Full Length cDNA Clones &
ORF-Adenoviral Expression System
User Manual

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Not for use in diagnostic procedures

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Components

The products may contain the following components.

1. **Catalog number (CHXXXXXX) : pEnter-ORF glycerol stock.** 500ul.
2. **Catalog number (AHXXXXXX): pAD-ORF glycerol stock.** 500ul.
3. **Catalog number (VHXXXXXX): ORF- adenovirus:** one 100 ul vial of 10^9 adeno-viral particles.

Storage

The products should be stored at -80ºC.

Safety Considerations

For the ORF adenovirus customers- follow the recommended NIH guidelines for all materials containing BSL-2 organisms.

For the ORF cDNA clone customers- follow the recommended NIH guidelines for all materials containing BSL-1 reagents.

Introduction

ViGene's full length cDNA clones feature proprietary cloning technology and unique vector design- pEnter Entry Vector System. The expression-ready ORFs can be shuttled into more than 30 destination vectors in a simple cut-and-paste approach in just 2-3 hours.

Key features

- 100% sequence-verified by NextGen Sequencing
- Expression guaranteed
- Ready to be shuttled into more than 30 destination vectors, including adenoviral & lentiviral vectors
- Contains puromycin gene for stable cell transfection
- Unique design to accommodate cDNA up to 30 kb
- The CMV promoter and a Kozak consensus sequence drive protein expression effectively

ViGene Biosciences' ORF-Adenoviral ORF expression system contains three sets of related products: **ORF shuttling system, pAD-ORF and premade ORF-Adenovirus.**

Recombinant adenoviruses are powerful and easy-to-use tools in gene delivery and expression. Several unique characters of adenoviral biology have made it the vector of choice for broad application. First, it is capable of infecting varieties of cell types, including dividing, non-dividing cells and stem cells. Moreover, high virus titer can be easily obtained. Furthermore, high titer of virus could achieve high infection rate and high expression level. Last but not the least, after entering the cells, the virus remains epichromosomal, thus the expression is transient and infection of recombinant adenovirus does not induce chromatin change in host cell. In ViGene Biosciences, we used the most common adenoviral vector, human adenovirus serotype 5, which is rendered replication defective by the deletion of E1 and E3 genes. The E1 gene is essential for the assembly of infectious virus particles, and it can be complemented during virus packaging process in HEK293T cell lines. And the E3 gene is dispensable. With the deletion of E1 and E3, adenoviral particles are capable of integrate of 7.5kb foreign DNA.

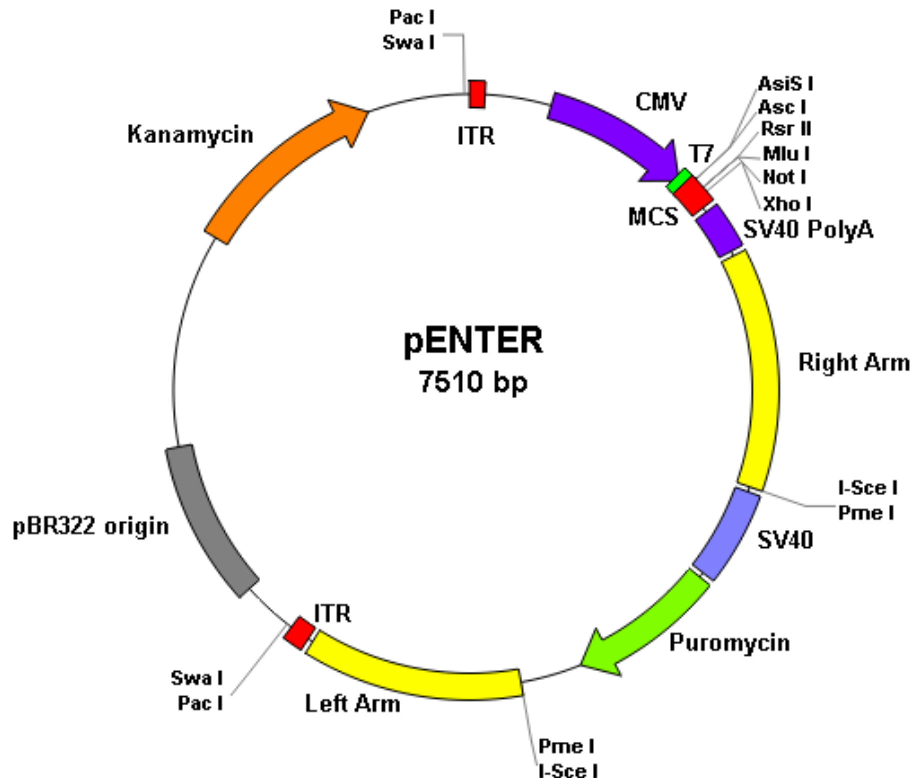
ORF plasmid shuttling system. Utilizing simple "cut and paste" cloning strategy, ViGene provides varieties of choices to express ORF with one or two different epitope tags or fluorescent markers. With four different combinations of endonuclease with rare sites inside genes, and with different selections between entry vector and destination vectors, ORF inserts could be easily transferred from

