Kobe University Repository : Kernel

PDF issue: 2025-12-05

# Association between adeno-associated virus genomic titers and intracellular plasmid levels

Masumi-Koizumi, Kyoko
Yuan, Yuzhe
Higashiyama, Kiyoko
Yusa, Keisuke
Uchida, Kazuhisa

(Citation)
Engineering Reports,6(1):e12704

(Issue Date)
2024-01

(Resource Type)
journal article

(Version)
Version of Record

(Rights)
© 2023 The Authors. Engineering Reports published by John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

(URL)
https://hdl.handle.net/20.500.14094/0100486182

Check for updates

Received: 13 January 2023 | Revised: 9 May 2023 | Accepted: 29 May 2023

DOI: 10.1002/eng2.12704

RESEARCH ARTICLE

Engineering Reports Open Access WILEY

# Association between adeno-associated virus genomic titers and intracellular plasmid levels

**Kyoko Masumi-Koizumi** | **Yuzhe Yuan** | **Kiyoko Higashiyama** | **Keisuke Yusa** | **Kazuhisa Uchida**

Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Japan

**Correspondence**
Kazuhisa Uchida, Graduate School of Science, Technology and Innovation, Kobe University, 7-1-49 MinatojimaMinamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan.
Email: kazuhisa.uchida@port.kobe-u.ac.jp

**Funding information**
Japan Agency for Medical Research and Development; JP22ae0201001

## Abstract

The recombinant adeno-associated viral (rAAV) vector is one of the most effective viral vectors in gene therapy because of its low immunogenicity, high transduction efficiency, broad tissue specificity, and long-term transgene expression ability. HEK293T cells are widely used to produce rAAV vectors, and acquisition of high rAAV-producing cells is one of the essential processes for higher rAAV vector production. To acquire such cells, a method to identify cells with high expression of transgene protein was developed; however, the relationship between high expression of transgene protein and increased production of rAAV has not been sufficiently studied to date. We used the fluorescent protein ZsGreen1 as a transgene, subdivided the adherent cells according to the intensity of transgene expression protein, and reconfirmed the relationship between rAAV vector production and the amount of intracellular plasmid. We found that the amount of rAAV produced was not correlated with the intensity of expression of the transgene protein but may be correlated with the amount of intracellular plasmid. We also found that cells with high expression of transgene protein might not necessarily produce large amounts of rAAV vector. Based on these results, it was suggested that intracellular plasmid copy number could be used as a new marker for cells producing high levels of rAAV vector.

**KEYWORDS**

adeno-associated virus, cell sorting, fractionation, genome titer, intracellular plasmid copies, transfection, transgene expression

## 1 | INTRODUCTION

In recent years, adeno-associated viral (AAV) vector has become a key tool in gene therapy. All genes of wild-type AAV have been removed from rAAV vector in gene therapy, resulting in relatively low endogenous immunogenicity, allowing persistent vector infection and long-term transgene expression.[1-3] One of the major issues associated with rAAV vector production is the high cost involved; therefore, it is necessary to reduce manufacturing costs in order to keep drug prices low.[4-8] In addition, a large amount of the viral vector is required;[9,10] for example, the current globally approved onasemnogene abeparvovec (Zolgensma) requires $1.1 \times 10^{14}$ vg/kg for children weighing 2.6 kg or more,[11,12] thus necessitating mass

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2023 The Authors. *Engineering Reports* published by John Wiley & Sons Ltd.

production of rAAV vectors. Therefore, to achieve efficient cost reduction in rAAV vector production and obtain as high an yield of rAAV vectors as possible, it is necessary to design high-yielding stocks of rAAV vector-producing cells.

A significant improvement in the evolution of rAAV vector production has been the introduction of the triple plasmid transfection method.[13-15] Usually, rAAV vectors are produced by co-transfection using three plasmids: pHelper, pRC, and pAAV-transgene.[16-19] Wild-type AAV is a dependoparvovirus and depends on helper virus proteins to provide essential genes for a productive infection.[20,21] Since the E1A/E1B genes are natively integrated into HEK293 or HEK293T cells,[22,23] other genes from adenovirus, namely, E2A, E4, and virus-associated (VA) genes must be carried by pHelper plasmids. The pRC plasmid carries the Rep and Cap of the wild-type AAV genes, with the Rep proteins playing a central role in AAV DNA replication and a leading role in vector genome packaging and the VP proteins compose the capsid of AAV.[17,19] The pAAV-transgene plasmid is usually introduced with the gene of interest, that is transgene, used for gene therapy, excised, and then replicated using Rep proteins, and finally packaged into the rAAV capsid. Gene of interest protein expression is not essential for rAAV vector production; however, similar to other plasmids, this plasmid also expresses the protein.

Gu et al. hypothesized that rAAV vector productivity by triple transfection shows a positive correlation with cell transfection efficiency; thus, the transfected cells were sorted to identify green fluorescent protein (GFP)-positive transfected cells.[24] However, the relationship between high expression of transgene proteins and increased production of rAAV vector has not been adequately studied. In this study, we compared transgene protein expression with rAAV titration using a cell sorter. We also employed *Zoanthus* sp. Green fluorescent protein (ZsGreen1) instead of the transgene of the rAAV2 vector to observe transgene protein expression in detail.

## 2 | MATERIALS AND METHODS

### 2.1 | Cells and culture medium

The AAVpro 293 T cell line (Takara Bio, Shiga, Japan) was used as the host cell line in the present study. The cells were maintained in T-flasks with high glucose Dulbecco's modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% HyClone™ Fetal Bovine Serum (GE Healthcare, UT, USA) and 1% penicillin–streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% $CO_2$ incubator.

### 2.2 | Triple plasmid transfection of AAVpro 293 T cells

The cells were plated at the cell density of $4.0 \times 10^4$ cells/cm$^2$ or $8.0 \times 10^4$ cells/cm$^2$ in 20 mL of complete growth medium in a T-75 flask for approximately 18–24 h and then transfected using the TransIT®-293 transfection reagent (Mirus Bio, Madison, WI, USA). The cells were transfected with 21 μg of total plasmid vectors (pRC2-mi342, pHelper, and pAAV-ZsGreen1 plasmid (Takara Bio) at a ratio of 1:1:1 (w/w)) and 63 μL of TransIT®-293 transfection reagent in 2 mL serum-free DMEM. Approximately 6 h after transfection, the culture medium was completely replaced with fresh DMEM containing 2% fetal bovine serum (FBS).

