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rAAV Vector Breakpoints Determined Using Single-Molecule, Modified Base Sequencing

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Recombinant adeno-associated virus (rAAV) is an important gene therapy vector due to its low immunogenicity and long track record for use in humans. As with all gene therapy systems, the quality of the material delivered is critical. The presence of empty or partially full capsids is one factor that affects rAAV quality. When rAAV is delivered to cells or analyzed *in vitro* after capsid disruption, the mixed ssDNA hybridizes to complementary partners, forming heteroduplex double stranded DNA molecules. Standard next generation sequencing (NGS)library preparation methods result in extension of DNA at pre-existing breakpoints, making their identification difficult. When there is no knowledge of breakpoint location, it is challenging to assess vector quality or to fix any sequence-dependent issues.

We have developed a NGS library preparation method that allows us to distinguish pre-existing DNA from any DNA added during NGS library preparation. Modified nucleotides are included in the library preparation so that any new DNA includes stretches of modified bases while the pre-existing ssDNA consists of natural, unmodified bases. Using the Sequel II system, the modified bases can be distinguished from unmodified bases, enabling breakpoint identification at high resolution. This method was used on a particular rAAV that was known to break during packaging, a phenomenon that can occur in AAV vectors of various serotypes, allowing us to precisely map the sites that were fracturing and measure the packaged lengths of rAAV ssDNA molecules.

These rAAV breakpoint data have allowed us to design better therapeutic vectors and generate more precise quality control data. Partially filled capsids have been an ongoing FDA concern and the new technology provides information that standard methods do not. This use of modified bases for localizing DNA breaks enables improved vector designs and provides better quality metrics for AAV vectors, resulting in higher quality gene therapy vectors.