



**Efficient Gene Knockdown via
Recombinant Adeno-Associated Virus (rAAV) Delivered shRNA
- A Technical Guide**

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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} vp/ml*.
2. **Large scale purified AAV.** 500ul of rAAV at 10^{13-14} vp/ml*.
3. **Customer Large scale purified AAV.** Custom amount (up to 10^{16} vp) of rAAV at 10^{13-14} vp/ml*.

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.

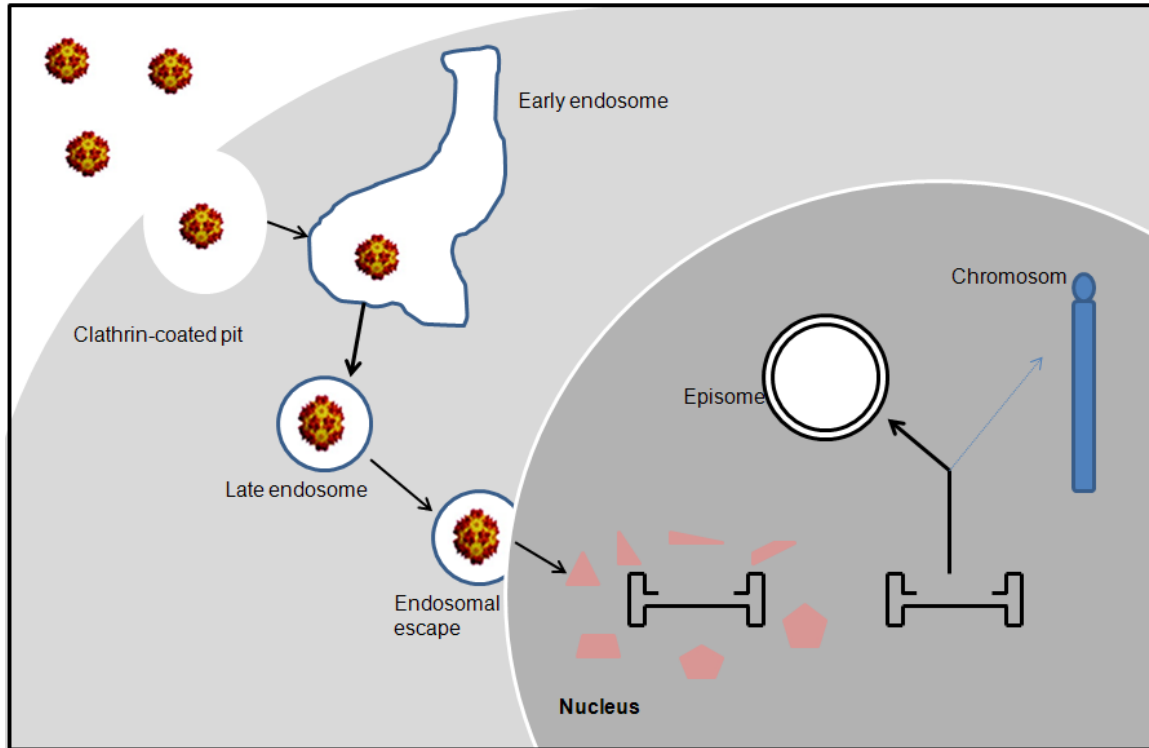
* viral particles (vp) ; viral genome (vg). In ViGene Biosciences our packaging efficiency is ~100%. So in this Manual we use vp and vg interchangeably.

Introduction

Short hairpin RNA (shRNA) is an artificial RNA molecule with a tight hairpin turn that, when expressed in cells or *in vivo*, can induce targeted gene knockout or knockdown via RNA interference. shRNA is a preferred mediator of RNAi especially for *in vivo* studies in that it has a relatively low rate of degradation and turnover. However, the delivery of shRNA to tissues *in vivo* via traditional plasmid transfection has been a bottleneck.

Thanks to the recent development, recombinant AAV vectors empower almost 100% delivery of shRNA into the targeted cell population or tissues while other systems could deliver genes to only a fraction of the cells. The efficiency of gene silencing through AAV gene delivery is much higher than those seen in other delivery systems. In this manual we set out to address main technical questions when one chooses to use AAV to deliver and express shRNA *in vivo*.

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*.

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 vp/ml without purification	10 ⁷ vp/ml without concentration	10 ⁷ vp/ml without concentration
Relative Transduction Efficiency	100%	70%	70%
Relative Foreign Gene Expression	High	Medium	Medium

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					X
AAV5			Lung alveolar cells	Neurons and glial cells	X		
AAV6	X	X	X				
AAV7		X		Neurons	X		
AAV8		X		Neurons	X	X	
AAV9	X		X	Neurons		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 AAV vector cloning service; the other is AAV packaging services.

