Electronic Supplementary Material (ESI) for ChemComm.

A method for quantitative detection of Cas12a ribonucleoproteins

Jie Qiao[a,b], Siyu Lin[a], Wenli Sun[a], Lixin ma[a] and Yi Liu[a,b]*

a. State Key Laboratory of Biocatalysts and Enzyme Engineering, Hubei University

b. Hubei Key Laboratory of Industrial Biotechnology, School of Life Sciences, Hubei University

Correspondence should be addressed to Prof. Yi Liu via yiliu0825@hubu.edu.cn

Materials and methods

Protein expression and purification

The gene encoding full-length Cas12a (LbCas12a) from *L. bacterium* (ND2006)[1] or AcrVA1[2] from *Moraxella bovoculi* was synthesized by Sangon Biotech (Shanghai, China). LbCas12a was fused with an N-terminal CL7 protein tag in addition to an HRC-3C proteinase recognition sequence between them, and the whole DNA fragment was cloned into the vector pET23a(+) (Invitrogen, USA)[3]. AcrVA1 is fused with an N-terminal 6xHis tag and the following maltose binding protein (MBP) in addition to a TEV proteinase recognition sequence. The above DNA fragment was cloned into the pET-23a(+) vector. All the constructed plasmids were confirmed by DNA sequencing. These plasmids were individually transformed into *Escherichia coli* BL21(DE3) cells for overexpression. Briefly, a single colony was picked and overnight grown in LB culture with ampicillin (100 $\mu g.ml^{-1}$). Then, the overnight cultures were transferred into 1-liter TB medium to an OD_{600} of 0.8, after which they were cooled on ice for 10 minutes before adding 0.5 mM IPTG and then incubated at 18^{o}C for 16 hours. The cells were harvested followed by resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1mM PMSF, 5 mM β-mecaptoethanol) in addition to proteinase inhibitor. For purification of Cas12a, the CL7 tagged Cas12a enzymes were loaded onto an Im7 column from TriAltus bioscience (Birmingham, AL USA). After washing two cycles of washing buffer I (20 mM Tris-HCl, pH 7.5, 300 mM NaCl), the CL7-3C protease was added to the column for in-column cleavage of the CL7 tags at 16^{o}C for three hours. Next, the Cas12a proteins were eluted with washing buffer II (20 mM Tris-HCl, pH 7.5, 500 mM NaCl). Finally, the Cas12a proteins were concentrated and stored at -80^{o}C in the storage buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM TCEP and 20% glycerin).

For purification of AcrVA1, the cell lysates were incubated with Ni-NTA resin (Qiagen, USA) for 1 hour. The resin was then washed with buffer A containing 10 mM imidazole and the target protein was eluted with buffer A containing 300 mM imidazole. Eluted proteins were digested overnight with TEV proteinase at 4^{o}C in a 14 kD dialysis bag against buffer B (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM TCEP and 5% glycerin). The digested proteins were loaded onto an MBP binding column (GE Healthcare, USA) and

eluted with buffer C (20 mM Tris-HCl, pH 7.5, 1 mM TCEP, 5% glycerin, 100 mM-1M KCl). The eluted protein was further concentrated before injected to a Superdex-G75 column (GE Healthcare, USA). At last, the AcrVA1 proteins were concentrated and stored at -80^{o}C in the storage buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM TCEP and 20% glycerin).

Preparation of crRNA and dRNA

The standard RNA samples including crRNA and dRNA were synthesized by GenePharma (Suzhou, China). The selected crRNA sequences are from the published works[4-6] and the detailed information was shown in Supplementary Table1.

Preparation of Cas12a RNPs *in vitro*

To reconstitute Cas12a RNP, the purified Cas12a protein was incubated with the synthesized crRNA *in vitro* at mole ratio of 1:1.5 under room temperature for 15 minutes. Then, we concentrated the *in vitro* assembled Cas12a RNPs by a 30 kDa Amicon Ultra centrifugal filter (Millipore), in which the excessive small molecular weight crRNAs without binding to Cas12a were gotten rid of by centrifugation. The concentration of Cas12a protein was quantified by typical BCA assay using Pierce™ BCA Protein Assay Kit (ThermoFisher).

