G0746
pFBAAVmcs
wtIRESeGFPBgHpA

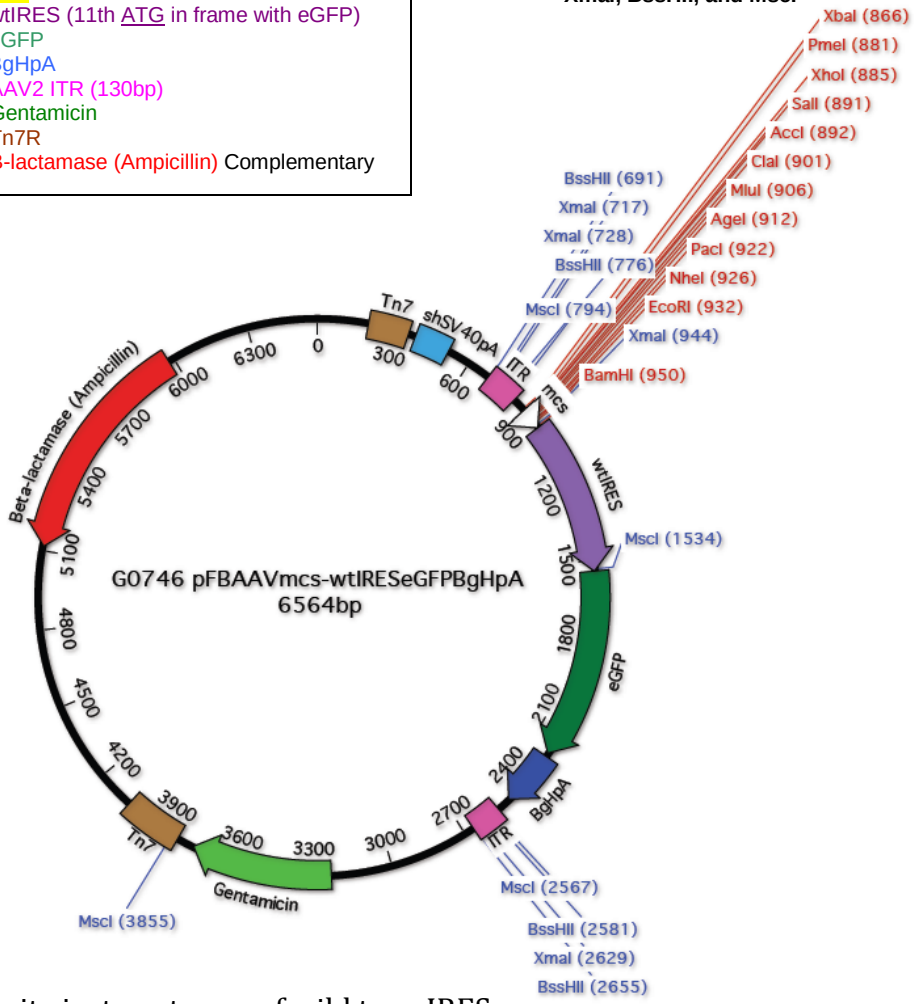
UNIVERSITY of IOWA CARVER COLLEGE OF MEDICINE
VIRAL VECTOR CORE

Plasmid Features: **6564bp**

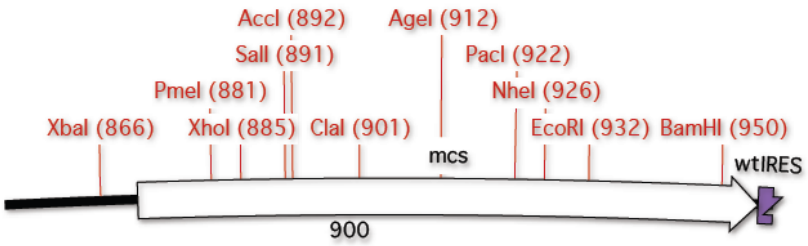
Coordinates	Feature
194-348	Tn7L
376-511	SV40pA Complementary
678-818	AAV2 ITR (141bp)
872-955	mcs
956-1542	wtIRES (11th ATG in frame with eGFP)
1543-2262	eGFP
2277-2490	BgHpA
2544-2673	AAV2 ITR (130bp)
3230-3763	Gentamicin
3830-4054	Tn7R
5118-5978	B-lactamase (Ampicillin) Complementary

