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FAQ About Adeno-Associated Virus (AAV)

1. What is recombinant AAV (rAAV)?

Adeno-associated virus (AAV) is a small, icosahedral, non-enveloped virus belonging to the *Parvoviridae* family. AAV does not encode its own polymerase, so its replication depends on host cell polymerase activity and the presence of helper viruses such as adenovirus or herpesvirus.

Recombinant AAV (rAAV) is an engineered version of AAV that lacks the viral *rep* and *cap* genes, which encode replication and structural proteins, respectively. Instead, *rep* and *cap* are replaced with a gene of interest flanked by inverted terminal repeats (ITRs) to create the transgene plasmid.

Helper-free rAAV production utilizes a helper plasmid that provides the necessary packaging components (E2A, E4, and VA RNA), enabling AAV packaging without the need for live adenovirus infection in HEK293 cells, which already express the essential E1A component.

2. How are rAAV produced (packaging process)?

In a helper-free AAV system, rAAV is produced by co-transfecting HEK293 cells with three plasmids: the transgene plasmid, a *rep/cap* plasmid, and a helper plasmid.

The **transgene** plasmid (containing the gene of interest flanked by AAV inverted terminal repeats, or ITRs), the **helper**-plasmid (providing adenoviral genes like E2A, E4, and VA RNA to support replication), and the **Rep-Cap** plasmid providing the replication (Rep) and capsid (Cap) proteins necessary for AAV genome replication and virion assembly. The rep gene encodes four proteins—Rep78, Rep68, Rep52, and Rep40; while the cap gene encodes three structural proteins: VP1, VP2, and VP3 that form the viral capsid. The p40 promoter, situated downstream of rep, controls the expression of the Cap proteins (VP1, VP2, VP3) from a single transcript with different start codons (ATG for VP1, ACG for VP2, and a downstream ATG for VP3). Rep52/40 facilitate packaging of the replicated single-stranded DNA into preformed capsids. The VP proteins assemble into capsids (VP1:VP2:VP3), aided by the assembly-activating protein (AAP) encoded within the cap ORF in an alternate reading frame.

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3. Why use rAAV?

rAAV is widely used for gene delivery due to its efficiency and safety. Key advantages include:

1) **Low Immunogenicity:** rAAV does not elicit strong immune responses in vivo.
2) **Broad Tropism:** Different AAV serotypes efficiently transduce various cell types.
3) **Non-Pathogenicity:** AAV is not associated with human disease and integrates into the host genome at very low frequencies, reducing the risk of insertional mutagenesis.
4) **Transduction of Both Dividing and Non-Dividing Cells:** Unlike some other viral vectors, AAV can infect quiescent cells.
5) **Long-Term Expression:** In non-dividing cells, AAV can mediate long-term gene expression due to episomal persistence.

4. Is rAAV safe? Can rAAV replicate after infection?

To date, AAV has not been linked to any human diseases. In rAAV systems, the plasmids used for packaging (cis-plasmid, helper plasmid, and *rep/cap* plasmid) do not share homologous regions, making recombination and subsequent replication highly unlikely.

Unlike the transgene plasmid, the Rep-Cap plasmid lacks ITRs to prevent its own packaging into AAV particles. Packaged AAV viral particles do not contain any packaging plasmids or replication components, meaning they cannot replicate after transduction into cells. Once inside the target cells, AAV cannot generate new viral particles.

5. What are the biosafety requirements for using AAV?

rAAV constructs that do not encode oncogenic genes and are produced without a helper virus can be handled under **Biosafety Level 1 (BSL-1)** conditions. However, if the vector carries potentially hazardous genes or is used in certain applications, **Biosafety Level 2 (BSL-2)** containment may be required.

6. What is the cloning capacity for rAAV?

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AAV has a packaging capacity of approximately **4.7 kb**, including the promoter, transgene, and polyadenylation signal. When the inserted DNA approaches this upper limit, packaging efficiency decreases significantly. Exceeding 4.7 kb can lead to truncated or incomplete vector genomes.

7. What are AAV serotypes, and how do I select the appropriate one?

AAV serotypes refer to natural or engineered variants of AAV that differ in their **capsid proteins**, which dictate the viral tropism (preference for specific cell types or tissues). There are over 12 naturally occurring AAV serotypes (AAV1–AAV12) and several engineered capsids (e.g., AAV-DJ) optimized for enhanced gene delivery.

Selecting the appropriate AAV serotype depends on the **target tissue or cell type (refer to table below)**. If unsure, an effective approach is to test multiple AAV serotypes carrying a GFP reporter gene to determine the most efficient one for your system.

