neDNA™, a Robust and High-Quality DNA Supply for rAAV Manufacturing

P340

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Introduction

Recombinant adeno-associated virus (rAAV) is used to develop therapies to treat genetic human diseases. The manufacture of rAAV relies on triple transfection of plasmid DNA (pDNA), a raw material that is expensive and difficult to obtain at industrial scale. At TAAV, we produce enzymatic DNA, neDNA™, as an alternative to plasmid.

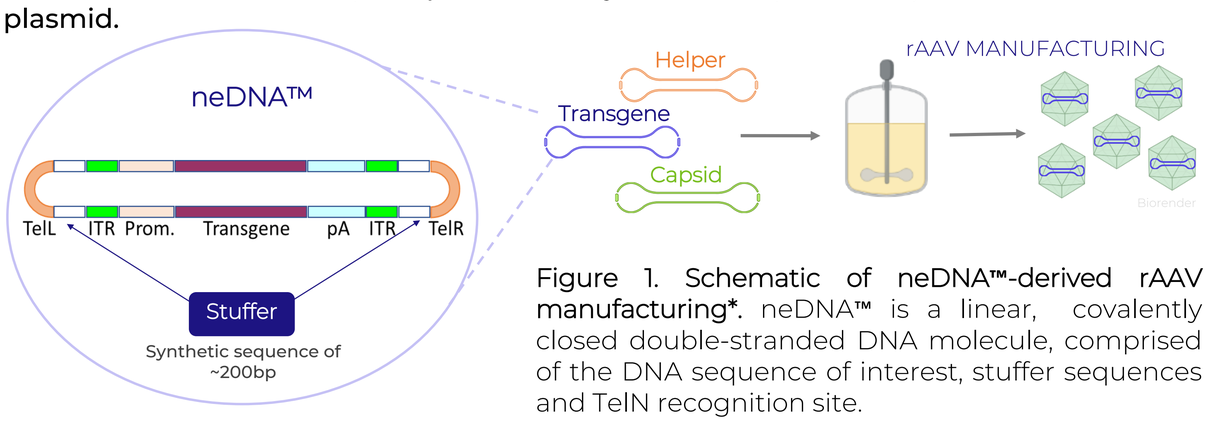


Figure 1. Schematic of neDNA™-derived rAAV manufacturing*. neDNA™ is a linear, covalently closed double-stranded DNA molecule, comprised of the DNA sequence of interest, stuffer sequences and TelN recognition site.

The current work aims to highlight the advantages of using neDNA™ as critical starting material to resolve the challenges in rAAV production.

POTENTIALLY SAFER

HIGHER YIELDS

SCALABLE & ROBUST

FASTER TO MANUFACTURE

rAAV Production* Using neDNA™ *Versus* Plasmid

*Data generated at AskBio

Figure 4. neDNA™ *versus* plasmid-derived rAAV Production. 30mL scale runs using plasmid *versus* neDNA™ were set up with T001 (CMV-Lux-2A-eGFP) Transgene, Rep-Cap, and XX680 Helper constructs. The ratios were fixed and concentration of total DNA was evaluated. All results were normalized to pDNA 100% condition for each serotype.

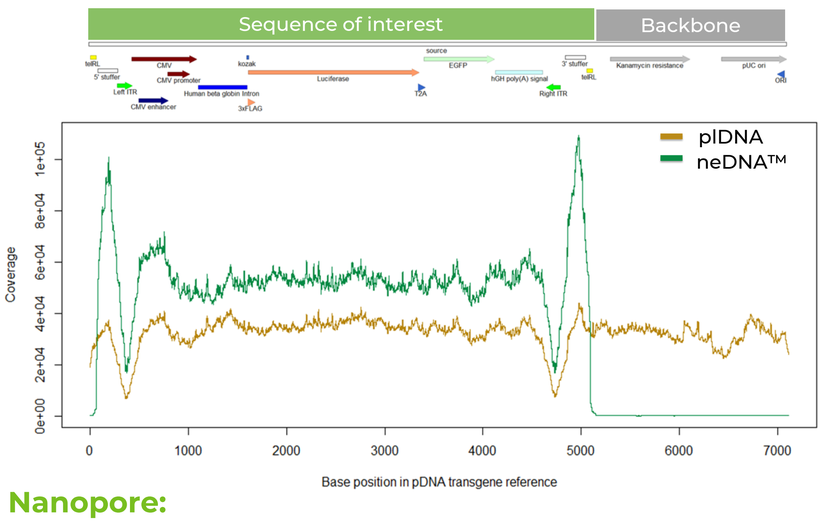
neDNA™-derived AAV-X and AAV-Y achieved **comparable to higher titers**, using **lower DNA quantity**.

