neDNA™ is a Robust Alternative to Plasmid DNA for AAV Production

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Introduction

The most popular method for production of recombinant adeno-associated virus (AAV) vectors is through transfection of three plasmids, one containing the gene of interest flanked with AAV inverted terminal repeats, one encoding the AAV rep and cap genes, and one encoding the adenovirus helper functions. However, the use of plasmid DNA leads to contamination of the AAV products with sequences of bacterial origin that may cause safety issues. As an alternative, these three sequence elements can be provided by other DNA starting materials. In this study, we evaluated the use of neDNA™, i.e., linear DNA molecules with closed ends that do not contain bacterial sequence elements. We compared side-by-side plasmids and neDNA™ for AAV production in the Pro10™ cell line, using different AAV capsid serotypes and productions scales. Vectors potencies were also compared in an in vivo study conducted in mice.

Materials and Methods

DNA starting materials. The plasmids used in the study are the adenovirus helper plasmid pXX680 (Xiao et al. 1998), plasmids encoding the AAV-2 Rep proteins and the capsid proteins of different AAV serotypes, and pTR-lux2A-GFP which contains a luciferase and GFP expression cassette flanked with AAV-2 inverted terminal repeats (ITR) sequences. Plasmids were manufactured by Aldevron (Fargo, ND). The neDNA™ used for comparison contained the same helper, rep-cap and lux2A-GFP sequences, respectively, and were manufactured by TAAV Biomanufacturing Solutions (San Sebastian, Spain).

AAV vectors production. The AAV vectors were produced through PEI-mediated transfection of Pro10™ cells (Grieger, 2006), using either three plasmids or three neDNA into 2-liters or 50-liters cell suspension cultures. Cells were harvested after 72 hours and disrupted using non-ionic detergent. AAV particles were purified from clarified lysates through POROS CaptureSelect AAVX or AAV9 affinity chromatography, then affinity eluates were adjusted to neutral pH and submitted to discontinuous iodixanol gradient ultra-centrifugation (Zolotukhin, 1999). Full AAV particles were collected from the interface of the 40 to 60% iodixanol layer and submitted to ion exchange chromatography using POROS 50HQ resin. Purified vectors were formulated into phosphate buffer saline containing 0.001% Poloxamer 188 through dialysis (2-liters scale) or tangential flow filtration (50-liters scale).

Vector genomes concentration. For vector genome titration, AAV samples were treated with DNase I at 37°C, then digested with proteinase K at 55°C followed by enzyme heat-inactivation at 95°C. AAV vectors genome concentrations were measured by droplet digital PCR (ddPCR, Bio-Rad) using primers and probe targeting the AAV2 ITR (Aurnhammer, 2012).

Viral particles concentrations. Total particles titers were measured using size exclusion chromatography combined with diode array detection in a high-performance liquid chromatography instrument (SEC-HPLC, Agilent). After loading of the AAV sample onto the column (Bio SEC-5 4.6x150mm, 500Å), an isocratic elution is applied to the column, allowing separation of high molecular mass species (HMMS) from AAV particles, which can be quantified by their UV absorbance. Particles titers are calculated using a standard curve prepared with an AAV standard of known concentration, as determined by ELISA, analyzed in the same run. In addition, calculation of the A260/A280 ratio allows evaluation of the ratio between full and empty particles and comparison of packaging efficiency between samples.

Full particles quantification. Cryo-TEM analyses of AAV samples for quantification of full and empty particles were performed by Vironova BioAnalytics AB (Stockholm, Sweden).

AAV vectors *in vivo* potency. Assessment of vectors potency in mice was performed using two different AAV serotypes, denominated AAV-X and AAV-Y, both produced at the 2L scale with the lux2a-GFP transgene using plasmid DNA or neDNA. The study was conducted at CIMA, University of Navarra (Pamplona, Spain). Animals were distributed in 10 groups and injected intravenously (iv) via the tail vein with AAV-X and AAV-Y vectors at a dose of 1E+12 vg/kg (n=6) or with the formulation buffer (n=4). Five groups were sacrificed 28 days after injection and the other 5 groups after 112 days. After injection, in vivo luciferase activity was measured on days 7, 14, 21, 28, 56, 84 and 112. Necropsy and organ sampling was performed after 28 and 112 days. Samples from different tissues were collected for protein extraction to measure ex vivo luciferase activity.

