
# Recombinant Adeno-Associated Virus (rAAV)

# Cloning and Virus Packaging Service Manual

Revision 201603.02



**RESEARCH USE ONLY.**

**Not for use in diagnostic procedures**

# Table of Content

## CONTENTS AND STORAGE

AAV stocks are supplied in liquid form at indicated titer. The storage solution is PBS with 0.001% F68. Store at -80°C. If desired, aliquot viral stock upon arrival, and store those aliquots at -80°C freezer immediately.

Dependent on different service types, the products may contain the following components.

1. **Small scale crude AAV.** 500ul of rAAV at 10^12-13 GC/ml. *****
2. **Large scale purified AAV.** 500ul of rAAV at 10^13-14 GC/ml. *****
3. **Customer Large scale purified AAV.** Custom amount (up to 10^16 GC) of rAAV at 10^13-14 GC/ml. *****

- GC/ml stands for AAV genome copies/ml measured by real-time qPCR in comparison with a standard reference plasmid with known genome copy number. For all the virus products from ViGene Biosciences Inc the titer virus is measured as genome copies /ml. In this Manual, we use GC interchangeably with vg (viral genomes) and vp (viral particles) as a unit for viral titers.

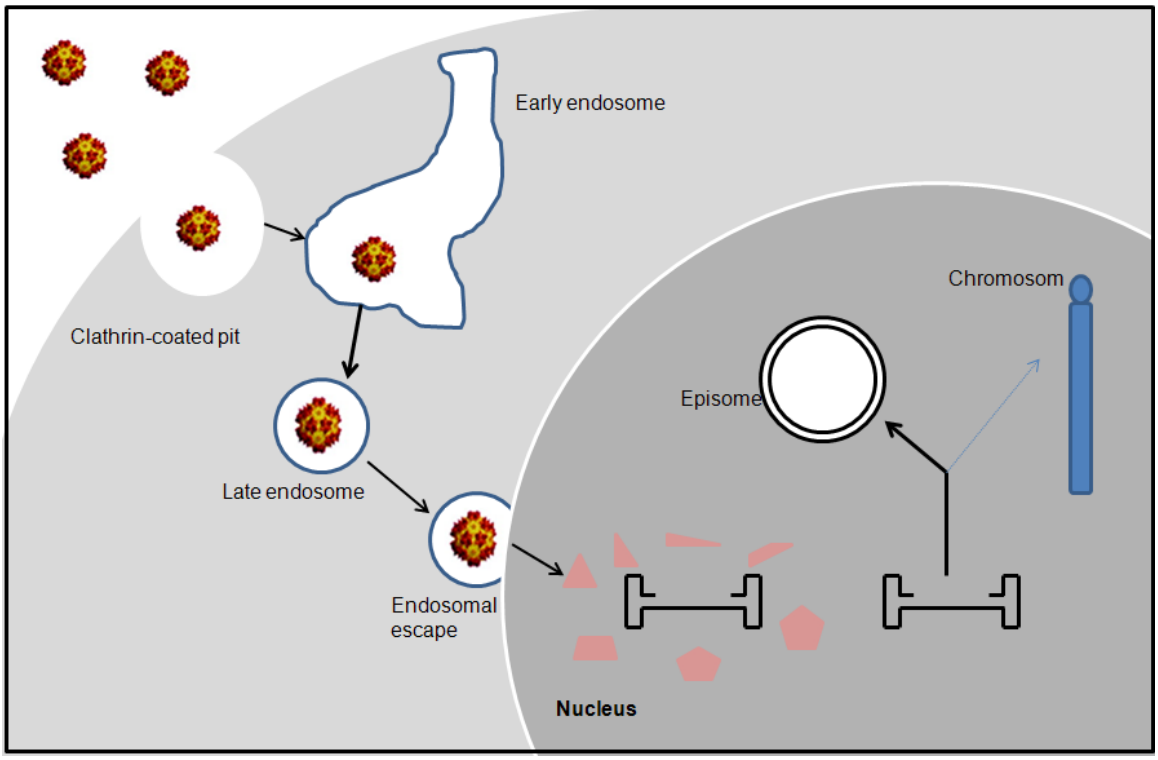
AAV clones are supplied in 5ug DNA in TE buffer of specified amount outlined in the Certificate of Analysis (CoA).

