User Instructions: AAV for In Vivo Applications

Content

The following table shows the components associated with custom ultra-purified adeno-associated virus (AAV). The specific AAV viral titer is provided in the certificate of analysis (COA) document.

Scale	Deliverable	Specification
Ultra-purified pilot	Custom virus	Concentrated virus (>10^{13} GC/ml, 4x25 ul)
	Control virus (optional)	Concentrated virus (>10^{13} GC/ml, 4x25 ul)
Ultra-purified medium	Custom virus	Concentrated virus (>10^{13} GC/ml, 10x50 ul)
	Control virus (optional)	Concentrated virus (>10^{13} GC/ml, 10x50 ul)
Ultra-purified large	Custom virus	Concentrated virus (>10^{13} GC/ml, 10x100 ul)
	Control virus (optional)	Concentrated virus (>10^{13} GC/ml, 10x100 ul)

Note: If the virus is used for in vitro cell cultures, ultra-purification is not required. For in vivo studies (i.e. animal studies), ultra-purification is essential to prevent strong immune responses via removing contaminants (such as defective particles, cell debris, and small amounts of media components). Furthermore, ultra-purification will concentrate the virus to a level suitable for in vivo experiments.

Storage and Handling

1. VectorBuilder's ultra-purified AAV is recommended for in vivo applications. Our ultra-purified AAV is stored in a PBS-based buffer.
2. Upon receiving, AAV should be stored at -80°C for long term storage (stable for at least 1 year), or -20°C for short term storage (stable for 2~3 weeks).
3. Thaw the vial of AAV on ice prior to use and keep it on ice during the experiment. Thawed AAV can be stored at 4°C for 1~2 weeks without significant loss of biological activity.
4. After thawing, AAV can be dispensed into smaller aliquots according to the quantity used in your experiment and then refrozen. If you need to dilute the virus, you may use PBS, but do so ONLY immediately prior to use.

CAUTION: Do not freeze and thaw your AAV sample multiple times. AAV can be frozen and thawed several times with minimal loss of activity but it is better to avoid this.

Safety Precautions

All AAV viruses from VectorBuilder consist of recombinant transgene sequences flanked by the AAV inverted terminal repeats (ITRs). The AAV ITRs, consisting of only 6% of the wild type AAV genome, are the only AAV specific sequences packaged into the virus particles. The removal of the majority of viral structural genes renders the virus replication defective and dependent on adenovirus helper provided in trans. The recombinant AAV viruses are generated in the presence of a helper plasmid, not helper virus. The viruses are generated by transient transfection of 293T cells using three plasmids (the cis ITR-containing plasmid, the trans plasmid encoding AAV replicase as well as capsid genes, and the adenoviral helper plasmid) which result in the pseudotyping of vector genomes with different serotype capsid proteins. The recombinant AAV viruses are based on wild type AAV virus which is non-pathogenic in human. Although the replication of wild type AAV is dependent on the presence of adenovirus or herpesvirus and will stably integrate into the host cell genome in the absence of helper virus, the recombinant AAV virus genomes remain primarily episomal in target cells and have a low (if any) frequency of integration. **We recommend that the viruses should be handled according to Biosafety Level 2 (BSL-2) criteria.** All handling, storage and disposal of biohazard waste must be in accordance with published and institutional criteria.

