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An amplification-free CRISPR-Cas12a assay for titer determination and composition analysis of the rAAV genome

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The viral genome titer is a crucial indicator for the clinical dosing, manufacturing, and analytical testing of recombinant adeno-associated virus (rAAV) gene therapy products. Although quantitative PCR and digital PCR are the common methods used for quantifying the rAAV genome titer, they are limited by inadequate accuracy and robustness. The clustered regularly interspaced short palindromic repeat (CRISPR)-Cas12a biosensor is being increasingly used in virus detection; however, there is currently no report on its application in the titer determination of gene therapy products. In the present study, an amplification-free CRISPR-Cas12a assay was developed, optimized, and applied for rAAV genome titer determination. The assay demonstrated high precision and accuracy within the detection range of 4×10^9 and 10^{11} vg/mL. No significant difference was observed between the Cas12a and qPCR assay results ($p < 0.05$, t test). Moreover, Cas12a exhibited similar activity on both single-stranded and double-stranded DNA substrates. Based on this characteristic, the titers of positive-sense and negative-sense strands were determined separately, which revealed a significant difference between their titers for an in-house reference AAV5-IN. This study presents the inaugural report of a Cas12a assay developed for the titer determination and composition analysis of the rAAV genome.

INTRODUCTION

The recombinant adeno-associated virus (rAAV) has been widely used in gene therapy as a vector to transfer genes into humans. Indeed, hundreds of rAAV products are currently in clinical trials worldwide, and at least seven have been approved for marketing.[1,2] The viral genome titer is used for clinical dosing, manufacturing, and analytical testing because of its high correlation with therapeutic benefit.[3] The rAAV genome titer is previously determined via quantitative PCR (qPCR). Over the past two decades, extensive efforts have been made to improve qPCR standard preparation, sample handling, and primer/probe design, among other improvements.[3–7] In particular, the field has been rapidly moving toward digital PCR (dPCR), a new PCR technique that can achieve superior accuracy and precision without standard curves.[8–10] Of note, PCR-based methods are favored because of their high sensitivity and suitability for testing trace DNA or RNA. In this process, a fluorescent signal is generated and detected when the target sequence is amplified, thereby facilitating quantitative analysis. It should be noted that the titers of rAAV preparations for clinical use are usually $>10^{12}$ vg/mL ($>10^{10}$ vg/mL for ophthalmic preparations).[11–13] To ensure test result accuracy, PCR-based methods require a moderate amount of DNA template. In particular, dPCR typically requires approximately a few thousand copies of the target sequence in the reaction system. Therefore, for the titer determination of rAAV products, multiple sample dilutions are usually required, which can lead to large errors. Meanwhile, the accuracy of PCR-based assays is limited by the inconsistency in amplification efficiency between the reference standard and test samples.[4,5,7] Although dPCR offers the unique advantage of absolute quantification, its performance is highly dependent on commercial systems. In fact, dPCR systems from different manufacturers differ in terms of instrument, reagent, consumables, and software, which can lead to differences in test results.[14,15] In addition, different sample preparation methods result in significant variance in test results. These factors necessitate the development of more accurate and robust assays for assessing the genome titer of rAAV products.

The AAV genome consists of a linear single-stranded DNA (ssDNA), either positive or negative sense, and it is widely believed that the positive and negative strands are packaged at similar frequencies.[16–18] However, this view has not been experimentally proven. Conventional PCR-based techniques do not distinguish

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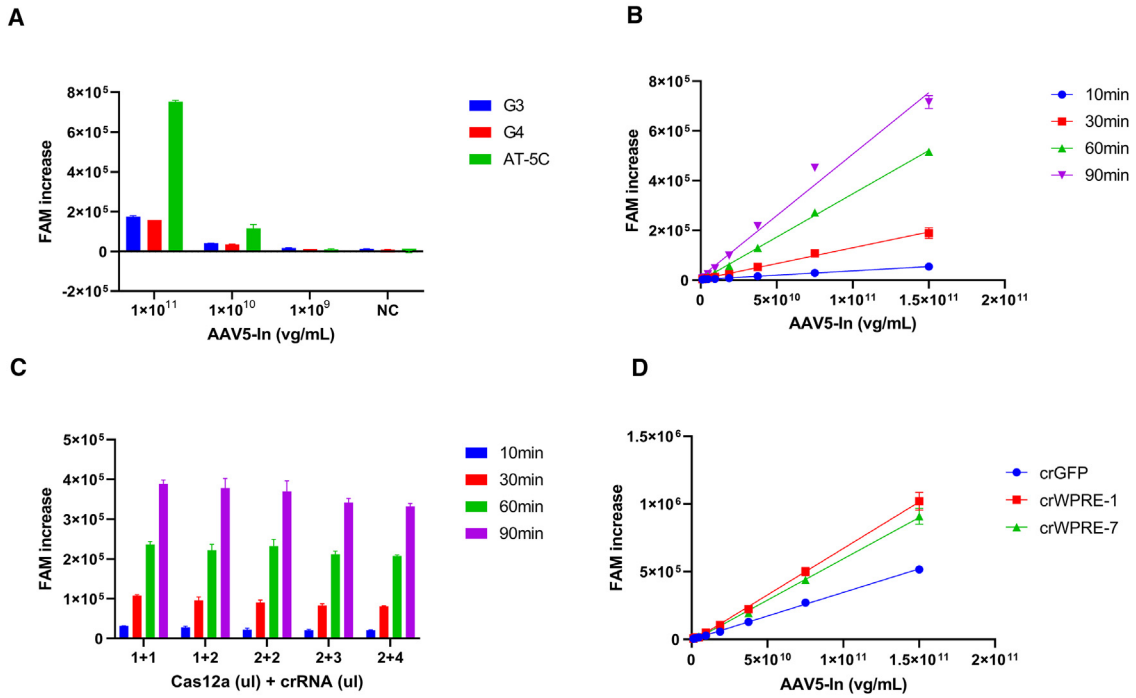

