Electronic Supplementary Information

Simultaneous detection of CaMV35S and T-nos utilizing CRISPR/Cas12a and Cas13a with Multiplex-PCR (MPT-Cas12a/13a)

Gaihua Cao[a], Jiangbo Dong[a], Xiaolong Chen[a], Peng Lu[b], Yifan Xiong[a], Lan Peng[c], Jiawei Li[a,b*], Danqun Huo[a,d*], Changjun Hou[a*]

[a] Key Laboratory for Biorheological Science and Technology of Ministry of Education, State and Local Joint Engineering Laboratory for Vascular Implants, Bioengineering College of Chongqing University, Chongqing 400044, PR China

[b] Chongqing University Three Gorges Hospital, Chongqing, 404000, PR China

[c] Chongqing Medical and Pharmaceutical College Basic Department, Chongqing, 401331, PR China

[d] Chongqing Key laboratory of Bio-perception & intelligent information Processing, School of microelectronics and Communication Engineering, Chongqing University, Chongqing, 400044, PR China

* Corresponding author. Tel.: +86 23 6511 2673; fax: +86 23 6510 2507.

E-E-mail addresses: houcj@cqu.edu.cn (C. Hou); huodq@cqu.edu.cn (D. Huo); LI_JIAWEI_1993@163.com (Jiawei Li)

S1. EXPERIMENTAL SECTION

S1.1. Reagents and instrumentations.

The PBI121-EGFP plasmid, primers, reporters, and crRNAs in Table S1 and Fig. S1 (Supporting information) were synthesized by Sangon Biotech (Shanghai, China). Lba Cas12a (100 μM), T7 RNA polymerase (50,000 U · mL^{-1}), and Ribonucleotide Solution Mix (NTP, 25 mM) were purchased from New England Biotechnology Co., Ltd (Beijing, China). And Cas13a protein was ordered from Beijing Kesin Biotechnology Co., LTD. PrimeSTAR® Max DNA Polymerase and 2×Premix Ex Taq (Probe qPCR) was obtained from TaKaRa. Plant Genome DNA Extraction Kit (DP305) and DNase/RNase-free water were purchased from TIANGEN Biotechnology Co., Ltd (Beijing, China). The 6×Loading buffer and 5×TBE buffer were provided by Sangon Biotech (Shanghai, China). The fluorescence spectrum was detected by LS-55 spectrophoto fluorometer (P. E. USA). The plants of *Solanum lycopersiucum Mill. var. Ailsa Craig.* contained wild type and transgenic type, which were preserved in our laboratory.

S1.2. The M-PCR of the CaMV35S and T-nos.

M-PCR for CaMV35S and T-nos was performed in 20 μL system, containing 10 μL PrimeSTAR® Max DNA Polymerase, 250 nM CaMV35S-F, CaMV35S-R, T-nos-F, and T-nos-R primers, 2 μL plasmid, and 4 μL DNase/RNase-free water. The PCR process was carried out at 95 ℃ for 5 min, followed by 30 cycles: 95 ℃ for 30 s, 53℃ for 15 s and 72 ℃ for 15 s, and finally extended at 72 ℃ for 10 min. The PCR products were detected by 2% agarose gel electrophoresis at 120 V for 20 min.

When CaMV35S or T-nos was detected alone, only corresponding primers were added and carried out the same PCR process.

S1.3. The transcription of the T-nos.

The mixture consisted of 1×RNA polymerase buffer, 500 U T7 RNA polymerase, 4 μL PCR products, and 2.5 mM NTP, which was incubated at 37 ℃ for 1 h.

S1.4. Detection by the MPT-Cas12a/13a for CaMV35S and T-nos.

The terminal signal detection of MPT-Cas12a/13a included 100 nM Cas12a and Cas13a, 100 nM crRNA-CaMV35S and crRNA-T-nos, 1×NEB buffer 2.1, 500 nM of DNA reporters, and 250 nM RNA reporters. It was added to the above system to incubate at 37 ℃ for 30 min. And then, the 78 μL of DNase/RNase-free water was added to the system for measurement by LS-55 spectrophoto fluorometer with an excitation wavelength of 480 nm. Therefore, Cas12a recognized DNA-CaMV35S to produce yellow fluorescence with a peak at 556 nm and Cas13a recognized RNA-T-nos to generate green fluorescence with a peak at 520 nm.

When CaMV35S was tested separately, the mixture containing 100 nM Cas12a and crRNA-CaMV35S, 1×NEB buffer 2.1, 4 μL PCR products and 500 nM DNA reporters. As T-nos was detected separately, the mixture included 100 nM Cas13a and crRNA-T-nos, 1×NEB buffer 2.1, 10 μL products of transcription and 250 nM RNA reporters, which all was kept at 37 ℃ for 30 min.