Recommended Tissue Tropism of AAV Serotypes

Tissue type	Recommended AAV serotypes
Smooth muscle	AAV1, AAV2, AAV3, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh10
CNS	AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9, AAVrh10, AAV-PHP.eB
PNS	AAV-PHP.S
Brain	AAV1, AAV2, AAV5, AAV7, AAV8, AAV-DJ/8
Retina	AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9, AAVrh10, AAV2.7m8
Inner ear	AAV1, AAV2, AAV6.2, AAV8, AAV9, AAV2.7m8
Heart	AAV1, AAV4, AAV5, AAV6, AAV8, AAV9, AAVrh10, AAV-DJ
Lung	AAV1, AAV3, AAV4, AAV5, AAV6, AAV6.2, AAV9, AAVrh10
Liver	AAV1, AAV2, AAV3, AAV6, AAV6.2, AAV7, AAV8, AAV9, AAVrh10, AAV-DJ, AAV-DJ/8
Pancreas	AAV1, AAV2, AAV6, AAV8, AAV9, AAVrh10
Kidney	AAV2, AAV4, AAV8, AAV9, AAVrh10, AAV-DJ, AAV-DJ/8
Adipose	AAV6, AAV8, AAV9
Testes	AAV2, AAV9
Spleen	AAV-DJ, AAV-DJ/8
Spinal nerves	AAV2-retro
Endothelial cells	AAV2-QuadYF

Note: The ITRs carrying your gene of interest (GOI) is from the AAV2 genome. Different serotypes are distinguished by the capsid protein.

Protocol for Mouse Tail Vein Injection

Intravenous (IV) injection via the lateral tail vein is an efficient means of transducing mouse tissues with AAV. Tail vein injections are preferred for their simplicity, as they do not require surgery or anesthesia. Other injection sites include the portal and jugular veins but these require surgery. Tail veins injections are more efficient at delivering AAV to tissues compared to portal vein injections.

To determine the optimal injection site and dose for your study, you could conduct pilot testing in your animals by using reporter AAV, such as EGFP-expressing AAV.

Materials

- Adult mice, 20 to 25 g, at least 6 weeks of age
- Ultra-purified AAV (a titer of $>10^{13}$ GC/ml is recommended)
- 70% ethanol
- Heat source
- Mouse restrainer
- Cotton gauze pads
- 0.5 ml syringe with 27 gauge 1/2 inch needle

Procedures

1. **Physical restraint**

   Mice can be physically restrained using a commercially available restraining device. Gently introduce the mouse to be injected into restrainer making sure the tail is exposed for further manipulations.

2. **Vasodilation**

   To induce peripheral vasodilation, warm the mouse by either dipping the tail in warm water (43°C) or placing the animal near a heat lamp for 5 to 10 minutes prior to injection.

   Note: When using a heat lamp, place the lamp about 15 to 25 cm above the cage and observe the mice for 5 minutes. Stop heating once the mice huddle together in one corner of the cage.

3. **Injection**

   - Making sure the mouse is placed in the restraining device in a stern position, slightly rotate the tail to visualize the lateral vein. Disinfect the injection site with 70% ethanol and wipe off excess alcohol with gauze pad.
   - Using a 0.5 ml syringe with a 27 gauge needle, draw up at least 0.1 ml of ultra-purified AAV particles. Carefully remove air from the needle.
   - Grasp the distal part of the tail and twist it slightly to the lateral vein's side (**Figure 1**). Insert the needle at a shallow angle (as the tail vein is relatively close to the surface) and inject 0.1 ml of diluted AAV into the vein. The injection should proceed smoothly without any resistance if the needle is correctly positioned, and the vein will transiently shift to a clear color. Stop injecting if any local swelling is observed, as in that case the virus is being injected into the tail tissue, not the vein.

   Note: Start your injection from the lower portion of the tail about 1/3 from the tip. This allows you to move up the tail if the previous injection was unsuccessful.

