Supporting Information

Locus-Specific Detection of Pseudouridine with CRISPR-Cas13a

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1. Materials and methods

Materials and chemicals

RNA oligonucleotides used in this work were synthesized from Takara and DNA primers used in this work were purchased from Genecreate, the sequences information were listed in Table S1. All chemicals were purchased from Thermo Scientific unless mentioned otherwise. The reverse transcriptase Superscript III was from Invitrogen™ and Oligo $d(T)_{25}$ Magnetic Beads for mRNA enrichment was from New England biolabs. The Cell Fast miRNA Extraction kit from ABclonal was used to extract and enrich small RNA. The dNTP (N = A, T, C, G) was purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The qPCR mix was purchased from YEASON. The GenCRISPR™ Cas13a (C2c2) was purchased from GenScript. DNA and RNA concentration was quantified by NanoDrop 2000c (Thermo Scientific, USA). The temperature control equipment for reverse transcription and chemical reaction was Thermal Cycler (BIO-RAD) and the Quantitative Real-time PCR is from Roche Diagnostics GmbH. The fluorescence signal was detected by the fluorescence Spectrometer from PerkinElmer. Gel Imaging was monitored with Pharos FX Molecular imager (Bio-Rad, USA). The pH value was measured by Mettler Toledo, FE20- Five Easy™ pH (Mettler Toledo, Switzerland).

CMC labeled Ψ and purification

In the CMC labeling, CMC (Sigma) and synthetic FAM labeled RNA Ψ-ssRNA was used in this experiment, its sequence was seen in Table S1. 500 ng Ψ-ssRNA and 0.5 μl EDTA (50 mM) were mixed and added Nuclease-Free water to 5 μl. After keeping the RNA solution at 80°C for 5 min, transferred it to ice immediately for 5 min. Then, added 90 μl CMC solution (210mg CMC dissolved in 90 μl BEU buffer) into the RNA solution and mixed well, keeping them at 37°C for 1 h. The BEU buffer is composed of 7 M urea, 4 mM EDTA (pH 8.0) and 50 mM Bicine (pH 8.5). After the treatment of CMC, the RNA solution was purified by ice ethanol precipitation method. Afterwards Na_2CO_3 solution (100 mM, pH=10.4) and the RNA solution were mixed in equal volumes (1:1) to remove CMC from U and G residues, then treated the new RNA mixture at 37°C for 3 h[1, 2].After that alkali treatment, we purified the RNA mixture by ice ethanol precipitation again. we used denaturing polyacrylamide gel electrophoresis (urea-PAGE) to segregated Ψ-ssRNA and Ψ-ssRNA-CMC to get purified Ψ-ssRNA-CMC and visualized by the fluorescence of FAM using Molecular Imager® ChemiDocTM XRS+ Imaging System (Bio-Rad) (Fig. S2).

CRISPR-Cas13a system specifically detected Ψ and the controlled trial for Ψ-ssRNA and U-ssRNA with and without Cas13a

4 nM crRNA (200 nM, 1 μl), 50nM quenched fluorescent reporter RNA (10 μM, 2.5 μL) and 4nM of input nucleic acid target RNA (Ψ-ssRNA, Ψ-ssRNA-CMC, U-ssRNA or U-ssRNA-CMC) were incubated together in 7.5 μl H_2O at room temperature for 5 min, respectively. And then 40 nM Cas13a (2 μM, 1 μL), 10 x reaction buffer (40 mM Tris-HCl, 60 mM NaCl, 6 mM $MgCl_2$, pH 7.3, 5 L) and 32 μl H_2O were added, keeping this mixture at 37 °C for 5 min, 65 °C for 10 min[3]. Then the fluorescence signal in the range of 510nm to 600nm of the RNA mixture was detected at excitation wavelength of 494nm by fluorescence analyzer (PerkinElmer) respectively. It is worth mentioning that, to verify the effectiveness of Cas13a, we have set a controlled trial for target Ψ-ssRNA and U-ssRNA with and without Cas13a. The experimental results showed that the group without Cas13a could not produce obvious fluorescence signal value, and the fluorescence intensity of the system with Cas13a was very high, which indicated the effectiveness of Cas13a for the CRISPR-Cas13a system (Fig. S1).

