User Manual

AAV-LC3 Autophagy Detection

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Safe Use of AAV

1. AAV related experiments should be conducted in biosafety level 2 facilities (BL-2 level).

2. Please equip with lab coat, mask, gloves completely, and try your best to avoid exposing hand and arm.

3. Be careful of splashing virus suspension. If biosafety cabinet is contaminated with virus during operation, scrub the table-board with solution comprising 70% alcohol and 1% SDS immediately. All tips, tubes, culture plates, medium contacting virus must be soaked in chlorine-containing disinfectant before disposal.

4. If centrifuging is required, a centrifuge tube should be tightly sealed. Seal the tube with parafilm before centrifuging if condition allowed.

5. AAV related animal experiments should also be conducted in BL-2 level.

6. AAV associated waste materials need to be specially collected and autoclaved before disposal.

7. Wash hands with sanitizer after experiment.

Storage and Dilution of AAV

Storage of AAV

Virus can be stored at 4°C for a short time (less than a week) before using after reception. Since AAV viruses are sensitive to freeze-thawing and the titer drops with repeated freeze-thawing, aliquot viral stock should be stored at -80°C freezer immediately upon arrival for long-term usage. While virus titer redetection is suggested before using if the AAV viruses have been stored for more than 12 months.

Dilution of AAV

Dissolve virus in ice water if virus dilution is required. After dissolving, mix the virus with medium, sterile PBS or normal saline solution, keeping at 4°C (using within a week).

Precautions

· Avoid AAV exposure to environmental extremes (pH, chelating agents like EDTA, temperature, organic solvents, protein denaturants, strong detergents, etc.)

· Avoid introducing air into the AAV samples during vortexing, blowing bubbles or similar operations, which may result in protein denaturation.

· Avoid repeated freezing and thawing.

· Avoid exposing to “regular” plastics (especially polystyrene or hydrophobic plastics) for prolonged periods in liquid phase. Most AAV viruses are very sticky and loss can occur if exposed to regular plastics, including tubes, cell culture plates, pipette tips, if not frozen. It is best to store AAV in siliconized or low protein binding tubes. Pluronic F-68 used at 0.01%-0.1% in the formulation buffer will minimize sticking if regular plastics are used.

· Avoid diluting AAV into low salt solution. Some AAV serotypes, such as AAV2, aggregates in low salt solution, which will be non-infectious.

Introduction of AAV

In Genemedi Biosciences, recombinant adeno-associated Virus (rAAV) Expression Systems are utilized in delivering and expressing shRNA, human ORF, CRISPR *in vitro* and *in vivo*.

Adeno-associated virus (AAV) is a small single strand DNA virus infecting human and some other primate species. Currently, AAV has not known to cause disease and only induces very mild immune responses. As a member of the family Parvoviridae, wild type AAV requires the assistance of adenovirus or herpesvirus to complete the duplication, which is the reason why it's called adeno-associated virus [1,2]. The wild-type AAV2 genome consists of the viral rep and cap genes (encoding replication and capsid genes, respectively), flanked by inverted terminal repeats (ITRs) that contain all the cis-acting elements necessary for replication and packaging. The genome of typical AAV2 is about 4800bp, consisting of two upstream and downstream open read frames (ORFs) which are between two inverted terminal repeats (ITR) comprising Rep and Cap (Figure 1). ITR is required for synthesis of complementary DNA strand, while Rep and Cap can be translated into various proteins, including AAV virus cycle essential protein Rep78, Rep68, Rep52, Rep40 and enveloped protein VP1, VP2, VP3, etc. [3].

The present recombinant AAV (rAAV) vectors are generated by replacing all of the viral genome between the ITRs with a transcriptional cassette of less than 5 kilobases in length. The resulting construct is then co-expressed with two other plasmids: 1) an AAV-RC plasmid that provides the Rep and Cap genes in trans (separate from the ITR/Transgene cassette) and 2) an AAV helper plasmid that harbors the adenoviral helper genes. AAV-293 cells are used as the packaging cell line since they provide the E1a protein in trans as well. By modifying the Rep and Cap genes, scientists can control the serotypes to guide the recombinant AAV infection towards certain tissues. This 3-plasmid co-transfection system liberates the need for adenovirus during AAV production, which greatly simplifies the purification process.

To date, a total of 12 serotypes of AAV have been described with their own unique traits and tropisms [4]. Concerning high safety, low immunogenicity, long-term expression of exogenous genes, AAV is thought to be the best gene delivery tool for gene function research *in vivo*.