Figure 1. Optimized experimental parameters of the Cas12a assay

(A) Performance of different ssDNA reporters. (B) Assay time of the Cas12a system. (C) Amount of Cas12a and crRNA in a 30 μL reaction system. (D) Dose-response curves with crRNAs targeting different regions or strands. The error bars represent the standard deviation of the mean.

between ssDNA and double-stranded DNA (dsDNA). Therefore, the composition of the rAAV genome remains unclear. The technique of clustered regularly interspaced short palindromic repeat (CRISPR) and its associated protein Cas12a, as an innovative new-generation genomic engineering tool, has been effectively used as a suitable DNA sensor to detect nucleic acids of interest.[19] Thus, CRISPR-Cas12a-based techniques are becoming increasingly important in industrial and medical applications because they are rapid, robust, sensitive, and inexpensive and can selectively detect viral DNA without additional sample purification or amplification.[20–23] In the current study, the feasibility of an amplification-free Cas12a assay was evaluated for the genome titer determination of rAAV products. For the Cas12a assay, CRISPR RNA (crRNA) is designed to specifically recognize a 20-nt target sequence upstream of the protospacer adjacent motif, a short-sequence TTTV.[24] In this assay, once the Cas12a/crRNA complex binds to the target sequence, the activated Cas12 enzyme cleaves the target (*cis*-cleavage) as well as the nearby nontarget ssDNA fluorescent probes (*trans*-cleavage). This results in the separation of the fluorophore from the quencher and the generation of fluorescence signals that correlate linearly with the amount of target sequence in the test system. In this study, the Cas12a assay was established, optimized, validated, and primarily verified on different AAV serotypes and then compared with conventional qPCR and dPCR assay results. The properties of Cas12a on ssDNA and dsDNA were also investigated and employed to identify the composition of the rAAV genome.

RESULTS

Development of an amplification-free Cas12a assay for rAAV genomic DNA

First, a sensitive amplification-free Cas12a system for AAV genomic DNA, comprising Cas12a, crRNA, ssDNA reporter, and reaction buffer, was established. The AAV samples contained a green fluorescence protein (GFP) sequence as the gene of interest, followed by a woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in the 3′ untranslated region (UTR). The Cas12a assay was designed to target GFP or the WPRE region, and key experimental parameters were optimized. Three ssDNA reporters (G3, G4, and AT-5C) were compared based on their reported efficiency in the Cas12a assay,[21,25] and the AT-5C reporter exhibited the best performance (Figure 1A). Of note, the increase in FAM fluorescence intensity (FAM increase) was significantly linearly correlated with the AAV5-IN genome titer at 60 min. However, at a longer time (such as 90 min), a nonlinear relationship was observed (Figure 1B). A combination of 1 μL Cas12a (1 μM) and 1 μL crRNA (1 μM) was sufficient to achieve the desired signal intensity in a 30 μL reaction system. Higher Cas12a and crRNA levels did not result in a better signal (Figure 1C). In addition, crRNAs targeting different regions or strands were designed and tested on AAV5-IN, and the results showed good dose-response relationships (Figure 1D).

A reference standard is required for a relative quantitative assay. In this context, linearized plasmids (L-plasmids) have been recently

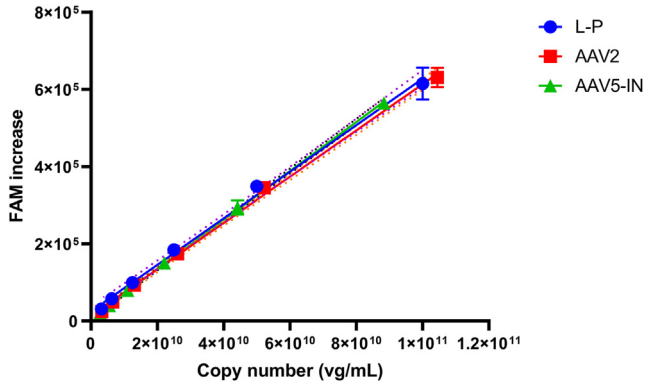

Figure 2. Dose-response relationships of linearized plasmid, AAV5-IN, and AAV2 using the Cas12a assay

Dotted lines represent 95% confidence intervals around the fitted lines. The error bars represent the standard deviation of the mean.