***In vitro* plasmid DNA cleavage assay**

The target plasmid DNA containing a 5'-TTTV-3' PAM was synthesized and inserted into a pET23a(+) vector. For a cleavage assay, the Cas12a RNP (300 ng) was diluted into the cleavage buffer (25 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5% glycerol). The plasmids (200 ng) were cleaved in the absence or presence of AcrVA1 (2 pM) in cleavage buffer. The mixture was incubated at 37^{o}C for 30 minutes and then the temperature was elevated to 85^{o}C for 5 minutes to stop the reaction. The cleavage products were analyzed by agarose gels and viewed by Goldview staining.

crRNA cleavage assay

The *in vitro* assembled Cas12a RNP (1.5 μg) was incubated in cleavage

buffer in the absence or presence of AcrVA1 (2.5 pM) at 37°C for 30 minutes. The reaction was stopped by adding TBE-urea loading buffer at 85°C for 10 minutes, and separated on a 12% (v/v) TBE-urea gel and visualized with SYBR Gold (Invitrogen, China) post-staining, imaged and quantified with a ChemiDoc (BioRad).

Stem-loop RT/Real-time qPCR

The stem-loop primers and Hairpin-it miRNA qRT-PCR detection kit were designed and ordered from GenePharma (Suzhou, China). To quantitatively detect Cas12a RNPs, the standard curve of dRNA was measured at first. Generally, the standard dRNA samples were diluted from 1 μM to 1 aM. Next, the reverse transcription reactions were performed according to the kit manual by employment of the above diluted dRNA samples and the specific stem-loop primers. Thereafter, the Real-time qPCR reactions were performed on a CFX Connect Real-time PCR Detection System (Bio-rad) and the dRNA molecules were detected by SYBR green fluorescent method, with the temperature regime consisting of a hot start of 94°C for 3 minutes, followed by 40 cycles of 94°C for 12 seconds and 62°C for 30 seconds. At last, the standard curve was drawn by Origin 8.0 using the concentrations of dRNA as the X-axis and the average Ct value as the Y-axis.

Initially, Cas12a RNPs were generated by mixing 200 nM Cas12a with 300 nM crRNA in reaction buffer (25 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5% glycerol) at room temperature for 15 minutes. Then, the Cas12a RNPs were concentrated by a 30 kDa Amicon Ultra centrifugal filter (Millipore). To detect the Cas12a RNPs in various diluted concentrations, AcrVA1 (20 pM) was added and the reactions were carried out at 37°C for 30 minutes. The temperature was then increased to 85°C for 10 minutes. In the next, the RT-qPCR assays were performed to detect the dRNAs. Notably, the annealing was carried out at 25°C for RT reaction, as it helps reducing RT of the free crRNA molecules.

Detection of Cas12a RNPs in whole cell extract (WCE)

The original HEK293T cell line was from Biovector NTCC Inc (Beijing, China). Cells were cultured in DEME supplemented with penicillin-streptomycin

and 10% fetal bovine serum (FBS) (Gibco, USA) at 37°C in humidified air with 5% CO_2. Cells cultured in a 12-well were harvested in 40 µL of lysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5% glycerol, 0.1% Triton X-100, supplemented with protease inhibitor cocktail), sonication for 20 minutes at 4^{o}C, and clarified via centrifugation. Then, 200 nM Cas12a and 300 nM crRNA were added to the supernatant WCE solution followed by incubation for 15 minutes at room temperature. RNP was diluted to the desired concentration using WCE and then incubated with AcrVA1 at 37^{o}C for 30 minutes followed by stopping the reaction at 85^{o}C for 10 minutes. Next, the above samples were treated with proteinase K (50 µg/mL) and the supernatant was collected after centrifugation. Finally, the stem-loop RT/Real-time qPCR assays were performed as mentioned above. To realize quality control and get the correction factor, a 10 pM pure dRNA was added to the WCEs and measured as an external reference.