Antibiotic Resistance: Ampicillin and Gentamicin
Bacterial Backbone: pFastBac™ (Invitrogen)

Note: To check the integrity of the AAV ITR's perform single restriction enzyme digestions with XmaI, BssHII, and MscI



Multiple cloning site just upstream of wild type IRES



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Maxi Digest

G0746 0.977ug/well 3-8-13
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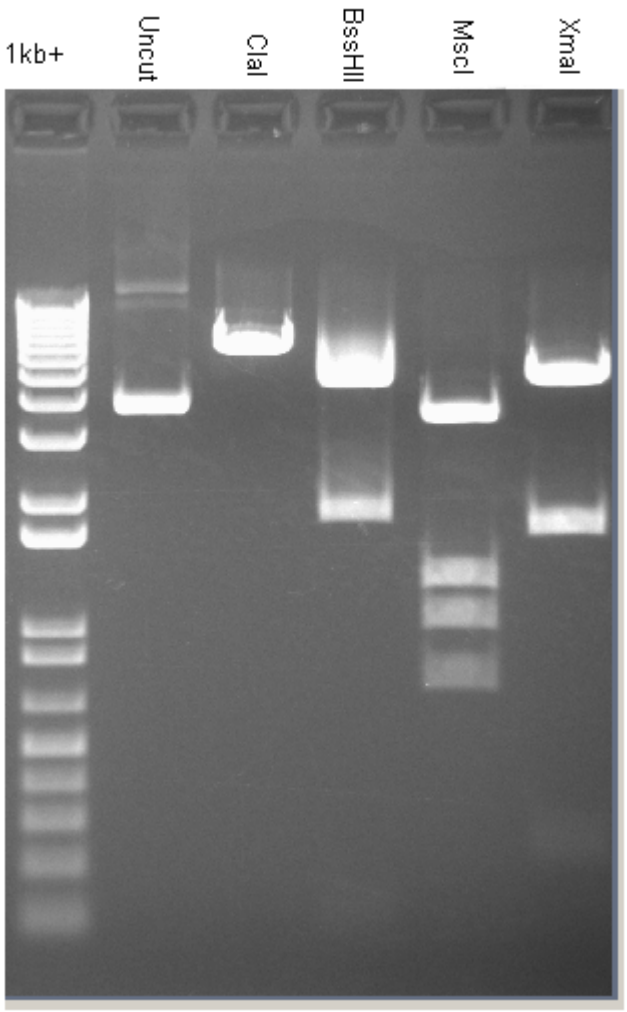
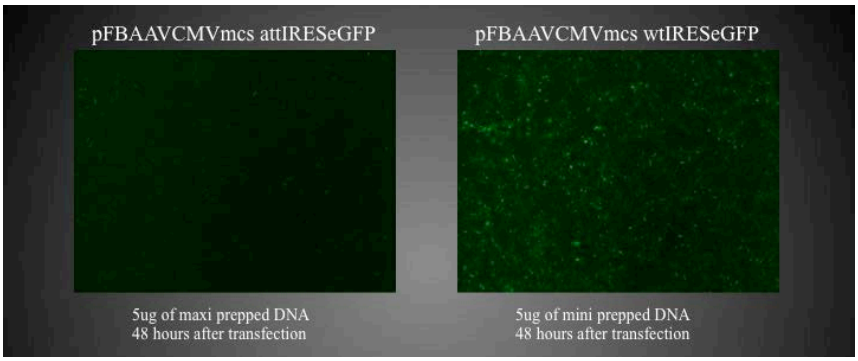