### 2.3 | Sample preparation

Three days after transfection, 250 μL of 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0) was added to 20 mL of the culture medium containing rAAV2-ZsGreen-producing cells and incubated for approximately 2 min. Twenty microliters of the detached cell suspension medium was dispensed and counted using a TC20 automated cell counter (Bio-Rad, Hercules, CA, USA). Another 100 μL of the detached cell suspension medium for use as a whole titer was then dispensed and centrifuged at 1800 × g at 4°C for 10 min. The supernatant was discarded and the cells were resuspended in 100 μL of 1× phosphate-buffered saline (PBS) (−). The sample was stored at −80°C until use. The remaining detached cells were collected in a sterile 50 mL centrifuge tube and centrifuged at 100 × g at 4°C for 5 min for flow cytometory. The supernatant was removed, and the cell pellet was washed in PBS (−) to remove any residual plasmids and transfection reagents. Finally, the cell pellet was resuspended in PBS (−) and sorted using the SH800S cell sorter (Sony Biotechnology Inc., San Jose, CA, USA).

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

## 2.4 | Flow cytometry and cell sorting using a flow cytometer

Samples were acquired on the SH800S cell sorter (Sony Biotechnology, San Jose, CA, USA). Before fractionation of ZsGreen1-positive cells, single cells were gated based on the forward scatter area (FSC-A) and forward scatter height (FSC-H) properties, followed by selection of intact cells based on back scatter area (BSC-A) and FSC-A scatter properties. Intact cells were also confirmed by LysoTracker® deep red fluorescent dye (Thermo Fisher Scientific, Waltham, MA, USA) and screened by a 638 nm-excitation laser in the SH800S cell sorter (data not shown).

Four hundred eighty-eight nm excitation laser for the fluorescein isothiocyanate (FITC) channel was used for the detection of ZsGreen1-positive cells, since ZsGreen1 excitation and emission maxima are 493 and 505 nm, respectively.[25] ZsGreen1-positive cells were identified and extracted based on the FITC fluorescence intensity and were subdivided into four fractions (L, M1, M2, and H fractions) expediently. Each fraction was sorted into 50,000 cells using one well of a 96-well plate in "ultra-purity" mode. The cells were collected into 1.5 mL centrifuge tubes and then centrifuged at 1800 × g at 4°C for 10 min. The supernatant was discarded and the cell pellet was washed in 1× PBS to remove impurities contained in the sheath solution (Beckman Coulter, Inc., Brea, CA, USA) used in the cell sorter. Finally, the cell pellet was suspended in 125 μL of 1× PBS, and the samples were stored at −80°C until use.

## 2.5 | Titrating rAAV using a droplet digital PCR

To titrate the rAAV genomes, 2 μL of each rAAV solution was added to 18 μL of DNase I solution (5 U, 40 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.05% Pluronic F68) and incubated at 37°C for 30 min. DNase I was inactivated by adding 1 μL of 0.5 M EDTA (pH 8.0), followed by incubation at 25°C for 5 min. The sample was heated at 95°C for 10 min to denature the AAV capsid. This sample was used as a pretreated sample. After proper dilution of the pretreated sample with 0.05% Pluronic F68 in tris-EDTA (TE) solution, 1 μL of the rAAV sample was mixed with 19 μL of reaction mixture with droplet digital PCR (ddPCR) Supermix for Probe (No 2′-deoxyuridine, 5′-triphosphate (dUTP)) following the instructions from Bio-Rad. After droplet generation, ddPCR was performed on a C1000 Touch Thermal Cycler (Bio-Rad). The ddPCR program was performed at 95°C for 10 min for enzyme activation, 40 cycles at 94°C for 30 s followed by 60°C for 1 min, and 98°C for 10 min for enzyme deactivation. The droplets were analyzed using a QX200 droplet reader and QuantaSoft™ (Bio-Rad). The ddPCR primers and probe for determining the rAAV titer are described in Table 1.

**TABLE 1** Primers and probes designed for ddPCR.

| | Primer name | Length | 5′–3′ |
|---|---|---|---|
| rAAV titer (ZsGreen1) | Forward | 23 | TTC GTG ATC ACC GGC GAG GGC AT |
| | Reverse | 23 | CCG TAC ATG AAG GCG GCG GAC AA |
| | Probe | 24 | [FAM[a]] AAC CTG TGC GTG GTG GAG GGC GGC [BHQ1[b]] |
| pRC2-mi342 plasmid copies (Rep) | Forward | 19 | CGG AGA AGC AGT GGA TCC A |
| | Reverse | 18 | ATT TGG GAC CGC GAG TTG |
| | Probe | 21 | [FAM] CCA GGC CTC ATA CAT CTC CTT [BHQ1] |
| pAAV-ZsGreen1 plasmid copies (Plasmid backbone) | Forward | 21 | CTT ACA GAC AAG CTG TGA CCG |
| | Reverse | 20 | CGT ATC ACG AGG CCC TTT CG |
| | Probe | 34 | [FAM] CAC CGT CAT CAC CGA AAC GCG C [BHQ1] |
| pHelper plasmid copies (Ad5E4) | Forward | 20 | CGT CCG GCG TTC CAT TTG GC |
| | Reverse | 20 | ACA TTC GGG CAG CAG CGG AT |
| | Probe | 23 | [FAM] TTG AGA CAG AGA CCC GCG CTA CC [BHQ1] |

[a] The 5 prime ends of the all probes were labeled with FAM.
[b] The 3 prime ends of all probes were labeled with Black Hole Quencher-1.

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

### 2.6 | Titrating rAAV using a median tissue culture infectious dose ($TCID_{50}$) assay

Titration of infectious rAAV2 particles based on ZsGreen1 fluorescence intensity was performed following the procedure for the rAAV infectious titer assay of the AAV2 reference standard stock available on the International Society of BioProcess Technology (ISBioTech) website (https://isbiotech.org/ReferenceMaterials/pdfs/AAV2_RSS_Infectious_titer_assays_V2.pdf). The $TCID_{50}$ transducing units (tu) were calculated from three independent assays.

### 2.7 | Monitoring intracellular plasmids in ZsGreen1-positive cells using ddPCR

Three independent ddPCR reactions were performed on QX200 Droplet Reader (Bio-Rad), C1000 Touch Thermal Cycler (Bio-Rad), and QuantaSoft™ (Bio-Rad). The plasmid copy number detection primers and the custom TaqMan probe used in this study were synthesized by Thermo Fisher Scientific (Table 1).