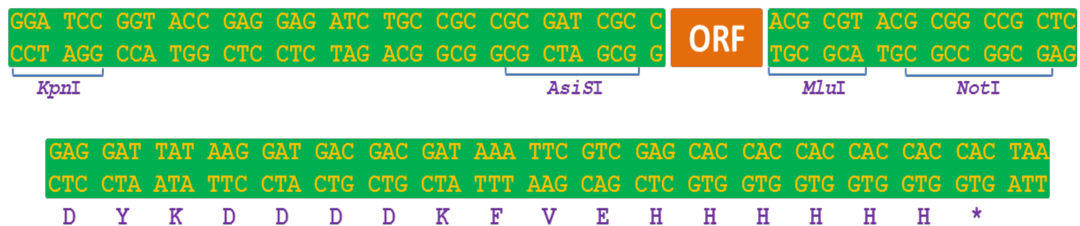
the entry vector to any of the destination vectors in two days. There are more than 30 destination vectors to help customers to achieve the best methods in overexpress the ORFs. For the detailed list of ViGene's destination vectors, please visit our web site, www.ViGeneBio.com. These destination vectors includes vectors with different promoters, viral vectors of adenovirus, lentivirus and adeno-associated virus, vectors to tag the epitope or fluorescent marker at the N' terminal or C' terminal of ORFs . In ViGene's pEnter vector, expression of ORF is driven by CMV promoter, is with Flag and His tag at the C' terminus of ORF, and with a SV40 poly (A) tailing signal. pENter contains a puromycin marker driven by SV40 promoter, which can be used to generate stable transfection. pEnter also contains two adenoviral ITR sequence and two Ad5 homologous sequences, which can be used to transfer ORF expression unit to adenoviral vector, pAD.

pAD-ORF is plasmid based ORF of gene expression system. Through recombination in E.coli, the ORF expression unit was transferred from the pEnter and most of the destination vectors to pAD, except the destination vectors of lentiviral and adeno-associate viral vectors. pAD is most commonly used human adenoviral vector , human adenovirus serotype 5, with E1 and E3 gene deletion. Plasmid of pAD-ORFs could be used to generate recombinant adenovirus in HEK293 cells.

ORF- Adenovirus is premade recombinant adenovirus. The adenoviral vector is generated through the recombination of ORF carrying pEnter and pAD. The express ORFs are with Flag and His tags at their C' termini. ViGene's premade ORF-Adenovirus provides the most efficient mode of gene delivery among all forms of ORF cDNA clones. It can be used *in vitro* and *in vivo* for ORF functional analysis.

pEnter vector map and features:

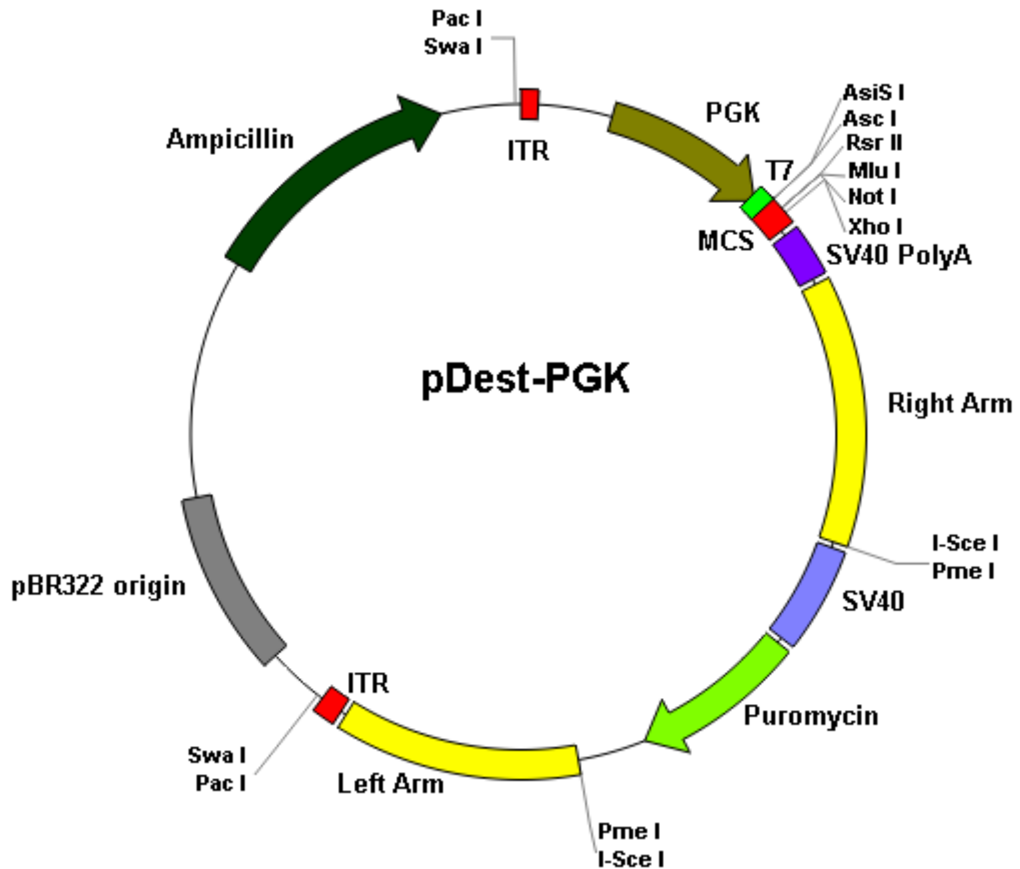


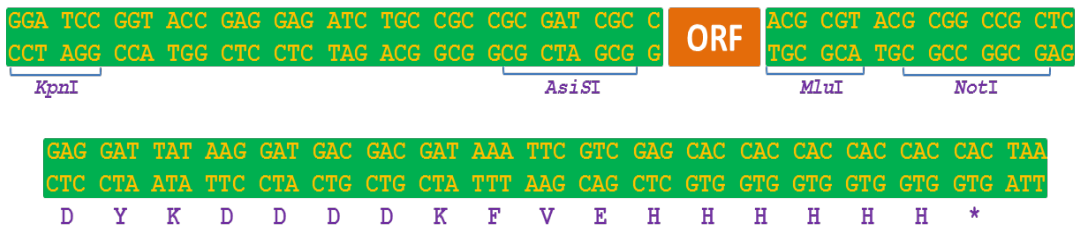


In most of case, ORF inserts are cloned between AsisI and MluI sites. In other rare case the combination of AsisI-RsrII, AsisI-NotI or AscI-MluI are used in the cloning. Please check our web site or the COA for specific clones. In the pEnter vector, ORF is fused with a Flag/His tag at its carboxyl terminus. The vector contains a kanamycin marker for bacterial selection. The SV40 driven puromycin marker can be used for stable clone selection in mammalian cells. Two ITRs and two Arm sequences are designed to generate recombinant adenoviral vector by recombination with pAD in E.coli and adenovirus in HEK293 cells. ViGene's pEnter 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.