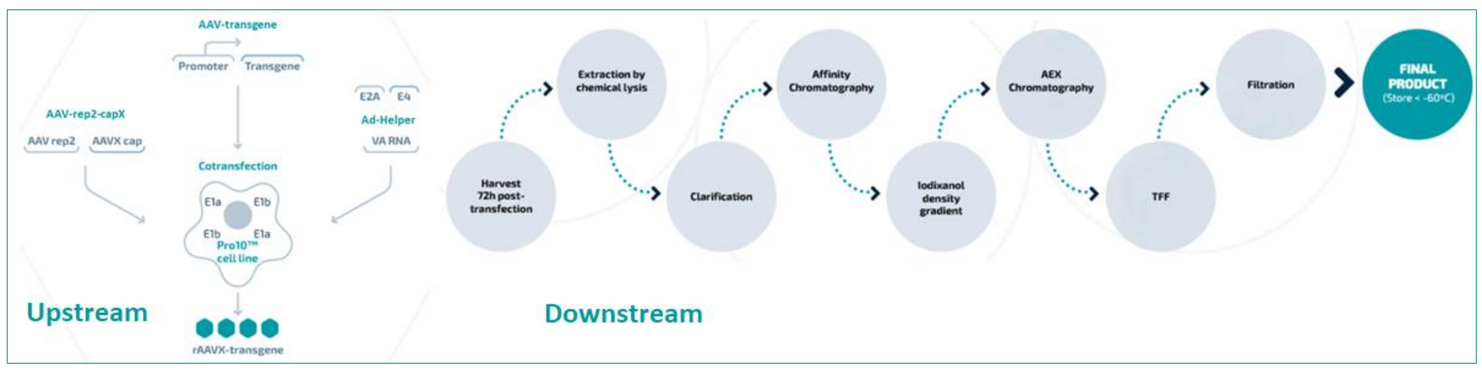

Figure 1. Schematic representation of the AAV manufacturing process. The DNA molecules used for triple-transfection of Pro10™ cells upstream can be either plasmid DNA or neDNA™. The downstream process doesn't change.

Results

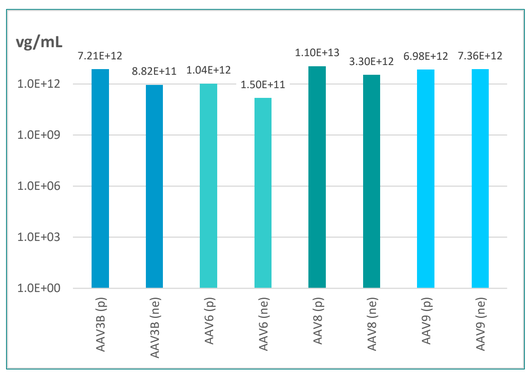

Figure 2. Vector genome titers of different AAV vector serotypes produced at the 2L scale. AAV vectors were produced in 2L bioreactors through transfection of plasmid DNA (p) or neDNA (ne), and after purification, vector genome (vg) titers were measured by ITR ddPCR in the final product.

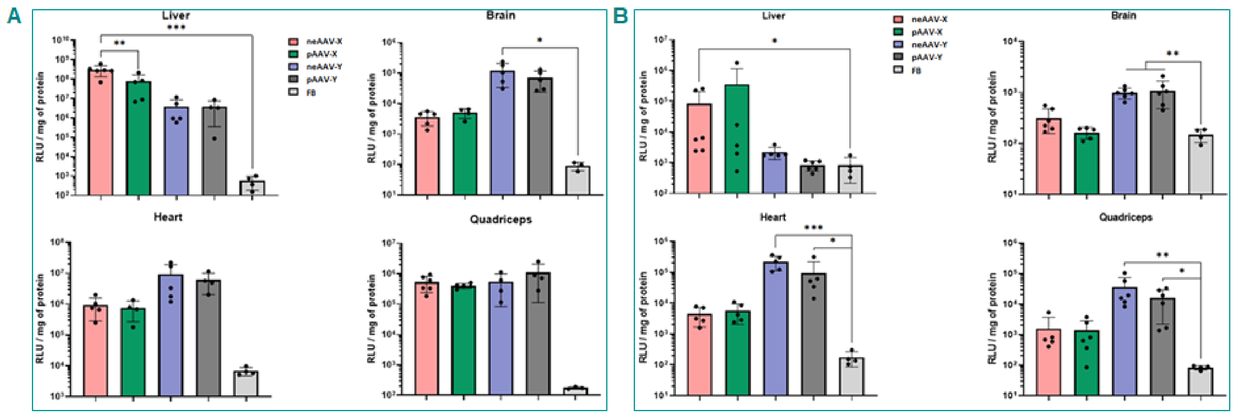

Figure 3. Analysis of *ex vivo* luciferase activity 28 (A) and 112 (B) days after vector administration in protein extracts obtained from liver, brain, heart, and quadriceps. Luciferase activity was normalized with the quantity of total proteins (in mg) and expressed as relative light units (RLU) . Data of individual animals and mean ± SD of each group are shown. One-way ANOVA followed by Holm-Šídák's multiple comparisons test was used if data followed normality and Kruskal-Wallis followed by Dunn's multiple comparisons test if they did not (* $p < 0.0332$, ** $p < 0.0021$ and *** $p < 0.0002$). The vectors neAAV-X and neAAV-Y were produced with neDNA, and pAAV-X and pAAV-Y were produced with plasmids. FB: formulation buffer.

