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COMMUNICATION

A method for quantitative detection of Cas12a ribonucleoproteins

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Cas12a ribonucleoprotein (RNP) is an RNA-guided CRISPR-associated nuclease used widely for genome editing and molecular diagnostics. Conventional detection methods rely on adopting antibody-based reagents that are expensive and lack of scalability, and moreover, only detect Cas12 enzyme rather than RNP which is the true effector. Here we describe a method for rapid and quantitative detection of the effective Cas12a RNPs by combined use of anti-CRISPR protein AcrVA1 and stem-loop RT-qPCR, achieving a limit of detection (LOD) of 1 fM in reaction buffer and 0.1 pM in biologically representative conditions.

In bacteria and archaea, cluster regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system provide adaptive immune against invading mobile genetic elements (MGEs)[1,2]. When infected by an MGE, bacteria utilize a ribonucleoprotein (RNP) complex that consists of a Cas nuclease guided by a CRISPR RNA (crRNA) to identify and destroy the target nucleic acid. In the last decade, Cas9 endonuclease has been most widely used for genome editing[3, 4]. Besides, Cas12a (previously named Cpf1) [5], adapting a distinct mechanism of activation and DNA cleavage, has been employed in developing plenty of powerful genetic tools[3, 6, 7]. Recently, Cas12a was found to possess a collateral trans-cleavage activity on non-specific single-stranded DNA (ssDNA)[8] after recognition of the target DNA. This property has been harnessed to develop high-sensitivity nucleic acid detection approaches[8, 9], showing great promise for clinical treatment and molecular diagnostics.

The ultimate goal of gene editing is to apply the CRISPR/Cas-associated technologies in clinical therapy. However, scientists and clinicians have nowadays come to realize that qualification of Cas RNPs in complex samples (e.g., cell lysates, blood specimens, tissues) is vital and the first requirement, since low doses of RNPs are insufficient for genome editing, while high doses of RNPs might lead to harmful off-target effects[10] and serious immune activation[11, 12]. Although the current CRISPR/Cas detection methods (e.g. Western-blot and ELISA) are effective, the problems of high-expense of anti-Cas antibodies and complex operation limit their clinical settings. More importantly, yet always been ignored, those methods only detect Cas proteins rather than RNPs which are the truly functional effectors. A latest work[13] reported an approach to capture Cas9 RNPs by anti-CRISPR protein AcrVA4 and measured the RNPs by electrochemical or fluorescent methods. The limit of detection (LOD) is 280 pM in reaction buffer and 8 nM in biological conditions. It's worth pointing out that this method still cannot distinguish the difference between gRNA-free Cas9 enzymes and functional Cas9 RNPs. Hence, developing a method to efficiently and inexpensively detect the effective Cas RNPs is urgently needed.

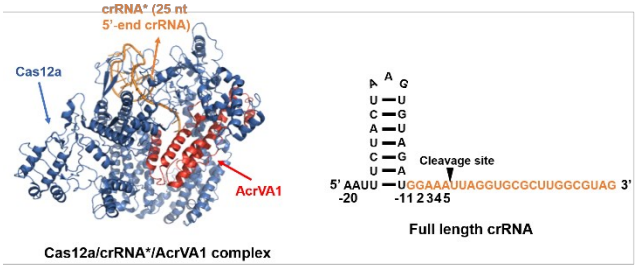


Fig. 1 Structure of LbCas12a/crRNA*/AcrVA1 complex (PDB: 6nme). Shown right is a schematic of the full length crRNA used in this study.

Lately, studies on anti-CRISPR proteins of Cas12a revealed an interesting board inhibitor protein AcrVA1[14, 15] (Fig. 1), which triggered multiple-turnover endoribonucleolytic cleavage of the target-recognition sequence of crRNA to irreversibly inactivate Cas12a RNP. Inspired by these findings, here we develop a method for rapid and quantitative detection of the effective Cas12a RNPs. The scheme of our method is illustrated in Fig. 2. The basis is that a 19 nt ssRNA (termed dRNA) was generated from truncation of the Cas12a-bound crRNA at position "+5" when an AcrVA1 bound to one

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Cas12a RNP. During the process, a Cas12a RNP only released one cleaved dRNA. It has been proven that the free crRNAs without binding to Cas12a will not be cleaved[14, 15]. Therefore, quantitative detection of Cas12a RNPs can be achieved by measuring the amounts of dRNA molecules instead.

