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Whitepaper

Addressing plasmid DNA challenges in large-scale manufacturing of recombinant adeno-associated virus and lentivirus using enzymatically generated dbDNA

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Executive summary

Advances in gene therapy have increased the use of recombinant adeno-associated viruses (rAAV) and recombinant lentiviruses (rLV) as gene delivery vectors, but challenges remain in scaling up their production due to reliance on plasmid DNA (pDNA). Traditional pDNA manufacturing methods face bottlenecks, including scalability issues and regulatory concerns related to bacterial contaminants and antibiotic resistance genes.

This study explores the use of doggybone DNA (dbDNA™), a synthetic alternative to pDNA, for producing rAAV and rLV. The results indicate that dbDNA is a promising solution for overcoming limitations associated with pDNA in large-scale viral vector manufacturing.

Key takeaways:

- pDNA production is hindered by scalability challenges and regulatory concerns over bacterial contamination and antibiotic resistance.
- dbDNA™, produced synthetically without bacterial intermediates, bypasses these concerns and enhances manufacturing efficiency.
- dbDNA™, demonstrated similar or improved rAAV and rLV production yields compared to pDNA while reducing transfection reagent usage.
- Purification of dbDNA-generated vectors matched pDNA performance, with no significant impact on vector quality or potency.
- dbDNA™, offers a scalable, safe alternative for large-scale viral vector manufacturing, meeting industry needs for more efficient production.

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Introduction

Significant advancements in cell and gene therapy over the past decade have established recombinant adeno-associated viruses (rAAVs) and recombinant lentiviruses (rLVs) as popular gene delivery vectors for treating various diseases. By mid-2023, six rAAV-based and eight rLV-based therapies had been approved worldwide, with more approvals expected.[1]

The most commonly used method to produce rAAV and rLV is through transient transfection of plasmid DNA (pDNA) carrying viral and therapeutic genes in production cells. Although improvements have been made over the years to meet the growing clinical demand, the supply of DNA continues to be a bottleneck for large-scale cGMP manufacturing. Additionally, there are regulatory concerns for using plasmids generated in bacteria e.g. the presence of antibiotic resistance genes and the immunogenic risk of CpG sequences to patients.[2]

An alternative to pDNA is the use of enzymatically generated DNA sequences such as Touchlight's synthetic DNA known as doggybone DNA (dbDNA™). dbDNA is synthesized using a proprietary rolling circle in vitro method thereby bypassing the concerns surrounding the use of bacteria. Moreover, Touchlight also offers several quality grades of synthetic DNA for development and commercial-scale manufacturing. With these features, dbDNA is a promising solution for the current supply-related bottleneck and concerns relating to generating pDNA using bacterial fermentation methods.[3]

The goal of this study was to assess the production and quality of rAAV and rLV generated using custom dbDNA synthesized using Thermo Fisher Scientific's pDNA sequences, which were optimized for rAAV and rLV production.

Materials and methods

Cell culture, transfection, and harvest

Gibco Viral Production Cells (Cat. No. A35347) and Gibco Viral Production Cells 2.0 (Cat. No. A49784) were used in the production of rLV and rAAV2 respectively. Suspension cells were thawed and cultured in production medium for a minimum of five passages. Cells were sub-cultured twice a week at cell densities suitable for 3-day or 4-day cultures in shake flasks. Cell densities were measured using the Vi-CELL BLU Cell Viability Analyzer (Beckman Coulter). Transfection was performed in shake flasks with culture volumes ranging from 50 mL to 800 mL. For rAAV production, cells were transfected with Thermo Fisher Scientific's control pDNA (Cat. No. A47672) or custom dbDNA using the Gibco AAV-MAX Transfection Kit (Cat. No. A51217). For rLV production, cells were transfected with in-house pDNA as the control or custom dbDNA using the Gibco LV-MAX Transfection Kit (Cat. No A35348). rAAV plasmid or dbDNA constructs encoded the emGFP transgene, the Ad5 helper genes and rep2cap2 packaging genes, whereas rLV constructs encoded for the CD19-CAR transgene and LP1, LP2, VSVG packaging genes.