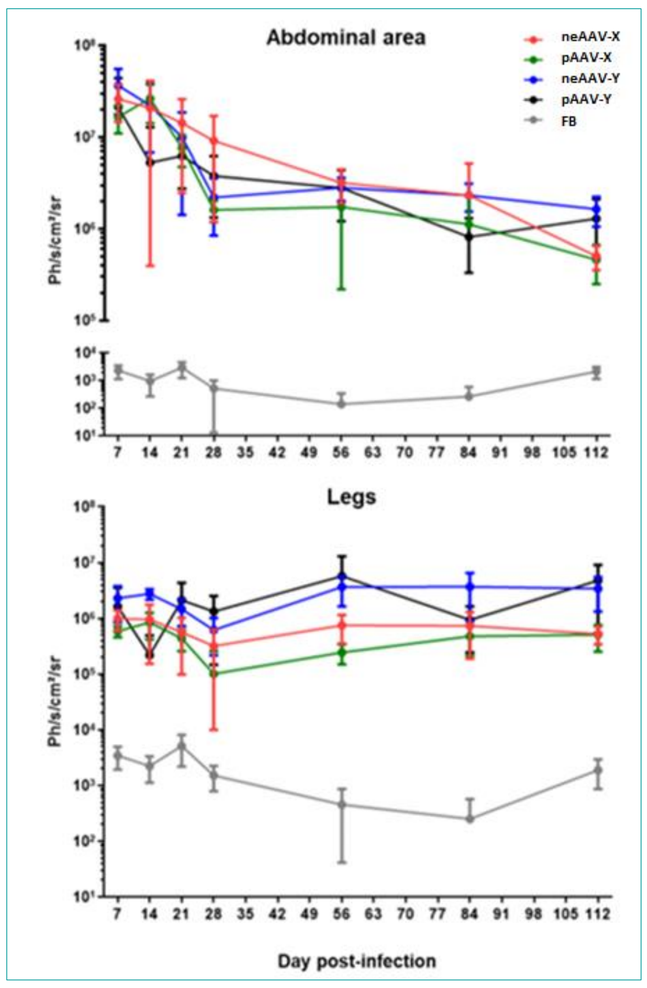

Figure 4. Analysis of *in vivo* bioluminescence. The luminescence signal produced by luciferase activity was measured in vector- or formulation buffer-injected mice over 112 days. Light emission from abdominal area and legs was quantified and plotted as mean ± SD for each group. The vectors neAAV-X and neAAV-Y were produced with neDNA, and pAAV-X and pAAV-Y were produced with plasmids. FB: formulation buffer.

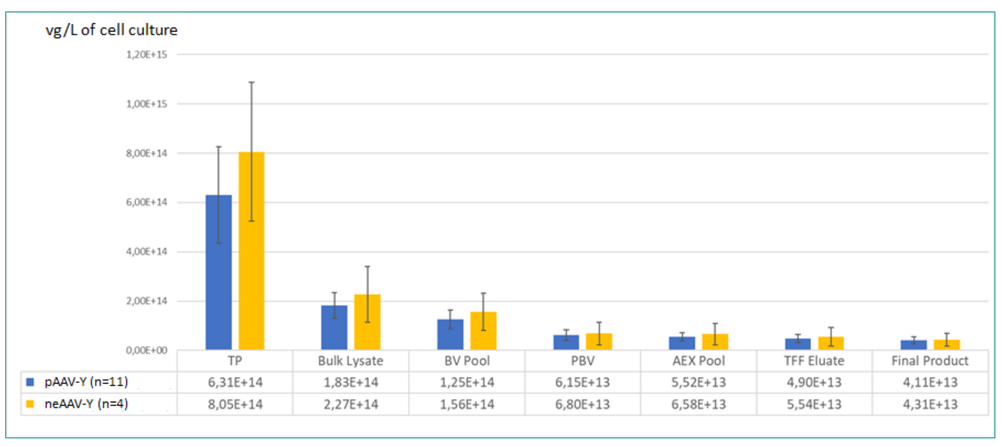

Figure 5. Mean vector genome titers of AAV-Y batches produced at the 50L scale throughout the manufacturing process. AAV vectors were produced in 50L bioreactors through transfection of plasmid DNA (pAAV-Y) or neDNA (neAAV-Y), and vector genome (vg) concentrations were measured by ITR ddPCR at each process step. Titers were calculated as vg per liter of cell culture. TP: transfection pool at harvest; Bulk Lysate: clarified lysate; BV Pool: bulk virus pool after affinity chromatography and pH adjustment; PBV: purified bulk virus after iodixanol gradient ultracentrifugation; AEX pool: anion exchange chromatography pooled fractions; TFF eluate: after buffer exchange to formulation buffer; Final Product: after sterile filtration.

