# Nanopore sequencing purity assessment of adeno-associated virus (AAV) genomes produced using enzymatically amplified linear dbDNA™

*K Staunton, DM Kuzeva, T Adie, T Merritt, S Kim, L Caproni, D McLaren and J Makin*
*Touchlight Genetics Ltd, Hampton, UK TW12 2ER*

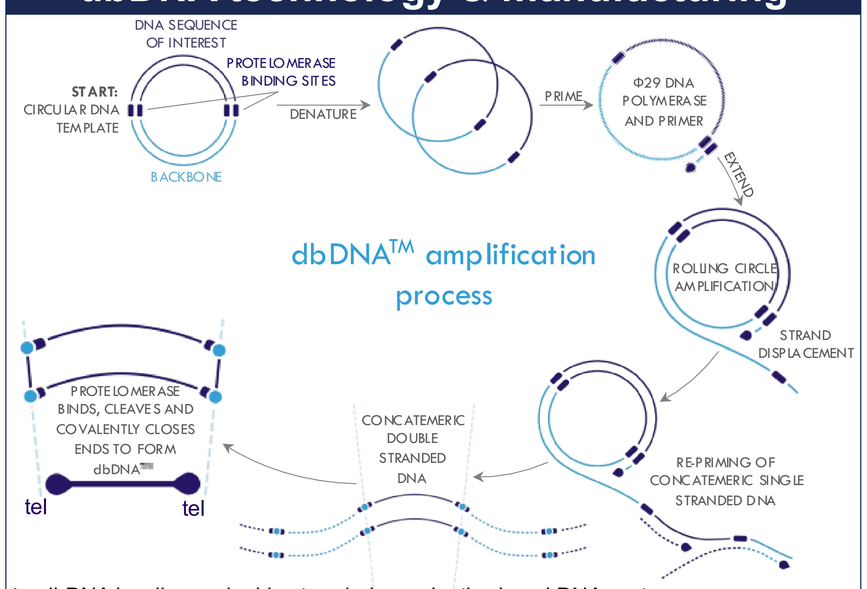
TOUCHLIGHT
DNA is our DNA™

Scan the QR code to download

## Abstract

Recombinant adeno-associated virus (rAAV) clinical translation remains constrained by significant manufacturing and safety challenges. One approach to address these limitations is to substitute the *E. coli* derived plasmid DNA with cell-free DNA. Touchlight's linear dbDNA™, an enzymatically amplified DNA construct, offers rapid, scalable generation of GMP-grade material in a fraction of the time of conventional plasmid DNA. dbDNA lacks bacterial sequences, making it an ideal DNA template for AAV production. To characterise the purity and quality of dbDNA-derived AAV vectors and plasmid-derived AAV vectors, nanopore sequencing was employed to profile the encapsidated DNA. Successful packaging of the ITR-to-ITR AAV genome using dbDNA was confirmed. Furthermore, the dbDNA-derived AAV population contained significantly fewer process- and product-related DNA impurities. While the reverse packaged plasmid sequences contained the antibiotic resistance and bacterial replication cassette, reverse packaged dbDNA comprised short inert stuffer sequences. Thus, dbDNA offers safety advantages over plasmid as an AAV template. Illumina sequencing confirmed full length ITRs are maintained during the production of dbDNA. Nanopore ITR characterisation of AAV genomes revealed high levels of intact ITRs in both dbDNA- and plasmid-derived AAV preparations. The dbDNA-derived AAV vectors contained lower levels of partial genomes compared to plasmid-derived AAV vectors. Thus, the high vector batch quality and low DNA impurity levels observed in dbDNA-produced AAVs further support their enhanced safety profile.

## dbDNA technology & manufacturing




- dbDNA is a linear, double stranded, covalently closed DNA vector.
- dbDNA is a minimal construct – no bacterial backbone sequences, no origins of replication, no antibiotic resistance genes.
- The amplification process for production of dbDNA is shown above.

