**Adeno-Associated Vectors**

Recombinant Adeno-Associated virus (AAV) belongs to the group of Parvoviruses, which has not been shown to be associated with any known symptoms or pathology in humans to date. AAV is commonly used in the preclinical and clinical settings due to its wide tropism, robust, long-term expression, and the low immunogenicity. Similar to the Lentiviral vector, the AAV can transduce non-dividing cells. In contrast to the wild-type AAV that is capable of integrating into the host cell genome, recombinant AAV depleted of the rep and cap genes is integration-defective. However, the integration of the AAV DNA into the host genome is still detectable at low frequency. The wild-type AAV genome is ssDNA, either positive- or negative-sensed, which is about 4.7 kbs long. The genome comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames (ORFs): rep and cap. Rep sequences encode proteins involving in viral replication, while the cap sequences encode capsid proteins: VP1, VP2 and VP3, which interact together to form a capsid of the vector.

AAV ITRs comprise 145 bases each. They are shown to be required for the replication of the AAV genome, providing self-priming sites. In addition, AAV ITRs are required for encapsidation of the newly generated AAV genome in the AAV shell. ITRs seem to be the only sequences that should be kept intact in the expression cassette next to the gene-of-interest. In fact, cap-rep genes can be delivered in trans by the AAV Helper cassette. Since, the AAV’s latency is rescued by gene products derived from Adenovirus or Herpes virus, the adenovirus helper factors, such as E1A, E1B, E2A, E4ORF6 and VA RNAs, should be provided by either adenovirus infection or
by transfecting into production cells as a third plasmid that provides these adenovirus helper factors. Given that HEK293 cells, a commonly used AAV production cells, already contain the E1A/E1b genes, the only helper factors that needed being delivered are E2A, E4ORF6 and VA RNAs.

To summarize, we utilize a triple transient transfection protocol for transfection which requires the use of (i) the adenovirus helper plasmid, (ii) the AAV helper plasmid and (iii) the inverted terminal repeat (ITR) transgene cassette plasmid (pITR), which contains only the ITRs from the wild-type AAV genome (see image below). The advantage to this system is that coinfection with adenovirus is not needed. Adenovirus helper cassette supplies the adenovirus proteins (E1A, E1B, E4 and E2A) along with the adenovirus virus-associated RNAs required for helper functions. AAV-Helper plasmid encodes the wild-type AAV genome lacking ITRs to circumvent packaging of the WT genome. The replicating rAAV genome from pITR is packaged into preformed empty capsids within the nucleus of producer cells. rAAV is then harvested from the nuclei of transfected cells after 48–72 h, followed by purification from the cell homogenate. The purified vector is then characterized for genome titer, infectious titer and transducing titer before in-vitro and in-vivo studies are conducted.

Our laboratory utilized a double round- CsCl gradient protocol for AAV purification- the only protocol that enables the physical separation of full particles (AAV containing a genome) from empty particles based on their differences in density. Another advantage of this protocol is that its use provides a possibility to purify all serotypes. We use real-time PCR method or fluorescent microscopy to assess the presence of viral genomes in AAV-fractions.
Advantages of using Adeno-Associated Vectors

1. Transduce dividing and non-dividing cells
2. Low immunogenicity
3. Long-term and strong transgenic expression
4. Safe (virus cannot replicate)
5. Easy to achieve high titers
6. Reduced risk of insertional mutagenesis
7. Stable ex-vivo