widely used as reference standards in qPCR-based quantitative analysis.[4,6,8] In the present study, a linearized pAAV-GFP-WPRE plasmid (L-plasmid) reference standard was prepared at a concentration of 5.0×10^{12} copies/mL, which was determined using GFP-targeting dPCR. The L-plasmid reference was maintained at 1.0×10^{13} vg/mL based on dsDNA composition. To ensure dose-response consistency between L-plasmid and AAV genomic DNA in the Cas12a assay, L-plasmid, AAV5-IN genomic DNA, and AAV2 genomic DNA were serially diluted and tested using the Cas12a assay with GFP-targeting crRNA (crGFP). All dose-response relationships showed a linear fit with an R^2 of >0.99, and the curves of AAV5-IN and AAV2 were parallel to those of L-plasmid (Figure 2), suggesting that it is feasible to use L-plasmid as a reference standard in the Cas12a assay. The developed Cas12a assay was then validated according to International Conference on Harmonisation (ICH) guideline Q2 (R2) for its specificity, range, accuracy, and precision.

Cas12a assay specificity

The specificity of a Cas12a assay depends on the recognition of the target sequence by crRNA. Thus, the effect of crRNA mismatches on the assay was investigated. Several crRNAs with mismatched bases were synthesized and tested to verify the specificity of the Cas12a assay. In WPRE-targeting crRNA (crWPRE)-7A, the original crRNA fully complementary to the target sequence, the original base A was replaced by C, G, or U (crWPRE-7G, -7C, and -7U) for a single-base mismatch, the original continuous bases GA were replaced by CU (crWPRE-7CU) for two-base mismatches, and the original continuous bases GAT were replaced by CUA (crWPRE-7CUA) for three-base mismatches. The results revealed that single-base mismatches resulted in a slight decrease in response, continuous two-base mismatches resulted in a significant decrease in response, and continuous three-base mismatches resulted in a complete loss of response (Figure 3C). These results indicated that the Cas12a assay may be sufficiently specific for the determination of the AAV genome titer.

Linear range, accuracy, and precision of the Cas12a assay

AAV5-IN DNA serial dilutions were assessed to verify the linear range of the developed Cas12a assay. The results showed that the FAM increase was linear relative to the genome titer when the genome titer was between 4×10^8 and 10^{11} vg/mL, with $R^2 > 0.99$ (Figures 3A and 3B). The limit of detection was approximately 4×10^8 vg/mL (0.66 pM). However, only when the expected titer was $>4 \times 10^9$ vg/mL did the recovery rate (the ratio of the estimated value to the expected value) reach the range of 80%–120% (Figure 3D), indicating that the limit of quantitation (LOQ) was approximately 4×10^9 vg/mL (6.6 pM). Next, several different serotypes of AAV genomic DNA samples were tested using the developed Cas12a assay directed by crGFP. The results are shown in Table 1. Both intra- and inter-assay relative standard deviations (RSDs) were <10%, demonstrating high precision. Overall, the assay showed high precision and accuracy within the linear range of 4×10^9 and 10^{11} vg/mL.

Different crRNA-directed Cas12a assays

AAV genomic DNA samples were tested using the Cas12a assay directed by crGFP or crWPRE (crWPRE-1). As shown in Figure 3E, although the estimated genome titers of the crWPRE-1 group were slightly lower than those of the crGFP group, no significant difference was observed between the two groups for each sample ($p < 0.05$, t test).

Comparison of Cas12a assay results with qPCR and dPCR

The AAV genomic DNA samples were tested using conventional GFP-targeting qPCR and dPCR methods. Of note, the L-plasmid reference was used as a standard to calculate the genome titers of the test samples in qPCR. To assess whether the state of the DNA substrate (such as ssDNA or dsDNA) had an effect on the test results, the effect of predenaturation was initially tested. For the denatured group, the test samples were heated at 96°C for 10 min, followed by rapid cooling on ice prior to testing. Figures 4A–4C show the Cas12a, qPCR, and dPCR test results of denatured or nondenatured AAV samples, respectively. Of note, a significant difference in the test results was found between the denatured and nondenatured groups for the Cas12a and dPCR assays ($p < 0.05$, t test). However, for qPCR, only AAV5-IN showed a significant difference in the test results between the two groups. Figure 4D shows the test results of the denatured group using the three methods. While the estimated genome titers from the Cas12a assay appeared the highest, no significant difference ($p < 0.05$, t test) was found between the Cas12a and qPCR assays.

Activity of Cas12a on ssDNA substrate and influence of the noncomplementary strand

Previous studies have shown that CRISPR-Cas12a has *cis*-cleavage activity on both dsDNA and ssDNA, but the mechanism of action has not been clearly elucidated.[26–28] In the current study, both positive-sense and negative-sense ssDNA of the 131-bp WPRE sequence were synthesized, and test samples containing different proportions of positive-sense and negative-sense ssDNA were prepared to investigate the activity of Cas12a on ssDNA. In this experiment, the Cas12a activity on the positive-sense ssDNA was directed