Expected Fragments

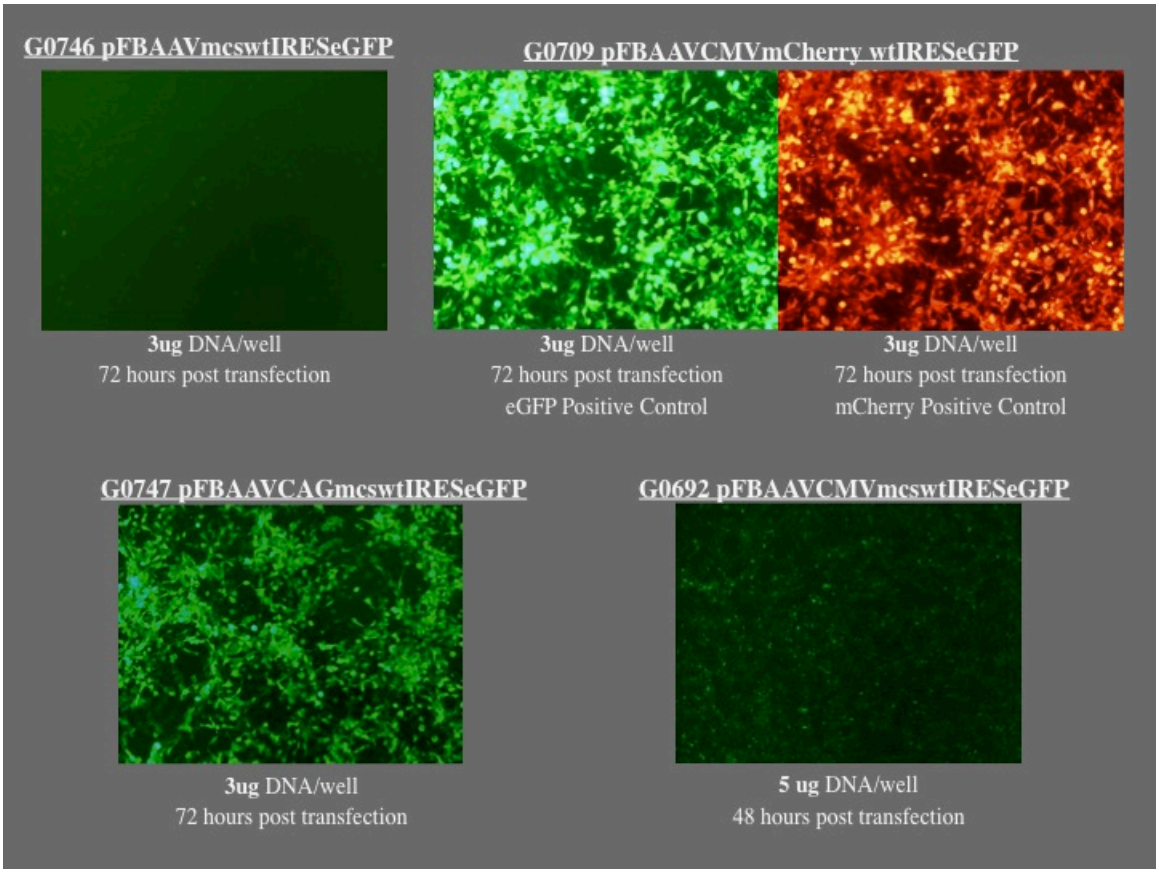
<u>pFBAAVmcs-wtIRESeGFP:</u>
Linear: ClaI (6564)
BssHII: 4600 1805 85 74
MscI: 3503 1288 1033 740
XmaI 4652 1685 216 11

About wild type IRES

University of Iowa Viral Vector Core IRES plasmids were re-cloned in 2013 to replace an attenuated EMCV IRES with the native wild type EMCV IRES for improved expression. There were two changes made to the attenuated IRES sequence to convert it back to the native IRES sequence. First, a single base pair was deleted in the A7 bifurcation loop to convert it back to the native A6 loop. Second, the IRES sequence was extended to include the native 11th and 12th ATGs. *Bochkov YA, Palmenberg AC. 2006.* Translational efficiency of EMCV IRES in bicistronic vectors is dependent both upon the IRES sequence and gene location.



Testing a particular sequence for IRES activity relies on a bicistronic reporter construct. An IRES segment drives translation of the downstream protein coding region best when it is located between two open reading frames as exampled below, Without an upstream open reading frame, the expression is present but weak as in the G0692 cloning plasmid.



Information and Cloning Suggestions for Working with pFBAAV Plasmids:

Characteristics of AAV:
Adeno-Associated virus (AAV) is a non-pathogenic helper dependant parvovirus. This is one of the most promising vehicles for gene delivery. Recombinant AAV vectors have predominantly episomal gene expression. AAV has long term expression in terminally differentiated cells. It can infect a wide range of cells, including non-dividing cells.

Insert Size:
The major disadvantage of the AAV vectors is the small genome, which limits the size of the transgene (from ITR to ITR) to about 4.7 Kb. Please consider the size of the promoter, polyA, any reporters, and your gene of interest when planning the cloning into an AAV shuttle vector. When the length of inserted DNA between the 2 ITRs is close the maximal allowed, i.e., 4.7Kb, the packaging efficiency decreases significantly.

