Use of long-read sequencing to iterate and optimise rAAV vector genome design

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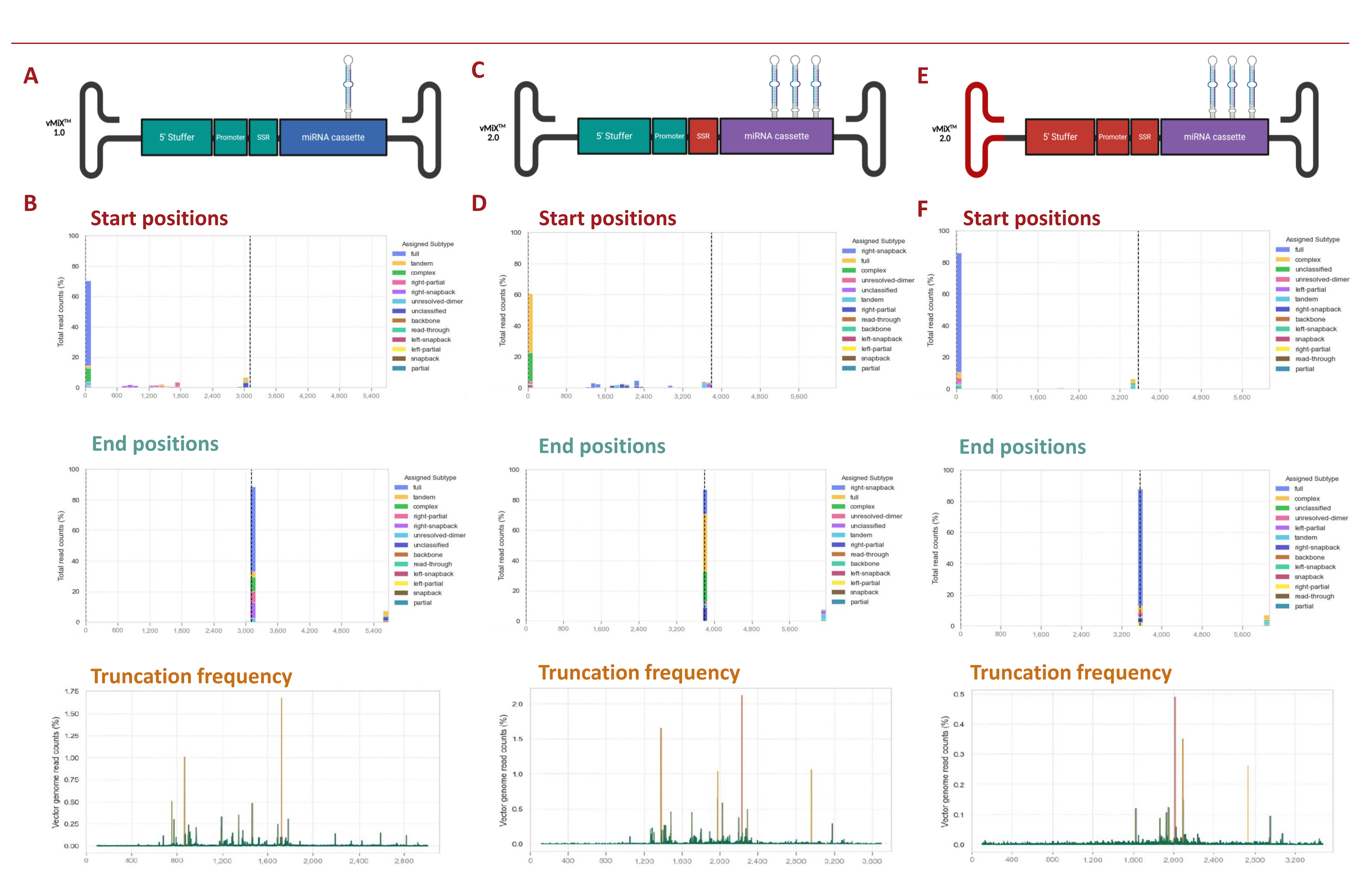
INTRODUCTION