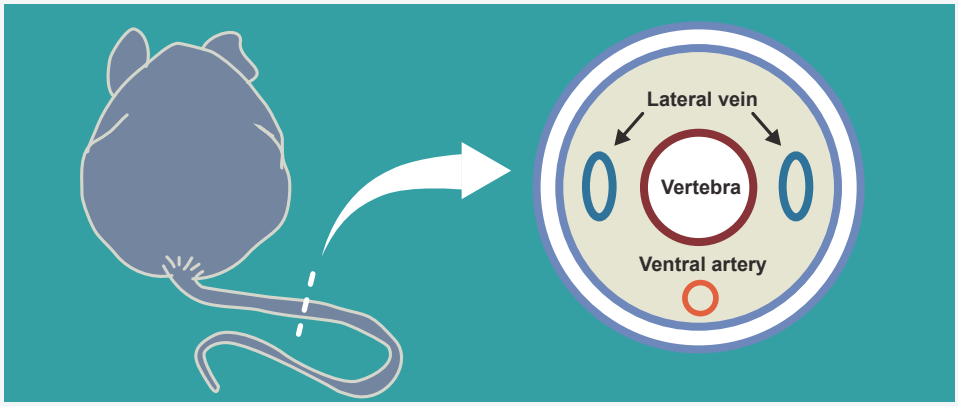

Figure 1. Diagram of a transverse sectional view of mouse tail lateral veins and ventral artery.

- Remove the needle and apply pressure with a clean gauze until bleeding stops.
- Return mouse to original cage.

4. Validation

AAV particles are predominantly sequestered by tissues following intravenous injection. To determine the efficiency of in vivo transduction, mice can be sacrificed at day 7 after virus infusion and targeting tissue(s) can be processed for analysis.

Anticipated Results

An example of successful in vivo transduction is shown in Figure 2.

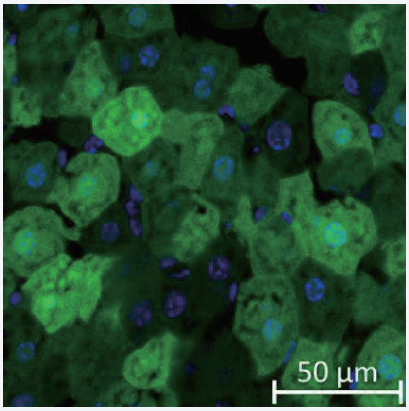

Figure 2. Mice were injected with the ultra-purified EGFP expressing AAV9 at a dose of 1 x 10^{13} GC/kg using the protocol of IV injection in this document. Ten days after the injection, the livers from injected animals were dissected for analysis. Images were taken by a confocal microscope. Green: EGFP. Blue: DAPI.

Protocol for Mouse Intra-Cerebroventricular Injection

Intra-cerebroventricular (ICV) injection is a procedure to deliver the ultra-purified AAV into the left and right lateral ventricles, followed by its diffusion into the central nervous system (CNS). The brain ventricles and CNS contain cerebrospinal fluid (CSF) which is produced in the two lateral ventricles. The CSF starts circulating from the lateral ventricles and eventually flows into the subarachnoid space (**Figure 3**). Since the subarachnoid space covers the entire brain, spinal cord and sacrum, the ICV injection can be used to introduce the virus sample or other therapeutic agents to the CNS without being obstructed by the blood-brain barrier.

To determine the optimal injection dose for your study, you could conduct pilot testing in your animals by using reporter AAV, such as EGFP-expressing AAV.