Most common destination vectors and features:

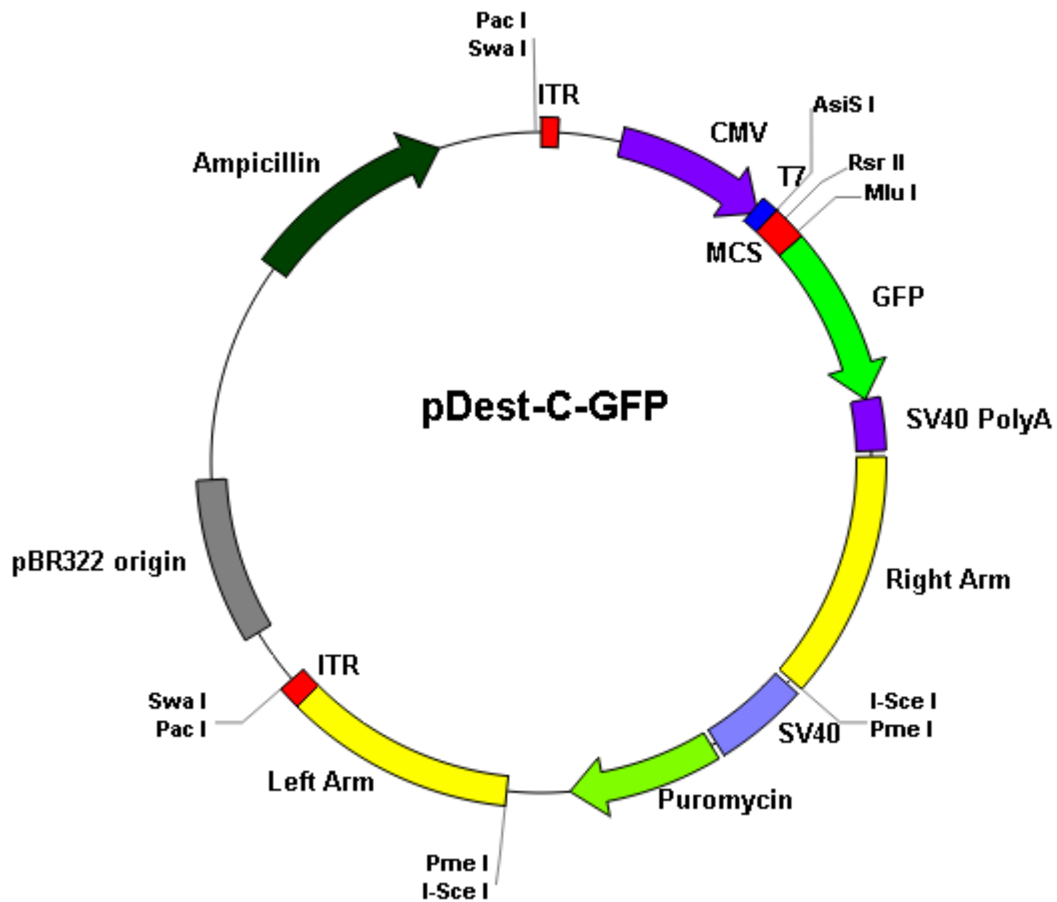
pDEST-PGK





Although CMV promoter usually is very strong in driving gene expression *in vitro* and *in vivo*, there are some reports suggested CMV promoter could be silenced from unknown reason. In order to address this problem, ViGene provides destination vectors PGK or EF-1a promoter. Both promoters have been reported to have good expression for *in vivo* and some cell lines where CMV is silenced. In pDest-PGK vector, amplicillin selection marker replaced the kenamycin selection marker and PGK promoter replaced CMV promoter in pEnter. Other elements and MCS remain the same in both vectors.

