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From Azenta Life Sciences

Engineering robust AAV vectors: Addressing ITR instability for effective cancer gene therapy

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Abstract

Introduction: Cancer remains one of the most challenging diseases to treat due to the complexity of tumor biology and barriers to effective drug delivery. Gene therapy using adeno-associated viruses (AAVs) offers a promising approach for oncology, particularly with the development of engineered capsids such as AAV-DJ, which demonstrate enhanced tissue penetration and targeted gene expression in tumor microenvironments. Importantly, AAV-based strategies are also being explored in neuro-oncology, where capsid variants capable of crossing the blood-brain barrier provide new opportunities for treating aggressive brain tumors such as glioblastoma. The AAV genome consists of a therapeutic transgene flanked by 145 bp inverted terminal repeats (ITRs), which are prone to mutations and deletions but also essential for viral packaging. While capsid engineering has improved tumor targeting and systemic delivery, ITR instability remains a critical challenge for consistent therapeutic outcomes.

Methods: In this study, we evaluated the impact of five common ITR mutations on vector integrity and performance. HEK293T cells were co-transfected with transfer, Rep/Cap, and helper plasmids for viral packaging. ITR mutations were confirmed via Sanger sequencing, and purified vectors were assessed for viral titers using qPCR and transduction efficiency via GFP fluorescence microscopy. Mutant ITRs were subsequently repaired to wild-type sequences to compare recovery of function. To model therapeutic relevance, AAV vectors were tested in tumor-bearing mice using immunofluorescence markers to assess transgene expression within cancerous tissues.

Results: Even minor ITR alterations, such as single base pair deletions, demonstrated significantly reduced viral titers and transduction efficiency, with more pronounced effects in constructs carrying large therapeutic cassettes. Although mutant vectors retained some tumor-targeting capability, wild-type ITR constructs achieved the highest efficiency.

Conclusion: This work underscores the critical role of ITR integrity in AAV-based oncology applications, including neuro-oncology. Even small sequence disruptions can compromise vector potency, highlighting the need for rigorous quality control during vector design and production. These findings inform the development of robust AAV platforms for cancer gene therapy, ensuring optimal delivery and therapeutic impact.

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AAV synthesis, cloning, packaging, and ITR sequencing workflow

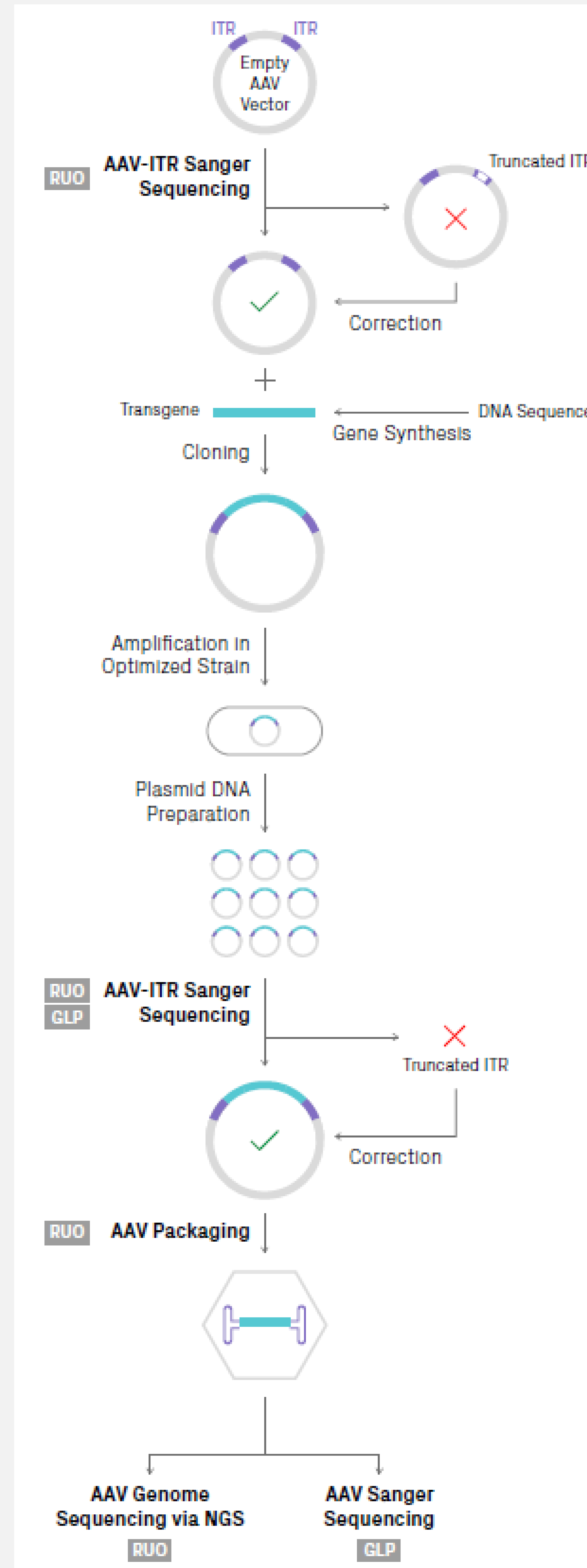

Figure 1. Full AAV synthesis and packaging workflow. GENEWIZ's AAV includes:

- 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.

