# Applying Next Generation Sequencing (NGS) to accelerate cell and gene therapy product development

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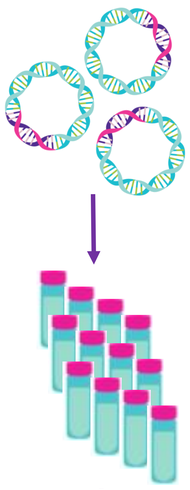
## Introduction

Viral vector mediated gene therapy (GT) vectors, such as AAV, are typically aimed at conditions for which no other treatment exists and for which there's urgent need. Viral vectors such as Lentivirus are also critical for the manufacture of many cell therapy products. Here, we outline where Next Generation Sequencing (NGS) can be applied during development and manufacture of cell and gene therapies to accelerate development and gain greater insights into product characteristics to de-risk the development process. Examples using both short-read and long-read NGS technology are provided.



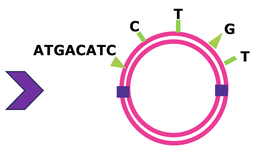
**Plasmid Quality Control**

NGS offers an off the shelf, rapid solution to obtain whole plasmid sequence information, with increased read depth to identify changes that may impact production of viral vectors.

**CASE STUDY**: Plasmids used in AAV manufacture


An unexpected low-level sequence variant was revealed during identity testing of our Client's AAV product using short-read NGS

As part of the investigation, plasmids used in manufacture were also analysed using NGS, which hadn't been part of the original QC testing




The same sequence variations were found in the plasmid. This demonstrates the value of using NGS for identity testing and applying consistently to both critical starting materials as well as final product, since this approach can detect low-level variants that may not be detected using Sanger sequencing.

**Plasmid Sequence Identification**

EMA and FDA Guidelines recommend full sequencing of plasmids used to produce gene therapy vectors

**Cell line characterization**

Cell banks used to manufacture viral vectors require extensive viral safety testing including broad spectrum virus detection assays. NGS is now recommended in key global regulatory guidelines as a replacement for traditional *in vivo* assays for adventitious virus detection. Fully validated GMP compliant NGS assays incorporating advanced bioinformatics analysis can provide viral safety assurance for cell lines used to manufacture AAV, Lenti- and other vectors. NGS enables broad-spectrum and high sensitivity virus detection which can be completed more rapidly than *in vivo* assays and avoids unnecessary animal testing which can also suffer from robustness issues.

**Viral safety assurance**

ICH Q5A (R2) and other key guidances now recommend NGS as an alternative *to in vivo* testing for cell banks

**Identity confirmation of vectors**

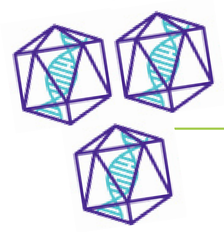
Sequencing of the entire vector is recommended in EMA and FDA guidelines*, however this can be challenging, especially for AAV vectors. NGS offers a solution for technically demanding sequencing needs such as ITR regions of AAV and provides extended depth of coverage and insights as compared with other sequencing methods, enabling variant calling, coverage of entire vector sequence as well as removing the need for custom primer design and production.

* Vectors under 40 Kbp (FDA)

AAV SEQUENCE DATA OBTAINED USING ILLUMINA® SHORT-READ TECHNOLOGY

AAV sequence variant characterization: Identification of possible insertions, deletions and single nucleotide changes with associated frequencies

| Position | Reference Base | Variant Base | #High-Quality Ref (fwd) | #High-Quality Ref (rev) | #High-Quality Var (fwd) | #High-Quality Var (rev) | Max # of Reads Supporting INDEL (IDV) | Raw Read Depth (DP) | % Variant Frequency | Variant Type |
|---|---|---|---|---|---|---|---|---|---|---|
| 5,702 | G | GTACGGCC | N/A | N/A | N/A | N/A | 1,047 | 1,298 | 80.66 | Insertion |
| 5,811 | C | T | 5,401 | 286 | 2,267 | 714 | N/A | 9,349 | 34.39 | Substitution |
| 5,856 | T | C | 7,306 | 517 | 3,798 | 1,997 | N/A | 18,848 | 42.55 | Substitution |
| 5,885 | T | A | 4,672 | 905 | 1,917 | 995 | N/A | 13,726 | 34.3 | Substitution |
| 5,885 | ATGACATC | T | N/A | N/A | N/A | N/A | 3,845 | 13,726 | 28.01 | Deletion |

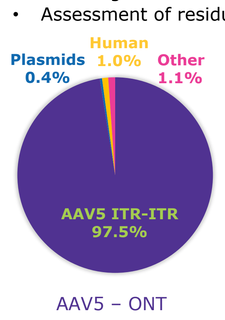


AAV vectors for *in vivo* gene therapy

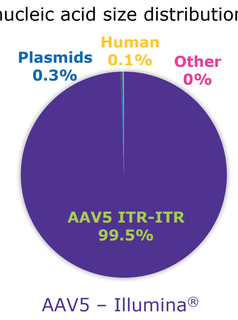
**Characterization of vectors: Encapsidated DNA impurities in AAV**