### 2.8 | Isolation and culturing of the M2 or H fraction cells

AAVpro293T cells in T-75 flasks were transfected with 21 μg of pAAV-ZsGreen1 plasmid and 63 μL of TransIT®-293 transfection reagent in 2 mL serum-free DMEM. Approximately 6 h after transfection, the culture medium was completely replaced with fresh DMEM containing 2% FBS. Three days post-transfection, 300,000 cells were collected using the SH800S cell sorter from M2 or H fractions and cultured in a 6 well plate (Zs-M2 or Zs-H fraction cells). Following expansion for 10 days in a T-75 flask, the cells from each fraction were again transfected with 21 μg of each plasmid vector at a ratio of 1:1:1 (w/w) and 63 μL of TransIT®-293 transfection reagent in 2 mL serum-free medium. Approximately 6 h after transfection, the culture medium was completely replaced with fresh DMEM containing 2% FBS. Three days post-transfection, 50,000 cells were collected using the SH800S cell sorter from the Zs-M2 or Zs-H fractions.

### 2.9 | Statistical analysis

Statistically significant differences among data sets were determined using Student's t-test. Statistical significance was set at $p < 0.05$. Correlation coefficient ($r$) between 0.3 and 0.7 indicated moderately positive linear relationships, and the correlation coefficient between 0.7 and 1.0 indicated strong positive linear relationships.[26]

## 3 | RESULTS AND DISCUSSION

### 3.1 | Fractionation of ZsGreen1-positive cells and evaluation of rAAV2 titer

When the plasmid is transfected into the cells, the proteins required for rAAV production are translated, the rAAV capsid is produced, the transgene is replicated between ITRs, and the rAAV vector genome is inserted into the capsid. Then, the transgene protein, which is originally unnecessary, is also translated at the same time (Figure 1). First, we compared transgene protein expression with rAAV titration using a cell sorter. To investigate the mechanism of intracellular rAAV2 vector production, we fractioned the ZsGreen1-positive cells into low, medium1, medium2, high ZsGreen1 protein expression fractions (i.e., L, M1, M2, and H fractions, respectively) according to the amount of ZsGreen1 protein expression and used a cell sorter to collect 50,000 cells from each fraction. In all four fractions, the difference in protein expression level was compared to the difference in the rAAV2 titer or intracellular plasmid copy number. An outline is shown in Figure 2. We used *Zoanthus* sp. green fluorescent protein (ZsGreen1) instead of the GFP in the transgene of rAAV2 vector to observe transgene protein expression in detail. ZsGreen1 has spectral characteristics almost identical to that of enhanced green fluorescent protein (EGFP),[25] and it can be used as a substitute for EGFP, which is highly toxic to mammalian cells.[27-29]

Triple plasmid transfection was conducted to produce rAAV2-ZsGreen1 in AAVpro293T cells, and 3 days post-transfection, the ZsGreen1-positive cells were extracted using a cell sorter. We focused on altering the initial cell density for preculture approximately 18–24 h before transfection and found no differences in the ratio of ZsGreen1-positive cells among the initial cell density of $4.0 \times 10^4$ cells/cm$^2$ or $8.0 \times 10^4$ cells/cm$^2$ (Figure 3A). The ZsGreen1 protein-positive

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

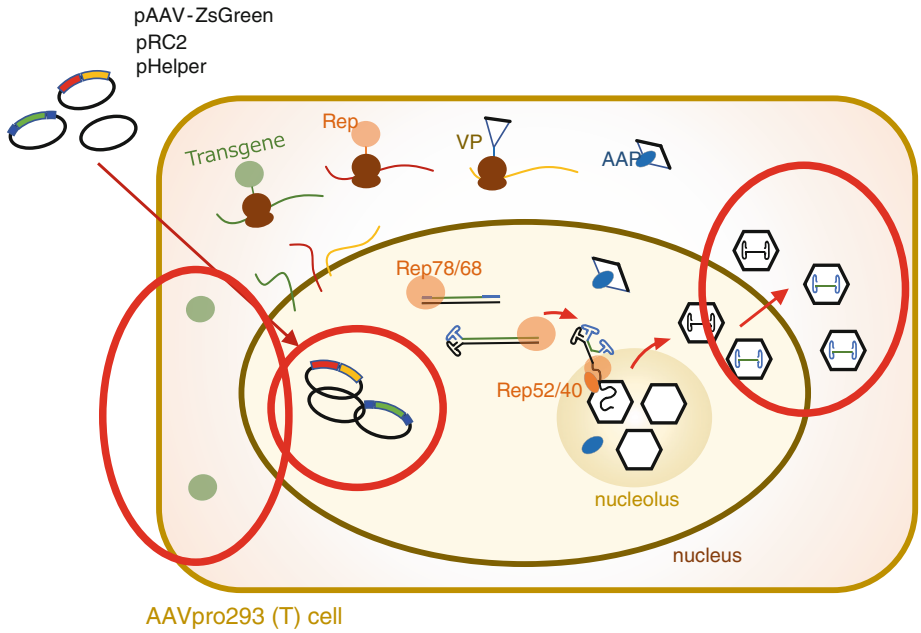


**FIGURE 1** Schematic representation of rAAV vector production by triple plasmid transfection. The red circles represent the monitoring factors in this assay, that is, transgene protein expression, rAAV genomic titer, and intracellular plasmid copy number. rAAV: recombinant adeno-associated virus