S1.5. Agarose gel electrophoresis.

Agarose gel electrophoresis was employed as an auxiliary proof to verify the products of M-PCR and transcription. The amplified products were mixed with 6×

loading buffer and added to 2% agarose gel. It carried out in 1×TBE at 120V for 20 min. An Azure Biosystems C150 (USA) was used to observe the gel images.

S1.6. Application of the MPT-Cas12a/13a to practical samples.

The capability of MPT-Cas12a/13a was evaluated using genomic DNA from the plants of Solanum lycopersiucum Mill. var. Ailsa Craig. DNA was extracted and purified from tomato leaves using Plant Genome DNA Extraction Kit. Two microliter DNA was detected by MPT-Cas12a/13a. Then, the existence of transgenic elements was determined by fluorescence color and intensity. In addition, 2% agarose gel electrophoresis was served as an auxiliary tool to verify the results.

S1.7. The qPCR for detecting the practical sample

The qPCR was performed on a QuantStudio 6 Flex instrument (Thermo Fisher Scientific) equipped with a 384-well block. The reaction consisted of a 12.5 μL 2×Premix Ex Taq (Probe qPCR), 400 nM of primers-qPCR, 400 nM of the probe, and 2 μL of the sample. The amplification program was set to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 1 min.

S2. Results and discussion

S2.1 The results of agarose gel electrophoresis for verifying the feasibility of the primers for CaMV35S and T-nos.

In theory, In the presence of CaMV35S and T-nos primers, 143 bp and 171 bp products were generated by PCR, respectively. And DNA-T-nos as the template for the transcription, produces a large number of 151 bp transcripts. In fact, Fig. 1(b) showed that the product size of CaMV35S was the same as the theory in channel 1. In

channel 3, there were two bands, which were DNA products and RNA products of T-nos. When two pairs of targets coexist, there were 3 bands in channel 5, which were T-nos-DNA, T-nos-RNA and CaMV35S-DNA products from top to bottom. No corresponding bands were present in the control group. This manifested that two sets of primers can be well applied in the amplification of CaMV35S and T-nos by PCR.

S2.2 Exploration of the interference between two signals of CaMV35S and T-nos

No cross between signals and non-interference between targets are necessary conditions for simultaneous detection. It has been proved that there was no crossover and interference between the Cas12a system of the CaMV35S and the Cas13a system of the T-nos in the feasibility section. Moreover, the excitation wavelengths for HEX and FAM were the same at 480 nm, but their emission wavelengths were different, 556 and 520 nm. The fluorescence spectra contained the peaks at 520 nm and 556 nm were obtained, when the slit for excitation and emission were set at 12 nm and 10 nm, respectively. Furthermore, the interference between the two signals of CaMV35S and T-nos was verified in Fig. S5. When the concentration of T-nos was fixed at 5×10^4 copies and the concentration of CaMV35S was increased, the fluorescence intensity at 520 nm did not change, while the fluorescence intensity at 556 nm kept increasing in Figure S5(a). Similarly, when the concentration of CaMV35S was fixed at 5×10^5 copies and the concentration of T-nos was increased, the fluorescence intensity at 520 nm increased, while the intensity at 556 nm did not change in Figure S5(b). These results demonstrated that the fluorescence signals corresponding to CaMV35S and T-nos did not interfere with each other.

S2.3 Optimizing reaction conditions

Optimal conditions were the key to ensure that Cas12a/Cas13a systems play a maximum role. Firstly, the Cas12a response system was established according to previously reported studies [1]. The shearing time of Cas12a was worth exploring because it was closely related to the occurrence of false positives and false negatives. Fig. S6(a) showed that the Δ F (Δ F was defined as the difference of fluorescence intensity between the test group and the control group) increased gradually over time. The Δ F reached the maximum at 30 min. Too long incubation time of the Cas12a could cause nonspecific cleavage, so 30 min was served as the optimum time of the Cas12a system. Next, transcription was a prerequisite for the Cas13a system to function. Fig. S6(b) showed the Δ F increased with the extension of transcription time and reached the maximum at 40 min. This demonstrated that the CRISPR/Cas13a system was saturated with transcripts produced within 40 min. So 40 min was the optimal time for transcription. Furthermore, the concentration of RNA reporters was the key factor for the Cas13a system. In Fig. S6(c), the Δ F was strongest at 250 nM. Too many RNA reporters had steric hindrance to Cas13a, which decreased the trans-cleavage activity. While, it was easy to saturate with few RNA reporters, leading to reducing the detection range. Therefore, 250 nM of RNA reporters was employed as the optimal concentration for subsequent reaction. Moreover, the cutting time of Cas13a was an important parameter. If the shearing time of Cas13a was too short, the fluorescence signal was not obvious enough. If the cutting time is too long, the non-specific cutting of Cas13a caused the background interference. In Fig. S6(d), the Δ F