Detection of delivered Cas12a RNPs within cells

The Cas12a and crRNA were incubated and delivered into HEK293T cells by lipofectamine CRISPRMAX transfection reagent (Thermal Fisher, USA) according to the standard genome-editing protocol[7]. In a typical experiment for a 24-well plane, ~4-9x10^4 cells in each well were incubated with 1.25 µg Cas12a RNP (~ 8 pmol). Thus, the theoretical concentration of Cas12a RNP in a well is 16 nM, assuming that the cells can absorb 100% RNPs. After 4 hours' post-transfection, the cells were replaced with DMEM (with 10% FBS and 1% antibiotics) culture and then collected at 0 hour, 6 hours, 12 hours, 24 hours, 48 hours, and 72 hours, respectively. The cells were lysed in the same way as mentioned above for the cells without transfection to obtain the WCEs. In the next step, the WCE solution was incubated with AcrVA1 at 37^{o}C for 30 minutes followed by stopping the reaction at 85^{o}C for 10 minutes. Then, the total RNAs of those samples were extracted by TRIZOL method[8]. Finally, the stem-loop RT/Real-time qPCR assays were performed to detect the dRNA molecules. To get the correction factor, a 10 pM pure dRNA was added to the WCE and measured as the external reference.

Detection of multiple Cas12a RNPs with distinct crRNAs

We tested the ability of our method to detect multiple Cas12a RNPs with distinct crRNAs in one experiment. As a proof of concept test, we designed three crRNAs including LY-A, LY-B and LY-C with different sequences. Firstly, we measured the concentration of recombinant Cas12a enzyme by BCA assay. Then, three different crRNAs were added into the Cas12a enzymes separately to assemble three Cas12a RNPs in vitro termed LY-A, LY-B and LY-C, respectively. Here, the initial amounts of Cas12a RNPs were determined by using the concentration of Cas12a enzymes instead. After transferring those RNPs into HEK293T cells at varying ratios, including 1:3:5, 1:2:7 and 1:6:4, we measured each Cas12a RNPs from the lysed cells by RT-PCR and analyzed their relative ratios, using U6 snRNA as the internal reference. It was noted that the concentration of LY-A detected by BCA assay in each experiment was 10 nM.

Statistical assay

Statistical analysis was performed with either software Origin 8.0 or Graphpad Prsim 8. Each experiment was repeated at least three times. The data were present as mean±SD.

References:

1. J. S. Chen, E. Ma, L. B. Harrington, M. Da Costa, X. Tian, J. M. Palefsky and J. A. Doudna, *Science*, 2018, **360**, 436-439.
2. K. E. Watters and C. Fellmann, *Science*, 2018, **362**, 236-239.
3. J. Qiao, W. Li, S. Lin, W. Sun, L. Ma and Y. Liu, *Commun. Biol.*, 2019, **2**, 161.
4. H. Zhang, Z. Li, C. M. Daczkowski, C. Gabel, A. D. Mesecar and L. Chang, *Cell. Host. Microbe.*, 2019, **25**, 815-826.e814.
5. G. J. Knott, B. W. Thornton, M. J. Lobba, J. J. Liu, B. Al-Shayeb and K. E. Watters, *Nat. Struct. Mol. Biol.*, 2019, **26**, 315-321.
6. B. Zetsche, J. S. Gootenberg, O. O. Abudayyeh, I. M. Slaymaker, K. S. Makarova, P. Essletzbichler, S. E. Volz, J. Joung, J. van der Oost, A. Regev, E. V. Koonin and F. Zhang, *Cell*, 2015, **163**, 759-771.
7. X. Yu and X. Liang, *Biotechnol. Lett.*, 2016, **38**, 919-929.
8. M. R. Green and J. Sambrook, CSH. Protoc., 2020, 2020, pdb.prot101659.