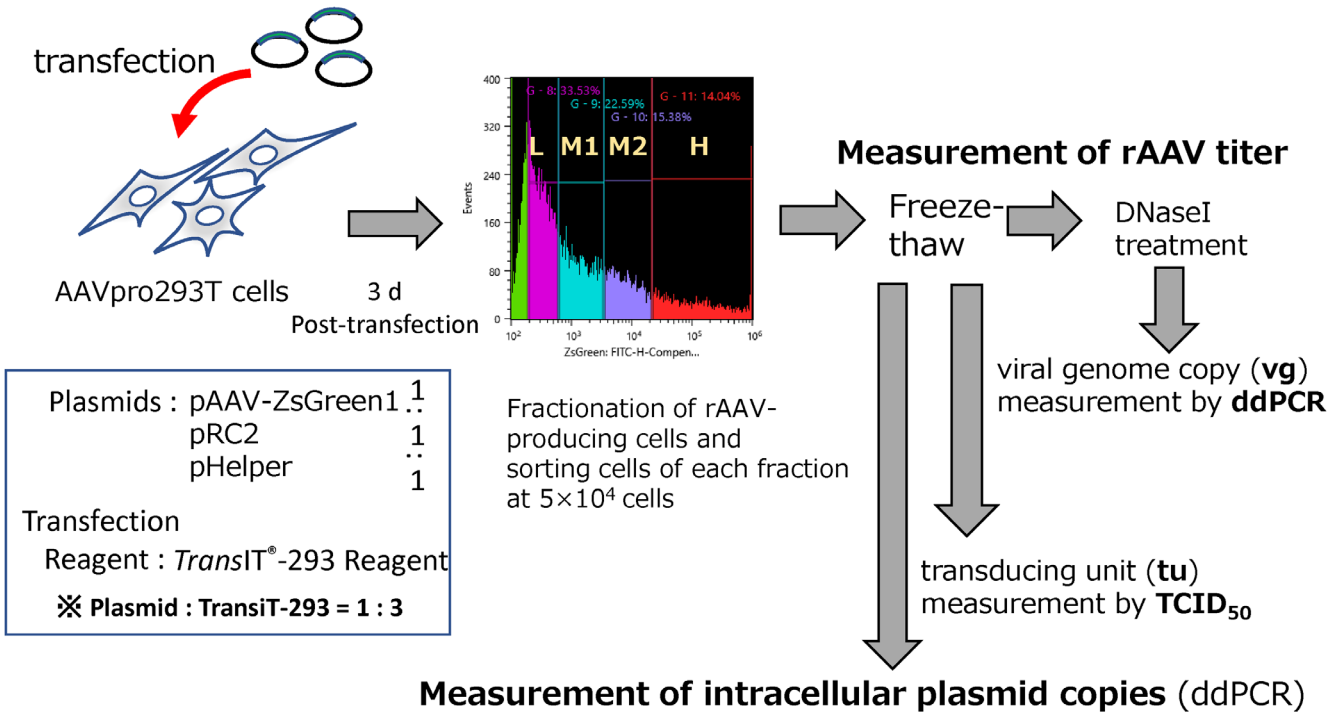


**FIGURE 2** A basic schematic representation of the analyzes of rAAV-producing cells in this assay. rAAV: recombinant adeno-associated virus

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

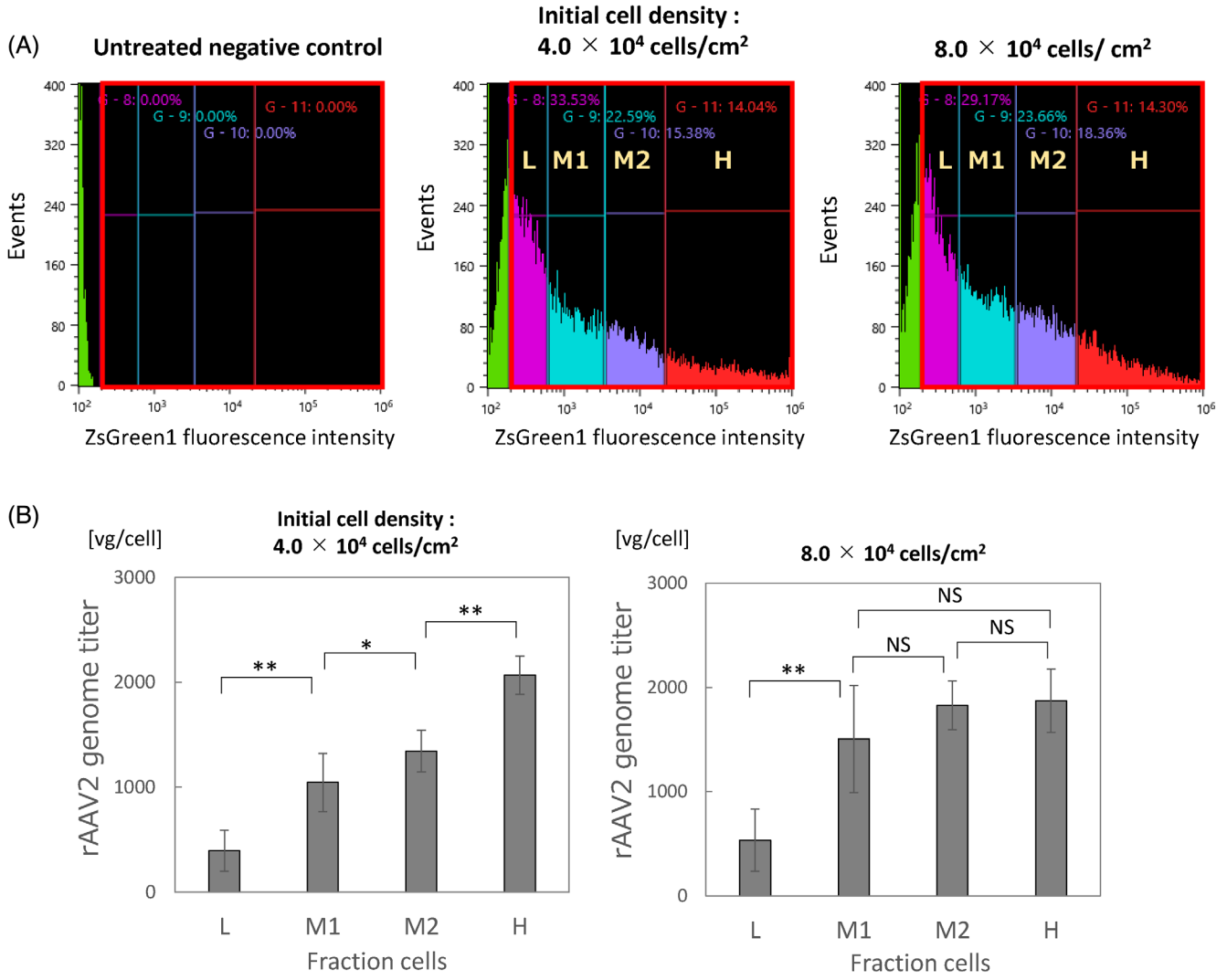


**FIGURE 3** Comparison of ZsGreen1 protein expression and rAAV genomic titers based on initial cell density. (A) FCM analysis of untreated negative control and three plasmid-transfected cells. The area in which the ZsGreen1-positive cells existed is indicated by an open red square. The ZsGreen1-positive cells were subdivided into 4 classes: L, M1, M2, and H based on the intensity of FITC (488 nm). (B) The result of rAAV genome titer (vg) for each fraction by ddPCR assay (*$p < 0.05$; **$p < 0.01$; NS, $p > 0.05$)