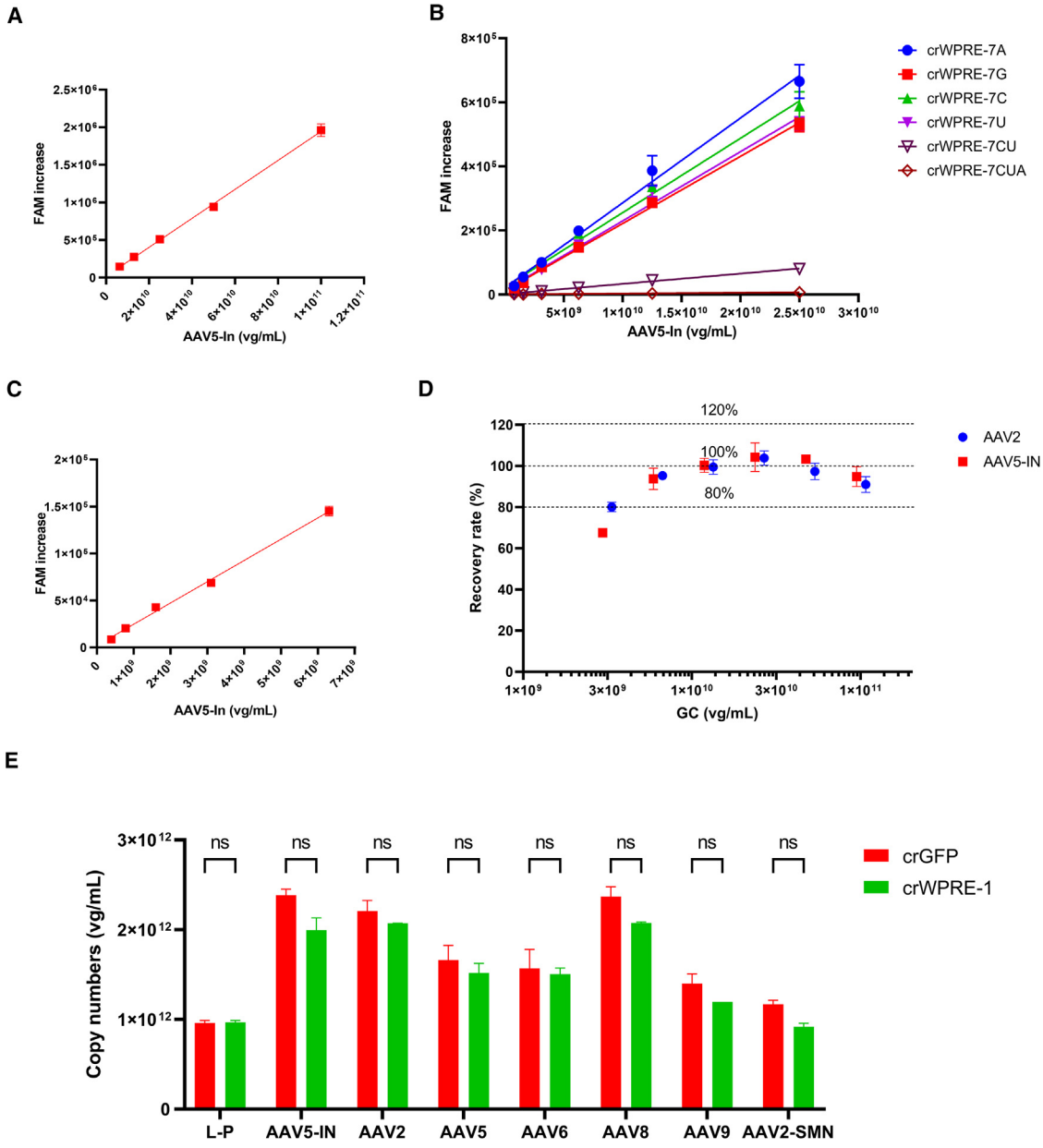

Figure 3. Performance evaluation of the developed Cas12a assay

(A and B) Linear range for AAV5-IN genomic DNA. (C) Evaluation of the specificity using crRNAs carrying different point mutations. (D) Quantitative range and accuracy of the Cas12a assay. (E) Comparison of the estimated genome titers in the crGFP- and crWPRE-1-directed Cas12a assays. The error bars represent the standard deviation of the mean. "ns" indicates "no significant difference between two groups" ($p < 0.05$, t test).

by crWPRE-1, whereas that on the negative-sense ssDNA was directed by crWPRE-2 (Figure 5A). The results revealed that Cas12a functioned when the ssDNA complementary to crRNA was present in the reaction system, regardless of the presence or absence of noncomplementary strands (Figures S1A and S1B). In addition, the activity of Cas12a remained essentially unchanged when the same amount of noncomplementary strands was added to the reaction system (Figures S1C and S1D). In fact, the signal decreased slightly as the amount of noncomplementary strands increased, suggesting that Cas12a exhibits similar activity on ssDNA and dsDNA and that excess noncomplementary strands have a negative effect on Cas12a activity. To validate this, the titers of samples with different proportions of positive-sense and negative-sense ssDNA were calculated using L-plasmid as a reference standard. As shown in Figure 5B, the Cas12a assay could effectively detect a 10% difference in the amount of the target strands.