**DO NOT FREEZE AND THAW REPEATEDLY.**

## Introduction

In ViGene 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 which infects humans and some other primate species. AAV is not currently known to cause disease and has very mild immune response. It can infect dividing and non-dividing cells. Further removal of the rep and cap from the vector has eliminated the AAV integrative capacity. Those features make rAAV ideal viral vector for gene therapy. To date, rAAV vectors have been used in many clinical trials in gene therapy, promising results have been achieved from Phase 1 and Phase 2 trials including, CFTR, Hemophilia B, Arthritis, and Parkinson's diseases. Figure 1 shows the cell entry and trafficking of rAAV.




After rAAV gets into the cells, it stays stable as episomal DNA. Expression of gene usually peaks in 5 to 10 days and can last several weeks or even months *in vivo.*

## Which viral vector to use- viral vector selection guide

When comparing three most popular viral vectors in gene delivery, you should take following consideration before you choose adeno-associated viral vector.

1. Do you need transient or stable gene expression?
2. Do you need to transduce dividing or non-dividing cells?
3. How important is potential immune response from your target cell?
4. How large is the gene of interest?

AAVs can infect dividing and non-dividing cells. It is low on cytotoxicity and immunogenicity, suitable for long term gene expression in non-dividing cells and short term in dividing cells with relative high gene delivery efficiency. But AAV vector has limited cloning capacity, the space between two ITRs is only 4.9kb, so your gene should be 3.5kb or less. Please refer to this table for choosing your viral vector system.

| | Adenovirus | Adeno-associated Virus (AAV) | Lentivirus |
|---|---|---|---|
| Genome | dsDNA | ssDNA | ssRNA (+) |
| Coat | Naked | Naked | Enveloped |
| Genome size | 38-39kb | 5kb | 9kb |
| Infection/tropism | Dividing and non-dividing cells | Dividing and non-dividing cells | Dividing and non-dividing cells |
| Host Genome Interaction | Non-integrating | Non-integrating | Integrating |
| Transgene expression | Transient | Potential long lasting | Long lasting |
| Packaging Capacity | 7.5kb | 4.5kb | 6kb |
| Immune Response | High | Very Low | Low |
| Relative Viral Titer | 10^11 GC/ml without purification | 10^7 GC/ml without concentration | 10^7 GC/ml without concentration |
| Relative Transduction Efficiency | 100% | 70% | 70% |
| Relative Foreign Gene Expression | High | Medium | Medium |

### AAV serotypes and native tropism- AAV selection guide

So far there are 11 AAV serotypes described, they all have different tropism and can infect cells from multiple diverse tissue types. Tissue specificity is determined by the capsid serotype. To select the right serotypes is critical in delivery of gene into different cells or tissues. The following table lists most popular rAAV serotype and their tropism.

| AAV Serotype | Tissue Tropism (X indicates recommended application) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Muscle | Liver | Lung | Brain | Retina | Pancreas | Kidney |
| AAV1 | X | | | Neurons and glial cells | X | X | |
| AAV2 | | | | | | | X |
| AAV5 | | | Lung alveolar cells | Neurons and glial cells | X | | |
| AAV6 | X | | X | | | | |
| AAV7 | X | | | Neurons | X | | |
| AAV8 | X | X | | Neurons | X | X | |
| AAV9 | X | X | X | Neurons | X | X | X |

If you cannot find enough information to decide which serotype of rAAV or which virus vector works best for your system, you can try our GFP-Virus Testing Kit, Cat# CT10001.

## AAV Related Service Details

We offer two services for AAV development and production. The first is AAC vector cloning service; the other is AAV packaging services.