cells were subdivided into four fractions, that is, L, M1, M2, and H fractions, based on ZsGreen1 fluorescence intensity (Figure 3A). We then examined the genome titer of 800 cells in each fraction using ddPCR. The rAAV2 genome titer at an initial cell density of $4.0 \times 10^4$ cells/cm$^2$ proportionally increased from the L fraction to the H fraction with the ZsGreen1 protein level (Figure 3B), whereas in case of higher initial cell density, the rAAV2 genome titer of each fraction reached a plateau; that is, it did not correlate with the ZsGreen1 protein level (Figure 3B). An infectious titer assay ($TCID_{50}$) was also performed using this system, and it was found that the rAAV titer using $TCID_{50}$ could be detected in 4000 cells theoretically. Investigation of the transduction unit of each fraction showed that the rAAV2 infectious titer per cell was almost the same in all fractions and did show any correlation with ZsGreen1 protein expression (Figure S1**)**. When successive rAAV2 genome titers for each fraction were monitored, the genome titer initially increased from the L to H fraction until about 2 days after transfection, while excessive cell density caused an imbalance in rAAV vector productivity of the cell (Figure S2). These results suggest that high-titer cells with rAAV vector may change depending on the initial cell density, culture days, and cell condition; that is, the transgene protein expression was not essential for rAAV vector production and not significant as a marker for the selection of cells producing high amounts of rAAV vector.

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

### 3.2 | Monitoring intracellular plasmid copy number in rAAV-ZsGreen1-positive cells

Some methods for measuring the amount of plasmid in rAAV vector production have so far been mainly used only to measure the amount of residual plasmid in rAAV vector.[30,31] In this study, we attempted to monitor the intracellular plasmid copy numbers in rAAV2-ZsGreen1-positive cells.

Four hundred cells in each fraction were used to monitor the intracellular plasmid copy number using ddPCR (Figure 2). When the initial cell density was $4.0 \times 10^4$ cells/cm$^2$, the number of copies of the intracellular plasmid increased from the L fraction to the H fraction, similar to that in case of the rAAV2-ZsGreen1 genome titer (Figure 4). However, when the initial cell density was doubled at $8.0 \times 10^4$ cells/cm$^2$, the plasmid copy number of each fraction reached a plateau similar to that in case of the rAAV2-ZsGreen1 genome titer, which was not correlated with the ZsGreen1 protein expression level (Figure 4). It is speculated that the reason for the constant intracellular plasmid levels in each fraction when the initial cell density was increased could be competition for the plasmid, because the amount of plasmid available was insufficient relative to the number of cells. Interestingly, the copy number ratio between the three plasmids in the cell was equal to the molar ratio of the three plasmids, even though they were transfected in a weight ratio at the time of transfection. Grieger et al. reported that higher rAAV genome titers were obtained using molar ratios of the three plasmids than using weight ratios;[16] It might stand the reason to use the molar ratio.

The rAAV genomic titer per cell, summarized in Figure 5 showed a strong correlation with intracellular plasmid copies (the correlation coefficient for the initial cell density of $4.0 \times 10^4$ cells/cm$^2$; 0.92, the correlation coefficient for initial cell density of $8.0 \times 10^4$ cells/cm$^2$; 0.97) rather than with the intensity of transgene protein expression (the correlation

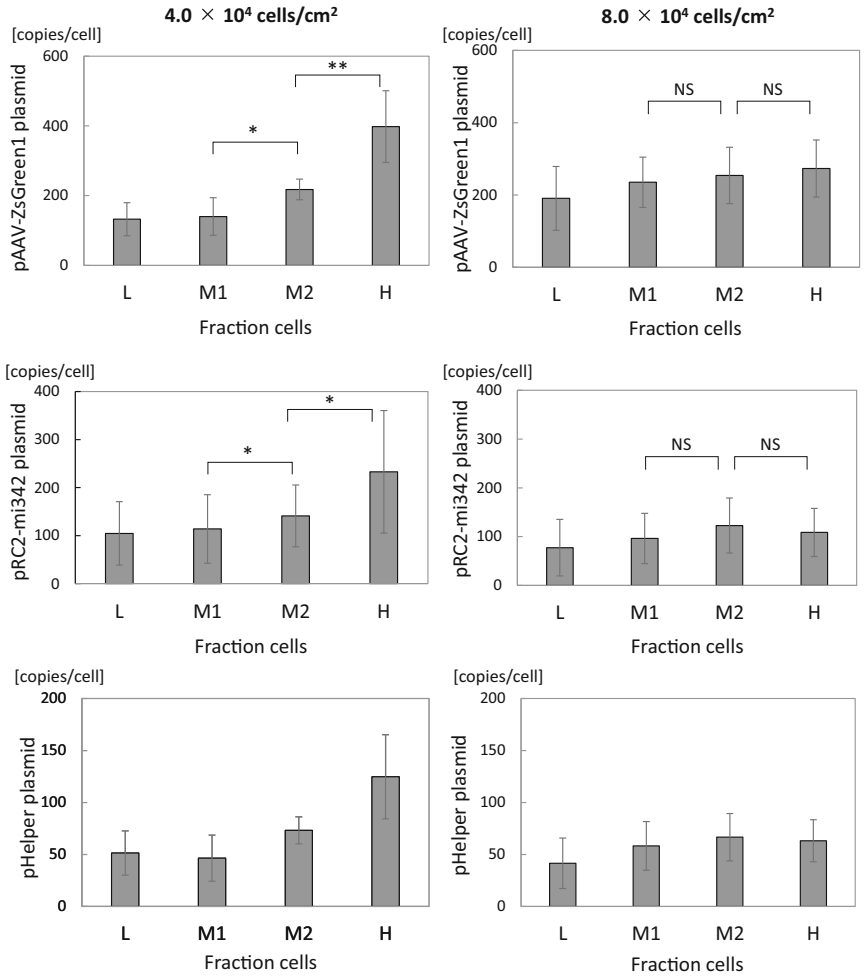


**FIGURE 4** Comparison of intracellular plasmid copy numbers in rAAV2-ZsGreen1-positive cells. Measurement of the residual intracellular plasmid copies for each fraction by the ddPCR assay (*$p < 0.05$; **$p < 0.01$; NS, $p > 0.05$).

