Comparative Analysis of Second Strand Synthesis Processes to Establish an rAAV Batch Quality Pipeline using Nanopore Sequencing

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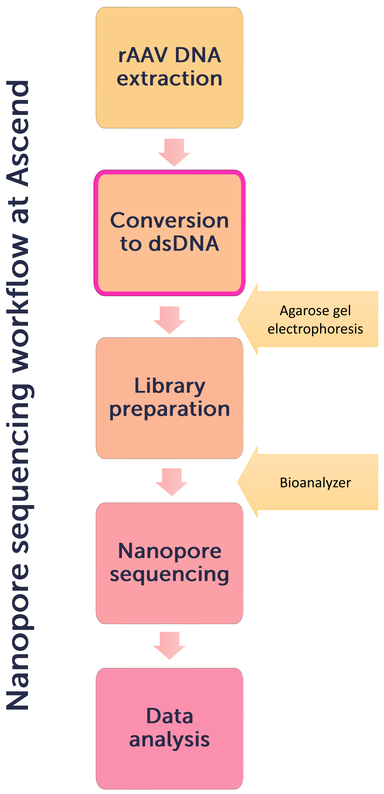
Introduction

During production, recombinant AAVs are equipped with heterogenous payloads that mainly derive from the desired vector cassette, but also from divergent sequences, so-called DNA impurities. Short and, more recently, long-read based NGS is becoming the method of choice for a comprehensive analysis of encapsidated DNA in AAV-based therapeutics. A crucial step in the process is the conversion of the viral ssDNA genome into dsDNA amenable for NGS library construction. The easiest method for conversion is the annealing of (+) and (-) strands[1]. Another described strategy is the synthesis of the second strand by DNA polymerase I using random primers[2]. A third approach, which was not focus of this work, is transposase-mediated direct library preparation without any dedicated DNA conversion step, relying on transposase activity on ssDNA and annealing[3]. Here, we describe a comprehensive, comparable analysis of various published and newly developed dsDNA conversion processes that we tested using the same sequencing chemistry.

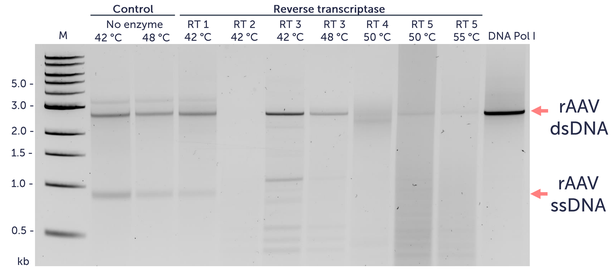
[1] Tam Tran et al. (2020), Molecular Therapy - Methods & Clinical Development, 18, 639-651.
[2] Lecomte et al. (2015), Molecular Therapy - Nucleic Acids, 4, e260,
[3] Radukic et al. (2020), NAR Genomics and Bioinformatics, 2, lqaa074

Nanopore sequencing workflow at Ascend



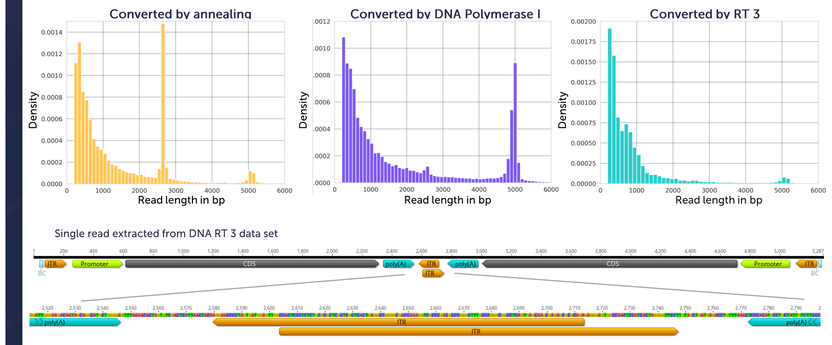
Unbiased conversion to dsDNA is crucial but distinct methods exists in the field

