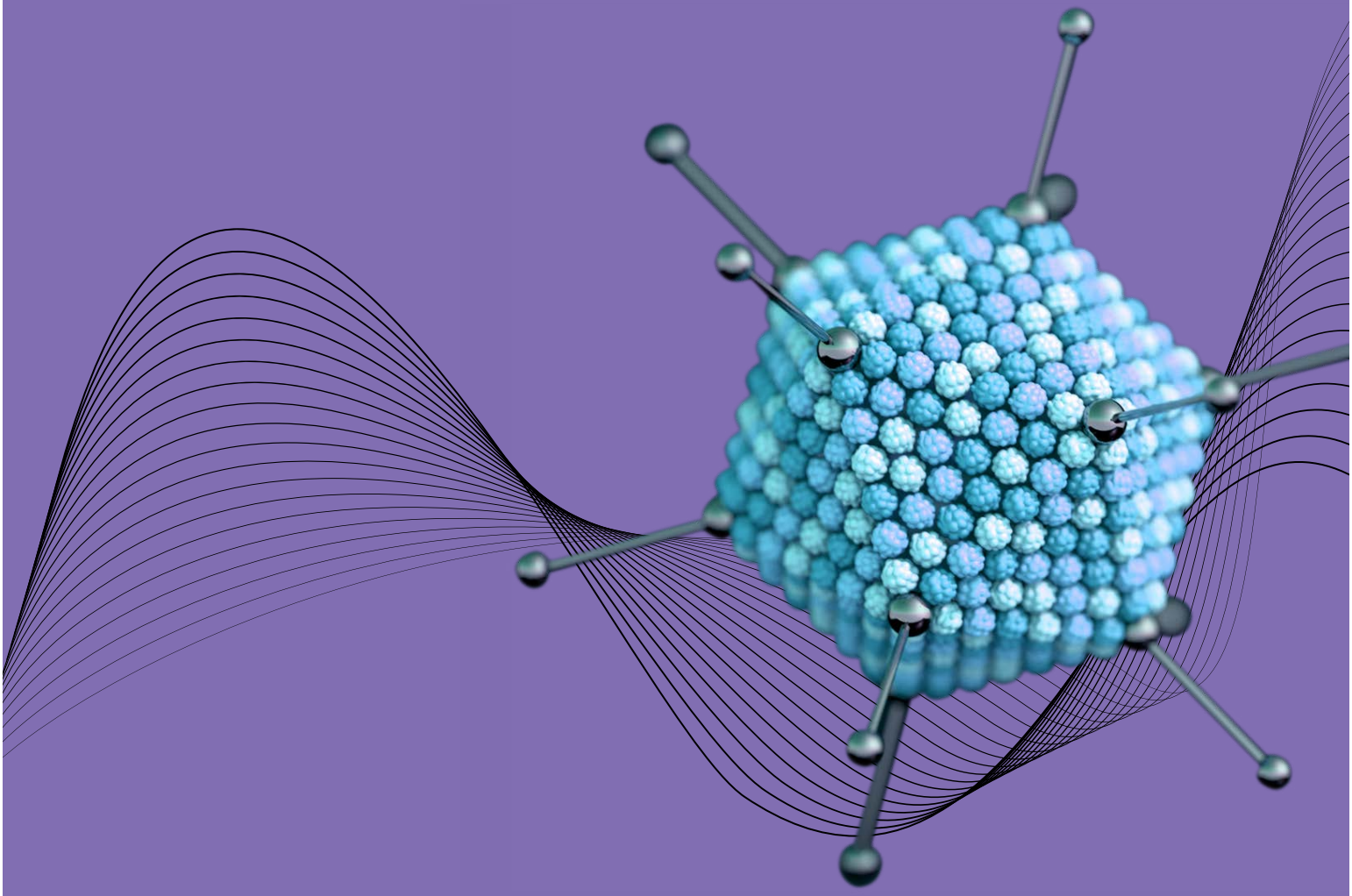
Genomics & Analytical Services

TECH NOTE

An Efficient and High-Fidelity Approach to AAV Plasmid Preparation



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Abstract

The inverted terminal repeat (ITR) regions in adeno-associated virus (AAV) constructs are highly unstable during propagation in bacteria. Spontaneous deletions in the ITR sequences are common during molecular cloning, and they can impact viral packaging of the recombinant AAV genome. Furthermore, these mutations are difficult to detect using traditional methods of DNA analysis. To address these challenges, Azenta Life Sciences developed a novel workflow for AAV plasmid preparation that enhances ITR integrity during propagation, repairs truncations, and uses a robust protocol to sequence ITR regions. This end-to-end solution, which includes transgene synthesis and cloning, delivers AAV plasmid preparations with high efficiency and quality.

Introduction

The adeno-associated virus (AAV) is a powerful vehicle for gene therapy; however, working with AAV vectors can be challenging. Each construct contains two inverted terminal repeat (ITR) sequences, usually 145 bp in length, that flank the gene of interest (Figure 1). The ITRs form highly stable T-shaped hairpins, which are critical for the replication and