- AVB's proprietary vMiX™ platform makes use of miRNA sequences, vectorised into a rAAV capsid, to target pathogenic genes. In the context of conditions such as amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and Alzheimer's disease (AD), vMiX™ offers a therapeutic approach to silence expression of deleterious genes and prevent neurodegeneration.
- Whilst the first-generation vMiX™ cassette (vMiX™ 1.0) is comprised of a single miRNA-encoding sequence, the second generation, vMiX™ multiplex (vMiX™ 2.0), contains three miRNA-encoding hairpins. This enables simultaneous knockdown of multiple gene targets, or augmented expression of a single miRNA species (Refer to poster P0169).
- Given the structural complexity of the vMiX™ multiplex cassette, we sought to evaluate its propensity for truncation during rAAV packaging. To address this, we applied a multi-modal analytical approach including:
    - Long-read PacBio sequencing for the identification genome truncation hotspots.
        - Bioinformatic analysis was supported by Form Bio.
    - CE-GI LIF analysis for the assessment of genome heterogeneity.
    - SEC-MALS analysis to gauge rAAV virion heterogeneity and titration.
- Redesigning the viral genome reduced truncation frequency, increased full-length genome packaging, and improved vector manufacturability. These findings highlight the utility of long-read sequencing and rational design for the optimisation of rAAV vector quality.