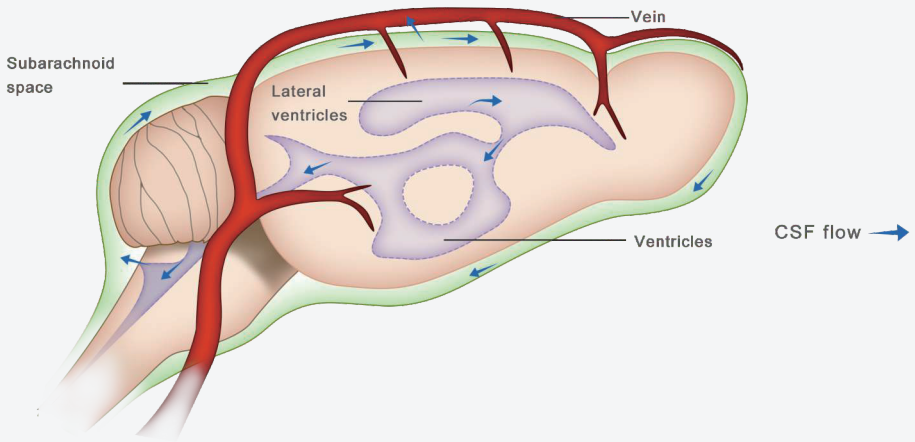

Figure 3. Diagram of cerebrospinal fluid circulatory pathway in mouse

Materials

- Newborn mice, 1~3 days of age
- Ultra-purified AAV (a titer of >10^{13} GC/ml is recommended)
- 70% ethanol
- Absolute ethanol
- Ice
- Dry ice
- Cotton gauze pads
- Heating pad or container
- Stereotaxic device
- 33~34 gauge 1/2 inch needle
- 10 ul syringe
- 15 cm pressure equalization (PE) tube

Procedures

1. Preparation of operating environment

- Wipe the operating table with 70% ethanol.
- Cool down the brain stereotaxic device by adding 5 ml of absolute ethanol in the groove of the operating table. Consistently add dry ice to the absolute ethanol to stabilize the temperature of the operating table at about 0°C. At the same time, the small heating pad is pre-warmed.

Note: To avoid the death of mice, the temperature of the operating environment should not be below 0°C.

2. Preparation of injection syringe

- Take a 33~34 gauge needle and sleeve it tightly with a PE tube, and then tighten the other side of the PE tube with a 10 ul syringe.
- Draw up 5 ul of ultra-purified AAV into the needle, and carefully remove air from the needle.
- Restrain the syringe and needle in the stereotaxic device.

3. Anesthesia

Anesthetize the mouse to unconsciousness by gently placing the mouse on ice for 4 minutes.

4. Physical restraint

After anesthesia, the head of the mouse can be physically restrained on the stereotaxic device gently.

5. Injection

5.1 Left ventricle

- Disinfect the mouse head with 70% ethanol and wipe off excess alcohol with gauze pad.
- Place the needle just above the lambda structure of the mouse (**Figure 4**). Record the indexes of x and y axis on the vernier scale.
- Place the needle just above the left ventricle (x + 1.2 mm, y + 1.6 mm). Then pierce the mouse skull with the needle by adjusting the z axis.
- Immediately after piercing the skull, withdraw the needle out of the mouse slowly.

Note: The z axis position of the mouse skull can be slightly shifted when being pierced due to the pressure from the needle. Withdrawing the needle immediately after piercing the skull can help the skull move back to the original position.

- Carefully put the needle back into the skull and move the needle down until the needle tip just touches the brain. Record the index of z axis (z_1) on the vernier scale.
- Lower the needle slowly for 2.5~3 mm so that the needle tip penetrates into the center of the left ventricle. The indexes on the vernier scale should be x + 1.2 mm, y + 1.6 mm, z_1 - 2.5~3 mm.
- Push the syringe slowly and inject 2.5 ul virus sample into the left ventricle. To prevent the AAV leaking from the brain, wait for 1 minute or even longer after injecting the virus sample.
- Slowly adjust the z axis to withdraw the needle from the mouse. This process should take about 2 minutes. Withdrawing the needle too fast may lead to AAV leakage.

5.2 Right ventricle

- Place the needle above the right ventricle (x - 1.2 mm, y + 1.6 mm). Then pierce the mouse skull with the needle by adjusting the z-axis.
- Immediately after piercing the skull, withdraw the needle out of the mouse slowly.

Note: The z axis position of the mouse skull can be slightly shifted when being pierced due to the pressure from the needle. Withdrawing the needle immediately after piercing the skull can help the skull move back to the original position.