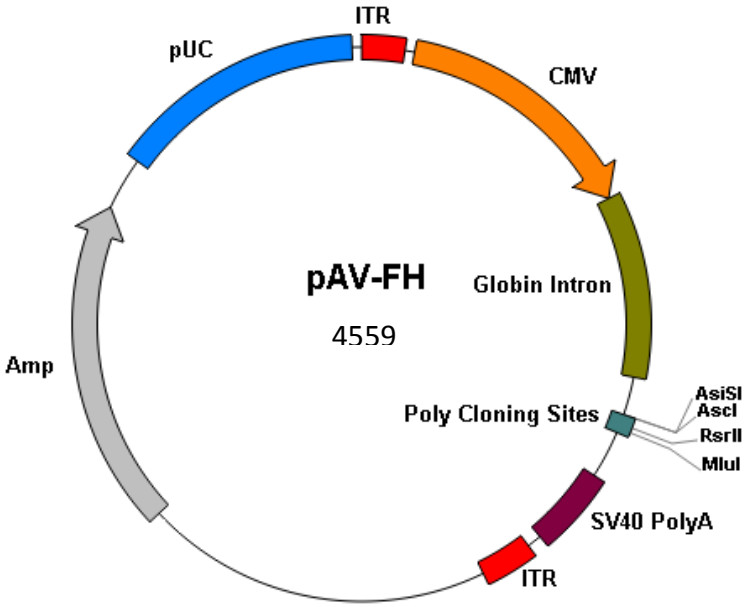
### AAV vector cloning services

#### Selections of rAAV vectors from ViGene Biosciences Inc.

Current ViGene Biosciences Inc. offers pAV-FH vector for gene expression and four vectors for shRNA expression.

## pAV-FH for gene expression

In most of case, ORF inserts are cloned between AsisI and MluI sites. In other rare case the combination of AsisI-RsrII, AsisI-NotI or AscI-MluI are used in the cloning. Please check our web site or the COA for specific clones. In the pAV-FH vector, ORF is fused with a Flag/His tag at its carboxyl terminus. The vector contains an ampicillin marker for bacterial selection. ViGene's pAV-FH vector is a mammalian ORF expression vector, dual tags of Flag and His could be used to detect and purify proteins expressed in mammalian cells.



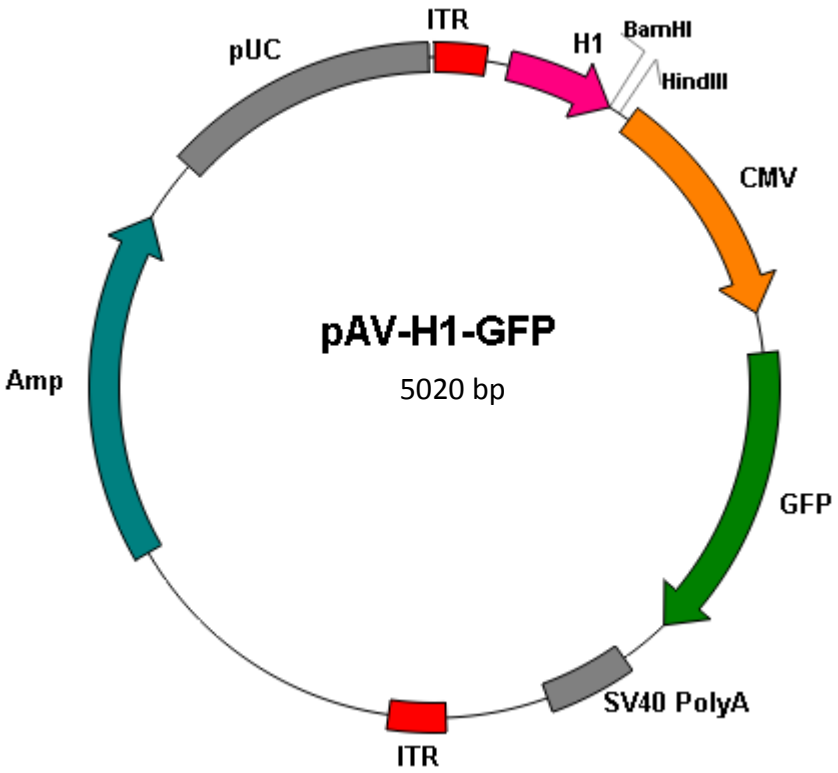

```
GGA TCC GGT ACC GAG GAG ATC TGC CGC CGC GAT CGC C   ORF   ACG CGT ACG CGG CCG CTC
CCT AGG CCA TGG CTC CTC TAG ACG GCG GCG CTA GCG G         TGC GCA TGC GCC GGC GAG
 KpnI                        AsiSI                       MluI       NotI

GAG GAT TAT AAG GAT GAC GAC GAT AAA TTC GTC GAG CAC CAC CAC CAC CAC CAC TAA
CTC CTA ATA TTC CTA CTG CTG CTA TTT AAG CAG CTC GTG GTG GTG GTG GTG GTG ATT
 D   Y   K   D   D   D   D   K   F   V   E   H   H   H   H   H   H   *
```