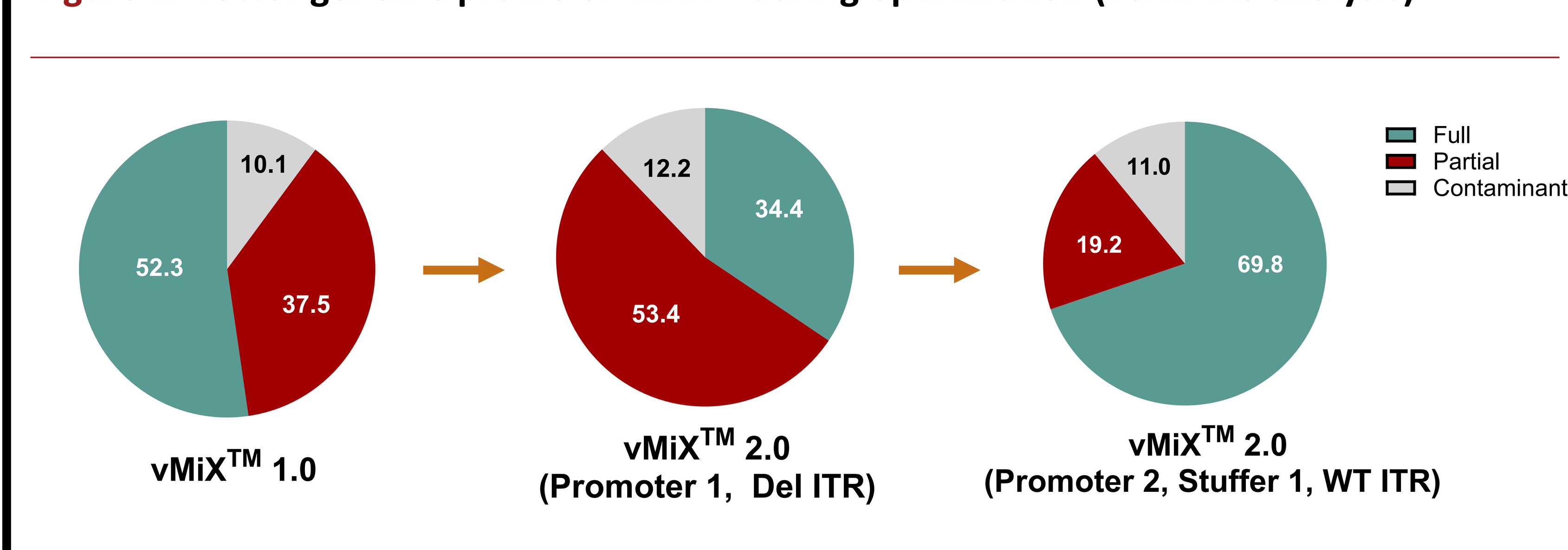
Vector design and optimisation

Figure 1: vMiX™ 1.0 and 2.0 designs and truncation hotspots



- The vMiX™ 1.0 cassette comprises a promoter, scrambled sequence (SSR), and a single miRNA-encoding hairpin. The cassette is flanked by AAV2-derived 5' and 3' ITR sequences; the former was believed to be sub-optimal (Figure 1A).
- Long-read PacBio sequencing of vMiX™ 1.0 rAAV genomes revealed truncation hotspots within the promoter, SSR, miRNA-encoding element, eliciting formation of partial genomes (Figure 1B).
- During optimisation of the vMiX™ 2.0 design, a triple hairpin configuration targeting Genes 1 and 2 were used (the third hairpin was maintained with a stuffer sequence).
- Incorporating the vMiX™ 2.0 triple hairpin configuration in the parental vMiX™ 1.0 backbone, gave rise to vector comprised largely of partial genomes, with only 35% of DNA-containing particles housing the full-length viral genome (Figure 1C, 1D).
- Optimisation of the vMiX™ 2.0 cassette included:
    - Optimisation of the 5' ITR sequence.
    - Modification of the SSR scrambled sequence to preclude putative hairpin formation.
    - Substitution of a GC-rich constitutive promoter (promoter 1) with an alternative neuronal promoter (promoter 2) displaying a lower GC content.
    - Insertion of an optimised stuffer sequence to increase the viral genome size from ~3.1kb to ~3.6-3.8kb – this sequence had been designed to exhibit a low CG-content, whilst containing few splice acceptor and donor sequences.
- The optimized cassette design (Figure 1E) was found to reduce the partial genome population, whilst increasing the proportion of virion particles containing the full-length payload (Figure 1F).
- Around 50% of the vMiX™ 1.0 vector was found to house the full-length payload, whilst the remaining 50% of virion particles contained partial genomes or other contaminant sequences (Figure 2).
- Optimisation of the vMiX™ 2.0 cassette increased the proportion of virion particles containing the full-length payload by 2-fold, whilst reducing the proportion of those containing partial genomes by around 2.5-fold.

Figure 2: Vector genome profile of vMiX™ during optimization (Form Bio analysis)