AAV Production:
The University of Iowa Viral Vector Core uses two systems for AAV production: A Triple Transfection System and the Baculovirus System.

The Triple Transfection System may be used with any pAAV plasmid including the pFBAAV plasmids. A pHelper plasmid isolated from adenovirus, a Rep/Cap plasmid, and the AAV ITR plasmid package containing the gene of interest are transfected into 40 x 150mm plates of adherent 293FT cells per prep. This system is more labor intensive for production and purification but can be completed within three to four weeks of receiving the plasmid.
The services provided for production are:

- Transfection.
- Large-scale amplification of AAV vectors.
- The AAV vector is purified using either CsCl or an iodixanol gradient followed by ion-exchange or affinity chromatography.
- A physical titer in viral genomes per ml (vg/ml) is assessed by QPCR.
- **Sample required**: 1500ug of high quality AAV plasmid expressing the gene of interest at a concentration greater than 0.25ug/ul per prep. One disadvantage to the investigator in the triple transfection system is having to provide 1500ug of DNA for each subsequent prep and the possibility of recombination during the maxi prep process. Each maxi prep should be digested and evaluated for integrity.

The Baculovirus System can only be used with pFBAAV plasmids with Tn7 transposable elements to incorporate the ITRs and gene of interest into the Bacmid DNA. *Please note: not all AAV plasmids contain the Baculo elements so this system may not be an option for everyone. This system also relies on the gentamicin selection marker within the transposable elements. The system was developed by Dr. Robert Kotin at the National Institute of Health. The system is described in the following publication: "*Insect Cells as a Factory to Produce Adeno-Associated Virus Type 2 Vectors"* Human Gene Therapy 13:1935-1943 (November 1, 2002). The Baculovirus System takes 5-6 weeks for production but has many advantages. The Baculovirus System can tolerate more toxic proteins driven by mammalian promoters as these promoters will not be active in the insect cells. This system is also generally more robust with higher titers, cost-effective, and less labor intensive for production. Once we have a Baculovirus stock of the investigator package, we can make additional preps readily and we have tested the Baculovirus stock to be stable for years.
The services provided for production are:

- Transposition of the gene interest into the DH10Bac bacmid DNA. Everything in the pFBAAV plasmid between the transposable elements (Tn7, ITR, Promoter, gene of interest, pA, ITR, gentamicin resistance, Tn7) is incorporated into the large

135,000kb bacmid DNA. Your plasmid must be gentamicin resistant for the transposition to work.

- Growth and midi prep preparation of Bacmid DNA.
- Transfection of the Bacmid DNA with gene of interest into Sf9 cells. Approximately 12mls of the P1 stage Baculovirus is harvested 5 days later.
- Titer of the Baculovirus P1 stock. We are able to make 1-3 preps of final AAV virus using the P1 stock depending on the titer of the stock and the desired MOI of infection for the final AAV prep.
- For multiple AAV virus preps the P1 stock is amplified to a larger volume P2 Baculovirus stock and titered.
- The final AAV virus is prepared in bioreactor bags on a rocker platform by dual infection with the package Baculovirus and the desired Rep/Cap Baculovirus into Sf9 cells. The Baculovirus provides pHelper function. The culture is harvested 3 days later.
- The AAV vector is purified using either CsCl or an iodixanol gradient followed by ion-exchange or affinity chromatography.
- A physical titer in viral genomes per ml (vg/ml) is assessed by QPCR.
- **Sample required**: 10ug of pFBAAV plasmid expressing the gene of interest at a concentration greater than 0.25ug/ul.

Bacterial Backbone:
All of our internal AAV plasmids contain Baculo compatible elements from Invitrogen pFastBac™. For information on the pFastBac backbone and the Baculovirus System, please review the Invitrogen manual:
http://tools.invitrogen.com/content/sfs/manuals/bactobac_topo_exp_system_man.pdf
**Please note: not all AAV plasmids are compatible with the Baculo system.

ITRs (Inverted Terminal Repeats):
The ITRs for AAV were engineered into the Invitrogen pFastBac backbone by Dr. Robert Kotin.