pDEST-C-GFP



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*

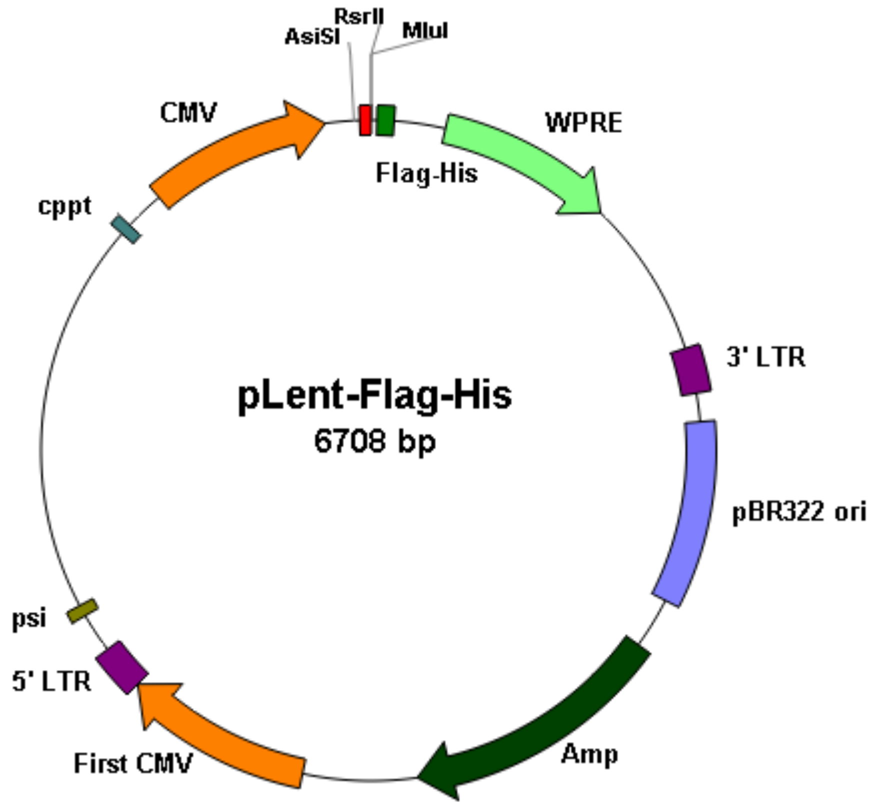
GAG ATG GTG AGC AAG GGC GAG GAG CTG ••• ••• CTC GGC ATG GAC GAG CTG TAC AAG TAA

CTC TAC CAC TCG TTC CCG CTC CTC GAC ••• ••• GAG CCG TAC CTG CTC GAC ATG TTC ATT

GFP

Fluorescent protein tags are very useful in tracing protein expression and location in vitro and in vivo. In addition to destination vectors with Flag, His, HA, Myc tags, ViGene provides destination vectors with fluorescent protein tags, such as GFP and RFP. All destination vectors from ViGene Biosciences are with ampicillin selection marker and with different tags either as N' terminal fusion or C' terminal fusion.

Lentiviral Destination vectors

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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   *
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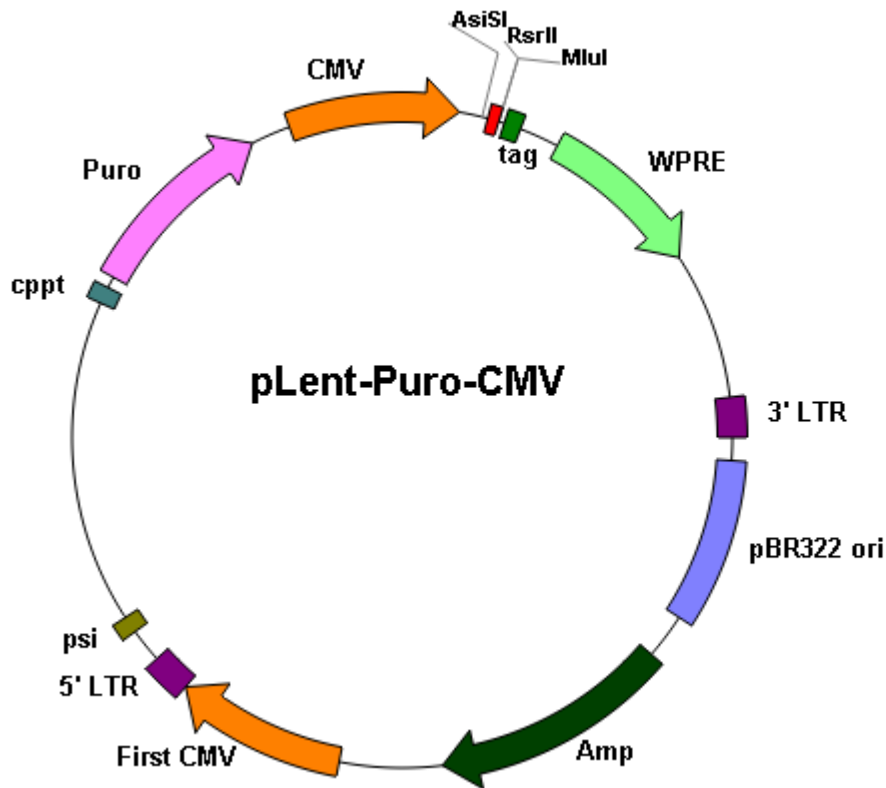
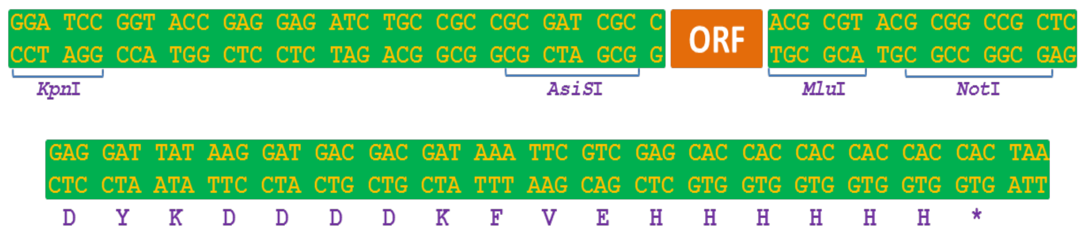