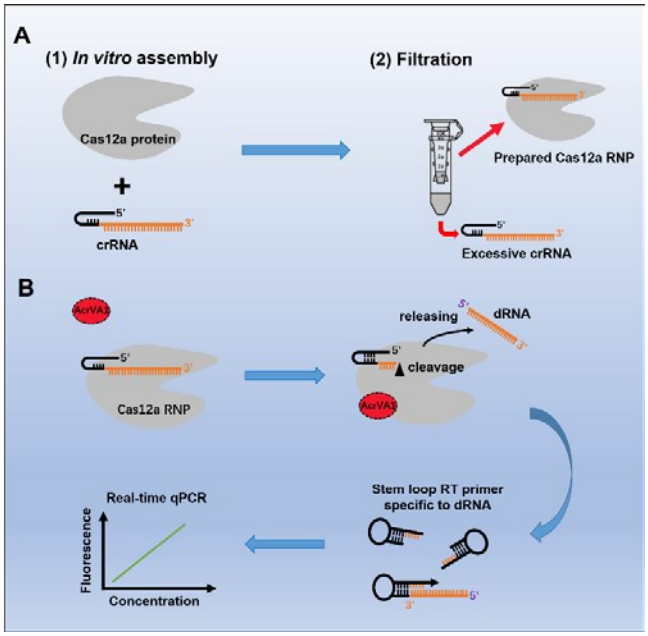


Fig. 2 The scheme of our method to detect the effective Cas12a RNPs. (A) *In vitro* preparation of Cas12a RNPs. The recombinant Cas12a proteins and synthetic crRNAs were assembled *in vitro* and filtrated by a 30 kDa Amicon Ultra centrifugal filter to get rid of excessive crRNA molecules; (B) Quantitative determination of the effective Cas12a RNPs by combined use of anti-CRISPR protein AcrVA1 and stem-loop RT/Real-time qPCR.

To verify our design, we firstly purified the recombinant LbCas12a and AcrVA1 proteins from *E. coli*. The Cas12a RNPs were generated by *in vitro* assembly of Cas12a enzymes and crRNAs at a mole ratio of 1:1.5 according to the protocol[15]. A cleavage assay on the plasmids containing the target plasmid DNA sequences was performed in the absence or presence of AcrVA1 (Fig. S1A, ESI†). The result was consistent with previous findings[15], confirming that the prepared Cas12a RNPs have nuclease activities and the addition of AcrVA1 completely blocked those activities. We also repeated the crRNA cleavage assay[14,15], demonstrating again that AcrVA1 triggered the specific crRNA truncation of Cas12a (Fig. S1B and Fig. S2, ESI†) to generate two RNA fragments including one 19 nt ssRNA termed dRNA similar to a micro RNA (miRNA). Currently, there are plenty of reliable methods for miRNA analysis[16], among of which RT-qPCR is widely considered as the gold standard[17, 18]. Therefore, here we chosed the sensitive stem-loop RT/ Real-time qPCR as the means to detect the dRNAs.

To quantitatively detect Cas12a RNPs, we initially measured the standard curve of synthetic dRNA molecules by RT-qPCR (Fig. 3A). The diluted standard dRNA samples showed a good linear range in seven orders from 1 fM to 1 nM. Next, the prepared Cas12a RNPs were incubated with AcrVA1 and measured by RT-qPCR using the specific stem-loop primers. The Ct values of Cas12a RNPs at various concentrations (1 fM-1 nM) showed a good correlation (Fig. S3, ESI †) in reaction buffer, achieving an impressive LOD of 1 fM. To further evaluate the validity of the method, five times of crRNAs were added into Cas12a proteins to prepare Cas12a RNPs, resulting in no changes of Ct (Fig. S4, ESI†). It has to be noted that we had concentrated the *in vitro* assembled Cas12a RNPs by a 30 kDa Amicon Ultra centrifugal filter (Millipore) before performing RT-qPCR assay, in which the excessive small molecular weight crRNAs without binding to Cas12a were gotten rid of by centrifugation. In this case, it can guarantee that the positive Ct responses are only aroused by the dRNAs but not from the free crRNAs.

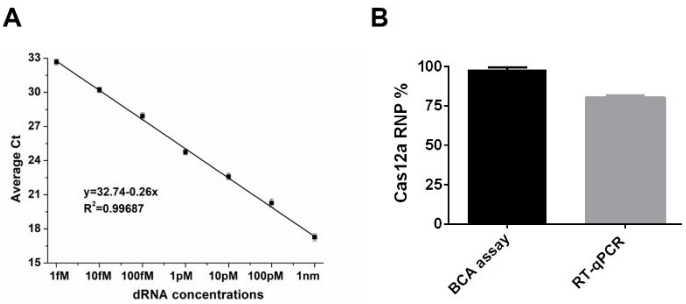


Fig. 3 (A) The standard curve of a representative synthesized dRNA. The dots indicate the mean data with SE bars. (B) The percentage of assembled Cas12a RNPs detected by BCA assay (red) or RT-qPCR (green). The columns show the mean data + SE. Note that all the experiments were carried out at three times.