## rAAV ShRNA vectors

1. **pAV-U6-GFP**
2. **pAV-U6-RFP**
3. **pAV-H1-GFP**
4. **pAV-H1-RFP**

To express shRNA with rAAV, ViGene provides the choices of either U6 or H1 promoter to drive the shRNA expression and GFP or RFP as mammalian expression marker, shown by the example map of pAV-H1-RFP.





### AAV Custom cloning services with pre-selected promoters

CMV is a very strong and the most commonly used promoter in driving gene expression in vitro and in vivo. CMV promoter drives ubiquitous gene expression in most tissue and cell types. Due to the methylation/silencing, expression by CMV promoter decreases in vivo after 10 to 20 weeks. For better tissue or cell type specific gene expression and for long term strong and stable expression, many different promoters are offered by ViGene Biosciences.

| Cat.# | Promoter | Size | Description |
|---|---|---|---|
| PM10001 | ALB | 2.4kb | Liver specific 10 timer stronger than CMV after 10 weeks |
| PM10002 | GFAP104 | 845bp | Hybrid of EF1a and GFAP |
| PM10003 | CAG | 944bp | Strong promoter, ubiquitous expression in vivo |
| PM10004 | CamKIIa | 1.2kb | Specific expression in excitatory neurons in the neocortex and hippocampus |
| PM10005 | EF1A | 1.2kb | Ubiquitous, weaker than CMV but better for in vivo |
| PM10006 | CK1.3 | 1.1kb | |
| PM10007 | CK0.4 | 217bp | Calcium/Calmodulin-dependent kinase II alpha |
| PM10008 | GFAP | 2.0kb | Specific in astrocyte |
| PM10009 | MBP | 1.3kb | Myelin basic protein promoter, efficient transduction of oligodendrocytes by adeno-associated virus type 8 vectors |
| PM10010 | EFFS | 253bp | A short version EF1A |
| PM10011 | TBG | 460bp | Homo sapiens serpin peptidase inhibitor, clade A |
| PM10012 | aMHC | 0.4kb | Mouse myosin heavy chain alpha promoter |
| PM10013 | cTNT | 702bp | Specifically transduce cardiomyocytes |
| PM10014 | Synapsin | 471bp | Specific in neuron |
| PM10015 | Mecp2 | 230bp | Truncated Mcep2 neuron specific |
| PM10016 | c-fos | 1.7kb | Activity-dependent promoter |
| PM10017 | MCK | 1.35kb | Muscle creatine kinase promoter/enhancer |
| PM10018 | UBC | 1.1kb | Ubiquitous, weaker than CMV but better for in vivo |
| PM10019 | PGK | 400bp | Ubiquitous, weaker than CMV but better for in vivo |
| PM10020 | Somatostat | 1.2kb | Restricting expression to GABAergic neuron |
| PM10021 | Rpe65 | 700bp | Retinal Pigment epithelium-specific expression in vivo and in vitro |
| PM10022 | Insulin1 | 1.0kb | Specific in beta- cells of the pancreas |
| PM10023 | 3Xenhancer McK | 728bp | Much stronger than CMV in muscle, inactive in nonmuscle cell lines and mouse liver |
| PM10025 | NSE | 1.3kb | Neuron-specific enolase promoter |

### FLEX-ON of Cre dependent inducible expression

Combine tissue specific promoters and Cre dependent FLEX-On inducible design can get more restricted tissue specific and temporal expression. In FLEX-ON system, gene is in reverse orientation down stream of promoter, and the gene is flanked by two oppositely orientated loxPs. In the absence of Cre, gene cannot be expressed and in the presence of Cre, gene expression can be turn on or induced.