Transfection details are listed in **Table 1** and **Table 2**. Cultures containing rAAV were harvested 3 days post-transfection and subjected to detergent lysis and nuclease treatment followed by clarification. Clarified samples were quantified for vector genome titer (VG titer) and capsid titer. Cultures containing rLV were harvested 2 days post-transfection and subjected to nuclease treatment followed by clarification. Clarified samples were quantified for infectivity (IU titer) and p24 particle titer.

Table 1. Transfection conditions evaluated for rAAV production

Tranfection parameter	A	B	C	D	E	F	G	H	Control
DNA type	dbDNA								pDNA
Molar ratio	#1							#2	
Total DNA (µg/mL)	0.3	0.3	0.9	1.2	1.2	1.5	1.5	1.2	1.5
AAV-MAX transfection reagent (µL/mL)	1.2	6	3.6	1.2	6	1.2	6	6	6

Table 2. Transfection conditions evaluated for rLV production

Tranfection parameter	A	B	C	D	E	F	G	H	Control
DNA type	dbDNA								pDNA
Molar ratio	#1							#2	
Total DNA (µg/mL)	0.3	0.3	0.7	0.7	0.9	1.5	1.5	0.7	2
LV-MAX transfection reagent (µL/mL)	0.9	6	0.9	6	3.45	0.9	6	6	6

Purification

Parallel purifications were conducted for rAAV and rLV generated using dbDNA and pDNA. rAAV purification was conducted using an affinity media whereas rLV purification was conducted using an anion exchange (AEX) media. For all purifications, clarified harvest was loaded onto the corresponding purification media followed by media wash and product elution steps. To evaluate rAAV purification performance, clarified harvest and elution samples were tested for vector genome titer and capsid particle titer. rAAV in the elution sample was assessed for infectivity by TCID50. For rLV purification assessment, clarified harvest and elution samples were tested for infectivity and particle titer. Purification performance was assessed by vector recovery in the elution step relative to the amount of vector loaded onto the purification media.

T-cell transduction

T-cells were isolated from apheresis material using the CliniMACS Prodigy™ with CliniMACS™ CD4 and CD8 microbeads. Post-isolated T-cells were counted and activated at a 1:1 ratio with CD3/CD28 Dynabeads™. Twenty-four hours after activation, transductions were performed using CD19- CAR rLV generated using clarified harvest produced using dbDNA or pDNA as described above. Modified T-cells were cultured in OpTmizer™ T-cell expansion media for 3 days and then quantified for CAR expression using the Attune Cytpix flow cytometer.

Vector genome titer

Samples were analyzed by Droplet Digital PCR (ddPCR), a method for performing digital polymerase chain reaction (PCR) that is based on water-oil emulsion droplet technology. The samples were treated with DNase I to remove any exogenous DNA. DNase was inactivated with heat and the treated samples were subjected to a Proteinase K digestion to release DNA within the viral capsid. Serial dilutions were then performed to bring the concentration of the samples to the range of the instrument. Diluted samples were combined with a master mix containing primers and probe specific to the region of interest. The diluted sample were fractionated into 20,000 droplets, and PCR amplification of the template molecules occurred in each individual droplet. Following PCR, each droplet was analyzed or read by an optical detection system to determine the fraction of PCR positive droplets in the original sample. These data were then analyzed using Poisson statistics to determine the target starting DNA template concentration in the original sample.

Capsid titer

The quantitation of AAV capsid in the preparation was based on an immunoassay using a two-antibody (sandwich) AAV2 Titration Enzyme-Linked Immunosorbent Assay (ELISA) kit (Progen Cat. No. PRATV). A monoclonal antibody specific for a conformational epitope on assembled AAV capsids is coated onto microtiter strips and is used to capture AAV particles. Captured AAV were detected in three steps; first, a biotin-conjugated monoclonal antibody to AAV was bound to the immune complex. In the second step, streptavidin-peroxidase conjugate bound to the biotin molecule from the biotinylated antibody. Lastly, the addition of a colorimetric substrate solution (Tetramethylbenzidine, TMB) resulted in a color reaction which was proportional to the amount of the specifically bound vector particles. The absorbance was measured spectrophotometrically at 450 nm. The AAV capsid titers were then quantified by comparing the absorbance of the test article to the absorbance of a standard curve generated by serial dilutions of a known concentration stock.