increased over time and reached the maximum at 30 min, so 30 min was the best reaction time of Cas13a. What's more, the ratio of Cas13a to Cas12a was a key parameter in dual-target detection. Fig. S6(e) showed when the concentration ratio was 50 nM:100 nM, the signal at 520 nm was in a weak position. Because the transcription was lagging and the content of Cas13a was low, the fluorescence intensity was relatively weak. When the ratio reached 50 nM: 200 nM, the fluorescence intensity of Cas12a at 556 nm slightly weakened, because too much Cas12a in the solution reduced the non-specific ssDNase activity. When it reached 100 nM: 100 nM, the peaks reached the best. This showed that the appropriate stoichiometry was crucial for the reaction. So 100 nM: 100 nM was the best ratio of Cas13a to Cas12a. In addition, the concentration ratio of RNA reporters to DNA reporters was crucial factor. The reporters with different concentrations were used in the experiment, such as 50 nM, 250 nM, and 500 nM. In Fig. S6(f), when the concentration ratio reached 50 nM: 50 nM, the fluorescence at 520 nm and 556 nm were weak. As it reached 250 nM: 250 nM, the fluorescence intensity was strengthened, but the fluorescence peak at 556 nm was not obvious, which indicated that 250 nM of the DNA reporters was relatively few. When the concentration reached 500 nM: 500 nM, the RNA reporters was so many that the trans-cleavage activity of Cas13a was decreased. However, the fluorescence intensity at 556 nm was enhanced, indicating that 500 nM of DNA reporters was more suitable for Cas12a. When the concentration reached 250 nM: 500 nM, the fluorescence intensity at 520 nm and 556 nm was strongest. Therefore, 250 nM: 500 nM was the optimal ratio of

RNA reporters to DNA reporters.

S2.4 Reproducibility and reproducibility of the MPT-Cas12a/13a.

Repeatability and reproducibility were two vital characteristics of strategies in practical applications. The experiments were repeated with the plasmid of concentration 5×10^5 copies for simultaneously detecting CaMV35S and T-nos. As shown in Fig. S7(a), the relative standard deviation (RSD) was 1.33% of T-nos and 1.39% of CaMV35S. The RSD was less than 5%, indicating that the MPT-Cas12a/13a had good reproducibility. In addition, Fig. S7(b) showed the fluorescence intensity was constant by multiple measurements for exploring repeatability. Therefore, the MPT-Cas12a/13a has excellent repeatability for simultaneously detecting CaMV35S and T-nos.

Table S1. The nucleic acid sequence in the study.

Oligo names	**Sequences (5'-3')**
CaMV35s-F	CCTCTGCCGACAGTGGTCCCAAAGATGGACC
CaMV35s-R	CCCTTACGTCAGTGGAGATATCACATCAATCC
T-nos-F	GAAATTAATACGACTCACTATAGG GTGAATCCTGTTGCCGGTCTTGCGATGATTATC
T-nos-R	TCTATCGCGTATTAAATGTATAATTGCGGGAC
CaMV35S-crRNA	AAUUUCUACUAAGUGUAGAUAAGACGUGGUUGGAACGU CUUCU
T-nos-crRNA	GUUUAGACCCCAAACAGGAGGACUAUGUUAAUUAUUAC AUGCUUAACGUAAUUCAA
DNA reporter	HEX-TAT TAT T-BHQ1
RNA reporter	FAM-rUrArUrArU-BHQ1
CaMV35s-qPCR-F	CGTCTACAAAGCAAGTGGATTG
CaMV35s-qPCR-R	TCTTGCGAAGGATAGTGGGATT
CaMV35s-probe	FAM-TCTCCACTGACGTAAGGGATGACGCA-QSY
T-nos-qPCR-F	GTCTTGCGATGATTATCATATAATTTCTG
T-nos-qPCR-R	CGCTATATTTTGTTTTCTATCGCGT
T-nos-probe	JUN-AGATGGGTTTTTATGATTAGAGTCCCGCAA-QSY
Interference 1	CGCACCCAGCAGTTTGGCCCGCCACACTTAGTAGAAATTC CTATAGTGAGTCGTATTAATTTC
Interference 2	ACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGAAGAGA AAGACCGCACCCAGCAGTTTGGCCCGCCCAAAATCTGT
Interference 3	GCGCGGGCCCCCAAAAAAAAAGCGGCCCCCCGCCTCAGC GCGCGGGCCCCCAAAAAAAAAGCGGCCCCCCGCCTCAGC CAGCAGTTTGGCCCGCCCAAAATCTGT
Interference 4	ATTGATACCATGAGCAGTTACGATCTACACTTAGTAGAAA TT CCCTATAGTGAGTCGTATTA

(The F and R referred to the Forward and Reverse primer)

Table S2. The sensitivity of this method was compared with other sensors for the GM assay.