Examining the sensitivity of CRISPR-Cas13a system for Ψ detection

Ψ-ssRNA and Ψ- SSRNA-CMC RNA solutions with concentrations of 4 nM, 3 nM, 2 nM, 1.5nM,500 pM and 200 pM were prepared, respectively. All concentration groups of RNA were incubated with crRNA and reporter RNA at room temperature for 5 min, respectively. And then Cas13a was added keeping this mixture at 37°C for 5 min, 65 °C for 10 min for subsequent fluorescence analysis. The fluorescence signal in the range of 510nm to 600nm of the RNA mixtures were detected at excitation wavelength of 494nm by fluorescence analyzer (PerkinElmer).

Quantitating Ψ in RNA by CRISPR-Cas13a

Ψ-ssRNA-CMC and Ψ-ssRNA were mixed with different molar ratio (0:10, 2:8, 4:6, 6:4, 8:2, 10:0). The mixture RNA was incubated with crRNA and reporter RNA at room temperature for 5 min, respectively. And then Cas13a was added keeping this mixture for at 37°C for 5 min, 65 °C for 10 min for subsequent fluorescence analysis.

Cell culture and RNA extraction

HEK293T cells were cultivated in Dulbecco's modified Eagle Medium (DMEM, high glucose) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Beijing Dingguo Changsheng Biotechnology Co., LTD., GA3502) at 37°C and 5% CO_2. Total RNA was extracted by using TRIzol reagent (Invitrogen, 15596018) according to the manufacturer's instructions. PolyA-mRNA was isolated from total RNA through one round of poly(A)+ selection with Oligo$(dT)_{25}$ magnetic beads (NEB, S1419S).

Detection of Ψ in18S RNA，28S RNA, mRNA, and snRNA with CRISPR-Cas13a system

we chose the position Ψ1248 in 18S RNA, Ψ3938 in 28S, Ψ519 in mRNA and Ψ72 to verify the performance of our method in real RNA samples. The total RNA and mRNA were fragmented by using RNA fragmentation reagent (Thermo Scientfic) at 70°C for 7.5 min. For Ψ519 detection, mRNA was firstly enriched and segregated from total RNA before fragmenting due to its low content in total RNA. To detect Ψ72, small RNA must be extracted and enriched using the Cell Fast miRNA Extraction kit from ABclonal. Then, the fragmented total RNA, fragmented mRNA, and small RNA should be divided into two parts each: one part is labeled with CMC to produce (+) CMC RNA, and the other part remains unlabeled, denoted as (-) CMC RNA. Mixed 1μg (+) CMC RNA or (-) CMC RNA with 1μl crRNA（10 μM）in 12 μl water solution at 65°C for 2 min, transferred them on the ice immediately. Then the RNA mixture was incubated with 1 μl Reporter RNA (200uM) on ice for 5min. Subsequently, 1 μl Cas13a (34.1 μM),5 μl 10 X reaction buffer and 32 μl H2O were added in and keeping at 37°C 5 min, 65°C, 10 min. The fluorescence signal in 510nm to 600nm for the (+) CMC RNA and (-) CMC RNA group were detected at excitation wavelength of 494nm by the fluorescence Spectrometer.

Reverse transcription

1 μg of the (+) CMC RNA or (-) CMC RNA and 1μl 5 μM RT primer (1:1 mix of reverse transcription primers for the corresponding target RNA and GAPDH reverse transcription primers, the sequences were seen in Table S1) were mixed and added H_2O to 10 μL, then treated them at 80°C for 2min and on ice for 2 min. Subsequently, 4 μL 5 × FS buffer, 2 μL 10 mM dNTP solution mix (NEB, N0447V), 0.6 μl 100mM $MgCl_2$, 1μl 0.1M DTT, 1 μL SuperScript III reverse transcriptase (SSIII, Thermo Scientific, 18090050), 1 μL Ribolock RNase Inhibitor (Thermo Scientific, EO0384) and 0.4μl H_2O were added into the RNA solution and mixed well. The mixture was firstly treated at 25°C for 3min, 42°C for 7min, 52°C for 30 min, then after adding 1 μl RNaseH (NEB, M0297L) keeping it at 37°C for 20 min, 70 °C for 5 min. The cDNA products were purified by DNA Clean and Concentrator-5 Kit (Zymo Research, D4004). The eluted cDNAs (20 μL) were stored at -20°C.