For more information visit AskBio poster P143: *Comparative analysis of neDNA™ and plasmid DNA for recombinant Adeno-Associated Virus (rAAV) Production*

Characterization of neDNA™ Final Product by High-Throughput Sequencing

Two high-throughput sequencing technologies, Next Generation Sequencing (NGS) with the Illumina platform and Nanopore, were used to confirm construct identity of neDNA™ final product. ITR-containing transgene neDNA™ final product was sequence interrogated and compared to the precursor template counterpart.

NGS: Illumina Platform



Condition	EGFP	Kan^R	Backbone (%)
pIDNA	32999.7	32882.1	99.64
neDNA™	51489.3	9.4	<1

Figure 2. Next Generation Sequencing characterization of neDNA™ final product *versus* precursor template (plT001-CMV-Lux-2A-eGFP). Illumina iSeq100 System obtained >33k as median coverage for precursor template and >50k as median coverage for neDNA™ (>Q13 = 74.13%).

Nanopore:

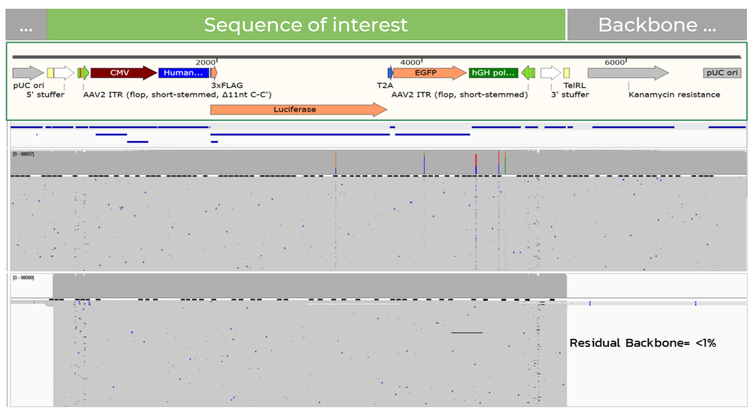


Figure 3. Nanopore characterization of neDNA™ final product *versus* precursor template (plT001-CMV-Lux-2A-eGFP). Nanopore obtained >275k total reads for the precursor template and >430k total reads for neDNA™ (>Q13=~95% accurate; reads are longer than 4.5kb) .

Both sequencing methods demonstrated that:

- In the manufacturing process of neDNA™, the DNA sequence is amplified with **high fidelity**, including complex molecular structures such as AAV-ITRs. With these analyses **no mutations** were detected in neDNA™ final product compared to the precursor template.
- In neDNA™ final product, **residual backbone is almost non-detectable (<1%).**

neDNA™ Workflow, Manufacturing and Specifications

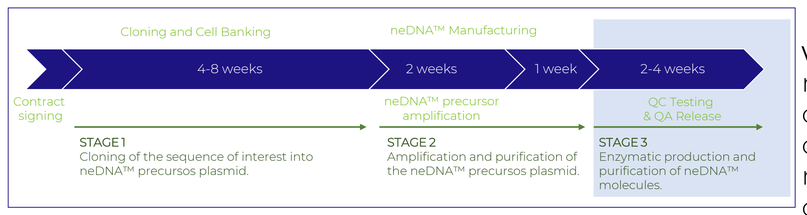
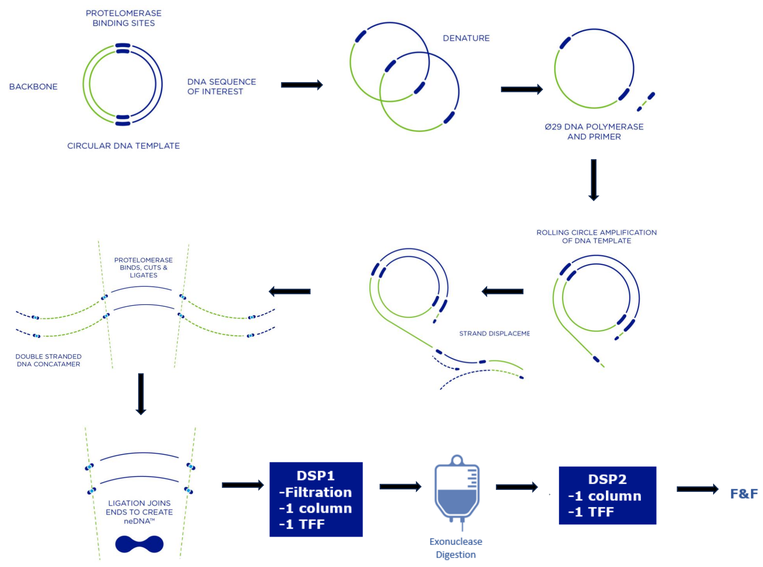


Figure 2. neDNA™ general workflow. neDNA™ manufacturing process consists of three different stages: cloning of the sequence of interest into neDNA™ precursor, amplification of the precursor template and manufacturing of the neDNA™.