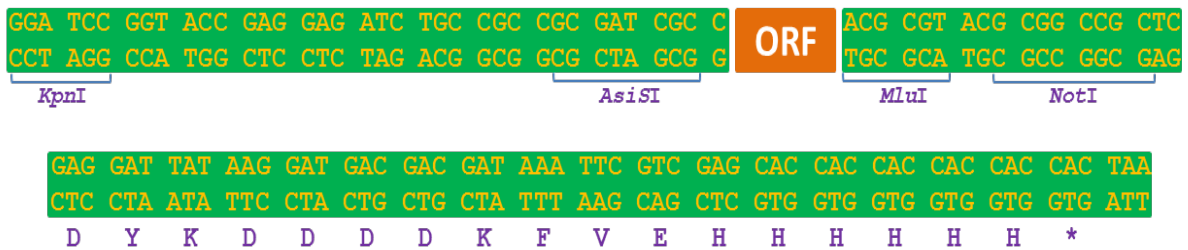
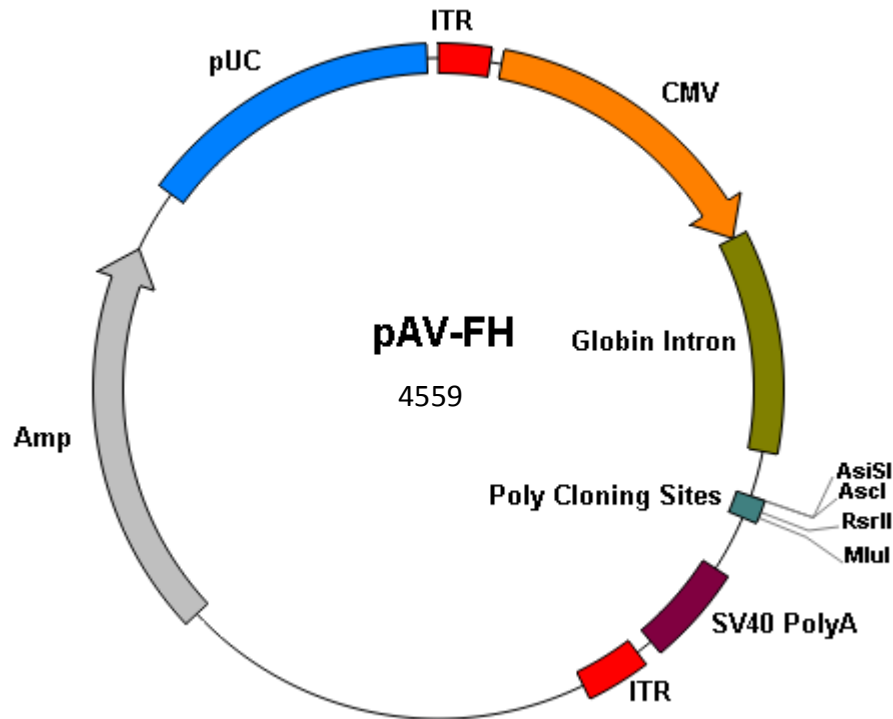
AAV vector cloning services

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 Mlul sites. In other rare case the combination of AsisI-RsrII, AsisI-NotI or AscI-Mlul 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.



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-GFP.

Cat.#	Promoter Name	Size	Description
PM10001	ALB	1.0kb	Liver specific 10 timer stronger than CMV after 10 weeks
PM10002	ApoE/AAT1	0.7kb	antitrypsin and cer/hepatic locus control region (HCR). Liver specific
PM10003	CAG	1.8kb	Strong promoter, ubiquitous expression in vivo
PM10004	CamKII	0.4kb	Specific expression in excitatory neurons in the neocortex and hippocampus
PM10005	EF1a	1.2kb	Ubiquitous, weaker than CMV but better for in vivo
PM10006	ELA1	0.3kb	Specific in pancreatic acinar cells, stomach, duodenum, and colon
PM10007	Enh358MCK	0.6kb	Specific in skeletal muscle fiber, cardiac muscle
PM10008	GFAP	0.7kb	Specific in astrocyte
PM10009	MBP	1.3kb	Specific in oligodendrocyte
PM10010	SST	0.9kb	Specific in pancreatic islets, gastrointestinal tract, nervous system, and thyroid gland
PM10011	TBG	0.8kb	Specific in liver
PM10012	aMHC	0.4kb	Specific in cardiomyocytes
PM10013	cTNT	0.4kb	Specific in cardiomyocyte muscle
PM10014	Synpsin	0.85kb	Specific in neuron
PM10015	hRPE	0.8kb	Specific in retinal pigment epithelium
PM10016	mIP1	1.2kb	Specific in beta- cells of the pancreas
PM10017	tMCK	0.7kb	Specific in muscle
PM10018	Ubc	1.2kb	Ubiquitous, weaker than CMV but better for in vivo
PM10019	PGK	0.4kb	Ubiquitous, weaker than CMV but better for in vivo