encapsidation of viral DNA[1]. These structural features can also wreak havoc on traditional cloning workflows. Spontaneous deletions in the ITR region plague DNA preparations of the transfer plasmid, and the formation of secondary structure stymies confirmatory sequencing. Hence, investigators are hit with a double whammy: AAV vectors prone to ITR truncations and these mutations are difficult to detect. Here, we discuss the challenges of propagating and validating AAV plasmids and demonstrate an optimized approach to the workflow.

The Challenge

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[1]. 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 (Figure 2).

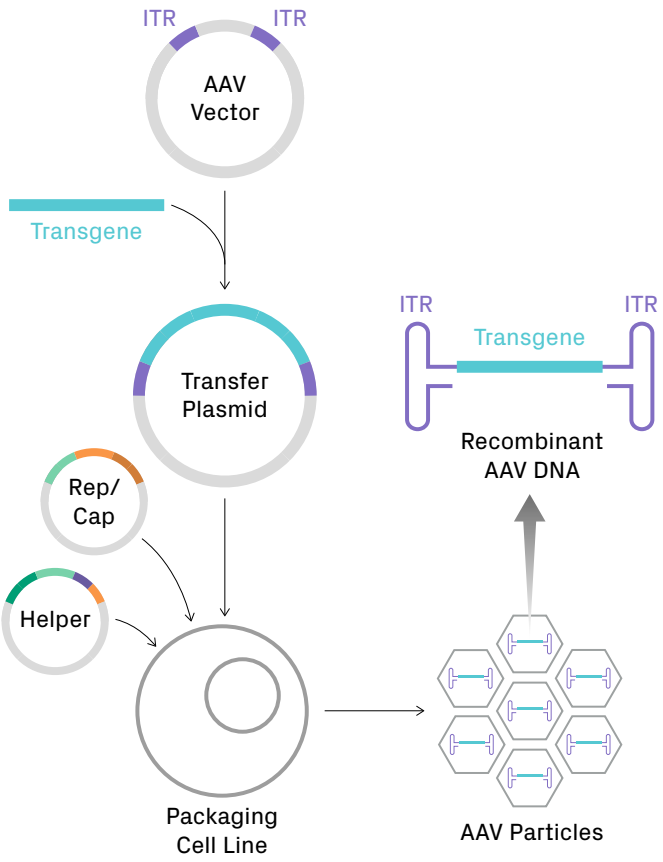

Figure 1. Production of recombinant AAV particles. A gene of interest is cloned into an AAV vector to create a transfer plasmid, which is then introduced into a eukaryotic cell line. Generating functional viral particles requires co-transfection with several viral genes, supplied by packaging plasmids (i.e. Rep/Cap and helper plasmids). Recombinant single-stranded DNA, containing the transgene flanked by inverted terminal repeat (ITR) sequences, is packaged into capsids. The ITRs form very stable hairpins, which are problematic for amplification and sequencing of AAV plasmids during the early steps of the workflow.

