of less than 10 copies, and the brightness was apparently visible for the single-copy template. For the sample without target DNA, there is no signal.

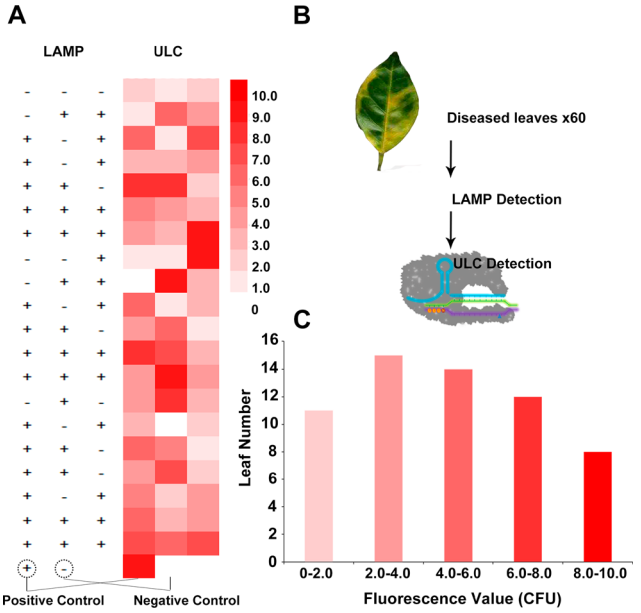


Figure 4. (A) Diagnosis of 60 diseased, one positive control and one negative control by normal LAMP (left) and ULC (right). Heatmap of ULC represents mean fluorescence values. (B) Scheme of 60 leaf samples detected by normal LAP and ULC. (C) A statistical distribution of the leaf samples based on their end point fluorescence values (CFU) after ULC detection.

identified and complementarily paired with guide RNA resulting in the activation of the single-strand cutting function of Cas12a enzyme. Each of the activated Cas12a enzymes can lead to a large number of fluorescent probes to be cut, thus greatly enhancing the signal. And as a result, the sensitivity was increased for detecting the single-copy level of DNA.

Real Sample Detection by ULC Platform. Then, we considered employing the ULC platform to detect real samples for evaluating its performance. Citrus huanglongbing (HLB) is known as the most destructive disease of citrus worldwide. *Candidatus* Liberibacter *asiaticus* (Las) is described as being associated with citrus HLB.[29,30] 60 diseased leaves infected with Las from 60 different citrus trees were employed as our samples. All of these samples have been tested with standard PCR assay described in our previous work.[31,32] A brief workflow is described in Figure 4B. The DNA extracted from a half-inch by a half-inch leaf mid-rib region was used as the template both for the experimental group with ULC assay and control group with normal LAMP. As for the experimental group, we plotted the fluorescence heat map of these 60 leaves based on the fluorescence values at the end of the ULC assay. These two results are shown in Figure 4A. From the results of normal LAMP, the detection rate was 70% (42/60). For the ULC assay, on the basis of the analysis above, once the end point fluorescence value (CFU) reached 3,000 or higher (Figure 2A), significant brightness could be observed by the naked eye. Therefore, here we set the fluorescence value (CFU) of 3,000 as the visual detection limit. Referring to the sensitivity evaluation, the initial template concentration of such a sample can be considered to less than 10 copies. Since there were large randomness and accidental error within this initial concentration range, we repeated evaluation for negative samples for further evaluation. Results showed that none of the negative samples could accumulate more than 2,000 fluorescence value (CFU). In this way, the detection rate of the ULC method was 75% (45/60). We then made a statistical distribution of these samples based on their end point fluorescence. As shown in Figure 4C, this may give a reference for evaluating their infection situation. On the basis of our sensitivity analysis of ULC, nearly 50% of the leaves might carry less than 10 initial copies. About one-third of the leaves were supposed to carry 10−100 bacteria. These conclusions were just purely analyzed from the perspective of experiment data, and there should still be a gap between them and the actual situation. And of course, these results demonstrate that the ULC method for DNA detection was indeed more sensitive than the normal LAMP method. And it can provide us with a visualized and anticontamination way for DNA sensing.

CONCLUSIONS

In summary, starting from the conjecture about uracil, we prove that Cas12a can recognize another new PAM, namely, UUUN, other than the widely reported TTTN. On the basis of this new finding, by combining with UDG enzyme and uracil−LAMP, we developed this new DNA detection platform named UDG-LAMP-CRISPR (ULC). This platform was capable of realizing ultrasensitive visualized DNA detection at a single-copy level and free from contamination. This greatly reduces the need for bulky machines and solves the problem of very disturbing amplicon contamination. Besides, this newly discovered PAM sequence of Cas12a enzyme can greatly improve its applicability and help us better understand its mechanism. The development of this visualized molecular diagnostic platform was supposed to bring us new ideas for wider application of CRISPR/Cas system, and it can also be an inspiration for launching a revolution on optimizing traditional molecular diagnosis methods.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b02554.

Specific sequences of all the oligonucleotides used in this work (Table S1); real-time curves and gel image of normal LAMP and uracil−LAMP (Figure S1); fluorescence records in UDG-based anticontaminant experiment (Figure S2); ULC platform schematic (Figure S3); specific layer situation of the reaction tube for ULC (Figure S4) (PDF)

Video S1 showing real-time recording of the visualized detection process of ULC (MP4)

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Notes

The authors declare no competing financial interest.

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