Comparative analysis of enzymes



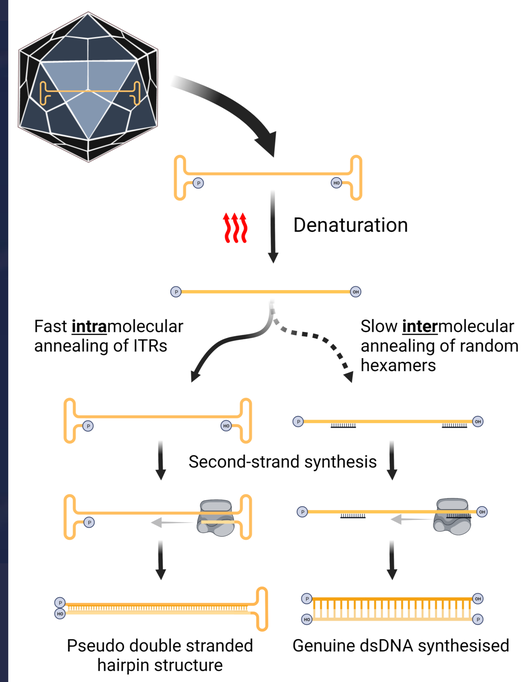
Different enzyme classes were tested for their ability to synthesize a second strand in the dsDNA conversion process. Next to previously published DNA polymerase I (DNA Pol I), reverse transcriptases (RTs) were tested for their ability to generate dsDNA. Reverse transcriptases are able to produce dsDNA from a ssDNA template. However, different length artefacts were detected at smaller sizes.

The synthesized double sized product is verified by Oxford Nanopore sequencing



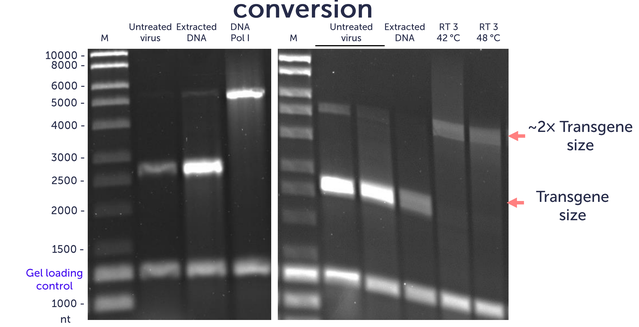
Nanopore sequencing on a R10.4.1 flow cell showed the double-sized product peak (~5100 bp) derived from second strand synthesis pre-dominantly primed by the 3' ITR. A single read with barcodes (BC) was extracted from the double sized fraction to confirm the presence of the ITR linked fragment which is the result of ITR-derived priming.cc

Model: ITR-mediated priming



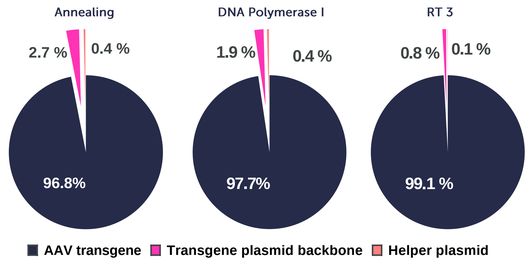
After extraction, viral DNA is denatured by heat to a linear structure. After cooling, the very fast intramolecular formation of the ITR hairpins outcompetes slower intermolecular annealing of random hexamers. The 3' ITR serves as a primer for enzyme-mediated second strand synthesis resulting in the formation of a pseudo double stranded hairpin structure. This is independent on the used enzyme class. Importantly, complementary data revealed that in the absence of ITR sequences hexamer priming is sufficient for efficient second strand synthesis.

Alkaline gel reveals a double-sized product after conversion



Alkaline gel electrophoresis was performed to verify the successful conversion into dsDNA. Next to the expected band at transgene size, a second fragment occurred in enzymatically converted dsDNA at approx. double the transgene size. Results look similar for both DNA polymerase I and reverse transcriptase.

Sequence mapping of distinct conversion methods reveals only subtle differences



Sequencing reads were processed in our in-house analysis pipeline including mapping to reference sequences using minimap2. Annealing leads to higher relative number of reads aligning to plasmid-derived impurities in comparison to enzymatic conversion. The hairpin sequence can still be accurately mapped to the reference sequences.

Summary

Taken together, we have established a robust rAAV nanopore sequencing process and evaluated the strength of different conversion methods. DNA conversion was achieved by annealing as well as by second strand synthesis with reverse transcriptase and DNA polymerase I. Nanopore sequencing and alkaline gel electrophoresis surprisingly suggested the formation of a pseudo-double stranded hairpin structure during conversion, that so far has been overlooked in literature. This indicated a strong priming activity of the fold back structure of the 3' ITR that dominates the conversion process. Understanding the molecular biology of this process in detail will allow us to further improve our bioinformatic pipeline, as each conversion method results in a process-dependent NGS data set with its own bias. Thus, having three independent methods provides us with the unique opportunity to obtain more reliable and comprehensive DNA impurity profiles than can be achieved by any single analysis. In summary, we believe that the comprehensive conversion process characterization empowers us to tailor sample preparation and data analysis to the aim of rAAV sequencing projects.

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