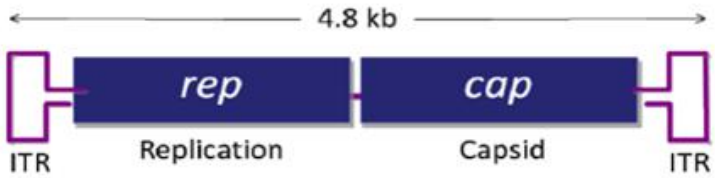

Figure 1. Schematic diagram of AAV2 genome structure.

Advantages of AAV for Gene Delivery and Expression

1. Superior biosafety rating

AAV is a naturally defective virus, requiring provision of several factors in trans for productive infection and has not been associated with any human disease. In our AAV production system, the AAV2 ITR sequences and rep/cap genes are present on separate plasmids that lack homology, preventing production of recombinant wild-type virus. These features give AAV a superior biosafety rating among gene delivery and expression vectors of viral origin.

2. Broad range of infectivity

AAV viruses infect a broad range of mammalian cells and have been used successfully to express human and non-human proteins. In contrast with other vectors of viral origin, AAV vectors have proven to be competent for gene expression in immunocompetent hosts.

3. High titer

Recombinant AAV can be produced at high titers of $\geq 10^7$ viral particles/ml with this protocol. Titers up to 10^{13} viral particles/ml after concentration have been published.

4. Infection does not require an actively dividing host cell

AAV can infect both dividing and non-dividing cells.

5. Long-term gene transfer potential

Recombinant AAV (rAAV) can be maintained in human cells, creating the potential for long-term gene transfer. In most cell populations, the viral genome typically remains epichromosomal, often forming concatemers, which are stable in slowly dividing or non-dividing cells, leading to long-term gene transfer and expression. Whereas in rapidly dividing cell populations, the AAV viruses can integrate into the host genome but not form concatemers, resulting in long-term gene expression in dividing cells, but this is a rare event. The integration occurs more frequently if an extremely high multiplicity of infection (MOI) of AAV is used or if infection occurs in the presence of adenoviral replicase, potentially supplied by the use of wild-type adenovirus. However, it will reduce the biosafety of the AAV system to increase integration events by using wild-type adenovirus.

AAV Serotypes and Native Tropism- AAV Selection Guide

Over the past decades, numerous AAV serotypes have been identified with variable tropism. To date, 12 AAV serotypes and over 100 AAV variants have been isolated from adenovirus stocks or from human/nonhuman primate tissues. Different AAV serotypes exhibit different tropisms, infecting different cell types and tissue types. So, selecting the suitable AAV serotype is critical for gene delivery to target cells or tissues.

Due to the exhibition of natural tropism towards certain cell or tissue types, rAAV has garnered considerable attention. Highly prevalent in humans and other primates, several AAV serotypes have been isolated. AAV2, AAV3, AAV5, AAV6 were discovered in human cells, while AAV1, AAV4, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 in nonhuman primate samples [5,6]. Genome divergence among different serotypes is most concentrated on hypervariable regions (HVRs) of virus capsid, which might determine their tissue tropisms. In addition to virus capsid, tissue tropisms of AAV vectors are also influenced by cell surface receptors, cellular uptake, intracellular processing, nuclear delivery of the vector genome, uncoating, and second strand DNA conversion [7].

In order to better improve the infection efficiency and specificity of AAV to target tissues, scientists have genetically modified the viral capsid, and generated mosaic vectors to create chimeric AAV by swapping domain's or amino-acids between serotypes [8,9], which may allow researchers to specifically target cells with certain serotypes to effectively transduce and express genes in a localized area [10].

Meanwhile, the ability of AAV to penetrate the blood-brain barrier in animals is greatly limited or improved. Traditionally, AAV could only be injected into the brain tissue by surgery for scientific research in the central nervous system, which greatly increased the difficulty of the experiment and affected the experimental results. Now the modified AAV serotype of PHP.B and PHP.eB can infect the whole brain through the blood-brain barrier by peripheral blood injection [11]. Most popular rAAV serotypes and their tropisms are listed in the following table1.

Table 1. rAAV serotypes and their tropisms.

AAV Serotype	Tissue tropism							
	CNS	Retina	Lung	Liver	Pancreas	Kidney	Heart	Muscle
AAV1	√	√			√		√	√
AAV2		√		√		√		
AAV3		√	√	√			√	
AAV4	√	√					√	
AAV5	√	√	√		√			
AAV6	√		√	√			√	√
AAV7				√				√
AAV8		√		√	√			√
AAV9	√		√	√			√	√
AAV-DJ		√	√	√		√		
AAV-DJ/8		√		√				√
AAV-Rh10	√		√	√			√	√
AAV-retro	√	√						√
AAV-PHP.B	√						√	√
AAV-PHP.eB	√							√
AAV-PHP.S	√						√	√

Introduction of Autophagy

Autophagy is a highly regulated homeostatic degradative process where cells destroy their own components via the lysosomal machinery and recycle them. This process plays both protective and deleterious roles in many diseases, including Alzheimer's disease, aging, cancer, infection and Crohn's disease.