The underlying mechanism of this phenomenon in bacteria is not well understood; secondary structure and strand slippage in the replication fork may play a role[2]. Since the loss of ITR sequences confers a growth advantage to the host bacteria, a mutated plasmid can rapidly amplify in a growing culture and become the dominant molecular species. Even with the best standard practices, AAV plasmid preparations can contain a significant fraction (5-15%) of plasmid DNA with mutated ITR sequences[3].

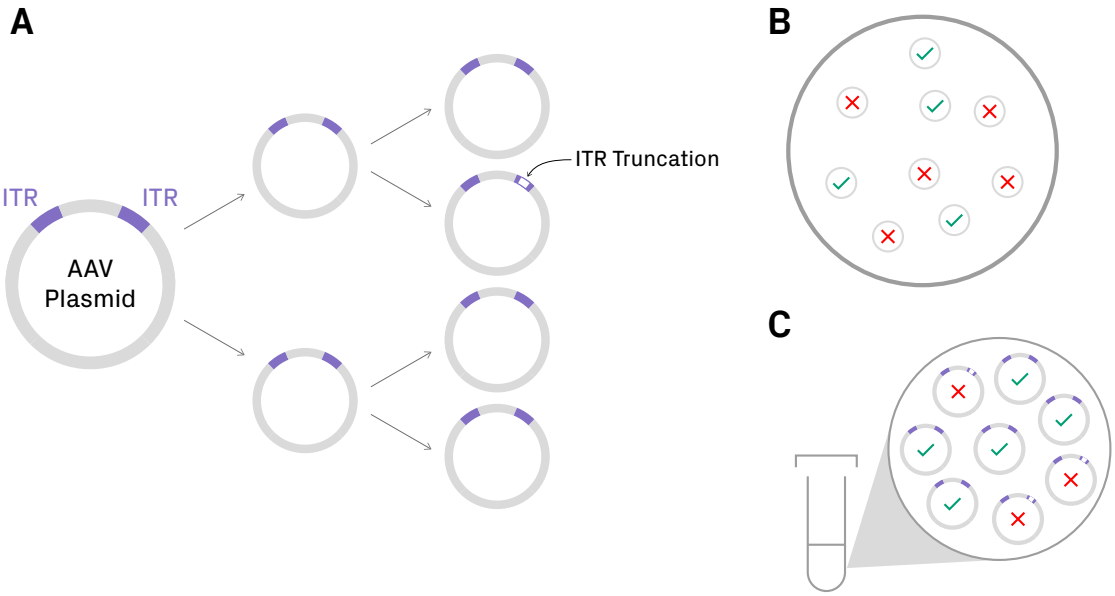

Figure 2. Plasmids with ITR sequences exhibit high instability. (A) Mutations and truncations frequently arise in the ITRs during propagation in bacterial cells. (B) When selecting colonies from an agar plate, many clones may contain the mutation. (C) When growing bacteria in liquid culture, the resulting plasmid preparation may contain a mixture of DNA molecules.

Disruption of ITRs can lead to reduced efficiency in viral packaging and increased variability in downstream experiments[4]. Therefore, measures must be taken to ensure that a plasmid prep is homogeneous with fully intact ITR regions. Recombination-deficient strains—such as JC8111, SURE2, Stabl3, XL10-Gold—are typically used when cloning AAV constructs, as they reduce the likelihood of ITR deletions[1]. However, depending on the construct, it can be very difficult to isolate a clone with fully intact ITRs using commercial strains. Plasmid preparation generally becomes more tedious with AAV constructs since more colonies need to be screened, assuming a correct clone can be obtained at all. Also, during scale-up, there is a greater chance that a mutation will arise, resulting in a mixed population of plasmids that can affect downstream experiments or require re-isolation of a clone.

Given the frequency of truncations that arise in AAV plasmids, it's critical to monitor the integrity of ITRs throughout cloning, especially prior to viral packaging. Sanger sequencing is the gold standard for plasmid sequence confirmation; however, standard protocols fail to read through ITR regions. The strong secondary structure of ITRs inhibits DNA polymerization, leading to a significant drop in signal intensity in chromatograms (Figure 3). Common strategies for sequencing difficult templates, such as adding dimethyl sulfoxide (DMSO) and/or betaine, are ineffective for ITRs[5,6].