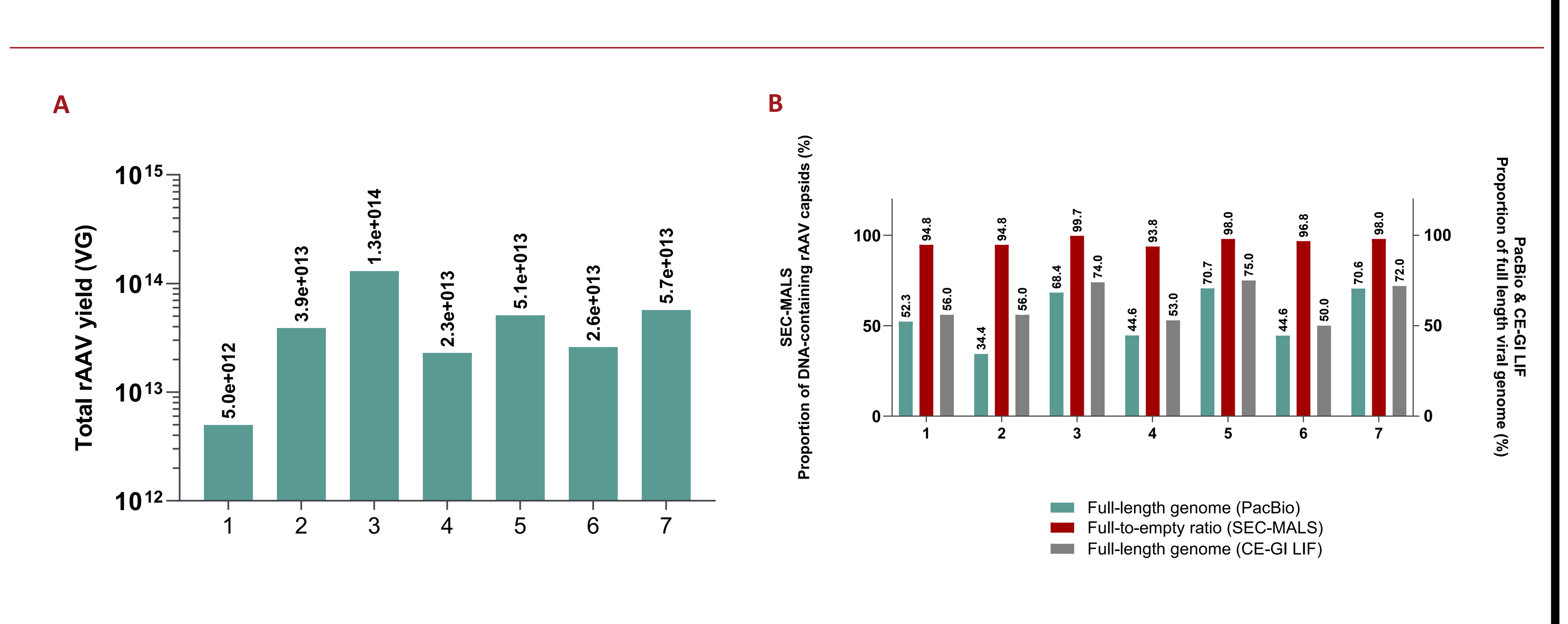


Vector iterations

Vector Number	vMiX™ 1.0 / 2.0	Promoter	5'ITR	SSR	5' Stuffer
1	1.0	Promoter 1	Sub-optimal		
2	2.0	Promoter 1	Sub-optimal	Modified	
3	2.0	Promoter 1	Optimised	Modified	
4	2.0	Promoter 2	Sub-optimal	Modified	Stuffer 1
5	2.0	Promoter 2	Optimised	Modified	Stuffer 1
6	2.0	Promoter 2	Sub-optimal	Modified	Stuffer 2
7	2.0	Promoter 2	Optimised	Modified	Stuffer 2