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

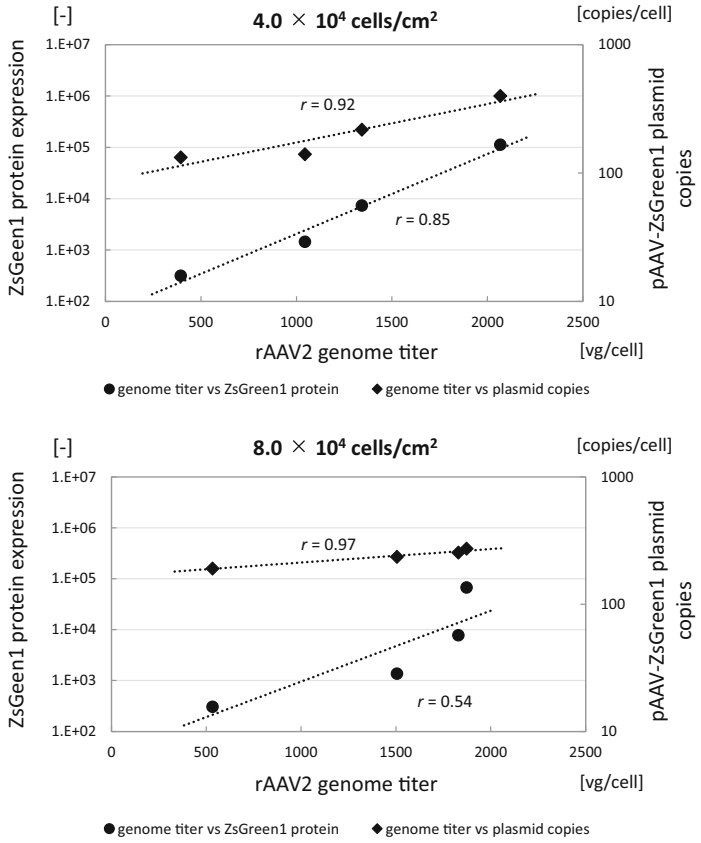


**FIGURE 5** The results of comparison between rAAV genome titer, ZsGreen1 protein expression level, and intracellular plasmid copies. The median value of the fluorescence intensity in each fraction for the ZsGreen1 protein expression level was determined. rAAV: recombinant adeno-associated virus; FCM: flow cytometer; FITC: fluorescein isothiocyanate; ddPCR: droplet digital PCR; NS: not significant

coefficient for $4.0 \times 10^4$ cells/cm$^2$; 0.85, the correlation coefficient for $8.0 \times 10^4$ cells/cm$^2$; 0.54) (Figure 5). Similar to genomic titers, intracellular plasmid copies also did not correlate with transgene protein expression levels at varying initial cell densities (the correlation coefficient for $4.0 \times 10^4$ cells/cm$^2$; 0.97, the correlation coefficient for $8.0 \times 10^4$ cells/cm$^2$; 0.72) (data not shown). Also, cells with high expression of transgene protein might not necessarily be cells producing high amounts of rAAV vector.

## 3.3 | Isolation and culturing of cells with high productivity

To further confirm whether the H fraction was a cell group with greater productivity, we cultivated the H fraction cell group with the M2 fraction cell group as a control, and studied the characteristics. The M2 and H fractions of pAAV-ZsGreen1-transfected cells were sorted and cultured in a 6 well plate (Figure 6A). Following expansion for 10 days in a T-75 flask, the cells from each fraction (Zs-M2 and Zs-H fraction cells) were transfected with three plasmid vectors for the production of rAAV2-ZsGreen1. Three days post-transfection, the Zs-M2 and the Zs-H fraction cells were analyzed by flow cytometer (FCM) and it was found that there was almost no difference in the ratio of ZsGreen1-positive cells between the Zs-M2 and Zs-H fraction cells (Figure 6B). The 50,000 cells of M2 and H fraction were again collected by cell sorter from Zs-M2 or Zs-H fraction cells, respectively, and the rAAV2 genome titer of the M2 or H fraction cells in each fraction was measured by ddPCR. The genome titer also did not differ between the two fractions. (Figure 6C). This suggests the possibility that even if the H-fractionated cell group is extracted, only high ZsGreen1-producing cells cannot be extracted, and a mixture of low and high ZsGreen1-producing cells are likely obtained. However, the AAVpro293T cells used in this study are adherent cells and differ considerably in gene expression and metabolic pathways from the serum-free conditioned suspension HEK293T and HEK293 cells currently used in industry.[32] Therefore, different results