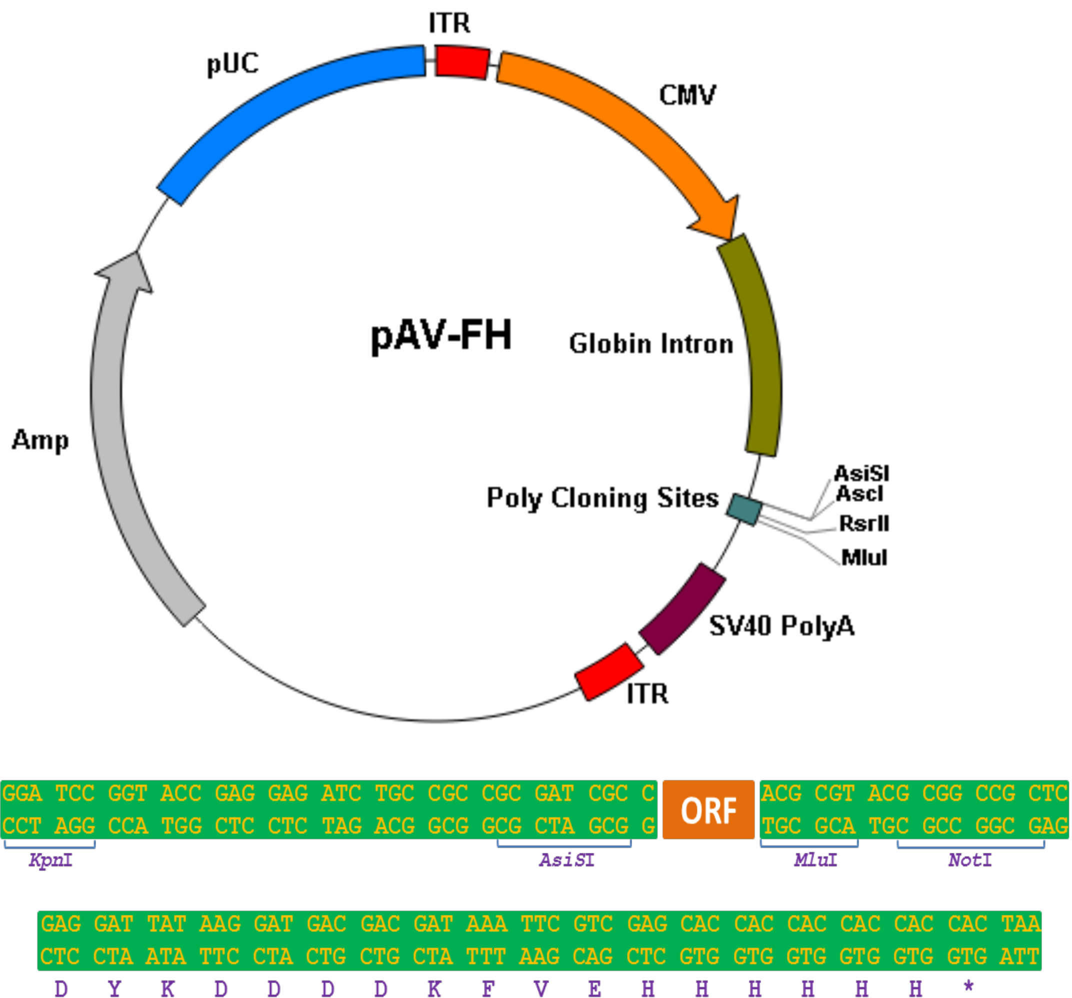


Lentiviral vectors are valuable tools in delivering gene in vitro and in vivo. Compared to adenovirus, lentivirus has the ability to integrate foreign genes into the genome of non-dividing cells. ViGene Biosciences provides lentiviral vectors with verities of tags and fluorescent markers. As pLent-Flag-His, most of our lentiviral vectors have the similar MCS as our pEnter vector and with ampicillin selection marker. Any ORF gene can be easily transferred from pEnter vector to our lentiviral vectors by “cut and paste” cloning method.

Lentiviral vectors can be easily used to generate stable transfection in vitro when compared to plasmid DNA. To facilitate the selection of stable transfection, we provide two lentiviral vectors with either puromycin or GFP selection marker, which is directly driven by 5’ LTR promoter for expression. In this two vectors, Customers can choose to express the ORF gene with a myc-flag tag by transferring the ORF inserts from pEnter by “cut and paste” method. If Customers choose to express the

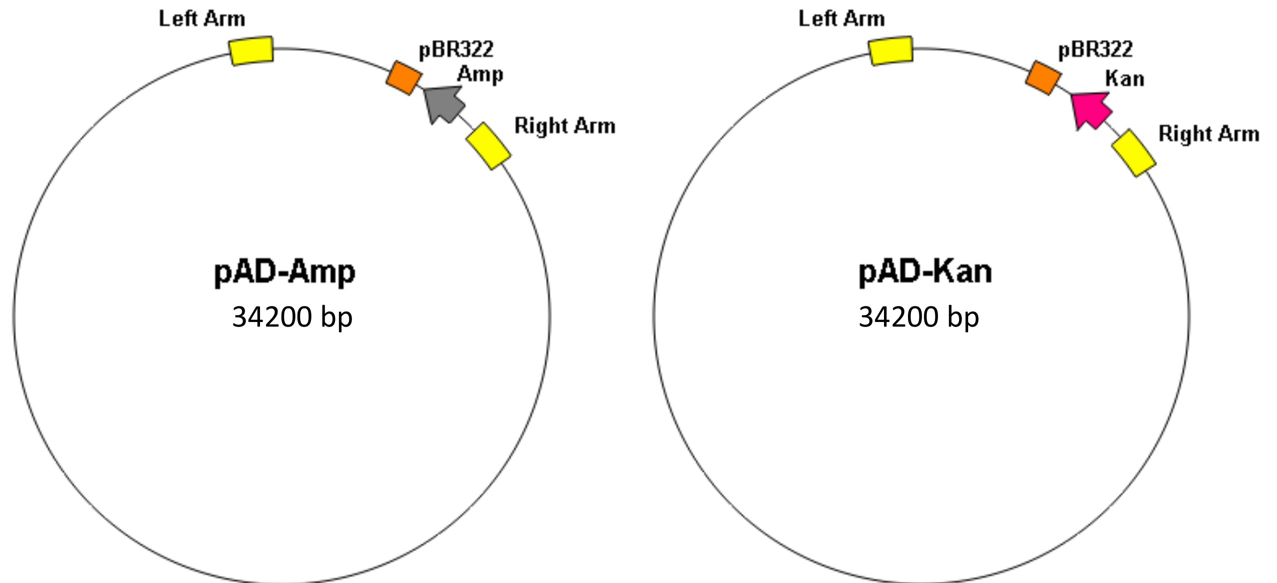
ORF gene with native stop codon, the ORF inserts from our pEnter can be PCRed out and clone into the destination vector, as a “copy and paste” cloning method.