Orientation and Updates:
The MCS and BgHpA were inserted in the antisense direction relative to the Invitrogen backbone and ITRs. Please note that the backbone and ITRs were re-sequenced and updated in 2012 and there are several changes. Please discard any older sequences. The nomenclature of 5'ITR (130bp) and 3'ITR (141bp) have been retained on some maps to identify the sequences. The orientation of the ITRs makes no difference to the formation of the viral vector.

Recombination:
Recombination is a possibility at both the transposable elements (Tn7L and Tn7R) and the Inverted Terminal Repeats (ITRs) in the pFBAAV plasmid. In order to check for and reduce the chance of recombination, we have the following recommendations.

Antibiotic Resistance:
The gentamicin resistant gene lies within the transposable elements and may be lost if the transposable elements recombine. We suggest using **both ampicillin and gentamicin** selection on plates and in your liquid media throughout the cloning process and during amplification to midi or maxi preps.

Ampicillin concentration: 100ug/ml final
Gentamicin Sulfate concentration: 7ug/ml final

E. Coli Competent Cell Recommendations:
We recommend using a stable E. coli strain such as **SURE2, Stable2, or Stable3** for transformation of your final plasmid product. These competent cell strains have been engineered to stop unwanted rearrangement events and lack the components of the pathways that catalyze the rearrangement and deletion of nonstandard secondary and

tertiary structures, including cruciforms (caused by inverted repeats) and Z-DNA, that occur frequently in eukaryotic cells. Cloning, however, can be difficult in these strains. We recommend DH5a competent cells for sub-cloning.

Quality Control ITR Digest:
We require a quality control digest of the plasmid upon submission to ensure that there is no recombination before vector production is started. The ITRs in the pFBAAV plasmids have several convenient restriction sites to determine whether the ITRs are intact without sequencing. Sequencing the ITRs can be difficult due to their hairpin secondary structure. We suggest a single digest each of **BssHII, MscI, and XmaI**. It is a good idea to also run un-cut and linearized plasmid next to these digests. Determine your expected fragment sizes for each digest and check carefully to see that you get what you expect and have no extraneous bands. Be sure to check your final midi or maxi product (not just your miniprep) as recombination is possible during any amplification process.

Sequencing pFBAAV plasmids and ITRs
The majority of the plasmid and insert can be sequenced normally. Sequencing of the ITRs is difficult but possible. Sequencing of the ITRs or other difficult secondary structures may be aided by requesting "Extended Denaturation" or by requesting "Betaine Chemistry for AAV ITRs" in the comments section at the University of Iowa Sequencing Core. Non-UI investigators should consult with their own sequencing facility for help if sequencing of the ITRs is desired. We routinely request "Extended Denaturation" for all AAV plasmid sequencing.

There is no one set of primers that will always sequence through the ITRs. The ITR primers listed below have been used with success in many cases. Try sequencing in both directions: from the plasmid in and from your insert out.

Suggested Sequencing Primers for a variety of pFBAAV plasmids:
Below is a general list of commonly used primers at the University of Iowa Viral Vector Core. Please check to make sure the primers work for the shuttle plasmid you have received. There are many free primer design programs available to design primers that will sequence your insert.

BgHpA For	TGAGGAAATTGCATCGCATTGTCT
BgHpA Rev	AGGAAAGGACAGTGGGAGTG
pFBAAV (130bp ITR)	GCCTTGCTGTTCTTCTACGG
pFB587F (141bp ITR)	CTCTACAAATGTGGTATGGCTG
pFB214F (141bp ITR)	GGGGTGGAAATGGAGTTT
pFB158F (141bp ITR)	CATAACAGGAAGAAAAATGCCCCG
EGFP-141Rev	GAACTTCAGGGTCAGCTTGCCGTA
EGFP-651For	TCACATGGTCCTGCTGGAGTT
IRES Rev	CTCACATTGCCAAAAGACG
mU6 For	ACAGACTTGTGGGAGAAGC
CMV Rev	GTAGGAAAGTCCCATAAGGTCA
CMV For	GTGGGAGGTCTATATAAGCAGAGCTCG
IRESfor	GTTGTGAGTTGGATAGTTGTGG

Please contact us with any questions:

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vectors@uiowa.edu

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Iowa City, IA 52242
Tel: (319) 335-6726
http://www.medicine.uiowa.edu/vectorcore/
*Updated 2-22-17 AN