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

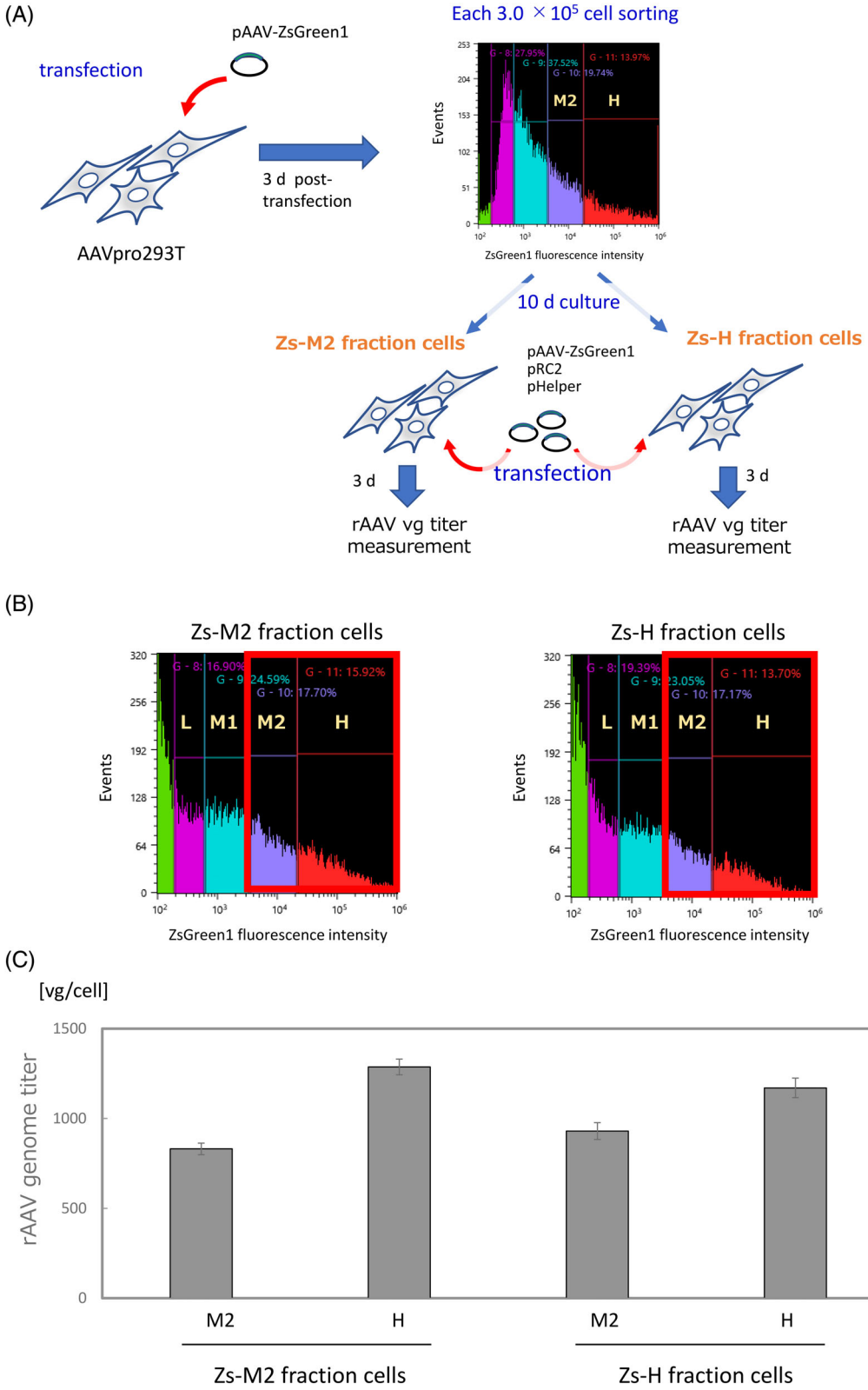


**FIGURE 6** Isolation and culturing of cells with high productivity. (A) A basic schematic representation of the analyzes of rAAV-producing cells in this assay. The M2 or the H fraction cells from pAAV-ZsGreen1 plasmid-transfected cells were sorted and cultured for 10 days (Zs-M2 or Zs-H fraction cells, respectively), and then three plasmid transfections were performed in each of the fraction cells. (B) FCM analysis of three plasmid-transfected Zs-M2 and Zs-H fraction cells 3 days after the transfection. The area where the cells were sorted and ddPCR analyzed is shown in the open red square. (C) The result of rAAV genome titer (vg) for the M2 and H fraction from Zs-M2 or Zs-H fraction cells by ddPCR assay. rAAV: recombinant adeno-associated virus; FCM: flow cytometer; ddPCR: droplet digital PCR

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

may be obtained when suspension of HEK293 cells are used. Gu et al. said that due to extensive cell death in the GFP high population, which is most likely attributed to GFP overexpression, they selected the viable GFP-medium cells for clonal cell isolation.[24] Since we used ZsGreen1 instead of GFP as the fluorescent protein in this study, cell death was hardly observed during the 10 days culture period. Interestingly, fluorescence microscopy results showed that ZsGreen1 protein expression did not decrease in the transfected cells even after 10 days (data not shown). Therefore, when ZsGreen1 protein is used as an index to isolate high-expressing cells as in Gu et al.,[24] ZsGreen1 protein contamination may pose an industrial problem.

## 4 | CONCLUSION

This study found that using adherent HEK293T cells, the rAAV genomic titer per cell showed a strong correlation with intracellular plasmid copy number rather than with the intensity of transgene protein expression. It was also found that cells with high expression of transgene protein might not necessarily produce large amounts of rAAV vector. Based on these results, it was suggested that intracellular plasmid copy number could be used as a new marker for cells producing high levels of rAAV vector.

### AUTHOR CONTRIBUTIONS

Kyoko Masumi-Koizumi conducted the experiments and drafted the manuscript. Kazuhisa Uchida formulated the experiments and secured funding for the study. Yuzhe Yuan, Kiyoko Higashiyama, Keisuke Yusa, and Kazuhisa Uchida supervised the study.

### ACKNOWLEDGMENTS

This research was supported by a grant from the Japan Agency for Medical Research and Development (AMED) (Grant number: JP22ae0201001).

### CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

### PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/eng2.12704.

### DATA AVAILABILITY STATEMENT

Data available on request from the authors.

### ETHICS STATEMENT

Human cells and plasmids used in this research were commercially available (Takara Bio, Shiga, Japan), and containment facilities and guidelines in this research conformed to those of Ministry of Education, Culture, Sports, Science and Technology.

### ORCID

*Kyoko Masumi-Koizumi* https://orcid.org/0000-0002-0184-9853
*Yuzhe Yuan* https://orcid.org/0000-0003-4886-1486
*Kiyoko Higashiyama* https://orcid.org/0000-0002-7196-892X
*Keisuke Yusa* https://orcid.org/0000-0003-3392-9871
*Kazuhisa Uchida* https://orcid.org/0000-0001-7360-4356

### REFERENCES

1. Naso MF, Tomkowicz B, Perry WL 3rd, Strohl WR. Adeno-associated virus (AAV) as a vector for gene therapy. *BioDrugs*. 2017;31(4):317-334. doi:10.1007/s40259-017-0234-5

