Biochemical Engineering Journal 230 (2026) 110144

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Regular article

Development of an inducible stable producer cell line for rAAV production

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ARTICLE INFO

Keywords:
Gene therapy
Synthetic biology
RAAV production
Stable producer cell line
Inducible circuit design
Transposon integration

ABSTRACT

Adeno-associated virus (AAV) is a widely preferred in vivo gene therapy vector for clinical trials due to its numerous advantages. However, manufacturing recombinant AAV (rAAV) using transient transfection methods poses significant challenges. Stable cell lines present a promising alternative for large-scale, continuous production, addressing the limitations of transient transfection and enabling more efficient rAAV manufacturing. In this study, we developed an inducible stable producer cell line by integrating transfer (GFP-containing) and assembly (Cap-containing) cassettes into a previously established inducible stable packaging cell line using transposon-mediated integration. The stable cell pool was initially evaluated for genome copy number of all viral components and rAAV production. Detection of genome titer signals in the pool confirmed successful integration, leading to the isolation of single clones. These clones were further screened for GFP and Cap genome copy numbers, with those exhibiting comparable or higher copy numbers than the stable pool undergoing comprehensive genome copy analysis for all viral components. The top-performing clone was identified and characterized in detail. Following doxycycline induction, genome titer (3.73E11 vg/L) and capsid titer (2.01E11 cp/L) were detected, confirming the successful establishment of the inducible stable producer cell line. This study highlights the feasibility of developing inducible stable cell lines for rAAV production through refactored viral vector components and a combination of site-specific and transposon-mediated integration approaches.

1. Introduction

The traditional rAAV production process is through triple plasmid transient transfection in HEK293 cells [1,2]. However, there are several drawbacks associated with transient production process, including complicated and expensive process consuming considerable amounts of transfection reagents and plasmid, the lot-to-lot variation of the products, and difficulty in scaling up [2–9]. In order to achieve consistent and scalable production, it is necessary to establish a stable cell line for rAAV production.

The major challenge in developing a stable producer is the cytotoxicity induced by continuous expression of rep and helper genes after stable integration [3,10]. Thus, in previous study, we successfully developed a stable packaging cell line with site specific integration of refactored and inducible Tet-on promoters controlled viral components [5]. To produce rAAV with this packaging cell line, transient transfection with transgene plasmid was required before doxycycline induction [5]. A comprehensive characterization from genomic, transcript, protein level and ability to produce rAAV was conducted for this cell line before and after doxycycline addition [5]. It was observed that the genome titer with one transgene plasmid transfection was ten-fold lower than that of rAAV products done with triple plasmid transfection [5]. Thus, additional efforts can be focused on optimizing gene expression level and timing for various viral components and minimizing the leakage of toxic genes to accomplish the aim of high titer rAAV production in the future.

In addition, the site-specific integration strategy has also been applied to establish a stable producer cell line in the previous study, with all viral components integrated sequentially [5]. Relative genome copy analysis of all viral components, including Rep68, Rep52, Cap, DBP, E4orf6, and GFP, in the stable cell pool indicated successful integration; however, no genome titer and only a very faint capsid titer signal were detected, thereby leading to the failure of stable producer cell line development [5]. Limited copy number of transgene cassette with site specific integration and low packaging efficiency could potentially contribute to this result [5]. Genome replication is one limitation: site

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https://doi.org/10.1016/j.bej.2026.110144
Received 6 October 2025; Received in revised form 13 February 2026; Accepted 24 February 2026
Available online 26 February 2026
1369-703X/

specific integration offers control over the number of integrated transgene copies [10,11], which must be significantly lower than the high copy numbers typically achieved during transient plasmid transfection. Genome encapsidation could be another bottleneck, with one study reporting only 0.3%-3% of total genomes were encapsidated [12]. Thus, we hypothesis that increasing the copy number of transgene and Cap gene can increase the likelihood of detecting genome and capsid titer signals.

DNA transposon-based integration is an effective method to enhance transgene integration levels and target transcriptionally active regions of host cell genome with greater affinity [11]. Additionally, transposon allows the development of stable cell factories with higher homogeneity compared to cell line generated by random integration [13]. Transposon system comprises a donor vector expressing gene of interest flanked by the ITRs, along with a helper plasmid expressing the transposases, which facilitates the transgene's transposition from donor plasmid into host cell genome [14,15]. Several commercially available transposase systems, including Sleeping Beauty [16,17], PiggyBac [18,19], and Leap-in [20,21], are widely used in biopharmaceutical research. Transposition efficiency and vector copy number vary greatly in different cell types [22], so it is worthwhile optimizing the co-transfection ratio of transposase to transposon. With a hyperactive transposase, optimized transposon, and optimal co-transfection ratio, 50–100 copies of the insert are expected to be stably integrated into the host genome. The genome titer is anticipated to increase as a result of the elevated copy number of GOI.

In this study, we aimed to develop a stable producer by conducting transposon mediated integration of transfer (containing GFP) and assembly (containing Cap) cassettes in isolated packaging cell line. Genome copy analysis of all viral components and rAAV production were initially evaluated in the stable cell pool. After confirming the genome titer signal detection in the stable pool, single clones were isolated. These clones were screened for their copy number of GFP and Cap. Clones with genome copy numbers comparable to or higher than those in stable pools were selected and tested for the genome copy of all viral components. Top clone was identified and fully characterized after all viral component genome copy evaluations. The detection of genome and capsid titer after doxycycline induction indicated the successful establishment of the stable producer cell line.