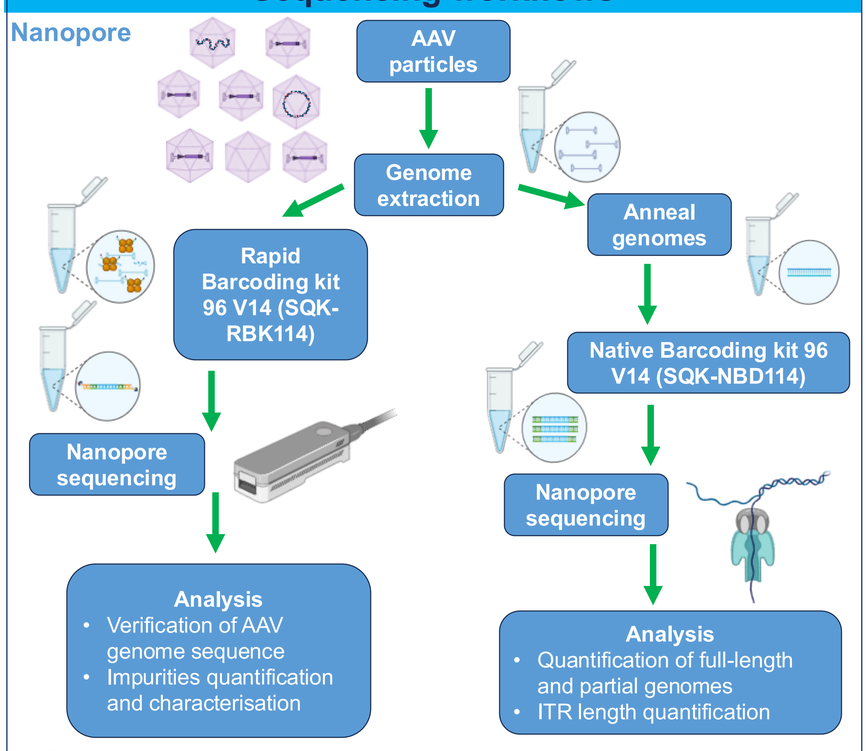

| Discovery | Research | Development | SmartGMP & GMP |
|---|---|---|---|
| Up to 1mg RUO grade 3-4 weeks* | 2.5mg to 55mg R&D grade 6 weeks* | 75mg to 5g R&D grade 4-6 weeks* | 75mg to 5g GMP grade 5-6 weeks* |

*Time from start of plasmid amplification

- Production is from mg to gram scale via a cell-free, high-fidelity enzymatic amplification process.

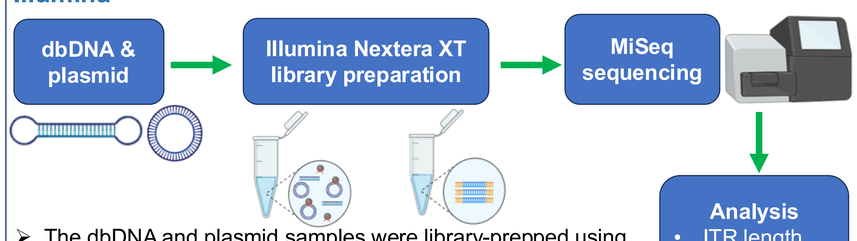
## Methods

### Sequencing workflows




- The AAV genomes were subject to transposome mediated- and ligated mediated-Nanopore library preparation and sequenced on R10.4.1 flow cells.