Method	Target	LOD	Ref.
RPA and fluorescent probes	CaMV35S and T-nos	86 and 126.84 copies	[2]
ddPCR	KMD and PLD	9 copies	[3]
Duplex RPA and lateral flow biosensor	CaMV35S and T-nos	10 copies	[4]
LAMP and TaqMan	T-nos	5 copies	[5]
RPA and CRISPR/Cas12a	CaMV35S and T-nos alone	10 copies	[6]
RPA and lateral flow test strip	CaMV35S and T-nos alone	100 copies and 50 copies	[7]
CRISPR/Cas12a, CRISPR/Cas13a, multiplex PCR, and transcription	**CaMV35S and T-nos at the same time**	**13 copies and 11 copies**	**This work**

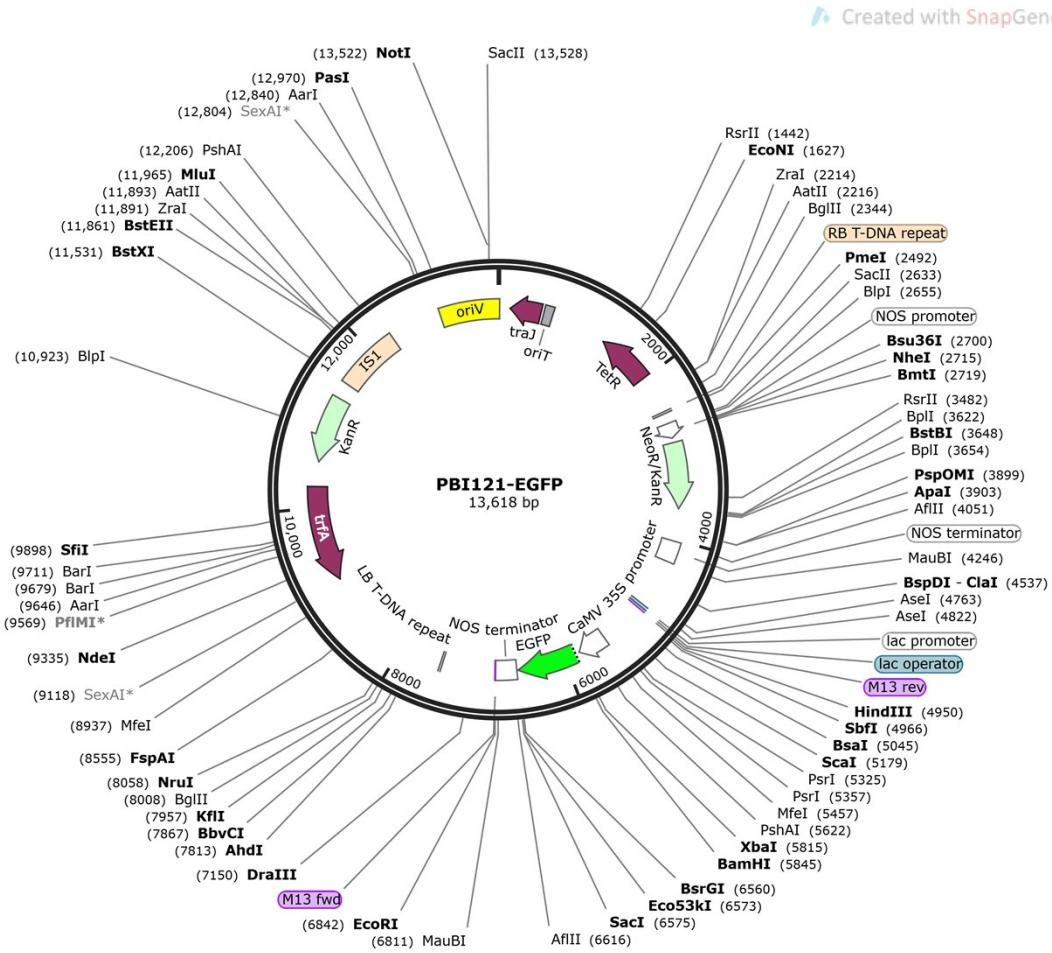

Figure S1. The profile of PBI121-EGFP plasmid.

