Genomic Characterization of AAV Products using Multiplex ddPCR and Nanopore Sequencing

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Introduction

Understanding the genome identity and integrity of AAV gene therapy products is important for understanding their efficacy, quality, stability, and safety. Identity of the AAV DNA product determines if mispackaging has occurred and integrity provides insight into whether the product is intact or fragmented. Both identity and integrity have the potential to impact quality and therapeutic effect. While Multiplex digital droplet PCR (ddPCR) has been developed as an effective analytical method to determine transgene integrity, it does not provide information about mispackaged DNA identity. In the reported study, we used the Next Gen sequencing method Nanopore, which utilizes ligand adaptor methods such as "shotgun" Tagmentation and targeted guide RNA, to show both transgene integrity and mispackaged DNA identity can be analyzed.

Materials and Methods

Multiplex ddPCR

Multiplex ddPCR measures the % of connected region with primer sets at the 5' and 3' ends of the gene of interest (GOI). Samples are diluted to obtain one genome per droplet to analyze whether the individual genome is intact. If the two fluorescently labeled targets on opposite ends of the GOI are positive, then the genome can be considered intact. Using this measurement, percent of intact product can be determined.

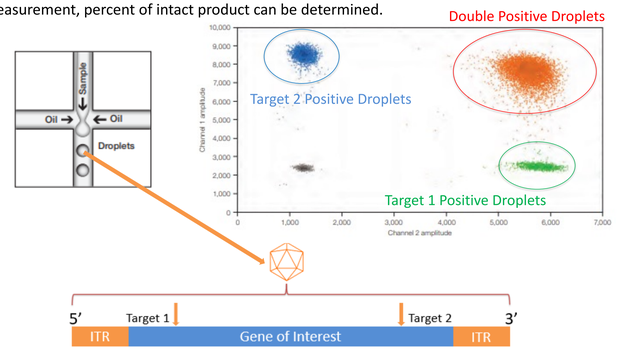

Figure 1. Workflow of Multiplex ddPCR: Samples are diluted to obtain approximately one capsid per droplet. The PCR sample is separated into oil droplets prior to DNA amplification.

Nanopore Sequencing

With Nanopore sequencing, long read sequencing is achieved by threading the DNA strand through a nano-scale pore so that each base is read by the instrument. The sequencing results of thousands to billions of bases can be aligned to provided reference sequences to determine packaged DNA identity. In the Tagmentation method, the DNA product is fragmented so adaptors can attach to read the bases. With the guide RNA method, the DNA sequence is targeted at specific sites - by targeting the ITRs of the genome, full ITR-ITR reading is achieved.

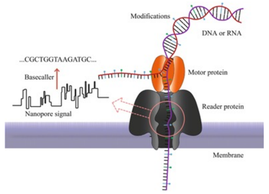

Figure 2. Workflow of Nanopore Sequencing: Bases of DNA strand are read by threading through a nano-scale pore.

Fragment Size Distribution Results

Fragment size distribution for each sample provides the number of sequences at a given sequence length. While the Tagmentation method compromises genome integrity through fragmentation, the guide RNA method does not. The guide RNA method sequencing results provide a fragment size distribution with full ITR-ITR reading that can be used to determine % Intact based on the GOI expected sequence length.

Tagmentation Method

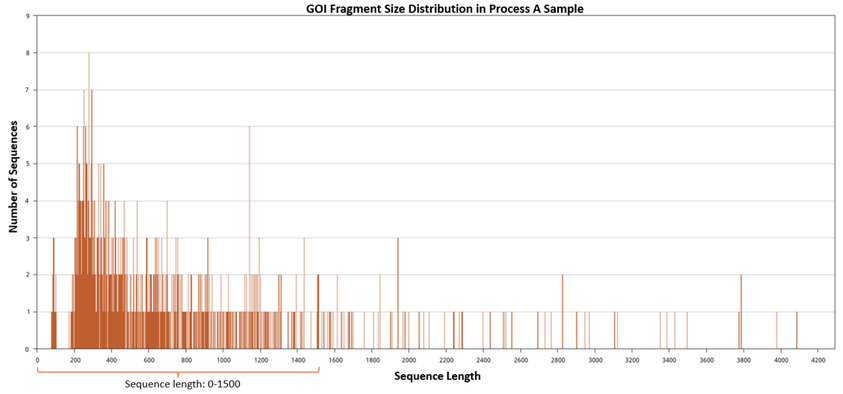

Figure 3. Fragment size analysis results using the Tagmentation method of a Process A Sample. Approximately 90% of fragments are below the length of ~1500 resulting in poor visualization of full length reads.