## AAV Virus Packaging Services

### Final Products Components and QC Standards

Unless specified otherwise, AAV viruses are produced from 10^9 Hek293 cells and are purified by Iodixanol gradient ultracentrifugation. Resulted virus are concentrated to 400ul with virus titer no less than 10^13 GC/ml. The purified virus is good for in vivo animal research.

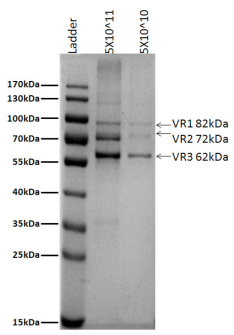
Vigene Biosciences Inc. provides the rAAV virus packaging services in a few formats.

1. Small sacle testing AAV packaging service. In this service, rAAV is packaged using 10^07 HEK293T cells. The virus is in crude cell lysate, without any purification or concentration. The titer is around 10^9-11 GC/ml.
2. Large scale purified rAAV packaging service. In this service, rAAV is packaged using 2.5X10^8 HEK293 cells. Viruses are purified by Iodixanol gradient ultracentrifugation. Resulted virus are concentrated to 400ul with virus titer no less than 10^13 GC/ml. The titer is around 10^12-15 GC/ml dependent on virus serotype and the size of insert.
3. Custom large scale purified rAAV service. The purity and virus are delivered per the specification. ViGene Biosciences can deliver up to 10^16 GC with virus titer no less than 10^13 GC/ml.

### The purity of the AAV virus

The AAV protein components are VR1 82kDa, VR2 72kDa and VR3 62kDa. So a good AAV purification should only show three major protein bands when the virus is analyze by SDA-PAGE. Following image showed the protein components

in our purified AAV virus. We guarantee the purity of the AAV virus based on the specification that we give for different services.

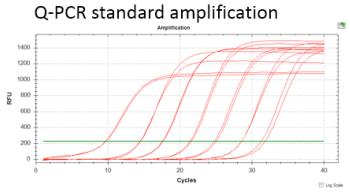



**Purity of purified AAV virus.** When 5X10^11 and 5X10^10 AAV-GFP virus particles were analyzed by SDS-PAGE and Coomassie blue staining. Only three virus proteins are in the purified virus sample.

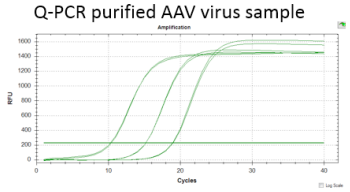
## The virus titer

The virus titer is determined by the viral genome copy number in 1ml sample by Q-PCR and compared to copy number standard samples. Following example is data for tittering AAV-GFP virus.

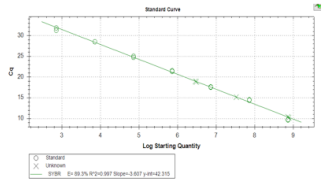
Q-PCR standard amplification



Q-PCR purified AAV virus sample



Q-PCR virus titer quantification curve



Q-PCR virus titer quantification

| Standard | Copy Number | Sample | dilution |
|---|---|---|---|
| Standard_1 | 7.2X10^2/ul | AAV GFP_1 | 10^2 |
| Standard_2 | 7.2X10^3/ul | AAV GFP_2 | 10^3 |
| Standard_3 | 7.2X10^4/ul | AAV GFP_3 | 10^4 |
| Standard_4 | 7.2X10^5/ul | | |
| Standard_5 | 7.2X10^6/ul | | |
| Standard_6 | 7.2X10^7/ul | | |
| Standard_7 | 7.2X10^8/ul | | |

Based on Q-PCR, the titer of this AAV-GFP virus is 7.2X10^13 vp/ml, which is average virus titer in our AAV production and purification

## Recommended protocol for in vitro cell transduction and in vivo animal injection

### In vitro cell transduction

Determine the MOI (multiplicity of infections)

- MOI means Multiplicity of Infection. MOI equals number of viral particles (vp) per cell. In other words, and MOI of 1 means infecting with 1 viral genome (vg) per cell. In ViGene Biosciences our packaging efficiency is ~100%. GC/ml stands for AAV genome copies/ml measured by real-time qPCR in comparison with a standard reference plasmid with known genome copy number. For all the virus products from ViGene Biosciences Inc the titer virus is measured as genome copies /ml. In this Manual, we use GC, vg and vp interchangeably as a unit for viral titers.