PCR amplification and real-time fluorescence quantification

The feasibility of our method to detect Ψ sites was verified by PCR amplification and real-time fluorescence quantification of the cDNA obtained from the reverse transcription of (+) CMC RNA or (-) CMC RNA group. In the PCR amplification experiment, 1 μl (+) CMC RNA or (-) CMC RNA cDNA, 1 μl corresponding Forward primer,1μl Reversed primer and 25 μl 2X Primer STAR premix were mixed, and then the (+) CMC RNA and (-) CMC RNA group were amplified with the certain and same number of cycles in the Thermal Cycler (BIO-RAD). Since, Ψ-CMC adduct can effectively block reverse transcription (RT) and cause RT products to be truncated, their amplification products were analyzed by agarose gel electrophoresis. In the qPCR amplification experiment, 2 μl (+)CMC RNA or (-)CMC RNA cDNA , 1 μl corresponding Forward primer, 1 μl corresponding Reversed primer, 10 μl 2X Hieff qPCR SYBR Green Master Mix (Yeasen) and 6 μl H_2O were mixed, and treated by qPCR with the following conditions: 95°C for 5 min; (95°C for 10 s; 60°C for 35 s) × 40 cycles; 95°C for 15 s; 60°C for 1 min; 95°C for 15 s (collect fluorescence at a ramping rate of 0.05°C/s); 4°C hold. And the amplification products were analyzed by using $2^{-\Delta CT}$. All assays were performed with at least two biological replicates and three technical replicates. The qPCR primers used in this study were listed in Table S1.

2.The controlled trial for Ψ-ssRNA and U-ssRNA with

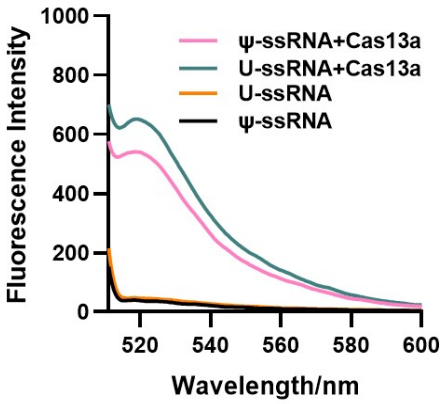

Figure. S1 Fluorescence spectra of the target Ψ-ssRNA and U-ssRNA with and without Cas13a.

and without Cas13a

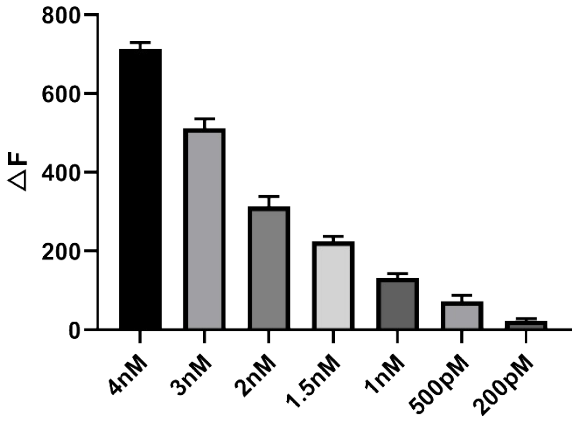

Figure. S2 Fluorescence changes of Ψ-ssRNA and Ψ- SSRNA-CMC at a concentration of 4 nM, 3 nM, 2 nM, 1.5nM,500 pM and 200 pM using CRISPR-Cas13a system (n=3 technical replicates; bars represent mean± SD).