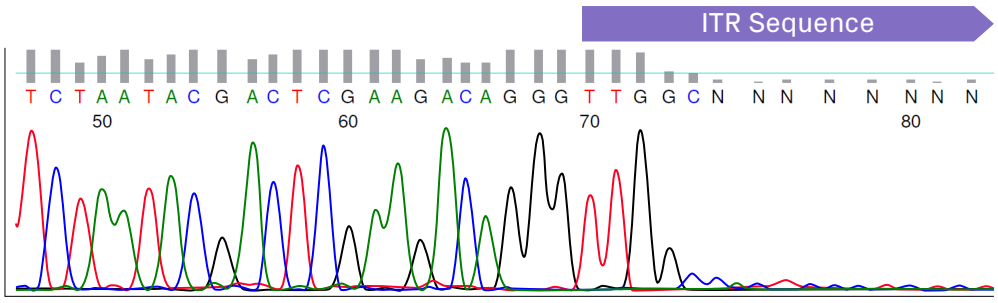

Figure 3. Sequencing chromatogram showing early termination of signal at the start of the ITR sequence. Standard protocols for Sanger sequencing fail to read through ITR regions.

Restriction digestions have traditionally been performed to screen for ITR deletions. The enzymes *SmaI* and *SrfI* are often used, as they cut within the C-C' arm of the ITR[3]. Given the limited resolution of gel electrophoresis, diagnostic digests fail to detect small deletions and point mutations outside the restriction site (Figure 4). In short, restriction enzyme analysis alone is insufficient to monitor ITR integrity in plasmids.

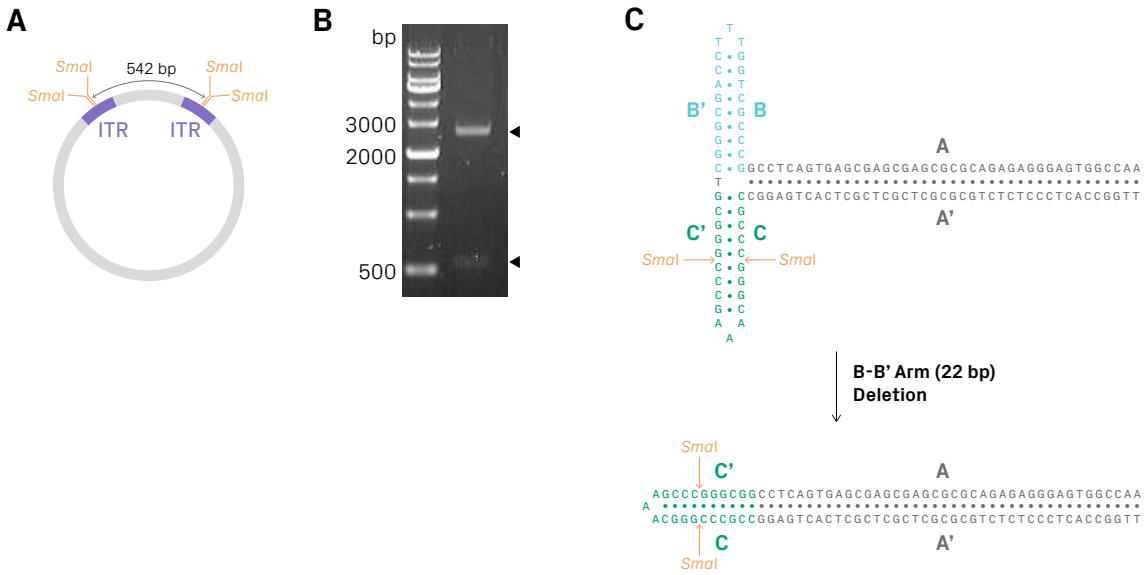

Figure 4. Restriction enzyme analysis fails to detect small deletions in ITRs. (A) Restriction map of an AAV plasmid in which *SmaI* cuts twice within the ITR region. (B) Gel of SmaI digestion showing bands of expected size. (C) The analyzed plasmid preparation has a deletion of the B-B' arm in one of the ITR regions, discovered through Azenta's AAV-ITR sequencing (see next section). Agarose gel electrophoresis does not have sufficient resolution to identify a loss of 22 bp.

