

Exploring the Role of Inverted Terminal Repeats in AAV Vector Performance for CNS Gene Therapy

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Abstract

Diseases of the central nervous system (CNS) are difficult to treat due to the blood-brain barrier (BBB), which limits therapeutic delivery. Gene therapy using adeno-associated viruses (AAVs) offers a promising solution, particularly with engineered capsids capable of crossing the BBB and enabling targeted CNS expression. The AAV genome contains a transgene flanked by 145 bp inverted terminal repeats (ITRs), which are essential for packaging but prone to mutations and deletions during bacterial propagation, leading to inconsistent outcomes. Despite advances in capsid engineering, ITR instability remains a significant challenge.

This study evaluated five common ITR mutations with varying levels of integrity and insert length. HEK293T cells were co-transfected with transfer, Rep/Cap (PHP.eB), and helper plasmids for viral production. ITR integrity was confirmed by Sanger sequencing. Following iodixanol purification, viral titers were measured by qPCR and transduction efficiency assessed via GFP fluorescence. Mutated ITRs were then repaired to wildtype sequences, and titer and fluorescence re-evaluated. CNS-targeted AAVs were also tested in vivo, with expression analyzed using immunofluorescence labeling (DAPI, NeuN, GFP) in mouse brain tissue.

Results showed partial or significant deletions in the 5'ITR reduced viral titers compared to wildtype, with greater reductions observed in constructs with larger deletions and transgene sizes. GFP expression similarly decreased in proportion to ITR disruption and cargo length. While mutant AAVs retained the ability to cross the BBB, wild-type vectors exhibited the highest transduction efficiency.

In conclusion, the 5'ITR plays a critical role in AAV vector performance. Even minor sequence changes, including single base pair deletions, can significantly impact viral yield and expression. These findings highlight the importance of maintaining ITR integrity throughout vector design and production to ensure optimal gene therapy outcomes.

AAV Synthesis, Cloning, Packaging and ITR Sequencing Workflow

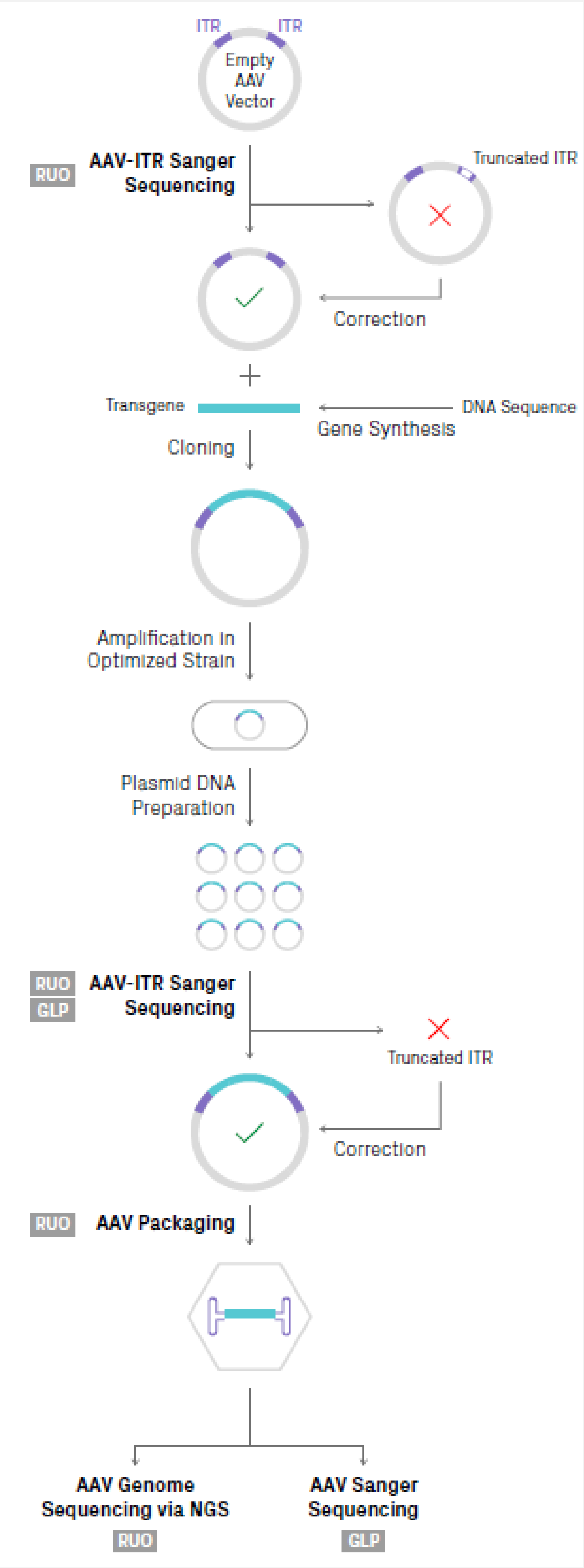


Figure 1. Full AAV Synthesis and Packaging Workflow. The GENEWIZ AAV workflow includes:

- 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

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 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.

