# Minimal DNA constructs for AAV vector productions with exceedingly high genomic purity

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## Introduction

Recently, the purity of recombinant AAV vectors used in gene therapy has come into sharp focus. Expanding gene therapy trials mandate investigation of safety aspects that might lead to unforeseen events in patients. AAV has been shown to frequently mispackage DNA sequences not intended to be part of the vector genome between the inverted repeats. These sequences can arise from the producer cell but most often they were found to originate from the producer plasmids and mainly the plasmid backbone. DNA constructs devoid of these bacterial sequences, like supercoiled Minicircles, offer a unique opportunity to nip the problem of genomic heterogeneity in the bud – by starting with precise molecules.

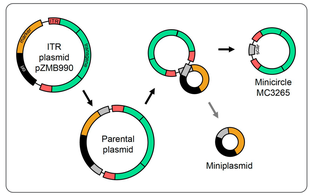



**Fig. 1:**
Minicircles are produced in *E. coli*, yielding a supercoiled product that is purified to high homogeneity. Here: pZMB990 encoding full-length ITRs was provided to PlasmidFactory where MC3265 was derived.

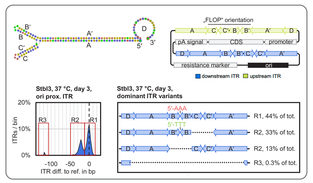



**Fig. 2:**
**What is challenging about maintaining wild type-length ITRs?**
Wild type-length ITRs (143 - 145 bp) are unstable on many plasmids, leading to deletions through a slippage mechanism (Radukic and Le et al., Nucleic Acids Research, 2025).

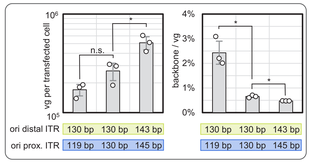



**Fig. 3: Why are wild type-length ITRs important?**
Wild type-length ITRs (143 - 145 bp) are important because they can increase the AAV production titer and reduce mispackaging.
Figure taken from Radukic and Le et al., Nucleic Acids Research, 2025.

**Providing wildtype ITRs is beneficial**

## Methods

Minicircles containing wild-type length AAV ITRs and an eGFPd2 reporter gene were produced in *E. coli* from their corresponding plasmids through *in vivo* intramolecular recombination and purified to homogeneity. HEK-293 cells were cultivated in 6- well plates and triple transfected with 5 pg/cell at a molar ratio of 1:1:1 (transfer:packaging:helper).Genomic titers were measured by qPCR based on GOI-specific primers. Purity was measured by detecting plasmid backbone sequences using specific primers. Capsid titers were measured by ELISA.

## Conclusion

Minicircle starting molecules with wild-type ITRs for AAV production are now available and combine advantageous properties for recombinant AAV vector production. These are expected to usher in a new age of safe and reliable gene therapy vectors based on the principle of quality in, quality out.

## Results

Replacement of the ITR plasmid alone with the corresponding Minicircle in a triple transfection protocol revealed a significant benefit in terms of vector genome yield and purity compared to the triple-plasmid combination reinforcing that backbone sequences coupled to the ITRs are the major source of DNA sequence heterogeneity in AAV vectors. Replacing only the packaging or the helper plasmid was detrimental to yield or purity. Only when all three plasmids were replaced with Minicircles could no mispackaged backbone sequence be detected and packaging efficiency was best of all conditions tested. In this combination however, gains in genomic titer were not observed. By carefully redesigning the packaging minicircle to include a larger intervening sequence in the untranslated region of AAV2 rep, possibly toning down its expression, genomic titer could be restored to the maximum level measured earlier.

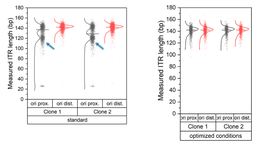



**Fig. 4:**
Maintenance of ITRs in the wildtype length during plasmid production

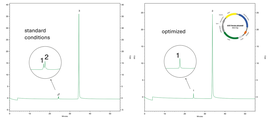
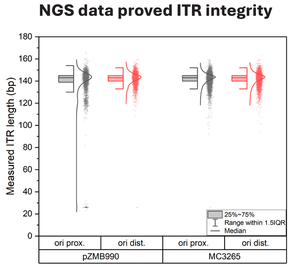



**Fig. 5:**
Capillary gel electrophoresis offers a quick in-process control method for verifying ITR homogeneity



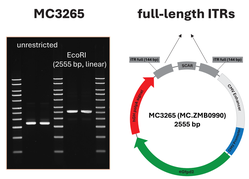
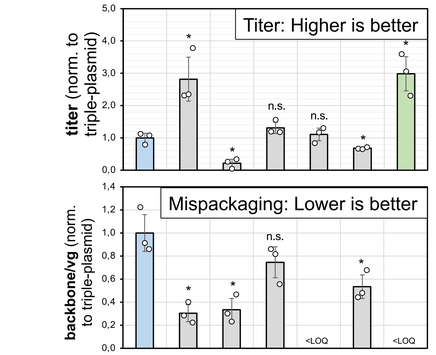



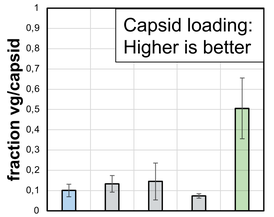

**Fig. 6:**
Successful production of a AAV transfer Minicircle containing wildtype length ITRs




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Full-ITR Vector **Plasmid** | x | | x | x | | x | |
| RepCap **Plasmid** | x | x | | x | | | |
| Ad5 Helper **Plasmid** | x | x | x | | | x | |
| Full-ITR **Minicircle** | | x | | | x | | x |
| RepCap **Minicircle** | | | x | | x | v2 | v2 |
| Ad5 Helper **Minicircle** | | | | x | x | | x |

**n.s.: not significant, * p<0.05, LOQ = 0.001**

**Fig. 7:**
Cumulative benefit in terms yield, purity and packaging efficiency when combining wildtype length ITRs with the Minicircle technology.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Full-ITR Vector **Plasmid** | x | | x | x | | | |
| RepCap **Plasmid** | x | x | | x | | | |
| Ad5 Helper **Plasmid** | x | x | x | | | | |
| Full-ITR **Minicircle** | | x | | | | | x |
| RepCap **Minicircle** | | | x | | | | x |
| Ad5 Helper **Minicircle** | | | | x | | | x |

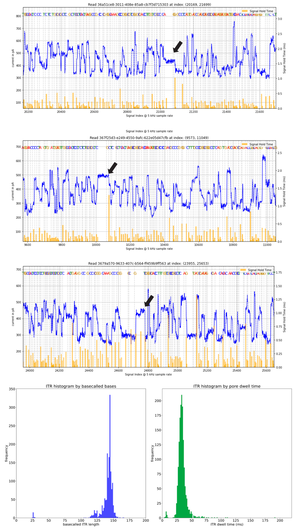



**Fig. 8:**
**Continuing efforts at optimising ITR length characterization through innovative application of nanopore sequencing technology.**
Plot of three raw nanopore signals (blue) of ITRs with their basecalled bases annotated at the respective stride (grey vertical line). From theses raw signals, the ITR dwell time in the pore can be calculated (in the range of milliseconds), which may be more representative of the ITR length compared to a length measurement based on the basecalled bases (Fig. 4). However, the sequencer apparently „gets stuck" at the ITR (block arrows), potentially due to secondary structures, leading to long hold times without a basecalled base (yellow bars and right-hand axis), which challenge this readout method.

**Combination of Minicircles and wtITRs** **Higher titer, higher purity, higher packaging efficiency**

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