Guide RNA Method

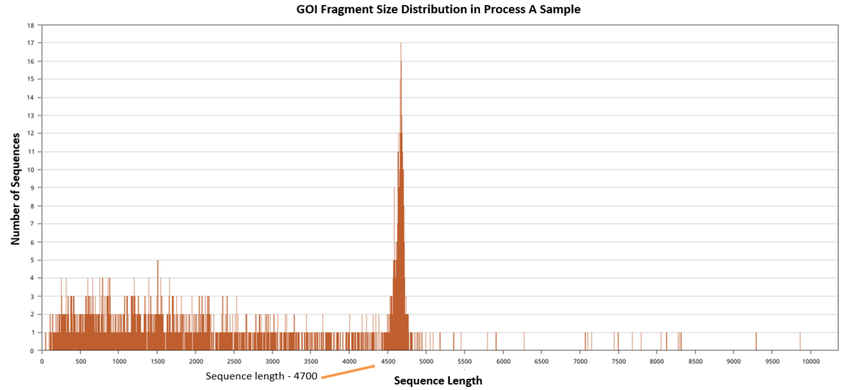

Figure 4. Fragment size analysis results using the guide RNA adaptor method of a Process A sample. Higher percent of sequences are seen at expected sequence length of 4700 providing better resolution of full-length genomes.

Multiplex ddPCR and Nanopore Results

Sample	Multiplex ddPCR % Intact	Multiplex ddPCR Extracted % Intact	Guide RNA Average % Intact
Process A Sample	41%	51%	39%
Process B Sample 1	56%	64%	27%
Process B Sample 2	54%	73%	43%

Table 1. Summary of Multiplex ddPCR and Nanopore Results. Multiplex ddPCR results calculated % Intact based on double positive droplets of samples. Multiplex ddPCR % Intact results report genome integrity of samples before extraction, while Multiplex ddPCR Extracted % Intact report results after extraction. Guide RNA method results calculated % Intact using the % of sequences at the expected sequence length of the gene compared to the total reads for the GOI.

% Alignment Results

The Tagmentation method provides better insight into DNA species identity and information on mispackaging. Identity of DNA product was determined by aligning to specific reference sequences to calculate the % mapped to each sequence.

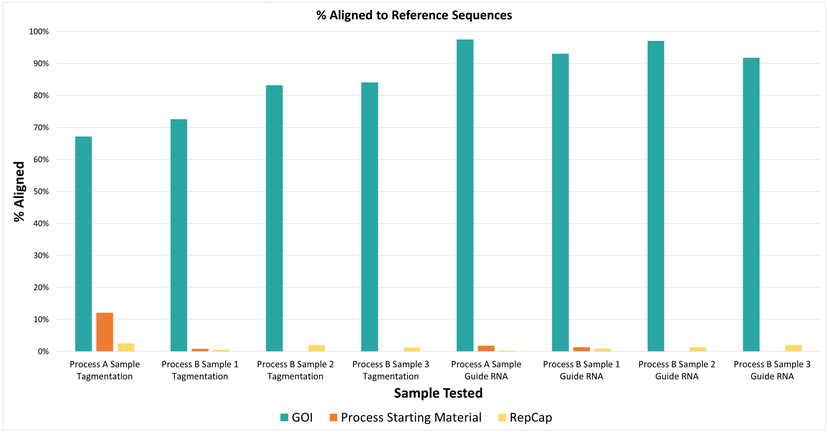

Figure 5. Process A and Process B Samples alignment to reference sequences shows the % Aligned to the GOI, RepCap, and Process Starting Material. Each alignment to reference sequences is performed using the Tagmentation method and guide RNA method to determine mispackaged DNA identity. The alignments show that the Process Samples tested did not contain significant amounts of Process Starting Material or RepCap.

Conclusions

The combined use of Multiplex ddPCR and Next Gen sequencing allowed for a more robust and accurate characterization of the packaged DNA length, integrity, and identity. Using these methods together, we were able to orthogonally determine the % Intact genome and that the processes used did not result in a significant amount of Process Starting Material or RepCap in the Process Samples. Although Multiplex ddPCR provided information on % Intact genome, Nanopore sequencing provided additional information on identity and integrity. While the guide RNA method did not compromise integrity, the Tagmentation method provided better DNA species identity and mispackaging information.

Reference

1. Leading Life Science Research & Clinical Diagnostics — Bio-Rad. (n.d.). Retrieved April 18, 2023, from https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf

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