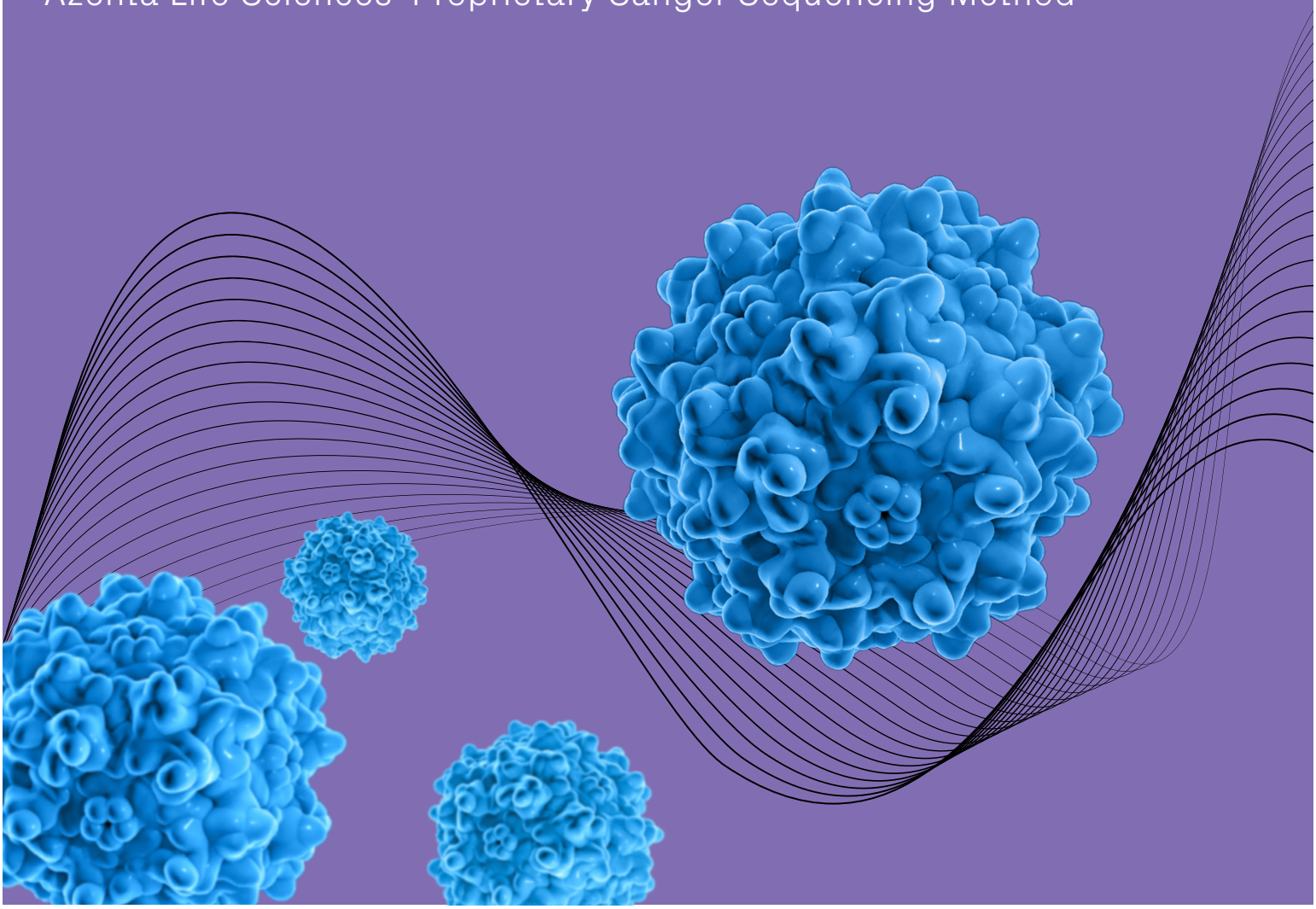
Genomics & Analytical Services

TECH NOTE

Reading Through the Inverted Terminal Repeats (ITRs) of Adeno-associated Virus (AAV)

Azenta Life Sciences' Proprietary Sanger Sequencing Method



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The inverted terminal repeat (ITR) regions of adeno-associated virus genomes form highly-stable secondary structures that pose major obstacles for sequence verification using existing technologies. To alleviate this issue, Azenta has developed a new proprietary Sanger sequencing method that provides unprecedented analysis of these intractable sequences enabling researchers to qualitatively assess the integrity of these ITR regions.