Table 1. Intra- and inter-assay precision of the Cas12a assay

Samples	Sample 1 (vg/mL)	Sample 2 (vg/mL)	Sample 3 (vg/mL)	Mean (vg/mL)	Intra-assay RSD (%)
L-P	9.81×10^{11}	1.08×10^{12}	1.1×10^{12}	1.05×10^{12}	6.0
AAV5-IN	1.6×10^{12}	1.77×10^{12}	1.69×10^{12}	1.69×10^{12}	5.0
AAV2	1.84×10^{12}	1.99×10^{12}	2.08×10^{12}	1.97×10^{12}	6.2
AAV5	1.44×10^{12}	1.64×10^{12}	1.64×10^{12}	1.57×10^{12}	7.3
AAV6	1.36×10^{12}	1.44×10^{12}	1.46×10^{12}	1.42×10^{12}	3.7
AAV8	$2E \times 10^{12}$	2.15×10^{12}	2.11×10^{12}	2.09×10^{12}	3.7
AAV9	1.09×10^{12}	1.11×10^{12}	1.09×10^{12}	1.10×10^{12}	1.1
AAV2-SMN	7.78×10^{11}	8.73×10^{11}	8.3×10^{11}	8.27×10^{11}	5.8
Samples	Test 1 (vg/mL)	Test 2 (vg/mL)	Test 3 (vg/mL)	Mean (vg/mL)	Inter-assay RSD (%)
L-P	1.05×10^{12}	1.09×10^{12}	1.05×10^{12}	1.06×10^{12}	1.9
AAV5-IN	1.69×10^{12}	1.76×10^{12}	1.71×10^{12}	1.72×10^{12}	2.2
AAV2	1.97×10^{12}	2.12×10^{12}	2.01×10^{12}	2.03×10^{12}	3.9
AAV5	1.57×10^{12}	1.68×10^{12}	1.71×10^{12}	1.65×10^{12}	4.2
AAV6	1.42×10^{12}	1.49×10^{12}	1.61×10^{12}	1.51×10^{12}	6.4
AAV8	2.09×10^{12}	2.21×10^{12}	2.30×10^{12}	2.20×10^{12}	4.8
AAV9	1.10×10^{12}	1.15×10^{12}	1.24×10^{12}	1.16×10^{12}	6.4
AAV2-SMN	8.27×10^{11}	8.55×10^{11}	8.55×10^{11}	8.46×10^{11}	1.9

Composition analysis of AAV genomes using the Cas12a assay

The ssAAV genome consists of a linear ssDNA, either positive or negative sense, and it has been postulated that both positive-sense and negative-sense strands are packaged at similar frequencies.[16] In the present study, the composition of the AAV genome was investigated using Cas12a with positive-sense strand-targeting crRNA (crWPRE-1) and negative-sense strand-targeting crRNA (crWPRE-2) and L-plasmid as a reference standard. As shown in Figure 6, AAV samples from the same production system (AAV2, AAV5, AAV6, AAV8, AAV9, and AAV2-SMN) exhibited no significant difference in the amount of positive and negative strands ($p < 0.05$, t test). However, the in-house reference AAV5-IN showed a significant difference in the amount of the two strands, demonstrating that the packaging efficiency of the two strands can be different, which may be caused by the production system, vector backbone, or other factors.

DISCUSSION

CRISPR-Cas12a-based nucleic acid detection methods have emerged as a prominent approach in molecular diagnostics.[22,23,29,30] In general, the Cas12a system can detect DNA molecules at the pM level.[31] Meanwhile, PCR and isothermal amplification techniques are often used to amplify the target sequence, thereby enhancing detection sensitivity.[29–31] In the present study, an amplification-free Cas12a assay was developed for the titer determination of the rAAV genome using a fluorescent ssDNA reporter. The LOQ of the developed assay was 4×10^9 vg/mL (approximately 6.6 pM), which is comparable to the performance of other reported amplification-free fluorescent Cas12a assays.[31–34] The sensitivity of the assay can be improved using certain methods. For example, the use of fluorescence readers with higher sensitivity could improve assay sensitivity. In this study, assay sensitivity was evaluated on the QuantStudio 5 (Q5) qPCR instrument. However, assays performed on the QuantStudio 7 Flex (Q7) qPCR instrument were 10 times more sensitive than those performed on the Q5. Meanwhile, multi-crRNA could also enhance signals and improve assay sensitivity (Figure 1D). It is worth noting that the genome titers of rAAV preparations for clinical use are typically $>10^{10}$ vg/mL.[11–13] This implies that a 10- to 100-fold dilution is sufficient for most AAV products currently used in the clinic and that multiple dilutions are not required. For lower-titer AAV samples such as intermediates, the sample can be concentrated prior to DNA extraction. Meanwhile, the specificity, precision, and accuracy of the assay were satisfactory for viral titer determination. With regard to titer calculation, it should be noted that the Cas12a assay targets only one strand. Therefore, if the AAV samples contain equal amounts of positive-sense and negative-sense strands, it is acceptable to double either of them. If not, the total copy number of positive-sense and negative-sense strands is recommended.