Destination of Adeno-Associate Vrial (AAV) vector



When compared to Adenovirus, AAV is a small virus and it causes very mild immune response. Most labs chose AAV as gene therapy vectors or in vivo animal research. AAV can deliver gene into both dividing and quiescent cells. Same as adenovirus, after entering the cells, the AAV virus remains epichromosomal. To accommodate the need, ViGene Biosciences provides one AAV vector for ORF gene expression. pAV-FH drives the ORF genes expression by CMV promoter, with SV40 polyA tail. The expressed ORFs are with a C’ terminal fused Flag-His tag. ORF inserts can be transferred from pEnter to pAV-FH by our “cut and paste” cloning method.

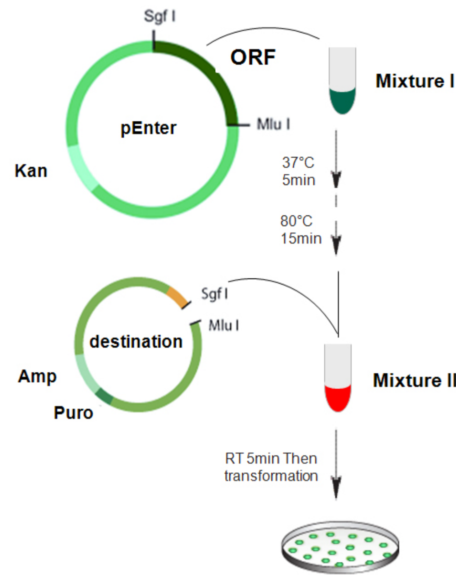
pAD vectors



pAD vectors contains human Ad5 adenoviral genome sequence with E1 and E3 deletion. In order to transfer the ORF genes into pAD vectors, researches have carry out recombination between pAD vectors and the liner DNA of either ORF in pEnter or in other destination vectors in recombination competent E. coli, such as BJ5183 cells. In ViGene Biosciences, we generated two pAD vectors. The pAD-Amp has the ampicillin selection marker, and is used in recombination with pEnter vector. The pAD-Kan has kanamycin selection marker, and is used in recombination with destination vectors.

Transfer ORF insert from pEnter to destination vector:

Within ViGene Biosciences ORF plasmid shuttling system, ORF inserts could be easily transferred from the entry vector to any of the destination vectors in two days. The transfer procedure is illustrated in following diagram.



1. The destination could be prepared in advance, or purchase the ready to use destination vectors from ViGene Biosciences.

2. Digest the ORF inserts from pEnter with right enzyme combination, for example, the most common AsisI- MluI combination in Mixture I:

Component	Volume
Restriction Digest Buffer	3µl
ORF in pEnter	5µl(500ng)
AsisI	0.6µl
Mlu I	0.6µl
Nuclease-Free Water	20.8µl

3. The digestion can be done in 5 minutes at 37 ºC. Then the enzymes should be inactivated in 80 ºC for 15 minutes.

4. Set up ligation reaction as in Mixture II:

Component	Volume
5 x Rapid Ligation Buffer	2µl
Mixtrue I	2µl (30ng)
Pre-prepared OriGene Vector	3µl (10ng)
T4 DNA Ligase (400/µl)	0.5µl
Nuclease-Free Water	2.5µl

5. Ligation reaction is done at room temperature for 5 minutes. And all the enzymes can be purchased from New England Biolabs.

6. Transform and incubate E.coli cells at 37 ºC over night.

7. Day3, DNA miniprepation, DNA digestion or sequencing to screen for correct clones.

Recombination:

Using either the pEnter or destination vectors as shuttle vectors, pAD-ORF clones can be generated in recombination competent E.coli, such as BJ5183 cells.

1. Digest the pEnter- ORF with PmeI enzyme.

Component	Volume
Restriction Digest Buffer	5µl
ORF in pEnter	7µl(700ng)
PmeI (from NEB)	1µl
Nuclease-Free Water	37µl

2. Incubate at 30 ºC for 2.5 hour then add 0.5 µl Alkaline phosphatase, Calf Intestinal (CIP) and incubate at 37 ºC for 30 minutes.
3. The digestion will generate two fragments, one at 1.2kb and the other one should be larger than 6.3kb dependent on the size of ORF inserts. Separate the two fragments by DNA agarose gel electrophoresis.
4. Recover the larger fragment with DNA gel extraction kit. Note: elute the DNA with 30 µl water instead of TE buffer at the last step.
5. Transform 30µl of BJ5183 electrical competent cells with 5 µl of recovered DNA by electroporation.
6. After electroporation, resuspend the cells in 800 µl of SOC medium and let the cell recover for 2 hours at 32 ºC shaking at 250-290 rpm.
7. Plate 150 µl of transformation on LB-Agar plate with 30µg/ml kanamycin and culture at 30-32 ºC for 24 hours.
8. Pool 20-30 colonies from the plate in 1-2 ml LB medium with 30µg/ml kanamycin and culture for 2-3 hours at 32 ºC.
9. Isolate plasmid DNA from the culture then transform 1µl of prepared DNA to DH5a and culture on LB-Agar plate with 30µg/ml kanamycin at 37 ºC for overnight to get rid of pAD-Amp plasmid.
10. Pick at least 6 colonies to screen for right recombination.
11. Verify the right recombination by PacI digestion and DNA sequencing. Dependent the sites of recombination, correct recombination will generate two fragments either 3.5kb and 34kb or 2.5kb and 34kb.