Introduction

Adeno-associated virus (AAV) is a single-stranded DNA virus, commonly used in gene therapy as a vehicle to transfer genetic material directly to the patient. At each end of the viral genome is a GC-rich element known as the inverted terminal repeat (ITR). ITRs play a key role in virus replication and encapsidation and are involved in viral genome integration and excision from the host genome. To produce recombinant adeno-associated virus (rAAV), mammalian or insect cells are typically transfected with two or three plasmids — one or two plasmids provide the AAV and adenoviral helper sequences and another (AAV plasmid) contains the expression cassette for the gene of interest flanked by the ITRs. Integrity of the ITR regions in AAV plasmid is important for rAAV production. Truncated ITRs can reduce the yield of full viral capsid production and increase generation of undesirable non- rAAV encapsidated DNA[1]. Unfortunately, AAV plasmid ITRs are frequently mutated during plasmid production in E. coli. Hence, a robust assay that confirms the integrity of ITR sequences of an AAV plasmid can provide tremendous value to rAAV quality control.

The Challenge

Although orientation and integrity of ITRs can be studied by restriction enzyme digestion, the low resolution of gel electrophoresis makes it difficult to detect point mutations or small deletions. Direct sequencing ITR regions can provide sufficient resolution, but the high GC-content and large (>100 bp) palindromic sequence that folds back into a T-shaped hairpin (Figure 1) hinder polymerases in commercially available Sanger cycle sequencing kits. The difficulty lies in two aspects: (1) sequencing enzymes are mutated thermophilic polymerases that, unlike their wild-type counterparts, have reduced processivity and are inhibited by hairpin structures, especially the extremely stable ITRs, and (2) these commercial kits use a deoxyribonucleoside triphosphate analog to achieve better peak resolution during capillary electrophoresis but the analog is not efficiently incorporated by DNA polymerases, especially in hairpins.

Amplifying the ITR regions via PCR and then sequencing the amplification products can yield better results, as secondary structure is less stable in linear templates[2]. However, this two-step method requires additional primer design and cycling, and its success is far from guaranteed as it strongly depends on the local sequences flanking the ITR regions. Moreover, one needs to develop two PCR assays for both ITR regions in every newly constructed AAV plasmid because the PCR assay is not universal.

Figure 1: Illustration of an AAV2 ITR Sequence. The ITR consists of two arm palindromes (B-B' and C-C'), and a longer stem palindrome (A-A'). D sequence is present only once at each end of the AAV genome.

The Solution

To overcome the challenge, Azenta has developed a new ITR sequencing method which improves sequencing signals and extends read lengths through ITR regions.

To test our ITR sequencing method and compare it to standard methods, we first sequenced a plasmid containing AAV2 ITRs using a BigDye® cycle sequencing kit (Thermo Fisher). As expected, the sequencing signal decreased abruptly at the start of the ITR hairpin (Figure 2), causing premature termination. Results could not be improved through addition of common additives, such as DMSO and/or betaine (data not shown).

On the other hand, the newly developed Azenta ITR sequencing method could sequence through the ITR hairpin and read the whole ITR region without significant loss of signal, achieving a read length similar to normal sequencing reactions (Figure 3). Additionally, the influence of primer binding sites on ITR sequencing qualities was also studied. We discovered that optimal sequencing quality is usually achieved when the primer binds 150 bp to 350 bp away from the ITR regions (data not shown).

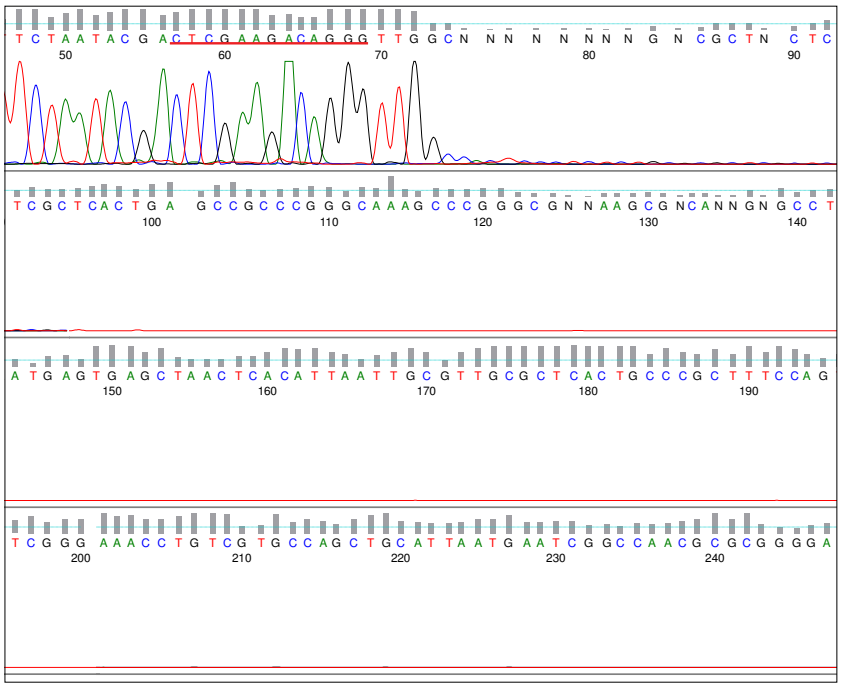

Figure 2: AAV2 ITR region sequenced with BigDye® cycle sequencing kit. The bases "TTGGC" after the red underlined sequence is the start of a 125-bp ITR hairpin which could not be sequenced successfully.

