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ITR Sequence is critical for AAV vector performance for CNS gene therapy

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Abstract

Introduction: Diseases of the central nervous system (CNS) are difficult to treat due to the blood-brain barrier preventing many therapeutic drugs from crossing. Gene therapy using adeno-associated viruses (AAVs) offers a promising solution with the advancement of new capsids including AAV-DJ, which has been demonstrated to cross the blood-brain barrier and achieve targeted and effective expression in CNS tissues. Additionally, AAVs are well suited for CNS treatments because they can achieve long-term gene expression with minimal immune response, and are ideal for conditions such as Alzheimer's, Huntington's, and Parkinson's diseases. The AAV genome consists of a transgene construct flanked by 145bp inverted terminal repeat (ITR) sequence. Previous studies have demonstrated that ITRs are essential for AAV packaging; however, they are prone to mutations and deletions during bacterial propagation, leading to inconsistent experimental results. While advances in capsid engineering have led to enhanced ability for AAVs to cross the blood-brain barrier, the instability of the ITRs remains a major concern.

Methods: The impact of five common ITR mutations, with varying degrees of integrity and insert length, were evaluated. HEK293T cells were co-transfected with the transfer, Rep/Cap (serotype AAV-DJ), and helper plasmids for viral packaging. AAV-ITR Sanger sequencing verified the ITR mutations in the transfer plasmid sequence in comparison to the wild-type. Following iodixanol purification, viral titers were assessed using quantitative PCR (qPCR), and transduction efficiency was evaluated through fluorescence microscopy of a GFP marker. To further investigate the effects of these mutations, each mutation was repaired to the wild-type sequence, and the titer and fluorescence were re-evaluated. To assess brain-wide transgene expression and the impact of ITR mutations, AAVs targeting the CNS tissues were evaluated using a triple-labeling immunofluorescence technique with DAPI, NeuN, and GFP to analyze AAV-mediated gene expression in the mouse brain.

Results: Recombinant AAV genomes with partial or significant deletion of the 5'-ITR had reduced titers compared to wild-type; even a single base pair deletion resulted in decreased titer. This drop in viral titer was more pronounced in constructs with significant ITR deletions and larger transgene cassette cargo. A marked decrease was also noted in GFP fluorescence, which correlated to the severity of the ITR deletion and transgene size. AAV mutant constructs retained the capability to cross the blood-brain barrier, with wild-type vectors achieving the highest efficiency.

Conclusion: This study demonstrates the critical role of the 5'-ITR in the efficacy of recombinant AAV vectors. Even minor alterations, such as single base pair deletions, can significantly affect viral titers and transduction efficiency. These findings provide valuable insights into the design of AAV vectors for gene therapy applications, emphasizing the need to carefully consider ITR integrity at all stages of design and development, and transgene size to maximize therapeutic potential.

AAV synthesis, cloning, packaging, and ITR sequencing workflow

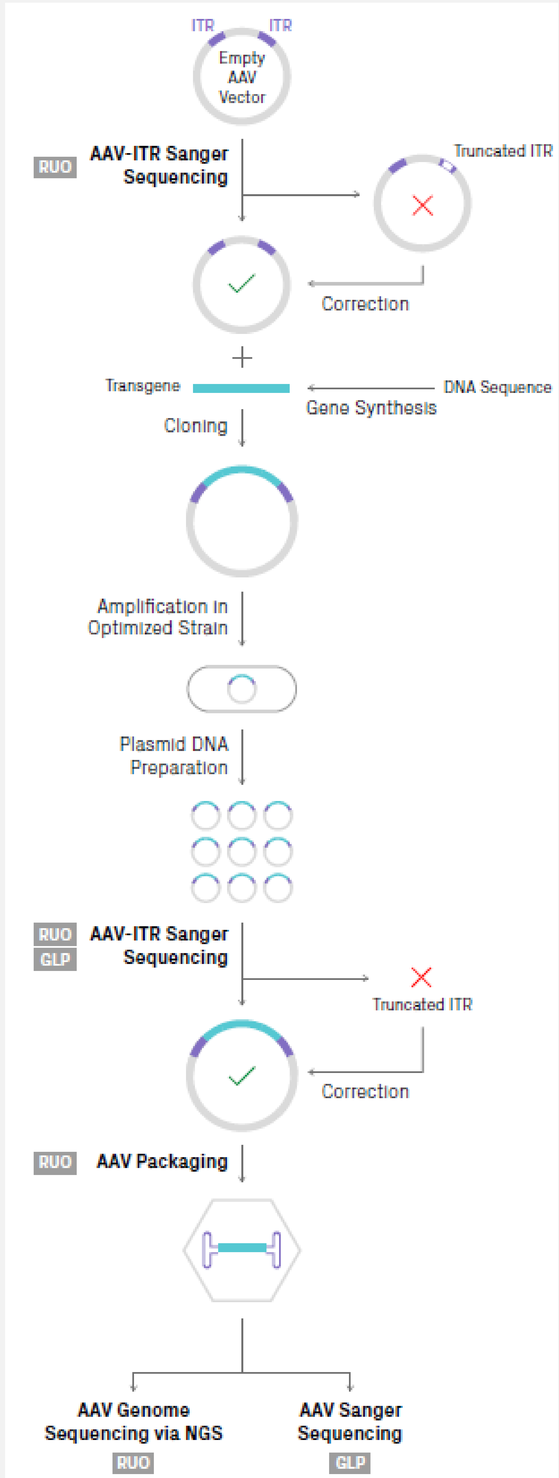

Figure 1. Full AAV synthesis and packaging workflow. GENEWIZ's AAV includes:

- ITR Sequencing: Ensures the integrity of ITR regions using advanced sequencing techniques
- Plasmid Synthesis: High-fidelity synthesis of transgene expression cassettes with cloning and sequence verification.
- Plasmid Preparation: Scalable production of AAV plasmids up to gram level yields.