For all the virus products from ViGene Biosciences Inc the titer virus is measured as virus particles or genome copies /ml. Although measurement of virus titer in GC/ml is reproducible in every lab, the real infection units could be very different when it's estimated in different experiments. Thus before you do your experiments, you have to estimate the Infection Units of virus in your experiments. In ViGene Biosciences Inc, we usually do a ten-fold serial dilution of virus starting with 1ul viral stock and ending in 10^8 GC/ml. Add the diluted the virus to your cells, 2-3 days later, based on how many cells been infected to calculate your infection units.

The goal is to get 100% of infection without causing any undesired effects. To determine this optimal concentration of virus for your study, we suggest you to conduct pilot testing in your cell line by using reporter AAV like AAV-GFP (ViGene Biosciences, catalog number # CV10003 through CV10009).

After you know the infection units of the viral stock and decide the MOI you are going to use in your experiments, dilute the viral stock with right MOI first.

Remove the original cell culture media, and add the above AAV-containing media to cell culture. Below is a general guideline for the amount of media used:
24-well plate: 0.2-0.3 ml
12-well plate: 0.5-0.8 ml
6-well plate: 2ml/well
60mm-plate: 3-4 ml/plate
10cm-plate: 8-12 ml/plate

Incubate cells with the virus-containing media for at least 6-12 hours. You don't have to exchange the virus-containing media for fresh media, but you can do it after 6-12 hours. It may take 3-7 days after the AAV infection to detect the gene over-expression.

Recommended protocol for in vitro cell transduction

1. Thaw the AAV virus on ice, and keep it on ice throughout the duration of the experiment.
2. AAV infection is cell type dependent. Some cell types exhibit low transduction efficiency, while others transduce very readily.

   When designing AAV transduction experiments, it is recommended to use different serotypes of a reporter vector such an AAV expressing GFP ((ViGene Biosciences, catalog number # CV10003 through CV10009) to determine optimal serotype for transduction of your tissue or cell culture.

3. Start cell transduction at MOI of 10^4 and 10 ^6 GC/cell when cells are readily transducible. With some cell lines a higher MOI might be needed. Look for the highest transduction with minimal cell death. With some cell lines, high transduction levels cannot be achieved.
4. Use the minimum concentration of FBS that the cells can withstand when performing the transduction. For example, HT1080 cells are maintained using media containing 10% FBS. Transductions are performed using media containing 2% FBS.
5. Use the minimum amount of media necessary to cover the surface of the plate. For example, transductions are performed in 6-well plates, 1 ml of media per well is used.
6. Look for expression at 24h, 48h, 72h and 96h, post transduction.

## In vivo animal use

- The recommended titer for in vivo animal injection is 10^11 GC per gram (body weight).
- Dilute the virus with PBS to achieve the appropriate GC number.
- Proceed to the intravenous injection or tail vein injection or localized tissue injection as demonstrated by labs (for references please visit http://www.vigenebio.com/delivery/AAV-Systems/).

## FAQ

**When should I use rAAV in my experiments?**

rAAV can deliver gene into dividing and nondividing cells for transient gene expression. It has been used in vivo and in vitro. Refer to our table when you are not sure which virual vector should you choose for your experiments. You also can purchase ViGene's "GFP virus testing kit". Cat# CT10001 to test in vitro or in vivo.

**What's the biosafety requirement for using AAV?**

Recombinant AAV constructs, in which the transgene does not encode either a potentially tumorigenic gene product or a toxin molecule and are produced in the

absence of a helper virus can be handled in Biosafety Level 1(BSL-1) facility. Otherwise it should be handled as biohazardous material under Biosafety Level 2 (BSL-2) containment. Please check with your Institutional Biosafety Committee or related NIH web site for detailed information, if you need more information.