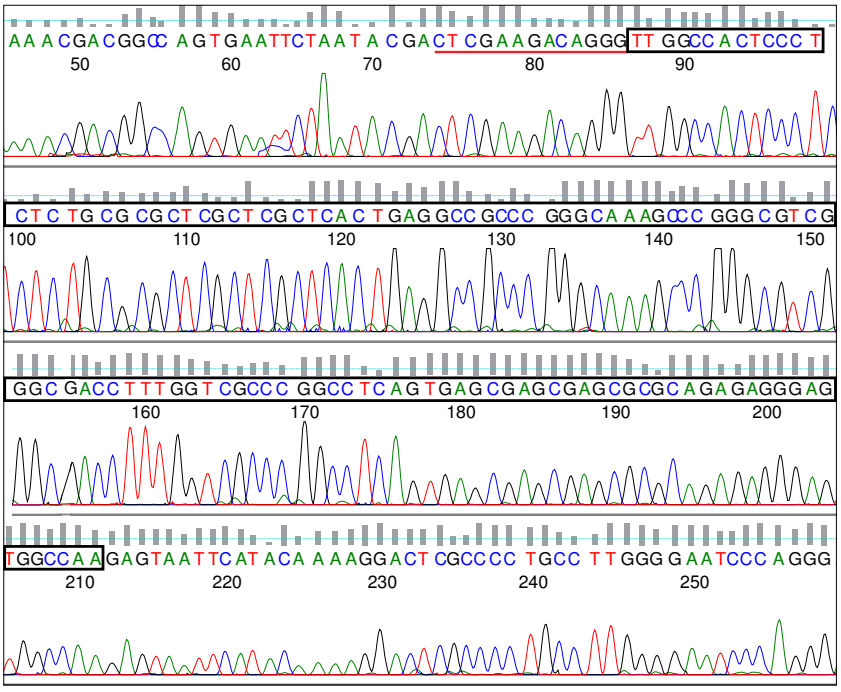

Figure 3: Chromatogram of AAV2 ITR region sequenced by Azenta ITR sequencing method. The sequence underlined in red represents the same sequence mentioned in Figure 2. The black box shows the full 125-bp ITR hairpin.

Qualitative Assessment of ITR Integrity in rAAV Plasmids

At present, rAAV plasmids are mostly propagated in bacteria. Unfortunately, ITRs are frequently rearranged by the homologous recombination systems in bacteria. One of the most common mutations observed in rAAV plasmids after bacterial propagation is the deletion of either B-B' or C-C' ITR arm (Figure 4). This deletion transforms the T-shaped ITR hairpin to have longer stems, making it even more stable than wild-type ITR. After sequencing hundreds of rAAV plasmids propagated in different bacterial cell lines, we showed that the Azenta ITR sequencing method can sequence through wild-type ITRs (Figure 4 and Figure 5A) and the more challenging truncated ITRs (Figure 5B). This helps in the qualitative assessment of the integrity of both ITR regions, even when the ITRs are partially truncated (Figure 5C).

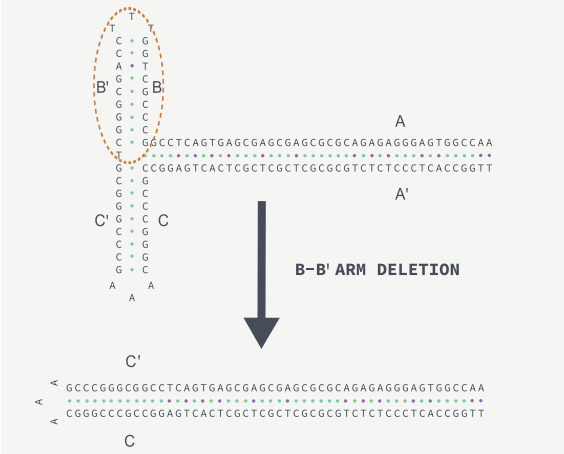

Figure 4: Illustration of the B-B' arm deletion in AAV2 ITR. The wild-type AAV2 ITR is a 125-bp T-shaped hairpin, and the truncated ITR is a 103-bp hairpin with much longer stems.