The Solution

Azenta Life Sciences 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 5A). Initial stocks of AAV plasmid are subjected to quality control via our proprietary AAV-ITR Sanger sequencing protocol, which can read through the challenging ITR regions. It is sufficiently robust to sequence through both wild-type and mutant ITRs, enabling detection of challenging ITR truncations in clonal and heterogeneous populations of plasmid DNA[6]. If mutations are found in the original plasmid stock, either ITR correction via synthesis and cloning of the affected region or isolation of the correct clone from a mixed population can be performed (Figure 5B). The transgene is synthesized *de novo*, verified by Sanger sequencing, and cloned into the AAV plasmid with clonal intact ITR regions. The resulting transfer plasmid is introduced into a proprietary strain of *E. coli* that has been specifically optimized to maintain ITR integrity during plasmid propagation. Bacterial cultures are grown, and plasmid DNA is isolated. The resulting DNA preparation is analyzed by AAV-ITR sequencing for final quality control.

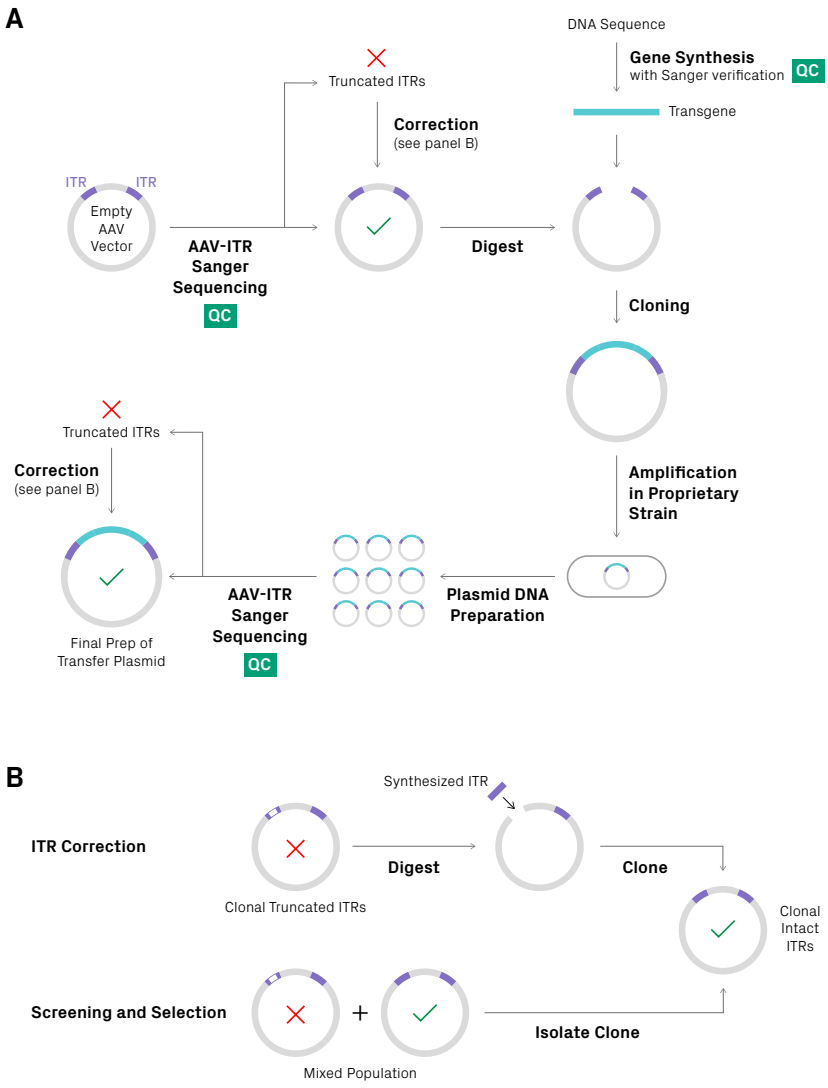

Figure 5. AAV plasmid synthesis. (A) Overall workflow. Sequencing-based quality control (QC) is performed at multiple stages of the process. The original stock of AAV plasmid is analyzed by Azenta's AAV-ITR Sanger sequencing to determine whether the population is clonal (i.e. homogeneous) and both ITR regions are fully intact. If mutations are detected after initial QC, corrective actions are available. The transgene is synthesized, sequence-verified, and cloned into the AAV vector. The resulting plasmid is replicated in a proprietary *E. coli* strain that is optimized for ITR stability. After plasmid DNA preparation, a final QC step using AAV-ITR sequencing is performed. (B) Methods to address AAV plasmids with truncated ITRs. For clonal truncations, the correct ITR sequence is synthesized and cloned it into the mutated plasmid, followed by amplification in the proprietary bacterial strain. This process is built on Azenta's gene synthesis technology and over a decade of experience synthesizing complex DNA sequences. For mixed populations, a new clone is screened and selected.

The Results

To evaluate the efficacy of our AAV plasmid preparation workflow, we compared ITR stability in our proprietary bacterial strains compared to commercial strains. Eight AAV plasmids with different vector backbones were introduced into the *E. coli* strains, and eight colonies per plasmid per strain were analyzed by AAV-ITR Sanger sequencing (Figure 6). The Azenta strains outperformed the three commercial strains, showing a higher percentage of clones with intact ITRs. The difference in ITR stability was more pronounced for the three most challenging plasmids: the commercial strains failed to generate a correct clone for at least two of the plasmids.

An additional forty-three plasmids were tested during the beta phase of our AAV plasmid preparation service. They were prepped to maxi- or giga-scale and analyzed by AAV-ITR sequencing. Forty-two plasmids (98%) had fully intact ITR sequences after the first attempt, demonstrating a high success rate for the workflow.