AAV Integrity is critical for high titer and transduction efficiency

Adeno-associated viruses (AAVs) are widely used as vectors for delivering genetic material due to their high efficiency and safety. However, working with AAV vectors can be challenging because of the instability of inverted terminal repeat (ITR) sequences, which are crucial for replication and encapsidation. The ITR regions in AAV plasmids are notoriously unstable. Their palindromic nature and high guanine-cytosine (GC) content make them prone to full or partial deletions during propagation in bacteria [2]. As a result, a significant portion of clones after transformation may contain mutated ITRs, and plasmid DNA preparations from a liquid culture may contain a mixed population. GENEWIZ has developed a novel workflow for AAV plasmid preparation that enhances ITR integrity during propagation and uses a robust, sensitive assay to analyze ITR sequences for mutations before and after DNA scale-up.

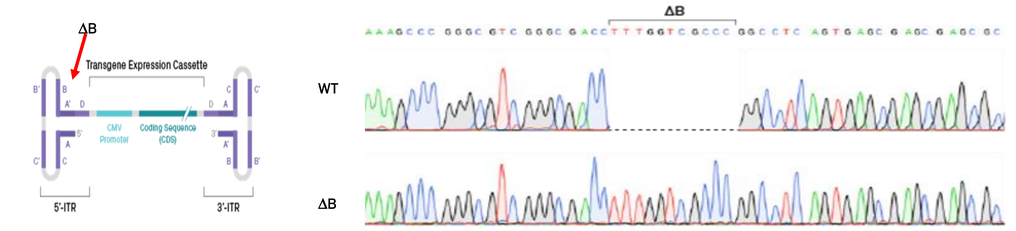

Figure 2: Schematic of transfer plasmid and sequence verification of ITR mutation. Transfer plasmid construction and ITR sequencing before and following ITR correction. Diagram of transfer plasmid cassette with ΔB-S = ITR deletion in a cassette with a gene S with GFP tag. Correction is verified using AAV-ITR Sanger sequencing

To evaluate the effects of partial ITR deletions on cell transduction, we compared viral titer and transduction efficiency as visualized by GFP in HEK293 cells (**Figure 1**). Deletions within the 5' ITR region significantly impaired transduction efficiency and titer was reduced ~35-40% compared to wildtype. PHP.eB is an engineered variant of the AAV9 serotype, specifically designed to enhance gene delivery to the central nervous system (CNS). The CNS was stained to evaluate the ability of AAVs produced to cross the blood-brain barrier (BBB) with a PHP.be serotype. The GFP expression was seen throughout the CNS.

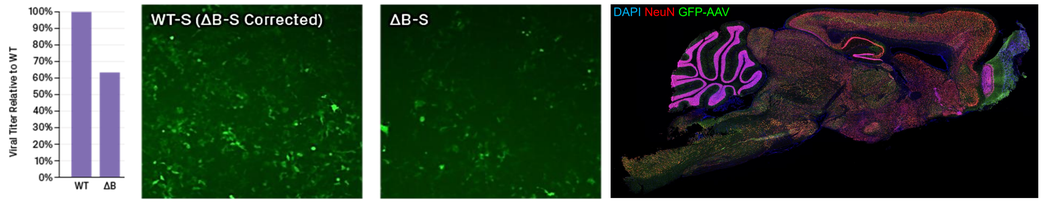

Figure 3: Evaluation of qPCR AAV Titer and transduction efficiency with immunostaining of CNS. Titer is determined using qPCR and transduction efficiency was visualized using eGFP for both WT and mutated AAVs. A triple-labeling immunofluorescence technique with DAPI, NeuN, and GFP was used to visualize the CNS.

PHP.eB is particularly useful for therapies requiring broad gene distribution in the CNS, such as treatments for neurodegenerative and neurodevelopmental disorders. CNS-targeted gene therapy, PHP.eB represents a significant advancement in overcoming the challenges of delivering genetic material across the blood-brain barrier. Our study demonstrates the critical role of the 5'-ITR in the efficacy of recombinant AAV vectors, highlighting that even minor alterations, such as single base pair deletions, can significantly affect viral titers and transduction efficiency. Further research is warranted to explore strategies for enhancing the functionality of modified AAVs while preserving their ability to efficiently deliver genetic material across the BBB.

Conclusions

These findings provide valuable insights into the design of AAV vectors for gene therapy applications, emphasizing the need to carefully consider ITR integrity and transgene size to maximize therapeutic potential.

References:
[1] Savy, A. et al. Impact of Inverted Terminal Repeat Integrity on rAAV8 Production Using the Baculovirus/Sf9 Cells System. Human Gene Therapy Methods 28, 277–289 (2017).
[2] Wilmott, P., Lisowski, L., Alexander, I. E. & Logan, G. J. A User's Guide to the Inverted Terminal Repeats of Adeno-Associated Virus. Human Gene Therapy Methods 30, 206–213 (2019).