P24 particle titer

The quantitation of rLV was based on an immunoassay using a two-antibody (sandwich) Enzyme-Linked Immunosorbent Assay (ELISA) method performed with the Lenti-XTM p24 Rapid Titer Kit (Takara Bio Cat. No. 631476). A monoclonal antibody specific for the p24 capsid protein was coated onto microtiter strips and was used to capture p24 particles. Captured p24 particles were detected in three steps; first, a biotin-conjugated monoclonal antibody for p24 was bound to the immune complex. In the second step, a streptavidin-peroxidase conjugate bound to the biotin molecule from the biotinylated antibody. Lastly, the addition of a colorimetric substrate solution (TMB) resulted in a color reaction which was proportional to the amount of specifically bound viral particles. The absorbance was measured spectrophotometrically at 450 nm. The p24 particle titers were then quantified by comparing absorbance of the test article to the absorbance of a standard curve generated by serial dilutions of a known concentration stock.

TCID50

HeLa RC32 cells (expressing AAV2 rep/cap), seeded in a 96-well plate, were infected with serial dilutions of vector in a TCID50 format. Cells were co-infected with Adenovirus 5 (Ad5). In the presence of Ad5, the vector replicates using the rep/cap proteins that are constitutively expressed in the HeLa RC32 cells. After incubation time, cells in each well were lysed and qPCR was performed to determine the vector genome copies in each well for each dilution of the vector. Spearman-Karber analysis was performed to determine the infectious titer of the vector. Cells containing Ad5 alone and medium alone were the two negative controls for the assay.

IU titer

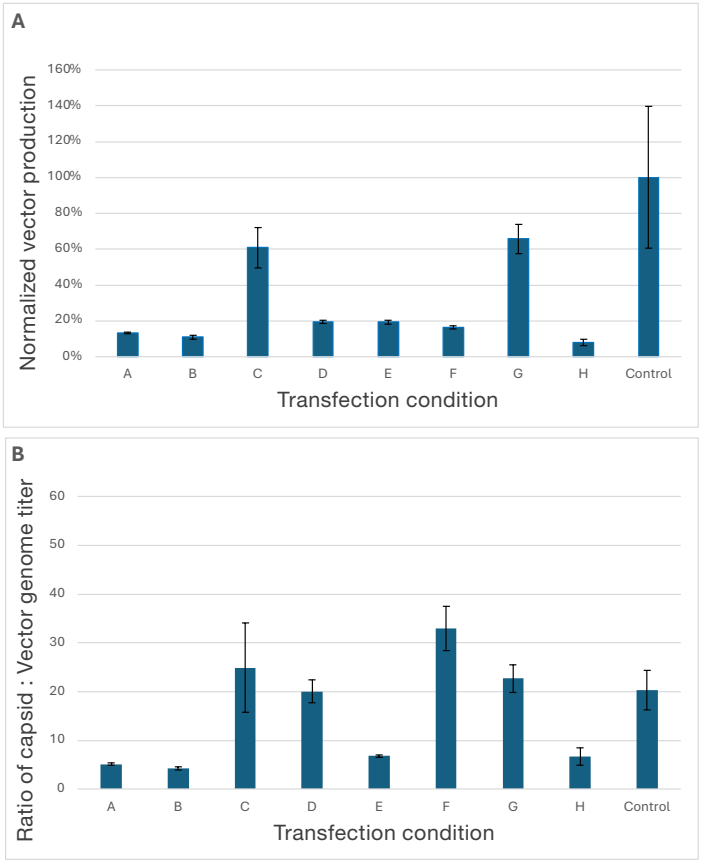
Replicate wells of a 96-well plate containing HEK-293T cells, seeded at 4.0E+04 cells /well, were infected with dilutions of samples and incubated at 37°C for 20 hours. After the incubation period, a DNA extraction solution was added to each of the wells and incubated through a series of temperatures to complete extraction and neutralize the lysis solution. Once inactivated, the presence of rLV and housekeeping gene in each well was determined using ddPCR. DNA extracted from the infected cells was analyzed by ddPCR using primers and probe specific to the target sequence in the test article and the housekeeping genes in the cells.