- Carefully put the needle back into the skull and move the needle down until the needle tip just touches the brain. Record the index of z axis (z_2) on the vernier scale.
- Lower the needle slowly for 2.5~3 mm so that the needle tip penetrates into the center of the right ventricle. The indexes on the vernier scale should be x - 1.2 mm, y + 1.6 mm, z_2 - 2.5~3 mm.
- Push the syringe slowly and inject 2.5 ul virus sample to the right ventricle. To prevent the AAV leaking from the brain, wait for 1 minute or even longer after injecting the virus sample.
- Slowly adjust the z axis to withdraw the needle from the mouse. This process should take about 2 minutes. Withdrawing the needle too fast may lead to the AAV leakage.

Note: To avoid the death of mice, mice should not be kept at low temperature for over 30 minutes.

6. Recovery

Right after the injection, place the mouse on the pre-warmed heating pad until the body temperature and the movement are restored.

7. Observation

Return the mouse to the cage with the mother mouse. Monitor the condition of the mice until starting further experiments.

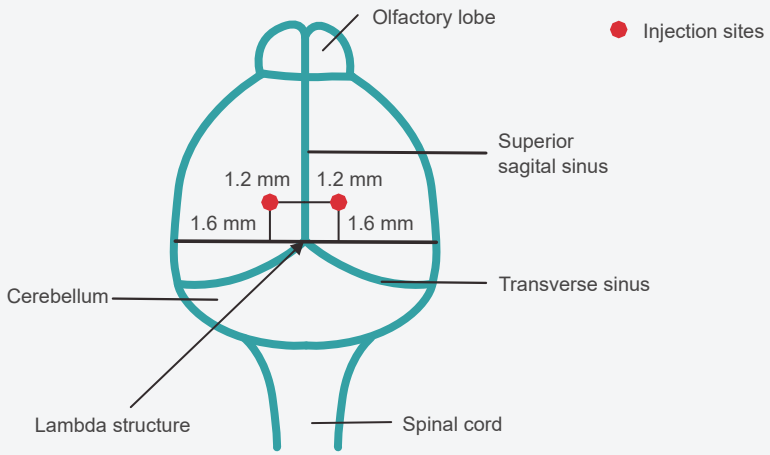

Figure 4. Diagram of the top view of mouse lambda structure and injection sites

Anticipated Results

An example of successful in vivo transduction is shown in Figure 5.

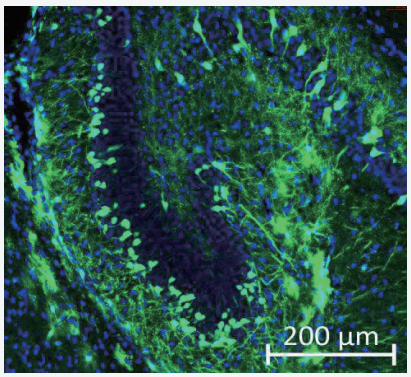

Figure 5. Mice were injected with the ultra-purified EGFP expressing AAV9 at a dose of 1 x 10^{13} GC/kg using the protocol of ICV in this document. Ten days after the injection, the brains from injected animals were dissected for analyses. Images of hippocampus were taken by a confocal microscope. Green: EGFP. Blue: DAPI.

Recommended Volume for Mouse In Vivo Injection

To transduce ultra-purified AAV to mouse tissues successfully, the following table lists the recommended injecting volumes for different injection methods.

Injection sites	Recommended volume
Lateral ventricles	5 ul
Nucleus accumbens	0.05~0.1 ul
Ventral tegmental area	
Hippocampus	
Jugular vein	≥100~250 ul
Tail vein	100~250 ul
Lung	10 ul/site, 5 sites in total
Abdominal cavity	200~250 ul
Vitreous humor	1~2 ul
Heart	1.5~3 ul/site, 5 sites in total
Muscle	10 ul/site, 4 sites in total

Note: For all tissues, the expression of the transgene carried by AAV can last for around 2~6 months. Usually, the peak expression can be detected at 2~4 weeks post-injection.