AAV Serotypes	**Tissues or Cell Types Preferences** (the most efficiently transduced tissues listed first)
AAV1	Skeletal Muscle, Heart ((vascular endothelial), cardiac muscle, CNS, Liver, Lung
AAV2	CNS (Brain), Retina, Kidney, Liver, Skeletal Muscle, Monocytes
AAV3	Liver, Kidney, Fibroblasts
AAV4	Retina, CNS (ependymal cells)
AAV5	Lung, CNS (brain and astrocytes), Retina, Ovary
AAV6	Lung (epithelial cells), Skeletal Muscle, Heart, Liver
AAV7	Skeletal Muscle, Liver, Heart
AAV8	Liver (hepatocytes), Heart, Skeletal Muscle, CNS, Kidney, pancreas
AAV9	CNS (can cross the blood-brain barrier), Heart (cardiac muscle), Skeletal Muscle, Liver, Lung
AAV10	CNS, Retinal ganglion cells, Heart, Skeletal Muscle,
AAV-DJ	Enhanced efficiency in CNS, Liver, CNS, and Kidney, Fibroblasts, muscle.

8. How is AAV titer determined?

The concentration of rAAV in a preparation is commonly measured in two ways:

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1) **Genome Titer (Genome Copies, GC/mL):**
   - Measured using **qPCR** or **Droplet Digital PCR (ddPCR)** by amplifying the transgene sequence or ITRs.
   - ddPCR is more precise than qPCR, as it avoids amplification biases.
   - A plasmid standard reference with a known titer should be included in each qPCR run.

2) **Physical Titer (Capsid Particles, VP/mL):**
   - Measured using **ELISA** with antibodies against AAV capsid proteins.
   - Quantifies both full and empty viral particles.
   - The full-to-empty particle ratio varies depending on factors such as transgene size, production system, and purification method.
   - The typical **full-to-empty ratio** is around **50 to 80%%** depend upon transfer plasmid integrity and AAV packaging protocols. This ration can be assessed more accurately using Mass spectrometry or Electron microscopy. However, those measures are not routinely performed.

9. How much AAV should I use for my experiment?

The optimal AAV dose depends on several factors, including **target cell/tissue type, AAV serotype, and desired expression levels**. A general starting point for **in vitro** experiments is **MOI (GC/cell) = 10^5**. For **in vivo** applications, typical doses range from **10^{11}–10^{13} GC per mouse**, depending on the delivery method and target tissue.

10. When can I expect to see gene expression after AAV transduction?

AAV-mediated gene expression takes longer to initiate compared to other viral vectors due to its **episomal nature** and reliance on cellular machinery. Expression typically begins within **1–14 days** post-transduction, with peak levels occurring around **2–4 weeks** after infection.

11. Can AAV be used for stable expression?

AAV primarily exists as an **episome** in the nucleus rather than integrating into the host genome. This means its expression is not permanent. However, AAV can mediate **long-term gene expression in non-dividing cells**. In rapidly dividing tissues, transgene expression may be transient due to loss of episomal AAV genomes over successive cell divisions.

12. Choose the Right vial products: Lentivirus, AAV, and Adenovirus.

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Selecting the appropriate viral vector depends on the specific needs of your research. Each vector type has unique advantages and disadvantages:

1) **Lentivirus:** Integrates into the host genome, ensuring stable gene expression but posing a risk of insertional mutagenesis. Suitable for long-term gene modification in dividing and non-dividing cells.
2) **AAV:** Non-integrative, offering long-term yet episomal gene expression with low immunogenicity. Ideal for in vivo gene therapy and long-term expression in non-dividing cells, with minimal immune response.
3) **Adenovirus:** High transduction efficiency with transient expression and strong immune response. Suitable for high-efficiency, transient expression in vitro and in vivo, but limited by immune response and cytotoxicity,

Comparison Table of Lentivirus, AAV, and Adenovirus

Feature	Lentivirus	AAV	Adenovirus
Integration	Yes	No (episomal)	No (episomal)
Cargo Capacity	~8 kb	~4.7 kb	~36 kb
Viron Size (nm)	80 ~ 100nm	~25 nm	90~100nm
Tropism	Broad (dividing & non-dividing cells)	Depends on serotype Broad (dividing & non-dividing)	Broad (dividing & non-dividing)
Expression Duration	Stable (integrates into genome)	Long-term (episomal)	Transient
Expression Peak time (promoter and cell type dependent)	24 hours to 72 hours	48 hours to a few weeks	24 hours to 72 hours
Titer	Moderate	High	Very High
Immune Response	Moderate	Low	High
Safety	Risk of insertional mutagenesis (BLS-2)	High (non-integrating) (BSL-1)	Lower than wild-type but still immunogenic (BSL-2)
Application	Gene	Gene therapy,	Vaccine development,

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	therapy, stable expression	research, long-term expression	high transient expression

13. How to destroy (disinfect) AAV?

Use 1/10 dilution of bleach to trash unwanted AAV.