Autophagy

Autophagy also known as type II cell-death, defines an evolutionarily conserved process of recycling, whereby damaged organelles and macromolecular substances are broken down into their constituent parts within the lysosomes, which is tightly regulated by the autophagy related gene (Atg). Three kinds of autophagy have been described to date: macroautophagy, microautophagy and chaperone-mediated autophagy.

Macroautophagy, also referred to as 'autophagy' in general, which consists of three main steps: 1) Induction and formation of phagophore; 2) Phagophore elongation and autophagosome formation; 3) Fusion, degradation and recycling (Figure 2). Members of the LC3 family play a key role in the maturation process of the autophagosome. LC3 precursors, diffusely distributed in the cytosol, are proteolytically processed to form LC3-I. Upon initiation of autophagy, C-terminal glycine of LC3-I is modified by addition of a phosphatidylethanolamine to form LC3-II, which translocates rapidly to nascent autophagosomes in a punctate distribution.

Microautophagy, mediated by direct lysosomal engulfment of the cytoplasmic cargo.

Chaperone-mediated autophagy (CMA), refers to the chaperone-dependent selection of soluble cytosolic proteins that are then targeted to lysosomes and directly translocated across the lysosome membrane for degradation.

AAV-mRFP-GFP-LC3 Biosensor

For autophagy study, Genemedi supplies autophagy biosensor, in which GFP and/or RFP tags are fused at the C-terminal of the autophagosome marker LC3, allowing to detect the intensity of autophagy flux in real-time with more accuracy, clarity and intuitiveness. These biosensors provide an enhanced dissection of the maturation of the autophagosome to the autolysosome, which capitalizes on the pH difference between the acidic autolysosome and the neutral autophagosome. The acid-sensitive GFP will be degraded in autolysosome whereas the acid-insensitive RFP will not. Therefore, the change from autophagosome to autolysosome can be visualized by imaging the specific loss of the GFP fluorescence, leaving only red fluorescence (Figure 2).

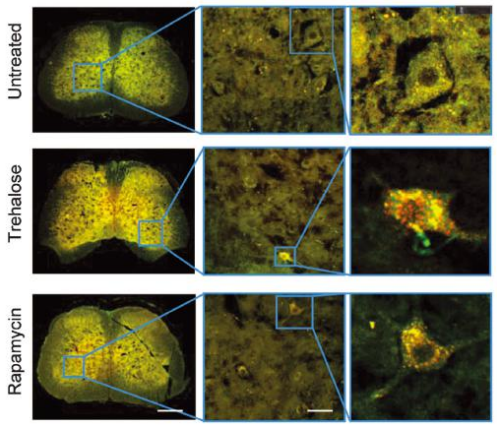

Figure 2. AAV-mRFP-GFP-LC3 indicates autophagy flux in brain tissue.

Taking advantage of RFP-GFP-LC3 and GFP-LC3 labeling system, Genemedi has launched the production service of AAV-RFP-GFP-LC3 and AAV-GFP-LC3, which can be used to observe autophagy flux and monitor the intensity of autophagy flux in real-time *in vivo* or *in vitro*. Besides, several AAV serotypes can be selected according to their tissue tropisms, which makes AAV-mRFP-GFP-LC3 the most effective tool for *in vivo* detection of autophagy flux.

Advantages of Genemedi AAV-LC3 Autophagy Flux Biosensor

Real time and quantitative *in vivo* autophagy flux detection.

High resolution, more accuracy and sensitivity, than traditional approaches.

Safety. The wild type Adeno Associated Virus (AAV) has not currently been known to cause disease, and further security of recombinant AAV is ensured after removal of most AAV genome elements.

Low immunogenicity. AAV causes a very mild immune response, lending further support to its apparent lack of pathogenicity.

Broad range of host and specificity targeting. AAV has the ability to infect both dividing and quiescent cells, allowing genetic material to be delivered to a highly diverse range of cell types. More than 12 AAV serotypes and a variety of capsid engineered AAV vectors can be selected according to their tissue tropisms.

Stable physical properties. AAV is still alive at 60℃ and resistant to chloroform.

Overall Experiment Procedure of AAV-LC3 Biosensor Production and Titer Detection

The AAV-LC3 autophagy flux biosensor virus can be packaged using AAV-293 cells, purified with iodixanol gradient ultracentrifugation method, and titer is detected by real-time quantitative PCR (QPCR) using primers targeting the AAV ITR. The detailed protocol can be consulted in the AAV User Manual on the Genemedi website.