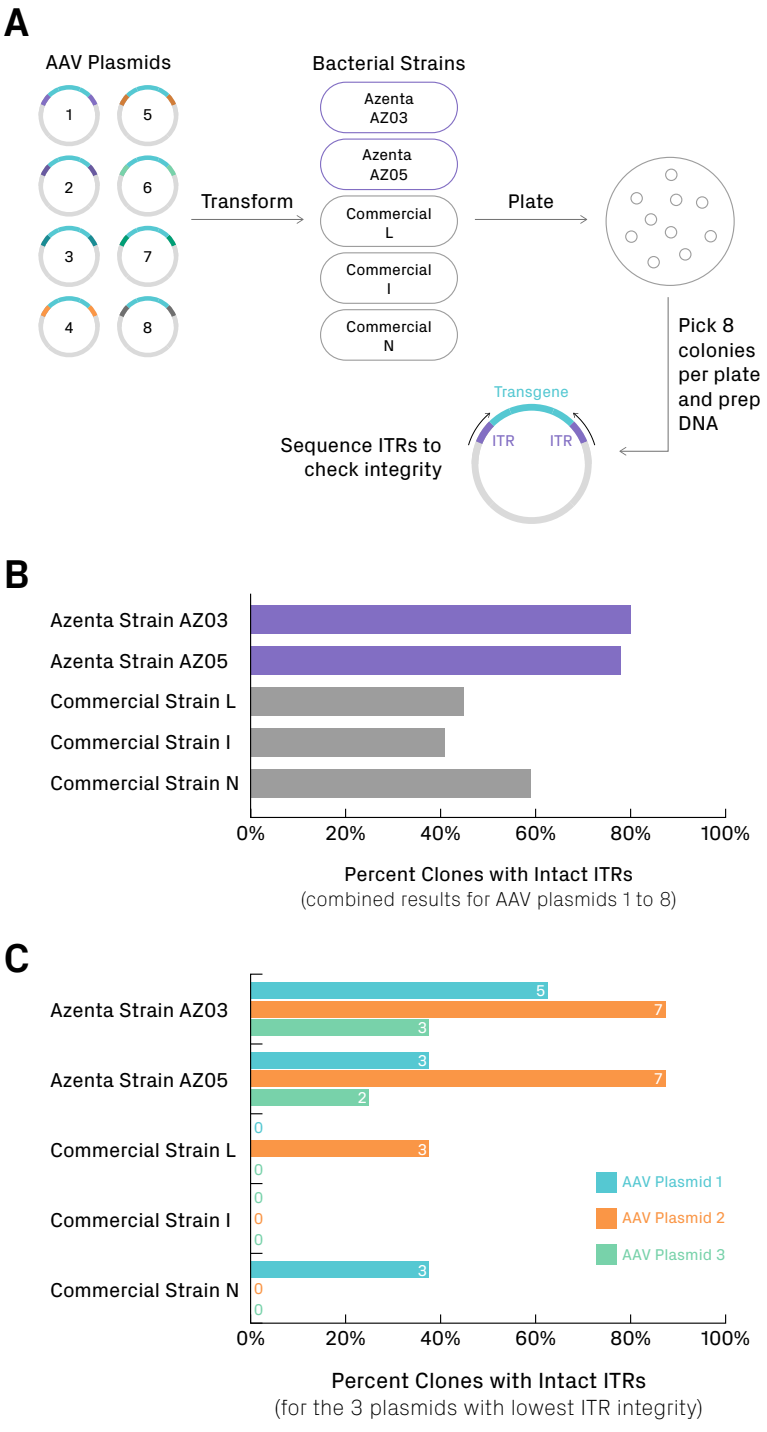

Figure 6. ITR integrity in bacterial strains. (A) Eight AAV plasmids with different backbones were tested in five strains. After transformation, eight colonies per plasmid per strain were picked and screened for ITR integrity using AAV-ITR Sanger sequencing. (B) Combined results for all eight AAV plasmids. (C) Results for the 3 plasmids with the lowest ITR stability. Values represent the number of successful clones out of eight selected. Overall, the proprietary Azenta strains outperformed the commercial strains, especially for the more challenging plasmids.

The full AAV plasmid synthesis workflow was tested by synthesizing a 689-bp gene and introducing it into two AAV vectors, each greater than 5 kb. A truncation discovered in the 5' ITR of one of the vectors was repaired via ITR correction prior to cloning (Figure 7). The final plasmid preparations for both constructs showed full sequence accuracy for the ITR regions and transgene.

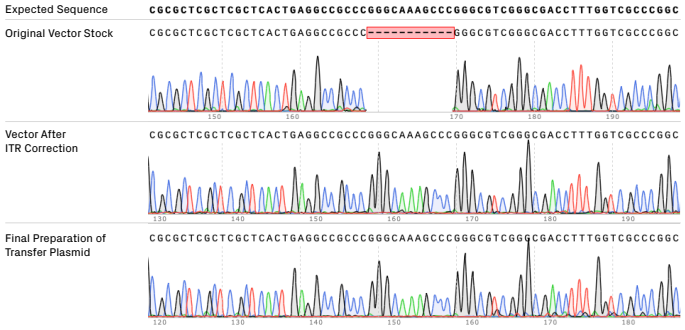

Figure 7. ITR correction. AAV-ITR sequencing revealed an 11-bp deletion in the 5' ITR region of an AAV vector. The truncation was successfully repaired using Azenta's ITR correction protocol. ITR integrity was maintained after introduction of the transgene and AAV plasmid preparation, as confirmed by AAV-ITR sequencing.

Conclusion

The structural features of ITR sequences pose significant quality control issues for investigators aiming to build and clone AAV constructs. Sequence accuracy of the transfer plasmid, especially the ITR regions and transgene, is essential for reproducible packaging and function of the viral DNA. Azenta's AAV plasmid synthesis method addresses these challenges by combining proven gene synthesis technologies, high-fidelity DNA replication in bacteria, and reliable confirmation of ITR sequences at single-nucleotide resolution. Our workflow significantly outperforms traditional methods for plasmid preparation and sequence verification, providing a powerful tool for AAV research.

LEARN MORE ABOUT AAV PLASMID SYNTHESIS →

References

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