DNA such as host cell DNA or plasmid may be co-packaged with the intended payload in AAV capsids. Non-therapeutic DNA impurities may impact the safety profile of AAV therapies by influencing the required dose of vector and immunogenic responses, hence characterization of the sequence identity and quantity of co-packaged DNA impurities is important for product characterization. Both short-read (e.g. Illumina® technology) and long-read (e.g. Oxford Nanopore Technology) NGS approaches provide valuable insights, and in combination, genome assemblies of longer length and deeper coverage can be created. Long-read technology provides additional information that can enhance understanding of packaged AAV genomes and DNA impurities, such as;

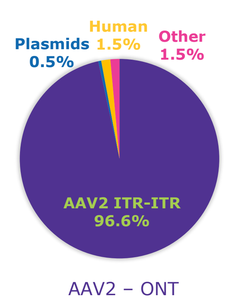
- AAV length distribution assessment and better ITR characterization
- Assessment of residual nucleic acid size distribution



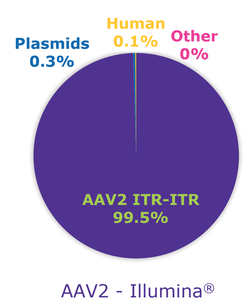

AAV5 – ONT




AAV5 – Illumina®




AAV2 – ONT



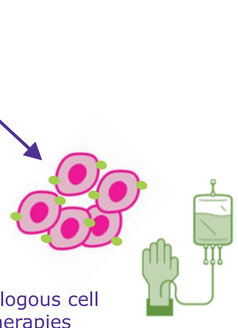

AAV2 - Illumina®

**Characterization of encapsidated DNA in AAV-2 and AAV-5 serotypes using short- and long–read NGS hybrid analysis.** The total proportions of DNA sequence mapped to either the expected AAV serotype (spanning the entire ITR-ITR region) or co-packaged impurities (host cell DNA, plasmid DNA) using each approach are shown

**METHOD:** The process of extraction and sequencing for challenging, primarily single-stranded AAV capsid contents was optimized. AAV-2 and -5 samples, consisting of full-capsid fractions only, were pre-treated with Benzonase® endonuclease prior to extraction.

- For short-Read Analysis: Extracted material was converted to cDNA, processed through an Illumina® compatible library preparation then sequenced on the NextSeq™2000 instrument.
- For long read processing: Extracted DNA was amplified, then libraries are prepared using the Ligation Sequencing Kit V14 for sequencing on the GridION® instrument.
- Short- and long-read sequence data was analyzed using a bioinformatics pipeline to map both AAV genome and residual DNA sequences.

**RESULTS:** Combining the two sequencing methods enabled deeper coverage of difficult to sequence regions such as ITRs than short read methods alone and recovered the contents of capsid payloads in clinically significant AAV serotypes. Removal of exogeneous sequences using Benzonase® endonuclease was very effective as >99% of the short-read sequences mapped to AAV associated genes and >96% in the long-read samples. Difficult to sequence ITR regions were captured effectively by the long-read approach, enabling coverage of the entire ITR sequences.



Autologous cell therapies

**Cell Therapy characterization and safety testing: Current and future applications of NGS**

### Lentiviral mediated autologous cell therapies

Lentiviral vector-mediated therapies exploit the ability to integrate into non-dividing cells to deliver and stably integrate transgenes to create cell therapies such as CAR-T. However, the site of integration may be considered a safety attribute as in rare cases, this could disrupt function of genes or even activate oncogenes. NGS is a useful tool to perform lentiviral integration site analysis (ISA), which is critical to investigate potential genotoxicity following random integration events. This is also considered an important aspect of investigating possible delayed adverse effects following treatment with gene therapy products.

- Long Term Follow-Up After Administration of Human Gene Therapy Products (FDA Guidance for Industry, January 2020)

### Gene editing therapeutics

Gene editing as a therapeutic strategy can be mediated by delivery of a variety of different editing systems e.g. CRISPR-Cas nucleases following delivery by viral or non-viral systems. Recent FDA guidance on Gene Editing outlines expectations for determining both on-target and off-target editing activity to understand potential genotoxicity following administration. NGS will be a critical component of the toolbox for safety testing of edited cells since guidance indicates use of methods that can detect low frequency events. The type, frequency and location of both on-and off target editing should be understood.

- Human Gene Therapy Products Incorporating Human Genome Editing (FDA Guidance for Industry January 2024)

### Human allogeneic cell therapies

Human allogenic therapies encompass a wide variety of different possible formats of cells, which may or may not have undergone gene editing, but can be used to treat multiple patients. Recent *draft* FDA guidance on Safety Testing of Human Allogeneic Cells (2024) recommends whole genome sequencing, necessitating NGS approaches for;

- Assessment of continuous cell lines used as therapy, which may accumulate mutations over repeated passage
- Assessment of expanded clones of edited cells, to evaluate potential mutations, genomic integrity as well as integration sites and on-/off-targeting events.

- Safety Testing of Human Allogeneic Cells Expanded for Use in Cell-Based Medical Products (**Draft** FDA Guidance for Industry, April 2024)

## Summary

- NGS has multiple applications during the development and manufacturing process of viral vector-mediated and cell therapies.
- It can help de-risk development when applied to gain in-depth insights into critical starting materials as well as final products, by providing sensitive and robust detection and characterization of sequence variants that may be missed using other approaches.
- By removing the need for custom primer design prior to sequencing, NGS can deliver faster results to help accelerate development of gene and cell therapies.
- Advanced sequencing technologies are increasingly referenced/recommended in regulatory guidances to improve characterization of critical starting materials, understand potential product impurities and gain safety insights for cell-based therapeutics.

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