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

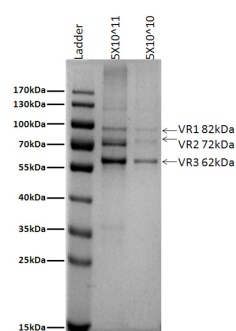
Unless specified otherwise, AAV viruses are produced from up to 10^{10} HEK293 cells and are purified by iodixanol gradient ultracentrifugation. Resulted virus are concentrated to 500ul with virus titer no less than 10^{13} vp/ml. The purified virus is good for in vivo animal research.

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

1. Small scale testing AAV packaging service. In this service, rAAV is packaged using 10^7 HEK293T cells. The virus is in crude cell lysate, without any purification or concentration. The titer is around 10^9 - 10^{11} vp/ml.
2. Large scale purified rAAV packaging service. In this service, rAAV is packaged using 2.5×10^8 HEK293 cells. Viruses are purified by iodixanol gradient ultracentrifugation. Resulted virus are concentrated to 500ul with virus titer no less than 10^{13} vp/ml. The titer is around 10^{12} - 10^{15} 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} vp with virus titer no less than 10^{13} vp/ml.

The purity of the AAV virus

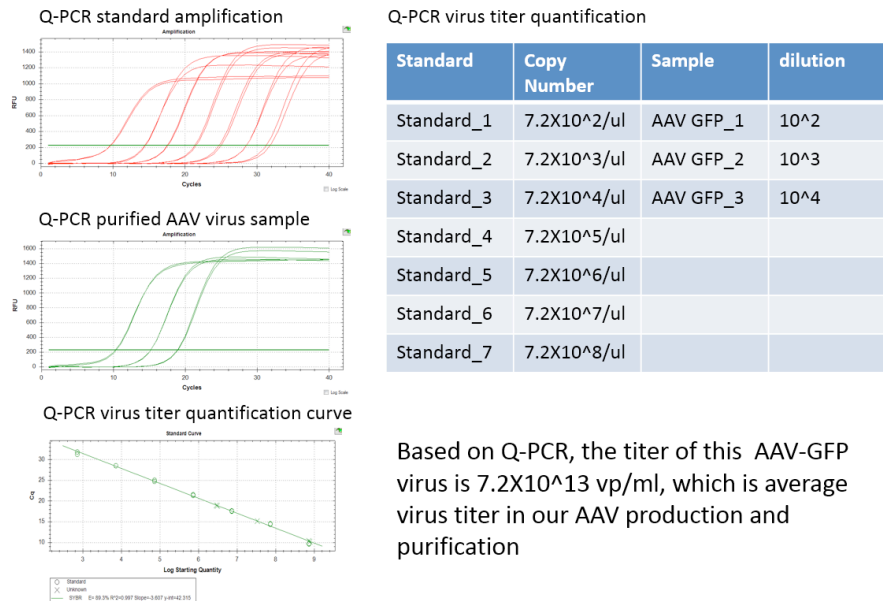
The AAV protein components are VP1 82kDa, VP2 72kDa and VP3 62kDa. So a good AAV purification should only show three major protein bands when the virus is analyze by SDS-PAGE. Following image showed the protein components in our purified AAV virus. We guarantee the purity of the AAV virus based on the specification that we give for different services.



Purity of purified AAV virus. When 5×10^{11} and 5×10^{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 titrating AAV-GFP virus.



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%. So in this Manual we use vp and vg interchangeably.

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 vp/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 10⁰⁸. 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 vg/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} vg per mouse or 2×10^9 vg/g (body weight)
- Dilute the virus with PBS to achieve the appropriate vg 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 viral 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 -80C. Once an aliquot is thawed it can be stored at 40C 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 maxprep 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?

The entire process takes approximately 4-5 weeks. If the customer could help on the 1st subcloning and Qigaen Mega prep for the cis AAV plasmid, the entire process will take about ~2-3 weeks.

How much AAV do I need?

- **Cell transduction**
 - Start cell transduction at MOI of 10^4 and 10^6 vg/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} vg per mouse or 2×10^9 vg/gram (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.

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