3.Examining the sensitivity of CRISPR-Cas13a system for Ψ detection

4.Isolation and identification of Ψ-RNA-CMC

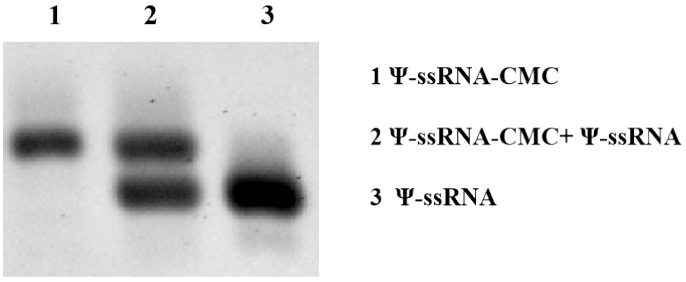

Figure. S3 Isolation and identification of Ψ-RNA-CMC. Lane1 is the RNA band of Ψ-ssRNA-CMC. Lane2 is the RNA band of the reaction mixture containg Ψ-ssRNA and Ψ-ssRNA-CMC. Lane3 is the RNA band of purified Ψ-ssRNA.

4.The sequences used for this study

Table S1. RNA and DNA sequences used in this study.

Name	Sequence
Ψ-ssRNA	5'-GCACUACΨAUCUAUGAAUCUCGAAUGUGAAGG-3'
U-ssRNA	5'-GCACUACUAUCUAUGAAUCUCGAAUGUGAAGG-3'
Reporter RNA	5'-FAM-UUUUU-BHQ1-3'
crRNA-ssRNA	5' -GGACCACCCCAAAAACGAAGGGG ACUAAAACAGAGAUUCAUAGAUAGUAG-3'
crRNA-Ψ1248	5'-GGACCACCCCAAAAACGAAGGGGACUAAAACAUCCCGUGUUGAGUCAAAUUAA-3'
crRNA-3938	5'-GGACCACCCCAAAAACGAAGGGGACUAAAACACCUCUUAUUCUACACCUCUC-3'
crRNA-Ψ519	5'-GGACCACCCCAAAAACGAAGGGGACUAAAACACAUUUUGUUAACACCGACAA-3'
crRNA-U1535	5'-GGACCACCCCAAAAACGAAGGGGACUAAAACAGAGCCAGUCAGUGUAGCGCG-3'
crRNA-Ψ72	5'-GGACCACCCCAAAAACGAAGGGGACUAAAACACGGGGTATTGGGAAAAGTTTTC-3'
Forward primer-Ψ1248	5'-AAGGGCACCACCAGGAGTGG-3'
Reversed primer-Ψ1248	5'-ATCCTGTCCGTGTCCGGGCC-3'
Forward primer-Ψ3938	5'-TGACTCTAGTCTGGCACGGT-3'
Reversed primer-Ψ3938	5'-GGGGACACCGGGGGGGCGCC-3'
Forward primer-Ψ519	5'-GCTTACACACTGGGTGTGAAACAA-3'
Reversed primer-Ψ519	5'-CTGTAGGGTGGCTCAGTGGA-3'
Forward primer-U1535	5'-GGTCTGTGATGCCCTTAGATGTCCG-3'
Reversed primer-U1535	5'-GCGCCTGCCGGCGTAGGGTA-3'
Forward primer-Ψ72	5'-GAGGTTTATCCGAGGCGCG-3'
Reversed primer-Ψ72	5'-TGCCAATGCCGACTATATTTCAAG-3'

Reference

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2. X. Li, P. Zhu, S. Ma, J. Song, J. Bai, F. Sun and C. Yi, *Nat Chem Biol*, 2015, **11**, 592-597.
3. Y. Chen, S. Yang, S. Peng, W. Li, F. Wu, Q. Yao, F. Wang, X. Weng and X. Zhou, *Chem Sci*, 2019, **10**, 2975-2979.