Multi-modal analytical assessment of rAAV vector quality

Figure 3: Vector titration and characterisation

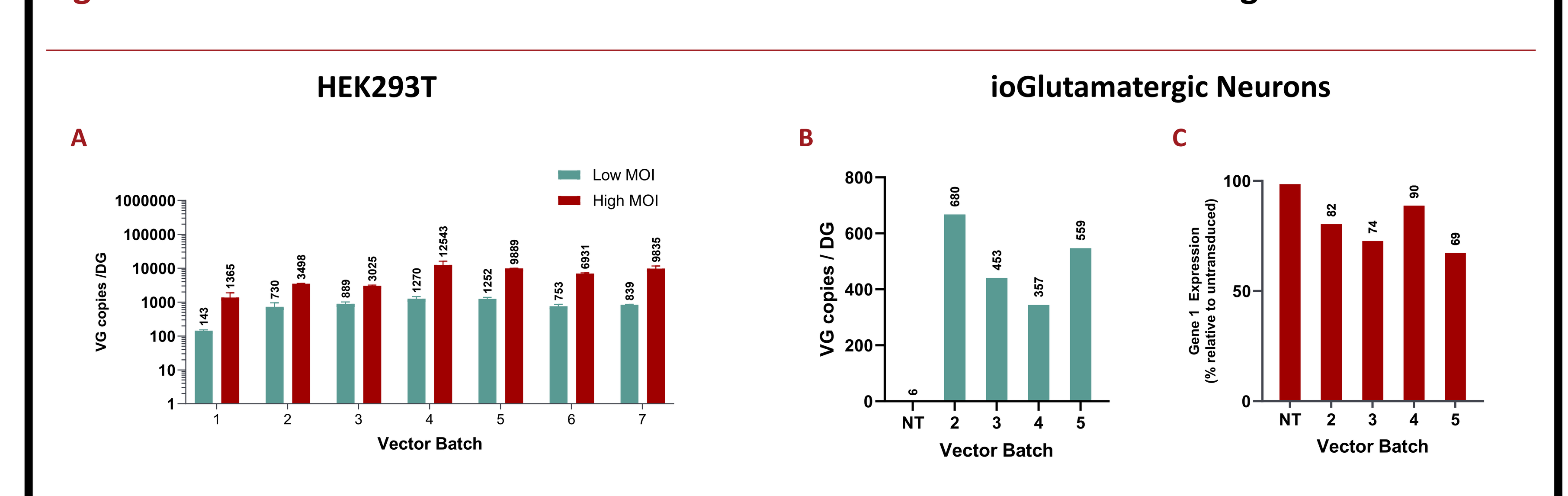


- During optimisation seven vector batches were generated, each housing different viral genome designs – see table above. Material was purified by ÄKTA-based affinity chromatography coupled with anion-exchange chromatography.
- Viral genome titre was determined for each purified vector batch via dPCR (Figure 3A). These vector batches were interrogated by means of CE-GI LIF, PacBio sequencing and SEC-MALS (Figure 3B).
- The proportion of DNA-containing rAAV particles housing the full-length payload, was found to be largely consistent between PacBio and CE-GI LIF analyses.
- Optimisation of the 5' ITR (sample 3 and 5) appeared to increase vector yield by at least 2-fold and consistently brought about an increase in the proportion of viral particles housing the full viral genome – this ranged from around 1.5-fold to almost 2.0-fold, as determined via PacBio sequencing.
- Substitution of the promoter (sample 5) was observed to have minimal impact on viral genome heterogeneity.
- Heterogeneity of the viral genome harbouring stuffer 1 or stuffer 2 was found to be largely analogous (sample 5 and 7).

In vitro assessment

- Vector batches were transduced onto HEK293T cells at matched MOIs, cells harvested 3-days post-transduction, and viral genome copies were quantified by dPCR. There appeared to be very little difference in viral genome copies, between vector harbouring the optimised and sub-optimal ITR sequences (Figure 4A). Given the neuronal specificity of promoter 2, target knock-down was not determined.
- Four of these vector samples (containing either promoter 1 or 2, downstream of either optimised or sub-optimal 5'ITR) were subsequently transduced onto human iPSC-derived glutamatergic neurons (ioGlutamatergic neurons, bit.bio), at matched MOIs, and harvested 7-days post-transduction. VG copy number per cell (VG/DG) and expression of Gene 1 were determined via dPCR and RT-PCR, respectively.
- Although there appeared to be some disparity in VG copy number (Figure 4B), viral genome harbouring the optimised 5' ITR sequence seemed to elicit a more pronounced knockdown of Gene 1, than the corresponding viral genome housing the mutated ITR (Figure 4C).

Figure 4: *In vitro* assessment of vMiX™ vector in HEK293T and ioGlutamatergic neurons



CONCLUSIONS

- Here, we have demonstrated the importance of long-read sequencing, together with SEC-MALS and CE-GI LIF, for rAAV vector genome optimisation. The former, in particular, is salient for the identification of truncation hotspots, which can be remedied by rational design.
- We have also demonstrated the importance of ITR sequence integrity for viral packaging. Optimisation of the 5' ITR was consistently found to bring about an impairment in packaging, whilst increasing propensity for truncation, compared to the equivalent vector containing optimised ITR sequence.
- When assessed in human iPSC-derived glutamatergic neurons, vector containing the optimised 5' ITR sequence was found to elicit a more pronounced gene knockdown, when compared to equivalent material harbouring the mutated ITR. Although we believed this phenomena could reflect changes in episome formation, the viral genome copy analysis failed to yield concordant results.

REFERENCES: Figure 1A, 1C and 1D were created using BioRender.com

ABBREVIATIONS: CE-GI LIF: capillary electrophoresis-laser-induced fluorescence genome integrity; **dPCR:** digital polymerase chain reaction; **HEK293T:** human embryonic kidney cells; **ITR:** inverted terminal repeat **kb:** kilobase; **miRNA:** microRNA; **MOI:** multiplicity of infection; **NT:** non-transduced; **rAAV:** recombinant adeno-associated virus; **RT-PCR:** reverse transcription-polymerase chain reaction; **SEC-MALS:** size-exclusion chromatography-multi-angled light scattering; **SSR:**; **VG/DG:** viral genome/diploid genome

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