Supplementary Table1. Sequences of the synthesized crRNAs and dRNAs.

crRNA: LY-A	5’AAUUUCUACUAAGUGUAGAUGGAAAUUAGGUGCGCUUGGCGUAG-3’
crRNA: LY-B	5’AAUUUCUACUAAGUGUAGAUGGAAAUUAGGUGCGCGGCUUAGCU-3’
crRNA: LY-C	5’AAUUUCUACUAAGUGUAGAUGAGAAGUCAUUUAAUAAGGCCACU-3’
dRNA-A	5’-UUAGGUGCGCUUGGCGUAG-3’
dRNA-B	5’-UUAGGUGCGCGGCUUAGCU-3’
dRNA-C	5’-GUCAUUUAAUAAGGCCACU-3’

Note: The highlight yellow color represents the complementary sequences specific to the target DNA.

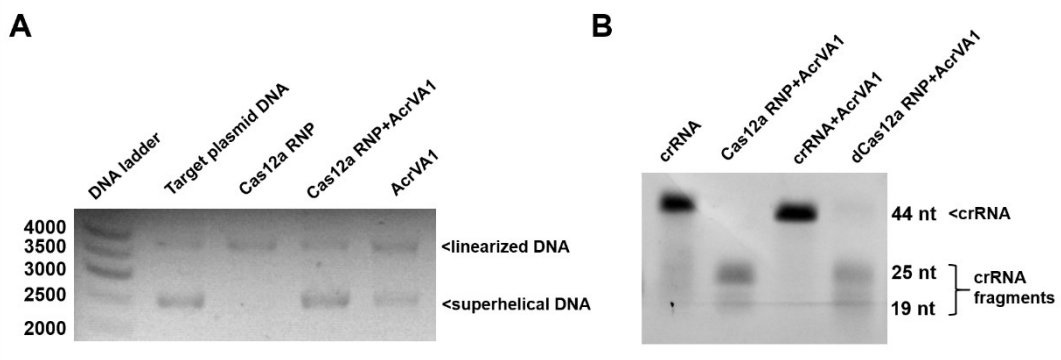

Fig. S1 (A) *In vitro* DNA cleavage assay for Cas12a RNP complexed with or without AcrVA1, as well as AcrVA1 only. (B) A 14% TBE-urea denaturing gel showing crRNA cleavage by AcrVA1-bound Cas12a RNP or dCas12a (D832A mutant) RNP.

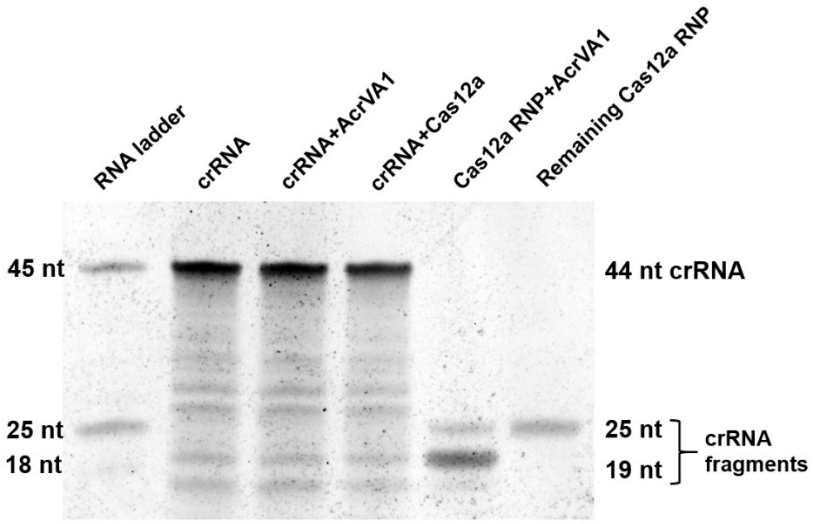

Fig. S2. A 14% TBE-urea denaturing gel showing crRNA cleavage by AcrVA1-bound Cas12a RNP. As shown, the crRNA with AcrVA1, or with Cas12a was not cleaved. Addition of AcrVA1 to the prepared Cas12a RNP showed two cleaved crRNA fragments. Then, we centrifuged this sample (Cas12a RNP+AcrVA1) by a 30 kDa Amicon Ultra centrifugal filter (Millipore) to get rid of the dRNAs (the 19 nt 3'-end crRNA). The band named "remaining Cas12a RNP" showed that a 25 nt 5'-end of crRNA fragment remains bound to the Cas12a.