**Is recombinant AAV replication deficient?**

For wild type AAV, replication is at extremely low efficiency, without the presence of helper virus, such as adenovirus. For recombinant adeno-associated virus produced these days, the replication and capsid genes are provided in trans (in pRep/Cap plasmid), and only the 2 ITRs of AAV genome is left and packaged into virion, while the adenovirus genes required are provided either provided by adenovirus or another plasmid, the likelihood for a recombinant AAV to replicate is theoretically impossible. This is in similar scheme to lentiviral vectors produced these days.

**What's the cloning capacity for recombinant AAVs?**

AAV has a packaging capacity of ~4.7Kb. Since the two ITRs of AAV is about 0.2-0.3Kb in total, the foreign DNA that could be introduced between these 2 ITRs should be <4.4Kb, which is much smaller than that of recombinant adenovirus (7.5Kb). In addition, when the length of inserted DNA between the 2 ITRs is close the maximal allowed, i.e., 4-4.4Kb, the packaging efficiency decreases significantly. For instance, for gene over-expression from cDNA, since the CMV-poly(A) element is about 1Kb, so the maximal allowable cDNA length is about 3Kb. In addition, if you are interested in GFP co-expression (from a separate expression cassette), given the additional CMV-EGFP-poly(A) is about 2 Kb, so the maximal cloning capacity for GFP co-expressing system is about 1.0-1.2Kb.

For double-stranded AAV (dsAAV), the capacity is only half of the single-stranded AAV (ssAAV).

**How many AAV Serotypes does ViGene Biosciences Offer?**

There are 11 different AAV serotypes have been reported so far. ViGene Biosciences provide the packaging services of AAV serotype 1, 2, 5, 6, 7, 8, 9. Total 7 different AAV serotypes.

**How stable are AAV vectors? How should they be stored?**

Stability studies carried out in house and by some colleagues show that purified AAV vectors are highly stable at temperatures of 4 C or less. We recommend aliquoting upon receipt and storing at -80 $^{o}$C . Once an aliquot is thawed it can be stored at 4$^{o}$C for short-term storage, e.g., 2-3 weeks, without significant loss of biological activity.

**What's the difference between physical and genomic particles?**

AAV genome particles relate to the viral particles that have been successfully packaged with the genome to be delivered. During the AAV packaging process many particles are formed lacking the genomic DNA, which lack the ability to transduce the cells they come into contact with and are therefore non-functional. As a researcher you probably want the concentration of functional AAV particles.

**What does a customer need to provide?**
If customer is going to order the clone from Vigene Bioscience, only the gene accession number from NCBI or catalog number from Vigene Bioscience is required. If customer provides AAV vector and using packaging services from ViGene Biosciences, 1mg DNA from maxi-prep at the concentration of 1mg/ml should be provided by customer.

**How long does the rAAV cloning, small scale and large scale AAV production take?**
If the 1 mg transfection grade quality plasmids can be supplied, small scale and large scale AAV production will take 1-2 weeks. If the customers requires Vigene to conduct subcloning and plasmid prep for the AAV plasmid, the entire process can take 4-5 weeks.

**How much AAV do I need?**

- **Cell transduction**
    - Start cell transduction at MOI of 10^4 and 10 ^6 GC/cell when cells are readily transducible. With some cell lines a higher MOI might be needed. Look for the highest transduction with minimal cell death. With some cell lines, high transduction levels cannot be achieved.
- **Animal injection**
    - The recommended titer for in vivo animal injection is 10^11 GC per mouse or 2X10^9 GC /g (body weight). For intravenous injections larger quantity of viruses may be needed in comparison to local injections.

## Biosafety Considerations:

Follow the recommended NIH guidelines for all materials containing BSL-1 organisms.

## LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by ViGene Biosciences. ViGene Biosciences shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

## ORDERING INFORMATION AND TECHNICAL SUPPORT

### Ordering

- Email: orders@vigenebio.com
- Toll Free (USA): 1-800-485-5808
- Telephone: 301-251-6638
- Fax: 301-251-6110

### Technical Support

- Email: custsupport@vigenebio.com
- Toll Free (USA): 1-800-485-5808
- Telephone: 301-251-6638
- Fax: 301-251-6110