Adenovirus packaging:

1. Digest the pAD- ORF with PacI enzyme.

Component	Volume
Restriction Digest Buffer	5µl

ORF in pEnter	20µl(2µg)
PacI (from NEB)	1µl
Nuclease-Free Water	24µl

2. Incubate at 37 ºC for 3 hour. Then the enzymes should be inactivated in 80 ºC for 15 minutes.

Note: The following procedure is suggested for T75 flasks and may be optimized to suit individual needs.

3. Seed 3-5 x 10^6 HEK293T cells in a T75 flask one day before transfection.
4. Transfect cells with 2ug liner pAD-ORF DNA.
5. 4-5 days after transfection, examine the monolayer twice per day under the microscope for CPE. When CPE is nearly complete (i.e. most cells rounded but not yet detached from the flask, usually it takes 7-14 days for CPE to be complete), harvest cells by pipetting media up and down to wash the infected cells from the flask into the media.
6. Pool cells and medium. Pellet cells by centrifugation at 1000g for 5 minutes. Remove supernatant, resuspend cell pellet in medium or in 10 mM Tris, pH 8.0, 100 mM NaCl. (0.25-0.5 mL per T75 flask).
7. Release the adenoviruses from the cell suspension with three freeze/thaw cycles. Centrifuge at 3000 g for 10 minutes to pellet the cell debris. Discard the pellet and save supernatant as viral stock.
8. The viral supernatant can be stored at -80°C or immediately purified or titered.

pEnter-ORF plasmid amplification:

Glycerol stock of pEnter-ORF or pAD-ORF should be streaked on LB-Agar plate with 30ug/ml kanamycin and culture at 37 ºC over night. Second day, at least 3 colonies should be picked for minipreparation. The sequencing pEnter-ORF or pAD-ORF from the 5' end should be performed with a primer whose priming site is located ~150 nt upstream of the polylinker.

Viral vector amplification:

Amplification of a virus stock is achieved by infection HEK 293T cultures with included MirAd microRNA precursor adenovirus. One round of amplification generally produces a 10-fold increase in titer.

Note: The following procedure is suggested for T75 flasks and may be optimized to suit individual needs.

1. Seed 3-5 x 10^6 cells in a T75 flask one day before infection.
2. Add 50% of the above Crude Viral Lysate to the cultuire. We recommend using a multiplicity of >0.5 PFU (plaque forming units) or enough viruses that cells demonstrate cytopathic effects (CPEs) within 48 hrs.
3. During 24 - 48 hr infection, examine the monolayer twice per day under the microscope for CPE. When CPE is nearly complete (i.e. most cells rounded but not yet detached from the flask), harvest cells by pipetting media up and down to wash the infected cells from the flask into the media.
4. Pool infected cells and medium. Pellet cells by centrifugation at 1000g for 5 minutes. Remove supernatant, resuspend cell pellet in medium or in 10 mM Tris, pH 8.0, 100 mM NaCl. (0.25-0.5 mL per T75 flask).
5. Release the adenoviruses from the cell suspension with three freeze/thaw cycles. Centrifuge at 3000 g for 10 minutes to pellet the cell debris. Discard the pellet and save supernatant as viral stock.
6. The viral supernatant can be stored at -80°C or immediately purified or titered.

Titering Adenovirus with quantitative-PCR:

qPCR method is a simple and high throughput method in estimating adenoviral particles in both crude lysate and purified adenovirus samples. This method is based on the quantitative real-time PCR amplification of specific adenoviral genome sequence. Within the linear range of quantification, the initial copy number of viral genome can be estimated when the Ct is compared with the Ct from known copy number plasmid.

1. Adenovial genomic DNA purification:

Adenoviral virions' DNA genome is surrounded by a capsid of structural proteins. It is preferred to disrupt this protein shell with proteinase K.

Component	**Volume**
Viral sample	5µl
Proteinase K (5µg/µl)	1µl
Nuclease-Free Water	4µl

I. Incubate at 37 ºC for 30 minutes. Then the enzymes should be inactivated in 95 ºC for 20 minutes.
II. Centrifuge at 3000g for 10 minutes, save the supernatant.

2. QPCR:

1. Template standard from ViGene Biosciences is 3.6X10^8 copies/ml. Series dilute the standard to 3.6×10^2-3.6×10^8 and use nuclease-free water as negative control.

2. Setup PCR reaction, 20 µl for each sample.

Component	**Volume**
2X SaberGreen Mix	10µl
Primer mix	1µl
Nuclease-Free Water	7µl
Treated viral sample or standard	2 µl(equivalent to 1µl initial viral sample)

Sample setting should be as in following table.