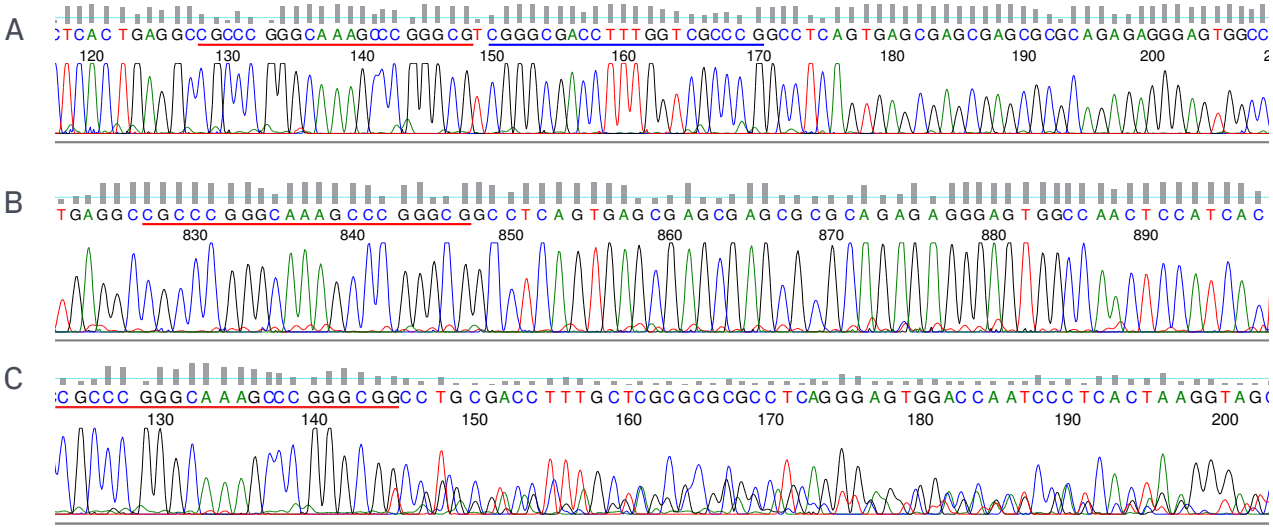

Figure 5: Analysis of the integrity of ITRs in rAAV plasmids using Azenta ITR sequencing method. A) Chromatogram of wild-type AAV2 ITR region sequenced by Azenta ITR sequencing method. The C-C' and B-B' arms are underlined in red and blue, respectively. B) Chromatogram of truncated AAV2 ITR region sequenced by Azenta ITR sequencing method. The sequencing results shows B-B' arm is deleted. C) Chromatogram of deleted AAV2 ITR region sequenced by Azenta ITR sequencing method. The sequencing results show the plasmid has a mixture of intact ITR and truncated ITR. The TTT peaks at 155-157 bp are consistent with the intact ITR sequence, whereas there is a subpopulation of template that lacks the B-B' arm.

Known Limitations of Azenta Life Sciences' ITR Sequencing Method

Some rAAV plasmids contain a homopolymeric G region preceding the 5' end of the ITR region (Figure 6A). Sequencing through homopolymeric G regions with existing Sanger sequencing methods has been very challenging, and the Azenta ITR sequencing method suffers from the same difficulty. The signal drops along the poly-G sequence and causes a premature termination without extending into the ITR hairpin (Figure 6B). Currently, no satisfactory solution is available to overcome the thermodynamic hurdles of a poly-G repeat combined with an ITR hairpin. However, the Azenta ITR sequencing method is capable of sequencing from the reverse direction (Figure 6A) through the ITR hairpin and poly-C repeat, improving data quality and read length in this region (Figure 6C).

Figure 6: Sequencing poly-G containing AAV plasmid by Azenta Life Sciences ITR sequencing method. A) Schematic showing the homopolymeric G before the 5' end of the ITR region; forward and reverse sequencing directions are shown by the black and green arrows. B) Chromatogram of homopolymeric G + ITR region sequenced from the forward direction by Azenta ITR sequencing method. C) Chromatogram of the homopolymeric G + ITR region sequenced from the reverse direction by Azenta ITR sequencing method.

Concluding Remarks

Here we discuss a newly developed direct Sanger sequencing method that reads through both intact and commonly mutated AAV ITRs. The Azenta ITR sequencing method is an effective tool for assessing the integrity of ITRs in AAV plasmids. It enables early detection of mutations in the ITRs, saving time and effort for troubleshooting downstream failures. We chose AAV2 ITRs for testing because AAV2 is the best characterized and most commonly used AAV serotype. We are currently testing our sequencing method on ITRs from other AAV serotypes, which have similar ITR sequences[3], and even other DNA viruses. This method may have broad applications for templates with thermodynamically stable hairpin structures.

References

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