Previously, the antibody-based detection methods including Western-blot and ELISA only detected Cas proteins rather than Cas RNPs that are essentially functional. All Cas proteins were assumed to be completely occupied by crRNAs when assembling them *in vitro*. However, we argue that was wrong. We quantified the pre-assembled Cas12a RNPs by determining the amounts of Cas12a proteins through BCA assay and defined that as 100% active (Fig. 3B). We then measured the same Cas12a RNP samples by RT-qPCR, which indicated that ~80% Cas12a RNPs were effective (Fig. 3B). The remaining 20% probably was partially or incorrectly assembled Cas12a RNPs without nuclease activities. Addition of excess crRNAs in Cas12a did not alter the results (Fig. S4, ESI†), further indicating that the amounts of Cas12 proteins cannot precisely represent Cas12 RNPs. This issue has been always overlooked in the previous works. Recently, we reported a streamline method for direct production of Cas9 and Cas12a RNPs from *E. coli* by co-expression of the Cas proteins and crRNAs[19]. The self-assembling Cas12 RNPs showed a remarkable stability in the absence of RNase inhibitors and enhanced genome editing efficiencies. Using the RT-qPCR method, we found that those self-assembling Cas12a RNPs have an assembly efficiency of close to 100%, suggesting that assembing Cas12a RNPs within *E. coli* cells is a better choice.

To assess the ability of harnessing AcrVA1 to enable specific detection of Cas12a RNPs in a biologically representative condition, the whole cell extracts (WCEs) were generated from HEK293T cells and mixed with varying amounts of Cas12a RNPs. There was no response from WCEs without Cas12a RNPs (Fig. 4A). In contrast, a good relation was observed between samples when increasing the RNP concentration from 0.1 pM to 1 nM, demonstrating a LOD of 0.1

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pM in complex sample matrices. According to the transfection protocol, the suggested working concentration of RNPs for cells is ~16 nM, which is far beyond the LOD. Thus, our method is sensitive enough to detect Cas12a RNPs. To gain the correction factor and evaluate the quality of experimental data, we additionally measured a WCE sample containing 10 pM pure dRNA as the external reference. Taken together, these results indicate the feasibility of our method in a biological matrix.

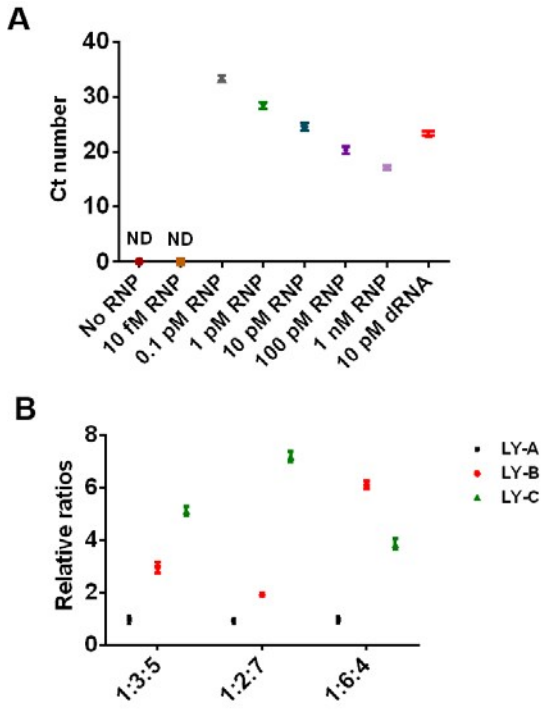


Fig. 4 (A) Change in Ct values response for various concentrations of Cas12a RNPs exposed to HEK293T whole cell extract. Noted that 10 pM dRNA standard sample was used as an external reference. (B) Detection of multiple different Cas12a RNPs that were tranferred into cells at varying ratios. Note that all the experiments were carried out at three times. The dots indicate the mean data with SE bars.