2. Materials and methods

2.1. Vector design and plasmids construction

PiggyBac transposon plasmid construction: PiggyBac transposon backbone with Long Terminal Repeats (LTR) were amplified via PCR from plasmid pTS1019_Tier3(PB) which was a gift from Martin Fussenegger (Addgene plasmid # 169649). The components of the AAV were generated either through synthesis by Twist Bioscience or PCR cloning from corresponding plasmids. Subsequently, they were assembled according to the vector design using the golden gate assembly method as shown in the previous packaging cell line development study [5]. Then the PiggyBac transposon sequences, selection genes, and well-prepared AAV components were assembled using NEBuilder HiFi DNA Assembly master mix (New England Biolabs, United States) followed by transformation using E. coli DH5α competent cells. Plasmids were verified by sequencing and prepared with the EndoFree plasmid maxi kit (Zymo, United States), according to the manufacturer's instructions. The information on the plasmids and primers used in cloning is listed in Supplementary Tables S1 & 2.

2.2. Cell culture, stable transfection, and single clone isolation

Packaging cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, United States) supplemented with 10% fetal bovine serum (Gibco, United States), 5 μg/mL blasticidin (InvivoGen, United States) and 200 μg/mL zeocin (InvivoGen, United States). The cells were cultured in T-flasks (GenClone, United States) with a working volume of 5 mL at 37 °C under 5% CO2 and passaged every 4 days. To establish stable cell pools, we transfected transposon plasmid and Hyperactive PiggyBac transposase plasmid (VectorBuilder, United States) at a 1:1 (w: w) using Lipofectamine 2000 (Invitrogen, United States) followed by 2 weeks of selection with antibiotics: 200 μg/mL zeocin (InvivoGen, United States), 15 μg/mL blasticidin (InvivoGen, United States) and 2 μg/mL of puromycin (Sigma-Aldrich, United States). The viable cell density (VCD) and cell viability were assessed using a Nova Flex2 Analyzer (Nova Biomedical, United States). The single-cell clones were isolated from the stable pools using limited dilution and seeded with 0.8 cells per well into 96-well plates.

2.3. Genome copy number analysis

The cell pellets from stable pools and single clones were collected for genomic DNA extraction. Genomic DNA was extracted using Quick-DNA Miniprep Plus Kit (Zymo, United States) following the manufacturer's instructions. qPCR was performed on genomic DNA samples using SYBR Green qPCR Master Mix (Thermo, United States) on the Bio-rad system (Biorad, United States). According to the manufacturer's instructions, qPCR reaction mixtures contained 2X SYBR Green master mix, 400 nM of forward and reverse primers, 20 ng of genomic DNA, and up to 20uL molecular biology water. Amplification was executed with the following conditions: 50 °C for 1 min; 95 °C for 10 min; 40 cycle: 95 °C for 15 s, 60 °C for 30 s, 72 °C for 1:30 min. The primer sequences used for Cap, Rep52/40, Rep78/68, E2a, E4orf6, codon-optimized green fluorescent protein (GFP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are listed in Supplementary Table S2.

2.4. RNA isolation for gene expression evaluation

The gene expression assay, including RNA isolation, cDNA reverse transcription and gene expression by RT-PCR, was previously described [23]. The comparative cycle threshold (2-ΔΔCt) method was used to analyze the transcript level fold changes between different conditions, in this case the induction of different doxycycline concentrations. The primer sequences used for Cap, Rep52/40, Rep78/68, E2a, E4orf6, codon-optimized green fluorescent protein (GFP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are listed in Supplementary Table S2.

2.5. rAAV vector production, rAAV preparation and analytical methods (genome titer, capsid titer)

Cells in the logarithmic growth phase were seeded at a density of 5E+ 5 cells per well in a 6-well plate. After a 24-hour incubation, induction was performed for rAAV production with the addition of 5 μg/mL dox. After 68 h post-induction, harvested cell cultures were aliquoted to 1 mL volume (cells and supernatant inclusive) in 1.5 mL centrifuge tubes either for immediate analysis or stored at −80°C for future analysis. The genome titer and capsid titer assays followed the previously published paper [4].

2.6. Statistical analysis

GraphPad Prism 9 was used for processing raw data and statistical analysis.