Results

rAAV production and purification

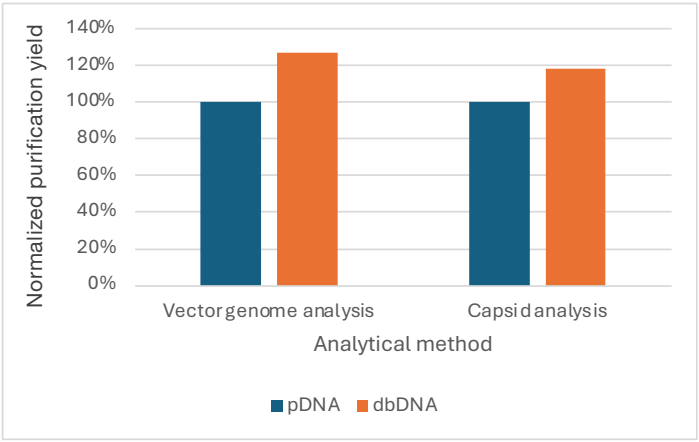
To assess rAAV2 production using dbDNA, several transfection conditions were evaluated and compared to pDNA as a control. Variable rAAV2 product yields and genome packaging profiles, measured by ratio of capsid titer to vector genome titer, were obtained across the various conditions as shown in **Figure 1A** and **Figure 1B**. The results from the clarified harvest conditions showed that molar ratios (Condition E vs Condition H), amounts of transfection reagent (Condition F vs Condition G) and total DNA (Condition B vs Condition G) impact rAAV2 production yields. Two transfection conditions (Condition C and Condition G) produced yields **(Figure 1A)** and genome packaging profiles comparable to the Control condition **(Figure 1B)**. One condition, Condition C, was purified by affinity chromatography and compared to the Control condition. Purification recovery evaluation by capsid titer and vector genome analysis showed comparable performance **(Figure 2)**. Infectious titer analysis of the eluates produced by Condition C (3.24E9 TCID50/mL) and the Control condition (1.12E9 TCID50/mL) verified production of infectious rAAV particles.

Figure 1. Comparison of rAAV2 production yields using dbDNA or pDNA



Various transfection conditions were used to produce rAAV2 using the AAV-MAX transfection kit. rAAV2 production was assessed in clarified harvest samples from ≥ 2 shake flasks by vector genome and capsid titer analysis. (A) Production yields using dbDNA were normalized to the vector genome titers obtained using pDNA (100%). (B) Assessment of portion of full particles by obtaining a ratio of capsid titer to vector genome titer.

Figure 2. Comparison of purification yields from clarified harvest containing rAAV2 produced using dbDNA or pDNA



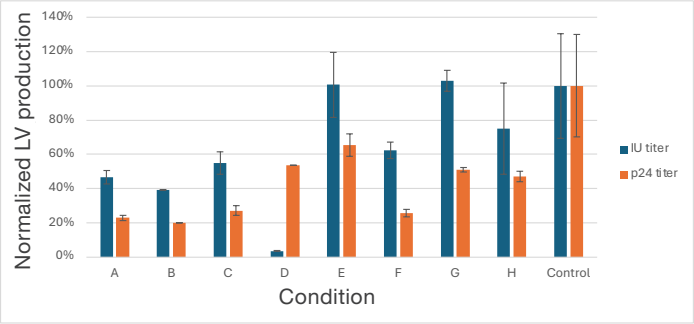
Vector genome analysis and capsid titer analysis were conducted on eluate and clarified harvest samples from the affinity purification (n=1). Purification recoveries were normalized to the pDNA purification recovery as a control (100%).

rLV production, purification and CAR T-cell production

To assess rLV production using dbDNA, several transfection conditions were evaluated and compared to pDNA as a control. Like rAAV, variable rLV yields were obtained across the different conditions as shown in **Figure 3**. The results showed that Condition E had yields comparable to the Control condition by infectious titer and p24 particle titer analysis. Clarified harvest produced using this condition underwent AEX purification. Comparison of product recovery based on infectious titer and p24 particle titer showed that harvest produced using dbDNA performs comparably to pDNA-produced harvest **(Figure 4)**.