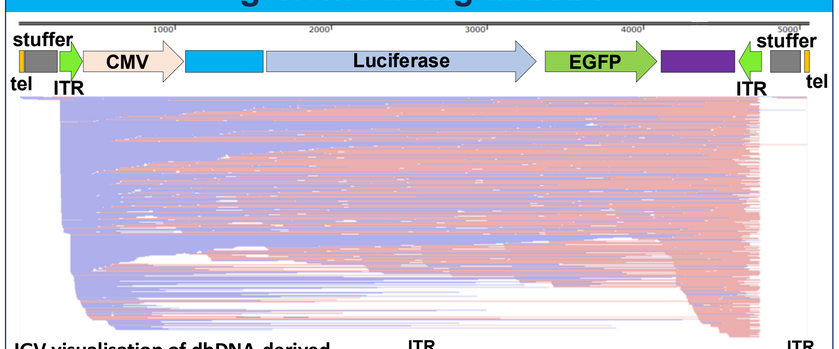
**Illumina**




- The dbDNA and plasmid samples were library-prepped using the Illumina Nextera XT kit and sequenced on the MiSeq using a 2x250 bp pared-end read configuration.

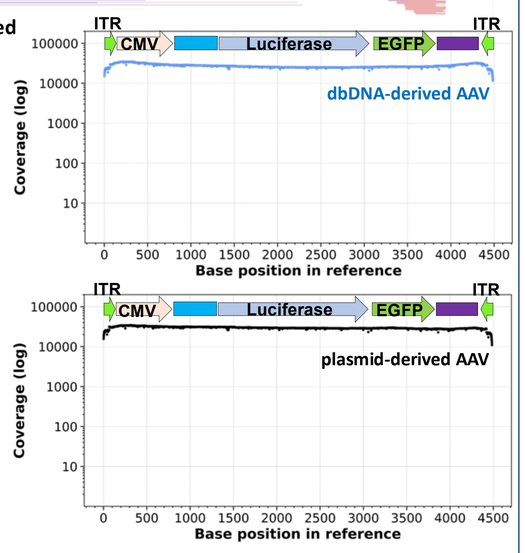
## Results

### Successful packaging of the ITR-to-ITR AAV genome using dbDNA



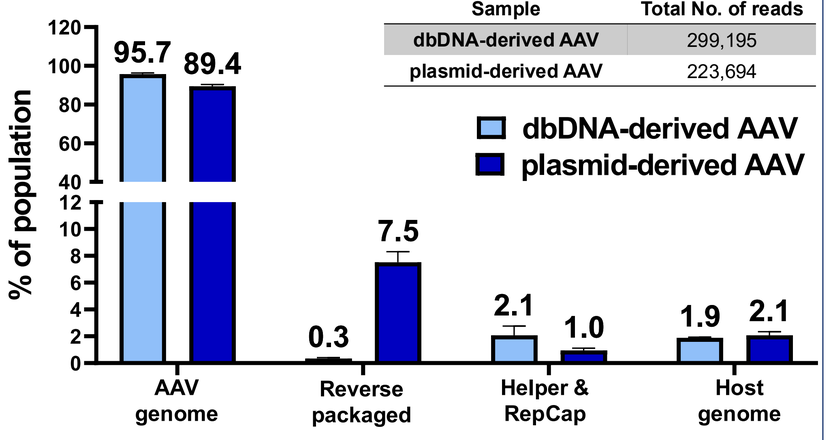

**IGV visualisation of dbDNA-derived AAV (1% of AAV genome reads)**

**Blue = forward strand (sense)**
**Red = reverse strand (antisense)**




- IGV visualisation of dbDNA-derived AAV genome reads confirmed ITR-to-ITR AAV genome was packaged instead of the entire dbDNA molecule (tel-to-tel), which would include the stuffers and protelomerase sites.
- Coverage graphs were identical for dbDNA- and plasmid-derived AAV genomes.