STD 0	STD 3.6×10^2	STD 3.6×10^3	STD 3.6×10^4	STD 3.6×10^5	STD 3.6×10^6	STD 3.6×10^7	STD 3.6×10^8
STD 0	STD 3.6×10^2	STD 3.6×10^3	STD 3.6×10^4	STD 3.6×10^5	STD 3.6×10^6	STD 3.6×10^7	STD 3.6×10^8
Viral sample 1	Viral sample 1	Viral sample 2	Viral sample 2	Viral sample 3	Viral sample 3	Viral sample 4	Viral sample 4

3. PCR protocol

4. Viral particles estimation.

Viral Particles (particles/ml) = Estimated number from standard X 1000

FAQ:

1. Does human ORF I purchased from ViGene Biosciences exactly match the reference sequence?

No, it does not always match to Refseq. Although most human ORFs provided by ViGene BioSciences match the reference sequence posted by NCBI, some ORFs from ViGene BioSciences contain single nucleotides polymorphisms (SNP), small in frame insertions or deletions. Human ORFs from ViGene Biosciences were subcloned either from clones in Mammalian Gene Collection (MGC), or from cDNA library, the difference may reflect the difference from different tissue and different individuals.

2. Are all human ORFs from ViGene Biosciences fully sequenced?

Yes, all the human ORF clones from ViGene Biosciences are fully sequenced. Each clone was first confirmed by ends sequences from Sanger sequencing, then was sequenced by next gene sequencing. The actual clone sequence is posted on our web site, www.vigenebio.com . Any sequence difference between ViGene's clone and the reference sequence is also posted. Before order, we strongly encourage customers to download our sequence and check the differences.

3. What is the safety consideration when using and handling

adenoviral vector?

mirAd adenoviral vector is replicating deficient human adenovirus serotype 5 and with deletion of the E1 and E3 genes. The biosafety office at your institution must be notified prior to use of adenoviral vector for permission and for further institution-specific instructions. BL2 conditions should be used at all time when handling the viral particles. All disinfection steps should be performed using fresh 10% bleach. Gloves should be worn at all times when handling adenoviral particles preparations and transducing cells.

4. **How do I determine whether adenoviral vector could be used for my gene delivery?**

You should take following consideration before you chose adenoviral vector. 1. Do you need transient or stable gene expression? 2. Do you need to transduct dividing or non-dividing cells? 3. How important is potential immune response from my target cell? 4. Will you use viral particles for in vivo or in vitro gene delivery?

Adenoviral vector can infect dividing and non-dividing cells. It is suitable for transient gene expression with high gene deliver efficiency, good for in vivo and in vitro gene delivery. But Adenoviral vector has relative higher immune response in target cells when compared to other viral vector system. Please refer to this table for choosing your viral vector system.

	Adenovirus	Adeno-associated Virus	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	10E11 without purification	10E12 without concentration	10E7 without concentration
Relative Transduction Efficiency	100%	70%	70%
Relative Foreign Gene Expression	High	Meidum	Meidum

5. **What is the difference between VP, PFU, and IFU? Which one should I use in designing experiment?**

Viral particles (VPs) represent the total number of viral particles (including live and dead ones). The percentage of dead viral particles can varies significantly because of the variations in virus preparation. Thus VP cannot be used in estimating functional virus amount.
PFU(plaque formation unit)reflects the amount of infectious viruses. In most cases, the VP/PFU ratio is 20:1 to 50:1. IFU(infectious unit) is equivalent to PFU. We suggest that you should use PFU and IFU in your experimental design in order to get more consistent results.

6. .**What are the methods in determine the viral titer?**

There are three mostly used methods in determine the adenoviral titer. 1. Plaque formation assay; 2. End-point dilution assay; and 3. BCA assay.

Plaque formation assay measure the concentration of infectious viruses (PFU), and is a biological assay. Plaque formation assay is done on a monolayer of HEK293 cells that are infected with diluted viruses. Viruses will propagate in infected cells, cause cytotoxicity effects and get released from infected cells. The released virus will infected neighboring cells, and the whole process will be repeated and lead to form holes or plaques on the cell monolayer. 0.5% agarose is laid on top of cells after the initial infection to prevent the diffusion of the viruses. Viral titer (PFU/ml) can be calculated based on the plaques observed. Plaque formation assay take up to 3 weeks.

End-point dilution assay is very similar to plaque formation assay, but much complicate. The formula for calculating the virus titer is also complicate.

BCA assays measure the concentration of Protein. It is only good for purified adenoviral vectors. In general 1ug protein = 1X109 VP.

7. **Should I purify and concentrate the viral particles with CsCl gradient ultra centrifugation?**

If the viral particles are used in in vitro cell cultures, double CsCl purification is not required. However, the contaminants from cell debris and culture medium

contribute greatly to the immune responses. Viral particles used in in vivo or animal studies should be purified and concentrated by CsCl purification in order to remove defective particles and other contaminants.

8. How to store adenoviral particles?

The viral particles are stable at -80°C for 6 months to a year.

9. What is the serotype of mirAd adenoviral particles?

MirAd adenoviral particles are human adenovirus serotype 5. With E1/E3 gene deletion.

10. What is RCAs and how to detect RCAs?

The 293 cell line contains adenovirus type 5 sequences which are homologous with sequences in mirAd adenoviral vector; Therefore, it is possible that replication competent adenoviruses (RCA) can emerge as a result of the rare recombinant of adenovirus and the genome of HEK293 cells.

The RCA can be tested in nonpermissive cell lines such as HeLa. Serial diluted adenoviral stocks are used to infect HeLa cells and the infected cells are incubated for 8 days. If after 8 days no cytopathology is apparent, recombinant adenoviral stocks can be assumed to be free of RCA.

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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- Toll Free (USA): 1-800-485-5808
- Telephone: 301-251-6638
- Fax: 301-251-6110

Technical Support

- Email: custsupport@vigenebio.com
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- Telephone: 301-251-6638
- Fax: 301-251-6110