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2. Wong KK Jr, Chatterjee S. Adeno-associated virus based vectors as antivirals. *Curr Top Microbiol Immunol*. 1996;218:145-170. doi:10.1007/978-3-642-80207-2_9
3. Zhao N, Liu DP, Liang CC. Hot topics in adeno-associated virus as a gene transfer vector. *Mol Biotechnol*. 2001;19(3):229-237. doi:10.1385/MB:19:3:229
4. Bolous NS, Chen Y, Wang H, et al. The cost-effectiveness of gene therapy for severe hemophilia B: a microsimulation study from the United States perspective. *Blood*. 2021;138(18):1677-1690. doi:10.1182/blood.2021010864
5. Mendell JR, Al-Zaidy SA, Rodino-Klapac LR, et al. Current clinical applications of in vivo gene therapy with AAVs. *Mol Ther*. 2021;29(2):464-488. doi:10.1016/j.ymthe.2020.12.007
6. Morrison C. $1-million price tag set for Glybera gene therapy. *Nat Biotechnol*. 2015;33(3):217-218. doi:10.1038/nbt0315-217
7. Qu G, McClelland A, Wright JF. Scaling-up production of recombinant AAV vectors for clinical applications. *Curr Opin Drug Discov Devel*. 2000;3(6):750-755. PMID: 19649903.
8. Ylä-Herttuala S. Glybera's second act: the curtain rises on the high cost of therapy. *Mol Ther*. 2015;23(2):217-218. doi:10.1038/mt.2014.248
9. Kay MA, Manno CS, Ragni MV, et al. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet*. 2000;24(3):257-261. doi:10.1038/73464
10. Manno CS, Chew AJ, Hutchison S, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood*. 2003;101(8):2963-2972. doi:10.1182/blood-2002-10-3296
11. Bitetti I, Lanzara V, Margiotta G, Varone A. Onasemnogene abeparvovec gene replacement therapy for the treatment of spinal muscular atrophy: a real-world observational study. *Gene Ther*. 2022;1–6. doi:10.1038/s41434-022-00341-6
12. European Medicines Agency: Assessment report. 2020 https://www.ema.europa.eu/en/documents/assessment-report/zolgensma-epar-public-assessment-report_en.pdf
13. Durocher Y, Pham PL, St-Laurent G, et al. Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells. *J Virol Methods*. 2007;144(1–2):32-40. doi:10.1016/j.jviromet.2007.03.014 Epub 2007 Apr 30.
14. Matsushita T, Elliger S, Elliger C, et al. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther*. 1998;5(7):938-945. doi:10.1038/sj.gt.3300680
15. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol*. 1998;72(3):2224-2232. doi:10.1128/JVI.72.3.2224-2232.1998
16. Grieger JC, Soltys SM, Samulski RJ. Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. *Mol Ther*. 2016;24(2):287-297. doi:10.1038/mt.2015.187
17. Urabe M, Shimazaki K, Saga Y, et al. Self-amplification system for recombinant adeno-associated virus production. *Biochem Biophys Res Commun*. 2000;276(2):559-563. doi:10.1006/bbrc.2000.3521
18. Wei J, Zhang M, Bu B, Zhang S, Xu J. Packaging and functional identification of recombinant adeno-associated virus encoding cdc2-siRNA. *J Huazhong Univ Sci Technol Med Sci*. 2008;28(6):626-629. doi:10.1007/s11596-008-0602-y
19. Maurer AC, Weitzman MD. Adeno-associated virus genome interactions important for vector production and transduction. *Hum Gene Ther*. 2020;31(9–10):499-511. doi:10.1089/hum.2020.069
20. Buller RM, Janik JE, Sebring ED, Rose JA. Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J Virol*. 1981;40(1):241-247. doi:10.1128/JVI.40.1.241-247.1981
21. Myers MW, Laughlin CA, Jay FT, Carter BJ. Adenovirus helper function for growth of adeno-associated virus: effect of temperature-sensitive mutations in adenovirus early gene region 2. *J Virol*. 1980;35(1):65-75. doi:10.1128/JVI.35.1.65-75.1980
22. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*. 1977;36(1):59-74. doi:10.1099/0022-1317-36-1-59
23. Louis N, Evelegh C, Graham FL. Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology*. 1997;233(2):423-429. doi:10.1006/viro.1997.8597
24. Gu B, Bhat V, Dong W, et al. Establishment of a scalable manufacturing platform for in-silico-derived ancestral adeno-associated virus vectors. *Cell Gene Ther Insights*. 2018;4(S1):753-769. doi:10.18609/cgti.2018.080
25. Telford WG, Hawley T, Subach F, Verkhusha V, Hawley RG. Flow cytometry of fluorescent proteins. *Methods*. 2012;57(3):318-330. doi:10.1016/j.ymeth.2012.01.003
26. Ratner B. The correlation coefficient: its values range between +1/−1, or do they? *J Target Meas Anal Mark*. 2009;17(2):139-142. doi:10.1057/jt.2009.5
27. Ansari AM, Ahmed AK, Matsangos AE, et al. Cellular GFP toxicity and immunogenicity: potential confounders in *in vivo* cell tracking experiments. *Stem Cell Rev Rep*. 2016;12(5):553-559. doi:10.1007/s12015-016-9670-8
28. Liu HS, Jan MS, Chou CK, Chen PH, Ke NJ. Is green fluorescent protein toxic to the living cells? *Biochem Biophys Res Commun*. 1999;260(3):712-717. doi:10.1006/bbrc.1999.0954
29. Richards B, Zharkikh L, Hsu F, Dunn C, Kamb A, Teng DH. Stable expression of Anthozoa fluorescent proteins in mammalian cells. *Cytometry*. 2002;48(2):106-112. doi:10.1002/cyto.10117
30. Smith PH, Wright JF, Qu G, Patarroyo-White S, Parker A, Sommer JM. Packaging of host cell and plasmid DNA into recombinant adeno-associated virus particles produced by triple transfection. *Mol Ther*. 2003;7(5):S348. doi:10.1016/S1525-0016(16)41342-0
31. Wright JF, Zelenaia O. Vector characterization methods for quality control testing of recombinant adeno-associated viruses. *Methods Mol Biol*. 2011;737:247-278. doi:10.1007/978-1-61779-095-9_11

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

32. Malm M, Saghaleyni R, Lundqvist M, et al. Evolution from adherent to suspension: systems biology of HEK293 cell line development. *Sci Rep*. 2020;10(1):18996. doi:10.1038/s41598-020-76137-8

## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Masumi-Koizumi K, Yuan Y, Higashiyama K, Yusa K, Uchida K. Association between adeno-associated virus genomic titers and intracellular plasmid levels. *Engineering Reports*. 2024;6(1):e12704. doi: 10.1002/eng2.12704

25778196, 2024, 1, Downloaded from https://onlinelibrary.wiley.com/doi/10.1002/eng2.12704 by Kobe University, Wiley Online Library on [17/01/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License