### Fewer process- and product-related impurities packaged in AAVs produced with dbDNA

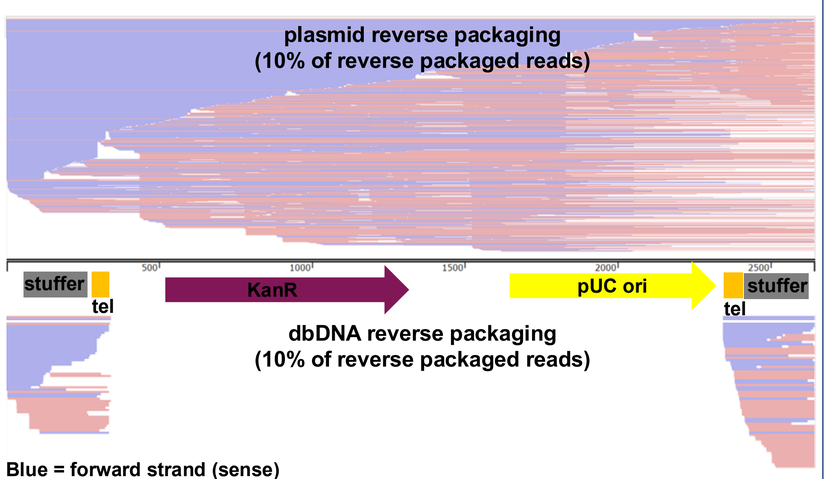



| Sample | Total No. of reads |
|---|---|
| dbDNA-derived AAV | 299,195 |
| plasmid-derived AAV | 223,694 |

Nanopore → FASTQ reads → QC filtered → Aligned to combined reference → Quantified aligned bases → % of pop.

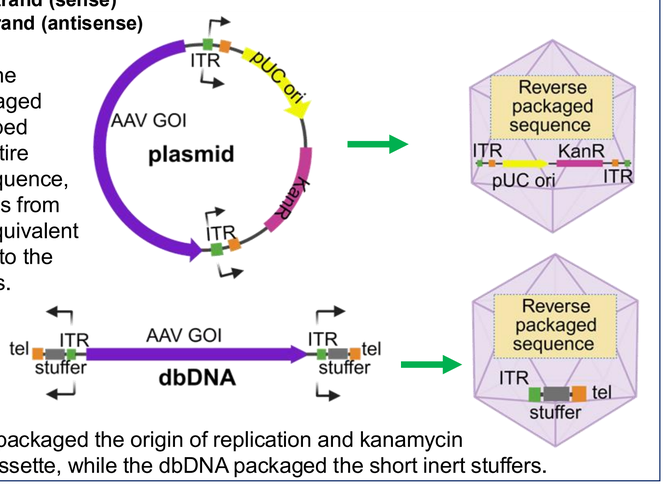
- Impurities were quantified within the dbDNA- and plasmid-derived AAV preparations.
- The dbDNA-derived AAV population contained predominantly vector genome (95.7%) with minimal reverse-packaged sequences. In contrast, the plasmid DNA-derived AAV contained 7.2% more reverse packaging.
- Low levels of helper, RepCap and host genome were detected in both samples.

### The reverse packaged dbDNA comprised short inert stuffer sequence, while the reverse packaged plasmid contained the bacterial replication and antibiotic resistance cassette.



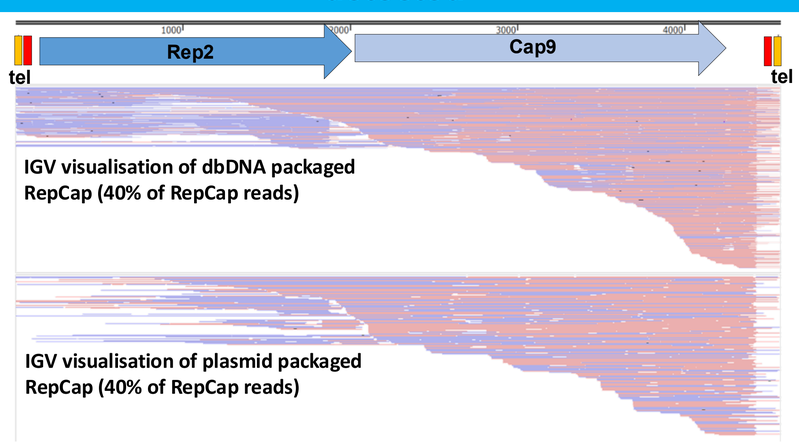

**Blue = forward strand (sense)**
**Red = reverse strand (antisense)**

- Reads from the reverse-packaged plasmid mapped across the entire backbone sequence, whereas reads from the dbDNA equivalent only mapped to the stuffer regions.