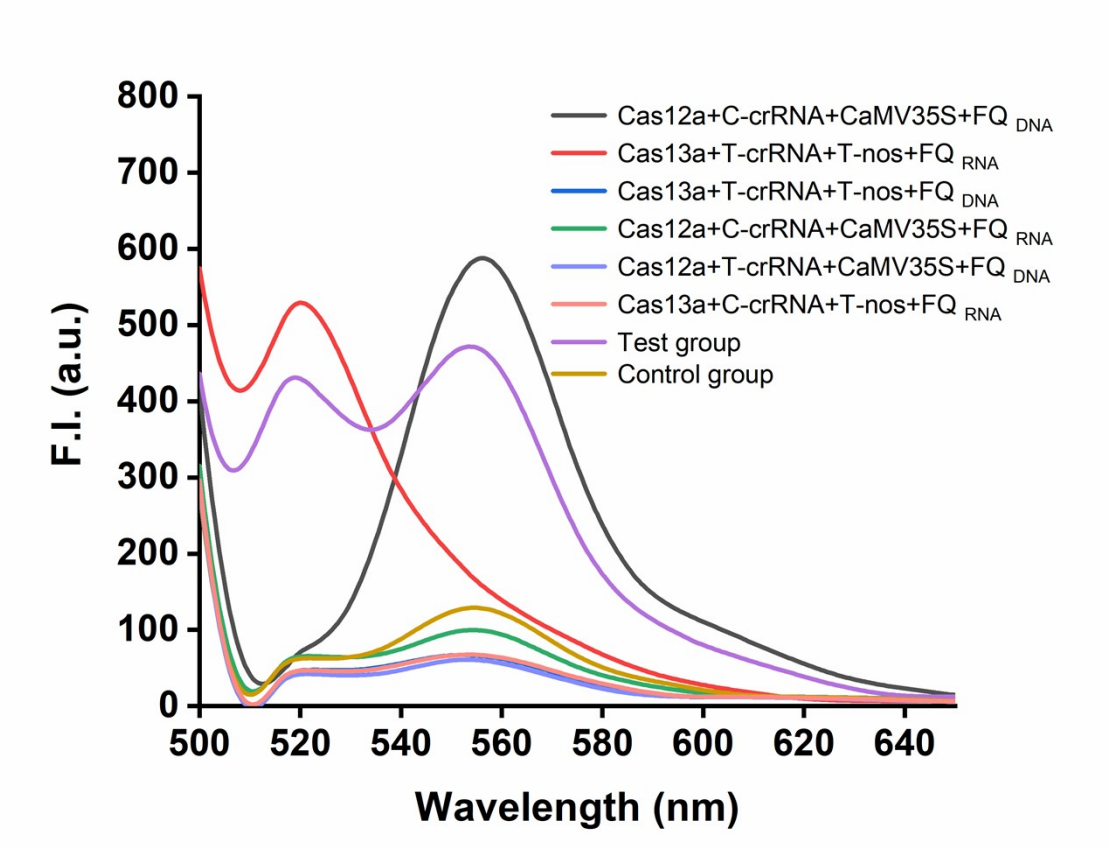

Figure S2. The feasibility of the method was verified. C-crRNA and T-crRNA refer to the crRNA of CaMV35S and crRNA of T-nos. FQ_{DNA} and FQ_{RNA} refer to the DNA reporter and RNA reporter.