3. Results

3.1. Strategies and workflow

Fig. 1 illustrates the overall integration strategy and workflow. The upper panel depicts the workflow used to develop the stable packaging cell line in the previous study [5], while the lower panel (highlighted in

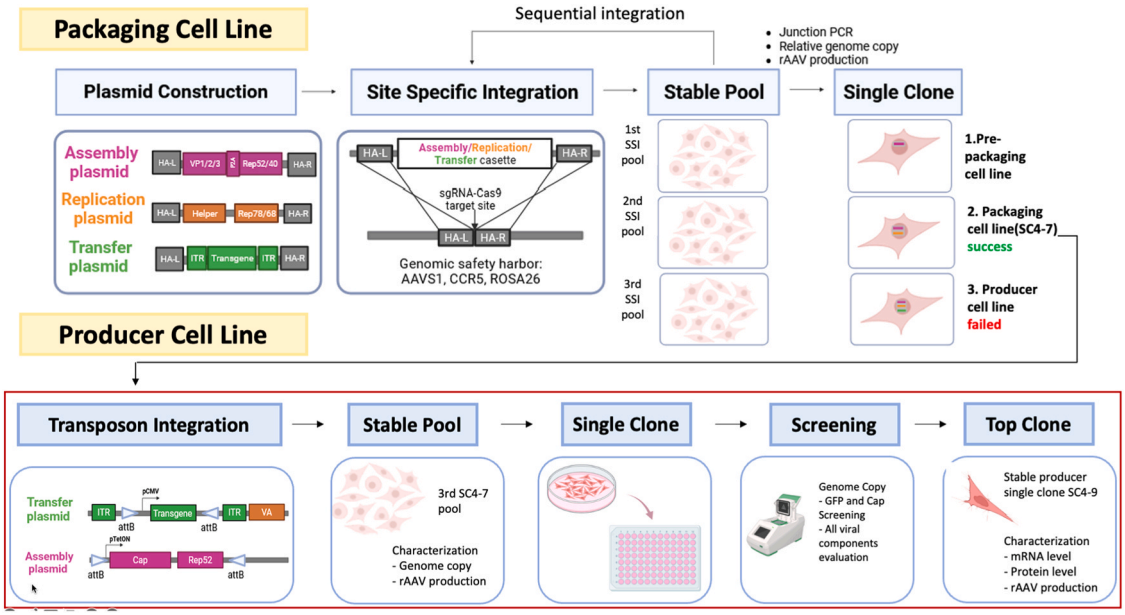

Fig. 1. Integration strategy and workflow for developing the stable producer cell line. The upper panel depicts the workflow used to develop the stable packaging cell line in the previous study [5], while the lower panel (highlighted in the red box) shows the workflow for generating the stable producer cell line in the current study.

the red box) shows the workflow for generating the stable producer cell line in the current study. Our goal was to establish a stable producer cell line by performing transposon-mediated integration of the transfer cassette (containing GFP/transgene) and the assembly cassette (containing Cap) into the isolated packaging cell line (highlighted as "SC4–7 packaging cell line" in Fig. 1) [5]. To achieve this goal, two strategies were evaluated for stable integration, one was to integrate the assembly and transfer transposon donor vector sequentially (later abbreviated as "3rd SC4–7 seq pool"), and another was to integrate the assembly and transfer transposon donor vector at one integration experiment (later abbreviated as "3rd SC4–7 double pool"). For simplicity, these two transfection strategies were not included in Fig. 1. The stable cell pools were generated after stable transfection of transposase and transposon donor vectors and antibiotic selection. Genome copy number of all viral components and rAAV productivity were first assessed for stable cell pools. After confirming the detection of viral genome and capsid titer signal, single clone isolation was further conducted.

The clones were screened for their GFP and Cap copy numbers. Clones with genome copy numbers comparable or higher than those in the stable pools were selected and assessed for the genome copies of all viral components. The top-performing clone was identified and thoroughly characterized, including genome copy analysis, genome/capsid titer, cytotoxicity, and cell line stability.

3.2. Plasmid construction

Transposon donor vectors containing transgene and assembly cassette were constructed. A few bacteria clones, typically 5–6, were selected for banking and plasmid extraction following Gibson assembly and transformation. Gel electrophoresis was utilized to verify and screen the size of the extracted plasmids from the clones. Clones displaying the correct plasmids size were sent out for the whole plasmid sequencing. Clones with confirmed sequences were filtered for transposon-mediated integration. The corresponding plasmid maps were provided in Supplementary Figure 1.

3.3. Stable pool evaluation

Reconstructed transfer and assembly donor cassettes were transfected along with hyperactive PiggyBac transposase plasmid in packaging cell line (2nd SC4–7). Both the double-transfection (3rd SC4–7 double pool) and sequential-transfection (3rd SC4–7 seq pool) approaches for delivering the reconstructed transfer and assembly donor cassettes were evaluated. In the double-transfection method, the transfer and assembly donor cassettes are co-transfected simultaneously, followed by two weeks of antibiotic selection. In the sequential-transfection method, the assembly donor plasmid is first integrated and selected for two weeks, after which the transfer donor plasmid is transfected and subjected to an additional two weeks of antibiotic selection. Antibiotic concentration, blasticidin, was elevated to 15 μg/mL to select cells with higher copy number of assembly cassettes. Puromycin (2 μg/mL) was used to select for cells containing transfer cassettes. After antibiotic selection, the stable pool was established and evaluated.