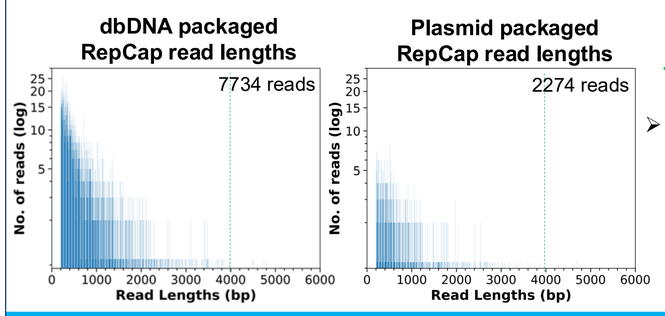



- The plasmid packaged the origin of replication and kanamycin resistance cassette, while the dbDNA packaged the short inert stuffers.

### Little to no full-length RepCap packaging was detected

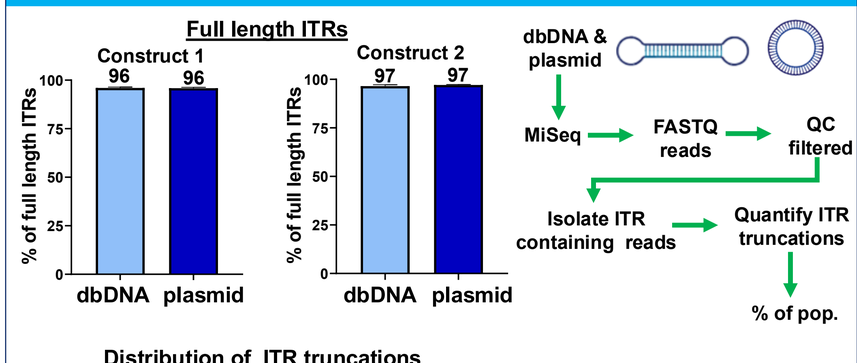


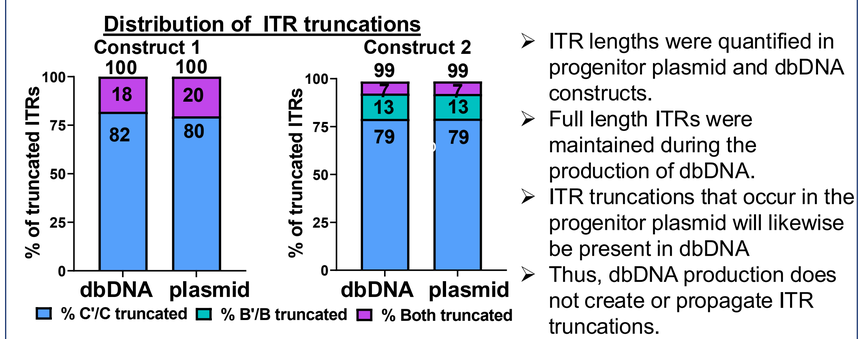

- IGV visualisation of RepCap reads from the dbDNA- and plasmid-derived AAV preparations. In both constructs, mostly Cap9 was packaged over Rep2.




- Lengths of reads that aligned to RepCap were plotted. Little to no full length RepCap was detected in this workflow.