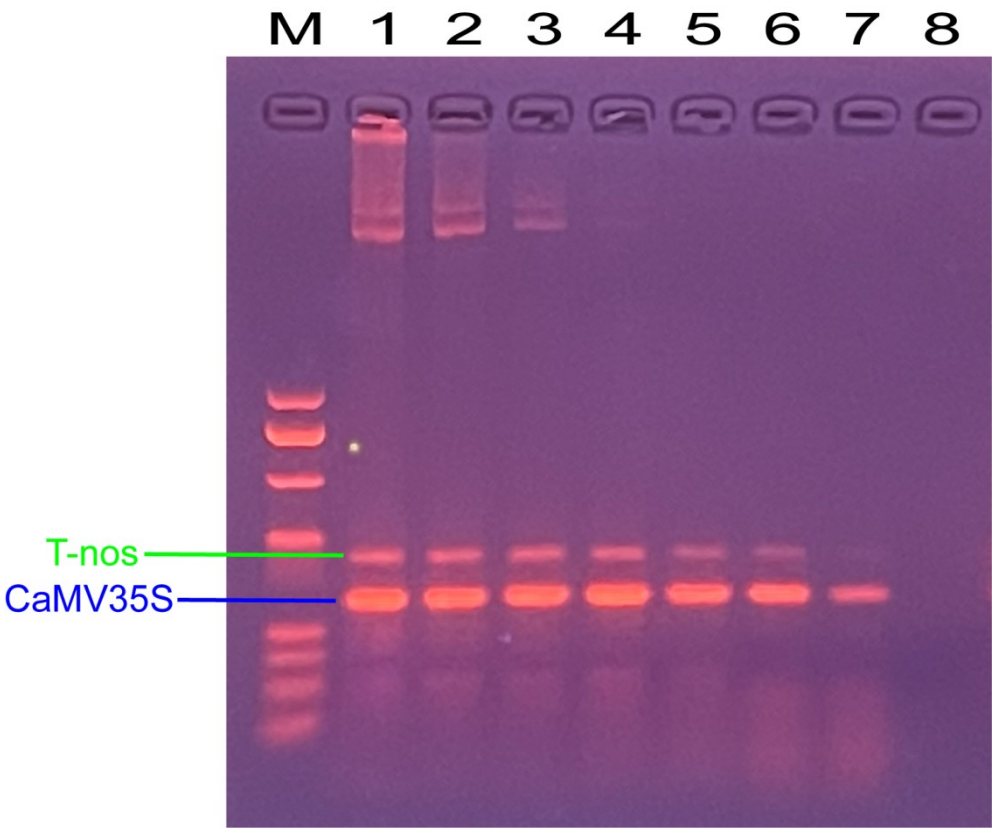

Figure S3. The sensitivity of CaMV35S and T-nos by multiplex PCR was determined by 2% agarose gel electrophoresis. From left to right were Marker, 5×10^8, 5×10^7, 5×10^6, 5×10^5, 5×10^4, 5×10^3, 5×10^2 copies, and the control group.

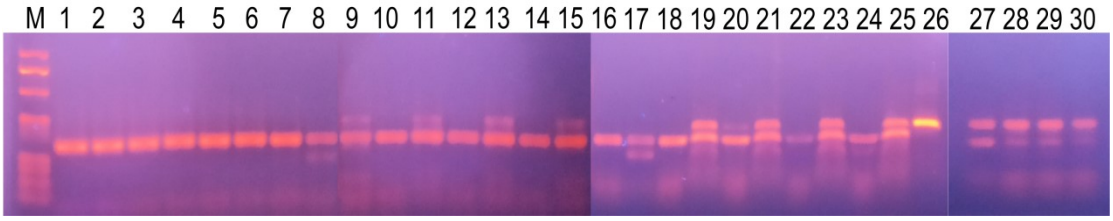

Figure S4. Nucleic acids from actual samples were detected by multiple PCR and 2% agarose gel electrophoresis.

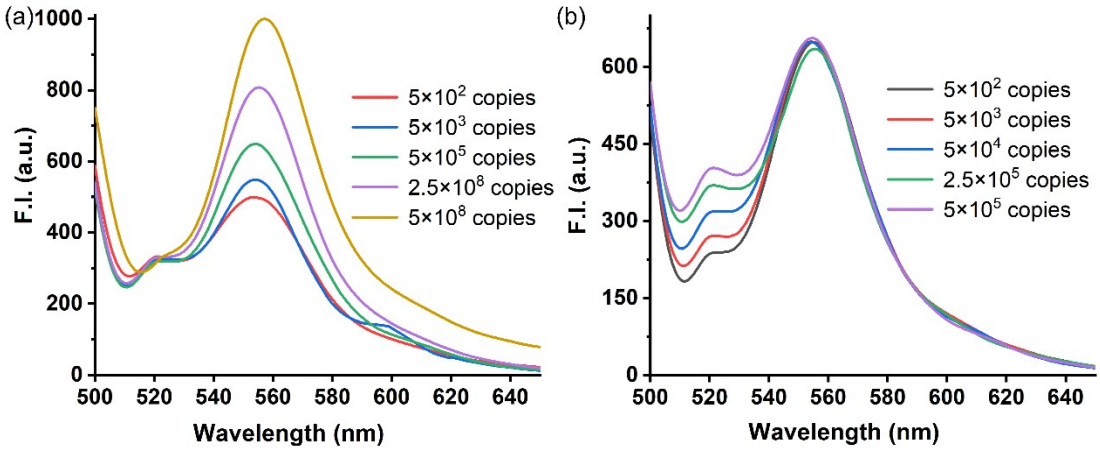

Figure S5. Interference exploration between two signals of CaMV35S and T-nos. (a) the fluorescence spectrum was obtained when the concentration of T-nos was fixed at 5×10^4 copies and the concentration of CaMV35S kept increasing from 5×10^2 to 5×10^8 copies. (b) the fluorescence spectrum was attained with increasing concentration of T-nos from 5×10^2 to 5×10^5 copies and fixed concentration of CaMV35S at 5×10^5 copies.