Genome copy analysis of all viral components for stable pool was conducted. The absolute Cq value for all viral genes were shown in Table 1. The relative fold change before and after transposon integration were demonstrated in Fig. 2(a) & (b). The viral components integrated from the replication cassettes had comparable genome copies in the stable cell pool as observed in the HEK293T packaging cell line (2nd SC4–7). This suggests that no replication genes were lost following transposon integration. Additionally, the copy number of Cap gene in stable pool increased up to 3.1-fold, compared to prior packaging cell line; and the copy number of GFP in stable pool increased up to 38-fold, compared to producer cell pool after three rounds of site-specific integration (SSI 3rd pool). It is noted that the genome copy data for the "SSI 3rd pool" were cited from the previous study [5]. Given that GAPDH served as the internal reference gene across all analyses, the referenced data are suitable for direct comparison.

rAAV production was then evaluated for the stable pool. After the addition of 5 μg/mL doxycycline, the stable cell pool achieved 1.4E+10 vg/L genome titer and 1.3E+11 cp/L capsid titer (Fig. 2(c) & (d)). For both transfection strategies (double and sequential), the Cap and GFP gene copy numbers showed minor differences, and the resulting genome titers and capsid titers were comparable (Fig. 2c & 2d).

Furthermore, the genome titer generated by the stable cell pool was much lower than that achieved by the packaging cell line after induction. The excessively high and potentially inaccurate genome titer in the packaging cells might result from the incomplete digestion of the replicated genome and transgene plasmids during the sample treatment [5]. Additionally, capsid titer in the stable pool was consistent with the

Table 1
Relative genome copy analysis summary of viral components for stable cell pools.

qPCR assay Cq	Internal Reference	Replication			Assembly		Transfer
n = 3	GAPDH	Rep68	DBP	E4orf6	Cap	Rep52	GFP
2nd SC4–7	25.20 ± 0.06	22.09 ± 0.12	22.03 ± 0.07	21.67 ± 0.12	23.79 ± 0.05	21.14 ± 0.05	
3rd SC4–7 seq pool	25.12 ± 0.02	22.34 ± 0.56	21.93 ± 0.05	21.50 ± 0.03	23.19 ± 0.02	20.78 ± 0.11	18.84 ± 0.08
3rd SC4–7 double pool	25.74 ± 0.05	22.45 ± 0.05	22.41 ± 0.07	21.98 ± 0.08	22.72 ± 0.13	20.88 ± 0.11	18.93 ± 0.09
SSI 3rd pool	24.47 ± 0.21	26.17 ± 0.17	24.81 ± 0.1	25.25 ± 0.01	23.4 ± 0.73	25.06 ± 1.11	22.92 ± 0.05

Footnote: 2nd SC 4–7: single clone, packaging cell line from prior work [5]; 3rd SC 4–7 seq and double pools: the transposon mediated producer cell pools generated by "sequential-transfection" and "double-transfection" strategies; SSI 3rd pool: site- specific integration mediated producer cell pool and Cq data is from from prior work [5].