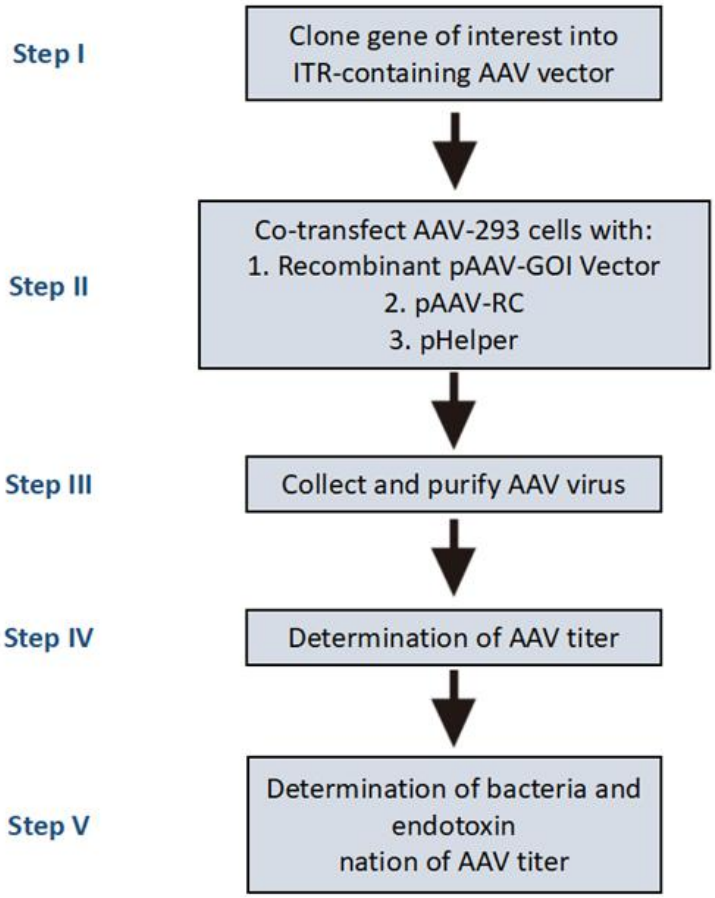

Figure 3. AAV packaging experiment flow chart.

AAV-LC3 Autophagy Flux Biosensor Virus Transduction in vitro

After virus titer detection, the AAV-LC3 autophagy flux biosensor can be tested *in vitro*. The detailed recommended protocol for *in vitro* cell transduction can be consulted from AAV User Manual. Infect primary cells, such as neuronal cells, with AAV-LC3 autophagy flus biosensor virus at confluency about 70%-80%. 24h post infection, refresh the medium. 96h post infection, perform live cell imaging with confocal microscopy and data analysis with ImageJ software.

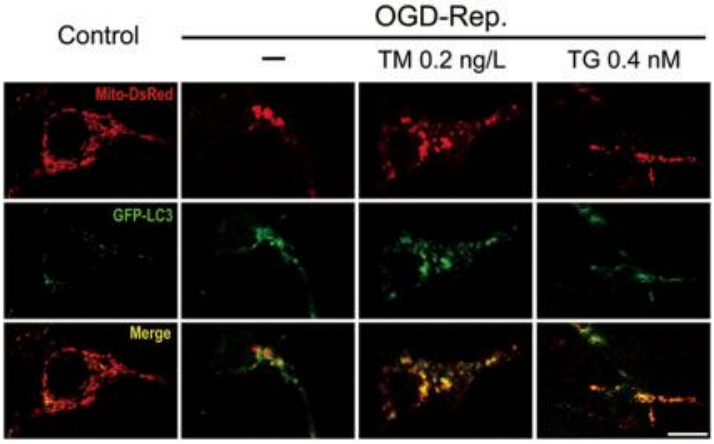

Figure 4. Neuronal cells were previously transfected with GFP-LC3 and Mito-DsRed by AAV vector infection [44].

AAV-LC3 Autophagy Flux Biosensor Virus Transduction in vivo

The successful validated AAV-LC3 autophagy flux biosensor virus can be delivered *in vivo*. Genemedi systematically organizes the corresponding optimal AAV serotype, gene delivery methods and injection volume for mouse and rat tissue infection, which can be found in the AAV User Manual.

About 3 weeks post infection, get the target tissues or organs from animals, embed with OCT, and carry out frozen section using a cryostat. Then fix the slices from frozen section with 4% paraformaldehyde and perform immunohistochemical staining. The autophagy flux can be detected with confocal microscopy and quantitated with the ImageJ software.

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