In the current study, a significant amount of dsDNA was present in AAV genomic DNA samples, which was determined by comparing the dPCR test results of denatured and nondenatured samples (Figure 4C). For L-plasmid, the copy number of the denatured sample was nearly twice that of the nondenatured sample, demonstrating that denaturation can effectively convert dsDNA into ssDNA. However, for AAV genomic DNA samples, the estimated titers of the denatured group were 2- to 3-fold higher than those of the nondenatured group. This result indicated that AAV genomic DNA samples contained varying degrees of multimers distributed in the same

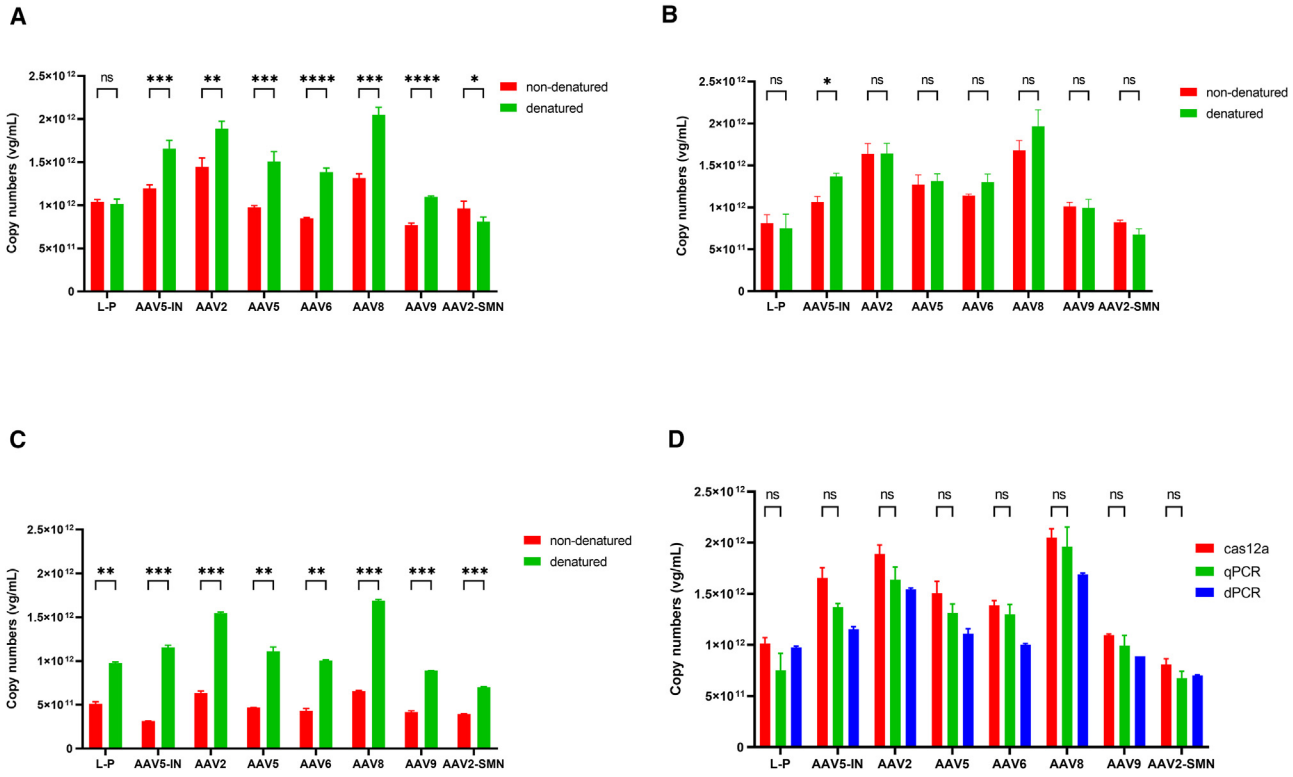

Figure 4. Comparison of Cas12a, qPCR, and dPCR assay results

(A) Results of Cas12a assay. (B) Results of qPCR assay. (C) Results of dPCR assay. (D) Summary of the estimated genome titers of denatured samples from the three assays. The error bars represent the standard deviation of the mean. "ns" indicates "no significant difference between Cas12a and qPCR," and asterisks indicate the level of significance ($p < 0.05$, t test).

chip well and that predenaturation dissociated the multimers into monomers, which were subsequently considered as multiple copies. However, denaturation not only converts dsDNA into ssDNA but also disrupts the spatial structure and exposes the target sequence to the Cas12a/crRNA complex. This can result in higher signals, which was confirmed by denatured samples showing higher genome

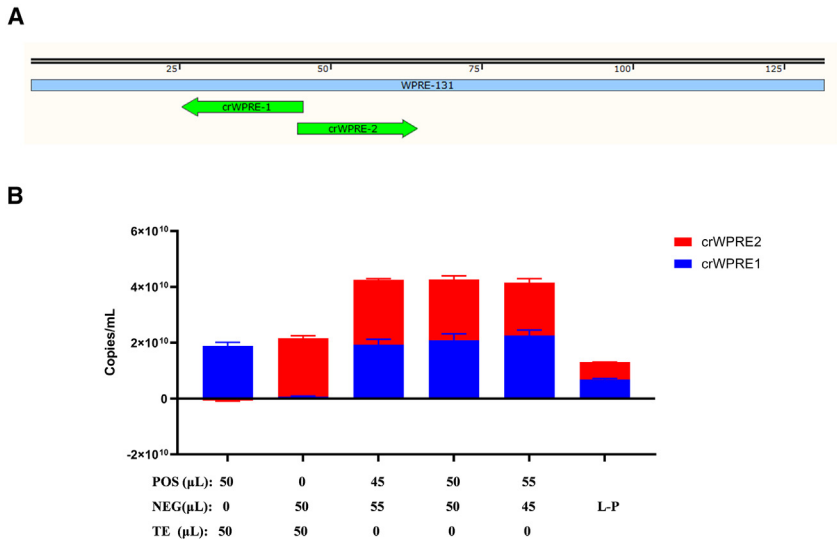

Figure 5. Evaluation of Cas12a activity on ssDNA and dsDNA substrate

(A) Distribution of crWPRE-1 and crWPRE-2. (B) Estimated copy numbers of samples comprising different proportions of positive (POS) strand and negative (NEG) strand using the Cas12a assay. The error bars represent the standard deviation of the mean.