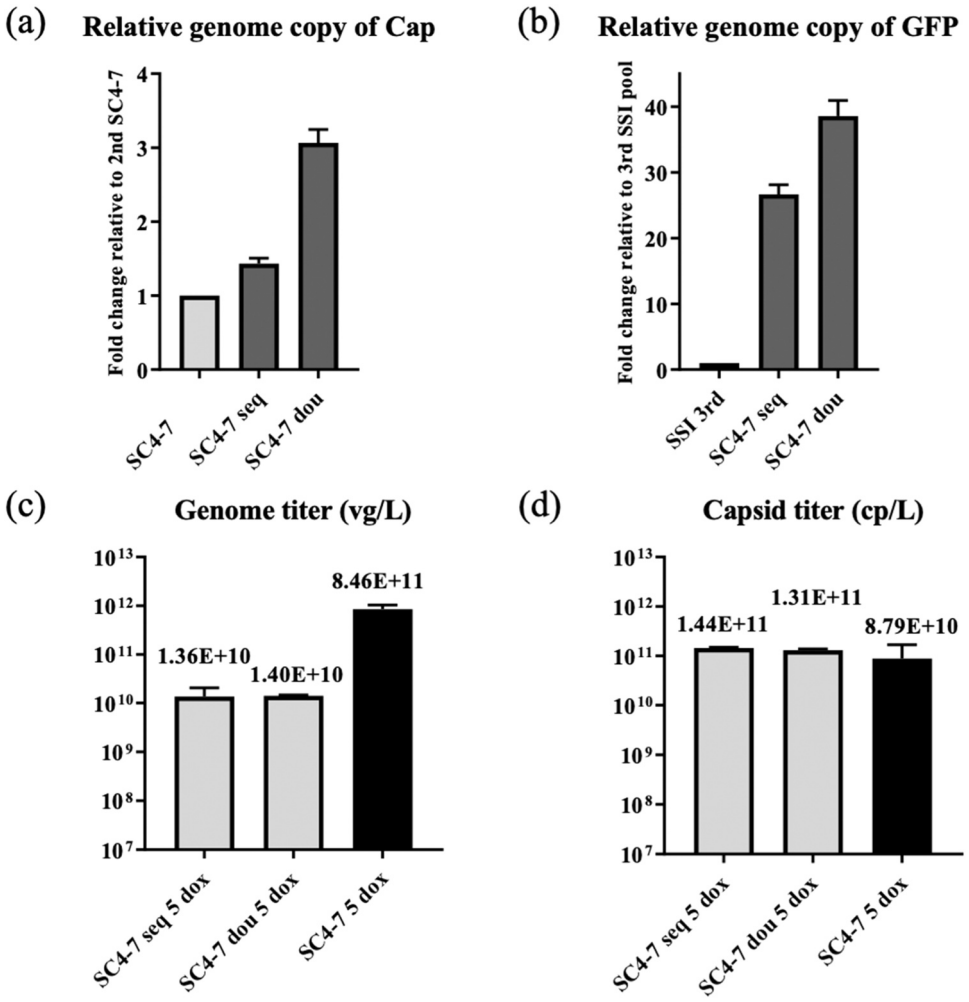

Fig. 2. (a) & (b) Relative genome copy analysis for Assembly (Cap gene) and Transfer (GFP) cassettes. SC4–7 is the packaging cell line developed in the previous study [5]. The SSI 3rd is the third-round site-specific integration stable cell pool developed in the previous study [5]. SC4–7 dou and SC4–7 seq are the 3rd SC4–7 double pool and 3rd SC4–7 seq pool respectively which were detailed specified in the results section. (c) Genome titer in vg/L harvest at 68 h after transfection (HPT). The SC4–7 5 dox is the packaging cell line with one plasmid transfection to produce rAAV with the addition of 5 μg/mL dox [5], and the SC4–7 5 dox genome titer data is from previous study [5]. (d) Capsid titer in cp/L at 68 HPT. The SC4–7 5 dox capsid titer data is from previous study [5].

results shown in the genome copy analysis, comparable and slightly higher than that in the packaging cell line.

3.4. Single clone isolation and genome copy screening

Given the comparable genome and capsid titer, and to simply the transfection process, we proceeded with a double transfection strategy (referred as "3rd SC4–7 double pool"), in which individual transposons containing the assembly and transfer cassettes were co-transfected together with the transposase plasmid, for single clone isolation and characterization. Approximately 500 wells were plated for single clone isolation. Out of 500 clones, 37 clones were expanded and screened for their genome copies of GFP and Cap. With transposon-mediated integration, we expect higher homogeneity compared to cell line generated by random integration. Fig. 3 showed the relative genome copies of GFP and Cap for all 37 clones, compared to these for the stable pool. The result suggested that only 3 clones out of 37 clones achieved comparable or higher genome copy of GFP and Cap than that in stable pool. 2 clones (SC4–9, SC1–8) along with other 2 randomly selected clones (SC2–2, SC2–3) were evaluated for their genome copies of all viral components, details shown in Table 2. It further confirmed that viral components (e.g. Rep68, DBP and E4orf6 from replication cassette) were retained from

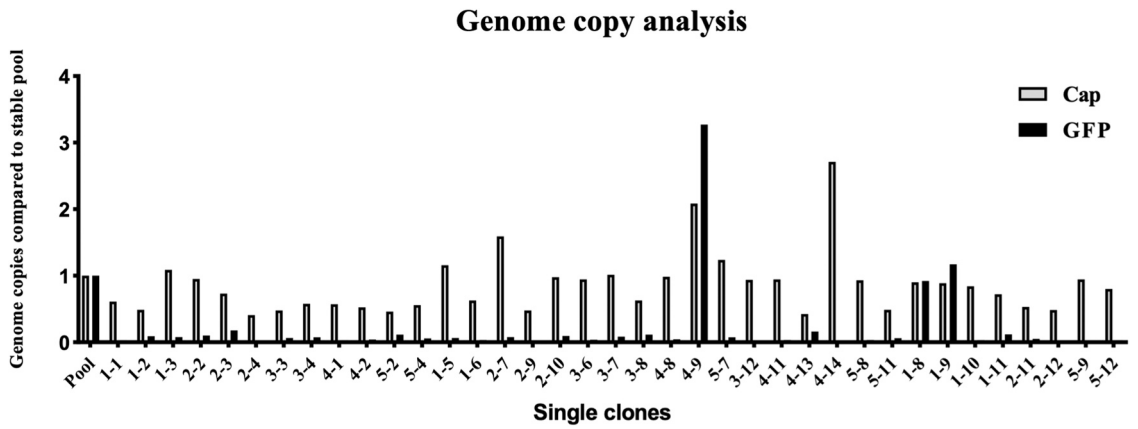

Fig. 3. Single clone screening via relative genome copy assays. The pool is the 3rd SC4–7 double pool, and others are single clones.

Table 2
Relative genome copy analysis summary of viral components for selected single clones.

qPCR assay Cq	Internal Reference	Replication			Assembly		Transfer
n = 3	GAPDH	Rep68	DBP	E4orf6	Cap	Rep52	GFP
3rd 2–2	24.72 ± 0.05	21.9 ± 0.04	21.62 + 0.00	21.25 ± 0.11	22.93 ± 0.02	20.51 ± 0.13	21.75 ± 0.02
3rd 2–3	24.57 ± 0.02	21.59 ± 0.07	21.76 ± 0.06	21.19 ± 0.03	23.25 ± 0.40	20.53 ± 0.10	20.81 ± 0.13
3rd 4–9	24.31 ± 0.07	21.18 ± 0.03	21.59 ± 0.06	21.04 ± 0.05	21.56 ± 0.05	19.43 ± 0.08	16.59 ± 0.00
3rd 1–8	24.09 ± 0.02	21.23 ± 0.06	21.21 ± 0.05	20.7 ± 0.04	22.29 ± 0.15	20.12 ± 0.00	18.16 ± 0.05
3rd SC4–7 double pool	25.74 ± 0.05	22.45 ± 0.05	22.41 ± 0.07	21.98 ± 0.08	22.72 ± 0.13	20.88 ± 0.11	18.93 ± 0.09

Footnote: Using transposon-mediated producer cell pools ("3rd SC 4–7 double pool") as a reference, the selected single clones (3rd 2–2, 2–3, 4–9, 1–9) were subjected relative genome copy analysis of viral components.

the packaging cell line and viral components (e.g. Cap and GFP) were comparable/higher than stable pool.