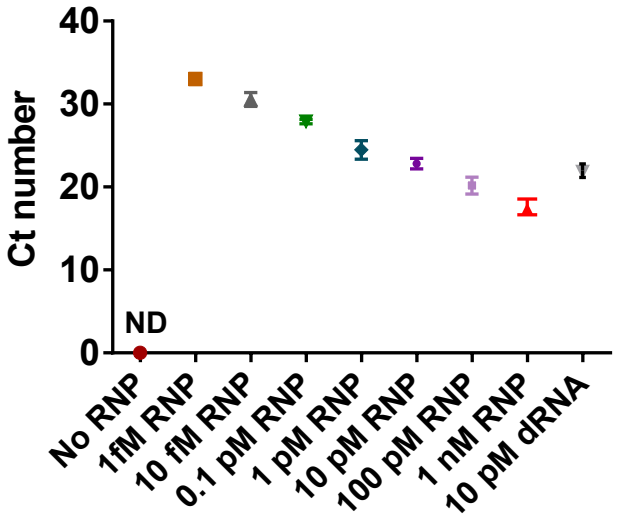

Fig. S3 Change in Ct values response for various concentrations of Cas12a RNP in reaction buffer. Noted that 10 pM dRNA standard sample was used as an external reference. All the experiments were carried out at three times. The dots indicate the mean data with SE bars.

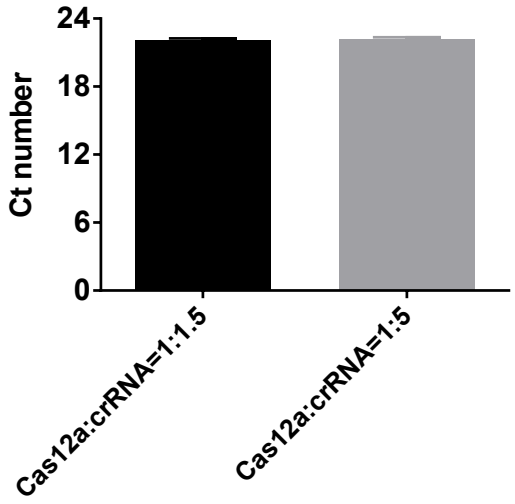

Fig. S4 Comparison of the assembled Cas12a RNPs that were made by mixture of Cas12a and crRNA at ratio 1:1.5 (red) and 1:5 (green). After assembling, the Cas12a RNPs were concentrated by a 30 kDa Amicon Ultra centrifugal filter to get rid of excessive crRNA molecules. The concentration of Cas12a protein was 10 pM for each sample. The columns show the mean data + SE. Note that all the experiments were carried out at three times.

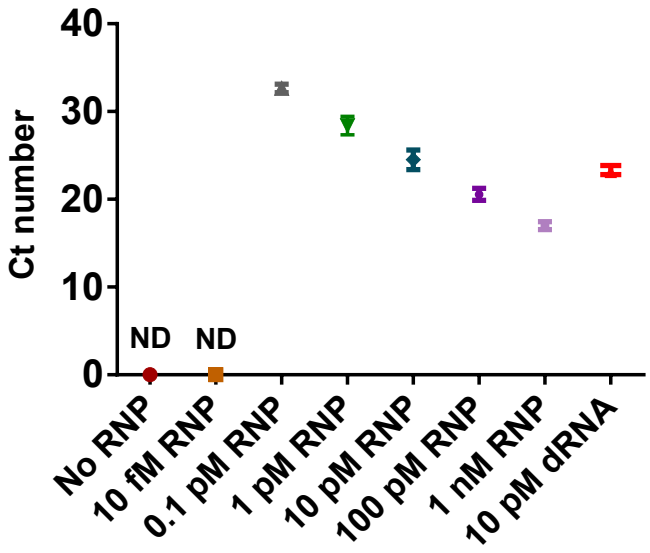

Fig. S5 Change in Ct values response for various concentrations of FnCas12a RNPs exposed to HEK293T whole cell extract. Noted that 10 pM dRNA standard sample was used as an external reference. All the experiments were carried out at three times. The dots indicate the mean data with SE bars.