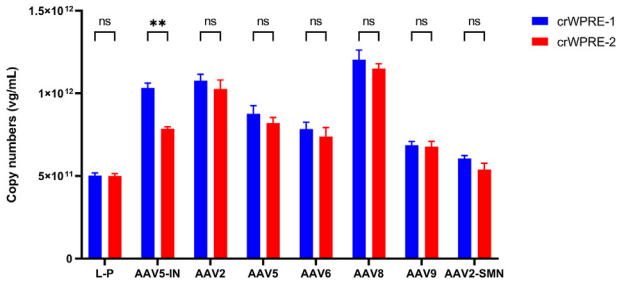

Figure 6. Test results of Cas12a assay with crRNA targeting the positive-sense strand (crWPRE-1) and negative-sense strand (crWPRE-2)

The error bars represent the standard deviation of the mean. "ns" indicates "no significant difference between two groups," and asterisks indicate the level of significance ($p < 0.05$, t test).

titers than nondenatured samples in the Cas12a assay. In theory, there should be no significant difference between the two groups in the qPCR assay. Nevertheless, a significant difference was observed for AAV5-IN, suggesting that its genome composition is distinct.

Shi-Yuan et al. found that the *cis*-cleavage activities of Cas12a on ssDNA were weaker than on dsDNA,[27] which is inconsistent with the results of the current study. Indeed, Cas12a was found to have similar activities on both dsDNA and ssDNA and slightly decreased activity with excess noncomplementary strands in the system. Based on this characteristic advantage, the composition of the rAAV genome was analyzed using the L-plasmid as a reference standard to assess whether the positive-sense and negative-sense strands were packaged at similar frequencies. AAV samples from the same production system (AAV2, AAV5, AAV6, AAV8, AAV9, and AAV2-SMN) showed no significant difference in the amount of positive-sense and negative-sense strands ($p < 0.05$, t test). However, the in-house reference AAV5-IN exhibited a significant difference in the amount of the two strands, demonstrating that the packaging efficiency of the two strands can differ. This difference may be attributed to many factors, such as the production system and the vector backbone, and these should be investigated by further studies.

Taken together, the developed Cas12a assay exhibited several unique advantages over conventional PCR-based assays. First, the assay is amplification free, which implies that the signal response is proportional to the amount of the target nucleic acid. Parallel dose-response relationships between the L-plasmid standard and the AAV genome avoid the error introduced by inconsistent amplification efficiencies in PCR, ensuring assay accuracy. Second, the assay does not require expensive reagents, special instruments, or tedious procedures, making it economical, reliable, and easily accessible.[35] Finally, the assay offers an ability to discriminate between positive-sense and negative-sense strands.[36] However, the Cas12a assay had a shortcoming similar to that encountered in PCR methods such as the inability to distinguish between intact genome and fragments. Furthermore, as a form of relative quantification, the accuracy of the Cas12a assay is dependent on the reference standard. Current methods for quantifying reference standards can hardly guarantee the authenticity and reliability of the assigned values. The adoption of uniform reference standards, such as international or national standards, could minimize inter-laboratory variation and increase the reliability and comparability of data generated by different laboratories.

To date, there have been no reports on the use of Cas12a-based genome titration for gene therapy products. The amplification-free Cas12a system developed in this study is a viable option for the titer determination of other viral gene therapy products. Furthermore, the activity of Cas12a on ssDNA can be employed to analyze the composition of positive-sense and negative-sense strand DNA in clinical samples.

MATERIALS AND METHODS

Materials

AAV5-IN, the in-house reference produced by Packgene Biotech (Guangzhou, China), was purified using affinity chromatography and iodixanol gradient ultracentrifugation. The plasmid pAAV-GFP-WPRE and six purified rAAVs were purchased from VectorBuilder (Shanghai, China). AAV2, AAV5, AAV6, AAV8, and AAV9 were packaged from pAAV-GFP-WPRE, while AAV2 carrying the survival motor neuron (SMN) protein was packaged from pAAV-SMN-GFP-WPRE. All AAV viruses were produced using the triple-transfection platform and contained a GFP sequence, followed by WPRE in the 3′ UTR.

ssDNA reporters and crRNAs

The ssDNA reporters AT-5C (5′FAM-TTATTCCCCC-3′BHQ1), G3 (5′FAM-GGTTGGTGTGG-3′BHQ1), and G4 (5′FAM-GGTTGGT GTGGTTGG-3′BHQ1) were synthesized by Sangon (Shanghai, China). In addition, crRNAs were synthesized by Genscript (Nanjing, China). The crRNA sequences are listed in Table S1.