3.5. Single clone 4-9 characterization and rAAV production

SC4–9 was selected as the top clone due to the high copy number of Cap and GFP gene (Table 2 and Fig. 2), and fully characterized for rAAV production. The addition of 5 μg/mL doxycycline inducible reagent activated the viral gene expression and initiated the rAAV production, following the prior packaging cell line development study [5]. The relative transcript level of these viral components was measured by RT-qPCR before and after induction. The transcript level of all viral components, controlled by inducible promoters, achieved 20–80-fold increase after doxycycline induction, as shown in Fig. 4.

The absolute Cq value for SC4–9 before induction shown in Table 3 demonstrated the detection of viral gene transcription without the addition of doxycycline and indicated the leakage of the designed inducible circuit. The vector production capability of this stable cell line was further evaluated by measuring genome and capsid titers. Genome titer reached 3.73E+11 vg/L and capsid titer reached 2.01E+11 cp/L after the addition of doxycycline, achieving 27-fold and 1.4-fold increase respectively compared to non-induction condition (Fig. 5). Limited increase in Cap expression before and after induction (Fig. 5(b)) aligned with the transcript level changes described in Fig. 4, with the Cap gene showing the smallest increase. Additional translation and viral protein assembly steps contributed to the nonlinear relationship between fold changes in transcript level and in capsid titer. Capsid titer was detected under the 0-dox condition, indicating leakage of the designed circuit in Fig. 5(b). Compared with previously reported packaging cell line [5], which shown minimal or undetectable capsid expression before induction, our current stable producer system exhibits significantly higher pre-induction capsid level. This is likely due to the higher genomic copy number of integrated assembly cassettes, increasing the probability of detectable baseline genome copy number, mRNA expression, and capsid production before induction. Limited

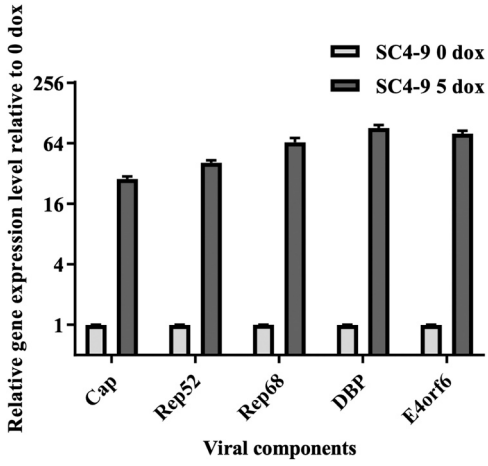

Fig. 4. Transcript level of viral genes fold change with 0 and 5 μg/mL dox induction at 68 HPT for producer single clone (SC4–9), normalized to housekeeping gene GAPDH. Key viral components include Cap, Rep52, Rep68, DBP, and E4orf6. Presented numbers for dox represent the actual concentration of inducers used in μg/mL. Data represent the mean and standard deviation of triplicate wells (n = 3).

amount of assembled capsids remained as a bottleneck for the low productivity of the established stable producer cell line. It was also noted that genome titer (3.73E+11 vg/L) was higher than capsid titer (2.01E+11 cp/L) (Fig. 5). The higher genome titer observed in this case is likely primarily due to differences in assay sensitivity at low-titer ranges. Specifically, trace amounts of excess transgene integrated into the host cell genome may not have been fully digested by benzonase and DNase, and were subsequently detected and amplified by the qPCR assay. This effect is further amplified in low-titer profiles due to the sensitivity of qPCR, whereas such trace amounts would be negligible at

Table 3
Transcript Cq value summary of all the viral components in the single clone producer cell line SC4–9 with 0 and 5 μg/mL doxycycline induction via RT-PCR assay.

RT-qPCR assay Cq	Internal Reference	Replication			Assembly	
n = 3	GAPDH	Rep68	DBP	E4orf6	Cap	Rep52
3rd SC 4–9 0 dox	16.98 ± 0.06	21.93 ± 0.02	22.81 ± 0.12	21.96 ± 0.09	21.9 ± 0.06	19.08 ± 0.14
3rd SC 4–9 5 dox	18.27 ± 0.03	17.2 ± 0.12	17.56 ± 0.08	16.91 ± 0.09	18.37 ± 0.01	15.05 ± 0.13