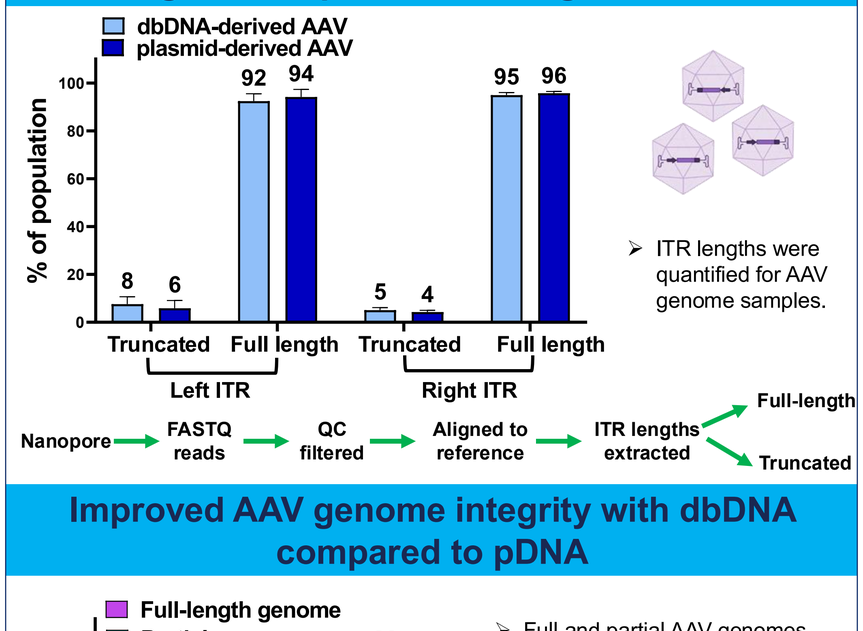
### Full length ITRs are maintained during production of dbDNA



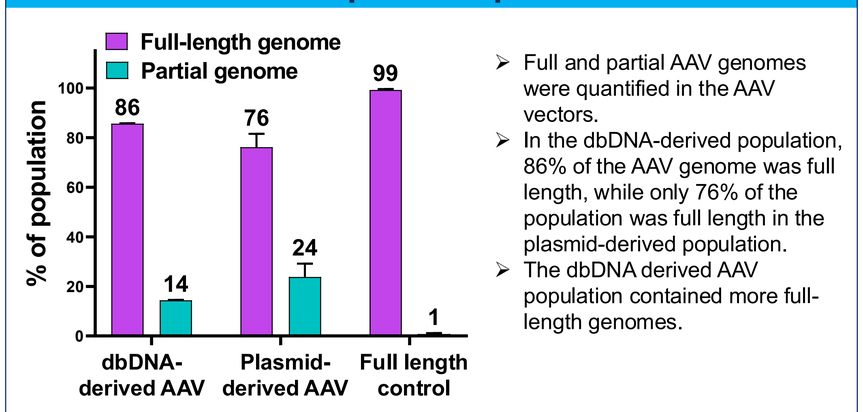

- ITR lengths were quantified in progenitor plasmid and dbDNA constructs.
- Full length ITRs were maintained during the production of dbDNA.
- ITR truncations that occur in the progenitor plasmid will likewise be present in dbDNA
- Thus, dbDNA production does not create or propagate ITR truncations.

### High levels of intact ITRs were detected in AAV genomes produced using dbDNA




- ITR lengths were quantified for AAV genome samples.

### Improved AAV genome integrity with dbDNA compared to pDNA




| Sample | Total No. of reads |
|---|---|
| dbDNA-derived AAV | 41,255 |
| plasmid-derived AAV | 82,607 |
| Full length control | 1,914,505 |

- Full and partial AAV genomes were quantified in the AAV vectors.
- In the dbDNA-derived population, 86% of the AAV genome was full length, while only 76% of the population was full length in the plasmid-derived population.
- The dbDNA derived AAV population contained more full-length genomes.

## Conclusion

The dbDNA derived AAV population contained fewer DNA impurities, higher percentage of full-length genomes and high levels of intact ITRs. While the reverse packaged plasmid contained the bacterial replication and antibiotic resistance cassette, reverse packaged dbDNA comprised short inert stuffers. Consequently, dbDNA provides safety advantages over plasmid-based templates for AAV production. The high vector batch quality and low levels of DNA impurities observed in dbDNA-derived AAVs further reinforce its improved safety profile.

## Funders and collaborators

CATAPULT Cell and Gene Therapy
UKRI Innovate UK
Complement THERAPEUTICS