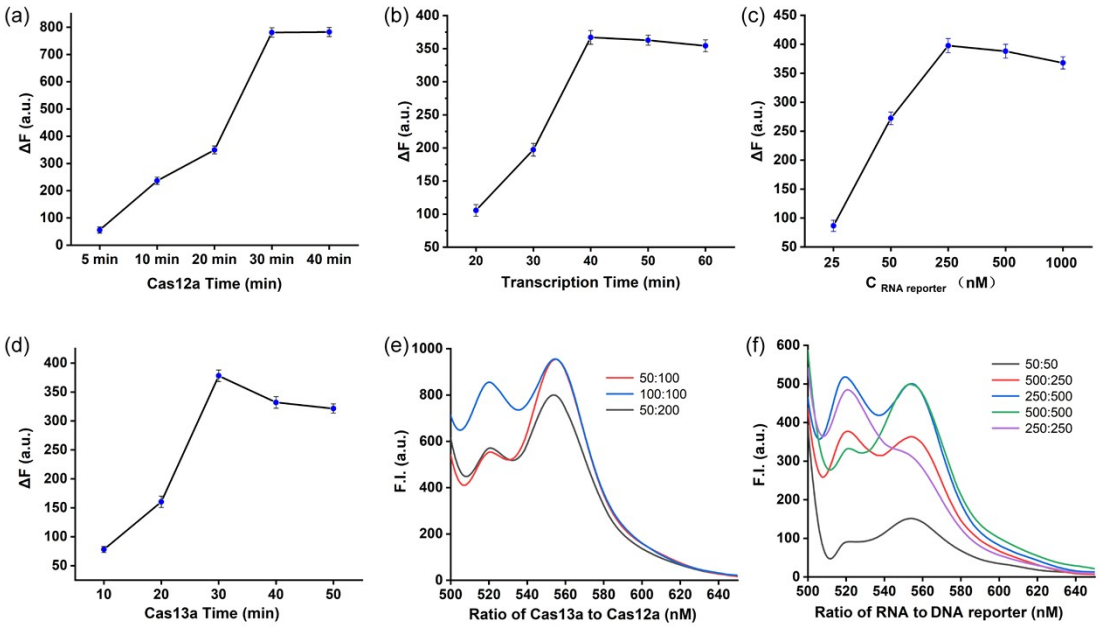

Figure S6. The conditional optimization of the MPT-Cas12a/13a including (a) the shearing time of Cas12a; (b) the transcription time; (c) the concentration of RNA reporters for Cas13a system; (d) the cutting time of Cas13a; (e) the ratio of Cas13a to Cas12a; (f) the ration of RNA reporters to DNA reporters.

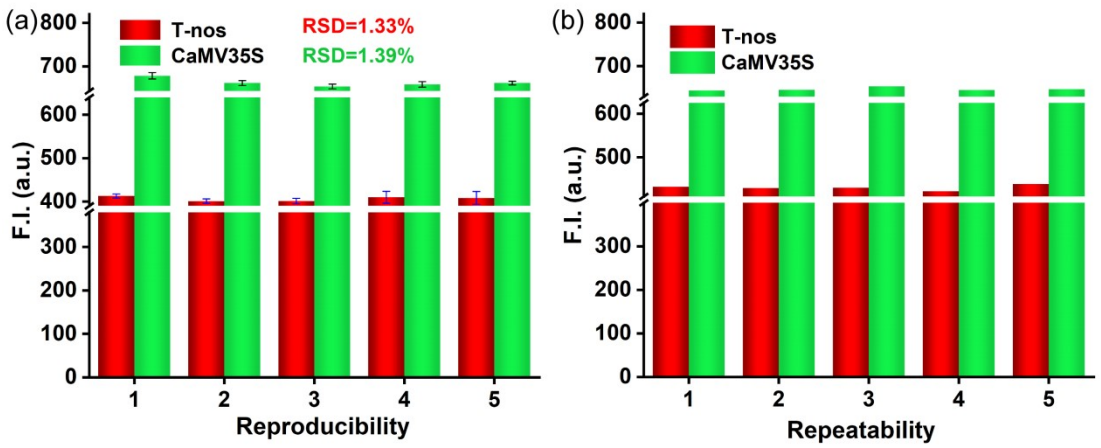

Figure S7. Reproducibility and repeatability of the MPT-Cas12a/13a for simultaneous detection of CaMV35S and T-nos.