Preparation of linearized pAAV-GFP-WPRE plasmid

First, the pAAV-GFP-WPRE plasmid was extracted and purified using the PureYield Plasmid Midiprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The purified plasmid was digested using the ScaI enzyme (NEB, Ipswich, MA, USA) at 37°C for 1 h, followed by inactivation at 80°C for 20 min. The L-plasmid was confirmed via 1% agarose gel electrophoresis, followed by copy-number determination using GFP-targeting dPCR as per the program described in the dPCR and qPCR section.

AAV sample preparation

AAV genomic DNA was prepared using a viral nucleic acid extraction kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. In brief, a 200 μL AAV virus sample was dissociated in a lysis buffer supplemented with proteinase K and carrier RNA (Poly A) at 72°C for 10 min. The viral nucleic acids were then separated and purified on a spin column and eluted with 200 μL TE buffer.

Table 2. Test sample constituents

Test sample	Positive-sense ssDNA (μL)	Negative-sense ssDNA (μL)	TE buffer (μL)
40POS	40	0	0
20POS	20	0	20
10POS	10	0	30
10POS+5NEG	10	5	25
10POS+10NEG	10	10	20
10POS+15NEG	10	15	15
10POS+20NEG	10	20	10
10POS+30NEG	10	30	0

Preparation of test samples comprising different proportions of positive-sense and negative-sense ssDNA

The positive-sense and negative-sense ssDNA of the 131 bp WPRE sequence (WPRE131) were synthesized by Sangon (Shanghai, China). Each ssDNA was diluted to 2.5 nM with PCR-grade water. The DNA test samples were prepared as described in Table 2 to investigate the activity of Cas12a on positive-sense ssDNA and the influence of negative-sense ssDNA. Likewise, to investigate the activity of Cas12a on negative-sense ssDNA and the influence of positive-sense ssDNA, test samples were prepared by exchanging the positive-sense and negative-sense ssDNA as shown in Table 2. The test samples were denatured at 96°C for 10 min and then annealed by slow cooling to 4°C at a rate of 0.1°C per second prior to the Cas12a assay.

Cas12a assay

EnGen Lba Cas12a (Cpf1) and NEBuffer r2.1 were purchased from NEB. Purified AAV genomic DNA was diluted in TE buffer and then denatured at 96°C for 10 min, followed by rapid cooling on ice. The total volume for the Cas12a reaction system was 30 μL, consisting of 3 μL 10× buffer, 1 μL AT-5C reporter, 1 μL Cas12a (1 μM), 1 μL crRNA (1 μM), 10 μL DNA sample, and 14 μL PCR-grade water. The components were thoroughly mixed, followed by fluorescence signal acquisition on the Q7 or Q5 real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The increase in FAM intensity was calculated by subtracting the FAM intensity at 1 min from the FAM intensity at 30 min.

dPCR and qPCR

TaqMan Universal Master Mix II (2×), Absolute Q DNA Digital PCR Master Mix (5×), and the QuantStudio Absolute Q MAP16 Digital PCR Kit, including MAP16 plates, gaskets, and Absolute Q Isolation Buffer, were purchased from Thermo Fisher Scientific. The forward primer (5′-GCTGGAGTACAACTACAAC-3′), reverse primer (5′-TGGCGGATCTTGAAGTTC-3′), and probe (5′VIC-CTTGATGCCGTTCTTCTGCTTGT-MGB3′) were synthesized by Sangon (Shanghai, China). The total volume for the qPCR reaction was 20 μL, consisting of 10 μL 2× TaqMan Universal Master Mix II, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 0.5 μL probe (10 μM), 5 μL template, and 3.5 μL PCR-grade water. PCR comprised two stages: initial denaturation at 95°C for 10 min and 40 cycles of denaturation and annealing at 95°C for 15 s and 55°C for 1 min, respectively. PCR and data acquisition were performed on the Q5 system. For dPCR, the total volume was 10 μL, consisting of 2 μL 5× Digital PCR Master Mix, 0.8 μL forward primer (10 μM), 0.8 μL reverse primer (10 μM), 0.4 μL probe (10 μM), 5 μL template, and 1 μL PCR-grade water. Of the mixture, 9 μL was added to MAP16 plates, followed by analysis on the QuantStudio Absolute Q Digital PCR System (Thermo Fisher Scientific). PCR comprised two stages: initial denaturation at 96°C for 10 min and 50 cycles of denaturation and annealing at 96°C for 15 s and 55°C for 1 min, respectively.

Data analysis and statistics

All of the statistical analyses were performed using GraphPad Prism 9.5 (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were performed using a multiple t test. p values <0.05 were deemed to be statistically significant.

DATA AND CODE AVAILABILITY

The authors confirm that the data generated during this study are included in this article and its supplemental information. Raw data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101304.

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AUTHOR CONTRIBUTIONS

L.Y. performed the experiments and wrote the manuscript; Y.Z., X.-c.S., and G.-y.W. assisted with the experiments and data analysis; Z.-h.F. provided financial support; J.-z.W. and C.-g.L. designed the experiments and revised the paper. All authors reviewed, edited, and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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