Cas12a has been successfully utilized to simultaneously edit or manipulate multiple genes20 in vivo. Additionally, Cas12a-based library screening has been broadly applied for functional gene analyses and cancer-related traits in cell lines. In those studies, Cas12a with diverse crRNAs were delivered together. To date, there are no detection methods that can distinguish multiple Cas12a RNPs simultaneously, because they all rely on recognition of Cas12a enzymes. Yet in some cases, we need to know the exact amounts of each Cas12a RNPs delivered in cells to guide gene editing. To resolve the problem, we applied the RT-qPCR method to analyze multiple Cas12a RNPs with different crRNAs in one experiment. For instance, we transferred three different Cas12a RNPs, including LY-A, LY-B and LY-C, into HEK293T cells at varying ratios. Meanwhile, we measured the U6 snRNA from cell lysates as the internal reference according to conventional miRNA detection assay16. The results (Fig. 4B) demostrate that our method is effective for detecting the relative ratios of each Cas12a RNPs. If needed, quantitative assays can be performed to determine the absolute concentration of each Cas12a RNPs. In addtion, AcrVA1 has been reported to be a board inhibitor for several Cas12a enzymes[15]. Here we tested our system for FnCas12a (*Francisella tularensis subsp. novicida U112*) and obtained good results (Fig. S5, ESI†), indicating that the method can be applied to detect other types of Cas12a RNP.

Unlike tranferring CRISPR plasmid or mRNA, direct delivery of Cas RNP for genome editing has obvious advantages such as reduced off-target effect, low toxicity, and superior cleavage efficiency in primary cells and stem cells, etc[21, 22]. It can eliminate the needs for transcription or translation of the payload, remove the risk of genomic integration, and is cell cycle independent. To prove these hypotheses, we monitored and compared the "window effectiveness" of Cas12a RNPs within cells between RNP and plasmid transfection mode. As shown in Fig. 5, the directly delivered Cas12a RNPs were rapidly degraded with time and barely detected after 24 hours. In contrast, the Cas12a RNPs were detectable after 12 hours' post-transfection and continuously expressed for 72 hours via plasmid delivery strategy. In this case, the amounts of Cas12a RNPs increased from 12 to 24 hours and then maintained at a relatively stable level. These results are in line with those observed by Western blotting analysis[23], providing evidence that RNP transfection has a much shorter retention time than plasmid transfection.

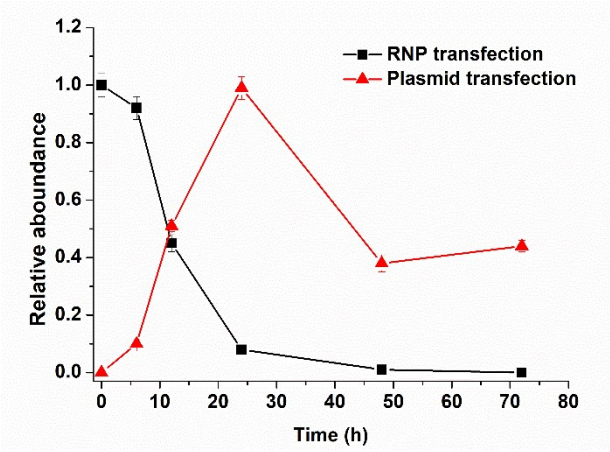


Fig. 5 Time-course analyses of Cas12a RNP within cells for genome editing via RNP transfection (black square) or plasmid DNA delivery (red triangle). Noted that only the relatively abundance of Cas12a RNP in each experiment is shown. All the experiments were carried out at three times. The dots indicate the mean data with SE bars.

Conclusions

In summary, we developed a method for rapid and cost-effective detection of the effective Cas12a RNPs by combined use of anti-CRISPR protein AcrVA1 and RT-qPCR, achieving a LOD of 1 fM in buffer and 0.1 pM in WCEs. Futher, we found that ~80% Cas12a RNPs were *in vitro* assembled by conventional method, indicating that determination of Cas RNPs by protein-based methods was exaggerated. More importantly, for the first time, we achieved simultaneously detect multiple Cas12a RNPs with different crRNAs. Together, this work provides a bioanalytical platform that takes advantage of newly-discovered AcrVA1 proteins as the capture reagent for sensitive detection of Cas12a RNPs in complex biological

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matrices, showing promises for guiding the CRISPR/Cas12a-based genome engineering, clinical treatment, and molecular diagnostics.

Conflicts of interest

All authors in this manuscript have declared no conflict of interest.

Acknowledgements

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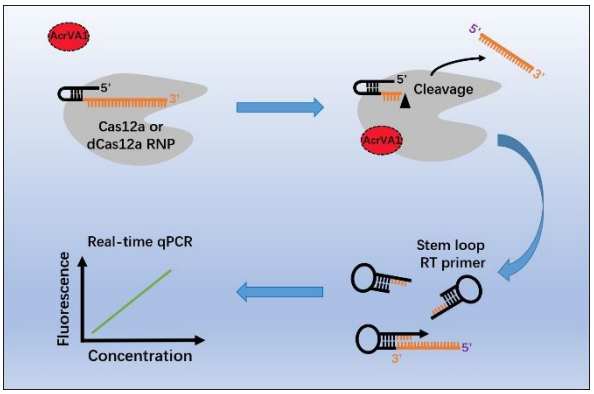
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