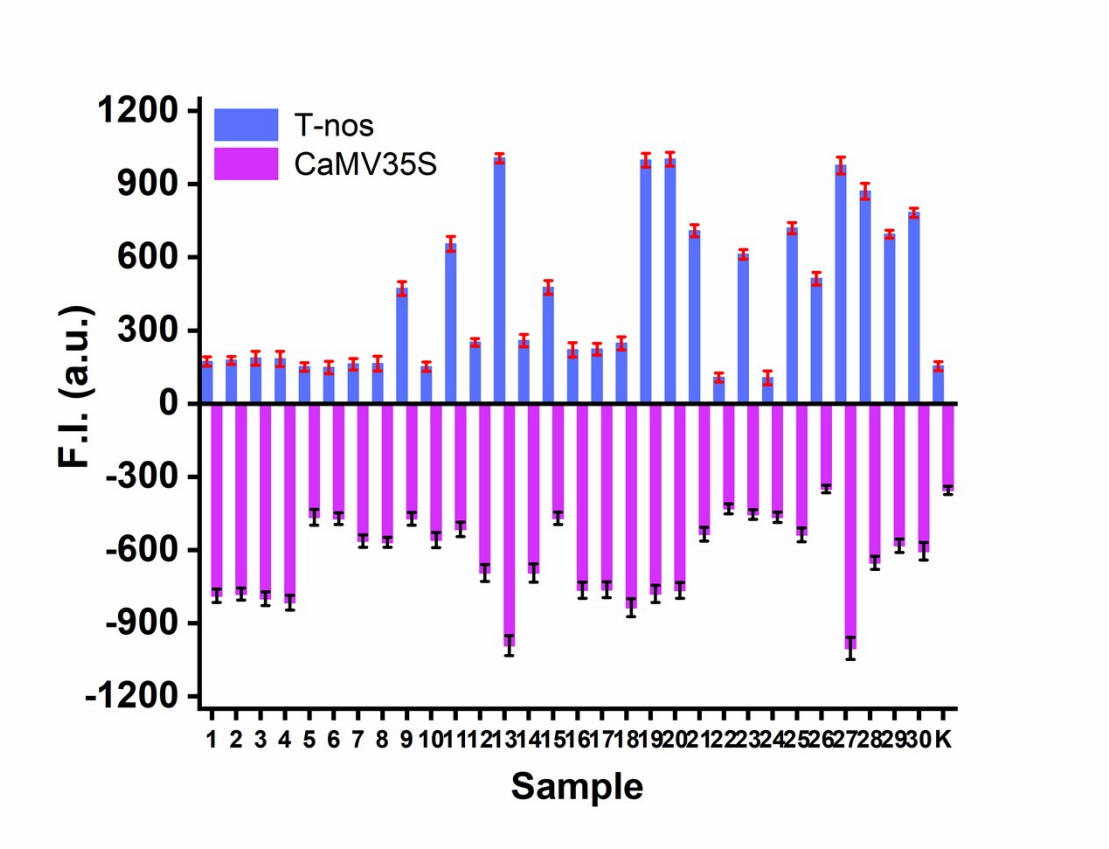

Figure S8. the fluorescence intensity of 30 samples detected by the MPT-Cas12a/13a.

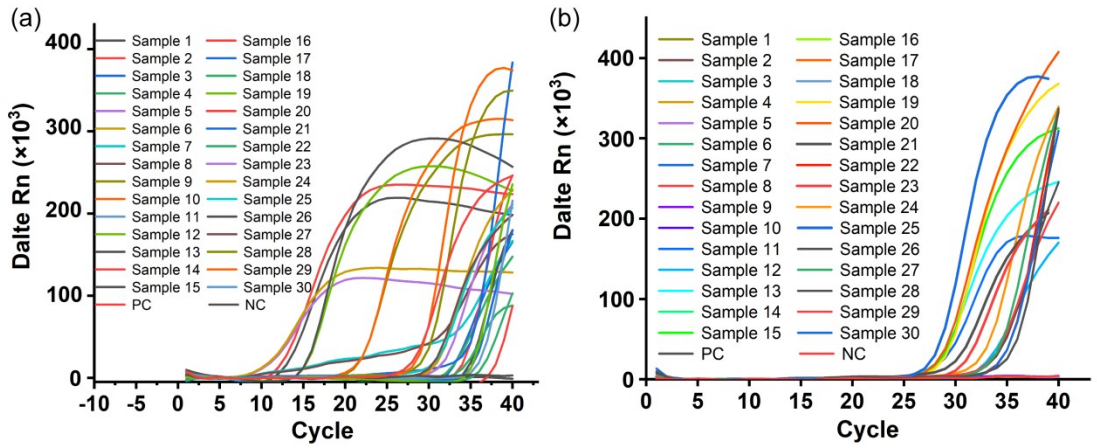

Figure S9. the qPCR results of the 30 samples. (a) the qPCR results for the CaMV35S. (b) the qPCR results for the T-nos.

Reference

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