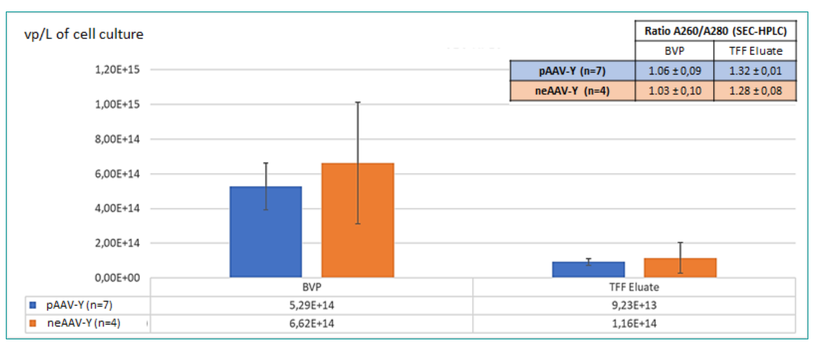

Figure 6. Mean viral particles titers of AAV-Y batches produced at the 50L scale at two process steps. AAV vectors were produced in 50L bioreactors through transfection of plasmid DNA (pAAV-Y) or neDNA (neAAV-Y), and viral particles titers (vp) concentrations were measured by SEC-HPLC after affinity chromatography and pH adjustment (BVP), and after anion exchange chromatography and buffer exchange by tangential flow filtration (TFF Eluate). Titers were calculated as vp per liter of cell culture. The embedded table on the right shows the mean A260/A280 ratio calculated from UV absorbance values.

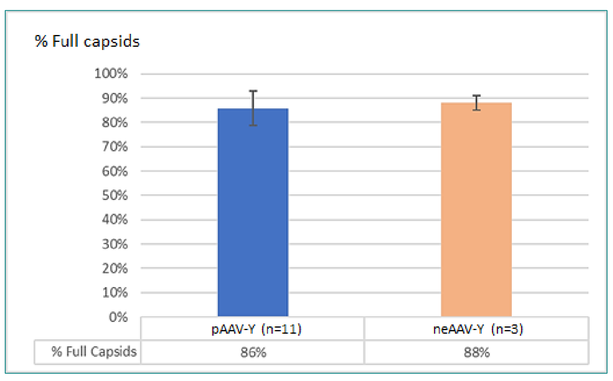

Figure 7. Mean percentage of full particles in AAV-Y batches produced at the 50L scale. AAV vectors produced through transfection of plasmid DNA (pAAV-Y) or neDNA (neAAV-Y). Purified vectors samples (final products) were submitted to cryo-TEM analysis for quantification of full and empty particles.

Discussion

The use of plasmid DNA and neDNA™ was compared for production of different AAV serotypes at the 2-liters scale in Pro10™ cells. Overall, the results show that both type of starting material provide similar yield in our process (figure 2), as well as similar vector quality based on A260/A280 ratio and in vitro infectivity (not shown). Following systemic injection in mice of two different AAV vector serotypes, denominated AAV-X and AAV-Y, vectors produced with neDNA™ show similar or even higher potency compared to those produced with plasmids, based on luciferase activity detected by bioluminescence *ex vivo* in different tissue samples (figure 3) and *in vivo* in live animals (figure 4). Finally, multiple batches of an AAV serotype denominated AAV-Y were produced at the 50-liters scale, showing that transfection of neDNA™ produces similar amounts of encapsidated vector genomes at different process steps (figure 5), and equivalent proportions of full particles during purification, as shown by both SEC-HPLC (figure 6) and cryo-TEM (figure 7) analyses.

Conclusion

Replacement of plasmid DNA with neDNA™ allows production of AAV vectors with similar yields and quality in a transient transfection-based system at different process scale. In addition, neDNA™ improves gene therapy vectors safety profile, since these synthetic DNA molecules that are produced through a cell-free enzymatic process are devoid of plasmid DNA or other contaminants of bacterial origin. In conclusion, our results confirm that neDNA™ offers a competitive alternative to plasmids for production of AAV vectors.

References

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