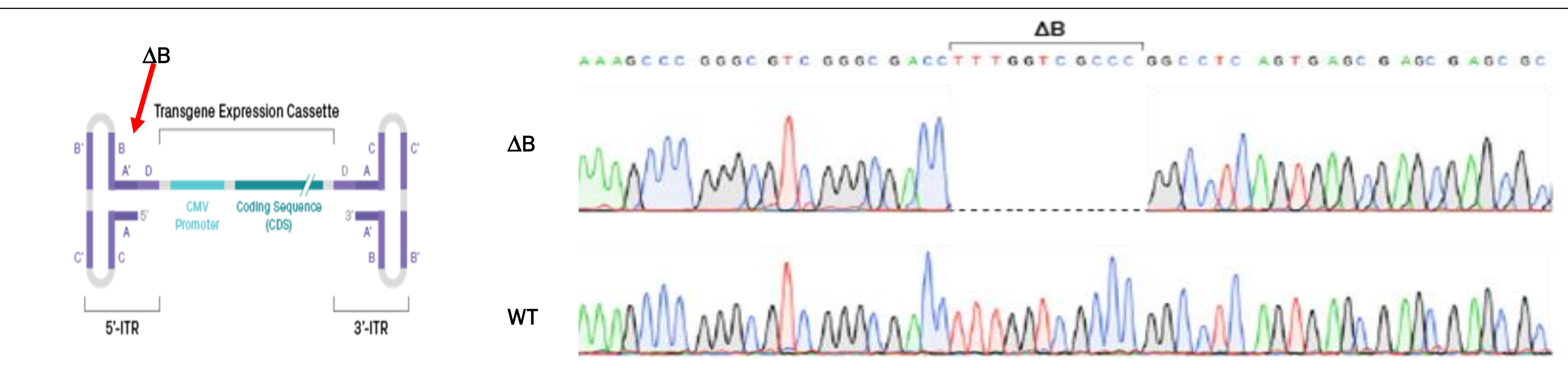


Figure 2: Schematic of Transfer Plasmid and Sequence Verification of ITR Mutation. Transfer plasmid construction and ITR sequencing before and following ITR correction. Diagram of transfer plasmid cassette with ΔB = ITR deletion in a cassette with a gene and GFP tag. Correction is verified using AAV-ITR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

To evaluate the effects of partial ITR deletions on cell transduction, we packaged wildtype and mutated ITR plasmids into PHP.eB, and compared viral titer and transduction efficiency, as visualized by GFP in HEK293 cells (Figure 2, 3). Deletions within the 5' ITR region significantly reduced titer by up to 40%, and impaired transduction efficiency, when compared to wildtype. PHP.eB is an engineered variant of the AAV9 serotype, specifically designed to enhance gene delivery to the CNS. The CNS was stained to evaluate the ability of AAVs produced to cross the BBB with a PHP.eB serotype. GFP expression was seen throughout the CNS, but with much lower efficiency in delivered AAVs with mutated ITRs.

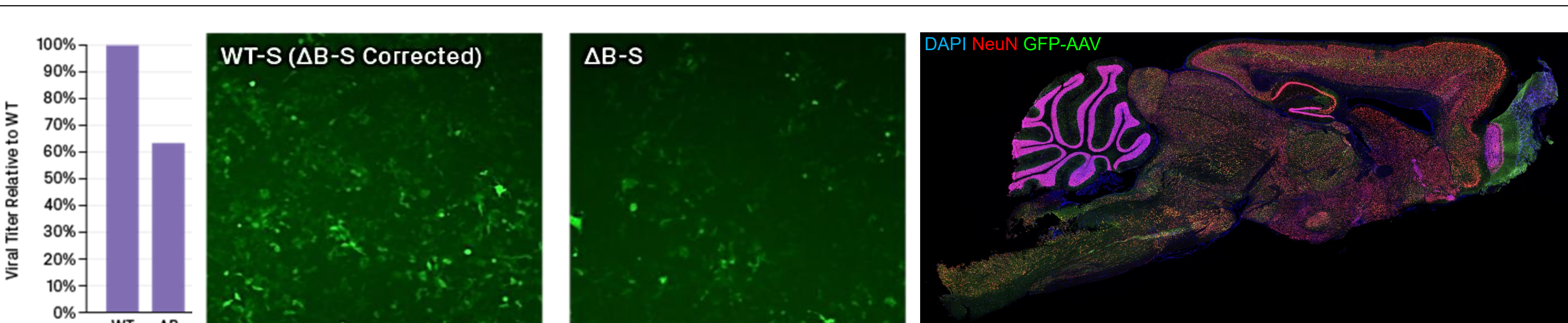


Figure 3: Evaluation of qPCR AAV titer and transduction efficiency with immunostaining of CNS. Titer was determined using qPCR and transduction efficiency was visualized using eGFP for both WT and mutated AAVs. A triple-labeling immunofluorescence technique with DAPI, NeuN, and GFP was used to visualize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

PHP.eB is particularly useful for therapies requiring broad gene distribution in the CNS, such as treatments for neurodegenerative and neurodevelopmental disorders. For 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, including 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.

Conclusion

These findings provide valuable insights into the design of AAV vectors for gene therapy applications, emphasizing the need to carefully consider ITR integrity o 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).