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# Genome-Wide Integration Assay for rAAV Mediated Homologous Recombination (HR) in Human Hepatocytes Demonstrated Precision of *In Vivo* Gene Editing Approach

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There have been many studies examining the integration of DNA arising from AAV and other viruses into sites throughout the human genome. Most current methods use amplification of sequences on both sides of the integration junction. Typically, specific primers directed to the virus being studied are combined with primers directed at non-specific adaptors ligated to genomic DNA near the junction. However, a variety of artifacts can obscure real integration signals, and these are often caused by a high degree of background amplification or from unintended ligation of free or truncated virus to random genomic DNA during processing. This is particularly a problem with assessing AAV-mediated HR integration sites because, at high doses, episomal AAV may be present at thousands of copies per cell and thus easily amplified or ligated inadvertently. False positive integration signals arising from the very high episomal background may occur more frequently than actual integration events. These issues have led to questions regarding the authenticity of some reported genomic integrations.

To overcome the high episomal background often observed with AAVs, and other known sources of artifactual “integrations”, we have modified previous protocols and combined them with new steps to eliminate the impact of excess vector genomes. Long-read sequencing has been employed to ensure that relevant viral and human genomic sequences are truly present on the same molecule, eliminating index hopping as an issue as seen with short-read sequencing. The mean length of molecules spanning the integration junction that we sequenced is approximately 2 kb and some molecules are over 6 kb. A positive control cell line has been generated with a single inserted DNA via CRISPR. Genomic DNA from this cell line is spiked at known fractional concentrations into genomic DNA from human hepatocytes engrafted in mice so that the frequency of real events can be estimated. These mice were treated with rAAV with a codon-optimized *PAH* gene and homology arms targeted for insertion into intron 1 of human *PAH*. Using these quantitative molecular methods, we are able to calculate the frequency of HR-mediated integration into the desired locus. We see no evidence of integration into any other genomic location, which further supports the precision of this gene editing approach.