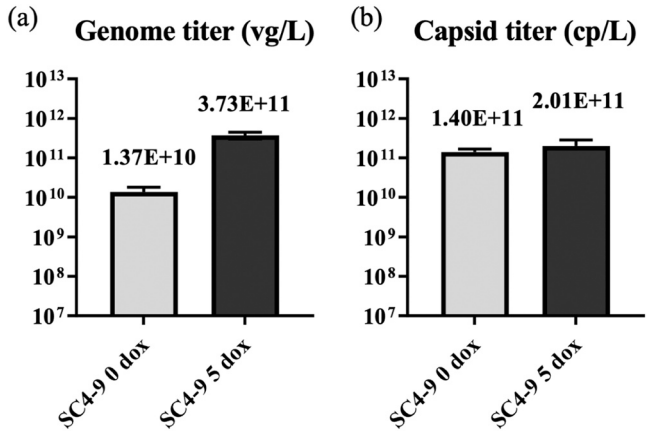

Fig. 5. Genome titer (a) and capsid titer (b) evaluation for producer single clone SC4–9 with 0 and 5 μg/mL dox induction. Error bars represent the standard deviation of biological triplicates (n = 3).

conventional transient transfection titers above 1 × 10^{13} vg/L, based on the validated qPCR assay we developed [4]. In addition, the sensitivity and limitations of the capsid ELISA assay at low titers should also be considered when interpreting the discrepancy between genome and capsid titers.

The cell growth performance and the cell line stability of the established stable producer cell line were further assessed, with results presented in Fig. 6. For growth performance (Fig. 6(a)), both the parental and producer cell line were seeded at the same initial density (5E+4 cells per well) in 12-well plates. Viable cell density (VCD) and cell viability were monitored daily over 7 days. SC4–9 exhibited much slower growth rate compared to the 293 T parental cells, indicating potential leakiness in viral component expression that may contribute to cytotoxicity. Regarding cell line stability (Fig. 6(b)), SC4–9 was maintained for over 10 passages, with genome titers evaluated every five passages. The results demonstrated comparable titers, remaining within a similar order of magnitude throughout the 10 passages.

4. Discussion

4.1. Rationale and progress compared to prior study

The purpose of this study is to establish a stable producer cell line for rAAV production. In the previous study, we were able to develop a stable packaging cell line with the design of an inducible circuit and site specific integration method [5]. The expression of viral components, such as Rep and helper genes, were controlled under an inducible circuit, to minimize the cytotoxicity. Viral components required for rAAV production were refactored to three cassettes based upon their functionality (replication, assembly, and transgene cassettes) and then integrated sequentially to pre-validated genome loci via site-specific integration [5]. The packaging cell line was derived after site specific integration of replication and assembly cassettes [5]. It requires additional transfection of transgene cassette for rAAV production and thus allows the easy switch between different transgenes [5]. Due to the large insertion size, only 2 clones out of total 38 clones got further confirmed with targeted integration [5]. The introduction of the doxycycline resulted in a significant increase in transcript levels of integrated viral components, yielding a genome titer of 8.46E+ 11 vg/L and a capsid titer of 8.79E+ 10 cp/L [5]. However, we failed to establish the stable producer cell line: after 3rd round of integration, it was observed that only limited amount of capsids was produced, and no genome titer signal was detected at all in the 3rd round integration stable pool [5]. This might result from inadequate viral replication due to the low transgene copy numbers.

Genome replication is the one key limitation, as site-specific integration produces significantly fewer genome copies compared to the high numbers achieved through transient plasmid transfection. The detection of capsid confirmed the capsid production, but the relatively low amount of assembled capsids along with limited copy number of transgenes might be the reason for no genome titer in the stable producer pool derived by multiple rounds of site-specific integration. The relatively low packaging efficiency, an inherent barrier in rAAV production, further contributes to the undetectable genome titer [12]. Thus here, we proposed to conduct additional transposon-mediated integration in the previous derived packaging cell line, integrating additional cap gene and transgene.

Although random integration emerges as the gold standard for generating stable cell lines, it is demonstrated that this approach introduces significant challenges to cell line development process, such as cell-to-cell variability [24], transgene rearrangement [25], loss of copy number [26], and lack of molecular mechanisms. Increased risk of inducing cell line instability through random integration indicates the necessity of developing and utilizing alternative technologies.

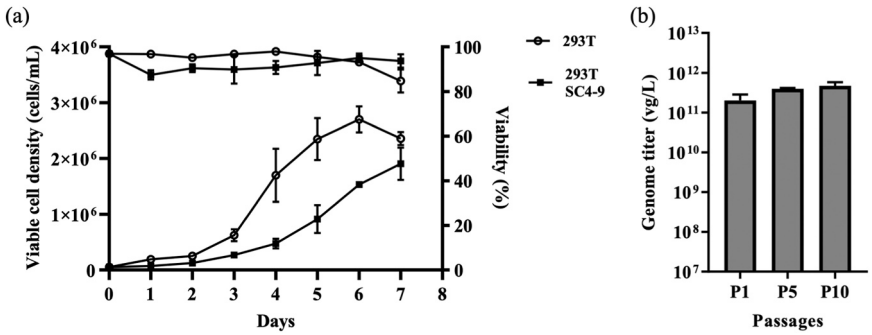

Fig. 6. Cell growth performance (a) and cell line stability (b) evaluation for stable producer single clone SC4–9. In Fig. 5(a), the bottom two curves represent viable cell density, while the top two curves depict cell viability. Error bars represent the standard deviation of biological triplicates (n = 3).