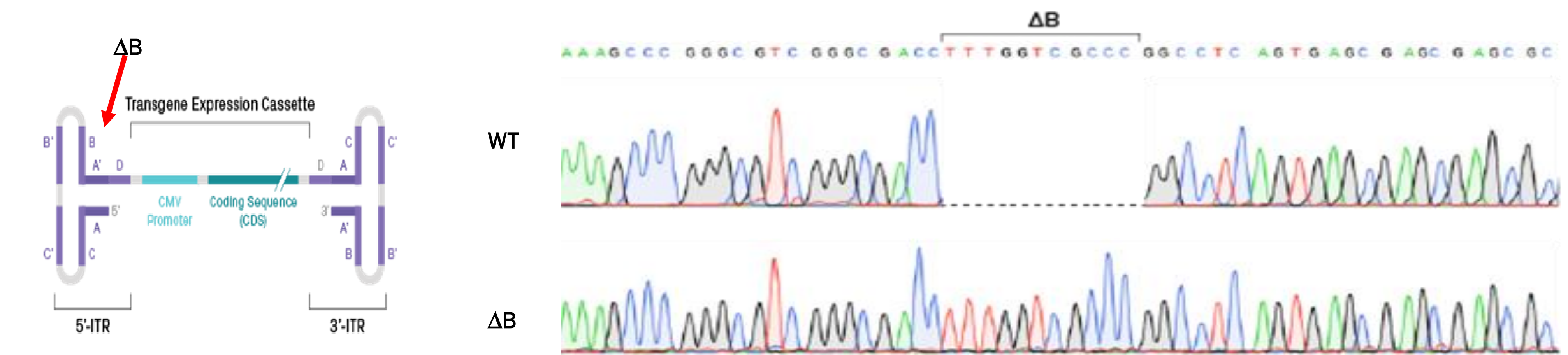

Figure 2: Schematic of transfer plasmid and sequence verification of ITR mutation. Transfer plasmid construction and ITR sequencing before and following ITR correction. Diagram of transfer plasmid cassette with ΔB-S = ITR deletion in a cassette with a gene S with GFP tag. Correction is verified using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

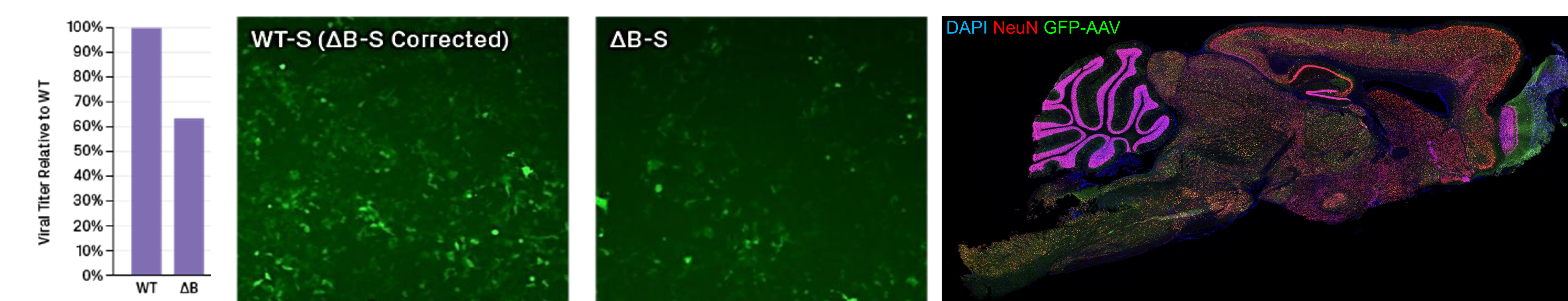

Figure 3: Evaluation of qPCR AAV Titer and transduction efficiency with immunostaining of CNS. Titer is determined using qPCR and transduction efficiency was visualized using eGFP for both WT and mutated AAVs. A triple-labeling immunofluorescence technique with DAPI, NeuN, and GFP was used to visualize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