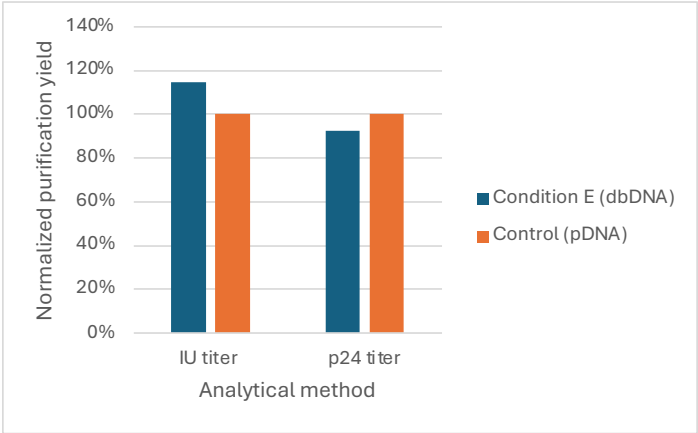
To assess the transduction efficiency of the rLV, production of CAR T-cells by T-cell transduction using rLV clarified harvest was evaluated by flow cytometry analysis using CAR marker antibodies. Comparison of CAR T-cells generated by transducing with clarified harvest produced using either dbDNA (Condition E) or the plasmid Control showed no difference in transduction efficiency **(Figure 5)**.

***Figure 3.* Comparison of rLV production yields using dbDNA or pDNA**



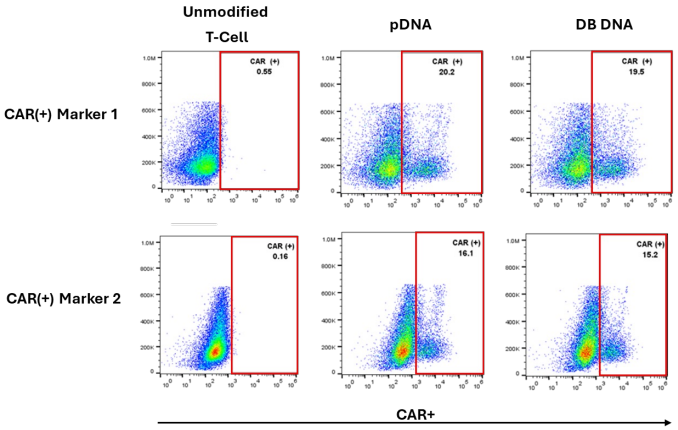
rLV production was assessed in clarified harvest samples from ≥ 2 shake flasks using infectivity and p24 particle titer analysis. Production yields using dbDNA were normalized to the plasmid control condition (100%).

Figure 4. Comparison of purification yields from clarified harvest containing rLV produced using dbDNA or pDNA



Infectious and particle titer testing was conducted on the clarified harvest and elution samples from the anion exchange purification (n=1). Purification recoveries were normalized to the pDNA purification recovery as a control (100%).

Figure 5. Generation of CAR T-cells from pDNA and dbDNA CD19-CAR rLV



CAR T-cells were generated from CD19-CAR rLV produced using either pDNA or dbDNA. CAR expression was measured 72 hours post-transduction using two different CAR marker antibodies and analyzed on the Attune CytPix. Flow cytometry histograms display an unmodified T-cell control and CAR T-cells transduced using pDNA produced rLV or dbDNA produced rLV, each measured using two different CAR markers.

Conclusions

Rapid solutions are required to overcome the current challenges associated with the use of pDNA in industrial manufacturing. This study assessed the use of dbDNA as an alternative to pDNA used in viral vector production. The results show that dbDNA generated comparable production yields to pDNA in viral vector production, using less total dbDNA and less transfection reagent. Additionally, no impacts were observed on purification process performance with the resulting purified vector product having strength similar to pDNA-generated product.

For vector production using pDNA or dbDNA, further optimization of transfection conditions can be implemented to reduce raw material amounts while increasing production yields. These approaches, combined with strategies to reduce material costs and cycle time, will address current gaps in viral vector production.

References

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