Transposase-mediated genomic integration is such a method that can allow increased level of transgene integration and better stability [27]. By utilizing a hyperactive transposase, an optimized transposon, and optimal co-transfection ratio, it is expected that 50–100 copies of the insert will stably integrate into the host genome. This increase in genome copy of gene of interest is anticipated to boost the genome and capsid titer accordingly.

Overall, transposon mediated integration of assembly and transgene cassettes in the packaging cell line increased the copy number of Cap gene up to 3-fold and GFP up to 38-fold. The antibiotic concentration was increased to further select for cells with a higher number of integrated assembly cassettes. The limited increase in Cap gene expression could be attributed to the presence of pre-existing site-specific integrations, hindering the effective selection of cells containing high copy numbers. The increase in genome copy of GFP transgene was as expected. One outstanding single clone was isolated and characterized for rAAV production. The isolated stable producer cell line was able to result in 3.73E+ 11vg/L genome titer and 2.01E+ 11 cp/L capsid titer, leaving significant room for improvement in the future study. This was only a proof-of-concept study aimed at stablishing the baseline producer cell.

4.2. Stable producer cell lines reported in the literature and advances

Similar synthetic biology approach has been applied before for rAAV production: multiple inducible promoters were used to control the expression of viral components; three separate modules were integrated into HEK293s cells by Leap-in transposase mRNA mediated transfection. The baseline they isolated was about two orders magnitude titer lower than traditional triple-based production [10]. Independent control over replication and packaging activity by varying the inducer levels and altering the viral components led to higher capsid titer [10]. To further understand the bottlenecks that limit the inducible cell line productivity, they conducted a comprehensive analysis of viral production kinetics using proteomics and other physical assays of viral components. They show that reducing the excessive expression of transgene resulted in higher genome and capsid titer. With additional optimization of induction profile and the addition of proteasome inhibitor, the rAAV produced reached the similar level as that in transient transfection [12]. Finally, multi-omics analysis further demonstrated that the balance of viral genome and capsid protein was key to productivity and quality. Thus sequential integration of multiple copies of cap gene allowed the cap gene expression, further boosting rAAV productivity, leading to the final inducible rAAV high producing cell line [28].

4.3. Future optimization and improvement

In our scenario, the titer achieved in the baseline producer cell was significantly lower than obtained by traditional transient transfection with commercially available plasmids. That indicates the potential for optimization and improvement in inducible system. With the use of a single type of Tet-on promoter, it can reduce the need for multiple inducible reagents [10] and simplify the process, but fine-tuning gene expression levels and timing to meet optimal production requirements becomes more complex. To better regulate the gene expression level and timing, the inducible promoters can be tuned by the number and the spacing between of tetO sites [29–31]. Protein expression control can be achieved by screening Kozak sequence variants and modulating the translational efficiency [32,33]. Additionally, a systematic characterization of rAAV production in inducible cell line can be conducted to identify potential factors that restrain the productivity. Corresponding tuning strategies can then be applied to increase the productivity of these synthetic cell lines.

More comprehensive characterization and method development are also needed, including detailed assessment of viral protein expression, AAV potency, and functional particle analysis. Additionally, analytical methods for genome and capsid titers should be further optimized to ensure accuracy and reliability.

5. Conclusion

In conclusion, this study successfully developed an inducible stable producer cell line for AAV production using a combination of site-specific and transposon-mediated integration approaches. Upon the addition of the inducing reagent, transcript levels of integrated viral components significantly increased, resulting in a genome titer of 3.73E+11 vg/L and a capsid titer of 2.01E+11 cp/L. However, cell growth was notably slower compared to the parental cells, likely due to the leakage of toxic viral genes. Stability testing confirmed that the cell line remained stable for at least 10 passages. Future research will focus on optimizing gene expression levels and timing for various viral components, as well as reducing toxic gene leakage, to further enhance the productivity and efficiency of the inducible rAAV-producing stable cell line.

CRediT authorship contribution statement

Qiang Fu: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Emily Doleh:** Methodology. **Yongdan Wang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Seongkyu Yoon:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Mark Blenner:** Supervision.

Author contributions

QF, YW and SY conceived the manuscript. QF and YW performed the experiments. QF and YW analyzed the data. QF and YW composed the manuscript. MB and SY provided input on data analyses. ED, MB and SY edited the manuscript. All authors read and approved the final manuscript.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

This work was funded and supported by the Advanced Mammalian Biomanufacturing Innovation Center (AMBIC) through the Industry–University Cooperative Research Center Program under the U.S. National Science Foundation (Grant number 2100075). The research project was also funded by NIH (Grant Number, R21EB036789). We would like to express our gratitude to all AMBIC member companies for their mentorship and their financial support. This work was also partially funded by the Massachusetts Life Science Center (MLSC). We also would like to thank the plasmids gift from Dr. Gyun-min Lee lab in Korea Advanced Institute of Science & Technology (KAIST). We also thank Mingli Hou from The UMass Lowell Core Research Facility (CRF) for her kind support.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2026.110144.

Data availability

Data will be made available on request.

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