Figure 3. neDNA™ manufacturing process. neDNA™ manufacturing process relies on different enzymatic and purification steps: rolling circle amplification (RCA) of the precursor template by Phi29 polymerase, precursor backbone processing by restriction enzymes, covalent closure of both ends by TelN protelomerase, and removal of residual DNA by exonucleases. The process includes chromatographic and tangential flow filtrations (TFF) to eliminate enzymes and DNA residuals.



*Technology for making neDNA™ is licensed from Touchlight IP Ltd.

Category	Parameter	Method	Advanced characterization
Appearance	Visual appearance	Visual inspection of color and particulates	
Quantity	Concentration	UV spectrophotometry 260nm	
Identity	Identity A- DNA sequence Identity B - Size confirmation	Sanger sequencing Image analysis- AGE densitometry	High Troughput Sequencing
Purity	Purity	Image analysis- AGE densitometry	HPLC
Impurities - Product related	Related Substances	Image analysis- AGE densitometry	
Impurities -Process related	Impurity A: Resitual TelN Impurity B: Residual Protein Content Impurity C: Relative Protein Content	ELISA microBCA UV 260/280 ratio	Analytical methods to detect Phi29 and Exonucleases
Microbiological contamination	Endotoxin Bioburden Sterility * Mycoplasma *	EP 2.6.14 Method D EP 2.6.12 EP 2.6.1 EP 2.6.7	
General tests	pH Osmolality	EP 2.2.3 EP 2.2.35	*Upon client's request

Residual Analyses: AskBio *in vivo* Stuffer Analyses

neDNA™ molecule presents a stuffer sequence (see Figure 1) that allows measurement of residual DNA in rAAV products by qPCR or Droplet Digital PCR (ddPCR). The stuffer is a synthetic sequence of around 200 bp. AskBio performed an *in vivo* experiment to determine whether the stuffer sequence adds promoter activity to the cassette.

Day 0 — Hydrodynamic injection

Day 1 — Organ/tissue collection

Analyses:
- Liver plasmid copy number
- Liver luciferase expression (mRNA)
- Luciferase protein activity

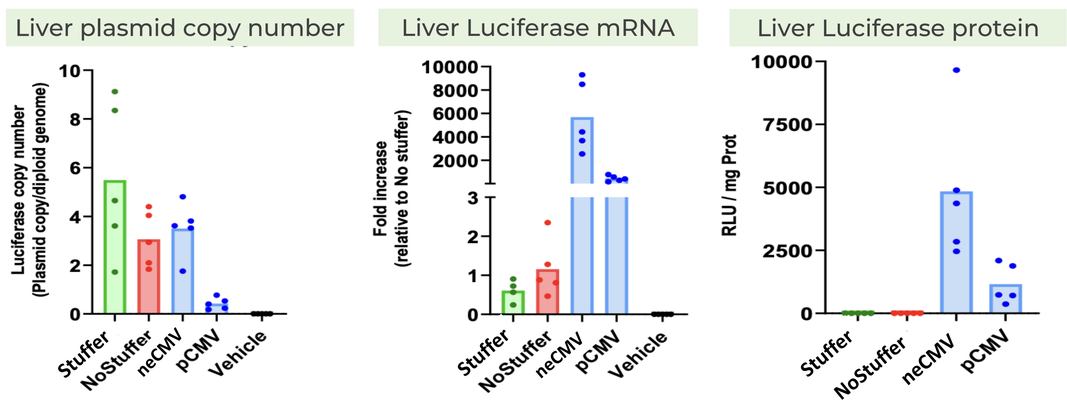


Figure 7. *In vivo* analyses performed by AskBio. Different luciferase constructs were directly inoculated: neDNA™ with and without stuffer and, as controls, neDNA™ and plasmid with CMV promoter.

- Constructs with and without stuffer sequence showed comparable results in terms of luciferase expression (mRNA). Furthermore, its expression was 5000 times lower than that driven by the CMV promoter.
- No luciferase protein activity was detected from the stuffer construct.

Stuffer does not add promoter activity to the cassette.

Conclusions

Data shown in the present study demonstrate that neDNA™ can resolve many of the challenges rAAV production presents, including:

- neDNA™ enzymatic process amplifies the precursor template with high fidelity, including complex molecular structures such as AAV-ITRs.
- Residual KanR is almost undetectable. High-throughput sequencing and qPCR analyses have consistently shown <1% KanR residual sequences in neDNA™.
- Using neDNA™ for rAAV manufacturing results in comparable to higher titers than plasmid-derived vectors.

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